



Isolation and Characterization of Halotolerant *Bacillus* sp with extra Cellular α -Amylase production Potential from Sambhar salt lake, India

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Abstract

Sambhar lake (27° 58 N, 75° 55 E) is a halo-alkaline playa with water density varying from 3-27 Be (Baume). The lake was explored for the diversity of Halophilic microbes. The study led to the isolation of a Moderate halophilic bacterial strain from brine samples of the lake. The isolate showed a growth range of 3-15% NaCl in alkaline medium and was designated as SL-3. The isolate was aerobic, gram +ve, spore forming, rod-shaped and motile. It also tested positive for catalase and oxidase. The isolate was tested for exo-enzyme production and found positive for α -Amylase and negative for lipase and protease production. The Isolates was not able to utilize citrate as a sole carbon source and failed to produce H₂S in TSI-medium. Carbohydrate fermentation revealed utilization of glucose, fructose and sucrose. Taxonomic Identification of isolate was confirmed by partial 16s rRNA gene sequence analysis that contained 1197 nucleotides. The phylogenetic study revealed that the isolate matches 100% with *Bacillus licheniformis* strain PS-6. Therefore the isolated bacterial strain belongs to phylum Firmicutes with low G+C contents and Genus *Bacillus*. Gene Bank Accession No. for the isolate is KP172152.

Keywords: Bacillus, phylogenetic study, sambhar lake, halophiles, α -amylase.

Introduction

Moderately halophilic bacteria growing optimally in media containing 3–15% (w/v) salts are widely distributed in hypersaline habitats. These organisms are of considerable interest owing to their biotechnological potential in the production of compatible solutes and hydrolytic enzymes¹. According to Kushner² though they are less exciting at first glance than the extreme halophiles, however can be more useful as they own the ability to grow over wide ranges of solute concentrations.

Halophilic studies in Indian context are limited to the marine salterns near Bhavnagar³, Lonar Lake⁴ and Peninsular coast⁵. These studies are mainly focused on aerobic red halophilic archaea. Sambhar salt lake, the largest inland lake of India, has also been explored mainly for halophilic archeal species^{6,7}. The aerobic eubacteria always remained understudied⁸.

All the Aerobic, spore-forming, moderately halophilic, Gram-positive rods shaped bacteria were originally assigned to the genus *Bacillus*, but with the advent of molecular methods and improved phenotypic approaches, they have been reclassified as new genera⁹⁻¹¹. Further molecular analyses of 16S rRNA gene sequences^{12,13} led to assigning large number of species and six phylogenetically distinct groups to the genus *Bacillus*. Recently the studies on *Bacillus* gained momentum as they are attracting interest because of their great biotechnological potential².

In the process of studying moderate halophiles of Sambhar salt

lake, a bacterial strain of Genus *Bacillus* was isolated and subjected to taxonomic characterization. Sambhar lake ecosystem is still under explored in regard of microbial studies and microbial studies with molecular approach need to be encouraged.

Material and Methods

Soil and water samples were collected from Jhapok dam area of Sambhar Salt Lake (Rajasthan, 26°58'N75°05'E) in sterile containers. In order to enrich moderate halophiles Nutrient broth supplemented with 3-15% NaCl was employed. 10 ml sample was added to 250 ml broth and incubated for 5-7 days at 35°C. 1 ml of enriched broth was spread cultured on Nutrient Agar with 3-15% NaCl. Bacterial colonies appearing after 7 days of incubation were re-streaked on fresh medium. Halophilic colonies were purified by repeated streaking on complete Salt Nutrient agar. The isolates were stored at 4 °C and sub-cultured at 15 days intervals.

Amylolytic activity of the isolates was screened by plate assay on starch nutrient agar plates containing gL⁻¹: starch 10.0; peptone 5.0; yeast extract 3.0; agar 30.0; NaCl 100.0. The pH tested was from 7.0 to 10.0. After incubation at 30 °C for 72h, the zone of clearance was determined by flooding the plates with iodine solution.

Results and Discussion

Results of Microbial and Molecular study: Sambhar Lake is the

largest single salt source in Rajasthan State. The initial source of brine in the lake is catchment water received by downpour annually, mostly during April to September every year¹⁴. Water is the most vital abiotic component of the lake ecosystem¹⁵. The total 348 mm rainfall was recorded in year 2013-14. Water temperature and Density fluctuated between 7-37°C and 3-27 Be (Baume). The water in Lake Basin turns into brine due to percolation and evaporation. The brine contains high salt concentration even up to saturation level. In nature, salinity is often associated with alkalinity. Alkaline saline lakes represent a unique habitat with a high pH and a variable salt concentration. Sambhar Lake exemplifies a halo-alkaline lake as the salinity and pH ranged from 0.53 to 162.21 ‰ and 6.54 to 8.87 respectively.

Hypersaline environments represents typical extreme habitats, where high salt concentration, alkalinity, and low oxygen stress may limit their biodiversity¹⁵, yet inhabitation of microorganisms in diverse hypersaline environments such as salt lakes^{16,17} solar salterns¹⁸, salt mines¹⁹ and brine wells²⁰ have been reported. The Sambhar lake brine also seemed lifeless; however in the effort to assess Halophilic microbial diversity from lake brine, strain belongs to 7 different genera were isolated. Out of these genera bacteria of genus *Bacillus* were the most frequent isolates. These isolates were capable of growing on 3-15% NaCl. One such isolate was further tested for biochemical and taxonomic characterization.

