

Comparative Growth Rate of Cyanobacteria from “Usar” Soil (saline/alkaline soils) with Respect to Pigments

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ABSTRACT

The pigment content in Blue-green algae is a specific feature of each species. The pigment variation is specific features among microalgae. The paper aim to analyze cyanobacterial extracts of different Usar soil of Azamgarh and Varanasi, Uttar Pradesh. The main object here is the importance of the blue green algae especially because of the pigments present in this class of algae. Pigments from natural sources are gaining more importance mainly due to health and environmental issues. Algae contain a wide range of pigments. Three major classes of pigments are chlorophylls, carotenoids (carotenes and xanthophylls) and phycobilins (Phycocyanin and phycoerythrin). Our present study investigates the efficiency for phycobiliprotein pigment production from four different cyanobacteria *Hapalosiphon* sp., *Phormidium* sp., *Anabaena* sp. and *Nostoc* sp. The harvested and dried biomass was subjected to extract pigments using different solvents. Thin Layer Chromatography was performed from extracted pigments using Acetone as extraction solvents. And running solvent especially for phycocyanin pigment was optimized and concluded that Petroleum ether and Acetone in the ratio of 7:3. This paper presents the information about the natural pigments of cyanobacteria and how they can be extracted and identified using different procedures and spectrophotometry. It emphasizes that the principal algal pigments are Phycobilins, Chlorophylls and Carotenoids.

Keywords: Cyanobacteria, cyanobacterial extracts, Cyanobacterial pigments, Phycobilins and Phycocyanin.

INTRODUCTION

Cyanobacteria are one of the major bacterial phyla and being large, widespread group inhabiting most of the world's environment. They are also one of the primary producers (Chisholm et al., 1988; & Waterbury et al.,

1979). Cyanobacteria and Blue green algae consist of Chlorophyll (green pigment) along with blue-green pigment known as Phycocyanin, which gives a distinct bluish green colour of many cyanobacteria (Whitton & Potts, 2000).

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Amongst the simplest of pigments, carotenoids are unbranched polyisoprenoid chains with conjugated double bond systems that may be linear or extend into terminal ring structures. Given their length (typically C40) and hydrophobicity, they are thought to have arisen in the Archaeobacteria as a structural reinforcement for the first cell membranes (Virshinin, 1999), and are now one of the most widespread and versatile lipid-soluble pigments in nature (Esteban et al., 2015). The structure and function of many of these carotenoproteins still requires elucidation, and many others are likely still to be identified, as there is no predictive method for recognizing carotenoid-binding pockets in proteins at the sequence level. On the other hand, much has been learned over the past decade about a group of water-soluble cyanobacterial carotenoproteins. Specific prediction of microcystin is due to the extracellular level of the fluorescent pigment Phycocyanin in the surface of water (Gerhardt & Bodermer, 2000; & Bastein et al., 2011) Major pigments of the microalgae which are used as pigments are carotenoids and phycobiliproteins. The pigments are characteristic of certain algal groups as indicated. Chlorophylls and carotenes are generally fat soluble molecules that can be extracted from thylakoid membranes with organic solvents such as acetone, methanol or dimethyl sulfoxide The phycobilins and peridinin, in contrast, are water soluble and can be extracted from algal tissues after the organic solvent extraction of chlorophyll in those tissues. Major objective in this study was Optimization, Screening, Selection and Extraction of Phycobiliproteins from four Cyanobacterial species *Hapalosiphone sp.*, *Phormidium sp.*, *Anabaena sp.*, and *Nostoc sp.*

