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# Reusability of Entrapped Cells of *Pseudomonas diminuta* for Production of 7-Aminocephalosporanic Acid

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## Abstract

Entrapped cells of *P. diminuta* were used for the production of 7-aminocephalosporanic acid (7-ACA), a key intermediate required for the production of most of the clinically used cephalosporin derivatives, i.e., semi-synthetic cephalosporins. The repeated batch production of 7-ACA with entrapped cells of *P. diminuta* in different carriers were carried out for six cycles at optimal conditions. It was found that 33% , 38%, and 47% of activity was lost with chitosan, gelatin, and agar, respectively as immobilizing supports after the sixth cycle of operation.

**Index Entries:** 7-ACA; CPC acylase; GL-7-ACA; GL-7-ACA acylase; immobilization; repeated batch production.

## Introduction

7-Aminocephalosporanic acid (7-ACA) is an important intermediate produced by deacylation of cephalosporin-C (CPC) in presence of industrially important enzymes known as CPC acylases. 7-ACA is used for the production of most of the semi synthetic cephalosporins *(1-3)*. Cephalosporin-C possesses weak antimicrobial activity, but substitutions on $C_3$ and $C_7$ positions of its β-lactam ring along with other structures generate semisynthetic cephalosporins with diversified antimicrobial activity, e.g., cefazolin, cefotoxime, cephamandole, cephaclor, and so on. The chemical methods of 7-ACA production are tedious, time consuming and involve multiple costly steps *(4,5)*, and hence, attempts have been made to produce 7-ACA by a biocatalytic process. Cephalosporin acylases are classified in to two groups depending on their substrate specificity: glutaryl 7-ACA

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acylases (GL-7-ACA acylases), also known as 7-β-(4-carboxybutanamido) cephalosporanic acid acylases *(1-3,6-11)*, which are involved in the three-step conversion of CPC to 7-ACA, and cephalosporin-C acylase (CPC acylase), which have been reported in a single-step conversion *(1,2)*. The main drawback in the production of 7-ACA by the three- step enzymatic process is the generation of hydrogen peroxide, which inactivates the enzyme D-amino acid oxidase *(12)*. A mutant of glutaryl 7-ACA acylase with a higher maximal activity on CPC as compared to GL-7-ACA that converts CPC to 7-ACA in a single-step enzymatic pathway has been developed *(13)*. In our earlier communications, the effects of different organic compounds on the biosynthesis of CPC acylase *(14)* and batch production of 7-ACA by different microorganisms *(15)* were established. Biotransformation has become a new tool in the modification of biotechnology and processes and is being studied for the substitution of the most of the chemical processes. The immobilization of microbial cells involved in the production of useful compounds is now gaining in importance over immobilized enzymes because it eliminates the need for the release of intracellular enzymes and thus succeeding purification steps. By immobilizing microbial cells, it is possible to maintain high cell concentration and high flow rates during continuous production and to reuse immobilized cells. Repeated batch immobilized cell systems have been used for the production and improvement of processes involved in the biosynthesis of a number of commercially important compounds including antibiotics, different enzymes, and so on *(16–18)*. We have already evaluated the production of 7-ACA by entrapped cells of *P. diminuta* and compared the production against the free cells *(19)*. The purpose of this investigation was to study the reusability of immobilized cells toward the production of 7-ACA.

## Materials and Methods

*Pseudomonas diminuta* NCIM 2865 was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. This microorganism was selected because of its CPC acylase activity in the single-step conversion of CPC to 7-ACA. The culture was maintained on medium containing 10 g $L^{-1}$ peptone; 10 g $L^{-1}$ yeast extract; 5 g $L^{-1}$ sodium glutamate; 2.5 g $L^{-1}$ sodium chloride; and 20 g $L^{-1}$ agar. The pH of the medium was adjusted to 7.0 before sterilization.

