Xylanase production by a wild strain of *Aspergillus nidulans*

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ABSTRACT. A wild strain of *Aspergillus nidulans* isolated from soil produce cellulase-free xylanase activity when developed on submerged cultures using corn cob powder as the main substrate. Maximum xylanase production (220 U/mL) was obtained when the strain was developed in mineral medium supplemented with 3% (w/v) corn cob for 6 days. SDS-PAGE revealed the occurrence of four isoxylanases with molecular weights of 50, 43, 20 and 18 kDa. Crude xylanase was resistant to acetone precipitation with 80% of recovery. The enzyme had optimal activity at pH values between 5.0 and 6.0 and temperature between 50-55ºC. The enzyme exhibited high stability under alkaline conditions and temperature up to 55ºC. It retained 50% and 23% of its activity when heated for 1 h at 60 and 65ºC, respectively.

Key words: *Aspergillus nidulans*, corn cob, submerged cultures, xylanases.

RESUMO. Produção de xilanases por uma cepa selvagem de *Aspergillus nidulans*. Uma cepa selvagem de Aspergillus nidulans isolada de solo produziu xilanase isenta de atividade celulolítica quando desenvolvida em meio submerso contendo sabugo de milho como principal substrato. Produção máxima de xilanase (220 U/mL) foi obtida quando a cepa foi desenvolvida em meio mineral suplementado com sabugo de milho a 3% (p/v) por 6 dias. Eletroforese com SDS revelou a ocorrência de quatro iso-xilanases, com pesos moleculares de 50, 43, 20 e 18 kDa. A xilanase bruta foi resistente à precipitação com acetona, apresentando atividade ótima em pH 5.0-6.0 e temperatura entre 50º e 55ºC. A enzima exibiu grande estabilidade quando exposta a condições alcalinas, e foi estável por várias horas quando exposta a temperaturas de até 55ºC, retendo 50% e 23% de sua atividade quando aquecida por 1 h a 60 e 65ºC, respectivamente.

Palavras-chave: *Aspergillus nidulans*, culturas submersas, sabugo de milho, xilanases.

Introduction

β-1,4 xylanases (1,4 β-D-xylan-xylanohydrolase, EC 3.2.1.8) catalyze the hydrolysis of xylan, the major component of hemicellulose of plant cell walls to xylo-oligosaccharides and xylose. A variety of microorganisms, including bacteria, yeasts and filamentous fungi, have been reported to produce xylanases (Coughlan and Hazlewood, 1993; Haltrich *et al*., 1996). The potential applications of xylanases with or without concomitant use of cellulase include the bio conversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juice and wine, and nutritional value improvement of silage and green feed.. In addition, cellulase-free xylanases can be used for the selective hydrolysis of the hemicellulose components in paper and pulp (Bajpai, 1997). The use of purified xylan as an inducer increases the cost of enzyme production. For this reason, different lignocellulosic residues, including wheat bran, wheat straw, corn cob and sugar cane bagasse, have been used as growth substrate in cultures to produce xylanases (Haltrich *et al*., 1996).

Several different species of *Aspergillus* have been reported to produce xylanases, including *A. niger*, *A. ochraceus*, *A. oryzae*, *A. awamori A. tamarii*, and *A. fumigatus* (Bailey and Poutanen, 1989; Haltrich *et al.,* 1996; Kadowaki *et al.,* 1997; Siedenberg *et al.,* 1998). Although *A. nidulans* has become a model system for studying mechanisms of control of gene expression in filamentous fungi, mainly due to knowledge acquired about its genetics, a few studies describe its ability to produce xylanase because this specie produces less xylanase when compared to other *Aspergillus* species (Pinaga *et al*., 1994; Fernandez-Espinar *et al*., 1992; Haltrich *et al*., 1996), The purpose of this work was to study the production of xylanase by a wild strain of *Aspergillus nidulans* isolated from soil on submerged cultures. An attempt was done to identify the number of different

iso-xylanases produced by the fungus under this condition.

