Investigation of phycobiliproteins in *Osmundea caspica (Laurencia)* (Zinova & Zaberzhinskaya) Maggs & L.M.McIvor Collected from the Coastal Waters of Nowshahr, Caspian Sea

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Abstract

Phycobiliproteins, including phycocyanin, allophycocyanin, and phycoerythrin, have diverse applications in food, cosmetics, and biomedical industries. Consequently, optimizing extraction conditions and identifying high-yielding species remain critical areas of study. The genus Osmundea is recognized for its significant phycobilin content. This study examines the macroalga *Osmundea caspica*, a member of the phylum Rhodophyta. The specimens of *O. caspica* were collected from the Caspian coasts of Sisangan in Mazandaran Province (Iran). The samples were lyophilized and subsequently ground after washing and removal of impurities. The extraction of phycobilins was evaluated using three solvents: distilled water, 100 mM phosphate-buffered saline (PBS), and 150 mM PBS (all adjusted to pH 7). Two distinct protocols: freeze-thaw at –20 °C for 24 hours and ultrasonication at a power of 70 W for 10 minutes. The results demonstrated that phycoerythrin exhibited the highest concentration among the extracted phycobilins, with an average of 0.0453 mg/mL, followed by phycocyanin (0.0067 mg/mL) and allophycocyanin (0.0018 mg/mL). Conversely, utilizing distilled water as the extraction solvent in conjunction with the Freeze-thaw Pre-treatment resulted in a greater extraction efficiency when compared to alternative methods. The results of one-way ANOVA showed that the differences in the mean concentrations and purity levels of phycobiliproteins among the extraction methods were statistically significant at the 0.05 level. For concentrations of phycocyanin (F:3.551, df: 5, P< 0.05), allophycocyanin (F: 23.984, df: 5, P< 0.05), phycoerythrin (F: 23.685, df: 5, P< 0.05), total phycobiliproteins yield (F: 18.489, df: 5, P< 0.05), purity of phycocyanin (F: 16.109, df: 5, P< 0.05), allophycocyanin (F: 34.155, df: 5, P< 0.05) and phycoerythrin (F: 25.353, df: 5, P< 0.05). This study presents promising results, particularly regarding the potential of phycoerythrin among the phycobiliproteins of the red alga *Osmundea caspica*, and offers a clear perspective for further exploitation of this species.

Keywords: Phycobiliproteins**,** Phycoerythrin, Algae, Rhodophyta, Caspian Sea

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Introduction

Marine biomass is recognized worldwide as a valuable carbon source, which can be used for food, feed, chemicals, and biopharmaceuticals of paramount industrial relevance (Merlo et al. 2021). Macroalgae comprise a group of marine algae classified into three major groups: green algae (*Chlorophyta*), brown algae (*Phaeophyta*), and red algae (*Rhodophyta*). The species diversity of macroalgae in the Caspian Sea is lower compared to the Persian Gulf. However, 13 species of brown algae and 25 species of red algae have been reported from this sea, which are typically distributed along rocky-coastal areas (Eshaghzadeh et al., 2023; Stepanian, 2016). In the Iranian coasts of the Caspian Sea, *O. caspica* is the only confirmed species of red algae. Previously, in past classifications, this species was categorized under the genus *Laurencia* (Eshaghzadeh et al., 2023). Red algae, along with cyanobacteria and cryptophytes, are among the primary sources of phycobiliproteins (PBPs).

