Liquid biphasic flotation for the purification of C-phycocyanin from *Spirulina platensis* microalga

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Abstract

Liquid biphasic flotation (LBF), an integrated process of liquid biphasic system (LBS) and adsorptive bubbles flotation, was used for the purification of C-phycocyanin from *S. platensis* microalgae. Various experimental parameters such as type of phase forming polymer and salt, concentration of phase forming components, system pH, volume ratio, air flotation time and crude extract concentration were evaluated to maximise the C-phycocyanin recovery yield and purity. The optimal conditions for the LBF system achieving C-phycocyanin purification fold of 3.49 compared to 2.43 from the initial LBF conditions was in polyethylene glycol (PEG) 4000 and potassium phosphate combination, with 250 g/L of polymer and salt concentration each, volume ratio of 1:0.85, system pH of 7.0, air flotation duration of 7 min and phycocyanin crude extract concentration of 0.625 %w/w. The LBF has effectively enhanced the purification of C-phycocyanin in a cost effective and simple processing.

Keywords: C-phycocyanin, liquid biphasic flotation; pigment; purification; *Spirulina platensis*

1.0 Introduction

The importance of high-value natural products that can be obtained from renewable source and their nutritional quality have been growing in recent times. Third generation feedstock like microalgae have shown great potential in the generation of valuable products depending on the strain (Chew et al., 2018a; Chew et al., 2018b). The cyanobacterium *S. platensis* has been on the spotlight for its great potential in the production of food and nutritional compounds, namely, proteins, carbohydrates, vitamins, fatty acids and food colorants (Kulshreshtha et al., 2008). One of the major pigments that can be extracted from *S. platensis* is C-phycocyanin (C-PC). C-PC is a natural blue pigment that is part of the phycobiliprotein group in the proteins present in *Spirulina*. C-PC is widely used in food processing and cosmetic industry and possess anti-oxidant, anti-inflammatory and anticancer activity. It can help to modulate lipid and carbohydrate metabolisms and control the occurrence of chronic diseases like obesity, cardiovascular disease and diabetes (Chew et al., 2017; Khan et al., 2005). These benefits have led to researchers putting focus on the development of an efficient process for the commercial extraction and purification of phycocyanin.

The main difficulties in phycocyanin purification involves the great number of processing steps whereby precipitation, centrifugation and dialysis are used in the initial purification, followed by ion-exchange chromatography and gel filtration chromatography in the final purification (Chew et al., 2019; Patil & Raghavarao, 2007). These long steps needed for phycocyanin purification will result in higher product yield loss, apart from posing difficulties in the scale-up process. The major portion of production costs arises from the purification steps, hence, a more efficient and economical large-scale

bioseparation method is required to help elevate the costly conventional processing for phycocyanin purification.

Liquid biphasic system (LBS), also known as aqueous two-phase system (ATPS), is an economical and effective downstream processing that has been widely used in the recovery of bioproducts. This technique is favoured for its simplicity, high extraction efficiency, biocompatibility, ease of operation, rapid processing time and feasibility for scale-up (Lee et al., 2017; Phong et al., 2018). LBS consists of two hydrophilic but immiscible solutions, made using either polymer/salt, alcohol/salt or other combinations. The partitioning behavior of biomolecules such as proteins in LBS can be affected by the type of raw materials used, concentration of the polymer/salt, volume ratio, temperature, pH of the system, biomass concentration and many more (Asenjo & Andrews, 2012; Chia et al., 2018). LBS has been successfully used for the downstream processing of C-PC from *Spirulina*, for example: ATP extraction with PEG and salt (Patil & Raghavarao, 2007), ATPS with ionic liquid and salt (Chang et al., 2018; Zhang et al., 2015), integrated ATPS with cell debris (Antelo et al., 2010) and vortex fluidic device-intensified ATP extraction (Luo et al., 2016). These processes have demonstrated the potential purification of C-PC using multiphase systems which are able to yield high recoveries with low cost operation and are suitable for food processes.

Liquid biphasic flotation (LBF) is an emerging bioseparation process for the extraction and purification of biomolecules (Sankaran et al., 2018). The LBF system is an integration of the conventional LBS and adsorptive bubbles flotation system, where the biphasic system is supported with air bubbling to transport the biomolecules from one phase to another. The surface-active compound of biomolecules present will adsorb onto

the surface of the ascending gas bubbles and be brought from the bottom aqueous phase to the top organic phase. LBF has been applied for the purification of various biomolecules which include lipase from *Burkholderia cepacia* (Sankaran et al., 2018; Show et al., 2013), proteins from microalgae (Chia et al., 2019), betacyanin from *Hylocereus polyrhizus* (Leong et al., 2018) and many more. The LBF which contains the benefits of both LBS and bubbles flotation, has shown to achieve high separation efficiency, ease of operation and environmental friendliness. This indicates that LBF has high prospects for the purification of biomolecules from microalgae.

