ISOLATION AND CHARACTERIZATION OF A THERMOPHILIC BACTERIUM, *GEOBACILLUS SP.* STRAIN MAS2, FROM A HOT SPRING OF BALOCHISTAN, PAKISTAN

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ABSTRACT

Till to date, there is no report on isolation of microorganisms from the Mahiwal hot water spring in Balochistan, Pakistan. In the current manuscript, we report on isolation and morphological, biochemical and molecular characterization of a thermophilic microorganism from this hot water spring. The isolated bacterial strain, MAS2, is rod shaped with 1 µm width and 5 µm length. It forms cream-colored slimy colonies. The strain has the ability to grow in the presence of air between 40 and 75 °C with an optimum growth temperature and pH of 65 °C and 6.5, respectively. It can utilize various sugars, hydrocarbons and carboxylic acids as sole carbon source. No additional salt is required for its growth, though relatively higher yield was achieved with the addition of 0.5% NaCl. The strain MAS2 is capable of producing several important extracellular enzymes of industrial use including an amylase, a lipase, an oxidase and a protease. The 16S rRNA gene sequence of strain MAS2 exhibited high homology with various species belonging to genus *Geobacillus*. The highest homology of 98.66% was observed with *Geobacillus kaustophilus*. However, the biochemical characteristics of the strain were more similar to *Geobacillus thermopakistaniensis* and *Geobacillus uzenensis*. These results indicate that strain MAS2 represents an unidentified species belonging to genus *Geobacillus*.

Keywords: Thermophiles; 16S rRNA sequence: *Geobacillus sp.* MAS2; biochemical characterization; extracellular enzymes.

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INTRODUCTION

Aquatic water resources accelerate economic growth with preserving and utilizing aquatic ecosystems comprising plants, animals and microorganisms (Mesut 2021). Microorganisms are one of the earliest living organisms that populated our planet earth. Among them, thermophilic microbes, whose optimal growth temperature is in the range of 45-75 °C, are found in deep sea vents, thermal springs, petroleum reservoirs and ocean basins habitats (Lebedinsky *et al.,* 2007). Thermal springs are scattered in all continents of the earth, even under the seas (Kauze *et al.,* 2006). They contain extremophiles that can grow at temperature above 90 °C and are called hyperthermophiles (Stetter, 2013). It is believed that the upper growth limit of some of the hyperthermophiles is 150 °C (Merino et al., 2019). Hyperthermophiles contain special metabolites with novel metabolic pathways that enable them to flourish in elevated temperatures (Schönheit and Schäfer, 1995; Sakuraba and Ohshima 2002).

Thermophilic bacteria and archaea are considered to be among initial life forms on the primitive earth (Kimura *et al.,* 2006). Genome sequence of numerous thermophilic bacterial (Takami *et al*., 2004; Siddiqui *et al*., 2014; Li *et al*., 2022; Sung *et al*., 2024) and archaeal domains (Bult *et al*.,1996; Fukui *et al*., 2005; Bridger *et al*., 2012) have been determined. These microorganisms produce hydrolytic enzymes that stay functional at elevated temperatures. Industrial processes require harsh reaction factors such as extremely high temperature, high or low basic and acidic pH and extreme ionic strength, and under these conditions, enzymes from mesophilic organisms are not optimally active. Extremozymes are usually suitable for such conditions, which can further be improved and produced in high amounts by protein engineering and recombinant production (Cabrera and Blamey, 2018; Bibi et al., 2018). A number of extremozymes are being applied for industrial applications. Although previously isolated thermophilic strains (Amo *et al*., 2002; Tayyab *et*

al., 2011; Siddiqui *et al*., 2014) are being used as sources of extremozymes, there is still a need to find and explore thermophiles from new locations and utilize their enzymes for industrial use. There is no report on isolation of thermophilic bacterial strains from the Mahiwal hot water spring, located in igneous rocks of Loralai area of Balochistan province. The hot springs emerging through igneous rocks contain mineral rich water and unique ecosystem. It is expected that the Mahiwal hot spring harbors unique extremophiles which have not yet been identified. Therefore, we selected this location for isolation and characterization of new thermophilic microorganisms.

