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Continuous Production of Thermostable α-Amylase by Immobilized Bacillus Cells in a Fluidized-Bed Reactor

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ABSTRACT

The exponential cells of *Bacillus cereus* immobilized in calcium alginate and spun into microcylindrical particles were used in a fluidized-bed reactor for continuous synthesis of thermostable α-amylase. The reactor was operated over a period of 30 d with a dilution rate of 0.33 h^{-1}, producing 1000–1200 U/mL of enzyme. The productivity of the reactor was in the range of 330–396 kU/h. A 20-fold increase in the productivity with respect to batch fermentation with free cells was attained.

Index Entries: Immobilization; fluidized bed; α-amylase; *Bacillus* cells; Ca-alginate.

INTRODUCTION

Hydrolysis of starch is conventionally accomplished by acid, which has been replaced in the recent years by specific starch hydrolyzing enzymes, namely α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3).

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In the early stage of hydrolysis, where α-amylase is used for the liquefaction of starch slurry, the temperatures are in the range of 85–100°C, thus necessitating higher thermal stability of enzyme. Many bacterial strains, such as *Bacillus licheniformis* (*1*) and *Bacillus amyloliquefaciens* (*2*), have been reported for the production of thermostable α-amylase. The use of immobilized cells for high-productivity fermentations have gained increased attention (*3*), and attempts have been made to produce extracellular enzymes by immobilized cells (*4–6*).

The present study has been focused on the continuous synthesis of thermostable α-amylase by the cells of *Bacillus cereus* immobilized in calcium alginate. For enhanced oxygenation, the gel was spun into microcylindrical fibers and operated in a fluidized-bed reactor. The long-term reactor stability over 30 d was achieved with consistent enzyme synthesis.

MATERIALS AND METHODS

Microorganism

The strain (RJ-8) used was isolated from local soil samples and characterized as *Bacillus cereus*. The strain was maintained on nutrient agar slants and stored at 4°C.

Growth Medium

The culturing medium was composed of (g/L) glucose 10; NH_4Po_4 5; yeast extract 1; K_2HPO_4 0.1; $MgSo_47H_2O$ 0.5; sodium citrate 0.5; $CaCl_2 \cdot 2H_2O$ 0.1; $FeSO_4 \cdot 7H_2O$ 0.1, and $MnSO_4 \cdot H_2O$ 0.1. The pH was adjusted to 7.2 prior to sterilization. The continuous fermentation with immobilized cells was carried out in a medium of the same composition as above, but phosphates were eliminated in order to prevent gel disintegration.

Large-Scale Culturing of the Strain

The strain was grown in a laboratory fermentor of 7-L capacity with a working vol of 5 L. Five hundred milliliters of preinoculum were grown in Erlenmeyer flasks for 16 h at 37°C on a rotary shaker for subculturing 4.5 L of medium in the fermentor. The medium was sterilized for 30 min at 121°C, and pH was adjusted to 7.2 before inoculation. The fermentor was operated at 37°C, and aeration and agitation were maintained at 1 VVM and 300 rpm, respectively. The culture was grown for 16 h until it reached late exponential phase.

Immobilization Procedure

The cell pellet obtained was resuspended in 0.85% sterile saline and used for immobilization. Equal amounts of cell suspension of known wet wt of cells and pasteurized sodium alginate slurry (6%) were mixed, and

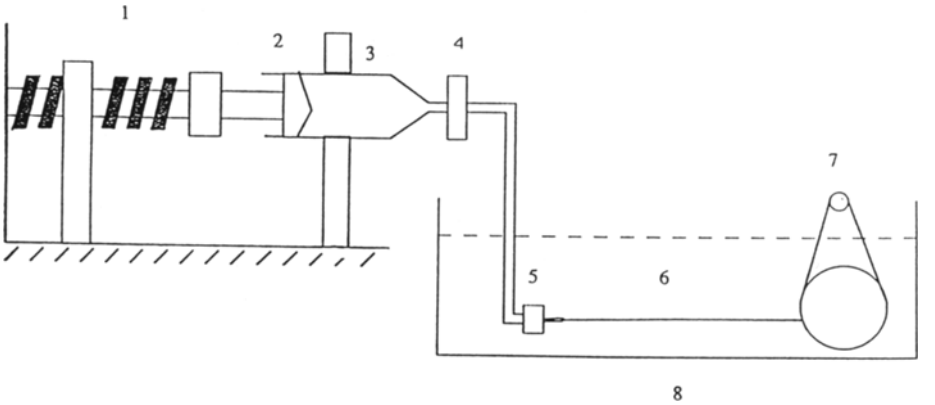

Fig. 1. Schematic diagram of the equipment for the preparation of alginate gel microfibers. 1. Drive, 2. plunger, 3. S.S. cylinder, 4. filter, 5. nozzle, 6. calcium chloride bath, 7. takeup drum, 8. fiber tow.

