



Production of xylanases by an *Aspergillus niger* strain in wastes grain

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ABSTRACT. Many fungi are used in order to extract products from their metabolism through bioprocesses capable of minimizing adverse effects caused by agro-industrial wastes in the environment. The aim of this study was to evaluate the xylanase production by an *Aspergillus niger* strain, using agro-industrial wastes as substrate. Brewer's spent grain was the best inducer of xylanase activity. Higher levels of xylanase were obtained when the fungus was grown in liquid Vogel medium, pH 5.0, at 30°C, during 5 days. The temperature for optimum activity was 50°C and optimum pH 5.0. The enzyme was stable at 50°C, with a half-life of 240 min. High pH stability was verified from pH 4.5 to 7.0. These characteristics exhibited by *A. niger* xylanase turn this enzyme attractive for some industrial applications, such as in feed and food industries. Additionally, the use of brewer's spent grain, an abundantly available and low-cost residue, as substrate for xylanase production can not only add value and decrease the amount of this waste, but also reduce xylanase production cost.

Keywords: agro-industrial waste, biochemical properties, filamentous fungi, xylanolytic enzymes.

Produção de xilanases por uma linhagem de *Aspergillus niger* em resíduos de grãos

RESUMO. Muitos fungos são utilizados com a finalidade de extrair produtos de seu metabolismo, por meio de bioprocessos capazes de minimizar efeitos nocivos que resíduos agroindustriais causam ao meio ambiente. O objetivo deste estudo foi avaliar a produção de xilanases por uma linhagem de *Aspergillus niger*, empregando resíduos agroindustriais como substrato. O bagaço de malte foi o melhor resíduo indutor da atividade xilanásica. Maiores níveis de xilanases foram obtidos quando o fungo foi cultivado em meio líquido de Vogel, pH 5,0, a 30°C, durante cinco dias. A temperatura ótima estabelecida para a atividade xilanásica foi a de 50°C e o pH ótimo 5,0. A enzima foi estável a 50°C, apresentando uma meia vida de 240 min. Elevada estabilidade enzimática foi verificada entre os pH 4,5 e 7,0. As características bioquímicas exibidas pela xilanase produzida por *A. niger* tornam esta enzima atraente para determinadas aplicações industriais, como as indústrias de ração animal e alimentícia. Adicionalmente, a utilização do bagaço de malte, um resíduo disponível em abundância e de baixo custo como substrato para a produção de xilanases poderá não somente adicionar valor a este resíduo, como também reduzir os custos de produção destas enzimas.

Palavras-chave: resíduos agroindustriais, propriedades bioquímicas, fungos filamentosos, enzimas xilanolíticas.

Introduction

Xylans are the main constituents of hemicellulose, the world's second most abundant resource after cellulose. This complex heteropolysaccharide consists of a main chain of 1,4- β -D-xylose monomers containing different substituents or ramifications. 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 (COLLINS et al., 2005; KHANDEPARKER et al., 2011; KULKARNI et al., 1999).

Xylanases (endo-1,4- β -D-xylan xylanohydrolase) are the most important enzymes responsible for the

xylans hydrolysis, which release long and short xylo-oligosaccharides, or those that only attack longer chains (KHENG; OMAR, 2005). These enzymes have applications in conversion of lignocellulosic substances to chemicals and fuels, animal feed digestion, food and textile industries, and as bleaching agents in the pulp and paper processing (KNOB et al., 2010; MOURE et al., 2006; POLIZELI et al., 2005). Filamentous fungi are widely used as enzyme producers and are generally considered as more potent xylanolytic enzymes producers than bacteria and yeast (KRISANA et al., 2005; POLIZELI et al., 2005).

In recent years, lignocellulosic substrates have received considerable attention because of their possible use in secondary fermentation processes.

