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Continuous Cellobiose Hydrolysis Using Self-Immobilized β-Glucosidase from *Aspergillus phoenicis* QM 329 in a Fluidized-Bed Reactor

KATI RÉCZEY,[1] HENRIK STALBRÅND,[2] INGRID PERSSON,[2] BÄRBEL HAHN-HÄGERDAL,[3] AND FOLKE TJERNELD*,[2]

[1]*Institute of Agriculture and Chemical Technology, Technical University of Budapest, H-1521 Budapest, Hungary; and Departments of* [2]*Biochemistry, and* [3]*Applied Microbiology, Chemical Center, Lund University, PO Box 124, S-221 00 Lund, Sweden*

ABSTRACT

Aspergillus phoenicis QM 329 was grown in the shape of beads in shake flasks and in an air-lift fermentor. The production of β-glucosidase started when the carbon source, glucose, was consumed. The β-glucosidase activity was retained in the beads at a pH below 6.0. The influence of bead diameter on enzyme activity and the pH and temperature optima for cellobiose hydrolysis has been studied. The enzyme-containing beads were used in a fluidized-bed reactor for continuous cellobiose hydrolysis, and a productivity of 2.0 g/L-h at a substrate conversion of 76% was obtained. The self-immobilized β-glucosidase is a stable and reusable enzyme with a half-life of 700 h when operating at 50°C and pH 4.8.

Index Entries: β-glucosidase; *Aspergillus phoenicis*; cellobiose hydrolysis; immobilization; fluidized-bed reactor.

INTRODUCTION

β-Glucosidase is one of the essential enzymes in the enzymatic conversion of cellulose. This enzyme converts the intermediate cellobiose

*Author to whom all correspondence and reprint requests should be addressed.

into glucose. High levels of β-glucosidase are important for the complete conversion of cellulose owing to the inhibition, by cellobiose, of the cellobiohydrolase and endoglucanase activities (*1,2*). When the cellulolytic enzyme system of *Trichoderma reesei* is used for cellulose hydrolysis, soluble β-glucosidase from *Aspergillus phoenicis* is added as a supplement in order to increase the rate of saccharification (*3*).

Soluble β-glucosidase from *A. phoenicis* has been immobilized on controlled pore alumina and used in hydrolysis of cellulosic materials (*4*). β-Glucosidase is cell-associated both in filamentous bacteria (*5*) and in fungi (*6,7*). The whole fungal mycelium of *Trichoderma* sp. E-58 has been immobilized by entrapment in calcium alginate beads in order to obtain an immobilized β-glucosidase activity (*8,9*). β-Glucosidase of *A. phoenicis* QM 329 has also been immobilized on chitosan, using the bifunctional agent glutaraldehyde (*13*).

In a previous paper (*10*), we have reported on the *in situ* immobilization of cell-associated β-glucosidase in mycelial pellets of *Aspergillus phoenicis* QM 329. The fungus was grown in the shape of beads in shake flasks and an air-lift fermentor. The retention of the β-glucosidase activity inside the beads was found to be dependent on the pH profile during cultivation, carbon source, inoculum size, and fermentor type. The beads could be air-dried and alcohol-sterilized with only minor loss of activity.

In this investigation, we present data on the hydrolysis of cellobiose by self-immobilized β-glucosidase. The activity of the cell-associated enzyme has been studied as a function of bead size, pH, and temperature. The self-immobilized β-glucosidase has been used for continuous hydrolysis of cellobiose in a fluidized-bed reactor. A constant glucose concentration was maintained in the effluent of the reactor for 70 h. The results show that β-glucosidase immobilized in mycelial beads of *A. phoenicis* is a stable enzyme that can be used for repeated conversions in a bioreactor.

MATERIALS AND METHODS

Organism

Aspergillus phoenicis QM 329 was obtained from the QM Culture Collection, Dept. of Botany, University of Massachusetts, Amherst, MA. The fungus was maintained on potato dextrose agar (PDA) slants at 27°C.

Medium

The nutrient medium composition was: glucose 10 g/L, proteose peptone no. 2 (DIFCO, Detroit, Michigan) 1 g/L, and citric acid 0.5g/L. After sterilization a stock solution of Vogel's medium, *N* (*11*) was added to the appropriate concentration. In the air-lift fermentor experiment, 0.75 mL antifoam (BDH silicone antifoam agent) was added.

