0273-2289/93/3803—0269$03.00

Simultaneous Production and Purification of *Bacillus subtilis* α-Amylase

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Received October 23, 1992; Accepted December 14, 1992

ABSTRACT

Production of α-amylase with *B. subtilis* CCM 2722 in an aqueous two-phase polyethylene glycol/dextran system integrated with product purification by affinity chromatography on crosslinked starch during cultivation was studied. The medium was drawn from the bioreactor to the external settler during fermentation. After phase separation in the settler the dextran-rich bottom phase with cells was returned to the bioreactor. The PEG-rich top phase was pumped to the column with crosslinked starch and returned to the bioreactor after α-amylase adsorption. The same volumetric productivities, 0.53 U/mL/h, were reached in both batch and described process, but total productivity of the latter method was much higher owing to shortening upstream and downstream processing time. The enzyme of 98% homogenity in 95% yield was obtained after its elution from the column.

Index Entries: Aqueous two-phase; *Bacillus subtilis;* simultaneous fermentation and adsorption; α-amylase; purification.

INTRODUCTION

Integration of bioconversion and downstream processing has received increased attention in recent years. It can be a way to make continuous bioprocesses and to improve total productivity. Various separation techniques have been used in combination with fermentation to form effective

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integrated systems, e.g., extraction with organic solvents (*3*), extraction in aqueous two-phase systems (*4*), adsorption (*5*), membrane techniques (*6*), and pervaporation (*7*).

Recently production of extracellular enzymes, mainly α-amylase (E.C. 3.2.1.1.), in aqueous two-phase systems has been reported (*8–10*). Polyethylene glycol (PEG) and dextran water solutions are described to form top and bottom phases of these systems. *Bacillus* cells are preferentially distributed in the dextran-rich phase and at the interface, and α-amylase is in both phases. Regular exchange of the top PEG-rich phase after its separation from the bottom one gives the possibility to perform the production in a semicontinuous mode (*11,12*). Effective enzyme isolation from the PEG-rich phase, developed in our laboratory (*13*), could use this method more effectively and could carry out microbial processes in a continuous mode.

The goal of this study is the continuous microbial production of α-amylase in the two-phase system integrated with continuous product removal by adsorption from the PEG-rich top phase on the modified starch column during cultivation.

MATERIALS AND METHODS

Materials

PEG 6000 was obtained from NCHZ (Nováky, CSFR) and Dextran D 250 (M_w = 225–250 kg/mol) from Biotika (Slovenská Lupca, CSFR). Modified starch for α-amylase adsorption was prepared in our laboratory by the method of Kuniak and Marchessault (*14*). 0.5 g of epichlorhydrine (Fluka, Buchs, Switzerland) and soluble starch (100 g) were added to 200 mL of stirred 0.01*M* NaOH. After 12 h the reaction was stopped by neutralization with 1*M* acetic acid. The adsorbent was separated by filtration and then washed with 100 mL of water, 50% ethanol, and 96% ethanol. Soluble starch and other chemicals of analytical grade were purchased from Lachema (Brno, CSFR).

Microorganisms and Media

Bacterial strain *Bacillus subtilis* CCM 2722 was obtained from the Czecho-Slovak Collection of Microorganisms (Brno. CSFR) and maintained on a nutrient agar. Liquid medium for α-amylase production by Yoo et al. (*15*) contained per liter of distilled water: 20 g soluble starch (sterilized separately), 1 g yeast extract (Fluka, Buchs, Switzerland) 1 g KH_2PO_4, 0.5 g Na_3-citrate (sterilized together), 0.1 g $MnSO_4 \times H_2O$, 0.5 g $MgSO_4 \times 7H_2O$, and 0.1 g $CaCl_2$ (sterilized together). For cultivation in the aqueous two-phase system, the nutrients were calculated as g/kg of medium. PEG was sterilized with the yeast extract and dextran with the inorganic salts. Sys-