Thus, various agro-industrial waste materials like corn meal, corn cob, wheat bran, wheat straw, rice straw, sugarcane bagasse and coffee by-products are being used as substrates for the production of fungal xylanases (MURUGAN et al., 2011). Brewer's spent grain (BSG) is the main residue of brewing industry, representing around 85% of total by products generated (ALIYU; BALA, 2011). Large amounts of BSG are produced and have accumulated in environment over the years. Since this waste is rich in cellulose and non-cellulosic polysaccharides, it has great potential for being used as xylanases inducer. However, BSG has received little attention as a low-cost by-product and its use is still limited, mainly as animal feed (MUSSATTO; ROBERTO, 2006).

Since many agro-industrial wastes are a potentially valuable resource for industrial exploitation, this work aimed to evaluate the xylanase production by an *Aspergillus niger* strain using different agro-industrial wastes, establish the best fungal growing conditions for xylanase production and biochemically characterize the enzymes produced.

Material and methods

Organism and growth

A. niger strain 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 (VOGEL, 1956) containing 1.5% (m v⁻¹) glucose and 1.5% (m v⁻¹) agar at 25°C for 7 days. Liquid cultures were prepared in the same medium containing 1% (w v⁻¹) 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 10⁷ spores mL⁻¹) and incubated at different conditions as indicated subsequently. All cultures were developed in duplicate and the results are presented through mean values.

Preparation of agro-industrial wastes

The agro-industrial wastes brewer's spent grain, soybean peel, corn straw, passion fruit peel, soybean waste, orange peel, pineapple peel, wheat bran, apple peel, sugarcane straw and sugarcane bagasse were obtained locally. The residues were prepared by exhaustive washing with distilled water, dried at 80°C for 24-48h and milled (35 mesh).

Enzyme preparations and assays

Cultures were harvested by vacuum 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 McIlvaine buffer pH 4.0. The suspension was centrifuged at 3.900 x g at 4°C and the supernatant was used as protein source.

Xylanase activity was assayed at 50°C using 1.0% (w/v) birchwood xylan in 50 mM sodium phosphate buffer pH 6.0. After 5 and 10 min. of incubation, the reaction was interrupted by the addition of 3,5-dinitrosalicylic acid (DNS) and the reducing sugars released were quantified (MILLER, 1959). For this, the sample was heated at 100°C for 5 min. and allowed to cool to room temperature in ice bath. After that, 2.5 mL of distilled water was added and the absorbance was determined in spectrophotometer at 540 nm. One unit of enzyme activity was defined as the enzyme amount that releases 1 µmol of reducing sugar equivalent to glucose, per ml, per min. Specific activity was expressed as unit per milligram of protein. All enzymatic assays were developed in triplicate and the results are presented through mean values.

Protein determination

Total protein was determined by modified Bradford method (SEDMAN; GROSSBERG, 1977), using bovine serum albumin (BSA) as standard. For this, the sample appropriately diluted was mixed with the Bradford reagent. The samples were then incubated at room temperature for 5 min. After that, the absorbance was determined in spectrophotometer at 595 nm.

Culture conditions for xylanase production

Enzyme production on different carbon sources

The Vogel's liquid medium was supplemented with various carbon sources at concentration of 1% (w/v). The inoculated flasks were incubated for 5 days, at 28°C, pH 6.5. Xylanase activity was determined in each case as described previously.

Effect of culture conditions, pH and temperature on xylanase production

The influence of incubation period on xylanase production was studied under submerged fermentation and stationary culture during 8 days. The effect of initial pH on the enzyme production was analyzed from 4.0 to 7.5 and the temperature influence was verified from 25 to 35°C.

Enzyme characterization

Temperature and pH optima

Xylanase activity was measured at 50°C in different pH values by the use of McIlvaine buffer from 3.0 to 7.0. The optimum temperature was determined by the incubation of the reaction mixture from 40 to 60°C and optimum pH.

Thermal and pH stability

For pH stability assay, the enzyme preparation was diluted (1:2) in McIlvaine buffer in a pH range from 3.0 to 7.0 and incubated at 4°C for 24h. To evaluate the thermal stability, the enzyme preparation was incubated at 50, 55, and 60°C at optimum pH determined above for different periods. The residual activity was determined in each sample, at the optima pH and temperature.

