Optimization of ω-3 Fatty Acid Production by Microalgae: Crossover Effects of CO_2 and Light Intensity Under Batch and Continuous Cultivation Modes

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Abstract

The microalga *Pavlova lutheri* is a potential source of economically valuable docosahexaenoic and eicosapentaenoic acids. Specific chemical and physical culture conditions may enhance their biochemical synthesis. There are studies relating the effect of CO_2 on growth; however, this parameter should not be assessed independently, as its effect strongly depends on the light intensity available. In this research, the combined effects of light intensity and CO_2 content on growth and fatty acid profile in *P. lutheri* were ascertained, in order to optimize polyunsaturated fatty acid production. The influence of the operation mode was also tested via growing the cultures by batch and by continuous cultivation. Higher light intensities associated with lower dilution rates promoted increases in both cell population and weight per cell. Increased levels of CO_2 favored the total lipid content, but decreased the amounts of polyunsaturated fatty acids. Mass productivities of eicosapentaenoic acid (3.61 ± 0.04 mg $\cdot L^{-1} \cdot d^{-1}$) and docosahexaenoic acid (1.29 ± 0.01 mg $\cdot L^{-1} \cdot d^{-1}$) were obtained in cultures supplied with 0.5% (v/v) CO_2, at a dilution rate of 0.297 d^{-1} and a light intensity of 120 μE $\cdot m^{-2} \cdot s^{-1}$.

Key words: photobioreactor — docosahexaenoic acid — eicosapentaenoic acid — polyunsaturated fatty acids

Introduction

The economic feasibility of fish hatchery systems strongly depends on improved knowledge of larval dietary requirements, such as those for ω-3 polyunsaturated fatty acids (PUFAs) (Sorgeloos et al., 1995). The importance of such PUFAs in feeding marine fish larvae has been rationalized (Sargent et al., 1994; Robin, 1995) on the basis of their incapacity to convert 18:3(n-3) into 20:5(n-3) at sufficiently high rates. As 20:5(n-3) promotes regular growth and high larval survival rates (Volkman et al., 1989), its inclusion in the daily diet as a standard nutritional requirement is urged. Microscopic algae that naturally contain large amounts of the aforementioned ω-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have thus attracted interest as primary sources of such nutrients for animals higher in the food chain. Although larvae and juveniles of most fish and crustaceans do not eat microalgae directly, they can be fed with zooplankton, that was previously enriched in PUFAs by ingestion of said microalgae. Because of the relatively high processing costs and time requirements associated with production of microalgal biomass in aquaculture facilities (Dunstan et al., 1993), it is convenient to maximize its PUFA content, which can be accomplished by engineering the culture conditions (Volkman et al., 1992). Carbon accounts for almost 50% of the overall dry weight of microalgal biomass (Becker, 1994). Microalgae that grow photolithotrophically use inorganic carbon sources to synthesize de novo their own organic carbon compounds (Kaplan et al., 1986). The most common inorganic sources used by those microorganisms include soluble CO_2 and HCO_3^- derived therefrom, although such other forms as H_2CO_3 and CO_3^{2-} may, via spontaneous chemical interconvertibility, produce the former (and metabolically more appropriate) carbon forms. Temperature, pH, and concentrations of several compounds (some of which may also be used as nutrients) control the rate of the reversible reactions that balance the elements of this family of inorganic compounds. Relationships between increase in concentration of available inorganic carbon

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and consequent change in biomass productivity differ between species (Tsuzuki et al., 1990; Negoro et al., 1991), and strongly depend on the overall amount of carbon added to the medium. Furthermore, such relationships also depend on the irradiance at the culture surface, the density of pigmentation, and the culture thickness; however, for cultures starting with identical viable numbers and assayed at identical physiologic states, only the light intensity at the surface will play a relevant role (Yongmanitchai and Ward, 1992). Because the microalga selected for this study operates photosynthetically, it converts inorganic CO_2 to organic carbon in the presence of light. Limitation in the supply of radiant energy constrains the rate of transport of inorganic carbon, so cells grown under light-limited conditions exhibit reduced capacity to accumulate CO_2 (Beardall et al., 1998). In fact, if light is scarce, microalgae will not exhibit a net intake of CO_2 from the surroundings, but will likely exhibit net excretion of CO_2 owing to respiration processes. Therefore, when attempting to determine the effects of carbon added to the culture medium, the light intensity prevailing should also be carefully considered.

