

## Evaluation of the enzymatic profile of microbial isolates from Lonar Soda Lake

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Alkaliphilic Gram positive bacteria were isolated and characterized from water and soil samples from alkaline Lonar lake, India. Total viable count of composite water and soil sample was found to be  $1.4 \times 10^5$  cfu/mL and  $1.7 \times 10^7$  cfu/gm, respectively. Thirty strains were isolated using different enrichment and isolation media. Alkaline protease assay was carried out to determine specific enzyme activity of partially purified alkaline protease. Ten alkaline protease producing isolates were identified as *Bacillus subtilis* LS-1, *Bacillus clausii* LS-2, *Bacillus megaterium* LS-3, *Bacillus licheniformis* LS-4, *Bacillus thuringiensis* LS-6, *Bacillus alcalophilus* LW9, *Alkalibacillus* sp. MGR8, *Bacillus horikoshii* MGR25, *Micrococcus* sp. MGR30 and *Alkalibacterium* sp. MGR11 on the basis of morphological, biochemical, physiological characterization, antibiotic sensitivity, enzyme profile and 16s rRNA sequencing. Out of these ten alkaline protease producers, nine isolates were belonged to phylum firmicute and one isolate belonged to phylum actinobacteria. Out of ten selected isolates, seven isolates were alkaline amylase producers. Two isolates were alkaline lipase and cellulase producers.

[**Keywords:** Alkaline protease, *Bacillus*, *Alkaliphiles*, Lonar crater, MEGA 6.06]

### Introduction

Proteases that have pH optima in the range of 8.0-11.0 are grouped under the category of alkaline proteases<sup>1</sup>. Among filamentous fungi, some *Trichoderma* sp. and molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are alkaline protease producers<sup>2,3</sup>. Alkaline protease from bacterial source like *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus novalis*, *Bacillus vireti*, *Bacillus soli*, *Bacillus bataveinsis*, *Bacillus drentensis*, *Bacillus clausii*, *Alcaligenes faecalis*, *Pseudomonas fluorescens* and *Aeromonas hydrophilia* are extensively exploited in different industries for various applications<sup>4,5,6</sup>.

The primary sources for occurrence of alkaliphiles are alkaline habitats like soil and water of alkaline lake ecosystems<sup>7</sup>. Lonar Crater Lake (Latitude 19°58', Longitude 76°31')<sup>8</sup> is popularly known for its hyper alkaline water and soil characteristics and thus being an original habitat for alkaliphiles<sup>9,10,11</sup>. Bioprospecting Lonar Lake, for isolation and identification of alkaline protease producers was attempted in our work.

### Materials and Methods

Five different soil samples and water samples were collected from Lonar Lake. In situ temperature of water sample was recorded. pH of water and soil samples were determined using a digital pH meter. Total viable count of composite water and soil sample was determined<sup>10</sup>. Four gram of composite air dried soil sample was added in a conical flask containing 20 mL of sterilized distilled water. The flask was heated in water bath for 20 min at 80 °C temperature with agitation<sup>12</sup>. Aliquots of diluted soil samples were prepared and spread on alkaline *Bacillus* agar plates<sup>13</sup>. These plates were incubated at 30 °C for 24 h. Composite water samples were inoculated in alkaline Furazolidone broth<sup>12</sup> (pH 10.0) and modified fermentation medium<sup>14</sup> (MF) (pH 10.0) and incubated at 30 °C temperature and 120 rpm agitation speed for 24 h in orbital shaking incubator. After incubation, 0.1 ml of diluted enriched samples from alkaline Furazolidone broth and MF medium was spread on alkaline Furazolidone agar<sup>12</sup> plates and Horikoshi-Akiba agar<sup>12</sup> plates respectively. These plates were

incubated at 30 °C temperature for 24 h of incubation period. Morphologically distinct colonies were isolated and cultivated on respective alkaline agar slants<sup>13,15</sup>.

Alkaline protease production was initially observed by inoculating selected isolates on alkaline casein agar plates (pH 10)<sup>4,16</sup>. After incubation of 24 h grown cultures were flooded with amido black solution and zone of clearance was observed<sup>17</sup>. Efficient alkaline protease producers were inoculated individually in Modified Fermentation Medium (MFM) comprising (g/L): casein 10.0, K<sub>2</sub>HPO<sub>4</sub> 0.5, KH<sub>2</sub>PO<sub>4</sub> 0.5 and Na<sub>2</sub>CO<sub>3</sub> 10.0 and incubated at 30 °C for 72 h at 100 rpm. Cultures were then centrifuged at 10,000 rpm for 10 min at 4 °C. The cell free supernatants were collected. Solid ammonium sulphate (70 %) was added in each supernatant. Enzyme precipitate were collected and dialyzed against 0.2 M glycine-NaOH buffer (pH 10.0). The dialyzed samples were used as partially purified enzyme. Quantitative alkaline protease activity assay by modified Anson's method<sup>18</sup>. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 µmole of tyrosine per minute, under assay conditions<sup>9,18</sup>. Total protein content of each dialyzed samples were determined using BSA as standard and specific enzyme activities were calculated<sup>19,20</sup>.

