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ULTRASTRUCTURE AND BIOCHEMICAL ANALYSIS OF *ANABAENA* AND *OSCILLATORIA sp.* (CYANOBACTERIA)

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ABSTRACT

Cyanobacteria are present abundantly in rice fields and are important in helping to maintain rice fields fertility through nitrogen fixation and involved in the photosynthesis. The present study is aimed to investigate the ultrastructure of cyanobacterial cell wall, biochemical and pigment analysis of blue green algae were isolated from the paddy field of Vallampadugai and Keerapalayam, Cuddalore District and maintained in BG11 medium for 10 days. Scanning Electron Microscopic (SEM), Trasmission Electron Microscopic (TEM) and biochemical studies such as total carbohydrates, total lipids, total protein, total free amino acid, and photosynthetic accessory pigment such as chlorophyll *a*, phycocyanin, phycoerythrin and allophycocyanin were observed in *Anabaena* and *Oscillatoria* sp. at 10^{th} day.

INTRODUCTION

Cyanobacteria are unique among the prokaryotes due to their capacity for oxygenic photosynthesis. An important feature of many cyanobacteria is their ability to fix atmospheric nitrogen both under free-living and symbiotic conditions. The species of Cyanobacteria which are known to fix atmospheric nitrogen are classified into three groups; heterocystous aerobic forms, aerobic unicellular forms and non-heterocystous filamentous forms. Furthermore, the nitrogen-fixing potentials of these organisms have also been crucial to ecosystems since 1969 (Wyatt and Silvey, 1969). They contain a diverse array of pigments, which have tremendous potential as natural dyes, antioxidants, nutritional and pharmaceutical supplements in bio industry (Glazer 1987; Dembitsky and Rezanka 2005)

Anabaena is a genus of filamentous cyanobacteria that exists as plankton. It is known for its nitrogen fixing abilities, and they form symbiotic relationships with certain plants, such as the mosquito fern (*Azolla*). *Anabaena i*s the first photoautotrophic cyanobacterium species, in which destructive photooxidative processes were described.

Oscillatoria is a genus of filamentous Cyanobacterium which is named for the oscillation in its movement and reproduces by fragmentation. *Oscillatoria* forms long filaments of cells which can break into fragments called hormogonia. The hormogonia can grow into a new, longer filament. Breaks in the filament usually occur where dead cells (necridia) are present. *Oscillatoria* uses photosynthesis to survive and reproduce. Each filament of Oscillatoria consists of trichome which is made up of rows of cells. The tip of the trichome oscillates like a pendulum.

The extensively utilized pigments in bioindustry, are the phycobiliproteins (comprising phycocyanin, phycoerythrin and allophycocyanin), which account for about 20% of total dry weight (Prasanna *et al*., 2007; Walsh, 1997) of many cyanobacteria. The phycobiliproteins represent the major photosynthetic accessory pigments in cyanobacteria, along with chlorophyll *a*. Phycobiliproteins are a family of highly soluble and reasonably stable fluorescent proteins derived from cyanobacteria. There are three basic types of biliproteins phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC).

Cyanobacteria have all three types of phycobilins: APC and PC are always present and PE is found in some organisms and not in others (Samsonoff and MacColl, 2001), but forms the most spectroscopically variable class of phycobiliproteins. Light energy absorbed by PE migrates first to PC, then to APC and finally to chlorophyll *a*. Phycocyanin is the major constituent of the phycobilisomes, while APC functions as bridge pigment between phycobilisomes and photosynthetic lamellae (MacColl, 1998). This research represents the first attempt to examine endomorphology of cyanobacteria with biochemical studies. But still there are many rice fields that remain unexplored in Keerapalayam and Vallampadugai rice fields of Cuddalore district has also received due attention it deserves. Hence, the present investigation was made to study the pigment and

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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.

MATERIAL 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 (fig1). The soil samples were collected from the paddy field

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 (Hayet and Folk, 1980).

