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Studies on *Anabaena* sp. NCCU-9 with special reference to phycocyanin

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Abstract

Phycocyanin is a major light harvesting pigment of cyanobacteria. Phycocyanin has gained importance in many biotechnological applications in food sciences, therapy, immunodiagnostics, cosmetics and pharmacological processes. Screening of cyanobacterial strain showed maximum phycobiliprotein in *Anabaena* NCCU-9. It was followed by optimization of extraction conditions. Sodium phosphate buffer, pH-7.5, supplemented with 0.15 M sodium chloride, freezing and thawing (-20 & 4°C) enhanced the total phycobiliprotein extraction. In this paper purification and characterization of phycocyanin: one of the important phycobiliprotein is being presented. Extraction, ammonium sulphate precipitation, dialysis, gel chromatography and SDS PAGE gave phycocyanin of 4.45 purity value and a band of 18.5 kDa.

Keywords: *Anabaena*, Extraction, Optimization, Phycobiliprotein, Phycocyanin, Purity value

Introduction

Phycobiliproteins are the major photosynthetic accessory pigments in cyanobacteria (blue-green algae, prokaryotic); rhodophytes (red algae, eukaryotic); cryptomonads (biflagellate unicellular eukaryotic algae) and cyanelles (endosymbiotic plastid-like organelles). Phycobiliproteins are brilliant-colored and water-soluble antennae-protein pigments organized in supramolecular complexes, called phycobilisomes (PBSs), which are assembled on the outer surface of the thylakoid membranes. The colors of phycobiliproteins originate mainly from covalently bound prosthetic groups that are

open-chain tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins. There are four main classes of phycobiliproteins – allophycocyanin (APC bluish green), phycocyanin (PC: deep blue), phycoerythrin (PE: deep red) and phycocyanobilin (PCB: orange). Cyanobacteria with high levels of specific phycobiliproteins are of commercial interest. The primary potential of these molecules are as natural dyes in food industry. A number of investigations have shown their health-promoting properties and pharmaceutical applications. Among different phycobiliproteins phycocyanin, is of greater importance because of its various biological and pharmacological properties. Recent studies have demonstrated antioxidant (Miranda et al. 1998) antimutagenic (Chamorro et al. 1996), antiviral (Ayehunie et al. 1998), anticancer (Chen et al. 1995; Schwartz et al. 1988), anti-allergic (Kim et al. 1998), immune enhancing (Qureshi et al. 1996), hepato-protective (Gonzalez et al. 1993), blood vessel relaxing (Paredes-Carbajal et al. 1997), neuro-protective (Romay et al. 1998), antitumor (Li et al. 2005), radical scavenging (Vadiraja and Madyastha, 2000) and anti inflammatory properties (Romay et al. 1999). Alzheimer's and Parkinson's can also be treated with phycocyanin (Rimbau et al. 2003). Blood lipid-lowering effects of phycocyanin also need special attention (Iwata et al. 1990). As of now the annual market of phycocyanin is around 5-10 million US dollars (Sekar & Chandramohan, 2008).

During screening of cyanobacteria for phycobiliproteins *Anabaena* NCCU-9 produced maximum phycobiliproteins (Hemlata & Fatma, 2009) and thus it was selected for extraction, purification and characterization of phycocyanin.

Materials & Methods

Materials

Sephadex G-25, G-100, Dialysis tubing (10 kDa cut out), lysozyme and standard protein molecular weight markers were obtained from Sigma (St. Louis, MO, USA). Rests of chemicals were procured from Hi-Media and Qualigens. All buffers and reagents used in this study were prepared in double distilled water and supplemented with 0.01% sodium azide.

Organism and culture conditions

The cyanobacterium *Anabaena* sp. was procured from the National Center for Culture Collection and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi. Cultures were raised in BG-11 Medium (Stanier et al, 1971). Sodium nitrate was excluded from the media as *Anabaena* is heterocystous form. The initial OD of the of the culture suspension was maintained 0.3 at 750 nm and were allowed to grow in light intensity provided by cool-white fluorescent tubes of 50 μ mol photons.m^{-2}.s^{-1} following 12:12 hour, light and dark regime at 30°C ± 1°C (Hemlata & Fatma , 2009). Cultures flasks were shaken manually to allow air and nutrients circulation.

