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Partial Purification and Characterization of Xylanase Produced by *Penicillium expansum*

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ABSTRACT

An extracellular xylanase was found to be the major protein in the filtrate culture of Penicillium expansum when grown on 0.3 % wheat bran, which showed no xylanase multiplicity. The enzime was partial purified by.ammonium sulfate fractioning, molecular exclusion chromatography, ultrafiltration and anion exchange chromatography. The protein eluation profile showed only one form of xylanase that was partially characterized. The activity of purified xylanase was optimal at pH 5.5 and 40 ^{0}C. The enzyme was stable at pH between 5.5 and 6.5 and temperatures between 20-40 ^{0}C. The enzyme showed a Km of 3.03 mM and Vmax of 0.027 μmol min^{-1} μg $^{-1}$ of protein. The enzymatic activity was increased 31 % by Mg^{2+} and 28 % by Al^{3+}.

Key words: Partial purification; xylan, xylanases; *Penicillium expansum*

INTRODUCTION

Microbial xylanases have important applications in the biodegradation of xylan, a biopolymer of lignocellulose biomass. In this process β-1,4-xylanase (EC 3.2.1.8) plays a key role. *Penicillium expansum* is a filamentous fungus that produces extracellular Xylanase (Ferreira-Filho et al., 1993). Xylanase has applications in the last years, much in the paper and cellulose manufacturing, as in the textile and food industries. (Godfrey and West, 1996). Yimbo et al., (1996) purified and characterized a cellulase-free-xylanase from *Aspergillus niger* Chandra et al., (1996) isolated *A. fisheri* Fxn1 for xylanase production and purification. The aim of the present study was to partially purify and characterize xylanase produced by *Penicillium expansum* to identify possible genetic polymorphism and to determine the physical and chemical parameters of the enzyme, evaluating its applicability in textile industry.

MATERIALS AND METHODS

Microorganism

A strain of *Penicillium expansum* isolated from forest seeds was used in this study.

Medium and Cultivation

The cultivation of *P. expansum* was conducted in unbuffered mineral medium with the following composition (g/L): K_2HPO_4, 0.62; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 1.1; pH 6.3; with yeast extract 0.06 % (w/v) and wheat bran 0.3 %

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(w/v) as the sole carbon source. The culture was incubated in an orbital shaker at 150 rpm, 25 ^{0}C for 120 h. The resulting mycelial mass was separated from the culture medium by filtration.

Enzyme Assay

The enzymatic assay was done according to Haltrich et al., (1993). β-1,4-xylanase activity was determined by estimating the xylose liberated from oat spelt xylan 1% (w/v) suspended in phosphate buffer 50 mM, pH 5.0. The reduction sugar was determined by the DNS method (Miller, 1959) using xylose standard sugar. One unit of enzyme activity was defined as the amount (µmol) of reducing sugar released per minute. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Purification

The culture filtrate was concentrated by lyophilization and resuspended in potassium phosphate buffer 0.05 M, pH 7.2. The crude enzyme was precipitated with ammonium sulfate at 60 % saturation, and the precipitate was collected by centrifugation at 8000g for 15 min. Crude enzyme precipitate was dissolved in potassium phosphate buffer, pH 7.2, 0.05 M, dialysed and applied 20 mL to exclusion column Sephadex G-25 (20x1000mm), pre-balanced in potassium phosphate buffer 0,05 M, pH 7.2. The fractions containing enzymatic activity were ultrafiltrated and applied 5 mL to anionic-exchange column DEAE-Sephadex A-50 (20x150mm), pre-balanced in potassium phosphate buffer 0,05 M, pH 7.2. A linear gradient of NaCl (0-0,5 M) was applied**.** Eluted fractions of 3 mL were collected for the determination of the xylanase activity and absorbance at 280 nm.

