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ORIGINAL ARTICLE

SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY DISPERSIVE SPECTROSCOPY (EDS) OF CYANOBACTERIA ISOLATED FROM CUDDALORE DISTRICT,TAMILNADU

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ABSTRACT

The present study is investigated to study the scanning electron microscopic study and Energy Dispersive Spectroscopy of different cyanobacteria isolated from the paddy fields of Keerapalayam and vallampadugai, Cuddalore District,Tamilnadu. Scanning Electron Microscope and Energy Dispersive Spectroscope were observed in two cyanobacteria such as in *Anabaena* and *Oscillatoria* sp. at 10th day. The present study shows the ultrastructural changes and various elemental composition in Anabaena and Oscillatoria.

Key Words: SEM, EDS, Cyanobacteria, *Anabaena, Oscillatoria*

1. INTRODUCTION

Cyanobacteria, also known as blue green algae, blue green bacteria or cyanophyta, that obtain their energy through photosynthesis. The name cyanobacteria come from the color of the bacteria. They are a significant component of the nitrogen cycle and an important primary producer in many areas of the aquatic ecosystem. The chemical composition of phytoplankton samples used for their calibration, which has been conventionally determined by bulk analysis techniques (Behrendt, 1990). The estimation of elemental concentrations for the whole sample without separation into separate constituents, *e.g.*, algae, detritus, small animals etc., inevitably. Such data on natural blooms of various microalgal species could contain errors due to the presence of other species and/or detritus particles in the analyzed samples. Therefore, in the present investigation, the elemental composition of certain blue green algaeis determined for individual species by X-ray microanalysis (XRMA) to avoid the above problem. XRMA has sufficient resolution to analyze single cyanobacterial cell within a mixed population (Liberton *et al.*, 2006) and involves the simultaneous determination of a comprehensive assessment of nutrient status (Sigee *et al.*, 1998; Baulina *et al.*, 2004; Marquardt and Palinska, 2006).

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Cyanobacteria are abundant throughout the world and contribute significantly to global primary productivity from which pytoplanktonic terrestrial components account for a large proportion (Marine *et al.*, 2004). The advent of the electron microscopy (McMullan, 1995) revealed that blue green algae were bacteria, presenting a well-defined submicroscopic organization with a lacking membrane-bound organelles. Previous cytological studies on blue-green algae, either with the light microscope or electron microscope, have established certainly that the cellular organization of these organisms is simpler and quite different from that of higher forms (Geitler, 1959). Kenyon *et al.* (1972) proposed that four types of fatty acid composition exist in cyanobacteria and demonstrated some correlations with morphological properties.

Various authors reported that different cyanobacteria containing intracellular elements; *Microcystis aeruginosa* (Sigee and Levado, 2000), *Anabaena* sp. (Krivtsov *et al.*, 2001), *Staurastrum planktonicum* (Sigee and Holland, 1997), *Spirulina platensis* and *Cyclotella meneghiniana* (El-Bestawy *et al.*, 1996). Patterns and dynamics of the intracellular elemental composition were shown to be dependent on specific combinations of ambient conditions (Krivtsov *et al.*, 1999c). Hence, the present investigation was made to study the pigment and biochemical analysis as well as the Scanning Electron Microscopic (SEM) and Ultrastructure of

the *Anabaena* and *Oscillatoria* sps were made to understand certain subcellular organelles.

2. MATERIALS AND METHODS

Description of Study area

Keerapalayam which is located 5 Km from Chidambaram towards West (11°24'N Latitude and 79°44'E Longitude) and Vallampadugai is located 4 Km from Chidambaram towards East (11°24'N Latitude and 79°44'E Longitude) Cuddalore District of Tamilnadu. The soil samples were collected from the paddy fields.

Culture of cyanobacterial species

The *Anabaena constricta* and *Oscillatoria curviceps* isolated from the paddy fields of Cuddalore District. The filamentous cyanobacterium *Anabaena constricta* and *Oscillatoria curviceps* were grown in culture tube at 30°C ± 2°C in the nitrogen-free form of BG-11 liquid and nitrogen medium respectively (Rippika *et al*., 1979) and pH was adjusted to 7.9. *Anabaena* sp. was grown in BG- 11 medium without nitrogen source and *Oscillatoria* sp. was cultured in BG-11 medium with nitrogen source.Their cultures were maintained in laboratory conditions in daylight fluorescent tubes for 16 h a day for 10 days. The cultured cyanobacterial species were used for further study.

