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Xylanolytic Enzyme Production by an *Aspergillus niger* Isolate

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ABSTRACT

Production of xylanolytic enzymes by an *Aspergillus niger* CCMI 850 isolate was investigated in batch cultures. The effect of the composition of a fermentation medium that did not include chemical inducers, on β-xylanase, β-xylosidase, α-L-arabinofuranosidase, and total cellulase activity was studied. With 4% xylan as the carbon source, about 65 U/mL of β-xylanase was obtained, whereas the total cellulase activity was undetectable, under the specified conditions. This β-xylanase activity represents the highest reported for a wild-type strain of *A. niger*. The effect of pH and temperature on the activity of β-xylanase was studied. Partial characterization of the β-xylanase showed that with insoluble birchwood as substrate the K_m and V_{max} were 0.3 m*M* and 19 μmol/min, respectively. Aspects of using the crude β-xylanase preparation for applications in the pulp and paper industry were discussed.

Index Entries: *Aspergillus niger*; xylanase; β-xylosidase; low cellulase production; pulp and paper industry applications.

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INTRODUCTION

Information on xylanases has been increasingly forthcoming in recent years because of their potential use in numerous industrial processes. These include bioconversion of lignocellulose-derived sugars into fuel (*1*), processing food including bread-making and clarification of beer and juice (2), as well as in the paper and pulp industry. In the latter industry, Viikari and coworkers first reported in 1986 that xylanases could be employed to enhance the bleachability of kraft pulps (*3*). In the space of a few years, this concept of xylanase-aided bleaching has translated into an economically viable and environmentally friendly technology that is presently being used in several industrial mills (4). The main enzymatic activities involved in this bioprocess have been shown to belong to the group of endo-β-xylanases (*5,6*).

Among the factors that determine the economic viability of using biocatalysts are enzyme productivity and fermentation-associated costs. Various approaches, including strain selection and mutation (*7*), optimization of fermentation medium (*8,9*), gene cloning, and in vitro mutagenesis (*10*) have been used to enhance xylanase production. Despite these advances the need remains for the isolation of novel strains able to produce in simple media the necessary enzyme(s) with high specific activity and operational stability.

This paper reports the production of high levels of xylanolytic enzymes by an *Aspergillus niger* isolate. The effect of the composition of the fermentation medium on the xylanolytic enzymes was investigated and this led to the development of a simple fermentation medium. Partial characterization of the key enzyme, endo-β-xylanase, is also reported.

MATERIALS AND METHODS

Microorganism and Media

Out of several fungal strains isolated in our laboratory, a strain isolated from a decaying specimen of the Mediterranean shrub *Cistus ladaniferus* and identified as *Aspergillus niger* var *usamii* was the most efficient at removing lignocellulosic substrates from the growth medium (*11*). Therefore this strain, denoted *Aspergillus niger* CCMI 850, was used throughout the present work. Stock cultures were maintained on malt agar slants. Spore suspensions were made from 5-d-old cultures grown at 30°C. Distilled water was added to each Petri dish and a suspension of spores was made by lightly brushing the mycelium. Spore suspensions at a final concentration of 10^7 spores/mL were used to inoculate each flask.

The medium was based on that described by Smith and Wood (*8*) and consisted of the following components (g/L): $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$ (anhydrous), 0.05; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot 4H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.005; $FeSO_4 \cdot 7H_2O$, 0.1; Na_2SO_4, 1; EDTA, 0.6; and NaH_2PO_4, 1.56. The nitrogen source was 1.4 g/L urea and carbon source is specified in the text.

Cultivation Conditions

Fermentations were carried out in 250-mL Erlenmeyer flasks containing 50 mL medium. Cultivations were carried out at 30°C with orbital shaking (150 rpm) for the length of time specified in the text.

Determination of Enzyme Activities

The activity of enzymes present in the extracellular broth was determined from the crude broth after filtration through several layers of gauze and followed by centrifugation at 10,000*g* for 15 min. Enzymatic assays were performed in triplicate, and each experiment was repeated at least 3 times. Results shown are from a typical experiment.

