

Enhancement of Phycobiliprotein Accumulation in Thermotolerant *Oscillatoria* sp. through Media Optimization

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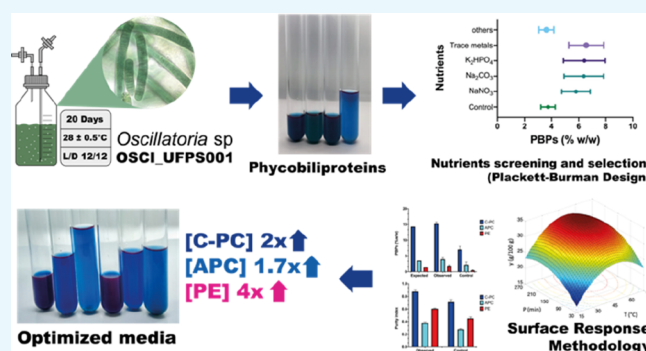
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ABSTRACT: Phycobiliproteins (PBPs) are a group of brilliant pigment proteins found in cyanobacteria and red algae; their synthesis and accumulation depend on several factors such as the type of strain employed, nutrient concentration, light intensity, light regimes, and others. This study evaluates the effect of macronutrients (citrate buffer, NaNO_3 , K_2HPO_4 , MgSO_4 , CaCl_2 , Na_2CO_3 , and EDTA) and the concentration of trace metals in BG-11 media on the accumulation of PBPs in a thermotolerant strain of *Oscillatoria* sp. The strain was grown in BG-11 media at 28 °C with a light:dark cycle of 12:12 h at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 15 days, and the effect of nutrients was evaluated using a Plackett–Burman Design followed by optimization using a response surface methodology. Results from the concentration of trace metals show that it can be reduced up to half-strength in its initial concentration without affecting both biomass and PBPs. Results from the Plackett–Burman Design revealed that only NaNO_3 , Na_2CO_3 , and K_2HPO_4 show a significant increase in PBP production. Optimization employed a central Non-Factorial Response Surface Design with three levels and four factors (3^4) using NaNO_3 , Na_2CO_3 , K_2HPO_4 , and trace metals as variables, while the other components of BG-11 media (citrate buffer, MgSO_4 , CaCl_2 , and EDTA) were used in half of their initial concentration. Results from the optimization show that interaction between Na_2CO_3 and K_2HPO_4 highly increased PBPs' concentration, with values of 15.21, 3.95, and 1.89 (% w/w), respectively. These results demonstrate that identifying and adjusting the concentration of critical nutrients can increase the concentration of PBPs up to two times for phycocyanin and allophycocyanin while four times for phycoerythrin. Finally, the reduction in non-key nutrients' concentration will reduce the production costs of colorants at an industrial scale and increase the sustainability of the process.



1. INTRODUCTION

Cyanobacteria are a group of photosynthetic prokaryotes that originated about 3.5 billion years ago. It can be found in different aquatic environments such as lakes, rivers,¹ and hot springs.² Cyanobacteria are considered one of the new sustainable biotechnological sources of various raw materials for the global pharmaceutical, food, clinical, and energy industries; among these metabolites, phycobiliproteins (PBPs) stand out for their full demand,³ especially for pharmaceutical industries.

PBPs are a family of hydrophilic brilliant pigment proteins that play a crucial role in harvesting photonic energy from solar radiation³ in cyanobacteria, red algae, cryptomonads, and cyanelles.⁴ Among phycobiliproteins, three categories stand out (Figure 1): phycocyanin (C-PC), allophycocyanin (APC), and phycoerythrin (PE).⁵ Each of these categories possesses a specific spectrum referred to as blue (610–620 nm), blue-green (650–655 nm), and pink (540–570 nm), respectively.⁶

Because of their nature, unique color, fluorescence, and antioxidant properties, PBPs are exploited in a wide range of applications such as dyes for the food industry (desserts, gums, jellies, and ice creams) and pharmaceuticals (eyeliners, lipsticks, and makeup) and even in the development of anticancer drugs.^{7–12}

The production, accumulation, and purity of phycobiliproteins in cyanobacteria are strongly regulated by various factors such as the strain to be used, light intensity and its wavelength,¹³ temperature, pH, and media composition.^{14–16} Generally, algae and cyanobacteria have been isolated and produced on specific culture media composed of inorganic

