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ORIGINAL PAPER

Optimized extraction of phycobiliproteins from *Arthrospira platensis*: quantitative and qualitative assessment of C-Phycocyanin, Allophycocyanin, and Phycoerythrin

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Abstract
Phycobiliproteins (PBPs) are light collecting pigments of cyanobacteria that attract growing interest for several industrial applications. Each step of the extraction process is crucial for yield, concentration and quality of obtained pigments. In the current work, we present an optimization scheme of major limiting steps for PBPs extraction from *Arthrospira platensis* biomass. As first step, the effects of pretreatment, extraction time, and separation conditions on the recovery of PBPs were compared. Subsequently, the influence of pH and concentration of the extraction buffer as well as the addition of preservatives (Polyethylene glycol (PEG), Magnesium chloride ($MgCl_2$), and Calcium chloride ($CaCl_2$)) was studied. In addition, the effect of the biomass type (dried vs wet) and its concentration in the extraction buffer was also investigated. Optimal extraction required the use of dry biomass at relatively low ratio (1:50, solvent:biomass), without previous treatment. The use of concentrated phosphate buffer (100 mM) at a neutral pH gave the highest PBPs recovery and concentration after 6 h of extraction followed with a separation at 6000 rpm during 15 min. Calcium chloride used at 1.5% improved by 30% both PBPs recovery and concentration in the crude extract. The optimized protocol allowed the recovery of 464.5 mg/g PBPs from *spirulina* biomass with concentration of 15.9 mg/ml. The crude PBPs obtained with this extraction method reduced the stable radical DPPH with a percentage scavenging activity of $86.45 \pm 1.2\%$. This protocol could reduce both PBPs time and cost extraction and is easily scalable for industrial application.

Keywords *Spirulina* · Phycobiliproteins · Extraction · Optimal parameters · DPPH

Introduction

Phycobiliproteins (PBPs) are light-harvesting pigments complexes found in cyanobacteria and red algae to collect light energy, since chlorophyll a has the highest absorption at 430 nm and 660 nm (Glazer 1994). Depending on their absorbing properties, PBPs are divided into 3 categories: Phycoerythrin (PE: pink-purple, λmax = 540–570 nm), Phycocyanin (PC: blue, λmax = 610–620 nm) and Allophycocyanin (APC: bluish-green, λmax = 650–655 nm), assembled in the phycobilisome located on the outer surface of thylakoid membranes (Kuddus et al. 2014).

The aforementioned proteins are used in food industry as natural coloring agents (soft candy, jellies and ice sherbets), for cosmetic applications (makeup and personal care products, perfumes, etc.) and for biomedical application (as fluorescent labeling reagent for flow cytometry, fluorescent immunoassay, immunohistochemistry, etc.). PBPs have also wide spectrum biological activities which increases their attractivity in the field of cosmetics, nutraceuticals and pharmaceuticals (Choi and Lee 2018; Hsieh-Lo et al. 2019).The cyanobacteria *Arthrospira* (*Spirulina*) sp. is the major source of PBPs whose concentration and proportion vary following the strain and culture conditions (de Jesus

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Raposo et al. 2013). Nevertheless, whatever the conditions, C-phycocyanin is the most abundant form of PBPs in *Spirulina* (Pan-utai and Iamtham 2019a; Fratelli et al. 2021).

Arthrospira platensis usually known as *Spirulina* is a microscopic filamentous cyanobacterium used in human diets by ancient civilizations (Sili et al. 2012). Nowadays, it is the most produced microalgae worldwide to being used as food supplement for its high content in proteins (up to 70%), vitamins (A, B and K complex) and pigments, mainly PBPs (Hsieh-Lo et al. 2019; Costa et al. 2020). Recently, the valuation of PBPs as high-added value product extracted from *Spirulina*'s biomass is gaining a growing interest. This is mostly due to the scientific results linking the biological activities of this microalgae to its PBPs content (Yang et al. 2021; Manirafasha et al. 2021; Mercier et al. 2022). Indeed, several studies have demonstrated that C-Phycocyanin and Phycoerythrin present antioxidant, anticarcinogenic, immunomodulatory and anti-inflammatory properties (de Jesus Raposo et al. 2013; Xie et al. 2015; Mercier et al. 2022). Nevertheless, only few data are available regarding biological activities of Allophycocyanin (Ge et al. 2006). In addition to their use in bioassays due to their fluorescent prosperities, PBPs are being used in agroindustry as natural colorants. C-Phycocyanin is the closest natural alternative matching the shade of Brilliant Blue FCF (Prasanna et al. 2007), while Phycoerythrin appears generally pink or red (Bermejo Román et al. 2002). Moreover, they have the added advantage of being water-soluble and safe (Soni et al. 2010).

Spirulina produces C-Phycocyanin (C-PC) as major PBPs followed by Allophycocyanin (APC) at a 10:1 ratio (Sotiroudis and Sotiroudis 2013). Some studies detected only small amounts of Phycoerythrin in *Spirulina* while others didn't detect it (Rizzo et al. 2015; Rodrigues et al. 2018).

Several protocols were used to isolate PBPs from *Spirulina*'s biomass and it was clear that each step is determining for yield and quality of the final product. Early protocols usually employed physical or chemical methods to destroy trichomes and extract PBPs by using water as major solvent (Doke 2005; Eriksen 2008). More recent works combined chemical and physical methods for cell walls disruption and introduced other methods like enzymatic cell wall digestion or supercritical CO_2 extraction (Tavanandi and Raghavarao 2019; Marzorati et al. 2020; Berrouane et al. 2022). The conclusion was that PBPs concentration and quality depend on key extraction conditions such as: cell wall disruption method, used solvent, extraction time and separation conditions. In addition, for a large-scale production, the scalability and the economic feasibility, are additional aspects that influence the extraction technique choice. Both on laboratory and industrial scales, limited information is available on PBPs key extraction factors affecting its recovery, quality and biological activities. In this way, we studied here the influence of each extraction step on PBPs recovery from *Spirulina's* biomass, it's purity and antioxidant activity as well as the proportion of C-PC, APC and PE in the crude extract.

