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Purification and some properties of low-molecular-weight extreme halophilic xylanase from *Chromohalobacter* sp. TPSV 101

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ABSTRACT

An extreme halophilic xylanase was purified from cultures of *Chromohalobacter* sp. TPSV 101 by ultrafiltration, hydroxylapatite and gel filtration chromatography. SDS-PAGE of the xylanase showed an apparent homogeneity and molecular weight of 15 kDa. The xylanase had maximum activity at pH 9.0 and 65 °C in the presence of 15–25% NaCl and was stable in the range of pH 7.0–9.0, temperature between 50 and 70 °C. The enzyme was stable at 50–65 °C for 1 h retaining 100% activity and by retaining 60% activity at 80 °C. The xylanase was completely inhibited by Hg^{2+} ions and was partially inhibited by Ca^{2+}, Cu^{2+} and Pb^{2+} ions, whereas Zn^{2+}, Mn^{2+} and Co^{2+} ions enhanced its activity. Both EGTA and EDTA enhanced its activity. It was active in solutions containing water-insoluble organic solvents and osmolytes. Kinetic experiments indicated that the enzyme had K_m and V_{max} values of 0.2 mg/ml and 1. 17 μmol/ml/min for oat spelt xylan. The major products of the oat spelt xylan hydrolysis were xylose and xylobiose; after prolonged incubation xylose was the major end product.

1. Introduction

Hemicelluloses are low-molecular-weight polysaccharides found in close association with cellulose and lignin in the plant cell wall and in contrast to cellulose, are branched heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, and galactose) and sugar acids. Xylan represents a large proportion of the hemicellulose present in woody and graminaceous plant tissues and are the most abundant polysaccharides found in nature apart from cellulose [1].

The application of thermophilic, psychrophilic, acidophilic, alkaliphilic and halophilic microorganisms in industrial processes opens up a new era in biotechnology. Each microbial group has unique features, which can be exploited for use in biotechnological industries. The main reason for selecting enzymes from extremophiles is their high stability and the reduced risk of contamination. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes [2].

While there have been numerous reports on extracellular xylanases from non-halophilic bacteria, very limited information is available on xylanases from halophilic species. Xylanolytic activities have been reported in halophilic archaeon *Halorhabdus utahensis* and halophilic strain CL8 [3,4], and also from the halotolerant species like *Bacillus halodurans* PPKS-2, *Nesterenkonia* sp., *Glaciecola mesophila* KMM 241 and *Bacillus* sp. NTU-06 [5–8]. Furthermore, exozymes from halophiles are not only interesting from the basic scientific viewpoint, they may also be of potential interest in many industrial applications and saline waste water treatment [9,10], owing to their stability and activity at low water activities [10,11].

In recent years, xylanases have received increasing attention due to their biotechnological uses and potential application in various industrial processes, such as bioconversion of lignocellulose material to fermentative products, clarification of juices, improvement of the consistency of beer and the digestibility of animal feed stock [12–15], and production of acidic xylooligosaccharides having potential pharmacological benefits [16]. One of the major potential applications of xylanases is the pulp and paper industry. The hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the use of chlorine as bleaching agent [12,17].

The present study reports the purification and characterization of extracellular, alkali-stable and thermostable xylanase from *Chromohalobacter* sp. TPSV 101. Earlier we have reported the optimum conditions for the production of halophilic xylanase from this strain by using agricultural residues [18].

Abbreviations: CTAB, cetyl trimethyl ammonium bromide; DMSO, dimethyl sulfoxide; DMF, dimethyl formamide; DTT, dithiothritol; EGTA, ethylene glycol tetra acetic acid; EDTA, ethyl diamine tetra acetic acid; LBG, Locust bean gum; SDS, sodium dodecyl sulphate.

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2. Materials and methods

All the chemicals used were of analytical grade.

2.1. Microorganism

Chromohalobacter sp. TPSV 101 was isolated from the solar evaporated saltern pond of Tuticorin, Tamilnadu, India. The organism was identified by biochemical tests and 16S rRNA sequence. The 16S rRNA sequence has been deposited in NCBI GenBank with accession number EU122306.

2.2. Growth and culture conditions

The organism was grown on agar plate composed of 0.1% (w/v) oat spelt xylan, 0.5% (w/v) peptone in mineral salt medium containing (g/l); NaCl, 200; $MgSO_4$, 10; KCl, 5; agar 20 and the pH was adjusted to 7.2. Cultures of *Chromohalobacter* sp. TPSV 101 were maintained on solid slants and as a glycerated stock at 4 °C and −20 °C, respectively.

