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Production, Extraction and purification of C-Phycoerythrin from marine cyanobacterium, *Phormidium persicinum* NTDP01

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Abstract:

Production, extraction and purification of C-Phycoerythrin pigment from a marine cyanobacterium, *Phormidium persicinum* NTDP01 was carried out in this study. Strain was grown in MN+ medium and molecularly characterized using 16S rRNA gene sequence. Pigments were extracted by means of freeze and thaw method and were characterized by spectral absorbance by UV-Vis spectrophotometer, spectrofluorometer and FTIR. The purity (A565/A280) of crude pigment was found to be 1.5. The crude pigment was concentrated by using a two step Ammonium sulphate precipitation, initially by 25% followed by 60%. It has been increased the purity ratio to 2.9. It was further purified by size exclusion chromatography using Sephadex G-150 column. Finally by purification through column chromatography an yield of 17.5 mg.g^{-1} C-Phycoerythrin with 5.9 purity ratio (*A*568/*A*280). The 16S rDNA sequences were deposited to GenBank with an accession number of KC859032.

Keywords: Cyanobacteria, C-Phycoerythrin, Ammonium sulfate precipitation, Size exclusion chromatography, FTIR.

1. Introduction

Cyanobacteria has worldwide attention for their possible use in mariculture, food, feed, fuel, fertilizer, colorant, production of various secondary metabolites including vitamins, toxins, enzymes, pharmaceuticals, pharmacological probes and pollution abatement (Thajuddin and Subramanian 2005). Phycobiliproteins are a family of light harvesting pigment protein complexes and they are widely found to exist in cyanobacteria, red algae and some cryptomonads. According to light absorption properties, the phycobiliproteins existing in cyanobacteria and red algae are commonly categorized in to four groups (1) Phycoerythrin (PE; λmax = 490- 570 nm); (2) phycocyanin (PC λmax = 610- 625nm); (3) Phycoerythrocyanin (PEC; λmax = 560- 600 nm) and (4) Allophycocyanin (APC λmax = 650- 660 nm). Phycobiliproteins, including phycoerythrin (C-PE), phycocyanin (C-PC) and Allophycocyanin (APC) are a class of chromo-proteins bearing covalently bound linear tetrapyrole chromophores (Glazer and Bryant 1975).

Cyanobacterial phycobiliproteins have gained much importance in the commercial sector, as they have various applications. Phycoerythrin is good for human health, having antioxidant, radical scavenging, anti-inflammatory, hepato- protective and anticancer properties. Other reported applications include, as food additives to enhance the colour of the flesh of Salmonid fish and to improve the health and fertility of cattle (Emodi A1978), synthesis of nanoparticles using C-PE, they could be used to make novel fluorescence probes for the ultrasensitive detection of DNA (Mubarak Ali *et al* 2012). Phycoerythrin is the most widely used in fluorescent probes and has quantum yields of 82-98% (Oi *et al*., 1982). Multinational companies commercializing the antibodies conjugated with are increasing day by day.

Although, several methods have been developed for the separation and purification of C-PE from cyanobacteria , the purity and the recovery is relatively low because , it is highly sensitive to light , oxygen and moisture , hence , it is needed to process it along with efficient preservatives. Due to their limited distribution and the difficulties in their purification, these pigments are rather expensive and obtaining them in pure form is a potentially attractive endeavor. Various method can be employed for extraction and purification of phycobiliproteins. A method that works well in one organism may not be the method of choice for another organism (Ranjith and Kaushik 2005). The present study was focused on the development of a simple protocol to extract and purify the phycoerythrin pigment from a marine cyanobacterium *Phormidium persicinum.*

2. Materials and Methods

2. 1. Culture and Growth:

Phormidium persicinum (NTDP01) was isolated from marine habitat in Gulf of Mannar, Tamilnadu, India.(9.27˚ N ,79.18˚ E). The organism was collected from the in surface of rocks in the sea shore and morphological features were examined by OPTICA simple Microscope. The organism was cultivated in MN^+ medium (Rippka *et a.*, 1979). Culture grown in 3L of MN+ medium having a salinity of 35ppt and pH 7.2 in an Erlenmeyer flask served as the inoculums. It was maintained at 22±1°C, under white fluorescent light (2000 Lux), with a light and dark cycle of 16/8 hours. The medium composition of MN+ was as follows :(chemical: g/l): $MgSo_4$ $7H_2O$ 0.4, K_2HPO_4 $3H_2O$ 0.02, $NaNo_3$ 0.75, $NaCO_3$ 0.02, $CaCl_2$ $2H_2O$ 0.02, Citric acid 3mg/l, Ferric ammonium citrate 3mg/l, EDTA (disodium salt) 5mg/l, Trace metals mix A-5, 1 ml/L. The composition of A5 Trace metal mix was as following

