Low-cost carbon sources for the production of a thermostable xylanase by *Aspergillus niger*

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ABSTRACT

A strain of the filamentous fungus *Aspergillus niger* was isolated and shown to possess extracellular xylanolytic activity. These enzymes have biotechnological potential and can be employed in various industries. This fungus produced its highest xylanase activity in a medium made up of 0.1% CaCO$_3$, 0.5% NaCl, 0.1% NH$_4$Cl, 0.5% corn steep liquor and 1% carbon source, at pH 8.0. A low-cost hemicellulose residue (powdered corncob) proved to be an excellent inducer of the *A. niger* xylanolytic complex. Filtration of the crude culture medium with suspended kaolin was ideal for to clarify the extract and led to partial purification of the xylanolytic activity. The apparent molecular mass of the xylanase was about 32.3 kDa. Maximum enzyme activity occurred at pH 5.0 and 55-60ºC. Apparent K$_m$ was 10.41 ± 0.282 mg/mL and V$_{max}$ was 3.32 ± 0.053 U/mg protein, with birchwood xylan as the substrate. Activation energy was 4.55 kcal/mol and half-life of the crude enzyme at 60ºC was 30 minutes. Addition of 2% glucose to the culture medium supplemented with xylan repressed xylanase production, but in the presence of xylose the enzyme production was not affected.

Keywords: *Aspergillus niger*. Xylanolytic enzymes. Thermostability. Agroindustrial residues.

INTRODUCTION

Endo-1,4-β-xylanase (EC 3.2.1.8) is the main enzyme in the xylanolytic complex and it acts on the main xylan chain to form xylose, xylobiose and other xylooligosaccharides (XOS). Xylanases have applications in various industries, such as the prebleaching of kraft pulps, the improvement of bread quality, the clarification of fruit juices and xylitol production (Beg et al., 2001; Juturu & Wu, 2012). More recently, XOS produced from xylan-containing lignocellulosic materials have been described as an emerging prebiotic that could be incorporated into many food products (Aachary & Prapulla, 2011).

Xylanolytic enzymes are mainly produced by bacteria and fungi (Biely, 1985). Among the microbial sources, filamentous fungi are of the greatest interest, as they secrete xylanase into the culture medium at much higher levels than those produced by yeasts or bacteria (Polizeli et al., 2005). Most of the xylanases used in industry are obtained from mesophilic organisms, but thermostable enzymes are very useful in diverse biotechnological processes, such as those in which high temperatures are required in order to increase the solubility of the substrates and/or the hydrolysis rate or even to reduce the viscosity of the mixture and minimize the risk of microbial contamination (Collins et al., 2005).

Although they can be produced industrially on semi-solid substrates, about 80 to 90% of xylanases are produced in liquid culture media (Polizeli et al., 2005). The use of agroindustrial residues, such as wheat bran, corncobs, sugarcane bagasse and similar substrates, to achieve better xylanase activity allows enzyme production costs to be cut, thereby improving the industrial xylan hydrolysis process both technically and economically (Lv et al., 2008).

This paper reports the use of low-cost agroindustrial residues (corncobs, sugarcane bagasse, wheat bran and soy husks) as carbon sources for the production of xylanase by *Aspergillus niger*. Physical and kinetic parameters of the enzyme secreted with birchwood xylan as the substrate were also studied.

MATERIALS AND METHODS

Xylanase was produced by *Aspergillus niger*. Kaolin, diatomaceous earth, birchwood xylan, oatspelt xylan, glucose, xylose, xylobiose and 3-5´-dinitrosalicylic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). A standard molecular-weight kit was acquired from GE Healthcare Life Sciences (Buckinghamshire, UK).

