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Single-Step Conversion of Cephalosporin-C to 7-Aminocephalosporanic Acid by Free and Immobilized Cells of *Pseudomonas diminuta*

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Abstract

7-Aminocephalosporanic acid (7-ACA), the starting material for the production of a number of clinically used semisynthetic cephalosporins, is produced by deacylation of cephalosporin-C. The production of 7-ACA was studied in various modes, at the optimal conditions using free and immobilized whole cells of *Pseudomonas diminuta.*

Index Entries: 7-ACA; CPC acylase; GL-7-ACA; GL-7-ACA acylase; immobilization.

Introduction

Cephalosporin acylases are industrially important enzymes, which hydrolyse cephalosporins to 7-aminocephalosporanic acid (7-ACA), a key intermediate required for the production of most of the clinically used cephalosporin derivatives, i.e., semisynthetic cephalosporins *(1–3)*. Cephalosporin-C (CPC), an important β-lactam antibiotic is exclusively produced by aerobic fermentation as such exhibits negligible antimicrobial activity but substitutions on C_3 and C_7 positions of β-lactam ring along with other structures generate semisynthetic cephalosporins with diversified antimicrobial activity, e.g., cefazolin, cefotoxime, cephamandole, cephaclor, etc. The chemical methods using iminoether *(4)* or nitrosyl chloride *(5)* for 7-ACA production are tedious, time consuming, and involve multiple cost-consuming steps. Therefore, attempts have been made to produce 7-ACA by a biocatalytic process. Cephalosporin acylases are of two types depending

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on their substrate specificity: glutaryl (GL) 7-ACA acylases, also known as 7-β-(4-carboxybutanamido) cephalosporanic acid acylases *(1–3,6–11)* and cephalosporin-C (CPC) acylases *(1,2)*. GL 7-ACA is involved in three steps conversion of CPC to 7-ACA, while CPC acylase is involved in a single-step conversion. In our earlier studies, the effects of different organic compounds on the biosynthesis of CPC acylase *(12)* and batch production of 7-ACA by different microorganisms *(13)* were established. The reports on production of 7-ACA with immobilized cells are very few and, therefore, the production of 7-ACA with immobilized whole cells of *Pseudomonas diminuta* has been studied in different carriers. The immobilization of microbial cells involved in the production of useful compounds is now gaining more importance than immobilized enzymes because it eliminates the need for the release of intracellular enzymes and, thus the succeeding purification steps. By immobilizing microbial cells, it is possible to maintain high cell concentration, high flow rates during continuous production, and reuse of immobilized cells. The whole cells of *P. diminuta* were immobilized by entrapment in different carriers, i.e., chitosan, gelatin, and agar, and then evaluated for the kinetic behavior. In this communication, the production of 7-ACA using free and immobilized whole cells of *P. diminuta* is reported and a comparison has also been made.

Materials and Methods

P. diminuta NCIM 2865 was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune (India). The microorganism was selected for the production of 7-ACA because of its CPC acylase activity involved in the single step conversion of CPC to 7-ACA. The microorganism was maintained on medium containing 10 g/L peptone, 10 g/L yeast extract, 5 g/L sodium glutamate, 2.5 g/L sodium chloride, and 20 g/L agar. The pH was adjusted to 7.0.

The medium used for the production of CPC acylase is of Shimizu et al. *(14)* supplemented with 0.15% glucose and an optimum concentration of glutaric acid (0.02%). The seed media used for the propagation of microorganism contained: 10 g/L beef extract, 10 g/L peptone, 5 g/L 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 4861*g* for 10 min. The supernatant was discarded and the cell pellet, after washing with buffer, was immobilized in different carriers such as chitosan, gelatin, and agar *(15–18)*, respectively by entrapment method and used for production of 7-ACA.

