0273-2289/92/3435—0261$02.40

Mycelia-Associated β-Xylosidase in Pellets of *Aspergillus* sps.

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ABSTRACT

A screening of 10 strains of *Aspergillus* for pellet formation and mycelia-associated β-xylosidase activity was performed in media containing glucose and glucose supplemented with methyl β-D-xylopyranoside. The aim was to produce an immobilized enzyme preparation. Three strains with high mycelia-associated β-xylosidase activity were investigated for enzyme leakage and enzyme stability: *A. terreus* QM 1991, *A. phoenicis* ATCC 13157, and *A. phoenicis* QM 329. The pellets of *A. phoenicis* QM 329 had the highest β-xylosidase activity (280 IU/g dry wt mycelia) after 333 h of incubation. From measurements of both cell-bound enzyme activity and the activity in solution, it could be concluded that for *Aspergillus phoenicis* QM 329 and ATCC 13157 the decrease in β-xylosidase activity bound to the pellets was owing to enzyme leakage. For *Aspergillus terreus* QM 1991, the decrease of pellet-bound β-xylosidase activity was the result of both leakage and enzyme deactivation at 50°C. β-Xylosidase in pellets of *A. phoenicis* QM329 hydrolyzes xylobiose and *p*-nitrophenyl β-D-xylopyranoside with the same rate of conversion.

Index Entries: β-xylosidase; *Aspergillus;* xylobiose hydrolysis; immobilization; mycelial pellets.

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INTRODUCTION

The enzyme β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) hydrolyzes xylo-oligosaccharides to xylose. The key role of β-xylosidase in the enzymatic hydrolysis of xylans into monomeric xylose has been demonstrated (*1–3*). The addition of β-xylosidase from *Trichoderma reesei* to a hydrolysis mixture containing xylanase and esterase resulted in a strong increase in the xylose yield from steamed birchwood xylan, as shown by Poutanen and Puls (*2*).

Methyl β-D-xylopyranoside has been used for the induction of xylan-degrading enzymes in strains of *Aspergillus* (*3*), and in the yeasts *Cryptococcus albidus* (*4*) and *Trichosporon cutaneum* (*5*). In these cases, an increase of β-xylosidase activity was observed in cultures after the addition of methyl β-D-xylopyranoside.

In previous papers, we have investigated the association of β-glucosidase to mycelial pellets of *Aspergillus phoenicis* QM 329 (*6,7*). An immobilized enzyme preparation was obtained without addition of immobilizing reagents. The retainment of the β-glucosidase activity inside the pellets depended on the carbon source, inoculum size, pH profile during cultivation, and fermentor configuration (*6*). The mycelial pellets were spontaneously formed in shake flask cultivations and in air-lift fermentations after inoculation with conidia. The size of the pellets could be varied with inoculum size (*7*). The pellets containing β-glucosidase were used in a fluidized-bed reactor for continuous cellobiose hydrolysis (*7*). Mycelial pellets with bound α-galactosidase have been used in large-scale applications in the sugar industry for raffinose hydrolysis in Japan (*8*) and the United States (*9*). The formation and structure of mycelial pellets have been reviewed by Metz and Kossen (*10*).

β-Xylosidase from *Aspergillus niger* has been immobilized to different carriers (*11*). Only by immobilizing on alumina using $TiCl_4$ as the binding agent could a high-immobilized activity be achieved (*12*). However, the stability of the enzyme was adversely affected by the immobilization. Decay rates were approx 10 times higher than those of soluble β-xylosidase as the same temperature (*12*).

We have investigated the association of β-xylosidase to mycelial pellets. This cultivation technique, which produces a self-immobilized enzyme, is very mild compared to conventional enzyme immobilization methods. It should be possible to obtain a durable immobilized enzyme preparation provided that the leakage of enzyme from the mycelial pellets is low and the enzyme has high stability. A screening of 10 strains of *Aspergillus* for pellet formation and β-xylosidase activity has been performed. The media contained glucose and glucose supplemented with methyl β-D-xylopyranoside. Three strains were selected for investigation of the stability of the immobilized enzyme preparations. The retainment of the enzyme in the

mycelial pellets was determined as well as the stability of the soluble enzyme at 50°C.

MATERIALS AND METHODS

Organisms

Aspergillus phoenicis QM 329 was obtained from the QM Culture Collection, Department of Botany, University of Massachusetts, Amherst, MA. The other strains of *Aspergillus* were obtained from the Technical University of Budapest, Hungary. The fungi were maintained on potato dextrose agar (PDA) slants at 27°C.