PBPs are water-soluble pigments. They are organized into complexes known as phycobilisomes, which are located on the outer surface of the thylakoid membrane (Kovaleski et al., 2022). This complex functions in light energy harvesting, as chlorophyll a exhibits maximum absorption at wavelengths of 430 nm and 660 nm (Glazer, 1994). Thus allowing the photosynthesis and the survival of living organisms even at low light intensities (Dumay et al., 2014). Phycobilisome captures light energy through its phycobilin chromophores and directs it towards reaction centers where it is converted into chemical energy (Roy et al., 2011). Based on their structure and properties, specifically on their radiation absorption ability, PBPs are divided into four main types (Pagels et al., 2019), include phycoerythrin (PE), which exhibits a pink-purple color and λmax = 540-570 nm; phycocyanin (PC), with a blue color and λmax = 610-620 nm; allophycocyanin (APC), with a blue-green hue and λmax = 650-655 nm (Lijassi et al., 2024) and Phycoerythrocyanin (PEC), with a magenta color and λmax = 560–600 nm (Munier et al., 2014). PE, with a total molecular weight around 240 kDa, can be classified into four classes: B-PE (Bangiophyceae PE, containing PEB only or containing PEB and phycourobilin), C-PE (cyanobacterial-PE), and R-PE (Rhodophyta-PE). Indeed, R-PE is recognized for its stability towards several denaturant agents, namely temperature and pH (Galland-Irmouli et al., 2000). As the main light-harvesting complexes, phycobilisomes represent one of the crucial factors of algae and cyanobacteria mass cultures' productivity. It has been demonstrated that phycobilisome truncation can enhance biomass accumulation under strong light (Kirst et al., 2014). On the other hand, under modest or low irradiance, the antenna truncation resulted in growth rates and biomass accumulation reduction (Kirst et al., 2014; Page et al., 2012). These proteins are utilized in the food industry as natural colorants (soft candy, jellies, and ice sherbets), as well as in cosmetic and biomedical applications (e.g., as fluorescent labels for flow cytometry, immunoassays, and more). Additionally, they exhibit a wide range of biological activities, including antioxidant, antibacterial, anticancer (Lijassi et al., 2024), anti-inflammatory,

neuroprotective, and immunomodulatory (Lauceri et al., 2019). PBPs aqueous extracts obtained from *Arthrospira platensis* (*Spirulina*) are approved by EFSA (Regulation (EU) No. 1333/2008 and No. 231/2012) as coloring foodstuff. The US FDA classifies PC (21CFR73.1530) as a food natural color additive (Lauceri et al., 2019). Various methods are available for disrupting the cell wall to extract phycobilins. Early protocols typically employed physical or chemical methods to destroy trichomes and extract PBPs by using water as a major solvent (Doke, 2005; Eriksen, 2008). These include repeated freeze-thaw cycles, ultrasonication, pressurized distilled water, microwave treatment, pulsed electric field, homogenization, and others. The solvents mentioned (distilled water, PPB, PBS, Tris-Cl buffer with sodium azide, and sodium chloride) are often used as buffers and diluents in various scientific techniques, particularly in biological and biochemical research. These solvents help maintain a stable pH, which is crucial for the stability and activity of biological molecules like proteins and nucleic acids. (Kovaleski et al., 2022). More recent works combined chemical and physical methods for cell wall disruption and introduced other methods like enzymatic cell wall digestion or supercritical CO_2 extraction (Marzorati et al., 2020; Berrouane et al., 2022). The cell wall of macroalgae consists of polysaccharides (agar and cellulose), which are an obstacle to cell rupture during the extraction of their bioactive compounds (Mittal et al., 2017). The concentration and quality of phycobilins depend on key extraction parameters, such as the method of cell wall disruption, the solvent used, extraction time, and separation conditions. The present study aimed to investigate the extraction of phycobilins, particularly phycoerythrin, from the red macroalga *O. caspica*, which is native to the southern coast of the Caspian Sea. Given the biological significance and commercial potential of phycobilins as natural pigments and fluorescent markers, this research sought to evaluate the efficiency of the extraction process and to explore the feasibility of utilizing *O. caspica* as a novel and sustainable source of high-value phycobiliproteins.

Material and methods

Sampling

The red macroalga *O. Caspica* was collected from shallow areas (approximately 20 to 40 centimeters deep) of the rocky

