Sustainable Food Technology

ROYAL SOCIETY
OF **CHEMISTRY**

PAPER
Pedro Geada *et al.*
Optimization of *Pavlova gyrans* biomass production and the
fatty acid profile using a two-step approach

Check for updates

Optimization of *Pavlova gyrans* biomass production and the fatty acid profile using a two-step approach†

Filipe Maciel, [ID] [a] Daniela Couto, [ID] [bc] Pedro Geada,*[ad] Hugo Pereira,[e] José Teixeira,[ad] M. Rosário Domingues,[bc] Joana Silva[f] and António Vicente [ID] [ad]

This work aimed to optimize and characterize the biomass production of *Pavlova gyrans* through a sequential multivariate approach. Among seventeen cultivation parameters, light intensity, $NaNO_3$, and $CuSO_4 \cdot 5H_2O$ were identified as the most significative factors ($p < 0.10$) for biomass productivity. Together with $NaH_2PO_4 \cdot H_2O$, these variables were further studied in order to maximize the biomass production of *P. gyrans*, with the optimum growth conditions identified as 700 $\mu mol_{photons}$ m^{-2} s^{-1} light intensity, 1500 mg L^{-1} $NaNO_3$, 6 μg L^{-1} $CuSO_4 \cdot 5H_2O$ and 40 mg L^{-1} $NaH_2PO_4 \cdot H_2O$. These conditions allowed a 3.8-fold increase in biomass production (2.26 g AFDW per L) and improved the biochemical profile ($p < 0.05$) when compared with the control (Walne's medium). Namely, it was increased the contents of protein (from 10.59 to 30.76% DW), PUFAs (from 37.13 to 47.11% TFA), $n - 3$ FAs (26.49 to 38.27% TFA), and DHA (from 5.73 to 10.33% TFA). The nutritional value of the microalga ($p < 0.05$) was also improved, decreasing its atherogenic (AI – from 1.34 to 0.93) and thrombogenic (TI – from 0.45 to 0.24) indices, while the hypocholesterolemic index (HI) increased from 0.96 to 1.63. This work highlights the importance of different growth conditions in *P. gyrans*, playing a relevant role in the large-scale implementation of this microalga with beneficial nutritional composition for human consumption.

Sustainability spotlight

Microalgae have been highlighted due to their high CO_2 biofixation, rapid growth and ability to thrive under a wide range of growth conditions (*e.g.* wastewater). Moreover, biomass enriched in high added-value compounds shows them as a valuable and contaminant-free source for food and nutraceutical applications. However, large-scale feasibility remains dependent on constraints such as optimization of biomass/biocompound production. In this work, multivariate optimization proved successful, identifying the optimal conditions that improved the biomass production with a higher content of omega-3 fatty acids and protein. Therefore, *Pavlova gyrans* is presented as a promising and sustainable source of nutritional lipids and protein, in line with the United Nations Sustainable Development Goals (SDG 2, 3, 9, 11, 12, 13, and 14).

1. Introduction

Long-chain polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA – C22:6 $n - 3$) and

[a]CEB – Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal. E-mail: pedrogeada@ceb.uminho.pt

[b]CESAM – Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Santiago University Campus, Aveiro, Portugal

[c]Mass Spectrometry Centre, LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Santiago University Campus, 3810-193 Aveiro, Portugal

[d]LABBELS – Associate Laboratory, Guimarães, Braga, Portugal

[e]GreenCoLab – Associação Oceano Verde, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[f]ALLMICROALGAE, Natural Products S.A., R&D Department, Rua 25 de Abril 19, 2445-287 Pataias, Portugal

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3fb00110e

eicosapentaenoic acid (EPA – C20:5 $n - 3$), are essential fatty acids (FAs) with important benefits for human and animal health. Several studies have highlighted the importance of their supplementation in human diet, mainly due to the associated antioxidant and anti-inflammatory properties and protective effect against various diseases,[1–3] as well as acting as an enhancer of the visual and cognitive functions of children.[4]

According to the European Food Safety Authority (EFSA), adults should have a daily intake of 250 to 500 mg of EPA and DHA.[5] Actually, 30% of EPA and DHA for human consumption is accomplished by ingestion of fish and seafood products. Several constraints have been described for these sources, such as the overexploitation of fish stocks, seasonality, lower content of EPA/DHA of aquaculture fish, and inefficient retention and elongation of these FAs in high trophic level organisms.[6,7] Moreover, western countries have been aware of the health consequences of the current diet enriched with pro-

inflammatory $n - 6$ FAs.[8] Thus, with an annual growth rate of 14.7% and an estimated market value of US$ 9.8 billion by 2025, it is crucial to develop and implement new sources of $n - 3$ FAs.[9]

Several oleaginous microalgae (lipid content > 20% w/w of dry weight (DW)) have been identified as a potential source of lipids for biotechnological applications. These microorganisms possess, as advantage, a rapid and controlled growth, higher plasticity allowing for their cultivation under a wide range of environmental conditions, higher productivities of high-added compounds (*e.g.*, $n - 3$ FAs, pigments),[10] and, unlike fish products, a contaminant-free food source.[11]

According to the algae species and nutrient conditions, it's possible to reach a lipid content of 53.2% DW with a $n - 3$ FA content varying from 0.06 to 9.32% DW.[11,12] Pavlovophyceae, a well-known class of microalgae commonly used as feed in aquaculture, have been pointed out as a promising source of EPA (1.8% DW) and DHA (1.32% DW) for nutraceutical and pharmaceutical applications.[13] Moreover, the absence of a cell wall and biomass enriched with biologically active compounds such as carotenoids (*e.g.*, fucoxanthin), essential amino acids, and bioactive sterols (5% DW), reflected on Pavlovophyceae's great bioactivity, digestibility and bioaccessibility, making them a suitable option for human diet and feed.[14–18]

To meet the increasing market demand for these high nutritional species, a thorough understanding of the effects of cultivation conditions on biomass production and the biochemical profile is fundamental, contributing to successfully implement their large-scale production in a cost-effective manner. Some studies have described the impact of salinity,[19] light intensity,[20] carbon source,[20–22] nitrogen availability and source,[15,21,23] phosphorus,[23] and micronutrient supplementation[24–28] on the growth performance and fatty acid profile of Pavlovophyceae species. However, most of these studies addressed the influence of a limited number of growth conditions.

Therefore, this study aimed to carry out a complete evaluation of those conditions on the biomass production and FA profile of the microalga *Pavlova gyrans*. To our knowledge, this is the first study where the growth conditions of this strain were fully optimized regarding its biomass production. For that, a sequential optimization procedure was carried out: (i) screening of growth parameters through a Placket–Burman (PB) design; (ii) optimization of the key factors using a rotatable central composite design (RCCD).

2. Materials and methods

2.1. Microalga and inoculum preparation

The non-axenic microalga strain *Pavlova gyrans* (RCC1553) was grown in a 2 L flat bottom flask with Walne's medium (500 mg L^{-1} $NaNO_3$) and a salinity of 30 psu under continuous aeration with a mixture of air and CO_2 (8 mL min^{-1} – Alicat Scientific, USA) to maintain the pH value in the range of 8.0 ± 0.5. *P. gyrans* cultures were grown at room temperature under continuous illumination (100 $\mu mol_{photons}$ m^{-2} s^{-1}). The remaining nutrient concentrations used in the inoculum preparation were those used under level 0 conditions, as presented in Table 1.

2.2. Experimental design

2.2.1. Plackett–Burman (PB) design. In the current study, PB design was used to assess the effect of 17 independent variables on biomass growth of *P. gyrans*.[29] Each variable was tested at three different levels (Table 1), accounting for 24 experiments with a unique combination of the 17 variables and the addition of six replicates at level 0 – as central points – to

Table 1 Growth conditions and the respective levels (−1, 0, and 1) tested in Plackett–Burman experiments, and the calculated effects of the independent variables on the biomass productivity (P_X, g AFDW per L per day) of *P. gyrans* ($p < 0.10$)

Growth parameters		−1	0	1	Effect	Standard error	t-Calc	p-Value	Significant
Inoculum size (g AFDW per L)	x_1	0.1	0.2	0.3	0.009	0.010	0.855	0.411	No
Salinity (psu)	x_2	20	30	40	−0.003	0.010	−0.269	0.793	No
Light intensity ($\mu mol_{photons}$ m^{-2} s^{-1})	x_3	150	450	750	0.065	0.010	6.346	0.000	Yes
Air flow (mL min^{-1})	x_4	600	800	1000	−0.002	0.010	−0.171	0.867	No
$NaNO_3$ (mg L^{-1})	x_5	250	500	750	0.047	0.010	4.587	0.001	Yes
$NaH_2PO_4 \cdot H_2O$ (mg L^{-1})	x_6	10	20	30	0.017	0.010	1.638	0.130	No
$Na_2H_2EDTA \cdot 2H_2O$ (mg L^{-1})	x_7	22.5	45	67.5	0.007	0.010	0.660	0.523	No
H_3BO_3 (mg L^{-1})	x_8	16.8	33.6	50.4	0.001	0.010	0.122	0.905	No
$FeCl_3 \cdot 6H_2O$ (mg L^{-1})	x_9	0.65	1.3	1.95	0.000	0.010	−0.024	0.981	No
$MnCl_2 \cdot 4H_2O$ (μg L^{-1})	x_{10}	180	360	540	−0.012	0.010	−1.149	0.275	No
$NaHCO_3$ (mg L^{-1})	x_{11}	170	652	1134	0.002	0.010	0.204	0.842	No
$ZnCl_2$ (μg L^{-1})	x_{12}	10.25	21	31.75	−0.010	0.010	−0.986	0.345	No
$CoCl_2 \cdot 6H_2O$ (μg L^{-1})	x_{13}	10	20	30	−0.008	0.010	−0.790	0.446	No
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (μg L^{-1})	x_{14}	4.5	9	13.5	−0.012	0.010	−1.214	0.250	No
$CuSO_4 \cdot 5H_2O$ (μg L^{-1})	x_{15}	10	20	30	−0.020	0.010	−1.980	0.073	Yes
Thiamin (μg L^{-1})	x_{16}	50	100	150	0.008	0.010	0.790	0.446	No
Cyanocobalamin (μg L^{-1})	x_{17}	2.5	5	7.5	0.001	0.010	0.138	0.892	No
			Mean		0.142	0.005	27.724	0.000	—
			Curvature		0.033	0.023	1.446	0.176	No

evaluate the repeatability of microalgae growth. Except for $NaNO_3$, nutrient concentration at level 0 represents the usual composition of Walne's medium.[30]

In addition, and according to the conclusions obtained in other studies with Pavlovophyceae species,[31,32] it was also decided to evaluate the effect of $NaHCO_3$ on the biomass production of *P. gyrans*. Table 2 displays the experimental matrix design for the 17 cultivation parameters considered. PB assays were performed in 1 L bubble column reactors (6.5 cm diameter and 43 cm height) using the inoculum as previously described. Individual stock solutions of the variables x_5 to x_{17} were prepared, sterilized by filtration (0.2 μm), and added to each reactor to reach the concentration described in Table 1.

