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Notes

Quantification and purification of C-phycocyanin from cyanobacterial strains *Anabaena* and *Phormidium*

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The cyanobacteria *Anabaena* and *Phormidium* are potential source of phycobiliproteins and C phycocyanin (C-pc). Here, we carried out extraction and purification of phycocyanin (PC) from the above selected cyanobacterial isolates using one-step anion exchange chromatography. Crude C-phycocyanins were extracted and concentrated by ammonium sulfate fractionation at saturation of 35%, then purified on a DEAE-sepharose with Fast Flow chromatography column having continuous pH gradient elution from pH 5.1 to 3.76. The process resulted in recovery of high purity C-pc from above cyanobacteria. The purity ratios (A620/A280) of phycocyanin reached 3.34 for *Phormidium* and 3.1 for *Anabaena*, respectively. The purity was further demonstrated and confirmed through fluorescence emission spectroscopy. The total recovery yield of pure C-pc was 14% after completion of the process, and the recovered pigment remained stable over a pH range of 4-9. This purification method for recovery of high purity pigment was fairly efficient compared to the existing methods. . As phycocyanin has higher antioxidant activity and hence, the above cyanobacterial strains *Anabaena* and *Phormidium* with considerable amount of C-pc, may serve to be a potential source as food supplement as well as for pharmaceuticals industries.

Keywords: Anion Exchange Chromatography, DEAE Cellulose, Phycobiliproteins

Cyanobacteria, also known as Blue green algae (BGA) are a class of gram negative bacteria which are considered to be the oldest form of life on the earth. They possess a wide range of coloured components included carotenoids, chlorophyll and phycobiliproteins[1]. Cyanobacterial diversity was initially discovered during the 19th century, and later was defined as a separate group of organisms. This classification is based on the morphology of the isolated strains and samples[2]. Cyanobacterial systematics has been altered using molecular markers of significantly 16S rRNA since the 20th century although it should be emphasized that the significance of morphological features is renowned. Hence, the taxonomic system of the cyanobacteria is especially combined with the morphological and molecular methods determined characterization of the cyanobacterial taxa[3].

Phycobiliproteins (PBPs) are large water supramolecular protein aggregates involved in light harvesting in these organisms and may comprise as much as 40-60% of the total soluble protein in these cells. These can be divided broadly into three classes based on their spectral properties: phycoeryhrin (λmax-565 nm), phycocyanin (λmax-620 nm) and allophycocyanin (λmax-650 nm). These are composed of two different kinds of polypeptide of which one is light (α, MW: 12-19 kD) and other is heavy (β, MW: 14-21 kD), and are generally present in equimolar amounts[4].

C-phycocyanin (C-pc) is a water-soluble light-harvesting protein, used in the cosmetic and pharmaceutical industries and in fluorescence labeling. Phycocyanin is also used as colourant in food (chewing gums, dairy products, ice and jellies). It is also shown to have therapeutic value (immuno-modulating activity and anti cancer activity). Owing to its fluorescence properties, it has gained importance in the development of phycofluor probes for immunodiagnostics[5]. Nurrahmah *et al.*[6] who exposed the biphasic action of C-pc in modulation of the inflammatory processes by influencing NF-κB and JNK proteins. The C-pc isolated from *Spirulina platensis* biomass has been reported to exhibit high level of biocompatibility and effective scavenging activity[7]. In view of the multiple uses of phycocyanin, the present study was undertaken to optimize extraction and purification of phycocyanin from selected potential cyanobacterial isolates[8]. We can also check the similarity analyses of phycocyanin operon locus gene cpcBA-IGS of both the strains. PBPs, mainly phycocyanin have been widely used as nutritional ingredients, natural dyes, florescent

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markers and pharmaceuticals, such as antioxidant and anti-inflammatory reagents. Phycocyanin is used as colourant in food (chewing gums, dairy products, gellies, etc) and cosmetic, such as lipstick and eye liners in Japan, Thailand and China[6]. It was also shown to possess immunomodulating activity and anticancer activity[6,9].