2.3. Enzyme production

The *Chromohalobacter* sp. TPSV 101 was grown aerobically at 37 °C in glycine–NaOH buffer (50 mM) of pH 9.0, containing 0.2% (w/v) xylan and 0.5% (w/v) skim milk powder as a nitrogen source, in above mentioned mineral salt medium excluding agar. The medium (50 ml) was taken in 250 ml Erlenmeyer flask; autoclaved at 121 °C for 15 min. Flasks were inoculated with 2 ml of 4 days old culture of *Chromohalobacter* sp. TPSV 101 and incubated for 6 days in an incubator shaker at 200 rpm. The contents were removed and centrifuged at 6200 × *g* for 10 min at 4 °C and the supernatant was used for the purification.

2.4. Xylanase assay

One ml of reaction mixture consisting of 500 μl of a 1% (w/v) substrate in 50 mM glycine–NaOH buffer (pH 9.0) containing 20% (w/v) NaCl and 500 μl of suitably diluted enzyme solution were incubated for 20 min at 65 °C. Oat spelt xylan, pectin, locust bean gum (LBG); starch and carboxymethyl-cellulose (low viscosity) were used as the assay substrates for xylanase, pectinase, mannanase, amylase and cellulase, respectively. The enzyme activity was determined by measuring the release of reducing sugars during the enzyme substrate reaction using dinitrosalicylic acid method [19]. One unit (U) of activity towards the substrates mentioned above was defined as 1 μmol of xylose, galacturonic acid, and mannose or glucose equivalent released per minute. β-D-Xylosidase activitiy was determined by measuring the release of *p*-nitrophenol from the *p*-nitrophenyl β-D-xyloside at 65 °C in 0.1 M Tris–HCl, pH 9.0, as described previously. One unit of activity is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min [20].

2.5. Enzyme purification

Cells were harvested by centrifugation at 6200 × *g* for 10 min at 4 °C. One litre culture supernatant was concentrated to 50 ml with a membrane protein concentrator with a molecular weight cut-off of 10 kDa (Vivacell 250). The concentrated enzyme solution was loaded on to a hydroxylapatite column (1 cm × 15 cm) equilibrated with 50 mM Tris–HCl buffer pH 7.5 containing 20% NaCl (buffer A). The column was washed with 40 ml of buffer A until no absorbance at 280 nm was detected in the eluent. The adsorbed proteins were eluted step wise with 45 ml of increasing concentration, i.e., 100, 200, and 300 mM of sodium phosphate buffer of pH 7.5 containing 20% NaCl (buffer B) at flow rate of 0.5 ml/min.

The active fractions containing the highest activity were pooled, concentrated, and passed through a gel filtration column Sephadex G-100 (1 cm × 65 cm). The column was pre-equilibrated and run with 50 mM of sodium phosphate buffer of pH 7.5 containing 20% NaCl at a flow rate of 0.2 ml/min of 1 ml fractions. The xylanase active fractions were pooled, concentrated and dialyzed against 50 mM glycine–NaOH buffer (pH 9.0) containing 20% (w/v) NaCl. This purified enzyme was used for further biochemical characterization. Protein content was determined by the method of Lowry et al. [21], with crystalline bovine serum albumin (Sigma) as a standard.

2.6. Gel electrophoresis and molecular weight determination

The purified xylanase was dialyzed over night against 50 mM glycine–NaOH buffer of pH 9.0 containing 10% (w/v) sucrose. The dialyzed sample was separated on non-denaturing electrophoresis slab gel (12% polyacrylamide) containing 10% sucrose as described by Cadenas and Engel [22]. After electrophoresis the slab gel was laid on the agar sheet containing 0.5% (w/v) xylan and 20% (w/v) NaCl in above mentioned buffer as replica plate and left for 2 h at 50 °C, the agar overlay was removed and stained with 0.2% (w/v) aqueous Congo Red solution [23]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12% polyacrylamide gel as described by Laemmli [24]. After electrophoresis, the protein gel was stained with Coomassie Brilliant Blue R-250.

2.7. MS/MS analysis of xylanase

The purified xylanase was excised from the gel and digested with trypsin, as described by Shevchenko et al. [25]. The resulting peptide mixture was analyzed on Agilent 6520 LC-ESI-MS Q-TOF system (Agilent, Boeblingen, Germany). The protein was first identified by searching the NCBI nonredundant database using the MASCOT MS/MS ion search, searched against all entries (Matrixscience, London). Further the peptide sequences obtained were submitted for the NCBI protein–protein blast and searched against bacterial xylanases.

