

Purification and characterization of thermoalkalophilic xylanase isolated from the *Enterobacter* sp MTCC 5112

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Abstract

Thermoalkalophilic *Enterobacter* sp MTCC 5112 was isolated from a sediment sample collected from the Mandovi estuary, west coast of India. This culture produced extracellular xylanase. The xylanase enzyme was isolated by ammonium sulfate (80 %) fractionation, and purified to homogeneity using size exclusion and ion exchange chromatography. The molecular mass of the xylanase was ~ 43 kDa. The optimal pH of the xylanase activity was 9, and at room temperature it showed 100% stability for 3 h at pH 7, 8 and 9. The optimal temperature for the enzyme activity was 100 °C at pH 9.0. At 80 °C and pH 9, 90 % of the enzyme activity was retained after 40 min. At 70 °C and 60 °C, the enzyme retained 64 % and 85 % of its activity after 18 h, respectively. While at 50 °C and pH 9 the enzyme remained stable for days. For xylan, the enzyme gave a K_m value of 3.3 mg/ml, and V_{max} value of 5000 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ when the reaction was carried out at 100 °C and pH 9. In the presence of metal ions such as Co^{+2} , Zn^{+2} , Fe^{+2} , Cu^{+2} , Mg^{+2} and Ca^{+2} the activity of the enzyme increased. Whereas, strong inhibition of the enzyme activity was observed in the presence of Hg^{+2} and EDTA. To the best of our knowledge this is the first report on the production of xylanase by this bacterium.

Keywords: *Enterobacter* sp; Xylanase enzyme; Thermophilic, Alkaliphilic; Purification

1. Introduction

Hemicelluloses are the most abundant heteropolymers in the nature. The two main types of hemicelluloses are xylan and glucomannan [38]. Xylan has a complex structure consisting of β -1,4 linked xylose residues in the backbone to which short side chains of o-acetyl, α -L arabinofuranosyl, D- α glucuronic and phenolic acid residues are attached [8]. A variety of microorganisms are reported to produce endoxylanases, that can degrade β -1,4 xylan in a random fashion, yielding a series of linear and branched oligosaccharide fragments [16,3]. These enzymes have been widely detected in fungi and bacteria [20,32]. From the biotechnological standpoint, xylanases have applications in baking, textile and paper and pulp treatment, production of ethanol, aroma, fruit juices and animal feed [2]. If xylanases have to be used for industrial applications then the enzyme need to be thermophilic and alkalophilic in nature. However, most of the xylanases known to date are optimally active at temperatures below 50 °C and are active in acidic or neutral pH [37,30,39]. Conversely, only a few xylanases are reported to be active and stable at alkaline pH and high temperature [27,10]. In view of this, there is a need to search for new sources of xylanases and their characterization, especially with extremophilic properties. In this study, we report on the isolation, purification and characterization of a thermophilic and alkalophilic xylanase from a newly isolated bacterium *Enterobacter sp.*

2. Materials and methods

2.1. Culture and growth conditions

A basal salt solution (BSS) supplemented with 0.5 % birchwood xylan was used to isolate xylan-utilizing bacteria from a sediment sample collected from the Mandovi estuary, west coast of India. Bacteria were grown on shaker incubator (100 rpm) at 50 °C for 48 h. The composition of the BSS medium (w/v) was as follows: NaCl, 30.00 g; KCL, 0.75 g; MgSO₄,7.00 g; NH₄Cl, 1.00 g; K₂HPO₄ (10%) 7.00 ml; KH₂PO₄(10%) 3.00 ml; Trace metal solution ,1.00ml; Distilled water 1000 ml; Trace metal solution has the following composition H₃B₀₃ , 2.85 g; MnCl₂ .7 H₂O, 1.80 g; FeS₀₄.7H₂O, 2.49 g; Na -

Tartarate, 1.77 g; CuCl₂, 0.03 g; ZnCl₂, 0.02 g; CoCl₂, 0.04 g; Na₂MoO₄.2H₂O, 0.02g ; Distilled water , 1000 ml. The pH of the medium was adjusted to 9.0 using 1N NaOH.

These isolates were screened for their ability to produce xylanase enzyme. One of the isolates that produced relatively good amounts of extracellular xylanase was selected for the present study. The isolate was purified and stored on ZoBell Marine Agar slants at 4 °C. The selected isolate was studied for morphological, physiological and biochemical characteristics following the standard procedures [34].

2.2. Growth and the production of enzyme

Pre-culture was grown as above and 1 ml was used to inoculate 100 ml of the BSS medium (pH 9) dispensed in 500 ml Ehrlenmeyer flasks. The flasks were incubated on a shaker incubator (100 rpm) maintained at 50 °C for 72 h. Culture samples were collected at 4 h intervals over the period of cultivation. The culture growth was monitored by measuring the turbidity at 540 nm. Immediately after collection the samples were centrifuged at 4 °C and 10, 000 g for 10 min. Supernatants were analysed for xylanase activity using the procedure described below.

2.3. Enzymatic assay

Xylanase activity was assayed using 1% birchwood xylan as the substrate. Xylan was dissolved in 50 mM Glycine-NaOH buffer (pH 9.0). The reaction mixture containing 10 µg of the enzyme and 2.5 mg of the substrate was incubated for 10 min at 100 °C in an incubator. The amount of the reducing sugars liberated was estimated following the 3, 5-dinitrosalicylic acid (DNS) method [23]. Xylose was used as a standard. The absorbance of reference sample (substrate solution incubated without enzyme and diluted enzyme solution in buffer) was deduced from the absorbance of the test sample. One unit of xylanase activity was expressed as µmol of reducing sugars (xylose equivalent) released in 1 min under the above conditions. Cellulase activity was assayed as above, using low-viscosity carboxymethylcellulose (1 %) in place of xylan.

2.4. Protein determination

Protein was estimated following the bicinchoninic acid method, and the bovine serum albumin was used as a standard [33]. The protein content of the chromatographic eluant was measured by monitoring the optical density at 280 nm.

2.5. Optimization of culture media for xylanase production

The concentrations of nutrients such as phosphate (0 to 3 mg/ml), nitrogen (0 to 10 mg/ml), yeast extract (0 to 3 mg/ml) and peptone (0 to 1.6 mg/ml) in the culture medium were varied to optimize xylanase production. The culture was grown for 48 h in the BSS medium supplemented with 0.5 % xylan wherein the concentration of one of the nutrients varied and xylanase activity was measured as above.

2.6. Preparation of enzyme

The culture was grown at 50 °C in a 5 l Ehrlenmeyer flask containing BSS medium (1L), pH 9 supplemented with 0.5% xylan as a source of carbon. The culture in the stationary growth phase (after 48 h) was centrifuged at 4 °C and 10,000 g for 10 min. The supernatant was collected and ultrafiltered using 10,000 MW cut off ultrafilter (Amicon, USA). To the ultrafiltered sample ammonium sulfate (80 %) was added to precipitate the enzyme. The sample was then kept at 4 °C for 12 h. The precipitate was recovered by centrifugation as above and the pellet obtained was dissolved in a minimum volume of 0.05 M Na-Phosphate buffer, pH 9, and dialysed against the same buffer.

