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Outdoor culture of *Isochrysis galbana* ALII-4 in a closed tubular photobioreactor

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Abstract

Growth and fatty acid composition of *Isochrysis galbana* ALII-4 in batch and semicontinuous outdoor culture in a closed tubular photobioreactor are reported. Specific growth rate at the exponential phase was 0.38 d^{-1}, higher than that obtained in previous indoor experiments. At biomass concentration above 2 g l^{-1} net biomass productivity obtained in three successive semicontinuous cultures was 0.32 g l^{-1} d^{-1}. A daily cyclic variation in the fatty acid profile was observed. From morning to afternoon, 14:0, 16:0 and 16:1*n*7 content increased, although it decreased during darkness renewing the cycle again with daylight. On the other hand, no daily variation was observed in polyunsaturated fatty acid content. The EPA generation rate during linear growth, with an average content of 2.56%, was 8.2 mg l^{-1} d^{-1}. The present study was conducted uninterruptedly in February and March, 1994, in Almería, Spain (latitude 36° 50′ N, longitude 2° 27′ W).

Keywords: Tubular photobioreactor; Outdoor culture; Polyunsaturated fatty acids; *Isochrysis galbana* ALII-4

1. Introduction

Microalgae provide an important source of dietary lipids to marine life and, directly or indirectly, to humans. Indeed, the active ingredients of fish oils used in medicine are the long-chain polyunsaturated fatty acids (LC-PUFAs) which ultimately originate in the microalgae in the food chain. In fact, oceanic phytoplankton represent the major source of these LC-PUFAs in the biosphere (Kyle, 1992). These *n*-3 (omega-3) class LC-PUFAs are made up primarily of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Environmental conditions of microalgal culture can have a profound effect on the fatty acid profile of biomass produced. Thus, with judicious selection of a microalga strain and the conditions of its cultivation, an oil, highly enriched in a particular LC-PUFA, may be obtained. The culture system is one of these factors affecting mi-

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croalga growth, and, therefore, the synthesis of such products.

Among the outdoor microalgae culture systems, closed tubular reactors seem to be a promising option (Gudin and Thepenier, 1986; Richmond et al., 1993; Torzillo et al., 1993), in contrast to open ponds, in which only a reduced number of species have been successfully grown on a large-scale (*Dunaliella, Spirulina, Chlorella*). Advantages and disadvantages of closed and open systems have been largely discussed elsewhere (Weissman et al., 1988; Tredici and Materassi, 1992). Here, we would like to stress the need for strictly controlled growth conditions, especially when a slow-growing alga used as a source of highly valuable chemicals may be contaminated by a fast growing species, causing the production process to fail.

Isochrysis galbana is a small naked flagellate widely used in aquaculture as a PUFA rich microalga. The isolate labelled ALII-4 was obtained in a phenotypic selection programme for an EPA rich strain of microalgae (López Alonso et al., 1992a,b). EPA content of 5% dry weight and EPA productivity of 15.26 mg l^{-1} d^{-1} were found in indoor chemostat cultures of *Isochrysis galbana* ALII-4 when the dilution rate was set at 0.0208 h^{-1} (Molina Grima et al., 1994).

A closed tubular photobioreactor was chosen as the most appropriate system for growing *Isochrysis galbana* ALII-4 outdoors. In the present paper, results from batch and semicontinuous outdoor culture of *Isochrysis galbana* ALII-4 are discussed.

2. Materials and methods

The microalga used was one of 42 isolates of a single strain of *Isochrysis galbana* obtained in a phenotypic selection programme carried out in our laboratory for an EPA-rich strain of microalgae (López Alonso et al., 1992a,b). This isolate (labelled ALII-4) is lodged with the Culture Collection of Algae and Protozoa (CCAP). The inoculum for the photobioreactor was grown under laboratory conditions in a 10-l stirred tank reactor at 20°C. The culture medium was: $NaNO_3$ 10 mM, $NaH_2PO_4 \cdot 2H_2O$ 0.1 mM, ferric citrate 20 μM, Cl_2Zn 1 μM, $CuSO_4 \cdot 5H_2O$ 0.1 μM, $CoCl_2 \cdot 6H_2O$ 0.1 μM, $Na_2MoO_4 \cdot 2H_2O$ 1 μM, EDTA-Na_2 29 μM, $Cl_2Mn \cdot 4H_2O$ 5 μM; vitamins were per l: thiamine 35 μg, biotin 5 μg and cyanocobalamine 3 μg. This composition was determined by a macronutrient optimization method based on a multifactorial response surface technique (unpublished data). Medium was autoclaved at 120°C at 1 atm. The culture was constantly illuminated by four Phillips (Eindhoven) TLD w/54 fluorescent lamps, providing a light intensity of 1.2×10^{16} quanta cm^{-2} s^{-1} on the vessel surface.

