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Extraction and purification of Phycoerythrin from *Anabaena variabilis* (CCC421)

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Abstract

A simple protocol for purifying phycoerythrin (PE) from *A. variabilis* (CCC421) was developed in this study involving ammonium sulfate precipitation followed by a single step anion exchange chromatography using DEAE-Cellulose-11 and Acetate buffer. Precipitation with 65% ammonium sulfate resulted in 85.81% recovery of PE with a purity of 2.81 (OD_{562}/OD_{280}) while 62.5% PE was recovered after chromatographic separation with a purity of 4.95 (OD_{562}/OD_{280}). SDS-PAGE analysis of purified PE showed the presence of two subunits viz. α and β with molecular weight of 16 kDa and 18 kDa respectively. Absorption spectra of purified PE showed maximum absorption at a wavelength of 565 nm characteristic to C-PE I group of phycoerythrins.

Key words: *Anabaena variabilis*, Ammonium sulfate precipitation, Anion exchange chromatography, Phycoerythrin, SDS-PAGE

Introduction

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 are very unique due to their oxygenic nature of photosynthesis which is very similar to higher plants. Like red algae and cryptomonads, blue green algae also contain Phycobiliproteins which serve as major accessory pigments during photosynthesis. Phycobiliproteins are large water soluble 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 (Bogorad, 1975). They are highly diverse in their structure and pigment composition in cyanobacteria, red algae and cryptomonads (Grossman et al., 1995; Guan *et al*., 2007). These brilliantly colored, proteins can be divided broadly into three classes based on their spectral properties: Phycoerythrin (λ_{max} ~565nm), Phycocyanin (λ_{max} ~620nm), and Allophycocyanin (λ_{max} ~650nm). A fourth Phycobiliprotein known as Allophycocyanin B (λ_{max} ~670nm) has also been shown to be present in cyanobacteria in low amounts (Glazer and Bryant, 1975). Light absorbed by Phycoerythrin is efficiently transferred to Phycocyanin, then to Allophycocyanin and ultimately to Chlorophyll. Most cyanobacterial Phycobiliproteins are composed of two different kinds of polypepetides of which one is light (α, MW- 12-19 kDa) and other is heavy (β, MW- 14-21 kDa). These chains are generally present in equimolar amounts (Bernard *et al*., 1992) and are generally organized as trimeric $(\alpha\beta)_3$ discs but larger aggregates like hexameric $(\alpha\beta)_6$ discs are also found (MacColl, 1998). The brilliant colors of Phycobiliproteins are mainly due to covalently bound prosthetic groups that are open-chain tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins. They are either blue colored phycocyanobilin (PCB), red colored phycoerythrobilin (PEB), yellow colored phycourobilin (PUB), or purple colored phycobiliviolin (PXB), also named cryptoviolin. These chromophores are generally bound to the polypeptide chain at conserved positions either by one cysteinyl thioester linkage through the vinyl substituent on the pyrrole ring A of the tetrapyrrole or occasionally by two cysteinyl thioester linkages through the vinyl substituent on both A and D pyrrole rings (Glazer, 1985). Cyanobacterial phycobiliproteins have gained considerable importance in the commercial sector, as they have several applications. The primary potential of these molecules are as natural dyes but a number of investigations have shown their health-promoting properties and broad range of pharmaceutical applications. Phycoerythrin is the most widely used Phycobiliproteins in fluorescent probes and has quantum yields of 82-98% (Oi *et al*., 1982). A number of multinational companies have commercialized antibodies conjugated with Phycoerythrin.

Hence, the present study was focused on optimization of a purification process to obtain high purity Phycoerythrin from a strain of *Anabaena variabilis* (CCC421).

Materials and methods

A. *Growth and maintenance of cultures*
Anabaena variabilis (CCC421) was procured 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 nitrogen free BG-11 media (Stanier *et al*., 1971) at 28 ± 2°C under a light intensity of 52-55 µmol photon m^{-2} s^{-1} and L: D cycles of 16:8 hours.

