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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 composition as Rhamnose, Fucose, Xylose, Mannose, Glucose and Galactose. It was evident from the study that the structural architecture of the extra cellular polysaccharide of Phormidium is highly complex in nature as common in algal system.

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

Introduction

Cyanobacteria are photoautotrophic organisms having prokarytic 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, controling 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 vareity 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).

Extra cellular polysaccharide extraction from Rhophycean and Pheophycean genera are extensively studied. (Arad *et al* 1988, Singh *et al* 2000, Shrestha *et al* 2004, Ramus 2008, Davis *et al* 2003, 2004,Wustman *et al* 1997 etc.). Among the cyanobacterial genera, strains like, *Nostoc*, *Anabaena*, *Arthrospira*, *Mastigocladus* etc have been studied so far (Helm et al 2000, Otero et al 2004, Xia et al 2001, Gloaguen et al 1999). Studies on algal EPS are performed extensively on the chemical, structural and rheological attributes (Belinger al 2010, Gloaguen et al 1999, Li et al 2001, Richert et al 2005). Influence of different growth factors, physical parameters, nutritional availability, aging etc. on the EPS production are some other key parameters, essential to be studied for proper exploitation.

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 1-1.5µm. The strain is maintained in batch culture mode under sterile conditions in Artificial Sea Nutrient III liquid medium, containing the salts (gmL^{-1}), NaCl 25gm; $MgCl_2$, $6H_2O$ 2 gm; KCl 0.5 gm; $NaNO_3$ 0.75 gm; K_2HPO_4,$3H_2O$ 0.02 gm; $MgSO_4$,$7H_2O$ 3.5 gm; $CaCl_2$ 0.5 gm; Citric Acid 0.003 gm; Ferric Ammonium Citrate 0.003 gm; EDTA 0.0005 gm; Na_2CO_3 0.02 gm; Trace Metal Mix A5 1 ml containing (mg mL^{-1}) H_3BO_3, 2.86 mg; $MnCl_2$,$4H_20$, 1.81 mg; Na_2MoO_4;$2H_2O$, 0.390 mg; $ZnSO_4$,$7H_2O$, 0.222 mg; $CuSO_4$;$5H_2O$, 0.079 mg and $Co(NO_3)_2$;$6H_2O$, 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) PO_4^- defficiency c) NO_3^- defficiency d) 10mM NO_3^- 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 extra-cellular polysaccharide,protein and chlorophyll estimation. Axenity of the cultures was checked by plating on agar medium and by microscopic observation

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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 starin, 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 dH_2O followed by 4M NaOH solution. Different procedures such as 1M NaCl, EDTA salt,0.1-0.2 H_2SO_4, were also tested. However, best result was obtained when extracted with dH_2O followed by 4M NaOH solution. Bio-mass was washed with ethanol and loosely bound polysaccharide fraction was extracted by dH_2O. The cell-free supernatant was separeted 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 1hour. 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 extrction which contained the intact cells was again washed with dH_20 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 dH_2O and 4M NaOH fractions were quantified by the Standard phenol-sulfuric acid method (Dubois *et al*,1956).

The polysachharides 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.

Monosachharide analysis:- Purified samples (1-2 mg) were hydrolyzed with 2N TFA at 120ºC 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 aquous 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

JEOL mass spectrophotometer (JEOLAX-500) with electron impact ionisation (EI) at 70 ev and ion source temparature 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 trifluroacetic 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-sliver nitrate reagent. In a separate experiment, the hyrolyzed sample was heated with anhydrous methanolic HCl in a sealed tube at 100^0C for 12 hours. The HCl was removed in a ratovapour 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 compund was analyzed as mentioned earlier. In both cases, strandard samples of glucuronic acid and glacturoinc acid was used for comparision. The galacuronic acid was estimated by using colourimentic method (REF) using m-hydroxy diphenyl (Blumenkratz *et al*,1973).