Results and discussion

Influence of the carbon source on xylanase production

In order to induce xylanase production, different agro-industrial wastes were evaluated (Table 1). Brewer's spent brain showed to be the best inducer for xylanase production by *A. niger* (3.63 ± 0.03 U mL⁻¹ and 41.34 U mg⁻¹ protein) and also provided the best fungal growth (1.50 ± 0.03 mg protein). The xylanolytic enzymes production using brewer's spent grain as substrate has been previously described only for *Penicillium janczewskii* (TERRASAN et al., 2010). However, in this work, the enzymes have not been biochemically characterized. The use of brewer's spent grain as substrate is promising, since large amounts of this waste are generated annually and discarded in the environment (ALIYU; BALA, 2011).

Table 1. Influence of some agro-industrial wastes on xylanase production by *Aspergillus niger*.

Carbon source (1% w v ⁻¹)	Enzymatic activity (U mL ⁻¹)	Specific activity (U mg protein ⁻¹)	Intracellular protein (mg)
Brewer's spent grain	3.63 ± 0.03	41.34	1.50 ± 0.03
Soybean hulls	3.40 ± 0.03	35.23	0.25 ± 0.02
Oat spelt xylan	3.34 ± 0.00	30.21	0.26 ± 0.00
Corn straw			
Passion fruit peel	2.55 ± 0.03	17.92	2.02 ± 0.00
Soybean waste	2.53 ± 0.00	19.16	0.31 ± 0.01
Orange peel			
Pineapple peel	2.35 ± 0.03	7.20	0.14 ± 0.01
Wheat bran	2.12 ± 0.01	8.96	0.06 ± 0.00
Apple peel	2.00 ± 0.01	3.67	0.16 ± 0.01
Sugarcane straw	1.82 ± 0.02	3.04	0.12 ± 0.00
Sugarcane bagasse	0.62 ± 0.00	1.21	0.04 ± 0.00
	0.51 ± 0.00	0.90	0.02 ± 0.00
	0.23 ± 0.00	0.31	0.04 ± 0.00

Media supplemented with soybean hulls and oat spelt xylan also exhibited high xylanase activity, corresponding to a 3.60 ± 0.03 U mL⁻¹ and 3.34 ± 0.00 U mL⁻¹, respectively. However, low intracellular proteins levels were observed, indicating that these substrates do not favored fungal growth, but were able to induce xylanase activity. The xylanase production at high levels by a strain of *A. niger* using soybean hulls as substrate was recently reported by Li et al. (2012). For other filamentous fungi, oat spelt xylan was the best inducer for xylanolytic enzymes (AHMED et al., 2012; KNOB; CARMONA, 2008). In general, higher levels of xylanolytic enzymes can be achieved with substrates derived from xylan. According to Kulkarni et al. (1999), xylanase activity is inducible and substrates from xylan play an important role in xylanase induction.

Low levels of xylanase activity and minimal fungal development were obtained in cultures supplemented with apple peels, sugarcane bagasse and sugarcane straw. Conversely, *Penicillium janthinellum*, *Thermoascus aurantiacus* and *Sporotrichum thermophile* were able to grow in the presence of sugarcane bagasse and produce higher levels of xylanase activity than those measured in this study (BAKRI et al., 2012; KATAPODIS et al., 2006; SILVA et al., 2005). Additionally, Irfan et al. (2010) and Irfan and Syed (2012) reported the xylanase production using sugarcane bagasse as substrate by other *A. niger* strain and *Trichoderma viride*, respectively. The different production levels observed among the lignocellulosic materials are probably related on differences in composition and the accessibility of the substrates to the fungi. Considering the high xylanase production obtained with brewer's spent brain, this agro-industrial waste was selected for the subsequent optimization experiments.