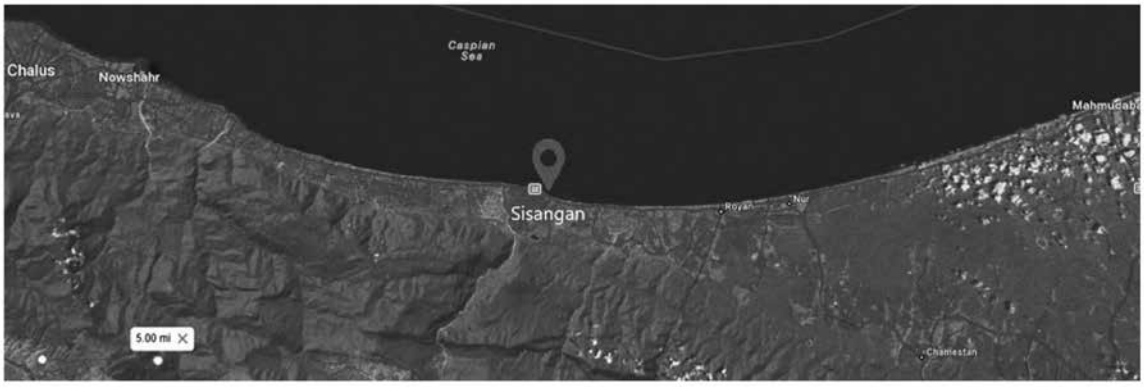

Fig. 1. Sampling location

shores in Sisangan, Nowshahr, Mazandaran Province, Iran, in mid-March (36.579133 N, 51.828014 E) (Figure 1). Sampling was carried out.

Preparation and Processing

The samples were washed with marine water and then distilled water to remove impurities, and then lyophilized in the dark for 24 hours. The samples were then ground using a porcelain mortar, and their weight was measured.

Phycoerythrin extraction using ultrasonication

To compare the effects of solvents in the extraction process, three solvents were used: distilled water, 100 mM PBS, and 150 mM PBS, all adjusted to pH 7, with a weight-to-volume ratio (W/V) of 1:25. Additionally, for cell disruption, the performance of intracellular content release was compared using sequential freeze-thaw cycles and ultrasonication methods. Accordingly, one set of samples underwent the freeze-thaw process, in which the samples were frozen at –20 °C for 24 hours and subsequently thawed at room temperature. while the second set was subjected to ultrasonication. During the ultrasonication process, the samples were placed in an ultrasonic device (Tosee Fanavari, 220-Iran) at a power of 70 W for 10 minutes. The freeze-thaw and ultrasonication procedures were repeated for three cycles. Between each cycle, the samples were vortexed for 2 minutes to enhance cell disruption and extraction efficiency. The solution was then filtered using filter paper (Fig. 2) and centrifuged (Universal 320R Hettich-Germany) at 8,000 rpm for 10 minutes at 4 °C. The supernatant was collected for spectrophotometric analysis.

Quantification of Extracted Phycobilins

The quantification of phycobilins was performed using a spectrophotometer (Lambda 25-Singapur) and modified equations (Lijassi et al., 2024). Thus, the amount of each of the PC, APC, and PE compounds was calculated using the following equations.

Phycocyanin *(*mg⁄ml*)*

$$= \frac{[(A620 - A720) - 0.474 \times (A652 - A720)]}{5.34}$$

Allophycocyanin *(*mg⁄ml*)*

$$= \frac{[(A652 - A720) - 0.208 \times (A620 - A720)]}{5.09}$$

phycoerythrin (*mg⁄ml*)

$$= \frac{[A562 - 2.41(PC) - 0.849(APC)]}{9.62}$$

The extraction yield of PBPs was estimated following the equation of (Silveira et al. 2007):

PBPs(mg/g)

$$= \frac{(PC + ACP + PE) * V}{DB}$$

V is the solvent volume (ml), and DB is dry biomass (g).

Purity was determined by using the formula below (Minkova et al., 2003):

PC Purity = $\frac{A620}{A280}$

APC Purity = $\frac{A652}{A280}$

PE Purity = $\frac{A562}{A280}$

Statistical analysis

The significant differences between mean values were evaluated using one-way analysis of variance (ANOVA). Tukey's test was performed with SPSS software (version 26.0) to determine whether there were any statistically significant differences at the $p< 0.05$ level.

Fig. 2. Initial purification of the algal extract was performed using ordinary filter paper

Results

The macroalga *O. caspica* (Fig. 3) was identified based on its morphological characteristics using microscopic images of internal structures and external morphological features (Rousseau et al., 2017) (Fig. 4).

The quantitative comparison of phycobilins indicates that PE had the highest concentration, with an average of 0.0453 mg/mL, followed by PC at 0.0067 mg/mL and APC at 0.0018 mg/mL, respectively (Figure 5). The yield of PBPs varied according to the extraction conditions: 1.05–1.76 mg/g (Table 1).

