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Research Article

EXTRACTION OF PHYCOCYANIN FROM SPIRULINA PLANTESIS USING SONICATION

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ABSTRACT

Spirulina platensis is found to be a good source of the phycobiliprotein, Phycocyanin (PC) and is commonly used as a dietary supplement and exhibits various pharmaceutical properties. In the current study, a simple efficient method for extracting PC from Spirulina platensis powder is described. This was done by means of two methods: sonication (using water and Na-Phosphate Buffer) and homogenization method. Extraction using sonication by water proved to be an efficient method. Crude PC obtained was purified using activated charcoal and chitosan, to go along with ammonium sulphate precipitation. Concentration of Phycocyanin yield varied from 0.24 mg/mL to 0.38 mg/mL, whereas extract purity varied from 0.5 to 0.8. Final product was lyophilized to obtain dry powder form of Phycocyanin. Characterization was carried out by performing SDS PAGE which confirmed the molecular mass by observing the bands at 16 kDa for the α-subunit and 18 kDa for the β-subunit. FTIR spectrum showed peaks at different functional groups.

INTRODUCTION

In Cyanobacteria (blue-green algae), the light-harvesting pigments embody chlorophylls, carotenoids as well as phycobiliproteins (Reis *et al*., 1998). The last ones are proteins having linear tetrapyrrole prosthetic teams, known as bilins, found not solely in blue-green algae however additionally in alga and crypto-monads (Bermejo *et al*., 2002). The Cyanobacteria (blue-green alga) Spirulina platensis has existed as a commercialized product in many countries for its utilization as a food and for remedial functions because of its valuable constituents, notably proteins plus vitamins. This\ cyanobacteria algae possess a good assortment of coloured compounds, carotenoids and phycobilliproteins.

Phycobiliproteins are a unit of pigments in cyanobacteria, alga and crypto monads. These proteins may be separated as pigment-protein complexes that are soluble in water and really fluorescent (Abalde *et al*. 1998). They're assembled into particles named phycobilisomes that are a unit hooked up in regular arrays to the external surfaces of the thylakoid membrane and act as major light harvesting pigments in these organisms (Sarada *et al*. 1999)

Phycocyanin is a blue coloured photosynthetic accentuated pigment that absorbs light at a concerning 620 nm and emits light at a concerning 640 nm. It's a phycobiliprotein with a molecular mass of approxminately 110 kDa containing 2 subunits. Unlike different phycobiliproteins (phycoerythrin and allophycocyanin), PC could be a major prominent pigment. (Vonshak 1997). This pigment is historically isolated from Spirulina. Spirulina has been rumored to own variety of beneficial properties, a number of that are attributed to PC. The extraction of phycobilliproteins includes cell break up and let loose of those proteins from inside the cell. (Silveira *et al*. 2007) By considering various vital biological uses of PC, the aim of this study was extraction of phycocyanin from the dry Spirulina powder using a simple yet efficient method and to purify the obtained product.

MATERIALS AND METHODS

Raw materials

The raw material for the entire extraction process, Medilina Spirulina Powder (dry) was procured from NB Laboratories, Nagpur. Each 1 kg of Medilina powder conatins: minerals (6-9%), proteins (55-59%), carbohydrates (15-20%), chlorophyll (1-1.7%), beta carotene (0.15-0.20%), vitamins (0.012-0.2%)

Chemicals used

For extraction and purification

Monosodium Phosphate and Disodium Phosphate, Activated Carbon, Chitosan and Ammonium Sulphate.

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For SDS PAGE

Acrylamide, Sodium dodecyl sulphate, Ammonium per sulphate, TEMED (Tetramethylethylenediamine), Tris Buffer.

Experimental Procedure

Two different extraction methods i.e. sonication and homogenization methods were used for extraction of Phycocyanin (PC) from Spirulina platensis dry powder.