Colony characters, Grams nature, motility, spore formation, pigmentation, arrangement of flagella were observed by using standard methods<sup>21,22</sup>. Alkaline protease producers were inoculated in modified alkaline Schaeffer medium<sup>7</sup> individually and after incubation for 15 days cultures were used for spore staining<sup>12,23</sup>. Sugar utilization pattern was determined by inoculating pure cultures of selected isolates in basal medium containing adonitol, arabinose, cellobiose, dextrose, fructose, galactose, glycerol, inulin, lactose, maltose, mannitol, mannose, mellibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose individually<sup>12,23,24,25</sup>. Extracellular hydrolyzing enzyme profile was evaluated by inoculating isolates in medium containing substrate like starch, gelatin, cellulose, urea, tween-80 and pectin individually. Catalase, oxidase and IMViC tests were also carried out<sup>24,26</sup>. SIM agar was used to determine motility and H<sub>2</sub>S production<sup>12,19,24</sup>. Antibiotic sensitivity of isolates was determined using Amikacin (30 µg), Ampicillin (10 µg), Cefoperazone (75 µg), Cefoxitin (30 µg),

Ceftazidime (30 µg), Ceftriaxon (30 µg),

Chloramphenicol (25 and 30 µg), Ciprofloxacin (5 µg), Cotrimoxazole (30 µg), Gentamicin (10 µg), Netillin (30 µg), Ofloxacin (5 µg), Penicillin (10 U), Penicillin G (1 U), Piperacillin (100 µg), Streptomycin (10 µg), Sulphatriad (30 µg), Teicoplanin (30 µg), Tetracycline (25 and 30 µg) and Vancomycin (30 µg) antibiotic discs<sup>24</sup> (HiMedia, India). Effect of pH, temperature, incubation period and NaCl concentration on growth of alkaline protease producers was determined. Effect of pH on growth was determined using 0.2 M of phosphate buffer (pH 6.0–8.0), glycine-NaOH buffer (pH 9.0–10.0), Na<sub>2</sub>HPO<sub>4</sub>-NaOH buffer (pH 11) and KCl-NaOH buffer (pH 12 and 13). Effect of temperature on growth was determined by incubating culture at various temperatures ranging from 10 to 70 °C. Effect of NaCl on growth was determined at various NaCl concentrations ranging from 0 to 10 %. Effect of incubation period on growth was determined by incubating inoculated isolates for various incubation periods ranging from 24 to 120 h<sup>9,12,24,26, 27,28,29</sup>.

Selected alkaline protease producers were subjected for 16s rRNA molecular analysis. DNA was isolated from bacteria using cell lysis method and 16s rRNA gene was amplified by PCR using pair of primers (Forward (27f) AGAGTTTGATCMTGGCTCAG and Reverse (1492r) TACGGYTACCTTGTTACGACTT). The amplified 16s rDNA PCR product was gel-purified using QIAquick Gel Extraction kit. and sequenced using ABI377 automated sequencer (Applied Biosystems, USA). The deduced sequence was compared with GenBank data using the BlastN search (<http://www.ncbi.nlm.nih.gov/blast>)<sup>10,25,29,30</sup>. Efficient alkaline protease producer was deposited at MCC, NCCS in Pune.

The relationships among sequences and their hypothetical common ancestors were estimated by constructing a phylogenetic tree. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.06 software<sup>31</sup>. Deduced 16s rDNA sequences of alkaline protease producers were aligned with other previously deposited closely related sequences from GenBank using Muscle program. Evolutionary history was inferred using the Neighbor-Joining method<sup>32</sup>. The evolutionary distances were computed using the Maximum Composite Likelihood method<sup>33</sup>. Distances were in the units of the number of base substitutions per site. All

positions containing gaps and missing data were eliminated. Bootstrap method was used for test of

phylogeny<sup>34</sup>. Number of bootstrap replications applied was 1000. Transition and transversion substitutions were included. Uniform rates were set among site. Homogeneous pattern was applied among lineages<sup>31</sup>.

