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Isolation of Halophilic Cyanobacteria from Saltpans of Eastern Suburbs of Mumbai

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ABSTRACT

Marine halophilic cyanobacteria were isolated from saltpan samples taken from Mulund salt station (19°9'24"N 72°58'4"E), Bhandup salt station (19°8'59"N 72°57'53"E) and Kanjurmarg salt station (19°7'49"N 72°56'37"E) of eastern suburbs of Mumbai, Maharashtra, India. Total of 8 species of cyanobacteria belonging to 4 families were isolated using different media viz. BG-11, Marine BG-11, ASN-III N^+, ASN-III N^- and Zarrouk's medium in order to select the medium best suited for respective strain. Isolates were further maintained as unialgal cultures. Cultural and morphological characteristics of isolates were studied and documented. Out of 8 species isolated, unicellular, non nitrogen fixing forms (3 species) were identified as *Synechocystis primigenia*, *Synechocystis salina* belonging to family Chroococcaceae, and *Chroococcidiopsis cubana* belonging to family Chroococcidiopsidaceae. Filamentous non-heterocystous forms (3 species) were identified as *Pseudanabaena limnetica* belonging to family Pseudoanabaenaceae, *Lyngbya salina* and *Oscillatoria salina* belonging to family Oscillatoriaceae whereas filamentous heterocystous forms (2 species) were identified as *Nostoc calcicola* and *Nostoc coerulum* belonging to family Nostocaceae.

INTRODUCTION

The potential of cyanobacteria for their scientific exploitation has been realized only in recent years. Marine cyanobacteria are considered to be most promising source of value added compounds for various industries. But only a few cyanobacterial strains have been well characterized or exploited commercially. Basic research is needed to identify new cyanobacterial strains of high value products (Thajuddin and Subramanian, 2005). It needs an extensive screening, which is expected to result in the discovery of better cyanobacterial strains of immense industrial interest. The systematics of cyanophycean should be based upon the information incorporated from both field studies and laboratory techniques. Various approaches have been adopted to develop efficient methods to isolate culture and purify fresh water BGA (Allen, 1952; Stanier *et. al*., 1971; Rippka, 1988; Castenholz and Waterbury, 1989; Ferris and Hirsch, 1991) as well as marine BGA (Waterbury and Willey, 1988). However, Choice of a suitable medium that would support normal growth of the organism without bringing in morphological changes is of prime importance in the study of the taxonomy of blue green algae in culture. In environment, cyanobacteria grow as a mixed population. Therefore it is essential to purify the individual type from the mixed population.

The remarkable adaptability of cyanobacteria to various habitats is well known (Hof and Fremy, 1933; Desikachary, 1959 and Fogg et al., 1973). There are reports proving that cyanobacteria could grow in salinities ranging from 0-99% (Prabhaharan, 1988). Salt pans of eastern suburbs of Mumbai are such hypersaline environments that represent a series of interconnected shallow water bodies with varying salinities. These saltpan lands fall under the high ecologically sensitive Coastal Regulation Zone-I and are protected under the Wetland (Conservation and Management) Rules 2010. However, the state government has proposed that the land may be used to build low-cost housing. Therefore, it is pertinent to make earnest attempts to study these sites for the resource potential and to protect these wetlands to conserve the biodiversity that they offer. Various cyanobacteria have been known to grow in these saltpans. However, they remained unexplored so far. Present work is first of its kind studying cyanobacterial diversity of these saltworks. In the present study for germplasm conservation, isolation, identification and further establishment of halophilic cyanobacterial species from salt pans of eastern suburbs of Mumbai, Maharashtra was carried out.

MATERIAL AND METHODS

The study sites

Salt pans of eastern suburbs of Mumbai are spread along the eight-kilometer stretch between Vikhroli and Mulund, Mumbai, Maharashtra. Algal samples were collected from three different salt stations viz., Mulund (19°9'24"N 72°58'4"E), Bhandup (19°8'59"N 72°57'53"E) and Kanjurmarg (19°7'49"N 72°56'37"E). These salt pans during high tide receive water from Thane creek which is further circulated through reservoirs, condensers and finally crystallizer pans where water is allowed to evaporate under natural sunlight to produce crude crystalline salt.

