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Original Article

Use of mining effluents for the production of algal-based colorants

Uso de efluentes mineros para la producción de colorantes a base de algas

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Abstract

In this research, a mining effluent was used to produce microalgal and cyanobacterial biomass to obtain red (carotenoids) and blue pigments (phycocyanin). Two strains were isolated from a hydrothermal source in Norte de Santander and grown in mining wastewater mixed with 50% BG-11 medium for the Osci_UFPS01 cyanobacterium and 50% with Bold Basal medium for the Chlo_UFPS01 microalgae. A carbon, nitrogen, and phosphorus experiment design was developed, and subsequent response surface analysis (RSM) was used to determine the optimal operating conditions for the formation of the products of interest. A notable decrease in pigment production was observed compared to that in the controls without mining wastewater. Overall, 45% of phycocyanin (C PC) per unit dry weight (DW) and 1,129% (w/w) of carotenoids were obtained in the cultures with a mining wastewater mixture in the final optimization processes.

Resumen

En esta investigación se utilizó un efluente minero para producir biomasa microalgal y cianobacterial para la obtención de pigmentos rojos (carotenoides) y azules (ficocianina). Se aislaron dos cepas de una fuente hidrotermal de Norte de Santander y se cultivaron en aguas residuales mineras mezcladas al 50% con medio BG-11 para la cianobacteria Osci_UFPS01 y al 50% con medio Bold Basal para la microalga Chlo_UFPS01. Se desarrolló un diseño experimental de carbono, nitrógeno y fósforo, y posteriormente se utilizó el análisis de superficie de respuesta (RSM) para determinar las condiciones de operación óptimas para la formación de los productos de interés. Se observó una notable disminución de la producción de pigmentos en comparación con la de los controles sin aguas residuales mineras. En conjunto, se obtuvo un 45% de ficocianina (C PC) por unidad de peso seco (PS) y un 1.129% (p/p) de carotenoides en los cultivos con mezcla de aguas residuales mineras en los procesos finales de optimización.

Keywords: acid mine drainage; Carotenoids; Cyanobacteria; Microalgae; Phycocyanin; Response surface.

Palabras clave: drenaje ácido de mina; Carotenoides; Cianobacterias; Microalgas; Ficocianina; Superficie de respuesta.

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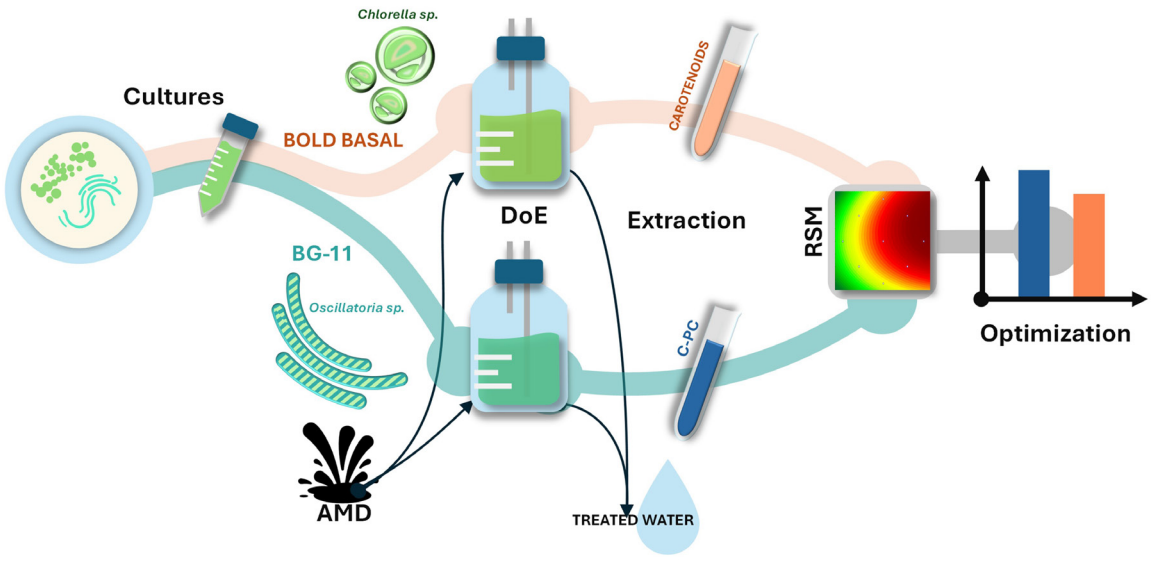
Why was it conducted?:
The study aimed to investigate the use of mining effluents for the production of microalgal and cyanobacterial biomass to obtain valuable compounds such as red pigments (carotenoids) and blue pigments (phycocyanin), while addressing the environmental issues caused by acid mine drainage (AMD).

What were the most relevant results?
The study isolated two strains from a hydrothermal source in Norte de Santander and cultivated them in mining wastewater mixed with BG-11 medium for the cyanobacterium Osci_UFPS01 and Bold Basal medium for the microalgae Chlo_UFPS01. The optimal conditions for phycocyanin production were determined using response surface analysis (RSM). The final optimization processes yielded 45% phycocyanin (C-PC) per unit dry weight (DW) and 1,129% (w/w) of carotenoids in the cultures with a mining wastewater mixture.

What do these results contribute?
This study contributes a methodology to valorize acid mine drainage (AMD) by converting it into biomass, thereby producing pigments and treating the contaminated effluent.

Graphical Abstract



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Introduction

Mining activities, involving the extraction of coal and metals, have significant adverse effects on the environment, primarily due to the creation of acid mine drainage (AMD) and subsequent fluctuations in pH levels ranging from 2 to 8. AMD is a byproduct of mineral oxidation upon exposure to water, leading to the formation of sulfuric acid, various metal ions (such as Cu, Cd, Ag, Pb, Sn, Zn, Fe, and Al), sulfates, bicarbonates, and other compounds. These pollutants have the potential to bioaccumulate in organisms residing in water bodies affected by AMD, posing risks to the ecosystem (1–4)

The use of microorganisms, especially photosynthetic ones, has been studied using swine, mariculture and synthetic wastewater, among others, as a culture medium to evaluate biomass production and the cultures' ability to remove nutrients (5–10). These developments hold importance due to the modern technologies being studied for the reuse of waste and the production of valuable products and energy (11–15). Cyanobacteria and/or microalgae are proposed to treat AMD since certain strains can grow naturally in these contaminated environments (16–19), and good removal percentages of biochemical demand (COD) and biological oxygen demand (BOD) can be obtained (20,21). The biomass produced in wastewater treatment has potential for use in the extraction of antioxidant compounds, which, despite the need for greater characterization to determine side effects due to their culture medium, have been shown to have beneficial effects on the metabolism of organisms such as *Drosophila melanogaster* (22).