In sonication method, Spirulina powder was taken into distilled water and sodium-phosphate buffer respectively, 1:25 (w/v), was irradiated at 20 kHz for 15 minutes. In homogenization method, Spirulina cells were pounded using a mortar and pestle in the presence of sodium-phosphate buffer at a pH of 7.0 and homogenized at an interval of 24 hours. The slurry resulting from both the methods was filtered and centrifuged at 5000 rpm for 15 minutes at 4℃ to remove the cell debris. The precipitate was discarded and the supernatant crude extract was saved for further purification

Analytical Procedure

The concentration of Phycocyanin (CPC) in terms of $mg.mL^{-1}$ was calculated by determining the optical densities at 652 and 620 nm, using the equation given by Bennett and Bogorad, 1973:

$$\text{CPC} = \frac{(\text{OD}_{620} - 0.474\ \text{OD}_{652})}{5.34}$$

Where CPC = Concentraion of Phycocyanin in $mg.mL^{-1}$, OD_{620} = Optical density at 620 nm, OD_{652} = Optical density at 652 nm The yield of extraction was calculated using the equation by Silveira *et al*., 2007:

$$\text{Yield} = \frac{\text{CPC} * \text{V}}{\text{DB}}$$

Where CPC = Concentraion of Phycocyanin in $mg.mL^{-1}$, V = Volume of solvent in mL, DB = Dry biomass in g.

The extract purity of phycocyanin as defined by Abalde *et. al*., 1998, was given by the equation:

$$EP = \frac{OD_{620}}{OD_{680}}$$

Where EP = Extract purity of Phycocyanin, OD_{620} – Optical density at 620 nm, OD_{680} = Optical density at 680 nm

Optical density at 620 nm signifies the phycocyanin concentration, while that at 680 nm is attributable to the total concentration of proteins in the solution (Liu *et al*., 2005).

Purification

The crude extract obtained was stirred on a magnetic stirrer with activated carbon coupled with chitosan (1%, w/v) for 15 minutes followed by centrifugation at a speed of 5000 rpm for 15 minutes. The supernatant was then collected and afterwards precipitated using ammonium sulphate (10% to 50%). Ammonium sulphate being added gradually, precipitation was done overnight at 4℃. The precipitate was filtered and subjected to lyophilization to obtain phycocyanin pigment. To examine the purity of the obtained pigment and to estimate its molecular mass, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified phycocyanin was performed.

RESULTS AND DISCUSSIONS

Extraction

Spirulina is exploited andsourced as a prominent quality protein predominantly for phycocyanin (Eriksen 2008), which is an essential cyanobacterial accessory pigment allowing a number of engineering applications. Optimizing the extraction along with purification steps is an important requirement to acquire phycobiliproteins from cyanobacterium.

The methods used to extract and purify proteins in general are also useful to apply to phycobiliproteins, but vary from one organism to another. The discharge of Phycocyanin is directly linked to cell rupture, but small algae such as Spirulina have resilient multilayered cell walls, making the extraction process difficult. The experimental results of the Phycocyanin concentration (CPC) and extract purity (EP) as a function of time, are shown in the following tables. The statistical analysis performed with data obtained after 24 hours of extraction showed that there was not any significant increase in the yield after 1 day. During experimentation period concentration of Phycocyanin varied from 0.24 mg/mL to 0.38 mg/mL, while the extract purity differed to some extent from 0.5 to 0.8

Sonication with water

In this method 30 grams of dry spirulina powder was mixed with 750 ml of distilled water and irradiated in a sonicator at 20 kHz for a time interval of 15, 20, 25 and 30 minutes. After sonication, it was kept at 4℃ overnight. Sample was filtered, centrifuged followed by determination of absorbance for concentration analysis. Table 1 shows concentration of extracted phycocyanin at different times.

