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Screening of Cyanobacteria for Phycobiliproteins and Effect of Different Environmental Stress on Its Yield

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Abstract Among 18 strains, cyanobacterium *Anabaena* NCCU-9 contained the highest amount of phycobiliprotein (91 mg/g dry cell weights). Therefore, the effects of various environmental stresses were investigated on its phycobiliprotein production potential. The ideal conditions observed were 30°C, 25 μmol photons/m^2/s, white light, pH-8, 16:8 light and dark regimes, nitrogen free medium and 10 mM sodium chloride. Among three pesticides studied malathion showed highest toxicity. Under heavy metal stress the order of toxicity was chromium > cadmium > lead > nickel > copper > zinc.

Keywords Cyanobacteria · Phycobiliprotein · Light · Temperature · Salinity · pH · Pesticide · Heavy metals

Phycobiliproteins are water-soluble naturally occurring light harvesting pigments commonly present in cyanobacteria and some eukaryotic algae (rhodophytes, cryptomonads, and glaucophytes). Phycobiliproteins are covalently attached linear tetrapyrrole chromophoric group called bilins or phycobilins because of their close structural relationship to the well-known bile pigments of human—bilirubin and biliverdins (Lemberge and Legge 1949). In algae, they are used as accessory or antenna pigments for photosynthetic light collection. They absorb energy in portions of the visible light spectrum that are poorly utilized by chlorophyll and convey the energy to chlorophyll-*a* at the photosynthetic reaction centre.

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Cyanobacteria can regulate their tetrapyrrole content and composition in response to environmental signals, such as nutrient availability, light intensity, light wavelength and temperature (Prassana et al. 2004). Natural colorants such as phycobiliproteins are gaining importance over synthetic ones, as they are nontoxic and non-carcinogenic. They are widely used in cosmetic industry and clinical/research laboratories as label for antibodies and receptors. Antioxidants, anti-inflammatory, neuroprotective and hepatoprotective properties are also exhibited by phycobiliproteins (Spolaore et al. 2006).

Present paper deals with the screening of 18 cyanobacterial strains for maximum phycobiliprotein yield as well as effect of different environmental conditions on its content in the best strain.

Materials and Methods

Anabaena NCCU-9, *A. variabilis* NCCU-441, *Aulosira fertillisma* NCCU-443, *Chrococcus* NCCU-207, *Calothrix brevissema* NCCU-65, *Cylindrospermum* NCCU-272, *Gloeocapsa gelatinosa* NCCU-430, *Hapalosiphon fontinalis* NCCU-339, *Lyngbya* NCCU-102, *Michrochaete* NCCU-342, *Nostoc muscorum* NCCU-442, *Oscillatoria* NCCU-369, *Plectonema* NCCU-204, *Phormidium* NCCU-104, *Scytonema* NCCU-126, *Spirulina platensis* NCCU-S5, *Tolypothrix tenuis* NCCU-122, *Westiellopsis prolifica* NCCU-339 were obtained from National Centre for Collection and Utilization of Blue Green Algae, Indian Agricultural Research Institute (IARI), New Delhi-110012.

Cultures were raised in BG-11 medium (Stanier et al. 1971) except *Spirulina platensis* in CFTRI medium (Venkataraman et al. 1982). Initial absorbances of cultures were adjusted to ±0.3 at 750 nm. The cultures were

allowed to grow at 30 ± 1°C under light intensity of 25 µmol photons/m^2/s following 12:12 hour light/dark regime for 27 days.

Impact of various environmental conditions like temperature, irradiances, coloured wavelength, photoperiods, pH, nutrient limitation, nitrogen supplements, salinity, pesticides and heavy metals stress were studied on phycobiliprotein content in best strain.

