Fractionation of phycocyanin and carbohydrate from *Spirulina platensis* using ionic liquid-based aqueous two-phase system

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**Abstract.** Microalgae have gained considerable attention due to their high-value biomolecules and potential applications in the pharmaceutical, food, medical and cosmeceutical field. However, the conventional biorefinery process of microalgae are costly, energy-intensive, and time-consuming. Aqueous two-phase system (ATPS) has emerged as a potential technique for the separation and fractionation of biomolecules in the biorefinery field. This study proposed the application of ionic liquid (IL) based ATPS for the fractionation of high-value phycocyanin and carbohydrates from a crude extract of *Spirulina platensis*. The biomass was first lysed by a high-speed homogenization with a solid to liquid ratio (S/L) of 1:10 and an operation time of 5 min to achieve optimum yields of multiple products. Next, the feasibility of several IL-based ATPS with phosphate/citrate buffer was evaluated by investigating the effects of cation, anion, and alkyl chain’s length of ILs. Among the IL-based ATPS, the system comprising of 1-butyl-1-methylpyrrolidinium dicyanamide and citrate buffer demonstrated the optimum phycocyanin extraction efficiency of 83.26 ± 0.05% at the top phase and a concurrent recovery of 73.89 ± 0.06% carbohydrate at the bottom phase. In this work, the IL-based ATPS performed better than conventional polymer-based ATPS. This work suggests that IL-based ATPS allows efficient fractionation of phycocyanin and carbohydrates.

1. **Introduction**

Recently, microalgae have been gaining substantial attention as a potential source of high-value biochemical compounds owing to their capability to accumulate proteins, antioxidants, antibiotics, vitamins, pigments, carbohydrates and lipids [1]. The blue-green microalgae *Spirulina* belongs to the Arthrospira genus and can be classified into two common species, namely, *Spirulina platensis* and *Spirulina maxima*. They have been marketed as healthy food ingredients and supplements in capsules, tablets, powders, and liquids form due to their high nutritional value. *Spirulina sp.* is a multi-filamentous photosynthetic cyanobacterium that is intensively investigated in the fields of medicine and food science due to its wide market potentials [2–3]. It is rich in proteins, vitamins, essential fatty acids, polysaccharides and pigments such as chlorophylls, β-carotene, xanthophylls and phycobiliproteins [3].

Phycobiliproteins are photosynthetic pigments that are accumulated in the cells which are attached to the thylakoid membranes of chloroplasts. They are categorized into three dominant classes which are phycocyanin, phycocerythrin and allophycocyanin [4–5]. *Spirulina sp.* is found to potentially produce high content of phycocyanin with a small quantity of allophycocyanin [6]. Phycocyanin is a water-soluble, non-toxic and blue-color pigment-protein complex with plenty of promising bioactivities. The
phycocyanin is comprised of α and β subunits with the molecular weights of 140 to 210 kDa. The number of α and β subunits that exist in the phycocyanin depends on the sources of species [7]. It is widely utilized as natural colour additives in food and cosmetic products, fluorescent markers, as well as probes and tracers in medical diagnosis, immunology, biological engineering, and other research fields. Moreover, a few studies reported the use of phycocyanin in pharmaceutical and medical applications, which serves as stimulants of the immune system, antioxidant, anti-inflammatory, anti-tumor, prevention, and treatment of some neurological disorders and neurodegenerative diseases such as Alzheimer’s and Parkinson’s [8]. Other than phycocyanin, Spirulina sp. also composed of 8 to 14% of carbohydrate on dry weight basis. However, the carbohydrate content in spirulina can be increased by varying the culture medium during the cultivation of microalgae. Carbohydrates in microalgae can be converted into biofuel such as bioethanol via fermentation processes [9–11].

Typically, biorefinery processes involve a series of intensive steps such as cell disruption, product extraction and purification. Cell disruption methods such as freezing and thawing, mechanical method, and sonication, followed by the purification approaches such as ammonium sulfate precipitation, ultrafiltration and chromatographic techniques (i.e., ion exchange, hydrophobic interaction and gel filtration) are generally used for the recovery of phycocyanin from biomass. However, the biorefinery of microalgae encounters challenges such as energy-intensive, time-consuming, costly processes and the difficulty of scaling up in the industry [12]. In addition to that, degradation of purity and loss of targeted products often occur in these tedious processing steps. Despite the high-value of microalgae as biochemical sources, the economic feasibility of production of phycocyanin derived from Spirulina sp. is still limited. In the interest of sustainability and cost competition in extraction and purification of phycocyanin from Spirulina sp., there is an urgent need to exploit a reliable, mild and scalable biorefinery process to eliminate the energy-intensive multiproduct steps. At this juncture, a product recovery platform which is environmentally beneficial and effective in term of high yield or purity is preferable.

