

**Isolation, purification and characterization of the
xylanase produced by *Arthrobacter* sp MTCC 5214
when grown in solid-state fermentation.**

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Abstract

Thermoalkalophilic *Arthrobacter* sp. produced extracellular xylanase, when wheat bran, rice husk, rice bran and bagassae were used as carbon source under solid state fermentation (SSF). 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 xylanase was ~ 20 kDa. Enzyme retained 100% activity at pH 7 and 8 for 24 hours. It was interesting to note that at higher pH such as 9, 10 and 11 the enzyme activity increased over the period of incubation. The optimum temperature for the enzyme activity was 100 °C at pH 9.0. At 80 °C and pH 9, half life of enzyme was 30 min. Half life of enzyme at 70 °C and at 60 °C was 18 h and 24 h respectively. While at 50 °C the enzyme retained 79 % of activity even after 48 hours. For xylan, the enzyme gave a K_m value of 0.9 mg/ml, and V_{max} value of 3571 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ 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} and metal chelator EDTA the activity of the enzyme increased. Whereas strong inhibition of the enzyme activity was observed in the presence of Hg^{+2} . These are some novel characteristics that make this enzyme potentially very effective for industrial applications.

Keywords: *Arthrobacter* sp; Wheat bran; Xylanase; Purification; Fermentation

1. Introduction

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall [1]. It is a heteropolymer with backbone of β -1,4-D-xylanopyranosyl residues and branches of neutral or uronic monosaccharides and oligosaccharides [2]. Xylanases (endo-1, 4-b-D-xylan xylanohydrolase; EC 3.2.1.8) degrade the xylan backbone into small oligomers. These enzymes are required for many applications such as bleaching of Kraft pulp, increasing the brightness of pulp, improving the digestibility of animal feed and for clarification of fruit juices etc. [3]. Cellulase free xylanases active at high temperature and pH are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds [4,5]. A treatment with xylanases can improve the chemical extraction of lignin from pulp [6]. This results in significant saving of chemicals required for bleaching thereby reducing the release of toxic chlorine compounds into the environment. However, the main problem faced by the pulp and paper industry while using enzyme treatment is the availability and cost of the enzyme. About 30-40% of the production cost of many industrial enzymes is accounted by the cost of growth substrate [7]. The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials as substrates [8].

The technique of solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water. These fermentation systems, which are closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites [9,10]. SSF offers distinct advantages over submerged fermentation including economy of the space, simplicity of the media, no complex machinery, equipments and control systems; greater compactness of the fermentation vessel owing to a lower water volume; greater product yields; reduced energy demand; lower capital and recurring expenditures in industry; easier scale up of processes; lesser volume of solvent needed for product

recovery; superior yields; absence of foam build-up; and easier control of contamination due to the low moisture level in the system [11,12,9,13,14,15]. In view of this, the main aim of the present study was to characterize alkalothermophilic xylanase produced by the selected culture when grown on SSF.

2. Materials and Methods

2.1. Culture and growth conditions

Enrichment technique with xylan as a sole source of carbon was used to isolate xylan utilizing bacteria. Using this technique bacterial cultures were isolated from a sediment sample collected from the Mandovi estuary, west coast of India. From these, one isolate which produced relatively more amounts of extracellular xylanases was selected for the present study [16]. The culture was purified, and was stored at 4 °C on ZoBell Marine Agar slants. Culture was studied for morphological, physiological and biochemical characteristics following the standard procedures [17].

The culture was grown at room temperature in 15 ml basal salt solution (BSS) containing 5 g of wheat bran as substrate (substrate to moisture ratio 1:3) for 7 days. The composition of the medium (w/v) was as follows: NaCl, 30.00 g; KCL, 0.75 g; MgSO₄, 7.00 g; NH₄Cl, 0.5 g; K₂HPO₄ (10%) 7.00 ml; KH₂PO₄(10%) 3.00 ml; Trace metal solution, 1.00ml; Distilled water 1000 ml; pH of the medium was adjusted to 9.0 using 1N NaOH. Trace metal solution has the following composition H₃B₃O₃ , 2.85 g; MnCl₂ .7 H₂O, 1.80 g; FeSO₄.7H₂O, 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.

2.2. Preparation of substrates

Wheat bran and rice husk were obtained from the local flour-mill while rice straw and bagassae were obtained from a local farmhouse in Goa. The substrates wheat bran, rice husk, rice straw, sugarcane bagassae were washed

2-3 times in distilled water, and then boiled with distilled water for 10-15 min. The water was then decanted and substrates were dried in an oven and powdered using mortar and pestle. The powder was then sieved using a 40 μ mesh and stored at -20 °C until used.

2.3. Effect of Carbon source on xylanase production

Effect of various carbon sources on the xylanase production was assessed by culturing the isolate in the MBSS medium (pH 9.0) at room temperature (28 ± 2 °C). Either of xylan, xylose, arabionse, glucose, sucrose, galactose, cellobiose and carboxymethylcellulose was used as carbon source (0.5 %) individually in liquid medium, while other carbon sources such as rice bran, rice husk, wheat bran, bagassae were used in the SSF culture medium. After 96 h of the culture growth xylanase activity was estimated.

2.4. Optimization of culture medium 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 SSF culture medium were varied to optimize xylanase production. This standardized medium was named as modified basal salt solution (MBSS) and used for the work described below. Xylanase activity was analysed after growing the culture for 4 days in the culture medium with wheat bran as a carbon source wherein the concentration of one of the nutrient was varied.

2.5. Production of enzyme

The culture was grown for 48 h in the MBSS as above this was used to inoculate six 100 ml flasks each containing 15 ml of MBSS medium and 5 g (substrate to moisture ratio 1:3) of either birchwood xylan, oat spelt xylan, rice husk, rice straw, wheat bran and bagassae. Culture was incubated at 28 ± 2 °C. Samples were removed at 12 h intervals over a period of 6 days. At each

sampling time 50 mg of spent solid substrate was removed under sterile condition and suspended in 10 ml of 50 mM glycine-NaOH buffer (pH 9), and vortexed thoroughly to extract the xylanase enzyme. The sample was centrifuged at 10,000 RPM for 10 min at 4 °C and the supernatant was used for the estimation of the xylanase enzyme activity as described below.

2.6. 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 µl of an appropriate dilution of the enzyme and 250 µl of the substrate, was incubated for 10 min in an incubator maintained at 100^o C. The amount of reducing sugars liberated was determined by using 3, 5-dinitrosalicylic acid method (DNS method) [18]. The absorbance of the reference samples (substrate solution incubated without enzyme and diluted enzyme solution in buffer) was deduced from the values of the test samples. 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.

2.7. Protein estimation

Protein was estimated by the method of bicinchoninic acid with bovine serum albumin as a standard [19]. The protein content of the chromatographic eluant was measured by monitoring the optical density at 280 nm.