Microorganism and culture conditions

Aspergillus niger FCUP1 was isolated from the rotting pulp of a tropical fruit (cupuaçu) by subculture on

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2% (w/v) agar with 4% (w/v) oat flour at 40ºC. Conidial suspensions were obtained from cultures grown on slants of the same medium at 40ºC for four days. After this period, the culture was maintained at 4°C. The liquid medium (M4;pH 8) was composed of 0.1% $CaCO_3$; 0.5% NaCl; 0.5% corn steep liquor; 1% carbon source and 0.25% gelatin (or 0.1% NH_4Cl in the case of modified M4). The low-cost carbon sources used were powdered corncobs, powdered sugarcane bagasse, wheat bran and powdered soy husks. Sometimes, oatspelt xylan, glucose and xylose were used. The medium was then inoculated with 10% (v/v) of a suspension of 10^7 conidia/ mL. Cultures were incubated in a rotary shaker at 40°C, 120 rpm, for 12-16 hours. The growth medium was vacuum filtered through paper and centrifuged at 7,400 *g* at 4°C for 20 min, as described by Goulart et al. (2005).

Intracellular β-xylosidase was extracted from *Humicola grisea* var. *thermoidea* as described by Almeida et al. (1995).

Clarification of culture medium and protein precipitation

Cultures were stopped at various times and filtered with Whatman No. 1 filter paper. The filtered medium was then clarified by adding kaolin, shaking gently at 4ºC for 30 minutes and filtering through filter paper under vacuum. A similar treatment was carried out with diatomaceous earth. Proteins in the clarified filtrate were precipitated by adding acetone (3:1 v/v) at 4ºC. After 12 hours at 4ºC, the material was centrifuged at 7,400 *g* for 20 minutes at 4ºC. Pellets were resuspended in Milli-Q purified water. Another method of precipitating and concentrating the proteins was tested: ammonium sulfate $(NH_4)_2SO_4$ (70% saturated solution) was slowly added to the filtrate at 4°C under mild stirring and the suspension was stored overnight. The mixture was then centrifuged as described above and the precipitated proteins suspended in milli-Q water and dialyzed against distilled water for 12 hours, the water being changed periodically. The resulting protein solutions were analyzed for protein content and xylanolytic activity.

Enzyme assays and protein determination

Xylanase activity was assayed with birchwood xylan as substrate. The quantity of reducing sugar produced was determined colorimetrically by reaction with 3-5´-dinitrosalicylic acid (Miller, 1959). The reaction mixture contained 1% (w/v) xylan in 50 mM sodium acetate buffer at pH 5.0 and a sufficient amount of enzyme to record a linear velocity against time. The enzyme unit (U) was defined as the amount of enzyme capable of producing 1 μmol of reducing sugar/minute. Specific enzyme activity was taken as the number of units of activity/milligram of protein. Assays were conducted at 40ºC, under constant shaking, with xylose as standard ($\varepsilon = 77$ M^{-1} cm^{-1}). Protein was determined by the Lowry method (1951) modified by Hartree (1972), with bovine serum albumin as standard.

Determination of optimal pH and temperature and kinetic constants

Optimal pH for xylan hydrolysis was determined at 60ºC in sodium acetate buffers for the pH range from 3.0 to 5.0, sodium phosphate for pH 6.0 to 7.0 and Tris-glycine for pH 8.0 to 9.0, all at 50 mM buffering species. Optimal temperature for xylan hydrolysis was determined in 50mM sodium acetate buffer at pH 5.0, at a series of temperatures from 25 to 90ºC. Apparent K_m and V_{max} were estimated by the Hanes-Woolf plot, using birchwood xylan as the substrate, as described by Segel (1975).

SDS-PAGE

Samples with xylanase activity were subjected to denaturing electrophoresis, based on Laemmli´s method (1970), using 10% polyacrylamide gel and a molecular weight standard kit consisting of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gels were developed by silver staining, as described by Heukeshoven & Dernick (1985).

Chromatographic separation of the hydrolysis products

The reaction medium consisted of 4 mL of 1% (w/v) xylan in 50 mM sodium acetate buffer at pH 5.0, to which was added 0.5 U of xylanase from *A. niger*, produced with powdered corncob as carbon source. In another experiment, the same conditions and mixture were used, and 0.5 U of β-xylosidase from *H. grisea* var. *thermoidea* was also added. Xylan hydrolysis products were analyzed by thin layer chromatography on silica gel G-60, using ethyl acetate-acetic acid-formic acid-water (9:3:1:4 v/v) as the mobile phase, as described by Fontana et al. (1988). Sugars were detected by spraying with 0.2% orcinol in sulfuric acid-methanol (1:9 v/v), with xylose and xylobiose as standards.

