Vol.48, n. 3 : pp. 327-333, May 2005
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

# Partial Purification and Properties of Cellulase-Free Alkaline Xylanase Produced by *Rhizopus stolonifer* in Solid-State Fermentation

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## ABSTRACT

Rhizopus stolonifer *was cultivated in wheat bran to produce a cellulase-free alkaline xylanase. The purified enzyme obtained after molecular exclusion chromatography in Sephacryl S-200 HR showed optimum temperature as 45º C and hydrolysis pHs optima as pH 6.0 and 9.0. Xylanase presented higher Vmax at pH 9.0 (0.87 μmol/mg protein) than at pH 6.0 and minor Km at pH 6.0 (7.42 mg/mL) than at pH 9.0.*

**Key words:** alkaline xylanase, *Rhizopus stolonifer*, solid-state fermentation

## INTRODUCTION

Xylan is the main carbohydrate in the hemicellulosic fraction of the vegetable tissues and forms an interface between the lignin and the other polysaccharides. They are mainly encountered in the secondary cellular wall, and their characteristic of adhesion helps to maintain the integrity of the cellular wall (Prade, 1996). Xylan is found in high quantity in hard wood (15 – 30% of the cellular wall), in annual plants (around 30%) such as corn, ramie and sugar cane as well as soft wood (10%) (Haltrich et al., 1996; Kulkarni et al., 1999; Kaya et al., 2000).

Xylanase (1,4-β-D-xylan xylanohydrolase EC 3.2.1.8) is the major component in a group of enzymes, which act depolymerizing the xylan molecules in monomers, which can be used by bacteria and fungi as a primary source of sugar. The products of the xylan hydrolysis (xylose, xylobiose, xylotriose and xylo-oligosaccharides) have possible applications in the chemical industry, nutritional industry, functional food and also in the production of alternative artificial sweetener with low rate of calories (xylitol). They can also be converted into liquid fuel and solvents (George et al., 2001). Xylanase has been used in bleaching during the paper production, resulting in reduced use of chemicals and resulting in a better brightness (Christov et al., 1999). It also has applications in the beverage industry, bakery and animal food (Kulkarni et al., 1999; Bhat, 2000). Xylanase has been a key in the maceration of vegetables, recuperation of oil in underground mines, extraction of flavors, pigments, vegetable oils and starch, and increase of the efficiency of production of agriculture silage.

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*Rhizopus stolonifer* belongs to the Zygomycota Phylum, Zygomycetes Class, Mucorales Order (Guarro et al., 1999; Ribes et al., 2000). Lipase of *Rhizopus sp* was used by Kolossváry (1996) for the optimization of the hydrolysis activity of the triglycerides. *R. oligosporus* was used by Ikasari and Mitchell (1996) for the production of acid protease in solid fermentation. Cordova (1998) used a strain of *R. rhizopodiformis* for the production of lipase from the sugar cane bagasse. Jin et al. (1999) used *R. oligosporus* for the production of the microbial biomass and glucoamylase from the residual water of starch processing; this fungus is also used in tempe production, a fermented food of Asiatic origin (Sparringa and Owens, 1999). Aikat and Bhattacharyya (2000) used *R. oryzae* for the production of protease in wheat bran medium. However, there is no account for the xylanase production by *Rhizopus stolonifer*. The aim of this work was to determine some properties of the xylanase produced by this fungus.

## MATERIALS AND METHODS

### Microorganism and cultivation conditions

The strain *Rhizopus stolonifer* CCT 7417 was isolated in our laboratory. The conidia were obtained from cultures maintained at 2% (w/v) agar and 4% (w/v) oat flour. After inoculation of 1 mL of suspension ($10^7$ spores/mL) in a solid medium containing 10 g of wheat bran and 20 mL of distilled water, the culture was grown at 35º C for 7 days.

After the growth, the medium was filtered in gaze and the filtrate was centrifuged for 20 min. at 4º C and 9000 rpm. To the filtrate 4% (w/v) of kaolin (Sigma) was added and homogenized. After 15 min. at room temperature, it was vacuum filtered and the proteins of the filtrate were concentrated with acetone.

### Enzymatic assay and proteins determination

Xylanase activity was determined through the reducing sugar produced following Miller's method (1959). The assays conditions were 1% (w/v) birchwood xylan Sigma in 50 mM sodium acetate buffer pH 5.0 at 35º C. One unit of enzyme was defined as the quantity of enzyme that released 1 μmol of reducing sugar per minute. The specific activity was expressed as unit of activity per milligram of protein. The concentration of proteins was determined by Lowry's method modified by Hartree (1972), using bovine serum albumin as standard.