2.8. Effect of pH, temperature, and salt concentration on activity and stability

The effect of pH on the activity of purified xylanase was measured in the range of 4.0–11.0, using the appropriate buffers at a concentration of 50 mM (4–6, sodium acetate; 7–8, Tris–HCl; 9–11, glycine–NaOH) under standard assay conditions. The effect of temperature on the purified enzyme was measured in the range of 30–90 °C. The activity of the purified enzyme was measured as above in the presence of 0–30% (w/v) NaCl.

The stability of xylanase at various pH values was determined by pre-incubating the enzyme at desired pH for 1 h at 65 °C. Relative enzyme activity was determined under the standard assay conditions. In order to monitor thermal stability, buffered samples of purified enzyme were incubated for 1 h at 50–90 °C. Aliquots were withdrawn and cooled on ice before assaying the residual enzyme activity under the standard assay conditions. The stability of the xylanase was determined in absence of NaCl by dialyzing the enzyme overnight against buffer containing 0% NaCl at 4 °C and the residual activity was determined under the standard assay conditions.

2.9. Effect of metal ions and chemical reagents

The effects of various salts, additives (1 mM final concentration) and detergents (0.1 and 1.0%) such as SDS, CTAB, Triton X-100 and

Tween-80 were investigated by pre-incubating the enzyme with different salts, additives and detergents in 50 mM glycine–NaOH buffer (pH 9.0) containing 20% (w/v) NaCl at 65 °C for 10 min under standard assay conditions. The degree of inhibition or activation of enzyme activity was expressed as a percentage of the enzyme activity in the control sample (no additives present).

2.10. Substrate specificity and kinetic properties

The substrate specificity of xylanase was tested against different sources of xylan (oat spelt, larch wood and birch wood) and other polysaccharides (cellulose, CMC, LBG, pectin and starch). Kinetic properties were determined by incubating the purified enzyme with increasing concentrations of oat spelt xylan (0.1–1.0%) using standard assay procedure. Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined from Lineweaver–Burke plot.

2.11. High performance liquid chromatography (HPLC) analysis of hydrolyzed products

The kinetics of product formation by xylanase activity was analyzed using oat spelt xylan as substrate under standard assay conditions. Samples corresponding to different incubation times were collected; the reaction was stopped by boiling and then centrifuged at 21,000 × *g* for 10 min. Each sample was then analyzed by HPLC analysis on a micro Bond pack Amino Carbohydrate column (4.1 mm × 300 mm). Samples (20 μl) were injected and eluted with acetonitrile: water (70:30 ratio) at a flow rate of 1 ml/min. The hydrolyzed products were detected using a refractive index detector. Xylose and xylobiose (Sigma) were used as standards.

2.12. Effect of organic solvents on xylanase activity

The activity of the purified xylanase was measured in the presence of 10% (v/v) concentration of different water-soluble and water-insoluble organic solvents under standard assay conditions.

2.13. Effect of osmolytes on activity of xylanase

The activity of the purified xylanase was measured at various concentrations (w/v) of osmolytes: betaine, sucrose, glycerol and mannitol in the absence of NaCl using oat spelt xylan as substrate at 65 °C and pH 9.0.

3. Results

3.1. Purification of xylanase

The total xylanase activity was eluted at 200 mM sodium phosphate buffer from the hydroxylapatite column. The enzyme was purified to near homogeneity through a Sephadex G-100 gel filtration column. The overall activity yield of the purified xylanase was 33% with specific activity of 5.37 U/mg of protein (Table 1). The xylanase activity in the native polyacrylamide gel was visualized by staining for activity as well as by Coomassie brilliant blue methods. The mobility of active xylanase band coincided with that of single protein band stained by Coomassie brilliant blue. The molecular mass of the purified xylanase was estimated to be 15 kDa by SDS-PAGE (Fig. 1).

