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Biochemical Studies of Purified Extracellular Xylanases from *Aspergillus versicolor*

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Abstract: Fungal xylanases from alkalo-thermophilic *Aspergillus versicolor* that hydrolyse xylan, had been purified and characterized. The purification procedure included ammonium sulphate precipitation and chromatography on gel filteration (Sephadex G-200 and G-100) combined with ion exchange chromatography on gel filteration (DEAE-sephadex). Three forms of extracellular xylanases XYI, XYII and XYIII are obtained. Their specific activities were 95.9, 83.33 and 106.7 unit g^{-1}. protein which represented 16.31, 14.17 and 18.15 fold purification over the crude extract, respectively. Characterization was carried out for all forms of xylanases. The K_m values are 0.66, 0.50 and 0.22 mg mL^{-1}. For XYI, XYII and XYIII, respectively. The optimum temperature for all xylanases was at 70°C except for XYII at 50°C. The optimum pH for XYII and XYIII were 10 and for XYI at pH 7. The effect of metal ions was examined. In conclusion, the present study indicate that the low molecular weight isoenzyme XYIII (31.6 kDa) produced from the alkalo-thermophilic *Aspergillus versicolor* is the most preferable isoenzyme due to its high pH and temperature optima (10 and 70°C) alkali-thermostable) accompanied with the highest affinity (K_m = 0.22 mg mL^{-1}) and specific activity (106.7).

Key words: Xylanase, exoenzyme, alkaophilic, thermophilic, baggase

INTRODUCTION

Xylans, the most plentiful of the hemicellulose, are present in the cell wall of all land plants and are particularly abundant in tissues that have undergone secondary thickening. They consists of a common backbone composed of β(1→4)-linked xylosyl residues, but differ in the side chains attached to it (Cesar and Mrsa, 1996).

Agroindustrial and food processing wastes are available in staggering quantities all over the world, which largely become a source of health hazard. The majority of these wastes contain hemicellulose (xylan 20-40%) (Rani and Nand, 1996; Lee *et al*., 2004). The use of these wastes for the production of strategic chemicals and fuel requires the hydrolysis. Because xylan is a major plant structural polymer, xylanases and the microorganisms that elaborate them could be used in food processing, paper, pulp sugar, ethanol, feed and agrofiber industries (Gomes *et al*., 1992). Xylanases are of great interest to the pulp and paper industry due to their bleach boosting properties helping to replace the use of damaging bleaching agent. A bleach booster is an enzyme facilitating lignin to be removed but unharmful to the paper pulp. The most advantageous working conditions for a bleach booster are high temperature and alkaline conditions. Therefore, an ideal xylanase should be able to function under the extreme conditions (Nissen *et al*., 1992).

The present study describes the purification of three forms of extracellular xylanases produced by the thermophilic fungus *Aspergillus versicolor*. These three forms of xylanases are purified and enzymatic characterizations are studied.

MATERIALS AND METHODS

Aspergillus versicolor was selected according to the temperature tolerance (thermophilic fungus) and alkalinity (alkalophilic) to extract thermostable alkaline xylanase enzyme from sugar cane bagasse.

Irach wood xylan, protein standard, sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), N, N, N`, N`-tetramethyl ethyen-di-amine (TMED), bromophenol blue, dithioeryhrital (DTE) and other various substrates were obtained from Sigma Chemical Co. St. Louis, Mo, USA.

Sephadex and biogel chromatographic materials were purchased from Pharmacia (UK) and Bio-Rad (USA), respectively. All other reagents used were of analytical grade.

Organism and growth conditions: The microorganism recorded in this study (*A. versicolor*) were isolated from sugar cane bagasse, collected after soaking in water 3 days and then fermentation was carried out by spraying with water and ammonia-solution to prepare a humid and alkaline media. The highest value of total xylanases

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isolated from *A. versicolor* was carried out on its optimized medium which consists of:- shaken culture containing xylan 1%; NH_4Cl_2 3 g L^{-1}; Na_2SO_4 1 g L^{-1}, K^+ ion 10 μg metal ion L^{-1}; pectin 10 g L^{-1}. KH_2PO_4 1 g L^{-1}, pH was adjusted at 10 at 50°C for 10 days incubation.