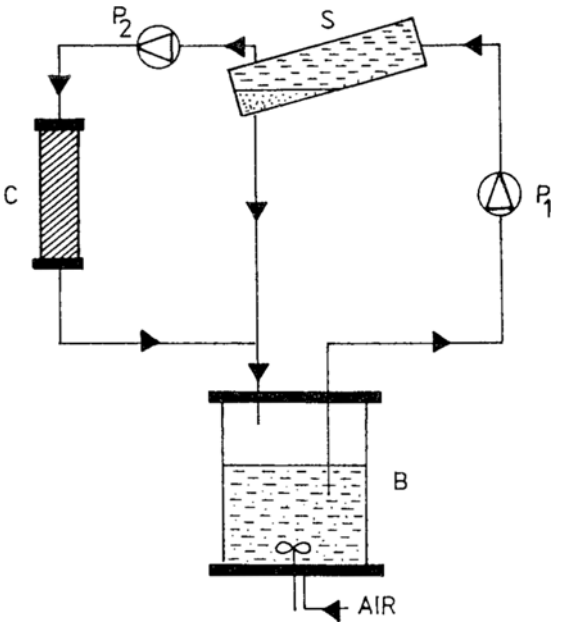

Fig. 1. Schematic diagram of experimental setup. Medium from the bioreactor (B) is drawn (P_1) to the settler (S). After phase separation, the top phase is pumped (P_2) to the column with the modified starch (C) where α-amylase is adsorbed. Then both phases are returned to the bioreactor.

tem PEG/dextran 10/3% (w/w) with a volume ratio of phases approx 5:1 (top:bottom) was used for all cultivations. Present starch (2%) interfered with the system, so the final volume ratio was 4:1.

Cultivation Conditions

Inoculum (30 mL) for bioreactor cultivations was prepared in a 200-mL flask in a rotary shaker at 180 rpm for 12 h. The fermentations were carried out in a 1-L stirred bioreactor LF-3 (Vývojové dílny CSAV, Prague, CSFR) with air-flow rate of 1 vvm and agitation rate of 400 rpm. Medium volume was 600 mL and the cultivation temperature was 37°C. The pH value was maintained at 7.0 by adding 2*M* NaOH or 1*M* H_2SO_4.

Simultaneous Process Study

Experimental setup is presented in Fig. 1. The settler and the column were autoclaved at 120°C after filling with medium top phase, and the bioreactor was without medium. Volume of the settler varied from 50 to 125 mL according to the medium flow rate (1.5–3 mL/min) in bypass. The column (16×4.8 cm) was filled with 100 g of the modified starch. Bypass was added to the bioreactor after 12 h cultivation. Settling and α-amylase adsorption were carried out at 22°C, cultivation at 37°C.

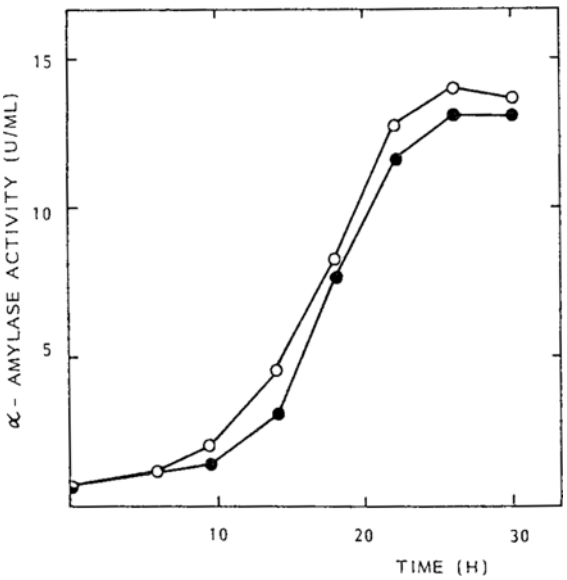

Fig. 2. Batch α-amylase production with *B. subtilis* in the aqueous two-phase system (●) and in the medium without phase polymers (○).