The remaining medium components and materials were sterilized by using an autoclave. The salinity in each reactor was adjusted using a concentrated stock solution of sea salt and quantified by using a salinity refractometer (Hanna HI96822). All experiments were performed at room temperature (25 ± 2 °C) under constant illumination provided by LED lighting (Primelux, Portugal). Photosynthetically active radiation (PAR) was measured at two points on the outer surface of the reactor (bottom and top) with a LI-250A light meter (LI-COR quantum

sensor Q44069). Cultures were continuously bubbled with different airflows in order to assess different turbulence levels (Table 1) and pH (8.0 ± 0.5) was kept stable by injection of 6 mL min^{-1} of CO_2.

2.2.2. Rotatable central composite design (RCCD). After the identification of the most significant independent variables for biomass production of *P. gyrans*, a RCCD was designed to determine the optimum growth conditions. The variables selected for optimization were light intensity (x_3), $NaNO_3$ (x_5), $NaH_2PO_4 \cdot H_2O$ (x_6), and $CuSO_4 \cdot 5H_2O$ (x_{13}). Although the effect of $NaH_2PO_4 \cdot H_2O$ was not considered significant in the PB design, this variable was also selected for optimization considering that its *p*-value was close to the 10% significance level ($p < 0.10$), and also due to its biological importance for microalgae cultivation.[23,33–35]

Thus, to optimize biomass production based on these four factors ($k = 4$), a 2^4 factorial design without replicates ($r = 1$) – with 8 axial points and 3 replicates at the central point (cp) – was performed, totaling 27 trials (ESI Table S1†). The different results produced in the RCCD were fitted to a second-order model by a multiple linear regression to predict the different responses (Y) related to microalgae growth performance and its biochemical composition, using the following equation:

Table 2 The experiments of PB matrix design with coded values of the seventeen growth conditions and the respective response for mean volumetric biomass productivity (P_X, g AFDW per L per day), and maximum biomass produced (X_{max}, g AFDW per L) calculated in the beginning of the stationary phase (*t*, days), during the *P. gyrans* growth

#E	Variable (x)																	*t*	P_X	X_{max}
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17			
1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	6	0.133	1.09
2	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	6	0.109	1.20
3	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	6	0.150	1.21
4	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	4	0.135	0.86
5	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	8	0.175	1.67
6	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	8	0.256	2.15
7	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	1	8	0.209	1.96
8	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	−1	10	0.121	1.31
9	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	−1	6	0.247	1.79
10	1	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	1	6	0.110	0.87
11	−1	1	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	−1	6	0.203	1.32
12	−1	−1	1	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	1	6	0.150	1.01
13	1	−1	−1	1	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	−1	8	0.138	1.41
14	1	1	−1	−1	1	1	−1	1	−1	1	1	1	1	1	−1	−1	−1	16	0.116	2.16
15	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	1	1	1	−1	−1	6	0.134	0.91
16	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	1	1	1	−1	6	0.123	0.85
17	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	1	1	1	14	0.101	1.71
18	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	1	1	12	0.099	1.30
19	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	1	6	0.131	1.10
20	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	1	10	0.077	0.87
21	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	1	6	0.178	1.18
22	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	−1	8	0.096	0.87
23	−1	−1	−1	−1	1	−1	1	−1	−1	1	1	−1	−1	1	1	−1	1	10	0.140	1.48
24	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	10	0.072	0.81
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.162	1.48
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.158	1.49
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.161	1.47
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.151	1.38
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.156	1.46
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0.162	1.47

$$Y = \beta_0 + \sum_j \beta_j X_j + \sum_{i<j} \beta_{ij} X_i X_j + \sum_j \beta_{jj} X_j^2 + \varepsilon \quad (1)$$

where X_i and X_j are the coded values for the independent variables, β_0 is the intercept coefficient, β_j is the linear coefficient, β_{jj} is the quadratic coefficient, and ε is the error of the model.[29]

The levels $(-\alpha, -1, 0, 1, \text{and} +\alpha)$ attributed to each factor were based on the outcomes of the PB design and are presented in Table 3. The inoculum size (x_1), air flow (x_4), and $NaHCO_3$ concentration (x_{11}) were fixed at level -1. In contrast, all the other variables that have shown a non-significant effect in the PB matrix were set at level 0 to not limit microalgae growth by nutrient deficiency. Cultivation procedures adopted were the same as described for PB experiments.

2.2.3. Validation test. The optimized growth conditions obtained after Section 2.2.2 were compared with the conventional Walne's medium formulation. In addition, two more formulations were tested (ESI Table S2†). One formulation (Lvl-1) aimed at understanding if the non-significant variables determined in the PB design could be used at level -1, instead of level 0 (Table 1), in the subsequent optimization steps. Another assay evaluated the importance of vitamin supplementation under the optimized growth conditions, in which no vitamins were added to the culture medium (Vit-). In order to eliminate the trace concentration of vitamins, the inoculum was washed twice under sterilized conditions. This process consisted of centrifuging the biomass (4000 rpm, 15 min, and 5 °C; Centurion Pro-Analytical CR7000, Chichester, UK) with further resuspension in autoclaved saline solution (30 psu). All conditions were performed in triplicate.

2.3. Growth analysis

Microalgal growth was monitored every 2 days by optical density (750 nm; Synergy HT, BioTek Instruments, Inc., U.S.A.) and its value converted to ash-free dry weight (AFDW), using the calibration curve previously determined: AFDW $(g\ L^{-1})$ = 0.8991 × $OD750$ + 0.0054, R^2 = 0.99. AFDW was determined by vacuum filtration of 10 mL of culture samples using pre-weighted glass filters (VWR) and their combustion in a muffle furnace (Nabertherm, Bremem, Germany) at 500 °C overnight. After filtration, samples were washed twice with 20 mL of ammonium formate (0.5 mol L^{-1}), dried at 105 °C in a convection oven (WTCBinder, Germany) for 24 h and weighed. Dried filters were then re-combusted at 500 °C overnight and re-weighed to determine the ash-free dry weight. AFDW determined along $P.$ $gyrans$ growth was used to assess the biomass production

parameters. Mean volumetric biomass productivity (P_X) was calculated using the equation:

$$P_X = \frac{AFDW_{t_1} - AFDW_{t_0}}{t_1 - t_0} \quad (2)$$

where t_0 represents the beginning of the growth (0 days) and t_1 was assumed as the first point of the stationary phase. Maximum biomass produced (X_{max}) was also calculated at time t_1. The experiments were stopped after reaching the stationary phase and full stabilization of the AFDW. Biomass was centrifuged at 5 °C (4000 rpm; 20 min – Centurion Pro-Analytical CR7000, Chichester, UK). The pellet was stored at -20 °C and later freeze-dried for biochemical characterization.

2.4. Biochemical characterization

2.4.1. Protein quantification. The protein content of $P.$ $gyrans$ was performed by elemental analysis of C, H, and N using a Vario el III (Vario EL, Elementar Analyser system, GmbH, Hanau, Germany).[36] As reported by Lourenço $et\ al.$,[37] the percentage of protein per dry weight was achieved after multiplying the total nitrogen (N) content by the conversion factor 4.78. The samples of the RCCD are presented as the mean of two replicates with deviation values representing the maximum and minimum values. The remaining values are presented as the mean values and standard deviation of three replicates.

2.4.2. Total lipids. The extraction and quantification of lipids were carried out by using the modified Folch method described by Couto $et\ al.$,[38] with some adjustments. Briefly, 10 mg of $P.$ $gyrans$ biomass and 2 mL of dichloromethane : methanol (2 : 1 v/v) solution were added to a glass tube. The suspension was homogenized by vortexing for 2 min. After the extraction, the suspension was centrifuged at 2000 rpm for 10 min and the organic phase was collected in a new glass tube. The biomass residue was extracted twice, until there was no more pigmentation in the solvent. The combined organic phases collected in the new glass tube were dried under a stream of nitrogen gas and dissolved in 2 mL of dichloromethane and 1 mL of methanol, followed by 1 min vortexing for complete re-solubilization.

Then, 0.75 mL of water were added and the mixture was vortexed for 2 min. The extracts were centrifuged at 2000 rpm for 10 min to separate the phases, with the lower phase (organic) being collected and transferred to a new glass tube. The remaining aqueous extract was re-extracted (with 2 mL of dichloromethane) two more times. The combined organic phases were dried under a nitrogen stream. The final dried lipid extract was re-suspended in dichloromethane, transferred to

Table 3 The abiotic factors and their respective levels tested in RCCD experiments

	$-2\ (-\alpha)$	-1	0	1	$2\ (+\alpha)$
Light intensity ($\mu mol_{photons}\ m^{-2}\ s^{-1}$), x_3	200	350	500	650	800
$NaNO_3$ (mg L^{-1}), x_5	500	750	1000	1250	1500
$NaH_2PO_4 \cdot H_2O$ (mg L^{-1}), x_6	10	20	30	40	50
$CuSO_4 \cdot 5H_2O$ ($\mu g\ L^{-1}$), x_{15}	0	5	10	15	20

a pre-weighed vial, dried with nitrogen, weighed, and stored at −20 °C. Total lipids were determined by gravimetry using three replicates for each set of growth conditions and presented as the percentage of dry weight.