Results

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

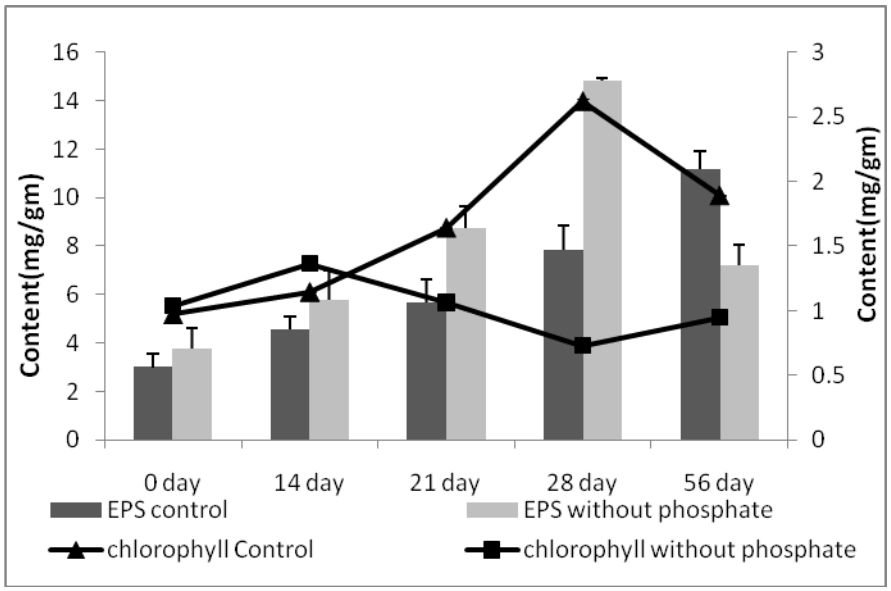

Fig 1a:- Variation in EPS and chlorophyll content in control and phosphate depleted condition with function of time

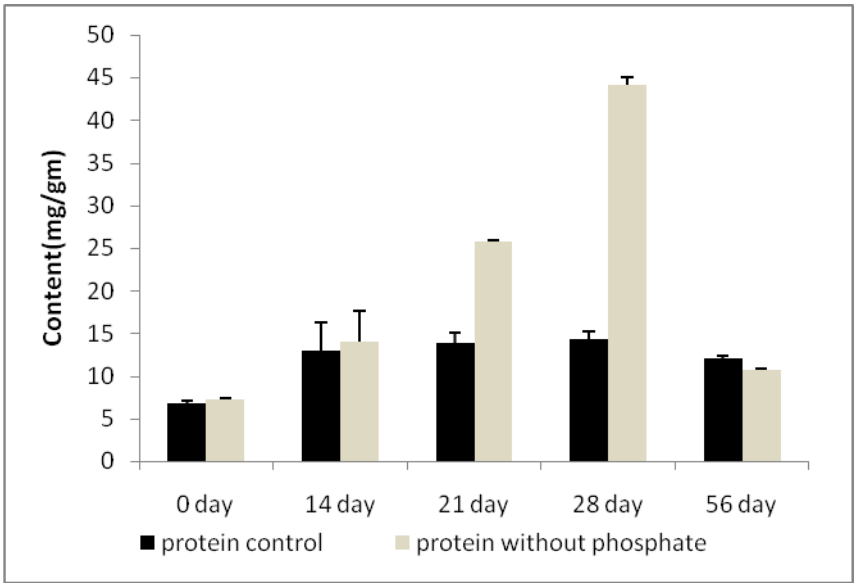

Fig 1b:- Variation in protein content in control and phosphate depleted condition with function of time

The results from nitrate depleted condition depicted that EPS content increased thrice the amount upto 28th 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 56th 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 28th days of exposure (Fig.4a). The chlorophyll content gradually decreased after 21st day, showing the ultimate death of biomass though protein content followed almost similar trend as in control. (Fig.4b).

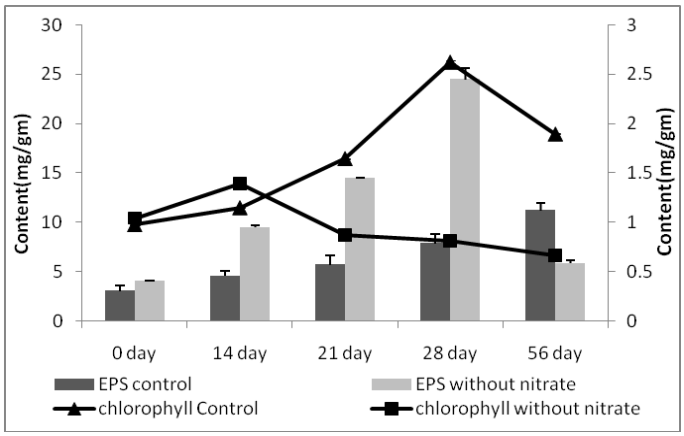

Fig 2a:- Variation in EPS and chlorophyll content in control and nitrate depleted condition with function of time