Medium and Shake Flask Cultures

The culture technique for mycelial pellet formation, which is described in our previous works (*6,7*), was used. The cultivation nutrient medium was: proteose peptone no. 2 (Difco, Detroit, MI) 1.0 g/L, citric acid 0.5 g/L, and Vogel's medium N (*13*). The glucose concentration was 5 or 10 g/L with or without addition of methyl β-D-xylopyranoside (Sigma Chemical Co., St. Louis,MO).

One hundred milliliters of medium in 1 L baffled conical flasks were inoculated with conidia from 7–14 days-old PDA slants suspended by vortexing in 4 mL of sterile 0.2% Triton X-100 solution (Merck, Darmstadt, Germany). The conidia concentration was determined by dilution on PDA dishes. The fungi were cultivated at 30°C and 150 rpm in an orbital shake incubator (Lab-line 3528, Lab-line Instruments Inc., Melrose Park, IL). The initial pH, 5.2, was not adjusted during the cultivations. The fungi grew in the form of mycelial pellets.

Screening

Ten strains of *Aspergillus* were grown in shake flasks on glucose (10 g/L) and on glucose (5 g/L) supplemented with methyl β-D-xylopyranoside (5 g/L). In the cultivations on glucose, samples were taken every 48 h. In the cultivations with addition of methyl β-D-xylopyranoside, samples were taken every 24 h. The β-xylosidase activity was determined in the culture fluid and in the intact pellets. The diameter of the pellets was measured, and the volume calculated. The pellet-associated activity is given in IU/cm^3 in order to show that it is based on the calculated pellet volume, and the activity in the culture fluid is given in IU/mL.

Sampling and Sample Preparation

Samples of 3–30 mycelial pellets and 0.5–10 mL culture fluid were withdrawn from the cultures and from the enzyme solutions in the stability experiments. The pellets were washed 3 times in 0.05*M* citrate buffer, pH 4.5. After each wash, the pellets were recovered on a Büchner funnel.

Enzyme Assays

The β-xylosidase activity was assayed with 5 m*M* *p*-nitrophenyl β-D-xylopyranoside (pNPX, Sigma Chemical Company, St. Louis, MO) in 0.05*M* citrate buffer, pH 4.5 (*2*). A 100-μL sample was added to 0.9 mL substrate solution and incubated for 5.0 min at 50°C. The β-xylosidase activity of the intact pellets was assayed by adding 1 pellet to 0.9 mL substrate solution and incubated at 50°C for 5.0–10.0 min. The reactions were terminated with 0.5 mL of 1*M* Na_2CO_3, and the absorbance was read at 400 nm (Shimadzu UV-240). A standard curve was obtained with *p*-nitrophenol (Sigma).

The pellets were also disrupted in 3 mL distilled water in a sonifier with a special microtip (Branson Sonic Power Co., Danbury, CT, model B-30). The output was 4.0, and 40% duty pulses were used. The sonication time was 3 min. The β-xylosidase activity was assayed as above.

Protein Assay, Dry Wt, and Sugar Analysis

Protein was determined by the method of Bradford (*14*) with bovine serum albumin as standard. The dry wt of the mycelia was determined at 110°C after washing in cold (4°C) distilled water. The samples were dried to a constant weight. Xylose, xylobiose, and glucose were determined by HPLC. The column was an Aminex HPX87H (Bio-Rad, Richmond, CA) with 0.005*M* H_2SO_4 as eluent at 60°C.

RESULTS

Screening for Pellet Formation and β-Xylosidase Activity

A screening of 10 strains of *Aspergillus* for pellet formation and mycelia-associated β-xylosidase activity was conducted. The cultivations were made on glucose and on glucose supplemented with methyl β-D-xylopyranoside. All strains formed pellets in both media. The size of the pellets was regulated by the inoculum size to be between 1–4 mm in diameter (Table 1). The size distribution within each cultivation was approx ±25% from the mean value, i.e., the pellets were of nearly uniform size. The maximum β-xylosidase activity associated with the mycelial pellets and the corresponding activity in the culture fluid are presented in Table 1.