2.4.3. Fatty acid analysis by gas chromatography-mass spectrometry (GC-MS). The transesterification of the total lipid extracts of *P. gyrans* was carried out according to the methodology described by Couto *et al.*[39] FA analysis was carried out on an Agilent 8860 gas chromatograph (Santa Clara, CA, USA) connected to an Agilent 5977B mass spectrometer (Agilent, Santa Clara, CA, USA), operating with electron impact ionization (70 eV). The remaining settings and conditions of operation were used as defined by Couto *et al.*[39] FAs were identified using commercial standards by comparing the retention time and spectral fragmentation patterns. The relative abundance of each FA was determined by using the equation:

$$\% \ \text{FA} = \frac{A_{\text{FA}}}{A_{\text{TFA}}} \times 100 \tag{3}$$

where A_{FA} is the integrated area of a single FA and A_{TFA} is the total area of all FAs identified.

2.4.4. Nutritional indices: hypocholesterolemic index (HI), atherogenic index (AI), and thrombogenic index (TI). The hypocholesterolemic (HI), atherogenic (AI), and thrombogenic (TI) indices were calculated from the FA composition (% TFA) according to the following equations:

$$\text{HI} = \frac{(C18:1 + C18:2 + C18:3 + C18:4 + C20:5 + C22:5 + C22:6)}{C14:0 + C16:0} \tag{4}$$

$$\text{AI} = \frac{(C12:0 + 4 \times C14:0 + C16:0)}{\left(\sum \text{MUFA} + \sum \text{PUFA}\right)} \tag{5}$$

$$\text{TI} = \frac{(C14:0 + C16:0 + C18:0)}{\left(0.5 \times \sum \text{MUFA} + 0.5 \times \sum n-6 \ \text{PUFA} + 3 \times \sum n-3 \ \text{PUFA}\right) + \left(\sum n-3 \big/ \sum n-6\right)} \tag{6}$$

where MUFAs and PUFAs are the monosaturated and polyunsaturated fatty acids, respectively.[40,41]

2.5. Statistical analysis

The significant variables in the PB design were selected using a 90% confidence level ($p < 0.10$). Second order models of the RCCD were obtained using a 90% confidence level ($p < 0.10$), and the quality of the fitted model was statistically evaluated by analysis of variance (ANOVA) and coefficient determination (R^2). PB and RCCD analyses were performed with the online software Protimiza Experimental Design (http://experimental-design.protimiza.com.br/). The experiments were performed in triplicate and their biochemical properties were analyzed by using differences among means using one-way ANOVA followed by Tukey's multiple comparison test at a 95% confidence level (software: GraphPad Prism version 8.0.2).

3. Results and discussion

3.1. Identification of significant factors for biomass productivity of *P. gyrans* (PB design)

The biomass productivities achieved in the PB design are presented in Table 2, whereas the calculated effects, standard errors, and *p*-value of the seventeen variables assessed are described in Table 1. Among the thirty assays performed, X_{max} ranged from 0.81 to 2.16 g AFDW per L (Table 2). Also, P_X varied between 0.072 and 0.256 g AFDW per L per day, which resulted in the identification of the three independent variables ($p < 0.10$) that greatly affected *P. gyrans* growth: light intensity, $NaNO_3$, and $CuSO_4 \cdot 5H_2O$ (Table 1).

Light intensity was identified as the most significant abiotic factor ($p < 0.001$), showing a positive calculated effect, meaning that P_X increased at higher light intensities within the tested interval. Although using a lower lighting range, Seoane *et al.*[42] corroborated the positive effect of light on the growth kinetics of eleven haptophytes.

The second most important variable was $NaNO_3$ ($p = 0.001$), favoring biomass production (positive effect). Nitrogen is a key nutrient due to its wide distribution in molecules (*e.g.* proteins, DNA, and RNA).[43] It is an important factor for both biomass production and regulation of total lipid content and the FA profile.[44] For instance, *Pavlova* sp. doubled its biomass production when supplemented with 400 mg L^{-1} $NaNO_3$,[15] while the biomass production of *D. lutheri* was positively affected until 160 mg L^{-1} $NaNO_3$, with the above concentration values decreasing its growth performance.[45]

In contrast with the abovementioned significant variables, $CuSO_4 \cdot 5H_2O$ ($p = 0.073$) presented a negative effect, hindering biomass production at higher concentrations. This micronutrient is an important cofactor for several metalloproteins responsible for redox homeostasis and photosynthesis;[46]

however, as reported for *Pavlova viridis*, when excessively supplied, it can lead to oxidative stress, inhibiting microalgae growth and triggering protective mechanisms.[26] This greater sensitivity was also described in other haptophytes, such as *Isochrysis galbana*, which presented a low EC_{50} value for copper.[47]

Although data from Table 1 excluded $NaH_2PO_4 \cdot H_2O$ concentration from the key factors involved in biomass production, this variable was also considered for further optimization through the RCCD. The main reasons for that option were the relative statistical importance ($p = 0.13$) and, mostly, the widely known biological role of phosphorus in microalgae growth and composition.[24,33,48] To not limit microalgae growth in the RCCD, the remaining non-significant variables, except for $NaHCO_3$, inoculum size and airflow (level −1), were set at level 0 in the RCCD.

3.2. Rotatable central composite design (RCCD)

3.2.1. Biomass growth analysis. The evaluation of optimal conditions for biomass production was carried out using a RCCD with the variables previously identified: light intensity (x_3), $NaNO_3$ (x_5), $NaH_2PO_4 \cdot H_2O$ (x_6), and $CuSO_4 \cdot 5H_2O$ (x_{15}) (ESI Table S1†). P_X ranged from 0.165 to 0.262 g AFDW per L per day, whereas X_{max} varied between 1.30 and 2.34 g AFDW per L (Table

4). Overall, the productivities achieved in the RCCD were higher and less variable when compared to those of the PB design (0.072 to 0.256 g AFDW per L per day), demonstrating the importance of the selected variables and their effects on growth performance.

As described in Table 5, and only using the significant terms ($p < 0.10$), these responses were used to calculate the regression coefficients and to generate a one-order model for P_X and a second-order model for X_{max}, each one as a function of coded independent variables. Analysis of variance (ANOVA) was carried out to assess the model's fitness. The mathematical model generated for the dependent variable P_X showed a low value of R^2 (32.0%), turning the production of contour curves unfeasible. This may be explained by the lower range of P_X values obtained.[49] On the other hand, X_{max} presented a satisfactory R^2 (84.1%) as well as $F_{calc} > F_{tab}$, which allowed the generation of the second-order model and subsequent contour curves, as presented in Fig. 1.

Although copper supplementation was reduced in this second optimization step, the increasing concentration of this micronutrient led to reduced biomass production of *P. gyrans*, as in the PB design. This behavior was more noticeable at higher concentrations (20 μg L^{-1}), being responsible for a reduction of about 0.3–0.4 g AFDW per L when compared with the optimal

Table 4 Results obtained in the RCCD experiments (#E) for the response variables: volumetric biomass productivity (P_X, g AFDW per L per day), maximum biomass produced (X_{max}, g AFDW per L), total lipids (% w/w), total saturated fatty acids (SFAs% TFA), total monosaturated fatty acids (MUFAs% TFA), total polyunsaturated fatty acids (PUFAs% TFA), total $n − 3$ fatty acids ($n − 3$ FAs% TFA), eicosapentaenoic acid (EPA% TFA) and docosahexaenoic acid (DHA% TFA), and protein content (% w/w). The values are the mean and standard deviation of three different analyses ($n = 3$), with the exception for the protein content whose values are the mean of two different analyses ($n = 2$) with the deviation representing the maximum and minimum values