the resultant dope was spun into microfibers of 100-μm diameter by pultrusion technique (*7*), where both pulling and extrusion of gel fibers were simultaneously accomplished. To produce the microfibers, the slurry was extruded through a cylinder provided with a nozzle (22 gage) into a large bath of 0.1-mm sterile calcium chloride. The instantly formed gel was guided on a rotating drum, provided at the other end of the bath. By adjusting the plunger speed and the superficial velocity of the drum, the desired diameter of the fiber was obtained. The schematic diagram of the equipment used for preparation of microfibers is shown in Fig. 1. The continuous microfiber tow, after curing for 4 h in the same calcium chloride bath, was cut into small fragments (3–4 mm) to obtain a mass of staple fiber fragments resembling microcylinders of 100-μm diameter and 3–4 mm length. The agglomeration of the fiber mass was eliminated by addition of a small quantity of Tween-80 (0.01%) in the suspended medium.

Batch Experiments

The medium for continuous operation of the fluidized-bed reactor was selected by incubating 1-g wet fibers (0.095 g dry biomass) in 100 mL of medium taken in 250-mL Erlenmeyer flasks. The flasks were kept on a rotary shaker for 24 h at 37°C. The media composition was varied to study the influence of each consistent in the original medium on enzyme synthesis.

Fluidized-Bed Reactor

A jacketed glass column of 7-cm diameter and 15-cm height provided with sintered glass bottom for uniform distribution of air was used for the operation of continuous reactor. The outlet was provided at a height of 7.5 cm. The empty volume of the reactor was 275 mL. Temperature of the

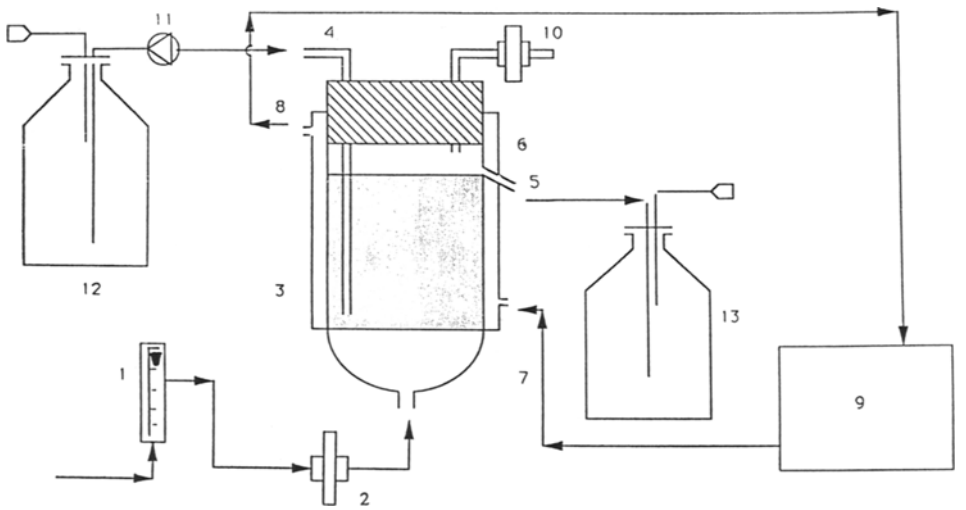

Fig. 2. The fluidized-bed reactor for continuous synthesis of α-amylase. 1. Rotameter, 2. inlet air filter, 3. jacketed column, 4. feed inlet, 5. effluent outlet, 6. fiber retainer, 7. water inlet, 8. water outlet, 9. thermostat control, 10. outlet air filter, 11. peristaltic pump, 12. sterile feed, 13. sterile container for effluent.

reactor was maintained at 37°C by circulating water in the outer jacket through a thermostat-controlled water bath. The reactor was filled with 35 g of wet fiber fragments containing 3.325 g cells (dry basis), and sterile medium was introduced from the top into the bottom section of the reactor. Air was admitted from the bottom through an air filter and rotameter. The air flow was maintained at 3–5 mL/min, which is just sufficient to fluidize the fibers. The effluent from the reactor was collected continuously in a sterile container. The whole reactor assembly was sterilized prior to fermentation and kept sterile throughout the period. The schematic diagram of the reactor is shown in Fig. 2. Samples were collected at every 24-h interval and analyzed for enzyme activity.

