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First record of *Halosarcinochlamys* sp. in India: Isolation, characterization, and phylogenetic analysis of a green hypersaline microalga from Mumbai saltpans

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ABSTRACT

The hypersaline saltpans of Mumbai, India have extreme environmental conditions which house very specific, yet unexplored diversity of microalgae. In the present study, we report the first record of *Halosarcinochlamys sp.* from the Indian subcontinent. The identification of the same was carried out based on the ultrastructural features and molecular phylogeny using 18S rRNA gene sequences. The microalga has minute, cocci shaped, motile cells with smooth surface and intracellular lipid globules; which was revealed through Scanning and Transmission Electron Microscopy (SEM/TEM). Molecular phylogeny analysis further placed the isolate in *Halosarcinochlamys* with considerable bootstrap values. The maximum parsimony tree showed that this strain of *Halosarcinochlamys* grouped with *H. cherokeensis*. One of the important outcomes of this study, is that the isolated strain can be used for mass scale cultivation in arid, semi-arid regions as it can withstand extreme salinity and can be utilised for potential biodiesel production because of its intracellular lipid reserves. The study highlights the importance of exploring saltpans and other halotolerant species for bioprospecting.

Keywords: *Chlamydomonadaceae*, Extremophiles, Solar salterns.

1. INTRODUCTION

One of the most hostile ecosystems on Earth is the hypersaline saltpan. The water in the saltpan often faces remarkable changes in salinity, which can sometimes exceed 400 PSU (Gawas & Kerkar, 2023). These habitats harbor many extremophilic microorganisms that have physiological adaptations for coping with high-salinity and high-temperature stress (Casero *et al*., 2021). These halophilic microalgae have been of special interest to scientists since they have a lot of potential for application in biotechnology (Oren, 2008). They can accumulate bioactive secondary metabolites, e.g., neutral lipids, that can be utilized as alternative feedstock for biodiesel production, and carotenoids, which have nutraceutical value (Navarro *et al*., 2023; Roy *et al*., 2023).

Recent studies focusing on identifying different microalgal species have been

conducted in parts of coastal Visakhapatnam, the Bay of Bengal, and other coastal areas of India (Borowitzka & Borowitzka, 1988; Jeyabalan *et al*., 2023). In spite of these studies, there is still a huge need to document hypersaline microhabitats in the country. There have been a few studies on some freshwater habitats (Lloyd *et al*., 2021) and a few marine habitats (Rashmi & Kejariwal, 2021) in Mumbai city, but the saltpan habitat remains mostly unexplored. Some of the most well-studied genera in these habitats are *Dunaliella* (Kim *et al*., 2022) and *Arthrospira* (Singh *et al*., 2023). However, combining data from light and electron microscopy with 18S rRNA gene sequences has revealed that there exists a lot of hidden diversity among rare and new species in hypersaline ecosystems and arid regions all around the world (Darienko *et al*., 2019; Salehipour-Bavarsad *et al*., 2023).

Among these less-studied organisms is *Halosarcinochlamys* from the Chlorophyceae class, which so far has been very rarely reported globally. The available sequences in public databases for this genus are limited to a few species (Tragin, 2017). The small, coccoid *Halosarcinochlamys* species have simple morphology that makes them hard to identify without the aid of molecular phylogeny. These microorganisms are a rare group that can serve as an important baseline for ecological monitoring. Ecologically, *Halosarcinochlamys sp*. acts as a primary producer in hypersaline habitats. It contributes to local biological productivity and serves as an important energy source for the organisms higher in the trophic web. They may also have industrial value and can be isolated from tropical hypersaline environments before future environmental changes affect these ecosystems (Eckmann *et al*., 2023). Studying these species may also help in understanding the ecology and diversity of halophilic microorganisms and lead to the discovery of new industrially relevant species (Paul & Mormile, 2017).

In the present study, a species of *Halosarcinochlamys* is reported for the first time from India. It was isolated from the hypersaline environment of Mumbai saltpans. Molecular phylogenetic studies on the isolate were carried out using the maximum parsimony method on the 18S rRNA gene sequence. The isolation and morphological studies of this hypersaline strain have added information about the geographic distribution of this genus. It also suggests the potential use of this species for biofuel production.

Figure 1. Environment surrounding the Mulund salt pan: an image acquired using Google Earth.

2. MATERIALS AND METHODS

Collection and Enrichment of Sample: Water samples were manually collected from Mulund saltpans (19°09'27.6" N 72°57'37.2" E) (Figure 1) and filtered through a 200 µm mesh to remove zooplankton and debris. The filtrate was enriched in 15 L fiberglass tanks containing F/2 medium and acclimatized to laboratory conditions for 3–4 weeks.

Isolation of Microalga*:* The microalga that was dominant in the lab-established culture was isolated using the serial dilution and the agar plating method. Serial dilution was done by aseptically diluting the culture ten-folds (10^{-1}–10^{-10}) (Fernandez-Valenzuela et al., 2021) and then incubating them under 14:10 h light–dark cycle for 2–4 weeks, until growth appeared in higher dilutions (10^{-5}–10^{-10}) (Lavens & Sorgeloos, 1996). After the algal growth was observed, the aliquots from these dilutions were streaked on 1% agar plates prepared in 150 ppt seawater supplemented with F/2 medium. These plates were kept inverted over a light source for up to 21 days. After that the distinct green colonies were examined under a stereomicroscope, picked up and were inoculated into 20ml sterile F/2 media (150 ppt).

