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Original Research

# Efficient methods for fast, producible, C-Phycocyanin from *Thermosynechococcus elongatus*

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**ABSTRACT:**

This article describes different protocols that enhance the extraction, isolation and purification of phycocyanin from the cyanobacterium, *Thermosynechococcus elongatus* as well as absorbance and fluorescence spectral characterization. A combination of enzymatic degradation by Lysozyme followed by high pressure showed a mild cell wall destruction except for the composition of thylakoid membrane compared with glass beads. The use of ammonium sulfate precipitation as the first purification step exhibited high efficiency in removing most of the protein contamination. The best purified phycocyanin was obtained after using the second purification step that could be ion exchange chromatography or sucrose gradient. Unexpected results that were not used earlier were obtained by sucrose gradient, where a large amount of highly pure phycocyanin was assembled compared with published methods. An evaluation of C-phycocyanin throughout the series steps of isolation and purification was achieved by using absorbance and 77K fluorescence spectral analysis. Besides a spectroscopical evaluation, SDS-PAGE, productivity, and $A_{620}/A_{280}$ values pointed to the purity and structural preservation of a purified complex. Compared with published methods, the existing method not only reduces purification time but also enhances the productivity of phycocyanin in its native structure.

The optimization of each purification step presented different purified phycocyanin levels; hence, it could be used not only by microbiologists but also by other researchers such as physicians and industrial applicants. In addition, this method could be used as a model for all cyanobacterial species and could be also used for Rhodophytes with some modifications.

**Keywords:**

$A_{620}/A_{280}$ value, C-PC purification, C-Phycocyanin, Cyanobacteria, Fluorescence Spectra, IEC, Phycobilines, Sucrose Gradient, *Thermosynechococcus elongatus*.

**Abbreviations**

**$A_{620}/A_{280}$:** Absorbance at 620 and 280 nm; **Amm Sulf. ppt:** Ammonium sulfate precipitate; **APC:** Allophycocyanin; **MCF-7: Michigan Cancer Foundation-7**, referring to the institute in Detroit where the cell line was established in 1973; **OD:** Optical density., **PBP:** Phycobilliprotein; **PC (C-PC):** Phycocyanin (phycocyanin from cyanobacteria); ***T. elongatus***: *Thermosynechococcus elongates;* **IEC:** Ion exchange column.

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Journal of Research in Biology

## INTRODUCTION

Blue green are one of oldest prokaryotic fossils (Schopf 2000) that have been known on the earth for more than 3.5 billion years. The traditional name 'blue-green algae' for Cyanophyceae is due to the presence of phycocyanin, allophycocyanin, and phycoerythrin, which mask the chlorophyll pigmentation. Most cyanobacteria are found in fresh water, whereas others are found in marines, in damp soil, or even in temporarily moistened rocks in deserts as well as in hot springs such as *Thermosynechococcus elongatus*. *T. elongatus* is considered a thermophilic obligate photoautotrophic organism that contains chlorophyll a, carotenoids, and phycobilins. For this reason, it has usually been used as a model organism for the study of photosynthesis; such as, X-ray structure of PSI and PSII (Sonoike and Katoh 1989; Zouni *et al.,* 2001; Jordan *et al.,* 2001; and Katoh *et al.,* 2001).

In addition, *Thermosynechococcus elongatus* has been postulated as the model organism of choice for structural studies. X-ray of photosystem I are studied by Jordan *et al.,* 2001 and photosystem II are studied by Ferreira *et al.,* 2004 and Loll *et al.,* 2005. A crystal structure of the cytochrome b6f complex has been determined from another thermophilic cyanobacterium, *Mastigocladus laminosus* (Kurisu *et al.,* 2003).

The thylakoid membrane of *Thermosynechococcus elongatus* attached to external light-harvesting structure known as the phycobilisome (PBS; reviewed by Adir 2005), which acts as a light-harvesting system for PSII and, to some extent, for PSI (Rögner *et al.,* 1996). The *Synechococcus elongatus* phycobilisome consists of allophycocyanin (APC) and C-phycocyanin (C-PC), along with the linker proteins (Adir, 2005). The bilin pigments are open-chained tetrapyrroles that are covalently bound to seven or more proteins. These chromophores are composed of the cyclic iron (heme) tetrapyrrole (Frankenberg and Lagarias 2003; Frankenberg *et al.,* 2003).

One function of PC is energy absorbance which is transferred by non-radiative transfer into APC and consequently into chlorophyll a, with an efficiency approaching 100%. In the absence or blocked the photosynthetic reaction center (RC), the PBPs are strongly fluorescent.

C-phycocyanin is composed of two subunits: the α-chain with one phycocyanobilin and the β-chain with two phycocyanobilins (Troxler *et al.,* 1981; Stec *et al.,* 1999; Adir *et al.,* 2001; Contreras-Martel *et al.,* 2007). In between, there are large amino-acid sequence similarities. The αβ subunits aggregate into α3 β3 trimers and further into disc-shaped α6 β6 hexamers, the functional unit of C-PC (Stec *et al.,* 1999; Adir *et al.,* 2001; Contreras-Martel *et al.,* 2007).