MATERIALS AND METHODS

Sampling: Mahiwal hot spring is located in the Tor Ghundi locality of the Shabozai area, Loralai division of Balochistan. Regionally it lies within Northern Sulaiman Foldbelt and is situated in the eastern vicinity of a Tor Ghundi igneous intrusive body. Small deposits of low weight travertine sedimentary lime stone of calcium compound and aragonite crystals of calcium carbonate are found in the vicinity of the spring (Malkani and Mahmood, 2016). The hot spring temperature ranges from ~75 to 80 °C and is highly sulfurous with high quantity of mineral elements that provide optimum conditions for survival of the microorganisms. Water samples were collected in clean aseptic autoclaved glass conical tubes. Physical (temperature) and chemical (pH) values of water samples were determined at the spot before shifting the laboratory using a Multipara Meter Water Quality Checker U-50 Series (Horiba, Japan). Elements in Mahiwal hot spring water sample were analyzed by atomic absorption spectrometer (Thermo Fisher Scientific, USA).

Enrichment and isolation of thermophilic bacterial strain: Water samples (10 mL) were used for enrichment in nutrient broth (100 mL), followed by incubation at 65 °C with continuous shaking at 200 rpm. After three days (72 h) enrichment, the culture was streaked on nutrient agar plates (1.5% tryptone, 1.4% agar, 1.0% yeast extract, and 0.5% NaCl) through serial dilution method and was incubated at 65 °C to obtain a pure culture (Al-Batayneh *et al.,* 2011). The pure culture, grown in LB medium, was stored at -20° C in 30% (final concentration) glycerol.

Morphological examination: For Gram staining examination, cells were grown at 65 °C for ~15 h. One drop heat fixed culture was stained with Sigma kit reagents and examined on a Niken DXM1200 camera fitted on phase contrast microscope. Transmission electron microscopy was performed for high resolution bacterial morphology determination with JEOL JEM-1010 transmission electron microscope, operating at 80 kV. For negative staining the bacterial culture was suspended in a drop of 5% uranyl acetate salt solution having pH 4.2 - 4.5. The suspension was filtered using a 0.22 µm disposable Polyether sulfone syringe filter on a specimen support sample holder carbon coated copper grid. The extra stain was wiped with a filter paper. The copper grid was dried in air for 1–4 min prior to micrographic observation (Oestreicher *et al*., 2012).

Biochemical characterization: Various biochemical investigations including biosynthesis of enzymes such as amylase, catalase, urease, protease, xylanase and oxidase were carried out according to the methods described by Church, 2016. The QTS-24 miniaturized identification system, manufactured by DESTO Laboratories, Karachi, Pakistan, was used to perform morphological and biochemical tests. All tests of QTS-24 system were performed according to the user instructions manual.

Physiological characteristics: The pH reliance of the "pure culture" was tested by growing it overnight (~15 h) in LB medium at 65 °C at various pH (5.5 - 9.0). Similarly, optimal growth temperature was determined at pH 6.5 under various incubation temperatures (from 40 to 80 °C). The saline conditions on growth was examined in nutrient broth containing 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6% NaCl (Harley and Prescott, 2002).

PCR amplification of 16S rRNA gene: The genomic DNA was isolated by growing pure culture of the bacterial strain MAS2 and gene amplification of 16S rRNA was carried out utilizing primer 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525R: AAGGAGGTGWTCCARCC-3' (Macrogen). The reaction mixture for PCR extension routinely contained 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 5U *Taq* DNA polymerase for elongation, and 1 µg of genomic DNA. PCR reaction mix contained 50 pmol quantity of each forward and reverse primers. Initial denaturation was achieved by heating the reaction mixture for 5 min at 94 °C. After that 30 cycles of denaturation (30 sec at 94 °C), annealing (30 sec at 56 °C) and extension (90 sec at 72 °C) were performed. Thereafter, an extension of 10 min was continued at 72 °C.

