Biol Res 26: 357-364 (1993)

Evaluation of penicillin acylase production by two strains of *Bacillus megaterium*

ANDRES ILLANES, RODRIGO TORRES,
OSVALDO CARTAGENA and ANDREA RUIZ

Escuela de Ingeniería Bioquímica,
Universidad Católica de Valparaíso, Valparaíso, Chile

Penicillin acylase is a key enzyme for the production of semisynthetic ß-lactam antibiotics. The intracellular enzyme from ***Escherichia coli*** *has been thoroughly studied and characterized. The extracellular enzyme from* ***Bacillus megaterium,*** *despite its potential advantages, has received less attention in the recent scientific literature. A comparative study is presented for the production of penicillin acylase with two strains of* ***Bacillus megaterium*** *in batch fermentation in previously optimized complex and defined media. The enzyme produced by the selected strain has been recovered, partially purified and its kinetic behaviour determined.*

INTRODUCTION

Penicillin acylase (PA; penicillin amidohydrolase; E.C.3.5.1.11) catalyzes the hydrolysis of penicillin G (PG) or V to phenylacetic acid (PAA) or phenoxyacetic acid and 6 aminopenicillanic acid (6APA), which is an obligate intermediate for the production of semisynthetic penicillins (Vandamme, 1988). The potentials of PA in the industrial and medical field have been recently outlined (Shewale and Sivaraman, 1989; Shewale *et al.*, 1990).

Most available information refers to the enzyme from *Escherichia coli*, which has been fully studied and characterized (Ospina *et al.*, 1992; Sudhakaran *et al.*, 1992). Less information is available on *Bacillus megaterium* PA, despite the facts that the enzyme from *B. megaterium* is easier to produce (Ishimura and Seijo, 1991) and exhibits better kinetic properties (Savidge and Cole, 1975). Furthermore, available information on *B. megaterium* PA production is rather old (Chiang and Bennett, 1967; Murao *et al.* 1975) and some published material has come only recently from industrially oriented research (Ishimura and Seijo, 1991).

PA from *B. megaterium* is induced by PAA (Chiang and Bennett, 1967) and it is specific for PG (Savidge and Cole, 1975), although it can also act over other ß lactam moieties like cephalosporins (Shewale *et al.*, 1990) and can catalyze the reverse reaction at low pH or at low water activity (Vandamme, 1981). There are substantial sequence and structural differences between the enzymes from *B. megaterium* and *E. coli* (Sudhakaram *et al.*, 1992). Even though PA from *B. megaterium* is considered extracellular, when the PA gene from *B. megaterium* was cloned into *E .coli*, the enzyme was not excreted, which was attributed to incorrect post-translational processing of the immature protein by *E. coli* (Meevootisom and Saunders, 1987).

B. megaterium is the only *Bacillus* species with reported PA activity and these strains have been the basis for the development of industrial strains, used formerly by Squibb in the U.S.A and presently by Toyo Jozo in Japan. Very little information is available on genetic improvement (Son *et al.*, 1982)), mutants or genetically engineered strains being unavailable because of industrial protection.

Correspondence to: Andrés Illanes, Escuela de Ingeniería Bioquímica, Universidad Católica de Valparaíso, Avenida Brasil 2147, Casilla 4059, Valparaíso, Chile. FAX: (56-32) 212-746.

Biol Res 26: 357-364 (1993)

The objective of this work is the assessment of the potential of two strains of *Bacillus megaterium* for the production of extracellular PA by submerged fermentation. The ultimate goal is the production of an immobilized enzyme suitable for industrial production of 6APA from PG.

Results are presented for the production of PA from two strains of *B. megaterium*, in complex and defined medium, which were previously optimized in terms of concentration and time of addition of PAA, which is the enzyme inducer, and concentration of inorganic phosphate (Pi), which proved to be a key component for enzyme excretion. The PA produced by the selected strain at selected fermentation conditions was recovered, partially purified and characterized.