Nowadays, Phycocyanin receives a lot of attention due to its potential in medical and pharmaceutical treatments as well as in food industries. Its antioxidant protection of DNA has been demonstrated by (Pleonsil and Suwanwong, 2013). It also promotes PC12 cell survival, modulates immune and inflammatory genes and oxidative stress markers in acute cerebral hypoperfusion in rats (Marín-Prida *et al.,* 2013), prevents hypertension and low serum adiponectin level in a rat model of metabolic syndrome (Ichimura *et al.,* 2013), exhibits an antioxidant and *in vitro* antiproliferative activity (Thangam *et al.,* 2013), and involves an apoptotic mechanism of MCF-7 breast cells either *in vivo* or *in vitro* induced by photodynamic therapy with C-phycocyanin (Li *et al.,* 2010).

For these reasons, a lot of attention is directed toward improving the purification of phycocyanin from several cyanobacterial organisms. The purification of C-phycocyanin from *Spirulina platensis* has been reported by Bhaskar *et al*., (2005); from *Anacystis nidulans* (Gupta and. Sainis 2010); and in aqueous phytoplankton by Lawrenz *et al.,* 2011.

Although all these represented evaluations were based on the ratio of $A_{620}/A_{280}$, which is suggested by

Bryant *et al.,* (1979) and Boussiba and Richmond (1979), this ratio does not save an optimum image of the presence of other impurities such as APC with C-phycocyanin, where the existence of APC does not strongly disturb this ratio. Purity ratios varied among publications: 4.3 (Minkova *et al.,* 2003), 3.64 (Niu *et al.,* 2007), 4.05 (Patil and Raghavarao 2007), 4.72 (Gupta and Sainis 2010), and more than four (Pleonsil and Suwanwong 2013).

This article displays the simple, fast, and effective protocol by which large scales of PC were purified.

## MATERIAL AND METHODS

### Culturing and assembly of *T. elongatus*

*Thermosynechococcus elongatus* cells were cultivated in BG-11 medium at 50 °C with a stream of 5% (v/v) $CO_2$ in air (according to Rippka *et al.,* 1979). Cells were grown in Polyamide flasks (2.5-L). 200-ml preculture cells were used for an inoculation of 2 L culture. The used white light was provided at about 100 $\mu E*m^{-2}*s^{-1}$. After incubation period, the cells were harvested in the exponential growth phase. The optical density at 750 nm was 2.5 - 3.

Cells were sedimented by centrifugation at 2000 g for 15 minutes (GSA-Rotor, Sorvall). The supernatant was removed. Cells in the pellet were washed once with MES buffer (20mM MES, 10 mM Magnesium chloride, and 10 mM Calcium Chloride) and then re-centrifuged at the same speed and conditions.

### Extraction of phycocyanin

The extraction of phycocyanin crude extract was performed in two steps. The first step was cell wall destruction, and the second step was isolation of phycocyanin from the thylakoid membrane. Two destruction techniques were applied. In both techniques, collected *T. elongatus* cells were suspended in 100 ml of MES containing Lysozyme buffer at pH 6.5 (20mM MES, 10 mM Magnesium chloride, and 10 mM Calcium Chloride and 0.2 % (w/v) Lysozyme). Stirring was applied at 37 °C for 30 minutes in the dark condition. In the first protocol, the cell wall was disrupted by applying 2000 psi pressure using Parr bomb at at 4°C for 20 minutes (El-Mohsnawy *et al.,* 2010). However, in the second protocol was done according to Kubota *et al.,* 2010, where *T. elongatus* cells were mixed with an equal volume of glass beads (0.5 mm of Glass Beads, Soda Lime, BioSpec Products), and then, the cells were exposed to 18 disrupted cell cycles (10s ec glass beads break and 2min 50sec pause) on a vortex mixer (BSP Bead-Beater 1107900, BioSpec Products).

Phycocyanin crude extract was collected by suspending the thylakoid membrane with HEPES buffer at pH 7.5 (20mM HEPES, 10mM $MgCl_2$, 10 mM$CaCl_2$, and 0.4 M mannitol) or with HEPES buffer at pH 7.5 containing 0.03% ß-DM and centrifugation at 3000 g at 4 °C for 10 min. The supernatant was collected, and pellets were exposed to an additional extraction step using the same buffer and centrifugation conditions. By using glass bead disruption, an additional isolation step was not required.

### Purification steps

### First purification step:

This step was preceded using two sequences of ammonium sulfate precipitation steps. Ammonium sulfate salts were added to the crude extract in HEPES buffer till it reached 20 %, was stirred at 4°C for 30 minutes followed by centrifugation of 6000 g at 4 °C for 15 min (Beckman -JA-14 Rotor). The pellets were discarded. Additional ammonium sulfate salts were added to the supernatant till they reached 50 % saturation and were stirred at 4°C for 60 minutes. Centrifugation of 12000 g at 4 °C for 30 min (Beckman -JA-14 Rotor) was used to sediment partial purified phycocyanin (El-Mohsnawy, 2013).

**Second purification step:**

Pellets were dissolved in HEPES buffer at pH 7.5 (20mM HEPES, 10mM $MgCl_2$, 6mM $CaCl_2$, and 0.4 M against HEPES buffer at pH 7.5 (20mM HEPES, 10mM MgCl2, 10mMCaCl2, and 0.4 M mannitol) for 6 hours before loading to IEC (POROS HQ/M).

