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Multivariable optimization process of heterotrophic growth of *Chlorella vulgaris*

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ABSTRACT

Microalgae have received increasing attention as one of the most promising feedstocks in the development of new healthier food products and different strategies have been attempted to improve their growth. However, the high production costs and low productivities, commonly associated with photoautotrophic growths, are still a big challenge. In this study, a two-step optimization strategy was carried out in order to maximize the biomass production of a *Chlorella vulgaris* strain used at industrial scale under heterotrophic conditions. From a total of 24 independent variables, which were studied simultaneously, 10 have presented a positive effect over X_{max}, while the remaining have shown to be negative. The amount of $(NH_4)_2SO_4$ (6.3 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.7 g L^{-1}), and $C_6H_{12}O_6$ (50% w/v) in the culture medium has revealed to be the only factors with a significant impact on biomass concentration, with optimum values of 25.5, 64.6, and 75 ml.L^{-1}, respectively. The optimized medium resulted in an improvement of the X_{max} by 99.6% when compared to the growth medium applied at industrial scale, proving the success of this strategy. Additionally, the carbohydrates production was enhanced by 48.0%.

1. Introduction

Microalgae are a group of unicellular and multicellular photosynthetic microorganisms – classified either as prokaryotic or eukaryotic – that comprises a broad variety of species (> 50,000). As consequence of such diversity, they can be found not only in aquatic environments but also in terrestrial ecosystems, living in a wide range of environmental conditions (Mata et al., 2010; Torres-Carvajal et al., 2017). The ability to effectively produce a panoply of bioactive compounds (e.g., lipids, proteins, carbohydrates, pigments, vitamins), places microalgae among the most promising feedstocks for multiple biotechnology sectors, such as food, feed, biofuels, agriculture, nutraceutics, cosmetics, or pharmaceutical. When used for food purposes, namely in the form of whole-biomass products, this group of microorganisms is frequently classified as "superfood", based on their balanced and rich biochemical composition that results in high nutritive value and health benefits (Vrenna et al., 2021). Additionally, considering the increasing world population

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and the predictions of an insufficient protein supply, huge efforts have been made in order to find new food alternatives. Microalgal biomass arises as a promising option since it exhibits higher protein contents and presents several other advantages when compared to its vegetable counterparts (e.g., soybean, chickpea), namely higher growth rates, no competition for arable land, more interesting nutritional composition, and the presence of amino acids – including the essential ones (Geada et al., 2021). However, microalgal proteins are not the only factor that makes these microorganisms so interesting and full of potential. Some species produce large quantities of polyunsaturated fatty acids (PUFAs) that are important for human beings – as the case of omega-3 (e.g., eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)) (Remize et al., 2021). PUFAs are commonly associated with anti-inflammatory activity, improvement of cardiovascular health, and a good development of children's brain, being currently incorporated in infant formulae (Enzing et al., 2014; Wiktorowska-Owczarek et al., 2015; Lafarga, 2019). Likewise, pigments, especially carotenoids (e.g., β-carotene, astaxanthin), are also constituents of microalgae that have gathered increasing attention as natural food colorants since they can be a source of provitamin A – which can be further converted into vitamin A – and display intrinsic anti-inflammatory and anti-oxidant properties (Enzing et al., 2014; Varela et al., 2015).

In general, microalgae cultivation modes can be classified as photoautotrophic, heterotrophic, and mixotrophic (Geada et al., 2017). Since they are inherently photosynthetic organisms, photoautotrophic growth is the most widely used metabolism (Assunção and Malcata, 2020). Under this mode, microalgae grow by absorbing solar light and fixing inorganic carbon (e.g., carbon dioxide (CO_2)), functioning as energy and carbon sources, respectively. However, this type of cultivation is conditioned since light availability – whether natural or artificial – can be limited. Furthermore, when high cell densities are attained, cultures might experience self-shading effect, as light penetration is inversely proportional to biomass concentration. This constraint frequently results in low biomass productivity, making photoautotrophic growth a challenging process in terms of cost-effectiveness (Ende and Noke, 2019; Hogg, 2013). The heterotrophic cultivation of microalgae has the potential to minimize the problems associated with photoautotrophic cultivation since this type of metabolism eliminates the light requirement and a significant improvement of the growth rate, cell density, and biomass productivity can occur (Ende and Noke, 2019). Another reason for the increased cell densities in this cultivation mode is due to the energy density of the carbon source; it is quite greater in an organic carbon (e.g., glucose, lactose, glycerol) than in CO_2 (Morales-Sánchez et al., 2017; da Silva et al., 2021). In addition, the use of wastewaters and low-cost by-products from food industry – containing significant amounts of organic matter and nutrients, including organic carbon – to produce microalgae can be an interesting solution to reduce the production costs and make this a more eco-friendly process at the same time (Ende and Noke, 2019; Jareonsin and Pumas, 2021; Murwanashyaka et al., 2020). Furthermore, with an optimized and successful cultivation process, it is possible to obtain high-density cultures that will allow for cheaper and easier downstream processing steps at large-scale.

With respect to optimization and modeling methodologies, there is an extensive number of options available. The One-Factor-At-a-Time (OFAT) approach, for example, is one of the most frequently used methods to assess the finest parameters and the precise effect of variables; however, it does not take into account interactions between variables and makes difficult to perform multiple runs at the same time since all the other factors are fixed while one variable is under study (Shah and Mishra, 2020). On the contrary, the use of statistically designed experiments – through Composite Rotational Design (CCDR), Placket-Burman Design (PBD), among others – allows studying all variables simultaneously and finding the effectiveness of each variable or even the most suitable mathematical models to adjust the experimental data, using a minimal number of experimental runs (Ido et al., 2018; Martins et al., 2021; Patil and Meti, 2018). This type of strategy has been increasingly applied in microalgae processes in order to optimize growth conditions and/or culture medium composition as means, for instance, of improving (by 3-fold) the biomass productivity (Kim et al., 2019) and/or the synthesis of specific compounds of interest, as the case of omega-3 fatty acids (from 5.6% to 16.72%) (Udayan et al., 2022). Despite the successful utilization of such methodologies, the number of factors tested simultaneously is sometimes rather low, not considering all the variables involved in the process (Chin et al., 2020).

The present study aimed at maximizing the heterotrophic growth of an industrial *Chlorella vulgaris* strain. For that purpose, a two-step optimization strategy based on Design of Experiment (DOE) tools was carried out in order to simultaneously identify the conditions of 24 independent variables – concentration of all the 20 culture medium components, as well as temperature, agitation speed, pH, and initial biomass concentration – that favored the production of microalgal biomass. Additionally, a comparison of the biochemical composition of *C. vulgaris* cells grown under the original and optimized conditions was performed to assess the impact of the optimization process on metabolites production.

2. Materials and methods

2.1. *Microalga strain and cultures maintenance*

The freshwater microalga *Chlorella vulgaris* 0002 CA used in all experiments was kindly provided by Allmicroalgae – Natural Products, S.A. and obtained from a cryopreserved vial of its private Culture Collection. Microalgal cultures were maintained in T-Flasks (under constant agitation and complete dark environment) containing a heterotrophic medium (FERM_MB) – with 4 different solutions: *i*) macronutrients (MB); *ii*) micronutrients (TM); *iii*) vitamins (VIT); and *iv*) glucose 50% w/v (as carbon (C) source) – previously developed by the company, as described by Trovão et al. (2020).