Extraction and quantification of phycobiliproteins

Anabaena cells were harvested by centrifugation (10,000g for 15 min). Adhere salts were removed by washing with double distilled water and then biomass was dried (50ºC). Biomass was homogenized with phosphate buffer and repeated freezing and thawing was done in dark. The mixture was subsequently centrifuged (10,000g for 20 min, 4ºC) to separate the phycobiliprotein containing clear supernatant. Absorbance of supernatants was measured at wavelengths 620, 652, and 562 nm (Varian CARY 500

Scan UV–VIS spectrophotometer) for C-phycocyanin, allophycocyanin and phycortyrhrin respectively (Sygelman & Kycia. 1978).

Optimization of phycobiliprotein extraction

Optimization of extraction protocol was done with respect to different buffers, pH, temperature, cell disruption technique and protein precipitating agents. Sodium acetate - Acetic acid buffer (pH-6.0), citrate buffer (pH-5.0), carbonate buffer (pH-9.6), potassium phosphate buffer (pH-7.0), sodium phosphate buffer (pH-7.0) of 0.1 M strength, tris buffer (pH-7.2), phosphate buffered saline and double distilled water were used. In the selected buffer effect of pH (6.0, 6.5, 7.0, 7.5 & 8.0) of buffer was checked on phycobiliprotein yield. Then a combination of lysozyme (100µg/ml) and 10 mM EDTA (Boussiba & Richmond. 1979), 0.10 M potassium chloride (Abalde et al. 1998) and 0.15 M sodium chloride (Minkova et al. 2003) and phosphate buffer saline were supplemented in selected buffer. It was followed by finding out effect of different cell disruption methods like homogenization (Oranda et al. 1978), sonication (Furuki et al. 2003), lytic enzyme-lysozyme (Bermejo *et al.* 2003), heat shock (Soni et al. 2006) and freezing and thawing (Dubos, 1937; Soni et al. 2006) on phycobiliprotein yield. An attempt has been made to find out correlation between freezing and thawing temperatures (0 & 4°C, 0 & 25°C, 4 & 25°C, -20 & 25°C & -20 & 4°C) on phycobiliprotein extraction. Different protein precipitation agents like poly ethylene glycol (20% with pinch of salt), polar solvents (acetone: equal volume; ethanol: 4 volumes), trichloroacetic acid (TCA), trichloroacetic acid in acetone (1:8:1) and 4M ice-cold saturated ammonium sulphate solution were compared for maximum recovery of phycobiliproteins

(Sadassivam & Manickam, 1991; Roe, 2000).

Purification and characterization of phycocyanin

100 g *Anabaena* cells were crushed with liquid nitrogen in pestle and mortar till whole biomass resulted in frozen powder and rapidly thawed by adding 100 ml sodium phosphate buffer above optimized buffer resulting leaching out of intracellular proteins including phycocyanin. The process of freezing and thawing was repeated till blue colored supernatant was obtained. Cell debris was removed by centrifugation and labeled as crude extract. Crude extract of phycocyanin was fractionally precipitated by ammonium sulfate first at 25 % and then 50 % saturation (Boussiba & Richmond. 1979). For this ammonium sulfate was added gradually in crude extract with continuous stirring. The resulting solution kept overnight and centrifuged (17,000 x g for 20 min). The precipitate obtained from 25 % saturation was discarded. The supernatant was further brought to 50% saturation in a manner similar to that of 30 % saturation. The precipitate obtained from 50% saturation contained mainly phycocyanin was dissolved in small quantity of sodium phosphate buffer (pH-7.0, 0.005 M) and subjected to dialysis overnight against 1000 times volume of the same buffer. The dialyzed solution after centrifuging was passed through Sephadex G-25 column (1.5 x 20 cm) pre-equilibrated and eluted with same buffer. The column was developed at a flow rate of 0.5 ml/min and elutions were collected in 1 ml fraction tubes. All those fractions having purity ratio of A_{615}/A_{280} > 3.0 were pooled together and again passed through Sephadex G-100 column (2.5 x 20 cm) pre-equilibrated and eluted with 0.005 M sodium phosphate buffer, pH-7 at 1 ml/ min. All the steps of purification are represented in (Fig.1). The products