Saturation of fatty acid residues can be induced by increases in CO_2 concentration during growth of some (but not all) species of microalgae; for example, Tsuzuki et al. (1990) reported decreases in the degree of unsaturation of fatty acid residues in *Chlorella vulgaris* when CO_2 supply was increased, although no differences could be found in *Porphyridium cruentum*. Consequently, increased amounts of inorganic carbon made available in the culture medium may, in a sense, improve biomass productivity; however, they may also decrease the amount of PUFA per cell; hence, an optimal compromise of these two parameters should be achieved in attempts to maximize PUFA productivity.

In the current research effort, the marine Prymnesiophyte *Pavlova lutheri*, commonly used in aquaculture, was selected and grown in both batch and continuous cultures. The influence of CO_2 concentration in the culture, coupled with the influence of light intensity at the culture surface (and dilution rate, where appropriate), were assessed in terms of biomass yield and fatty acid profile.

Materials and methods

Organism and Growth Conditions. *Pavlova lutheri* (Droop) Green (SMBA 60) was obtained from Instituto Português de Investigação Marítima (IPIMAR), Portugal.

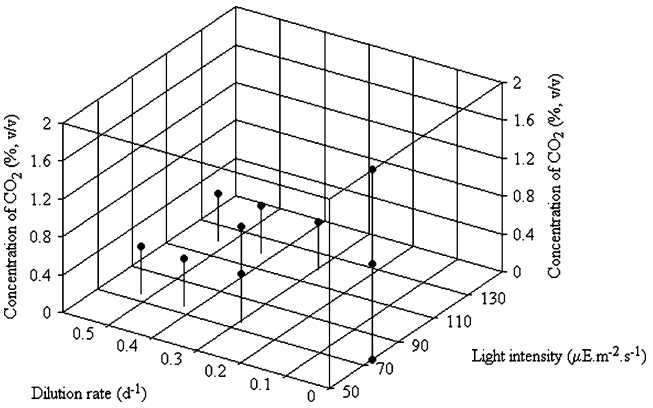

Fig. 1. Experimental values of CO_2, light intensity, and dilution rate tested during experiments performed under both batch and continuous modes. Experimental values of CO_2 range between 0% and 2% (v/v); experimental values of light intensity range between 75 and 120 $\mu E \cdot m^{-2} \cdot s^{-1}$; and dilution rates range between 0 (i.e., batch mode) and 0.523 d^{-1}.

Cells were grown in artificial seawater (ASW) culture medium, buffered with Tris-HCl, with modifications as described elsewhere (Carvalho and Malcata, 2000). The pH of the culture was adjusted to approximately 8.0 before autoclave sterilization.

Cultivation Systems. In batch experiments, 2-L glass flasks were maintained at 20 ± 0.5°C by a water bath, and subjected to a sustained continuous light cycle provided by a bank of cold fluorescent lamps (Philips TLD 30W/84), located on top and laterally; light intensity was measured as photosynthetic active radiation (PAR) with a LI-190SA Quantum Sensor (LI-COR). Aeration was provided via bubbling the inoculated media with filtered plain air, or filtered air enriched with CO_2 at appropriate levels, at a constant flow rate of 22.5 $L \cdot h^{-1}$, duly monitored by flow-meters (Aalborg). The pH was adjusted daily. Cells were submitted to a light intensity of 75 $\mu E \cdot m^{-2} \cdot s^{-1}$, and supplied with air containing 0%, 1%, or 2% (v/v) CO_2. All experiments started with equivalent inoculum sizes, and were run in duplicate.

In continuous mode experiments, a 2-L fermenter (Braun) was employed; temperature was computer-controlled, whereas light intensity was provided by the lamp system described for the batch mode. The pH was constantly monitored and adjusted to approximately 8.0 by injection of either acid or alkali into the culture medium. Experiments were performed at 2 light intensities (75 and 120 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 3 dilution rates (0.297, 0.426, and 0.523 d^{-1}); furthermore, the effect of CO_2 was also studied at 2 levels (0.5% and 1% [v/v]), for one dilution rate. Figure 1 depicts all aforementioned experimental conditions.

Analytic Methods. Ash-free dry weight (AFDW) biomass was determined by filtering 10 ml of the culture through preconditioned Whatman GF/C glass fiber filter, drying at 100°C to constant weight, and heating at 550°C to volatilize organic compounds. Cells were counted with a Neubauer Improved bright-line hemocytometer (Superior), using appropriate dilutions, under an Olympus CH-2 microscope.

