Purification, Characterization, and Activity Evaluation of Allophycocyanin from *Thermosynechococcus elongatus*

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Abstract: Nowadays treatment of most serious diseases is switched to biological natural products. Most applications use complete organisms (e.g. *Spirulina platensis*). Recently, phycobilins have a lot of attention in pharmaceutical discipline (e.g. anti-oxidant reagent, anti-inflammatory activity, reduces drug induced hepatotoxicity and nephrotoxicity and protective effects on liver and kidney). This manuscript describes for the first time different developed methods for optimum extraction, purification, spectroscopical characterization and activity evaluation of allophycocyanin from *Thermosynechococcus elongatus*. Compared to combination of Lysozyme followed by high pressure (2000 psi), glass beads destruction showed high efficiency in isolation of allophycocyanin crude extract. The use of two series of ammonium sulfate saturation (20% and 50%) was found to be effective pre-treatment for further purification. Simple sucrose gradient exhibited almost the same purity and A_{650}/A_{280} value compared to chromatographic purification of allophycocyanin. Purification by hydroxyl-appetite column showed the best purity and the highest A_{650}/A_{280} value. The fluorescence emission spectra at 77K showed two peaks at 662 nm and 686 nm, which gave strong evidence to presence of trimeric and monomeric form; accordingly it indicates the mild purification methods. The enhancement O_2-uptake by photosystem 1 due to the presence of allophycocyanin proved to the ability of purified allophycocyanin to energy *in vitro*. Compared to the limited published methods, the existing method describes efficient protocols for chromatographic and non-chromatographic purification of allophycocyanin from *Thermosynechococcus elongatus*. The main advantages of presented manuscript are the reduction of the purification time, high purity and omitting sodium azide. Since these methods could be used as a model, the present work paves the way for wide medical applications from other cyanobacterial species.
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1. Introduction

Cyanobacteria are considered one of the oldest known fossils (Schopf, 2002). *Thermosynechococcus elongatus* is an obligate photoautotrophic organism, has an extrinsic light-harvesting structure known as the phycobilisomes (Adir, 2005), which act as a light-harvesting system for PSII and to some extent for PSI (Rögner *et al.*, 1996). Since the relative phycobilin content varied among different species and changing in environmental conditions, routine quantification of these pigments may provide useful information about photoacclimation in the phytoplankton (Kamiya and Miachi, 1984; Hauschild *et al.*, 1991; Lewitus and Caron, 1991; Kana *et al.*, 1992; Sciandra *et al.*, 2000). Due to presence of high amount of phycobilins the green colour is masked. Allophycocyanin (APC) is one of the phycobiliproteins expressed in cyanobacteria, which contains covalently bound chromophores, and thus, they are valuable as fluorescent probes with a molecular weight of 104 kDalton (Hu *et al.*, 2006). APC is made up of alpha and beta subunits and is present as a trimer $(\alpha\beta)3$. Compared to trimeric form, the monomer, $\alpha\beta$, has a lower fluorescence quantum yield. The chemically cross-linked APC trimer is much more stable than the native protein, but still retains comparable spectroscopic properties (MacColl *et al.*, 2003).

The crystal structure of allophycocyanin has been demonstrated by Murray *et al.* (2007), and Marx and Adir (2013), who pointed to presence of allophycocyanin in trimeric form *in vivo*.

In vivo, allophycocyanin acts to absorb energy as well as transfer non-radiative into chlorophyll a, with an efficiency approaching 100%. Because of its high emission quantum yields, allophycocyanin is considered ultra-sensitive fluorescent tracer. It is significantly more sensitive than conventional organic fluorophores and has been used in applications such as flow cytometry, homogeneous FRET assay and immunoassays (Kronick, 1986).

