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Research Article

Purification of Phycocyanin from *Spirulina platensis* Using Natural Deep Eutectic Solvents with Varied Hydrogen Bond Donor

Eirene Tentua[1], Safrina Dyah Hardiningtyas[1]*, and Iriani Setyaningsih[1,2]

[1]Department of Aquatic Product Technology, Faculty of Fisheries and Marine Science, IPB University, Bogor, Indonesia
[2]Division of Marine Biotechnology, Center for Coastal and Marine Resources Studies, IPB University, Bogor, Indonesia

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*) Corresponding author:
E-mail: safrina_dyah@apps.ipb.ac.id

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Abstract

Phycocyanin, a pharmacologically potent blue pigment extracted from *Spirulina platensis*, requires optimal purity for effective utilization. Traditional purification methods, although effective, are impeded by time and cost constraints. Addressing this challenge, aqueous two-phase systems (ATPs) incorporating natural deep eutectic solvents (NaDES) emerge as promising alternatives. These systems offer operational simplicity and cost-effectiveness, ensuring efficient purification with high purity and optimal recovery of phycocyanin. This study investigated the impact of various types of hydrogen bond donors (HBD) within choline chloride-based natural deep eutectic solvents (NaDES) on the purification of phycocyanin. Phycocyanin was extracted from *Spirulina platensis* biomass through ultrasonication and purified using an aqueous two-phase system with various HBD of NaDES, including urea, glycerol, and citric acid. The experimental design adhered to a completely randomized design. The initial purity index and yield of crude extract phycocyanin were 0.54±0.02 and 12.79±0.27%, respectively. The NaDES system with ChCl:citric acid exhibited superior performance, demonstrating a high purity index and recovery (2.3-fold, 71.83±2.36%) compared to ammonium sulfate (1.5-fold, 70.15±4.10%). The obtained phycocyanin was partially pure compared to commercial phycocyanin (purity index: 1.60), as indicated by SDS-PAGE. Moreover, the antioxidant activity of phycocyanin was enhanced post-purification, evident in the IC_{50} value of 40.54 ppm. In summary, organic acid-based NaDES has proven effective in increasing the purity and achieving a significant recovery percentage of phycocyanin compared to conventional ammonium sulfate methods. The antioxidant activity of phycocyanin was enhanced after purification. These results indicate the promising potential of NaDES-based ATP systems for producing functional protein-based ingredients, exemplified by phycocyanin.

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1. Introduction

Spirulina platensis, a microalga from the phylum Cyanobacteria, can synthesize a pigment known as phycocyanin. This distinctive blue pigment originates from the phycobiliprotein group and consists of two subunit proteins, globular α and β chains, with molecular weights ranging from 112 kDa to 280 kDa (Figueira *et al*., 2018) its large-scale purification remains problematic and expensive. The aim of this work was to establish the best process for obtaining C-phycocyanin of different purities for different applications. The first step of this study was the maximization of the ultrafiltration process. Under the best ultrafiltration conditions, we evaluated the application of ultrafiltration, precipitation, and ion exchange chromatography (expanded and fixed beds. Phycocyanin exhibits anticancer, antioxidant, antibacterial, antidiabetic, anti-inflammatory, and immunomodulatory effects (Grover *et al*., 2021; Nege *et al*., 2020; Prabakaran *et al*., 2020). These potential health effects were positively correlated with the purity of phycocyanin. Generally, pure phycocyanin is obtained through a multi-stage process involving ultrasonication, precipitation with ammonium sulfate, dialysis, and ion-exchange chromatography (Jaeschke *et al*., 2021), ultimately resulting in specific phycocyanin yield, concentration, and purity levels. Although effective, this method is inefficient owing to its time-consuming nature and high processing costs. Therefore, an appropriate purification method is needed to obtain pure phycocyanin, maximize the extraction yield, and minimize the processing costs.

Aqueous two-phase systems (ATPs) refer to systems formed when combinations of hydrophilic solutes, such as polymers or a combination of polymers and specific salts, demonstrate incompatibility in aqueous solutions above critical concentrations (Ahmed *et al*., 2021). ATPs are categorized into five types: polymer–polymer, polymer–salt, alcohol–salt, micellar, and ionic liquid-based. Among these, IL-based ATPs can form a transparent solution without emulsification, despite being difficult and expensive to prepare (Nie *et al*., 2022). As an alternative, deep eutectic solvents (DES) have emerged as a new generation of solvents that form eutectic mixtures through hydrogen bond interactions. Natural deep eutectic solvents (NaDES) are derivatives of DES that are deemed natural, drawing their components from primary metabolite groups. Choline chloride (ChCl) is frequently used as a hydrogen bond acceptor (HBA) among quaternary salts because of its cost-effectiveness and ease of extraction from biomass (Jurić *et al*., 2021). Simultaneously, hydrogen bond donor (HBD) components typically originate from three groups: amines, alcohols, and acids (Singh *et al*., 2021). NaDES has numerous advantages over conventional separation methods such as cost-effectiveness, environmental sustainability, and degradability (Bowen *et al*., 2022).

