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Studies of Repeated Fed-Batch Fermentation of Cephalosporin C in an Immobilized Cell Bioreactor

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Acremonium chrysogenum was immobilized in ionotropic gel beads to develop semi-continuous production of cephalosporin C (CPC). Barium alginate beads were more stable than calcium alginate or strontium alginate beads in chemically defined media. The gel stability of Ba-alginate was further increased by cross-linking with polyethyleneimine (PEI). The presence of carboxymethyl cellulose inside Ba-alginate beads did not reduce mass transfer resistance. Ba-alginate microbeads that had little diffusion limitation increased CPC production rate 1.6 fold higher than that of normal beads. CPC fermentation with immobilized cells in Ba-alginate microbeads was performed continuously for 40 days by way of repeated fed-batch operations. Mathematical modeling was developed to describe the repeated fed-batch fermentation system. Results of the computer simulation agreed well with the experimental data, which made it possible to predict an optimal feeding rate that could maximize total CPC productions.

The most important advantage of an immobilized cell bioreactor over a free cell bioreactor is perhaps the availability of continuous process with high dilution rates, and hence high productivity, since the cells cannot be washed out from the reactor. Despite this advantage of the immobilized cell bioreactor, the development of a continuous process for antibiotic production has been rather slow. This may be due to the technical difficulties in maintaining high yields of antibiotic production. The major drawback of cell immobilization is the restriction of substrate transport inside the particles by diffusion (7, 11). Oxygen diffusion is especially important for the production of cephalosporin C (CPC), an industrially important antibiotic, in an immobilized cell system (2, 5). It has been reported that such a diffusion limitation of oxygen could be overcome by decreasing the diameter of the gel beads (10) or by improving internal pore sizes of gel matrices (9).

Calcium alginate gel is one of the most widely used carriers for cell immobilization. The Ca-alginate gel matrix has, however, a drawback that is easily dissolved in culture media containing high concentrations of monovalent cations. Replacement of calcium ions with barium (8) and strontium ions (4) or cross-linking alginate molecules with polyethyleneimine (1) has been reported to increase the stability of alginate gel matrix. To develop a continuous bioprocess of CPC fermentation, the present paper describes the modification of alginate gel matrices that increased gel stability without generating further diffusion limitation and the expression of a mathematical model developed for a repeated fed-batch fermentation system.

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Key words: cephalosporin C, repeated fed-batch fermentation, immobilized cell bioreactor, mathematical modeling

MATERIALS AND METHODS

Microorganism

A wild-type *Acremonium chrysogenum* (KCTC 1220) was purchased from Korean Collection for Type Cultures and incubated in a complex medium containing yeast extract (10 g/l), glucose (16 g/l), and peptone (10 g/l) at 28°C, 160 rpm.

Culture Conditions

A. chrysogenum cells were cultured in 500 ml Erlenmeyer flasks at 28°C (200 rpm) in chemically defined media, which contained per liter NH_4Cl 7.5 g, KH_2PO_4 10 g, K_2HPO_4 5 g, DL-methionine 3 g, glucose 10 g, sucrose 24 g, and a trace element solution (135 ml). The trace element solution contained in 1 liter, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ 94 mg, $CuSO_4 \cdot 5H2O$ 7.4 mg, $CaCl_2 \cdot 2H_2O$ 7.4 mg, $ZnSO_4 \cdot 7H_2O$ 27 mg, $MnSO_4 \cdot 2H_2O$ 27 mg, and $MgSO_4 \cdot 7H_2O$ 27 mg. Sugars, phosphates, and trace elements were sterilized separately. Repeated fed-batch fermentation with immobilized cells was carried out at 28°C in a 5 l fermentor (Model HDF-300, Marubishi Co. Ltd., Japan) using minimal

production media containing per liter DL-methionine 3 g, yeast extract 5 g, and glucose 10 g. Oxygen tension was kept over 30% of saturation in the culture.