2.7. Gel filtration chromatography

The crude enzyme extract was applied to a Sephadex G-200 column (65 x 1.5 cm). Elution of the enzyme was carried out with 0.05 M Na-Phosphate buffer (pH 9.0) at a flow rate of 2 ml/min. Each fraction was analysed for protein and xylanase activity. The active fractions were pooled and dialysed against the same buffer and concentrated using rotary vacuum evaporator at 40 °C.

2.8. Ion exchange chromatography

Concentrated enzyme (2 ml) was loaded onto a anion exchange DEAE Sepharose FF (Sigma-Aldrich Co., USA) column (15 mm diameter and height 100 mm). Equilibration and elution were performed first with 0.05 M Na-phosphate buffer to remove unbound proteins and then with a linear salt gradient from 0 to 3 M NaCl at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and analysed for xylanase activity and protein content. Active fractions were pooled and concentrated, and then purified using cation exchange CM Sepharose FF (Sigma-Aldrich Co., USA) column as above. The active fractions were pooled, and were used for the studies described below.

2.9. Kinetic determinations

In order to determine the K_m and V_{max} , 22 μ g of purified xylanase enzyme was added to the test tubes each with 250 μ l of 50 mM glycine-NaOH buffer, pH 9 containing various amounts of xylan (0.5 mg to 8.0 mg). The reaction mixture was incubated at 100 °C for 10 min and the enzyme activity was measured as above. The kinetic constants K_m and V_{max} were estimated following the method of Lineweaver and Burk [21].

2.10. Hydrolysis studies

To 50 ml of 1 % xylan prepared in 50 mM glycine-NaOH buffer, pH 9.0, xylanase enzyme (0.45 μ g, 90 U/ml) was added, and the mixture was incubated at 70 °C. Samples (5 ml) were removed periodically at 0 h, 3 h, 6 h and 12 h. The unused xylan was precipitated using isopropanol. The precipitated xylan was removed by centrifugation (10,000 g, 10 min) and the supernatant containing the hydrolysed products was collected. The supernatant was concentrated to ~ 500 μ l using rotary vacuum evaporator and made to a known volume. Hydrolysis products were detected by thin layer chromatography (TLC). Concentrated sample was applied onto TLC plates (0.25-mm layers of silica gel F 254, Merck, India). Separation of the components was achieved using acetone : *n*-butanol : H₂O (8:1:1 by vol) mixture as a solvent system. Compounds were detected by spraying with ethanol-sulphuric acid mixture followed by heating at 150 °C for 5 min. D-xylose was applied as standard.

2.11. *Molecular mass determination*

The molecular mass of the purified xylanase was estimated by SDS-PAGE electrophoresis. The SDS-PAGE (12%) was performed as described by Laemmli, [18] using medium range (14.3 to 97.4 kDa) molecular weight markers (Banglore Genei Pvt., India). Proteins were visualized by staining with Coomassie brilliant blue.

2.12. *Zymogram analysis*

Zymogram for xylanase was carried out using SDS-PAGE electrophoresis as described by Kubata *et al.* [17]. After electrophoresis, the gel with separated proteins was washed using 50 mM Glycine- NaOH buffer (pH 9.0). Washed gel was then laid over a pre-cast 0.5% (w/v) agar gel containing 0.5% (w/v) birchwood xylan in 50 mM glycine-NaOH buffer, pH 9.0. Agar plates were incubated for 10 min at room temperature. The polyacrylamide gel was removed while the agar gel was immersed in 5.0% (w/v) Congo red dye for 30 min and washed with 1.0% (w/v) NaCl to visualize zones of clearance corresponding to xylanase activity.

2.13. *Effect of temperature on xylanase activity and stability*

The optimal temperature for the purified xylanase (90 U/ml) was obtained by assaying the enzyme activity at different temperatures. The enzyme activity at temperatures from 50°C to 140 °C was determined by incubating the enzyme(10 µg; 90 U/ml) along with 250 µl (2.5 mg) of the substrate for 10 min at respective temperature using a incubator. The temperature effect on the activity of the enzyme was further reconfirmed using a water bath (50°C to 100 °C) and /or oil bath (> 100 °C). In order to assess the stability, the enzyme solution (5 ml) was incubated at 50 °C, 60 °C, and 70 °C for 48 h. Whereas, the enzyme was incubated at 80 °C, 90 °C and 100 °C for 40 min using incubators maintained at respective temperatures. Sub-Samples were removed at definite time intervals over the period of incubation. The residual enzyme activity was measured following the procedure described above.

2.14. Effect of pH on xylanase activity and stability

The relative xylanase activity using 1% (w/v) Birchwood xylan was determined at various pHs. The pH range used varied from 4 to 11. Three different buffers (0.05 M) were used. Citrate buffer was used for pH 4 to 6. Phosphate buffer was used for pH from 6 to 8 and glycine-NaOH buffer was used for pH from 8 to 11. To test the pH stability, the purified enzyme (90 U) was diluted 100 fold using respective buffers having pH ranging from 4 to 11 as described above and were incubated for 24 h at room temperature. The residual enzyme activity was estimated at 3 h intervals during the 24 h period of incubation.

2.15. Effect of metal ion concentration

Enzyme was incubated with 1 mM solution of Ca^{+2} , Mg^{+2} , Hg^{+2} , Fe^{+2} , Cu^{+2} , Mn^{+2} , Co^{+2} and Zn^{+2} and metal chelator such as EDTA for 1 h at room temperature (28 ± 2 °C). Residual activity was measured using enzyme assay described above.

2.16. Amino acid composition of xylanase enzyme

A known amount of the purified xylanase enzyme was taken in an ampoule to which 2 ml of 6N HCl was added. The ampoule was flushed with nitrogen gas before the ampoule was sealed. Hydrolysis of the samples was carried out at 110 °C for 24 h. The hydrolyzate was then neutralized with 6N NaOH and centrifuged. The supernatant was evaporated to dryness under vacuum. The residue was dissolved in 2ml of HPLC grade distilled water, and filtered through a 0.4 μm polycarbonate filter (Nuclepore, USA). A suitable aliquot was then taken for the analysis of amino acids. Na-phosphate buffer (0.05M) in place of enzyme was processed as above and used as blank.

Prior to HPLC analysis, pre-column reaction of amino acids with o-phthalaldehyde (OPA) and 2-mercaptoethanol at pH 9.5 was carried out to form fluorescent derivatives. The Shimadzu HPLC model 1 consisting of a degasser, auto injector, column oven and fluorescent detector was used. A reverse-phase Shim-Pack HRC-ODS column (4.6 mm ID, 15 cm, 5 μm) with a ODS guard column (4.6 mm ID, 4.5 cm, 10 μm) and a binary solvent system were used to separate amino acids. A binary

solvent system consisting of 0.05M sodium acetate and 3 % of tetrahydrofuran, pH 6.5 as solvent A and HPLC grade methanol as solvent B was used to separate amino acids. The best separation of amino acids was obtained with a flow rate of 1.5 ml min^{-1} using a 40 min gradient elution program beginning with 12 % and ending with 100 % solvent B, with a number of isocratic steps in the program.