The aim of this study was to investigate the potential of integrating LBS and adsorptive bubbles flotation system in a combined LBF the purification of C-PC from *S. platensis*. The LBF study was conducted by optimizing the operating conditions and evaluating the yield and separation efficiency of C-PC. The parameters that were investigated include types of PEG and salts, concentration of PEG and salt, volume ratio, pH of the system, air flotation time and crude extract concentration. The molecular weights of the C-PC purified were established through SDS-PAGE tests.

2.0 Materials and methods

2.1 Materials

Dipotassium hydrogen phosphate [K_2HPO_4], potassium dihydrogen phosphate [KH_2PO_4], ammonium sulphate [$(NH_4)_2SO_4$], magnesium sulphate [$MgSO_4$], sodium dihydrogen phosphate [NaH_2PO_4], Polyethylene glycol (PEG) 4000, PEG 8000, phosphate buffer and sodium chloride [NaCl] were purchased from R&M chemicals (Malaysia). PEG 2000 was

purchased from Alfa Aesar (UK). Dried *S. platensis* powder was purchased from Charming & Beauty Co. Ltd. (Taipei, Taiwan). All chemicals used were of analytical grade.

2.2 Preparation of crude phycocyanin extract

S. platensis dried powder were dissolved in phosphate buffer and disrupted using an ultrasonicator (Elma, Germany) at 35 kHz (40% amplitude) for 5 min. The suspension was then centrifuged at 6500 rpm for 10 min. The resulting supernatant was separated from the cell debris and stored at 4°C for further purification studies.

2.3 Liquid biphasic flotation technique

The LBF apparatus used was adapted from the work by Leong et al. 2018 (Leong et al., 2018). The LBF system consists of a glass filter funnel of 20 cm length and 2 cm diameter with a sintered glass disk (Grade G4 porosity) installed at the bottom of the funnel. Air was supplied using a pump and air bubbles of average size 5-15 µm were generated when the air passed through the sintered glass disk. Figure 1 illustrates the schematic diagram and concept of phycocyanin purification from *Spirulina* crude extract using PEG/salt-based LBF. The LBF of predetermined properties was generated by initially adding 300 g/L PEG solution (20 ml) into 250 g/L of potassium salt solution (20ml) premixed with 0.5 %w/w crude phycocyanin extract. The aqueous mixture was aerated with air flow of 0.5-0.75 vvm (volume of gas per volume of medium per min) for a duration of 5 min. The mixture was then transferred to a centrifuge tube and allowed to settle and separate into two phases for 30 min. One-variable-at-a-time (OVAT) approach was used to investigate the effects of different parameters on the purification of phycocyanin from the

microalgae. The operating parameters of the LBF system such as types of PEG, type of salt, concentration of PEG, concentration of salt, volume ratio of salt to polymer, pH of the system, air flow duration and concentration of crude extract were optimized for the phycocyanin purification. The initial settings and variables of the LBF system are listed in Table 1.

2.4 Analytical procedures

2.4.1 Determination of C-phycocyanin concentration

C-PC concentration was determined according to Bennett and Bogorad (1973) (Bennett & Bogorad, 1973) using Equation (1), where CPC is the C-phycocyanin concentration (mg/ml), OD_{615} is the optical density of the sample at 615 nm and OD_{652} is the optical density of the sample at 652 nm:

$$CPC = \frac{OD_{615} - 0.474 \times OD_{652}}{5.34} \qquad \text{Equation (1)}$$

The purity of C-PC (PT) was calculated spectrophotometrically according to Abalde et al. 1998 (Abalde et al., 1998) using Equation (2), where OD_{620} is the optical density of the sample at 620 nm, which represents the maximum absorption of C-PC, and OD_{280} is the optical density of the sample at 280 nm, which represents the total proteins in the solution. This purity is an indication of the pureness of C-PC extract with respect to other contaminating proteins. The purification factor (PF) is calculated by using the purity of the sample divided by the purity of the crude extract (Equation (3)):

$$PT = \frac{OD_{620}}{OD_{280}} \qquad \text{Equation (2)}$$

$$PF = \frac{PT_{top}}{PT_{crude}} \qquad \text{Equation (3)}$$

The partition coefficient (K) was calculated using Equation (4), where CPC_t and CPC_b are the concentration of C-phycocyanin (mg/ml) in the top and bottom phases, respectively.

$$K = \frac{CPC_t}{CPC_b} \qquad \text{Equation (4)}$$

The volume ratio (V_r) is the ratio between the volumes of the top and bottom phases and can be calculated using Equation (5):

$$V_r = \frac{V_t}{V_b} \qquad \text{Equation (5)}$$

The recovery yield (RC, %) of the C-PC from the sample was calculated using Equation (6):

$$RC = \frac{K \times V_r}{1 + K \times V_r} \qquad \text{Equation (6)}$$

2.4.2 Gel electrophoresis

Ultrafiltration units (Amicon Ultra, Millipore) were used for the separation of PEG from the C-PC solution prior to gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Deutscher's method (Deutscher, 1990), using a 30% polyacrylamide slab gel, running in a vertical gel electrophoresis unit. Electrophoresis was run at 50 V, 12.5 mA, for 3–4 h. The gel was

stained with a solution containing 0.05 %w/w Coomassie Brilliant Blue R250 and destained using deionized water.

