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Effect of isothermal and thermal diffusion on aqueous two-phase extraction for the purification of C-phycocyanin from *Spirulina platensis*

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Abstract

Aqueous two-phase extraction (ATPE) is effective for the purification of C-phycocyanin (CPC) from *Spirulina platensis*. Polyethylene glycol and potassium phosphate was used to purify the CPC. The influence of various different temperatures (dT) of process on purity (EP) partitioning and diffusion coefficient was evaluated. The optimal conditions for CPC purification was found at dT 25°C. CPC purity increased to 2.337 from an initial purity of 1.106. The concentration and recovery yield were found to be the highest at dT 25°C (13.932 g/L and 91.18, respectively), which was significantly higher than the conventional process. The dT affected the fluid viscosity variable, potentially due to a phenomenon which decreases the viscosity of the mixture and enhances the solvent solubility and diffusion capacity. The present work presented the diffusion coefficient in ATPE at various dT of the process. The isothermal diffusion coefficient (D_i) significantly increased to dT. The Soret effect (S_T) and thermal diffusion coefficient (D_T) were obtained at a high dT, but it should not be greater than 25°C. Hydrophobicity plays an important role in the thermal diffusion behaviour. A high diffusion coefficient is expected to result in the purification process due to high purity and efficiency.

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Introduction

Phycobiliproteins are the major photosynthetic pigments in cyanobacteria. C-phycocyanin (CPC) is the major component of the phycobiliprotein family. CPC is a type of blue-coloured protein with great commercial and industrial significance, and is formed by two subunits of α and β. In addition to being widely used in the food and cosmetics industries (Moraes *et al.,* 2011), CPC has also been used as a fluorescent marker in biomedical research and as a therapeutic agent in oxidative stress-induced diseases. CPC purity is recognised as a good indicator of preparation purity, especially where other protein contaminants may be involved in the preparation processes. CPC purities of 0.7 is considered to be food grade, 1.5 as cosmetics grade, 2.5 as drug and food supplement, 3.9 as reactive grade, and greater than 4.0 as analytical grade (Rito-Palomares *et al.,* 2001).

Several methods have been developed to extract and purify CPC, such as precipitation, ion-exchange chromatography, and gel-filtration chromatography, but these are tedious and time-consuming (Niu *et al.,* 2007; Liao *et al.,* 2011; Chaiklahan *et al.,* 2011). The major drawback of almost all such methods is the large number of steps involved, high cost, low loading quantities, low recovery rates, and complexities and difficulties in up-scaling. Aqueous two-phase extraction (ATPE) is an effective purification technique for downstream processing. It is a better alternative to existing methods, especially in the early processing stages, in terms of scaling up to an industrial scale. Most ATPE-related researches on CPC purification focuses on process optimisation involving a varying type of salt, volume ratio, and molecular weight of polyethylene glycol, PEG (Patil *et al.,* 2008; Chethana *et al.,* 2015). The most common methods for phase separation are gravity and centrifugation. The small different densities between the two phases is an obstacle to the application of ATPE. Majority of CPC is gathered in the PEG phase. The viscosity of the PEG was increased by increasing the mass fraction of PEG. Therefore, it is important to use low viscosity and low density in the purification processes. Temperature variations can cause viscosity and density changes through natural convection. If the density is variable for a general fluid, a buoyancy force will arise, thus decreasing the mass transfer resistance, and improving the extraction efficiency. Therefore, the present work aimed to increase the efficiency of the process by experimenting in systems with different temperatures that affect the diffusion coefficient.