Culture Establishment and Maintenance: The microalga that was grown in 20ml media was further upscaled to 2L culture bottles gradually with continuous mild aeration. Cultures were maintained at 150 ppt salinity in F/2 media under 4000 lux white fluorescent

light with a 14:10 h light-dark cycle (Lavens & Sorgeloos, 1996). Sub-culturing was done every three weeks, and they were monitored routinely by microscopic examination to ensure unialgal purity.

Preparation of Culture Media*:* The microalgal cultures were maintained in artificial seawater (150 ppt), prepared by dissolving 150g sea salt in minimum amount of Milli-Q water and then topping up to a final volume of 1 Litre. Salinity was verified each time using a salinometer. F/2 medium was prepared using the standard method and was autoclaved before being added to the prepared seawater (Guillard *et al*., 1962).

Identification of Microalga: We identified the isolated microalga using microscopy and molecular phylogeny.

Microscopy*:* Three types of microscopy were used to identify the isolated microalga.

1. Compound microscopy: Cell morphology was examined at 400x and 1000x magnification using a Kyowa Getner binocular microscope.
2. Scanning Electron Microscopy (SEM): Scanning Electron Microscopy (SEM) was performed using a Quanta 200 ESEM. The cultured cells were sampled and mounted on double-sided carbon tape coated on aluminum stubs. These cells were then dried at room temperature and loaded into the SEM. SEM was performed in low vacuum without any pre-treatment of the sample, using an FEI QUNATA 200 SEM with EDS (Product version 3.0.13) (Arora *et al*., 2013).
3. Transmission Electron Microscopy (TEM): The microalga cells were pelleted and then fixed by rinsing several times in 2% glutaraldehyde in seawater containing 0.1 M cacodylate buffer (pH 7.0). The cells were then fixed overnight in 1% cold osmium tetroxide, rinsed in 0.1 M cacodylate buffer for 10 min, and the dehydrated by passing through ethanol series (30 mins each in 50 and 70% ethanol, followed by 100% ethanol for 1 hour at room temperature). Following dehydration, cells were impregnated using resin infiltration. First, the cells were washed with propylene oxide twice for 10 minutes each. Epon resin was later used with propylene oxide in a ratio of 1:1 for 30 minutes, followed by 1:3 ratio for 30 minutes. The embedding medium was then replaced with fresh 100% resin at room temperature, and the cells were transferred 5 hours later to an embedding dish for polymerization at 60 °C overnight. Sections were cut with a diamond knife which was mounted on an RMC MT-XL ultramicrotome. The sections were stretched with chloroform to eliminate compression and mounted on copper grids. The sections were then further analyzed with a transmission electron microscope, Model JEOL JEM-1010 (Arora *et al*., 2013).

Molecular Phylogeny

Molecular identification was performed by amplifying the partial 18S rRNA gene using primers 18S13 and NS3, followed by maximum parsimony analysis.

1. *DNA extraction and quantification:* Genomic DNA was extracted using the HiPurA Bacterial Genomic DNA Purification Kit (MB505) and quantified with a Shimadzu UV-1800 spectrophotometer. DNA was stored at −20°C for further use.
2. *PCR amplification***:** PCR was carried out in a 50 µL reaction using 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase in a Bio-Rad T-Personal 48 thermal cycler. Thermocycling for the 18s13/NS3 primers included an initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 35 sec, and 72°C for 1.5 min, with a final extension at 72 °C for 5-7 min. PCR reagents and primers were sourced from Bangalore GeNei and BioServe, respectively. The resulting PCR products were resolved on a 1% agarose gel (SeaKem). The PCR product was purified using the PureLink Quick Gel Extraction Kit (K2100-12) and sequenced on an Applied Biosystems 3730xl DNA Analyzer using primer 18S13. Sequences were analyzed using BLASTn at the National Centre for Biotechnology Information, and a phylogenetic tree was constructed with eleven sequences using MEGA6.
3. Maximum Parsimony Analysis: Evolutionary relationships were inferred using the Maximum Parsimony method with 1000 bootstrap replicates. Branches supported by <50% bootstrap values were collapsed, and support values were indicated on the nodes. The tree was generated using the Close-Neighbor-Interchange algorithm with search level 1 and random addition of sequences (10 replicates). The analysis included 11 nucleotide sequences, with all gap and missing positions removed, yielding 392 final positions. Phylogenetic analysis was performed using MEGA6 (Felsenstein *et al*., 1985).