obtained at every step of purification were analyzed for total protein, total phycobiliprotein and phycocyanin content and subjected to both absorbance and fluorescence spectral scanning. SDS–PAGE was performed according to Laemmli et al. (Laemmli et al. 1970) using 15% polyacrylamide slab gel, run at 50V, 12.5 mA. Proteins standard was of medium range 97–14 kDa was used as molecular weight markers and visualized by staining with coomasie brilliant blue G-250.

Results and Discussion

One of the most important requirements for obtaining phycobiliproteins from cyanobacteria is selection of extraction and purification protocol. A purification procedure that works well for a phycobiliprotein from one organism may not be the method of choice for the corresponding phycobiliprotein from another organism. For, this reason the phycobiliprotein extraction and purification were compared in *Anabaena* in present investigation.

Optimization of Phycobiliprotein extraction

Selection of suitable buffer for maximum phycobiliproteins extraction is very crucial (Viskari & Colyer, 2003) as these ensure that proteins are not denatured due to shift in pH (Roe 2000). During comparison of various buffers, sodium phosphate buffer w as found as best buffer with 100.5 $\pm$0.22 mg/g phycobiliprotein, while double distilled water extracted least 83.6 $\pm$0.17 mg/g. (Table 1). The lowest yield in distilled water may be due to its incapability to lyse the cell wall reducing seepage of phycobiliprotein pigment. Soni et al. (2006) and Silveira et al. (2007) have also found sodium phosphate buffer (pH-7.0) as best for phycocyanin extraction in *Oscillatoria* and *Spirulina* Sodium phosphate buffer is also being reported to have inhibition property to metabolic enzymes providing

protein stability for several weeks at 4°C (Roe 2000).

Table 1 Effect of different buffers on the extraction of total phycobiliproteins in *Anabaena* sp.

Buffer (0.1M)	Total phycobiliprotein (mg/g)	SD
Acetate buffer (pH-6.0)	92.4	±0.20
Carbonate buffer (pH-9.6)	84.5	±0.4
Citrate buffer (pH-5.0)	90.1	±0.13
Double distilled water	83.6	±0.171
Potassium phosphate buffer (pH-7.0)	97.2	±0.161
Sodium phosphate buffer (pH-7.0)	100.5	±0.223
Tris chloride buffer (pH-7.2)	89.3	±0.131

In the best buffer pH of on phycobiliproteins extraction was also studied and maximum extraction was achieved at pH-7.5 yielding 114.5 ±0.005 mg/g phycobiliproteins (Table 2). pH above or lower pH-7.5 resulted reductions in phycobiliprotein extraction. Findings of Sarada et al. (1999) is accordance with the present study. Actually extreme buffer's pH cause internal electrostatic attraction by changing the charge on protein giving net positive charge and at this stage protein open up and bound solvent is lost, resulting denaturation of protein (Roe 2000).

Table 2 Effect of varying pH of sodium phosphate buffer in extracting total phycobiliprotein in *Anabaena* sp.

Selected buffer (0.1M)	Total phycobiliprotein (mg/g)	SD
Sodium phosphate buffer (pH-6.0)	105.7	±0.200
Sodium phosphate buffer (pH-6.5)	102.4	±0.207
Sodium phosphate buffer (pH-7.0)	110.0	±0.223

Sodium phosphate buffer (pH-7.5)	114.5	±0.058
Sodium phosphate buffer (pH-8.0)	104.1	±0.105

Further addition of sodium chloride to the selected buffer (sodium phosphate buffer, pH-7.5) up to 0.15 M enhanced the phycobiliptotein extraction capacity up to 131.8 ±0.005 mg/g (Table 3). Minkiva et al. (2003) and Rossano et al. (2003) had also shown that externally supplemented sodium chloride (0.15 & 0.1 M) along with phosphate buffer had improved extraction of phycocyanin in *Spirulina* and phycoerythrin in *Carrolina.* This suggested that Na and Cl ions may play some crucial role in phycobiliprotein extraction.