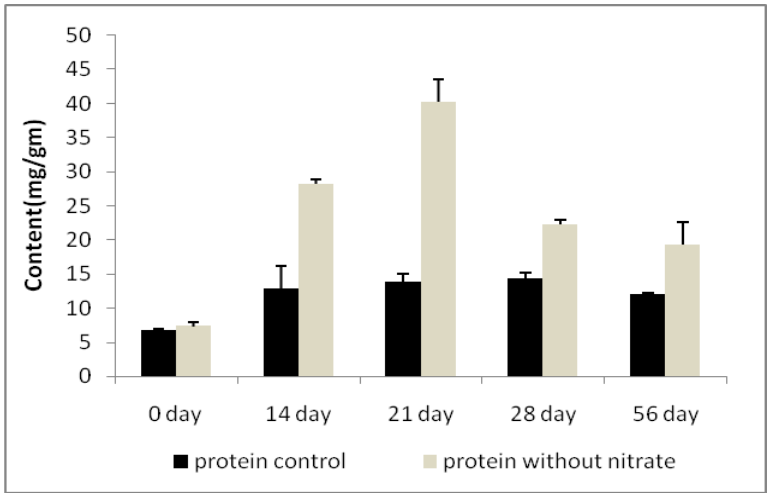

Fig 2b:- Variation in protein content in control and nitrate depleted condition with function of time.

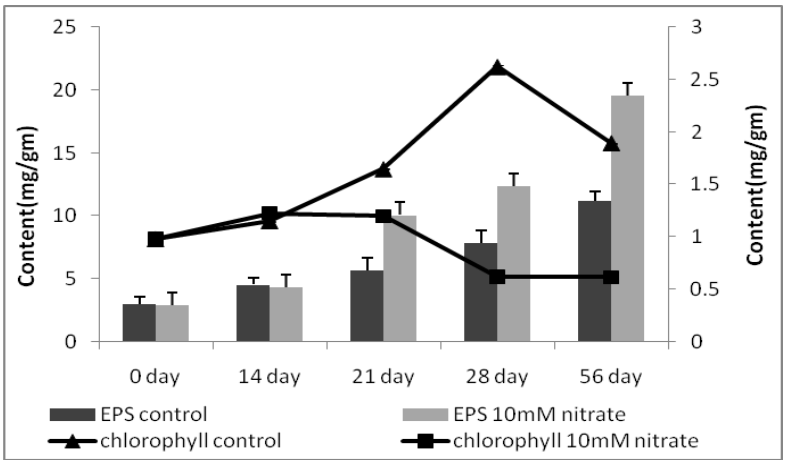

Fig 3a:- Variation in EPS and chlorophyll content in control and excess nitrate (10 mM) condition with function of time

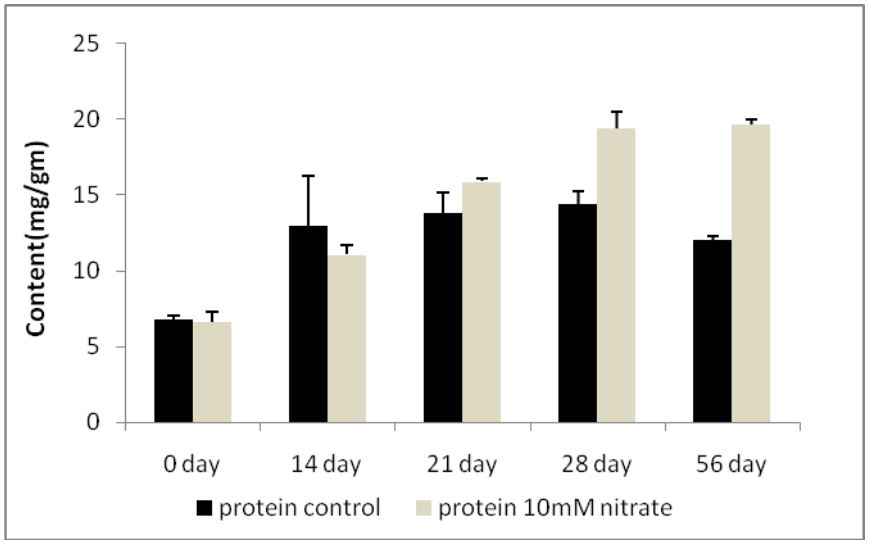

Fig 3b:- Variation in protein content in control and excess nitrate (10 mM) condition with function of time.