2.2. *Experimental work*

All the solutions and glassware utilized were previously autoclaved at 121 °C for 40 min, being the laboratory experiments performed using a working volume of 100 ml in 250 ml Erlenmeyer flasks with a cotton wool plug. After inoculation, the Erlenmeyer flasks were placed in a temperature-controlled orbital shaker (IKA KS 4000i control, Staufen, Germany) using the culture conditions identified in Tables 1 and 2. Two samples were collected per day in order to monitor cultures growth and pH, which was measured using a pH meter (Hanna HI 2210, Padova, Italy) and adjusted,

Table 1 – Low (–1) and high (1) levels of the factors tested in the PBD28, as well as the conditions of the central points (CPs), with the corresponding units and nomenclature.

Factors	Units	Symbol	Low (–1)	CP (0)	High (1)
Temperature	°C	X_1	25	30	35
pH	-	X_2	6	7	8
Agitation speed	rpm	X_3	150	200	250
Initial optical density (750 nm)	-	X_4	0.02	0.06	0.10
K_2HPO_4	ml L^{-1}	X_5	24.97	48.49	72.01
$NaH_2PO_4{\cdot}H_2O$	ml L^{-1}	X_6	23.46	59.37	95.29
$(NH_4)_2SO_4$	ml L^{-1}	X_7	21.31	44.74	68.18
$MgSO_4{\cdot}7H_2O$	ml L^{-1}	X_8	9.37	26.65	43.94
$C_6H_8O_7{\cdot}H_2O$	ml L^{-1}	X_9	4.34	8.50	12.66
$CaCl_2{\cdot}2H_2O$	ml L^{-1}	X_{10}	9.58	19.15	28.73
$C_6H_{12}O_6$	ml L^{-1}	X_{11}	19.98	39.96	59.95
$CuSO_4{\cdot}5H_2O$	ml L^{-1}	X_{12}	4.01	8.01	12.02
H_3BO_3	ml L^{-1}	X_{13}	5.01	10.67	16.34
$ZnSO_4{\cdot}7H_2O$	ml L^{-1}	X_{14}	4.87	9.74	14.61
$MnSO_4{\cdot}H_2O$	ml L^{-1}	X_{15}	5.01	10.73	16.45
$Na_2MoO_4{\cdot}H_2O$	ml L^{-1}	X_{16}	1.94	3.79	5.63
$NiCl_2{\cdot}6H_2O$	ml L^{-1}	X_{17}	9.26	20.06	30.86
$FeSO_4{\cdot}H_2O$	ml L^{-1}	X_{18}	4.03	7.91	11.80
Thiamine-HCl	ml L^{-1}	X_{19}	2.22	4.45	6.67
D-Biotin	ml L^{-1}	X_{20}	6.14	9.21	12.28
Cyanocobalamin	ml L^{-1}	X_{21}	7.75	13.28	18.81
Calcium Pantothenate	ml L^{-1}	X_{22}	1.26	1.89	2.52
p-Aminobenzoic acid	ml L^{-1}	X_{23}	4.38	6.56	8.75
PIPES buffer	ml L^{-1}	X_{24}	25	50	75

Table 2 – Different levels of the significant factors (i.e., (NH4)2SO4, $MgSO_4{\cdot}7H2O$, and C6H12O6) tested in the CCRD.

Factors	Units	Experimental values – Coded level				
		-α	-1	0	1	α
$(NH_4)_2SO_4$	ml L^{-1}	1.53	7.50	16.25	25	30.97
$MgSO_40.7\,H_20$	ml L^{-1}	29.77	40	55	70	80.23
$C_6H_{12}O_6$	ml L^{-1}	41.36	55	75	95	108.64

whenever necessary, with 1 M HCl or 5 M NaOH. When reaching the stationary phase, cultures were centrifuged at 4000 rpm for 20 min (Centurion Pro-Analytical CR7000, Chichester, United Kingdom) and the pellet was frozen (–20 °C) for later lyophilization. Regarding the supernatant, it was also frozen and then analyzed for evaluation of the consumption of the C source under each culture condition tested.

The optimization process was carried out based on Design of Experiments methodologies, particularly using the Protimiza Experimental Design software (http://experimental-design.protimiza.com.br). Due to the high number of independent variables involved in this study, 24, the rationale of the optimization process was as follows: *i*) firstly, a screening step (Section 2.2.1.), using a PBD, was carried out in order to assess the effect of each variable over microalgae's growth, as well as to identify the statistically significant ones; *ii*) after selecting the significant variables, an optimization step (2.2.2.) was performed, using a CCRD, for determination of the culture conditions that allow for biomass concentration maximization; *iii*) the optimal conditions were tested (2.2.3.) and compared to the original conditions (applied by the company) using assays that were run in parallel.

2.2.1. *Screening step (PBD)*

The PBD was employed to assess the impact of the 24 independent variables (factors), tested simultaneously, over the response variables – maximum biomass concentration (X_{max}) and productivity (P_{max}) – and select those that have shown a significant effect. Given the number of factors under study, each of the variables was applied at low (–1) and high (1) levels (Table 1), resulting in a total of 28 assays (PBD28) and 3 additional experiments named Central Points (CPs) – as shown in Table 3. The culture conditions of the CPs correspond to the intermediate values between the levels – 1 and 1 of each independent variable. These are the growth conditions currently applied by Allmicroalgae – Natural Products, S.A. in the heterotrophic cultivation of *C. vulgaris* 0002 CA. All the independent variables selected to proceed with the optimization process have shown a significant effect on biomass production at a confidence level higher than 90%.

2.2.2. *Optimization step (CCRD)*

The CCRD was employed to optimize the most significant medium components identified through the experiments carried out at the PBD28 (Section 2.2.1.), namely the concentrations of $(NH_4)_2SO_4$ (X_7), $MgSO_4{\cdot}7H_2O$ (X_8), and $C_6H_{12}O_6$ (X_{11}). These independent variables were studied at 5 different

Table 3 – Matrix of PBD28 for evaluation of independent variables with coded values, where "– 1" corresponds to the minimum value, "1" to the maximum value, and "0" to the intermediate value of the input parameter range.

