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Optimization,Purification and characterization of Phycocyanin from *Spirulina platensis*

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Abstract

A method for stably purifying a functional dye, phycocyanin from Spirulina platensis was developed by freezing and thawing method. Among various buffers used for phycocyanin yield efficiency sodium phosphate buffer (pH 7 and 0.1 M) was found to be most suitable for highest yield. During comparison of various buffers, sodium phosphate buffer was found as best buffer with 146 ±0.265 mg/g phycobiliprotein. In the best buffer pH of on phycobiliproteins extraction was also studied and maximum extraction was achieved at pH-7.0 yielding 146 ±0.265 mg/g phycobiliproteins. The isolated phycocyanin from this process also showed highest purity of 3.2 based on absorbance of 0.026 at 280 nm and 0.065 at 615nm. Freezing at –20^{0}C for 3hr and thawing at 4^{o}C for 5min was found to be optimal for highest phycocyanin yield. A polypeptide unit of the phycocyanin was isolated, and their molecular weight was 14 kDa, respectively. These weights are consistent with those of previous reports. This finding suggests that the freezing and thawing process enabled isolation of a stable, highly purified phycocyanin with a short treatment 3cycle (-20^{o}C with time interval 3 h) as 146 mg/g whose value is 8% to 10% higher than is the case from another conventional separation method using phosphate buffer.

I. Introduction

Cyanobacteria are economically important microorganism and have potential to produce a large array of natural products and have a tremendous application in agriculture, industry, pharmaceuticals and biomedical research [Rastogi and Sinha, 2009]. The Cyanobacterium *Spirulina* has been commercialized in several countries for its use in health foods and for therapeutic purposes due to its valuable constituents particularly proteins and vitamins [Benneman, 1988].

Spirulina platensis is an alkaliphilic Halobacteria that lives in tropical and subtropical lakes in Africa and in Central and South America. The growing awareness of importance of natural colours especially food and cosmetic colourants has placed great demand on biological sources of natural colours. 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.

Phycobiliproteins are natural pigments in red and blue-green algae. When purified, they are fluorescent, stable, and water soluble. They could be utilized as a natural pigment for the food, drug and

cosmetic industries to replace the currently used synthetic pigments that are suspected of being carcinogens. Phycobiliproteins are used as colourants in food (chewing gums, dairy products, ice sherbets, jellies, etc.) and cosmetics such as lipstick and eyeliners in Japan, Thailand and China (It was also shown to have therapeutic value by immuno-modulating activity and anticancer activity (Iijima and Shimamatsu, 1982).

Recent studies have demonstrated antioxidant (Miranda et al. 1998) anticancer (Chen et al. 1995; Schwartz et al. 1988), hepato-protective (Gonzalez et al. 1993), radical scavenging (Vadiraja and Madyastha, 2000).

II. Materials and Methods

Organism and culture conditions

The cyanobacterium spirullina 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). 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 30^0C ± 2^0C (Hemlata & Fatma , 2009). Cultures flasks were shaken manually to allow air and nutrients circulation.

Extraction and quantification of phycobiliproteins

Spirullina 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 nm (Varian CARY 500 Scan UV–VIS spectrophotometer) for C-phycocyanin.

Optimization of Phycocyanin 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), sodium phosphate buffer (pH-7.0) of 0.1 M strength, 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. 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), and freezing and thawing (Soni et al. 2006) on phycobiliprotein yield.

An attempt has been made to find out correlation between freezing and thawing temperatures (0 & 4^0C, 0 & 25^0C, 4 & 25^0C, -20 & 25^0C & -20 & 4^0C) 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

1.5 g cells were crushed with liquid nitrogen in pestle and mortar till whole biomass resulted in frozen powder and rapidly thawed by adding 1.5 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 10 % and then till 100 % 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 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. (1970) using 12% 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.

Preparation of sample buffer: 2.0ml of glycerol+2.0ml of 0.5Tris (pH6.8) +0.1ml of SDS+0.2mlmercaptoethanol+0.4m 0f 0.05% bromophenol blue (0.05 gm dissolve in 100 ml distilled water) +2.4ml of distilled water.

III. Result and discussion

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

Among various buffers used for phycocyanin yield efficiency sodium phosphate buffer (pH 7 and 0.1 M) was found to be most suitable for highest yield. (Table1)

During comparison of various buffers, sodium phosphate buffer was found as best buffer with 137 ±0.265 mg/g phycobiliprotein, while double distilled water extracted least 88 ±0.108 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) have also found sodium phosphate buffer (pH-7.0) as best for phycocyanin extraction in *Spirulina.* Sodium phosphate buffer is also being reported to have inhibition.

