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Research Note

Effect of Cell Concentration on the Acylation of Penicillin G Enzymatic Reaction in Immobilized Cells

M. Barin[1], M. Otadi[2], F. Khorasheh[3,*] and A. Kheirolomoom[4]

Abstract. *E.coli was immobilized by entrapment within calcium alginate beads using cell suspensions of different concentrations. The immobilization procedure resulted in beads with a homogeneous cell distribution, referred to as a Non-overlapping Cell (NC) configuration. If cells within the beads were allowed to grow, the NC configuration would transform into a Clustered Cell (CC) configuration as a result of cell growth. Enzyme activity and substrate conversion were obtained for NC and CC configurations of different cell density, using penicillin G acylation by penicillin G acylase, to produce 6-amino penicillanic acid. Enzyme activity and conversion were found to depend on both cell concentration and configuration. Beads with NC configuration resulted in higher enzyme activity and conversion compared with CC configurations of similar cell density.*

Keywords: *E-coli; Immobilized cells; Penicillin G acylation; Cell distribution.*

INTRODUCTION

Immobilized cell systems provide an alternative approach to carrying out some biochemical processes. Entrapment of cells within a matrix limits cell movement and allows for easier control of cell recovery from the reaction mixture and reuse of the immobilized cells [1]. The main goal in the immobilization of microbial cells is the long lasting usage of preplasmic enzymes in continuous reactors or their repeated use in batch operations. The stability of the immobilized cells within a matrix is, therefore, an important consideration for successful operations [2]. Other important factors, which affect the reaction rate and product yield in immobilized cell systems, are cell concentration and cell configuration within the matrix. Various cell configurations within the matrix include non-overlapping cells, overlapping cells and clustered cells, which are representative of low cell density, high cell density, and the growing of immobilized cell systems, respectively [3]. In a recent study [3], Monte Carlo simulations of nutrient diffusion and reaction in immobilized cell systems indicated that rate constants in such systems depend on the cell fraction and cell distribution within the matrix. Cell configurations ranging from a non-overlapping homogeneous distribution to overlapping and clustered cells with a heterogeneous distribution were considered in these simulations. The Monte Carlo simulations indicated that for a given cell fraction under diffusion-limited conditions an increase in the homogeneity of the cell distribution resulted in an increase in the rate constants. The objective of the current study is to experimentally investigate the effect of cell concentration and distribution on reaction rates for an immobilized cell system.

The immobilized cell system used in this study is E.coli entrapped in calcium alginate for Penicillin G Acylase (PGA) hydrolysis to produce 6-amino penicillanic acid (6-APA). PGA is an enzyme that hydrolyses penicillin G (Pen G) to 6-APA in an aqueous solution. This product is the common precursor in the synthesis of semi-synthetic penicillin [4]. Most microorganisms can produce this enzyme, among which E.coli has been shown to be the most promising. Immobilized cell

1. *Biotechnology Group, Science and Research Campus, Islamic Azad University, Tehran, Iran.*
2. *Biotechnology Group, Tehran Central Branch, Islamic Azad University, Tehran, Iran.*
3. *Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, Iran.*
4. *Department of Chemical Engineering, University of California at Davis, California, USA.*
*. *Corresponding author. E-mail: khorashe@sharif.edu*

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systems have been reported extensively in the literature. One of the earliest systems was the production of acetic acid by acetobacter immobilized on wood. The first articles regarding experimental immobilized systems were published in the 1950's and the first industrial system utilizing an immobilized enzyme was used for the production of L-methionine by a kinetic racemic resulotion [5]. The use of immobilized Pen G acylase from E.coli for the production of 6-APA was shown to be economical, compared with the free enzyme, because of its repeated use [6]. Calcium alginate gel was introduced as a suitable matrix to entrap microbial cells [7,8]. E.coli was immobilized by entrapment in calcium alginate for the acylation of pen G [9] and parameters such as temperature, pH and substrate concentration were optimized for this reaction [10,11]. In the present study, E.coli was immobilized in a calcium alginate gel to provide two different cell configurations. Non-overlapping cell (NC) configurations were obtained by the entrapment of cells with no further nutrients fed to the cells after immobilization. On the other hand, the Clustered Cell (CC) configurations were obtained by providing nutrients to the cell after initial immobilization so that cell growth would cause clustering. The effect of cell concentration and configuration on Pen G acylation was subsequently investigated.