Cell Immobilization

Free cells were harvested and washed with phosphate buffer. The washed cells were concentrated to be about 50 g cell dry wt/l and then mixed with sodium alginate (Yakuri Chemical Co., Japan), yielding a total of 1.5% (w/v) Na-alginate solution. The cell-alginate mixtures were dropped through a needle (No. 18 gauge) to various hardening solutions containing 2% (w/v) of $CaCl_2$, $BaCl_2$, or $SrCl_2$. Polyethyleneimine (PEI)-hardening solutions were prepared by mixing 1.0 ml of 50% PEI (MW50,000, Sigma) with 99 ml of each hardening solution. Carboxymethyl cellulose (CMC)-alginate beads were made by dropping 0.5% (w/v) CMC and 1% (w/v) Na-alginate mixtures to each PEI-hardening solution. Alginate beads were cured for 1 hour at 25°C. The average diameter of the normal beads was 3.0±0.3 mm. Micro-alginate beads were prepared by blowing sterile air in the outer tube when alginate mixtures were dropped into hardening solutions through the inner pore of a dual nozzle. The average diameter of the microbeads was 0.5±0.2 mm.

Analysis

Glucose was determined using DNS (6). Colorimetric assay of CPC was performed using procedures described previously (2). The colorimetric determination was confirmed by HPLC (Tosoh Co., Japan) using C18 column (ODS-80, Tosoh Co., Japan) and mobile phase (0.1 M glycine/HCl, pH 2.5, and methanol 10%, flow rate 0.5 ml/min). Purified CPC used as a standard was kindly donated by Dr. Young-Hwan Ko (Cheil Foods & Chemicals Inc., Korea).

Electron Microscopy

Alginate beads were freeze-dried overnight. After being sliced with a razor, the thin-sectioned gel beads were coated using a Pt-ion coater and observed using a Scanning Electron Microscope (Hitachi S-4100, Japan).

RESULTS AND DISCUSSION

Improvement of Alginate Gel Stability

The major drawback of Ca-alginate beads is that they are easily dissolved in cultures containing high concentrations of monovalent cations. To improve the unstable Ca-alginate beads, it has been reported that Ba-alginate (8) or Sr-alginate (4) beads were used. Since chemically defined media prepared for free cells of *A. chrysogenum* contained high concentrations of monovalent cations such as ammonium and potasium, the Ca-alginate matrix would be easily dissolved. To compare the alginate gel stability of three ionotropic gels (Ba-, Ca-, and Sr-alginate), long-term stability was estimated by culturing immobilized cells in chemically defined media. As listed in Table 1, Ca-alginate and Sr-alginate beads were completely disrupted within 9 and 15 days, respectively. But Ba-alginate beads were not disrupted even after 25 days. The extent of cell leakage and cell viabilities were very similar among these ionotropic gels, indicating that barium ions did not give any negative effect to the immobilized cells and increased the alginate gel stability by about 3 folds of magnitude compared with calcium ions.

Table 1. Long-term stability of alginate beads in chemically defined media.

Ionotropic gel normal beads	Bead stability (day)	Outgrowing cells (g/l)/day	Initial CPC production rate (mg/l)/h
Ba-alginate	> 25	0.64	11.2
Ca-alginate	< 9	0.47	9.8
Sr-alginate	< 15	0.52	11.9

Improvement of Diffusion Limitation

It was reported that the use of polyethyleneimine (PEI) in making alginate gel beads increased gel strength because the positive charges of PEI molecules were easily crosslinked with the negative charges of alginate molecules (1). PEI-modified alginate beads, however, caused increased mass transfer resistance due to the shrinkage of internal pores (3). Thus, in an attempt to reduce the diffusion limitation of PEI-modified alginate beads, CMC-Ba-alginate beads crosslinked with PEI were examined. When beads were freeze-dried overnight, PEI-modified CMC-Ba-alginate beads showed a hollow internal structure as compared with the PEI-modified Ba-alginate beads (Fig. 1), indicating that CMC parts within Ba-alginate gels were dried out. Accordingly, it was suggested from this observation that the presence of CMC in alginate gel beads might cause to reduce mass transfer resistance by improving internal pore structures. To measure the effect of CMC in alginate gels on the efficiency of mass transfer in CPC fermentation, *A. chrysogenum* cells were immobilized in Ba- and Ca-alginate matrices containing CMC and cultured in 500 ml Erlenmeyer flasks. When batch fermentations were repeated three times with the immobilized cells, PEI-modified Ba-alginate beads gave the best results both in terms of gel stability and CPC productivity as shown in Fig. 2. It seems that CMC did not make a contribution to improving mass transfer of substrates and/or products. This may be due to the fact that CMC also forms an ionotropic gel with divalent cations such as barium and calcium ions although the strength of the CMC gel was much

