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Biomass production and biochemical variability of the marine microalga *Isochrysis galbana* in relation to culture medium

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Abstract

We have studied the autotrophic growth of the marine microalga, *Isochrysis galbana* Parke, in a batch photobioreactor, comparing five different culture media and analysing the influence of each on growth kinetics as well as on the fatty-acid composition and protein content of the biomass. All the experiments were performed at 15°C, with the culture medium at pH 8.0, a specific rate of air supply of $1\,v\,v^{-1}\,min^{-1}$ and a continuous illumination of 40–43 $W\,m^{-2}$. The results show no parallel between good nutritional characteristics and high values of the kinetic parameters. Nevertheless, a compromise between the nutritional factors and growth kinetics could be provided by Ukeles medium, which provided a biomass with a good composition in polyunsaturated fatty acids (quotient $n3/n6=3.2$), an adequate protein content (25.3%) and relatively high values, although not the highest registered, for maximum specific growth rate ($\mu_m=0.018\,h^{-1}$) and biomass productivity ($1.9\times10^{-3}\,kg\,m^{-3}\,h^{-1}$).

Keywords: Microalgae; Photobioreactor; *Isochrysis galbana*; Fatty acids; Protein; Culture medium

1. Introduction

Unicellular marine algae constitute a renewable resource currently under exploitation. Prominent among their characteristics is their higher($n-3$) fatty-acid composition, attributed with notable properties such as energetic components and essential nutrients for animal feed, and, in some cases, certain microalgae can supply fatty acids for human consumption [1].

Among marine microalgae, *Isochrysis galbana* is a haptophyceae which, for its good nutritive characteristics (especially in relation to polyunsaturated fatty-acid composition), is of substantial interest in aquaculture, principally to feed mollusk larvae, as well as fish and crustaceans in the early stages of growth [2–4].

In the production of biomass with certain desired characteristics, the composition of the culture medium is a fundamental factor. The relationship between the nutrients used and the composition of the biomass is known [5]. On the other hand, the culture medium affects the specific growth rate and the maximum level of biomass production. Deficiency in the medium with respect to a given nutrient can cause the alga to adapt its metabolism to new external conditions. In general, modifications in the culture medium change the biochemical composition of the biomass, fundamentally proteins, lipids, carbohydrates and pigments.

In the present work, we study the autotrophic growth of *Isochrysis galbana* Parke in a batch photobioreactor, comparing five culture media and analysing the influence of each on growth kinetics as well as on the fatty-acid composition and protein content of the biomass produced.

2. Experimental details

2.1. Microorganisms

The experiments were performed with the marine microalga *Isochrysis galbana* Parke, obtained from the 'Culture Center for Microalgae and Protozoa', Oban, UK.

2.2. Culture media

The five culture media used were: Guillard [6], Ukeles modified according to Fábregas and Herrero [7], the medium of Ben-Amotz [8], the commercial medium Algal-1, and the synthetic medium S88 [9].To the media Guillard and Ukeles, we added Tris(hydroximethyl)aminomethane chlorohydrate

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Table 1
Composition of media for culture of *Isochrysis galbana*

Nutrient	Culture medium ($mg\,l^{-1}$)				
	Guillard (f/2)	Ukeles	Ben-Amotz	S-88	Algal-1
N (as nitrate)	12.353	28.000	70.000	13.848	39.375
P (as phosphate)	1.125	3.098	6.200	2.222	4.200
Fe	0.6538	0.9310	0.1954	0.5022	1.785
Zn	0.004481	0.06519	2.290	0.005006	0.09270
Mn	0.04940	0.05496	0.5490	0.05005	0.07700
Mo	0.002485	0.09596	0.4797	0.0005157	0.09800
Co	0.002972	0.005944	0.01770	0.0005039	0.008000
Cu	0.002547	0.006366	0.01908	0.004988	0.008500
Thiamine HCl	0.1000	0.0350	0.0350	0.0500	0.0280
Biotin	0.0005	0.0050	0.0050	–	0.0014
Cyanocobalamin	0.0005	0.0030	0.0030	0.0001	0.0014

(Tris-HCl) to buffer the media. The medium Algal-1 was supplied by Nutrición Avanzada, S.A. (La Coruña, Spain).

