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Production and characterization of xylanase from *Bacillus licheniformis* S3 isolated from hot spring

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Abstract

Xylanases (EC 3.2.1.8) are hemicellulases responsible for the catalysis of xylan, a major component of hemicellulose and the second largest renewable biomass. It is applied in various industries such as paper and pulp, biofuel, baking, detergents, animal feeds and textile. The current study was focused on screening of hot spring samples, collected from Skardu, Pakistan, for novel xylanases producing microbial strains. A bacterium designated as strain S3 was isolated that could effectively breakdown xylan. It was found to be the specie of genus *Bacillus* based on morphological, biochemical analysis, while 16S rRNA gene sequencing results indicated 99% similarity with *Bacillus licheniformis*. Various physical and chemical conditions were statistically optimized using Design-expert software for maximum production of xylanase. Xylanase produced under these optimized physical and chemical conditions, was purified to homogeneity by size exclusion column chromatography using Sephadex G-100. The molecular weight was found to be approximately 28 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The specific activity of the purified xylanase was up to 39 U/mg with a 4.38-fold purification and 58% yield. The Km and Vmax of S3 xylanase were 8.6 $mgmL^{-1}$ and 43.71 $\mu molmg^{-1}min^{-1}$, respectively. The activity exhibited by *B. licheniformis* xylanase was found to be acellulolytic with stability at wide temperature (40°C-60°C) and pH (5.0 to 10.0), and stimulated by $CaCl_2$, $FeSO_4$, $FeSO_4$, $CdCl_2$ and $MgSO_4$, while inhibited by $HgCl_2$ and $CuSO_4$. Furthermore, the enzyme was resistant to most of the proteases tested. Since the enzyme was stable at wide pH range (5.0-10.0) and showed resistance to Ni, Cd, Zn and Co at 10 mM concentration that represents its efficiency and potential application in paper and pulp, food and feed industries.

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Introduction

Hemicellulases are enzymes that hydrolyze hemicellulose – the second most abundant renewable biomass (Irfan *et al.*, 2016), representing 20–35% of overall biomass, after cellulose. Among the hemicellulose, xylan is the most plentiful polysaccharide and have a key role in plant cell wall integrity as it is present in the interface between cellulose and lignin (Collins *et al.*, 2005). Xylanases are the key enzyme in hydrolysis of xylan as they catalyze β-1-4 linkages of the xylan polymer, resulting xylo-oligosaccharides – substrate for β-xylosidases (Basit *et al.*, 2018). Xylanases are produced by variety of organisms but microorganisms such as Bacteria, Fungi, and Yeast are the prominent sources (Lombard *et al.*, 2013). The ecological niches of these organisms are diverse and wide spread and found from mesophilic to extreme environments. The typical habitat of xylanases producers is the environment where plant materials are accumulated.

Enzymes must be active under the harsh conditions of the industrial processes in order to make them applicable for industrial use. Mesophilic enzymes are unstable in the hostile conditions and therefore, not well studied for such applications (Demirjian *et al.*, 2001). The discovery of extremophilic organisms, organisms which thrive in the extreme environment of pH, temperature, salt, etc., revealed that they produce unique enzymes that are functional under extreme conditions allowing the advancement of additional industrial processes. Extreme environments are explored and continued to explore for novel industrial enzymes all over the world. Several thermophilic and hyper-thermophilic xylanase producing microorganisms have been reported from diverse sources, including terrestrial and marine solfataric fields, hot pools, thermal springs and self-heating decaying organic debris (Vieille & Zeikus, 2001).

The major barriers in the scale up of enzymes to industrial levels are; short half-life, sensitive to inhibitors and narrow range of temperature and pH stability. There are no universal enzymes which have all these capabilities. Different applications demand different characteristics for enzymes and enzyme with a single character cannot be applicable in all processes. It is suggested that, extreme thermophiles that produce xylanase are few and search for organism with high yield of enzyme with desired characteristics is still being pursued (Haki & Rakshit, 2003). Screening of thermophiles is of special interest as a source of novel xylanases (Touzel *et al.*, 2000) for their major application in pre-bleaching of Kraft pulp (Christov *et al.*, 1999).

The natural extreme environment serves as a potential source for extremozymes. To overcome problems in biotechnological application, such environment should be explored for novel enzymes. Several extreme environments are also found in Pakistan that are yet unexplored. These environments may serve for novel xylanases producing strains that may have potential biotechnological applications. In the present study, bacterium that was labelled S3 was determined for xylanase production. The strains were identified by 16S rRNA gene sequence analysis. Placket-Barmen design was used to optimize components and production parameters for optimum xylanase production. Enzymes were produced from both strains under the optimized conditions, precipitated with ammonium sulfate, dialyzed and purified by column chromatography. Molecular masses of the purified enzymes were determined using SDS-PAGE and the purified enzymes were further characterized.

Materials and methods

Screening of the isolates for xylanases activity

Soil and water samples were collected from the Chotron hot spring, shigar valley district Skardu in Gilgit-Baltistan, Pakistan. Enrichment was done using oat spelt xylan (Sigma Chemicals, Germany) as a sole carbon source. Culture was grown on agar plates containing 0.5% (w/v) Birchwood xylan, after incubation, plates were flooded with 0.5% Congo red solution for 20 mins and then distained with 1MNaCl for 20 mins. The colonies showing clear zone of hydrolysis were picked and used for xylanase

production. One bacterial isolate with highest xylanolytic activity was designated as S3 and ultimately chosen for further study.

Identification of the xylanase producing bacterial isolate

S3 isolate was identified on the basis of cultural, morphological and biochemical properties along with 16S rRNA sequencing. 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 & Nei, 1987).

