Int.J.Curr.Microbiol.App.Sci (2016) *5*(2): 215-224

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 2(2016) pp. 215-224**
Journal homepage: http://www.ijcmas.com

Original Research Article doi: http://dx.doi.org/10.20546/ijcmas.2016.502.024

Isolation and Characterization of a Halophilic Cyanobacterium *Euhalothece* SLVH01 from Sambhar Salt Lake, India

Harshil H. Bhatt[1], Renu Pasricha[2] and Vivek N. Upasani[3]*

[1]Department of Biotechnology, Kadi Sarva Vishwavidyalaya, Gandhinagar-382015, India
[2]National Centre for Biological Sciences, Bangalore-560065, India
[3]Department of Microbiology, M. G. Science Institute, Navrangpura, Ahmedabad, India

**Corresponding author*

Keywords

Sambhar Salt Lake, soda lake, halophilic Cyanobacterium, *Euhalothece* SLVH01

Article Info

Accepted:
13 January 2016
Available Online:
10, February 2016

A B S T R A C T

Soda lakes or alkaline hypersaline lakes are distributed world-wide mainly in deserts. They are a microbiologist's delight and treasure. Microorganisms inhabiting saline environments and requiring a minimum 3-5 (% w/v) NaCl for growth are referred to as halophiles. They are classified as slight, moderate, and extreme halophiles depending on their salt (NaCl) requirements. Sambhar Salt Lake in Rajasthan is India's largest soda lake with salinity ranging from 2-4 (% w/v) to saturation, and pH (8-12) depending on the season as well as location. Microscopic studies of the brine samples revealed the presence of 4-5 algal species. We have enriched halophiles by Winogradsky technique and isolated a halophilic cyanobacterium from this extreme environment. Based on morphological, physiological and 16S rRNA sequencing studies one species has been identified as *Euhalothece* SLVH01.This is the first report on the isolation and characterization of this haloalkaliphilic microalga (cyanobacterium) from this lake.

Introduction

Some of the well-known soda lakes that have been explored for microbial diversity include: The Kenyan-Tanzanian Rift Valley lakes (Lake Magadi, Lake Bogoria, Lake Natron, etc.); Owens Lake, Lake Texcoco, Albert Lake, etc., in the Americas; Chita and Bikal region, Loonar Lake, Tafusu Lake, Sambhar Salt Lake, etc., in Asia (6,17).

Sambhar Salt Lake is the largest soda lake in India with 190 sq. kms area and situated in the gaps of Aravali mountain range (9,12). The water from this lake is an important source of salt (NaCl) and other chemicals since ancient times. The chemical composition and isolation of haloalkaliphilic archaea and algae from this lake has been reported (15,17). The cyanobacteria or

prokaryotic algae belonging to the *Cyanophyceae* are considered to be responsible for the presence of oxygen in the atmosphere and have evolved during the Precambarian era (20). Microalgae are the primary producers in this lake and serves as food for flamingos, other birds and zooplankton. Low algal biomass and/or its quality results, decrease in the number of lesser flamingos in Kenyan Rift Valley lakes. A rise in the population of *Anabaenopsis* causes death of flamingos (11). Their lysis at higher salinity releases nutrients for the growth of extremely halophilic bacteria (6.17). The microbial ecology of soda lakes has been reviewed by Grant et. al. (7). Besides the cyanobacteria and eukaryotic algae, anoxygenic phototrophs and halophilic archaea are the predominant microorganisms in this environment. The isolation of *Ectothiorhodhospira*, *Natronobacterium* and *Natronococcus* species from the African Rift Valley lakes (8,16) triggered an interest on the research related to the diversity and applications of haloalkaliphiles from desert lakes. The halophilic microalgae reported from saline and alkaline lakes include *Anabaenopsis arnoldii, Chloroflexus, Chroococcus, Dunaliella salina, Spirulina sp./Arthrospira platensis, Synechococcus sp.,* etc. (6,7). Some of these algae such as *Spirulina* spp. and *Dunaliella salina*, are used as a source of food supplement, β-carotene, glycerol, biofuel, essential omega fatty acid production, etc. Many reports are available on diversity of bacterial and archaeal species from soda lakes, but algal diversity is still a thrust area.