MATERIALS AND METHODS

The present study has been carried out at the Department of Botany, U.P. College, Varanasi to analyze the pigment extracted from different sources. Three soils sampling sites were identified for the collection of soil samples. These soil site are located around Azamgarh

and Varanasi district of Uttar Pradesh. The site selected for this present study includes of two districts. The one site districts Varanasi (Bhawanipur) is located at an elevation of 80.71 meters (264.8 ft) and coordinates 25.28°N 82.96°E. The temperature between 22 and 46°C (72-115°F) in the summers. In the wenter from December to February temperature bellow 5°C (41°F) are not uncommon. The average rainfall is 1110 mm (44 inch) The second districts is Azamgarh where two site selected Bunda and Alipur has an average elevation of 64 meter (209 ft) and its geographical are coordinates 26°3'36" North 8°11'10"East. The temperature and rainfall like as a Varanasi districts. Three areas are selected from districts Varanasi and Azamgarh, Varanasi selected one site Bhawanipur and Azamgarh selected two sites Alipur and Bunda (Figure 1). Soil sample was collected from 'Usar' field during May 2018 to April 2019. The Cyanobacterial cultures for this study such as *Haplosiphone sp.*, *Phormidium sp.*, *Anabaena sp.* and *Nostoc sp.* Media and pH were selected rather than other factors and optimized because they play an important role in nutrition and its abiotic factors. Temperature was maintained at 29°C (room temperature), and Light (yellow fluorescent lamp), 12:12 (Light and Dark) respectively.

Preparation of unialgal cultures:

The environmental samples were used to isolate single algae by serial dilution and plating and streaking methods. Out of several samples 12 pure cultures were obtained out of which 4 samples were found to be the blue greens which could be identified.

Maintenance of cultures:

The unialgal cultures were maintained in the laboratory conditions in the culture room. The unialgal cultures were kept at 16/8 light and dark period and 28±.2 o C temperature, then they were identified according to keys given in Desikachary (1959) and Geitler (1932). 2 ml of cultures samples were taken for the extraction process of pigments. The pigments extracted from the collected algae were - Chlorophyll, Carotenoides and the most

important, the Phycobilins (C/R-Phycocyanin). The extraction of chlorophylls and carotenes were done using the three solvents- methanol, acetone and ethanol. Empirical correlations for total carotenoids evaluation by spectrophotometry are less common in the literature (M. Henriques, A. Silva & J. Rocha, 2007). Firstly, 0.05 – 0.5 g of thallus was taken out and weighed, secondly it was transferred to mortar and ground in 5 mL 0.1M phosphate buffer with a pH of 6.8, with acid-washed sand. Then it was centrifuged for 10 min at 1,000 x g. After this, the mixture was transferred to 25 mL volumetric flask and increased up to volume. Then the phycobilin concentration was determined using the correlations mentioned by Kursar and Alberte (1983). The concentration in millilitres is calculated according to the above mentioned correlations, and then the concentration of these pigments was also calculated in per gram of the algae according to the relation given by Maria Rio A. Naguit and Wilson L. Tisera, (2009). Algae were estimated for different pigment content such as chlorophyll, carotenoid, phycobilins. There are several methods to extract the pigments from these algae but maximum are based upon spectrophotometrical analysis with the following steps:

- a) Separation of algal cells from the supernatant
- b) Extraction of pigments by the help of an organic solvent
- c) Spectrophotometric determination of the pigments in the extract.

Estimation of Pigments

Major pigments such β -carotene, Chlorophyll a and Chlorophyll b were estimated. Along with these major pigments Phycocyanin a specialized and cyanobacterial specific pigment was also considered mainly for this work. Pigments such as Chlorophyll a, b and β -carotene were estimated photometrically by the following methods; Jeffrey and Humphrey method, (1975) and MacKinney method, (1941) respectively using different wavelengths by UV-visible spectrophotometer. Phycocyanin was measured photometrically by analyzing in different wavelengths at 620, 652 and 562 nm

(Siegelman & Kycia method, 1978). Along with the pigments, Growth rate curve of cyanobacteria was also analysed at 690 nm.