Preparation of extracellular crude enzyme: Solid ammonium sulphate was added in small amounts to the clear filtrate to bring salt concentration at 85%. The mixture was allowed to stand in cold (5°C) for 48 h and the precipitate was collected by centrifugation at 5,000 rpm for 20 min. The precipitate was dissolved in 0.05M sodium citrate buffer (pH 6.2) and stirred for 30 min at room temperature. Any insoluble were then removed by centrifugation (Somkuti and Bable, 1968). Dialyzing the enzyme preparation overnight at 5°C using 0.05 sodium citrate buffer of pH 6.2 carried out elimination of excess salt.

Assay of xylanase enzyme: Xylanase was assayed as described by (Poutanen and Puls, 1988) using 0.01% birch wood xylan in 0.05 M sodium citrate buffer, pH 6.2 was used as substrate. Xylanase activity was measured by incubating 1 mL of an approximately diluted enzyme solution (1:250) with 1 mL of the substrate for 5 min at 50°C. The reaction was stopped by heating for 10 min at 100°C and then rapidly cooled (Ganju *et al*., 1989; Van Laere *et al*., 1999) and determining the reducing sugar released by the method Nelson (1944) and Smogyi (1952). A xylanase unit was defined as the release of one μmol of reducing sugar as a xylose equivalent/minute under the specified conditions.

Determination of protein: Protein was measured according to the method of Bradford (1976) using bovin serum albumin as the standard.

Xylanase purification: The crude extract of extracellular xylanase was prepared by filtering the broth through four layers of gauze and then through prechilled puchner. The solution was then rotated in refrigerated centrifuge at 20,000 rpm to remove the muddy matter. The protein content and the extracellular xylanase activity were assayed for this crude extract preparation.

Ammonium sulphate precipitation: Fungal crude extract was precipitated by 80% ammonium sulphate at 4°C. The precipitate formed was suspended in 0.05 M sodium citrate buffer (pH 6.2). Each precipiced was dissolved in 10 mL 0.05 M citrate buffer pH 6.2, placed in a dialysis bag and dialyzed against distilled water in a refrigerator for 48 h. The dialysis was repeated against sucrose for another 48 h.

The dialyzed enzyme sample was then incorporated into the DEAE-sephadex column (1.6×55 cm) equilibrated with 0.05 M sodium citrate buffer for pH 6.2. A constant feeding peristaltic pump was used. The exchanged material was eluted with a stepwise gradient of sodium chloride ranging from 0.0 to 0.2 M prepared in 0.05 M citrate buffer of pH 6.2 at a flow rate of 60 mL h^{-1} and 5 mL per fraction.

The eluted fractions were applied in small amount of citrated buffer at pH 6.2 to sephadex G-100 column (1.3×90 cm) and protein fractions exhibiting xylanase activity were eluted and designated XYI, XYII and XYIII.

Molecular weight determination: The molecular weight of the purified enzymes was determined by gel filtration on sephadex G-200 column. The calibration curve of sephadex G-200 gel between log M.Wt. of the standard proteins versus relative elution volume (Ve/Vo) was used. Also the molecular weight was determined by SDS-PAGE according to the method of Laemmli (1970).

Characterization and factors affecting the activity of purified enzymes: The effect of enzyme concentration, substrate concentration, effect of pH value, effect of reaction temperature were investigated. Also the kinetic of constant (K_m) and thermostability were determined for all purified isoenzymes.

Statistical analysis: The data generated in these studies were suitable for the least significance difference (LSD) by Duncan method (SAS Institute, 1982).