Amino acids were fluorometrically detected at an excitation wavelength of 328 nm and an emission wavelength of 450 nm. The identification of individual amino acids in the samples was determined by comparing the retention times of peaks in the samples with those of the standard solutions. Concentrations of the amino acids were calculated from peak areas relative to a known internal standard and a standard calibration using data handling system available with the instrument. The derivatizing procedure used does not react with secondary amino acids like proline and hydroxyproline, so these were not quantified in the samples. The method showed a relative standard deviation of $< 10 \%$ for individual amino acids detected and quantified.

All the glassware used was cleaned by soaking in 10 % HCl for 24 h and rinsing with distilled water. Finally all the glassware was rinsed with methanol and dried in a hot air oven.

3. Results and discussion

3.1. Culture, growth pattern and xylanase production

The culture was isolated from a sediment sample collected from the Mandovi estuary, west coast of India. Culture showed good growth at $50 \text{ }^\circ\text{C}$ in BSS medium of pH 9 supplemented with 0.5 % birchwood xylan. Its ability to produce xylanase enzyme was further confirmed when it formed orange digestion halos on birchwood xylan plates when treated with Congo red and washed with 1M NaCl. The isolate is Gram-negative, short rods, facultative anaerobe, motile, oxidase negative and catalase positive. The isolate utilized glucose, lactose, xylose, mannitol, arabinose and fructose. However, it did not use sucrose and mannose. Isolate produced pink colonies when grown on MacConkey agar. It can hydrolyze starch and citrate but not gelatin (Table 1). On the basis of these characteristics, the culture was tentatively identified as *Enterobacter* sp

[34]. The culture was deposited with the Institute of Microbial Technology Chandigarh, India, and was assigned a culture collection number of MTCC 5112.

Table 1
Morphological, physiological and biochemical characteristics
of *Enterobacter* sp MTCC 5112

Tests	Results
Color	White
Shape	Rods
Gm stain	Gm -ve
Motility	+
Oxidase	-
Catalase	+
Hugh-Leifson's test	Facultative anaerobe
Spore staining	No spores
Gelatin hydrolysis	-
Starch hydrolysis	+
Nitrate reduction	+
MR	-
VP	+
Growth at 41 °C	+
Simmons Citrate	+
Arginine dehydrolase	+
Sugar fermentation:	
Glucose	A
Lactose	A
Xylose	A
Mannitol	A
Arabinose	A
Sucrose	-
Mannose	-
Fructose	A
Utilization of carbon source	
Glucose	+
Lactose	+
Xylose	+
Mannitol	+
Arabinose	+
Sucrose	-
Mannose	-
Fructose	+
Arginine hydrolase	+
Ornithine decarboxylase	-
Lysine decarboxylase	-
TSI	Acid/Acid
MacConkey Agar	Pink colonies
Genus Identified	<i>Enterobacter</i> sp

The time course of growth and the production of extracellular xylanase were studied using BSS medium (Fig. 1). Soon after inoculation the culture started growing and reached a highest biomass at 36 h (Fig. 1). After this the culture growth started decreasing until 56 h and did not change much thereafter. Conversely, the xylanase

production by the culture increased gradually and reached a highest value at 56 h of the incubation period and remained same thereafter.

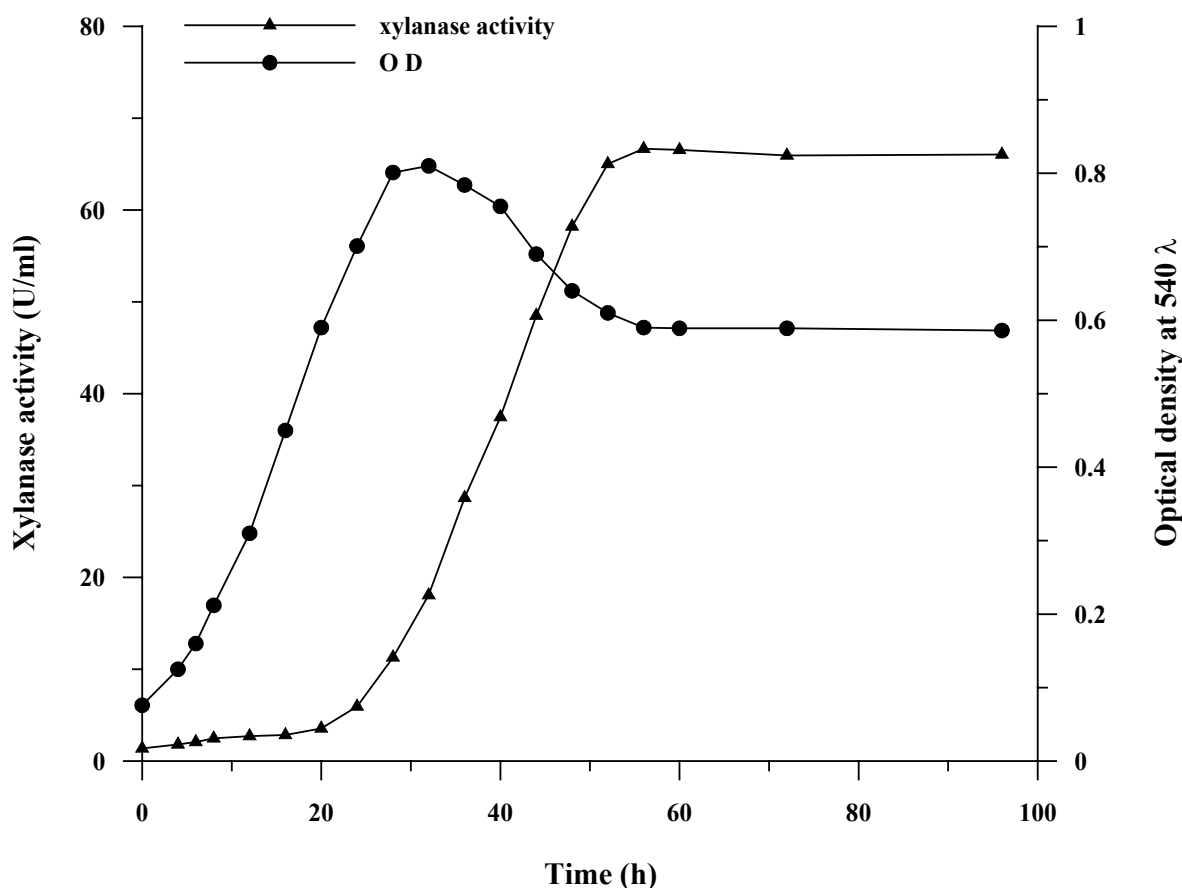


Fig.1. Time course of growth and enzyme production by *Enterobacter* sp. MTCC 5112 . Cells were grown at 50 °C in the BSS medium, pH 9 supplemented with birchwood xylan as a sole source of carbon (0.5 %). Growth was monitored by measuring optical density at 540 nm. Xylanase activity was assayed from the cell free supernatant as described above.

3.2. Optimization of growth medium for xylanase production

Nutrient concentrations in the growth medium influenced the production of xylanases by the bacterium. *Enterobacter* sp showed highest xylanase production when the growth medium was supplemented with peptone (0.45 mg/ml), yeast extract (2 mg/ml), ammonium chloride (1.25 mg/ml) and potassium phosphate (0.35 mg/ml) (Fig. 2a to 2d).