## Results and Discussion

Temperature of Lonar water sample was 28 °C and 10. Water and soil samples were found alkaline in pH having pH value 10.0. Total viable bacterial count of composite water and soil samples were  $1.4 \times 10^5$  cfu/mL and  $1.7 \times 10^7$  cfu/gm respectively. After 24 h of incubation period total 55, 35 and 42 colonies were appeared on alkaline *Bacillus* agar, alkaline Furazolidone agar and Horikoshi-Akiba agar plates respectively. Ten morphologically distinct colonies were selected from each medium. Of the total 30 selected isolates, 10 isolates showed zone of clearance on alkaline casein agar plates (Figure 1) and designated as LS-1, LS-2, LS-3, LS-4, LS-6, LW9, MGR8, MGR11, MGR25 and MGR30. Five isolates viz. LS-1, LS-2, LS-3, LS-4 and LS-6 were selected from alkaline *Bacillus* agar medium, four isolates viz. LW9, MGR8, MGR11 and MGR25 were selected from Horikoshi-Akiba agar medium and one isolate MGR30 was selected from alkaline Furazolidone agar medium. Specific activity of partially purified alkaline protease was recorded for all 10 isolates.



Fig. 1- Primary screening for alkaline protease (caseinase) production using amido black staining method

The highest specific activity (97.22 U/mg) was derived from isolate LS-1 followed by isolates LS-2, LS-3, LS-4, LS-6, LW9, MGR8, MGR11,

MGR25 and MGR30 (Table 4).

Colonies of isolate MGR30 were pigmented and observed orange colored (Table 1). Out of 10 alkaline protease producers, nine were Gram positive rods and one was Gram positive cocci. Out of 10 alkaline protease producers, 8 isolates were motile and spore former whereas 2 isolates were non motile and non spore former (Table 2). Among 21 different sugars used, all isolates utilized dextrose and ribose except MGR8 and MGR30 respectively (Table 3). Of the total 10 alkaline protease producers, all isolates were catalase positive whereas seven were oxidase positive (Table 4). Optimum pH, temperature and incubation period required for growth of all alkaline protease producers was recorded 10.0, 30 °C and 48 h respectively (Table 5). All the isolates have showed sensitivity to various antibiotics (Table 6). Isolate MGR8 showed resistance to Amikacin (30 µg/disc) and Tetracycline (30 µg/disc). Isolates MGR30 and MGR11 were resistant to Penicillin G (1 Unit/disc). Isolates LS-1 and LW9 have showed resistance to Teicoplanin (30 µg/disc) and Netillin (30 µg/disc) respectively.

The sequenced PCR product of different base pair lengths were subjected to BLAST analyses. Phylogenetic analyses of these sequences revealed a range of identities to several groups of bacteria (Figure 2). The result of phylogenetic analysis showed distinct clustering of the isolates and confirmed the results of the sequence similarity analysis (Figure 2). Isolates LS-1, LS-2, LS-3, LS-4, LS-6, LW9, MGR8, MGR25, MGR30 and MGR11 showed identity with *Bacillus subtilis* M50 (99 %), *Bacillus clausii* U1032 (99 %), *Bacillus megaterium* X14 (99 %), *Bacillus licheniformis* ATHE9 (98 %), *Bacillus thuringiensis* SLS3 (99 %), *Bacillus alcalophilus* IARI-IHD-22 (100 %), *Alkalibacillus* sp. 20-13 (100 %), *Bacillus horikoshii* DL25 (100 %), *Micrococcus* sp. SNN-LL-11 (100 %) and *Alkalibacterium* sp. I-5 (100 %) respectively. Alkaline protease producers were identified as *Bacillus subtilis* LS-1, *Bacillus clausii* LS-2, *Bacillus megaterium* LS-3, *Bacillus licheniformis* LS-4, *Bacillus thuringiensis* LS-6, *Bacillus alcalophilus* LW9, *Alkalibacillus* sp. MGR8, *Bacillus horikoshii* MGR25, *Micrococcus* sp. MGR30 and *Alkalibacterium* sp. MGR11 on the basis of morphological, biochemical, physiological characterization, antibiotic sensitivity, enzyme profile and 16s rRNA sequencing. Sequences from isolates LS-1, LS-2,