Trial Number	Independent Variables																								Responses	
	x1	x2	x3	x4	x5	x6	x7	x8	x9	x10	x11	x12	x13	x14	x15	x16	x17	x18	x19	x20	x21	x22	x23	x24	X_{max} ($g\,L^{-1}$)	P_{max} ($g\,L^{-1}\,d^{-1}$)
1	1	-1	1	1	1	1	-1	-1	-1	-1	1	-1	-1	-1	1	-1	-1	1	1	1	-1	1	-1	1	11.36	2.38
2	1	1	-1	1	1	1	-1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	5.03	1.27
3	-1	1	1	1	1	1	-1	-1	-1	1	-1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	5.54	1.11
4	-1	-1	-1	1	-1	1	1	1	1	-1	-1	1	-1	1	-1	-1	-1	1	1	-1	1	1	1	-1	5.99	2.18
5	-1	-1	-1	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	6.45	2.35
6	-1	-1	-1	-1	1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	-1	-1	1	1	1	-1	1	8.50	1.22
7	1	1	1	-1	-1	-1	1	-1	1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	-1	1	0.20	0.20
8	1	1	1	-1	-1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1	1	1	1	-1	1	1	-1	0.56	0.37
9	1	1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	-1	1	-1	-1	-1	1	1	-1	1	1	18.70	3.74
10	1	1	-1	1	-1	1	1	-1	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	-1	-1	-1	1	0.22	0.51
11	-1	1	1	1	1	-1	1	1	-1	1	1	-1	1	1	1	-1	-1	-1	-1	-1	1	1	-1	-1	0.68	0.23
12	1	-1	1	-1	1	1	-1	1	1	-1	1	1	1	1	1	-1	-1	-1	1	-1	-1	-1	1	-1	9.06	1.39
13	1	-1	1	1	1	-1	1	-1	1	-1	-1	-1	1	-1	1	1	1	1	-1	-1	1	-1	1	-1	5.80	2.87
14	1	1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	0.46	0.56
15	-1	1	1	1	-1	1	-1	1	1	-1	-1	-1	-1	1	1	1	1	1	-1	1	-1	1	-1	-1	6.13	1.55
16	1	-1	1	1	-1	1	1	1	-1	1	1	1	-1	-1	-1	1	-1	1	-1	-1	1	-1	-1	1	12.72	2.67
17	1	1	-1	1	1	-1	-1	1	1	1	1	1	-1	-1	-1	1	1	-1	1	-1	-1	1	-1	-1	10.38	2.21
18	-1	1	1	-1	1	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	-1	0.17	0.07
19	-1	1	-1	-1	-1	1	-1	-1	1	1	1	-1	1	-1	1	1	-1	1	1	-1	1	1	1	1	7.75	1.01
20	-1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	1	-1	1	1	-1	1	1	1	3.20	1.07
21	1	-1	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	-1	1	1	-1	1	1	1	1	1	5.71	2.30
22	-1	-1	1	-1	1	-1	-1	-1	1	1	-1	1	1	1	-1	1	-1	1	-1	-1	-1	1	-1	1	4.01	1.01
23	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	-1	9.46	1.19
24	-1	1	-1	1	-1	-1	-1	1	-1	-1	1	1	1	-1	1	-1	1	1	-1	-1	-1	-1	1	1	14.81	2.61
25	-1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	-1	1	1	1	-1	1	1	1	-1	-1	-1	5.94	1.82
26	1	-1	-1	1	-1	-1	-1	-1	1	1	1	-1	1	1	-1	-1	1	1	1	1	1	-1	-1	-1	5.04	0.88
27	-1	1	-1	-1	1	-1	1	-1	-1	-1	1	1	-1	1	1	1	-1	1	1	1	1	-1	-1	-1	0.25	0.09
28	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	6.23	2.09
CP1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.84	2.51
CP2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.99	3.13
CP3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.99	2.55

levels (-α, −1, 0, 1, α), where the values of (-α) and (α) were – 1.68 and 1.68, respectively (Table 2). Based on such considerations, an experimental matrix of 14 assays – combining the 3 significant factors simultaneously – and 4 CPs was formulated (Table 6). The remaining independent variables, previously reported as non-significant for microalgal growth, were kept at an intermediate concentration/level (the same applied for the CPs of PBD28 – Table 1). All the independent variables, as well as the respective interactions, that have shown a significant effect on biomass production at a confidence level higher than 90%, were selected to determine the model equation of the coded variables (Eq. (3)). Through Eq. (3), it was possible to identify the predicted value for X_{max} under optimal conditions.

2.2.3. *Validation assays*

The validation of CCRD results was accomplished by growing *C. vulgaris* in triplicate under the optimal conditions predicted by the model. The biomass concentration obtained was analyzed statistically (Section 2.6.) in order to understand whether the experimental results matched the predicted ones. In parallel, three independent replicates were carried out using non-optimized conditions (i.e., standard growth conditions applied at Allmicroalgae – Natural Products, S.A.) – Section 2.2.1. – to perform a comparison with optimal cultures. In addition to the evaluation of differences in terms of growth kinetics, a biochemical characterization was also done in order to understand the impact of the optimization process on metabolites production.

2.3. *Determination of growth kinetics*

Microalgae growth was followed by optical density (OD) at 750 nm and used for dry weight estimation (*X*, g. L^{-1}) according to the following calibration curve (1):

$$X = 1.0702 \times OD + 0.069 (R^2 = 0.993) \quad (1)$$

Dry cell weight was measured by gravimetric determination, where 2 ml of culture was filtered through GF/C filter paper (Whatman, Maidstone, UK), washed with equal volume of water, and dried at 105 °C for 24 h.

Biomass productivity (P, g. $L^{-1}.d^{-1}$), was used to analyze the performance of microalgae subject to the different conditions tested, being obtained by Eq. (2):

$$P = (x_2 - x_1)/(t_2 - t_1) \quad (2)$$

where x_1 and x_2 are the biomass concentration at the time points t_1 and t_2, respectively.

2.4. *Glucose concentration measurement*

To evaluate the amount of glucose present in the growth medium at the end of the assay, an adaptation of the Bernfeld method (DNS Reagent Method) was used (Bernfeld, 1955). D-Glucose solution was used as standard. The DNS solution was composed of 10 g.L^{-1} of 3,5 – Dinitrosalicylic Acid and 300 g.L^{-1} of Sodium Potassium Tartrate. 500 µl of supernatant resulting from microalgae cultures (Section 2.2.) were mixed with 500 µl of DNS solution. Samples were incubated at 100 °C for 5 min. After cooling, these mixtures were measured at 540 nm in 96-well plates using a microplate absorbance reader Synergy™ HT Multi-detection Microplate Reader (Bio-Tek Instruments, Inc., USA).

2.5. *Biochemical characterization*

2.5.1. *Protein quantification*

Total nitrogen content was analyzed using the Kjeldahl method. Nitrogen content was determined after acid digestion using a digestion block (TecatorTM Digestor 2508, FOSS, Denmark). In this digestion, 500 mg of lyophilized biomass was used to initiate the treatment. A conversion factor of 6.25 was then applied to estimate crude protein content from total nitrogen (Gougoulias et al., 2022; Xie et al., 2017).

2.5.2. *Lipid quantification*

The extraction of lipids from lyophilized biomass was performed following the Bligh and Dyer method with some modifications (Bligh and Dyer, 1959). 1 ml of a mixture of solvents dichloromethane/methanol (2:1 v/v) was added to 10 mg of lyophilized biomass. This mixture was vortexed for 2 min and then centrifuged for 10 min at 2000 rpm (Hettich Mikro 120, Tuttlingen, Germany). The organic phase was pipetted to a pre-weighed glass tube and the biomass residue was re-extracted 2 more times. After this, the resultant organic phase was dried under a stream of nitrogen gas. To remove the non-lipid contaminants, the initial extract was re-dissolved in a mixture of dichloromethane, methanol, and water, using 2 ml, 1 ml, and 0.75 ml respectively. The mixture was centrifuged again. The organic phase was pipetted to a new pre-weighed tube. To finish the procedure, the organic phase was dried under nitrogen stream and weighed.

2.5.3. *Carbohydrate quantification*

The method used to quantify carbohydrates was performed according (Castro-Ferreira et al., 2022), determining structural carbohydrates and acid-insoluble residue in biomass, where glucuronic acid, glucose, xylose, arabinose, mannose, and fucose were used as standard (0.2–2.5 g.L^{-1}). 300 mg of lyophilized pellets (Section 2.2.) was subjected to a two-step acid hydrolysis. In the first step, 3 ml of sulfuric acid, H_2SO_4 (72%), was added to the pellet, in a bath at 30 °C for 1 h, under manual stirring. In the second step, a dilution to 4% H_2SO_4 was made to the first hydrolysate and treated for 1 h at 121 °C in autoclave (Sanyo Labo Autoclave MLS3020, Japan). Acid insoluble lignin was gravimetrically determined after vacuum filtration using crucibles (Gooch crucibles porosity grade 3) and dried overnight at 105 °C. The remaining autoclaved hydrolysis solution was filtered (0.22 µm FilterBio® PES syringe filter) and analysed by high-performance liquid chromatography (HPLC). The conditions used in the HPLC analysis were as follows: refractive Index (RI) detector, Aminex HPX-87 H column at 60 °C with a mobile phase of 0.005 mol.L^{-1} H_2SO_4 at a flow rate of 0.6 ml.min^{-1}.