3.2. Effect of pH

The enzyme showed activity over a wide pH range with an optimum pH 9.0 in presence of 20% NaCl at 65 °C (Fig. 2) and was stable

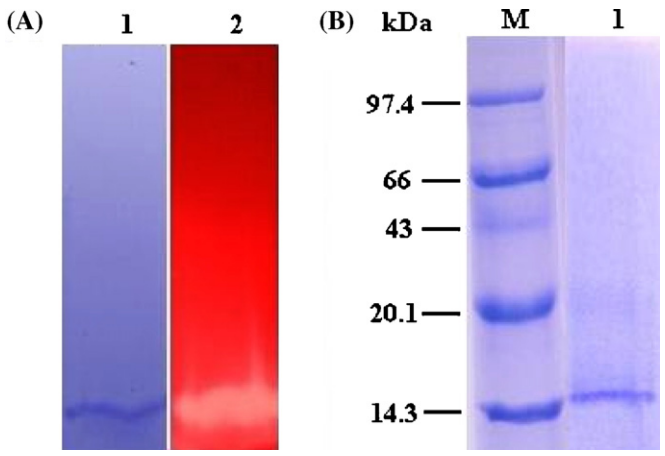

Fig. 1. Native PAGE and SDS-PAGE pattern of purified xylanase *Chromohalobacter* sp. TPSV 101. (A) Lane 1, Coomassie brilliant blue-stained native-PAGE gel of xylanase preparation, lane 2, zymogram analysis of xylanase. (B) Lane M, molecular weight markers: phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa); lane 1, SDS-PAGE pattern of purified xylanase from *Chromohalobacter* sp. TPSV 101.

in a pH range of 7.0–9.0 for 1 h. The activity declined at acidic conditions (55% inactivation at pH 5.0 and 85% inactivation at pH 4.0), but in an alkaline range the enzyme was relatively stable, with only 30% loss of activity at pH 10.0 (Fig. 2).

3.3. Effect of temperature

The activity of the xylanase was measured at various temperatures and the optimum temperature was 65 °C (Fig. 3A). Regarding the stability of xylanase at different temperature, it was found to be stable at 50–65 °C for 1 h retaining 100% activity and by retaining 60% activity at 80 °C.

3.4. Effect of NaCl

Optimal xylanase activity was detected in the range of 15–20% NaCl and more than 90% of the activity was observed at 30% NaCl (Fig. 3B). The xylanase retained more than 60% of its activity in absence of added NaCl. Furthermore the effect of NaCl was studied

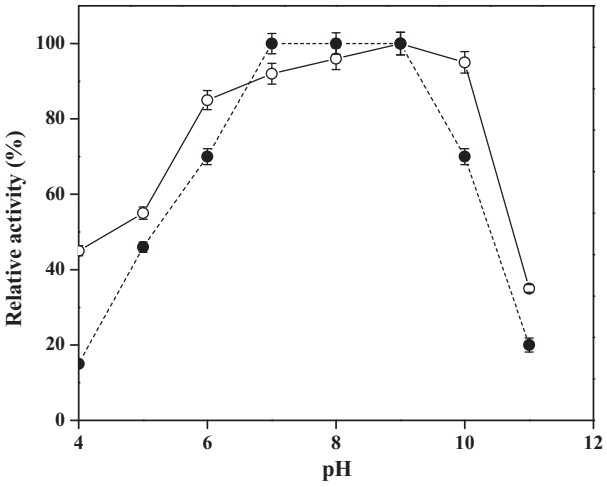

Fig. 2. The effect of pH on the activity (solid lines) of purified xylanase from *Chromohalobacter* sp. TPSV 101. For determining the pH stability (dotted lines), xylanase was incubated in different buffers of pH ranging from 4.0 to 11.0 for 1 h and relative activity was determined under standard assay conditions. Each value is an average of triplicate determination.

Table 1
Summary of the purification of xylanase from the supernatant of the culture *Chromohalobacter* sp. TPSV 101.

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude	406.6	800	0.50	1	100
10 kDa membrane	373.2	286	1.30	2.6	92
Hydroxylapatite	244	113	2.1	4.3	60
Gel permeation (G-100)	134.3	25	5.37	10.6	33

Table 2
Effect of salts and additives on the purified xylanase from the strain *Chromohalobacter* sp. TPSV 101.

