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ONE-STEP PURIFICATION AND CHARACTERIZATION OF CELLULASE-FREE XYLANASE PRODUCED BY ALKALOPHILIC *BACILLUS SUBTILIS* ASH

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ABSTRACT

The present study describes the one-step purification and characterization of an extracellular cellulase-free xylanase from a newly isolated alkalophilic and moderately thermophilic strain of *Bacillus subtilis* ASH. Xylanase was purified to homogeneity by 10.5-fold with ~43% recovery using ion-exchange chromatography through CM-Sephadex C-50. The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 23 kDa. It showed an optimum pH at 7.0 and was stable over the pH range 6.0-9.0. The optimum temperature for enzyme activity was 55 °C. The purified xylanase did not lose any activity up to 45 ºC, however, it retained 80% and 51% of its activity after pre-incubation at 55 ºC and 60 ºC, respectively. The enzyme obeyed Michaelis-Menton kinetics towards birch wood xylan with apparent K_m 3.33 mg/ml and V_{max} 100 IU/ml. The enzyme was strongly inhibited by Hg^{2+} and Cu^{2+} while enhanced by Co^{2+} and Mn^{2+}. The purified enzyme could be stored at 4 ºC for six weeks without any loss of catalytic activity. The faster and economical purification of the cellulase-free xylanase from *B. subtilis* ASH by one-step procedure together with its appreciable stability at high temperature and alkaline pH makes it potentially effective for industrial applications.

Key words: Alkalophilic, *Bacillus subtilis*, Purification, Xylanase

INTRODUCTION

Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8) is a hydrolytic enzyme involved in depolymerization of xylan, the major renewable hemicellulosic polysaccharide of plant cell wall. It is produced by bacteria (11, 19, 37, 41), fungi (29, 34, 41), actinomycetes (33) and yeast (26). Recently, interest in xylanase has markedly increased due its wide variety of biotechnological applications such as pre-bleaching of pulp, improving the digestibility of animal feed stocks, modification of cereal-based stuffs, bioconversion of lignocellulosic material and agro-wastes to fermentable products, clarification of fruit juices and degumming of plant fibers (15, 21, 44) etc. Cellulase-free xylanases active at high temperature and pH are gaining importance in pulp and paper industry as they reduce the need for toxic chlorinated compounds making the bleaching process environment-friendly (40, 43).

The industrial application of xylanase may be limited by its high cost of production. The use of cost-effective agro-residues as substrates in solid state fermentation may reduce

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the cost of enzyme production substantially (37). Solid state fermentation offers several advantages over submerged fermentation including simplicity of media, greater product yield, easier scale up of process, economy of space, no complex machinery, equipment and control systems etc (2, 14). In order to investigate the biotechnological applications of this enzyme, it would be desirable to purify and characterize it. Presently, purification and characterization costs are becoming important issues in modern biotechnology as the industry matures and competitive products reach the market. Although, purification of microbial xylanase has been reported by several investigators using a combination of two or more standard enzyme purification techniques yet it would be advantageous to develop a one-step purification procedure so as to reduce the enzyme cost. In this study, we report a single step purification and characterization of an extracellular cellulase-free xylanase from an alkalophilic and moderately thermophilic strain of *Bacillus subtilis* ASH 7414 isolated from soil.

MATERIALS AND METHODS

Microbial strain and its growth conditions

The xylanase-producing bacterial strain used in this study was isolated from soil sample collected locally from Kurukshetra, India, by using enrichment technique with wheat bran as a source of carbon. The organism was identified as *Bacillus subtilis* ASH 7414 on the basis of its morphological, physiological and biochemical characteristics by the Institute of Microbial Technology (IMTECH). It was assigned the accession number MTCC 7414. The culture was maintained at 4 ºC on nutrient agar medium (in g/l: peptone, 5.0; beef extract, 3.0; NaCl, 5.0; pH 7.0; 2 % agar).

Xylanase production and extraction

Xylanase was produced by *B. subtilis* ASH 7414 in solid-state fermentation. Erlenmeyer flasks each containing 10g wheat bran and 25 ml mineral salt solution (in g/l: $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.4; pH 7.0) were autoclaved at 1.05 kg/cm^2 for 45 min and cooled. After inoculation with 10% (v/w) of 18 h old inoculum, the flasks were incubated at 37 ºC for 72 h. The flasks were gently tapped intermittently to mix the contents. The incubator was humidified by keeping a tray containing sterile distilled water.