In recent studies, ChCl-based DES have been demonstrated to be effective and environmentally friendly for the selective separation of proteins. According to Wang *et al*. (2020), the ChCl:urea (1:2) combination significantly increases the purity index of phycocyanin after purification, rising from 1.27 in the crude extract to 3.307; the recovery value only reaches 63.04%. Among alcohol groups, ChCl:glycerol (1:1) exhibits an enhanced extraction yield of 60% for soy protein (Chen *et al*., 2021). Meanwhile, from the acid group, ChCl:citric acid (1:1) shows a wheat protein extraction recovery of 70% (Cannavacciuolo *et al*., 2023). These studies indicate the ability of urea, glycerol, and citric acid as potential HBD as components to purify proteins. Despite these notable advancements, there remains a rarely explored area concerning the representation of each HBD group in NaDES for phycocyanin purification.

The primary objective of this study was to investigate the influence of different HBD types in ChCl-based NaDES for phycocyanin purification. Expected outcomes include the production of pure, sustainable, and cost-effective phycocyanin, with an additional exploration of its impact on antioxidant activity. By filling the gap in understanding the representation of each HBD group in NaDES, this research aims to contribute significantly to the advancement of phycocyanin purification methodologies. Ultimately, the study envisions broader applications of purified phycocyanin in various industries, capitalizing on its pharmacological potential.

2. Materials and Methods

2.1 Material

The main material used in this study was *Spirulina platensis* cultivated at the Laboratory of Biotechnology, Department of Aquatic Product Technology, IPB University. Other materials used were seawater, Walne fertilizer, distilled water, ammonium sulfate (Merck, Germany), choline chloride (Himedia, USA), urea (Vivantis, Malaysia), citric acid (Himedia, USA), glycerol (Himedia, USA), potassium phosphate dibasic anhydrous (Himedia, USA), commercial phycocyanin (Tokyo Chemical Industry, Japan), 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, USA), Japan medical science visking tube (Monotaro, Japan), 30% Acrylamide/Bis Solution 37.5:1 (Biorad,

USA), Bio-Safe™ Coomassie Stain (Biorad, USA).

2.1.1 Ethical approval

This study does not require approval because it does not use experimental animals.

2.2 Cultivation and Harvesting of Spirulina plaensis

The cultivation of *S. platensis*, following the method outlined by (Setyaningsih *et al*., 2013), involved using 15 ppt seawater supplemented with Walne media. In this process, a glass container containing 10 L of 15 ppt seawater has 0.1% (v/v) Walne media added, and *S. platensis* seeds were mixed into the seawater at a proportion of 20% (v/v). The *S. platensis* culture was then exposed to illumination from a tubular lamp (Philips, 40 W) with an intensity of 3000 lux and subjected to aeration for 24 hours. Monitoring the growth of *S. platensis* was done by measuring the optical density (OD) once a day using a spectrophotometer (VIS-723G) at a wavelength of 670 nm until the OD value reached ≥0.5 (Afriani *et al*., 2018). Harvesting took place after 14 days of cultivation using a 400-mesh nylon filter, and the wet biomass obtained was subsequently dried at 5–8°C for 48 hours.

2.3 Synthesis of NaDES

The components of the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) of the DES are detailed in Table 1. Deep eutectic solvents (DES) were formulated by blending HBA and HBD components in appropriate molar ratios. The resulting mixture was stirred at 80°C at about 500 rpm until a homogeneous transparent liquid is achieved, approximately 15 minutes.

Table 1. Components used in the synthesis of natural deep eutectic solvents.

HBA	HBD	Molar Ratio	Abbreviation
Choline chloride	Urea	1:2	ChCl:U
Choline chloride	Citric acid	1:1	ChCl:CA
Choline chloride	Glycerol	1:1	ChCl:G

2.4 Extraction of Phycocyanin

The extraction of phycocyanin from *S. platensis* biomass followed the ultrasonication method as described by Gustiningtyas *et al*. (2020). The dry biomass of *S. platensis* was dissolved in demineralized water at a ratio of 1:24 (v/v) and subjected to ultrasonicator bath (Branson 1510) with a frequency of 42 kHz for 20 minutes at a temperature of 4°C. The sample was then centrifuged at 11200×g at 4°C for 15 minutes using a centrifuge (Ohaus FC5718R). Phycocyanin purification involved two different methods, namely the precipitation method and the use of natural deep eutectic solvents.