The biomass concentration, C, was estimated from the optical density of the culture, which was measured at 530 nm wavelength with a 1-cm light path in a HITACHI U-1000 spectrophotometer. The relationship between biomass concentration and optical density (OD) was previously:

$$C\ (g\ l^{-1}) = 0.232\ OD_{530}\ (r^2 = 0.992).$$

To determine the biomass dry weight, duplicate known volumes of the algal culture were centrifuged at 4000 rpm and washed with 0.5 N HCl and distilled water to remove non biological materials such as mineral salt precipitates, lyophilized during 3 d, and then weighed. Samples were ashed at 470°C in a muffle furnace to obtain the ash-free dry weight (AFDW) which was 88.2% of the total dry weight.

The photon-flux density of photosynthetically active radiation (PAR) at the culture surface was determined with a quantum scalar irradiance meter (QSL-100 Biospherical Instruments Inc., San Diego, CA).

The outdoor culture medium was the same as that used in the inoculum, but was sterilized by filtration through 1 μm- and 0.22 μm-bore Millipore filters. The pH was set at 8.00. Culture temperature, pH and dissolved oxygen (DO_2) were measured on-line and biomass concentration was measured several times a day. Nutrients were added to the culture throughout the culture period to assure excess nutrient requirements and avoid nutrient limitation. The evaporation rate in the culture which was topped with distilled water every morning, was never higher than 200 ml d^{-1}.

The present study was conducted uninterruptedly in February and March, 1994, in Almería (Southeast of Spain; latitude 36° 50′ N, longitude 2° 27′ W) where the excellent solar irradiance is attractive for microalgal biotechnology.

Fresh centrifuged wet biomass was used for fatty acid analysis. Methylation was done by direct transesterification following the Lepage and Roy method (1984) with modifications by García Sánchez et al. (1993). The analysis of methyl esters was carried out by GLC, using a 30-m capillary column of fused silica (SP2330, Supelco, Bellefonte, PA, USA), with an internal diameter of 0.25 mm, 0.20 μm standard film, split ratio of 100:1, and a flame ionization detector. Supelco PUFA-1, PUFA-2 and PUFA-3 patterns were used for the determination of retention times. Sigma patterns of main fatty acids for this microalgae were used for response factor determination. Nonadecanoic acid was used as an internal standard to quantify fatty acid content in biomass.

The reactor

The culture system is shown in Fig. 1. The photobioreactor is based on an external loop airlift. The airlift pump (riser, degasser and downcomer) is 3 m high. The external loop is the solar receiver consisting of Plexiglas transparent tubes (80.8 m long, 3 cm external diameter and 2.6 cm internal diameter) connected by joints of the same material. The tubes, positioned horizontally over the ground, are connected to the airlift system. The top and wall of the degasser were sealed with rubber bungs through which liquid and gas inlets and outlets and temperature, dissolved oxygen and pH sensors were passed. The culture volume was about 50 l. The 8.6 m^2 solar receiver was submerged in a thermostatic water pool. Temperature was controlled at 20°C.

pH in the culture was measured with an Ingold pH glass probe and controlled by the automatic addition of pure CO_2. The dissolved-oxygen content above air saturation in seawater was measured by an Ingold polarographic probe. An ML-

