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A COMPARISON OF PHOTOPIGMENTS AND CHARACTERIZATION OF PHYCOCYANIN IN CYANOBACTERIA ORIGINATING FROM DIFFERENT HABITATS

AKHILESH P. SINGH,[1] RAVI K. ASTHANA[1] AND SUBHASHA NIGAM*[2]

[1]Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India
[2] Amity institute of Biotechnology, Amity University, Sec 125 Noida, India

ABSTRACT

Cyanobacterial diversity under environmental extremes led us to screen Chl *a*, carotenoids d and phycocyanin in cyanobacteria *Nostoc muscorm* (mesophile), *Mastigocladus laminosus* (thermophile) and *Nostoc* 593 (Antarctic) grown at 30, 40 and 20°C, respectively. Maximum amount of Chl *a*was recorded in *N muscorum* (6.34 µg mg^{-1} dry weight) over *Nostoc* 593 (6.29 µg) and *M. laminosus* (4.62 µg).While for carotenoids, the sequence changed to *Nostoc* 593 (4.35 µg) > *M. laminosus* (4.08 µg) > *N. muscorum* (2.48 µg). Phycocyanin followed the same pattern as carotenoids. Maximum amount of phycocyanin was recorded in *Nostoc* 593 with 154 µg over *M. laminosus* with 84.7 µg and *N. muscorum* with 64.52 µg mg^{-1}dry wt. Phycocyanin was purified to homogeneity and confirmed further by SDS-PAGE. Pure phycocyanin yielded two subunits with apparent molecular mass of 14 and 15 kDa each. It is anticipated that cyanobacteria may be the ideal resource of such antioxidant proteins from the biotechnological viewpoint.

KEYWORDS: **Antarctic; mesophilic; thermophilic; cyanobacteria; phycocyanin; purification**

*Corresponding author

SUBHASHA NIGAM
Amity institute of Biotechnology, Amity University, Sec 125 Noida, India

INTRODUCTION

Cyanobacteria can also be termed extremophilic, as these inhabit a variety of extremes including low temperature (-2^{0}C) [1] and high temperature (75^{0}C) among others although with a sluggish growth[2]. The constitution of cyanobacterial photosynthetic apparatus is environmentally induced[3]. For example, temperature acclimation of the light harvesting efficiency is generally associated with increased photosynthetic pigment(s) [4], and pigment amount and growth temperature are directly linked. By contrast, in some algae, and higher plants, chlorophyll content is inversely related to growth temperature[5]. Changes in the organization of photosynthetic pigment in response to growth temperature have also been reported in the green alga *Chlorella* and the cyanobacterium *Anabaena* [6] There are suggestions that polar cyanobacteria decrease concentrations of Chl *a* and other light-harvesting pigments as the survival strategy [4]. Decreasing temperature also enhanced carotenoid content but lowered Chl *a* [7] Also, the role of carotenoids as a screening pigment and protectant has been associated with this increase. Carotenoids quench excess photochemical energy and thereby protect photosynthesis from the synergistic effect of low temperature and high irradiance [7, 8.] The extracellular UV absorbing pigment scytonemin has multiple roles, functioning as UV sunscreen and antioxidant [9]. Phycobilisomes, the large supramolecular aggregates attached to the thylakoid membrane of cyanobacteria, function in light harvesting and energy migration [10] Cyanobacteria only contain chl *a*, and almost 50% of the photosynthetic light required, is captured by phycobiliproteins and the latter are also used as N reserve during nutritional-stress [11.] carotenoids Phycocyanin is an effective scavenger for various reactive oxygen species *in vivo* [12] and thus has therapeutic properties mainly as the antioxidant effective 20 times over ascorbic acid [13]. It is also used as anti-inflammatory [14]and hepatoprotective agent [15]. The major application of phycobiliproteins is in fluorescent techniques. Recent studies have implicated them in immunomodulating and anticarcinogenic testings and as natural dyes for feed and cosmetics [9]. The cyanobacteria offer the potential resources of phycobiliproteins as ~ 20% of the cell dry weight is phycocyanin. There is a strong correlation between phycocyanin concentration and cyanobacterial biovolume within and acreoss ponds [16]. Owing to the biotechnological potential of phycocyanins, and the advantages that the cyanobacteria offer, we attempted to compare the photopigments in general with special emphasis on isolation purification and partial characterization of phycocyanin from *M. laminosus* (thermophile), *N. muscorum* (mesophile) and *Nostoc* 593 (Antarctic strain), respectively.