**Sucrose gradient**

Sucrose gradient was prepared by dissolving 20 % (w/v) sucrose in HEPES buffer at pH 7.5 (20mM HEPES, 10mM MgCl2, and 10 mMCaCl2). 12 ml of sucrose solution was poured into each centrifuge tube (SW40-Rotor ultracentrifuge, Beckman) followed by freezing and slowly thawing overnight at 10°C. 100 µl of $OD_{620}$ nm 6 suspensions were slowly dropped onto the top of sucrose gradients. After centrifugation at 36000 rpm for about 12 hours at 4°C (SW40-Rotor ultracentrifuge, Beckman), two identical bands were detected. The lower band (phycocyanin) was collected for further investigation.

**Ion Exchange Chromatography (IEC)**

POROS HQ/M column was used as IEC for the second purification step. The column was equilibrated by 8 CV of IEC equilibration buffer (20 mM MES, pH 6.5, 10mM MgCl2, and 10 mMCaCl2) before loading the phycocyanin suspension. After loading the samples, washing occurred for 5 CV. The gradient from 0 to 200 mM MgSO4 with a step at 35 mM that was carried out for the elution of purified C-phycocyanin complex. Purified phycocyanin was eluted at 23 mM MgSO4. Purified phycocyanin was concentrated by centrifugation at 3000 r/min for 40 min at 4°C using an Amicon 10,000 Dalton weight cut-off.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed according to Schägger and Von Jagow (1987). Briefly, 6 µl of phycocyanin ($OD_{620}$ nm 3) was mixed with sample buffer. Then, the mixture was injected into SDS-PAGE (12% Acrylamide). The electrophoresis was carried out by applying a current of 100 mA for 30 min, and then, the current was reduced to 60 mA until the samples reached the edge of the gel. After electrophoresis, SDS-PAGE was fixed by incubation in a mixture of 50 % methanol and 10% acetic acid for 20 min. The gel was stained with Coomassie Brilliant Blue reagent (0.2% (w/v), Coomassie Brilliant Blue R, 40% (v/v) methanol, and 7 % (v/v) acetic acid) for an additional 20 min. The gel was destained by immersing the gel in a mixture of 30 % (v/v) methanol and 10 % (v/v) acetic acid for 8–12 hours.

**Absorption spectral analysis**

1 ml of crude or purified phycocyanin complexes was diluted in buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 10 mM CaCl2, and 0.5 M mannitol) till it reached a maximum $OD_{620}$ nm of 0.2–0.8 before measuring the absorption spectra from 250 to 750 nm. While thylakoid pellets were diluted to $OD_{680}$ nm of 1.2-2. Two spectrophotometers are used according to the purpose of measurements. For fast evaluation of the efficiency of each purification step, 2 µl of sample was used (NanoDrop ND-1000 Spectrophotometer). 500 µl samples were used in case of Shimadzu UV-2450 or Beckman Du7400. Phycocyanin concentration was estimated according to an equation suggested by Bennett and Bogorad 1973; Bryant et al. 1979:

$$PC\ (mg.ml) = \{A_{620} - (0.7*A650)\}/\ 7.38$$

**Fluorescence emission spectra at 77 K**

Fluorescence emission spectra were performed in an SLM-AMINCO Bauman, Series 2 Luminescence spectrometer (Schlodder *et al.,* 2007). Phycocyanin complex was diluted to $OD_{620}$ nm 0.05 buffer containing 20 mM HEPES, pH 7.5, 10 mM MgCl2, 10 mM CaCl2, and 60 % glycerol. The diluted sample was frozen to 77 K by gradual immersion in liquid nitrogen. 580 nm of actinic light was used for excitation. Fluorescence emission spectra were monitored in the range from 600 to 800 nm with a step size of 1 nm and a bandpass filter of 4 nm.

**RESULTS:**

The purification of phycocyanin from *T. elongatus* cells was achieved via several steps, so the optimization of each step was required to enhance the productivity as well as the purity of phycocyanin. The scheme shown in Figure 1 illustrates the summary steps of extraction and purification of phycocyanin.

**Cell destruction and extraction of crude extract.**

Two different techniques have been used for cell destruction: combination of 0.2 % Lysozyme with pressure (2000 psi) or combination of 0.2 % Lysozyme with glass-beads vortex. 0.2 % Lysozyme with pressure (2000 psi) exhibited mild destruction of the cell wall while keeping the thylakoid membrane in its native structure, even the attached phycobilisomes. After cell destruction, the crude extract was isolated using HEPES (pH 7.5) buffer or HEPES (pH 7.5) containing 0.03 % ß-DM. Both crude extracts exhibited different spectroscopical behavior. On the other hand, glass beads destroyed the cell wall and thylakoid membrane, so centrifugation led to sedimentation of the largest photosynthetic complexes. Figure 2a, b shows the absorbance comparison between Lysozyme + HEPES, Lysozyme + HEPES containing 0.03 % ß-DM, and extraction by glass beads. It is obvious that the use of glass-bead destruction yielded a large amount of allophycocyanin which has a maximum absorbance at 650 nm, in addition to small peaks at 680 nm for PSI and 673 nm for PSII that also have a maximum absorbance of nearly 440 nm. The absorption spectrum at 650 nm proves the contamination of C-phycocyanin by a large amount of allophycocyanin, whereas the absorbance at 280 nm proves the presence of an additional large amount of non-colored proteins. Extraction by HEPES buffer showed a small shoulder at 650 nm, compared with the same buffer containing ß-DM. A remarkable peak at 440 nm and small shoulders were observed at 650 nm and 680 nm in case of HEPES buffer containing ß-DM, which confirmed the contamination with PS (I and II) complexes. It should be pointed out that the high absorbance value of HEPES buffer containing ß-DM compared with other treatments may reflect the ability of ß-DM to dissolve large amounts of protein which do not have absorption spectra in visible regions. However, high contamination of crude extract by allophycocyanin in case of using glass beads did not exhibit a big difference in $A_{620}/A_{680}$ value (Table 1) compared with HEPES extraction.