The algal biomass harvested through centrifugation, washed and dried overnight (50°C) for phycobiliproteins extraction. It was extracted in potassium phosphate buffer (pH-7) till coloured supernatant obtained from the pellet through repeated freezing and thawing method. The absorbencies of phycobiliproteins containing supernatant were measured at 562, 615 and 652 nm using phosphate buffer as a blank. For estimation, following equations were used and expressed in mg/g per dry weight (Siegelman and Kycia 1978).

$$\text{Phycocyanin (PC)} = \{A_{615} - (0.474 \times A_{652})\}/5.34 \quad (1)$$

$$\text{Allophycocyanin (APC)} = \{A_{652} - (0.208 \times A_{615})\}/5.09 \quad (2)$$

$$\text{Phycoerythrin (PE)} = \{A_{562} - (2.41 \times \text{PC}) - (0.849 \times \text{APC})\}/9.62 \quad (3)$$

$$\text{Total phycobiliprotein} = \text{PC} + \text{PE} + \text{APC} \quad (4)$$

The experimental data are presented as mean ± SD of three replicates. All analysis was conducted using Graphpad Prism version-5.0 (Graph Pad software, San Diego, CA, USA). Stastical analysis of the data was done by one way analysis of variance (ANNOVA). Dunnett's multiple comparison test was used in experimental setup with control in which significant difference at a level of significance of 0.01, 0.001 and 0.0001 ($p < 0.01$, $p < 0.001$, $p < 0.0001$) and 'ns' for non significant are represented, while post tests for linear trend method was used in experiment setup without control.

Results and Discussion

Being a tropical country, India has great diversity in its cyanobacterial resources that can be exploited at commercial level. But, research in the field of cyanobacterial phycobiliproteins is very nascent and need adequate attention. The prices of phycobiliproteins products are US$ 3 to US$ 25 mg^{-1} for native pigment but they can reach US$ 1,500 mg^{-1} for certain cross linked pigments (with antibodies or other fluorescent molecules). In the near future (next 5 years) its price are likely to grow by 20% annually (Sekar and Chanramohan 2008).

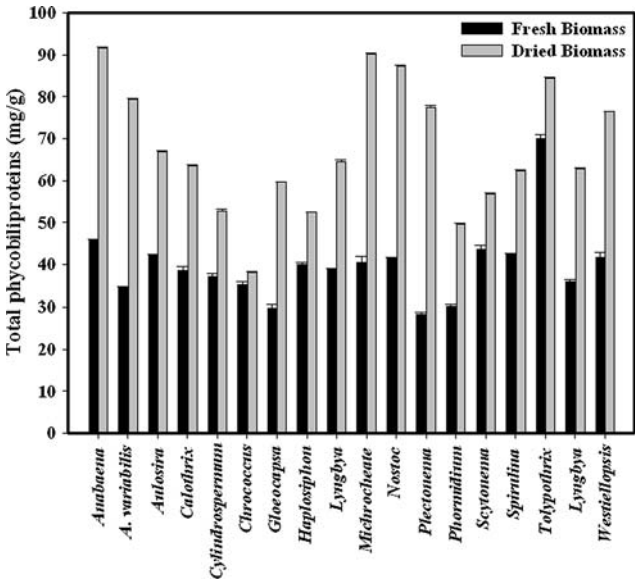

Fig. 1 Total phycobiliproteins in fresh and dried biomass of different cyanobacteria (*error bars* = ±SD)

Screening of cyanobacterial strains for maximum phycobiliproteins was done using fresh and dried biomass. Dried biomass gave good amount of phycobiliproteins as compared to fresh biomass (Fig. 1). So, further studies were conducted using dried biomass. *Anabaena* NCCU-9 gave highest phycobiliprotein content in dry weight (91.54 mg/g) and it was almost equal in *Michrochaete* NCCU-342 (90.05 mg/g). In *Anabaena variabilis* NCCU-441 we have found 79.31 mg/g phycobiliproteins. Other workers have reported comparatively very low amount of phycobiliprotein in *Anabaena.* e.g. 22.57 mg/g in *Anabaena sphaerica* (Yan et al. 1997) and 23.67 mg/g in *Anabaena* ATTGK-A1 and 21.28 mg/g in *Anabaena* NS-TGK-A1 (Gopalswamy and Kannayan 2002). Analysis of variance revealed $r^2 = 0.0003$, $p < 0.0001$, post test for linear trend (n = 18).

Our findings on effect of various environmental conditionals (temperature, light intensity and colour), pH, photoperiod, nitrogenous sources, salinity, pesticides and heavy metals) on the phycobiliprotein yield of best strain *Anabaena* NCCU-9 are as follow.