METHODS

Organisms and culture conditions

Two strains of *Bacillus megaterium* from the American Type Culture Collection (ATCC) were selected for PA production. Strain ATCC 14945 was maintained by monthly subculture in casein hydrolyzate-glucose agar slants at pH 7.0 and 4° C. A fresh stock of liophylized cells was used every month for strain ATCC 14946, and subculturing was done in the meantime, as in the case of strain 14945.

Fermentations were carried out batchwise in an orbital shaker at 250 rpm and 30° C in 1 liter flasks with 250 ml of medium at pH 7.0 with 10% v/v inoculum, representing an initial concentration of cells of approximately 0.1 g liter^{-1}.

Table I presents the composition of complex and defined media, designed under carbon limitation, with all other nutrients in an excess of 30%. These media were previously optimized, in terms of level of inorganic phosphate (Pi) and level and time of addition of PAA, as previously reported (Illanes *et al.*, 1992). Complex medium was designed, on the basis of that reported by Murao *et al.* (1975), for a cell growth of 2 g liter^{-1}, but glucose was not included since it was determined to be detrimental for enzyme production, by delaying the induction of PA by PAA (data not shown), probably because of catabolite repression. Defined medium was designed, on the basis of that reported by Acevedo and Cooney (1973), for a cell growth of 1.5 g liter^{-1}. The concentration of Pi was in excess of its requirement as a nutrient and buffer, since, although the enzyme is reported extracellular, a high concentration of Pi was required for excretion, a substantial portion of the activity remaining cell-associated at lower concentrations (Illanes *et al.*, 1992). PAA was added at the onset of fermentation at a concentration of 1.5 g liter^{-1}. Best fermentation temperature and pH were 30° C and 7.0 respectively, as previously determined (Illanes *et al.*, 1992). All reagents were of analytical or microbiological grade. Glucose, phosphates and other mineral salts were obtained from Merck (Darmstadt, Germany); histidine, PAA, 6APA and PG potassium salt, from Sigma (St Louis, Missouri, USA); and casein

TABLE I

Compositions of complex and defined media for penicilin acylase production

	Concentration	
Medium	Complex	Defined
Glucose (g liter^{-1})		1.4
Casein hydrolyzate (g liter^{-1})	3.4	
Histidine (g liter^{-1})		1.5
Phosphate (in buffer)[a] (g liter^{-1})	21.6	21.6
Phenylacetic acid (g liter^{-1})	1.5	1.5
Salts solution (ml liter^{-1})[b]	1	10

[a] Ratio of KH_2PO_4 to $Na_2HPO_4x2H_2O$ determined to match pH 7.0.

[b] Composition of salts solution in g liter^{-1}: $MgCl_2x6H_2O$ 7.53; $CaCl_2x6H_2O$ 5.51; $FeCl_3x6H_2O$ 1.45; $ZnCl_2$ 0.063; $CuCl_2x2H_2O$ 0.08; $Na_2MoO_4x2H_2O$ 0.068; $CoCl_2x6H_2O$ 0.121; $MnCl_2x4H_2O$ 0.107; Na_2SO_4 5.33.

hydrolyzate from Difco (Detroit, Michigan, USA).

Enzyme characterization

For the purpose of recovery and characterization of the PA produced with the selected strain, the fermentation broth was microfiltered to remove the cells and the permeate was diafiltered and ultrafiltered to concentrate the enzyme. These operations were conducted in a crossflow hollow fiber unit from A/G Technology, with a total area of 0.046 m^2. Fibers were $7.5 \cdot 10^{-4}$ m in diameter, with pore average size of $4 \cdot 10^{-7}$ m in the case of microfiltration, and molecular cutoff value of 100,000 daltons, in the case of ultrafiltration.