Salt/additives	Concentration (1 mM)	Relative activity (%)[a]
None	0	100 ± 1.52
$MnSO_4$	1	134 ± 2.0
$CuSO_4$	1	79 ± 2.6
$CaCl_2$	1	84 ± .0
$ZnCl_2$	1	125 ± 0.57
$PbNO_3$	1	86 ± 1.0
$HgCl_2$	1	00
$CoCl_2$	1	121 ± 1.19
$NiCl_2$	1	98 ± 0.57
EGTA	1	132 ± 2.0
EDTA	1	128 ± 1.15
DTT	1	88 ± 1.17
1,10-Phenanthroline	1	68 ± 1.52
Urea	1	79 ± 1.50
SDS	0.1%	85 ± 1.1
	1%	61 ± 1.5
CTAB	0.1%	86 ± 1.6
	1%	63 ± 1.2
Triton X-100	0.1%	86 ± 1.5
	1%	64 ± 1.3
Tween 80	0.1%	86 ± 1.5
	1%	65 ± 1.4

[a]Each value is an average of triplicate determination.

on the stability of the xylanase by dialyzing the enzyme for 24 h against the buffer containing 0% NaCl. The xylanase was relatively stable in absence of NaCl retaining more than 50% of its residual activity after 4 days and lost its complete activity after 1 week (data not shown).

3.5. Effect of salts, chemical reagents and detergents

Xylanase activity was completely inhibited by Hg^{2+}, partially by Cu^{2+}, Ca^{2+}, and Pb^{2+} respectively, to the extent of 21, 16 and 14% (Table 2), and Mn^{2+}, Zn^{2+} and Co^{2+} enhanced the activity by 34, 25 and 21%, respectively. Among the additives tested 1,10-phenanthroline, DTT and urea inhibited the activity by 32, 12, and 21%, respectively. On the other hand, relative increase of xylanase activity was observed in the presence of EGTA and EDTA. Xylanase retained more than 85% of the residual activity among the (0.1%) detergents tested, whereas at 1%, it retained more than 60% of the residual activity (Table 2).

3.6. Substrate specificity and kinetic properties

Xylanase from *Chromohalobacter* sp. TPSV 101 showed maximum activity with oat spelt xylan followed by larch wood xylan (92%) and birch wood xylan (69%). No activity was detected against other carbohydrates tested such as CM-cellulose, locust bean gum, starch and pectin. However *p*-nitrophenyl-β-D-xyloside hydrolyzing activity was detected and found to be 38 ± 1.2 U. The K_m and V_{max} values of oat spelt xylan were 0.2 mg/ml and 1.17 μmol/min, respectively (Fig. 4).

3.7. HPLC analysis

The products obtained at various stages of the hydrolysis of oat spelt xylan by xylanase were examined. At the early stages of reaction (60 min) xylanase hydrolyzed oat spelt xylan to yield xylose and xylobiose (Fig. 5A). After prolonged (10 h) incubation the major hydrolysis product was xylose (Fig. 5B).

3.8. Effect of organic solvents

The effect of different organic solvents was tested in the presence of 20% NaCl and was found to be more active in presence of water-insoluble organic solvents such as benzene and n-hexane, whereas most of the water-soluble organic solvents partially inhibited the xylanase activity. On the other hand DMF inhibited 90% of the residual activity (Fig. 6).

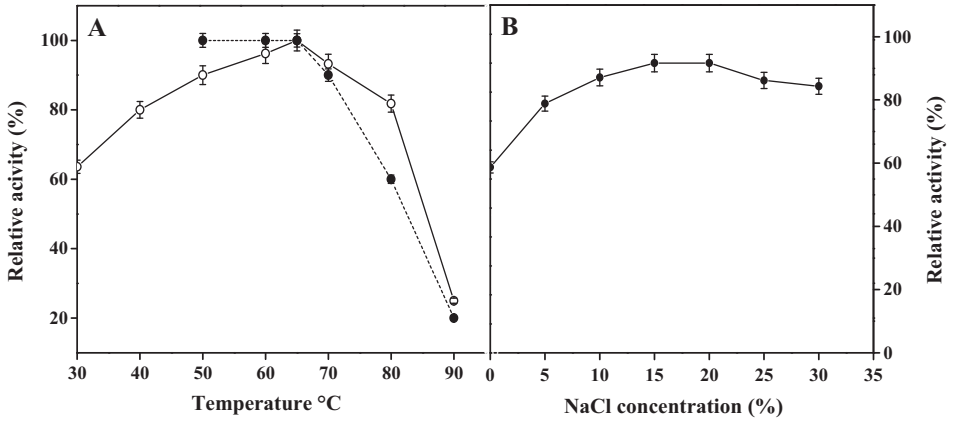

Fig. 3. (A) The effect of temperature on the activity (solid lines) of purified xylanase. For determining the thermostability (dotted lines), xylanase was incubated at 50–90 °C for 1 h and relative activity was determined at 65 °C under standard assay conditions. (B) The effect of NaCl on the activity of purified xylanase. Each value is an average of triplicate determination.