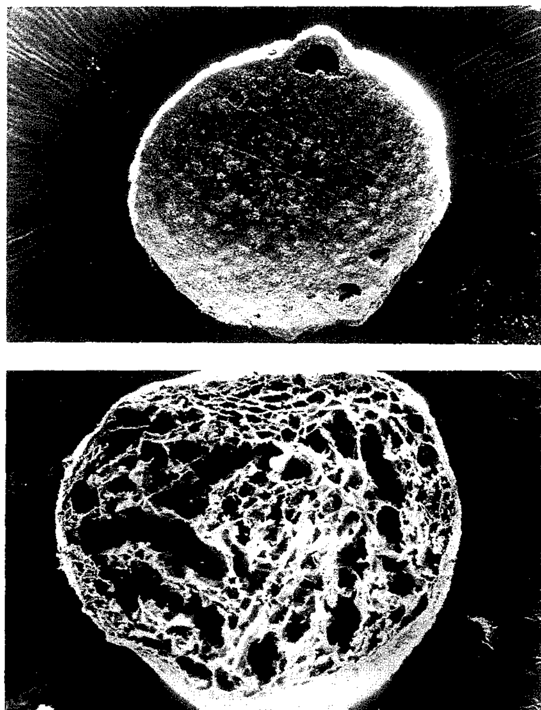

Fig. 1. Electron microscopic photos of Ba-alginate beads (diameter=3 mm).

Beads were freeze-dried overnight before pictures being taken: (Top) PEI-modified Ba-alginate bead (40× magnification) and (Bottom) PEI-modified CMC-Ba-alginate bead (23× magnification).

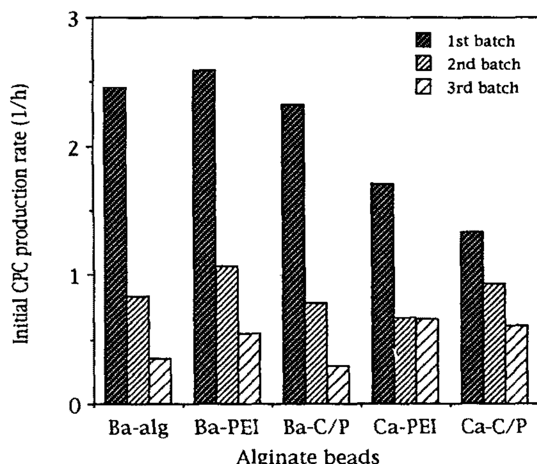

Fig. 2. Repeated CPC batch fermentations using normal alginate beads.

Immobilized cells were cultured in chemically defined media. Ba-alg =Ba-alginate bead, Ba-PEI=Ba-alginate bead modified by PEI, and Ba-C/P=CMC-Ba-alginate bead modified by PEI. Ca-alginate beads were completely dissolved in the first batch and not used. Ca-PEI=Ca-alginate bead modified by PEI and Ca-C/P=CMC-Ca-alginate bead modified by PEI.

weaker than that of the alginate gel. Hence, PEI-modified Ba-alginate beads, with a diameter of about 3.0 mm, were selected for further study.