Characterization

Effect of temperature and pH on xylanase activity was measured at 20 and 70 ^{0}C and pH 4.0-9.0, respectively. The pH was adjusted with NaOH 2 M or HCl 2 M. The effect of ions Mg^{2+} and Al^{3+}, applied as $MgSO_4$ (1 mM), and $AlPO_4$ (1 mM) on xylanase activity was studied by addition of the ions to the substrate xylan at optimal pH and temperature. Kinetic constants for xylanase were determined using concentrations varying between 1.0 and 60.0 mg/mL of xylan substrate in potassium phosphate buffer 0,05 M, at pH 5.5 and optimal temperature. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined using the method of double reciprocals (Lineweaver and Burk, 1934). All xylanase activity values are provided as the means of three replication.

RESULTS AND DISCUSSION

The elution profile of proteins showed a single form of xylanase, which was partially characterized (Figs 1-2). An activity peak was eluted during the lineal gradient of NaCl (0,1 M).

The profile of elution in the DEAE-Sephadex A50 suggested that only one xylanase was produced. Electrosphoresis in SDS polyacrylamide gel (12 %, stained with silver nitrate) showed that a good purification (Fig 3).

The activity of partially purified β-1,4-xylanase was optimal at pH 5.5 and 40 ^{0}C. Similar results were observed for other microorganisms. *P. chrysogenum* (Haas et al., 1992) and *A. ficheri* Fxn1 (Chandra et al., 1996) also presented xylanases with maximum activities at similar pH. Kitamoto et al. (1999) found a pH of 5.0 as optimum for a xylanase produced by *A. oryzae*. Sherief (1990) found an optimal pH of 5.0 for a xylanase of *A. flavipes*. The xylanases of fungal origin usually show optimal activity around 50 ^{0}C, being inactivated at 65 ^{0}C (Gaspar et al., 1997). Kitamoto et al. (1999) observed an optimal temperature of 60 ^{0}C for the xylanase produced by *A. oryzae*, whereas Sherief (1990) found an optimal temperature of 55 ^{0}C for a xylanase produced by *A.flavipes*.

The xylanase was stable at temperatures between 20 and 40 ^{0}C. The activity dropped to less than 48% between 50 and 70 ^{0}C. The same happened to polygalacturonases (PG) produced by *Trichoderma resei* QM 9414, which was inactivated when maintained during one hour above 45 ^{0}C (Dekker, 1993). Thermal stability however, to higher temperatures could be increased after the addition of 1 mg/mL of of bovine serum albumin (BSA) prevening the enzymatic inactivation significantly.

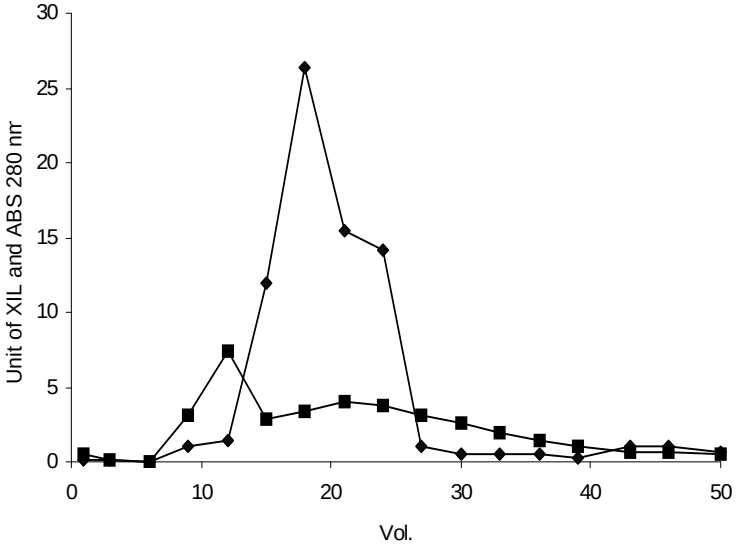

Figure 1 - Elution profile by molecular exclusion chromatography in the Sephadex G-25. (♦) xylanase activity (u); (■) absorbance at 280 nm.

