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Factorial optimization of upstream process for *Cyanobium* sp. pigments production

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Abstract

Cyanobacteria-based pigments, such as carotenoids and phycobiliproteins, have emerged in the last few years as products with great economical interest. However, only the production of a few strains has been optimized for large-scale productions. As photosynthetic components, pigments have their synthesis modulated by abiotic factors, such as pH, temperature and salinity, which can lead to a huge impact on cyanobacteria production. This work aimed the optimization of biomass and pigments production by *Cyanobium* sp. LEGE 06113, using a factorial Box-Behnken design for three abiotic factors—temperature (20–30 °C), pH (6.0–9.0) and salinity (NaCl, 10–30 g L^{-1}). Biomass, photosynthetic activity, carotenoid and phycobiliprotein productivity and antioxidant capacity of acetonic and aqueous extracts were measured over time and plotted into quadratic models. Results revealed that temperature and pH had a more significant impact than salinity on *Cyanobium* sp. metabolism and it was possible to determine a significant quadratic model for all evaluated parameters. According to the factorial modelling, the optimal condition for biomass, carotenoids and phycobiliprotein productivity was obtained at 20 °C, pH 9.0 and 10 g L^{-1} of NaCl, as subsequently confirmed in experimental trials, with an observed productivity of 127.12 ± 1.30 mg_{DW} $L_{culture}^{-1}$ day^{-1} for biomass; 2.04 ± 0.51 mg_{carot} $L_{culture}^{-1}$ day^{-1} for total carotenoids; and 4.14 ± 0.71 mg_{phyco} $L_{culture}^{-1}$ day^{-1} for total phycobiliproteins.

Keywords Cyanobacteria · Box-Behnken design · Carotenoids · Phycobiliproteins · Antioxidant capacity · Abiotic factors

Introduction

Cyanobacteria are a great potential source of pigments when compared with high plants as they reach a much higher concentration of biomass in a short period of time, need smaller areas for cultivation and do not compete for arable soil. Moreover, the diversity of compounds produced by these organisms contributes to their market applicability (Olaizola 2003). In an industrial scenario, pigments from cyanobacteria and microalgae represent the main source of revenues when compared with other components, such as lipids, proteins and carbohydrates; mainly due to their high added value in the food, cosmetics and healthcare industries (Ruiz et al. 2016). The cyanobacteria-based natural product market is growing, yet limited by the bioprocess constrains, since the cost of production is still too high when compared with other sources such as plants (Guedes et al. 2011a). On this matter, a few solutions have been presented for reduction of production costs, including the prospection for new strains with better pigment content and the optimization of culture conditions, increasing pigment productivity (Rizwan et al. 2018).

In cyanobacterial cells, pigments are responsible for light harvesting and photosynthesis through energy transfer to the reaction centres. Chlorophylls (Chl), carotenoids and phycobiliproteins are the main groups of pigments and form the so-called antennae, pigment complexes located in the thylakoid membranes

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(Masojdek et al. 2007). Also, carotenoids and phycobiliproteins act as non-enzymatic antioxidants, being one of the first cell defences against oxidative damage (Müller and Mu 2001).

The synthesis of pigments is influenced by both biotic and abiotic factors. Changes in the pH, light, temperature, salinity, nutrient availability and other processing factors modulate the productivity and the composition of biomass (Juneja et al. 2013). Cyanobacteria quickly respond to changes in the environment, adjusting their metabolism and consequently changing their chemical composition (Guedes et al. 2011b). For that reason, the optimization of the growth conditions of specific strains has been studied in order to increase biomass and bioactive compound production.

The most important factor for all living organisms is temperature; it influences the metabolic processes and cell composition (Hemlata and Fatma 2009), and for cyanobacteria, it may affect the growth rate, cell size and nutrient requirements (Juneja et al. 2013). The optimal growth temperature and temperature tolerance range vary from strain to strain (Hemlata and Fatma 2009).

pH is also a key parameter in cyanobacteria production; it defines the solubility and availability of CO_2 and other essential nutrients. Cyanobacteria, in general, grow in neutral to alkaline pH (7.0–9.0); however, although changes in pH can affect their metabolism and inhibit growth, cyanobacteria are able to adapt and thus grow in other pH ranges (Juneja et al. 2013).

Another important factor to consider in marine cyanobacteria production is salinity, and in the case of artificial media, NaCl is usually used to adjust the salinity. Similarly, to the former parameters, salinity affects the growth rate and biochemical composition of the cells (Juneja et al. 2013). Cyanobacteria acclimate better to salinity than higher plants, which have a more restricted salt tolerance range (Sudhir and Murthy 2004).

Taking this into account, the main goal of this work was to optimize biomass and pigment production of *Cyanobium* sp. LEGE 06113, a poorly studied cyanobacterium that has already shown to have potential biotechnological interest, as producer of antitumoral hierridin B and antimalarial hierridin C (Leão et al. 2013; 2018). *Cyanobium* sp. is a unicellular marine cyanobacterium, and it has a few advantages for a possible industrial application, - it does not form agglomerates nor biofilm and is able to produce a variety of bioactive pigments (Pagels et al. 2020).

By evaluating the effects of the interaction of temperature, pH and salinity, on growth, pigment production, photosynthetic activity and antioxidant capacity of *Cyanobium* sp., it is expected to define the optimal conditions for the production of this cyanobacterium, thus increasing its biotechnological potential.

Material and methods

Cyanobacterium source

Cyanobium sp. LEGE 06113 was obtained from LEGE Culture Collection (LEGE-CC) and kept at 25 °C, using a culture medium and a modified marine BG11 medium with addition of NaCl (20 g L^{-1}), with pH set at 7.5 (Allen 1968; Pagels et al. 2020). Light was provided by fluorescent lamps (BIOLUX, OSRAM) with an intensity of 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and a light/dark cycle of 16:8 h. Constant air flow was also assured at 0.75 L L^{-1} min^{-1}.

Experimental design and growth conditions

Aiming an optimization on growth and pigment production of *Cyanobium* sp., a factorial Box-Behnken design was performed with three abiotic factors: temperature (T; 20–30 °C), pH (6.0–9.0) and NaCl concentration (NaCl; 10–30 g L^{-1}). The three factors and their minimum and maximal values were defined by preliminary tests and bibliographic data. The design and model were created and analysed in the Design-Expert 9.0 software (Stat-Ease, Inc., USA) (Montgomery 2017). In total, 13 design points were performed in triplicate (Table 1), and a quadratic model was determined for each measured parameter: biomass, carotenoids, phycobiliproteins, antioxidant compound productivity and photosynthetic efficiency.