Aqueous two-phase system (ATPS) has been explored as an alternative to isolate and fractionate the high-value compounds from microalgae [11,13]. ATPS has benefits over conventional methods such as short processing time, energy efficient as well as easier operation and scaling up in industry. Nevertheless, several authors reported that the conventional ATPS consisting of polymer and salt was constrained by the limited polarity range between two phases and resulted in unfavourable selectivity of desired biomolecules. In contrast, ionic liquid (IL) has emerged as a promising phase former to replace conventional polymer in ATPS. The tunable properties of IL can establish an ATPS with higher flexibility in designing the polarity range between two phases by pairing with appropriate cation and anion. IL-based ATPS (IL-TPS) can provide a mild environment and achieve higher selectivity which reduce the intensive purification steps, thus minimizing the degradation of phycocyanin purity. To date, only a few reports on employment of IL-ATPS in extraction and fractionation or purification of high-value compounds from microalgae are available [2,14]. Santos et al. [15] and Suarez Ruiz et al. [16] investigated the selective and mild separation of protein and carbohydrate from the crude extract of microalgae. These findings showed that the migration of protein to the top phase while carbohydrates to the bottom phase, proving the successful application of IL-ATPS in fractionation of compounds from crude extract of microalgae.

To the best of the authors’ knowledge, numerous studies have reported the use of conventional polymer-based ATPS to fractionate and purify phycocyanin. However, only one study reported the use of IL containing bromide anion as phase forming components for the isolation of phycocyanin from Spirulina sp.. Aiming at validating the concept of multiproduct biorefinery, this study implements various combination of ILs and inorganic salts to investigate the feasibility of IL-ATPS for fractionation of phycocyanin and carbohydrate as well as phycocyanin purification from Spirulina sp. crude extract. The effects of type of salts, ILs cation alkyl chain length and different ILs anions on the biomolecule fractionation and purification of phycocyanin were discussed. Prior to fractionation and purification, biomolecules extraction operating conditions such as solid biomass to liquid ratio and the homogenization time were evaluated to attain optimum biomolecules yield and phycocyanin purity ratio.
Lastly, the result of IL-based ATPS with best performance was compared with conventional polymer-based ATPS.

2. **Methodology**

2.1. **Materials**

Spray-dried *Spirulina sp.* was obtained from the Algae International Sdn. Bhd. located in Negeri Sembilan, Malaysia. ILs used in this work including 1-ethyl-3-methylimidazolium bromide, [C$_2$min]Br (purity=99%), 1-butyl-3-methylimidazolium bromide, [C$_4$min]Br (purity=99%), 1-ethyl-3-methylimidazolium dicyanamide, [C$_2$min]DCN (purity>98%), 1-butyl-3-methylimidazolium dicyanamide, [C$_4$min]DCN (purity>98%), 1-ethyl-3-methylimidazolium thiocyanate, [C$_2$min]SCN (purity>98%), and 1-butyl-1-methylpyrrolidinium dicyanamide, [C$_4$mpy]DCN (purity>98%) were purchased from Sigma-Aldrich. Potassium citrate tribasic monohydrate, C$_6$H$_5$O$_7$K$_3$ (purity >99%), citric acid, C$_6$H$_8$O$_7$ (purity>99%), potassium phosphate dibasic, K$_2$HPO$_4$ (purity >99%) and monopotassium phosphate, KH$_2$PO$_4$ (purity>99%), polyethylene glycol 4000 (PEG4000), Brilliant Blue G-250, ethanol (purity >95%), phosphoric acid, H$_3$PO$_4$ (purity=85wt%), sulfuric acid, H$_2$SO$_4$ (purity>99%), glucose standard, bovine serum albumin (BSA), acetonitrile, ACN (HPLC grade), trifluoroacetic acid, TFA, phycocyanin from *Spirulina sp.* were also procured from Sigma Aldrich. Bicinchoninic acid assay (BCA) test kit was sourced from Thermo Fisher Scientific.

2.2. **Preparation of crude extract from microalgae**

Extraction of biomolecules was performed via homogenization method as demonstrated in previous literature with modifications [17,18]. Spray-dried *Spirulina sp.* powder was mechanically disrupted using ultra-turrax homogenizer (IKA dispersion S18N-19G) at 12000 rpm in the presence of distilled water as extraction solvent. The experiment was conducted in a water bath to maintain the temperature of crude extract at 25±1 °C under dark condition since phycocyanin is photosensitive and thermo-sensitive biomolecules. The resulting supernatant was centrifuged at 10000 rpm for 15 min at 4 °C to remove cell debris. The operating parameters, which were solid biomass to liquid (S:L) ratio and the homogenization time were investigated using one factor at-a-time (OFAT) method. The S:L ratio varied at 0.200, 0.100, 0.067 0.050, 0.040 g/ml while the homogenization time was manipulated from 0 to 20 min by collecting the samples at the interval time of 5 min. The concentration of phycocyanin, total protein and carbohydrate were analysed and the supernatant was stored at 4 °C for further use.

