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Research Article

ISOLATION, PHYSIOCHEMICAL CHARACTERIZATION AND CULTIVATION OF SALT TOLERANT MICROALGAL SPECIES FROM MARAKKANAM SALT PAN, TAMIL NADU, INDIA

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A B S T R A C T

The present work deals with the Salt water samples and biodiversity of Salt Tolerant Microalgal Species from Marakkanam Salt Pan, Tamil Nadu, India. Salt-loving microorganisms are called halophiles. They include fungi, diatoms, bacteria, algae and cyanobacteria. Microalgae are classified based on the production of pigmentation, namely the chlorophytes or green algae (chlorophyll a and b), the rhodophytes or red algae (chlorophyll a and phycobilins), and chromophytes or yellow-brown algae (chlorophyll a, c, and absence of chlorophyll b). Halophilic microorganisms are balance their cytoplasm osmotically with accumulation of molar concentrations of potassium and chloride. Salt which act as a substrate for intracellular enzymatic process. The sample collection was made during winter season in the month of November, 2017. The samples were subjected to physico - chemical analysis, microalgae were examined in the algal biotechnology laboratory, university of madras and identified the following algae *Navicula* sp., *Spirogyra* sp., *Phormidium* sp., *Oscillatoria* sp., *Stauroneis* sp., *Lyngbya* sp.,*Cymbella* sp., *Nitzschia* sp., *Nostoc* sp., *Amphora* sp., *Anabaena* sp., *Placoneis* sp., *Tolypothrix* sp., *Chroococcus* sp., and *Haematococcus* sp.were recorded. Algae are described with photographs.

INTRODUCTION

Microorganisms are distributed among three primary related groups such as Archaea, Bacteria and Eucarya.

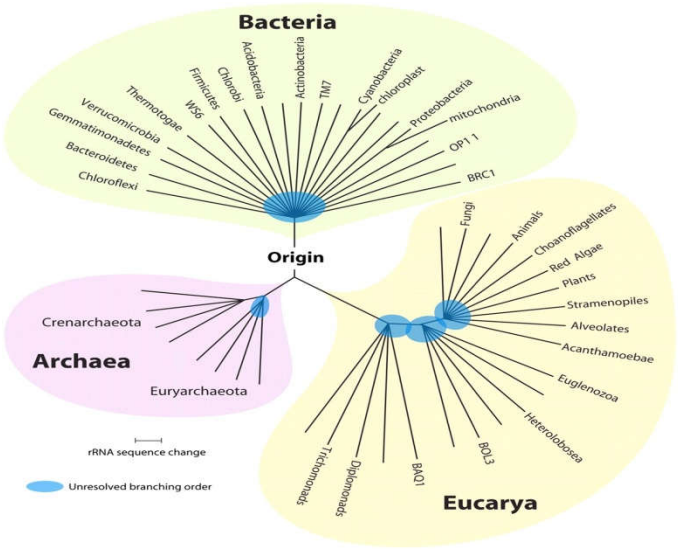

Fig 1 Phylogenetic Tree of Life (Courtesy : Brent Grissom)

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marine water, surface of moist rocks, hypersaline lakes, coastal dunes, saline deserts, salt marshes, inland salt seas and springs.

Synthesis of Microalgae biocompounds (lipids, proteins, carbohydrates, pigments, and polymers, carotenoids, phycobilins, polyunsaturated fatty acids, proteins, polysaccharides, vitamins, and sterols other chemicals) have high nutritional value and therapeutic functions. Microalgae considered as a rich source of secondary metabolites with potential application in the various fields like pharmacological, medical, aquaculture, agriculture, cosmetics, and biofuel. Salt pans are rectangular or square covered with soil-base and protected by mud bunds. The collected sea water is exposed to sunlight and makes the brine finally obtained the crude crystalline salt. Common salt is impure sodium chloride; it contains varying amounts of sodium, potassium, calcium, chloride, sodium bicarbonate and magnesium. Salt-loving microorganisms are called halophiles. It is further classified, depends on the concentration of salt environment such as non halophilic, Sunlight halophile, Moderate halophile, Borderline extreme halophile, Extreme halophile, halotolerant, haloversatile. They include fungi, diatoms, bacteria and cyanobacteria, which occur as free forms or associations with mats. Halophilic archaebacteria, cyanobacteria, diatoms, microalgae are play an important role in a natural ecosystem, marine environment, C_2 fixation, N_2 fixation, microbial activities and their *importance* to the biosphere and *economic importance* of halophilic microorganisms producing secondary metabolites. There are two types of hypersaline environments;

Thalassohaline and Athalassohaline. Athalassohaline waters are marine derived; similarly, to that of sea water. Solar salterns are examples for athalassohaline. Here, sea water is evaporated using sun light in the production of sea salt.