Material and methods

Spirulina biomass production

Spirulina platensis used is a local linear strain isolated from a natural lac near Rabat (North of Morocco). Culture was carried out on *spirulina* medium (Aiba and Ogawa 1977) in open raceway ponds measuring $3 \times 1 \times 1$ m (LxlxH) with an approximate volume of 600 L, under outdoor conditions, in a semi-continuous mode (Fig. 1). Biomass was harvested at 1.5 g/L and separated from medium by mechanical filtration system (50 µm mesh). Obtained biomass was weither at 45 °C during 12 h or immediately stored at 4 °C for wet form.

Optimization of PBPs extraction protocol

First, a simple PBPs extraction protocol, commonly used in our laboratory was followed. *Spirulina* dry biomass (ratio 1:24 w:v) was resuspended in 100 mM potassium phosphate buffer, pH 7. The extraction was carried out for 3 h at room temperature (RT) under stirring at 150 rpm. Broken trichomes as well as other cells debris were removed by centrifugation at 4000 rpm for 15 min at 4 °C and supernatant recuperated was the crude PBPs extract. All experiments were performed in triplicate (Fig. 2).

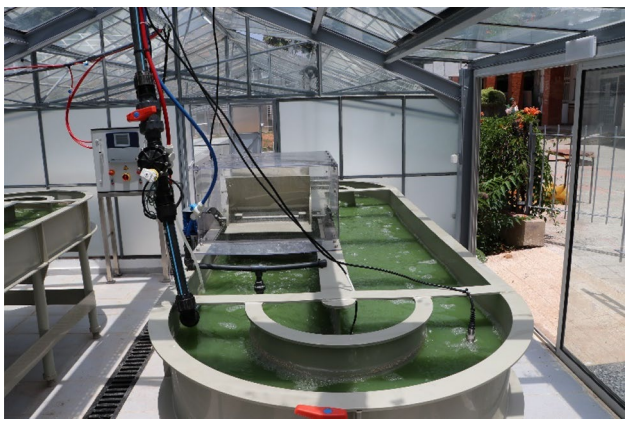

Fig. 1 *Spirulina* culture in open raceway ponds. *Pilot microalgal station of Faculty of Sciences, Rabat. (Morocco)*

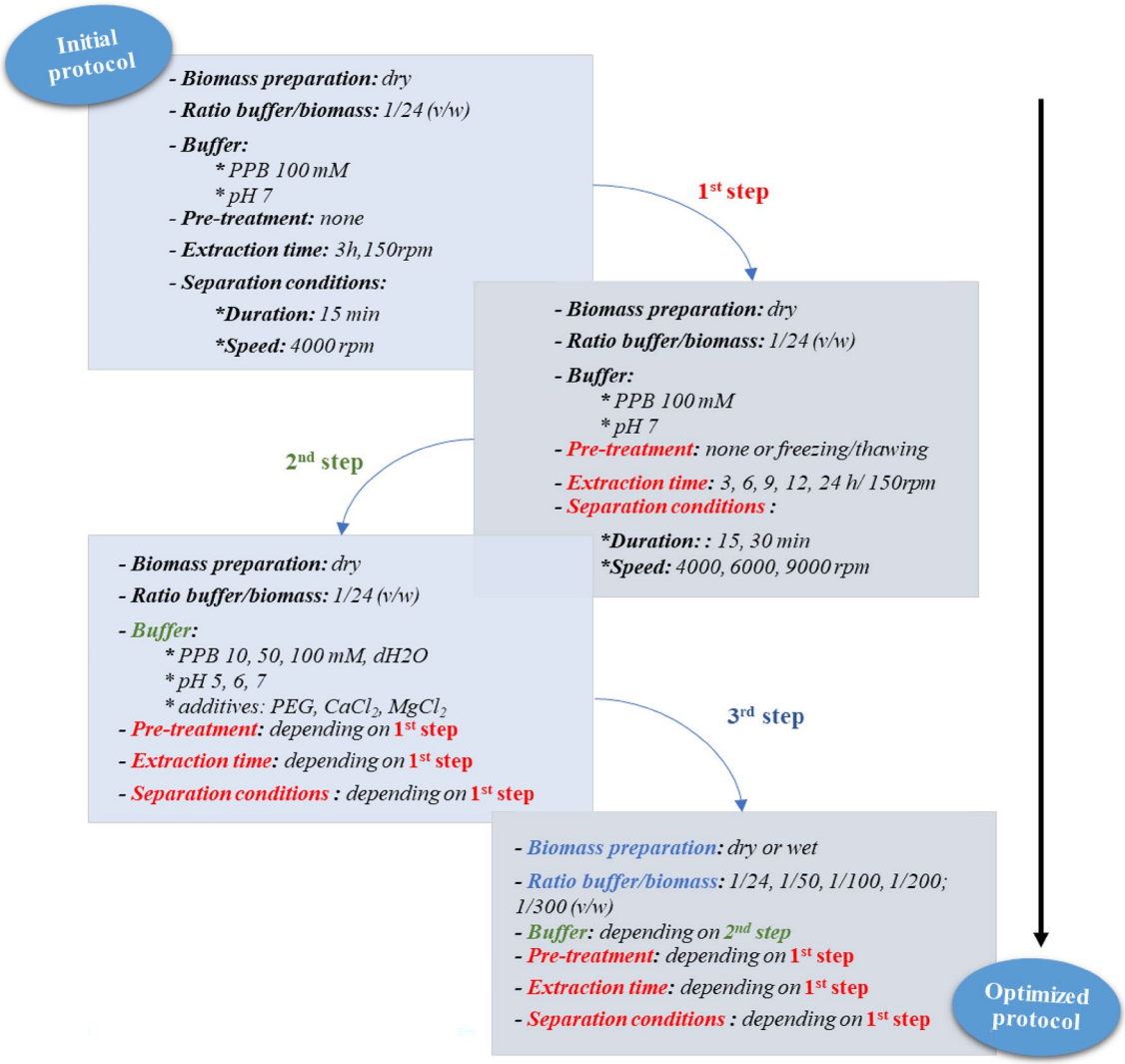

Fig. 2 Scheme of optimization steps during the PBPs extraction

Step 1: The effect of pretreatment was studied by testing a cycle of freezing (– 20 °C/ 12 h)/thawing (25 °C/ 3 h) under different extraction times by stirring the biomass/extraction buffer mixture during: 3, 6, 9, 12 and 24 h. The recovery of PBPs from biomass debris and broken trichomes was carried out at 3 centrifugation speeds (4000, 6000 and 9000 rpm) during 15 and 30 min.