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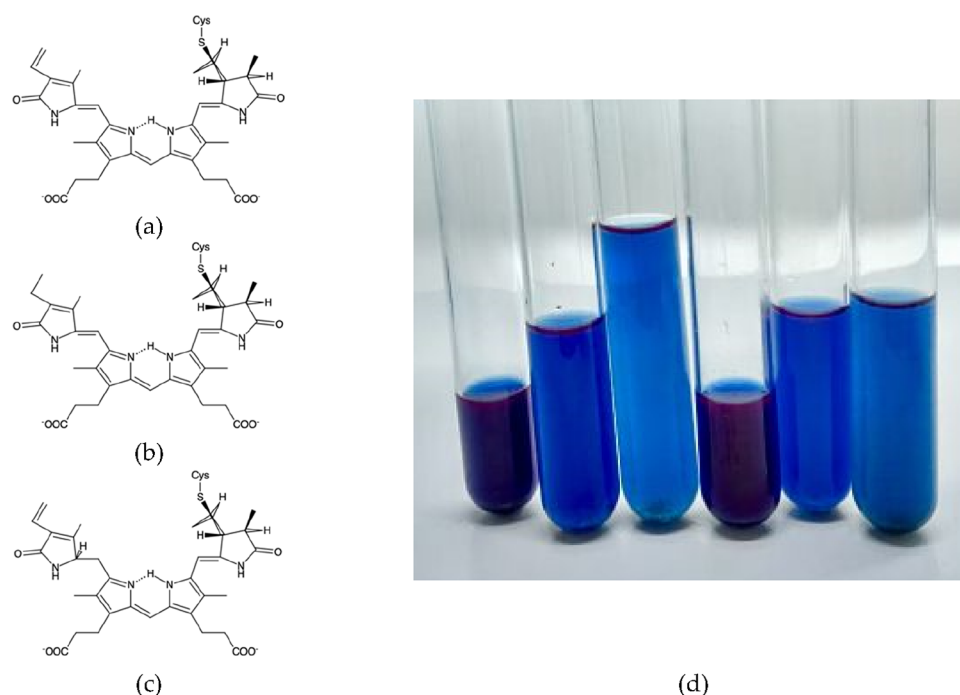


Figure 1. Structure of chromophores found in phycocyanin (a), allophycocyanin (b), and phycoerythrin (c) of cyanobacteria. Extract from *Oscillatoria* sp. OSCI_UFPS001 (d).

salts.¹⁷ Therefore, it is possible to modulate (or artificially adjust) the final concentration of PBPs.

Media composition plays an essential role in microalgal and cyanobacterial growth and byproduct (lipids, carotenes, etc.) synthesis, affecting the total biomass productivity.¹⁸ Generally, growth media such as BG-11,¹⁹ Bold's Basal medium,²⁰ Chu-10 medium,²¹ and Zarrouk medium²² were initially designed to produce as much biomass as possible from the strains and not for the overproduction of a specific metabolite.

Carbon and nitrogen are the most crucial nutrients for growth of microalgal and cyanobacterial biomass.²³ In most algae, nitrogen is an important nutrient that regulates not only biomass production but also the accumulation of lipids and carotenoids.²⁴ However, in cyanobacteria, nitrogen plays a key role in cell viability since it regulates the synthesis of PBPs, which in turn work as the main storage of nitrogen within the cell.²⁵ Other nutrients such as calcium chloride, trace metal mix, and citric acid have proven to modulate the accumulation of PBPs;²⁶ however, the effect on the synthesis and accumulation of PBPs may depend on the unique biology of the selected strain. Therefore, the tuning of nutrient concentration seems to be a critical step to enhance the production of specific metabolites such as PBPs. The conventional method used for improving the overproduction of a specific metabolite by one variable at a time²⁶ is an expensive and time-consuming process.¹⁸ A response surface methodology (RSM) is a simple and precise method that allows the user to optimize operational conditions for the system evaluated without affecting production cost.^{27,28} However, limited studies using the RSM to enhance growth and PBP production have been done. Therefore, the current study investigates the effect of macronutrients (citrate buffer, NaNO₃, K₂HPO₄, MgSO₄, CaCl₂, Na₂CO₃, and EDTA) and trace metal concentration from BG-11 media on the accumulation of phycobiliproteins in a thermotolerant strain

of *Oscillatoria* sp. (OSCI_UFPS001), as it shows a high concentration of PBPs, especially C-PC.

2. RESULTS

2.1. Selection of Relevant Media Components.

Cyanobacteria constitute a group of photosynthetic microorganisms with morphological and physiological characteristics capable of responding to extreme changes by irradiation, nutrient limitation, salinity, and pH. Therefore, they are considered biotechnological sources to produce phycobiliproteins, carotenoids, and proteins of economic interest.¹⁶

According to the Pareto charts (Figure 22a–d), NaNO₃, Na₂CO₃, and K₂HPO₄ affect the production of biomass and PBPs (C-PC, APC, and PE). On the other hand, only the biomass was affected by other variables such as CaCl₂, EDTA, and MgSO₄. The only factor that affects neither biomass nor PBPs was citrate buffer.

2.2. Selection of Trace Metal Concentration. According to the results, by reducing the concentration of micronutrients by 50%, the biomass (Figure 3a) remained relatively constant (0.6–0.58 g/L); however, using 25% less of concentration, the biomass was considerably reduced up to 0.48 g/L. In contrast to biomass, PBP concentration and purity were not affected by reducing the concentration of micronutrients (Figure 3b,c).

2.3. Optimization by the Response Surface Methodology. Based on the results from the previous stage, four factors (NaNO₃, Na₂CO₃, K₂HPO₄, and trace metals) were used in the next step of the process for the optimization of PBP production. The other components of BG-11 media (citrate buffer, MgSO₄, CaCl₂, and EDTA) were used in half concentration. Evaluation was done using a Non-Factorial Response Surface Design with four factors, three levels, and two central points. The resolved design can be found in Table 1.