Extraction of pigments

The optimized culture conditions were established to grow the four cyanobacterial cultures in four flasks containing 25 ml of media for 16 days and the whole culture was allowed to centrifuge at 8000 rpm for 10 min and obtained cyanobacterial biomass served as a source for extraction of pigments. The wet biomass was grounded well using Mortar and Pestle for 5 min. allowed to break the cells physically and subjected to Sonication (Equitron Ultrasonic cleaner) at 53 KHz for 30 min. in room temperature (29°C). After successive centrifugation, at 10,000 rpm for 5 min. the pellet formed cell debris were discarded and supernatant can be used as the crude pigment complex. One ml of crude extract from all the four cyanobacteria was taken in each of two ml eppendorf tubes, in which 1 ml of 90% Acetone was added and kept overnight for complete extraction of all the pigments. Then the Acetone extract subjected to centrifugation at 10,000 rpm for 5 min. the supernatant was allowed to run as a loading sample on TLC sheet.

Results

Media and pH were optimized for all the four cyanobacteria, in which BG11 and pH 7.0 were concluded to be the best when compared to all the other three media and four pH respectively for all the four cyanobacteria *Hapalosiphon sp.*, *Phormidium sp.*, *Anabaena sp.* and *Nostoc sp.* When comparing the growth rate analysis of all the four Cyanobacteria, in which *Hapalosiphon sp.* and *Anabaena sp.* showed to be reaching high optical density in photometric analysis than *Phormidium sp.* and *Nostoc sp.* it is not that *Phormidium sp.* and *Nostoc sp.* are not suitable for BG11 but growth rate was propelled in *Hapalosiphon sp.* and *Anabaena sp.* Thus *Hapalosiphon sp.* and *Anabaena sp.* are considered to yield high biomass. Quantitative analysis of pigments-UV-Vis. Spectrophotometer β -carotene, Chlorophyll content are found high quantity in *Hapalosiphon sp.* and *Anabaena sp.* compared to *Phormidium sp.* and *Nostoc sp.*

Hapalosiphon sp. (188.1 mg ml⁻¹) showed high content of β -carotene, Chlorophyll content than *Anabaena sp.* *Hapalosiphon sp.*, *Anabaena sp.*, *Nostoc sp.*, and *Phormidium sp.*, Chlorophyll content respectively 13.39 μ g/ml, 11.67 μ g/ml, 4.67 μ g/ml and 1.55 μ g/ml (Fig-2) and Carotenoid content are 188.1 mg/ml, 153.12 mg/ml 72.01 mg/ml and 69.02mg/ml (Fig-3). Phycocyanin was estimated at 620 nm, 652 nm and 562 nm which consist of Phycocyanin, Allophycocyanin and Phycoerythrin respectively. Phycocyanin, Allophycocyanin and Phycoerythrin was abundant in both

Hapalosiphone sp. (3.16 mg ml⁻¹, 2.89 mg ml⁻¹ and 2.36 mg ml⁻¹) and *Anabaena sp.* (2.58 mg ml⁻¹, 2.62 mg ml⁻¹ and 2.69 mg ml⁻¹, respectively) compared to all the other cyanobacterial species (Fig-4).

Qualitative analysis of pigments-Thin Layer Chromatography (TLC) Different types of solvents are used to optimize the exact extraction method of pigments. In which Phycocyanin associated with Chl. a and Chl. b, thus a combination of solvents with various ratios were used. Petroleum ether and Acetone are found to be the best solvents for extraction of Phycocyanin at the ratio of 7:3.