These beneficial features of photosynthetic microorganisms are due to their ability to produce extracellular and intracellular antibiotic and antioxidant compounds, which have great potential as biocontrol agents and medicines (23). These microorganisms have light-collecting supramolecules (phycobilisomes) located in the membrane of the thylakoid and are composed of antenna pigments called phycobiliproteins (PBPs), whose characteristics confer them with potential for use as fluorescent and antioxidant agents (24). Antioxidant compounds can prevent and delay the damage caused by reactive oxygen species (ROS). ROS are highly reactive molecules produced by anaerobic metabolism, and under normal conditions, they regulate metabolic pathways. Phycocyanin (PC) is an antioxidant pigment in the family of phycobiliproteins, has a blue color, and is part of the photosynthetic system of cyanobacteria, red algae, and some protists. The production of phycocyanin from cyanobacteria (C-PC) has drawn the attention of the industry since C-PC has anticancer characteristics, such as that obtained with *Oscillatoria tenuis*, which has been proven to have a high antioxidant capacity and a cytoprotective effect on carcinogenic cells (25). C-PC has been shown to have neuroprotective (26) and hepatoprotective effects since it prevents renal cell damage caused by mercury (27). The production, extraction, and purification of C-PC have been studied using Zarrouk, BG-11, and BZ-11 diazotrophic media (BG-110) for strains such as *Arthrospira platensis* (28) and *Nostoc* sp. NK in BG-110 to increase C-PC production (29). The resistance of these microorganisms to other adverse effects that can induce the production of C-PC should also be considered, which is why the use of residual mining effluents as a substrate for microorganisms is an interesting alternative not only for the treatment of AMD but also for the production of photosynthetic biomass.

Materials and methods

Strains isolation process

The strains were obtained from a thermal spring situated at the major seminary of Cucuta, Norte de Santander. Initial cultivation was performed in petri dishes using BG-11 medium at pH 7 for cyanobacteria (for phycocyanin production) and Bold Basal medium at pH 7 for microalgae (for carotenoid production). The growth of these strains was facilitated under a light intensity of 1800 lumens, with a 24-hour photoperiod using cold fluorescent lamps, maintaining a temperature of 30°C ± 2. This cultivation process was repeated until the pure growth of each microorganism was visually confirmed under a microscope. Subsequent strain identification was carried out through microscopic examination following the isolation of microalgae, utilizing the taxonomic keys outlined by Rivera et al.(30) and Baker (31).

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Biomass production

The isolated strains were initially maintained in petri dishes. Subsequently, a portion of the biomass from these plates was carefully scraped off and transferred to 1.5 mL microtubes containing liquid culture medium. These microtube cultures were allowed to grow for a period of 10 days.

After the initial growth phase in microtubes, the biomass content was transferred to 15 mL plastic conical tubes filled with fresh liquid culture medium. These larger volume cultures were then incubated for an additional 15 days sufficient growth . Visual cues were used to assess the growth of the cultures. In the case of microalgae, the color of the culture medium in the tubes transitioned to a distinct green hue. For cyanobacteria, the formation of colonies resembling those observed in the original petri dish cultures was noted.

This step-wise approach, starting from petri dish cultures and gradually scaling up to larger volumes, facilitated the successful propagation of the isolated microorganisms while maintaining their characteristic growth patterns and pigmentation.

Physicochemical characterization of the water from AMD

The sampling process followed the methodology described by the Institute of Hydrology, Meteorology and Environmental Studies (IDEAM), and the analyzed parameters were biochemical oxygen demand (BOD5), chemical oxygen demand (COD), suspended volatile solids (SSV), total suspended solids (SST), total solids (ST), sedimentable solids (SS), pH, nitrates (NO_3), phosphates (PO_4) and ash, total acidity, total alkalinity, total hardness and calcium hardness.

Biomass quantification

Biomass measurements were carried out via the dry weight (DW) method using Pall Corporation 47 mm fiber filters

Phycocyanin quantification

The modified method of Bennett A & Bogorad L. (32) was used: the biomass and filter residue obtained from the dry weight step were destroyed subjected to mechanical disruption in a 15 mL conical tube using a Heidolph Vortex Reax Top shaker for a duration of 15 minutes. This process was facilitated by the addition of 10 mL of a 0.05 M phosphate buffer solution, comprising a mixture of potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4), adjusted to a pH of 6.8. Following disruption, the resulting mixture was refrigerated at 5 °C for a period of 24 hours to allow for complete extraction of the desired compounds. The phycobiliprotein quantification process was performed by centrifuging the tubes (15 minutes at 5000 rpm) and reading them in a GENESYS10S UV–Vis spectrophotometer. The concentrations of phycocyanin (C-PC), allophycocyanin (APC), and phycoerythrin (PE) and the purity of C-PC were calculated using equations 1, 2, 3 and 4, respectively (32,33):

$$C-PC\left(\frac{g}{L}\right)=\frac{A_{620}-0.474\,A_{652}}{5.34} \quad (1)$$

$$APC\left(\frac{g}{L}\right)=\frac{A_{652}-0.208A_{620}}{5.09} \quad (2)$$

$$PE\left(\frac{g}{L}\right)=\frac{A_{562}-2.41\,(PC)-0.849(APC)}{9.62} \quad (3)$$

$$Purity\,C-PC=\frac{A_{620}}{A_{280}} \quad (4)$$

Carotenoid quantification

The pigment content was quantified using a modified protocol based on the method described by Přibyl et al. (34). The extraction process commenced with the disruption of dry biomass cells using

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glass beads and 3 mL of phosphate buffer (pH 7.4). This step was carried out using a HEILDOLPH Vortex Reax top shaker for a duration of 10 minutes. The resulting mixture was then subjected to centrifugation at 3000 rpm for 10 minutes. To ensure accuracy, this extraction procedure was performed in duplicate.

Following centrifugation, chloroform (6 mL) was employed as the extraction solvent, and the obtained solution was left to stand for 24 hours at a temperature of 18 °C. The carotenoid content was determined spectrophotometrically by measuring the absorbance of the chloroform extract at a wavelength of 464 nm using a GENESYS10S UV–Vis spectrophotometer. Subsequently, the concentration of carotenoids was calculated using the following formula:

$$Carotenoids\left(\frac{mg}{L}\right) = \frac{A_{464} - 0.0222}{0.0325}$$

Cyanobacteria cultures

Phase 1. Incubator cultures

Experiments were conducted in 500 mL Erlenmeyer flasks, utilizing only 250 mL of BG-11 culture medium per reactor. The flasks were sealed with stoppers and placed in an incubator equipped with orbital shaking capabilities. The temperature was maintained at 35 ± 2 °C, and the shaking speed was set at either 100 rpm or 150 rpm, depending on the experimental condition.

Phase 2. Cultures in aerated reactors

The strains exhibiting superior phycocyanin and biomass yields identified in phase 1 were selected for further cultivation in specialized aerated reactors. These reactors were blue screw-cap flasks equipped with a stopper, air filter, and injector to facilitate aeration. Each reactor was prepared by inoculating 50 mL of biomass into a total liquid medium volume of 250 mL (BG-11) within a 500 mL capacity vessel.

Cultivation conditions included a 12-hour light/12-hour dark photoperiod, with a light intensity of 1800 lumens provided by cold fluorescent lights. Sterile air was continuously supplied at a rate of 12 L/min to ensure optimal growth. The temperature was maintained at 30±2 °C, and the pH was controlled at 7±0.2 throughout the cultivation period.

Biomass quantification was conducted by measuring dry weight, achieved by filtering 50 mL of the reactor contents (with five filtrations/samples performed per reactor). After a fifteen-day incubation period post-inoculation, the production of nitrate, phycocyanin (C-PC), allophycocyanin (APC), and phycoerythrin (PE) was assessed. Additionally, the purity of the phycocyanins was determined to evaluate the quality of the produced compounds.

This optimized cultivation strategy aimed to maximize phycocyanin production while ensuring robust biomass growth under controlled environmental conditions, setting the stage for efficient and high-quality pigment extraction processes.