Optimization of culture conditions using Design Expert

Plackett-Burman (experimental design) adopts that between different variables there is no interaction in the considered range. And for screening a single linear approach is acceptable. It is a factorial fractional design. To identify different variable that affect xylanase production Plackett-Burman design was used. 11 variables were screened in 15 runs (Table 1) and insignificant ones were removed so as to attain a smaller, adaptable set of factors. Table 1 show the PlackettBurman experimental design matrix in which each column represents variables and each row represents an experiment. Design-Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA) was used for evaluating the experimental data.

Xylanase assay

The enzyme activity was measured by Dinitro Salicylic Acid (DNS) method (Irfan *et al.*, 2016). The xylanolytic activity of the crude xylanase was studied by incubating the reaction mixture 400 µl of 1 % (w/v) xylan solution (as a substrate) in phosphate buffer (pH 7.0) with 100 µl of crude xylanase at 60°C for 10 min. The reaction was then terminated via addition of 500 µl DNS reagent and kept at temperature of 99˚C for about 15 min and absorbance of the solution was measured at 540 nm using spectrophotometer. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1 µm of xylose per ml per minute under the specific conditions of assay.

Purification of xylanase

Ammonium sulfate precipitation

All purification steps were performed at 4°C. The crude enzyme preparation was subjected to ammonium sulfate precipitation, and the harvested culture was centrifuged at 10,000 rpm for 10 min at 4°C. Ammonium sulfate was added slowly to the cell-free culture at 60 % saturation to precipitate the xylanase with continuous stirring. The precipitated xylanase was separated by centrifugation at 10,000 rpm for 10 min and the resultant pellet was dissolved in 100 mM phosphate buffer pH 7.0.

Sephadex G-100 column chromatography

The precipitated protein collected by centrifugation (10,000 rpm 10 min) was dissolved in 100 mM phosphate buffer (pH 7.0). A 3.0 mL sample from ammonium sulfate precipitation was slowly loaded on a Sephadex G-100 column (100 cm _ 1.0 cm) and equilibrated with 100 mM phosphate buffer (pH 8.0). The protein was eluted at a flow rate of 0.33 mL/min, and 3 mL fractions were collected. All active fractions were gathered and concentrated to 0.5 mL by ultrafiltration and examined for xylanase activity and protein content.

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS PAGE)

Molecular weight and purity of S3 xylanase was determined by SDS PAGE analysis by the method (Laemmli, 1970). The purified xylanase enzyme was mixed with loading dye (0.5 M Tris–HCl pH 8.0, 6 % (w/v) 2-β-marcaptoethanol, 10 % (v/v) Glycerol, 6 % (w/v) SDS and 0.3 % (v/v) Bromophenol blue and heated in water bath for 5 min before use. xylanase enzyme was run with Thermo Scientific PageRuler Prestained Protein Ladder (10–170 kDa) at 120 V for

2–2.5 hours. the gel was stained with Coomessie Brilliant Blue for 30 min following destained for about 2.5 h.

Characterization of xylanase

Purified enzyme was characterized and the relative activity of the enzymes was calculated using the following formula:

$$\text{Residual Activity (\%)} = \frac{\text{Activity (U/mg) of enzyme}}{\text{Activity (U/mg) of the original enzyme}} \times 100$$

Effect of temperature and pH

The purified S3 xylanase was analyzed for activity at different temperature ranges (30°C to 80°C) for 10 minutes, and the relative activity was calculated. The purified S3 xylanase was analyzed for activity at different pH (3.0–11.0) by using different buffer systems i.e. 0.05M acetate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0 – 8.0), and Glycine NaOH buffer (pH 9.0 – 11.0). Enzyme assay was performed at their optimum temperature for 10 minutes and the relative activity was calculated.

Temperature and pH stability

Thermo-stability of purified S3 xylanase was determined by incubating enzymes with substrate at different temperatures for 3 hours with 30 minutes interval. Activity was calculated and expressed in term of relative activity. pH stability of purified S3 xylanase was determined by incubating enzymes in different pH buffers (pH 4.0- 10.0) without substrate at temperature of 4˚C for 3 hours with 30 minutes' interval. Substrate was then added and incubated at optimum temperature for 10 mins. Activity was calculated and expressed in term of relative activity.

Effect of metals, surfactants, detergents and organic solvents

The purified enzyme preparations were further analyzed for metal ions as metal ions have significant effect on activity of enzyme. Different metal ions were analyzed for studying effect on xylanase activity i.e. ($FeCl_2$), ($CoCl_2$), ($CuCl_2$), ($CaCl_2$), ($MgCl_2$), ($ZnCl_2$), (NaCl), ($NiCl_2$), ($HgSO_4$), (Cr) and (Ar). Metals were used in 2 and 10 mM concentration and the effect of metals were expressed in term of relative activity.

Effect of Different surfactants was analyzed by incubating S3 xylanases with surfactants at a concentration of 0.5–1.0%. These include SDS, EDTA, Tween 20, Tween 40, Tween 60, Tween 80, Cetyl trimethylammonium bromide (CTAB), Polyethylene glycol (PEG), Sodium laureth sulfate (SLES) and Triton X-100. Enzyme assay was performed at their respective optimum conditions and the relative activity was calculated.

Effect of different organic solvents was analyzed by incubating S3 xylanases at a concentration of 15%. These include ethanol, methanol, acetonitrile, acetone, ethyl acetate, propanol, DMSO, butanol, formaldehyde and glycerol. Enzyme assay was performed at their respective optimum conditions and the relative activity was calculated.

Kinetics studies and substrate specificities of S3 xylanase

Vmax and Km of S3 xylanase was determined. Different substrate concentrations were prepared ranging from 0.5–35 mg/mL. Enzyme assay was performed under optimum condition for 5 minutes followed by DNS treatment. Data from Enzyme assay were plotted according to the Line weaver–Burk method (Lineweaver & Burk, 1934).