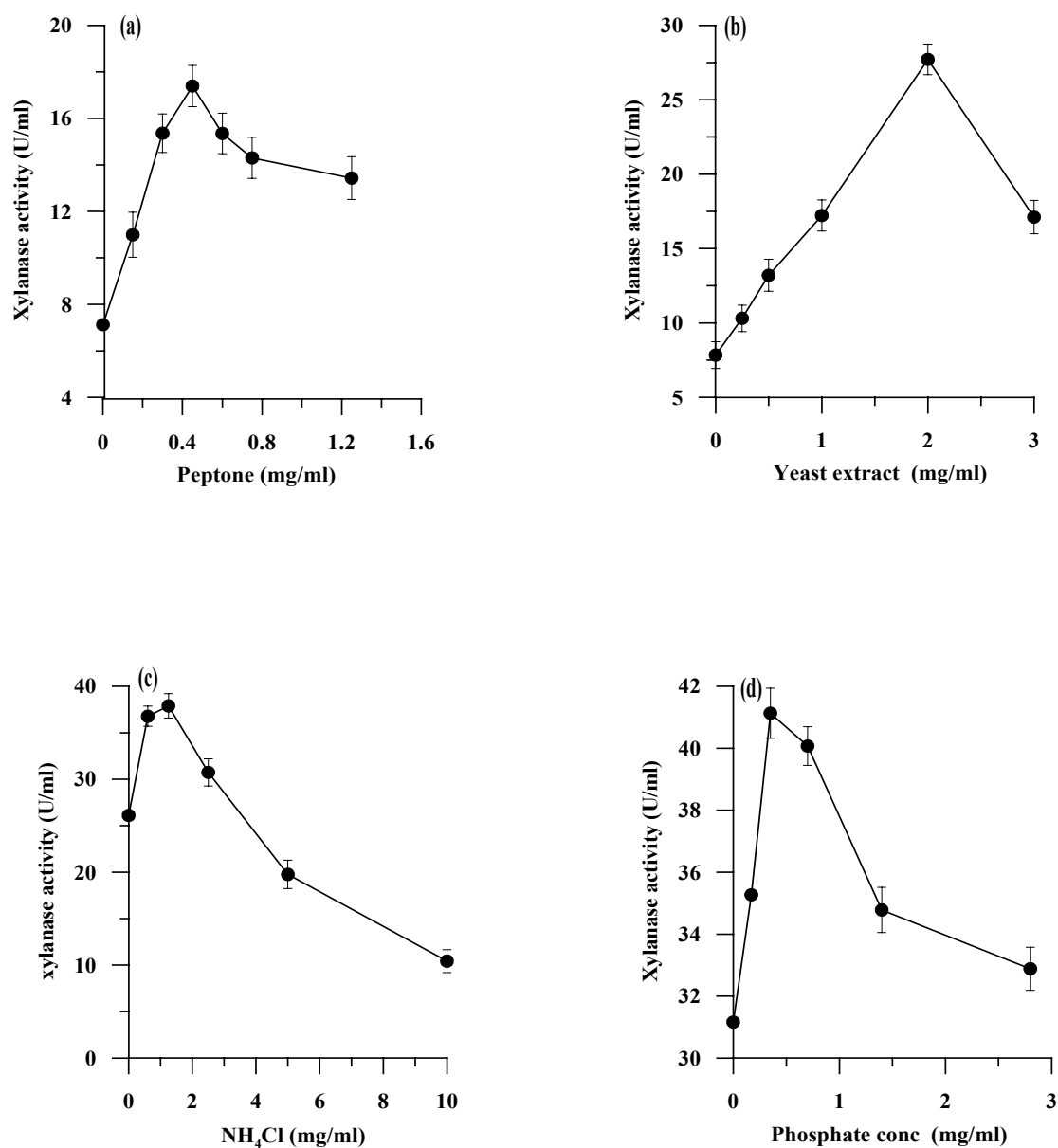


Fig.2. Effect of peptone (a), yeast extract (b), ammonium chloride(c) and phosphate(d) on the production of xylanase by *Enterobacter* sp. MTCC 5112.

3.3. Purification of xylanase

The enzyme isolation, purification and characterization were performed at room temperature (28 ± 2 °C). The extracellular xylanase was purified to homogeneity from the culture filtrates of *Enterobacter* sp grown on BSS medium. A summary of the purification steps is presented in Table 2.

Table 2

Purification steps of xylanase enzyme isolated from *Enterobacter* sp MTCC 5112

Purification steps	Xylanase activity (U)	Proteins (mg)	Specific activity (U/mg)	Purification fold	Yield
Filtrate	48360	1090	446	1	100
(NH ₄) ₂ SO ₄ Precipitation	31360	78	3982	9	64.8
Sephadex G-200	30170	23	13020	29	62.3
DEAE Sepharose FF	20900	8	26050	58	43.2
CM Sepharose FF	17170	3.8	44940	100	35.5

The xylanase enzyme was purified by a four-step chromatographic procedure. The enzyme was eluted with a blunt peak when passed through Sephadex G-200 column (Fig. 3a). The enzyme was further purified using ion exchange chromatography. Two peaks of proteins with xylanase activity could be separated using DEAE-Sepharose column (Fig. 3b). The first peak, representing the major portion of xylanase activity was further purified using CM-Sepharose column that resulted in only one protein peak with xylanase activity (Fig. 3c). The overall level of recovery was ~35% while 100-fold purification of xylanase was achieved with specific activity of 44,940 U/mg.

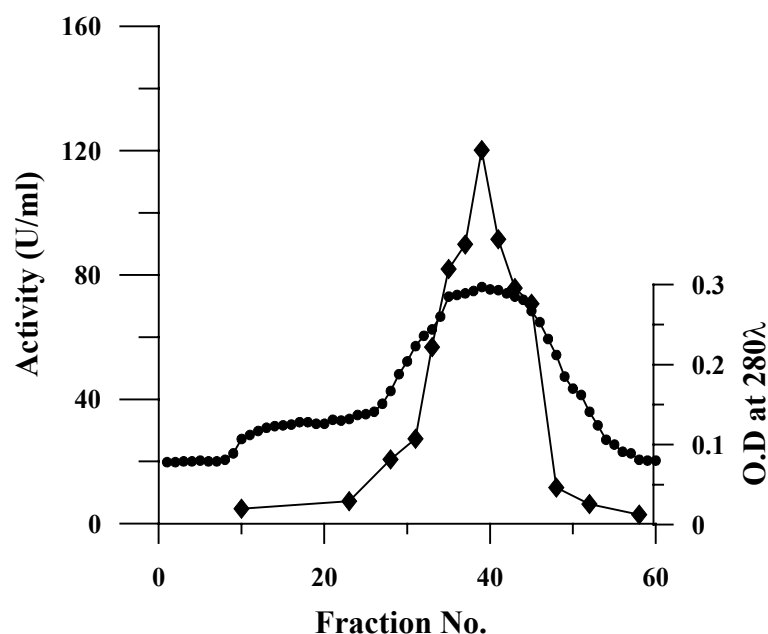


Fig. 3a. Elution profile of xylanase from *Enterobacter* sp on Sephadex G-200. (◆) Fractions containing xylanase activity. (■) Protein profile at 280 nm.

