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Cloning, Purification and Characterization of a Cellulase-Free Xylanase from *Geobacillus thermodenitrificans* AK53[1]

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Abstract—*Geobacillus thermodenitrificans* AK53 *xyl* gene encoding xylanase was isolated, cloned and expressed in *Escherichia coli*. After purifying recombinant xylanase from *G. thermodenitrificans* AK53 (GthAK53Xyl) to homogeneity by ammonium sulfate precipitation and ion exchange chromatography, biochemical properties of the enzyme were determined. The kinetic studies for GthAK53Xyl showed K_M value to be 4.34 mg/mL (for D-xylose) and V_{max} value to be 2028.9 μmoles mg^{-1} min^{-1}. The optimal temperature and pH for enzyme activity were found out to be 70°C and 5.0, respectively. The expressed protein showed the highest sequence similarity with the xylanases of *G. thermodenitrificans* JK1 (JN209933) and *G. thermodenitrificans* T-2 (EU599644). Metal cations Mg^{2+} and Mn^{2+} were found to be required for the enzyme activity, however, Co^{2+}, Hg^{2+}, Fe^{2+} and Cu^{2+} ions caused inhibitor effect on it. GthAK53Xyl had no cellulolytic activity and degraded xylan in an endo-fashion. The action of the enzyme on xylan from oat spelt produced xylobiose and xylopentose. The reported results are suggestive of a xylanase exhibiting desirable kinetics, stability parameters and metal resistance required for the efficient production of xylobiose at industrial scale.

Keywords: xylanase, *Geobacillus thermodinitrificans*, thermostable, GthAK53Xyl (recombinant xylanase)

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One of the most important sources of renewable energy is hemicellulose biomass. It is chiefly composed of xylan, mannan and galactan [1] and is the second most abundant natural polysaccharide. Xylans are heteropolymers made up of a linear chain of β-D-xylopyranose residues with β-(1,4) linkages substituted with sugars (arabinose, xylose, galactose, etc.), glucuronic acids, and other groups (e.g., acetyl, feruloyl, p-coumaryl). Xylan is among the most important hemicellulose components of the plant cell wall [2]. It is generally known that the complete hydrolysis of xylan requires the synergistic activities of several hydrolytic enzymes, mainly xylanase, β-xylosidase and additional enzymes such as α-l-arabinofuranosidase, α-d-glucuronidase, and acetyl xylan esterase [3]. Among these enzymes, the most important one is endo-β-1,4-xylanase (EC 3.2.1.8) which cleaves internal glycosidic bonds in xylan to create short xylo-oligosaccharides [4]. Majority of identified xylanases belong to glycoside hydrolase (GH) families 10 and 11 while others belong to GH families 5, 7, 8, 16, 26, 43, 52 and 62 [5]. Xylanases have captured much of the attention because of their potential applications in agro-industrial processes such as bioconversion of hemicellulosic biomass into fermentative sugars, pulp bleaching, bleach boosting, improvement of baking properties in bread making, enhancement of the digestibility of animal feed, clarification of fruit juices and xylitol production [5].

Large scale industrial production of enzymes is associated mainly with the substrate as well as depends upon the conditions prevailing in that particular industry where enzyme is required. The industrial demand of thermostable xylanases has not yet been met adequately. Moreover, xylanases are also required for hydrolyzing abundantly available and renewable ligniocellulosic biomass in order to produce xylooligosaccharides and fermentable sugars that are used as probiotics and for bioethanol production [6].

In consideration with the facts mentioned above, the present study aims to clone, purify and characterize extracellular thermophilic xylanase produced by *Geobacillus thermodenitrificans* AK53 (GthAK53Xyl) and to test it for cellulolytic activity. This xylanase was found to have the ability to degrade xylan into xylooligosaccharides.

[1] The article is published in the original.

MATERIALS AND METHODS

Substrates and chemicals. The chemicals were purchased from Fluka Chemie AG (Switzerland), Merck (Germany), Sigma-Aldrich (USA) and Acumedia Manufacturers, Inc. (USA). The Wizard genomic DNA purification kit, Wizard plus SV minipreps DNA purification system, *Taq* DNA polymerase, dNTP, and all restriction enzymes were obtained from Promega Corp. (USA). All chemicals were reagent grade. SDS-PAGE molecular mass standards (10–250 kDa) were purchased from New England Biolabs (UK).

Strains, vectors and media. *Escherichia coli* BL21 (DE3), *E. coli* JM101 and pET28a (+) vector were gently supplied from Karadeniz Technical University, Molecular Biology Laboratory (Turkey). *E. coli* containing recombinant plasmids was cultured according to the method of Karaoglu [7].

Screening of xylanolytic strains. Water and soil samples were collected from the hot springs of Azad Kashmir (Pakistan). Enrichment was performed using oat spelt xylan (Sigma-Aldrich, USA) as a sole carbon source. Twenty five bacterial strains were screened for xylanolytic ability.

Phenotypic characteristics. One prominent isolate with high xylanolytic activity was selected and then identified on the basis of morphological, cultural and biochemical properties [8] along with 16S rRNA sequencing.

Phylogenic analysis. The partial 16S rRNA sequences were recovered on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST tool. Similar sequences were downloaded in FASTA format. ClustalW2 program was utilized to perform multiple alignment of sequences and calculations of levels of sequence similarity. The obtained phylogenetic tree was analyzed for closely related organism. Neighbor-joining method [6] was used to deduce evolutionary history.