Shake Flask Cultures

One-hundred milliliters of medium in 1 L baffled Erlenmeyer flasks in a shaking water bath (175 rpm) at 28°C was inoculated with 2–7 mL conidia suspension at concentrations between 6×10^5 and 1×10^6 conidia/mL. The initial pH, 5.2, was not adjusted. For inoculation conidia from 7–14-d-old slants were suspended in sterile distilled water. The concentration was determined by dilution on PDA dishes.

Air-Lift Fermentor Cultures

The air-lift fermentor used has been described previously (*12*). It was constructed from a Pyrex glass cylinder with an inner diameter of 70 mm and a height of 550 mm. The bottom of the reactor was a porous glass filter with a pore size of 20–40 μm. Agitation and aeriation was performed by letting sterile air through the filter at a rate of 0.5 VVM. Medium (1500 mL) was inoculated with 35 mL conidia suspension (3.6×10^6 conidia/mL), prepared as described. The temperature was maintained at 28°C. The initial pH, 5.2, was not adjusted.

pH and Temperature Optimum

The pH and temperature optimum investigation was performed by adding 30 uniform beads, with a diameter of 2 mm, to a 15 mL cellobiose solution at 50g/L in a 25 mL plugged Erlenemeyer flask placed in a shaking water bath. Samples during the first 3 h were analyzed by HPLC. The conversion rate during this time was found to be almost linear. The activity was calculated as μmol glucose produced/min from the slope during the initial 3 h. The stability of the enzyme at different temperatures was studied by following the described conversions over 48 h. The cellobiose was dissolved in 0.05*M* sodium acetate buffer at pH 4.0 and 4.8. At pH 5.4 and 6.0, the cellobiose was dissolved in 0.05*M* sodium phosphate buffer. The temperature experiments were performed at pH 4.8 in 0.05*M* sodium acetate.

Cellobiose Hydrolysis in a Fluidized-Bed Reactor

The beads used were cultivated in the described medium in the air-lift fermentor and were approximately uniform with a diameter of 2 mm. A schematic diagram of the fluidized-bed reactor is shown in Fig. 1. The reactor was constructed as follows: a column (250 mm in length and 15 mm inner diameter) with a short tapered entrance section in the bottom. On top of the column there was a conical 60 mm long expansion section (in order to let the biocatalyst beads fall back into the column), followed by a uniform cylinder section with an inner diameter of 36 mm and a length of 120 mm. From the latter section, two side arms were placed for recirculating flow and overflow outtake. In the batch, as well as the con-

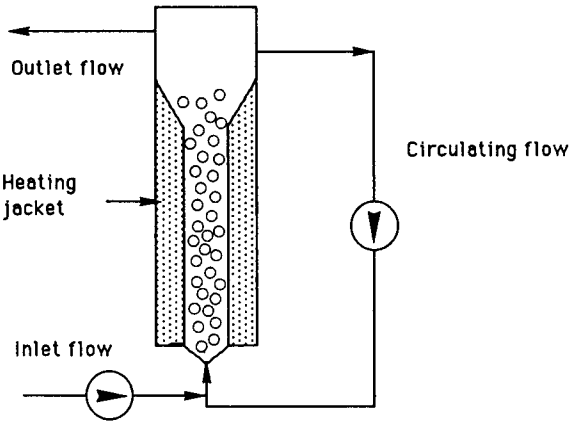

Fig. 1. Schematic diagram of the fluidized-bed reactor.

tinuous hydrolysis, a recirculating flow was used (2mL/min) for agitation and to keep the beads fluidized. The temperature was held at 50°C with a heating-water jacket. The substrate cellobiose was dissolved in 0.05*M* acetate buffer, pH 4.8.

Enzyme Assay

The β-glucosidase activity was assayed with 10 m*M* *p*-nitrophenyl β-D-glucopyranoside (Sigma, St. Louis, MO) in 0.05*M* acetate-buffer, pH 4.8, as substrate. A 1.4 mL substrate solution and 0.1 mL sample were incubated at 50°C, and the reaction was terminated after 10 min with 3 mL 0.1*M* NaOH. The absorbance was read at 400 nm. A standard curve was obtained with *p*-nitrophenol (Sigma).

