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Cephalexin Synthesis Using Immobilized *Xanthomonas citri* Cells

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Abstract

For continuous production of cephalexin, whole cells of *Xanthomonas citri* were immobilized by entrapment in polyacrylamide gel and kappa-carrageenan gel. It wasfound that cells immobilized with kappa-carrageenan showed better thermal stability compared to those immobilized by polyacrylamide gel. The cells immobilized with kappa-carrageenan were treated with glutaraldehyde and hexamethylenediamine to prevent gel destruction during prolonged operation. By immobilizing intact cells, the optimal temperature for the synthetic enzyme reaction shifted higher by 8°C and the optimal pH became broader around 6.2. In continuous operation, the immobilized cells retained better operational stability at 25 than at 37°C, and also showed maximal conversion up to 83% at 25°C.

Index Entries: Cephalexin, synthesis using immobilized cells; synthesis, of cephalexin using immobilized cells; immobilized cells, in cephalexin synthesis; *Xanthomonas citri* cells, cephalexin synthesis using immobilized; polyacrylamide gel immobilization, of *Xanthamonas citri* cells; κ-carrageenan immobilization, of *Xanthomonas citri*.

Introduction

During the last decade, many methods for immobilizing microbial cells have been developed (*1*, *2*). The use of immobilized cells for biotransformation of organic compounds has several advantages compared with immobilized enzyme: (i) high retention of enzyme activity during immobilization, (ii) good operational stability

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of immobilized cells, and (iii) low cost for preparation of enzyme source. Kappa-carrageenan has been suggested as a good entrapping matrix for the immobilization of microbial cells (*3–9*). Immobilization with kappa-carrageenan provides good enzyme stability and retention of enzyme activity because of mild preparation conditions, high viability of microbial cells, and high diffusivity.

For penicillin amidase, *Escherichia coli* cells were immobilized using polyacrylamide gel (*10, 11*), calcium alginate (*11*), polyurethane (*11*), and epoxide (*11*) for production of 6-aminopenicillanic acid from benzylpenicillin. Cellulose acetate was used as a support material for preparation of ampicillin and amoxicillin from 6-aminopenicillanic acid (*12*). A similar synthesizing enzyme produced from *Xanthomonas citri* has been investigated for the synthesis of cephalexin from D-α-phenylglycine methyl ester (PGM) and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) (*13–19*). This enzyme differs from the others in its substrate specificity (*18*).

X. citri cells containing cephalexin-synthesizing enzyme were immobilized using polyacrylamide and kappa-carrageenan; these support materials were then compared. The immobilized enzyme was biochemically characterized and optimal reaction conditions were determined. Cephalexin productivity in a continuous enzyme reactor system was also evaluated.

Materials and Methods

Materials

The D-α-phenylglycine methyl ester (PGM) was prepared from D-α-phenylglycine (PG) using thionyl chloride in methanol. The antibiotics, 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) and cephalexin (CEX), were kindly supplied from Chong Keun Dang (Seoul, Korea). Other chemicals used were analytical reagents grade.

Preparation of Immobilized Microbial Cells

A mutant of *Xanthomonas citri* (IFO 3835) was used throughout this work. The culture medium and fermentation condition used were the same as those described previously (*15*). The fermented broth was centrifuged, washed, again centrifuged at 8000 rpm for 10 min, and the cell cake obtained then immobilized.

The immobilized cells entrapped in polyacrylamide gel were prepared by the method of Chibata et al. (*20*). For entrapping intact cells in kappa-carrageenan, a mixture of 8 g cells in 8 mL physiological saline solution (37°C) and 2.0 g of kappa-carrageenan in 40 mL of physiological saline solution (50°C) was poured into cold 0.3*M* potassium chloride solution through a nozzle with an 1 mm orifice at a constant speed to give a uniform diameter of noodle shaped gel. After soaking in cold 0.3*M* potassium chloride solution to increase gel strength, the gel was cut into cylindrical pellet shapes. For hardening, the gel containing immobilized cells was treated with glutaraldehyde and hexamethylenediamine (HMDA) in 0.5*M* so-

dium phosphate buffer (pH 6.2) using the same method reported by Nishida et al. (*7*).