RESULTS

The results represent the mean of at least three experiments. Error did not exceed 5% in any assay.

Xylanase production

A wild-type fungal strain, denominated FCUP1, was isolated from decomposing fruit and identified by molecular taxonomy as *Aspergillus niger* van Tieghem, at the André Tosello Foundation, Campinas, SP, Brazil (OS 120017). The fungus produced and secreted thermostable extracellular xylanase when grown on various low-cost carbon sources. The specific activities secreted depended on the culture medium, carbon source and initial pH of the culture. Table 1 shows the results achieved when the fungus was grown in M4 medium at various initial pH on powdered corncob as the carbon source. The best specific

activities were obtained in M4 medium after 16 hours of culture at an initial pH of 7.0 and 8.0 (0.170 and 0.174 U/mg of protein, respectively).

Table 1. Effect of varying pH and culture growth time on the production of xylanase by *A. niger* FCUP1 in medium M4 at 40°C with powdered corncob as carbon source.

Culture time (hours)	Initial-final pH	Protein (mg/mL)ª	Xylanase activity (U/mL)ª	Specific xylanase activity (U/mg protein)ª
	4.0 - 6.1	2.86	0.042	0.015
	5.0 - 6.4	2.21	0.145	0.066
12	6.0 - 6.7	2.19	0.148	0.068
	7.0 - 6.8	2.21	0.119	0.054
	8.0 - 7.1	2.14	0.162	0.076
	4.0 - 5.2	2.01	0.267	0.133
	5.0 - 5.9	2.43	0.365	0.150
16	6.0 - 6.5	2.50	0.378	0.152
	7.0 - 6.8	3.26	0.555	0.170
	8.0 - 7.1	2.64	0.459	0.174

ª Values are the mean of at least three experiments, with standard deviation lower than 5%. Composition of the M4 medium: 0.1% $CaCO_3$, 0.5% NaCl, 0.25% gelatin, 0.5% corn steep liquor and 1% powdered corncob.

Under similar conditions, but with powdered sugarcane bagasse, wheat bran and powdered soy husks as carbon sources, the best xylanolytic activities were 0.029, 0.083 and 0.036 U/mg of protein, respectively, at initial pH 6.0.

The specific activity of the enzyme produced in the modified M4 culture medium, in which gelatin was replaced as nitrogen source by NH_4Cl, is shown in Table 2. At an initial pH of 8.0, with powdered corncobs as the carbon source, the specific activity at 16h (0.362 U/mg protein) was twice that in the original medium (0.174 U/mg protein).

Table 2. Effect of pH and culture growth time on the production of xylanase by *A. niger* FCUP1 in modified medium M4 at 40°C, with powdered corncob as carbon source.

Culture time (hours)	Initial-final pH	Protein (mg/mL)a	Xylanase activity (U/mL)a	Specific xylanase activity (U/mg protein)a
	4.0 - 6.3	1.32	0.062	0.047
	5.0 - 6.7	1.14	0.074	0.065
12	6.0 - 8.1	0.94	0.018	0.019
	7.0 - 7.2	0.41	0.091	0.223
	8.0 - 7.5	0.39	0.096	0.243
	4.0 - 6.0	0.99	0.300	0.303
	5.0 - 6.3	0.97	0.297	0.305
16	6.0 - 6.6	1.19	0.295	0.246
	7.0 - 7.2	1.40	0.434	0.309
	8.0 - 7.5	1.36	0.493	0.362

ª Values are the mean of at least three experiments, with standard deviation lower than 5%. Composition of the modified M4 medium: 0.1% $CaCO_3$, 0.5% NaCl, 0.1% NH_4Cl, 0.5% corn steep liquor and 1% powdered corncob.

Using the modified M4 medium and sugarcane bagasse, wheat bran and powdered soy husk as carbon sources, after 16 hours of culture, the best specific activities were 0.106 U/mg of protein, at an initial pH of 5.0, and 0.302 U/mg protein and 0.096 U/mg of protein at an initial pH of 8.0, respectively.