The medium of Shimizu et al. *(20)* supplemented with 0.15% glucose and optimum concentration of glutaric acid (0.02%) is used for the production of CPC acylase. The seed media used for the propagation of microorganism contained 10 g $L^{-1}$ beef extract; 10 g $L^{-1}$ peptone; 5 g $L^{-1}$ sodium chloride; pH was adjusted to 7.0 before autoclaving for sterilization. The seed inoculum was prepared by growing the bacteria for 24 h at 30°C in an orbital shaker set at 200 rpm. The inoculum was transferred to production medium at 5% (v/v). The well-grown cells from the production medium were harvested by centrifugation at 4860*g* for 10 min. The supernatant was

discarded and cell pellet after washing with phosphate buffer (0.01 *M*, pH 7.0) was immobilized in different carriers such as chitosan, gelatin and agar *(21–23)*, respectively by entrapment method.

The cell growth (biomass) was determined by measuring the optical density at 660 nm and converted to dry cell weight (DCW) using a standard curve. Glucose concentration was measured by dinitrosalicylic acid (DNS) method of Miller *(24)*. The enzyme activity was determined by mixing entrapped cells of *P. diminuta* (3.2 mg entrapped dry cell weight in the reaction mixture) with 7.5 mg/mL of cephalosporin-C (0.016 m*M*, Sigma, >90% purity) in 0.1 *M* potassium phosphate buffer (pH 7.2) and incubated at 34°C for 9 h at 100 rpm. The 7-ACA formed after deacylation of CPC was determined by the colorimetric method of Marrelli *(25)* and by thin-layer chromatography with solvent system butanol-acetic acid-water in the ratio of 4: 1: 5. The 7-ACA used for standard is of Himedia with 98% purity. The activity of CPC acylase enzyme is expressed in terms of unit (U), which is defined as micromole of 7-ACA formed per milliliter.

## Results and Discussion

In order to evaluate the reusability of immobilized whole cells in different carriers, the production of 7-ACA was studied for six cycles. Each cycle of batch production was of 9 h duration *(19)*. The results are shown in Figs. 1–3 for all the carriers used for entrapment process.

The production of 7-ACA by entrapped cells of *P. diminuta* in first batch run was considered as 100% (9.0 $\mu M/mL \times 10^{-2}$). The production of 7-ACA decreases with increasing the number of cycles and it was found that 67%, 62%, and 53% of enzymatic activity was regained with chitosan, gelatin, and agar, respectively as immobilizing supports after the sixth cycle of operation. The loss of activity of immobilized cells in the repeated batch production of 7-ACA was probably due to continuous agitation of immobilized cells, resulting in the modification of gel structure, and also due to the leakage of cells.

The production of 7-ACA was also carried out using CPC fermentation broth as substrate after concentrating the broth (Crude CPC 1.2 mg/mL), and the results are given in Figs. 4–6.

The experiments were further designed in such a manner that the entrapped whole cells after each run were kept in the enzyme synthesis medium *(20)* for 6 h so that the whole cells might regain their original activity. It was observed during the repeated batch production of 7-ACA with entrapped cells using CPC fermentation broth as substrate, that the loss of enzyme activity was regained to a large extent by providing the enzyme synthesis medium to the entrapped cells at the end of each batch (Table 1). The production of 7-ACA was low as compared to the pure substrate. This decrease in production of 7-ACA using crude CPC fermentation broth as substrate might be due to presence of lower amount of CPC and other intermediates of cephalosporins not hydrolyzed by cephalo-

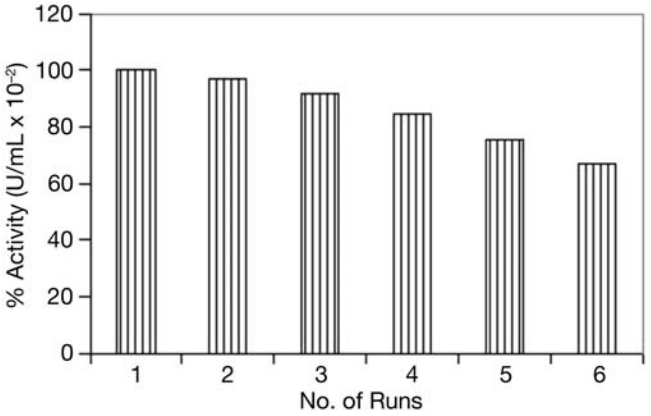


Fig. 1. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in chitosan.