Table 1 Classification of microbes based on their salinity tolerance

Category	Salt Range (molarity)	Optimum salt Concentration (molarity)	Examples
Non halophilic	0-1.0	< 0.2	Most freshwater Microorganisms
Sunlight halophile	0.2-2.0	0.2-0.5	*Dunaliella salina*
Moderate halophile	0.4-3.5	0.5-2.5	*Amphora coffeaeformis*
Borderline extreme halophile	1.4-4.0	2.0-3.0	*Haloarchaeon Natrinema sp*
Extreme halophile	2.5-5.2	>3.0	*Aphanothece halophytica*
Halotolerant	0->1.0	<0.2	*Halomonas elongate*
Haloversatile	0->3.0	0.2-0.5	*Dunaliella parva*

The 1M NaCl is require for the growth of halophile. Halotolerance organisms is living in high concentration of salt. The halobacteria have two primary energy sources, a electron transport chain and purple membrane. When a substrate is oxidized or when the purple membrane absorbs light, protons are ejected from the cell and an electrochemical potential is generated. Halotolerance organism and their organelles studies which gives the fundamental knowledge of biochemical reaction and convert the biological energy transportation mechanisms, biophysical problems and cell signaling. There are two different important mechanisms occurred in halophilic potassium and chloride osmosis process are carried out by intra cellular enzyme and extra cellular enzyme. Salt act as a substrate the proteins should maintain their proper conformation and activity at near-saturating salt concentrations. *Nitzschia* is a marine diatom, produce the neurotoxin called as domoic acid, a toxin causes the illness to human by consuming of amnesic shellfish.

Algal taxonomy starts in early part of the 19th century and recognizes the four different classes of algae namely chlorophyceae, Phaeophyceae, Rhodophyceae, cyanophyceae groups. M.O.P.Iyengar, Father of Indian Algology. Monographs on different groups of algae were published (Him, 1900; Forel, 1901; West 1912; Bristol *1920;* Smith, 1924; Hustedt, 1930; Geitler 1932; Fritsh 1945; Geittler, 1932; Iyengar, 1933; Pascher 1939; Prescott1951; Krishnamurthy 1954; Ganapati 1956; Desikachary 1959; Randhawa, 1959; Venkatraman, 1961; Pal 1962; Ramanathan 1964; Philipose, 1967; Anand, 1978; Zhafer 1986; Prasad and Misra 1992). The taxanomic groups of fresh and marine algal ecosystem (Elumalai *et al*., 2011, 2013 and Sakthivel *et al*., 2012). Middle of 1980‘s; the first phylogenic construction of green plants from 5.8S rDNA sequences (Hori *et al*.,1985; Hori and Osawa, 1987), soon followed by 18S and 28S rDNA sequence analyses (Gunderson *et al*.,1987).

Sample Collection

The sample collection was made during winter season in the month of November, 2017. 1000 ml of water samples and visible cyanobacterial mats specimens were collected from different places of Marakkanam salt pan and immediately water parameters (The temperature, P^{H}, Conductivity, TDS, DO, Calcium, salinity, Silica, Sodium, Potassium, and Chloride) were analyzed by field portable water analyzing YSI-Instrument. The collected cyanobacterial samples were transferred to Erlenmeyer flasks containing BG11 medium and Algal culture maintenance in Algal biotechnology laboratory, University of Madras. The micro algal samples were isolated by using serial dilution, spread plate and streak plate method. These isolated Cyanobacterial species were cultured in BG11medium under 3000 lux light intensity with static condition, for 12 h under illumination and 12 h under darkness. cyanobacterial cultures were harvested approximately after a production period of 21days. The sampling places were designated as A, B, C, D, E, F ,G and H.

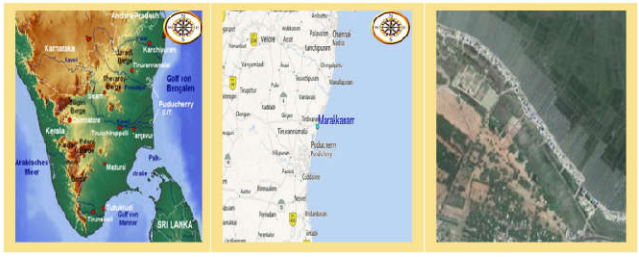


Fig 2 Map of Tamil Nadu, Marakkanam highlighting the sampling sites and satellite map

(The latitude of Marakkanam is 12.186952 and the longitude is 79.927895. The gps coordinates of 12° 11' 13.0272" N and 79° 55' 40.4220" E.it hasan average elevation of 11 meters height, that is equal to 36 feet.)

