

RESEARCH ARTICLE

## Characterization, Phylogenetic Analysis and Potential Applications of Heterotrophic Bacteria Inhabit Sand Dunes of Thar Desert, India

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### Abstract

Microorganisms inhabit extreme environments are novel in terms of phylogeny and also valuable source of extracellular hydrolytic enzymes. The present study focused on isolation and characterization of moderately thermophilic heterotrophic bacteria from Thar Desert using culture dependent method and assessment of their potential for production of industrially important enzymes. Screening of bacteria from sand dune samples collected from arid region of India led to isolation of total eight moderately thermophilic bacterial strains. All strains were characterized morphologically, biochemically and 16S rRNA gene sequencing analysis. The phylogenetic analysis of strains revealed that these strains affiliated to genera *Bacillus* (5 strains), *Geobacillus* (2 strains) and *Aeribacillus* (1 strain), which were able to grow between 25-60 °C. The present study documented the occurrence of species *Geobacillus thermodenitificans*, *Bacillus paralicheniformis* and *Aeribacillus pallidus* in sand dunes of Thar Desert, India. These moderately thermophilic strains showed an ability to produce one or more extracellular enzymes like amylase, protease, lipase and asparaginase. Thus, the strains isolated from Thar Desert could be potential candidates for industrial applications. The present study showed that bacterial strains exist in the same ecological niche might have ability to produce distinct enzymes.

**Keywords:** 16S rRNA gene, *Bacillus*, Phylogenetic analysis, Thar Desert, Moderately thermophiles.

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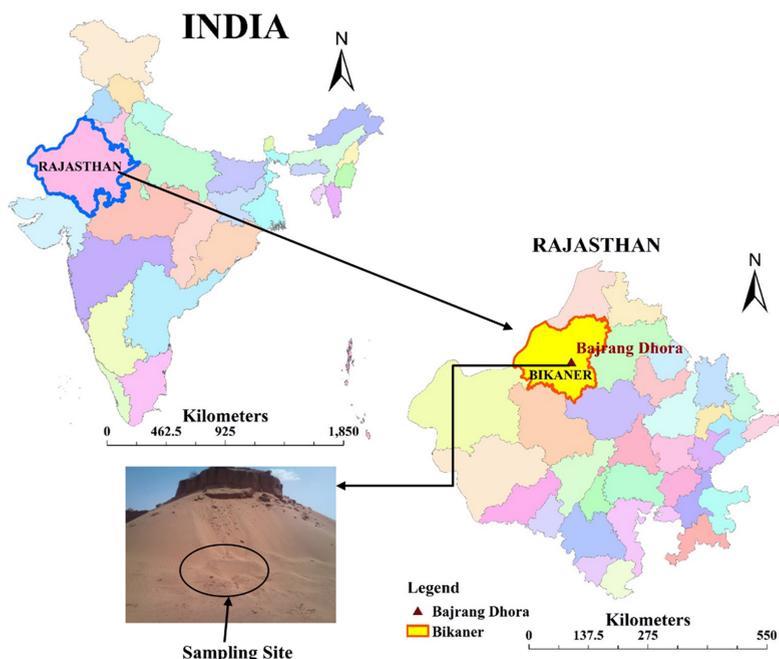
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## INTRODUCTION

Temperature is one of the most important factor for classification of the bacteria. The optimal temperature for growth of mesophilic bacteria is around 37 °C, whereas, for moderately thermophilic bacteria, it is about 45-55 °C for moderately thermophilic bacteria, 60-80 °C for thermophilic bacteria and above 80 °C for hyperthermophilic bacteria.<sup>1-3</sup>. The enzymes produced by mesophilic bacteria have limited stability at high temperature. In the recent years, thermophilic bacteria are paid more attention because they are the potential source of thermostable extracellular hydrolytic enzymes. Thermostable protease enzyme Alcalase from *Bacillus licheniformis* is commercially used in processing of soy meal, protein-fortified beverages, etc.<sup>4</sup>. Likewise, thermostable cellulases are able to avoid neutralization step in bioprocess conversation to improve lignocellulose conversion to biofuels<sup>5</sup>. Moreover, thermostable enzymes reduce the risk of contamination at higher temperatures and work against many solvents, detergent, acidic and alkaline pH in different industrial bio-processes<sup>6,7</sup>. Thermophilic bacteria thrive in harsh environmental conditions like as geothermal vents, hot springs and desert soil<sup>8</sup>.