3.0 Results and Discussion

3.1 Effect of PEG molecular weight

The LBF study was performed using polyethylene glycol of different molecular weights, namely PEG 2000, PEG 4000 and PEG 8000. The phase compositions were set at 300 g/L of polymer and 250 g/L of salt for a system with polymer/salt volume ratio of 1:1. The influence of PEG molecular weight on the recovery yield and purification fold of C-PC are shown in Table 2. PEG 4000 obtained the highest yield (RC) at 81.2% while PEG 2000 obtained the best purification fold (PF) of 2.60. The RC for PEG 2000 was the lowest while for PF the lowest was found at PEG 8000. The increase in PEG molecular weight will lead to less hydroxyl groups present, hence, increasing the top phase hydrophobicity. By adding similar concentrations of polymer, the higher molecular weights of PEG will induce higher chain lengths of the polymer that results in less free volume in the top phase for proteins. This indicates that lower molecular weight of PEG will have more free volume and hence more proteins will be present at the top phase (Patil & Raghavarao, 2007), where a lower concentration of C-PC was found at the top phase of PEG 2000. On the other hand, increasing the PEG molecular weight from 4000 to 8000 facilitated the partitioning of proteins and C-PC to the bottom phase as there are less space for proteins at the top phase, resulting in a lower yield and purity. Higher molecular weight of PEG also has lower solubility and creates a thicker solution that may reduce the effectiveness of the air bubbles from transporting the C-PC to the top phase. The purity of both PEG 2000 and PEG 4000

does not vary significantly but the yield of PEG 2000 is much lower compared to PEG 4000. In view of this, PEG 4000 was selected for further experiments.

3.2 Effect of types of salt

The selection of suitable salts for the purification of C-PC can affect the partitioning of proteins to the top phase. Different salts like ammonium sulphate, magnesium sulphate, dipotassium hydrogen phosphate and sodium dihydrogen phosphate were studied in the polymer/salt LBF. PEG 4000 was the polymer used as the upper phase for all the salt combinations (Table 2). The highest RC was found for ammonium sulphate while the highest purity was obtained by dipotassium hydrogen phosphate. The lowest yield and purity were observed when magnesium sulphate was used as the salt in LBF. It is essential to select the salt which can provide an initial pH that is higher than the pI of C-PC to allow for better partitioning. Ammonium sulphate has been commonly used for the precipitation of proteins in protein and enzymes purification process. The high RC of ammonium sulphate could be attributed to the sulphate ions which interact well with water, hence, forming good hydration capacity and affinity for water that increases protein yield. However, much of the undesired proteins are also partitioned to the top phase, leading to lower purity of C-PC. Both ammonium sulphate and magnesium sulphate produced a system of pH 4.5-5.5, which may have caused the C-PC to be unstable. Phosphate salts, on the other hand, are able to provide a comfortable phase environment (pH 6.0-8.0) for the proteins in LBF system, resulting in better purity of C-PC and good recovery yield. Patil and Raghavarao (2007) reported that among the salts tested (phosphate, sulphate, citrate), phosphate salts showed the maximum purity as well (Patil & Raghavarao, 2007).

Comparing both phosphate salts (Dipotassium hydrogen phosphate and sodium dihydrogen phosphate), dipotassium hydrogen phosphate showed higher RC and PF, hence, the PEG/potassium phosphate system was selected for subsequent experiments.

3.3 Effect of polymer and salt concentration

Changes in the polymer and salt concentration will directly influence the phase composition of the biphasic system. These changes will affect the interfacial tension, density and viscosity of the system due to the variation occurring in solute partitioning. The partitioning of C-PC was studied by adjusting the polymer concentration in the range of 200-400 g/L and the salt concentration in the range of 200-350 g/L. Figure 2(a) shows the effects of polymer concentration on the C-PC recovery yield and purification fold. Increasing the polymer concentration above the initial setting of 300 g/L showed a lower RC and PF. Lowering the polymer concentration down to 200 g/L showed an increase in both the RC and PF for 250 g/L but decreased at 200 g/L. 250 g/L of polymer concentration produced the best RC (86.3%) and PF (2.63). Increasing the polymer concentration will increase the volume of the top phase, which encourages the partitioning of contaminating proteins to the top, this would lead to a lower purity of C-PC compounds in the top phase (Chethana et al., 2015). The higher concentration of polymer also tends to cause foaming during the bubbling process in the LBF system, where the top polymer phase will be reduced as the solution foams at the top. Foaming is less apparent when the lower concentration of polymer is used. The decrease in polymer concentration will result in less free volume available for the protein and hence C-PC will be more concentrated at the top. However, too little free volume would result in the insufficient space for C-PC to be

partitioned to the top. Less polymer concentration also results in lower top phase volume whereby the air bubbles flow through a longer distance of the bottom phase, adsorbing more contaminating proteins to the top phase before dispersing at the surface. Based on the findings, polymer concentration of 250 g/L was selected for the next study.