MATERIALS AND METHODS

Materials

Lyophilized E.coli ATCC 11105 and penicillin G potassium salt were obtained from DSMZ Co. (Germany) and Antibiotic Sazi Iran Co., respectively. Other materials such as Tris-HCl, ammonium chloride, yeast extract, phenyl acetic acid, calcium chloride, magnesium sulfate $7.H_2O$, mono- and di-basic sodium and potassium, sodium alginate and nutrient agar were obtained from Merck AG (Darmstadt, Germany).

Cell Suspension Production

A mutant strain of E.coli, ATCC 1115, was added (2.5% w/v) to the pre-culture of a yeast extract at pH 7 and was incubated for 24 hours in a shaker at room temperature at a shaking speed of 220 rpm. The main culture is obtained by adding the resulting pre-culture (10% v/v) to a medium that is composed of (w/v) phenyl acetic acid (0.2%), yeast extract (0.5%), NH_4Cl (0.1%), K_2HPO_4 (0.1%), KH_2PO_4 (0.01%) and $MgSO_4.7H_2O$ (0.02%). The shaking was carried out at 220 rpm at 24°C for 48 hr [12]. The pH of the medium was adjusted to 7.0 using 1 M NaOH solution. These conditions were found to maximize enzyme production in the cells. The cell mass was separated from the main culture using a tubular bowel centrifuge at 13000 rpm at a temperature range of 0-4°C for 40 minutes, and was subsequently transferred to a 100 mM phosphate buffer at pH 7.8. In order to obtain five cell suspensions with different cell concentrations, 10% v/v pre-culture was added to five shake flasks, each containing 100 ml of the main culture. After centrifuging, the cell mass obtained from the flasks was transferred to different volumes of buffer, resulting in five different cell suspensions with corresponding cell concentrations of 37.40, 31.16, 23.37, 18.70, and 9.35 g/l, respectively.

Cell Immobilization in Calcium Alginate

Sodium alginate powder was added (4% w/v) to 100 mM Tris-HCL buffer of pH 7. The sodium alginate powder was added very slowly to the buffer solution in order to prevent flocculation. Stirring was performed by a magnetic stirrer for one hour to obtain a homogeneous sodium alginate solution. The cell suspension was then slowly added (volume ratio of 0.75) to the sodium alginate solution. The resulting mixture was added drop-wise to a 0.15 M calcium chloride solution using a syringe placed 10 cm above the solution. Each drop led to the formation of a calcium alginate bead, inside which the cells were entrapped. The beads were stabilized by stirring for one hour and were stored at 4°C for subsequent use. This immobilization procedure led to a homogeneous distribution of non-overlapping cells (NC) within the beads. Prior to use in experiments, the beads were washed with distilled water and Tris-buffer (100 mM, pH 7). A fixed number of beads (150) were used in each of the subsequent experiments.

Hydrolysis of Penicillin G Potassium Salt

The hydrolysis of penicillin G potassium (Pen G K) salt with immobilized PGA is carried out in a 50 ml stirred batch reactor to produce 6-APA [13]. The amount of enzyme that can produce 1μmol of 6-APA per minute from a specified substrate concentration at a specified temperature and pH is referred to as the enzyme activity and the fraction of the substrate that reacted with the enzyme and was converted into the product is the conversion ratio.

Evaluation of Enzyme Activity by pH-Stat Method

The pH-stat method is based on the titration of phenyl acetic acid produced during the hydrolysis of Pen G K. To evaluate enzyme activity, Pen G K (2% w/v) was added to 50 ml of Tris-buffer (100 mM, pH 7.5). The temperature of the system was kept at 45°C. 150 calcium alginate beads, inside which cells

containing PGA had been entrapped, were washed and subsequently added to the Pen G K substrate solution. The product of Pen G K hydrolysis is acidic, causing the solution pH to drop as the reaction proceeds. The solution was titrated with 100 mM NaOH in order to keep the pH constant at 7.5. Enzyme activity and conversion ratios were obtained from the amount of NaOH used in the titration [14].