The β-glucosidase activity associated with the beads was assayed by adding two uniform beads (washed in 0.05*M* acetate buffer, pH 4.8) to a 1.4 mL substrate solution and treated as above. The activity was calculated as IU/cm^3. One international unit is defined as one μmol *p*-nitrophenol produced/min under the assay conditions.

Glucose and Cellobiose Analysis

Glucose and cellobiose were determined by HPLC using a Shimadzu LC-6A chromatograph. The column was a Bio-Rad HPX-87P with water as eluent at a flow rate of 0.4 mL/min at 60°C. The glucose concentration during the cultivations was determined with Boerhinger and Mannheim's UV-method glucose-kit.

Biocatalyst Stability Under Reactor Conditions

The beads with self-immobilized β-glucosidase were kept at 50°C, pH 4.8 (0.05*M* acetate buffer) for 1196 h. The conversion rate was determined at the start and after 161 and 1170 h. This was done by emptying the reac-

tor and separating the beads from the liquid on a Büchner funnel and finally readding the beads with fresh cellobiose solution (50 g/L). The conversion under batch conditions was followed during the initial 2 h by HPLC analysis. The activity was calculated from the slope, as in the pH and temperature optimum investigations.

RESULTS AND DISCUSSION

We have previously reported that *A. phoenicis* QM 329 can grow in the shape of beads in shake flasks and an air-lift fermentor (*10*). With glucose and soluble starch as carbon sources, beads with the highest β-glucosidase activity and the best stability were produced.

Cultivation of *A. phoenicis* in Air-Lift Fermentor

When *A. phoenicis* QM 329 is cultivated in a regular impeller-stirred fermentor, the fungus does not grow in the shape of beads (*10*). This is probably owing to the shear damage to the mycelium caused by the impeller. It was previously observed that fungal mycelia could grow as beads in air-lift fermentors (*12*). In Fig. 2, the time course of a cultivation of *A. phoenicis* in an air-lift fermentor is shown. The cultivation conditions were chosen from results obtained in shake-flask cultures (*10*). The initial pH (5.2) and carbon source (glucose) were chosen for optimal retention of the β-glucosidase activity inside the beads and the production of beads of high stability.

The concentration of carbon source in the medium and the pH were measured (Fig. 2A). The β-glucosidase concentration was measured both inside the beads and in the medium (Fig. 2B). The minimum in the pH profile is exactly at the point where all the glucose in the medium is consumed. At this point, production of β-glucosidase starts. This indicates that the enzyme is a secondary metabolite that is produced after the initial growth phase of the fungus. As long as the pH is below 6.0, very little enzyme is released into the medium. When the pH increases above 6.0, extracellular β-glucosidase is released.

Bead Diameter

The influence of the bead diameter on enzyme activity was studied in beads obtained in shake flasks inoculated with varying amounts of conidia (Fig. 3). By increasing the inoculum size up to 10^5 conidia/mL, it was possible to reduce the diameter down to 2 mm. With a reduced average diameter of the beads, an increased β-glucosidase activity per unit volume is obtained (Fig. 3). The highest enzyme activity was obtained with beads of an average diameter of 2 mm.