Material and methods

Microorganism, media and culture conditions

Aspergillus nidulans was isolated from soil. In the lab, the organism was maintained on potato dextrose agar slants at 30ºC. The spore suspensions were prepared by adding 10 mL of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop. Enzyme production was studied in 250 ml Erlenmeyer flasks containing 50 mL of mineral solution (Montenecourt and Eveileigh, 1977) and the appropriate carbon source. One mililiter of spore suspension (10^4 spores) was used as the inoculum. The cultures were incubated at 30ºC in a rotary shaker at 120 rpm for 8 days. The cultures were harvested by filtration and the filtrates were saved as sources of crude extracellular enzymes.

Enzyme assays

Xylanase activity was determined by measuring the reducing sugar by the dinitrosalicylic acid method (Miller, 1959), using D-xylose as the standard. The enzyme assay was carried out at 50ºC, using 1.0% (w/v) birchwood xylan in 50 mM citrate buffer, pH 5.4, as substrate. An enzyme unit (U) is the amount that releases 1 μmol of reducing sugars per min. The same method was used to determine the cellulolytic activity (CMCase), using carboxymethylcellulose (CMcellulose) as substrate. The protease activity of the culture filtrates was assayed using 1% (w/v) casein in 0.1 M sodium phosphate buffer, pH 7.0, as substrate. One ml of the casein solution was incubated at 40ºC for 1 h with 1.0 mL of the suitably diluted culture filtrate. The reaction was stopped by the addition of 2.0 mL of 0.3 M trichloroacetic acid. After mixing in a vortex mixer, the tubes were centrifuged at 3,000 rpm for 10 min. The tyrosine released was estimated by Lowry's method using a tyrosine standard curve. One unit of enzyme activity (U) is the amount that releases 1 μmol of tyrosine per min. Reducing sugar amounts were quantified by using the dinitrosalycilic method.

Effect of pH and temperature on xylanase activity and stability

The optimal pH and temperature were determined assaying the enzyme at various pH in McIlvaine buffer (pH 2.8-8.1), and at temperature ranging from 30 to 70ºC. The pH stability was investigated by incubating the enzyme at different pH for 4 h at 40ºC. The remaining activities were measured under standard conditions. To pH superior than 8.0, the experiments were done by using 0.1 M glycine buffer. Thermal stability was investigated by incubating the enzyme at 30, 55, 60 and 65ºC for different times. Immediately afterwards the enzyme was immersed in an ice bath and then the residual activity was determined under standard conditions.

SDS-PAGE and xylanases detection

Sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS-PAGE) was carried out using a 10% gel and low molecular mass standards from 14 to 66 kDa (Laemmli, 1970). The xylanase activity in the polyacrylamide gel after the electrophoresis was detected using remazol brilliant blue (RBB) dyed xylan (Sigma) as the substrate (Kadowaki *et al*., 1997).

Results

A. nidulans was grown in 250 mL Erlenmeyer flasks containing 50 mL mineral medium supplemented with different carbon sources (1%, w/v) for 5 days. Xylanase, CMCase and protease activities were determined in the early stationary growth phase. As shown in Table 1, low xylanase and no measurable amounts of CMCase were observed after growth on glucose, xylose, lactose, cellobiose and CMCellulose. High xylanase activities were detected when the microorganism was grown in commercial xylan (from birchwood and from oat spelt) and in lignocellulosic residues (corn cob, wheat bran, wheat straw, and sugar cane bagasse). A very significant improvement in the production of xylanase without concomitant increase in the CMCase and protease activities was observed when the substrate concentration in the media was increased from 1 to 3-5%. Low CMCase ($\angle$ 0.05 U/mL) and protease ($\angle$ 50 U/mL) activities were detected in these cultures, except in wheat bran cultures, where proteolytic activity up to 200 U/mL were found in the culture filtrates. For this reason, corn cob was the substrate selected to study the effect of cultivation time in the production of enzyme (Figure 1). The maximum peak of xylanase production occurred at 6 days of cultivation, while very low CMCase and protease activities were detected, even after long time of cultivation.