Enzyme Assay

Activity of α-amylase was determined by measuring reducing sugars with alkaline 3,5-dinitrosalicylate (*16*) after 5 min hydrolysis of starch at 40°C and pH 6.5. One α-amylase unit was defined as the amount of enzyme that releases 1 μmol of reducing sugars (such as glucose) from 0.5% starch solution in 1 min.

Electrophoresis

The purity of the isolated α-amylase was checked by SDS-polyacrylamide gel electrophoresis as described by Laemmli (*17*). Gel was stained with a 0.1% Coomassie blue R solution (in 50% trichloracetic acid) and destained with 7% acetic acid.

RESULTS AND DISCUSSION

Batch Process

Batch α-amylase production with *B. subtilis* CCM 2722 in the two-phase system and the normal medium are compared in Fig. 2. The time courses of both cultivations were similar. The phase polymers exhibit minimal effect on cell growth and productivity. In the control, maximum enzyme activity of 13.8 U/mL (volumetric productivity 0.53 U/mL/h), and

in the mixed phase of the two-phase system 13.2 U/mL, were reached after 26 h. However, α-amylase activity in the PEG-rich top phase was lower, because its actual partition coefficient varied between 0.20–0.23 during cultivation owing to the enzyme adsorption on particularly precipited starch in the dextran-rich phase, which was previously described (*11,12*). Since the volume of the PEG-rich phase formed about 83% of the total volume at the end of the batch cultivation, more than 50% of α-amylase activity was in this phase and could be isolated after phase separation.

Simultaneous Production and Purification

The setup illustrated in Fig. 1 was used for simultaneous microbial production and purification of α-amylase. The mixed phase from the bioreactor was drawn to the settler, where phase separation was performed. Choice of a medium retention time in settler is a very important factor. It must be sufficient to achieve phase separation, but long settling could cause a change of cell properties, because oxygen limitation occurs. On the basis of previous works (*9,12*) a time of 40 min was singled out, when change of production properties *Bacillus* cells was not observed. Retention time was controlled by the settler volume and medium flow rate. Better results were achieved at a temperature of 22°C than at 37°C, probably owing to lower metabolic activity of cells during this process. Phase separation before α-amylase adsorption is necessary, since contact between the fermentation broth and the adsorbent results in unspecific binding of cells to a sorbent (*1*).

The PEG-rich top phase containing a negligible number of cells were then pumped on the column head. In the column, α-amylase from the top phase was bound by affinity binding to crosslinked starch. This material had as high efficiency as much more expensive dextrin-bound affinity gels (*18,19*). Almost 100% enzyme adsorption was reached under the conditions of this experiment (22°C, concentration of PEG in the top phase higher than 10% w/w). At concentration > 10% PEG was described to have a positive effect on adsorption of the *B. subtilis* α-amylase to starch (*13*). No activity of the enzyme was registered on the column outflow. The PEG-rich phase from the column and the dextran-rich one from the settler were returned to the bioreactor.

The time courses of α-amylase production integrated with its isolation at different medium recirculation rates are shown in Fig. 3. First batch cultivation was carried out for 12 h. Intensive cell growth was observed during this phase. The peak cell density was 3.6 g/L after 12 h when cell growth declined as in the batch process. Then the bypass was started and α-amylase activity dropped at all medium flow rates. It could probably be caused by its adsorption in the column and/or second lag-phase. After this phase the α-amylase activity increased and maximum enzyme level was reached between 24–28 h. Then a gentle decrease and/or steady-state appeared.