#E	P_X	X_{max}	Total lipids	SFAs	MUFAs	PUFAs	$n − 3$ FAs	EPA	DHA	Protein content
1	0.262	1.57	21.95 ± 3.02	48.80 ± 1.31	18.22 ± 0.23	32.98 ± 1.12	26.63 ± 0.90	16.79 ± 0.72	5.11 ± 0.17	18.37 ± 0.13
2	0.196	1.57	20.75 ± 2.17	62.23 ± 1.40	22.73 ± 0.62	15.05 ± 1.42	11.79 ± 1.18	6.62 ± 0.55	3.52 ± 0.43	19.64 ± 0.04
3	0.240	1.92	20.53 ± 1.25	43.29 ± 1.69	18.63 ± 1.31	38.08 ± 0.84	32.26 ± 0.63	19.28 ± 1.11	6.80 ± 0.28	27.30 ± 0.47
4	0.249	1.99	20.76 ± 0.69	63.64 ± 2.78	20.73 ± 1.21	15.63 ± 1.64	12.60 ± 1.25	7.11 ± 0.72	3.75 ± 0.41	29.91 ± 0.52
5	0.205	1.64	28.34 ± 2.85	44.65 ± 0.27	20.44 ± 0.16	34.92 ± 0.11	27.80 ± 0.08	17.67 ± 0.16	5.40 ± 0.09	18.04 ± 0.29
6	0.167	1.67	24.57 ± 2.02	56.88 ± 0.48	25.50 ± 0.09	17.62 ± 0.56	13.98 ± 0.50	8.99 ± 0.43	3.06 ± 0.31	17.79 ± 0.37
7	0.239	1.91	25.66 ± 1.80	46.37 ± 1.72	19.65 ± 1.26	33.97 ± 2.21	27.80 ± 2.29	18.35 ± 2.27	4.74 ± 0.07	28.49 ± 0.21
8	0.211	2.11	22.09 ± 0.41	62.78 ± 2.30	22.68 ± 0.84	14.54 ± 1.59	11.54 ± 1.18	7.33 ± 0.70	2.79 ± 0.33	25.23 ± 0.35
9	0.217	1.30	22.81 ± 1.49	45.82 ± 1.79	18.54 ± 0.37	35.64 ± 2.02	29.43 ± 1.75	18.84 ± 1.10	5.31 ± 0.47	22.51 ± 0.31
10	0.255	1.53	19.95 ± 1.90	68.95 ± 2.05	18.59 ± 1.10	12.46 ± 1.34	9.49 ± 0.98	5.35 ± 0.65	2.84 ± 0.29	15.61 ± 0.20
11	0.215	1.72	20.04 ± 1.08	42.40 ± 4.73	18.33 ± 0.64	39.28 ± 4.10	33.14 ± 3.57	20.14 ± 2.37	5.52 ± 0.66	37.57 ± 0.28
12	0.228	1.82	20.87 ± 0.77	61.26 ± 3.57	21.95 ± 1.36	16.79 ± 2.69	13.47 ± 2.25	6.70 ± 0.84	4.86 ± 1.28	33.25 ± 1.02
13	0.183	1.46	24.12 ± 1.29	44.93 ± 2.23	21.01 ± 0.49	34.06 ± 2.04	27.67 ± 1.68	16.80 ± 1.08	6.26 ± 0.36	24.65 ± 0.66
14	0.166	1.66	25.23 ± 1.76	58.76 ± 0.11	25.28 ± 0.40	15.96 ± 0.51	12.36 ± 0.36	7.70 ± 0.31	2.86 ± 0.08	17.43 ± 0.34
15	0.165	1.65	17.59 ± 1.31	44.15 ± 2.98	22.59 ± 0.22	33.26 ± 3.10	27.38 ± 2.82	16.39 ± 2.09	4.30 ± 0.80	35.47 ± 0.94
16	0.220	1.76	22.46 ± 1.38	60.76 ± 1.49	23.72 ± 0.23	15.52 ± 1.42	12.26 ± 1.18	7.52 ± 0.58	2.99 ± 0.42	28.65 ± 0.09
17	0.209	1.67	24.46 ± 0.97	45.54 ± 0.80	18.69 ± 0.44	35.76 ± 0.83	29.44 ± 0.73	18.72 ± 0.58	5.27 ± 0.14	27.73 ± 0.03
18	0.234	1.87	23.38 ± 2.10	61.65 ± 2.68	23.45 ± 0.87	14.91 ± 1.81	12.05 ± 1.50	7.64 ± 0.97	2.89 ± 0.42	19.71 ± 0.02
19	0.222	1.33	23.27 ± 2.30	59.28 ± 1.84	23.02 ± 0.55	17.69 ± 1.48	13.48 ± 1.13	8.67 ± 0.78	3.06 ± 0.22	14.51 ± 0.10
20	0.234	2.34	17.45 ± 1.28	56.37 ± 0.53	23.21 ± 0.80	20.42 ± 0.28	16.11 ± 0.25	9.73 ± 0.13	4.27 ± 0.17	28.61 ± 1.83
21	0.227	1.36	18.73 ± 2.51	59.50 ± 0.85	22.77 ± 0.35	17.72 ± 0.59	13.97 ± 0.52	7.74 ± 0.37	4.08 ± 0.30	37.71 ± 0.63
22	0.245	1.96	24.22 ± 2.72	61.10 ± 4.12	20.23 ± 2.49	18.67 ± 2.37	14.31 ± 1.96	8.83 ± 1.08	3.68 ± 0.68	18.53 ± 0.21
23	0.225	1.80	23.09 ± 2.61	42.47 ± 0.24	18.89 ± 0.34	38.64 ± 0.57	32.07 ± 0.44	20.13 ± 0.31	6.17 ± 0.08	24.41 ± 0.41
24	0.199	1.59	21.17 ± 0.95	39.09 ± 0.92	20.06 ± 0.78	40.85 ± 0.89	33.80 ± 0.72	20.32 ± 0.51	6.91 ± 0.16	32.09 ± 0.87
25	0.234	1.87	21.16 ± 0.07	64.60 ± 0.63	22.56 ± 0.35	12.84 ± 0.35	9.70 ± 0.32	6.09 ± 0.14	2.37 ± 0.17	21.45 ± 0.30
26	0.240	1.92	21.90 ± 2.30	59.47 ± 2.51	22.04 ± 0.98	18.49 ± 1.56	14.17 ± 1.19	9.28 ± 0.74	3.16 ± 0.37	22.14 ± 0.61
27	0.236	1.89	22.10 ± 1.46	56.71 ± 1.73	22.83 ± 0.06	20.46 ± 1.78	16.07 ± 1.49	10.08 ± 0.68	4.04 ± 0.74	23.52 ± 0.19

Table 5 Analysis of variance (ANOVA) with the percentage of explained variance (R^2), $F_{calculated}$ and $F_{tabulated}$, at the 10% significance level, for the responses: mean volumetric biomass productivity (P_X, g AFDW per L per day), maximum biomass (X_{max}, g AFDW per L), total lipids (% w/w), protein content (% w/w), saturated fatty acids (SFAs% TFA), monosaturated fatty acids (MUFAs% TFA), polyunsaturated fatty acids (PUFAs% TFA), total of $n-3$ FAs ($n-3$% TFA), eicosapentaenoic acid (EPA% TFA) and docosahexaenoic acid (DHA% TFA)[a]

	R^2	F_{calc}	F_{tab}
P_X (g AFDW per L per day) = $0.22 - 0.01x_6 + 0.01x_3x_{15}$	32.0	5.66	2.54
X_{max} (g AFDW per L) = $1.82 + 0.06x_3 + 0.19x_5 + 0.07x_6 - 0.05x_6^2 - 0.08x_{15} - 0.04x_{15}^2$	84.1	17.59	2.09
Protein content (% w w^{-1}) = $22.36 - 1.71x_3 + 5x_5 - 1.95x_6 + 1.37x_6^2 + 1.91x_{15} + 1.40x_{15}^2 - 1.60x_3x_{15}$	84.44	14.73	2.06
Total lipids (% w w^{-1}) = $21.61 + 0.64x_3^2 - 1.22x_5 + 1.39x_6 - 0.64x_{15} + 0.57x_3x_5 + 0.77x_3x_{15} - 0.70x_5x_6 - 0.68x_6x_{15}$	84.25	12.04	2.04
SFAs% TFA = $59.62 + 6.96x_3 - 1.49x_3^2 - 4.69x_{15}^2 + 1.50x_5x_6$	88.20	41.10	2.22
MUFAs% TFA = $21.94 + 1.39x_3 + 0.75x_6 - 0.67x_{15}^2$	58.50	10.81	2.34
PUFAs% TFA = $18.07 - 8.35x_3 + 1.83x_3^2 + 5.43x_{15}^2$	91.97	87.83	2.34
$\sum n-3$ FAs% TFA = $14.06 - 7.06x_3 + 1.71x_3^2 + 4.76x_{15}^2$	91.27	80.11	2.34
EPA% TFA = $8.60 - 4.55x_3 + 1.13x_3^2 + 2.89x_{15}^2 + 0.73x_3x_6$	92.29	65.85	2.22
DHA% TFA = $3.65 - 0.90x_3 - 0.25x_6 + 0.72x_{15}^2 - 0.43x_5x_6$	82.39	25.84	2.22

[a] x_1: inoculum size (g AFDW per L); x_2: salinity (psu); x_3: light intensity ($\mu mol_{photons}$ m^{-2} s^{-1}); x_4: air flow (mL min^{-1}); x_5: NaNO$_3$ (mg L^{-1}); x_6: NaH$_2$PO$_4\cdot$H$_2$O (mg L^{-1}); x_7: Na$_2$H$_2$EDTA$\cdot$2H$_2$O (mg L^{-1}); x_8: H$_3$BO$_3$ (mg L^{-1}); x_9: FeCl$_3\cdot$6H$_2$O (mg L^{-1}); x_{10}: MnCl$_2\cdot$4H$_2$O (μg L^{-1}); x_{11}: NaHCO$_3$ (mg L^{-1}); x_{12}: ZnCl$_2$ (μg L^{-1}); x_{13}: CoCl$_2\cdot$6H$_2$O (μg L^{-1}); x_{14}: (NH$_4$)6Mo$_7$O$_{24}\cdot$4H$_2$O (μg L^{-1}); x_{15}: CuSO$_4\cdot$5H$_2$O (μg L^{-1}); x_{16}: thiamin (μg L^{-1}); x_{17}: cyanocobalamin (μg L^{-1}).

CuSO$_4\cdot$5H$_2$O concentration range, <12 μg L^{-1} ($\approx$3 μg L^{-1} Cu^{2+}), shown in Fig. 1. Purbonegoro et al.[50] also described a reduction in the cell density of *Pavlova* sp. when supplemented with 13 μg L^{-1} Cu^{2+} and a growth inhibition of 95.47% with 98 μg L^{-1} Cu^{2+}.

Concentrations ranging from 0 to 12 μg L^{-1} of CuSO$_4\cdot$5H$_2$O and from 24 to 49 mg L^{-1} of NaH$_2$PO$_4\cdot$H$_2$O led to the optimal X_{max} of *P. gyrans*. Fig. 1a also shows a lower X_{max} at higher copper and lower phosphorus levels. According to Rocha et al.,[51] phosphorus-limited cells presented lower tolerance to Cu, mainly due to changes in membrane permeability and intracellular polyphosphate content.