There are some difficulties in phycocyanin extraction because of multilayered cell walls and large amounts of contaminants. Several methods have been reported to successfully purify C-pc, but these methods comprised multiple steps and are time consuming, which may lead production costs to increase and limit their widespread application[10]. Recently, Demirel & Sukatar[11] have demonstrated extraction and purification of C-pc from hot spring cyanobacteria in two stages i.e., ammonium sulfate saturation and dialysis, gel filtration and ion exchange chromatography

In present study, we attempted one-step anion exchange chromatography with pH gradient elution for purification of C-Phycocyanin with high purity and recovery from *Anabaena* and *Phormidium* sp. in easy steps. The purity of C-pc was further demonstrated and analyzed by HPLC.

Material and Methods

Growth and maintenance of culture

Two cyanobacterial cultures viz. (*Anabaena* sp.) and (*Phormidium* sp.) were chosen from the Culture Collection of Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), IARI, New Delhi-12, India. Cultures were maintained in chemically defined BG-11 media at 28±2°C under a light intensity of 52-55 μmol photon m^{-2} s^{-1} and L: D cycles of 16:8 h[12]. Protocol was also standardized for higher mass production[13].

Extraction

Homogenized log phase (15 days old) culture around 500 mL was centrifuged at 5000 rpm to obtain pellet, then pellet was suspended in 100 mL of 20 mM acetate buffer containing 0.002 M sodium azide and 50 mM sodium chloride (pH 5.10). C-pc was extracted by repeated freezing (–20°C) and thawing at 25°C until the blue colour becomes in acetate buffer (Step I). Cell debris was removed by centrifugation at 5000 rpm for 15 min and the extract thus obtained was termed as crude extract. Amount of C-pc was measured as described by and purity was determined by using the following formulae[14].

Purity = OD_{620}/OD_{280}

Purification

The crude extract was subjected to a single step precipitation using 65% $(NH_4)_2SO_4$ (Bio Xtra, >99%; Sigma- Aldrich) and kept overnight at 4°C. The pellet was recovered by centrifugation at 27000 rpm for 15 min at 4°C and dissolved in 10 mL of the same extraction buffer and termed as ammonium sulfate extract (ASE). 10 mL of ASE was dialysed against the extraction buffer using dialyses membrane (Dialyses membrane-70, MWCO; 12-14 kD) procured from Hi-Media. Dialyses was performed twice against 1000 mL extraction buffer at 25°C and again dialyzed against 1000 mL of extraction buffer at 4°C overnight. The resultant extract was recovered from the dialyses membrane and filtered through 0.45 µm filter[15].

Anion exchange chromatography

For anion exchange chromatography, we used DEAE-Cellulose from Sisco Research Laboratory (SRL). A column (31×8×3 cm) was prepared for purifying the phycocyanin, and equilibrated with 155 mL of acetate buffer (pH-5.10). In column we placed dialyzed filtered sample (10 mL). A linear gradient of acetate buffer with pH ranging from 3.76 to 5.10 was used to develop the column and elutes were collected in 5 mL fractions[16]. Flow rate was kept 20 mL h^{-1}. Absorption spectrum was also determined by scanning the sample in the range of 310-760 nm using Specord 200 spectrophotometer (Analytikjena, Germany) Fig. 1.

