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Characterization Of A Novel Hydrolytic Enzyme Producing Thermophilic Bacterium Isolated From The Hot Spring Of Azad Kashmir-Pakistan

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ABSTRACT

A thermophilic bacterium (TP-2) was isolated from the Tatta Pani hot spring in Azad Kashmir and was characterized using phenotypic and genotypic characters. The strain developed cream colored, round, smooth, flat and slimy colonies while the cells were Gram positive rods that ranged in size from about 2.1-3.6 µm to 0.2-0.3 µm in width. Sequence analysis of its 16S rRNA gene showed that isolate TP-2 had 89% homology with *Geobacillus debilis.* It grew within pH range of 5.5 to 8.5 with optimum growth at pH 7.0. The isolate showed optimum growth at 65ºC and gave positive results for gelatin hydrolysis (GEL), ortho nitrophenyl-β-D-galactopyranosidase (ONPG), and nitrate production and produced acid from sucrose, glucose and maltose. It utilized glucose, fructose, maltose, lactose, sucrose, xylan, starch, filter paper and carboxymethylcellulose as sole carbon source. Isolate TP-2 produced significant amount of industrially important enzymes i.e. extracellular α-amylase, CMCase, FPase, Xylanase, Protease and Lipase and intracellular CMCase and FPase.

Keywords: Tatta Pani, characterization, enzyme production, *Geobacillus*

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INTRODUCTION

Microorganisms occupy all possible sites in which life survives, ranging from ideal environments for growth and reproduction to those signifying extreme environments. Extremophiles are adapted to live and grow in conditions like extreme salinity, temperature, pH and absence of oxygen. Thermophilic bacteria grow optimally at temperatures ranging from 45°C to 70°C, and hence, it is likely to isolate them from numerous habitats like deep-sea hydrothermal vents, deep ocean-basin cores, petroleum reservoirs and artificially hot environments like fermented compost (Rahman et al. 2004; Lebedinsky et al. 2007). One of the natural habitats of the thermophilic bacteria is the hot spring. Hot springs are springs that are geothermally heated. Hot springs are located all over the earth, on all continents and even beneath the seas and oceans (Kauze et al. 2006).

Thermophiles have gained a great consideration since they are not usually denatured at high temperature, yet they are active at elevated temperature (Lee et al. 1999; Beg et al. 2000). Thermostable enzymes, isolated chiefly from thermophilic microorganisms, are commercially important because of their inherent stability that is maintained under harsh industrial processes conditions as well as benefits for accomplishing the procedures at high temperature (Demirjian et al. 2001).

In recent times, molecular phylogenetic methods utilizing the small-subunit rRNA gene have been used to study the bacterial diversity of varied hot springs (Reysenbach et al. 2000). Thermophilic bacteria have been characterized by phenotypic and genotypic means from numerous geothermal regions of the World, comprising Italy (Maugeri et al. 2001), Turkey (Gul-Guven et al. 2008), Bulgaria (Derekova et al. 2008), Iceland (Marteinsson et al. 2001), Yellowstone National Park (Boomer et al. 2009), China (Lau et al. 2009), India (Sharma et al. 2009) and Greece (Sievert et al. 2000).

Tatta Pani hot spring located in Azad Kashmir, Pakistan has not been explored yet from the microbiological aspects. The objective of this work was to isolate and characterize thermophilic bacteria from Tatta Pani hot spring using genotypic and phenotypic methods and to check the biotechnological potential of the isolates.

MATERIALS AND METHODS

SAMPLE COLLECTION

Water samples were collected separately in sterile thermal glass containers and in aseptic culture tubes containing LB medium (1% NaCl, 0.5% yeast extract, 1% tryptone, pH: 7.0). These tubes were placed into the water in the same place for two hours and taken to the lab for additional processing. *In-situ* (temperature) and *ex-situ* (pH, EC, soluble anions and cations) analysis of water samples was carried out to characterize the hot springs (Khalil et al. 1998).

ISOLATION OF BACTERIAL STRAINS

Thermophilic bacteria were isolated through serial dilution method. Water sample (100 µL) was added to the LB media and incubated at 65ºC. Pure culture was obtained by repeated streaking on LB agar plates followed by incubation at 65ºC (Khalil et al. 1998).

MORPHOLOGICAL CHARACTERIZATION

Colony morphology characteristics of isolates were observed from 48 h culture on LB agar with respect to color, shape, margin, internal structure, elevation and configuration. Cell characteristics of isolate were studied by Gram's staining. Scanning electron microscopy was done commercially from University of Karachi.

PHYSIOLOGICAL CHARACTERIZATION

Optimum temperature for growth was determined by incubating the isolate in LB medium at different temperatures (30-90ºC) with 5ºC interval. Optimum pH was determined by incubating the isolate overnight at 65ºC±1ºC by altering the initial pH of the LB medium from 4.0 to 10 with either 1N NaOH or 1N H_3PO_4. The ability of the isolate to grow at different NaCl concentration was examined by incubating the isolate in LB medium (pH: 7.0) containing different amount of NaCl ranging from 0-5% (w/v). Growth was checked by measuring optical density (O.D.) at 600 nm using spectrophotometer (Nair and Surendran 2004).