Effects of culture conditions on xylanase production

Cultivation conditions are essential for the successful production of an enzyme, and optimization of parameters such as period of cultivation, pH and temperature are important for a process development. In standing culture, with brewer's spent brain as carbon source, the highest xylanase production as unit per volume was obtained in 5 days old cultures (4.00 ± 0.07 U mL⁻¹) (Figure 1A). In relation to specific activity, those maxima were observed at 4 and 6 days old cultures. The highest *A. niger* growth, measured by the intracellular protein concentration, occurred at 5 days (Figure 1B), showing that xylanases were expressed during the exponential

phase, reaching the stationary phase. According to Kulkarni et al. (1999), xylanases are usually expressed at the end of the exponential phase and the harvesting time is correlated to the medium under consideration. Contrary to this study, in which the enzyme production and fungal growth were correlated, Knob and Carmona (2008) verified that high biomass production was not related to high levels of xylanases produced in *Penicillium sclerotiorum*.

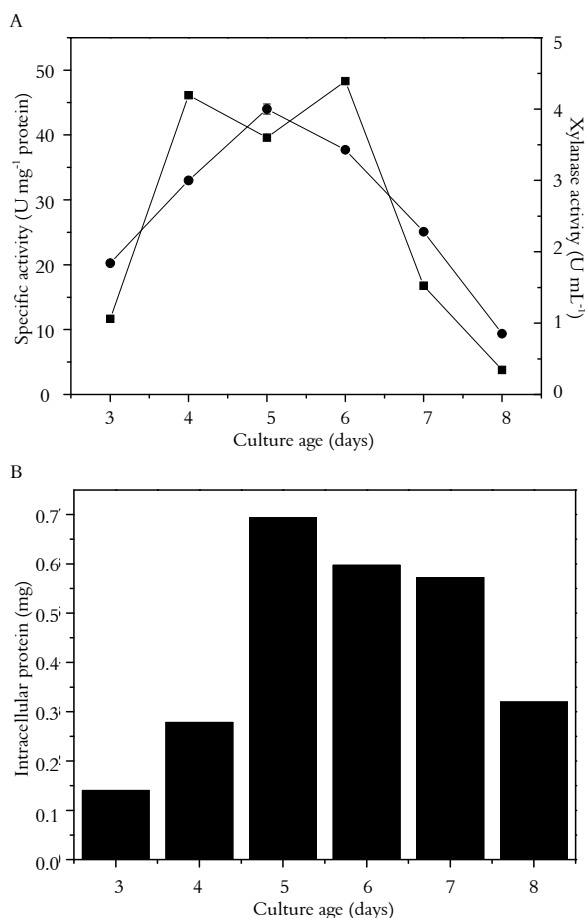


Figure 1. Influence of culture time on the xylanase production by *A. niger* (A) and on its growth (B). Culture conditions: Vogel medium with 1% (w/v) brewer's spent grain, pH 6.5 at 28°C. (■) specific activity (U mg protein⁻¹); (●) xylanase activity (U mL⁻¹).

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 *A. niger* cultivation is showed in the Figure 2A. Xylanase activity was detected in all pH evaluated. The highest activity per unit volume was observed at initial pH 5.0, corresponding to the values of 4.72 ± 0.04 U mL⁻¹.