Table 1 Concentration of Phycocyanin at different time intervals for sonication using distilled water

Sample no.	*Time (min)*	*λmax (nm)*	*O.D at λmax*	*O. D. at λ = 652 nm*	*CPC Concentration (mg/mL)*
1	15	618.2	1.585	0.163	0.2455
2	20	618.2	1.585	0.23	0.2745
3	25	618.2	1.585	0.477	0.2964
4	30	618.2	1.585	0.803	0.3633

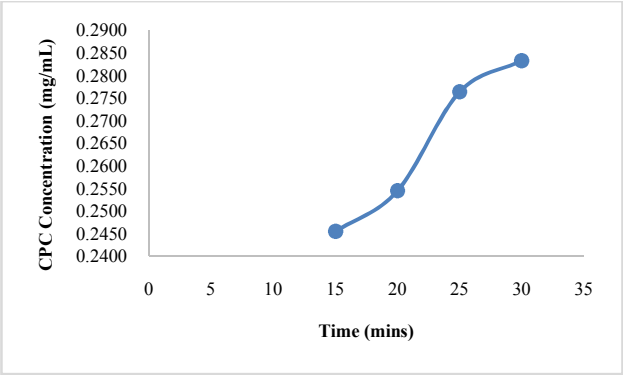

Figure 1 CPC vs time pot for sonication using water

For extraction of phycocyanin by sonication using water, from the results (figure 1), it was observed that concentration of phycocyanin was 0.2455 mg/mL at 15 minutes and it increases with sonication time i.e from 15 mins to 30 mins. At 30 minutes the concentration was 0.3633, after which there was certainly no significant increase in the concentration.

Sonication using sodium phosphate buffer

In this method 30 grams of dry spirulina powder was mixed with 750 ml of sodium-phosphate buffer and irradiated in a sonicator at 20kHz for a time interval of 15, 20, 25 and 30 minutes. After sonication, it was kept at 4℃ overnight. Sample was filtered, centrifuged followed by determination of absorbance for concentration analysis. Table 2 shows concentration of extracted phycocyanin at different times.

Table 2 Concentration of Phycocyanin at different time intervals for sonication using sodium-phosphate Buffer

Sample no.	*Time (min)*	*λmax (nm)*	*O.D at λmax*	*O. D. at λ = 652 nm*	*CPC Concentration (mg/mL)*
1	15	617	1.49	0.112	0.2490
2	20	617	1.49	0.116	0.2587
3	25	617	1.49	0.144	0.2662
4	30	617	1.49	0.272	0.2891

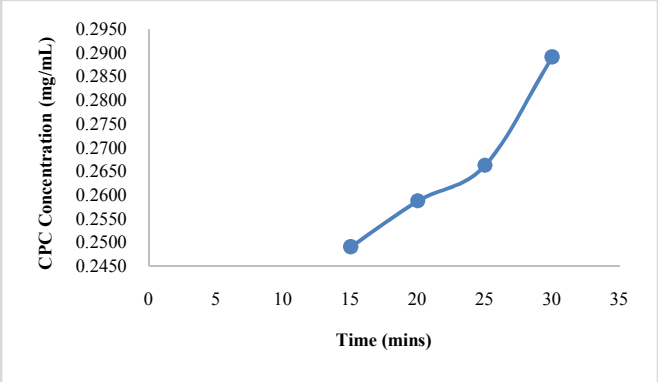

Figure 2 CPC vs time plot for sonication using buffer

For extraction of phycocyanin by sonication using buffer, from the results (figure 2), it was observed that concentration of phycocyanin was 0.2490 mg/mL at 15 minutes and it increased with sonication time i.e from 15 mins to 30 mins. At 30 minutes the concentration was 0.2891, after which there was certainly not significant increase in the concentration.

Homogenization

In this method 30 grams of dry spirulina powder was mixed with 750 ml of distilled water and taken into a mortar and pestle in which it was homogenized for a time interval of 15, 20, 25 and 30 minutes. After sonication, it was kept at 4℃ overnight.

