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Xylanase Production by *Penicillium sclerotiorum* and its Characterization

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Abstract: Recently, xylanases have expanded their use in many processing industries, such as pulp and paper, food and textile. The production of xylanase by the fungus *Penicillium sclerotiorum* under submerse cultivation was investigated. Oat spelts xylan and wheat bran were the best inducers of xylanase activity. Optimal xylanase production was obtained in liquid Vogel medium, pH 6.5, at 30ºC, under stationary condition during 5 days. The temperature for optimum activity was 50ºC and optimum pH 4.5. The enzyme was stable at 40ºC, with a half-life of 72 min. and when it was incubated at 45 and 50ºC, the half-life was 8 min and shorter than 4 min, respectively. High pH stability was verified from pH 3.5 to 7.5. This enzyme showed interesting characteristics for some process, such as pulp and paper industry, because *P. sclerotiorum* produces low cellulase level, as well as in food industries.

Key words: Xylanase Production · *Penicillium sclerotiorum*

INTRODUCTION

Xylan, after cellulose, is the most abundant polysaccharide present in wood, agricultural and several agro-industrial wastes. This complex heteropolysaccharide consists of a main chain of 1,4-β-D-xylose monomers containing different substituents or ramifications [1, 2]. The substituents including arabinofuranosyl, glucuronyl and acetyl groups show a pronounced influence on its chemical and structural properties and also on the enzymatic degradability of xylan in lignocelluloses [3, 4].

In nature, xylan is completely hydrolyzed to monosaccharides by the synergistic action of different enzymes [2]. The xylan-degrading system include endo-1,4-xylanases (1,4-β-xylan xylanohydrolase; EC 3.2.1.8), which release long and short xylo-oligosaccharides, or those that only attack longer chains and β-D-xylosidase (1,4-β-xylan xylohidrolase; EC 3.2.1.3.7), which remove D-xylose residues from short xylo-oligosaccharides [5, 6]. Enzymes such as β-arabinosidase, β-glucuronidase, ferulic acid esterase and acetyl xylan esterase are very important for the removal of side chain groups from xylan, especially when the fragments of the cleaved heteroxylans present high proportions of branched substituents [7].

Xylanases are produced by many different fungi and bacteria. From an industrial point of view, filamentous fungi are interesting producers of these enzymes due to xylanases releasing and their easy cultivation [8, 9]. These enzymes are commercially used in the pulp and paper, food, beverage, textile and animal feed industries [1, 10]. Moreover, xylanases show immense potential for increasing the production of several valuable products like xylitol and ethanol in a most economic way [11]. Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium* and *Talaromyces* sp. [10, 12].

The *Penicillia* are mostly saprophytic in nature and numerous species are of particular value for humanity [13]. The production of xylanolytic enzymes by *Penicillia* has been explored in a number of species. According to Chávez *et al*. [14], *Penicillia* constitute a rich source of enzymes for the biodegradation of xylan. Among 80 strains isolated from Brazilian soil at the Ecological Station of Juréia-Itatins in the Mata Atlântica region, the strain of *Penicillium sclerotiorum* attracted attention by producing xylanase in high levels, with low cellulolytic activity. In this paper, we report the production and characterization of xylanase secreted by this fungus.

MATERIALS AND METHODS

Organism and growth: *P. sclerotiorum* used in the present work is available in the Culture Collection of Environmental Studies Center-CEA/UNESP, Brazil. It was cultivated for conidia production on Vogel's solid medium [15] containing 1.5% (m/v) glucose and 1.5%

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(m/v) agar at 25ºC for 7 days. Liquid cultures were prepared in the same medium containing 1% (w/v) of the carbon source mentioned and the pH was adjusted for each experiment. Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 1.0 mL of spore suspension (5 x 10^7 spores/mL) and incubated at different conditions as indicated subsequently. All cultures were developed in duplicate and the results are presented through mean values.

Enzyme preparations and assays: Cultures were harvested by filtration. The filtrate was assayed for extracellular activity and protein. The mycelium was washed with distilled and sterilized water, frozen and ground with sand in 50 mM sodium phosphate buffer pH 6.0. The slurry was centrifuged at 3.900 *g* at 4°C and the supernatant was used as intracellular enzymes source.

