MICROBIAL ENZYMES :

STUDIES ON PENICILLIN V ACYLASE (EC 3.5.1.11)

FROM *Bacillus Sphaericus*

A THESIS
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DECLARATION

Certified that the work incorporated in the thesis "Studies on Penicillin V Acylase (EC 3.5.1.11) from *Bacillus sphaericus*" submitted by Mrs. Archana V. Pundle was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in this thesis.

H Sivaraman

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Research Guide

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ABBREVIATIONS.

6-APA	6-Aminopenicillanic acid.
PDAB	p-dimethylaminobenzaldehyde.
CSL	Corn steep liquor.
DEAE-Sephadex	Diethylaminoethyl-Sephadex.
Tris	Tris(hydroxymethyl)amino-methane.
PAGE	Polyacrylamide gel electrophoresis.
SDS	Sodium dodecylsulphate.
NBS	N-bromosuccinimide.
TNBS	2,4,6-Trinitrobenzenesulphonic acid.
PMSF	Phenylmethanesulphonyl fluoride.
pHMB	p-hydroxymercuribenzoate
βME	ß-mercaptoethanol.
DTT	Dithiothreitol.

ABSTRACT

ABSTRACT

INTRODUCTION

Penicillin acylases (penicillin amidohydrolase, EC 3.5.1.11) are industrially important enzymes which catalyse the hydrolysis of penicillins to give the side chain acids and 6-aminopenicillanic acid (6-APA), the key intermediate in the production of semisynthetic penicillins. The enzymes are grouped according to their side chain preference or specificity. Penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin) acylases preferentially catalyse the cleavage of the respective penicillins, while ampicillin acylases specifically catalyse the cleavage of ampicillin. Molecular aspects and the applications of penicillin G acylases from several bacterial sources have been extensively documented in the literature. Relatively little is known, however, about penicillin V and ampicillin acylases.

PRESENT INVESTIGATION

The present thesis embodies work on the production, purification and characterization of penicillin V acylase from *Bacillus sphaericus* NCIM 2478 and the immobilization of whole cells of the *B. sphaericus* for 6-APA production.

Enzyme Production

Bacillus sphaericus NCIM 2478 was selected as the microbial source for the isolation of penicillin V acylase after screening several isolates and standard type

cultures.

Penicillin V acylase production by *B. sphaericus* on a cornsteep liquor (CSL)-minerals medium under shake-flask conditions was standardised for minerals requirement and environmental parameters. The enzyme remains cell-bound under the conditions used. Growth and production of the enzyme were shown to be enhanced on supplementation of the CSL-minerals medium with wheat bran extract, the overall enhancement in productivity being more than 70% in presence of 1% (w/v) bran or an equivalent of its aqueous extract.

Enzyme Purification

The enzyme from *B. sphaericus* was extracted from harvested cells after sonication and purified by a procedure involving streptomycin sulphate treatment for removal of nucleic acids; removal of inactive proteins by precipitation at pH 4; ammonium sulphate fractionation; ion-exchange chromatography on DEAE-Sephadex A50; and repeated hydrophobic chromatography using octyl-Sepharose. The final preparation was homogeneous on polyacrylamide gel electrophoresis (PAGE), SDS-PAGE and isoelectric focussing in polyacrylamide gel.

Kinetic Properties of the Pure Enzyme

The purified protein had a specific activity of about 30 $\mu mol.min^{-1}.mg^{-1}$ towards penicillin V at 40°C and pH 5.8 in presence of DTT (5 mM).

The K_m of the enzyme towards penicillin V

was shown to be 10 mM. Relative susceptibilities of some ß-lactam antibiotics and of a side chain derivative to enzyme catalysed hydrolytic cleavage indicated a narrow range of specificity, particularly for the phenoxyacetyl moiety, both the side chain structure and 6-APA moiety being required for high rates of cleavage. The purified enzyme had high thermal stability, 80% activity being retained after exposure to 70°C for 2 h at pH 5.8.

Molecular Aspects

The M_r of the native enzyme determined by gel filtration gave a value of 138,000. SDS-PAGE of the enzyme dissociated by heating with SDS gave a single band of M_r 35,000, confirming that the enzyme is a homotetramer.

Amino acid composition: Amino acid composition of the enzyme was analysed and the amino acid sequence of 3 residues from the N-terminus was determined. The amino acid residues content and the sequence at the N-terminus differed from the data deduced earlier by Olsson and Uhlen (1986) from the nucleotide sequence of the *pac* gene.

Secondary structure: Gross features of the secondary structure of the enzyme were deduced from the far UV-CD spectrum at neutral pH. The molecule had approximately 5% α-helix, 20% ß-sheets, 42% ß-turns and 33% random coil.

Chemical modification: Chemical modification studies were carried out to determine the nature of the amino acid residues at or near the active site of the *B. sphaericus*

penicillin V acylase.

Modification of lysine residue : Kinetics of inactivation of the enzyme from *B. sphaericus* by 2,4,6-trinitrobenzene sulphonate (TNBS) at pH 8 were investigated. The inactivation of the hydrolytic activity followed pseudo-first order kinetics and indicated the presence of a single lysine residue per active unit of the enzyme. Protection against inactivation by TNBS was provided by the substrate and by the products. Circular dichroism measurements showed no gross conformational changes on modification of the lysine residues by TNBS. Further, titration of lysines with acetic anhydride at pH 6 showed a linear dependence of the extent of inactivation on the number of lysyl groups modified, the modification of 4 of these leading to the total loss of activity. Penicillin V (500 mM) protection led to modification of 1 lysine residue with retention of about 75% of initial activity.

These findings would indicate the presence of a single lysine residue at or near the active site of each subunit.

Modification of serine: Incubation of the purified enzyme with phenylmethanesulphonyl fluoride (PMSF) at pH 7 resulted in inactivation of the hydrolytic activity of the enzyme. Substrate as well as the products protected the enzyme from inactivation indicating the presence of serine at or near the active site. The reaction followed pseudo-first order and the reaction kinetics showed the likely involvement of a single essential serine residue per active

unit of the enzyme.

Modification of cysteine residues: Treatment of the pure enzyme with the chemical modifier p-hydroxymercuribenzoate at pH 4.5 resulted in loss of hydrolytic activity of the enzyme. Far-UV CD spectrum remained unchanged. The kinetics of inactivation showed a rapid initial inactivation followed by a slower rate of activity loss. Penicillin V and phenoxyacetic acid partially protected against inactivation.

Applied Aspects

Attempts to immobilize the enzyme through adsorption on synthetic ion exchange resins and hydrophobic polymers followed by crosslinking with glutaraldehyde resulted in enzyme inactivation.

Immobilized Whole Cells: Whole cells of *B. sphaericus* entrapped in porous low-gelling agarose beads, retained cell-bound activity, 3 mm diameter beads expressing about 75% of the activity of the free suspended cells. Experiments on the effect of cell loading showed that 20% wet cells (w/v) was optimal. Immobilized whole cells were reused 10 times for the hydrolysis of 2% w/v penicillin V potassium salt, the immobilized system being operationally stable over the cycles of use tried.

The Thesis is presented in 6 parts:

PART I. GENERAL INTRODUCTION:

Reviews the earlier relevant work on penicillin acylases and related enzymes.

PART II. SCREENING MICROBIAL ISOLATES AND STANDARD TYPE CULTURES FOR PENICILLIN V ACYLASE PRODUCTION:

Describes the screening of some microbial isolates and standard type cultures and the selection of *Bacillus sphaericus* NCIM 2478 as the source for penicillin V acylase production.

PART III. PRODUCTION OF PENICILLIN V ACYLASE FROM *BACILLUS SPHAERICUS*:

Comprises standardization of shake-flask conditions for penicillin V acylase production by *B. sphaericus* NCIM 2478.

PART IV. PURIFICATION AND CHARACTERIZATION OF PENICILLIN V ACYLASE FROM *BACILLUS SPHAERICUS* :

Describes the purification procedure developed for obtaining a homogeneous enzyme preparation and the characterization of some of the molecular and kinetic properties of the purified enzyme.

PART V. STUDIES ON CHARACTERIZATION OF ACTIVE-SITE RESIDUES OF *BACILLUS SPHAERICUS* PENICILLIN V ACYLASE BY CHEMICAL MODIFICATION:

Reports work on the chemical modification of the enzyme with amino acid specific reagents and the evidences for lysine, serine and cysteine as putative active-site residues.

PART VI. STUDIES ON IMMOBILIZED WHOLE CELLS OF *BACILLUS SPHAERICUS* WITH PENICILLIN V ACYLASE ACTIVITY:

Reports breifly work on the immobilization of whole cells of *B. sphaericus* and the application of the immobilized system for conversion of penicillin V to 6-aminopenicillanic acid.

REFERENCES are listed collectively at the end of these PARTS.

PART I

INTRODUCTION

I.1 ENZYME CATALYSED CLEAVAGE OF PENICILLINS

Penicillin acylases (penicillin amidases; penicillin amidohydrolases EC 3.5.1.11) catalyse reversibly the hydrolytic cleavage of the side chain amide bond of penicillins to give the ß-lactam nucleus, 6-aminopenicillanic acid (6-APA) and the corresponding carboxylic side chain acids. Two other classes of enzymes act on the penicillin molecule. These are the **ß lactamases (penicillin ß lactam hydrolases EC 3.5.2.6)** which catalyse the opening of the ß lactam ring and the **acylCoA:isopenicillin N acyltransferases** which catalyse the acyl-CoA mediated substitution of L-α-aminoadipyl side chain of isopenicillin N with non-polar side chains such as phenylacetyl or phenoxyacetyl group. The acyltransferases also function as isopenicillin N amidohydrolase (Montenegro *et al.*, 1990; Tobin *et al.*, 1990; Aharnowitz *et al.*, 1992). The acyltransferases catalyse the final step in the biosynthesis of penicillins in the presence of side chain precursors and of 6-APA in their absence.

I.2. PENICILLIN ACYLASES (EC 3.5.1.11)

Historically, Sakaguchi & Murao (1950); and Murao (1955) were the first to report on the enzymatic side chain

cleavage of penicillin. Mycelia of *Penicillium chrysogenum* and of *Aspergillus oryzae* were shown to cleave penicillin G to give phenylacetic acid and a compound which was later identified as 6-APA (Erickson and Bennett, 1965; Murao and Kashida, 1961). The identification of 6-APA as a product of the hydrolytic cleavage followed the earlier work of Kato (1953) and Batchelor *et al.* (1959) who identified 6-APA in the fermentation medium of *P. chrosogenum* in the absence of penicillin side chain precursors.

Extensive screening of microorganisms for penicillin acylase production followed the identification of 6-APA, the key intermediate in the manufacture of semisynthetic penicillins, as a product of the enzyme-catalysed cleavage of biosynthetic penicillins. Several isolates of bacteria, actinomycetes, fungi and yeasts were shown to produce the enzyme, the activity produced remaining intracellular or cell-bound in some cases while in others the activity was produced either extracellularly or in both modes. Enzyme production in many instances was found to be strain-dependent and not to be a general property of the species (Vandamme and Voets, 1974).

I.2.i Types of Penicillin Acylases

Penicillin acylases differ in their penicillin substrate preference or specificity and have been grouped under three types on the basis of this criterion (Fig.1.1).

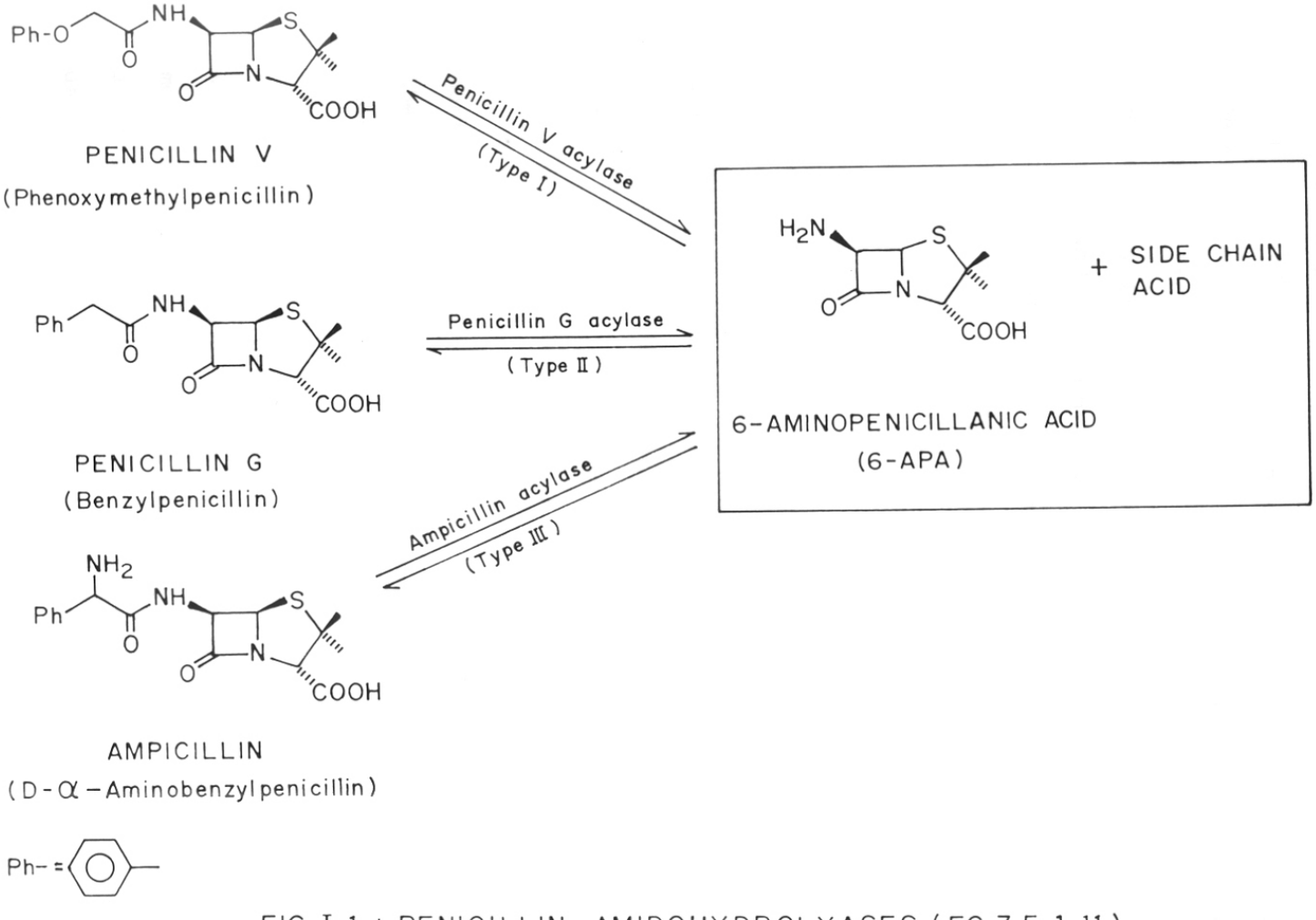


FIG. I·1 : PENICILLIN AMIDOHYDROLYASES (EC 3.5.1.11)

The types are:

(a) **Penicillin V (phenoxymethylpenicillin) acylases** (described earlier as type I) which catalyse the hydrolysis of penicillin V preferentially. Although these were earlier considered to be mainly of fungal origin, several bacteria and actinomycetes have been shown later to produce this type of the enzyme activity.

(b) **Penicillin G (benzylpenicillin) acylases** (grouped earlier as type II) which catalyse the hydrolysis of penicillin G preferentially. Penicillin G acylases are mainly of bacterial origin.

(c) **Ampicillin (D-α-aminobenzylpenicillin) acylases (type III)** which catalyse the hydrolysis of ampicillin specifically. Ampicillin acylase activity has been found hitherto only in *Pseudomonads.*

I.2.ii Physiological Role

The physiological role of penicillin acylases is still unclear. The early belief was that the enzymes could confer resistance towards penicillins produced by fungi in the natural habitats (Holt and Stewart, 1964 b). This was considered later as unlikely on account of the low affinity the acylases have for the penicillins and in the case of penicillin G acylase, the alkaline pH for the cleavage activity (Sutherland, 1964; Cole and Sutherland, 1966). A more favoured hypothesis is that the enzymes probably have a role in the microbial utilization of the aromatic derivatives in plant detritus present in soil (Valle *et*

al., 1991). This may be the case of penicillin G acylase-producers on account of the low substrate specificity and a preference for phenylacetyl derivatives such as phenylacetyl L-amino acids, phenylacetamide rather than for penicillin G. Penicillin V acylases, however, show a narrower specificity range and in many cases a marked preference for the presence of 6-APA moiety in the substrate. The acidic pH optimum of several penicillin V acylases might also support the earlier hypothesis; particularly in the case of the non-β-lactamase producers.

I.2.iii Industrial Applications

Penicillin acylases are important industrial enzymes used extensively in the production of 6-APA, the key intermediate in the manufacture of semisynthetic penicillins. Under appropriate conditions of pH, they catalyse the hydrolysis of the biosynthetic penicillins almost quantitatively. Currently, the earlier chemical route for the cleavage of the side chain has been displaced by the enzymatic route using immobilized enzyme systems.

The industrial importance of the penicillin acylases has been responsible for extensive literature on the production and the applications of the enzymes. The many earlier reviews that have been published include those by Hamilton-Miller (1966), Cole (1967), Vandamme and Voets (1974), Cole *et al.* (1973), Savidge & Cole (1975), Vanderhaeghe (1975), Abbott (1976), Vandamme (1980), Sudhakaran and Borkar (1985), Mahajan (1984), Francetic

(1988), Shewale and SivaRaman (1989), Shewale *et al.* (1990), Sudhakaran *et al.* (1992) and Shewale (1992).

The **GENERAL INTRODUCTION** to the present thesis reviews briefly the screening procedures used in the isolation of microbial enzyme producers; representative examples of microbial producers of the three types of penicillin acylase activities; penicillin acylase (*pac*) gene cloning for enhancing levels of enzyme activity; the purification, kinetic and molecular properties of penicillin V acylases in particular; and some immobilized microbial whole cell and enzyme systems with penicillin V acylase activity.

1.2.iv Screening Methods

Several methods have been used in screening for penicillin acylase producing microorganisms. The procedure using penicillin-resistance as a screen often proved ineffectual due to the relatively wider distribution of β-lactamase activity in microorganisms compared to the side chain cleavage activity (Holt and Stewart, 1964). This limitation is also encountered in the approach of selective growth on a mineral medium with penicillin or the side chain acid as the sole source of carbon and testing for the hydrolysis of acyl-amino acids (Kameda *et al.*, 1961). This difficulty is partially overcome through addition of toluene to the cells grown on media containing penicillin before assay of activity (Nara *et al.*, 1971). In early investigations selective growth on corn steep liquor and other penicillin fermentation media have also been used for

screening penicillin acylase producers (Batchelor *et al.*, 1961; Haung *et al.*, 1963). Mineral media supplemented with side chain amide, acetylglycine, glycylglycyl-glycine as a sole source of carbon and nitrogen had been employed by Vandamme and Voets (1973) and Vandamme (1973) for the selective growth of penicillin G acylase-producers.

A more reliable and rapid procedure has been the use of chromogenic substrates as a screen. Walton (1964) used this approach in soil enrichment procedure and in screening of isolates for penicillin G acylase production with the chromogenic substrate, N-phenylacetyl-4-nitroanilide, a colourless substrate which produces yellow 4-nitroaniline on cleavage. Other chromogenic substrates used for isolation of penicillin G acylase producers have been 6-nitro-3-phenylacetamido benzoate (NIPAB) (Kutzbach and Rauenbusch, 1974) and 2-nitro-4-phenylacetamide bennzoate (Nys *et al.*, 1977). Olsson *et al.* (1985) have used 6 -nitro-3-phenoxyacetamidobenzoate in place of NIPAB in screening for penicillin V acylase positive clones. Visualisation of the activity has also been achieved through derivatization of a product of hydrolysis as in the case of the product of the hydrolysis of the substrate, phenylacetyl-4-aminobenzoate which lights up as a red spot on diazotization and coupling with H-acid (1-amino-8-hydroxy naphthalene-3, 6-disulphonic acid) (Szewczuk *et al.*, 1980).

A widely used microbiological screening method is the

use of *Serratia marcescens* ATCC 27117 which is sensitive to 6-APA and not to penicillin V and penicillin G (Oostendorp, 1972; Meevootisom *et al.*, 1983). In the *S. marcescens* overlay technique, 6-APA production is seen as a clear zone against the red lawn of *S. marcescens* growth.

I.2.v Occurrence

Penicillin acylases are produced by several microorganisms. Enzyme production shows wide variation among strains within the species (Vandamme and Voets, 1974). Enzyme production in the wild type is generally induced in the presence of side chain or side chain derivatives. Tables 1.1, 1.2 and 1.3 include examples of microorganisms that produce the different types of penicillin acylases. The list is representative and not comprehensive, as a very large number of enzyme producers have been reported in the literature.

(a) Penicillin V Acylases

Penicillin V acylases are produced by several bacteria and fungi (Table I.1). This is contrary to the earlier assumption that the enzyme is produced mainly by fungi (Hamilton-Miller, 1966). Penicillin acylase producing bacteria include *Bacillus sphaericus*, *B. subtilis*, *Erwinia aroideae* and *Arthrobacter* sp.

E. aroideae (Vandamme and Voets, 1975), *B. sphaericus* (Carlsen and Emborg, 1982) and *Beijierinckia indica* var. *Penicillanicum* (Sudhakaran and Shewale, 1990) produce the enzyme constitutively. Enzyme production in all these

TABLE 1.1: MICROORGANISMS THAT PRODUCE PHENOXYMETHYL PENICILLIN ACYLASE

Organism	References
BACTERIA	
Achromobacter (NCIB 9424)	Cole (1964)
Beijierinckia indica var penicillianicum	Sudhakaran & Shewale (1990)
Bacillus sphaericus NCTC 10338	Carlsen & Emborg (1981)
Erwinia aroidea	Vandamme & Voets (1974)
Pseudomonas acidovorans	Carlsen & Emborg (1982)
Pseudomonas diminuta	Carlsen & Emborg (1982)
Micrococcus ureae KY 3769	Nara *et al.* (1971)
Streptomyces lavendulae BRL 198	Batchelor *et al.* (1961)
Streptomyces ambofaciens SPSL-15	Nara *et al.* (1971)
Streptoverticillium	Borisov *et al.* (1984)
Sreptomyces netropis 2814	Haupt & Thrum (1967)
Nocardia globerula KY 3901	Nara *et al.* (1971)
YEASTS	
Rhodotorula glutinis	Vandamme & Voets (1973)
Saccharomyces, Cryptococcus Trichosporon	Batchelor *et al.* (1961)
Zygosaccharomyces Debaromyces, Torula	Cole (1966, 1967)

Table I.1 cont.

Organism	References
FUNGI	
Fusarium sp. SKF 235	Sudhakaran & Shewale (1993)
Fusarium semitectum	Brandl (1972); Waldschmidt-Leitz & Bretzel (1964)
Fusarium moniliformae AYF 255	Vandamme & Voets(1974)
Pleurotus ostreatus NRRL, 3501, 3824 (Bovista plumbea)	Schneider & Roe hr (1976)
Penicillium chrysogenum Q 176	Sakaguchi & Murao (1950)
Penicillium chrysogenum A 9342	Claridge *et al.* (1963)
Penicillium chrysogenum Wis 49408	Erickson & Bennett (1965)
Cephalosporium species	Cole (1966)
Cp. acremonium ATCC 11550	Dennen *et al.* (1971)
Aspergillus niger sp.	Vandamme & Voets (1974)
Emericellopsis minim (Stolk) IMI 69015	Cole & Rolinson (1961)
Botrytis cinerea	Batchelor *et al.* (1961)
Gibberella fugikuroi	Vasilescu *et al.* (1969)

TABLE I.2: MICROORGANISMS THAT PRODUCE BENZYLPENICILLIN ACYLASE

Organism	References
BACTERIA	
Escherichia coli ATCC 11105	Bauer *et al.* (1971)
E. coli NCIB 9465	Holt & Stewart (1964a)
E. coli NCIB 8134, 8879, 8949	Cole (1967)
Escherichia sp.	Rolinson *et al.* (1960)
Proteus rettgeri ATCC 9919, 9250	Cole (1967)
P. rettgeri FD 13424	Huang *et al.* (1963)
P. morganni KY 4035 KY405	Okachi *et al.* (1973)
Bacillus subtilis var. niger	Claridge *et al.* (1960)
Bacillus megaterium ATCC 14945 (extracellular)	Chiang & Bennett (1967)
Alcaligenes faecalis BRL 1237, 1238	Cole & Sutherland (1966)
Azotobacter chroococcum Beij C12 Pr.	Lepidi *et al.* (1970)
Flavobacterium	Huang *et al.* (1963)
Xanthomonas sp.	Huang *et al.* (1963)
Rhodopseudomonas spheroides	Nara *et al.* (1971a)
Pseudomonas aeruginasa	Okachi *et al.* (1973)
Pseudomonas sp.	Huang *et al.* (1960)

Table I.2 cont.

Organism	References
Streptomyces ambofaciens SPSL-15	Nara *et al.* (1971a)
Kluyvera citrophila KY 3641	Nara *et al.* (1971a); Okachi *et al.* (1973)
Arthrobacter viscosus ATCC 15294	Ohashi *et al.* (1988)
Nocardia FD 46973, ATCC 13635	Huang *et al.* (1960)
Mycobacterium phlei	Claridge *et al.* (1960)
FUNGI	
Neurospora crassa	Rossi *et al.* (1973)

TABLE I.3: MICROORGANISMS THAT PRODUCE AMPICILLIN ACYLASE

Organism	References
Pseudomonas melanogenum KY 3987, KY 4030, KY 4031	Okachi *et al.* (1973)
Pseudomonas ovalis KY 3962	Okachi *et al.* (1973)

cases have been reported to be from β-lactamase-negative strains. Several Pseudomonad species have been reported to be penicillin V acylase producers (Lowe *et al.*, 1981). These include *P. acidovorans* and *P. diminuta*. The enzyme production has been reported to be induced by phenoxyacetic acid and that β-lactamase activity is co-produced.

Several genera of fungi have been reported to be penicillin V acylase-producers (Vandamme and Voets, 1974; Sudhakaran and Borkar, 1985). Enzyme production in most cases is associated with the mycelia, the activity being produced intracellularly. The enzyme from *Pleurotus ostreatus* (*Bovista plumbea*) (Stoppock *et al.*, 1981; Stoppock and Wagner, 1983) has been reported to be used industrially for 6-APA production from penicillin V (Savidge, 1984).

Several strains of Fusarium have been reported to produce penicillin V acylase (Doctor *et al.*, 1964; Lowe *et al.*, 1986; Vandamme, 1980; Thadani *et al.*, 1972; Sudhakaran and Shewale, 1993a). The enzyme activity has been reported to be present in the spores of *Fusarium conglutinans* (Singh *et al.*, 1969) and of *F. moniliforme* (Vandamme *et al.*, 1971).

Among the actinomycetes, *Streptomyces lavendulae* BRL 198 ATCC 13644 has been reported to produce penicillin V acylase even in the early work on 6-APA production (Batchelor *et al.*, 1961, Robinson *et al.*, 1960). *Streptomyces ambofaciens* SPSL-15 reportedly produces an

intracellular penicillin G acylase and an extracellular penicillin V acylase (Nara *et al.*, 1971). Most of the actinomycetes produce penicillin V acylase constitutively (Vandamme, 1977; 1980).

Among the yeasts, *Candida*, *Rhodotorula*, *Torula*, *Trichosporon* and *Saccharomyces* have been reported to produce penicillin V acylase (Batchelor *et al.*, 1961; Cole, 1966; 1966; 1967; Vandamme, 1973; Hunkava *et al.*, 1984).