Aqueous extracts from some cyanobacteria species showed antiviral activity (Carlucci *et al.*, 1997; Ayehunie *et al.*, 1998; Fabregas *et al.*, 1999;

Serkedjieva *et al.*, 2000). Nowadays phycocyanin has a lot of attention due to its potential in medical and pharmaceutical treatments. On the other hand, the main uses of allophycocyanin are acting as labelling, where it is conjugated with proteins such as antibodies and other molecules. Recently, allophycocyanin was found to inhibit enterovirus 71-induced cytopathic effects, viral plaque formation, and viral-induced apoptosis (Shih *et al.*, 2003). Kota *et al.*, 2007 pointed to the role of allophycocyanin in controlling hepatitis virus (HCV), where time fluorescence resonance energy driven from allophycocyanin able to inhibit virus core dimerization. Compared to C-Phycocyanin, medical treatments by allophycocyanin are very limited. One of these reasons could be the low amount of allophycocyanin content compared to phycocyanin and / or its purification difficulties. So the aim of present work is to develop methods for optimum purification and characterization of allophycocyanin from *Thermosynechococcus elongatus* with evaluation its ability to energy transfer to *in vitro*.

2. Material and methods

2.1. Cultivation and cells assembly of *T. elongatus*

T. elongatus cells were cultivated in BG-11 medium (according to Rippka *et al.*, 1979) at 50 °C in a stream of 5 % (v/v) CO_2 in air. *T. elongatus* cells were grown in photobioreactors 2.5 L Polyamide flasks. 200 ml preculture cells were inoculated into 2 L culture. White light was provided at about 100 $\mu E*m^{-2}*s^{-1}$. After about 3 days, cells were harvested in exponential growth phase at OD_{750nm} of 2.5-3. Cells were collected by centrifugation at 2000 g for 15 minutes (GSA-Rotor, Sorvall). The supernatant was discarded and the cells were washed once with MES buffer (20mM MES, 10mM $MgCl_2$ and 10 $mMCaCl_2$) and recentrifuged as mentioned before.

2.2. Extraction of allophycocyanin crude extract

Extraction of allophycocyanin crude extract was achieved in three steps; cell wall destruction; the 2nd step was washing to remove as much as possible of C-phycocyanin; and the 3rd step was isolation of allophycocyanin in crude extract. Two effective destruction techniques have been used. In both techniques, collected *T. elongatus* cells were suspended in 100 ml MES buffer pH 6.5 (20mM MES, 10mM $MgCl_2$ and 10 $mMCaCl_2$) containing 0.2 % (w/v) Lysozyme and stirred at 37 °C for 30 minute in dark condition. By 1st techniques, cells were disrupted by applied pressure using Parr bomb at 2000 psi at 4 °C for 20 minute (El-Mohsnawy *et al.*, 2010). After cell destruction, thylakoid membrane was collected by centrifugation at (3000 g, 4 °C for 15 min) followed by washing by HEPES buffer (20mM HEPES, 10mM $MgCl_2$ and 10 $mMCaCl_2$,) and recentrifuged at the same speed and time. Allophycocyanin crude extract was isolated by suspending the thylakoid membrane (Chl. Conc. 0.5 mg/ml) in 60 ml HEPES buffer and homogenized by glass homogenizer for five times. Centrifugation was applied at 3000 g, 4 °C for 15 min. Pellets containing thylakoid membrane was discard and supernatant containing allophycocyanin crude extract was collected for purification. While in 2nd techniques; *T. elongatus* cells were mixed with an equal volume of glass beads (0.5 mm Glass Beads, Soda Lime, BioSpec Products) and then the cells were exposed to 18 cyclic cell disrupted (20sec. each with intervals of 100 sec pause) on a vortex mixer (BSP Bead-Beater 1107900, BioSpec Products) operated at maximum speed. Allophycocyanin crude extract was separated in supernatant by centrifugation at 3000 g, 4 °C for 15 min (Kubota *et al.*, 2010).