The cells were grown in the production medium for 36 h; cell growth (biomass) was determined by measuring the optical density at 660 nm and converted to dry cell weight (DCW) using a standard curve *(14)*. Glucose concentration was measured by dinitrosalicylic acid (DNS) method of

Miller *(19)*. Protein concentration was determined by the method of Lowry et al. *(20)* using bovine serum albumin as standard. The enzyme activity was determined by mixing 5 mg/mL of cephalosporin-C in 0.1 *M* phosphate buffer (pH 7.0) with 3.2 mg of lyophilized cells for 3 h at 37°C. The reaction was stopped by adding 4% (v/v) acetic acid and centrifuged at 4861*g* for 10 min. The 7-ACA formed after hydrolysis of CPC was determined by the colorimetric method of Marrelli *(21)* and by thin layer chromatography with solvent system butanol–acetic acid–water in the ratio of 4:1:5. 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

It has been found that the cephalosporin-C acylase enzyme is intracellular in nature because the activity is restricted only within the cells and not in the broth. Preliminary experiment during the characterization of CPC acylase enzyme showed that the pH of 7.4 and temperature of 37°C were optimal for maximum production of 7-ACA *(13)*. Furthermore, the experiments were performed to optimize the substrate concentration, production of 7-ACA by whole cells (intact cells) and cell free extracts of *P. diminuta,* optimization of different parameters for immobilized whole cells, and batch production of 7-ACA using immobilized cells of *P. diminuta* in different immobilizing agents.

Figure 1 shows the growth kinetics of *P. diminuta* carried out in a 2-L environmentally controlled bioreactor (Bioengineering, A.G.). The samples were withdrawn aseptically at different time intervals and analyzed for dry cell mass, glucose concentration, and enzyme activity. After a period of 6 h where no significant increase in cell mass is evident (lag phase), a period of rapid growth ensues where the cell mass increases exponentially with time (exponential phase). The maximum cell mass (dry cell weight) was obtained during the end of exponential phase. The concentration of glucose declines rapidly and exhausted at approx 20 h. It is also clear that the synthesis of enzyme starts only in the beginning of exponential phase of the growth. No activity was observed during the lag phase of the growth and reached its maximum value in the stationary phase.

Table 1 represents the effect of substrate concentration on the production of 7-ACA at the optimal conditions mentioned earlier. It has been observed that the production of 7-ACA increases with an increase in CPC concentration (substrate) up to 7.5 mg/mL and no appreciable raise in 7-ACA content was observed above this substrate concentration.

Again, the velocity of above mentioned reactions were calculated for various substrate concentrations and plotted in Fig. 2. A double reciprocal plot of V against S (computed from Fig. 2) was further determined and shown in Fig. 3. The values of K_m and V_{max} calculated are 1.25 m$M \times 10^{-2}$ and 5.0 U $\times 10^{-2}$, respectively.

In another experiment, the production of 7-ACA was carried out by using different modes of cells (whole cells, as well as cell-free extracts

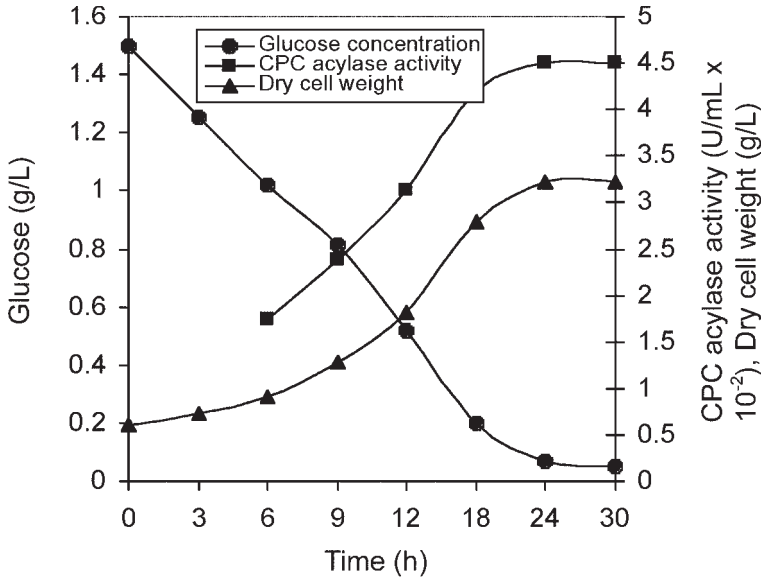

Fig. 1. Growth kinetics and CPC acylase activity of *P. diminuta*.