2.8. Preparation of enzymes

Ehrlenmeyer flask (500ml) containing 10 g of wheat bran and 30 ml of MBSS, pH 9 was sterilized, cooled and inoculated with pre-grown culture of the isolate. The flasks were incubated at room temperature. The culture was harvested in the stationary growth phase i.e. after 4 days. The content of the

flask was suspended in 100 ml of 50 mM glycine-NaOH buffer (pH 9) vortexed thoroughly and centrifuged (10,000 RPM for 10 min, 4 °C). The enzyme was precipitated from the culture supernatant by adding ammonium sulfate to 80 % saturation. This was left overnight and the precipitate collected by centrifugation at 10,000 RPM for 10 min. The precipitate obtained was dissolved in phosphate buffer (50 mM, pH 8.0) and dialysed against the same buffer for 24 h. Dialysis was carried out using cellulose tubing (molecular weight cut off 13,000 kDa).

2.9. 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 xylanase activity. The active fractions were pooled and dialysed against the same buffer and concentrated using rotary vacuum evaporator at 40 °C.

2.10. 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) at a flow rate of 0.5 ml/min. 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. 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 used for the studies described below.

2.11. Kinetic determinations

Initial reaction rates of wheat bran hydrolysis were determined at different substrate concentrations ranging from 0.5 mg to 8 mg of wheat bran/ml of 50 mM

glycine-NaOH buffer, pH 9 at 100 °C. The kinetic constants K_m and V_{max} were estimated following the method of Lineweaver and Burk [20].

2.12. Hydrolysis studies

To 50 ml of wheat bran suspension (1% of wheat bran in 50 mM glycine-NaOH buffer pH 9.0), 40 µg of xylanase enzyme (1276 U/g) were added and incubated at 70 °C. Samples (5 ml) were removed at 0 h, 3 h, 6 h and 12 h. The unused polysaccharide from the sample was precipitated using isopropanol. The precipitate was removed by centrifugation and the supernatant containing the hydrolyzed products was collected. The supernatant was concentrated to ~ 500 µl using rotary vacuum evaporator and made to a known volume. Hydrolysis products were detected by thin layer chromatography (TLC). TLC (TLC plates, 0.25-mm layers of silica gel F 254, Merck, India) was performed using the mixture of acetone : *n*-butanol : H₂O (8:1:1 by vol) 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.

2.13. Molecular mass determination

The molecular mass of the purified xylanase was estimated by SDS-PAGE electrophoresis. SDS-PAGE (12%) was performed as described by [21] 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.

2.14. Zymogram analysis

Zymogram for xylanase was carried out using SDS-PAGE electrophoresis as described by Kubata et al. [22]. 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 50mM 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.

2.15. Effect of Temperature on xylanase activity and stability

The optimal temperature for the purified xylanase (240 U/ml) was obtained by assaying the enzyme activity at different temperatures (28 °C to 110 °C) using a incubator. At each temperature, the enzyme (10 µg; 240 U/ml) along with 250 µl (2.5 mg) of the substrate was incubated for 10 minutes. In order to assess the stability, the enzyme solution (5 ml) was incubated at 50 °C, 60 °C, and 70 °C for 48 h, and at 80 °C, 90 °C and 100 °C for 40 min using a incubator maintained at the respective temperature. Sub-Samples were removed at definite time intervals over the period of incubation. The residual enzyme activity was measured following the procedure described above.

2.16. 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 6 to 8 and glycine-NaOH buffer was used for pH from 8-11. To test the pH stability, the purified enzyme (240 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.

2.17. *Effect of Metal ion concentration*

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

2.18. *Amino acid composition of xylanase enzyme*

Purified xylanase enzyme was taken in the 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*-phthaldialdehyde (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

3.1 Culture and growth conditions

The culture was isolated locally from a sediment sample collected from the Mandovi estuary, west coast of India. Culture showed good growth at $28\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ in MBSS 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 positive, pluromorphic, facultative anaerobe, motile, oxidase positive, catalase positive and reduced nitrate to nitrite (Table 1).

3.2. Effect of carbon source on xylanase activity

Xylose induced the production of xylanase enzyme as compared to other sugars, but relatively less than that obtained with birchwood xylan. When the xylanase production by other agricultural residues was compared, it was seen that wheat bran gives better yield compared to others (Table 2).

3.3. Growth and the production of enzyme

The time course of growth and the production of extracellular xylanase by the culture were studied using different carbon sources such as wheat bran, rice husk, rice straw, bagassae, oat spelt xylan and birch wood xylan. The xylanase production and growth pattern by the culture for each carbon source differed. When grown on birch wood xylan the culture gave maximum xylanase activity in the early stationary growth phase (48h) of the bacterium. While in the case of other substrates, the maximum xylanase activity was detected in the late stationary phase (Fig. 1). Xylanase activity was maximum when the culture was grown on birch wood xylan followed by oat spelt xylan and wheat bran. Whereas, when grown on rice husk, rice straw and bagassae the xylanase production was relatively poor (Fig. 2).

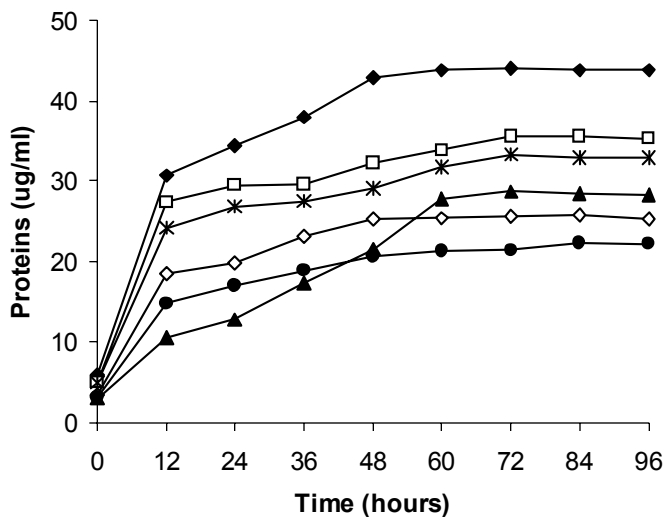


Fig. 1. Time course of growth profiles of *Arthrobacter* sp (measured as proteins) when grown in solid state fermentation using different substrates: (◆) Birch wood xylan ; (□) Oat spelt xylan; (△) Rice husk; (*)Wheat bran; (◇)Rice straw ;(●) Bagassae.