2.3. **Morphology and optical analysis of Spirulina sp.**

The dried cell debris from homogenization and *Spirulina sp.* powder were immersed in 1% glutaraldehyde phosphate buffer solution for 3 h. Then, dehydration of samples was conducted using a series of ethanol solutions with concentrations of 10, 30, 50, 70, 90 and 100% for 5 min for each concentration and the resulting samples were dried under room condition [19]. The samples were analysed using Scanning electron microscopy (SEM) and microscope to study the effect of homogenization on the optical and morphology changes of samples.

2.4. **Construction of phase diagram**

Phase diagram comprising of different ILs and salts buffer such as phosphate buffer and citrate buffer were constructed using cloud point method at room temperature and atmospheric pressure. The aqueous solutions of ILs and salts with known concentration were prepared gravimetrically (± 10$^{-4}$ g) and were used for the determination of saturation curves. Salt buffer solution was added dropwise into the IL solution under constant stirring and the addition of salt solution was stopped once the solution turned cloudy, which indicated the formation of biphasic region. To detect the formation of monophase regime, distilled water was added until the solution turned clear. The weight of salt solution and distilled water added were measured and recorded. The weight composition of ILs and salts in ATPS were calculated and binodal curves of IL-ATPS were plotted [20]. The experimental steps were repeated to attain a
number of cloud points that enable the formation of binodal curve which correlates to Merchuk equation as in equation (1):

\[ w_T = A \exp \left[ B w_B^{0.5} - C w_B^3 \right] \]  

(1)

where \( w \) refer to weight fraction and subscript T and B represent top phase (ILs) and bottom phase (salts) while A, B, C are constant of Merchuk parameter [21]. The mixture points with volume ratio of 1 were selected based on the plotted phase diagram and to be used in further ATPS step.

2.5. Fractionation of biomolecules using ATPS

Phosphate buffer consisting of K$_2$HPO$_4$ and KH$_2$PO$_4$ and citrate buffer consisting of C$_6$H$_5$O$_7$K$_3$ and C$_6$H$_8$O$_7$ were prepared at pH 7 and pH 6, respectively, by mixing the salts at appropriate ratios. Different combinations of ATPS comprising of ILs, namely [C$_2$min]Br, [C$_4$min]Br, [C$_2$min]DCN, [C$_4$min]DCN, [C$_2$min]SCN and [C$_4$mpy]DCN, and the salt buffers were prepared gravimetrically by adding an appropriate amount of IL, salts and crude extract into the centrifuge tube. Distilled water was added into the mixture to bring the final weight of the system to 1 g. To allow the contact between phase components and crude extract, the mixture was stirred gently in a vortex mixer and was centrifuged at 2000 rpm for 5 min to facilitate the phases separation. The top and bottom phases were separated carefully using a micropipette. To compare IL-based ATPS with conventional polymer-based ATPS, a system composing of PEG4000 and salt buffer were also prepared. To avoid interference, the reference system for each trial was prepared without the addition of crude extract. The volume of the top and bottom phase were measured while phycocyanin and carbohydrate presence at the top and bottom phase were quantified [22]. For all systems, the volume ratio was fixed at 1±0.1.

2.6. Spectrophotometric estimation of phycobiliproteins

The concentrations of phycocyanin in crude extract, top phase and bottom phase were determined by measuring the absorbance spectrum at 620 and 652 nm using UV-vis spectrophotometer in accordance with the equation (2) [23].

\[ C_{PC} \left( \frac{mg}{mL} \right) = \frac{[(A_{620} - 0.70 \left( A_{652} \right))]}{7.38} \]  

(2)

The corrective equations developed by were implemented in the cases which exhibited the presence of chlorophyll as shown in equation (3) and (4) [24].

\[ A_{620} = 1.012(A_{620}) - 0.215(A_{675}) \]  

(3)

\[ A_{652} = 1.038(A_{652}) - 0.256(A_{675}) \]  

(4)

where \( C_{PC} \) represents the phycocyanin concentration, \( A_{620} \) is the peak absorbance of phycocyanin, \( A_{652} \) is the maximum absorbance of allophycocyanin and \( A_{675} \) indicates the presence of chlorophyll. The purity ratio of phycocyanin, \( P \) was calculated using equation (5):

\[ P = \frac{C_{PC}}{C_P} \]  

(5)

where \( C_P \) is the concentration of total protein and \( C_{PC} \).