RESULTS

The results presented in Table 1 showed that 2.47 fold increase in specific activity was manifested for extracellular xylanase over the crude extract and the recovered activity was 73.30% of the crude extract. By DEAE-sephadex (Fig. 1), three isoenzymes of extracellular xylanase XYI, XYII and XYIII were resolved. Their specific activities were 79.69, 73.65 and 96.15 units mg^{-1} protein which represented 13.55, 12.54 and 16.35 fold purification over the crude extract with 11.57, 8.03 and 11.34% recovery, respectively. The lyophilized fraction from DEAE-Sephadex column was applied to Sephadex G-100 (1.3×90 cm), which was pretreated and equilibrated with 0.05 M citrate buffer of pH 6.2. The active fractions of the three peaks of extracellular xylanase were pooled. By sephadex G-100, their specific activities were 95.90, 83.33 and 106.7 units mg^{-1} protein, which represented 16.31, 14.17 and 18.15 fold purification over the crude extract with 10.09, 6.80 and 7.85% recovery, respectively.

Table 1: Purification scheme of alkalo-thermophilic *A. versicolor* extracellular xylanases

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (Units mg^{-1})	Purification fold	Recovery (%)
Crude extract	1869.00	11023	5.88	-	100.00
Ammonium sulfate and dialyses	557.10	8077	14.50	2.47	73.30
DEAE-Sephadex					
Peak A	16.00	1275	79.69	13.55	11.57
Peak B	12.00	885	73.75	12.54	8.03
Peak C	13.00	1250	96.15	16.35	11.34
Sephadex G-100					
Peak A	11.60	1113	95.90	16.31	10.09
Peak B	9.00	750	83.33	14.17	6.80
Peak C	8.11	865	106.70	18.15	7.85

