

Purification And Characterization Of Acido-Thermophilic Xylanase From *Aspergillus Terrus*

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Abstract: Purification and characterization of extracellular xylanase from *Aspergillus terrus* was recorded. The enzyme was purified to homogeneity by salting out with ammonium sulphate, dialysis and passage through gel chromatography resins (Sephadex G-200, Sephadex G-100 columns) followed by anion exchange chromatography (Diethylaminoethyl Sephadex column). The purified enzyme resulted in 516.4 fold increase over the crude extract exhibited a specific activity of 175.6 unit/mg protein with the recovery of 30.6 %. Two criteria for the purity of the purified *A. terrus* extracellular xylanase were used. DEAE-sephadex column (final stage of purification) resulted in a single sharp peak of *A. terrus* pure xylanase. The second criterion was given by applying SDS-PAGE electrophoresis technique. The molecular weight of *A. terrus* extracellular xylanase was 33 KDa. Studying factors affecting the activity of the purified xylanase were determined. An optimum temperature and pH for the acidothermophilic purified xylanase were 50 °C at pH 4, respectively.

Key words: Xylanase, *Aspergillus*, Purification, Characterization.

INTRODUCTION

Xylan displays a large polydiversity and polymolecularity. This corresponds to their being present in a variety of plant species and to their distribution in several types of tissues and cells. All land plant xyans are characterized by β -1,4-linked-D-xylopyranosyl main chain, which carry a variable number of neutral or uronic monosaccharide substituents or short oligosaccharide side chains (Kango, 2010). Xylanases play important physiological roles in plant tissue, because they involved in fruit softening, seed germination, and plant defense mechanisms (Geweely *et al.*, 2006). The production of microbial xylanase has attracted great interest due to their potential application in chemical, pharmaceutical, food and paper industries (Turner *et al.*, 2007).

Filamentous fungi are useful producers of xylanase because they are capable of producing high levels of extracellular enzymes and can cultivated very easily. On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp. (Pal and Khanum, 2011). Many reports on xylanases from *Aspergillus* spp. and other microorganisms are available (Evstatieva *et al.*, 2010). The production and extraction of xylanase has been reported by Pal and Khanum, (2010). Studies on the isolation and characterization of xylanases always prove beneficial, but to study their properties and to determine their effectiveness, it is necessary to obtain sufficient quantities of xylanase in a pure form. Keeping these points in view, the present work is planned to study the purification and properties of extracellular acidophilic xylanase from *Aspergillus terrus*.

MATERIAL AND METHODS

Test organism:

Aspergillus terreus used in this study was isolated from sugar cane bagasse.

Cultivation of *A.terrus*:

The medium composed of (g/l) xylan, 10; pepton, 5; yeast extract, 5; KH_2PO_4 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5. The medium was brought up to 1000 ml with distilled water. Three replicates were used for each treatment. The flasks were incubated at 30 °C for 10 days. Thereafter, the culture medium was filtered through filter paper to separate the mycelium from the culture filtrate. The activity of extracellular xylanase was determined in the culture filtrate.

Enzyme Assay:

Xylanase was assayed as described by Poutanen and Puls, (1988) using 0.01% birch wood xylan as substrate in 0.05M sodium citrate buffer at pH 6. Xylanase activity was measured by incubating 1 ml of 1:250 diluted enzyme solution with 1ml of the substrate for 5 min at 50 °C (Uchino and Nakane, 1981). The reaction was stopped by heating for 10 minutes at 100 °C and then rapidly cooled (Van Laer *et al.*, 1999). The released reducing sugar was determined according to the method described by Nelson, (1944) and Somogyi, (1952). A

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xylanase unit was defined as the release of one μmol of reducing sugar as a xylose equivalent / minute under the specified conditions.