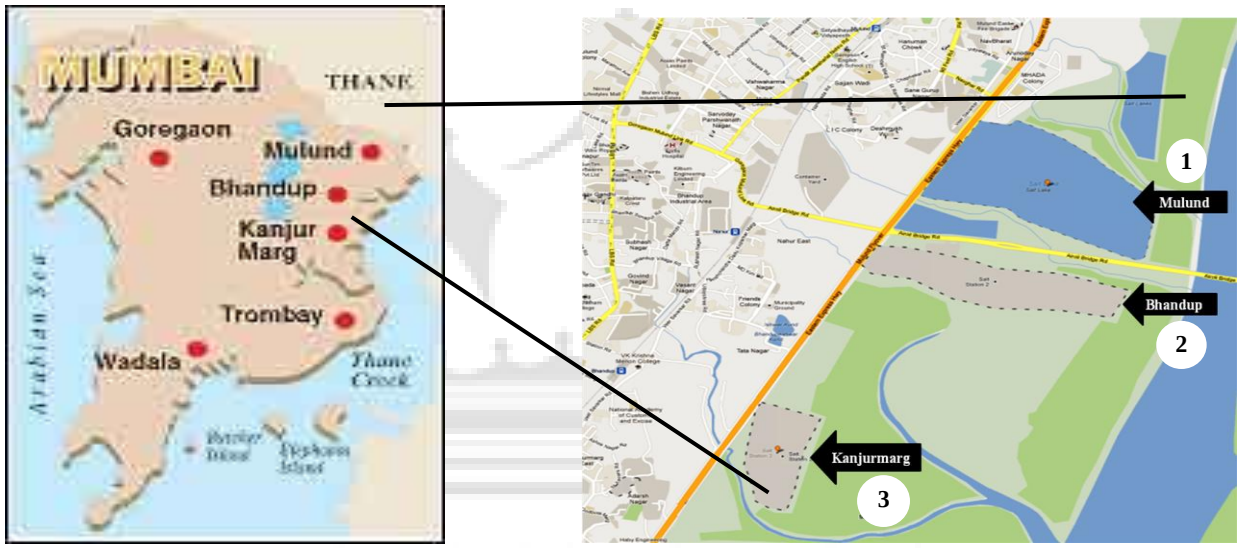

Fig. 1. Sampling sites of eastern suburbs of Mumbai (1-3):1. Salt station-1-Mulund, 2. Salt station-2-Bhandup, 3. Salt station-3- Kanjurmarg.

Collection and processing of samples

The water samples and cyanobacterial mat samples were collected *in situ* in disposable zip lock polyethylene bags and/or screw cap bottles. Mat samples were washed carefully with sterile distilled water in order to remove soil and/or mud attached to the mats. Site of the collection, salinity, temperature and pH of salt pan water were measured *in situ* and recorded. A small amount of algal material from the mat was picked up and transferred to sterile capped tubes containing sterile distilled water. The tube was then well shaken to break the clumps of the mat.

Isolation and Identification

Water samples and mat samples collected were observed under compound microscope. Small portion of well shaken suspension was taken on the slide and clean trichomes were marked and picked up for further isolation. Enrichment and isolations were carried out on different culture media viz. BG-11, Marine BG-11, ASN-III N^+, ASN-III N^- and Zarrouk's medium (Zarrouk, 1966; Waterbury, 1976 and Rippka et al., 1979).Pure cultures of cyanobacteria were obtained by three techniques viz. enrichment, serial dilution and direct streak plate method. For samples showing unicellular algal forms, serial dilution method was employed. In the serial dilution method, the inoculum was prepared by mixing the samples with different autoclaved media and then cultures were surface spreaded on agar plates. For mat samples, streak plate method was employed in which mat samples were directly streaked on sterile agar plates. The plates were incubated on racks in a culture room at temperature 20 ± 2ºC and in light 2000 lux provided by cool-white fluorescent tubes with a light regime of 16L 8D.

