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Optimization of Phycobiliprotein Solubilization from a Thermotolerant *Oscillatoria* sp.

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Abstract: The present study evaluated the effect of multiple variables (drying time, drying temperature, biomass/solvent ratio, glass beads/biomass ratio, extraction time, and extraction speed) in the solubilization of three different phycobiliproteins (C-PC, APC, and PE) from a thermotolerant *Oscillatoria* sp. The strain was grown in BG11 media (28 °C, light: dark cycle of 12:12 h at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 20 days) and the experiments were conducted according to a two-level randomized factorial design with six center points (38 runs). Results show that biomass/solvent ratio, glass beads/biomass ratio, and extraction time, are the most significant variables in the extraction of all three proteins, whereas the glass beads/biomass ratio and extraction time significantly affect their purity. The optimized conditions allow a statistical increase in the concentration of C-PC, APC, and PE extracted from the biomass; however, the purity was lower in comparison with the expected value. The latter occurs due to a larger biomass/solvent ratio and longer extraction times, which enhanced the solubility of other hydrophilic metabolites (proteins and carbohydrates, etc.).

Keywords: surface response methodology; allophycocyanin; phycocyanin; phycoerythrin; drying; biomass/solvent ratio; cell lysis

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1. Introduction

The global demand for organic and sustainable food products has risen over the past decade. Furthermore, as a result of the expansion in the world's population, it is vital to investigate and identify alternative food sources [1] that comply with national and international regulations [2]. Microalgae and cyanobacteria are now considered promising sources of natural molecules (such as carbohydrates, proteins, lipids, etc.) suitable for food, feed, cosmetic, and pharmaceutic applications [3–11]. Among those components, phycobiliproteins (PBPs) have gained popularity in the food and pharmaceutical sectors [12]. PBPs are a group of brilliant-colored proteins responsible for photosynthetic activity in different microbial groups such as cyanobacteria, cryptophytes, red algae, and glaucocystophytes [2]. PBPs are composed of several classes called phycocyanin (C-PC), allophycocyanin (APC), and phycoerythrin (PE). Each of these classes possesses a unique spectrum referred to as blue (610−620 nm), blue-green (650−655 nm), and pink (540−570 nm) [3,7,13]. Due to its antioxidant and anti-inflammatory properties, and its flexibility to be used by various industries such as the medical, pharmaceutical, cosmetic, and food industries, phycocyanin (C-PC) is one of the most expensive dye proteins on the market, with an estimated value of 5000–33,000 USD per gram [8]. C-PC has shown great success in the food sector as a replacement for its synthetic counterparts such as Patent Blue V and Brilliant Blue FCF (E133) [12].

Once the cyanobacterial biomass has been harvested, it must undergo physical and chemical processes to efficiently extract the different metabolites concealed within the cell [9]. Over the last ten years, other authors have identified several critical factors that affect PBPs' extraction yield and purity grade [10]. The most notable factors are drying or water content [14–16], temperature [2,8–11,16–19], biomass/solvent ratio [8–10,14,15,18–22], mixing speed [14,15,19–21], and mixing time [8–11,14,15,17–20,23].

The cyanobacterial and algal biomass is known for its high humidity content (up to 80%) biomass. This high humidity can significantly reduce its shelf life and thus reduce the quality of the metabolites of interest [16]. Therefore, it is critical to remove excess water totally (or partially) [24]. In the case of phycobiliproteins, temperatures higher than 45 °C degrees can reduce the concentration and quality of these pigments by up to 50% (*w*/*w*) [25]. However, as pointed out by Pez Jaeschke et al. [1], the information available on the use of biomass form (fresh, frozen, oven-dried, or freeze-dried) is still unclear. Another factor that influences the extraction efficiency is the destruction time and speed. Since the extraction solvent employed is an aqueous buffer, other hydrophilic substances such as carbohydrates and total proteins will be diluted into the buffer, affecting PBPs' final purity [26]. Moreover, longer extraction times will eventually increase the system's temperature, which will degrade the PBPs [12].