Study of utilization of different carbon sources by the isolate was also checked. Mineral salts broth (0.02% $MgSO_4 \cdot 7H_2O$; 0.25% NaCl; 0.001% $FeSO_4 \cdot 7H_2O$; 0.1% $(NH_4)_2SO_4$; 0.15% KH_2PO_4; pH:7.0) with 1% of different carbon sources, like sucrose, maltose, lactose, fructose, glucose, carboxymethylcellulose (CMC), xylan, starch,

wheat bran extract and filter paper (0.05 g) was added to test tubes. These were inoculated with overnight grown culture (20 µL) of the isolate followed by incubation at 65°C for 48 h. Growth of the culture was determined by measuring the optical density at 600 nm using spectrophotometer.

BIOCHEMICAL CHARACTERIZATION

Oxidase test, catalase test and QTS-20 tests were performed following the method of Wiegel and Ljungdahl (1981), Tarrand and Groschel (1982) and Macfaddin (2000).

MOLECULAR CHARACTERIZATION

Genomic DNA was isolated after Kronstad et al. (1983). 16S rRNA gene was amplified by PCR using the following primers

"9F 5′-GAGTTTGATCCTGGCTCAG-3′
1510R 5′- GGTTACCTTGTTACGACTT-3′"

PCR reaction was carried out in a 50 µl reaction mixture, using DNA (1 µl; 50 ng), primers (l µ1 each; 10 µM), dNTPs (5 µl; 2.5 mM), PCR buffer (5 µl; IX), magnesium chloride (5 µl; 25 mM) and *Taq* polymerase (l µl; 2.5 U). Initial denaturation of template was carried at 94°C (5 min), followed by 35 cycles of 94°C (30 sec), 54°C (30 sec) and 72°C (90 sec) and one final cycle of extension at 72°C (10 min). The PCR product was analyzed by agarose gel electrophoresis (1%). The PCR product was excised from the gel and purified using Novagen spin prep gelmelt kit. The purified 16S rRNA gene was sequenced using 3130xl Genetic Analyzer. Sequence database similarity searches of the 16S rRNA gene sequence were done using the BLAST tool of GenBank. Alignment of 16S rRNA gene sequences was done using software Clustal W and observed by Jalview. Phylogenetic analysis was done using neighbor-joining method using the software MEGA 5.0 (Saitou and Nei 1987; Altschul et al. 1990; Thompson et al. 1994; Waterhouse et al. 2009). The stability of relationships was evaluated by carrying out bootstrap analysis of the neighbor-joining data based on 1000 resamplings.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The 16S rRNA gene sequence of isolate TP-2 has been submitted in the GenBank with accession no. JQ284017.

BIOTECHNOLOGICAL POTENTIAL

Production of intracellular and extracellular enzymes was studied by incubating the isolate in different media reported by Mandels and Reese (1957), Srivastava and Baruah (1986), Chen et al. (2004), Lee et al. (1999), Sharma et al. (2007) and Kim et al. (1998) for the production of CMCase & FPase, α-amylase, protease, lipase, xylanase and phytase, respectively.

Extraction of Extracellular and Intracellular Enzyme

Following incubation for particular time interval, the fermented broth was centrifuged at 10,000 rpm (4ºC) for 15 min. The supernatant obtained was utilized as extracellular enzyme. After centrifugation of the fermented broth, the cells were washed two times with 0.85% NaCl (5 ml). The wet weight of the pellets was recorded and the pellets were placed at –20°C before sonication. Frozen cells were thawed in potassium phosphate (50 mM)-EDTA (5 mM); pH: 7.0 buffer and the final cell concentration was kept at 20% wet weight/volume and then cells were broken by sonication on ice, using sonicator UP 400S at 70 amplitude for 30 cycles (30 sec on/50 sec off). The mixture was centrifuged at 12,000 rpm for 10 min at 4ºC and the supernatant was utilized as intracellular enzyme.

Enzyme Assay

CMCase, α-amylase, FPase, Lipase, protease, xylanase and phytase were estimated by the methods of Mandels et al. (1976), Rick and Stegbauer (1974), Ghose (1987), Mustranta (1992), McDonald and Chen (1965), Bailey et al. (1992) and Harland and Harland (1980) and Heinonen and Lahti (1981), respectively.

STATISTICAL ANALYSIS

Significance difference among replicates was shown in the form of probability ($p \leq 0.05$) values using the software Costat (Snedecor and Cochrane 1980).

RESULTS AND DISCUSSION

Tatta pani is located in Kotli, Azad Kashmir (Pakistan) and Tatta Pani hot springs are positioned on the right bank of the river Poonch and can be approached from Kotli by covering a distance of 26 km. Heat produced due to the occurrence of sulphur in Patala shales, friction alongside the Main Boundary Thrust and deep circulation of meteoric water underneath the surface is the possible cause of heat giving rise to hot springs in Tatta Pani, Azad