Clarification of culture medium

In order to reduce interference in the levels of protein and specific xylanolytic activity, the filtrates of cultures grown in modified M4 medium were subjected to various treatments: filtration with kaolin or diatomaceous earth, precipitation with acetone or ammonium sulfate. All the treatments except precipitation with ammonium sulfate afforded a clear extract and an increased specific activity. The best treatment was filtration in kaolin, which raised the specific activity 1.32-fold. Filtration in diatomaceous earth and precipitation with acetone improved the specific activity 1.25-fold and 1.2-fold, respectively. SDS-PAGE was performed to follow the crude extract clarification process (Figure 1). The protein band in lanes 2 (kaolin) and 3 (diatomaceous earth), corresponds to the thermostable xylanase. The apparent molecular mass was estimated to be 32.3 kDa. Subsequent assays were carried out only with the crude extract treated with kaolin.
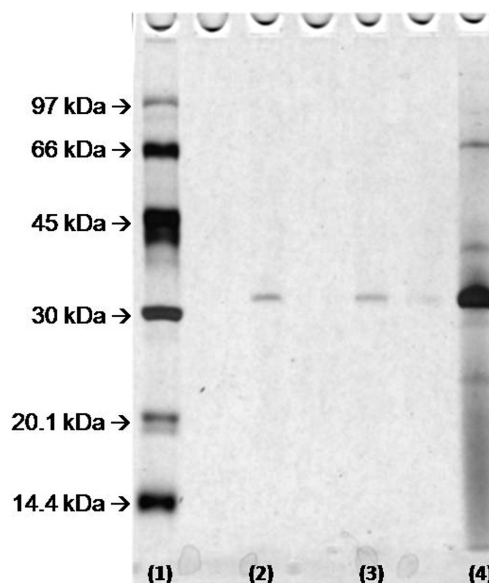

Fig.1. SDS-PAGE of *A. niger* FCUP1 xylanase extract. Lane 1: Molecular mass standard; Lane 2: crude extract filtered with kaolin; Lane 3: crude extract filtered with diatomaceous earth; Lane 4: crude extract.

Effect of temperature and pH on the activity and stability

The optimal temperature for xylanase activity in the kaolin-filtered crude extract was 55-60ºC and 78% of maximal activity was found in the range from 45 to 65ºC (Figure 2A). The most favorable temperature range for the hydrolysis of xylan by the *A. niger* FCUP1 xylanase was comparable to that of xylanases from thermophilic fungi,

such as *Humicola grisea* var. *thermoidea* (Monti et al., 1991; Monti et al., 2003) and *Thermomyces lanuginosus* (Puchart et al., 1999).

The best pH for activity of xylanase from *A. niger* FCUP1 was estimated as 5.0 (Figure 2B), in agreement with those described for other microbial xylanases (Sunna & Antranikian, 1997; Carmona et al., 1998; Polizeli et al., 2005; Milagres et al., 2005; Fengxia et al., 2008). However, the xylanase from *Aspergillus niger* Z1 has an optimal pH at 7.5 (Coral et al., 2002), suggesting that our strain is different from their isolate or, as this fungus can secrete multiple xylanase forms (Wong et al., 1988), that the enzyme studied here could be another isoform.

The enzyme showed good stability up to 55 °C. At this temperature, 100% of its activity was preserved for at least 60 minutes (Fig. 3A) and its half-life at 60°C was 12 minutes (Fig. 3B), a value close to the 15 minutes described for xylanases from *Aspergillus* sp. (Gawande & Kamat, 1998) and *Aspergillus versicolor* (Carmona et al., 1998). The activation energy, calculated by linear regression of the Arrhenius plot, was 19.05 kJ/mol (4.55 kcal/mol).
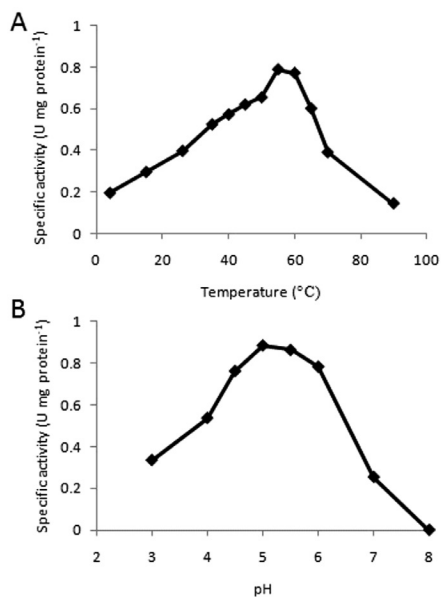