Total lipids were determined gravimetrically according to Bligh and Dyer (1959). Fatty acid methyl esters (FAMEs) were obtained by direct transesterification of previously freeze-dried samples, according to the method described by Lepage and Roy (1984), after the modifications introduced by Cohen et al. (1988), using acetyl chloride as derivatization agent and heptadecanoic acid as internal standard. FAMEs were analyzed with a gas chromatograph (Hewlett Packard 5890), equipped with a flame ionization detector and a polar, 60-m fused silica capillary column Supelcowax-10 (Supelco). The oven temperature was programmed to increase from 170° to 220°C at a rate of $1°C \cdot min^{-1}$; the injector and detector temperatures were 250° and 270°C, respectively; helium was used as carrier gas. Pure standards of free fatty acids (Sigma) were used for fatty acid identification, which was based on comparison of peak retention times of samples and standards under similar elution conditions. Peak areas were quantified by automatic integration (Hewlett Packard 3395), and calculations were performed according to the AOCS Official Method Ce 1b-89 (Firestone, 1994). Fatty acids were named using the code *i*:*j*(n–*k*), where *i* indicates the total number of carbon atoms, *j* the number of double bonds, and *k* the position of the last double bond counted from the terminal methyl group.

Statistical Analyses. Analyses of variance (ANOVA) for the overall data set, and Fisher's protected least significant difference (PLSD) tests for pairwise comparison, were performed with the software StatView (Abacus Concepts).

Results and Discussion

Biomass Yield. The evolution of AFDW throughout incubation time for the batch cultures of *P. lutheri*, under the various concentrations of CO_2, is plotted in Figure 2. The average values of AFDW (and corresponding standard deviations) by 18 days were 0.365 ± 0.028, 0.540 ± 0.035, and 0.385 ± 0.035 $g \cdot L^{-1}$, for cultures supplied with 0% (control), 1%, and 2% (v/v) CO_2, respectively. (It should be noted that the cultures which were not supplied with extra CO_2 had access to some inorganic carbon, as air contains approximately 0.04% [v/v] CO_2.)

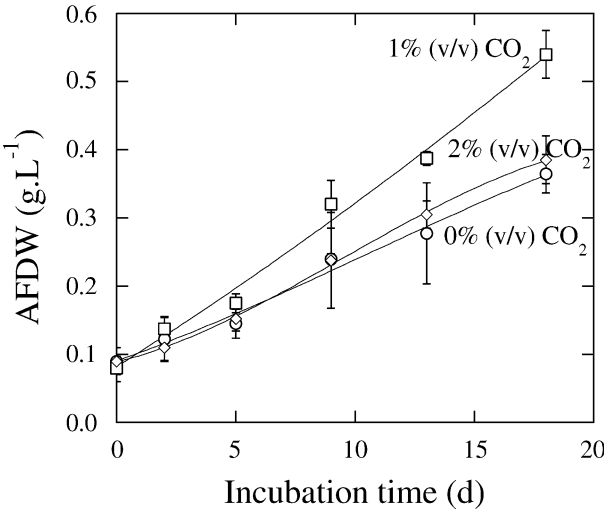

Fig. 2. Evolution of ash-free dry weight (AFDW) (average and 95% error bars) throughout incubation time, at various concentrations of CO_2, in the experiments run under batch mode.

Early during incubation there were no significant differences in terms of biomass between the cultures supplied with the different amounts of CO_2; however, an inflexion in this trend was noticed by 9 days. The highest results were obtained in the cultures grown with 1% (v/v) CO_2. In order to determine whether these results are statistically different from those obtained at the other 2 levels of CO_2 throughout the whole incubation period, the data were submitted to ANOVA and Fisher's PLSD tests with respect to CO_2 effect. It was thus concluded that the data pertaining to the AFDW obtained at 1% (v/v) CO_2 were statistically different (at the 5% significance level) from those obtained at 0% and 2% (v/v) CO_2, by 18 days of incubation.