2.5.4. *Ash content*

Total ash was determined by the weigh difference before and after the combustion of the biomass. The biomass was placed in small ceramic cups with a pre-determined weight (50 mg of biomass) and heated for 8 h at 550 °C using a muffle furnace (Nabertherm N3P, Lilienthal, Germany). The combustion resultant was weighed again (Trovão et al., 2020).

2.6. *Statistical analyses*

The experiments of validation test (Section 2.2.3.) were performed in triplicate. Mean values and standard errors were calculated from triplicates and used in corresponding tables

and graphical representations (Table 7 and Fig. 2). Statistical analyses of experimental data were performed using the GraphPad Prism 8.0.2 software (Dotmatics, UK). One-way analysis of variance (ANOVA), coupled with Tuckey's post hoc test, was used to determine any statistically significant differences at a confidence level of 95% between mean values of the biomass concentration and biochemical characterization obtained under different culture conditions.

3. Results and Discussion

3.1. *Screening of the 24 independent variables*

The growth of microalgae is influenced by several abiotic factors, such as nutritional and environmental parameters. Therefore, if optimized, these parameters are able to significantly enhance biomass production. Taking into account these facts, a PBD28 was applied in order to assess the impact of 24 independent variables on *C. vulgaris* grown under heterotrophic conditions and select the meaningful ones. Consequently, all the nutrients present in the FERM_MB medium (20 compounds), along with temperature, agitation speed, pH, and initial inoculum concentration, were tested, while maximum biomass concentration, X_{max}, and productivity, P_{max}, were considered and analyzed as response variables. The assays performed to assess the combined effect of these 24 factors on growth-related parameters are shown in Table 3.

The results have shown a considerable variation of the two dependent variables considering the different combinations of operational and nutritional parameters tested. In this sense, the highest X_{max} – 18.70 $g.L^{-1}$ – was observed in run number 9, followed by run number 24, with the maximum concentration of 14.81 $g.L^{-1}$. Regarding the minimum values of X_{max} obtained in this matrix PBD28, runs number 18 and 7 presented a biomass concentration of 0.17 $g.L^{-1}$ and 0.20 $g.L^{-1}$, respectively. Besides these two runs, other combinations of the studied parameters (i.e., 8, 10, 11, 14, and 27) led to a X_{max} below 1 $g.L^{-1}$, suggesting that the nutrient conditions applied might have caused inhibition of *C. vulgaris* growth either by shortage or excess of some elements concentration (Gonçalves et al., 2017; Sakarika and Kornaros, 2017). With respect to the P_{max}, this variable presented a 53.4-fold variation, ranging between 0.07 $g.L^{-1}.d^{-1}$ and 3.74 $g.L^{-1}.d^{-1}$. This relatively significant variation was clearly influenced by the limited growth obtained using several cultivation conditions of the PBD28, as stated previously.

Considering the statistical analysis summarized in Table 4, increasing the nutrients available in the medium has shown, in several cases, positive effects on the growth of microalgae; however, if the nutrient concentration reaches a certain value, it can cause inhibitory effects due to nutrient overload. This was also possible to observe in the present study, since some of the constituents of the medium had a negative effect on the biomass. A study carried out by Li et al. (2018) evaluated the effect of excessive amount of P on the heterotrophic growth of *Chlorella regularis*. No considerable changes were detected on both cells' growth and viability using concentrations ranging from 5.4 to 45 mg-$P.L^{-1}$ – in the present work, P concentration varied between 15.9 and 55.2 mg-$P.L^{-1}$. However, for higher amounts, the authors observed a slight decrease in cell density when using 150 mg-$P.L^{-1}$, while a drastic reduction (of approximately 40%) was reported for the greatest concentration applied (250 mg-$P.L^{-1}$). In summary, the authors concluded that higher P

Table 4 – Estimated effects of each independent variable tested on the response variables X_{max} and P_{max}. The symbol * represents the factors with p-value ˂0.1.

Factor	X_{max}				P_{max}			
	Effect	S.D	t-value	*p*-value	Effect	S.D	t-value	*p*-value
Mean	6.08	0.54	11,30	0.0001	0.06	0.01	11.31	0.0001
Curvature	7.72	3.46	2.23	0.0760 *	0.11	0.03	3.06	0.0279 *
T	1.36	1.08	1.27	0.2614	0.01	0.01	1.14	0.3058
pH	-2.04	1.08	-1.90	0.1163	-0.03	0.01	-2.74	0.0406 *
Agitation speed	-0.16	1.08	-0.15	0.8894	0.00	0.01	-0.01	0.9899
Initial OD	1.17	1.08	1.09	0.3269	0.02	0.01	1.88	0.1184
K_2HPO_4	-1.68	1.08	-1.56	0.1787	-0.01	0.01	-0.78	0.4694
$NaH_2PO_4{\cdot}H_2O$	0.45	1.08	0.42	0.6931	-0.01	0.01	-0.85	0.4348
$(NH_4)_2SO_4$	-4.36	1.08	-4.05	0.0098 *	-0.03	0.01	-2.72	0.0419 *
$MgSO_4{\cdot}7H_2O$	2.99	1.08	2.78	0.0390 *	0.03	0.01	2.61	0.0475 *
$C_6H_8O_7{\cdot}H_2O$	0.46	1.08	0.43	0.6868	0.00	0.01	0.41	0.6980
$CaCl_2{\cdot}2H_2O$	-1.51	1.08	-1.40	0.2207	-0.02	0.01	-1.52	0.1878
$C_6H_{12}O_6$	3.84	1.08	3.57	0.0160 *	0.00	0.01	0.16	0.8798
$CuSO_4{\cdot}5H_2O$	-1.07	1.08	-0.99	0.3669	-0.01	0.01	-0.60	0.5767
H_3BO_3	-2.02	1.08	-1.87	0.1197	-0.02	0.01	-2.03	0.0983 *
$ZnSO_4{\cdot}7H_2O$	-1.53	1.08	-1.42	0.2141	-0.02	0.01	-1.47	0.2010
$MnSO_4{\cdot}H_2O$	-0.19	1.08	-0.18	0.8650	0.00	0.01	0.03	0.9799
$Na_2MoO_4{\cdot}H_2O$	1.59	1.08	1.47	0.2006	0.02	0.01	1.63	0.1638
$NiCl_2{\cdot}6H_2O$	-0.55	1.08	-0.51	0.6319	0.00	0.01	-0.44	0.6799
$FeSO_4{\cdot}H_2O$	-0.63	1.08	-0.59	0.5838	0.00	0.01	0.05	0.9598
Thiamine-HCl	-1.86	1.08	-1.72	0.1453	-0.02	0.01	-1.59	0.1724
D-Biotin	-1.13	1.08	-1.05	0.3416	0.00	0.01	-0.46	0.6621
Cyanocobalamin	0.38	1.08	0.35	0.7374	0.01	0.01	0.62	0.5605
Calcium Pantothenate	-0.89	1.08	-0.83	0.4467	-0.01	0.01	-1.27	0.2590
p-Aminobenzoic acid	1.87	1.08	1.73	0.1436	0.02	0.01	1.70	0.1504
PIPES Buffer	2.07	1.08	1.92	0.1130	0.01	0.01	1.23	0.2723

Table 5 – Glucose consumption in each assay of the matrix PBD28.