Scanning electron microscopic (SEM) study

For SEM study the *Oscillatoria* and *Anabaena* sps were fixed in primary fixative 3% glutaraldehyde. The fixed samples were given 3 washes thoroughly in 0.1 m phosphate buffer (pH 6.8) they were dehydrated through a graded series of alcohol 10-15 minutes interval at 4°c

upto 70%. Then 90 and 100% alcohol were kept in room temperature at 2-3 h interval. Then dehydrated samples treated with critical point drier (CPD) were on a stub and the specimens were examined with joel JSM-56010 with INSA-EDS and electromicrograph were taken selectively from the computer screen.

3. RESULTS

Scanning electron micrograph of different cyanobacteria

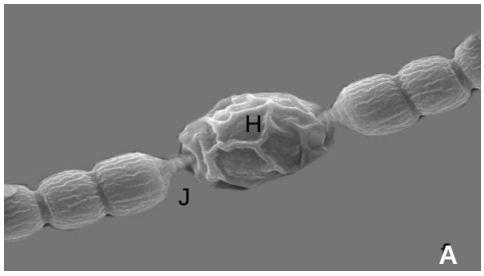
Fig. A and B shows the Scanning Electron micrograph of *Anabaena* and *Oscillatoria*, *Osillatoria* showed filaments was 15- 20 µm and cell of filaments was cylindrical, trichome single, slightly bent, mucilaginous layer is covered the trichome, septum are distinctly seen, and in anabaena cells colourless, sub-spherical, 5-6 µm wide, 5-9 µm long, with gas vacuole, end cell conical akinetes forming rows of distant from the heterocyst wall smooth or with fine warts, colourless mucilage sheath is covered the entire filament. Heterocyst was clearly seen with thick mucilage sheath covering. The heterocyst is markedly thicker than that of the vegetative cell and it is differentiated into three layers. Outer loosely fibrous layer, middle homogenous layer and inner laminated layer which cover the cell wall of the heterocyst. All the layers of the outer envelope surround the heterocyst except at the junction of the heterocyst with the vegetative cell.

Elemental composition of cyanobacteria

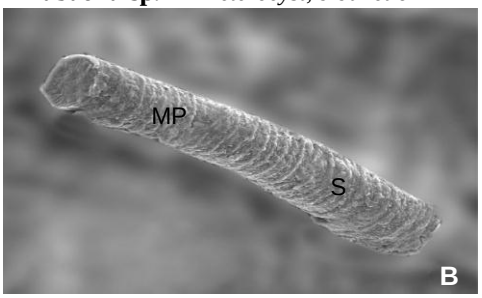
Table 9 shows the presence of different chemical elements in *Anabaena and Oscillatoria. Anabaena* contains eight elements in the following order Si>Mg>Ca>Zn>Mn>N>P>K. *Oscillatoria* contains nine chemical elements in the following order Si>Mg>Ca>Mn>Zn>N>K>S>P. Among this Si, Mg and Ca was more when compared to other elements.There are nine elements were present in algal samples. Among the Nine elements, Magnesium was present in maximum in all the species.

Table 1. Elemental composition weight in (%) of different cyanobacteria

S. No.	Name of the chemical elements	Name of the species *Anabaena* sp.	Name of the species *Oscillatoria* sp.
1	Calcium (Ca)	14.05	13.30
2	Magnesium (Mg)	16.03	15.42
3	Zinc (Zn)	13.15	14.05
4	Potassium (K)	1.10	04.01
5	Manganase (Mn)	13.12	11.15
6	Silicon (Si)	26.12	27.03
7	Phosphorous	2.20	2.02
8	Sulphur	-	3.01
9	Nitrogen	14.23	10.02