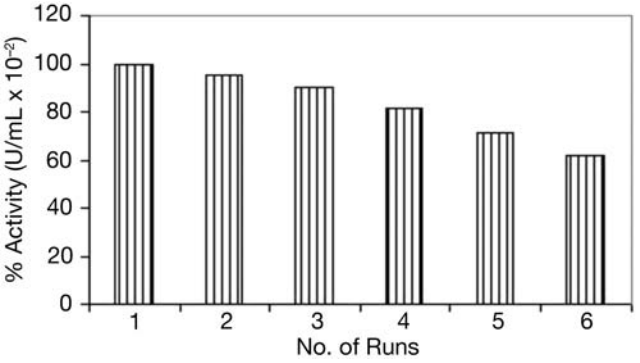


Fig. 2. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in gelatin.

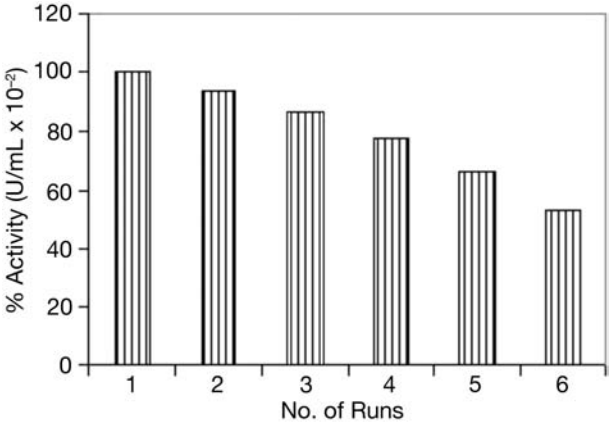


Fig. 3. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in agar.

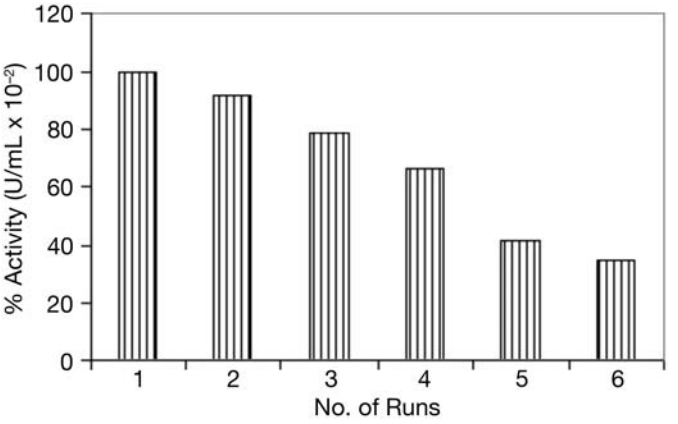


Fig. 4. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in chitosan using CPC fermentation broth as substrate.

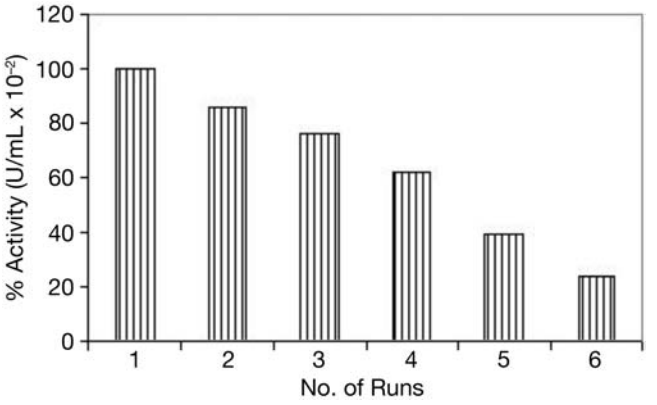


Fig. 5. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in gelatin using CPC fermentation broth as substrate.