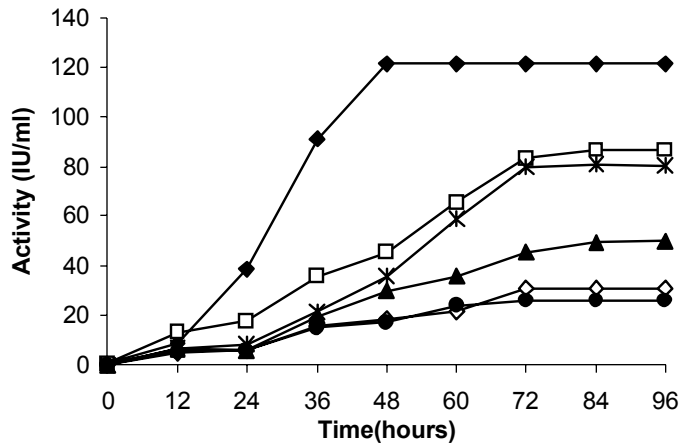


Fig. 2. Time course profiles of enzyme production by *Arthrobacter* sp. in solid state fermentation using different substrates: (◆) Birch wood xylan ; (□) Oat spelt xylan; (△) Rice husk; (*)Wheat bran; (◇)Rice straw ;(●) Bagassae, with wt./vol. ratio of 1:3. Xylanase activity was assayed from the cell free supernatant fluid at 100 °C

3.4. Optimization of growth medium for xylanase production

The culture grew well on wheat bran, rice husk, rice bran and bagassae. It also produced substantial amounts of xylanase while using these substrates. However, when grown on wheat bran the production of the enzyme was highest. Therefore, further studies were done using this carbon source. Nutrient concentration in the growth medium influenced the production of xylanase by the bacterium. The culture showed highest xylanase production when the growth medium was supplemented with peptone (0.3 mg/ml), yeast extract (2 mg/ml), ammonium chloride (1.25 mg/ml) and potassium phosphate (0.35 mg/ml) (Fig. 3a to 3d).

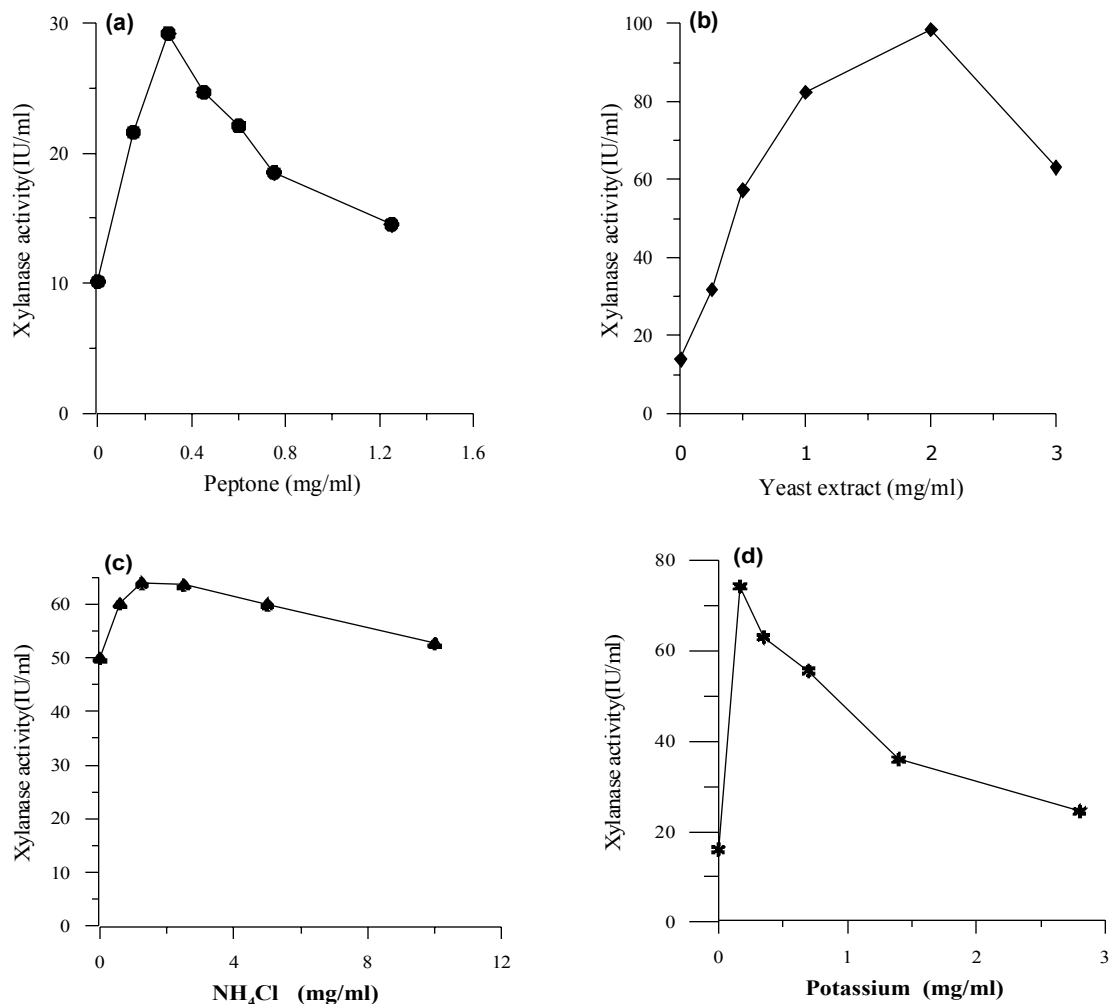


Fig. 3. Effect of peptone (a), yeast extract (b), ammonium chloride(c) and phosphate(d) in the culture medium on xylanase production by *Arthrobacter* sp.

3.5. Purification of xylanase

The enzyme was isolated, purified and characterized at room temperature (28 ± 2 °C). The extracellular xylanase was purified to homogeneity from the culture filtrate when grown on wheat bran. A summary of purification procedures is presented in Table 3.

Xylanase enzyme produced by the culture when grown on wheat bran was purified by a four-step chromatographic procedure. The enzyme was eluted as a blunt peak when passed through Sephadex G-200 size exclusion

chromatographic column (Fig. 4a). The enzyme was further loaded onto the ion exchange columns. One Major peak and three minor protein peaks were seen. Of these, one major protein peak and one minor peak showed xylanase activity. The major protein peak was then separated using DEAE-Sepharose chromatography (Fig. 4b). Here two peaks were observed. The first peak, representing the major portion of xylanase activity was further purified on cation exchange chromatography. The elution profile on CM-Sepharose resulted in only one pool of xylanase (Fig. 4c). The overall level of recovery was 14% while 21-fold purification of xylanase was achieved with specific activity of 1697 IU/gm.