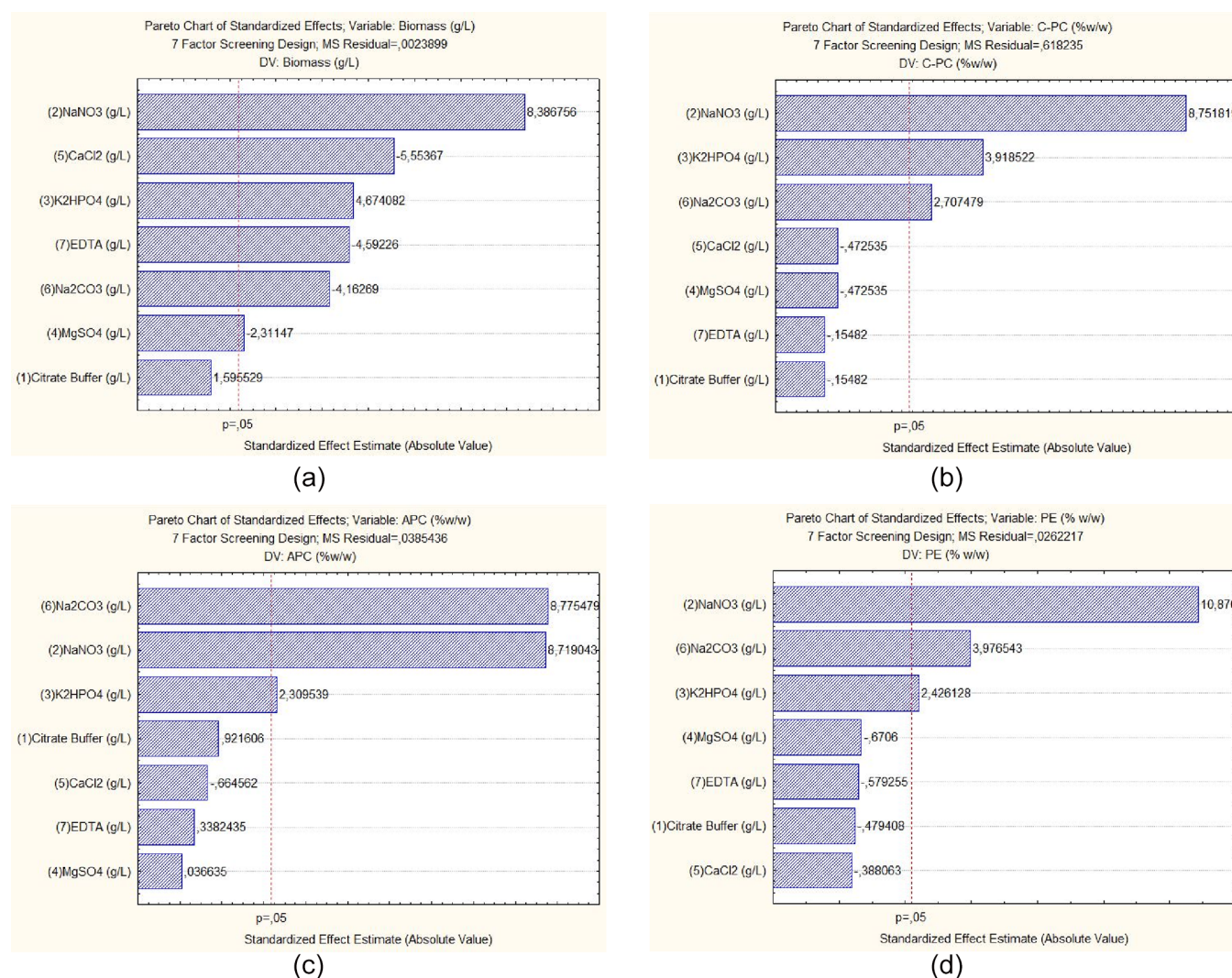


Figure 2. Pareto charts for the effects of different nutrients on biomass (a), C-PC (b), APC (c), and PE (d) in *Oscillatoria* sp.

The experimental data concerning the effect of concentration of NaNO₃, Na₂CO₃, K₂HPO₄, and trace metals on the production of C-PC, APC, and PE were fitted on two models: linear (L) and quadratic (Q). ANOVA illustrates that not all the factors studied affect the synthesis of PBPs. Table 2 summarizes the most significant factors ($p = 0.05$). In the case of C-CP, trace metals, NaNO₃, and K₂HPO₄ affect its synthesis. APC is only affected by trace metals and Na₂CO₃. Finally, PE is affected by trace metals, Na₂CO₃, and NaNO₃. Similarly, it was found that the interaction between the four variables (NaNO₃/Na₂CO₃, NaNO₃/K₂HPO₄, NaNO₃/trace metals, Na₂CO₃/K₂HPO₄, Na₂CO₃/trace metals, and K₂HPO₄/trace metals) affects the production of PBPs; however, the interaction Na₂CO₃/K₂HPO₄ substantially increases the final concentration of C-PC, APC, and PE.

Figure 4a–c presents the response surface plots for the effect of Na₂CO₃/K₂HPO₄ on the accumulation of C-PC, APC, and PE. According to the results, relatively larger concentrations of Na₂CO₃ (>4 mL/L) and normal concentrations of K₂HPO₄ (1 mL/L) substantially increase the accumulation of the three phycobiliproteins studied.

