Production of Phycocyanin Natural Blue Dye of Algal Origin and Evaluation of Different Extraction and Purification Methods

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Abstract

Phycocyanin's potential as a natural dye is indeed promising, especially considering the harmful side effects associated with artificial food colors. Its high antioxidant properties make it a valuable ingredient in various industries, including food, pharmaceutical, and beauty. Phycocyanin, a blue pigment extracted from *Spirulina platensis*, is a phycobiliprotein known for its diverse pharmacological benefits. Its natural origin and functional properties make it a promising alternative to synthetic food colorants, and continued research into its applications may provide safer and healthier options for consumers. The comparison of the freeze-thaw and phosphate buffer extraction methods in this study sheds light on the efficiency and effectiveness of different extraction techniques for obtaining phycocyanin. The exploration of salt concentrations as a means to enhance purity index and product yield provides valuable insights into optimizing extraction processes. Additionally, the use of chitosan, activated charcoal, and sodium citrate for purification further demonstrates the importance of refining and purifying phycocyanin for various applications. Overall, the study's findings contribute to the understanding of extraction and purification methods for phycocyanin, offering potential strategies for improving the quality and yield of this natural pigment.

The outcomes of this research project indicate that the purity index of phycocyanin obtained through the freeze-thaw method surpasses that achieved via the phosphate buffer method. Moreover, the results from the sodium chloride salt method, when compared to the control, demonstrate that the purity of phycocyanin can be enhanced with increasing concentrations of salt, reaching up to1 M. Furthermore, activated charcoal has been identified as the most effective substance for phycocyanin purification, significantly enhancing the purity of the blue color among three purification methods evaluated, which include chitosan, activated charcoal, and sodium citrate. Under optimal conditions, the extracted phycocyanin exhibits the highest concentration of 5.12 mg/mL, a purity index of R: 1.17, and a production efficiency of 11.8%. Future studies should aim to scale up the optimized extraction and purification processes, eval-

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uating the economic feasibility for industrial production, and evaluate the stability and functionality of the purified phycocyanin within specific food and cosmetic applications.

Keywords: Blue-green algae, *Spirulina platensis*, Purity index, Production efficiency

Introduction

The growing demand for natural ingredients has spurred the food, pharmaceutical, and cosmetic industries to seek alternatives to synthetic colorants (Ghosh et al., 2022). Microalgae represent a sustainable source of valuable natural pigments, including phycocyanin, astaxanthin, chlorophyll, and carotenoids. These pigments are not only safer but also offer enhanced health benefits derived from their antioxidant and anti-inflammatory properties, unlike their synthetic counterparts (Vinothkanna and Sekar, 2020; Mirbagheri Firoozabad and Nateghi, 2025). Furthermore, microalgae cultivation requires less water and land compared to conventional agriculture and can contribute reduce greenhouse gas emissions, positioning it as an environmentally friendly platform for producing high-value compounds (Omokaro et al., 2025). Among microalgae, the cyanobacterium *Spirulina platensis* is the primary commercial source of the natural blue pigment, C-phycocyanin (C-PC). *Spirulina* is a nutrient-dense organism, with a high protein content (up to 70% dry weight) and a rich profile of vitamins, minerals, and essential fatty acids, making it a popular dietary supplement (Matufi et al., 2020). Beyond its nutritional value, *Spirulina* and its predominant pigment, C-PC, have been investigated for their therapeutic potential in treating chronic conditions such as cancer and immune diseases, largely due to their potent antioxidant and anti-inflammatory activities (Subramaiam et al., 2021; Vilahur et al., 2022).

C-PC is a phycobiliprotein, a class of brightly colored, fluorescent pigment proteins that constitute the light-harvesting phycobilisomes in cyanobacteria and red algae. These complexes are crucial for photosynthesis, as they absorb light energy and transfer it to reaction centers (Hsieh-Lo et al., 2019). Phycobiliproteins are categorized based on spectral properties; C-PC, the major phycobiliprotein in *Spirulina*, has a maximum absorbance between 610 and 620 nm (Payne et al., 2025) and can constitute up to 20% of the dry weight under optimal growth conditions (Chini Zittelli et al., 2022). This high abundance, combined with its unique color and fluorescence, makes C-PC highly valuable for applications in biotechnology, medicine, and as a natural colorant.

