



# A new culture medium based on genetic algorithms for *Isochrysis galbana* production relevant to hatcheries

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## Abstract

The nutrient content of a commercial seawater culture medium for growing the microalga *Isochrysis galbana* was optimized using a stochastic strategy based on genetic algorithms. For this, 210 experiments spread over seven generations were carried out. This strategy reduced the number of assays by more than 90% compared to a factorial design involving the optimization of twelve nutrients simultaneously. The optimized medium outperformed the reference medium in all aspects. The genetic algorithm strategy achieved a polyunsaturated fatty acids (PUFAs) productivity of 7.8 mg L<sup>-1</sup> day<sup>-1</sup> in a continuous culture of *I. galbana*, corresponding to an increase of 15% compared to the commercial formulation. Carotenoids, on the other hand, increased by 50% d.w. In addition, PUFA yields were significantly improved, which allowed us to reduce the requirement of several nutrients, for instance, N (25%), Mo (20%), Mn (60%), Co (60%), and Cu (60%).

**Keywords** Microalgae · Medium optimization · Genetic algorithm · *Isochrysis galbana* · Aquaculture · PUFAs · Fucoxanthin

## Introduction

In recent decades the use of microalgae as a food supplement and as functional additives in marine aquaculture has grown rapidly. Species commonly used for animal feed in aquaculture include the genera *Chlorella*, *Isochrysis*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Tetraselmis*, *Dunaliella*, *Scenedesmus*, *Thalassiosira*, and *Skeletonema*. The supply of microalgae is fundamental to certain aquaculture stages. Thus, microalgae are used to feed copepods, rotifers, and *Artemia*, which in turn are commonly used as the main live prey during the rearing of many larval marine species. Important progress has recently been made to allow for the direct use of microalgae as feed in all the mollusk life stages (Sirakov et al. 2015), as well as in the larval or juvenile stages of some fish (Vizcaíno et al. 2014). Raw microalgae biomass has been evaluated as feed for seabass (*Dicentrarchus labrax*), an alternative to oil and fishmeal, with substitution levels higher than 10% and 36%, respectively (Cardinaletti et al. 2018), or even 50% in the case

of red drum (*Sciaenops ocellatus*) (Perez-Velazquez et al. 2018). Accordingly, microalgae are generating great interest, not only because of their protein content, essential amino acids, and PUFAs, but also because of the presence of other compounds that give them an additional role as a functional ingredient for feed. In fact, numerous studies describe positive effects on muscle growth, coloration and oxidative state, intestinal ultrastructure and functionality, stress response, and disease resistance (Vizcaíno et al. 2014, 2016). From this perspective, using microalgae as a functional ingredient at a low inclusion level is seen as a promising strategy for improving the growth and general condition of the fish. Carotenoids are used in aquaculture as functional additives. Fish, like other animals, cannot synthesize carotenoids, so incorporating them to achieve natural pigmentation depends on their intake. Microalgal biomass has proven to be a valuable source of natural pigments (Camacho-Rodríguez et al. 2016; Sun et al. 2019). Microalgal carotenoids have been shown to carry out important biological functions in various fish species (Pereira da Costa and Campos Miranda-Filho 2020) due to their antioxidant properties (Pham et al. 2014; Vizcaíno et al. 2014), acting as an immune system modulator and influencing skin muscle pigmentation (Pham et al. 2014; Sales et al. 2020).

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Therefore, the main requirements that a microalga species must currently satisfy for use in aquaculture are a high total lipid content (13–33%, dry weight), a considerable accumulation of polyunsaturated fatty acids (PUFAs) (Huerlimann et al. 2010; Camacho-Rodríguez et al. 2013; Vizcaíno et al. 2014), and a high pigment content. For this last requirement, chlorophylls, carotenoids, and phycobiliproteins are sought (Lubián et al. 2000). *Isochrysis* is well known as a source of different valuable pigments such as fucoxanthin, diadinoxanthin, and  $\beta$ -carotene (Borowitzka 2013; Ahmed et al. 2014) and a good potential source of docosahexaenoic acid (DHA) (Tzovenis et al. 1997). Consequently, for decades, *Isochrysis galbana* has been used worldwide in the aquaculture industry. For instance, Sukenik and Wahnon (1991) evaluated the biomass quality of this species three decades ago, with special attention to the lipid content, for its possible use in aquaculture.

To supply the microalgae, the aquaculture industry standard has been to use pond or bag systems, where the biomass is produced with no controlled conditions applied. Due to the low productivity and the high cost entailed in producing the necessary microalgae in situ, microalgae production is considered the main bottleneck to aquaculture industry development (Muller-Feuga 2000; Norsker et al. 2011; Acien et al. 2012). Given their critical role in aquaculture, more efficient microalgal biomass production systems using photobioreactors have been implemented in recent years (Naumann et al. 2013; Taelman et al. 2013).