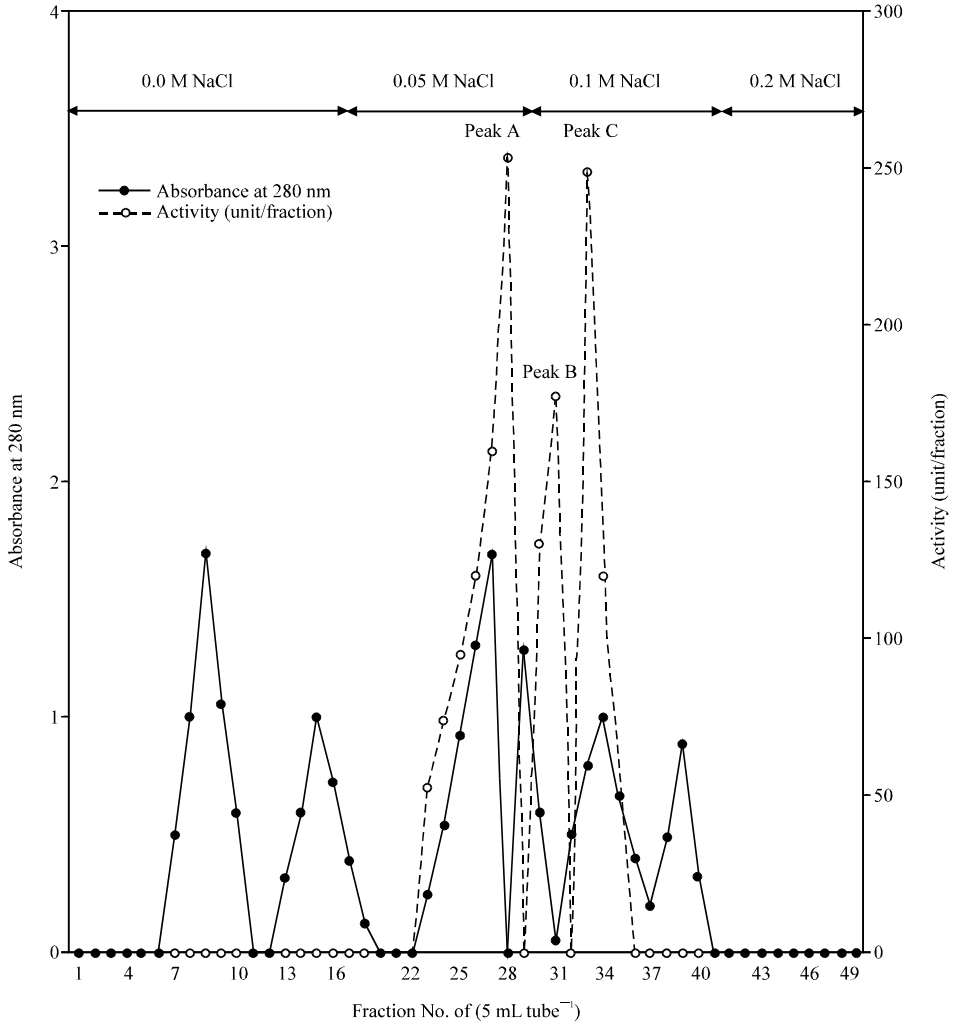

Fig. 1: Typical elution profile for the behavior of *A. versicolor* extracellular xylanase on DEAE-Sephadex column (1.6×55 cm) previously equilibrated with 0.05 M citrate buffer, pH 6.2 at a flow rate of 60 mL h^{-1}

Estimation of molecular weight: The three isoenzymes of extracellular xylanses were dialyzed against excess distilled water for 24 h at 4°C and the molecular weight was determined by gel filtration on Sephadex G-200 column (85×2.5 cm) equilibrated with 0.05 M sodium citrate buffer of pH 6.2 and by electrophoresis. The protein was excluded from the column at a rate of 30 mL h^{-1}. The voide volume (Vo) was determined using blue dextran.

The plot of relative elution volumes (Ve/Vo) versus logarithms of known molecular weights of standard proteins (phosphorylase B, 97.40 kDa; bovine serum

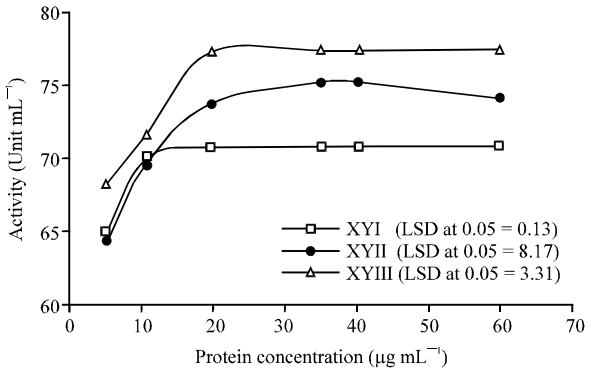

Fig. 2: Effect of xylanase protein concentration on the activity of the purified *A. Versicolor* extracellular xylanases XYI, XYII and XYIII

albumin, 67.00 kDa, soybean trypsin inhibitors, 21.05 kDa and lysozyme, 14.40 kDa) has been done. The molecular weight of *A. versicolor* extraceullar xylanases (XYI, XYII and XYIII), were determined to be 42.0, 40.0 and 32.0 kDa.

The determined molecular weight by gel filtration through Sephadex G-200 was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of standard protein (Serum albumin, 66.40 kDa, glutamin dehydrogenase, 55.60 kDa, maltose-binding protein MBP2, 42.70 kDa and lactate dehydrogenase, 36.50 kDa). The estimated molecular weight of *A. versicolor* extracellular iso-enzymes XYI, XYII and XYIII were 42.70, 40.50 and 31.60 kDa, respectively.

Characterization of pure extracellular xylanases: The active fractions of extracellular xylanases from sephadex G-100 were dialyzed, pooled, lyophilised and used for the following experiments.

Effect of enzyme concentration: Figure 2 show that there is a direct relation between the activity of extracellular xylanases and the quantity of protein.

Effect of substrate concentration: A hyperbolic relationship between the activities of purified extracellular xylanases and xylan concentration was obtained up to 5.80 mg mL^{-1}, above this concentration, the isoenzymes showed non significant changes as shown in Fig. 3.

According to Woolf plot, the K_m value of pure extracellular xylanases was found to be 0.66, 0.50 and 0.22, respectively as in Fig. 4.

pH optima: The pH profile for purified *A. versicolor* xylanases were assayed at different pHs from 3-11.9 by using different buffer solutions. Figure 5 reveals that the extracellular xylanases XYI, XYII and XYIII reach to its maximal value (40.50, 41.98 and 37.90 unit mL^{-1}) at pH (7, 10 and 10, respectively), inhibition inactivity was obtained at pH 3 and 11.9.