Step 2: Optimal conditions from step 1 were applied to investigate the effect of the extraction buffer on PBPs yield and composition. First, 3 concentrations of potassium phosphate buffer (PPB) pH 7 were used: 10, 50 and 100 mM and water served as control. The effect of pH was further investigated by adjusting PPB pH to 5, 6 and 7. Finally, the effect of some additives (used as to destabilize cell walls or to stabilize PBPs) was studied: 1) Polyethylene glycol (PEG) 300 (30% w:w),PEG 6000 (30% w:w), 2) $CaCl_2$ (1.5% w/v) and 3) $MgCl_2$ (1.5%; w/v). All additives were incorporated to the extraction buffer before its use.

Step 3: With the optimal conditions found in previous steps, the effect of biomass type (dry vs. wet) as well as its concentration in the buffer were investigated. For each type of biomass, several concentrations (ratio extraction buffer:biomass) were tested (v/w):: 1:24, 1:50, 1:100,1:150, 1:200 and 1:300. One L of extraction buffer was used for all conditions.

Qualitative and quantitative PBPs analysis

PBPs composition and concentration were estimated following the equations of modified by (Zavřel et al. 2018):

$$C-Phycocyanin(C-PC)(mg/ml) = \frac{[(A620-A720)-0.474\times(A652-A720)]}{5.34} \quad (1)$$

$$Allophycocyanin\,(ACP)\,(mg/ml) = \frac{[(A652-A720)-0.208\times(A620-A720)]}{5.09} \quad (2)$$

$$Phycoerythrin\,(PE)\,(mg/ml) = \frac{[A562-2.41(CPC)-0.849(ACP)]}{9.62} \quad (3)$$

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

$$PBPSs(\text{mg/g}) = \frac{(PC+ACP+PE)*V}{DB}$$

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

PBPPs purity

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

$$\text{C-PC Purity} = \frac{A620}{A280};\text{APC Purity} = \frac{A652}{A280};\text{PE Purity} = \frac{A562}{A280}$$

Where A280, A618, A652 and A562 are the maximum absorbance of the total protein, C-PC, APC and PE, respectively.

Measurement of the DPPH Free Radical-Scavenging Activity of PBPs extracts

The antioxidant scavenging activity against α.α-diphenyl-β-picrylhydrazyl radical (DPPH) was evaluated using the previously described method by Kang et al. (2004). First, 2 ml PBPs extract were mixed with 1 ml of DPPH in ethanol solution (0.02 mM) and DPPH served as the control. After 30 min of incubation in darkness at RT, the absorbance at 517 nm was measured. Antioxidant activity (AA) was evaluated following the equation:

AA (%) = (Control absorbance − Sample absorbance)/(Control absorbance) × 100.

Statistical analysis

The significant difference between mean values was assessed by one-way analysis of variance (ANOVA). Tukey test was carried out using SPSS 20.0 software to determine whether there was any significant difference at the level of $p < 0.05$.

Results and discussion

Optimization of PBPs extraction

Phycobiliproteins (PBPs) present in Spirulina, are composed of C-phycocyanin (C-PC), Allophycocyanin (APC) and Phycoerythrin (PE) at different proportions. In all experiments C-PC was the major component of PBPs extracted from used *Spirulina*. The concentration of the three components varied according to the extraction conditions: 1.05–8.52 mg/

Table 1 Effect of pretreatment and extraction time on PBPs extraction yieldand concentration

Pre-treatment	Extraction time (h)	PBPs concentration (mg/ml)			Ratio	Yield (mg/g)
		C-PC	APC	PE	C-PC:APC:PE	PBPs
Without pre-treatment	3	$1.41^{b} \pm 0.13$	$0.49^{b} \pm 0.18$	$0.12^{c} \pm 0.02$	1:0.3:0.08	$84.83^{c} \pm 11.63$
	6	$2.53^{a} \pm 0.09$	$1.77^{a} \pm 0.19$	$0.92^{a} \pm 0.05$	1:0.7:0.36	**$232.25^{a} \pm 6.24$**
	9	$2.48^{a} \pm 0.26$	$1.54^{a} \pm 0.17$	$0.37^{b} \pm 0.05$	1:0.6:0.14	$183.92^{b} \pm 16.35$
	12	$2.44^{a} \pm 0.06$	$1.14^{a} \pm 0.19$	$0.11^{c} \pm 0.05$	1:0.7:0.04	$154.51^{b} \pm 10.55$
	24	$1.21^{b} \pm 0.54$	$0.25^{b} \pm 0.13$	$0.10^{c} \pm 0.00$	1:0.2:0.08	$65.11^{c} \pm 17.25$
Freeze/Thaw	3	$1.25^{b} \pm 0.22$	$0.43^{b} \pm 0.24$	$0.12^{c} \pm 0.01$	1:0.4:0.09	$71.27^{c} \pm 18.73$
	6	$2.17^{a} \pm 0.06$	$1.23^{a} \pm 0.19$	$0.13^{c} \pm 0.03$	1:0.5:0.05	$147.75^{b} \pm 16.68$
	9	$2.07^{a} \pm 0.06$	$1.29^{a} \pm 0.11$	$0.11^{c} \pm 0.01$	1:0.6:0.05	$144.91^{b} \pm 17.12$
	12	$1.07^{b} \pm 0.09$	$1.55^{a} \pm 0.29$	$0.11^{c} \pm 0.05$	1:1.4:0.1	$155.98^{b} \pm 12.26$
	24	$1.05^{b} \pm 0.03$	$0.14^{b} \pm 0.60$	$0.10^{c} \pm 0.00$	1:0.1:0.09	$53.87^{c} \pm 7.26$

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 separation conditions on PBPs extraction yieldand concentration