(b) Penicillin G Acylases

Penicillin G acylase is produced mainly by Gram negative bacteria and only a few examples of the production of the enzyme by fungi have been reported in the literature (Table 1.2). *Bacillus megaterium* (Chiang and Bennett, 1967) and *Arthrobacter viscosus* (Ohashi *et al.*, 1988) produce the enzyme extracellularly, while in the other bacterial sources listed in Table I.2, the enzyme is retained cell-bound. The most extensively studied source is *Escherichia coli* in which the enzyme activity is retained in the periplasmic space.

(c) Ampicillin Acylases

Pseudomonas species are the only sources reported to date to produce ampicillin acylase activity. The enzymes are apparently specific towards ampicillin and have no activity towards penicillin V and penicillin G.

I.2 vi.Strain Improvement for Penicillin Acylase Production

Penicillin V Acylase

Strain improvement has been effected both by

mutagenesis and by cloning penicillin acylase (*pac*) gene, the latter being reported only in the case of bacterial enzymes. Penicillin V acylase production by *Beijierinckia indica* var. *Penicillanicum* mutant UREMS-5 has been reported to be 70% higher than that by the parent strain (Sudhakaran and Shewale, 1990; Ambedkar *et al.*, 1991). The mutant was obtained through successive UV, γ- irradiation and ethylmethane sulphonate treatment.

The gene encoding penicillin V acylase from *Bacillus sphaericus* had been cloned and expressed in *E. coli* and *B. subtilis* (Olsson *et al.*, 1985). The expression in *B. subtilis* carrying the recombinant plasmid (pOH38) was approximately two-fold higher than the parent *B. sphaericus* strain. In contrast, the *E. coli* strain carrying recombinant plasmids (pOH3 and pOH35) produced lower activities than the parent strain.

Penicillin G Acylase

Extensive reports are available for cloning of penicillin G acylase *pac* gene and its homologous as well as heterologous expression (Shewale and SivaRaman, 1989). Standard recombinant DNA technology does not result in high overproduction of activity in the case of the heterodimeric penicillin G acylases. This has been attributed to the post-translational pathway of maturation of the gene product (discussed under Section I.viiib). In the case of *E. coli*, *K. citrophila* and *P. rettgeri* an inactive precursor polypeptide has been shown to be secreted into

the periplasmic space where the active heterodimeric (αβ) enzyme accumulates after proteolytic processing. The saturation of the secretion across the periplasmic membrane due to the limited number of secreting channels has been assumed to limit the formation of active enzyme (Valle *et al.*, 1991). The alternate approach of separately expressing the genes coding for α and β subunits of the enzyme molecule and reassembling the heterodimeric enzyme *in vitro* poses problems of low activity recovery (Lindsay and Pain, 1991). Burtscher and Schumacher (1992) have reported the reconstitution *in vivo* of penicillin G acylase activity from the separately expressed subunits. Genes coding for α and β subunits were put into *E. coli* hosts in separate plasmids and the activity was found to be reconstituted in the cytoplasm. The activity could only reach levels close to that of the wild type strain used.

I.2.vii Purification

(a) Penicillin V Acylases

Only a few reports in the literature claim even a partial purification of penicillin V acylases from microbial sources. Contaminating activities make the substrate susceptibility data determined with crude and partially purified enzyme preparations unreliable.

The partial purification of the enzyme from *Bacillus sphaericus* NCTC 10338 was reported by Carlsen and Emborg (1982). The cells were lysed with lysozyme + EDTA, and the activity was precipitated with ammonium sulphate at 40%

saturation followed by gel filtration through Sephadex G-200 and ion exchange chromatography on DEAE-Sephadex A50. The final preparation had a specific activity of 20 µmol. $min^{-1} \cdot mg^{-1}$ towards penicillin V at pH 6.8 and 42°C. The molecular weight estimated by gel filtration was about 140,000. The preparation yielded several immunoreactive fractions in cross-immunoelectrophoresis. The K_m for penicillin V was reported to be 140 mM. Phenoxyacetic acid acted as a noncompetitive inhibitor (K_i 30 mM) and 6-aminopenicillanic acid as a competitive inhibitor (K_i 9 mM).

The enzyme from *Bacillus sphaericus* ATCC 14577 (NCTC 10338) had been purified to near homogeneity by Olsson *et al.* (1985). The purification steps included extraction of enzyme after disruption of cells by sonication followed by centrifugation and ammonium sulphate precipitation of the activity in the supernatant at 70% saturation, gel filtration through Sephadex G-200 and chromatography on DEAE- Sephadex A50 column. The final preparation analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) revealed a major band with molecular weight of about 35,000 corresponding to more than 95% of total protein. The molecular weight of the native enzyme estimated by gradient electrophoresis and by gel filtration indicated a molecular weight 135,000 - 140,000, suggesting that the enzyme is a homotetramer. Isoelectric focussing showed a major band corresponding to pI of 4.8. The optimum pH for the hydrolysis of penicillin

V was 5.8. The purified protein had a specific activity of 20.2 umol.min^{-1}. mg^{-1} towards penicillin V at pH 5.8 and 37°C. The K_m of the enzyme was found to be 11 mM, a value significantly lower than the value of 140 mM reported by Carlsen and Emborg (1982). Among the products of the hydrolytic cleavage reaction, phenoxyacetic acid was found to be a noncompetitive inhibitor with K_i of 25 mM and 6-APA was reported to be a competitive inhibitor with K_i of 50 mM.

The purification of the enzyme from *E. aroideae* has been described by Vandamme and Voets (1974). The cell-free extract obtained after the addition of lysozyme was treated with streptomycin sulphate for removal of nucleic acids, centrifuged and the supernatant fractionated by ammonium sulphate precipitation, chromatography on Cellulose-123 and gel filtrations through Sephadex G-25 and G-100. The purified enzyme which had a specific activity of 39 µmol.min^{-1}.mg^{-1} at pH 5.6 showed two bands on cellulose acetate electrophoresis, both bands having acylase activity. The molecular weight of the enzyme preparation was estimated to be 62,000 by thin layer gel chromatography. The enzyme had only very weak activity towards penicillin G and had no activity in the reverse synthetic reaction with sodium phenoxyacetate and 6-APA in the pH range of 4 to 8. The enzyme showed no activity towards phenoxymethylpenicilloic and phenoxymethylpenilloic acids, indicating high specificity towards penicillin V and

a requirement of both side chain and intact 6-APA moieties for its activity.

Preparation of the enzyme from an actinomycete *Streptoverticillium,* has been reported by Borisov *et al.* (1984). The enzyme was purified by gel filtration through Sephadex G-25, CM-cellulose column chromatography followed by gel filtration through Sephadex G-25 and ion-exchange chromatography on DEAE-toyopearl 650 M. The enzyme was homogeneous by PAGE, isoelectric focussing (pI $>\cdot$ 9.0) and ultracentrifugation (5.30 S). The molecular weight estimated by gel filtration was 65,000.

Several reports on the partial purification of fungal penicillin V acylases have appeared in literature. Waldschmidt-Leitz and Bretzel (1964) partially purified the intracellular enzyme from *Fusarium semitectum* and a 300-fold purification was reported. Extracts of dried mycelium were fractionated by DEAE-cellulose treatment, gel filtration on Sephadex G-25 and fractional precipitation with acetone followed by chromatography on Amberlite IRC-50. The purified enzyme preparation was ultracentrifugally homogeneous and had a molecular weight of 65,000. The enzyme contained two Zn atoms per molecule. The optimum pH for hydrolytic action was 7.5.

The enzyme from *Bovista plumbea* (*Pleurotus ostreatus*) in which the activity is present constitutively was purified 220-fold by Schneider and Roehr (1976). The purification procedure involved gel filtration through

Sephadex G-25, chromatography successively on DEAEcellulose, Biogel P-200 and hydroxyapatite. K_m of the enzyme towards penicillin V was 1.67 mM at pH 7.5 and 30°C. The molecular weight of the enzyme was reported to be 83,000.

(b) **Penicillin G Acylases**

For the sake of comparison, a few examples of the purification of some penicillin G acylases are summarized briefly below:

E. coli penicillin G acylase, the most studied of the penicillin acylases, was the first of this class of enzymes to be obtained homogeneous and crystalline (Kutzbach and Rauenbusch, 1974). The procedure involved extraction of cells of *E. coli* ATCC 11105, adjustment of pH to 5, fractionation of the supernatant on SE-Sephadex/CM-Sephadex and DEAE-Sepharose columns followed by crystallization from 0.45 saturation ammonium sulphate at pH 6. The crystalline enzyme was homogeneous in PAGE but showed the presence of a main band and several minor bands on isoelectric focussing, all components possessing enzyme activity. The presence of multiple active components in purified *E. coli* enzyme has also been reported by Kasche *et al.* (1984).

The *E. coli* enzyme exhibits broad specificity, hydrolysing side chain derivatives such as phenylacetylamide and phenylacetyl L-amino acid derivatives at higher initial rates than benzylpenicillin (Cole, 1964;

Kutzbach and Rauenbusch, 1974). Among the penicillins, the enzyme catalyses the cleavage of penicillin G at more than 10-fold higher initial rates than that of penicillin V and 5-fold higher rates than that of ampicillin (Kutzbach and Rauenbusch, 1974).

Purification of the *E. coli* enzyme through hydrophobic chromatography has also been reported (Mahajan and Borkar, 1984; Sudhakaran and Shewale, 1987; Karyekar and Hegde, 1991).

The *Proteus rettgeri* enzyme was purified by Daumy *et al.* (1985a) through a procedure involving treatment of the cell extract with protamine sulphate, the supernatant being fractionated by ammonium sulphate precipitation, ion exchange chromatography and gel filtration.

Barbero *et al.* (1986) have reported the purification of the *K. citrophila* enzyme after cloning of its *pac* gene into *E. coli* (pYKH5). Cells of the recombinant *E. coli* were shocked with 33 mM EDTA + 20% sucrose, lysed with distilled water and the aqueous extract fractionated by ammonium sulphate precipitation, gel filtration and DEAE-cellulose column chromatography.

The enzyme from *A. viscosus*, which unlike the ones described above, is extracellular, has been purified by Ohashi *et al.* (1988). The activity in the culture broth was adsorbed with calcium phosphate gel containing Celite; the adsorbed enzyme extracted, precipitated with ammonium sulphate and fractionated through chromatography on

hydroxyapatite, CM-Sephadex and DEAE-cellulose followed by gel filtration.

The *B. megaterium* enzyme, which is also produced extracellularly, has been purified by Chiang and Bennett (1967) through Celite adsorption, extraction with 24% ammonium sulphate and concentration in vacuum to precipitate the enzyme, followed by CM-cellulose chromatography and a repetition of the earlier steps.

I.2.viii **Kinetic Properties of Penicillin V and Penicillin G Acylase**

For purposes of comparison, some of the kinetic properties of penicillin V and penicillin G acylases from different sources are summarized in Tables 1.4 and 1.5, respectively.

The pH optima reported for penicillin V acylases from bacterial sources, namely *Bacillus sphaericus* and *E. aroideae* range from 5.6 to 6.8 for the hydrolytic reaction. In contrast, penicillin G acylases from the bacterial sources *E. coli, K. citrophila* and *B. megaterium* show a pH optimum in the alkaline range for the hydrolysis of the substrate, the reported values being 8.1 for the purified enzyme from *E. coli* ; 8.0 for *K. citrophila* enzyme and 8 - 9 for the enzyme from *B. megaterium.* Among the bacterial penicillin G acylases, however, the enzyme from *A. viscosus* has an optimum pH of 6-7 for the hydrolytic reaction. Most of the penicillin V acylases from actinomycetes and fungal source have an optimum pH in the neutral to alkaline range

TABLEI 4: GENERAL KINETIC PROPERTIES OF PENICILLIN V ACYLASES IN HYDROLYSIS OF PENICILLIN V

Enzyme source (purity)	Speci. acti. ($\mu mol.min^{-1}mg^{-1}$)	pH opt. imum	K_m (mM)	Product inhibition		Reference
				Phenoxyacetic acid	6-aminopenicillanic acid	
BACTERIA						
B. sphaericus (partial)	20	6.8	140	noncompetitive (K_i 30 mM)	competitive (K_i 9 mM)	Carlsen & Emborg (1982)
B.sphaericus (> 95%)	20.2	5.8	11	noncompetitive (K_i 25 mM)	competitive (K_i 20 mM)	Olsson *et al.* (1985)
E. aroideae (2 components on cellulose acetate electrophoresis; both active)	39	5.6	35	-	-	Vandamme & Voets (1974)
Nocardia sp.	-	8.0	-	-	-	Huang *et al.* (1963)
N. globerula	-	7.4	-	-	-	Nara *et al.* (1971)
Streptomyces ambofaciens	-	7.4	-	-	-	Nara *et al.* (1971)
S. lavendulae (partial)	-	9.0-10.0	10.3-12.5	No inhibition at 4 mg/ml	Marked inhibition at 4 mg/ml	Hamilton-Miller (1966); Batchelor *et al.* (1961)
Streptoverticillium (homogeneous)	39	8.0	-	-	-	Borisov *et al.* (1986)

Table I.4 cont..

Enzyme source (purity)	Speci. acti. ($\mu mol.min^{-1}mg^{-1}$)	pH opt. imum	K_m (mM)	Product inhibition		Reference
				Phenoxyacetic acid	6-aminopenicillanic acid	
YEASTS						
Rhodotorula glutinis (crude)	-	6.5	5.1			Vandamme & Voets (1973)
FUNGI						
Pleurotus ostreatus	-	7.0	5.0-10.0	240 mM	125 mM	Stoppock *et al.* (1981)
P. ostreatus 220 fold purified	26.45	7.5	1.67	No inhibition	No inhibition	Schneider & Roehr (1976)
Fusarium moniliforme	-	8.0	5.75			Vandamme *et al.* (1971)
F. oxysporum (partial)	4.8	8.0	1.8-5.2	Competitive	Non-Competitive	Lowe *et al.* (1986)
F.semitectum	-	7.5-8.0	2.5-2.82	-	-	Brandl (1965)
F. semitectum BC 805 (purified 20 fold)	-	7.5	4.75	-	-	Baumann *et al.* (1971)
Penicillium chrysogenum	-	8.0	16.7	-	-	Spencer & Maung (1970)

TABLE I.5 : GENERAL KINETIC PROPERTIES OF PENICILLIN G ACYLASES IN HYDROLYSIS OF PENICILLIN G

Enzyme source (purity)	Speci. activi. ($\mu mol.min^{-1}mg^{-1}$)	pH opt. imum	K_m (mM)	Product inhibition		Reference
				Phenoxyacetic acid	6-aminopenici-llanic acid	
E. coli ATCC 11105 (crystalline)	23* at pH 7.5 37°C	8.1	0.02	competitive (K_i 0.2 mM)	noncompetitive (K_i 15 mM)	Kutzbach & Rauenbusch (1974)
K. citrophila/ *E. coli* (pYKHS) clone	30 at pH 8.0 37°C	8.0	-	-	-	Barbero *et a* (1986)
A.viscosus ATCC 15294	67 at pH 7 37°C	6.0-7.0	0.42	-	-	Ohashi *et al.* (1988)
B. megaterium ATCC 14945	32 at pH 8.7 37°	8.0-9.0	4.5	competitive (K_i 0.45 mM)	noncompetitive (K_i 26 mM)	Chiang & Bennett (1967)

*Calculated from reported activity towards 6-nitro-3 phenylacetamido benzoate.

(7.0 - 10.0) for substrate cleavage.

In regard to K_m values of penicillin V acylases, variations are observed even in the values reported for the enzyme from equivalent strains of *Bacillus sphaericus*, a K_m value of 140 mM being reported by Carlsen and Emborg (1982) for the partially purified enzyme from strain NCIC 10338 of the organism and a value of 11 mM being reported by Olsson *et al.* (1985) for a relatively pure enzyme preparation from the corresponding ATCC 14577 strain of the organism. K_m values reported for the enzymes from actinomycetes and fungi vary from 1.67 mM to 12.5 mM (Table I.4). The K_m values of penicillin G acylase from bacteria vary from 0.02 mM reported for the enzyme from *E. coli* to 4.5 mM for the enzyme from *B. megaterium* (Table I.5).

In regard to product inhibition, phenoxyacetic acid has been reported to be a noncompetitive inhibitor of penicillin V acylase from *B. sphaericus* (Carlsen and Emborg, 1982; Olsson *et al.*, 1985) and 6-APA to inhibit competitively. The enzyme from the fungal source, *Pleurotus ostreatus* apparently shows no product inhibition (Schneider and Roehr, 1976).

In the case of penicillin G acylases, the side chain acts as a competitive inhibitor and 6-APA as noncompetitive inhibitor in the enzymes from *E. coli* and from *B. megaterium* (Table I.5).

I.2.ix. **Molecular Aspects**

(a) **Molecular Weight and Subunit Structure**

Relatively few penicillin V acylases have been characterised in regard to their molecular properties. Examples documented in the literature for purified penicillin V acylases are summarised in Table I.6 along with the corresponding data for penicillin G acylases, ampicillin acylases and glutaryl 7-aminocephalosporanic acid (GL 7-ACA) acylases.

As described earlier, penicillin V acylase from *B. sphaericus* has been reported to be a homotetramer (M_r 140,000 assembled from 4 identical subunits of (M_r ≈ 37,500) (Olssen and Uhlen, 1986). The enzyme from *E. aroidea* has been reported to have a lower molecular size (M_r 63,000), no data being available for any quaternary structure (Vandamme and Voets, 1975). The enzyme from the actinomycete *Streptoverticellium* which had been purified to homogeneity has been reported to have a M_r of 64,000 (Borisov *et al.*, 1984).

Among the fungal enzymes, data in the literature describe only M_r values. The enzyme from *Pleurotus ostreatus* has been reported to have M_r of 80,000 and that from *Fusarium semitectum* to be of Mr 65,000. The enzyme from *F. semitectum* which was ultracentrifugally homogeneous (5.5S) was shown to contain 2 Zn^{2+} per mole, removal of the metal resulting in its inactivation (Waldschmidt-Leitz and Bretzel, 1964). The enzyme from *F. oxysporum* was shown to

TABLE 1.6: MOLECULAR WEIGHTS OF HOLOENZYMES AND SUBUNITS OF PENICILLIN AND CEPHALOSPORIN ACYLASES

Enzyme	Holoenzyme	Subunit		Reference
		α	β	
PENICILLIN V ACYLASES				
B. sphaericus NCTC 10338	140,000			Carlsen & Emborg (1982)
B. sphaericus ATCC 14577	135,000-140,000	35,500-37,500 (homotetramer)		Olsson *et al.* (1985) Olsson & Uhlen (1986)
E. aroideae	62,000			Vadamme & Voets (1975)
Pleurotus ostreatus	88,000			Schneider & Roehr (1976)
Fusarium semitectum	65,000			Waldschmidt-Leitz & Bretzel (1964)
F. semitectum	67,000			Bauman *et al.* (1971)
F. oxysporum	190,000	63,000 - 65,000 (trimer)		Lowe *et al.* (1986)

Table I.6 cont.

Enzyme	Holoenzyme	Subunit		Reference
		α	β	
PENICILLIN G ACYLASES				
B. megaterium	120,000	-	-	Chiang & Bennett (1967)
E. coli	85,500	20,500	65,000	Schumacher *et al.* (1986)
K. citrophila	85,200	23,600	61,600	Barbero *et al. (1986)*
P. rettgeri	90,000	24,500	66,000	Daumy *et al.* (1985a,b)
A. viscosus	81,000	24,000	60,000	Ohashi *et al.* (1988)
CEPHALOSPORIN ACYLASES				
GL 7-ACA Acylase				
P. melanogenum sp. GK 16	70,000	16,000	54,000	Matsuda & Komatsu(1985)
GL 7-ACA Acylase I *Pseudomonas* sp. SE 83	58,100	38,000	19,900	Matsuda *et al.*(1987a,b)
GL 7-ACA Acylase II				
Pseudomonas sp. SE 83	83,600	25,400	58,200	Matsuda *et al.*(1987a,b)
AMPICILLIN ACYLASE				
P. melanogenum	146,000	72,000 (homodimer)		Kim & Byun (1990)

have M_r of 190,000 (Lowe *et al.*, 1986). The *F. oxysporum* enzyme which was only partially purified was reported to be trimeric.

Among the penicillin G acylases, the enzyme from *B. megaterium* has been shown to have a molecular weight of 120,000 but no reports are available on its quarternary structure. The enzyme which is produced extracellularly, was reportedly one of the earliest to be used commercially for 6-APA production in a process developed as a Bentonite adsorbed system by E.R. Squibb & Sons, Inc. (Henser *et al.*, 1969).

In contrast to the *B.megaterium* enzyme, penicillin G acylases from *E. coli* (Bock *et al.*, 1983a,b; Schumacher *et al.*, 1986), *K. citrophila* (Barbero *et al.*, 1986), *P. rettgeri* (Daumy *et al.*, 1985a, b) and *A. viscosus* (Ohashi *et al.*, 1988) have all been shown to be heterodimers of M_r 81,000-90,000 with subunit components of M_r 20,000-25,000 (α) and M_r 60,000 - 66,000 (ß). The GL 7-ACA acylases investigated hitherto have been shown to be structurally similar to these penicillin G acylases in being heterodimers (Table I.6).

Ampicillin acylase from *Pseudomonas melanogenum* has been reported to have a homodimeric structure, the holoenzyme having a molecular weight of 146,000 and a subunit size of M_r 72,000 (Kim and Byun, 1990 a).

(b) **Post-translational Pathway of Maturation of Heterodimeric Pencillin G Acylases and GL 7-ACA Acylases**

The heterodimeric (α,β) structures of the bacterial penicillin G and GL 7-ACA acylases set out in Table I.6 have been shown to be the result of post-translational processing. The earliest of these to be described and the first to be shown in a procaryote was of penicillin G acylase of *E. coli* ATCC 11105 by Bock and coworkers (Schumacher *et al.*, 1986; Sizmann *et al.*, 1990). This followed the identification of a precursor, membrane-bound polypeptide of M_r 95,000 which cross-reacted immunologically with antibodies raised against the purified enzyme (Bock *et al.*, 1983a; b). The two subunits α and β of penicillin G acylase from *E. coli* were shown to be processed from the M_r 95,000 precursor polypeptide which was enzymatically inactive. Evidences were based on nucleotide sequencing of the structural penicillin acylase (*pac*) gene and correlation of the *pac* gene sequence with the primary structures of the two subunits of the active enzyme. The plasmid constructs lacking sequences coding for specific domains of precursor protein or leading to the formation of mutant penicillin G acylase precursors were used for elucidating gene/protein relationship *in vitro*. These studies indicated the following:

The structural *E. coli pac* gene contains 4 domains, nucleotide positions 1-78 coding for a signal peptide, positions 79-705 coding for the α subunit, positions 706-

867 coding for a spacer endopeptide and positions 868-2538 coding for the ß subunit. The following were shown to be the steps in the post-translational modification of the precursor protein:

(a) Transport of the precursor protein across the cytoplasmic membrane into the periplasmic space followed by removal of the 26 amino acid signal peptide.

(b) The polypeptide chain comprising α subunit, spacer endopeptide and ß subunit domains undergoes proteolytic cleavage at the N-terminus of the ß subunit (557 amino acids).

(c) Removal of the spacer peptide from the α subunit (209 amino acids) in two or three stages of proteolytic cleavage.

The precursor protein and its intermediate cleavage products were shown to be enzymatically inactive till the final formation of the heterodimer (αß). The spacer peptide function has been suggested to be that of a intrapeptide chaperon responsible for correct folding of α, ß subunits to yield the active enzyme (Sizmann *et al.*, 1990; Lindsay and Pain, 1990; 1991).

Essentially similar pathways have been shown to occur in the maturation of the enzymes from *K. citrphila* (Garcia and Buesa, 1986; Barbero *et al.*, 1986), *P. rettgeri* (Daumy *et al.*, 1986) and *A. viscosus* (Ohashi *et al.*, 1988; Konstantinovic *et al.*, 1994). The active enzymes remain in the periplasmic space in *E. coli*, *K. citrophila* and *P.*

rettgeri but is secreted extracellularly in *A. viscosus*.

In a recent report, a hybrid penicillin G acylase structural *pac* gene encoding for the α subunit and the spacer endopeptide from *K. citrophila* and for the β subunit of *E.coli* had been constructed and cloned in *E. coli* (Piotraschke *et al.*, 1994). The processing pathway of maturation was shown to lead to the formation of a hybrid enzyme which was catalytically active.

The heterodimeric GL 7-ACA acylases have also been shown to be derived from a single precursor polypeptide. They however differ from the heterodimeric pencillin G acylases in the absence of a spacer endopeptide. In addition, except for the precursor of GL 7-ACA acylase of *Pseudomonas* sp. GK 16, the precursors of GL 7-ACA acylases I and II of *Pseudomonas* sp SE-83 are also devoid of the signal peptide domain.

The structural features of the precursor protein domains of the heterodimeric enzymes described above are summarized in Tabel I.7.

I.2.x **Primary Structure**

Penicillin V Acylase

The only amino acid sequence data of penicillin V acylase is for the enzyme from *B. sphaericus*, a homotetramer built up of identical subunits of M_r 37,500 and 338 amino acid residues (Olsson and Uhlen, 1986). The sequence was derived from the nucleotide sequence of its gene (Fig. I.2) The derived sequence was shown to have no

TABLE I.7: PROTEIN DOMAINS OF PRECURSOR POLYPEPTIDES OF PENICILLIN G ACYLASES AND GL 7-ACA ACYLASES

Enzyme	Number of amino acid residues				Reference
	single peptide	α subunit	Spacer peptide	β subunit	
PENICILLIN G ACYLASES					
E. coli	26	209	56	557	Schumacher *et al.* (1986)
K. citrophila	26	209	54	555	Barbero *et al.* (1986)
P. rettgeri	20	208	56	555	Ljubijankic *et al.* (1992)
A. viscosus	26	208	31	537	Ohashi *et al.* (1988) Konstantinovic *et al.* (1994)
GL 7-ACA ACYLASES					
GL 7-ACA acylase					
Pseudomonas GK16	29	169	Absent	Not available	Matsuda *et al.* (1985)
GL 7-ACA acylase I					
Pseudomonas sp. SE 83	Absent	336	Absent	181	Matsuda *et al.*(1987)
GL 7-ACA acylase II					
Pseudomonas SE83	Absent	238	Absent	535	Matsuda *et al.* (1987)

M-L-G-C-S-S-L-S-I-R-T-T-D-D-K-S-L-F-A-R-T-M-D-F-T-M-E-P-A-S 30

K-V-I-I-V-P-R-N-Y-G-I-R-L-L-E-K-E-N-V-V-I-N-N-S-Y-A-F-V-G-M 60

G-S-T-D-I-T-S-P-V-L-Y-D-G-V-N-E-K-G-L-M-G-A-M-L-Y-Y-A-T-F-A 90

T-Y-A-D-E-P-K-K-G-T-T-G-I-N-P-V-Y-V-I-S-Q-V-L-G-N-C-V-T-V-D 120

D-V-I-G-K-L-T-S-Y-T-L-L-N-E-A-N-I-I-L-G-F-A-P-P-L-H-Y-T-F-T 150

D-A-S-G-E-S-I-V-I-E-P-D-K-T-G-I-T-I-H-R-K-T-I-G-V-M-T-N-S-P 180

G-Y-E-W-H-Q-T-N-L-R-A-Y-I-G-V-T-P-N-P-P-Q-D-I-M-M-G-D-L-D-L 210

T-P-F-G-Q-G-A-G-G-L-G-L-P-G-D-F-P-P-S-A-R-F-L-R-V-A-Y-W-K-K 240

Y-T-E-K-A-K-N-E-T-E-G-B-T-N-L-F-H-I-L-S-S-V-N-I-P-K-G-V-P-L 270

T-N-E-G-K-T-D-Y-T-I-Y-T-S-A-M-C-A-Q-S-K-N-Y-Y-F-K-L-Y-D-N-S 300

R-I-S-A-V-S-L-M-A-E-N-L-N-S-Q-D-L-I-T-F-E-W-D-R-K-Q-D-I-K-Q 330

L-N-Q-V-N-V-M-S.