The concentrated and partially purified PA preparation was characterized kinetically. Activity profiles were determined by measuring initial rates, while stability was determined in terms of the activity remaining after 3 hours in the case of pH and one hour in the case of temperature. Kinetic parameters were determined at the selected pH and temperature in double reciprocal plots by measuring initial rates at varying concentrations of PG, PAA and 6APA. When the effects of PG or PAA were under study, rates were calculated on the basis of 6APA production; when the effect of 6APA was studied, rates were calculated on the basis of PAA production.

Methods of analysis

PA activity was determined as the initial rate of PG hydrolysis, measured by the rate of 6APA production. When the effect of 6APA on enzyme kinetics was studied, the rate of PAA production was measured. 6APA was determined according to Balasingham *et al.* (1972) and Shewale *et al.* (1987). The analysis is based on the measurement of optical density at 415 nm, which reflects the formation of a Schiff base when 6APA reacts with p-dimethylaminobenzaldehyde. A calibration curve was done, in which the initial rate of chemical reaction was correlated linearly with the concentration of 6APA. PAA was assayed by gas chromatography according to Niedermayer (1964), in a Perkin Elmer Sigma 3 gas chromatograph, using a column with 10% diethyleneglycol and 2% concentrated H_3PO_4.

One international unit (iu) of PA was defined as the amount of enzyme that hydrolyzes 1 μmol of PG per min at 30° C, pH 8.7 and 10 g $liter^{-1}$ of PG. For the determination of PA activity, samples were centrifuged at 17500 • g by 15 min in a Sorvall RC-5B high-speed centrifuge and the cell-free supernatant was assayed.

Protein was determined according to Bradford (1976). Glucose was determined as reducing sugars, by the method of Miller (1959). Cell concentration was determined using calibration curves between cell dry weight and optical density at 650 nm. Calibration curves were done for each fermentation medium.

RESULTS

Fermentation kinetics

Kinetics of fermentation for both strains in complex medium is presented in Figure 1 and that in defined medium, in Figure 2. In all cases, PA was growth-associated. No diauxic growth was observed in the defined medium, where glucose did not repress enzyme synthesis. On the contrary, in complex medium, glucose acted as a repressor, delaying the onset of PA synthesis (data not shown). Growth levels were slightly higher than design values, since PAA was used by the cells not only as an enzyme inducer but as a carbon source, which was not considered for media design. This has been conclusively proved in a separate experiment, in which PAA was used as the only carbon source.

A summary of the results obtained with both strains in complex and defined media is presented in Table II. The reported values represent the higher activities, obtained at the onset of the stationary phase. Results in terms of PA production and productivity were significantly better in complex than in defined medium, for both strains. The values of volumetric activity, specific activity and specific productivity were 45, 46 and 56% higher for strain 14945, and 230, 200 and