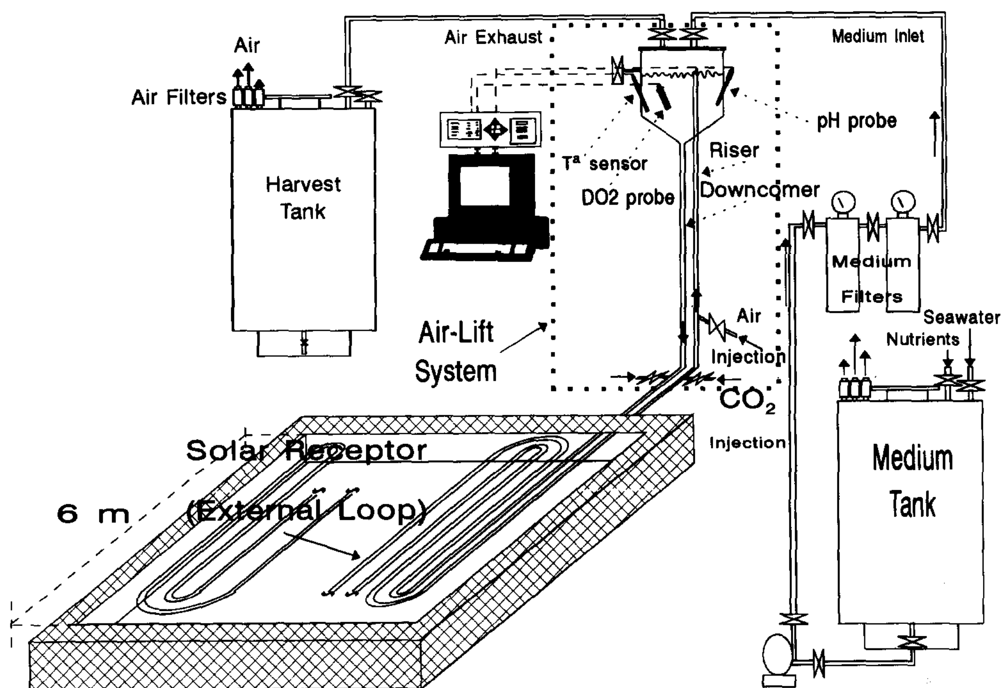


Fig. 1. Scheme of outdoor culture system.

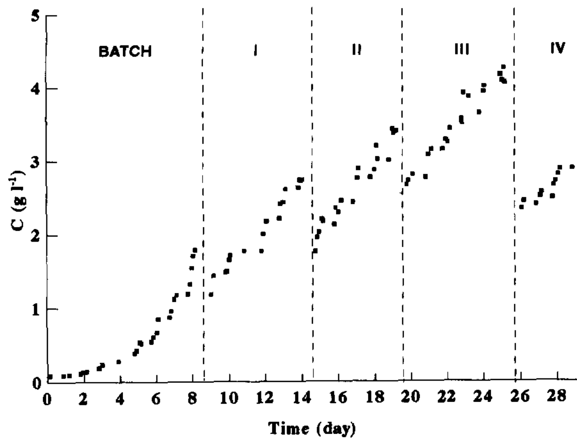


Fig. 2. Variation of biomass concentration against culture time.

4100 control-unit (New Brunswick Scientific, USA), connected to a computer was used for data acquisition and control.

The operating speed was set at 30 cm s^{-1} (turbulent flow) to prevent alga cells from settling and ensure favorable light in the system.

3. Results

Development of the biomass concentration during the experiment is represented in Fig. 2, where the different stages of the culture are pointed out. After inoculation, a lag phase was observed followed by exponential growth during the next 7 d. The specific growth rate averaged over the whole culture time (i.e., over 24 h d^{-1}) was 0.38 d^{-1} (0.016 h^{-1}, $n = 29$, $r^2 = 0.9986$).

Biomass concentration at the end of the exponential growth reached 1.7 g l^{-1}. A semicontinuous culture was then initiated by dilution every 4 d, increasing the initial biomass concentration in each stage from 1.19 g l^{-1} to 2.66 g l^{-1} in order to study the effect on productivity. After each harvest, biomass concentration rose linearly to a maximum of 4 g l^{-1}. These results are shown in Table 1.

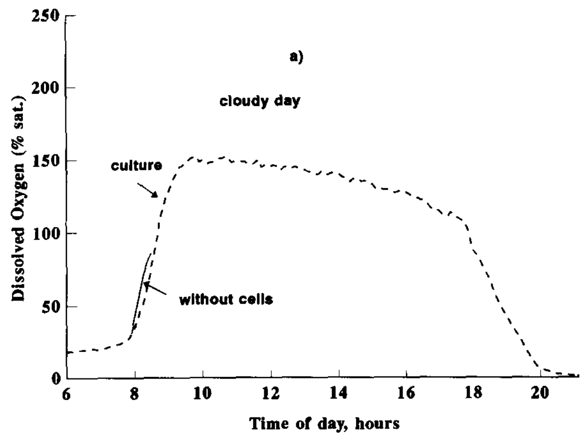
Changes in dissolved oxygen concentration, DO_2, during the daylight cycle are shown in Fig. 3 for a cloudy and a sunny day. At sunrise, a

Table 1

Influence of initial biomass concentration on net biomass productivity of *Isochrysis galbana* ALII-4 cultivated outdoors in a tubular photobioreactor

Stage	Concentration interval (g l^{-1})	Net productivity (g l^{-1} d^{-1})	r^2
I	1.19–2.73	0.28	0.9589
II	1.76–3.04	0.32	0.9456
III	2.66–4.15	0.32	0.8598

I (days 9 to 14) first semicontinuous.
II (days 15 to 19) second semicontinuous.
III (days 20 to 25) third semicontinuous.