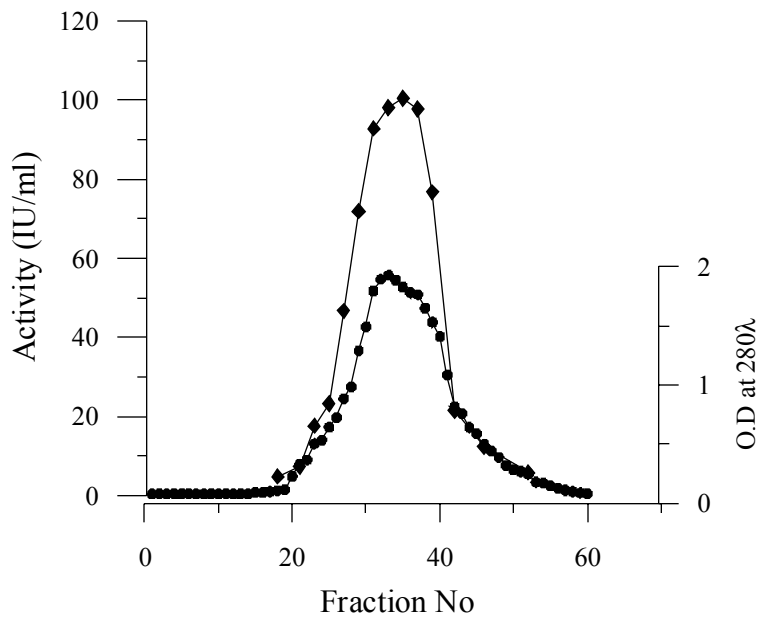


Fig. 4a. Elution profile of xylanase on Sephadex G-200. (◆) Fractions containing xylanase activity. (●) Protein profile at 280 nm.

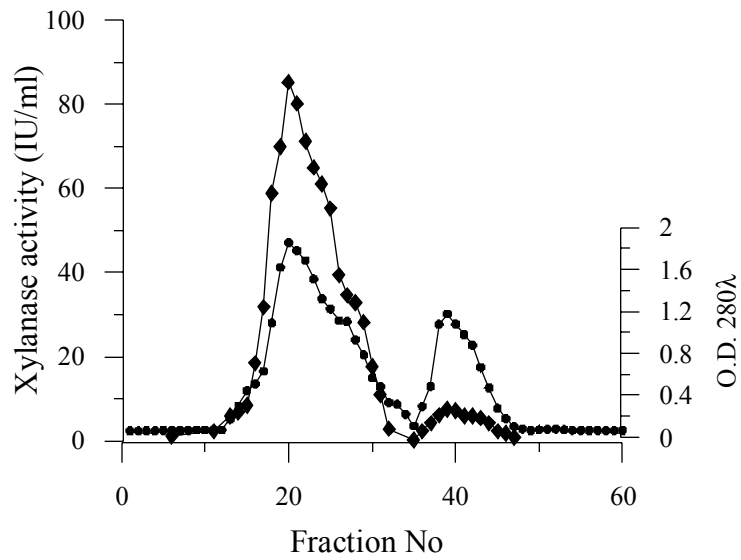


Fig. 4b. Elution profile of xylanase on DEAE-Sepharose (◆) Fractions containing xylanase activity. (■) Protein profile at 280 nm.

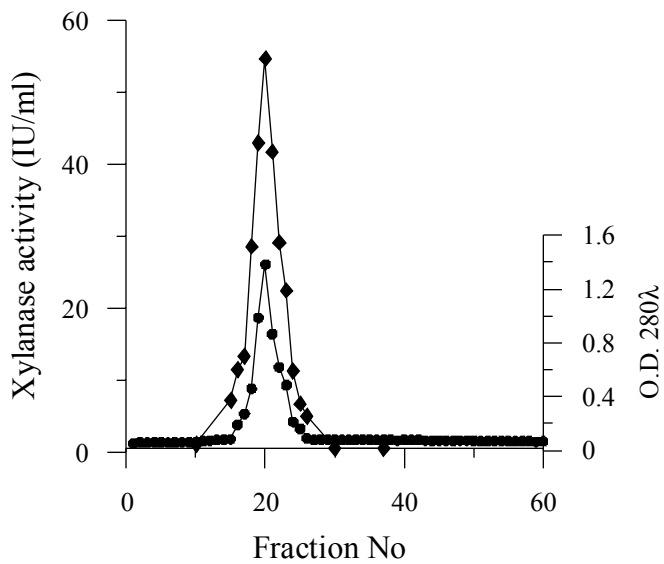


Fig. 4c. Elution profile of xylanase on CM-Sepharose. (◆) Fractions containing xylanase activity. (■) Protein profile at 280 nm.

3.6. Molecular weight determination and zymogram analysis

The purified enzyme showed a single protein band on SDS-PAGE. The molecular mass of denatured xylanase, estimated from the relative mobility of proteins on SDS-PAGE (Fig. 5) was ~ 20 kDa. Zymogram of xylanase enzyme

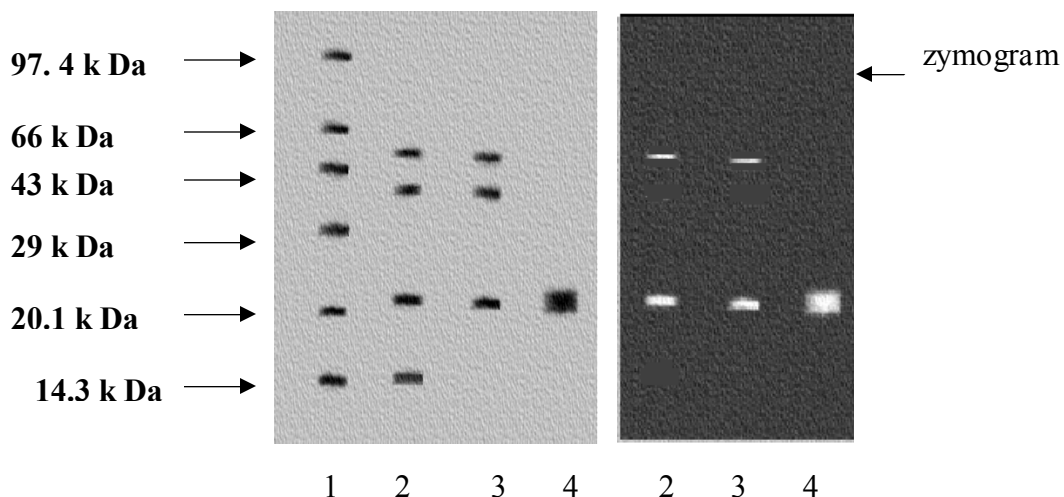


Fig. 5. SDS-PAGE analysis of purified xylanases from *Arthrobacter* sp. Lane 1; Molecular markers, Lane 2; Crude enzyme, Lane 3; After column chromatography, Lane 4; 10 µg of purified xylanase enzyme

showed a prominent activity band corresponding to ~ 20 kDa in lane 4 where purified enzyme was applied, while at lane 2 and lane 3 two bands were visible in which one corresponding to pure enzyme was more prominent (Fig.5).