Table 3 presents the values for X (Na₂CO₃ in mL/L) and Y (K₂HPO₄ in mL/L) used for the validation of the proposed concentrations. In this scenario, NaNO₃ and trace metals were

used at 1.5 g/L and 0.5 mL/L, respectively, while the other components of BG-11 media (citrate buffer, MgSO₄, CaCl₂, and EDTA) were used in half concentration.

The contents of PBPs of *Oscillatoria* sp. grown on BG-11 media without any modification were 7.02, 2.23, and 0.45 (% w/w), respectively (Figure 5a). However, under the optimized conditions, the concentration of PBPs was significantly higher than the control, with values of 15.21, 3.95, and 1.89 (% w/w), respectively, which is 2.12-, 1.77-, and 4.17-fold higher for the three PBPs studied. Another critical result to highlight is the increase in the purity index of PBPs (Figure 5b). In the case of C-PC, purity increased from 7.0 to 8.7; in APC, it increased from 0.28 to 0.38, and the purity of PE increased from 0.45 to 0.6. It should be noted that in this work, any of the extracts were not subjected to any purification process.

3. DISCUSSION

Oscillatoria sp. is a genus of cyanobacteria that presents an unbranched filamentous growth with mucilaginous sheaths (Figure 6). It can be identified by its characteristic movement of oscillating trichomes, which are composed of short cells (usually several times shorter than its width).²⁹ In recent years, different strains of *Oscillatoria* sp. have been studied for their capacity on PBP production. Soni *et al.*³⁰ isolated a strain of

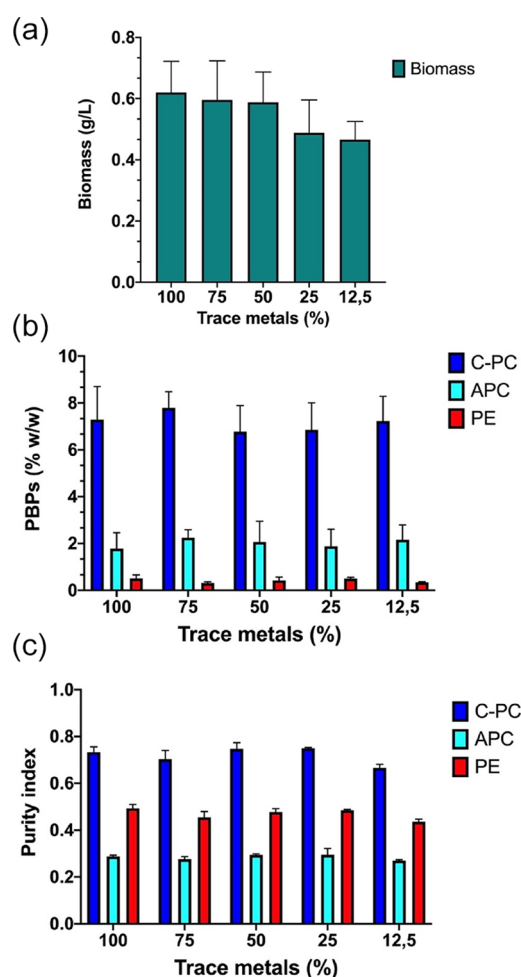


Figure 3. Production of biomass (a) and PBPs (b) and its purity (c) under the reduced concentration of trace metals from BG-11 media.

Oscillatoria quadripunctulata with a purity index of 0.85 in the crude extract. More recently, Chittapun *et al.*³¹ proved the capacity of a strain of *Oscillatoria okeni* to obtain high-purity extracts (0.68–1.65) using different extraction solutions. Other studies focused on the inhibitory capacity of C-PC from *Oscillatoria* sp. over pro-inflammatory enzymes such as lipoxygenase³² and their antioxidant capacity and antiproliferative activity against human cancer cells through apoptosis.^{33,34} Therefore, this study could allow exploring the potential of developed processes at an industrial scale to produce nutraceutical and pharmaceutical compounds for human health.

3.1. Relevant Media Components. The adjustment in the concentration of the nutrients of the culture medium (especially N, P, K, and C) is a critical step to improve the production of specific metabolites. The carbon source is a critical element since the cyanobacterial biomass can be up to 50% (w/w) of carbon.³⁵ Therefore, the correct carbon source added into the media, plus an optimal concentration of carbon, allows the accumulation of metabolites of interest.³⁶ Sodium carbonate is the preferred carbon source on different culture media for cyanobacterial production, not only because it is used for pH adjustment, but it also assists in the maintenance and adaption of the microorganism to the autotrophic culture condition.³⁷ In their work Johnson *et al.*³⁷ found that a reduction in inorganic carbon (0.008 g/L Na_2CO_3) promotes