The Thar Desert is large arid and semi-arid region in north-western part of India. It occupies about 2.34 million km<sup>2</sup> area encompassing Rajasthan, Haryana Gujarat and South-Western Punjab<sup>9</sup>. Sandy soil of desert is a key factor that makes the mobile and fixed sand dunes by aeolian processes. Generally sand dunes represent the extreme drought condition that is hostile for the existence of life<sup>10</sup>. Thus, only extremophilic microorganisms can survive in such kind of environments. A few studies have been carried out to find out microbial diversity in Thar Desert<sup>11-13</sup>. Efforts have been made to explore unculturable bacterial diversity in Thar Desert soils by denaturing gradient gel electrophoresis<sup>14</sup>. The earlier studies from different arid regions of Thar Desert indicate the presence of potential thermophilic bacteria which are good source of lipase and amylase enzymes<sup>13,15</sup>. The study conducted on screening of extracellular enzymes from *Yuhushiella* sp. TD 032 isolated from rocky soil of Thar Desert but later it was found to be mesophilic bacterium<sup>16</sup>. The Thar Desert region of India is unique habitat with high temperature in summer and low temperature in winter and poor in availability of water because of sandy soil that make the desert soil an extreme ecosystem. Considering presence of these harsh conditions



**Fig. 1.** Location of sampling sites at Bajrang Dhora, Thar Desert, Bikaner, Rajasthan, India. (Outline map source: Google map)

and paucity of work on culturable thermophilic bacteria and their ability for production of extracellular enzymes, the present study was undertaken to isolate and characterise strains of eubacteria using conventional methods and 16S rRNA gene sequencing. The work was extended for investigation of industrially important enzymes from isolated moderately thermophilic strains to evaluate their potential applications.

## MATERIALS AND METHODS

### Site description, sample collection and physico-chemical analysis

Thar Desert also known as Great Indian Desert covers about 2.34 million km<sup>2</sup> area within India and mainly Bikaner, Barmer, Jaisalmer and Jodhpur districts of Rajasthan encompassing 61% boundary of Thar Desert. It is a large arid region dominated by fixed and mobile sand dunes. The sand dune samples were collected from Bajrang Dhora (28°03'18.41" N 73°15'58" E) which is located almost 11 km away from Bikaner (Rajasthan), India (Fig. 1). Samplings were carried out in the month June, 2014 and February 2015. Sand dune samples were collected from 10 cm depth of dune surface with the help of sterile spatula and transferred in to sterilized zip lock polythene bags and transported aseptically to laboratory. Physico-chemical conditions (temperature, pH, conductivity, total dissolve solids and salinity) of the soil during sampling were determined at the sampling site by multi-parameter PCSTestr<sup>TM</sup>35 (Eutech instrument, Oakton, Singapore).

### Isolation and purification of bacterial isolates

Serial dilution method was used for bacterial isolation. Sandy soil sample (1 g) was suspended in 100 mL of sterilized distilled water, slightly homogenized and spreaded onto nutrient agar plates. The plates were incubated at 55 °C for 24 h<sup>13</sup>. The colonies grown on the plates were purified by successive streaking on nutrient agar plates. Isolated bacterial strains were then subsequently stored at 4 °C in refrigerator for further study.

### Determination of optimum temperature and pH for growth

The physiological growth studies were carried out in the nutrient broth medium as described previously<sup>17</sup>. The temperature range for optimum growth was determined by incubating

the isolates from 25 to 60 °C. The effect of pH on growth of isolates was tested with the pH range from 4.0 to 10.0. All growth tests were done at 50 °C for 24 h and the growth was measured at OD<sub>600</sub> nm by using spectrophotometer (HALO DB-30 UV-Visible double beam, Dynamica, Singapore).

### Morphological studies of colony and cell

The appearance of colony such as shape, colour, opacity, margin and elevation was observed by magnifying lens (10X) after the growth of isolated strains on nutrient agar plates. The cell size was measured using ocular micrometer. Gram staining was performed using standard<sup>18</sup>. The various morphological features of bacterial strains were observed at 1000X using Axio Lab.A1 compound microscope (Carl Zeiss, Germany).

### Biochemical characterization

A series of biochemical tests were performed at 50 °C for 24 h at pH 7.0 using standard protocols<sup>19</sup>. Briefly, catalase activity was determined by adding a 3 % (w/v) H<sub>2</sub>O<sub>2</sub> solution on colonies of isolates. Oxidase test was done by placing a loopful bacterial isolate on oxidase disc (Himedia, India) and the change in color was observed. Indole test was performed in tryptone broth after adding 0.5 ml Kovac's reagent and waited for color change. Methyl Red (MR) and Voges-Proskauer (VP) tests were performed for acid production ability of strains. Utilization of citrate by isolates was observed on Simmons citrate agar (Himedia, India) medium slants. Urease enzyme production from urea was determined with suspension of bacterial strains inoculated in Christensen's urea broth (CUB). Reduction of nitrate was determined in nitrate broth medium. Fermentation of carbohydrates was performed using triple sugar iron agar medium.