Besides more efficient culture systems, the polyunsaturated fatty acid contents in microalgae can be enhanced by several strategies including the manipulation of the environmental parameters and using media formulations containing different extracts or growth stimulators (Hoffmann et al. 2010; Rohani-Ghadikolaei et al. 2012). However, nutrient availability and the medium formulation are the most important aspects to consider when the fatty acid and carotenoid contents are to be enhanced (Carvalho et al. 2006). The nutritional requirements vary depending on the species and its application. For example, in a culture destined for biofuel production, the objective is to accumulate fatty acids with the lowest possible level of unsaturation, establishing the maximum level of PUFAs at 1% (Chisti 2007), while in aquaculture, a balance is sought between the PUFAs and protein. Therefore, for each application a tailored formulation must be obtained.

Media formulation optimization using genetic algorithms (GA) is an efficient search strategy that allows the culture medium to be improved in a faster way than using a factorial design. One cannot avoid the large number of experiments needed for a traditional strategy, even without considering the interaction between nutrients. In contrast, a GA methodology can be set to conduct a reasonable number of cultures at a time, and improvements are found within a few

generations (Link and Weuster-Botz 2006; García-Camacho et al. 2011; Camacho-Rodríguez et al. 2015). This method has been used previously for the optimization of macroorganism growth (Marteijn et al. 2003) and also for microbial processes, with satisfactory results.

The aim of this paper was to determine the nutritional requirements for the growth of a previously selected marine microalgal strain using a stochastic search strategy through genetic algorithms. This strategy was based on optimizing the content of each nutrient in a previous culture medium (ALGAL; Aqualgae S.L., A Coruña, Spain), which is used to grow microalgae destined for aquaculture. PUFAs were selected as the objective function although carotenoids were also determined. Finally, the enhanced medium was validated in continuous culture to check the feasibility of using it for the large-scale culture of *I. galbana*.

## Materials and methods

### Microorganism and culture maintenance

The prymnesiophyte *Isochrysis* aff. *galbana* (CCAP 927/1) was obtained from the Culture Centre for Algae and Protozoa (Cambridge, UK). Cultures (inocula) were aseptically maintained in photoautotrophic mode in 1-L Erlenmeyer flasks, with a liquid volume of 700 mL at  $25 \pm 2$  °C. The flasks were continuously illuminated with an incident irradiance of  $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , using white light lamps (Philips PL-32 W/840/4p). As the culture medium, the commercial marine formulation ALGAL was selected although the  $\text{KNO}_3$  was reduced to  $0.4 \text{ g L}^{-1}$ . The original ALGAL medium formulation is  $0.8 \text{ KNO}_3$ ;  $0.027 \text{ NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ;  $0.0104 \text{ C}_6\text{H}_5\text{FeO}_7 \cdot \text{H}_2\text{O}$ ;  $0.00115 \text{ Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ;  $0.00084 \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$ ;  $0.0006 \text{ ZnCl}_2$ ;  $0.0001 \text{ CoCl}_2 \cdot 6\text{H}_2\text{O}$ ;  $0.00015 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$ ;  $0.011 \text{ EDTA}$ ;  $0.00208 \text{ Thiamine}$ ;  $0.000027 \text{ Biotin}$ ;  $0.000019 \text{ Cyanocobalamin}$  (all quantities are in  $\text{g L}^{-1}$ ) (Aqualgae S.L., A Coruña, Spain). The macronutrient solution, previously sterilized in an autoclave at  $126$  °C for 20 min, was added to natural seawater. The micronutrient solution was sterilized by filtering through a  $0.22\text{-}\mu\text{m}$  filter (Whatman GF/F, UK).

### Genetic algorithm: culture conditions and medium composition

Assays were started using nutrient-depleted inoculum. The batch cultures were performed in this way for 14 days to assure that the macronutrients were consumed. One hundred-milliliter Erlenmeyer flasks with an effective volume of 60 mL were used. The flasks were agitated in an orbital shaker at 100 rpm without bubbling, under continuous illumination ( $166 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and at a temperature

of  $25 \pm 2^\circ\text{C}$ . Culture growth was evaluated after seven days. In a previous work (Camacho-Rodríguez et al. 2020), several assays were carried out to find the inhibiting concentration (upper limit) for each individual component included in the commercial ALGAL medium. The range of concentrations tested for each nutrient is shown in Table 1.  $\text{CO}_2$  mass transfer was calculated using an empirical equation for  $k_L a$  in shaken flasks (Veljkovic et al. 1995). Values of  $154 \text{ h}^{-1}$  implied  $\text{CO}_2$  supply of  $1.6 \text{ mmol CO}_2 \text{ h}^{-1} \text{ L}^{-1}$ . Thus,  $\text{CO}_2$  mass transfer was similar to the one obtained in typical PBRs (Rubio et al. 1999).

