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# Some growth studies on hyper EPS producing synechococcus spp. From different Industrial and coastal regions of Gujarat, India

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## Abstract

*Cyanobacteria are simplest form of algae, a representing of plant kingdom. They are widely distributed over land, water and often in environments where no other vegetation can exists. They've also distinction of being the oldest known fossils and account almost 8% of total population of bacteria in water bodies. Polysaccharides are renewable resources representing an important class of polymeric materials of biotechnological interest, offering a wide variety of potentially useful products to mankind. Extracellular polysaccharide of microbial origin have a novel functionality, reproducible physico-chemical properties, stable cost and supply, and so are better alternative to polysaccharides of plant and higher algal origin. Cyanobacteria are better suited than macro algae or higher plants, since they exhibit high growth rate and are more amenable to manipulation of conditions for enhancing growth or EPS production. Synechococcus spp. was isolated from different coastal and industrial regions of Gujarat and its growth kinetics were studied by using internationally recommended medium at various light intensities and production of the Exopolysaccharides was carried out under controlled growth conditions. Qualitative and quantitative estimations of total carbohydrates and proteins were carried out using Duboi's method and Folin-lowrey's method respectively. Total carbohydrates and proteins concentrations were measured at regular time interval of 5 days and gradual increase in concentration (Ranging from 539 µg/l to 588 µg/l ) of carbohydrates and (Ranging from 72 µg/l to 109 µg/l ) of proteins were found between 25 to 38 days. SDS-PAGE was carried out for further identification of proteins and two moieties of proteins were found having molecular weight of 65 kDa and 30 kDa respectively.*

**Keywords:** Cyanobacteria, synechococcus spp., EPS, SDS-PAGE.

## Introduction

Blue-green algae are photosynthetic bacteria, they appear like algae and are called 'Cyanobacteria, Blue-green bacteria or Cyanophyta, Cyanoprokaryotes[1,2]. They have the distinction of being the oldest known fossils, more than 3.5 billion years old, cyanobacteria are still around. Cyanobacteria are very small organisms and can be seen under the microscope as a single cell or large accumulation of cells (colonies) or strings of cells (trichomes)[3]. They are group of gram negative, unicellular to multicellular, coccoid, branched to filamentous autotrophic to heterotrophic, psycrophiles to thermophiles, acidophilic to alkalophilic, fresh water to marine and domestic water organisms[4].

Cyanobacteria include unicellular and colonial species[3]. The colonies may form filaments, sheets or even hollow balls. Some filamentous colonies show the ability to differentiate into several different cell types: vegetative cells, the normal, photosynthetic cells that are formed under favorable growing conditions; akinetes, are the climate-resistant spores that may form when environmental conditions become harsh; and thick-walled heterocysts, which contain the enzyme nitrogenase, vital for nitrogen fixation[5,6].

Synechococcus (Greek-'Synechos'=in succession, 'kokkos'=berry) is a unicellular cyanobacterium that is very wide spread in marine as well as in fresh water environment. Its size varies from 0.8 - 1.5µm. Synechococcus sp, the prokaryotic autotrophic picoplankton of tropical region, are preferentially found in well-lit surface waters where it can be very abundant (1000 to 200000 cells/ml)[2]. We have selected Synechococcus spp of fresh water having little bit higher salt and mineral concentration, significantly found at coastal regions of Gujarat.

Polysaccharides that are secreted from cells are known as Exopolysaccharides (EPS). In other words, these molecules are not held in association with the cells as are cell wall polysaccharides[1,7]. Instead, these molecules are released from the cells[4,12].

Exopolysaccharides were hydrolyzed by 2M TCA (Tri Chloro Acetic Acid) at $110^0$ C for 90 min. sand precipitated by compounds like Cetylpyridinium chloride, Isopropanol, Ethanol, or Methanol to an aqueous solution containing polysaccharide[8,9]. Pellets of precipitated polysaccharide can be washed and resuspended in double distilled water, buffers such as Phosphate buffered saline or Tris, or other aqueous solutions[1,10].