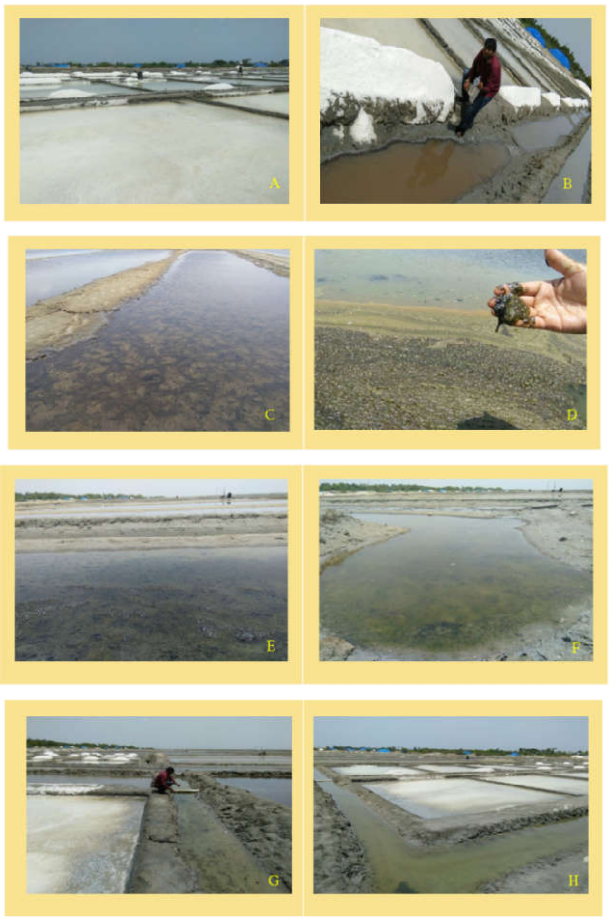


Fig 3 sampling place of microalgae from Marakkanam salt pan

MATERIALS AND METHODS

Culture Media Preparation

Table 2 Chemical Composition of BG11 Media for the Culture of Microalgae (Allen and stanier 1968 and Rippka *et.al*., 1979)

Reagents	Per liter (g)
$NaNo_3$	1.5
$K_2HPO_4.3H_2O$	0.004
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.027
Citric acid ($C_6H_8O_7$)	0.006
Ammonium Ferric Citrate ($C_6H_8O_7$ .nFe.nNH_3)	0.006
EDTA Na_2Mg	0.001
Na_2CO_3	0.02
Microelement stock solution	1ml

Microelement Stock Solution

Reagents	Per liter (g)
H_3BO_3	2.860
$MnCl_2.4H_2O$	1.810
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$CO(NO_3)_2.6H_2O$	0.0494
P^H	= 7.2

Preparation of BG11Solid medium

The BG11 broth chemical ingredients with agar powder (1.5 gm/lts) were suspended in distilled water and 5% of NaCl was added. The medium was completely dissolved and sterilized by autoclaving at 15 Lbs (121ºC) for 15 minutes.

Pure Culture Technique

Inoculation: Spread Plate Method

Serial Dilution method

Seven test tubes containing 9 ml of distilled water were taken, to that first tube 1 ml of sample was added and labeled as 10^{-1}. The sample was mixed. 1.0 ml of the sample taken from the previous test tube (10^{-1}) was transferred to the next test tube and labeled as 10^{-2}. The same procedure is continued so that serial dilution is attained up to 10^{-8}. 1 ml of aliquot was drawn from the last tube and discarded. Then 0.1 ml of samples from all dilutions was transferred in to BG11 agar medium plates.

In spread plating, an L- rod was sterilized in 80% ethanol and exposed to the alcohol lamp. BG11 agar medium plate was placed on the top of the plate spinner. The sterilized L-rod was used to spread the sample evenly across the agar surface in all BG11 agar medium plates. The agar was allow to absorb the sample for 10 minutes. The innoculated plates were placed in incubator for 15 days at 25ºC, and then stored in the refrigerator. Observations were made in the following week and the colony forming unit per ml (CFU/ml) of each dilution was calculated.