The optimization of the culture medium composition was carried out through a four-stage cyclic genetic algorithm based on the creation of new populations through “genetic manipulation” (García-Camacho et al. 2011). For this, GALOP software (Institute of Biotechnology, Jülich, Germany) was used (Beste et al. 1997). The first stage of this process consisted of randomly choosing a first generation (randomization) within the limits established in the nutrient concentration range (Table 1). The twelve components of the culture medium were the variables encoded in binary character strings. The media formulation is known as the “chromosome” for each experiment. For the subsequent generation, crosses were made based on the best chromosome obtained from the previous generation. The productivity of the PUFAs (PPUFAs) was selected as the selection criterion. The “Roulette” selection method (Muffler et al. 2007), with an exchange rate or genetic crossing of 0.95 and a mutation probability of 0.01, was selected. In each generation, fifteen duplicate individuals were evaluated in parallel (a total of 30 cultures).

### Comparison of the best media for continuous culture in PBRs

Once the culture medium formulation was optimized by means of the genetic algorithm, *I. galbana* was continuously cultivated to compare the best medium obtained from the original ALGAL medium using this strategy; the nitrate level was set to an initial concentration of 4 mM (Camacho-Rodríguez et al. 2020). These experiments were carried out in indoor bubble columns with a 1.8 L capacity, an inner diameter of 0.07 m, and a height of 0.5 m. The PBRs were illuminated simulating a circadian cycle with a maximum incident irradiance of  $1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (12:12 h; light:dark) using white light lamps (Philips PL-32 W/840/4p). Six lamps were placed vertically aligned around the outside surface of the cylindrical vessel. Aeration was supplied to the base of the column at 0.5 vvm. The temperature was maintained at  $25.0 \pm 0.5^\circ\text{C}$  using refrigerated water as the cooling agent. The pH was maintained in the 7.8 to 8.0 range by injecting carbon dioxide on demand. The experiments started with

**Table 1** Nutrient concentration range considered for the batch culture media formulation, the optimized commercial ALGAL medium formulation, and the optimal medium (Generation 7) formulation for the continuous culture found in this work

	KNO <sub>3</sub>	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	C <sub>6</sub> H <sub>5</sub> FeO <sub>7</sub> ·H <sub>2</sub> O	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	MnCl <sub>2</sub> ·4H <sub>2</sub> O	ZnCl <sub>2</sub>	CoCl <sub>2</sub> ·6H <sub>2</sub> O	CuSO <sub>4</sub> ·5H <sub>2</sub> O	EDTA	<sup>1</sup> BI	<sup>2</sup> B7	<sup>3</sup> B12
Concentration range (batch culture) [g L <sup>-1</sup> ]	0–0.8	0–0.06	0–0.01	0–0.0012	0–0.0006	0–0.0001	0–0.00015	0–0.01	0–0.002	0–3·10 <sup>-5</sup>	0–2·10 <sup>-5</sup>	
Concentration range (continuous culture) [g L <sup>-1</sup> ]	G-7	0.32	0.054	0.0021	0.00017	0.00038	0.00002	0.00003	0.00376	0.00125	2.1·10 <sup>-5</sup>	4·10 <sup>-7</sup>
	<sup>4</sup> N-ALG-AL	0.4	0.027	0.0052	0.00042	0.00031	0.00005	0.000074	0.0047	0.001	1.35·10 <sup>-5</sup>	9.4·10 <sup>-6</sup>

<sup>1</sup> Tiamine <sup>2</sup> Biotin <sup>3</sup> Cyanocobalamin <sup>4</sup> N-optimized ALGAL (Camacho-Rodríguez et al. 2020)

a biomass concentration of  $0.17 \text{ g L}^{-1}$  in discontinuous mode. When the biomass reached a concentration of  $1 \text{ g L}^{-1}$ , the continuous mode started. The dilution rate was fixed at  $0.3 \text{ day}^{-1}$  since this value was previously determined to be optimum for the N-ALGAL medium under these conditions (Camacho-Rodríguez et al. 2020).