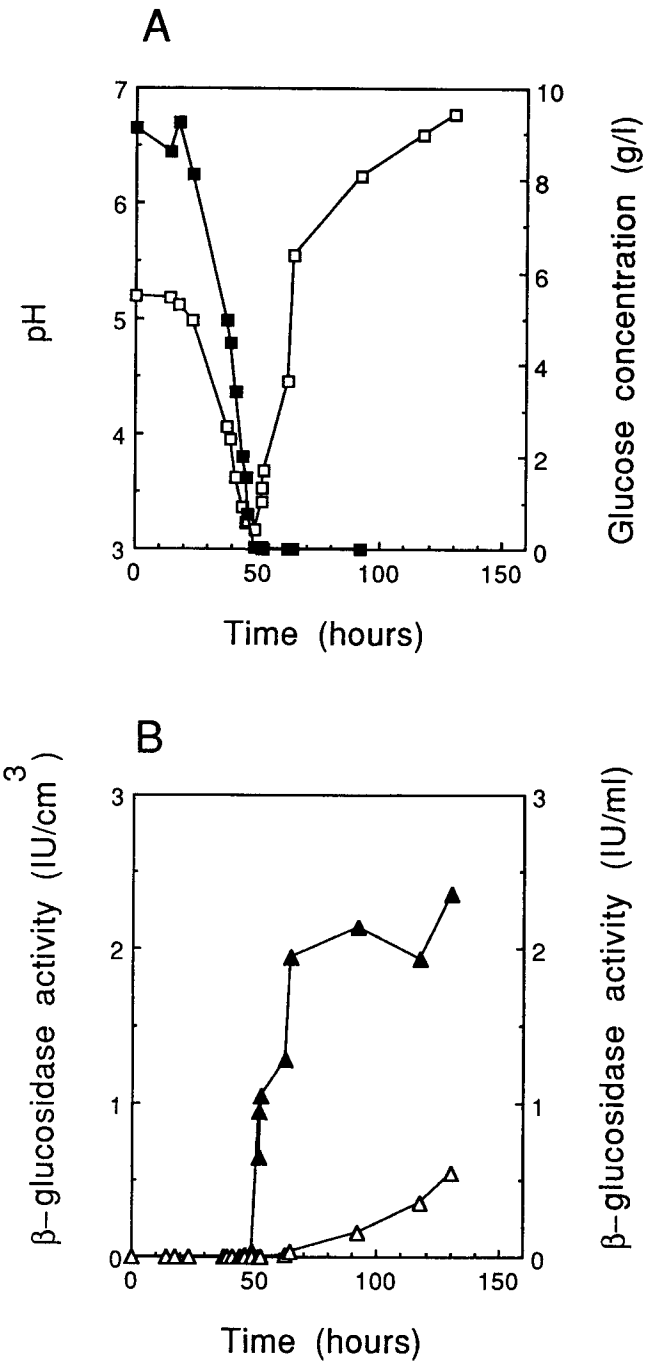

Fig. 2. Cultivation of *A. phoenicis* QM 329 in an air-lift fermentor. The working volume was 1500 mL. The carbon source was glucose at 10 g/L. A 35 mL inoculum was used at 3.6×10^6 conidia/mL. (A) The change in pH (□) and glucose concentration (■). (B) The β-glucosidase activity determined with the pNP assay in the beads (IU/cm^3) (▲) and medium (IU/mL) (△).

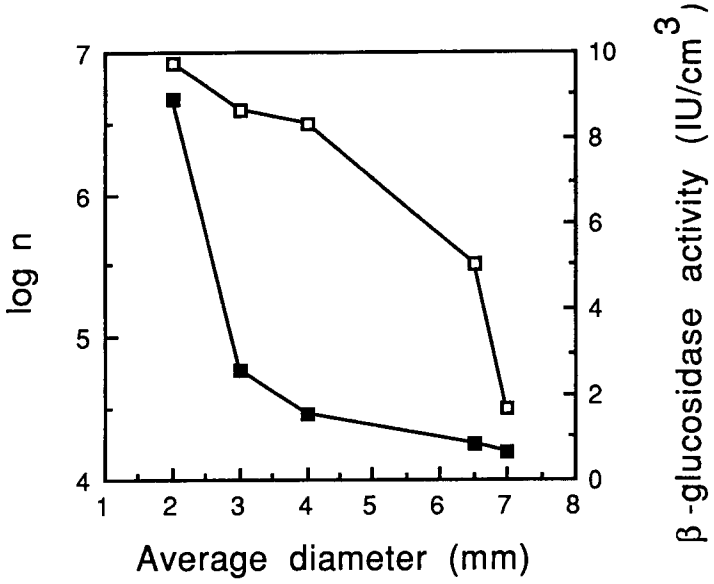

Fig. 3. β-Glucosidase activity (■) in beads of *A. phoenicis* and inoculum size (□) plotted vs average bead diameter. The beads were cultivated in shake-flasks (1 L) with 100 mL working volume at 28°C and 175 rpm. The inoculum size (n) was varied between 3.1×10^4 and 8.4×10^6 conidia/100 mL. The enzyme activity was the maximum in each cultivation and was determined with the pNP assay.