Cloning of 16S rRNA gene, sequencing and phylogenetic analysis: The final PCR amplified 16S rRNA gene fragment was purified by gel electrophoresis and ligated in pTZ57R/T plasmid. *Escherichia coli* DH5α cells were transformed by using this ligation mixture. Positive clone was obtained by blue/white screening of the colonies. Recombinant plasmid DNA was isolated from the white colonies. The purified DNA was double digested with *Eco*R1 and *Hin*dIII restriction enzymes. After confirmation of the positive clone, DNA sequencing was performed (Sanger *et al.,* 1977). Basic Local

Alignment Search Tool (BLAST) program (Altschul *et al.*, 1990) was used for bacterial strain homology searches. Phylogenetic and molecular evolutionary analyses were performed by using MEGA version 11.1. The neighbor-joining method (Saitou and Nei, 1987) was applied for the creation of phylogenetic tree.

Statistical analysis: Statistical analysis was conducted using SPSS Modeler 18 freely available at https://www.ibm.com/support/pages/downloading-ibm-spss-modeler-180. Quantitative data were presented as mean ± SD on three independent experiments.

Accession number of 16S rRNA gene sequence: The 16S rRNA gene sequence of strain MAS2 was submitted to GenBank data base under accession number LC616852.1.

RESULTS AND DISCUSSION

Isolation of microorganism: For the isolation of bacterial strain water samples were taken from Mahiwal hot spring located in the mountains in Loralai division of Balochistan, Pakistan (30°22´N 68°36´E). The sampling site is shown in Fig. 1A and location map in Fig. 1B. The pH and temperature at the collection spot were 7.1 and 80 °C, respectively. Collected samples were brought to the laboratory for enrichment. Prior to enrichment, composition of the collected water sample was analyzed by atomic absorption spectrometer. High amount of sulfur (382 ppm), calcium (220 ppm), iron (124 ppm) and magnesium (75 ppm) was found in the collected water sample (Table 1). The high concentration of these metals could be due to the surrounding igneous rocks rich of calcium carbonate, fluorite (CaF_2), iron, magnesium and zinc. Other elements such as Na, K, Cl, P and Zn were present in the range of 12-200 ppm.

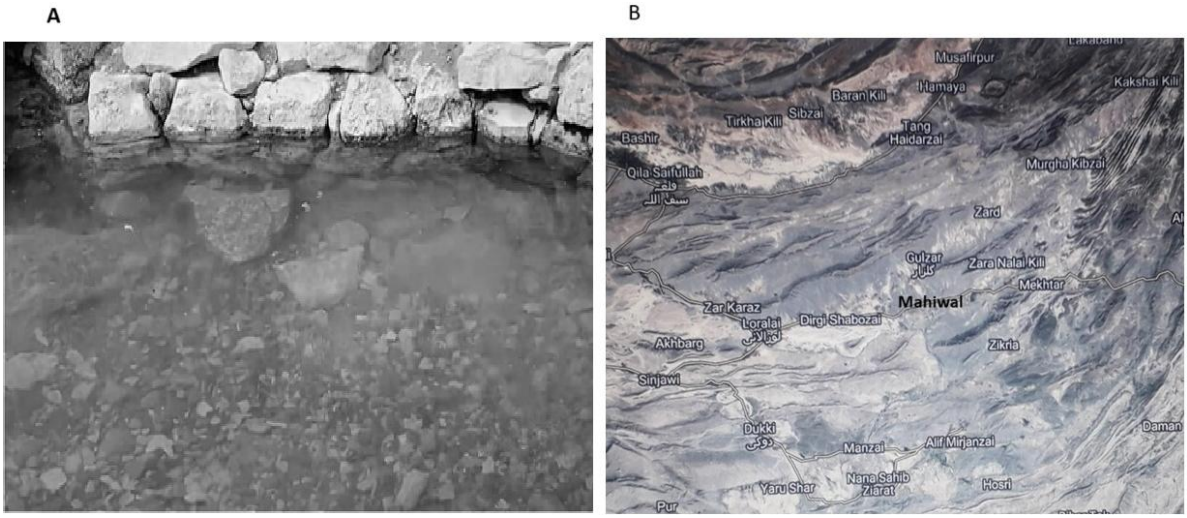

Fig. 1. A) A photograph of the Mahiwal hot water spring on mountains near Loralai, Balochistan, Pakistan (place of sampling). B) Location map of Mahiwal hot spring.