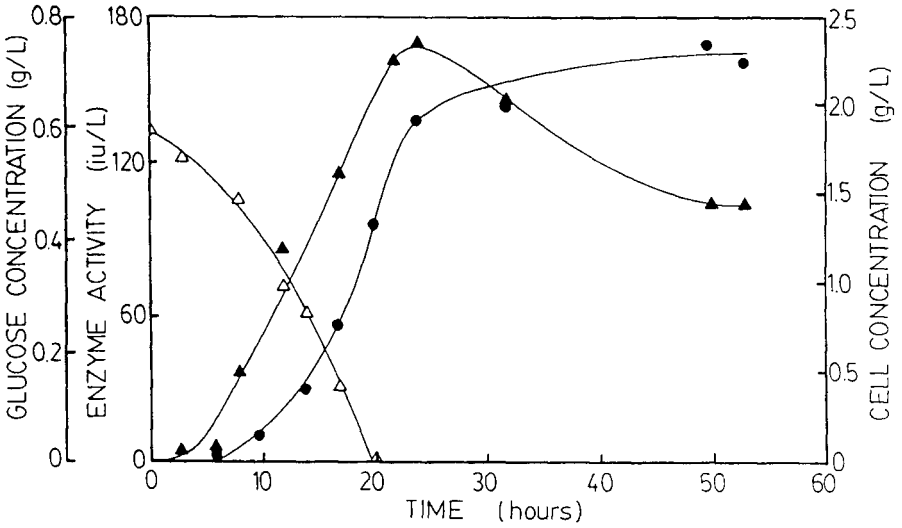

Fig. 1: Kinetics of PA production in complex medium. (●): PA activity, iu liter^{-1}; (▲): cell growth, g liter^{-1}; (△): glucose concentration, g liter^{-1}.

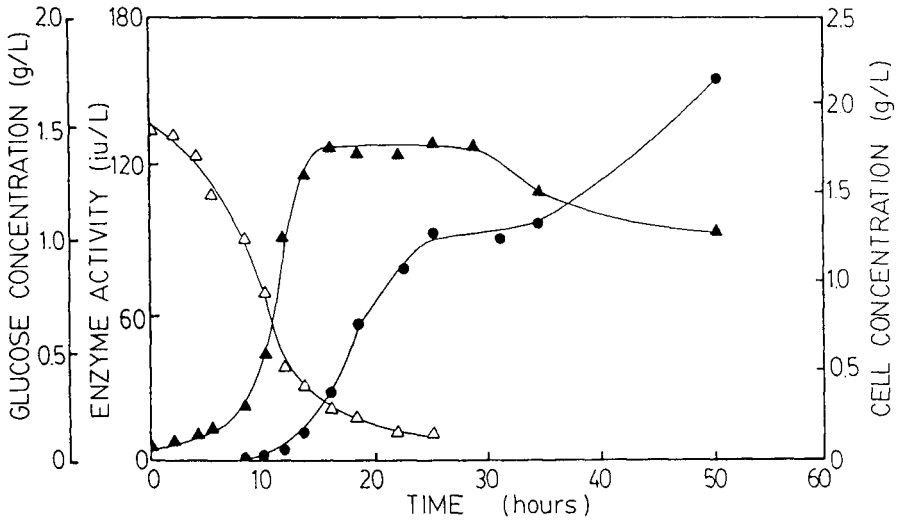

Fig. 2: Kinetics of PA production in defined medium. (●): PA activity, iu liter^{-1}; (▲): cell growth, g liter^{-1}; (△): glucose concentration, g liter^{-1}.

70% higher for strain 14946. These differences are highly significant. In complex medium, volumetric enzyme production was slightly lower in strain 14945 than in strain 14946, but specific activity and enzyme productivity were somewhat higher. In defined medium, strain 14945 was superior in all aspects, with much higher values of enzyme volumetric production and enzyme productivity. Results obtained for PA production are better than those reported in similar systems, when compared in an equal cell growth basis (Acevedo and Cooney, 1973; Son *et al.*, 1982).

Enzyme production and characterization

PA produced in complex medium with strain 14945 was recovered by microfiltration, and then the cell-free broth was diafiltered and concentrated by ultrafiltration. The final enzyme product obtained had 1840 iu liter^{-1} and 21.5 iu mg^{-1} protein. Initial enzyme specific activity was 9.0 iu mg^{-1} protein,

TABLE II

Fermentation parameters for *B. megaterium* ATCC 14945 and 14946 in complex and defined media

Medium	Complex		Defined	
Strain	14945	14946	14945	14946
$\mu(h^{-1})$	0.24	0.29	0.29	0.24
A (iu liter^{-1})	229	255	158	77
a_P (iu mg^{-1} protein)	9.2	8.4	6.3	2.8
a_X (iu g^{-1} cell)	157	139	102	40
Q_P (iu mg^{-1} protein • h^{-1})	0.25	0.22	0.16	0.13
Q_X (iu g^{-1} cell • h^{-1})	4.4	2.9	1.9	0.6