2.3. Purification steps

Pre-chromatographic purification: The ammonium sulfate precipitation has been suggested by Rito-Palomares *et al.* (2001); and Song *et al.*(2013) was modified. The crude extract was mixed with ammonium sulfate salts to reach 20 % saturation followed by stirring at 4 °C for 30 minutes. Centrifugation was applied at 6000 g at 4 °C for 15 min (Beckman -JA-14 Rotor). Ammonium sulfate salts were further added to supernatant to reach 50 % saturation and then stirring applied at 4 °C for 60 minutes. Allophycocyanin was sediment by centrifugation at 12000 g at 4 °C for 30 min (Beckman -JA-14 Rotor). Pellets were divide into two parts; the 1st was suspended in HEPES buffer pH 7.5 (20mM HEPES, 10mM $MgCl_2$ and 10 $mMCaCl_2$) for sucrose gradient and ion exchange purification. The 2nd was suspended in MES buffer pH 6.5 (20mM MES, 10mM $MgCl_2$, 10 $mMCaCl_2$ and 400 mM mannitol) containing 1.5 M ammonium sulfate for hydrophobic interaction purification.

Purification by sucrose gradient

Sucrose gradient was prepared by dissolving of 20 % (w/v) sucrose in HEPES buffer pH 7.5 (20mM HEPES, 10mM $MgCl_2$ and 10 $mMCaCl_2$). 12 ml sucrose solution was poured to each centrifuge tube (SW40-Rotor ultracentrifuge, Beckman) followed by frozen and slowly thawed overnight at 10 °C. 100 µl of $OD_{650\ nm}$ 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 upper band (allophycocyanin) was collected, concentrated before further investigations.

Ion Exchange Chromatography (IEC)

Before loading, sample was dialyzed and stirred in HEPES buffer pH 7.5 for 4 hours. Dialyzed buffer was changed 2 times after 1 and 2 hours.

POROS HQ/M was equilibrated by 6 CV of IEC equilibration buffer (20 mM HPEES pH 7.5, 10mM $MgCl_2$ and 10 mM$CaCl_2$) before loading phycocyanin suspension. After loading the samples, washing was applied for 5 CV. The gradient step 0 – 200 mM $MgSO_4$ with 2CV step at 35 mM was carried out for elution the purified allophycocyanin complex. Purified allophycocyanin was eluted at 50 mM $MgSO_4$. It was concentrated before further investigation.

Purification by HIC:

This chromatographic purification step was performed with a PerSeptive Biocad 700 E chromatography system (Applied Biosystems, BioRad), using POROS-50OH column. Column was equilibrated by 5CV equilibration buffer (20mM MES, 1.5M $(NH_4)_2SO_4$, 10mM $MgCl_2$, 10 mM$CaCl_2$ and 0.4 M mannitol) followed by loading crude extract pellets dissolved in 1.5 M ammonium sulfate MES buffer pH 6.5. Column was washed by equilibration buffer for 6 CV before eluting. Allophycocyanin was eluted through gradient of 1.5 to 0 M ammonium sulfate in MES pH 6.5. Pure allophycocyanin was eluted at 950 mM ammonium sulfate. Then it was dialyzed against MES buffer pH 6.5 for 4 hours (Changing buffer was required after 1 and 2 hours) and concentrated before further investigations.

Absorption spectral analysis

1 ml of crude or purified phycocyanin complexes were diluted in buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.4 M mannitol) before measuring the absorption spectra 250 to 750 nm. Two spectrophotometers are used (Shimadzu UV-2450 or Beckman Du7400). Allophycocyanin concentration was estimated according to equation suggested by Bennett and Bogorad 1973.

$$\textbf{APC (mg.ml)} = \{A_{650} - (0.208 * A_{620})\} / 5.09$$

2.4. Evaluation of allophycocyanin

Fluorescence emission spectra at 77 K

Fluorescence emission spectra were performed in SLM-AMINCO Bauman, Series 2 Luminescence spectrometer according to Schlodder *et al.* (2007). Phycocyanin complex was diluted to $OD_{650\ nm}$ 0.05 buffer containing (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 60 % glycerol). The diluted sample was frozen to 77 K by gradually immersing in liquid nitrogen. 580 nm actinic light was used for excitation. Fluorescence emission spectra were monitored in the range of 600 to 800 nm with a step size of 1 nm and a bandpass filter of 4 nm.