Centrifugation		PBPs concentration (mg/ml)			Ratio	Yield (mg/g)
Speed (rpm)	Time (min)	C-PC	APC	PE	PC:APC:PE	PBPs
4000	15	$2.53^{b} \pm 0.09$	$1.77^{b} \pm 0.09$	$0.92^{b} \pm 0.05$	1:0.7:0.36	$218.33^{b} \pm 2.60$
	30	$2.55^{b} \pm 0.20$	$1.84^{b} \pm 0.07$	$0.87^{b} \pm 0.10$	1:0.7:0.34	$219.71^{b} \pm 2.06$
6000	15	$3.34^{a} \pm 0.07$	$2.34^{a} \pm 0.08$	$1.22^{a} \pm 0.10$	1:0.7:0.37	**$287.73^{a} \pm 4.18$**
	30	$3.23^{a} \pm 0.02$	$2.31^{a} \pm 0.10$	$1.25^{a} \pm 0.07$	1:0.7:0.39	$283.59^{a} \pm 5.77$
9000	15	$3.27^{a} \pm 0.06$	$2.3^{a} \pm 0.05$	$1.27^{a} \pm 0.06$	1:0.7:0.39	$285.96^{a} \pm 3.98$
	30	$3.18^{a} \pm 0.03$	$2.32^{a} \pm 0.08$	$1.24^{a} \pm 0.03$	1:0.7:0.39	$281.47^{a} \pm 3.43$

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

Table 3 PBPs yield and concentration obtained with different solvent/biomass ratios

Biomass	Solvent (L):biomass (g) ratio	PBPs concentration (mg/ml)			Yield (mg/g)
		C-PC	APC	PE	PBPs
Dry	1:24	$4.60^{d} \pm 0.31$	$2.11^{e} \pm 0.62$	$1.45^{e} \pm 0.30$	$360.23^{b} \pm 10.58$
	1:50	$8.52^{c} \pm 1.08$	$4.02^{e} \pm 1.37$	$3.39^{d} \pm 0.29$	**$464.50^{a} \pm 17.39$**
	1:100	$15.60^{b} \pm 4.65$	$8.27^{d} \pm 2.57$	$3.72^{d} \pm 0.43$	$250.59^{c} \pm 80.87$
	1:150	$21.27^{b} \pm 3.25$	$11.68^{c} \pm 3.21$	$5.44^{c} \pm 0.37$	$266.16^{c} \pm 50.72$
	1:200	$29.65^{b} \pm 5.67$	$20.30^{a} \pm 7.50$	$9.40^{b} \pm 0.41$	$273.34^{c} \pm 32.12$
	1:300	$41.46^{a} \pm 4.83$	$27.38^{a} \pm 4.66$	$13.47^{a} \pm 0.29$	$230.07^{c} \pm 63.09$
Fresh	1:24	$5.34^{d} \pm 2.11$	$4.12^{e} \pm 2.30$	$2.70^{e} \pm 1.19$	$232.32^{c} \pm 57.52$
	1:50	$18.42^{b} \pm 0.99$	$20.91^{a} \pm 0.66$	$9.40^{b} \pm 0.30$	$231.54^{c} \pm 22.49$
	1:100	$18.43^{b} \pm 0.56$	$6.73^{d} \pm 0.43$	$3.34^{d} \pm 0.23$	$288.15^{c} \pm 21.84$
	1:150	$20.61^{b} \pm 1.31$	$7.79^{d} \pm 0.43$	$3.44^{d} \pm 0.34$	$270.14^{c} \pm 68.63$
	1:200	N/A	N/A	N/A	N/A
	1:300	N/A	N/A	N/A	N/A

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

ml, 0.14–4.02 mg/ml and 0.10–3.39 mg/ml for C-PC, APC and PE, respectively. Moreover, PBPs recovery was between 53.8 and 664.5 mg/g depending on the used protocol.

Step 1 optimization

Table 1 showed the effect of pretreatment under different extraction times on PBPs recovery. The highest PBPs yield was obtained without pretreatment and with an extraction time of 6 h (232 mg/g vs. 84 mg/g for the control 'no pretreatment and 3 h of extraction') and the use of longer extraction times diminished the PBPs extraction efficiency. Tan et al. (2020) showed also that longer extraction times (24 h and more) decreased the extraction efficiency of phycobiliproteins from *Spirulina* biomass. This can be due to a pigments denaturation under used conditions since other studies extracted efficiently PBPs after 24 h of incubation (Vali Aftari et al. 2015; Pan-utai and Iamtham 2019a).

After 6 h of extraction, the crude extract contained 2.53, 1.77 and 0.92 mg/ml of C-PC, APC and PE, respectively in a ratio of 1:0.7:0.36 (Table 1). In addition, the evaluation of the effect of these conditions on PBPs composition (ratio C-PC:APC:PE) indicated an increase of APC and PE concentrations in the crude extract at 6 h, allowing a best equilibrium between these phycobiliproteins. However, longer extraction times didn't improve obtained results. The pretreatment used decreased extraction efficiency to 155 mg of PBPs by g of biomass (Table 1) and after an extraction time of 12 h, the crude extract contains more APC than C-PC (1.5 vs. 1 mg/ml, receptively). Here, the effect of the extraction time was less visible before 24 h. Previous work found no variation in the proportion on the three pigments in the crude extract depending on the extraction time with a ratio of 1:0.07:0.08 C-PC:APC:PE (Pan-utai and Iamtham 2019a). APC and PE percentages were lower than obtained in the current study.

In general, freezing–thawing has been widely used for cyanobacteria cell disruption during PBPs extraction (Tan et al. 2020; Yu 2017; Ores et al. 2016; Xie et al. 2015; Moraes et al. 2011). Controversial results were obtained and extraction efficiency depended on the number of freezing–thawing cycles and additional combination with

physical or chemical methods. In our experimental conditions, the use of a pretreatment prior to PBPs extraction didn't improve yield and a lot of debris remained in the extract. Moreover, high polysaccharides content was observed in hydrolysates in such condition, (data not shown) which could be hindering for downstream process. It has been previously demonstrated that polysaccharides affect the structure and stability of proteins including PBPs by forming electrostatic interactions (Li et al. 2021; Zhao et al. 2022). This may explain the difficulty to extract PBPs in the presence of large peptidoglycans that have not been broken during the drying biomass period.