Table 1
Screening of β-Xylosidase Activity in Strains of *Aspergillus* *

Strain of *Aspergillus*	Medium: glucose			Medium: glucose+βMX		
	Mycelia-associated IU/cm³	Culture fluid IU/mL	Time, h	Mycelia-associated IU/cm³	Culture fluid IU/mL	Time, h
A. phoenicis ATCC 13157	0.07 (3)	0.03	168	4.37 (3)	0.01	96
A. phoenicis QM 329	0.11 (3)	0.04	168	5.28 (4)	0.03	120
A. phoenicis ATCC 15555	0.03 (3)	0.02	192	1.19 (4)	3.52	159
A. niger ATCC 46890	0.52 (1)	0.06	192	5.71 (3)	5.33	159
A. niger BKM F-1305	0.13 (2)	0.05	192	1.05 (3)	5.29	159
A. niger T 2413	0.22 (2)	0.05	192	0.35 (4)	0.03	94
A. terreus QM 1991	0.21 (1)	0.02	168	5.23 (1)	0.14	50
A. terreus QKI 16/5	0.20 (2)	<0.01	192	0.71 (4)	<0.01	150
A. terreus BKM F-699	0.01 (4)	<0.01	192	1.04 (4)	0.01	98
A. oryzae OKI 154	0.01 (4)	<0.01	192	3.35 (4)	0.02	96

*The media contained glucose (10 g/L) and glucose (5 g/L) plus methyl β-D-xylopyranoside (βMX, 5 g/L). The maximum values for the mycelia-associated β-xylosidase activity (assayed with intact pellets) are given with the corresponding activity in the culture fluid. The diameter (mm) of the pellets is given in parenthesis.

The activity in the glucose-grown cultures was low. When cultivated on glucose, the strains of *Aspergillus niger* showed a slightly higher activity in comparison to the others. In two strains, the activity was also low when the medium was supplemented with methyl β-D-xylopyranoside (*A. niger* T 2413 and *A. terreus* QKI 16/5). In all other strains, both the mycelia-associated β-xylosidase activity and the activity in the culture fluid were significantly enhanced by the addition of methyl β-D-xylopyranoside.

Methyl β-D-Xylopyranoside as Inducer for β-Xylosidase Activity

The role of methyl β-D-xylopyranoside as the inducer for β-xylosidase activity was studied in cultivations of *Aspergillus phoenicis* QM 329. Cultivations were made with glucose and with glucose supplemented with methyl

β-D-xylopyranoside. The time-course of glucose consumption is shown in Fig. 1A, the β-xylosidase activity associated with the mycelial pellets in Fig. 1B, and the β-xylosidase activity in the culture fluid in Fig. 1C. After glucose had been consumed, the activity of β-xylosidase associated with the mycelial pellets and the specific activity in the culture fluid increased rapidly in the methyl β-D-xylopyranoside-supplemented culture. After a peak at 70 h the specific activity in the culture fluid decreased. Only a minor decrease was observed for the mycelia-associated enzyme activity.

Enzyme Leakage and Stability

Three strains with high mycelia-associated β-xylosidase activity and low activity in the culture fluid were selected on the basis of the cultivations presented in Table 1. The selected strains were *A. phoenicis* ATCC 13157, *A. phoenicis* QM 329, and *A. terreus* QM 1991. Pellets from the three strains cultivated on glucose 5 g/L and methyl β-D-xylopyranoside 5 g/L were harvested after 70 h of cultivation. The pellets were carefully washed three times with cold (4°C) 0.05*M* citrate buffer, pH 4.5. After washing, the pellets were kept at 50°C in 0.05*M* citrate buffer, pH 4.5, on a rotary shake incubator at 150 rpm. The β-xylosidase activity bound to the mycelia and the leakage of β-xylosidase into the solution was measured throughout a time period of 333 h. The mycelia-associated enzyme activity was measured after sonicating the pellets (Fig. 2). All three strains exhibited a decrease of mycelia-bound enzyme activity during the incubation. The decrease in terms of percentage was largest in *A. terreus* QM 1991. After 333 h, 39% of the initial activity remained bound to the pellets. The corresponding values for *A. phoenicis* ATCC 13157 and *A. phoenicis* QM 329 were 64 and 44%, respectively. The pellets of *A. phoenicis* QM 329 had the highest β-xylosidase activity (280 IU/g dry wt mycelia) after 333 h of incubation.