Phase 3. Cultures in aerated reactors with a mixture of AMD

The strain selected for cultivation was *Oscillatoria sp.*, designated as Osci_UFPS01. This strain was cultured in aerated reactors containing acid mine drainage (AMD) at varying concentrations of 10%, 30%, 50%, and 70% (v/v) in BG-11 medium. The reactors were equipped with caps featuring three holes, with two designated for outlet purposes and one serving as the air inlet, utilizing a 253 mm Pasteur pipette as the air injector. Cotton plugs were used to replace the air filters.

The assembly of the reactors was conducted both with unadjusted pH conditions and with the pH adjusted to 7 using 0.1 M NaOH. Prior to filtration, the reactors underwent homogenization by magnetic stirring on a Thermo Scientific heating plate set at maximum speed (1200 rpm) for approximately 30 minutes. This process ensured thorough mixing and uniform distribution of the culture components within the reactors.

doi: 10.25100/iyc.v26i1.13752

Phase 4. The culture was optimized with a mixture of mining water

To enhance C-PC production, a central non-factorial design was implemented over a 15-day period, with phosphorus, carbon, and nitrogen concentrations serving as variables (as detailed in Table 1). The experiments were conducted in 500 mL blue cap flasks containing a 1:1 mixture of sterile wastewater and BG-11 culture medium. Prior to inoculation, the mine's residual water was allowed to settle, pH was adjusted to 7, and subsequently utilized in the experiments. Inoculation of the strains was carried out until reaching a cell density ranging from 0.1 to 0.2. The experimental setup comprised 17 reactors, including two controls, with each experiment maintaining the specified variables determined by the STATISTICA 7 software. The phosphate and sodium carbonate solutions utilized mirrored those conventionally found in BG-11 culture media.

Table 1. Variables to evaluate the production of phycocyanin.

Nutrient	K_2HPO_4	PO_4	Na_2CO_3	C	$NaNO_3$	NO_3
	(mL/L)	(ppm)	(mL/L)	(ppm)	(g/L)	(ppm)
Low level	0.5	10.90	2	1,13	0.75	547.14
Center	0.75	16.36	4	2,27	1.5	1094.27
High level	1	21.81	6	3,40	2.25	1641.41

Microalgae cultures

Phase 1. Growth in aerated reactors

The isolated microalgal strain, designated as Chlo_UFPS01, was cultivated in 500 mL blue screw-top flasks equipped with stoppers, air filters, and injectors. A total of 50 mL of the inoculum was added to 200 mL of BG-11 culture medium, resulting in a final working volume of 250 mL in each reactor. The cultures were subjected to a 12:12 light/dark photoperiod, with a light intensity of 1800 lumens provided by cold fluorescent lamps. Sterile air was supplied at a flow rate of 12 L/min, and the temperature was maintained at 30°C ± 2°C. The pH of the culture medium was adjusted to 7 ± 0.2.

To monitor biomass production, 50 mL samples were withdrawn from each reactor and filtered (five filtrations/samples per reactor). The dry weight of the biomass was then determined. Additionally, the concentrations of nitrate and carotenoids were quantified throughout the cultivation process.

This controlled cultivation setup in photobioreactors allowed for the optimization of growth conditions, ensuring consistent and reproducible results for the Chlo_UFPS01 strain while enabling the measurement of key parameters such as biomass, nitrate, and carotenoid levels.

Phase 2. Growth in reactors with medium mixed with AMD

Chlo_UFPS01 was grown in aerated reactors with AMD at concentrations of 10%, 30%, 50% and 70% (v/v) in a Bold Basal reactor with the pH adjusted to 7.

Phase 3. The culture was optimized with a mixture of mining water

The Chlo_UFPS01 microalgal strain was inoculated into sterile 500 mL blue cap flasks, each containing 250 mL of a 1:1 mixture of sterile residual water and Bold-Basal culture medium. This cultivation setup was performed in accordance with the experimental design guidelines outlined in Table 2.

The use of 500 mL flasks allowed for the scale-up of the culture volume while maintaining sterile conditions. The 1:1 ratio of DAM residual water to Bold-Basal medium provided the necessary nutrients and growth factors for the Chlo_UFPS01 strain to thrive in a controlled environment.

By following the experimental parameters specified in Table 2, the authors ensured consistency and reproducibility in the cultivation of Chlo_UFPS01, enabling the collection of reliable data for subsequent analysis and optimization of pigment production.

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Table 2 Variables to evaluate carotenoid production.

Nutrient	K_2HPO_4	PO_4	Na_2CO_3	C	$NaNO_3$	NO_3
	(mL/L)	(ppm)	(mL/L)	(ppm)	(g/L)	(ppm)
Low level	6	97.81	0.2	22.644	6	109.43
Center	7	114.11	0.3	33.966	7	127.67
High level	8	130.41	0.4	45.288	8	145.90

Results and discussion

Physicochemical characterization of AMD

The results of the physicochemical analysis are presented in Table 5. We observe that the parameters that do not comply with the Colombian resolution 0631 of 2015 are total suspended solids (TSS) and pH.

Cyanobacteria cultures

Phase 2. Cultures in aerated reactors

The aerated experiments produced more biomass, due to the additional sources of oxygen and nitrogen provided by the air stream. As shown in Figure 1, the C-PC production of Osci_UFPS01 increased dramatically compared to that in the preliminary experiments, reaching 301.4 mg/L C-PC and thus exceeding the production of all the strains analyzed, including *Spirulina maxima*, whose production was approximately 13 mg/L C-PC grown in Zarrouk media.

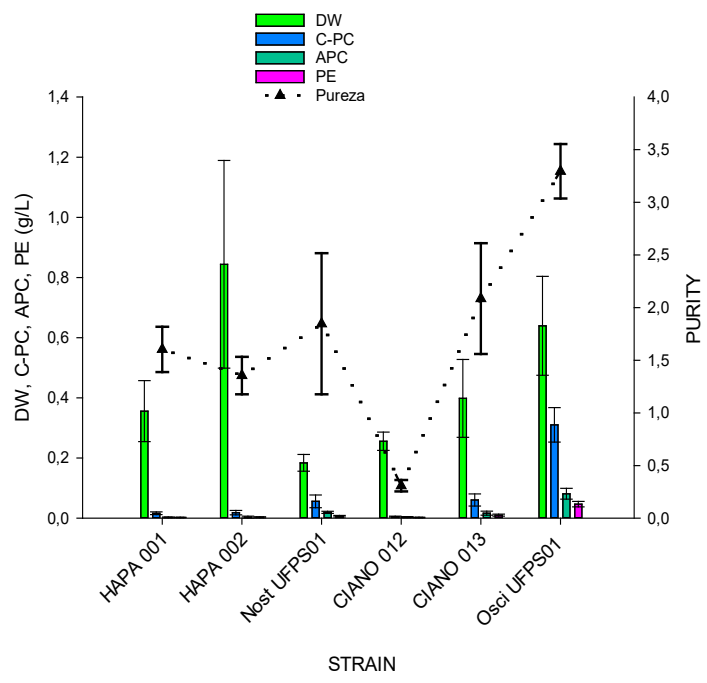

Figure 1. Biomass production, C-PC, purity, APC, and PE in aerated reactors

The C-PC concentration measured for *S. maxima* in the present study was lower than the values reported by Prates et al. (35) and Dejsungkranont et al. (36) in their respective control experiments. Prates et al. (35) reported a C-PC concentration of 46.36 mg/L, while Dejsungkranont et al. (36) reported a value of 84.55 mg/g. The discrepancy in C-PC concentrations between the current study and the aforementioned studies can be attributed to differences in the culture conditions. employed. Specifically, Prates et al. (35) utilized a tubular bioreactor and compressed air injection

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at 0.05 vvm, while Dejsungkranont et al. (36) bubbled the culture with 2% CO2 in air (v/v) at a flow rate of 11 L h− 1.