A pre-inoculum was grown for 7 days in the central point condition (T, 25 °C; pH, 7.5; NaCl, 20 g L^{-1}) in order to standardize culture adaptation. Each design condition was tested in batch triplicate cultures for 18 days in glass flat-bottom round flasks with 1800 mL of medium and initial optical density of 0.1 ($OD = A_{680nm}–A_{750nm}$). Light intensity of 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, light/dark cycle of 16:8 h and

Table 1 Box-Behnken matrix for the three variables (T, pH and NaCl) and respective levels for *Cyanobium* sp. batch experiments

Run	T (°C)	pH	NaCl (g L^{-1})
1	20	6.0	20
2	20	7.5	10
3	20	7.5	30
4	20	9.0	20
5	25	6.0	10
6	25	6.0	30
7	25	7.5	20
8	25	9.0	10
9	25	9.0	30
10	30	6.0	20
11	30	7.5	10
12	30	7.5	30
13	30	9.0	20

continuous air flow with a rate of 0.75 L L^{-1} min^{-1} were set constant in all conditions. The pH was kept stable by the addition of buffers—MES (15 mM, PanReac AppliChem) for pH 6.0, HEPES (20 mM, PanReac AppliChem) for pH 7.5 and CHES (10 mM, PanReac AppliChem) for pH 9.0. Nine sampling days were set (0, 1, 4, 6, 8, 11, 13, 15 and 18) for culture monitoring along time and all the referred parameters were evaluated in each one.

Biomass quantification

Biomass was assessed by dry weight (DW), determined (in duplicate) by filtering a known volume of culture through preconditioned GF/C glass fibre filters (Whatman, UK) and further dried at 100 °C for 24 h. In the conditions grown at 10 g L^{-1} NaCl, 3 mL of culture were filtered and in the conditions grown at 20 and 30 g L^{-1} NaCl, to dismiss the NaCl interference, 3 mL of culture were previously diluted in distilled water to 10 g L^{-1} NaCl and the total volume was filtered. The biomass productivity (P_X) was determined using the DW experimental data. P_X (mg_{DW} L^{-1} day^{-1}) was calculated according to $P_X(t) = (X_t - X_0)/t$, where t is the sampling time and X_0 the initial biomass concentration at the beginning of the experimental period (Guedes et al. 2011b).

In vivo chlorophyll *a* fluorescence monitoring

Photosynthetic activity was evaluated by in vivo chlorophyll *a* fluorescence monitoring with pulse-amplitude modulation (PAM) using a Junior-PAM/White fluorometer (Walz, Germany). For that, 2 mL of the culture were centrifuged and the medium removed to allow biomass concentration. Then, the biomass was incubated in darkness for 15 min. Before the measurement, the sample was exposed to a pulse of far-red light (0.8 s). For the maximal quantum yield (F_v/F_m), the culture was exposed to a basal measurement to estimate the minimum fluorescence (F_o), followed by a saturating pulse of 0.8 s (> 4000 $\mu mol_{photons}$ m^{-2} s^{-1}) to obtain maximal fluorescence from PSII (F_m) and the F_v/F_m (Figueroa et al. 2013).

Pigment characterization and quantification

Sample preparation

For pigment characterization and quantification, acetonic and successive aqueous extractions were performed, for carotenoids and phycobiliproteins, respectively. For that, 15 mL of each batch culture were sampled and centrifuged at 2000 × *g* for 15 min. The supernatant was discarded and the pellet resuspended in 5 mL of 100% acetone. For control of the acetonic extraction process and quantification of carotenoids, 100 μL of β-carotenol internal standard (170 mg L^{-1}; Sigma) were added. Extraction was performed in a bead beater (6 series of 8000 rpm for 30 s with 45 s pause). After, the extract was centrifuged at 2000 × *g* for 15 min and the supernatant was stored at − 20 °C for further carotenoid characterization and quantification.

For phycobiliprotein extraction, a successive aqueous extraction was then performed by resuspending the remaining pellet with 5 mL of distilled water and using the bead beater set as referred before. Then the extract was centrifuged at 2000 × *g* for 15 min and the supernatant was used for phycobiliprotein quantification.

Carotenoids

The carotenoids' profile was established following a method described by Guedes et al. (2011b), by high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection, in a Waters Alliance 2695 (USA) equipment. A column heater (Waters, USA) was used during the analyses in order to maintain a constant temperature of 25 °C. The extracts were resuspended in 200 μL of acetone/acetonitrile (9:1) before analysis.

The stationary phase was constituted by a 4 × 250-mm Purospher Star RP-18e (5 μm) column (Merck) and the mobile phase by the HPLC-grade solvent ethyl acetate (solvent A), and acetonitrile/water 9:1 (*v*/*v*) (solvent B). A flow rate of 1 mL min^{-1} was set, and the solvent gradient was installed to obtain 0–31 min (0–60% A); 31–36 min (60% A); 36–38 min (60–100% A); 38–43 min (100% A); 43–50 min (100–0% A); and 50–55 min (0% A). Spectra data from all peaks were collected in the range from 250 to 750 nm. The compounds were identified by comparing the retention times and the UV-visible spectrum with those of authentic standards. The elution times of the authentic standards were lutein 12.4 min, zeaxanthin 12.8 min, echinenone 24.6 min and β-carotene 32.5 min. Standards (HPLC grade) were purchased from Extrasynthese (lutein, zeaxanthin and β-carotene) and from Sigma (echinenone). The calibration curves, correlation coefficient (R^2), limit of detection (LOD = 3 × So/b) and limit of quantification (LOQ = 10 × So/b, where *So* is the standard deviation of signal-to-noise ratio and *b* is the calibration plot slope, for the analysed carotenoids are shown in the Table 2. The results were expressed in terms of productivity of total carotenoids in mg per litre of culture per day (mg_{carot} $L_{culture}^{-1}$ day^{-1}).

Phycobiliprotein quantification

For phycobiliprotein quantification, the aqueous extract absorbance was read at 615 and 654 nm. The phycobiliprotein quantification was calculated as described by Bennett and Bogorad (1973) using the following formulas: phycocyanin (PC) = $(A_{615} - 0.474 \times A_{652})/5.34$; allophycocyanin (APC) = $(A_{652} - 0.208 \times A_{615})/5.09$. The results were expressed in

Table 2 Calibration curves of authentic standards used for carotenoid profiling

Standards	Calibration curve (μg mL^{-1})	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Lutein	$y = 133772885*x - 22683$	0.999	0.882	2.675
Zeaxanthin	$y = 1399289886*x - 119637$	0.998	0.315	0.954
β-carotene	$y = 290230023*x + 172819$	0.999	1.935	5.864
Echinenone	$y = 61438587*x + 12034$	0.999	0.507	1.538

LOD = limit of detection; LOQ = limit of quantification

terms of productivity of total phycobiliproteins in mg per litre of culture per day ($mg_{phyco}\ L_{culture}^{-1}\ d^{-1}$).