This is regarding the close of absorption spectra between allophycocyanin and phycocyanin (650 and

**Table 1 a: Summary of purity of phycocyanin (expressed as $A_{620}/A_{280}$ ratio), productivity (expressed as percent to crude extracts), and required periods for each step.**

| Step | $A_{620}/A_{280}$ ratio | Productivity % | Estimation Time |
|---|---|---|---|
| Crude HEPES | 1.02909 ± 0.08229 | 100 | 30.0 min. |
| Crude ß-DM | 0.26732 ± 0.05131 | 100 | 30.0 min. |
| Crude Beads | 1.09185 ± 0.07352 | 100 | 30.0 min. |
| After Amm Sulf. ppt | 3.49497 ± 0.11303 | 92 | 2.0 hours |
| After IEC | 4.51656 ± 0.03006 | 76 | 7.5 hours |

**Table 1 b:**

| Step | $A_{620}/A_{280}$ ratio | Productivity % | Estimation Time |
|---|---|---|---|
| After concentration | 2.59960 ± 0.24710 | 93 | 30.0 min. |
| After Sucrose gradient | 4.40767 ± 0.03941 | 85 | 8.0 hours |

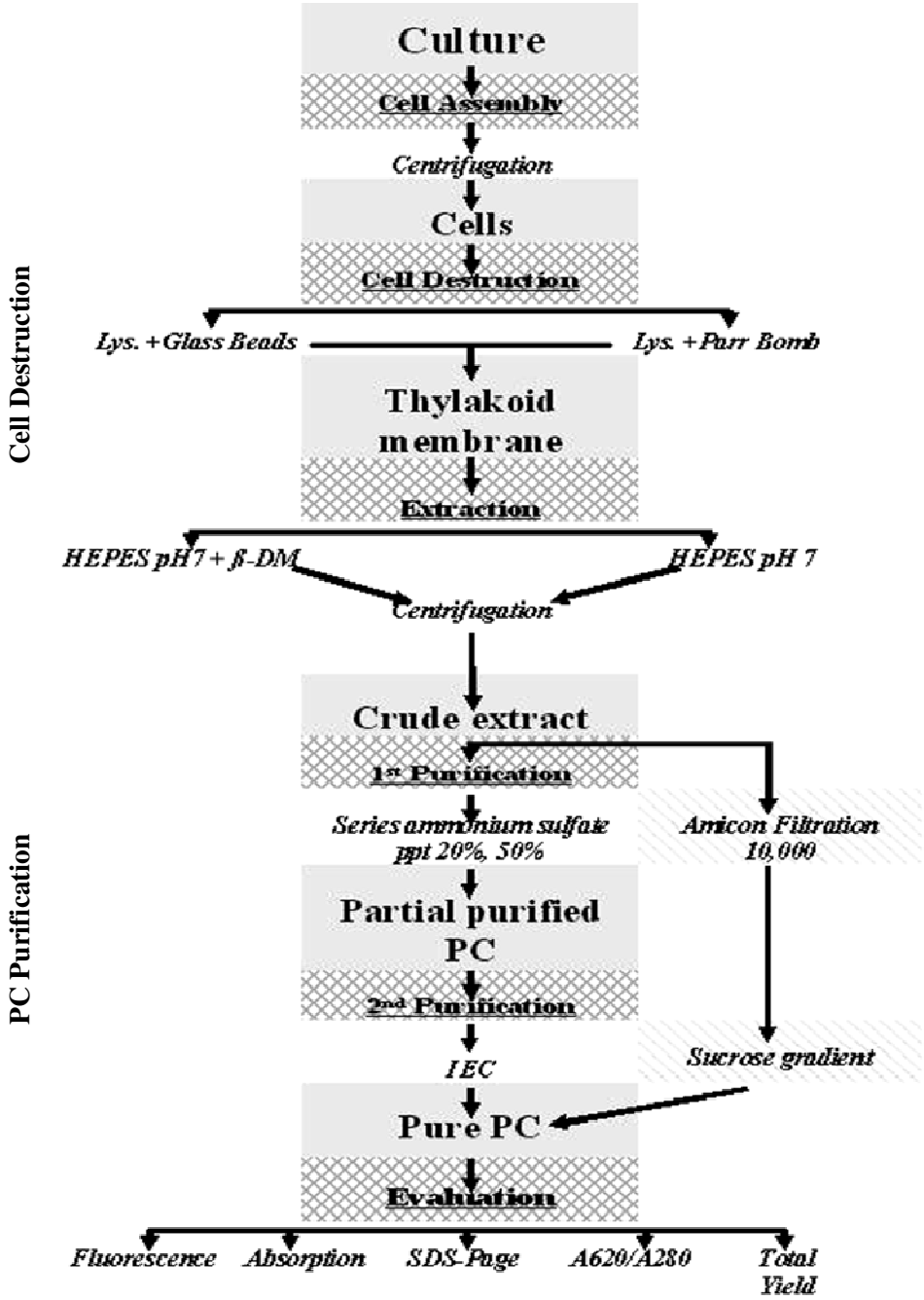


**Figure 1: Scheme shows different isolation and purification steps for phycocyanin purification. During the first purification step, two series of ammonium sulfate precipitation were applied.**