Strake Plate Method

Inoculation in BG11 agar medium

The BG11agar medium was prepared and poured in to the sterile Petri dish and allowed to solidify. The petri dish was held in the left hand between the fingers and the thumb near the flame. The loop was held in the right hand, flamed and then cooled. The lid of the petri plate was opened and the sample organism was inoculated by means of streaking. The petri plates were sealed with paraffin tape. The petri plates were incubated at 25ºC for the growth of theorganisms. The changes were observed after 12 -15 days.

RESULT AND DISCUSSION

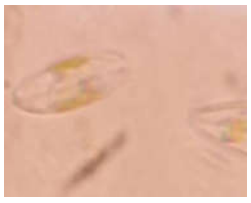
In this present investigation, 7 species of Marinecyanobacteria distributed in marakkanam salt pans were recorded. Total of three genera belonging to eleven families such as *Naviculaceae, Oscillatoriaceae, Stauroneidaceae, Cymbellaceae, Bacillariaceae, Characeae, Nostocaceae, Catenulaceae, Gomphonemataceae, Chroococcaceae, Haematococcaceae* namely; *Navicula* sp., *Spirogyra* sp., *Phormidium* sp., *Oscillatoria* sp., *Stauroneis* sp., *Lyngbya* sp.,*Cymbella* sp., *Nitzschia* sp., *Nostoc* sp., *Amphora* sp., *Anabaena* sp., *Placoneis* sp., *Tolypothrix* sp., *Chroococcus* sp., and *Haematococcus* sp. were reported from the present study from the salt pan sites.

The samples were subjected to physico- chemical analysis, Temperature, Conductivity, pH @ 25°C, Calcium, Dissolved Oxygen, ORP, values high in sample C . Total Alkalinity, Silica, Dissolved Oxygen, Sodium, Potassium values high in sample A. Chloride, Total Dissolved Solids values high in sample B.

Morphological identification of microalgal species from marakkanam salt pan

Empire	Eukarya
Kingdom	Chromista
Phylum	Bacillariophyta
Class	Bacillariophyceae
Order	Naviculales
Family	*Naviculaceae*
Genus	*Navicula*

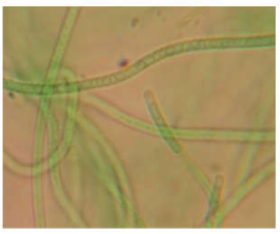
Systematic-position: *Navicula* sp.



Navicula cells have two chloroplasts lying along each side of girdle, each with a single rod shaped pyrenoid along their length (only visible in girdle view). Cells are unaccompanied or individual, size – length (12-50 µm) width (7-17µm) Shape – Asymmetrically biraphid.

Empire	Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Oscillatoriales
Family	*Oscillatoriaceae*
Genus	*Phormidium*

Systematic-position: *Phormidium* sp.

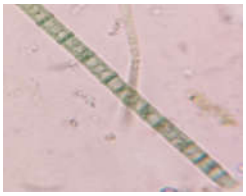


Phormidium usually forms flat, slimy mats of tangled filaments. The filaments are long, cylindrical, and may be

curved or spiralled. Thin, firm, colorless sheaths adhere closely to out growths or appendages on *Phormidium* cells. The apical cells may have calyptra with more pointed, narrow or sperical than the other cells.

Empire	**Prokaryota**
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Oscillatoriales
Family	*Oscillatoriaceae*
Genus	*Oscillatoria*

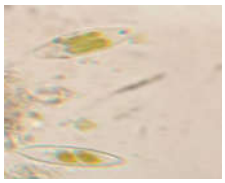
Systematic-position: *Oscillatoria* sp.



Oscillatoria trichome consists of single row of cells. *Ocillatoria* have broader trichomes not enclosed by sheath. The trichomes are straight, slightly undulating, or coiled, and are made up of disk-shaped cells wider than they are long. In some species the end cells can be rounded or tapered. This genus is named for the gliding, rotating, or oscillating motion of the filament around its axis.

Empire	**Eukarya**
Ingdom	Chromista
Phylum	Bacillariophyta
Class	Bacillariophyceae
Order	Naviculales
Family	*Stauroneidaceae*
Genus	*Stauroneis*

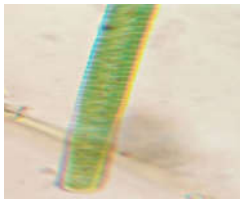
Systematic-position: *Stauroneis* sp.