## Material and Methods

Sea water, fresh water, and sewage water samples are collected from different regions of Gujarat table-1. General techniques for the sampling given by the procedures listed in the recommendation from the Department of Environmental Protection (DEP): Standard Operating Procedures (SOPs) were followed to collect aqueous Cyanobacterial samples[11,7]. Only amber glass bottles were used to collect aqueous samples. Clean, proper containers with no defects for samples. There are so many methods available for the sampling of fresh water as well as marine water Cyanobacteria. Among all those techniques we have used three techniques as noted below.

**Direct scrubbing from the stone with Cyanobacterial layer:** In this method samples are directly scrubbed from the solid surface i.e. rocks and walls by the mean of presterilized scalpel. This technique should be handled with care because it may lead to breaking down of algal filaments. After the scrubbing, the cells/filaments are directly collected into the same water sample and immediately used for further procedure (within 1-2 days).

**Scrubbing from Wall:** This technique is the best method for the collection of Cyanobacteria and algae which are float on the surface of water as blooms or scum the wall having moisture[12]. In this technique, the algae are directly collected from the wall by scrubbing. And this sample is preserved into fresh water or Mill Q water (for 1-2 days). We have used this technique for sample collection at Junagadh, and Veraval, and Himatnagar.

**Water scum sample :** Water scum sample technique is the best method for the collection of Cyanobacteria and algae, having the capacity to formation bloom on to the water surface. With the help of wide mouth polycarbonate bottle or glass bottle collect surface grab sample within the top 5-10 cm of the water which is having the Cyanobacterial bloom on its surface. Samples were not collected by skimming the surface. After samples were collected, with the help of Nicrome wire give loop transfer of the filaments in the cultivation medium. This technique gave promising results then the other which we have used. This technique was used for sample collection from Junagadh, Veraval, Amreli, Vadodara and Himatnagar. The organism were grown in various recommended medium described in the Isolation and cultivation. Other methods were also described in isolation and cultivation and results.

**Isolation and Cultivation:** Isolation and cultivation were carried out followed by the sampling, for that below mentioned internationally recommended medium. BG-11 Medium[9,6,13], Castenholtz D Medium[4,3,8,10], Gorham's Medium [5,6], ASN-I Medium[11,8,9], Bold Basal Medium[11,10],

Among all above listed medium BG-11 medium gave luxurious growth followed by Castenholtz D medium and Gorham's medium. Expected growth was not found in ASN-I medium, which is actually designed for the growth of true marine *Cyanobacteria*. Bold Basal medium gave the difficulty of moisture loss and excessive evaporation hence the last two medium were less frequently utilized.

BG-11 medium was modified further due to constrain of some specific ingredients like Ferric ammonium citrate and antibiotics like Ampicillin (100µg/ml), Streptomycin (40 µg/ml) and antifungal - Chlorohexidine (60 µg/ml) to overcome the contamination of autotrophic bacteria and fungi[9,8,7]. 10% of the inoculum was inoculated in highly sterile condition and the flask was kept in an illuminated shaker Incubator (12/12 hr light dark cycle), 25$^{o}$ C till desirable cell density is attained. Some requires long period of 30 days for attaining adequate growth. Table-2 [figure 1-6] shows some of the isolates.

Pure culture of *Cyanobacteria*l spp. were obtained by sterile transfer of cells from liquid medium to solid medium and solid medium to again liquid medium. After considerable growth, microscopy was carried out and structures were confirmed with the standard ones. To maintain the purity of culture, cultures were transferred to fresh medium, for that four novel techniques were applied as mentioned below.

capillary transfer technique, serial dilution technique, transfer with help of glass beads, transfer with the help of filter paper.

**EPS Production and Purification:** The EPS production was carried out in 2.01 Erlenmeyer flask containing 400 ml of BG-11 (Modified) Medium, having less amount of Nitrogen source with respect to carbon source[7,9]. For inoculation 10% of inoculums inoculated, to achieve the growth and these flasks were kept at 28°C, humidity was also maintained by using wet filter paper. Light/dark cycle of 12/12 hours was necessary for better growth and agitation was also provided by rotary shaker at 60 rpm twice in day. These flasks were incubated at least for 25 days for to achieve adequate growth for EPS production. For the extraction of Extracellular Polysaccharides, cells were separated by centrifugation at 15000 rpm at 15°C for 40 min., an d then supernatant was concentrated 1/4th of its total volume by keeping it in an oven at 60° C for 10-12 hours.