619 nm, respectively). It could be concluded that the extraction with HEPES buffer was the best kind of extraction. Re-dissolving the thylakoid membrane in HEPES buffer not only enhanced the extraction of phycocyanin but also increased the amount of allophycocyanin. Absorption spectra of thylakoid membrane pellets exhibited no significant differences between phycocyanin extracted by HEPES buffer and that extracted by HEPES buffer containing ß-DM, whereas a remarkable reduction was observed in the absorbance at 440 nm and 680 nm in case of extraction by HEPES buffer only (Figure 3a). These results are

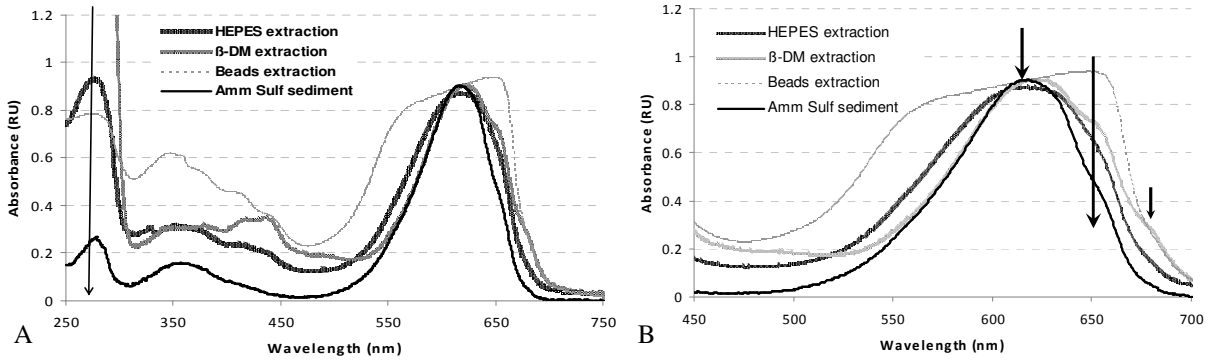


**Figure 2 : Absorption spectra of crude extracts by different conditions and after ammonium sulfate precipitation. 500 µl samples were measured by Shimadzu UV-2450 spectrophotometer. Absorption spectra 250-750 (A), absorbance 550-700 (B)**

supported by 77K fluorescence spectra (Figure 3b), where a high peak was observed at 647 nm for both isolation steps; whereas higher peaks were detected at 664 nm, 686 nm, and 733 nm for PSI. These spectra point to the presence of more allophycocyanin, PSII, and PSI in case of isolation by buffer containing ß-DM.

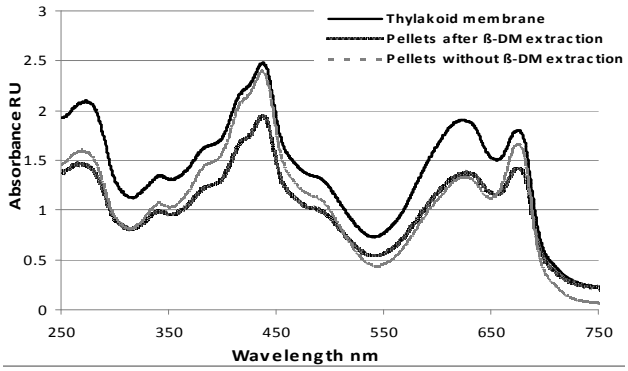


**Figure 3 a: Absorption spectra of pellets after different extraction conditions. Pellets were suspended in HEPES 7.5 buffer till they reached an $OD_{680}$ of 1.5–2. 500-µl samples were measured by a Shimadzu UV-2450 spectrophotometer.**

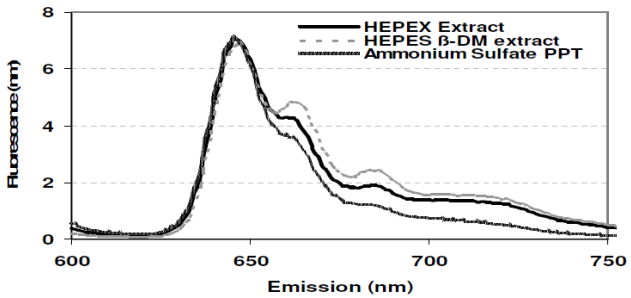


**Figure 3 b: 77K fluorescence emission spectra of different extraction conditions compared with ammonium sulfate precipitation. Samples were diluted with HEPES 7.5 buffer containing 60 % glycerol to $OD_{620}$ = 0.05. The applied actinic light was 580 nm.**

**Purification**

**Ammonium sulfate precipitation**

Phycocyanin crude extract containing other impurities (allophycocyanin, photosystem complexes, and other soluble proteins) was exposed to two series of ammonium sulfate precipitation. In the first step (20% ammonium sulfate), large hydrophobic proteins were sedimented; whereas after the second step, phycocyanin was precipitated. A remarkable reduction in the absorbance at 650 nm, 440 nm, and 280 nm (Figure 2a b) was observed, which proves the high efficiency of these two steps to remove most of the dissolved and large hydrophobic contaminated proteins. These results were supported by $A_{620}/A_{280}$ value (3.494 ± 0.113) as shown in Table 1. This value is considered quite high, indicating the purity of phycocyanin.