Hence our research was focused on studying the microalgal flora and photosynthetic bacteria of Sambhar Salt Lake by enriching these organisms by Winogradsky column. This resulted in the isolation of a cyanobacterium strain from the brines (water samples). Based on morphological, physiological and 16S rRNA sequencing the isolate was identified as *Euhalothece* SLVH01.

Materials and Methods

Sample Collection

Brine, soil and salt samples were collected from Devyani region, Sambhar Salt Lake, Rajasthan and were brought to the laboratory in sterile bottles (500ml). Samples were examined under microscope for presence of microalgae. The samples were stored at 4^{o}C in a refrigerator.

Isolation and Cultivation

Winogradsky column was developed with soil and brine samples from this lake by incubating it for several months in sunlight. The enriched algal culture was inoculated into 250ml of BG 11 medium (Himedia, India) which consisted of (g/l): sodium nitrate, 1.5; dipotassium hydrogen phosphate, 0.0314; magnesium sulphate, 0.036; calcium chloride dehydrate, 0.0367; sodium carbonate, 0.020; disodium magnesium EDTA, 0.001; citric acid, 0.056; ferric ammonium citrate, 0.006; final pH was adjusted to 8.0. Incubation was carried out at 30°C, 200 lux light with the photoperiod 16:8 (light: dark ratio). Growth was observed at 5 days interval. Serial dilution technique and combinations of different antibiotics were used to obtain pure culture.

Studies on Morphology and Growth Conditions

Photomicrographs of the isolate were taken with a Leica bright field microscope DM2500 with camera attachment (45X magnification). The effect of NaCl (%w/v) 5.0, 10.0 and 15.0g/100 ml; pH: 8.0, 9.0 and 10.0; and temperatures 25°C, 30°C

and 40°C were studied to optimize growth conditions. Culture was incubated as described earlier for 14 days, cells were harvested by centrifugation at 6000 rpm and total wet biomass was calculated. Pigments were extracted from the cyanobacterial biomass in the solvents acetone and methanol for chlorophyll analysis. Spectra were taken using UV-Vis spectrophotometer Shimadzu 700.

FAME Analysis

Fatty Acid Methyl Esters (FAME) was prepared by direct trans-esterification method (1). Sample was treated with 1.0 ml toluene and 5% HCl in methanol and then homogenized and vortexed at low speed. Sample was kept at 70°C for 2 hours in a water bath. The solution was allowed to cool at room temperature and neutralized with 6% K_2CO_3. FAMEs were extracted with hexane, centrifuged and the supernatant was preserved (hexane extract) at 4-10^{0}C. GC analysis was carried out at SICART, Vallabh Vidhyanagar, Gujarat (GC: Perkin Elmer Auto system XL with FID detector).

Transmission Electron Microscopy (TEM)

TEM was carried out at Microscopy Lab., NCBS, Bangalore. Cells collected by centrifugation were first fixed overnight with the primary fixative glutaraldehyde (2.5%, containing 3.0g% NaCl). Post fixation was done with 1.0 g% of Osmium tetroxide. Staining was carried out with 2g% Uranyl acetate and washing with Milli Q water, respectively. The samples were dehydrated in graded ethanol series. Samples were embedded in resin 812 and polymerized at 70°C for 1-2 days. Images were captured on Tecnai G2 Spirit BioTWIN electron microscope.

Table.1 The 16S rRNA sequences from GenBank (NCBI) and their accession numbers.