When supplementing cultures of *Phaeodactylum tricornutum* with gaseous CO_2, Yongmanitchai and Ward (1991) also described increases in biomass up to a concentration of 1% (v/v) of that gas, and a decrease afterward. In a well-mixed batch dense culture, cells may have access to reasonably constant amounts of CO_2, but the availability of energy (i.e., light) will decrease with culture age, owing to its increasing density; consequently, microalgae will be more frequently subjected to darkness, and there will be less CO_2 uptake from the surroundings, so that it may eventually become net excreted because of the respiration process. A likely explanation for the lack of growth enhancement of photosynthetic microalgal cells in the presence of an enhanced CO_2 supply derives from the fact that, after a certain threshold of biomass, the amounts of CO_2 and light available in the bulk of the culture are no longer linearly correlated. The fact that 2% (v/v) CO_2 did not lead to

higher biomass productivity than 1% (v/v) lies probably on the affinity of microalgae for inorganic carbon during photosynthesis, which is reduced when the concentration of CO_2 is increased (Tsuzuki et al., 1990), a phenomenon probably related to decreases in the activity of carbonic anhydrase, and concomitant easier saturation of its active site with inorganic carbon. In fact, numerous experiments have demonstrated that high CO_2 concentrations (i.e., 1% [v/v] or above) can repress the synthesis of carbonic anhydrase, and hence constrain the CO_2-concentrating mechanisms in microalgae (namely, the pathway underlying active transport of inorganic carbon, which concentrates CO_2 at the active site of the carbon-fixing enzyme ribulose biphosphate carboxylase-oxygenase) (Beardall et al., 1998). As CO_2 levels exceeding 1% (v/v) did not promote further increases in biomass yields, the levels of CO_2 tested in the continuous experiments were deliberately not above that threshold.

Results of biomass yield (in terms of cell number and AFDW) and biochemical composition (in terms of lipid content), both pertaining to the continuous experiments, are depicted in Table 1. The highest values for both cell number and organic cell weight (i.e., AFDW) were obtained at the highest light intensity and lowest dilution rate tested. Statistical analysis of the results confirms that both values are statistically different from the remaining ones. Hence, one can state that higher light intensities promote increases in population and biomass (in terms of individual cell weight). The experimental results obtained at higher levels of CO_2 clearly demonstrate that this processing parameter was not limiting, as there was no improvement in biomass when the carbon level was raised.

The biomass was higher in batch cultures (0.540 ± 0.035 g $\cdot$ L^{-1}) than in the equivalent continuous cultures ($0.385 + 0.007$ g $\cdot$ L^{-1}); however, if one considers the experimental results in terms of productivity, the yield obtained in batch cultures by 18 days (0.030 g $\cdot$ L $\cdot$ d^{-1}) is considerably lower than the equivalent yield obtained when operating continuously (0.114 g $\cdot$ L^{-1} $\cdot$ d^{-1}). If one considers batch cultures as pseudocontinuous cultures with dilution rates asymptotically tending to zero, the aforementioned results agree with previous reports of proportional relationships between dilution rate and net biomass productivity (Molina-Grima et al., 1994b).

The effect of light intensity on the biosynthesis of lipids is positive, a result in agreement with reports from Sukenik et al. (1993a) regarding *Nannochloropsis* sp. However, the internal amounts of lipids (in picogram per cell) were higher under higher rates of dilution, whereas better results were

Table 1. Average Values (and Corresponding Standard Deviations) of Biomass and Lipid Yields, in Experiments Run under Continuous Mode*

CO_2 concentration (%)	0.5	0.5	0.5	0.5	0.5	1
Light intensity ($\mu E \cdot m^{-2} \cdot s^{-1}$)	75	75	120	120	120	75
Dilution rate (d^{-1})	0.297	0.426	0.523	0.297	0.426	0.523	0.297
Cell number (cell $\cdot$ mL^{-1})	$(1.7 \pm 0.1)\times10^{7}$	$(7.9 \pm 0.6)\times10^{6a}$	$(2.7 \pm 0.2)\times10^{6}$	$(2.5 \pm 0.1)\times10^{7}$	$(7.4 \pm 1.7)\times10^{6a,b}$	$(7.5 \pm 1.5)\times10^{6a,b}$	$(5.9 \pm 0.2)\times10^{6b}$
AFDW (g $\cdot$ L^{-1})	0.565 ± 0.021	0.267 ± 0.006^{a}	0.235 ± 0.007^{a}	0.900 ± 0.035	0.318 ± 0.022^{b}	0.335 ± 0.024^{b}	0.385 ± 0.007
Lipid content (pg $\cdot$ $cell^{-1}$)	3.0 ± 0.9^{a}	$4.3 \pm 0.6^{a,b,c}$	13.3 ± 2.0	5.3 ± 0.1^{b}	5.3 ± 0.2^{c}	2.11 ± 0.26	8.3 ± 1.6
Lipid content (mg $\cdot$ L^{-1})	50.8 ± 16.3^{a}	34.0 ± 5.0^{b}	36.3 ± 5.3^{b}	132.5 ± 3.5	$39.5 \pm 1.4^{a,b}$	15.8 ± 2.0	$48.5 \pm 9.6^{a,b}$
Productivity (g $\cdot$ L^{-1} $\cdot$ d^{-1})	0.168 ± 0.002	0.114 ± 0.001	0.123 ± 0.001	0.267 ± 0.003	0.135 ± 0.002	0.175 ± 0.003	0.114 ± 0.001

*Values followed by the same letter are statistically equal to each other.