Analytical Procedure

A 20-mL volume of substrate solution containing 20 m*M* of 7-ADCA and 60 m*M* of PGM in 0.1*M* sodium phosphate buffer (pH 6.2) was added to 2 g of immobilized cells and the reaction mixture was incubated at 37°C for 10 min with shaking. The reaction was stopped by removing the gels by filtration, and the amount of CEX produced was determined (*15*). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol CEX/min at 37°C and pH 6.2 when 20 m*M* of 7-ADCA and 60 m*M* of PGM were employed as substrates.

Continuous Operation of Immobilized Cell Reactor

The immobilized cell reactor was prepared by packing 12 g of immobilized cells (wet weight basis) into a column reactor (id = 2.2 cm, l = 10 cm). The substrate solution containing 5 m*M* of 7-ADCA and 15 m*M* of PGM in 0.1*M* sodium phosphate buffer (pH 6.2) was equilibrated to operational temperature and passed through the column at various flow rates regulated by a syringe pump (Cole-Parmer Instrument Co., USA). After a steady state was reached, the conversion of 7-ADCA to CEX was measured.

Results and Discussion

Immobilization of X. citri *Cells*

In order to improve the retained enzyme activity and operational stability, the preparation conditions of immobilized *X. citri* cells within kappa-carrageenan and polyacrylamide gel were investigated. In the case of immobilization within polyacrylamide gel, the gel hardness and its pore size are influenced by the amount of acrylamide monomer to cell concentration, and are dependent upon the degree of crosslinking, which is affected by the ratio of acrylamide monomer to *N,N'*-methylene-bis-acrylamide (BIS) (*2*). The highest enzyme activity was foundwhen 200 mg/mL of cell concentration (wet weight basis) was entrapped into a polg/mL of acrylamide monomer and 8 mg/mL of BIS(Table 1). But, the enzyme stability of entrapped cells in polyacrylamide was notvery good, at least for operating the column reactor continuously for a prolongedperiod, in spite of the report of Sato et al. (*10*) that *E. coli* cells containing penicilimmobilized within polyacrylamide gel showed goodoperational stability as long as 42 d at 30°C and 17 d at 40°C. This may be attributed to the different enzyme stabilities. The half-life of soluble enzyme from *E. coli* was over 1 d at 37°C (*21*) whereas that of soluble enzyme from *X. citri* was only 2.2–2.3 h at 40°C (*18*).

Because of gelling temperature of kappa-carrageenan as high as 45°C (*8*), the thermal deactivation of enzyme activity in intact cells may be critical to activity retention during immobilization. As a preliminary experiment, the thermal stability of enzyme activity in intact *X. citri* cells was investigated (Fig. 1). This result

TABLE 1
Comparison of Immobilization Methods: kappa-Carrageenan and Polyacrylamide

Immobilization	Retained activity, units/5g gel	Activity retention, %	Half-life, 37°C
Kappa-carrageenan			
without hardening	58	47	—
with hardening, 7.6 mg/mL of glutaraldehyde and 8.8 mg/mL of HMDA	39	31	5.5 d
Polyacrylamide			
0.5 g/ml of acrylamide and 8 mg/ml of BIS	56	45	32 h