Sr. No.	Organisms	Gene Bank Accession number
1	*Euhalothece* sp. **SLVH01**	**KC924847.1**
2	*Euhalothece* sp. MPI 95AH10	AJ000709.1
3	*Euhalothece* sp. MPI 95AH13	AJ000710.1
4	*Euhalothece* sp. Z-M001	EU628548.1
5	*Euhalothece* sp. BDU 130911	KM350247.1
6	*Euhalothece* sp. BDU 130192	KF498709.1
7	*Euhalothece* sp. BDU 130913	KM350246.1
8	*Euhalothece* sp. MPI 96N304	AJ000713.1
9	*Euhalothece* sp. MPI 96N303	AJ000712.1
10	*Cyanothece* sp. PCC 7418	AJ000708.1
11	*Halothece* sp. PCC 7418	NR_102451.1
12	*Halothece* sp. PCC 7418 (1)	AF296872.1
13	*Aphanothece naegelii* KCTC AG10042	AY121354.1
14	*Aphanothece naegelii* KCTC AG10041	AY121353.1
15	*Xenococcaceae* cyanobacterium CENA331	KT731151.1
16	*Cyanobacterium* GI-1	JN202625.2
17	*Chroococcidiopsis* sp. CCMP1991	JF810072.1
18	*Synechococcus* sp. HOG	AF448075.1
19	*Gloeothece* sp. KO68DGA	AB067580.1
20	Uncultured cyanobacterium clone WP3	JN122739.1
21	Uncultured cyanobacterium clone LL31B	EF106405.1

16S rRNA Sequencing and Phylogenetic Tree

The genomic DNA was isolated from the algal culture and PCR amplification was carried out using cyanobacterial 16S rRNA primers CYA359F (GGGGAATYTTCC GCAAT- GGG) and CYA781R (GACTAC TGGGGTATCTAATCCCATT) as described by Nubel Garcia-Pichel and Muyzer (14). PCR product was sequenced using ABI 3130 automated sequencer (Applied Biosystems) at Chromous Biotech Pvt. Ltd., Bangalore.

In all twenty one (21) 16S rRNA sequences (including our isolate) from GenBank, NCBI for *Euhalothece* and related genera/strains were used for phylogenetic analysis (Table 1). Neighbour-joining (N-J) method was used to construct the phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA6) software.

Results and Discussion

Sampling and Enrichment

Samples were collected from Devyani kyars (pans), Sambhar Salt Lake, Rajasthan (Fig. 1). Although soda lakes provide extreme growth conditions, our results show that it is rich in microbial diversity, especially that of haloalkaliphilic microalgae at low to moderate salt concentration. The colour of the brines/water in the kyars changed with season as it brings about physicochemical changes that allow succession of diverse microbial species.

Diversity of microalgae was examined microscopically from natural samples and the enrichment culture obtained by Winogradsky's column. The samples contained mainly oval, coccoid, spiral, filamentous and rod-shaped microalgae. An alga in tetrad form was also observed. Various shapes of bacteria were also abundant.

Fig. 1. Sample collection sites, Devyani Kyars, Sambhar Lake, India

Studies on Cultivation, Morphology and Growth

The enriched culture in BG 11 was observed after incubation at 30°C for 14 days. The contamination by bacteria especially actinomycetes was inhibited by the addition of three antibiotics (Nalidix acid 10mg/l, Trimethoprim 20 mg/l and Cycloheximide 20 mg/l). Pure culture of an oval to rod-shaped cyanobacterium was obtained by serial dilution and designated as strain SLVH01 (Fig. 3).