Since most of the works published in recent years focused on the analysis of one or two factors, the objective of this study was to evaluate for the first time the effect of multiple factors (drying, biomass/solvent ratio, biomass/glass beads ratio, mixing speed and time) on the extraction yield and purity of phycobiliproteins (C-CP, APC, and PE) from a thermotolerant strain of *Oscillatoria* sp. with a high concentration of those colorant proteins.

2. Materials and Methods

2.1. Strain

Oscillatoria sp. OSCI_UFPS001 was isolated from a thermal spring in Cucuta (Colombia) and kept at the INNOValgae collection (UFPS, Cúcuta, Colombia) (https://www.innovalg.com accessed on 20 April 2020). The strain was cultured in a 2 L tubular glass flask with 1.3 L of BG-11 media (composition in g/L: citric acid 0.006; $NaNO_3$ 1.5; $K_2HPO_4 \cdot 3H_2O$ 0.04; $MgSO_4 \cdot 7H_2O$ 0.075; $CaCl_2 \cdot 2H_2O$ 0.036; Na_2CO_3 0.02; $MgNa_2EDTA \cdot H_2O$ 0.01; and 1 mL of trace element solution (in g/L: H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$ 1.81; $ZnSO_4 \cdot 7H_2O$ 0.222; $NaMoO_4 \cdot 2H_2O$ 0.39; $CuSO_4 \cdot 5H_2O$ 0.079; Co $(NO_3)_2 \cdot 6H_2O$ 0.049 with pH 7.0 ± 2) [27]. The strain was mixed through the injection of filtered air with 1% (*v*/*v*) CO_2 at a flow rate of 0.78 L min^{-1}, with a light:dark cycle of 12:12 h at 100 µmol·m^{-2}·s^{-1}, 27 ± 1 °C, for 20 days.

2.2. Experimental Design

The evaluation of six factors that affect the extraction yield and purity of PBPs were studied using a 2-level randomized factorial design with six center points (38 runs), using Design-Expert® software (version 13.0, Stat-Ease Inc., Minneapolis, MN, USA). Table 1 shows the levels studied for each factor.

Table 1. Variables evaluated with their levels for the extraction of PBPs.

Variables	Coded Name	Low Level (−1)	High Level (+1)
Drying time (h)	A	18	30
Drying temperature (°C)	B	40	60
Biomass/solvent ratio (%)	C	0.125	0.5
Glass beads/biomass ratio (%)	D	5	15
Extraction time (min)	E	10	30
Extraction speed (rpm)	F	1000	1500

2.3. Culture Conditions

For each experiment, *Oscillatoria* sp. was cultured (in triplicate) in 500 mL flasks with a working volume of 250 mL of BG-11 culture media. Each flask was mixed through the injection of filtered air with 1% (*v*/*v*) CO_2 at a flow rate of 0.15 $L_{air} \cdot min^{-1}$, with a light:dark cycle of 12:12 h at 100 µmol·m^{-2}·s^{-1}, 27 ± 1 °C, for 20 days.

2.4. Biomass and PBPs Quantification

After 20 days, each flask was removed from the air inlet and allowed to self-flocculate, due to the filamentous morphology of *Oscillatoria* sp., the cyanobacteria precipitate in 20 min. The concentrated biomass was transferred to a 500 mL plastic flask and centrifuged at 3500 rpm (20 °C, 20 min), and the supernatant was withdrawn. The pellet was poured into non-stick food-grade silicone molds. The different samples were dried using a food-grade dehydrator (Hamilton Beach® 32100a). The mass obtained was recorded using a digital balance. The dehydrated biomass was subjected to extraction (one sample plus 5 replicates) using the method described by Zuorro et al. [28]. Briefly, a known amount of dried biomass was mixed with a volume of cold phosphate buffer solution (0.05 M, pH 6.8) and a known amount of glass beads (0.5 mm diameter) according to the experimental design. The solution was mixed using an automatic vortex (Multi Reax, Heidolph, Germany). The mixture was stored in a refrigerator to promote the solubilization of the phycobiliproteins (4 °C, 24 h). PBPs were separated from cell debris by centrifugation (3400 rpm, 30 min, 20 °C). The deep blue supernatant was collected and measured in a spectrophotometer at different wavelengths (620, 652, 562, and 280 nm). The concentration of C-PC, APC, and PE was calculated using the Equations (1)–(3) described by Bennett and Bogorad [29]. The purity of C-PC, APC, and PE was determined using the Equations (4)–(6) proposed by Patil [30] and Antello et al. [31]. The average of the results obtained for each experiment was used to perform the ANOVA analysis according to the Design-Expert® software.