Transmission electron microscopic (TEM) study

For ultrastructural studies, cells were fixed in 2% glutaraldehyde in the culture medium (pH 6.8) for 3 h at 4°c. They were rinsed with the medium and post fixed in 1% Os04 in distilled water for 2h at 4°c. Fixed cells were embedded in 0.1% (w/v) agar and dehydrated in a graded ethanol series followed by three changes of absolute ethanol at room temperature, followed by three changes of absolute ethanol and propylene oxide for 15 min, and finally passed through two changes of propylene oxide for 15 min each. The cells were infiltrated and embedded in spurr's epoxy resin(spur 1969) and polymerized at 70°c for 48 h. Thin serial sections were cut using a dimond knife on an RMC MT-6000 ultramicrotome (Boeckeler Instruments, Inc, Tuc-son,Az) or a sorvall MT2-B ultramicrotome (Kendro, Newton,CT) and collected on farmwar coated copper slot grids. The sections were strained sequentially with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a zeiss omega 912 or a JEOL 100 CX 11 transmission electron microscope.

Estimation of chlorophyll *a*

The estimation of chlorophyll a was done by the method of McKinney's (1941).The cultures were taken and centrifuged at 7000xg for 10 minutes. The pellets were washed with distilled water, suspended in 4 ml of 80% methanol and vortexed thoroughly. Then the tubes were covered with aluminum foil to prevent solvent evaporation and incubated in a water bath set at 60°C for 1 hour in dark with occasional shaking. After 1 hour the contents were taken, cooled and centrifuged at 7000 xg for 5 minutes. The supernatant was saved and the above procedure repeated once again to ensure complete extraction of the pigments. The pooled supernatant was made upto a known volume with 80% methanol. The absorbance was measured at 663 nm using a spectronic 20 against methanol as blank.

Estimation of Phycobiliproteins

The estimation of phycobiliproteins was done by the method of Moreno *et al.*, (1994). The cultures were centrifuged at 7000 xg for 10 minutes. The pellets were washed with distilled water, suspended in 3 ml of phosphate buffer (0.05 M) and homogenized. The contents were freeze thawed, repeated and centrifuged at 7000 xg for 5 minutes. The supernatant was stored in refrigerator. The above procedure was repeated to ensure complete extraction. The absorbance of the pooled supernatant was measured at 562 nm, 615 nm and 652 nm to quantify phycoerythrin, phycocyanin and allophycocyanin respectively against phosphate buffer as blank

Estimation of carbohydrates

The estimation of carbohydrates was done by the method of Dubois *et al.*, (1956). The cultures were centrifuged at 7000 xg for 10 minutes. The supernatant was discarded and 20 mg of pellets was taken in a test tube. Then it was hydrolyzed with 2 ml of Con. H_2SO_4 were added and mixed thoroughly. The color developed was measured at 490 nm using Spectronic 20 against glucose as blank.

Estimation of Total Proteins

The estimation of total protein was done by the method of Lowry *et al.*, (1951). The cultures were centrifuged at 7000 xg for 10 minutes. From the pellets 20 mg was treated with 10% TCA and centrifuged at 10,000 xg for 10 minutes. The resulting pellet was resuspended in 0.1N NaOH and boiled for 30 minutes, cooled and then centrifuged to eliminate light scattering materials. The supernatant was made upto known volume. To 0.1 ml of supernatant, 0.9 ml of distilled water and 5 ml of alkaline copper reagent were added and allowed to stand for 10 minutes, finally 0.5 ml of Folin-ciocalteau reagent added. The absorbance was measured after 30 minutes in Spectronic 20 against the Bovin Serum Albumin as blank

Estimation of Free amino acids

The estimation of free amino acids was done by the method of Jayaraman (1981). The cultures were centrifuged at 7000 xg for 10 minutes. From the pellets, 20 mg was homogenized in 80% ethanol. The homogenate was centrifuged at 7000 xg for 5 minutes. The clear supernatant was made upto a known volume. From this, 1 ml was pipette out into a test tube and diluted with 4 ml of distilled water. To this 1 ml of ninhydrin reagent was added and kept in boiling water bath for 15minutes. Then the tubes were cooled and 1 ml of 50% ethanol was added. The purple colour was measured at 540 nm using a spectronic 20. Standard graph was prepared by using a mixture of alanine, aspartic acid, tryptophan, proline and lysine.

Estimation of total Lipids

The estimation of total lipids was done by the method of Sato, (1988). The cultures were taken and centrifuged at 7000 xg for 10 minutes. From the pellet, 20 mg was taken, homogenized with extraction solvent (Chloroform: 2:1 (v/v)) and filtered through filter paper. The filtrate was vortexed with sodium sulphate to remove moisture. Then it was taken in a pre-weighed bottle and kept this overnight at room temperature in dark place. The dried extracts were reweighed and total lipids were estimated by subtracting the initial weight from the final weight. The amount of total lipid expressed as mg/g $^{-1}$dry weight.