The results of one-way ANOVA showed that the differences in the mean concentrations (Table 1) and purity levels (Table 2) of phycobiliproteins among the extraction methods were statistically significant at the 0.05 level. For concentrations of phycocyanin (F:3.551, df: 5, P< 0.05), allophycocyanin (F: 23.984, df: 5, P< 0.05), phycoerythrin (F: 23.685, df: 5, P< 0.05), total phycobiliproteins yield (F: 18.489, df: 5, P< 0.05), purity of phycocyanin (F: 16.109, df: 5, P< 0.05), allophycocyanin (F: 34.155, df: 5, P< 0.05) and phycoerythrin (F: 25.353, df: 5, P< 0.05).

According to the results presented in Table 1, the combination of the freeze–thaw pretreatment and 150 mM PBS as the extraction solvent was significantly more effective for phycocyanin (PC) compared to other methods. In the case of allophycocyanin (APC), the freeze–thaw pretreatment combined with distilled water yielded the highest recovery. For phycoerythrin (PE), the freeze–thaw pretreatment in combination with both distilled water and 150 mM PBS demonstrated superior performance relative to the other

extraction approaches. Overall, the freeze–thaw pretreatment coupled with distilled water—and to a lesser extent with 150 mM PBS—proved to be more efficient than other extraction methods for the total recovery of phycobiliproteins.

According to Table 2, the purity of phycoerythrin (PE), consistent with the concentration results, was significantly higher when the freeze–thaw pretreatment was combined with either distilled water or 150 mM PBS, compared to the other extraction methods.

Fig. 3. *Osmundea caspica* habitat collected from Nowshahr, Iran

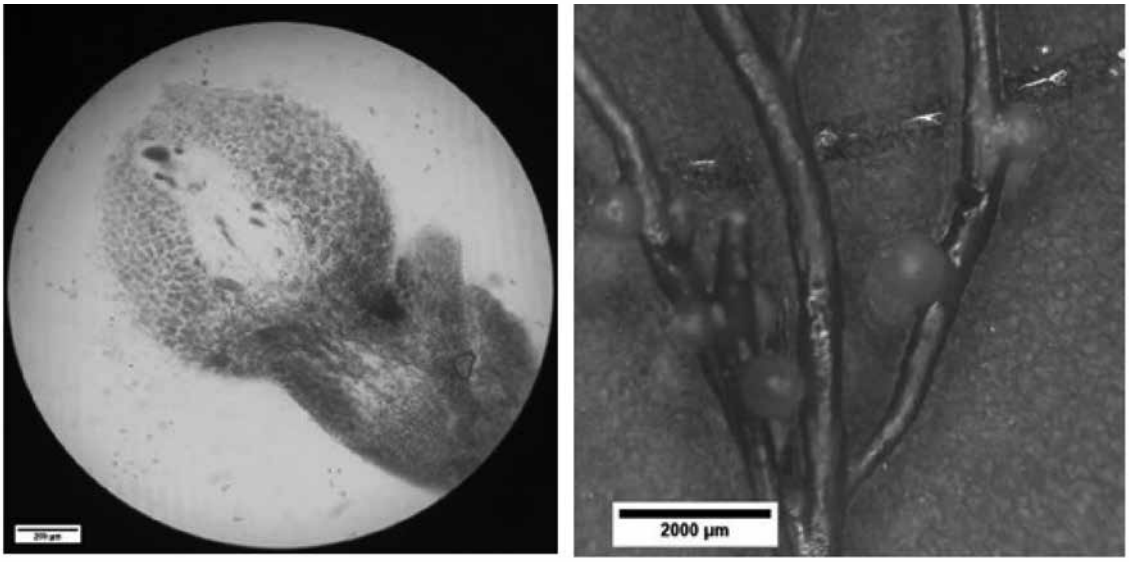

Fig. 4. Internal structure (left) and external morphology (right) of the cystocarp.

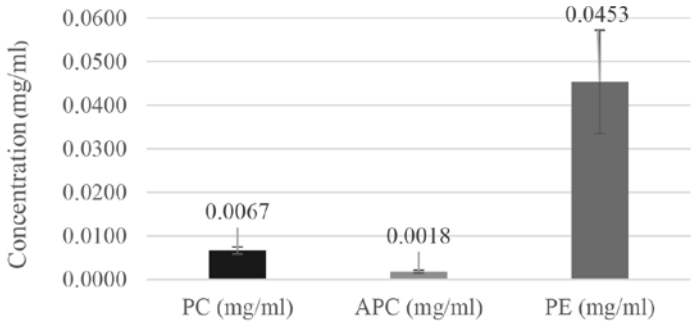

Fig. 5. Comparison of the concentrations of extracted phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) from *Osmundea caspica*

