JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CLONING, PURIFICATION AND CHARACTERIZATION OF HALOTOLERANT XYLANASE FROM *Geobacillus Thermodenitrificans* C5

*[1]Muhammad Irfan, [2]Halil Ibrahim Guler, [1]Aamer Ali Shah, [3]Fulya Ay Sal, [2]Kadriye Inan, [3]*Ali Osman Belduz*

Address(es):
[1]Departmet of Microbiology, Faculty of Biological Sciences, Quaid I Azam University, Islamabad, Pakistan.
[2]Department of Molecular Biology and Genetic, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey.
[3]Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey.

*Corresponding author: belduz@ktu.edu.tr

doi: 10.15414/jmbfs.2016.5.6.523-529

ARTICLE INFO

Received 18. 2. 2015
Revised 13. 1. 2016
Accepted 14. 1. 2016
Published 1. 6. 2016

Regular article

OPEN ACCESS

ABSTRACT

High levels of extracellular xylanase activity (994.50 IU/ml) produced by *Geobacillus thermodenitrificans* C5 originated gene was detected when it was expressed in *E. coli* BL21 host. Thermostable xylanase (GthC5Xyl) was purified to homogeneity and showed a molecular mass of approximately 44 kDa according to SDS-PAGE. The specific activity of the purified GthC5Xyl was up to 1243.125IU/mg with a 9.89-fold purification. The activity of GthC5Xyl was stimulated by $CoCl_2$, $MnSO_4$, $CuSO_4$, $MnCl_2$ but was inhibited by $FeSO_4$, Hg, $CaSO_4$. GthC5Xyl showed resistant to SDS, Tween 20, Triton X-100, β- Mercaptoethanol, PMSF, DTT, NEM and DEPC, SDS, and EDTA. A greater affinity for oat spelt xylan was exhibited by GthC5Xyl with maximum enzymatic activity at 60°C and 6.0 pH. The activity portrayed by GthC5Xyl was found to be acellulytic with stability at high temperature (70°C-80°C) and low pH (4.0 to 8.0). Xylobiose and xylopentose were the end products of proficient oat spelt xylanase hydrolysis by GthC5Xyl. High SDS resistance and broader stability of GthC5Xyl proves it to be worthy of impending application in numerous industrial processes especially textile, detergents and animal feed industry.

Keywords: *Geobacillus thermodenitrificans*, thermostable GthC5Xyl enzyme, recombinant xylanase

INTRODUCTION

Hemicellulose in plant cell walls contain xylan as the major component which accounts for one third of the earth's total renewable organic carbon source **(Cheng *et al.*, 2014).** These hetero-polysaccharides are primarily composed of xylose subunits linked by β-1, 4-glycosidic bonds which forms the backbone. This backbone further contains different side groups such as acetyl groups, methyl groups and other sugar molecules **(Collins *et al.*, 2005).** As xylan has a complex and intricate structure, it requires a combination of enzymes for its complete breakdown. These enzymes include endo-1, 4-β-xylanase, β-xylosidase, acetylxylan esterase, arabinose and glucuronidase (Khandeparker and Numan, 2008) out of which the most potent and thus most important one are the endo-1, 4-b-xylanases **(Li *et al.*, 2014).** Xylan is degraded to short xylo-oligosaccharides of various lengths by the action of these enzymes. Xylanases conjoin with β -xylosidases (EC 3.2.1.37) for completely hydrolyzing xylan to xylose monomers. There are various benefits of carrying out xylan hydrolysis at high temperatures by utilizing thermostable enzyme. These advantages include increased reactant and products solubility due to low viscosity, a higher mass transport rate, a decreased hazard of contamination by mesophilic microorganism and half-lives at elevated temperatures leads to better hydrolysis **(Bhalla *et al.*, 2014).** One of the important concern that still persists is the enzyme stability while thermal processing. The changes in the 3D structural confirmation of enzymes at high temperature lead to enzyme inactivation **(Bankeeree *et al.*, 2014).**
There is a frequent isolation and identification of *Geobacillus* from various sources because of its ability to produce thermostable endo-xylanases for xylan hydrolysis. Examples of *Geobacillus* strains producing xylanases with potential industrial applications are novel thermostable endo-xylanase from *Geobacillus sp.* WSUCF1**(Bhalla *et al.*, 2014),** *Geobacillus stearothermophilus* 1A05583 **(Yan Wang *et al.*, 2013)** producing xylanase, *Geobacillus thermodenitrificans* TSAA1 producing Thermostable and Alkalistable Endoxylanase (Verma *et al.*, 2013), novel thermophilic xylanase from *Geobacillus thermodenitrificans* **JK1 (Gerasimova and Kuisiene, 2012),** *G. thermoleovorans* producing highly thermophilic endoxylanase **(Verma and Satyanarayana, 2012),** thermophilic *Geobacillus* sp. 7 1**(Canakci *et al.*, 2012)** producing an alkali-stable endoxylanase.

For application in industry, it is necessary for xylanase to be acellulolytic **(Goswami *et al.*, 2014)** and requiring minimum downstream processing for its production. The aim of this study was to carry out the heterologous expression of *Geobacillus thermodenitrificans* xylanase gene in *E. coli* for increased xylanase production as well as to secrete this expressed enzyme via *E. coli* in the medium to minimize its downstream processing for industrial application. Purification and biochemical characteristic's analysis was further carried out.

MATERIALS AND METHODS

Bacterial Strains, Substrates, Vectors and Chemicals

The chemicals were obtained commercially from Merck A.G. (Darmstadt, Germany), Sigma Chem. Co. (St. Louis, MO, USA), Fluka Chemie A.G. (Buchs, Switzerland), and Acumedia Manufacturers, Inc. (Baltimore, Maryland, USA), Aldrich-Chemie (Steinheim, Germany). The, Wizard Plus SV Minipreps DNA Purification System, Wizard Genomic DNA Purification Kit, dNTP, restriction enzymes and *Taq* DNA Polymerase were obtained from Promega Corp. (Madison, WI, USA). All chemicals were reagent grade and all solutions were made with deionized and double distilled water. *E. coli* JM101: *E. coli* BL21 (DE3): pET28 (a) + were gently provided by Karadeniz Technical University, Molecular Biology Laboratory. The method of Karaoglu was used for culturing of recombinant *E. coli* (Karaoglu *et al.*, 2013).

Screening and Phylogenic Analysis for Xylanase Producing Bacteria

Water and soil samples were collected from the Garam Chashma hot springs of Chitral KPK Pakistan. Enrichment was done using oat spelt xylan (Sigma Chemicals, Germany) as a sole carbon source. Twenty bacterial strains were screened for xylanolytic ability. One prominent isolate was selected and then identified on the basis of cultural, morphological and biochemical properties **(Sneath, 1994)** along with 16S rRNA sequencing (Supplementary Table 1).
NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was utilized to recover the partial 16S rRNA via BLAST tool. Similar sequences were then downloaded in FASTA format. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using ClustalW2 program. Analysis for

closely related organisms was carried out by using obtained phylogenetic tree. Evolutionary history was deduced by Neighbor joining method **(Saitou and Nei, 1987).**

Amplification, Sequencing and Bioinformatics Analysis of Xylanase

Wizard Genomic DNA Purification Kit was used to isolate Genomic DNA. Xylanase gene was amplified by using XyGeoT-F: (5'CTAgCTAgCATgTTgAAAAgATCgCgAAAAg-3') having *Nhe*I and XyGeoT-R: (5'- CCCAAgCTTTCACTTATgATCgATAATAgCCCA-3') having *Hind*III restriction sites in defined PCR conditions. Thermo Cycler (Bio-Rad, Hercules, CA) was used for amplification of xylanase encoding gene. Initial denaturation was at 94℃ for 3 minutes followed by 36 cycles (denaturation (94℃ for 1 min), annealing (62℃ for 40 sec), and extension (72℃ for 90 sec) were performed in a PCR vial containing 50 µl of reaction volume, and final extension was done at 72℃ for 8 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 ampicillin plate. The white colonies were picked up and confirmed for the insert by double digestion. Two clones having the insert were processed for sequencing.
Nucleotides and their deduced amino acids were analyzed by using BLASTn and BLASTp programmers respectively (http://www.ncbi.nlm.nih.gov/BLAST). CLUSTALW program was used for carrying out multiple sequence alignment of xylanase (http://www.ebi.ac.uk/clustalW) and MEGA 4.0 (with minimum evolution) was used for phylogenetic analysis and dendogram construction of xylanase.