Table 2. Average Values (and Corresponding Standard Deviations) of Percentage Fatty Acid Residues by 18 days, in Experiments Run under Batch Mode

	Concentration of CO_2 (%[v/v])		
Fatty acid	*0*	*1*	*2*
16:0	16.76 ± 2.23	25.91 ± 1.15	21.24 ± 1.32
16:1(n-7)	18.93 ± 2.87	26.38 ± 1.12	28.33 ± 1.42
18:0	1.60 ± 0.53	0.78 ± 0.18	1.52 ± 0.11
18:1(n-9)	1.67 ± 0.31	2.55 ± 0.08	2.95 ± 0.25
18:1(n-7)	2.64 ± 0.03	3.11 ± 0.07	2.86 ± 0.20
18:2(n-6)	0.93 ± 0.08	1.82 ± 0.33	2.05 ± 0.37
18:3(n-6)	0.00 ± 0.00	0.58 ± 0.03	0.49 ± 0.03
18:3(n-3)	0.93 ± 0.18	0.97 ± 0.04	1.18 ± 0.08
18:4(n-3)	5.17 ± 0.23	4.35 ± 0.08	5.05 ± 0.27
20:5(n-3)	16.97 ± 1.13	15.55 ± 0.07	14.68 ± 1.14
22:6(n-3)	10.79 ± 0.08	7.44 ± 0.70	7.18 ± 0.27

obtained under lower dilution rates in terms of weight per culture; note that Reis et al. (1996) had already reported higher lipid levels (on AFDW basis) when lower rates of dilution were employed. Increase of total lipid contribution in slowly growing cultures appears to be a normal feature of eukaryotic cells (Reis et al., 1996).

The internal amounts of lipids were favored by increases in CO_2 availability, although such an effect was overridden in terms of culture biomass because of the lower levels of cells attained under higher contents of CO_2. Chrismadha and Borowitzka (1994) also reported increases in the cellular lipid content of *P. tricornutum*, as a result of adding 5% (v/v) (CO_2) to the culture.

Fatty Acid Profile. The average fractional amounts of the major fatty acids in *P. lutheri* cells grown under 0%, 1% and 2% (v/v) CO_2 (in batch mode) are presented in Table 2. The dominant fatty acid residues found were palmitic (16:0), palmitoleic (16:1(n-7)), octadecatetraenoic (18:4(n-3)), eicosapentaenoic (20:5(n-3)), and docosahexaenoic (22:6(n-3)) acids, in agreement with reported results (Volkman et al., 1991; Yongmanitchai and Ward, 1991; Dunstan et al., 1993).

Significant positive correlations between CO_2 concentration and fatty acid percentage concentrations were found for palmitoleic and oleic (18:1(n-9)) acids, as well as for linoleic (18:2(n-6)) acid; significant negative correlations were observed for the longer chain PUFA DHA. In general, positive correlations were observed between the concentration of CO_2 and the percentage concentrations of less unsaturated fatty acids, whereas negative correlations were found for longer chain PUFAs. These findings are consistent with reports by Tsuzuki et al. (1990), who tested batch cultures of microalgae under CO_2 supplies ranging from 0.04% (approximate concentration in the atmosphere) up to 2% (v/v). As CO_2 is used by cells during photosynthesis, which takes place in the photosynthetic apparatus (namely, chlorophyll, carotenoids, and membrane-associated lipids), there is no need for cells to pass energy to the photosynthetic apparatus as long as the surrounding environment is saturated with CO_2 and other parameters such as light intensity are limiting. Hence, a relatively higher amount of the carbon fixed by cells will be allocated to synthesis of triacylglycerols, with concomitant increase in the total amount of saturated fatty acids. Sato et al. (2003) also reported that saturation levels of fatty acids in *Chlorella kessleri* are determined by the balance between de novo synthesis of fatty acids and desaturation of preexisting ones, depending on CO_2 concentration. Furthermore, correlations between the induction of the CO_2–concentrating mechanism and the acceleration of fatty acid desaturation have also been proposed (Pronina et al., 1998).