1. Endo-β-xylanase, hereafter referred to as either β-xylanase or simply xylanase (EC 3.2.1.8: β-1,4-xylan xylanohydrolase) activity was determined by incubating 0.5 mL of the appropriately diluted supernatant and 0.5 mL of 1% birchwood xylan (Sigma, St. Louis, MO) in 0.05*M* citrate buffer, pH 4.5. The mixture was incubated at 50°C for 30 min, and the reducing sugars were assayed using the dinitrosalicyclic reagent (*12*). One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar, expressed as xylose equivalent per min at 50°C and pH 4.5.
2. β-Xyloxidase (EC 3.2.1.37) activity was measured by incubation of the enzyme extract with 0.9% (w/v) *p*-nitrophenyl-β-D-xylopyranoside (Sigma) as substrate in 0.05*M* citrate buffer, pH 3.0 at 65°C. After 30 min the reaction was stopped by the addition of 2% (w/v) sodium carbonate. The *p*-nitrophenol released was determined spectrophotometrically at 410 nm, and activity was defined as the release of one μmol of *p*-nitrophenol/min.
3. α-L-Arabinofuranosidase (EC 3.2.1.55) was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl α-L-arabinofuranoside (Sigma) according to published procedures (*13*).
4. Total cellulolytic activity (FPase) (EC 3.2.1.4) was determined using the methodology recommended by IUPAC (*14*) using a strip of Whatman (Maidstone, England) No. 1 filter paper as substrate.

Table 1
Effect of C Source on Accumulation of Xylanolytic Enzymes by *Aspergillus niger*[a]

C source	Enzyme Activity (U/mL)			
	β-Xylanase	β-Xylosidase	α-Arabinofuranosidase	FPase[c]
Xylan	6.44	5.12	0.40	0.02
Spent grain	4.82	ND[b]	ND[b]	0.03
Isopam-42	1.07	0.49	0.42	0.01
CSL	0.05	0.20	0.49	0.00

[a] All C sources were used at a concentration of 1% (w/v). Enzymatic activities were determined using crude filtrates following cultivation for 7 d, under conditions described in Materials and Methods.
[b] ND = Not determined.
[c] Units as defined by IUPAC.

RESULTS

Effect of Carbon Source on Xylanolytic Enzyme Production

The effect of soluble as well as nonsoluble carbon sources on the extracellular xylanolytic enzyme production by *Aspergillus niger* CCMI 850 was investigated in shake flask cultures. The sugar composition of the soluble Isopam-42 (COPAM) was 52% dextrose, 42% fructose, and 6% other sugars, whereas the corn steep liquor (CSL) contained about 3.5% reducing sugars. The results presented in Table 1 show the activities for β-xylanase, β-D-xylosidase, α-arabinosidase, and FPase using 1% of the appropriate substrate. It can be seen that birchwood xylan was more effective than either Isopam-42 or CSL in inducing both β-xylanase and β-xylosidase activity. Neither β-xylosidase nor α-arabinofuranosidase activity were significantly affected by the amount of xylan present in the cultivation medium (results not shown). The use of Isopam-42 gave a maximum of 1.38 U/mL β-xylanase and 0.49 U/mL β-xylosidase, whereas CSL resulted in the production of less than 1 U/mL β-xylanase and low levels of β-xylosidase. The α-arabinofuranosidase activity did not appear to be critically dependent on the type of carbon source used. Negligible amounts of FP activity were observed using the soluble carbon sources. The time courses for the accumulation of β-xylanase and FPase using different amounts of insoluble C sources are presented below.

Time Course of β-Xylanase Production Using Xylan and Spent Grain

The time course for β-xylanase accumulation as a function of different amounts of birchwood xylan is shown in Fig. 1A. It can be seen that the β-xylanase produced increased in proportion to the amount of substrate