Amplification and sequencing of xylanase gene. Genomic DNA isolation was performed using the Wizard genomic DNA purification kit according to the manufacturer's directions. The extracted DNA was used for the amplification of the xylanase gene using XyGeoT-F: (5'CTAgCTAgCATgTTgAAAAgATCgCgAAAAg-3') and XyGeoT-R: (5'-CCCAAgCTTTCACTTATgATCgATAATAgCCCA-3') primers having *Nhe*I and *Hind*III sites in forward and reversed primers, respectively. The primers were designed using the conserved domain sequence of GthAK53Xyl available in NCBI database. The xylanase encoding gene was amplified using defined PCR conditions in a thermal cycler (Bio-Rad, USA). Initial denaturation was performed at 95°C for 3 min followed by 36 cycles of denaturation (94°C for 1 min), annealing (62°C for 1 min), and extension (72°C for 1 min 30 s) in a PCR vial containing 50 μL of reaction volume. Final extension was done at 72°C for 7 min. The amplified gene was cloned into pGEM®-T easy vector and positive clones were selected using blue white selection on 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, isopropyl β-D-1-thiogalactopyranoside, and LB-ampicillin agar plates. The white colonies were picked up and confirmed for the insert analysis by double digestion. Three clones having the insert were processed for sequencing.

Bioinformatics analysis. BLASTn and BLASTp programs were used for the analysis of nucleotides and their deduced amino acids, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment of xylanase was carried out using CLUSTALW program (http://www.ebi.ac.uk/clustalW) and phylogenetic analysis and dendrogram construction for the GthAK53Xyl was performed using MEGA 6.0 (with minimum evolution).

Construction and expression of the recombinant vector pET_GthAK53Xyl. Recombinant vector pET_GthAK53Xyl was constructed using the above primers having *Nhe*I and *Hind*III restriction sites compatible to pET28a (+) vector. The full-length xylanase gene was first amplified and then digested with *Nhe*I and *Hind*III. The digested product was purified by gel extraction and ligated into already digested and purified pET28a (+) vector using T4 DNA ligase overnight at 16°C. The ligated products were transformed into *E. coli* JM101 competent cells using heat shock method [8]. The positive clones were picked from LB agar plates with kanamycin (50 μg/mL) and confirmed by double digestion of the putative plasmids with respective restriction enzymes. Three plasmids having xylanase gene was sequenced using the Taq DyeDeoxy terminator cycle sequencing kit according to the manufacturer's instructions, and analyzed with Model 370A automated sequencer (Applied Biosystems, Inc., USA). Plasmids having the same xylanase sequences were processed for expression. The recombinant vector pET_GthAK53Xyl was transformed into *E. coli* BL21 (DE3) cells. Five clones were grown in LB broth overnight and 1% (vol/vol) of this was used as inoculum to cultivate *E. coli* BL21 (DE3) cells. The expression of xylanase gene was induced by 1 mM IPTG at OD_{600} of 0.5–0.6. The induced cells were further cultivated at 37°C for 20 h for higher expression of the protein.

Purification of the recombinant xylanase from *G. thermodenitrificans* AK53. After 20 h incubation at 37°C, the culture was reaped by centrifugation at 1300 *g* for 15 min and suspended in 100 mM Na-phosphate buffer (pH 7.0). Culture supernatant was precipitated using 60% ammonium sulphate. The obtained precipitate was dissolved in 100 mM Na-phosphate buffer (pH 7.0). Precipitate was dialyzed against the same buffer overnight and loaded on a column (1.5 × 50 cm) of DEAE-Sepharose pre-equilibrated with 20 mM Na-phosphate buffer (pH 7.0). The column was washed with 250 mL of the same buffer at flow rate of 1 mL/min. The enzyme was eluted with linear gradient of (0–0.5 M) NaCl in same buffer. The active frac-

tions were collected and concentrated by ultrafiltration (30000 MWCO filters, Sartorious, Germany).

Homogeneous enzyme test. To test the GthAK53Xyl purity a reverse phase C-18 column (4.6 × 250 mm; Merck, Germany) of HPLC System 600 Waters (Waters Corp. USA) was employed. The solvent system acetonitrile-water (70 : 30 vol/vol) at a flow rate of 0.5 mL/min was utilized for the separation of sample components. OD OD_{280} was read using a highly sensitive photodiode array detector (996 Waters).

SDS–PAGE, zymogram analysis and protein identification. The fractions containing GthAK53Xyl activity were analyzed by SDS-PAGE and zymography as described by Liao et al. [7]. SDS-PAGE was performed using an 11% polyacrylamide gel with a 5% stacking gel with the mini-protean II system (Bio-Rad, USA) according to Laemmli [9]. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). For the zymogram analysis, briefly, after the separation of the enzyme samples by SDS-PAGE, the zymogram gel was soaked in 0.1% oat spelt xylan at 37°C for 1 h in 2.5% (vol/vol) Triton X-100 to remove the SDS and re-nature the proteins in the gel, which was then washed thoroughly in MilliQ water (Millipore, USA) and incubated at 70°C for 20 min in 100 mM Na-phosphate buffer (pH 7.0). The gel was submerged in 0.1% (wt/vol) Congo red solution at 37°C for 30 min and destained with l.0 M NaCl until pale-red hydrolysis zones appeared against a red background. The reaction was stopped by dipping the gel into 5% acetic acid. To identify the protein sequence, a homology search was performed using Mascot (http://www.matrixscience.com). The partial amino acid sequence was used to identify analogous proteins through a BLAST search of the NCBI protein sequence database. Amino acid homology alignment of the predicted XYN11A with other highly homologous known xylanases was carried out.