High performance liquid chromatography

Phycobiliprotein subunit separation by HPLC was performed using a reversed phase Discovery BIO Widepore C_5 (Supelco, Sigma Aldrich) column (250×4.6 mm i.d.) packed with 5 µm porous silica

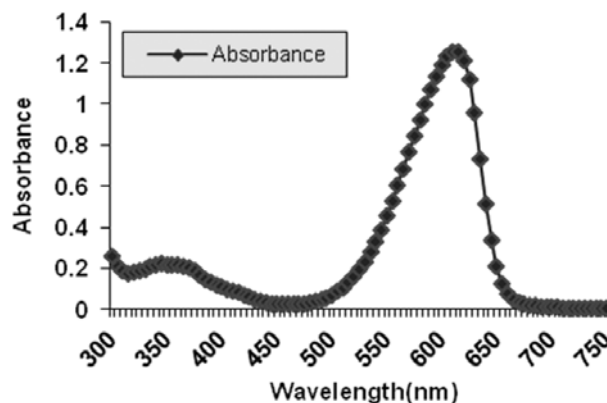

Fig. 1 — Absorption spectrum of purified phycocyanin

particles (300 Å pore diameter). This column was operated at a flow rate of 1.0 mL/min for optimum separation efficiency. All solutions were filtered through 0.5 µm membrane filter and degassed by bubbling with helium before use. Optimization of chromatographic separations was performed using a Alliance system (Waters) with 2695 separation module with auto-sampler consisting of a Waters 2998 Photo Diode-Array detector (PDA) and Waters 2475 Multi λ fluorescence detector. Excitation and emission wavelength for fluorescence detector was set at 580 and 640 nm. The Discovery BIO Widepore C_5 column was pre-equilibrated with 20% (v/v) aqueous acetonitrile (ACN) solution containing 0.1% (v/v) trifluoroacetic acid (TFA). 20 µL sample (200 µg mL^{-1}) was injected and elution was performed using a linear gradient from 20 to 100% (v/v) aqueous ACN (containing 0.1% TFA) in 45 minutes[17]. Both PDA and fluorescence detector was connected in series for detection of biliprotein subunits. Figs 2 and 3.

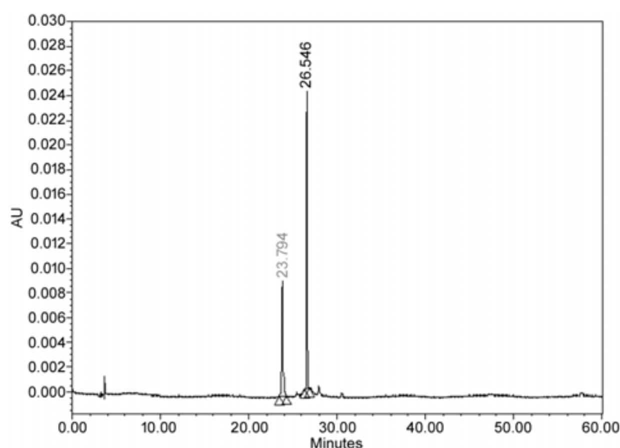

Fig. 2 — Reverse phase HPLC profile of phycocyanin.

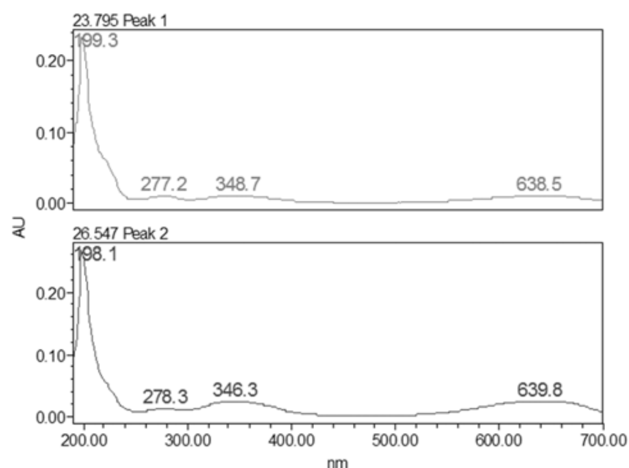

Fig. 3 — Absorbtion spectra of α-subunit (RT-23.795) and β-unit (RT-26.547)