Xylanase activity was resistant to acetone precipitation. About 80% of initial activity was recoverred after precipitation procedure. Some biochemical properties of crude xylanase were determined. Crude xylanase was most active between pH 5.0-6.0 and the enzyme was stable in

pH range 3.5 to 10 after 4 hours of incubation at 40ºC (Figure 2). The optimum temperature was 50-55ºC (Figure 3A). The enzyme was stable at temperature up to 55ºC, and it retained 50% and 23% of its initial activity when heated for 1 h at 60 and 65ºC, respectively (Figure 3B). The crude xylanase system was characterized by electrophoretic techniques. SDS-PAGE showed the presence of at least four isoxylanases, with molecular weights of 50, 43, 20 and 18 kDa.

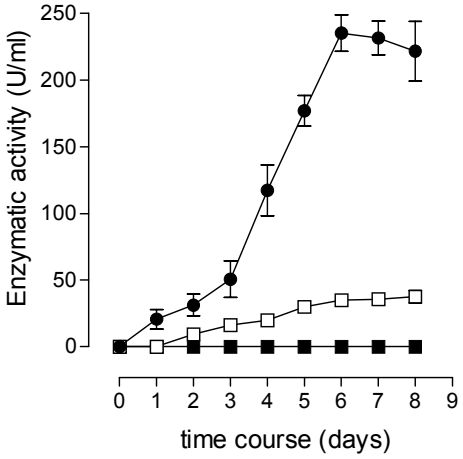

Figure 1. Time course of xylanase production by *Aspergillus nidulans* on submerged cultures with 3 % corn cob as substrate. (●) xylanase activity; (❑) protease activity; (■) CMCase activity. Error bars are SD of the mean.

Table 1. Effect of carbon source on the production of xylanase, CMCase and protease by *Aspergillus nidulans*

Carbon source (%, w/v)	Xylanase (U/ml)	CMCase (U/ml)	Protease (U/ml)
Soluble sugars at 1%			
Glucose	7±2	nd	23
Xylose	8±3	nd	21
Lactose	9±4	nd	13
Cellobiose	11±4	nd	19
Polysacharides			
Birchwood xylan 1%	35±5	0.023	29
Birchwood xylan 3%	32±7	0.021	31
Oat spelt xylan 1%	45±5	0.029	21
Oat spelt xylan 3%	38±3	0.026	25
CMCellulose 1%	12±3	0.031	16
CMCellulose 3%	15±3	0.030	10
Lignocellulosic materials			
Corn cob 1%	66±6	0.039	30
Corn cob 3%	205±20	0.032	36
Corn cob 5%		210±27	0.043
Wheat bran 1%	73±10	0.047	110
Wheat bran 3%	189±21	0.051	190
Wheat bran 5%	269±31	0.040	204
Wheat straw1%	44±6	0.031	35
Wheat straw 3%	65±9	0.031	43
Wheat straw 5%	103±14	0.023	37
Sugar cane bagasse 1%	52±5	0.031	33
Sugar cane bagasse 3%	91±10	0.029	51
Sugar cane bagasse 5%	130±16	0.035	45

The cultures were developed at 30° C, and 120 rpm for 5 days. Values are given as means ± SD of three replicated cultures; nd= not detectable

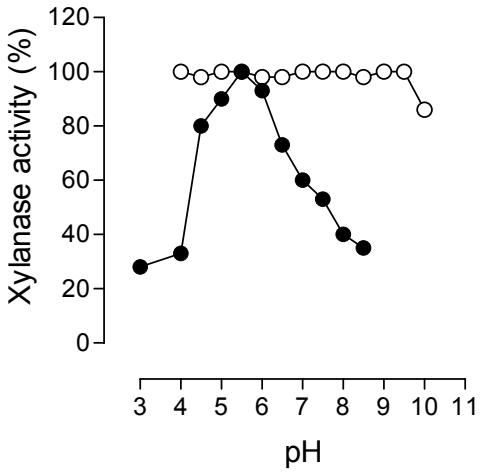

Figure 2. Effect of pH on activity (●) and stability (❍) of xylanase from *A. nidulans*