### Screening of isolates for extracellular hydrolytic activities

#### Extracellular protease production

The qualitative screening of protease enzyme production by isolates was achieved using skim milk in nutrient agar medium with following components (g l<sup>-1</sup>): skim milk, 10; peptic digest of animal tissue, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5; and agar, 15 and pH adjusted to 7.0 before autoclaving. Isolates were streaked on medium plates. A zone of hydrolysis around the colonies was considered as an evidence of proteolytic activity<sup>20</sup>.

### **Extracellular amylase production**

The amylase production was determined by growing the isolates on soluble starch containing nutrient agar medium with the following constituents (g l<sup>-1</sup>): soluble starch, 10; peptic digest of animal tissue, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5; and agar, 15 (pH adjusted to 7.0 before autoclaving). Isolates were streaked in straight lines on medium plates. After incubation, individual plates were flooded with Gram's iodine solution (3% (w/v), KI and 0.3% (w/v) iodine). A clear zone around the growth indicated hydrolysis of starch<sup>21</sup>.

### **Extracellular lipase production**

Lipase activity of the isolates was detected by screening for turbid halo or zones of precipitation around colonies growing on nutrient agar plates containing 1% Tween-80<sup>22</sup>.

### **Extracellular asparaginase production**

The ability of isolates to produce asparaginase was determined using nutrient agar medium with L-asparagine. The composition of the test medium was as follows (g l<sup>-1</sup>): L-asparagine, 20; glucose, 2; potassium chloride, 0.5; magnesium sulfate heptahydrate, 0.5; monopotassium phosphate, 1.52; agar, 20 and phenol red, 0.09 and pH 7.0. Clear pink zone around the colonies after the incubation of petri plates indicated the asparaginase production<sup>23</sup>.

### **Extracellular inulinase production**

The production of inulinase enzyme by isolates was screened using inulin agar medium plates containing following constituents (g l<sup>-1</sup>): ammonium sulfate, 0.5; magnesium sulfate heptahydrate, 0.2; inulin, 2; monopotassium phosphate, 3; agar, 20 and pH 7.0. After incubation, plates were flooded with Lugol's iodine solution for 3 to 5 minutes and washed with distilled water. The colorless zone around the colonies indicated inulinase enzyme production<sup>24</sup>.

### **Extracellular cellulase production**

Cellulolytic activity of isolates was detected on carboxymethylcellulose (CMC) agar plates. Isolates were streaked on CMC agar medium plates and incubated at 50 °C for 24 h to allow the secretion of cellulase. After the incubation, plates were flooded with congo red (1% w/v) for 15 min. Subsequently, congo red solution was discarded and 1M NaCl solution added on plates for 15 min. The hydrolysis zone

around the colonies indicated the cellulolytic activity<sup>6</sup>.

### **Genomic DNA extraction**

Genomic DNA of isolates was extracted using modified freeze/thaw method<sup>25</sup>. Briefly, bacterial culture was pelleted out at 9,000 g for 5 min and lysis solution (500 µl) added (mixed gently by inverting 3-6 times) and incubated at -20 °C for 15 minutes. Later, tubes were incubated at 70 °C for 15 min in water bath and this step was repeated. Cells were suspended in 600 µl of Chloroform and gently emulsified by inverting tubes for 3-5 times and centrifuged at 9,000 g for 3 min. Upper aqueous phase containing DNA was transferred to a new tube and equal amount of Propan-2-OL (iso-propyl alcohol) was added and mixed gently by inversion at room temperature and centrifuged at 9,000 g for 3 min. Supernatant was removed completely and DNA pellet was dissolved in 100 µl of 1.2 M NaCl solution. 300 µl of absolute cold ethanol added and the DNA was precipitated in 10 min at -20 °C. Later, the tubes were centrifuged at 9,000 g for 5 min. Ethanol was removed and pellet washed once with 70% cold ethanol and tubes were centrifuged at 9,000 g for 5 min. Lastly, the pellet was dissolved in 50 µl deionized water (pH 7.0) by gentle vortexing and kept at -20 °C for further use.

### **16S rRNA gene Sequencing and phylogenetic analysis**

The 16S rRNA gene of genomic DNA was amplified using PCR master mix (Genei™ Company) with the universal primers 27F and 1495R<sup>26</sup>. The PCR thermal cycling conditions were as follows: Hot start at 98 °C for 5 min, followed by 35 cycles denaturation at 95 °C for 30 s, primer annealing at 56 °C for 45 s, elongation at 72 °C for 1.45 min with final extension at 72 °C for 5 min. Amplified genomic DNA was purified with GeneJET PCR purification kit (Thermo Fisher) as per the manufacturer's protocol. Purified products were sequenced commercially (Eurofins Genomics India, Bangalore) to get partial sequences 16S rRNA gene.