Substrate specificity of S3 xylanase was performed for different substrate for 10 minutes and the assay was performed as described earlier. Different substrates which were used are birchwood xylan, beechwood xylan, oatspelt xylan, arabinoxylan, wheat straw, rice husk, sugar cane baggase, soluble starch, avicel CMCand different pNP containing substrates.

Effect of proteases on S3 xylanase stability

The resistance behavior with different proteases was analyzed in presence of 10 µg/mL of *Tritirachium album* proteinase K, bovine pancreas trypsin and chymotrypsin, and *Clostridium histolyticum* collagenase. For this purpose, the purified xylanase (150 µg/mL) was pre-incubated in presence of the

different proteolytic activities in 100 mM sodium phosphate buffer at optimum condition for 60, 120and 200 minutes. The residual xylanase activity was measured at the standard conditions. The enzyme, pre-incubated under the same conditions in the absence of proteases, was used as control.

Shelf life determination of S3 xylanase

Shelf life was determined for 20 weeks at room temperature and 4˚C for S3 xylanase, and the residual activity were calculated after specific time periods.

Result

Isolation and screening of xylanase producing isolates

In the present study, isolate S3 was screened for xylanolytic activity on nutrient agar plates supplemented with 0.5% beechwood xylan. The strain showed xylanolytic activity by formation of prominent clear zone of hydrolysis around colony by Congo red method. The Congo red method is widely used for xylanase screening and has also been reported by some earlier researchers (Nihalani & Satyanarayana 1992; Gessesse & Gashe, 1997).

Table 1. Experiment design of Placket-Burman and observed response of strain *Bacillus licheniformis* S3 for xylanases production.

Run	Factors g/l											Response
	1	2	3	4	5	6	7	8	9	10	11	U/mg
	NaCl	InoculumSize %	KH2PO4	MgSO4	Sodium Citrate	Xylan	Peptone	K2HPO4	Glucose	CaCl2	yeast extract	
1	20.00	0.50	2.00	2.00	0.20	14.00	14.00	8.00	4.00	0.02	0.30	2.9
2	12.50	1.25	1.02	1.02	0.40	11.00	11.00	6.00	6.00	0.03	0.50	2.7
3	12.50	1.25	1.02	1.02	0.40	11.00	11.00	6.00	6.00	0.03	0.50	3
4	20.00	0.50	0.05	0.05	0.60	8.00	14.00	8.00	4.00	0.04	0.70	3.1
5	20.00	0.50	2.00	2.00	0.60	8.00	8.00	4.00	8.00	0.02	0.70	2.5
6	12.50	1.25	1.02	1.02	0.40	11.00	11.00	6.00	6.00	0.03	0.50	2.5
7	5.00	0.50	2.00	0.05	0.60	14.00	8.00	8.00	8.00	0.04	0.30	3.7
8	5.00	2.00	0.05	2.00	0.60	8.00	14.00	8.00	8.00	0.02	0.30	2
9	5.00	2.00	2.00	2.00	0.20	8.00	8.00	8.00	4.00	0.04	0.70	4.2
10	20.00	2.00	2.00	0.05	0.20	8.00	14.00	4.00	8.00	0.04	0.30	3.5
11	5.00	0.50	0.05	2.00	0.20	14.00	14.00	4.00	8.00	0.04	0.70	2.4
12	5.00	0.50	0.05	0.05	0.20	8.00	8.00	4.00	4.00	0.02	0.30	2.4
13	5.00	2.00	2.00	0.05	0.60	14.00	14.00	4.00	4.00	0.02	0.70	2.9
14	20.00	2.00	0.05	2.00	0.60	14.00	8.00	4.00	4.00	0.04	0.30	1.8
15	20.00	2.00	0.05	0.05	0.20	14.00	8.00	8.00	8.00	0.02	0.70	0.4

Identification of the isolate S3

The strain S3 was rod shaped, spore forming and tentatively identified as *Bacillus* sp. based on its morphological and biochemical characteristics (Table 2). Subsequently, the comparison of the 16S rRNA gene nucleotide sequence of the strain S3 with other 16S rRNA genes sequences of closely related strains by EZ-Taxon server showed that the strain S3 has 99% similarities with *Bacillus licheniformis* strain (KX812811) and *Bacillus licheniformis* isolate SK02 (KX173846) The sequence of 16S rRNA gene of *Bacillus licheniformis* S3 strain was deposited in GenBank with accession number (MG800782) (Fig 1).

Optimization of Media Components for Production of xylanase by Statistical Analysis (Placket Burman Design)

Placket-Burman design was used for the optimization of different nutritional factors. Temperature and pH was separately optimized and maximum specific activity was observed at 40°C (4.07U/mg) after 24 hours incubation. Maximum enzyme production was achieved at pH 5.0 (4.77 U/mg). In Placket-Burman design total Eleven (11) factors were analyzed i.e., carbon sources (xylan, glucose), nitrogen sources (peptone, yeast extract) different salts (K_2HPO_4, $Na_3C_6H_5O_7$, KH_2PO_4, NaCl and $MgSO_4.7H_2O$) metal ($CaCl_2$) and inoculum. 15 runs were provided by

experiment design and maximum xylanase production was achieved at run#9 by strain S3. Response was measured in terms of specific activity exhibited by xylanase. Factors that affecting the fermentation process of strain S3 is given by the software.

Table 2. Physiological and biochemical characteristic of *Bacillus licheniformis* S3.