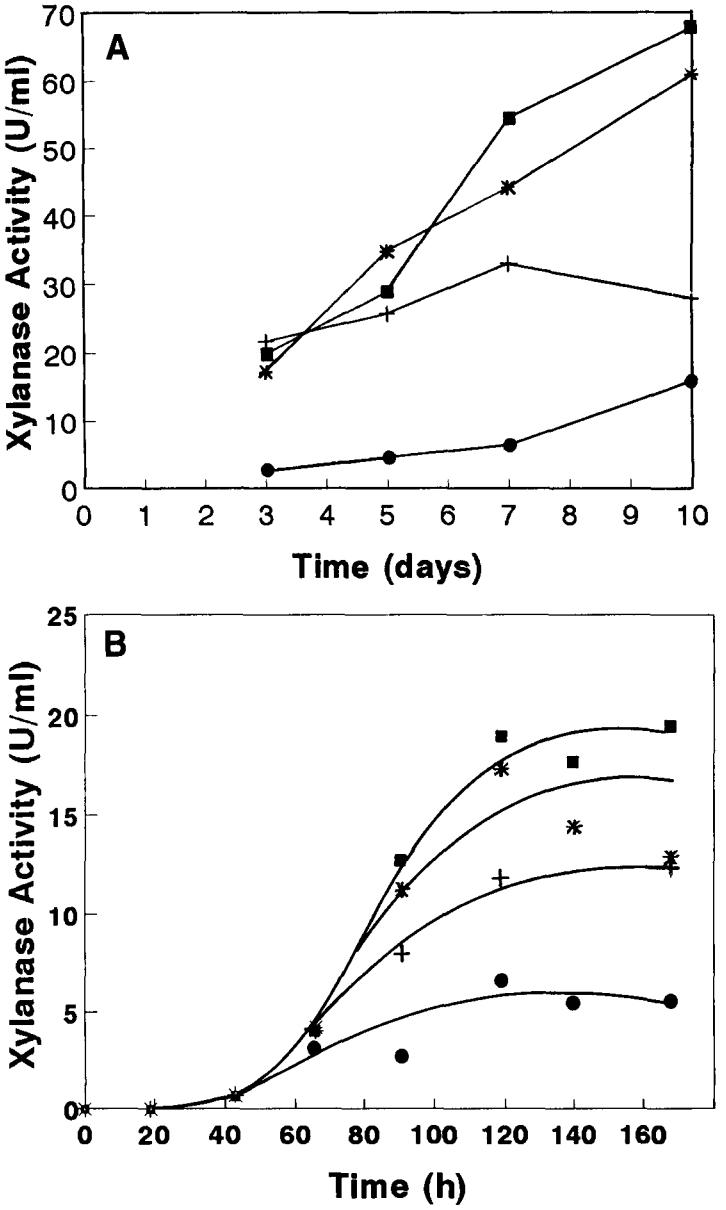

Fig. 1. Effect of xylan (A) and spent grain (B) amount on β-xylanase production by *A. niger* as a function of cultivation time. The amount of C source used was 1% (●), 2% (+), 3% (*), and 4% (■).

used up to about 4%. With 4% of this substrate, about 66 U/mL of β-xylanase was produced following 10 d of cultivation. This represented a relatively small (about 10%) increase above the amount produced in the presence of 3% xylan and did not justify the use of amounts above 4% xylan.

The effect of using different amounts of distiller's spent grain on extracellular β-xylanase activity is shown in Fig. 1B. It can be seen that the β-xylanase activity produced increased in proportion to the amount of spent

grain used. Following 120 h of cultivation, use of 3% of the substrate resulted in production of about 18 U/mL of β-xylanase, which represented a threefold increase compared to that produced in the presence of 1% spent grain. Further increase in the amount of spent grain to 4% gave only a small increase in activity. When spent grain was employed, the activity peaked at about d 5, whereas longer cultivation time was required with xylan as inducer. However, the use of xylan as compared with spent grain, resulted in a more than threefold increase in β-xylanase activity.

Time Course of Total Cellulolytic Activity Using Xylan and Spent Grain

The data, summarized in Fig. 2A, show that when birchwood xylan was employed, relatively high levels of total cellulolytic activity (FPase) were observed during the early stage of cultivation, which was dependent on the amount of substrate used. However, a sharp decrease in activity was observed by d 5, which by d 10 was reduced to undetectable levels.

The time course profile for FP activity in the presence of spent grain (Fig. 2B) contrasted with that using xylan in that the activity increased as a function of time and peaked by about d 4. On further cultivation the FP activity was still present in proportion to the amount of spent grain used. The sustained FP activity is not surprising, since the spent grain had a composition of about 20% cellulose, which would be expected to induce cellulases.

PARTIAL CHARACTERIZATION OF *A. Niger* β-XYLANASE

Effect of pH on β-Xylanase Activity

The results presented in Fig. 3 show that the β-xylanase displayed maximal activity at pH 4–6. Either decreasing or increasing the pH by 2 U away from this optimal range resulted in a 60% decrease in the activity.

Determination of Temperature Optimum and Stability

The effect of temperature on xylanase activity is shown in Fig. 4. It can be seen that the enzyme displayed a wide temperature optimum, with maximal activity at about 55°C.

