0273-2289/00/87/0135/$11.50

Immobilized *Streptomyces clavuligerus* NP1 Cells for Biotransformation of Penicillin G into Deacetoxycephalosporin G

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Received October 1, 1999; Revised December 1, 1999; Accepted December 1, 1999

Abstract

An investigation was conducted to determine whether immobilized resting cells of *Streptomyces clavuligerus* NP1, entrapped on a polymeric matrix, are able to perform oxidative ring expansion of benzylpenicillin into deacetoxycephalosporin G by virtue of their deacetoxycephalosporin C synthase ("expandase") activity. Cells entrapped in polyethyleneimine-barium alginate (1.5%) were able to sustain activity for at least four 2-h cycles, whereas free resting cells were inactive after the second cycle. Although entrapped cells exhibited lower oxidative ring expansion activity than free resting cells, immobilization may offer storage stability, recyclability, and operational stability for biotransformation of penicillins to cephalosporins, thus contributing to the development of a biological means for the production of the important industrial intermediate 7-aminodeacetoxycephalosporanic acid.

Index Entries: Penicillins; cephalosporins; biotransformation; immobilized cells; β-lactam antibiotics.

Introduction

For bioprocessing purposes, an increase in the stability of biocatalysts (enzymes and cells) is quite often a result of immobilization *(1–4)*. This technology is an attractive alternative to expensive and polluting multistep chemical processes, allowing recycling of expensive enzymes and cofactors, and coordinating multistep enzymatic processes into a single operation.

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There have been several studies on the use of cells and/or enzymes in the production of penicillins and cephalosporins *(5–10)*. Biosynthesis of cephalosporin C using immobilized cells of *Streptomyces clavuligerus* NRRL 3585 was accomplished by Freeman and Aharonowitz *(11)*. Jensen et al. *(12)* reported on the immobilization of β-lactam synthesizing enzymes from the same strain. None of these previous studies used penicillin substrates other than the normal intermediate penicillin N, such as penicillin G (benzylpenicillin). Recently, however, we have elucidated conditions allowing ring expansion of penicillin G and many other penicillins by cells and extracts of *S. clavuligerus (13–15)*. The present study focuses on the biotransformation of benzylpenicillin into deacetoxycephalosporin G by gel-entrapped cells of *S. clavuligerus* strain NP1 containing the enzyme deacetoxycephalosporin C synthase ("expandase"). Strain NP1 is a mutant of strain NRRL 3585 that produces only a trace of β-lactam antibiotics *(8)*.

Materials and Methods

Materials

Alginic acid sodium salt was from Aldrich (Milwaukee, WI). Agarose Type VII (low gelling temperature 50% polyethyleneimine [PEI], penicillin G, ascorbic acid, and α-ketoglutaric acid were from Sigma (St. Louis MO). Deacetoxycephalosporin G was a gift from Antibioticos, S.p.A. (Leon, Spain). Bactopenase was from Difco (Detroit, MI).

Culture Conditions

A seed culture of *S. clavuligerus* mutant NP1 (ATCC 700751) was made by thawing a frozen preparation and inoculating 40 μL into 40 mL of MST medium (containing 90 m*M* MOPS buffer, pH 7.0; 1% starch; and 3% trypticase soy broth medium without dextrose) (BBL Becton Dickinson, Cockeysville, MD) in 250-mL baffled flasks. The flasks were incubated for 2 d at 30°C and 250 rpm. One milliliter of the seed culture was used as inoculum for the main culture, which contained 80 mL of MST medium in 500-mL unbaffled flasks. The flasks were incubated for 24 h at 30°C and 250 rpm.

Preparation of Resting Cells

Cells were harvested by centrifugation at 14,000*g* for 15 min at 4°C and washed twice with cold deionized water. Washed cells were resuspended in 10 mL of water, giving a concentration (wet wt) of about 300 mg/mL.

Preparation of PEI–Barium Alginate–Entrapped Cells

Resting cells from a 1-d-old culture of *S. clavuligerus* NP1 (2 g of wet wt) were directly resuspended in 20 mL of 1.5% (w/v) sodium alginate solution. The resulting sodium alginate cell suspension was placed into a 10-mL plastic syringe with a 26.6-gage needle. The suspension was added

drop by drop into a slowly stirring hardening solution of 2% (w/v) barium chloride containing 1% (w/v) PEI, giving beads of about 1.5–2.0 mm in diameter. The beads were filtered on a plastic net, washed twice with water, suspended in 0.05 *M* MOPS buffer (pH 6.5) to a volume of 100 mL, and stored at 4°C until used (usually within 5 h).

