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RESEARCH ARTICLE

Extracellular Polysaccharides of the cyanobacterium- *Leptolyngbya tenuis*: Structural characterization and its compositional changes during stress exposure

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Abstract

Economically important and widespread marine cyanobacterium *Leptolyngbya tenuis* produces significant amount of extraellular polysaccharidic material (EPS).This prokaryotic biopolymer has vast significance in understanding of different interesting activities like biofilm formation, bio-remediation of metal wastes etc. Biotechnology industries like food, cosmetic and medicine industries are positively influenced by this type of polymer. The ability of this polymer to change its chemical composition due to stress exposure is another fascinating feature. To understand these properties better, bound exopolysaccharides produced by cyanobacterial strain *Leptolyngbya tenuis* has been analysed by Gas chromatography, 2D nuclear magnetic resonance spectroscopy (NMR) and Mass spectrometry. Analysis of monosaccharidic composition of EPS was done from the biomass in control condition, as well as subjected to stress conditions like, variations in nitrate, phosphate and salinity level of the growth medium. The results indicated the presence of six sugars (fucose, galactose, glucose, mannose, rhamnose) showing glucose as predominant one in normal growth medium, but in stress exposed biomass synthesis of 'new' (not present in control) pentose sugar arabinose was observed and rhamnose disappeared in all stress exposed conditions.NMR studies for structure determination revealed three fractions, of which, partial structure of two were determined in this study by 2D NMR and ESI-MS analysis.

INTRODUCTION

Production of exopolysaccharides (EPS) or additional surface structure is a conspicuous process of cyanobacteria – the most primitive prokaryotic plants of earth, appeared 3.5 billions years ago. Cyanobacterial EPS release is the result of the physiological process related to photoassimilation of carbon (Viera and Myklestad 1986).Therefore, several cyanobacterial strains possess additional surface structures, outside their outer membrane, differing in thickness, consistency and appearance. Structurally they are of three types- namely, capsules, sheath and slime (De Philippis and Vincenzine 1998). Among these, capsule and sheath give a definite shape of the thallus, whereas slime is almost amorphous. The prokaryotic biopolymers are important materials for their varied interesting activities like biofilm or colony formation and are responsible for adhesion to solid surface. Having a strong metal chelation capacity or presence of sulphur in the polymer, their potential use in different field of biotechnology like, food industry, waste water treatment and medicine industry are well appreciated now-a-days. (Sutherland, 1990; Pulz &

Koehler, 1994; Weiner, 1997; De Philippis & Vincenzini 1998).Owing to the attracting future, productivity and chemical nature of EPS from a number of cyanobacterial taxa have been studied so far (Bertocchi *et.al* 1990; Flaibani *et. al.,* 1989; Helm, *et al*., 2000; Huang *et.al* 1998; Morvan *et.al* 1997; Vincenzini *et.al*. 1990; 1993). But most of the works have been done with soluble RPS or released polysaccharides in the medium, which are not very cost effective for collection and exploitation for biotechnological purposes. Furthermore, to utilize this polymer in different biotechnological fields, a detailed knowledge about the biosynthetic mechanism and structural attributes of the polymer is of critical importance. In our previous publication we have tried to understand the variations in bound EPS production in *Phormidium tenui* (collected from NFMC) exposed to different stress conditions related to other growth parameters (Chakraborty et.al 2012). In the present communication, structural characterization of bound EPS of *Leptolyngbya tenuis* (Komarek*)* by NMR study, together with changes in monosaccharidic sugar composition in stress exposed biomass have been investigated to know further about the unique properties of this cyanobacterial EPS.

2. Materials and Methods

2.1 Cyanobacterial culture set up: - The marine cyanobacterial strain was collected from coastal areas of West Bengal and unialgal cultures were developed. Initially it was inoculated in 1.5% solid agar plates in aseptic condition. Agar plates were then incubated at 298.15 K, 16:8 light dark cycles, until colonies develop. With the help of proper microscopic studies, the cyanobacterial strain was identified and confirmed to be axenic. After establishment of individual culture on solid media, they were transferred to liquid media.

2.2 Media preparation:- The strain is filamentous, multicellular, having breadth 1-1.5µm and was maintained in batch culture mode under sterile conditions in Artificial Sea Nutrient III liquid medium, containing the salts (KgL^{-1}), NaCl 0.025kg; $MgCl_2$, $6H_2O$ 0.002 kg; KCl 0.0005 kg; $NaNO_3$ 0.00075 kg; $K_2HPO4,3H_2O$ 0.00002 kg; $MgSO_4,7H_2O$ 0.0035 kg; $CaCl_2$ 0.0005 kg; Citric Acid 0.000003 kg; Ferric Ammonium Citrate 0.000003 kg; EDTA 0.0000005 kg; Na_2CO_3 0.00002 kg; 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;2H2O, 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 were 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}.

2.3 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- deficiency c) NO_3- deficiency d) 10mM NO_3- conc. e) 0.9M NaCl conc. Bio-mass were harvested at regular intervals of 7 days from 14 days after inoculation (acclimatization period). Axenity of the cultures was checked by plating on agar medium and by microscopic observation.

2.4 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, Chattopadhyay *et al* 2007) for better extraction.

The modified procedure is given in flowchart as follows-

Bio-mass was first refluxed with acetone in a Soxhlet apparatus for 3 hrs

↓

Bio-mass was taken out and air-dried

↓

EPS was extracted with double distilled water (1:100) at 90ºC for 3hrs under constant stirring

↓

This process was repeated twice

↓

Residue was separated from the extract by filtration through glass filter

↓

The filtrate was concentrated and subjected to dialysis

2.5 Purification:-The recovered polymer was re-dissolved in water and further purified by repeated precipitation with ethyl alcohol (4vol).The final precipitate was then subjected to lyophilization. The amount of extracellular polysaccharide was quantified by the Standard phenol-sulfuric acid method (Dubois *et al*, 1956).

2.6 Differential interference contrast microscopy: DIC, also known as Nomarski Interference Contrast (NIC) or Nomarski microscopy has been used to identify the peptidogycan layer around the cyanobacterial sample. This illumination technique using an inverted microscope (TiU Eclipse, Nikkon) has been used to enhance the contrast. DIC images has been acquired by separating a polarized light source into two orthogonally polarized mutually coherent parts which are spatially displaced (sheared) at the sample plane, and recombined before going to the camera (DS-Ri1, Nikkon). The interference of the two parts at recombination is sensitive to their refractive index and geometric path length. Using such differences the images were taken (Fig 1).

2.7 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. (Fig 2)

2.8 Monosachharide analysis: - Purified samples (1-2 mg) were hydrolyzed with 2N TFA at 393.15K 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 473.15K was used. For resolution, DB-5MS capillary column (0.25 mm x 0.25 μ x 30 m) was used using temperature programming (423.15K-2min-278.15K/min-473.15K -10min). Analyses were carried by using a HP-5 column equipped with Agilent Chemstation software.

2.9 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 393.15K**.** The acid was removed under reduced pressure in a rotavapour and traces of acid were 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 373.15K for 12 hours. The HCl was removed in a ratovapour and traces of acid were 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 colourimentic method (REF) using m-hydroxy diphenyl.

3. Partial structure determination of the EPS:-

3.1 Gel permeation chromatography:-

For hydrolysis 20 mg of EPS was taken and mixed with 1N TFA. It was then heated at 393.15K in a sealed tube for 1 hour. After that the crude mass was co-evaporated with water under reduced pressure to free excess TFA. The residue was dissolved in 1ml double distilled water and subjected to Gel permeation chromatography.

Gel permeation chromatography was performed using Bio-Gel P-2 fine and medium (45-90 μm hydrated bead diameter, 100x1.0 cm) for the purification of oligosaccharide and desalting.

For all size exclusion chromatography, glass distilled and degassed water was used as eluent. Eluents were monitored using Shimadzu refractive index detector (RID-10A) and LKB 2238 SII UV detector fitted with a dual channel recorder (LKB-Bromma 2210). Fractions (2.5 ml) were collected using a fraction collector (Pharmacia Frac-100).

3.2 Methylation analysis:-

The oligosaccharide (2 mg) was dried over P_2O_5 overnight and was dispersed in absolutely dry dimethyl sulphoxide (1 ml) with continuous stirring at room temperature or at a slightly elevated temperature (333.15K) when necessary. Finely powdered sodium hydroxide (40 mg) was made using morter and pestle in an atmospheric bag under nitrogen atmosphere and was added to the solution under anhydrous condition. The mixture was stirred vigorously for 30 min at room temperature under nitrogen atmosphere. The solution was then cooled to 288.15K in an ice-water bath and dry and distilled methyl iodide (1 ml) was added drop wise by a syringe under anhydrous condition. The mixture was stirred at 288.15K for 15 min and then at room temperature for further 15 min. The reaction mixture was again

cooled to 288.15K and chilled water (1 ml) was added to it. Sodium hydroxide was then neutralized with 25% cold aqueous acetic acid under cold condition and the methylated OS was extracted with chloroform (3 x 5 ml). The chloroform extracts were combined and the solvent was removed under reduced pressure. Trace of DMSO was removed under high vacuum. The methylated OS was then purified by passing through Sephadex LH-20 column (30x1 cm) and using acetone:chloroform (1:1, v/v) as eluent. Appropriate fractions were collected, pooled, evaporated to dryness to give methylated oligosaccharide. Complete methylation was confirmed by the absence of hydroxyl group by I.R. spectroscopy and the partially methylated sugars were finally identified as their partially methylated partially acetylated alditols by GLC.

3.3 NMR analysis:-

One dimensional ^{13}C NMR spectroscopy was performed using Bruker DPX-600 spectrometer in the Fourier transform mode, with or without proton coupling at 75 MHz. Spectra were recorded in $CDCl_3$ at room temperature.

One dimensional ^{1}H NMR spectroscopy was carried out with Bruker DPX-600 spectrometers. The samples (3-4 mg each) were dissolved in $CDCl_3$ and lyophilized. This procedure was repeated thrice to ensure complete deuterium exchange. Finally, the materials were dissolved in $CDCl_3$ and the spectra were recorded at $25^{O}C$ at room temperature.

Two dimensional DQF-COSY, TOCSY, NOESY, HSQC and HMBC NMR spectra of OS (in D_2O) were recorded using a Bruker 600 MHz instrument at $25^{O}C$.

3.4 Mass-Spectroscopy:

3.4.1 Electrospray-Mass spectroscopy (ESI-MS)

Ion cyclotron resonance Fourier transform ESIMS was performed on a Micromass ZQ instrument (Waters). The OS was dissolved in chloroform at a concentration ~20 ng μL^{-1} and sprayed at a flow rate 2 µl min^{-1}. Capillary entrance voltage was set to 3.0 kV and drying gas temperature to 393.15K.

3.5 Determination of absolute configuration of sugars:-

To the dried OS (~2 mg) in a sealed tube, S-(+)-2-butatanol (0.5ml) and catalytic amount of TFA (10 µl) were added under anhydrous condition and heated at 373.15K for 16 h with occasional shaking. The solutions were then concentrated to dryness under reduced pressure in rotavapour and traces of acid was removed by repeated co-evaporation with minimum volume of methanol at 35°C. Finally, 2-butatanol was completely removed under high vacuum and the sample was dried over P_2O_5. The butyl glycosides were then acetylated using anhydrous pyridine and acetic anhydride [1:1 (v/v), 1 ml] at 363.15K for 45 min. The reagents were removed in a rotavapour under reduced pressure and traces of pyridine and acetic anhydride were removed by co-evaporation with dry toluene. D-glucose, D-galactose were also converted to S-(+)-2-butyl glycoside derivatives by using same procedure as described above. The absolute configuration of the sugars were then ascertained by Glc using suitable column by comparison with S-(+)-2-butyl glycosides of authentic standards. The entire sugar residues have been found to have D configuration.

4 Results and Discussion:

4.1 Differential interference contrast microscopy analysis:-

Some strains of cyanobacteria are well-known for their capability to excrete mucilaginous material. Indeed, several cyanobacterial strains possess, outside their outer membrane, additional surface structures, mainly of a polysaccharidic nature, that comprise a wide variety of outermost investments differing in thickness, consistency and appearance. These structures, in spite of the rather arbitrary terminology sometimes used, can be referred to as sheaths, capsules and slimes. The sheath is somewhat as a thin, electron dense layer loosely surrounding cells or cell groups. The DIC image (Plate 1a, b) of *Leptolyngbya* shows such thin sheath like structure hence confirming the presence of extracellular polysaccharide.

4.2 Fourier transform infrared spectroscopy analysis:-

Peaks in FT-IR spectra appeared in the range of 3300-3400 cm^{-1} corresponds to hydroxyl groups present in the polysaccharide of the experimental cyanobacteria (Fig 1). Peaks appeared in 1380 or 2125 cm^{-1} was may be due to C-H bending vibration, the absence of azide was confirmed by chemical analysis. The C=O groups of uronic acids occurred at 1608-1650 cm^{-1}. A sharp peak at 618 cm^{-1} indicated the presence of unsaturation in the polysaccharide of *Leptolyngbya tenuis.*

4.3 Monosachharide analysis:-

The EPS of *Leptolyngbya tenuis* from control biomass was found to be comprised of six identifiable and two non-identifiable monosachharides. Fucose, rhamnose, xylose, glucose, galactose and mannose were present in control condition (Table-1, Fig. 2). It was observed that unlike the other species of *Leptolyngbya* studied earlier, where

galactose was the main component sugar, in *L. tenuis* glucose was produced in maximum amount. In nitrate depletion (Fig 3) and in excess nitrate stress (Fig 4), significant amount of arabinose synthesis took place, especially in excess nitrate. In other stress conditions also, like phosphate depletion (Fig 5), and excess salinity (Fig 6), arabinose was recorded though absent in control condition, On the other hand, rhamnose was recorded in control but absent in all other stress conditions.