Construction and Expression of the Recombinant Vector pET_GthC5Xyl

Complete xylanase gene was amplified and purified by gel extraction and ligation was done into pGEM®-T Easy Vector. Heat shock method **(Belduz *et al.*, 1997)** was used to transformed the ligated product in *E. coli* JM101 competent cells.
Recombinant vector pET_GthC5Xyl having *Nhe*I and *Hind*III restriction sites compatible to pET28a (+) vector was constructed. The xylanase gene cloned pGEM®-T Easy Vector was isolated and digested with respective restriction enzymes. Xylanase gene product was purified by gel extraction and ligated into already purified and digested pET28a (+) vector using T4 DNA ligase overnight at 16°C. Heat shock method **(Belduz *et al.*, 1997)** was used to transform the ligated product in *E. coli* JM101 competent cells. The positive clones were established by double digestion of the recombinant plasmid with specific restriction enzymes. Two plasmids having xylanase gene was sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit according to the manufacturer's instructions, and analyzed with an Applied Biosystems (Macrogen, Korea) Model 370A automatic sequencer. The recombinant vector pET_GthC5Xyl was transformed into *E. coli* BL21 (DE3) cells. Four clones were grown in LB broth overnight and 1% (v/v) of this was used as inoculum to cultivate *E. coli* BL21 (DE3) cells. 1 mM IPTG was used for induction of the recombinant xylanase at OD_{600nm} of 0.5–0.6. For higher expression of xylanase the induced cell were cultivated for 20 h.

Purification of the Recombinant GthC5Xyl

After higher expression of recombinant xylanase the culture was centrifuged for 15 minutes at 10,000 rpm and suspended in 50 mM sodium phosphate buffer. 60% ammonium sulphate was used for precipitation of enzyme and then DEAE affinity chromatography was used to purify the enzyme. The obtained 60% precipitates were loaded on a column (1.5 × 50 cm) of DEAE-Sepharose pre-equilibrated with 10 mM sodium phosphate buffer pH 6.0. The column was washed with 1000 ml of the same buffer at flow rate of 0.5 ml/min. After washing, column was eluted with linear gradient of (0.55 M) NaCl in sodium phosphate buffer. The active fractions were combined and concentrated by ultrafiltration (Sartorious, 30000 MWCO filters).
GthC5Xyl enzyme purity was checked by 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).The solvent system acetonitrile-water (70:30) at a flow rate of 0.5 ml/min was employed for the separation of sample components. Absorbance was read at 280 nm using a highly sensitive photo-diode array (PDA) detector (996 Waters).

SDS-PAGE, Zymogram Analysis and Protein Identification

The fractions containing xylanase activity were analyzed by SDS-PAGE and zymography as described by Liao *et al.* **(Karaoglu *et al.*, 2013**; **Liao *et al.*, 2012).** SDS-PAGE was performed using an 11% (w/v) polyacrylamide gel with a 5% stacking gel with the Mini-Protean II system (Bio-Rad, Hercules, CA) according to Laemmli (1970). The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA). For the zymogram analysis, briefly, after performing SDS-PAGE the zymogram gel was soaked for 30 minutes in 2.5% (v/v) Triton X-100 to remove the SDS and re-nature the proteins in the gel, which was then washed thoroughly in 50 mM Phosphate buffer (pH 6) and incubated at 60 °C for 30 min in 1% xylan. The gel was flooded in 0.1% (w/v) Congo red solution for 15 min and destained with l M NaCl until hydrolysis zones appeared against a red background. The reaction was then stopped by dipping the gel into 5% acetic acid solution. To classify 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 GthC5Xyl with other highly homologous known xylanases was carried out.

Activity Assay, Determination of Protein Concentration and Kinetics of Xylanase

Dinitrosalicylic acid (DNS) method **(Miller, 1959)** was used for studying the activity of purified GthC5Xyl via measuring the reducing sugar release from oat spelt xylan. All xylanase assays was performed with 100 mM sodium phosphate buffer pH. 6.0 (unless otherwise specified). The xylanase assay was carried out by incubating suitable diluted enzyme with 1% xylan in 100 mM sodium phosphate buffer for 20 minutes at 60ºC. 3,5-dinitrosalicylic acid reagent (DNSA) was used for the estimation of liberated sugars. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of reducing sugar under the assay conditions using oat spelt xylan as the substrate. Bradford method (1976) was used for determining protein concentration. In this procedure, Bovine serum albumin was used as standard **(Bradford, 1976).** Kinetic parameters V_{max} (µmol/min/mg) and K_m were determined by Michaelis-Menten plots of specific activities at multiple xylan concentrations varying between 0.5 mg/ml to 30 mg/ml.

Determination of pH and Temperature Effects on Activity and Stability of GthC5Xyl

The effect of temperature on activity of GthC5Xyl was determined spectrophotometrically using xylan as substrate. By using the method previously described enzyme activity was assayed over a range of temperatures from 40°C to 100°C. Results were expressed as relative activity (%) obtained at optimum temperature. GthC5Xyl temperature stability was determined by incubating enzyme at 40, 50, 60, 70, 80, 90 and 100°C for 200 min and then measuring the residual activity.
pH optimum was determined at 540_{nm} and 60°C by using buffer solutions of different pH. Results were expressed as relative activity (%) obtained at optimum pH. pH stability of enzyme was determined by incubating enzyme at each pH value for 200 min at 60°C and then measuring the residual activity.

Activator and Inhibitor Effects of Metal Ions on GthC5Xyl Activity

For the determination of activation and inhibition effect of various metal ions, the enzyme was incubated for 20 min with 1, 5 and 10 mM of bivalent metal ions such as, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Fe^{2+} and Cu^{2+} at optimum reaction conditions. GthC5Xyl activity was defined 100% without metal ions and residual activity (%) was determined spectrophotometrically (Table 2).

Analysis of Hydrolysis Products and Shelf life Determination of Xylanase

For analysis of xylanase hydrolysis product purified GthC5Xyl was mixed with 100 mM sodium phosphate buffer (pH 6.0) containing 1% (w/v) xylan and incubated for 10 h at 60°C. For the removal of insoluble materials samples were centrifuged for 12 min at 3000 g. TLC pates were then spotted with 3 µl aliquots. Chromatography by ascending method was then performed on silica gel 60 F_{254}TLC plates (Merck) with n-butanol, acetic acid and water (2:1:1) containing solvent system. Plates were then sprayed with 5% (v/v) sulfuric acid in ethanol and then heated at 120°C for about 10 min for the sugar detection. Shelf life determination was done by keeping GthC5Xyl in refrigerator at 4°C as well as at room temperature. Samples were then removed at different intervals and residual activity was determined for 16 weeks.