A : Volumetric activity of PA.
a_P : Specific activity referred to total protein excreted.
a_X : Specific activity referred to cell mass.
Q_P : Specific enzyme productivity referred to excreted protein.
Q_X : Specific enzyme productivity referred to cell mass.

therefore a purification factor of 2.4 was obtained. Although higher levels of purity have been reported for PA from *B. megaterium* and we have obtained specific activities as high as 40 to 50 iu/mg protein by ion-exchange chromatography, yields are low to be considered for production purpose. On the other hand, specific activity of the ultrafiltered broth is acceptable for conducting further studies of immobilization, to produce an industrial quality PA catalyst. Therefore, this enzyme preparation has been characterized as the starting point for the production of an immobilized PA.

Activity and stability profiles are presented in Figure 3 with respect to temperature (a) and pH (b). Optimum pH was 8, and the enzyme was almost fully stable in the pH range from 5 to 9 after incubation at 37° C. However, the enzyme was markedly unstable at the optimum temperature of 57° C. The enzyme was fully stable after one hour of incubation at temperatures up to 40° C at pH 8.5; it lost 60% of its initial activity at 50° C and was almost completely inactivated at temperatures higher than 60° C. At 37° C, the enzyme was fully stable and activity was 75% of the optimum. Therefore, kinetic parameters were determined at pH 8 and 37° C. Results are presented in Figure 4, from where the following values were determined for the kinetic parameters: K_m = 2.0 mM; K_{PG} = 376 mM; K_{PAA} = 23 mM; K_{6APA} = 56 mM and V_{max} = 3018 µM min^{-1} (31 µmoles mg protein^{-1} min^{-1}).

DISCUSSION

To select the most suitable strain for PA production, two strains of *Bacillus megaterium* were characterized. Selection was not simple. Although strain 14945 was far superior in defined medium, where PA volumetric production and specific activities more than doubled those of strain 14946, results were significantly better for both strains in complex medium, where no substantial differences were observed. In complex medium, volumetric PA production by strain 14946 was somewhat higher, but specific activities and productivities were lower. Since these parameters make significant differences in a production scheme, strain 14945 was selected. It has to be pointed out, however, that strain 14946 produced significantly higher levels of PA than strain 14945 in complex and complete (with glucose) Murao's medium, where PA synthesis by strain 14945 was severely repressed by the presence of glucose, but not in the case of strain 14946. In that medium, volumetric PA production by strain 14946

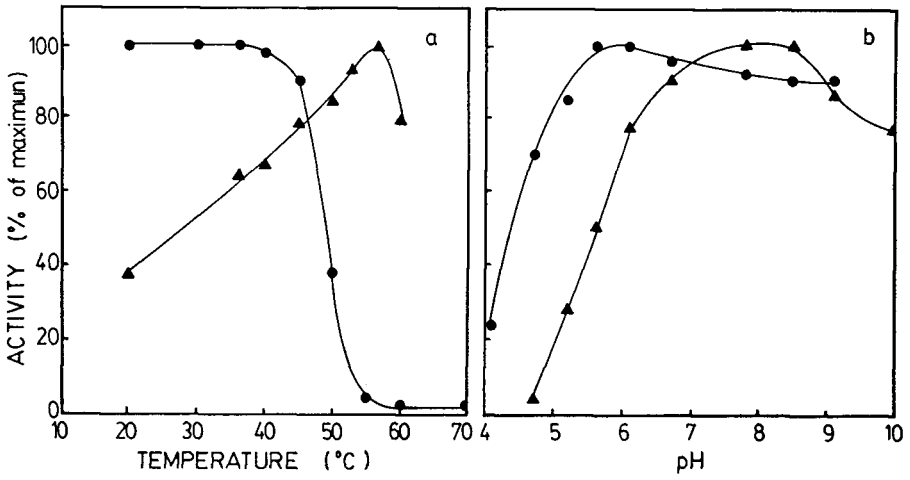

Fig. 3: Characteristics of partially purified penicillin acylase from *Bacillus megaterium* ATCC 14945. a) Temperature profiles for enzyme activity (▲) and stability (●), expressed as % of maxima. Activity determined at pH 8.5. Stability determined as residual activity after 1 h incubation at pH 8.5. b) pH profiles for enzyme activity (▲) and stability (●), expressed as % of maxima. Activity determined at 37° C. Stability determined as residual activity after 3 h incubation at 37° C.