Energy transfer (Oxygen-uptake of PS1)

The evaluation of allophycocyanin to energy transfer was recorded using purified PS1 complexes. Trimeric PS1 complex was purified according to El-Mohsnawy *et al.* (2010). O_2-uptake in response to illumination of PS1 using the FIBOX 2 (PreSens) was applied in the presence and absence of purified allophycocyanin. The system was calibrated by using oxygen saturated water as 100 % oxygen and solution of $Na_2S_2O_3$ as 0 % oxygen. The mode using was µMol O_2/L. 3 µg chlorophyll containing PS1 samples were mixed with PS1-activity buffer pH 7.5 (30 mM HEPES, 3 mM MgCl2, 50 mM KCl, 330 mM mannitol and 0.03 % ß-DM), 80 µM dichlorophenolindophenol and 50 µM Na-ascorbate as ED and 0.5 mM methyl viologen and 10 µg/ml allophycocyanin. Mixture was completed into a final volume of 1 ml. The calibration and measurements were done at 30 °C. The reagents were stirred in dark for 2 min before illumination by 2500 µE $m^{-1}s^{-1}$ white light. The average rate of O_2-uptake before illumination was subtracted from that after illumination. The activity of PS1 was calculated using the following equation.

$$\textbf{O}_2\ \textbf{Uptake} = \Delta Sx60x1000/Cx1000 = \mu Mol\ O2\ mg\text{-}Chl^{-1}\ h^{-1}$$

3. Results:

Since purification of allophycocyanin from *T. elongatus* cells required several steps, the optimization of each step was found to be important for enhancement the productivity and reducing the contamination.

3.1 Cell destruction and isolation of crude extract.

Two different techniques was used for cell destruction; by the 1st, cells were incubated in 0.2 % Lysozyme followed by pressure inducing (2000 psi). The 2nd was based to incubation in 0.2 % Lysozyme followed by glass-beads vortex. Since 0.2 % Lysozyme with pressure (2000 psi) exhibited mild destruction, the supernatant was rich by phycocyanin. So washing thylakoid membrane was very important to remove as much as possible from phycocyanin, so it gave a main peak at 618.5 nm and minute shoulder at 652 nm. Figure 1 shows absorption spectra of allophycocyanin crude extract that have been isolated either by Lysozyme with pressure or Lysozyme with glass-beads. Extraction with enough amount of HEPES buffer enhanced isolation of allophycocyanin so the main peak shifted to be at 628 nm and a big shoulder at 652 nm was observed. Very interesting results were obtained when destruction occurred by glass-beads, where large amount of allophycocyanin was observed. Moreover, the main peak was red shifted to be at 657 nm. It could be concluded that: destruction by glass beads was very effective to isolate most of allophycocyanin contents.

3.2. Purification

Pre-purification was found to be very important to remove some contaminated protein

specially photosystem complexes and small soluble protein. During series ammonium sulfate precipitation, large hydrophobic proteins were sediment at 20% ammonium sulfate, while allophycocyanin were separated from small soluble proteins by precipitation at 50%. The main problem was contamination by C-phycocyanin, which was overcome by one of the following purification techniques.

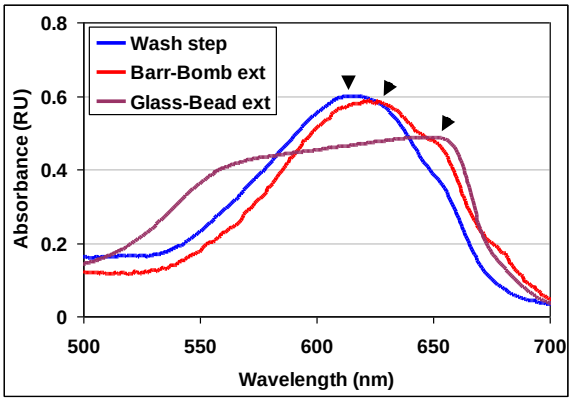

Figure 1: Absorption spectra of crude extracts by different condition. 500 µl samples were measured by Shimadzu UV-2450 spectrophotometer. Absorption spectra 500-700