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Materials and methods

Preparation of crude CPC extracts

All the experiments involved the utilisation of *Spirulina platensis* which was cultivated by a smart control (Jaturonglumlert *et al.,* 2017; Kaewdam *et al.,* 2019). Cells were harvested and washed twice with distilled water. Then, 5 g of biomass was mixed with 25 mL of 0.1 M sodium phosphate buffer (pH = 7.0), and frozen at -10°C overnight, before being thawed at room temperature for about 30 min. Ultrasound-assisted extraction (Thaisamak *et al.,* 2019) was performed. Next, the samples underwent centrifugation at 3,500 rpm for 30 min to remove cell debris, then the supernatant (blue colour) was collected, and the CPC concentration (g/L) of the crude extract was calculated by absorbance with UV/Vis spectrophotometer at wavelengths of 620 and 652 nm, using Eq. 1 (Ruangyot *et al.,* 2016):

$$CPC = \frac{A_{620} - 0.474A_{652}}{5.34} \quad \text{(Eq. 1)}$$

Aqueous two-phase extraction

Polyethylene glycol 4000 MW (PEG 4000) and potassium phosphates (K_2HPO_4:KH_2PO_4 = 1.82:1 to obtain the required pH 7.0) are the type of phase system of ATPE (Patil *et al.,* 2006). PEG 4000 (6%, w/v) and potassium phosphate (15%, w/v) was mixed with distilled water and later mixed with the crude extract. The mixture was stirred for about 30 min to equilibrate and allow for phase separation. The cylinder was set up in the equipment as shown in Figure 1A for 2 h, allowing phase separation to occur at the specified temperatures (dT = 0 [control], 15, 25, and 35°C), in which dT was the different temperature between top (T_1) and bottom (T_2). Pure CPC extracts after separation are shown in Figure 1B. The CPC and total protein concentrations in each phase were analysed to estimate the purity (EP), purification factor (PF), and partition coefficient (K) of the process, using Eqs. 2, 3 and 4, respectively.

$$EP = \frac{A_{620}}{A_{280}} \quad \text{(Eq. 2)}$$

where, A_{620} and A_{280} = absorbance of the sample at 620 and 280 nm, respectively. This relationship is indicative of the CPC extract purity with respect to most forms of contaminating proteins (Chethana *et al.,* 2015). Absorbance at 620 nm indicates the CPC concentration, while absorbance at 280 nm indicates the total protein concentration in the solution.

$$PF = \frac{EP_p}{EP_c} \quad \text{(Eq. 3)}$$

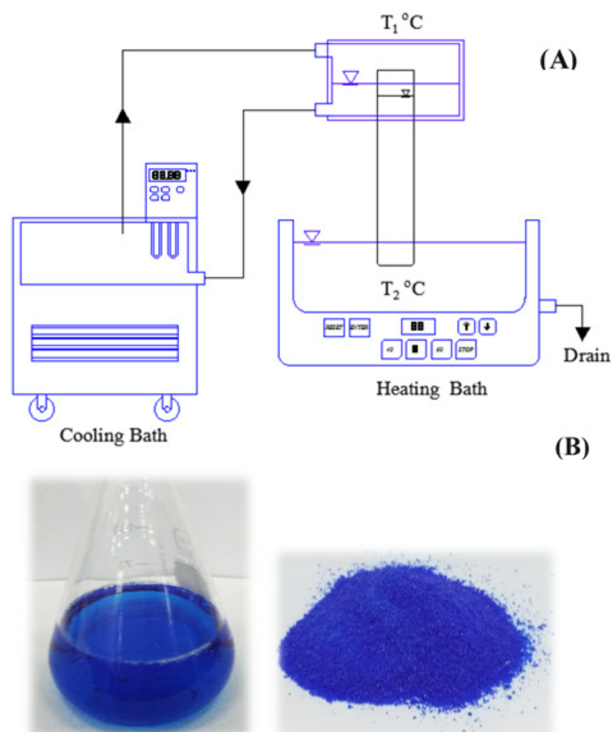

Figure 1. (A) the experimental setup, and (B) pure CPC after aqueous two-phase extraction.

where, PF = purification factor, EP_c = crude extract purity, and EP_p = extract purity after the purification process.

$$K = \frac{CPC_T}{CPC_B} \quad \text{(Eq. 4)}$$

where, K = partition coefficient, CPC_T and CPC_B = CPC concentration in the top phase and bottom phase, respectively.