The enzyme leakage was measured by assaying the β-xylosidase activity in solution during incubation of the pellets (Fig. 3). For *A. phoenicis* ATCC 13157 and *A phoenicis* QM 329, the mycelia-associated β-xylosidase activity lost during incubation was recovered in the fluid. For *A. terreus* QM 1991, a loss of total activity was noticed; after an incubation of 333 h, the original total activity of 2.39 IU mycelia bound had decreased to 1.41 IU distributed as 0.94 IU mycelia bound and 0.47 IU in solution. Thus, the total activity had decreased to 59% of the initial. The total activity remained around 100% for *A. phoenicis* ATCC 13157 and *A. phoenicis* QM 329.

Stability of Soluble Enzyme at 50 °C

The stability of the soluble β-xylosidase at 50°C was investigated. The culture broth from cultures cultivated for 160 h on glucose supplemented with methyl β-D-xylopyranoside was diluted in 0.05*M* citrate buffer, pH 4.5. The solutions were kept at 50°C on a shake incubator at 150 rpm. The

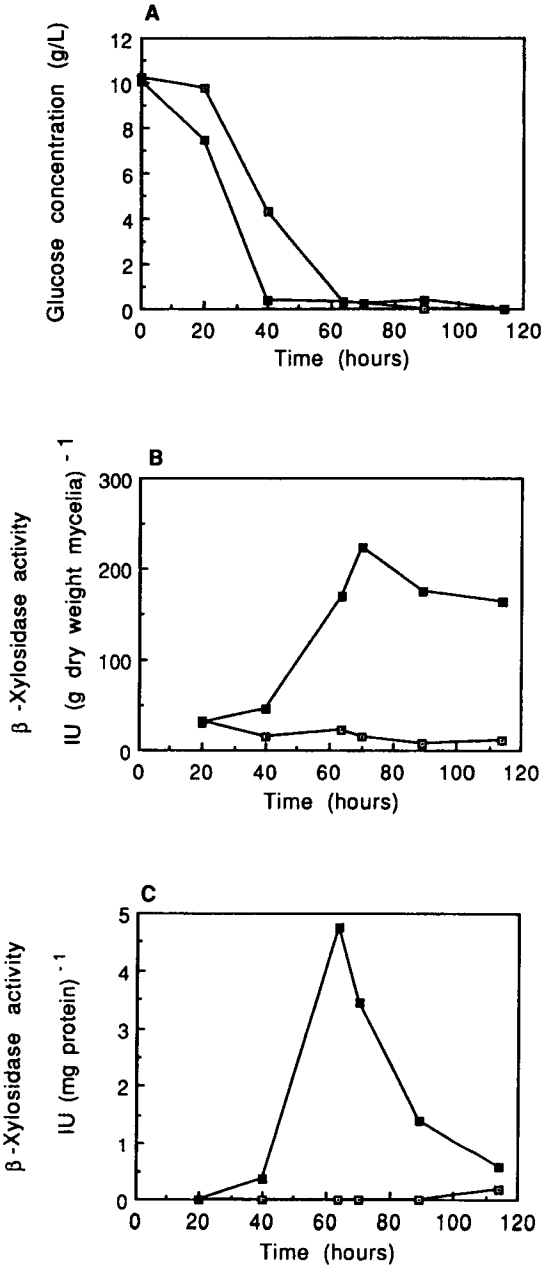

Fig. 1. Cultivation of *A. phoenicis* QM 329 on glucose (10 g/L) (□) and on glucose (10 g/L) plus methyl β-D-xylopyranoside (5 g/L) (■). **A.** Glucose concentration in the culture fluid. **B.** The β-xylosidase activity associated with the mycelial pellets. The enzyme activity was measured after sonicating the pellets. **C.** The specific β-xylosidase activity in the culture fluid.

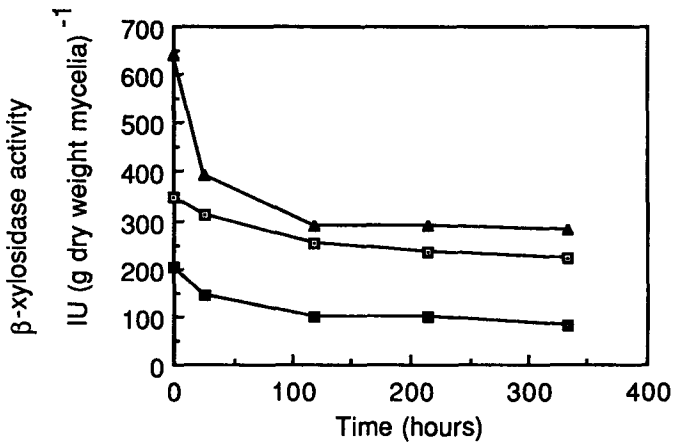

Fig. 2. The cell-associated β-xylosidase activity during incubation of mycelial pellets at 50°C, pH 4.5, 0.05*M* citrate buffer, on a shaker at 150 rpm. The enzyme activity was measured after sonicating the pellets. The pellets are from *A. phoenicis* QM 329 (▲), *A. phoenicis* ATCC 13157 (□), and *A terreus* QM 1991 (■).