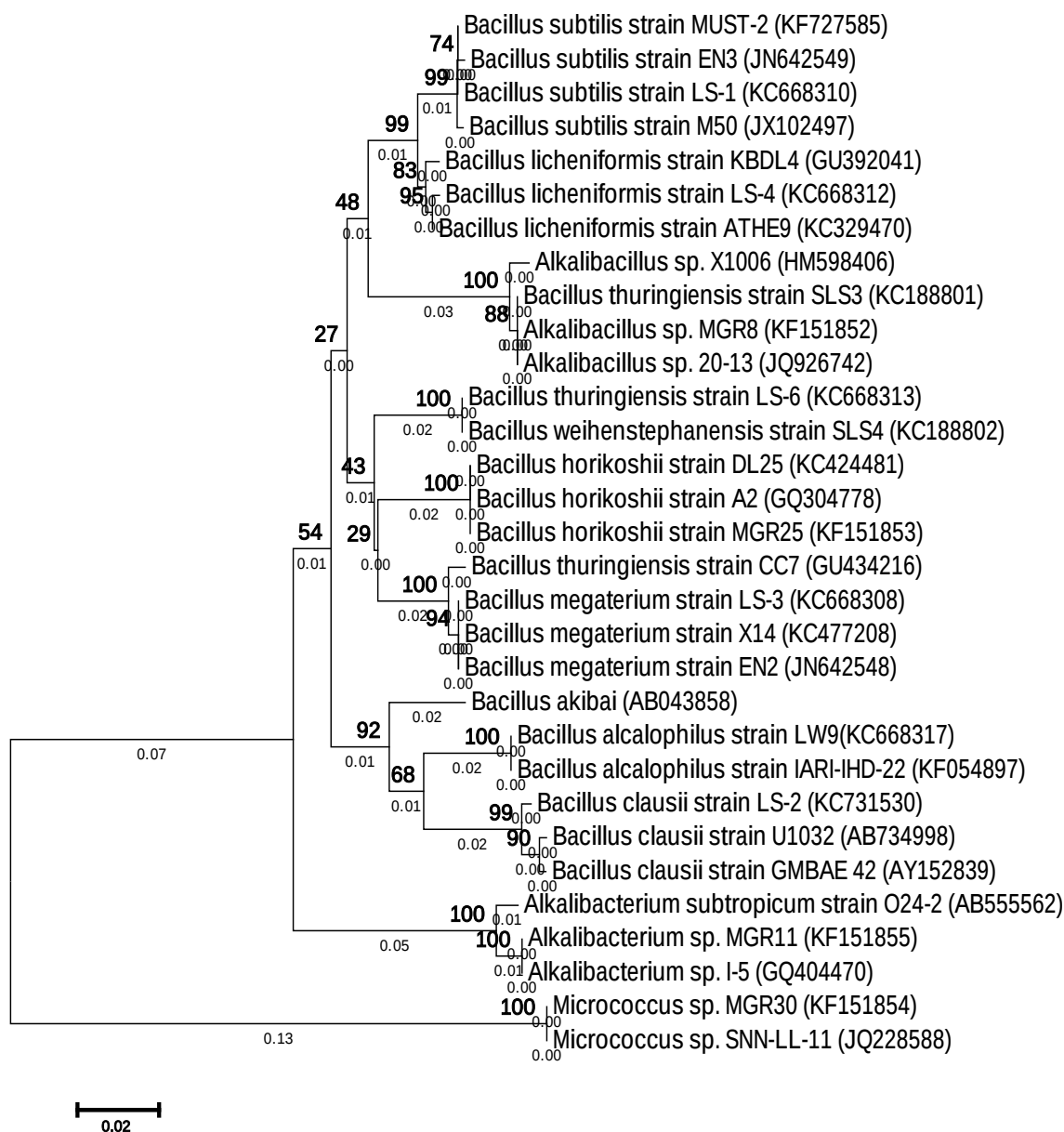


Fig. 2- Evolutionary relationships of taxa

The optimal tree with the sum of branch length = 0.52764926 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 31 nucleotide sequences for construction of phylogram. There were a total of 608 positions in the final dataset.

LS-3, LS-4, LS-6, LW9, MGR8, MGR25, MGR30 and MGR11 were deposited in GenBank 16s rDNA repository under the accession numbers KC668310, KC731530, KC668308, KC668312, KC668313, KC668317, KF151852, KF151853, KF151854 and KF151855 respectively. Isolation and identification of *Alkalibacillus* sp., *Bacillus horikoshii*, *Micrococcus* sp. and *Alkalibacterium*

sp. were earlier reported from soda saline-alkaline soil (Daqing, China), sediment from alkaline crater lake (Lonar), water sample (Lonar Lake) and soda lake sediment (Inner Mongolia, Lake Xiarinur) respectively. Of all the alkalophilic microorganisms, members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline proteases and in this context we

have isolated and identified eight different alkaline protease producing *Bacillus* sp. Isolation and identification of *Bacillus thuringiensis* is the

first time reported by us from Lonar Soda Lake. Phylogenetic tree has illustrated in Figure 2.