$$\text{PC}\ [\text{g}/\text{L}] = \frac{\text{OD}_{620} - 0.474(\text{OD}_{652})}{5.34} \quad (1)$$

$$\text{APC}\ [\text{g}/\text{L}] = \frac{\text{OD}_{652} - 0.208(\text{OD}_{620})}{5.09} \quad (2)$$

$$\text{PE}\ [\text{g}/\text{L}] = \frac{(\text{OD}_{562} - 2.41(\text{P} - \text{PC}) - 0.849(\text{APC}))}{9.62} \quad (3)$$

$$\text{PC}\ [\text{purity}] = \frac{\text{OD}_{620}}{280} \quad (4)$$

$$\text{APC}\ [\text{purity}] = \frac{\text{OD}_{652}}{280} \quad (5)$$

$$\text{PE}\ [\text{purity}] = \frac{\text{OD}_{562}}{280} \quad (6)$$

3. Results

3.1. Effect of Multiple Parameters on the Concentration of PBPs

The results of the ANOVA analysis (Table 2) on the effect of multiple variables (drying time (A), drying temperature (B), biomass/solvent ratio (C), glass beads/biomass ratio (D), extraction time (E), and extraction speed (F)) in the extraction of phycobiliproteins (C-PC, APC, and PE) from *Oscillatoria* sp. biomass shows that in the case of C-PC, the F-value obtained (85.78) implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise; therefore, the results obtained did not happen by chance.

The *p*-values obtained for the different variables analyzed show that drying time, drying temperature, biomass/solvent ratio, glass beads/biomass ratio, extraction time, and extraction speed (A, B, C, D, E, and F, respectively) are statistically significant (p-value < 0.05) variables that affect the extraction efficiency. Another interesting result obtained is that several interactions between the analyzed variables (AB, BC, BE, BF, CE, DF, EF, ABD, ABE, ABF, ACD, ADE, ADF, and AEF) are significant model terms. The Lack of Fit F-value of 1.32 implies the Lack of Fit is not substantial relative to the pure error, which means that the model fits.

In the case of allophycocyanin (APC), an F-value of 13.38 implies the model is significant, with only a 0.01% chance that an F-value this large could occur due to noise.

Table 2. Analysis of variance (ANOVA) of the model obtained for C-PC, APC, and PE extraction.

		Sum of Squares	Df	Mean Square	F-Value	*p*-Value
C-PC (g/L)	Model	47.83	27	1.77	85.78	<0.0001 *
	Curvature	1.48	1	1.48	71.73	<0.0001 *
	Residual	0.1859	9	0.0207		
	Lack of Fit	0.0956	4	0.0239	1.32	0.3760 **
	Pure Error	0.0903	5	0.0181		
	Cor Total	49.49	37			
APC (g/L)	Model	92.28	23	4.01	13.38	<0.0001 *
	Curvature	1.21	1	1.21	4.03	0.0658
	Residual	3.90	13	0.2997		
	Lack of Fit	1.51	8	0.1886	0.3948	0.8834 **
	Pure Error	2.39	5	0.4776		
	Cor Total	97.38	37			
PE (g/L)	Model	302.89	11	27.54	18.54	<0.0001 *
	Curvature	16.17	1	16.17	10.89	0.0029
	Residual	37.14	25	1.49		
	Lack of Fit	34.80	20	1.74	3.73	0.0748 **
	Pure Error	2.33	5	0.4666		
	Cor Total	356.20	37			

	R^2	Adj R^2	Pred R^2	Adq Pr	Std. Dev.	Mean	C.V. %
C-PC g/L	0.9961	0.9845	0.8699	355.217	0.1437	2.72	5.28
APC g/L	0.9595	0.8878	0.7133	135.805	0.5475	4.81	11.39
PE g/L	0.8908	0.8427	0.7281	169.951	1.22	7.56	16.11

* Significant. ** Not Significant.