Xylanase activity assay. The activity of GthAK53Xyl was measured by the release of reducing sugars from oat spelt xylan following the dinitrosalicylic acid method [10]. The xylanase assay was carried out by incubating suitably diluted enzyme with 1% xylan in 100 mM Na-phosphate buffer (pH 5.0) at 60°C for 20 min. The liberated sugars were estimated using 3,5-dinitrosalicylic acid reagent. One unit of xylanase was defined as the amount of enzyme that liberates 1 μmol of reducing sugar as xylose under the assay conditions using oat spelt xylan as the substrate.

Determination of protein concentration. Protein concentration was determined by the method of Bradford [11]. BSA was used as a standard.

Effect of the temperature on GthAK53Xyl activity and stability. Using xylan as substrate the effect of temperature (over the range from 40 to 100°C) on GthAK53Xyl activity was determined spectrophotometrically. The results were then expressed as relative activity (%) obtained at optimum temperature. For the determination of temperature stability of GthAK53Xyl the residual activity was measured after incubating enzyme for 200 min at 40, 50, 60, 70, 80, 90 and 100°C.

Effect of pH on GthAK53Xyl activity and stability. Enzyme's pH optimum was measured at 70°C using the buffer solutions of different pH values. The following buffers (100 mM) were used: Na-acetate (pH 5.0–6.0), K-phosphate (pH 6.0–7.0), Tris-HCl (pH 7.0–9.0) and glycine-NaOH (pH 9.0–10.0). The results were expressed as relative activity (%). For determination of the pH stability of enzyme, it was incubated at each pH value and 70°C temperature for 200 min, and then the residual activities were measured.

Effect of some metal ions on GthAK53Xyl activity. Bivalent metal ions Zn^{2+}, Mn^{2+} and Mg^{2+} were reported to be activators of xylanase [12]. The effect of various metal ions was assayed on GthAK53Xyl activity under optimum reaction conditions. To find activator effect the enzyme solution was pre-incubated with 1, 5 or 10 mM Zn^{2+}, Mn^{2+} or Mg^{2+}. To reveal the inhibitor effect of various metal ions on GthAK53Xyl activity enzyme was made devoid of its own metal ions by dialysis and then enzyme solution was pre-incubated with 1, 5 or 10 mM Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Fe^{2+} or Cu^{2+}. 100% xylanase activity was defined for enzyme without metal ions and residual (%) activity was assayed.

Analysis of hydrolysis product from oat spelt xylan. GthAK53Xyl after purification was mixed with 100 mM Na-phosphate buffer (pH 5.0) containing 1% (wt/vol) xylan and incubated at 70°C for 12 h. Samples were then taken and centrifuged at 3000 g for 10 min to remove insoluble materials. Aliquots of 3 μL were spotted on the TLC plates. Chromatography was then performed by the ascending method on silica gel 60 F_{254} TLC plates (20 cm × 20 cm), Merck, Germany) with a solvent system consisting of n-butanol, acetic acid and water (2 : 1 : 1, vol/vol/vol). For detection of sugars, plates were sprayed with 5% (vol/vol) sulfuric acid in ethanol and then heated for about 10 min at 120°C.

Determination of the GthAK53Xyl shelf life. For the determination of shelf life, enzyme was kept both at room temperature and at 4°C. For 12 weeks, samples were withdrawn at different intervals and residual activity of xylanase was determined.

RESULTS

Isolation and identification of bacteria. Twenty five bacterial strains isolated from the hot springs of Azad Kashmir (Pakistan) were screened for xylanolytic ability. Bacterial strains, which formed clear halos around their colonies on xylan agar plates, were picked up for further studies. The strain that showed the largest zone of hydrolysis around the colony proved its xylanolytic ability. It was identified as *G. thermodenitrificans* AK53 on the basis of various morphological and biochemical characteristics along with 16S rRNA sequencing.

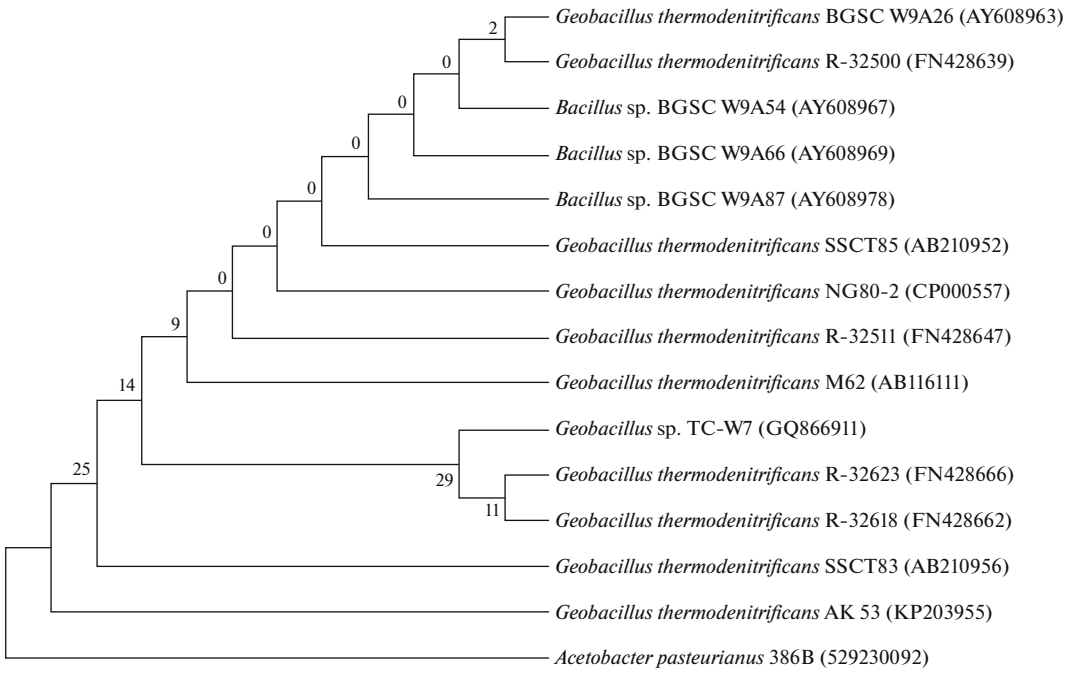

Fig. 1. Phylogenetic tree demonstrating the location of *G. thermodenitrificans* AK53.