Table 1: Distribution of various elements found in the water sample.

Element	Concentration (ppm)
Ca	220 ± 5
Mg	75 ± 3
Na	200 ± 5
K	55 ± 2
Cl	118 ±3
P	14 ±1
C	28 ±1
S	382 ±7
Fe	124 ±3
Zn	12 ±1

Morphological characterization: In order to obtain a pure strain, enriched culture was flecked on nutrient plates followed by incubation at 65 °C. By repeated flecking on culture plates a pure culture of thermophilic bacterium was obtained. Round colonies with creamy white color were formed after 24 h of incubation. After Gram staining cells appeared pinkish violet, instead of violet (Fig. 2). Some species of Gram-positive bacteria, such as *Geobacillus kaustophilus*, decolorize easily to appear as Gram-negative, though they possess cell wall similar to Gram positive bacteria (Cuebas *et al*., 2011). In order to determine the cell size and morphology, cells were examined under transmission electron microscope. The cells appeared rod shaped with ~5 µm length and ~1 µm width (Fig. 3). These values are close to *Geobacillus uzenensis* which is reported to be 4.7−8 µm in length and 0.9−1.3 µm in width (Nazina *et al*., 2001). Long and thin flagella were seen on the surface of the strain MAS2. Flagella are intricate and complex structures composed of multiple proteins and act as rotary motor for bacterial motility towards favorable environments or away from unfavorable ones. Motile *Geobacillus* species such as *Geobacillus thermoglucosidasius* and *G. uzenensis* contain flagella while nonmotile thermophilic bacterial species belonging to *Geobacillus* genus such as *G. kaustophilus* and *Geobacillus thermoleovorans* do not have flagella (Nazina *et al*., 2001).

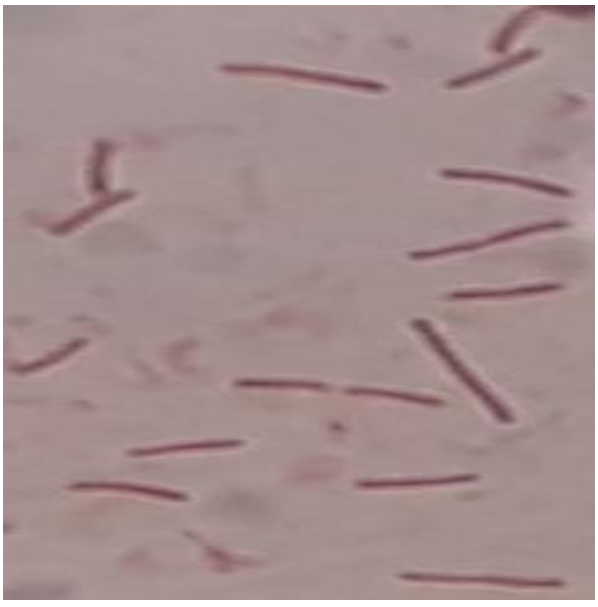

Fig. 2. Result of Gram staining under light microscope with a magnification 1000x. Cells showed decolourization of Gram staining similar to several *Geobacillus* species.

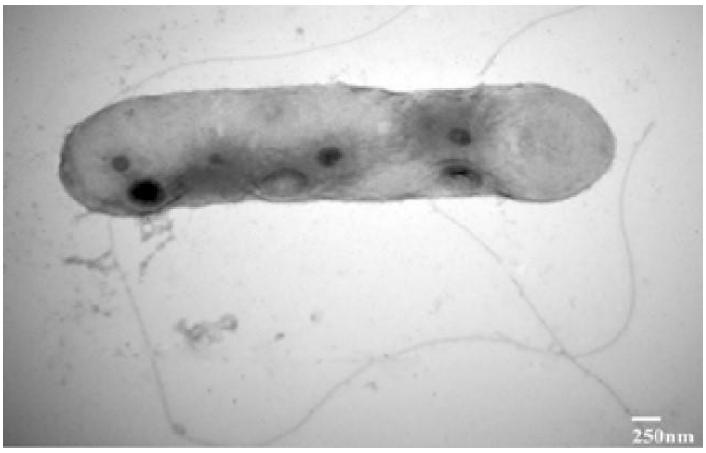

Fig. 3. Transmission electron microscopic photograph of *Geobacillus* strain MAS2 with a magnification 550,000x.