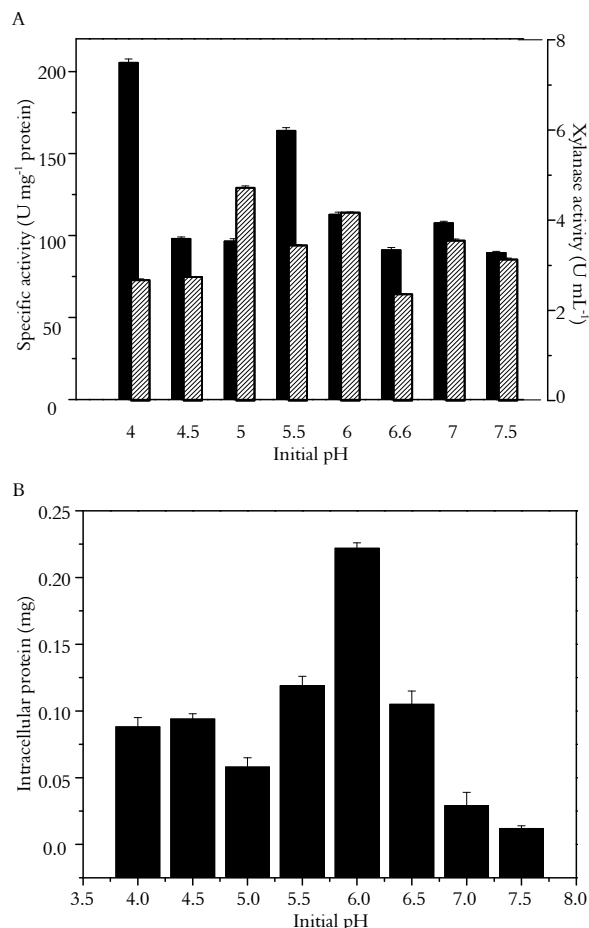


Figure 2. Effect of initial pH on xylanase production by *A. niger* (A) and on its growth (B). Culture conditions: Vogel medium with 1% (w/v) brewer's spent grain for five days, at 28°C and pH 6.5. (■) specific activity (U mg protein⁻¹); (▨) xylanase activity (U mL⁻¹).

In relation to specific activity, higher levels of production were observed when the culture medium pH was adjusted to pH 4.0, corresponding to 205.4 ± 0.12 U mg⁻¹ protein. With rare exceptions, xylanase production by filamentous fungi occurs in cultures with an initial pH under 7.0. According to Madlala et al. (2001), most xylanases presents optimal activity in pH between 5.0 and 7.0 and among the acidophilic xylanases, the majority of them showed high activity only under slight acid conditions. *Trichoderma harzianum* (ISIL; NILUFER, 2005) and *P. janczewskii* (TERRASAN et al., 2010) showed enhanced xylanase production at pH 5.0 and 5.5, respectively. *A. niger* could grow in media with initial pH between 4.0 and 7.5, with maximum growth in the range of pH 5.5 to 6.5 (Figure 2B). This result clearly indicates the acidophilic nature of this fungus.

The effect of temperature on xylanase production by *A. niger* is presented in Figure 3A. The highest xylanase activity was verified at 25°C,

corresponding to 5.49 U mL^{-1} and $206.33 \pm 0.20 \text{ U mg protein}^{-1}$. Similarly, maximum xylanase production by *Trichoderma viride* was achieved at 25°C (MEENAKSHI et al., 2008). In the same temperature, the higher fungal biomass production was also observed (Figure 3B), indicating the mesophilic character of this strain. When cultures were incubated at 30°C , there was a slight reduction in the levels of xylanases produced ($4.76 \pm 0.06 \text{ U mL}^{-1}$) and the fungal growth declined. At 35°C , the xylanases production and fungal growth were considerably decreased, when compared to those observed at 25°C .