The similarity search of 16S rRNA gene sequences of isolated strains was done through the Eztaxon database<sup>27</sup> and parallel search also performed using NCBI database using BLASTN ([www.ncbi.nlm.nih.gov/blastn](http://www.ncbi.nlm.nih.gov/blastn)) to retrieve closely related sequences for phylogenetic analysis.

Initially, a large group of sequences (>1100 bp) was selected and later a smaller subset was employed for analysis based on the relatedness of the sequences and excluding taxa of uncertain affiliation. The multiple sequences alignment was performed using programme MUSCLE<sup>28</sup> inbuilt in the phylogenetic analysis software MEGA 7<sup>29</sup>. The aligned sequences were further refined manually to obtain unambiguous data. Finally, the sequences

for the edges of the maximum likelihood tree. *Planococcus citreus* (X62172) was chosen as the outgroup.

#### Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank (NCBI database) under accession numbers MH560567-MH560574.

## RESULTS

### Physico-chemical properties of sand dunes samples of Thar Desert

In the present study, the physico-chemical properties of sand dune samples named Sp1S, Sp2S, Sp3S and Sp4S (June 2014), Sp2W, Sp3W and Sp4W (February 2015) from Thar Desert were determined, as shown in Table 1. The Thar Desert sand dunes in both seasons represent the variation in temperature between 55 to 25 °C. The sand samples showed properties from neutrophilic to slightly alkaliphilic (pH 7.6 to 8.4). The electrical conductivity (µs) and total dissolved solids (ppm) in the sand samples were found to be more in winter season in comparison to summer season (Table 1).

#### Colony and cell morphology

The present study revealed that all isolated strains (1S, 2AS, 2BS, 3S, 2AW, 3W and 4W) were motile, gram positive rods, spore forming and arranged in chain form which indicated that isolates belonged to *Bacillus* species. All isolated strains showed growth on nutrient agar plate within 24 h of incubation at 50 °C. Morphologically, all isolates showed some variation in shape, size, color, light transmission, margin, opacity, elevation of the colonies (Table 2 & 3). Isolates were found

**Table 1.** Physicochemical analysis of Sand Dune Samples of Thar Desert

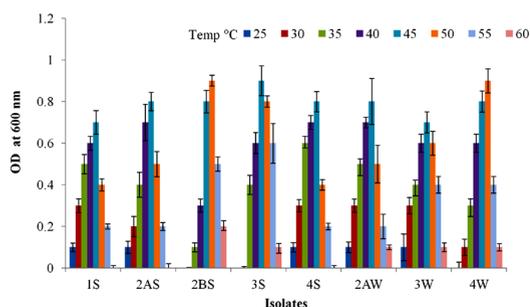
| Sample | pH  | Salinity (ppm) | Conductivity (µs) | TDS (ppm) |
|--------|-----|----------------|-------------------|-----------|
| Sp1S   | 7.6 | ND             | 0.05              | 0.02      |
| Sp2S   | 7.5 | ND             | 0.11              | 0.06      |
| Sp3S   | 7.7 | ND             | 0.05              | 0.02      |
| Sp4S   | 7.8 | ND             | 0.05              | 0.03      |
| Sp2W   | 8.2 | 40             | 0.07              | 50.5      |
| Sp3W   | 8.3 | 52.4           | 0.1               | 71.5      |
| Sp4W   | 8.4 | 77.8           | 0.155             | 110       |

ppm=part per million; µs=microsiemens; ND=not detected

of type strains and other bacterial strains mostly allied to sequences of the present study were employed for a synoptic view of the phylogenetic tree. The phylogenetic tree using 16S rRNA gene fragment of 1117 nucleotides was constructed by the maximum likelihood (ML) method using the program MEGA 7<sup>29</sup> applying a T92+G model of nucleotide substitution, which gave the best fit to this dataset according to the Bayesian Information Criterion (BIC). Bootstrapping of 1000 replications was calculated to find out confidence values

**Table 2.** Morphological features of isolates

| Isolate name | Shape             | Colour        | Opacity | Elevation | Margin   | Light transmission |
|--------------|-------------------|---------------|---------|-----------|----------|--------------------|
| 1S           | Irregular         | Creamy- white | Rough   | Raised    | Undulate | Opaque             |
| 2AS          | Circular- large   | Creamy-white  | Rough   | Flat      | Entire   | Opaque             |
| 2BS          | Circular-small    | Creamy-white  | Smooth  | Raised    | Entire   | Opaque             |
| 3S           | Irregular         | White         | Rough   | Raised    | Undulate | Opaque             |
| 4S           | Circular-moderate | Creamy-white  | Smooth  | Raised    | Entire   | Opaque             |
| 2AW          | Irregular         | White         | Rough   | Flat      | Curled   | Translucent        |
| 3W           | Circular-large    | Creamy-white  | Smooth  | Raised    | Entire   | Translucent        |
| 4W           | Circular-moderate | White         | Smooth  | Raised    | Entire   | Translucent        |