The conditions of PBPs separation from broken trichomes (centrifugation speed and time) were also important to optimize the extraction yield as well as PBPs concentration in the extract. Indeed, the increase of centrifugation speed to 6000 rpm improved both the extraction yield and PBPs concentration by 30%, independently from the separation time (Table 2). Nevertheless, no additional improvement was obtained with faster separation.

Step 2 optimization

Phycobiliproteins are water soluble and the use of water can easily extract them. Nevertheless, it has been demonstrated that their concentration and stability were improved after use of some extraction buffers like potassium phosphate buffer (PPB), protic ionic liquid (*N*-methyl-2-hydroxyethylammonium acetate (2-HEAA) + *N*-methyl-2-hydroxyethylammonium formate (2-HEAF) or Tris–HCl (Poojary et al. 2016; Ores et al. 2016; Rodrigues et al. 2019).In addition, PBPs are pH sensitive since it affects their quaternary structure causing denaturation (Camara-Artigas et al. 2012; González Ramírez et al. 2014).In general, a maximal PBPs stability is reached in pH from 5.5 to 7 and decreased out of this range (Chaiklahan et al. 2012). When extraction was carried out with water, about 120 g of PBPs were extracted per g of biomass with a final concentration in the extract close to 3 mg/ml (Fig. 3). The use of phosphate buffer improved significantly both results in a dose dependent manner. Indeed, phosphate buffer at 100 mM allowed to extract about 300 mg/g of PBPs with a concentration of 6.8 mg/ml. This increased by 150 and 120% PBPs recovery and

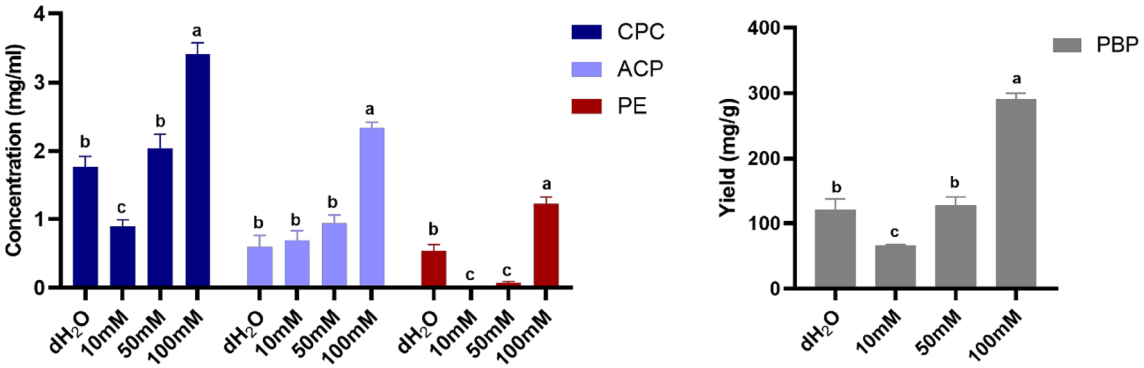

Fig. 3 Effect of the extraction buffer concentration on PBPs yield and concentration. Data with different letters are significantly different ($p < 0.05$)

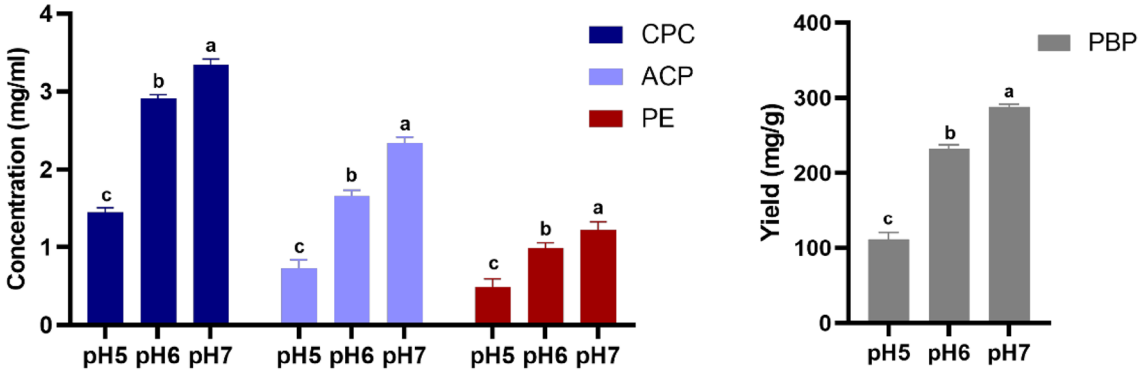

Fig. 4 Effect of phosphate buffer pH on PBPs yield and concentration. Data with different letters are significantly different ($p < 0.05$)

concentration, respectively, in comparison with water as extraction buffer. Controversial results were found regarding phosphate buffer vs. water efficiency. Some authors obtained better PBPs concentration with phosphate buffer while others with distilled water (İlter et al. 2018; Tan et al. 2020). In our study, high buffer concentration increased PBPs recovery. Similar results were found with sodium phosphate buffer (Pan-utai and Iamtham 2019b).

The buffer's pH affected both PBPs concentration and extraction yield (Fig. 4) in the same manner. With acid pH (5), protein extraction yield and concentration were reduced to half (Fig. 4). Previous studies obtained an optimal PBPs (specially C-Phycocyanin) extraction from *spirulina*'s at pH 7, while no detailed studies were found for Allophycocyanin and Phycoerythrin (Doke 2005; Li et al. 2020). In this way, it has been demonstrated that pH affects conformational structure charge of C-PC (Chang et al. 2018; Cottas et al. 2021). The use of concentrated phosphate buffer (100 mM) at pH7 for phycobiliproteins extraction, mainly C-PC, was previously described and seems to give good results due to its high biocompatibility with proteins (Ajayan et al. 2012; Pan-utai and Iamtham 2019b; Cottas et al. 2021).

The optimized extraction buffer was used to study the effect of some additives on PBPs extraction yield and concentration. No significant improvement was obtained excepted with1.5% $CaCl_2$ that increased by more than 30% PBPs yield and concentration (Fig. 5). Calcium chloride is used in agroindustry for stabilization and precipitation of specific components. And it is also used for cell disruption through a change of osmotic pressure, enabeling it to efficiently be used for C-PC extraction and conservation (Cisneros and Rito-Palomares 2004; İlter et al. 2018). A previous work obtained a good C-PC extraction efficiency by using 1.5% $CaCl_2$ (İlter et al. 2018). In such conditions, a darkest blue and concentrated C-PC extract was obtained, who's also occurred in our experimentation (Fig. 6).