Xylanase was extracted from the above bacterial bran with 100 ml of sodium phosphate buffer (0.05 M, pH 7.0) by gently squeezing through a wet muslin cloth followed by centrifugation at 10,000 x g for 30 min at 4ºC. The clear supernatant (crude extract) was used for xylanase purification.

Xylanase assay

Xylanase activity was assayed by measuring the amount of reducing sugars liberated from birchwood xylan using 3, 5-dinitrosalicylic acid (28). The reaction mixture (1.0 ml) containing 0.5 ml of 1 % birchwood xylan (prepared in 0.05M sodium phosphate buffer, pH 7.0) as substrate, 0.02 ml of appropriately diluted enzyme extract and 0.48 ml of sodium phosphate buffer (0.05M, pH 7.0) was incubated at 55 ºC for 5 min and then the reaction was terminated by adding 3.0 ml of 3, 5-dinitrosalicylic acid reagent. A control was run simultaneously which contained all the reagents but the reaction was terminated prior to the addition of enzyme extract. The test tubes were placed in a boiling water bath for 10 min. After cooling to room temperature, the absorbance of the resulting red color was measured against the control at 540 nm using a double beam spectrophotometer (Systronics 2202, India). One unit of xylanase activity was defined as the amount of enzyme catalyzing the release of 1 µmol/min of reducing sugars equivalent to xylose under the specified assay conditions. Cellulase activity was assayed as above, using low viscosity carboxymethylcellulose (1%) in place of xylan as substrate. All the experiments were carried out independently in triplicate and the results presented are mean of the three values.

Protein estimation

Protein was estimated by the Lowry's method using bovine serum albumin as standard (27). The protein content of the chromatographic fractions was measured by monitoring the optical density at 280 nm in a double beam spectrophotometer.

Purification of xylanase

The crude enzyme extract was chromatographed on CM-Sephadex C-50 column (20 cm × 2.0 cm), pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was run at a flow rate of 40 ml/h. The bound proteins were eluted using a continuous gradient of 0-1.0 M NaCl. Fractions of 5 ml each were collected and analyzed for protein content and xylanase activity. The fractions containing enzyme activity were pooled and concentrated using Amicon ultrafiltration cell membrane (10 kDa cut off).

Checking of enzyme homogeneity

The purity of the enzyme was checked by performing 12% SDS-PAGE as described by Laemmli (22). Protein bands were visualized by staining with coomassie brilliant blue R-250. A reverse phase C-18 column (4.6 x 250 mm; E. Merck, Germany) of High Performance Liquid Chromatography (HPLC System 600 Waters, Waters Corporation, Massachusetts, USA) was also employed to test the enzyme purity. The sample components were separated using the solvent system acetonitrile-water (70:30) at a flow rate of 0.5 ml/min. A highly sensitive photo-diode array (PDA) detector (996 Waters) was set to read the absorbance at 280 nm.

Characterization of the purified enzyme

Molecular weight determination

Molecular weight (MW) of the purified xylanase was estimated by SDS-PAGE and gel filtration. SDS–PAGE was performed as described above using molecular weight markers viz. lysozyme (14.3 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (46 kDa) and BSA (66 kDa). Gel filtration chromatography was performed on a Sephadex G-100 column (90 cm x 0.8 cm), pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.0. The purified enzyme was loaded onto top of this column, which was run at 15 ml/h and fractions of 3 ml each were collected. A mixture of gel filtration protein molecular weight markers viz. carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (205 kDa) was also separated on the same column under identical conditions and their elution volumes were determined. A standard graph was then plotted between V_e/V_o on x-axis and log MW on y-axis for calculation of molecular weight of the purified xylanase.

Determination of K_m and V_{max}

Initial reaction rate of birchwood xylan hydrolysis was determined by varying its concentration in the range of 1 to 15 mg/ml in standard xylanase assay and the double reciprocal plot was drawn. The kinetic parameters K_m and V_{max} were estimated from this plot (25).