Purification of Xylanase:

The crude extract of xylanase was prepared by filtering the broth through four layers of guaze and then through prechilled puchner. The solution was then rotated in refrigerated centrifuge at 20,000 r.p.m. to remove the muddy matter. Supernatant was designated as crude extract. The precipitate was dissolved in 10 ml 0.05M citrate buffer of pH 6.2, placed in a dialysis bag and dialyzed against distilled water in a refrigerator for 48 hours. Dialysis against sucrose for 48 hour was then carried out to elute excess water inside the bag. After complete dialysis, the protein content and extracellular xylanase activity were determined. The dialyzed fractions of xylanase was chromatographed on Sephadex G-200 column followed by Sephadex G-100 column (1.3 x 90cm). The pooled lyophilized active fractions obtained from the previous columns were concentrated with ammonium sulphate, dialyzed and applied, in small amount of buffer at pH 6.2, to DEAE-Sephadex column (1.6x 55cm) and the exchanged material was eluted in succession with stepwise gradient of 0.0- 0.2 M NaCl prepared with the same buffer. Five - ml fraction of protein was eluted and collected at a flow rate of 60ml /hour. This combination was established by Ganju *et al.* (1989) and Anand *et al.* (1990). The protein content and activity of xylanase were determined for each fraction. The fractions showing the highest enzyme activity were pooled and lyophilized for further investigation.

Protein Determination:

Protein concentration was determined spectrophotometrically at 260 nm by the method of Segel (1968).

Estimation of the Molecular Weight of the Purified Xylanase:

Sodium dodecyl-sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.5% acrylamide gel according to the method of Laemmli, (1970). Xylanase was visualized by silver staining as described by Wray *et al.* (1981). A protein sample (12 μl) containing about 10-15 μg protein was mixed with 3 μl of sample buffer and heated in boiling water bath for 5 min followed by centrifugation. The molecular mass standards (maltose-binding proteins (MBP2), 42.7 KDa., lactate dehydrogenase, 36.5 KDa and soybean trypsin inhibitor, 21.5 KDa) were used.

Characterization of the Purified Xylanase:

The prepared reaction mixture of substrate and pure xylanase was incubated at the following temperatures: 20°C 30°C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C for 5 minutes. Control was made using denatured inactivated enzyme. Xylanase activity was assayed as described before. Different pH values ranging from 2-10.9 were tested on xylanase activity. Three different buffer systems were used: citrate phosphate buffer solution for pHs (2- 7); boric acid-borax buffer solution for pH 8 and carbonate-bicarbonate buffer solution for pH 10-10.9. Incubation of the reaction mixture was incubated at the optimum temperature for 5 minutes, after which xylanase activity was measured.

Statistical Analysis:

The data were analyzed using the least significance difference (L.S.D.) at 0.05 by Duncan method, (Sas Institute, 1982).

RESULTS AND DISCUSSIONS

Purification of Extracellular Xylanase:

The specific activity of xylanase in the crude extract of filtrate was 0.34 unit/mg (Table 1). The highest precipitation was obtained by 80% ammonium sulphate followed by dialyses against distilled water and then against sucrose to concentrate the activity of the crude extract of extracellular xylanase, where the specific activity raised to 7.9 fold over the crude xylanase extract. This increase in activity is presumably by removal of low molecular weight inhibitors, possibly phenolic substance released from the growth substrate. From the elution profile of Sephadex G-200 column chromatography in Fig. 1 and Table 1, it can be seen that *A. terreus* xylanase was eluted from the column in several peaks of xylanase activity accompanied with several peaks of protein. Two peaks of activity (A and B) were collected through a column chromatography on Sephadex G-100 (Fig. 2). The active fraction after the second purification step were collected and applied to DEAE-Sephadex column. Finally, one pure peak of purified xylanase with the specific activity of 175.6 unit/mg proteins accompanied with an enrichment of about 516.4 fold increase over the crude extract and recovery of 30.6 % were obtained (Table 1 and Fig 3). This increase in purification fold may indicated a high xylanase content in the original fungal extract relative to other proteinaceous compounds. This may be expected because the isolated fungal species have high xylanase activity. The study provided better results in terms of purification fold as well

as recovery in comparison to some of the earlier published reports from various microorganisms. Khandeparkar and Bhosle (2006) reported purification of xylanase to 21 fold with 14% recovery. Purification of *A. niger* xylanase was purified 36.97 fold by Pal and Khanum, (2011).

