Isolation, Identification and Characterization of a Thermo-halotolerant

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Aeribacillus Pallidus SHJP4, from the Arid Region of Rajasthan

Jagdish Parihar1 and Ashima Bagaria1*

1Department of Physics, Manipal University Jaipur, Jaipur-303007, Rajasthan, India

*Correspondence: ashima.bagaria@jaipur.manipal.edu

Abstract

The bacterial strain SHJP4 was isolated from the soil samples of arid and semi-arid zones of Rajasthan. SHJP4 experimentally confirmed to be a gram positive, rod shaped, aerobic, motile and endospore forming. The optimum growth was seen at 55°C and at alkaline pH. The strain was able to adapt itself in the medium containing 5% NaCl. The DNA was isolated from the culture and checked on 1.2% agarose gel. After amplification and sequencing by 16S rRNA the strain was identified as *Aeribacillus pallidus* based on nucleotide homology and Phylogenetic analysis and Bayesian. The strain SHJP4 is deposited to NCBI with accession number - MK296526.

Key words: Polyextremophiles, Extremozymes, Thermophile, Halotolerant, Aeribacillus pallidus.

1. Introduction

The microbial population found on earth is mainly mesophilic. Interestingly, some microbes grow at extreme conditions of temperature, salt concentration, pH, pressure, water and nutrient availability etc. which are classified as thermophiles, halophiles, barophiles and xerophiles respectively [1]. Thermophiles are the most popular category of extremophiles, where the members can withstand high temperatures without affecting their activity [2] [3]. Due to high stability at extreme temperatures these microbes are in high demand for various industrial purposes [4] [5] [6]. Most of these thermophiles are found in hot water springs, deserts, volcano pits and deep ocean floors. The microbes that belong to the other category of halophiles, grow in saline or hyper saline environment. They have been explored extensively for their salt tolerant genes and carotenoid production that have potent applications in industries like cosmetic, optoelectronic devices, treatment of saline and hypersaline wastewaters, bioplastics and biofuel etc. [7] [8].

The arid regions are dry and have low water and nutrients content. The alkalinity and high...
salt concentration of the soil provides ideal conditions for thermophiles, alkaliophiles and halophiles to flourish. [9] [10] [11] [12] [13] [14, 15]. The adaptability and stability of the extremophilic bacteria (a thermophile, in the current study) is attributed to the thermophilic proteins with stable structure, complicated membrane lipids, and altered metal ions that increase the stability of the proteins [16] [17]. Halophiles, the other class of extremophilic bacteria, can survive under extreme salt concentrations. They are found to accumulate K+ and Cl− in their cells to preserve the osmotic stability achieved by the bacteriorhodopsin membrane bound proton-pump action. The elevated water potential within the cell protects the cellular proteins from denaturation in hypersaline conditions subsequently stabilizing the cellular architecture. The protein formed by halophiles is adjusted to a strong salt environment that encourages the folding of the riparophilic peptide chain and constitutes a functional protein that regulates the enzymatic activity. [18] [19]. The enzymes extracted from these extremophiles such as lipase, xylanase, pectinase, protease, amylase, catalase are high in demand for various industrial and biotechnological purposes [20-27]. The current study focuses on the identification and characterization of thermophilic-halotolerant bacteria from soil samples of arid zones of Rajasthan.

2. Materials and Methods

2.1 Sample Collection

The soil samples were collected from 11 predefined spots in aspect of high soil temperature that is 55°C of Sidhar village, near Desert national Park, Jaisalmer (Latitude: 26° 13’ 40.8” N; Longitude: 70° 38’ 24.6” E), Rajasthan, India, in June 2017 (Figure 1). The samples were stored in sterile zip lock bags and transferred to laboratory for further processing.

![Figure 1: This map shows a) arid and semi-arid regions of Rajasthan. b) Sampling site.](image-url)
2.2. Physicochemical Profiling of Soil Samples

The collected soil samples were pooled and filtered through 2mm mesh and studied for physical and chemical properties of soil like pH, TDS, temperature, macronutrients (carbon, phosphorous and potassium) and micronutrients (iron, copper, zinc and manganese) with standard methods [28]. The chemical characteristics like total organic carbon (TOC) was determined by rapid titration method. The phosphorus, potassium and micronutrients content (iron, copper, zinc and manganese) were measured by spectrophotometer, flame photometer and atomic absorption spectroscopy (Agilent Technologies, 200 Series AA), respectively. The physical characteristics of the samples such as pH and TDS were evaluated by multi parameter tester [29] [30] [31]. For all components analysed in this research, analytical uncertainties are < 5%.

