C-phycocyanin production by halotolerant cyanobacteria

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Abstract

Cyanobacteria are considered to be the potential source of secondary metabolites and pigments. The present study is an attempt to evaluate natural biodiversity of halotolerant cyanobacteria and screening for phycocyanin (CPC) pigment of commercial value. Cyanobacterial cultures were isolated from diverse locations including sulfur spring of Unapdeo (Dist: Jalgaon, MS), soda lake of Lonar (Dist: Buldhana, MS), saline soils and fresh waters by enrichment in presence of sodium chloride (200 mM) from Pune and Jalgaon district. Cultures were characterized for their nitrogen fixation ability, tolerance to salt (sodium chloride, 200–1000 mM). The cultures were also screened for phycocyanin production. Attempt been made to extract and purify C-phycocyanin from selected halotolerant cyanobacterial isolates. Partially purified C-phycocyanin showed absorbance maxima at 620 nm. The recovery efficiency of C–phycocyanin from crude extract was above 60%. Among the isolates tested, *Oscillatoria limosa, Synechocystis* sp. and *Nostoc commune* were found to be efficient for C-phycocyanin production.

Key words: *C-Phycocyanin, Halotolerant cyanobacteria, Phycobiliprotein, saline soil*

Introduction

Phycobiliproteins are globular proteins of low molecular weight found in some species of algae and cyanobacteria. They form light-harvesting antenna complexes and act as photosynthetic accessory pigments in microalgae. Phycobiliproteins in association with the outer surface of the photosynthetic lamellae are the constituents of the photosystem-II light harvesting apparatus (Patel *et al.,* 2005). Phycobiliproteins are divided into three main classes: phycoerythrins (PEs, λ_{max} 540–570 nm), phycocyanins (PCs, λ_{max} 610–620 nm) and allophycocyanins (APC, λ_{max} 650– 655 nm) (Reis *et al.,* 1998). Phycobiliproteins participate in an extremely efficient energy transfer chain in photosynthesis.

Phycobiliproteins are soluble in water and can be easily isolated as proteins pigment complexes (Patel *et al*., 2005). Extraction of phycocyanin could be achieved with physical treatment of cell wall disruption such as homogenizer, sonication, cell mill with glass beads and hand grinding (Furuki *et al.,* 2003). Various methods have been employed to isolate cyanobacterial phycobiliproteins which include aqueous extraction, proteolysis, fractional precipitation and chromatography (Bhaskar *et al.,* 2005; Patel *et al.,* 2005; Chen *et al.,* 2006; Soni *et al.,* 2006). A method for stably purifying a phycocyanin from *Spirulina platensis* has been developed, which comprised of hexane extraction with high pressure (Seo *et al.,* 2013).

The present investigation reports screening of halotolerant cyanobacterial cultures for their phycocyanin contents. Selection of cultures could be made from the screening data for possible commercial exploitation of the cultures.

Materials and Methods

Sample collection

Soil samples were collected from agricultural fields of Pune and Jalgaon district region by removing the surface debris from four to five randomly selected spots in a field and scraping about 100 g soil from the upper 1 cm layer from each spot. After thorough mixing, 200 g representative sample from each field was stored in polythene bags. Water samples were collected from sulfur spring of Unapdeo (Dist: Jalgaon), soda lake of Lonar (Dist: Buldhana) and fresh water samples from Pune and Jalgaon district region.

Enrichment and isolation of halotolerant cyanobacteria

Samples were enriched in BG-11, Chu-10 and Gerloff's medium containing 200 mM sodium chloride (Kaushik, 1987). The enrichment flasks were incubated under continuous light illumination with fluorescent tubes at 24±2 °C. Growth of cyanobacteria was judged on the basis of visual observations (7 day incubation). Cyanobacterial colonies were isolated by streaking the enriched sample on respective growth medium-agar plates. Visibly distinct cyanobacterial colonies were reinoculated for further purification. In case of filamentous cultures, selective inoculation of single filament under aseptic conditions was used as the method of isolation.