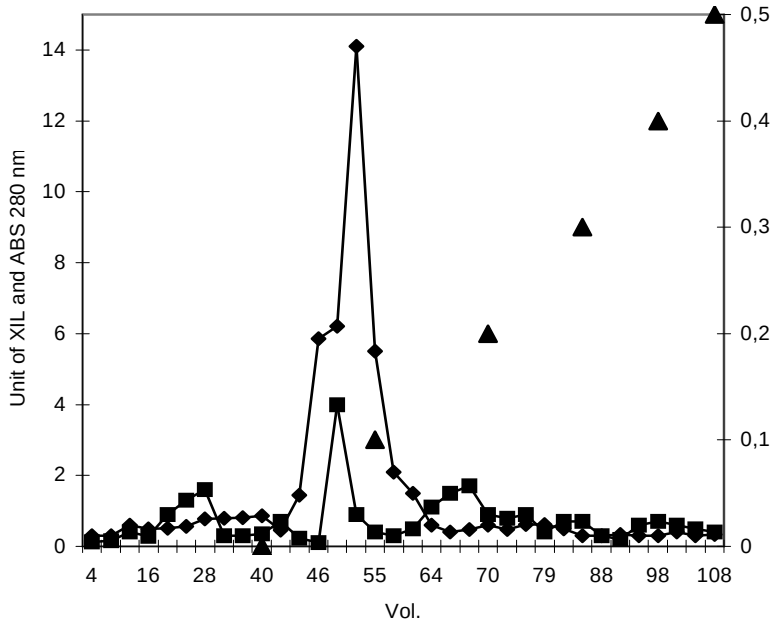

Figure 2 - Elution profile by anionic exchange chromatography in the DEAE-Sephadex A-50. (♦) xylanase activity (u); (■) absorbance at 280 nm; (▲) NaCl gradient 0-0,5 M.

Table 1 - Resume of stages of the partial purification of xylanase of *P. Expansum.*

Stage	Protein (µg)	Activ. (U)	Specific activity	Yield (%)	Purif. Factor
Supernatant	190000	50321	0.26	100	1
$(NH_4)_2SO_4$	29000	20621	0.71	49.2	2.7
Gel Filtration	3600	3050	0.84	5.7	3.2
Ultrafiltration	2170	2600	1.19	4.8	4.5
Ion Exchange	374	725	1.93	1.3	7.4

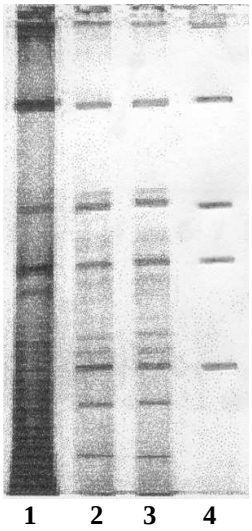

Figure 3 - Electrophoresis in SDS-polyacrylamide ge1 l2 %, stained with silver nitrate. 1. Supernatant of the culture; 2. After molecular exclusion colunm; 3. After ultrafiltration; 4. After ionic exchange column.

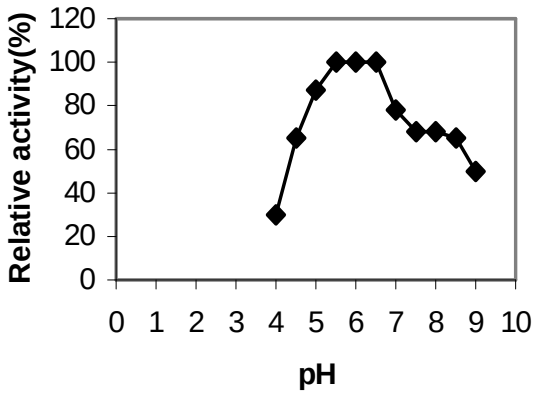

Figure 4 - Stability of xylanase to pH. Relative activity was determined at pH 5.5, 40 ^{0}C, after 1 h.

The xylanase was stable at pH between 5.5-6.5. The enzyme retained about 78, 87 and 65 % of its optimal activity at pH 7.0, 5.0 and 4.5, respectively. The activity dropped to 68 % of its optimal value at pH between 7.5 and 8.5; to 50 % at pH 9.0, and to 30 % at pH 4.0 (Fig 4). The

xylanases of fungal origin are more active at pH that oscillates between 3.5 and 5.5 but are quite stable over a wide pH range (3.0 to 10.0). On the other hand, the optimal pH of the bacterial xylanases oscillates among pH 5.0 and 7.5 (Dekker, 1993). The xylanase produced from alkali-tolerant Thermophiles was shown to be stable at pH 5.5-9.5 (strain SP) and pH 6.0-7.5 (strain BC) after 30 minutes of incubation at 60 ^{0}C (Plamen et al., 1997).