Thermal inactivation of the xylanase was studied by preincubating the enzyme extract for various times at 40°C (results not shown) and at 50°C (Fig. 5), then cooling and carrying out the standard assay procedure described in Methods. The enzyme was very stable at 40°C so that no significant decrease in activity was observed following incubation for 180 min. However, on incubation at 50°C, the β-xylanase activity was stable

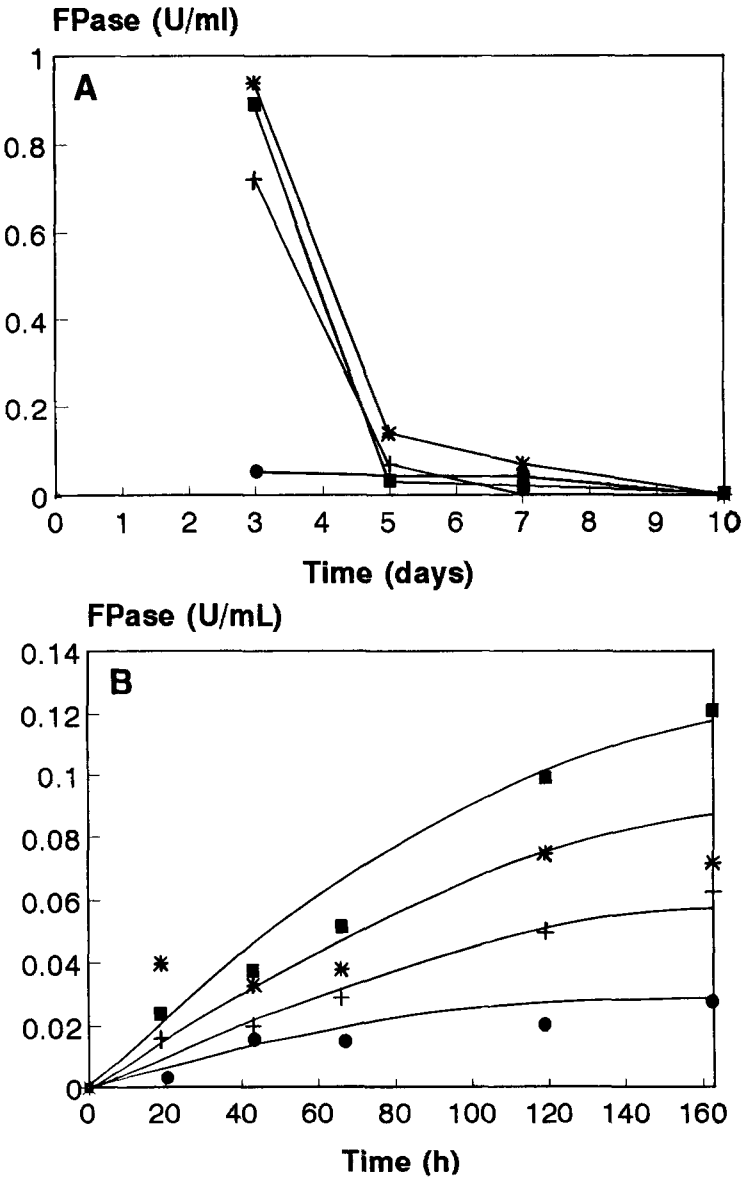

Fig. 2. Effect of xylan (A) and spent grain (B) amount on total cellulolytic activity by *A. niger* as a function of cultivation time. The amount of C source used was 1% (●), 2% (+), 3% (*), and 4% (■).

during the initial 120 min, but thereafter there was a decrease in activity so that after 180 min about one-third of the activity was lost. That the instability was in part owing to the presence of proteolytic enzymes is suggested by the data obtained in the presence of phenylmethanesulphonyl fluoride (PMSF, 1 m*M*). It can be seen that including the serine protease inhibitor, PMSF, markedly enhanced the stability of the β-xylanase preparation of 50°C.