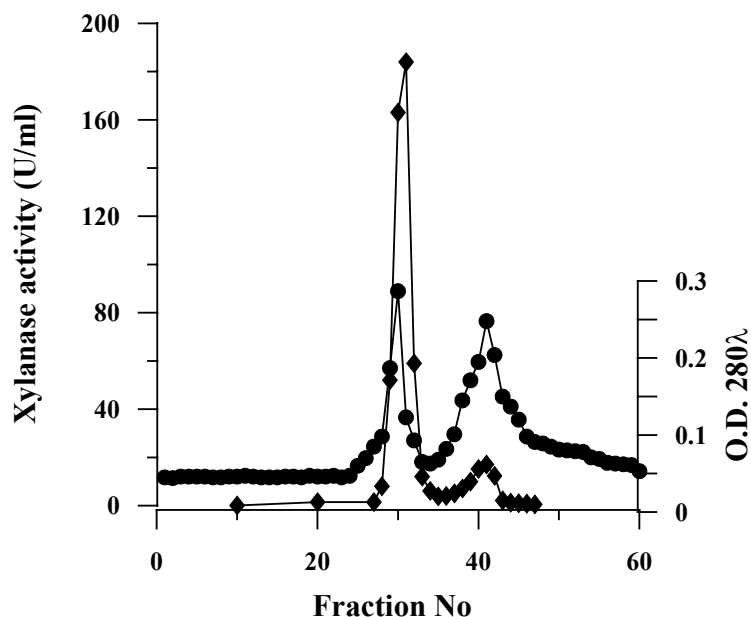


Fig. 3b. Elution profile of xylanase from *Enterobacter* sp on DEAE-Sepharose .
 (◆) Fractions containing xylanase activity. (●) Protein profile at 280 nm.

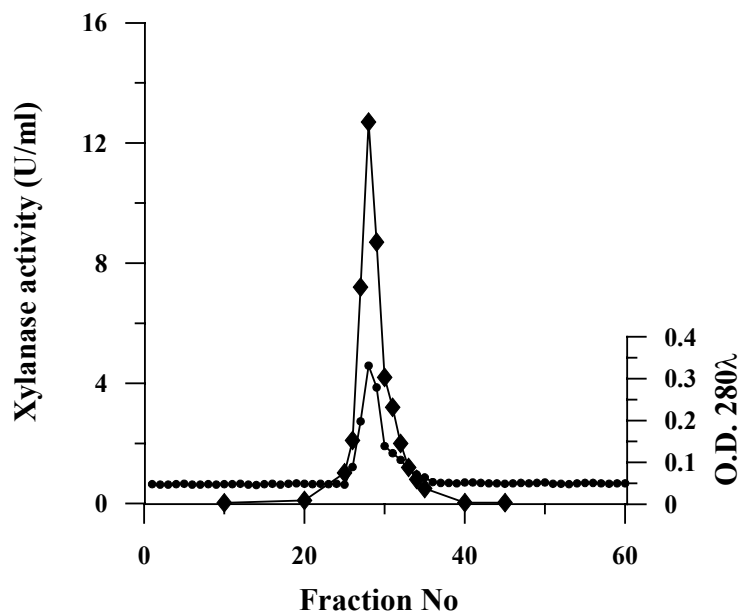


Fig. 3c. Elution profile of xylanase from *Enterobacter* sp on CM-Sepharose.
 (◆) Fractions containing xylanase activity. (●) Protein profile at 280 nm.

3.4. Molecular weight determination and Zymogram analysis

The purified enzyme showed a single protein band on SDS-PAGE (Fig. 4). The molecular mass of the denatured xylanase estimated from the relative mobility of the standard proteins on SDS-PAGE (Fig. 4) was ~ 43 kDa. Zymogram of the xylanase

enzyme showed a prominent activity band corresponding to ~ 43 kDa. Xylanase obtained from *Bacillus* sp *TAR-1* had a molecular weight of 40 kDa [24]. Whereas, a high molecular weight (340 kDa) xylanase was produced by *Bacillus subtilis* [31].

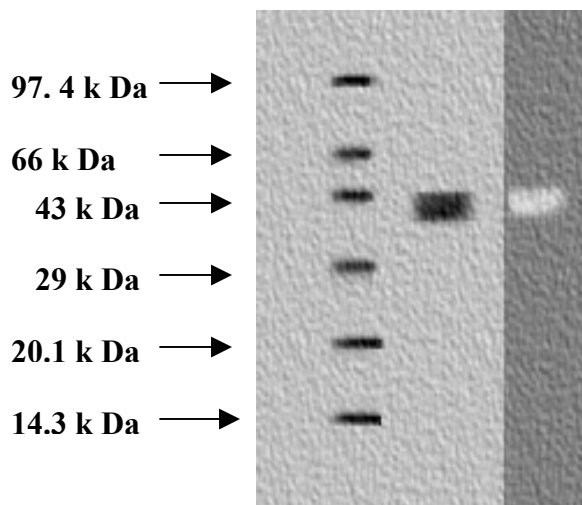


Fig.4. SDS-PAGE analysis of purified xylanase from *Enterobacter* sp MTCC 5112. Lane 1; Molecular markers, Lane 2; 10 µg of purified xylanase enzyme, Lane 3; zymogram of purified xylanase.

3.5. Kinetic parameters

The K_m value was obtained from Lineweaver-Burke plot of xylanase activity at 100 °C using various concentrations of birchwood xylan as a substrate. The K_m was 3.3 mg/ml, and the V_{max} was 5000 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (Figs. 5 & 6). The affinity of xylanase enzyme towards xylan was similar to the xylanases isolated from other bacteria such as *Bacillus* sp *NCIM 59* [9] and *Bacillus* sp *41M* [24]. Whereas, xylanase of *Enterobacter* sp showed better affinity for xylan than that reported for xylanases of some other bacteria. For example, xylanase isolated from *Staphylococcus* sp showed less affinity (K_m of 4 mg/ml) for xylan [12].

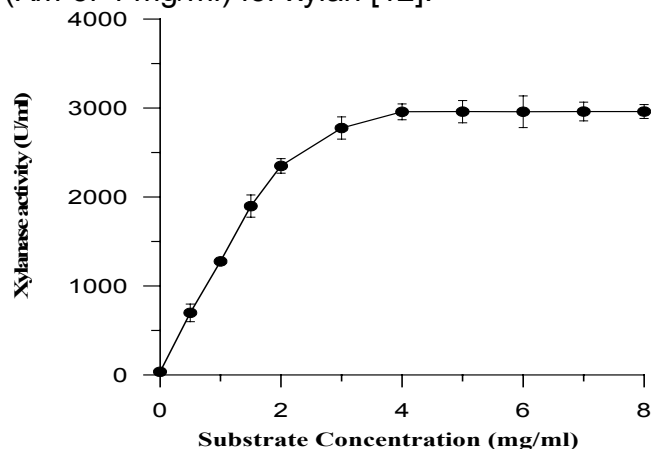


Fig.5. Effect of substrate concentration on the activity of the xylanase enzyme produced by *Enterobacter* sp MTCC 5112.