Approximately a third of the known enzymes possess metals as part of their structures (Conn and Stumpf, 1987). The effect of the Mg^{2+} and Al^{3+} was shown in Table 2. Mg^{2+} increased the activity of the enzyme by 31% and Al^{3+} increased the activity of the enzyme by 28%.

For xylanases of *A.ficheri* Fxn1, Mg^{2+} (10 mM) didn't show any effect whereas $AlCl_3$ (10 mM) decreased the activity by 95% (Chandra et al., 1996). Ghareib (1992) demonstrated that Zn^{2+}, Cu^{2+}, K^{+} and Co^{2+} increased the xylanolitic activity of *A. terreus*, but $HgCl_2$, 2,4-dinitrophenol (DNP) and diamino ethylene acetic acid (EDTA) strongly inhibited the activity at 1mM.

Table 2 - Ions effect about the activity of the xylanase of *P. expansum* partially purified.

Composed added (1 mM)	Relative activity (%)
Control (without addition)	100
$MgSO_4$	131
$AlPO_4$	128

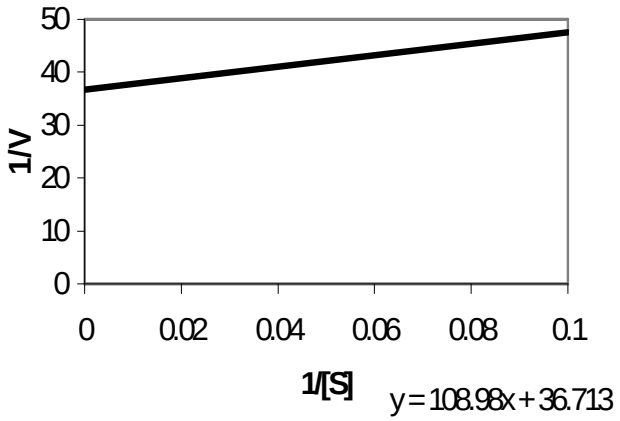

Figure 5 - Kinetics of the xylan hydrolysis for xylanase. Graphic representation of Lineweaver-Burk.

The (K_m) and (V_{max}) were calculated to be 3.03 mM and 0.027 µmol min^{-1} µg $^{-1}$ of protein, respectively (Fig 5). This was in the same range of value of Km and V_{max} of the main xylanase of alkali tolerant *A.fischeri* Fxn1 (Chandra et al., 1996), which was 4.88 mM and Vmax 0.058 µmol min^{-1} µg $^{-1}$ of protein. On the other side, the values of the Km of the xylanase of *Acrophialophora nainiana* were 40.9 mM and 16.1 mM, respectively (Salles et al., 2000).

CONCLUSION

Based on the above results, it was concluded that *P.expansum* was a potentially interesting producer of a single form of a xylanolitic enzyme.

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RESUMO

Uma xilanase extracelular foi encontrada como a principal proteína na cultura filtrada de *Penicillium expansum* quando cultivado em farelo de trigo 0,3 %, a qual não mostrou multiplicidade. A enzima foi parcialmente purificada por fracionamento com sulfato de amônia, cromatografia de exclusão molecular, ultrafiltração e cromatografia de troca aniônica. O perfil de eluição das proteínas mostrou uma única forma de xilanase, sendo esta parcialmente caracterizada. A atividade da xilanase purificada foi ótima em pH 5.5 e à temperatura de 40 ^{0}C. A enzima foi estável em pH entre 5,5 e 6,5 e à temperatura entre 20-40 ^{0}C. A enzima apresentou Km de 3,03 mM e Vmax de 0,027 μmol min^{-1} μg^{-1} de proteína. A atividade enzimática foi aumentada 31 % por Mg^{+2} e 28 % por Al^{+3}.

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