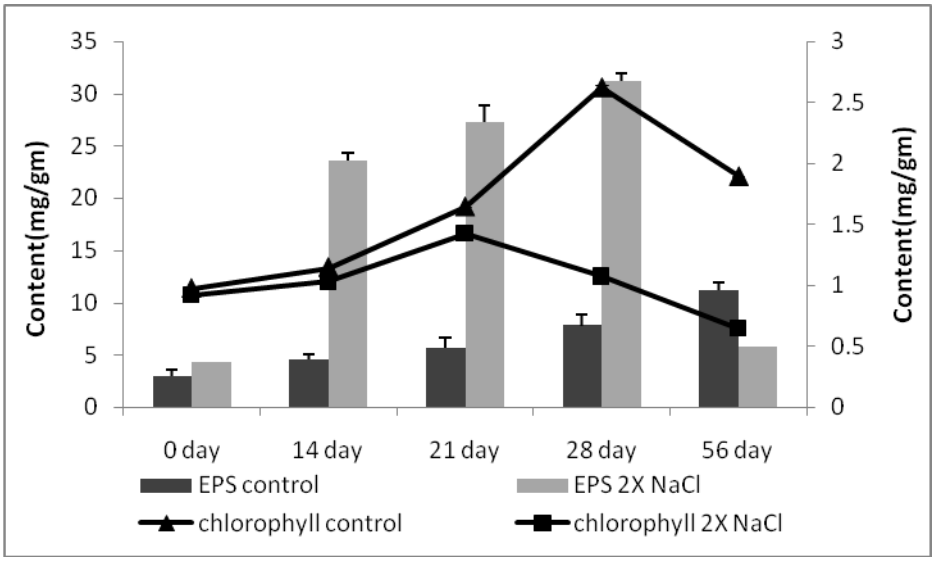

Fig 4a:- Variation in EPS and chlorophyll content in control and salinity stress (0.9M NaCl) with function of time

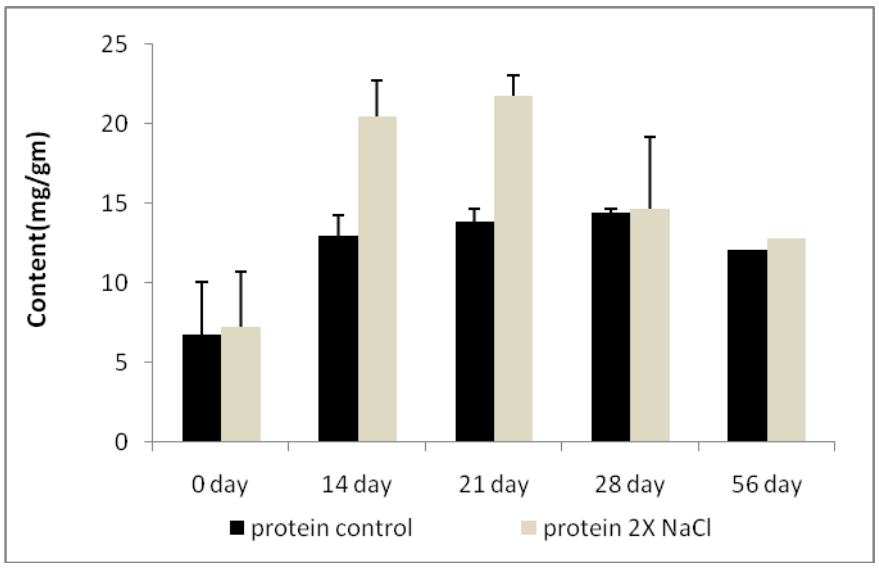

Fig 4b:- Variation in protein content in control and salinity stress (0.9M NaCl) with function of time.