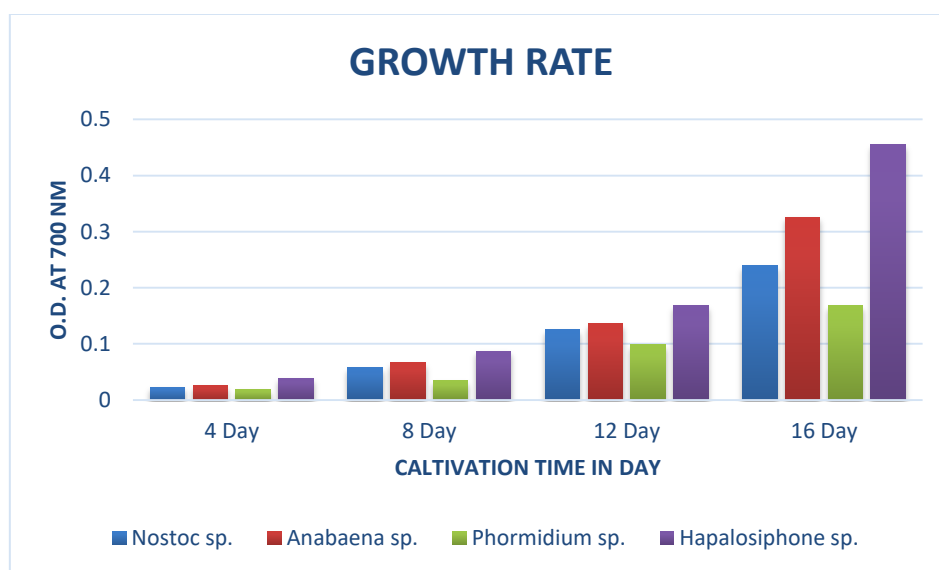


FIG. 1: SHOWING THE GROWTH ANALYSIS CURVE OF FOUR DIFFERENT CYANOBACTERIA- *Nostoc sp.* *Anabaena sp.*, *Phormidium sp.*, and *Hapalosiphon sp.*

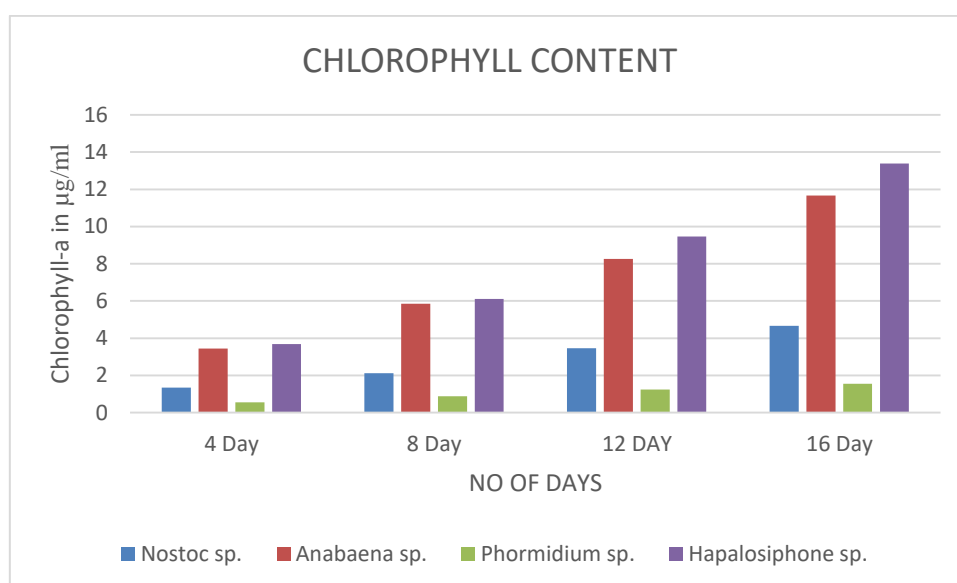


FIG. 2: Showing Chlorophyll content at 4th, 8th, 12th and 16th days of four different Cyanobacteria- of four different Cyanobacteria- *Nostoc sp.* *Anabaena sp.*, *Phormidium sp.*, and *Hapalosiphon sp.*