However, the commercial use of C-PC is limited by challenges in downstream processing. Efficient extraction and purification are crucial steps that directly influence the yield, purity, and cost of the final product. The tough cell wall of Spirulina requires effective disruption methods to release C-PC. Common techniques include freeze-thaw cycles, bead milling, homogenization, and chemical extraction. Each method has trade-offs: while freeze-thaw often produc-

es high-purity extracts, it is time-consuming and energy-intensive (Jaeschke et al., 2021). Mechanical methods such as bead milling, are quick but can co-extract impurities such as chlorophyll and cell debris, making subsequent purification more chalenging. Chemical extraction with buffers is simple but may yield dilute extracts with lower initial purity (Soto-Sierra et al., 2018; Ferreira-Santos et al., 2020; Zhang et al., 2015). Therefore, a purification step is necessary to achieve the high purity required for sensitive applications like pharmaceuticals. Techniques such as ammonium sulfate precipitation, chitosan treatment, and activated charcoal adsorption are used, but there is no consensus on their relative effectiveness. Importantly, no standardized protocol exists for extracting and purifying C-PC, as the best methods depend on the algal strain and product specifications. Thus, systematic comparisons are essential to develop efficient and scalable processes. This study aims to fill this gap by comparing the efficiency of freeze-thaw and phosphate buffer extraction methods for obtaining C-PC from *Spirulina platensis*. It also evaluates three purification strategies—chitosan, activated charcoal, and sodium citrate treatment—to improve the purity and yield of the final product. The results will provide valuable insights for optimizing the downstream processing of C-PC, thereby facilitating its broader application as a natural blue colorant and nutraceutical.

Material and methods

Growth and culture conditions

Spirulina platensis was obtained from the Yazd University's algae bank and cultivated to generate sufficient biomass for analysis. The cyanobacterium was grown in a modified Zarrouk medium (pH ~9.5) with the following composition of the medium per liter of distilled water: 1.0 g $NaHCO_3$, 1.5 g K_2HPO_4, 1.5 g $NaNO_3$, 1.1 g urea, 1.15 g $FeSO_4$, 1.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, and 1.5 g K_2SO_4. For initial culture, 20–25 mL of *S. platensis* stock culture was inoculated into 1 L Erlenmeyer flasks containing autoclaved Zarrouk liquid medium (sterilized at

Fig. 1. Layers in freeze-thaw cycles

120 °C for 20 min) (Vonshak et al., 1982). Cultures were incubated at room temperature under a 12 hours' light to 12 hours' dark cycle. For large-scale biomass production, the culture was scaled up to a 70 L aquarium maintained under the same conditions. Biomass was harvested during the final growth phase for subsequent extraction procedures.

Optimization of extraction procedures

Freeze-Thaw extraction

The efficacy of repeated freeze–thaw cycles for extracting C-phycocyanin (C-PC) from *S. platensis* was investigated. This method disrupts cells through the formation and melting of intracellular ice crystals (D'hondt et al., 2017). Harvested biomass was mixed with distilled water at a 5:1 water-to-biomass ratio (w/w). To determine the optimal freezing duration, two freezing times were tested: 3 h and 24 h. Based on the results of this initial test, a 3-hour freezing period was selected for further optimization. Subsequently, the impact of adding salt to the slurry was evaluated by testing 0.7 M and 1 M salt concentrations, alongside a salt-free control. Following the cycles, samples were centrifuged at 5,000 rpm for 15 min to pellet cell debris (Figure 1). The supernatant was then collected, and its absorbance was measured spectrophotometrically within a range of 200–700 nm using a spectrophotometer (Analytik Jena 210, Germany) to determine C-PC concentration and purity. The optimal freeze–thaw condition was selected based on the highest yield and purity of C-PC (Figure 1).

Phosphate buffer extraction

C-PC was also extracted using phosphate buffer, which facilitates cell wall disruption and dissolution of water-soluble phycobilins. A sterile phosphate buffer solution was prepared and autoclaved. Subsequently, 3 g of dry *S. platensis* powder was dissolved in 100 mL of this buffer. The mixture was agitated at 150 rpm for three hours to facilitate extraction (Oliveira et al., 2008).

Purification

Crude extracts from both methods were clarified by filtration through 20-µm filter paper followed by centrifugation at 14,000 rpm for 20 minutes. The clarified extracts were then purified using one of three methods: chitosan flocculation, activated charcoal, and sodium citrate precipitation (Figure 2).

Fig. 2. Removing cell debris via filter paper

Chitosan flocculation

Chitosan was added to the clarified extract at a final concentration of 0.5% (v/v) and incubated for 20 min. The mixture was magnetically stirred at 4 °C for 20 min and then centrifuged at 8,000 rpm for 15 min at 4 °C. The resulting supernatant was analyzed by spectrophotometry between 200–700 nm (Fekrat et al., 2019).