FIG. I.2

Amino acid sequence of penicillin V acylase from *Bacillus sphaericus* deduced from nucleotide sequence data of pac gene (Olsson and Uhlen 1986).

homology to the sequences of the heterodimeric penicillin G acylases.

Penicillin G Acylase

As described earlier, the amino acid sequences of the precursor polypeptides of penicillin G acylase from *E. coli* (Schumacher *et al.*, 1986), *K. citrophila* (Garcia and Buesa, 1986), *P. rettgeri* (Ljubijankic *et al.*, 1992), *A. viscosus* (Poitrascheke *et al.*, 1994) have been derived from the nucleotide sequences of their *pac* genes. Partial N-terminal amino acid sequences of α and β subunits of penicillin G acylases from *A. viscosus* has also been reported from amino acid sequencing (Ohashi *et al.*, 1988). The data indicate close homology in all cases. The sequence homologies strongly suggested that these penicillin G acylases evolved from a common ancestral protein. However, the amino acid sequences show no homology with the sequence of the penicillin V acylase suggesting that the two classes of enzyme have different phylogenetic origins.

I.2.xi **Mechanism of Action and Active-Site Residues**

No information is available on these aspects in the case of penicillin V acylases. A brief discussion of the data regarding the mechanism of action and the active-site residues of penicillin G acylases is presented below:

Kinetics of the enzyme-catalysed reaction indicated the involvement of an acyl-enzyme intermediate (Konecny, 1981 a; b). The presence of an active-site hydroxyamino acid residue was established from phenylmethane

sulphonylfluoride (PMSF)-inactivation, the serine reagent totally inactivating an approximately equimolar amount of enzyme from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgeri* (Daumy *et al.*, 1985b) or *K. citrophila* (Martin *et al.*, 1991). The essential PMSF-sensitive residue in the enzyme from *P. rettgeri* was shown by Daumy *et al.* (1985b) to be in the large β subunit from subunit complementation experiments. In this study, the untreated enzyme and the PMSF-inactivated enzyme were dissociated separately with urea, the subunits separated by PAGE, isolated and reassociated in different combinations. The product of the reassociation of the small α subunit from the untreated enzyme with the large β subunit isolated from PMSF-inactivated enzyme was inactive, while that of the α from the PMSF-inactivated and β from the untreated enzyme was active.

The presence of the PMSF-sensitive residue in the β subunits of the *E. coli* and of the *K. citrophila* enzymes was shown through the use of ^{35}[S]-PMSF and PAGE of the inactivated enzyme and assay of gel slices for radioactivity (Slade *et al.*, 1991; Martin *et al.*, 1991). In an effort to locate the PMSF-sensitive residue, Sizmann *et al.* (1990) showed that serine 838 (precursor polypeptide numbering) was not involved since the site-directed mutagenesis of the serine to cysteine did not abolish activity. Slade *et al.* (1991) and Martin *et al.* (1991) used the strategy of site -directed chemical mutagenesis to

locate the essential hydroxyamino acid residues in the enzymes from from *E. coli* and *K. citrophila*, respectively. The PMSF-inactivated enzymes in both the cases were converted to the thiol enzymes by treatment with potassium thioacetate and subsequent removal of the acetyl moiety. Since the native enzymes from *E. coli* and *K.citrophila* have no cysteine residues, the newly derived cysteine residues were identified and located by tagging with iodo -[2-^{3}H] acetic acid. SDS-PAGE electrophoresis and sequence analysis of the tagged enzyme showed that the amino terminal serine -290 residue had been the one which was converted to S-carboxymethyl-cysteine. The mutant enzyme had less than 0.02% of the catalytic activity of the native enzyme. The unexpected location of the essential serine residue at the N-terminal of the β subunits led to the suggestion that the serine-290 might probably lie close to the active-site of the penicillin G acylase, which favours its selective modification. An alternate possibility that was suggested was that the acetylthiol group could have migrated during the preparation of the thiol enzyme on account of the possibility of serine-290 being spatially located in the proximity of an initially modified residue.

The involvement of an hydrophobic environment at or near the active site of *E. coli* penicillin G acylase has been deduced from different lines of investigation. Among these are the reports of Klyosov (1977a,b) that the *E. coli* enzyme is inhibited by aliphatic alcohols, the inhibitory

effects varying with the hydrophobicity of the ligand. Marquez *et al.* (1988) have reported that the binding of penicillin G sulfoxide, a substrate analogue that functions as a competitive inhibitor, results in changes in circular dichroism of aromatic residues. The possibility of a specific hybrophobic interaction was shown by Kutzbach and Rauenbusch (1974). Only PMSF which structurally resembles the side chain of penicillin G could inactivate the enzyme stoichiometrically, while the serine reagent, diisopropyl-fluorophosphate, could inactivate only to the extent of <5%. Site-directed mutagenesis of Met 168 to Ala in the α subunits of the enzyme from *E. coli* (William and Zugel, 1985) and from *K. citrophila* (Martin *et al.*, 1990; Preito *et al.*, 1990) have been shown to alter the substrate specificity of these enzymes.

Kinetic evidences have indicated the presence of an arginine residue at or near the active-site of penicillin G acylase from *E. coli* (Prabhune and SivaRaman, 1990). Evidence has also been obtained for the possible involvement of two essential histidine residues per subunit of the homodimeric ampicillin acylase from *Pseudomonas melanogenum* (Kim and Byun, 1990b).

I.2.xii **Applied Aspects**

Immobilized Penicillin V Acylases

The industrial production of 6-APA, the key intermediate in the manufacture of semisynthetic penicillins, has been mainly through the enzyme route

using immobilized preparations of partially purified penicillin acylases (Vandamme, 1988; Burnett and Heckler, 1989). The sustained interest in penicillin acylases is due mainly to the expected steep increase from the estimated 8000 tons annual global production of 6-APA some ten years ago (Poulsen, 1984). This is primarily because ß-lactams are the most extensively used antibiotics in treatment of bacterial infections and semisynthetic penicillins continue to find wide application as therapeutic agents.

More than 87% of the 6-APA produced by the enzyme route is estimated to be through the use of immobilized penicillin G acylase (Vandamme, 1988). This is despite some of the intrinsic advantages in using suitable penicillin V acylases with penicillin V as substrate. These advantages are mainly due to the optimum pH of most of the penicillin V acylases being in the range of 6 to 7 compared to the alkaline range of 7.8 to 8.5 for most of the penicillin G acylases. The stability of penicillin V, specially in the 6-7 pH range, is greater than that of penicillin G which undergoes more rapid spontaneous degradation, particularly at the alkaline optimal pH range of penicillin G acylases.

The need to use an immobilized biocatalyst system in 6-APA technology is discussed briefly below:

(a) The economic benefit of repeated reuse in batch mode.

This is particularly so in the case of cell-bound

enzymes due to the high cost of enzyme isolation and purification from contaminating cellular proteins,

(b) Exclusion of allergenic macromolecules from the product,

(c) Rapid conversions at high catalyst levels in order to minimize the losses due to spontaneous degradation of the relatively unstable substrates. This would not be economically feasible if high levels of soluble enzymes are used without the possibility of recovery, and

(d) Continuous operation for high volumetric productivity.

A number of immobilization procedures and an extensive range of matrices have been used for the preparation of immobilized penicillin acylases. Immobilization techniques include physical adsorption, physical adsorption followed by crosslinking, covalent attachment, intermolecular cross linking through the use of bi- or multifunctional reagents, fibre entrapment and entrapment within gels (Savidge, 1984; Vandamme, 1988; Shewale and SivaRaman, 1989; KatchalskiKatzir, 1993).

Whole cells carrying penicillin acylase activity have also been immobilized. This approach makes the costly and tedious process of enzyme isolation and purification unnecessary, the disadvantage being however the relatively low levels of activities present in whole cells compared to purified enzyme preparations.

The extensive literature, particularly in patents,

would make any comprehensive review beyond the scope of the present introduction. For this reason, only a few representative examples of immobilization of whole microbial cells containing penicillin V acylase activity and of the isolated enzyme are discussed here (Table I.8).

Despite the relatively low levels of activity in whole cells compared to isolated and partially purified enzyme preparations, a large number of immobilized whole cell systems with penicillin V acylase activity have been described in the literature, particularly in patents. In contrast, most immobilized penicillin G acylase systems are of the isolated and partially purified enzyme (Shewale and SivaRaman,1989). This is probably due to the higher stability of penicillin V compared to penicillin G under operating environmental conditions which would permit the extended periods of time required for the conversion of the substrate to 6-APA by low-activity biocatalyst systems.

TABLE 1.8: IMMOBILIZED PENICILLIN V ACYLASE SYSTEMS

Organism	Method	Reference
BACTERIA		
E. aroideae ATCC 25206	Entrapment in cellulose tri-acetate fibre	Fleming *et al.* (1974; 1975; 1977)
Streptomyces gardeneri	Entrapment in polyacrylamide gel	Chibata *et al.* (1974, 1976)
Streptomyces griseus	Entrapment in poly-acrylamide gel	Chibata *et al.* (1974, 1976)
YEASTS		
Cryptococcus	Cells Crosslinked with glutaraldehyde	Vojtisek *et al.* (1989)
FUNGI		
Pleurotus osreatus	Shredded mycelium entrapped in chitosan and matrix cross-linked with glutaraldehyde	Kluge *et al.* (1982)
M. pulchella	Mycelium entrapped in alginate + polyacrylamide	Singh *et al.* (1988)
Fusarium sp	Spores entrapped in polyacrylamide gel	Linko & Linko (1983)
F. oxysporum	Cells agglutinated by polyethylenimine	Lowe *et al.* (1986)
IMMOBILIZED ENZYMES		
BACTERIA		
Bacterium NRRL 11240	Enzyme in broth precipi-tated with polyethyleni-mine and crosslinked	G estrelius (1980)

Table I.8 cont.

Organism	Method	Reference
Streptomyces griseus	Enzyme adsorbed on Bentonite	Heuser *et al.* (1969)
Streptomyces noursi	Enzyme adsorbed on Bentonite	Heuser *et al.* (1969)
YEASTS		
Rhodotorula glutinis	Enzyme entrapped in polyacrylamide gel	Vandamme & Voets (1973)
FUNGI		
Pleurotus ostreatus	Entrapment of enzyme in cellulose acetate fibres and polyacrylic resins.	Brandl *et al.* (1973); Brandl & Knauseder (1975)
Fusarium sp.	Adsorption on Amberlite CG-50 followed by cross-linking with glutaraldehyde	Sudhakaran & Shewale (1993)
Fusarium sp.	Intermolecular cross-linking of enzyme with glutaraldehyde	SivaRaman *et al.*(1976a)
Fusarium sp.	Covalent binding to CNBr-activated cellulose	SivaRaman *et al.*(1976b)

PART II

SCREENING MICROBIAL ISOLATES AND STANDARD TYPE CULTURES FOR PENICILLIN V ACYLASE PRODUCTION

SUMMARY

Several isolates of bacteria, yeast and fungi from soil, air and sea water as well as some standard type cultures of microorganisms were screened for penicillin V acylase activity in a sequence of three stages. The initial stage comprised screening by *S. marcescence* overlay technique, the second stage comprised visual assay of 6-APA produced on cleavage of penicillin V by aliquots of whole cells using PDAB and the third stage comprised the spectrophotometric assay of 6-APA produced from penicillin V by whole cells. The following three organisms were found to produce relatively high whole cell activity: *Bacillus* sp. (isolate), *B. sphaericus* (NCIM 2478) and *Rhodotorula aurantica* (NCIM 3425), the activities in $\mu mole.h^{-1}.g^{-1}$ packed cells being 290, 1160 and 190; respectively. *R. aurantica* has not been reported hitherto to be a penicillin V acylase producer.

Extracts of sonicated cells of the three cultures had approximately similar specific activities (0.104 - 0.16 $\mu mol.min^{-1}mg^{-1}$ protein). Fractionation of the individual extracts with ammonium sulphate showed that unlike the fraction from *B. sphaericus* (NCIM 2478), the ammonium sulphate fractions obtained from the *Bacillus* isolate and the yeast *Rhodotorula aurantica* lost activity on storage. On the basis of these results *B. sphaericus* (NCIM 2478) was

used as a source material for the isolation and characterization of the enzyme.

INTRODUCTION

As described in the GENERAL INTRODUCTION, several bacteria, yeasts and fungi have been shown to produce penicillin V acylase activity, wide variations being reported among the strains of the various species. A preliminary survey was made in the present studies to identify a wild-strain producing high levels of enzyme activity.

Isolates and standard type cultures of bacteria, yeast and fungi were screened preliminarily for penicillin V acylase production before attempting the isolation of a homogeneous enzyme preparation and characterization of its kinetic and molecular properties. Enzyme production by the isolates and standard type cultures was compared with that of known penicillin V acylase producers for identifying a suitable source material.

MATERIALS AND METHODS

Materials

Beef extract and yeast extract were obtained from Himedia, India; tryptone was obtained from Difco Laboratories, USA; p-dimethylaminobenzaldehyde (PDAB) and methanol were obtained from Qualigens India.

Penicillin V (phenoxymethyl penicillin), 6-aminopenicillanic acid (6-APA) and corn steep liquor were

gifts from Hindustan Antibiotics Ltd., Pune, India.

All other chemicals were commercially available high purity or analytical grade compounds. All media were prepared in distilled water and all buffers were prepared in glass-distilled water.

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, Pune, India.

Methods

Microbial isolates: Microorganisms were isolated from soil, air and sea water. Soil samples were obtained from both the open ground around the laboratory as well as those obtained by a colleague from an altitude of 5000-6000 m while trekking in Sikkim. The microbial contaminants in air were obtained by exposing petri plates with various nutrient media to the laboratory atmosphere. Samples of sea water were provided by Dr. Aditi Pant from collections made in the open intertidal sea off the coast of Goa.

Culture media for isolation of microbes

Bacterial cultures were isolated using the following media:

(a) **Medium A** : Nutrient broth containing (gL^{-1}) peptone 5, beef extract 3 and NaCl 5 ; pH adjusted to 7.0.

(b) **Medium B** : A complex medium used for penicillin G

acylase production by *E. coli* (Prabhune and SivaRaman, 1990): (gL^{-1}) 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, $(NH_4)_2SO_4$ 1 and $MgSO_4.7H_2O$ 0.2; pH adjusted to 7.5.

(c) **Medium C**: reported by Carlsen and Emborg (1981) for penicillin V acylase production by *B. sphaericus* having the following composition: NaH_2PO_4 10 mM, $(NH_4)_2SO_4$ 50 mM, KCl 10 mM, $MgSO_4$ 4 mM, $CaCl_2$ 120 µM, $ZnSO_4$ 75 µM, $FeSO_4$ 300 µM, $MnCl_2$ 150 µM, $CuSO_4$ 15 µM, $CoCl_2$ 30 µM, H_3BO_3 15 µM, $(NH_4)_6MoO_{24}$ 0.03 µM, CSL (50% total solids) 40 g/L.

(d) **Medium D**: Minerals with penicillin V as sole source of carbon. The mineral components were those used by Carlsen and Emborg (1981), penicillin V 1% (w/v), pH 6.5.

Actinomycetes were isolated using **Medium E**: soluble starch 40, yeast extract 25, NaCl 20, $FeSO_4$ 0.25, KH_2PO_4 3, $CaCO_3$ 5, casein hydrolysate; 0.4% (w/v); pH adjusted to 6.8.

Yeast cultures were isolated using **Medium F**: (gL^{-1}) glucose 20, malt extract 3, yeast extract 3 and peptone 5; pH adjusted to 6.5. Tetracycline (500 μgL^{-1}) was added just before use to suppress bacterial growth.

Fungal isolates were obtained using potato infusion **Medium G**: (gL^{-1}) glucose 20, and potatoes (infusion from 200 g potatoes) pH adjusted to 4.5. Tetracyclinee (500 μgL^{-1} was added just before use.

Medium H: The medium reported by Stoppock and Wagner (1983) for penicillin V acylase production by *Pleurotus ostreatus* (*Bovista plumbea*) was also used for isolation of fungi: (gL^{-1}) KH_2PO_4 1, $MgCl_2.H_2O$ 0.5, $Ca(NO_3)_2.4H_2O$ 0.5, $FeSO_4.7H_2O$ 0.05 g, yeast extract 10, sucrose 20 (autoclaved separately), pH 7.0. Tetracycline 500 μgl^{-1} was added just before use.

Media were made upto volume with distilled water when isolates were made from soil and air. Artificial sea water of the following composition was used when isolates were made from marine samples. Composition of artificial sea water: (gL^{-1}) NaCl 24.53, $CaCL_2.2H_2O$ 1.54, KBr 0.1, NaF 0.003, KCl 0.7, H_3BO_3 0.03, Na_2SO_4 4.09, $NaHCO_3$ 0.2, $SrCl_2.6H_2O$ 0.017, $MgCl_2.6H_2O$ 11.1.

All the culture media contained 2% agar when solid media in petri plates or slants were used.

For isolation from soil samples, about 1g soil was suspended in sterile, distilled water (10 ml), 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°C for 24 - 48 h. Isolated colonies of microorganisms obtained in petri plate were subcultured on slants and these isolates were then screened individually for penicillin acylase production.

The species identification of isolates was done on morphological characteristics. In addition to the isolates, both randomly selected standard cultures of

microorganisms and a few known penicillin V acylase producers such as *B. sphaericus* (NCIM 2478) and several strains of *R. glutinis* were screened for enzyme production.

Screening Procedures

Screening was done in 3 successive stages:

Stage I

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

In a typical procedure, the isolate was point inoculated at the centre or within a sector of petri plate (9 cm dia) containing the basal solid (2% agar) growth medium. After overnight incubation at 30°C, the plate was overlaid with 5 ml of soft nutrient agar containing 10 $mg.ml^{-1}$ penicillin V and 2 ml of 18 h culture of *S. marcescens* ATCC 27117 grown in nutrient broth. After the overlay had hardened, the plate was incubated at 28°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 activity. (Plate I)

Stage II

Cultures that showed significant zones of clearance were grown in liquid media of the composition described

earlier for cultivation of bacteria, yeast and fungi. 12 ml liquid medium was dispensed in a boiling tube, a loopful of cells was inoculated in the sterile medium and was incubated for 24 h at 30°C on a rotary 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.*, 1987). The cells were harvested by centrifugation on a Microfuge. Both pelleted cells and an aliquot of the supernatant were assayed for penicillin V acylase activity.

Assay of penicillin V acylase activity of isolates

Cells were resuspended in 1 ml 0.1 M citrate buffer, pH 5.8 containing penicillin V (20mg/ml). The reaction mixtures were incubated for 1 h at 40°C. An aliquot was added to 2 ml citrate-phosphate buffer, pH 2.5 and an equal volume of the 6-APA colour reagent, p-dimethylaminobenzaldehyde (PDAB) (Bomstein and Evans, 1965) was added to it. The colour reagent was prepared by dissolving 1 g PDAB in 170 ml of methanol containing 0.1% hydroquinone.

The penicillin V acylase producers are assessed on the visual gradation of the intensity (0 to ++++) of yellow colour formed after 2 min.

Stage III

The cultures that showed high (≥ +++) activity were then assayed for production of 6-APA from penicillin V as follows:

Depending on the isolate (bacteria, yeast or fungus),

the appropriate liquid medium was prepared and dispensed into a set of boiling tubes and 250 ml conical flasks containing 12 ml and 50 ml medium, respectively.

The inoculum was developed in the boiling tube containing the medium by transferring a loopful of the isolate to be tested, the tubes were then incubated at 30°C for 24 h on a rotary shaker at 150 rpm. The inoculum was transferred to the 250 ml conical flask containing the same medium and incubated for 24 - 48 h at 30°C on a rotary shaker at 150 rpm.

The cells were harvested by centrifugation on a Sorvall RC-5B refrigerated centrifuge at 6000 x g for 20 min at 4°C. The cells were washed twice with 0.01 M potassium phosphate buffer, pH 6.5. 0.1 g packed wet cells were suspended in 1 ml of penicillin V solution (20 mg/ml) in 0.1 M sodium citrate buffer (pH 5.8) and incubated for 1 h at 40°C.

0.1 ml sample was removed after the incubation period and transferred into 1 ml citrate-phosphate buffer, pH 2.5 to quench the hydrolytic reaction. The clear supernatant obtained after centrifugation on a microfuge was made up to a final volume of 2 ml to which 2 ml PDAB colour reagent was added. 6-APA formed was estimated by measuring the absorbance at 415 nm of the yellow-coloured complex formed.

Activity of cell-free extracts

Cells of cultures which showed high activities at

Stage III were disrupted by sonication. Cells were suspended in 0.05 M potassium phosphate buffer, pH 6.5 (3 $ml.g^{-1}$ wet cells and sonicated for a total period of 8 min (4 exposures of 2 min each) at 0-4°C using Biosonic III sonic oscillator (Bronwill Scientific Co., USA). Cell debris was removed by centrifugation at 6000 x g and 4°C for 30 min. Aliquots of clear supernatant were assayed for penicillin V acylase activity.

Assay of penicillin V acylase

Enzyme activity was measured by determination of 6-APA formed with PDAB. The assay system contained 2% w/v potassium salt of penicillin V substrate in 0.1 M sodium citrate, pH 5.8 at 40°C.

1 unit (U) of enzyme is defined as the amount of enzyme catalysing the hydrolysis of 1 µmol substrate in 1 min under assay conditions.

Protein was determined by the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The results of screening for penicillin acylase production are summarised under the three stages described in **Materials and Methods**

Stage I

Among the several isolates, 9 bacterial cultures and 7 fungi showed significant activity in Stage I of screening. None of the yeast isolates showed any measurable activity.

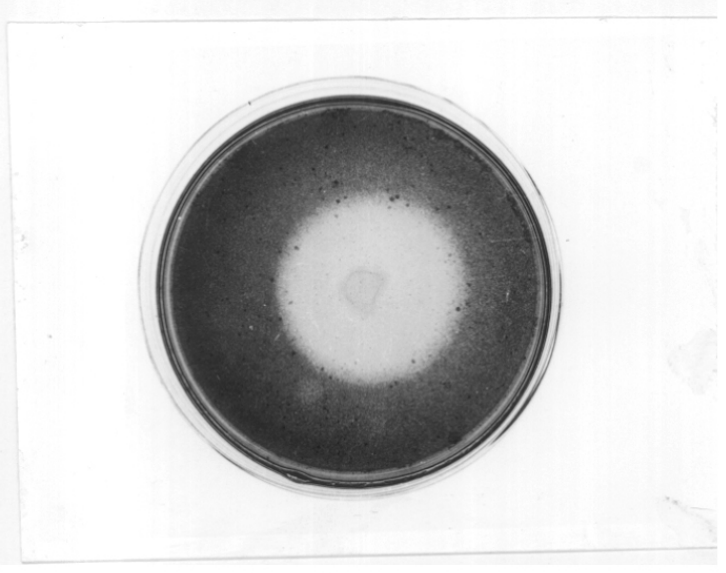


PLATE II.1

Plate II.1. Plate shows zone of clearance (++++) by *R. aurantica* in *S. marcescens* overlay tecnique.

Among the randomly chosen standard type cultures, the following showed activity at this stage of screening. These include, 4 bacterial cultures, 12 yeasts and 4 fungi.

Stage II

The activities of isolates and standard type cultures are set out in Table II.1 and II.2, respectively. The activities in all cases were cell-bound, the supernatants being inactive.

Stage III

As it can be seen from Tables II.1 and II.2, an isolate of *Bacillus* species, which had in addition a flocculent property, and two standard type cultures, namely *Rhodotorula aurantica* (NCIM 3425) and *Bacillus sphaericus* showed promising activity. These were taken up to Stage III of screening. The results at Stage III are shown in Table II.3.

As it is evident from Table II.3, the known penicillin V acylase producer *Bacillus sphaericus* (NCIM 2478) showed the highest activity of 1160 $\mu mol.h^{-1}g^{-1}$ wet cells compared to the corresponding values of 290 and 190 of *Bacillus* sp. (isolate) and *R. aurantica* NCIM 3425, respectively. In all the cases, the addition of inducer, phenoxyacetic acid made no significant difference to enzyme production.

Although whole cells of bacillus isolate and the yeast *R. aurantica* were less active than *B. sphaericus* NCIM 2478 sonicates of all the cultures were assayed for activity to determine whether the whole cell activity differences were

TABLE II.1: PENICILLIN V ACYLASE ACTIVITY OF WHOLE CELLS OF ISOLATES BY VISUAL ASSAY OF 6-APA WITH PDAB REAGENT

Microorganism	Source	Medium used	Penicillin V acylase activity
BACTERIA			
Bacillus sp. Y	Soil from high altitude	A (15°C)	-
Bacillus sp. a)	Soil	D	-
Bacillus sp. b)	Soil	B	-
Staphylococcus	Soil	A	-
Bacillus sp. X	Marine water	B	-
Bacillus sp. y	Marine water	B	-
Bacillus sp. V^+	Air	B	+++
Actinomycete sp. 88-2-2	Soil	F	-
Actinomycetes sp. 90-1-30	Soil	F	-
FUNGI			
Aspergillus sp.	Soil	H	-
Fusarium sp.	Air	G	+
Penicillium	Soil	H	-
A mushroom sp.	Soil	H	++
Albizzia	Soil	H	-
Elusine smut	Soil	H	-
Dicanthium smut	Air	H	-

TABLE II.2: PENCILLIN V ACYLASE ASSAY OF STANDARD TYPE CULTURES BY VISUAL ASSAY OF 6-APA WITH PDAB REAGENT

Microorganism	NCIM	Medium	Penicillin V acylase activity
BACTERIA			
Bacillus sphaericus	2478	C	++++
Arthrobacter viscosus	2451	B	-
Pseudomonas acidovarans	2861	C	-
Bacillus sp.	0059	A	-
YEAST			
Rhodotorula glutinis	3168	F	-
Rhodotorula glutinis	3169	F	-
Rhodotorula glutinis	3170	F	-
Rhodotorula glutinis	3353	F	+
Rhodotorula glutinis	3379	F	-
Rhodotorula aurantica	3425	F	+++
Rhodotorula graminis	3426	F	-
Filobasidium capsuligenium	3368	F	-
Filobasidium capsuligenium	3553	F	+
Leucosporidium toruloides	3537	F	+
Rhodosporidium toruloides	3547	F	-
Tremella mesentrica	3530	F	-

Table II.2 cont.

Microorganism	NCIM	Medium	Penicillin V acylase activity
FUNGI			
Aspergillus flavus	541	G	+
Absidia blakesleena	889	G	-
Absidia blackesleena	890	G	-
Sclerotium rolfsii	1084	H	-

TABLE II.3: PENICILLIN V ACYLASE ACTIVITY OF WHOLE CELLS BY SPECTROPHOTOMETERIC ASSAY OF 6-APA PRODUCED

Microorganism	Source	Penicillin V acylase activity $\mu mol.h^{-1}g^{-1}$ wet cells
Bacillus sp.	Isolate	290
Bacillus sphaericus	NCIM 2478	1160
Rhodotorula aurantica	NCIM 2478	190

due only to differences in the cell permeability. The sonic extracts were assayed for penicillin V acylase activ ity and fractionated by ammonium sulphate precipitation after preliminary pilot runs for determining ammonium sulphate saturations for precipitation of the activity. The results are shown in Table II.4.

It can be seen from Table II.4 that the bacillus isolate and the standard yeast culture *Rhodotorula aurantica* are almost comparable to the known enzyme produc- er *B.sphaericus* in their intracellular enzyme levels. This is the first report of *Rhodotorula aurantica* being a peni- cillin V acylase producer.