Amplification and sequencing of xylanase gene. The gene encoding xylanase was amplified with previously described primer having 1200 bp from *G. thermodenitrificans* DNA as a template having translational initiation code ATG and termination code TGA.

Construction and expression of the recombinant vector pET_GthAK53Xyl. The cloning of xylanase gene into pET28a (+) vector was confirmed by double digestion of the recombinant vector with respective restriction enzymes. The construct pET_GthAK53Xyl was transformed and successfully expressed in *E. coli* BL21 (DE3) cells. A higher GthAK53Xyl production was achieved by inducing the expression of xylanase with 1 mM IPTG at 37°C.

Bioinformatics analysis. The isolate was confirmed as *G. thermodinitrificans* AK53 strain. The sequence of 16S rRNA gene of *G. thermodinitrificans* AK53 strain was deposited in GenBank with accession number (KP203955). Comparative 16S rRNA gene sequence analysis showed that the strain studied is phylogenetically most closely affiliated to the genus *Geobacillus*. The phylogenetic tree revealed that *G. thermodinitrificans* AK53 strain is closely associated (99.0%) with *G. thermodenitrificans* SSCT83 (AB210956) as shown in Fig. 1.

The deduced amino acid sequence contained more (Asp+Glu) negatively charged residues and no Cys residues were found in the xylanase. *In silico* analysis demonstrated the aliphatic index of 37. BLASTp analysis of amino acid sequence exposed extensive similarity with various endoxylanases of GH10 family and displayed a high homology with enzymes from *G. thermodenitrificans* JK1 (JN209933) and *G. thermodenitrificans* T-2 (EU599644) followed by other *Bacillus* and *Geobacillus* spp. (Fig. 2). Secondary and tertiary structure of GthAK53Xyl has been proposed using the available crystal structure of xylanase (PDB ID, 1HIZ chain A) from *G. thermodinitrificans*. The secondary structure contained a total of 13 β- sheets and 11-α helices along with 5 sharp turns. Three-dimensional structure, obtained from PyMol PDB viewer, showed catalytically important residues, Glu1 in conserved regions in "bowl" shaped structure of GH10 xylanase (Fig. 3).

Purification of the GthAK53Xyl. GthAK53Xyl was purified by ammonium sulfate precipitation and ion exchange chromatography on DEAE-Sepharose (Table 1). The yield of the purified xylanase was 138% with a specific activity of 1113.5 U/mg protein and an overall purification fold of 7.3. The GthAK53Xyl was eluted from DEAE-Sepharose column by 0.6 M NaCl (Fig. 4). The eluted protein appeared as a single band on 15% SDS-PAGE. The molecular weight of GthAK53Xyl was determined against denaturing protein markers and found to be of 45 kDa. The purity of the purified enzyme was confirmed by SDS-PAGE and reverse phase HPLC on C-18 column. The purified enzyme showed a single band in SDS-PAGE gel (Fig. 5a) and revealed a single peak at a retention time of 2.5 min on HPLC chromatogram (Fig. 5b) indicating that it was homogeneous.

Effects of temperature and pH on GthAK53Xyl activity. The purified GthAK53Xyl exhibited activity over a broad range of temperatures (40–100°C) and pH (3.0–

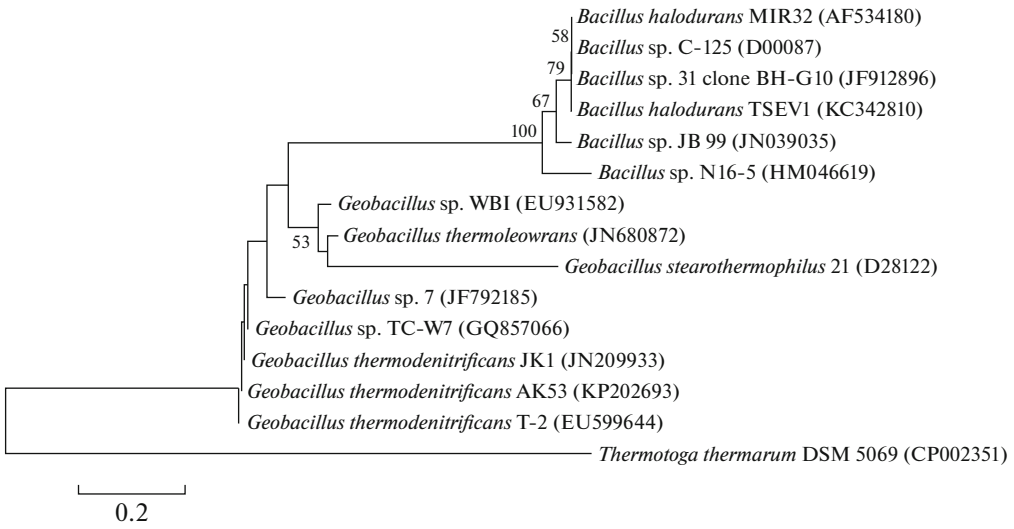

Fig. 2. Phylogenetic relationship of GthAK53Xyl with other xylanases available at NCBI database.