B. *Extraction and estimation of Phycobiliproteins*
500 mL of homogenized log phase (14 days old) culture was centrifuged at 4000 rpm to obtain pellet. The pellet was suspended in 100 mL of 20 mM Acetate buffer containing 50 mM Sodium

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Chloride and 0.002 M Sodium Azide (pH 5.10). Phycobiliproteins were extracted by repeated freezing (-20^{o}C) and thawing (room temperature) until the cell mass becomes greenish. Cell debris were removed by centrifugation at 5000 rpm for 10 minutes and the extract thus obtained was termed as crude extract. Amount of Phycoerythrin was measured as described by Bennett and Bogorad (1973) and purity was determined using the following formulae-

$$\text{Purity} = OD_{562} / OD_{280}$$

C. Purification

The crude extract was subjected to a single step precipitation using 65% $(NH4)_2SO_4$ (BioXtra, >99%: Sigma-Aldrich) and kept overnight at 4^{o}C. The pellets were recovered by centrifugation at 27000 g for 15 minutes at 4^{o}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 Dialysis membranes (Dialysis membrane-70, MWCO- 12-14 kDa) procured from HiMedia (India). At firs,t dialysis was performed twice against 1000 mL extraction buffer at room temperature and again dialysed against 1000 mL of extraction buffer at 4^{o}C for overnight. The resultant extract was recovered from the dialysis membrane and filtered through 0.45 µm filter (Sartorious).

DEAE-Cellulose 11 from Sisco Research Laboratory (SRL) was used for Anion exchange chromatography using.. 30cm X 2cm column was prepared for purifying the Phycoerythrin protein. Column was equillibriated with 150 mL of Acetate buffer (pH 5.10). 10 mL of dialysed and filtered sample was placed on the top of the DEAE-Cellulose column with the help of a syringe. 50 mL of Acetate buffer (pH 5.10) was then applied to the column to wash out any unbound protein. A linear gradient of Acetate buffer with a pH ranging from 3.76 to 5.10 was used to develop the column and eluates were collected in 5 mL fractions. Flow rate was kept at 20 mL/hr.

D. Determination of absorption spectra

Absorption spectra was determined by scanning the sample in a range of 300-750 nm wavelengths by Specord 200 spectrophotometer (Analytikjena, Germany).

E. Poly Acrylamide Gel Electrophoresis

A 7.5% continuous PAGE under non-denaturing conditions was carried out to reconfirm the purity of the phycoerthrin. 15% denaturing SDS-PAGE was also carried out as described by Laemeli (1970). In both the cases bands were visualized by Coomassie Blue staining. Molecular weight of the purified protein was determined by running Novex Sharp pre-stained Protein Marker along with the sample.

Results

Anabaena has been mostly exploited for Phycocyanin (Eriksen, 2008) which is another important cyanobacterial accessory pigment having a number of industrial applications. But exploitation of *Anabaena* for phycocrythrin production is very limited according to the literatures available till date. Although a number of reports are available for purification and characterization of Phycoerythrins from different cyanobacterial and red algal strains (Schoenleber *et al*., 1984; Swanson *et al*., 1991; Federspiel and Scott, 1992; Wilbanks and Glazer, 1993; MacColl *et al*., 1996; Rossano *et al*., 2003, Miearas and Wall, 1968; Bryant, 1982; Reis *et al*., 1998; Tchernov *et al*., 1999), only a few reports from India (Ranjitha and Kaushik, 2005; Tripathi *et al*., 2007; Parmar *et al*., 2011) are available for purification of PE from cyanobacteria.