Table 2: Thermostability of the purified *A. versicolor* extracellular xylanases after 30 min incubation periods. Residual activity was determined under standard assay conditions

	Loss of activity (%)		
	Extracellular xylanases		
Temperature (°C)	XYI	XYII	XYIII
30	81.0d	4.0a	78.0d
40	59.0c	00.0a	67.0d
50	52.0c	70.0b	44.0c
60	39.0b	78.0bc	29.0b
70	00.0a	87.0cd	00.0a
80	81.0d	100.0d	90.0e
90	100.0e	100.0d	100.0e
Statistical analyses (LSD at 0.05)	8.1	16.2	11.1

Means followed by the same letter(s) are non significant

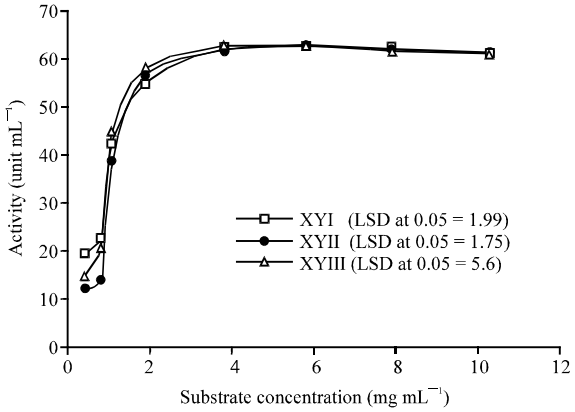

Fig. 3: Effect of xylanase protein concentration on the activity of the purified *A. versicolor* extracellular xylanases XYI, XYII and XYIII

Effect of reaction temperature: The results in Fig. 6 showed that the optimum temperature of extra cellular XYI and XYIII was at 70°C the maximum activities were 58.9 and 78.7 unit mL^{-1}, respectively, while XYII gave its maximum activity (81.5 unit mL^{-1}) at 50°C.

Thermostability of purified *A. versicolor* xylanases can be illustrated in Table 2. Extracellular xylanase XYII lost 100% of its activity after 30 min exposure to 80°C. The results also show that the purified XYI and XYIII lost about 80.5 and 90.3%, respectively and complete inactivation of all enzymes was attained at 90°C.

Effect of metal ions: Addition of Ba^{2+} and Pb^{2+} ions at concentrations of 10^{-2} and 10^{-3} M revealed an

Table 3: Effect of metal ions on the activity of the purified xylanase enzymes

	Extracellular xylanases (unit mL^{-1})					
	XYI		XYII		XYIII	
Metals	10^{-2} M	10^{-3} M	10^{-2} M	10^{-3} M	10^{-2} M	10^{-3} M
Ni^{2+}	44.1e	43.1d	40.70d	38.9d	40.8de	38.4de
Zn^{2+}	37.5d	37.0d	40.30d	35.3d	39.1de	35.1de
Ba^{2+}	163.1a	160.4a	167.10a	160.3a	149.9a	141.1a
Cd^{2+}	40.8d	40.1d	41.03d	38.1d	36.4e	33.1e
Sr^{2+}	42.6d	40.9d	36.20d	32.9d	42.9d	38.9de
Li^{2+}	39.0d	37.1d	157.40b	151.3b	139.3b	130.2b
Pb^{2+}	151.9b	141.4b	39.90d	35.8d	44.6d	41.3d
K^{+} (control)	118.8c	118.6c	118.80c	118.6c	118.8c	118.6c
Statistical analyses (LSD)	0.01 = 10.5	0.01 = 14.2	0.01 = 8.9	0.01 = 11.8	0.01 = 7.6	0.01 = 10.2
	0.05 = 7.6	0.05 = 10.3	0.05 = 6.5	0.05 = 8.6	0.05 = 5.5	0.05 = 7.5