Thus extracted polysaccharides (supernatant) were precipitated by using varieties of organic solvents i.e. Methanol, Isopropyl alcohol, Acetone and Dimethyl sulfoxide. But among all of above Isopropyl alcohol was best suited for precipitation, giving maximum yield with floating precipitate on the surface precipitated by gradual addition of cold Isopropyl alcohol or Ammonium sulfate to the supernatant and kept at 4$^{0}$C overnight. The precipitates were re-dissolved in buffer or double distillated water. This

process was repeated for to purify the polysaccharides. Total carbohydrates and protein content was measured by quantitative analysis of carbohydrates and protein i.e. Duboi's method and Folin-Lowrey's method and further protein moieties were analyzed by SDS-PAGE[13].

## Results and Discussion

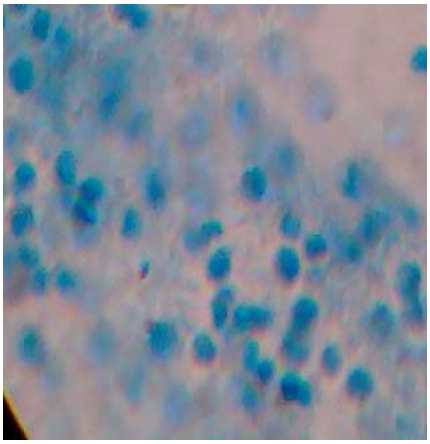
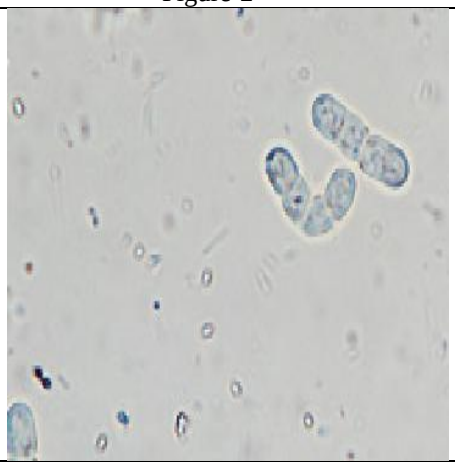
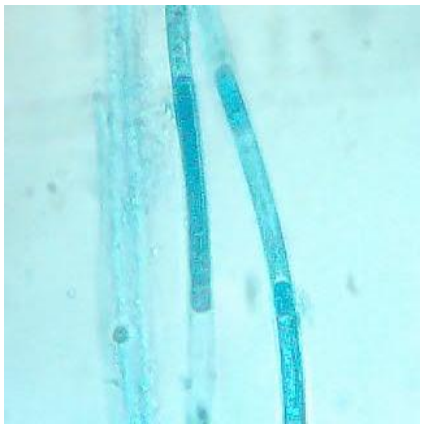
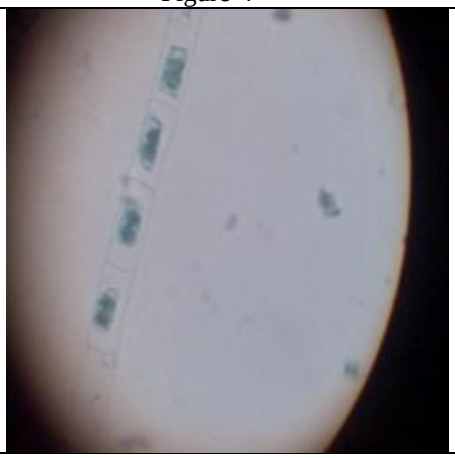
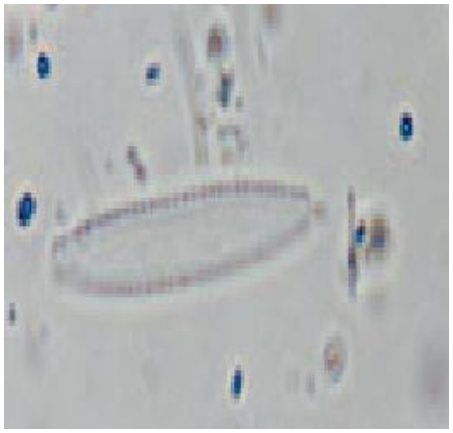
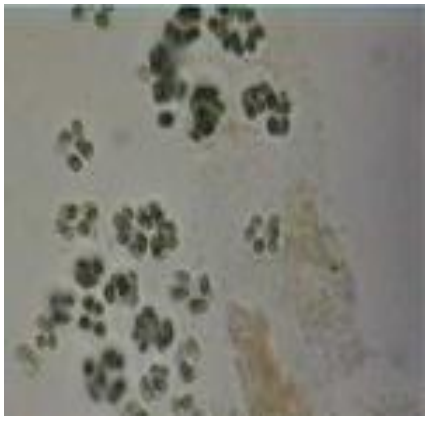
Gorham's medium recommended by ATCC for the better growth of Synechococcus spp. but in our case we won't be able to get promising results in Gorham's medium. Comparing the optical density it is observed that, the optical density was 1/3rd after 17th day of incubation. The max optical density (0.073nm) was observed at 17th day. Of the two medium D medium was second best and optimum optical Density was observed 0.16. Max growth response observed on modified BG-11 medium on 17th day incubation which is 0.178 graph-1. This data proves that BG-11(modified) medium gave better growth compare to others and so it was selected for further studies. We have grown Synechococcus spp. in different growth medium, among all medium BG-11 gives maximum growth. So for further optimization of growth of Synechococcus spp. we were tried to grow Synechococcus spp. under different light intensities and study their effects on the growth[3,5].