Antioxidant capacity assessment

For monitoring of antioxidant capacity by the $ABTS^{\bullet+}$ assay, an acetonic and a successive aqueous extraction were performed as follows: 1 mL of each batch was centrifuged at 2000 *xg* for 5 min and the pellet was resuspended and homogenized in 1 mL of 100% acetone. Cells were then broken in a bead beater (8000 rpm, 6 series of 30 s with 45 s pause) and the supernatant collected for further analysis. To extract water-soluble compounds, the remaining pellet was resuspended in 1 mL of distilled water and the extract performed using the same beat beater settings mentioned before. The extract was centrifuged, and the supernatant collected for further analysis. Before performing the assay the acetone extracts were dried in a Rotavapor and resuspended in the same volume of distilled water with 20% DMSO. The antioxidant capacity assessment was evaluated in triplicate through the $ABTS^{\bullet+}$ assay as described by Guedes et al. (2013). For the determination of the antioxidant capacity of the extracts, a calibration curve with Trolox was established and the antioxidant capacity expressed as Trolox equivalents (TE) per DW of biomass ($mg_{TE}\ g_{DW}^{-1}$). The productivity of antioxidant compounds was then calculated and expressed as TE per litre of culture per day ($mg_{TE}\ L_{culture}^{-1}\ day^{-1}$).

Model validation

In order to validate experimentally the quadratic model, a batch experiment was set with the optimal conditions of T, pH and NaCl obtained from the Box-Behnken design. All the assays for biomass, carotenoids, phycobiliproteins, antioxidant compound productivity and photosynthetic efficiency were performed as described before. The observed values were compared with the predicted model and also to a non-optimized condition in which the cyanobacterium was kept before optimization (Pagels et al. 2020) set at T = 25 °C; pH = 7.5; NaCl = 20 g L^{-1}.

Statistical analysis

Significance levels for each evaluated parameter were obtained by the analysis of variance (ANOVA), using the Design-Expert 9.0 software. A lack-of-fit test was applied to compare the residues of the model and the observed results. The model is validated whenever the statistical significance was higher than 0.05 in the lack-of-fit test.

Results

Factorial model

The Box-Behnken design of thirteen runs was able to provide a significant quadratic model ($p < 0.01$) for all the studied parameters (Table 3). The three factors studied influenced the parameters in different ways and specific results will be detailed in the following sections.

Biomass productivity

The maximum biomass productivity for each condition was calculated and modelled on the factorial design. The obtained equation (Table 3) was plotted and biomass productivity surface response is shown in Fig. 1.

The values for biomass productivity under tested conditions ranged from 16.33 ± 1.53 to 110.33 ± 0.58 $mg_{DW}\ L_{culture}^{-1}\ day^{-1}$. By the quadratic model, the optimal condition was found at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}, with a predicted maximum productivity of 122.66 ± 1.77 $mg_{DW}\ L_{culture}^{-1}\ day^{-1}$.

Regarding the influence of the three factors individually, some trends appear on the biomass productivity of *Cyanobium* sp. In terms of temperature, it can be set that the lower the temperature, the higher is the biomass productivity. When it comes to pH, in the range studied, an alkaline pH seems to increase the growth. Additionally, in terms of NaCl, a less evident trend was found in the present study which means that the NaCl influenced the biomass productivity less effectively. Nevertheless, lower salinities seem to induce a slight increase on biomass production of *Cyanobium* sp., and

Table 3 Model analyses for all measured parameters. The quadratic model is set for temperature (T), pH and salinity (NaCl)

Parameter	*p* value	R^2	Quadratic model
P_X (mg_{DW} $L_{culture}^{-1}$ day^{-1})	< 0.01	0.79	− 0.5471 + 0.0104 T − 0.0046 [NaCl] + 0.1302 pH + 0.00007 T*[NaCl] − 0.00093 T*pH − 0.00013 [NaCl]*pH − 0.00015 T^2 + 0.00008 $[NaCl]^2$ − 0.0055 pH^2
F_v/F_m	< 0.01	0.96	− 0.8564 + 0.0149 T − 0.0015 [NaCl] + 0.2026 pH + 0.00002 T*[NaCl] − 0.0010 T*pH − 0.0001 N*pH − 0.00003 T^2 + 0.00005 $[NaCl]^2$ − 0.0098 pH^2
TC (mg_{carot} $L_{culture}^{-1}$ day^{-1})	< 0.01	0.79	+ 4.5485 − 0.9113 T − 0.2746 [NaCl] + 2.6901 pH + 0.0069 T*[NaCl] + 0.0214 T*pH + 0.00003 [NaCl]*pH + 0.1089 T^2 + 0.0020 $[NaCl]^2$ − 0.1922 pH^2
TPB (mg_{phyco} $L_{culture}^{-1}$ day^{-1})	< 0.01	0.81	− 31.1664 + 1.2292 T − 0.3185 [NaCl] + 5.4474 pH + 0.0062 T*[NaCl] − 0.0075 T*pH − 0.0045 N*pH − 0.0287 T^2 + 0.0043 $[NaCl]^2$ − 0.2906 pH^2
AOX-A (mg_{TE} $L_{culture}^{-1}$ day^{-1})	< 0.01	0.85	− 4.6600 − 0.3428 T + 0.0293 pH − 0.0004 T*[NaCl] − 0.0196 T*pH − 0.0030 [NaCl]*pH + 0.0094 T^2 + 0.0002 $[NaCl]^2$ − 0.1020 pH^2
AOX-W (mg_{TE} $L_{culture}^{-1}$ day^{-1})	< 0.01	0.81	− 5.1639 + 0.0643 T − 0.030313 [NaCl] + 1.3217 pH + 0.0014 T*[NaCl] − 0.0083 T*pH − 0.0010 [NaCl]*pH − 0.0010 T^2 + 0.00006 $[NaCl]^2$ − 0.0636 pH^2

P_X = productivity of biomass; F_v/F_m = maximum PSII photochemical efficiency; TC = total carotenoids; TBP = total phycobiliproteins; AOX = antioxidant compounds (A = acetonic extract and W = aqueous extract); *p* value = probability value and R^2 = correlation coefficient

that is why the optimal condition was set as NaCl of 10 g L^{-1}.