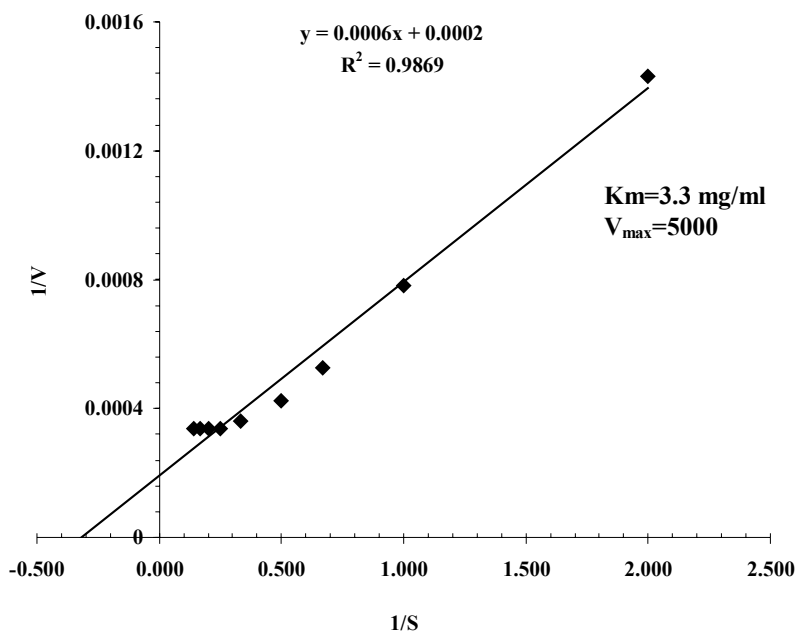


Fig.6. Double reciprocal plot for determining the V_{\max} and K_m values of xylanase against birchwood xylan at 100°C for xylanase enzyme of *Enterobacter* sp MTCC 5112.

3.6. Analysis of hydrolytic products

Hydrolysis of birchwood xylan was carried out at 70°C at different time intervals using pure xylanase as the enzymatic source (Fig.7). In the early stages of the reaction

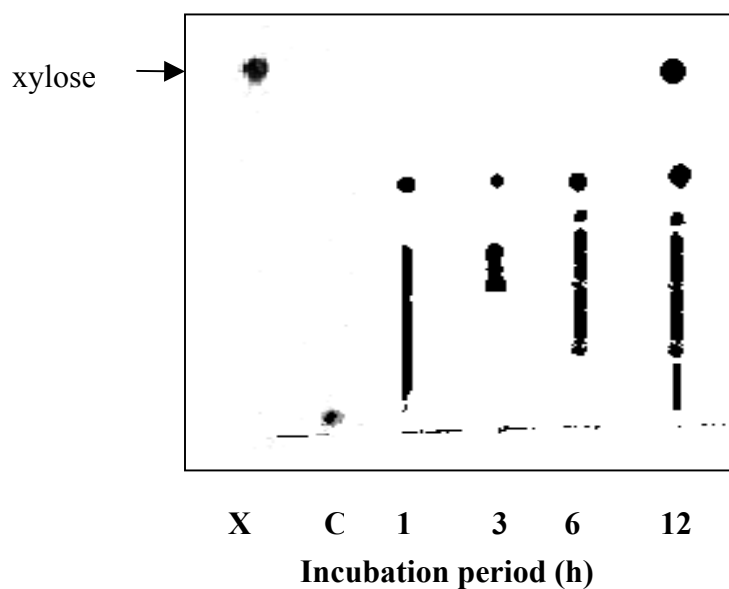


Fig.7. TLC analysis of the hydrolysis products released from birchwood xylan by xylanase enzyme from *Enterobacter* sp MTCC 5112. Position of standards (X), control (C) and time of incubation time (h) are indicated.

a large number of intermediate products were obtained but there was no trace of xylose until 6 h of the incubation period. However, at 12 h following incubation xylose was detected as one of the hydrolysis products. These results indicated that initially xylanase cleaved the substrate to liberate xylooligosaccharides and then the resulting oligosaccharides were probably cleaved to form xylose.

3.7. Effect of temperature on the activity and stability of xylanase

The optimal temperature of xylanase under standard assay condition was 100°C (Fig.8). At 100 °C the enzyme was stable for 10 min and thereafter the stability decreased with the increase in the incubation period and only 9 % of the activity was retained after 40 min of the incubation period. At 90°C 11 % and at 80 °C 90 % of the enzyme activity was retained after 40 min, respectively. At 70 °C and at 60 °C, enzyme was quite stable and retained 64 % and 85 % of its activity, respectively after 18 h. Whereas, the enzyme remained stable for several days at 50 °C (Fig. 9a&b). To the

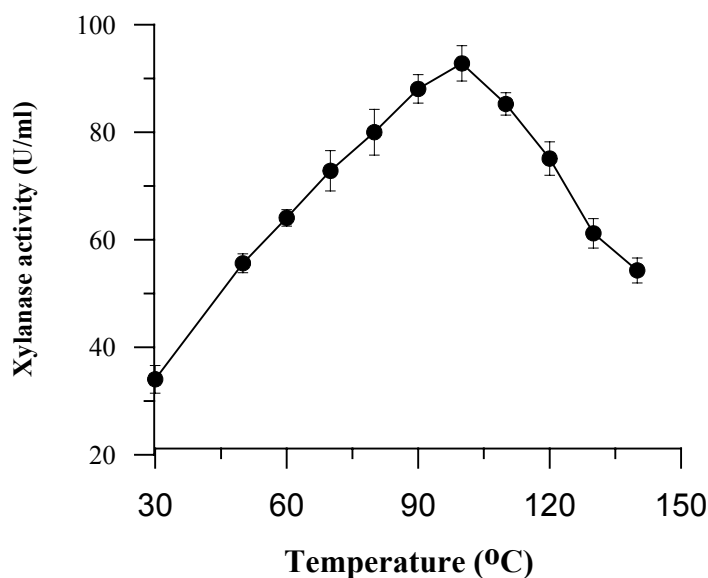


Fig.8. Effect of temperature on the activity of xylanase of *Enterobacter* sp. *Enterobacter* sp. was grown in BSS medium supplemented with xylan (pH 9.0) for 48 h at 50 °C. Xylanase activity in the culture filtrates of xylan medium was assayed at different temperatures and at pH 9 (50mM glycine-NaOH buffer).