MATERIALS AND METHODS

(i) Organisms and growth conditions

The diazotrophic cyanobacterium *Mastigocladus laminosus* was isolated from hot springs at Gangnani, Uttarkashi, India, *Nostoc mscorum* ISU (*Anabaena* ATCC27893), the kind gift from R. Haselkorn, USA and *Nostoc* 593, the Antarctic strain from Dr. Paul A. Broady, New Zealand and were grown in modified Chu-10 medium [17], free from any combined nitrogen sources under continuous tungsten plus fluorescent illumination (14.4 W/m^2) at 40±1^{O}C, 30±1^{O}C and 20±1^{O}C, respectively.

(ii) Measurement of growth (k) and generation time (g)

The specific growth rate constant (k) and generation time (g) were calculated as described by Kratz and Myers [18.]

(iii) Dry weight determination

Cells were concentrated by centrifugation, washed and dried (60^{O}C) to constant weight (expressed as gl^{-1}).

(iv) Protein estimation

Protein was estimated by the method of Lowry et al. (1951) as modified by Herbert et al. (1971) using lysozyme (Sigma, USA) as standard.

(v) Extraction and determination of photo pigments

A known volume (10ml) of cyanobacterial culture (7d old), centrifuged (3,000 rpm), the pellet suspended in 10 ml acetone (80%, v/v) and incubated overnight in dark ($4^{O}C$). The supernatant was used to measure Chlorophyll *a* (665 nm) and carotenoids (460 nm) in Spectronic-20 colorimeter (Bausch and Lomb, USA). Phycocyanin from the remaining residue was extracted in deionized water through 2-3 cycles of freezing and thawing, and the absorbance read at 620 nm. Pigment concentration (mg g^{-1} biomass d wt.) was calculated using absorbance coefficients 82.04 for Chl *a,* 200 for carotenoid [19] and 7.5 for phycocyanin [20], respectively according to the equation $\alpha = D/dc$ where D = optical density of the pigment, d = inside path length of the cuvette (cm) and c = pigment concentration (gl^{-1}).

(vi) Isolation, purification and partial characterization of phycocyanin

Phycocyanin in bulk was isolated as described by Boussiba and Richmond [21]. Approximately 7g (wet weight) of cyanobacterial biomass as suspended in 200ml 0.1 M Na-phosphate buffer (pH 7.0) containing 100 µg ml^{-1} lysozyme and 10 mM EDTA and enzymatic disintegration of cell wall was brought about in a shaking bath at $30^{O}C$ (24 h). The slurry was then centrifuged for 1 h at 40,000xg ($4^{O}C$) to remove cell debris, yielding a clear blue supernatant. Blue supernatant containing phycocyanin was precipitated in $(NH_4)_2.SO_4$ (50%) and recovered by centrifugation (10,000xg, 10 min). The clear supernatant was discarded and the blue precipitate dissolved in a small volume of 0.0025 M Na-phosphate buffer (pH 7.0) followed by dialysis against the same buffer using dialysis tube (Sigma, USA). Absorption spectrum of the blue pigment as recorded using ATI-UNICAM V_2.O UV-VIS spectrophotometer, UK. The dialyzed blue pigment was then loaded in a gel filtration assembly using sephadex G-100 (Sigma, USA) column (1.5x20 cm). The main fraction was eluted and pooled following a step-wise elution with phosphate buffer of increasing ionic strength at pH 7.0. The fraction with highest 620/280 > (4.2) ratio, eluted between 2.5 mM-70 mM represented phycocyanin. The elute was subjected to fluorescence spectral analysis using Perkin-Elmer LS-5B spectrofluorimeter (USA) equipped with a constant temperature cell holder