Activated charcoal treatment

This procedure mirrored the chitosan method. but it used 10 mL of extract with 5% (w/v) activated carbon. This treatment was noted to enhance the chromatic properties of the solution (De Matos Fernandes et al., 2010).

Sodium citrate precipitation

Sodium citrate was added to a phycocyanin solution at a concentration of 0.5 g per 100 mL. Acting as a stabilizer and chelating agent, sodium citrate enhances the refinement of phycocyanin. The mixture was centrifuged at 4,500 rpm to remove impurities, resulting in a concentrated and purified product in the supernatant (Mogany et al., 2019).

Drying methods

Purified phycocyanin was subjected to three drying methods to assess their impact on stability and yield. These methods included freeze-drying, cold drying (conducted in a refrigerated, dark setting), and oven drying at 35 °C. The phycocyanin content and purity index of each dried sample were measured and compared. The cold-drying process required approximately two days to achieve complete desiccation (Kuhnholz et al., 2024).

Analytical procedures

The concentration and purity of phycocyanin during all extraction and purification stages were measured using a spectrophotometer scanning from 200-700 nm. The C-PC concentration (mg/mL) was calculated using the equations below (Bennett et al., 1973):

$$C-PC=\frac{OD_{620}-0.474\ OD_{652}}{5.34}$$

The purity index was calculated as the ratio of the absorbance at 620 nm to that at 280 nm.

Purity= OD620 /OD280

The extraction yield of phycocyanin (mg/g dry biomass) is calculated using the formula stablished by Silveira et al. (2007).

$$\text{Yield}=\frac{(C-PC)V}{DB}$$

Where V represents the volume of the solvent (mL) and DB is the mass of the dry biomass (g).

Results

The optimization of the freeze-thaw extraction process identified a three-hour freezing period combined with three complete freeze-thaw cycles in the presence of sodium chloride (NaCl) as the most effective protocol. This study evaluated the impact of NaCl concentration on phycocyanin purity by comparing 1 M and 0.7 M solutions to a salt-free control. The results show that the inclusion of salt significantly enhanced phycocyanin purity compared to the control. Furthermore, a higher NaCl concentration (1 M) yielded a product with greater purity of phycocyanin compared to the lower concentration (0.7 M), which has a purity index of 0.94. The extract obtained using the 1 M NaCl protocol exhibited an intense blue col-

or and achieved the highest purity index of 1.17 (Fig. 3). The control showed a purity index of 0.77.

Comparison of salt concentrations

The optimal process involved three freeze-thaw cycles (freezing at -20°C and thawing at 4°C) using a 1 M NaCl solution, which yielded the highest purity index (Figure 4). The purity of phycocyanin extracted under different conditions is detailed in Figure 5.

Comparison of phycocyanin purification methods

Three purification methods activated charcoal, sodium citrate, and chitosan- were evaluated. Among these, activated charcoal treatment was the most effective, resulting in the greatest increase in phycocyanin purity (Fig. 5).

A comprehensive summary of all techniques is shown in Table 1. The combined freeze–thaw and sodium citrate purification method achieved a high purity index of 1.03. However, the most effective method for obtaining high-purity phycocyanin was freeze-thaw with 1 M NaCl solution, which yielded a purity index of 1.17 at a concentration of 3.8 mg/mL, though with an extraction efficiency of 6%. In terms of production yield, the phosphate buffer extraction method followed by a combined chitosan and activated charcoal purification step was most effective. This method achieved a higher concentration (3.54 mg/mL) and extraction efficiency (11.8%), although with a lower purity

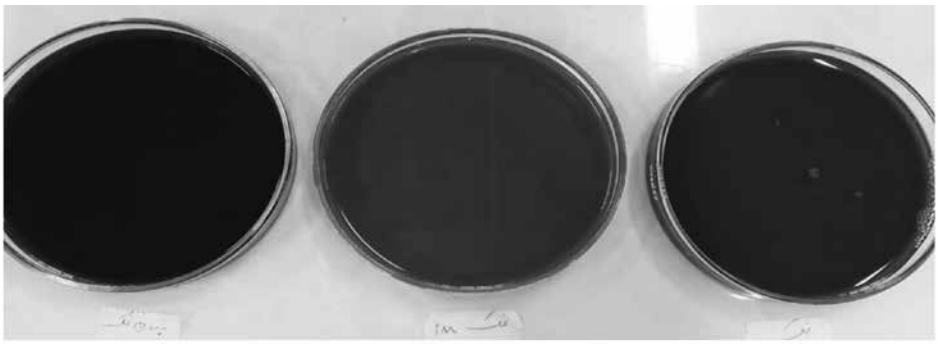

Fig. 3. A: Phycocyanin extraction without salt, B: Extraction with 1M salt, and C: extraction with 0.7M salt