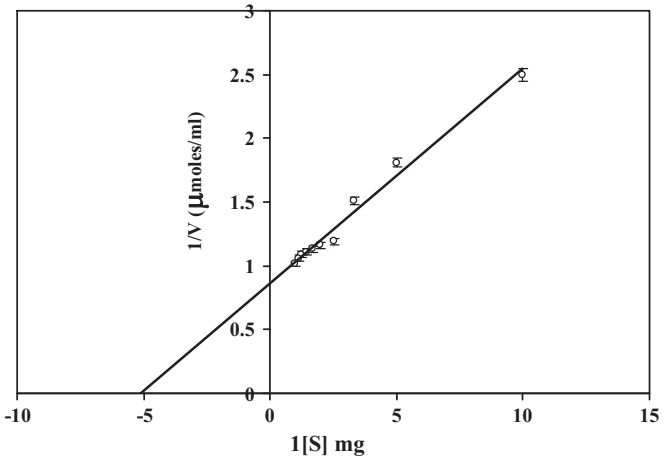

Fig. 4. Lineweaver–Burke plot for xylanase purified from the *Chromohalobacter* sp. TPSV 101. Xylanase activity was measured under standard assay conditions. Each value represents the mean ± S.E. of three independent experiments.

3.9. Effect of osmolytes on the activity of xylanase

The effect of different concentrations of osmolytes on the activity of xylanase in the absence of NaCl was determined. The xylanase was active retaining more than 90% of the activity at 10% sucrose, betaine, glycerol and mannitol (Fig. 7).

3.10. MS/MS identification

Although MS/MS ion search in mascot did not give any hit against known xylanases, further we searched in NCBI protein–protein against bacterial xylanases, blast search gave the four peptides matching to the different bacterial and fungul xylanases such as *Cellvibrio japonicus*, *Vibrio* sp. Ax-4, *Cellulomonas fimi*, *Streptomyces olivaceoviridis*, *Clostridium thermocellum*,

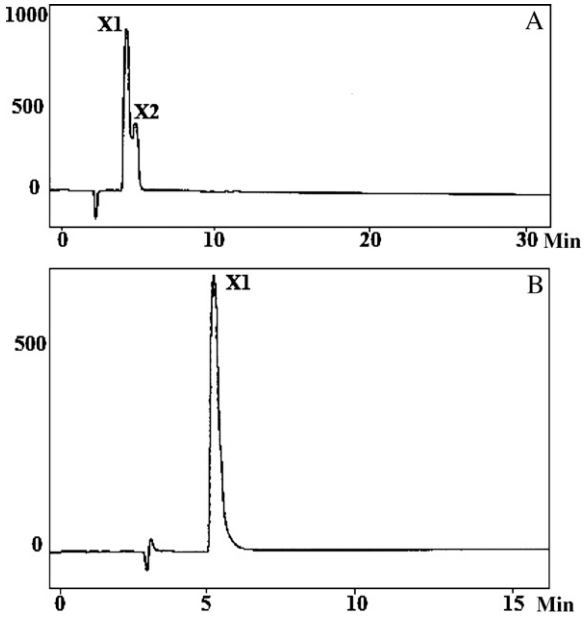

Fig. 5. HPLC profiles of the products released by the action of xylanase from *Chromohalobacter* sp. TPSV 101. Oat spelt xylan was treated with xylanase under standard assay conditions for 24 h at 50 °C. Samples were removed and analyzed for various xylooligosaccharides: hydrolysis pattern after (A) 1 h, (B) 10 h incubation. X1 = xylose, X2 = xylobiose.