Effect of temperature on activity and stability of xylanase

The optimum temperature for the purified xylanase activity was determined by assaying the enzyme activity at different temperatures ranging from 30-65 ^oC. To investigate the thermal stability of the purified xylanase, an aliquot of the enzyme was pre-incubated at different temperatures (30-60 ^oC) for 10 min at pH 7.0, quickly chilled and assayed for its residual activity at 55 ^oC.

Effect of pH on activity and stability of xylanase

The effect of pH on xylanase activity was studied by carrying out the enzyme assay using buffers of different pH ranging from 4.0 to 10.0. Three different buffers (each at 0.05M) viz. citrate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0) and Tris-HCl (pH 8.0-10.0) were used for this study. To test the pH stability of the enzyme, an aliquot of the purified enzyme was pre-incubated with the above mentioned buffers of different pH (4.0 – 9.0) for 10 min at room temperature followed by measurement of xylanase activity using sodium phosphate buffer (0.05M, pH 7.0). The residual activity (%) at each pH was calculated.

Effect of metal ions

The purified enzyme was incubated with various metal salts viz. $ZnCl_2$, $CaCl_2$, $HgCl_2$, NaCl, KCl, $FeCl_3$, $MgCl_2$, $CoCl_2$, $CdCl_2$, $NiCl_2$, $CuCl_2$ and $MnCl_2$ at a final concentration of 1mM for 5 min at room temperature and then xylanase

activity was assayed. The residual activity (%) was then calculated.

Determination of shelf life

The shelf life of the purified enzyme was determined by keeping it in a refrigerator (4°C) and at room temperature. Enzyme samples were withdrawn at different intervals up to 10 weeks and the residual xylanase activity was determined.

RESULTS AND DISCUSSION

Microbial strain and its growth conditions

Bacillus subtilis ASH is an alkalophile being capable of growing at pH values up to 11.0. It is a moderate thermophile with minimum, optimum and maximum temperature for growth at 15, 37 and 55ºC, respectively (37).

Enzyme activity

The bacterium produced high levels of xylanase (8,964 U/g dry wheat bran) in solid state fermentation under optimized conditions. The enzyme was cellulase-free as the crude extract was devoid of cellulase activity. Lignocellulosic materials have been used earlier by various researchers for production of xylanase but with lower activity (3, 9, 12).

Purification of xylanase

Xylanase produced by *B. subtilis* ASH in solid state fermentation on wheat bran was purified to apparent homogeneity by a single step chromatographic procedure. After loading the crude extract onto the column of CM-Sephadex C-50, the enzyme eluted as a single peak in bound fractions at 0.675 M NaCl as shown in Fig. 1. The fractions containing enzyme activity were pooled and concentrated using Amicon ultrafiltration cell membrane (10 kDa cut off). The purity of the purified enzyme was checked by SDS-PAGE and reverse phase HPLC on C-18 column. The purified enzyme showed a single band in SDS-PAGE gel indicating that it was homogeneous (Fig. 2). HPLC chromatogram of the purified enzyme also revealed a single peak at a retention time of 2.513 min confirming that it was a pure preparation (Fig. 3). The yield of the purified xylanase was 43.05% with a specific activity of 1275.5 IU/mg and an overall purification fold of 10.5 (Table 1).