Cells symmetrical to the apical and transapical axes.Valves elliptical to lanceolate. Apices rounded to capitate. Raphe straight. slight ridge or groove on a surface, visible and flecked.Central area with bow tie shaped stauros comprising of the non-striated, thickened central nodule and usually extending to the valve margin.

Empire	**Prokaryota**
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Oscillatoriales
Family	*Oscillatoriaceae*
Genus	*Lyngbya*

Systematic-position: *Lyngbya* sp.



Lyngbya is a unicellular , Filamentous; thick, rarely solitary, rarely tangled into free clusters of coiled filaments, firm sheaths, which are sometimes layered or stratified and brownish coloured, opened at the ends; asexually reproduction (hormogonia); barrel-shaped discoid, cell content blue-green, olive green, yellowish, brownish or pinkish, with coiled tylakoids, situated more or less over the whole cell content; thickened cap on the terminal cell of a cyanobacterial filament. Heterocytes and akinetes absent.

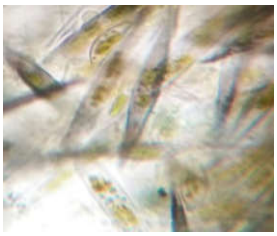
Empire	**Eukarya**
Kingdom	Chromista
Ylum	Bacillariophyta
Class	Bacillariophyceae
Order	Cymbellales
Family	*Cymbellaceae*
Genus	*Cymbella*

Systematic position: *Cymbella* sp.

Cells solitary, free-floating or attached at the ends of gelatinous stalks or confined in branched gelatinousmass; in girdle view somewhat rectangular with smooth girdles, intercalary bands absent; in valve viewasymmetrical longitudinally, lunate or more rarely elliptical, rhombic or naviculoid, dorsal surface convex, ventral surface usually concave sometimes havingmedian gibbosity; axial area narrow gradually widening towards centre; central areawith or without dots; raphe thin or thick straight or curved, eccentric, usuallyplaced towards ventral sidewith well defined nodules; striae lineate or punctate,transverselyplaced, parallel or slightly radial sometimes becoming convergent towards poles,chormatophores single, expanded, plate like.

Empire	**Eukarya**
Kingdom	Chromista
Phylum	Bacillariophyta
Class	Bacillariophyceae
Order	Bacillariales
Family	*Bacillariaceae*
Genus	*Nitzschia*

Systematic-position: *Nitzschia* sp.



Nitzschia cell contains two chloroplasts located in the each pole (very occasionally >2 chloroplasts, in few marine species). Solitary cells, Freely floating, Size – Length (10-15 µm) width (3 µm). Shape – Valves are linear with concave margin (Pennales).

Empire	Eukaryota
Kingdom	Plantae
Phylum	Chlorophyta
Class	Charophyceae
Order	Charales
Family	*Characeae*
Genus	*Spirogyra*

Systematic-position: *Spirogyra* sp.

Spirogyra is unbranched with cylindrical, filaments are connected an end to end. The chloroplasts are ribbon shaped, notched edge or sawlike teeth and spirally arranged. Two types of conjugation (ladder and lateral conjugation) are seen.

Empire	Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Nostocales
Family	*Nostocaceae*
Genus	*Nostoc*

Systematic-position: *Nostoc* sp.

It is filamentous form of both terrestrial and aquatic habitats. Trichome resemling a string of beads. Large colonies of closely packed trichomes enclosed by its own mucilaginous sheath. Cells are rounded or oval cells. At frequent intervals along the trichome terminal or in intercalary position heterocysts are found.

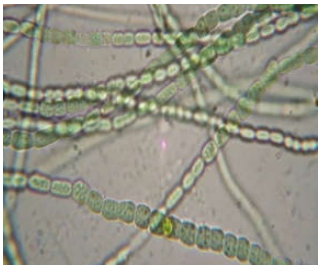
Empire	Eukaryota
Kingdom	Chromista
Phylum	Bacillariophyta
Class	Bacillariophyceae
Order	Thalassiophysales
Family	*Catenulaceae*
Genus	*Amphora*

Systematic-position: *Amphora* sp.

Cells are solitary and chloroplasts are attached to the inner side of the frustule. The pyrenoid can be observed clearly at the center of the cell. Cells are attached to substrate, but they are usually motile. The girdle bands are not plicate and four girdle bands can be observed. Valves are small and lunate, with a convex dorsal margin. The valve length is 15.0 to 19.2 µm, and the valve width is 5.2 to 7.9 µm. The raphe is well-marked and slightly deflected at the median. The marginal ridge is not pronounced. The ventral striae are composed of single areolae. The size of the areolae increases towards the mid-valve and they converge toward the ends. Striae are interrupted by the central area, which is conspicuous at the center of the valve.