2.7. Total protein content determination

Total protein content was quantified by BCA method. BSA was used as protein standard for plotting of calibration curve. In a 96-well microplate, 10 µL of sample was mixed with 200 µL of BCA working reagent. The microplate was shaken for 30 s and incubated for 15 min at 37 °C. After the incubation period, the mixture in the microplate was cooled for 5 min and their absorbance was measured at 562 nm using a microplate reader and the total protein concentration in crude extract, top phase and bottom phase were determined [15].
2.8. Total carbohydrates content determination

Total carbohydrate contents were analysed by phenol sulfuric acid method adapted from Santos et al [15]. Firstly, 80 μL of sample, 150 μL of 5% (w/v) of an aqueous phenol solution and 1 mL of concentrated H₂SO₄ were pipetted into a long test tube. The test tube was stirred under dark environment for 10 min in 100 ºC water bath. Quantification was carried out by measuring the absorbance of the yellow-orange colour sample at 490 nm using the spectrophotometer. A calibration curve (0.05–0.3 mg/mL) was established using glucose as the predominant sugar standard in the *Spirulina sp.*

2.9. Calculation and statistical analysis

Extraction efficiency, EE of each biomolecule at the top and bottom phase was evaluated based on equation (6) and (7) [15].

\[
EE_{iT} = \frac{C_{iT}V_T}{C_oV_o} \times 100\% \quad (6)
\]

\[
EE_{iB} = \frac{C_{iB}V_B}{C_oV_o} \times 100\% \quad (7)
\]

where, \(C_{iT}\) and \(C_{iB}\) represent the concentration of biomolecules while subscript \(i\) represents phycocyanin, total proteins and carbohydrates at the top and bottom phase, respectively. The purification factor, \(P_f\) of each ATPS system was calculated using equation (8)

\[
P_f = \frac{P_A}{P_B} \quad (8)
\]

where \(P_A\) denotes phycocyanin purity ratio after ATPS and \(P_B\) represents the phycocyanin purity ratio before ATPS. The volume ratio of the top and bottom phases can be calculated based on equation (9):

\[
V_R = \frac{V_T}{V_B} \quad (9)
\]

where \(V_R\) indicates the volume ratio, \(V_T\) is the volume of top phase while \(V_B\) is the volume of bottom phase. Triplicate independent runs were conducted for each extraction parameters study and fractionation runs. The mean values and standard deviation were evaluated.

3. Results

3.1. Extraction of biomolecules

In this work, rotor-stator-based homogenization method was employed to disrupt the cell wall of biomass by rapidly moving the inner rotor. The strong shear stress applied on the cells attributed to the disruption of the rigid cell wall [25]. Eventually, phycocyanin, carbohydrate and total protein could be discharged from cell into the extraction solvent. Upon preliminary test of crude extraction, a blue green color extract was observed instead of typical blue color, which signifying the release of chlorophylls from the cells. Similar observation was revealed by Syrpas et al. [17] This would induce interference and deviation in phycocyanin content estimation with spectrophotometric analysis due to the overlapping of absorbance bands of phycobiliproteins and chlorophyll \(a\) (Chl\(a\)). The presence of Chl\(a\) could be further verified by the existence of peak absorbance around 675 nm. Lauceri et al. [24] claimed that the contamination of phycobiliprotein by Chl\(a\) was possible when the extraction method was not sufficiently mild. The extreme extraction process triggered the release of undesired biochemical compounds from the cytoplasm or other cell organelles. In fact, equation (2) proposed by Bennett and Bogorad [23] did not consider the presence of Chl\(a\) in the crude extract and the interference was generally neglected in other studies. In order to compute a more accurate phycocyanin quantification, the corrected equations (3) and (4) that were developed by Lauceri et al. [24] were included in this study. Additionally, allophycocyanin was not detected in the crude extract due to its small quantity as compared
to phycocyanin [26]. To obtain optimum biomolecules yield, the effect of extraction conditions such as S:L ratio and homogenization time were investigated at room temperature. Although it was reported that higher stability of phycocyanin could be achieved at 4 ºC, it was suggested to conduct the experiment at room temperature with regard to the perspective of economic viability and industrial scale-up [27].

Figure 1 displays the effect of solid biomass to solvent ratio on yield of phycocyanin, carbohydrate, total protein and phycocyanin purity ratio. The lower S:L ratio indicates the presence of more solvent in the extraction process. As the S:L ratio decreased from 0.200 to 0.100 g/ml, the trend demonstrates a drastic increase in phycocyanin yield from 39.85±3.43 mg/g to 101.20±3.48 mg/g. The phycocyanin yield gradually increased at ratio of 0.067 g/ml and remained unchanged when the S:L ratio was further decreased to 0.04 g/ml. At highest S:L ratio, the solvent volume available for contact between solvent and biomass became saturated, in which their interaction became limited and caused an incomplete extraction of phycocyanin from dry biomass, leading lower phycocyanin yield [28]. It can also be observed that the suspension was highly concentrated due to the presence of high amount of biomass at highest S:L ratio. With decreasing ratio, higher amount of solvent available for the contact between solvent and biomass thus increasing the driving force for mass transfer and diffusion of solvent into cells, thereby, the desorption of phycocyanin from the cells into the solvent was enhanced [29]. The finding was in agreement with literature which reported that the presence of more solvent resulted in better extraction of phycocyanin from Spirulina sp. [18,30]. Nevertheless, the purity ratio of phycocyanin shows a decreasing trend from 0.611±0.03 to 0.396±0.02 with a decreasing S:L ratio. In this study, the phycocyanin purity ratio is defined as the ratio of concentration of phycocyanin to concentration of total protein. The decline in purity ratio can be explained by the increase in availability of solvent for extraction of total protein from the biomass [18]. The result can be evidenced by increasing total protein yield trend with decreasing S:L ratio as described in figure 1. For carbohydrate extraction, a maximum yield of 103.55±1.02 mg/g of carbohydrate was obtained at S:L ratio of 0.100 g/ml and further decrease in the ratio did not result in significant change in the yield. The result was in good accordance with the works of literature which stated that the polysaccharides extraction yield increased when the solvent available increased, and was remained unchanged beyond a certain ratio [29,31]. The results described that the effect of S:L ratio is an essential parameter to take into consideration. Considering the phycocyanin yield and purity, the S:L ratio of 0.100 g/ml was chosen for further study due to its maximum phycocyanin yield and phycocyanin purity ratio as well as comparable total protein and carbohydrate yield.