Means followed by the same letter(s) are non significant

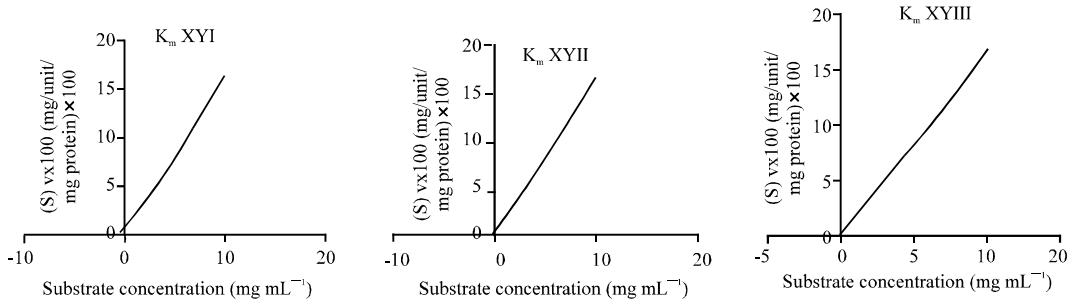

Fig. 4: Determination of kinetic constant (K_m) of the purified *A. versicolor* extracellular xylanases (XYI, XYII and XYIII) by woolf plot

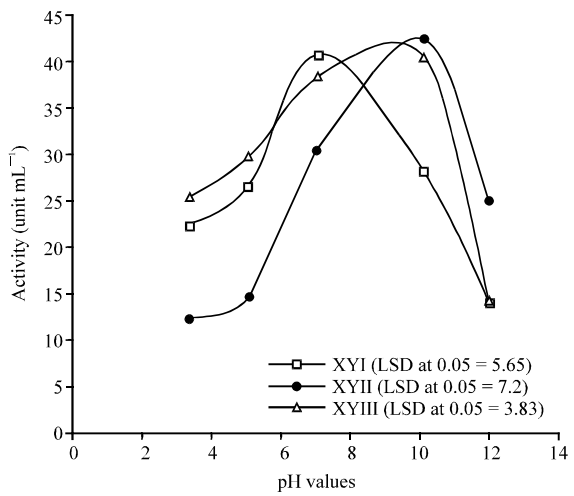

Fig. 5: Effect of initial pH values of the reaction mixture on the activity of the purified *A. versicolor* extracellular xylanases (XYI, XYII and XYIII). Three different buffer systems were used, citrate phosphate buffer solution for pH (3-7); carbonate-bicarbonate buffer solution of pH 10 and glycin NaCl-NaOH buffer solution for pH (11.9)

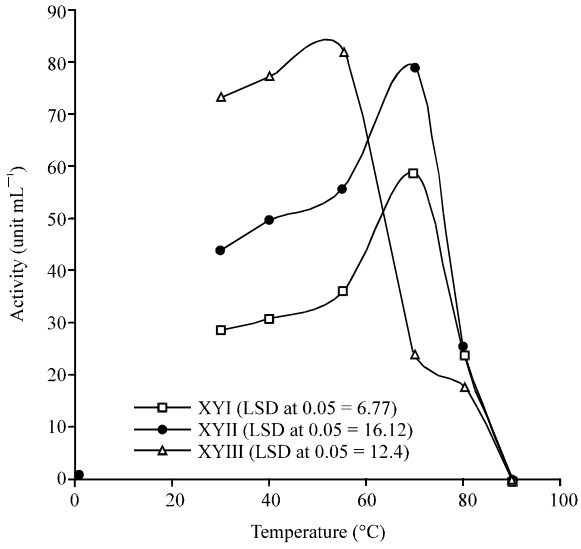

Fig. 6: Effect of reaction temperature on the activity of the purified *A. versicolor* extracellular xylanases (XYI, XYII and XYIII) after 5 min incubation time

accelerating effect on extracellular xylanase XYI as compared with control (K^{+}), while the inhibition was detected with Ni^{2+}, Sr^{2+}, Cd^{2+}, Li^{2+} and Zn^{2+} at 10^{-2} and 10^{-3} M. Activation of extracellular xylanases XYII and XYIII was shown by both Ba^{2+} and Li^{2+} at 10^{-2} and 10^{-3} M, however the inhibition of both isoenzymes was detected by Cd^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+} and Sr^{2+} at 10^{-2} and 10^{-3} M Table 3.

DISCUSSION

The last two decades have expressed an active scientific and technological development of research on utilization of waste materials as a renewable base.