The concentration of salt was altered while keeping the polymer concentration constant. The effects of salt concentration on the C-PC recovery yield and purification fold are shown in Figure 2(b). The increase in salt concentration up to 350 g/L led to much lower RC and lower PF, on top of that, decreasing the salt concentration to 200 g/L also led to a large decrease in RC and PF. Increase in the salt concentration will decrease the top phase volume, creating less free space for proteins and C-PC, hence, reducing the yield and purity of C-PC. The high concentrations of salt also caused slight precipitation of C-PC. The higher volume of bottom phase would contain more C-PC and this has significantly affected the yield. At lower salt concentrations (200 g/L), the top phase volume is higher, this indirectly also increase the free volume which would be taken by C-PC and the contaminating proteins. Coupled with the gas flotation effect, more contaminating proteins would be brought to the top phase and cause a lower purity of C-PC. Chethana et al. (2015) reported that the increase in salt concentration led to the significant increase in the change in free volume, resulting in increased partitioning of C-PC and contaminating proteins to the top phase (Chethana et al., 2015). Hence, 250 g/L of salt concentration was selected to remain for the subsequent experiments.

3.4 Effect of volume ratio

The phase volume ratio can alter the volume in each phase in a liquid biphasic system, leading to changes in the concentration, yield and purity of biomolecules recovered. The volume ratio of bottom to top phase was adjusted from 1:0.7 to 1:1.5 to observe the concentration of the targeted protein in each phase (Figure 3(a)). As the volume ratio increases, the yield of C-PC increased, while the purity decreased. The highest PF (2.91) was found at the volume ratio of 1:0.85, which showed an 11% increase from the initial setting at 1:1, although the RC dropped slightly by 3.4%. Lower RC and PF were observed in the lowest volume ratio used, and despite having the best RC for volume ratio of 1:1.5, the PF was much lower compared to the volume ratio of 1:0.85. The increase in volume ratio will result in a higher volume of top phase, which allows for both C-PC and the contaminating proteins to concentrate there. This would increase the yield of C-PC but reduce its purity as there will be a lot of contaminating proteins in the top phase. On the other hand, decreasing the volume ratio will lead to lower volume for the top phase and a higher total salt concentration in the system, meaning that there will less free volume for both the C-PC and proteins. The influence of salt on the partitioning is caused by the non-uniform distribution of the salt in both phases through the difference in electric potential. This facilitates the movement of proteins to the other phase through electrostatic attraction or repulsion (Nagaraja & Iyyaswami, 2015). A lower volume in the top phase would also allow for less volume for the ascending bubbles to release the C-PC in the top phase, where the bubbles will likely bring more contaminating proteins from the bottom to the top, leading to a lower purity. The PF of the volume ratio 1:0.85 was the highest, despite its RC being slightly lower. This volume ratio was selected for the further experiments.

3.5 Effect of pH

The extraction pH is one of the vital parameters that produces substantial effects on the efficiency of C-PC separation. The changes in the pH value of the system can affect the net charge of C-PC, the charge distribution on the C-PC surface, zeta potential and conformational structure change (Chang et al., 2018). The pH of the system was adjusted by using various combination of potassium salts to make a final pH in the range of 6-9. The pH values range were selected based on the isoelectric point of C-PC, which is 5.8, as there needs to be a balance between the positive and negative charges of the ionic groups of a protein (Antelo et al., 2010). Figure 3(b) shows the influence of pH of the system on the RC and PF of C-PC partitioning. The highest C-PC recovery and purity was observed at pH 7, with pH 8 and 9 showing comparable purity but slightly lower yields. pH 6 showed the lower yield and purity of the C-PC. The partitioning of C-PC to the top of the system depicts that C-PC is more soluble in the PEG rich phase, where it will be more concentrated depending on the charge-charge interaction. The pH value higher than the pI of C-PC with negative charge will make the C-PC favour the top polymer phase, while the other proteins with positive charges will partition to the bottom phase. Patil and Raghavarao (2007) specified that at pH 6, the partitioning of C-PC was due to the surface properties rather than net charge as the difference of isoelectric point of C-PC and the pH was very small. While for pH of 7 and 8, the C-PC would be negatively charged and thereby attracted by the PEG-rich phase (Patil & Raghavarao, 2007). This means that C-PC would get partitioned to the top phase and the contaminating proteins would go to the opposite phase (bottom phase). Further increase in pH to 9 will lead to C-PC denaturation and conformational changes as

even the *Spirulina* cells would be unable to survive at high pH. This is also supported by the fact that C-PC is mostly found to be stable at pH of 6 to 8 (Antelo et al., 2008). Higher pH will also increase the free volume in the top phase, allowing more contaminating proteins to go to the top. The pH of 7 proved to be the best in terms of RC and PF of C-PC and hence, this pH was selected for the subsequent experiment.

