Publications


Unusual habitat of algae

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Abstract

In a recent survey of algal diversity from different habitat of Eastern India, 3 algal genera commonly occurring in aquatic habitat and in free living condition, viz. *Chlorococcum* and *Cladophora* of Chlorophyceae and *Euglena* of Euglenophyceae were recorded away from their usual habitat. *Chlorococcum* was found in endozoic condition within slug (mollusc without shell), *Euglena* in endophytic condition within the leaf of the pteridophytic genus *Selaginella* of Eastern Himalaya region and *Cladophora* was found to grow in terrestrial condition near the bank of the river Matla of the marine region of Sundarban mangrove forest of India.

Key words- Unusual habitat/ Endozoic/ Endophytic/ Sundarban/ Autotrophic/ Heterotrophic

Introduction

The algae are of widespread occurrence in moist situations as tree trunks, walls, wood, rocks and damp soil where they frequently occur as an extended stratum consisting of either a single species or a mixture of species. In addition to these there are some other algae growing on some unusual habitats. These algae includes those growing endophytic in other plants, leaf epiphytes, on mollusces, calcareous rocks, snow algae, thermal algae, epizoic algae and certain others (Smith, 1950). The habitats of algae are mainly classified into three categories viz. aerial habitats, aquatic habitats and unusual habitats. Aerial algae have been defined as algae that obtain their water wholly or in large part from moisture in the air. Strictly aerial algae are found on the bark and leaves of trees, on wood, stones, and rock and on soil surface. Most of these algae belong to the class Chlorophyceae. *Protococcus*, *Trentepohlia* and *Prasiola* are conspicuous members of the aerial flora (Smith, 1950). Aquatic algae are generally growing on floating waters, ponds, lakes, pools, ditches, bogs and swamps. The algae of running water are more diversified than those of any other aquatic habitats and include a larger percentage of species restricted to the particular habitat. The main algal classes of aquatic habitat are Chlorophyceae, Bacillariophyceae, Chrysophyceae, Euglenophyceae etc. Finally algae growing on unusual habitats are broadly classified into the following categories- cryophytes or snow algae, thermal algae, halophytic algae, lithophytes, epiphytes and symbiotic algae (Sambramury, 2005). Some macroalgal habitat ecology was studied by Nyberg (2007). Smith (1950) reported some fresh water algae on different aquatic sources from the United States.

Material and methods

Algal samples were collected in free-living condition from the aquatic habitats of Eastern Himalaya and Sundarban. The specimens were divided into two parts, one part was preserved in 4% formalin (v/v) solution for voucher collection and microscopic study whereas the other part was taken in a polythene bag for culture and cultivation in the laboratory. The samples preserved in formalin solution were taken for slide preparation using 20% glycerine (v/v). Digital photographs were taken in Carl Zeis Axiostar Plus microscope by Canon Power Shot 500D Camera. Unialgal cultures were set up using Bold Basal Medium (Bold, 1942) to induce the reproductive structures for proper identification. The genera were identified using proper monographs (Smith, 1950; Prescott, 1976; Kargupta et al., 1992 and Krishnamurthy, 2000).

Results

Taxonomic description of the genera

1. *Chlorococcum infusionum* (Schrank) Menegh (Pl 1, Figs. 9-11)

Smith, 1950, p.224, fig. f & g.

New Habitat: Endozoic growth in molluscs, brackish water, Bakkhali, Sundarban, India (N 20° 01.935’, E 088° 80.955’).

Free living, unicellular, green, cells are solitary, sometimes embedded in a gelatinous matrix, striking variation in size shows between various cells when the alga grows in an expanded stratum. Young cells...
are thin walled and spherical or somewhat compressed, old cells have thick walls that are often irregular in outline, the thickened portion of a wall are often distinctly stratified, young cells are 40-125 μm in diameter and mature cells are 150-210 μm in diameter, chloroplasts of young cells are parietal massive cups, completely filling the cell except for a small hyaline region at one side, they contain one pyrenoid, as a cell increases in size, the chloroplast usually becomes diffuse and contains several pyrenoids, the cells are uninucleate until shortly before reproduction.

2. Cladophora nitellopsis Boergesen (Pl 1, Figs. 4-8)

Boergesen, 1939, figs. 11-13; Dixit, 1970, p. 106; Nizamuddin & Begum, 1973, figs. 38-39; Krishnamurthy, 2000, p.146, fig. 21H.

New habitat: Marine, Hamanbere Island, Sundarban, India (N 22° 00.117´, E 088° 42.609´).