3. RESULTS & DISCUSSION

Morphology of microalga: The morphology of the isolated strain was observed under light microscopy (Figure 2). The microalga cells were spherical, with a diameter of 2- 5 µm (Figure 2a), and were motile, having two isokont flagella. The cells showed distinct green coloration. Some cells formed chains, where individual cells within the short chains maintained the same diameter, with overall chain

lengths extending from 6 to 10 μm, depending on the number of linked cells (Figure 2b). Some cells were observed undergoing division and containing 3-6 daughter cells within the mother cell (Figure 2c). It has been studied that some species of Chlamydomonas have a distinct mode of cell division (asexual cycle), wherein they form palmelloid colonies of adherent, non-motile cells in nature and in culture conditions (Hanschen *et al*., 2017). Palmelloid colony formation of this type was observed during light microscopic examination of the microalga in the present study.

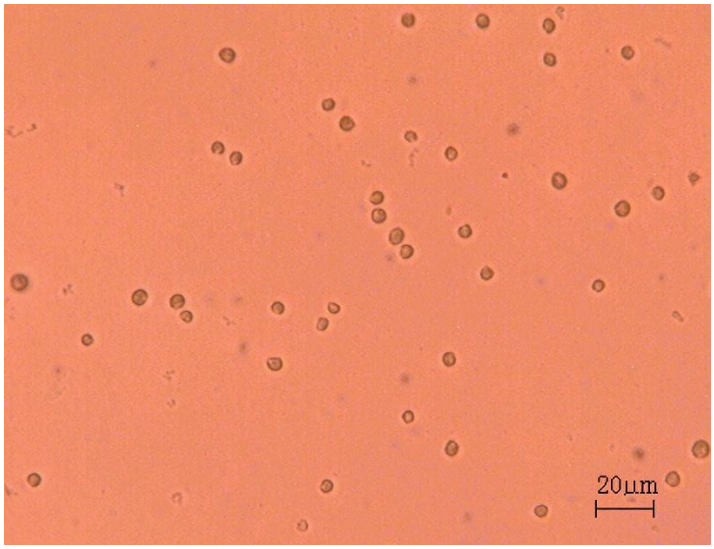

Figure 2. Light microscopy images of isolated microalga. **2a:** Image of microalga showing cocci shaped distinct green cells under imaging microscope. The scale towards right indicates a scale bar of 20µm.

Figure 2b. Compound microscope image of microalga at 40X magnification. The red circle indicates chain formation of the microalga cells.

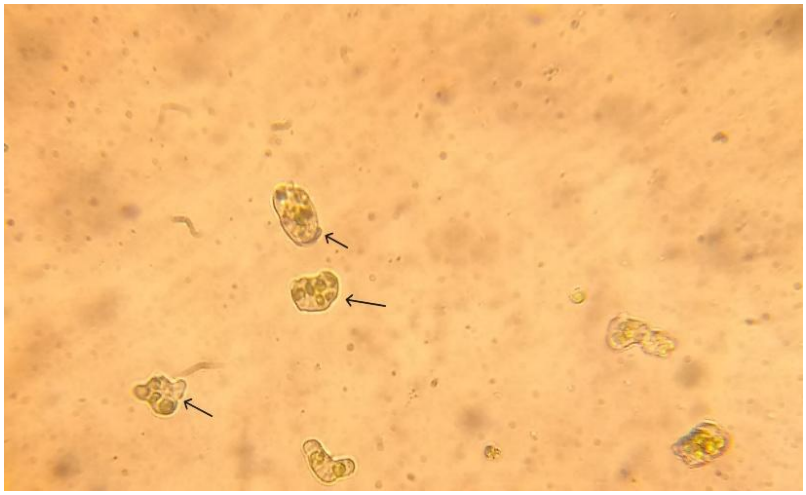

Figure 2c. Microalga showing palmelloid colony formation. Arrows indicate 3-6 non-motile daughter cells present in the mother cell wall.

The morphological characteristics, for the isolated strain of microalga included, the presence of two flagella at the apical tip. The Scanning electron microscopic image (SEM) of the microalga showed that the size of the microalga varied from 2- 5 microns (Figure 3 a, b, and c). Most of the cells were spherical in shape; however, some of the cells appeared to be elongated (Figure 3c). It also showed the presence of a flagellar pit at the apical region of the microalgal cell (Figure 3d). The transmission electron microscopic images (TEM) revealed the presence of cell organelles like a nucleus (N), pyrenoid (P), chloroplast (Ch), starch granules (S) and lipid body (LB) and a distinct flagellar pit (FP) (Figure 4a). From these findings, it could be concluded that the present microalga was a eukaryotic green alga with flagella. Green algae are characterized by several distinct features, many of which are also shared with the land plants (Hoek *et al.*, 1995; Graham *et al.*, 2009). The chloroplasts are enclosed by a double membrane with thylakoids grouped in lamellae and contain chlorophyll *a* and *b* along with a set of accessory pigments such as carotenes and xanthophylls (Leliaert *et al.*, 2012). This microalga showed chloroplasts with distinct thylakoid membranes, seen as stacks in the transmission electron micrograph (Figure 4a). Pyrenoids, when present in green algae, are embedded within the chloroplast and are surrounded by starch, which is the main reserve polysaccharide (Leliaert *et al.*, 2012). The microalga in the present study had a dark pyrenoid at the periphery of the cell accompanied by a chloroplast at its side (Figure 4a). The microscopic analysis of live cells of the isolated microalga showed the presence of two flagella of equal length.