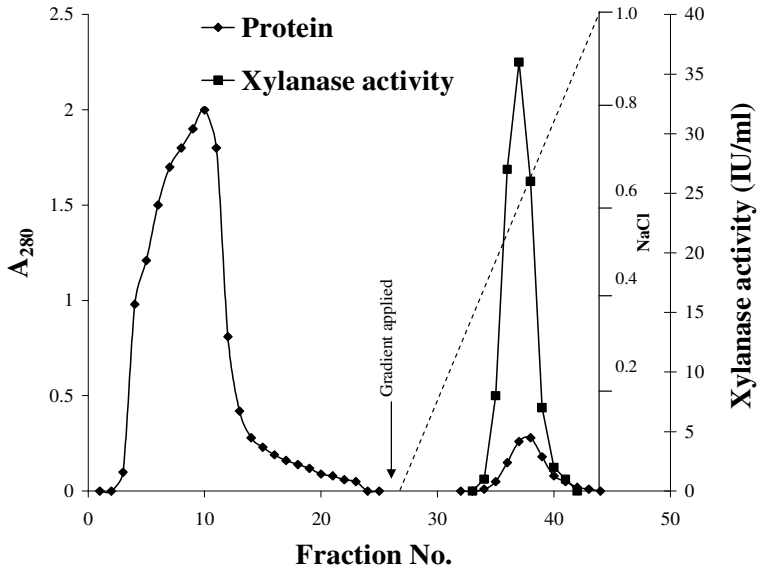

Figure 1. Purification of xylanase by ion-exchange chromatography through CM-Sephadex C-50 column (20 × 2.0 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.0. The bound proteins were eluted using a linear gradient of 0-1.0 M NaCl at a flow rate of 40 ml/h. Fractions of 5 ml each were collected and assayed.

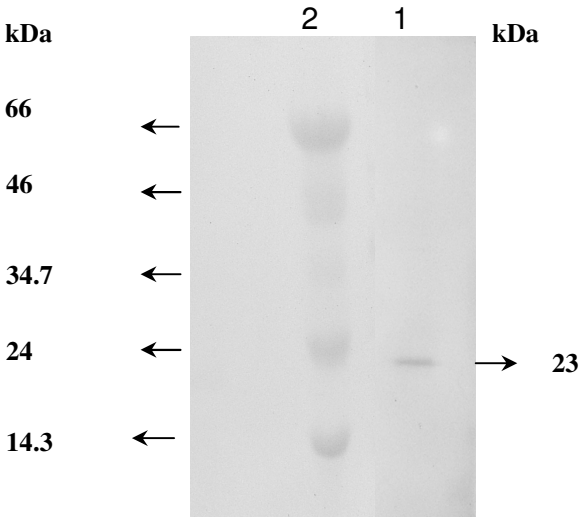

Figure 2. SDS-PAGE analysis of the purified xylanase from *Bacillus subtilis* ASH. Lane 1: purified xylanase; Lane 2: molecular weight markers viz. lysozyme (14.3 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (46 kDa) and BSA (66 kDa). Electrophoresis was performed using 12% polyacrylamide gel. The gel was stained with coomassie brilliant blue R-250.

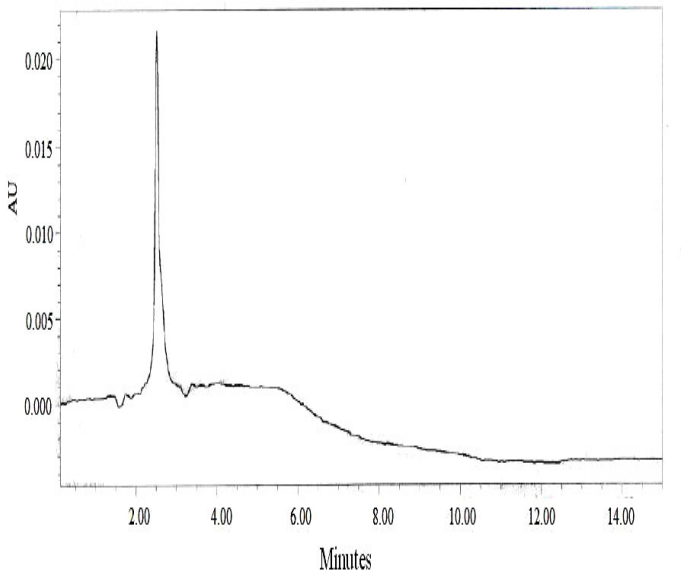

Figure 3. HPLC profile of the purified xylanase from *Bacillus subtilis* ASH using a reverse phase C-18 column (4.6 x 250 mm). The purified enzyme (10 µl) was injected into the loop of column and eluted with acetonitrile-water (70:30) at a flow rate of 0.5 ml/min. A photo-diode array (PDA) detector was set to read the absorbance at 280 nm.

Table 1. Purification of xylanase from *Bacillus subtilis* ASH. The enzyme was produced in solid state fermentation using wheat bran and the crude extract obtained after 72 h incubation was subjected to purification.