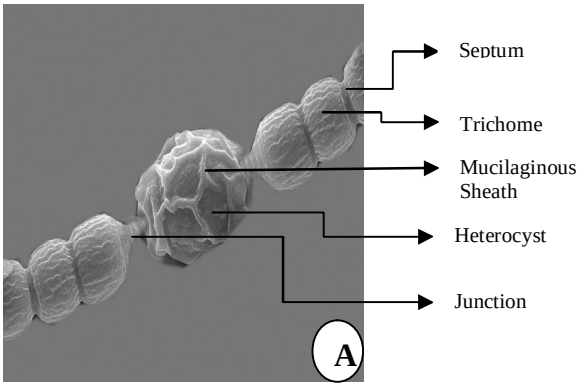
Statistical analysis

The data were statistically analyzed and the results were expressed as means ±SE of 3 independent replicates. The ANOVA is used to analyze the statistical significance.

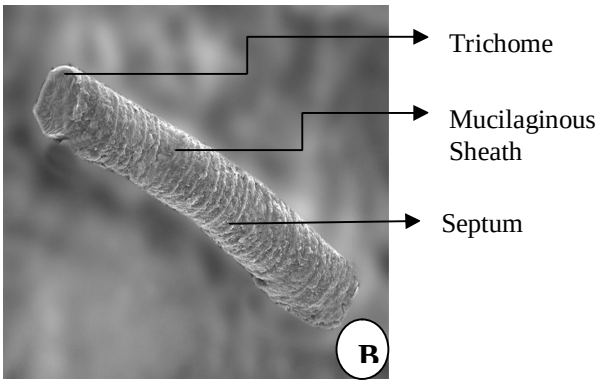
RESULTS

Scanning electron microscopic stidies (SEM) of Anabaena and Oscillatoria

Fig. 1(a,b) shows the Scanning Electron micrograph of *Anabaena* and *Oscillatoria* sp. showed trichome was long, the cell sizes were distinct. The diameter of filaments was 15- 20µm 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



Anabaena



Oscillatoria

Fig. 1 Scanning Electron Micrograph

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 fiberous layer, middle homogenous layer and inner laminated layer which cover the cell wall of the heterocyst (Fig 2a). All the layers of the outer envelope is surrounded by mucilage layer except at the junction of the heterocyst with the vegetative cell.

Ultrastructure of Anabaena and Oscillatoria

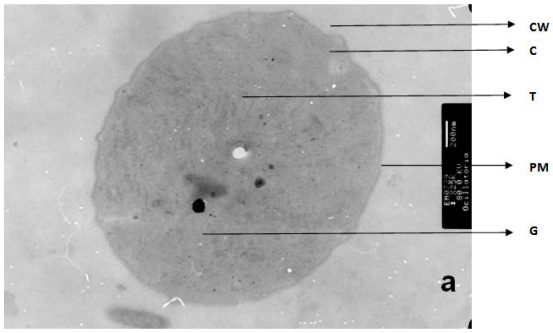
Fig.2 (a,b) shows the ultrastructure of *Oscillatoria* and *Anabaena* sp. respectively. The *Anabena* constricta shows 4 distinct layers. L_1 inner most layer of the cell. It is electron transparent layer. L_2 is a electron transluescent layer. L_3 is also electron transparent layer. L_4 is the outermost layer of the cell. Next to layer 4 there are dense mucilage is present. The mucilage is colloidal in nature. Inner to the L_1 there is thick covering named plasma membrane is clearly visible. Inside this covering there are 11 cross septum is present, and incipient formation of round shaped nucleus is present. This is the evidence for the prokaryotic cell. Thylakoids were arranged peripherally, running parallel to the cytoplasmic membrane. Cell wall is present in both species. In ultrastructure of Oscillatoria sp (Fig.2a). the thylakoid are seen within the cell. Plasma membrane is also seen. Peptidoglycon granule and α granule are seen in some area. Peripheral aggregates of the carboxysomes were also observed. The cell cytoplasmic membrane and the cell wall outer membrane were clearly seen as typical three-layer membranes. The surface of outer membrane contained depositions of capsular substance in the form of finely dispersed material.

The level of chlorophyll a

Fig.3 shows the level of chlorophyll *a* observed in *Anabaena constricta* (1.27±0.04mg/ml) and *Oscillatoria curviceps* (1.01±0.07mg/ml) at 10th day. The level of chlorophyll a was more in *Anabaena consticta than Oscillatoria curviceps*.