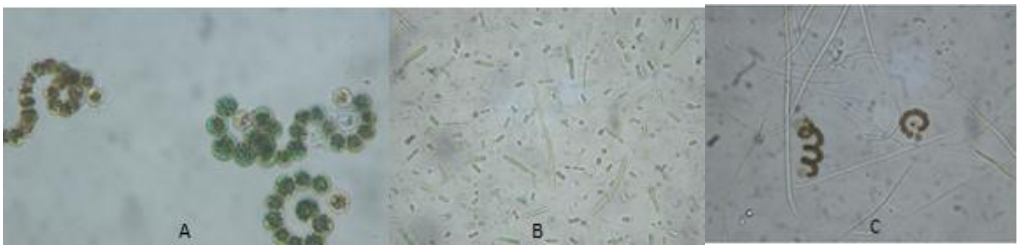

Fig. 2. Photomicrographs of microalgal species observed in the enriched culture by Winogradsky column. A. Spiral species (*Anabaenopsis sp.*) with heterocysts; B. Filamentous, coccoid and rod shaped; C. Filamentous and spiral forms.

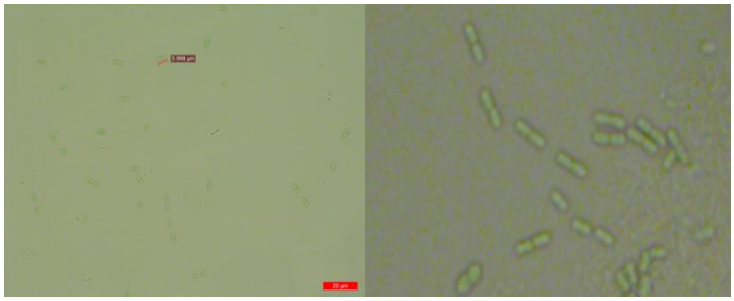

Fig. 3. Photomicrograph of *Euhalothece sp.*SLVH01

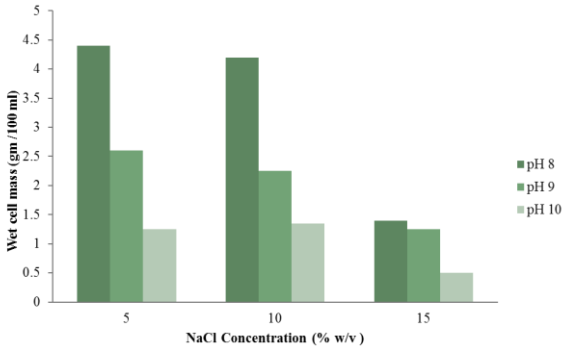

Fig. 4. Growth of *Euhalothece* SLVH01 at different salt and pH.

Table.2 Qualitative FAMEs analysis by GC of hexane extract of SLVH01 strain.

Sr.No.	Fatty Acid	% content
1	Lauric Acid (12:0)	4.68
2	Myristic Acid (14:0)	2.97
3	Palmitic Acid (16:0)	8.53
4	Palmitoleic Acid (16:1)	-
5	Stearic Acid (18:0)	-
6	Oleic Acid (18:1)	6.63
7	Linoleic Acid (18:2)	3.67
8	Linolenic Acid (18:3)	-
9	Arachidic Acid (20:0)	5.32
10	Erucic Acid (22:1)	8.02
11	Unknown fatty acids	60.18

The microalgal strain grew optimally at 5%w/v, NaCl and pH 8.0 at temperature 30^0C (Fig. 4). The strain grew well upto 10%w/v NaCl and pH 8.0, however further increase in NaCl and pH had adverse effect on its growth. No growth was obtained in absence of NaCl and pH 7.0.

FAME Analysis

This is the first report on the fatty acid composition of a *Euhalothece* strain. Table 2 indicates higher content of saturated fatty acids as compared to unsaturated fatty acids. The major fatty acids present were palmitic

acid, oleic acid and erucic acid. However, it was surprising to note that in this preliminary study about 60% of the fatty acids were unknown. It contains the essential fatty acid linoleic acid (Omega 6 fatty acid) amount (3.63%).

TEM Studies

The cells of SLVH01 were prokaryotic, oval or rod-shaped (2.0 to 5.0-6.0 µm). There is a single outer membrane structure and cell-wall was not observed. It shows lengthwise pattern of thylakoids in fascicles. The development of a daughter cell attached with parental cell was observed. Intracellular carboxysome granules and osmiophylic eyespot globules were present (Fig. 5). Osmiophylic eyespot globules have been reported in the apical cells of *Leptolyngbya* spp. (2).