![Figure 1. Effect of solid biomass to liquid ratio on biomolecules yield and phycocyanin purity ratio at constant homogenization time.](image-url)
The effect of homogenization time on biomolecules yield and phycocyanin purity ratio is illustrated in figure 2. As depicted in figure 2, all biomolecules yield show a drastic increase with the increasing of homogenization time from 0 min to 5 min. The sharp increment in the yield at initial time was due to large concentration gradient of biomolecules between the solvent, which caused more phycocyanin, carbohydrates and total protein to diffuse out from the cells into the exterior solvent. Nevertheless, the further extension of homogenization time did not trigger significance change in phycocyanin and carbohydrate yield. This phenomena indicated that there was mass transfer limitation between the cell and the solvent when the solvent became saturated with biomolecules due to continuous exposure of biomass to solvent [28]. However, the trend for total protein yield was differed from phycocyanin and carbohydrate yielded. It can be seen that total protein yield was increased gradually with prolonged homogenization time. The high protein content in *Spirulina sp.* caused the yield to increase when the biomass was continuously exposed with the solvent. Summing up, an optimum yield of 110.30±1.07 mg/g of phycocyanin, 289.00±1.70 mg/g of total protein, 30.74±1.02 mg/g of carbohydrate with phycocyanin purity ratio of 0.656±0.01 were attained at S:L ratio of 1:10 and homogenization time of 5 min. These conditions were chosen for preparation of crude extract in further ATPS study.

![Figure 2. Effect of homogenization time on biomolecules yield and phycocyanin purity ratio at constant solid to liquid ratio.](image)

Figure 3 and 4 depict the optical structure and morphology features of *Spirulina sp.* before and after homogenization. From figure 3a and 3b, it can be observed that the helical shape filament of *Spirulina sp.* is broken down into smaller fragments after subjecting to high shear force during homogenization. As portraited in figure 4a, the morphology feature of *Spirulina sp.* is characterized by intact and smooth cell wall surface before the homogenization. After homogenization, the cell surface is found to be altered, and rough cell surface is observed in figure 4b. This indicated that homogenization method was an effective cell disruption method which allowed the release of biomolecules from the phycobilisomes of the *Spirulina sp.* In the study of Ilter *et al.* [30], they concluded that homogenization was the most appropriate method in comparison to ultrasonication and microwave-assisted extraction. By using homogenization method, a maximum phycocyanin yield of 74.74 mg/g was achieved in their work at S:L ratio of 0.32% (w/w), speed of 7000 rpm and homogenization time of 18.41 min. Previously, Tavanandi *et al.* [18] achieved phycocyanin yield of 109.03 mg/g and purity of 0.8 by a combination of
ultrasonication and freezing and thawing method (2 cycles) at S:L ratio of 1:6 and ultrasonication time of 2.5 min. Tavanandi et al. [32] also achieved the highest phycocyanin yield of 98.24 mg/g and purity ratio of 1.09 by a combination of ultrasonication and enzyme-assisted extraction method. The use of ultrasound-assisted extraction by Bachchhav et al. [33] on the extraction of phycocyanin followed by B-carotene also showed a phycocyanin yield of 67 mg/g at 80% amplitude and duty cycle of 66%. Although a lower phycocyanin purity ratio was attained, it can be concluded that the homogenization method used in the present study obtained a phycocyanin yield with 10.79 to 32.23% higher than that was obtained in other studies [18, 32–33].

Figure 3. Morphology feature of Spirulina sp, at 400 × magnification (a) Before homogenization (b) After homogenization.

Figure 4. Optical image of Spirulina sp. at 3000 × magnification, (a) Before homogenization (b) After homogenization.