RESULTS

Isolation and Identification of Bacteria

Bacterial strains, which formed clear halos around their colonies on xylan agar plates, were picked up for further studies. The isolate was confirmed as *Geobacillus thermodenitrificans* C5 with partial 16S rRNA sequencing having a length of 1419 bp nucleotide. The sequence was deposited in GenBank (Accession No. KP203956). The Fig. 1 shows phylogenetic relation of isolate. It shows a very close relation with *Geobacillus thermodenitrificans* subsp.*calidus* F84b (EU477773) and *Geobacillus thermodenitrificans* subsp. *thermodenitrificans* NG80-2 (CP000557) (Fig. 1) having 99% similarity.

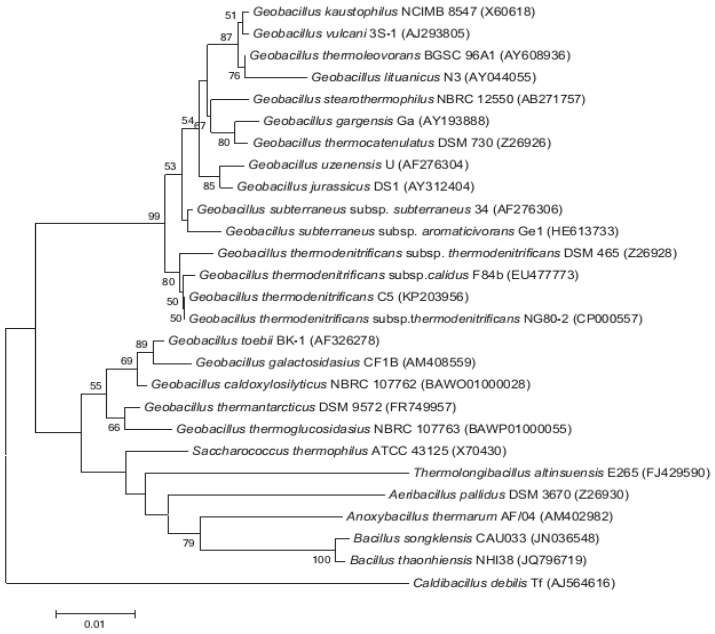

Figure 1 Phylogenetic tree of *Geobacillus thermodenitrificans* C5. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA 5.0.

Cloning and Sequence Analysis of Xylanase

Xylanase encoding gene having 1230 bp was amplified by using *G. thermodenitrificans* DNA as template with translational initiation codon ATG and termination codon TGA. Confirmation of xylanase gene cloning in pET28 (+) vector was done by recombinant vector double digestion with *Nhe*I and *Hind*III restriction enzymes. A successful transformation and expression of the construct pET_GthC5Xyl was carried out in *E. coli* BL21 (DE3) cells. Xylanase expression was induced with 1 mM IPTG at 37°C and higher production of recombinant xylanase was achieved.

Bioinformatics Analysis

Deduced amino acid sequence of xylanase from *Geobacillus thermodenitrificans* C5 having showed no presence of cysteine amino acid residues but an excess of (Asp+Glu) negatively charged residues. Aliphatic index of 37 was demonstrated by the *in silico* analysis. A wide resemblance with various GH10 family endoxylanases and high homology with other *Bacillus* sp and *Geobacillus* sp was depicted by amino acid sequence analysis with BLASTp (Fig. 2). Available crystal structure of xylanase (PDB ID, 1HIZ chain A) from *G. thermodinitrificans* was utilized for proposing GthC5Xyl secondary and tertiary structures. A total of 11 α helices along with 5 sharp turns and 13 β sheets were found in secondary structure. Important catalytic residues Glu was found within the conserved region present inside a "bowl" shaped structure of GH10 xylanase via 3D structure obtained from PyMol PDB viewer. Phylogenetic relationship of GthC5Xyl of *G. thermodenitrificans* C5 with other xylanases available at NCBI database showed maximum identity with xylanase of *Geobacillus* sp. TC-W7 (GQ857066) and *G. thermodenitrificans* strain JK1 (JN209933).

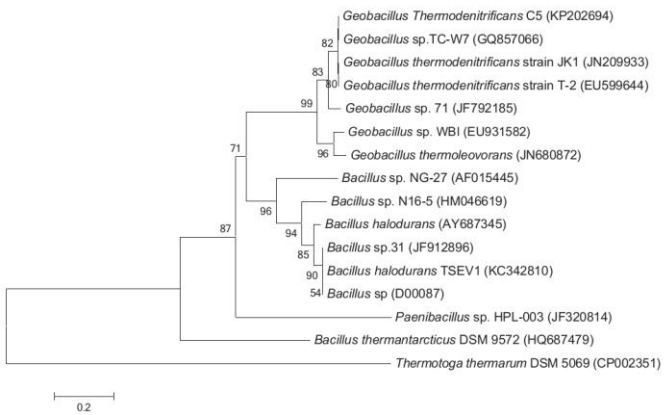

Figure 2 Phylogenetic relationship of *xyl* of *G. thermodenitrificans* C5 with other xylanases available at NCBI database. Neighbor-joining tree showed maximum identity with xylanase of *Geobacillus* sp. TC-W7 (GQ857066) and *G. thermodenitrificans* strain JK1 (JN209933)

Purification of the GthC5Xyl

Elution of recombinant GthC5Xyl was done by DEAE affinity chromatography column with 0.55 M of NaCl (Supplementary Fig. 2). The protein eluted was visualized as a single band on 15% SDS- PAGE which indicated its homogeneous nature (Fig. 3). GthC5Xyl's molecular weight was found out to be approximately 44 kDa. This was confirmed by denaturing protein markers. SDS-PAGE and reverse phase HPLC on C-18 column were used to check the purity of purified protein. A single peak was revealed at retention time of 2.5 min by HPLC chromatogram confirming the purity of preparation (Fig 4) with control of *Bacillus subtilis* (Supplementary Fig. 3). Purified xylanase yield was 176.28% with a specific activity of 1243.12 IU/mg and an overall purification fold of 9.89 (Table 1).

Table 1 Summary of GthC5Xyl purification steps

Purification step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification Yield
Cell extract	4.49	564.1286	125.6411	100	1
Precipitate	3.03	714.4617	235.7959	126.6487	1.876742
DEAE-Sepharose	0.8	994.5004	1243.125	176.2897	9.894258

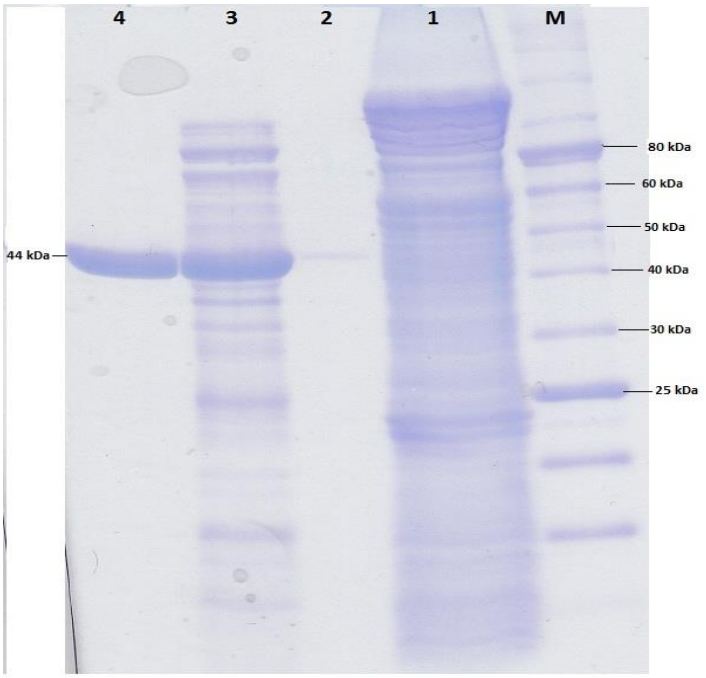

Figure 3 SDS-PAGE showing purified recombinant *Xyl* enzyme obtained from ion-exchange column chromatography by DEAE Sepharose. M, SDS-PAGE molecular mass standards 10–250 kDa New England Biolab; 1, culture supernatant; 2, Induced supernatant; 3, ammonium sulfate precipitation; 4, Purified GthC5Xyl