Photosynthetic activity

The measurement of the photosynthetic activity allows to monitor the status of the culture and also to verify the efficiency of growth and photosynthetic metabolism. The photosynthetic activity was measured every sampling day and the maximum rate F_v/F_m was plotted to the factorial model. The quadratic equation was significant (Table 3) and the response surface data is shown in Fig. 2. The F_v/F_m values range between 0.143 ± 0.003 and 0.303 ± 0.003. From the quadratic model, the optimal condition was found at T = 30 °C, pH = 9.0 and NaCl = 10 g L^{-1}, with a predicted maximum F_v/F_m of 0.299 ± 0.009. Contrary to biomass, the highest F_v/F_m was found in a higher temperature.

Pigment productivity

As photosynthetic organisms, cyanobacteria are dependent on the light-harvesting complex to obtain energy and be able to perform photosynthesis. Changes on growth conditions led to different types of acclimation and consequently to changes in the pigment composition, in order to become more photosynthetically efficient.

In terms of pigments, the profile and content of carotenoids and phycobiliproteins were measured every two sampling days, and the maximum productivity of total carotenoids and total phycobiliproteins were used for the factorial modelling. The response surface plots from the quadratic model (Table 3) are shown in Fig. 3 (carotenoids) and Fig. 4 (phycobiliproteins).

The productivity of carotenoids ranged from 0.27 ± 0.02 and 2.80 ± 0.08 mg_{carot} $L_{culture}^{-1}$ day^{-1}, being the higher carotenoid productivity values found in the late exponential

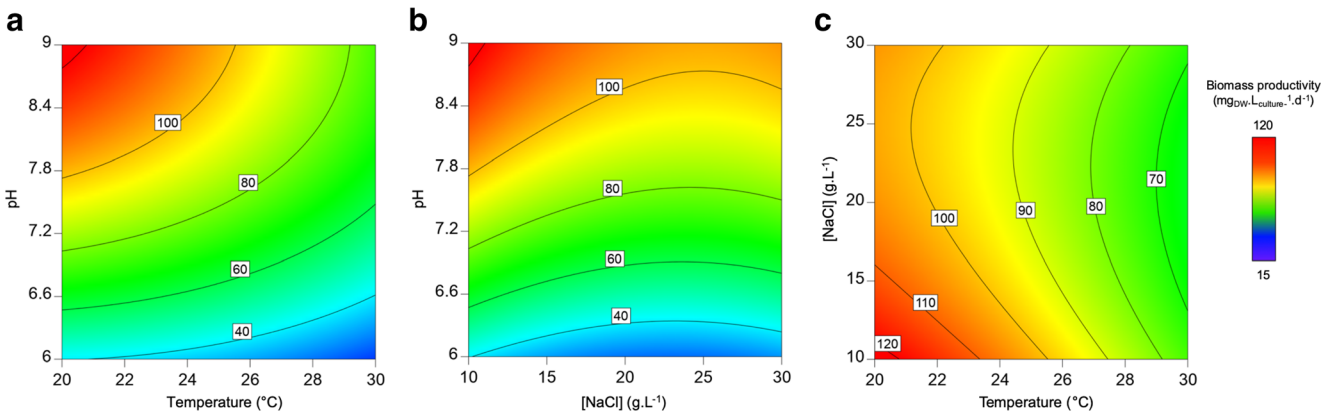

Fig. 1 Response surface plots for maximum biomass productivity (mg_{DW} $L_{culture}^{-1}$ day^{-1}) of *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 g L^{-1}); (**b**) pH *versus* NaCl in constant T (20 °C); (**c**) NaCl *versus* T in constant pH (9.0)

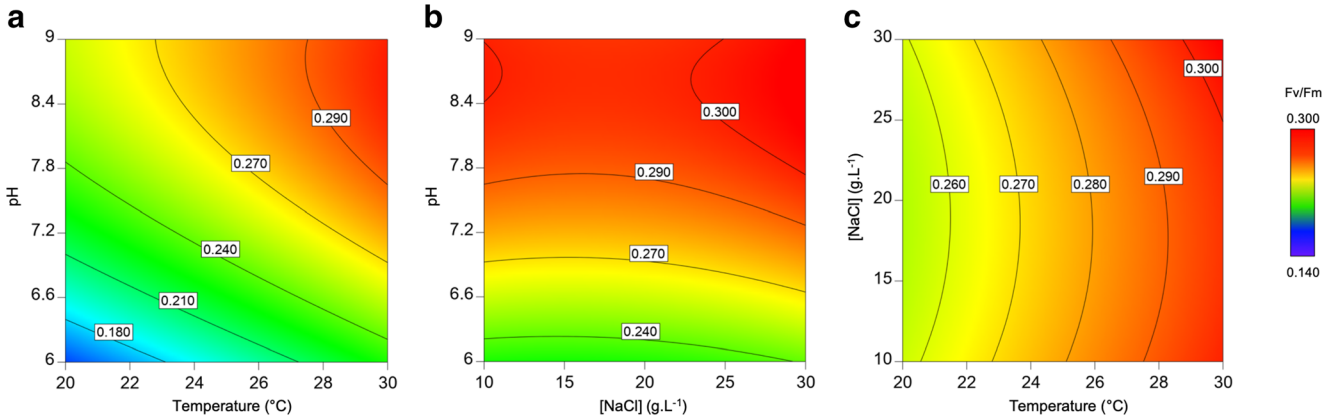

Fig. 2 Response surface plots for maximum F_v/F_m in *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 $g.L^{-1}$); (**b**) pH *versus* NaCl in constant T (30 °C); (**c**) NaCl *versus* T in constant pH (9.0)

growth phase. From the quadratic equation, the optimal condition would achieve a carotenoid productivity of 2.17 ± 0.69 $mg_{carot}\ L_{culture}^{-1}\ day^{-1}$ at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}. The predicted optimal value for carotenoid productivity was lower than the found experimentally in the tested conditions; this difference may be due to the statistical fit of the model. The three factors seem to influence the productivity of carotenoids, although it is notable that the interaction of temperature and NaCl had less impact.

In terms of phycobiliproteins (Fig. 4) productivity, it ranged from 0.14 ± 0.02 to 4.26 ± 0.03 $mg_{phyco}\ L_{culture}^{-1}\ day^{-1}$, being the higher values of phycobiliproteins productivity found also in the late exponential growth phase. The optimal condition was also obtained at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1} according to the quadratic model, with a maximum productivity of 4.14 ± 0.71 $mg_{phyco}\ L_{culture}^{-1}\ day^{-1}$. All the three factors had great influence on phycobiliproteins production. As the main pigments on cyanobacteria, these compounds are highly regulated by changes in the environmental conditions in order to guarantee the best metabolic fitting in stress conditions.