Test	Result
Colony	Dull, opaque, adherent colonies
Gram reaction	Gram-positive
Shape of cells	Rod
Motile	+
Spore formation	+
Catalase activity	+
Oxidase activity	+
Starch	+
Citrate utilization	-
H_2S	-
Indole	-
Voges Proskauer	+
Methyl red	-
Cellulose	-
Nitrate reduction	+
Esculin	+

The capability of the model was analyzed using analysis of variance (ANOVA) which was confirmed by Fisher's statistical analysis and the results are showed in Table 3. The Model F-value of 38.56 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Curvature F-value" of 0.42 implies the curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space is not significant relative to the noise. There is a 54.25% chance that a "Curvature F-value" this large could occur due to noise.The "Lack of Fit F-value" of 0.45 implies the Lack of Fit is not significant relative to the pure error. There is a 77.70% chance that a Lack of Fit F-value this large could occur due to noise.

Table 3. ANOVA for selected factorial model Analysis of variance for S3 xylanase.

Source	Sum of Squares	df	Mean Square	F Value	Prob>F
Model	10.80	7	1.54	38.56	0.0001
A-NaCl	0.96	1	0.96	24.08	0.0027
B-InoculumSize	0.40	1	0.40	10.08	0.0192
C-KH2PO4	4.81	1	4.81	120.33	< 0.0001
F-Xylan Conc	1.08	1	1.08	27	0.0020
G-Peptone	0.27	1	0.27	6.75	0.0408
J-Glucose	0.65	1	0.65	16.33	0.0068
K-CaCl2	2.61	1	2.61	65.33	0.0002
Curvature	0.017	1	0.017	0.42	0.5425
Residual	0.24	6	0.040		
Lack of Fit	0.11	4	0.028	0.45	0.7770
Pure Error	0.13	2	0.063		
Cor Total	11.05	14			

Xylanase Purification and SDSPAGE electrophoresis

Crude S3 xylanase was collected by centrifugation. The supernatant containing S3 xylanase was precipitated with ammonium sulfate at 60% saturation. Precipitates were dissolved in 100 mM sodium phosphate buffer and dialyzed against the same buffer overnight. Partially purified sample was subjected for further purification by Gel Filtration Chromatography technique. Maximum specific activity of strain S3 xylanase was observed in fractions 8–14 with a maximum specific activity of 1.52U/mL at fraction 12 (Fig 2).

Table 4. Purification profile of xylanase produced from *Bacillus licheniformis* S3.

Purification Steps	Activity (U/ml)	Protein (mg/ml)	SA (U/mg)	Yield (%)	Purification Fold
Supernatant	5.48	0.60	9	100	1
Precipitated	3.79	0.79	11	69	1.22
Sephadex 100	3.2	0.08	39.42	58	4.38

The yield of the purified xylanase was 58% with a specific activity of 39.42 U/mg proteins and an overall purification fold of 4.38 (Table 4). The molecular weight of S3 xylanase was found to be approximately 28 KDa as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The single bands obtained indicate monomeric nature of the enzyme (Fig 3).

Characterization of Xylanase

Purified S3 xylanase was characterized for different parameters known to effect enzyme activity.

Table 5. Effect of metal ions, surfactant and detergents on purified S3 xylanase activity.

metals	2mM	10 mM
Control	100	100
$CaSO_4$	101	119
$CaCl_2$	101	145
$FeSO_4$	101	142
$CuSO_4$	61	3
$CoCl_2$	88	82
$NiSO_4$	102	105
K_2SO_4	99	125
$ZnSO_4$	110	95
$CuCl_2$	55	27
KCl	101	144
$CdCl_2$	106	114
Na_2SO_4	110	104
$MgSO_4$	111	132
NaCl	97	118
$NiCl_2$	99	72
$HgCl_2$	3	2
Urea	103	90
surfactant and detergents		
	0.5%	1%
Tween 20	107	106
Tween 40	104	105
Tween 60	105	104
Tween 80	103	98
Triton 100x	101	97
CTAB	96	82
SDS	33	11
PEG	100	108
SLES	97	91

Effect of temperature and pH on activity of xylanase

The optimum temperature of S3 xylanase was turned out to be 55˚C, while more than 70 % of initial activity was retained from 40–70˚C (Fig 4a). S3 xylanase exhibiting optimal activity around pH 7.0 and retained more than 60% of initial activity at pH 4.0–10.0 (Fig 4c).

Temperature and pH stability

To assess the thermal stability of S3 xylanase, the enzyme was incubated at 40°C-80°C for 3 hours in 100 mM phosphate buffer (pH 7.0). The enzyme was stable from 40°C to 60°C for 3 hours and more than 68% activity was retained as the said temperature (Fig 4b). The enzyme was quite stable in term of its pH stability and retained initial activity of more than 70% from 5.0-9.0 pH for 3 hours (Fig 4d).

Effect of metals, surfactants, detergents and organic solvents

Effect of different metals on S3 xylanase was analyzed at concentration of 2 mM and 10 mM. Monovalent cations, such as Li^+ and Na^+, did not significantly affect xylanase activity; On the contrary, K^+ exhibited a marked stimulating effect, at 10 mM concentrations, enhancing the activity, as chloride salt, by 44%. Bivalent cations such as Ca^{2+} and Mg^{2+} increase the activity by 19% and 31% respectively at 10mM concentration. Strong inhibitory effect was observed for $HgCl_2$ and Cu^{2+} at both concentrations. Although the activity of S3 xylanases was not significantly influenced in the presence of metals. Most metals have not inhibited the activity of S3 xylanase. S3 xylanase are quite stable in the presence of metals especially against Fe (Table 5).

Table 6. Stability of S3 xylanase in the presence of various organic solvents.