In most green algae, the cells are flagellate and isokont, although they may differ in length. They have firm cell walls with a fiber matrix generally composed of cellulose (Leliaert *et al*., 2012). The general morphological features of the microalga, including two equal-length flagella, a pyrenoid, a spherical structure, and a nucleus, indicated that it belonged to Domain: Eukaryota, Kingdom: Viridiplantae, Division: Chlorophyta, Class: Chlorophyceae, and Order: Chlamydomonadales (cells with two equal-length flagella). The Order Chlamydomonadales is further divided into two families: 1) Chlamydomonadaceae and 2) Dunaliellaceae. Members of the genus *Dunaliella* are unicellular, biflagellate, and wall-less green algae. *Dunaliella* has been the subject of debate regarding its phylogenetic position. Ettl *et al.* (1981) considered the absence of a typical cell wall as evidence against the inclusion of *Dunaliella* with other anteriorly biflagellate unicells that he placed in the class Chlamydophyceae.

Consequently, Ettl *et al.* (1981) separated *Dunaliella* from the walled flagellates (i.e., order Chlamydomonadales, class Chlamydophyceae) into the order Dunaliellales (class Chlorophyceae). Although Melkonian *et al.* (1990) did not adopt the broad concept of the classes Chlorophyceae and Chlamydophyceae, they did recognize the order Dunaliellales as separate from Chlamydomonadales, within the class Chlorophyceae (Melkonian, 1990). Thus, the main difference between *Dunaliella* and *Chlamydomonas* is the presence of a cell wall in the latter, which is absent in the former. Therefore, *Dunaliella* is morphologically similar to *Chlamydomonas*, with the main difference being the absence of a cell wall in *Dunaliella.* A cell wall was observed in the isolated microalga, as seen in the transmission electron micrograph (Figures 4a and 4b). Other than these characteristic features, some cells were also observed in the division stage, which occurred by the formation of a furrow and a thick mucilage sheath covering the dividing daughter cells (Figure 4c). This is another characteristic feature of Chlamydomonas, where cell division occurs through palintomy rather than by binary fission; that is, cells grow to many times their original size without dividing and then undergo several rounds of

rapid cell division with little or no growth between successive divisions (Sleigh *et al*., 1989). The resulting offspring emerge from the mother cell wall and begin their lives as separate unicellular organisms. A similar cell division pattern was observed in the microalga in the present study. Thus, from the morphological analysis, it was concluded that the present microalga belonged to the Chlamydomonadaceae family.