Overall monosaccharide composition of the EPS of the *Leptolyngbya* showed that they were composed of different monomers and numbers of monomers were usually more than four. The monosaccharides found were all pentoses and hexoses. Presence of galactose was common in in both control and stress condition. Other monosachharides varied both quantitatively and qualitatively when subjected to different stress conditions.

The cyanobacterial EPS are complex heteropolysaccharides, with 75% of the polymers composed of six or more different kinds of monosaccharides. The results obtained are more or less similar with the earlier reports of cyanobacterial samples. This feature contrasts with the polymers synthesized by other bacteria or macroalgae, which contain a lower number of different monomers, usually, less than four (De Philippis & Vincenzini 1998). To date, up to 12 different monosaccharides have been identified in cyanobacterial EPS, the hexoses like, glucose, galactose, mannose and fructose, the pentoses like ribose, xylose and arabinose, the deoxyhexoses, fucose, rhamnose and methyl rhamnose, and the acidic hexoses, glucuronic and galacturonic acid (De Philippis & Vincenzini 1998,2003; De Philippis et al.2001). In cyanobacterial EPS, glucose is most frequently recorded monosaccharide, although there are polymers where other sugars, such as xylose, arabinose, galactose or fucose, are present at higher concentrations than glucose (Tease et al. 1991; Bender et al. 1994; Gloaguen et al. 1995; Fischer et al. 1997; De Philippis & Vincenzini 1998, 2003; Parikh & Madamwar 2006).

In different species belonging to *Leptplyngbya*, previously examined, mannose was an important component of both RPS and CPS i.e released and cell wall polysaccharides (Gloaguen et al. 1995; Morvan et al. 1997); in contrast this hexose was totally absent for the strain studied by Nicolaus (1999) whereas in *L.tenuis* mannose was absent only in excess nitrate condition.

Monosaccharide compositions of cyanobacterial EPS showed high degree of variations in stress conditions. A few number of available reports indicated that these responses were also very much species specific and may even vary in different culture set ups. Ozturk et al (2010) found that the ratio of glucose to other monosaccharides decreased following cyanobacterial exposure to NaCl but in *L.tenuis*, glucose ratio increased. Briefly, it can be said that synthesis of pentose and hexose sugar is dependent on experimental factors and these variable compositions of polysaccharides may be important for industrial applications.

In general, the carbohydrate polymer composition was altered in nutrient-limited conditions, indicating that nutrient depletion used to influence the biosynthetic pathways (Piedras et al 2010). It is known that the flow of carbon to different biosynthetic pathways is modulated through regulation of several enzyme activities (Konopka 1980). Generally in all plants, glucose is the primary photosynthetic product and then other sugars are synthesized. In higher plants UDP-D-glucose functions as a donor of glucose for the synthesis of cellulose at the plasma membrane (Amor *et al*, 1995). UDP-D-galactose is also generated from UDP-D-glucose via a freely reversible 4-epimerization reaction involving an enzyme based 4-keto intermediate (Maitra and Ankel 1971).Therefore the present study revealed that galactose is synthesized during EPS production in control condition and the production of galactose was comparatively higher. Galactose synthesis remained almost same in all stress conditions except in nitrate depleted condition where it increased to a considerable amount.

Again UDP-L-arabinose is synthesized from UDP- D xylose via a freely reversible 4-epimerization reaction by the activity of UDP-D xylose-4-epimerase. Arabinose content was also found to be enhanced in excess nitrate stress in *L.tenuis*. Hence, for this study it can be assumed that different enzymes play a crucial role for the observed variations in the monosaccharide composition of cyanobacterial EPS especially in stress conditions. A further strain-specific detailed study of genes and metabolic pathways and the genes involved in the production of EPS in cyanobacteria is required for better understanding.

4.4 Estimation of uronic acid:-

Uronic acids were detected and the ratio of glucuronic acid and galacturonic acid was determined by GLC as their completely silylated derivatives (Fig.7). Total uronic acid present in the samples was estimated by colourimetic method.

Uronic acids of the EPS was found to be glucouronic and galactouronic acid in the ratio of 1:2.

Uronic acids of the EPS samples were quantified using the method of Blumenkranz et al (1973) using m-Hydroxybiphenyl reagent. From the standard curve, the amounts of uronic acids present in the EPS of the samples were measured.

According to the earlier reports, the anionic nature of the polymers was ensured by the presence of one or two acidic sugars, namely, glucuronic and galacturonic acids. However, it has to be stressed that it is quite difficult to obtain

reliable quantitative data for uronic acids from hydrolyzed polymers, owing to their easy degradation (Cesaro *et al* 1990). The presence of uronic acids in cyanobacterial EPS may be considered quite usual (Vincenzini *et al* 1990) whereas there are too few data concerning the presence of sulfate and pyruvate groups to draw a general picture.
The use of cyanobacterial or microalgal biomass in remediation processes, such as the removal of heavy metals from polluted waters, has been investigated under several aspects, but in most cases only strains devoid of capsules were adopted (Wilde & Benemann, 1993; Garnham, 1997). On the other hand, cyanobacterial cells surrounded by thick polysaccharidic capsules or slimy investments should possess a larger number of binding sites for metal ions compared to non-capsulated strains. Indeed, most cyanobacterial polysaccharides have abundant uronic acid subunits (De Philippis & Vincenzini, 1998) which, owing to their carboxyl groups (Urrutia, 1997), may efficiently bind metal ions. Thus, the presence of ample amount of uronic acids in thre EPS of the studied sample seems quite promising for metal chelation.

4.5 Partial structure determination of the EPS of *Leptolyngbya tenuis*

Among the four experimental taxa, *Leptolyngbya tenuis* has been found to produce maximum EPS in most of the experimental conditions. So, the exocellular polysaccharide produced by this strain was characterized partially by the help of different techniques. The EPS was found to be composed of six identifiable and two non-identifiable monomers (Fuc, Rham, Xyl, Man, Gal, Glu) and two uronic acids (GalA and GlcA). Oligosaccharides have been isolated using Gel permeation chromatography and partial structure was determined by a combination of 1D and 2D NMR, mass spectrometry, sugar composition and linkage analysis. The polysaccharide was observed to be composed of three fractions as revealed by permeation chromatography.

4.5.1 Gel permeation chromatography:-

After gel permeation of the EPS sample three fractions were obtained. The fractions were evaporated to dryness and dried over P_2O_5. It was then acetylated using standard protocol (Acetic anhydride: pyridine = 1:1, 5 ml). The mass thus obtained was then dissolved in $CDCl_3$ and subjected to NMR analysis.

4.5.2 Methylation analysis:-

The sugars were finally identified as their partially methylated, partially acetylated alditols by GLC. The results obtained are given below

4.5.2.1 Analysis of the 1st fraction

Sugars	Linkages	% composition
2,3,4,6-Me_4-Glc	Glc-(1→	1
2,3,4-Me_3-Glc	→6)-Glc-(1→	1
2,3,6-Me_3-Glc	→4)-Glc-(1→	1

In the methylated samples, three partially methylated sugars namely, 2,3,4,6-tetra-O-methyl-D-glucitol, 2,3,4 tri-O-methyl-D-glucitol and 2,3,6-tri-O-methyl-D-glucitol were detected.

When compared with the sugar analysis data, it was found that in the methylation analysis, all three glucose residues are present in approximate equimolar amount.

4.5.2.2 Analysis of the 2nd fraction

Sugars	Linkages	% composition
2,3,4,6-Me_4-Gal	Gal-(1→	1
2,3,6-Me_3-Gal	→4)-Gal-(1→	1
2,3,6-Me_3-Glc	→4)-Glc-(1→	1

In the methylated OS (second fraction), three partially methylated sugars namely, 2, 3, 4, 6-tetra-O-methyl-D-galacitol, 2,3,6-tri-O-methyl-D-galaccitol and 2,3,6-tri-O-methyl-D-glucitol were detected.

When compared with the sugar analysis data, it was found that in the methylation analysis, two different galactose and one glucose residues are present in approximate equimolar amount.

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4.5.2.3 Analysis of the 3rd fraction

The GLC analysis of the 3rd fraction did not show presence of any sugar molecules. It may contain some organic and inorganic compounds other than known sugar residues. Similar observation was made in NMR and ESI spectra.

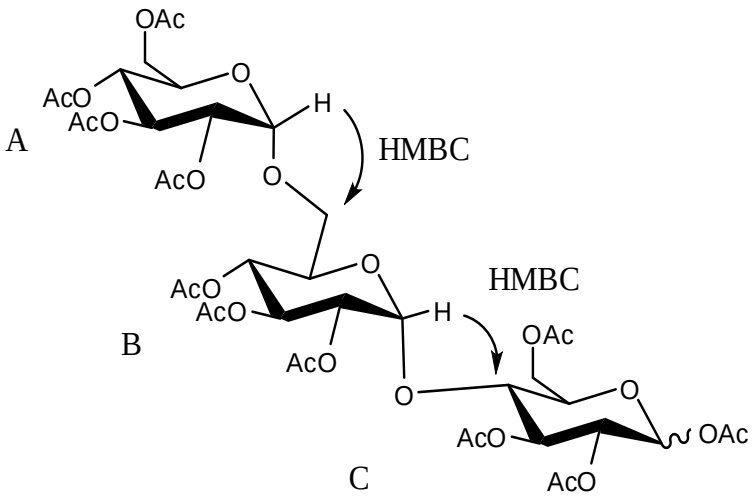
4.5.3 NMR and Mass analysis of each fraction:-

4.5.3.1 Analysis of the 1st fraction

One dimensional and two dimensional spectra were recorded in a Bruker 600 MHz/150 MHz instrument at 25 °C using $CDCl_3$.

In ^1H NMR spectrum (Fig 8) peaks appeared in the range of δ = 1.9-2.4 ppm were due to the methyl protons of acetyl groups. The ring protons appeared in the range of 3.5-6.3 ppm. Similarly in ^{13}C NMR (Fig 9) peaks appeared in the range of δ = 20.58-20.94 ppm were due to the methyl carbons of acetyl groups. The ring carbons appeared in the range of δ = 61.74-95.99 ppm. The corresponding quaternary carbons of the acetyl groups appeared in the range of 168.97-170.03 ppm. However for detailed analysis, HMBC (Fig 10), HSQC (Fig 11) and COSY (Fig 12) analysis of the sample was carried out.

From HSQC experiment it was confirmed that the anomeric proton and carbon appeared at 5.15, 5.16, 5.36 ppm and 95.98, 95.79, 95.57 ppm respectively. The ring methylene carbon signals of the sugars appeared between 61.74-75.00 ppm. The formation of trisaccharide was confirmed by ESI-MS experiment (Fig 24). The excess carbon signal at anomeric region may be due to the formation of both α, β isomer, formed during acetylation (^{13}C NMR expanded fig, not included). The C-6 of each three molecule appeared at 61.74, 62.72 and 62.82 ppm which were further confirmed by DEPT-135 experiment. The exact confirmation and linkages of three glucose residues were determined by 2D experiments. From methylation analysis it was confirmed that three glucose residues are linked via 1→6 and 1→4 linkages. In HMBC experiment the anomeric proton of 'A' showed strong correlation with C-6 of sugar 'B'. It was further confirmed as two H-atoms attached to the C-6 of 'B' showed strong correlation with C-1 of 'A'-sugar. In the same experiment H-1 of sugar 'B' showed strong correlation with C-4 of 'C' sugar. All the sugar molecules were linked by α-glycosidic linkages which can be confirmed by the coupling constant of anomeric proton of sugar residues of 'A' and 'B'.



4.5.3.2 Analysis of the 2nd fraction

One dimensional and two dimensional spectra were recorded in a Bruker 600 MHz/150 MHz instrument at 25 °C using $CDCl_3$.

In ^{1}H NMR spectrum (Fig. 13) peaks appeared in the range of δ = 1.99-2.23 ppm was due to the methyl protons of acetyl groups. The ring protons appeared in the range of 3.68-6.25 ppm. Similarly in ^{13}C NMR (Fig. 14) peaks appeared in the range of δ = 20.51-20.91 ppm was due to the methyl carbons of acetyl groups. The ring carbons appeared in the range of δ = 61.18-101.33 ppm. The corresponding quarternary carbons of the acetyl groups appeared in the range of 168.98- 170.52 ppm. However for detailed analysis, HMBC (Fig.15), HSQC (Fig 16) and COSY (Fig. 17) analysis of the sample was then carried out.

From HSQC experiment it was confirmed that the anomeric proton and carbon appeared at 4.51, 4.44, 6.25 ppm and 89.02 and 100.6 ppm respectively. The ring methylene carbon signals of the sugars appeared between 61.18, 61.6 and 62.77 ppm. The formation of trisaccharide was confirmed by ESI-MS experiment (Fig.25). The C-6 of each three molecule appeared at 61.18, 61.6 and 62.77 ppm which was further confirmed by DEPT-135 experiment. The exact confirmation and linkages of three sugar residues were confirmed by 2D experiments. From methylation analysis it was confirmed that three sugar residues are linked *via* 1→4 and 1→4 glycosidic linkages. It was further confirmed by HMBC experiment. In HMBC experiment, the

H-1 of 'A' showed strong correlation with C-4 of 'B'sugar and H-1 of sugar 'B' showed strong correlation with C-4 of sugar 'C'. All three sugar molecules have β-configuration which was confirmed by NOE-experiment. Anomeric proton of three sugar molecules did not show any spatial correlation with corresponding H-2, however have found to show strong correlation in COSY experiment, signifying the all three sugars have β-configuration.