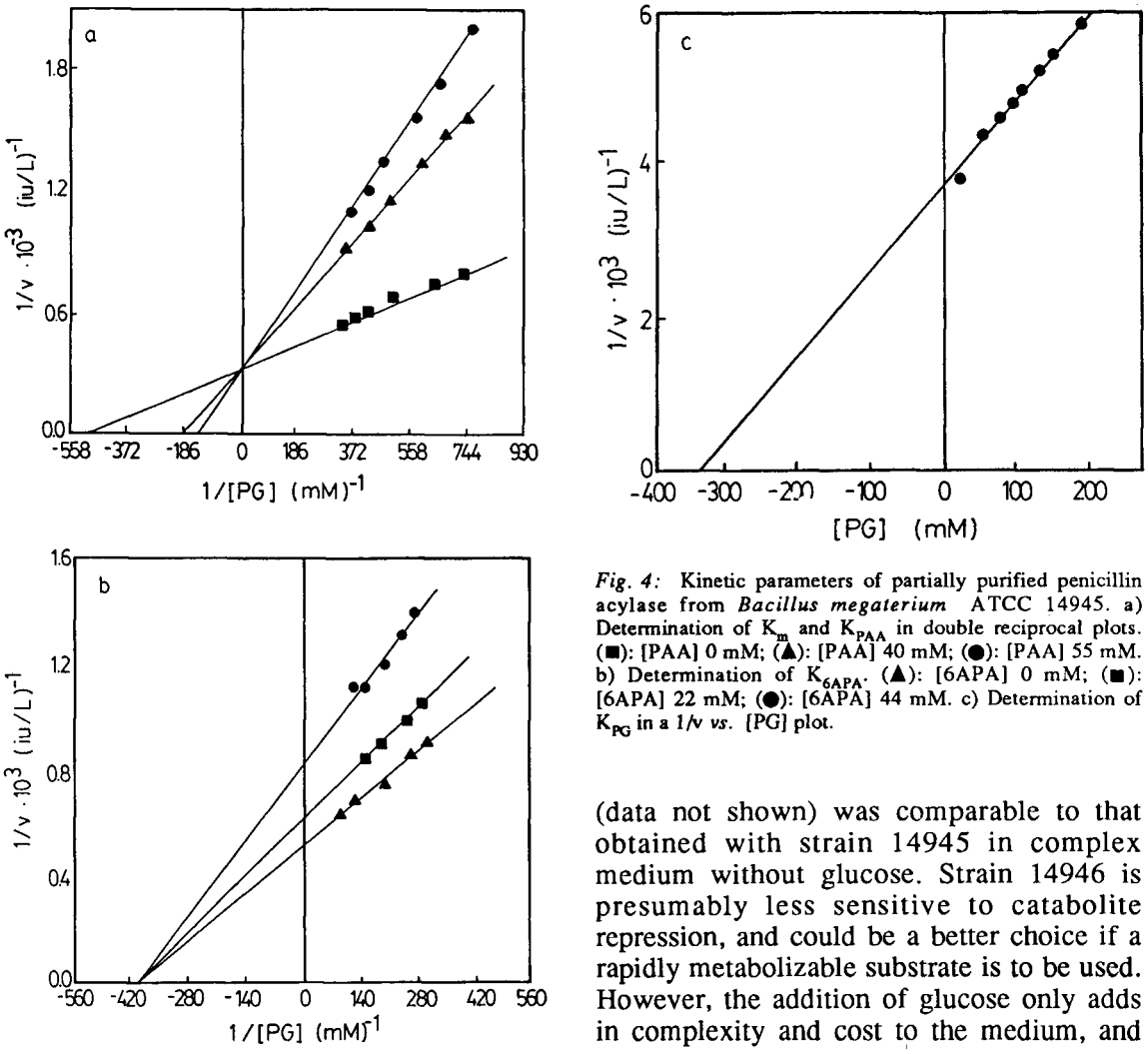

Fig. 4: Kinetic parameters of partially purified penicillin acylase from *Bacillus megaterium* ATCC 14945. a) Determination of K_m and K_{PAA} in double reciprocal plots. (■): [PAA] 0 mM; (▲): [PAA] 40 mM; (●): [PAA] 55 mM. b) Determination of K_{6APA}. (▲): [6APA] 0 mM; (■): [6APA] 22 mM; (●): [6APA] 44 mM. c) Determination of K_{PG} in a 1/v *vs.* [PG] plot.

(data not shown) was comparable to that obtained with strain 14945 in complex medium without glucose. Strain 14946 is presumably less sensitive to catabolite repression, and could be a better choice if a rapidly metabolizable substrate is to be used. However, the addition of glucose only adds in complexity and cost to the medium, and

specific growth rate is lower with glucose, which gives no real advantage in selecting strain 14946 in Murao's complete and complex medium as the production system. In addition, strain 14946 was unstable and hard to maintain by agar subculturing, needing always a fresh liophilized stock, which was not the case for strain 14945.

During downstream processing of the fermentation broth containing the enzyme, a 2.4 fold purification was obtained during ultrafiltration, because of selective retention of PA, whose molecular weight has been estimated in 120 kDa (Savidge and Cole, 1975). Membrane separations are readily scalable operations in which the enzyme is not subjected to harsh conditions, being a very good alternative when no intense purification is required as is the present case.

The pH profiles of the partially purified PA exhibited broad and overlapping ranges for enzyme activity and stability, which is a very good behaviour that can be even improved by immobilization. Since one of the key factors in industrial PA utilization is pH control (Ospina *et al.