2.2. DNA extraction :

Extraction of genomic DNA from the organism was carried out as outlined by (Smoker and Barnum 1998). 1ml of grown cultures were centrifuged at 10,000 rpm for 5 minutes and the pellet was collected, and washed using STE buffer (NaCl – 50mM; Tris-HCl - 50mM; EDTA - 5mM) and suspended in 500µl of STE buffer. Followed by 20µl of lysozyme (10mg/ml) was added and incubated in water bath at 55°C for 30 minutes. After incubation, 10µl of proteinase K (10mg/ml) and 20µl of 10% SDS was added and incubated in water bath at 55°C for 30 minutes. The mixture was then cooled in ice and extracted with equal volume of phenol: chloroform: Isoamyl alcohol mixture (25:24:1) and centrifuged at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was separated with care and equal volume of 4M Ammonium acetate and two volumes of Isopropanol were added. Mixture was again centrifuged at 14,000 rpm for 15 minutes and the supernatant was decanted and the pellet was washed with 70% ethanol, dried. Finally, the DNA pellet was dissolved in 100µl of TE buffer and stored at 20°C until further use. The loading dye was added to each DNA samples and the samples were transferred to separate wells in 0.8% agarose gel and after loading, the electrophoresis was carried out using 1X TAE buffer at a constant supply of 100 V for 30 minutes. The bands were documented under UV-gel documentation system.

PCR amplification was performed for the respective two samples of purified DNA using CYA 106 (5' - CGG ACG GGT GAG TAA CGC GTGT- 3') and CYA 781 (5' - GAC TAC TGG GGT ATC TAA TCC CA T - 3') primers. The polymerase chain reactions conditions include initial denaturation of template DNA was achieved at 94^{o}C for 2 minutes. Further denaturation was carried out at 94^{o}C for 5 sec; annealing at 47^{o}C for 10 sec, elongation at 72^{o}C for 30 sec and a final elongation at 70^{o}C for 7 min for 40 cycles. Amplified products were isolated by electrophoresis on 1.2% agarose gel using 1X TAE buffer at a constant supply of 100 V for 30 minutes. Again, resolved bands were documented. Sequencing was done with amplified samples with respective forward and reverse primers and sequences were deposited to GenBank.

2.3. C-phycobiliproteins extraction and estimation.

Cyanobacterial culture was harvested with plankton mesh of around 30 microns and washed with distilled water. It was followed by washing with HEPES buffer twice. The buffer washed culture was then subjected to extraction using PBS buffer 0.05M ,pH 7.5. One gram of biomass was resuspended in PBS buffer. Extraction with PBS was done by repeated freezing and thawing until the cell turns green color. A freeze and thaw cycle of 6/2 hours was employed respectively for the complete extraction. After each thawing, the pigments were extracted and filtered using Whatman filter cloth and centrifuged at 10,000 g for 5 mins at 4°C in a cooling centrifuge (Fingfan, china). The supernatant was pooled and treated as crude phycobiliproteins (Phycocyanin, Allophycocyanin. Phycoerythrin). The collected pigment was kept in dark at -10°C. The amount of C-Phycobiliproteins was measured as described by (Bennet and Bogorad 1973) and purity was determined using the following formulae

Purity = OD 562/ OD 280. The Absorbance at 565-568 nm indicated the concentration of C-Phycoerythrin pigment while that the 280 nm was due the whole concentration of protein in the sample.

2.4. Determination of Absorption Spectra.

The absorbance spectra was determined by scanning the sample in the range of 200-800 nm wavelengths by a UV-Vis spectrophotometer(Cary 60, Agilent Technologies, USA).

C-Phycocyanin (mg/ml) = $[A_{620}$- 0 :474(652)]/5.34

Allophycocyanin(mg/ml) = $[A_{652}$ – 0: 208(A_{620})]/ 5.09

C-Phycoerythrin(mg/ml) = [(A_{568} – 2.41(PC) – 0.849(APC)]/9.62

2.5. Purification:

2.5.1. Ammonium sulfate Precipitation

The extracted crude pigment was subjected to a two step precipitation using 20% $(NH_4)_2SO_4$ and kept overnight at -20˚C. Then it was centrifuged at 10,000g for 10 minutes and the supernatant was discarded. The precipitated pellet was subjected to a second step of precipitation using 40% $(NH_4)_2SO_4$ and it was also incubated at -20˚C overnight, then the solution was centrifuged at 10,000g for 5 minutes. The pellet obtained was dissolved in 10 ml of potassium phosphate buffer (0.05M, pH 7.2).