3.7. Kinetic parameters

The K_m value was obtained from Lineweaver-Burke plot of xylanase activity at 100 °C using various concentrations of xylan as substrate. The K_m of xylanase for wheat bran was 0.9 mg/ml, and the V_{max} was 3571 µmol min⁻¹ mg⁻¹ (Figs. 6 & 7).

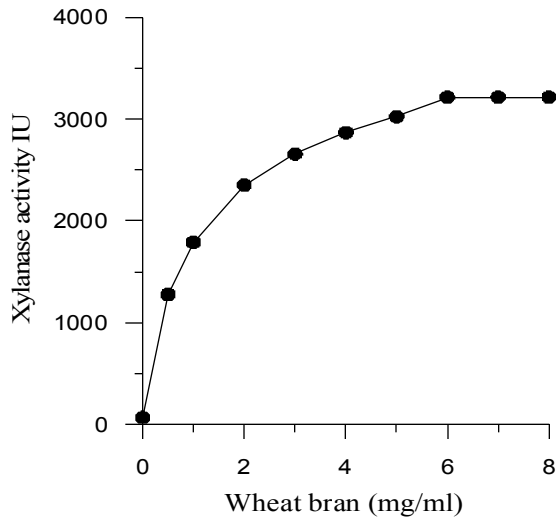


Fig. 6. Effect of substrate concentration (wheat bran) on the activity of the xylanase enzyme produced by *Arthrobacter* sp from wheat bran.

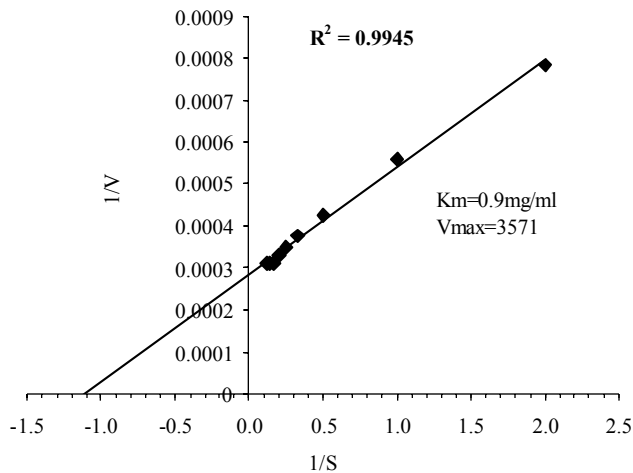


Fig. 7. Double reciprocal plot for determining the V_{max} and K_m values of xylanase against birchwood xylan at 100 °C for xylanase enzyme of *Arthrobacter* sp from wheat bran.

3.8. Analysis of hydrolytic products

Hydrolysis of wheat bran was carried out using pure enzyme at 70 °C at different time intervals (Fig. 8). In the early stages of the reaction some intermediate products were obtained. At 6 h following incubation xylose was detected. At 12 h following incubation xylose was relatively more abundant compared to other hydrolyzed products.

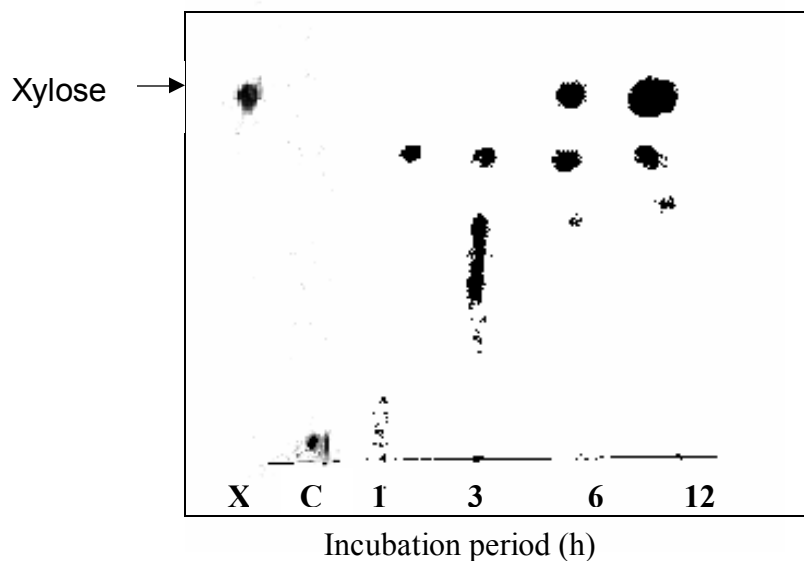


Fig. 8. TLC analysis of the hydrolysis products released from birchwood xylan by xylanase enzyme from *Arthrobacter* sp. MTCC 5214. Position of standards (X) and time of incubation time (h) are indicated.

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

The optimum temperature of xylanase enzyme was 100 °C (Fig. 9). It

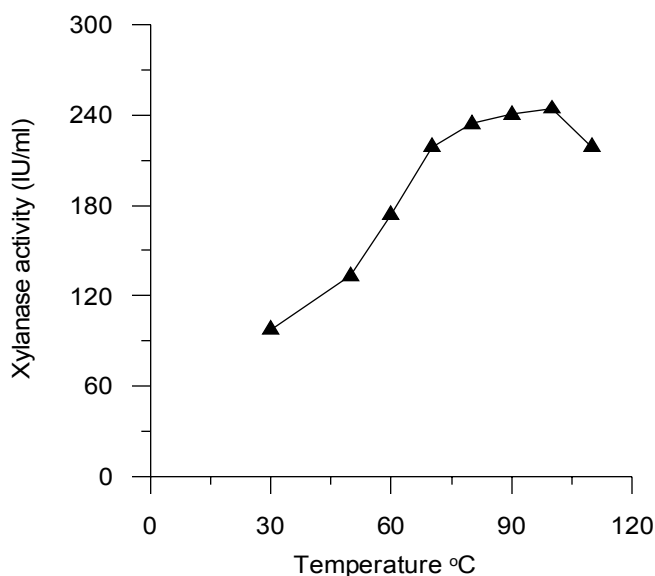


Fig. 9. Effect of temperature on the activity of xylanase of *Arthrobacter* sp. grown in BSS medium supplemented with wheat bran (pH 9.0) for 96 h at room temp. Xylanase activity in the culture filtrates of xylan medium was assayed at different temperatures and at pH 9 (50mM glycine-NaOH buffer).

was seen that at 100 °C, 75% of enzyme activity was retained after 5 min. At 90 °C 50% of enzyme activity was retained after 9 min, while at 80 °C, 50% of the

enzyme activity retained after 30 min. At 70 °C and at 60 °C 50% activity retained after 18 h and 24 h, respectively. While at 50 °C, the enzyme retained 79 % of its activity even after 48 hours (Fig. 10a & b).