Concerning the effect of light intensity on X_{max}, regardless of the interaction with the other variables (x_5, x_6, and x_{15}), its increase always promoted higher growth of *P. gyrans*, showing that there was no apparent photoinhibition under the conditions tested (Fig. 1b, c and f). From Fig. 1b, c, and f, the light conditions considered ideal for X_{max} should be above 650 $\mu mol_{photons}$ m^{-2} s^{-1}. However, this feature is highly species-dependent; *D. lutheri*, for instance, has been reported as having higher growth performance under medium light intensity (100 $\mu mol_{photons}$ m^{-2} s^{-1}),[20] while *Acutodesmus* sp. showed the highest growth rate at 500 $\mu mol_{photons}$ m^{-2} s^{-1}.[52]

The addition of NaH$_2$PO$_4\cdot$H$_2$O to the RCCD greatly affected the biomass produced by *P. gyrans*. In fact, the increased range of values in the RCCD allowed understanding that biomass production was favored by the highest values of phosphorus, with optimum values ranging from 27 to 47 mg L^{-1}. Similarly, *P. pinguis* grown under higher loadings of phosphorus promoted higher biomass production and productivity.[33]

A similar trend was observed for NaNO$_3$, with higher X_{max} reached at the highest concentration (1500 mg L^{-1}). *P. pinguis* also showed high nitrogen uptake and storage with increasing nitrogen supplementation, from 140 to 1752 μmol L^{-1}.[53] However, the highest nitrogen level did not increase the final cell density, possibly due to light limitation during growth, which was 3 to 11 times lower than in the present work. In opposition, *D. lutheri* showed growth inhibition when NaNO$_3$ concentration exceeded 160 mg L^{-1}, possibly due to enzyme-mediated production of ammonia and nitrite.[45]

3.2.2. Biochemical characterization

3.2.2.1. Lipid and protein content. The biomass produced from the RCCD was analyzed concerning its lipid and protein contents (Table 4). The lipid content varied between 17.45 (trial 20) and 28.34% (trial 5), whereas the protein concentration ranged from 15.61 (trial 10) to 37.71% (trial 21). For both protein and lipid contents, all the independent variables were statistically significant ($p < 0.10$; Table 5). Considering the analysis of variance in Table 5 (R^2 and $F_{calc} > F_{tab}$), the second-order models achieved were considered adequate for the responses "total lipids" and "protein content".[49] The effects of the independent variables on these responses are illustrated in the contour curves of ESI S1.†

Briefly, total lipids increase by decreasing NaNO$_3$ concentration (x_5), with a common optimal region for concentration values below 600 mg L^{-1}. Likewise, copper concentration (x_{15}) exerted a negative impact on lipid accumulation. The opposite behavior was found for NaH$_2$PO$_4\cdot$H$_2$O (x_6), with increased lipid accumulation observed at higher concentrations of phosphorus in the medium, reaching a higher lipid content when NaH$_2$PO$_4\cdot$H$_2$O ranged from 45 to 50 mg L^{-1}. Regarding the importance of light intensity (x_3), its quadratic effect and the interaction of this factor with the other independent variables over the total lipids of *P. gyrans* were noteworthy. Overall, higher lipid accumulation was promoted at low and high light intensities, a trend that was well defined when assessing the interaction between the independent variables light and NaNO$_3$.

Regarding the protein content of *P. gyrans*, both the concentration of cooper and nitrogen revealed a positive effect. Nitrogen-replete conditions enhanced the production of protein – reaches 40% w/w in the range of 1400–1500 mg L^{-1} of NaNO$_3$ – attesting its vital role in protein and glycerolipid synthesis.[54] High light intensity decreased the protein content, which only exceeded 32% w/w under low and medium light intensities, namely, from 200 to $\approx$ 400 $\mu mol_{photons}$ m^{-2} s^{-1} (ESI Fig. S1†).

© 2023 The Author(s). Published by the Royal Society of Chemistry
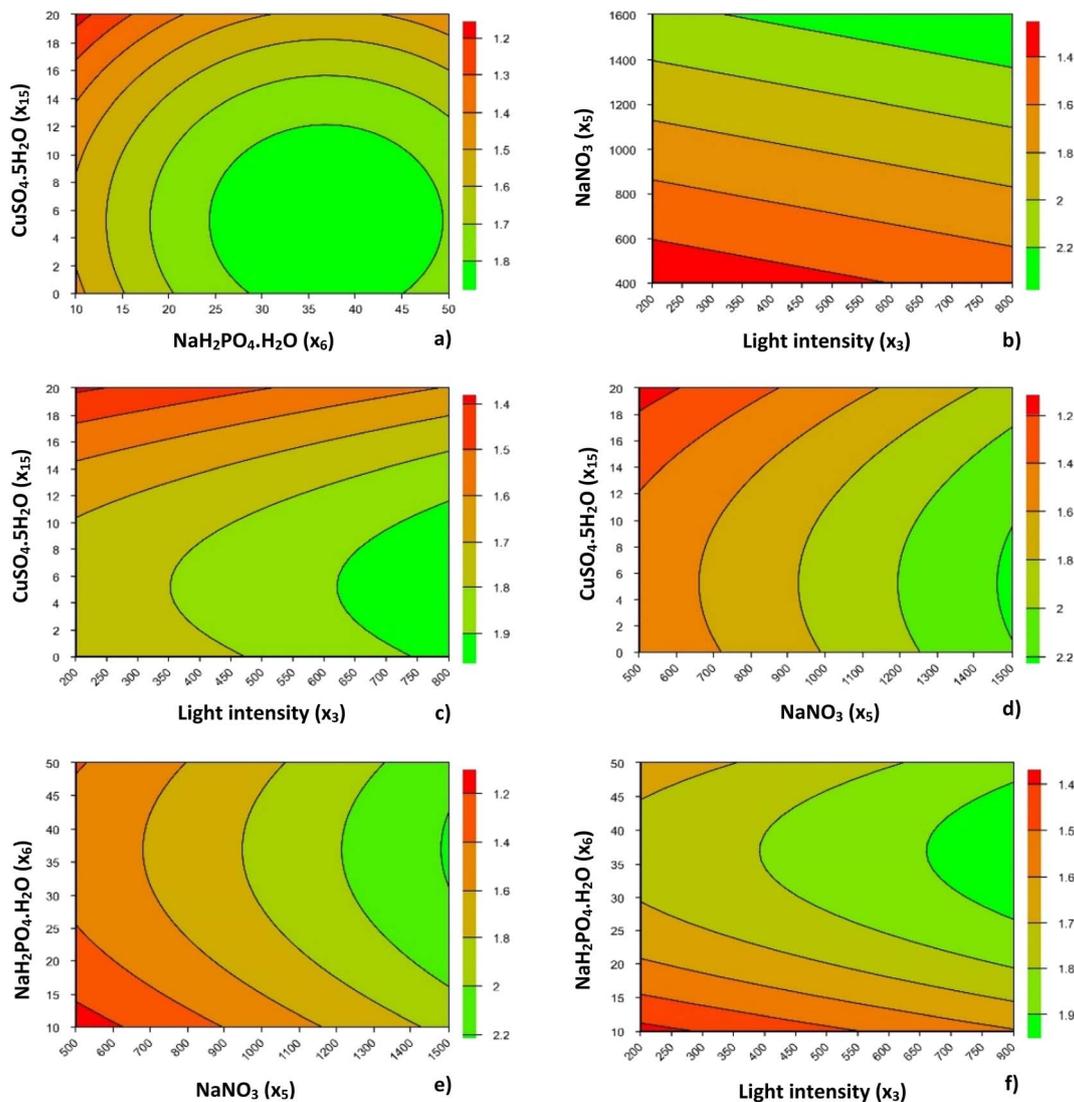
Fig. 1 Contour curves for dependent variable X_{max} (g AFDW per L), illustrating the interactions between $CuSO_4 \cdot 5H_2O$ and $NaH_2PO_4 \cdot H_2O$ (a), $NaNO_3$ and light intensity (b), $CuSO_4 \cdot 5H_2O$ and light intensity (c), $CuSO_4 \cdot 5H_2O$ and $NaNO_3$ (d), $NaH_2PO_4 \cdot H_2O$ and $NaNO_3$ (e), and NaH_2-$PO_4 \cdot H_2O$ and light intensity (f).

Concerning $NaH_2PO_4 \cdot H_2O$, it was concluded that, generally, the use of larger concentrations of phosphorus led to a decrease in the protein content of *P. gyrans*. In fact, higher concentrations of this compound (>35% w/w) were only reached when the growth medium was supplemented with a lower concentration of phosphorus (10–20 mg L^{-1}).