3.6 Effect of air flotation time

The air flotation time contributes significantly in the LBF system as this parameter affects the area of air-water interface per unit volume of aqueous solution with time (Sankaran et al., 2018). Besides that, the size of air bubbles are also worth considering as it relates to the available interfacial area for gas-liquid mass transfer. Extremely small bubbles will coalesce into larger bubbles to overcome the interfacial tension (Bi et al., 2010). In this work, the sintered glass disk of G4 porosity size was used as the performance of this porosity size range (5-15 µm) was found to be suitable and effective for the biphasic flotation study (Show et al., 2013). The aeration rate was set to be around 0.5-0.75 vvm to avoid the foaming of the polymer phase due to high aeration rate. Different durations of flotation time from 1.5 min to 9 min were evaluated for the LBF system on C-PC partitioning. Figure 4 shows the influence of air flotation time of the system on the C-PC recovery yield and PF. The yield of C-PC increases as the flotation time increased from 1.5 min to 5 min, then the yield drops slightly after 5 min of flotation time. The purity of C-PC also observes the same trend, increasing up to 7 min and dropping significantly at 9 min. The highest RC (88.0%) was found at 5 min with PF of 3.09, while the highest PF (3.16) was found for 7 min, with a RC of 86.9%. Increasing the flotation time increases the

transportation of the C-PC from the bottom phase to the top phase, however, too long a duration will also encourage the partitioning of contaminant proteins to the top. Lower flotation time are insufficient for effective C-PC partitioning to the top phase, while higher flotation time showed comparable yields but a reduction in purity of C-PC. Other research works on flotation also showed that extending the flotation time will result in a slight decrease on yield and separation efficiency, indicating that there was no need to extend the flotation time for too long periods (Leong et al., 2018; Phong et al., 2018). Air flotation time of 7 min showed the highest purity and comparable C-PC yield with the initial setting of 5 min, hence 7 min was selected as the suitable flotation time for the next experiment on biomass concentration, where extension of the flotation time beyond 7 min was deemed unnecessary.

3.7 Effect of crude extract concentrations

The partitioning behavior of the proteins will be affected by the amount of feedstock in the system. Crude extract concentrations which are too high will result in a more viscous solution which may cause difficulties in the separation process, while lower crude extract concentration makes the process less effective as the total product recovery would be lower (Chia et al., 2019). The crude extract concentration was investigated in the range of 0.25 to 0.75 %w/w. From Figure 5, the concentration of 0.625 %w/w gives the highest RC (90.4%) and PF (3.49). The increase and decrease in crude extract concentration both showed increasing C-PC recovery but lower purity. The lowest yield and purity was found for concentration of 0.375 %w/w. At lower crude extract concentration, there will be more space available for both C-PC and the undesired proteins, hence the yield is higher

but a drop in purity was observed. Lower crude extract concentration is also not favourable as the production output of C-PC will be lower and this makes the purification process less cost-effective. With increasing crude extract concentration, the yield of C-PC increases, and the purity of C-PC increases as well up to 0.625 %w/w, then it decreases at 0.75 %w/w. The higher concentration of C-PC with higher crude extract concentration requires more work from the air bubbles to transport the C-PC to the top phase. As both the C-PC and contaminating proteins are present in large volumes, higher proportions of the proteins will be transported to the top phase, leading to a lower purity. In this study, the crude extract concentration of 0.625 %w/w showed the best RC and PF, compared to the initial setting of 0.5 %w/w. This is likely due to the available free volume in the top phase that can accommodate additional C-PC content, resulting in a slightly higher C-PC yield and purity in the top phase. The crude extract concentration of 0.625 %w/w was selected as the most suitable condition for the LBF study.

3.8 SDS-PAGE

SDS-PAGE analysis of C-PC purity from the crude extract of *S. platensis* was performed to determine the molecular weights of the C-PC subunits. The purified top phase of the LBF system were subjected to gel electrophoresis as shown in Figure 6. Lane A indicates the molecular marker, lane B indicates the crude extract of C-PC and lane C shows the C-PC after LBF process. From the SDS-PAGE, the C-PC shows two bands in the range of 18 and 20 kDa. The bands present are in accordance with other works done by purifying C-PC from *S. platensis*, which shows that C-PC are apparent in the band range of 18 and 20 kDa (Chethana et al., 2015; Patil & Raghavarao, 2007). After LBF, the majority

of the contaminant proteins present in the crude extract have been partitioned to the bottom phase, resulting in less bands in the lane C compared to Lane B. This shows the potential of LBF for the effective purification of C-PC from microalgae.