(vii) SDS-polyacrylamide gel electrophoresis of purified phycocyanin

Phycocyanin purified from the three target strains was subjected to SDS-PAGE analysis as described by Laemmli [22] using separating gel 10% (w/v) and stacking acrylamide gel 5% (w/v) (Sigma). The requisite volume of acrylamide solution, Tris-buffer, SDS solution and water was mixed, aerated followed by the addition of $(NH4)_2$ SO4 (0.1% w/v) and 5 µl of TEMED (Sigma) per 10 ml gel solution. To the protein samples, $1/5^{th}$ volume of 5 x gel loading dye was added and boiled for 3-5 min. Electrophoresis was carried out at 100V for stacking and 200V for resolving (separating) gel using running buffer. Protein markers (36-14 kDa, Banglore Genei, India) were run to account for the molecular weight of phycocyanin subunits. The gel was then stained with a Coomassie Brilliant Blue (R 250) according to Jackman [23]. For a better contrast, the gel was subjected to silver staining according to [24].

(viii) Statistical analysis

Statistical analysis was done according to Zar [25]. One-way ANOVA test has been used for the significant difference among the mean levels of different pigments at the respective growth temperature optima of the test strains. Multiple comparisons [Student- Newman- Keuls; (SNK) test] have been applied to find out the pairwise significant differences.

RESULTS

1. Comparision of photopigments and general growth

The data in Table 1 account for general growth of the cyanobacterial strains grown at their respective temperature optima along with the amount of Chl *a*, phycocyanin and carotenoids. *M. laminosus* growing at $40^{O}C$, had the lowest Chl *a* level (4.62 µg mg^{-1} dry wt.) followed by a rise in the pigment level to 6.34 µg in the

mesophilic *N. muscorum* (30°C) thus amounting to an almost 1.5-fold rise. It is also evident that Chl *a* amount in *N. muscorum* was on top of the rest two as for *Nostoc* 593 (growing at 20°C), it was slightly less (6.29 µg). Regarding carotenoids, *Nostoc* 593 had the maximum amount (4.35 µg mg^{-1} d wt) followed by *M. laminosus* (4.08 µg) and *N. muscorum* (2.48 µg). Estimations of phycocyanin put *Nostoc* 593 as the efficient producer because the level reached 154.0 µg followed by *M. laminosus* (84.7 µg), thus amounting to a reduction very close to 2-fold and the least in *N. muscorum* (64.52µg). The overall comparison presents the sequence of Chl *a* amount as: *N. muscorum* > *Nostoc* 593 > *M. laminosus*; for carotenoids, the sequence changed to *Nostoc* 593 > *M. laminosus* > *N. muscorum*. For phycocyanin, the sequence was in the increasing order as: *Nostoc* 593 > *M. laminosus* > *N. muscorum*. The data, therefore, indicate high synthesis of phycocyanin in *Nostoc* 593 followed by the thermophile *M. laminosus*. Another point of significance was that the *Nostoc* 593 (Antarctic) is slow growing (generation time, 70.58 h), the pace improved in thermophilic *M. laminosus* (33 h) and achieved it maximum in *N. muscorum* (26 h).

Table 1

Comparison of photopigments and growth of the targeted cyanobacterial strains at their respective temperature optima (after 7 days).