Kashmir. *In-situ* measurements of temperature showed that the hot spring had temperature ranging from 79-86°C. The hot spring had nearly neutral pH of 6.93. Khan et al. (1999) noted pH and temperature 1.70-1.86 and 56-60°C, respectively for these hot springs. *Ex-situ* characterization of water sample was done to determine the chemical composition of the hot spring (Table 1). The key cation in the Tatta Pani hot springs was found to be Na^+ (208 mg/L). EC value and TDS were recorded to be 462.6 µS/cm and 231.3 mg/L, respectively. Khan et al. (1999) also stated Na^+ as the major ion in Tatta Pani hot spring, having concentration of 110 to 120 ppm. Total dissolved solids (TDS) and electrical conductivity (EC) were found to be 3911 to 4183 ppm and 7020 to 9560 µS/cm, respectively. Khan et al. (1999) study displayed a little variation in the water characterization results, they recorded low pH and temperature and higher TDS and EC values as compared to the current findings. This variation might be due to the difference in time period of study and due to the change in concentration of various ions and amount of sulfur that resulted in shift in pH from extremely acidic to neutral. The change in temperature detected may be because of different sampling points (distance from the source) or this change might be because of the earthquake that occurred in 2005.

Table 1: Composition of water in Tatta Pani hot spring

Type of Study	Result
Mg^{+2} (mg/L)	0.91
K^+ (mg/L)	2.9
Na^+ (mg/L)	208
Ca^{+2} (mg/L)	4.5
Cl^- (mg/L)	58.3
HCO_3^- (mg/L)	0.71
CO_3^{-2} (mg/L)	0.05
NO_3^{-1} (mg/L)	26.23
TDS (mg/L)	231.3
S.A.R	23.31
COD (mg/L)	8.2
BOD5 (mg/L)	16.9
Temperature (°C)	79-86
EC (µs/cm)	462.6
pH	6.93

A thermophilic bacterium (TP-2) was isolated from Tatta Pani hot spring in Azad Kashmir. Single colonies were identified and the pure culture was isolated by repeated streaking on LB agar plates incubated at 65°C. Colony morphology characteristics were examined from over 48 h culture on LB agar. Colonies were flat, cream colored and slimy with smooth margins while the cells were Gram positive rods (Fig. 1). *Geobacillus* sp. strain GWE1 formed white, convex and circular colonies while cells were gram positive rods (Daniela et al. 2013). *Geobacillus* sp. NMS2 isolated from water and soil samples from a hot spring in Sri Lanka formed yellow colored, round, convex and smooth colonies while the cells were rod shaped that stained positive with Grams staining (Mathew and Rathnayake 2014). *Geobacillus thermoleorans* strain Rekadwadsis isolated from hot spring in Unkeshwar formed light yellow, circular, convex and smooth colonies whereas the cells were long Gram positive rods (Rekadwad 2015).

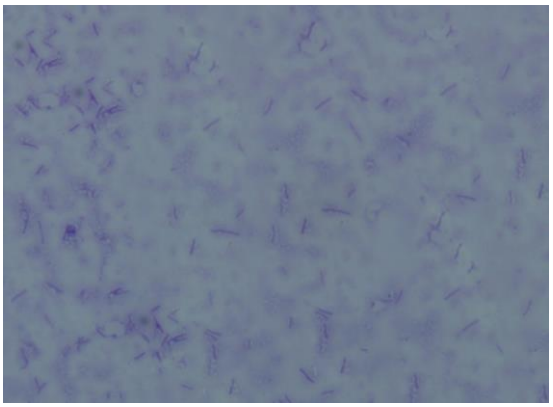

Figure1: Gram's staining of the isolate TP-2

Scanning electron microscopy results showed that the cells have smooth surface and ranged in size from 0.2-0.3 µm in width to 2.1-3.6 µm in length (Fig. 2). The length and diameter of cells of *G. debilis*, *G. thermoglucosidasius* and *G. stearothermophilus* ranged in size from 1.0-14.2 µm to 0.5-1.0 µm, <3.0 µm to <0.9 µm and 2.0-3.5 µm to 0.5-0.9 µm, respectively (Banat et al. 2004; Nazina et al. 2001; Sung et al. 2002). The length and diameter of cells of *Geobacillus* sp. strain GWE1, *Geobacillus* sp. NMS2, *Geobacillus thermoleorans* strain Rekadwadsis ranged in size from 8.0 to 0.8-1.0 µm, 8-10 to 1 µm and 6.0-1.0 µm, respectively (Daniela et al. 2013; Mathew and Rathnayake 2014; Rekadwad 2015**)**