The residual medium of the Osci_UFPS01 reactor exhibited a dark brown coloration attributed to the synthesis of an exopolysaccharide. Studies by Li et al. (37) on *Oscillatoria* sp. strains have indicated that the motility of these cyanobacteria is facilitated by surface fibrils and the continuous release of extracellular silt through a network of pores. Cyanobacteria belonging to this group are known to produce sulfated heteropolysaccharides as extracellular flocculants, often bound to fatty acids and proteins.

Upon release into the environment, capsular and exopolysaccharides from these cyanobacteria may contain a variety of monosaccharides such as xylose, arabinose, fucose, rhamnose, galactose, glucose, mannose, and uronic acid, with specific composition varying among strains. Notably, the yield of exopolysaccharides achieved by Osci_UFPS01 surpassed that reported for *Oscillatoria quadripunctulata* by Soni et al. (38) and *Oscillatoria tenuis* by Thangam et al. (25). Based on absorbance measurements at 620 nm and 650 nm, concentrations of 274.3 mg/L and 146 mg/L of C-PC were reported in the crude extract, respectively.

This observation highlights the significant exopolysaccharide production capacity of Osci_UFPS01 compared to related Oscillatoria strains, as supported by the quantitative data obtained from the absorbance readings.

The hydrothermal isolate Nost_UFPS01 produced 56.3 mg/L of C-PC (C-phycocyanin), which is relatively low compared to the values reported by Lee et al. (29). In their study, Lee and colleagues obtained C-PC concentrations close to 650 mg/L in diazotrophic BG-11 cultures and 850 mg/L in non-diazotrophic BG-11 cultures of *Nostoc* sp. NK.

Interestingly, Lee et al. also observed that *Nostoc* sp. NK spontaneously releases phycobiliproteins, including C-PC, from the cells into the culture medium when previously grown in diazotrophic medium and then stored in the dark at 5°C. This phenomenon suggests that environmental factors, such as nitrogen availability and light conditions, can significantly influence C-PC production and release in *Nostoc* species.

The lower C-PC yield obtained with Nost_UFPS01 in the present study, representing only about 6% of the concentrations reported by Lee et al., highlights the need for further optimization of cultivation conditions to enhance phycocyanin production by this hydrothermal isolate. Factors such as nitrogen sources, light intensity, and temperature should be investigated to unlock the full potential of Nost_UFPS01 as a C-PC producer.

Phase 3. Cultures in aerated reactors with a mixture of AMD

Figure 2 illustrates the production of dry weight (DW), C-PC, purity, APC, and PE after a 15-day period. The data reveals a correlation between DW and the concentration of acid mine drainage (AMD) in the culture medium, indicating an increase in DW with higher AMD concentrations. However, the production of pigments and their purity exhibit contrasting trends. Specifically, C-PC, APC, and PE decrease with elevated residual water percentages, suggesting a unique positive influence induced by solutes present in AMD on biomass production. This phenomenon leads to a reduction in pigment production by the cells.

In the control group, DW production was recorded at 0.640 g/L, with C-PC levels at 300 mg/L. In comparison, reactors containing mixed AMD displayed DW production of 0.53 ± 0.04 g/L and C-PC levels of 293±30 mg/L for the 10% (v/v) reactor, which demonstrated the highest phycocyanin concentration. The 30% and 70% (v/v) reactors exhibited similar C-PC production levels (176 ± 30 mg/L and 171±30 mg/L, respectively), yet displayed varying biomass concentrations (0.765 ± 0.1 g/L and 1.210 ± 0.1 g/L, respectively). Notably, the reactor with a 50% water mixture yielded C-PC levels of 193±20 mg/L and DW production of 1,124±0.13 g/L, indicating suitability for further experimentation.

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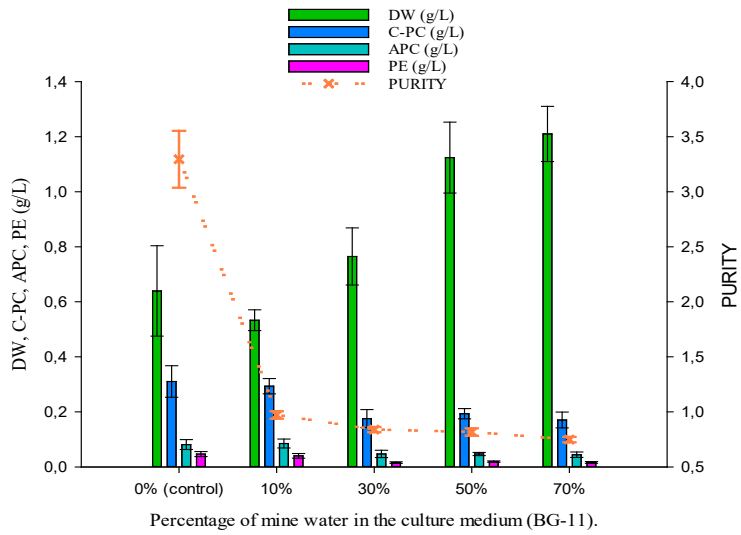

Figure 2 DW, C-PC, APC, PE and purity as a function of the AMD concentration in the medium

Phase 4. Culture optimization with a mixture of mining water

In reactor 14, the highest phycocyanin production was achieved, exhibiting a purity of 0.89±0.095 and a C-PC (g/L) to DW (g/L) ratio of 39.63±8.5%, resulting in yields of 190±0.033 mg/L of C-PC and 0.482±0.02 g/L of dry weight. Notably, the purity of C-PC in the control reactor experienced a significant decrease compared to previous experimental runs.

Statistical analysis conducted using the STATISTICA 7 software revealed that the interaction involving $NaNO_3$ had a substantial impact on the percentage of C-PC and APC, as depicted in the Pareto diagram (Figure 3). Furthermore, surface response graphs (Figure 4) were generated to assess pigment and biomass production trends in the experimental setup.

These findings highlight the significance of reactor conditions, particularly the influence of $NaNO_3$ interaction, on phycocyanin and allophycocyanin production, underscoring the importance of optimizing nutrient parameters for enhanced pigment yields in the cultivation system.