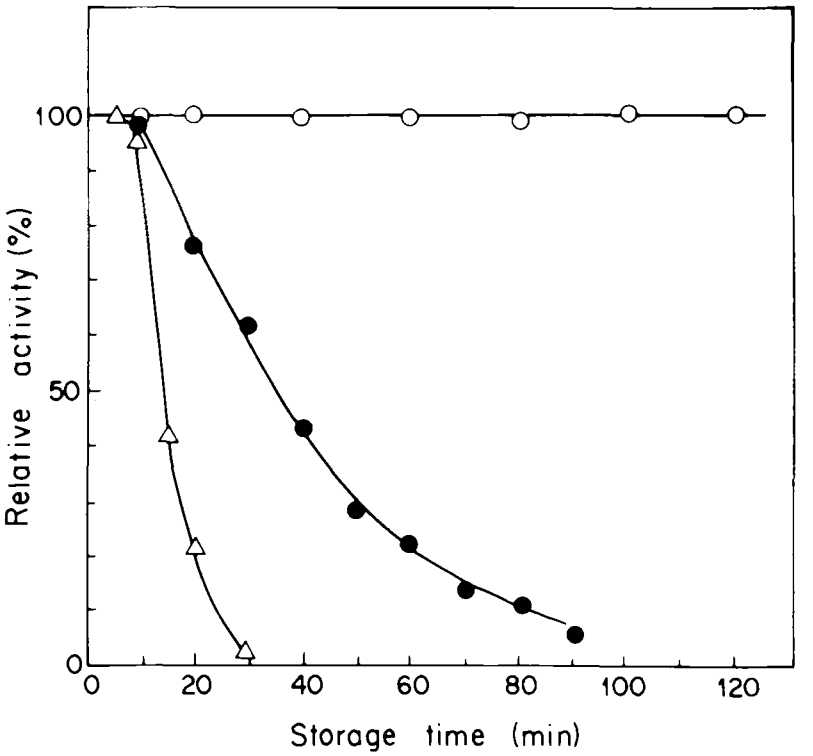

Fig. 1. Thermal stability of cephalexin synthesizing enzyme activity in intact free *Xanthomonas citri* cells at 37°C (○), 45°C, (●), and 50°C (△).

suggested that the mixing of cell suspension and kappa-carrageenan solution should be finished within about 5–10 min because of rapid deactivation of enzyme activity beyond 10 min at 45°C. The retained enzyme activity of kappa-carrageenan was about the same as that of polyacrylamide without hardening or crosslinking of the gel (Table 1). When treated with glutaraldehyde and HMDA for

better gel strength, the retained enzyme activity of kappa-carrageenan-immobilized gel was somewhat reduced, but the operational stability was significantly improved, as was reported by Tosa et al. (*4*) and Nishida et al. (*7*). In all other experiments, the cells of *X. citri* entrapped in hardened kappa-carrageenan gel were used as immobilized cells.

Enzyme Activity of Immobilized X. citri *Cells*

To find the optimal condition for the production of CEX using immobilized *X. citri* cells, some properties of immobilized cells were investigated and compared with those of intact cells and soluble enzyme. Although the maximal enzyme activity of immobilized cells was achieved at pH 6.2, the range of optimal pH was broader than intact cells or soluble enzyme (Fig. 2). Optimal temperatures for immobilized cells, intact cells, and soluble enzyme were 45, 37 and 35°C, respectively, as shown in Fig. 3. The activation energies for soluble enzyme, intact cells, and immobilized cells were estimated as 7.15, 8.25 and 9.74 kcal/mol, respectively. The broadening of optimal range for enzyme activity may be attributed to the mild envi-