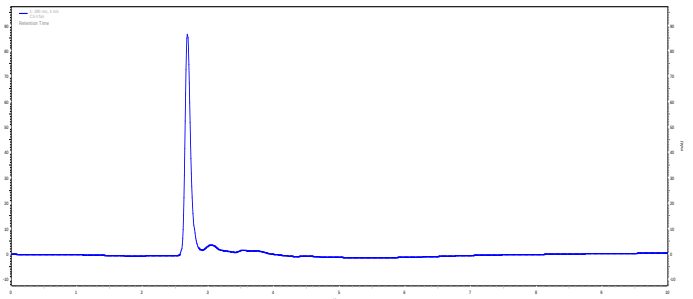

Figure 4 HPLC profile of the purified xylanase from *Geobacillus thermodenitrificans* C5 using a reverse phase C-18 column (4.6 x 250 mm) HPLC chromatogram of the purified enzyme shows a single peak at a retention time of 2.5 min confirming that it was a pure preparation.

Biochemical Characterization of Recombinant Xylanase

The purified GthC5Xyl exhibits activity over a broad range of temperature (40–90°C) and pH (3.0–9.0) with optimum temperature at 60°C (Fig. 5) while optimum pH was 6.0 (Fig. 6). The recombinant GthC5Xyl retained more than 80 % activity after exposure to 60°C for 200 min, and retained more than 70%

activity after exposure to 70°C and 50°C for 200 min (Fig. 7). The enzyme retained more than 80% activity after 3 h at various pH values (6.0, 7.0 and 8.0) (Fig. 8).

In order to verify the effect of substances on GthC5Xyl activity, the purified GthC5Xyl was incubated in the presence of several metallic ions, and detergents such as, sodium dodecyl sulfate (SDS), tetrasodium ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and β-mercaptoethanol, at 1 mM, 5 mM and 10 mM concentrations (Table 2). In general, the GthC5Xyl activity was enhanced with increased concentration of the substances used. Hg^{2+} and $FeSO_4$ were strong inhibitors of the GthC5Xyl while $CaSO_4$ partially inhibit GthC5Xyl at higher concentration. The GthC5Xyl has been inhibited by $AlCl_3$, $ZnSO_4$, $MgCl_2$ and MgSO4 at 10 mM concentration (Table 3). CsCl inhibited the GthC5Xyl at 5.0 mM concentration only. $CoCl_2$, AgNO3, $MnSO_4$, $CaCl_2$, KCl, $CuCl_2$, $MnCl_2$ and $CuSO_4$ does not show inhibitory effect on GthC5Xyl.

The GthC5Xyl is quite stable in the presence of the detergents tested but half of its activity is inhibited in the presence of 1% CTAB concentration and more than 80% activity is retained even at 1% concentration of Tween 20. Triton X-100 and Tween 40 inhibit enzyme at higher concentration only.

The effect of NaCl on activity of GthC5Xyl revealed that the activity was increased with increasing concentrations till 0.8 M. At 1 M of NaCl, relative activity was 101% which sharply decreased to 71 % at 1.5 M of NaCl (Table 3).

Table 2 The effects of various metal ions, detergents and inhibitors on GthC5Xyl

Control	**100%**		
	1 mM 0.1% (v/v)	5 mM 0.5% (v/v)	10 mM 1% (v/v)
Metal Ions			
$AgNO_3$	91.24	126.81	111.35
CsCl	105.16	85.05	89.69
$MnSO_4$	135.43	157.75	107.48
$AlCl_3$	107.71	110.57	81.96
$CoCl_2$	125.27	120.63	116.76
$ZnSO_4$	93.83	91.24	81.96
$MgSO_4 \cdot 7H_2O$	107.48	98.20	71.13
$CuSO_4$	115	105	92
$FeSO_4$	88	74	66
$MnCl_2$	139	124	125
LiCl	101	97	93
$CaCl_2$	105	101	91
$MgCl_2$	115	98	77
$CaSO_4$	94	88	81
Hg	57	30	
KCl	106	92	91
$CuCl_2$	105	130	101
Inhibitors			
EDTA	104	90	79
TSC	103	103	102
β- Mercaptoethanol	96	97	97
NEM	102	100	74
DEPC	104	102	79
DTT	106	107	113
PMSF	100	86	76
NBS	100	73	54
Detergents			
Tween 20	99	96	84
Tween 40	90	81	63
CTAB	84	46	43
SDS	96	91	85
Triton X-100	98	87	73

Table 3 Effect of NaCl on purified xylanase GthC5Xyl

Salt Concentration	**Xylanase activity %**
0	100±0.08
0.2 M	101 ±0.07
0.4 M	104±0.10
0.6 M	105±0.20
0.8 M	106±0.15
1 M	101±0.22
1.2 M	98±0.30
1.5 M	71±0.24

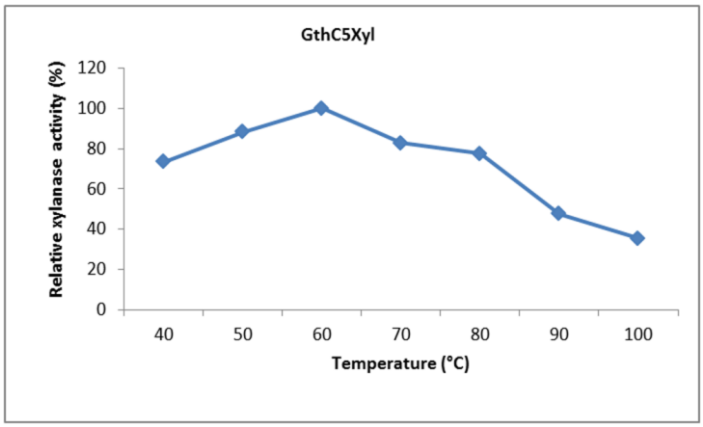

Figure 5 The effect of temperature on the activity of purified xylanase.

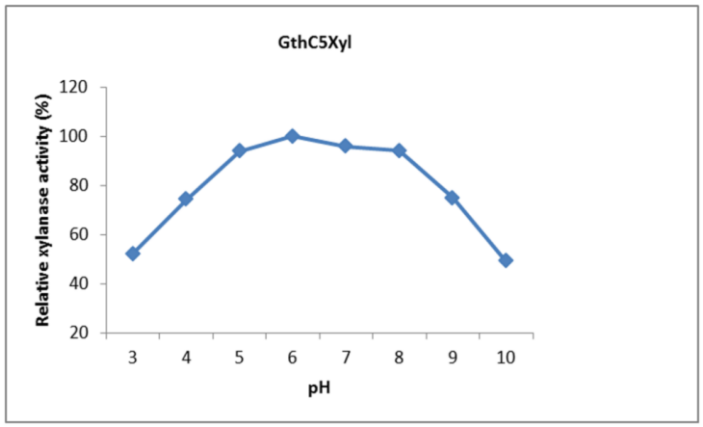

Figure 6 The effect of pH on the activity of purified xylanase.