The microalgae content of both classes of biocompounds have been described as being interconnected and strongly dependent on the nutritional composition of the growth medium. For instance, the optimal growth conditions (*e.g.*, light and copper level) resulted in better growth performance that may induce faster and higher nutritional deficiencies (*e.g.*, nitrogen availability). Thus, under these conditions, a metabolic change occurs increasing the production of reserve compounds (lipids), with a concomitant decrease in protein synthesis.[55,56]

3.2.2.2. Fatty acid analysis.
The FA profile (% TFA) obtained from the RCCD is presented in Table 4. The different combinations of light intensity, $NaH_2PO_4 \cdot H_2O$, $NaNO_3$, and $CuSO_4$-$\cdot 5H_2O$ had an impact on the content of FAs in biomass, leading to a wide range of values, for example, for PUFAs (12.46–40.85% TFA), SFAs (39.09–68.95% TFA) or EPA content (5.35–20.32% TFA). Using the set of responses and the respective significant terms ($p < 0.10$), a mathematical model with coded independent variables was generated for each dependent variable of Table 4. According to ANOVA, apart from MUFAs, all dependent variables related to FA composition showed a high R^2 value and respected the $F_{calc} > F_{tab}$ condition, attesting the fitness of all second-order models (Table 5). The contour curves of DHA and EPA (% FA/TFA) are presented in ESI Fig. S2.†

According to Table 4, the growth conditions tested promoted a significant impact on EPA content, the value of which ranged between 5.35 and 20.32% TFA. The variation in DHA levels

occurred in a narrower range of values, varying between 2.37 and 6.91% TFA. The production of EPA and DHA was strongly reduced with increasing light intensities, especially at medium and high light (ESI Fig. S2†). Guihéneuf et al.[20] also reported a reduced content of $n - 3$ FAs and PUFAs – mainly EPA – in P. lutheri when increasing light intensity from 20 to 340 $\mu mol_{photons}$ m^{-2} s^{-1}. Such behavior occurs since high light intensities are linked to a decreased unsaturation degree of the FAs from different lipid classes.[57]

For both EPA and DHA, $NaNO_3$ showed no significant effect ($p > 0.10$); just its interaction with $NaH_2PO_4 \cdot H_2O$ was proven significant over DHA production ($p < 0.10$) – Table 5. According to the calculated coefficients (Table 5), there was a negative correlation between the phosphorus supply and DHA level. This result is in agreement with the variation of DHA content of P. pinguis, which suffered a slight decrease when increasing phosphorus loading from 36.60 to 72.40 μmol L^{-1}.[33]

On the other hand, copper showed a quadratic effect for both FAs, reaching their highest contents at the limits of the tested range. Considering the biomass production (Fig. 1), the lowest EPA and DHA composition was achieved at intermediate copper concentrations (ESI Fig. S2†), i.e., close to the range of values where P. gyrans produced the highest biomass levels (X_{max}). Thus, EPA and DHA contents might be indirectly affected by the copper content through its influence on the growth performance of microalgae, which determines the nutritional properties of P. gyrans growth conditions and, consequently, its biochemical composition.[21]

3.3. Validation test

3.3.1. Biomass growth analysis. In order to validate the optimal conditions of the independent variables x_3, x_5, x_6, and x_{15}, described in Section 3.2, a validation assay was performed – Fig. 2.

The main goal was to validate the mathematical model developed (Table 5) and to compare the growth performance of

Fig. 2 Growth of P. gyrans, ash-free dry weight (g AFDW per L), in the validation assays: optimized conditions, Opt, control/Walne's medium, Con, medium without vitamins, Vit-, and assay with the variables considered non-significant in PB design fixed at level −1, Lvl-1. Data from sampling time (a1) and (a2) were used in the biomass growth analysis. Samples (b) in Opt assay were used in lipid analysis.

P. gyrans in the optimal formulation with control conditions (Walne's medium) – ESI Table S2.† Supplementary assays were also carried out to evaluate: (i) the impact of the absence of vitamins and (ii) if all the non-significant variables identified in PB design could be set at their minimum level. However, due to practical issues, an assay with control conditions (Walne's medium) was also conducted under optimal light intensity (700 $\mu mol_{photons}$ m^{-2} s^{-1}).

ESI Table S3† and Fig. 2 show that a very distinctive growth profile was reached with the optimized growth factors. While optimized conditions allowed a constant growth from day 2 to 10, reaching a maximum biomass of 2.26 g AFDW L ($p < 0.05$), in all the other experiments microalgae cultures ceased their growth on day 4, with the control reaching 0.59 g AFDW L. The two-step optimization adopted here yielded a significant 3.8-fold improvement in the biomass production of P. gyrans (ESI Table S3†). Considering that both trials were performed at the same light intensity, this result seems to be related to the increased supplementation of $NaNO_3$ and $NaH_2PO_4 \cdot H_2O$, and the limitation of $CuSO_4 \cdot 5H_2O$ in the growth medium and, to a lesser extent, probably due to the addition of an extra source of inorganic carbon ($NaHCO_3$).[21,58]

Opt and Con formulations present a N : P molar ratio of 63 : 1 and 9 : 1, respectively, suggesting a nitrogen limitation in Con. N and P concentrations in Opt are 15 and 2 times higher than those in Con. The optimal N : P ratio described here is different and higher than those presented by P. pinguis (24 : 1 and 12 : 1;[33] 26 : 1 and 48 : 1 [53]) and Dunaliella tertiolecta (45 : 1 with a three-fold increase on N and P supply),[59] both grown using f/2-Si medium under low lighting. The N : P ratio has been claimed as an important parameter to manage microalgal growth and its biochemical composition. However, its impact strongly depends on the microalgae species and is affected by the study approach performed (uni- or multivariate), nutrient composition and ratios, and other abiotic factors such as lighting, which limits the definition of a common optimal N : P value for microalgae.[33] This limitation was observed in the RCCD experiment, where nine N : P ratios were tested producing a diverse and unrelated range of X_{max} and biochemical compounds.

Using the coded values for the independent variables, the mathematical models (Table 5) were used to predict the response values for X_{max}, total proteins, total lipids, EPA, and DHA (% FA/TFA). The predicted values and associated errors between experimental and predicted data are displayed in ESI Table S4.† Due to their low relative error, the predicted values for X_{max} (RE −3.69%) and total protein (RE 0.60%) are in accordance with the experimental values, attesting the fitness of the model. In contrast, the deviation from experimental values for total lipids, and especially for EPA and DHA, was higher, accounting for relative errors of 22.10, 64.39, and 82.54%, respectively.

The assay without vitamins confirmed the crucial role played by these nutrients in P. gyrans. Its growth was severely delayed after the second day and ceased by the fourth day, with a maximum biomass produced similar to the control ($p > 0.05$) (ESI Table S3†). The inability to grow in vitamin-free
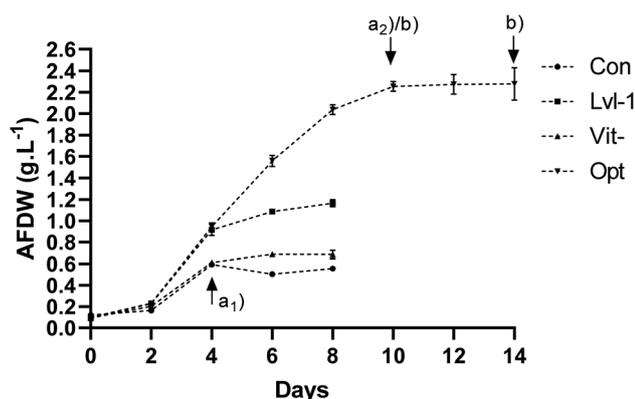
environments is in accordance with that described for other haptophytes such as *D. lutheri*[24] and *T. lutea*,[60] in which B_{12} dependency was underlined.[60]

Regarding the Lvl-1 assay, these conditions produced the second highest X_{max}, with a significative difference from the remaining conditions tested ($p < 0.05$) – ESI Table S3† and Fig. 2. Despite being presented in PB design as independent variables with no significant effects on X_{max} ($p > 0.10$), reducing these growth parameters at the same time inhibited *P. gyrans* growth from the fourth day onwards. Similarly, in previous studies, mineral limitation in the culture medium negatively affected the biomass production of *D. lutheri*, especially when deprived of Ca, Fe, Mn, Zn or Co.[24] Some of these nutrients are important cofactors for enzymatic processes involved in protein synthesis, carbon fixation, and chlorophyll production in microalgae,[61,62] which may be severely affected under limited mineral conditions.

3.3.2. Biochemical analyses

3.3.2.1. Lipid and protein contents. *P. gyrans* resulting from the validation assays reached a protein content of 45.86, 42.11, 30.76, and 10.59% w/w under the growth conditions of Vit-, Lvl-1, Opt, and Con, respectively (Table 6). The assays Vit- and Lvl-1 had similar protein amounts, with significant differences ($p < 0.05$) for Opt and Con. In addition to increasing biomass production, the optimized growth conditions also allowed a 3-fold increase in protein content compared to the control ($p < 0.05$). Despite the variety of formulations, the highest protein

values were achieved in the assays that shared the optimal value of nitrogen, phosphorus, and copper.

In terms of lipid content, the experiments Lvl-1, Opt, and Con showed the highest value, ranging from 26.73 to 28.3% w/w (Table 6). The absence of vitamins significantly decreased the amount of lipids compared to the other conditions ($p < 0.05$), totaling only 19.83% w/w. The effect of time was also assessed in the lipid content and FA profile of optimum growth conditions – Table 6. For that, two different samples were taken throughout *P. gyrans* growth: one was taken after 10 days (Opt.i) and the second one at the end of microalgae growth (Opt – 14 days) – sample (b) in Fig. 2. Increasing the sampling time for 4 days enabled an increment of 5% in lipid content ($p < 0.05$), reaching 28.3% w/w at the end of the growth.

3.3.2.2. Fatty acid analysis. The FA profiles of *P. gyrans'* biomass produced in the validation experiment are presented in Table 6. In general, thirteen FAs (>1% TFA) were identified, of which five stand out most prominently (>10% TFA): C14:0, C16:0, C16:1 $n - 7$, C20:5 $n - 3$ and C22:6 $n - 3$. The FA profile described here is in accordance with that of other Pavlovophyceae species, such as *P. viridis*,[63] *P. pinguis*[14] and *D. lutheri*.[31] Among the identified FAs, a common feature of *P. gyrans* was verified: a high level of both EPA and DHA – which reached a maximum value of 27.03% and 11.65% TFA, respectively.