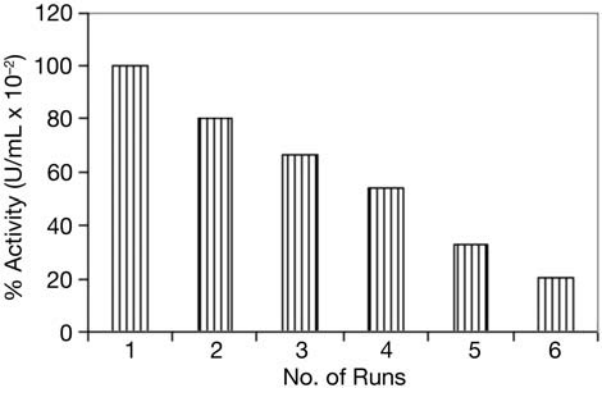


Fig. 6. Percentage activity of CPC acylase enzyme with immobilized cells of *P. diminuta* in agar using CPC fermentation broth as substrate.

Table 1
Percentage Activity and Percentage Regaining Activity of CPC Acylase by Immobilized Cells of *Pseudomonas diminuta* During Repeated Batch Production of 7-ACA Using CPC Fermentation Broth as Substrate

| No. of runs | % Activity | | | % Regaining activity | | |
|---|---|---|---|---|---|---|
| | Chitosan | Gelatin | Agar | Chitosan | Gelatin | Agar |
| 1. | 100 | 100 | 100 | 100 | 100 | 100 |
| 2. | 91.4 | 86.1 | 80.5 | 95.7 | 96.0 | 94.4 |
| 3. | 79.3 | 76.2 | 66.7 | 87.1 | 86.1 | 80.6 |
| 4. | 66.4 | 62.4 | 54.2 | 75.0 | 71.0 | 59.7 |
| 5. | 41.4 | 38.6 | 33.3 | 54.3 | 47.5 | 40.3 |
| 6. | 25.0 | 23.8 | 20.8 | 33.6 | 28.7 | 26.4 |

Table 2
Percentage Residual CPC During Batch Study of 7-ACA Production With Entrapped Cells of *Pseudomonas diminuta* in Different Carriers

| Time (h) | Residual CPC (%) | | |
|---|---|---|---|
| | Chitosan | Gelatin | Agar |
| 0 | 100 | 100 | 100 |
| 1 | 94.9 | 96.0 | 97.0 |
| 3 | 90.6 | 92.9 | 94.2 |
| 6 | 85.3 | 87.0 | 89.3 |
| 9 | 72.9 | 75.1 | 79.1 |

sporin-C acylase enzyme and also constraint provided by broth to the immobilized whole cells.

Table 2 depicts the biotransformation of CPC to 7-ACA in presence of entrapped cells of *P. diminuta* and it was noticed that maximum conversion took place with the cells immobilized in the chitosan. The Rf values calculated for CPC and 7-ACA are 0.089 and 0.241, respectively.

## Conclusions

The reusability of entrapped whole cells is investigated and it has been observed that at the end of sixth cycle of operation, approx 30% to 45% of enzymatic activity is lost using pure CPC as substrate. This loss of enzymatic activity could be regained by supplying the nutrient medium to the immobilized cells at the end of operation of each batch as performed with the crude CPC. Thus, the immobilized cells (IC) system provides more

efficient way for the biotransformation of CPC to 7-ACA and hence, the overall production of 7-ACA could be increased.

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