4.0 Conclusion

The purification of C-phycocyanin from *S. platensis* was performed using LBF, which is a combination of LBS and adsorptive bubbles flotation with the incorporation of bubbling effect into the system. The optimized conditions for the LBF was found to be: PEG 4000 and potassium phosphate combination, polymer and salt concentration of 250 g/L each, bottom to top volume ratio of 1:0.85, system pH of 7.0, air flotation period of 7 min and crude extract concentration of 0.625 %w/w. The maximum C-PC recovery and purification fold of the optimized LBF system was found to be 90.4% and 3.49, respectively.

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Declaration of interest:

None

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Table 1: Operating parameters of LBF system for C-phycocyanin purification from *S. platensis*.

Step No.	Operating parameter	Initial condition	Variables	Unit
1	Type of PEG	-	PEG 2000, PEG 4000, PEG 8000	-
2	Type of salts	K_2HPO_4	$(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, NaH_2PO_4	-
3	Concentration of PEG	300	200, 250, 300, 350, 400	g/L
4	Concentration of salt	250	200, 250, 300, 350	g/L
5	Volume ratio	1:1	1:0.7, 1:0.85; 1:1, 1:1.25, 1:1.5	-
6	pH	9	6, 7, 8, 9	-
7	Air flotation time	5	1.5, 3, 5, 7, 9	min
8	Crude extract concentration	0.5	0.25, 0.375, 0.5, 0.625, 0.75	%w/w

Table 2: Effect of polymer molecular weight and salts type on the C-PC recovery yield and purification fold from *S. platensis.*

Polymer type	Salt type	Volume ratio	C-PC recovery yield (%)	Purification fold
Types of polymer				
PEG 2000	K_2HPO_4 – phosphate	1.02	66.5 ± 0.83	2.60
PEG 4000	K_2HPO_4 – phosphate	0.95	81.2 ± 0.28	2.43
PEG 8000	K_2HPO_4 – phosphate	1.08	75.3 ± 0.13	2.19
Types of salt				
PEG 4000	$(NH_4)_2SO4$ – sulphate	1.00	93.5 ± 0.52	1.63
PEG 4000	$MgSO_4$ – sulphate	1.76	66.4 ± 1.01	1.22
PEG 4000	NaH_2PO_4 – phosphate	1.97	74.7 ± 0.14	1.76

Figures:

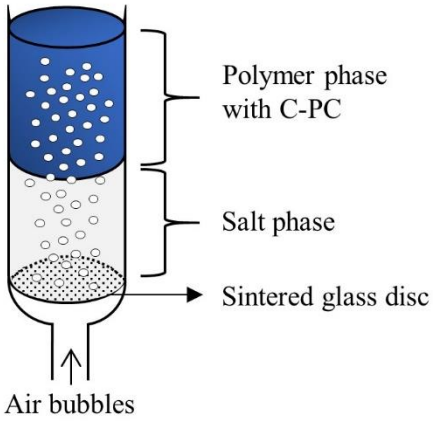

Figure 1: Schematic diagram of the PEG/salt-based LBF for the purification of phycocyanin from *Spirulina*. The flotation effects created with the air bubbles assist in the upwards flow of phycocyanin from the bottom aqueous salt solution to the PEG-rich top phase.

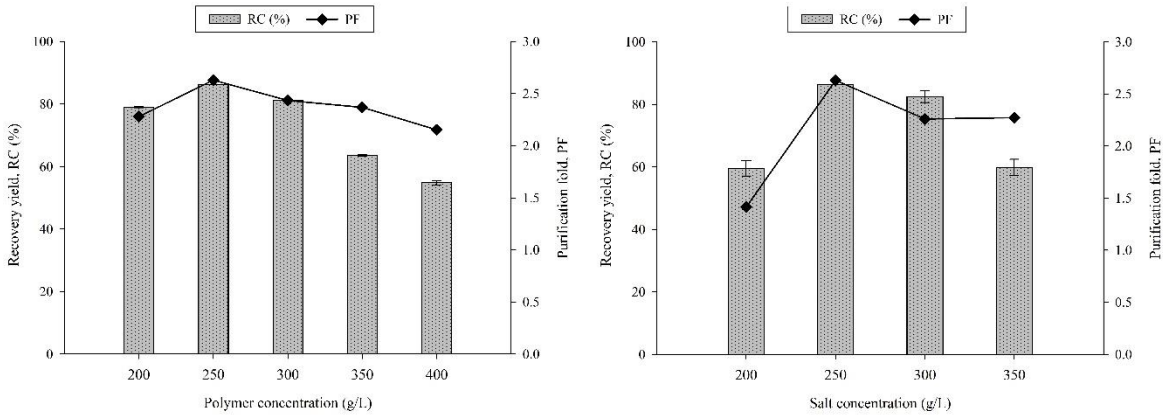

Figure 2: Effect of polymer and salt concentration (g/L) on the C-PC recovery yield and purification fold from *S. platensis*. (a) Variation in polymer concentration; (b) Variation in salt concentration.