2.5 Purification of Phycocyanin

The precipitation method for phycocyanin purification followed the procedure outlined by Gustiningtyas *et al*. (2020). The supernatant was precipitated with 50% ammonium sulfate for 2 hours, followed by centrifugation at 1792×g at 4°C for 20 minutes. The concentration of samples was achieved using a dialysis bag with a molecular weight limit of 12-14 kDa. The dialysis process extended over 3 days, with water changes every 4 hours at a temperature of 4°C.

The purification of phycocyanin through ATPs with NaDES followed the procedure outlined by Wang *et al*. (2020). 0.5 mg of phycocyanin crude extract, 3.25 g of DES, and 5 mL of K_2HPO_4 (0.90 g/mL) were introduced into 50 mL vials and stirred at 25°C for 2 hours. The mixture was then centrifuged at 2000 rpm for 10 minutes to yield two liquid-liquid phases. The DES-rich phase is collected and subjected to centrifugation at 12000 rpm for 10 minutes, resulting in a precipitate that was redissolved in distilled water. The phycocyanin produced was analyzed by measuring absorbance at 615, 620, and 652 nm, to determine its characteristics, including concentration, purity index, and recovery. The concentration, purity index, and recovery of phycocyanin were calculated using the Bennett and Bogobad (1973) equation as follow:

$$\text{PC (mg/mL)} = \frac{(\text{OD } 620) - 0{,}474\ (\text{OD } 652)}{5{,}34} \quad \ldots \text{Eq 1}$$

$$\text{Purity Index} = \frac{\text{OD } 615}{\text{OD } 280} \quad \ldots \text{Eq 2}$$

$$\text{Recovery (\%)} = \frac{\text{PC (mg/ml)} \times \text{collected volume (mL)}}{\text{PC initial extract (mg/ml)} \times \text{initial volume (mL)}} \times 100\% \quad \ldots \text{Eq 3}$$

2.6 Viscosity

The viscosity of the Deep Eutectic Solvent (DES) was determined using a digital viscometer (Brookfield RV DV-E230), with measurements con-

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ducted at a temperature of 298.15 K, as specified in the study by Mjalli and Naser (2015). In the experimental procedure, 200 mL of both the chitosan solution and water-soluble chitosan were added separately to a beaker. The viscometer spindle was then gradually lowered until immersed in the sample, and the resulting viscosity value was observed on the viscometer display.

2.7 Phase Forming Ability

The phase diagram determination utilized the cloud point titration method at room temperature, following the approach outlined in the study by (Xu *et al*., 2020). 1 mL of the DES was measured and placed into a 15 mL centrifuge tube. Subsequently, 1.0 g/mL K_2HPO_4 solution was incrementally added drop by drop to the centrifuge tube, and the mixture was shaken until it became cloudy, indicating the formation of the DES/K_2HPO_4. The mass of the added K_2HPO_4 solution was recorded. Distilled water was then added drop by drop until a single phase was achieved, with the mass of water recorded after each addition, capturing the difference in mass before and after adding water. This entire process was repeated iteratively to gather sufficient data for constructing a liquid equilibrium phase diagram. The mass fractions of K_2HPO_4 and DES solutions were represented on the X-axis and Y-axis, respectively.

2.8 Protein Profile Analysis using SDS-PAGE

Protein profile analysis on phycocyanin was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The analysis was carried out by applying 20 µl of protein onto a 15% (v/v) running and 6% stacking polyacrylamide slab gel (30% Acrylamide/Bis Solution, 37.5:1, Biorad) with a thickness of 1.5 mm. The gel was prepared using Laemmli buffer. Before loading, samples underwent pre-incubation with a loading buffer consisting of 50 µl β-mercaptoethanol and 950 µl Laemmli sample buffer (1:1) for 5 minutes at 95°C. Gel electrophoresis was conducted at room temperature using Mini-Protean TGX Precast Gels, and protein bands were visualized through staining with Bio-Safe™ Coomassie Stain (Biorad). The molecular weight of subunits was determined by calibrating the gel using molecular weight markers.

2.9 Analysis of Antioxidant Activity

The antioxidant activity of pure phycocyanin was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, as outlined by (Sharma and Bhat, 2009). For this analysis, methanol was utilized to dilute the samples at concentrations of 5, 15, 30, 45, 60, and 75 ppm. Subsequently, 600 µL of the sample solution was dispensed into 200 µL of DPPH (200 µM), and the absorbance was measured at a wavelength of 517 nm using spectrophotometer (VIS-723G). The percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of sample}} \times 100\% . \quad \text{Eq 4}$$

2.10 Analysis Data

The experimental design model employed in this study was a Completely Randomized Design (CRD), incorporating various phycocyanin purification methods. The objective was to identify the optimal purification method based on the analysis of physical properties, including purity index, concentration, and recovery of phycocyanin. The data evaluation revealed significant values, prompting the execution of the Duncan test using Statistical Process for Social Science (SPSS) version 29.0.