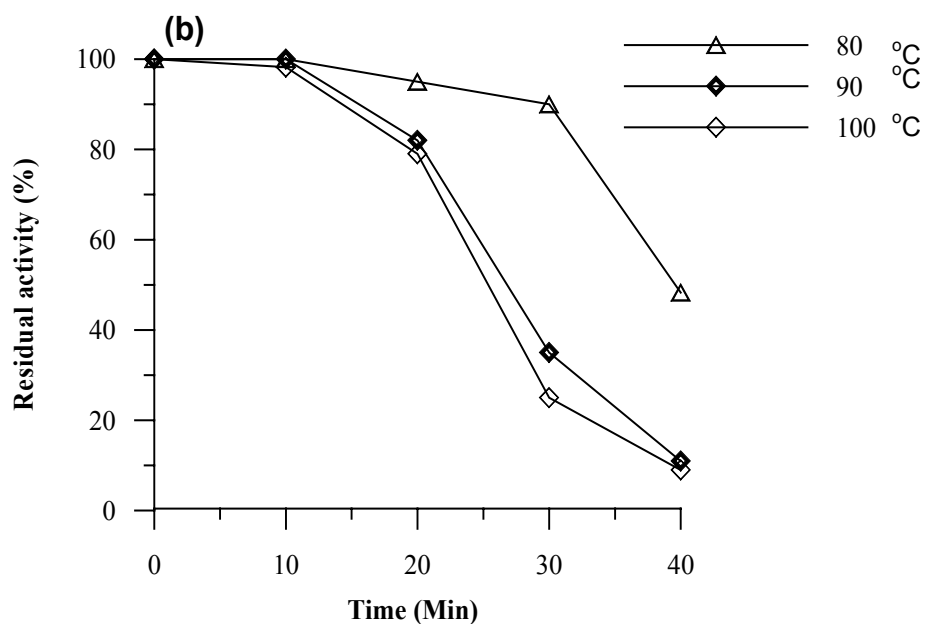
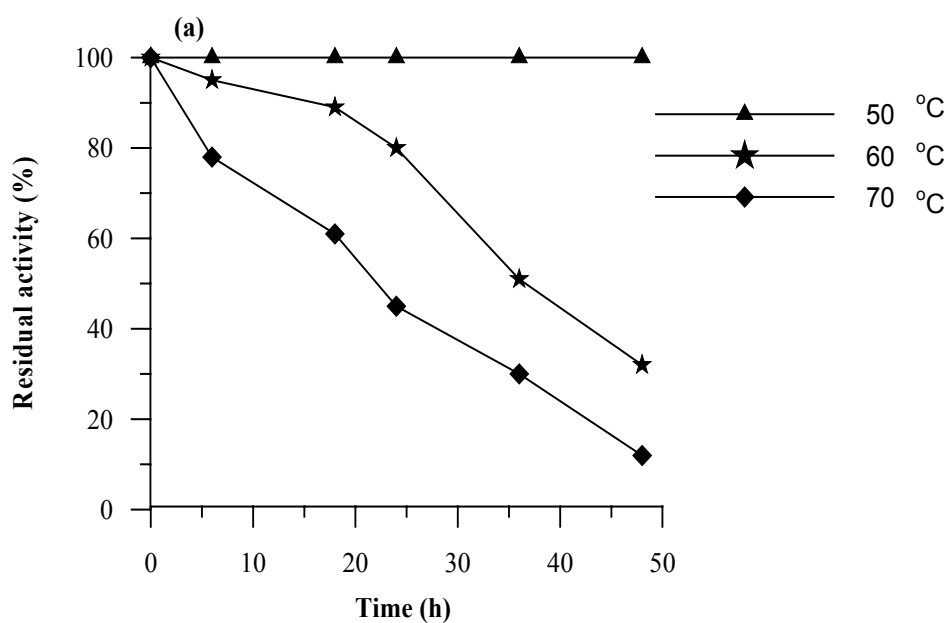


Fig.9. Thermal stability of *Enterobacter* sp xylanase. The pure enzyme was incubated in 0.05M Glycine-NaOH buffer (pH 9) at 50 °C (▲), 60 °C (★), and 70 °C (◆)(a) and at 80 °C (△), 90 °C (◆) and 100 °C (◇) (b) for different time intervals and residual activity was determined.

best of our knowledge there are no published reports on xylanase exhibiting optimal activity at 100 °C when the reaction buffer of pH 9.0 was used. However, Xyn A and Xyn B from *Thermotoga maritima* showed optimal temperature of 92 °C and 105 °C, respectively at pH 6 [40]. Similarly, xylanase isolated from *Thermotoga neapolitana* had optimal temperature of 102 °C at pH 5.5 [42]. Xylanase isolated from *Bacillus thermoantarcticus* [19] had a temperature optimum of 80 °C and the thermal stability of the enzyme was almost similar to that of *Enterobacter* sp used in the present study. However, *Bacillus thermoantarcticus* had a pH optimum of 5.6, while *Enterobacter* sp had pH optimum of 9. Xylanase obtained from *Bacillus circulans* AB 16 [10] also had temperature optimum of 80 °C but the enzyme was less stable as it retained 70 % of its activity at 65 °C after 2 hours. While xylanases reported by other workers showed highest activity at 60 °C when the pH was < 9.0 [14,35,26,29,24,41,28,12,31,30,36].

3.8. Effect of pH on the activity and stability

The optimal pH of the purified xylanase of the *Enterobacter* sp was 9 (Fig.10). Xylanases isolated from many other bacteria showed highest activity at or near neutral pH. For example, xylanases isolated from *Bacillus* sp WI-I, *Bacillus* sp WI-II, *Bacillus* sp W2I-I, *Bacillus* sp W2-II and *Bacillus* sp YC 335 all exhibit highest activity at pH 6.0 [25,26]. Similarly, xylanase produced by *Bacillus sterothermophilus* showed optimal activity at pH 6 to 7 [15], while xylanase of *Bacillus subtilis* had pH optimum of 5.6 [31]. Conversely, only a few xylanases isolated from *Bacillus* sp C-59-2 [14], *Bacillus* sp NCL 87-6-10 [35] and *Staphylococcus* sp [12] showed highest activity at pH 9.0.

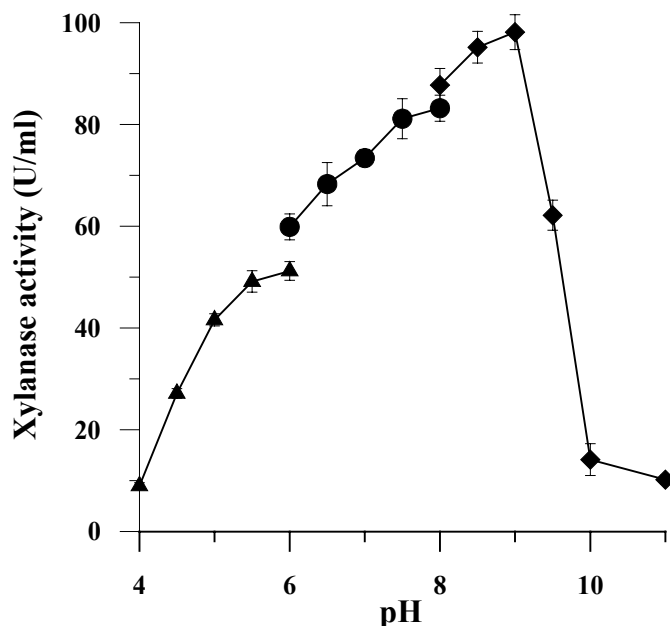


Fig.10. pH profile of *Enterobacter* sp MTCC 5112 xylanase. Enzyme activity was measured at 100 °C and different pH values. The buffers used, are : ▲ , citrate; ● , phosphate; ◆ ,glycine-NaOH.