For providing sun light the inoculated flasks were kept on the corridor were bright direct sun light was achieved. Flasks were kept at 8'oclock in the morning and taken out at 6'oclock, and then dark cycle was provided by keeping the system in dark. During the study in one of the experiment on *Synechococcus Spp.* we have used following various lights intensities for growth studies for 25 days. Sunlight, Red light: 650 nm, blue light: 475 nm,green light: 510 nm.

**Table-1**
**Sampling sites**

| Sample | City | Location | Observed spp. |
|---|---|---|---|
| **A** | Junagadh | Jadeshwar Lake Sudarshan Lake | *Ocillatoria spp., Cyanothacea spp., Spirogyra spp, Anabaena spp., Ulothrix spp., Chorococcus spp., Synechococcs spp., Lichens.* |
| **B** | Amreli | Kamnath Lake<br>Thebi River Bank | *Cyanothacea spp., Spirogyra spp., Diatoms, Chlorococcus spp.,*<br>*Microcystis spp., Anacystis spp., Lichens.* |
| **C** | Kutchchh | Hamitsar Lake | *Anabeana spp., Ulothrix spp., Chlorella spp.* |
| **D** | Rajkot | Shaper<br>Aaji Dem-II<br>Nyari Dem<br>Municipal Waste Water Treatment Plant, Madhapar | *Gleocapsa spp., Spirogyra spp., Lichens, Microcystis spp., Diatoms (Nitzia spp.),*<br>*Chlorococcus spp.* |
| **E** | Himatnagar | Hathmati River Bank | *Volvox spp., Spirogyra spp., Microcystis spp., Clamidomonas Spp.,* |
| **F** | Bhavnagar | Sea-cost | *Trichodesmium spp., Synechocystis spp., Chlorococcus spp.* |
| **G** | Veraval | Chaupati (Somnath)<br>Waste Water Nearby Reyon Industry | *Synechocystis spp., Chorococcus spp., Synechococcs spp.* |

**Table-2**
**Figure 1-6 Isolates**

| Figure-1 | Figure-2 | Figure-3 |
|---|---|---|
|  |  |  |
| *Synechococcus spp.* | *Chlorococcus spp.* | *Ocillatoria spp.* |
| | | |
| Figure-4 | Figure-5 | Figure-6 |
|  |  |  |
| *Spirogyra spp.* | *Diatoms (Unidentified) Nichtia?* | *Gleocapsa spp* |

