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Ampicillin Biosynthesis by Immobilized Enzyme

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Abstract□Ampicillin was synthesized from 6-aminopenicillanic acid (6-APA) and D-α-phenylglycine methyl ester by using ampicillin synthesizing enzyme from *Pseudomonas melanogenum* (IAM 1655). The whole cell enzyme was immobilized by entrapping it in the polyacrylamide gel lattices. The polymer used in the enzyme entrapment was made from 150 mg per ml of acrylamide monomer and 8 mg per ml of N,N'-methylenebisacrylamide. About 200 mg/whole cell enzyme was mixed in the polymer for entrapment. The maximal activity retention after immobilization was 56%. The optimal pH values for the whole cell enzyme and the immobilized whole cell enzyme were 6.0 and 5.9, respectively. The optimal temperature for the enzyme activity were the same for both type of preparations. The enzyme stabilities against pH and heat increased for immobilized whole cell enzyme. Immobilized cell was more stable especially in the acidic condition while both type were found to be very susceptible to thermal inactivation at a temperature above 40°C. The kinetic constants obtained from Lineweaver-Burk plot based on two substrate reaction mechanism showed somewhat higher value for immobilized whole cell enzyme as compared to the whole cell enzyme: the Km value for 6-APA were 7.0 mM and 12.5 mM while Km values for phenylglycine methyl ester were 4.5 mM and 8.2 mM, respectively. Using the immobilized whole cell enzyme packed in a column reactor, the productivity of ampicillin was studied by varying the flow rate of substrate solution. At the space velocity, SV, 0.14 hr^{-1} the conversion was 45%. Operational stability found in terms of half life was 30 hr at SV=0.2 hr .

Keywords□β-Lactam antibiotic—Pennicillin—Ampicillin—biosynthesis by an immobilized enzyme of *Pseudomonas melanogenum* use of 6-aminopenicillanic acid

Some penicillin amidases (penicillin amidohydrolase, E. C. 3.5.1.11) that hydrolyze penicillin to 6-aminopenicillanic acid (6-APA) also catalyze the reverse reaction, namely, the synthesis of penicillin derivatives from the 6-APA and appropriate side chains.[1]

Kaufmann *et al.*[2] first demonstrated the enzymatic acylation of a penicillin acylase from *Escherichia coli*. Since then, many workers reported on several microorganisms having penicillin amidase. Cole *et al.*[3] reported the various factors affecting the synthesis of benzylpenicillin, DL-α-hydroxy benzylpenicillin and ampicillin by the acylase of *E. coli*. Nara *et al.*[4] and Okachi *et al.*[5] screened the ampicillin producing microorganism, *Kluyvera citrophila,* and *Pseudomonas melanogenum,* respectively. Especially the ampicillin synthesizing enzyme from *P. melanogenum* has been known to be an interesting penicillin amidase which shows activity only for the synthesis of ampicillin and cephalexin which contain a side chain of D-phenylglycine[6]. Marconi *et al.*[7] used the immobilized form of penicillin acylase from *E. coli.* entrapped in cellulose triacetate fibers to produce ampicillin and amoxycillin from 6-APA and phenylglycine methyl ester and

hydroxyphenylglycine methyl ester, respectively.

In recent years, immobilized whole cell enzymes have been used as enzyme preparations because of good stability of the whole cell enzyme[7,8,9]. Using the whole cell enzyme of *P. melanogenum* entrapped in the polyacrylamide gel, the reaction kinetics, optimal reaction conditions, and the performance of continuous enzyme reactor system were studied and the results reported in this paper.

MATERIALS AND METHODS

Microorganism and Cultivation

Mutants of *Pseudomonas melanogenum* (IAM 1655) preserved as lyophilized vials was used in all experiments. The preparation of inoculum and the culture were carried out by the same method as that of Okachi *et al.*[5]

Enzyme Assay

Ampicillin was determined by the method of Smith *et al.*[10] One unit of enzyme activity was expressed as one μmole of product formed per hour. The initial concentrations of 6-APA and D-phenylglycine methyl ester were 46 mM and 138 mM, respectively unless indicated otherwise.