Results and Discussion

Anabaena and *Phormidium* are used as a high quality protein mainly for phycocyanin, which is an important cyanobacterial accessory pigment having wide industrial applications. Although a number of reports are available for extraction and purification of phycocyanin from cyanobacterial strains[18,19] but theyhave many loopholes and methods are so expensive and the purity of phycocyanin is very less. Extraction and purification of phycocyanin, was completed in four major steps: crude extract preparation (Step I), ammonium sulfate precipitation (Step II), dialyses (Step III) and anion exchange chromatography (Step IV). After ammonium sulphate precipitation separation, PC with maximum purity was eluted as a bright blue coloured solution at pH 3.76 with purity 3.11 and 3.34 for *Anabaena* and *Phormidium*, respectively (Tables 1 and 2). The absorption spectra of the purified PC showed a prominent peak at 620 nm (Fig. 1). In order to further characterize the PC purified from BGA, reverse phase HPLC was performed using C5 column (Fig. 2). The PDA detector set at 620 and 226 nm revealed two major peaks at 23.795 and 26.547 min. When absorption spectra of these two chromatogram peaks were critically analyzed, it was found that A620:A280 for the first peak RT = 23.795 min (Fig. 3A) was

Table 1— Purity and Recovery ratio of phycocyanin content in *Anabaena* and *Phormidium*

Steps	Volume (mL)	Yield (µg/mL)	Total PC (µg)	Recovery (%)	Purity
Anabaena					
Crude extract	100	70	7000	100	0.68
Ammonium sulphate precipitation	30	226.66	6800	97	1.15
Dialysis	30	163.3	4900	72	1.39
Column chromatography	5	591 (3 conc. pooled) 197 (µg/mL)	2955 (µg/15 mL) 985 (µg/5mL)	60	3.11
Phormidium					
Crude extract	100	55	5500	100	0.44
Ammonium sulphate precipitation	30	150	4500	81	1.26
Dialysis	30	110	3300	79	1.35
Column chromatography	5	525 (3 conc. pooled) 175 (µg/mL)	2625 (µg/15 mL) 875 (µg/5mL)	45	3.34

Table 2— Purity and Recovery ratio of phycocyanin content in *Anabaena* and *Phormidium*

	Anabaena					*Phormidium*				
	Volume (mL)	Yield (µg/mL)	Total PC (µg)	Recovery (%)	Purity	Volume (mL)	Yield (µg/mL)	Total PC (µg)	Recovery (%)	Purity
Crude extract	100	70	7000	100	0.68	100	55	5500	100	0.44
Ammonium sulphate precipitation	30	226.66	6800	97	1.15	30	150	4500	81	1.26
Dialysis	30	163.3	4900	72	1.39	30	110	3300	79	1.35
Column chromatography	5	591 (3 conc. pooled)	2955 (µg/15 mL)	60	3.11	5	525 (3 conc. pooled)	2625 (µg/15 mL)	45	3.34
		197 (µg/mL)	985 (µg/5mL)				175 (µg/mL)	875 (µg/5mL)		

approximately 1, which is due to the presence of one phycocyanobilin (PCB) chromophore, thus indicating that this peak corresponds to a subunit of PC, while the A620:A280 for the second peak RT = 26.547 min (Fig. 3B) was approximately 2, which is due to the presence of two PCB chromophores, and therefore this peak corresponds to b subunit of PC.

Conclusion

The purification results suggest an easy and efficient method for purification of C-phycocyanin (C-pc) from these cyanobacterial strains *Anabaena* and *Phormidium*. This C-pc is a source of supplement material for various applications in food, cosmetics, medicine and biotechnology, and hence, useful to nutracueticals and pharmaceuticals based companies.

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Conflict of Interest

Authors declare no conflict of interest.

Authors contribution

Dr Devendra Kumar prepared and designed the concept and experimentation. Dr Dolly Wattal Dhar, Priyanka Nehra and Mr Sudhir Saxena executed the research work.

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