10.0) with optima at 70°C (Fig. 6a) and pH 5.0 (Fig. 7a), respectively. The recombinant enzyme retained more than 90% activity after exposure to 70°C for 200 min (Fig. 6b). On the other hand, GthAK53Xyl retained more than 80% activity after 3 h incubation at various pH values (5.0, 6.0 and 7.0) (Fig. 7b).

Effects of metal ions, inhibitors and detergents on GthAK53Xyl activity. The activity of the purified GthAK53Xyl was tested in the presence of several metal ions, inhibitors and detergents at different concentrations (Table 2). Metal cations Mg^{2+} and Mn^{2+} were found to be required for the enzyme activity. On the other hand, Co^{2+}, Hg^{2+}, Fe^{2+} and Cu^2 were reveled as strong inhibitors of the xylanase. The GthAK53Xyl has been pointedly inhibited by $CoCl_2$, $FeSO_4$, $CuCl_2$ and $CaSO_4$ even at very low concentration of 1 mM. $AlCl_3$, $ZnSO_4$, and $CuSO_4$ inhibited the enzyme at higher concentration (5.0 mM) only. Trisodium citrate (TSC) enhanced the enzymatic activity. β-mercaptoethanol, DTT and EDTA did not have adverse effect on enzyme activity, while diethylpyrocarbonate (DEPC), N-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF) caused inhibitory effect only at higher concentration (5.0 mM) (Table 2). The xylanase is quite stable in the presence of the detergents tested but half of its activity was inhibited in the presence of 1% cetyltrimethylammonium bromide (CTAB) concentration and more than 80% activity is retained even at 1% Tween 20 and 1% Tween 40.

Substrate specificity and kinetic parameters. The xylanase activity of GthAK53Xyl was evaluated with various substrates at 70°C (pH 5.0) for 20 min to determine the enzyme specificity. GthAK53Xyl showed specificity toward polymeric xylan, but not to other substrates such as insoluble xylan, carboxymethyl cellulose, filter paper, avicel, pNP-α-glucopyranoside,

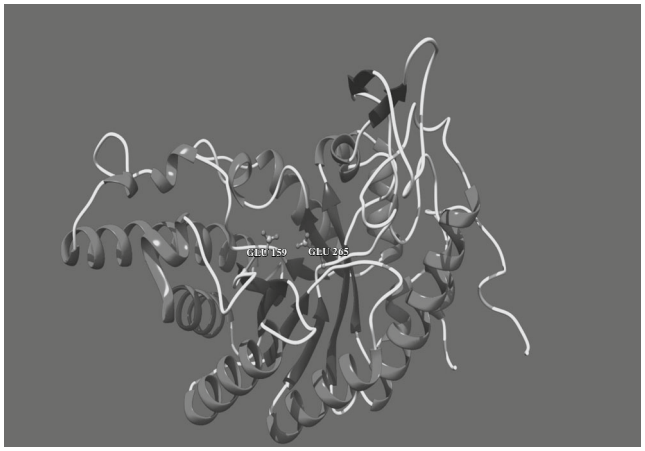

Fig. 3. The proposed structure of GthAK53Xyl.

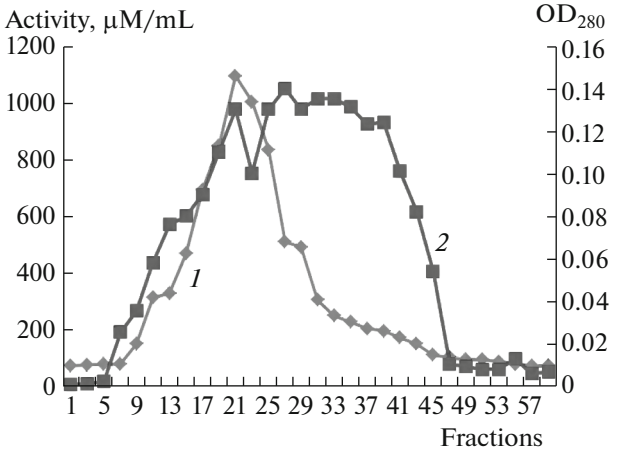

Fig. 4. DEAE-Sepharose chromatography of the endoxylanase produced by *G. thermodenitrificans* AK53. *1*—activity; *2*—protein.

Table 1. Purification of GthAK53Xyl

Purification step	Total protein, mg	Total activity, U	Specific activity, U/mg protein	Yield, %	Purification, -fold
Cell extract	4.58	695.4	151.5	100	1
Ammonium sulfate precipitation	2.90	821.8	282.7	118	1.8
DEAE-Sepharose	0.85	952.7	1113.5	138	7.3

pNP-β-xylopyranoside, pNP-β- galactopyranoside, pNP-α-L-arabinofuranoside, pNP-acetate and pNP-α-D- xylopyranoside.

The K_M and V_{max} of GthAK53Xyl (for oat spelt xylan) were found to be 4.34 mg/mL and 2028.9 μmoles mg^{-1} min^{-1}, respectively.

Mode of hydrolysis. The mode of action of purified GthAK53Xyl was analyzed by TLC of the hydrolyzed product of oat spelt xylan. As shown in Fig. 8, the enzyme released a range of xylooligosacchrides from xylan. The main products were xylobiose and xylopentose.