Purification by sucrose density gradient

Although sucrose gradient is simple separation technique, it exhibited high efficiency in separation of allophycocyanin from other contaminations. Sucrose gradient was prepared as described in material and methods. After centrifugation, two distinct bands were observed. The upper band was allophycocyanin while the lower was C-phycocyanin (Figure 2).

It is important to point that purification by sucrose fractionation showed not only high A_{650}/A_{280} value (4.368) but also quite high productivity (54.64%).

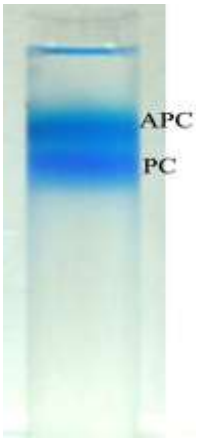

Figure 2: Sucrose density gradient of concentrated crude extract. 20% sucrose was frozen and slowly thawed at 10 °C. 500µl of concentrated crude extract was poured on the tope of sucrose gradient and exposed to centrifugation overnight at 36K in SW40-Rotor ultracentrifuge, Beckman

Purification by ion exchange chromatography

It should be announced that dialysis of allophycocyanin was very important for binding the protein in the column. Figure 3 shows the elution gradient of $MgSO_4$ (0-200 mM) with step at 35 mM, where C-phycocyanin was removed. Pure allophycocyanin was eluted at 50 mM magnesium sulfate. It was clear that 35 mM $MgSO_4$ step was essential for the purity of allophycocyanin. The duration of this step was reversely proportion to existing C-phycocyanin contamination. This method produced high A_{650}/A_{280} value (4.54) with moderate production value (51.35%).

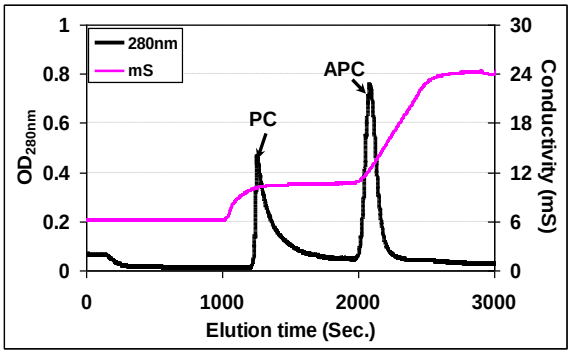

Figure 3: Elution profile of purified allophycocyanin using IEC (Poros HQ/M). Column was equilibrated by 6 CV of HEPES 7.5 buffer before loading. Allophycocyanin was eluted at 50 mM $MgSO_4$. Step at 35 mM $MgSO_4$ was applied for 2CV to remove C-phycocyanin contamination.

Purification by hydrophobic interaction chromatography.

Pellets of partial purified allophycocyanin were suspended MES buffer pH 6.5 containing 1.5 M ammonium sulfate. Figure 4 shows the elution profile of purified allophycocyanin. Unexpected results were obtained under this condition. Allophycocyanin selectively bounded to the column, while C-phycocyanin passed through the column. For this reason, washing the column was found to be very important to remove all contamination. A gradient (1.5-0M) ammonium sulfate was used to elute pure allophycocyanin at 950 mM ammonium sulfate. Compared to other purification methods, HIC was found to be the most efficient one, where the highly purified allophycocyanin was obtained (A_{650}/A_{280} value = 4.74) with high productivity (65.46 %).