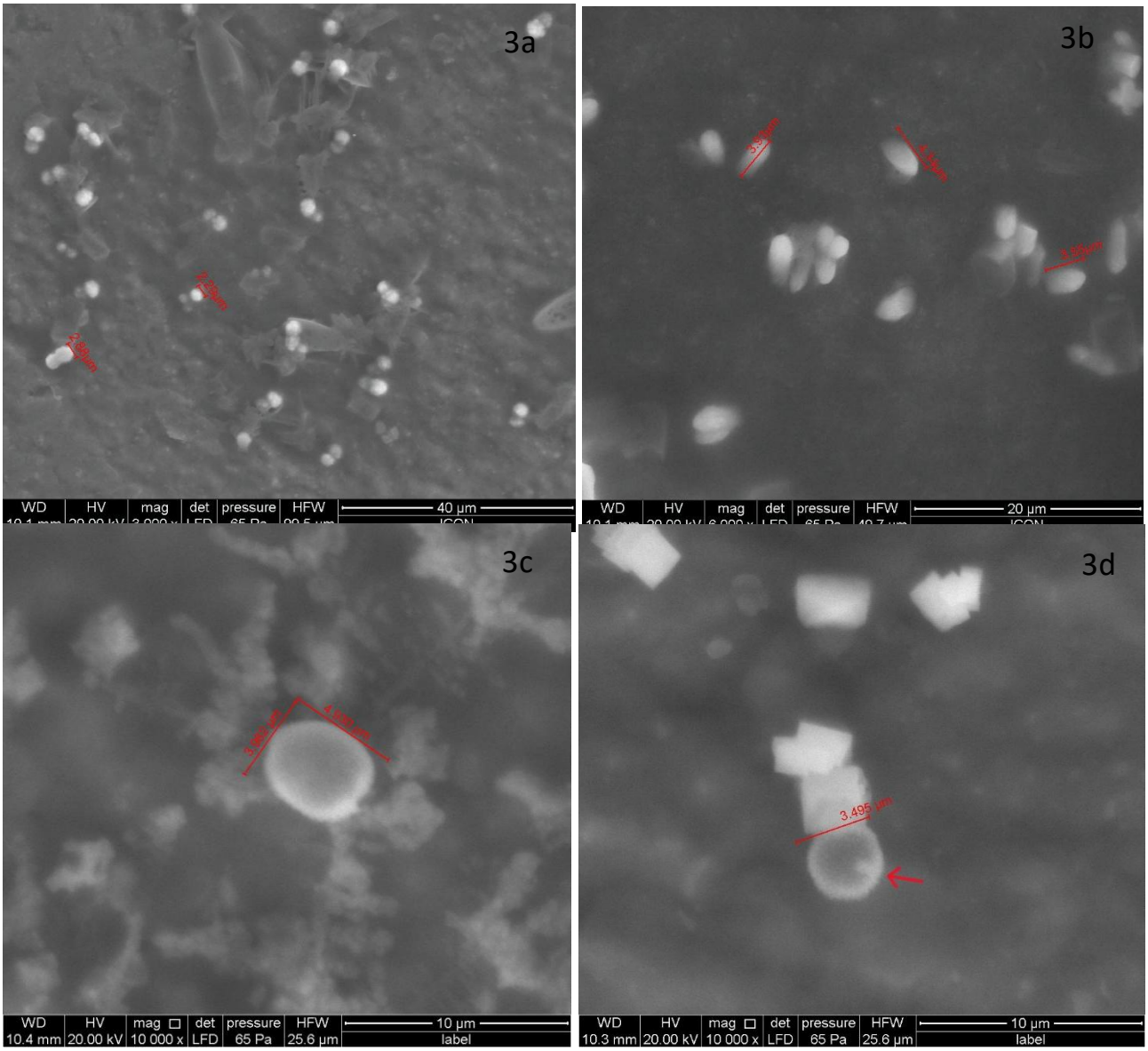

Figure 3. Scanning electron microphotographs (SEM) of microalga cells. **3 a, b and c** indicating cells size and shape of the microalga cells. **3 d** Microalga cell with flagellar pit, indicated by a red arrow.

The transmission electron microscopy results also revealed the presence of lipid globules in the isolated microalga (Figure 4a and 4b). Accumulation of intracellular starch or lipid bodies during high-stress conditions is a well-known trait of certain microalgae, especially the ones which are found in extreme environments (Shi *et al*., 2020; Varshney *et al*., 2014). In a recent study, similar adaptation was found in *Valeriella persica* which is a coccoid green alga inhabiting hyper-arid soil. It was found to accumulate dense lipid globules for surviving in the stress conditions (Salehipour-Bavarsad *et al*., 2023).

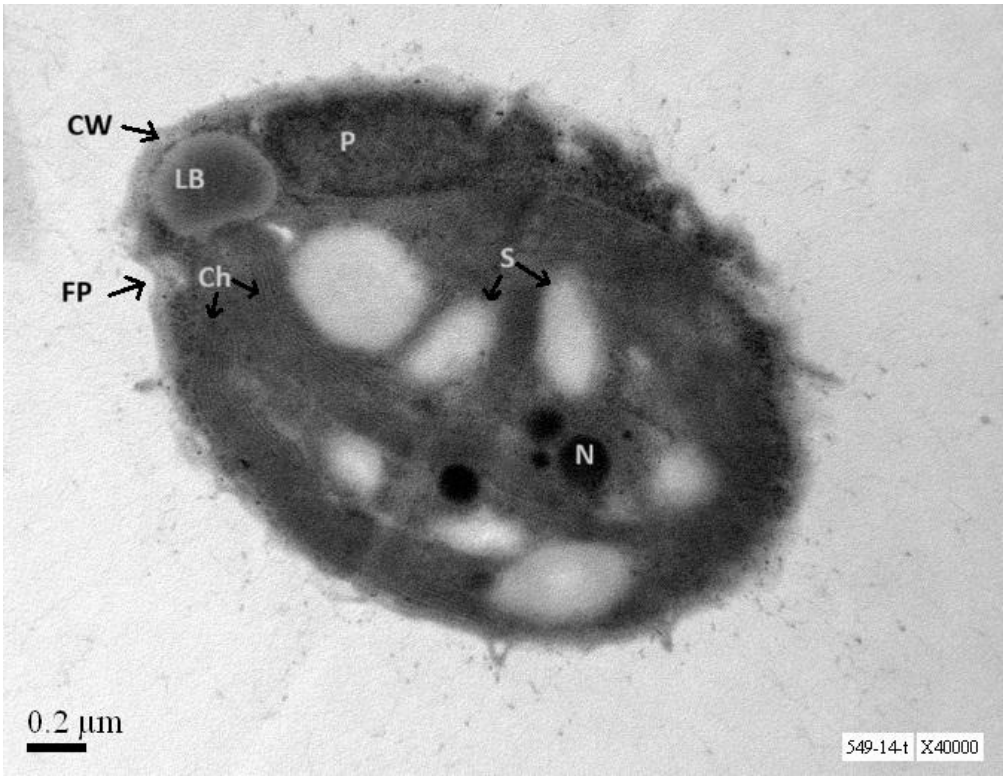

Figure 4a. Transmission electron microphotograph of the microalga whole cell, Bar= 0.2 µm (Abbreviations: Ch= chloroplast, LB= Lipid body, S= starch granules, P= pyrenoid, N= nucleus, FP= flagellar pit, CW= cell wall)