Chemicals

Acrylamide monomer, N,N′-methylenebisacrylamide (BIS) and D-phenylglycine were purchased from Aldrich Chemical Co. Inc. N,N,N′,N′-Tetramethylethyldiamine used was the product of Merck Co. and potassium persulfate was obtained from Mallinkrodt Co. 6-APA was supplied from Wyeth Laboratories, Ltd (U.S.A.). D-α-phenylglycine methyl ester was prepared by the method of esterification of amino acids.

Immobilization

One gram of intact cells (wet weight) was suspended in 4 ml of 0.1 M phosphate buffer solution (pH 6.0). To the suspension acryl amide monomer (0.75 g), BIS (40 mg), 5% N,N,N′,N′-tetramethylethyldiamine (0.5 ml), 2.5% potassium persulfate (0.5 ml) were added, and the mixture was mixed at 4°C under the nitrogen gas for 30 min. In this case, an optimal ratio between the monomer and crosslinking agent was selected for the maximum retention of enzyme activity. The resulting gel was pulverized in the mortar and washed with saline solution to remove the unreacted monomer and loose cells which were not tightly entrapped.

RESULTS

Immobilization

The concentrations of acrylamide monomer and BIS were varied and the immobilized enzyme activity was compared (Table I). The

Table I: Relationship between polymer content and activity of entrapped *P. melanogenum*[a]

Monomers		Immobilized cells	
Acrylamide (mg/ml gel)	BIS (mg/ml gel)	Activity (mM/hr/g gel)	Activity (%) retention
250	8	1.0	14
200	8	1.6	18
150	8	4.9	56
100	8	4.0	44
50	8	2.5	28
150	4	1.8	21
150	12	4.4	49

a) 200 mg/ml of intact whole cells (wet weight) was used.

enzyme activity of immobilized cells was highest when 200 mg per ml of cell concentration (wet weight) was entrapped into polymer which was made from 0.15 g acrylamide monomer per ml and 8 mg per N,N′-methylenebisacrylamide per ml.

Effect of pH

The pH dependence of the enzyme activity is shown in Fig. 1. The optimal pH shifted slightly to the acidic region. The optimum pH of the intact whole cell enzyme was 6.0 and that of immobilized whole cell enzyme was 5.9. Also the pH stability of immobilized whole cell enzyme was higher than that of whole cell enzyme judging from the slightly broader peak (Fig. 1).

Effect of Temperature

The temperature dependence of the enzyme activity was practically the same as that of the intact whole cell enzyme(Fig. 2). Both opti-

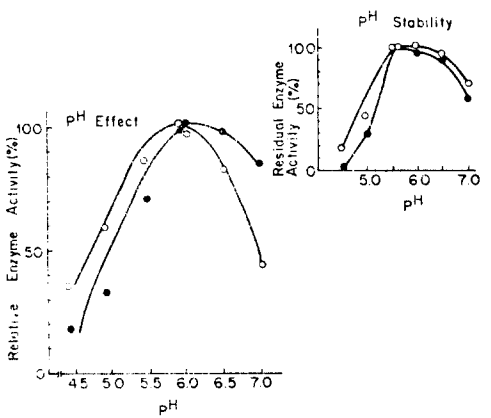

Fig. 1: The effect of pH on enzyme activity and the pH stability (inset,the whole cell enzyme was incubated in 0.1 M phosphate buffer at a given pH for 2 hr at 35°C, followed by the activity assay under the standard assay conditions). Intact whole cell enzyme(●) and immobilized whole cell enzyme(○).

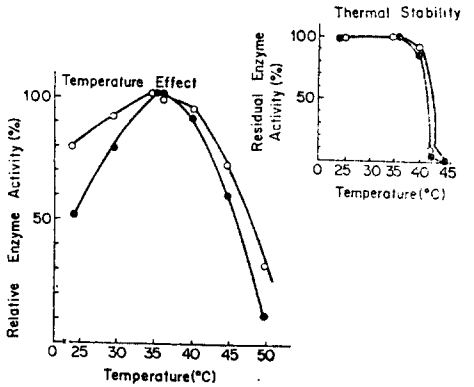

Fig. 2: The effect of temperature on enzyme activity and thermal stability (inset, the whole cell enzyme was incubated in 0.1 M phosphate buffer at a given temperature for 2 hr at pH 6.0, followed by the activity assay under the standard assay conditions). Intact whole cell enzyme (●) and immobilized whole cell enzyme (○).