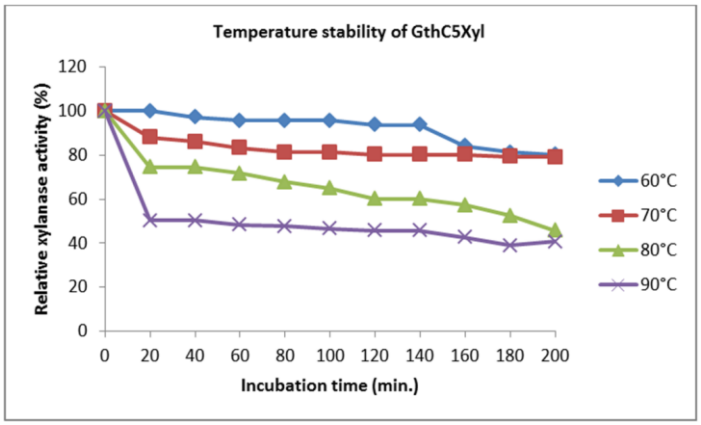

Figure 7 The effect of temperature on the stability of purified xylanase.

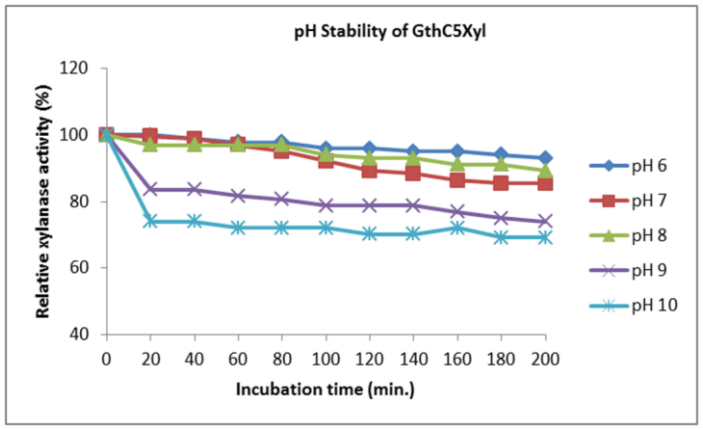

Figure 8 The effect of pH on the stability of purified xylanase

Substrate Specificity, Kinetic Parameters, Mode of Hydrolysis and Determination of Shelf Life of Xylanase

Recombinant GthC5Xyl enzyme activity was evaluated at 60°C and pH 6.0 for 20 min with various substrates for the determination of enzyme specificity (Supplementary Table 2). Specificity of the recombinant enzyme was towards polymeric xylan source and attacked no other substrate such as insoluble xylan, carboxymethyl cellulose, Avicel, filter paper, pNP-β- Xylopyranoside, pNP-α- L-arabinofuranoside, pNP-α- glucopyranoside, pNP- β- galactopyranoside, pNP-α-D- Xylopyranoside and pNP- acetate.

The K_m and V_{max} of GthC5Xyl (for oat spelt xylan) were 3.9084 mgml−1 and 1839.86 μmolmg−1min−1, respectively (Supplementary Fig. 1). TLC of the oat spelt xylan hydrolyzed product was done for the analysis of purified GthC5Xyl mode of action (Fig. 9). A range of xylooligosacharides were released by the action of GthC5Xyl on xylan. Xylopentose and xylobiose were the main products released.

No activity was lost by purified GthC5Xyl when stored for 12 weeks at 4°C but after that decline was observed. After 16 weeks, 90% of initial activity was retained by the enzyme which is an important fact for its industrial application. Conversely enzyme remained completely stable for five weeks at room temperature but showed 80% and 70% residual activity after storage for 10 and 12 weeks, respectively.

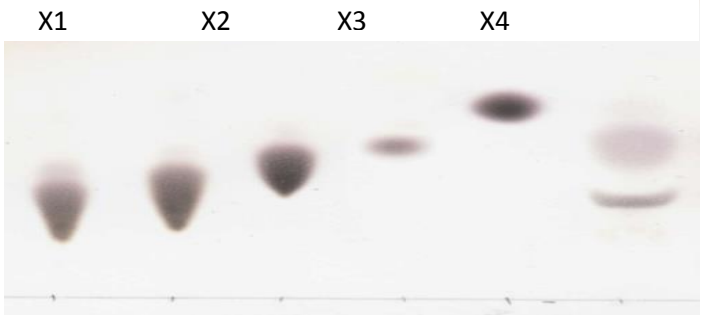

Figure 9 TLC analysis for hydrolysis products released from oat spelt xylan by xylanase from *Geobacillus thermodenitrificans* C5, 1: GthC5Xyl; X5: D-xylose; X4: xylobiose; X3: xylotriose; X2: xylotetraose; X1: xylopentose

DISCUSSION

Hemicellulose is a promising renewable raw material source with immense potential. Its annual production and wastage is humongous and if utilized sensibly it has numerous uses to offer. Xylanase, being regarded as industrially most important enzyme, if employed prudently in industry it can yield clean processes, low energy consumption and higher yields **(Kashyap *et al.*, 2014).** High concentration of salts, surfactants and solvents are required in most of the industrial processes. For instance biofuel production requires ethanol tolerant xylanase, for the bioremediation of industrial waste water contaminated with solvents requires solvent and salt resistant xylanase and deinking of recycled paper requires solvent and surfactant resistant xylanase. Moreover, solvent tolerance enables the enzyme to be recovered, recycled and reused **(Shafiei *et al.*, 2011**; **Ibara *et al.*, 2012**; **Shin *et al.*, 2004**; **Juturu and Wu, 2012**; **Kashyap *et al.*, 2014).**

In industry, the most treasured property of an enzyme is considered to be its thermostability **(Cheng *et al.*, 2014).** So, to study the strategies that an enzyme employs to become thermophilic is an intense area of research. The extraordinarily high specific activity depicted by the recombinant xylanase obtained from Geo*bacillus thermodenitrificans* C5 makes it a worthy candidate for further research.

In the presently conducted study xylanase gene was isolated from Geo*bacillus thermodenitrificans* C5 and then cloned and expressed in *E. coli* BL21 (DE3) host. The expression of this cloned gene of xylanase into the extracellular medium of *E. coli* is the most substantial part of this study which can be employed industrially for commercial xylanase production. One of the interesting facts is the greater xylanase activity of clone in *E.coli* over that of Geo*bacillus thermodinitrificans* C5 strain which may be is the result of higher protein expression. The enzyme produced by *E. coli* functionally active and proficiently degrades oat spelt xylan even in SDS-PAGE gel.

GthC5Xyl optimal reaction temperature was found to be 60ºC which is found to be even higher than that of *Paenibacillus* sp. 12-11 (55ºC) **(Zhao *et al.*, 2011).** GthC5Xyl also retained 77% of its maximum activity at 80ºC and 82 % of its maximum activity at 70ºC (Fig. 5). GthC5Xyl thermal stability was assessed by incubating the enzyme for 200 min at temperatures from 40ºC to 100ºC. The enzyme retained more than 70% of its original activity when incubated for 200 min at temperatures ranging from 40ºC to 60ºC and still retained 79% and 45% of its original activity when incubated for 200 min at 70ºC and 80ºC respectively (Fig. 7). This thermal stability is far better than most of the bacterial xylanases that are previously reported **(Shi *et al.*, 2013**; **Subramaniyan and Prema, 2000**; **Zhao *et al.*, 2011**). Maximum activity of purified GthC5Xyl was found at pH 6.0 which is lower than that of XynG1-1 (7.5) from *P. campinasensis* G1-1 **(Zheng *et al.*, 2012).** Purified enzyme retained 52 % of its activity at pH 3.0 (Fig. 6) and was found stable for 180 min at pH between 5.0 and 8.0 with retaining about 73% activity (Fig. 8). These facts revealed that GthC5Xyl has much higher stability at lower pH than most of the bacterial xylanases reported **(Beg *et al.*, 2001**; **Subramaniyan and Prema, 2000**; **Zhao *et al.*, 2011)**. The stability of this enzyme at higher temperature (60ºC-80ºC) and lower pH renders it worthy for countless industrial applications such as bioenergy conversion, food industry and animal feed.