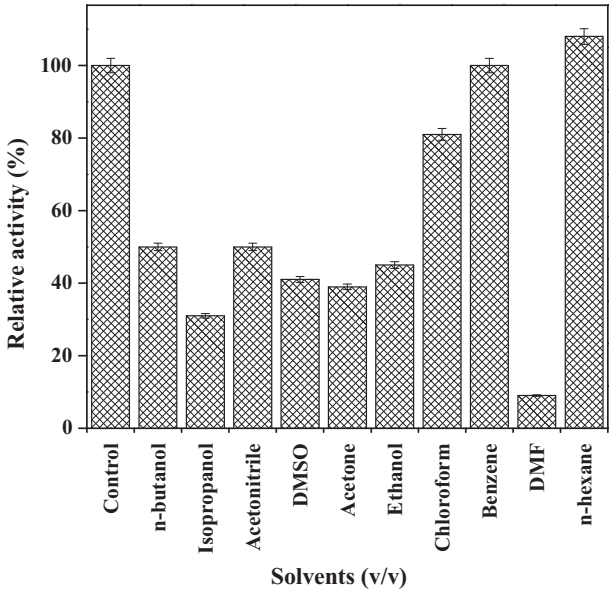

Fig. 6. The effect of organic solvents on the activity of purified xylanase from *Chromohalobacter* sp. TPSV 101 at 65 °C, pH 9.0, in the presence of 20% NaCl. Each value is an average of triplicate determinations.

Bacillus sp. *Ng*-27, *Trichodrema reesei* and *Scytalidium acidophilum*, the results are summarized in Table 3.

4. Discussion

A classical method to isolate enzymes with new industrial capabilities is to screen microorganisms that inhabit extreme environments. A group of these extremophiles are halophilic microorganisms and among them, moderately halophilic bacteria have been studied rarely. Since cell-free extract of *Chromohalobacter* sp. TPSV 101 contains cellulase-free xylanase and the same was purified to near homogeneity. The apparent purity was demonstrated by SDS-PAGE and zymogram techniques, and the molecular weight of the xylanase was about 15 kDa on SDS-PAGE. However the molecular weight of xylanases from halophilic *H. utahensis* (45 and 67 kDa) and strain CL 8 (43 and 62 kDa) [3,4] were found to be higher than the present xylanase.

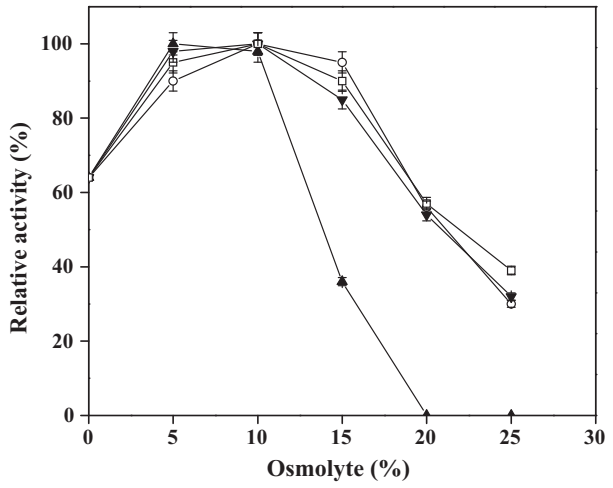

Fig. 7. The effect of osmolytes on the activity of purified xylanase from *Chromohalobacter* sp. TPSV 101 at 65 °C, pH 9.0, in the absence of NaCl. (○) Betaine, (▲) mannitol, (▼) glycerol, and (□) sucrose (w/v). Each value is an average of triplicate determinations.

Table 3
Identification of tryptic peptides of xylanase from *Chromohalobacter* sp. TPSV 101.

Amino acid sequence	Similar sequence in the database of the NCBI blast	Protein	Organism
MLGIIGGSGLYNLPGIK	S LY L GI	Cellulosomal xylanase Z	*Clostridiumt thermocellum*
	LYNL	Alkali thermostable F10 xylanase	*Bacillus* sp. *Ng*-27
	GGSG Y	Endo-1,4-β-xylanase	*Scytalidium acidophilum*
NAQLPDGATLGLALVSPKNGEVLALVGQK	ALV S NGEV	β-1,3-xylanase	*Vibrio* sp. Ax-4
	TLGL	C-Terminal xylan binding domain	*Cellulomonas fimi*
	G TL A++ NG	Endo-1,4-β-xylanase	*Streptomyces olivaceoviridis*
	AQ G TLG	Endo-1,4-β-xylanases	*Trichodrema reesei*
	DG TL GL	Endo-1,4-β-xylanase precursor	uncultured bacterium
IITDSFGNAVAKENLISLK	II FG V EN S + K	Thermostable xylanase	*Thermoascus aurantiacus*
	I DS F VA EN	Xylanase	*Cellulomonas fimi*
	AK NL + S	Native Xylanase10c	*Cellvibrio japonicus*
	AKEN	Endo-1,4-β-xylanase	Uncultured bacterium
VPVNQRGGISV	VPVNQRGGISV	Native Xylanase10c	*Cellvibrio japonicus*

Up to now there is no sequence information about the halophilic xylanase. This could be the one possible reason for the low sequence coverage and homology. These results suggest that the proteins from the halophilic microorganisms differ from that of the mesophilic proteins.