Analysis

The α-amylase activity was estimated by measuring the decrease in the intensity of blue color of starch–iodine complex with known quantity of fermented sample (*8*). The mixture was incubated for 10 min at 95°C at a pH of 6.9. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 5 mg of starch in 10 min under the assay conditions.

RESULTS AND DISCUSSION

Batch Experiments

Various batch experiments were performed, changing the composition of the original medium by eliminating each constituent at a time.

Table 1
Effect of Media Composition on Enzyme Activity

S. no.	Medium composition[a]	Enzyme activity, U/mL 24 h
1.	Glucose alone	NIL
2.	Glucose + yeast extract	271
3.	Glucose + salts	917
4.	Glucose + salts + yeast extract	989
5.	Glucose + phosphates	720
6.	Glucose + phosphates + yeast extract	781
7.	Complete medium	1016

[a]The concentration of each constituent in the medium was given in Materials and Methods.

Table 1 shows the various combinations tried. One hundred milliliters of sterile medium in a 250-mL flask were inoculated with 1 g of fiber fragments and incubated for 24 h on a rotary shaker at 37°C. The immobilized cells could not synthesize the enzyme in the medium containing glucose (1%) alone, but the addition of 0.1% yeast extract has improved the cell metabolism by producing 271 U/mL. The influence of salts (*see* Materials and Methods) has been found to be considerable, and a threefold increase in enzyme activity was noticed (989 U/mL). The elimination of phosphates did not affect the enzyme activity. Phosphates in the medium were generally avoided to prevent the disintegration of the alginate gel beads (*6*) in operating cell-immobilized systems with alginate gel matrix. Hence, the medium for continuous fermentation experiments contains all the constituents of the original medium except NH_4PO_4 and K_2HPO_4.

Preliminary Experiments in Fluidized-Bed Reactor

The continuous production of an α-amylase was carried out in a fluidized-bed reactor (Fig. 2) at different feed flow rates to study the enzyme synthesis profile. The reactor was operated at each flow rate for 24–48 h to ensure steady-state conditions. The dilution rate was calculated on the basis of total volume to the reactor and found to be in the range of 0.116–0.465 h^{-1}. Samples were collected in steady-state conditions and analyzed for enzyme activity. The reactor performance in terms of enzyme output was calculated as productivity (kU/L/h) and plotted in Fig. 3 at various dilution rates. The productivity increased with increase in dilution rate up to 0.33 h^{-1} and subsequently decreased. This trend was similar to our earlier work with immobilized bacillus cells for α-amylase synthesis (*6*) and immobilized yeast cells for ethanol production (*9*). At a high dilution rate, the contact time between medium and immobilized cells decreased, resulting lower enzyme yield and productivity.

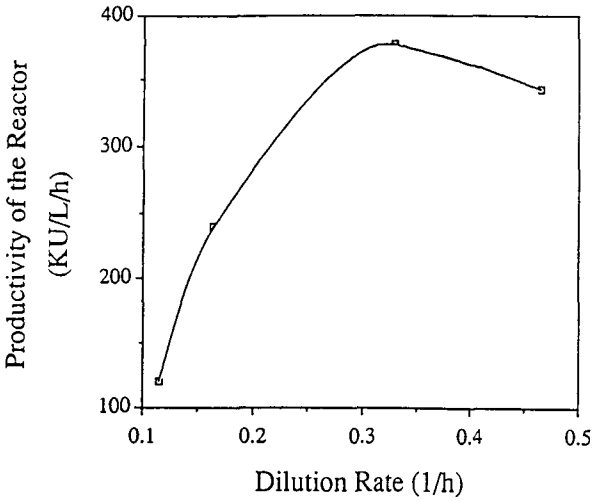

Fig. 3. Reactor productivity at various dilution rates.