The recovery yield (RC, %) of the CPC from the sample was calculated using Eq. (5) (Chew *et al.,* 2019), where, V_r = volume ratio, and in Eq. 6, V_T and V_B = volumes in the top and bottom phase, respectively:

$$RC = \frac{K \times V_r}{1 + K \times V_r} \times 100 \quad \text{(Eq. 5)}$$

$$V_r = \frac{V_T}{V_B} \quad \text{(Eq. 6)}$$

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is an electrophoresis method that allows protein separation by mass. The method of Deutscher (1990) was followed using a 1 mm thick, and 15% polyacrylamide slab gel (5% stacking gel). Electrophoresis was run at room temperature at 150 V for approximately 90 min. The distances of buffer and bands were measured from the beginning

of the separation gel. The bands were visualised with Coomassie brilliant blue R250. The size of the phycocyanin subunit bands were determined using a Thermo Scientific Page Ruler Pre-stained Protein Ladder, a molecular weight with a broad range of 16 - 127 kDa (AccuProtein marker Chroma-I from Enzmart Biotech Co., LTD).

Phase partitioning

Pure PEG 4000 and potassium phosphate salt were used to prepare the biphasic systems in a 100 mL cylinder. The mixture was allowed to settle for 2 h at different temperatures (dT = 0 [control], 15, 25, 35°C) to obtain clear phase separation and reach equilibrium. A thermostatic water bath was used to control the temperature with a precision of ± 0.01 K (UC-150, Sturdy Industrial, Taiwan). Each resultant phase was collected with a volumetric pipette, and salt concentration was determined by a conductivity meter. The salt calibration curve was prepared with a known concentration of salt with suitable dilutions.

Determination of PEG concentration was performed by measuring the refractive index using a hand refractometer (N-1α ATAGO Co. Ltd, Japan). Calibration curves were prepared with a known concentration of PEG in the homogeneous regions of the binodal diagram of the individual ATPE (Nagaraja and Iyyaswami, 2015).

Prediction of diffusion coefficients

Estimation of the isothermal diffusion coefficient (Di) for CPC in both phases of ATPE utilised the correlation for protein diffusion coefficients proposed by Novak *et al.* (2015), which is based on the solute's molecular weight (M) and radius of gyration (R_G) as shown in Eq. 7:

$$D_i = \frac{6.85\times10^{-15}\times T}{\eta\cdot\sqrt{M^{1/3}R_G}} \quad \text{(Eq. 7)}$$

where, η = dynamic viscosity of the solvent in Pa.s (dependent on operating temperature), and T = temperature in K. Meanwhile for CPC, M and R_G were 18500 Da and 54.1 Å, respectively (Thaisamak *et al.,*2020)

In 1879, the Swiss scientist, Charles Soret, discovered that a salt solution contained in a tube with the two ends at different temperatures generated a salt flux and temperature gradient, resulting in steady-state conditions in a concentration gradient. Although German scientist, Ludwig, described the same phenomenon several years before in a one-page report, the name "Soret effect" is usually attributed to the mass separation induced by temperature gradients (Platten, 2006). The Soret coefficient (S_T) in binary systems is defined as shown in Eq. 8:

$$S_T = \frac{D_T}{D_i} \quad \text{(Eq. 8)}$$

where, D_T = thermal diffusion coefficient.

$$J_x(\text{x,t}) = -\rho \text{D}_i \frac{\partial c}{\partial x} - \rho D_T c(1-c)\frac{\Delta T}{a} \quad \text{(Eq. 9)}$$

Using this approximation, if the concentration difference remains small, the thermal diffusive contribution is ruled out of Eq. 9 (Costesèque *et al.,* 2004), but is reintroduced via the boundary conditions that also uses the same approximation. Then, Eq. 9 becomes Eq. 10:

$$\frac{\partial c}{\partial t} = \text{D}_i \frac{\partial^2 c}{\partial x^2} \quad \text{(Eq. 10)}$$

The particular solutions of the diffusion equation, besides the stationary one is written as $c_\infty = c(x, t=\infty)$ are typically of the form:

$$c(x,y) = (A_n \cos\lambda_n x + B_n \sin\lambda_n x)\,\text{e}^{-\lambda_n^2 D_t} \quad \text{(Eq. 11)}$$