*, 1992), this characteristic is highly desirable. The enzyme is however sensitive to high temperatures, being its activity extremely unstable at optimal temperature. Fortunately, activity decay was mild at temperatures below the optimum, being possible to obtain a high percentage of maximal activity at temperatures in which the enzyme was quite stable. This behaviour should be improved significantly by immobilization.

PA was inhibited competitively by PAA, totally and non competitively by 6APA, and mildly acompetitively at high concentrations of PG. This kinetic behaviour agrees with published results for similar systems (Ryu *et al.*, 1972; Lee and Ryu, 1982; Ospina *et al.*, 1992). Kinetic properties of the partially purified PA are good: the Michaelis constant for PG, K_m, is low, while the values of the inhibition constants K_{PAA}, K_{6APA} and KPG are one to two orders of magnitude higher than K_m, and compare favourably to other values reported for *B. megaterium* PA (Chiang and Bennett, 1967). This is encouraging for the studies that follow, which consider enzyme immobilization in activated chitosan matrices, according to an already developed methodology (Illanes *et al.*, 1990a, 1990b).

ACKNOWLEDGEMENTS

This work was supported by Grant 91-0530 from the Chilean National Fund for Scientific and Technological Research (FONDECYT). The authors are deeply grateful to Ms Marlene Vásquez and Ms Iris Reyes, and wish to recognize their invaluable laboratory work and contribution to the ongoing research.

REFERENCES

ACEVEDO F, COONEY C (1973) Penicillin amidase production by *Bacillus megaterium*. Biotechnol Bioeng 15: 493-503.

BALASINGHAM K, WARBURTON D, DUNNILL P, LILLY M (1972) The isolation and kinetics of penicillin amidase from *Escherichia coli*. Biochim Biophys Acta 276: 250-256.