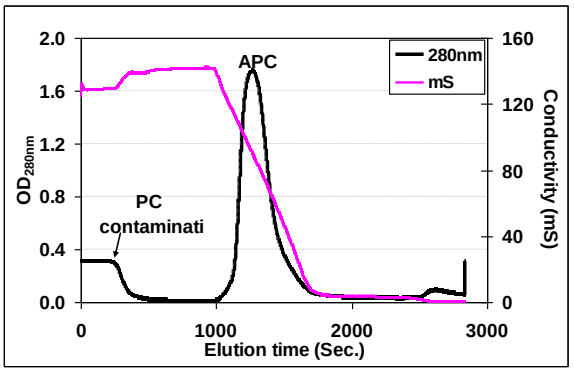

Figure 4: Elution profile of purified allophycocyanin using HIC (Poros 50OH). Column was equilibrated by 5 CV of equilibration buffer. Allophycocyanin (APC) was eluted at 950 mM $(NH_4)_2SO_4$.

3.3. Evaluation purified allophycocyanin

Evaluation of the purification of allophycocyanin did not stop at the level of A_{650}/A_{280} values and total yield, while it extended to include spectroscopical analysis and activity estimation. Room temperature absorption spectra of allophycocyanins purified by sucrose gradient, IEC, and HIC exhibited typical behaviour, where only one peak was detected at a maximum absorbance of 650 nm and shoulder at 596 nm, while, an obvious reduction in the absorbance at 355 nm and 280 nm was observed (Figure 5).

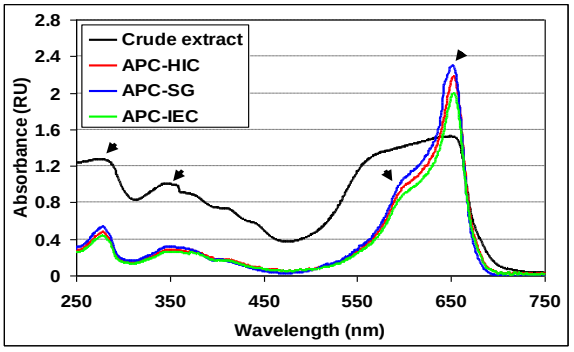

Figure 5: Absorption spectral comparison of purified allophycocyanin after HIC, IEC purifications and sucrose gradient to crude extract. The main peak and small shoulder were observed at 653 nm and 596 nm respectively. 500 µl samples were measured by Shimadzu UV-2450 spectrophotometer.

77K emission fluorescence spectral investigations of allophycocyanins purified by sucrose gradient, IEC, or by HIC exhibited two peaks at 662 nm and 686 nm (Figure 6). Peaks at 650 nm and 735 nm that detected in crude extract were disappeared which reflected the purity of the complex**.** It should be pointed that the fluorescence behaviour differed to that of *Arthrospira platensis***.**

Since the efficiency of these new purification methods should be based on the productivity and purity, these parameters were estimated and summarize in Table 1. A_{650}/A_{280} ratio of crude extract showed low value (1.33) that indicated presence of high impurities. These impurities were discarded after purifications steps to reach 4.37, 4.54 and 4.74 for sucrose gradient, IEC and HIC purification respectively. Productivity of purified allophycocyanin after purification showed high productivity in case if HIC purification (65%) compared to sucrose gradient (54.6%) and IEC purification (51%).

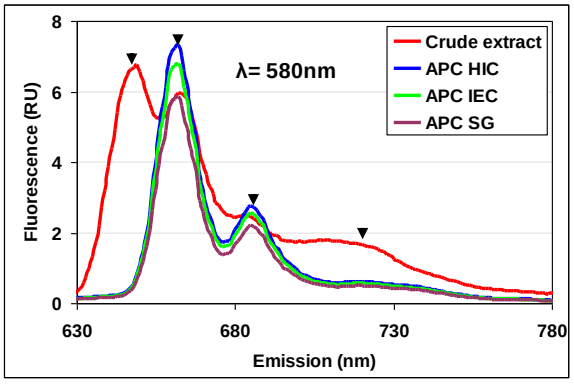

Figure 6: 77K fluorescence emission spectra of phycocyanin purified by ammonium sulfate precipitation, IEC and sucrose gradient and that precipitated by ammonium sulfate. Samples were diluted with HEPES 7.5 buffer containing 60 % glycerol to OD620 = 0.05. The applied actinic light was 580 nm