3. Results and Discussion

3.1 Characterization of NaDES

Viscosity and pH are crucial physical characteristics of DES, especially for evaluating the flow ease of DES and their utility in phycocyanin purification. Table 2 presents the viscosity and pH values of choline chloride paired with various HBD, such as urea, glycerol, and citric acid. Experimental results demonstrate that ChCl:U exhibits higher viscosity compared to ChCl:G and ChCl:CA at their respective molar ratios, suggesting the greater viscosity or flow resistance tendency of ChCl:U DES.

Table 2. Physical Characterization of NaDES.

Parameter	ChCl:U	ChCl:G	ChCl:CA
Viscosity (cP)	515.5±6.36	275.5±0.71	302±1.41
pH	7.9±0.04	8.205±0.02	4.455±0.05

The viscosity of DES correlates with the molecular structure and hydrogen bonding interactions, which can be explained by the relative hydrogen bonding capacity of each HBD. Urea, with four hydrogen bonds, exerts a stronger influence compared to

glycerol and citric acid, each contributing three hydrogen bonds. ChCl:U exhibits a stronger hydrogen-bond network in the bulk compound. As discussed earlier, the partially retained hydrogen bond network of urea in ChCl:U is reflected in its significant viscosity. This study is consistent with the findings of Stefanovic *et al.* (2017), who observed that ChCl:U had the highest viscosity at room temperature (1200 mPa.s), far exceeding that of glycerol and ethylene glycol.

The noticeable pH differences among the three DES systems reflect the varying acid-base properties of their constituent components. In this context, the influence of urea and glycerol on ChCl:U and ChCl:G, respectively, on pH may not be dominant, resulting in more limited differences. However, the presence of citric acid in ChCl:CA appears to have a more significant impact, substantially lowering the pH. This highlights the pH sensitivity of DES to their molecular composition, wherein the presence of acidic compounds can significantly shift the pH balance.

3.2 Phase Behavior of NaDES on the ATP System

Liquid-liquid equilibrium data are essential for the design of water-based two-phase extraction processes. In this study, K_2HPO_4 was selected as the phase-separating salt due to its high solubility in water and strong phase-forming ability. K_2HPO_4 demonstrated superior phase separation performance compared to other inorganic salts such as K_2CO_3 and Na_2CO_3 (Yao *et al.*, 2017). A concentration of 0.9 g/mL K_2HPO_4 exhibited significant two-phase formation with increasing DES (Xu *et al.*, 2015).

The phase diagram was determined at a temperature of 28°C, and from the visualization in Figure 2, it is evident that the ability of DES to form phases can be ranked as follows: ChCl:U > ChCl:CA > ChCl:G. Variations in the phase-forming ability of DES are related to the degree of affinity of DES for water molecules (Freire *et al.*, 2012). In general, the lower the affinity for water, the smaller the amount of salt required to induce two-phase separation, resulting in a binodal curve closer to the axis and a broader biphasic region. In this context, the pattern of phase change behavior, as outlined in the study by Wang *et al.* (2020), indicates that with an increasing amount of NaDES ChCl:U, the volume of the upper phase increases, and the volume ratio between the upper and lower phases also increases. Conversely, the volume of the upper phase decreased with increasing K_2HPO_4 concentration. The findings of this study indicate that alterations in phase constituents can lead to modifications in the characteristics of the phases. Consequently, it can be inferred that competition for water molecules exists between these two phases.

3.3 Crude Extract of Phycocyanin from Spirulina platensis

The growth of *S. platensis* was visually monitored throughout the cultivation process, as depicted in Figure 1a. The recorded optical density (OD) value at the harvesting stage was 0.54, surpassing the threshold of 0.5 associated with the rapid growth phase of *Spirulina* (Afriani *et al.*, 2018). Analysis of *Spirulina* biomass characteristics revealed filament lengths ranging from 650 to 2500 µm (Figure 1b), contributing significantly to overall biomass production. Additionally, the dis-