The protocol developed in this study has four major steps viz. preparation of crude extract (Step I), 65% ammonium sulfate precipitation (Step II), dialysis (Step III) and anion exchange chromatography (STEP IV) using DEAE-Cellulose-11 and Acetate buffer. This extraction and purification protocol developed is efficient enough to obtain high purity PE as evident from the high final purity (OD_{562}/OD_{280}=4.95) and high (62.5%) recovery (Table 1). Precipitation of phycobilliproteins with 65% saturation of ammonium sulfate resulted in 85.81% recovery with a purity of 2.80, although there was no significant increase in purity after dialysis (Table 1). During the chromatographic separation, PE with maximum purity was eluted as a bright pink coloured solution at pH 3.9 (Figure 1a & 1b). From crude extract to purified PE, the purity was increased by almost 7 times which also indicated the efficiency of the method to obtain high purity Phycoerythrin. The absorption spectra of the purified PE showed a prominent peak at 565 nm (Figure 3). Noticeably there was no peak corresponding to phycocyanin or allophycocyanin. The purity as determined by spectrophotometric analysis was reconfirmed by the presence of a single band during native gel electrophoresis (Figure 3). SDS-PAGE analysis of the purified PE revealed two bands of 16 kDa and 18 kDa corresponding to α and β subunits respectively (Figure 4).

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Table 1: Stepwise purification of phycoerythrin from *A. variabilis* (CCC421)

Steps	Volume (ml)	PE (µg/ml)	Purity of PE (OD_{562}/OD_{280})	Recovery of PE (%)
Crude extract	100	48	0.69	100
Ammonium Sulfate precipitation	10	410	2.80	85.81
Dialysis	10	320	2.83	78.05
DEAE-Cellulose column chromatography	5	400	4.95	62.50

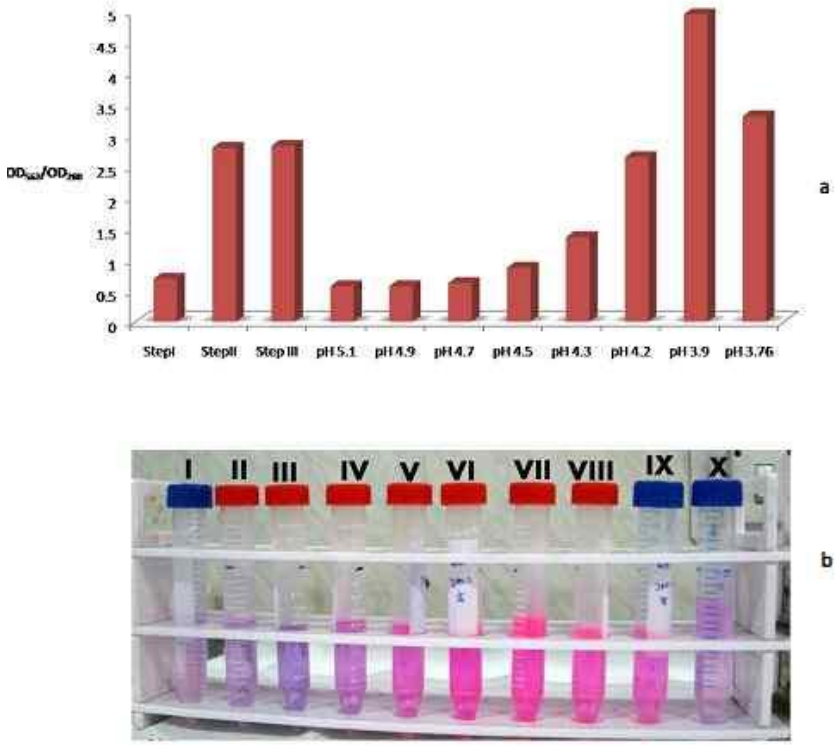

Figure1: a. Purity of phycoerythrin attained at different steps viz. Step I- Crude extract, Step II- Ammonium sulfate precipitation, Step III- Dialysis and elution of the column using Acetate buffer with a linear gradient of pH from 3.76 to 5.1. b. Coloured eluates obtained during developing the column. I- eluate at pH 5.1, II- eluate at pH 4.9, III- eluate at pH 4.7, IV- eluate at pH 4.5, V- eluate at pH 4.3, VI- eluate at pH 4.1, VII & VIII- eluates at pH 3.9, IX & X- eluates at pH 3.76