4.5.3.3. Analysis of the 3rd fraction

One dimensional and two dimensional spectra were recorded in a Bruker 600 MHz/150 MHz instrument at 25 °C using $CDCl_3$.

In ^{1}H NMR spectrum (Fig.21) peaks appeared in the range of δ = 1.8-2.1 ppm was due to the methyl protons of acetyl groups. The ring protons appeared in the range of 3.1-5.4 ppm. Similarly in ^{13}C NMR (Fig. 22) peaks appeared in the range of δ = 20.14-22.54 ppm was due to the methyl carbons of acetyl groups. The ring carbons appeared in the range of δ = 50.36-105.28 ppm. From ^{13}C NMR spectrum it was also found that the anomreic carbons appeared at 82.74, 88.93, 92.09, 94.49, 98.18, 99.28, 101.44, 103.28 ppm, confirming the presence of eight sugar residues.

4.6 Electrospray-Mass spectroscopy (ESI-MS)

4.6.1 Analysis of the 1st fraction

ESI-MS spectra showed a peak (m/z) at 988.97 [M+Na] and 1005.06 [M+K] indicating the sample was a peracetylated trisaccharide (Fig.24).

4.6.2 Analysis of the 2nd fraction

ESI-MS spectra showed a peak (m/z) at 988.9 [M+Na] and 1005.01 [M+K] indicating the sample was a peracetylated trisaccharide (Fig.25)

4.6.3 Analysis of the 3rd fraction (crude)

ESI-MS spectra showed peaks (m/z) at 144.04 [M_1+Na], 172.97 [M_2+Na], 189.01[M_3+ Na], 206.95 [M_4+Na], 304.96 [M_5+Na], 334.97 [M_6+Na], 346.95 [M_7+Na], 364.97 [M_8+Na] (Fig. 26).

4.7 Determination of absolute configuration of sugars

The absolute configuration of the sugars were then ascertained by GLC using suitable column by comparison with S-(+)-2-butyl glycosides of authentic standards. The entire sugar residues have been found to have D-configuration.

Similar structural study of cyanobacterial EPS was done by a number of authors. Identification of oligosaccharide from capsular polysaccharide of *Mastigocladus laminosus* was done by Gloaguen et al (1995) and they reported 7 different monosccharides. Acidic hydrolysis of purified polysaccharide led to the isolation four oligosaccharidic fractions. NMR spectroscopic studies of two of the four fractions allowed them to be identified as: alpha-GlcA-(1-->2)-alpha-GalA-(1-->2)-Man and alpha-GlcA-(1-->2)-alpha-GalA-(1-->2)-beta-Man-(1-->4)-beta-Gal-(1-->2)-Rha. In another study, EPS produced by *Cyanospira capsulata* was also analyzed by NMR spectroscopy by Garazzo et al (1998).The oligosaccharides released, have been isolated by weak anion exchange and aqueous size exclusion chromatography. It was further characterized by a combination of 1D and 2D NMR, Mass spectrometry, sugar composition and linkage analysis. The polysaccharide has an octasaccharide repeating unit. The polysaccharide extracted from *Aphanothece sacrum* was analyzed by Okajima-Kaneko et al (2007). From their ^{1}H NMR study it revealed that dimethylated fucose unit was the major sugar. The combination of sulfate group and fucose in the prokaryotic polysaccharide was first evidenced by the direct spectroscopic studies. In the present study, it was observed that the first fraction of the extracted EPS contained three glucose residues. Two galactose and one glucose unit were found in second fraction. The third fraction indicated the presence of rhamnose, xylose, fucose, mannose along with glucose and galactose.

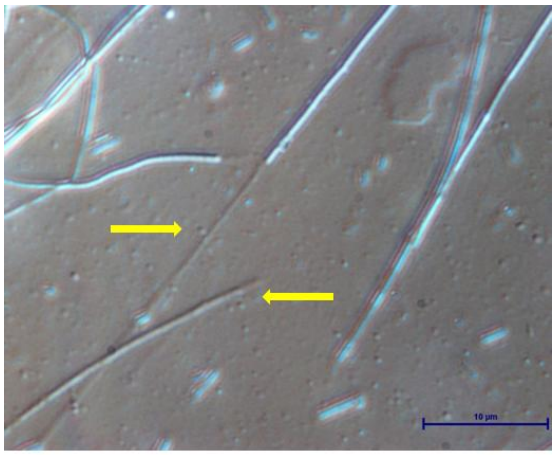


Plate 1a

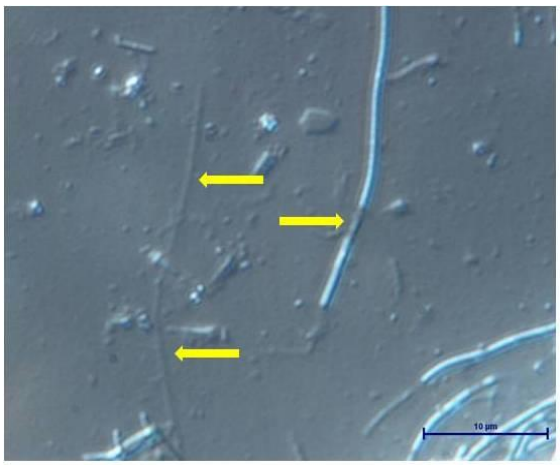


Plate 1b

Plate 1a. DIC images of *Leptolyngbya tenuis* showing sheath like structure.

Plate 1b. DIC images of *Leptolyngbya tenuis* showing sheath like structure.

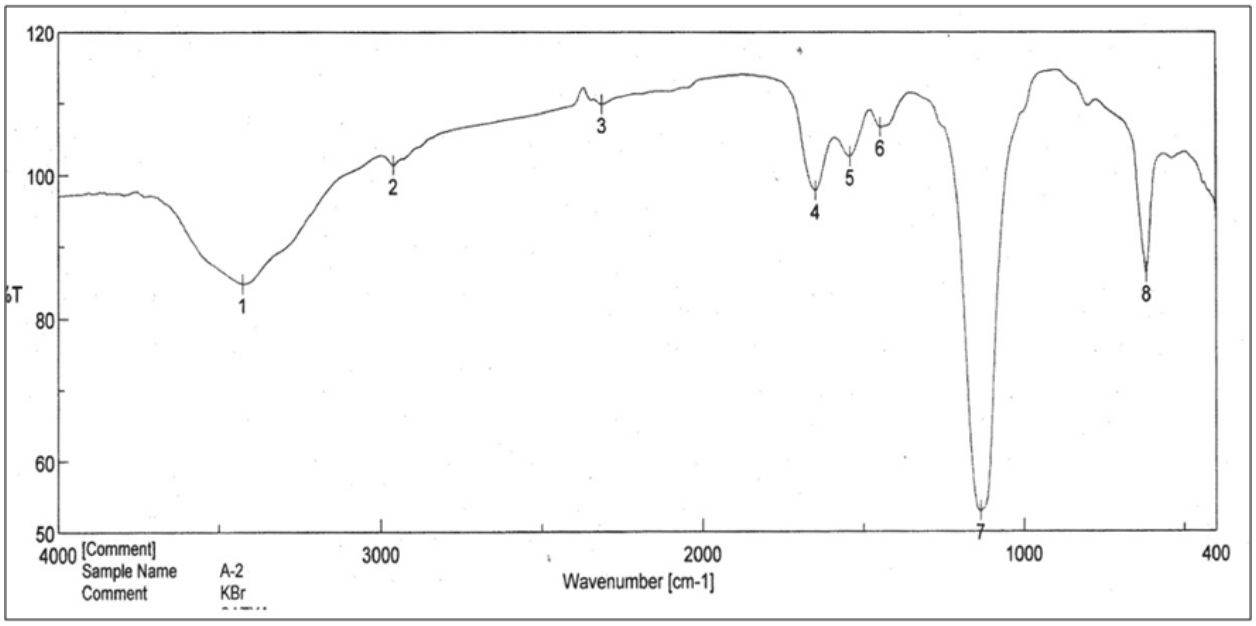


Fig 1

Fig 1. Fourier transform infrared spectra of the EPS of *Leptolyngbya tenuis* in control condition.

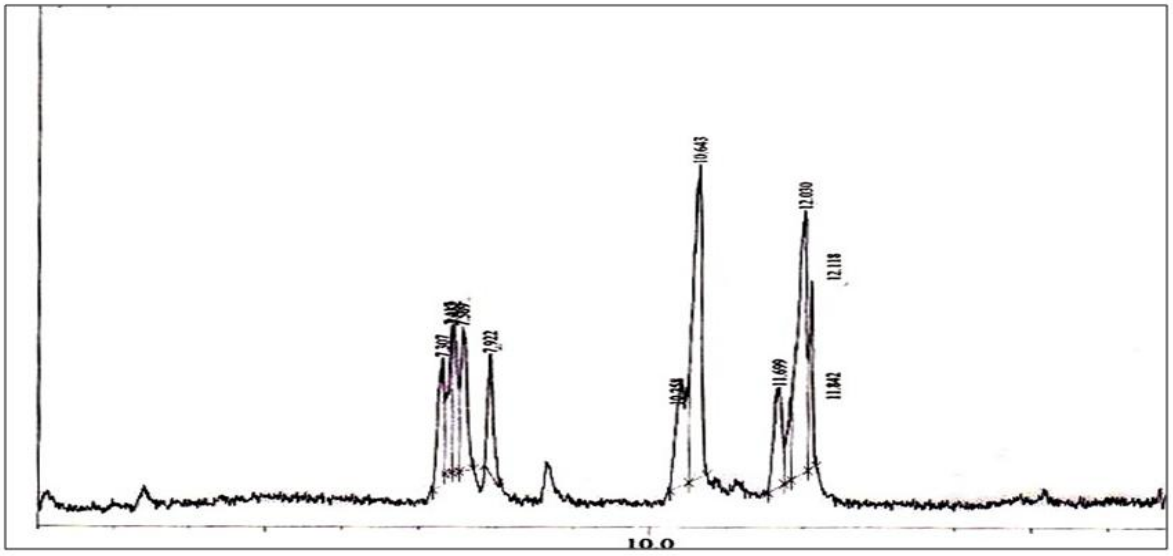


Fig. 2

Fig 2. Gas Chromatogram of the EPS of *Leptolyngbya tenuis* in control condition.

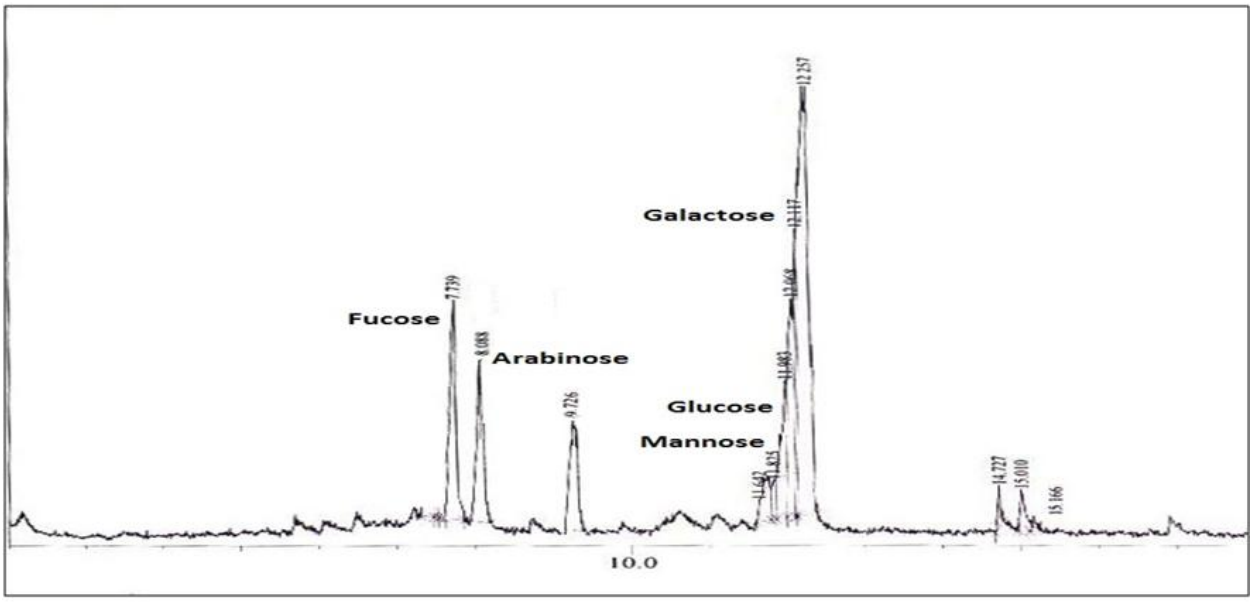


Fig.3

Fig 3. Gas Chromatogram of the EPS of *Leptolyngbya tenuis* under nitrate depletion.