Halotolerance of cyanobacteria

To test the salt tolerance cultures were subjected to various concentrations of sodium chloride (100-1000 mM) in sterile respective medium at pH 7.4. The flasks were incubated for 15 day at 24±2ºC under 1800-2000 lux light intensity. Growth of a culture was assessed on the basis of visible growth.

Extraction of photosynthetic pigments

For extraction of CPC 15 days old cyanobacterial cells were harvested by centrifugation at 3000 ×*g* for 5 min. Cell pellet was washed with 10 mM phosphate buffer (pH 6.5) and resuspended in five volumes of the same buffer. To release phycobiliproteins, the suspension was subjected to repeated freeze-thaw cycles of -20°C and 4°C temperature shocks. The suspensions were further exposed to ultrasonication at 20 KHz frequency (Sonics and Materials Inc., USA) for 2 min. The cell mass was separated by centrifugation at 5000 ×*g* for 20 min and extraction procedure was repeated thrice. The phycocyanin content of pooled supernatants was estimated using spectrophotometric analysis. The amount of phycocyanin in the sample was calculated using the following equation (Bennett and Bogorad, 1973).

$$\text{CPC (mg/ml)} = [A_{620} - 0.474\, A_{652}]/5.34$$

Partial purification of CPC

Partial purification was performed as per Patel *et al.* (2005). The purification was done in the dark at 10-15°C using 10 mM phosphate buffer (pH 6.5) containing 0.01% sodium azide. Appropriate amount of finely powdered ammonium sulphate was added gradually to the cell-extract with continuous stirring for 1 h. The crude cell extract was fractionated by precipitation with ammonium sulphate first at 25% and then at 50% saturation. The precipitate from 25% saturation was discarded and the supernatant was further brought to 50% saturation of ammonium sulphate and allowed to stand for 4 h at 4°C. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 30 min at 4°C and resuspended in phosphate buffer and dialyzed overnight at 4°C against the same buffer. The purification of CPC was tracked by determining optical density of fractions at 280, 620 and 652 nm using UV-visible spectrophotometer (Shimadzu, Japan).

Phycocyanin production under different conditions

Freshly-grown cultures were (10 ml) inoculated in 100 ml BG-11 medium in 250 ml Erlenmeyer flasks. Samples (5 ml) were withdrawn at an interval of 48 h and analyzed for phycocyanin as described earlier. To check the effect of nitrogen starvation on growth and synthesis of phycocyanin, cultures were inoculated in BG-11 medium lacking nitrogen. Then nitrogen source was introduced in the flask on the 4th day. The effect of salinity on production of phycocyanin was checked in BG-11 medium amended with varying concentrations of sodium chloride (100-1000 mM). For estimating the effect of irradiation on phycocyanin synthesis, the cultures were cultivated in BG-11 medium at varying light: dark cycles (8, 12, 18 and 24 h light period) in a day using fluorescent tubes giving 2000 lux. All the experiments were performed in triplicates at pH 7.4 and 24±2°C. Appropriate positive and negative controls were run for each experiment simultaneously.

Results

Screening of halotolerant cyanobacteria for phycocyanin production

In all 40 isolates tolerating ≥400 mM sodium chloride, were screened for phycocyanin contents (Table 1). Out of the 40 cultures, 24, 8, 3 and 4 isolates had shown tolerance to 400, 500, 600 and 700 mM salt concentrations, respectively. There was only one isolate that could tolerate 800 mM salt. The set of cultures comprised 5 filamentous heterocystous and 30 filamentous nonheterocystous morphology, while, there were 5 unicellular cultures. Genus *Oscillatoria* was dominant among the genera contributing 16 isolates in the set of 40 cultures.

The distribution of cultures on the basis of % w/w CPC content is depicted in Fig. 1. It can be seen that the number of isolates with CPC content between 4.1 – 5.0 % and 5.1 – 6.0 % were 14 and 11, respectively. 10% of isolates shown >7 % w/w CPC content. The maximum synthesis of CPC (9.61%) was obtained for the isolate, NMU-84 *Synechocystis* sp. Cultures, *Oscillatoria limosa*, *Synechococcus elongatus* and *Nostoc commune* were the next best producers of CPC containing 8.96, 8.63 and 8.34 % CPC of cell dry weight, respectively.