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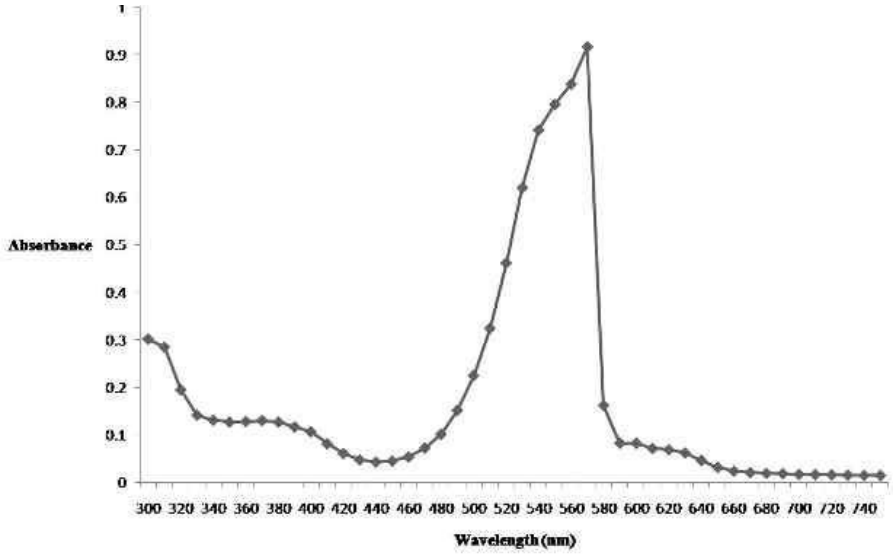

Figure 2: Absorption spectra of the purified phycoerythrin.

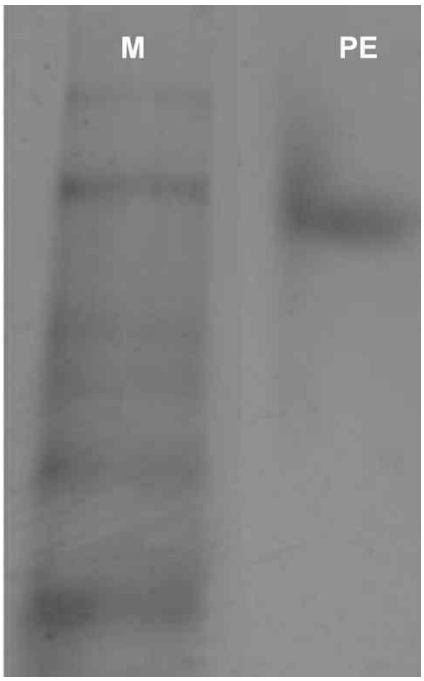

Figure 3: Single band of phycoerythrin obtained during the native PAGE. M- Protein molecular weight marker, PE- Purified phycoerythrin

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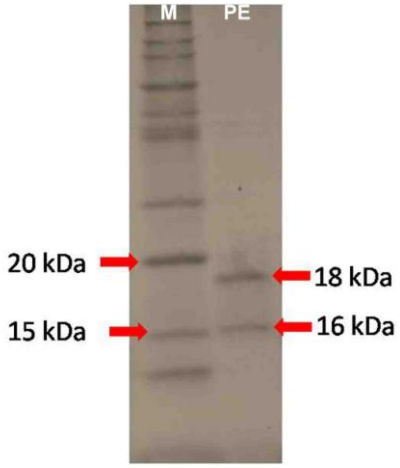

Figure 4: SDS-PAGE analysis of purified phycoerythrin. M- Protein molecular weight marker, PE- Purified phycoerythrin

Discussion

As mentioned earlier, phycoerythrin has immense potential for use in pharmaceutical and food industries. But due to their limited distribution and difficulties to obtain high purity phycoerythrin, purification of this pigment has become a challenging endeavor. Particularly in cyanobacteria, there is really a dearth of effective and efficient methodologies for purifying phycoerythrin.