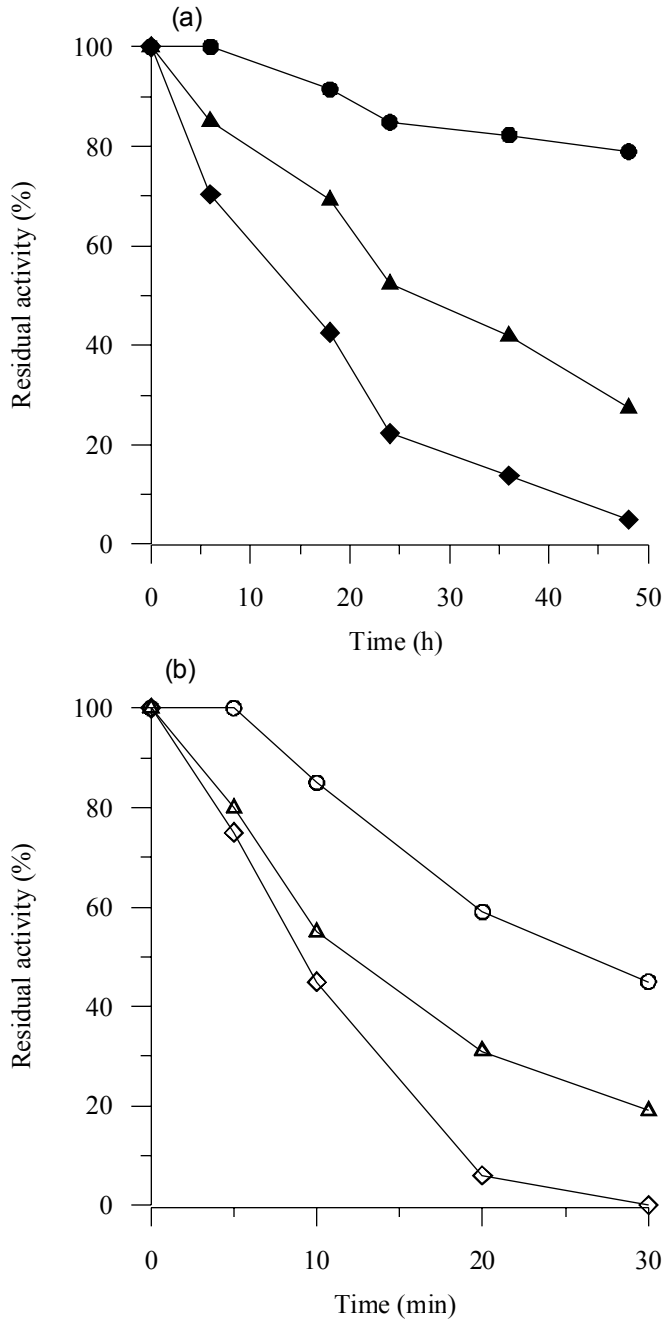


Fig.10 Thermal stability of *Arthrobacter* sp. xylanase (a &b). The pure enzyme was incubated in 0.05M Glycine-NaOH buffer (pH 9) at (●) 50 °C, (▲) 60 °C, (◆) 70 °C, (○) 80 °C, (▲) 90 °C and (◇) 100 °C for different intervals and residual activity was determined.

3.10. Effect of pH on the activity and stability

The optimum pH of the purified xylanase of the culture was 9 (Fig. 11).

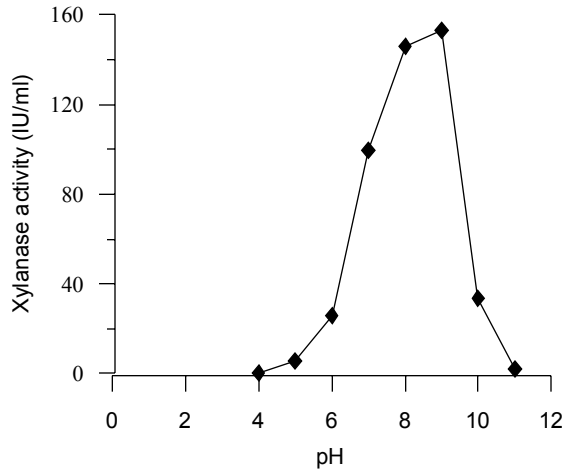


Fig. 11. Effect of pH on the activity of xylanase of *Arthrobacter* sp. was grown in MBM medium supplemented with wheat bran (pH 9.0) for 48 h at 50 °C . Xylanase activity in the culture filtrates of xylan medium was assayed at different temperatures and at pH 9 (50mM glycine-NaOH buffer).

Enzyme retained 100% of its activity at pH 7 and 8 for 24 h. Interestingly, at higher pH such as 9, 10 and 11 the enzyme activity increased over the period of incubation (Fig. 12).

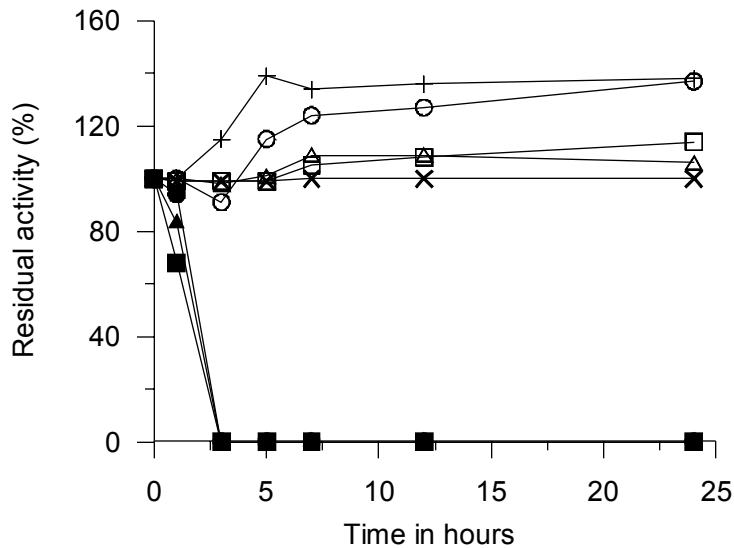


Fig.12. Effect of pH on xylanase stability. The enzyme was diluted with different buffers of varying pH such as (●) pH 4, (■) pH 5, (▲) pH 6, (△) pH 7, (×) pH 8, (□) pH 9, (⊙) pH 10 and (+) pH 11 and incubated at 50 °C up to 24 hours. Residual activity was assayed at pH 9 and 100 °C. Buffers used were citrate, phosphate and glycine-NaOH.

3.11. Effect of metal ions on the xylanase activity

Xylanase activity was assayed in the presence of metal ions. The xylanase activity was greatly elevated by the addition of CO^{+2} , Zn^{+2} , Fe^{+2} , Cu^{+2} , Mg^{+2} and Ca^{+2} ions. (Table 4). In contrast, the xylanase activity was strongly inhibited by Hg^{+2} ions.