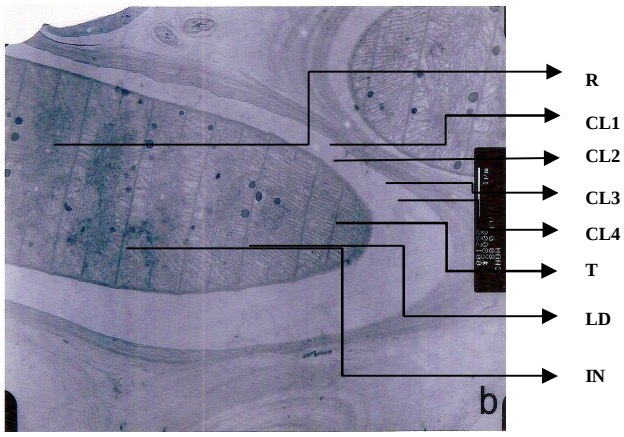
The level of biliproteins

Fig.3 shows the level of phycophiliprotein (phycocyanin, phycoerythrin, allophycocyanin). Among the pigments phycocyaninn was more in *Anabaena constricta* (5.0±0.06 mg/ml)) than *Oscillatoria curviceps* (3.4±0.05mg/ml) when compared to phycoerythrin and allophycocyanin.



CW-Cell Wall;C-Carboxysome;T-Thylakoid;PM-Plasma Membrane;G-Granule

Fig 2(a) Transmission Electron Micrograph of Oscillatoria



R-Ribosome;CL1-Cell Wall Layer1; CL2-Cell Wall Layer2; CL3-Cell Wall Layer3; CL4-Cell Wall Layer4;T-Thylakoid;LD-Lipid Droplet; IN-Incipient Nucleus

Fig 2(b) Transmission Electron Micrograph of Anabaena

The level of biochemical parameters

The Fig.4 shows the biochemical studies (carbohydrates, total protein, free amino acids and total lipids) observed in the *Anabaena constricta* and *Oscillatoria curviceps* cultured in BG11 medium. The protein content was more (1.99±0.09) than the total lipids (1.46±0.06), carbohydrates (1.28±1.02) and free amino acids (0.812±0.07) in *Anabaen aconstricta*. But in *Oscillatoria curviceps*, the level of total carbohydrates was high (3.14±0.9) when compared with total lipids (1.58±0.04), total protein (1.24±0.07) and total free amino acids (0.742±0.04).

DISCUSSION

The ultrastructure of cyanobacterial cells has been studied for several decades. However, the morphological details of the intracellular membranes in these organisms have

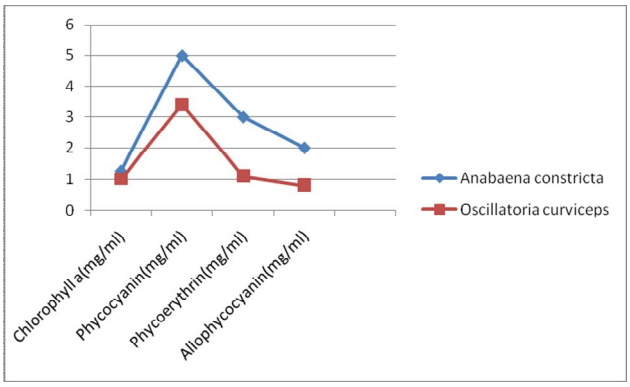


Fig 3 shows the pigment variations of Anabaena and Oscillatoria sp.

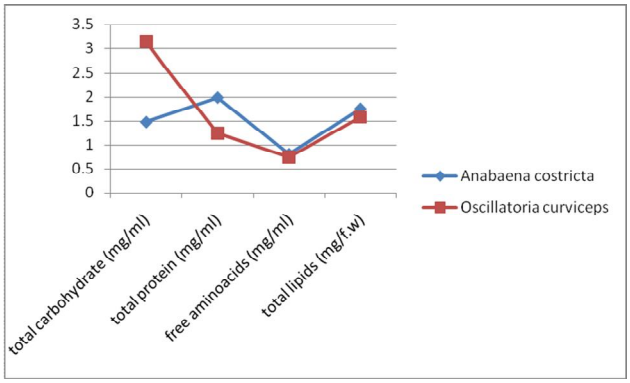


Fig. 4 Shows the biochemical parameters of Anabaena and Oscillatoria sp.