No significant inhibition of GthC5Xyl activity occurred in the presence of different metallic ions however, it was prudently inhibited by Hg^{2+} and partially by $AlCl_3$ and $ZnSO_4$. As oxidation of indole ring occurs by Hg^{2+} it is possible that the enzyme gets inhibited by the reaction of Hg^{2+} with tryptophan residues **(Zhang *et al.*, 2007).** $MnCl_2$ and $CoCl_2$, showed no inhibitory effect on the enzyme which is similar to *Geobacillus* sp. 71 **(Canakci *et al.*, 2012).** However Mn strongly inhibited xylanase isolated from *Bacillus halodurans* **(Mamo *et al.* 2006/31)** but it differs from *Geobacillus* sp. 71 as it gets inhibited by $ZnSO_4$ whereas *Geobacillus* sp. 71 is resistant to it.

$CuCl_2$ also showed no inhibitory effect on GthC5Xyl activity. This property differed it from xylanases isolated from *Geobacillus thermodenitrificans* TSAA1 **(Verma *et al.*, 2013),** *Plectosphaerella cucumerina* **(Zhang *et al.*, 2007),** *Geobacillus thermoleovorans* **(Verma and Satyanarayana, 2012),** *Thermotoga thermarum* **(Shi *et al.*, 2013),** *Penicillium glabrum* **(Knob *et al.*, 2013),** *Penicillium sclerotiorum*,**(Knob and Carmona, 2010),** *Aspergillus ficuum* AF-98 **(Lu *et al.*, 2008)** which are inhibited by Cu^{2+}. Mn^{2+}, Co^{2+}, $AgNO_3$, and $CuCl_2$ were found to be activity stimulators of GthC5Xyl. Xylanases isolated from *Streptomyces olivaceoviridis* A1 **(Wang *et al.*, 2007)** and *Bacillus subtilis* strain R5 **(Jalal *et al.*, 2009)** had been reported to be stimulated by Fe^{2+}. Recombinant xylanase GthC5Xyl differed from them as it is inhibited by Fe^{2+}.

Sodium dodecyl sulfate (SDS) is a known protein denaturant which strongly inactivates most of the proteins. Even low concentration of SDS deactivates most of the xylanases. Only a few enzymes are reported in literature which show resistance against high concentrations of SDS. For instance xylanase BSX from alkalophilic *Bacillus* sp. NG-27, GH11 xylanase from symbiotic *Streptomyces* sp. TN119 and GH 10 xylanase XynAHJ3 from *Lechevalieria* sp. HJ3, have been reported to retain over 100% of their activities in the presence of SDS **(Bhardwaj *et al.*, 2010**; **Zhou *et al.*, 2012**; **Zhou *et al.*, 2011**; **Zheng *et al.*, 2013).** More than 85% of activity is retained by GthC5Xyl even at 1% SDS concentration and 96% and 91% at 0.1% and 0.5% of SDS concentration respectively which is far better than that of *Geobacillus thermodenitrificans* TSAA1 showing 91% at 0.1% and 72% at 0.5% of SDS concentration (Verma *et al.*, 2013). While xylanase from *Geobacillus* sp. MT-1(Wu *et al.*, 2006), *Bacillus thermantarcticus* **(Lama *et al.*, 2004)** and *Paenibacillus* sp. NF1 **(Zheng *et al.*, 2014)** is significantly inhibited even at 0.02% concentration of SDS. While xylanase from *Melanocarpus albomyces* **(Gupta *et al.*, 2014)** is partially inhibited even at low concentration 0.02% of SDS having 85% of its residual activity. Moreover, GthC5Xyl's activity was also not affected by the presence of the chelating agent EDTA which proposed that metallic cations are not required for its activity. Similar to this *Geobacillus stearothermophilus* and *Bacillus halodurans* xylanases were not inhibited by EDTA, while *Geobacillus* sp. 71 was inhibited by EDTA. CTAB and Triton X can only inhibit the activity of GthC5Xyl at high concentrations.

Substrate specificity and kinetic parameter determination of GthC5Xyl showed that it only exhibits xylanolytic activity. The thin layer chromatography analysis of oat spelt xylan hydrolysis product specified it to be endo-xylanase. Like many other xylanases, the major end products of GthC5Xyl hydrolysis of oat spelt xylan were xylobiose and xylopentose. Acellulytic xylan has added advantages in high quality pulp production. Our results showed that GthC5Xyl has cellulase-free nature.

Up to 1.2 M salt concentration is tolerated by GthC5Xyl with maximum activity of 0.8 M. Similar results were also seen with *Gordonia* sp **(Kashyap *et al.*, 2014).** *Glaecicola mesophila* KMM241 has been reported to secrete xylanase that is active at low temperatures and is salt tolerant as well **(Guo *et al.*, 2009).** *Glaecicola mesophila* retained 90% residual activity at 2.5 M NaCl where as its optimal activity was described at 0.5 M NaCl. Whereas *Bacillus pumilus* xylanase has optimum activity at 1.2 M NaCl (Menon *et al.*, 2010). *Bacillus subtilis* cho40, a marine bacterium has highest activity at at 0.5 M NaCl **(Khandeparker *et al.*, 2011).** However marine bacteria generally secrete halo tolerant enzymes. Xylanase produced by these bacteria have not been characterized for their tolerance to solvents or detergents. All these observations indicated to the fact that xylanase obtained from *Geobacillus thermodenitrificans* C5 has the utmost conditions to resist the harsh conditions of industrial processes and meet the industrial demands.

CONCLUSION

The present work reports the expression, characterization of halo thermostable xylanase from bacterium *Geobacillus thermodenitrificans* C5. It also addresses the property of xylanase such as stability in broad pH range, temperature, NaCl concentration and resistant to SDS. Thus, this strain could be good contender for

different biotechnological applications under extreme conditions. Further, improvements in enzyme production using optimization parameters by statistical approach and use in biobleaching are in progress.

Acknowledgement: Authors are thankful to TUBITAK (2216 Research Fellowship Programme) and Karadeniz Technical University, Turkey to support the project and provide necessary infrastructure to carry out the present research work.

REFERENCES

BAI, Y., WANG, J., ZHANG, Z., YANG, P., SHI, P., LUO, H., MENG, K., HUANG, H., YAO, B. 2010. A new xylanase from thermoacidophilic Alicyclobacillus sp. A4 with broad-range pH activity and pH stability. J Ind Microbiol Biotechnol, 37(2), 187–194. http://dx.doi.org/10.1007/s10295-009-0662-4

BANKEEREE, W., LOTRAKUL, P., PRASONGSUK, S., CHAIAREEKIJ, S., EVELEIGH, D.E., KIM, S.W., PUNNAPAYAK, H. 2014. Effect of polyols on thermostability of xylanase from a tropical isolate of *Aureobasidium pullulans* and its application in prebleaching of rice straw pulp. *SpringerPlus*, 3, 1-37. http://dx.doi.org/10.1186/2193-1801-3-37

BEG, Q.K., KAPOOR, M., MAHAJAN, L., HOONDAL, G.S. 2001. Microbial xylanases and their industrial applications: a review. *Appl Microbiol Biotechnol*, 56, 326-338. http://dx.doi.org/10.1007/s002530100704

BELDUZ, A.O., DEMIRBAG, Z., DULGER, S., COSKUNCELEBI, H. 1997. The Effect of a CO_2-Saturated $CaCl_2$ Solution on the Transformation Efficiency of Escherichia coli JM 109 with an M 13 Derivative. *Turk J Biol*, 21, 133-139.