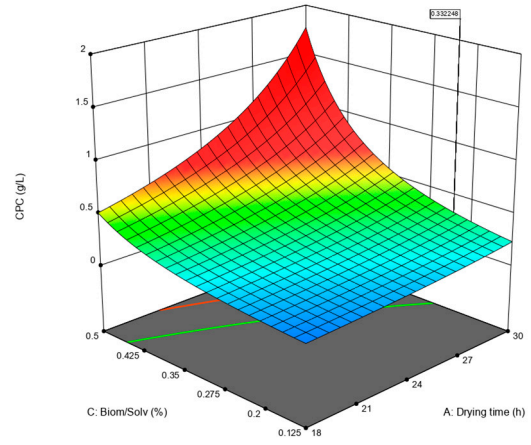
In the extraction of APC, the statistically significant variables (p-value < 0.05) were biomass/solvent ratio, glass beads/biomass ratio, and extraction time (C, D, and E, respectively). Moreover, different interactions between the variables were found to be statistically significant (AB, BC, CE, DF, ABD, ABF, ACD, ADE, and ADF). The Lack of Fit F-value of 0.39 implies the Lack of Fit is not significant relative to the pure error, which means that the model fits.

Finally, the analysis of the extraction of phycoerythrin (PE) shows that an F-value of 18.54 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Drying time, biomass/solvent ratio, glass beads/biomass ratio, extraction time, and extraction speed (A, C, D, E, and F, respectively) and several interactions (BF and ADE) are the most important terms affecting the extraction of the colorant protein PE. The Lack of Fit F-value of 3.73 implies the Lack of Fit is not significant relative to the pure error, which means that the model fits.

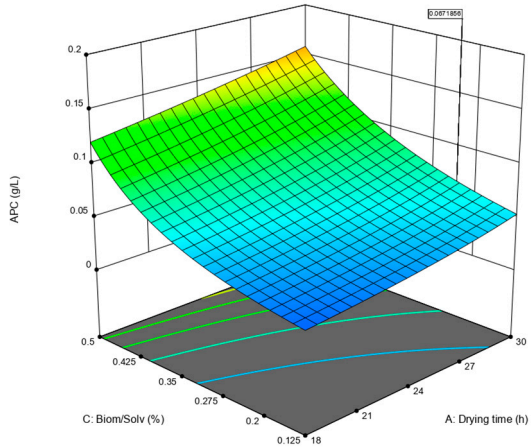
3.2. Effect of Multiple Parameters on the Purity of PBPs

One of the most critical responses in the extraction of phycobiliproteins is their purity. Since PBPs are proteins, the selected extraction method will inevitably allow the solubilization of other groups of proteins that will reduce the quality of the extract. The results of the ANOVA analysis (Table 3) on the purity of PBPs shows that, in the case of C-PC, the F-value obtained (16.17) for this protein implies the model is significant. There is only a 0.04% chance that an F-value this large could occur due to noise. In this case, the purity of C-CP is affected only by glass beads/biomass ratio, extraction time (D, E, respectively), and several interactions (AB, AE, BC, BE, BF, EF, ABC, ABD, ABE, ABF, ACD, ADE, and ADF); in this case, these terms are considered to be significant model terms. The Lack of Fit F-value of 0.09 implies the Lack of Fit is not significant relative to the pure error, which means that the model fits. The different surface responses (three-dimensional plots) obtained from the extraction of C-PC, APC, and PE are shown in Figure 1. According to the results, a high

biomass/solvent ratio of up to 0.5% w/v coupled with 30 h of drying using a food-grade dehydrator maximizes the extraction of the studied PBPs.