remained elusive. Meanwhile, biochemical data have begun to elucidate the important role of the plasma membrane in photosystem biogenesis (Keren *et al.*, 2005, Zak *et al.*, 2005).The present study shows that the Anabaena and Oscillatoria contain thylakoid membranes. The thylakoid membranes in cross sections appear as thin bilayers with a distinct luminal space of approximately 5-7 nm across in most cells. The anabaena and Oscillatoria appear to progress around the cell as concentric layers of proximal to the plasma membrane were also seen to form large loops and whorls in the interior of the cell. The thylakoid membrane arrangement in dividing cells was also examined in Anabaena and Oscillatoria.

In Anabaena sp. thylakoids were arranged peripherally, running parallel to the cytoplasmic membrane. In Anabaena sp. Nucleoid and Plasma membrane are seen. Lipid droplets are all area. Sheath and wall layer are present in Anabaena sp. In ultrastructure of Oscillatoria sp. the thylakoid and nucleoid are also seen within the cell. Plasma membrane is also seen. Peptidoglycon granule and α granule are seen in some area. Within the cells there were distinguished zones of nucleoid with peripheral aggregates of ribosomes, the carboxysomes, and glycogen depositions (granules). Tyagi (1973) found that heterocyst formation depends on the availability of carbon intermediaries and ATP. The former are supplied by photosynthesis and the latter by oxidative metabolism. Tyagi and Ahluwalia (1978) observed that the different wavelengths of the visible spectrum, the red light (630-680nm) was most

effective in maxuimum heterocyst production in anabaena dolibum. The cell cytoplasmic membrane and the cell wall outer membrane were clearly seen as typical three-layer membranes. The surface of outer membrane contained depositions of capsular substance in the form of finely dispersed material. Casamatta *et al.* (2005) found congruence between inferred phylogenetic relationships among strains and their thylakoid arrangement.

Baulina *et al.*, (2004) observed that the characteristic feature of these distortions is reflected by dramatic changes in ultrastructure of thylakoids. The lack of a unitary three-layer structure typical of photosynthetic membranes, which was revealed with a transmission electron microscopy, points to the impairment of the phospholipid bilayer in consistency with the conclusions reached (Schmetterer *et al.*, 1983). It is noteworthy that the destructive alterations in ultrastructure of thylakoid membranes in *A. variabilis* were observed already during the lag period preceding bleaching of Chlorophyll, Carotenoids and Phcobilisomes. The outer membrane was found to be the most resistant cell structure. On the other hand, the peptidoglycan layer of cell wall lost its rigidity and turned perforated with numerous holes and narrow canals (Baulina *et al.*, 1982). Baulina *et al.*, (2004) observed that the ultrastructural changes in Anabaena variabilis and *Synechococcus elongates.* They also observed that thylakod, cell wall, granules, lipid layer and plasma membrane in both species.

Smarda *et al.*, (2002) observed that regular polygonal pattern of cell surface in the cyanobacteria. Liberton *et al.*, (2006) stated that on the basis of serial sectioning reconstructions that the thylakoid membranes are arranged inside the cell as concentric cylinder underlying the plasma membrane, thereby creating a separate and isolated cytosolic compartment in the interior of the cell. Nierzwicki-Bauer *et al.*, (1983a) reported that the thylakoid membrane furthermore contact the plasma membrane points along the long axis of the cell. They also observed that the areas of contact between plasma membrane and thylakoid may provide a mean of communication between the plasma membrane and interior cytosol.

The present study shows the level of phycocyanin was more than phycoerythrin, allophycocyanain at 10^{th} day of culture. The present study shows the chlorophyll *a* content is lower than phycocyanin, phycoerythrin, allophycocyanain which may be due to N_2 fixing cyanobacteria. Kaushik (2000) reported that among various cyanobacterial strains, heterocystous forms *Anabaena variabilis*, *Aulosira fertilissima*, *Hapalosiphon* sp. and *Tolypothrix tenuis* can be exploited for phycobilins, Badrish *et al.*, (2006) observed the pycocyanin in Oscillatoria sp. Mishra *et al.*, (2004) observed that highest chlorophyll content in Anabaena sp. in the soil.