Bioconversion Reaction

The standard reaction mixture (10 mL) contained 0.05 *M* Tris-HCl buffer (pH 7.4), 8.0 m*M* KCl, 8.0 m*M* $MgSO_4 \cdot 7H_2O$, 4.0 m*M* ascorbic acid, 1.8 m*M* $FeSO_4 \cdot 7H_2O$, 1.28 m*M* α-ketoglutaric acid, 200 mg of penicillin G, and 4.0 mL of free-cell suspension containing 1.4 g (wet wt) of cells, or 3.4 g (wet wt) of cell-containing barium alginate beads containing the same weight of cells. The order of addition of the components was as previously described *(7)*. The reaction started when penicillin G was added to the reaction mixture, which was incubated at 30°C and 220 rpm for 1–12 h. Experiments were reproducible to the extent of ±15%.

Bioassay of Product Formation

Product formation was estimated by the paper disk–agar diffusion bioassay as previously described *(13)*, using deacetoxycephalosporin G as standard and penicillinase to destroy the substrate penicillin G. *Escherichia coli* Ess *(16)*, a β-lactam supersensitive mutant, was used as the assay microorganism.

Results

Immobilized vs Free Resting Cells

The oxidative ring expansion of benzylpenicillin by free and entrapped resting cells was compared. As shown in Fig. 1, immobilized cells were able to perform the expandase reaction although more slowly and less extensively than free cells. Both types of cells virtually ceased production at 2 h. This is similar to the cessation of activity previously reported by Adrio et al. *(14)* with cell-free extracts.

Effect of Biomass Concentration on Product Formation by Immobilized Cells

Different biomass amounts (2, 4, and 6 g wet wt) were suspended in 20 mL of alginate solution and PEI–barium alginate–entrapped cells were prepared and tested for ring expansion. Figure 2 shows that increasing biomass concentration resulted in increased product formation. Again, the reaction virtually ceased after 2 h.

Activity During Sequential Cycles of Reaction and Washing

Free and immobilized resting cells were allowed to carry out oxidative ring expansion for 2 h. Then the entire reaction mixture was centrifuged at

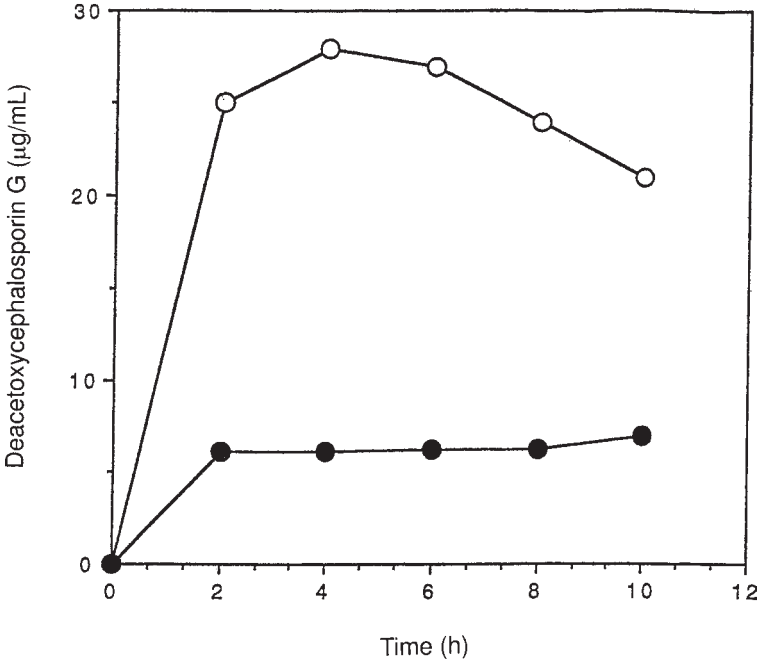

Fig. 1. Comparison of expandase reaction using free resting cells (○) vs cells entrapped in PEI–barium alginate (●).