Biochemical characterization: The biochemical characterization results indicated that strain MAS2 could produce acid by utilizing a number of sugars including glucose, maltose, sucrose, inositol and rhamnose. However, it could not produce acid from L-arabinose and mannose. It was observed that strain MAS2 was able to hydrolyze starch and gelatin but could not hydrolyze citrate and urea. It possessed oxidase and catalase activities. Similarly, strain MAS2 could liberate nitrogen gas from nitrate (Table 2). The characteristics presented in Table 2 indicate that strain MAS2 is a member of genus *Geobacillus* and is more close to species *G. kaustophilus*. On the other hand, strain MS2 contains flagella while *G. kaustophilus* does not. Previous studies have also shown that some traits of a strain resembled to one species while the others were more similar to another species (Tayyab *et al*., 2011).

Table 2. Characteristics that differentiate *Geobacillus* sp. MAS2. from the closely related species of the genus *Geobacillus*

Characteristics	1	2	3	4	5	6	7	8
Cell width (um)	1.0	0.9	1.5		0.5	0.8-1	.9-1.3	0.5-1
Cell length (um)	5.0	3.0	3.5		3-7	2.5-6	4.7-8	1.5-2.5
Motility	+	-	-	+	+		+	ND
Production of acid from								
Adonitol	+	+	V	-	-	-	-	ND
L-arabinose	-	-	-	+	-	+	+	+
Inositol	+	+	-	+	-	+	-	ND
Rhamnose	+	-	-	+	+	ND	-	V
Sorbitol	+	-	-	-	+	ND	-	ND
Glucose	+	+	ND	+	ND	ND	ND	ND
Mannose	-	+	ND	-	ND	ND	ND	ND
Maltose	+	+	ND	+	ND	ND	ND	ND
Sucrose	+	+	ND	-	ND	ND	ND	ND
Melibiose	ND	ND	ND	+	ND	ND	ND	ND
Raffinose	ND	ND	ND	+	ND	ND	ND	ND
Hydrolysis of								
Starch	+	+	+	+	+	ND	+	V
Gelatin	+	+	-	+	-	+	+	ND
Urea	+	-	ND	-	+	ND	-	-
Citrate	-	+	+	-	-	ND	-	V
Malonate	ND	ND	ND	+	ND	ND		ND
Utilization of								
Formate	ND	V	ND	ND	ND	ND	-	ND
Acetate	ND	-	ND	ND	-	ND	+	ND
Lactate	ND	-	ND	ND	-	ND	+	ND
Formation of glucose	-	-	+	ND	-	ND	-	ND
Methyl red test	ND	ND	ND	ND	-	ND	-	+
Denitrification	+	ND	+	+	ND	+	-	ND
Oxidase	+	+	+	+	ND	-	+	ND
Catalase	+	+	+	+	ND	ND	+	ND
NaCl conc. for growth (% w/v)	0-3.5	0-= 5	0-1.5	0-4	1.5	0-4	0-4	0-3
pH range for growth	5.5-8	6-8	6.5-8.5	6-9	6.5-8.5	5-9	6.2-8	6-8
Temperature for growth (°C)	40-75	37-68	35-70	ND	42-69	50-70	45-65	45-70

Table 2. The numbers are indicated as: 1, *G. thermoloralaiensis* MAS2; 2, *G. kaustophilus*; 3, *G. thermoleovorans*; 4, *G. thermopakistaniensis* MAS1; 5, *G. thermoglucosidasius*; 6, *G. zalihae*; 7, *G. uzenensis*; 8, *G. thermodenitrificans*. Symbols: +, growth or activity detected; -, growth or activity could not be detected; ND, growth or activity did not determine; V, stands for variable within the group. Data presented from the current study (*G. thermoloralaiensis* MAS2); Tayyab *et al,* (2011) (G. SBS-4S/MAS1); Nazina *et al,* (2001) (*G. kaustophilus*; *G. thermoleovorans*; *G. thermodenitrificans*; *G. uzenensis*; *G. thermoglucosidasius*) and Rahman *et al* (2007) (*G. zalihae*).