(**a**)



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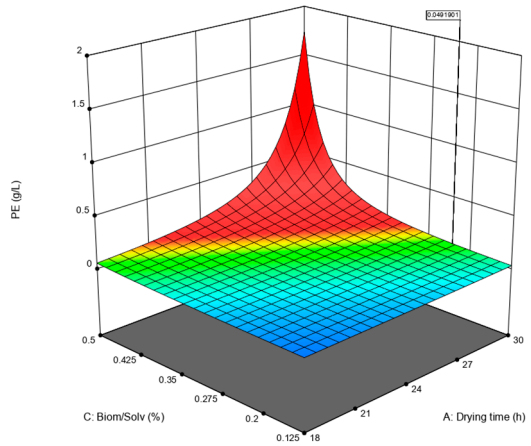


(**c**)

Figure 1. Surface response (three-dimensional plots) of the model equation fitted to the data on extraction concentration (g/L) of C-PC (**a**), APC (**b**), and PE (**c**).

Table 3. Analysis of variance (ANOVA) of the model obtained for phycobiliprotein purity.

		Sum of Squares	df	Mean Square	F-Value	*p*-Value
C-PC Purity	Model	0.5172	29	0.0178	16.17	0.0004 *
	Curvature	0.0237	1	0.0237	21.50	0.0024
	Residual	0.0077	7	0.0011		
	Lack of Fit	0.0003	2	0.0001	0.0914	0.9141 **
	Pure Error	0.0074	5	0.0015		
	Cor Total	0.5486	37			
APC Purity	Model	0.2874	26	0.0111	4.16	0.0113 *
	Curvature	0.0046	1	0.0046	1.73	0.2172
	Residual	0.0266	10	0.0027		
	Lack of Fit	0.0015	5	0.0003	0.0599	0.9961 **
	Pure Error	0.0251	5	0.0050		
	Cor Total	0.3187	37			
PE Purity	Model	1.15	21	0.0550	11.24	<0.0001 *
	Curvature	0.1099	1	0.1099	22.47	0.0003
	Residual	0.0734	15	0.0049		
	Lack of Fit	0.0333	10	0.0033	0.4152	0.8890 **
	Pure Error	0.0401	5	0.0080		
	Cor Total	1.34	37			

	R^2	Adj R^2	Pred R^2	Adq Pr	Std. Dev.	Mean	C.V. %
C-PC Purity	0.9853	0.9244	0.8468	236.165	0.0332	0.0921	36.04
APC Purity	0.9153	0.6951	0.6887	115.801	0.0516	0.3667	14.06
PE Purity	0.9402	0.8565	0.6753	176.953	0.0699	1.47	4.77

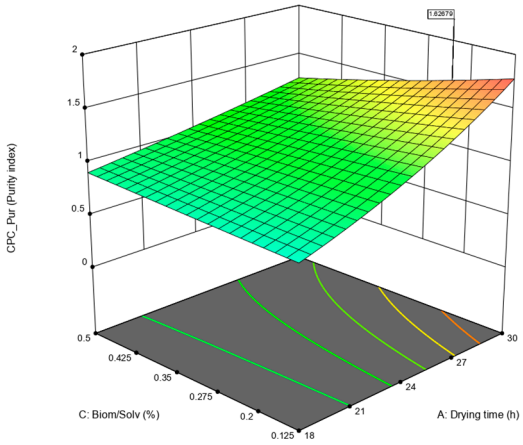
* Significant. ** Not Significant.

In the case of APC, an F-value of 5.37 implies the model is significant, with only a 0.21% chance that an F-value this large could occur due to noise. According to the *p*-values obtained for the different model terms, only glass beads/biomass ratio, extraction time (D and E, respectively), and several interactions between the analyzed variables are significant model terms (AB, AE, BC, BF, ABC, ABD, ABF, ACD, and ADE). The Lack of Fit F-value of 0.05 implies the Lack of Fit is not significant relative to the pure error, which means that the model fits.