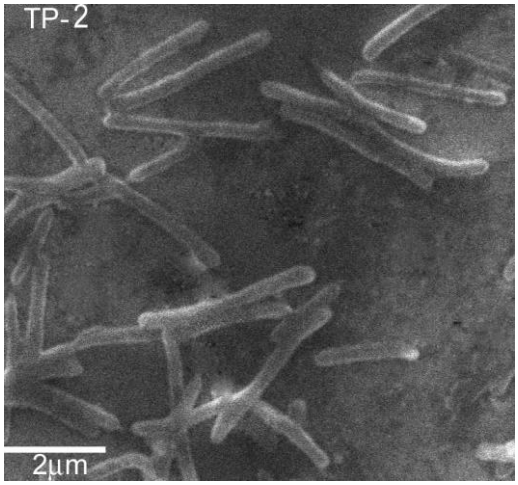

Figure 2: Scanning Electron Micrograph of isolate TP-2

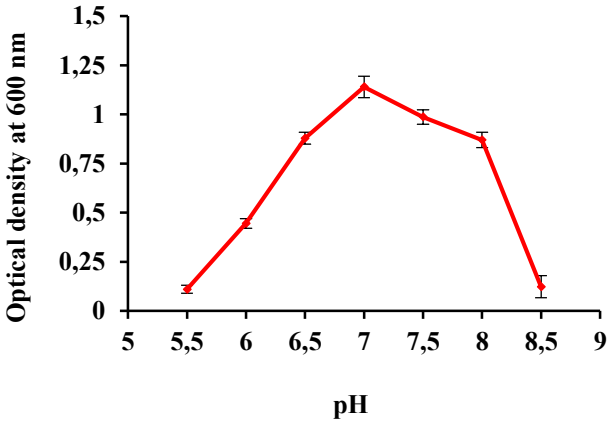

Figure3: Effect of pH on the growth of isolate TP-2. ± indicates the standard deviation among the three parallel replicates.

Physiological and biochemical characters are significant as they give indications for selection of efficient strains for additional studies principally of applied value. Various range of pH (4.0-10) was tested to record the pH optimal for the growth of the isolate (Fig. 3). Maximum growth of isolate (TP-2) was recorded at pH 7.0. The isolate grew at pH 5.5 to 9.0 while no growth was observed below pH 5.5 and above 9.0. The isolate was able to survive at both slightly alkaline and acidic environments. Though the isolate preferred a neutral pH range, it adopted itself to survive at slightly basic and acidic pH conditions in the environment. Sharma et al. (2009) isolated *Geobacillus* spp. from the Soldhar hot spring site (India) that can tolerate pH range of 4.0-11. Thermophilic bacteria that grew well at pH ranging from 7.5-8.5 were isolated from the hot springs of Saudi Arabia (Khalil 2011). *Geobacillus* sp. SG 01 displayed optimum growth at pH 7.0 while no growth was observed at pH values higher than 9.5 (Yang et al. 2013). *Geobacillus pallidus* p26 isolated from Pasinler hot spring, Erzurum, Turkey grew optimally at pH 7.5 (Celik et al. 2014). *Geobacillus* sp. 12AMOR1 exhibited growth within the pH range of 5.5-9.0 with optimal growth within the wide pH of 6.5-8.0 (Wissuwa et al. 2016).

Minimum, optimum and maximum growth temperature for the isolate was noted by incubating the isolate at different temperature (30-90ºC). The isolate displayed good growth within temperature range of 50-65ºC with maximal growth observed at 65ºC (Fig. 4). No growth was observed below 40ºC while the upper limit for growth with respect to temperature was around 75ºC. The temperature specificity of the isolate indicated that it is thermophilic in nature since it required 65ºC for optimum growth and was unable to grow outside the temperature range of 40-75ºC. Thermophilic bacteria that showed good growth between 45-85ºC were isolated from hot springs of Fiji (Narayan et al. 2008). *G. thermoglucosidasius* grew within the temperature range of 37-68ºC (Nazina et al. 2001). *Geobacillus toebii* showed growth within the temperature range of 45-70ºC with optimum growth at 60ºC (Sung et al. 2002). *Geobacillus* sp. strain GWE1 showed growth within the temperature range of 60-80ºC while optimum growth was observed at 70ºC (Daniela et al. 2013). *G. thermoleovorans* DA2 isolated from Southern Sinai, Egypt was able to grow within the temperature range of 50–80ºC displaying optimal growth at 65∘C (Fotouh et al. 2016). *Geobacillus thermoleorans* strain Rekadwadsis also showed optimal growth at 65ºC (Rekadwad 2015).

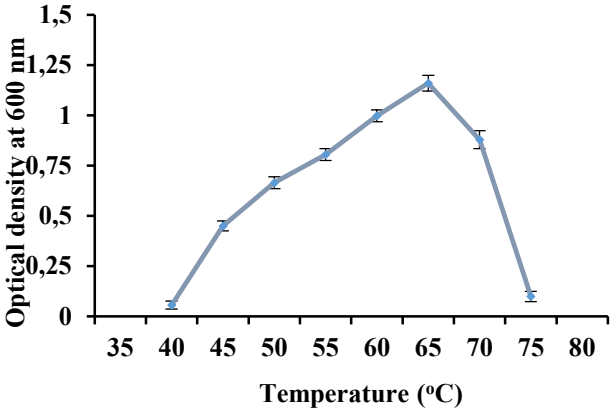

Figure4: Effect of temperature on the growth of isolate TP-2. ± shows the standard deviation among the three parallel replicates.

Isolate TP-2 displayed growth at NaCl concentrations ranging from 0-3.5% while optimal growth was observed at 0.5% NaCl concentration (Fig. 5). *Geobacillus* sp. isolated from a hot spring in Gilgit grew optimally in the absence of NaCl while it was incapable of growing at 5% NaCl (Tayyab et al. 2011). *G. vulcani and G. thermodenitrificans* exhibited growth at NaCl concentration (0-3.0%) whereas *G. uzenensis* and *G. thermocatenulatus* displayed growth at NaCl concentration (0-4%) (Golovacheva et al. 1975; Suzuki et al. 1983; Caccamo et al. 2000; Nazina et al. 2001). *Geobacillus* sp. SG 01 presented growth at NaCl concentration (0-2%) with optimal growth at 1% NaCl (Yang et al. 2013). *Geobacillus pallidus* p26 grew within the NaCl range of 2-5% (Celik et al. 2014).