Phylogenetic Analysis

The 16S rRNA was amplified and sequencing data of the 770 bp product was analyzed by BLAST tool and the strain was identified and designated as *Euhalothece* SLVH01. Sequence was submitted to Gene Bank, accession number: KC924847.1. The phylogenetic tree revealed that *Euhalothece* SLVH01 was closely related with four strains mentioned in cluster one, as shown in Fig. 6.

Samples were collected from shallow solar salt pans, where salinity was 5-10%, as at higher salt concentrations the microalgae lysed, providing nutrients for the growth of halophilic bacterial strains (12, 18). We were successful in enrichment of phototrophs by Winogradsky Column (Fig. 2). After 6-8 months there was an increase in the algal community mainly cyanobacteria as also reported by Mikhodyuk et. al. (13). The column turned from red-pink to green on prolonged incubation, showing the succession of cyanobacteria. This inspired us for the isolation and identification of haloalkaliphilic microalga from the enrichment culture. Isolation of *Euhalothece species* has been reported from Lake Magadi and Eilat, Israel (10, 13).

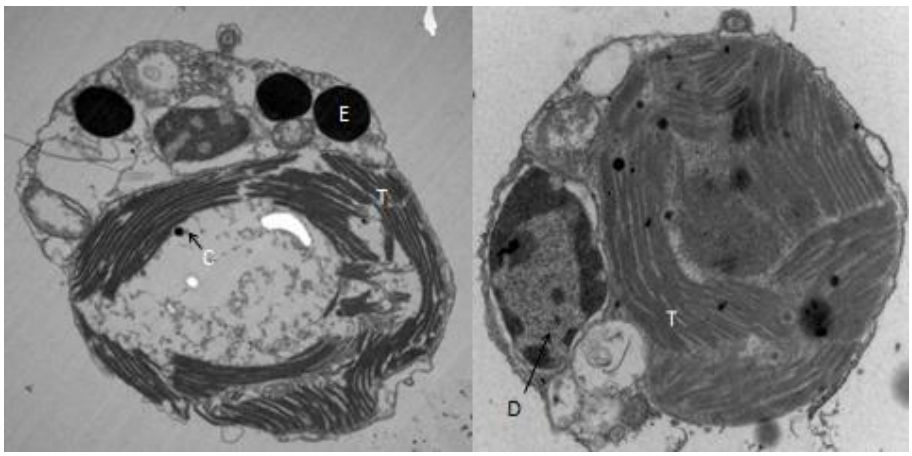

Fig.5 TEM image of SLVH01 strain (Longitudinal section). T: thylakoids pattern, D: daughter cell, C: carboxysome, E: eyespot globules