Unialgal cultures were maintained by repeated sub-culturing of species to its respective fresh sterile culture medium every 3-5 weeks or as and when required. Stock cultures were maintained on sterile agar slants in quadruplicates as well as in 100ml Erlenmeyer flasks containing 50ml of sterile liquid medium in a culture room at temperature 20 ± 2ºC and in light (2000 lux) provided by cool-white fluorescent tubes with a light regime of 16L 8D and for shake flask cultures, on orbital shaker with speed 100rpm.

In order to identify lab isolates, they were examined microscopically for their morphological features that aid in identification and then photographed using trinocular research microscope with camera attachment (Tucsen CMOS). Identification was done using standard reference taxonomic publications such as Geitler (1925), Desikachary (1959) and Komárek & Anagnostidis (1998, 2005).

RESULTS AND DISCUSSION

Isolation studies

Total eight different species were isolated and further maintained as unialgal cultures in the laboratory. Out of eight species isolated, unicellular, non nitrogen fixing forms (3 species) were

identified as *Synechocystis primigenia, Synechocystis salina* belonging to family Chroococcaceae, and *Chroococcidiopsis cubana* belonging to family Chroococcidiopsidaceae. Filamentous non-heterocystous forms (3 species) were identified as *Pseudanabaena limnetica* belonging to family Pseudoanabaenaceae, *Lyngbya salina* and *Oscillatoria salina* belonging to family Oscillatoriaceae whereas filamentous heterocystous forms (2 species) were identified as *Nostoc calcicola* and *Nostoc coerulum* belonging to family Nostocaceae.

During isolation studies, it was observed that *Synechocystis primigenia, Synechocystis salina* and *Chroococcidiopsis cubana* were growing well on marine BG-11 medium containing 2.5% NaCl compared to normal BG-11 medium without NaCl. Filamentous non-heterocystous species namely, *Pseudanabaena limnetica* and *Oscillatoria salina* showed maximum growth on ASN-III N^+ containing 2.5% NaCl. However, Zarrouk's medium also supported their growth to a lesser extent. *Lyngbya salina* showed grew well on ASN-III N^+ containing 2.5% NaCl whereas it showed lesser growth on marine BG-11 containing 2.5% NaCl. Heterocystous strains viz. *Nostoc coerulum* and *Nostoc calcicola* grew equally well on ASN-III N^+ and ASN-III N^- media. However, presence of heterocyst was prominent when they were grown on ASN-III N^- medium (**Table. 1**). Bhatt *et al.* (2016) isolated and characterize halophilic cyanobacterium *Euhalothece* SLVH01 from Sambhar Salt Lake, Rajasthan, India. The strain was enriched and further isolated on BG-11 medium supplemented with 5-15 % (w/v) NaCl. They observed that strain grew optimally at 5% (w/v) NaCl. It grew well even at 10% NaCl. Nagle *et al.* (2009) attempted to isolate few species of *Oscillatoria, Lyngbya, Synechococcus* and *Phormidium* on ASN-III N^+ and ASN-III N^- media. Our isolation results are in agreement with these reports.

Morphological and Cultural characteristics

The pattern of growth while culturing and morphological features of each isolate was studied. Differential movement of organisms on agar plates and differential migration of organisms through agar was observed.

1. *Synechocystis primigenia* Gardner Komárek & Anagnostidis 1998,P.140,Fig. 156- **(Plate. 1, Figure. 1-4)**

Cells perfectly globular, without visible gelatinous envelopes, dark blue green, 0.7-1 µm in diameter. Solitary or two together. In Petri-plate culture as well as agar slant culture, it was found to be growing in the form of small colonies and/or in the form of patches formed along the line of streak. Diameter of the colonies varied from pinpoint to 1 mm. colonies were dark bluish green, with smooth appearance. It formed homogenous suspension culture in liquid medium.