Table 3 Effect of different salts on the extraction of total phycobiliprotein in selected sodium phosphate buffer in *Anabaena* sp.

Selected buffer (pH-7.5)	**Total phycobiliprotein (mg/g)**	**SD**
Lysozyme & EDTA	104.0	±0.287
Phosphate buffered saline	110	±0.093
Potassium Chloride (0.15M)	117.9	±0.103
Sodium Chloride (0.10M)	131.8	±0.057

Normally cyanobacterial cell walls are thick and extremely resistant (Stewart & Farmer, 1984). It acts as physical barrier for the release of phycobiliprotein into the extracting medium. So, optimization of cell disruption method is also very important for the maximum extraction. In present study, it was observed that freezing and thawing method was the best for cell disruption and produced 128 ±0.09 mg/g phycobiliproteins

(Table 4). Freezing and thawing method was have been the most efficient way to extract phycobiliprotein in various cyanobacteria such as *Microcystis* (Padget & Krogman, 1987), *Synechococcus* (Abalde et al. 1998), Spirulina (Doke 2005), Nostoc muscorum (Ranjitha & Kaushik, 2005) than other used disruptive methods, whereas this method was found to be poorest in red algae *Porphyridium cruentum* (Bermejo et al. 2003). This technique is mild and non-denaturing. The ice crystals formed during freezing rip the cell wall and cell membrane and released the phycobiliprotein into the extracting medium (Soni et al. 2006).

Table 4 Effect of different cell disruption method on the extraction of total phycobiliprotein in *Anabaena* sp.

Selected buffer (0.1M)	Total phycobiliprotein (mg/g)	SD
Homogenization	30.0	±0.287
Sonication	100.0	±0.142
Lysozyme	85.0	±0.121
Heat Shock	55.0	±0.287
Freezing and thawing	128.2	±0.098
Nitrogen lysis	124.5	±0.156

Standardization of temperatures for freezing and thawing temperatures has revealed that temperature also plays an important role in the extraction process. Freezing at –20 ºC and thawing at 4ºC appeared as optimal temperature for highest phycobiliproteins

extraction (Table 5). Similar observations were also being reported in Synechococcus, Spirulina and Oscillatoria for phycobiliprotein extraction (Abalde *et al.* 1998; Doke, 2005; Soni *et al.* 2006).

Table 5 Effect of freezing and thawing temperatures on the extraction of total phycobiliproteins in *Anabaena* sp.

Temperatures (Freezing and thawing)	**Total phycobiliprotein (mg/g)**	**SD**
0°C & 4°C	80	±0.32
0°C & 25°C	60.12	±0.392
4°C & 25°C	32	±0.332
-20°C & 25°C	92	±0.299
-20°C & 4°C	128	±0.129

For phycobiliprotein precipitation various precipitating agents (PEG, ethanol, acetone, TCA, ammonium sulfate) were compared. Among these ammonium sulfate was turned out to be the best (Table 6). Protein precipitation using ammonium sulphate is cheap, best and reliable method as it precipitates readily and also prevents denaturation of protein due to its low heat of solublization and bacteriostatic effect (Roe 2000).

Table 6 Weight of total phycobiliprotein precipitate from *Anabaena* sp. concentrated by various precipitation methods.