2.3. Isolation of Extremophilic Bacteria

The Nutrient agar and broth medium (Himedia) was used to isolate the bacteria. The nutrient agar media was prepared of pH 7.00 then autoclaved. Phosphate buffer saline (PBS) pH 7.00 was used as dilution media. The soil samples were pooled and serially diluted up to 10^-8 in dilution medium in three replicates. Inoculum (0.1mL) from each 10^-8 diluted replicate tube was spread on nutrient agar plate and incubated at 37°C for 24 h. After growth observation, the single colony was picked up on the basis of colony morphology and inoculated in nutrient broth medium and incubated at 37°C for 24 h. [32]

2.4. Growth on Different Temperature

To check the sustainability of pure isolates on higher temperature bacteria are incubated at different temperatures such as 37°C, 45°C, 50°C and 60°C respectively. After 24 h the growth observed, and the optical density (OD) was measured at 600nm using spectrophotometer (Shimadzu). [33]

2.5. Morphological and Biochemical Characterization

The isolated pure cultures were screened for various biochemical tests like Gram’s staining, Endospore staining [34], Indole test, Citrate test, Starch hydrolysis, Deaminase, Motility and Catalase. [35]

2.6. Salt Tolerance Test
The pure isolate was tested for tolerance against different concentration of NaCl salt ranges from 1-8% (w/v). The pure isolate inoculated in nutrient broth media containing NaCl salt with concentration. The growth was observed by taking the optical density (OD) at 600nm.

2.7. pH Sensitivity Test
For pH sensitivity test, the media was prepared with different pH ranges from pH 4-9 and the pure isolate inoculated in media with different pH. The ability of bacteria to tolerate different range of pH was observed after 24 hours of incubation on 60°C by measuring the growth or turbidity shown in culture tubes at 600nm.

2.8. Molecular identification
For molecular identification, the genomic DNA was extracted from isolated pure culture SHJP4 using QIA amp DNA Mini Kit (Qiagen). The quality and quantity of DNA was evaluated on 0.8% agarose gel and nanodrop (Thermo Scientific, Germany). The extracted bacterial DNA was stored at -80°C until used.

2.9. 16S rDNA Gene Amplification
Isolated DNA from pure culture SHJP4, was amplified with 16S rRNA universal primer (8Fand 1492R) using Veriti® 96 well Thermal Cycler (Model No. 9902). The PCR reaction components PCR buffer (10X), forward and reverse primer (10mM), dNTP mix(10mM), Dream Taq DNA polymerase (1.5U) and up to 100ng DNA template were uniformly mixed and reaction carried out for 35 cycles under normal PCR cycling conditions. The amplified PCR products were gel purified with GeneJET Gel extraction kit (Cat#K0691) as per the manufacturer's instructions (Thermo Scientific, Germany) and subjected for sequencing of 16S rDNA region (Eurofins Genomics, India, Pvt. Ltd.). The received sequences were verified in Editseq software and both strands were aligned. The aligned sequences were then analysed in BLAST tool for the sequence similarities with other sequences and were submitted to NCBI GenBank using BankIt submission tool.

2.10. Phylogenetic Analysis
The evolutionary history was inferred using the Neighbor-Joining method [36]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed [37]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the
Maximum Composite Likelihood method [38] and were in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 1410 positions in the final dataset [39]. Evolutionary analyses were conducted in MEGA 10.

3 Results and Discussions

3.1. Physicochemical Profiling of Soil Samples

The soil samples were collected from Sihdar village near Desert National Park, Jaisalmer and the identification of their physical properties like TDS, pH and temperature and chemical properties like micro and macronutrient contents were determined. The results revealed the concentration of various nutrients like carbon, phosphorus, potassium, zinc, copper, iron and manganese present in the soil which is shown in Table 1. The accuracy of the results was checked by performing the experiments in triplicates.

Table 1. Soil profiling

| S. No. | Properties          | Soil Parameters | Observations |
|--------|---------------------|-----------------|--------------|
| 1.     | Physical Properties | a) pH           | 8.25         |
|        |                     | b) TDS          | 0.08 ppm     |
| 2.     | Chemical properties |                 |              |
| A.     | Macronutrients      | a) C            | 0.21 ppm     |
|        |                     | b) P            | 40.0 ppm     |
|        |                     | c) K            | 299 ppm      |
| B.     | Micronutrients      | a) Zi           | 0.54 ppm     |
|        |                     | b) Cu           | 4.28 ppm     |
|        |                     | c) Fe           | 0.24 ppm     |
|        |                     | d) Mn           | 2.74 ppm     |

3.2. Isolation of Extremophilic Bacteria
Thermophilic microorganisms have the versatility to get by in extreme conditions. Several researchers had validate that such ability might be because of their changes at cell and subcellular level. In our current study soil samples collected from arid regions of Rajasthan, India was explored for thermophilic microorganisms. In the present study, we isolated a total of 25 bacterial isolates on 37°C named as SHJP1 to SHJP25.