3.2. Fractionation of phycocyanin and carbohydrate using IL-based ATPS
In this study, kosmotropic buffer salts such as phosphate buffer at pH 7 and citrate buffer at pH 6 were utilized as phase-forming components to maintain the pH of the system during partitioning of biomolecules. Kosmotropic salts are designated as water-structuring salts which can stabilise and precipitate protein as well as promote salting-out effect. The addition of kosmotropic salts into the IL solution leads to entropic effect which is contributed by formation of water-kosmotropic ions complexes. The high affinity of kosmotropic ions towards water causes dehydration of hydrophilic IL and a decrease in its solubility in aqueous solution [34]. When the salt and IL reach certain concentration,
hydrophilic IL containing anions with less water structuring characteristic and hydrophobic cation are separated and salted out [35–36].

In an ATPS, the partitioning behaviours of biomolecules are complex and could be driven by several interactions such as hydrophobic interaction, electrostatic interaction, hydrogen bonding interaction, van der Waals force and steric effects, depending on types of biomolecule [37–39]. The properties of protein such as isoelectric charges, hydrophobicity, solubility and size could influence the type of interaction involved during the partitioning [16]. A few studies have explored the domination of hydrophobic interaction between the phase former in polymer-based ATPS [40]. Nevertheless, the partitioning behaviour of biomolecules in IL-based ATPS seems to be more complicated due to the presence of cation and anion of IL which contributes to a wide range of polarity. Figure 5 shows the extraction efficiency of phycocyanin (top phase), total protein and carbohydrate (bottom phase) as well as the purification factor of phycocyanin using ATPS composing of ILs and salts. Purification factor is an important parameter for the measurement of the effectiveness of purification platform by comparing the purity ratio of phycocyanin before and after ATPS process.

![Figure 5. Fractionation of phycocyanin and carbohydrate using various ILs and salt buffers.](image)

3.2.1. Effect of inorganic salts. The effect of inorganic salts on the partitioning behaviour of phycocyanin and carbohydrates are depicted and compared in figure 5. Total protein at bottom phase were also estimated to study the migration of contaminated protein which could affect the purity ratio of phycocyanin. The result indicated that the ATPS comprising of citrate buffer resulted in higher phycocyanin extraction efficiency and purification factor at top phase. The good partitioning behaviour and purification of phycocyanin can be explained based on the pH associated with each ATPS, its biocompatibility and salting-out effect. The pH of the system played a major role in partitioning behaviour of biomolecules, especially phycocyanin and total protein since pH could greatly influence their net charge, charge distribution, zeta potential and conformation structure change of phycocyanin and protein [41]. In current study, it was portrayed that higher phycocyanin extraction efficiency was
found when using citrate buffer at pH 6. However, the result was in disagreement with another study which indicated that the optimum pH of 7 for extraction of phycocyanin with extraction efficiency and partition coefficient of 97.8% and 20.46, respectively, using ATPS consisting of K_3HPO_4 and [C_5min]Br [2]. Chew et al. [41] also disclosed that working pH of 6 triggered unfavourable phycocyanin purity ratio and yield when compared with working pH 7 in ATPS formulated with PEG and K_3HPO_4. They claimed that phycocyanin becomes more negatively charge and being partitioned more effectively as the pH of the system increases, thereby the positively charge cation of IL tends to undergo electrostatic interaction with the negative charge of phycocyanin [41]. In this context, it is worth mentioning that the isolectric point (PI) of phycocyanin varies between 4.6 to 5.2 [42]. When the pH above its PI, phycocyanin becomes negatively charges and interacts electrostatically with the cation of ILs [41]. At pH 6, there was insignificant charge on phycocyanin surface and the protein was in neutral state. For this reason, it was assumed that the surface properties or hydrophobic effect are the crucial effect on the partitioning behaviour of phycocyanin instead of electrostatic interaction in this study. The result was conformed with the disclosure of Patil et al. [43], which reported a greater purity ratio of phycocyanin at pH 6 using PEG4000 and potassium phosphate. The authors also claimed that the interaction that happened for partitioning behaviour of phycocyanin at this working pH was due to surface properties instead of electrostatic charge. Apart from that, the pH of ATPS also affects the stability of phycocyanin since it is heat, light and pH-sensitive biomolecules. Several studies revealed that a higher solubility of phycocyanin at neutral working pH. It might suffer from structural conformation change and degradation at acidic and alkaline pH [41,44–45]. According to Chentir et al. [46], phycocyanin tends to dissociate and aggregate in the form of various oligomer structures such as monomers, trimers, hexamers and higher degree of aggregation, depending on the working pH ionic strength and concentration. At working pH range between 4.5 to 6, phycocyanin was predominantly retained their hexameric structure, thus entraining their higher spectrum property and maximum stability [46]. At higher pH, phycocyanin undergoes denaturation as they dissociate from hexamers to trimers [47]. This was reported by Adam and Kao [48–49] whom revealed that the existence of phycocyanin in hexamer form was 77% at pH 6 and 18% only at pH 7. Several authors postulated that maximum phycocyanin stability were achieved at pH of 6 [46,47]. Moreover, citrate salt is well-known for its environmental friendly characteristic and biocompatible with biomolecules, thus offering a conducive environment for phycocyanin [16]. It was inferred that the presence of citric acid in citrate buffer aided in maintenance of stability since citric acid has been touted as good preservative for food-grade phycocyanin [45]. By referring to extraction efficiency of total protein, it was deduced that ATPS comprising of citrate buffer has higher ability to partition total protein to bottom phase, thus giving rise to higher purification factor of phycocyanin at top phase.