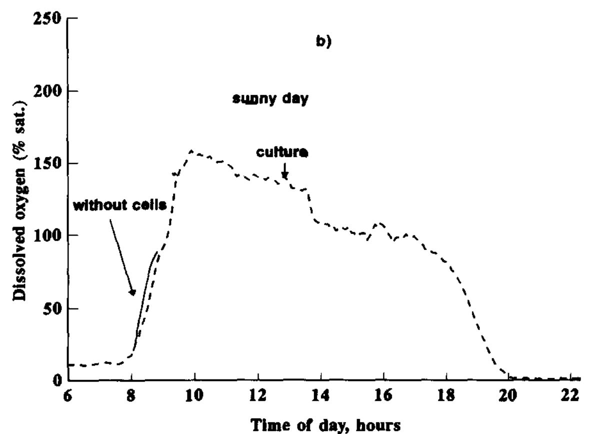


Fig. 3. Dissolved oxygen concentration evolution during the daylight cycle for a cloudy and a sunny day. pH was set at 8.00.

sharp increase in DO_2 concentration up to a maximum of 150% saturation at 10:00 h was observed. This was followed by a continuous decrease until sunset more marked on sunny days. Finally, in order to increase carbon availability, the pH control setpoint was lowered to 7.65, which was found to affect DO_2 behavior (Fig. 4). First of all, the maximun DO_2 reached was 200% saturation followed by a tableau during the period of highest solar radiation. Secondly, DO_2 remained at around 50% at night, while at pH = 8 it dropped to zero.

Throughout the culture period, fatty acids were analyzed at different times of day and on different days (see Table 2). At exponential growth, total fatty acid content was lower than that obtained in indoor exponential cultures (Molina Grima et al., 1994), especially 16:1*n*7 and EPA. After the first harvest, when biomass concentration rose to 2 g l^{-1}, analyzes were carried out daily and no increase of fatty acid content was observed despite, the lower growth rate during this stage of culture. During the second semicontinuous period, three analyzes were made daily at different times of the day. From morning to afternoon, 14:0, 16:0 and 16:1*n*7 content increased,

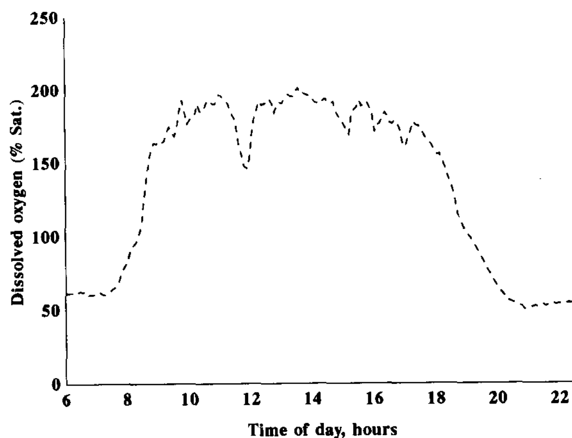


Fig. 4. Dissolved oxygen concentration evolution during the daylight cycle for a sunny day. pH was set at 7.65.

Table 2

Fatty acids profiles of *Isochrysis galbana* ALII-4 grown outdoors for different culture days and times of day