In this work, the productivity of pigments and antioxidant compounds were used instead of their content in the cell. The reason is that in a biotechnological perspective, the amount of the final product has a greater impact on the decision-making than the biomass composition, e.g. in the case of carotenoids, the optimal condition for carotenoid content has a productivity 2.0-fold lower than the referred condition for maximum carotenoid productivity. In the case of phycobiliproteins, the productivity decreases 1.3-fold in the optimal phycobiliproteins content condition. These reductions on productivity are due to reduction on growth and biomass production, which are not compensated with the increase on content. In fact, it is indeed necessary to have a balance between content and biomass production, and that is the reason why specific productivities have more impact on decision-making.

Antioxidant capacity

Cyanobacteria are extremely flexible organisms, capable of acclimating to different kinds of stresses. In nature, these organisms are exposed to variations on light, temperature, salinity, pH, heavy metals and nutrient availability. The response to those changes in environmental conditions is made mainly by changing the metabolic content and functioning of the cell.

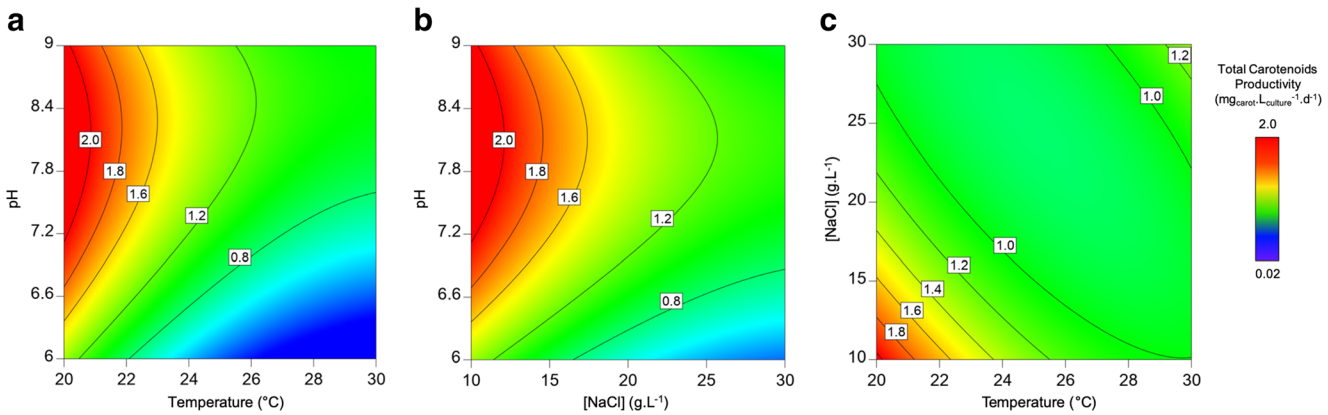

Fig. 3 Response surface plots for maximum total carotenoids productivity ($mg_{carot}\ L_{culture}^{-1}\ day^{-1}$) of *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 g L^{-1}); (**b**) pH *versus* NaCl in constant T (20 °C); (**c**) NaCl *versus* T in constant pH (9.0)

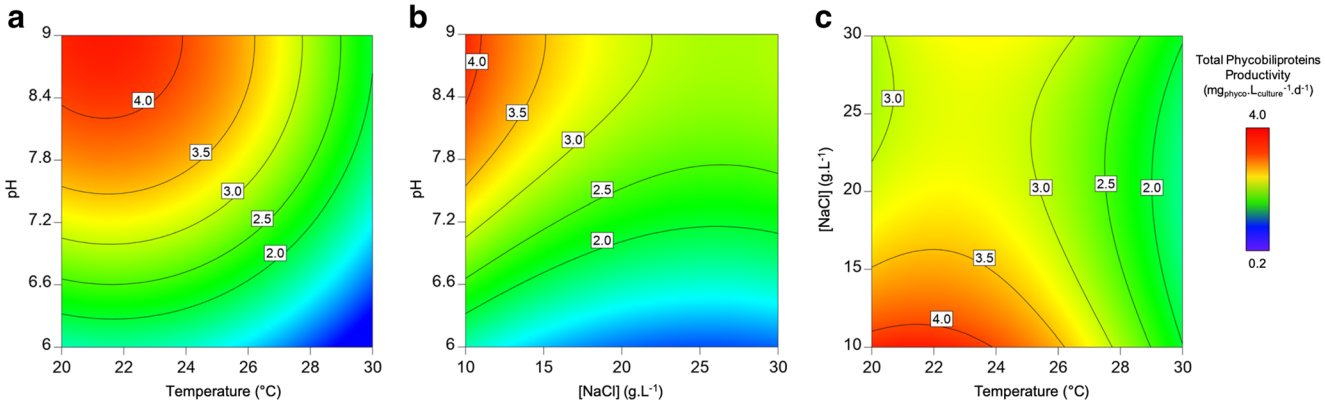

Fig. 4 Response surface plots for maximum total phycobiliproteins productivity (mg_{phyco} $L_{culture}^{-1}$ day^{-1}) of *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 g L^{-1}); (**b**) pH *versus* NaCl in constant T (20 °C); (**c**) NaCl *versus* T in constant pH (9.0)

The first defence against abiotic stress are antioxidant components, both enzymatic and non-enzymatic (Babele et al. 2019). The antioxidant potential of *Cyanobium* sp. was evaluated in both acetone and water extracts, in order to determine the radical scavenging capacity of lipophilic (including carotenoids) and hydrophilic components (including phycobiliproteins), respectively.

The antioxidant capacity was measured every sampling day and the maximum antioxidant equivalent productivity was used for the factorial modelling. The response surface plots from the quadratic models (Table 3) are represented in Fig. 5 (acetonic extracts) and Fig. 6 (water extracts).

In terms of acetonic extracts (Fig. 5), antioxidant productivity ranged from 0.26 ± 0.01 to 1.54 ± 0.15 mg_{TE} $L_{culture}^{-1}$ day^{-1}, being the higher productivity values found in the middle exponential growth phase. The optimal condition, found with the quadratic model for this parameter, was at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}. The predicted value for optimal antioxidant productivity is 1.55 ± 0.19 mg_{TE} $L_{culture}^{-1}$ day^{-1}. Thus, the antioxidant capacity seems to be influenced by the three abiotic factors, but in a more effective way by temperature and pH. The antioxidant capacity of acetonic extracts is probably due to carotenoids and other compounds (such as fatty acids and some phenols). These compounds allow a greater maintenance of the main metabolism in the cyanobacterium, helping on the growth of the culture.

Furthermore, the water extract antioxidant productivity (Fig. 6) ranged from 0.11 ± 0.01 to 0.82 ± 0.12 mg_{TE} $L_{culture}^{-1}$ day^{-1}, with the higher productivity values also found in the middle exponential growth phase. The optimal value obtained from the factorial optimization was found at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}, also the same optimal condition for the acetonic extracts. The predicted value for maximum antioxidant productivity in aqueous extracts was 0.84 ± 0.11 mg_{TE} $L_{culture}^{-1}$ day^{-1}. The antioxidant capacity of aqueous extracts comes mainly from phycobiliproteins, some phenols and possibly peptides.