Beads with Clustered Cell

The beads with Clustered Cell (CC) configurations were obtained by allowing cell growth within beads containing entrapped non-overlapping cells. The beads with the NC configuration were placed in a growth medium with the following composition (w/v): yeast extract 0.5%, NH_4Cl 0.1%, phenyl acetic acid 0.2%, $MgSO_4 7H_2O$ 0.02% and $CaCl_2$ 0.5%. Cell growth within the beads would result in a clustered configuration.

Determination of Cell Density in Beads

To determine the total number of immobilized cells within the beads, five beads were selected at random from a sample. The selected beads were dried using a freeze drier and were weighed. The dried beads were dissolved in a solution of 100 mM phosphate buffer at pH 7. This solution was used to produce solutions with different dilution ratios. The cells within the diluted solutions were allowed to grow in nutrient agar as the poor plate and subsequently placed on petri dishes at 37°C for 24 hours. Cell density was obtained by colony counts on petri dishes [15].

NC and CC Configurations with Similar Cell Density

When beads immobilized with NC configuration are placed in the growth medium, cell growth would result in an increase in the cell density of the resulting CC configuration. The following procedure was employed to obtain NC and CC configurations with approximately equal cell densities. Three different volumes (1 ml, 1.5 ml and 2 ml) of cell suspension were diluted to a total volume of 3 ml by addition of the phosphate buffer. Three ml of the undiluted cell suspension as well as 3 ml of each of the diluted cell suspensions were each mixed with 4 ml of sodium alginate solution and the immobilization procedure was employed to obtain beads with NC configuration and different cell density. The cell growth for beads immobilized with diluted cell suspensions resulted in CC configurations with a higher cell density than the original NC configuration. Colony counts would indicate which of the subsequent CC beads had the closest cell density to the NC beads that resulted from the original undiluted cell suspension.

RESULTS AND DISCUSSION

Enzyme activity and fractional conversion ratios for NC immobilized beads are presented in Figures 1a and 1b, respectively, for different cell concentrations and reaction times up to 4 hours. The results indicate that both enzyme activity and conversion increase with increasing cell concentration upto an optimum cell concentration of 23.37 g/l, beyond which both enzyme activity and conversion decrease with increasing cell concentration. The number of cells entrapped within the beads increase with increasing cell concentration of the cell suspension used for immobilization. At high cell concentrations, many of the cells entrapped within the interior of the beads do not encounter the