In Table 1, the compositions of the five culture media are compared in relation to their concentration in each of the most important chemical elements. Only for the vitamin content is the concentration of the three components detailed. It should be emphasized that, in all cases, the real concentrations were slightly higher than those indicated; given the use of enriched media, seawater contributes an additional quantity of each of these elements. Only in the synthetic medium, S-88, did the real concentrations of the medium coincide with those listed in Table 1.

In terms of macronutrients, the richest medium was Ben-Amotz, and the poorest Guillard medium. In general, the Ben-Amotz medium proved richer in micronutrients, except for Fe content. Also, this medium stands out for its high Zn content. In vitamins, the commercial media Algal-1 and S-88 contained the lowest concentrations.

2.3. Experimental installation

The experimental installation (Fig. 1) consisted fundamentally of four photobioreactors equipped with a thermal jacket and with a lid having two orifices: the central orifice was used for the aerator and the other for measurement and control of the pH and for an air outlet.

2.4. Preparation of the inoculate and preculture

Before beginning each experiment, we performed a preculture in two phases. The first phase was performed in 10-ml test tubes, adding 2 ml of inoculate to 6 ml of culture medium, maintaining the tubes 7 days under continuous illumination at room temperature. The second phase was conducted in the bioreactor by adding the contents of five test tubes to 100 ml of the culture medium, for a total of 140 ml and kept at 15°C for 7 days. With this stepped process, we attained an adequate initial concentration and similar characteristics in all the cultures.

2.5. Procedure

Each culture began with the addition of 360 ml to each photobioreactor for a total volume of 500 ml. The illumination was continual at 40–43 $W\,m^{-2}$, a specific-air-supply rate of 1 $v\,v^{-1}\,min^{-1}$ at 15°C and an initial pH of 8.0. Both the culture medium and the air supply were sterilized by filtration through cellulose-nitrate membranes with a pore size of 0.2 μm. In each culture, the pH was measured upon sampling, although it was not adjusted to its initial value. In general, the pH was found to rise slightly over the course of the culture, the variation being <1.

Over the course of each experiment, we determined the quantity of biomass produced. At the end of each experiment, we determined the crude-protein concentration and the fatty-acid composition of the lipid fraction of the biomass.

2.6. Analytical methods

Growth was determined by absorbance measurements of the cell suspension at 660 nm. The biomass concentration, x, in $g\,l^{-1}$ was calculated by the expression:

$$x = 0.226A_{660} - 5.39 \times 10^{-3} \quad (1)$$

The crude-protein content was determined by measuring the total nitrogen by element analysis of the biomass and multiplying this value by 6.25 [10].

The content and nature of the fatty acids of the biomass were determined from the lipid fraction [11]. The methylation of the fatty acids were performed using a mixture of acetyl chloride–methanol at a proportion of 5:1 (v:v). The methyl esthers were analysed in a Hewlett–Packard 5930A gas chromatograph equipped with a flame ionization detector. A 30-m-long capillary column of high-polarity fused silica was used. The column had an inside diameter of 0.25 mm and a film 0.2 μm thick. The temperature programme was as follows: initial oven temperature of 150°C for 8 min; then an increase in temperature of 3°C min^{-1} to 190°C; finally the oven maintained isothermically for 25 min. Both detec-