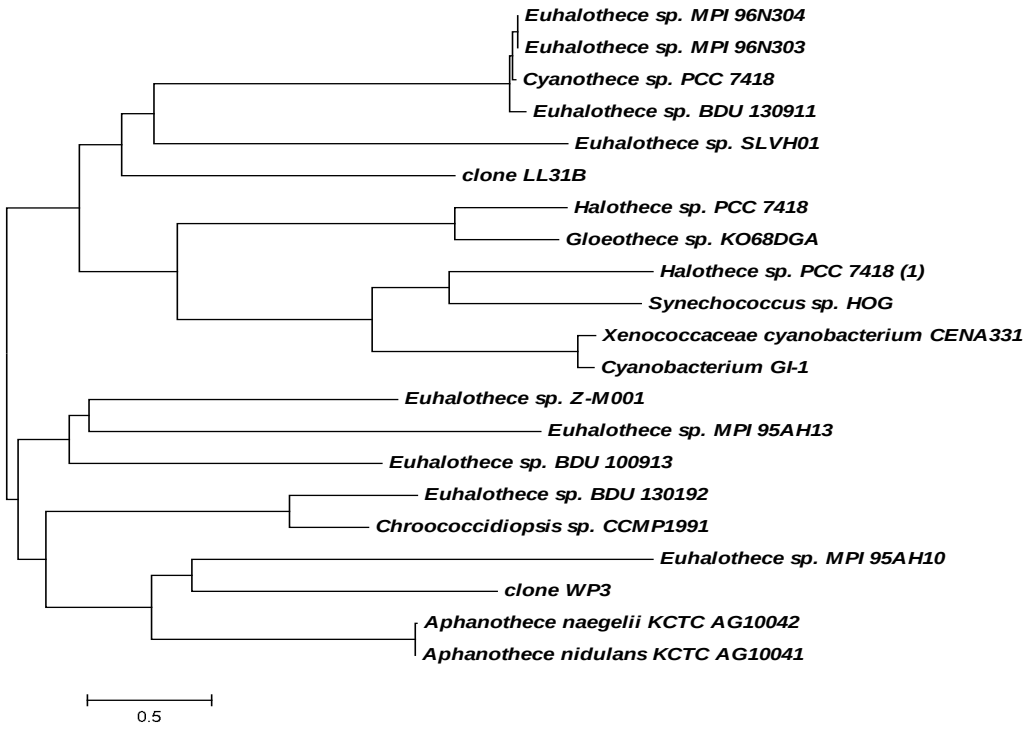

Fig. 6. Phylogenetic tree showing position of SLVH01 strain

Garcia-Pichel et al. (4) reported that the morphology of the unicellular cyanobacteria belonging to "*Euhalothece*" and "*Halothece*" was diverse and dependent on the culture conditions. Cell widths varied from 2.8 to 10.3 µm. The cells of SLVH01 strain were oval to rod-shaped, dividing, singly or pairs, size 2.0 to 5.0-6.0 µm. The cyanobacterium SLVH01 strain was moderately halophilic and alkaliphilic and grew optimally in BG 11 medium, as compared to other media used for algal cultivation. The growth of this strain in liquid medium was turbid, uniform, dark green after 10-15 days of incubation, and did not form sediment or clumps. In our attempts to isolate on solid media, no growth was observed by streaking, but the strain formed colonies within the medium in case of pour plate method. UV-Vis spectroscopy revealed the presence of Chlorophyll *a* and *b* in the strain, in the methanol and acetone extracts.

Our preliminary qualitative data of FAMEs analysis of SLVH01 strain, showed the presence of 60% unknown fatty acids content (as only 10 fatty acid standards were used), and therefore further studies need to be carried out to know the exact lipid profile. Omega 6-linoleic acid content was less.

Most of the cyanobacteria produce mucilages sheath, which accumulate around the cells or trichome to form an envelope or sheath that allows the cells to aggregate and form colonies. This feature was absent in the TEM images SLVH01 strain. The cyanobacteria belonging to the *Euhalothece* group 1 possess parallel, lengthwise arranged thylakoids through the whole cell volume with distinct or indistinct fascicles (3).

The phylogenetic analysis with 20 related cultures resulted in their grouping into four (4) clusters as follows: (I) *Euhalothece sp.*