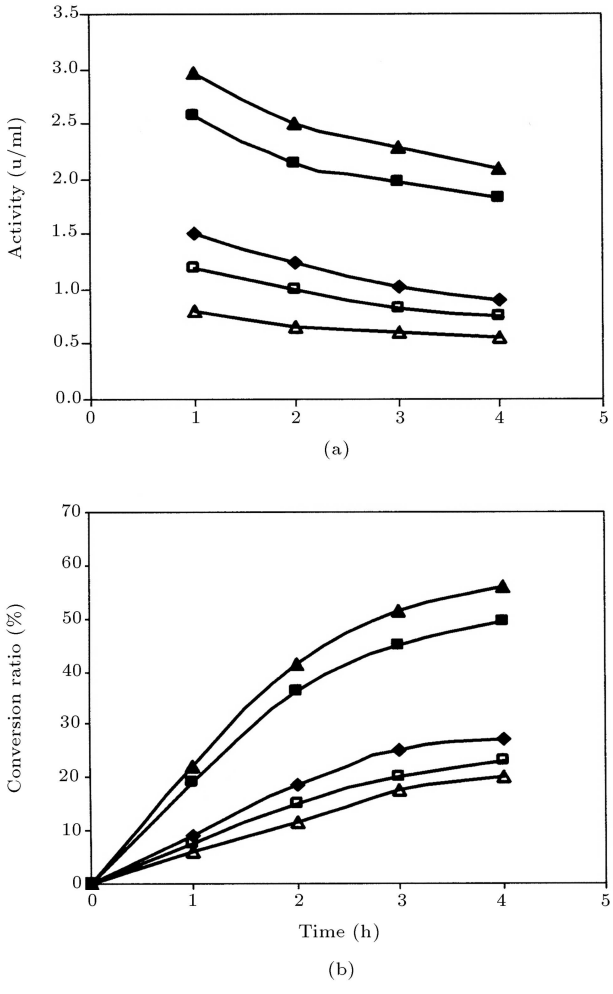

Figure 1. Enzyme activity (a) and conversion ratio (b) for NC immobilized beads of different cell concentrations: 9.35 g/l (open triangle), 18.7 g/l (filled square), 23.37 g/l (filled triangle), 31.16 g/l (filled diamond), 37.4 g/l (open square).

diffusing substrate within the beads, as the substrate is consumed by the cells closer to the exterior surface of the beads.

Figures 2a and 2b represent enzyme activity and fractional conversion after 4 hours, respectively, as a function of the cell concentration for beads with NC and CC configurations. As indicated above, the NC configuration shows an optimum cell concentration at which enzyme activity and fractional conversion reach a maximum. In the case of CC configuration, however, both enzyme activity and conversion slightly decrease with increasing cell density. In CC configurations, interior cells within each cluster are not exposed to the substrate and do not participate in the reaction. At cell concentrations higher than the optimum value, enzyme activity and conversion for NC structures decline to a level comparable with CC structures. At low cell concentrations, however, the CC configuration showed slightly higher enzyme activity and conversion. This was due to the fact that, at low cell density, the number of cells entrapped in beads with NC configurations was lower than the corresponding CC configurations, due to the cell growth.

The results of the colony counts for NC and different CC configurations, using a cell suspension with a concentration of 23.37 g/l, are presented in Table 1. The colony counts were repeated 3 times; each time using 5 beads selected at random. The 1 ml cell suspension, diluted with 2 ml of buffer solution after immobilization and subsequent cell growth, had resulted in beads with a CC configuration of approximately equal cell density compared with the NC configuration. Other CC configurations had even higher cell density. Figures 3a and 3b represent enzyme activity and conversions, respectively, for the NC and the three different CC configurations reported in Table 1. As seen in these figures, The CC configurations resulting from the most diluted cell suspension had the highest enzyme activity and conversion among the CC structures. Furthermore, enzyme activity and conversions for the NC configuration are higher than those for the CC configuration of similar cell density.