Table 1. Resolved Design of Four Factors and Three Levels with Two Center Points

experiment	NaNO_3 (g/L)	Na_2CO_3 (mL/L)	K_2HPO_4 (mL/L)	trace metals (mL/L)
8	1.0	6	0.7	0.3
2	0.4	2	0.7	0.3
26	0.7	4	0.23	0.9
4	0.4	6	0.7	0.7
9 (C)	0.7	4	0.23	0.5
23	0.7	4	0	0.5
21	0.7	0	0.23	0.5
11	0.4	2	0.7	0.7
20	1.3	4	0.23	0.5
10	0.4	2	0.08	0.3
13	0.4	6	0.7	0.3
18 (C)	0.7	4	0.23	0.5
24	0.7	4	0.85	0.5
6	1	2	0.7	0.7
12	0.4	6	0.08	0.7
1	0.4	2	0.08	0.7
7	1	6	0.08	0.7
22	0.7	8	0.23	0.5
17	1	6	0.7	0.7
3	0.4	6	0.08	0.3
25	0.7	4	0.23	0.1
15	1	2	0.7	0.3
14	1	2	0.08	0.7
19	0.1	4	0.23	0.5
5	1	2	0.08	0.3
16	1	6	0.08	0.3

the synthesis of accessory pigments in a strain of *Nostoc* sp. This statement contrasts with the results obtained in this work since 0.16 g/L Na_2CO_3 (20 times more) was needed to improve PBP production. A possible explanation for this phenomenon is the origin of the two strains, while Johnson *et al.*³⁷ employed a strain from a lake, the strain used in this study was originally isolated from a hot spring with high carbonate contents (data not shown).

Nitrogen is the second most required nutrient for the biomass and metabolite production, just after carbon,⁴ and the second most abundant nutrient on algae and cyanobacteria.³⁵ It plays a critical role in the overproduction of PBPs since nitrogen is stored within PBPs. Therefore, under nitrogen shortage, cells selectively degrade their PBP storage.^{38,39} Typically, cyanobacteria and algae prefer NH_4^+ rather than NO_3^- sources; however, due to its high toxicity, nitrate is preferred over ammonia for PBP production.⁴⁰ Several studies concerning the optimization of PBP production using different nitrogen sources on a wide range of cyanobacteria (including nitrogen-fixing strains) report high concentrations of NaNO_3 in their culture media (0.75–4.3 g/L) (Table 4). In the present work, 1.5 g/L NaNO_3 increases the production of PBPs. Phosphorous is one of the most important macronutrients for cyanobacterial growth, and its content varies from 0.05% up to 3.3% (w/w).⁴¹ P is used for the formation of several organic molecules, such as RNA and DNA, ATP, and membrane phospholipids.⁴² Unlike carbon, nitrogen and phosphorous are expensive nutrients derived from fossil phosphate rocks.³⁶ On the production of PBPs, the concentration of K_2HPO_4 varies from species, from 0.04 to 0.75 g/L. Most species evaluated for the overproduction of

Table 2. Analysis of Variance of Quadratic Model for C-PC, APC, and PE Production

	sum of squares	df	mean square	F value	p-value	
C-PC (% w/w) $R^2 = 0.963$						
(A) NaNO ₃ (g/L)	14.8568	1	14.8568	8.58584	0.004262	significant
(B) Na ₂ CO ₃ (mL/L)	0.0187	1	0.0187	0.01078	0.917518	
(C) K ₂ HPO ₄ (mL/L)	10.4787	1	10.4787	6.05572	0.015707	significant
(D) micronutrients (mL/L)	106.0142	1	106.0142	61.26643	<0.000001	significant
BC	28.2680	1	28.2680	16.33630	0.000109	significant
APC (% w/w) $R^2 = 0.964$						
(A) NaNO ₃ (g/L)	0.20976	1	0.209755	0.96771	0.327804	
(B) Na ₂ CO ₃ (mL/L)	3.48559	1	3.485587	16.08083	0.000122	significant
(C) K ₂ HPO ₄ (mL/L)	0.03979	1	0.039794	0.18359	0.669296	
(D) micronutrients (mL/L)	6.29052	1	6.290518	29.02143	0.000001	significant
BC	2.49803	1	2.498033	11.52472	0.001011	significant
PE (% w/w) $R^2 = 0.926$						
(A) NaNO ₃ (g/L)	0.143036	1	0.143036	4.81829	0.030649	significant
(B) Na ₂ CO ₃ (mL/L)	0.174440	1	0.174440	5.87615	0.017281	significant
(C) K ₂ HPO ₄ (mL/L)	0.087924	1	0.087924	2.96178	0.088580	significant
(D) micronutrients (mL/L)	1.448332	1	1.448332	48.78810	<0.000001	significant
BC	0.185073	1	0.185073	6.23430	0.014289	significant

PBPs use the concentration from BG-11 (0.04 g/L). Other components such as glycine (carbon source and precursor of δ -aminolevulinic acid) and sodium glutamate (nitrogen source) have improved the maximum yield of cell biomass and PBP production on *Oscillatoria* sp. BTA170.⁴³

3.2. Trace Metal Concentration. Trace elements or micronutrients greatly influence the growth and production of metabolites in different species of microalgae and cyanobacteria. These are required in minimal amounts from micro-, nano-, or picograms per liter. The most important are iron, cobalt, zinc, nickel, manganese, copper, boron, vanadium, and molybdenum;⁴⁴ in general, these elements are supplied as a mix of nutrients. According to the literature, different investigations have been carried out to find out the effect of some micronutrients on the growth and content of pigments in some species of cyanobacteria; for example, the importance of four microelements (Fe III, Cu II, Zn II, and Mo VI) was investigated in *Arthrospira platensis*, where it was concluded that they greatly influence pigment accumulation and biomass production.⁴⁵ Isao *et al.*⁴⁶ found that iron limitation caused a reduction in cellular chlorophyll and carotenoid concentration. On the other hand, Rueter and Petersen⁴⁷ reported that high Fe contents promote growth of cyanobacteria in lakes.