Optimal growth conditions produced noticeable differences compared to the control. The modifications carried out on growth conditions, mainly those on $NaNO_3$, $NaH_2PO_4 \cdot H_2O$, and

Table 6 Values for total lipids (% w/w), protein content (% w/w), FA composition (% of TFA), hypocholesterolemic index (HI), atherogenic index (AI), and thrombogenic index (TI) of *P. gyrans* from the validation assays: optimized conditions on days 10 (Opt.i) and 14 (Opt), control/Walne's medium, Con, medium without vitamins, Vit-, and assay with the variables considered non-significant in PB design fixed at level −1, Lvl-1. Values are the mean and standard deviation of three replicates ($n = 3$). Different letters indicate significant differences between the treatments (one-way ANOVA, $p < 0.05$, followed by Tukey's test)

		Opt.i (10 days)	Opt (14 days)	Con	Vit-	Lvl-1
Lipids (% w/w)		22.33 ± 0.32^a	28.30 ± 0.95^b	27.10 ± 0.96^b	19.83 ± 0.68^a	26.73 ± 3.25^b
Protein (% w/w)		—	30.76 ± 4.37^a	10.59 ± 0.32^b	45.86 ± 0.83^c	42.11 ± 0.45^c
% TFA	C14:0	11.33 ± 0.47	10.57 ± 0.89	11.55 ± 1.36	8.87 ± 1.13	9.41 ± 0.91
	C16:0	17.73 ± 0.21	19.32 ± 2.63	29.62 ± 2.96	20.25 ± 2.15	17.73 ± 0.21
	C16:1 $n - 7$	13.92 ± 1.94	17.88 ± 1.52	17.1 ± 1.49	8.83 ± 0.58	14.21 ± 0.50
	C16:2 $n - 4$	1.02 ± 0.05	0.99 ± 0.36	0.39 ± 0.05	0.66 ± 0.04	0.96 ± 0.15
	C18:0	3.46 ± 0.63	2.75 ± 0.32	2.21 ± 0.40	7.45 ± 1.39	3.38 ± 0.86
	C18:1 $n - 9$	0.85 ± 0.06	1.15 ± 0.09	1.59 ± 0.22	1.05 ± 0.20	1.13 ± 0.13
	C18:1 $n - 7$	1.11 ± 0.51	1.21 ± 0.15	0.81 ± 0.14	4.13 ± 0.83	1.82 ± 0.13
	C18:2 $n - 6$	1.74 ± 0.52	1.24 ± 0.26	2.03 ± 0.37	0.28 ± 0.05	2.16 ± 0.16
	C18:3 $n - 3$	0.78 ± 0.03	0.69 ± 0.15	0.42 ± 0.05	0.67 ± 0.17	1.18 ± 0.13
	C18:4 $n - 3$	7.04 ± 0.80	6.56 ± 0.62	3.25 ± 0.46	7.77 ± 0.97	6.70 ± 0.33
	C20:5 $n - 3$	24.33 ± 1.03^{ac}	20.69 ± 1.61^{ad}	17.09 ± 1.65^{bd}	27.03 ± 2.41^{ce}	23.37 ± 0.51^{ae}
	C22:5 $n - 6$	7.36 ± 0.82	6.60 ± 0.11	8.21 ± 0.99	4.30 ± 0.29	6.32 ± 0.52
	C22:6 $n - 3$	9.30 ± 0.80^a	10.33 ± 0.30^{ad}	5.73 ± 0.62^b	8.71 ± 0.80^a	11.65 ± 0.83^{cd}
	SFAs	32.53 ± 1.03^{ac}	32.65 ± 2.25^{ac}	43.37 ± 2.25^b	36.57 ± 2.46^a	30.51 ± 1.36^c
	MUFAs	15.88 ± 2.29^{ac}	20.24 ± 1.35^b	19.5 ± 1.62^{ab}	14.01 ± 1.28^{cd}	17.16 ± 0.44^{abd}
	PUFAs	51.59 ± 1.54^a	47.11 ± 3.24^a	37.13 ± 3.5^b	49.42 ± 3.45^a	52.33 ± 1.59^a
	$n - 3$ FAs	41.46 ± 0.47^{ac}	38.27 ± 2.57^a	26.49 ± 2.33^b	44.19 ± 3.20^{cd}	42.89 ± 1.08^{ad}
	$n - 6$ FAs	9.10 ± 1.35	7.84 ± 0.36	10.25 ± 1.24	4.57 ± 0.32	8.47 ± 0.66
	$n - 3/n - 6$	4.62 ± 0.72	4.88 ± 0.15	2.59 ± 0.1	9.66 ± 0.32	5.08 ± 0.28
HI		—	1.63 ± 0.21^a	0.96 ± 0.15^b	1.85 ± 0.18^a	2.00 ± 0.12^a
AI		—	0.91 ± 0.05^a	1.34 ± 0.12^b	0.88 ± 0.03^a	0.80 ± 0.06^a
TI		—	0.24 ± 0.03^a	0.45 ± 0.06^b	0.24 ± 0.03^a	0.21 ± 0.01^a

$CuSO_4 \cdot 5H_2O$ concentrations, resulted in a significant increase ($p < 0.05$) in the total PUFA content ($\approx 10\%$ TFA) and $n - 3$ FAs ($\approx 12\%$ TFA), with a concomitant decrease of $\approx 11\%$ in total SFAs ($p < 0.05$). Particularly, a substantial improvement in the DHA content was evident, which rose from 5.73 to 10.33% TFA ($p < 0.05$), with a similar trend for EPA – increased from 17.09 to 20.69% TFA ($p > 0.05$). Regarding the evolution of the FA profile of Opt between the onset of the stationary (Opt.i) phase and its end (Opt), no significant variation was noticed, except for the total MUFAs ($p < 0.05$) that increased with culture's aging.

The composition of *P. gyrans* grown with limited micronutrients presented a similar FA profile to the optimal conditions. Although a lower concentration of a significant number of nutrients hindered biomass production, this change in culture conditions did not greatly affect the FA composition. In fact, the comparison between Opt, Lvl-1, and Con assays allows us to assume that the production of FAs by *P. gyrans* was strongly dependent on individual or combined availability of nitrogen, phosphorus, and copper. The amount of nitrogen available has been reported as a crucial factor involved in lipids and FAs modelling in microalgae. Under N-replete conditions, microalgae are not stressed, preventing lipid membrane remodeling, in which an increased accumulation of neutral lipids (*e.g.* TAGs), at the expense of polar lipids, is promoted. Polar lipids are the main components of photosynthetic membranes in microalgae, which are the main sink of PUFAs and may explain the relatively high levels of EPA and DHA, as well as the lower content of SFAs on *P. gyrans*.[21,63]

The trial without supplementation of vitamins presented the highest value of EPA compared to Con and Opt (14 days) ($p < 0.05$). Furthermore, important variations were also obtained in five other FAs (statistical analysis was not carried out). Namely, Vit- had the lowest level of C16:1 $n - 7$, C18:2 $n - 6$, and C22:5 $n - 6$, and the highest level of C18:0 and C18:1 $n - 7$.

3.3.2.3. Nutritional indices. Taking into account the FA composition, the impact of validation conditions on the nutritional of the lipid fraction of biomass was estimated by the calculation of the hypocholesterolemic (HI), atherogenic (AI), and thrombogenic indices (TI) (Table 6). These indices allow the categorization of food products according to their healthiness for human diet, especially the likelihood to develop cardiovascular disease (CVD), which is usually associated with the accumulation of lipid plaques in the circulatory system (atherogenicity), clot formation in blood vessels (thrombogenicity), and cholesterol increase in blood (hypocholesterolemia).[41] In general, a FA composition rich in saturated residues, in contrast to unsaturated FAs, has pro-atherogenic, pro-thrombogenic, and hypercholesterolemic activity, promoting high AI and TI indices, and a reduced HI index.[41,64]

There was a significant variation ($p < 0.05$) in the values of AI, TI, and HI compared to the Con experiment (Table 6). The control condition produced the highest values for AI (1.34) and TI (0.45) and the lowest for HI (0.96). Opt assay yielded 0.93, 0.24, and 1.63 for AI, TI, and HI, respectively. This variation amongst the nutritional indices arises from the considerable increase of C16:0 (29.62% TFA) in Con (Table 6), a SFA considered pro-atherogenic, pro-thrombogenic, and

hypercholesterolemic, in contrast to microalga biomass enriched with $n - 3$ FA (38.27% TFA) and PUFAs (47.11% TFA) from Opt.[41] Similarly, the ratio $\sum n - 3 / \sum n - 6$ (% TFA) almost doubled in Opt (4.88) when compared to Con (2.59).

The nutritional values here reported for optimal growth conditions are in accordance with those of other microalgae. Aussant *et al.*[40] assessed the nutritional value of eight microalgae species and stated that the average values for AI, TI, and HI indices were 0.492–1.424, 0.197–0.836, and 0.523–2.014, respectively. Thus, considering that a healthy diet should be characterized by high intake of $n - 3$ FAs, low values of AI and TI, and relatively high values of HI,[8,64] *P. gyrans* cultured under optimum conditions presents itself as a potential source of healthy lipids for human consumption.

4. Conclusions

The present work studied the importance of seventeen growth parameters on the biomass production and biochemical composition of *Pavlova gyrans* through a sequential optimization procedure. It allowed the identification of the key variables for microalgae growth and understanding their impact and their interactions both on the growth performance and biochemical composition of *P. gyrans*. Optimized conditions substantially improved the final X_{max} and biochemical composition of biomass, namely the content of PUFAs and protein. These findings highlight the importance of tailoring *P. gyrans* growth conditions as a key strategy to identify and develop promising sources of healthy lipids with very interesting nutritional indices for human consumption.