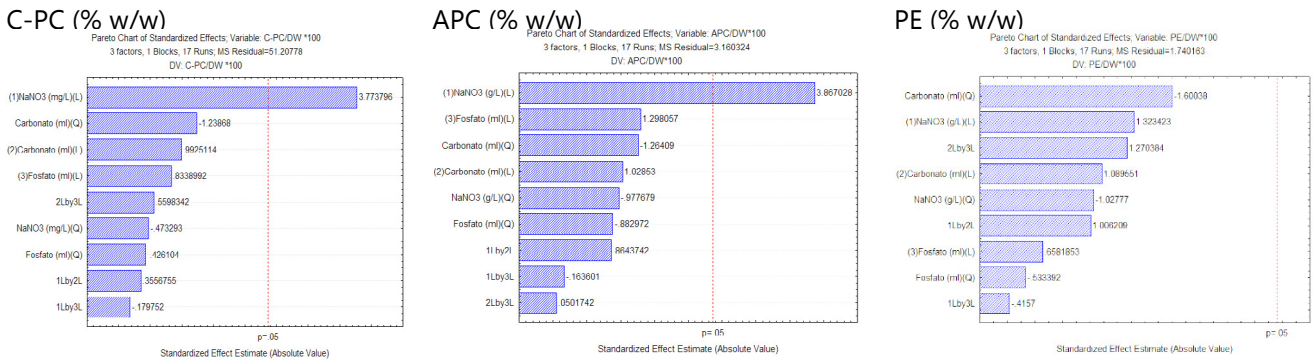

Figure 3 Pareto chart for Osci_UFPS01

The presence of AMD had a direct negative impact on biomass production after a 15-day cultivation period. Interestingly, this inhibition appeared to primarily affect the growth rate rather than the overall potential for total biomass production of Osci_UFPS01. Despite the presence of AMD in some reactors, growth was still evident after the 15-day period, whereas in the control reactors without AMD, the strain exhibited signs of discoloration.

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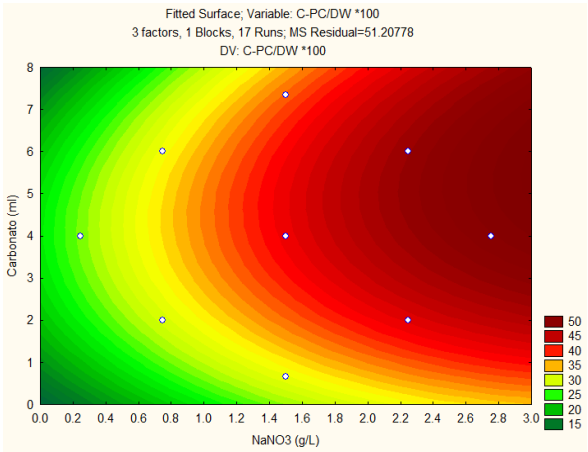
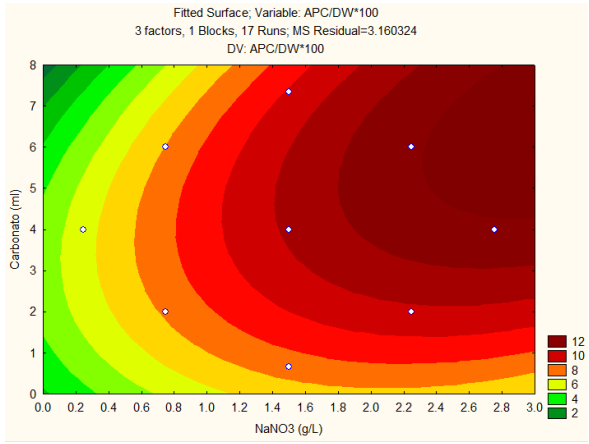
Remarkably, purities of up to 0.9 were attained in experiments involving a mixture containing AMD. This suggests that while AMD may hinder growth rates, it does not necessarily impede the overall biomass production potential of Osci_UFPS01, highlighting the resilience of the strain under adverse conditions.

a) % C-PC



b) % APC



c) % PE

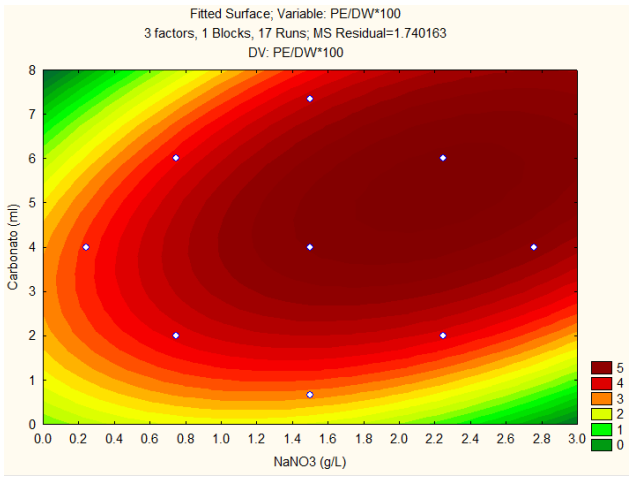


Figure 4 Response surface graphs for the experiments with Oscillatoria sp.

According to the response surface plot, at a mean phosphorus concentration (0.75 mL of the K_2HPO_4 stock solution), the percentage production of C-phycocyanin (C-PC) per unit of dry weight (DW) increased to 50% at concentrations exceeding 2.2 g/L $NaNO_3$ and 3 mL of Na_2CO_3. The percentage of allophycocyanin (APC) reached 12% at concentrations ranging from 2.8 to 3.0 g/L $NaNO_3$ and 4 to 6 mL of Na_2CO_3. Furthermore, the percentage of phycoerythrin (PE) attained 5% at concentrations between 1.6 and 2.8 g/L $NaNO_3$ and 3 to 5 mL of Na_2CO_3.

The following equations were derived to predict the percentages of C-PC, APC, and PE, where X represents the concentration in grams of $NaNO_3$ and Y denotes the volume in milliliters of the Na_2CO_3 solution (the number at the end of the equation is the error term):

$$\%C-PC = 4.5618889197466 + 14.598124983093 * X - 1.8049794032073 * X^2$$

$$+ 3.2529090217226 * Y - 0.6643006507269 * Y^2 + 0.59990933216839 * XY$$

$$- 2.4254614136054 * 0.75 * x + 2.8327771229723 * 0.75 * Y + 7.3113305$$

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$$\%APC = -3.7003699122271 + 4.2268563796414 * X - 0.92626629782189 * X\text{^}2 + 1.0046385321818 * Y - 0.16841459005952 * Y\text{^}2 + 0.3621857510719 * XY - 0.54841127883985 * 0.75 * X + 0.063071167352759 * 0.75 * Y + 6.5399345$$

$$\%PE = 1.0568889821739 + 2.3229171464158 * X - 0.72254632634233 * X\text{^}2 + 0.10257706718479 * Y - 0.15821718961931 * Y\text{^}2 + 0.31285766744181 * XY - 1.0340194137713 * 0.75 * X + 1.1849909139598 * 0.75 * y + 0.212976917$$

The optimal pigment values were calculated from equations 6, 7 and 8, with which the final assembly was carried out to verify that the values of $NaNO_3$, Na_2CO_3 and K_2HPO_4 determined by the program were adjusted to the real behavior of the microorganism using a 50% AMD mixture. These values are shown in Table 3.

Table 3 Concentrations of N, C, and P and the expected percentage of C-PC in the Osci_ UFPS01 culture

$NaNO_3$ (g/L)	NO_3 (ppm)	Na_2CO_3 (ml/L)	C (ppm)	K_2HPO_4 (mL/L)	PO_4 (ppm)	Expected percentage of C-PC
3	2188.54	4	2,27	0.75	16.36	52.05

Following a 15-day growth period of the optimized reactor (Figure 5), pigment production and quality assessments were conducted on the residual medium. The growth pattern of the cyanobacteria in the optimized reactor closely resembled that of the control group; however, the residual medium did not exhibit a dark coloration as observed in some cases. Quantitative analysis revealed that the optimized reactor yielded pigment percentages of 45.0% for C-PC, 10.8% for APC, and 8.9% for PE. The discrepancies between the obtained percentages and the calculated values were minimal, with actual values measuring at 52.046% for C-PC, 11.809% for APC, and 4.597% for PE. These results indicate a high degree of accuracy and consistency in pigment production within the optimized reactor system.