Determination of shelf life of xylanase. The purified enzyme did not lose any activity when stored at 4°C for 12 weeks but thereafter, a decline was observed. The GthAK53Xyl retained more than 90% of initial activity after 10 weeks which would be important for its

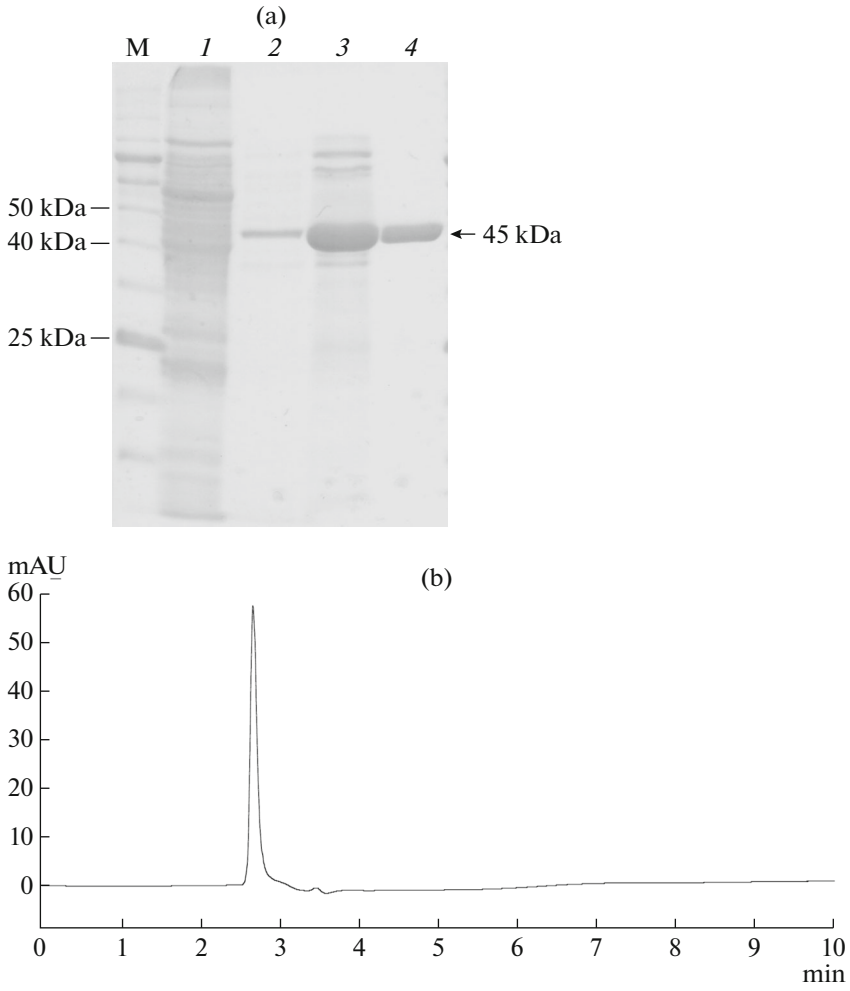

Fig. 5. SDS-PAGE (a) of crude and purified GthAK53Xyl (an arrow). M—SDS-PAGE molecular mass standards 10–250 kDa; *1*—culture supernatant; *2*—induced supernatant; *3*—ammonium sulfate precipitate; *4*—eluate from DEAE-Sepharose. (b)—HPLC profile of the purified GthAK53Xyl.

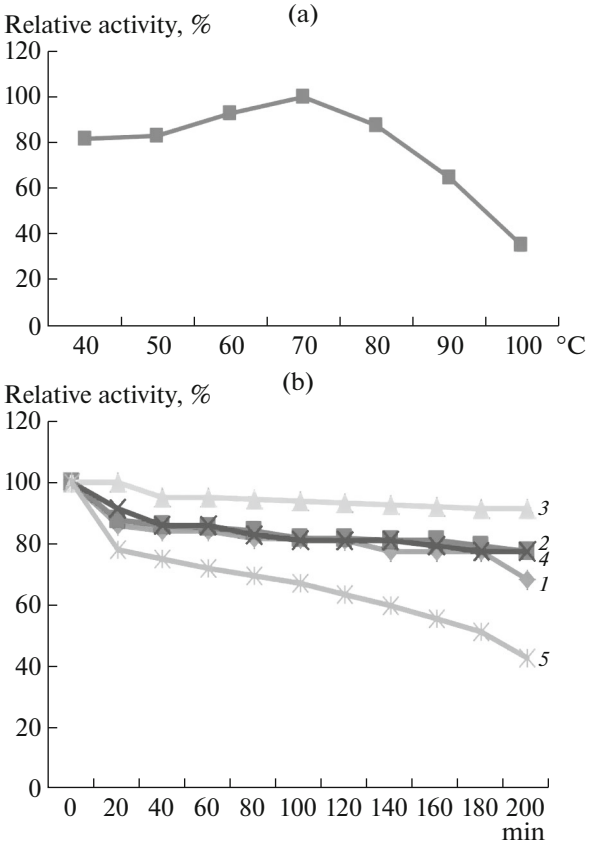

Fig. 6. The effect of temperature on the activity (a) and stability (b) of purified GthAK53Xyl. b: *1*—50°C; *2*—60°C; *3*—70°C; *4*—80°C; *5*—90°C.