3.12. Effect of additives

In order to improve the thermostability of xylanase at higher temperature, the effect of the polyols (2M) additives such as ethylene glycol (C2), glycerol (C3), sorbitol and mannitol (C4) was studied at 80 °C. All the polyhydroxy additives had a positive effect on xylanase stability (Fig. 13).

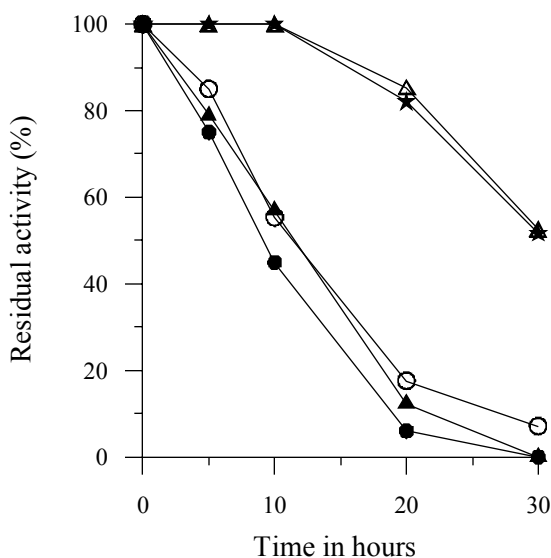


Fig.13. Thermal stability of (■) xylanase enzyme at 80 °C and in presence of (▲) ethylene glycol, (○) glycerol, (▲) mannitol and (★) sorbitol. 5 IU of xylanase enzyme was incubated in 0.05 M Glycine buffer (pH 9) in the presence of additives.

The thermostabilizing effect of polyol was proportional to its molecular size, which can be correlated to the number of hydroxyl groups per polyol molecule. An increase in the concentration of sorbitol and mannitol brought about an increase in its protective effect (Fig. 14).

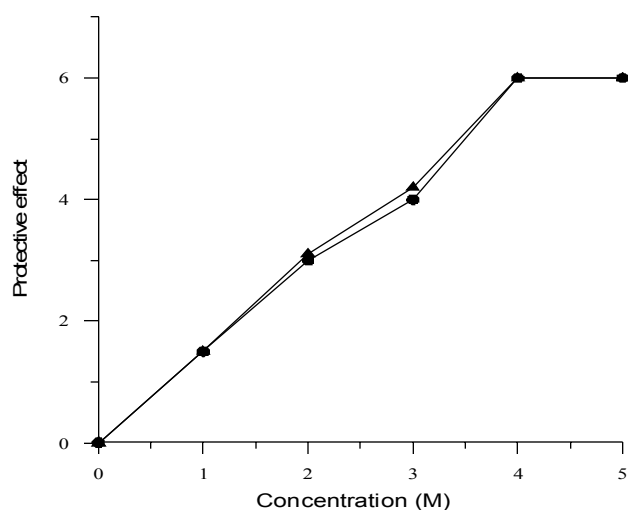


Fig.14 Effect of different concentrations of (●) sorbitol and (▲) mannitol on the thermostability of xylanase at 80 °C. The effect of sorbitol and mannitol on the thermostability is measured in terms of protective effect, which can be defined as the ratio of xylanase half-life in the presence and absence of additive.

3.13. Amino acid composition

The amino acid composition of the xylanase produced by the culture when grown on wheat bran in solid state fermentation is presented in Table 5. The major amino acids in the enzyme were Aspartic acid (11%), Alanine (11%), Glycine (9%), Lysine (8.5%), Serine (7%), Leucine (6.9), Valine (6.8%), while Asparagine, Tyrosine, Ornithine were minor.

4. Discussion

The characteristics of the culture (Table 1) are similar to those described for *Arthrobacter* sp in the Bergey's Manual of Systematic Bacteriology [18]. Based on these characteristics the culture was tentatively identified as *Arthrobacter* sp and was deposited with the Institute of Microbial Technology Chandigarh, India. The culture was assigned a culture collection number MTCC 5214. *Arthrobacter* sp used in the present study is novel, as to the best of our knowledge, there are no published reports on the production of xylanase enzyme by this culture. Furthermore, the outstanding feature of this culture is that it produced reasonably good amounts of alkalophilic and thermophilic xylanase.

In *Arthrobacter* sp the amount of enzyme produced and the time required for the maximum production of enzyme differed with each carbon source. The enzyme production on birchwood xylan was maximum and also the time required for optimum enzyme production was relatively less (48 h). When grown on agricultural residues the enzyme production was maximum on wheat bran and the time for maximum enzyme production was 72 h. *Aspergillus niger* when grown on rice straw as a solid state fermentation showed maximum xylanase production after 120 h following incubation [23].

A number of substrates are known to induce xylanase production. Xylose induced the production of the enzyme. It appears that low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers [24].

The molecular weight of purified xylanase enzyme from *Arthrobacter* sp was ~20 kDa. Xylanase obtained from *Bacillus* sp strain K-1 [25] also has a molecular weight of 23 kDa. In case of *Bacillus* sp 41M molecular mass of xylanase was 36 kDa [26]. While xylanase isolated from *Bacillus subtilis* [27] is reported to have a high molecular weight of 340 kDa.

Xylanase enzyme from *Arthrobacter* sp showed highest activity at high pH (9) and temperature (100 °C). To the best of our knowledge, there are no reports on xylanase enzyme having optimal temperature of 100 °C at pH 9.0. However, Xyn A and Xyn B isolated from *Thermotoga maritima* showed optimum temperature of 92 °C and 105 °C, respectively at pH 6 [28]. Similarly, xylanase isolated from *Thermotoga neapolitana* had optimum temperature of 102 °C at pH 5.5 [29]. Xylanase isolated from *Bacillus coagulans* BL69 grown on soyabean residue [30] showed activity over a wide range of temperatures (45-75 °C), however the thermal stability of the enzyme was very poor. This enzyme retained only 34 % of its activity after 5 min of incubation at 70 °C. *Bacillus subtilis* produced a xylanase, which had highest activity at pH 6.0 and 60 °C temperature, when the culture was grown on oat spelt in solid fermentation [26]. Xylanase obtained from *Streptomyces actuosus* A-151 grown on rice bran

showed optimum temperature of 60-70 °C [31]. *Bacillus* sp. JB-99 grown on rice bran in solid state fermentation produced xylanase with optimum activity at 50 °C [32].