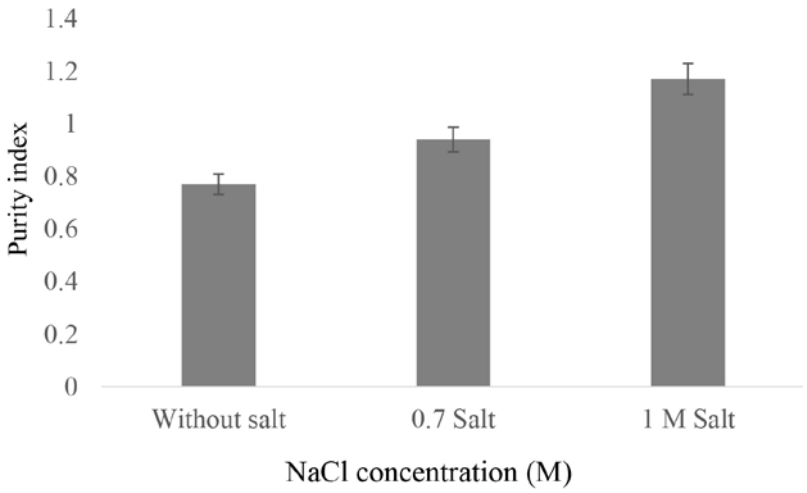

Fig. 4. Purity index of phycocyanin after centrifugation in different NaCl concentrations by the freeze-thaw method

index of 0.64. This finding is consistent with previous studies indicating that chitosan and activated charcoal can enhance phycocyanin purity index (Fekrat et al., 2019).

Comparison of drying methods

Analysis of the purity index showed that freeze-drying (vacuum lyophilization) was the most effective method, producing the highest quality powder with an R-value of 0.60. Refrigeration and oven drying resulted in lower purity levels, with R-values of 0.51 and 0.41, respectively (Fig. 6). Therefore, for optimal dry powder production, lyophilization is the preferred method.

In summary, the most effective protocol identified was a triple freeze-thaw extraction using a 1 M sodium chloride solution, which achieved a purity index of 1.17. The purity of phycocyanin can be further improved after extraction with activated charcoal, with the optimal treatment (5% w/v) resulting in a purity index of 1.06. For final powder

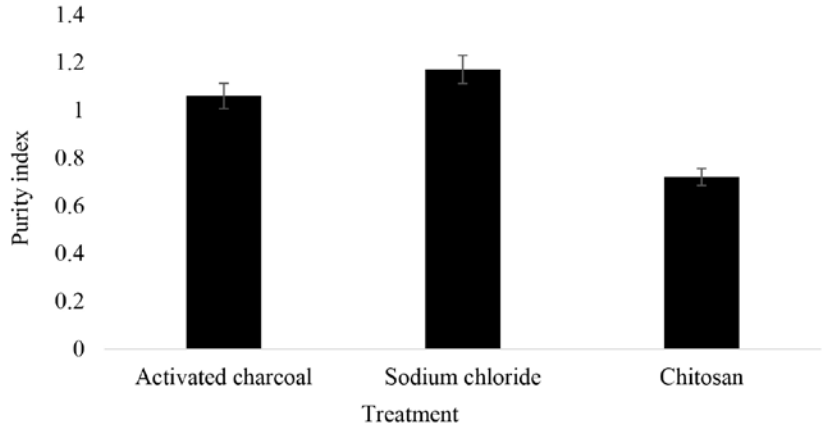

Fig. 5. Purity index of results of three phycocyanin purification methods, including activated charcoal, sodium citrate, and chitosan

Table 1. Evaluating the purity index, concentration, and production efficiency of different extraction and purification methods

Extraction	Purification	Purity Index	Concentration (mg/ml)	Product (Yield)
Freeze-thaw	–	0.77	3.6	45 mg/g Efficiency: 4.5%
Freeze-thaw	Activated charcoal and chitosan	0.78	5.12	80 mg/g Efficiency: 8%
Freeze-thaw	Sodium chloride	1.17	3.8	60 mg/g Efficiency: 6%
Freeze-thaw without sodium chloride	Chitosan	0.72	3.8	76 mg/g Efficiency: 7.6%
Freeze-thaw without sodium chloride	Activated charcoal	1.06	3.8	76 mg/g Efficiency: 7.6%
Freeze-thaw without sodium chloride	Sodium citrate	1.03	3.8	45 mg/g Efficiency: 7.6%
Phosphate buffer	–	0.58	1.69	33.56 mg/g Efficiency: 5.63%
Phosphate buffer	Activated charcoal and chitosan	0.64	3.54	118 mg/g Efficiency: 11.8%

production, lyophilization is recommended. This study successfully established a method to achieve a high purity index (R: 1.06), though further studies could potentially improve color purity even more.