Table 1. Effect of pretreatment and solvent on PBPs extraction yield and concentration

Pre-treatment	solvent	PBPs concentration (mg/ml)			Ratio	Yield (mg/g)
		PC	APC	PE	PC:APC:PE	PBPs
Freeze-thaw	distilled water	0.0070^{ab}±0.0002	0.0023^{a}±0.0001	0.0611^{a}±0.0006	0.11:0.04:1	1.7596^{a}±0.0178
	PBS 100 mM	0.0055^{b}±0.0001	0.0012^{d}±0.0000	0.0437^{b}±0.0000	0.13:0.03:1	1.2594^{b}±0.0032
	PBS 150 mM	0.0075^{a}±0.0012	0.0017^{bc}±0.0002	0.0579^{a}±0.0077	0.13:0.03:1	1.6783^{a}±0.2290
ultrasonication	distilled water	0.0066^{ab}±0.0006	0.0021^{ab}±0.0000	0.0332^{b}±0.0047	0.20:0.06:1	1.0462^{b}±0.1312
	PBS 100 mM	0.0069^{ab}±0.0001	0.0018^{bc}±0.0000	0.0371^{b}±0.0009	0.19:0.05:1	1.1455^{b}±0.0268
	PBS 150 mM	0.0067^{ab}±0.0006	0.0016^{c}±0.0002	0.0370^{b}±0.0048	0.18:0.04:1	1.1337^{b}±0.1400

Data (calculated from triplicate experimental values ± standard deviation) in the same column with different letters are significantly different ($p < 0.05$)

Table 2. Effect of the extraction process on purity

Pre-treatment	solvent	Purity		
		PC	APC	PE
Freeze-thaw	distilled water	0.0167^{a}±0.0006	0.0092^{bc}±0.0005	0.1755^{a}±0.0043
	PBS 100 mM	0.0108^{b}±0.0007	0.0052^{d}±0.0004	0.1086^{b}±0.0064
	PBS 150 mM	0.0167^{a}±0.0027	0.0080^{c}±0.0013	0.1670^{a}±0.0212
Ultrasonication	distilled water	0.0193^{a}±0.0010	0.0122^{a}±0.0008	0.0978^{b}±0.0131
	PBS 100 mM	0.0184^{a}±0.0005	0.0105^{ab}±0.0005	0.1099^{b}±0.0023
	PBS 150 mM	0.0162^{a}±0.0008	0.0086^{c}±0.0004	0.1059^{b}±0.0122

Data (calculated from triplicate experimental values ± standard deviation) in the same column with different letters are significantly different ($p < 0.05$)

In contrast, the highest purity was achieved for allophycocyanin (APC) using ultrasonic pretreatment in combination with distilled water. A comparative evaluation of the obtained results with previous studies on red macroalgae is presented in Table 3.

Discussion and Conclusion

According to existing scientific reports, *O. caspica* is the only confirmed species of red macroalgae along the southern coasts of the Caspian Sea (Eshaghzadeh et al., 2023). For many years, the red macroalga of the Southern Caspian Coast was considered a species of the genus Laurencia. However, a molecular study conducted in Azerbaijan led to the reassignment of this species from *Laurencia* to *Osmundea* (Rousseau et al., 2017). Despite this taxonomic revision, no molecular studies have been conducted on this species along the Iranian coasts of the Caspian Sea. This highlights a research gap, suggesting the necessity of molecular investigations to confirm its classification in Iranian waters. Furthermore, there is significant potential for broader research efforts aimed to exploring additional species of red macroalgae along the Southern Caspian Coastline.

The absorption spectra of PBPs may vary significantly among different species of algae and cyanobacteria, and even between strains of the same cyanobacterial genus. Therefore, specific wavelengths and absorption coefficients used to determine phycobilins for particular strains are generally not applicable to other strains (Zavřel et al., 2018). This study is no exception in this regard; however, it utilizes the standard methods from previous studies, with an awareness of the potential errors specific to the species *O. caspica*. The optimal extraction method depends on the type of phycobiliprotein, the type of algae, and the operational conditions. One common approach for extracting molecules is the use of solvents. PBPs are hydrophilic proteins. Therefore, common solvents used for their extraction are water or buffers, which also serve to control the pH of