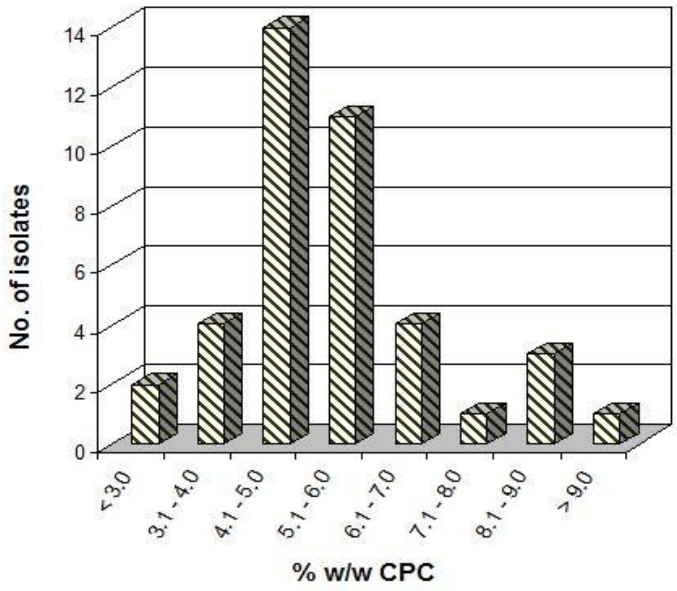

Fig: 1. Distribution of cyanobacterial isolates on the basis of % w/w CPC content

Fig. 2 shows the distribution of halotolerant cultures as a function of absorption ratio, A_{620}/A_{652}. A maximum number of isolates (17) was found to be distributed in the class of 1.41 – 1.60. The numbers of cultures were further decreased on both side of this class. Distinct absorption ratios (A_{620}/A_{652}) of 2.07, 2.18 and 2.59 were obtained for three cultures viz. *Anabaena doliolum, Oscillatoria limosa* and *Synechocystis* sp., respectively. On the basis of both the parameters, *Synechocystis* sp. emerged as the most efficient CPC producer among the cultures screened.

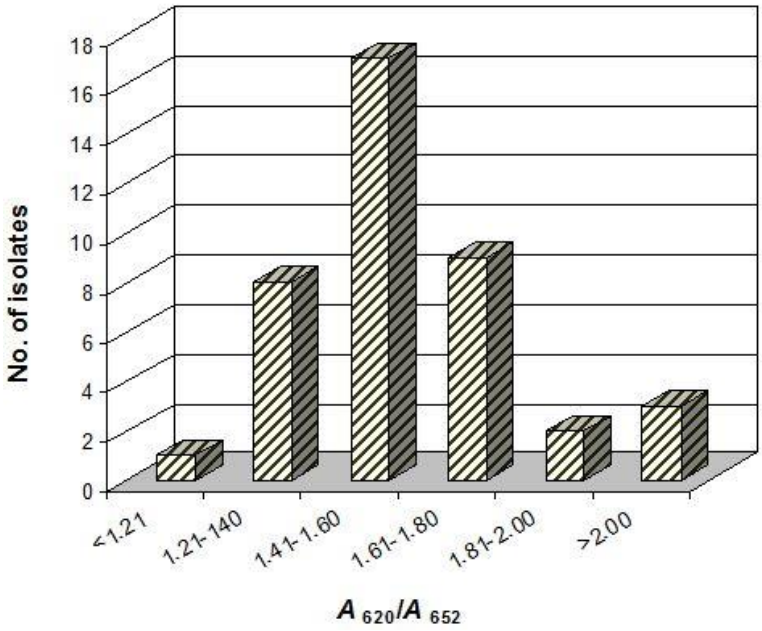

Fig: 2. Distribution of cyanobacterial isolates on the basis of ratio, A_{620}/A_{652}

Partial purification of CPC

The data on partial purification of CPC from three cultures selected from the screening program viz. *Synechocystis* sp., *Oscillatoria limosa*, and *Nostoc commune* are given in Table 2. The purity ratio as well as the separation factor was found to have increased in fractional precipitation with ammonium sulphate. The loss of CPC in the first fractionation at 25% saturation concentration of ammonium sulphate was <10% for all the cultures. After two steps of purification, the percent

recovery of phycocyanin was to the tune of 83, 75 and 61% in the cases of *Oscillatoria limosa*, *Nostoc commune* and *Synechocystis* sp., respectively. A maximum separation factor, 3.7 was achieved for *Synechocystis* sp.