Although the sonicates showed almost comparable specific activities in all three cases, the ammonium sulphate fraction obtained from *Bacillus sphaericus* was significantly higher than of the *Bacillus* isolate and the yeast. Further unlike the enzyme from *Bacillus sphaericus*, the enzymes from the *Bacillus* isolate as well as the yeast showed loss in activity on storage. Attempts to stabilise the activity in these cases through the addition of bovine serum albumin (5 mg/ml) or of protease inhibitor, the neutralised extracts from horse gram (*Dolichos biflorus*), to the sonicate were unsuccessful.

Based on these results, the known penicillin V acylase producer, *Bacillus sphaericus* (NCIM 2478) was chosen as a source material for further studies.

TABLE II.4: PENICILLIN V ACYLASE ACTIVITIES OF SONICATES AND AMMONIUM SULPHATE FRACTIONS OF SONIC EXTRACTS

Organism	*Bacillus* sp. isolate	*Bacillus sphaericus* NCIM 2478	*Rhodotorula aurantica* NCIM 3425
	Specific activity ($\mu mole.min^{-1}mg^{-1}$)		
Sonicate	0.104	0.16	0.14
$(NH_4)_2SO_4$ Saturation Fraction	0.177 (0.2-0.8S)	0.32 (0.3-0.6S)	0.25 (0.2-0.55S)

(Figures in parenthesis indicate ammonium sulphate saturation

PART III

PRODUCTION OF PENICILLIN V ACYLASE BY BACILLUS SPHAERICUS

SUMMARY

Production of penicillin V acylase by *Bacillus sphaericus* NCIM 2478 was standardized. The organism produced high levels of penicillin V acylase (20 $\mu mol.min^{-1}g^{-1}$ packed cells) when grown in batch mode on a rotary shaker at 120 rpm for 20 h at 25°C and an initial pH of 7.5 on the corn steep liquor (CSL)-minerals medium formulated by Carlsen and Emborg (1981). Deletion of NaH_2PO_4, KCl, $MgSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$ and $(NH_4)_6Mo_7O_{24}$ from the CSL-minerals medium made no difference to growth or levels of the intracellular enzyme activity produced.

Supplementation of the CSL-minerals medium with starch, chitosan, glucose, galactose, sucrose, lactose, xylose and mannose did not have any effect on growth or enzyme production. Replacement of corn steep liquor with peptone, tryptone, malt extract or yeast extract resulted in marked reductions in growth and specific activity of whole cells. In the CSL-minerals medium the enzyme was produced constitutively, phenoxyacetate (1 $mg.ml^{-1}$) inhibiting both growth and enzyme production. Supplementation of the CSL-minerals medium either with 1% w/v whole wheat bran or its aqueous extract resulted in more than 70% increase in enzyme productivity.

Optimal growth and enzyme production on both CSL-minerals and CSL-wheat bran extract-minerals media under

shake-flask conditions were obtained when the initial pH was 7.5, the temperature 25°C and the period of fermentation 20 h with continuous agitation at 120 rpm on a rotary shaker.

The organism grown on the CSL-minerals media had no β lactamase activity.

INTRODUCTION

The production of microbial penicillin acylase activity is regulated by nutrients and growth conditions. For most bacterial sources of the enzyme, complex organic constituents such as yeast and malt extracts, corn steep liquor, soyabean meal and protein hydrolysates have been used or included in the medium (Huang *et al.*, 1963; Cole *et al.*, 1966; Savidge and Cole, 1975; Plaskie *et al.*, 1978; Okachi and Nara, 1972). The presence of the side chain carboxylate of the penicillin molecule induces enzyme production in wild strains of *E. coli* and other sources in which the activity is not produced constitutively (Kaufman and Bauer, 1968; Levinton *et al.*, 1967). Catabolite repression by glucose and acetate has been reported in *E. coli*, glucose repressing enzyme production completely (Vojtisek and Slezak, 1975). High levels of dissolved oxygen have also been shown to repress enzyme synthesis by *E. coli* (Kleiner and Lopatev, 1972; Vojtisek and Stezakm 1975). c-AMP has been reported to stimulate enzyme production by *E. coli* and to overcome repression by glucose in *E. coli* (Geng and Shaikh, 1976) and in *K. citrophila*

(Takesava *et al.*, 1972; Shimizu *et al.* 1975a,b).

B. sphaericus NCIM 2478 grown on the corn steep liquor (CSL)-minerals medium of Carlsen and Emborg (1981) had been shown by us to be the most productive source of penicillin V acylase activity among several microbial isolates and standard type cultures that had been screened (**Part II of the Thesis**). Standardization of environmental conditions and the effect of modifications of the CSL-minerals medium and of supplementation of the modified medium with complex organic additives on enzyme production by *B. sphaericus* NCIM 2478 are presented in this **Part of the Thesis.**

MATERIALS AND METHODS

Corn steep liquor (CSL; 50% dry solids content) was a gift from Hindustan Antibiotics Ltd., Pune. CSL was pretreated by suspension of 50 g of liquor in 80 ml of water; the diluted solution was adjusted to pH 7 with NaOH, made upto 100 ml; steamed for 1 h, cooled and clarified by centrifugation.

Wheat bran was obtained from a local mill. Wheat bran extracts were obtained by autoclaving a suspension of the wheat bran in water (5 $ml.g^{-1}$) at 20 psi for 20 min, cooling the suspension and filtering through a sintered funnel to give a clear, opalescent filtrate. The residue was reextracted as before and the extracts pooled and added to the CSL-minerals medium as described in the text. The extraction procedure solubilized about 20% w/w of the whole bran.

Sources of other nutrients and chemicals are described in the earlier **Part II of the Thesis.**

Microorganism

Bacillus sphaericus NCIM 2478 was obtained from the National Collection of Industrial Microorganisms, Pune. The organism was maintained routinely on slants containing (gL^{-1}): tryptone, 5; yeast extract, 3; beef extract, 3; agar, 20, pH 7, and was subcultured once every month.

Fermentation Medium

Initial experiments were carried out using the CSL-minerals medium described by Carlsen and Emborg (1981). The medium (designated as Medium A) contained: NaH_2PO_4, 10 mM; $(NH_4)_2SO_4$, 50 mM; KCl, 10 mM; $MgCL_2$, 4 mM; $CaCl_2$, 120 µM; $ZnCl_2$, 75 µM; $FeCl_2$, 300 µM; $MnCl_2$, 150 µM; $CuSO_4$, 15 µM; $CoCl_2$, 30 µM; H_3BO_3, 15 µM; $(NH_4)_6MoO_{24}$, 0.03 µM; CSL (50% dry weight), 40 $g.L^{-1}$. The pH of the medium was adjusted with NaOH to values in the range of 6.5-8.0 in steps of 0.5 units. 50 ml aliquots of the medium were dispensed into 250 ml conical flasks and sterilised at 20 psi for 20 min.

Fermentations were carried out under shake-flask conditions and optimized in regard to agitation rate, initial pH, temperature, period of growth, dispensing volume and inoculum size. Cells freshly grown in the fermentation media were used as inocula.

The effects of varying levels of CSL as well as of deleting individual mineral components of the medium on

growth and penicillin V acylase production were determined under the optimized conditions of batch fermentation. Effect of replacing CSL by complex organic nutrients such as peptone, tryptone, casamino acids, malt extract, yeast extract was also determined. A medium containing the optimum level of CSL supplementation and containing only such mineral supplements which affected growth and enzyme production (designated as Medium B) was used in further studies in which the effects of additives such as chitosan, wheat bran or wheat bran extracts were studied.

Enzyme Assay

Penicillin V acylase activity determination was carried out at pH 5.8 and 40°C using 2% (w/v) penicillin V as described by Olsson *et al.* (1985). The 6-APA formed was estimated spectrophotometrically by p-dimethylamino-benzaldehyde method of Bomstein and Evans (1965) as modified by Sudhakaran and Shewale (1990). The standard curve of 6-APA is shown in Fig. III.1. Cell suspensions were used for assay of cell-bound activity. Under shake flask conditions, the broth had no detectable activity.

One unit (U) of the enzyme is defined as the amount of enzyme catalyzing the hydrolysis of 1 µmole of substrate.min^{-1} under the assay conditions.

Enzyme Extraction

Cells harvested by centrifugation were washed twice with 0.05 M potassium phosphate buffer, pH 6.5, suspended in the same buffer (4-5 $ml.g^{-1}$ packed wet cells) and

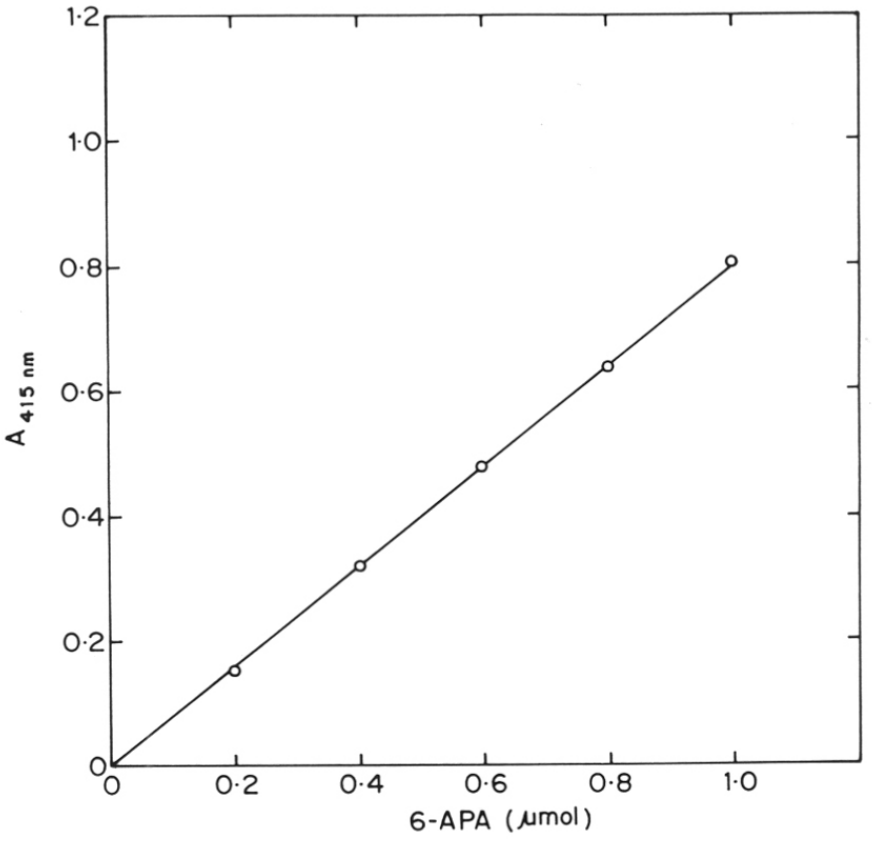


FIG. Ⅲ·1

Fig III.1. Standard curve of 6-Aminopenicillanic acid estimation with p-dimethylaminobenzaldehyde.

disrupted by sonication (20 Kc, 300 W) at 4°C for a total period of 10-12 min,2 min at a time, using a Biosonic III sonic oscillator (Bronwill Scientifc Co., USA). The suspension was centrifuged, cell debris discarded and aliquots of the clear supernatant were assayed for enzyme activity and protein content. Protein was determined according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

The absence of β lactamase activity was checked in sonicated cells by the iodometric titration procedure of Ross and Callaghan (1975). The activity was absent under all the fermentation conditions used.

Optimization Studies

Effect of pH

Medium used was the CSL-minerals Medium A. pH of the medium was adjusted from 6.5-8 in steps of 0.5. *B. sphaericus* was inoculated with one subculture into 250 ml conical flasks containing 150 ml of the medium and grown on a rotary shaker at 120 rpm and 30°C for 20 h. Cells were harvested by centrifugation at 8000 x g and 4°C.

Effect of Growth Temperature

B. sphaericus was inoculated after one subculture into 50 ml Medium A of pH 7.5 contained in 250 ml conical flasks. The flasks were incubated separately at temperatures in the range 22°C - 38°C for 20 h on a rotary shaker at 120 rpm.

Effect of Dispensing Volume

Amount of medium dispensed in 250 ml conical flasks was 25 ml, 50 ml, 100 ml and 150 ml. Corresponding volumes dispensed in 1 L conical flasks were 100 ml, 200 ml, 400 ml and 600 ml flasks. Flasks were incubated at 120 rpm at 25°C. Cells were harvested after 20 h, weighed, sonicated and tested for enzyme activity.

Effect of Inoculum Size

Inocula were grown 24 h in CSL-minerals Medium B, pH 7.5. Inoculum sizes tried varied from 5%-20% of the volume of the medium. The calculated absorbance of the inoculum at 600 nm was 6.84 equivalent to 0.012 g cells.ml^{-1}

Effect of Corn Steep Liquor Concentration

Corn steep liquor (50% solids) concentration was varied from 20 - 80 $g.L^{-1}$.

Effect of Phenoxyacetate

1 $mg.ml^{-1}$ of phenoxyacetic acid was added to Medium A maintaining the initial pH of the medium 7.5.

Media Optimization

Salts were deleted individually from the CSL-mineral salts Medium A and the effect on cell growth and penicillin acylase activity production were determined. A modified CSL-minerals medium (Medium B) which contained 40 $g.L^{-1}$ CSL (50% solids) and from which the nonessential minerals were deleted was used for further studies.

The effect of supplementation of Medium B individually with various carbon sources like starch, glucose, sucrose,

galactose, lactose, mannose, chitosan, wheat bran or wheat bran extract was determined.

RESULTS AND DISCUSSION

Growth and Enzyme Production on Medium A

Growth as well as cell bound penicillin acylase production in Medium A were determined at 25°C and at varying initial pH values and at different periods of time. Initial pH in the range 6.5-8 resulted in approximately the same final pH of 7.90 - 8.15 at the end of a period of 24 h. Optimal growth and activity at 25°C were obtained when the initial pH of the medium was 7.5 and the period of fermentation was 20 h (Fig. III.2)

Effect of temperatures in the range of 22°C - 38°C was then studied using Medium A at an initial pH of 7.5, Cell yield and the production of penicillin acylase activity were highest at 25°C and at the end of 20 h period, low activities being obtained below 24°C and above 32°C at the corresponding period of 20 h (Fig. III.3).

The following conditions were found to be optimal for Medium A at initial pH of 7.5 and at 25°C under shake flask conditions: dispensing volume, 50 ml in a 250 ml conical flask or 200 ml in a 1 L conical flask gave the best result; inoculum size 1 x 10^6 cells grown for 16 h in 5 ml of Medium A for 50 ml of the medium and 10% of volume of medium was best suited for growth and enzyme production.

The effect of varying levels of CSL on growth and cell-bound enzyme activity is shown in Table III.1. It can

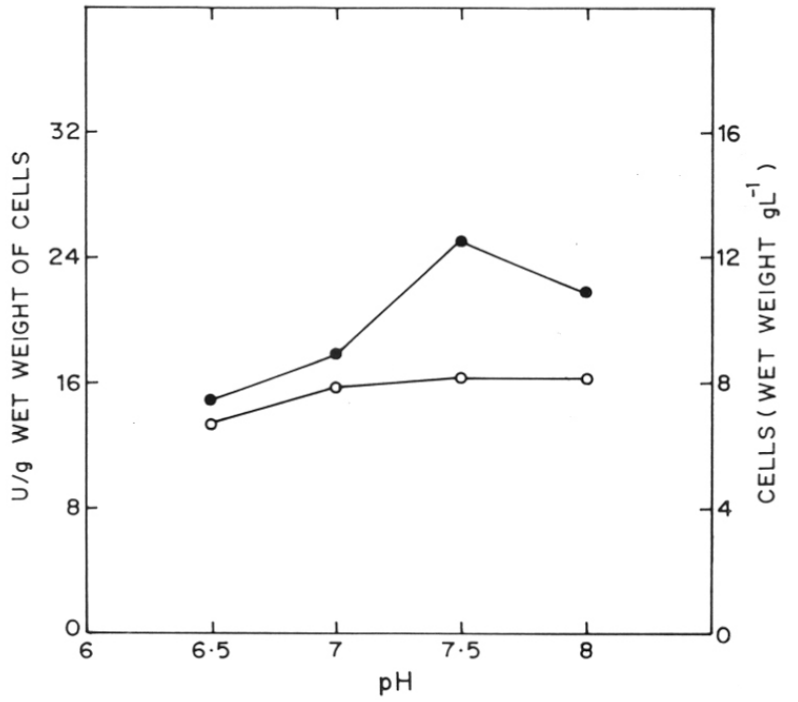


FIG. III·2

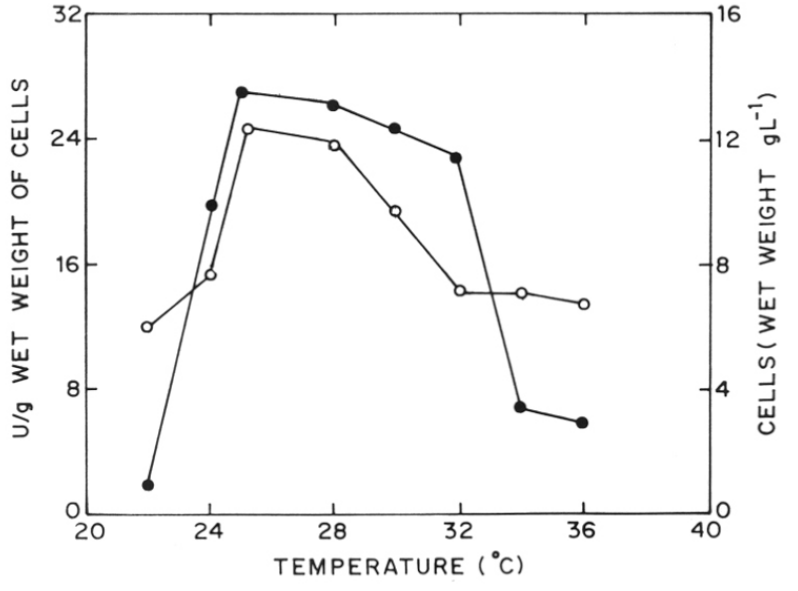


FIG. III·3

Fig III.2. Effect of initial pH on growth (-o-) and cell-bound penicillin V acylase (-●-) of *B. sphaericus*. CSL-minerals Medium A, 20 h at 25°C and 120 rpm.

Fig III.3. Effect of temperature on growth (-o-) and penicillin acylase activity (-●-) of *B. sphaericus*.. CSL-minerals Medium A, initial pH 7.5, 20 h and 120 rpm.

TABLE III.1: EFFECT OF VARIOUS LEVELS OF CSL ON GROWTH AND ON PENICILLIN V ACYLASE PRODUCTION BY *B. SPHAERICUS*. MEDIUM CONTAINED VARYING CONCENTRATIONS OF CSL AT INITIAL pH OF 7.5; YIELD AFTER 20 h AT 25°C,120 rpm. CELL PASTE HAD 20% DRY SOLIDS CONTENT

CSL (50% solids) $g.L^{-1}$	Cells (Wet weight) $g.L^{-1}$	Penicillin acylase activity $\mu.g^{-1}$ cell paste
20	6.0	18.6
40	10.2	21.2
60	10.8	14.0
80	11.8	5.0

TABLE III.2: EFFECT OF PHENOXYACETATE ON CELL YIELD AND ENZYME PRODUCTION BY *B. SPHAERICUS*; INITIAL pH 7.5, 25°C AT 120 rpm FOR 20 h

	Cells (wet wt.) gL^{-1}	Penicillin acylase activity $\mu mol.g^{-1}$ cell paste
Medium A	10.1	20.1
Medium A + Phenoxyacetic acid (1 $mg.ml^{-1}$)	8.3	17.5

be seen from Table III.1 that growth and enzyme production at 40 $g.L^{-1}$ of CSL (50% solids), which is similar to the amount used by Carlsen and Emborg (1981) gave optimal results.

The effect of phenoxyacetate on cell yield and enzyme production is shown in Table III.2. As seen from Table III.2, in the CSL-minerals medium, the enzyme is produced constitutively, phenoxyacetate (1 $mg.ml^{-1}$) supplementation inhibiting both growth and enzyme production.

The substitution of CSL in Medium A with complex organic nutrients like peptone, tryptone, casamino acids, malt extract and yeast extract resulted in markedly lowered growth and enzyme yield (Table III.3).

Deletion of individual mineral components of Medium A indicated that the following components had no significant effect on enzyme production as compared to the complete medium: Na_2HPO_4, KCl, $MgSO_4$, $CaCl_2$, $FeSO_2$ and $CuSO_4$ (Table III.4). These nonessential minerals were therefore deleted in the modified Medium B. Table III.5 summarizes the effect of deletion of all the nonessential mineral components on growth of *B. sphaericus* and on enzyme production.

Effect of supplementation of CSL-minerals medium with complex organic compounds on growth and enzyme production

Supplementation of Medium B with chitosan (1%), peptone (0.3%), tryptone (0.3%), casamino acids (0.3%) or yeast extract (0.3%) did not affect growth and enzyme

TABLE III.3 : GROWTH AND PENICILLIN ACYLASE PRODUCTION BY *B.SPHAERICUS* ON MINERAL SALTS MEDIUM CONTAINING COMPLEX ORGANIC NUTRIENTS; INITIAL pH 7.5; YIELD AFTER 20 h AT 25°C, 120 rpm.

Nutrient added to mineral salts	Final pH	Cell yield (Wet wt. $g.L^{-1}$)	Penicillin acylase activity ($\mu mol.min^{-1}g^{-1}$ wet cells)
Corn steep liquor (CSL (40 $g.h^{-1}$) (Medium A)	8.4	7.1	18.0
Peptone (Difco) (0.5%)	8.3	3.1	4.0
Peptone (Bengal Chem) (0.5%)	8.2	2.3	4.4
Peptone (Loba) (0.5%)	7.3	3.4	2.5
Peptone (Hi-media) (0.5%)	8.2	2.9	2.4
Peptone Sarabhai (0.5%)	8.3	2.9	2.7
Tryptone (0.3%	7.9	3.2	2.7
Casamino acids (0.3%)	7.5	1.6	0.4
Malt extract (0.3%)	7.2	0.4	0.3
Yeast extract (0.3%)	8.6	2.8	1.3

TABLE III.4: EFFECT OF DELETION OF INDIVIDUAL MINERAL COMPONENTS FROM CSL-MINERALS MEDIUM A. INITIAL pH 7.5; 20 h AT 25°C, 120 rpm

Component	Final pH	Cell yield (Wet wt. $g.L^{-1}$)	Penicillin acylase activity ($\mu mol.min^{-1}g^{-1}$ wet cells)
None	8.1	6.2	19.9
NaH_2PO_4	8.2	6.1	26.3
$(NH_4)_2SO_4$	8.2	6.2	7.7
KCl	8.0	6.0	18.6
$MgSO_4$	8.0	6.1	22.9
$CaCl_2$	7.8	6.7	25.0
$ZnSO_4$	7.8	6.3	16.0
$FeSO_4$	7.9	6.3	19.6
$MnCl_2$	7.9	7.5	13.0
$CuSO_4$	7.9	7.0	19.0
$CoCl_2$	7.8	7.1	13.6
H_3BO_3	7.8	7.1	12.4

TABLE III. 5: EFFECT OF DELETION OF NONESSENTIAL MINERALS FROM MEDIUM A. MEDIUM B CONTAINED CSL-MINERALS AS IN MEDIUM A EXCEPT THAT NaH_2PO_4, KCl, $MgSO_4$, $CaCl_2$, $FeSO_4$, $CuSO_4$ AND $(NH_4)_6Mo_2O_{24}$ WERE DELETED. INITIAL pH 7.5, 25°C AND 20 h AT 120 rpm.

Component	Final pH	Cell yield (Wet wt. $g.L^{-1}$)	Penicillin acylase activity ($\mu mol.min^{-1}g^{-1}$ wet cells)
Medium A	8.45	0.74	21.26
Medium B	8.48	0.71	22.91

TABLE III.6: EFFECT OF SUPPLEMENTING CSL-MINERALS MEDIUM B WITH WHEAT BRAN OR WHEAT BRAN EXTRACT. INITIAL pH 7.5, 25°C AND 20 h PERIOD OF GROWTH

Supplementation	Cell (dry wt.) $g.L^{-1}$	Penicillin V acylase activity	
		(U.g dry^{-1} cells)	(U.mg^{-1} protein)
Nil	1.80	100	0.17
Wheat bran (1%)	2.24	135	0.23
Wheat bran extract (equivalent to 0.5% bran)	1.94	119	0.20
Wheat bran extract (equivalent to 1.0 bran)	2.34	134	0.23
Wheat bran extract (equivalent to 2.0% bran)	2.40	137	0.23
Wheat bran extract Equivalent to 3.0% bran)	2.38	137	0.23

production.

Table III.6 summarizes the effect of supplementation of the modified medium from which the nonessential minerals had been deleted (Medium B) with wheat bran or wheat bran extract.

Since the complete separation of cells from the bran posed problems, and as bran extracts were also effective in enhancing cell and enzyme yields, the effects of varying levels of supplementation were determined only with the extract. Supplementation with extract equivalent to 1% whole bran enhanced cell yield approximately 30% and cell bound activity about 33% equivalent to more than 70% increase in yield of total activity. Increasing supplementation level upto 3% equivalent of whole bran increased yields only slightly.

The profiles of pH change, growth and penicillin V acylase production by *B. sphaericus* in CSL-minerals Medium B and the medium supplemented with wheat bran extract (1% equivalent to 1% whole bran) are set out in Fig. III.4. Carlsen and Emborg (1982) have reported production of 0.092 $U.ml^{-1}$ after 20 h fermentation in Medium A by *B. sphaericus* ATCC 14577 at 25°C and without control of pH. In the present study, Medium B yielded 0.18 $U.ml^{-1}$ in the absence of wheat bran supplementation and 0.32 $U.ml^{-1}$ in the presence of wheat bran extract after 20 h at 25°C. Enhancement in enzyme yield on supplementation of the fermentation medium with additives such as extract of wheat

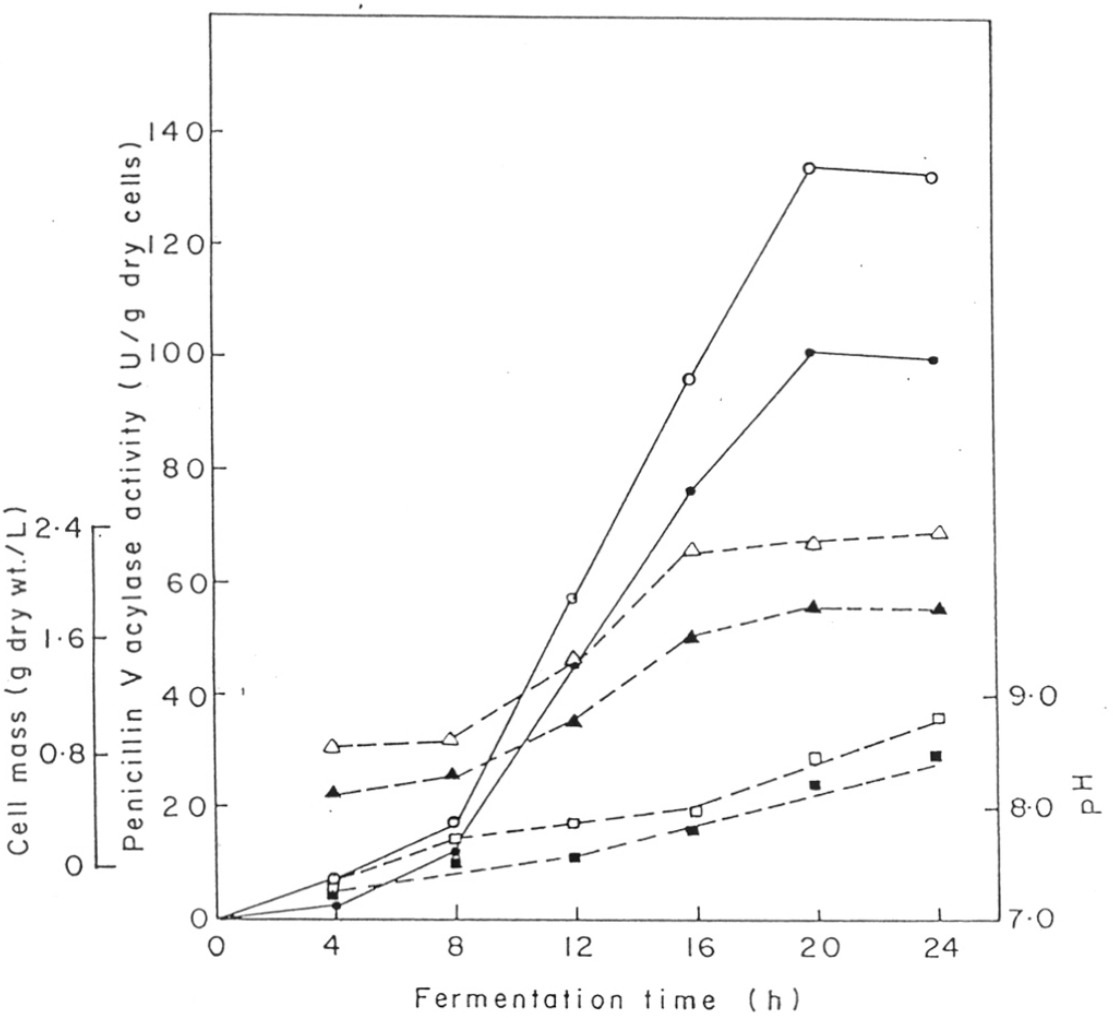


Fig. III·4 Comparison of penicillin V acylase production (-○●-), growth (-△▲-), and change in pH (-□■-) during fermentation of CSL-minerals medium by *B. sphaericus* in the presence (-○△□-) and absence (-●▲■-) of wheat bran extract equivalent to 1 % whole bran. Temperature, 25°C; 120 rpm

bran, a cheap agricultural by product, has industrial importance.