***Anabaena* sp.** *H– Heterocyst; J-Junction*



***Oscillatoria* sp.** *MP-Mucopeptide,S-Septum*

Most of the elements showed considerable variation between samples with percentage of major elements calcium (Ca) and magnesium (Mg) ranging from 13.30 to 14.25 and 15.42 to 16.36% in weight respectively. The calcium

composition shows 13.30 and 14.05 in *Oscillatoria and Anabaena* respectively and magnesium shows 15.42>16.03 in *Oscillatoria and , Anabaena*. zinc ranging from 13.15 to 14.23. Zinc concentration was more in *oscillatoria* (14.05) than *Anabaena* (13.15). P and K ranging from 2.02 to 4.35% and 04.01 to 1.35% respectively. The phosphorus compositions of *Anabaena and* were 2.20 and 2.02, respectively and potassium were 1.10 and 04.01in two algal species. Manganese ranging from 11.15 to 14.22%. Manganese composition was more in *Anabaena* (13.12) than *Oscillatoria* (11.15). Si showed high variable reach very high level ranged from 26.12 to 28.52 %. The silicon concentrations were 26.12>27.03 in *Anabaena* and *Oscillatoria* species respectively. S showed 1.24 to 3.01%. The sulphur concentration in *Osillatoria* was 3.01%. Nitrogen concentrations present in algal samples ranged from 7.41 to 14.23%. The N concentrations were 10.02 and 14.23 % in*Oscillatoria* and *Anabaena* respectively.

4. DISCUSSION

Scanning Electron Microscopic (SEM) study is to observe endomorphology of microorganism, which is helpful in determining the differentiation of the cell wall and cellular inclusion. The instrument is capable of generating three-dimensional images for analysis of topographical features. When used in conjunction with EDS the analyst can perform an elemental analysis on microscopic sections of the material or contaminants that may be present.

The present study shows the scanning electron micrograph (SEM) of *Oscillatoria* and *Anabaena* sps. *Oscillatoria* showed trichome was long, the cell sizes were distinct. The diameter of filaments was 15-20 µm and cell of filaments was cylindrical, trichome single, slightly bent, mucilaginous layer is covered the trichome, septum are distinctly seen, and in anabaena, cells colourless, sub-spherical, 5-6 µm wide, 5-9 µm long, with gas vacuole, end cell conical akinetes forming rows of distant from the heterocyst wall smooth or with fine warts, colourless mucilage sheath is covered the entire filament. Cefali *et al*. (2002) and Koch *et al*. (1982) observed that the cell wall of *Anabaena cylindrica* also seemed to be expanded and stretched at the junction of the septum and nascent pole in response to tension created by osmotically derived hydrostatic pressure. However, the shape of the cell was found to be generally preserved and deformations of the cell surface were found to be almost absent.

Venter *et al.* (2003) observed that scanning electron microscopic study in the *Oscillatoria simplicissima* which represents the filaments of this cyanobacteria was 8-12 µm width and also reported that the straight, unbranched trichome is dark blue green, covered with a thin hyaline sheath and is not attenuated or capitated at the apices and terminal cells are hemispherical with a slightly thickened membrane on the outer cell envelope. Hader and Hoiczyk (1992) observed that cell envelope to describe all the cell layers outside the cytoplasmic membrane.

It might be useful at this point to compare briefly the cell structure of blue-green algae with cells of other groups. The cell surface of blue greens as seen from our electron micrographs consists usually of a double layer of unit membranes within which there is a dense material of variable thickness forming the wall or inner investment. The cell divides through extension inwards of this material analogous to a closing diaphragm until a continuous septum is formed. In *Oscillatoria brevis* the inner investment may be missing and the cell border is formed by a double membrane about 200 Å thick (Lefort, 1960).

Grilli *et al*. (1993) observed the SEM micrographs in *Chroococcidiopsis.* They show a thick multilayered envelope rich in polysaccharides that surround the cell cytoplasm and the colonies of organisms. This layer could act as a shield, slowing down desiccation and ameliorating the extreme external conditions. The heterocysts are covered by a thick cell envelope, which limits gas diffusion into the cell and consists of distinct polysaccharide and glycolipid layers (Walsby, 1985; Murry and Wolk, 1989; Thamizh Selvi and Sivakumar, 2012a). Bhadauriya *et al.* (2007) reported the SEM of *Anabaena cylindrica*. Similar morphological changes have also been reported in microorganisms (Cefali *et al*., 2002).

Mitra *et al.* (2012) observed that the sample revealed the presence of green filamentous, unbranched trichome with only vegetative cells containing red crystals of photosynthetic pigment, phycoerythrin It was identified as *Oscillatoria* sp. a gram negative cyanobacteria further confirmed by SEM study (Diestra *et al*., 2004). The organism had a cell diameter of 7.056 micrometer studied under compound microscope while from SEM study the diameter was found to be 6.73 micrometer. Krivtsov *et al*. (1998) modeled the competition between *Stephanoidiascus rotula* and *Asterionella formosa* in Rostherne Mere during the spring of 1996. They showed that the earlier increase and collapse of the *S. rotula* population resulted from its higher maximum growth rate and half saturation constant of Si uptake. Coincidence of the *S. rotula* population decline, on the one hand, with a decrease in intracellular concentrations of certain elements (Si, P, K) and, on the other hand, with a substantial rise in Ca was similar to the case of *Asterionella formosa* reported by Krivtsov *et al.* (2000).

