

# Isolation and Identification of Haloalkaliphilic Archaeal Isolates from a Soda Lake in India

Gopal N. Kalambe<sup>1</sup>, Vivek N. Upasani<sup>2</sup>

Research Student, Department of Microbiology, JJTU, Vidyanagari, Jhunjhunu, Rajasthan, India

Associate Professor, Department of Microbiology, M. G. Science Institute, Ahmedabad, Gujarat, India<sup>2</sup>

**ABSTRACT:** Although an extreme environment, the hypersaline ecosystems are rich in microbial diversity for biotechnological applications. The aerobic, gram-negative, pleomorphic rods and cocci, chemoorganotrophic archaea belonging to the family *Halobacteriaceae* within the Phylum *Euryarchaeota* have been extensively studied in the last few decades. They have provided new insights for survival under high salinity and/or alkalinity. They occur in both marine as well as inland saline regions mainly in solar salterns. The soda lakes are intermittent saline lakes that contain sodium carbonate / bicarbonate (soda) alongwith sodium chloride that are found in desert regions of the world for example, Lake Magadii (Kenya), Wadi Natrun lakes (Egypt), Sambhar Salt Lake (India), etc. Several halobacterial strains were isolated from the hypersaline brines collected from Sambhar Lake, Rajasthan. Three of the isolates were selected based on differences in colony/cultural characteristics and identified phylogenetically based on 16S rRNA sequence homology studies. Two isolates were identified as strains identical to *Natronobacterium* sp. and one as *Natrialba* sp.

**KEYWORDS:** *Natronobacterium*, *Natrialba*, Haloarchaea, Sambhar Lake.

## I. INTRODUCTION

The extremely halophilic archaea belonging to the family *Halobacteriaceae* (Phylum *Euryarchaeota*) are commonly found in the hypersaline environments such as salt lakes, salt ponds, marine salterns and soda lakes. Haloarchaea living in such harsh environment cope up with salinity stress by higher intracellular KCl concentration and synthesis of compatible solutes such as glycerol and glycine betaine. Some methanogens also belong to the group haloarchaea. The haloalkaliphilic archaea survive in an environment with two extremities, namely, high pH (>9.0) and salt concentration (>3.0M NaCl) [1]. The halobacteria contain ether linked lipids based on the lipid core 2, 3-di-O-phytanyl-sn-glycerol (C<sub>20</sub>-C<sub>20</sub>-diether). Ross et al. [2] demonstrated that the extreme haloalkaliphiles also possess 2-O-sesterterpanyl-3-O-phytanyl-sn-glycerol (C<sub>20</sub>-C<sub>25</sub>-diether), and 2,3-di-O-sesterterpanyl-sn-glycerol (C<sub>25</sub>-C<sub>25</sub>-diether) lipid cores. *Natronobacterium* and *Natronococcus* were the first two genera described by Tindall et al. [3] to include haloalkaliphilic archaea isolated from saline and alkaline lakes.

There has been an exponential increase in the number of recognized haloarchaeal genera and species that have been studied by both culture-dependent and culture-independent methods [4]. Several new genera for example the genus *Natrialba*, *Natronorubrum*, *Natronomonas*, etc. have been described in the Bergey's Manual of Systematic Bacteriology, vol. I, [5]. According to Gupta et al. [6] based on conserved signature proteins and insertions/deletions that have been used as new phylogenetic markers, the class *Halobacteria*, within the phylum *Euryarchaeota*, consists of three orders: *Halobacteriales*, *Haloferacales* and *Natrialbales*. Recently, a new halophilic archaeal class "*Nanohaloarchaea*" has been proposed based on *de novo* genome assembly and metagenomic studies on surface waters of an Australian hypersaline Lake Tyrrell (LT) [7]. The biotechnological applications of the exoenzymes, pigments, compatible solutes from these extremophiles have been reviewed extensively [8, 9]. The present study includes the identification of three haloarchaeal isolates from Sambhar Lake, India based on 16S rRNA sequence analysis.