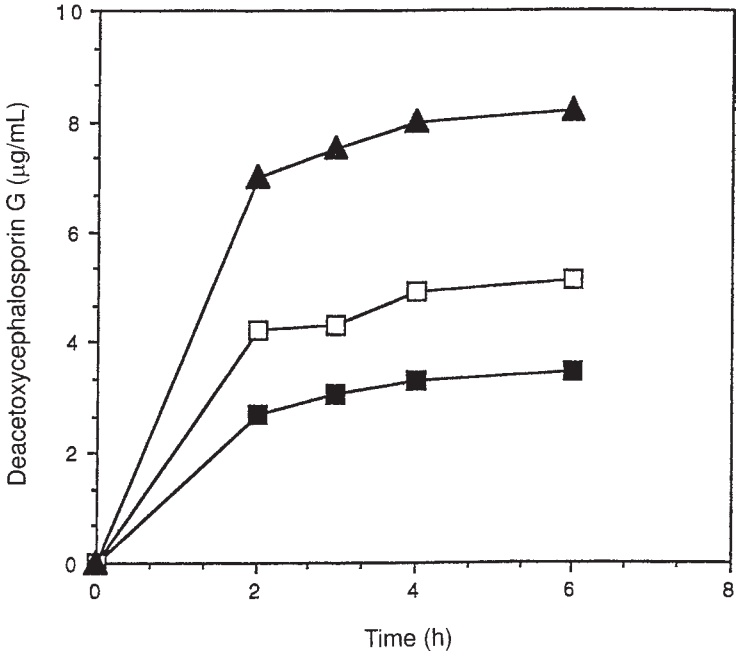

Fig. 2. Effect of biomass concentration on expandase reaction using entrapped resting cells. A constant amount of cell-entrapped beads (3.4 g of wet wt/10 mL of reaction mixture) containing 0.4 g of cells (wet wt) (■); 0.78 g of cells (□); and 1.2 g of cells (▲).

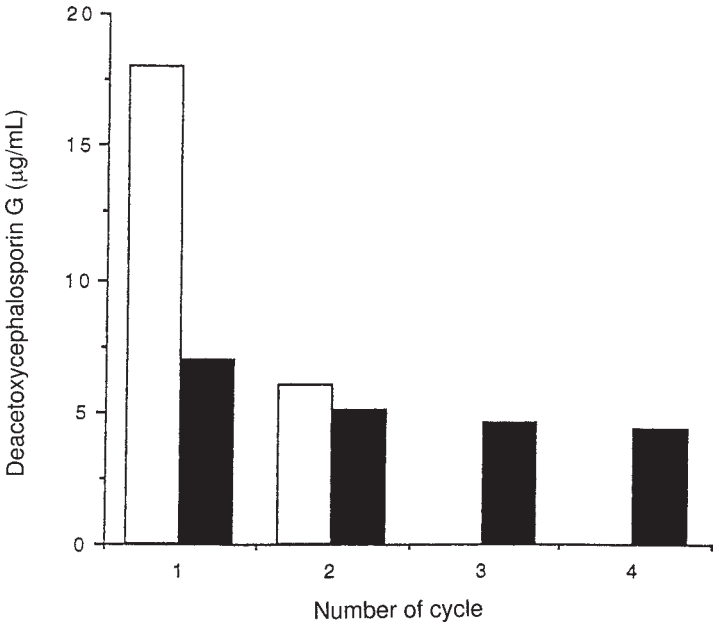

Fig. 3. Deacetoxycephalosporin G produced during sequential 2-h cycles of incubation with washing of cells between cycles using free (□) and immobilized cells (■).

3500*g* and 4°C for 5 min. The cells were washed with 0.05 *M* MOPS buffer (pH 6.5) and recentrifuged. The expandase reaction was again initiated. Two-hour cycles were done up to four times with assays of product formation done at the end of each cycle. As indicated in Fig. 3, the activity of free cells was reduced by about 60% from the first to the second cycle, and completely lost after the second cycle. By contrast, immobilized cells experienced only a small reduction in activity at each cycle and still had activity through four cycles.