Table 1
Effect of Substrate Concentration on the Production of 7-ACA

Substrate (mg/mL)	7-ACA (m*M*/mL × 10^{-2})
1.25	2.72
2.50	4.45
5.00	6.89
7.50	9.74
10.00	9.88
12.50	9.94
15.00	10.20

obtained by sonication and lysozyme treatment) and the results obtained are presented in Fig. 4. The cells were lyzed with lysozyme (0.1%) at room temperature for 1 h and by means of ultrasonication at 4°C with intermittent pulsing (Sonicator, Virsonic 475, Virtis Company Inc.). The clear lysate after centrifugation was used for production of 7-ACA. It is observed from Fig. 4 that the production of 7-ACA by whole cells of *P. diminuta* increases with time and becomes almost constant after 5 h of incubation, whereas, with sonicated and lysozyme treated cells, the concentration of 7-ACA increases with time up to 4 h and becomes constant thereafter. Figure 4 also indicates that the maximum production of 7-ACA is achieved with cell-free extracts of lysozyme treated cells followed by sonication as compared to whole cells. The lower concentration of 7-ACA using whole cells is due to

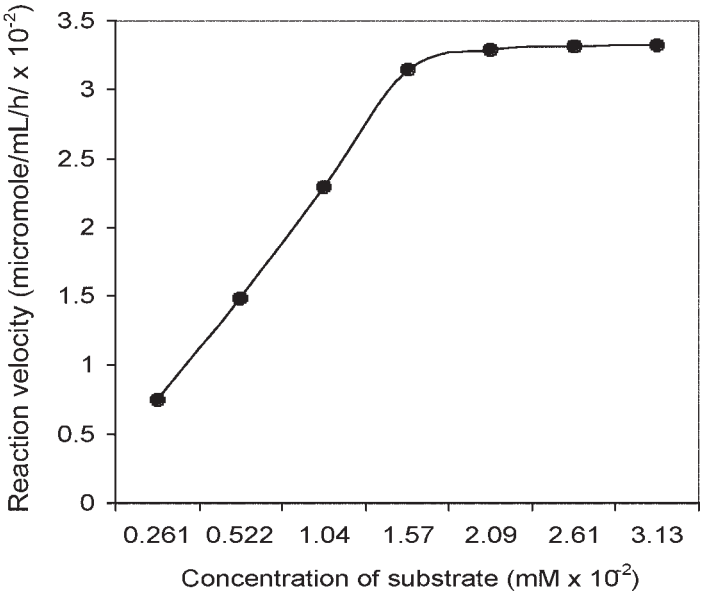

Fig. 2. Plot of reaction velocity and substrate concentration.

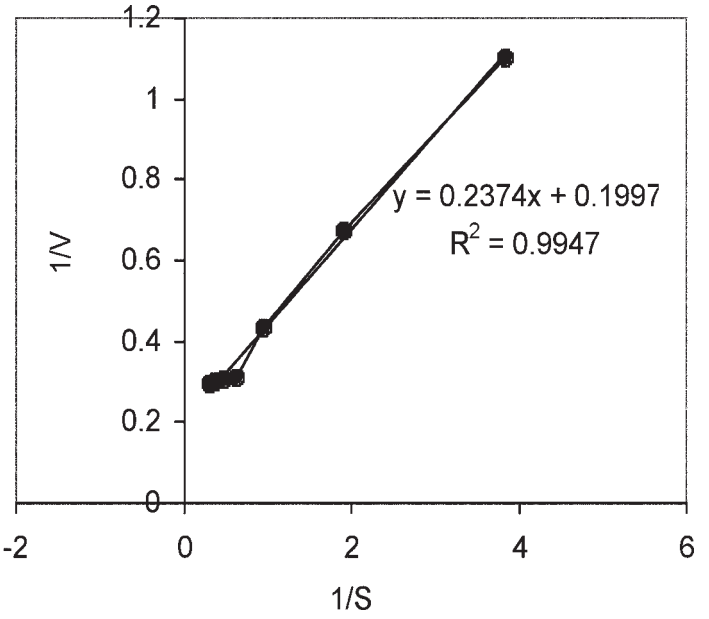

Fig. 3. Double reciprocal plot of V and S.

the resistances offered by cell wall and the membrane of cells, hindering the reaction between the enzyme and substrate. This also proves the intracellular nature of enzyme CPC acylase.