Table 1- Morphological characters of bacterial isolates from Lonar lake

| Characters of isolates | <i>Bacillus subtilis</i> LS-1 | <i>Bacillus clausii</i> LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i> LS-4 | <i>Bacillus thuringiensis</i> LS-6 | <i>Bacillus alcalophilus</i> LW9 | <i>Alkalibacillus</i> sp. MGR8 | <i>Bacillus horikoshii</i> MGR25 | <i>Micrococcus</i> sp. MGR30 | <i>Alkalibacterium</i> sp. MGR11 |
|------------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|------------------------------------|----------------------------------|--------------------------------|----------------------------------|------------------------------|----------------------------------|
| Size (mm)              | Round                         | Undulate                     | Round                           | Rhizoid                            | Round                              | Round                            | Round                          | Round                            | Round                        | Irregular                        |
| Margin                 | 2.5                           | 3.0                          | 3.0                             | 3.8                                | 2.5                                | 3.0                              | 3.0                            | 2.8                              | 2.0                          | 2.0                              |
| Elevation              | Entire                        | Filamentous                  | Entire                          | Fimbriate                          | Entire                             | Entire                           | Undulate                       | Entire                           | Entire                       | Undulate                         |
| Consistency            | Raised                        | Slightly raised              | Raised                          | Flat                               | Raised                             | Semi-raised                      | Semi-raised                    | Raised                           | Convex                       | Semi-raised                      |
| Density                | Brittle                       | Brittle                      | Brittle                         | Brittle                            | Brittle                            | Brittle                          | Brittle                        | Brittle                          | Brittle                      | Brittle                          |
| Surface                | Opaque                        | Opaque                       | Opaque                          | Opaque                             | Opaque                             | Opaque                           | Opaque                         | Opaque                           | Opaque                       | Opaque                           |
| Color                  | Glowing                       | Smooth                       | Shiny                           | Dull                               | Smooth                             | Smooth                           | Smooth                         | Shiny                            | Shiny                        | Rough                            |
| Pigmentation           | N                             | N                            | N                               | N                                  | N                                  | N                                | N                              | N                                | P                            | N                                |

Abbreviations: N: Non pigmented, P: Pigmented

Table 2- Microscopic features of bacterial isolates from Lonar lake

| Characters of isolates | <i>Bacillus subtilis</i> LS-1 | <i>Bacillus clausii</i> LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i> LS-4 | <i>Bacillus thuringiensis</i> LS-6 | <i>Bacillus alcalophilus</i> LW9 | <i>Alkalibacillus</i> sp. MGR8 | <i>Bacillus horikoshii</i> MGR25 | <i>Micrococcus</i> sp. MGR30 | <i>Alkalibacterium</i> sp. MGR11 |
|------------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|------------------------------------|----------------------------------|--------------------------------|----------------------------------|------------------------------|----------------------------------|
| Cell shape             | Rod                           | Rod                          | Thick Rod                       | Straight rod                       | Rod                                | Rod                              | Long rod                       | Rod                              | Spherical                    | Slender rod                      |
| Cell length (µm)       | 2.0                           | 2.2                          | 2.2                             | 2.3                                | 2.5                                | 2.6                              | 2.9                            | 2.4                              |                              | 3.0                              |
| Cell width (µm)        | 0.6                           | 0.5                          | 1.0                             | 0.5                                | 1.0                                | 0.5                              | 0.6                            | 0.6                              | D=1.0                        | 0.5                              |
| Sporulation            | +                             | +                            | +                               | +                                  | +                                  | +                                | +                              | +                                | -                            | -                                |
| Position of spore      | C                             | ST                           | C                               | C                                  | C                                  | ST                               | C                              | ST                               | -                            | -                                |
| Motility               | M                             | M                            | M                               | M                                  | M                                  | M                                | M                              | M                                | NM                           | NM                               |
| Gram reaction          | +                             | +                            | +                               | +                                  | +                                  | +                                | +                              | +                                | +                            | +                                |
| Flagella arrangement   | PF                            | PF                           | PF                              | PF                                 | PF                                 | PF                               | PF                             | PF                               |                              |                                  |

Abbreviations: PF: Peritrichous flagella, ST: Sub-terminal, T: Terminal, C: Central, M: Motile, NM: Non motile, D: Diameter, +: spore former, -: Non spore former, +: Gram positive.