Table 1. Screening of halotolerant cyanobacteria for C-phycocyanin production.

Sr. No.	Isolate	Identification	Salt tolerance (mM)	CPC (% w/w)	A_{620}/A_{652}
1	NMU-4	*Lyngbya commune*	400	3.87	1.16
2	NMU-6	*Oscillatoria amphibian*	400	6.48	1.76
3	NMU-7	*Gloeothece samoensis*	500	4.87	1.60
4	NMU-8	*Oscillatoria salina*	400	4.52	1.59
5	NMU-10	*Phormidium fragile*	400	6.27	1.62
6	NMU-13	*Lyngbya corticola*	400	2.92	1.52
7	NMU-14	*Oscillatoria tenuis*	700	3.48	1.30
8	NMU-16	*Oscillatoria salina*	800	4.38	1.52
9	NMU-18	*Synechococcus elongates*	400	8.63	1.75
10	NMU-19	*Aphanocapsa thermalis*	400	6.29	1.81
11	NMU-21	*Phormidium tenue*	400	4.86	1.46
12	NMU-27	*Synechococcus lividus*	500	2.77	1.50
13	NMU-28	*Arthrospira platensis*	600	4.72	1.23
14	NMU-29	*Oscillatoria amphibian*	400	5.86	1.75
15	NMU-30	*Spirulina platensis*	400	5.22	1.53
16	NMU-31	*Oscillatoria limosa*	500	8.96	2.18
17	NMU-33	*Spirulina major*	600	4.94	1.33
18	NMU-34	*Oscillatoria salina*	700	6.86	1.80
19	NMU-36	*Trichodesmium erythraeum*	400	7.50	1.78
20	NMU-37	*Phormidium tenue*	400	4.22	1.40
21	NMU-41	*Oscillatoria salina*	400	5.25	1.70
22	NMU-44	*Anabaena doliolum*	400	5.97	2.07
23	NMU-45	*Oscillatoria ravi*	500	4.11	1.43
24	NMU-47	*Oscillatoria amoena*	700	3.17	1.51
25	NMU-48	*Microcystis robusta*	500	4.01	1.52
26	NMU-49	*Oscillatoria tenuis*	400	5.97	1.67
27	NMU-51	*Oscillatoria amphibian*	700	4.50	1.49
28	NMU-54	*Oscillatoria chlorine*	500	4.82	1.48
29	NMU-55	*Phormidium tenue*	400	5.07	1.32
30	NMU-56	*Oscillatoria martini*	400	4.80	1.34
31	NMU-60	*Anabaena doliolum*	400	5.01	1.48
32	NMU-61	*Oscillatoria protens*	600	5.57	1.51
33	NMU-33	*Spirulina major*	600	6.04	1.69
34	NMU-68	*Lyngbya puteali*	500	6.00	1.57
35	NMU-70	*Lyngbya lutea*	400	5.56	1.41
36	NMU-71	*Lyngbya major*	500	4.63	1.41
37	NMU-72	*Scytonematopsis* sp.	400	3.79	1.33
38	NMU-84	*Synechocystis* sp.	400	9.61	2.59
39	NMU-59	*Nostoc commune*	400	8.34	1.92
40	NMU-96	*Calothrix javanica*	400	4.97	1.22

Table 2. Partial purification of CPC from selected halotolerant cyanobacteria.