Model validation

In order to validate the quadratic model and the optimal conditions found in the Box-Behnken factorial, the optimal condition for simultaneal maximization of biomass, carotenoids and phycobiliprotein productivity was performed. This

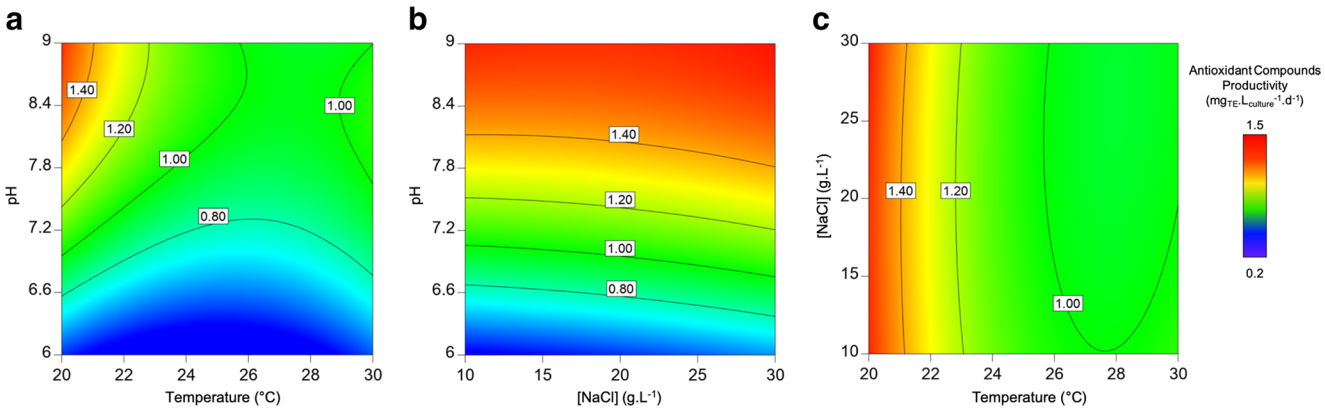

Fig. 5 Response surface plots for maximum antioxidant compound productivity (mg_{TE} $L_{culture}^{-1}$ day^{-1}) of acetonic extract of *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 g L^{-1}); (**b**) pH *versus* NaCl in constant T (20 °C); (**c**) NaCl *versus* T in constant pH (9.0)

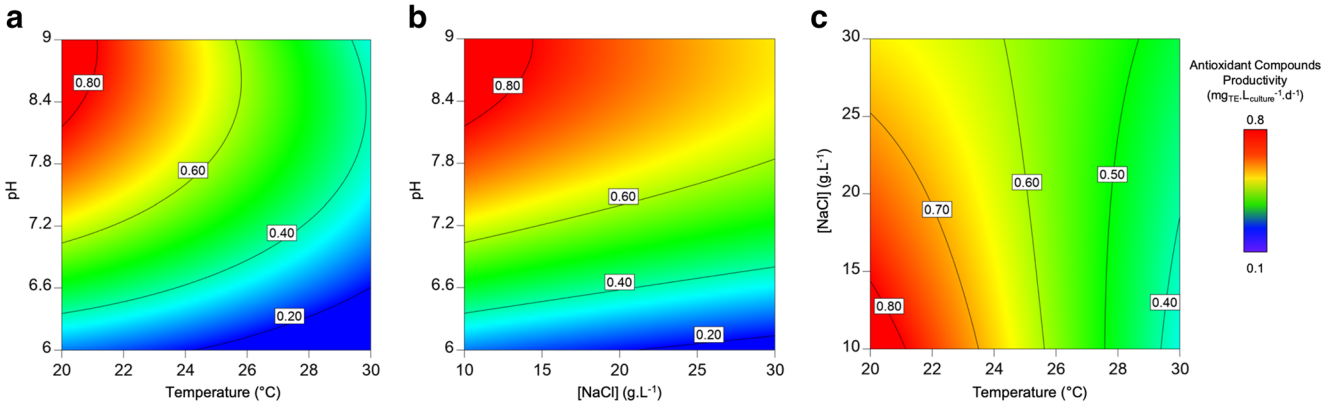

Fig. 6 Response surface plots for maximum antioxidant compound productivity ($mg_{TE}\ L_{culture}^{-1}\ day^{-1}$) of aqueous extract of *Cyanobium* sp., showing (**a**) pH *versus* temperature (T) in constant NaCl (10 g L^{-1}); (**b**) pH *versus* NaCl in constant T (20 °C); (**c**) NaCl *versus* T in constant pH (9.0)

resulted in a triplicate batch in the following conditions: temperature = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}. The predicted and observed values are shown in Table 4.

From the obtained results in the optimal condition, it is possible to validate the model to biomass, carotenoids and phycobiliprotein productivity, and also to photosynthetic activity and aqueous extract antioxidant capacity. The only parameter that had statistical differences was the antioxidant potential of acetonic extracts. The limitation of statistical methods for biological optimization is dependent on the high variability in biological systems.

Table 5 shows the fold change between the optimized condition and the standard condition of the culture collection, used in previous studies with the same strain (Pagels et al. 2020). The optimization performed with the Box-Behnken design had increased the biomass, antioxidant capacity of water extracts and phycobiliprotein productivity by more than 1.5-fold and increased the carotenoid productivity by 2.7-fold; on the other hand, no statistical differences ($p > 0.05$) were found in the antioxidant capacity of acetonic extracts and photosynthetic activity.