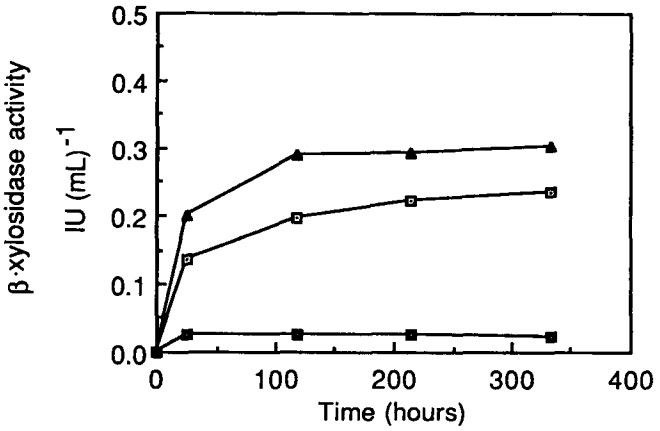

Fig. 3. The β-xylosidase activity in the solution during incubation of mycelial pellets of *A. phoenicis* QM 329 (▲), *A. phoenicis* ATCC 13157 (□), and *A. terreus* QM 1991 (■). Conditions are the same as in Fig. 2.

β-xylosidase activity throughout a time period of 333 h was measured (Fig. 4). A slight decrease in activity was observed for *A. phoenicis* ATCC 13157 and *A. phoenicis* QM 329 with 85.1 and 92.4% of the initial activity remaining after 333 h, respectively. However, for *A. terreus* QM 1991 the β-xylosidase activity was reduced to 50.8% of the initial activity after 333 h.

Hydrolysis of Xylobiose Using Self-Immobilized β-Xylosidase

Aspergillus phoenicis QM 329 was chosen for a study of xylobiose hydrolysis by self-immobilized β-xylosidase. Two batch hydrolysis were performed, one with 5 m*M* *p*-nitrophenyl β-D-xylopyranoside (pNPX)

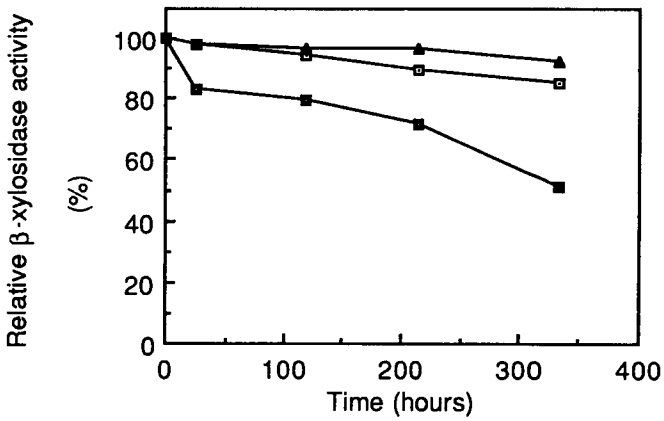

Fig. 4. Stability of soluble β-xylosidase from three strains of *Aspergillus*. The enzyme solutions were incubated at 150 rpm, 50°C, pH 4.5 (0.05*M* citrate buffer), on a shaker at 150 rpm. The culture broth from cultivations for 160 h on glucose (5 g/L) with addition of methyl β-D-xylopyranoside (5 g/L) was used. The initial activity (set to 100%) was for *A. phoenicis* QM 329 (▲) 0.17 IU/mL, for *A. phoenicis* ATCC 13157 (□) 0.22 IU/mL, and for *A. terreus* QM 1991 (■) 0.13 IU/mL.

and one with 5 m*M* xylobiose. The pH was 4.5 (0.05*M* citrate buffer), and the temperature was 50°C. Ten pellets from a 70-h culture, washed 3 times in buffer, were used in 3mL substrate on a shake incubator at 150 rpm. The activity of the pellets was 600 IU/g (dry wt), and 0.5 mg (dry wt) of pellets were added. The diameter of the pellets was 2 mm.