Filaments subdichotomous in the lower parts, alternately branched above, cells of main axis cylindrical, 125 μm in diameter and 180-250μm long, with thick cell walls up to 25 μm, branches in lower parts at long intervals but in upper parts, frequent, almost one branch from each cell, cells of branches 75 μm in diameter, 7-10 times as long, with cells 45 μm thick, 2-3 times as long.

3. Euglena gracilis Klebs (Pl 1, Figs. 1-3)

Smith, 1950, p.353; Prescott, 1982, p. 393, pl. 85, fig. 17.

New habitat: Fresh water, endophytic in Selaginella leaf, Eastern Himalaya, India, (N 27° 03´, E 88° 19´).

Unicellular, green, uniflagellate free swimming cells are continually changing in shape as they move through water, the cells are fusiform to acicular and with the posterior and more or less pointed, the single flagellum is bifurcate at its lower end and with a granular swelling at the point of branching, cells are 5-20 μm long and 3-15 μm broad and having an eyespot at the anterior end, the chloroplasts are numerous and discoid to band shaped, they may be with or without pyrenoids, division may take place while the cells are motile or after they have come to rest, division in the motile condition is longitudinal and begins at the anterior end.

Plate 1: Showing some unusual habitat of algae

Fig 1. Euglena gracilis in the leaf margin cells of Selaginella sp (20μm); Fig 2. Euglena cells growing endophytically within the stomata of Selaginella leaf (20μm); Fig 3. Isolated Euglena from Selaginella leaf in laboratory cultural condition (20μm)

Fig 4. Cladophora nitellopsis in the terrestrial zone of Hamanbere Island, Sundarban (2cm); Fig 5. Showing formation of grass like mat under simple microscope (2cm); Fig 6. Showing branching pattern under compound electron microscope (200μm)
Discussion

Endophytic *Euglena* population became prominent within sterilized hyaline leaf tissue (Inoculum) of *Selaginella* sp, during the tissue culture processing (Pl 1; figs. 1 & 2). The *Cladophora* population formed a green mat like grass land (Pl 1; fig. 4), much above the sea level on Hamanbere Island (7 to 10 ft) of Sundarban delta. The inundation of algal bed is not very regular, except the spring tide and neap tide waters. The endophytic *Euglena* grew well both in autotrophic and heterotrophic media, though growth rate of endozoic *Chlorococcum* was very slow.

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Chemical characterization and the stress induced changes of the extracellular polysaccharide of the marine cyanobacterium, *Phormidium tenue*

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**Abstract**

The cyanobacterial strain *Phormidium tenue* was subjected to different stress conditions like culture aging, phosphate and nitrate depleted condition, excess nitrate (10mM) and salinity (0.9M NaCl). Excess nitrate and salinity used were just the double amount, required for normal concentration. Growth was monitored by Chlorophyll content and total protein level in relation to polysaccharide production. Remarkable increase in EPS contents were noted in all stresses given, which were almost 1.7, 2, 3 and 4 times more in excess nitrate condition, phosphate depleted, nitrate depleted and high salinity respectively. It was also observed that aging is an important factor for increasing EPS production. Exocellular polysaccharide (EPS) from this marine cyanobacterium was characterized using GLCMS and stress induced variation in total production was studied in batch culture mode. The neutral sugar composition of *Phormidium* biomass was identified by gas liquid chromatography showing monosaccharide compositional analysis.

Key words:- Culture aging, Extra cellular polysaccharide, Filamentous cyanobacteria, Gas-chromatography, Nutritional stress, Phormidium tenue.

**Introduction**

Cyanobacteria are photoautotrophic organisms having prokaryotic cell structure share many characters with bacteria in spite of the fact that their photosynthetic metabolism resembles that of aerobic photosynthetic eukaryotic algae. Many cyanobacteria are able to synthesize outermost slimy investments and to release polysaccharidic material into the culture medium during cell growth. These polysaccharides, are of enormous interest in view of their possible uses in several industrial applications. This EPS with high biotechnological potential is much easier to exploit further, unlike the plant system.

Cell wall polysaccharides have been proved to be helpful in energy storage, in maintaining the structural integrity and mechanical strength, controlling of osmotic pressure, buffer layer that protects against drought and infective organisms such as viruses,bacteria, fungi (Arad 1988,1999; Kloareg and Quatrano 1988; Lapsin and Prici 1995; Arad and Richmond,2004). Among the algal (including cyanobacteria) bio-chemicals of commercial importance, algal polysaccharides vary in structural and functional properties based on the type of organism and the growth conditions. Polysaccharides from algal sources have been found to possess a variety of biological activities which may find application in cosmetic,food and pharmaceutical industries (Morris et al 2001,Chen et al 1994;Tache et al 2000; Berteau and Mulloy 2003).