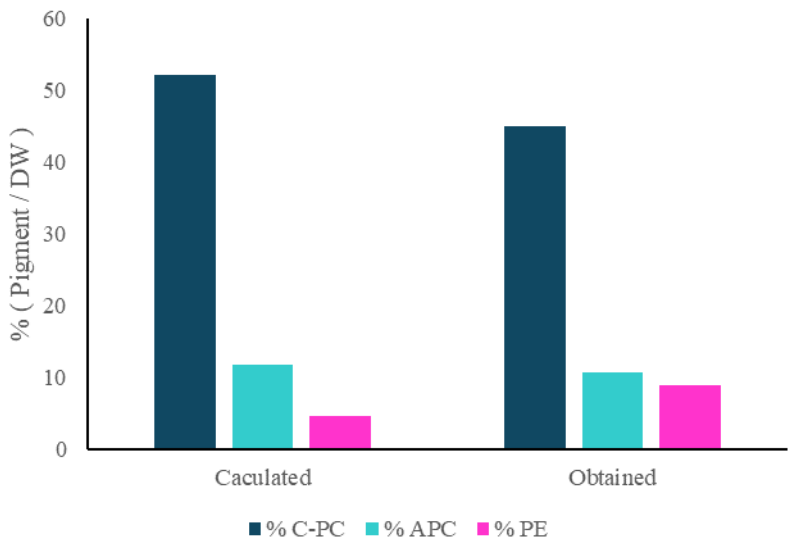

Figure 5 Percentages of C-PC, APC and PE calculated by the response surface equation and the experimental data obtained from the optimized reactor.

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While literature on cyanobacteria cultivation in wastewater for pigment production is limited, the optimization process with acid mine drainage (AMD) resulted in a C-phycocyanin (C-PC) concentration of 89 mg/L. This yield is notably lower compared to previous studies conducted without AMD, as reported by Soni et al. and Thangam et al. This decrease in pigment production is attributed to the presence of toxic solutes in the AMD, as highlighted by Aduvire O. (2006). Despite this challenge, the strain shows significant potential for C-PC production in BG-11 cultures both in the absence and presence of AMD.

Microalgae cultures

Phase 2. Cultures in reactors with medium mixed with AMD

The factorial analysis results are presented in Figure 6, depicting the experimental data for dry weight (DW, g/L) and carotenoid content (% w/w). Biomass production increased from 0.5 DW g/L in the control cultures to 0.98 DW g/L in the experimental design. This enhancement was attributed to the addition of an external carbon source, as no significant difference was observed between the control media (CM) and the modified Bold Basal medium (CBB). This suggests that the presence of acid mine drainage (AMD) did not substantially influence biomass or carotenoid production.

Carotenoid yields were higher in the control cultures, reaching 77,855 mg/L for CM and 89,363 mg/L for CBB. In contrast, carotenoid production in the reactors supplemented with sodium carbonate (Na2CO3) as a carbon source was lower, ranging from 60.7 mg/L to less than or equal to this value. Consequently, the control cultures exhibited carotenoid contents up to 3% (w/w), while the reactors with added carbon source had carotenoid levels below 1.5% (w/w).

These findings indicate that while the addition of a carbon source enhanced biomass production, it had a negative impact on carotenoid yields compared to the control cultures. The presence of AMD did not significantly alter the production of either biomass or carotenoids under the tested conditions.

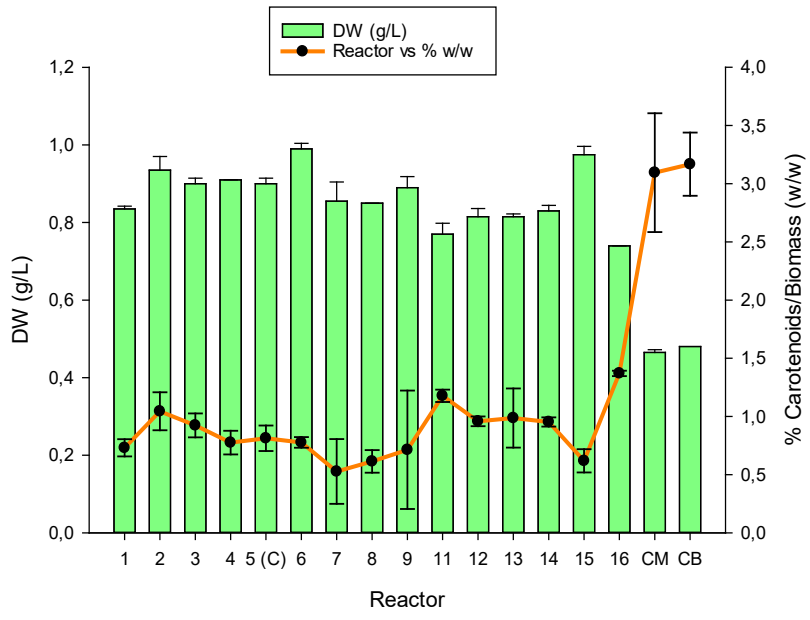

Figure 6 Biomass and percentage w/w of carotenoids produced in the experiments by the Chlo_UFPS01 strain.

The Pareto plots presented in Figure 7 reveal that the most significant interaction is the % w/w due to the carbon factor. However, the dry weight (DW) is not significantly influenced by the interaction of any of the variables studied.

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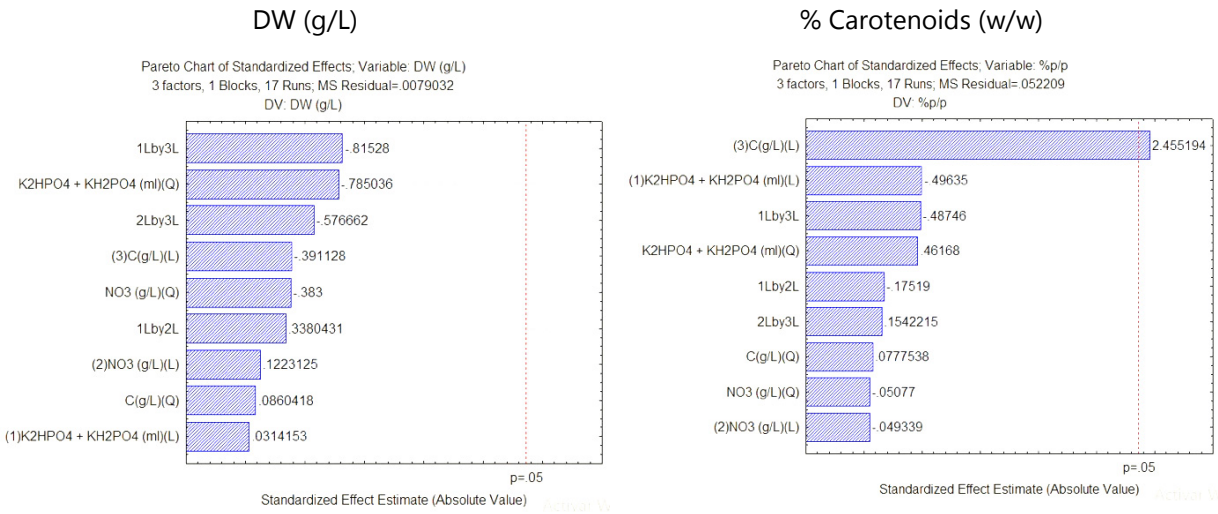

Figure 7 Pareto charts for experiments performed with Chlo_UFPS01

The response surface graphs (Figure 8) generated from the experimental design data revealed the optimal concentrations of phosphorus (P) and carbon (C) for biomass and carotenoid production. The mean concentration of $NaNO_3$ was maintained at 7 mL/L of the stock solution.