## Analytical procedures

The monitoring of growth was carried out measuring optical density (750 nm). In the continuous cultures, the biomass concentration was determined daily. Samples were periodically taken for biomass dry weight determination. The culture samples were centrifuged at 6640 rcf for 5 min. Then they were washed with 0.5 M ammonium bicarbonate (Zhu and Lee 1997) with 5 mL of this solution was used per g of wet biomass. Two washes were performed to remove the salts. The biomass samples were freeze-dried and stored at  $-20^\circ\text{C}$ . The fatty acid content was determined by gas chromatography using an adaptation of the method of Rodríguez-Ruiz et al. (1998). In brief, 8 mg of biomass was ground up with an equivalent mass of alumina. It was then methylated by adding 1 mL of hexane, 50  $\mu\text{L}$  of a standard grade acid solution (C19:0 nonadecanoic acid), and 1 mL of the methylating mixture (acetyl chloride-methanol 5:100 v:v). To carry out the methylation, the solution was heated in a thermoblock at  $105^\circ\text{C}$  for 20 min and stirred once with a vortex shaker after 10 min. The sample was allowed to cool to room temperature before adding 1 mL of distilled water. Then, it was centrifuged at 3500 rpm for 5 min. The hexane phase was analyzed by gas chromatography. Similarly, the carotenoid content was determined by HPLC using the saponification-extraction method described by Cerón-García et al. (2018). To do this 5 mg of biomass was mixed with 5 mg of alumina and an equal volume of glass beads (0.5 mm glass beads, BioSpec Products Inc., USA) in 1 mL of a three-component solution containing hexane (18%), ethanol (76%), and KOH (10% mass fraction) in water (6%). Once the three-component mixture had been added, the saponification was performed for 2 min in a mini-bead-beater (MiniBeadBeater-16, Model 607EUR, BioSpec Products Inc., USA). It was then centrifuged for 2 min at 12,000 rpm in an Eppendorf centrifuge (Eppendorf AG 22,331, Germany) and 800  $\mu\text{L}$  (approx.) of the supernatant was transferred to the chromatography vials. The pigment content was measured directly in an HPLC-fluorescence detector (RF-10A XL, Shimadzu, Japan) using the method described by Cerón et al. (2007). Analytical standards of fucoxanthin and diadinoxanthin were acquired from DHI (Denmark), while  $\beta$ -carotene was obtained from Sigma Chemical Co. (USA).

## Kinetic parameters

The biomass productivity ( $P_b$ ,  $\text{g L}^{-1} \text{ day}^{-1}$ ) was calculated by Eq. 1 as the difference between the initial and final biomass concentration ( $C_X$ ,  $\text{g L}^{-1}$ ), divided by the cultivation time ( $t$ , days):

$$P_b = \frac{C_{X\text{final}} - C_{X\text{initial}}}{t} \quad (1)$$

The productivity of the PUFAs ( $P_{\text{PUFAs}}$  ( $\text{mg L}^{-1} \text{ day}^{-1}$ )) was calculated as:

$$P_{\text{PUFAs}} = P_b \cdot Y_{\text{PUFAs}/X} \quad (2)$$

where  $Y_{\text{PUFAs}/X}$  is the PUFA yield of the biomass ( $\text{g PUFAs g}^{-1}$  biomass).

The productivity of carotenoids ( $P_{\text{carot}}$  ( $\text{mg L}^{-1} \text{ day}^{-1}$ )) was calculated according to Eq. 3:

$$P_{\text{carot}} = P_b \cdot Y_{\text{carot}/X} \quad (3)$$

where  $Y_{\text{carot}/X}$  is the carotenoid yield of the biomass ( $\text{g carot g}^{-1}$  biomass).

The yields for the PUFAs and the carotenoids with respect to the main substrates (nitrogen and phosphorus) achieved in the continuous cultures were calculated according to Eqs. 4 and 5, as the product of  $Y_{\text{PUFAs}/X}$  and  $Y_{\text{carot}/X}$ , respectively, and the concentration of biomass in the culture ( $C_X$ ,  $\text{g L}^{-1}$ ) divided by the concentration of the nutrient in the medium ( $C_S$ ,  $\text{g L}^{-1}$ ):

$$Y_{\text{PUFAs}/S} = Y_{\text{PUFAs}/X} \cdot \frac{C_X}{C_S} \quad (4)$$

$$Y_{\text{carot}/S} = Y_{\text{carot}/X} \cdot \frac{C_X}{C_S} \quad (5)$$

## Economic evaluation

An economic balance was made based on the optimum formulation in which the cost of each nutrient, as well as the requirements of each, has been calculated. The cost of each component is based on the technical grade product at laboratory size (100–1000 g). The reference value was that of the ALGAL medium, which was around 1€ per L. The aim was to determine if the productivity improvements due to the new formulation could imply higher or lower cost. As components have very different market prices, the medium cost is more sensitive to changes of some components. A detailed description of the procedure can be found elsewhere (Camacho-Rodríguez et al. 2020).

## Statistical analysis

All experiments were carried out in triplicate. The experimental results were given as the average values of the three independent experiments and their standard deviation. Statistical data analyses were performed using the Statgraphics Centurion XVI software package. The normality and homogeneity analyses were performed using the Kolmogorov–Smirnov and Levene tests, respectively. The average values of  $P_b$  and  $P_{PUFAs}$  have been determined with their corresponding standard deviations. For this, a one-way ANOVA was conducted on the average values of  $P_b$  and  $P_{PUFAs}$  in each experiment. A confidence level of 95% and  $p$  values  $< 0.05$  were considered statistically significant.