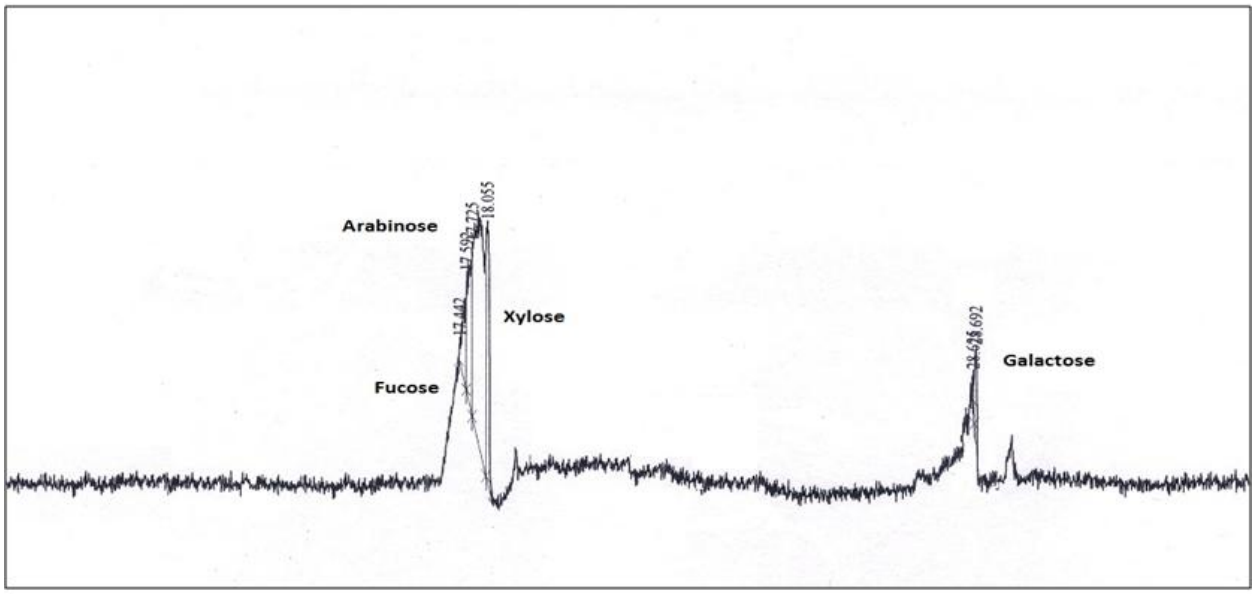


Fig 4

Fig 4. Gas Chromatogram of the EPS of *Leptolyngbya tenuis* under excess nitrate stress.

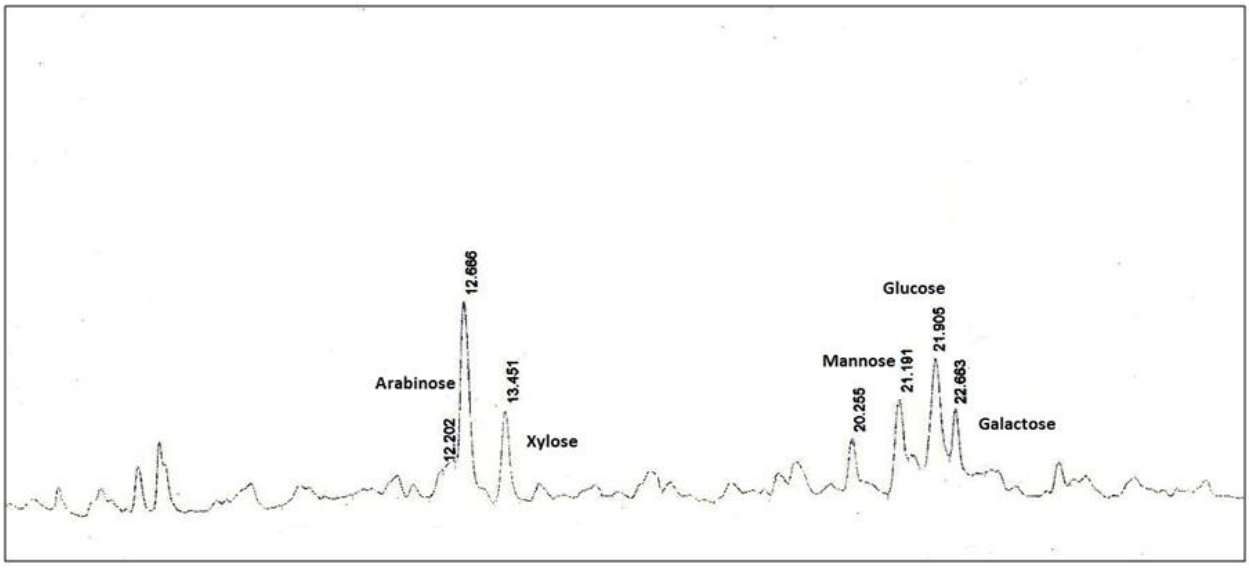


Fig 5

Fig 5. Gas Chromatogram of the EPS of *Leptolyngbya tenuis* under phosphate depletion.

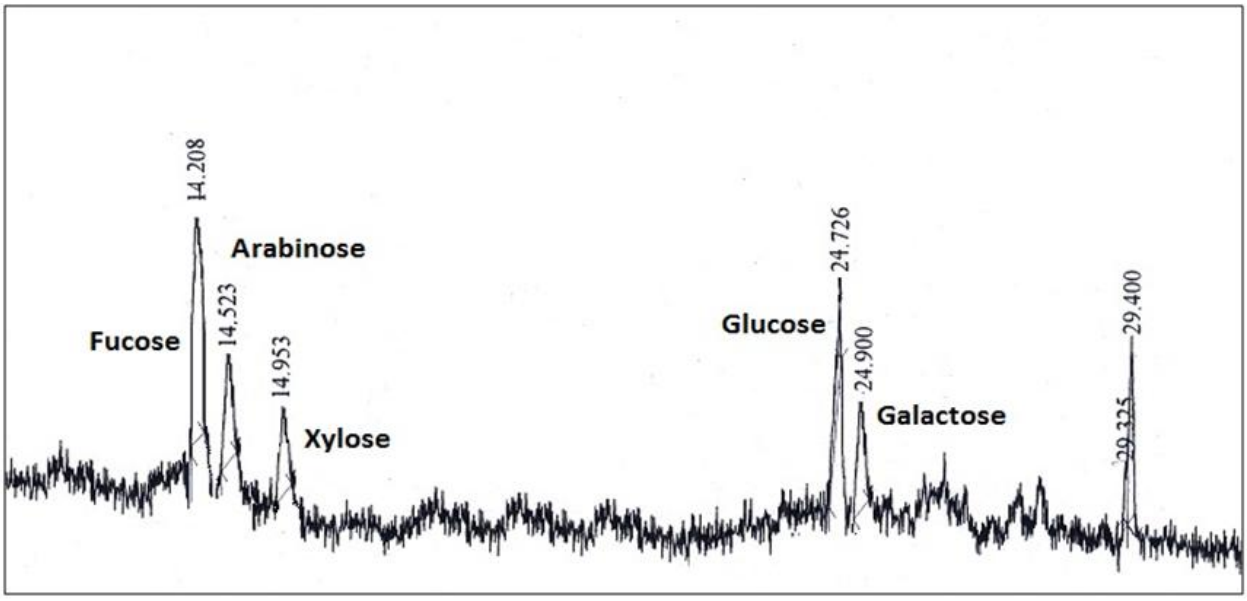


Fig 6

Fig 6. Gas Chromatogram of the EPS of *Leptolyngbya tenuis* under double salinity.

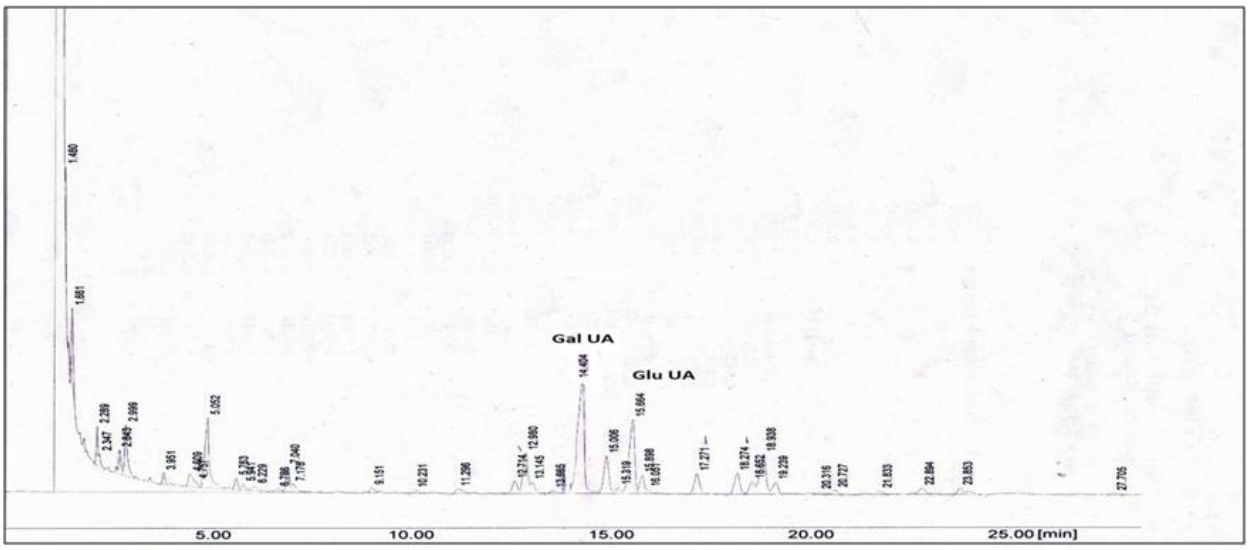


Fig 7

Fig 7:-Gas Chromatogram of the EPS of *Leptolyngbya tenuis* in control condition for the determination of uronic acids

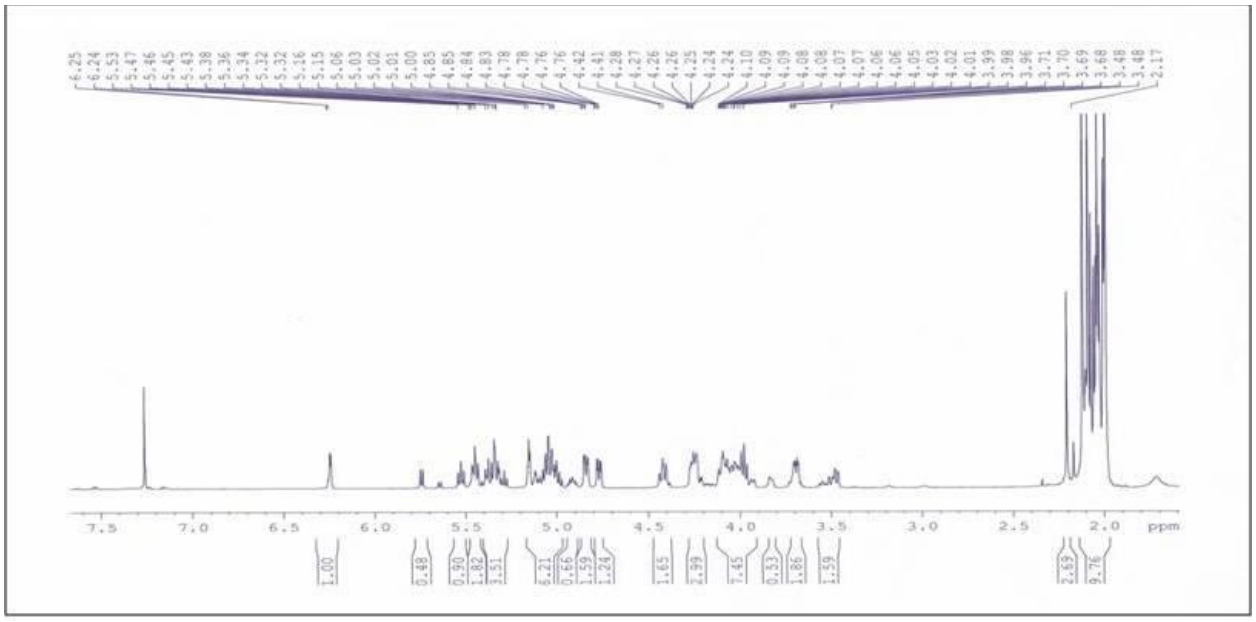


Fig. 8

Fig 8. ^{1}H NMR spectra of 1st fraction (extended spectra not included)

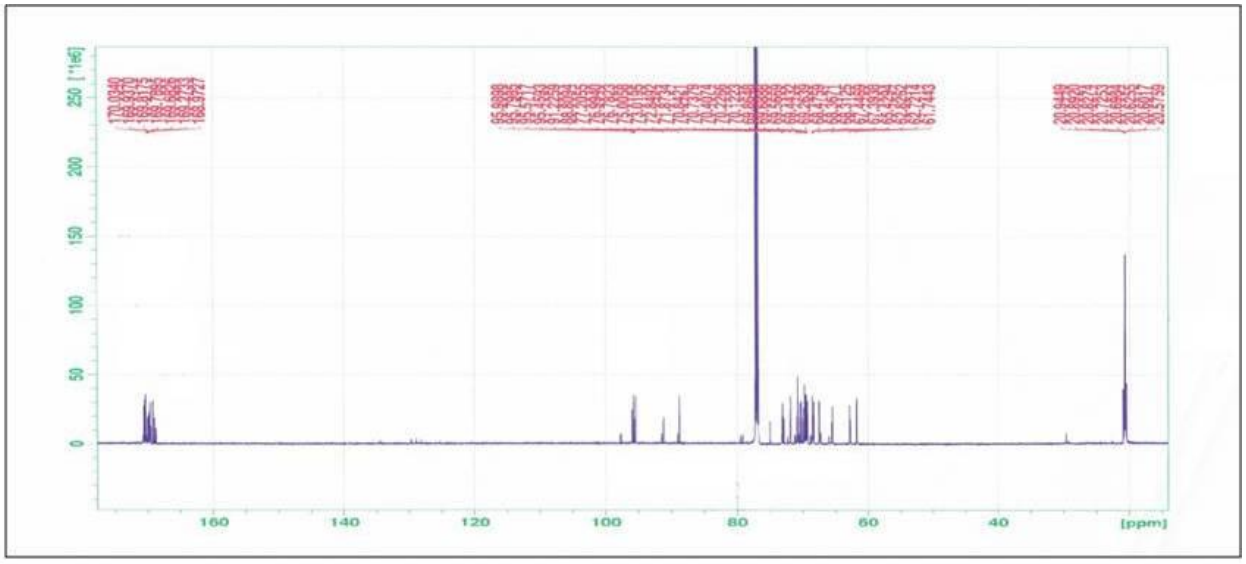


Fig 9

Fig 9. ^{13}C NMR spectra of 1st fraction (same)