Purification stage	Absorption ratio						% Recovery of CPC		
	Purity ratio (A_{620}/A_{280})			Separation factor (A_{620}/A_{652})					
	S	*O*	*N*	*S*	*O*	*N*	*S*	*O*	*N*
Cell extract	0.65	0.75	0.76	2.5	2.18	1.92	100	100	100
Precipitation 30% $(NH_4)_2SO_4$	0.95	0.9	0.82	2.8	2.6	2.4	90	93	96
Precipitation 50% $(NH_4)_2SO_4$	1.25	3.0	1.69	3.7	2.9	2.8	61	83	75

S: *Synechocystis* sp., O: *Oscillatoria limosa*, N: *Nostoc commune*, CPC: C-phycocyanin

Effect of nitrogen-starvation on phycocyanin synthesis

The data on effect of nitrogen starvation and deprivation on the synthesis of phycocyanin by *Synechocystis* sp. are presented in Fig. 3. The culture failed to grow well under nitrogen deprived of conditions. The adverse effect of nitrogen deprivation was recovered on reintroduction of nitrogen source in the medium on the 4th day of incubation. However, the growth and biosynthetic recovery upon readdition of nitrogen did not match control conditions.

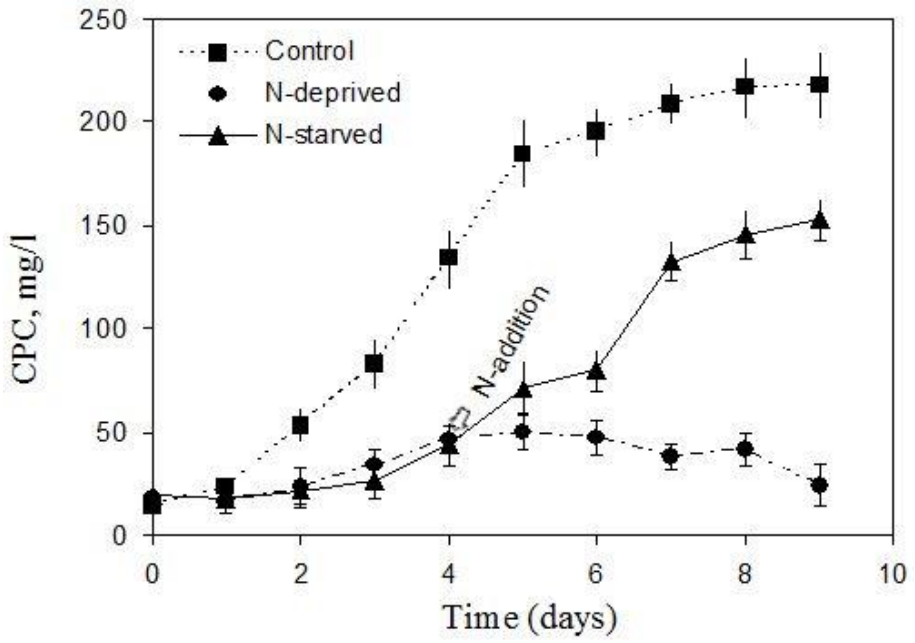

Fig: 3. Effect of nitrogen starvation conditions on CPC synthesis

Effect of salinity on phycocyanin synthesis

Fig. 4 shows the synthesis of CPC by *Synechocystis* sp. in presence of increased salt concentrations. An increase in CPC content could be noticed with increase in salt-concentration from 100 mM to 300 mM. However, inhibitory effect on CPC synthesis was seen at salt concentration of 400 mM. The experimental results revealed that 300 mM was the optimum salt concentration for CPC production.