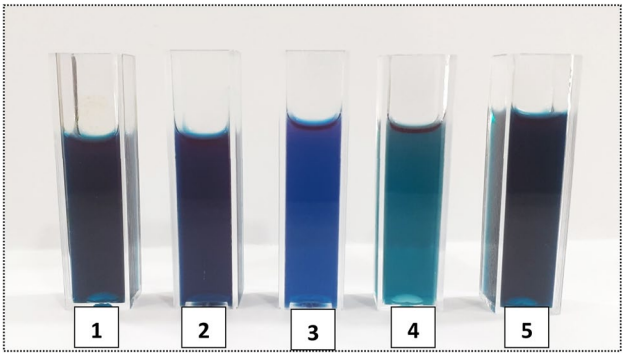

Fig. 6 Crude PBPs extract colors obtained with additives:1: control (100 mMPPB, pH7), 2:+1.5%$MgCl_2$, 3:+1.5%$CaCl_2$, 4:+PEG300 and 5:+PEG6000

The PEG is widely used for cell disruption mainly during electroporation and few studies used it to extract and purify C-PC from *spirulinaV* (Patil and Raghavarao 2007; Antelo et al. 2015). The system is designed to allow the target molecule to partition to the more soluble phase, whereas contaminants partition to the opposite phase, enabling simultaneous extraction and purification (Fratelli et al. 2021). For this reason, we studied the effect of two PEG molar masses on PBPs extraction. PEG300 reduced drastically PBPs yield and concentration, while PEG6000 gave no difference with the control (Fig. 6). Previous work found a positive correlation between PEG molecular mass and C-PC extraction yield that was 81% highest with PEG4000 in comparison with PEG2000 (Chew et al. 2019). Antelo et al. (2015) found also an effect of the PEG concentration on extraction with optimal recovery with PEG6000 (Antelo et al. 2015).

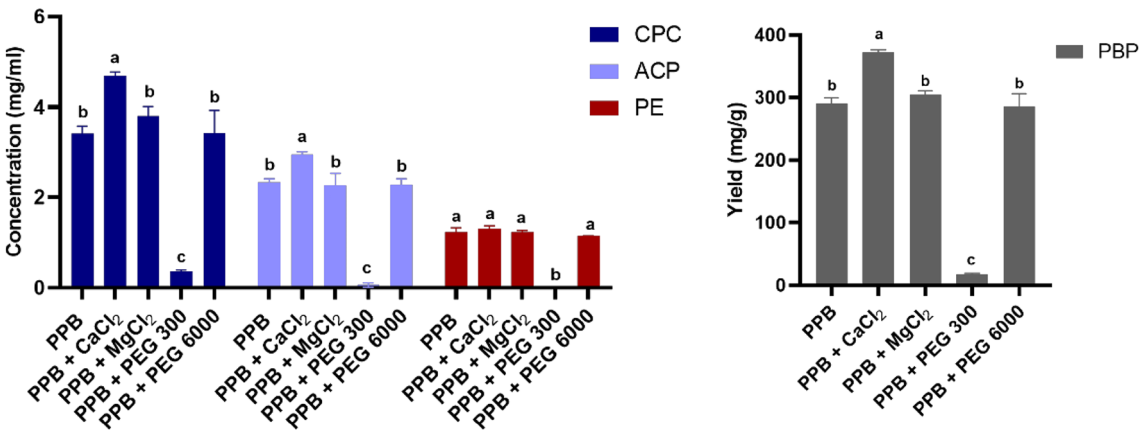

Fig. 5 Effect of buffer additives on PBPs yield and concentration. Buffers used herewas PPB100 mM, pH7 (control), or+1.5% $CaCl_2$,+1.5% $MgCl_2$,+PEG300 and+PEG6000

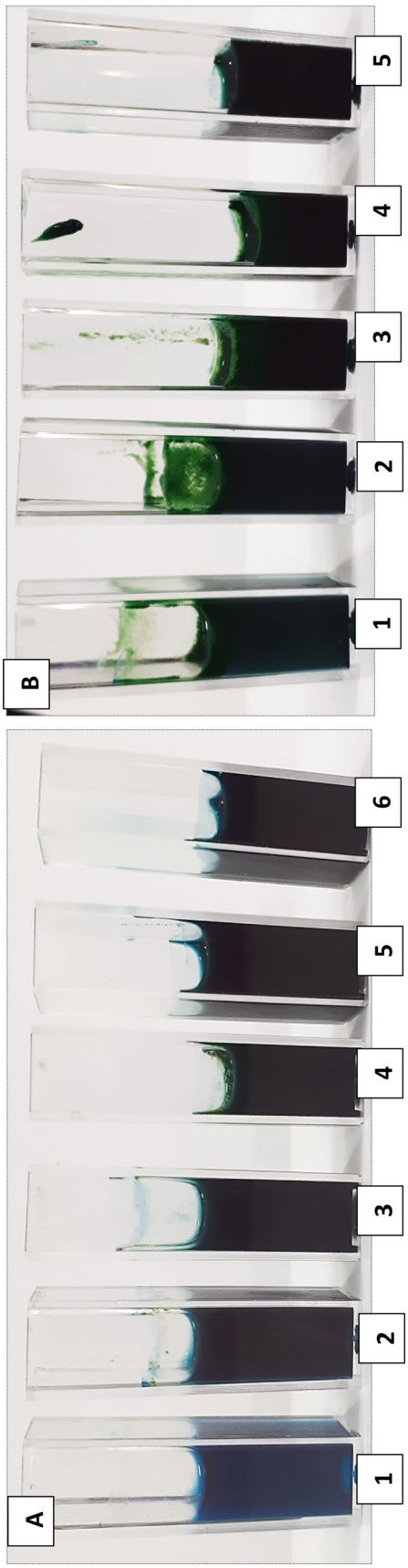

Fig. 7 Effect of biomass type and solvent:biomass ratio on color and texture of PBPs extract. **A** dry biomass/ **B**: fresh biomass; 1/ 1:24, 2/ 1:50, 3/ 1:100 4/ 1:150, 5/ 1:200 and 6/ 1:300