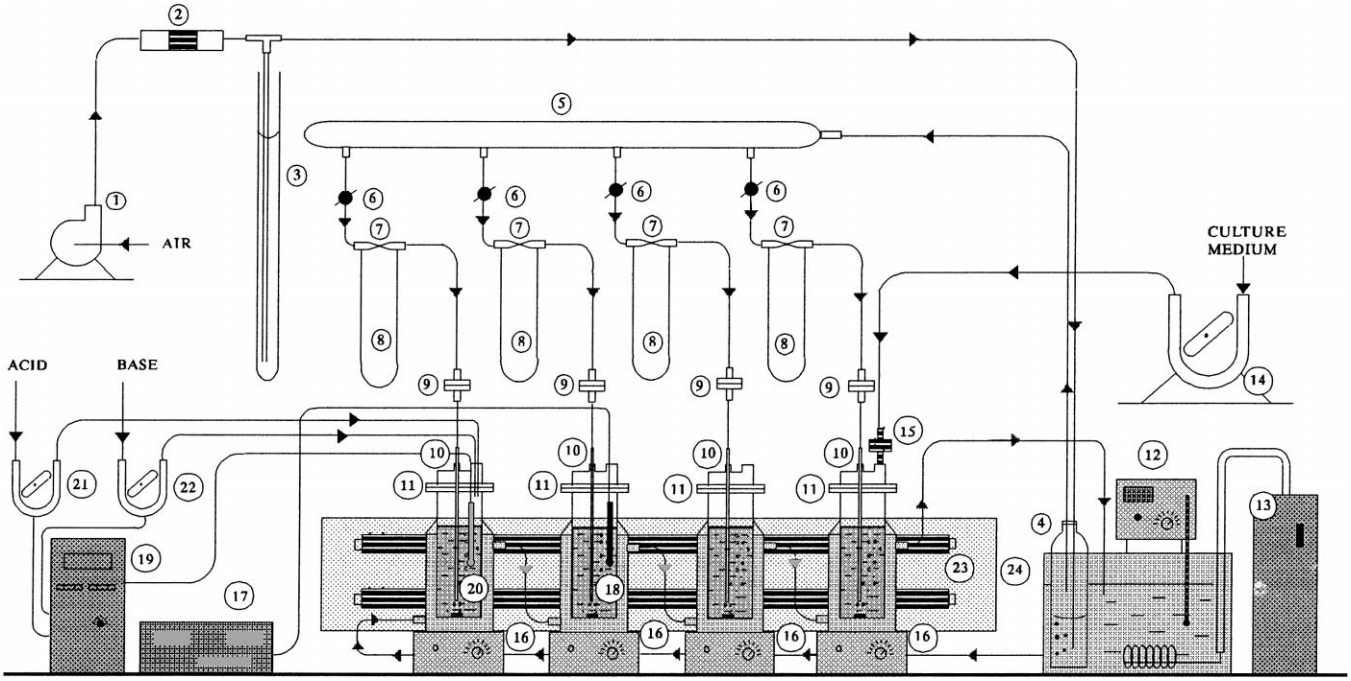

Fig. 1. Experimental installation: 1, compressor; 2, filtre; 3, stabilizing column; 4, washing flask; 5, distributor; 6, valve; 7, diaphragm; 8, manometers; 9, filtres; 10, aerifiers; 11, photobioreactor, 12, bath with thermostat; 13, refrigerant; 14, peristaltic pump; 15-filter; 16, magnetic stirrers; 17, pH meter; 18, combined electrode; 19, pH controller; 20, combined electrode; 21 and 22, peristaltic pumps; 23, fluorescent tubes; and 24, reflecting screen.

tor and injector temperatures were 220°C. In each analysis, 1.4 μl of methyl esther solution was injected into the chromatograph. The standards used were rapeseed-oil mix and PUFAS-1 of Supelco, catalogue numbers 4-7017 and 4-7033, respectively.

3. Results and discussion

From the experimental results, the kinetic parameters maximum specific growth rate (μ_m) and biomass productivity (P_B) were determined. In addition, at the end of each culture, calculations were made of the protein content and fatty-acid composition of the lipid fraction of the biomass.

3.1. Kinetic parameters

In all the experiments, the lag (or adaptation) phase was practically negligible, and from the beginning of the culture there was an exponential phase of a certain duration, for which the maximum specific growth rate was determined by a minimum-square fit to the equation:

$$\ln\left(\frac{x}{x_0}\right) = a + \mu_m t \quad (2)$$

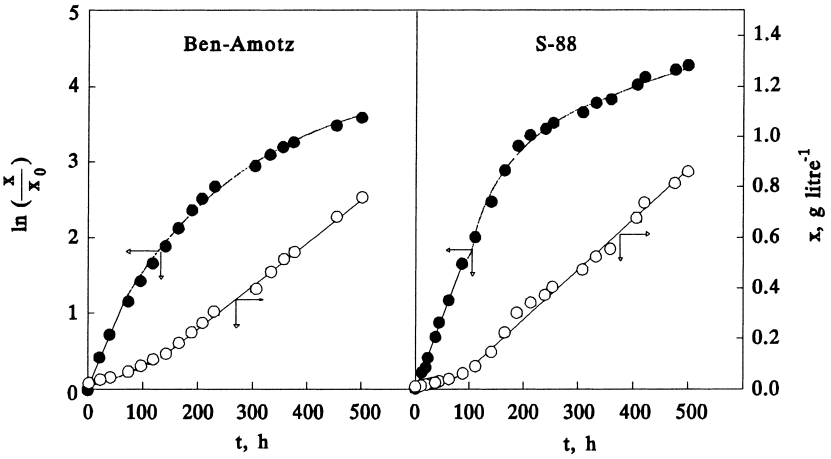

Fig. 2. Growth curves (●) and biomass concentration (○) against time for the experiments corresponding to the Ben-Amotz and S-88 mediums.

The Neperian logarithm of dimensionless biomass concentration, $\ln(x/x_0)$, was represented against time (t) for each of the culture media. For example, Fig. 2 shows the growth curves for two of the experiments. According to Eq. (2), in the event that the lag phase was completely negligible, the ordinate at the origin (a) would be null. In the entire series of experiments, these values remained very close to zero.

The maximum specific-growth values, for each of the experiments, together with the linear regression coefficients, r^2, are listed in Table 2. The highest values of μ_m, close to $0.02\,h^{-1}$, were achieved in the culture media S-88 and Algal-1, while the lowest value, $0.015\,h^{-1}$ corresponded to Ukeles medium buffered with 4.13 mM of Tris-HCl.

With respect to the order of magnitude of the μ_m values, it should be indicated that other marine microalgae of aquacultural interest, such as *Tetraselmis* sp. and *Skeletonema costatum*, under very similar conditions and a temperature of 15°C, have given values far higher (*Tetraselmis*, μ_m=0.04 h^{-1}, [12]) and lower (*Skeletonma*, μ_m=0.01 h^{-1}, [13]) than in *Isochrysis galbana*.

After the exponential phase, cell growth continued but at a lower rate. During this long phase of slowing growth, the biomass values fit a linear relationship of the type:

$$x = c + P_B t \quad (3)$$

In this equation, P_B ($g\,l^{-1}\,h^{-1}$) represents biomass productivity and is a parameter characteristic of this long period in which it is possible to admit linear growth. For example, the same Fig. 2 shows a representation of the biomass concentration against time for two experiments, in which the long period of linear variation in biomass contrasts with that corresponding to the exponential phase.

The P_B values calculated are also listed in Table 2, indicating the starting time of the linear growth (t_L, h) and the biomass levels (x, $g\,l^{-1}$) at that time for each experiments. This linear growth period, after the exponential phase, is characteristic of the bioprocesses controlled by physical stages. In this particular case, the kinetic control of the bioprocess may reside in the transference of carbon dioxide in the cell suspension or to insufficient illumination in the culture on reaching a certain cell density. Although the two factors may exert an influence, in this case, it appears that the transference of CO_2 is the fundamental cause of growth limitation. In this sense, it was found experimentally that the beginning of the linear phase coincided with the greatest rise in the pH of the culture, and it is known that the CO_2 solubility declines with the pH.

The greatest P_B value was reached in the culture using the Ukeles medium buffered with Tris-HCl at a concentration of 4.13 mM, also the culture that gave the lowest value for the maximum specific growth rate.

3.2. Protein and fatty-acid composition

The crude-protein content was determined in each experiment at a time near the stationary phase of the culture. These results were expressed in percentages by dry-biomass (%P_r) of the culture (Table 3). Clearly, the highest percentages of protein were attained with the mediums Ben-Amotz (37%) and Algal-1 (30.3%), which are also characterized by a greater concentration of nitrogen, especially in the form of nitrate.

With regard to other algae, the data of Becker [10] show that the percentages in protein of *Isochrysis* are substantially lower than in the species *Spirulina* (50–70%) or in freshwater microalgae, which are characterized by their high protein concentrations, as in *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (50–60%), even lower than in other marine microalgae, such as *Tetraselmis maculata* (52%), *Dunaliella salina* (57%) or *Dunaliella tertiolecta* (30–70%) [14]. The highest percentage recorded, 37%, was of the same order as that reached in the cultures of other marine microalgae, *Skeletonema costatum*, using operational conditions more favourable for growth, as well as a medium formulated specially for this diatom [15].