PART IV

PURIFICATION AND CHARACTERIZATION OF PENICILLIN V ACYLASE FROM BACILLUS SPHAERICUS

SUMMARY

B.sphaericus NCIM 2478 produces high levels of penicillin V acylase intracellularly. A purification procedure was developed for obtaining pure enzyme from *B. sphaericus*. The procedure involved sonication of cells, removal of nucleic acids by streptomycin sulphate, precipitation of inactive proteins by pH adjustment, fractional precipitation with ammonium sulphate, DEAE-Sephadex chromatography followed by a repeated hydrophobic-interaction chromatography on octyl-Sepharose. The overall yield was about 15%. The homogeneity of the preparation was established by polyacrylamide gel disc electrophoresis (PAGE), SDS-PAGE and isoelectric focussing (IEF), a single, sharp protein band being obtained in all three procedures. This is amongst the few reports of a homogeneous penicillin V acylase preparation and the first of the enzyme from *B. sphaericus*.

The molecular weight of the purified enzyme determined by gel filtration was 138,000 and the subunit M_r 35,000 by SDS-PAGE confirming the homotetrameric structure reported by Olsson and Uhlen (1986) for a partially purified *B. sphaericus* penicillin V acylase preparation. The pure enzyme had a specific activity of about 30 $\mu mol.min^{-1}.mg^{-1}$ protein in the hydrolysis of penicillin V at pH 5.8 and 40°C. The pI of the enzyme was found to be 4.8. The K_m of

the enzyme towards penicillin V was found to be 10 mM.

The optimum pH for the enzyme activity was found to be between 5.4-5.8 and the optimum temperature to be 70°C. The enzyme retained about 80% of its initial activity at 70°C for 2 h at pH 5.8. The enzyme showed relatively high specificity towards its substrate, penicillin V sulfoxide being hydrolysed at 15% and penicillin G at 7% of the rate of hydrolysis of penicillin V. Phenoxyacetamide was hydrolysed at 40% the rate of penicillin V hydrolysis. Ampicillin as well as several other semisynthetic penicillins and cephalosporins C and G were virtually resistant to hydrolysis.

Divalent metal ions such as Ca^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} inhibited enzyme activity while DTT and β mercaptoethanol markedly enhanced the rate of hydrolysis of penicillin V.

The enzyme did not catalyse the synthesis of penicillin V from 6-APA and the methyl ester of phenoxyacetic acid.

The amino acid composition of the purified protein and the amino acid sequence of three residues from the amino terminus were determined. Both the composition and sequence were at variance from the primary structure deduced by Olsson and Uhlen (1986) from the nucleotide sequence of the *pac* gene of *B. sphaericus*.

Gross secondary stuctural features of the enzyme molecule were deduced from its far UV-CD spectrum.

INTRODUCTION

Only a few penicillin V acylases have been obtained even in a partially purified state and even fewer have been characterized for their molecular properties. This has been discussed in the **GENERAL INTRODUCTION** to the Thesis. The present studies were, therefore, initiated to purify the enzyme from *Bacillus sphaericus* NCIM 2478 which had been found to produce the highest activity among several isolates and standard type cultures screened preliminarily. In contrast to the limited data available for penicillin V acylases, several penicillin G acylases from bacterial sources have been obtained pure and characterized extensively and in most cases shown to differ from the known penicillin V acylases in being heterodimers.

The purification of penicillin V acylase to homogeneity from *B. sphaericus* NCIM 2478 is described in this **Part of the Thesis.** Some of the kinetic and molecular properties of the enzyme are also described.

MATERIALS AND METHODS

Materials

DEAE-Sephadex A50, Sephadex G-200, Octyl-Sepharose CL-4B and Blue Dextran 2000 were obtained from Pharmacia, Sweden. Streptomycin sulphate, penicillin V sulphoxide, penicillin G, cephalosporin C, cephalosporin G were

obtained from Hindustan Antibiotics Ltd., Pune. Ampicillin, amoxycillin and carbenicillin were from HiMedia, India. Methicillin, cloxacillin, dicloxacillin, sodium dodecyl sulphate (SDS) and 1,4-dithiothreitol (DTT) were obtained from Sigma, USA. The gel filtration molecular weight markers such as carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase and β-amylase besides the kit of molecular weight markers for SDS-gel electrophoresis comprising α lactalbumin (M_r 14,200), trypsin inhibitor (M_r 20,100), trypsinogen (M_r 24,000), carbonic anhydrase (M_r 29,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), egg albumin (M_r 45,000) and bovine serum albumin (M_r 66,000) were also from Sigma.

Acrylamide, methylene bisacrylamide and N,N,N',N'-tetramethylethylenediamine were from Eastman Organic Chemicals, USA. N-Bromosuccinimide (NBS), ninhydrin, Coomassie Blue staining dyes, and Tris were from Sigma. β mercaptothanol (βME) was from Fluka, Switzerland. Phenoxyacetamide (procedure of Kao and Ma, 1930) and methyl ester of phenoxyacetic acid were synthesized in the laboratory.

Other chemicals were from sources described in the earlier Parts of the Thesis.

Methods

Isolation and Purification of Penicillin V acylase from *Bacillus sphaericus* NCIM 2478

Growth of Cells

B. sphaericus cells were grown in the modified CSL-minerals medium described in **Part III of the Thesis.** The cells were harvested after 20 h of growth by centrifugation at 6,000 x g for 30 min in a Sorvall RC-5B refrigerated centrifuge at 10°C. Cells were washed once on the centrifuge with 0.05 M potassium phosphate buffer, pH 7.5.

All subsequent steps were carried out at 4°C.

Cell-free Extract

Batches of about 25 g packed cells were worked up at a time. Cells were suspended in 0.05 M potassium phosphate buffer, pH 6.5, containing 10 mM EDTA (3.5 $ml.g^{-1}$ packed cells) and disrupted by sonication in an ice bath five times for 2 min each on a Biosonic III sonic oscillator (Bronwill Scientific Co., USA) at 20 KHz, 300 W. Cell debris was removed by centrifugation at 10,000 x g for 30 min and discarded.

Treatment with Streptomycin Sulphate

The cell-free extract was adjusted to a protein concentration of 10 $mg.ml^{-1}$ with 0.05 M potassium phosphate buffer, pH 6.5, containing 10 mM EDTA and treated under stirring with streptomycin sulphate to a final concentration of 0.7% (w/v). The stirring was continued for a period of 1 h, after which the precipitated nucleic acids were removed by centrifugation at 10,000 x g for 30 min.

pH Treatment

The pH of the clear supernatant from the streptomycin sulphate step was brought down to 4.5 with dropwise addition of 1 M acetic acid under stirring and centrifuged at 10,000 x g for 15 min. The precipitate which was inactive was discarded. The supernatant was treated by careful addition of 1 M Tris under stirring to readjust the pH to 6.5.

Ammonium Sulphate Fractionation

The supernatant from the pH treatment step was subjected to fractional precipitation by slow addition of finely ground ammonium sulphate under stirring. The fraction which precipitated at 0.3 saturation was removed by centrifugation at 10,000 x g for 30 min and discarded, the supernatant containing the activity was treated further with ammonium sulphate till 0.6 saturation. The fraction which precipitated between 0.3-0.6 saturation was collected by centrifugation at 10,000 x g for 30 min. The supernatant was discarded and the precipitate which contained the activity was dissolved in a minimum volume of 0.05 M potasssium phosphate buffer, pH 6.5, containing 10 mM EDTA and dialysed overnight with one change against 100 volumes of 0.05 M potassium phosphate buffer, pH 7.5, containing 10 mM EDTA. The dialysate was centrifuged and undissolved solids were discarded.

DEAE-Sephadex A50 Chromatography

The clear, centrifuged dialysate from the ammonium

A50 column (3 x 78 cm) which was previously equilibriated with 0.05 M potassium phosphate buffer, pH 7.5, containing 10 mM EDTA. The column was washed overnight with 120 ml of the phosphate-EDTA buffer containing 0.05 M KCl at a flow rate of 8 $ml.h^{-1}$. The washings had no enzyme activity. The enzyme was eluted with a linear concentration gradient of the buffer containing KCl (0.3 M - 1.5 M) using a Pharmacia Gradient Mixer with 250 ml each in the buffer reservoirs. Fractions (3-4 ml) were collected on an automatic fraction collector at a flow rate of 15 $ml.h^{-1}$. Aliquots of the fractions were assayed for enzyme activity. Fractions (250-400 ml) containing penicillin V acylase activity were pooled.

Octyl-Sepharose CL-4B Chromatography

The pooled fractions from DEAE-Sephadex chromatography were treated with solid ammonium sulphate to a final concentration of 24% (w/v) and loaded on the octyl-sepharose column (2.5 x 25 cm) previously equilibriated with 0.1 M potassium phosphate buffer, pH 6.0, containing 24% (w/v) ammonium sulphate. The column was washed with about 30 ml of 0.01 M potassium phosphate buffer, pH 6.0, containing 24% ammonium sulphate. The column was washed further, first with 150 ml of 0.01 M phosphate buffer, pH 6.0, containing 5% (w/v) ammonium sulphate and then with 150 ml of 0.01 M phosphate buffer, pH 6.0, containing 3% (w/v) ammonium sulphate. A flow rate of 8 $ml.h^{-1}$ was used for all the washings. All washings were inactive. The

activity was finally eluted with 0.001 M potassium phosphate buffer, pH 6.0. 2 ml fractions were collected at a rate of 8 $ml.h^{-1}$. The activity eluted between 80-110 ml.

The fractions containing activity were pooled, treated with ammonium sulphate to a final concentration of 24% (w/v) and the solution was reloaded on a preequilibriated octyl-Sepharose column (2.5 x 10 cm) and again washed successively first with the buffer containing 24% (w/v) ammonium sulphate (10 ml), then with buffer containing 5% (w/v) ammonium sulphate (80 ml) followed by buffer containing 3% (w/v) ammonium sulphate (80 ml). The enzyme was eluted finally with the 0.001 M potassium phosphate buffer, pH 6.0 buffer containing no ammonium sulphate. Fractions of 1.5-2.0 ml were collected at a flow rate of 6 $ml.h^{-1}$. The activity eluted in fractions between 30-50 ml. The fractions were pooled and concentrated by ultrafiltration using an Amicon unit with PM-10 membrane.

The purified enzyme was stable to storage at -20°C.

Enzyme Assay

Hydrolytic Activity

During enzyme purification, estimation of enzyme activity indicated enhancement in presence of ß-mercapto-ethanol (ßME) or DTT. The use of thiol compound in the assay system has been reported by Vandamme and Voets (1975) for the enzyme from *E. aroideae*.

Determination of the activity of penicillin V acylase in the hydrolysis of penicillin V was carried out with 2%

(w/v) potassium salt of penicillin V in 0.1 M sodium citrate buffer, pH 5.8, containing 5 mM DTT. The progress curves of hydrolysis were linear upto 20% hydrolysis of substrate. Activity determinations were done within this range. 6-APA formed was estimated with PDAB by the spectrophotometric method described earlier in **Part II of the Thesis.**

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

Synthetic Activity

The ability of purified penicillin V acylase to catalyse the reverse reaction was tested with phenoxyacetyl methyl ester and 6-APA at various pH values in the range 4.0-7.0 in steps of 1 unit. The reaction mixture contained 8 mg 6-APA and 40 mg phenoxyacetyl methyl ester in 1 ml 0.05 M citrate-phosphate buffer of the particular pH, pH adjustments being made with 1 N NaOH. The reaction mixture was maintained at 25°C and treated with enzyme (about 100 µg). Aliquots were removed after 1 h and 22 h, diluted and assayed for 6-APA to monitor synthesis of penicillin V.

Polyacrylamide Gel Electrophoresis (PAGE)

Disc electrophoresis of the native enzyme was carried out according to Davis (1964) using 7% polyacrylamide gel. Electrophoresis runs were carried out at room temperature with a current of 4 mA per tube applied till bromophenol blue, the tracking dye, had migrated till the bottom of the

gel.

Protein bands were visualized by staining the gels with Coomassie Brilliant Blue G-250 instant staining reagent (Reisner *et al.*, 1975). The reagent comprised 100 mg Coomassie Brilliant Blue G-250 dissolved in 70% perchloric acid (7 ml) and diluted with addition of 100 ml of glass distilled water, the solution being filtered before use.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in tube gels according to the procedure of Laemmli (1970). 7.5% polyacrylamide gels were prepared containing 0.1% SDS. The enzyme was dissociated by heating in a boiling water bath in presence of 1.25% (w/v) SDS and 1.25% (v/v) βME. The solution was cooled, made dense with sucrose and tracking dye was added. Sample (100 µl) containing 100 µg protein was loaded on the tube gel. Protein bands in tube gels were visualized by staining the gels with Coomassie Brilliant Blue R-250 in acetic acid and destaining with a mixture of methanol:acetic acid:water (4:1:5 volume).

Isoelectric Focussing (IEF)

Isoelectric focussing in polyacrylamide gel was performed according to the method of Vesterberg (1972). The electrofocusing in 10% polyacrylamide gel was carried out in duplicate in tube gels using Ampholine buffer of pH 3-10 range (Pharmacia Inc., Sweden). A constant current of 12 mA and an initial voltage of 600 V was applied for 18 h

at 10°C. At the end of the run, the gels were removed, one was stained for protein using Coomassie Brilliant Blue R-250 and subsequently destained using a mixture of methanol:acetic acid:water (4:1:5 volume). The other was cut into small segments which were sequentially put into tubes for elution with glass distilled water. The eluates of the gel slices were checked for pH and enzyme activity.

Molecular Weight Determination

Molecular weight determination of native enzyme was carried out according to the method of Andrews (1965) by gel filtration on a Sephadex G-200 column (1 x 100 cm) using β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000) and carbonic anhydrase (M_r 29,000) as marker proteins. 0.05 M Potassium phosphate buffer, pH 6.5, was used as elution buffer.

Marker proteins (2 mg each) were loaded separately on the column. Fractions of 2 ml each were collected at a flow rate of 6 $ml.h^{-1}$. The void volume (V_o) of the column was determined with Blue Dextran 2000 (M_r 2,000,000) and locating it by noting the midpoint of the absorbance maximum at 280 nm. The elution volume (V_e) for each marker protein was determined by measuring the buffer volume eluted upto the midpoint of the elution peak of the particular protein. The concentrated purified enzyme was loaded separately. A standard curve was plotted of V_e/V_o *versus* log molecular weight of standard proteins. The molecular weight of the enzyme was determined from the

standard curve obtained with the marker proteins.

Determination of Molecular Weight of Subunits

The subunit molecular weight of the enzyme was determined by running SDS-PAGE slab gel electrophoresis (Laemmli, 1970) using a mixture of prestained standard marker proteins comprising bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000),trypsin inhibitor (M_r 20,000) and α-lactalbumin (M_r 14,200). The enzyme sample was dissociated with 1% SDS in presence of 2.5% v/v β mercaptoethanol. The treated sample was heated in a boiling water bath for 3 min, cooled and made dense with addition of sucrose (10%) then mixed with bromophenol blue, the tracking dye. 5 µg of the treated enzyme was loaded in a well adjacent to the well in which the mixture of marker proteins was loaded. After the end of the electrophoresis run, protein bands were visualized by silver staining according to the procedure described by Rabilloud *et al.* (1988).

Amino Acid Analysis

The analysis was done by the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. Sample of purified enzyme (85 µg) was hydrolysed under standard hydrolysis conditions using 6 N HCl at 100°C for 22 h. Protection was done for cysteine, methionine and tyrosine using proper protecting reagents. An aliquot of the hydrolysate was

analysed on the Pharmacia-LKB Alpha Plus Amino Acid Analyser. Calculations of concentration of amino acid residues in nanomoles was provided by CCMB.

Estimation of Tryptophan

Tryptophan was determined by titration with N-bromosuccinimide (NBS) as described by Witkop (1961) and Spande and Witkop (1967). 400 µg purified enzyme in 0.05 M sodium acetate buffer, pH 4.0, was taken to dryness in vacuo and the residue dissolved in 1 ml of a saturated solution of urea in 0.05 M sodium acetate buffer, pH 4.0, taken in a 1 ml capacity cuvette (10 mm light path). The absorbance at 280 nm was read against a blank prepared under identical conditions but containing no enzyme. The solution was left till no further change in absorbance was observed. 10 µl aliquots of 10 mM NBS were added to both the cuvettes till no further decrease in absorbance at 280 nm was recorded. The number of trp residues (n) per mole subunit of enzyme was calculated from the relationship:

$$n = \frac{1.31 \times \Delta A_{280}}{5500 \times \text{molarity of subunit}}$$

where ΔA_{280} is the decrease in absorbance at 280 nm, 5500 is the molar extinction coefficient of tryptophan at pH 4.0, and 280 nm and 1.31 is an empirical factor based on oxidation of model tryptophan peptides (Patchornik *et al.*, 1958). M_r 35,000 was used for calculating molarity.

Amino Acid Sequence

The N-terminal amino acid sequence of three residues was also carried out by CCMB, Hyderabad. The sequencing was done on an Applied Biosystems-430 A instrument using Edman's procedure.

Circular Dichroism

Far UV-circular dichroism (CD) analysis was carried out at the Indian Institute of Science, Bangalore, on a Jasco J 500 A spectropolarimeter at 20°C in the range of 200-240 nm. The CD measurements were done in a 1 mm cell. Enzyme concentration was about 500 $\mu g.ml^{-1}$ of purified enzyme.

Mean residue ellipticity values were calculated using

$$[\theta]_{MRW} = \frac{\Psi(\lambda) \times MRW}{10 \times l \times C}$$

where MRW = mean residue weight (115)

l = path length in cms

C = concentration of proteins in $gm.ml^{-1}$

The analysis of the CD spectrum was done using unintelligent search method for minimum error making use of data from 15 proteins for estimates of gross secondary structural features.

RESULTS

Enzyme Purification

The results of purification of penicillin V acylase

from *B. sphaericus* NCIM 2478 in a typical batch are summarized in Table IV.1.

The specific activity of the purified enzyme varied from 28-34 $\mu mol.min^{-1}.mg^{-1}$ at pH 5.8 and 40°C. The final recovery was about 15% of the initial activity in the cell extract.

The purified enzyme was electrophoretically homogeneous and moved as a single band in PAGE, SDS-PAGE and in IEF as can be seen from Fig. IV.1 A & B and Fig. IV.2. IEF indicated a pI value of 4.8.

TABLE IV.I: SUMMARY OF PURIFICATION OF PENICILLIN V ACYLASE FROM *B. SPHAERICUS* NCIM 2478 (24 g PACKED CELLS)

Purification step	Volume ml	Protein $mg.ml^{-1}$	Activity $\mu mol.min^{-1}ml^{-1}$	Speific activity $\mu mol.min^{-1}ml^{-1}$	Yield %
Cell-free extract	230	14	4.6	0.33	100
Streptomycin sulphate	230	13	4.4	0.34	96
pH treatment	240	10	4.0	0.4	91.7
0.3 - 0.6 Saturation Ammonium Sulphate	46	22	14.6	0.64	63.5
DEAE-Sephadex	24	12	19	1.6	43.0
Octyl-Sepharose (I)	17.5	2	11.8	6.0	19.5
Octyl-Sepharose (II)	2.2	2.3	69.2	29.2	14.4

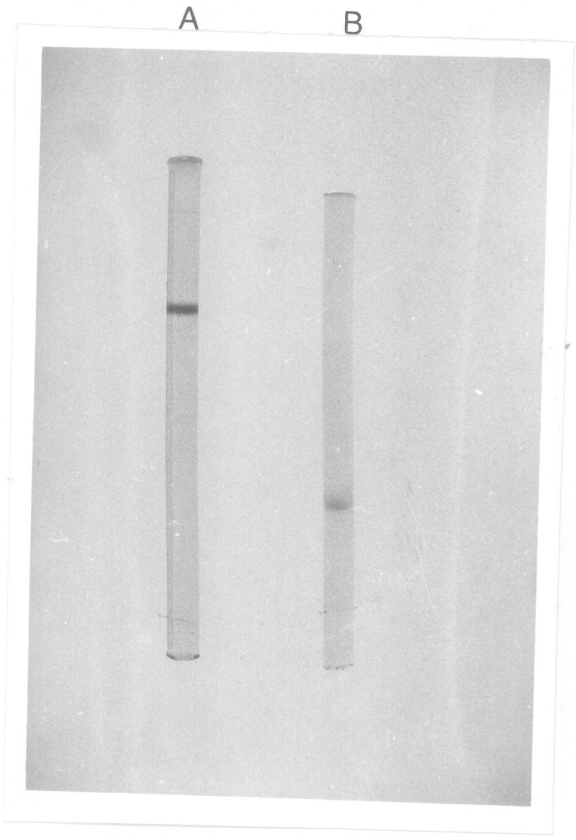


FIG IV. 1

Fig IV.1. (A) Polyacrylamide gel electrophoresis of purified penicillin V acylase from *B. sphaericus*.

(B) SDS-polyacrylamide gel electrophoresis of purified penicillin V acylase from *B. sphaericus*.

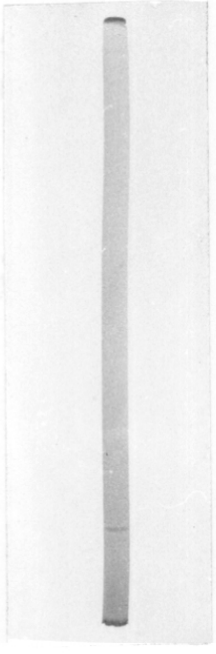


FIG IV.2

Fig IV.2. Isoelectric focussing of purified penicillin V acylase from *B. sphaericus* in polyacrylamide gel; pH range 3 - 10.

Properties of Purified *B. sphaericus* Penicillin V Acylase

The homogeneous enzyme preparation with specific activity of about 30 $\mu mol.min^{-1}.mg^{-1}$ was used in these studies.

Hydrolytic Reaction

pH optimum

The dependence of the enzyme activity on pH of the test medium for the cleavage of penicillin V is shown in Fig. IV.3 The pH-activity profile showed optimum activity between 5.4-5.8. The optimum value reported by Olsson *et al.* (1985) was pH 5.8. A pH optimum of 6.8 had been reported by Carlsen and Emborg (1982) at marked variance from the values obtained by Olsson *et al.* (1985) and in the present work. In the present work the activity was found to decrease sharply at values above pH 5.8, the value at 6.8 being only 15% of the maximum. A similar decrease had also been reported by Olsson *et al.* (1985).

Optimum temperature

The optimum temperature for hydrolytic cleavage of penicillin V was found to be as high as 70°C (Fig. IV.4). Carlsen and Emborg (1982) had reported optimal activity at temperatures of 40° - 60°C, the profile of the temperature-activity curve flattening in this range and dropping sharply above 60°C.

Temperature Stability

The temperature stability of the purified enzyme was determined by incubating a solution of the enzyme (100

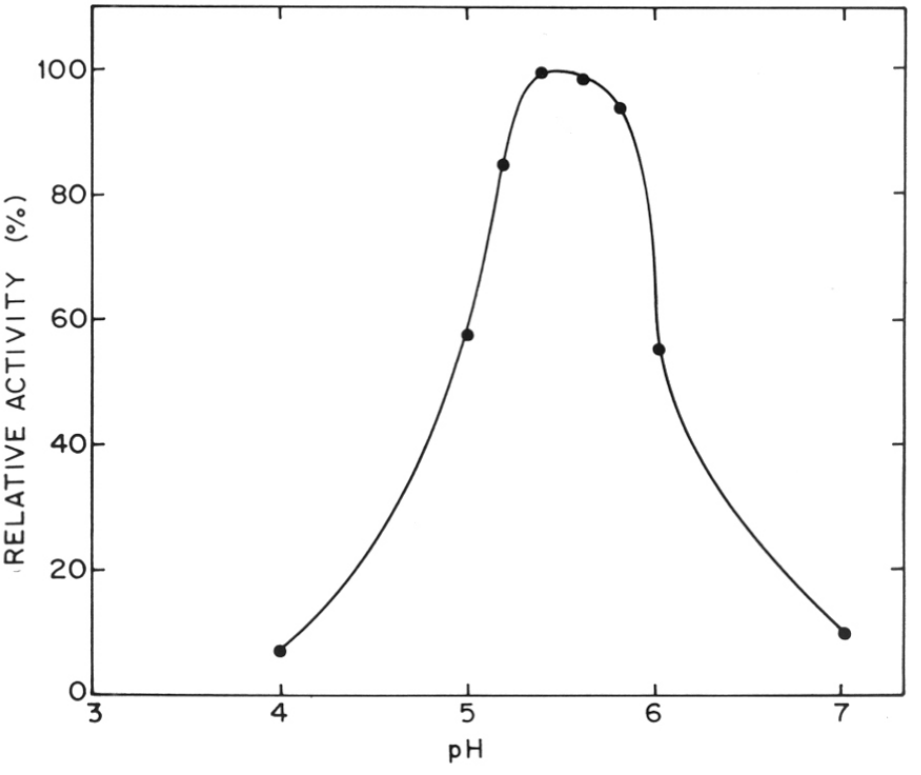


FIG. IV·3

Fig IV.3. Effect of pH on hydrolytic activity of *B. sphaericus* penicillin V acylase. Buffers used was 0.05 M citrate-phosphate buffers.