To achieve a maximum dry weight (DW) of 0.95 g/L, the concentrations of K_2HPO_4 and KH_2PO_4 stock solutions should be between 7.5 and 9 mL/L, while the Na_2CO_3 concentrations should range from 0.1 to 0.15 g/L. Values higher than 0.45 g/L of Na_2CO_3 were found to be detrimental to biomass production.

On the other hand, to maximize the carotenoid content (w/w) at 1.4%, the optimal conditions were between 5 and 5.5 mL/L of the K_2HPO_4 and KH_2PO_4 stock solutions and greater than 0.45 g/L of Na_2CO_3. Based on these findings, two bioreactors were set up for further optimization experiments, both with 5 mL/L of the K_2HPO_4 and KH_2PO_4 stock solutions, but with different Na_2CO_3 concentrations of 1.0 and 0.5 g/L (Table 4).

These results demonstrate the importance of optimizing nutrient concentrations, particularly phosphorus and carbon sources, to achieve maximum biomass and carotenoid production in the microalgal cultures. The response surface methodology provided valuable insights into the interactive effects of these variables on the desired outcomes.

a) DW (g/L)

b) % Carotenoids (w/w)

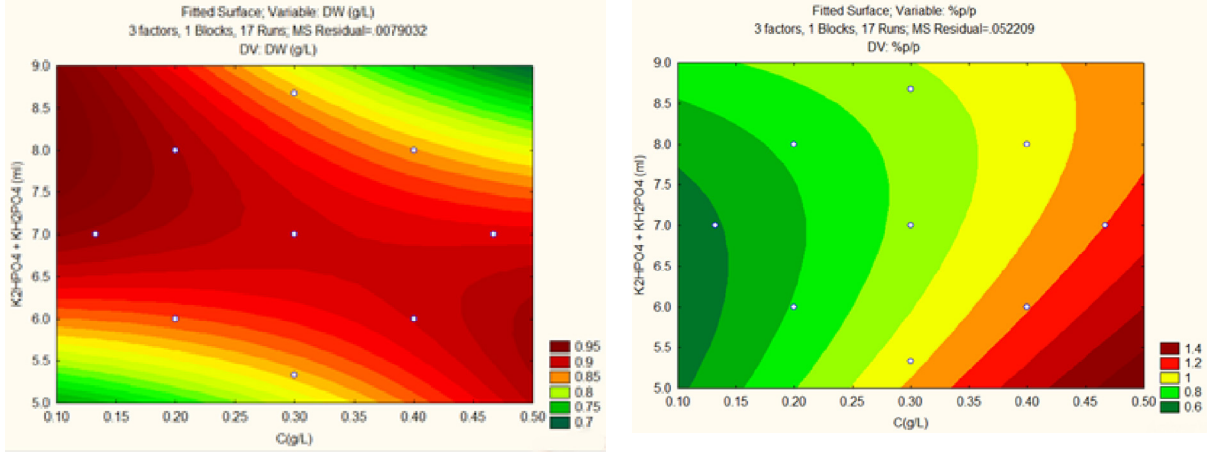

Figure 8 Response surface graphs for the experiments developed with Chlo_UFPS01

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The equations obtained from the response surfaces used to optimize the cultivation of AMD are presented below, where the value of X corresponds to the g/L of Na_2CO_3 and Y to the mL/L of the stock solutions of K_2HPO_4 and KH_2PO_4.

$$W\left(\frac{g}{L}\right) = -1.0061819532424 + 0.29615503627186 * Y - 0.020921266233767 * Y^2 + 2.8306319102345 * X + 0.22930194805186 * X^2 + 0.010625000000001 * 7.* Y - 0.25625000000001 * YX - 0.18125000000001 * 7.* X + 0.380781649$$

$$\%\, Carotenoids\ \left(\frac{P}{P}\right) = 0.78074513888654 - 0.25627677266931 * Y + 0.031623522343061 * Y^2 + 3.086089772363 * X + 0.53258741202192 * X^2 - 0.014152622699261 * 7.0 * Y - 0.39379159030069 * YX + 0.12458696184803 * 7.0 * X + 0.580849051$$

From equations 9 and 10, the optimal values for biomass production and carotenoid percentage were determined, and the assembly of two reactors with different Na_2CO_3 conditions was carried out:

Table 4 Concentrations of N, C, and P and expected results of DW and %w/w of carotenoids for the optimization of the Chlo_UFPS01 culture

Reactor	Stock solutions of K_2HPO_4 and KH_2PO_4 (ml/L)	PO_4 (ppm)	Na_2CO_3 (g/L)	C (ppm)	Stock solution of $NaNO_3$ (mL/L)	NO_3 (ppm)	DW result (g/L)	Expected Carotenoid Result (%w/w)
1	5	81.51	0.5	56.61	7	127.67	0.90	1.50
2	5	81.51	1	113.22	7	127.67	1.21	2.90

In the optimization, it was found that only reactor 1 resembled the values calculated by equations 9 and 10, with values of 0.84 DW g/L and 1.13% (w/w), while the values of reactor 2 were far from those calculated by the equations, with values of 0.65 DW g/L and 0.76% (w/w) (Figure 9).

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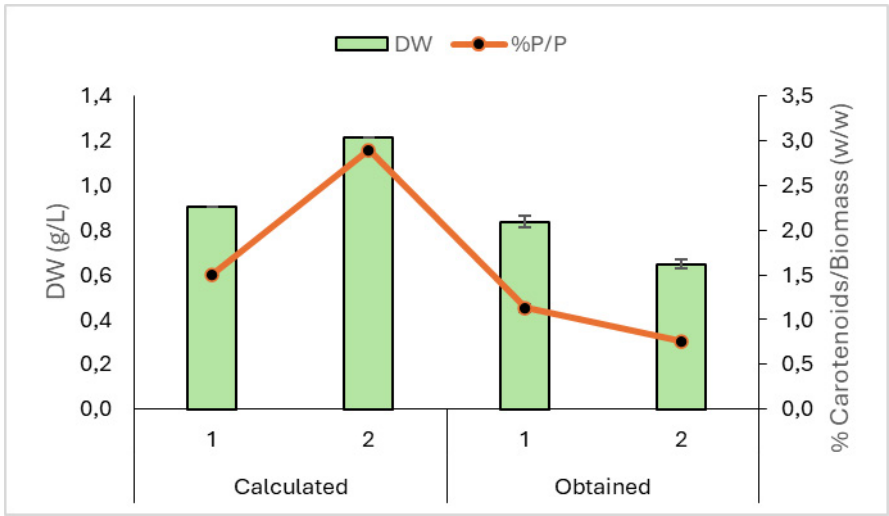

Figure 9 DWs (g/L) and percentages of carotenoids calculated by the response surface equation and those obtained in the optimized reactors.

In comparison to the findings reported by Barajas Solano et al., (40), the biomass production obtained in the current study was generally lower. In their work, Chlorella vulgaris cultures aerated with CO_2 injection exhibited enhanced biomass and chlorophyll levels. Specifically, they observed biomass concentrations of 1,416 g/L and 2,199 g/L, along with chlorophyll concentrations of 40 mg/L and 70 mg/L, at $NaNO_3$ concentrations of 0.59 mM and 1.18 mM, respectively. It is noteworthy that these $NaNO_3$ concentrations are lower than the standard concentration used in Bold Basal medium (2.94 mM).