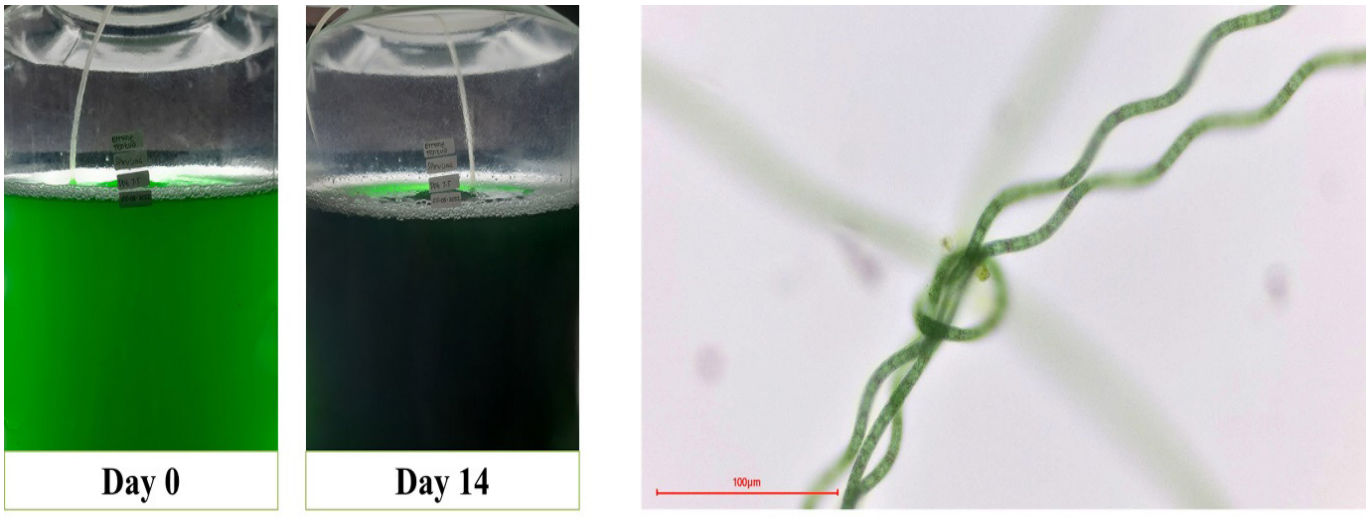

Figure 1. *Spirulina platensis*: (a) *S. platensis* culture, (b) *S. platensis* cell.

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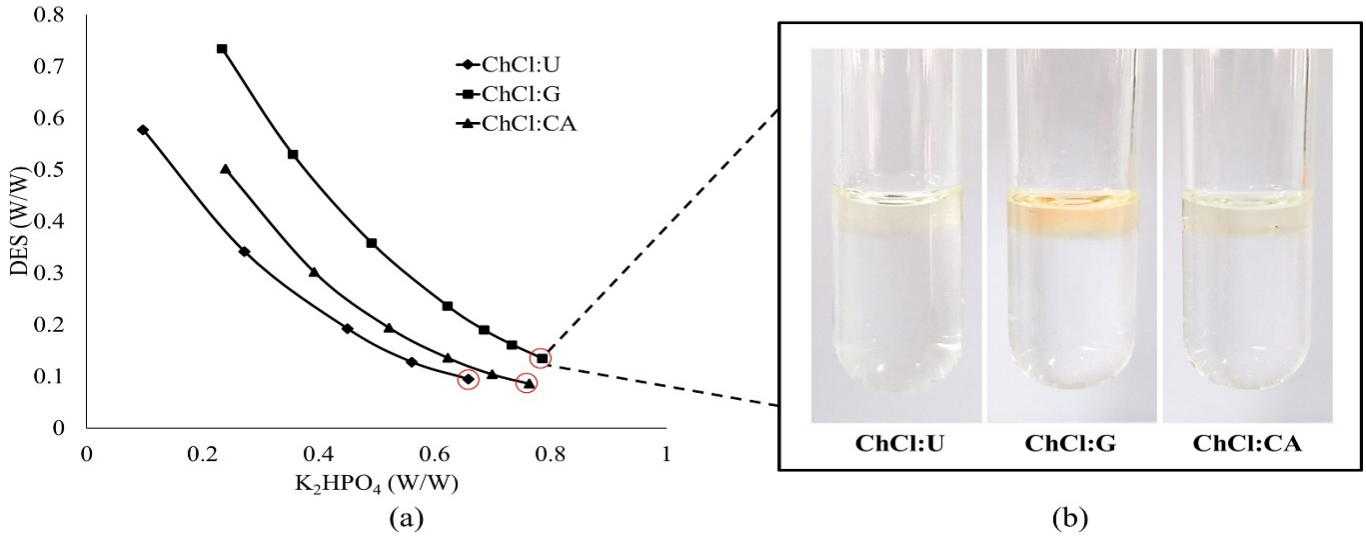

Figure 2. Aqueous two-phase system: (a) equilibrium phase forming, (b) appearance formed during the equilibrium phase of DES and K_2HPO_4. Note: the red circle indicates the endpoint of the equilibrium phase.

tinctive spiral morphology inherent to *Spirulina* cells was observed, representing a characteristic feature of this microorganism. The produced biomass, in both wet and dry forms, exhibited substantial yields of 1.73±0.44 g/L and 0.21±0.06 g/L, respectively, with a moisture content of 6.50±0.15% in dry biomass. These findings underscore the efficiency of the cultivation process in yielding significant *Spirulina* biomass under optimized growth conditions.