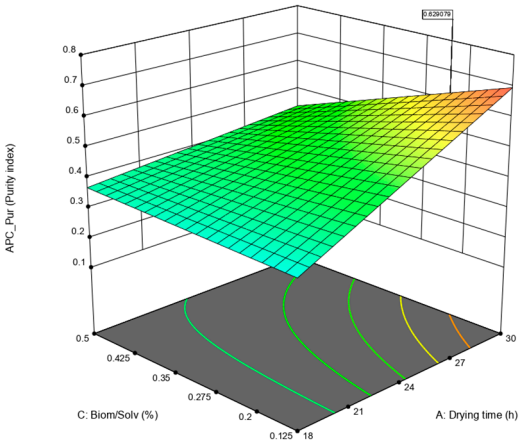
Finally, the analysis of the extraction of PE shows that an F-value of 13.75 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Drying time, glass beads/biomass ratio, extraction time (A, D, and E, respectively), and several interactions (AB, AE, BC, BE, BF, DE, ABC, ABD, ABE, ABF, ACD, ADE, and ADF) are the most significant terms affecting the extraction of the colorant protein PE. The Lack of Fit F-value of 0.36 implies the Lack of Fit is not significant relative to the pure error, which means that the model fits.

The surface responses (three-dimensional plots) of the model equation fitted to the data on the purity index of the extracted C-PC, APC, and PE are shown in Figure 2. Unlike the extraction results, the purity of PBPs is favored with a lower biomass/solvent ratio of 0.2 % w/v (or lower), and 30 h of drying using a food-grade dehydrator can effectively enhance the purity of the extracted PBPs.

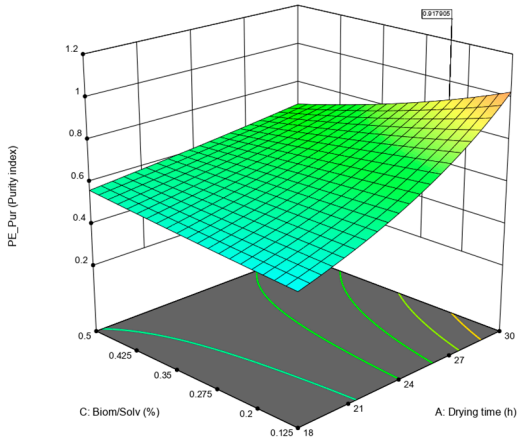
Table 4 represents the highest scenario that maximizes C-PC, APC, and PE concentration and purity using all the different variables analyzed. To test the viability of the proposed method, *Oscillatoria* sp. was grown in three 2 L flasks (1.2 L of working volume, 0.72 L·min^{-1} of filtered air, 12:12 h light:dark cycle at 100 µmol·m^{-2}·s^{-1}, 27 ± 1 °C, for 20 days). The biomass obtained was prepared according to Table 3, with 12 samples (one experiment with 11 replicates).



(**a**)



(**b**)



(**c**)

Figure 2. Surface response (three-dimensional plots) of the model equation fitted to the data on purity of C-PC (**a**), APC (**b**), and PE (**c**).

Table 4. Variables for optimal biomass concentration on both strains were studied.

Coded Name	Variable	Units	Value
A	Drying time	h	30
B	Drying temperature	°C	40
C	Biomass/Solvent ratio	% *w*/*v*	0.26
D	Glass beads/Biomass ratio	% *w*/*v*	14.9
E	Extraction time	min	30
F	Extraction speed	rpm	1486
Z_1	C-PC		0.38
Z_2	APC	g/L	0.073
Z_3	PE		0.064
Z_4	C-PC		1.56
Z_5	APC	Purity Index	0.64
Z_6	PE		0.87

The results obtained for the concentration and purity of C-PC, APC, and PE were analyzed using an unpaired *t*-test (Figure 3). The analysis shows that the concentration of C-PC, APC, and PE under the conditions presented in Table 4 were statistically higher than expected (<0.0001). However, the purity of the obtained extracts was lower than expected. The latter indicates that the proposed factors can effectively enhance the solubility of C-PC, APC, and PE; however, they also increase the solubility of other proteins (globular proteins, etc.), which in turn reduce the purity of PBPs.