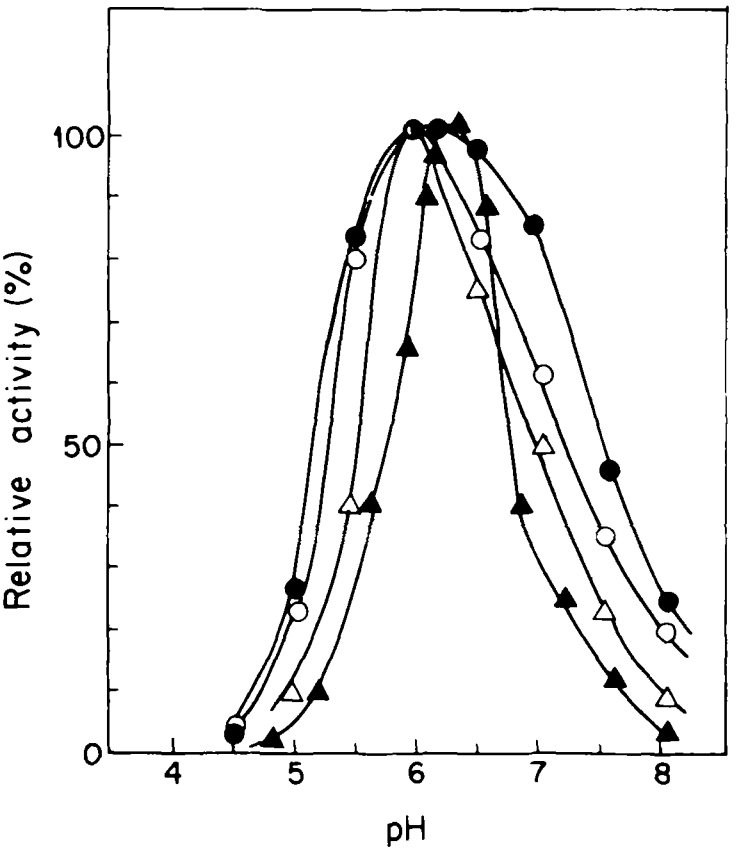

Fig. 2. pH dependence of cephalexin-synthesizing enzyme activity: immobilized cells at 25°C (●) and 37°C (○), free cells at 37°C (△), and soluble enzyme at 37°C (▲). The data for soluble enzyme was reproduced from Kato et al, (*18*).

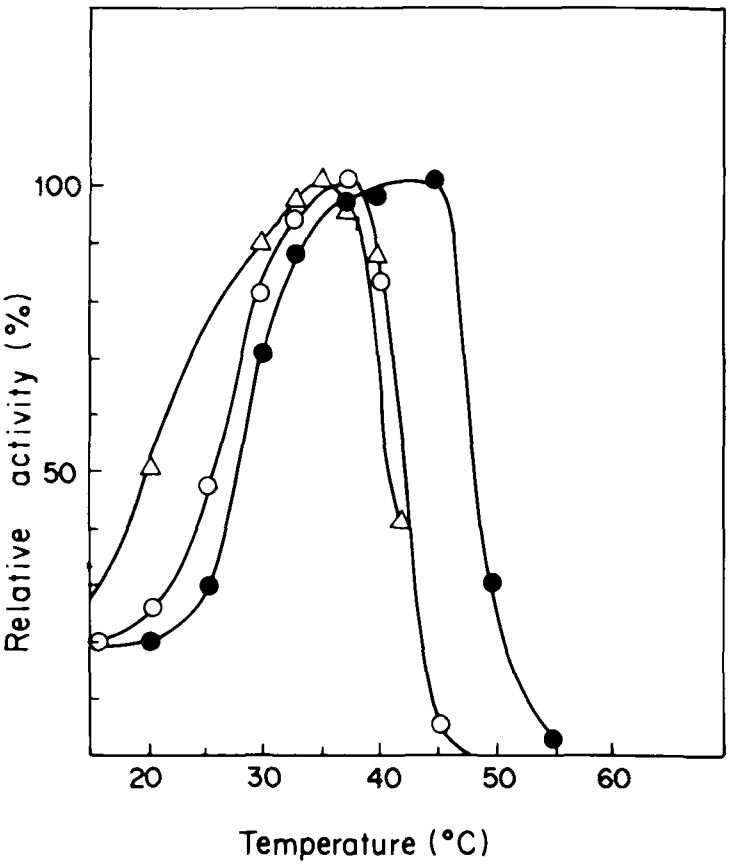

Fig. 3. Temperature dependence of cephalexin synthesizing enzyme activity of immobilized cells (●), free cells (○), and soluble enzyme (△). The data for soluble enzyme was reproduced from Kato et al. (*18*).

ronment of the vicinity of immobilized cells and the diffusion barrier effect of matrices different from those in the bulk phase (*2*).

Continuous Production of Cephalexin by Immobilized X. citri *Cells*

Rhee et al. (*15*) reported that conversion was more rapid for a higher enzyme concentration of intact cells loaded, but a lower maximal was achieve. This means that long operation time with high loading of intact cells may cause a decrease in CEX productivity. In order to operate a column reactor for continuous production of CEX with immobilized *X. citri* cells, the relationship between the retention time and the conversion ratio of 7-ADCA to CEX was determined (Fig. 4). For operation at 37°C, the maximal conversion was 60% at a residence time of 1.2 h, and for operation at 25°C, the maximal conversion as high as 83% was achieved at a residence time of 6 h. The above results show that rapid reaction rates of enzyme due to reaction temperature (this work) or a large amount of loaded enzyme (*15*) may cause a low maximal conversion of 7-ADCA to CEX in spite of rapid conversion.