Effect of pH on the stability of xylanase was studied at room temperature. Xylanase enzyme of *Enterobacter* sp showed good pH stability compared to other xylanases reported in the literature. It retained 100 % activity at pH 7, 8 and 9 for 3 h, while 70 % activity was retained after 12 h. The enzyme activity decreased greatly, and lost most of its activity in acidic pH range after 3 h. At pH 10 and 11 the enzyme retained ~ 58 % and 18 % of its activity, respectively after 12 h incubation (Fig. 11). At pH 9 xylanase isolated from *Bacillus* sp AR-009 [11] retained 54 % activity after 1 h which is very much less compared to our results. Whereas, xylanase from other *Bacillus* sp [7] showed 100 % stability at pH 7 but the enzyme was less stable at higher pHs and not at all active at pH 10.

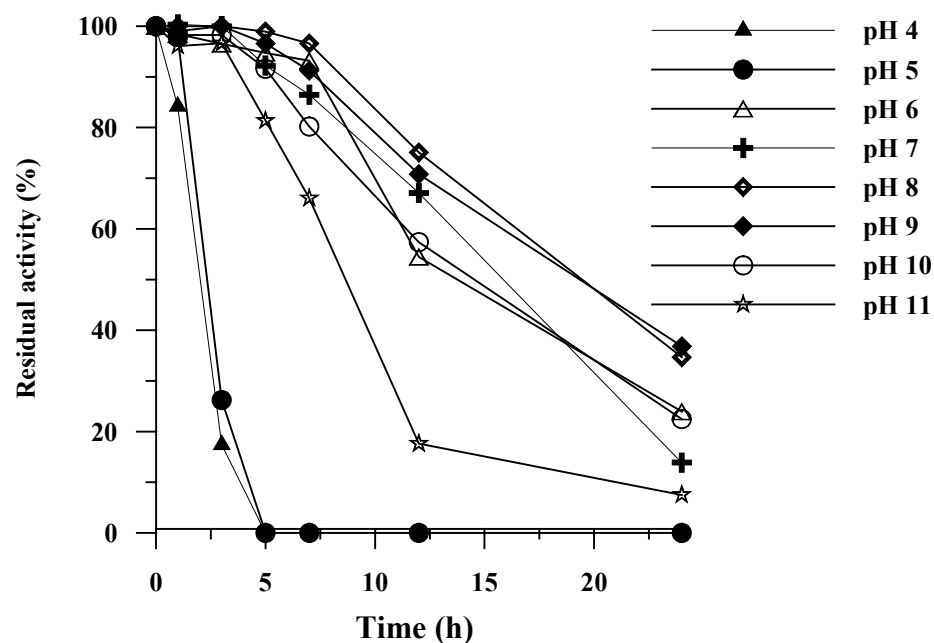


Fig.11. Effect of pH on xylanase stability. The enzyme was diluted with different buffers of varying pH values and incubated at room temperature over a period of 24 h. Residual activity was assayed at pH 9 and 100 °C. Buffers used were citrate, phosphate and glycine-NaOH

3.9. Effect of metal ions on the xylanase activity

Xylanase activity was assayed in the presence and absence of metal ions, and a metal chelator (EDTA). Both Hg^{+2} and EDTA inhibited the activity of the xylanase enzyme. The inhibition of the enzyme by Hg^{+2} ions may be due to its interaction with sulphhydryl groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme. Other workers also have reported similar results [1,6,7]. Nevertheless, cysteine was not detected in our enzyme. Therefore, it was not possible to identify the exact reason for the observed inhibitory effect of Hg^{+2} ion on the enzyme activity. However, our results are similar to those reported by others. For example, Nakamura *et al.* [24] did not detect the presence of cysteine in their enzyme but still observed strong inhibition of the enzyme activity in the presence of Hg^{+2} . The inhibition of xylanase activity in the presence of EDTA suggests that metals are needed for the

Table 3
Effect of metal ion on the activity of the xylanase enzyme produced by *Enterobacter* sp MTCC 5112

Metal ions (1 mM)	Residual activity (%)
None	100
Ca ⁺²	131
Mg ⁺²	156
Zn ⁺²	163
Co ⁺²	202
Fe ⁺³	142
Cu ⁺²	131
Mn ⁺²	107
Hg ⁺²	20
EDTA	11

enzymatic reaction. This was further confirmed by the observed increase in xylanase activity in the presence of Co⁺², Zn⁺², Fe⁺², Cu⁺², Mg⁺² and Ca⁺² (Table 3). It is not certain from these studies whether these ions/agents could be binding to the enzyme, causing conformational changes that resulted in increased enzyme activity, or the xylanase requires a metal ion on the active site. Further work using more inhibitors, their analogous and combinations thereof would be necessary to ascertain the mode of action of xylanase.

3.10. Amino acid composition

The amino acid composition of the xylanase from *Enterobacter* sp is presented in Table 4. Thermophiles and alkalophiles tend to have more salt bridges and slightly more hydrogen bonds than acidophilic and mesophilic xylanases. The bulky side chain of arginine offers a possibility for several hydrogen bonds and also for salt bridges. As a result increased abundance of arginine is generally found in the thermophilic xylanases as compared to xylanases of acidophiles and mesophiles. Amino acid composition of the xylanase from *Enterobacter* sp MTCC 5112 showed relatively higher abundance (as mole %) of arginine compared to that observed in xylanases from *Bacillus* sp strain BP-23 [4] and *Bacillus subtilis* [31] which are less thermostable. On the other hand, arginine content is similar to that found in the thermophilic xylanase produced by *B. amyloliquefacens* [5].

Thermophilic enzymes have relatively higher content of polar amino acids compared to acidophilic and mesophilic enzymes. The increase in fractional polar surface results in added hydrogen bonding to water that contributes to the greater stability of thermophilic enzyme [13]. Polar -amino acids (50.33 mole %) were relatively more abundant in the xylanase produced by *Enterobacter* sp MTCC 5112 suggesting their importance in enzyme stability.

Table 4

Amino acid composition (mol. %) of xylanase from *Enterobacter* sp MTCC 5112 and its comparison with other bacterial xylanases

Amino acid residue	<i>Bacillus</i> sp strain BP-23	<i>B. amyloliquefaciens</i>	<i>Bacillus subtilis</i>	<i>Enterobacter</i> sp MTCC 5112
Asx	14.3	13.8	NR	-
Asp	NR	NR	10.73	12.22
Thr	4.4	12.1	8.97	0.70
Ser	6.0	8.4	12.04	6.25
Glx	12.5	7.8	6.20	-
Gly	9.7	14.5	18.70	16.51
Ala	8.2	4.1	7.69	10.69
Cys	0	0.0	1.45	-
Val	4.9	8.6	4.80	7.02
Met	1.4	0.2	1.09	ND
Ile	5.3	3.8	3.18	5.11
Leu	6.5	3.4	3.48	6.00
Tyr	3.5	8.2	4.38	2.04
Phe	3.9	2.4	1.10	3.46
Lys	4.9	1.5	4.99	8.72
His	1.6	1.8	2.06	-
Arg	3.3	6.0	3.32	5.79
Pro	4.9	3.4	5.81	-
b-Ala	NR	NR	NR	0.03
g-ABA	NR	NR	NR	0.54
Orn	NR	NR	NR	1.30

NR= not reported ; - = not present

To the best of our knowledge, this is the first report on the production of xylanase by *Enterobacter* sp. Further the bacterium produced a novel thermophilic and alkalophilic xylanase. The thermophilic and alkalophilic enzyme isolated from the *Enterobacter* sp appears to have potential for industrial applications.

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