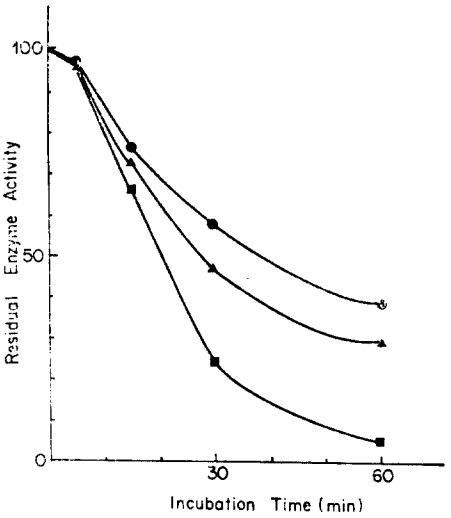

Fig. 3: The effect of Mg^{++}ion addition on the thermal stability of the whole cell enzyme. Intact whole cell enzyme (■), the immobilized whole cell enzyme (▲), and the immobilized whole cell enzyme with 20 mM Mg^{++} (●).

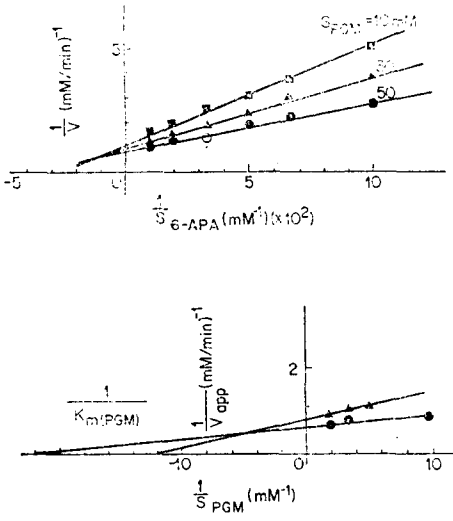

Fig. 4: Determination of kinetic constants.
(4-A): 1/V vs. $1/S_{6\text{-APA}}$ at a fixed S_{PGM}(■S = 10 mM, ▲ 30 mM, and ● 50 mM)
(4-B): The intercept on the ordinate in Fig. 4-A is replotted against $1/S_{PGM}$ for the immobilized (▲) and intact (●) whole cell enzyme.

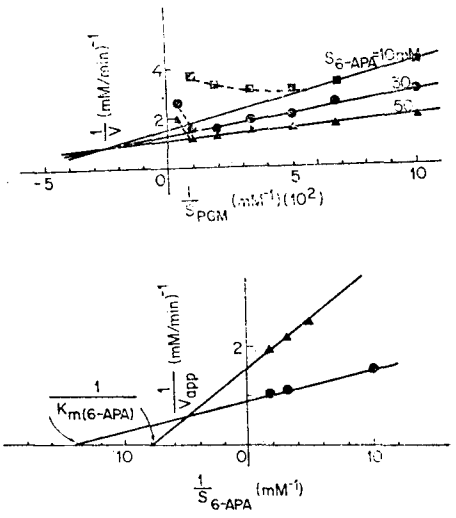

Fig. 5: Determination of kinetic constants.
(5-A): 1/V vs. $1/S_{PGM}$ at a fixed $S_{6\text{-APA}}$ (■S $_{6\text{-APA}}$ = 10 mM, ● 30 mM, and ▲ 50 mM)
(5-B): The intercepts on the ordinate in Fig. 5-A is replotted against $1/S_{PGM}$ for the immobilized (▲) and intact (●) whole cell enzymes.

mum temperatures for the intact cell and the immobilized cell were 35°C. The immobilized whole cell enzyme was only slightly more stable than the intact whole cell enzyme against the changes in temperature. The optimal temperature found was 35°C (Fig. 2). Since the enzyme activity is very low beyond 45°C, the heat stabilities were examined in the presence of substrates at this temperature. The addition of 20 mM Mg^{++} improved somewhat the heat stability of immobilized whole cell enzyme(Fig. 3). The activation energy was estimated as 8.43 Kcal/mole for the intact whole cell enzyme and 6.15 Kcal/mole for the immobilized whole cell enzyme. Theoretically the activation energy of immobilized enzymes can be reduced to one half that of soluble enzymes due to the diffusional limitation.