BERGE, A.C.B., ATWILL, E.R., SISCHO, W.M. 2005. Animal and farm influences on the dynamic of antibiotic resistance in faecal *Eschrichia coli* in young dairy calves. *Preventive Veterinary Medicine*, 69, 25-38. http://dx.doi.org/10.1016/j.prevetmed.2005.01.013

BHALLA, A., BISCHOFF, K.M., UPPUGUNDLA, N., BALAN, V., SANI, R.K. 2014. Novel thermostable endo-xylanase cloned and expressed from bacterium *Geobacillus* sp. WSUCF1. *Bioresour Technol*, 165, 314–318. http://dx.doi.org/10.1016/j.biortech.2014.03.112

BHARDWAJ, A., LEELAVATHI, S., MAZUMDAR-LEIGHTON, S., GHOSH, A., RAMAKUMAR, S., REDDY, VS. 2010. The critical role of N- and C-terminal contact in protein stability and folding of a family 10 xylanase under extreme conditions. *PLoS One*, 5(6), 13-47. http://dx.doi.org/10.1371/journal.pone.0011347

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem*, 72, 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

CANAKCI, S., CEVHER, Z., INAN, K., TOKGOZ, M., BAHAR, F., KACAGAN, M., FULYA A.S., BELDUZ, AO. 2012. Cloning, purification and characterization of an alkali-stable endoxylanase from thermophilic *Geobacillus* sp. 71. *World J Microbiol Biotechnol*, 28,1981–1988. http://dx.doi.org/10.1007/s11274-011-1000-3

CHENG, Y.S., CHEN, C.C., HUANG, C.H., KO, T.P., LUO, W., HUANG, J.W., LIU, J.R., GUO, R.T. 2014. Structural analysis of a glycoside hydrolase family 11 xylanase from *Neocallimastix patriciarum*: insights into the molecular basis of a thermophilic enzyme. *J Biol Chem*, 289, 11020-11028. http://dx.doi.org/10.1074/jbc.m114.550905

COLLINS, T., GERDAY, C., FELLER, G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Rev*, 29, 3-23.

Gerasimova, J., Kuisiene, N. 2012. Characterization of the Novel Xylanase from the Thermophilic *Geobacillus thermodenitrificans* JK1. *Microbiol*, 81, 418–424. http://dx.doi.org/10.1016/j.femsre.2004.06.005

GOSWAMI, G.K., KRISHNAMOHAN, M., NAIN., V., AGGARWAL, C., RAMESH, B. 2014. Cloning and heterologous expression of cellulose free thermostable xylanase from *Bacillus brevis*. *SpringerPlus*, 3, 1-20.

GUO, B., CHEN, X.L., SUN, C.Y, ZHOU, B.C, ZHANG, Y.Z. 2009. Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo-b-1, 4-xylanase from marine *Glaciecola mesophila* KMM 241. *Appl Microbiol Biotechnol*, 84, 1107–1115. http://dx.doi.org/10.1007/s00253-009-2056-y

GUPTA, G., SAHAI, V., GUPTA, R.K. 2014.Thermal stability and thermodynamic of xylanase from *melanocarpus albomyces* in presence of polyols and salts. *Bioresour*, 9(4), 5801-5816. http://dx.doi.org/10.15376/biores.9.4.5801-5816

IBARA, D., MONTE, M.C, BLANCO, A., MARTINEZ, A.T, MARTINEZ, M.J. 2012. Enzymatic deinking of secondary fibers: cellulases/hemicellulases versus laccase-mediator system. *J Ind Microbiol Biotechnol*, 39, 1–9. http://dx.doi.org/10.1007/s10295-011-0991-y

JALAL, A., RASHID, N., RASOOL, N., AKHTAR, M. 2009. Gene cloning and characterization of a xylanase from a newly isolated *Bacillus subtilis* strain R5. *J Biosci Bioeng*, 107 (4), 360–365. http://dx.doi.org/10.1016/j.jbiosc.2008.12.005

JUTURU, V., WU, J.C. 2012. Microbial xylanases: engineering, production and industrial applications. *Biotechnol Adv*, 30(6), 1219–1227. http://dx.doi.org/10.1016/j.biotechadv.2011.11.006

KARAOGLU, H., YANMI, D., FULYA A.S., CELIK, A., CANAKCI, S., BELDUZ, A.O. 2013. Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2 T that displays a high level of activity and thermal stability. J Mol Catal B: *Enzym*, 97, 215-224. http://dx.doi.org/10.1016/j.molcatb.2013.08.019

KASHYAP, R., MONIKA., SUBUDHI, E. 2014. A novel thermoalkaliphilic xylanase from Gordonia sp. is salt, solvent and surfactant tolerant. *J Basic Microbiol*, 54, 1–8. http://dx.doi.org/10.1002/jobm.201400097

KHANDEPARKER, R., NUMAN, M.T. 2008. Bifunctional xylanases and their potential use in biotechnology. *J Ind Microbiol Biotechnol*, 35, 635-644. http://dx.doi.org/10.1007/s10295-008-0342-9

KHANDEPARKER, R., VERMA, P., DEOBAGKAR, D. 2011. A novel halotolerant xylanase from marine isolate *Bacillus subtilis* cho40: gene cloning and sequencing. *N Biotechnol*, 28(6), 814–821. http://dx.doi.org/10.1016/j.nbt.2011.08.001

KNOB, A., BEITEL, S.M., FORTKAMP, D., TERRASAN, C.R.F., FERNANDO DE ALMEIDA, A. 2013. Production, Purification, and Characterization of a Major *Penicillium glabrum* Xylanase Using Brewer's Spent Grain as Substrate. *BioMed Research Int*, 10, 11-55. http://dx.doi.org/10.1155/2013/728735

KNOB, A., CARMONA, E.C. 2010. Purification and characterization of two extracellular xylanases from *Penicillium sclerotiorum*: a novel acidophilic xylanase. *Appl Biochem Biotechnol*, 162(2), 429–443. http://dx.doi.org/10.1007/s12010-009-8731-8

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4. *Nature*, 227(5259), 680–685. http://dx.doi.org/10.1038/227680a0

LAMA, L., CALANDRELLI, V., GAMBACORTA, A., NICOLAUS, B. 2004. Purification and characterization of thermostable xylanase and β-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus. Res Microbiol*, 155, 283–289. http://dx.doi.org/10.1016/j.resmic.2004.02.001

LI, X.R., XU, X., XIE, J., YI, Q.F., LI, W., DAI, R., QIAO, D.R, CAO, Y., CAO, Y. 2014. Thermostable Sites and Catalytic Characterization of Xylanase XYNB of *Aspergillus niger* SCTCC 400264. *J. Microbiol Biotechnol*, 24(4), 483–488. http://dx.doi.org/10.4014/jmb.1307.07086

LIAO, H., XU, C., TAN, S., WEI, Z., LING, N., YU, G., RAZA, W., ZHANG, R., SHEN, Q., XU, Y. 2012. Production and characterization of acidophilic xylanolytic enzymes from *Penicillium oxalicum* GZ-2. *Bioresour Technol*, 123(2), 117–124. http://dx.doi.org/10.1016/j.biortech.2012.07.051

LU, F., LU, M., LU, Z., BIE, X., ZHAO, H.,WANG, Y. 2008. Purification and characterization of xylanase from *Aspergillus ficuum* AF- 98. *Bioresour Technol*, 99(13), 5938–5941. http://dx.doi.org/10.1016/j.biortech.2007.10.051