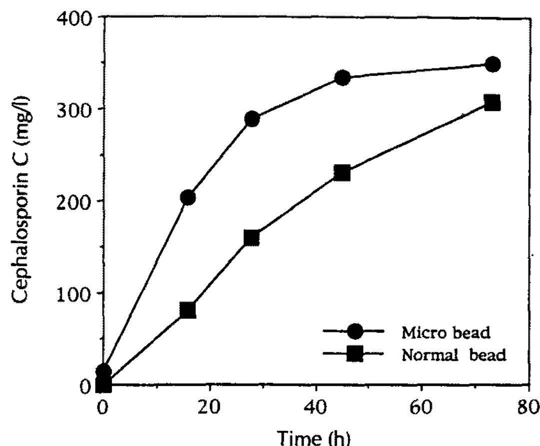

Fig. 3. Effect of Ba-alginate bead size on CPC production.

The average diameter of microbead was 0.5±0.2 mm and of normal bead 3.0±0.3 mm.

It was reported that only cells placed within 200 nm from the alginate bead surface remained active (10). Thus, in order to keep all immobilized cells alive, PEI-modified Ba-alginate microbeads, with a diameter of about 0.5 mm, were prepared. The efficiency of the microbeads can be seen in Fig. 3 where CPC production performed with cells immobilized in both normal beads and microbeads were compared. Initial CPC production rates with microbeads increased 1.6 fold higher than that of normal beads. When initial CPC production rates of microbeads were compared with those of free cells altering glucose concentration of minimal production media, CPC production rates between free and immobilized cells were very similar as shown in Fig. 4, which strongly indicates that cells entrapped in microbeads had little diffusion limitations of substrates. Therefore, cells immobilized in microbeads were used for the study of CPC fed-batch fermentation.

Repeated Fed-batch Fermentation

CPC fermentation with immobilized cells in microbeads was initially performed in a batch operation with 1.5 l of minimal production media in a 5-l fermentor. When glucose concentration in culture medium decreased below 2 g/l, batch fermentation was switched to fed-batch fermentation with a constant feeding rate, 0.015 l/h. When that working volume reached 3.5 liters, microbeads settled down and 2 l of supernatant cultures were removed using a peristaltic pump from the bioreactor. Fresh medium then began to be supplied with the same feeding rate (0.015 l/h). Fig. 5 shows that CPC concentrations could be maintained at about 0.6 g/l, which is very similar with the maximum CPC concentration of free cells cultured in

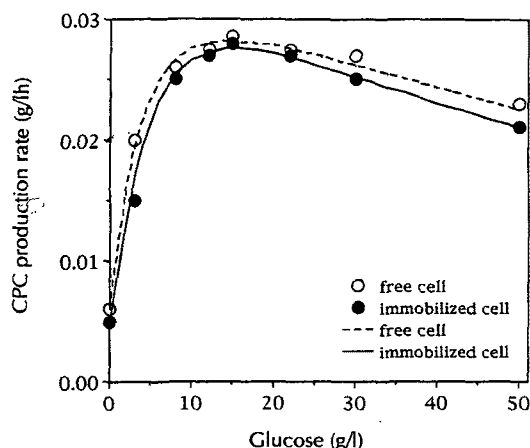

Fig. 4. Comparison of CPC production rates between free and immobilized cells in microbeads.

Each line was obtained from a substrate inhibition kinetic model, $V = V_{max}S/(K_m+S+S^2/Ki)$.

	Immobilized cell	Free cell
V_{max} (1/h)	0.045	0.040
K_m (g/L)	4.8	3.1
K_i (g/L)	48.0	70.0

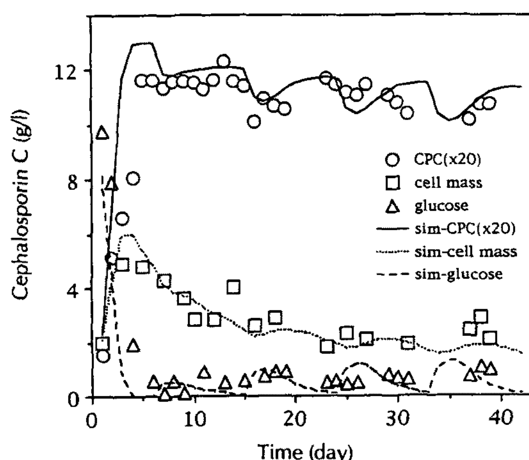

Fig. 5. Comparison of the experimental data (open symbols) and the modeling data (lines) of repeated CPC fed-batch fermentations.