2. *Synechocystis salina Wislouch* Komárek & Anagnostidis 1998, P. 141, Fig. 161- **(Plate.1, Figure. 5-8)**

Cells spherical, solitary or two together. Dark blue green in color with homogeneous content.
In Petri-plate culture as well as agar slant culture, it was found growing in the form of small colonies and/or patches formed along the line of streak. Diameter of the colonies varied from pinpoint to 1-2 mm. colonies were dull green to olive green, with smooth and sticky appearance. It formed homogenous suspension culture in liquid medium.

3. *Chroococcidiopsis cubana* Komárek et Hindak- Komárek & Anagnostidis 1998, P. 423, Fig. 552- **(Plate.1, Figure. 9-12)**

Cells solitary or in more or less spherical or irregular groups, sometimes in large aggregates. Cells are green when young while turning dark brown upon maturity. Cells are 17-30 µm in diameter, with thin, firm, colorless sheath. Sheath splits during the liberation of daughter cells. Cells show irregular division producing baeocytes. Cell contents are homogeneous or sometimes granulated.

In Petri-plate and agar slant culture, it was found to be growing in form of colonies formed along the line of streak. Diameter of the colony varies from pinpoint to 2 mm. As growth proceeds, colonies adjacent to each other unite for form small brown patches on the agar surface. Colonies were dark brown to reddish brown in color with uneven margin and amorphous rough texture. In liquid cultures, it grows as a homogenous granular suspension of cells.

4. *Pseudanabaena limnetica* (Lemm.) Komárek Komárek & Anagnostidis 2005, P. 84, Fig. 60;- **(Plate.1, Figure. 13-16)**

Trichomes bluish green, solitary, straight or slightly curved, motile, 1-2 µm broad, slightly constricted at the cross walls, cross walls hyaline, translucent, without aerotopes, not attenuated at the ends, occasionally with thin mucilaginous envelope or sheath. Cells long, cylindrical, 2-3 µm broad, 8-12 µm long, cell contents homogenous, apical cells rounded.

In Petri-plate and agar slant culture, many filaments of variable length were found growing after 2 weeks of inoculation. In solid cultures, *Pseudoanabaena* showed a tendency to burrow into the agar as well as to grow on the agar surface. Short filaments produced probably as a result of the natural breakage of long filaments. Short filaments were found growing at the periphery of the growing area. The filaments developed radially during the first few days but then because of the crowding of the filaments, the direction of the growth got diversified, as a result filaments grew all over the agar surface. In liquid cultures, it grew as a homogenous mass of filaments of dark bluish green color. Filaments have a tendency to emerge out of the liquid surface and creep on the sides of the culture vessels.

***5. Lyngbya salina* Kützing ex Starmach** Komárek & Anagnostidis 2005, P. 618, Fig. 938 - **(Plate.1, Figure. 17-20)**

Thallus dark bluish green, filamentous. Filaments solitary, straight, sheathed. Sheath thin, colorless. Trichome not constricted at the cross walls, terminated with wide rounded apical cell. Cells are discoid, broader than long. Cells 13 µm broad and 2 µm long. Cell contents granular. In Petri-plate and agar slant culture, filaments developed after 20 days of inoculation. Filaments exhibited a radial spreading growth in all the possible directions from the site of inoculation giving star like appearance on the surface of agar. Bundles of filaments were formed on the agar surface instead of uniform spreading of the filaments. Color of the filaments was dark bluish green with rough leathery texture. In liquid cultures, filaments initially attached at the bottom of the flask, later formed floating clumps. It does not develop homogenous suspension culture in liquid media.

***6. Oscillatoria salina* Biswas** Komárek & Anagnostidis 2005, P. 601, Fig. 906- **(Plate.1, Figure. 21-24)**

Thallus membranous, blue green. Trichomes more or less straight, 4-5 µm wide, not constricted at the cross walls, end of the trichome attenuated. Cells up to 2 µm long, cell contents homogenous, apical cells elongated, hyaline and pointed.