# International Journal of Innovative Research in Science, Engineering and Technology

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Vol. 6, Issue 6, June 2017

The research work in this paper is divided in two stages. 1) Isolation and characterization of haloalkaliphilic archaea 2) Phylogenetic identification of the haloarchaeal isolates. The paper is organized as follows. Section II describes materials and methods for isolation, morphological and cultural characterization, cultivation and phylogenetic analysis of isolates. In Section III the experimental results showing cultural characteristics, growth requirements, phylogenetic trees and discussion is included. Finally, Section V presents conclusion.

## II. MATERIALS AND METHODS

### Organism and growth conditions

The haloalkaliphilic archaea were isolated from brines (hypersaline water) collected from Sambhar Lake situated (Longitude 75°05'E Latitude 26°58'N) in Rajasthan, India. The samples were enriched and the isolates were obtained on modified Brown medium containing 20 (%w/v, NaCl), pH 9.0, at 37°C as described by Upasani and Desai [10]. The isolates were preserved on slants at 4-5°C in refrigerator.

### Cultural characteristics

The colony characteristics, gram reaction, morphology and motility were studied by routine microbiological procedures.

### Growth requirements

The effect of salt (%w/v, NaCl) (1.0, 5.0, 10.0, 15.0, 20.0 and 25.0), and pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0) was studied by inoculating actively growing culture (2.0ml) in 100ml medium and incubation at 37°C on environmental shaker. The growth was measured at 610nm after 10 days.

### Phylogenetic analysis

The isolates were grown on agar slants of modified Brown medium. The gene(s) encoding 16S rRNA was amplified using the PCR method of Emble [11], as modified by McGenity and Grant [12]. The forward amplification primer was: 27F, TCCGGTTGATCCTGCCGGAG (positions 8-27), and the reverse amplification primer was: 1525R, AAGGAGGTGATCCAGCC (positions 1541-1525) and sequenced at Chromus Biotech Limited, Bangalore. Similar sequence homologs were obtained by BLAST search. The evolutionary relationship of the isolates was inferred using the Neighbour-Joining method [13]. 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 evolutionary distances were computed using the Maximum Composite Likelihood method [14], and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted using MEGA7 [15].

## III. EXPERIMENTAL RESULTS AND DISCUSSION

Three isolates designated as CS6, S6 and S14 of haloalkaliphilic archaea were selected based on cultural characteristics that were isolated on modified Brown's medium from the brines collected from Devyani Kyars (salt pans) at Sambhar Lake, Rajasthan. After fifteen days of incubation (15) the colonies of CS6 were very small (pinpoint), convex, translucent and red pigmented; colonies of S6 were medium, convex, irregular margin, mucoid, translucent and red pigmented; whereas the colonies of S14 were large, convex, mucoid, opaque and pink pigmented (Figure 1).

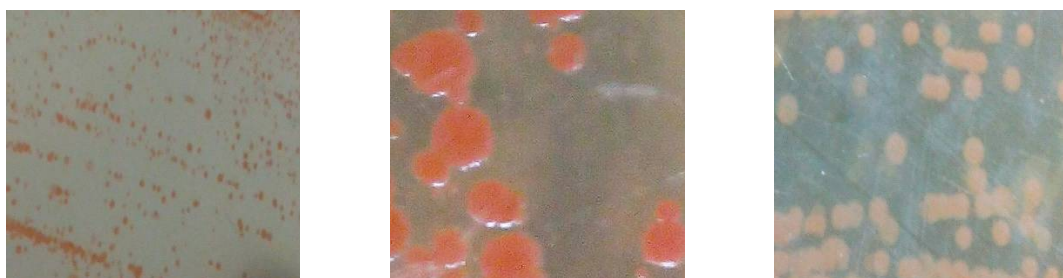


Fig. 1. Cultural characteristics of haloalkaliphilic isolates (L to R): CS6, S6 and S14 (magnification of all the three images is different).