Figure 5 shows the pH activity profile of immobilized whole cells of *P. diminuta* carried out by varying the pH of 0.1 *M* phosphate buffer in the

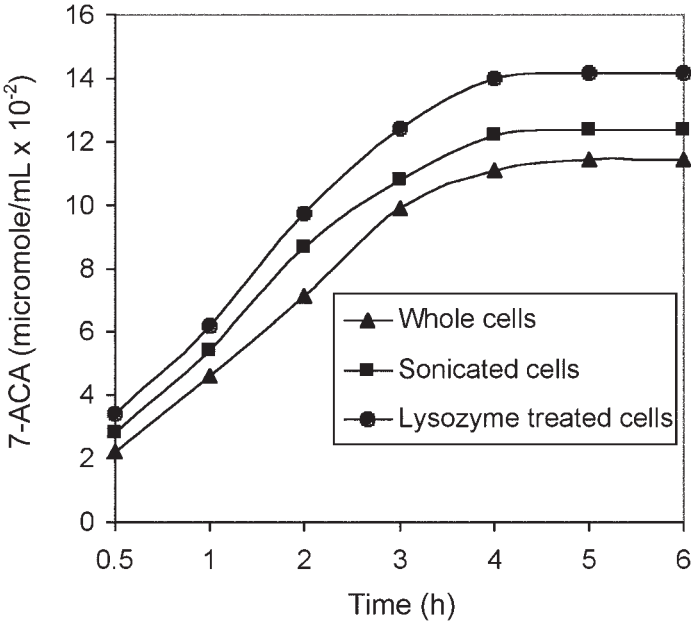

Fig. 4. Production of 7-ACA by whole cells and cell free extracts of *P. diminuta*.

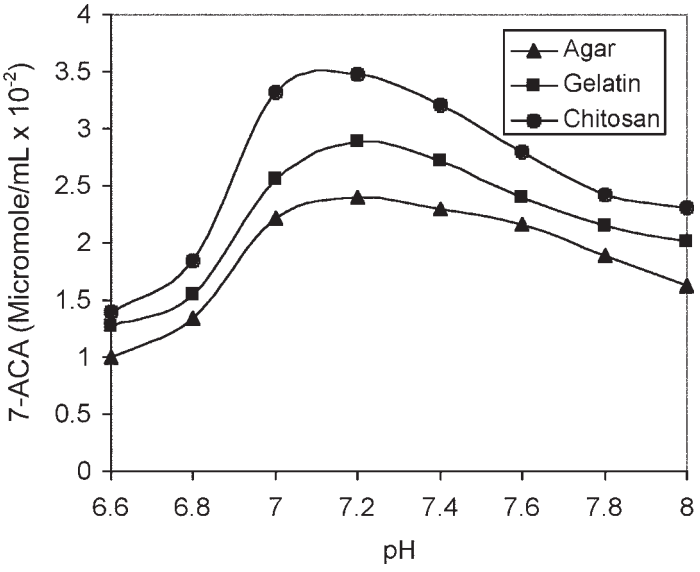

Fig. 5. Effect of pH on the production of 7-ACA by immobilized cells of *P. diminuta*.

range of 6.6 to 8.0. The production of 7-ACA rose with increase in pH of the buffer from 6.6 to 7.2 and then decreased. The maximum production was observed with chitosan, whereas the least production was reported with agar. Thus, the optimal pH for the production of 7-ACA with immobilized whole cells is 7.2. The difference in activity of enzyme with different immo-