Purification step	Total activity (IU)	Total Protein (mg)	Specific activity (IU/mg protein)	Recovery (%)	Purification fold
Crude extract	**4000**	**33.0**	**121.2**	**100.0**	**1.0**
CM-Sephadex C-50	**1722**	**1.35**	**1275.5**	**43.05**	**10.5**

One-step chromatographic procedure for purification of xylanase from *B. subtilis* ASH is rapid and economical which is desirable for its industrial application. Other research groups reported purification of xylanase by employing a combination of two or more different methods including salt fractionation, ion-exchange, gel filtration and hydrophobic interaction chromatography (1, 5, 7, 16, 24, 29, 35, 36). The overall purification in the present study using one step was higher than that reported from *Bacillus amyloliquefaciens* (7) and *Bacillus circulans* (36) using a multistep sequence of purification. Further, recovery of the purified enzyme from *B. subtilis* ASH following one step protocol was better than that obtained by using multistep protocol (1, 5, 16, 24). However, grouping of two or more techniques resulted in higher purification fold (1, 5, 16, 24, 35) or recovery (7).

Molecular weight determination

The purified enzyme showed a single protein band on SDS-PAGE corresponding to a molecular mass of 23 kDa (Fig. 2). The purified enzyme eluted as a single peak when applied to Sephadex G-100 column (Fig. 4) with a molecular weight 23 kDa as calculated from the standard graph (Fig. 5). An identical molecular mass obtained by gel filtration chromatography and SDS-PAGE suggested that the enzyme was a monomer consisting of a single polypeptide chain.

The molecular weight of *B. subtilis* ASH xylanase was identical to the enzyme reported from *B. circulans* AB16 (10), *Bacillus* sp. strain TAR-1 (42), *Bacillus licheniformis* A99 (1), *Arthrobacter* sp. (16), *Paecilomyces themophila* (23) and *Streptomyces cyaneus* SN32 (33). However, it was higher than 5.5 kDa as observed by Bastawde (4). In contrast, some workers documented a higher molecular weight than that observed in the present study (5, 19, 20, 38, 39). Low molecular weight xylanases are preferred for commercial application in paper and pulp industry as they penetrate throughout the pulp fibres more effectively to hydrolyze xylan making the fiber structure more permeable to extraction of lignin by chemicals (21).

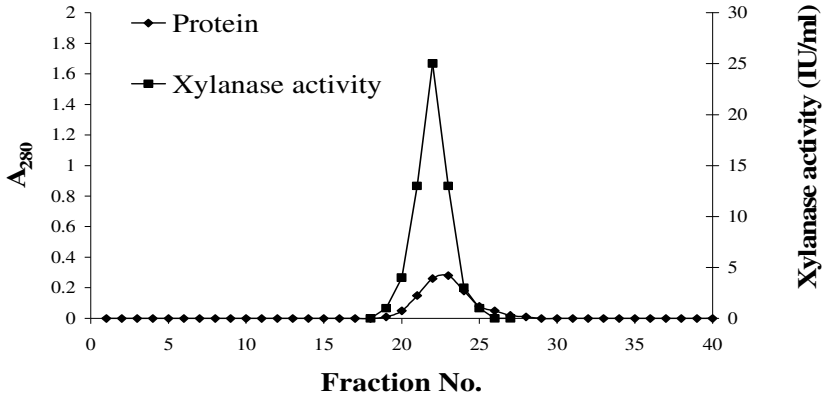

Figure 4. Elution profile of the purified xylanase through Sephadex G-100 column (90 x 0.8 cm) eluted with 50 mM sodium phosphate buffer, pH 6.0 at 15 ml/h. Fractions of 3 ml each were collected and analyzed for enzyme activity and protein.