In the present study, variation in total EPS production, related to other growth parameters of *Phormidium tenue* in long term controlled batch culture mode was studied in different nutritional condition. Chemical characterization of the neutral sugars present in the EPS was performed by Gas Liquid Chromatographic study.

**Materials and Methods**

**Organism and Culture Conditions:-**

The cyanobacterial strain *Phormidium tenue* was procured from National Facility for Marine Cyanobacteria (NFMC), Tiruchirapalli, Tamilnadu, India. The cyanobacterial strain is filamentous, multicellular organisms, having breadth 1–1.5 μm. The strain is maintained in batch culture mode under sterile conditions in Artificial Sea Nutrient III liquid medium, containing the salts (g L⁻¹): NaCl 25 gm; MgCl₂ 6H₂O 2 gm; KCl 0.5 gm; NaNO₃ 0.75 gm; K₂HPO₄ 3H₂O 0.02 gm; MgSO₄ 7H₂O 3.5 gm; CaCl₂ 0.5 gm; Citric Acid 0.003 gm; Ferric Ammonium Citrate 0.003 gm; EDTA 0.0005 gm; Na₂CO₃ 0.02 gm; Trace Metal Mix A5 1 ml containing (mg mL⁻¹): H₂BO₃, 2.86 mg; MnCl₂·4H₂O, 1.81 mg; Na₂MoO₄·2H₂O, 0.390 mg; ZnSO₄·7H₂O, 0.222 mg; CuSO₄·5H₂O, 0.079 mg and Co(NO₃)₂·6H₂O, 0.0494 mg. The pH was maintained at 7.5 after sterilization. The culture sets are maintained by regular transfers into fresh liquid medium at 20°C in 16/8 hour light/dark cycle under cool fluorescent light having light intensity 20-30 μmol photons m⁻² s⁻¹.

**Experimental design:-**

Each experimental set is inoculated with known amount of live bio-mass of exponential growth phase. One set was maintained as control. The sets are subjected to different stresses like a) culture aging b) PO₄ deficiency c) NO₃ deficiency d) 10mM NO₃ conc. e) 0.9M NaCl. Bio-mass were harvested at regular intervals of 7 days from 14 days after inoculation (acclimatization period). Samples were taken in triplicate from one culture for extracellular polysaccharide, protein and chlorophyll estimation. Axenity of the cultures was checked by plating on agar medium and by microscopic observation.

**Extraction of EPS:-**

Most of the extraction procedure so far reported, dealt with EPS which were released in the culture medium. The main obstacle faced here is that the filamentous algal strain, do not release EPS in this way. The extraction procedure was modified from the standard protocols (Li et al 2001, Helm et al, 2000) for better extraction. For chemical characterization and biochemical assay, biomass of known weight were taken and EPS were extracted first with dH₂O followed by 4M NaOH solution. Different procedures such as 1M NaCl, EDTA salt, 0.1-0.2 H₂SO₄ were also tested. However, best result was obtained when extracted with dH₂O followed by 4M NaOH solution. Bio-mass was washed with ethanol and loosely bound polysaccharide fraction was extracted by dH₂O. The cell-free supernatant was separated by centrifugation and 90% ethanol (3times) was added and kept overnight in 4°C. The precipitated polysaccharide was collected by centrifugation. The residual biomass was treated with 4M NaOH at 90°C for 1 hour. The cell-free supernatant was collected by centrifugation and to it, 90% ethanol (3times) was added and kept overnight in 4°C. The precipitated polysaccharide was collected by centrifugation and washed with alcohol till it was free from residual alkali. The residue after 4M NaOH extraction which contained the intact cells was again washed with dH₂O to remove alkali, grinded in presence of 10% TCA and kept overnight in 4°C. The amount of extracellular polysaccharide present in the precipitate from dH₂O and 4M NaOH fractions were quantified by the Standard phenol-sulfuric acid method (Dubois et al,1956).

The polysaccharides were further purified by repeated precipitation with ethyl alcohol. The sugars present in the purified polysaccharides were analyzed by GLC-MS, Chlorophyll (Arnon, 1949) and protein (Lowry et al, 1951) were estimated following standard protocols.

**Fourier transform infrared spectroscopy:-**

Fourier transform– infrared spectroscopy (FT-IR) were performed on KBr plate. FT-IR spectra were recorded on a Jasco 410 instrument, with a resolution of 4 cm⁻¹. Spectra were obtained in the 4000-400 cm region.