Strain	Chlorophyll *a* (µg mg^{-1} d wt.)	Phycocyanin (µg mg^{-1} d wt.)	Carotenoids (µg mg^{-1} d wt.)	Specific growth rate (k)	Generation time (h)
M. laminosus	4.62±0.11	84.70±0.50	4.08±0.04	0.03	33
N. muscorum	6.34±0.44	64.52±0.48	2.48±0.03	0.04	26
Nostoc 593	6.290± 0.22	154.00±1.50	4.35±0.17	0.0142	70.58
F-value	33.94***	7266.69***	292.83***	-	-

Multiple comparisons (Student-Newman-Keuls values)

Strains	Chlorophyll *a*	Phycocyanin	Carotenoids
M. laminosus vs. *N. muscorum*	7.23***	25.91***	19.05***
M. laminosus vs. *Nostoc* 593	7.02**	88.96***	3.20
N. muscorum vs. *Nostoc* 593	0.21	114.87***	22.26***

***P<0.01**

****P<0.001**

(ii) Isolation purification and partial characterization of phycocyanin

Variation in phycocyanin level of the three cyanobacterial strains belonging to different habitats tempted us to go for isolation, purification and characterization of the pigment. Figs. 1,2,3 represent absorption spectra of partially purified blue pigment from *M. laminosus*, *N. muscorum* and *Nostoc* 593, respectively. However, an extra shoulder at 651 nm in Fig. 1 in case of *M. laminosus* and *N. muscorum* (Fig. 3, 674 nm) indicated partial purification of phycocyanin. The blue pigment's characteristic excitation and emission wavelengths during fluorescence are also presented (Table 2). The emission signatures at 653, 650 and 651 nm for *M. laminosus*, *N. muscorum* and *Nostoc* 593 reflect the extent of phycocyanin purity. The SDS-PAGE (silver stained) pattern of purified phycocyanin from the three target strains indicated two bands corresponding to subunits of phycocyanin in the mol.wt range of 14 kDa and 15 kDa, respectively (Fig.4).

Table 2

Excitation and emission pattern of phycocyanin isolated from the target cyanobacterial strains

Strain	Fluorescence	
	λ_{ex} (nm)	λ_{em} (nm)
Mastigocladus laminosus	345	653
Nostoc muscorum	342	650
Nostoc 593	343	651

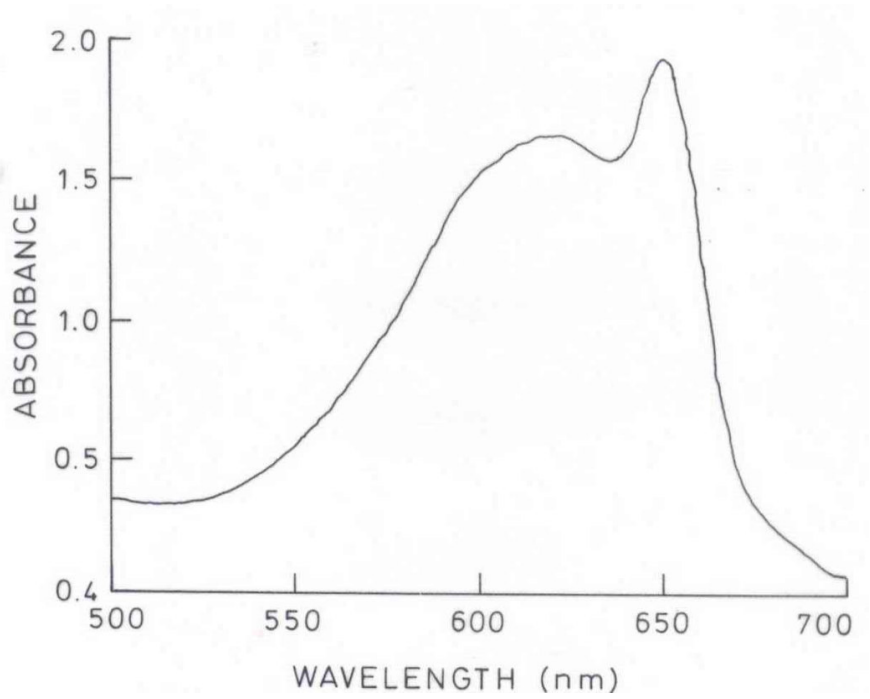

Figure1
Absorption spectrum of phycocyanin from Mastigocladus laminosus in 0.1 M phosphate buffer pH 7.0 (ammonium sulphate precipitated and dialyzed)