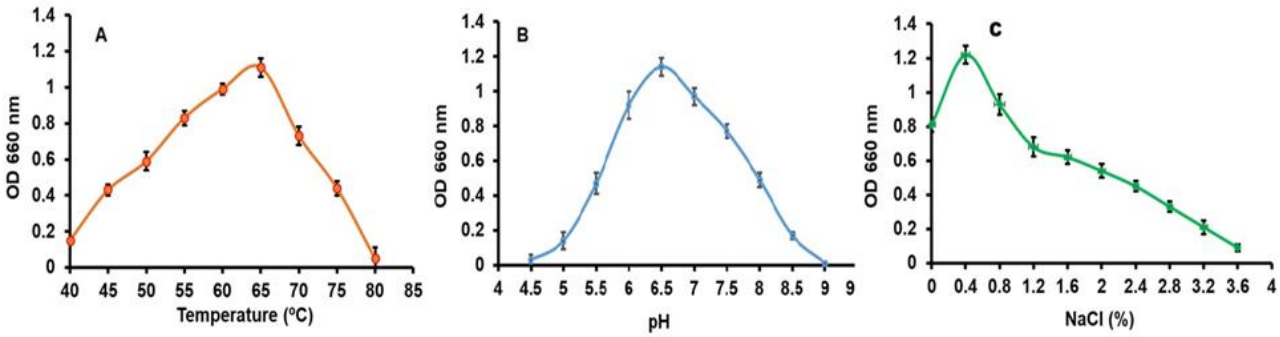

Fig. 4. Growth profile of strain MAS2. A) Effect of temperature on the growth of MAS strain. B) Effect of pH on the growth of MAS strain. C) Influence of salinity on the cell growth. Cells were cultivated either in LB medium (A and B) or in nutrient broth (C). Standard deviation among three replicates is indicated by ± symbol.

Optimal growth conditions: In order to know the optimum growth conditions such as temperature, pH and salt concentration the strain MS2 was cultivated at various temperatures, pH and NaCl concentrations. The strain was able to grow between 40 and 75 °C with an optimal growth temperature at 65 °C (Fig. 4A). Similarly, it could grow between pH 5.0 and 8.5 with optimal growth at pH 6.5 (Fig. 4B). It was able to grow well without addition of NaCl, however, slight increase in growth was observed with the addition of NaCl at a final concentration of 0.5%. Addition of higher concentrations of NaCl inhibited the growth of the strain (Fig. 4C).

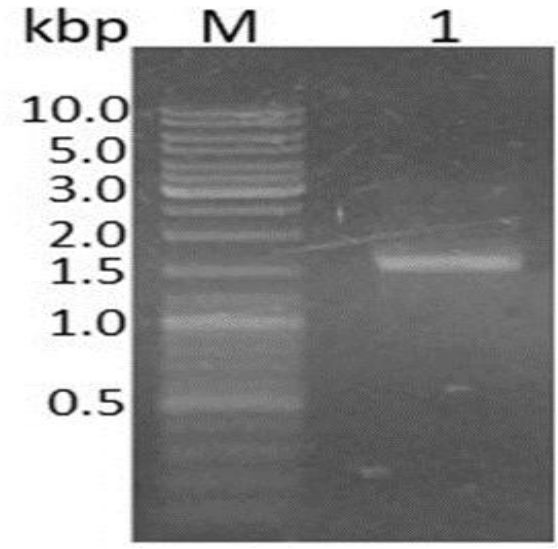

Fig. 5. Analysis of PCR amplified 16S rRNA gene fragment on 1% agarose gel. Lane M, standard marker; lane 1, 1.6 kbp PCR amplified 16S rRNA gene fragment.