**Monosaccharide analysis:-** Purified samples (1-2 mg) were hydrolyzed with 2N TFA at 120°C for 2 h in sealed glass tube to produce monosaccharides. For the detection and estimation of sugar by GLC as their alditol acetates, the liberated monosaccharides were reduced with sodium borohydride followed by neutralization with aqueous acetic acid to adjust its pH to 4. The resulting alditol was acetylated and traces of the reagents were removed by repeated co-evaporation with dry toluene. The neutral sugars were analyzed as alditol acetates by GLC-MS analysis. A Hewlett Packard 5890 plus GC tandemly linked to a
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

JEOL mass spectrophotometer (JEOLAX-500) with electron impact ionisation (EI) at 70 eV and ion source temperature at 200°C was used. For resolution, DB-5MS capillary column (0.25mm, 0.25μ, 30m) was used using temperature programming (150°C-2min-5°C/min-200°C-10min). Analysis were carried by using a HP-5 column equipped with Agilent Chemstation software.

**Uronic acid estimation:** Galacturonic acid was detected by paper chromatography and GLC. The sample (5 mg) was hydrolyzed by 2N trifluoroacetic acid (2 ml) in a sealed tube at 120°C. The acid was removed under reduced pressure in a rotavapour and traces of acid was removed by co-distillation with water. The sample was then analyzed by paper chromatography using solvent [acetic acid-water-pyridine-ethyl acetate, 1:3:5:5 (v/v)]. The spots were visualized by using alkaline-silver nitrate reagent. In a separate experiment, the hydrolyzed sample was heated with anhydrous methanolic HCl in a sealed tube at 100°C for 12 hours. The HCl was removed in a rotavapour and traces of acid was removed by repeated co-distillation with anhydrous methanol. The resulting methy glycoside methyester of uronic acid was acetylated as describe above. The resulting compound was analyzed as mentioned earlier. In both cases, standard samples of glucuronic acid and glucuronic acid was used for comparison. The galacturonic acid was estimated by using colourimetric method (REF) using m-hydroxy diphenyl (Blumenkratz *et al*, 1973).

**Results**

The results clearly showed that extracellular polysaccharide production changed in both control and different stress conditions given. In control condition, EPS content gradually increased up to 56 days of culture. In phosphate depleted condition, the EPS production also increased but with a greater rate and was maximum in after 28th days which was almost double compared to the control. Growth was measured by estimating chlorophyll content, increased up to 28 days of culture in control set, whereas the experimental biomass growth rate decreased after 14 days and remained almost stationary up to 56th day (Fig.1a). Protein content increased a bit throughout the experimental tenure in control, and a gradual increase was observed in total protein content up to 28th day under phosphate depleted condition (Fig.1b).
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

The results from nitrate depleted condition depicted that EPS content increased thrice the amount upto 28\textsuperscript{th} days of exposure, whereas chlorophyll content followed the similar trend as in phosphate depleted condition (Fig.2a). Protein content soared high by 3 times after 21st days in nitrate depleted condition (Fig.2b). Under excess nitrate (10 mM) condition, the EPS production was greatly enhanced by three times upto 56\textsuperscript{th} day. Chlorophyll content went up upto 14 days and then remain almost static (Fig.3a) whereas protein content showed a similar trend but a higher rate than control. (Fig.3b). The EPS production greatly elevated under salt stress condition. It reached almost 4 times after 28\textsuperscript{th} days of exposure (Fig.4a). The chlorophyll content gradually decreased after 21\textsuperscript{st} day, showing the ultimate death of biomass though protein content followed almost similar trend as in control. (Fig.4b).
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

Fig 2b: Variation in protein content in control and nitrate depleted condition with function of time.

Fig 3a: Variation in EPS and chlorophyll content in control and excess nitrate (10 mM) condition with function of time.
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

**Fig 3b:** Variation in protein content in control and excess nitrate (10 mM) condition with function of time.

**Fig 4a:** Variation in EPS and chlorophyll content in control and salinity stress (0.9M NaCl) with function of time.
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

A broad peak in FT-IR spectra (Fig.5) appeared in 3426 cm⁻¹ region corresponds to hydroxyl groups present in the polysaccharide. Peaks appeared in 1135-1542 cm⁻¹ was due to C-H bending vibration. The C=O absorption of uronic acids occurred at 1651 cm⁻¹. A sharp peak at 618 cm⁻¹ may be due to the presence of unsaturation within the polysaccharide. The GLC-Mass spectrum of all the monosaccharides are shown in Fig 6. Analysis of the neutral sugars were carried by by GLC-MS analysis using a HP-5 column equipped with Agilent Chemstation software. The constituent sugars were found to be Rhamnose, Fucose, Xylose, Mannose, Glucose and Galactose in 2:3:2:3:8:2 ratio in the exopolysaccharides of *Phormidium*. The glucuronic acid (60%) was estimated by colourimetric assay.