Temperature is undoubtedly the most fundamental factor for all living organisms as it affects metabolic processes and biochemical composition of cells. The optimal growth temperature and tolerance to the extreme values usually vary from strain to strain. Sudden temperature changes exert stress on the organisms. At high temperature, deficiency of oxygen, which is much less soluble in hot than in cold water, may be the proximal cause of stress (Brock 1978). In present study, optimum temperature for phycobiliproteins was obtained at 30°C (Table 1). There was

23.6% decrease at 20°C, 16.4% at 25°C, 18.7% at 35°C and 38% at 40°C observed when compared with maximum phycobiliprotein yielding temperature (30°C). Analysis of variance revealed $r^2 = 0.1273$, $p < 0.0001$, post test for linear trend (n = 5). Other workers have reported 37°C as optimum for *Arthronema africanum* (Chaneva et al. 2007), 36°C for *Synechococcus* (Sakamoto and Bryant 1998) and 25°C for red algae *Audoinella pygmaea, Batrachospermum delicatulum, Comsopogon coureuleus* (Zucchi and Neechi 2001).

The effect of light intensity at different experimental irradiance showed 25 µmol photons/m^2/s as best for phycobiliprotein production in *Anabaena* NCCU-9 (Table 1). Analysis of variance revealed $r^2 = 0.27$, $p < 0.0001$, post test for linear trend (n = 4). Light irradiance of 25 µmol photons/m^2/s was also reported to be optimal for *Synechococcus* NKBG 042902 (Takano et al. 1995), *Spirulina subsalsa, S. maxima* (Tomasseli et al. 1995, 1997) and *Synechocystis* (Hong and Lee 2008). Light intensity of 12.5 µmol photons/m^2/s was found to be optimal for *Nostoc* UAM 206 (Poza-Carrion et al. 2001) and *N. muscorum* (Ranjitha and Kaushik 2005). Red algae generally prefer higher irradiance e.g. 40 µmol photons/m^2/s by *Gracilaria tenuispitata* (Carnicas et al. 1999); 65 µmol photon/m^2/s by *Audounella*, *Batrachospermum* and *Comsopogon* (Zucchi and Neechi 2001). A high light intensity 150 µmol photons/m^2/s was found to be optimum for phycobiliprotein production in the cyanobacterium *Arthronema africanum* (Chaneva et al. 2007). It has been suggested that cyanobacteria prefer low light intensities and stimulate phycobiliprotein synthesis (Grossman et al. 1993) because of their low specific maintenance energy rate and their pigment composition (Mur and Elema 1983). Not only this, low irradiances actually broadens the overall light absorption band in such a way that the balance of light energy distribution between the two photo systems is maintained that optimizes the rate of light energy conversion (Wyman and Fay 1986).

Table 1 Effect of different environmental conditions on total phycobiliproteins in *Anabaena* NCCU-9

Conditions	Total phycobiliprotein
Temperature (°C)	
20	97.01***
25	106.13***
30	127.02***
35	105.30***
40	78.64***
Light intensity (µmol photons/m^2/s)	
12.5	97.24***
25	124.95***
36.5	101.88***
50	82.88***
Light colour	
White	125.95***
Blue	107.80***
Yellow	101.57***
Red	93.87***
Green	71.65***
pH	
4.0	75.72***
6.0	82.35***
7.0	97.35***
8.0	102.24***
10.0	82.23***
Photoperiod (Light: dark regime)	
12:12	114.29***
16:08	122.81***
08:16	105.21***
10:14	109.06***
Dark	52.29***
Light	39.09***

Probability of error is represented by asterisk (*) for * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$

The order of suitable chromatic regime for the phycobiliprotein was found to be White > blue > yellow > red > green (Table 1), suggesting that coloured light play no stimulatory effect on phycobiliprotein production in *Anabaena* NCCU-9. Analysis of variance revealed $r^2 = 0.958$, $p < 0.0001$, post test for linear trend (n = 5). However, it is reported that red light stimulate phycobiliprotein production in *Anacystis nidulans* (Lonneborg et al. 1985), *Synechococcus* (Takano et al. 1995), *Calothrix* 7601 (Liotenberg et al. 1996), *Nostoc* UAM 206 (Poza-Carrion et al. 2001) and *N. muscorum* (Ranjitha and Kaushik 2005), while blue light positively affect phycobiliprotein production in red algae *Chondrus crispus* (Franklin et al. 2002); *Porphyra lecosticta* (Tsekos et al. 2002); *Halymenia floresii* (Godinez-Ortega et al. 2008); *Rhodella reticulata* (Mihova et al. 1996).