## Results

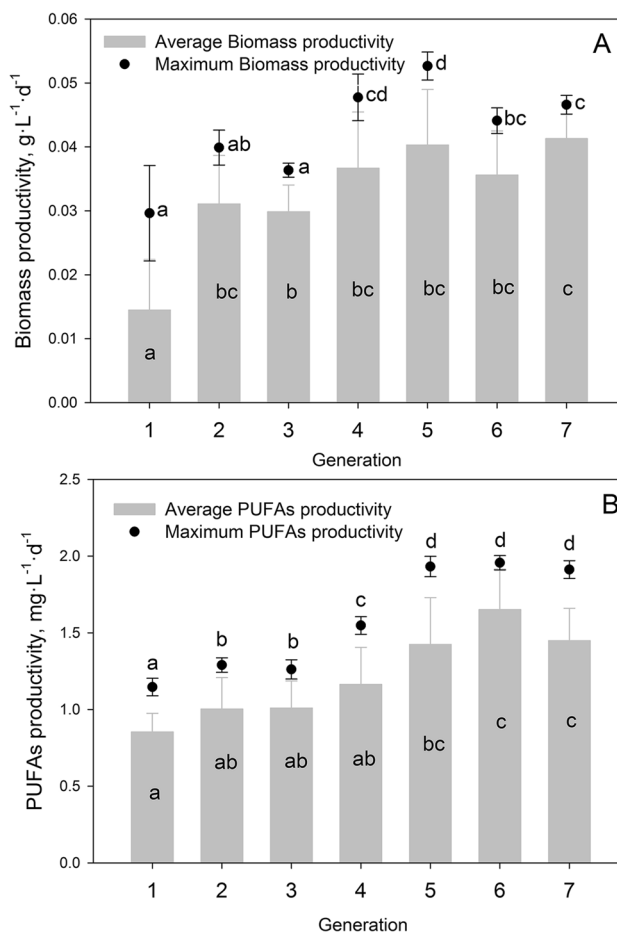
### Culture optimization process

In Fig. 1A, a significant increase in the average biomass productivity value for each successive generation ( $p = 0.00001$ ) can be seen. The software determined the medium formulations for each generation based on the best result from the previous generation.

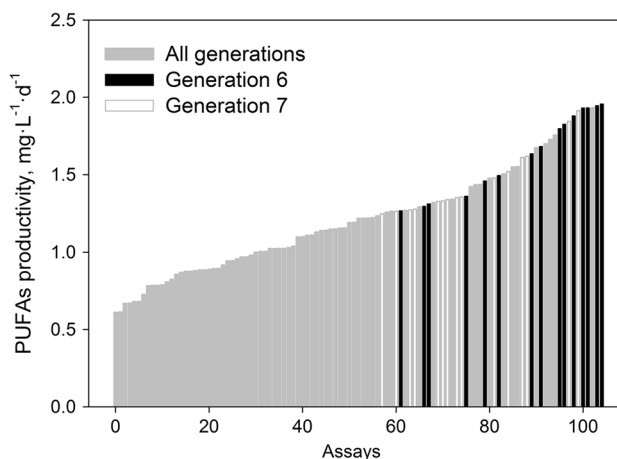
From the average of all the mediums within a generation, the maximum biomass productivity value ( $56 \text{ mg L}^{-1} \text{ day}^{-1}$ ) was reached in generation 5, and it was almost three times higher than the best value achieved in the initial generation. Similarly, in Fig. 1B, we can see the average PUFA productivity values for all the experiments, as well as the maximum value in each generation. Each generation (up to the sixth) produced an enhancement compared to the previous generation (significant differences with a 95% confidence level,  $p < 0.05$ ). The maximum intergenerational PUFA productivity average was reached in generation 6, an average value of  $1.65 \text{ mg L}^{-1} \text{ day}^{-1}$ . The maximum individual value was also achieved in a culture medium from this generation, with a value of  $1.96 \text{ mg L}^{-1} \text{ day}^{-1}$ . The improvement obtained in the PUFA productivity in this generation was twice that of the initial value (Fig. 1B).

Figure 2 shows the values obtained for the productivity of the PUFAs in all the experiments carried out over the 7 generations. The values achieved in generations 6 and 7 have been highlighted to show that no improvement was observed compared to the values obtained in the previous three generations. That is why a decision was made to terminate the genetic algorithm optimization at this point.

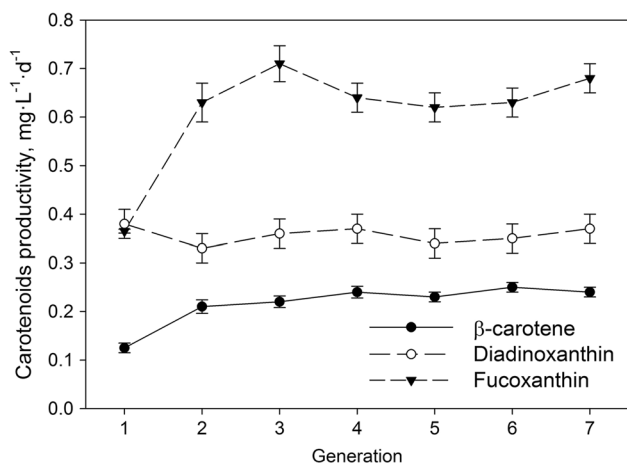
As can be seen in Fig. 3, the values of the main *I. galbana* carotenoids (fucoxanthin, diadinoxanthin, and  $\beta$ -carotene) have been represented; these correspond to the experiments with the best culture media for each generation (according to the selection criteria).