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All the isolates were gram-negative, pleomorphic rods and motile. The cells of CS6 were small rods, whereas those of S6 and S14 were slender rods. All the three isolates grew optimally between 20.0 - 25.0 (% w/v, NaCl) and pH 9.0-11.0. Thus, based on the cultural and physiological characteristics the three isolates were presumed to be haloalkaliphilic archaeal isolates and further identified by phylogenetic studies. The 16S rRNA sequences of these isolates have been deposited with GenBank with accession nos. MF150019.1 to MF150021.1.

The identification of haloalkaliphilic archaea based on phenotypic and lipid composition is difficult, and hence the use of molecular techniques such as 16S rRNA homology studies and DNA-DNA hybridization are crucial for this purpose [16, 17].

Table 1 includes the details of the 16S rRNA sequences selected based on BLAST results for construction of the phylogenetic tree. Based on the phylogenetic studies the isolate CS6 was identified as *Natronobacterium* species with 99% identity with the haloalkaliphilic species isolated from the former Lake Texcoco in Mexico, namely *Natronobacterium texcoconense* B23 [18] within the cluster consisting of *Natronobacterium gregoryi* SP2 (Figure 2).

Table 1. Details of the GenBank sequences selected for phylogenetic analysis of isolate CS6.

Name	Accession no.	identity
<i>Natronobacterium gregoryi</i> SP2	NR_113531.1	99%
<i>Natronobacterium gregoryi</i> SP2_1	NR_102442.1	99%
<i>Natronobacterium gregoryi</i> SP2_2	CP003377.1	99%
<i>Natronobacterium gregoryi</i> SP2_3	NR_028223.1	99%
<i>Archaeon</i> WN32	KT459359.1	99%
<i>Natronobacterium</i> sp. S4FS3Drt	KY053865.1	99%
<i>Natronobacterium texcoconense</i> JCM 17655	AB935412.1	97%
<i>Natronobacterium texcoconense</i> B23	NR_109480.1	97%
<i>Natronococcus amylyticus</i> Ah-36	NR_028217.1	95%
<i>Natronococcus amylyticus</i>	D43628.1	95%
<i>Halobiforma nitratreducens</i> C231	NR_028153.1	95%
<i>Halopiger</i> sp. IHH4	KC430941.2	95%
<i>Halobiforma lacisalsi</i> AJ5	CP019285.1	94%
<i>Halobiforma lacisalsi</i> JCM 12983	NR_113500.1	94%
<i>Halopiger djelfimassiliensis</i>	NR_133030.1	94%

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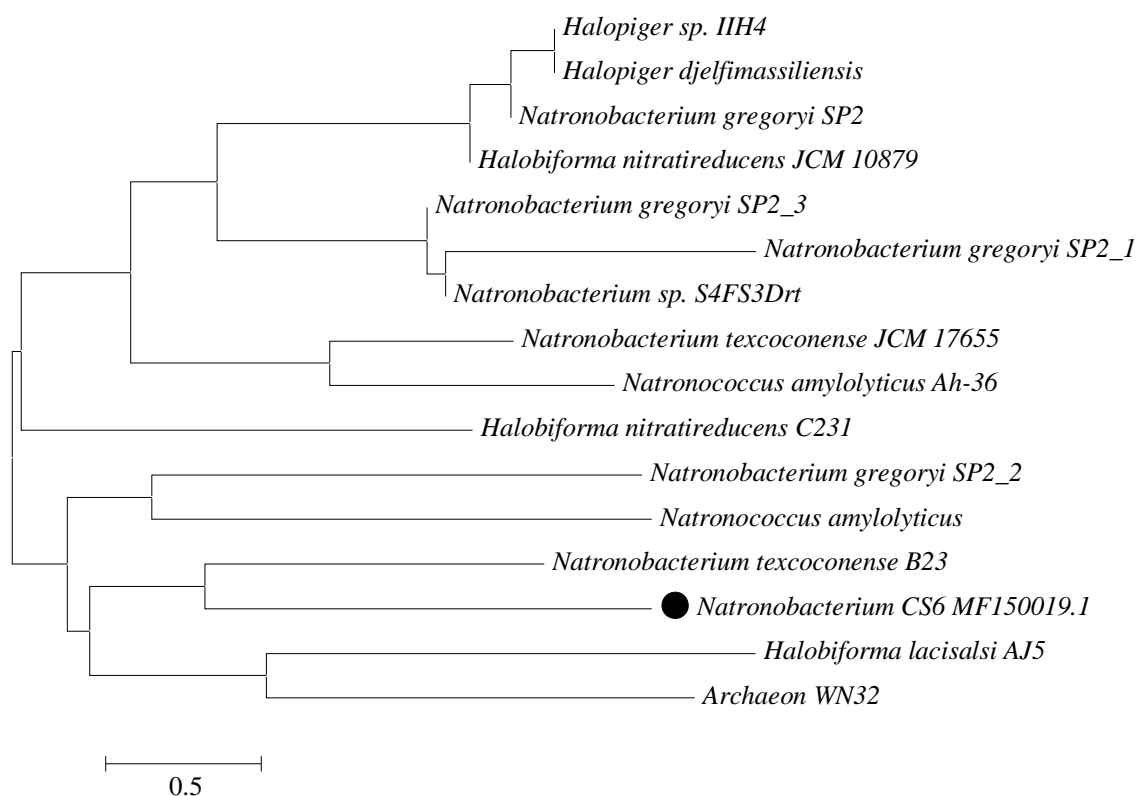