Solvent 10%	Residual activity %			
	30 min	60 min	90 min	120 min
Ethanol	91	87	85	79
Methanol	88	87	83	32
Acetonitrile	63	57	46	32
Acetone	77	70	43	23
Ethyl acetate	87	83	70	21
Propanol	70	65	59	22
DMSO	83	69	42	37
Butanol	39	42	41	23
Formaldehyde	118	114	110	110
Glycerol	102	97	95	84

The influence of various detergents at 1 % concentration on the S3 xylanase activity was investigated. CTAB and SLES have shown somehow negative effect on strain S3 xylanase at 1% concentration. Xylanase from S3 was highly stable after 2-hour incubation in formaldehyde and glycerol at 10% concentration. S3 xylanase was partially affected at concertation of 10% by acetonitrile, acetone, ethyl acetate, DMSO and propanol, while S3 xylanase is sensitive to butanol at 10% concentration, after 2-hour incubation (Table 6). No significant effect of 1% surfactant including; Tween 20, Tween 40. Tween 60, Tween 80, Triton-100X, CTAB, PEG and SLES, was observed on S3 xylanase. The effect of potential inhibitors or activators such as β marcaptoethanol, PMSF, and urea was also examined. PMSF β marcaptoethanol, and urea did not substantially affect the enzymatic activity.

Kinetics studies and Substrate specificities

The *Km* and *Vmax* value for strain S3 xylanase was calculated by Line weaver and Burk (1934) plot. Strain S3 xylanase was incubated with varying concentration of substrate and specific activity was analyzed. The *Km* and *Vmax* of Strain S3 for Beachwood xylan was 8.6 mg/mL and 43.71µmolmg^{-1}mL^{-1}min.

Substrate specificities

Strain S3 xylanase showed highest activity on beech-wood xylan followed by oat-spelt and birch-wood xylan. However, in case of arabinoxylan, rice-husk

and sugarcane-bagasse comparatively less activity was observed (Table 7).

Shelf life

The purified xylanase from *B. licheniformis* S3 was 100% stable for 6 weeks at 4°C. No significant decrease was observed in the activity after week 8 at 4˚C while more than 10% lost in the activity was found at room temperature. After 16 weeks, it was concluded that xylanase from *B. licheniformis* S3 has retained 88 and 56% activity 4˚C and room temperature respectively (Table 8).

Table 7. Relative activities of S3 xylanase on different xylan types, other polysaccharides and pNP substrates.

Substrates	Relative activity %
Birchwood xylan	89
Beechwood xylan	100
Oat spelt xylan	95
Arabinoxylan	61
Wheat straw	58
Rice husk	55
Sugar cane baggase	52
Soluble starch	0
Avicel	0
CMC	0
Lichenan	3
pNP-a-D-galactopyranoside	0
Laminarin	0
pNP-acetate	0
pNP-a-D-mannopyranoside	0
pNP-a-L-arabinopyranoside	0
pNP-b-D-glucopyranoside	0
pNP-b-D-xylopyranoside	0
pNP-b-D-cellobioside	0
pNP-b-D-galactopyranoside	0
pNP-a-L-arabinofuranoside	0

Resistance of S3 xylanase to proteases

The purified S3 xylanase exhibited good resistance to proteases as demonstrated by the residual activity values detected upon incubation up to 200 minutes with 10 µg/mL of collagenase, trypsin, chymotrypsin, and proteinase K. In particular, S3 xylanase activity remained almost unchanged after pre-incubation for 100 minutes with collagenase and for 70 minutes with trypsin, while, after 1 hours, the residual activity was 88 % with proteinase K, and 75 % with chymotrypsin respectively. More than 70% initial activity was retained by S3 xylanase in the presence of all protease except chymotrypsin for 200 minutes. After 200 minutes chymotrypsin decreases the activity up to 75%.

Discussion

Discovery of extremophiles have revolutionized the industrial world by shifting from chemical processes toward enzymatic processes. Enzymes are renewable and approved safe in terms of biodegradation, handling and side product formation.

The industrial processes are most often hostile and it is considered that enzymes from extremophiles – extremozymes, might be incredible candidates due to

their ability to withstand and remain active in the harsh conditions. Xylanase has drawn increasing attention in the last two decades. At present, the most commonly used application of xylanase can be seen in the bleaching of pulp for paper production. With the help of xylanase numerous bleaching steps and the dose of chemical consumption can be reduced (Techapun *et al.*, 2002). The other most important applications of xylanases are considered in the baking industry, animals feed and biofuels production. Until now, xylanases are reported from diverse organisms and sources but microorganisms are the predominant group among these. Extremophiles are considered the best source for industrial enzymes as their enzymes are adopted to active in the extreme conditions. Natural extreme environments are present across the world and a variety of xylanases have been reported from fungi and bacteria isolated from various extreme environments. However, still there is a need of industrial enzymes with improved properties. Natural thermal extreme environments are also present in Pakistan that is current focus of our study.

Table 8. Shelf life profile of purified S3 xylanase.

Weeks	S3 xylanase RA%	
	4°C	Room temperature
2	100	100
4	100	100
6	100	95
8	98	87
10	96	76
12	93	68
14	89	61
16	88	56
18	86	52
20	82	47