Although the absorption spectra and $A_{620}/A_{280}$ value pointed to pure phycocyanin, the emission fluorescence spectra showed the presence of some contamination (Figure 3b), where fluorescence emission spectra at 664 nm and 686 nm were detected apart from 647 nm, which indicates the presence of a few contaminations of allophycocyanin in phycocyanin crude extracts.

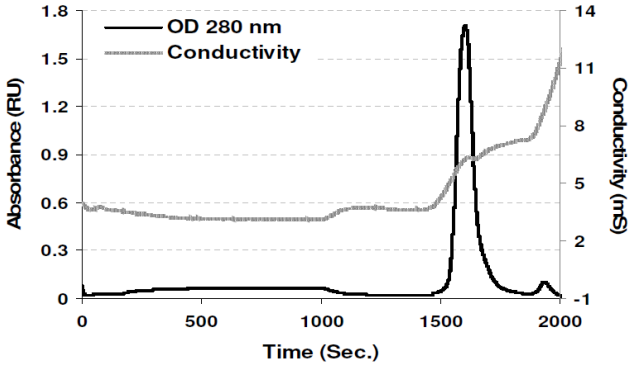


**Figure 4: Elution profile of purified phycocyanin using IEC (Poros HQ/M). The column was equilibrated by 8 CV of HEPES 7.5 buffer before loading. PC was eluted at 35 mM of $MgSO_4$.**

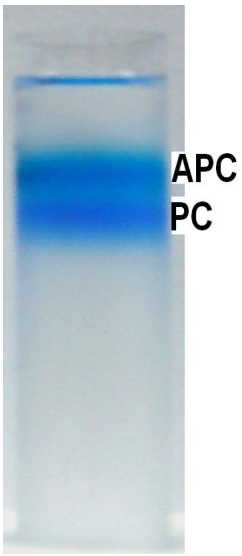


**Figure 5: Sucrose density gradient of concentrated crude extract. 20% sucrose was frozen and slowly thawed at 10 °C. 100 µl of $OD_{620}$ nm 6 suspensions were slowly dropped onto the top of sucrose gradients and centrifuged at 36000 rpm for about 12 hours at 4°C (SW40-Rotor ultracentrifuge, Beckman).**

**Second purification steps.**

Since purification by ammonium sulfate precipitation did not reach an optimum $A_{620}/A_{280}$ value, C-phycocyanin extract needs an additional purification step. A chromatographic step has been applied to reach an optimum value.

**Purification by IEC**

After 50% ammonium sulfate precipitation, the pellet was dissolved in HEPES buffer followed by dialysis against HEPES buffer for 8 hours. Changing of dialysis buffer was done after 2 hours. POROS HQ/M column was equilibrated with HEPES buffer before loading partial purified phycocyanin. Figure 4 shows the elution gradient of $MgSO_4$ (0-150 mM) with a step at 35 mM that was used to elute highly purified phycocyanin. Pure phycocyanin was eluted at 35 mM of magnesium sulfate. Phycocyanin complex was desalted and concentrated to $OD_{619}$ = 3. Quite a high $A_{620}/A_{280}$ value (4.516 ± 0.03) was obtained.

**Purification by sucrose density gradient**

Although the chromatographic purification presented a highly purified and large yield of C-phycocyanin, sucrose gradient was found to be a fast and effective step for the same purpose. Sucrose gradient was prepared as described in the "Materials and Methods" section. A highly contaminated crude extract-derived glass-bead extraction step was concentrated using a 10,000 Amicon tube before being dropped directly onto the top surface of the sucrose gradient tube. After centrifugation, two distinct bands were observed. The lower one was C-phycocyanin, and the upper one was allophycocyanin (Figure 5). The phycocyanin band was collected, washed by HEPES buffer, and concentrated to $OD_{619}$ = 3 before storing it.

**Phycocyanin evaluation of both methods**

Evaluation of the purification of C-phycocyanin did not stop at the level of $A_{620}/A_{280}$ values and total yield, whereas it extended to be investigated spectroscopically and by SDS-gel PAGE. Room temperature absorption spectra of C-phycocyanin purified by IEC and sucrose gradient exhibited almost the same behavior, where only one peak was detected at a maximum absorbance of 619 nm; whereas a reduction in the absorbance at 355 nm and 280 nm was observed. Moreover, the small shoulder at 650 nm disappeared.