The xylobiose, xylose, and pNPX concentrations are shown in Fig. 5. The insert shows the concentration of produced *p*-nitrophenol compared with the conversion of xylobiose expressed as the xylose concentration divided by two. The curves show a high degree of similarity. The rate of conversion for 30 min in the two hydrolysis experiments was calculated from the slopes in the inserted figure (Fig. 5). The rate of xylobiose conversion for 30 min was 91.7 μmol (min L)$^{-1}$. The rate of *p*-nitrophenyl β-D-xylopyranoside hydrolysis for the same time period was 93.9 μmol (min L)$^{-1}$.

The β-xylosidase activity in the solution during the xylobiose hydrolysis experiment in Fig. 5 was assayed at 15 min and 30 min. At 15 min, the activity was < 0.01 IU/mL, and at 30 min, the activity was 0.01 IU/mL. The maximum conversion for 0.010 IU/mL of β-xylosidase is calculated to 10 μmol (min L)$^{-1}$ corresponding to 11% of the total rate of xylobiose hydrolysis (91.7 μmol [min L]$^{-1}$). Thus, at least 89% of the rate of xylobiose hydrolysis during 30 min was owing to β-xylosidase activity associated with the mycelial pellets.

DISCUSSION

It has been demonstrated by Dekker (*1*) and by Poutanen and Puls (*2*) that β-xylosidase plays an important role in the total hydrolysis of xylan

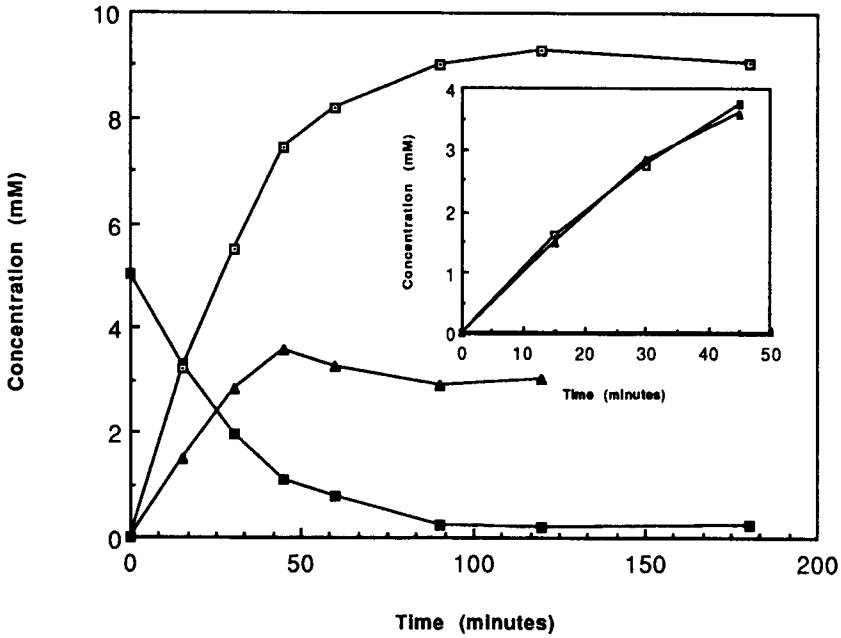

Fig. 5. Hydrolysis of xylobiose and *p*-nitrophenyl β-D-xylopyranoside by self-immobilized β-xylosidase in pellets of *A. phoenicis* QM 329. The initial concentrations of xylobiose and pNPX were 5 m*M*. The concentrations of xylobiose (■) and xylose (□) were determined by HPLC. The concentration of *p*-nitrophenol (▲) was determined by absorbance at 400 nm. The inserted figure shows the *p*-nitrophenol and xylose production during the initial 45 min. The xylose concentration has been divided by two in the inserted figure.

to xylose. The xylose yield in the hydrolysis of solubilized xylan is better correlated with the level of β-xylosidase than with that of xylanases (2).