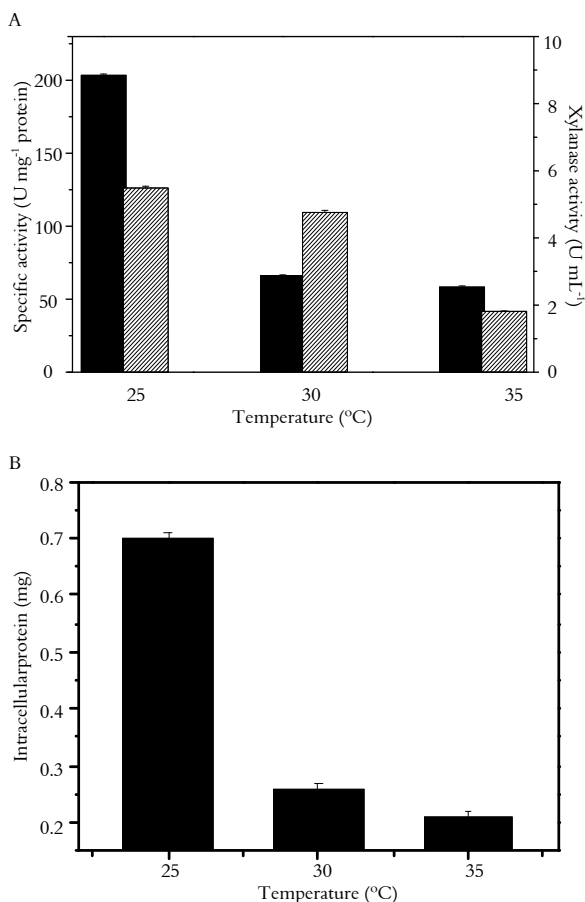


Figure 3. Effect of temperature on xylanase production by *A. niger* (A) and on its growth (B). Culture conditions: Vogel medium with 1% (w/v) brewer's spent grain for five days, at 28°C and pH 5.0. (■) specific activity (U mg protein^{-1}); (▨) xylanase activity (U mL^{-1}).

Properties of extracellular crude xylanase

This study revealed that the best pH for this xylanase activity was between 5.0 and 6.0 (Figure 4A). According to Polizeli et al. (2005), several xylanases produced by *Aspegillus* species showing optimal pH between 4.0 and 6.0. Exceptions are the xylanases produced by *Aspegillus nidulans*, which

presented optimal activity at pH 8.0. The lower pH optimum for xylanase activity were reported for the enzymes produced by *P. sclerotiorum*, which showed maximal activity at pH 2.5 (KNOB; CARMONA, 2008) and those produced by *Penicillium* sp. 40, which had optimal activity at pH 2.0 (KIMURA et al., 2000). However, most of the acidophilic xylanase shows high activity in slight acid conditions.

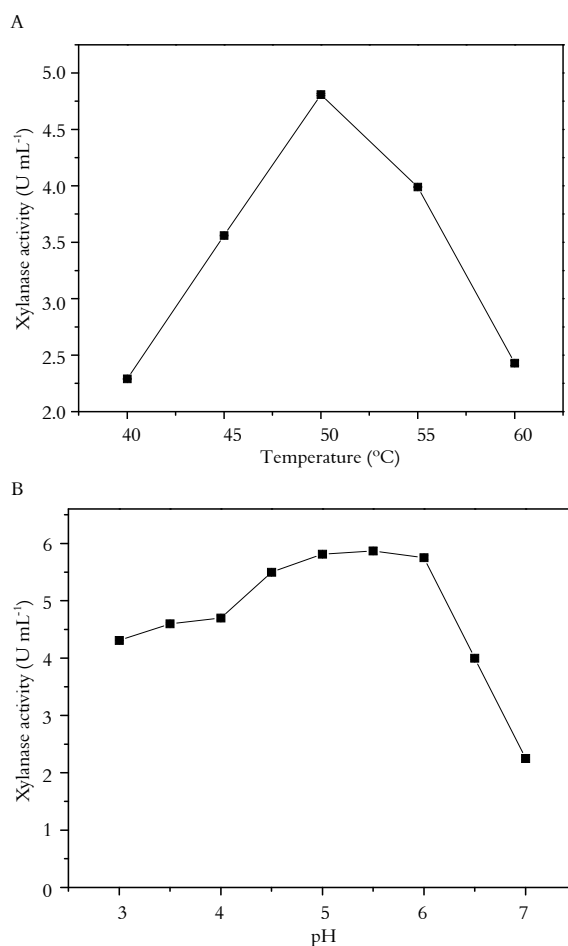


Figure 4. Influence of temperature (A) and pH (B) on the xylanase produced by *A. niger*. Assay conditions: McIlvaine buffer pH 6.5 (A); McIlvaine buffer from 3.0 to 7.0; 50°C (B).

The optimum temperature for xylanase activity was 50°C , corresponding to $4.81 \pm 0.01 \text{ U mL}^{-1}$ (Figure 4B). Likewise, xylanases produced by other *Aspegillus* species showing maximal activity at 50°C (POLIZELI et al., 2005). Usually, xylanases from filamentous fungi show optimum temperature between 40 and 55°C (DOBREV; ZHEKOVA, 2012; KNOB; CARMONA, 2008; QUERIDO et al., 2006). Nevertheless, other fungal xylanases show optimum temperature at 60°C or above this temperature (FAWZI, 2010; LEE et al., 2009).