Discussion

Cyanobacteria are able to grow in a great range of temperatures and have acclimation mechanisms to withstand such changes. In nature, variation in temperature happens daily and seasonally, with different thermic amplitudes depending on the zone of the globe. Temperature plays a crucial role in membrane stability and lipidic composition of cyanobacterial cell. At lower temperatures, polyunsaturated fatty acids become prevalent in the fatty acid profile, while at higher temperatures, the membrane fluidity is maintained by saturated

Table 4 Responses for optimal condition (predicted and observed) for the culture of *Cyanobium* sp. based on the Box-Behnken design

Parameter	Predicted value	Observed value	*p* value
P_X ($mg_{DW}\ L_{culture}^{-1}\ day^{-1}$)	122.67 ± 17.71	127.12 ± 1.30	0.676
F_v/F_m	0.257 ± 0.009	0.255 ± 0.003	0.724
AOX – A ($mg_{TE}\ L_{culture}^{-1}\ day^{-1}$)	1.55 ± 0.19	0.92 ± 0.06	0.001
AOX – W ($mg_{TE}\ L_{culture}^{-1}\ day^{-1}$)	0.84 ± 0.11	0.97 ± 0.07	0.122
TC ($mg_{Carot}\ L_{culture}^{-1}\ day^{-1}$)	2.04 ± 0.51	2.09 ± 0.25	0.986
Lutein	0.05 ± 0.02	0.06 ± 0.00	0.929
Zeaxanthin	0.25 ± 0.07	0.28 ± 0.01	0.789
Echinenone	0.15 ± 0.05	0.12 ± 0.01	0.537
β-carotene	0.72 ± 0.17	1.00 ± 0.13	0.101
TPB ($mg_{phyco}\ L_{culture}^{-1}\ day^{-1}$)	4.14 ± 0.71	3.98 ± 0.07	0.708
Phycocyanin	2.94 ± 0.50	2.79 ± 0.03	0.841
Allophycocyanin	1.20 ± 0.22	1.18 ± 0.05	0.978

Predicted and observed values were compared in an ANOVA analysis and the statistical validation is present in form of *p* value (identical when $p > 0.05$)

P_X = productivity of biomass; F_v/F_m = maximum PS II photochemical efficiency; AOX = antioxidant compounds (A = acetonic extract and W = aqueous extract); TC = total carotenoids and TBP = total phycobiliproteins

Table 5 Comparison between optimized (T = 20 °C; pH = 9.0; NaCl = 10 g L^{-1}) and non-optimized (T = 25 °C; pH = 7.5; NaCl = 20 g L^{-1}) condition for the production of *Cyanobium* sp.

Parameter	Non-optimized condition	Optimized condition	Fold increase
P_X (mg_{DW} $L_{culture}^{-1}$ day^{-1})	69.01 ± 1.73	127.12 ± 1.30	1.84
F_v/F_m	0.257 ± 0.002	0.255 ± 0.003	n.s.
AOX – A (mg_{TE} $L_{culture}^{-1}$ day^{-1})	0.86 ± 0.05	0.92 ± 0.06	n.s
AOX – W (mg_{TE} $L_{culture}^{-1}$ day^{-1})	0.53 ± 0.01	0.97 ± 0.07	1.83
TC (mg_{Carot} $L_{culture}^{-1}$ day^{-1})	0.77 ± 0.04	2.09 ± 0.25	2.71
TPB (mg_{phyco} $L_{culture}^{-1}$ day^{-1})	2.50 ± 0.07	3.98 ± 0.07	1.59

P_X = productivity of biomass; F_v/F_m = maximum PSII photochemical efficiency; AOX = antioxidant compounds (A = acetonic extract and W = aqueous extract); TC = total carotenoids and TBP = total phycobiliproteins; n.s., no statistical differences ($p > 0.05$)

fatty acids (Paliwal et al. 2017). Furthermore, pH plays a fundamental role in cyanobacteria metabolism and physiology. pH influences nutrient uptake, carbon availability and photosynthetic activity rate (Shruthi and Rajashekhar 2014). Likewise, salinity—in the case of artificial media, NaCl concentration—is fundamental for ion regulation, osmotic balance and metabolic activity (Kannaujiya et al. 2017). Salt stress combines ionic and osmotic stresses, leading also to an oxidative stress—however, many of the salt tolerance mechanisms are yet not clarified (Cepoi 2019).

Growth and photosynthetic activity

Cyanobium sp. growth was not inhibited in any of the tested conditions. In terms of temperature, the range in which the organism is able to survive is called thermal niche. In this study, *Cyanobium* sp. was able to grow from 20 to 30 °C, with the optimal temperature 20 °C. By reaching the upper critical temperature, the organism becomes exposed to heat stress, inducing inhibition on the photosynthetic apparatus and thereafter inducing cell death (Nalley et al. 2018). The thermal niche and optimal temperature are species dependent, although, in general, cyanobacteria are able to grow in a wide range of temperatures, as in the case of *Leptolyngbya* sp. HS-16 and HS-36, isolated from a hot spring (c.a. 40 °C) that could grow in a range from 20 to 50 °C (Prihantini et al. 2019). The versatility of a strain to adapt to a wider range of temperatures allows its cultivation in different areas or climatic conditions.

On the other hand, the photosynthetic activity in *Cyanobium* sp. was higher at a higher temperature (30 °C). A heat stimulation of photosystem I has already been described in *Arthrospira* sp., where temperatures between 30 and 40 °C increased the photosynthetic capacity, upregulating PSI (Zhang and Liu 2016) and in *Synechococcus* sp., where photosynthetic activity is higher during summer, when compared with other seasons (Kim et al. 2018). In fact, growth and photosynthetic activity are not always correlated, since cyanobacteria have other mechanisms that allow growth in a suboptimal photosynthetic condition, e.g. non-photochemical quenching (Müller and Mu 2001).

When it comes to pH, in the range studied, an alkaline pH seems to increase the growth and biomass production in *Cyanobium* sp. as well as the photosynthetic activity. In general, cyanobacteria tend to grow better in neutral to alkaline environments, although the range also varies between species (Yang et al. 2018). A similar trend was found for *Cyanobacterium aponinum* that could grow in a pH range from 3.0 to 8.0, achieving higher productivity in more alkaline conditions (Meng et al. 2018). Regarding photosynthetic activity, pH 9.0 also induced better conditions for photosynthesis; this response is mainly due to the light-harvesting complex composition and the production of antennae pigments. The same positive effect of higher pH occurred in *Synechococcus obtusiusculus*, with a higher photosynthetic activity in neutral to alkaline environment, with optimal pH of 8.0 (Cabello et al. 2015). Additionally, the tolerance to high pH can be used to overcome contaminations, such as other microalgal species, in cyanobacterial productions (Yang et al. 2018).

Moreover, in terms of NaCl, a less evident trend was found in the present study, which means that the NaCl influenced the biomass productivity in a less effective way. Nevertheless, lower salinities seem to induce a slight increase on biomass production and photosynthetic activity of *Cyanobium* sp., and the optimal condition was set as NaCl = 10 g L^{-1}. The amount of salt needed in the media is very species dependent, due to the variability of these organisms' habitat, distribution and ability to adapt to extreme environments. Some studies have found similar responses to NaCl concentration as those found herein, as in the case of *Synechocystis* sp. CCNM 2501, a cyanobacterium isolated in brackish waters, which was able to survive in a NaCl range from 0 to 60 g L^{-1}, but had its productivity affected negatively with the increase on salt concentration, with the optimal concentration of 0 g L^{-1} (Paliwal et al. 2015).