β-Xylosidase can be induced in strains of *Aspergillus* with methyl β-D-xylopyranoside (Table 1). For *Aspergillus phoenicis* QM 329, the β-xylosidase activity in the culture fluid and associated with the mycelium is more than 10 times higher in cultivations that include methyl β-D-xylopyranoside when compared to a cultivation only on glucose (Fig. 1). In the yeast *Trichosporon cutaneum,* the total cell-associated and the extracellular β-xylosidase activity increased 10–20-fold upon the addition of 10 m*M* methyl β-D-xylopyranoside in glucose pregrown cells (5). A two- to fivefold increase in extracellular β-xylosidase activity has also been observed in strains of *A. oryzae, A. foetidus, A. fumigatus,* and *A. terreus* when either Solca-floc cellulose or wheatbran media were supplemented with methyl β-D-xylopyranoside (3).

Fungal glycosidases with both β-glucosidase and β-xylosidase activity have been reported for *Stachybotrys atra* (15) and *Aspergillus fumigatus* (16). A cell-wall-bound enzyme with both activities has been reported for the yeast *Cryptococcus albidus* (17). The specificity and the cellular location of the cell-associated β-xylosidase presented here are currently under investigation.

Three strains with high mycelial-associated activity and low extracellular activity of β-xylosidase were selected for investigation of enzyme leakage and enzyme stability. The mycelial pellets were kept at 50°C and pH 4.5 without substrate present (Figs. 2 and 3). From measurements of the total enzyme activities in the pellets and in solution, it can be concluded that for *A. phoenicis* ATCC 13157 and *A. phoenicis* QM 329 enzyme leakage is the main cause of the decrease in mycelial-associated activity. The soluble β-xylosidase of the *A. phoenicis* strains was found to be stable for 330 h at 50°C and pH 4.5. For *A. terreus* QM 1991, on the other hand, a decrease of 49% in soluble β-xylosidase activity was observed. This decrease in total β-xylosidase activity in the pellets of *A. terreus* QM 1991 was concluded to be owing both to enzyme leakage and to enzyme deactivation at 50°C.

Ten carriers for β-xylosidase immobilization have been investigated by Oguntimein and Reilly (*11*). The source of β-xylosidase was *Aspergillus niger*. The best results were obtained with the β-xylosidase bound to aluminia with $TiCl_4$ as binding agent and to alkylamine porous silica with glutaraldehyde. To alumina, 40–45 U/g could be bound with an efficiency of approx 70%. The highest stability of β-xylosidase bound to aluminia was at pH 4–4.5 (*12*). However, the stability was adversely affected by this immobilization method. Our results show that, with the self-immobilization technique, a high amount of β-xylosidase activity can be bound to the carrier (the mycelial pellets). The highest activity achieved was 640 IU/g in pellets of *Aspergillus phoenicis* QM 329. After 333 h at 50°C, 280 IU/g remained bound to the pellets.

In this investigation, the assays of β-xylosidase activity in the mycelial pellets and in solution were made with the artificial substrate *p*-nitrophenyl β-D-xylopyranoside. In order to compare the activity of the enzyme utilizing this substrate and the natural substrate xylobiose, the experiments shown in Fig. 5 were made. The self-immobilized β-xylosidase hydrolyzes xylobiose and *p*-nitrophenyl β-D-xylopyranoside at the same rate for 30 min. Thus, the same β-xylosidase activity in mycelial pellets of *A. phoenicis* QM 329 was found for the conversion of the natural substrate xylobiose as for *p*-nitrophenyl β-D-xylopyranoside. It should be possible to use β-xylosidase immobilized in mycelial pellets in a process for the conversion of soluble xylan, e.g., produced in steam pretreatment of wood (*18*), to xylose.

CONCLUSIONS

β-Xylosidase activity was induced in eight out of ten investigated strains of *Aspergillus* by supplementing glucose media with methyl β-D-xylopyranoside. Three strains had high mycelia-associated and low extracellular β-xylosidase activity, which made it possible to obtain mycelial

pellets with self-immobilized β-xylosidase. In the strains *Aspergillus phoenicis* QM 329 and ATCC 13157, the decrease in β-xylosidase activity bound to pellets stored at 50°C and pH 4.5 was owing to enzyme leakage. For *Aspergillus terreus* QM 1991, the decrease of pellet-bound β-xylosidase activity was owing both to leakage and to enzyme deactivation at 50°C. Self-immobilized β-xylosidase in pellets of *A. phoenicis* QM 329 hydrolyzes the natural substrate xylobiose and the artificial substrate *p*-nitrophenyl β-D-xylopyranoside with the same rate of conversion.

ACKNOWLEDGMENT

This investigation was supported by the Swedish National Energy Administration.

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