Trial number	Glucose consumption (%)	Trial number	Glucose consumption (%)	Trial number	Glucose consumption (%)
1	86.66 ± 0.61	**12**	77.26 ± 0.96	**23**	94.33 ± 0.62
2	98.54 ± 0.00	**13**	97.41 ± 0.26	**24**	86.30 ± 0.68
3	98.74 ± 0.14	**14**	1.90 ± 2.27	**25**	98.58 ± 0.07
4	98.71 ± 0.07	**15**	98.84 ± 0.12	**26**	36.10 ± 2.33
5	98.72 ± 0.05	**16**	96.62 ± 0.58	**27**	0.00 ± 0.00
6	64.79 ± 1.60	**17**	67.95 ± 0.21	**28**	99.06 ± 0.43
7	9.03 ± 2.93	**18**	0.00 ± 0.00	**CP1**	98.18 ± 0.20
8	0.00 ± 0.00	**19**	41.16 ± 0.88	**CP2**	98.33 ± 0.13
9	98.32 ± 0.13	**20**	0.00 ± 0.00	**CP3**	98.25 ± 0.08
10	0.00 ± 0.00	**21**	97.82 ± 0.12		
11	1.37 ± 1.38	**22**	97.53 ± 0.16		

concentrations might inhibit growth and hinder the uptake of C and N sources, inflicting severe morphological damages on microalgae cells. On the contrary, Shrestha et al. (2020) analyzed the effect of N concentration in heterotrophic growths of *Chlorella kessleri* – concentration ranged between 0 and 30 mM – and observed that growth limitation occurs – being almost null – when the amount of N in the medium was lower than 0.3 mM. N–limiting strategies are commonly used to increase lipid yields since, under N limitation, algal cells utilize N to synthesize functional proteins and C to make carbohydrates and lipids (Richardson et al., 1969; Sakarika and Kornaros, 2017). However, this approach usually comprises a reduction of the maximum biomass concentration (Shrestha et al., 2020). Although the excess or limitation of nutrients is commonly associated with negative effects in microalgae growth, other works indicate that stresses induced by shortage of some elements might, in fact, enhance biomass concentration and productivity. During the limitation of sulfur (S) element in heterotrophic cultures of *C. vulgaris*, for instance, Sakarika and Kornaros (2017) reported a X_{max} of 2.69 g.L.$^{-1}$, while low P and N concentrations resulted in a X_{max} of 9.81 and 11.12 g.L.$^{-1}$, respectively. Based on their results, the authors therefore concluded that high density cultures can be achieved with low P and/or N concentrations, as happened in the case of runs 9 and 24.

In order to choose which factors were more significant, the *p*-value was analyzed. In this sense, three factors screened for the biomass production of *C. vulgaris* – X_7 ($(NH_4)_2SO_4$), X_8 ($MgSO_4{\cdot}7H_2O$), and X_{11} ($C_6H_{12}O_6$) – had significant effect ($p < 0.1$) on X_{max}, with a confidence level of 90%. In heterotrophic growths, the organic carbon source is one of the dominant factors to produce high density cultures. Therefore, one of the parameters evaluated in this study was the glucose consumption in each growth. At the end of each trial, a quantification of the remaining glucose in the culture medium was carried out (Table 5). In general, glucose was totally consumed in the assays where lower initial concentrations of glucose (50% w/v) were tested (19.98 ml.L^{-1}). On the other hand, it is possible to conclude that most of the experiments with the highest initial glucose concentration (59.95 ml.L^{-1}), presented greater non-consumed glucose – which might also be connected to the lack of other nutrients in the growth medium, hindering glucose uptake by microalgae. Nonetheless, analyzing the results of matrix PBD28 (Table 3), one can clearly see that the highest X_{max} values were obtained when higher glucose amounts were provided to the cultures, as the case of runs number 9, 16, and 24. However, a study performed by Liang et al. (2009) observed an inhibitory effect of glucose on the growth of *C. vulgaris* for concentrations higher than 10 g.L^{-1} (1% w.v^{-1}). This limitation was not verified in the present study, where the glucose concentration applied ranged from 10 g.L^{-1} (corresponding to level "–1" of PBD28) to 30 g.L^{-1} (corresponding to level "1″ of PBD28). Similarly, other studies devoted to the optimization of heterotrophic media have shown that the growth of *Chlorella* species was not inhibited when these microalgae were supplemented with high concentrations of glucose (30 – 40 g.L^{-1}) (Isleten-Hosoglu et al., 2012; Jin et al., 2021; Kim et al., 2019). Since the highest glucose concentration did not appear to be inhibitory to the *C. vulgaris* used in the present study and this parameter has shown a significant positive effect on X_{max}, the values selected to be tested in the optimization phase, using the CCDR methodology (Section 3.2.), were higher.

Regarding the P element, obtained by the microalgae through the K_2HPO_4 and $NaH_2PO_4{\cdot}H_2O$ present in the medium, it is a vital nutrient for cell survival that cannot be replaced by any other element, being essential for many physiological and biological processes (Su, 2021; Wu et al., 2021). However, in the present study, K_2HPO_4 showed a negative effect over cell concentration; Jeon et al. (2014) obtained similar results during their optimization process of the BG11 medium for heterotrophic growth of *C. vulgaris* as the same compound presented negative effects as well. The excess of P ions was previously shown to compete with iron and manganese uptake by plants (Heintze, 1968; Welter et al., 2013) – which is also likely occur in microalgae. Being these two elements essential to their growth (Liu et al., 2018), this can explain the negative effect of K_2HPO_4 over the X_{max} (Table 4). In the case of $MgSO_4{\cdot}7H_2O$, one of the significant factors (Table 4), Jeon et al. (2014) have reported significant and positive effects of its concentration on cell concentration, which is in agreement with the results obtained in the present study.

Concerning the other response variable, P_{max}, the statistical analysis (Table 4) showed that four factors had a significant effect ($p < 0.1$): X_2 (pH), X_7 ($(NH_4)_2SO_4$), X_8 ($MgSO_4{\cdot}7H_2O$), and X_{13} (H_3BO_3). Among these factors, three showed a negative effect, X_2, X_7, and X_{13} (effect of –0.03 for the first two variables and –0.02 for the last one), indicating that an increase in their value/concentration can lead to a decrease of P_{max}. The other factor, X_8, had a positive effect (of 0.03) on P_{max}. One of the strategic areas for improving microalgae production is to focus on the interrelation of nutrients with biomass and the accumulation of some target metabolite. Macronutrients have to be present in the medium in large quantities while micronutrients, even in much lower amounts, are essential for the maintenance of

the microorganism (Ghafari et al., 2018). Relating the significant variables obtained for the two responses analyzed (X_{max} and P_{max}), two of them coincided and obtained the same effect (a negative effect in the case of $(NH_4)_2SO_4$ and a positive effect of $MgSO_4{\cdot}7H_2O$). Additionally, besides pH, another nutrient appeared with a significant effect over P_{max}, the boron element (B) – through H_3BO_3. A research work carried out by Yan et al. (2022) studied the effect of B on the heterotrophic biomass production of *Chlorella regularis* in order to develop a new biological methodology for the removal of this element from industrial wastewater. The authors observed that this element had a negative effect since an increase of its concentration induced a decrease both in the maximum cell concentration and the growth rate. This negative effect is in agreement with the results obtained in the present study. Despite the negative effect observed, the presence of B in the culture medium is of the utmost importance since this element plays a significant role in the enzymatic mechanism of microalgae, bonding mainly to proteins and carbon-containing components depending on the level of stress (Yan et al., 2022).