Xylanase activity was assayed at 50°C using 1.0 % (w/v) birchwood xylan (Sigma) in 50 mM sodium phosphate buffer pH 6.0. The reducing sugars released were quantified by the dinitrosalicylic acid method according to Miller [16], using xylose as standard. One unit of enzyme activity was defined as the enzyme amount that releases 1 µmol of reducing sugar per min. Specific activity was expressed as unit per milligram of protein.

Protein determination: Protein concentration was determined by Lowry *et al*. [17] with bovine serum albumin as standard.

Enzyme production on different carbon sources: The Vogel's medium was supplemented with various carbon sources at concentration of 1% (w/v). The inoculated flasks were incubated for 5 days at 28ºC under agitation (120 rpm). Xylanase activity was determined in each case as described previously.

Effect of culture conditions, pH and temperature on xylanase production: The culture conditions influence on incubation period was studied under standing culture during 12 days and under shaking culture (120 rpm) during 8 days. The effect of initial pH on the enzyme production was analysed from 2.5 to 8.5 and the temperature influence was verified from 15 to 30°C.

Enzyme characterization

Optimum pH and temperature xylanase activity: Enzyme activity was measured at 50ºC in different pH values by the use of McIlvaine buffer from 3.0 to 8.0. The optimum temperature was determined by the incubation of the reaction mixture from 15 to 70ºC and optimum pH.

Stability of xylanase at different temperatures and pHs: Crude enzyme preparation was dilluted in McIlvaine buffer (1:2) in a pH range from 3.0 to 8.0 and incubated at 4ºC for 24 h. The crude extract was incubated at 40, 45 and 50°C in optimum pH determined above for different periods. The residual activity was determined in each sample, at the pH and temperature optimum for the enzyme.

RESULTS AND DISCUSSION

Influence of the carbon source on xylanase production: In order to induce xylanase production, different substrates as pure carbohydrates and some natural substrates were tested (Table 1). Among the pure carbohydrates used, only oat spelts xylan induced xylanase production. According to Kulkarni *et al*. [18], xylanase activity is inducible and substrates from xylan play an important role in xylanase induction. For others species of genus *Penicillium*, xylan also showed to be the best inducer [19, 20]. Enzyme activity was not detected with glucose, xylose, maltose, celobiose and

Table 1: Influence of some pure carbohydrates on xylanase production by *P. Sclerotiorum*

Carbon source (1 % w/v)	Intracellular protein (mg)	Enzymatic activity (U/ml)	Specific activity (U/mg protein)
Glucose	1.58±0.19	ND	ND
Xylose	1.30±0.09	ND	ND
Maltose	1.09±0.07	ND	ND
Lactose	0.13±0.01	ND	ND
Sucrose	2.12±0.05	ND	ND
Celobiose	1.44±0.12	ND	ND
Avicel	0.04±0.00	ND	ND
CM-cellulose	ND	ND	ND
Oat spelts xylan	0.47±0.02	7.82±0.25	24.51±0.20

Average and standard deviation of two cultures, ND: Not Detectable

Table 2: Effect of different agricultural and agro-industrial wastes on xylanase production by *P. Sclerotiorum*

Carbon source (1 % w/v)	Intracellular protein (mg)	Enzymatic activity (U/ml)	Specific activity (U/mg protein)
Sugar-cane bagasse	ND	ND	ND
Wheat bran	1.03±0.15	7.50±0.06	21.71±0.6
Oat bran	0.80±0.03	3.58±0.05	19.51±0.9
Rice straw	0.01±0.00	ND	ND
Soybean meal	0.06±0.00	ND	ND
Corn cobs	0.03±0.00	0.89±0.02	3.61±0.16
Citrus pectin	0.59±0.07	ND	ND
Orange bagasse	0.56±0.04	ND	ND