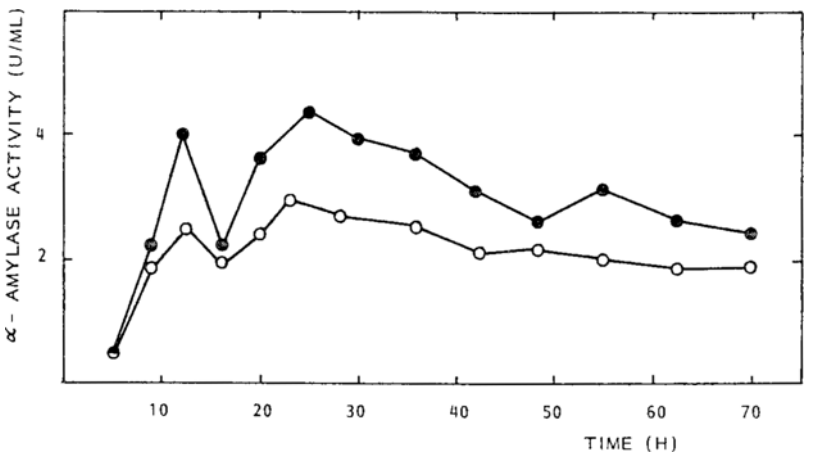

Fig. 3. α-Amylase activity in top PEG-rich phase after phase separation during the simultaneous process. Medium recirculation rates 1.5 mL/min (●) and 3.0 mL/min (○).

Bypass flow rate played a significant role in the process. The best results were achieved between 1.5–3.0 mL/min. These values mean dilution rates in the bioreactor of $D = 0.3$–0.15 h^{-1}. The highest volumetric productivity 0.53 U/mL/h was reached at $D = 0.30$ h^{-1}. Many authors reached the highest volumetric productivities at similar dilution rates, e.g., Cheng et al. (*20*) in continuous culture of *B. caldolyticus* at $D = 0.29$ h^{-1}, Groom et al. (*21*) with adsorbed *B. amyloliquefaciens* at $D = 0.345$ h^{-1}, and Ruten and Daugilis (*22*) in two stage fermentor with *B. amyloliquefaciens* and $D = 0.18$ h^{-1}. Unlike some continuous processes (*20,22*) the method of simultaneous α-amylase production and isolation with recycled biomass, where product is formed and separated continuously, has the advantage that a reversion of the microorganism to lower producing variant did not occur.

The same value of the volumetric productivity 0.53 U/mL/h was reached in the batch cultivation and the cultivation integrated with the enzyme isolation, but owing to shortening upstream and downstream processing time the total productivity of the latter method was 2 times higher than the former one when this time was assumed. In addition two purification steps were carried out in this process: cell separation and affinity adsorption of the product. A concentrated α-amylase of 98% protein purity (determined by gel electrophoresis) was obtained in 95% yield after elution with 0.05*M* phosphate buffer, pH 7.0, from the column (Fig. 4).

The volumetric productivity could be improved by an increase of partitioning of the enzyme to the PEG-rich phase (*23,24*). Kim and Yoo (*10*) described increasing the α-amylase partition coefficient by using a lower mol wt dextran and adding sodium sulphate to the system. The use of some other substrates instead of the starch that precipitated in the dextran-rich phase and particularly adsorbed α-amylase could also help to improve partitioning.

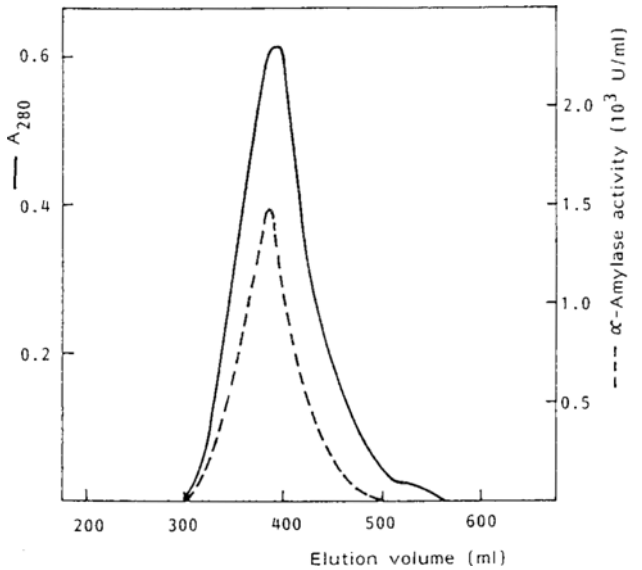

Fig. 4. Elution profile of α-amylase from crosslinked starch column (16× 4.8 cm) after simultaneous process at $D = 0.3\ h^{-1}$. Elution was realized with 0.05*M* phosphate buffer, pH 6.5. Absorbance was measured in diluted (5x) samples.