In addition, at the end of each experiment, in the stationary phase, we determined the fatty-acid content of the lipid fraction of the biomass produced.

The results for each medium are shown in Table 4. The highest percentages correspond to myristic, oleic and palmitic acid, and, when Ukeles 2 and Ben-Amotz media were used, docosahexanoic acid, also. Fig. 3 gives the total percentage of saturated, monounsaturated, polyun-

Table 2
Kinetic parameters

Culture medium	μ_m (h^{-1})	r^2	t_l (h)	x ($g\,l^{-1}$)	P_B 10^3 ($g\,l^{-1}\,h^{-1}$)
Guillard	0.0166	0.999	166	0.22	1.91
Guillard[a]	0.0165	0.999	91	0.059	0.89
Ukeles	0.0184	0.999	140	0.10	1.85
Ukeles[b]	0.0158	0.995	117	0.12	1.69
Ukeles[a]	0.0154	0.999	166	0.24	2.25
Ben-Amotz	0.0157	0.989	117	0.11	1.71
S-88	0.0194	0.996	111	0.090	1.91
Algal-1	0.0204	0.996	91	0.097	1.93

[a] Buffered with a concentration of 4.13 mM Tris-HCl.
[b] Buffered with a concentration of 2.06 mM Tris-HCl.

Table 3
Protein content of the biomass

Medio de cultivo	Pr (%)
Guillard	12.4
Guillard[a]	20.6
Ukeles	25.3
Ukeles[b]	24.1
Ukeles[a]	19.4
Ben-Amotz	37.0
S-88	27.4
Algal-1	30.3

[a] Buffered with a concentration of 4.13 mM Tris-HCl.
[b] Buffered with a concentration of 2.06 mM Tris-HCl.

Table 4
Fatty-acid composition

Fatty acid	Guillard	Guillard[a]	Ukeles	Ukeles[b]	Ukeles[a]	Ben-Amotz	S-88	Algal-1
14:0	38.9	27.8	15.1	13.0	28.8	15.6	31.3	30.4
14:1 (*n*−5)	–	2.8	3.2	tr[c]	–	tr[c]	–	3.1
16:0	25.2	20.5	17.1	13.5	21.6	14.0	17.5	22.8
16:1 (*n*−7)	–	1.1	tr[c]	–	–	tr[c]	tr[c]	tr[c]
16:2 (*n*−4)	tr[c]	2.0	7.7	7.4	tr[c]	7.9	–	4.4
18:0	2.9	2.6	tr[c]	–	tr[c]	tr[c]	3.1	3.1
18:1 (*n*−9)	19.6	10.4	21.7	14.5	20.6	16.2	28.7	12.3
18:2 (*n*−6)	3.4	3.3	6.3	tr[c]	5.5	tr[c]	5.7	3.8
18:3 (*n*−3)	tr[c]	3.5	4.9	7.7	tr[c]	8.3	4.7	3.7
18:4 (*n*−3)	6.3	5.9	tr[c]	tr[c]	4.2	tr[c]	3.6	6.2
20:1 (*n*−9)	tr[c]	5.5	6.4	7.1	7.7	6.8	tr[c]	tr[c]
20:2 (*n*−6)	3.8	0.9	tr[c]	6.5	–	6.6	tr[c]	–
22:1 (*n*−9)	–	tr[c]	3.4	5.0	6.1	4.4	–	–
22:6 (*n*−3)	tr[c]	9.7	15.3	25.3	5.6	20.2	5.2	10.3

[a] Buffered with a concentration of 4.13 mM Tris-HCl.
[b] Buffered with a concentration of 2.06 mM Tris-HCl.
[c] Traces.

saturated, essential and *n*−3 higher polyunsaturated fatty acids (Σ*n*−3 HUFA) against the culture medium used in each experiment. In addition, Fig. 3 shows the *n*−3/*n*−6 index of each culture; this index represents the quotient between the fatty acids corresponding to the groups *n*−3 and *n*−6, and, according to Webb and Chu [16], is a parameter that can be considered to estimate the nutritional value of the microalgae. According to these authors, when the *n*−3/*n*−6 quotient is within the 2–5 range, the biomass being produced in the culture can have acceptable nutritional quality.