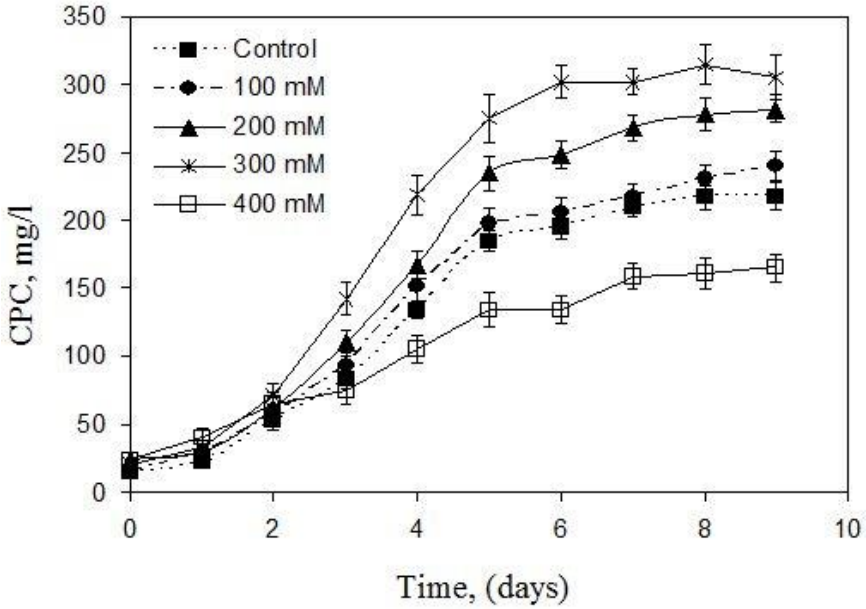

Fig: 4 Effect of salinity on CPC synthesis

Effect of light: dark cycles on phycocyanin synthesis

The data on CPC production as a function of irradiance-period (light:dark cycle) are presented in Fig. 5. When the culture, *Synechocystis* sp. was exposed to varying light periods i.e. 8, 12, 16 and 24 h in a day, deflation in production of CPC was noticed. In comparison with continuous irradiation, CPC synthesis increased at 16:8 h light: dark cycle. However, the CPC content showed dependence on light intensity at light: dark cycles of 8:16 h and 12:12 h, exhibiting decrease in CPC content with decreasing period of irradiance.

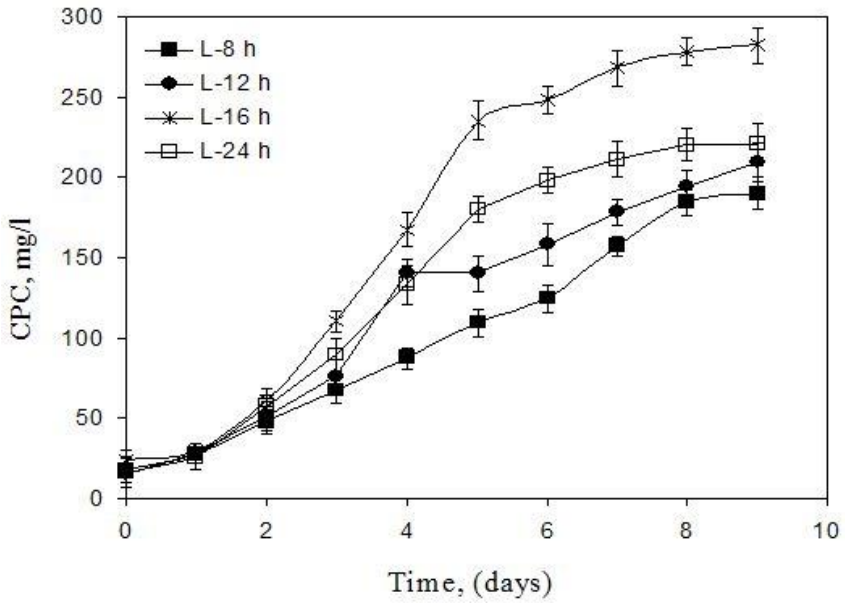

Fig: 5. Effect of light exposure cycles on CPC synthesis

Discussion

Cyanobacteria have recently attracted the attention of scientists in connection with their biotechnological significance. However, the economic exploitation of this group of organisms in an open system seems to have been limited because of lack of multiple stress-tolerant strains (Singh and Kshatriya, 2002). Stress-tolerant cyanobacterial cultures producing biotechnologically important phycocyanin could be the ideal organisms. Salt-tolerant cyanobacteria hold a great future-potential for growing and harvesting them in open saline ecosystem. Water-soluble phycobiliproteins found in cyanobacteria are highly fluorescent by virtue of their covalent bond, linear tetrapyrrole chromophores known as bilins. Phycocyanin, a major phycobiliprotein constitutively produced by many cyanobacteria, holds several promising applications in diagnostics, biomedical research, and therapeutics (Soni *et al*., 2008).