In this study, a pure preparation of extracellular xylanases has been obtained by the thermo-alkalophilic A. versicolor grown on xylan medium composed of: 1% Xylan, 0.3% ammonium chloride, 0.1% sodium sulphate, 10 μg metal ion L^{-1} K^{+} and 10 g L^{-1} pectin at optimum pH and temperature (10 and 50°C, respectively) for 10 days in shaking incubator were used. The increased specific activity to 2.47 fold over the crude extract by ammonium sulphate is presumably by removal of low molecular weight inhibitors, possibly phenolic substances released from the growth substances.

Purification of extracellular xylanase on DEAE-Sephadex reveals that the organism secretes more than one form of xylanase into the medium, which led to the separation of the three-xylanase activities. Such three fractions containing xylanase XYI, XYII and XYIII were with 13.55, 12.54 and 16.35 fold increase over the crude extract, respectively. Each of the concentrated protein fraction containing xylanase XYI, XYII and XYIII was desalted and chromatographed on Sephadex G-100 column resulting in an increase of 16.31, 14.17 and 18.15 fold over the crude extract, respectively. This increase in purification fold may indicated a high xylanases content in the original extract relative to other proteinaceous compounds. This may be expected because the selected species have high xylanolytic activity.

Multiple forms of xylanases have been reported in various mesophilic fungi (John and Schmidt, 1988; Wood and McCrae, 1986; Wong *et al*., 1986; Frederick *et al*., 1985), from thermophilic fungus (Anand *et al*., 1990; Ari and Kamalam, 1985) and from *Susarium oxysporum* (Christakopoulos *et al*., 1996).

Estimation of the apparent molecular weights were carried out in the present work for extracellular xylanases XYI, XYII and XYIII (42, 40 and 32 kDa, respectively), by using gel filtration and 42.7, 40.5, 31.6 for XYI, XYII, YIII, respectively by using SDS-PAGE methods.

The extracellular xylanase enzyme from thermophilic fungus HG-1 had a molecular weight of 33 kDa by SDS-PAGE and 31 kDa by gel filtration (Ishihara *et al*., 1997). Two xylanases (24 and 34 kDa) were produced by *Coprinus psychromorbidus* (LRS 067) from straw and wood (Inglis *et ai*., 2000).

Alkali-tolerant *Aspergillus fischeri* Fxn1 produced two extracellular xylanases and the major xylanase molecular weight was 31 kDa (Raj and Chandra, 1996). Also, Acidic xylanase enzyme XYL1 from *Fusarium oxysporum* had molecular weight of 40 kDa upon SDS PAGE (Ruiz *et al*., 1997). Rodrigues *et al*. (1999) reported that the molecular weight of xylanase removed from crude extract of the fungus *Penicillium janthinellum* was 20.1 kDa.

Blanco *et al*. (1997) isolated two forms of the extracellular xylanase from the thermophilic actinomycete *Thermomonospora alba*, the molecular weight of 48 and 38 kDa, were produced respectively that differed in their cellulose-binding ability.

Characterization of the purified extracellular xylenases in the present study revealed that, with respect to pH profiles, the optimum pH for XYII and XYIII was 10 and for XYI was at pH 7.

Decline in xylanases activities with the rest of pH could result from the formation of improper ionic forms of substrate and enzyme or from inactivation of the enzyme or from a combination of these effects.

Three major xylanases XylA, XylB and XylC from *Aspergillus kawachii* have pH optima of 6.7, 4.4 and 3.5. Other properties were studied and these three xylanases were found greatly different in their properties (Ito *et al*., 1992).

The pH extracellular xylanase from thermophilic fungus HG-1 isolated from a compost heap was stable in the range from 2 to 12 at 30°C (Ishihara *et al*., 1997).

The optimum pH of the purified extracellular xylanase from alkali-tolerant *Aspergillus fischeri* Fxn1 was 6.0. pH stability ranged from 5 to 9.5 (Raj and Chandra, 1996).