As the objective of the PBD28 was to determine which physical parameters and/or nutrients concentration were most significant in the growth of *C. vulgaris*, considering the discussion throughout this Section (3.1.) and the fact that the "curvature" for P_{max} (shown in Table 4) had the lowest *p*-value among all the parameters analyzed – indicating that the optimal point is close to the conditions applied in the CPs (level "0") –, it was decided to proceed with the optimization step (Section 3.2.) solely taking into account the response X_{max}, having the concentrations of $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and $C_6H_{12}O_6$ as the independent variables under study. Furthermore, it was also analyzed which variable had a positive or negative effect in order to readjust the range of concentrations to be applied in the CCRD. Thus, if the effect was negative, the values to be tested in the modulation would be lower than those applied in the PBD28; on the other hand, if the effect was positive, the values would be greater. As all the other factors had no significant effect on biomass production, their nutrients concentrations and values (in the case of physical parameters) were kept constant and at an intermediate concentration/level (the same applied for the CPs of PBD28 – Table 1) during the optimization process (Section 3.2.).

3.2. *Optimization process of the significant variables*

As mentioned in the previous section, after the screening step of all the 24 independent variables was concluded, the factors that have shown significant impact over X_{max} were selected – concentrations of $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and $C_6H_{12}O_6$ – and underwent an optimization process based on CCRD methodology. CCRD was therefore utilized as a means of determining which conditions (considering these 3 nutrients) should be applied in order to maximize biomass concentration. Each independent variable was tested at 5 coded levels – "– 1.68″, "– 1", "0", "1″, and "1.68″ –, resulting in 14 different combinations plus 4 CPs, as shown in Table 6.

The X_{max} varied considerably in this set of experiments, being the lowest obtained in run number 9 (3.17 $g.L^{-1}$) and the highest in run 10 (20.37 $g.L^{-1}$). Analyzing the conditions applied in these two runs, it is possible to observe that the main difference was in the concentration of $(NH_4)_2SO_4$, since the other variables under study remained at the intermediate point of the CCRD matrix (level "0") (Table 6). The concentration of $(NH_4)_2SO_4$ in run number 9 was at the level "– 1.68″ while in run number 10 it was at level "1.68″, suggesting that the concentration at the lowest level (0.01 $g.L^{-1}$, corresponding to 1.53 $ml.L^{-1}$) was insufficient for a significant growth of *C. vulgaris*. Regarding the glucose, the highest concentration applied in this matrix (level "1.68″ with concentration of 54.32 $g.L^{-1}$, corresponding to 108.64 $ml.L^{-1}$) did not induce a higher X_{max}, probably indicating a nutritional overload of this compound. The C/N molar ratio is an important parameter and one of the most critical nutritional factors to the heterotrophic growth of microalgae, since it can influence all the growth-related parameters and the biosynthesis of some metabolites (Abreu et al., 2022; Gao et al., 2021; Jin et al., 2021). Overall, the ratios applied in each assay ranged from 17:1–383:1. However, for the trials with the

Table 6 – Matrix of the CCRD with coded values of each independent variable and the corresponding Xmax and glucose consumption obtained.

Trial Number	Independent Variables			Response	Glucose consumption (%)
	$(NH_4)_2SO_4$	$MgSO_4{\cdot}7H_2O$	$C_6H_{12}O_6$	X_{max} ($g.L^{-1}$)	
1	-1	-1	-1	12.34	69.57 ± 1.47
2	1	-1	-1	18.38	98.92 ± 0.06
3	-1	1	-1	10.19	66.11 ± 1.65
4	1	1	-1	18.80	98.91 ± 0.05
5	-1	-1	1	9.37	22.74 ± 3.81
6	1	-1	1	18.10	80.01 ± 3.18
7	-1	1	1	7.36	10.67 ± 1.99
8	1	1	1	19.87	57.11 ± 0.63
9	-1.68	0	0	3.17	8.42 ± 3.70
10	1.68	0	0	20.37	93.64 ± 0.09
11	0	-1.68	0	17.69	79.95 ± 0.37
12	0	1.68	0	16.92	77.17 ± 1.05
13	0	0	-1.68	13.70	99.23 ± 0.05
14	0	0	1.68	13.43	19.55 ± 7.19
CP1	0	0	0	20.30	62.99 ± 0.78
CP2	0	0	0	19.58	79.39 ± 1.70
CP3	0	0	0	16.90	75.45 ± 0.88
CP4	0	0	0	19.68	82.28 ± 8.27

lowest X_{max} (i.e, runs numbered as 1 (57:1), 3 (57:1), 5 (99:1), 7 (99:1), 9 (383:1), and 14 (52:1)), the C/N ratios were higher than 50. In a study reported by Jin et al. (2021), using heterotrophic cultures of *Chlorella sorokiniana*, different ratios were tested, being the optimum 16:1. Higher ratios (> 30) resulted in slower biomass growth with lower maximum biomass concentration and greater amounts of unconsumed glucose. Similar results were obtained by Singhasuwan et al. (2015) since three different C/N ratios – 29:1, 63:1, 95:1 – were applied for the growth of *Chlorella* sp. and the 29:1 was the one where the highest biomass production was obtained. On the contrary, an increase of the ratio towards the highest value tested led to a reduction by more than 50% as consequence of N limitation, following the same logic of the authors of the previous study (Jin et al., 2021). Comparing with the results of the present study, the assay that reached the highest X_{max} (run number 10) applied a C/N molar ratio of 19:1, which is aligned with the results of the previously discussed works.

Regarding the $MgSO_4{\cdot}7H_2O$, all microalgae species have an absolute need for this element since it is a major source of S for proteins and chlorophyll and also magnesium for chlorophyll. In the case of S, it is associated with a carbon-fixation enzyme (i.e., Rubisco) and thus biomass production, which may be connected to the need of increasing the concentration of $MgSO_4{\cdot}7H_2O$ in the growth medium (Jeon et al., 2014). Proving this fact, $MgSO_4{\cdot}H_2O$ had a positive effect on PBD28 (Table 4) and the optimum point predicted by the model (Eq. (3)) was close to level "1", one of the highest tested concentrations during the CCRD methodology (Table 2). Furthermore, given the results obtained, this compound showed a significant and positive synergistic effect with $(NH_4)_2SO_4$. Another positive synergistic effect obtained in this modeling was between $(NH_4)_2SO_4$ and $C_6H_{12}O_6$. These two compounds are strongly interconnected in the heterotrophic growth of microalgae since they are two of the main elements in their nutrition, being the C/N ratio commonly selected in order to maximize cell growth or some specific metabolite, as discussed earlier.

Based on the results from CCRD experiments (Table 6), it was possible to identify the most suitable conditions in order to favor *C. vulgaris* growth under heterotrophic conditions, as shown in Fig. 1.