In the best buffer pH of on phycobiliproteins extraction was also studied and maximum extraction was achieved at pH-7.0 yielding 137 ±0.265 mg/g phycobiliproteins (Table 1). pH above or lower pH-5.0 resulted reductions in phycobiliprotein extraction. Earlier reports of Sarada et al (1999) are 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. 1. Optimization of different buffers for phycoyanin yield .

Different buffer used	Yield of phycocyanin (mg/g)	SD
Acetate buffer (pH-6.0)	98.00	±0.161
citrate buffer (pH-5.0)	104.00	±0.228
sodium phosphate buffer (pH-7.0)	**137.00**	±0.265
double distilled water	88.00	±0.108

Table 2:- Evaluation of extraction efficiency at different time exposure of Freezing and Thawing cycle:

Sample no.	Time period for Freezing(F) and Thawing (T) cycle	Temperature		Time Duration		Extraction efficiency of phycocyanin (mg/g)
		Freezing(F)	Thawing (T)	Freezing(F)	Thawing (T)	
01	1 cycle	-20^{0}C	4^{0}C	1 hr	3 min	102.00
02	2 cycle	-20^{0}C	4^{0}C	2 hr	4 min	107.00
03	3 cycle	-20^{0}C	4^{0}C	3 hr	5min	146.00
04	4 cycle	-20^{0}C	4^{0}C	4 hr	6 min	115.00

Further for eavulation of phycocyanin by using freezing and thawing cycle at different time exposure (1-4 hr) the maximum extraction efficiency was measured for 3 hr freezing and thawing at 5 min .

Standardization of temperature and time period for freezing and thawing has revealed that both play an important role in the extraction process. Freezing at –20^{0}C for 3hr and thawing at 4ºC for 5min was found to be optimal for highest phycocyanin yield.

Freezing and thawing method 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)

Optimization of phycocyanin purity ratio.

The purification of crude extract was done by fractional precipitation using ammonium sulphate with different concentrations from 10% and 100%. It was observed that phycocyanin got precipitated and purity ratio was improved to a considerable level and has been increased to 3.2 times. (Table 3).

Table 3:- **Determination of phycocyanin purity after different steps of purification from *Spirullina platensis*:-**

S.No	Steps of Purification	Phycobilin protein (mg/ml)	Phycocyanin mg/ml	Purity (A615/A280)
1.	Crude extract	46.52	88	0.87
2.	Ammonium sulphate	35.38	67	2.18
3.	Dialysis	25.11	43	2.36
4.	Sephadex G-50	27.09	55	3.2

For phycobilliprotein precipitation ammonium sulfate was used. Protein precipitation using ammonium sulfate 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).

The purification of crude extract involves fractional precipitation with 10% to 100% ammonium sulphate which is particularly useful in salting out unwanted proteins and at the same time to concentrate phycocyanin

Fig 1: - Purified phycocyanin after dialysis

SDS PAGE:-

The successive purified fractions from each step were run on SDS PAGE and the protein bands appeared, one band (14 KDa) corresponding to phycocyanin was observed. In our manuscript polypeptide units of the phycocyanin was isolated, and their molecular weights was 14 kDa, respectively. These weights are consistent with those of previous reports. Moreover, the phycocyanin isolated using the freezing and thawing process separation method had polypeptide subunits that migrated to the same location on the gel and had the corresponding molecular weights as the earlier previous report (Santiago et al, 2004). This finding suggests that the freezing and thawing process enabled isolation of a stable, highly purified phycocyanin with a short treatment 3cycle (-20^{o}C with time interval 3 h) as 146 mg/g **(Table 1)** whose value is 8% to 10% higher than is the case from another conventional separation method using phosphate buffer. The isolated phycocyanin from this process also showed the highest purity of 2.500 based on absorbance of 0.026 at 280 nm and 0.065 at 615 nm. Phycocyanin was found to remain from the original mixtures after being extracted, based on SDS-PAGE analysis, using fewer chemical components and increasing the structural stability of polypeptide subunits.

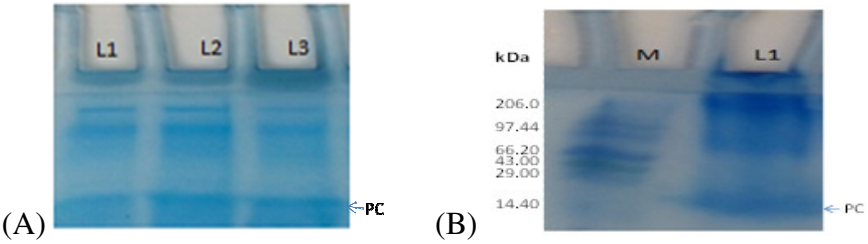

Fig: - 12% SDS PAGE purification of phycocyanin
(A) L1: Crude extract L2: Ammonium sulphate precipitation
L3: Dialyzed protein
(B) M: Protein molecular weight Marker (KDa)
L1: Purified phycocyanin with Sephadex- G 50

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