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PRODUCTION OF 6-AMINOPENICILLANIC ACID BY IMMOBILIZED PLEUROTUS OSTREATUS+

Michael Kluge[1], Joachim Klein[1] and Fritz Wagner[2]

[1] Institute of Chemical Technology, Technical University Braunschweig, Hans-Sommer-Strasse 10, D 3300 Braunschweig, Fed. Rep. of Germany

[2] Chair of Biochemistry and Biotechnology, Technical University Braunschweig, Konstantin-Uhde-Str. 8, D 3300 Braunschweig, Fed. Rep.Germany

SUMMARY

6-Aminopenicillanic acid from penicillin V is produced by *Pleurotus ostreatus* immobilized by entrapment in a chitosan matrix. In these carriers the cell concentration increases after network formation by irreversible shrinking of the biocatalyst. Specific activity of the biocatalyst for the hydrolysis reaction is 1,31 $\mu mol.min^{-1}$. (g wet weight of catalyst)$^{-1}$ corresponding to a relative activity of 38%. Catalytic half-life of immobilized *Pl. ostreatus* is 25 days compared to 2.5 days for free suspended cells.

INTRODUCTION

6-Aminopenicillanic acid (6-APA) can be obtained by enzymatic penicillin amidohydrolysis of penicillin G, which is a good substrate for bacteria (Klein and Wagner 1980, Sato *et al.*, 1976) or penicillin V, which is favourably hydrolyzed by fungi (Stoppok *et al.*, 1980, Stoppok *et al.*, 1981). Enzymatic hydrolysis maintains the β-lactam ring, which is responsible for the antibiotic effect, so that 6-APA can be used as basis for new semisynthetic antibiotics. Immobilization of whole cells avoids isolation of the enzyme and gives a biocatalyst with better handling, high mechanical stability, and a catalytic half-life, which is longer than for free cells. A suitable immobilization method is selected for the fungus *Pleurotus ostreatus.*

MATERIALS AND METHODS

MATERIALS

P. ostreatus (Stoppok *et al.*, 1981) was cultured by E. Stoppok. Chitosan was purchased from KYOWA OIL & FAT CO. LTD. Tokio/Chiba, Japan. Penicillin V.K was a commercial sample.

IMMOBILIZATION

Before immobilization the pellets of *P. ostreatus* had to be slashed. 10 g pellets were suspended in 10 ml NaCl solution (0.9%) and stirred by a turbomixer. The mycelium was filtered and washed repeatedly with small portions of NaCl solution (0.9%). Loss of activity after all these operations was about 25%.

The immobilization was performed according to a procedure developed in our laboratory (Vorlop and Klein 1981). 1.5 g chitosan were mixed with 130 ml

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hot water stirring powerfully and dissolved within 1 h by adding 0.5 ml acetic acid (100%). The resulting pH of this solution was 5.5. Then 2.5 g shredded mycelium were moistened with 2.5 g glycerin and chitosan acetate solution was added little by little giving 50ml suspension. The biocatalyst was prepared by dropping the suspension through a cannula (0.9 mm diameter) of a syringe into 500 ml of a solution, which solidifies the suspension droplets immediately. The precipitating solution was Na tripolyphosphate (2%) or $K_4[Fe(CN)_6].3H_2O$ (3%), whose pH values were adjusted to 5.5 by phosphoric acid or HCl. The particle size of the biocatalyst usually was 2-3 mm, but particles in the range 1-2 mm could also be obtained by blowing the droplets from the top of the cannula with a concentric stream of air.

In Na tripolyphosphate solution the pH was shifted to 8.3 with NaOH after 30 min. Thereby chitosan was discharged and the particles shrank. After 4 h the biocatalyst was filtered and washed repeatedly with water.

In $K_4[Fe(CN)_6].3H_2O$ solution the pH was not shifted and the biocatalyst was filtered after 1 h and washed repeatedly with water. Drying in air at 20°C let the particle shrink irreversibly to a diameter of 0.8 mm.

Biocatalysts cross-linked by ions are stable in basic solutions but dissolve in acid solutions. To cross-link chitosan covalently the biocatalyst was put into 0.1 M phosphate buffer (pH 7.0) with addition of glutaraldehyde (0.5%) for 3 h.

ANALYTICAL PROCEDURE

Specific activity of the mycelium is related to a g dry weight, which was determined at 40°C in a vacuum drier. After shredding of the mycelium 75% of the original enzymatic activity could be immobilized. Relative activity of immobilized cells is related to this value. Before testing for chitosan biocatalyst activities the pH of the matrix had to be adjusted to the required value by stirring the biocatalyst in a pH-stat device in phosphate buffer until self-titration of the matrix was complete.

Specific activity of immobilized cells was tested by pH-stat titration of 6-APA in 0.1 M phosphate buffer (pH 7.0) with 4% penicillin V at 37°C. Measurement values were corrected for hydrolysis of the β-lactam ring in accordance to reaction conditions.

Wash-out of enzymes from immobilized cells during immobilization was tested at standard reaction conditions by adding penicillin V to a sample of filtered precipitating solution. Comparing with a blank test no enzymatic activity was found.

Wash-out of enzymes during reaction was tested after the biocatalyst had been filtered. About 1% of the immobilized activity had been washed-out during 24 h.