Isolate S3 was isolated from hot springs and screened for xylanases production. The 16S rRNA sequence analysis indicated that strain S3 is closely similar (99%) to *Bacillus licheniformis strain* (KX812811). *Bacillus licheniformis* strains were also isolated from agriculture wastes (Bajaj & Manhas, 2012), biosulphidogenic bioreactor, compost (Archana *et al.*, 1999) and from decaying wood for xylanase production. The pH of the medium is sound recognized to affect many enzymatic processes and transport of numerous components across the cytoplasmic membrane. Most of the known microbial xylanases are optimally produced at pH 5.5–9.5 (Kumar & Satyanarayana, 2014). The optimum temperature and pH for xylanases production was found 40˚C and pH 5.0 after 24 hours of incubation for *B. licheniformis* S3, Damiano *et al.*, 2003 reported optimum xylanases production for *B. licheniformis* after 72 hours incubation at 50˚C (Damiano *et al.*, 2003). These differences may be due to inoculum size or high diffusion and reaction rate of S3 isolate for efficient xylanases production. Short incubation time and high thermostable enzymes production at mesophilic conditions are desirable and economical for a microbial candidate in terms of industrial application. In this case, *B. licheniformis* S3 is a more suitable candidate as it has short incubation time compared to above reported similar strains. Analytical processes required a high stage pure enzyme, while purity is not the pre-requisite of industries like food processing, detergent as well as paper and pulp due to economic reasons. However, it is necessary to exclude certain other unwanted proteins. The strain S3 xylanase was precipitated at 60% concentration of ammonium sulfate effectively. Ammonium sulfate method is widely reported in the literature for xylanases precipitation (Chanwicha *et al.*, 2015; Kallel *et al.*, 2015; Kocabaş *et al.*, 2015;

Ninawe *et al.*, 2008; Zhu *et al.*, 2012; Irfan *et al.*, 2016). Purification by Sephadex G-100 column chromatography increased specific activity from 9.0-39.42 Umg-1 (58% yield) with 4.38 purification fold for *B. licheniformis* S3 xylanase. The purified enzyme has a molecular weight of 28 kDa. Archana and Satyanarayana 2003 reported a 45 kDa monomeric xylanase from *B. licheniformis* A99 (Archana & Satyanarayana, 2003). Two proteins having molecular weight 21 and 45 kDa with xylanases activity have been reported from *B. licheniformis* SVD1 (Van *et al.*, 2009).

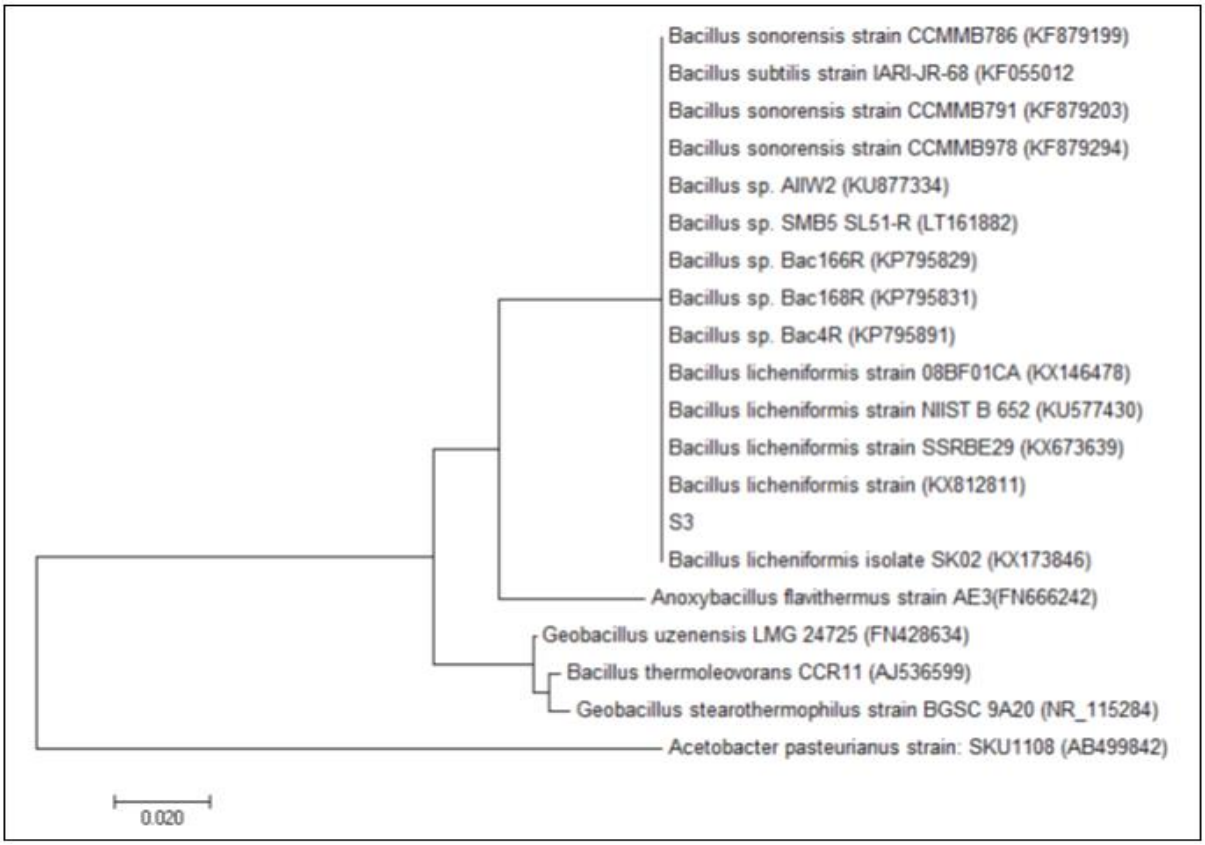

Fig. 1. Phylogenetic analysis of *Bacillus licheniformis* S3, phylogenetic tree was constructed using MEGA 6.0 software with NJ method.

The optimum temperatures of purified S3 xylanases were 55 ˚C. S3 xylanase retained 68% of initial activity at 40- 60˚C for 3 hour. Archana and Satyanarayana 2003 reported xylanase from *B. licheniformis* A99 having optimum temperature of 60˚C while unstable at 70˚C. However, a high thermostable xylanases was reported from *B. licheniformis* P11C that was thoroughly stable after 1 hour incubation at 40–100˚C (Bajaj & Manhas, 2012). S3 xylanase have a broad range of pH activity (4.0–10.0) with optimum at 7.0. More than 70% activity was retained by S3 xylanase from pH 5.0-9.0. *Bacillus halodurans* TSEV1 has optimum pH of 9.0 (Kumar & Satyanarayana, 2014). pH stabilities of S3 xylanase was better than *Paenibacillus* sp. NF1, which is stable between pH 4.0–7.0 (Zheng *et al.*, 2014). S3 xylanase exhibiting broad pH stability profile from acidic to alkaline pH (4.0–10.0) and its stability in a high temperature range make it worthy of different industrial application, such as food and feed industry, bread making, paper making and bioenergy conversion. Impurities such as metal ions, which exist in the different industrial wastes, can potentially interfere the activity of xylanases.