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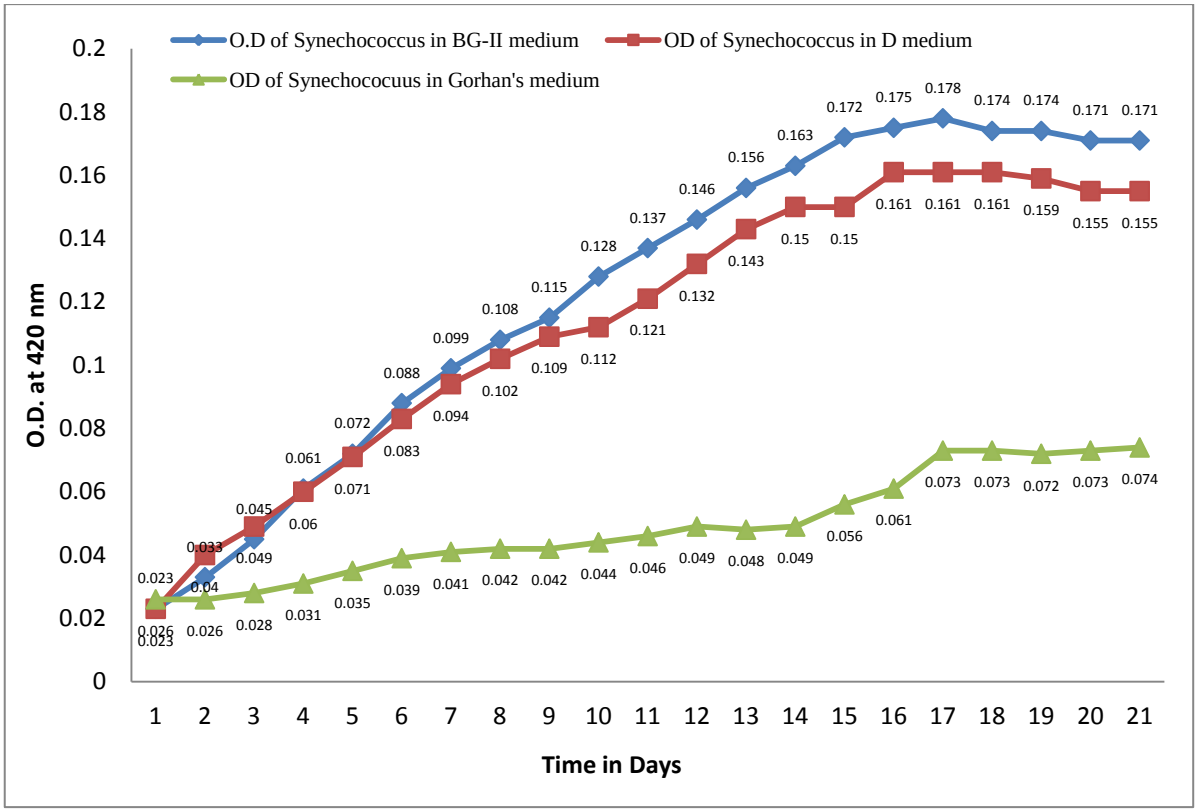