Effect of Type of Matrix

Other polymeric matrices such as agarose (4% [w/v]) and κ-carrageenan (3% [w/v]) were also evaluated but neither gave activity. This may be owing to reaction impedance resulting from strong diffusional limitations. Such external and internal barriers would be expected to reduce catalytic efficiency.

Discussion

The results obtained demonstrate the capacity of resting cells entrapped in PEI–barium alginate to perform oxidative ring expansion of benzylpenicillin to deacetoxycephalosporin G. Product formation rate was higher with free than with entrapped resting cells, probably owing to strong diffusional limitations that prevent the expandase enzyme from binding the substrate and cofactors and/or preventing oxygen transfer to the interior of the beads.

With free cells, diffusional limitation and mass transfer problems are less important than with immobilized systems. Similar behavior was reported by Freeman and Aharonowitz *(11)*, who compared free and immobilized cells of *S. clavuligerus* NRRL 3585 in linear polyacrylamide gels for the formation of cephalosporin C in fermentations. They observed that free cells produced higher levels of cephalosporin C than immobilized cells. We found that cells entrapped in alginate were capable of performing the expandase reaction in four consecutive batch runs, whereas the activity of the resting cells was completely lost after two cycles of activity and washing. Although we still do not understand the basic mechanism for activity loss *(15)*, we are encouraged by the stability of the entrapped cell preparation. Further work will be focused on improving the activity of the immobilized cell preparation.

Acknowledgments

We thank Aiqi Fang for advice and technical assistance with the preparation of the manuscript. We also thank Antibióticos for financial assistance and for supplying the deacetoxycephalosporin G and Ermanno Bernasconi for encouragement. We acknowledge the Organization of American States Scholarship Department, Washington, DC, and the Instituto Tecnólogico y de Estudios Superiores de Monterrey-Campus Monterrey, México, for financial support to M. A. Báez-Vásquez, during his postdoctoral work at Massachusetts Institute of Technology.

References

1. Fukui, S. and Tanaka, A. (1982), *Annu. Rev. Microbiol.* **36,** 145–172.
2. Takata, I., Kayashima, K., Tosa, T., and Chibata, I. (1982), *J. Ferment. Technol.* **60,** 431–437.
3. Vandamme, E. J. (1983), *Enzyme Microb. Technol.* **5,** 403–416.
4. Bahulekar, R., Ayangar, N. R., and Ponrathnam, S. (1991), *Enzyme Microb. Technol.* **13,** 858–868.
5. Demain, A. L. (1983), in *Antibiotics Containing the β-Lactam Structure*, Demain, A. L. and Solomon, N. A., eds., Springer-Verlag, Heidelberg, pp. 189–228.
6. Kupka, J., Shen, Y., Wolfe, S., and Demain, A. L. (1983), *Can. J. Microbiol.* **29,** 488–496.
7. Shen, Y., Wolfe, S., and Demain, A. L. (1984), *J. Antibiot.* **37,** 1044–1048.
8. Mahro, B. and Demain, A. L. (1987), *Appl. Microbiol. Biotechnol.* **27,** 272–275.
9. Zhang, J. and Demain, A. L. (1992), *Appl. Biochem. Biotechnol.* **37,** 97–110.
10. Demain, A. L. and Fang, A. (1995), *Actinomycetologia* **9,** 98–117.
11. Freeman, A. and Aharonowitz, Y. (1981), *Biotechnol. Bioeng.* **23,** 2747–2759.
12. Jensen, S. E., Westlake, D. W. S., and Wolfe, S. (1984), *Appl. Microbiol. Biotechnol.* **20,** 155–160.
13. Cho, H., Adrio, J. L., Luengo, J. M., Wolfe, S., Ocran, S., Hintermann, G., Piret, J. M., and Demain, A. L. (1998), *Proc. Natl. Acad. Sci. USA* **95,** 11,544–11,548.
14. Adrio, J. L., Cho, H., Piret, J. M., and Demain, A. L. (1999), *Enzyme Microb. Technol.* **25,** 497–501.
15. Báez-Vásquez, M. A., Adrio, L. J., Piret, J. M., and Demain, A. L. (1999), *Appl. Biochem. Biotechnol.* **81,** 145–152.
16. Kohsaka, M. and Demain, A. L. (1976), *Biochem. Biophys. Res. Commun.* **70,** 465–473.