Finally, the general solution, which is the sum of all the particular solutions, is:

$$c(x,y) = c_\infty(x) + \sum_{n=1}^{\infty} A_n \cos\left(\frac{n\pi x}{a}\right)\exp\left(-n^2\frac{t}{\tau_D}\right) \quad \text{(Eq. 12)}$$

with:

$$c_\infty(x) = c_0 + \frac{D_T}{D_i}c_0(1-c_0)\frac{\Delta T}{h}\left(\frac{h}{2}-x\right) \quad \text{(Eq. 13)}$$

and:

$$A_n = -\frac{4}{n^2\pi^2}\frac{D_T}{D_i}c_0(1-c_0)\Delta T \quad \text{(with n odd)} \quad \text{(Eq. 14)}$$

Knowing $c(x,y)$, the concentration difference between the bottom and the top $c(o,t)$ - $c(h,t)$ within the cell is:

$$\frac{\Delta c(t)}{\Delta T} = \frac{D_T}{D_i}c_0(1-c_0)\left[1-\frac{8}{\pi^2}\sum_{n\ odd}\frac{e^{-n^2\frac{t}{\tau_D}}}{n^2}\right] \quad \text{(Eq. 15)}$$

with:

$$\tau_D = \frac{(V/A)^2}{\pi^2 D_i} \quad \text{(Eq. 16)}$$

Therefore, it is necessary to collect experimental variations of concentration from initial to separation completely. The measured values are then divided by the corresponding ΔT and the experimental curve is drawn. Next, a curve fitting procedure using statistical

software is used to set two parameters, the Soret coefficient (S_T) and mass diffusion time (τ_D) of Eq. (15), and adjusted until Eq. (15) fits the experimental curve. So, the S_T and τ_D values are evaluated using the mathematical fitting procedure.

The enhancement factor (EF) was used to quantify the thermal diffusion effect. EF is defined by the ratio of the diffusion coefficient of the conventional process (D_0) and the diffusion coefficient of aqueous two-phase extraction coupled with different temperatures (D_{dT}) (summation of isothermal and thermal diffusion coefficient). The equation is defined as:

$$EF = \frac{D_{dT}}{D_0} \quad \text{(Eq. 10)}$$

Results and discussion

Effect of different temperature (dT) on ATPE process

Experiments were performed at three levels of dT (15, 25, and 35°C), while the other parameters were kept constant (PEG 4000 with saturation 6% [w/v], potassium phosphates 15% [w/v], pH 7.0, total volume 100 mL, and separation time of 2 h). Figure 2A shows the results of the effect of dT on CPC concentration in the top phase at complete separation. The highest concentration was found at dT 25°C (13.932 g/L), significantly higher than the conventional method. According to the result in Figure 2B, the highest purity and yield values were 2.337 and 91.18, respectively, which were obtained under the condition of dT at 25°C (Table 1).

The reason for this is a potential phenomenon which decreases the viscosity of the mixture and enhances the solvent solubility and diffusion capacity (Zhang *et al.,* 2015). Meanwhile, when the dT was greater than 35°C, the purity of CPC extracted in ATPE of PEG 4000 and potassium phosphates decreased sharply due to an observed significant reduction in the absorption strength at 620 nm, due to the characteristic absorption band of the CPC protein. Conversely, this means that the hydrogen bonding which interacted between the surface water of protein was destroyed at high temperatures.

SDS-PAGE analysis was used to confirm the purity of CPC and is shown in Figure 2C. Lane 1 indicates the molecular marker, while Lane 2 shows the pure CPC which is clearly visible in a band. This might be that during ATPE, the majority of the contaminant protein present in the crude extract was partitioned at the bottom phase, resulting in increased CPC purity. The molecular weights of CPC show two bands, α and β, with approximate sizes of 18 and 19 kDa, respectively. These results are similar to those obtained by Patil *et al.* (2006) and Chethana *et al.* (2015) who presented the molecular weight of CPC from *S. platensis* by SDS-PAGE.