In Petri-plate and agar slant culture, filaments developed after 2 weeks of inoculation. It was interesting to note that, filaments were found to move or glide towards the incident light source. Color of the filament was bright bluish green with soft and sticky texture. Small filaments resulted from the breakage of long filaments, growing towards the edge of the surface. As growth proceeds, filaments spread all over the agar surface. In liquid culture, filaments attached at the bottom initially and later aggregated into clumps. It does not produce homogenous culture in the liquid media. Allen (1952) also reported same surface shunning phenomenon of *Oscillatoria* in solid cultures.

7. *Nostoc coerulum* Lyngbye ex Born. et Flah. Desikachary, 1959, P. 388- **(Plate.1, Figure. 25-28)**

Thallus mucilaginous, Trichomes densely entangled, flexuous, 4-5µm broad, unbranched, sheath thin, delicate, colorless and coalescing. Cells short, barrel-shaped, appearing squarish under magnification, 2-3 µm long, heterocysts spherical, mainly intercalary, sometimes terminal. Akinetes not visible.

In Petri-plate and agar slant culture, it was found growing in the form of patches with rough, uneven margin and dull brown color. Appearance of the patch was sticky due to presence of mucilaginous sheath. It showed wide range of morphological variations under culture conditions, simulating many allied forms. Trichomes appeared short, straight or flexuous. Owing to thick mucilaginous sheath, showed growth on the surface of agar and has not shown surface shunning. In liquid cultures, it was found growing in the form of small aggregates of cells. Sometimes also found attached at the bottom and to the side walls of the flask. Similar reports on morphological variations under culture conditions were reported for *Anabaena cycadae* by Goyal and Venkatraman (1964).

8. *Nostoc calcicola* Bréb. Ex Born et Flah. Komárek 2013, P. 983, Fig. 1280-**(Plate.1, Figure. 29-32)**

Thallus mucilaginous, slightly diffluent, expanded olive green. Filaments loosely arranged, sheath indistinct, Trichomes 3-5 µm wide, dark olive green, cells barrel shaped, spores are spherical, 4-5 µm broad. Heterocyst intercalary.

In Petri-plate and agar slant culture, it was found to be growing in the form of dark brown colored colonies. Diameter of the colonies varies from 1-3 mm. As the growth proceeds, small colonies unite to form big patches of the growth on the agar surface. It has not shown surface shunning phenomenon. Colonies were sticky in appearance. Trichomes appeared long, straight or flexuous. In liquid culture, it was found growing as small aggregates of cells.

CONCLUSION

As a result of this study, it was found that all the cyanobacterial strains isolated from saltpan samples are halophilic. This indicates that saltpans of eastern suburbs of Mumbai offer untapped reservoir of halophilic marine cyanobacteria. These strains being indigenous in origin can be brought under mass cultivation in outdoor environments and can further be utilized as a most promising source of value added compounds of industrial importance.

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Table 1: Growth of lab isolates on different culture media

		Media				
Sr. No.	**Name of the organism**	**BG-11**	**Marine BG-11**	**ASN-III N^+**	**ASN-III N^-**	**Zarrouk's Medium**
1	*Synechocystis primigenia*	+	++	-	-	-
2	*Synechocystis salina*	+	++	-	-	-
3	*Chroococcidiopsis cubana*	+	++	-	-	-
4	*Pseudoanabaena limnetica*	-	-	++	-	+
5	*Lyngbya salina*	-	+	++	-	-
6	*Oscillatoria salina*	-	-	++	-	+
7	*Nostoc coerulum*	-	-	++	++	-
8	*Nostoc calcicola*	-	-	++	++	-