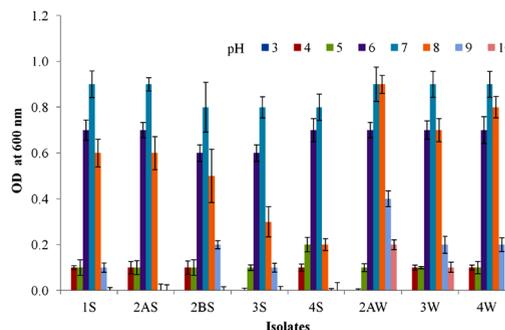
**Fig. 2.** Growth profile of isolates at various temperature range 25-60 (°C) for 24 h.

basically in two different shapes; circular and irregular. The colonies of isolates were white and creamy- white in color. The cell size varied in the range of width 0.1-0.5  $\mu\text{m}$  and length 0.3-1.6  $\mu\text{m}$ . Colonies of strains 1S, 2AS, 2BS, 3S and 4S were observed to be opaque and strains 2AW, 3W and 4W formed translucent colonies on nutrient agar plates.

#### Physiological and biochemical characterization

Physiological parameters such as temperature and pH were optimized for the growth of all investigated strains (Fig. 2 & 3). The optimum temperature range for growth of all the strains was determined by incubating at 25 to 60 °C for 24 h. It was noted that strains 1S, 2AS, 4S and 3W were able to grow between 25-55 °C with optimum temperature of 45 °C. Strains 2BS, 3S, 2AW and 4W were able to grow between 30-60 °C with optimum temperature of 50, 45, 45 and 50 °C respectively. The optimum pH range for growth of all strains was observed 7.0-8.0. All strains showed maximum growth at slightly alkaline condition.

The physiological and biochemical characteristics of strains observed by classical methods (Table 3) showed similarity with phylogenetically related strains. Strain 1S and 4S exhibited similar characteristics to phylogenetically close *Bacillus subtilis* subsp. *inaquosorum* as it was gram positive, rod shaped catalase positive, nitrate reduction positive, citrate positive, motile and optimum growth 45 °C. The strain 2BS showed alike characteristics to *Aeribacillus pallidus* as it was small rod shaped, gram positive, motile, catalase positive, oxidase negative, unable to degrade starch and casein and optimum growth at 50 °C. Likewise, the strain 2AS was phylogenetically related to *Bacillus subtilis* hswx88



**Fig. 3.** Growth profile of isolates at pH range 3-10 for 24 h in nutrient broth medium.

which showed features such as gram positive, motile, indole negative, nitrate reduction positive, starch hydrolysis positive, urease production negative. The strains 3S and 4W showed similar characteristics as *Geobacillus thermodenitrificans* subsp. *calidus*, as it was gram positive, rod shaped, motile, catalase positive, oxidase positive, nitrate reduction positive, amylase production positive and optimum growth was observed at 45 °C and 50 °C respectively. The 2AW and 3W strains exhibited similarity with *Bacillus paralicheniformis*, as they were gram positive, rod shaped, motile, catalase positive, nitrate reduction positive, able to hydrolysis of starch, casein and optimum growth at 45 °C.

#### Production of extracellular hydrolytic enzymes

The isolated strains were tested for their ability to produce six industrially important hydrolytic enzymes (Table 4). Interestingly, it was noted that except 2BS and 3S all other strains showed multi hydrolytic enzyme production ability. The strains 1S, 2AS, 2BS, 3S, 4S, 2AW, 3W and 4W were able to produce amylase enzyme. The enzymes such as protease, lipase and cellulase were produced by 1S, 2AS, 4S, 2AW, 3W and 4W. Nevertheless, inulinase enzyme production was only shown by strains 2AS, 2AW and 3W. It is worth to mention that strain 2AW and 3W were able to produce asparaginase enzyme along with other enzymes.