### Molecular exclusion chromatography

Samples concentrated with acetone were applied to a Sephacryl S-200 HR column (51 x 1.8 cm) and eluted with 50 mM sodium acetate buffer pH 5.0. Fractions of 2 mL were collected with flow rate of 10 mL/h. Proteins of each fraction were detected by absorbance at 280 nm and the enzymatic activity was determined. The fractions with xylanase activity were pooled and this sample was used in the subsequent experiments.

### Polyacrylamide gel in denaturing conditions

The sample with xylanase activity from the Sephacryl S-200 HR was submitted to 12% SDS-PAGE, according to Laemmli (1970), using the Amersham Biosciences molecular mass pattern, consisting of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

### Optimum temperature and pH determination

The optimum temperature of birchwood xylan hydrolysis was determined at 22 to 90º C in 50 mM sodium acetate buffer pH 5.0. The optimum pH of hydrolysis was determined using McIlvaine buffers from pH 2.9 to 8.0 and glycine-sodium hydroxide for pH 8.6 to 10.0 at 45º C.

### Kinetic parameters (Km and Vmax) determination

The substract concentrations varied from 0.5 to 20 mg/mL in 50 mM sodium acetate buffer pH 5.0 and glycine-sodium hydroxide buffer pH 9.0 at 45º C.

### Chromatographic separation of the hydrolysis products

The products of the xylan hydrolysis were analyzed by thin-layer chromatography on silica gel (Silica Gel 60 AL TLC Merck), using solvent system composed of ethyl acetate, acetic acid, formic acid and water (9:3:1:4 v/v), according to Fontana et al., 1988. The hydrolysis products were detected with 0.2% of orcinol (w/v) in sulfuric acid and methanol (10:90 v/v). Xylose and xylobiose (Sigma) were used as standards.

## RESULTS AND DISCUSSION

### Molecular exclusion chromatography

Fig. 1 presents the enzymatic activity of the eluted fractions as well as the proteins concentration. Two groups of proteins were separated and identified as P-I (proteins without xylanolytic activity) and P-II (proteins with xylanolytic activity). An increase of 55% in the xylanase specific activity was reached in the group P-II (Table 1). Monti et al. (1991) reported 169.35 U/mg protein for xylanase of *Humicola grisea*; Christov et al. (1999), using several species of *Aspergillus* obtained 547.4 U/mL for a xylanase of *A. foetidus* (ATCC 14916); Duarte et al. (1999) isolated several strain of bacteria, with 41.1 U/mg protein for a xylanase of the "B" strain; Ghanem et al. (2000) used several forms for the purification of a xylanase of *A. terreus* and obtained a final specific activity of 183.29 U/mg protein. Heck et al. (2002) reported 5.19 U/mg protein for xylanase of a strain of *Bacillus subtilis;* Damaso et al. (2002), using *Thermomyces lanuginosus* (IOC-4145), obtained 85 U/mg protein for the xylanase activity in a filtered culture. Coelho and Carmona (2003) reported 107.43 U/mg protein for xylanase, using *A. giganteus*. The specific activity of xylanase determined in our work was lower than the range cited in the literature; however, no cellulase activity was found in the fraction P-II, using cellulose microcrystalline Sigma 1% (w/v).

The electrophoresis profile of the samples obtained after molecular exclusion chromatography is shown in Fig. 2, indicating partial purification of the sample P-II. The ion exchange resins DEAE-Trisacryl and S-Sepharose were also used with differente buffers and pHs, however, xylanase did not link to any of them under the assay conditions, suggesting that this enzyme was a glycoprotein (Boyer, 1993). The purification of this enzyme could be done through electroelution technique, used by Monti et al. (2003) to purify the form I xylanase from *Humicola grisea* var. thermoidea.

### Determination of temperature and pH optimum

The optimum temperature of partially purified xylanase was observed in the interval between 40 and 60º C (Fig. 3). Values of optimum temperature of xylanase hydrolysis vary according to the producing microorganism. The xylanase produced by different cultures had different temperature optima, such as for *Humicola grisea* var. thermoidea presented 70º C (Monti et al., 1991); *A. versicolor* 55º C (Carmona et al., 1998). *A. terreus* 40º C, *A. niger* 70º C, *B. stearothermophilus* 75º C. However, 50 to 55º C seems to be the range of optimum temperature of xylanases (Uhlig, 1998).

The optimum pH of xylan hydrolysis was determined at 45º C, using McIlvaine buffer systems and glycine-sodium hydroxide. The results obtained revealed two optima pHs clearly different, with maxima at pH 6.0 and 9.0 (Fig. 4). This indicated the presence of more than one xylanases in fraction P-II. The optimum pH for xylanases cited in the literature varied between 4.0 and 5.5 (Uhlig, 1998); however, some bacterial xylanases showed optimum activity between pH 6.0 and 7.0. These results are interesting as the thermostable and alkaline xylanases have good scope because the enzymatic bleaching of kraft pulp needs these enzymes with such characteristics (Subramaniyan and Prema, 2000; Duarte et al., 1999; Christov et al., 1999). Thus, the alkaline xylanase studied in this work could be utilized with this purpose.