The influence of pH on the phycobiliprotein of cyanobacteria has received little attention. At pH-2 and 12 *Anabaena* NCCU-9 became white. However, it could comfortably grow over the pH range (6–10). The maximum phycobiliprotein was achieved at pH-8 (Table 1). Analysis of variance revealed $r^2 = 0.215$, $p < 0.0003$, post test for linear trends (n = 5). There was 4% decrease at pH-7, 19% at pH-6 and 10 and 26% decrease with pH-4 when compared with pH-8. The increase in external pH-7–9 significantly increased the total phycobiliprotein content in *Nostoc* sp. UAM 206 (Poza-Carrion et al. 2001). pH-8 was found to be optimum for *Synechocystis* (Hong and Lee 2008).

The effect of photoperiod adjusted by different light: dark regime 8:16, 16:80, 12:12, 10:14, 24:00 and 00:24

hour, revealed that 16:8 light: dark regime as the optimal photo-period for the production of phycobiliprotein in *Anabaena* NCCU-9 (Table 1). There were 7% decrease at 12:12, 10% decrease at 10:14, 15% decrease at 8:16, 58% decrease in complete dark and 69% decrease at continuous light when compared with maximum yielding photoperiod (16:8). Analysis of variance revealed $r^2 = 0.772$, $p < 0.0003$, post test for linear trends (n = 6). Similar observation is also reported in the cyanobacterium *Calothrix elenkenii* by (Prassana et al. 2004) and in red algae *Audoinella pygmaea, Batrachospermum delicatulum* and *Comsopogon coureuleus* (Zucchi and Neechi 2001).

Cyanobacteria have special requirements regarding nitrogen sources, NO_3^-, NH_4^+ or carbamide, $NaNO_3$ (Richmond 1986). *Anabaena* NCCU-9 produced highest amount of phycobiliprotein under nitrogen free environment (Table 2). Percent decrease in total phycobiliprotein in presence of ammonia was 80% ($p < 0.0001$) and 92% ($p < 0.0001$) at 1 and 2 mM, respectively; whereas in urea supplementation 59% ($p < 0.0001$), 75% ($p < 0.001$) and 88% ($p < 0.0001$) decrease was determined in 1, 2 and 3 mM, respectively. While under nitrate supplementation 52% ($p < 0.001$), 74% ($p < 0.0001$), 79% ($p < 0.0001$) and 82% ($p < 0.0001$) was observed at 1, 2, 3 and 4 mM, respectively. Similar findings are also reported by other workers. In *Anabaena* 7120 the amount of phycobiliprotein exceeded in nitrogen-free media than nitrate grown cultures (Loreto et al. 2003). While, *Fischerella* produced more phycobiliprotein under nitrate grown cells than nitrogen free media and ammonium grown cells (Soltani et al. 2007). Cyanobacterial cells do not grow on urea as a sole source of nitrogen (Sakamoto and Bryant 2001). Like our observation, ammonium is also reported to be most toxic resulting in growth inhibition, even cell death in *Nostoc, Nodularia spumigena, Calothrix sp*. PCC7601, and *Spirulina platensis,* respectively (Rajni and Subramaniam 1990; Liotenberg et al. 1996; Belkin and Boussiba 1991).

In our study lowest concentration of NaCl (10 mM) increased the phycobiliprotein content in *Anabaena* NCCU-9 as compared to control but further increase in salt concentration resulted in gradual decrease (Table 3). One way of analysis of variance showed 8% increase at 10 mM ($p < 0.0001$), 4% decrease at 50 mM ($p < 0.0001$), 29% decrease at 100 mM ($p < 0.0001$), 37% decrease at 150 mM (ns), 59% decrease at 200 mM ($p < 0.0001$) and 80% decrease was observed at 250 mM ($p < 0.0001$). It has been suggested that rapid entry of sodium ions might result in detachment of phycobilisomes from the thylakoid membranes that lead to reduction in photosynthesis (Rafiqul et al. 2003), energy transfers from phycobiliproteins to PSII reaction centre (Schubert et al. 1993; Verma and Mohanty 2000) and uptake of other mineral nutrients, such as K^+, Ca^{2+} and Mn^{2+} (Hasegawa et al. 2000).