#### Phylogenetic analysis

16S rRNA gene nucleotide sequencing data of representative strains were analyzed for similarity with BLAST search (<http://www.ncbi.nlm.nih.gov>). The sequencing results revealed that the genus *Bacillus* was predominant rather than *Geobacillus* and *Aeribacillus* in sand dunes

**Table 3.** Biochemical characterization and phylogenetic relatedness of strains isolated from Thar Desert

| Characteristics                          | 1S   | 2AS   | 2BS                          | 3S  | 4S   | 2AW                                       | 3W                                 | 4W  |
|--|--|---|------------------------------|---|--|---|------------------------------------|---|
| Gram staining                            | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| Cell size (width, length $\mu\text{m}$ ) | 0.1-0.3x<br>1-2                                    | 0.2-0.3x<br>1-3                                 | 0.5-0.8x<br>1-4              | 0.5-0.8x<br>1-8   | 0.1-0.4x<br>1-3                                  | 0.2-0.3x<br>1-5                           | 0.3-0.6x<br>1-3                    | 0.4-0.8x<br>1-6   |
| Motility                                 | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| Endospore                                | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| Temperature range (°C)                   | 25-55  | 30-55   | 30-60                        | 30-60   | 25-55  | 25-60                                     | 30-60                              | 30-60   |
| pH range                                 | 6-8  | 6-8   | 6-8                          | 6-8   | 6-8  | 6-11                                      | 6-10                               | 6-8   |
| Indole                                   | -  | -   | -                            | -   | -  | -   | -                                  | -   |
| Triple sugar Iron                        | +  | +   | -                            | -   | +  | +w  | +                                  | +   |
| MR                                       | -  | -   | -                            | -   | +  | -   | +                                  | -   |
| VP                                       | +  | +   | -                            | -   | +  | +   | +                                  | +   |
| Nitrate                                  | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| H <sub>2</sub> S                         | -  | -   | -                            | -   | -  | -   | -                                  | -   |
| Citrate                                  | +  | +   | -                            | -   | +  | +   | +                                  | +   |
| Catalase                                 | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| Oxidase                                  | +  | +   | +                            | +   | +  | +   | +                                  | +   |
| <b>Phylogenetic similarity</b>           | <i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> | <i>Bacillus subtilis</i> subsp. <i>subtilis</i> | <i>Aeribacillus pallidus</i> | <i>Geobacillus thermode-nitrificans</i> subsp. <i>calidus</i> | <i>Bacillus subtilis</i> subsp. <i>inaquorum</i> | <i>Bacillus parali</i> <i>cheniformis</i> | <i>Bacillus paraliche-niformis</i> | <i>Geobacillus thermode-nitrificans</i> subsp. <i>calidus</i> |

+ indicates positive; - indicate negative; +w indicates weakly positive

**Table 4.** Screening of industrially putative enzymes from isolated strains.

| Strain no. | Amylase | Protease | Cellulase | Asparaginase | Inulinase | Lipase |
|------------|---------|----------|-----------|--------------|-----------|--------|
| 1S         | +       | +        | +         | -            | -         | +      |
| 2AS        | +       | +        | +         | -            | +         | +      |
| 2BS        | +w      | -        | -         | -            | -         | -      |
| 3S         | +       | -        | -         | -            | -         | -      |
| 4S         | +       | +        | +         | -            | -         | +      |
| 2AW        | +       | +        | +         | +            | +         | +      |
| 3W         | +       | +        | +         | +            | +         | +      |
| 4W         | +       | +        | +         | -            | -         | +      |

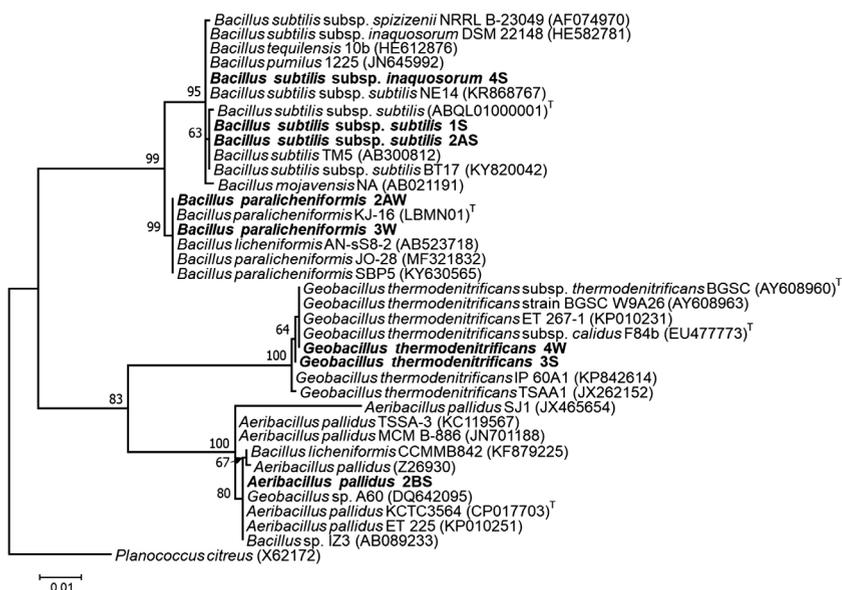
+ indicates positive; - indicates negative; +w indicates weakly positive

of Thar Desert. The five strains (1S, 2AS, 4S, 3W and 2AW) were found to belong to genus *Bacillus*. Other two strains (3S, 3W and 4W) were related to genus *Geobacillus* and one of strains (2BS) was affiliated to genus *Aeribacillus* (Fig. 4).