A. chrysogenum cells were immobilized in PEI-modified Ba-alginate microbeads. CPC concentrations were used after the actual data being multiplied by 20 times. Cell mass indicates cell dry weight of outgrowing cells.

the same environmental conditions, during four consecutive fed-batch fermentations. Cells entrapped in microbeads remained active for more than 40 days. It was observed that immobilized cells were leaked from the alginate gel matrices but the leaked cells did not grow well due to the low concentration of glucose, which was maintained at 1-2 g/l during fermentation. Mathematical modeling was constructed to describe the repeated fed-batch fermentation system. The mass

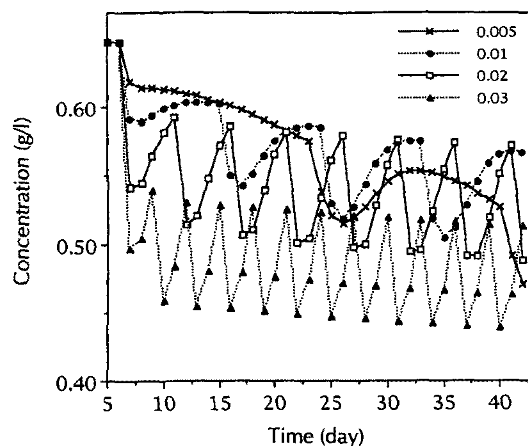

Fig. 6. Simulation results of repeated CPC fed-batch fermentations at different initial feeding rates (l/h).

balances of glucose (S), total cell mass of free and immobilized cells (X), and CPC (P) over a bioreactor yielded the following equations:

$$\frac{dV}{dt} = F \tag{1}$$

$$\frac{d(VS)}{dt} = -\{\mu X(1-\alpha\mu X) - \beta t^{\varepsilon}\}\frac{V}{Y_{XS}} + FS_f \tag{2}$$

$$\frac{d(VX)}{dt} = \{\mu X(1-\alpha\mu X) - \beta t^{\varepsilon}\}V \tag{3}$$

$$\frac{d(VP)}{dt} = \{(\frac{\mu}{Y_{XP}} + \delta)X - \gamma t^{\varepsilon}\}V \tag{4}$$

where F is the feeding rate, 0.015 l/h; V; the working volume, Sf; the feeding glucose concentration, 10.0 (g/l); μ; the specific cell growth rate, $\mu=\mu_m S/(K_s+S)$, K_s; the Monod constant; and t; the fermentation time. The classical Monod equation was chosen for the cell formation rate equation because it was seen in Fig. 4 that no glucose inhibition occurred at low concentration of glucose. Kinetic parameters and yield coefficients (Y_{xs} and Y_{px}) were experimentally determined in batch fermentations as follows: K_s=4.8 (g/l), μ_m=0.045 (1/h), Y_{xs}=0.6 (g cells/g glucose), and Y_{px}= 37.0 (mg CPC/g cells). Other kinetic constants were determined by trial and error using the fourth-order Runge-Kutta-Gill method: α=0.1, β=0.0036, γ=0.00023, δ=0.0005, ε=0.25. The simulation results agreed well with the experimental data when cell mass and CPC were considered to be self-degraded along with the fermentation time. Therefore, it was possible to predict CPC productions at different feeding rates using the modeling equations as shown in Fig. 6. As a feeding rate increased, CPC concentrations oscillated at a lower level: With the initial feeding rate of 0.005 l/h, CPC concentrations remained relatively high but there were only 2 change-overs of fresh medium. With the initial

feeding rate of 0.03 l/h, CPC concentrations decreased but there were 12 change-overs yielding a greater production volume. An optimal feeding rate for the maximization of total CPC amounts produced for the period of 40 days was calculated to be about 0.025 l/h in this repeated fed-batch fermentation system.

In conclusion, Ba-alginate microbeads modified by PEI were very effective in developing a semi-continuous bioprocess of CPC fermentation where the performance results could be predicted quantitatively by the numerical integration of ordinary differential equations.

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