Discussion

Phycocyanin, a blue pigment derived from blue-green algae like *Spirulina*, is well-known for its fluorescence and antioxidant properties. This phycobiliprotein can be extracted using various cell wall disruption techniques, but the cost of obtaining and purifying phycocyanin remains a significant challenge due to the complex purification processes involved. Currently, there is no universally accepted method for isolating and purifying phycocyanin, which highlights the need for cost-effective techniques that can produce sufficient quantities of phycocyanin for various applications in healthcare and manufacturing.

Numerous extraction techniques have been used in both industrial and laboratory settings, including ultrasound, high hydrostatic pressure, ultracentrifugation, ultra-homogenization, and extraction with water, organic solvents, and inorganic solvents. Among these methods, the use of water for separation of the cell membrane is widely practiced in global industries. This process involves sequential freezing and thawing of *Spirulina* in a liquid solution to disrupt the cell membrane (Mirbagheri Firoozabad and Nateghi, 2025). The most effective method for extracting and purifying phycocyanin, in terms of achieving the highest concentration and purity index, was found to be the freeze–thaw technique combined with a 1 M sodium chloride solution. This method yielded a purity index of 1.17, a concentration of 3.8 mg/ml, and an extraction efficiency of 6%, outperforming other techniques and therefore recommended for obtaining high-quality phycocyanin. This research is comparable to that of Ameri et al. (2018), and the purity of phycocyanin obtained by the freeze-thaw method is very similar in both studies, with the yield increasing to 60 mg/g in this study. Further research shows that the freeze-thaw technique, combined with a 1 M Tris-HCl buffer, yields about 11.34% of dry cell weight in phycocyanin, which is a significantly higher amount compared to previous

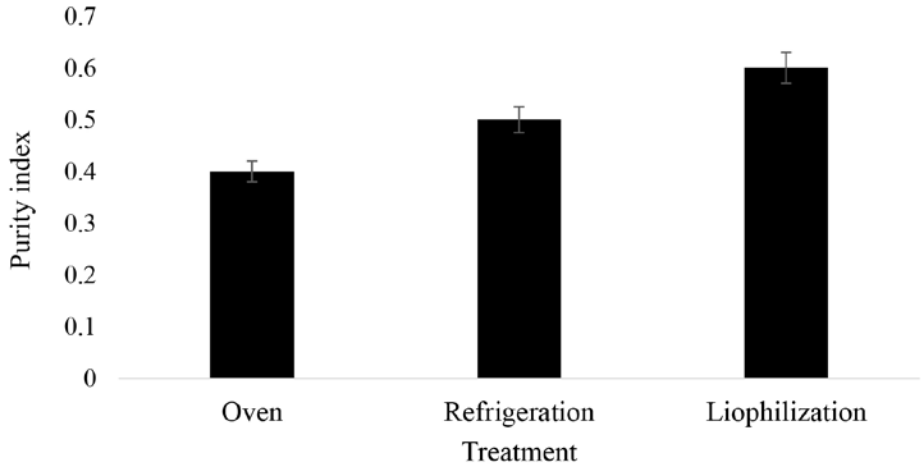

Fig. 6. Purity index of results of phycocyanin drying methods

studies (Pispas et al., 2024). It is recommended that this buffer be used as a lysis buffer for extracting of phycocyanin from Spirulina. Additionally, research suggests that enzyme treatment may be more effective than freeze-thaw methods or mineral solvents for extracting phycocyanin, and its use is recommended for future studies (Safari et al., 2020). Based on the research by Kumar et al. (2014), it is also suggested to use dialysis membranes and anion exchange chromatography to increase the purity to 2.93 and 4.58, respectively. Future studies should focus on scaling up the optimized extraction and purification process, evaluating the economic feasibility for industrial production, and assessing the stability and functionality of the purified phycocyanin in specific food and cosmetic matrices.

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