The order of pesticide toxicity on phycobiliprotein was found to be as follows malathion > alphamethrin > chlorpyriphos (Table 4). The drastic decrease in phycobiliprotein under malathion stress was associated with rapid bleaching, while chlorpyriphos stressed cultures were slow at growth and least bleached. One way analysis of variance showed 3% ($p < 0.0001$), 61% ($p < 0.0001$), 72% ($p < 0.001$), 77% ($p < 0.0001$) and 81% ($p < 0.0001$) decrease at concentration of 0.003%, 0.006%, 0.009%,

Table 3 Effect of salt stress on total phycobiliproteins of *Anabaena* NCCU-9

NaCl (mM)	Total phycobiliproteins
Control	124.59
10	135.73***
50	121.68***
100	88.94***
150	79.53^{ns}
200	51.73***
250	25.82***

Probability of error is represented by asterisk (*) for * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and 'ns' for non significant respectively superscripted to the data in tables

Table 2 Effect of different nitrogen sources on total phycobiliproteins *in Anabaena* NCCU-9

Concentration (mM)	Ammonium	Urea	Nitrate
Control	127.5	127.5	127.5
1	26.05***	52.8 ***	60.63**
2	10.68***	31.42**	33.85***
3	–	21.29***	26.56***
4	–	–	20.72***
5	–	–	–

Probability of error is represented by asterisk (*) for * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$. Whereas (–) shows no growth

Table 4 Effect of different pesticides on total phycobiliproteins in *Anabaena* NCCU-9

Concentration (%)	Chlorpyriphos	Alphamethrin	Malathion
Control	92.52	92.52	92.52
0.003	85.11***	89.93***	87.54***
0.006	82.61***	85.8***	35.87***
0.009	77.93***	50.81***	25.81**
0.012	40.78***	32.08***	20.01***
0.015	31.82***	20.93***	17.59***

Probability of error is represented by asterisk (*) for * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$

0.012% and 0.015% malathion, respectively. Whereas under alphamethrin pesticide stress 1% ($p < 0.0001$), 5% ($p < 0.0001$), 44% ($p < 0.0001$), 65% ($p < 0.0001$) and 77% ($p < 0.0001$) decrease was observed at 0.003%, 0.006%, 0.009%, 0.012% and 0.015% pesticide, respectively. While 4% ($p < 0.0001$), 9% ($p < 0.0001$), 14% ($p < 0.0001$), 55% ($p < 0.0001$) and 66% ($p < 0.0001$) decrease was associated with chlorpyriphos pesticide at 0.003%, 0.006%, 0.009%, 0.012% and 0.015% pesticide, respectively. The inhibitory effect of pesticide on phycobiliprotein is also reported by many phycobiologists e.g. in *Anabaena* under lindane stress (Babu et al. 2001), in *Plectonema boryanum* under endosulphan (Prasad et al. 2005), in *Anabaena variabilis* under arozin, alachlor and butachlor (Singh and Dutta 2005), in *Anabaena dolilolum* under cypermethrin (Mohapatra et al. 2003, in *Synechocystis* PCC6803 under organoposphate (Mohapatra and Scheiwer 2000), in *Nostoc linckia* under endosulphan stress (Satish and Tiwari 2000). The decreased phycobiliprotein content was reported earlier in *Nostoc sphaeroids* (Xia 2005) and *Anabaena variabilis* (Battah et al. 2001) in presence thiobencarb pesticide stress. It is suggested that the external localization of phycobiliprotein on intracellular thylakoid membrane give more exposure of pesticides causing more damaging effect on phycobiliproteins resulting in their detachment (Prasad et al. 2005). The reduction in phycobiliproteins may be due to inhibition of pigment synthesis or accelerated degradation by reactive oxygen species at the various sites of photosynthetic electron transport chain.