Table 3- Biochemical characteristics of bacterial isolates from Lonar lake

| Isolates →<br>Sugars ↓                    | <i>Bacillus subtilis</i><br>LS-1 | <i>Bacillus clausii</i><br>LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i><br>LS-4 | <i>Bacillus thuringiensis</i><br>LS-6 | <i>Bacillus alcalophilus</i><br>LW9 | <i>Alkalibacillus</i><br>sp. MGR8 | <i>Bacillus horikoshii</i><br>MGR25 | <i>Micrococcus</i> sp.<br>MGR30 | <i>Alkalibacterium</i><br>sp. MGR11 |
|-------------------------------------------|----------------------------------|---------------------------------|---------------------------------|---------------------------------------|---------------------------------------|-------------------------------------|-----------------------------------|-------------------------------------|---------------------------------|-------------------------------------|
| Dextrose                                  | +                                | +                               | +                               | +                                     | +                                     | +                                   | -                                 | +                                   | +                               | +                                   |
| Fructose                                  | +                                | -                               | +                               | +                                     | +                                     | -                                   | -                                 | -                                   | -                               | +                                   |
| Lactose                                   | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Sucrose                                   | +                                | -                               | +                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Mannitol                                  | +                                | +                               | +                               | +                                     | +                                     | +                                   | +                                 | +                                   | -                               | -                                   |
| Maltose                                   | +                                | -                               | +                               | +                                     | +                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Xylose                                    | +                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | +                                   |
| Arabinose                                 | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Galactose                                 | -                                | -                               | +                               | +                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Glycerol                                  | +                                | -                               | +                               | +                                     | -                                     | +                                   | -                                 | -                                   | -                               | -                                   |
| Cellobiose                                | -                                | -                               | +                               | +                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Sorbitol                                  | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Melibiose                                 | -                                | +                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Mannose                                   | +                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Trehalose                                 | +                                | -                               | +                               | -                                     | +                                     | -                                   | +                                 | -                                   | -                               | -                                   |
| Ribose                                    | +                                | +                               | +                               | +                                     | +                                     | +                                   | +                                 | +                                   | -                               | +                                   |
| Salicin                                   | -                                | -                               | +                               | +                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Rhamnose                                  | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | +                               | -                                   |
| Inulin                                    | -                                | -                               | +                               | -                                     | -                                     | -                                   | -                                 | +                                   | -                               | -                                   |
| Adonitol                                  | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Raffinose                                 | -                                | -                               | +                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Gas and<br>H <sub>2</sub> S<br>production | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Indole test                               | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| MR test                                   | -                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| VP test                                   | +                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | -                                   | -                               | -                                   |
| Citrate test                              | +                                | -                               | -                               | -                                     | -                                     | -                                   | -                                 | +                                   | -                               | -                                   |

Table 4- Enzyme profile of bacterial isolates from Lonar lake

| Enzymes             | <i>Bacillus subtilis</i> LS-1 | <i>Bacillus clausii</i> LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i> LS-4 | <i>Bacillus thuringiensis</i> LS-6 | <i>Bacillus alcalophilus</i> LW9 | <i>Alkalibacillus</i> sp. MGR8 | <i>Bacillus horikoshii</i> MGR25 | <i>Micrococcus</i> sp. MGR30 | <i>Alkalibacterium</i> sp. MGR11 |
|---------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|------------------------------------|----------------------------------|--------------------------------|----------------------------------|------------------------------|----------------------------------|
| Catalase            | +                             | +                            | +                               | +                                  | +                                  | +                                | +                              | +                                | +                            | w+                               |
| Oxidase             | +                             | +                            | +                               | +                                  | +                                  | +                                | -                              | -                                | +                            | -                                |
| Alkaline protease   | +                             | +                            | +                               | +                                  | +                                  | +                                | +                              | +                                | +                            | +                                |
| Amylase             | +                             | +                            | +                               | +                                  | +                                  | +                                | -                              | +                                | -                            | -                                |
| Cellulase           | +                             | -                            | +                               | -                                  | -                                  | -                                | -                              | -                                | -                            | -                                |
| Urease              | w+                            | -                            | -                               | -                                  | -                                  | -                                | -                              | +                                | -                            | -                                |
| Lipase              | +                             | -                            | +                               | -                                  | -                                  | -                                | -                              | -                                | -                            | -                                |
| Pectinase           | w+                            | -                            | -                               | -                                  | w+                                 | -                                | -                              | -                                | -                            | -                                |
| Specific activity * | 97.22                         | 97.05                        | 96.87                           | 94.11                              | 93.93                              | 90.32                            | 88.33                          | 82.75                            | 78.57                        | 71.15                            |