### 3.3. Growth on Different Temperature

All the 25 bacterial isolates were subjected to grown on different temperature, but all bacteria were failed to grow on above 37°C except isolate SHJP4. Only one isolate SHJP4 were able to grow on temperature 37°C, 45°C, 55°C and 60°C. The bacterial isolates inoculated and incubated overnight for different temperature and growth was observed. The optical density (OD) was measured at 600nm with spectrophotometer. The growth curve of bacterial isolates at different temperatures are shown in Figure 2. It shows that the optimum temperature for growth is 55°C. The capacity of the bacteria to sustain at 55°C temperature showed to be moderate thermophile.

![Figure 2.](image-url)  
**Figure 2.** Effect of temperature on growth.

### 3.4. Morphological and Biochemical Characterization

The morphology of the isolated bacterial strain confirms that SHJP4 is gram positive, motile and endospore forming. The strain formed opaque and circular colonies having regular margins, on nutrient agar plates and was tested positive for the enzymes like deaminase, catalase, tryptophanase and negative for caseinase and amylase.

**Table 2. Morphological and Biochemical Characterization**
3.5. Salt Tolerance Test

The salt tolerance of bacteria isolates was checked on nutrient broth medium with 0–8\% (w/v) NaCl at 55 °C. The observation shows maximum growth at 5% NaCl and beyond this value growth decreased. The optimum salt concentration is 4% NaCl (Table 3).

Table 3. Salt Tolerance Test

| S.No | Characteristics | SHJP4 |
|------|-----------------|-------|
| 1    | Shape           | Rod   |
| 2    | Colour          | Cream |
| 3    | Opacity         | +     |
| 4    | Gram stain      | +     |
| 5    | Endospore stain | +     |
| 6    | O₂              | Aerobe|
| 7    | Motility        | +     |
| 8    | DNA G+C content (mol %) | 56 |

3.6. pH Sensitivity Test

The effect of pH on the growth of bacterial isolates were observed. The results showed that maximum growth was achieved at pH 8.25 (P ≤ 0.05) while optimum pH was obtained at pH 8 beyond optimum pH values decreased. Bacterial growth declined at acidic pH significantly. This suggests that these bacterial isolates are more active in alkaline pH (Table 4).
### Table 4. pH Sensitivity Test

| S.No | pH | SHJP4 |
|------|----|-------|
| 1    | 4  | -     |
| 2    | 5  | -     |
| 3    | 6  | +     |
| 4    | 7  | ++    |
| 5    | 8  | ++++  |
| 6    | 9  | -     |
| 7    | 10 | -     |

#### 3.7. Molecular Identification

The molecular characterization of the bacterial isolate SHJP4 was carried out using 16S rDNA molecular technique. The amplification of the isolated DNA with 16S rDNA Specific Primer, (8F and 1492R) exhibited a single discrete PCR amplicon band of around 1500 bp on 1.2% agarose gel (Figure 3).

![1.2% agarose gel showing single 1500 bp of 16S rDNA amplicon. Lane 1: 1Kb DNA ladder; Lane 2: 16S rDNA amplicon of SHJP4](image)

#### 3.8. Phylogenetic Analysis

Phylogenetic analysis was carried out using neighbour joining method and SHJP4 showed 99% similarity with *Aeribacillus pallidus*, strain: UICC B-80 based on nucleotide homology (Figure 4). The 16S rDNA sequence isolated from SHJP4 was submitted to NCBI (accession number MK296526). On the basis of morphological and molecular characterization the bacterial isolate identified as *Aeribacillus pallidus*. 
Conclusion

The current study reveals that the soil samples from arid regions of Rajasthan are a rich source of thermo-halotolerant microbes and to best of our knowledge this is the first of its kind reported from this region. The strain SHJP4 possesses certain enzymatic activities namely, deaminase, tryptophanase catalase. This has been observed in the biochemical analysis reported in the paper. Deaminase are the enzymes that are involved in deamination process, where an amine group is removed via hydrolysis. Deamination can be deleterious if it causes any change in protein formation. Various structural studies have been carried out on deaminase [40]. This opens an area of exploration of the 3-D structural features of the strain under study, which would further add to the mechanism of adaptation to extreme biomes. In 2011, *Aeribacillus pallidus* had been characterized as thermophiles from Tao Dam hot spring, Thailand. Similar study had described various opportunities for the synthesis of non-aqueous peptides [41]. Contributing towards the research on *Aeribacillus pallidus*, which is isolated mainly from hot water springs, deserts, marsh lands and lands contaminated from industrial waste water [42] [43] [41, 44]. In other study showed the lipase catalytic activity used for formulations of detergent and to treat oily wastewater [23] [24]. Furthermore, the thermostability of the enzymes secreted by these isolated bacteria suggests their biotechnological potential are studied extensively by many researchers [20, 22, 25-27, 35]. The promising results obtained can be exploited to find the optimal conditions for large scale production and characterization of the thermostable enzymes secreted by SHJP4.

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Conflict of Interest
The authors declare no conflict of interest.

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