Step 3 optimization

The extractability of total phycobiliproteins was strongly affected with biomass ratio. When dry biomass was used, a positive correlation was obtained between extraction solvent: biomass ratio and PBPs yield and concentration (Fig. 7). The yield improved by 22% in comparison with initially used ratio (1:24) and PBPs concentration in the extract reached 15 mg/ml (Table 3). However, beyond a ratio of 1:50, biomass remained in the extract and could not be removed despite several centrifugation cycles (Fig. 7). We suppose that 50 g/l here was near to saturation point. The available bibliography used a wide range of ratios extraction solvents: biomass with controversial results. Tan et al. (2020) extracted a maximum of PBPs (97 mg/g) from *Spirulina* when a ratio of 1:5 was used. This remains less concentrated than our tested ratios. Other works showed that both total PBPs and C-PC were best extracted with highest (1:60 and 1:80) biomass concentration (Silveira et al. 2007; Pan-utai and Iamtham 2019a). In such conditions, close to our optimal ratio (1:50), about 16 mg/g PBPs were extracted while we extracted more than 400 mg/g.

We were not able to obtain a clean PBPs crude extract from fresh biomass. As shown in Fig. 7, biomass could not resuspend correctly to start proteins extraction process, even at the lowest used ratio. The initial biomass type (dry or fresh) seems influence the PBPs extraction efficiency and purity. İlter et al. (2018) obtained high C-PC when dry biomass was used (41.8% vs. 11.2% for fresh biomass). The same protocol was used satisfactory by Tavanandi et al. (2018) for C-PC extraction (Tavanandi et al. 2018). Nevertheless, other authors preferred fresh biomass that gave best results and avoided pigment losses (Manirafasha et al. 2016). The results seem depend on the used *Spirulina* strain and the pre-extraction conditions. Indeed, biomass drying can affect phycobiliproteins content and activity, while fresh biomass, highly perishable, can lead to their degradation during the storage. The choice may depend finally on several factors such as used strain and PBPs extraction and valuation industrial scheme. In the current work, we used a Moroccan linear *Arthrospira* strain. A recent study demonstrated that such strain has longer cell wall and higher peptidoglycan content, that can explain the difficulty to extract PBPs from fresh biomass (Zhao et al. 2022).

Effect of extraction process on purity

– C-Phycocyanin, Allophycocyanin and Phycoerythrin are generally graded according to its purity ratio, which corresponds to the contamination of the extract by proteins, other than PBPs. A purity of 0.7 is considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Rito-Palomares et al. 2001). Table 4 shows

Table 4 Effect of extraction process on purity

1ststep				
Pre-treatment	Extraction time (h)	Purity		
		C-PC	APC	PE
Without pre-treatment	3	$0.51^{a} \pm 0.13$	$0.11^{a} \pm 0.03$	$0.20^{a} \pm 0.06$
	6	$0.42^{a} \pm 0.09$	$0.14^{a} \pm 0.04$	$0.19^{a} \pm 0.10$
	9	$0.40^{a} \pm 0.12$	$0.10^{a} \pm 0.02$	$0.29^{a} \pm 0.11$
	12	$0.52^{a} \pm 0.05$	$0.12^{a} \pm 0.06$	$0.23^{a} \pm 0.15$
	24	$0.46^{a} \pm 0.10$	$0.11^{a} \pm 0.04$	$0.23^{a} \pm 0.09$
Freeze/Thaw	3	$0.39^{a} \pm 0.14$	$0.09^{a} \pm 0.04$	$0.18^{a} \pm 0.11$
	6	$0.50^{a} \pm 0.09$	$0.10^{a} \pm 0.06$	$0.28^{a} \pm 0.13$
	9	$0.39^{a} \pm 0.13$	$0.11^{a} \pm 0.03$	$0.29^{a} \pm 0.08$
	12	$0.49^{a} \pm 0.10$	$0.09^{a} \pm 0.04$	$0.28^{a} \pm 0.07$
	24	$0.42^{a} \pm 0.08$	$0.12^{a} \pm 0.03$	$0.19^{a} \pm 0.12$
Centrifugation		Purity		
Speed (rpm)	Time (min)	C-PC	APC	PE
4000	15	$0.44^{b} \pm 0.08$	$0.11^{b} \pm 0.04$	$0.28^{b} \pm 0.13$
	30	$0.50^{b} \pm 0.09$	$0.11^{b} \pm 0.03$	$0.29^{b} \pm 0.08$
6000	15	$0.73^{a} \pm 0.02$	$0.31^{a} \pm 0.10$	$0.54^{a} \pm 0.07$
	30	$0.76^{a} \pm 0.03$	$0.31^{a} \pm 0.11$	$0.52^{a} \pm 0.07$
9000	15	$0.70^{a} \pm 0.06$	$0.30^{a} \pm 0.05$	$0.57^{a} \pm 0.06$
	30	$0.74^{a} \pm 0.04$	$0.33^{a} \pm 0.09$	$0.55^{a} \pm 0.07$
2^{nd}step				
Buffer concentration		Purity		
Type	[C] mM	C-PC	APC	PE
dH_2O		$0.50^{b} \pm 0.09$	$0.10^{b} \pm 0.06$	$0.28^{b} \pm 0.13$
PPB	10	$0.73^{a} \pm 0.02$	$0.31^{a} \pm 0.10$	$0.54^{a} \pm 0.07$
	50	$0.74^{a} \pm 0.07$	$0.34^{a} \pm 0.08$	$0.52^{a} \pm 0.05$
	100	$0.71^{a} \pm 0.03$	$0.31^{a} \pm 0.09$	$0.55^{a} \pm 0.07$
Buffer pH		Purity		
Type	pH	C-PC	APC	PE
PPB	5	$0.73^{a} \pm 0.02$	$0.31^{a} \pm 0.10$	$0.54^{a} \pm 0.07$
	6	$0.74^{a} \pm 0.07$	$0.34^{a} \pm 0.08$	$0.52^{a} \pm 0.10$
	7	$0.75^{a} \pm 0.02$	$0.33^{a} \pm 0.10$	$0.50^{a} \pm 0.07$
Additives		Purity		
Type	Additive	C-PC	APC	PE
PPB		$0.73^{b} \pm 0.04$	$0.31^{c} \pm 0.09$	$0.54^{b} \pm 0.03$
PPB	$CaCl_2$	$\mathbf{0.94^{a} \pm 0.02}$	$\mathbf{0.76^{a} \pm 0.03}$	$\mathbf{0.89^{a} \pm 0.05}$
	$MgCl_2$	$0.84^{b} \pm 0.07$	$0.54^{b} \pm 0.08$	$0.52^{b} \pm 0.10$
	PEG_{300}	$0.68^{b} \pm 0.09$	$0.55^{b} \pm 0.00$	$0.51^{b} \pm 0.03$
	PEG_{6000}	$0.93^{a} \pm 0.07$	$0.64^{b} \pm 0.11$	$0.52^{b} \pm 0.08$
3^{rd}step				
Biomass	S (L): B (g) ratio	Purity		
		C-PC	APC	PE
Dry	1:24	$0.91^{a} \pm 0.10$	$0.72^{a} \pm 0.02$	$0.81^{a} \pm 0.07$
	1:50	$0.94^{a} \pm 0.05$	$0.76^{a} \pm 0.03$	$0.80^{a} \pm 0.05$
	1:100	$0.80^{a} \pm 0.07$	$0.74^{a} \pm 0.07$	$0.09^{a} \pm 0.02$
	1:150	N/A	N/A	N/A
Fresh	1:24	N/A	N/A	N/A