Effect of different temperature (dT) on phase partitioning in the ATPE process

The partition coefficient (K) of purification increased monotonically with different temperatures, as shown in Table 1. To fully understand this behaviour, it is important to investigate the effect of the different temperatures on the CPC concentrations. Then, the concentration of salt and PEG in both phases can be determined using a conductivity meter and a refractometer, respectively. A set of experiments were subsequently conducted to generate binodal curves at different temperatures varying from 15 to 35°C. Binodal curves obtained by plotting the concentrations of PEG and phosphate salt in the top and bottom phases are shown in Figure 3, in which the binodal moved away from the origin when the temperature increased, causing the tie line length (TLL) to decrease. This can result in a faster and more complete separation due to preferential binding of the water molecules to the polar salt surface instead of the PEG at a higher temperature difference. These results have also been observed by several authors such as Sé and Aznar (2002), Gautam and Simon (2006), and Carvalho *et al.* (2007).

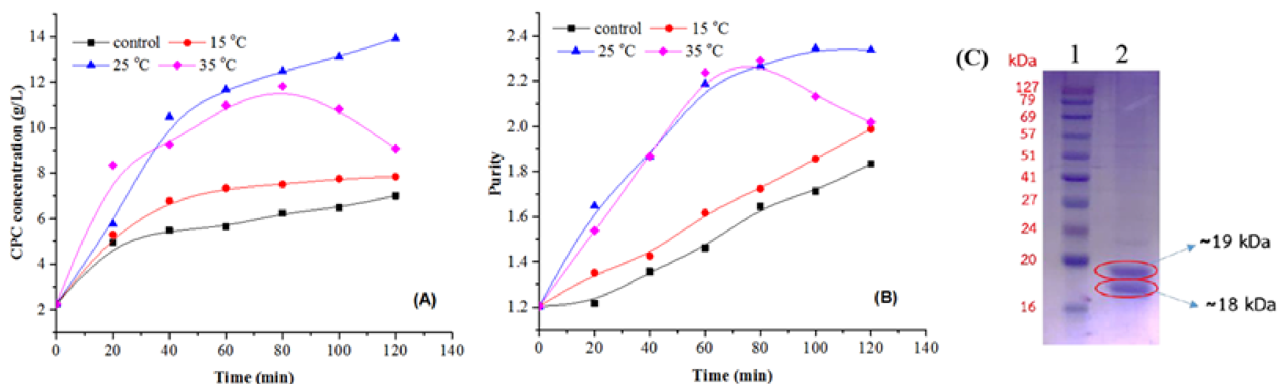

Figure 2. (A) Concentration profiles of CPC in top phase, and (B) purity of CPC in top phase, and (C) SDS PAGE of CPC. Lanes 1 and 2 were molecular marker and pure CPC, respectively.

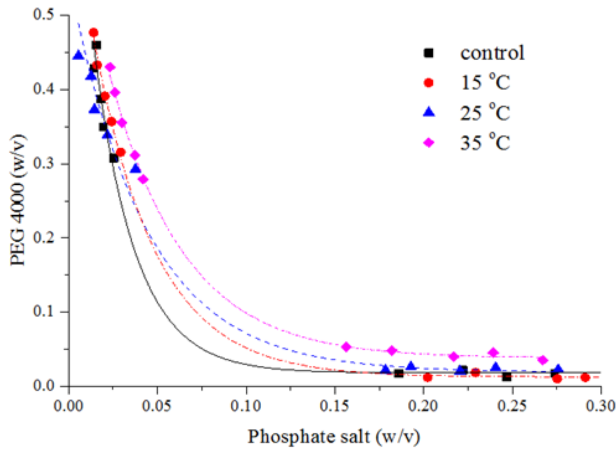

Figure 3. Phase diagram of PEG4000 and potassium phosphates in aqueous two-phase extraction.

Differential temperature on diffusion coefficients

Table 2 shows the experiments to determine the isothermal diffusion (from Eq. 7) thermal diffusion and Soret coefficients (from Eq. 15) of aqueous two-phase extraction, the corresponding Soret coefficients, mass diffusion times, and deduced isothermal diffusion and thermal diffusion coefficients.