77K emission fluorescence spectral investigations of phycocyanin purified by IEC or fractionated by sucrose gradient exhibited only one peak

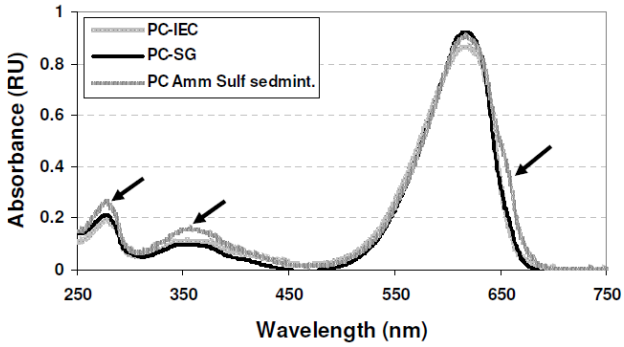


**Figure 6 a: Absorption spectra of purified phycocyanin after ammonium sulfate precipitation, IEC purification, and sucrose gradient. A partial purified phycocyanin was used to visualize the difference at 650 nm. 500-µl samples were measured by a Shimadzu UV-2450 spectrophotometer.**

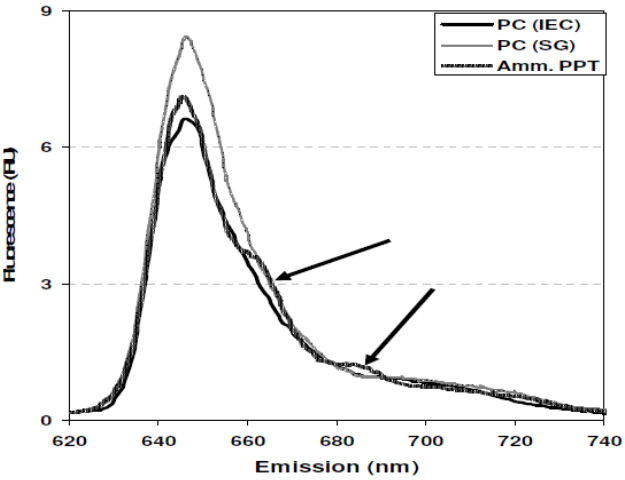


**Figure 6 b: 77K fluorescence emission spectra of phycocyanin purified by ammonium sulfate precipitation, IEC, and sucrose gradient, and these were precipitated by ammonium sulfate. Samples were diluted with HEPES 7.5 buffer containing 60 % glycerol to $OD_{620}$ = 0.05. The applied actinic light was 580 nm.**

at 647 nm; whereas shoulders at 664 nm and 686 nm disappeared (Figure 6b). These results supported absorbance results and indicated the purity of the complex. With regard to the $A_{620}/A_{280}$ value, purification by IEC and sucrose gradient produced 4.5 and 4.4 (Table 1a &b). These values pointed to high-quality C-phycocyanin. As shown in Figure 7, the SDS-gel electrophoresis page, alpha, and beta phycocyanin subunits are visualized without any additional contamination. These results provided high evidence for the efficiency of the presented methods.

A summary evaluation of chromatographic and sucrose gradient methods are shown in Tables 1a and 1b. There were no significant differences in $A_{620}/A_{280}$ values, whereas the total productivity was high in case of the sucrose gradient. In addition, a significant reduction in purification time was observed in case of the sucrose gradient.

## DISCUSSION

The extraction and the purification of C-phycocyanin have been reported for different cyanobacterial species using several steps. These protocols required longer time and more equipment. To reach an optimum PC complex (large amount, pure, and in a short time), the production of C-phycocyanin passed through 2 main steps. The first step was the isolation of PC, and the second one was purification. Each step was monitored spectroscopically in order to achieve high efficiency.

Since the cyanobacterial cell wall is composed of peptidoglycan with an external lipopolysaccharide layer such as gram-negative bacteria, the design of cell destruction is very important, by which the cell wall is destroyed while keeping the thylakoid membrane in its native structure. As shown in the "Results" section, a combination of Lysozyme with 2000 psi was effective and mild. These results were in agreement with Gan *et al.,* (2004) for *Spirulina sp.*, Santos *et al.,* (2004) for *Calothrix sp.*, and Gupta and Sainis (2010) for *Anacystis nidulans*. The use of a combination of Lysozyme and glass beads was very strong and caused the destruction of both the cell wall and the thylakoid membrane, resulting in a huge amount of contamination, especially allophycocyanin. These contaminations extended to include photosystem complexes in case of using a buffer containing ß-DM. It should be pointed out that further

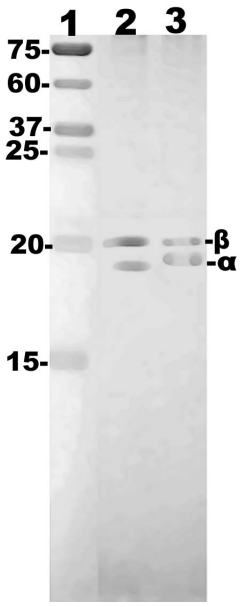


**Figure 7: SDS-gel PAGE of purified phycocyanin. Lane 1 marker protein, lane 2 phycocyanin purified by sucrose gradient and lane 3 phycocyanin purified by IEC.**

extractions by HEPES buffer enhanced the isolation of the remaining C-phycocyanin, in addition to a large amount of allophycocyanin. There was an inverse relationship between the repetition of extraction and PC isolation, whereas a direct relationship has been recorded with regard to allophycocyanin (El-Mohsnawy, 2013). A model in Figure 8 illustrates a comparison between different isolation conditions. It could be concluded that a combination between Lysozyme and high pressure (2000 psi) with HEPES buffer was ideal for phycocyanin isolation with a low contamination. Different C-phycocyanin purification conditions have been widely investigated. A combination of two or more purification steps were usually applied till they reach a high $A_{620}/A_{280}$ ratio. A combination of ultrafiltration charcoal adsorption and spray drying was used to obtain C-PC with $A_{620}/A_{280}$ of 0.74 and a yield of 34%, whereas additional chromatographic steps were included to purify