A pH stability study is an essential part of an enzyme characterization before it can be exploited commercially. The xylanase produced by *A. niger* maintained its stability over a broad of pH evaluated (Figure 5A). Less than 50% of activity was verified in pH 4.0 and 7.5, while high stability (above 50%) was observed from 4.5 to 7.0. Usually, microbial xylanases are stable over a wide pH range 3.0-10.0 (KULKARNI et al., 1999).

Thermal stability is an interesting property of enzymes because it is of great industrial importance (EIJSSINK et al., 2005). Then, enzyme stability analyses were carried out. The crude xylanase from *A. niger* was incubated without substrate at 50, 55, and 60°C (Figure 5B). The half-life ($T_{1/2}$) at 50°C was 240 min. At 55°C, $T_{1/2}$ was 150 min. and at 60°C it was 40 min. The *A. niger* xylanase is more thermostable than many fungal xylanases, such as those from *Aspergillus niger* B03 (DOBREV; ZHEKOVA, 2012).

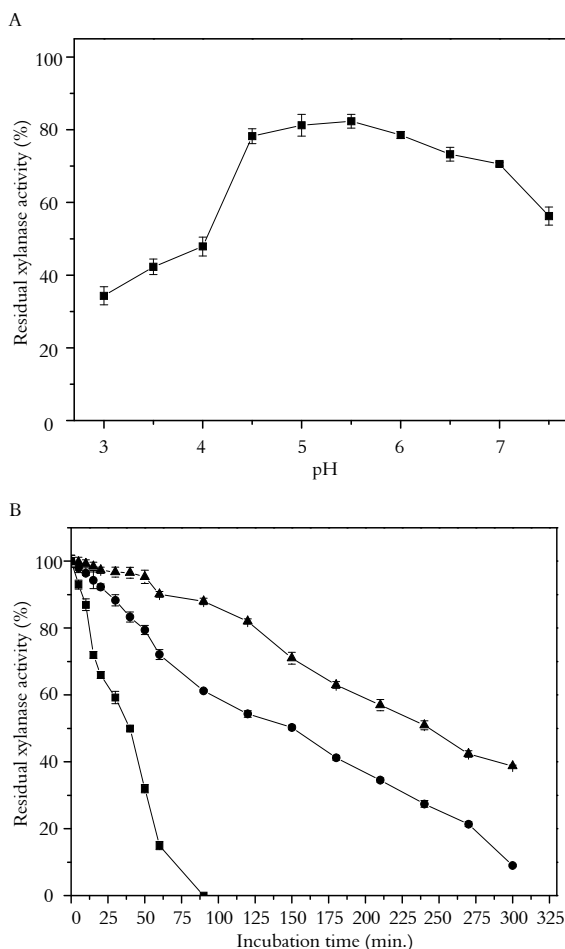


Figure 5. pH (A) and thermal (B) stabilities of the xylanases produced by *A. niger*. The enzymatic preparation was incubated without substrate with McIlvaine buffer from pH 3.0 to 7.0 at 4°C for 24h (A); The enzymatic preparation was incubated at (▲) 50, (●) 55 and (■) 60°C, without substrate (B). In both assays, the residual xylanase activity was assayed with McIlvaine buffer, pH 5.0, at 50°C.

Conclusion

In this study, an *A. niger* strain was able to produce xylanase using brewer's spent grain as substrate. The *A. niger* xylanase is a novel enzyme, being active at acid pH with an optimum at 5.0 and stable in acid pH range. It showed optimum activity and considerable stability at 50°C. These characteristics make it potentially useful in some biotechnological processes as animal feed and food industries. Additionally, the use of brewer's spent grain as substrate for xylanase production can not only add value and decrease the amount of this waste, but also reduce the xylanase production cost.

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