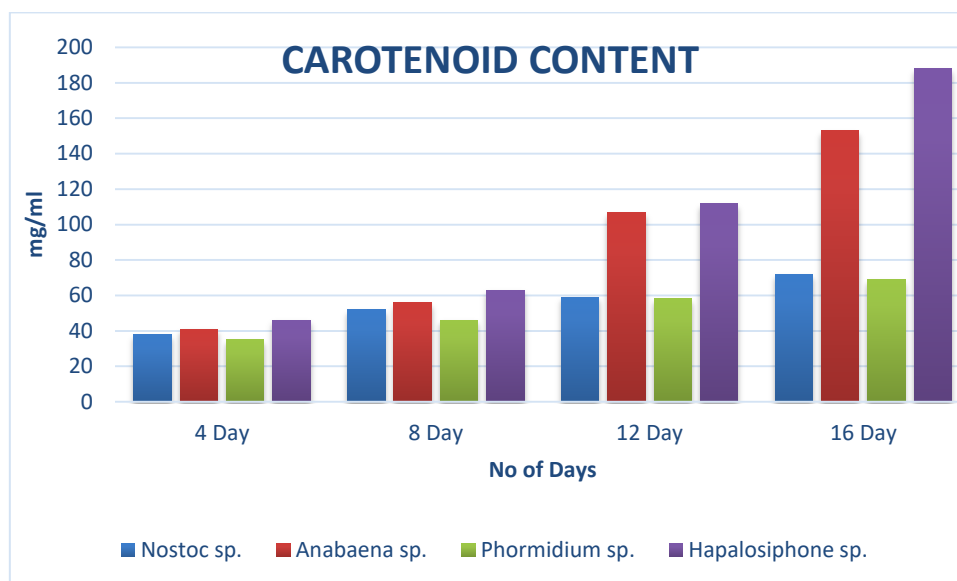


Fig. 3: Showing Carotenoid Content at 4th, 8th, 12th and 16th days of four different Cyanobacteria- Nostoc sp. Anabaena sp., Phormidium sp., and Hapalosiphon sp.

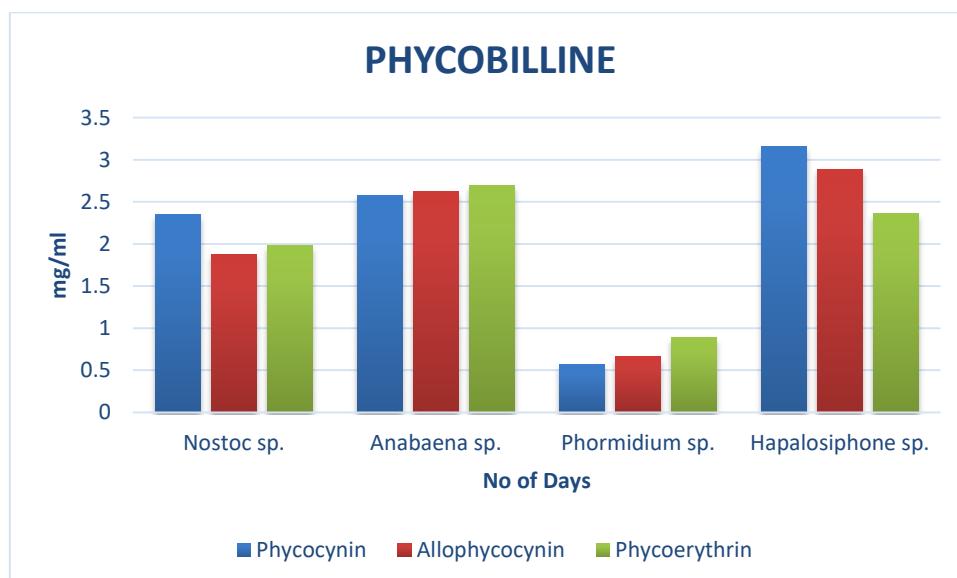


Fig.4. Quantitative analysis of Phycobiliproteins from different cyanobacteria Nostoc sp. Anabaena sp., Phormidium sp., and Hapalosiphon sp