Properties of Cell-Associated β-Glucosidase of *A. phoenicis* QM 329

Studies of enzyme activity as a function of pH and temperature were conducted in order to find the optimal conditions for the use of the β-glucosidase containing beads in enzyme reactors. In Fig. 4, the initial cell-associated enzyme activity (at 50°C) is measured as a function of pH. The optimum pH is about 4.8. Between pH 4.0 and 5.4, there is more than 50% of the maximum activity. The pH optimum for *A. phoenicis* QM 329 β-glucosidase in solution has been reported to be 4.3 (*3*). In the investigagation of Sundstrom et al. (*4*), both free and immobilized β-glucosidase from *A. phoenicis* were found to have pH optima around 3.5.

The initial cell-associated enzyme activity was measured as a function of temperature (Fig. 5). The optimum initial enzyme activity was obtained at 50°C. The enzyme activity is slightly lower at 60 and 70°C. In Fig. 6, the hydrolysis of cellobiose with *A. phoenicis* beads in shake flasks at different temperatures is shown. This experiment measures both the activity and stability of the enzymes during 48 h of hydrolysis. At 70°C, the enzymes are unstable, and at 40°C, the enzyme activity is too low to obtain a high degree of conversion. Optimal stability and activity were obtained at 50 and 60°C.

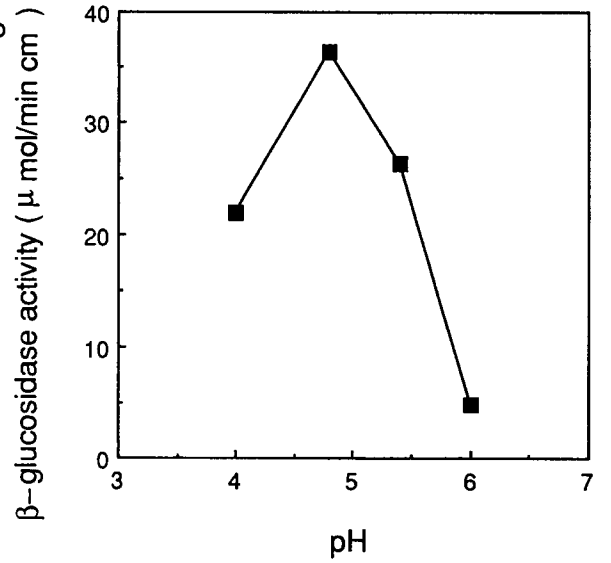

Fig. 4. Cell-associated β-glucosidase activity as a function of pH. The enzyme activity was measured as initial (3 h) cellobiose hydrolysis at 50°C.

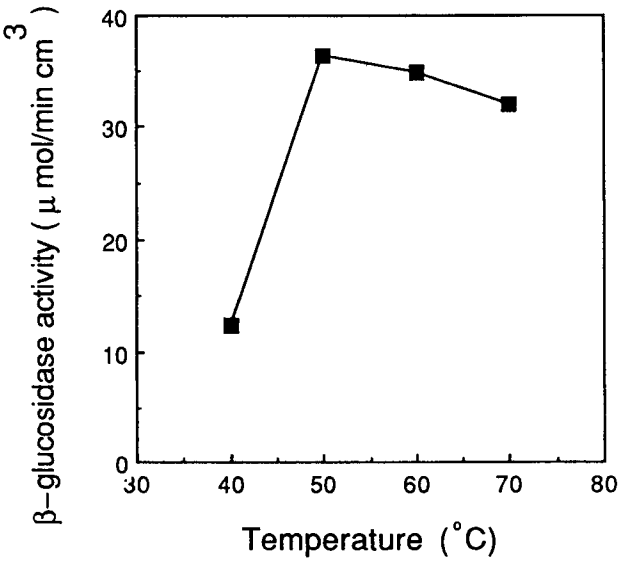

Fig. 5. Cell-associated β-glucosidase activity as a function of temperature. The enzyme activity was measured as initial (3 h) cellobiose hydrolysis. The pH was 4.8 with 0.05*M* Na-acetate buffer.

Cellobiose Conversion in a Fluidized-Bed Reactor

The beads formed by *A. phoenicis* are probably not rigid enough to be used in a packed-bed reactor. A fluidized-bed reactor (*see* Fig. 1) was used, which allowed the beads to be kept in the reactor at optimal conditions for cellobiose hydrolysis (50°C, pH 4.8).