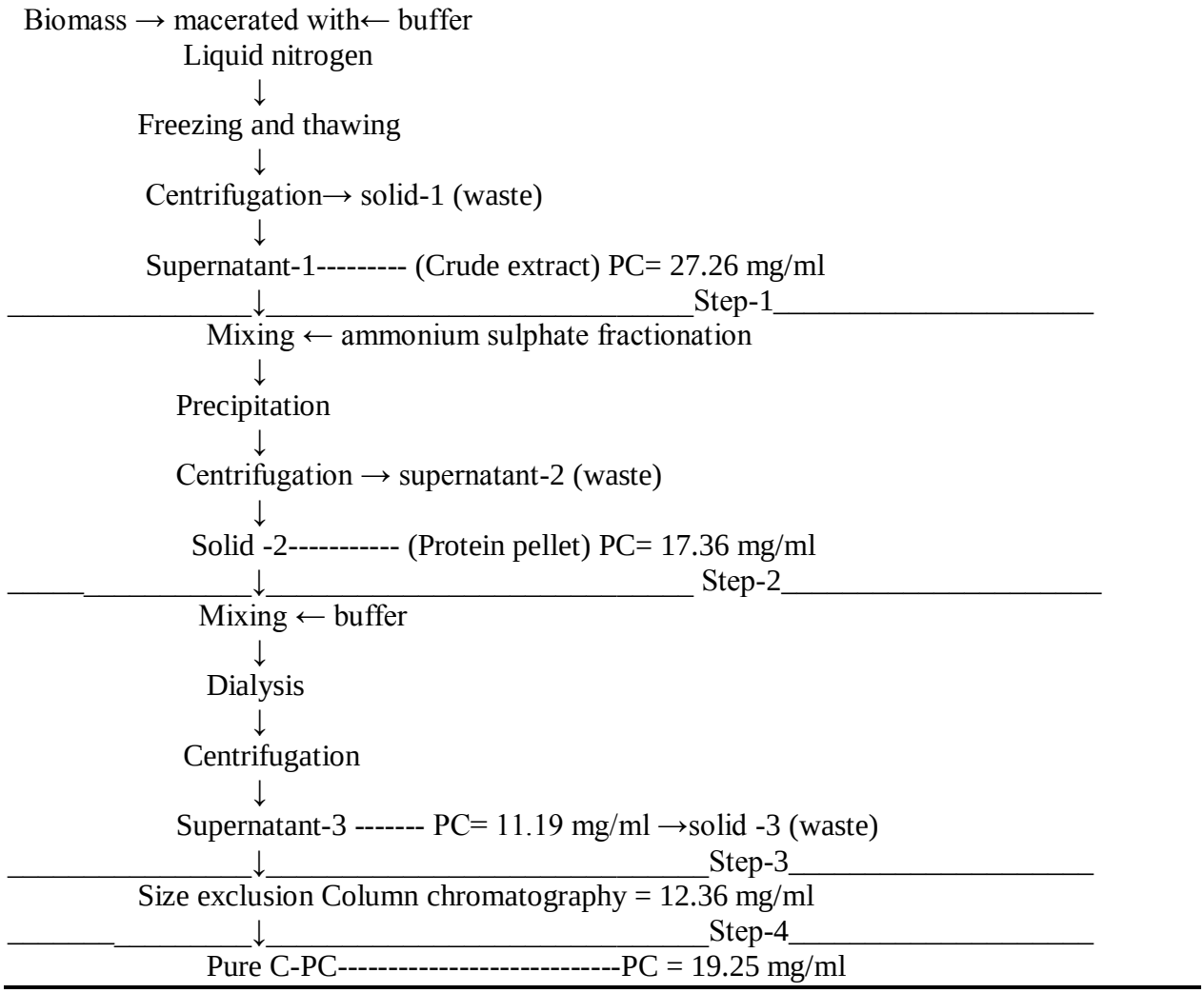
Precipitation method	Total phycobiliprotein precipitate (g/10 ml)
Poly ethylene glycol	0.196
Acetone	0.222
Ethanol	0.216
Trichloroacetic acid	0.230
Trichloroacetic acid in acetone	0.235
Ammonium sulphate	0.333