Kinetic Study

The apparent kinetic constants were determined from Lineweaver-Burk plots based on the two substrate reaction kinetics. The one substrate concentration was chosen in the range of 10 to 200 mM when the other substrate concentration was fixed. The kinetic constants obtained show somewhat higher values for the immobilized whole cell enzyme as compared to the whole cell enzyme : K_m values for 6-APA were 7.0 mM and 12.5 mM while K_m values for PGM were 4.5 mM and 8.2 mM, respectively (Figs. 4 and 5). The K_m

values for ampicillin were 2.5 mM and 4.3 mM. Using these kinetic constants, the reversible two substrate kinetic equation was derived for a batch reactor system.

$$V=\left[V_f\left\{A.B-P/K_{eq}\right\}/K_A.K_B\right]\Big/ \left[1+\frac{P}{K_P}+\frac{A.P}{K_A.K_P}+\frac{A}{K_A}+\frac{B}{K_B}+\frac{A.B}{K_A.K_B}\right]$$

where A and B represent the concentration of phenylglycine methyl ester and 6-APA, respectively, and P, the concentration of ampicillin. The conversion is defined as $X=P/B_0=(A_0-A)/B_0=(B_0-B)/B_0$.

Continuous production of ampicillin

The relationship between the flow rate of substrate solution and the amount of ampicillin produced in the effluent of the immobilized enzyme column reactor was investigated. With the decrease in space velocity (SV) (i.e. increase in space time), the concentration of ampicillin increased. At SV= 0.14 hr^{-1} the maximum conversion of 6-APA to ampicillin reached 45% (Fig. 6). The operational stability of immobilized whole cell enzyme was examined by passing through the substrate solution at SV=0.2 hr^{-1}. The half life of the immobilized enzyme found was 30 hr (Fig. 6).

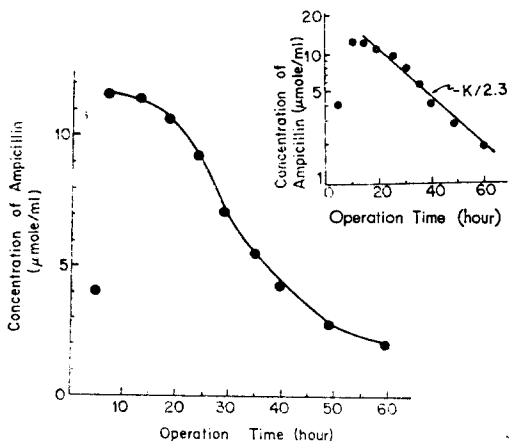

Fig. 6: The concentration of ampicillin in the effluent of continuous reactor vs. operation time under the condition of 0.2 hr^{-1} space velocity and 35°C. The inset: semilogarithmic plot of Fig. 6 showing a first order decrease of productivity.

DISCUSSION

The principal factors affecting the activity of the entrapped whole cell enzyme in the polymer gel were the amount of acrylamide and the degree of BIS in the acrylamide relative to the cell concentration. The former influences on the hardness of the gel lattice and the latter has an effect on the pore size of gel particle in which cells are entrapped. It is known that the substance that has inactivating effect on the enzyme activity is monomer itself.[11] Experimental results suggested that the enzyme system for ampicillin synthesis could be partially inactivated by the polymerizing reagents, especially acrylamide monomer, and the internal diffusion resistance substantially reduces the activity of immobilized whole cell enzyme.

The slight shift in the optimal pH could be due to the microenvironmental effect. The polymer lattice has the positively charged amino groups, and the apparent pH in the bulk solution is slightly lower. As ampicillin was being formed, the pH of the reaction mixture was lowered. This was favorable to immobilized whole cell enzyme because of a

higher stability at acidic condition.

It is also known that temperature stability of an enzyme depends on the pH, ionic strength and substrate concentration. Substrates has some protection effect against heat deactivation. The fact that activation energy is lower for the immobilized whole cell enzyme could be attributable to diffusion barrier.[12)]

In this case, the apparent activation energy approaches the arithmetic mean of the activation energies of the chemical reaction and diffusion. The increased apparent K_m values in the immobilized whole cell enzyme seem to be influenced by the internal and/or external diffusion barrier and repulsive electrostatic forces between substrate and the charged groups in the polymer matrices.

The conversion to ampicillin from 6-APA achieved was about 45% in continuous reactor system. This is not a good yield compared to the chemical synthesis but could become competitive when the process is further improved. For the purpose of a long-term continuous operation the enzyme stability should be considerably improved.

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