**Table 1 -** Specific xylanase activity after molecular exclusion chromatography.

| Sample | Protein | Xylanase activity | |
|---|---|---|---|
| | mg/mL | U/mL | U/mg protein |
| Crude extract | 2.23±0.01 | 0.82±0.02 | 0.37±0.01 |
| Sephacryl S–200 HR | 0.16±0.01 | 0.09±0.01 | 0.57±0.02 |

Results represent the average of three experiments.

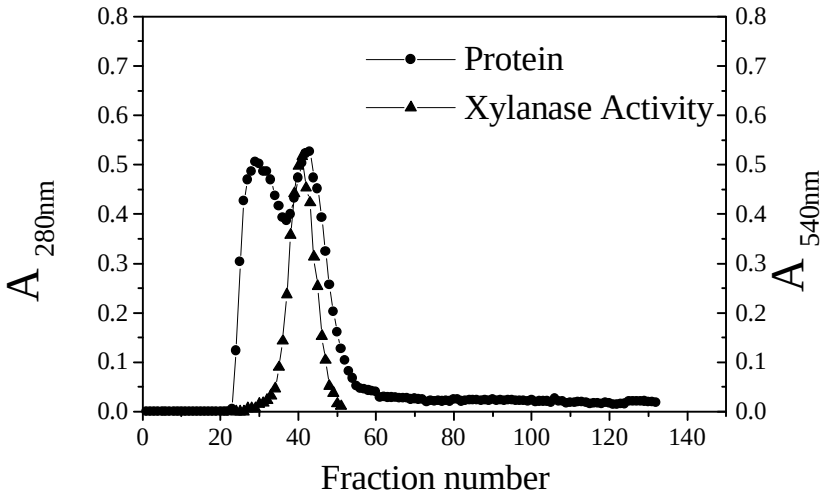


**Figure 1 -** Elution profile of the proteins and xylanase activity produced by *Rhizopus stolonifer* in Sephacryl S–200 HR The column (50 x 1.8 cm) was eluted with 50 mM sodium acetate buffer pH 5.0; fractions of 2 mL were collected in a flow rate of 10 mL/h.

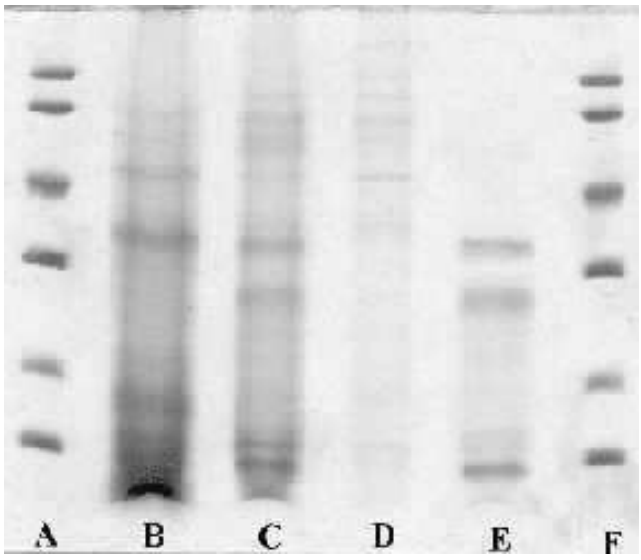


**Figure 2 -** SDS–PAGE 12% of samples obtained after Sephacryl S–200 HR. A and F: LMW kit Amersham Biosciences; B: crude extract; C: crude extract precipitated with acetone; D: fraction P-I; E: fraction with xylanolytic activity (P-II).