Fig. 2. Phylogenetic tree constructed using the neighbour-joining method using complete 16S rRNA sequences for isolate CS6. The optimal tree with the sum of branch length = 17.13123285 is shown. There were a total of 1180 positions in the final dataset

The details of 16S rRNA sequences used for phylogenetic analysis of isolate S6 is given in Table 2, and based on the phylogenetic analysis using these sequences the isolate was identified as a species belonging to the genus *Natronobacterium* and closely related to a cultured (*Archaeon* WN32) and two uncultured archaeons (41-1 and 38B-1) (Figure 3).

Table 2. Details of GenBank sequences selected for phylogenetic analysis of isolate S6.

Name	Accession no.	Identity
<i>Archaeon</i> WN32	KT459359.1	99%
<i>Uncultured archaeon</i> 41-1	AF424774.1	99%
<i>Uncultured archaeon</i> 38B-1	AF424771.1	99%
<i>Natronobacterium sp.</i> S4FS3Drt	KY053865.1	99%
<i>Natronobacterium texcoconense</i> JCM 17655	AB935412.1	97%
<i>Natronobacterium texcoconense</i> B23	NR_109480.1	97%
<i>Halobiforma nitratireducens</i> C231	NR_028153.1	95%
<i>Halopiger sp.</i> IIH4	KC430941.2	95%
<i>Halopiger sp.</i> IIH1	KC430938.2	95%
<i>Natronococcus aibiensis</i>	AY277581.1	94%
<i>Natronococcus roseus</i> CG-1	NR_117208.2	94%