On the other hand, the results presented in figure 5 shows that all ATPSs composing of citrate buffer exhibited better carbohydrate extraction efficiency at salt-rich bottom phase. The higher salting-out effect of citrate buffer plays a crucial role in migration of carbohydrate to bottom phase and phycocyanin to the IL-rich top phase [22]. The citrate buffer with trivalent anion (C_6H_5O_7^-) reflects a greater salting-out effect than phosphate buffer consisting of monovalent and bivalent anions (H_3PO_4^-/HPO_4^{2-}). The high charge of salt anion contributes to stronger electric field and causes alignment of dipole of water molecules around the surrounding of anion, leading to hydration of salt over ILs [35]. At the same time, the hydrogen bond interaction was established between carbohydrate and water since carbohydrates have high polarity due to presence of numerous hydroxyl groups. This caused the exclusion of carbohydrates to the bottom phase and hence less free water molecules at the bottom phase causing the phycocyanin to migrate to the IL-rich top phase [2,39].

3.2.2. Effect of Ionic liquids nature. The effects of ionic liquid nature which were anion, cation and cation alkyl chain length of ILs on the partitioning behaviour of biomolecules are as shown in figure 5. From figure 5, the extraction efficiency of phycocyanin for all ATPS with citrate buffer in descent order are [C_5min]Br > [C_4mpy]DCN ≥ [C_2min]Br > [C_5min]DCN ≥ [C_4min]DCN > [C_2min]SCN while the purification factor showed decreasing trend of purity ratio of [C_5min]Br > [C_4min]DCN > [C_4mpy]DCN

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The ATPS with citrate buffer and phosphate buffer exhibited similar phycocyanin extraction efficiency and $P_f$ trends as well as carbohydrate extraction efficiency with descent order of: $[\text{C}_4\text{mpy}]\text{DCN} > [\text{C}_4\text{min}]\text{DCN} > [\text{C}_4\text{min}]\text{SCN} > [\text{C}_2\text{min}]\text{DCN} > [\text{C}_2\text{min}]\text{Br} > [\text{C}_2\text{min}]\text{SCN} > [\text{C}_2\text{min}]\text{DCN} > [\text{C}_2\text{min}]\text{Br}$.

From the performance trend, the effect of anions with increasing extraction efficiency of phycocyanin was $[\text{SCN}]^->[\text{DCN}]^->[\text{Br}]^-$. The finding suggests that the effect of anions on extraction performance is highly dependent on their hydrophilicity. In this sense, the hydrophilic $[\text{Br}]^-$ anion has highest hydrogen bond acceptor ability than $[\text{DCN}]^-$ and $[\text{SCN}]^-$, thereby, $[\text{Br}]^-$ possessed a stronger interaction with the surrounding aqueous solution. Hence, the ILs with $[\text{Br}]^-$ anion resulted in superior phycocyanin extraction and purification performance as a consequence of stronger interaction IL and water-soluble phycocyanin molecules. This result was agreed with Martins et al. [50], which proved that the use of hydrophilic ILs was effective in extraction of phycobiliproteins such as phycocerythrin, phycocyanin and allophycocyanin. On the other hand, with an increasing number of cyanamide groups from $[\text{SCN}]^-$ to $[\text{DCN}]^-$, the hydrogen bonding interaction with water was enhanced and thus increasing IL hydrophilicity [51]. The finding was in close agreement with the solvatochromic parameter $\beta$ reported by Batista et al. [52], whom found that the value of $\beta$ of $[\text{C}_4\text{min}]\text{DCN}$ was higher than $[\text{C}_4\text{min}]\text{SCN}$. This indicated that the hydrogen bond acceptor ability of anion $[\text{DCN}]^-$ was stronger than $[\text{SCN}]^-$ thus leading to greater phycocyanin extraction efficiency [52]. In contrast, the carbohydrate extraction efficiency at bottom phase showed an opposite trend. As the hydrophilicity of ILs anion increased, the ability of carbohydrate to form hydrogen bond with ILs at the top phase increased. This could lead to lower carbohydrate extraction efficiency at the bottom phase [39]. The hydrophobic ILs anion which were $[\text{SCN}]^-$ and $[\text{DCN}]^-$ lead to higher enrichment of total carbohydrate to bottom phase due to their higher propensity migration toward more hydrated phase. Santos et al. [15] reported similar opposite trend against protein partitioning.