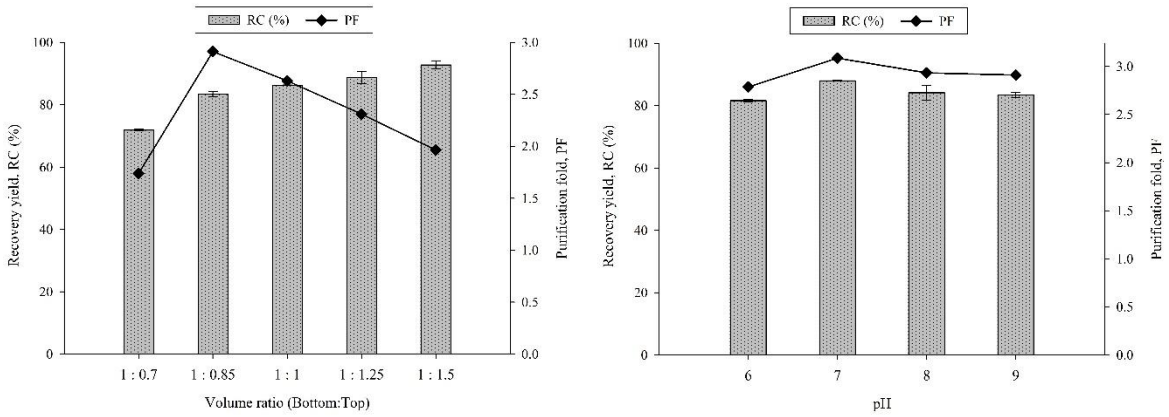

Figure 3: Effect of volume ratio and pH of the system on the C-PC recovery yield and purification fold from *S. platensis*. (a) Variation in volume ratio; (b) Variation in pH of the system.

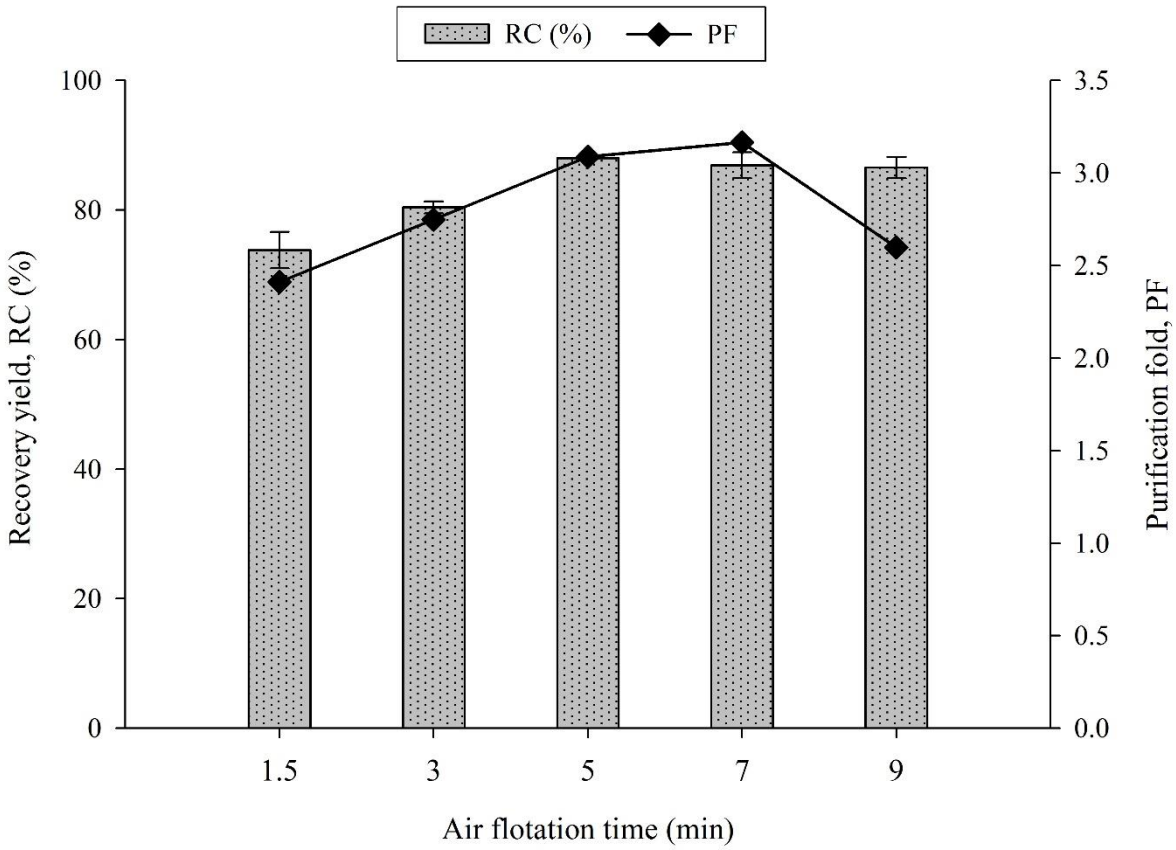

Figure 4: Effect of air flotation time (min) on the C-PC recovery yield and purification fold from *S. platensis*.