BRADFORD M (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 72: 248-254.

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

ILLANES A, ZUÑIGA M, RUIZ A (1990a) Immovilización de lactasa microbiana. Arch Biol Med Exp 23: 159-164.

ILLANES A, RUIZ A, ZUÑIGA M, AGUIRRE C, O'REILLY S, CUROTTO E (1990b) Immobilization of lactase for the continuous hydrolysis of whey permeate. Bioprocess Eng 5: 257-262.

ILLANES A, GENTINA JC, ACEVEDO F, REYES I, TORRES R, RUIZ A, CARTAGENA O (1992) Induction of penicillin acylase synthesis with *Bacillus megaterium* in complex and defined media. Abstr 9th Internat Biotechnol Symp, Washington. p 376.

ISHIMURA F, SEIJO H (1991) Immobilization of penicillin acylase using porous polyacrylonitrile fibers. J Ferment Bioeng 71: 140-143.

LEE S, RYU D (1982) Reaction kinetics and mechanisms of penicillin amidase: a comparative study by computer simulation. Enzyme Microb Technol 4: 35-38.

MEEVOOTISOM V, SAUNDERS J (1987) Cloning and expression of penicillin acylase genes from overproducing strains of *Escherichia coli* and *Bacillus megaterium*. Appl Microbiol Biotechnol 25: 372-378.

MILLER G (1959) Use of dinitrosalicylic reagent for determination of reducing sugars. Anal Chem 31: 426-429.

MURAO S, SAKAGUCHI K, KONO K (1975) Process for preparing 6-APA by *Bacillus megaterium*. U.S. Patent 3.145.395.

NIEDERMAYER A (1964) Determination of phenylacetic acid in penicillin fermentation media by means of gas chromatography. Anal Chem 36: 938-939.

OSPINA S, LOPEZ-MUNGUIA A, GONZALEZ R, QUINTERO R (1992) Characterization and use of a

penicillin acylase biocatalyst. J Chem Tech Biotechnol 53: 205-214.

RYU D, BRUNO, C, LEE, B VENKATASUBRAMANIAN K (1972) Microbial penicillin amidohydrolase and the performance of a continuous enzyme reactor system. In: TERUI G (ed) Fermentation Technology Today. Tokio: Society of Fermentation Technology. pp 307-314.

SAVIDGE T, COLE, M (1975) Penicillin acylase (bacterial). Meth Enzymol 43: 705-721.

SHEWALE J, KUMAR K, AMBEDKAR G (1987) Evaluation of determination of 6-amino penicillanic acid by p-dimethylamino benzaldehyde. Biotechnol Tech 1: 69-72.

SHEWALE J, SIVARAMAN (1989) Penicillin acylase: enzyme production and its application in the manufacture of 6-APA. Process Biochem 24: 146-154.

SHEWALE J, DESPHANDE B, SUDHAKARAN V, AMBEDKAR S (1990) Penicillin acylases: Applications and potentials. Process Biochem 25: 97-103.

SON H, MHEEN T, HAN M (1982) Studies on microbial penicillin amidase (IV): the production of penicillin amidase from a partially constitutive mutant of *B. megaterium*. J Gen Appl Microbiol 28: 281-291.

SUDHAKARAN V, DESPHANDE B, AMBEDKAR S, SHEWALE J (1992) Molecular aspects of penicillin and cephalosporin acylases. Process Biochem 27: 131-143.

VANDAMME E (1981) Use of microbial enzyme and cell preparations to synthesise oligopeptide antibiotics. J Chem Tech Biotechnol 31: 637-659.

VANDAMME E (1988) Immobilized biocatalysts and antibiotic production: biochemical, genetical and biotechnical aspects. In: MOO-YOUNG M (ed) Bioreactor Immobilized Enzymes and Cells. London: Elsevier Applied Science. pp 261-286.