Table 1: Purification scheme of *Aspergillus terreus* xylanase:

Purification steps	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Purification fold	Recovery (%)
Cell free-Supernatant	720.0	832.0	286.0	0.34	1	100
Amm. Sulphate precipitate	130.0	320.0	233.0	0.73	2.1	81.4
Dialyzed protein	52.0	78.0	210.0	2.69	7.9	73.4
Sephadex G-200 column chromatogram	8.0	10.0	192.0	19.2	56.4	67.1
Sephadex G-100 column chromatogram	5.0	3.0	165.0	55.0	161.7	57.6
DEAE-Sephadex	1.5	0.5	87.8	175.6	516.4	30.6

Table 2: Effect of temperature on the activity of the purified *A. terreus* xylanase

Temperature (°C)	<i>A. terreus</i> purified xylanase (unit/ml)
20	11.3
30	22.9
40	30.5
50	37.1
60	28.0
70	12.0
80	-
LSD at 0.05	5.2

Table 3: Effect of pH on the activity of the purified *A. terreus* xylanase

pH	<i>A. terreus</i> purified xylanase (unit/ml)
2	11.4
4	45.40
6	32.90
7	25.10
8	18.0
10	8.0
10.9	-
LSD at 0.05	12.5

Estimation of the Molecular Weight of the Pure Extracellular Xylanase Produced by *A. Terreus*:

The tested purified enzyme seemed to be homogeneous because it migrated as a single band on SDS-PAGE which formed by a single polypeptide chain with a molecular mass of about 33 kDa (Fig. 4). Xylanases of almost similar molecular mass have been reported from some *Aspergillus* strains (Evstatieva *et al.*, 2010) and *Aspergillus ceasptosus* (Sandrim *et al.*, 2004).

Characterization of Pure *A. terreus* Xylanase:

The results in Table 2 show that the optimum temperature for extracellular pure xylanase was at 50 °C, where the maximum significant activity of the pure thermophilic xylanase was 37.1 unit/ml. On further increase in temperature, the enzyme activity declined gradually. The optimum temperature of pure xylanase of *Aspergillus* was reported to vary between 45 and 60 °C (Krisana *et al.*, 2005). A thermostable xylanase was purified and characterized by Fawzi, (2010).

The tested purified enzyme showed relatively high activity (45.4 unit/ml) under acidic conditions with an optimum of pH 4.0 (Table 3), near to the optimum pH 4.8 for the *Aspergillus* xylanase that recorded by Ohta *et al.* (2001). The optimum pH was 5 in the similar experiments on some fungal xylanases (Naveen *et al.*, 2006 and Goyal *et al.*, 2008). However, xylanase from *Aspergillus niger* was most active at pH 6, and the activity decreased as the pH was increased (Chidi *et al.*, 2008). A pH stability study is an essential part of any enzyme characterization before it can be exploited commercially. Thus, the tested xylanase was acidophilic. Purification and characterization of acidophilic xylanase was recorded by Kimura *et al.*, (2000). Xylanase from *Aspergillus nidulans* has been found stable in the pH range of 3.5–10.0 (Reis *et al.*, 2003).

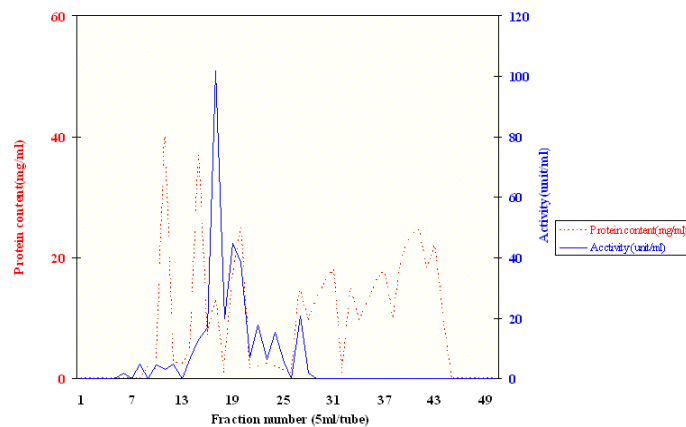


Fig. 1: Typical elution profile for the behaviour of *A. niger* xylanase on Sephadex G-200.