Table 3. Comparison of the methods and results of the present study with conventional studies

solvent	APC (mg/g)	PC (mg/g)	PE (mg/g)	Reference
distilled water	0.06	0.17	1.53	(Data obtained from the Freeze-thaw Pre-treatment)
PBS 100 mM	0.03	0.14	1.09	
PBS 150 mM	0.04	0.19	1.45	
PBS 100 mM	0.27	0.25	0.37	(Sudhakar et al. 2015)
Distilled water	0.34	0.28	0.5	
Sea water	0.18	0.14	0.36	
PBS 25 mM	*	*	0.84	(Eshaghzadeh et al. 2023)
PBS 50 mM	*	*	0.86	
PBS 100 mM	*	*	0.97	
PBS 20 mM	0.04	0.027	1.57	(Karuppannan et al. 2024)

*: (No data)

the environment. These solvents include sodium phosphate buffer, acetate buffer, citrate buffer, carbonate buffer, Tris-HCl buffer, and ethylenediaminetetraacetic acid (EDTA) (Kovaleski et al., 2022). Sharmila et al. (2017) tested extraction methods using various buffers and pH levels, demonstrating that phosphate buffer (pH 7.2) combined with freeze-thawing at temperatures between -20 °C and -25 °C yielded the best results. Sintra et al. (2021) used phosphate buffer for C-PC extraction and reported a 90% recovery rate. Nguyen et al. (2016) compared different concentrations of phosphate buffer (20 mM, 50 mM, and 0.1 M) with tap water and distilled water, finding that the 20 mM phosphate buffer with pH 7.1 showed the best results for PE in *Mastocarpus stellatus*. Sudhakar et al. (2015) investigated the extraction of PE and PC from *Gracilaria crassa* using distilled water, seawater, and phosphate buffer (0.1 M). The results demonstrated that distilled water performed best for extracting PE (0.35 mg/g) and PC (0.18 mg/g). Based on these studies, one of the objectives of the present study was to investigate the effect of the solvent on the extraction process. The difference in the amount of extracted PE compared to other phycobilins, as well as the relatively higher efficiency of distilled water as a solvent for extraction, aligns with the findings of similar studies (Sudhakar et al., 2015; Karuppannan et al., 2024). The superior performance of distilled water in extracting phycobilins compared to PBS solutions can be analyzed from several perspectives. One key factor is the difference in osmotic pressure between the extracellular environment and the intracellular space, which is higher in distilled water than in saline solutions. This increased osmotic pressure can lead to greater cell turgescence, facilitating the release of intracellular components. Additionally, PBPs are hydrophilic and exhibit higher solubility in pure aqueous environments like distilled water compared to saline solutions. Furthermore, extraction techniques such as ultrasonication and freeze-thaw cycles may be more effective in distilled water, as its salt-free and purer nature prevents interference from ionic interactions, thereby enhancing the efficiency of the extraction process. On the other hand, the freeze–thaw pretreatment, which has been employed in most similar studies, has proven to be a more efficient method compared to ultrasonication. Although Pereira et al. (2020) and Mittal et al. (2019) reported favorable outcomes for ultrasonication or its combination with maceration, their findings focused on specific red algae species and may not be generalized to all biomass types. Moreover, in studies such as that by Sharmila et al. (2017), the freeze–thaw method—particularly at lower temperatures (−20 °C to −25 °C)—showed comparable or superior performance in terms of pigment recovery. In our experimental conditions, the freeze–thaw method not only provided higher purity and yield of phycobiliproteins but also preserved their structural integrity more effectively. Additionally, it required no special equipment and maintained a gentle processing environment, minimizing the risk of denaturation. Accordingly, the present study identifies the freeze–thaw pretreatment combined with distilled water

as the most effective overall approach for phycobiliprotein extraction. Factors influencing the extraction of phycobilins include species potential, initial preparation, and the type of solvent-extraction protocol. Numerous studies have examined and confirmed the significance of each of these factors (Lijassi et al., 2024; Eshaghzadeh et al., 2023; Sudhakar et al., 2015; Karuppannan et al., 2024). The findings of the present study are no exception to these three factors. Hence, to improve the extraction of phycobilins, broader comparative studies across different species, initial preparation processes, and more refined modifications in the choice of solvent or protocol are suggested. Given the high demand for PBPs in various industries, research and development in improving extraction and purification methods continues to ensure the sustainable and economic utilization of these natural resources.

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