2.5.2. Size exclusion chromatography

Further purification was carried out by means of column chromatography. The precipitated solution dissolved in PBS was loaded on a Sephadex G-150 column (50 cm x 1.5 cm bed height 27 cm). The column was equilibrated at a flow rate of 2ml/min with 0.05M of potassium phosphate buffer (pH 7.2). Colored fractions were eluted and collected in screw cap tubes. Each collected fraction was analyzed by UV – Vis photospectroscopy. Purity of different fractions were analyzed by the A565/A280. Purified Phycoerythrin solution obtained in buffer was dialyzed against distilled water and then stored at -20˚C for further characterization.

2.6. FTIR Analysis :

The purified C-phycoerythrin was characterized by FTIR spectroscopy (Spectrum Two, Perkin Elmer, USA). The IR spectra of purified phycoerythrin was recorded within the range of 4000–600 cm^{-1} to record the IR spectra of purified phycoerythrin.

2.7. Fluorescence emission spectrum:

The fluorescence emission spectrum of purified Phycoerythrin was recorded by using a spectroflurometer with an excitation wavelength of 575nm. All the measurements were recorded at room temperature.

2.8. Results and Discussion

2.8.1. Culture identification

The isolated culture was morphologically identified as *Phormidium persicinum* with the help of standard monographs. Molecular characterization was done by the amplification of 16S rRNA gene. The sequenced were analyzed and deposited in the GenBank database and was given an Accession number KC859032.

2.8.2. Extraction of C-Phycoerythrin

Even though cyanobacteria are known to have various potential pigments the extraction and purification of it assumes great importance. A simple methodology which yields maximum extraction of the phycobiliproteins is very important for the efficient utilization of the products.

Proteins are usually soluble in water because they have hydrophilic amino acids on their surface that attracts water molecules (Mishra *et al*., 2010). Extraction of phycobiliproteins was carried out by means of freeze-thawing. This method is much simpler than many other methods like ultrasound, use of Lysozyme, acetone and Triton-X 100 (Saxena 1988, Techernov *et al*., 1999).

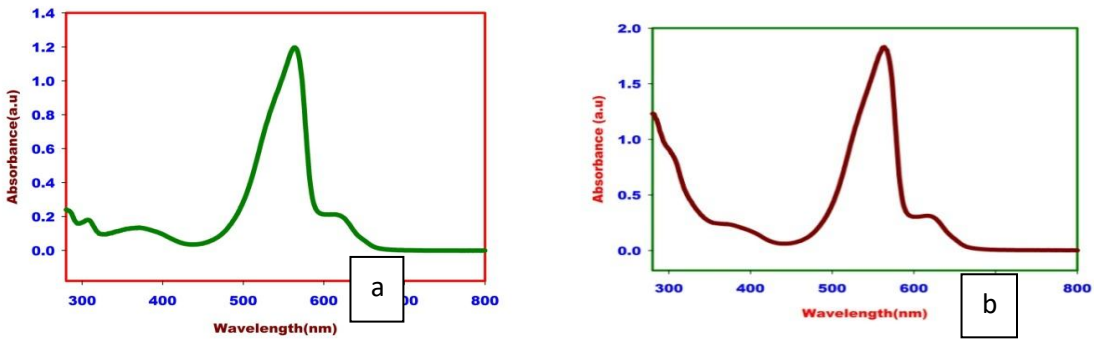
2.8.3. Ammonium sulfate Precipitation

Ammonium sulfate is highly water soluble in low temperature, which prevents the bacterial growth and help the protein purification and concentration. Here ammonium sulphate was used for the precipitation of C-Phycoerythrin which increases the purity of the product. Spectrophotometric purity of C-Phycoerythrin from *Phormidium persicinum* at each stage of purification was analyzed (Table 1). The crude extract as well as the precipitation purified extract also have subjected to purity ratio analysis. The spectrum showing the absorbance of crude extract was depicted and the same extract after the two step ammonium sulfate precipitation initially 25% followed by 60% were shown in (Fig.1) and (Fig.3) respectively. From the purity ratio analysis it was evident that ammonium sulfate precipitation has highly removed the other proteins and increased the purity level. The purity ratio of the crude extract has increased from 1.52 to 2.91 (Table.1). A purity ratio of 0.93 for the crude extract and 2.1 for the purified one has been reported by (Mishra *et al.,* 2011, Hillol Chakdar and Sunil Pabbi 2012) reported a purity ratio of 0.69 for the crude extract and 2.8 for the purified one respectively. Our results found to be superior to all these reports with higher purity ratio of the C-Phycoerythrin.