Abbreviations: \*: Specific activity of alkaline protease (caseinase) (U/mg) = Enzyme activity / Protein content of enzyme.  
w+: weakly positive,                      +: Positive test,                      -: Negative test,

Table 5- Physiological attributes of bacterial isolates from Lonar lake

| Characters                       | <i>Bacillus subtilis</i> LS-1 | <i>Bacillus clausii</i> LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i> LS-4 | <i>Bacillus thuringiensis</i> LS-6 | <i>Bacillus alcalophilus</i> LW9 | <i>Alkalibacillus</i> sp. MGR8 | <i>Bacillus horikoshii</i> MGR25 | <i>Micrococcus</i> sp. MGR30 | <i>Alkalibacterium</i> sp. MGR11 |
|----------------------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|------------------------------------|----------------------------------|--------------------------------|----------------------------------|------------------------------|----------------------------------|
| Temperature growth range (°C)    | 20-50                         | 20-50                        | 20-50                           | 20-50                              | 20-40                              | 20-40                            | 20-40                          | 10-40                            | 20-30                        | 20-30                            |
| Optimum temperature (°C)         | 30                            | 30                           | 30                              | 30                                 | 30                                 | 30                               | 30                             | 30                               | 30                           | 30                               |
| pH growth range                  | 8-12                          | 8-12                         | 8-12                            | 8-12                               | 8-12                               | 8-13                             | 8-13                           | 8-12                             | 8-10                         | 8-10                             |
| Optimum pH                       | 10                            | 10                           | 10                              | 10                                 | 10                                 | 10                               | 10                             | 10                               | 10                           | 10                               |
| NaCl required for optimum growth | -                             | -                            | -                               | -                                  | -                                  | -                                | 2 %                            | -                                | 1 %                          | 1 %                              |
| NaCl tolerance (%)               | 0-7                           | 0-8                          | 0-7                             | 0-6                                | 0-5                                | 0-6                              | 0-9                            | 0-8                              | 0-5                          | 0-5 %                            |
| Optimum growth period (h)        | 48                            | 48                           | 48                              | 48                                 | 48                                 | 48                               | 48                             | 48                               | 48                           | 72                               |