Stage	Culture time (d)	Time of day	Photon flux (quanta × 10^{-17} cm^{-2} s^{-1})	C (g l^{-1})	14:0	16:0	16:1*n*7	18:4*n*3	20:5*n*3	22:6*n*3
Exp	7	11:30	1.80	1.33	0.92	1.11	0.83	0.63	2.22	1.14
I	11	12:00	2.25	2.00	1.07	1.14	1.06	0.73	2.53	1.29
	12	11:00	2.10	2.41	1.02	1.06	0.77	0.65	2.17	1.19
	14	16:00		2.73	0.96	1.17	0.93	0.62	2.12	1.02
II	16	19:00		2.44	1.07	1.26	1.13	0.76	2.50	1.18
	17	10:30	1.00	2.43	0.99	1.14	0.84	0.82	2.53	1.11
	17	16:30	1.40	2.75	1.04	1.28	1.08	0.75	2.41	1.12
	17	19:00	0.10	2.89	1.03	1.28	1.11	0.75	2.54	1.12
	18	10:15	0.55	2.77	1.04	1.18	0.81	0.85	2.64	1.37
	18	15:00	2.15	2.87	1.21	1.44	1.12	0.81	2.72	1.32
	18	19:00	0.07	3.02	1.05	1.17	1.03	0.75	2.51	1.18
	19	10:00	0.76	3.01	0.83	0.90	0.60	0.70	2.30	1.10
	19	14:00	1.25	3.43	0.97	1.09	0.83	0.72	2.27	1.11
	19	16:00	1.55	3.37	1.02	1.13	0.91	0.70	2.36	1.05
III	26	17:00	1.80	2.50	1.20	1.70	1.25	0.69	2.95	1.22
	27	09:00	0.30	2.49	0.95	1.13	0.74	0.72	2.84	1.19
	27	11:00	1.70	2.65	1.01	1.32	0.88	0.75	3.02	1.20
	27	13:15	2.20	2.71	1.03	1.33	0.99	0.69	2.85	1.13
	27	16:30	2.00	2.80	1.12	1.51	1.13	0.71	2.79	1.10
	27	19:00	0.26	2.88	1.12	1.53	1.19	0.73	2.92	1.14
	28	10:00	0.46	2.89	1.02	1.29	0.82	0.77	2.87	1.24

Results are expressed as dry weight percentages. Photon flux and biomass concentration at sample time are given.

although a slight decrease was observed in late evening when solar irradiance went down to below 1×10^{16} q cm^{-2} s^{-1}. On the other hand, no variation in polyunsaturated fatty acid content was observed during the same intervals. Furthermore, the same fatty acid profiles were found in late evening (19:00 h) for three consecutive days (16, 17 and 18) highlighting the cyclic variation of the fatty acid content. Another interesting feature is the decrease in short-chain fatty acid content during the dark period, especially 16:1*n*7.

In the last stage of the experiment (day 27) with pH set at 7.65, fatty acids were analyzed during daylight, from 09:00 to 19:00 h, with solar intensities of 3.0×10^{16} q cm^{-2} s^{-1} and 2.6×10^{16} q cm^{-2} s^{-1}, respectively, and biomass concentration of from 2.49 to 2.88 g l^{-1} (Table 2). The diurnal increase of short-chain fatty acid content was strenghthened as well as the invariance in polyunsaturated fatty acid content.

4. Discussion

The experiment reported here is one of the first experiences in outdoor culture of *Isochrysis galbana* ALII-4 and constitutes the initial step in testing the potential of this strain for mass production.

The closed-tube technology enables a wide variety of species to be grown under controlled conditions of high solar irradiance and steady temperature. On the other hand, the use of air-lift pumps prevents mechanical stress in cells, a factor which contributes to the succsess of such photobioreactors (Gudin and Chaumont, 1991).

I. galbana ALII-4 has demonstrated suitable growth under outdoor conditions, with a net specific growth rate of 0.38 d^{-1} in the exponential growth phase. Nonetheless, by considering only the daylight period (9 h) the theoretical specific growth rate becomes 1.01 d^{-1} (0.042 h^{-1}) which agrees with the daily specific growth rates measured on days 5 and 8, 0.97 d^{-1} and 1.08 d^{-1}, respectively. Such a high specific growth rate was not obtained under laboratory conditions, although in a previous work, the maximun specific growth rate at light saturation for this strain was predicted to be 0.042 h^{-1} (Molina Grima et al., 1993a).

The linear increase of biomass concentration, *C*, with culture time during the semicontinuous procedure suggested a linear growth phase for which net biomass productivity could be calculated as the slope of curve *C* vs. time (see Table 1). Regardless of initial biomass concentration, net productivity was around 0.32 g l^{-1} d^{-1} which enables the reactor to be operated at high cell densities with the corresponding advantage for harvest.

In indoor light-limited chemostats of this strain, the highest biomass productivity for a 24-h light period was 0.31 g l^{-1} d^{-1} at low biomass concentrations (0.6 g l^{-1}; Molina Grima et al., 1994). Thus, despite daily variation of solar irradiance and difference of scale, this productivity was maintained outdoors. As far as we know, the only reference of an outdoor culture of *Isochrysis galbana* in tubular reactors is by Richmond et al. (1993), who reported output rates of 1500 mg dry weight l^{-1} d^{-1} in preliminary experiments in a tubular reactor made of 28 mm inner diameter glass tubes. Nevertheless, it was not specified whether the strain was T-ISO or *I. galbana* Parke. Under strong irradiance, the former yields higher output rates than the latter, according to Ewart and Pruder (1981), who reported that these two strains have very different responses to light intensity and temperature changes.