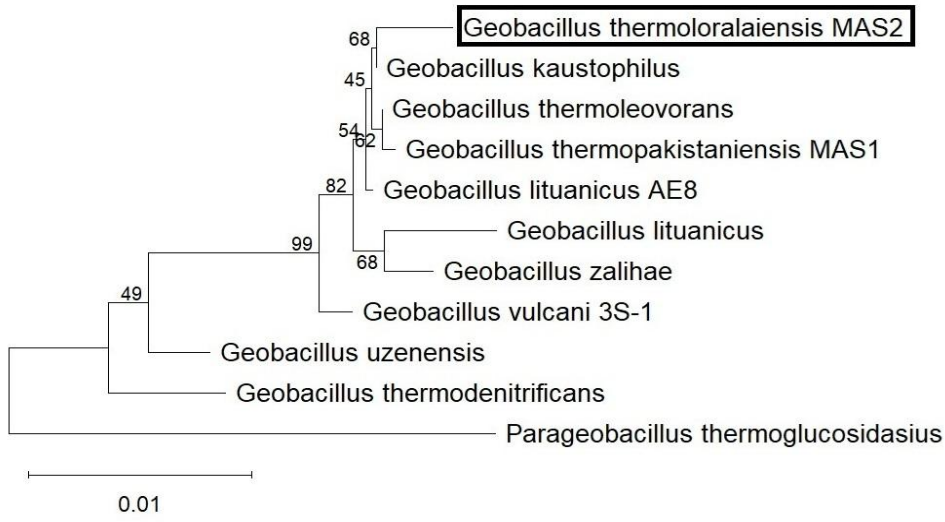

Fig. 6. Phylogenetic analysis of strain MAS2 and its closely related strain belonging to genus *Geobacillus* established using 16S rRNA gene sequences. The branch points indicate bootstrap values. Evolutionary distance of 0.01 change per nucleotide is indicated with the scale bar. 16S rRNA sequence accession numbers of various *Geobacillus* species are as follows: *G. thermoloralaiensis* MAS2, LC616852.1; *G. kaustophilus*, AY608934.1; *G. lituanicus*, AY044055.1; *G. thermoleovorans*, FN428693.1; *G. thermopakistaniensis* MAS1, AB306519.1; *G. zalihae*, AY166603.1; *G. thermodenitrificans*, NR043021.2; *G. uzenensis*, AY608959.1; *Parageobacillus thermoglucosidasius*, NR043022.2; *G. lituanicus* AE8, FN666246.1; *G. vulcani* 3S-1, NR025426.1.

Molecular characterization: The genomic DNA of the strain was isolated and used as template for PCR amplification of 16S rRNA gene. Amplification of the gene was performed by using universal forward (27F) and reverse (1525R) primers. The PCR resulted in amplification of approximately 1.5 kb DNA fragment (Fig. 5), which was ligated in pTZ57R/T plasmid and bacterial *Escherichia coli* cells DH5α were used for transformation. Recombinant plasmid DNA was purified and DNA sequencing was performed. The 16S rRNA sequence (1473 nt) of strain MAS2 was obtained and analyzed using NCBI BLAST search which indicated that the strain MAS2 was related to thermophilic genus *Geobacillus*. Based on the 16S rRNA sequence, the nearest homologue of strain MAS2 was *G. kaustophilus* with an identity of 98.66%. Utilizing the highly similar 16S rRNA sequences of related microorganisms, a phylogenetic tree was generated by neighbor-joining method (Fig. 6). The constructed tree displayed the closest resemblance of strain MAS2 with *G. kaustophilus*. Although strain MAS2 exhibited high similarity with *G. kaustophilus*, however it contained long and thin flagella on the surface which are not reported for *G. kaustophilus.* Such flagella are reported in *G. thermoglucosidasius* and *G. uzenensis.* These findings demonstrate that MAS2 is a distinct strain of genus *Geobacillus*.

Conclusion: A thermophilic strain MAS2 was isolated from the Mahiwal hot spring located in Balochistan, province of Pakistan. Biochemical characteristics of strain MAS2 are similar to *G. uzenensis* and *G. thermopakistaniensis*. However, 16S rRNA sequence put it adjacent to *G. kaustophilus* (98.66% identity). Therefore, we propose MAS2 a distinct strain belonging to genus *Geobacillus*. We designate it as *Geobacillus thermoloralaiensis* MAS2 sp. nov.

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Authors contributions: NT, OAS and MAS performed research work. OAS collected samples from Mahiwal hot spring. MAS and NR wrote and edited the manuscript.

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