Average and standard deviation of two cultures, ND: not detectable

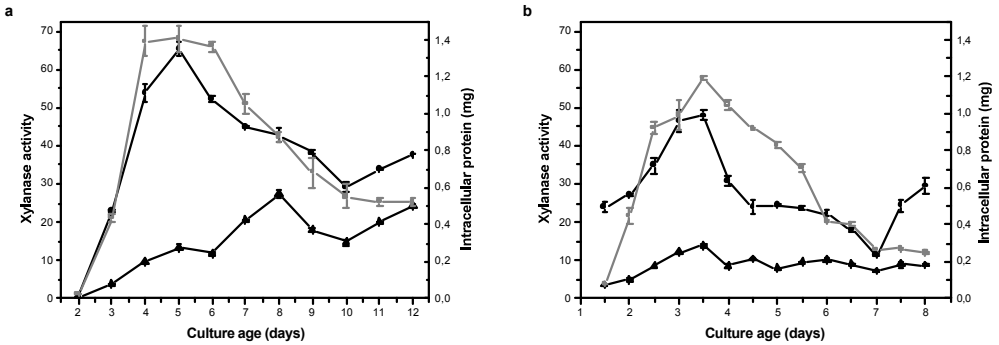

Fig. 1: Time-course of xylanase production by *P. sclerotiorum* in stationary (a) and shake culture at 120 rev min^{-1} (b). Culture conditions: Vogel medium with xylan 1 % (w/v), at 28 ^{o}C and pH 6.5. (▲) xylanase activity (U/ml), (●) specific xylanase activity (U/mg of protein); (■) intracellular protein (mg)

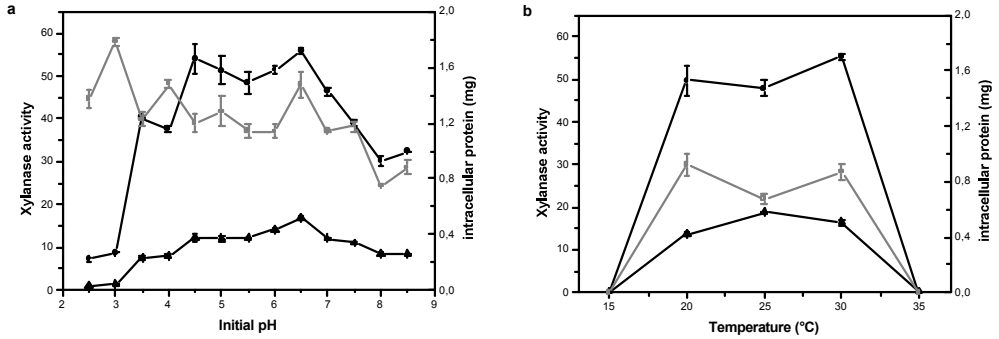

Fig. 2: Effect of initial pH (a) and temperature (b) on xylanase production by *P. sclerotiorum*. Culture conditions: Vogel medium with 1% xylan (w/v) under stationary condition for 5 days at 28ºC (a) and pH 6,5 (b). (▲) xylanase activity (U/ml), (●) specific xylanase activity (U/mg of protein); (■) intracellular protein (mg)

sucrose as carbon source (Table 1), showing that this enzyme is non-inducible with these easily metabolizable sugars. Besides, *P. sclerotiorum* xylanase synthesis also can be affected by carbon catabolite repression, as verified in other filamentous fungi [21].

Among the agricultural and agro-industrial wastes, wheat bran showed to be the best inducer for xylanase production by *P. sclerotiorum* as for units of activity per volume and for specific activity as well. *Penicillium expansum*, *Penicillium* sp. ZH-30 and *Penicillium chrysogenum* xylanases also were induced by this carbon source [22-24]. Wheat bran induced very similar values of xylanolytic activity per volume as those obtained with oat spelts xylan. Carmona *et al*. [25] observed the same result in liquid cultures of *Aspergillus versicolor*. However, in this work, higher specific activity was verified in the presence of xylan, due to the minor amount of proteins released in the