The relative fatty acid contents recorded in continuous cultures are given in Table 3. Increases in the rate of dilution produced statistically significant increases in the percentage concentration of oleic acid in the experiments run at lower levels of light, and increases in the percentage concentrations of γ-linolenic (18:3(n-6)) and α-linolenic (18:3(n-3)) acids, in the experiments run at higher levels; for the latter levels of light, octadecatetraenoic acid and EPA decreased in percentage content with dilution rate. With regard to the effect of CO_2 on the fatty acid profile, only oleic acid showed statistically significant enhancement with increased amounts of carbon (at constant light intensities and dilution rates); percentages of EPA and DHA decreased, although not significantly. Although high levels of CO_2 have been shown to affect the fatty acid residue profile of microalgae, reports on the effects observed are not consistent: whereas Chrismadha and Borowitzka (1994) observed decreases in EPA with CO_2

Table 3. Average Values (and Corresponding Standard Deviations) of Percentage Fatty Acid Residues, in Experiments Run under Continuous Mode

CO_2 (%, v/v)	0.5	0.5	0.5	0.5	0.5	0.5	1
Light intensity ($\mu E \cdot m^{-2} \cdot s^{-1}$)	75	75	75	120	120	120	75
Dilution rate (d^{-1})	0.297	0.426	0.523	0.297	0.426	0.523	0.297
Fatty acid							
16:0	12.18 ± 0.90	12.81 ± 0.44	10.36 ± 1.13	16.28 ± 3.04	18.02 ± 5.51	14.14 ± 1.36	15.00 ± 0.11
16:1(n-7)	17.75 ± 1.21	19.76 ± 0.74	14.96 ± 1.65	18.04 ± 2.29	20.20 ± 6.32	23.88 ± 2.26	16.96 ± 0.14
18:0	2.30 ± 0.01	2.00 ± 0.26	3.45 ± 0.16	4.31 ± 6.20	6.51 ± 10.25	1.03 ± 0.31	4.47 ± 0.12
18:1(n-9)	2.25 ± 0.08	1.67 ± 0.51	3.00 ± 0.17	2.28 ± 0.25	2.29 ± 1.04	1.71 ± 0.15	3.88 ± 0.05
18:1(n-7)	1.48 ± 0.09	1.40 ± 0.04	1.79 ± 0.30	2.49 ± 0.16	2.88 ± 0.65	3.08 ± 0.10	0.70 ± 0.99
18:2(n-6)	0.65 ± 0.02	0.46 ± 0.04	0.80 ± 0.05	1.83 ± 0.11	2.33 ± 0.64	2.30 ± 0.29	0.47 ± 0.01
18:3(n-6)	Trace	Trace	Trace	0.49 ± 0.03	0.43 ± 0.13	1.13 ± 0.21	Trace
18:3(n-3)	0.99 ± 0.09	0.56 ± 0.01	0.58 ± 0.01	1.18 ± 0.22	0.60 ± 0.15	2.76 ± 2.36	0.96 ± 0.01
18:4(n-3	5.90 ± 0.27	6.25 ± 0.18	4.75 ± 0.13	7.02 ± 1.11	4.17 ± 1.29	4.00 ± 0.30	4.87 ± 0.04
20:5(n-3)	13.99 ± 0.64	14.39 ± 0.37	10.51 ± 0.15	17.06 ± 2.45	10.48 ± 3.31	11.07 ± 1.12	11.70 ± 0.16
22:6(n-3)	5.78 ± 0.28	5.62 ± 0.21	5.38 ± 0.20	6.09 ± 0.81	5.84 ± 1.83	6.64 ± 0.23	4.54 ± 0.06

addition in cultures of *P. tricornutum*, Yongmanitchai and Ward (1991) found that CO_2 addition increased the content of EPA in the same species. Distinct environmental parameters are known to exert interdependent effects on a culture, leading to different results in apparently equivalent situations (Carvalho and Malcata, 2003); consequently, those (putatively) inconsistent results were probably due to differences in the remaining experimental conditions.