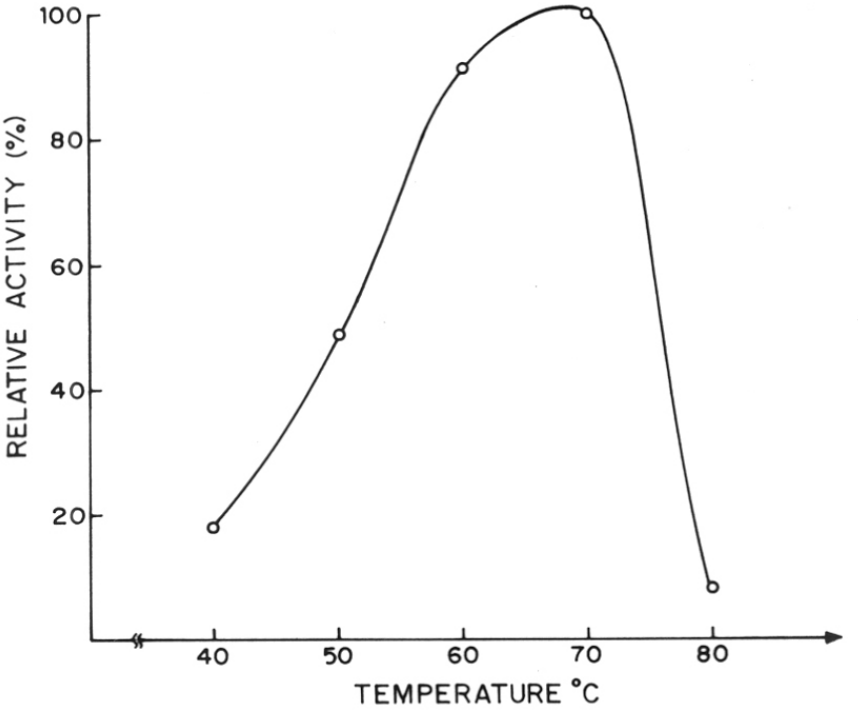


FIG. Ⅳ·4

Fig IV.4. Effect of temperature on hydrolytic activity of *B. sphaericus* penicillin V acylase. Assayed in 0.1 M sodium citrate buffer, pH 5.8. Values expressed relative to maximum activity.

$\mu g.ml^{-1}$) in 0.1 M citrate buffer, pH 5.8, at temperatures of 40°C - 80°C for a period of 2 h and assaying aliquots for activity at 30 min, 1 h and 2 h periods of heat treatment. Activity assays were carried out at 40°C. The stability data is shown in Fig. IV.5. It can be seen that even at 70°C almost 80% of initial activity was retained after a 2 h period of exposure. At 80°C, however, rapid inactivation lead to retention of only about 20% of the initial activity after 30 min and less than 5% after 2 h period of exposure.

Effect of Divalent Metal Ions, EDTA, DTT and βME

Effect of divalent metal ions and EDTA, DTT and βME were determined at 5 mM concentrations of the salts, 5 mM EDTA and 1-5 mM DTT and 70 mM βME. The enzyme (85 µg) was incubated at 25°C in the presence of test substances in 0.1 M sodium citrate buffer, pH 5.8 for 15 min before assay of enzyme activity in the test system containing no DTT or βME. The results are set out in Table IV.2.

It can be seen from Table IV.2 that except for Mg^{2+} all the other divalent metal ions tested inhibited activity to varying extents. EDTA had no effect. However 5 mM DTT and 70 mM βME enhanced the activities about 100%. Higher levels of the thiol compounds had no further activation effect. Similar effects were obtained when 5 mM DTT was included in the test system and no preincubation was done.

The test system used for activity determination in the purification procedure therefore contained 5 mM DTT.

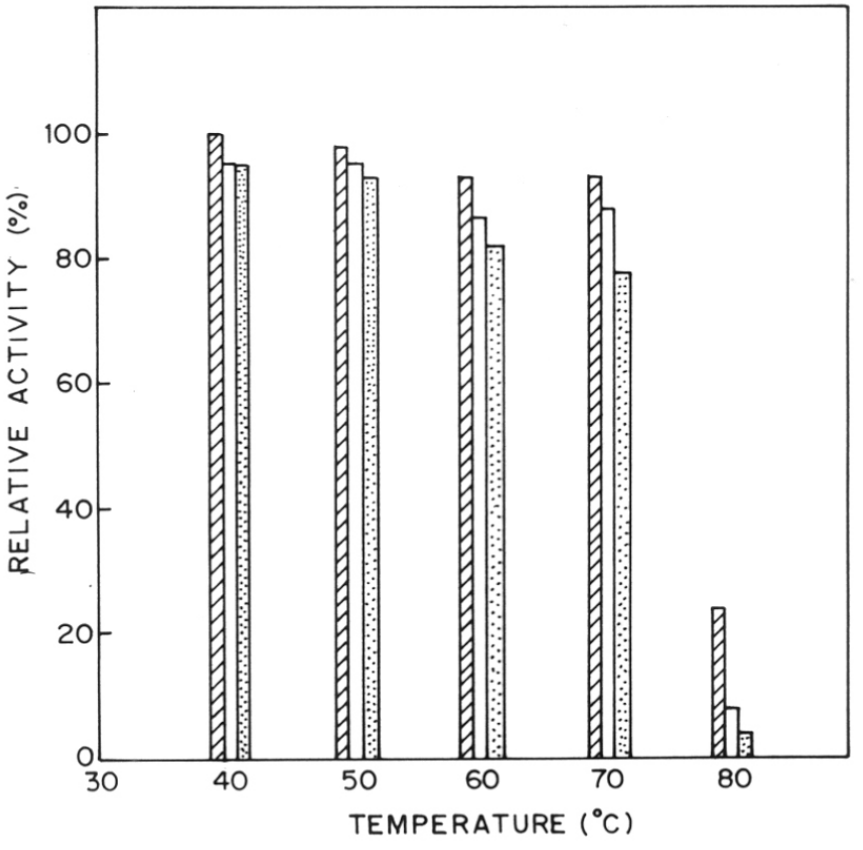

RELATIVE ACTIVITY (%)
100
80
60
40
20
0
30
40
50
60
70
80
TEMPERATURE (°C)

FIG. IV·5

Fig IV. 5. **Temperature stability of *B. sphaericus* penicillin V acylase.** The enzyme (100 µg ml^{-1}) in 0.1 M sodium citrate buffer, pH 5.8, was incubated at indicated temperatures. Aliquots were assayed at 25°C after 30 min (hatched columns), 1 h (open columns) and 2 h (dotted columns) exposures. Enzyme retained initial activity after 30 min at 40°C. Residual activities shown relative to the initial activity.

TABLE IV.2 : PENICILLIN V ACYLASE (85 µg) WAS PREINCUBATED FOR 15 MIN AT 25°C IN 1 ml 0.1 M SODIUM CITRATE BUFFER (pH CONTAINING THE SALTS (5 mM), EDTA, DTT or βME. ASSAY SYSTEM CONTAINED NO THIOL COMPOUNDS

Test Compound	Relative Rates of Penicillin V hydrolysis (%)
None	100
$MgSO_4$	95.30
$CuCl_2$	72.7
$CoCl_2$	64.2
$ZnSO_4$	71.2
$FeSO_4$	9.6
$CuSO_4$	12.4
EDTA	105.3.
DTT (1 mM)	140
DTT (5 mM)	195
βME (70 mM)	180

Kinetic Aspects

K_m was determined by Lineweaver-Burk double reciprocal plot of enzyme activity *versus* penicillin V concentration. The K_m for the hydrolytic cleavage of penicillin V was found to be 10 mM (Fig. IV.6). The substrate concentration range was 2-20 mM at pH 5.8 and 40°C. The K_m value of the enzyme is in agreement with the value of 11 mM reported by Olsson *et al.* (1985) but significantly lower than the value of 140 mM reported by Carlsen and Emborg (1982). Olsson *et al.* (1985) had used a pH of 5.8 and 37°C, while Carlsen and Emborg (1982) had carried out the experiment at pH 6.8 and 42°C. The differences in the pH values could contribute to the widely differing K_m values reported by Carlsen and Emborg (1982). The present studies were carried out at pH 5.8 and the K_m of 10 mM is in agreement with the value obtained by Olsson *et al.* (1985) at the same pH.

Substrate Specificity

The hydrolytic activities of the enzyme towards various penicillins and cephalosporins and phenoxyacetamide are shown in Table IV.3 All substrates were tested at a concentration of 10 $mg.ml^{-1}$, except phenoxyacetamide which was tested at 4 $mg.ml^{-1}$ concentration due to the lower solubility of the compound. Phenoxyacetamide hydrolysis was monitored by ninhydrin assay of the NH_3 released (Moore and Stein, 1948; 1954).

The enzyme exhibited a relatively high specificity for the side chain structure, penicillin G being hydrolysed at

TABLE IV.3 : RELATIVE RATES OF HYDROLYSIS OF B-LACTAM ANTIBIOTICS AND A SIDE CHAIN DERIVATIVE BY B.SPHAERICUS PENICILLIN V ACYLASE.

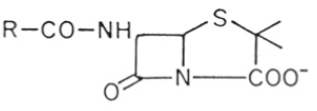
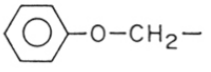
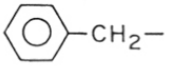
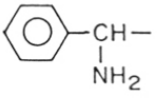
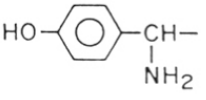
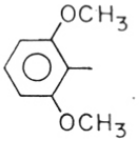
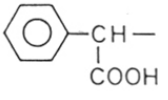
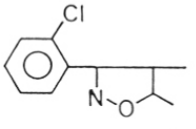
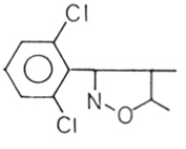
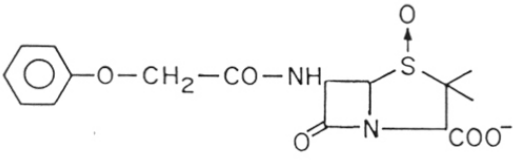
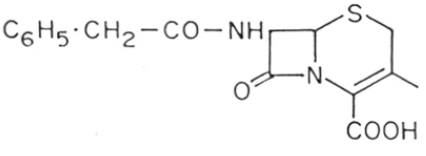
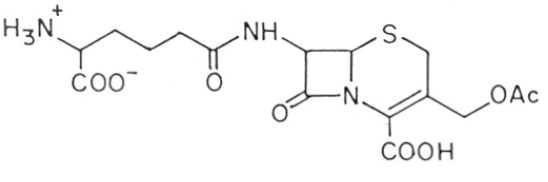
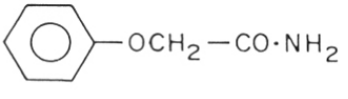
Substrate	Relative rates of hydrolysis (%)
PENICILLINS 	
Penicillin V R = 	100
Penicillin G R = 	6-7
Ampicillin R = 	0
Amoxycillin R = 	0
Methicillin R = 	0
Carbenicillin R = 	1
Cloxacillin R = 	0·8
Dicloxacillin R = 	2

TABLE IV.3 : RELATIVE RATES OF HYDROLYSIS OF B-LACTAM ANTIBIOTICS AND A SIDE CHAIN DERIVATIVE BY B.SPHAERICUS PENICILLIN V ACYLASE.

Substrate	Relative rates of hydrolysis (%)
PENICILLIN V SULPHOXIDE 	15
CEPHALOSPORINS	
Cephalosporin G 	1
Cephalosporin C 	0
Phenoxy acetamide 	40·7

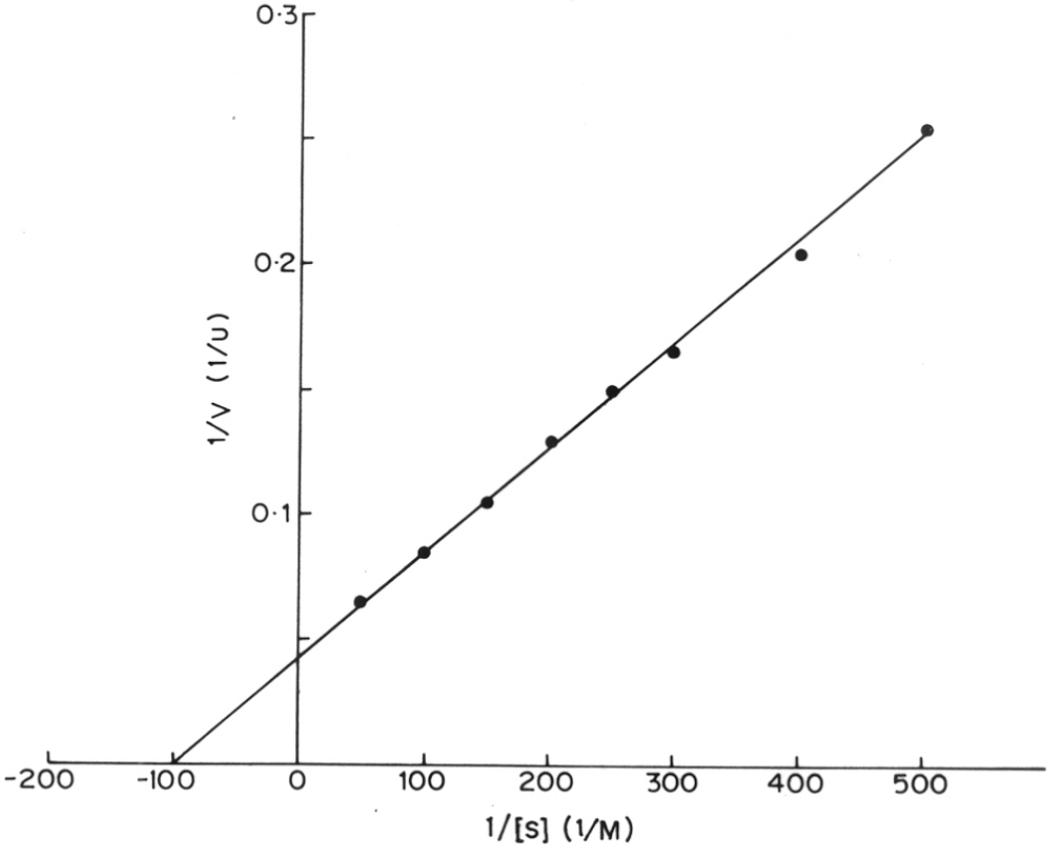


FIG. IV·6

Fig IV.6. Lineweaver-Burk plot for *B. sphaericus* penicillin V acylase. 0.1 M sodium citrate buffer, pH 5.8, 40°C.

less than 10% of the rate of hydrolysis of pencillin V. Ampicillin, amoxycillin, methicillin were resistant to hydrolytic cleavage, while carbenicillin, cloxacillin and dicloxacillin were hydrolysed at less than 2% of the rate of the preferred substrate. Amongst the cephalosporins, cephalosporin C which has a D-α-amino-adipyl side chain was resistant to cleavage. Cephalosporin G which contains the phenylacetyl side chain was cleaved at about 1% of the rate of cleavage of penicillin V. A derivative of the side chain of penicillin V, namely phenoxyacetamide, was cleaved at about 40% of the rate of cleavage of penicillin V. In the case of penicillin G acylase from *E. coli* the corresponding side chain amide derivative, phenylacetamide was found by Kutzbach and Rauenbusch (1974) to be hydrolysed 60% faster than penicillin G. Penicillin V acylase from *B. sphaericus* in contrast shows high rates of hydrolysis only when both the side chain of the substrate and the 6-APA moiety are present in the molecule. Derivatives of 6-APA nucleus also lowered the rate of hydrolysis, penicillin V sulphoxide being hydrolysed at 15% of the rate of hydrolysis of penicillin V. High specificity for both the side chain and the intact β lactam ring structure had been reported by Vandamme and Voets (1975) for the purified penicillin V acylase of *E. aroidae.*

Synthetic Reaction

No measurable disappearance of 6-APA was observed in

the test of the ability of the *B. sphaericus* penicillin V acylase enzyme to catalyse synthesis of penicillin V acylase from phenoxyacetyl methyl ester and 6-APA at pH values in the range 4-7. The methyl ester of phenoxyacetic acid was used in place of the free acid as the higher-energy form of the side chain acid had been shown to enhance synthetic rates in the case of penicillin G acylases (Okachi *et al.*, 1972; 1973a; Takesawa *et al.*, 1972; Nara *et al.*, 1972).

The enzyme from *E. aroideae* had also been reported by Vandamme and Voets (1975) to be inactive at pH range of 4-8 in the synthetic reaction. This is in contrast to the penicillin G acylases which catalyse the hydrolytic reaction at alkaline pH and the synthetic reaction at acidic pH (Bauer; . 1970; Self *et al.*, 1969; Nara *et al.*, 1971 b).

Molecular Weight Determination

Native protein

The results of determination of M_r of the native penicillin V acylase from *B. sphaericus* by gel filtration is shown in Fig. IV.7. The value estimated from the calibration curve obtained with molecular weight markers was 138,000.

Enzyme Subunits

Determination of the molecular weight of the enzyme subunits from the calibration curve obtained with standard molecular weight markers by SDS-PAGE is shown in Fig. IV.8

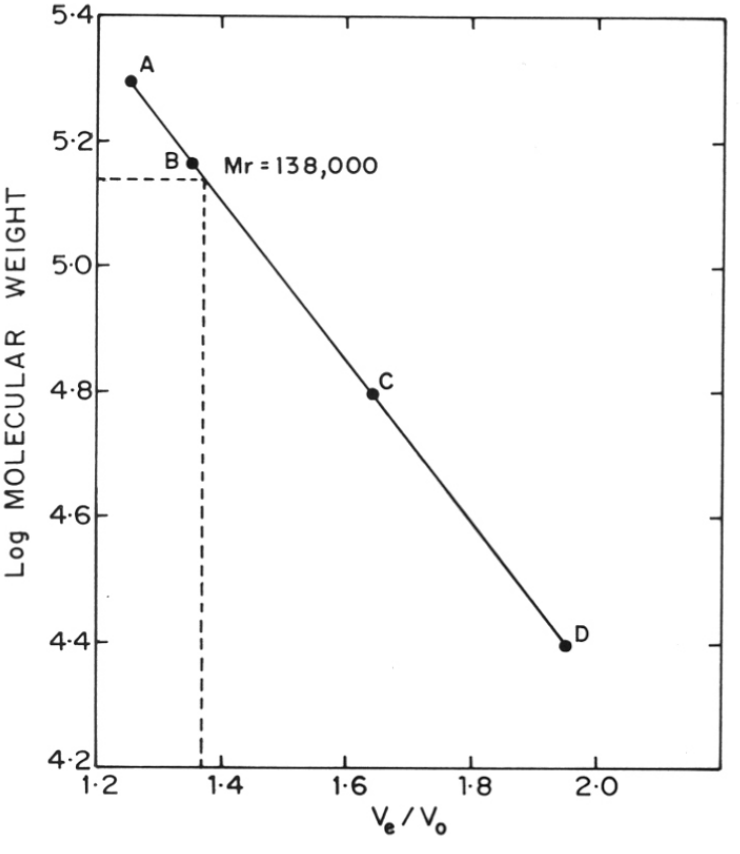

5·4
5·2
5·0
4·8
4·6
4·4
4·2
Log MOLECULAR WEIGHT
A
B
Mr = 138,000
C
D
1·2
1·4
1·6
1·8
2·0
Ve / Vo

FIG. IV·7

Fig IV.7. M_r determination of penicillin V acylase from *B. sphaericus* by gel filtration chromatography. Standard curve obtained with molecular weight markers:
ß-amylase, (M_r 200,000) (A);
alcohol dehydrogenase, (M_r 150,000) (B);
bovine serum albumin,(M_r 66,000) (C);
carbonic anhydrase (M_r 29,000) (D).
Graphical estimate of M_r shown by dotted lines.

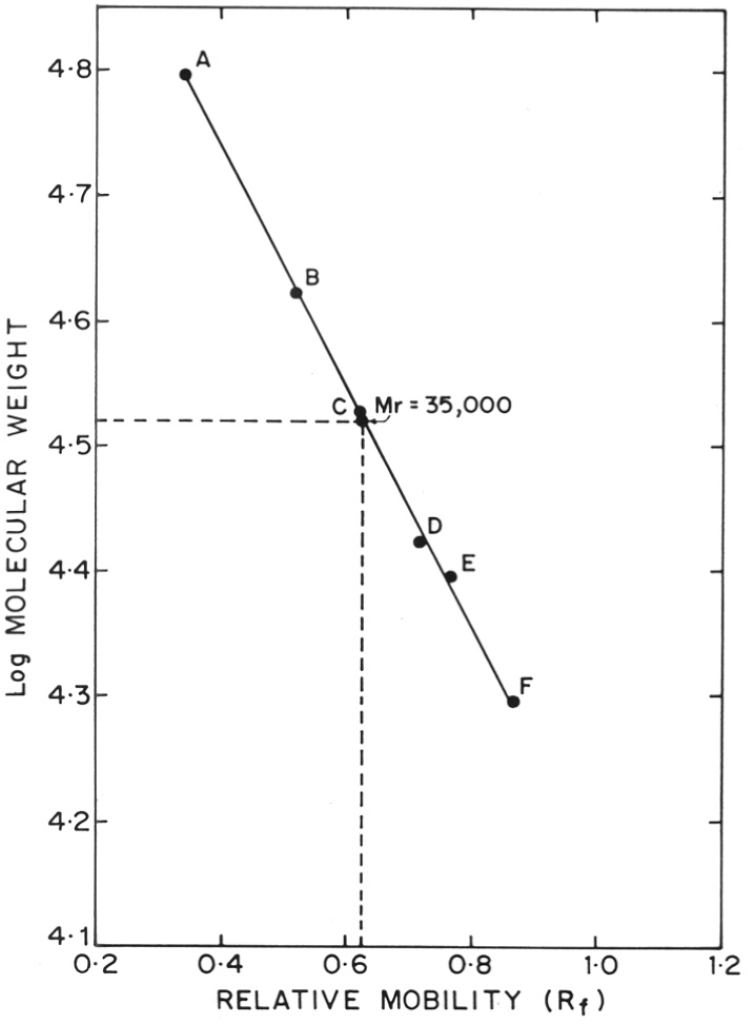

4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
Log MOLECULAR WEIGHT
A
B
C
Mr = 35,000
D
E
F
0·2
0·4
0·6
0·8
1·0
1·2
RELATIVE MOBILITY (Rf)

FIG. IV·8

Fig IV.8. **M_r determination of *B. sphaericus* penicillin V acylase subunit** by SDS-PAGE. Standard curve obtained with 7.5 % gel and molecular weight markers:

bovine serum albumin (M_r 66,000), (A);

egg albumin (M_r 45,000), (B);

glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000) (C);

carbonic anhydrase (M_r 29,000) (D);

Trypsinogen (M_r 24,000) (E);

lactalbumin (M_r 14,200) (F).

The M_r estimate from the standard curve was 35,000. SDS-PAGE had showed a single sharp band (Fig.IV,1B). These evidences would show that the enzyme is a homotetramer, the native enzyme molecule (M_r 138,000) having 4 identical subunits of M_r 35,000. Similar results had been reported by Olsson *et al.* (1985) from a preparation of about 95% purity.

Amino Acid Composition

In the estimation of tryptophan by NBS titration according to the method of Spande and Witkop (1967) A_{280nm} value of 0.162 was obtained for a 0.4 mg sample of protein. Based on subunit M_r value of 35,000, the estimated number of tryptophan residues/subunit = 3.3.

Table IV.4 shows the amino acid composition of the subunit of *B. sphaericus* penicillin V acylase in terms of number of residues/mole subunit. The data were calculated for a molecular weight of 35,000 and the analysis of tryptophan by NBS titration and the values obtained for the other amino acids by analysis of the acid hydrolysate of the protein. Values were approximated to the nearest integer, the differences in almost all cases being not more than $\pm$ 0.3. For the sake of comparison, the number of residues/subunit computed from the amino acid sequence deduced by Olsson and Uhlen (1986) is included in Table IV.4 The amino acid sequence was deduced by Olsson and Uhlen (1986) from the nucleotide sequence analysis of the *B. sphaericus* gene coding for its penicillin V acylase (see

TABLE IV.4: AMINO ACID RESIDUES CONTENT OF SUBUNIT OF PENICILLIN V ACYLASE FROM *BACILLUS SPHAERICUS*

Residue	Present work*	Olsson and Uhlen (1986)#
Gly	34	26
Ala	24	18
Val	21	24
Leu	28	28
Ile	18	24
Asp	{ }	20
	{39}	
Asn	{ }	22
Glu	{ }	13
	{31}	
Gln	{ }	12
Lys	17	19
Arg	11	10
His	4	4
Trp	3	3
Tyr	16	18
Phe	12	12
Pro	25	16
Ser	15	23
Thr	17	31
Cys	2	3
Met	5	12
Total	322	338
M_r	35,000 (assumed)	37,459 (predicted)

*Number of residues/subunit calculated from amino acid analysis of purified enzyme.
#Number of residues/subunit computed from amino acid sequence deduced from nucleotide sequence of gene encoding the enzyme.

Fig. I.2).

As can be seen from Table IV.4, marked differences are observed between the values obtained for several amino acid residues in the present work from actual amino acid analysis of the purified protein and that deduced from the nucleotide sequence of the *pac* gene.

Amino Acid Sequence

The sequence of 3 residues from the amino terminus penicillin V acylase showed the following. H_2N-Gln-Gly-Asn-. The amino terminal sequence of penicillin V acylase of *B. sphaericus* deduced by Olsson and Uhlen (1986) from the necleotide sequence of its gene shows NH_2-Met-Leu-Gly- as the three amino terminal residues and the -Gln-Gly-Asn- sequence is not seen in the entire sequence (Fig. I.2).

The reasons for the discrepancies in the amino acid analysis and the amino acid sequence data are not apparent except that purified protein had been used in the present work and the published report is from a sequence deduced from nucleotide sequencing. A possible reason for the differences in the determined and the deduced data is stated under **DISCUSSION**.

Secondary Structure

Gross features of the secondary structure of the enzyme were deduced from the far UV-CD spectrum at neutral pH (Fig. IV.9) revealed 5% α helix, 20% β sheets, 42% β turns and 33% random coil.

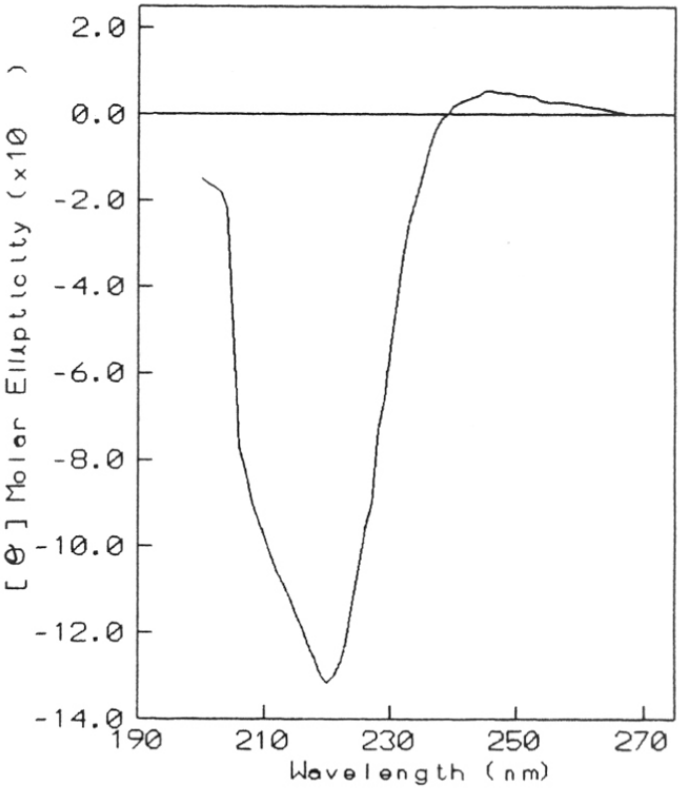

2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
[Θ] Molar Ellipticity (x10)
190
210
230
250
270
Wavelength (nm)

Fig. IV.9

Fig IV.9. The CD spectrum of purified native penicillin V acylase from *B. sphaericus*.