Fig. 2. (A) Effect of temperature on xylanase activity. Reactions performed with 0.54% (w/v) birchwood xylan in 50 mM sodium acetate buffer (pH 5.0) with constant shaking. (B) pH profile of *A. niger* FCUP1 xylanase (60 °C, sodium acetate buffer for pH 3.0-5.0, sodium phosphate buffer for pH 6.0-7.0 and Tris-glycine buffer for pH 8.0-9.0).
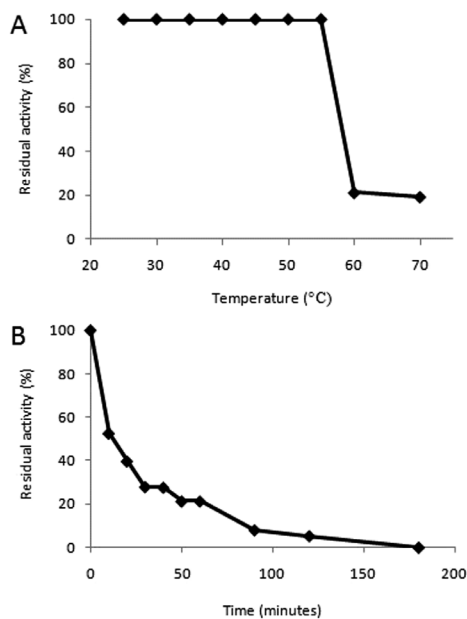

Fig. 3. (A) Thermal stability of xylanase incubated in 50 mM sodium acetate buffer at pH 5.0 for 1 h at various temperatures. (B) Thermal inactivation of xylanase incubated in 50 mM sodium acetate buffer (pH 5.0) at 60°C for various times.

Effect of carbon source

To establish the effect of the carbon source on the production of xylanase, *A. niger* FCUP1 was grown in a modified M4 medium with various carbon sources and in a medium with no carbon source, for 16 hours (Table 3). The results demonstrated that glucose acts as a repressor of the synthesis of xylanases in this microorganism. Full (100%) inhibition of enzyme production was achieved with 2% glucose in the culture medium. In the presence of glucose, the final pH of the culture medium after 16 hours was slightly acidic, between 3.9 and 4.6. However, the presence of xylose in the culture caused no inhibition of xylanase activity. When 1% xylose was used, specific activity was 94% of that obtained with only xylan as carbon source. In the presence of 1.0% xylan and 0.5% xylose, there was a 5% increase in specific activity, and a 3% increase in the presence of 1.0% xylan and 1.0% xylose. These results suggest that extracellular xylanase activity in this fungus is induced by xylan and xylose (up to 1%). However, at 2%, xylose partially represses the enzyme.

Table 3. Xylanase activity in culture filtrates and cell growth of *A. niger* FCUP1 on M4 plus various carbon sources at 40°C for 16 hours.

Carbon source	Final pH	Protein in filtrate (mg/mL)[a]	Xylanase activity (U/mL)[a]	Specific xylanase activity (U/mg protein)[a]	Inhibition (-) / induction (+)[b]
None	6.6	1.07	0	0	-100%
Xylan 1%	7.4	0.698	0.388	0.556	0
Glucose 1%	4.2	0.594	0.025	0.042	-92.4%
Xylose 1%	6.9	1.090	0.574	0.527	-6%
Xylan 1% + glucose 0.5%	6.6	0.714	0.368	0.515	-7.4%
Xylan 1% + glucose 1%	4.6	0.658	0.063	0.096	-82.7%
Xylan 1% + glucose 2%	5.3	0.970	0	0	-100%
Xylan 1% + xylose 0.5%	7.0	0.940	0.550	0.585	+5.2%
Xylan 1% + xylose 1%	6.1	1.173	0.672	0.573	+3.1%
Xylan 1% + xylose 2%	6.7	1.710	0.294	0.172	-30.9%