As a result, the optimal growth conditions predicted by the model were the following: 25.50 $ml.L^{-1}$ of $(NH_4)_2SO_4$, 64.60 $ml.L^{-1}$ of $MgSO_4{\cdot}7H_2O$, and 75.00 $ml.L^{-1}$ of $C_6H_{12}O_6$. Additionally, considering a confidence level of 90% (p-value < 0.1), it was also able to determine the significant variables of the process, as well as synergistic interactions between them, and, consequently, set the model's Eq. (3):

$$X_{max} = 19,10 + 4,75x_1 - 2,51x_1^2 - 0,56x_2^2 - 1,88x_3^2 + 0,80\,x_1x_2 + 0,82x_1x_3 \quad (3)$$

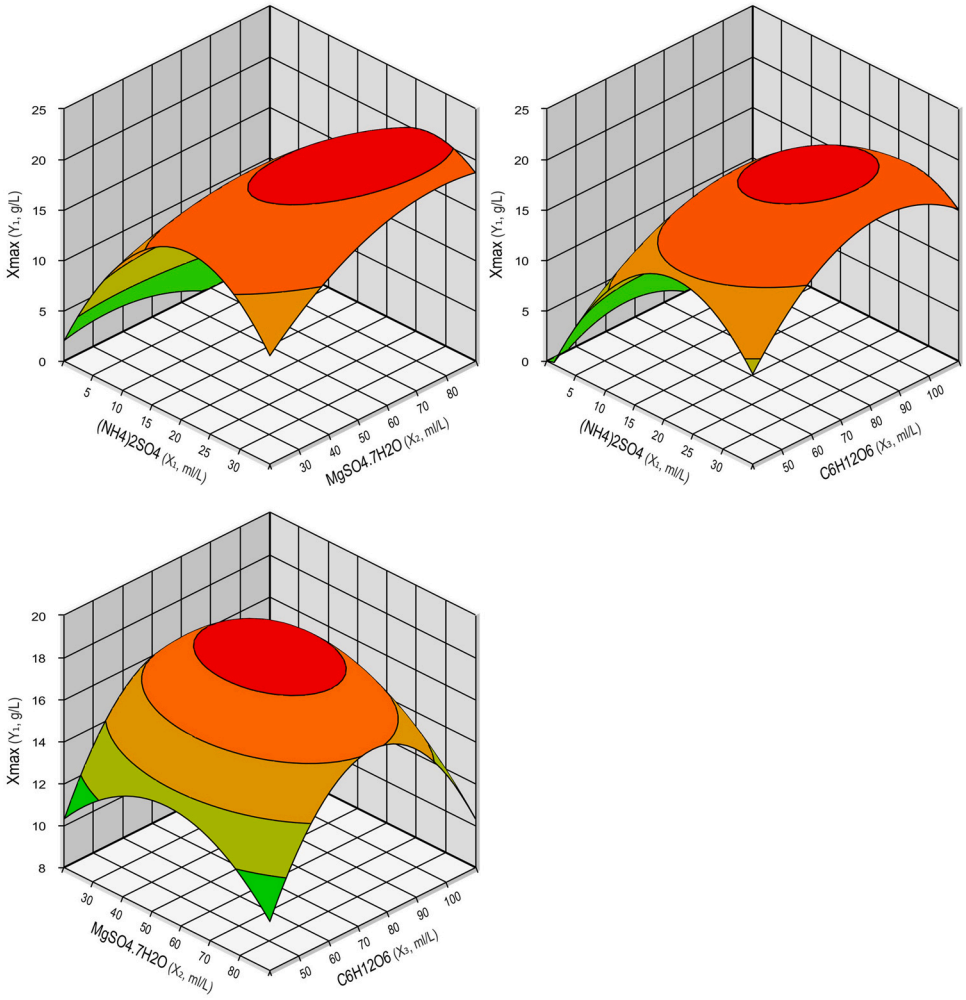

Fig. 1 – Three-dimensional surface plots for X_{max} showing the interactive effects of the tested independent variables on the heterotrophic growth of C. vulgaris. (A) $(NH_4)_2SO_4$ vs $MgSO_4{\cdot}7H_2O$; (B) $(NH_4)_2SO_4$ vs $C_6H_{12}O_6$; (C) $MgSO_4{\cdot}7H_2O$ vs $C_6H_{12}O_6$.

where x_1, x_2, and x_3 represent the concentrations of $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and $C_6H_{12}O_6$, respectively.

The coefficient of determination (R^2) was applied as an indicator to demonstrate the preciseness of models for experimental data. The model developed in the present study exhibited a R^2 of 97.01%, meaning that 97.01% of the variance of X_{max} was explained by the independent variables tested. This was an excellent result since a regression model with a high R^2, above 90%, is considered to have a strong significant correlation (Saengwong et al., 2018).

3.3. *Validation of the model*

The optimization of culture conditions for the growth of *C. vulgaris* using the CCDR design provided an efficient medium by combining the nutrients with the most significant effects. Thus, to confirm the goodness of the model obtained, the biomass production was validated experimentally by performing a trial, in triplicate, under the optimal predicted medium composition by the model (Eq. (3) and Fig. 1).

3.3.1. *Biomass production enhancement*

According to the model represented by Eq. (3), the maximum biomass concentration of *C. vulgaris* under the optimized conditions was estimated to be 21.62 ± 0.77 $g.L^{-1}$, as shown in Fig. 2. Experimentally, using the optimized growth medium, a maximum biomass concentration of 20.10 ± 0.84 $g.L^{-1}$ was obtained. Statistically, no significant differences were found, demonstrating that this result was in agreement with the model's prediction (Fig. 1). On the other hand, under Allmicroalgae's original conditions, containing 44.74 $ml.L^{-1}$ of $(NH_4)_2SO_4$, 26.65 $ml.L^{-1}$ of $MgSO_4{\cdot}7H_2O$, and 39.96 $ml.L^{-1}$ of $C_6H_{12}O_6$, the X_{max} did not go beyond 10.07 ± 0.11 $g.L^{-1}$. Comparing the performance of *C. vulgaris* using the optimized and the Allmicroalgae's conditions, it is possible to observe that, through this two-step optimization strategy, an increment of 99.60% has occurred in maximum biomass concentration, resulting from an increase of 88% and 142% for $C_6H_{12}O_6$ and $MgSO_4{\cdot}7H_2O$, respectively, and a decrease of 76% for $(NH_4)_2SO_4$. Another important aspect of this optimization was the confirmation that all the glucose was consumed, both in the optimized medium and in the FERM_MB medium – (99.40 ± 0.02)% and (99.35 ± 0.02)%, respectively –, indicating that the supplemented concentration did not cause any negative effect on growth.

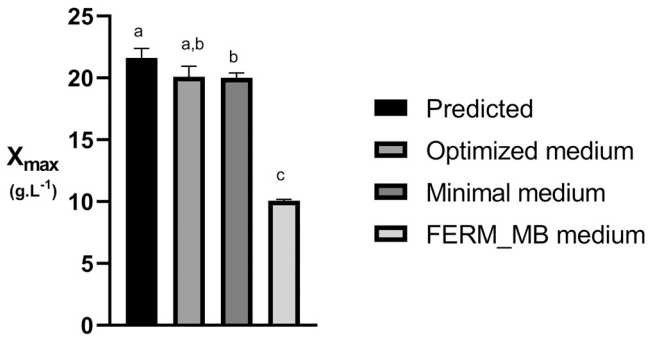

Fig. 2 – Maximum biomass concentration, X_{max}, predicted by the model using the optimal conditions and obtained experimentally for the different conditions tested in the validation step (optimized, minimal, and FERM_MB media). Different letters indicate significant differences between the values ($p < 0.05$).