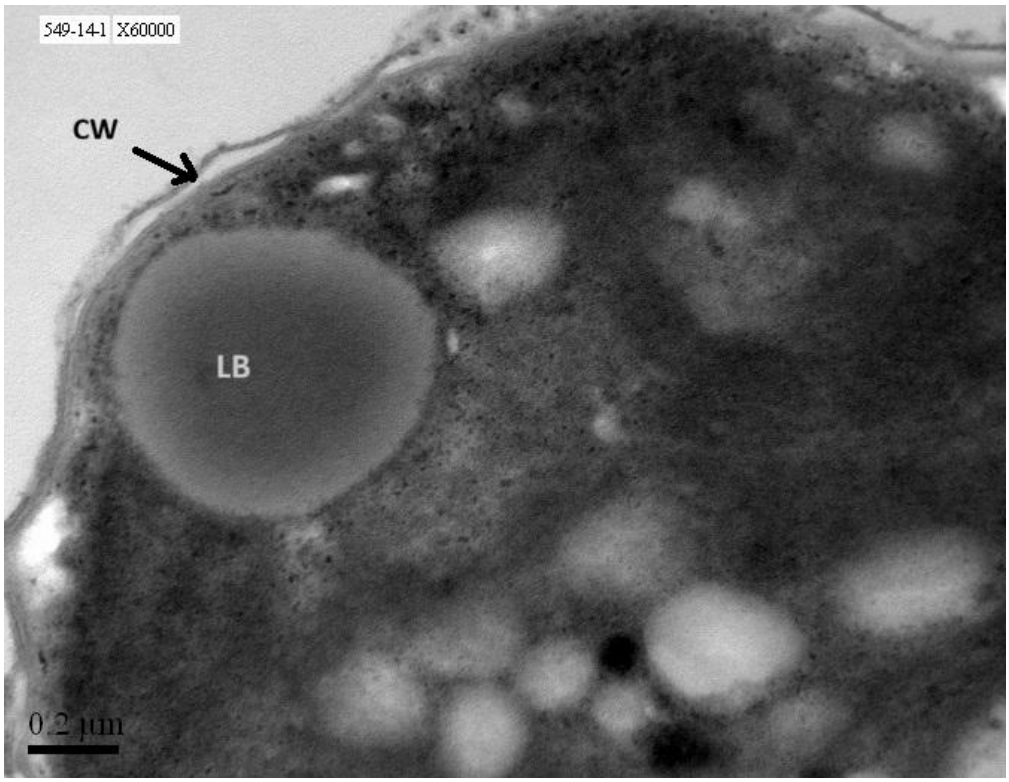

Figure 4b. Transmission electron microphotograph of microalga cell showing CW=cell wall and LB= Lipid body Bar= 0.2 µm

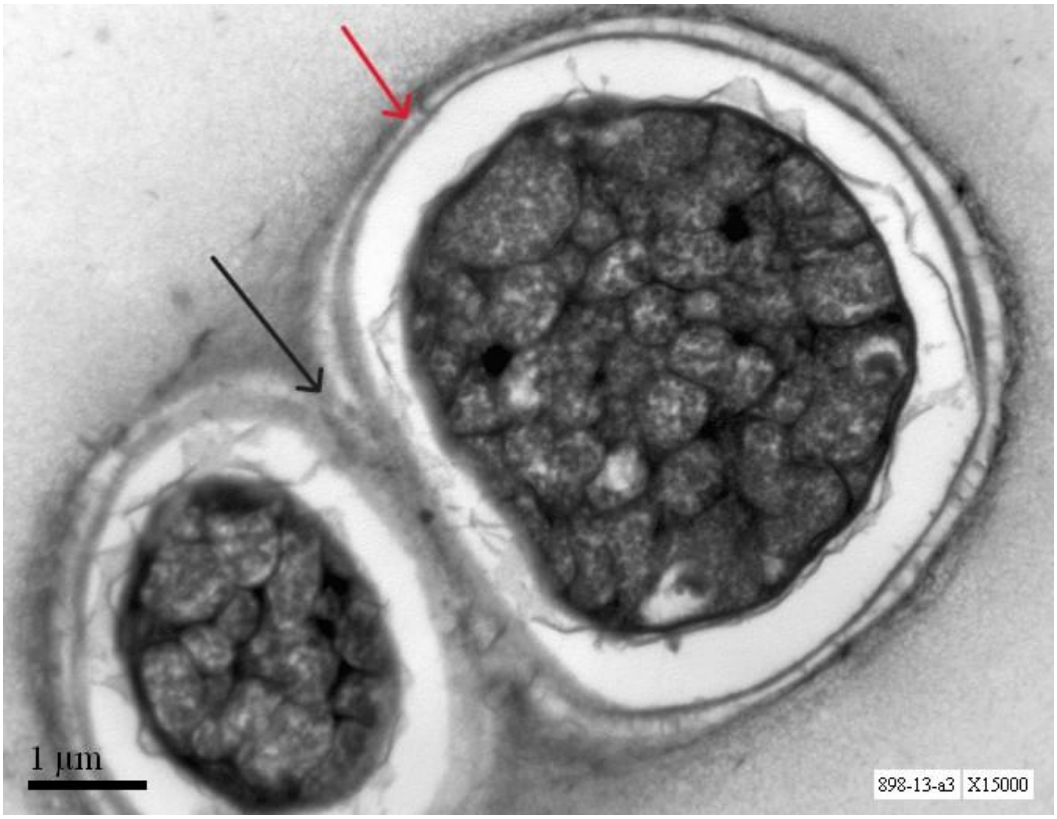

Figure 4c. Transmission electron microphotograph of Microalga showing cell division, where black arrow indicated furrow formation and red arrow indicates mucilage covering. Bar= 1 µm