The present experimental data revealed that, the optimum reaction temperature of *A. versicolor* extracellular xylanases was 70°C for XYI and XYIII while at 50°C for XYII. At 80°C the enzyme activity was reduced by about 80.5, 100 and 90.3% for XYI, XYII and XYIII, respectively. This result suggests that thermostability of extraxylanases may due to the presence of disulfide bonds, which leads to more heat stability.

The temperature optima for xylanase I and II isolated from thermophilic fungus *Chaetomium thermophile* were 70 and 60°C, where Xylanase I lost 50% of its activity in 60 and 20 min, whereas xylanase II lost 50% of its activity in only 30 and 8 min, respectively (Ganju *et al*., 1989).

Ghareib and Nour-El-Dein (1992) showed that heating of the xylanase enzyme preparation purified from *Aspergillus terreus* THOM at 60°C for 1 h resulted in a 82.61% loss of activity. After exposing to 90°C for 10 min, xylanase retained 4.28% of its original activity.

The optimal conditions for activity of *Trichoderma* xylanases range from 45-65°C and from pH 3.5-6.5 as reported by (Wong and Maringer, 1999).

In the present study the Michaelis-Menten constant (K_m) for *A. veriscolor* extracellular XYI, XYII and XYIII

0.66, 0.50 and 0.22 mg mL^{-1}, respectively. In this respect, the K_m of xylanase from *Trichoderma koningii* was determined with the soluble fraction of oat spelt xylan as substrate, was 0.70 mg mL^{-1} (Huang *et al*., 1991). Okeke and Obi (1993) reported that the K_m value for oat spelts xylan was 7.7 mg mL^{-1} for prufied xylanase enzyme from *Arthrographis* sp. An extracellular xylanase from thermophilic fungus was purified and the K_m values for birchwood xylan and oat-spelt xylan were 8.3 and 20 mg mL^{-1}, respectively (Ishihara *et al*., 1997).

The purified extracellular xylanase from alkali-tolerant *Aspergillus fischeri* Fxn 1 had a K_m of 4.88 mg mL^{-1} (Raj and Chandra, 1996).

In the present experimental results, the influence of several metal ions on xylanases system revealed that Ba^{2+} and Pb^{2+} were stimulatory for XYI, while the inhibition was detected by Zn^{2+}, Li^{2+}, Cd^{2+}, Sr^{2+} and Ni^{2+}. The activation of XYII and XYIII, was shown by both Ba^{2+} and Li^{2+}, while the inhibition was detected by addition of Cd^{2+}, Zn^{2+}, Ni^{2+}, Sr^{2+} and Pb^{2+}. Non competitive inhibition by metal ions may be by reaction with a functional group such as hydroxyl group or with a metal atom in a prosethetic group in the active site of xylanase enzymes.

Ishihara *et al*. (1997) found that extracellular xylanase enzyme activity was strongly inhibited by SDS and partially by Hg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+} and iodoacetic acid. Significant inhibition of the xylanase enzyme purified from *Arthrographis* sp. strain was observed with Mn^{2+}, Hg^{2+}, Cu^{2+} or Ag^{+} but not with Ba^{2+}, Ca^{2+} or Co^{2+} (Okeke and Obi, 1993).

Alkali-tolerant *Aspergillus fischeri* Fxn1 produced two extracellular xylanases. The activity was inhibited (95%) by $AlCl_3$ (10 mM). This enzyme appears to be novel and will be useful for studies on the mechanism of hydrolysis of xylan by xylanolytic enzymes (Raj and Chandra, 1996).

In conclusion, the present study indicate that the low molecular weight isoenzyme XYIII (31.6 k.Da.) produced from the alkalo-thermophilic *Aspergillus versicolor* is the most preferable isoenzyme due to its high pH and temperature optima (10 and 70°C) alkali-thermostable) accompanied with the highest affinity (K_m = 0.22 mg mL^{-1}) and specific activity (106.7). These results agree with Nissen *et al*. (1992), who stated that the most advantageous working conditions for application of xylanase in a bleach booster are high temperature and alkaline conditions. Xylanases are of great interest to the pulp and paper industry due to their bleach boosting properties helping to replace the use of damping bleaching agent and unharmful to the paper pulp. Therefore, an ideal xylanase should be able to function under the extreme conditions.

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