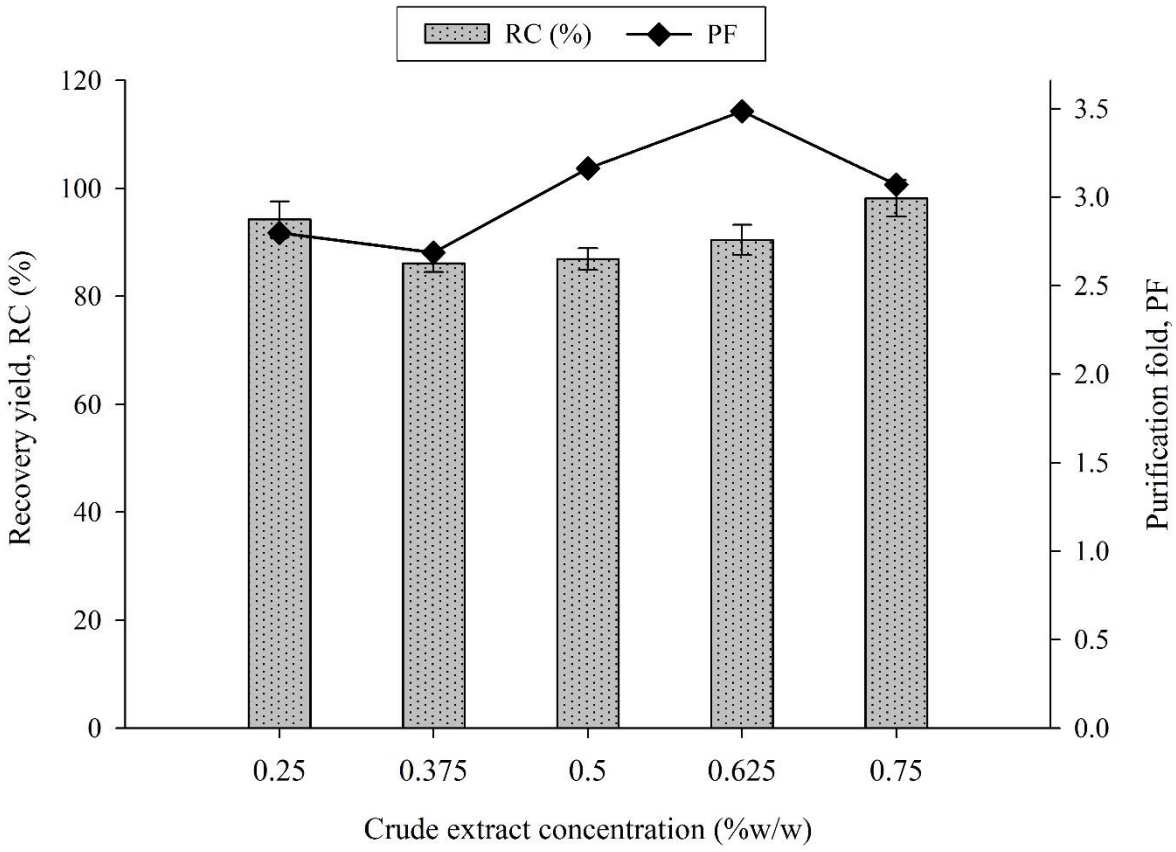

Figure 5: Effect of crude extract concentration (%w/w) on the C-PC recovery yield and purification fold from *S. platensis*.

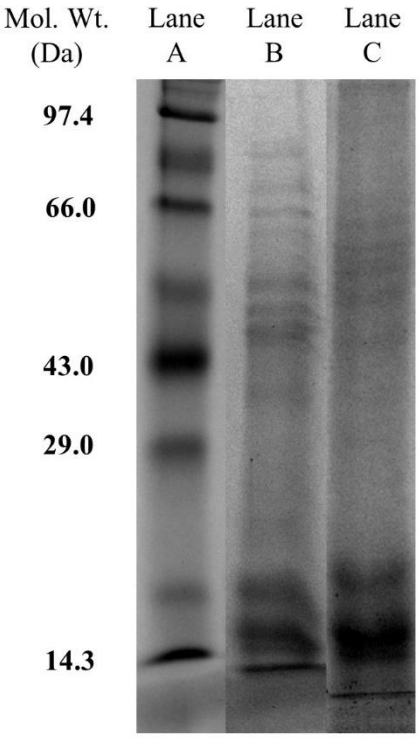

Figure 6: SDS-PAGE of C-phycocyanin: Lane A indicates the molecular marker; lane B indicates the crude extract of C-PC; and lane C shows the C-PC after LBF process.