Extraction and purification of phycocyanin

The purity of phycocyanin plays a significant role in commercial applications and is generally evaluated using the absorbance ratio of A_{615}/A_{280} where A_{615} represents maximum peak height for phycocyanin and A_{280} indicates contamination of aromatic amino acid rich proteins. A purity of 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Rito-Palmares 2001). Only a few methods are reported which could achieve analytical grade phycocyanin. The purity of crude *Anabaena* phycocyanin came to be 0.87. It is noticed that crude extract from organisms other than *Spirulina* have low purity values e.g. 0.85 in *Oscillatoria quadripunctatis* (Soni et al. 2007), 0.43 in *Synechococcus* (Abalde et al. 1998) and 0.4 in *Calothrix* (Santeigo-Santos et al. 2003), whereas, Minkova et al. (2007) found purity value up to 0.87 in *Arthronema africanum* that is equal to our strain *Anabaena* NCCU-9. Different workers have reported different purity values for crude extract in *Spirulina platensis*, 1.14 (Zhang &

Chang, 1999), 0.97 (Bhasker et al. 2005), 0.95 (Minkova et al. 2003).

Fig. 1: Flow chart for purification of phycocyanin from cyanobacterium *Anabaena*.



The purification of crude extract involves fractional precipitation with 25% and 50% ammonium sulfate which is particularly useful in salting out unwanted proteins and at the same time to concentrate phycocyanin (Baussiba and Richmond. 1979). Supplementation of 25 % ammonium sulphate gave no precipitation and thus was subjected to 50% ammonium sulphate saturation. At this step phycocyanin got precipitated and improves the purity ratio to a considerable level and increased to 2.21.

Different workers have reported different purity values ranging from 1.26-3.1 at 50% ammonium sulfate saturation in *Calothrix, Phormidium, Oscillatoria, Lyngbya, Aphanozomenon-flos-aquae* and *Arthronema africanum* strains. For further purification removal of phycocyanin is compulsory and for this dialysis was carried out and improved the purity value up to 2.53 that comes under food grade pigment category (Table 7). It was further purified by passing through sephadex G-25 column. This increased purity ratio to 3.9. Phycocyanin less than 4 purity value is not considered pure (especially for applications as a fluorescent label) and further purification is needed (Borowitzka 1994). Therefore the fraction needed further purification. The fractions of sephadex G-25 showing maximum absorption at 615 nm were further passed on sephadex G-100 column. Now the purity value of eluant increased to 4.45. Earlier reports showed purity values of 3.31-3.5 for *Calothrix* (Duerring et al. 1991; Santiago-Santos et al. 2004), 4.43-4.52 for *Phormidium* (Patel et al. 2005; Soni et al. 2008), 4.59 for *Lyngbya* (Patel et al. 2005) and 4.85 for *Synechococcus* (Abalde et al. 1998). Purity values 4.02-6.13 have been reported for *Spirulina* by different workers (Baussiba & Richmond, 1979; Zhang & Chen. 1999; Minkova et al. 2003; Bhaskar et al. 2005; Patel et al. 2005; Patil et al. 2008). The purity of phycocyanin was further confirmed by absorption spectral and fluorescence spectral scanning (Fig. 2a-2e). The successive purified fractions from each step were run on SDS PAGE and the contaminating protein band disappeared. Only one band (18.5 kDa) corresponding to α subunit was observed (Fig. 3). Both higher and lower molecular weight α subunit were also being reported from various cyanobacteria e.g. 17 kDa in *Calothrix* (Santiago-Santos et al. 2004), *Spirulina* (Patel et al. 2005) and Arthronema

africanum (Minkova et al. 2007), 18.5 kDa in Chrocosidopsis sp. (Hayashi et al. 1997) and *Aphanozomenon-flos-aquae* (Benedditi et al. 2006). 18.9 kDa in *Phormidium* (Patel et al. 2005), 19.5 kDa in *Spirulina fusiformis* (Minkova et al. 2003) and 15 kDa in *Lyngbya* (Patel et al. 2005). Blue colored phycocyanin pigment with single absorption maximum at 615 nm and fluorescence emission at 647 nm, confirmed that purified phycocyanin is C-phycocyanin (www.prozyme.com).