In this study, following 65% ammonium sulfate precipitation, we have achieved a recovery of 85.81% with a purity of 2.81 which is greater than that reported by others. Ranjitha and Kaushik (2005) reported 85% recovery of phycoerythrin with a purity of 2.89 after 55% ammonium sulfate precipitation from *Nostoc muscorum* while around 80% recovery of PE content with a purity ratio of around 1.5 for young and old cultures from three cyanobacteria viz. *Phormidium* sp. A27DM, *Lyngbya* sp. A09DM and *Halomicronema* sp. A32DM has been after the treatment of crude extract with 70% ammonium sulfate (Parmar *et al.*, 2011). It is pertinent to mention here that the purity achieved after ammonium sulfate precipitation is high enough for using the phycoerythrin in foods and feeds. Scaling up this process for large scale production of food and feed grade phycoerythrin may help the industries engaged in production of natural colorants. During chromatographic separation a purity of 4.95 was achieved which is higher or comparable to that reported by Tchernov *et al*. (1999), Reis et al. (1998) and Parmar *et al*. (2011) although Ranjitha and Kaushik (2005) achieved a purity of 8.12 from *Nostoc muscorum*. Tripathi *et al*. (2007) also reported a purity of 5.25 from *Lyngbya arboricola* using a procedure involving acetone precipitation, gel filtration in addition to ammonium sulfate precipitation and DEAE-Cellulose column chromatography. The recovery (62.5%) was however not as high as reported by others (Ranjitha and Kaushik, 2005; Parmar *et al*., 2011). On the other hand, the whole process described in this study involving single step chromatographic separation is simple and comparable or better than the other existing methods for purification of Phycoerythrin as it does not involve any additional purification steps like membrane filtration, gel filtration or use of organic solvents (Reis *et al*., 1998; Tchernov *et al*., 1999, Tripathi *et al*., 2007).

C-PE generally exist in hexameric state i.e. $(\alpha\beta)_6$. The α and β subunits vary with their originations in molecular mass from 15 kDa to 22 kDa. Galzer and Cohen-Bazirre (1971) reported α and β subunits of 20 kDa and 22 kDa from *Aphanocapsa* sp. (strain 6701) while Bennett (1972) reported α and β subunits of 18.3 kDa and 20 kDa from *Fremeyella diplosiphon*. Ranjitha ad Kaushik (2005) also reported α and β subunits of 19.4 kDa and 16.5 kDa from *Nostoc muscorum*. The molecular mass of the α (16 kDa) and β (18 kDa) subunits obtained in this study are in compliance with the earlier reports. Cyanobacterial phycoertythrin or C-PE is generally divided into two subtypes viz. C-PE I and C-PE II. The most common C-PE-I exhibiting absorption spectra with maximum absorbance near 565 nm (Tripathi *et al*., 2007). C-PE-II from *Synechococcus* strains WH8020 and WH8103 had absorption maxima at 495 and 543, and 492 and 543, respectively (Ong and Glazer, 1991). The phycoerythrin protein purified from *A. variablis* (CCC421) showed an absorbance maxima of 565 nm characteristic to the group I phycoerythrin.

Amidst a large array of natural products produced by cyanobacteria, phycobiliprotins seem to be most colorful and attractive components due to their potential biotechnological and industrial application. Selection of suitable cyanobacterial culture and simple and economic process for purification of pigments hold the key to their future exploration for the benefit of the mankind. In this context, the present study may help the researchers and entrepreneurs to develop strategies for large scale production of phycoerythrin. However, toxicological studies must be carried out to assess their biotechnological feasibility for commercial production

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