MAMO, G., HATTI-KAUL, R., MATTIASSON, B. 2006. A thermostable alkaline active endo-b-1-4-xylanase from Bacillus halodurans S7: Purification and characterization. *Enzyme Microb Technol*, 39, 1492–1498. http://dx.doi.org/10.1016/j.enzmictec.2006.03.040

MENON, G., MODY, K., KESHRI, J., JHA, B. 2010. Isolation, purification, and characterization of haloalkaline xylanase from a marine *Bacillus pumilus* strain. *Biotechnol Bio-process Eng*, 5, 998–1005. http://dx.doi.org/10.1007/s12257-010-0116-x

MILLER, G.R. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem*, 31, 426–428. http://dx.doi.org/10.1021/ac60147a030

SAITOU, NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4, 406–425. http://dx.doi.org/10.1016/j.ympev.2008.01.019

SHAFIEI, M., ZIAEE, A.A., AMOOZEGAR, M.A. 2011. Purification and characterization of an organic-solvent-tolerant halophilic a-amylase from the moderately halophilic *Nesterenkonia* sp. Strain. F. *J Ind Microbiol Biotechnol*, 38, 275–281. http://dx.doi.org/10.1007/s10295-010-0770-1

SHI, H., ZHANG, Y., LI, X., HUANG, Y., WANG, L., WANG, Y., DING, H., WANG, F. 2013. A novel highly thermostable xylanase stimulated by Ca2+ from *Thermotoga thermarum*: cloning, expression and characterization. *Biotechnol Biofuels*, 6, 26-30. http://dx.doi.org/10.1186/1754-6834-6-26

SHI, P., CHEN, X., MENG, K., HUANG, H., BAI, Y., LUO, H. 2013. Distinct actions by Paenibacillus sp. strain E18 alpha-*L*arabinofuranosidases and xylanase in xylan degradation. *Appl Environ Microbiol*, 79, 1990-1995. http://dx.doi.org/10.1128/aem.03276-12

SHIN, YO., WAHNON, D., WEBER, M.E., VERA, J.H. 2004. Precipitation and recovery of xylanase using surfactant and organic solvent. *Biotechnol Bioeng*, 86, 698–705. http://dx.doi.org/10.1002/bit.20080

SNEATH, P.H.A 1994. Endospore forming Gram positive rods and cocci," in *Bergey's Manual of Systematic Bacteriology*, Hensyl WM Ed, Williams & Wilkins, Philadelphia, Pa, USA, 9th edition, 1994. http://dx.doi.org/10.1016/s0723-2020(11)80095-0

SUBRAMANIYAN, S., PREMA, P. 2000. Cellulase-free xylanases from Bacillus and other microorganisms. *FEMS Microbiol Lett*, 183, 1-7. http://dx.doi.org/10.1111/j.1574-6968.2000.tb08925.x

VERMA, D., ANAND, A., SATYANARAYANA, T. 2013. Thermostable and Alkalistable Endoxylanase of the Extremely Thermophilic Bacterium

Geobacillus thermodenitrificans TSAA1: Cloning, Expression, Characteristics and Its Applicability in Generating Xylooligosaccharides and Fermentable Sugars. *Appl Biochem Biotechnol*, 170, 119–130. http://dx.doi.org/10.1007/s12010-013-0174-6

VERMA, D., SATYANARAYANA, T. 2012. Cloning, expression and applicability of thermoalkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. *Bioresour Technol*, 107, 333–338. http://dx.doi.org/10.1016/j.biortech.2011.12.055

WANG, S-Y., HU, W., LIN, X-Y., WU, Z-H., LI, Y-Z. 2012. A novel cold-active xylanase from the cellulolytic myxobacterium *Sorangium cellulosum* So9733-1: gene cloning, expression, and enzymatic characterization. *Appl Microbiol Biotechnol*, 93(4), 1503–1512. http://dx.doi.org/10.1007/s00253-011-3480-3

WANG, Y., FENG, S., ZHAN, T., HUANG, Z., WU, G., LIU, Z. 2013. Improving catalytic efficiency of endo 1, 4-xylanase from *Geobacillus stearothermophilus* by directed evolution and H179saturation mutagenesis. *J Biotechnol*, 168, 341– 347. http://dx.doi.org/10.1016/j.jbiotec.2013.09.014

WANG, Y., ZHANG, H., HE, Y., LUO, H., YAO, B. 2007. Characterization, gene cloning, and expression of a novel xylanase XYNB from *Streptomyces olivaceoviridis* A1. *Aquaculture*, 267 328–334. http://dx.doi.org/10.1016/j.aquaculture.2007.03.005

WU, S., LIU, B., ZHANG, X. 2006. Characterization of a recombinant thermostable xylanase from deep-sea thermophilic *Geobacillus* sp. MT-1 in East Pacific. *Appl Microbiol Biotechnol*, 72, 1210–1216. http://dx.doi.org/10.1007/s00253-006-0416-4

ZHANG, G.M., HUANG, J., HUANG, G.R, MA, L.X, ZHANG, X.E. 2007. Molecular cloning and heterologous expression of a new xylanase gene from *Plectosphaerella cucumeria*. *Appl Microbiol Biotechnol*, 74, 339–346. http://dx.doi.org/10.1007/s00253-006-0648-3

ZHAO, Y., MENG, K., LUO, H., YANG, P., SHI, P., HUANG, H. 2011. Cloning, expression, and characterization of a new xylanase from alkalophilic *Paenibacillus* sp. *J Microbiol Biotechnol*, 21, 861-868. http://dx.doi.org/10.4014/jmb.1102.02024

ZHENG, F., HUANG, J., YIN, Y., DING, S. 2013. A novel neutral xylanase with high SDS resistance from Volvariella volvacea: characterization and its synergistic hydrolysis of wheat bran with acetyl xylan esterase. *J Ind Microbiol Biotechnol*, 40, 1083–1093. http://dx.doi.org/10.1007/s10295-013-1312-4

ZHENG, H.C., SUN, M.Z., MENG, L.C., PEI, H.S., ZHANG, XQ., YAN, Z., ZENG, W.H., ZHANG, J.S., HU, J.R., LU, F.P., SUN, J.H. 2014. Purification and Characterization of a Thermostable Xylanase from Paenibacillussp. NF1 and its Applicationin Xylooligosaccharides Production. *J Microbiol Biotechnol*, 24(4), 489–496. http://dx.doi.org/10.4014/jmb.1312.12072

ZHENG, HC., LIU, Y.H., LIU, X.G., HAN, Y., WANG, J.L., LU, F.P. 2012. Overexpression of a *Paenibacillus campinasensis* xylanase in *Bacillus megaterium* and its applications to biobleaching of cotton stalk pulp and saccharification of recycled paper sludge. *Bioresour Technol*, 125, 182-187. http://dx.doi.org/10.1016/j.biortech.2012.08.101

ZHOU, J., GAO, Y., DONG, Y., TANG, X., LI, J., XU, B., MU, Y., WU, Q., HUANG, Z. 2012. A novel xylanase with tolerance to ethanol, salt, protease, SDS, heat, and alkali from *actinomycete Lechevalieria* sp. HJ3. *J Ind Microbiol Biotechnol*, 39(7), 965–975. http://dx.doi.org/10.1007/s10295-012-1113-1

ZHOU, J., SHI, P., ZHANG, R., HUANG, H., MENG, K., YANG, P., YAO, B. 2011. Symbiotic *Streptomyces* sp. TN119 GH 11 xylanase: a new pH-stable, protease- and SDS-resistant xylanase. *J Ind Microbiol Biotechnol*, 38(4), 523–530. http://dx.doi.org/10.1007/s10295-010-0795-5