Different temperatures had a significant effect on diffusion coefficient, as observed in Table 2, which shows that the isothermal diffusion coefficient (D_i) at dT of 25°C and the conventional method were 2.281 × 10^{-12} and 2.104 × 10^{-12} m^2/s, respectively. The Soret coefficient and thermal diffusion were found to be highest at dT of 25°C. This is why the ATPE coupled with different temperature technique is an effective driving force for the mass transfer of the CPC concentration. It might be relative importance of the diffusion coefficient due to

Table 1. Effect of different temperature on concentration and purity in ATPE.

dT	Control	15°C	25°C	35°C
CPC Concentration (g/L)				
Top phase	7.012 ± 0.29	7.840 ± 0.54	13.932 ± 1.25	9.069 ± 0.84
Bottom phase	1.975 ± 0.23	1.112 ± 0.25	0.758 ± 0.14	1.028 ± 0.36
Partition coefficient (K)	3.550	7.050	18.380	8.822
EP of CPC				
Top phase	1.839 ± 0.04	1.889 ± 0.29	2.337 ± 0.20	1.918 ± 0.33
Bottom phase	0.812 ± 0.05	0.856 ± 0.18	0.569 ± 0.13	0.746 ± 0.17
Purification factor (PF)	1.663	1.708	2.113	1.734
V_r and RC				
Volume of top phase (V_T, mL)	48 ± 2	39 ± 3	36 ± 3	43 ± 4
Volume of bottom phase (V_B, mL)	52 ± 3	61 ± 2	64 ± 3	57 ± 3
Volume ratio (V_r)	0.92	0.64	0.56	0.75
Recovery yield (RC, %)	76.62	81.84	91.18	86.94

Table 2. The mass diffusion times, Soret coefficient, the isothermal diffusion coefficient, and the thermal diffusion coefficient of ATPE.

dT	τ_D (min)	S_T (1/K)	D_i (m^2/s) × 10^{-12}	D_T ($m^2/s{\cdot}K$) × 10^{-13}	EF
control			2.104		-
15°C	23.325	0.162	2.210	3.580	1.220
25°C	52.797	0.211	2.281	4.813	1.313
35°C	16.753	0.192	2.351	2.398	1.231

temperature variations. Schimpf and Semenov (2000) extended the relations between the thermal diffusion coefficient and viscosity, in which reducing the viscosity, and resulted in the thermal diffusion coefficient increase. Therefore, the ATPE coupled with different temperatures affects viscosity and buoyancy forces, thereby resulting in improved mass transfer efficiency between the processes.

The value of isothermal diffusion and thermal diffusion coefficients were different in each experiment. From their values, it is possible to deduce that the enhancement factor (EF) is the factor that characterises the efficiency of a process compared with the conventional method, as shown in Table 2. The different temperature at 25°C was enhanced around 30% from the conventional method. This result indicates that different temperature not only affects efficiency of the ATPE process, but also enhances the efficiency of the transport phenomena during the process. This finding is similar to Chan *et al.* (2003) who concluded that the Soret coefficient and isothermal diffusion coefficient are driven by temperature gradients and concentration gradients.

Conclusion

Aqueous two-phase extraction (ATPE) with combined different temperatures (dT) is a new finding and is suitable for C-phycocyanin purification. The optimal different temperatures of the process were achieved by ATPE conducted at dT of 25°C. Furthermore, the present work successfully investigated the isothermal and thermal diffusion of the purification process using ATPE. The isothermal diffusion coefficient (D_i) significantly increased with increased dT. The Soret effect (S_T) and thermal diffusion coefficient (D_T) were obtained at a high dT, but this should not go beyond 25°C. It can thus be concluded that the Soret effect (S_T) and thermal diffusion coefficient (D_T) were good parameters for explaining C-phycocyanin purification efficiency. Further studies should be undertaken into the steps of purification, backward purification, or using continuous process to further increase purity.

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