[a] Values are the mean of at least three experiments, with standard deviation lower than 5%. Composition of the modified M4 medium: 0.1% $CaCO_3$, 0.5% NaCl, 0.1% NH_4Cl, 0.5% corn steep liquor and 1% carbon source, with initial pH 6.0.
[b] Percent change in specific activity relative to standard induced culture (with 1% xylan).

Kinetic studies

K_m and V_{max} of the xylanase were estimated, with birchwood xylan as the substrate. Xylanase in the kaolin-clarified extract exhibited typical Michaelis-Menten kinetics, with an apparent K_m of 10.41 mg/mL ± 0.28 mg/mL and V_{max} of 3.32 U/mg protein ± 0.05 U/mg protein. This K_m is consistent with published values for xylanase with the same substrate, which generally range from 1 to 8 mg/mL.

Thin-layer chromatography (TLC) of the xylan hydrolysis products

Xylan was incubated with the enzyme for several time periods and hydrolysis products were analyzed by TLC. Xylobiose, xylotriose, xylotetraose and xylopentaose were identified at short xylan hydrolysis times (30 to 120 minutes). At 1,080 minutes, xylose, xylobiose and xylotriose were detected, with a concomitant reduction in xylotetraose and xylopentaose levels (Fig. 4A). These results are noteworthy for the observation of the direct release of xylose by extracellular enzymes secreted by this strain. By contrast, extracellular xylanolytic enzymes from microorganisms such as *Thermoanaerobacter ethanolicus* (Shao & Wiegel, 1992), *Aspergillus versicolor* (Carmona et al., 1998), *Humicola grisea* var. *thermoidea* (Monti et al., 1991) and *Rhizopus stolonifer* (Goulart et al., 2005) did not produce xylose as a hydrolysis product.

Fig. 4B displays the TLC plate of the xylan hydrolysis products obtained with a mixture of the clarified xylanase extract from *A. niger* FCUP1 and crude mycelial extract of *Humicola grisea* var. *thermoidea*, known as a good producer of intracellular xylosidase (Almeida et al., 1995). The photograph reveals the production of xylose and xylobiose after a short hydrolysis (30 minutes) and a reduction in xylotetraose and xylopentaose at 1,080 minutes.

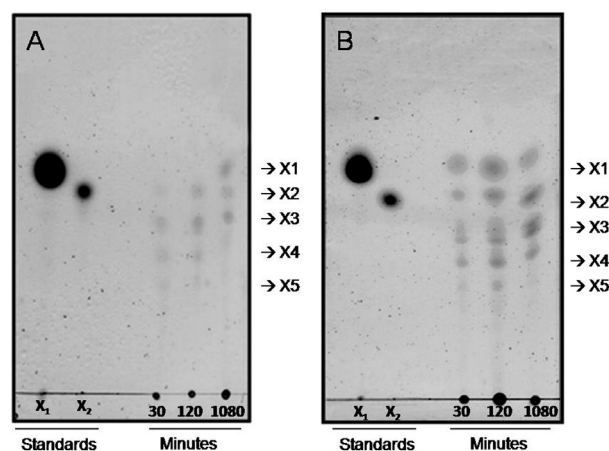Fig. 4. Thin layer chromatograms of birchwood xylan products at three different hydrolysis times in 50 mM sodium acetate buffer (pH 5.0). X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose. Stain for XOS: orcinol-sulfuric acid. (A) *A. niger* FCUP1 clarified culture filtrate. (B) A. niger FCUP1 clarified filtrate plus crude mycelial extract of Humicola grisea var. thermoidea.

DISCUSSION

Filamentous fungi described in the literature are able to produce a range of different xylanases in submerged fermentation (Polizeli et al., 2005). In recent years, considerable effort has been spent in isolating thermostable fungi that are good producers of xylanolytic enzymes. The enzyme produced here showed excellent stability up to 55°C, which is important in industrial processes that require high temperatures, such as the bleaching of kraft paper, or even to increase the solubility of the substrates and to avoid microbial contamination in the production of xylose and XOS.