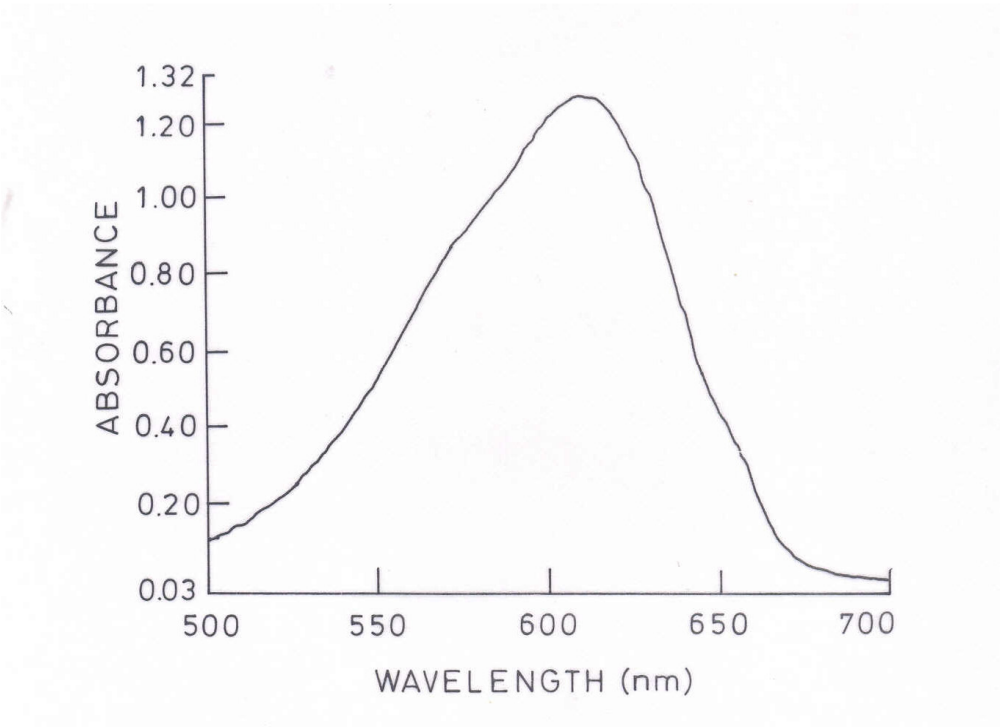

Figure 2
Absorption spectrum of phycocyanin from Nostoc muscorum in 0.1 M phosphate buffer pH 7.0 (ammonium sulphate precipitated and dialyzed)