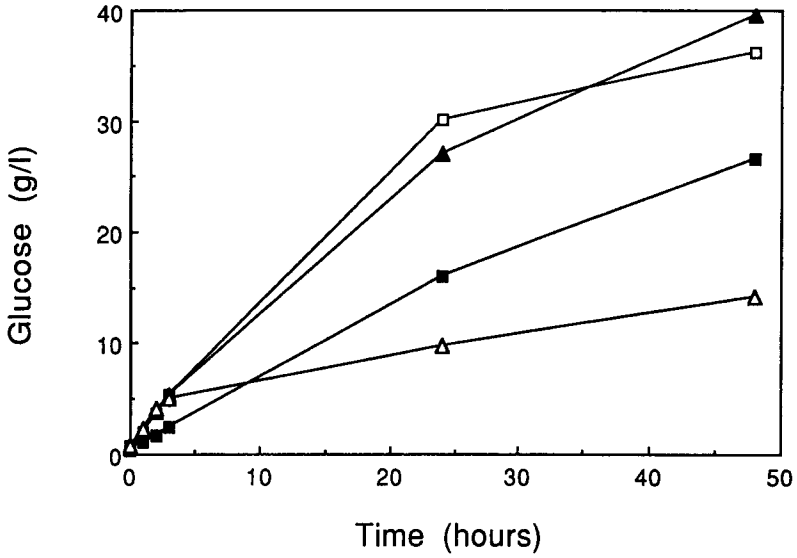

Fig. 6. Cellobiose hydrolysis with beads of *A. phoenicis* at different temperatures: (■) 40, (□) 50, (▲) 60, and (△) 70°C. The hydrolysis was made in shake-flasks at pH 4.8 in 0.05*M* Na-acetate. The initial cellobiose concentration was 50 g/L. Glucose was measured with HPLC.

In Fig. 7, two repeated batch conversions of cellobiose in the fluidized-bed reactor are shown. The enzyme containing beads were added at the start to a total enzyme activity of 87 IU. The average bead diameter was 2 mm. The total working volume in the reactor was 140 mL. Cellobiose was added at the start to a concn. of 50 g/L. After 21 h, a conversion of 85% of the cellobiose was obtained. At 26 h, cellobiose was added to the reactor to a final concn. of 43 g/L. At 48 h, 70% of the added cellobiose had been converted to glucose. The rate of glucose production in the reactor was 87 μmol/min during the first 2 h. After the cellobiose addition at 26 h, at an accumulated glucose concn. of 45 g/L, the rate of glucose production was 40 μmol/min. The decrease in the rate of glucose production is probably owing to inhibition of the cell-associated β-glucosidase by the product glucose. Product inhibition of β-glucosidase from *A. phoenicis* (*13*) and *T. viride* (*14*) has been shown. From the K_m (0.8 m*M* cellobiose) and K_i (1.8 m*M* glucose) for the free enzyme (*13*), it can be estimated that glucose at 45 g/L would reduce the initial rate of glucose production by a factor of 0.54, which is close to the observed value of 0.46.

Continuous Cellobiose Hydrolysis in a Fluidized-Bed Reactor

Cellobiose was continuously hydrolyzed by self-immobilized β-glucosidase in the fluidized-bed reactor over 72 h (Fig. 8). The conditions are listed in Table 1. The average diameter of the *A. phoenicis* beads was 2