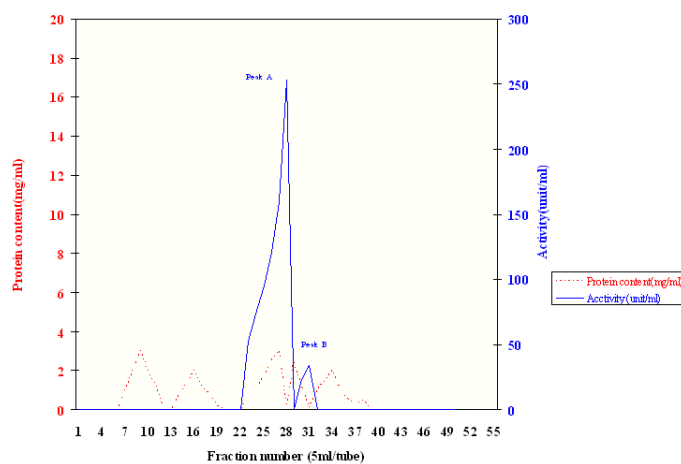


Fig. 2: Typical elution profile for the behaviour of *A. niger* xylanase on Sephadex G-100.

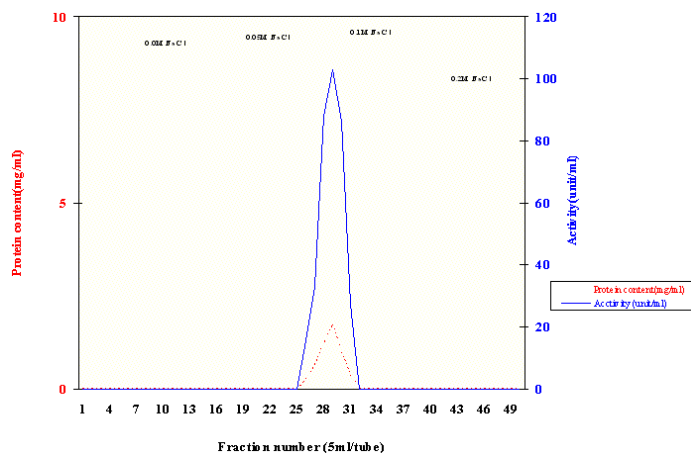


Fig. 3: Typical elution profile for the behaviour of *A. niger* xylanase on DEAE-Sephadex.

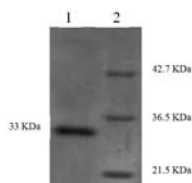


Fig. 4: SDS-PAGE analysis of purified xylanase. Lane 1: purified xylanase; lane 2: molecular mass marker.

Conclusion:

In conclusion, scientific and biotechnological development of research has expressed the utilization of waste materials like sugar cane bagasse as a renewable base. The present study indicates that the purified xylanase produced from the *Aspergillus terreus* may be preferable enzyme due to its high specific activity (175.6 unit/mg) resulted in 516.4 fold increase over the crude extract exhibited a recovery of 30.6 %. The tested purified xylanase was used in industrial field and biotechnology helping in the pretreatment of paper pulps to reduce the dependence on chlorine used for bleaching in brightening process to produce a large amount of the enzyme at the lowest cost, rather than the use of high expensive chemicals, where the most advantageous working conditions prevailing for application of xylanase in biotechnology at high temperature. Therefore, an ideal xylanase should be able to function under the extreme conditions.

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