MPI 96N304, *Euhalothece sp.* MPI 96N303, *Euhalothece sp.* BDU 130911, *Euhalothece sp.*SLVH01 and *Cyanothece sp.* PCC 7418; (II) *Halothece sp.* PCC 7418 and *Gloeothece sp.* KO68DGA; *Halothece sp.* PCC 7418 (1), *Synechococcus sp.* HOG, *Xenococcaceae* cyanobacterium CENA 331 and *Cyanobcterium* GI-1; (III) *Euhalothece sp.* Z-M001, *Euhalothece sp.* MPI 95AH13 and *Euhalothece sp.* BDU 100913; (IV) *Euhalothece sp.* BDU 130192, *Chroococcidiopsis sp.* CCMP1991, *Euhalothece sp.* MPI95AH10, Clone WP3, *Alphanothece naegelii* KCTC AG10042 and *Alphanothece nidulans* KCTC AG10041. Clone LLB31 and *Euhalothece sp.* BDU 100913 appeared as individual entity in cluster one and cluster three respectively. In the Bergey's Manual of Systematic Bacteriology (vol. 1, 2001), the phylogenetic trees included on the basis of cyanobacterial 16S rRNA sequences have a "fan-like" appearance that shows the evolution of oxygenic photosynthesis during a brief period. Based on the distance trees constructed by Neighbour-joining method and Maximum Likelihood method the cyanobacteria have been classified into fourteen (14) clusters (21). Accordingly, the strains belonging to the genus *Euhalothece* belong to the Cluster VII. The halotolerant unicellular strains**.** They are moderately thermophilic and mainly found in marine and euryhaline habitats. The cyanobacterium isolate SLVH01 belongs to the Form-genus V. Cyanothece, Cluster 3, as it grows best at NaCl concentration greater than 5.0 %w/v (slight growth at 15%w/v), however it differs from the two strains PCC 7418 and PCC 9711 isolated from solar evaporation ponds (4,5) as they are moderately alkaliphilic growing well at pH between 8-10.0. A haloalkaliphilic *Euhalothece* strain Z-M001, has been isolated from the saline and alkaline water samples from Lake Magadii (13). However, our strain SLVH01 and Z-M001 belong to different clusters (Fig. 6).

Mycosporin- like amino acids (MAAs) has been reported mainly in macro and microorganisms of marine environments. MAAs absorbs UV light and thus it protects the cell from UV radiation. Mycosporine-2-Glycine and 2-(E)-3-(E)-2,3-dihydroxyprop-1-enylimino-mycosporine-alanine has been reported from *Euhalothece sp.* isolated from hypersaline saltern pond in Eilat, Israel (10,19). MAA was not detected in our isolate (unpublished results).

This is the first report of isolation and characterization of a haloalakliphilic cyanobacterium belonging to the genus *Euhalothece* from the saline waters of Sambhar Lake, India.

Acknowledgement

We are grateful to the M. G. Science Institute and Ahmedabad Education Society for providing lab facilities. We acknowledge the support of DBT grants under Star College Scheme; for TEM at C-CAMP; and Institute of Life Sciences (Ahmedabad University) for photomicrography.

References

1. Alves, S. P., Carbita, A. R. J., Fonseca, A. J. M. & Bessa, R. J. B. 2008. Improved method for analysis in herbage based on direct transesterification followed by solid-phase extraction. J. Chrom. 1209:212-219.
2. Barsanti, L. & Gualtieri, P. 2014. Algae: Anatomy, Biochemistry and Biotechnology, 2nd ed. CRC press, FL, USA, 51 pp.
3. Cepak, V. & Komarek, J. 2010. Cytomorphology of six halotolerant