Previous studies have demonstrated that supplementing Bold Basal medium with alternative carbon sources can significantly enhance biomass yields. Estévez-Landazábal et al. (41) reported that the utilization of acetate and residual glycerol from biodiesel production as carbon substrates resulted in notable increases in biomass production. Their findings indicated biomass yields of 3.4 g/L with 20 mM acetate and 3.15 g/L with 1% v/v glycerol, surpassing the biomass production of 1.75 g/L observed in control cultures using standard Bold Basal medium.

These comparative analyses suggest that optimizing nutrient concentrations, particularly nitrogen sources, and supplementing growth media with suitable carbon substrates could potentially improve biomass production and pigment yields in the microalgal and cyanobacterial strains investigated in the current study.

In a study by González-Delgado et al. (42), cultures of Chlorella vulgaris supplemented with carbonate showed a significant relationship between sodium nitrate (1.96 mM) and potassium phosphate (2.11 mM) in terms of biomass production (2.17 g/L), as indicated by the Pareto diagram. However, this finding differs from the results obtained for the Chlo_UFPS01 strain in the current study, where the carbon source (carbonate) had a direct effect on the %w/w of carotenes but not on biomass production. This discrepancy can be attributed to differences in cultivation conditions between the two studies.

The use of wastewater as a growth medium for microalgae has shown promising results in terms of carotenoid production. Rodrigues et al. (43) reported successful cultivation of Phormidium autumnale in industrial residues, while Ge et al. (44) observed increased biomass and carotenoid production using wastewater from anaerobic digestion in wastewater treatment plants and glycerol from lipid transesterification processes. Furthermore, Chen et al. (45) found that incorporating nutrient-rich ash and combustion gases generated in biomass power plants into the BG-11 culture medium enhanced the biomass production of Chlorella sp. by up to 35%. However, in the present study, the use of acid mine drainage (AMD) for cultivating microalgae did not significantly impact the production of both biomass and carotenoids, as observed in the experimental design. These findings suggest that carotenoid production in the Chlo_UFPS01 strain is more closely related to the carbon source.

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The extraction methodology and solvent synergy play a crucial role in obtaining carotenoids. Damergi et al. (46) used 2-methyltetrahydrofuran (MTHF), a renewable heterocyclic organic compound, for the first time in carotenoid extraction, which yielded 45% of the total carotenoids extracted from Chlorella vulgaris. Interestingly, this percentage increased to 66% when a 1:1 mixture of methanol and MTHF was employed. The application of various extraction techniques, as reported in previous studies (11,28,47–50), has the potential to significantly increase pigment yields by up to 92%. Additionally, the implementation of complementary chromatic adaptation in experiments conducted by Johnson et al. (51) and Lee et al. (29) has demonstrated promising effects on pigment production and their spontaneous release into the culture medium under diazotrophic conditions.

Future research should also explore the production of exopolysaccharides by the Osci_UFPS01 cyanobacterial strain. Furthermore, there is a limited number of studies investigating microalgal cultures in acid mine drainage (AMD) media. One such study by Torres et al. (20) focused on the bioremediation potential of microalgae but did not optimize for biomass production and carotenoid yields.

In summary, the strategic application of extraction techniques and complementary chromatic adaptation could significantly enhance pigment yields. Additionally, exploring exopolysaccharide production by Osci_UFPS01 and optimizing microalgal growth in AMD media for biomass and carotenoid production are promising avenues for future research.

Final characteristics of the residual medium with AMD

Table 5 presents a comparison of the physicochemical properties of the 50% AMD residual medium utilized in the final optimization stages and the characteristics of the acid mine drainage generated from mining operations. Following the optimization process with the strains, notable changes were observed in the water composition. Specifically, the total suspended solids decreased significantly from 350 mg/L to 25 mg/L when utilizing Osci_UFPS01. Moreover, the pH level exhibited a substantial increase from 1.22 to 8.62 with the same strain, leading to significant alterations in total acidity and total alkalinity.

Typically, untreated AMD necessitates primary treatment, such as settling, to meet the regulatory discharge standards and pH requirements outlined in Colombian regulations. This approach aligns with the recommendations proposed by Torres et al. in their study conducted at the mines of the CI company Minas la Aurora S.A.S, emphasizing the importance of effective treatment methods to address the environmental impact of mining activities.

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Table 5 Physicochemical characterization of the residual medium of each of the final optimizations

Parameter	Units	Osci_ UFPS01	Chlo_UFPS01 (reactor 1)	Chlo_UFPS01 (reactor 2)	AMD	Resolution 0631 of 2015
Total Solids (ST)	mg/L	584±3	2124±13	2556±16	4533.33±177.19	NA
Suspended Volatile Solids (SSV)	mg/L	17.5±0.09	20±0.15	17.5±1.36	57±17	NA
Total Suspended Solids (SST)	mg/L	25±0.2	230±2	30±0.4	352±52	50
Fixed Suspended Solids (SSF)	mg/L	7±0.05	210±1.5	12±1.12	295±57	NA
Settling Solids (SS)	ml/L	0	0	0	12±2	2
Biochemical Oxygen Demand (BOD_5)	mg/L	2.4	5.4±0.85	7.8±0.85	12.0±0.60	50
Chemical Oxygen Demand (COD)	mg/L	NR	NR	NR	NR	150
Hydrogen potential (pH)	pH	8.62	9.7	9.86	1.22	6 a 9
Conductivity	µs	5.87	2.16	2.89	2.14	NA
Temperature (T)	°C	26	26	26	26.6	NA
Total Dissolved Solids (tds)	ppm	4.12	1.52	2.03	1.94*	NA
Salinity	ppm	2.53	1.21	1.64	1.13*	NA
Nitrates (NO_3)	ppm	1520	29	44	72±5	AyR
Phosphates (PO_4)	mg/L	0.359	0.539	0	0	NA
Turbidity	FAU	44	39	25	209	NA
Ashes	g/L	3.66±1.26	1.148±0.1	1.588±0.23	2.088±0.1	NA
Total Acidity	mg $CaCO_3$/L	0	0	0	150±2	AyR
Total alkalinity	mg $CaCO_3$/L	145.6±10	500±36.8	980±40	0	AyR
Total hardness	mg $CaCO_3$/L	332±35	80±10	82±10	470±20	AyR
Hardness Ca	mg $CaCO_3$/L	200±40	40±5	20±3	100±10	AyR
Hardness Mg	mg $CaCO_3$/L	132±10	40±7	62±10	370±20	AyR

Note: NR: Not registered, NA: not applicable, AyR: Analysis and Report. Values with an * have ppt units.

Conclusions

The Osci_UFPS01 strain isolated from Norte de Santander produces 300 mg/L C-PC under normal BG-11 conditions; however, its production decreased below 90 mg/L C-PC when the medium was mixed with DAM residual water. However, this process still results in good pigment production. The extraction methodology and solvent synergy play a vital role in carotenoid extraction efficiency. Various extraction techniques have been shown to enhance pigment yields significantly. Future research avenues include exploring exopolysaccharide production by Osci_UFPS01 and optimizing microalgal growth in AMD media for improved biomass and carotenoid production. The study underscores the importance of tailored nutrient optimization for maximizing pigment yields in microalgal and cyanobacterial cultivation systems.

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