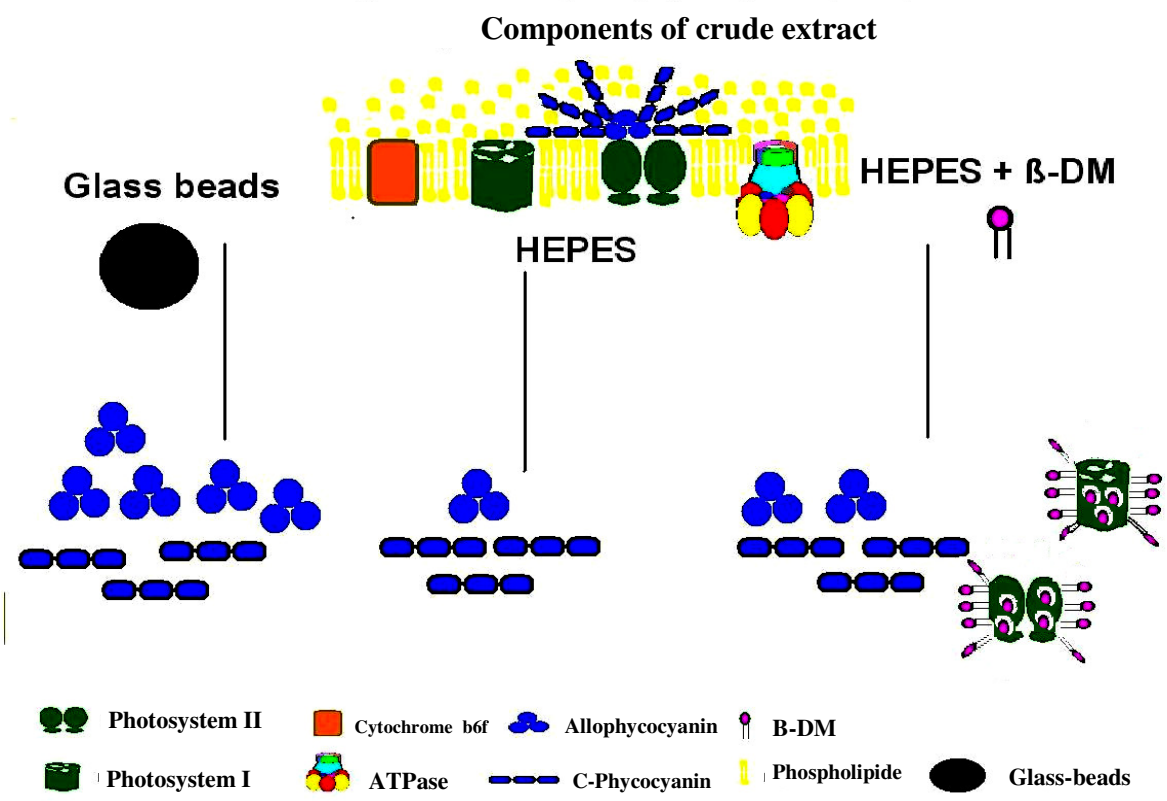


**Figure 8: Model illustrates the major protein isolated as a result of different extraction conditions. This model is based on the results of absorbance and 77k fluorescence spectral analysis.**

C-PC to $A_{620}/A_{280}$ of 3.91 with a yield of 9% (Herrera *et al.,* 1989). This method was improved by Gupta and Sainis (2010) and reached 2.18 and 4.72, respectively. Combination of ammonium sulfate with chromatographic purification has been used for obtaining C-phycocyanin in different purity levels and recommended by Rito-Palomares *et al.,* 2001 and Song *et al.,* 2013. On the other hand, the use of two-phase aqueous extraction followed by chromatographic purification was recently reported by Soni *et al.,* 2008. Although it produced extremely pure C-phycocyanin with $A_{620}/A_{280}$=6.69, the total yield was affected. In the present work, two strategies have been applied. The first one was based on two steps: ammonium sulfate precipitation followed by chromatographic purification (IEC). The second strategy was based on the concentration of crude extract followed by sucrose gradient fractionation. Through concentration of crude extract was considered important not only for concentration C-phycocyanin but also for the removal of the small-molecular-weight soluble protein.

To evaluate this new purification step (sucrose gradient), a highly contaminated PC crude extract (Lysozyme with glass beads) was concentrated by an Amicon 10,000 centrifugation tube and exposed to sucrose density gradient fractionation. The astonishing results were recorded by the sucrose gradient that gave almost the same purity and a much better yield.

After several optimization sequences, it could be recommended that the digestion of *T. elongatus* cell wall by Lysozyme and the exposure to high pressure (2000 psi) followed by PC extraction by HEPES buffer once or twice was found to be the best condition for the isolation of partial pure PC. This crude extract should be concentrated through an Amicon 10,000 centrifugation tube before fractionation by the sucrose gradient. Isolation and purification should be quick, reliable, and efficient; so, absorbance and fluorescence spectra facilitated the purity of C-phycocyanin, thus enabling the optimization of each step. Several advantages of the sucrose gradient method are that it reduces the amount of lost PC complex during purification sequences, produces a highly purified complex ($A_{620}/A_{280}$ value), and reduces time; thus, it could be considered a standard model that is applied in different cyanobacteria species and too simple not to be used by specialists.

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