coccoid cyanobacteria using DAPI fluorescent and transmission electron microscopy, compared with molecular data. Fottea 10(2):229–234.
4. Garcia-Pichel, F., Nubel, U. & Muyzer, G. 1998. The phylogeny of unicellular, extremely halotolerant cyanobacteria. Arch. Microbial. 169:469-482.
5. Garlick, S., Oren, A. & Pedan, E. 1977. Occurrence of facultative anoxygenic photosynthesis among filamentous and unicellular cyanobacteria. J. Bacteriol. 129:623-629.
6. Grant, W. D. & Tindall, B. J. 1986. The Alkaline saline environments. In Herbert, R. A. & Codd, G. A. (eds.) Microbes in extreme environments. Academic Press, London, pp. 25-54.
7. Grant, W., Jones, B., Duckworth, A. & Owenson, G. 1998. Microbial diversity of soda lakes. Extremophiles 2:191-200.
8. Imhoff, J. F. & Trüper, H. G. 1977. Ectothiorhodospira halochloris sp. nov., a new extremely halophilic phototrophic bacterium containing bacteriochlorophyll b. Arch. Microbiol. 130:238-242.
9. Jakher, G., Bhargava, S. & Sinha, R. 1990. Comparative limnology of Sambhar and Didwana lakes (Rajasthan, NW India) Hydrobiologia 197 (1):245-256.
10. Kedar, L., Kashman, Y. & Oren, A. 2002. Mycosporine-2-glycine is the major mycosporine-like amino acid in a unicellular cyanobacterium (Euhalothece sp.) isolated from a gypsum crust in a hypersaline saltern pond. FEMS Microbiol. Letters 208:233–237.
11. Krienitz, L. & Kotut, K. 2010. Fluctuating Algal food populations and the occurrence of lesser flamingos (Phoeniconaias minor) in three Kenyan Rift Valley Lakes. J. Phycol. 46:1088-1096.
12. Kumar, S. 2008. Conservation of Sambhar Lake- An important waterfowl habitat and a Ramsar Site in India. Proceedings of TAAL2007-The 12th World Lake Conference. 1509-1517.
13. Mikhodyuk, O. S., Gerasimenko, L. M., Akimov, V. N., Ivanovsky R. N., & Zavarzin, G. A., 2008. Ecophysiology and polymorphism of the unicellular extremely natronophilic cyanobacterium Euhalothece sp. Z-M001 from Lake Magadi. Microbiol. 77:717-725.
14. Nubel, U., Garcia-Pichel, F. & Muyzer, G. 1997. PCR Primers to amplify 16S rRNA genes from cyanobacteria. Appl. envi. microbial. 63:3327–3332.
15. Sharma, P., Agarwal, V., Mohan, M. K., Kachhwaha, S., Kothari S. L. 2012. Isolation and Characterization of Dunaliella Species from Sambhar Lake (India) and its Phylogenetic Position in the Genus Dunaliella Using 18S rDNA, Natl. Acad. Sci. Lett. 35:207-213.
16. Tindall, B. J., Ross, H. N. M. & Grant, W. D. 1984. Natronobacerium, gen. nov. and Natronococcus, gen. nov., two new genera of haloalkaliphilic archeobacteria. Syst. Appl. Microbiol. 5:41-57.
17. Upasani, V. & Desai, S. 1990. Sambhar Salt Lake: chemical composition of brines and studies on haloalkaliphilic archeobacteria. Arch. Microbiol. 154:589-593.
18. Upasani, V. N. 2008. Microbiological studies on Sambhar Lake (Salt of Earth) Rajasthan, India. In Proceedings of TAAL2007-The 12th World Lake Conference. 448-450.
19. Volkmann, M., Gorbushina, A., Kedar, L. & Oren, A. 2006. Structure of euhalothece-362, a novel red-shifted

mycosporine-like amino acid, from a halophilic cyanobacterium (Euhalothece sp.). FEMS Microbiol Letters 258:50–54.

20. Wilmotte, A., Turner, S., Peer, Y. Van de, & Pace, N. R. 1992. Taxonomic study of marine oscillatoriacean strains (Cyanobacteria) with narrow trichomes: II. Nucleotide sequence analysis of the 16S ribosomal RNA. J. Phycol. 28: 828-838.
21. Wilmotte, A. & Herman, M. 2001. Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In Boone, D. R. & Castenholz, (eds.) Bergey's Manual of Systematic Bacteriology. 2nd ed., vol. I Springer, New York, 487-493 pp.

How to cite this article:

Harshil H. Bhatt, Renu Pasricha and Vivek N. Upasani. 2016. Isolation and Characterization of a Halophilic Cyanobacterium Euhalothece SLVH01 from Sambhar Salt Lake, India. *Int.J.Curr.Microbiol.App.Sci.*5(2): 215-224. doi: http://dx.doi.org/10.20546/ijcmas.2016.502.024