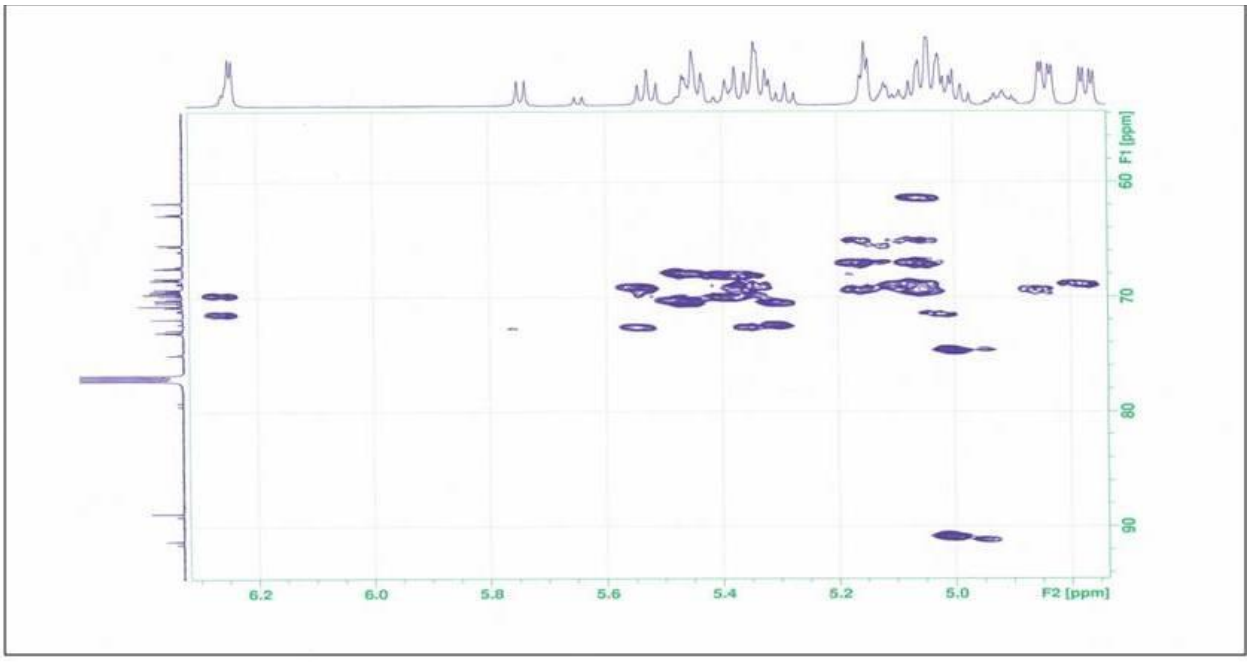


Fig 10

Fig 10. HMBC spectra of 1st fraction (same)

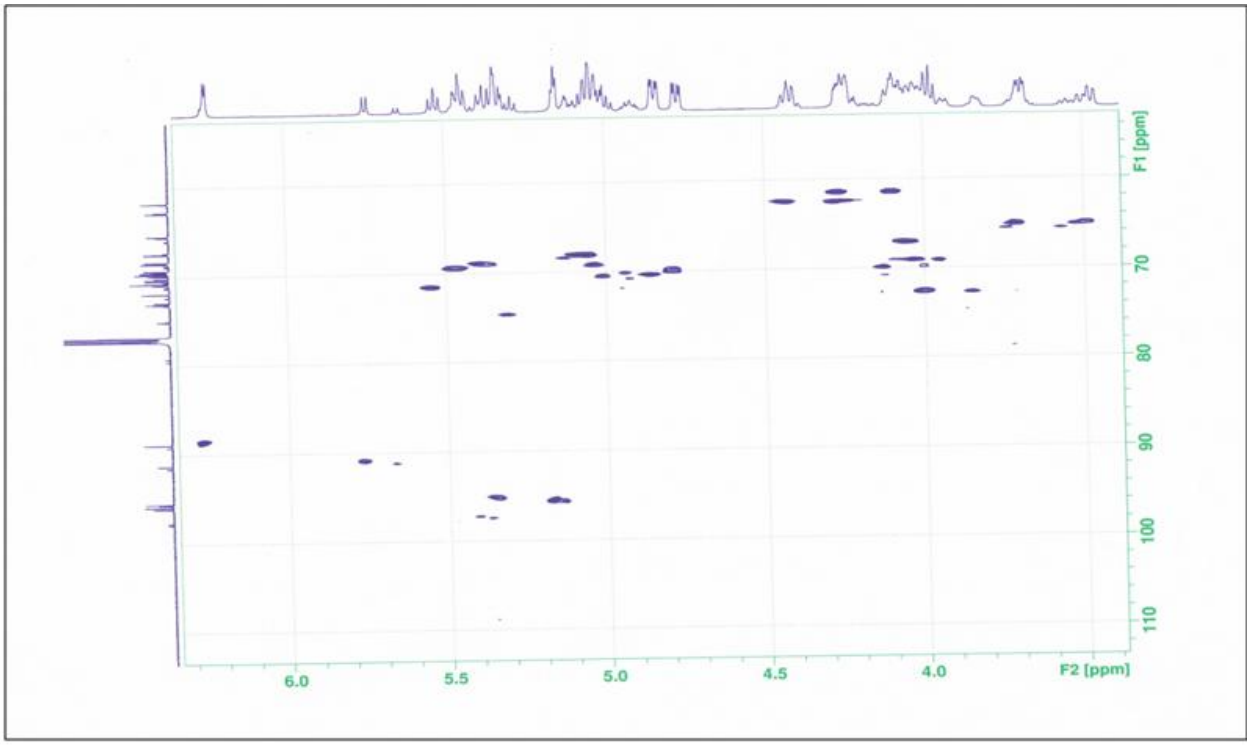


Fig 11

Fig 11. HSQC spectra of 1st fraction (same)

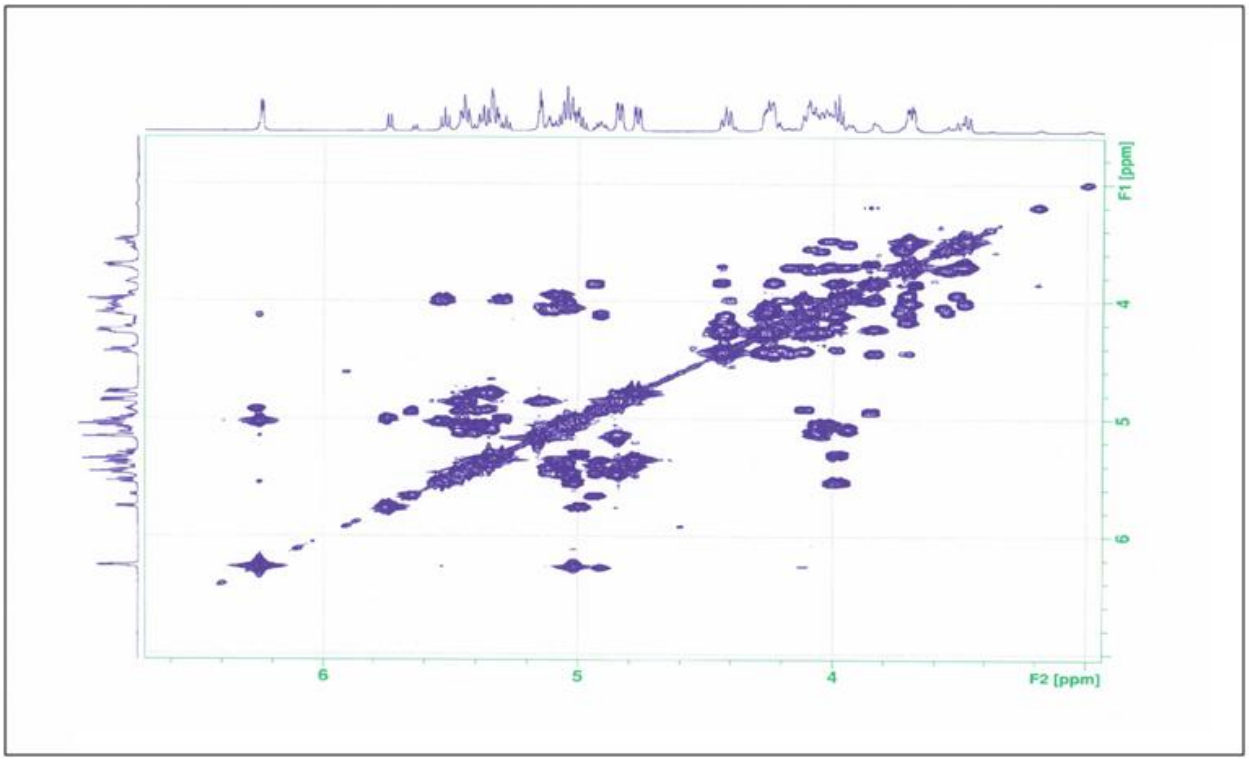


Fig 12

Fig 12. COSY spectra of 1st fraction (same)

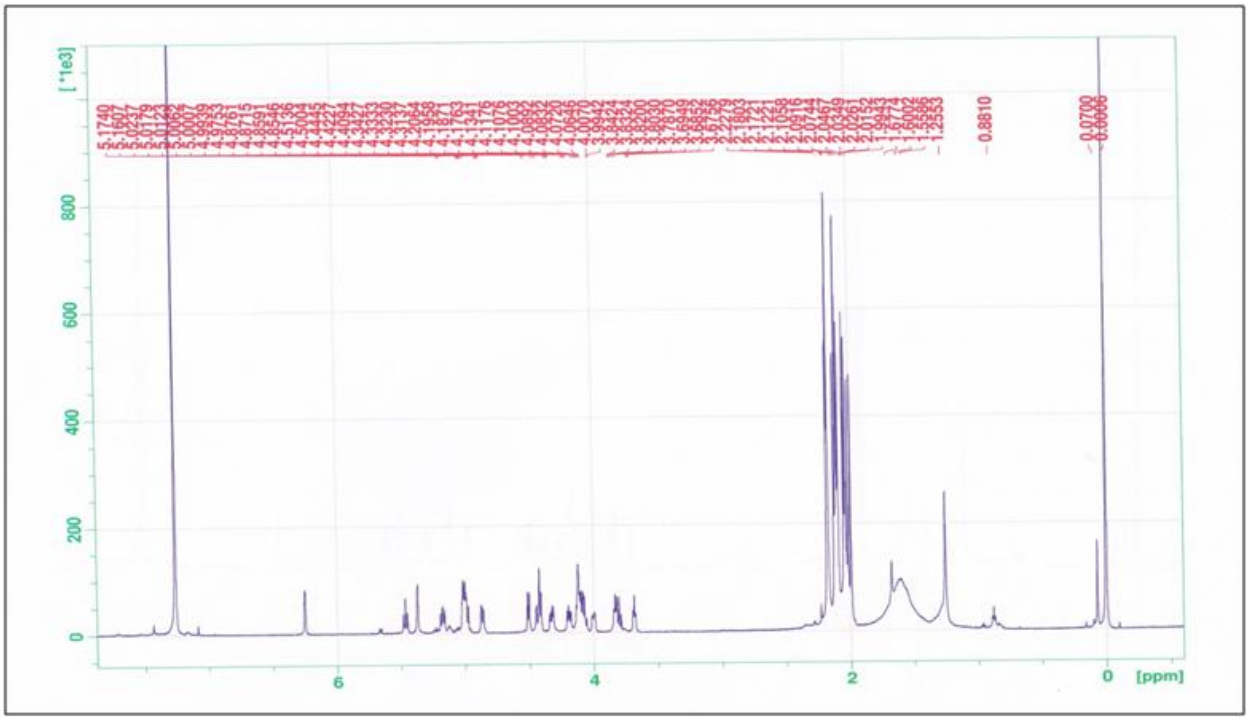


Fig 13

Fig 13. ^{1}H NMR spectra of 2nd fraction (same)