Table 3 Concentration of Phycocyanin at different time intervals for homogenization

Sample no.	*Time (min)*	*λmax (nm)*	*O.D at λmax*	*O. D. at λ = 652 nm*	*CPC Concentration (mg/mL)*
1	15	618	1.561	0.368	0.2287
2	20	618	1.561	0.613	0.2366
3	25	618	1.561	0.628	0.2379
4	30	618	1.561	0.717	0.2597

Sample was filtered, centrifuged followed by determination of absorbance for concentration analysis. Table 3 shows concentration of extracted phycocyanin at different times.

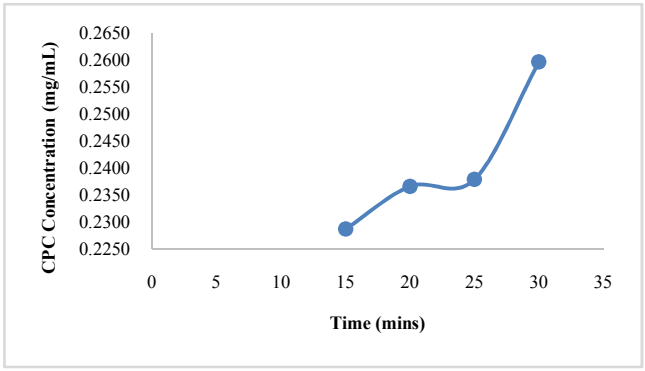

Figure 3 CPC vs time plot for homgeization

For extraction of phycocyanin by homogenization, from the results (figure 3), it was observed that concentration of phycocyanin was 0.2287 mg/mL at 15 minutes and it increased with sonication time i.e from 15 mins to 30 mins. At 30 minutes the concentration was 0.2597, after which there was certainly no significant increase in the concentration.

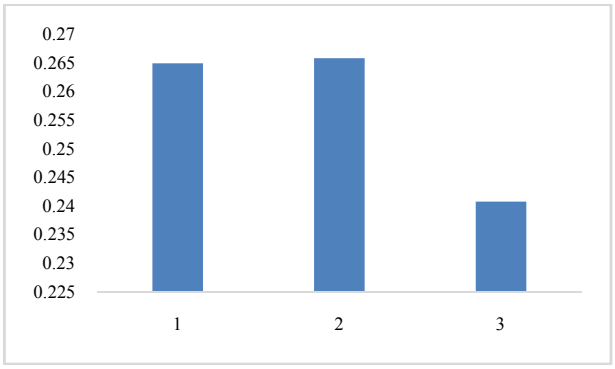

Figure 4 Comparison of extraction methods used in current study

In the cyanobacterium, spirulina plantesis, the disruption of samples homogenized with a mortar and pestle was far-off less efficient than sonication of cells. (Figure 4)

Hence, sonication method was considered to be more effective when compared to homogenization as it gave a better yielded concentration of phycocyanin after an extraction period of 24 hours.

Purification

The purity of extracted phycocyanin was established by using the ratio of the absorbance of sample at 620 nm and absorbance of sample at 680 nm. (Abalde *et al*. 1998)

The absorbance at 620 nm (A_{620}) is relative to the amount of pigment, and that at 280 nm (A_{280}) is relative to some amino acids present in the proteins in the solution. The samples of phycocyanin having an A_{620}/A_{280} ratio more than 0.7 are regarded as food grade. The samples having a ratio of 3.9 are regarded as reactive grade, whereas values more than 4.0 are regarded as analytical grade.

Only a few methods are reported which could achieve analytical grade phycocyanin. It is noticed that crude extract from bacteria 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.