In present study heavy metals (Pb^{2+}, Cr^{6+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+}) resulted in phycobiliprotein reduction (Table 5). The decline in phycobiliprotein content was highest in presence of chromium ($p < 0.0001$) and it was followed by cadmium ($p < 0.0001$), lead ($p < 0.0001$), nickel ($p < 0.0001$), copper ($p < 0.0001$) and zinc ($p < 0.0001$) metal ions at 0.05, 0.1, 0.5, 1.0 and 1.5 mM, respectively. In all heavy metal concentrations used in present study showed significant value ($p < 0.0001$). This decline at higher concentrations of metal ions could be due to an enhanced need of metabolic energy for responses associated with adaptive mechanism of various enzymes (Andrade et al. 1994). In *Microcytis aureginosa,* the phycobiliprotein content decreased under even at 2 μM cadmium metal stress (Zhou et al. 2006). But, in *Michrochaete tenera* phycobiliprotein content increased four times in presence of lead metal (Zaccaro et al. 2000).

The effect of nutrient limitation was studied by removing iron, phosphorus and sulphur from the culture medium individually and the order of nutrient limitation was found to be sulphur > phosphorus > iron (Table 6). One way analysis of variance showed 10% ($p < 0.0001$), 19% ($p < 0.0001$) and 32% ($p < 0.0001$) decrease at iron, phosphorus and sulphur limitation, respectively. Phycobilisomes are the poor source of sulphur containing amino acids, despite that upon sulphur starvation cyanobacteria degrades their phycobiliprotein content (Schwarz and Grossman 1998). In *Synechocystis* PCC 6803 sulphur deprived cultures show rapid degradation of phycobiliproteins than phosphorus limited cultures (Richaud et al. 2001). In nitrogen fixing cyanobacteria decrease in phycobiliprotein under nutrient limitation is said to be due to loss of photosynthetic membranes (Bhaya et al. 2000).

Under iron limitation culture was green and healthy but under sulphur limitation chlorosis of cells was seen resulting in slow growth. In phosphorus limitation the culture colour changed from green to brownish green. Nutrient deprivation based reduction in phycobiliprotein

Table 6 Effect of nutrient limitation on total phycobiliproteins of *Anabaena* NCCU-9

Nutrition limitation	Total phycobiliproteins
Control	118.80
Iron	106.79***
Phosphorus	95.85***
Sulphur	80.88***

Probability of error is represented by asterisk (*) for * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$

Table 5 Effect of different heavy metal salts on total phycobiliproteins in *Anabaena* NCCU-9

Concentration (mM)	Chromium	Cadmium	Lead	Nickel	Copper	Zinc
Control	99.7	99.7	99.7	99.7	99.7	99.7
0.05	80.8***	84.34***	90.42***	96.5***	97.20***	99.2***
0.10	76.5***	83.52***	88.41***	94.7***	95.30***	97.42***
0.50	72.6***	79.20***	85.48***	89.0***	90.50***	93.54***
1.00	60.05***	71.51***	75.57***	80.5***	85.7***	86.51***
1.50	55.6***	60.02***	69.6***	72.3***	75.3***	79.32***

Probability of error is represented by asterisk (*) for * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$

content has been reported to be associated with dramatic changes in colour from bluish green to yellowish green (Allen and Smith 1969). In *Synechococcus* sp. phosphorus (Ihlenfeldt and Gibson 1975), sulphur (Schmidt et al. 1982; Jensen and Rachlin 1984) and iron (Sherman and Sherman 1983) deprivation resulted bleaching. In sulphur deprived cells of *Synechococcus* a gene called nblA synthesize a protein NblA which initiate the degradation of phycobilisomes and to a lesser extent in phosphorus limited cultures (Collier and Grossman 1994). Another gene nblB encode a polypeptide with similarities to phycocyanin lyases enzyme that catalyzes the covalent bond formation between linear tetrapyrolle and phycobilisomes (Dolganov and Grossman 1999).

It can be concluded that cyanobacterium *Anabaena* NCCU-9 produced maximum phycobiliprotein (91.54 mg/g) which could be enhanced by changing culture conditions. Maximum enhancement in phycobiliproteins was observed when cultures grown at 30°C temperature, white colour, 25 μE photons/m^2/s light intensity, pH-8, 16:8 light and dark regimes, nitrogen free medium, 10 mM sodium chloride without pesticide and heavy metal exposure.

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