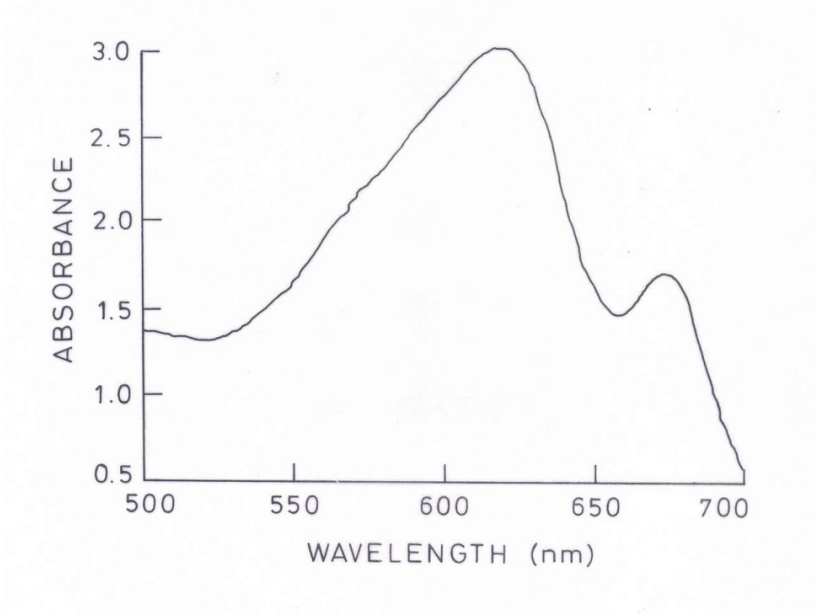

Figure 3
Absorption spectrum of phycocyanin from Nostoc 593 in 0.1 M phosphate buffer pH 7.0 (ammonium sulphate precipitated and dialyzed)

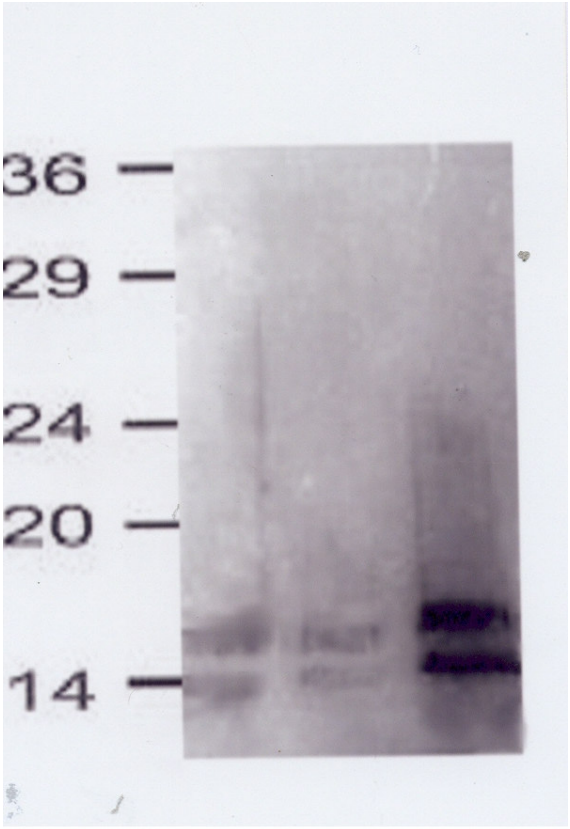

Figure 4
SDS-polyacrylamide gel electrophoresis showing protein bands of the purified phycocyanin from the target strains, lane-1 (M. laminosus), lane-2 (N. muscorum) and lane-3 (Nostoc 593) (silver stained).