In this study, ultrasonication was employed as a methodological approach to extract phycocyanin, a blue pigment found in *Spirulina platensis*. The ultrasonic extraction process relies on the utilization of high-frequency sound waves to disrupt microalgae cells. This method efficiently breaks cell walls, which is a crucial step for releasing intracellular components, especially phycocyanin (Duangsee *et al.*, 2009). The obtained yield, concentration, and purity index of the phycocyanin extract were 12.79±0.27%, 2.56±0.05 mg/mL, and 0.54±0.02, respectively. A study conducted by Hardiningtyas *et al.* (2022) found that applying ultrasonication for 10 minutes resulted in a phycocyanin extract yield of 10.65±1.82%. This indicates an enhanced extraction efficiency compared to prior research, highlighting the effectiveness of ultrasonication in extracting bioactive compounds like phycocyanin.

3.4 Effect of Various Hydrogen Bond Donors-NaDES on Phycocyanin Characteristics

In this study, an aqueous two-phase system (ATPs) based on NaDES and K_2HPO_4 was utilized as a method for purifying phycocyanin. NaDES consists of an HBA and an HBD, forming strong hydrogen bond interactions crucial for system stability and biomolecule selectivity (Farias *et al.*, 2020). ChCl, acting as an HBA, forms a stable solvent system when combined with HBD, such as urea, glycerol, and citric acid. The experimental results demonstrated the effectiveness of the NaDES and K_2HPO_4-based ATP system in separating phycocyanin from the mixed solution. After purifying phycocyanin, two phases are formed within the ATP system; the upper phase contains NaDES and phycocyanin, while the lower phase contains K_2HPO_4 and impurities (Figure 3). This is supported by Iqbal *et al.* (2016), who explained that proteins tend to accumulate in the top phase due to the ionic strength in the salt-rich phase, which affects the interaction of protein in the ATP system. In the purification process, NaDES forms aggregates or structures surrounding the protein, which means that phycocyanin-NaDES complexes were formed in the NaDES-rich phase. This process may occur because of interactions between the NaDES components and proteins, which enhance the efficiency of separating the protein from impurities. This is consistent with previous research showing that the conformation of phycocyanin exhibits similar results throughout the purification using NaDES and the formation of aggregates in the DES-rich phase (Wang *et al.*, 2020). The pure phycocyanin was then obtained by centrifugation to separate it from NaDES (Figure 4).

The recovery of phycocyanin from NaDES showed the highest value when using the combination of ChCl with citric acid (1:1), reaching 71.83±2.76%

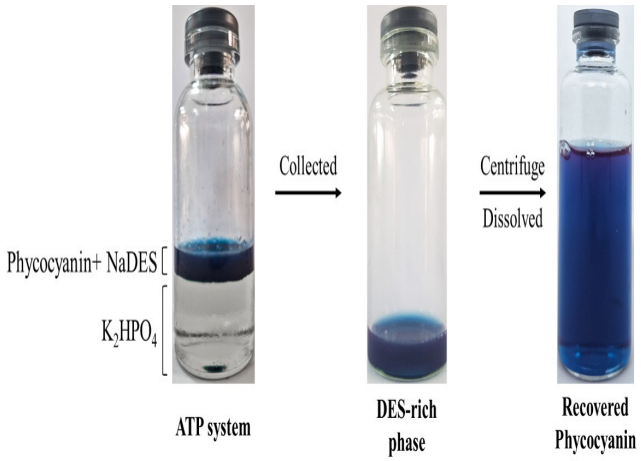

Figure 3. Purification of phycocyanin using NaDES - based ATP system.

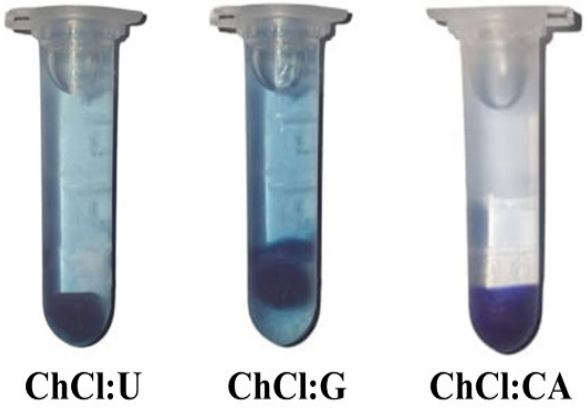

Figure 4. Post-separation of phycocyanin from residual NaDES.