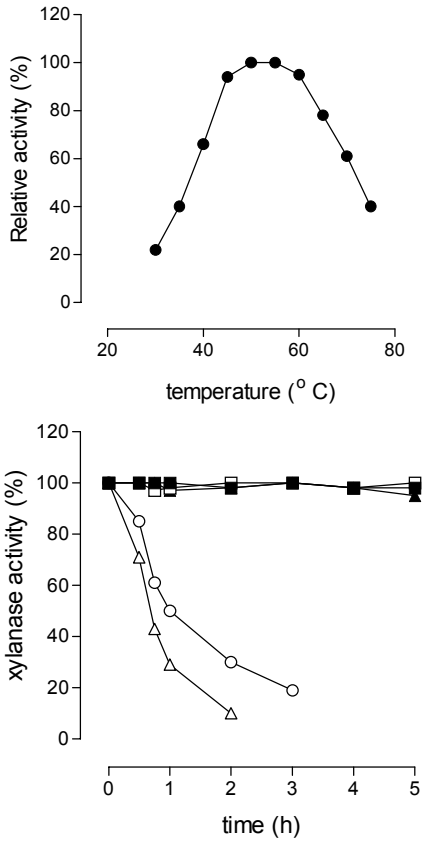

Figure 3. Effect of temperature on activity and stability of xylanase from *A. nidulans*. In A: Relative activity at different temperature; In B: The enzyme was incubated at 0 (■), 30 (❑), 55 (▲), 60 (❍) and 65° C (Δ) in 0.05 M citrate buffer, pH 5.4. At different time intervals the aliquots were withdrawn, and residual activities were measured under standards conditions

Discussion

The inductive nature of production of xylanase by *A. nidulans* was suggested by the high levels of enzyme found in cultures with xylan or lignocellulosic residues as substrate, whereas very low xylanase activities were detected on glucose, xylose, cellobiose and lactose cultures. The supplementation of cultures with 3-5% of lignocellulosic material improved the production of xylanase. The decreasing in the production of xylanase when high amounts of xylan was offered as carbon source, may be due to catabolite repression, likewise described for other xylanolytic microorganisms (Kadowaki *et al*., 1997).

The strain of *A. nidulans* used in this work was able to produce levels of xylanase activity superior to those produced by other strain of *A. nidulans* (Fernandez-Espinar *et al.,* 1992; Pinaga *et al*., 1994; Haltrich *et al*., 1996; Taneja *et al*., 2002). In addition, the maintenance of xylanase activity after 8 days of cultivation, suggests that the enzyme produced by the fungus was stable and no proteolytic activity was co-produced by the fungus in the medium used in this work.

The condition of temperature and pH for maximum xylanase activity was similar to other *Aspergillus* sp xyanases (Haltrich *et al*., 1996; Bajpai 1997). However, the xylanases described here presented a rare stability in a large range of pH. Recently, a strain of *A. nidulans* able to produce a xylanase optimally active at pH 8.0 has been documented (Taneja *et al*., 2002). High activity and stability at alkaline pH is a very desirable property to use xylanases in selective hydrolysis of the hemicellulose components in paper and pulp (Bajpai, 1997).

The occurrence of multiple xylanases in a microorganism immediately raises questions concerning the functions and origins of each iso-enzyme. Multiple xylanases production have been reported in numerous microorganisms (Wong *et al*., 1988) and could be caused by several factors, such as different mRNA processing, partial proteolysis or differences in the degree of amidation and glycosilation. Multiple forms of xylanases differ in stability, catalytic efficiency, and absorption and activity on substrates (Maheshwari *et al*., 2000). Different types of xylanases might allow the microorganisms to use a wider range of substrates (Wong *et al*., 1988) or allow their diffusion into the plant cell walls of highly variable structure (Prabhu and Maheswari, 1999).

In this work, we described some basic information for the production of high titles of xylanase by a wild strain of *A. nidulans* isolated from soil. Its xylanase activity is cellulase-free and can be produced on cultures using a very cheap growth substrate.

Acknowledgments

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