3.3. Optimization by the Response Surface Methodology. The traditional method for optimizing medium involves one factor at a time in which each component is changed one by one, keeping other factors constant. This traditional approach is very tedious and time-consuming. Moreover, a biological system is a time-consuming process that is affected by several factors simultaneously.⁴⁷

The RSM is an interesting approach for the optimization of biomass and specific metabolites. Over the years, this method has been proven to be an interesting statistical tool for better culture media in strains such as *Ankistrodesmus falcatus* KJ671624,⁴⁸ *Auxenochlorella protothecoides*,⁴⁹ *Botryococcus braunii*,²⁴ *B. braunii* LB572,⁵⁰ *Chlorella* sp.,⁵¹ *Chlorellaproteo-*
thecoides,⁵² *Chlorella sorokiniana* Mb-1,⁵³ *Chlorella vulgaris*,⁵⁴ *C. vulgaris* 1803,⁵⁵ *Chlorococcum oleofaciens*,¹⁷ *Desmodesmus armtus*,⁵⁶ *Dunaliella salina*,⁵⁷ *C. pyrenoidosa*,⁵⁸ *Scenedesmus* sp.

ASK22,⁵⁹ *Scenedesmus obliquus*,^{60,61} and *Spirulina* sp.⁶² In PBP research, the application of RSM has been focused on their extraction and purification.^{53–71} Until now, there are few reports such as the works of Singh *et al.*²⁶ and Mogany *et al.*⁷² that have focused on the effect of multiple nutrients on biomass production and PBP content.

Typically, N and C are the most common factors evaluated because in photosynthetic organisms, up to 50% of the fixed carbon (in the form of CO₂) is destined for nitrogen fixation²⁴ while high concentrations of nitrogen promote the synthesis of PBPs; however, factors such as phosphate, sodium, magnesium, and trace metals are less obvious choices. Kumar *et al.*⁷³ reported that K₂HPO₄ (0.2 g/L) and trace metals (0.5 mL/L) enhance PBP production in *Anabaena variabilis* CCC421. Singh *et al.*²⁶ evaluated the interaction of trace metals with NaNO₃ and citric acid and found that the optimal concentration is between 0.9 and 1 mL/L. On the other hand, Mogany *et al.*⁷² found that the interaction between trace metals, MgSO₄, and NaNO₃ positively affects the production of PBPs, with concentrations of 10 mL/L. This extreme difference (10 times higher than BG-11) can be due to the unique strain used in the research.

4. CONCLUSIONS

The results of this study indicate that the Plackett–Burman Design coupled with the RSM is a robust methodology for identifying critical nutrients for PBP overproduction in *Oscillatoria* sp. Culture media are designed for cyanobacteria to grow without any possible starvation; therefore, in order to increase the production of specific metabolites, most salts are not required in larger quantities. The development of an optimized concentration of the nutrients from BG-11, especially NaNO₃, Na₂CO₃, K₂HPO₄, and trace metals (with half-concentration of citrate buffer, MgSO₄, CaCl₂, and EDTA), resulted in an increase of 2.12-, 1.77-, and 4.17-fold for C-PC, APC, and PE. These results allow reducing the nutrient loss on PBPs' industrial-scale production. Future studies should be directed at providing further insights into the possible interaction of nutrient availability and abiotic factors