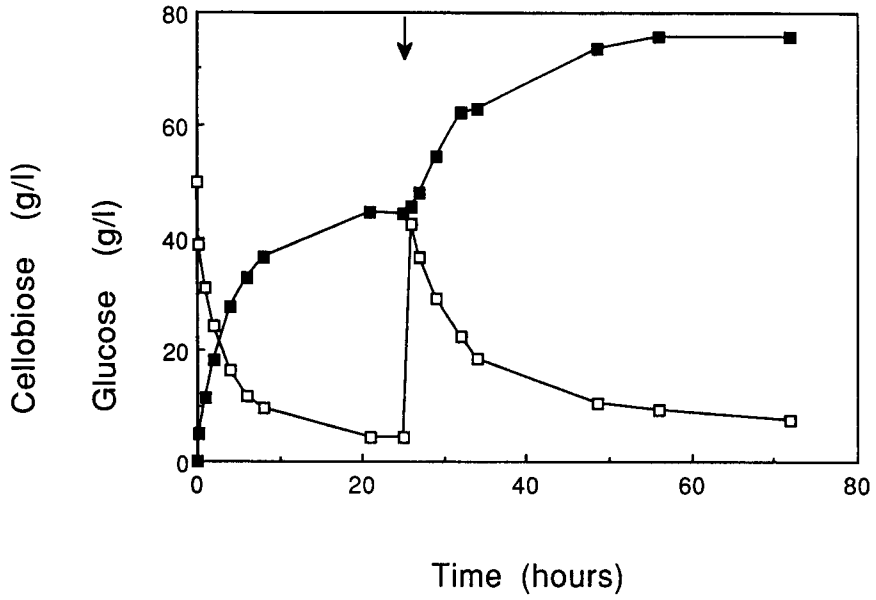

Fig. 7. Cellobiose hydrolysis with beads of *A. phoenicis* in a fluidized-bed reactor. The working volume was 140 mL, and the enzyme activity was 87 IU. The initial cellobiose concentration was 50 g/L. Cellobiose (□) and glucose (■) were analyzed by HPLC. At 26 h (→), cellobiose was added to 43 g/L. The temperature was 50°C and the pH 4.8 (0.05*M* Na-acetate).

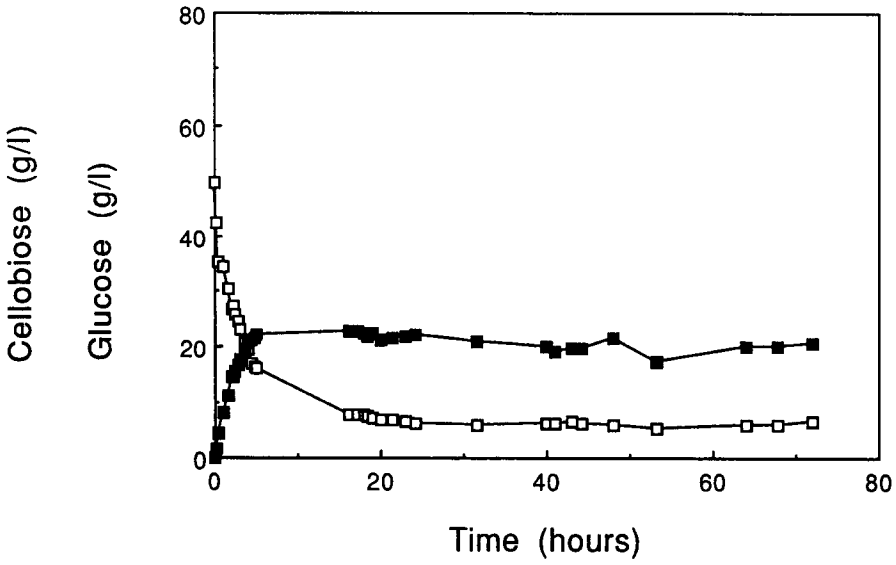

Fig. 8. Continuous cellobiose hydrolysis with beads of *A. phoenicis* in a fluidized-bed reactor. The temperature was 50°C and the pH 4.8 in 0.05*M* Na-acetate. The other conditions are listed in Table 1. Cellobiose (□) and glucose (■) were analyzed by HPLC.

Table 1
Continuous Cellobiose Hydrolysis
in a Fluidized-Bed Bioreactor with Self-Immobilized
β-Glucosidase from *Aspergillus phoenicis*

Reactor volume, mL	160
Initial enzyme activity, IU	85
Initial cellobiose concentration, g/L	50
Inlet cellobiose concentration, g/L	25
Outlet average glucose concentration, g/L	20
Outlet average cellobiose concentration, g/L	6
Average flow rate, mL/h	15,8
Experiment duration, h	72
Dilution rate, h^{-1}	0,1
Productivity during steady-state, g/L-h	2,0
Conversion during steady-state, %	76
Total conversion, %	72
Total processed volume, mL	1105

mm, and their total enzyme activity was 85 IU. The cellobiose concentration at the start of the reaction was 50 g/L. During the initial 2 h, the reactor was run under batch conditions. A continuous feed flow of cellobiose at 25 g/L was started at 2 h, together with the outlet flow.