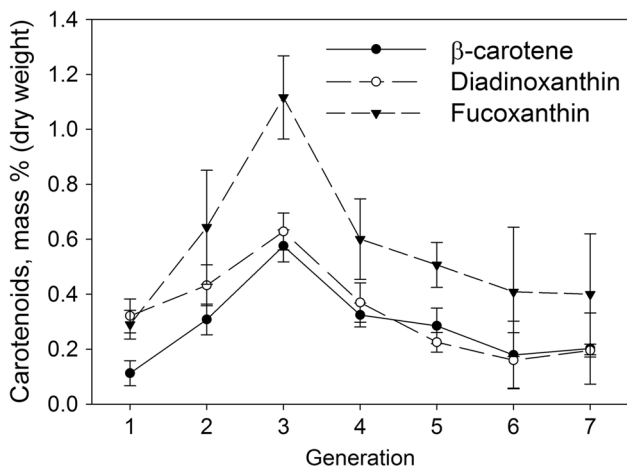
**Fig. 1** Average and best productivity in each generation of the optimization process. (A) Biomass productivity; (B) PUFA productivity. Different letters indicate significant differences ( $p$ -value  $< 0.05$ ) within each treatment



**Fig. 2** PUFA productivity in all the experiments carried out during the optimization process



**Fig. 3** Productivity of carotenoids (fucoxanthin, diadinoxanthin, and β-carotene) for the best culture media in each generation



**Fig. 4** Content of carotenoids (fucoxanthin, diadinoxanthin, and β-carotene) expressed in mass % (dry weight) for the best culture media in each generation

Diadinoxanthin productivity did not suffer significant differences for the different media tested, obtaining an average value of 0.37 mg L<sup>-1</sup> day<sup>-1</sup>. However, with respect to the β-carotene and fucoxanthin contents, a significant increase in productivity was observed after the first generation, although after the second and third generation,

no significant variation could be appreciated between generations.

In Fig. 4, the percentage in mass has been plotted for the best culture media in each generation. As one can see, the biomass containing the highest carotenoid content was found in the third generation. From G-4, steady composition was observed for all the main *I. galbana* carotenoids.

### Continuous culture with the best culture media

The best culture medium proposed by the genetic algorithm obtained in generation 7 was further evaluated under continuous culture conditions. It was compared with the nitrogen-optimized (4 mM of N) ALGAL medium. The biomass productivity values were almost identical (0.212 and 0.211 g L<sup>-1</sup> day<sup>-1</sup>) for the best G-7 and N-optimized ALGAL media (Table 2). There was no significant difference in the biomass productivity improvement between the two media since the ALGAL medium was previously improved (with nitrogen) and our objective function here was the PUFA content. Under continuous mode, the biomass cultivated using the best G-7 medium achieved an accumulation of 3.69% (dry weight), 15% higher than that achieved with the N-optimized ALGAL medium, which was 3.22% (Table 2). Therefore, the productivity of the PUFAs obtained with the best G-7 medium was also 15% higher than that achieved with the N-optimized ALGAL medium, with values of 7.8 and 6.8 mg L<sup>-1</sup> day<sup>-1</sup>, respectively (Table 2). This value of 7.8 implies a PUFA content of 42%, based on the total fatty acids. With respect to carotenoids, the accumulation of fucoxanthin when using the best G-7 medium increased from 0.54 to 0.81% (dry weight) compared to using the N-optimized ALGAL medium (Table 2). In the case of diadinoxanthin, the increase achieved was similar, from 0.05 to 0.08% (dry weight) with the best G-7 medium (Table 2). The β-carotene content was not affected by varying the culture medium composition. As a result of the higher fucoxanthin and diadinoxanthin contents, the total carotenoid content was 50% higher in the best G-7 medium compared to the N-optimized ALGAL medium (Table 2). If we compare the results of the different culture modes, we find that the continuous mode favored the accumulation of carotenoids. With the best G-7 medium, the biomass produced in continuous

**Table 2** Biomass and PUFA productivities (in mg·L<sup>-1</sup>·d<sup>-1</sup>), accumulation of PUFAs and carotenoids in the biomass (% dry mass), and the total carotenoids (% dry mass) for *I. galbana* grown in con-

tinuous mode, comparing the best medium proposed by the genetic algorithm (best of G-7) and the N-optimized ALGAL commercial medium (Camacho-Rodríguez et al. 2015)

Medium	% PUFAs	P <sub>B</sub>	P <sub>PUFAs</sub>	% fucoxanthin	% diadinoxanthin	% β-carotene	% total carotenoids
Best G-7	3.69	0.212	7.8	0.81	0.08	0.04	1.04
N-ALGAL	3.22	0.211	6.8	0.54	0.05	0.04	0.69