Carotenoids and acetonic extract antioxidant capacity

Carotenoids are lipophilic pigments present in photosynthetic organisms and are part of the light-harvesting complex. Carotenoids are an important defence in stress situations for cells; the induction of carotenogenesis comes mainly through light conditions, but also through changes on the photosynthetic apparatus, since those compounds are responsible for light harvesting and also energy dissipation in the form of heat.

When it comes to the antioxidant capacity of acetonic extracts, carotenoids and other lipophilic compounds (such as fatty acids and some phenolic compounds) are the main active components. These compounds allow a greater maintenance of the main metabolism in the cyanobacterium, helping on the growth of the culture. In this study, antioxidant compound productivity was optimal at T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}. The same condition was set optimal for carotenoid production. Lipophilic antioxidant compounds, such as carotenoids and polyunsaturated fatty acids (PUFAs), have been shown to be influenced in different ways by abiotic factors. In most cases, low temperatures are related to an increase in PUFAs accumulation and to the activation of superoxide dismutase (SOD) (Cepoi 2019).

Regarding specifically the carotenoid production, temperature had a huge effect on carotenoid production by *Cyanobium* sp. with a great variation between the optimal and worst conditions. On the other hand, some cyanobacteria are not even affected by changes on temperature, as in the case of *Arthronema africanum* that ranges from 15 to 50 °C, keeping the content of carotenoids constant (Chaneva et al. 2007). Concerning pH, the positive effect of a higher pH in *Cyanobium* sp. carotenoid production was expected, as in general, alkaline conditions appear to increase the productivity of carotenoids in cyanobacteria (Juneja et al. 2013).

Furthermore, in the case of salinity, *Cyanobium* sp. was negatively affected by the increase of NaCl. This effect was also observed in *Synechocystis* sp. CCNM2501, grown in a range of NaCl from 0 to 60 g L^{-1}, with optimal condition for carotenoid production at 10 g L^{-1}, reducing the amount of zeaxanthin and echinenone by any increase in salt concentration (Paliwal et al. 2015).

Phycobiliproteins and aqueous extract antioxidant capacity

Phycobiliproteins are the main group of pigments in cyanobacteria, and in some cases, these compounds can represent more than half of the cell proteins (Bryant et al. 1979). In the cell, phycobiliproteins are responsible for light energy transfer to the photosystem and, like carotenoids, have an important role in the antioxidant defence of the cell. In fact, the antioxidant capacity of the aqueous extracts can come mainly from phycobiliproteins, but also from phenolic compounds and peptides. Valuta et al. (2015) found a correlation between the content of phycobiliproteins and $ABTS^{\bullet+}$ assay values for aqueous extracts of *Nostoc linckia*.

The negative effect of increasing temperature on phycobiliprotein production in *Cyanobium* sp. is opposed to the general trend in cyanobacteria, which usually associates a higher production of these pigments to high temperatures (≥ 30 °C) (Pagels et al. 2019) although the results for *Cyanobium* sp. were similar to the ones found for *Synechococcus* sp. PCC 7002 with optimal condition set at 22 °C (Sakamoto and Bryant 1998).

In terms of pH and salinity, *Cyanobium* sp. followed the overall response for cyanobacteria, with higher phycobiliprotein production under high pH and low NaCl (Pagels et al. 2019). The relation between high concentrations of NaCl and a decrease on phycobiliprotein content is due to a detachment of phycobilisomes induced by accumulation of intracellular sodium ions and inhibition of the energy transfer from the phycobilisomes to the photosystem (Kannaujiya et al. 2017; Chentir et al. 2018).

Box-Behnken as a tool for optimization of cyanobacterial production

Overall, although the use of cyanobacteria as a source of pigments for industrial applications is increasing, there are still constraints regarding the economic aspects of the production of these organisms. Some of the main issues contemplate the high costs of production. To overcome some of these constrains, optimization of culture conditions is usually attempted in order to increase the pigment productivity and to reduce production costs. Therefore, in this work, the optimization of temperature, pH and NaCl for *Cyanobium* sp. biomass, carotenoids and phycobiliprotein production was achieved by a Box-Behnken factorial design. The interaction of the three abiotic factors tested on *Cyanobium* sp. cultures was significant for all parameters studied, although temperature and pH seemed to take a more important role on *Cyanobium* sp. metabolism than NaCl, at least in the studied range of parameters. Factorial models for cyanobacterial production optimization are still scarce and related to different factors and metabolites than the ones herein discussed, although the use of factorial models has been encouraged due to their accuracy and versatility for different goals (e.g. Chentir et al. 2018).

The use of factorial statistic optimization allows observation of the influence of the interaction of studied factors, as well as their individual influence. In addition, it is possible to generate a higher-order response surfaces with a reduced number of experimental trials. The Box-Behnken model, together with central composite design, appears as an ideal tool for a more accessible and cost-efficient optimization of cyanobacterial cultures. The models allow not only to obtain

the ideal condition of cultivation but can help in the decision-making of a large-scale production, evaluating the advantages and disadvantages of a suboptimal condition that can occur depending on the weather conditions. Moreover, the comparison between the non-optimized and optimized conditions (Table 5) shows an increase of more than 1.5-fold in biomass productivity, antioxidant capacity of W extracts and phycobiliproteins production, and 2.7-fold in carotenoids production, without compromising the photosynthetic productivity, which is very significant in the culture maintenance.

Furthermore, in an optimal scenario, it would be possible to use the biomass for a range of products, in a biorefinery process. The total revenues generated by the different coproducts in a biorefinery could increase the economic feasibility of cyanobacteria-based applications.

In the present study, the best condition for simultaneous *Cyanobium* sp. biomass and pigment production is T = 20 °C, pH = 9.0 and NaCl = 10 g L^{-1}. The quadratic models for all parameters, except for antioxidant capacity of acetonic extracts, were confirmed by the model validation under the optimal condition. In the optimal set conditions, biomass, carotenoid and phycobiliprotein productivity reached a maximum of 127.12 ± 1.30 mg_{DW} $L_{culture}^{-1}$ day^{-1}, 2.09 ± 0.25 mg_{carot} $L_{culture}^{-1}$ day^{-1} and 3.98 ± 0.07 mg_{phyco} $L_{culture}^{-1}$ day^{-1}, respectively. The results obtained are expected to give fundamental bases for the biotechnological valorisation of *Cyanobium* sp.; however, further studies are still necessary for its application on industrial level, as scale-up methodologies and downstream processing.

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Authors' contributions FP, DS, HMA and ACG conceived and designed the research. FP and DS conducted the experiments. FP, DS, GL and HMA performed the analytical measurements. FP and DS wrote the manuscript. ISP, VV and ACG were responsible for the supervision and funding acquirement. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflicts of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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