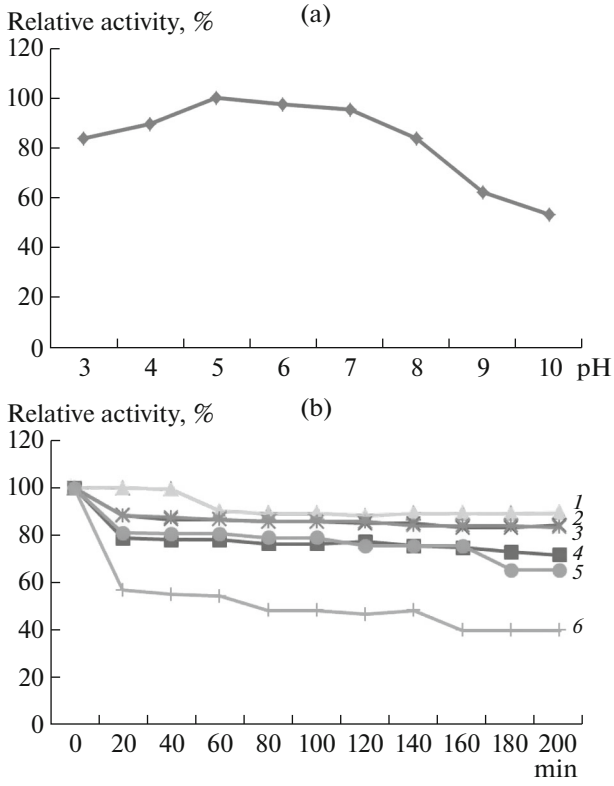

Fig. 7. The effect of pH on the activity (a) and stability (b) of purified GthAK53Xyl. b: *1*—5.0; *2*—6.0; *3*—7.0; *4*—4.0; *5*—8.0; *6*—pH 9.0.

application. On the other hand, at room temperature, the enzyme was completely stable for 5 weeks but showed 80 and 70% residual activity after storage for 10 and 12 weeks, respectively.

DISCUSSION

Microorganisms are well known to have adapted to the environment by producing extracellular enzymes which are expected to form nutrients by degrading complex macromolecules into soluble monomers for supporting the growth of the producing microbes. Isolate AK53 from the Tattapani hot spring of Azad Kashmir (Pakistan) was revealed to be a strain of *G. thermodenitrificans* based on biochemical analysis and 16S rRNA sequencing. Xylanase produced by the bacterium displayed optimal activity and stability at elevated temperatures (50–80°C) and under neutral to slightly acidic conditions (pH 5.0–7.0). The xylanase gene cloned from *G. thermodenitrificans* is of 1200 bp like those of *Bacillus* and *Geobacillus* spp. [13, 14].

In this study, GthAK53Xyl gene was cloned to pET-28a (+) expression vector and expression, purification and characterization of recombinant xylanase was carried out. Enzyme activity was determined according to the revealed D-xylose amount. after the enzyme reaction.

Both, the activity and stability of GthAK53Xyl were tested in the range pH 3.0–10.0. The enzyme exhibited the highest activity around pH 5.0. It was observed that GthAK53Xyl was active and stable at 70°C in a broad pH range from 4.0 to 8.0. The pH stability of the enzyme is very important for the prediction of its storage conditions. As GthAK53Xyl did not lose activity after being stored at different pH values, it

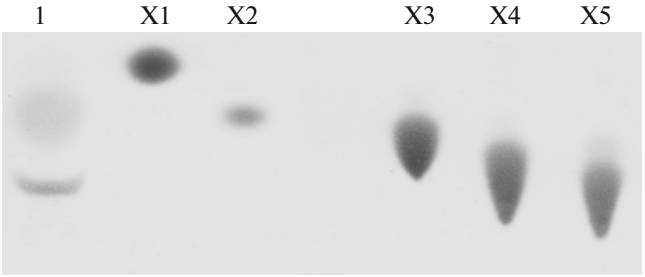

Fig. 8. TLC analysis for hydrolysis products released from oat spelt xylan by GthAK53Xyl. 1—sample; X1—D-xylose; X2—xylobiose; X3—xylotriose; X4—xylotetraose; X5—xylopentose.

Table 2. The effect of various metal ions, reagents and detergents on GthAK53Xyl acitivity

Metal Ions*	1 mM	5 mM	10 mM
Co^{2+}	88	60	51
Ag^{+}	108	101	80
Cs^{+}	117	107	104
Al^{3+}	99	84	48
Mn^{2+} (SO_4)	144	109	100
Zn^{2+}	116	82	68
Mg^{2+} (SO_4)	110	99	97
Ca^{2+} (Cl_2)	108	98	89
Fe^{2+}	89	46	27
K^{+}	108	109	111
Li^{+}	108	111	124
Cu^{2+} (Cl_2)	88	72	65
Mn^{2+} (Cl_2)	99	94	86
Ca^{2+} (SO_4)	89	71	60
Mg^{2+} (Cl_2)	124	99	94
Cu^{2+} (SO_4)	99	86	62
Hg^{2+}	54	27	
Reagents			
DTT	98	96	89
TSC	118	115	114
EDTA	89	80	70
β-mercaptoethanol	99	98	98
DEPC	94	89	68
NEM	90	89	61
PMSF	80	71	58
Detergents	0.1%	0.5%	1%
SDS	91	86	84
CTAB	85	53	49
Tween 20	96	89	82
Tween 40	90	86	80
Triton X-100	107	84	74

* Control—100%, values are given in% of control.

can be stored under conditions with a broad range of pH for a long period of time.

GthAK53Xyl exhibited optimum activity at 70°C. Most of the previously studied *G. thermodenitrificans* xylanases have been reported to operate at an optimum temperature of 70°C [13, 14]. Xylanases from some other microorganisms have higher temeprature optima, e.g 85°C for enzymes of *Streptomyces* sp. and *Bacillus sp*, 95°C for enzyme of *Thermotoga neapolitana* [15, 7]. On the other hand, xylanases from *Paenibacillus* sp. DG-22, *Geobacillus* sp. 70PC53, *Bacillus firmus*, *Thermobifida fusca*, and *Bacillus* sp. NG-27 were optimally active in the temperature range between 60 and 70°C [16, 17].