Table 6- Antibiotic Sensitivities of bacterial isolates from Lonar lake

| Isolates→<br>Antibiotics↓ | <i>Bacillus subtilis</i> LS-1 | <i>Bacillus clausii</i> LS-2 | <i>Bacillus megaterium</i> LS-3 | <i>Bacillus licheniformis</i> LS-4 | <i>Bacillus thuringiensis</i> LS-6 | <i>Bacillus alcalophilus</i> LW9 | <i>Alkalibacillus</i> sp. MGR8 | <i>Bacillus horikoshii</i> MGR25 | <i>Micrococcus</i> sp. MGR30 | <i>Alkalibacterium</i> sp. MGR11 |
|---------------------------|-------------------------------|------------------------------|---------------------------------|------------------------------------|------------------------------------|----------------------------------|--------------------------------|----------------------------------|------------------------------|----------------------------------|
| Amikacin (30 µg)          | 2.1                           | 2.5                          | 2.4                             | 2.2                                | 2.4                                | 2.5                              | R                              | 2.7                              | 2.9                          | 2.8                              |
| Ampicillin (10 µg)        | 1.9                           | 1.8                          | 1.7                             | 1.9                                | 1.8                                | 1.7                              | 1.5                            | 1.0                              | 2.0                          | 2.1                              |
| Cefoperazone (75µg)       | 3.5                           | 3.4                          | 3.5                             | 3.8                                | 3.2                                | 3.6                              | 3.3                            | 3.4                              | 3.5                          | 3.2                              |
| Cefoxitin (30 µg)         | 3.4                           | 3.2                          | 3.5                             | 3.7                                | 3.3                                | 3.1                              | 3.0                            | 3.2                              | 3.1                          | 3.7                              |
| Ceftazidime (30 µg)       | 2.3                           | 2.1                          | 2.7                             | 2.5                                | 2.3                                | 2.4                              | 2.1                            | 2.6                              | 2.4                          | 2.2                              |
| Ceftriaxon (30 µg)        | 1.9                           | 1.5                          | 2.0                             | 1.9                                | 2.4                                | 2.7                              | 2.9                            | 3.0                              | 2.1                          | 2.4                              |
| Chloramphenicol (25 µg)   | 1.9                           | 2.5                          | 2.9                             | 2.5                                | 3.0                                | 3.3                              | 3.5                            | 3.2                              | 3.1                          | 3.1                              |
| Chloramphenicol (30 µg)   | 2.3                           | 2.5                          | 4.1                             | 3.2                                | 3.1                                | 3.5                              | 3.9                            | 3.5                              | 3.4                          | 3.5                              |
| Ciprofloxacin (5 µg)      | 1.2                           | 1.5                          | 1.6                             | 1.7                                | 1.8                                | 1.9                              | 2.0                            | 2.2                              | 2.5                          | 2.2                              |
| Cotrimoxazole (30 µg)     | 1.9                           | 2.2                          | 2.4                             | 2.9                                | 2.2                                | 2.9                              | 3.0                            | 2.2                              | 2.5                          | 2.8                              |
| Gentamicin (10 µg)        | 2.0                           | 2.5                          | 2.8                             | 2.9                                | 2.0                                | 3.0                              | 3.2                            | 3.4                              | 3.2                          | 3.1                              |
| Netillin (30 µg)          | 1.5                           | 1.4                          | 2.5                             | 2.7                                | 2.6                                | R                                | 2.4                            | 2.7                              | 2.7                          | 2.1                              |
| Ofloxacin (5 µg)          | 2.3                           | 2.1                          | 2.2                             | 2.4                                | 2.3                                | 2.6                              | 2.3                            | 2.1                              | 2.3                          | 2.4                              |
| Penicillin (10 U)         | 1.9                           | 2.4                          | 3.0                             | 3.1                                | 2.9                                | 2.8                              | 2.5                            | 2.7                              | 2.8                          | 1.9                              |
| Penicillin G (1 U)        | 1.5                           | 2.1                          | 2.5                             | 2.6                                | 2.4                                | 2.1                              | 2.0                            | 1.8                              | R                            | R                                |
| Piperacillin (100 µg)     | 2.4                           | 2.1                          | 2.6                             | 2.8                                | 2.9                                | 2.1                              | 3.9                            | 4.0                              | 3.8                          | 4.0                              |
| Streptomycin (10 µg)      | 2.8                           | 2.5                          | 2.7                             | 3.1                                | 3.1                                | 3.3                              | 3.2                            | 3.1                              | 3.5                          | 3.2                              |
| Sulphatriad (30 µg)       | 2.4                           | 2.3                          | 2.1                             | 3.3                                | 3.2                                | 3.4                              | 2.4                            | 2.7                              | 2.6                          | 2.2                              |
| Teicoplanin (30 µg)       | R                             | 1.3                          | 1.5                             | 1.5                                | 1.8                                | 1.9                              | 2.4                            | 2.1                              | 1.9                          | 2.0                              |
| Tetracycline (30 µg)      | 2.4                           | 1.9                          | 2.3                             | 2.6                                | 2.7                                | 2.4                              | R                              | 2.8                              | 2.6                          | 2.0                              |
| Tetracycline (25 µg)      | 2.0                           | 1.8                          | 1.9                             | 1.7                                | 1.9                                | 2.0                              | 2.1                            | 2.3                              | 2.0                          | 1.8                              |
| Vancomycin (30 µg)        | 2.5                           | 2.6                          | 2.8                             | 2.1                                | 2.2                                | 2.9                              | 2.1                            | 2.6                              | 2.5                          | 2.2                              |
| Abbreviation:             | R:                            | Resistant;                   | Size                            | of                                 | zone                               | is                               | given                          | in                               | centimeter                   |                                  |

Out of ten alkaline protease producers, nine isolates belonged to phylum firmicute and one

isolate belonged to phylum actinobacteria. *Bacillus subtilis* LS1 was deposited under accession number MCC2183 at Microbial Culture Collection (MCC) division at National Center for Cell Science (NCCS), Pune in Maharashtra, India.

### Conclusions

Our study describes the existence of cultivable bacterial diversity of alkaline protease producers from Lonar lake. Alkaline protease producing strains were isolated and identified as *Bacillus subtilis*, *Bacillus clausii*, *Bacillus megaterium*, *Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus alcalophilus*, *Alkalibacillus* sp., *Bacillus horikoshii*, *Micrococcus* sp. and *Alkalibacterium* sp. from Lonar soda lake. Partially purified alkaline proteases derived from these alkaliphilic and halotolerant bacterial isolates exhibited remarkable specific enzyme activity. Partially purified alkaline protease from *Bacillus subtilis* LS-1 showed highest specific enzyme activity. The results obtained from our studies revealed that, besides protease identified bacterial isolates can also be exploited for production of biotechnologically important and industrially demanding alkaline biocatalysts like amylase, gelatinase, cellulase, lipase and pectinase.

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