Evaluation the efficiency of purified allophycocyanin to energy transfer *in vitro* was monitored using the O_2 consumption by PS1 using white light and artificial electron donor and acceptor. O_2 consumption was estimated at light intensity of (2500 $\mu Em^{-1}s^{-1}$). O_2 uptake was jump from 963 µMol O_2/L to 1005 µMol O_2/L in case of absence and presence of allophycocyanin respectively**.**

Table 1: Estimation the purity of phycocyanin (expressed as A_{650}/A_{280} ratio) and the productivity (expressed as percent to crude extracts).

Step	A_{650}/A_{280} ratio	Productivity %
Crude Extract	1.13 ± 0.086	100
After SG	4.37 ± 0.054	54.64 ± 5.1
After IEC	4.54 ± 0.02	51.35 ± 2.78
After HIC	4.74 ± 0.03	65.46 ± 3.24

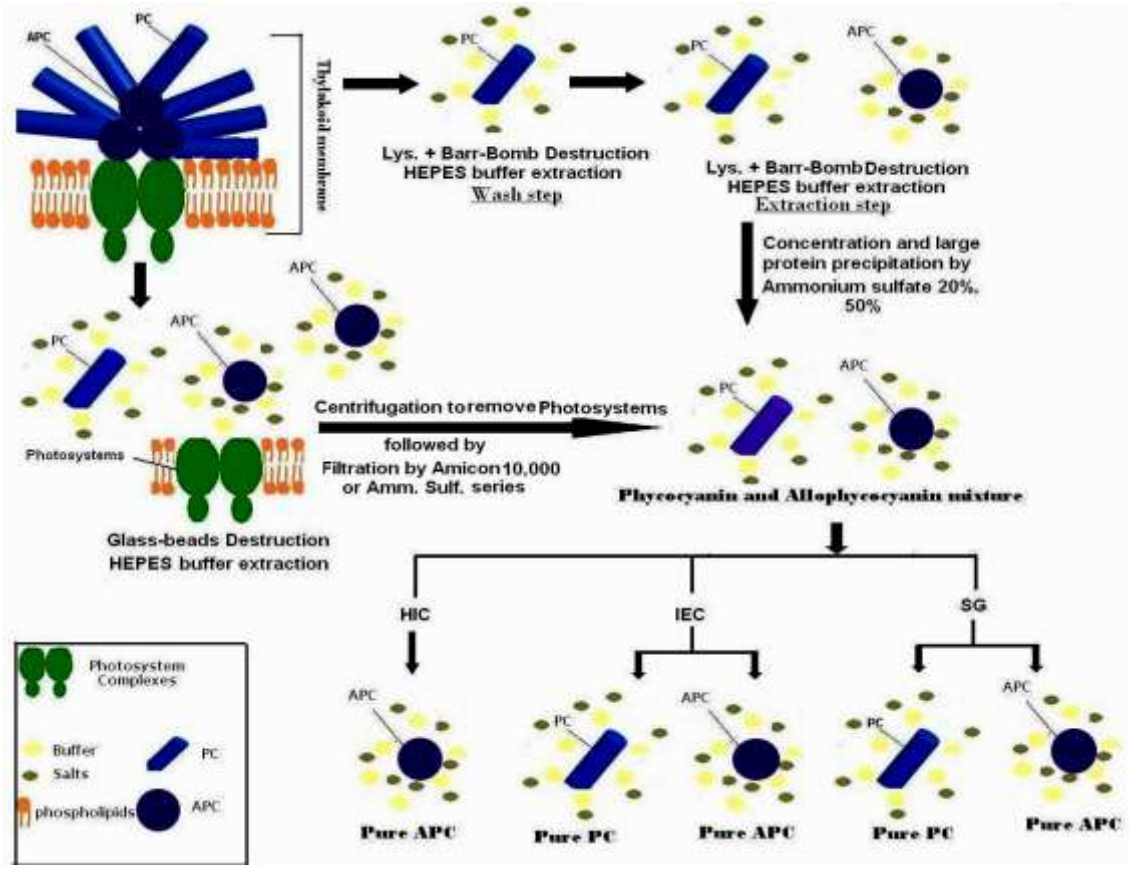

Figure 7: Diagram illustrates the steps of extraction and purification of allophycocyanin from *Thermosynechococcus elongatus*.