Kenyon *et al.* (1972) proposed that four types of fatty acid composition exist in Cyanobacteria and demonstrated some correlations with morphological properties. Caudales and Wells (1992) investigated the cellular fatty acids of benthic Cyanobacteria belonging to the genera Anabaena and Nostoc and found significant differences between those genera. Kruger *et al.* (1995) evaluated the taxonomic importance of fatty acid composition at the genus and subgenus level by analyzing the fatty acid composition of different Microcystis isolates and other members of the order Chroococcales. The present study concludes that pycocyanin is more than chlorophyll and among the biochemical substances the carbohydrate content is higher in oscilatoria which promotes the soil fertility in the paddy fields.

Lipid biomarker studies have been used extensively to characterize complex microbial communities from a variety of environments, including marine, freshwater, hydrothermal, hypersaline and cold habitats (Thiel *et al.*, 1997; Cowen *et al.*, 2003; Jahnke *et al.*, 2004; Fang *et al.*, 2006, Zhang *et al.*, 2007). Lipids are an integral part of membranes, as well as structural and storage compounds in all prokaryotes and eukaryotes. Certain lipids diagnostic of specific organism classes have been identified, such as the mid-chain methyl heptadecanes diagnostic of cyanobacteria (Shiea *et al.*, 1990; Kenig *et al.*, 1995; Köster *et al.*, 1999).

In the present study the level of carbohydrate, protein, free amino acids, lipids, were observed in *Anabaena consticta* and *Oscillatoria curviceps.* Yandigeri *et al.*, (2010) suggests that the results on total soluble proteins and total carbohydrates increased due to addition of phosphate. They also reported that the higher amounts of total soluble protein may be attributed to high amount of available phosphate content, which is present at higher concentration. Li *et al.*, (2009) reported that the reducing sugars increased rapidly within hours after development began, suggesting that this stage may be the critical phase for the synthesis of carbohydrates and the fast growth of the cyanobacterium. This suggests that carbohydrates, especially non-reducing sugars, were the main components of the sheath.

Watanabe (1951) recorded the presence of aspartic acid, glutamic acid and alanine the only free amino acids in the culture solutions of *Calothrix brevissima.* The liberation of these amino acids has also been reported in *Nostoc* sp. and *Anabaena azollae* (Venkataraman et al., 1963).Singh and Trehan (1973) also reported production of amino acids like aspartic acid, glutamic acid, proline and valine from *Aulosira fertilissima* and *Anacystis nidulans* and they found that the maximum liberation of the amino acids in the culture medium took place during the lag and stationary phase of algal growth. Mohon and Mukerji(1978) in their study attempted to detect the amino acid secretion from various N_2 fixing and non N_2 fixing cyanobacteria and concluded that in non N_2 fixing blue green algae, *Anacystis nidulans*, the type of amino acids in culture filtrates were different from those of N_2 fixing i.e. *Cylindrospermum* sp. and *Anabaenopsis raciborskii.*

Misra and Kaushik (1989b) in his study reported that a variety of amino acids were liberated during the growth phase of cyanobacteria. The presence of threonine,

glutamic acid, proline and valine were well established in external growth medium of *Nostoc muscorum.* In addition to this glycine, serine was also liberated. They also reported the presence of lysine, cystine and isoleucine from *Hapalosiphon fontinalis* in addition to that observed in *Nostoc*. Chen et al., (2008) reported growing culture *of Azolla* whole plants under controlled conditions, *Anabaena azollae* accounts for nearly 16% of the total chlorophyll and protein of the association (Ray *et al*., 1979), as well as the optimum contents of phycobiliproteins, phycoeyanin, phycoerythrin, and allophycocyanin (Tyagi *et al*., 1980). Gribovskaya *et al.*, (2009) reported that the protein, carbohydrates and lipids were found in the Oscillatoria sp. Mishra *et al*., (2004) reported that the protein and carbohydrates were present in Anabaena sp. and Calothrix sp. in the soil.

CONCLUSION

In Scanning Electronmicrospic studies of anabaena showed that specialized thick-walled resistant cell formed generally in some heterocystous blue green algae, they are longer then the vegetative cell frequently they are developed singly next to a heterocyst at the base of a trichome. The ultrastrucutural evidence of the vegetative cell wall increases several times in size and accumulate food reserves (cyanophycean granules). This is accompanied by the secretion of a thick multilayered spore wall envelope surrounding the inner investment. This endomorphology studies clearly revealed that presence of thylakoid and incipient formation of nucleus. This results support that presence of phycocyanin, phycoerythrin and allophycocyanin and the accumulation of biochemical substances in the *Oscillatoria* and *Anabaerna* sps.

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