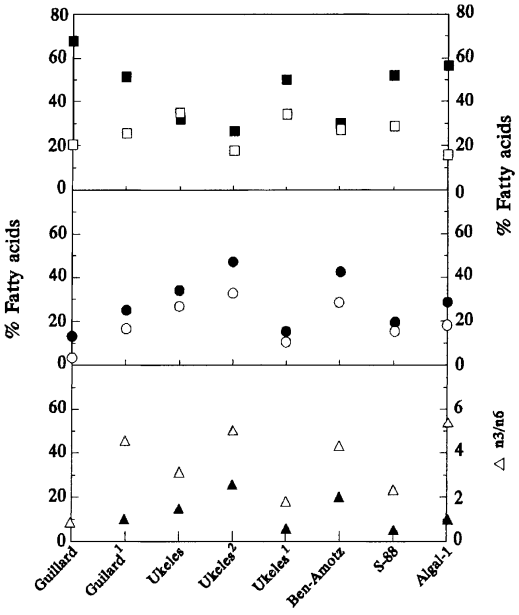

Fig. 3. Variation in fatty-acid content of the lipid fraction in the biomass with composition of the culture medium: (■) % saturated fatty acids; (□) % monounsaturated; (●) % polyunsaturated; (○) % essential; (▲) % Σ*n*-3 HUFA; (△) *n*−3/*n*−6 relationship.

Of all the media assayed, Ukeles-2 reached the highest percentage of polyunsaturated fatty acids (46.9%, of which 25.3% was docosahexanoic acid), followed by Ben-Amotz (43%). The highest percentages in essential fatty acids and Σ*n*−3 HUFA (33 and 25.3%, respectively) corresponded to Ukeles-2, and the next highest to Ben-Amotz (28.5 and 20.2%, respectively). It should be pointed out that neither in the operational conditions assayed nor in the culture media used was the acid 20:5 *n*−3 detected, although under certain conditions it is characteristic of some species of *Isochrysis* (20). In this sense, our results coincide with those of Ben-Amotz (8) and Servel et al. [21] for *Isochrysis galbana* but differ from those of Vazhappilly and Chen [20]. Additionally, on comparing the different media used, we found a wide variation in the content of the acid 22.6 *n*−3, perhaps related to the effective maintenance of the pH of the culture, although this is not clearly appreciable in our results. With reference to docosahexanoic acid, 22:6 (*n*−3), it should be indicated that this acid is highly important for the growth and development of the oyster *Grassostrea gigas* [17] and in general for the feeding of oyster larvae. In addition, studies by different researchers have shown that this acid, together with 20:5 (*n*−3) are essential for the growth and maintenance of marine fish [18,19]. Thus, it is worthwhile emphasizing the high percentages of docosahexanoic acid achieved with Ukeles-2 and Ben-Amotz mediums.

On the other hand, the Ukeles medium gives rise to a greater percentage of monounsaturated fatty acids (34.7%), although it is notable that the synthetic medium S-88 gave 28.7% corresponding only to oleic acid, 18:1 (*n*−9). In addition, the high percentage (38.9%) of myristic acid, 14:0, in the culture medium Guillard deserves mention.

The relationship between the fatty-acid fractions *n*−3/*n*−6 showed values of between 2 and 5 for the Ukeles, Guillard-1, Ben-Amotz and S-88 media. As pointed out above, this would indicate that the biomass produced in

these cultures have acceptable nutritive value. In addition, the Ukeles medium provided an $n-3/n-6$ relationship close to the value 3.1, considered indicative of a biomass having excellent nutritional value [16].

On the basis of our experimental results, we conclude that no clear parallel can be established between the good nutritional characteristics of the biomass and the high values of kinetic parameters. However, a compromise between the nutritional properties and growth kinetics could be achieved by the use of Ukeles medium, which provides good fatty-acid composition and an adequate protein content (25.3%) as well as relatively high values (although not the highest attained) of specific growth and biomass productivity.

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