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Vol. 6, Issue 6, June 2017

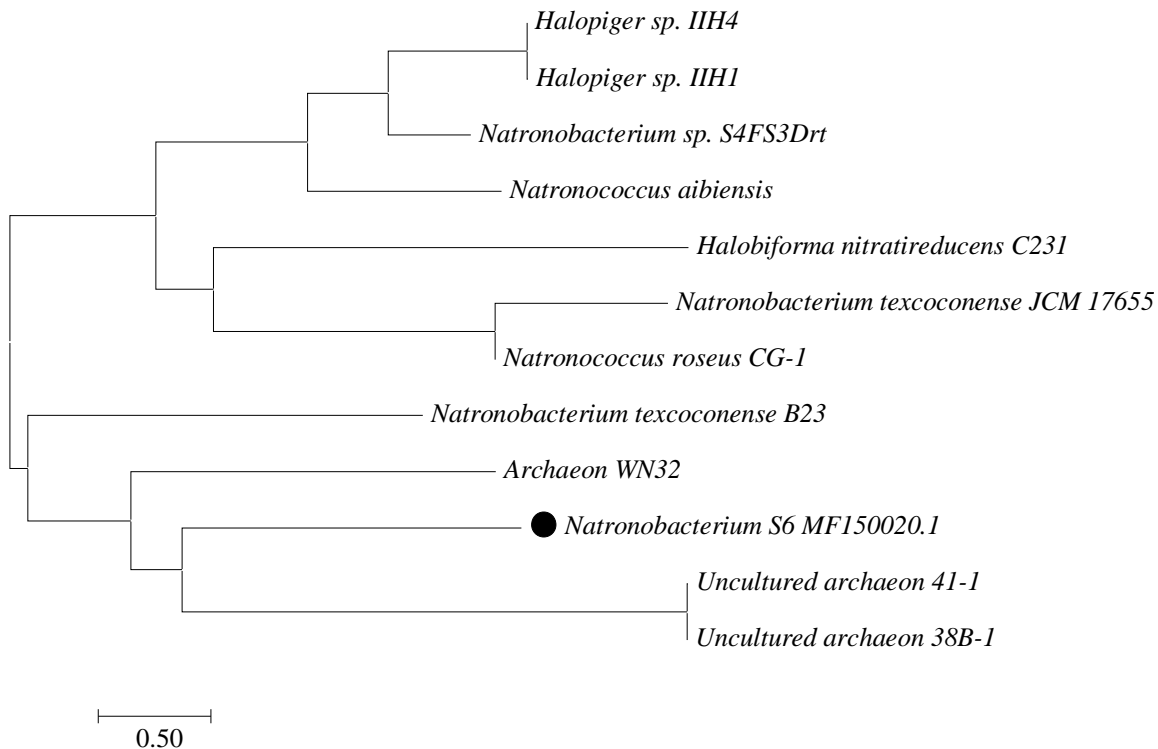


Fig. 3. Phylogenetic tree constructed using the neighbour-joining method using complete 16S rRNA sequences for isolate S6. The optimal tree with the sum of branch length = 15.82130881 is shown. There were a total of 1180 positions in the final dataset

Based on the 16S rRNA sequences selected from BLAST search (Table 3) and the phylogenetic analysis (Figure 4) the third isolate S14 was identified as a member of the genus *Natrialba*, and was closely related to *Natrialba wudunaoensis* Y21 isolated from soda lakes in Inner Mongolia Autonomous Region, China [19]. The first haloalkaliphilic isolate from Sambhar Lake was reported by Upasani and Desai [10] and currently recognized as *Natrialba* SSL1 (ATCC 43988, earlier as *Natronobacterium*), its genome has been sequenced and produces hydrolytic enzymes such as amylase, lipases, protease and bacteriocin [20, 21, 22, 23]. However, isolate S14 is not closely related to *Natrialba* SSLVU01 and *Natronobacterium* SSL1 (ATCC 43988) reported earlier.

Table 3. Details of GenBank sequences selected for phylogenetic analysis of isolate S14.

Name	Accession no.	Identity
<i>Natrialba chahannaensis</i> JCM 10990	NR_113520.1	99%
<i>Natrialba chahannaensis</i> WNHS9	KP828441.1	99%
<i>Natrialba chahannaensis</i> WNHS4	KP828440.1	99%
<i>Natrialba chahannaensis</i> WNHS20	KP828442.1	99%
<i>Natrialba chahannaensis</i> WNHS2	KP788716.1	99%
<i>Natrialba chahannaensis</i> w	KP676930.1	99%
<i>Natrialba chahannaensis</i> C112	NR_028181.1	99%
<i>Natrialba wudunaoensis</i> Sua-E42	EU672838.1	99%
<i>Natrialba chahannaensis</i> Sua-CS1	EU672837.1	99%
<i>Natrialba wudunaoensis</i> Y21	NR_114682.1	99%
<i>Natronobacterium</i> sp. ATCC 43988	D88256.1	99%

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<i>Natrialba</i> sp. SSLVU01	KJ123850.1	99%
<i>Natrialba hulunbeirensis</i> JCM 10989	NR_113521.1	99%
<i>Natrialba hulunbeirensis</i> WNHS14	KP765047.1	99%
<i>Natrialba hulunbeirensis</i> X21	NR_114688.1	99%