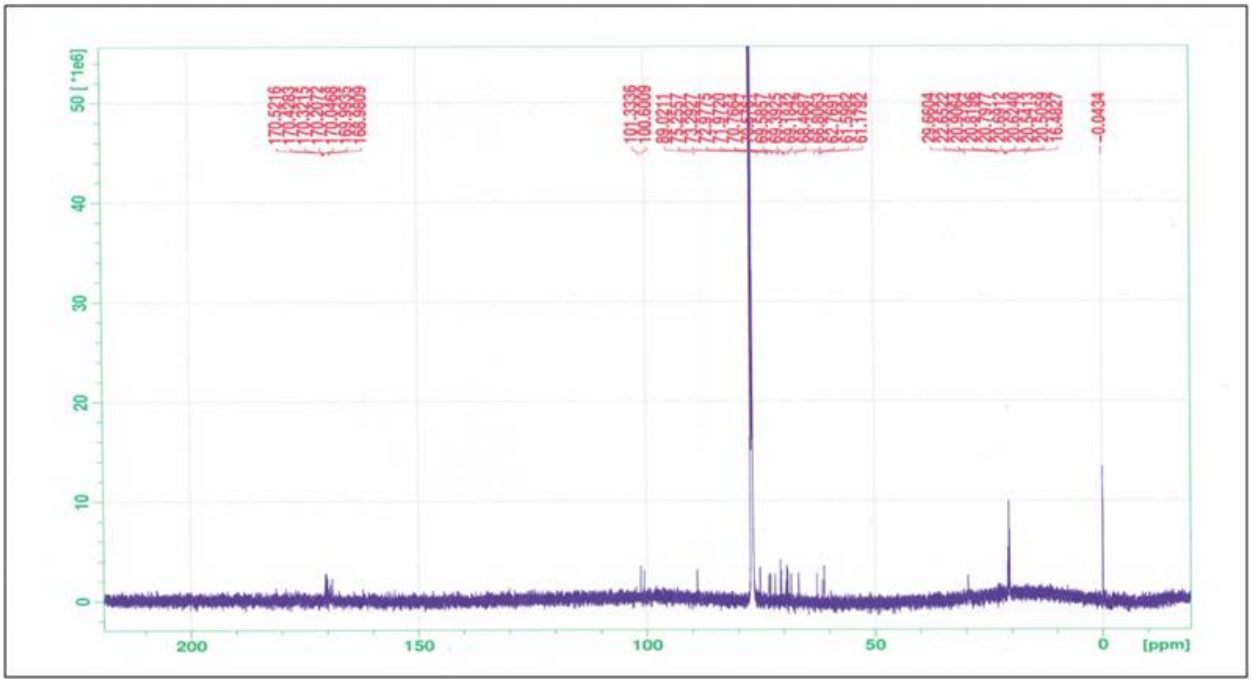


Fig 14

Fig14. ^{13}C NMR spectra of 2nd fraction (same)

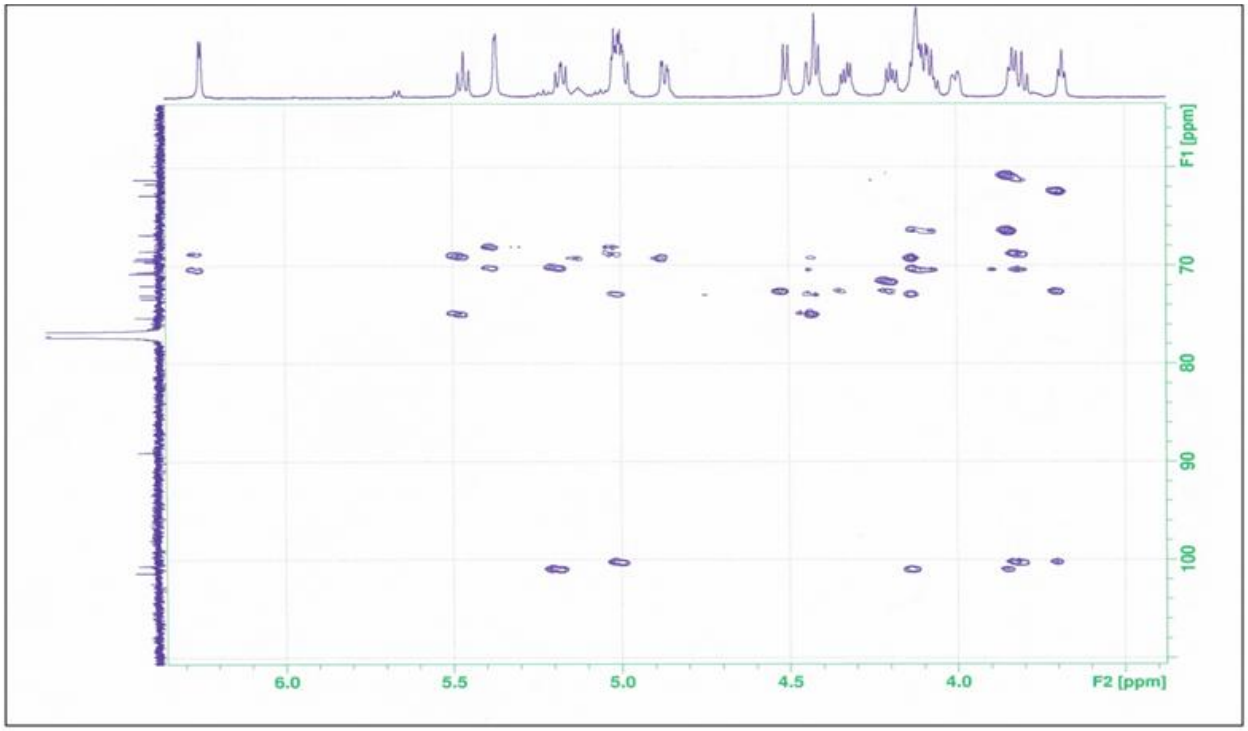


Fig 15

Fig 15. HMBC spectra of 2nd fraction (same)

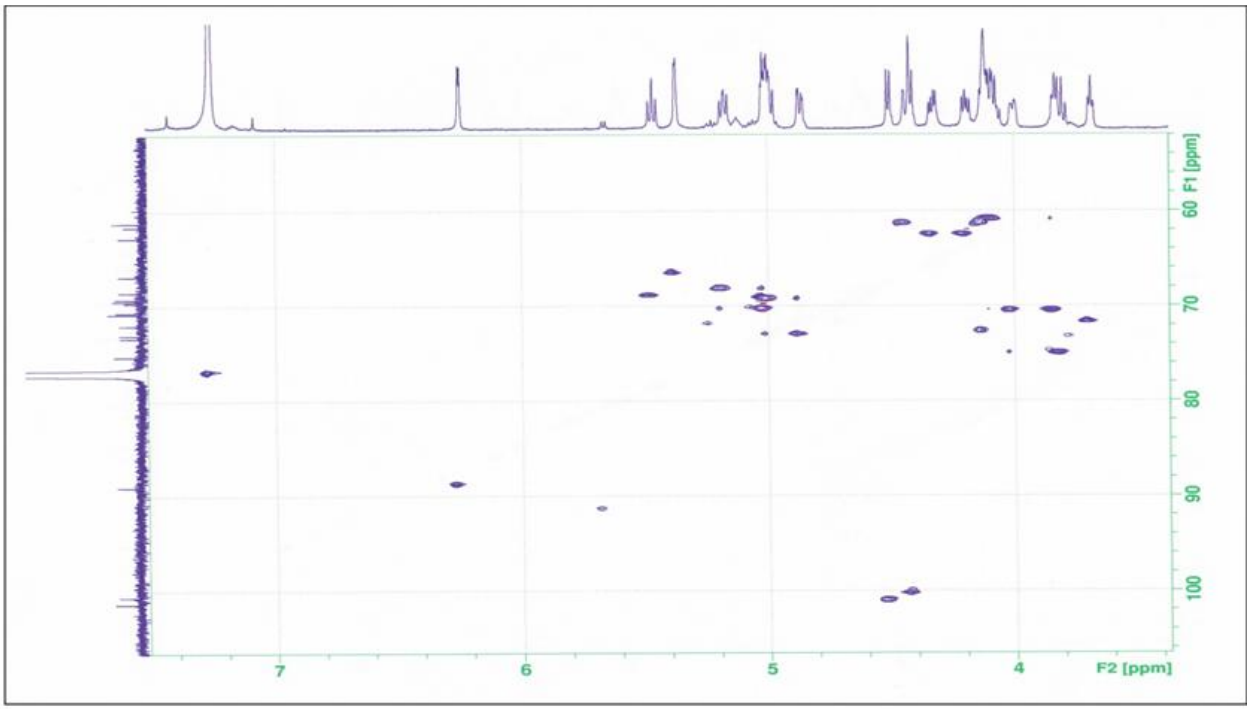


Fig 16

Fig 16. HSQC spectra of 2nd fraction (same)

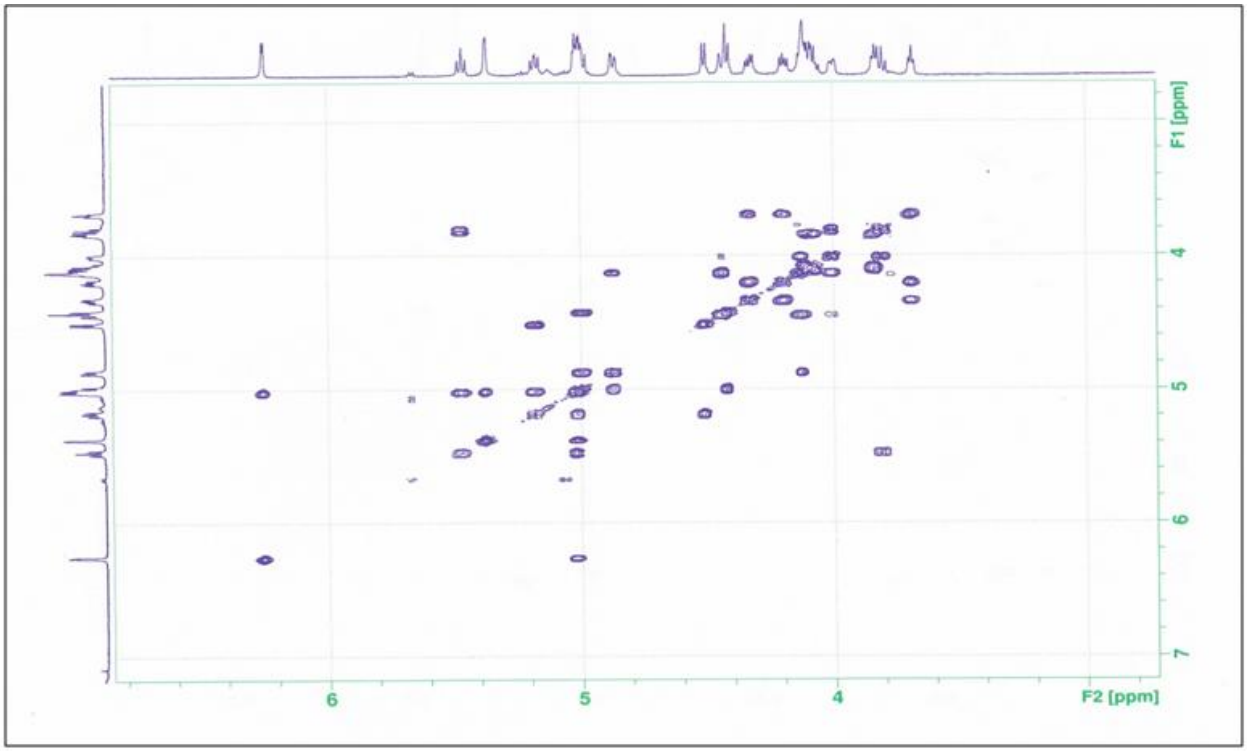


Fig 17

Fig 17. COSY spectra of 2nd fraction (same)

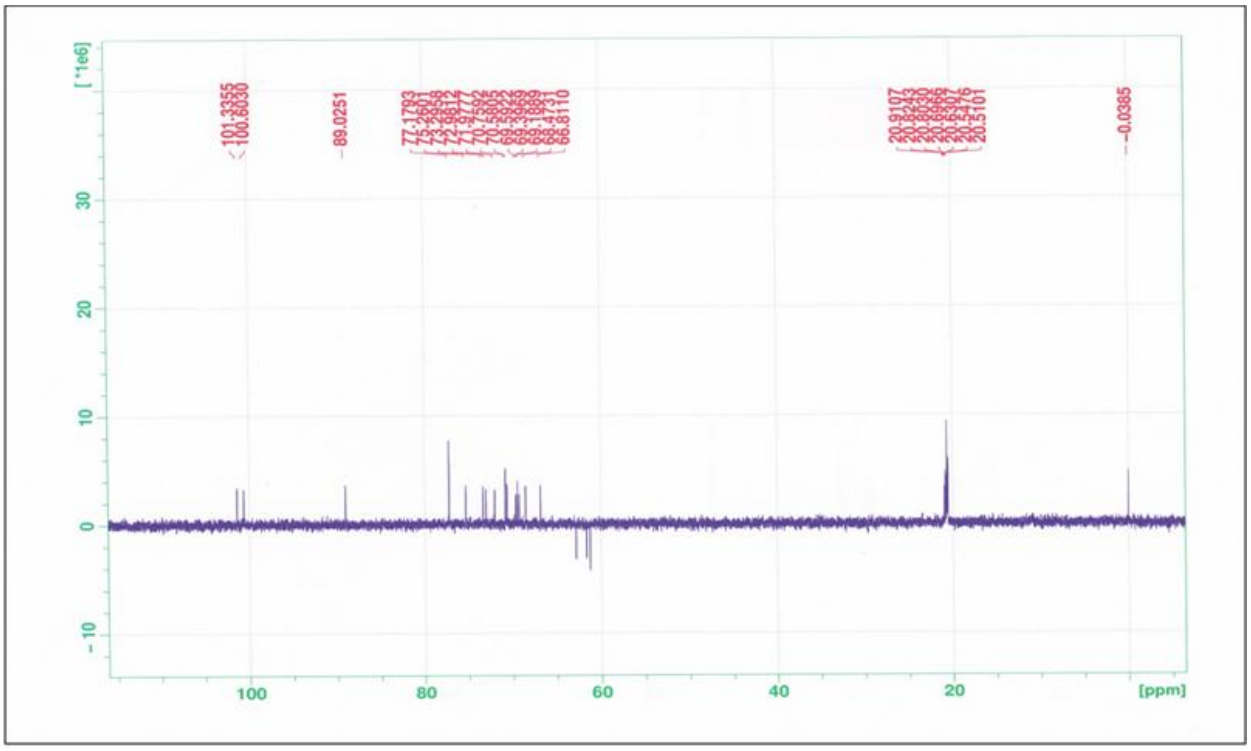


Fig 18

Fig 18. DEPT-135 spectra of 2nd fraction (same)