Table 4 Extract Purity of Phycocyanin from (1) sonication with water, (2) sonication with Buffer, and (3) Homogenization

Time (min)	*EP (1)*	*EP (2)*	*EP (3)*
15	0.5847	0.5636	0.5241
20	0.6983	0.6932	0.6471
25	0.7492	0.7581	0.7112
30	0.8899	0.8662	0.8772

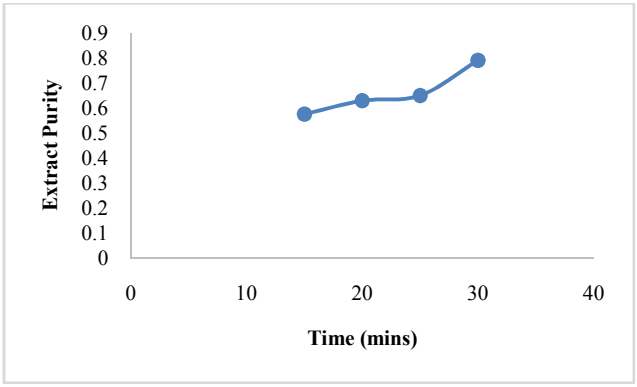

Figure 5 EP vs time plot for sonication using distilled water

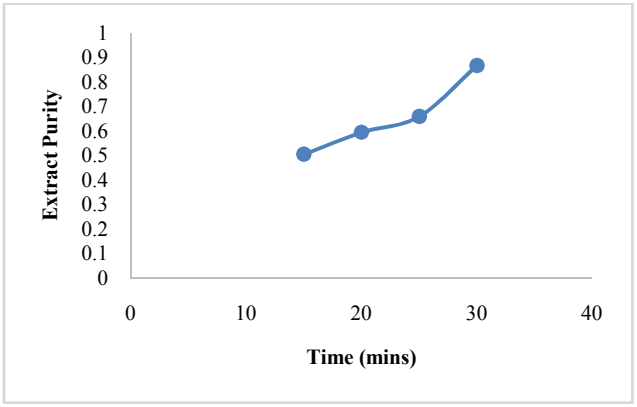

Figure 6 EP vs time plot for sonication using buffer

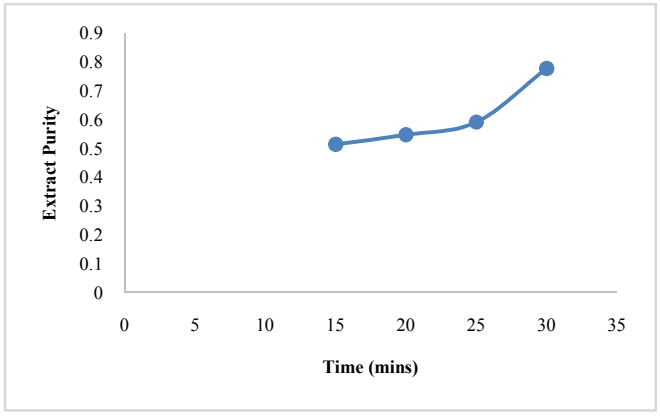

Figure 7 EP vs time plot for homogenization

The above graphs show that the extract purity of phycocyanin increases with increase in extraction time.

The current sample under examination gave an average extract purity of 0.7136 which is nearing the studies carried out by Minkova *et al*. (2007) who found purity value up to 0.8

Confirmation of phycocyanin by observing emission spectrum

Emission spectrums of the samples were recorded from 550 nm to 700 nm in UV-Vis Spectrophotometer. It showed maximum peaks of fluorescence emission at approximately 620 nm, confirming that the extracted product is phycocyanin.

Absorbance spectra of C-PC showed a pick on 620 nm as shown in figures below.