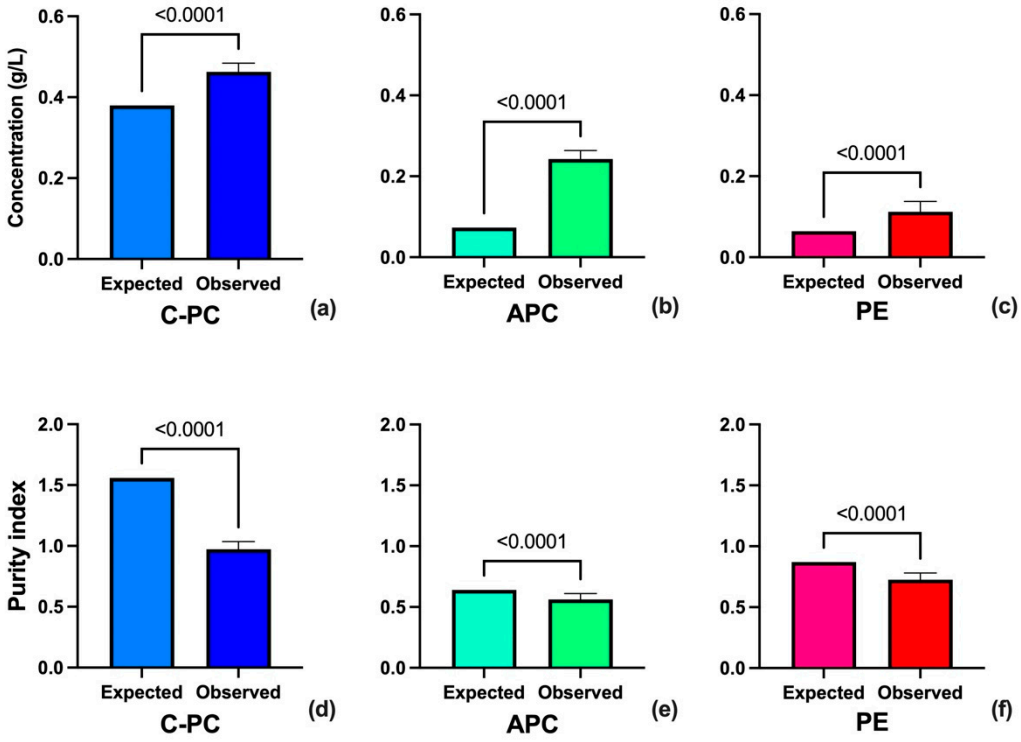


Figure 3. Unpaired *t*-test between the expected and expected results from extraction (**a**–**c**) and purity (**d**–**f**) of C-PC, APC, and PE.

4. Discussion

In recent years, PBPs obtained from cyanobacteria and red algae have become a dye of industrial interest [2]. The main source of these proteins are *Arthorspira platensis* [2,4,9,10,12,15,18,20,21], and *A. máxima* [19]; therefore, the extraction protocols have been specifically designed for this genera. More recently, another group of species has been evaluated to produce different PBPs, such as *Anabaena cylindrica* [11], *Cyanidium cal-*

darium [23], *Cyanidioschyzon merolae* NIES-3377 [17], *Gracilaria gracilis* [21], *Nostoc commune* TUBT05 [8], *Oscillatoria* sp. BTA170, [22], and *Oscillatoria okeni* TISTR8549 [8].