The effect of different metal ions on GthAK53Xyl activity was unique among all the characterized xylanases of *Geobacilli* [13]. Xylanases of the other *Geobacilli* were inhibited by Mn^{2+} or it had no effect on the activity, while xylanase of *G. thermodenitrificans* JK1 was slightly stimulated by these ions. In contrast, Mn^{2+} ions at low concentration stimulated GthAK53Xyl in the presence of SO_4^{2-} ions. Co^{2+}, Hg^{2+}, Fe^{2+} and Cu^{2} were reveled as strong inhibitors of the enzyme studied. Similarly, *Penicillium sclerotiorum* and *Aspergillus ficuum* xylanases were introverted by these ions [18–20]. The inhibition by Hg^{2+} seems to be a universal property of xylanases, demonstrating the presence of thiol groups of Cys residues in active sites or around them [20]. The inhibition of GthAK53Xyl by Hg^{2+} confirmed the presence of Trp residues in the active site, since oxidation of indole ring disrupts the interaction [14]. The inhibition of GthAK53Xyl by Cu^{2+} is similar to those of *Bacillus* sp. YJ6, *Staphylococcus* sp. SG-13, *Plectosphaerella cucumerina, Cellulosimicrobium* sp. MTCC 10645, *Aspergillus versicolor*, *A. ficuum* AF-98 and *G. thermodenitrificans* TSAA1 [14, 21–26]. Xylanase of *G. thermodenitrificans* JK1 was inhibited even at low concentration of Zn^{2+} while GthAK53Xyl was inhibited only at higher concentration of this ion.

The GthAK53Xyl was inhibited by N-bromosuccinimide (N-BS) only at high concentration (10 mM), where xylanase from other *Geobacillus* species was inhibited even at low concentration [14]. N-BS, Trp modifier, reduced xylanase activity at 5 and 10 mM; this is an evidence for the role of aromatic amino acid residues in the active site [27]. The xylanases were quite stable in the presence of the detergents tested in *Geobacillus* sp., *G. thermodenitrificans* TSAA1 [14], *G. thermoleovorans*, sp. MT-1 and *Dictyoglomus thermophilum* Rt46B.1 [21, 28]. The slight inhibitory effect of different detergents (besides CTAB) on GthAK53Xyl (Table 2) is similar to other microbe xylanases [21]. Tweens (40 and 60) and Triton X-100 inhibited GthAK53Xyl only at high concentration and these results are comparable to those in *Aspergillus awamori* [22]. Even at 1% SDS the enzyme retained more than 80% residual activity while xylanase from *Bacillus*, *Geobacillus* [14] and *Paenibacillus* sp. NF1 was strongly inhibited in presence of this detergent [5].

Almost all known xylanases of *Geobacilli* belong to the GH10 family [29]. GthAK53Xyl was also found to belong to this family. It was ~45 kDa in size, while xylanolytic enzymes of other *Geobacilli* were 30, 35, 36, and 40 kDa [30, 28]. 43 kDa xylanase of *G. thermodenitrificans* JK1 [13] and 47 kDa enzyme of *G. thermodenitrificans* TSAA1 [14] were the most similar in size to GthAK53Xyl. However, the xylanase

Table 3. Comparison of properties of recombinant xylanases from 3 different strains of *G. thermodenitrificans*

Strains	pH optima	Tem. optima, °C	Thermostability	pH stability, min	References
G.thermodenitrificans JK1	6.0	70	T1/2 (70°C), 0.5 h	Not determined	[13]
G. thermodenitrificans TSAA1	9.0	70	>85% activity retained at 70°C for 3 h; T 1/2 (80°C), 10 min	>90% activity retained for 3 h at pH 5.0	[14]
G.thermodenitrificans AK53	5.0	70	>90% activity retained at 70°C for 3 h	>85% activity retained for 3 h at pH 5.0	Present investigation

studied differed from these enzymes in their pH and temperature optima as well as pH stability and thermostability (Table 3).

As cellulase activity may result in poor fiber mechanical strength, xylanase should be free from cellulolytic activity for paper and pulp treatment [31]. Thus, the cellulase-free xylanase has an advantage in the production of high quality pulp. Our result indicated that GthAK53Xyl had a cellulase-free nature and can be used in the production of high quality pulp.

Substrate specificity and kinetic parameters of GthAK53Xyl revealed that the enzyme contained no other enzyme activity. The result of TLC analysis of the hydrolysis products of oat spelt xylan indicated the enzyme as endoxylanase (Fig. 8). Xylobiose and xylopentose were the major end products of hydrolysis. The K_M of the enzyme was within the range for K_M of other microbial xylanases (0.14–14 mg/mL) [14] and closely resemble to the K_M of *Pichia stipites* xylanase (4.2 mg/mL [32]). The K_M value of GthAK53Xyl with oat spelt xylan as substrate was lower than those for the xylanases isolated from other microorganisms, such as *Aspergillus niger* SCTCC 400264 [33], *Scytalidium acidophilum* [34], *Bacillus* sp [24], *Aspergillus usamii* E001 [35], *Paenibacillus* sp. NF1 [5].

In summary, due to biochemical properties, high activity, profile of produced xylooligosaccharides and resistance to some detergents and metals, GthAK53Xyl is an attractive candidate for the production of xylooligosaccharides from the paper and pulp and food industries as compared to other xylanases from *G. thermodenitrificans*.

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