RESULTS AND DISCUSSION

Table 1 shows the specific activity of biocatalysts obtained by different

immobilization methods. Cell concentration in the chitosan carrier becomes advantageously high, since this matrix shrinks irreversibly. Relative activity depends on the loss of activity during immobilization and on the method of immobilization. Relative activity as well as absolute activity increases in the following sequence:

polyphosphate/glutaraldehyde < hexacyanoferrate < polyphosphate

Time dependence of activity of free cells as well as of immobilized cells was tested by consecutive batch reactions in penicillin V solution (4%) without buffer. To reduce the swelling of the matrix which is caused by the pH-gradient of 6-APA inside the matrix the pH of the solution was changed to 7.3. Conversion within 24 h fell from 81% at the first day to 40.5% at the 25th day. After each run of 24 h duration the penicillin V solution was replaced by fresh one.

TABLE 1 *Pleurotus ostreatus* immobilized in chitosan

crosslinking agent	specific activity of shredded mycelium	cell conc.	specific activity of biocat-alyst	relative activity
	o	x	+	(%)
polyphosphate-glutaraldehyde	29.2	0.176	0.83	16.1
hexacyanoferrate	29.2	0.134	1.03	26.3
polyphosphate	33.2	0.103	1.31	38.0

o : $\mu mol \cdot min^{-1} \cdot (g\ dry\ weight\ of\ cells)^{-1}$

x : $(g\ dry\ weight\ of\ cells) \cdot (g\ wet\ weight\ of\ catalyst)^{-1}$

+ : $\mu mol \cdot min^{-1} \cdot (g\ wet\ weight\ of\ catalyst)^{-1}$

The catalytic half-life was only 2.5 days for free suspended cells compared to 25 days for immobilized cells in chitosan cross-linked by polyphosphate. The biocatalyst treated with glutaraldehyde had a catalytic half-life of only 10 days (Fig. 1).

The decay constant k_d was calculated by the following equation

$$v'_t = v'_{t_o} \cdot e^{-k_d \cdot t} \qquad \text{(Equ.1)}$$

The catalytic half-life of 25 days of the biocatalyst cross-linked by polyphosphate corresponds to a decay constant $k_d = 0.028\ (1.day^{-1})$. Productivity within this period (Equ..2) calculated by integration of (Equ.1) was 27 mmol 6-APA . (g wet weight of catalyst)$^{-1}$ or 5.8 g 6-APA. (g wet weight of catalyst)$^{-1}$

$$P_{t_{1/2}} = \frac{v'_{t_o}}{2 \cdot k_d} \qquad \text{(Equ.2)}$$

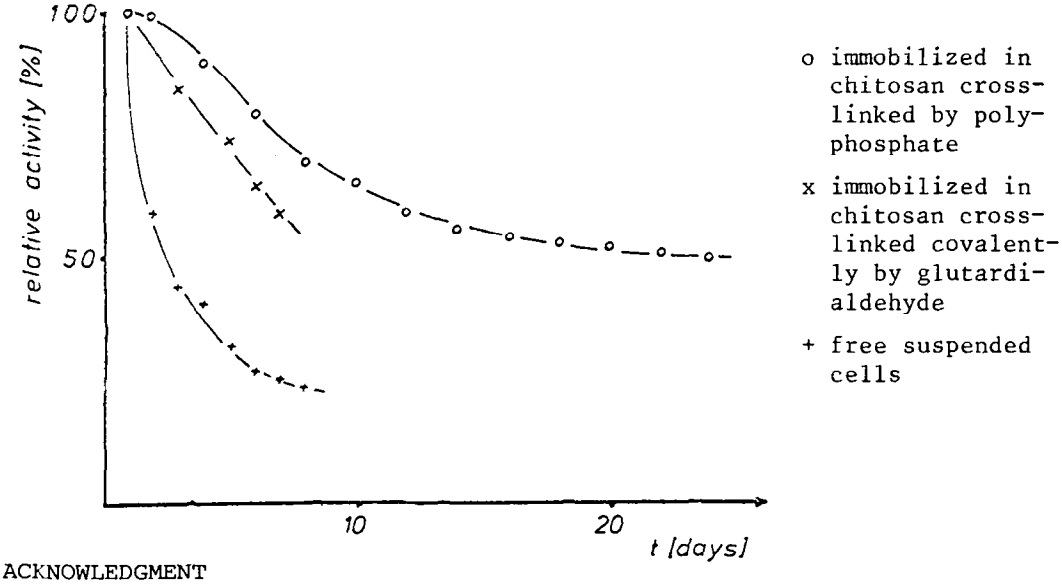
Immobilization enhances the catalytic half-life of cells tenfold. Considering the relative activity of 38% of immobilized cells the productivity within this period increases by a factor of 3.8 compared to free cells.

Cross-linking of chitosan by glutaraldehyde gave an insoluble biocatalyst with high mechanical stability. Disadvantages of this method were lower activity and shorter catalytic half-life and thus much lower productivity.

In order to estimate the influence of diffusional limitation on the reaction, diffusion of penicillin V inside the biocatalyst was measured without interference of reaction. After enzyme had been denaturated by heat the biocatalyst was stirred in 0.11 M penicillin V solution for 120 min. Wash-out of penicillin V was measured by photometry (276 nm) (Klein and Washausen 1979).

Calculation of catalytic effectiveness factor showed that there was no diffusional limitation using this strain of P. ostreatus. The influence of diffusional limitation became dominant, when a strain with high specific activity was immobilized. In this case the specific activity became strongly particle size dependent. On reducing the particle size to a diameter of 0.5 mm, diffusional limitation was decreased and the resulting specific activity increased to 30 $\mu mol.min^{-1}.(g\ wet\ weight\ of\ catalyst)^{-1}$

Fig. 1 Time dependence of catalytic activity of free and immobilized cells of Pl. ostreatus (4 % penicillin V; pH 7,3; 37 °C; each run 24 h)



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