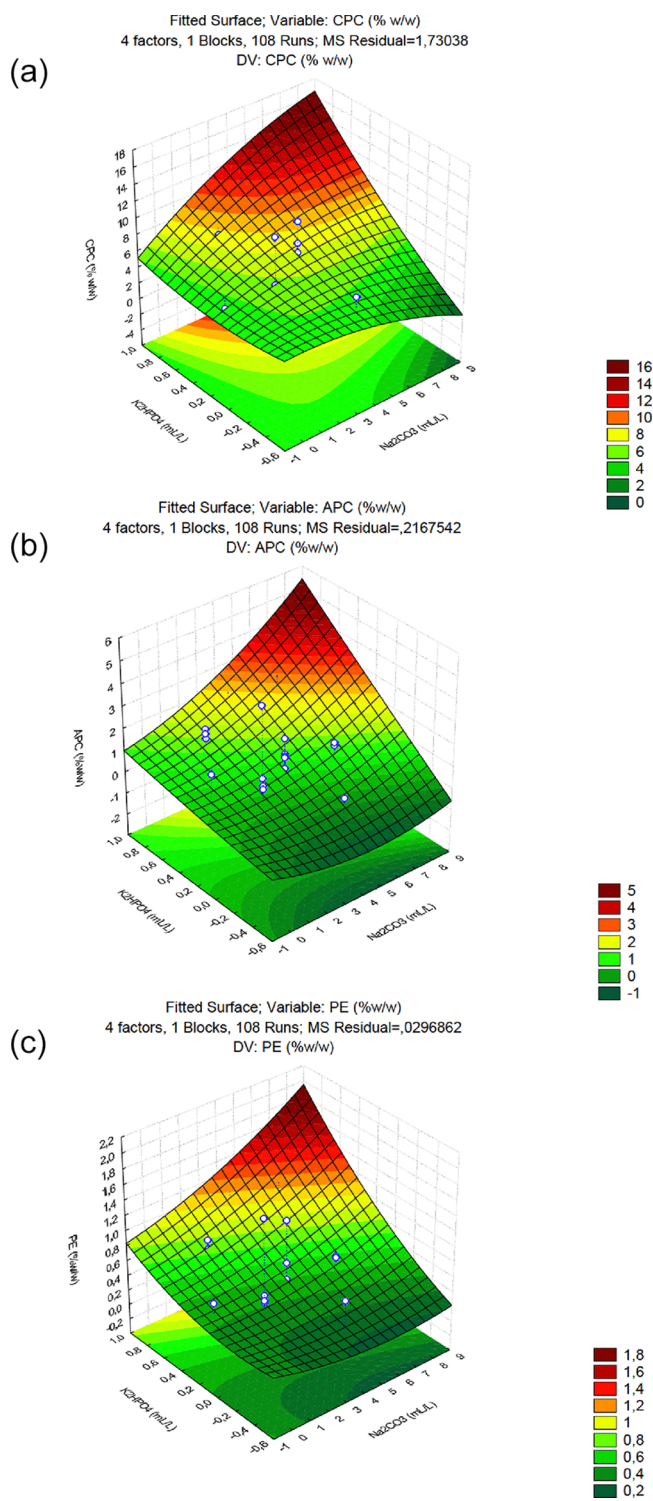


Figure 4. Surface response and Pareto charts for C-PC (a), APC (b), and PE (c).

such as light regimes (light:dark cycle, intensity, and light quality). Finally, the optimized conditions can allow the industrial-scale production of nutraceutical and pharmaceutical compounds for human health.

5. MATERIALS AND METHODS

5.1. Strain. *Oscillatoria* sp. OSCI_UFPS001 was isolated from a thermal spring in Cúcuta (Colombia) and kept at

Table 3. Variables for Optimal PBP Concentration on *Oscillatoria* sp.

label	variable	value
X	Na ₂ CO ₃ (mL/L)	6
Y	K ₂ HPO ₄ (mL/L)	1
Z _{C-CP}	concentration (% w/w)	14.3
Z _{ACP}	concentration (% w/w)	3.5
Z _{PE}	concentration (% w/w)	1.5

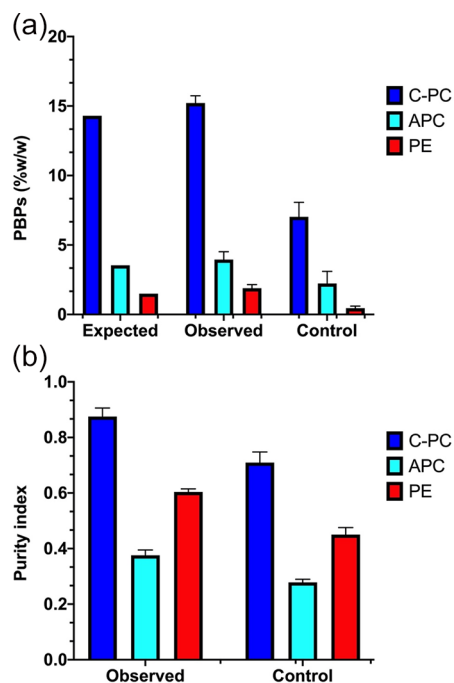


Figure 5. Expected vs observed concentration of PBPs (a) and observed purity of PBPs (b) after optimization of culture media.

INNOValgae collection (UFPS, Colombia). The strain was grown in a tubular glass flask with a culture volume of 2 L containing BG-11 media.¹⁹ The strain was mixed through the injection of filtered air with 1% (v/v) CO₂ at a flow rate of 0.78 L min⁻¹ and a light:dark cycle of 12:12 h at 100 μmol m⁻² s⁻¹ for 15 days.

5.2. Selection of Relevant Media Components. The effects of seven nutrient stock (citrate buffer, NaNO₃, K₂HPO₄, MgSO₄, CaCl₂, Na₂CO₃, and EDTA) from BG-11 media were determined using a Plackett–Burman design with two center points and three replicates (30 runs)⁸⁰ (Table S). The concentrations of biomass (expressed as g/L) and C-PC, APC, and PE (expressed as % w/w) were selected as the response variable and subjected to analysis of variance (ANOVA). Pareto charts were employed for the selection of influencing variables.

5.3. Selection of Trace Metal Concentration. To prove the possible effect of the micronutrient concentration on biomass and PBPs' final production, several experiments were carried out using reduced quantities of micronutrient mix of BG-11 media (75%, 50%, 25%, and 12.5%).