(Figure 5), with a purity index of 1.25±0.08 (Figure 6). The high recovery value is presumed to be due to dif ferences in the final pH of the system after mixing with K_2HPO_4 0.9 mg/ml. The optimal pH for phycocyanin purification from *S. platensis* falls within the range of 6-6.9 (Liao *et al*., 2011; Patil and Raghavarao, 2007). The pH of systems with ChCl:CA DES approached neutrality (pH 6.14), conducive to phycocyanin stability. In contrast, systems with ChCl:U and ChCl:G tended to be alkaline (pH >8), potentially denaturing proteins, as observed by the change in phycocyanin color post-purification. In alkaline environments, the protein component of phycocyanin tends to unfold, leading to alterations in the shape and structure of the chromophores (Adjali *et al*., 2022). Additionally, the use of urea as an HBD has the potential for significant denaturation at high concentrations, causing structural changes in proteins and resulting in the loss of biological activity due to the loss of the original structure (Almarza *et al*., 2009). Structural changes in proteins in

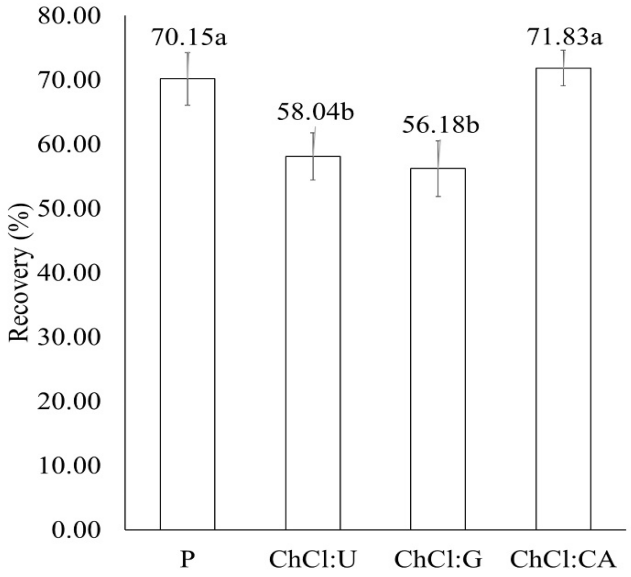

Figure 5. Recovery of phycocyanin from ATPs and precipitation. Different superscripts show significant differences ($p < 0.05$).

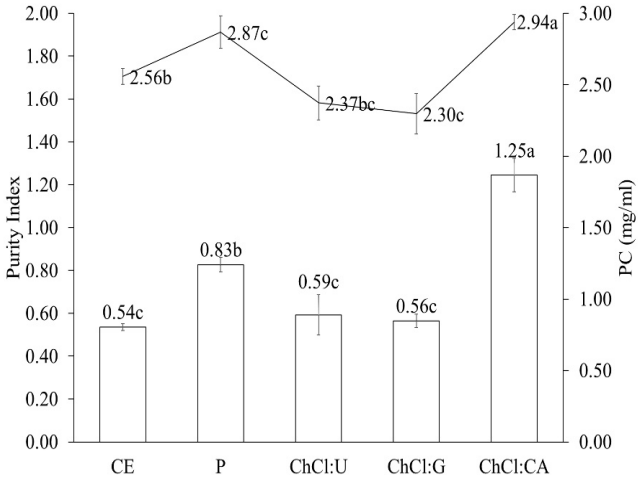

Figure 6. Purity index and concentration of phycocyanin. Note: CE: crude extract, P: precipitation. Different superscripts show significant differences ($p < 0.05$).

the ChCl:U and ChCl:G systems also lead to difficulties in collecting phycocyanin from NaDES, resulting in low recovery and purity index. The effectiveness of this purification was demonstrated by measuring the absorbance at wavelengths of 620 and 280 nm (Supplementary 1). A wavelength of 280 nm detected total protein in the sample, whereas a wavelength of 620 nm was the peak absorption for phycocyanin. The decrease in absorbance at 280 nm indicated a reduction in total protein in the sample after purification. However, the absorbance of ChCl:CA at 280 nm was still higher than that of commercial phycocyanin (TCI), suggesting that purified phycocyanin remained partially pure.

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The purification results were confirmed using SDS-PAGE analysis. Molecular weight analysis of phycocyanin from *S. platensis* revealed two distinct polypeptides: the α-unit with a lower molecular weight and the β-unit with a higher molecular weight. Specificaly, the molecular weight of purified phycocyanin was within the range of 15-20 kDa (Figure 7). In this study, the α-subunit polypeptide had a molecular weight of approximately 16 kDa, and the β-subunit had a molecular weight of approximately 17 kDa, which is consistent with previous research findings (Kumar *et al*., 2014). Gel electrophoresis post-purification showed clearer protein band patterns than the crude extract, with the disappearance of several bands, indicating the successful removal of contaminants and impurities, enhancing the purity of the obtained phycocyanin. However, the result after purification still showed visible bands between 20-25 kDa, indicating that the purification process did not completely remove the impurities. This suggests that further optimisation is required to achieve a purity level comparable to that of commercial phycocyanin.