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 occured 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 glacuturonic acid (60%) was estimated by colourimetric assay.

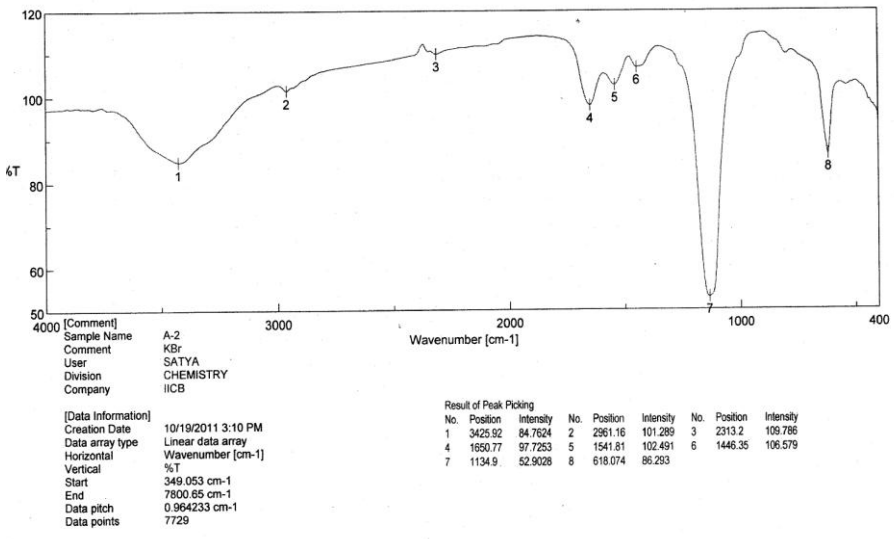

Fig 5:-FT-IR spectrum (400-4000 cm^{-1}) of pure exopolysachharide of *P. tenue*.

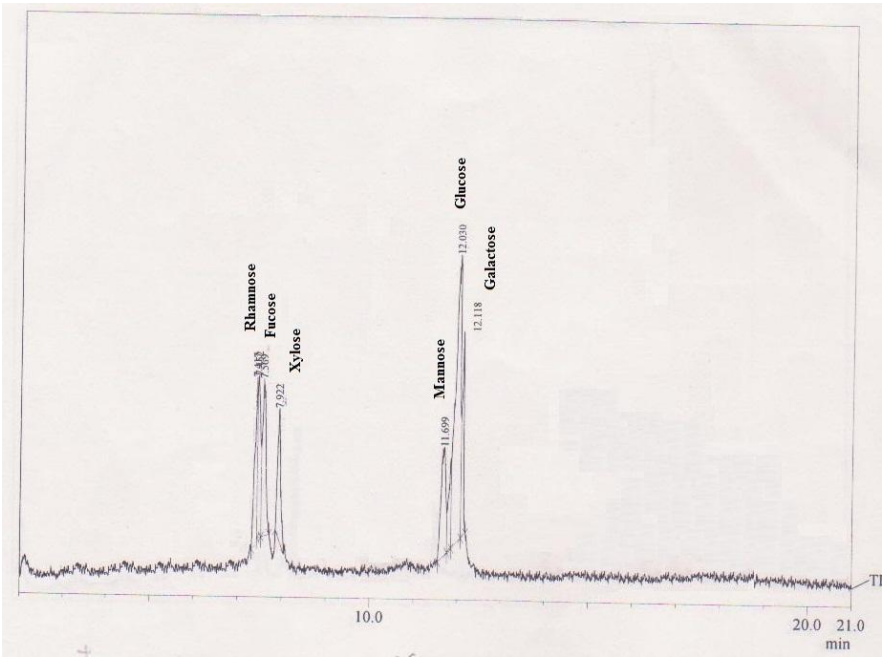

Fig 6:- Total ion chromatogram of the alditol acetates showing the neutral sugar composition.

Discussion

The present knowledge of cyanobacterial polysaccharides indicates that there lies a great oppportunity, 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 *Cyanothece* (De Philipps 1993). Growth decreased after 14 days of exposure in phosphate defficiency. 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 defficiency also resulted in 3 fold increase of EPS production. Nitrogen starvation has well been described as a condition that enhances EPS synthesis in *Cyanothece* (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 architechture 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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