The concentration of cellobiose and glucose was measured in the top of the fluidized-bed reactor. Samples were taken at a position just before the outlet flow from the reactor. The concentration of glucose in the effluent increased to 22.5 g/L after 5 h and was then stabilized in a steady-state condition at an average concn. of 20 g/L (Fig. 8). The outlet flow rate was varied between 13.6 to 16.8 mL/h, corresponding to a dilution rate of 0.08–0.10 h^{-1}. At this dilution rate, the cellobiose concentration in the effluent approached a steady-state value of 6 g/L. The degree of conversion during steady-state conditions was 76%. The degree of conversion during the whole run was 72%. The productivity was 2.0 g/L-h, and a total volume of 1105 mL was processed in the reactor.

When cell-associated β-glucosidase of *Trichoderma* sp. E-58, entrapped in alginate beads, was used in a packed-bed reactor, the maximum productivity obtained was 5.4 g/L-h at a substrate conversion of 47% (*9*). Our results with the self-immobilized β-glucosidase of *A. phoenicis* shows that a productivity of half that reported in (*9*) can be obtained though a much higher substrate conversion was achieved.

Biocatalyst Stability Under Reactor Conditions

The stability of the cell-associated β-glucosidase of *A. phoenicis* was investigated (Fig. 9). After the continuous conversion, shown in Fig. 8, the enzyme containing beads were kept in the reactor at 50°C and pH 4.8. A batch conversion of cellobiose at 50 g/L was then performed starting at 161 h. The initial hydrolysis rate of cellobiose was reduced to only 87% of

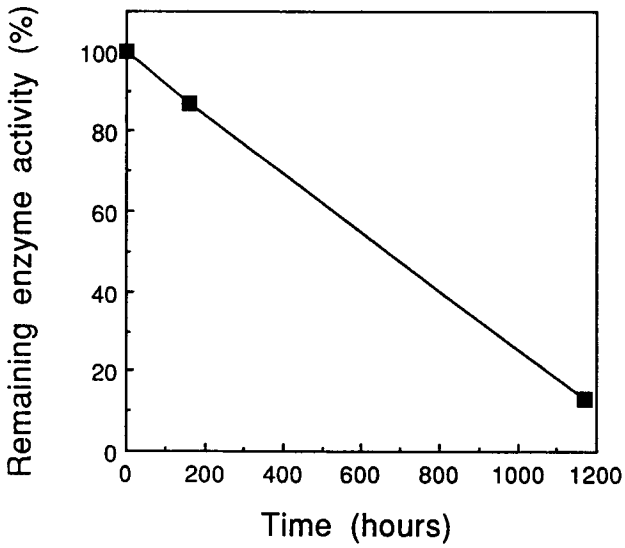

Fig. 9. Stability of β-glucosidase in beads of *A. phoenicis* in a fluidized-bed reactor. The beads were kept in the reactor at 50°C and pH 4.8 (0.05*M* Na-acetate). The initial rate of cellobiose hydrolysis was measured at 0, 161, and 1170 h.

the original rate. The degree of conversion was 87% after 23 h, which is almost equivalent to the result obtained with fresh beads (*see* Fig. 7). When the beads had been kept in the reactor for 1170 h, a new batch hydrolysis was performed. The initial rate of cellobiose hydrolysis had decreased to 13% compared with the rate at the start of the experiment. Based on these results, the half-life of the cell-associated β-glucosidase was estimated to be approximately 700 h. This is in the same order of magnitude as the half-life of cell-associated β-glucosidase of *Trichoderma* entrapped in alginate beads, which was found to be approximately 1100 h in a packed-bed reactor (*9*).

CONCLUSIONS

Aspergillus phoenicis QM 329 can be cultivated in the shape of beads in shake-flasks and in an air-lift fermemtor. β-Glucosidase is retained in the beads at a pH below 6. The self-immobilized β-glucosidase can be used for continuous cellobiose hydrolysis in a fluidized-bed reactor. The productivity was 2.0 g/L-h at a cellobiose conversion of 76%. The half-life of the self-immobilized β-glucosidase was 700 h at 50°C and pH 4.8.

ACKNOWLEDGMENTS

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