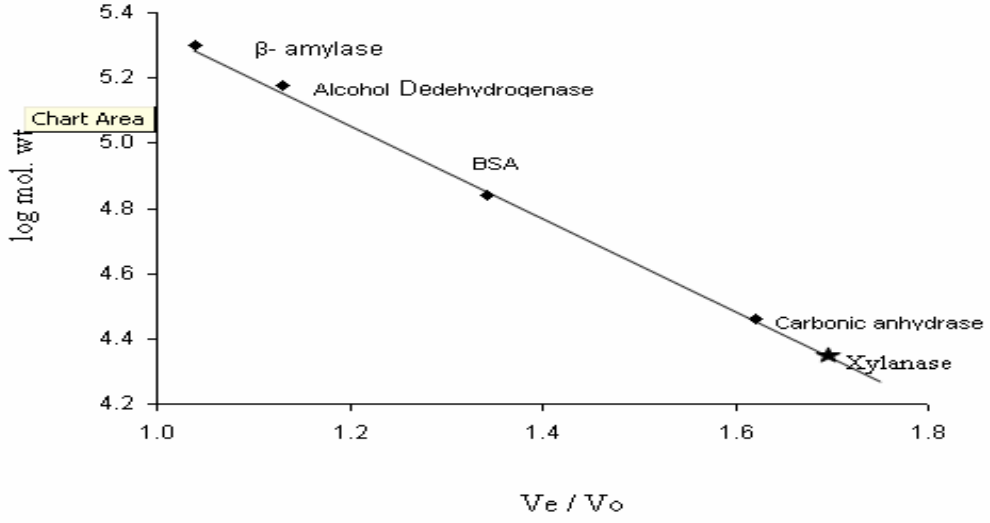

Figure 5. Determination of molecular weight of the purified xylanase from *Bacillus subtilis* ASH by gel filtration through Sephadex G-100 column. The elution volume of the purified enzyme and molecular weight marker proteins viz. carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (205 kDa) was determined. A graph was plotted between Ve/Vo and log MW.

Determination of K_m and V_{max}

The activity of the purified xylanase from *B. subtilis* ASH, measured at various concentrations of birch wood xylan (1-15 mg/ml) as substrate, exhibited a rectangular hyperbolic response thus obeying Michaelis-Menton Kinetics. The apparent K_m and V_{max} values obtained from the Lineweaver-Burk plot (1/v versus 1/[S]) were 3.33 mg/ml and 100 IU/ml, respectively. These values were comparable to those for xylanases isolated from *Bacillus* sp. strain 41-1 (31), *Bacillus* sp NCIM 59 (32), *B. circulans* Teri-42 (36), *Aspergillus niger* (29) and *Thermomyces lanuginosus* (24). However, some xylanases were found to have lower K_m ranging from 0.025-1.7 mg/ml (8, 16, 23, 38).

Effect of temperature on xylanase activity and stability

Measurement of xylanase activity at different temperatures showed that the activity increased up to 55 ºC and then declined progressively retaining 57% of the residual activity at 65 ºC. The optimum temperature of the purified xylanase from *B. subtilis* ASH was 55 ºC as the enzyme was most active at this temperature (Fig. 6). Thermostability study of the purified enzyme revealed that it did not lose any activity up to 45 ºC. However, it retained 80% and 51% of its activity after pre-incubation at 55 ºC and 60 ºC respectively (Fig. 7). Significant enzyme stability at higher temperatures would be important for its industrial application. Purified xylanases exhibiting optimum temperature in the range of 50-55 ºC have been reported from several *Bacillus* and *Streptomyces* sp. (1, 6, 38, 44). However, some xylanases showed higher temperature optima (5, 7, 16, 18, 24).

Effect of pH on xylanase activity and stability

The purified enzyme showed more than 90% activity in the pH range 6.5 to 7.5 with maximum at pH 7.0. It was active at alkaline pH values although, to a lesser extent than at pH 7.0 (Fig. 8). The enzyme retained 80%, 28% and 10% activity when assayed at pH 8.0, 9.0 and 10.0, respectively. Maximum pH stability of the purified xylanase from *B. subtilis* ASH was between pH 6.0-7.5. On pre-incubation for 10 min at pH 8.0 and 9.0, the residual enzyme activity was 85% and 50%, respectively (Fig. 9). A similar pH optimum was reported for xylanase isolated from *Bacillus spp.* (7, 18, 30, 36), *Staphylococcus* sp. (13), *Paecilomyces themophila* (23) and *Thermomyces lanuginosus* (24). However, some xylanases exhibited optimum pH at 6.0 (5, 33). Xylanase from alkalophilic *Bacillus sp.* strain 41M-1 showed a broad pH activity profile in the range of pH 4.0-11.0 (31). The pH stability of the purified thermoalkalophilic xylanase of *Bacillus sp.* ranged from 6.0–10.5 (36). A considerable stability of *B. subtilis* ASH xylanase at alkaline pH values makes it potentially effective for use in industry.