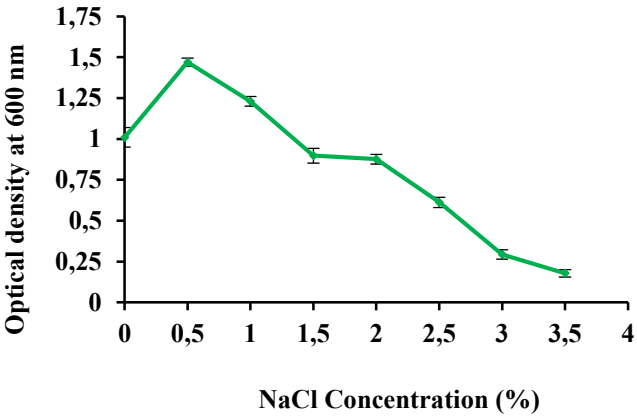

Figure 5: Effect of NaCl concentration on the growth of isolate TP-2. ± indicates the standard deviation among the three parallel replicates.

Isolate TP-2 was catalase and oxidase positive. Biochemical characterization of the isolate using QTS-20 test strips revealed that isolate TP-2 gave positive result for gelatin hydrolysis, ortho-nitrophenyl β-D-galactopyranoside (ONPG), nitrate reduction and produced acid from sucrose, maltose and glucose while gave negative results for other tests such as tryptophan deaminase, sodium malonate, sodium citrate, indole production, lysine decarboxlase, ornithine decarboxylase, urea hydrolysis, H_2S production, arginine dihydrolase, acetoin production (VP), and acid production from sorbitol, arabinose, mannose, inositol and rhamnose following incubation at 65°C for 24 to 48 h. *Geobacillus toebii* HBB-218 isolated from hot spring in Turkey and *Geobacillus thermoleorans* Strain Rekadwadsis isolated from Unkeshwar hot spring were found to be catalase and oxidase positive (Ozdemir and Biyik 2012; Rekadwad 2015)

Several carbon sources i.e., glucose, lactose, fructose, sucrose, maltose, starch, xylan, wheat bran extract and carboxymethylcellulose (CMC), at a final concentration of 1% (w/v) and filter paper (0.05 g) were also screened for their ability to support the growth of the isolate in mineral salt medium after 48 h of incubation at 65°C (Fig. 6).

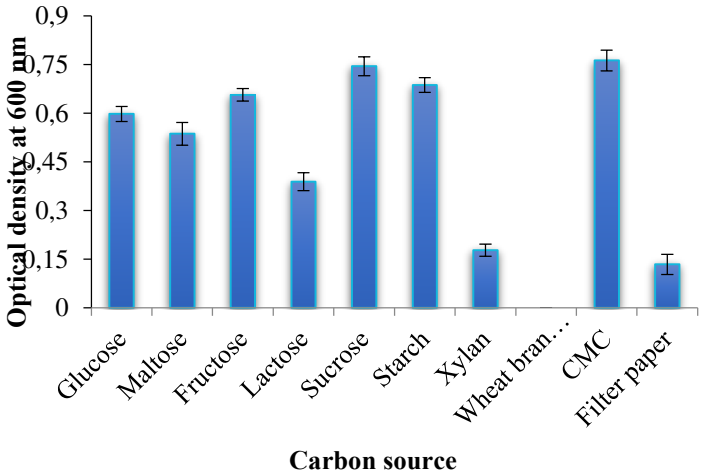

Figure 6: Growth of isolate TP-2 on different substrates. ± indicates the standard deviation among the three parallel replicates.

Results showed that the isolate showed good growth on all the carbohydrates tested as sole carbon source except wheat bran extract. Maximum growth of the isolate was observed on CMC amended mineral salt medium followed by sucrose and starch. The ability of the isolate to degrade different carbohydrates could be explained by

inclination of microbial societies toward living at low organic content in such environments and development of adjustable systems for uptake of any available food (Derekova et al. 2008). Examination of carbohydrate degrading activity of the isolate demonstrated that the isolate could be considered as possible source of biotechnologically valuable enzymes such as cellulases, amylase and xylanase. *G. thermodenitrificans* SG-01, SG-02 and SG-03 gave positive result for utilization of fructose, fructose and sucrose and sucrose, respectively (Yang et al. 2013). However, *Geobacillus* sp. strain GWE1 was unable to grow on fructose and xylan (Daniela et al. 2013). *Geobacillus* sp. NMS2 was able to ferment glucose, sucrose, maltose and lactose (Mathew and Rathnayake 2014). *G. thermoleorans* strain Rekadwadsis utilized fructose, lactose, sucrose and maltose as carbon source (Rekadwad 2015).