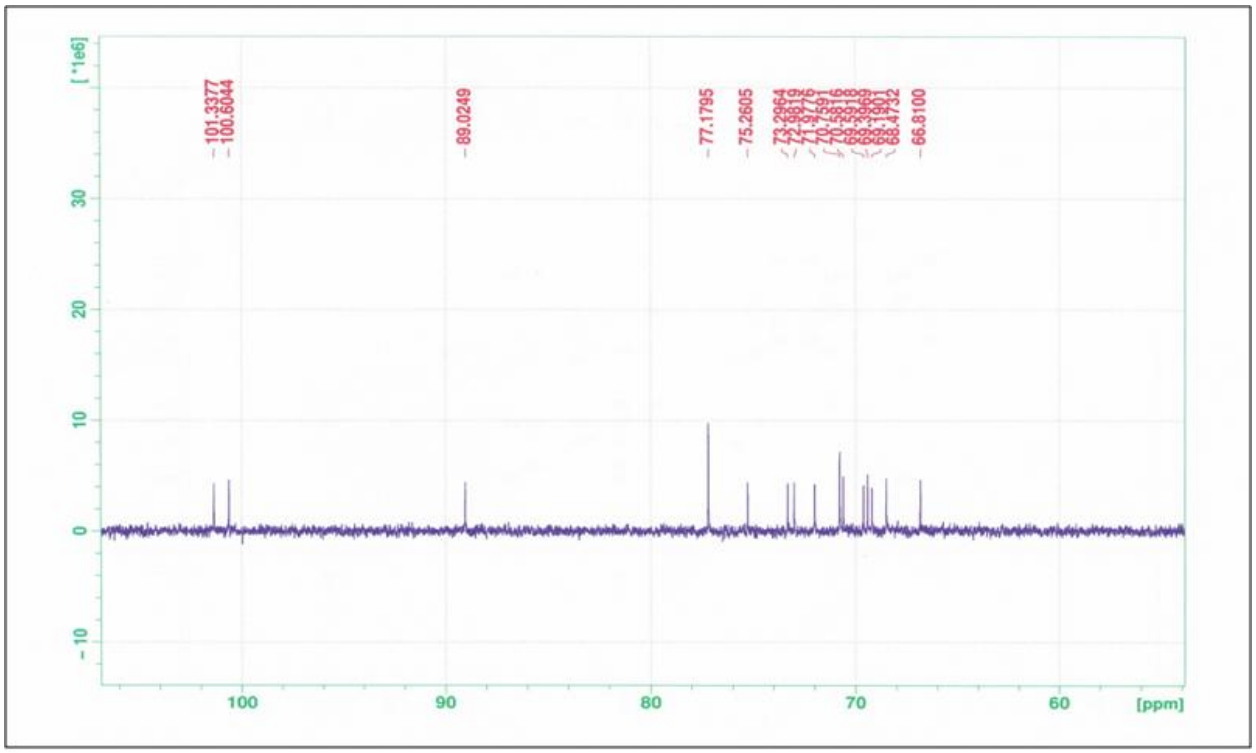


Fig 19

Fig 19. DEPT-90 spectra of 2nd fraction (same)

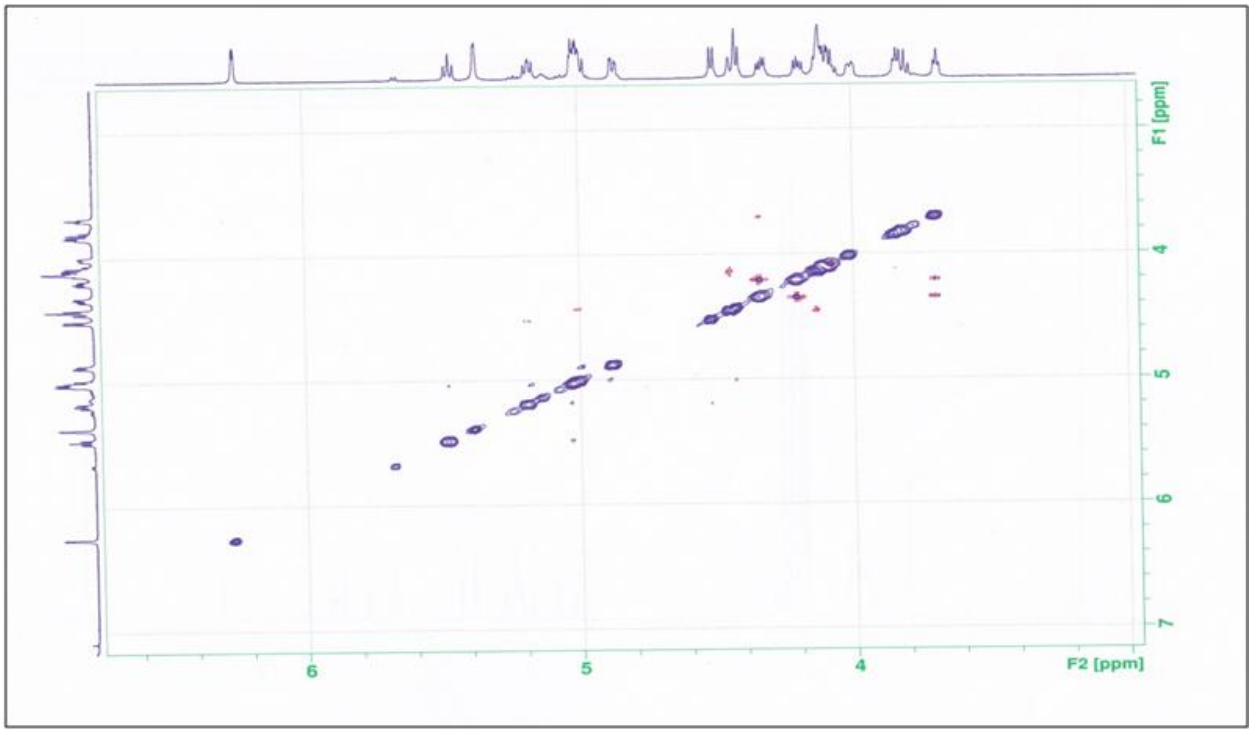


Fig 20

Fig20. NOE spectra of 2nd fraction (same)