+: indicates growth ++: indicates maximum growth -: indicates No growth

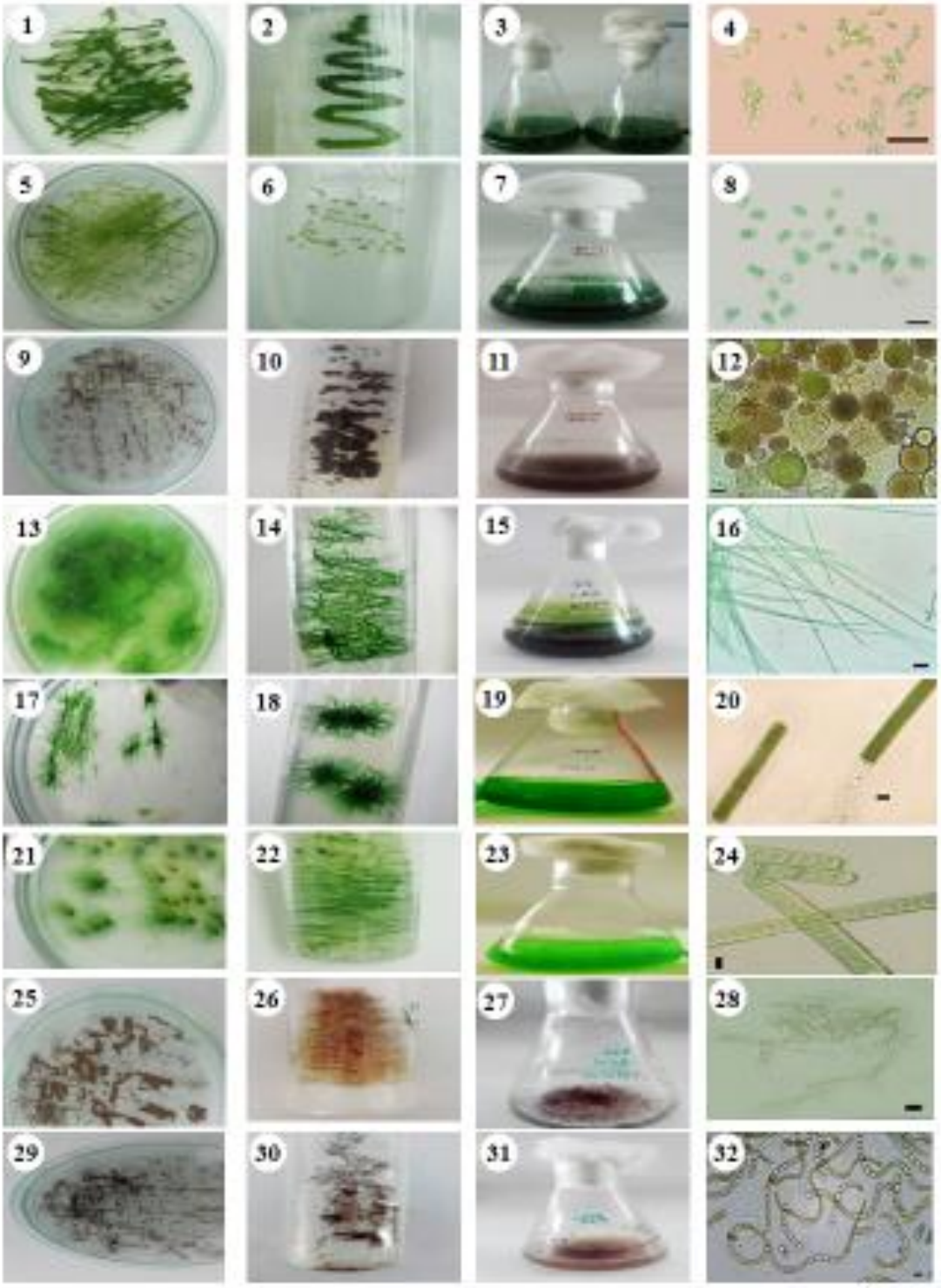

Plate 1. Figs. (1-32). Isolation and establishment of pure cultures of halophilic cyanobacteria from saltpans of eastern suburbs of Mumbai. 1-4: *Synechocysis primigenia*; 5-8: *Synechocystis salina*; 9-12: *Chroococcidiopsis cubana;* 13-16: *Pseudoanabaena limnetica;* 17-20: *Lyngbya salina;* 21-24: *Oscillatoria salina;* 25-28: *Nostoc coerulum*; 29-32: *Nostoc calcicola*

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