DISCUSSION

Microalgae are the rich sources of pigments which are high value due its medicinal properties. Cyanobacteria are Blue green algae as the name indicates the blue green was due to the presence of phycocyanin pigment in it. Blue green algae can also produce pigments such as β -carotene and Chlorophyll pigments as in microalgae, but Phycocyanin is a specialized pigment present only in Cyanobacteria. Phycobiliproteins are coloured proteins, employed in the absorption of light

and transfer to the reaction centre of PSII (Glazer, 1984). From the total amount of proteins present in the cells of cyanobacteria, Phycobiliproteins alone constitutes 60 % of it (Bogard, 1975). In our study, β -carotene was present in larger amount than the other pigments in all the four cyanobacteria. Phycobiliproteins are the next to β -carotene present in larger amount. C-Phycocyanin has free radical active sites in it; this was the first report by analyzing in Electron spin resonance spectra (ESR), thus Phycocyanin can perform

free radical scavenging property. And Lyngbya C-Phycocyanin has higher peroxy radical scavenging activity than Spirulina and Phormidium C-Phycocyanin with an IC₅₀ value of 6.63 μ M (Patel et al., 2006). Anti-cancer activity in humans and animals were also reported from C- Phycocyanin (Pardhasaradhi et al., 2003). Synechococcus sp. WH8102, in high temperature growth led to bountiful production of PBS pigments (Mackey et al., 2013). Pigments were extracted using different solvents; Acetone was used to extract all the proteins from the cyanobacterial biomass. TLC showed a distinct band of Phycobiliproteins in Haploshiphon sp. from both BG11 and CFTRI media, but in Anabaena sp. and Nostoc sp. the bands were drifted slightly. Petroleum ether and Acetone in the ratio of 7:3 was concluded to be the best solvent mixture for separation of phycobiliproteins. TLC a qualitative method is a traditional and innovative method of extraction of Phycobiliproteins rather than Ammonium sulphate fractionation and Dialysis are the method used globally. Phycocyanin content of six cyanobacteria in batch cultures from dried biomass were 144.2 μ g mg⁻¹ in Anabaena sp. (Antarctic), 82.8 μ g mg⁻¹ in Anabaena sp. (Tropical), 136.4 μ g mg⁻¹ in Nostoc sp. (Ant.), 88.6 μ g mg⁻¹ in Nostoc sp. (Tro.), 162.8 μ g mg⁻¹ in Phormidium sp. (Ant.) and 95.5 μ g mg⁻¹ in Phormidium sp. (Tro.) (Shukia et al., 2008). Anabaena sp. and Arthospora platensis have produced 14-23.5 and 0.82-1.32 m⁻² day⁻¹, respectively while growing in Raceway ponds (Jiménez et al., 2003). *Hapalosiphon sp.* and *Anabaena sp.* found to produce high yield of Phycocyanin from this study.

CONCLUSION

Four cyanobacteria, in which three are heterocyst's and one is nonheterocystes were used to optimize for the high yield of pigment Phycocyanin. During optimization *Hapalosiphon sp.* was found to show result in BG11 media thus, *Hapalosiphon sp.* is a marine cyanobacterium. Optimization of pH recruits a uniform media for all the four

cyanobacteria which was BG11. All the four cyanobacteria were grown well in pH 7.0. While analyzing the production of phycocyanin, *Hapalosiphon sp.* and *Anabaena sp.* was the best when compared to *Phormidium sp.* and *Nostoc sp.* Solvent extraction method was established to extract the pigments from the biomass obtained. Solvent used to run TLC were optimized for separation of Phycocyanin and can be useful to isolate using Column chromatography for further studies. Analysis of phycocyanin screening and selection leads to select the cyanobacteria *Hapalosiphon sp.* and *Anabaena sp.* to yield high biomass to yield expected amount of phycocyanin. The conclusion of the present study is that *Hapalosiphon sp.* a "Usar" soil cyanobacterium. The cyanobacterial strains, such as *Anabaena* (both freshwater and halotolerant) are found to show good growth and synthesized high yield of phycobiliproteins in comparison to other marine cyanobacterial strains. The filamentous cyanobacterial forms like *Anabaena sp.* was recorded as maximum phycocyanin, marine *Hapalosiphon sp.* (3.16 mg/ml) was having highest phycoerythrin. *Anabaena sp.* was reported to acquire more amounts of allophycocyanin (2.62 mg/ml) of phycobiliproteins.

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