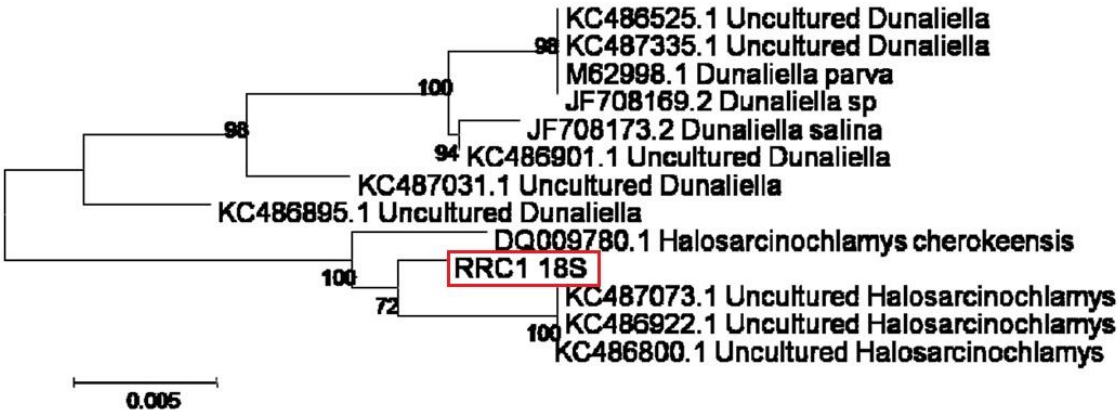

Figure 5. Phylogenetic tree of isolated microalga (labelled here as RRC1 18S, indicated by red outline) and closely related species based on 18s rRNA. The tree was constructed using the Maximum Parsimony method. The numbers at nodes indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees for values greater than 50%.

Sequence and phylogenetic analysis

The taxonomic identity of the Mumbai isolate was confirmed through a polyphasic approach, mainly by 18S rRNA gene sequencing. Phylogenetic analysis was determined using the maximum parsimony method in MEGA6, and the resulting tree was constructed using the neighbor-joining algorithm. It placed the isolated microalga within the *Halosarcinochlamys* clade, showing the closest evolutionary

affinity to *Halosarcinochlamys cherokeensis*. Following DNA extraction, purification, and sequencing, a 1032 bp sequence was obtained and designated as 'RRC1 18S'.

The RRC1 18S microalgal sequence in this study is deposited in the NCBI GenBank database under accession number KR871012. BLAST was applied to identify regions of similarity between biological sequences. The results showed that the microalga should indeed be assigned to the family *Chlamydomonadaceae* since it showed 99% similarity to *Halosarcinochlamys cherokeensis* strain SPMO BS 18S ribosomal RNA gene, partial sequence, uncultured and uncultured *Halosarcinochlamys* isolate LT41_c02 18S ribosomal RNA gene, partial sequence. Furthermore, it showed 96% similarity to *Dunaliella parva* (Figure 5). Dunaliella and related genera form a group of wall-less, halotolerant green flagellates that often appear closely allied to the Chlamydomonadaceae, although sequence analyses indicate they belong to distinct clades (González *et al.*, 2001). The genus *Halosarcinochlamys* is comparatively rare. It was first isolated from a 20-L carboy stored brine stock made from collected salts at the Great Salt Plains, Oklahoma, USA. One of the isolates of *Halosarcinochlamys* was also cultured by Heidelberg et al. from hypersaline Lake Tyrrell, Australia, during their study of eukaryotic microbial diversity (Heidelberg *et al.*, 2013). Another recent reference to this microalga was found in Lake Chokrak, Russia (Selivanova *et al*., 2026). This isolate dominated at high salinities ranging from 190 to 316 ppt.

We isolated the present microalga from the hypersaline environment of the Mumbai salt pans, and it grows at salinities as high as 150 ppt. Based on morphological and molecular phylogenetic analyses, we conclude that the isolated microalga belongs to the genus Halosarcinochlamys.

Considering this combination of halotolerance, distinct cell morphology, and its phylogenetic position, we establish the identity of the isolated microalga within the taxonomic framework given below:

Taxonomic treatment

Systematics

- Domain: Eukaryota
- Kingdom: Viridiplantae
- Division: Chlorophyta
- Class: Chlorophyceae
- Order: Chlamydomonadales
- Family: Chlamydomonadaceae
- Genus: *Halosarcinochlamys*

Isolate**:** *Halosarcinochlamys* sp. (RRC1 18S)

Material examined

A microalgal strain belonging to *Halosarcinochlamys sp*. was obtained from hypersaline water sampled at the Mulund saltpans in Mumbai, Maharashtra, India. The isolate was obtained by serial dilution, then agar plating, and was cultured in F/2 medium at 150 ppt salinity with a 14:10 h light: dark cycle (Lavens & Sorgeloos, 1996). The microalga was then examined using light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and molecular characterization through partial 18S rRNA gene sequencing.