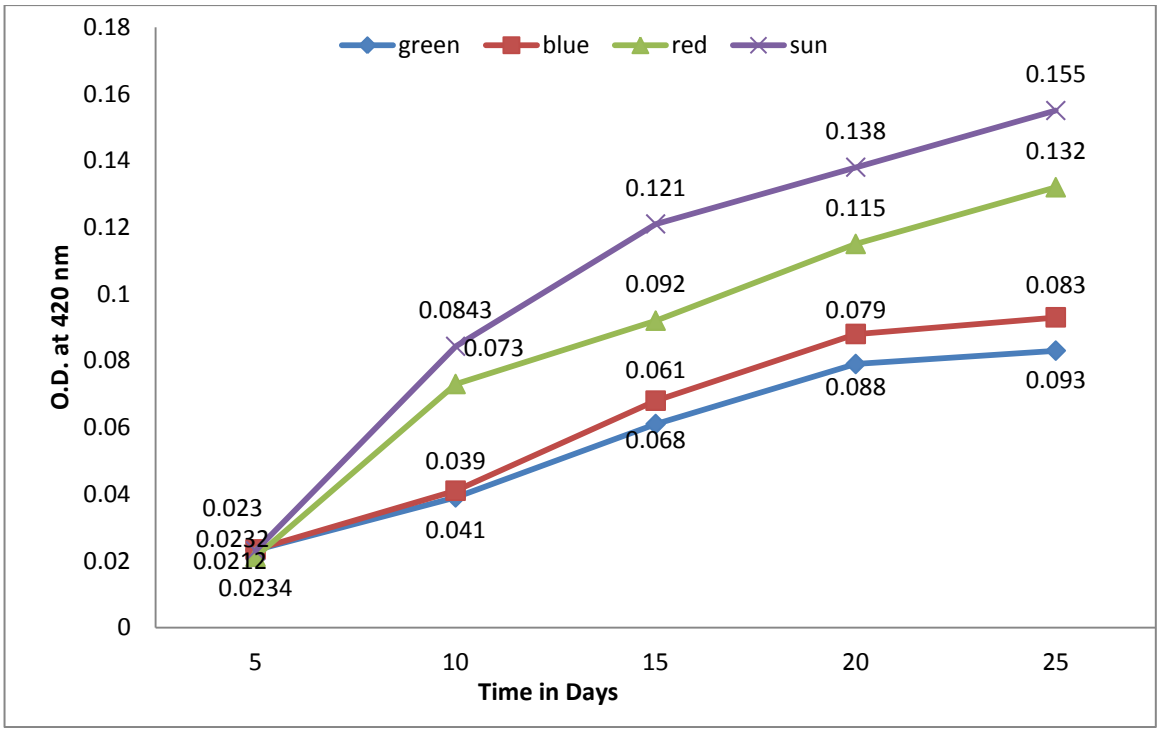
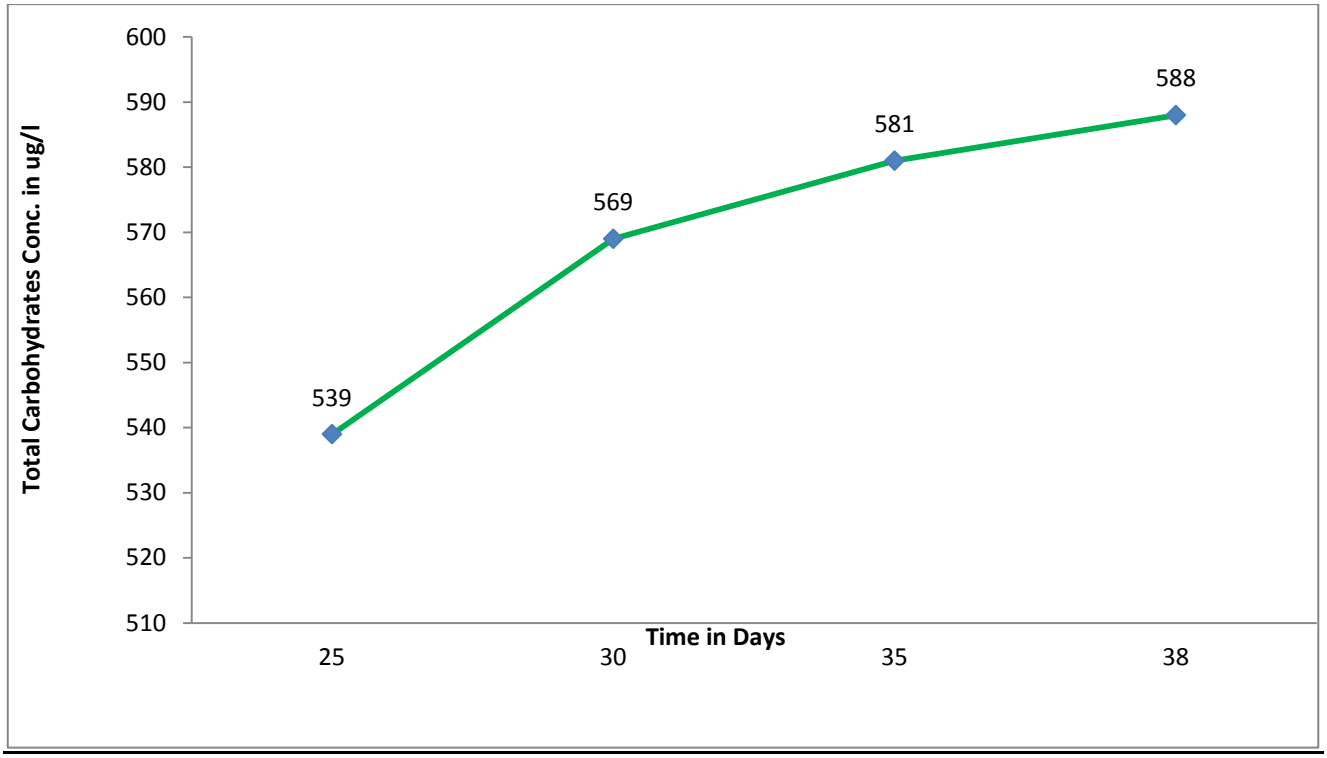