The response of the cells to daily cyclic variations in light intensity is illustrated in Fig. 3 for two different types of weather conditions. In the dark, before 07:00 h, absorption of atmospheric O_2 in the airlift riser is balanced by O_2 consumption by cell respiratory activity causing a constant DO_2 value. Early in the morning, DO_2 rises due to adaptation of the cell to light which causes minor O_2 consumption in the transition from respiration to photosynthesis. At breakday, respiratory activity should be still greater than initial photosynthesis, giving rise to a net O_2 consumption instead of net O_2 generation. This is demonstrated by comparing the increase in DO_2 in the culture with the more pronounced increase in DO_2 from physical absorption of atmospheric O_2 in the absence of cells (see Fig. 3), which was

measured during experiments to determine the overall volumetric oxygen mass transfer coefficient, K_{la}, following the method of Molina Grima et al. (1993b). The O_2 transfer rate is related to K_{la} by the following equation:

$$\frac{dDO_2}{dt} = K_{la}(DO_2^*-DO_2) + \text{Generation} - \text{Consumption} \quad (1)$$

where DO_2 and DO_2^* are the instantaneous and saturation (or equilibrium) O_2 dissolved concentrations in the liquid repectively. Oxygen generation and consumption are nihil in the absence of cells, so that, the minor increase in DO_2 with algae is due to transfer from air minus the net O_2 uptake by respiration.

When photosynthesis becomes intensively active, O_2 supersaturation appears to reach a maximum followed by a slow decrease, diurnal decrease being faster on sunny days than on cloudy days, which could be due to photoinhibition. But, as shown in Fig. 4, this behaviour was not observed (at pH = 7.65), e.g., carbon availability could be photosynthesis-limiting at pH = 8.00. In the morning, when photosynthesis starts, carbon is supplied by reserves in the culture medium through equilibrium displacement: $HCO_3^- \leftrightarrow CO_2 + OH^-$, and pH increases from 7.60 to 8.00. At this pH, CO_2 injection is activated and carbon consumption is then limited by the pure CO_2 supply rate. At pH = 7.65 CO_2 availability is greater than at pH = 8.00, giving rise to the difference between Figs. 3 and 4. Furthermore, DO_2 was higher at night with pH set at 7.65 than at 8.00, which could be due to more intensive photosynthetic activity in daylight and consequently lower respiratory activity at night, as cells have enough energy reserves from photosynthetically produced ATP and NADH.

It is well known that fatty acid synthesis may be qualitatively and quantitatively affected by several environmental conditions. Parameters, such as media composition, temperature, light intensity, lighting cycle and age of the culture play major roles in the biosynthesis and accumulation of fatty acids (Álvarez, 1989; Bajpai and Bajpai, 1993).

The fatty acid variation presented in Table 2 could be explained by the different roles of the fatty acids in cell metabolism, as stated previously (Molina Grima et al., 1994). 14:0, 16:0 and 16:1*n*7 are the main components of neutral lipids (storage lipids) and the initial step in the path of fatty acid synthesis (Hodgson et al., 1991). Thus, their contents are subjected to the daily cyclic variation of environmental conditions, that is solar radiation. On the other hand, polyunsaturated fatty acids are structural lipids, mainly found in glycolipids and phospholipids (Molina Grima et al., 1994), and therefore, their contents are more related to state of growth than short-term environmental variation. Another fact worthy of mention is the increase observed in EPA content at pH = 7.65 in comparison to pH = 8.00 cultures from an average of 2.56% to 2.89%, which is statistically significant, as confirmed by one-way analysis of variance (significant level 0.0001, 95% confidence interval).

EPA generation rate during linear growth, with an average content of 2.56%, was 8.2 mg l^{-1} d^{-1} which is twice as low as that obtained in indoor continuous cultures, as biomass productivities are the same but EPA content was 2-fold higher (5% dry weight) in the study of reference (Molina Grima et al., 1994). Thus, opimization of outdoor culture could significantly increase EPA productivity to equal indoor results.

There is scant literature on EPA production in outdoor systems. Notwithstanding, it is interesting to compare these results with those obtained by Cohen et al. (1988) with the red alga *Porphyridium cruentum*, which had an EPA content of 1.3% in summer, but with productivity 10-times lower than that reported in the present paper.

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