4. Discussion

Allophycocyanin is an efficient energy absorbent and converter to photosystem complexes *in vivo*. Since there are limiting publications dealing with the purification of allophycocyanin, this work acted to describe biochemical approach for purification of allophycocyanin from *Thermosynechococcus elongatus*. The main problem was to balance between increasing the isolated allophycocyanin and reduction the other impurities. To achieve optimum allophycocyanin production, the purification of C-phycocyanin passed through 2 main steps. The first step was the cell wall destruction and allophycocyanin isolation, and the second one was purification. Although a combination of Lysozyme with 2000 psi was effective and mild for cell wall destruction (Gan *et al.*, 2004; Santiago *et al.*, 2004; and Gupta and Sainis, 2010), the use of a combination of Lysozyme and glass beads was very effective for allophycocyanin extraction. The main reason was the strongest destruction effect of glass beads that extended to the thylakoid membrane leading to liberation of most allophycocyanin content. The use of Lysozyme with 2000 psi resulted in more phycocyanin and minor amount of allophycocyanin that required additional washing step. A model in Figure 7 summarizes the steps with illustration the amount of allophycocyanin to other impurities based on the spectroscopical analysis. A pre-purification step was required to remove the impurities in allophycocyanin crude extract reaching high A_{650}/A_{280} ratio. Ammonium sulfate precipitation combined with a variety of chromatographic C-phycocyanin purification published by Rito-Palomares et al. (2001), and Song et al. (2013) was modified into two series ammonium sulfate precipitation steps followed by chromatographic purification. A large molecular weight protein was sediment in the 1st step (20%), while allophycocyanin was precipitated leaving other small soluble protein in the 2nd step (50%). Filtration via Amicon 10,000 centrifugation tube was also exhibited a considerable partial purification results. These pre-purification treatments enabled fast and effective purification of allophycocyanin. Chromatographic purification of allophycocyanin from *Arthrospira platensis* have been reported using size-exclusion chromatography by Parmar *et al.*(2010) for, anion exchange chromatography with continuous pH gradient by Yan *et al.*(2011), hydroxylapatite chromatography by Su *et al.* (2010) and Fan *et al.*

(2012). Here, through one chromatographic step or sucrose gradient, a highly purified allophycocyanin have been produced in short time. Evaluation spectroscopically, or A_{650}/A_{280} values pointed to the quality of purified complex. Long isolation and purification period as well as using sodium azide were critical problems in the previous purification of allophycocyanin by Shih *et al.* (2003), which gives the present work obvious advantages. Fluorescence spectra of purified allophycocyanin showed two peaks at 662 nm and 686 nm that evident the presence of monomeric forms. This result came in agreement with Csatorday (1984), who reported the spectrum difference between APC trimer and its monomer is very large. When APC monomers aggregate to trimer, the absorption spectrum has a 40-nm red shift.

Enhancement the O_2-uptake in the presence of purified allophycocyanin indicated the efficiency of allophycocyanin to energy transfer *in vitro*. It could be due to light absorbance at 650 nm, where low chlorophyll absorbance and energy emission at 662 nm and 686 nm where maximum absorbance of chlorophyll.

Finally, it could be recommended that the use of glass-beads followed by series of 20% and 50% ammonium sulfate saturation were found to be successful treatment before purification either simply by sucrose gradient or chromatographic purification. High A_{650}/A_{280} value and enhancement the O_2-uptake proved the purity and activity of purified allophycocyanin.

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