The effect of light intensity often depended on the dilution rate: the contents of octadecatetraenoic acid and EPA decreased significantly with light intensity at intermediate dilution rates, whereas those results were not statistically affected at extreme dilution rates; palmitoleic, oleic, and α-linolenic acids increased significantly in content with light only at the highest dilution rate, whereas octadecenoic (18:1(n-7)), linoleic, and γ-linolenic acids increased consistently with light at all dilution rates considered. Molina-Grima et al. (1994a) also described decreases in the content of EPA with increasing light, in batch cultures of *Isochrysis galbana*. It is widely accepted that low levels of light intensity bring about increases in the amount of thylakoid membranes, thus promoting synthesis of its lipid constituents—galactolipids (which contain a high percentage of EPA). Therefore, maximum productivity of EPA is expected when cells are grown in a nutrient-replete medium and under light-limiting conditions (Sukenik and Carmelli, 1989; Sukenik et al., 1993b). In our experiments, EPA reached its highest percentage level at high light intensity (120 $\mu E \cdot m^{-2} \cdot s^{-1}$) and at the lowest dilution rate (0.297 d^{-1}); nevertheless, this value is not statistically different from that obtained under the lowest light intensity, with other experimental conditions being equivalent; therefore, under these conditions of dilution rate and CO_2 added, light intensity is not a critical parameter in cellular composition of EPA. The highest percentage content of DHA was obtained at high light intensity (120 $\mu E \cdot m^{-2} \cdot s^{-1}$) and high dilution rate (0.523 d^{-1}); this value is statistically different only from that obtained in the experiment run at 1% (v/v) CO_2.

The productivities of EPA and DHA under the various experimental conditions tested are grouped according to the amount of CO_2 added (Figure 3) and

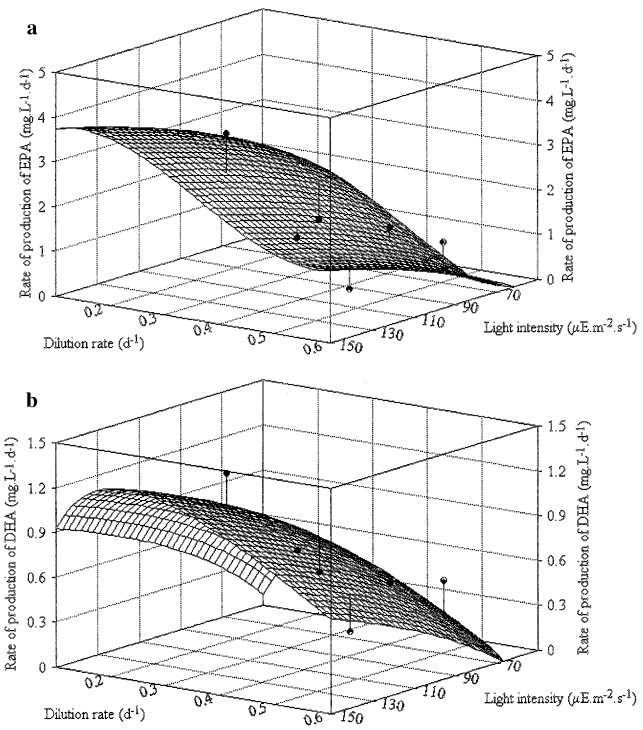

Fig. 3. Rates of production of EPA (a) and DHA (b), in the experiments run under continuous mode, at a constant concentration of CO_2 (0.5%, v/v), and variable dilution rates and light intensities.

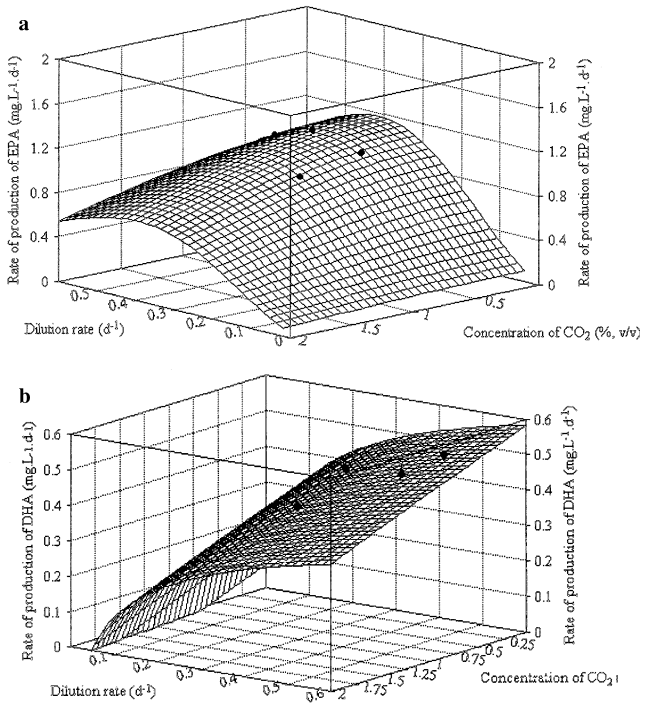

Fig. 4. Rates of production of EPA (a) and DHA (b), in the experiments run under continuous and batch modes, at a constant level of light intensity (75 $\mu E \cdot m^{-2} \cdot s^{-1}$), and variable dilution rates and CO_2 concentrations.