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

the effect of the extraction process on crude PBPs extract purity:

- *1st step:* The extraction time is a critical factor for PBPs recovery. However, it didn´t impact the purity ratio, as demonstrated by our study. Gorgich et al. (2020) showed similar results, without improvement of purity ratio after 24h of orbital shaking. On the other hand, the increase of centrifugation speed to 6000 rpm improved purity ratio from 0.44, 0.11, 0.28 to 0.73, 0.31, 0.54 for C-PC, APC and PE, respectively. The separation speed applied induced a good separation of the PBPBs from debris and impurities resulting in a purity ratio improvement.
- *2nd step*: Buffer concentration and pH value showed no significant effect on the purity ratio. Indeed, even if the buffer concentration and pH are directly implicated in the stability of proteins, they may not necessarily improve phycocyanin purity. Vali Aftari et al. (2015) also found that pH value had no significant effect on the purity ratio. Nevertheless, the addition of additives to the buffer solution sems have a great effect on purity ratio. For example, $CaCl_2$ and PEG_{6000} allowed reach the food-grade standard.
- *3rd step*: In this extraction stage, no significant influence of the solvent: biomass ratio was noted on the purity ratio. However, the increase in biomass presents challenges for optical density measurements due to the complexity of separating the solvent from the biomass.

In conclusion, even if several extraction conditions impact significantly the PBPs purity, the improvement of this indicator to other grades seems complicated and need additional steps. Higher PBPs purity ratio can be improved by purification techniques such as precipitation, ultrafiltration and dialysis as well as chromatographic (Yu et al. 2017; Ashaolu et al. 2021).

The results obtained in this study showed that all tested factors influenced phycobiliproteins PBPs extraction. The optimized protocol **[Extraction buffer** (PPB 100 mM, pH 7 + 1.5%$CaCl_2$) used to extract PBPs from **dried biomass** with a **ratio of 1:50**, for **6 h** followed with a centrifugation at **6000 rpm for 15 min**)] allowed to extract 464.5 mg/g of PBPs with a concentration of 16 mg/ml (52% C-PC, 25% APC and 23% PE) and food grade extract (0.94 C-PC, 0.72 APC, 0.81 PE). Several PBPs extraction protocols have been described in literature. For example, the combination of a protic ionic liquid (*N*-methyl-2-hydroxyethylammonium acetate (2-HEAA) + *N*-methyl-2-hydroxyethylammonium format (2-HEAF)), mechanical agitation and thermal heating (35 °C) were more performant than sodium phosphate buffer to extract PBPs with a concentration of 3.95 mg/ml (Rodrigues et al. 2019). Enzyme assisted methods were also used and mainly combined to other technics like ultrasound or microwave (Vali Aftari et al. 2015; Tavanandi et al. 2018). Ultrasounds assisted extraction carried out for 3 h at 60 kHz was also used and gave higher PBPs concentrations (15.2 mg/ml) in the crude extract (Choi and Lee 2018). Nevertheless, the cost and safety (with regard to PBPs stability) of these technics is still discuses.

Antioxidant activity

The crude PBPs extract obtained under optimal conditions exhibited a remarkable 86.45 ± 1.2% inhibition of DPPH radicals. This outcome compares favorably to the findings of Fekrat et al. (2019), who, using solvent extraction with potassium phosphate buffer, achieved a 82% reduction in DPPH radicals after 4 h of stirring at 400 rpm at room temperature and subsequent centrifugation at 18,000 × g for 10 min. In contrast, PBPs extracted with alternative solvents or under different conditions, as reported by Rodrigues et al. (2019) and Pan-utai and Iamtham (2019a), demonstrated lower DPPH radical inhibition, typically near 60%. However, the study of Seo et al. (2013) showed that phycocyanin isolated using the high-pressure process and hexane separation method achieved a substantial 83% removal rate of oxygen free radicals. In summary, the DPPH radical inhibition achieved under the optimal conditions in our study highlight the effectiveness of our extraction approach.

Conclusions

Even if phycobiliproteins are water soluble, their extraction from *Spirulina*'s biomass is a delicate process. Indeed, each step of the extraction protocol is determining for yield and quality of extracted PBPs. Results showed that, for studied *Spirulina* strain, the use of dry biomass (50 g/l) without pretreatment gave better results. Moreover, a concentrated phosphate buffer added with 1.5% $CaCl_2$ and a relatively short extraction time (6 h) improved the PBPs recovery. High PBPs yield was obtained with the optimized protocol (464 mg/g) and their concentration reached 15.9 mg/ml. The developed method is easily scalable for phycobiliproteins at large scale for further applications.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest related to funding or otherwise.

Ethical approval As no human or mammalian subjects were involved in this research, no ethical approval was required.

Consent for publication All authors consent to publication.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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