Genomic DNA was isolated from the isolate TP-2 and utilized for the amplification of 16S rRNA gene fragment using 9F and 1510R primers. The purified 16S rRNA gene fragment was successfully recovered for TP-2 (Fig. 7). The purified PCR product was approximately 1.5 kb in size corresponding to the group of bacteria. Partial sequence of 16S rRNA gene fragment (936 bp) was obtained for TP-2 using an automated sequencer. 16S rRNA gene sequence analysis indicated that the isolate was distantly related to thermophilic *Geobacillus* group and the 16S rRNA gene sequence was quite distinct from the data available in the databases. The partial 16S rRNA gene sequence showed a similarity of 89% to *Geobacillus debilis* and 83% similarity to *Geobacillus thermoglucosidasius* and *Geobacillus toebii* (Fig. 8). *Geobacillus debilis* is a Gram negative bacterium that does not utilize starch. It is incapable of producing acid from sucrose, maltose and glucose but few strains give acid from arabinose and mannitol. It displayed growth within narrow temperature range of 50-75ºC as compared isolate TP-2 (45-75ºC) and gave positive result for arginine dihydrolase. Moreover, the cells of isolate TP-2 were smaller in diameter (0.2-0.3 µm) as compared to *G. debilis* (0.5-1.0 µm) (Banat et al. 2004) (Table 2). *Geobacillus thermoglucosidasius* produces acid from mannitol, inositol, sorbitol and rhamnose and is urease positive. It does not hydrolyze gelatin. *Geobacillus toebii* does not hydrolyze starch and gelatin and produces acid from inositol (Suzuki et al. 1983; Priest et al. 1988; White et al. 1993; Caccamo et al. 2000; Sung et al. 2002; Banat et al. 2004).

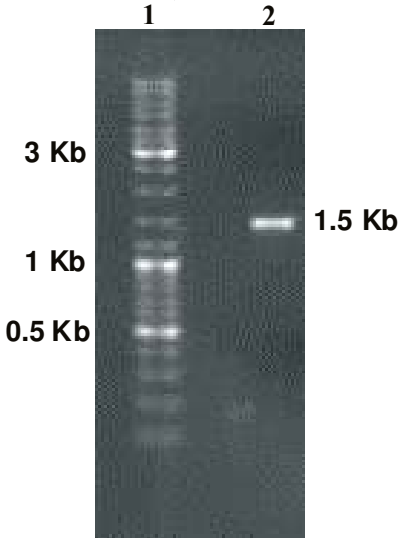

Fig. 7: Purified 16S rRNA gene fragment of the isolate TP-2. Lane 1 ladder, lane 2 purified 16S rRNA gene

A phylogenetic tree made by aligning 16S rRNA gene sequences of type strains taken from GenBank, NCBI and sequence of the isolate TP-2 displayed similar relationship (Fig. 9). The separated phylogenetic relationship, low sequence homology and distinct phenotypic and biochemical characteristics of isolate TP-2 that were not confined to any specific group in the phylum Firmicutes suggest that it may indicate a novel strain.