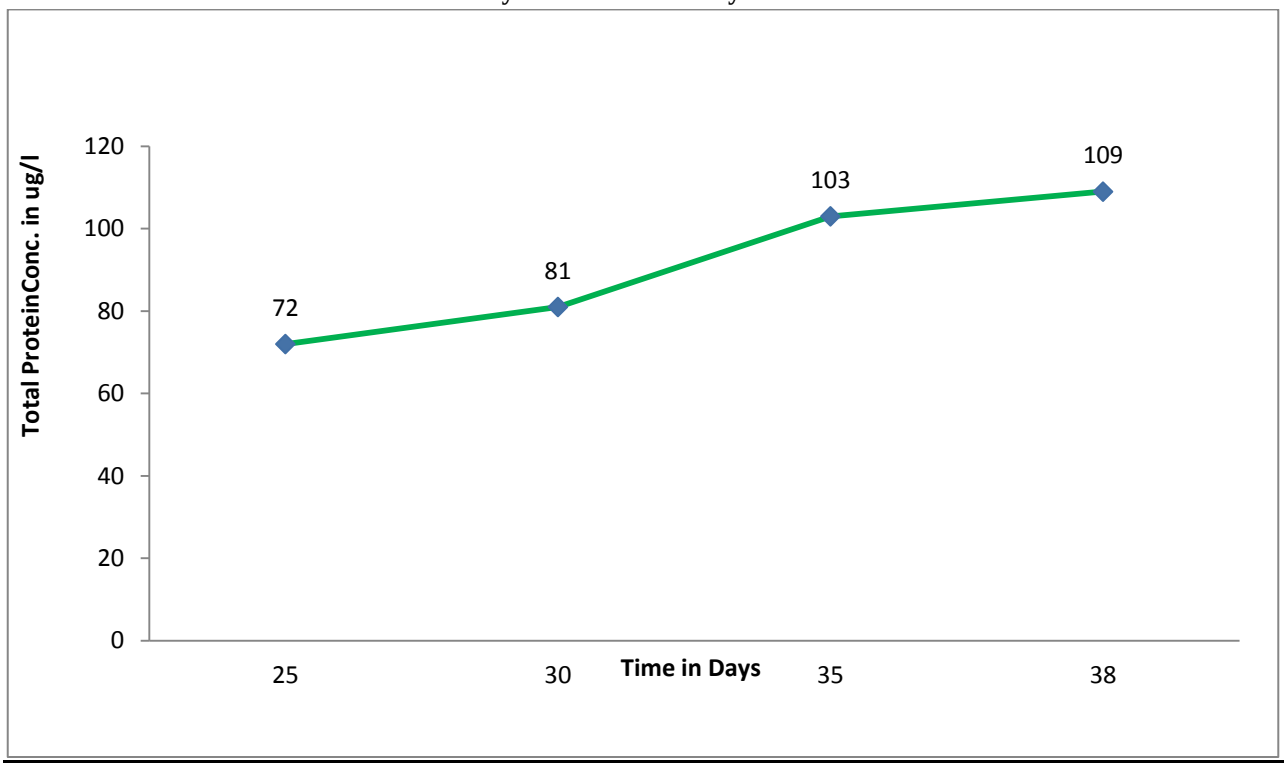

**Graph-1**
**Comparative Growth Curve studies of *Synechococcus spp.***




**Graph-2**
**Effect of Various Light Intensities on the Growth of *Synechococcus Spp*. in BG-11 Medium**




**Graph-3**
**Total Carbohydrate Estimation by Duboi's Method**




**Graph-4**
**Total Protein Estimation by Folin-Lowrey's Method.**

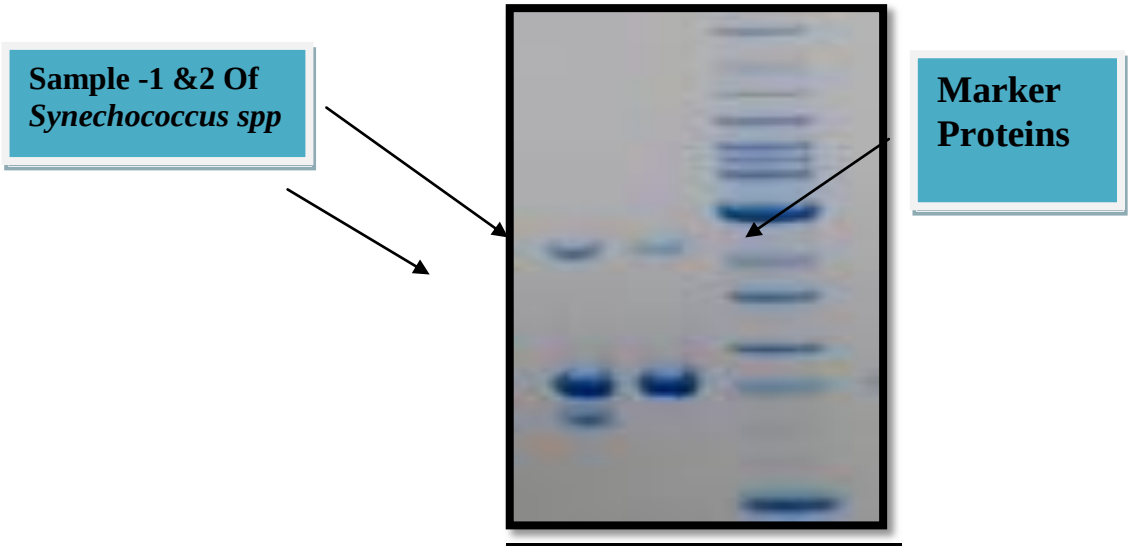



**Figure-7**
**SDS – PAGE**

The graphical representation graph - 2 proves that, the *Synechococcus spp*. shows maximum growth in presence of Sun light as compared to other lights i.e. blue, red, and green. Hence we have confirmed that, Sun light is necessary for maximum growth while the second most promising growth is observed in presence of Red light. Under optimum growth condition blue light also support the growth but here the lag phase is longer than the red light. A dead slow growth is observed in presence of green light in other words, Green light partially support the growth.

Total sugar and Proteins content of the EPSs were calculated by using Duboi's method and Folin-lowrey's method. By performing its qualitative analysis of EPS sample by on the basis of different tests we found reducing and non-reducing sugar present in the EPS sample. And on the basis of different references we conclude that these were sugars like, glucose, mannose, xylose and ribose present. Total carbohydrates and proteins concentrations were measured at regular time interval of 5 days and gradual increase in concentration (ranging from 539 µg/l to 588 µg/l ) of carbohydrates and (Ranging from 72 µg/l to 109 µg/l ) of proteins were found between 25 to 38 days graph - 3 & 4. SDS-PAGE was carried out for further identification of proteins and two moieties of proteins were found having molecular weight of 65 kDa and 30 kDa respectively figure - 7.

## Conclusion

Total sugar and proteins content of the EPS were calculated by using Duboi's method and Folin-lowery's method. By performing its qualitative analysis of EPS sample by on the basis of different tests we found reducing and non-reducing sugar present in the EPS sample. And on the basis of different references we conclude that these were sugars like, glucose, mannose, xylose and ribose present. The proteins so obtained were subjected to SDS-PAGE and the results, the molecular weight of protein which were obtained, were of 65 kDa and 30 kDa respectively.

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