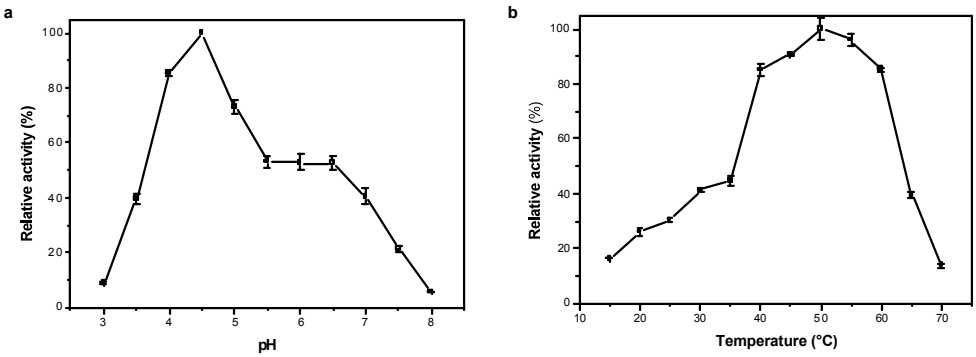

Fig. 3: Influence of pH (a) and temperature (b) on xylanase activity from *P. sclerotiorum.* Culture condition: Vogel medium with xylan 1 % (w/v) under stationary condition for 5 days, pH 6.5. Xylanase activity was assayed with McIlvaine buffer at 50°C (a) and with McIlvaine buffer pH 4.5 (b)

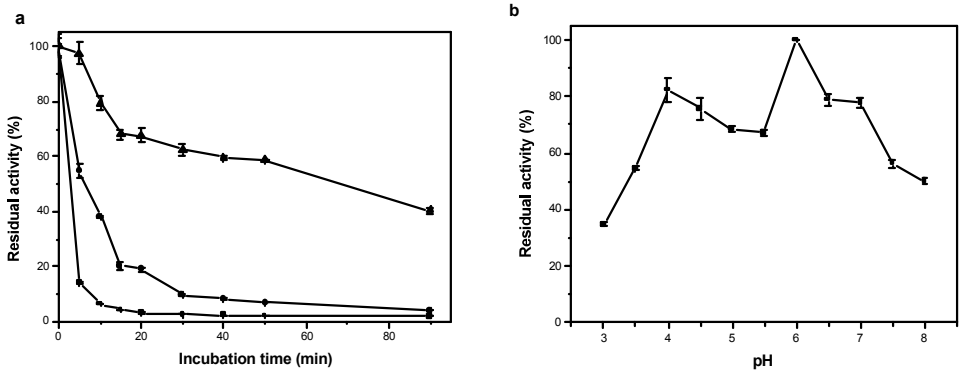

Fig. 4: Thermal (a) and pH (b) stability of xylanase activity from *P. sclerotiorum.* (a) The crude filtrate was incubated at (▲) 40, (●) 45 and (■) 50°C without substrate and the residual xylanase activity was assayed with McIlvaine buffer, pH 4.5, at 50ºC. (b) The crude filtrate was incubated without substrate with McIlvaine buffer at 4°C for 24 h and the residual xylanase activity was assayed with McIlvaine buffer, pH 4.5, at 50ºC

medium, than those detected in the presence of wheat bran. Media with oat bran exhibited lower xylanase activity than that observed with wheat bran. A low level of xylanase activity was verified with corn cobs. Absent or no significant levels of xylanase activity were obtained in cultures supplemented with all others substrates tested, such as orange bagasse, citrus pectin and soybean meal. *P. sclerotiorum* was not able to grow in the presence of sugar-cane bagasse and carboximethylcellulose (CM-cellulose). Avicel, rice straw, corn cobs and soybean meal provided minimal fungal development . According to previously published work, *P. sclerotiorum* produce low levels of cellulase [26], what may explain the fact that this fungus could not grow in the presence of some cellulosic predominant sources. In such case, this enzyme can be applied in biotechnological processes where the presence of cellulose is undesired. In all cases the values of intracellular activity (data not shown) were lower than those obtained extracellularly, as observed in most of the xylanases reported in the literature by Querido *et al*. [22], Carmona *et al*. [25] and Tanaka *et al*. [27]. Thus, the carbon source used in the subsequent experiments was oat spelts xylan.

Effects of culture conditions on xylanase production: In standing culture, with oat spelts xylan as carbon source, the highest extracellular xylanase production was obtained in 8 and 5 days old cultures (27.21 U/ml and 65.29 U/mg of protein) (Fig. 1a). In shaking condition (Fig. 1b), those maxima were observed at 3.5 days, corresponding to the values of 13.82 U/ml and 47.84 U/mg of protein. The highest *P. sclerotiorum* growth, measured by the intracellular protein concentration, occurred at 5 days in standing culture and 3 days in shaking culture (Fig. 1). In shaking condition, xylanase was expressed during the exponential phase and in standing condition, xylanase was expressed during the stationary phase, reaching the decline phase. According to Kulkarni *et al*. [18], xylanases are usually expressed at the end of the

exponential phase and the harvesting time is correlated to the medium under consideration.