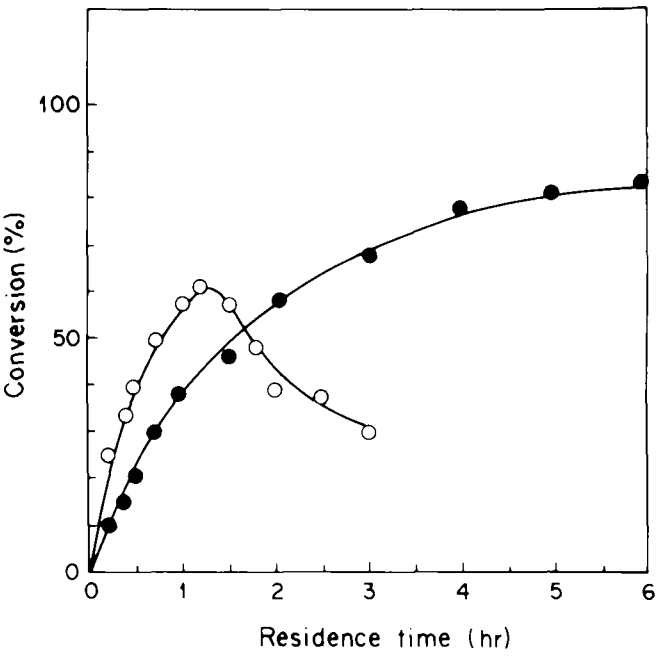

Fig. 4. Relationship between mean residence time and conversion of 7-ADCA to CEX by immobilized cells when operated continuously at 25°C (●) and 37°C (○).

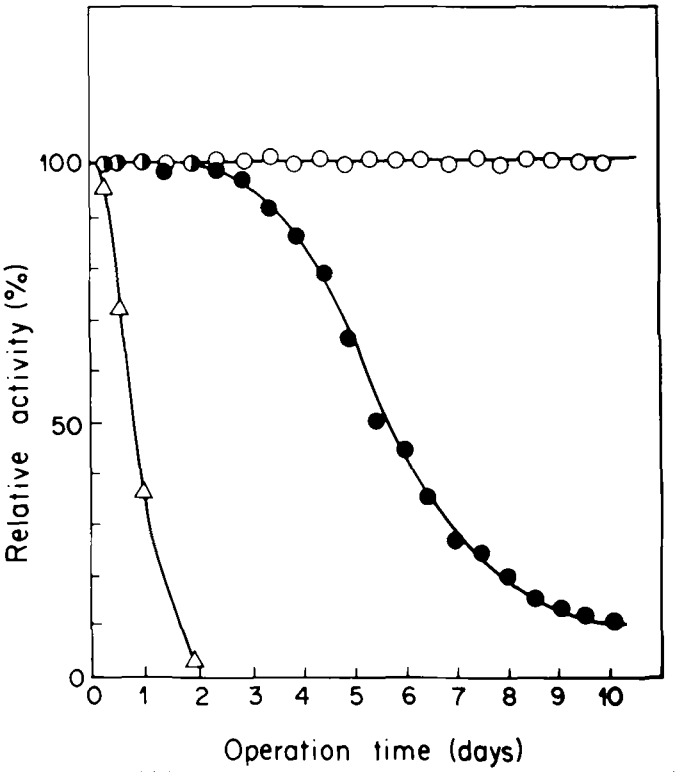

Fig. 5. Operational stability of continuous packed column reactor using immobilized cells at 25°C (○), 37°C (●), and 45°C (△). The relative activity was calculated based on the initial reaction rate at each temperature.

In this reactor system, the operation at 25°C was more favorable than at 37°C because of high conversion in the former case.

For a prolonged operation of column reactor, it is important that enzyme activity should be maintained without thermal deactivation. Figure 5 shows that the half-lives of immobilized cells at 45 and 37°C during operation were 18 h and 5.5 d. At room temperature (25°C), it was observed that the enzyme activity of immobilized cells did not show any decrease during 10 d. From the above results, it can be concluded that the operation of column reactor of immobilized *X. citri* cells within kappa-carrageenan at 25°C is more suitable for high CEX production caused by the kinetic properties of the enzyme reaction and operational stability of immobilized cells.

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