Habitat and Distribution

The present microalga was found in the hypersaline habitat of Mulund Saltpans, Mumbai, Maharashtra, India (19°09'27.6" N 72°57'37.2" E). It is the first documented record of the genus *Halosarcinochlamys* from the Indian subcontinent. Microalgae from this genus have been reported previously from hypersaline habitats of North America, Australia (Heidelberg *et al.*, 2013) and Russia (Selivanova *et al*., 2026).

Morphological Description

The vegetative cells of isolated microalga were green, motile and predominantly spherical with a diameter of 2-5 μm. Two isokont flagella originated from the anterior end through a distinct flagellar pit. The microalga was also found to form short chains. During the asexual stage of life cycle the microalga showed formation of palmelloid colonies having three to six non-motile daughter cells enclosed within parental wall. The ultrastructural studies revealed a typical cellular organization with distinct nucleus, chloroplast, pyrenoid, starch granules and cell wall. Some cells also showed lipid bodies. The microalga showed multiple fission type of cell division with furrow formation and the daughter cells enclosed in a thick mucilaginous sheath.

Molecular characterization

The microalga's 18S rRNA gene was sequenced to confirm its taxonomic identity. A 1032 bp sequence was obtained and designated 'RRC1 18S'. The sequence was later deposited in NCBI GenBank with KR871012 as its accession number. BLAST and Maximum Parsimony analysis revealed 99% sequence similarity with *Halosarcinochlamys cherokeensis* and uncultured *Halosarcinochlamys* isolates.

The present study shows the first recorded occurrence of *Halosarcinochlamys* in India, thus expanding its known geographical distribution. It also highlights the importance of exploring the understudied hypersaline environments of our country.

The present study documents the first record of the hypersaline green microalga *Halosarcinochlamys* sp. in India, specifically isolated from the solar saltpans of Mumbai. This finding extends the known geographical range of this rare genus—previously recorded only at limited sites in North America, Australia (Tragin, 2017), and Russia (Selivanova, 2026)—to the tropical hypersaline ecosystems of the Indian subcontinent. The study provided definitive identification of the isolate. The morphological and ultrastructural analysis revealed cocci-shaped cells (~2 μm ± 0.5) with a flagellar pit, nucleus, prominent lipid and starch granules and a distinct cell wall. The phylogenetic analysis confirmed that the isolated microalga (RRC1 18S) belonged to the *Halosarcinochlamys* clade. Combining methods like microscopy and phylogenetic analysis becomes important for identifying some small organisms which have a relatively simple morphological structure (Darienko *et al*., 2019; Santos *et al*., 2017). In the present study both the methods proved to be essential for identification of the isolated microalga.

Furthermore, the presence of prominent lipid globules in the TEM analysis of *Halosarcinochlamys* sp. suggests its potential for biotechnological applications. Recent studies have highlighted the importance of the trade-off between growth and lipid accumulation in microalgae. Some of these microalgae can accumulate triacylgycerols (TAGs) in stress conditions (Navarro *et al*., 2023; Zhu *et al*., 2022). For example, *Halamphora coffeaeformis*, isolated from semi-arid habitat, has proven to be a better source of TAGs producer with higher lipid productivity as compared to the established soyabean oil (Navarro *et al*., 2023). The microalga examined in this study can survive in highly saline conditions, which reduces the reliance on freshwater for its maintenance and upscaling. This makes it an ideal candidate for sustainable biodiesel production. (El-Sheekh *et al*., 2024; Jeyabalan *et al*., 2023).

4. CONCLUSION

The identification of *Halosarcinochlamys* sp. from Mumbai' saltpans, in the present study, emphasizes on the need to explore such extreme habitats as they inhabit rare microalgal diversity. Future studies pertaining to the isolated microalga, should be directed towards understanding its culture conditions to increase its lipid content for biodiesel applications.

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Author contributions

Dr. Gargi Rane: Performed Collection, single cell culture preparation, standardization and maintenance of microalga, Light Microscopy, Analysis of SEM, TEM and sequencing and phylogenetic data, Study concept preparation manuscript preparation.
Dr. Madhavi Indap: Contributed to study concept preparation, manuscript preparation and critically revised the manuscript for intellectual concept.

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Conflict of Interest

The authors declare that they have no conflicts of interest, competing financial interest or personal relationship that could have influenced the work reported in this paper.

Informed consent

Not applicable.

Ethical approval & declaration

In this article, as per the algae regulations followed in the Foundation for Ecological Security, District-Anand, Gujarat, India; the authors observed the new distribution record of *Halosarcinochlamys sp.* From Mumbai, Maharashtra, India. The ethical guidelines for algae & National Biodiversity Authority Guidelines are followed in the study for observation, identification and experimentation.

Data and materials availability

All data associated with this study are present in the paper.

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