Krivtsov *et al.* (2002) analyzed that the intracellular elemental associations in *S. rotula* cells relates to conditions of sufficient nutrients. Previous studies of intracellular elemental composition of other representatives of the genus are also limited (Sommer, 1991). Krivtsov *et al.* (2003) observed the intracellular elemental concentration such as P, S, K, Na, Cl, Ca, Mg and Si in the *S. rotula.* Trichomes may be uniseriate, with cell division exclusively perpendicular to the trichome axis, or multiseriate in more differentiate types, where cells also have the ability to divide longitudinally. Cells of many genera produce slimy, colloidal substances external to the cell wall, composed of hydrated polysaccharides (de Vecchi and Grilli-Caiola, 1986).

Several members of orders within the filamentous cyanobacteria exhibit the greatest level of morphological and cellular differentiation. In the nostacales and stigonemateles, the heterocytes and akinetes develop. Heterocysts develop from vegetative cells and may be solitary in pairs or in rows (Komarek and Anagnostidis, 1989). They produce cell walls, which are thick, multilayered

and apparently gas tight (Stewart, 1980). Heterocytes synthesis the enzyme nitrogenase, which enables fixation of gaseous nitrogen from the atmosphere under aerobic conditions, which are maintained within the heterocysts (Wolk, 1982). The morphology (shapes) of the heterocysts and their position in the trichomes are apparently genetically predetermined, but their frequency in the trichomes depends on nitrogen supply in the environment (Kohl *et al*., 1987).

Akinetes are resting cells that develop from solitary cells in several members of the nostocales and stigonemateles (Hindak, 2001). Akinetes possesses thick cell walls and an accumulation of photosynthetic assimilates increases during their development (Sutherland *et al*., 1985b). The morphology of akinetes, their shapes, size, position within the trichomes, cell wall characteristics and mode of germination apparently are genetically determined features (Komarek and Anagnostidis, 1989).

Energy dispersive X-ray microanalysis is a popular method for the determination of trace elements in geological and environmental samples. Energy dispersive spectroscopy (EDS) is used to analyze the various elements present in the organism. EDS is an analytical technique which utilizes x-rays that are emitted from the specimen when bombarded by the electron beam to identify the elemental composition of the specimen. EDS analysis device, is possible to identify elements in soil and organisms. Energy dispersive spectroscopy which relies on the investigation of a sample through interactions between electromagnetic radiation and matter, analyzing X-rays emitted by the matter in response to being hit with charged particles. Its characterization capabilities are due in large part to the fundamental principle that each element has a unique atomic structure allowing X-rays that are characteristic of an element's atomic structure to be identified uniquely from one another.

The chemical composition of algal samples is conventionally determined by bulk analysis techniques leading to estimates elemental concentrations for the whole samples (Behrendt, 1990). X-ray microanalysis (XRMA) is important to ensure that X-rays are derived entirely from the specimen or other sources. The degree of beam penetration into the cells depends on the accelerating voltage of the electron probe and density of the specimen (Boekestein *et al*., 1980). This will reach the central part of the algal cells without over penetration into underlying support film, resulting in X-ray emission data that are representative of the main body of the cell. The absence of elemental characteristic peaks in XRMA spectra from clear areas of support film indicates that there is no detection of extraneous elements and that elements detected in cells are from genuinely present.

Cyanobacteria are also known to increase soil fertility by enhancing the available N and P levels (Singh and Bisoyi, 1989). Some species of cyanobacteria were capable of fixing elemental nitrogen to ammonia and solubilizing of insoluble phosphate reserves of soils. Thus, these organisms were capable of providing N and P to rice crop (Kaushik, 1995). The present results were in agreement with the results of Kulaev (1979), who reported that polyphosphate containing granules were actually present in living hyphae and are not artifacts caused by fixation or staining of cells.