The CD measurements were performed in a 1 mm cell at enzyme concentration of 500 $\mu g.ml^{-1}$.

DISCUSSION

As stated in the GENERAL INTRODUCTION, very few reports in the literature claim the purification to homogeneity of penicillin V acylases. Amongst the few are the enzyme from *E. aroideae*, the purified enzyme showing two active bands in cellulose acetate electrophoresis and having a specific active of 39 $\mu mol.min^{-1}mg^{-1}$ (Vandamme and Voets, 1975) and the enzyme from the actinomycete *Streptoverticillium* which was shown to be homogeneous by PAGE, IEF and ultracentrifugation and which had a specific activity also of 39 $\mu mol.min^{-1}.mg^{-1}$ (Borisov *et al.*, 1986).

Olsson *et al.* (1985) have reported the partial purification of penicillin V acylase from *B. sphaericus* ATCC 14577 by a procedure involving ammonium sulphate fractionation (0.3-0.7 saturation), gel filtration through Sephadex G-200 and chromatography on DEAE-Sephadex A50. The reported specific activity of the purified preparation was 20.2 $\mu mol.min^{-1}.mg^{-1}$ at pH 5.8 and 37°C. The purified preparation reportedly showed a major band (> 95%) in SDS-PAGE and a major band corresponding to pI 4.8 in IEF. The overall recovery of the activity was 9% after a 15.4-fold purification.

Carlsen and Emborg (1982) had earlier reported the purification of the enzyme from *B. sphaericus* NCTC 10338 (ATCC 14577) by precipitation at 0.4 saturation ammonium sulphate, gel filtration through Sephadex G-200 and chromatography on DEAE-Sephadex A50 column. The purified

preparation had a specific activity of 20 $\mu mol.min^{-1}.mg^{-1}$ at pH 6.8 and 42°C. The preparation showed 8 separate immunoreactive fractions in cross immunoelectrophoresis. Recoveries of activity had not been reported by the authors.

In the present work, the enzyme has been purified from *Bacillus sphaericus* NCIM strain 2478. The NCIM strain 2478 was originally procured from NCIB (strain 9370) and corresponds to strain NCTC 10338 (ATCC 14577) used by the previous authors. The culture had been maintained routinely at NCIM with subculturing once a month over a period of 22 years by NCIM, Pune. The purification procedure in the present work involved 2 steps of hydrophobic chromatography on octyl Sepharose columns in addition to a pH adjustment step, ammonium sulphate fractionation and column chromatography on DEAE-Sephadex A50. The final step of purification yielded an enzyme with a specific activity of about 30 $\mu mol.min^{-1}.mg^{-1}$, a value significantly higher than 20 $\mu mol.min^{-1}.mg^{-1}$ reported by the earlier workers. The activity yield obtained in the present work (15%) is also markedly higher than that reported by Olsson *et al.* (1985). The homogeneity of the purified enzyme obtained in the present work had been established by PAGE, SDS-PAGE and IEF, single protein bands being obtained in all the procedures.

The activity of the purified enzyme obtained in the present work was enhanced markedly by thiol compounds,

approximately 100% enhancement being observed in presence of DTT (5 mM) or βME (70 mM). The involvement of an essential cysteine residue in the enzyme, reported in the next Part of the Thesis, would explain the activation in the presence of thiol compounds, possibly by maintaining the reduced state of the essential cysteine. Since EDTA had no effect on the activity of the enzyme, scavenging of divalent metals by the thiol compounds may not be involved.

The pure enzyme also showed high temperature stability, 2 h exposure at 70°C reducing the specific activity by about 20% only. The high temperature stability is probably due to the low α helix content (5%) and the high content β-sheet (20%) and β-turns (42%) deduced from the far UV-CD spectrum at neutral pH. This has been suggested by Marquez *et al.* (1988) as the possible reason for the structural stability of penicillin G acylase from *K. citrophila* which was shown to have 11% α helix, 44% β-sheet and 11% β-turns from its CD spectrum.

As stated earlier, the amino acid composition and amino acid sequence of the N-terminus of the purified enzyme determined in the present work is markedly different from the data deduced by Olsson and Uhlen (1986) from the nucleotide sequence of the *pac* gene. In the deduced sequence, the codon used to initiate translation and the codon preference analysis were based as stated by the authors, on assumptions which were strongly suggestive but not conclusive. The variations could not be due to strain

differences as the *B. sphaericus* NCIM 2478 is a type culture which was obtained originally from NCIB as a strain equivalent to NCTC 10338 (ATCC 14577). *B. sphaericus* ATCC 14577 has been used in the studies by Olsson and Uhlen (1986).

PART V

STUDIES ON CHARACTERIZATION OF ACTIVE SITE RESIDUES OF BACILLUS SPHAERICUS PENICILLIN V ACYLASE BY CHEMICAL MODIFICATION

SUMMARY

Incubation of penicillin V acylase from *B. sphaericus* with the lysine-modifying reagent, 2,4,6-trinitrobenzene sulphonic acid (TNBS) resulted in inactivation. The kinetics of inactivation indicated the presence of a single essential moiety per active unit of the enzyme. Penicillin V fully protected the enzyme against inactivation while phenoxyacetate and 6-APA did so partially. A comparison of the far-UV CD spectra of the modified and the native enzymes showed that the modification caused no changes in gross conformation.

Titration of lysyl residues with acetic anhydride at pH 6 showed a linear dependence of the extent of inactivation on the number of lysyl groups modified, complete inactivation resulting from the modification of 4 lysine residues per mole of the homotetrameric enzyme.

Penicillin V protected the enzyme partially against acetic anhydride inactivation.

The results indicate that a putative single essential lysine residue is present at or near the active-site of each subunit of the homotetrameric enzyme. The enzyme was also inactivated by the serine-modifying reagent phenylmethanesulphonyl fluoride (PMSF). Kinetics of the inactivation indicated the presence of a single essential serine per active unit of enzyme. Penicillin V partially

protected the enzyme against inactivation while phenoxyacetate decreased inactivation to a much lower extent, indicating the possible presence of the serine residue at the catalytic site.

The enzyme was inhibited by the cysteine-modifying reagents, p-hydroxymercuric benzoate (pHMB) at pH 4.5 and iodoacetamide at pH 7.0. Plots of the logarithm of residual activity *versus* time of contact with pHMB as the inhibitor (0.5 - 5 µM) showed rapid initial linear decrease followed by a slower linear phase. The order of the initial reaction from uncorrected values indicated that approximately 1.25 µmoles modifier had reacted with each active unit of the enzyme in the initial stage of inactivation. Both penicillin V and phenoxyacetic acid partially protected against inactivation by pHMB. Far-UV CD measurements showed that modification with pHMB had not caused any change in the gross structural features of the native enzyme.

INTRODUCTION

No information is available in the literature on the mechanism of action and the active-site residues of penicillin V acylases. In contrast, penicillin G acylases have been investigated in greater detail in regard to these aspects. The various evidences for the involvement of an acyl-enzyme intermediate in the mechanism of penicillin G acylases have been summarized in the **GENERAL INTRODUCTION** of the Thesis. These include the kinetic evidences pre-

sented by Konecny (1981 a,b) and the complete inactivation of the purified penicillin G acylases from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgeri* (Daumy *et al.*, 1985b) and *K. citrophila* (Martin *et al.*, 1991) by equimolar amounts of phenylmethanesulphonyl fluoride (PMSF). The essential hydroxyamino acid was identified as the serine located at the N-terminus of the large ß-subunit in the heterodimeric (αß) enzymes of *E. coli* (Slade *et al.*, 1991) and *K. citrophila* (Martin *et al.*, 1991) by site-directed chemical mutagenesis. Kinetic evidences for the presence of an arginine residue at or near the active site of *E. coli* penicillin G acylase was provided by Prabhune and SivaRaman (1990). In the case of the homodimeric ampicillin acylase from *P. melanogenum*, Kim and Byun (1990 b) have reported the presence of two putative essential histidine residues per subunit of the enzyme.

In the present work, chemical modification studies were carried out to probe essential amino acid residues in penicillin V acylase from *B. sphaericus*.

MATERIALS AND METHODS

Materials

2,4,6-Trinitrobenzene sulphonic acid (TNBS) was obtained from BDH, India. Acetic anhydride, N-acetylimidazole, phenylmethanesulphonyl fluoride (PMSF), diethylpyrocarbonate, iodoacetamide and citraconic anhydride were obtained from Sigma, USA. Absolute alcohol was from Fluka, p-chloromercuribenzoate was obtained from

National Chemical Laboratory, Pune.

Methods

The enzyme was purified to homogeneity as described in **Part IV of the Thesis.**

Preliminary experiments to test the effects of some amino acid specific reagents on penicillin V acylase from *B. sphaericus* were carried out. Treatment with following reagents showed no effect on enzyme acticity: diethylpyrocarbonate (500 µM) at pH 6.5 for modification of histidine (Gold and Segal, 1964), N-acetylamidazole (1 mM) at pH 7.5 for tyrosine (Riordan *et al.*, 1965). Inactivation was observed, however, when the enzyme was treated with 2,4,6-trinitrobenzene sulphonic acid (TNBS) at pH 8.0 for modification of lysine, PMSF for serine at pH 7 and p-hydroxymercuribenzoate (pHMB) at pH 4.5 for cysteine. The effects of these reagents were therefore studied in greater detail.

Treatment with Lysine-Modifying Reagents

2,4,6-Trinitrobenzene sulphonic acid (TNBS) was used as the lysine-specific modifying reagent at pH 8.0, essentially according to the procedure of Habeeb (1966). The purified penicillin V acylase from *B. sphaericus* was incubated in the dark at 25°C with TNBS (0-5 mM) in 0.05 M potassium phosphate buffer, pH 8.0. Aliquots were withdrawn at various time intervals for assay of residual hydrolytic activity of the enzyme. The enzyme incubated without TNBS served as control.

Titration of Lysine Residues with Acetic Anhydride

Acetylation of amino groups of penicillin V acylase was carried out according to the method of Frankel-Conrat (1957). The enzyme (800 µg) in 4 ml 0.05 M acetate buffer, pH 6.0, was treated at room temperature with a total volume of 20 µl of acetic anhydride. The reagent was added in successive instalments, taking care to maintain the pH at 6.0 with 1 N NaOH. After every addition of acetic anhydride, a sample was removed and assayed for residual activity as well as for the number of amino groups modified. Enzyme incubated under identical conditions without addition of acetic anhydride served as control. The total number of amino groups in the native and the modified enzyme was estimated by the method of Habeeb (1966).

Treatment with Serine-Specific Reagent

For chemical modification of penicillin V acylase a 10 mM stock solution of phenylmethanesulphonyl fluoride (PMSF) in dry ethanol was used. The treatment conditions were essentially similar to those used by Kutzbach and Rauenbusch (1974), Slade *et al.*, (1991) and Gold and Fahrney (1964). The purified penicillin V acylase was incubated at 25°C in 0.05 M potassium phosphate buffer, pH 7.0, and treated with PMSF (0 - 400 µM). Aliquots were withdrawn at stated intervals of time for enzyme assay. The reaction mixture containing no PMSF served as control.

Effect of Stoichiometric Amounts of PMSF on Enzyme Activity

The enzyme was incubated for 30 min at 25°C in 0.05 M potassium phosphate buffer, pH 7.0, in presence of 1.5 - 400-fold molar excess of PMSF. Molar equivalent was calculated on M_r 138,000 of the enzyme (**Part IV of the Thesis**).

Treatment with Cysteine-Modifying Reagent

The reagent p-hydroxymercuribenzoate (pHMB), was prepared according to the procedure described by Boyer (1954). p-Chloromercuribenzoate was suspended in water and treated with sufficient 1 N NaOH to dissolve the bulk of the material and the small amount of insoluble matter was removed by centrifugation. The solution was precipitated with 1 N HCl, centrifuged and the precipitate again dissolved in dilute NaOH. The product was precipitated twice more, washed repeatedly with distilled water on the centrifuge and dried *in vacuo* over P_2O_5. A weighed sample of the purified preparation was suspended in water, treated with NaOH to pH 11 and the pH adjusted to 4.5 with HCl, diluted with an equal volume of 0.1 M sodium acetate buffer, centrifuged and the clear supernatant decanted, assayed spectrophotometrically (Boyer, 1954) and diluted with 0.05 M sodium acetate buffer, pH 4.5, to 100 µM concentration (stock solution).

The purified enzyme was incubated at 25°C with pHMB (0 - 5 uM) in 0.05 M sodium acetate buffer, pH 4.5. Aliquots were withdrawn for assay of enzymatic activity.

Kinetics of Inactivation

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

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

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

where K is the second-order rate constant, (M) the molar concentration of the modifier and n is the average order of the reaction with respect to the concentration of the modifier. K_{app} can be calculated from a semi-logarithmic plot of the enzyme activity remaining as a function of time.

The order of the reaction (n) can be obtained experimentally by determining K_{app} at a number of concentrations of the modifier. A plot of log K_{app} *versus* log (M) should give a straight line with a slope equal to n, where n is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme - inhibitor complex (Levy *et al.*, 1963; Ramakrishna and Benjamin, 1981; Marcus *et al.*, 1976).

Protection Against Inactivation

Protection of the enzyme against inactivation by TNBS, PMSF and pHMB was tested with the substrate, penicillin V (50 mM final concentration), and the products of its hydrolytic cleavage, phenoxyacetic acid (50 mM final concentration) and 6-APA (25 mM final concentration).

The compounds were tested at the stated final concentrations and were added immediately before the addition of the modifying reagents. The compounds were tested at the pH used for the modification using the appropriate buffer system.

Enzyme Assay

Enzyme assay for the relative activities for native and modified enzyme was carried out in absence of any added thiol compounds. The assay system was similar to the one described in **Part III of the Thesis.**

Protein was assayed as described earlier according to the procedure of Lowry *et al.* (1951).

Circular Dichroism (CD)

Far UV-CD measurements at pH 7.0 were carried out for monitoring any gross conformational changes brought about by the modification. The determinations were carried out at the Indian Institute of Science, Bangalore, as described in **Part IV of the Thesis.**

RESULTS

Inactivation of *B. sphaericus* penicillin V acylase by the lysine-modifying reagent TNBS

Incubation of the penicillin V acylase from *B. sphaericus* at pH 8 with TNBS inactivated the enzyme rapidly. Plots of the logarithm of residual activity *versus* time of contact with the reagent were straight lines throughout the test period of 20 min at all the concentrations of modifier that were used, indicating that

the rates of inactivation followed pseudo first-order kinetics (Fig. V.1). The residual enzyme activity was about 25% of the initial activity with 5 mM reagent after 20 min exposure. In the absence of the modifier, no loss in activity was observed.

The reaction order (n) with respect to the lysine modifying reagent was determined from the plot of the logarithm of the apparent first-order rate constant, K_{app}, *versus* the logarithm of the reagent concentrations (Fig. V.1 inset) and the value of n = 1.1 for TNBS indicated that the loss of enzyme activity resulted from the reaction of one lysine per active unit of enzyme.

Protection Against Inactivation by TNBS

Table V.1 summarises the data on protection of *B. sphaericus* penicillin V acylase against inactivation by TNBS.

TABLE V.1: PROTECTION OF *B. SPHAERICUS* PENICILLIN V ACYLASE AGAINST INACTIVATION BY TNBS. Test compounds were added immediately before the addition of the modifying reagent. Enzyme (70 $\mu g.ml^{-1}$) was treated at 25°C and pH 8.0 as indicated in the Table and samples were withdrawn after 20 min for assay of enzyme activity.

Treatment	Enzyme activity (% Initial activity)
None	100
TNBS (5mM)	24
Penicillin V (50 mM) + TNBS (5 mM)	98
Phenoxyacetate (50 mM) + TNBS (5 mM)	60
6-APA (25 mM) + TNBS (5 mM)	50

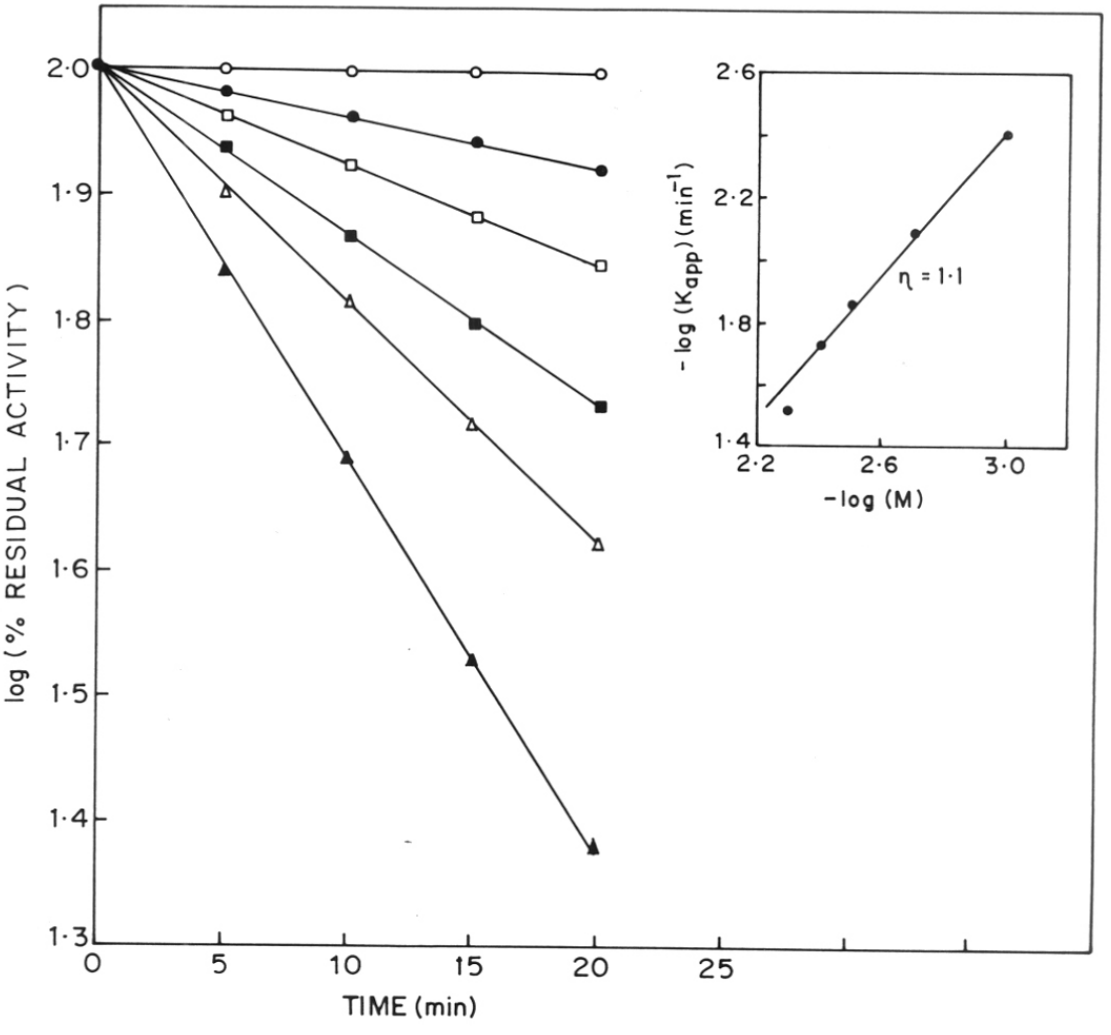

log (% RESIDUAL ACTIVITY)
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
0
5
10
15
20
25
TIME (min)
- log (Kapp) (min-1)
2·6
2·2
1·8
1·4
η = 1·1
2·2
2·6
3·0
- log (M)

FIG. V·1

Fig V.1. Inactivation of penicillin V acylase from *B. sphaericus* by lysine-modifying reagent TNBS (2,4,6-trinitrobenzenesulphonic acid).
Concentration of 2,4,6-trinitrosulphonic acid: 0 mM (-o-), 1 mM (-●-), 2 mM (-□-), 3 mM (-■-), 4 mM (-△-), 5 mM (-▲-).
The enzyme 70 $\mu g/m^{-1}$ was incubated in the dark at 25°C in 50 mM potassium phosphate buffer, at pH 8.0, and varying concentrations of the inhibitor.
Inset: Determination of the order of the reaction with respect to 2,4,6-trinitrobenzene sulphonic acid.

Penicillin V (50 mM) protected the enzyme fully, almost 98% of the initial activity being retained after 20 min. Protection by phenoxyacetate and 6-APA was partial 60% and 50% initial activity, respectively, being reatined compared to 24% in the absence of the test compounds.

The results would indicate that the reactive lysine is located at or near the active site of the enzyme since the substrate and products protect against inactivation by the lysine specific reagent.

Titration of Lysine Residues using Acetic Anhydride

The enzyme was inactivated by both citraconic anhydride in 1,4-dioxane at pH 8 as well as by acetic anhydride at pH 6, successive additions of aliquots of both modifiers progressively inactivating the enzyme till about 5% residual activity. Titration of available amino groups was carried out with acetic anhydride (Oppenheimer *et al.* (1966).

The lysine residues in *B. sphaericus* penicillin V acylase were acetylated with the stepwise addition of acetic anhydride at pH 6 as described under **Materials and Methods**. The total number of available lysine residues was estimated according to the method of Habeeb (1966). The plot of residual activity *versus* number of lysine ϵ-amino groups modified was linear throughout the titration upto about 20% residual activity(Fig. V.2). Extrapolating to zero activity gave a value of about 4 lysine residues per mole of enzyme. The data would support the kinetic

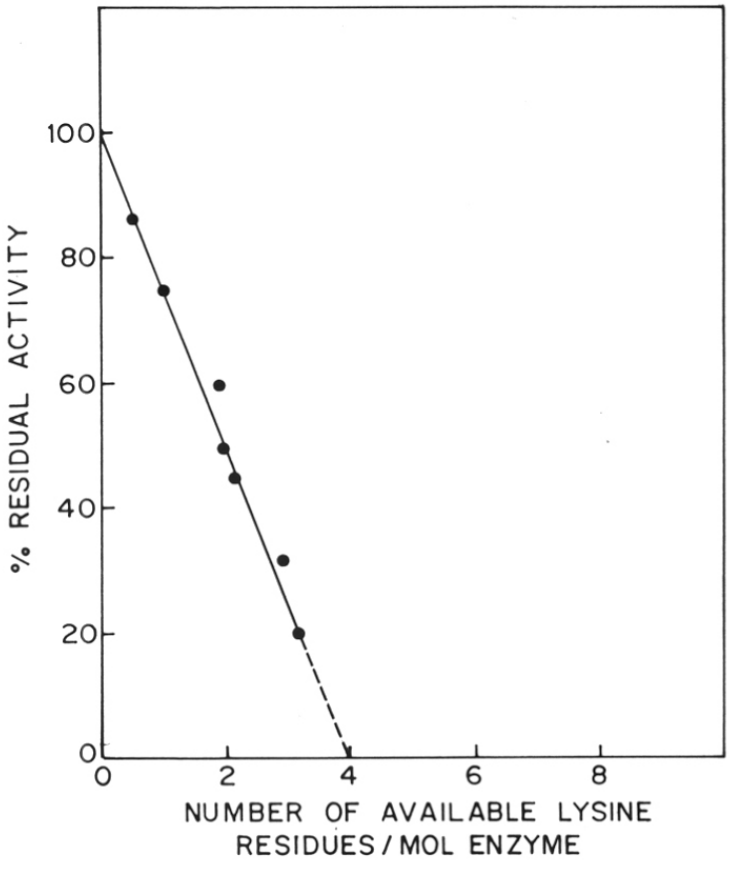

% RESIDUAL ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
NUMBER OF AVAILABLE LYSINE
RESIDUES / MOL ENZYME

FIG. V·2

Fig V.2. Titration of lysine residues with acetic anhydride.

The lysine residues of *B. sphaericus* penicillin V acylase were acetylated with stepwise addition of acetic anhydride as described in Materials and Methods.

evidence that the homotetramer has 4 essential lysines, one per subunit.

Protection against inactivation by acetic anhydride was tested by addition of aliquots of acetate anhydride to 266 µg enzyme in 1 ml 0.05 M potassium phosphate buffer at 25°C, both in the absence of penicillin V and the presence of 500 mM penicillin V. The amount of acetic anhydride added was sufficient to give 21.6% residual activity in absence of substrate. The corresponding value in presence of penicillin V was 74.9% residual activity. Estimations of the number of NH_2-groups modified indicated that 0.92 groups/mole holoenzyme were modified in presence of substrate, conditions under which about 75% initial activity was retained. The result is in agreement with the titration data in the absence of the substrate (Fig. V.2).

C.D. Measurements

Far-UV CD spectral analysis of the native penicillin V acylase from *B. sphaericus* and of the enzyme modified by TNBS with about 20% residual activity is depicted in (Fig. V.3). Modification did not result in any detectable spectral change indicating that the inactivation by the reagent was not caused by any non-specific modification leading to changes in gross conformation.

Inactivation of *B. sphaericus* Penicillin V Acylase by Serine Modifying Reagent

Phenylmethanesulphonyl fluoride (PMSF) rapidly inactivated the enzyme at pH 7.0 (Fig. V.4). The enzyme

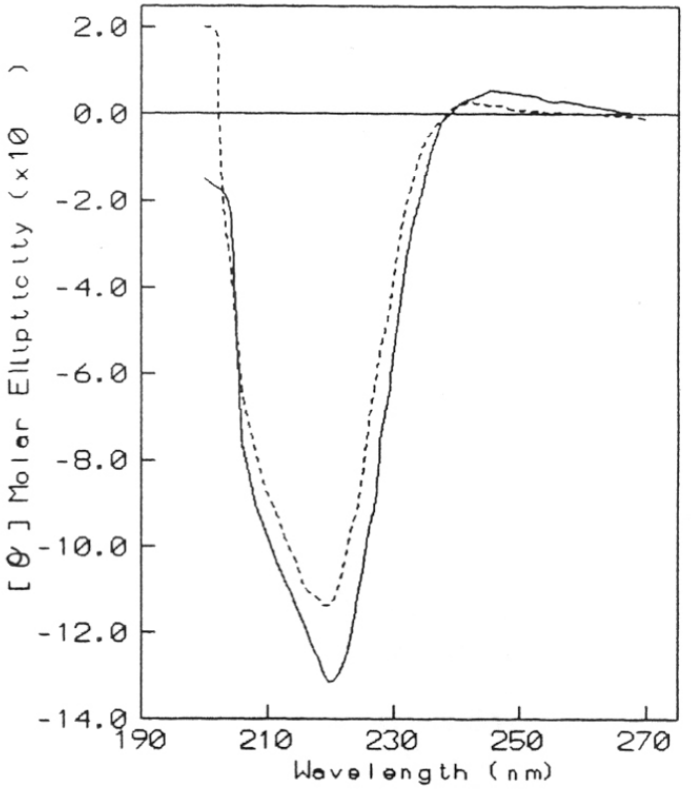

2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
190
210
230
250
270
[θ] Molar Ellipticity (x10)
Wavelength (nm)

Fig. V.3

Fig V.3. The CD spectra of native and TNBS-treated penicillin V acylase from *B. sphaericus*.

The Cd measurements were performed in a 1 mm cell at an enzyme concentration of 500µg.ml^{-1}. Native enzyme (——) and TNBS-treated (----).

activity in presence of 400 uM PMSF was 30% at the end of 20 min test period, while the activity of the enzyme in absence of PMSF remained unchanged. The plot of the logarithm of the residual activity *versus* time of incubation was linear upto the end of the test period at all the concentrations of PMSF used, indicating pseudo first-order kinetics of inactivation by the reagent.