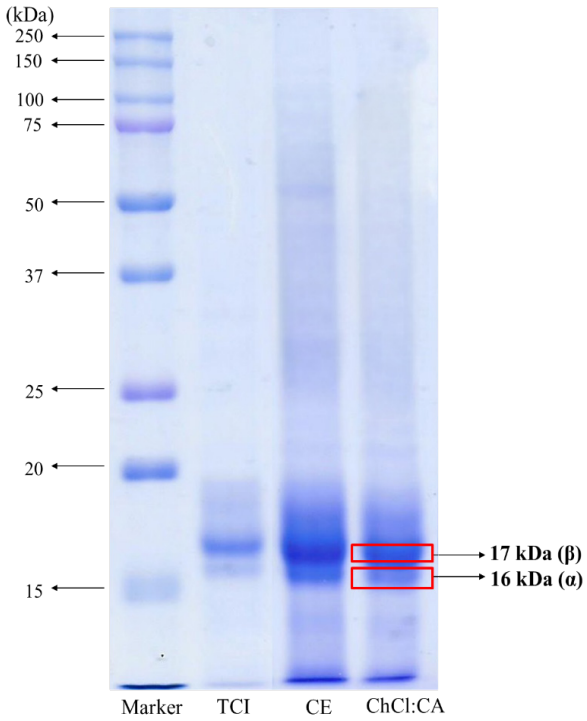

Figure 7. SDS-PAGE profile of phycocyanin. Note: TCI: commercial phycocyanin (Tokyo Chemical Industry), CE: crude extract, ChCl:CA: purification using NaDES choline chloride-citric acid.

3.5 Antioxidant Activity of Phycocyanin

The antioxidant activity of phycocyanin was evaluated using the DPPH method to assess its ability to scavenge free radicals. As an antioxidant compound, phycocyanin donates electrons or hydrogen to transform DPPH into DPPH-H, a non-radical compound (Renugadevi *et al*., 2018). The color change from purple to yellow and the decrease in absorbance at 517 nm reflected this reduction.

In this study, the antioxidant activity of phycocyanin, both in crude and purified forms, showed IC_{50} values of <50 ppm, categorizing it as a very strong antioxidant. The purification of phycocyanin can enhance its antioxidant activity, as evidenced by the IC_{50} value. The best treatment for phycocyanin purification, achieved using ATP-based DES ChCl:CA, resulted in an IC_{50} value of 40.54 ± 0.84 ppm (Figure 8). This value is lower than previous studies using water-extracted phycocyanin from *S. platensis*, with IC_{50} values around 158.3 ppm (Agrawal *et al*., 2021) and 197.67 ppm (Suzery *et al*., 2017), respectively. These results indicate that purified phycocyanin exhibits strong antioxidant activity, possibly associated with a high level of double bond conjugation. This conjugation can effectively stabilize and neutralize DPPH radicals (Wada *et al*., 2013).

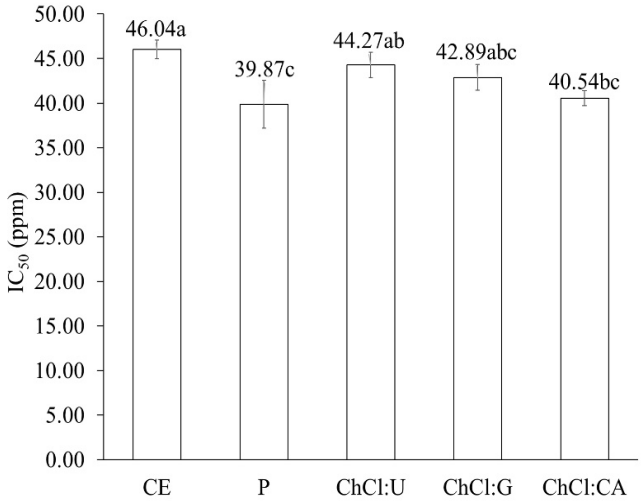

Figure 8. Antioxidant activity of crude extract and purified phycocyanin. Different superscripts show significant differences ($p < 0.05$).

4. Conclusion

The research underscores the effectiveness of utilizing a Natural Deep Eutectic Solvent (NaDES)-based aqueous two-phase system for phycocyanin purification from *Spirulina platensis*. The combination of ChCl:CA (1:1) emerges as the superior purification method, exhibiting the highest purity index 1.25±0.08 and recovery rate 71.83±2.76%. This purification approach holds promise for obtaining

pure and bioactive phycocyanin, suggesting potential applications in food industries, and warrants further exploration for large-scale production optimization.

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Authors' Contributions

All authors contributed to the final manuscript. ET; conceptualized the idea, designed the methodology, conducted formal analysis and investigation, and drafted the manuscript. SDH; contributed to conceptualizing the idea, resourcing the project, and reviewing and editing the manuscript. IS; contributed to conceptualizing the idea, securing funding, as well as reviewing and editing the manuscript. All three authors collaborated on writing the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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