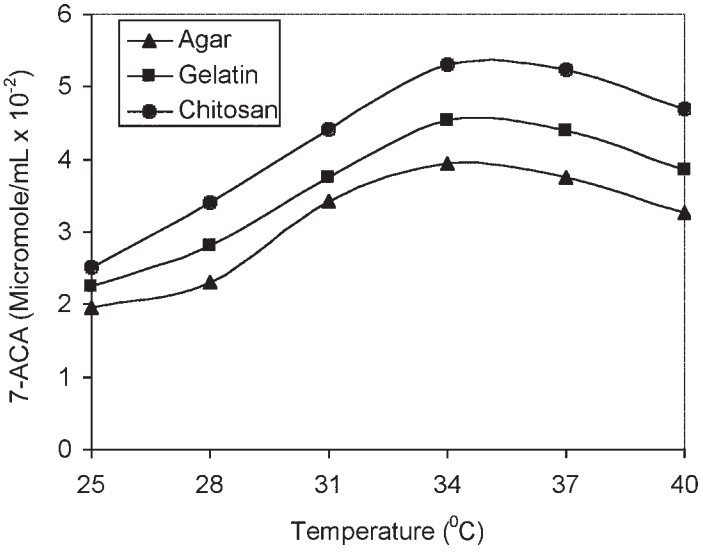

Fig. 6. Effect of temperature on the production of 7-ACA by immobilized cells of *P. diminuta*.

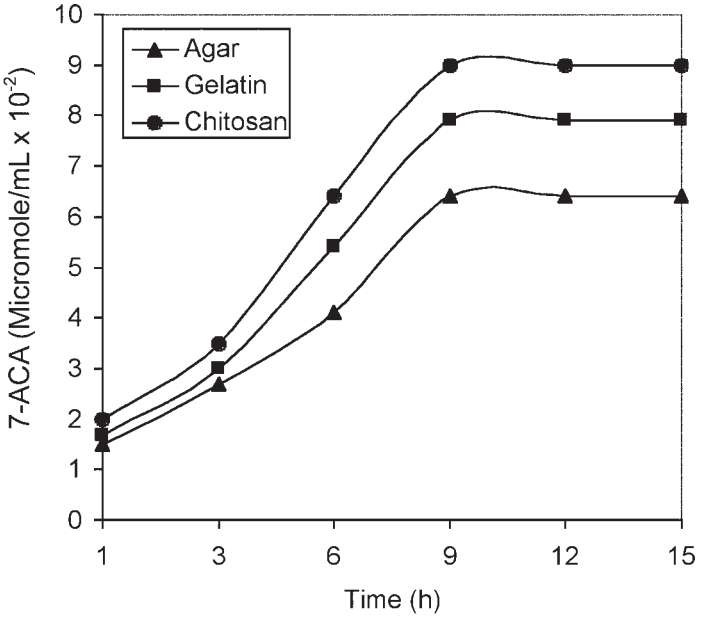

Fig. 7. Production of 7-ACA by immobilized cells of *P. diminuta*.

bilizing supports might be owing to any one of the causes; structural differences of matrix, method of entrapment and other associated phenomenon.

The effect of temperature on the production of 7-ACA using immobilized cells of *P. diminuta* in different carriers was carried out as standard assay conditions by varying the temperature of incubation from 25°C to 40°C and the temperature activity profile is shown in Fig. 6. It is clear from

the figure that maximum production of 7-ACA was obtained at 34°C (optimum temperature) with immobilized whole cells. The temperature activity profile is broadened compared to free cells. This might be due to the increase in the thermal stability when cells are immobilized.

The batch production of 7-ACA utilizing immobilized whole cells of *P. diminuta* in different carriers was carried out at the optimal conditions and the results obtained are shown in Fig. 7. It indicates that the production of 7-ACA increases with time and becomes constant after 9 h of incubation. Maximum production of 7-ACA was observed with the support chitosan followed by gelatin, whereas the least 7-ACA production was found with agar as a carrier. The production of 7-ACA using immobilized cells is lower than that of free cells. It can be explained on the basis of mass transfer limitation of substrate CPC to the immobilized cells. The production of 7-ACA is 76% in chitosan, 67% in gelatin, and 55% in agar as compared to the free cells.

Conclusions

In this communication, the production of 7-ACA using different modes of cells was investigated and a comparison between the performance of free cells and immobilized whole cells have been made. The yield of 7-ACA was higher with lysozyme treated and sonicated cells as compared to whole (intact) cells. The production with immobilized cells was lower than that of free cells, which might be the result of the diffusion barrier. The maximum production of 7-ACA was obtained with chitosan, followed by gelatin and agar as immobilizing agents.

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