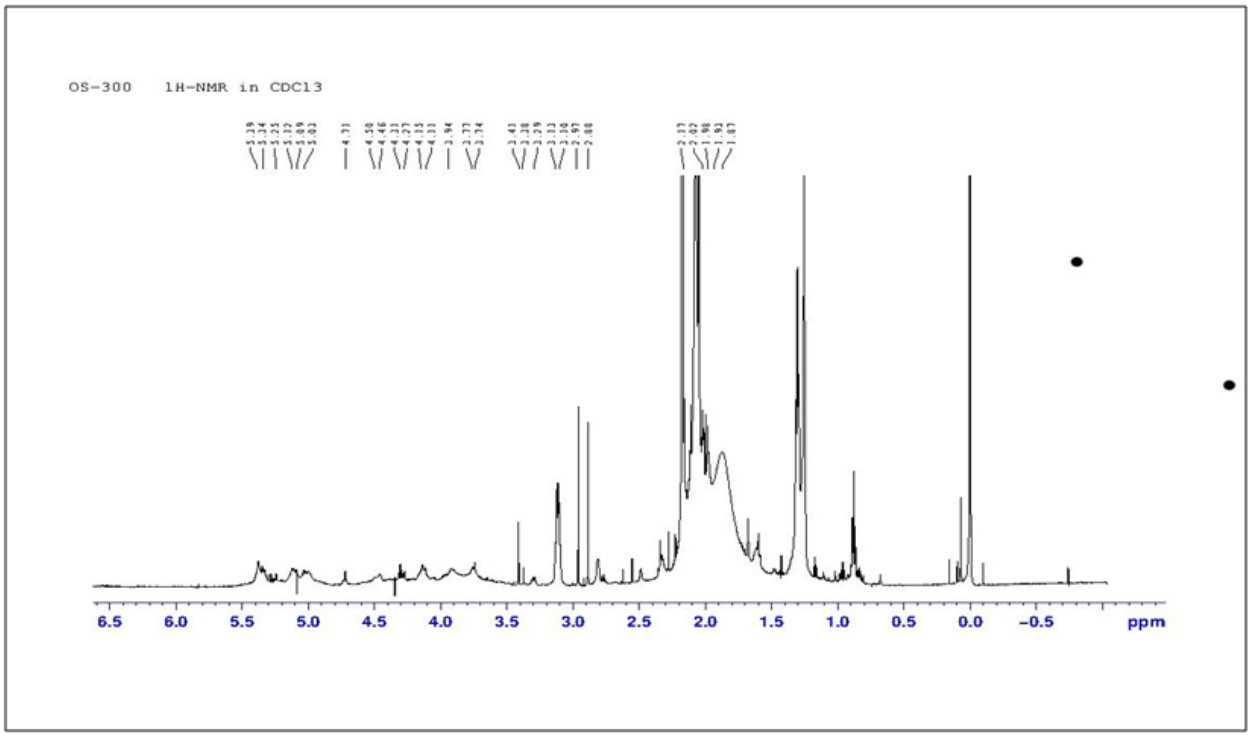


Fig 21

Fig 21. ^{1}H spectra of 3rd fraction

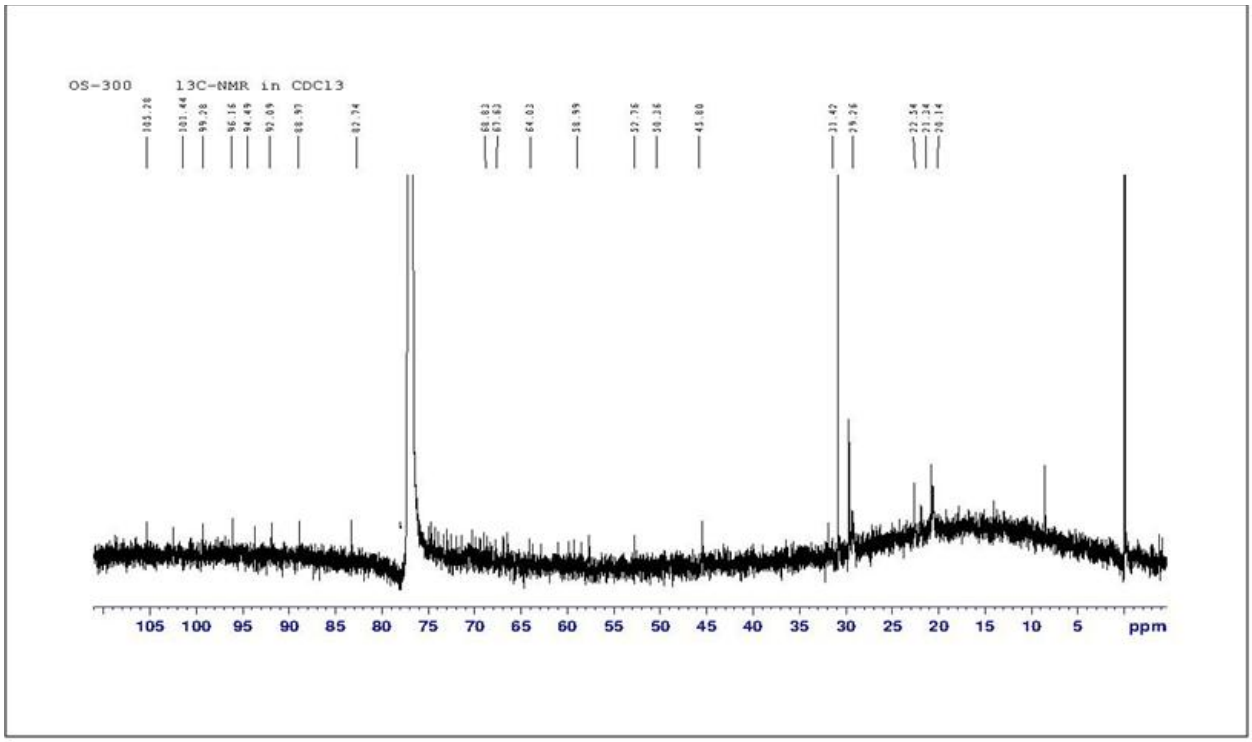


Fig 22

Fig22. ^{13}C spectra of 3rd fraction

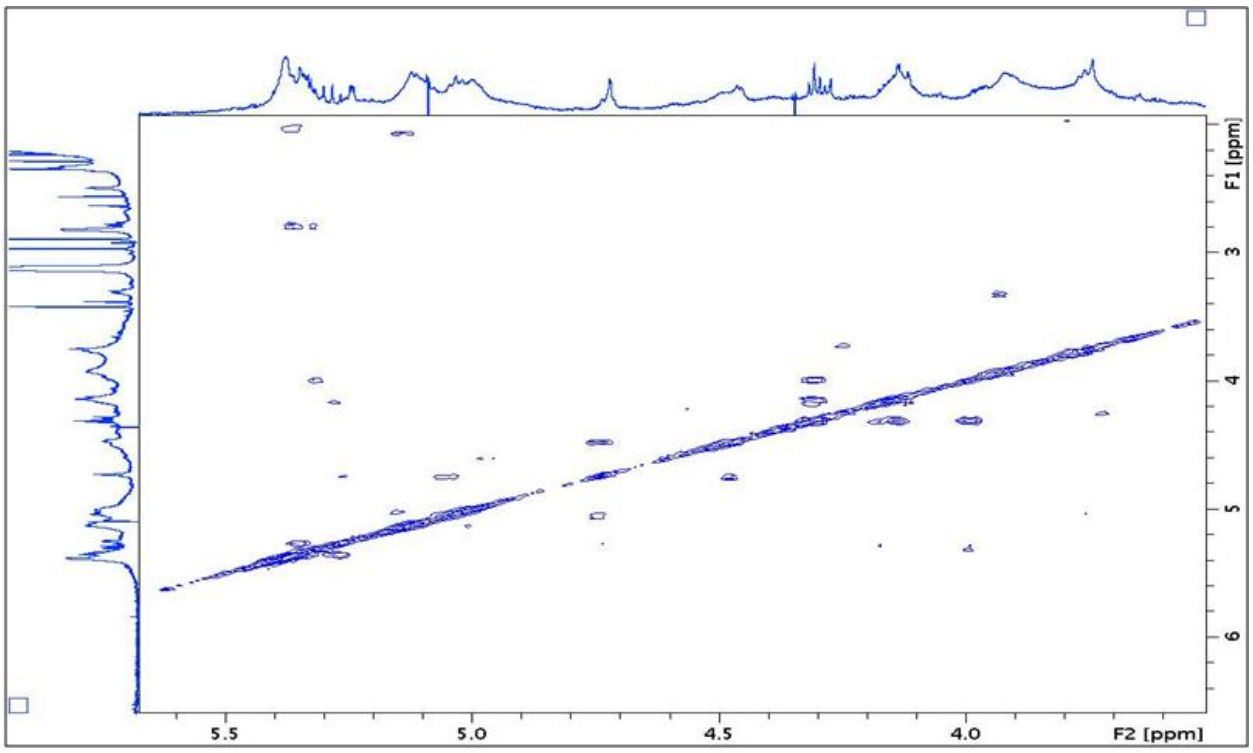


Fig 23

Fig 23. COSY spectra of 3rd fraction

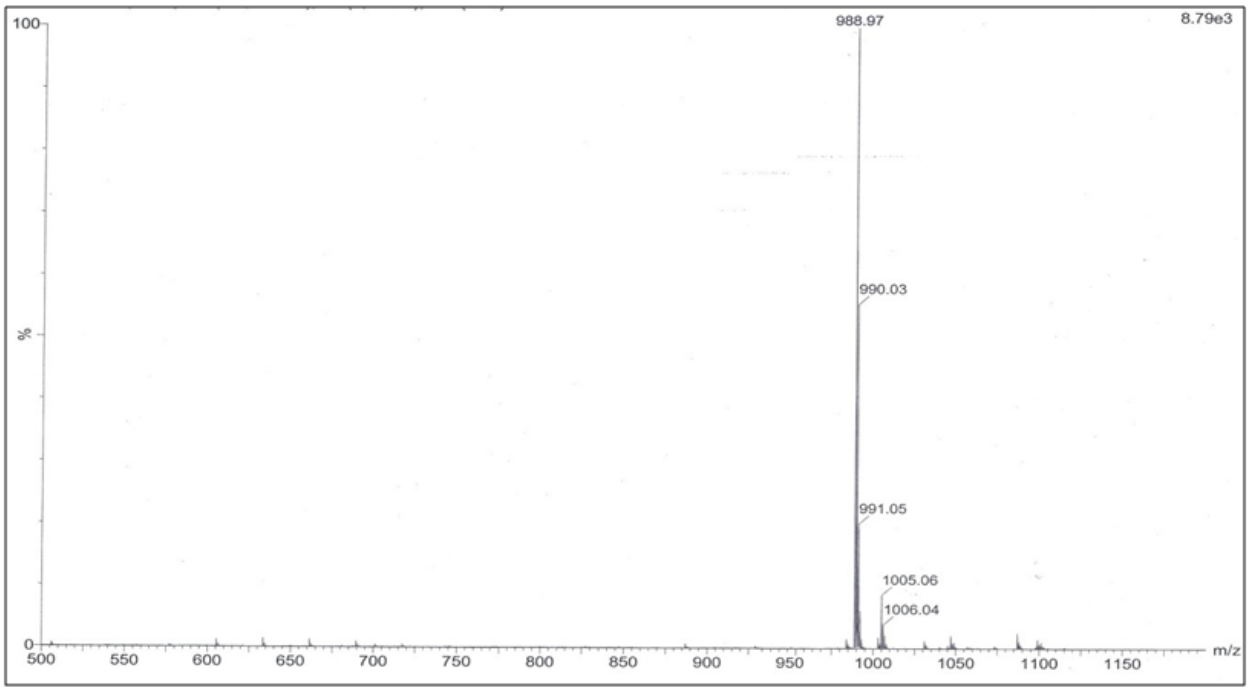


Fig 24

Fig 24. ESI-MS spectra of 1st fraction

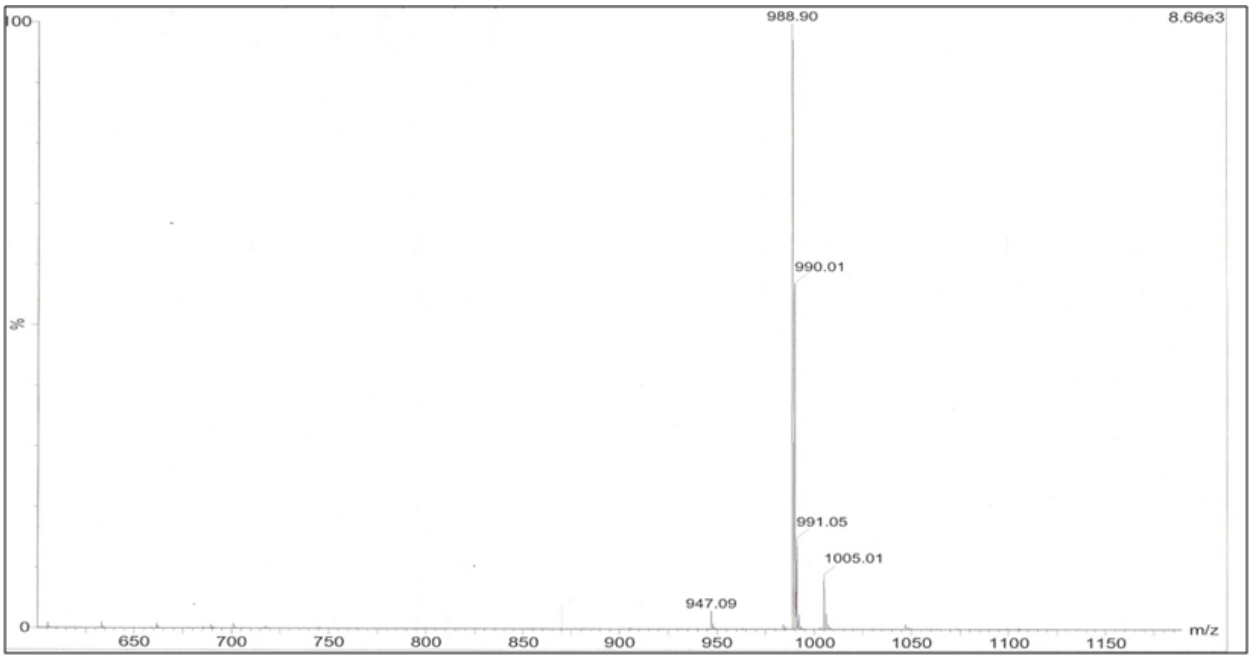


Fig.25

Fig 25. ESI-MS spectra of 2nd fraction

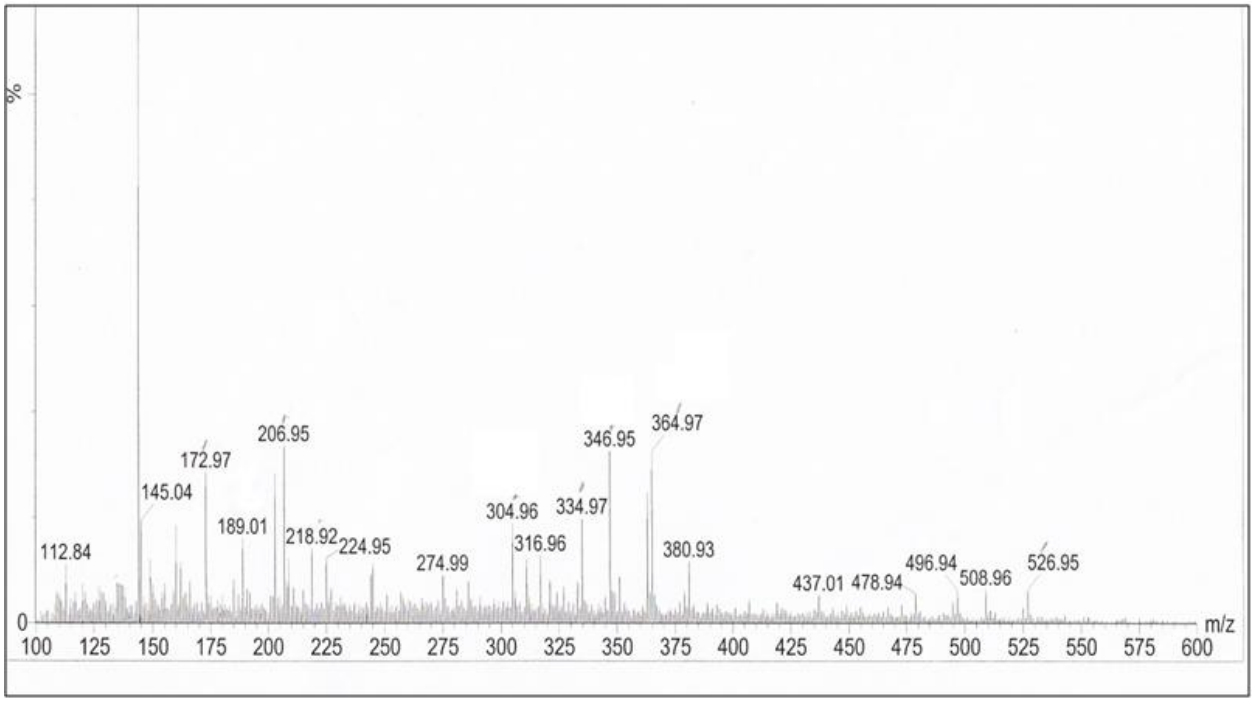


Fig. 26

Fig 26. ESI-MS spectra of 3rd fraction

Table 1. Variations of monosaccharides present in *Leptolyngbya tenuis*[a]

Different stress conditions	Ara	Fu	Gal	Glu	Man	Rham	Xy	Unidntfd
Control (14 days exposure)	-	1	1	4	1	1	1	4+3
Nitrate depletion (-NO_3)	4	6	7	2	1	-	-	2
Excess nitrate stress(10mM)	11	5	1	-	-	-	3	-
Phosphate depletion(-PO_4)	2	-	1	1	1	-	1	1
Double Salinity stress (1.25mM)	2	6	1	5	-	-	1	5

[a] The values are the ratio on the basis of the % availability of the sugars

Table 2:-Percentage of uronic acids present in the EPS of the cyanobacterial samples

Sample	**Uronic acid (µg/ml)**
Leptolyngbya tenuis	65

5. Conclusion

Compared to other polysaccharides like bacterial ones, studies on the cyanobacterial polysaccharides are relatively few in number. Structural complexities of cyanobacterial polymers like general presence of two different uronic acid residues, more than four manosaccharidic units, complex branching often renders great difficulties to elucidate the complete structure. The structural information is urgent not only for fundamental reasons but also critical in biotechnological sense. In this study, the cyanobacterium *Leptolyngbya tenuis*, the polysaccharide is scarcely soluble in water thus imparting a great challenge to the purification of it.

Although the monosachharide compositions of EPS of a number of species have been elucidated, the variation in that composition due to stress exposure is needed to study further. In the present study, not only the compositional ratios had changed, but the occurrence or disappearance of certain sugars had been observed. To understand this

stress induced changes, further detailed studies on the biochemical steps of EPS biosynthesis have become necessary. Moreover unlike the most other studies which dealt with released EPS (RPS) of cyanobacteria, this current investigation investigated the loosely bound EPS of *Leptolyngbya tenuis* where a different approach was needed to extract the polymer from the organism. It is also intriguing that if the synthetic mechanism of RPS is different from that of cell bound EPS (CPS) as in most of the cases the monosaccharide composition of RPSs were different than that of CPSs.

Acknowledgement-

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