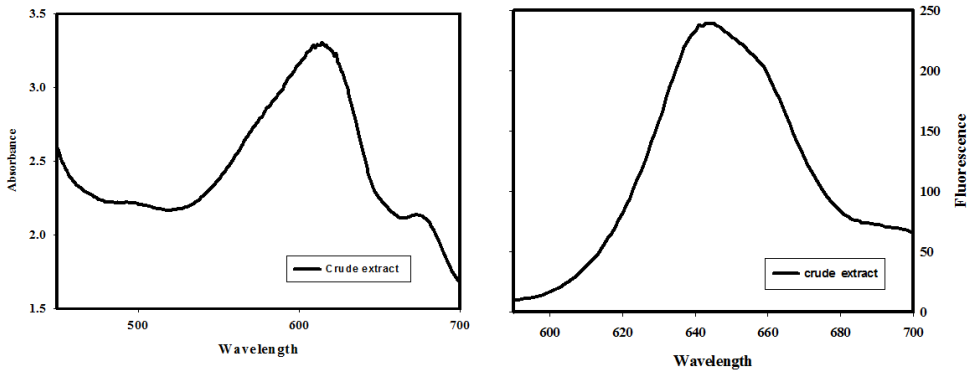


Fig. 2(a) Absorbance and emission spectra of crude extract from *Anabaena*

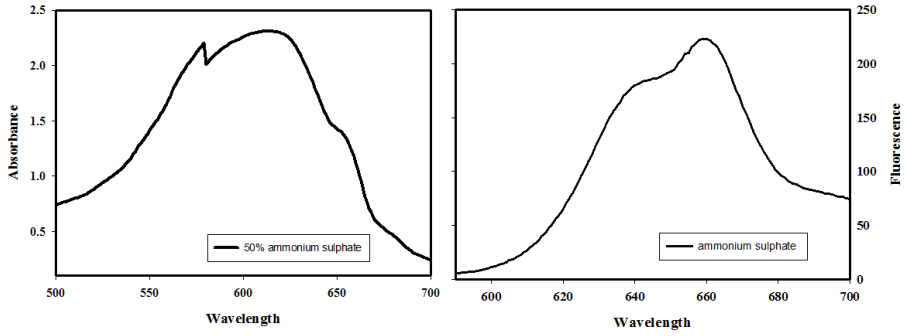


Fig. 2 (b) Absorbance and fluorescence spectrum of 50 % ammonium sulphate from *Anabaena*