Biochemical properties of the enzyme such as temperature and pH profile revealed a moderately thermoactive and alkalitolerant character. These characters are higher than the xylanase from *H. utahensis* and strain CL 8 [3,4]. The enzyme was found to be thermostable in nature at high temperature, but its stability decays gradually at temperature above 80 °C. A similar range of optimal temperature has been reported for a halophilic xylanase of *Bacillus* sp. NTU-06 [8].

The xylanase was active over a broad range of salt concentrations (10–30%) retaining more than 90% of the activity. This type of halotolerance has been observed in extracellular xylanases and amylase from *H. utahensis* and *Chromohalobacter* sp. TVSP 101, respectively [3,26]. Whereas halophilic xylanase from *Thermoanaerobacterium saccharolyticum* NTOU1 and *B. halodurans* PPKS-2 displayed optimal activity at 12% NaCl [5,27]. Similarly amylases from marine haloalkaliphilic *Saccharopolyspora* sp. A9 displayed its optimal activity at 11% NaCl [28]. The effect of different metal ions on the activity of xylanase showed that the divalent metal ions Cu^{2+}, Ca^{2+}, and Pb^{2+} inhibited enzyme activity and other divalent cations Mn^{2+}, Zn^{2+} and Co^{2+} stimulated the enzyme activity. Similar results were reported in halotolerant xylanase from marine isolate *Bacillus subtilis* cho40 [29] and strain CL 8 [4]. The addition of EDTA and EGTA did not affect the activity, suggesting that no metals are required for the enzymatic reaction.

Purified xylanase catalyzed the hydrolysis of oat spelt xylan producing exclusively xylobiose and xylose after prolonged incubation (2–24 h); xylose was the main product. Similar mode of action was observed by *Bacillus stearothermophilus* T-6 and *Trichoderma viridae* [30,31]. The release of xylose was also reported (for xylanase) from *Bacillus* sp. strain SPS-0 and *Aspergillus oryzae* [32,33]. Hence this enzyme can be used in the hydrolysis of xylan-containing hemicellulosic materials to yield D-xylose, which can be converted into a variety of bioproducts.

Introducing an organic solvent reduces the polarity of the medium surrounding the enzyme molecules and the enzymes are usually inactivated in response to the increase in hydrophobic environment [34]. Xylanase from *Chromohalobacter* sp. TPSV 101 was active in most of the water-soluble organic-solvents tested. Similar results were reported for protease from halophilic strain *Salinivibrio* sp. AF-2004 [34], whereas amylases from halophilic strain *Haloarcula* sp. S-1 are inhibited by ethyl alcohol and acetone [35]. The xylanase was 100% active in the presence of water-insoluble organic solvents such as n-hexane and benzene similar to that of amylases from halophilic strain *Haloarcula* sp. S-1. The effect of organic solvents on the activity and stability of halophilic enzymes is not universal and seems to depend on the properties of the solvents and/or the particular enzyme [36]. This is the first report from halophilic xylanase that has been tested in organic media. The xylanase was active in osmolytes such as sucrose, betaine, and glycerol. Similar results were reported in haloalkaliphilic serine protease from *Halogeometricum* sp. TTS101 [37]. Osmolytes stabilized the xylanase in the absence of added NaCl. However, these solutes have cytoprotective properties, such as antioxidation and stabilization of proteins that go beyond simple compatibility and vary from solute to solute [38].

There are many reports on xylanases of mesophilic and thermophilic microorganisms [31,39,40], but only two reports have appeared on halophilic xylanases, produced by *H. utahensis* and halophilic strain CL8 which are optimally active at 15% NaCl and 5.8% NaCl, respectively [3,4]. However, the xylanase reported here is distinct from other mesophilic and halophilic xylanases in terms of salt concentration, pH and temperature.

Xylanase purified from *Chromohalobacter* sp. TPSV 101 is a novel enzyme, as it was active at moderate to extreme catalytic conditions such as high temperature, pH and NaCl and could be used in the paper and pulp industry to modify pulp properties by removing hemicelluloses without disturbing the cellulose fiber strength of paper products. Earlier we have shown that this enzyme has a potential application in the production of xylose and xylobiose from the agricultural waste [18].

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