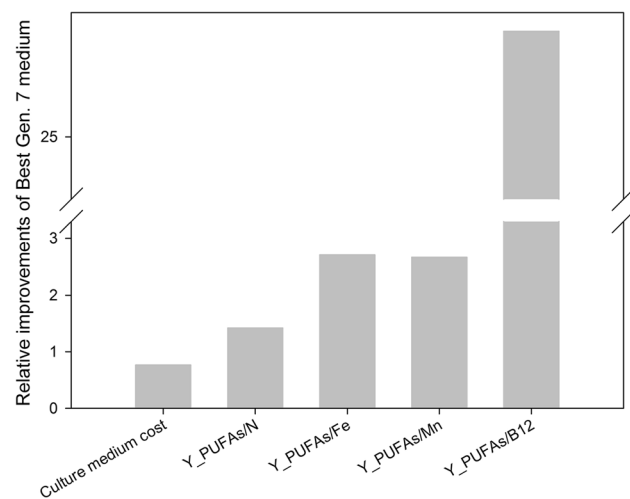
mode contained 1.04% carotenoids; this is a 20% improvement compared to batch mode.

### Economic balance: culture media composition

The concentration of each nutrient in the best G-7 and N-optimized ALGAL media tested under continuous culture mode is shown in Table 1. Important differences can be observed between them. Based on this, the cost of each ingredient has been calculated. The market price and the requirement of each ingredient were used to estimate a medium cost (see the “Materials and methods” section). Thus, a cost saving of around 25% was obtained for the algorithm-optimized medium compared to the N-optimized ALGAL medium (see Fig. 5). Figure 5 shows the PUFA yield ( $Y_{\text{PUFAS}/X}$ , gPUFAs/gX) with respect to several nutrients, including nitrogen (as the main macronutrient) and micronutrients such as iron, manganese, and cyanocobalamin (vitamin B12). The maximum PUFA yield for the best G-7 medium has been relativized in relation to the N-optimized ALGAL medium. The improvement in the PUFAs/N yield was 42%; in the cases of Fe and Mn, the yield improved by around 3 times, whereas for B12, it improved by almost 27 times.

### Discussion

Culture medium formulation is a critical factor in bioprocesses since it affects both microalgal growth and composition. Culture optimization using genetic algorithms is



**Fig. 5** Relative improvement in medium cost and PUFA yield with respect to the nutrients ( $Y_{\text{PUFAs}/X}$ ) obtained in a continuous culture of *I. galbana* comparing the best G-7medium (genetic algorithm) with the N-optimized ALGAL. Medium costs were calculated based on technical grade nutrients (100–1000 g)

usually conducted for the sole purpose of optimizing cell growth (e.g., Marteiijn et al., 2003; García-Camacho et al., 2011; Camacho-Rodríguez et al., 2015;). The product in our study is not directly linked to growth; instead, the objective function we aimed to maximize was the productivity of the PUFAs, although we also monitored the accumulation of carotenoids. The maximum attained biomass productivity ( $56 \text{ mg L}^{-1} \text{ day}^{-1}$ ) improved importantly the result of our internal reference. Lin et al. (2007) achieved similar biomass productivity values,  $57.4 \text{ mg L}^{-1} \text{ day}^{-1}$ , but a lower PUFA content, with *I. galbana* grown in Walne’s medium. The conditions that favor the accumulation of PUFAs (primary metabolites) are not necessarily the same conditions that induce the accumulation of carotenoids. However, one can observe that in each successive generation, culture growth was favored at the same pace as the primary metabolite (PUFAs) accumulation (Fig. 1A and B). Carotenoids are secondary metabolites, so stress conditions in the culture are required for their accumulation to increase. Within the first 3 generations, an important increase in total carotenoids productivity was also observed in our study. However, there were no further improvements from this G3. Fucoxanthin contributed almost exclusively to this increase. Conversely, carotenoids mass fraction decrease from G3 (where a maximum was observed). Nevertheless, the carotenoid results of final generations are in the range of the referenced values for this species and were higher than our control culture. For instance, Ahmed et al. found around 0.5% of carotenoids for *I. galbana* (Ahmed et al. 2014), whereas Sun et al. (2019), in their comparative analysis of 16 different *I. galbana* strains, reported higher values (0.9–1.6%). In our case, the formulations assayed showed a highly variable total carotenoid content, ranging between 0.4 and 2.3%.

The positive results obtained in batch cultures carried out in shaken flasks maintained in the continuous assay since more PUFAs (15%) and carotenoids (50%) were produced if we compared the proposed medium to the N-ALGAL. As expected, for the proposed medium the scaled-up continuous culture improved the productivity of the batch culture ( $0.212$  vs  $0.056 \text{ g L}^{-1} \text{ day}^{-1}$ ). This is a considerable biomass productivity. For instance, Patil et al. (2007), evaluating *I. galbana* grown in the “Z8” medium, achieved significantly lower biomass productivity values ( $0.16 \text{ g L}^{-1} \text{ day}^{-1}$ ) and a PUFA content of 39.9% (of the total fatty acid content).