Table1. Determination of spectrophotometric purity of C-Phycoerythrin from *Phormidium persicinum* NTDP01 at each stage of purification

	Crude extract	1st Ammonium sulfate precipitation	2nd Ammonium sulfate precipitation	Size exclusion chromatography	Ion Exchange Chromotography
Volume of sample	50ml	40ml	40ml	20ml	20 ml
A652	0.2410	0.2130	0.0214	0	0
A620	0.4939	0.4539	0.0954	0	0
A565	0.8474	0.8250	0.7825	0.6589	0.7142
Dilution factor	1:10	1:10	1:10	1:10	1:10
Allophycocyanin(mg/ml)	0.0023	0.0023	0.0020	0	0
C-Phycocyanin(mg/ml)	0.0157	0.0150	0.0110	0	0
C-Phycoerythrin (mg/ml)	0.0841	0.0898	0.0854	0.0875	0.0870
Purity ratio A565/A280	1.05	1.52	2.92	5.95	5.97
Total C-PE	42.05	35.92	34.16	17.5	17.8

Fig.1 Absorption spectrum of C-Phycoerythrin after (a) 60% Ammonium sulfate precipitation (b) 25% Ammonium sulfate precipitation.



2.8.4. Size exclusion chromatography

The C-PE precipitated with 60% saturation was further purified by size exclusion chromatography. It helps to eliminate some high molecular weights, unwanted proteins form the extracted Phycoerythrin if any. Sephadex G-150, size exclusion chromatography column was equilibrated with 50mM phosphate buffer (pH 7.2). The 60% precipitated C-PE was dissolved in 5 ml of buffer and was used for gel filtration chromatography. 2 ml of fraction was collected and further analyzed for its purity. The purity ratio was achieved by this process was 5.9 (Fig.2). The absorption spectra of the purified fraction obtained from the size exclusion chromatography showed low absorption in the region of 280nm, thus reflecting high purity of the PE in the elute 5.9. This ratio also was found to be higher when compared to few other previous reports which have achieved a purity ratio of 5.32 and 4.95 under the same conditions (Mishra *et al*., 2011, Hillol Chakdar and Sunil Pabbi 2012). Total amount of pure phycoerythrin obtained was 17.5 mg/g with a purity ratio of 5.9 (*A*568/*A*280), this is higher than the reported yield 13mg/g (Mishra et al 2011).

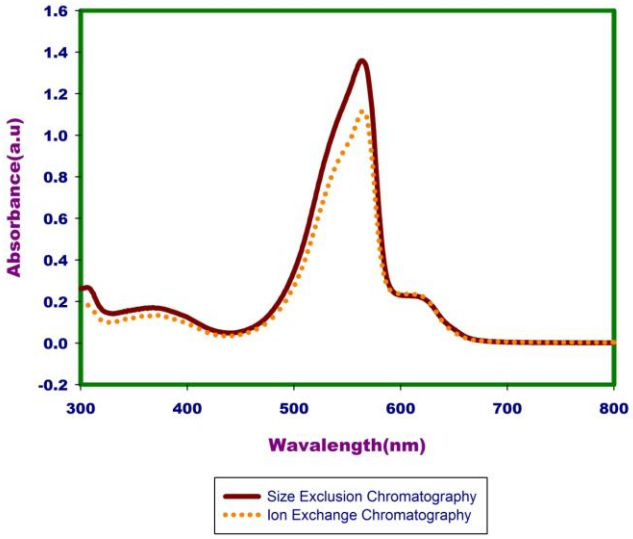


Fig.4 Absorption spectrum of C-Phycoerythrin after Size exclusion chromatography and ion exchange chromatography.