The reaction order (n) with respect to the serine-modifying reagent determined from the plot of K_{app} *versus* log reagent concentration gave a value of 1.10 (Fig. V.4 inset). This value would indicate that the modification of a single serine residue results in the inactivation of an active unit of enzyme.

Protection against Inactivation by PMSF

Table V.2 summarizes data on protection of *B. sphaericus* penicillin V acylase against inactivation by PMSF (400 µM).

About 78% activity was retained after 20 min at 25°C in the presence of penicillin V (50 mM) compared to 26% in the absence of substrate. Phenoxyacetate (50 mM) provided markedly lower extent of protection, residual activity being 38% at the end of 20 min. This might indicate that the essential serine is involved in substrate hydrolysis as shown in the case of penicillin G acylases.

Effects of Stoichiometric Amounts of PMSF on Enzyme Activity

Attempts to titrate the essential serine residues with

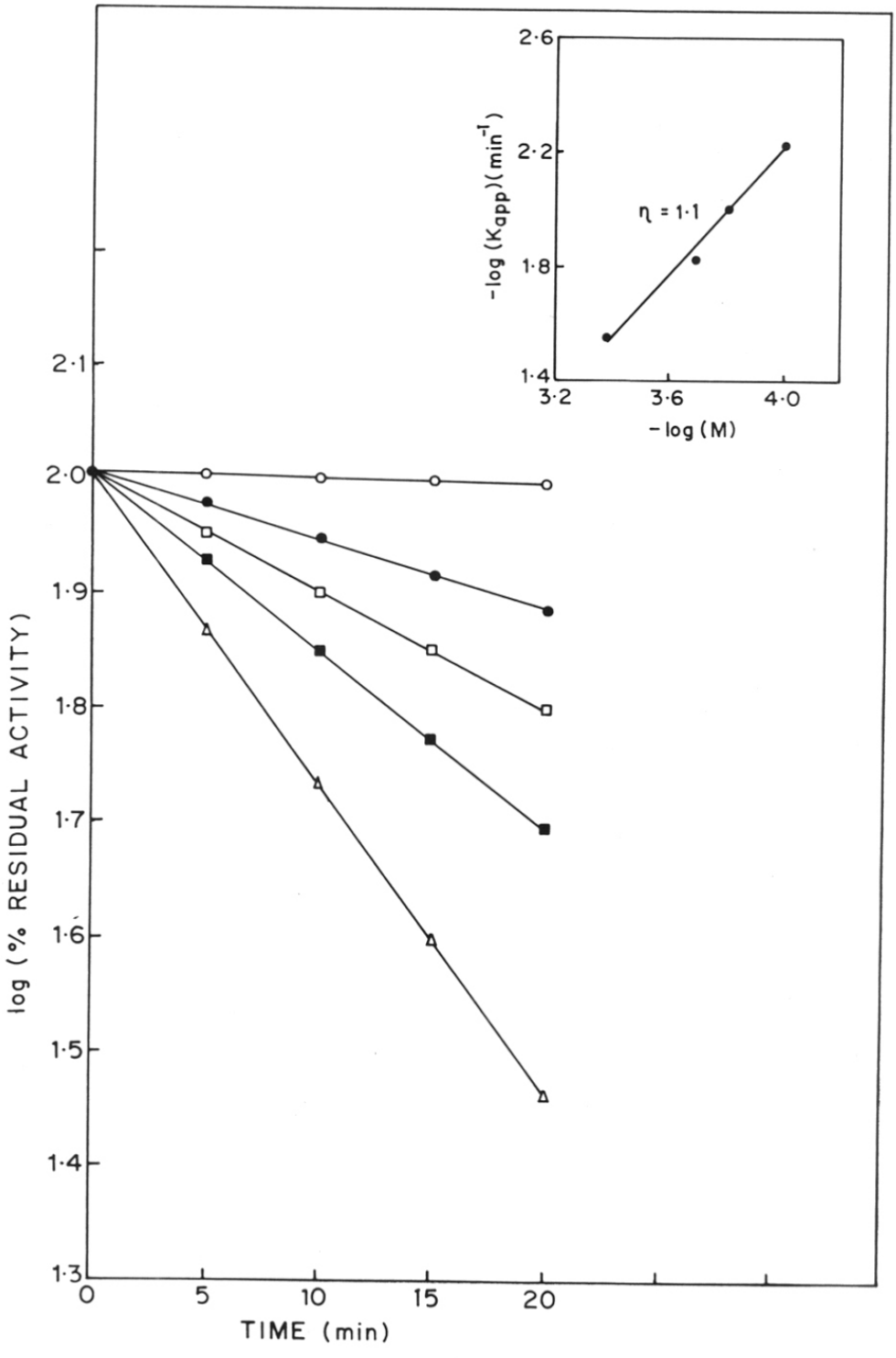

2·6
2·2
1·8
1·4
-log (Kapp)(min-1)
η = 1·1
3·2
3·6
4·0
-log (M)
2·1
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
log (% RESIDUAL ACTIVITY)
0
5
10
15
20
TIME (min)

FIG. V·4

Fig V.4. Inactivation of penicillin V acylase from *B. sphaericus* by serine-modifying reagent phenylmethanesulphonyl fluoride (PMSF).
Concentration of phenylmethanesulphonyl fluoride:
0 µM (-o-), 100 µM (-●-), 150 µM (-□-), 200 µM (-■-), 400 µM (-Δ-).
The enzyme 80 µg/ml was incubated at 25°C in 50 mM potassium phosphate buffer, pH 7.0 and varying concentrations of the inhibitor.

PMSF were made using stoichiometric amounts of the reagent

TABLE V.2: PROTECTION OF *B. SPHAERICUS* PENICILLIN V ACYLASE AGAINST INACTIVATION BY PMSF.

Test compounds were added immediately before the addition of the modifying reagent. Enzyme (80 $\mu g.ml^{-1}$) was treated at 25°C and pH 7.0 as indicated in the Table and samples were withdrawn after 20 min for assay of enzyme activity.

Treatment	Enzyme activity (% Initial activity)
None	100
PMSF (400 µM)	26
Penicillin V (50 mM) + PMSF (400 µM)	77.5
Phenoxyacetate (50 mM) + PMSF (400 µM)	38

In the case of penicillin G acylase from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgeri* (Daumy *et al.* 1985b) and *K. citrophila* (Martin *et al.*, 1991) had shown that approximately 1.3 moles of the reagent completely inactivates a mole of the heterodimeric enzyme. In the present work, an amount of the reagent equivalent to approximately 4-molar excess of reagent per mole of a homotetramer resulted only in approximately 5% inactivation. Even a 400-molar excess reduced activity only about 30%.

Treatment with Cysteine Modifying Reagent

Penicillin V acylase from *B. sphaericus* was inactivated rapidly both by pHMB (10 µM) and by iodoacetamide (500 µM) in the dark at pH 7.0 in 0.05 M potassium phosphate buffer, residual activities in both

cases being about 20% after a period of 30 min at 25°C. Since the former reagent inhibited at high dilutions, detailed studies were carried out with pHMB.

The purified enzyme was incubated at 25°C with pHMB (0 - 5 μM) in 0.05 M sodium acetate buffer, pH 4.5 (Boyer and Segal, 1954).

The plots of the logarithm of the residual activity *versus* time of contact with the reagent were biphasic, an initial rapid inactivation being followed by a slower rate of inactivation indicating the possibility of atleast two different cysteine residues reacting with the modifier, one at a slower rate than the other. Amino acid analysis of the hydrolysate had shown the presence of two cysteine residues per subunit of the enzyme (Table IV.4).

The reaction order n was determined from the plot of the logarithm of the first-order rate constant K_{app} of the initial fast reaction *versus* the log of reagent concentration (Fig. V.5 inset). The K_{app} values were from uncorrected values, not accounting for the contribution by the slower reacting moiety. The slope n = 1.25 might indicate the involvement of more than one cysteine residue per active unit of the homotetramer.

Protection against Inactivation by pHMB

Table V.3 includes the data on protection of penicillin V acylase against pHMB (20 μM) in presence of penicillin V and phenoxyacetate (Table V.3).

The residual activities after 10 min at pH 4.5 and

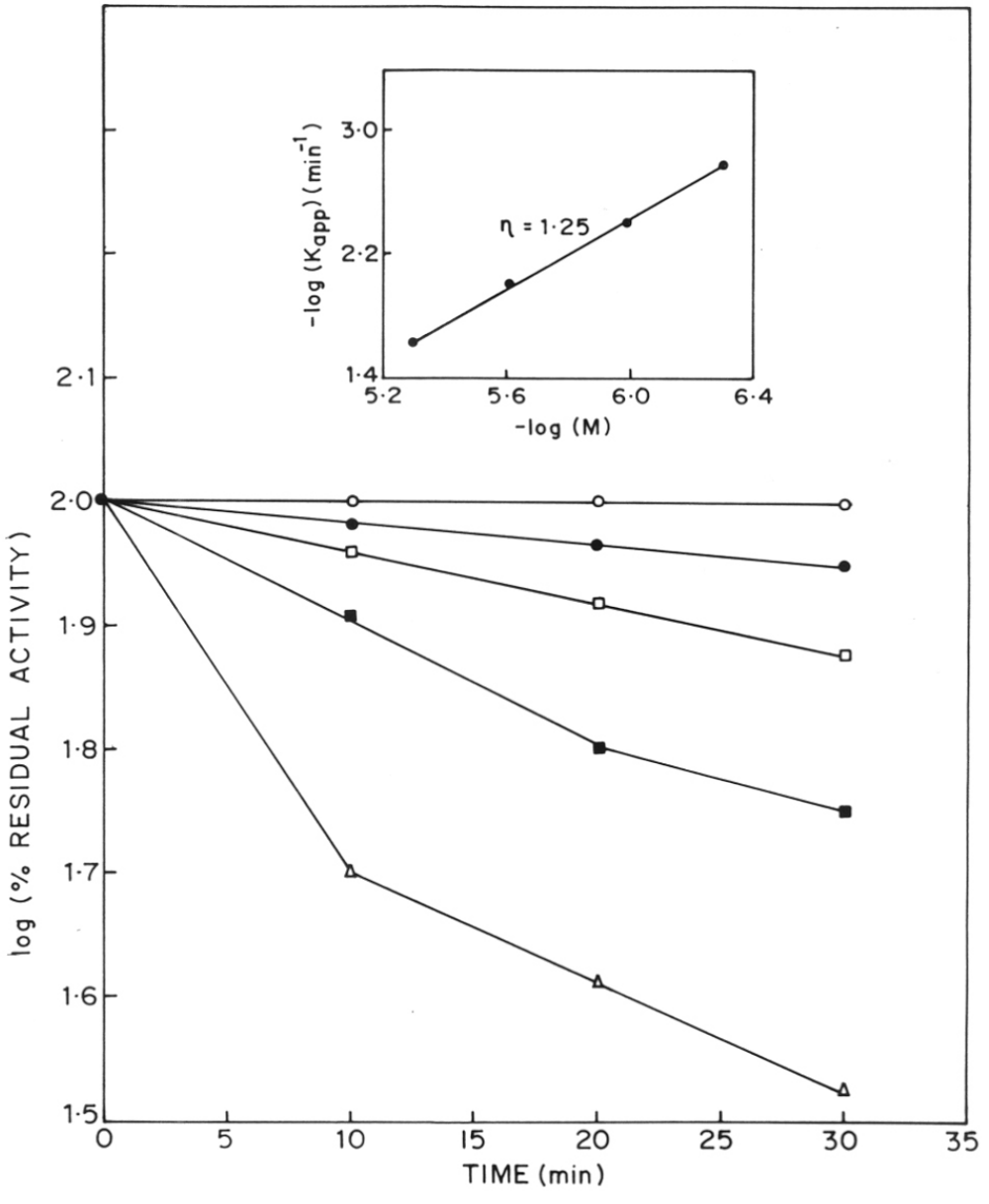

log (% RESIDUAL ACTIVITY)
2·1
2·0
1·9
1·8
1·7
1·6
1·5
0
5
10
15
20
25
30
35
TIME (min)
-log (Kapp) (min-1)
3·0
2·2
1·4
η = 1·25
5·2
5·6
6·0
6·4
-log (M)

FIG. V·5

Fig V.5. Inactivation of penicillin V acylase from *B. sphaericus* by cysteine-modifying reagent, p-hydroxymercuribenzoate.

Concentration of p-hydroxymercuribenzoate: 0 µM (-o-), 0.5 µM (-●-), 1µM (-□-), 2.5 µM (-■-), 5.0 µM (-Δ-).

The enzyme 100 µM/ml was incubated at 25°C in 50 mM sodium acetate buffer, pH 4.5, and varying concentrations of the inhibitor.

25°C was 36% in the absence of penicillin V and phenoxya

cetic acid, the corresponding values in presence of sub

TABLE V.3: PROTECTION OF *B. SPHAERICUS* PENICILLIN V ACYLASE AGAINST INACTIVATION BY pHMB.

Test compounds were added immediately before the addition of the modifying reagent. Enzyme (100 $\mu g.ml^{-1}$) was treated at 25°C and pH 4.5 as indicated in the Table and samples were withdrawn after 40 min for assay of enzyme activity.

Treatment	Enzyme activity (% initial activity)
None	100
pHMB (20 µM)	36
Penicillin V (50 mM) + pHMB (20 µM)	76
Phenoxyacetate (50 mM) + pHMB (20 µM)	61

strate (50 mM) and phenoxyacetic acid (50 mM) being 76% and 61%, respectively. The results indicated partial protection against inactivation by the modification of cysteine residues in the presence of the substrate and of the side chain.

CD Measurements

Far-UV CD spectral analysis showed no changes on modi fication of the enzyme with *p*HMB (Fig. V.6) indicating inactivation is not due to any gross nonspecific conformation change.

DISCUSSION

The mechanism of action and the nature of the essential amino acid residues at or near the active-site of

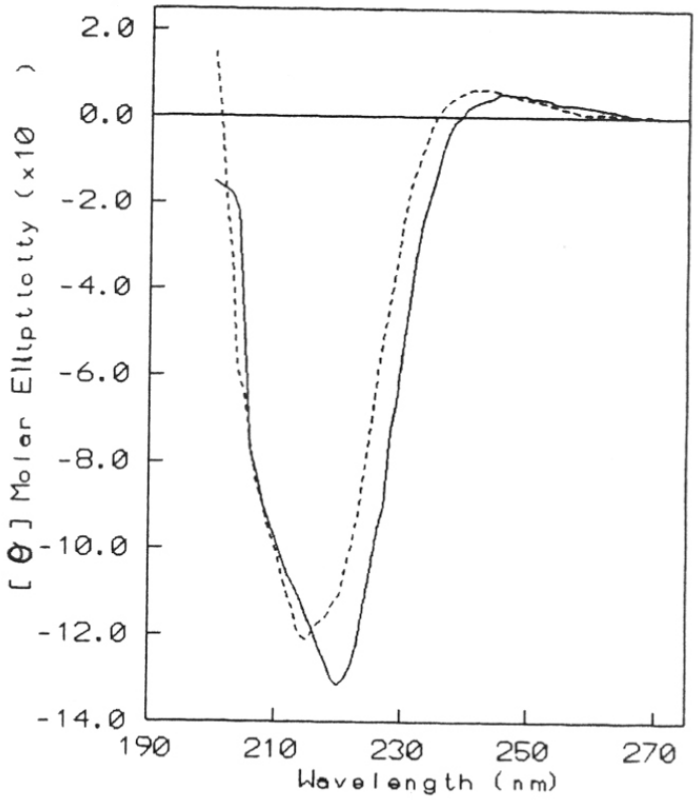

2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
[θ] Molar Ellipticity (x10)
190
210
230
250
270
Wavelength (nm)

Fig. V.6

Fig V.6. The CD spectra of native and pHMB treated penicillin V acylase from *B. sphaericus*.

The CD measurements were performed in a 1 mm cell at enzyme concentration of 500 $\mu g.m^{-1}$.
Native enzyme (——) and
pHMB-treated enzyme (----).

penicillin V acylase have not been reported earlier in the literature. The present studies indicate for the first time the involvement of a single lysine residue at or near the active site of each subunit of the homotetrameric penicillin V acylase from *B. sphaericus*. The evidences are the kinetics of inactivation by TNBS, protection from TNBS inactivation by substrate and the titration of essential lysine residues with acetic anhydride, which indicated that complete enzyme inactivation required the modification of four lysine moieties per mole enzyme by acetylation. All the evidences indicate a single essential lysine per active unit of the homotetramer, the active unit probably being each subunit. Evidences have been provided for the presence of single arginine residues in the heterodimeric penicillin G acylase from *E. coli* (Prabhune and SivaRaman, 1990) and of two essential histidine residues in the homodimeric ampicillin acylase from *Pseudomonas melanogenum* (Kim and Byun, 1990 b). Several enzymes acting upon anionic cofactors and substrates have been shown to have essential basic amino acid residues, possibly required for cofactor or substrate binding (Riordan *et al.*, 1977). The present work extends this to penicillin V acylase where an essential lysine has been shown to be present at or near each of the active-sites of the penicillin V acylase from *B. sphaericus*.

Penicillin G acylase catalysed hydrolysis of its substrate has been shown to be through the formation of a

putative acyl-enzyme intermediate involving an essential serine at the catalytic site, the essential serine being located at the N-terminus of the β-subunit of the heterodimeric enzymes from *E. coli* (Slade *et al.*, 1991) and of *K. citrophila* (Martin *et al.*, 1991). Approximately equimolar amounts of PMSF have been shown to inactivate completely penicillin G acylases from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgeri* (Daumy *et al.*, 1985 b) and *K. citrophila* (Martin *et al.*, 1991). In the present work a putative essential-serine residue had been shown to be present in the active unit of homotetrameric *B. sphaericus* penicillin V acylase from the kinetics of inactivation by PMSF and protection against inactivation in presence of substrate. Attempts to titrate the essential hydroxyamino acid with PMSF indicated that a 4-molar excess of the reagent inactivated the enzyme by less than 5%. The unexpectedly low percentage of inactivation in the case of penicillin V acylase could possibly be attributed to the side-chain specificity of the enzyme and the presence of phenylacetyl group and not of the phenoxyacetyl group in the modifying reagent, PMSF. As seen in **Part IV of the Thesis** penicillin G is hydrolysed only at about 7% the rate of hydrolyis of penicillin V. The failure of a 4-molar excess of a PMSF to inactivate penicillin V acylase to any more than 5% is in keeping with the observation of Kutzbach and Rauenbusch (1974) that while an approximately equimolar amount of PMSF inactivates *E. coli* penicillin G acylase

completely, a 4.4 molar excess of the serine reagent diisopropyl fluorophosphate leads to only 5% inhibition and a 75 molar excess to 16% inhibition.

Penicillin V acylase from *B. sphaericus* has been shown in the present work to be inactivated by the cysteine-modifying reagents, pHMB and iodoacetamide, partial protection against inactivation by the former reagent being provided by both penicillin V and phenoxyacetic acid. The kinetics of inactivation of the enzyme by pHMB indicated the presence of both fast reacting and slow reacting residues, probably a single one of the former being involved per active unit of the enzyme. The suggestion that -SH group(s) may be present at or near the active-site of penicillin V acylase of *B. sphaericus* had been made by Carlsen and Emborg (1982) based on the observation that iodoacetate (33 mM) inhibited the enzyme completely and tetrathionate (33 mM) to the extent of 97%; the latter inactivation and not the former being reversed by βME (6 mM).

PART VI

STUDIES ON IMMOBILIZED WHOLE CELLS OF BACILLUS SPHAERICUS WITH PENICILLIN V ACYLASE ACTIVITY

SUMMARY

Efforts to immobilize isolated penicillin V acylase of *B. sphaericus* by adsorption on ion exchange resin and stabilization through treatment with glutaraldehyde resulted in extensive loss of activity. Whole cells of the organism entrapped in porous low-gelling agarose beads, however, retained cell-bound activity, 3 mm diameter beads expressing about 75% of the activity of the free suspended cells. Experiments on effect of cell loading showed that 20% wet cells (w/v) was optimal. Immobilized whole cells were reused 10 times for the hydrolysis of 2% w/v penicillin V potassium salt, the immobilized system being operationally stable over the cycles of use tried.

INTRODUCTION

The importance of penicillin acylases as industrial enzymes have been stressed in the GENERAL INTRODUCTION.

Immobilized enzymes or whole cells with penicillin acylase activity, particularly the former, are used in industrial processes. Among the types of penicillin acylases, more than 87% of 6-APA production is estimated to be through the use of penicillin G acylases, particularly, the enzyme from *E. coli* which has a pH optimum of about 8, a pH at which penicillin G undergoes spontaneous degradation. Penicillin acylases with an optimum pH in the acidic range

of about 6 - 7 would therefore have an obvious advantage. Further, penicillin V which has higher stability than penicillin G would offer added advantage as raw material for the production of 6-APA, the key intermediate in the manufacture of semisynthetic penicillins. Penicillin V acylase from *B. sphaericus* has an optimum pH of 5.4 to 5.8, high temperature stability and higher specific activity compared to purified *E. coli* penicillin G acylase. Despite these advantages, there are no reports in the literature on the application of the enzyme for 6-APA manufacture.

The present studies were therefore initiated to obtain immobilized systems of either the isolated enzyme or whole cells of *B. sphaericus*.

MATERIALS AND METHODS

Materials

Low-gelling H agarose was obtained from Pharmacia, Sweden; Protanal type LF 120 sodium alginate was obtained from Protan AS, Norway.

Methods

Initial experiments on enzyme immobilization by adsorption on ion-exchange resins such as Indion 48-R and stabilization of the activity by crosslinking with glutaraldehyde resulted in immobilized systems with low activity. The reason could probably have been the involvement of putative lysine residues at or near the active site (**Part V of the Thesis**). The use of some macroporous hydrophobic polymers prepared in the Polymer

Division of the Laboratory indicated low expression of the bound activity. Further experiments were therefore carried out with whole cells entrapped in porous agar beads.

Growth of Organism

B. sphaericus was grown on the modified CSL-minerals medium reported in **Part III of the Thesis.** Yield of packed cells was about $10g.L^{-1}$.

Entrapment of Whole Cells in Open-Pore Agar Beads

Open pore gel beads with the entrapped *B. sphaericus* cells were obtained by the selective leaching of Ca-alginate from calcium alginate - agarose composite beads. The procedure was essentially similar to that described by Rao *et al.* (1986) for entrapment of yeast cells.

A slurry of a weighed amount of packed *B. sphaericus* cells in 100 ml of an aqueous solution containing 2% sodium alginate and 2% agarose at 35 - 40°C was dropped into a stirred solution of 2% $CaCl_2$ and the beads were removed after 30 min agitation at room temperature. Ca-alginate was leached out of the composite beads by washing with 0.05 M potassium phosphate buffer, pH 6.5, until the washings were clear. The beads were then washed with distilled water. This treatment had been shown by scanning electron microscopy in our laboratory to give a porous matrix (Rao *et al.*, 1986).

Hydrolysis of Penicillin V

Water-jacketed, cylindrical, stirred reactors maintained at 40°C were used for conversion of 2% (w/v)

penicillin V potassium salt to 6-APA in batch mode. The pH of the reaction system was maintained at 5.4 - 5.8 by continuous addition of N aqueous ammonia. The completion of the hydrolytic cleavage reaction was apparent from the cessation of ammonia consumption. The contents of the reactor including beads were made upto volume, mixed thoroughly and an aliquot was removed for assay of 6-APA.

Assay of Penicillin V Acylase Activity

Suspensions of free cells of *B. sphaericus* were assayed as described in **Part III of the Thesis.**

Immobilized enzyme and whole cell systems were assayed for penicillin V acylase activity in stirred, water-jacketed vessels maintained at 40°C. The assay medium was similar to that used for cell suspensions.

Effect of Treatment of *B. sphaericus* Cells with Detergents

Effects of some commercially available detergents as well as of toluene on cell-bound penicillin V acylase activity of *B. sphaericus* cells was determined. Cell suspensions of 100 mg packed cells.ml^{-1} distilled water containing the test compounds were agitated at 150 rpm for 60 min at 30°C. The cells were recovered by centrifugation, washed once on the centrifuge with 0.05 M potassium phosphate buffer, pH 6.0, and assayed for activity. Controls without test substances were run simultaneously.

Reuse of *B. sphaericus* Cells Immobilized in Porous Agarose

18 g packed cells in 90 ml bead volume (3 mm diameter beads) were added to an aqueous solution of penicillin V and the total volume made upto 160 ml with distilled water, penicillin V being sufficient to give a final concentration of 2% w/v of the substrate. The pH was maintained at 5.4 - 5.8 with continuous addition of 1 N NH_4OH under agitation. The temperature was maintained at 40°C in a water-jacketed reactor agitated with an overhead stirrer.

RESULTS AND DISCUSSION

Effect of Bead Size on Activity of Immobilized Whole Cell System

Beads of different average diameters (3-8 mm) were prepared through use of nozzles of various internal diameters Beads of 1 mm average diameter were obtained by application of compressed air and use of nozzles with the finest orifice. The results are summarized in Table VI.1.

TABLE VI.1: RELATIVE EFFECTS OF BEAD DIAMETER ON ACTIVITY OF IMMOBILIZED SYSTEM

Average bead diameter	Relative Activity (%)
1 mm	100
3 mm	74
5 mm	59
7 - 8 mm	47

As expected, the beads of the least diameter (1 mm)

showed highest activity. Although open pore beads were used, increase in bead diameter to 3 mm, lowered effective activity to about 75%. In practice the repeated use of 1 mm beads lead to material losses. Beads of 3 mm diameter were therefore used in further studies. The initial specific activity of the cells immobilized in 3 mm dis was estimated to be about 1000 $\mu mol.g^{-1}$ wet $cells.h^{-1}$ compared to about 1375 $\mu mol.g^{-1}$wet $cells.h^{-1}$ for free cells. The calculated value of expression of cell bound activity in 3 mm dia bead was about 73%.

Effect of Cell Loading

Effect of different cell loading in 3 mm dia beads on the initial hydrolytic activity is shown in Table VI.2.

TABLE VI.2 EFFECT OF CELL LOADING ON RELATIVE ACTIVITY OF 3 mm DIAMETER BEADS OF POROUS AGAROSE.

g.wet cells in 100 g beads	Relative Activity (%)
30	100 (assumed)
20	94
10	75
5	31

It can be seen from the Table that the optimum cell loading is about 20% wet cells, increase to 30% showing almost similar activity.

Table VI.3 shows the effect of test substances on specific activity of whole cells of *B. sphaericus*.

TABLE VI.3: EFFECT OF PRETREATMENT OF WHOLE CELLS OF *B. SPHAERICUS* ON CELL-BOUND ACTIVITY.

Test Substance	Specific Activity $\mu mol.g^{-1}$ wet cells.h^{-1}
None	1503
CTAB (0.1% w/v)	1225
Oxgall (0.5% w/v)	1215
SDS (0.1% w/v)	1011
Brij - 35 (0.1% w/v)	1403
Triton X-100 (0.1% v/v)	484
Toluene (0.1% v/v)	1390
Tween-80 (0.1% v/v)	1175

As can be seen from the Table, pretreatment with none of the test substances enhanced expression of cell-bound activity. This could either be due to the failure of the substances to permeabilize the cells or the permeabilized cells could have leaked activity. The latter could have been controlled by crosslinking with glutaraldehyde if the enzyme activity had not been sensitive to such a treatment. Intact, untreated cells immobilized in porous agarose were used in batch mode for repeated reuse in hydrolysis of penicillin V.

Reuse of *B. sphaericus* Cells Immobilized In Porous Agarose

The batch operations were carried out till about 95% penicillin V potassium salt was hydrolysed. The conversion

period under the conditions described in Materials and Methods was about 3.5 h. Recovery of the beads and repeated reuse after washing free from products showed that the beads were operationally stable over the 10 reuses tried, no increase in the period for the completion of hydrolysis being observed.

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