5.4. Culture Conditions. Each experiment was done in 500 mL flasks with a working volume of 250 mL of culture media. Each flask was mixed through the injection of filtered air with 1% (v/v) CO₂ at a flow rate of 0.78 L min⁻¹ and a light:dark cycle of 12:12 h at 100 μmol m⁻² s⁻¹ for 15 days.



Figure 6. *Oscillatoria* sp. OSCI_UFPS_001, filamentous cyanobacteria isolated from a thermal spring in the city of Cúcuta (Colombia).

Table 4. Comparison of NaNO₃, K₂HPO₄, and Carbon Source Concentrations Used to Increase PBP Concentration

strain	NaNO ₃ (g/L)	carbon source (g/L)		K ₂ HPO ₄ (g/L)	biomass (g/L)	PBPs	reference
<i>Anabaena</i> sp. PCC 6803	1.5	Na ₂ CO ₃	0.02	0.04		C-PC: 10% (w/w)	73
<i>A. variabilis</i> CCC421	0	Na ₂ CO ₃	0.02	0.2		C-PC: 408.5 (mg/L)	74
<i>A. platensis</i> EGEMACC 30	2.5	NaHCO ₃	16.8	0.04	1.56	PBPs: 22.3% (w/w)	66
<i>Euhalothece</i> sp.	1.67	Na ₂ CO ₃	0.02	0.04		C-PC: 0.045 (g/g)	72
<i>Nostoc</i> sp.	1.5	Na ₂ CO ₃	0.008	0.04	0.625	C-PC: 0.13 (g/g)	37
<i>Phormidium</i> sp. EGEMACC 72	1.5	Na ₂ CO ₃	0.02	0.04	1.18	PBPs: 18.53% (w/w)	66
<i>Phormidium ceylanicum</i>	4.5	Na ₂ CO ₃	0.02	0.04	0.97	C-PC: 0.73 (g/L)	26
<i>Phormidium rubidum</i> A09DM	0.75	Na ₂ CO ₃	0.02	0.75			75
<i>Pseudoscillatoria</i> sp. EGEMACC 74	1.5			0.04	0.87	PBPs: 18.9% (w/w)	66
<i>Synechocystis</i> sp. PCC 7120	1.5	Na ₂ CO ₃	0.02	0.04		C-PC: 6.5% (w/w)	74
<i>Spirulina maxima</i>	2.5	CO ₂	0.03% v/v	0.5	3.75	C-PC: 2.51% (w/w)	76
	2.5	NaHCO ₃	16.8	0.5		APC: 4.11% (w/w)	
						C-PC: 0.08 (g/L)	77
						APC: 0.06 (g/L)	
						PE: 0.02 (g/L)	
	2.5	NaHCO ₃	16.8	0.5	4.82	C-PC: 0.53 (g/L)	78
<i>Trichromus</i> sp. IMU26	0	Na ₂ CO ₃	0.02	0		CPC: 4% (w/w)	79
						APC: 3% (w/w)	
						PE: 5% (w/w)	
<i>Oscillatoria</i> sp. OSCI_UFPS001	1.5	Na ₂ CO ₃	0.16	0.04	0.59	CPC: 15.2% (w/w)	this study
						APC: 3.9% (w/w)	
						PE: 1.8% (w/w)	

Table 5. Plackett–Burman Design for the Seven Nutrient Stock from BG-11 Media

level	citrate buffer (mL/L)	NaNO ₃ (g/L)	K ₂ HPO ₄ (g/L)	MgSO ₄ (g/L)	CaCl ₂ (g/L)	Na ₂ CO ₃ (g/L)	MgNa ₂ EDTA (g/L)
low (–)	0.5	0.5	0.02	0.0375	0.018	0.01	0.0005
high (+)	1.0	1.0	0.04	0.075	0.036	0.02	0.001

5.5. Biomass and PBP Quantification. After 15 days, 50 mL samples were removed from each flask (by triplicate) and centrifuged at 3400 rpm for 20 min and the supernatant was withdrawn. The pellet was resuspended in 20 mL of distilled water, filtered on precombusted CF/C glass fiber filters, and dried overnight at 60 °C in an oven containing a bed of silica gel. The dried filters were stored in a desiccator until constant weight. The mass of cyanobacteria was recorded using a digital balance. The filtered biomass was suspended in 10 mL of cold

phosphate buffer solution (0.05 M, pH 6.8) and approximately 1 g of glass beads (0.5 mm diameter), and the solution was vortexed at maximum speed for 10 min. 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 supernatant (deep blue) was collected and measured using a spectrophotometer at different wavelengths, i.e., 620, 652, 562,

and 280 nm. The concentration of C-PC, APC, and PE was calculated using eqs 1–3 described by Bennett and Bogorad.⁸¹

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

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

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

The purities of C-PC, APC, and PE were determined using eqs 4–6 proposed by Patil *et al.*⁸² and Antelo *et al.*⁷

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

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

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

5.6. Optimization by the Response Surface Methodology. The optimization of biomass and PBPs production was further refined by using the central Non-Factorial Response Surface Design with two central points on software STATISTICA 7.0 (Statsoft).

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