## DISCUSSION

The extreme environments which exist on Earth are the rich source of variety of microorganisms with special characteristics. Low water availability and high temperature during

summer and diurnal fluctuations make the Thar Desert an extreme environment for microbial life<sup>11</sup>. In the present study, total eight moderately thermophilic bacterial strains were screened out from different sand dune samples of Thar Desert, Bikaner, Rajasthan using conventional studies such as morphological, physiological, biochemical characterization and molecular identification by 16S rRNA gene sequencing. The results obtained from effect of different temperature range (25-60 °C) revealed that all studied strains were belonged



**Fig. 4.** Maximum likelihood phylogenetic tree of partial sequences of 16S rRNA gene. The strains isolated in the present study are indicated as bold. T denotes “type species”. Bootstrap values >50 are only mentioned on the branch of the tree.

to moderately thermophilic. The 16S rRNA gene sequence similarity (1419 bp) exhibited that isolated strains were related to different species of bacilli such as 1S and 4S to *Bacillus subtilis* subsp. *inaquosorum*<sup>30</sup>; 3S and 4W to *Geobacillus thermodenitrificans* subsp. *calidus*<sup>31</sup>; 2BS to *Aeribacillus pallidus*<sup>32</sup>; 3W and 2AW to *Bacillus paralicheniformis*<sup>33</sup> and 2AS to *Bacillus subtilis* hswx88<sup>34</sup>.

The results obtained in the present study for biochemical characterization such as oxidase, catalase, MR-VP, citrate utilization, indole production, triple sugar iron utilization, nitrate reduction were consistent with earlier thermophilic bacteria isolated from various geothermal areas. Moreover, biochemical characters of moderately thermophilic bacteria vary from strain to strain even if they are isolated from same ecological niche as revealed from our study and previous findings<sup>13,17,35,36</sup>.

The hydrolytic properties of thermostable enzymes have long driven in many industries such as food, cosmetic, agriculture, pharmaceutical, leather because they increase the rate of reaction, reduces the risk of contamination, increases reactant solubility, increases the stability of enzyme<sup>37</sup>. Among of thermophilic bacteria of genus *Bacillus* and *Geobacillus* are dominant

for the production potential thermostable enzymes<sup>15,30,38</sup>. Nevertheless, many thermophilic bacteria are still unexplored which possess ability to produce variants of thermostable enzymes. It is obvious from the findings of the present study that strains isolated from sand dunes of Thar Desert are able to produce at least one and more than two hydrolytic enzymes. Worldwide, thermophilic bacteria having hydrolytic activities were mostly found in hot spring<sup>17,35</sup>. Moreover, greater hydrolytic activity was observed in gram positive thermophilic bacteria rather than gram negative from different hot springs<sup>6,35,36</sup>. The extracellular thermostable enzymes of thermophilic bacteria have great interest for both fundamental research and industrial application<sup>39</sup>. The number of thermophilic bacteria affiliated to genus *Bacillus* sp. and *Geobacillus* sp. like *Bacillus licheniformis*, *Anoxybacillus* sp. NBY46, *Bacillus mycoides* NBM19, *Bacillus pumilus* NBM31, *Bacillus* sp. NBM43, *Bacillus subtilis* NBM48, *Geobacillus* sp. TP-2, *Aeribacillus* sp. O3, *Bacillus* sp. O2, O7, O8, O14, O17 and O19 have been isolated from different geothermal springs and reported as producer of various thermostable hydrolytic extracellular enzymes such as amylase, protease, cellulase, gelatinase, xylanase, lecithinase and lipase<sup>6,17,40</sup>.

The enzyme asparaginase has been reported as a source of high therapeutic agent for acute lymphocytic leukemia and Hodgkin's lymphomas and also able to reduce acrylamide level commercial fried foods<sup>22,41</sup>. Recently, the asparaginase enzyme of *Bacillus licheniformis* was used to treat acute lymphoblastic leukemia (ALL) and *in vitro* evaluation of anti-cancerous properties<sup>42,43</sup>. In the previous studies, asparaginase production has been reported from thermophilic bacteria belonging to *Bacillus* sp. HB8<sup>44</sup>, *Bacillus subtilis* hswx88<sup>33</sup>, *Geobacillus thermoleovorans* N7 originated mostly from hot springs. In our investigations, only two strains (2AW and 3W) belonging to *Bacillus paralicheniformis* showed ability to produce asparaginase. To the best of our knowledge, this is the first report to show asparaginase production by *Bacillus* strains originated from sand dunes of Thar Desert.