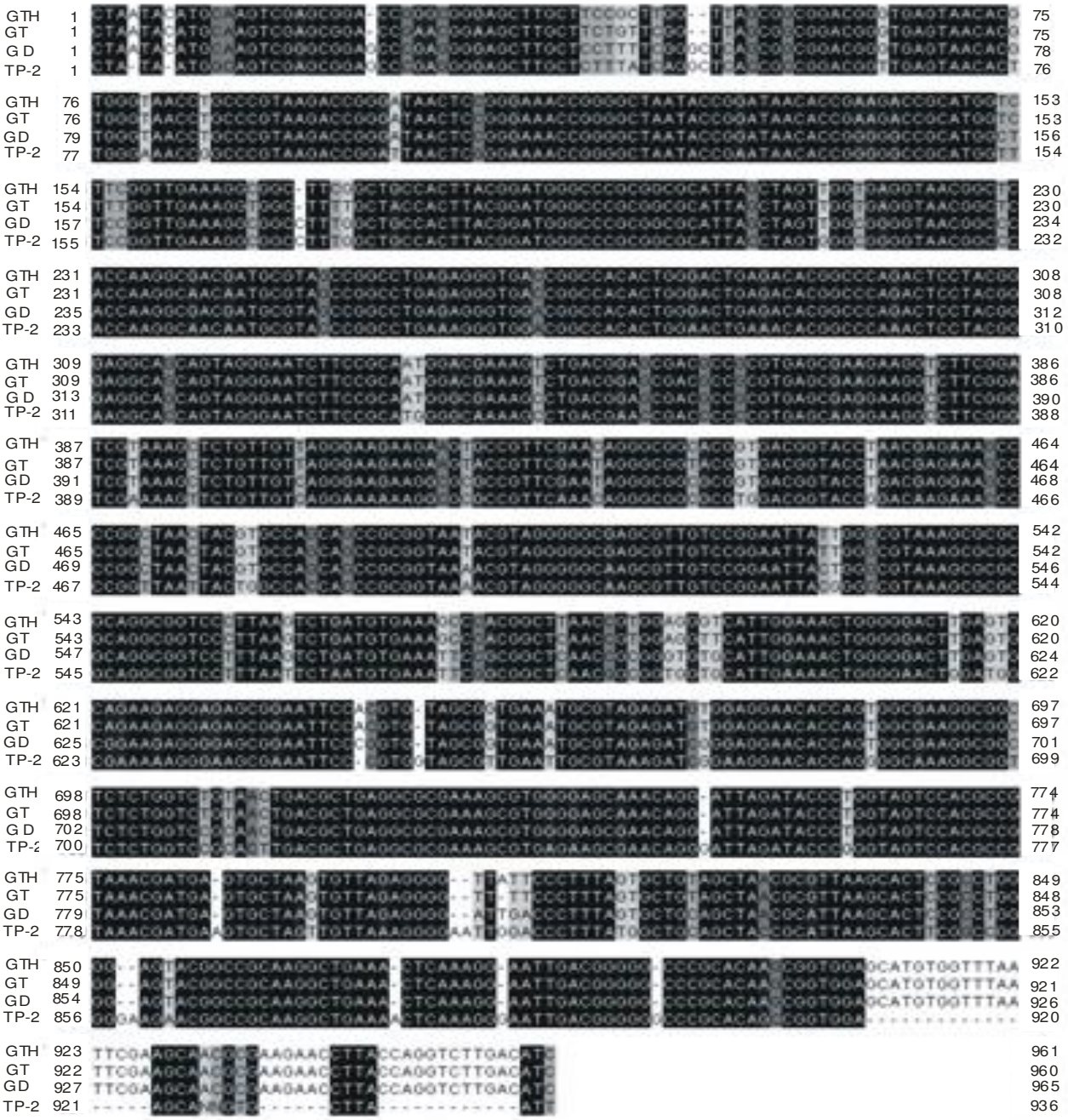

Figure8. Jalview representation of multiple sequence alignment of isolate TP-2 16S rRNA gene partial sequence with 16S rRNA gene sequences of closely related strains. GTH, *Geobacillus thermoglucosidasius*; GT, *Geobacillus toebii*; GD, *Geobacillus debilis*; TP-2, Isolate from the present study. Black shaded areas indicate identities in all sequences whereas other identities are presented as grey shaded areas.

Table 2: Comparison of characteristics of isolated strain (TP-2) with other related strains

Characteristics	Isolate TP-2 (This study)	*Geobacillus* sp. strain GWE*	*G. debilis*[€]
Cell length (µm)	**2.1-3.6**	**0.8**	**1.0-14.2**
Cell width (µm)	**0.2-0.3**	**0.1**	**0.5-1.0**
Motility	+	-	+
Temperature range (°C)	**45-75**	**60-80**	**50-70**
pH range	**5.5-8.5**	**3.0-8.0**	**ND**
NaCl range (%)	**0-3.5**	**0-2**	**ND**
Catalase	+	**ND**	**ND**
Oxidase	+	-	**ND**
Grams Reaction	+	+	-
ONPG	+	**ND**	+
Sodium citrate	-	**ND**	-
Lysine dehydrogenase	-	**ND**	-
Arginine dihydrolase	-	**ND**	+
Ornithine decarboxylase	-	**ND**	-
H_2S production	-	**ND**	-
Urea hydrolysis	-	**ND**	-
Acetoin production	-	**ND**	-
Gelatin hydrolysis	+	-	**d**
Nitrate Reduction	+	+	-
Acid Production from			
Glucose	+	+	-
Maltose	+	-	-
Sucrose	+	**ND**	-
Mannose	-	-	-
Arabinose	-	+[w]	**d**[w]
Rhamnose	-	**ND**	-
Sorbitol	-	**ND**	**d/w**
Inositol	-	**ND**	-

+, positive; - negative; ND, not determined, +[w] weakly positive; d, positive in some strains
*Daniela et al. (2013); [€] Banat et al. (2004)