Author contributions

Filipe Maciel: conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review & editing. Daniela Couto: conceptualization; data curation; formal analysis; investigation; methodology; writing – review & editing. Pedro Geada: conceptualization; writing – review & editing. Hugo Pereira: conceptualization; investigation; resources; writing – review & editing. José Teixeira: funding acquisition; resources; writing – review & editing. M. Rosário Domingues: conceptualization; resources; supervision; writing – review & editing. Joana Silva: conceptualization; resources; supervision; writing – review & editing. António Vicente: conceptualization; funding acquisition; project administration; resources; supervision; validation; writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

References

1 M. Peet and C. Stokes, *2005, Drugs*, 2005, **65**, 1051–1059.

2 P. M. Kris-Etherton, W. S. Harris and L. J. Appel, *Circulation*, 2002, **106**, 2747–2757.

3 M. Fotuhi, P. Mohassel and K. Yaffe, *Nat. Clin. Pract. Neurol.*, 2009, **5**, 140–152.

4 M. P. Judge, O. Harel and C. J. Lammi-Keefe, *Am. J. Clin. Nutr.*, 2007, **85**, 1572–1577.

5 EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), *EFSA J.*, 2012, **10**(7), 1–48.

6 B. D. Glencross, D. R. Tocher, C. Matthew and J. Gordon Bell, *Fish Physiol. Biochem.*, 2014, **40**, 1213–1227.

7 H. A. Hamilton, R. Newton, N. A. Auchterlonie and D. B. Müller, *Nat. Food*, 2020, **1**, 59–62.

8 A. P. Simopoulos, *Biomed. Pharmacother.*, 2002, **56**, 365–379.

9 BCC Research (b), *Global Omega 3 Market Size and Growth Forecast to 2025*, https://www.bccresearch.com/partners/verified-market-research/global-omega-3-market-size-and-forecast.html, (accessed 11 April 2022).

10 F. Guihéneuf, V. Mimouni, G. Tremblin and L. Ulmann, *J. Agric. Food Chem.*, 2015, **63**, 1261–1267.

11 S. P. Slocombe, Q. Zhang, M. Ross, A. Anderson, N. J. Thomas, Á. Lapresa, C. Rad-Menéndez, C. N. Campbell, K. D. Black, M. S. Stanley and J. G. Day, *Sci. Rep.*, 2015, **5**, 1–17.

12 K. I. Reitan, G. Øie, H. Jørgensen and X. Wang, *J. Appl. Phycol.*, 2021, **33**, 3831–3842.

13 V. Patil, T. Källqvist, E. Olsen, G. Vogt and H. R. Gislerød, *Aquacult. Int.*, 2007, **15**, 1–9.

14 T. Fernandes, A. Martel and N. Cordeiro, *Sci. Rep.*, 2020, **10**, 1–11.

15 A. Kanamoto, Y. Kato, E. Yoshida, T. Hasunuma and A. Kondo, *Mar. Biotechnol.*, 2021, **23**, 331–341.

16 S. M. Tibbetts and S. J. J. Patelakis, *Aquaculture*, 2022, **546**, 737236.

17 F. Ahmed, W. Zhou and P. M. Schenk, *Algal Res.*, 2015, **10**, 210–217.

18 S. M. Tibbetts, S. J. J. Patelakis, C. G. Whitney-Lalonde, L. L. Garrison, C. L. Wall and S. P. MacQuarrie, *J. Appl. Phycol.*, 2020, **32**, 299–318.

19 J. P. Cañavate and C. Fernández-Díaz, *Eur. J. Phycol.*, 2021, 1–21.

20 F. Guihéneuf, V. Mimouni, L. Ulmann and G. Tremblin, *J. Exp. Mar. Biol. Ecol.*, 2009, **369**, 136–143.

21 F. Guihéneuf and D. B. Stengel, *Mar. Drugs*, 2013, **11**, 4246–4266.

22 K. M. I. Bashir, S. Mansoor, N. R. Kim, F. R. Grohmann, A. A. Shah and M. G. Cho, *Ann. Microbiol.*, 2019, **69**, 353–368.

23 B. Huang, V. Mimouni, E. Lukomska, A. Morant-Manceau and G. Bougaran, *J. Phycol.*, 2020, **56**, 908–922.

24 A. P. Carvalho, I. Pontes, H. Gaspar and F. X. Malcata, *Enzyme Microb. Technol.*, 2006, **38**, 358–366.

25 A. P. Carvalho and F. X. Malcata, *Cryptogam.: Algol.*, 2000, **21**, 59–71.

26 M. Li, C. Hu, Q. Zhu, L. Chen, Z. Kong and Z. Liu, *Chemosphere*, 2006, **62**, 565–572.

27 M. Li, Q. Zhu, C. wei Hu, L. Chen, Z. li Liu and Z. Ming Kong, *J. Environ. Sci.*, 2007, **19**, 1330–1335.

28 X. Huang, L. Wei, Z. Huang and J. Yan, *J. Appl. Phycol.*, 2014, **26**, 105–114.

29 M. I. Rodrigues and A. F. Iemma, *Experimental Design and Process Optimization*, CRC Press, 1st edn, 2014.

30 R. A. Andersen, *Algal Culturing Techniques*, Elsevier Academic Press, 2005.

31 F. Guihéneuf and D. B. Stengel, *Algal Res.*, 2017, **23**, 113–125.

32 F. Guihéneuf, L. Ulmann, G. Tremblin and V. Mimouni, *Eur. J. Phycol.*, 2011, **46**, 143–152.

33 T. Fernandes and N. Cordeiro, *J. Appl. Phycol.*, 2022, **34**, 757–775.

34 I. Dahmen-Ben Moussa, H. Chtourou, F. Karray, S. Sayadi and A. Dhouib, *Bioresour. Technol.*, 2017, **238**, 325–332.

35 J. P. Cañavate, I. Armada and I. Hachero-Cruzado, *J. Plant Physiol.*, 2017, **214**, 28–38.

36 H. Pereira, J. Páramo, J. Silva, A. Marques, A. Barros, D. Maurício, T. Santos, P. Schulze, R. Barros, L. Gouveia, L. Barreira and J. Varela, *Sci. Rep.*, 2018, **8**, 5112.

37 S. O. Lourenço, E. Barbarino, P. L. Lavín, U. M. Lanfer Marquez and E. Aidar, *Eur. J. Phycol.*, 2004, **39**, 17–32.

38 D. Couto, T. Melo, T. A. Conde, M. Costa, J. Silva, M. R. M. Domingues and P. Domingues, *Algal Res.*, 2021, **53**, 1–15.

39 D. Couto, T. Melo, T. A. Conde, A. S. P. Moreira, P. Ferreira, M. Costa, J. Silva, R. Domingues and P. Domingues, *Food Chem.*, 2022, **375**, 1–10.

40 J. Aussant, F. Guihéneuf and D. B. Stengel, *Appl. Microbiol. Biotechnol.*, 2018, **102**, 5279–5297.

41 J. Chen and H. Liu, *Int. J. Mol. Sci.*, 2020, **21**, 1–24.

42 S. Seoane, M. Zapata and E. Orive, *J. Sea Res.*, 2009, **62**, 286–294.

43 Y. Su, *Sci. Total Environ.*, 2021, 762.

44 J. H. Janssen, J. Kastenhofer, J. A. de Hoop, P. P. Lamers, R. H. Wijffels and M. J. Barbosa, *Algal Res.*, 2018, **33**, 125–132.

45 S. H. Kim, I. Y. Sunwoo, H. J. Hong, C. C. Awah, G. T. Jeong and S. K. Kim, *Bioprocess Biosyst. Eng.*, 2019, **42**, 1517–1526.

46 L. Kong and N. M. Price, *Metallomics*, 2020, **12**, 1106–1117.

47 B. Debelius, J. M. Forja, Á. DelValls and L. M. Lubián, *Ecotoxicol. Environ. Saf.*, 2009, **72**, 1503–1513.

48 L. M. Blank, *Curr. Opin. Biotechnol.*, 2012, **23**, 846–851.

49 M. S. Sadahira, M. I. Rodrigues, M. Akhtar, B. S. Murray and F. M. Netto, *Food Hydrocolloids*, 2016, **58**, 1–10.

50 T. Purbonegoro, Suratno, R. Puspitasari and N. A. Husna, in *IOP Conference Series: Earth and Environmental Science*, Institute of Physics Publishing, 2018, vol. 118.

51 G. S. Rocha, A. T. Lombardi and M. da G. G. Melão, *Ecotoxicol. Environ. Saf.*, 2016, **128**, 30–35.

52 P. Varshney, S. Sohoni, P. P. Wangikar and J. Beardall, *J. Appl. Phycol.*, 2016, **28**, 2631–2640.

53 T. Fernandes and N. Cordeiro, *Biomass Bioenergy*, 2022, **158**, 1–12.

54 J. Jia, D. Han, H. G. Gerken, Y. Li, M. Sommerfeld, Q. Hu and J. Xu, *Algal Res.*, 2015, **7**, 66–77.

55 J. C. Nzayisenga, X. Farge, S. L. Groll and A. Sellstedt, *Biotechnol. Biofuels*, 2020, **13**(4), 1–8.

56 F. F. Chu, P. N. Chu, X. F. Shen, P. K. S. Lam and R. J. Zeng, *Bioresour. Technol.*, 2014, **152**, 241–246.

57 A. C. Guedes, L. A. Meireles, H. M. Amaro and F. X. Malcata, *JAOCS, J. Am. Oil Chem. Soc.*, 2010, **87**, 791–801.

58 M. Nayak, W. I. Suh, B. Lee and Y. K. Chang, *Energy Convers. Manage.*, 2018, **156**, 45–52.

59 R. Jafarpour, S. Vahdat, F. Asadi, H. K. Dardashti, B. Zarei and A. Dadkhah, *J. Appl. Phycol.*, 2023, **35**, 1553–1563.

60 C. Nef, S. Jung, F. Mairet, R. Kaas, D. Grizeau and M. Garnier, *Sci. Rep.*, 2019, **9**, 1–11.

61 G. R. Rout and S. Sahoo, *Rev. Agric. Sci.*, 2015, **3**, 1–24.

62 N. Farhat, A. Elkhouni, W. Zorrig, A. Smaoui, C. Abdelly and M. Rabhi, *Acta Physiol. Plant.*, 2016, **38**(145), 1–10.

63 X. Huang, Z. Huang, W. Wen and J. Yan, *J. Appl. Phycol.*, 2013, **25**, 129–137.

64 Y. A. Attia, M. A. Al-Harthi, M. A. Korish and M. M. Shiboob, *Lipids Health Dis.*, 2015, **14**(136), 1–8.