Morphological and biochemical observations: The reported isolate showed yellowish, convex colonies with entire margins and glossy surface. It grew over a range of 3-15% salt with optimum growth at 10%. The isolate tested catalase and oxidase positive. Optimum temperature varied between 30-35°C. It gave negative results for H₂S production and Citrate utilization. The bacterial isolate showed motility and appeared as Gram +ve, rods. Various sugar fermentation tests were carried out in media containing 10% NaCl. The isolate could utilize only glucose, fructose and lactose out of the 10 types of sugar media. Nitrate reduction test gave positive results with the isolate.

Plate Assay for Exo-enzymes production: The moderate halophilic isolate was screened for the characteristic halophilic property of exo-enzyme secretion. The bacterial strain was unable to produce lipase and protease. However strong amylolytic activity was shown at 10% NaCl.

Taxonomic Characterization: DNA isolation by phenol-chloroform Extraction Method: The isolate suspected to be *Bacillus* was subjected to DNA isolation by phenol-chloroform extraction method. It is a liquid-liquid extraction method that separates mixtures of molecules based on the differential solubility's of the individual molecules in two different immiscible liquids.

Amplification of 16s ribosomal genes: The isolated DNA

templates were subjected to 16s r-DNA amplification, using the primers 18F and 1492R as described by Weisburg *et al.*²¹. Optimized PCR reaction and thermal cycling conditions used for the primers, described by Weisburg *et al.*²¹

Phylogenetic Identification: The identification of phylogenetic neighbor was initially carried out by the BLAST and mega BLAST programmes. BLAST results of 1197 nucleotides showed 100% similarity with *Bacillus licheniformis strain PS-6*.

Nucleotide sequence accession number: The 16s rDNA sequence was submitted in Gene Bank and was allotted Accession No. **KP172152**.

Discussion: The above mentioned results showed that the isolated bacterial strain is a member of Genus *Bacillus* and is moderate halophilic in nature as it showed growth in media with 3-15% salt concentration. Genus *Bacillus* is comprised of Gram-positive, rod-shaped (bacillus), bacterial species that have the ability to be aerobic or anaerobic. The Genus is a member of phylum Firmicutes that includes low-G+C group.

A number of moderately halophilic *Bacillus* species have been isolated from hypersaline environments²²⁻²⁶. Rahman (2005)¹⁴ reported that Sambhar brine mainly contains *Bacillus* and *Halobacterium*, and reported *Bacillus subtilis*, *B. amyloliquefaciens*, *B. sphaericus*, *B. licheniformis* from the lake. The occurrence of *Bacillus licheniformis* in the lake was reported to 78% by Rahman¹⁴. Banat²⁷ reported organic hydrocarbon degrading potential in *B. licheniformis*.

Sambhar lake is a haloalkaline lake and brine samples always showed high pH. *Bacillus licheniformis* prefers alkaline growth range²⁸. Since the isolated bacillus strain was capable of growing in twin extremities of pH and salinity, it can be categorized as halo-alkaliphilic. Survival strategies, robust biocatalysts and unique metabolic capabilities of these organisms can be a great source of novel commercial applications. Members of the order *Bacillales* shows great metabolic versatility and ability to grow under physico-chemical extremes, which make them survive in almost every environment on earth from the stratosphere³⁰ to the deep subsurface³¹. This ubiquity is, in part, attributed to their ability to form resilient spores that can be transported over long distances²⁹. Sahoo reported heavy metal tolerance in *B. licheniformis*³².

The *Bacillus* strain under the study showed amylolytic potential by producing extra-cellular amylase, while it tested negative for protease and lipase production. However *Bacillus subtilis* growing optimally at 10% NaCl, showing protease activity has been isolated from Sambhar lake³¹. Bacterial enzymes are the ones that are having wide variety of industrial applications. Among these enzymes, α -amylase has high demand³². α -Amylases from *Bacillus* species have potential worthwhile applications in conversion of starch to sugar syrup, production

of cyclodextrin³³ and in other industries such as pharmaceutical, textile etc; therefore, these enzymes account for about 30% of the world's enzyme production³⁴. *Bacillus diposauri*, *Halobacillus sp*, *Haloferax mediterranei*, *Halothermothrix sp* are known to be good producer of a amylase. However, the production of α -amylases depends on the strain, composition of media, metal ions, pH, temperature, moisture and oxidative stress^{35,36}.

Conclusion

The study concludes that the bacterial strain isolated from Sambhar salt lake is a Halotolerant, aerobic, gram +ve, spore forming, rod-shaped and motile strain belonging to phylum Firmicutes and Genus Bacillus with low G+C contents. Molecular analysis of 16s r-RNA showed 100% similarity with Bacillus licheniformis strain PS-6. Gene Bank Accession No. for the isolate is KP172152. In conclusion, escalating discoveries of halophilic bacteria from Sambhar lake, with immense biotechnological and industrial potential, demands a more extensive study and more importantly conservation of the lake.

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