DISCUSSION

The background of habitat specific adjustments of algae in the Arctic ice [1], Antarctic [26] motivated us to know the fate of selected photopigments in the mesophilic, Antarctic and thermophilic cyanobacterial representatives. Limited information is available on cyanobacterial growth vs. temperature [2]. The latter investigators reported that growth temperature optima varied considerably among the 27 polar cyanobacterial isolates, and for the two extreme examples: (a) *Phormidium subfuscum* growing in 5-25^{O}C range had optimum at 15^{O}C, while in (b) *Phormidium tenue* the specific growth increased between 5-35^{O}C suggesting that the two cyanobacterial strains were thermo-tolerant rather than adapted. The observations compiled in Table1 provide a summary of general growth of three cyanobacterial targets at their respective growth temperature optima. The general growth of *Nostoc* 593 was slowest (doubling time, 70.58 h) followed by *M. laminosus* (33 h) thus presenting a contrast with the mesophilic *N. muscorum* (26 h). The observations although restricted to only three cyanobacteria in the present case, do collectively reflect that mesophiles possess high level of the main photopigment Chl *a*, as the amount for the two 'extremophiles' fell in the lower range; the most affected one was *M. laminosus* (4.62 µg mg^{-1} d wt). The carotenoid levels were contrary to Chl *a* in *N. muscorum* that had the minimum (2.48 µg), while the two 'extremophiles' had almost the same pigment amount (4.21 µg, average). The latter two also had high levels of phycocyanin with the maximum of 154.0 µg in *Nostoc* 593 followed by *M. laminosus* (84.70 µg). The mesophilic *N. muscorum* in such comparisons, had the upper limit of this pigment to just 64.52 µg. Therefore, it is tempting to suggest that a rise in carotenoids and phycocyanin level was strain specific. Algae tend to be more prone to photoinhibition at temperatures low or high than the optima [27]. The increase in carotenoid: Chl *a* ratio at supraoptimal temperatures also reflects the faster degradation of Chl *a* relative to carotenoids [7]. Generally, photosynthesis limits growth at low temperature in such a way that algal cells tend to direct resources away from the synthesis of light harvesting components [28]. These investigators also suggested that decrease in the phycocyanin: Chl *a* or the carotenoids: Chl *a* ratio was controlled primarily by variations in cellular Chl *a* biosynthesis. However, further investigations are needed to unravel the impact of decreasing temperature on the pigment synthesis and also the architecture of the phycobilisomes. A marked variation in amounts of phycocyanin among the three strains selected, aroused interest in the purification of this photopigment for further characterization. The presence of shoulders at 651 nm for *M. laminosus*.(Fig. 1) or at 674 nm for *Nostoc* 593 (Fig. 3) indicates qualities of the partially purified phycocyanin. The emission wavelengths for this pigment signify the extent of phycocyanin purification through gel-filtration as this forms the basis to differentiate based on the optical signals. Similar signals were also obtained in *Synechococcus leopoliensis* and *Anabaena flosaquae* [29]. Genera like *A. flosaquae* and *S. leopoliensis* displayed very distinct emission signatures, most likely attributable to differences within phycobilin pigments. Such emission signatures were also used for discriminating phycobilin-containing and non-phycobilin-containing species. Fig. 4 shows the outcome of SDS-PAGE for phycocyanin purified from the three cyanobacterial targets used. The adjoining bands indicate two subunits with molecular weight 14kDa and 15 kDa, and the complete heterodimer of 29 kDa. The molecular weight of β subunits was found to be 24.4 kDa for all the C-PC, whereas for α subunits it was 17,19.1, and 15.2 kDa for *Spirulina*, *Phormidium*, and *Lyngbya* spp. C-PC, respectively [30.] The molecular weight of intact phycocyanin is 215 kDa, whereas the denaturating gel electrophoresis showed presence of two bands of 19 and 20 kDa, indicating the characteristic (αβ) 6-subunits assembly of phycocyanin as in *Oscillatoria quadripunctulata* [31]. The other report in *Calothrix* sp. indicated two sub-units with apparent molecular mass of 21-17 kDa

each [32]. In other words, ranges reported are in close proximity with the present data and collectively suggest that all the target strains have heterodimers in their subunit makeup of phycocyanin as applicable to other cyanobacteria, and exposure to different temperature regimes might affect their relative distribution/configuration. It may also be speculated that the structure and number of phycobilisomes could be responsible for the altered level of phycocyanin depending on the habitat in a strain-specific manner. This idea gets support from the work on *S. platensis* that a decrease in the number of phycobilisomes could be well correlated with the decrease in cellular phycocyanin and allophycocyanin content for cells shifted from low to high light [33].Some study has revealed the utilization of certain strains of cyanobacteria for the production of biologically active compounds particularly antioxidants [34]. Further work on phycobilisomes as well as gene(s) governing the overproduction of phycobilins in a praticular strain, would possibly open the vistas of biotechnological potential of overproduction of phycobilins.

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