Ca was the main counter ion in polyphosphate granules (Grellier *et al*., 1989) proposed a linkage between the phosphorus and calcium metabolism in fungal cells. Calcium is essential for growth, cell division and enlargement. It is a component of cell membranes. Calcium is an important micronutrient in an aquatic ecosystem. Magnesium is important for chlorophyll, which is important in photosynthesis. Purohit and Saxena (1990) magnesium/Calcium Ions behave like to ion exchange reaction and influence. The presence of calcium and magnesium along with their carbohydrates and sulphates make soil hard.

Zinc promotes growth hormone and enzymes system and its necessary for chlorophyll production and also the formation of starch and carbohydrate. Zinc occurs in many forms in natural waters as free ions zinc hydroxide, sulphate and zinc carbonate. Silicon is an important to strengthen the cell walls and improving health and productivity of organism. Silicon element presents in all the cyanobacteria and in the present study, this provides rigidity and strengthening of the cell wall. It enhances the physiological availability of zinc in microalgae.

Phosphorus is important in plant bioenergetics and is needed for the conversion of light energy to chemical energy during the photosynthesis. It can also be used to modify the activity of various enzymes by phosphorylation and can be used for cell signaling. Phosphorus is limited in moist soils because it is released very slowly from insoluble phosphates. Nitrogen is an essential component of all proteins and taken up by plants from the soil in the form of NO_3^-. It is important for normal plant growth and component of amino acids, protein which include nucleic acids, enzymes light harvesting pigments and chlorophyll. It is of special importance because soil nitrogen is result of biological action and is due to nitrogen fixation by blue green algae.

Potassium assists in the processes, which ensure carbon assimilation and the transport of photosynthesis through organism for storage of sugars, proteins and growth metabolism. Manganese is part of enzymes and help in photosynthesis and metabolic function of organisms. The present study shows that the cyanobacteria contain manganese. This may catalyze the photolysis. Sulphur is a structural component of some amino acids and vitamins and essential in the manufacturing of chloroplast. It can enter into the soil through agricultural waste and aquatic animal waste.

In the present study, the various chemical elements observed in *Anabaena, Oscillatoria, Azolla* and *Anabaena Azolla*. This study shows elements normally present as diffusible ions and cations (Na, K) were not present in equimolar concentration in any most cases. The imbalance between these soluble ions may be compensated by the presence of bound ions (PO_4) and detectable organic charged molecules. Fagerbakke *et al*. (1991) reported that variations in the relative concentration of Na and K have also been noted in algal cells in the present study. The results showed clear peaks of divalent cations (Ca, Mg, K, Zn and Mn) and monovalent anions (P, N, Si and S). Most of the elements showed considerable variation between samples

with percentage of major elements P and K ranging from 4 to 10% and 4.2 to 8% respectively. S showed 5.2 to 7.6%. Si showed highest variable reach very high level. Other samples showed wide variations. The major cations such as Ca, Mg, K, Zn, Mn were showed marked variations in *Anabaena, Oscillatoria, Azolla* and *Anabaena Azolla* species.

The major elements detected in *Anabaena* cells correspond to those seen in other freshwater algae (Sigee and Holland, 1997). The wide variation in elemental concentrations within the studied micropopulations was considerably greater than 3-4% limits of the technique accuracy (Ingram and Ingram, 1980; Lyman *et al*., 1990) and reflects a genuine variation within the samples. This variation has also been noted for other phytoplankton samples (Sigee *et al*, 1998), and may relate to differences such as microenvironment, physiology and cell cycle. Krivtsov *et al*. (1999c) demonstrated a remarkable ability of *Anabaena* to concentrate certain elements in its cells far in excess of its immediate requirements. Storage of potentially limiting P in polyphosphate bodies is well documented for cyanobacteria (Clay, 1992).

Growth *of Azolla* depends on the presence of sufficient concentrations of key nutrients. The most important macronutrients are K, Ca, Mg, and P (Watanabe, 1982). The effect of nutrient deficiencies on growth and nitrogen-fixation rates of *Azolla pinnata* were reported by Yatazawa *et al*. (1980). The threshold concentration of P for growth is 0.5 mM. The reported minimum P requirement of *Azolla* is 0.4% of its dry weight (Ganesh *et al.*, 2011).

The results obtained in this study demonstrate that XRMA can give a detailed characterization of particular algal constituents, providing informations on elemental concentration, ionic balance in the cells and statistical associations between individual elements and groups of elements. XRMA also permits the direct comparison of elemental composition of single species with environmental levels, allowing elemental changes in the algal populations.

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