In term of purification performance, both $[\text{DCN}]^-$ and $[\text{SCN}]^-$ exhibited close value of purification factor with non-ideal performance. The hydrophobicity of ILs which correlates to their toxicity can affect the stability of phycocyanin in ILs. With increasing hydrophobicity of ILs, the toxicity increases and this could cause degradation of phycocyanin during the partitioning [53]. The hydrophobic character of ILs with $[\text{SCN}]^-$ and $[\text{DCN}]^-$ anions had negatively affected phycocyanin purity ratio owing to their high toxicity. The purification factor of phycocyanin not only accounted for its partitioning behaviour but also enrichment of total protein at bottom phase. In ATPS composing of citrate buffer, it was found that the total protein extraction efficiency at bottom phase followed an descending order of: $[\text{C}_4\text{min}]\text{Br} > [\text{C}_4\text{min}]\text{DCN} > [\text{C}_4\text{mpy}]\text{DCN} > [\text{C}_4\text{min}]\text{Br} > [\text{C}_2\text{min}]\text{DCN} > [\text{C}_2\text{min}]\text{SCN}$. It can be seen that IL containing $[\text{Br}]^-$ anion tended to partition more total protein to bottom phase when compared with $[\text{DCN}]^-$ and $[\text{SCN}]^-$.

Moreover, the effect of cation which were imidazolium and pyrrolidinium on the fractionation of biomolecules was also studied. It was found that $[\text{C}_4\text{bmpy}]\text{DCN}$ shows a higher extraction efficiency of all biomolecules and phycocyanin purification than $[\text{C}_4\text{min}]\text{DCN}$. It could be inferred that the good result of $[\text{C}_4\text{bmpy}]\text{DCN}$ was associated with good stability of phycocyanin in pyrrolidinium-based ionic liquid. Bui-le et al. [54] proved that protein retains its secondary structure and tertiary structure in pyrrolidinium-based ionic liquid which was verified by presence of negative band at particular wavelength and maximum absorbance associated with the protein chromophore using circular dichroism spectrum and UV-vis spectrum analysis.

In addition, the cation alkyl chain length of ILs with anions $[\text{DCN}]^-$ and $[\text{Br}]^-$ were varied from $n = 2$ to 4 to evaluate their effects. By referring to figure 5, it can be seen that the carbohydrate and phycocyanin extraction efficiency and its purification factor increase with IL cation alkyl chain length. The finding was consistent with the study of Chang et al. [2] which reported an increase in extraction efficiency of phycocyanin as the cation alkyl chain length increased. The author claimed that the partitioning behaviour of phycocyanin accounted for both hydrophobic interaction and electrostatic interaction. As aforementioned, the driving force for the fractionation of phycocyanin is mainly contributed by hydrophobic interaction or surface properties. The increase in cation alkyl chain length
enhanced the hydrophobic interaction between the IL cations and the hydrophobic residues on the surface of phycocyanin. This caused an increase in phycocyanin extraction efficiency at top phase. Furthermore, the ILs with longer alkyl chain length has higher ability to promote the migration of carbohydrate and total protein to the bottom phase as depicted in figure 5, thus resulting in higher purity ratio of phycocyanin. As the hydrophobicity of IL increased with cation alkyl chain length, the hydrogen interaction between carbohydrate and water in salt-rich phase was intensified.

By looking at the overall performance of each ATPS, [C$_4$mpy]DCN and citrate salt was chosen as the best ATPS constituents for the fractionation of phycocyanin and carbohydrate to top and bottom phase, separately. In this ATPS, optimum extraction efficiency of 83.26±0.05% phycocyanin and 73.89±0.06% of carbohydrate were achieved. Despite [C$_4$min]Br exhibited higher phycocyanin and purification performance, this system only recovered 48.23±0.02% of carbohydrate at the bottom phase. In ATPS comprising of [C$_4$mpy]DCN and citrate salt, simultaneous fractionation of phycocyanin and carbohydrate to opposite phase can be achieved. When comparing the IL-based ATPS and conventional polymer-based ATPS as displayed in figure 5, it is reflected that IL-based ATPS performed more effectively for fractionation of multiple products.

4. Conclusion

In this work, the implementation of various ILs-based ATPS for fractionation of biomolecules from *Spirulina* crude extract as well as purification of phycocyanin were investigated. The results suggested that the use of biocompatible citrate buffer at pH 6 provided a more conductive environment for phycocyanin and resulted in high salting-out effect in contrast to phosphate buffer which was generally used in other studies. This work postulated that ILs-based ATPS are not touted as good purification platform for phycocyanin since the non-ideal purification factor of 1.29 was achieved during purification. However, it can be concluded that ILs-based ATPS is a promising method for the fractionation of biomolecules which can achieve the multiproduct biorefinery concept. Moreover, [C$_4$mpy]DCN and citrate buffer were inferred as best phase forming components due to its high phycocyanin and carbohydrate recovery of 83.26±0.05% and 73.89±0.06% at the top and bottom phase, respectively. Lastly, the phycocyanin recovery of this system is higher than those obtained in conventional polymer based ATPS. Therefore, the application of ILs-based ATPS is substantially envisaged in biorefinery field.

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