Examination of the macroscopic morphology of the *P. sclerotiorum* mycelium in stationary and shaked cultures revealed that in the former, the hyphae formed a freely dispersed mycelia, whereas in the latter, pellets were formed. It is well known that fungal morphology is influenced by agitation and that the formation of many products depends on the morphological structure of the macroscopic growth of filamentous fungi [28, 29]. In some cases, pellets formation is a prerequisite for secondary metabolites production, as citric acid and some fungal enzymes, such as polygalacturonase, glucoamylase or β-glucosidase. In others, as well as in this study, freely dispersed mycelium is preferred to higher metabolite production [30, 31]. Such factors probably explain the better xylanase production in standing than in shaked culture. For this reason, the subsequent experiments were carried out under stationary condition.

Temperature and pH are important environmental parameters that determine growth rates of microorganisms and significantly affect the level of xylanases produced. The influence of pH culture on xylanase production during *P. sclerotiorum* cultivation is showed in the Fig. 2a. Xylanase activity was detected in all pH evaluated. The highest activity was observed at initial pH 6.5, corresponding to the values of 16.93 U/ml and 55.76 U/mg of protein. With rare exceptions, xylanase production by filamentous fungi occurs in cultures with an initial pH under 7.0. *Penicillium purpurogenum* [32] and *Penicillium janthinellum* [33] presented highest levels of xylanolytic activity at pH 5.5 and *Penicillium* sp. ZH-30 [23] at pH 6.0.

P. sclerotiorum could grow in media with initial pH between 2.5 and 8.5 (Fig. 2a), with maximal growth in the range of 3.0 to 4.0. This result clearly indicates the acidophilic nature of this fungus.

The effect of temperature on xylanase production by *P. sclerotiorum* is presented in Fig. 2b. The highest xylanase activity per unit volume was verified at 25ºC, while the maximum value of specific activity was obtained at 30ºC, corresponding to 18.92 U/ml and 55.42 U/mg of protein, respectively. Milagres *et al*. [34] reported that 30°C is the best culture temperature for xylanase production by *P. janthinellum* FM-5 and Haas *et al*. [35] obtained highest xylanase activity from *P. chrysogenum* at 28ºC.

The highest growth was verified at 20°C, indicating the mesophilic character of this strain. Nevertheless, lower values of xylanase activity per volume unit were obtained at 20ºC. According to Aiba *et al*. [36], at lower temperature, the transport of substrate across the cells is decreased and lower yield of products are attained. *P. sclerotiorum* was not able to grow at 35ºC, a particular feature of this fungal specie [37].

Properties of extracellular crude xylanase: This study revealed that the best pH for this xylanase activity was around 4.5 (Fig. 3a). Studies carried out with *Penicillium* spp. [34, 38] as well with others fungal species [39, 40] also concluded that the most suitable pH value for xylanase activity was within the acid region.

The optimum temperature for xylanase activity was 50°C (Fig. 3b). Similarly, in other studies with *Penicillium* spp., it was concluded that the optimum temperature varied between 40 and 50°C [20, 38]. Besides, others fungal xylanases show optimum temperature at 50ºC [41, 42].

Thermal stability is an interesting enzymes property due to the great industrial importance [43]. Then, enzyme stability analyses were carried out. The crude xylanase from *P. sclerotiorum* was incubated without substrate at 40, 45 and 50°C (Fig. 4a). The half-life ($T_{1/2}$) at 40°C was 72 min. At 45°C, $T_{1/2}$ was 8 min and at 50°C it was shorter than 4 min. An increase of thermal stability would be interesting and could be achieved with directed-site mutagenesis.

The xylanase produced by *P. sclerotiorum* maintained its stability over a broad of pH evaluated (Fig. 4b). Less than 50% of activity was verified in pH 3.0 and 8.0, while high stability (above 50 %) was observed from 3.5 to 7.5, with two major peaks at pH 4.0 and 6.0.

The xylanase from *P. sclerotiorum* was a novel enzyme, being active at acidic pH with an optimum at 4.5 and was stable in acid and neutral pH range. It showed optimum activity at 50ºC and moderate stability at 40ºC. These are desirable properties for application in the pulp and paper, as well as in food industries.

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