Likewise, inulinase enzyme producing thermophilic bacteria have great industrial interest because this enzyme is used to hydrolyse inulin for production of fuel ethanol<sup>45</sup>. The strains LT-2, LT-4, LT-5, LT-6, LT-7, LT-8 (*Bacillus licheniformis*) and LMa-9 (*Aneuribacillus migulanus*) isolated from sediment and water samples of geothermal areas in Patagonia, Argentina<sup>46</sup> and the strain *Bacillus* sp. SG7 isolated from thermal water samples from the region of Velingrad, Bulgaria was able to produce extracellular inulinase<sup>47</sup>. Interestingly, the strains (2AW and 3W) those were able to produce asparaginase also showed potential to produce extracellular inulinase. Besides these strains, 2AS also exhibited positive test for this enzyme. So far, none of study described the inulinase producing bacteria isolated from Thar Desert, India. The ability of production of amylase, protease, lipase, asparaginase, inulinase and cellulase enzymes by strains isolated from Thar Desert indicates their potential use in different industries like textile, food, pharmaceutical, detergent, leather, agrochemicals, etc.

On the basis of phylogenetic analysis, the strains 1S and 4S were closely related to type strain *B. subtilis* subsp. *inaquosorum* (CP013984) with 99.92 and 100% respectively<sup>29,46</sup>. The sequencing results revealed that the genus *Bacillus* was predominant rather than *Geobacillus* and *Aeribacillus* in sand dunes of Thar Desert. The four strains (1S, 2AS, 4S and 2AW) were found to

be belonged to genus *Bacillus*. The strains 1S and 4S were closely related to type strain *B. subtilis* subsp. *inaquosorum* (CP013984) with 99.92 and 100% respectively. The phylogenetic tree consists of two main clades and each clade is divided into two sub-clades. Two distinct sub-clades of *Bacillus* genus were observed in the phylogenetic tree (Fig. 4). One sub-clade virtually intricate group of closely related species including *Bacillus subtilis* subsp. *subtilis*. Most of the sequence from isolates originated from India are yet unpublished and needs further validation (data not shown). The present study described the morphological, biochemical and production of hydrolytic enzymes of strain related to *Bacillus subtilis* subsp. *subtilis*. Another sub-clade consisted sequences related to *Bacillus licheniformis* with the type strain of *B. paralicheniformis*<sup>32</sup>. It is clearly indicated from our results that earlier reported species belong to *paralicheniformis* instead of *licheniformis*.

The Thar Desert soil is an evidence of ecological niches of *Aeribacillus pallidus* as the strain 2BS showed the closeness to type strain KCTC3564 of *Aeribacillus pallidus*. Most of the strains of genus *Aeribacillus* isolated from hot springs, geothermal oil field and desert soils<sup>48,49</sup>. The strain *Aeribacillus pallidus* SAT4 was isolated from Thar Desert, Pakistan used to produce antibacterial peptide<sup>50</sup>. The sequence of 16S rRNA gene was not included in tree because of a short sequence (432 bp) available in GenBank. The alkalistable carbonic anhydrase producing *Aeribacillus pallidus* TSHB1 has been isolated from Choti Anhoni hot spring of Pipariya, Madhya Pradesh, India<sup>51</sup>. These findings reveal that *Aeribacillus* is widely distributed in different habitat.

The strains 3S and 4W were closely related to type strain F84B of *Geobacillus thermodenitrificans* subsp. *calidus* isolated from well-pipeline sediment sample in Kizilcahamam, Turkey<sup>30</sup>. Recently, most of the strains of this genus were isolated from compost, deep surface oil reservoirs, hot springs and soils<sup>36,52,53</sup>. *Geobacillus thermodenitrificans* strains have also been isolated from Tattapani hot spring sediment, North West Himalayas and from Damodar river, India<sup>54</sup>. In the earlier studies, a few of other strains were reported from intestine of a mackerel (fish) and rhizosphere of plant<sup>55,56</sup>. The present study exhibited the

occurrence of *Geobacillus thermodenitrificans* in sandy soil of Thar Desert of India.

The present study revealed that Thar Desert is a treasure for moderately thermophilic bacterial diversity. The presence of strains related to species such as *Geobacillus thermodenitrificans*, *Bacillus paralicheniformis*, *Bacillus subtilis* and *Aeribacillus pallidus* in Thar Desert of India can tolerate two extreme conditions (high temperature and aridity). The investigated strains were able to produce thermostable amylase, cellulase, protease, inulinase, asparaginase and lipase enzymes. Thus, microorganisms thriving in such harsh environments should be explored extensively for phylogenetic studies and also for their biotechnological applications.

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#### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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