Although the activity of S3 xylanase was not significantly influenced in the presence of metals. $HgCl_2$ inhibited S3 xylanase even at low concentration (2mM), indicating the presence of tryptophan amino acid in the binding site of xylanase.

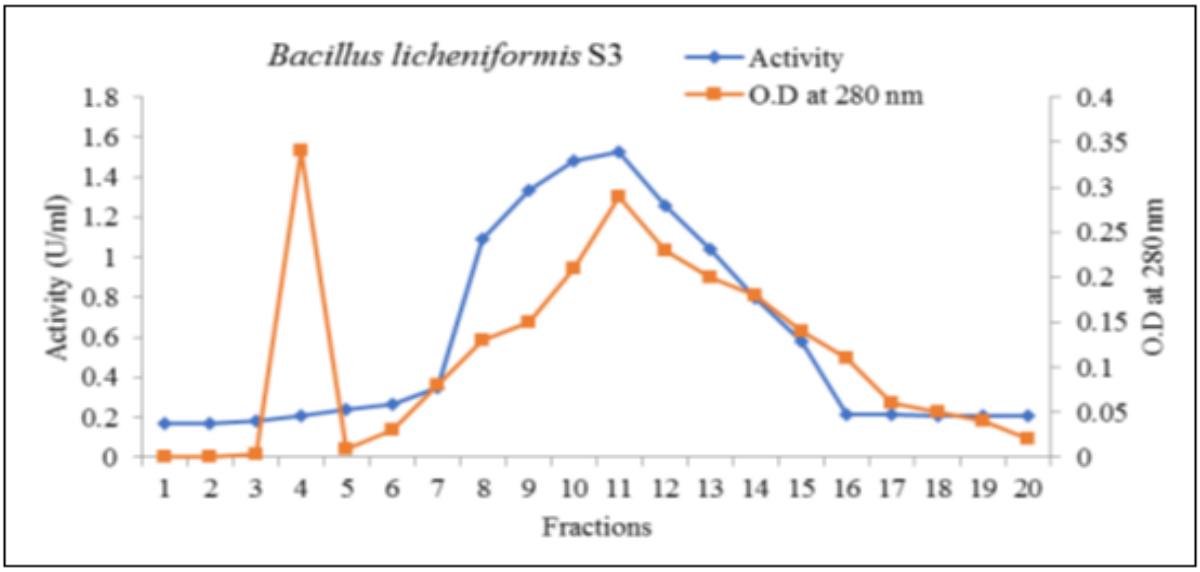

Fig. 2. Elution profile of strain *B. licheniformis* S3 xylanase on Sephadex G-100 chromatography. The profile revealed single peak of xylanase enzyme activity indicating absence of multiple forms.

The oxidative nature of Hg metal can inhibit the enzyme activity completely as it is tryptophan modifier. Similar results that indicated inhibition of xylanase by Hg were also reported in the literature (Irfan *et al*., 2016; Marcolongo *et al*., 2015).

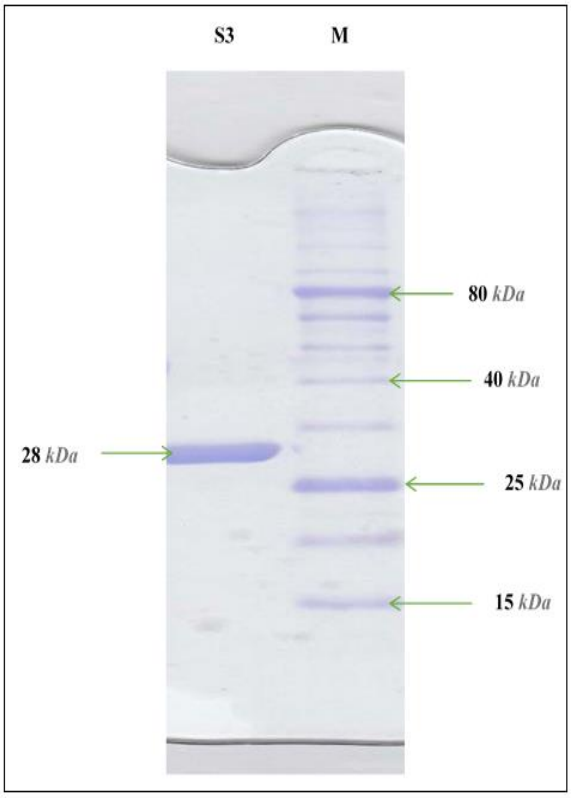

Fig. 3. SDS-PAGE analysis during purification steps of xylanases from strain S3. The purified xylanase after Sephadex G-100 chromatography revealed a single band.

Most metals did not inhibit the activity of S3 xylanase; however, Cu salt inhibited the enzyme activity at a concentration of 10 mM. S3 xylanase is quite stable in the presence of metals especially against Fe. Previously isolated xylanase from *Geobacillus thermodenitrificans* A333 was strongly inhibited in the presence of Fe ion due to oxidizing nature of Fe ions.