There are only few bacterial xylanases reported till date with pH optima of < 9.0 when grown on SSF. Xylanase isolated from *Bacillus subtilis* which was grown on SSF using oat spelt xylan showed maximum activity at pH 6.0 [27]. Xylanase isolated from *Bacillus licheniformis* A99 [33] had the pH optimal of 7.0. Similarly, xylanase isolated from *Bacillus coagulans* BL69 grown on soyabean residue [30] showed a pH optimum of 7.0. In view of these observations the properties of the xylanase produced by *Arthrobacter* sp are much more superior than those reported for xylanases isolated from other bacteria. Moreover, the stability of xylanase enzyme from *Arthrobacter* sp was better as compared to the other bacterial xylanases. For example, xylanase isolated from *Streptomyces actuosus* [31] grown on rice bran retained 80% of its activity in the pH range of 5 to 8 after 30 min period of incubation. This is very much less compared to that obtained for the xylanase produced by *Arthrobacter* sp.

Xylanase activity increased in the presence of Co^{+2} , Zn^{+2} , Fe^{+2} , Cu^{+2} , Mg^{+2} and Ca^{+2} ions. In contrast, the xylanase activity was strongly inhibited by Hg^{+2} ions. The inhibition of the enzyme activity 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. 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. [34] 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} ions. Further work using more inhibitors, their analogous and combinations thereof would be necessary to ascertain the mode of action of xylanase.

The stability of xylanase enzyme increased with the addition of the polyols additives. Sorbitol as well as mannitol provided maximum protection at a 4M concentration. These compounds showed similar effect on xylanase isolated

from *Thermomonospora* sp [35]. Polyols have the capability to form hydrogen bonds that play key role in supporting the native conformation and stabilization of the protein.

k_m (0.9 mg/ml) of the xylanase of *Arthrobacter* sp for wheat bran observed is similar to those reported for the xylanases isolated from other bacteria. For example, xylanase isolated from *Bacillus subtilis* [27] and *Bacillus* sp. strain TAR-1 [36] showed a k_m value similar to that observed for xylanase of *Arthrobacter* sp. Xylanase enzyme of *Arthrobacter* sp. also gave a better V_{max} than that reported for xylanase isolated from *Aeromonas cavie* ME-1 [22] and *Bacillus* sp. strain 41m-1 [34].

In the hydrolysis experiment the presence of xylose was detected at 6h following incubation. The abundance of xylose increased over the period of immersion. These results indicated that, initially xylanase cleaved the substrate to liberate xylooligosaccharides and then the resulting oligosaccharides were probably cleaved to form xylose.

Arginine is more abundant in thermophilic xylanases. Amino acid composition of xylanase from *Arthrobacter* sp showed relatively higher mole % arginine compared to less thermostable xylanases isolated from *Bacillus* sp strain BP-23 and *Bacillus Subtilis* [37,38]. On the other hand, arginine content in the xylanase of *Arthrobacter* sp is similar to that found in thermophilic xylanase produced by *B. amyloliquefacens* [39]. Similarly, 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 [40]. Polar -amino acids (50.63 mole %) were relatively more abundant in the xylanase produced by *Arthrobacter* sp MTCC 5214 suggesting their importance in enzyme stability.

In summary, *Arthrobacter* sp produced a novel thermoalkalophilic xylanase when grown on SSF using wheat bran as a substrate. These properties make this enzyme potentially very effective for industrial applications.

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Table 1
Morphological, physiological and biochemical
Characteristics of *Arthrobacter* sp. MTCC 5214

Tests	Results
Colour	Off white
Shape	Pluromorphic
Gm stain	Gm + ve
Motility	+
Oxidase	+
Catalase	+
Hugh-Leifson	Facultative anaerobe
Spore staining	No spore
Esculin hydrolysis	+
ONPG	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Urease	-
Simmons Citrate	+
Diamination(TDA)	-
Nitrate reduction	+
Vogous Prauskaur	-
Methyl red	-
Sugar fermentation:	
Indol	-
Melonate	-
Arabinose	-
Xylose	Alk
Adonitol	Alk
Rhamnose	Alk
celliobiose	Alk
Meliciose	Alk
Sacchrose	A
Trehalose	Alk
Glucose	A
Lactose	Alk
Fructose	Alk
Bacteria Identified	<i>Arthrobacter</i> sp.

Table 2

Effect of different carbon sources on xylanase activity

Soluble substrate	Activity ($\mu\text{mol}/\text{min}/\text{ml}$)
Arabinose	18
Cellobiose	1.0
Carboxyl methyl cellulose	1.2
Galactose	4.3
Glucose	14.5
Sucrose	11.4
Xylose	21
Birchwood xylan	121.3
Oat spelt xylan	45.58
Solid material (SSF)	
Wheat bran	35.70
Rice husk	25.04
Rice bran	18.64
Bagassae	17.29

Table 3

Purification steps of xylanase enzyme isolated from *Arthrobacter* sp. grown on wheat bran

Purification steps	Xylanase activity (IU)	Proteins (mg)	Specific activity (IU/mg)	Purification fold	Yield
Filtrate	316595	3953	80.1	1	100
(NH ₄) ₂ SO ₄ Precipitation	236220	1458	162.0	2	74
Sephadex G-200	198380	702	282.6	3.5	62
DEAE Sepharose FF	156360	352	444.2	5.5	49
CM Sepharose FF	44140	26	1697.7	21	14

Table 4
Effect of metal ions on the activity of the xylanase enzyme produced by *Arthrobacter* sp.

Metal ions (1 mM)	Residual activity (%)
None	100
Ca ⁺²	154
Mg ⁺²	154
Zn ⁺²	161
Co ⁺²	175
Fe ⁺³	193
Cu ⁺²	212
Mn ⁺²	177
Hg ⁺²	27

Table 5

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

Amino acid residue	Bacillus sp. strain BP-23	B. amyloliquefacens	Bacillus subtilis	Arthrobacter sp.
Asx	14.3	13.8	NR	-
Asp	NR	NR	10.73	11.02
Thr	4.4	12.1	8.97	0.35
Ser	6.0	8.4	12.04	7.10
Glx	12.5	7.8	6.20	-
Gly	9.7	14.5	18.70	9.24
Ala	8.2	4.1	7.69	11.48
Cys	0	0.0	1.45	-
Val	4.9	8.6	4.80	6.82
Met	1.4	0.2	1.09	ND
Ile	5.3	3.8	3.18	5.14
Leu	6.5	3.4	3.48	6.99
Tyr	3.5	8.2	4.38	2.24
Phe	3.9	2.4	1.10	3.08
Lys	4.9	1.5	4.99	8.53
His	1.6	1.8	2.06	-
Arg	3.3	6.0	3.32	6.52
Pro	4.9	3.4	5.81	-
b-Ala	NR	NR	NR	0.33
g-ABA	NR	NR	NR	0.47
Orn	NR	NR	NR	2.09
Reference	Blanco et al. 1995	Salles et al. 2000	Berenger et al. 1985	Present study

NR= not reported ; - = not present