Typically, most studies in this area evaluate the effect of two or a maximum of four variables at a time. Moreover, most of these works employ One Factor at a Time (OFAT) experimental processes, increasing the experimentation time required. Design of Experiments (DoE) is a series of statistical tools that improve the number of the experiments needed to obtain a statistically significant response [32–35]; in recent years, its application in biotechnology has proven to be a valuable tool to reduce experimental costs and improve the quality of the results [36–40]. One example of the application of DoE in the optimization of PBP extraction is the work reported by Sintra et al. [11], where the researchers evaluated the effect of solid–liquid ratio, time of extraction, and temperature in the extraction of C-PC and chlorophylls in *Anabaena cylindrica* using the response surface methodology (RSM). In another study, Ferreira-Santo et al. [10] found that the application of ohmic heating (OH) at shorter times (44 °C and 30 min, respectively) allowed a significant increase in C-phycocyanin extraction yield compared to conventional heating and freeze-thawing. Another example is the experiment reported by Tavanandi et al. [18], where they analyzed the effect of ultrasonication with thawing and maceration. Other more advanced research, such as the work of Ilter et al. [14], examined the impact of up to five variables (homogenization rate (rpm), amplitude (%), microwave power (W), biomass/solvent ratio (%), and extraction time of classical, ultrasound and microwave extraction) in the extraction of C-PC using central composite rotatable design (CCRD).

In the case of the PBP extraction, the common factors affecting the efficiency of C-PC, APC, and PE were biomass/solvent ratio (C), glass beads/biomass ratio (D), and extraction time (E). By comparison, the most repetitive interaction found was drying time–glass beads/biomass ratio–extraction time (ADE). In the case of the PBP purity, the common factors affecting the efficiency of C-PC, APC, and PE were glass beads/biomass ratio (D) and extraction time (E). In addition, the most repetitive interactions found were AB, AE, BC, BF, ABC, ABD, ABF, ACD, and ADE, where drying time is the most repetitive factor (A). One exciting result obtained from the ANOVA analysis for the extraction of PBPs is that only C-PC and PE showed a fit for the curvature model. The latter may occur due to the high content of both proteins in comparison with APC in the thermotolerant strain of *Oscillatoria* sp. The same result was obtained in analyzing the purity of all three PBPs.

In each of the papers mentioned previously, the authors employed freeze-dried or spray-dried biomass. Since PBPs are sensitive to high temperatures (>45 °C) [25], these drying processes are preferred. To the best of the author's knowledge, this is the first time a food dehydrator has been used for the drying process of cyanobacterial biomass for PBP extraction. A food dehydrator is an inexpensive piece of equipment that can be acquired in almost every country. This research showed excellent results in maintaining the quantity and quality of PBPs while removing the moisture within the cyanobacterial biomass using longer times and mild temperatures (30 h and 40 °C, respectively). Another poorly evaluated variable is the glass beads/biomass ratio; the latter occurs because when glass beads are employed, they are commonly added in excess to better destroy cyanobacterial or algal biomass.

This research shows that a glass beads/biomass ratio of 15% *w*/*w* enhances biomass contact with the beads, allowing better PBP solubilization. Another variable worth mentioning is the destruction time; according to the results, longer times increase the content of PBPs extracted from the cells but reduce their purity. These results are congruent with those reported by Pott et al. [26], who found that longer times will eventually solubilize other proteins and hydrophilic metabolites such as carbohydrates. Another variable that affects the extraction time is the biomass/solvent ratio. According to the results, larger proportions of biomass in the buffer will increase the final content of PBPs but decrease their purity. One possible explanation for this behavior between PBP concentration and purity can be found in the drying time–glass beads/biomass ratio–extraction time (ADE) interaction; however, it is necessary to explore this interaction using other experimental

design methods, such as central composite design, to determine if both the concentration and purity of PBPs can be optimized in parallel.

5. Conclusions

This study indicates that the Design of Experiments is a robust methodology for identifying critical variables that enhance the extraction and purity of PBPs in *Oscillatoria* sp. The obtained results prove that, in the case of the PBP extraction, the common factors are biomass/solvent ratio, glass beads/biomass ratio, and extraction time, whereas the factors affecting purity are glass beads/biomass ratio and extraction time. The optimized conditions allowed the concentration of extracted C-PC, APC, and PE to be significantly increase; however, the purity of the proteins was lower than expected. The latter occurs due to a larger biomass/solvent ratio and longer extraction times, which enhance the solubility of other hydrophilic metabolites (proteins and carbohydrates, etc.). Future studies should provide further insight into the optimization of the interaction between the drying time, glass beads/biomass ratio, and extraction time variables.

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