**Fig 4b:** Variation in protein content in control and salinity stress (0.9M NaCl) with function of time.

**Fig 5:** FT-IR spectrum (400-4000 cm⁻¹) of pure exopolysaccharide of *P. tenue*. 
Stress induced changes of the extracellular polysaccharide of *Phormidium tenue*

Discussion

The present knowledge of cyanobacterial polysaccharides indicates that there lies a great opportunity, if the different parameters influencing the productivity of the exopolymer is better understood for bio-technological exploitation. A very important feature for cyanobacterial polysaccharides is that in some cases, the production changes during cell growth due to the presence or absence of certain factors. Several conditions like, energy availability and the C:N ratio, controlling the production of the cyanobacterial EPS have been identified (De Philippis & Vincenzini, 1998; Li et al., 2002). The role of nutritional factors influencing the production of cyanobacterial EPS is also an interesting field to study further.

In the present study, age of culture play an important role in increasing EPS content of *Phormidium* biomass. Probably nutritional stress condition is the main controlling factor of producing EPS in batch culture condition of cyanobacteria. Jones and Yopp (1979) also found that the extracellular carbohydrates increased with the age of cultures of *A. halophytica*. It was also corroborated from the previous study that many algae produce polysaccharides, mainly when they enter stationary growth phase (Hellebust, 1974).

Phosphate limitation almost doubled the EPS production. Similar results were also obtained in *Synechococcus* (Roux, 1996) and in *Cyanothecae* (De Philippis 1993). Growth decreased after 14 days of exposure in phosphate deficiency. As previously reported (Healey, 1982) no certain relationship has been found between growth rates and phosphate concentrations in the present study. The relationship between the available amounts of phosphate and the production of EPS is also not clearly understood, as the overall effect might be dependent on a set of interlinked variables such as the amount of phosphate, nitrate and sulphate (Grillo & Gibson, 1979, Sara Pereira et al. 2009).

In *P. tenue*, nitrogen deficiency also resulted in 3 fold increase of EPS production. Nitrogen starvation has well been described as a condition that enhances EPS synthesis in *Cyanothecae* (De Philippis et al., 1993), *Nostoc* (Otero & Vincenzini, 2003), probably because this contributes to the increase in the C: N ratio, which plays a critical role in the production of exopolysaccharide (Cho et al. 2001). Elevated C:N ratio results in ample availability of carbon for the incorporation into the
exopolymers, thus producing more EPS (Otero & Vincenzini, 2003; Kumar et al., 2007, Sara Pereira et al. 2009). Growth was elevated at initial stage but afterwards it deceased as a result of nutrient limitation. Excess nitrogen affects the EPS production possibly in the opposite way. The result obtained in this study, also supports the same depicting that the EPS production did not amplify much as compared to the other stresses given. Excess nitrate do not affect EPS significantly probably because it is more metabolisable source of nitrogen compared to ammonium or urea which significantly induces EPS production (Roux et al., 1996). Interestingly, growth was almost linear upto 21st day and started to decline while EPS production began to enhance. Therefore, from the observed data and earlier reports (Roux et al., 1996), it can be stated that an increase in nutrient availability would not affect the EPS production; however, an increase in biomass would be expected.

The data obtained from the salinity (0.9M) stress in P. tenue showed great increase in EPS production which is almost 4 fold. It is well-known that extra cellular polysaccharide functions as an osmotic solute protecting membranes from desiccation (Chen et al., 2006). Under salt stress, cyanobacteria exports large amounts of EPS which improves salt tolerance and carbohydrate metabolism (Chen et al., 2003). During the experimental tenure, salinity stress became lethal in long term exposure resulting in the death of bio-mass.

In the present experiment, protein content also got enhanced or remained unchanged in all stress conditions, compared to that of control, indicating that the carbohydrate synthesis and protein formation, both were not hindered but stimulated due to stress exposure.

From the composition of the neutral sugars, it was evident that the structural architecture of the extra cellular polysaccharide is highly complex in nature similar to algal systems (Sara Pereira et al. 2009). It contained 6 different sugars as Rhamnose, Fucose, Xylose, Mannose, Glucose and Galactose in the ratio of 2:3:2:3:8:2.

In conclusion, different responses in polysaccharide production were observed under different stress conditions in Phormidium tenue. These results indicate that polysaccharide production, triggered by diverse conditions may be due to different mechanisms of polysaccharide synthesis. Thus, the strain can be well utilized as a source of EPS for biotechnological purposes.

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