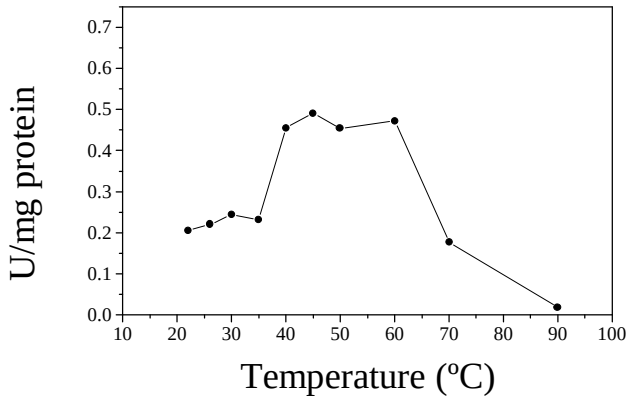


**Figure 3 -** Optimum temperature determination for the birchwood xylan hydrolysis.

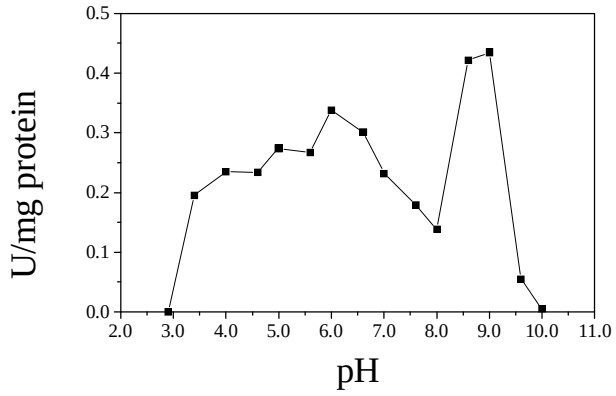


**Figure 4 -** Optimum pH determination for the birchwood xylan hydrolysis.

**Determination of Km and Vmax**

The results showed the values of 7.42 mg/mL for Km and 0.47 μmol/mg protein for Vmax, whereas the values determined in glycine-sodium hydroxide buffer pH 9.0 were 10.0 mg/mL and 0.87 μmol/mg protein, respectively.

**Thin-layer chromatography of the xylan hydrolysis products**

The products released at pH 6.0 and 9.0, were xylotriose and xylo-oligosaccharides (Fig. 5). However, after 120 min. at pH 9.0 it was clearly possible to observe the presence of xylobiose, which did not occur at pH 6.0. These results, once again, suggested the existence of isoforms in P-II fraction. After 120 min. reaction, traces of xylose were obtained, indicating β-xylosidase activity. These results showed this enzyme as endo-xylanase.

## ACKNOWLEDGEMENTS

Antonio José Goulart thanks Capes – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial support. We thank Dirlene Attili de Angelis for the identification of the strain.

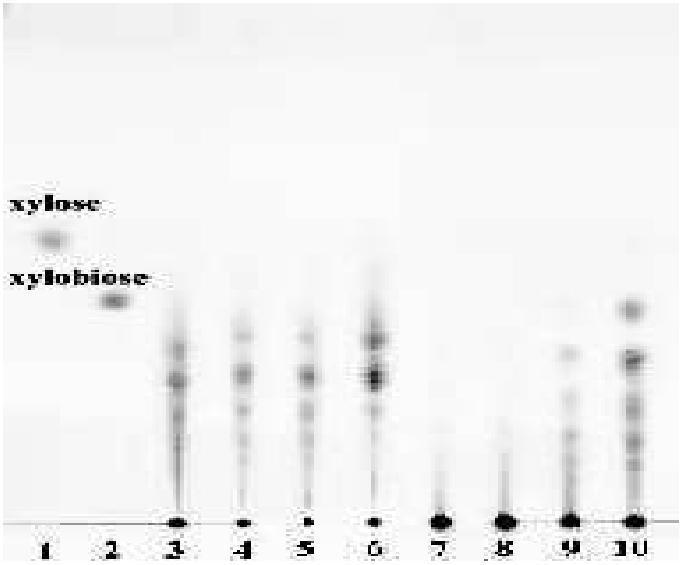


**Figure 5 -** Chromatographic profile of hydrolysis products of birchwood xylan in pH 6.0 (lines 3 to 6) and 9.0 (lines 7 to 10). Line 1: xylose; line 2: xylobiose; line 3: 10 min.; line 4: 20 min.; line 5: 40 min.; line 6: 120 min.; line 7: 10 min.; line 8: 20 min.; line 9: 40 min.; line 10: 120 min.


## RESUMO

*Rhizopus stolonifer* foi cultivado em meio de farelo de trigo para produzir uma xilanase alcalina celulase-free. Uma amostra parcialmente purificada desta enzima foi obtida após cromatografia de exclusão molecular em Sephacryl S–200 HR. A temperatura ótima de hidrólise determinada (45º C) está dentro do intervalo citado na literatura (45º C a 60º C) para xilanases microbianas. Quanto ao pH ótimo, a amostra obtida apresentou atividades máximas em pH 6,0 e 9,0. Estes dados diferem da literatura, uma vez que o pH ótimo citado para a maioria das xilanases estudadas varia entre 4,0 e 5,5. De acordo com os estudos cinéticos realizados, a xilanase apresentou maior Vmax em pH 9,0 (0,87 μmol/mg proteína) e menor Km em pH 6,0 (7,42 mg/mL). Os dois pHs ótimos determinados podem indicar a presença de isoformas desta enzima. Estes dados são interessantes pelo fato de que enzimas xilanolíticas alcalinas celulase-free podem ser utilizadas para o biobranqueamento da polpa na indústria de papel.


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Received: September 18, 2003;
Revised: February 17, 2004;
Accepted: September 20, 2004.