However, to make microalgae production more economically viable, it is necessary to reduce, for example, the production costs associated with the growth medium to be used. Therefore, in addition to the validation of the optimal conditions predicted by the model, another experimental condition was evaluated (minimal medium). Maintaining the optimal concentration of the three significant compounds ($(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, and $C_6H_{12}O_6$), a growth was tested (in triplicate) with the reduction of the concentration of all the non-significant compounds of the medium that have shown a negative effect over X_{max} (Table 4) to the minimum values tested in the PBD28 (level "–1") (Table 1). Through this assay, a maximum concentration of 20.02 ± 0.39 $g.L^{-1}$ was obtained (Fig. 2). Statistically, this concentration did not show significant differences ($p > 0.05$) when compared to the X_{max} attained experimentally for the optimized medium, suggesting that the reduction of the concentration of non-significant macro- and microelements is possible without compromising the heterotrophic growth of *C. vulgaris*. Regarding glucose consumption, this was also fully consumed. This is a particularly interesting information for industrial-scale processes since the reduction of these compounds might represent an improvement of the process cost-effectiveness and, eventually, greater profit.

3.3.2. *Impact on biochemical composition of C. vulgaris biomass*

To evaluate the impact of the three conditions tested for model validation – optimized, FERM_MB, and minimal media – on *C. vulgaris* composition, a biochemical characterization was performed, as shown in Table 7.

Regarding protein content, both the optimized and minimal media have presented a reduction – of 57% and 60%, respectively – when compared to FERM_MB medium, with statistically significant differences found between all the conditions tested ($p < 0.05$). Analyzing the three media applied in the validation phase (Section 3.3.), a significant reduction of the amount of the supplied N was imposed in the case of both the optimized and minimal media. Since the element N is a major component of proteins and its availability in the culture medium, as well as the balance with C (in this case, organic C (glucose)), are relevant factors for protein accumulation (Abreu et al., 2022; Fernandes et al., 2016; Ji et al., 2014), this can explain the decrease of the protein content – in comparison to the FERM_MB medium. The studies conducted by Xie et al. (2017) and Ji et al. (2014) have also reported that *C. vulgaris* synthesizes more protein when growing in media with higher amounts of N source, reducing its biosynthesis in N-limiting environments. The possible reason for this decrease in protein synthesis can be attributed to the fact that N-limited microalgal cells generally accumulate reserve metabolites, such as carbohydrates and lipids, rather than proteins (Xie et al., 2017). Another reason for these low protein concentrations is related to the nutritional mode used in the present study (i.e., heterotrophy). As mentioned in Section 1, the energy densities of organic C sources (e.g., glucose) are relatively higher than, for example, an inorganic source (CO_2). This increase in energy density can induce an excess of energy in the biological system favoring an accumulation of reserve metabolites – because excess organic C and energy are redirected to lipid and carbohydrates accumulation instead of protein accumulation – without compromising microalgae growth (Abreu et al., 2022). Furthermore, the goal of the present study was to

Table 7 – Biochemical composition of Chlorella vulgaris grown in different media. Data are reported as means ± standard deviation of triplicates. Different letters represent significant differences between values within the same column ($p < 0.05$).

Growth medium	Protein (% (w/w))	Carbohydrates (% (w/w))	Lipids (% (w/w))	Ash (% (w/w))
Optimized medium	17.86 ± 0.31[a]	50.45 ± 0.13[a]	15.08 ± 0.43[a]	6.50 ± 0.26[a]
FERM_MB medium	41.70 ± 0.28[b]	34.09 ± 1.13[b]	21.56 ± 1.53[b]	10.37 ± 0.27[b]
Minimal medium	16.63 ± 0.17[c]	49.59 ± 0.95[a]	13.71 ± 1.21[a]	5.17 ± 0.16[c]

maximize biomass growth rather than protein (or other metabolites of interest) production and, thus, biomass harvesting was just performed after reaching X_{max} (i.e., at stationary phase), which is clearly beyond the production peak of certain compounds (e.g., protein).

Analyzing the carbohydrates content, both the optimized (50.45 ± 0.13%) and minimal (49.59 ± 0.95%) media showed significant higher concentrations compared to the FERM_MB medium (34.09 ± 1.13%) ($p < 0.05$). Between the optimized and the minimal medium, no statistically significant differences were observed ($p > 0.05$). On the other hand, the lipid content of the biomass grown in the FERM_MB medium obtained the highest value (21.56 ± 1.53%), with statistically significant differences ($p < 0.05$) when compared to the other media, once the optimized and minimal media have induced a reduction of approximately 30% and 36%, respectively – no statistically significant differences were found between them. As mentioned before, in the presence of excessive organic C and energy, metabolites synthesis is directed towards the accumulation of lipids and carbohydrates, being the main strategies applied for the purpose either nitrogen and phosphorus starvation or carbon enhancement (Fernandes et al., 2016; Palmucci et al., 2011). Given the low concentration of protein in the biomass and the sum of both carbohydrates and lipids content (reserve metabolites) in the optimized and minimal media (≈ 66% and 63%, respectively), this phenomenon is likely to have occurred in the present study. In both media, the reduction of N concentration and increase of C amount enhanced carbohydrates production and decreased lipid content, showing that these conditions tend to favor the metabolism of *C. vulgaris* towards carbohydrates accumulation.

Finally, concerning the amount of ash, all the media presented statistically significant differences ($p < 0.05$), with the FERM_MB medium showing the highest content, followed by the optimized medium and lastly the minimal medium. This result was somehow expected, particularly in the case of the minimal medium, as its composition has a poorer concentration in terms of inorganic compounds – as consequence of the reduction of some non-significant compounds concentrations – comparing to the other media.

Based on the aforementioned results, the minimal medium may still be a good alternative to the industrial sector due to the significant reduction of the concentration of compounds present in the culture medium (i.e., lower production costs) and no loss in biomass quantity and quality (when compared to the optimized medium).

4. Conclusions

The two-step optimization process, comprising the simultaneous variation of 24 independent variables, allowed determining their impact over growth-related parameters. In the case of X_{max}, three screened factors – X_7 ($(NH_4)_2SO_4$), X_8 ($MgSO_4{\cdot}7H_2O$), and X_{11} ($C_6H_{12}O_6$) – have presented a significant effect ($p < 0.1$), with a confidence level of 90%. The increase of $MgSO_4{\cdot}7H_2O$ and $C_6H_{12}O_6$ concentrations demonstrated the potential to positively affect microalgae growth, whereas a negative impact was found with $(NH_4)_2SO_4$. As for the P_{max}, the statistical analysis showed that four factors played a significant role ($p < 0.1$): X_2 (pH), X_7 ($(NH_4)_2SO_4$), X_8 ($MgSO_4{\cdot}7H_2O$), and X_{13} (H_3BO_3). Among these, the pH, $(NH_4)_2SO_4$, and H_3BO_3 indicated that an increase in their value/concentration could lead to a decrease of P_{max}. On the contrary, $MgSO_40.7H_2O$ had a positive effect on P_{max}, as happened in the case of X_{max}.

The optimization step, solely oriented to the maximization of the biomass production, allowed determining the optimal growth conditions predicted by the model – 25.50 ml.L^{-1} of $(NH_4)_2SO_4$, 64.60 ml.L^{-1} of $MgSO_4{\cdot}7H_2O$, and 75.00 ml.L^{-1} of $C_6H_{12}O_6$ – and defining the model's equation (Eq. (3)), through which was possible to find out that the $(NH_4)_2SO_4$ (both linear and quadratic terms), the $MgSO_4{\cdot}7H_2O$ and the $C_6H_{12}O_6$ (quadratic terms), and the synergistic interactions between $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$ and between $(NH_4)_2SO_4$ and $C_6H_{12}O_6$ were statistically significant to X_{max}. By applying these optimal conditions, the X_{max} obtained doubled when compared to FERM_MB medium, attaining a similar value to the minimal medium. With these results, the optimization process has proven to be a success since it has substantially improved the biomass concentration of microalgal culture, the main goal of the present work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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