The results of screening experiments in present study have indicated diversity in quantity and quality of photosynthetic pigments in cyanobacteria. In the light of many commercial applications of phycocyanin in food and pharmaceutical industry, the purity of the pigment plays a major role. The pharmaceutical industry demands a highly pure phycocyanin with A_{620}/A_{280} ratio of 4 and food industry demands a ratio of 2 (Bhaskar *et al.*, 2005). In an industrial process, phycocyanin is extracted using aqueous extraction, however, has unfavorable aspects at the stage of aqueous extraction (Furuki *et al*., 2003). For consistency and assurance of high product-quality of the phycocyanin itself and/or phycocyanin-including products, there is a need to establish a method for extracting phycocyanin rapidly and selectively. Ultrasonication used to extract phycocyanin from *Synechocystis* sp. providing certain advantages to overcome the limitations of aqueous extraction. Patel *et al*. (2005) have described an efficient single step chromatographic method for purification of CPC. It involves a multistep treatment of the crude extract by fractional precipitation with ammonium sulfate, followed by ion exchange chromatography. Purity ratios (A_{620}/A_{280}) of 4.42, 4.43 and 4.59 have been obtained for *Spirulina*, *Phormidium* and *Lyngbya*, respectively. Purification phycocyanin from *Oscillatoria quadripunctulata* with purity ratio of 3.31 has been reported by Soni *et al.* (2006). The recovery efficiency of CPC from crude extract was above 68%. In another study, enhancement in phycobiliprotein extraction has been reported using sodium phosphate buffer (pH 7.5) supplemented with 0.15 M sodium chloride and freezing and thawing (-20 and 4°C) in *Anabaena* sp. (Hemlata *et al.*, 2011). Maximum 85.81% recovery of phycoerythrin with a purity of 2.81 (OD562/OD280) using ammonium sulfate precipitation followed by a single step anion exchange chromatography and 62.5% recovery after chromatographic separation with a purity of 4.95 (OD562/OD280) has been reported by Chakdar and Pabbi (2012) for *Anabaena variabilis*.

In present study, CPC synthesis in *Synechocystis* sp. was found to be inhibited in nitrogen deprived conditions. Nutrient and co-factor deprivation, for instance nitrogen, sulphur, phosphorus, iron, or copper, represents another source of environmental stress. The best-studied degradation processes in cyanobacteria, in response to nutrient deprivation, are the degradation of phycobilisomes in the absence of nitrogen or phosphorus and plastocyanin in the absence of copper (Sauer *et al*., 1999). In another study, screening of 18 cyanobacterial cultures and effect of various environmental stresses on synthesis of phycobiliproteins has been studied for *Anabaena* sp. and optimized the conditions as 30°C, 25 µmol/m2/s white light, pH 8, 16:8 light and dark regimes, nitrogen free medium and 10 mM sodium chloride (Hemlata and Fatma, 2009) . Dhiab *et al.* (2007) have reported the effect of salt concentration on growth, fluorescence, photosynthetic activities and pigment content of cyanobacterium *Arthrospira platensis*. It has been observed that high NaCl concentration induces an increase of the growth, photosynthetic efficiency (α), and phycobilin/chlorophyll ratio. The effect of salinity on growth, photosynthetic parameters and nitrogenase activity in estuarine planktonic cyanobacteria, has been reported by Kaushik (1989). *Anabaenopsis*, *Anabaena* and the two *Nodularia* strains rapidly responded to salinity by increasing their maximum photosynthetic rates.

Conclusion

Stress tolerant cultures have more advantages in the development of open culture systems for industrial production of metabolites. Halotolerant cyanobacterial cultures isolated from diverse habitats were screened for their ability to produce CPC. Partial purification involving ultrasonication and freezing-thawing yielded >61% recovery of CPC. CPC synthesis was increased with salinity and irradiance period. Stress tolerant cultures have provided a promise to develop mass scale cultivation, extraction and complete purification of CPC.

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