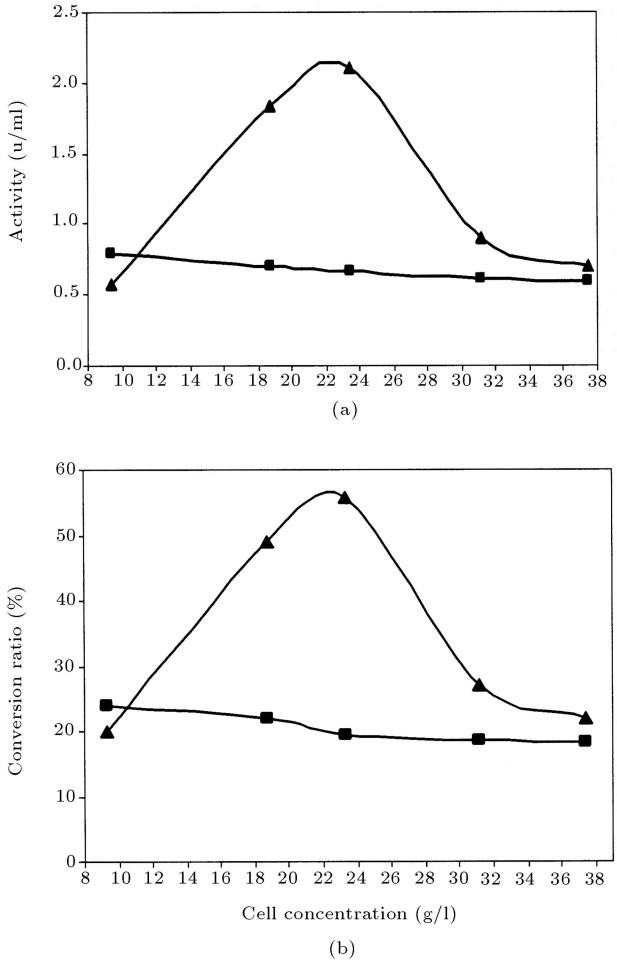

Figure 2. Enzyme activity (a) and conversion ratio (b) as a function of cell concentration with NC (filled triangle) and CC (filled square) configurations after 4 hours.

CONCLUSION

Enzyme activity and fractional conversion in immobilized cell systems depend on cell concentration and configuration within the beads. Immobilized beads with NC configurations showed higher enzyme activity and conversion compared with CC configurations of similar cell density.

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Table 1. Colony number for NC and different CC configurations in 5 beads of a cell suspension with concentration of 23.37 g/l.

Experiment No.	1	2	3
Colony number ($*10^7$) in NC form with [3(ml)S^a + 4(ml)A^b]	18	17	18
Colony number ($*10^7$) in CC form with [1(ml)S+2(ml)B^c + 4(ml)A]	17	17	19
Colony number ($*10^7$) in CC form with [1.5(ml)S+1.5(ml)B+4(ml)A]	25	23	25
Colony number ($*10^7$) in CC form with [2(ml)S+1(ml)B+4(ml)A]	34	33	36

a: Cell Suspension; *b*: Sodium Alginate; *c*: Buffer.

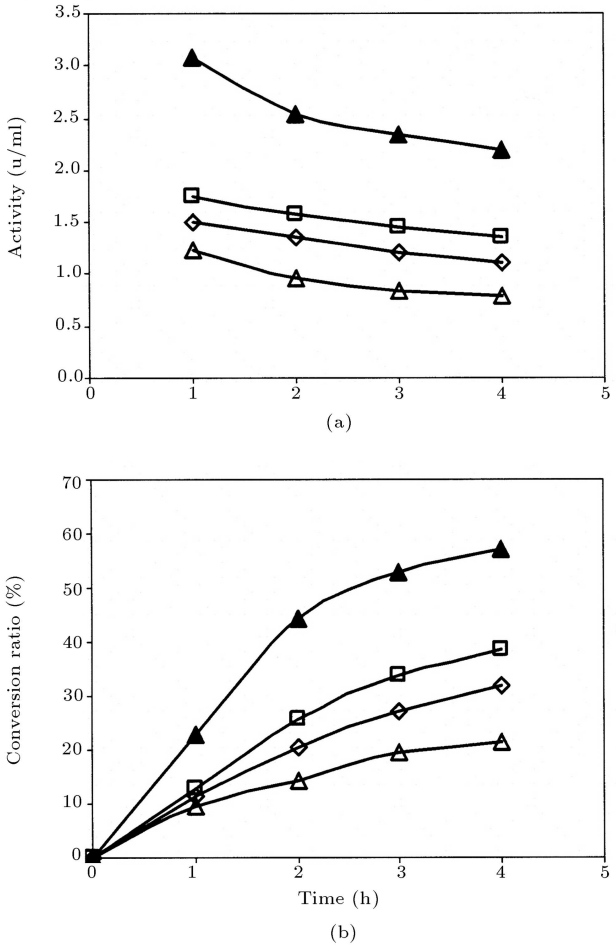

Figure 3. Enzyme activity (a) and conversion ratio (b) for the NC (filled triangle) and the three different CC configurations: [1(ml)S^a + 2(ml)B^b + 4(ml)A^c] (open square), [1.5(ml)S+1.5(ml)B+4(ml)A] (open diamond), [2(ml)S+1(ml)B+4(ml)A] (open triangle). *a*: Cell Suspension; *b*: Buffer; *c*: Sodium Alginate.

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