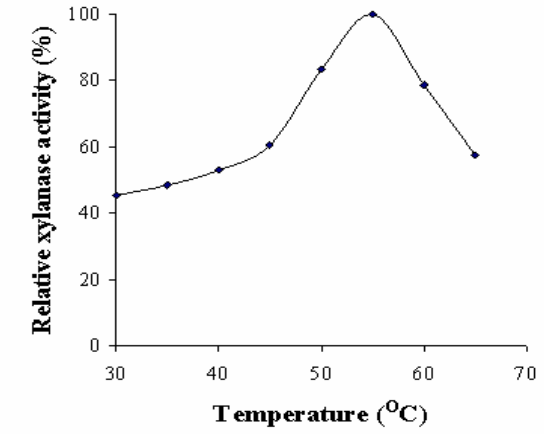

Figure 6. Temperature optima of the purified xylanase from *Bacillus subtilis* ASH. The enzyme activity was assayed at different temperatures ranging from 30-65 °C and relative xylanase activity (%) was calculated.

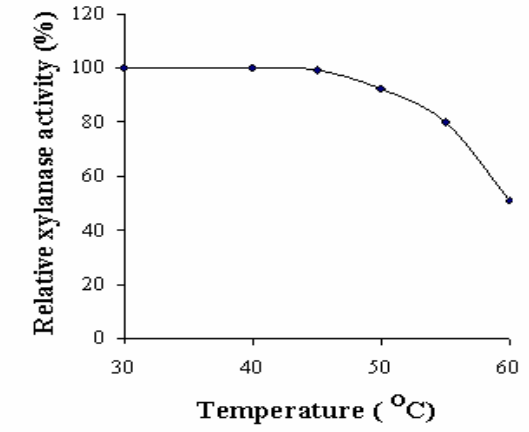

Figure 7. Thermostability of the purified xylanase from *Bacillus subtilis* ASH. The purified enzyme was pre-incubated at different temperatures (30-60 °C) for 10 min at pH 7.0, quickly chilled and assayed for its residual activity at 55 °C.

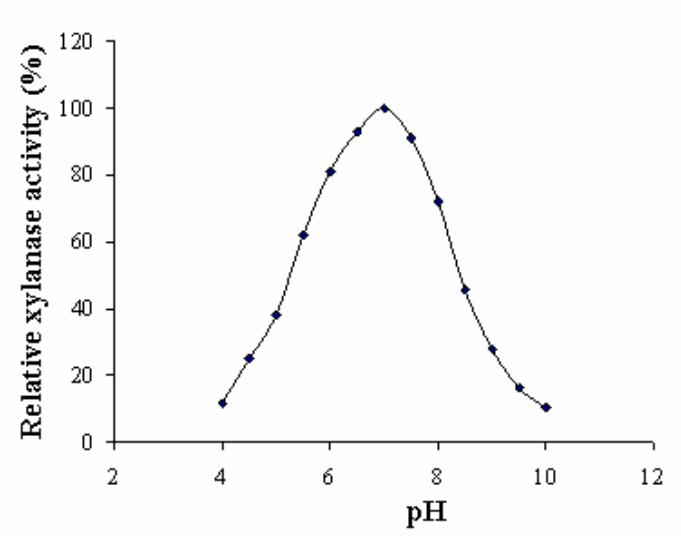

Figure 8. pH optima of the purified xylanase from *Bacillus. subtilis* ASH. The enzyme activity was determined at different pH using citrate (pH 4.0-6.0), phosphate (pH 6.0-8.0) and Tris-HCl (pH 8.0-10.0) buffers.