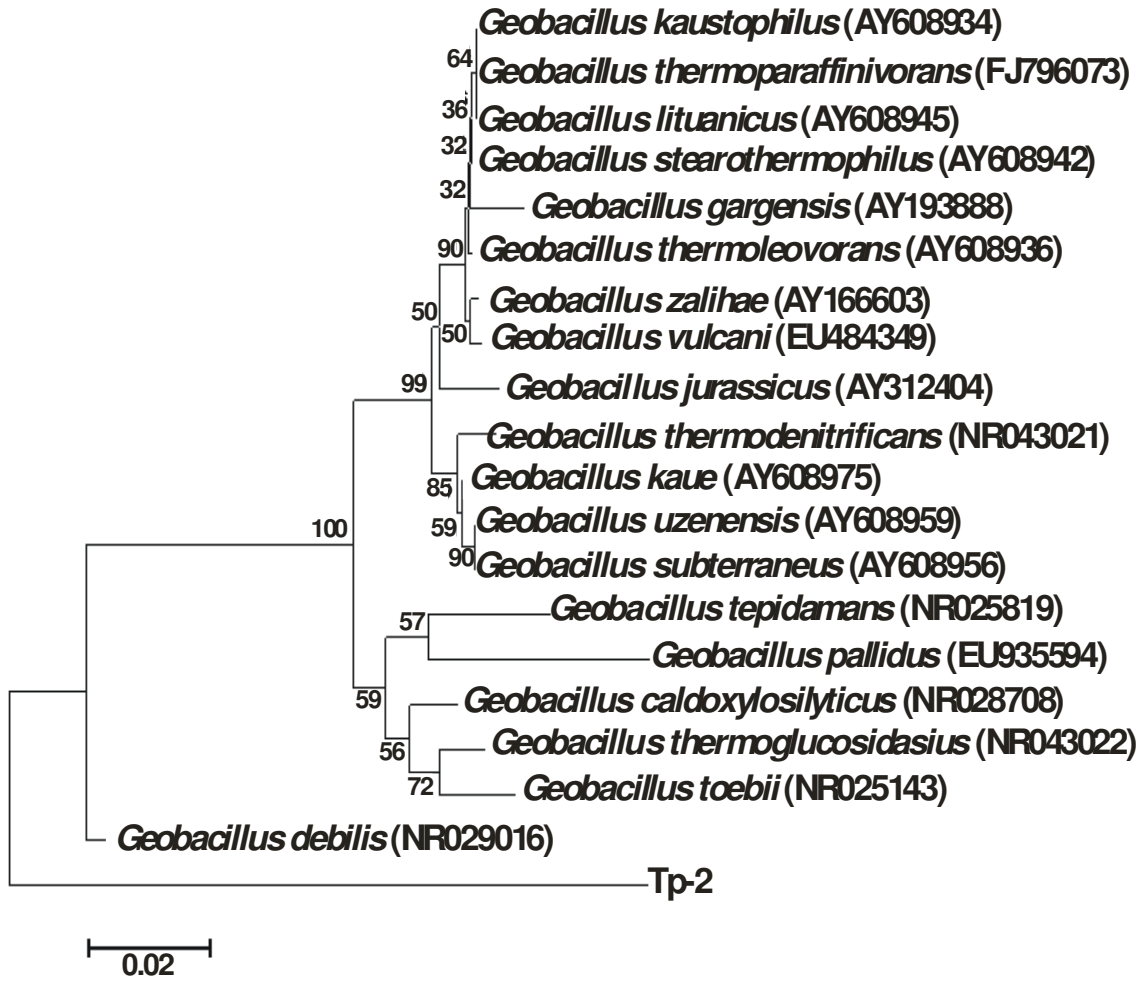

Figure 9: Phylogenetic tree showing evolutionary relationship between isolated strain and some reference strains. The number at the branch nodes indicates bootstrap values (%) built on 1000 replications. The accession numbers are shown in parentheses. The scale bar denotes 0.02 nucleotide substitutes per position.

Thermophiles are modified to live at high temperature in hot springs. These microorganisms synthesize characteristic biocatalysts that work under extreme conditions analogous to those predominant in numerous industrial processes. Enzymes from thermophiles, hence, have found great attention in industrial applications (Haki and Rakslit 2003). Isolate TP-2 gave significant production of extracellular enzymes such as α-amylase (0.654 U/ml/min), CMCase (0.086 U/ml/min), FPase (0.008 U/ml/min), Xylanase (0.475 U ml^{-1} min^{-1}), Protease (0.089 U ml^{-1} min^{-1}) and Lipase (0.21 U ml^{-1} min^{-1}). Isolate TP-2 also gave intracellular production of CMCase (0.017 U g^{-1} min^{-1}) and FPase (0.003 U g^{-1} min^{-1}). Information about the production of enzymes by *Geobacillus* sp. is scarce and no work has been published regarding the production of these enzymes by *G. debilis*. *G. thermoleovorans* DA2 produced thermoalkaliphilic lipase that was optimally active at 60ºC (Fotouh et al. 2016). *Geobacillus thermoleorans* strain Rekadwadsis produced extracellular amylase (8, 578 U/mL) that was optimally active at 68ºC. *Geobacillus kaustophilus* PW11, *Geobacillus toebii* PW12, *Geobacillus thermoleovorans* PW13 and *Geobacillus toebii* PS4 isolated from Tattapani hotspring of Himachal Pradesh, India gave extracellular cellulase activity that was optimum at 80-90ºC and pH 6.0-8.0 (Sharma et al. 2015). The lipase activity given by isolate TP-2 was more than the maximum lipase activity (0.3 U L^{-1}) given by the thermophilic *Bacillus* sp. IHI-91(Becker et al. 1997) and maximum lipase activity (0.15 U ml^{-1}), reported for *Geobacillus zalihae* T1 (Rahman et al. 2007). Extracellular xylanase and CMCase activities of isolate TP-2 were higher than the

xylanase activities (300-400 U/L) reported for *G. thermoleovorans* (Sharma et al. 2007) and CMCase/cellulose activities reported for *Geobacillus* sp. even under optimized conditions (Tai et al. 2004; Abdel-Fattah et al. 2007; Rastogi et al. 2009). The thermophilic nature of isolate TP-2, profiling of the bacterial carbohydrates metabolism and significant production of industrially important enzymes makes it an interesting candidate for biotechnological applications.

CONCLUSION

Isolate TP-2 isolated from Tatta Pani hot springs is a highly potent strain that give significant production of enzymes i.e., α-amylase, lipase, protease, CMCase, xylanase and FPase making it a promising strain for use in industries because of its industrially important thermostable enzymes.

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Erratum

In Article "Characterization Of A Novel Hydrolytic Enzyme Producing Thermophilic Bacterium Isolated From The Hot Spring Of Azad Kashmir-Pakistan", with the number of DOI: http://dx.doi.org/10.1590/1678-4324-2016150662, published in journal Brazilian Archives of Biology and Technology, vol. 59, the 01 page.

That read:
"**Sana Zahoor[1]*; Huhammad Mohsin Javed[2]; Masroor Ellahi Babar[1].**"

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