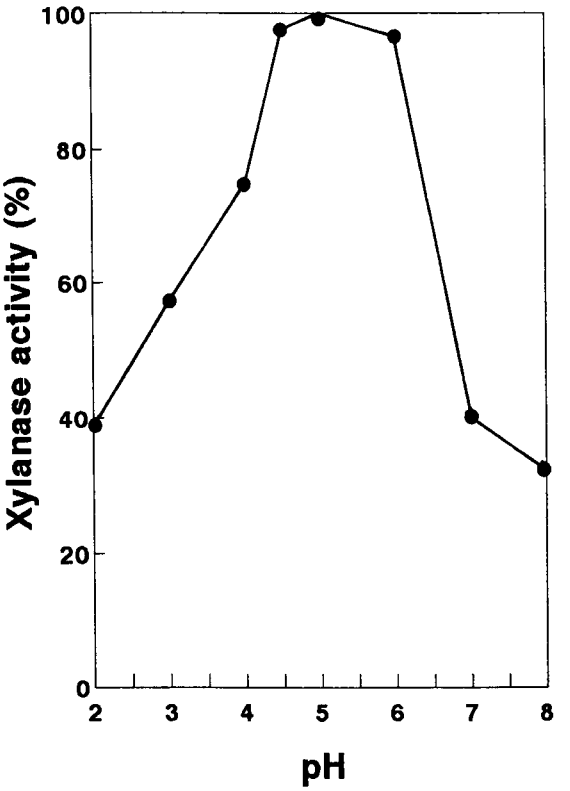

Fig. 3. Effect of pH on the activity of β-xylanase at 50°C. The enzyme activity is expressed relative to the maximal value.

Determination of Kinetic Parameters

Birchwood xylan of varying concentrations was incubated with appropriately diluted crude enzyme extract, and allowed to incubate for up to 25 min at 50°C. Figure 6 shows a Hanes-Woolf plot used to determine the kinetic parameters for the degradation of the insoluble substrate. The K_m was found to be 0.3 m*M* and the V_{max} value was 19.1 μmol/min for the insoluble birchwood xylan.

DISCUSSION

The *A. niger* CCMI 850 isolate described in the present work produced high levels of xylanolytic enzymes using a simple fermentation medium that did not include chemical inducers such as β-methyl-D-xylopyranoside, often used to enhance xylanase activity. The activity of the xylanolytic enzyme of major interest in enhancing the bleachability of pulps, β-xylanase, was to the best of our knowledge, the highest reported for a wild-type

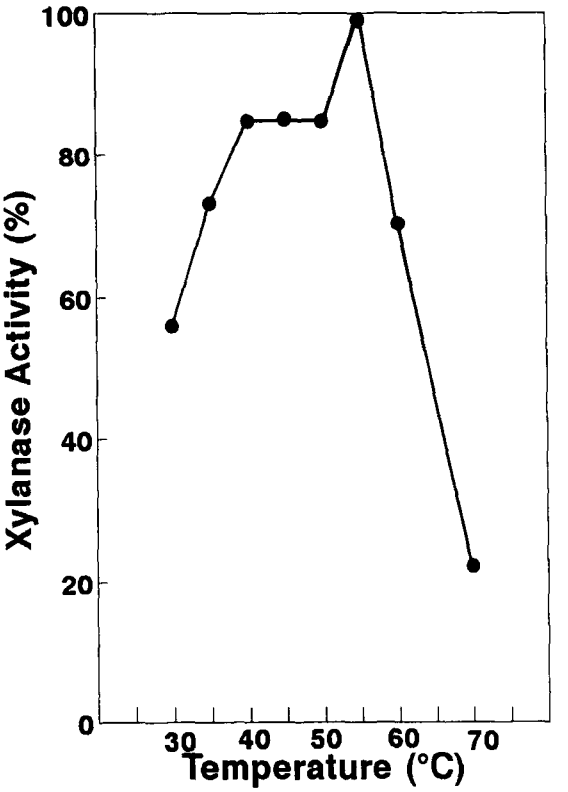

Fig. 4. Effect of temperature on the activity of β-xylanase at pH 4.5. The enzyme activity is expressed relative to the maximal value.

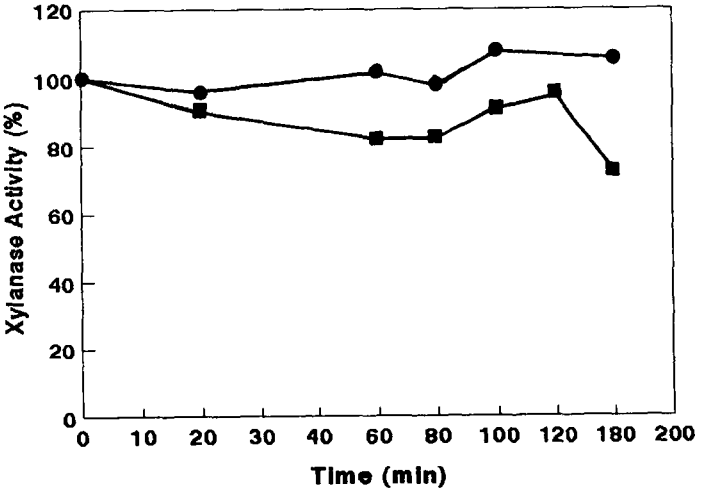

Fig. 5. Temperature stability of the β-xylanase extract. The crude enzymatic extract was preincubated at 50°C for the appropriate length of time in the absence (■) and presence of (●) 1 m*M* PMSF, and subsequently assayed for activity.