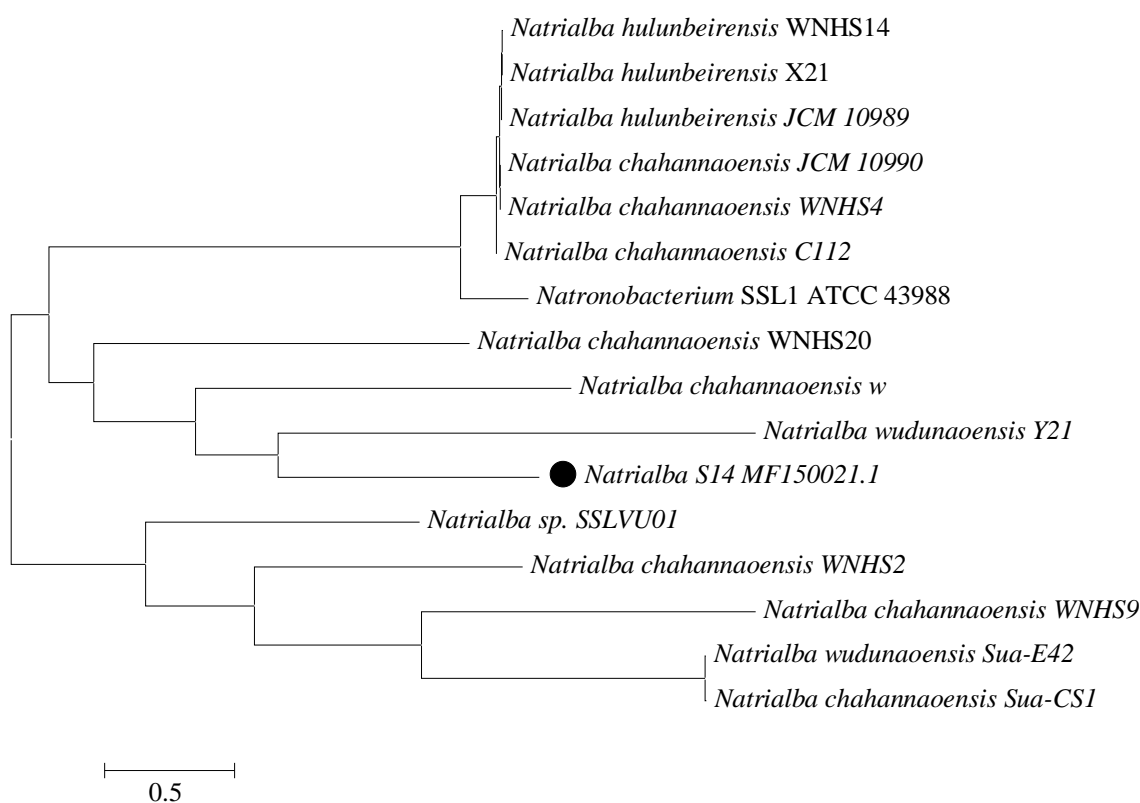


Fig. 4. Phylogenetic tree constructed using the neighbour-joining method using 16S rRNA sequences for isolate S14. The optimal tree with the sum of branch length = 14.72868707 is shown. There were a total of 1358 positions in the final dataset

Thus, the present study reconfirms the presence of haloalkaliphilic archaea belonging to the genera *Natronobacterium* and *Natrialba* as dominant haloarchaea in this extreme environment.

## IV. CONCLUSION

The soda lakes worldwide have been explored for the isolation and characterization of mainly haloarchaea and the anoxygenic phototrophic bacteria. We were able to isolate and identify three haloalkaliphilic archaeal isolates. Based on 16S rRNA sequence homology studies the isolates were belonging to the genus *Natronobacterium* (CS6 and S6) and *Natrialba* (S14). Further biochemical and physiological characterization will provide more information on their biotechnological applications.

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Vol. 6, Issue 6, June 2017

## V. ACKNOWLEDGEMENT

The authors are grateful to M. G. Science Institute (Ahmedabad Education Society) for providing lab facilities, and the assistance by Harshil H. Bhatt for phylogenetic analysis.

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