In addition (Marcolongo *et al*., 2015), S3 xylanase was among the few reported xylanases endowed with the capability to tolerate different metals concentration. Xylanase from S3 was highly stable after 2-hour incubation in formaldehyde and glycerol at 10% concentration. S3 xylanase was partially affected at concertation of 10% by acetonitrile, acetone, ethyl acetate, DMSO and propanol. Different modulators and chemicals did not have any inhibitory effect on xylanase, CTAB partially inhibited the enzyme at higher concentration (1%). Xylanase activity was not inhibited by chelating reagents (EDTA, EGTA), but it was partially inhibited by -reducing agents DTT and marcaptoetahnol, indicating that disulfide bonds are essential to protect the binding side in S3 xylanase. Inhibition by reducing agent was also reported for *Caldicoprobacter algeriensis* sp. novel strain TH7C1 xylanase (Amel *et al*., 2016). S3 xylanase was also found to be protease-

resistant and, even if the core mechanism of this performance is not completely clear, the insensitivity to proteolytic dose could be clarified with thermostability and extracellular localization (Marcolongo *et al.*, 2015). This relevant characteristic makes S3 xylanase useful for potential applications in food and textile industry. S3 xylanase has a Km value of 8.6 mg/mL and Vmax of 43.71 μmolmg^{-1}min^{-1}, with no cellulose activity. Basit (2018) also reported xylanase from *Myceliophthora thermophila* having Km value of 8.80mg/mL (Basit *et al.*, 2018). The km value is lower than that of *Bacillus brevis* (11.53 mg/mL) xylanase against Soluble Oat Spelt Xylan (Goswami & Rawat, 2015).

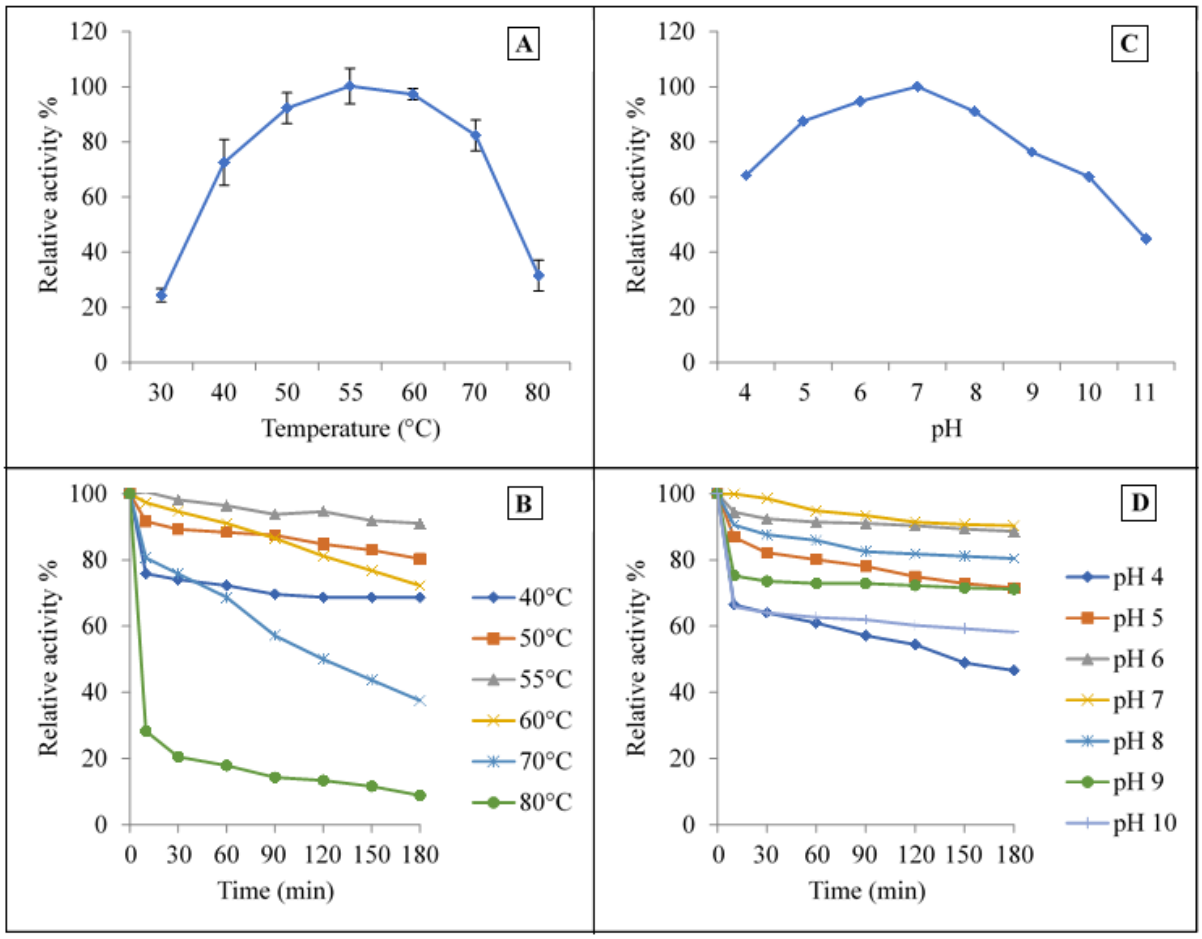

Fig. 4. Effect of temperature (A) Activity (B) stability of S3 xylanase, and Effect of pH (C) Activity (D) stability of S3 xylanase respectively. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining the stability, the activity of the enzyme without any treatment was taken as 100%.

The high catalytic profile of S3 xylanase indicates that it proficiently degrades Beech wood xylan followed by oat spelt xylan and Birchwood xylan. S3 xylanase was active at moderate-extreme conditions (High temperature, pH, detergents, and metals) and could be the contender in the paper and pulp industry to modify pulp properties by hydrolyzing hemicelluloses without affecting the cellulose fiber.

Conclusions

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