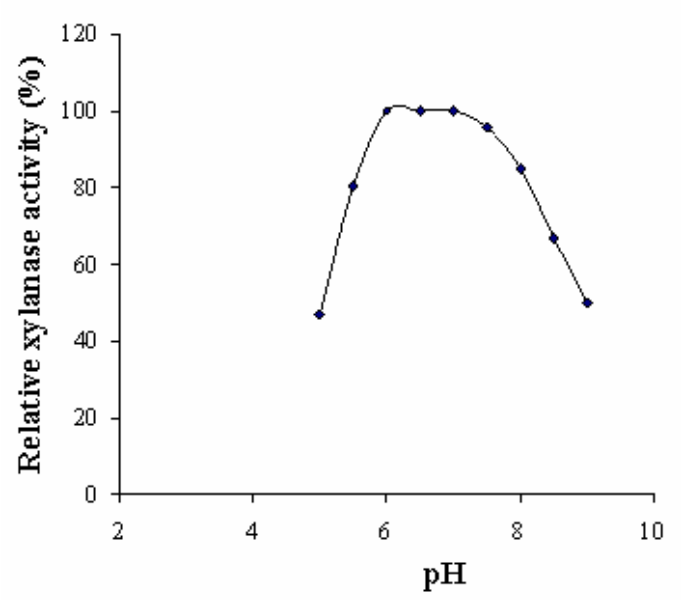

Figure 9. pH stability of the purified xylanase from *Bacillus subtilis* ASH. The purified enzyme was pre-incubated with citrate (pH 4.0-6.0), phosphate (pH 6.0-8.0) and Tris-HCl (pH 8.0-9.0) buffers for 10 min at room temperature followed by measurement of xylanase activity using sodium phosphate buffer (0.05M, pH 7.0). The residual activity (%) at each pH was calculated.

Effect of metal ions on xylanase activity

Xylanase activity was strongly inhibited by Hg^{2+} which might be due to its interaction with sulfhydryl groups present on the enzyme. Cu^{2+} ions were also found to be inhibitory causing 30% inhibition of enzyme activity (Table 2). Some other metal ions like Na^{2+}, K^{+}, Ca^{2+}, Ni^{2+} and Zn^{2+} decreased the enzyme activity but to a very less extent. In contrast, xylanase activity was enhanced in the presence of Co^{2+} and Mn^{2+} with 20% and 85% increase as compared to the control. $HgCl_2$ has earlier been reported to completely inhibit the activity of xylanase from different sources (5, 16, 17, 36). An increase in xylanase activity in the presence of $MnCl_2$ has also been reported by several workers (5, 13, 19).

Table 2. Effect of metal ions on the activity of purified xylanase from *Bacillus subtilis* ASH. The purified enzyme was incubated with various metal salts at 1mm concentration for 5 min at room temperature and the xylanase activity was assayed.

Metal ion (1 mM)	Xylanase activity (IU/ml)	Xylanase activity (% of control)
Control	85.3	100.0
$ZnCl_2$	73.6	86.3
$CaCl_2$	71.6	84.0
$HgCl_2$	4.9	5.7
NaCl	75.5	88.5
KCl	71.7	84.1
$FeCl_3$	78.7	92.3
$MgCl_2$	72.1	84.5
$CoCl_2$	102.4	120.0
$CdCl_2$	73.5	86.2
$NiCl_2$	70.5	82.6
$CuCl_2$	59.6	69.9
$MnCl_2$	157.8	185.0

Determination of shelf life of xylanase

The purified enzyme did not lose any activity when stored at 4°C for six weeks but thereafter, a decline was observed. The enzyme retained 80 % its initial activity after 10 weeks which would be important for its application. On the other hand, at room temperature, the enzyme was completely stable for three weeks but showed 60 % and 50 % residual activity after storage for 7 and 10 weeks, respectively.

To sum up, an extracellular, cellulase-free xylanase has been produced in high titer by *B. subtilis* ASH in solid state fermentation using wheat bran, a cost-effective agro-residue. This enzyme was purified to homogeneity in a single step by cation exchange chromatography on CM-Sephadex C-50. The characteristics of the purified enzyme have been studied. The one-step procedure for xylanase purification reported in the present study is rapid and economical. This protocol together with the characteristics of the purified enzyme such as significant stability at higher temperatures and alkaline pH make it potentially effective for industrial applications.

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