CONCLUSION

Microbial α-amylase production in aqueous two-phase systems integrated with product purification by affinity chromatography was successfully tested. The volumetric productivity of the microbial process was the same, but total productivity was much higher than in the batch process and, moreover, a enzyme of high purity was obtained. The method could also be applicable to some other extracellular enzymes or secondary metabolites.

ACKNOWLEDGMENTS

The authors want to thank P. Gemeiner for valuable consultation, and V. Heriban and V. Sládková for technical assistance.

REFERENCES

1. Larsson, M. and Mattiasson, B. (1984), *Chem. Ind.* **12**, 428.
2. Wielen, L. A. M. van der and Luyden, K. Ch. A. M. (1992), *Curr. Opinion Biotechnol.* **3**, 130.
3. Busche, R. M. (1991), *Appl. Biochem. Biotechnol.* **28/29**, 605.

4. Larsson, M., Arasaratnam, V., and Mattiasson, B. (1989), *Biotechnol. Bioeng.* **33,** 758.
5. Wang, H. Y., Palanki, S., and Hyatt, G. S. (1989), *Appl. Microbiol. Biotechnol.* **30,** 115.
6. Boyaval, P., Corre, C., and Terre, S. (1987), *Biotechnol. Lett.* **9,** 207.
7. Groot, W. J., Reyer, M. C. H. den, Baart de la Faille, T., Lans, R. G. J. M. van der, and Luyben, K. Ch. A. M. (1991), *Chem. Eng. J.* **46,** B1.
8. Andersson, E. and Hahn-Hagerdahl, B. (1988), *Appl. Microbiol. Biotechnol.* **29,** 329.
9. Alam, S., Wiegand, W. A., and Hong, J. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 421.
10. Kim, S. H. and Yoo, Y. L. (1991), *J. Fermn. Bioeng.* **71,** 373.
11. Andersson, E., Hahn-Hagerdahl, B., and Johansson, A. C. (1985), *Enz. Microb. Technol.* **7,** 333.
12. Stredanský, M., Svorc, R., Sturdik, E., and Dercová, K. (1991), *J. Biotechnol.* **27,** 81.
13. Stredanský, M., Kremnický, L., and Sturdik, E. (1991), *Biotechnol. Lett. Tech.* **7,** 60.
14. Kuniak, L. and Marchessault, R. H. (1972), *Staerke* **24,** 110.
15. Yoo, J. Y., Cadman, T. W., Hong, J., and Hatch, R. T. (1988), *Appl. Microbiol. Biotechnol.* **28,** 426.
16. Bernfeld, P. (1955) in *Methods Enzymology,* vol. 1, Colowick, S. P., and Kaplan, N. O., eds., Academic, New York, pp. 149–158.
17. Laemmli, U. K. (1970), *Nature* **227,** 680.
18. Monma, M., Mikuni, K., and Kainuma, K. (1988), *Biotechnol. Bioeng.* **32,** 404.
19. Turchi, L. S. and Becker, T. (1987), *Curr. Microbiol.* **15,** 203.
20. Cheng, C. Y., Yabe, I., and Toda, K. (1989), *J. Fermn. Bioeng.* **67,** 176.
21. Groom, C. A., Daugilis, A. J., and White, B. N. (1988), *Appl. Microbiol. Biotechnol.* **28,** 8.
22. Rutten, R. and Daugilis, A. J. (1987), *Biotechnol. Lett.* **9,** 505.
23. Albertsson, P.-A. (1985), *Partition of cell particles and macromolecules.* 3rd Ed., Wiley, New York.
24. Andersson, E. and Hahn-Hagerdahl, B. (1990), *Enz. Microb. Technol.* **12,** 242.