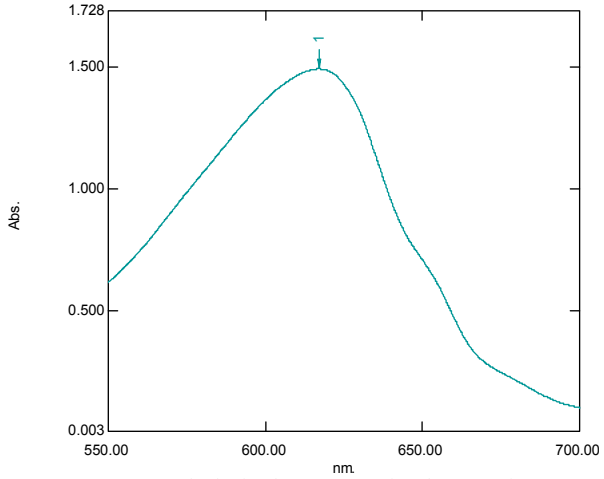

Figure 8 Peak obtained at ~620 nm for phycocyanin

Determination of molecular subunits

To test the purity of phycocyanin pigment, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) of the purified phycocyanin sample was performed.

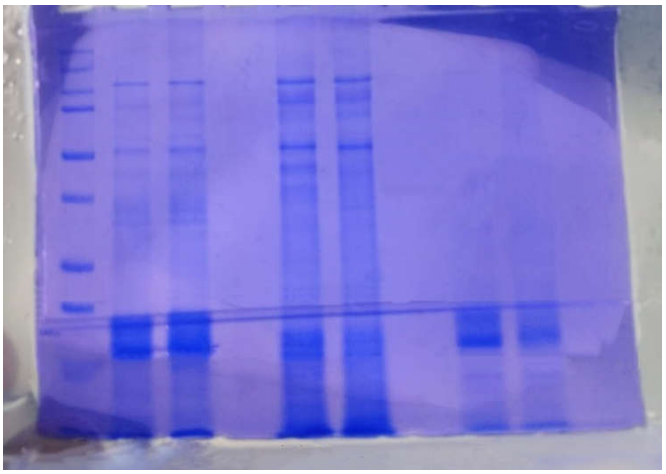

Figure 9 Bands obtained from SDS-PAGE at 16 & 18 kDa corresponding to the two subunits of phycocyanin

The final gel showed two bands representingα and β subunits. As seen in the figure, bands were at ~16 kDa for the α-subunit and ~18 kDa for the β-subunit.

Determination of functional groups

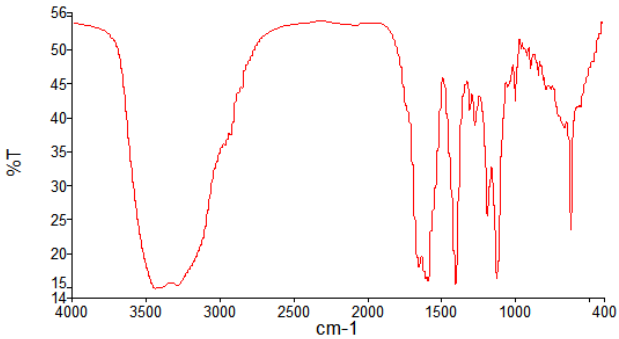

Figure 10 FTIR Spectrum for phycocyanin

FT-R spectrum was recorded in the range of 400-4000 cm^{-1}. The peak observed in the range of 1000 to 1400 cm^{-1} indicates presence of C-O bond. The peak for C-H bond is absorbed anywhere in the range 2800 to 2900 cm^{-1}. The peak found in the range of 1500 to 1800 cm^{-1} gives the presence of C = O bond. The O-H bond is observed in a range of 2500 to 3500 cm^{-1}.

CONCLUSIONS

The current assessment makes it clear that it is convenient to use sonication technique for extraction together with precipitation with ammonium sulphate for purification. The current work illustrates a suitable method for the extraction of phycocyanin from the cyanobacteria Spirulina platensis. Water was selected as the extracting agent, because it yielded high phycocyanin concentration. Moreover, it is a low cost extracting agent. The concentration of extracted Phycocyanin varied from 0.24 mg/mL to 0.38 mg/mL, while the extract purity varied slightly from 0.5 to 0.8.The bands obtained after SDS PAGE for both crude extract of phycocyanin and purified phycocyanin remained identical, hence proving that the molecular structure of phycocyanin continued to be unaffected.

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