Nutrient efficiency in synthesizing the PUFAs, expressed as the yield of PUFAs with respect to the different nutrients ( $Y_{\text{PUFAs}/X}$ , gPUFAs/gX), is an important economic parameter that can be used to select the most appropriate sources of a certain nutrient (Acién et al. 2012). Besides, it can be used to compare different formulations on the basis of a certain objective function. If we consider the composition of the media evaluated under continuous culture mode (Table 2), we can see that the nitrogen concentration in both media

increased significantly with respect to the reference medium. However, for the best G-7 medium, a similar biomass was obtained with 25% less nitrogen. This might be related to the fact that a PUFA productivity of  $7.8 \text{ mg L}^{-1} \text{ day}^{-1}$  was obtained for the best G-7 medium, which was 15% higher than that for the N-optimized ALGAL medium. Regarding the phosphorus concentration in the culture medium, the optimal value at which the genetic algorithm converged was  $0.054 \text{ g L}^{-1}$ ; this is twice the concentration used by the N-optimized ALGAL medium ( $0.027 \text{ g L}^{-1}$ ). In similar studies where microalgal medium were optimized, the phosphorus concentration also increased significantly (Gallardo Rodríguez et al. 2009; García-Camacho et al. 2011; López-Rosales et al. 2015).

Micronutrients were also significantly changed during the optimization process. Micronutrients are essential for growth although their influence is not always easy to establish. Their requirements are influenced by interactions with other nutrients and culture conditions such as the sources or availability of nutrients, the light intensity, and the competition between different nutrients within the same metabolic pathway. All of these factors regulate cellular metabolism, which can lead to the accumulation of certain metabolites that can be further used (Procházková et al. 2014). Chen et al. (2011) carried out a daily determination of the micronutrient concentrations in a *Dunaliella tertiolecta* culture medium, finding measurable consumption in a few of them (i.e., Mo and Co). However, much of the initial content remained after 7 days of cultivation. This gives us an idea of the importance of these micronutrients for microalgal growth, but it also shows that they are formulated to excess on many occasions. Although micronutrients are formulated at low concentrations, their cost can be quite important. For instance, iron requirements, which are relatively high, were shown to influence the final medium cost (Stefels and van Leeuw 1998). The optimized G-7 medium allowed us to reduce the iron requirement 2.5 times without reducing the biomass or PUFA productivity. Similarly, the genetic algorithm significantly reduced the concentration of other micronutrients (see Table 2). In the present study, the Mo content in the optimized G-7 medium was 20% lower than in the N-optimized ALGAL medium. This reduction was even greater in the cases of Mn, Co, and Cu, all of which had a 60% lower content than in the ALGAL medium. Nevertheless, not all the micronutrient concentrations were reduced; for instance, the Zinc content was slightly increased.

The effect of group B vitamins on microalgal growth has been extensively studied although their mechanisms of action are not totally clear. Fábregas et al. (1998) found that *Haematococcus lacustris* needs thiamine, biotin, and cyanocobalamin (although requirements are low), while Gong and Chen (1997) found that these vitamins had no effect on growth in the same species. Based on the results

obtained in the present study, we can conclude that vitamin B12 is necessary for the growth and accumulation of PUFAs in *I. galbana*, since the media formulation that lacked it showed poor performance. The thiamine and biotin content increased slightly in the best G-7 medium with respect to the commercial medium, although the cyanocobalamin content could be reduced 20 times without affecting biomass or PUFA productivity.

## Conclusions

The use of a stochastic search strategy, in this case a genetic algorithm, has allowed us to formulate a new culture medium for *Isochrysis galbana* that improves the PUFA content both in batch and continuous mode. The biomass and carotenoid productivity were also improved for this species with respect to the reference medium (nitrogen-optimized ALGAL). During the genetic algorithm optimization, 210 individual cultures were carried out. This strategy reduced the number of assays by more than 90% compared to a factorial design with twelve nutrients and twelve levels. The new medium proposed by the genetic algorithm increased the productivity of the PUFAs in continuous culture by 15%, reducing the cost of biomass production by 24% compared to the N-optimized ALGAL medium. Carotenoids increased by 50% (d.w.) in the proposed medium, although other formulations assayed increased by only 2.3% (d.w). We showed that a reduction in several nutrient concentrations, such as nitrogen (by 25%), molybdenum (by 20%), and manganese, cobalt, and copper (by 60% each), did not reduce PUFA and carotenoid productivity, but actually increased it. Consequently, a richer *I. galbana* biomass destined for aquaculture can be produced at lower cost.

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**Data availability** All data generated or analyzed during this study are included in this published article.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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