In a variety of culture media, *A. niger* FCUP1 shows a preference for inorganic nitrogen (NH_4Cl) in the production of thermostable extracellular xylanase, on which the best xylanolytic activity (total and specific) was produced at initial pH 8, after 16 hours. The use of low-cost carbon sources such as corncob is of great interest for the production of enzymes, especially in Brazil, where the disposal of lignocellulosic waste is incalculable and still unexploited, although the potential for reuse of the biomass is well known.

Surprisingly, filtration with kaolin was shown to be a simple and effective method for partial purification, by adsorbing almost all of the other proteins, but not the xylanase. This is a very early method of enzyme purification (Dixon & Kodama, 1926), little used nowadays.

Among fungi, there are various types of mechanism of enzyme induction. Carbon catabolite repression by glucose seems to be a consensus in xylanase biosynthesis. However, while the production of xylanase is induced by xylose in some fungi, others do not show the same behavior. This variation is observed even within the same species, including *Aspergillus* sp. This kind of induction is due to some effect not yet fully elucidated at the transcriptional level of xylanase gene expression (Rizatti et al., 2008). Some work has been published on the regulation of xylanases in filamentous fungi. Mach-Aigner et al. (2010) demonstrated that the xylanase induction/repression in *Trichoderma reesei* is modulated by the carbon catabolite repressor Cre1, which causes a reduced transcription of xylanase-encoding genes that depends on the xylose concentration. Also, this influence could be exerted indirectly by antagonizing Xyr1, the main activator of most hydrolase-encoding genes.

Our results also demonstrated that the xylanolytic enzyme alone produced xylose, but in synergy with an intracellular β-xylosidase from *Humicola grisea* var. *thermoidea*, xylan hydrolysis improved and there was an earlier production of xylose. Thus, according to the desired end product, both enzymes may be used together or separately, thus controlling the appearance of XOS with reaction time.

The enzyme described has desirable properties for in various industrial sectors, for instance the bleaching of kraft paper, the animal feed industry or XOS production for future use as prebiotics in the food industry. Therefore, further studies must be carried out, to enhance the production and specific activity of the enzyme. Furthermore, since *Aspergillus* spp. are known to produce several depolymerizing enzymes and isoforms, the full purification of xylanase will be carried out to confirm the existence of a single protein.

RESUMO

Fontes de carbono de baixo custo para a produção de xilanase termoestável por Aspergillus niger

Uma linhagem do fungo filamentoso *Aspergillus niger* foi isolada e apresentou atividade xilanolítica extracelular. Estas enzimas possuem grande potencial biotecnológico e podem ser aplicadas em diversas indústrias. O fungo produziu sua maior atividade de xilanase em um meio contendo $CaCO_3$ 0,1%, NaCl 0,5%, NH_4Cl 0,1%, 0,5% água de maceração de milho e 1% de fonte de carbono, em pH 8,0. Um resíduo lignocelulósico de baixo custo (sabugo de milho em pó) mostrou ser um excelente indutor do complexo xilanolítico em *A. niger*. A filtração do extrato cru com caulim foi ideal para a clarificação do extrato e levou à purificação parcial da enzima. A massa molecular aparente da xilanase foi de 32,3 kDa. A máxima atividade da enzima ocorreu em pH 5,0 e a 55-60ºC. O K_m aparente foi de 10,41 ± 0,282 mg/mL e a V_{max} foi de 3,32 ± 0,053 U/mg proteína, utilizando-se xilana *birchwood* como substrato. A energia de ativação foi de 4,55 kcal/mol, e a meia-vida da enzima a 60ºC foi de 30 minutos. A adição de 2% de glicose ao meio de cultura suplementado com xilana reprimiu a produção de xilanase, mas em presença de xilose a produção da enzima não foi afetada.

Palavras-chave: *Aspergillus niger*. Enzimas xilanolíticas. Termoestabilidade. Resíduos agroindustriais.

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