Empire	Eukaryota
Kingdom	Bacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Nostocales
Family	*Nostocaceae*
Genus	*Anabaena*

Systematic-position: *Anabaena* sp.



Anabaena has arranged in a single row, layer, or series unis, straight, curved, or coiled. Trichomes are out growths or hair like appendages on *Anabaena* cell, blue-green to yellow-green coloured was observed and the cells are look like a spherical, round, cylindrical, or bent and string of beads. Absence of mucilage wall, intercalary, solitary heterocysts spaced fairly regularly along the filament. Heterocysts are specialized cells, function as the sites for N_2 fixations under aerobic conditions The akinetes is a enveloped spore, spherical, round, cylindrical or curved in shape, and are sometimes present near the heterocysts.

Empire	Eukaryota
Kingdom	Chromista
Phylum	Bacillariophyta
Class	Bacillariophyceae
Order	Cymbellales
Family	*Gomphonemataceae*
Genus	*Placoneis*

Systematic-position:*Placonies* sp.

The valve outlines are elliptical to linear-elliptical and the ends shortly rostrate . Raphe filiform with rather close-standing central pores. Terminal fissures heteromorphous.Axial area narrow, linear. Stigmata lacking. Central area somewhat variable in shape by several striae alternating shorter and longer.

Empire	Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Nostocales
Family	*Tolypothrichaceae*
Genus	*Tolypothrix*

Systematic-Position:*Tolypothrix* sp.

Trichomes are out growths or appendages on Tolypothrix cell with a thick mucilaginous wall, uniseriate or unbranched, falsely branched (initiated at a heterocyst or between two vegetative cells). Heterocysts are specialized cells, function as the sites for nitrogen fixation under aerobic conditions and akinete is an enveloped, thick-walled, non-motile, dormant cell formed by filamentous cells, asexual reproduction mainly by hormogonia Which germinate at both ends or hormocysts.

Empire	Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Chroococcales
Family	*Chroococcaceae*
Genus	*Chroococcus*

Systematic-position: *Chroococcus* sp.

Chroococcus usually forms small groups of cells which can either be free floating or attached. Cells have distinct sheaths which may be reformed after each cell division resulting in a multilayered sheath. Only planktonic species have gas vacuoles. Planktonic species do not tend to have distinct sheaths since surface layers are often confluent with the surrounding mucilage. Cells 2–58 µm in diameter. Easily confused with Gloeocapsa. Cyanophyta.

Empire	Prokaryota
Kingdom	Plantae
Phylum	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	*Haematococcaceae*
Genus	*Haematococcus*

Systematic-position: *Haematococcus* sp.

Haematococcus cells are round or spherical, rarely oval and Size is 8–30 µm in diameter. *Haematococcus* cells contain a thick mucilaginous wall. The *haematococcus* cells appear red because the chloroplast are masked with red haematochrome. The chloroplast is cup-shaped with one to four pyrenoids. There are often protoplasmic extensions into the wide cell wall. The cells are often noticed in an encysted state giving them red. Occurs in small water bodies and snow as well as bird baths which it may red. Chlorophyta.

Table 3 Physicochemical parameter of water sample from Marakkanam salt pan

Parameters	Sample A	Sample B	Sample C
Temperature	28.5°C	28.4°C	28.6 °C
Conductivity	534000µs/cm	599800µs/cm	306400 µs/cm
pH @ 25°C	7.1mg/l	7.2 mg/l	7.5 mg/l
Calcium as Ca	310 mg/l	413 mg/l	974 mg/l
Total Alkalinity as CaCO3	506 mg/l	326 mg/l	146 mg/l
Chloride as Cl^-	165353 mg/l	187857 mg/l	83166 mg/l
Total Dissolved Solids	368400 mg/l	419800 mg/l	199100 mg/l
Silica as SiO2	3.9 mg/l	0.46 mg/l	3.1 mg/l
Dissolved Oxygen	3.6 mg/l	5.2 mg/l	5.9 mg/l
ORP	-6.5mV	-13.5 Mv	-30.6 Mv
Potassium as K	5850 mg/l	4880 mg/l	1420 mg/l
Sodium as Na	100450 mg/l	160720 mg/l	47630 mg/l

Fig4 Algal culture maintenance in Algal biotechnology laboratory at University of Madras

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