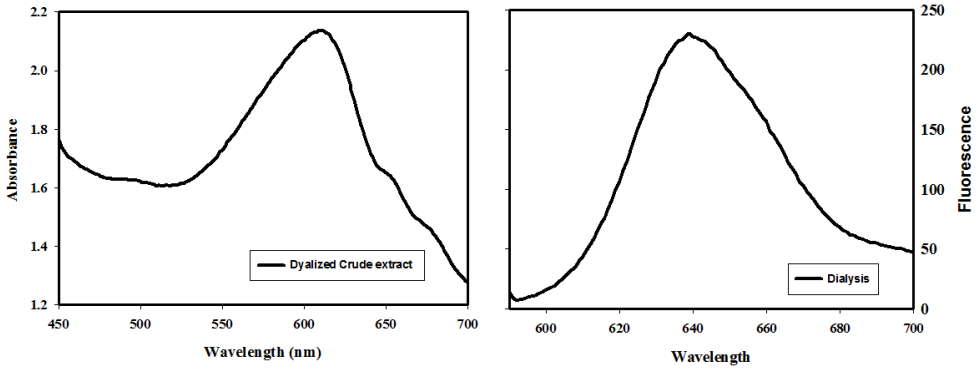


Fig. 2 (c) Absorbance and fluorescence spectrum after dialysis in *Anabaena*

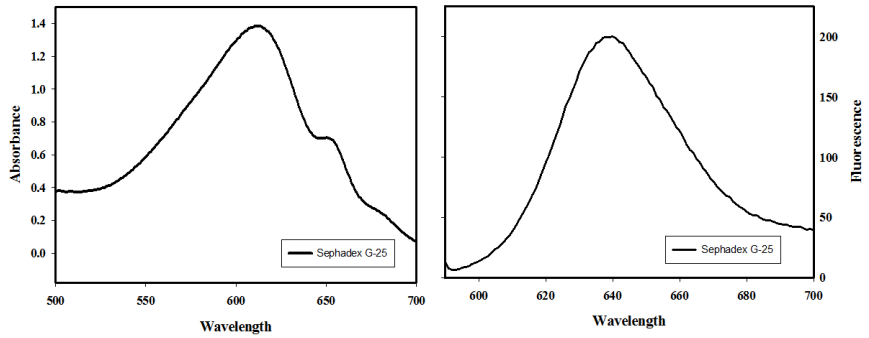


Fig. 2(d) Absorbance and emission spectra of sephadex G-25 from *Anabaena*

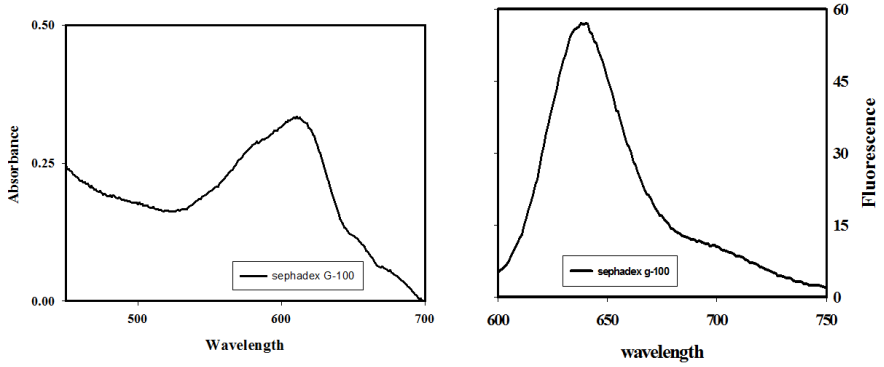


Fig. 2(e) Absorbance and Fluorescence of elution from Sephadex G-100

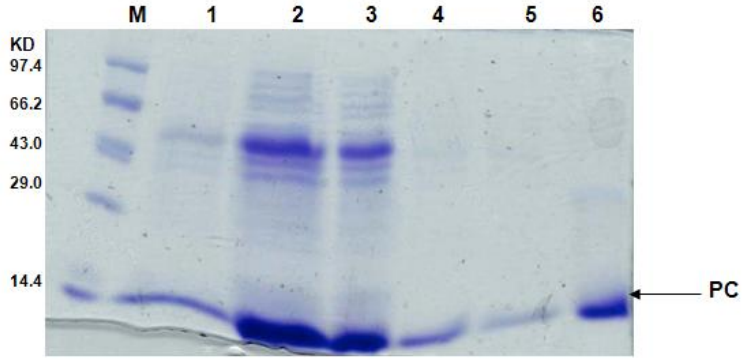


Fig. 3 15% SDS-PAGE at each stage of purification of phycocyanin (PC) from *Anabaena* M: protein molecular mass marker standard; 1: crude extract; 2: ammonium sulphate precipitation; 3: dialyzed protein 4: Sephadex G-25; 5: Sephadex G-100; 6: after lyophilization

Table 7 Determination of spectrophotometric purity of phycocyanin after different steps of purification from *Anabaena sp.*

Steps of Purification	**Phycobiliprotein (mg/ml)**	**Phycocyanin (mg/ml)**	**Purity (A_{615}/A_{280})**	**Total protein (mg/10 g)**
Crude extract	36.54	27.26	0.87	46.42
Ammonium sulphate	25.34	17.36	2.12	38.38
Dialysis	15.72	11.19	2.53	25.67
Sephadex G-25	18.81	12.36	3.24	22.54
Sephadex G- 100	17.11	19.25	4.45	20.26

In conclusion, the present work describes an efficient method of extraction and purification of analytical grade phycocyanin from *Anabaena* NCCU-9. The purity ratio of phycocyanin at the end of the process was 4.45. To our knowledge there is no report of

an extraction and purification of phycocyanin from *Anabaena*.

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