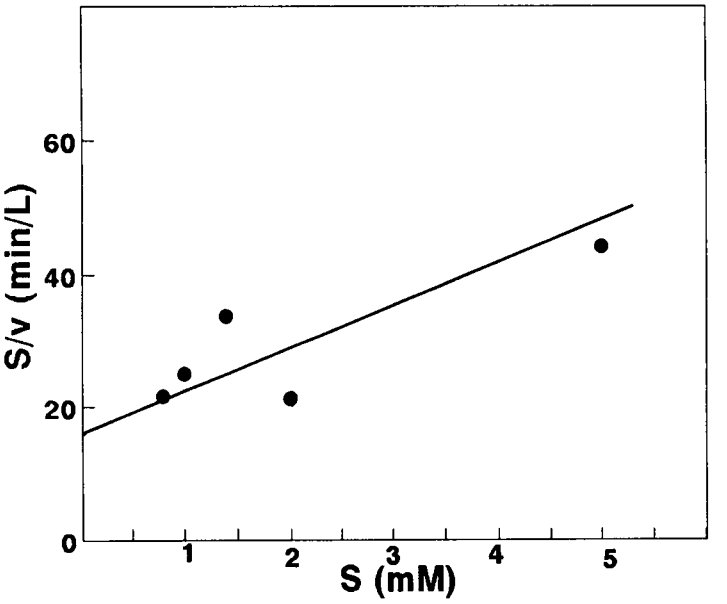

Fig. 6. Hanes-Woolf plot for the determination of kinetic parameters. The β-xylanase was incubated with varying amount of birchwood xylan at 50°C for up to 25 min, as described in Materials and Methods.

strain of *A. niger*. The approx 65 U/mL of xylanase produced by the *A. niger* isolate was about threefold higher than the amount produced by the strain selected by Conrad (*15*) following screening of 15 *A. niger* strains from the CBS collection. The xylanase produced by the present isolate using 4% xylan was about tenfold higher than that observed with *A. niger* NCIM 1207 (*16*), in which a similar substrate and concentration was used. Bailey and Poutanen (*17*) also described a xylanase overproducing strain of *A. niger*, in which 49 U/mL (820 nkats/mL) and 18 U/mL (310 nkats/mL) of xylanase were obtained using wheat bran and Solka floc cellulose, respectively, as the carbon source. It should be noted, however, that strains of *Aspergilli* other than *niger*, are capable of producing even higher amounts of xylanases (reviewed in *18*).

The broad pH optimum (pH 4–6) and temperature optimum of about 55°C observed for the β-xylanase described in the present study was within the range reported for several other strains of *A. niger* (*see 19*). It was, however, more stable than that reported for *A. niger* strain 110.42 CBS (*15*) where a 50% loss of activity was observed following 40 min treatment at 50°C. Kinetic studies appeared to indicate that the β-xylanase from the present isolate had a higher apparent affinity for the insoluble substrate than other previously reported strains (*7*), although it is necessary to be cautious in comparing data obtained with crude preparations.

The low levels of cellulolytic activity accumulated by the present isolate compared with other *A. niger* strains (*20*) makes it potentially suitable for use in the pulp and paper industry as the deleterious effect of cellulases on pulp viscosity is avoided. We have reported in preliminary studies the use enzymatic extracts produced by the *A. niger* isolate in biopulping studies (*21*) as well as in the biobleaching of raw eucalyptus pulp (*22*).

The various xylanolytic enzymes shown to be present in the isolate, make this organism suitable for studies on the biodegradation of lignocellulosic materials. By manipulation of the cultivation medium, it has been shown that different amounts of hemicullulases can be induced. The optimal level of hemicellulases and/or cellulases required clearly depends on the particular target application. Work on the purification of the xylanolytic enzymes is presently underway in order to investigate the role of the different hemicellulolytic enzymes in the degradation of substituted xylans. This may be expected to lead to more efficient application of these enzymes in various bioprocesses including their use as bleaching aids.

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