2.8.5. Spectral analysis of C-PE

The absorption spectra of the purified protein showed maxima at 567 nm it showed that the purified Phycoerythrin contained only PEB, while phycourobilin was found to be absent, since at 498 nm no peak was found, which commonly represents phycourobilin. There is also no evidence of either PC and APC, because of there were no peak at 620 and 562 nm. Fluorescence emission spectrum of purified C-Phycoerythrin shows maximum fluorescence at 580nm (Fig.3). PE is highly useful in the laboratory as fluorescence based indicator to mark for the presence of cyanobacteria and also for labeling the antibodies in a technique called immunofluorescence. Phycoerythrin fluoresces in a spectral region that is distinct from the region of emission of the simple organic dyes commonly used as fluorescent indicators generally emit light at 587nm (Malcolm and Julie 1998). The purified C-Phycoerythrin obtained from *Phormidium persicinum* was characterized by FTIR spectroscopy shown Fig.4. IR spectra showed protein specific amide I band at 1637 cm^{-1} (C = O stretching) and 3255 cm^{-1} (N=H bending). The sharp amide band at 1637 and 3255 cm^{-1} for C-Phycoerythrin indicates the alpha helix as the major element of its secondary structure.

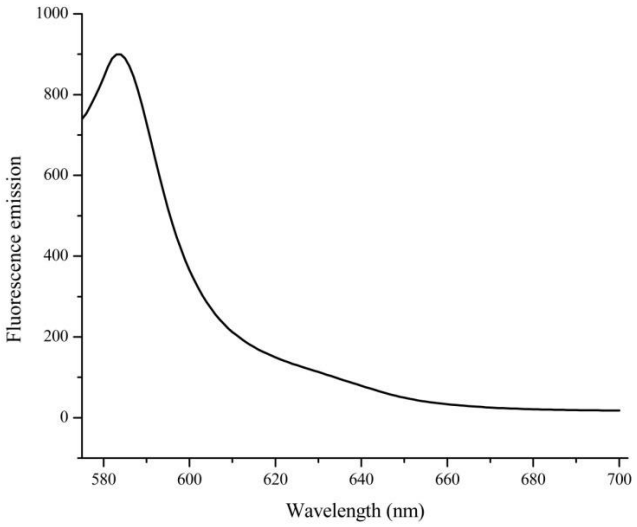


Fig.3 Fluorescence emission spectrum of purified C-Phycoerythrin

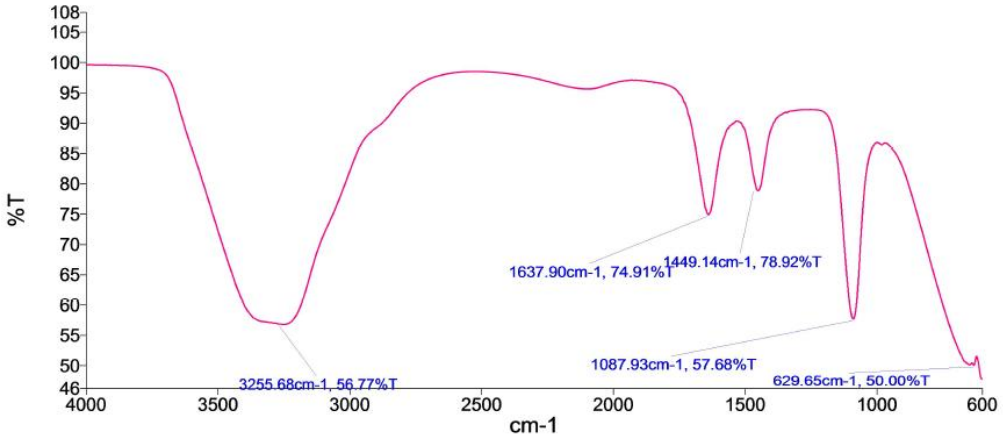


Fig.4 FTIR spectra of purified C-Phycoerythrin from *Phormidium persicinum*

2.9. Conclusion

Amidst a large array of natural products from cyanobacteria, phycobiliprotiens seem to be most colorful and attractive due to their vast biotechnological, industrial and medical applications. In this study, a marine cyanobacterium *Phormidium persicinum* NTDP01 instituted as an alternative potential natural source of C-Phycoerythrin and developed an efficient pigment extraction and purification method without using any extra chemicals like lysozyme, rivonal acetone and TritonX. This cyanobacterial strain has an industrial potential to produce Phycoerythrin because of its filamentous nature, is easier to harvest than the other unicellular cyanobacteria. Freezing and thawing is found to be a better process than other chemical and mechanical processes of extraction of pigments. Further, by purification through column chromatography an yield of 17.5 mg C-Phycoerythrin of 5.9 purity ratio (*A*568/*A*280) from 1 gm dry biomass have been achieved. Selection of strain and feasible method for extraction and purification of pigments hold the key to their exploration for the benefit of mankind. Hence *Phormidium*

persicinum NTDP01 with an inevitable capability of producing C-Phycoerythrin can be considered as a potential candidate for industrial scale production.

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