LONG-TIME OPERATION OF FLUIDIZED-BED REACTOR

The continuous production of α-amylase was performed in a fluidized-bed reactor. The feed flow rate was maintained at 90 mL/h (dilution rate 0.33 h^{-1}). Samples were withdrawn at regular intervals and analyzed for enzyme activity, which remained in the range of 1000–1200 μ/mL over a period of 30 d (Fig. 4). Although the effluent was clear most of the days, on few occasions (9th and 18th d), the turbidity rapidly increased indicating excessive cell leaching. It was also observed that the enzyme activity decreased in subsequent days. The lowering of the enzyme activity can be attributed to the excessive cell leaching, and subsequent recovery of the activity may be owing to sporulation and regeneration of the cells. The reactor productivity varied from 330–396 kU/L/h. In our earlier work with immobilized *Bacillus* cells in a packed reactor for α-amylase synthesis, the productivity decreased from an initial value of 100 to 35 kU/L/h in 16 d of operation (*6*). The productivity of the batch fermented with free cells was found to be on the order of 20 kU/L/h (*10*), which has been considerably improved by immobilizing cells and operating a fluidized-bed reactor.

The enhanced enzyme activity and long-term stability of the present system can be attributed to improved oxygenation of the cells. Varying the geometry of the gel from spherical to cylindrical and using a fluidized-bed reactor had definitely improved the cell performance. The thermostability of the enzyme from the present culture has been reported elsewhere (*10*).

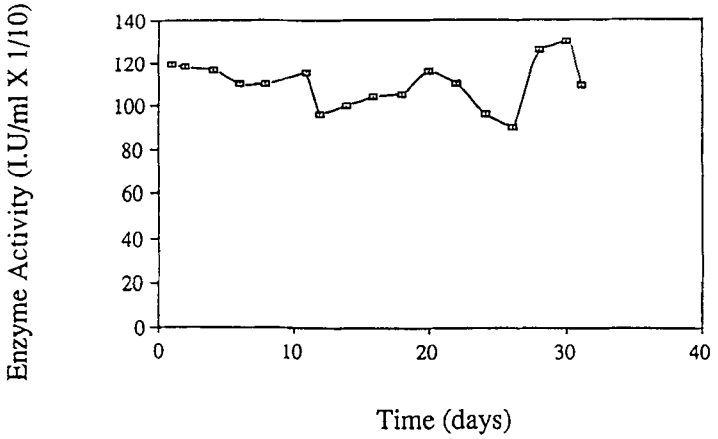

Fig. 4. Continuous production of α-amylase.

CONCLUSIONS

It has been demonstrated that a fluidized-bed reactor containing *Bacillus cereus* cells entrapped in alginate gel in the form of microcylinders can be operated over a period of 30 d with a productivity in the range of 330–396 kU/L/h without decrease in the enzyme activity. The improved performance has been the result of enhanced oxygenation of the immobilized cells.

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REFERENCES

1. Morgan, F. J. and Priest, F. G. (1981), *J. Appl. Bacteriol.* **50,** 107.
2. Young, J. Y., Theodore, W. C., Juan, H., and Randloph T. H. (1988), *Biotechnol. Bioeng.* **31,** 357.
3. Ramakrishna, S. V., Prema, P., and Sai, P. S. T. (1991), *Bioprocess Eng.* **6,** 117-121.
4. Kokubu, T., Karube, I., and Suzuki S. (1978), *Eur. J. Appl. Microbiol. Biotechnol.* **5,** 233-240.

5. Abraham, T. E., Jamuna, R., and Ramakrishna, S. V (1991), *Starch/Starke* **43,** 113–116.
6. Jamuna, R. and Ramakrishna, S. V. (1992), *Enzyme Microb. Technol.* **14,** 36–41.
7. Baker, M. R., Emery, A. N., and Easthope, C. J. (1987), in *Proceedings 4th European Congress of Biotechnology,* Weijssel, O. M., Vandermeer, R. R., and Luybenkich, A. M., eds. 1, 218–221.
8. Young, J. Y., Juan, H., and Randolph, T. H. (1987), *Biotechnol. Bioeng.* **30,** 147–151.
9. Ramakrishna, S. V., Sreedharan, V. P., and Prema, P. (1988), in *Bioreactor Immobilized Enzymes Enzymes and Cells,* MooYoung, M., ed., Elsevier Publishers, Amsterdam, pp. 251–260.
10. Jamuna, R., Bindu, C., and Ramakrishna, S. V. (1991), *Starch/Starke* **43,** 28–32.