the light intensity supplied (Figure 4). Continuous cultures accounted for higher productivities of both fatty acids than batch cultures did. Although the highest cellular content of PUFAs was found in experiments run at high dilution rates, the highest productivities were again attained at the lowest dilution rates, similar to the trends prevailing for the total lipid content. In fact, increases in the cellular concentrations of unsaturated fatty acids when the dilution rate is increased do not necessarily lead to increases in the productivity of those unsaturated fatty acids, because, as dilution rate increases, the biomass yield decreases concomitantly, thus leading to lower numbers of viable cells (although each cell possesses a higher amount of unsaturated fatty acids). Light intensity promoted increases in both EPA and DHA productivities at lower dilution rates, but the effect was reversed at the highest dilution rate. Although the total lipid content was markedly higher when CO_2 was added, the PUFA productivity was not enhanced; hence, considering the high cost of CO_2 for commercial algal culturing devices, there is no apparent benefit in adding extra CO_2 when attempting to maximize the yield of PUFAs. Mass productivities of both EPA (3.61 ± 0.04 $mg \cdot L^{-1}.d^{-1}$) and DHA (1.29 ± 0.01 $mg \cdot L^{-1} \cdot d^{-1}$) were higher in cultures subjected to 0.5% (v/v) CO_2, a dilution rate of 0.297 d^{-1}, and a light intensity of 120 $\mu E \cdot m^{-2} \cdot s^{-1}$; these values were statistically different from the remaining ones, at the 5% significance level.

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References

1. Beardall J, Johnston A, Raven J (1998) Environmental regulation of CO_2-concentrating mechanisms in microalgae. Can J Bot 76, 1010–1017
2. Becker EW (1994) *Microalgae: Biotechnology and Microbiology*. (Cambridge, U.K.: Cambridge University Press)
3. Bligh WJ, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37, 911–917
4. Carvalho AP, Malcata FX (2000) Effect of culture media on production of *Pavlova lutheri*. Cryptog Algol 21, 59–71
5. Carvalho AP, Malcata FX (2003) Kinetic modeling of the autotrophic growth of *Pavlova lutheri:* study of the combined influence of light and temperature. Biotechnol Prog 19, 1128–1135
6. Chrismadha T, Borowitzka MA (1994) Effect of cell density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. J Appl Phycol 6, 67–74
7. Cohen Z, Vonshak A, Richmond A (1988) Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum:* correlation to growth rate. J Phycol 24, 328–332
8. Dunstan GA, Volkman JK, Barret SM, Garland CD (1993) Changes in the lipid composition and maximisation of the polyunsaturated fatty acid content of three microalgae grown in mass culture. J Appl Phycol 5, 71–83
9. Firestone D (1994) *The Official Methods and Recommended Practices of the American Oil Chemists' Society* (Champaign, Ill.: American Oil Chemist' Society)
10. Kaplan D, Richmond AE, Dubinsky Z, Aaronson S (1986) *CRC Handbook of Microalgal Mass Culture*. (Boca Raton, Fla.: CRC Press)
11. Lepage G, Roy C (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25, 1391–1396
12. Molina-Grima E, Camacho FG, Pérez JAS, Sanchéz JLG (1994a) Biochemical productivity and fatty acid profiles of *Isochrysis galbana* Parke and *Tetraselmis* sp. as a function of incident light intensity. Proc Biochem 29, 119–126
13. Molina-Grima E, Pérez JAS, Camacho FG, Fernandez FGA, Sevilla JMF, Sanz FV (1994b) Effect of dilution rate on eicosapentaenoic acid productivity of

Phaeodactylum tricornutum UTEX640 in outdoor chemostat culture. Biotechnol Lett 16, 1035–1040
14. Negoro M, Shioji N, Miyamoto K, Miura Y (1991) Growth of microalgae in high CO_2 gas and effects of SO_X and NO_X. Appl Biochem Biotechnol 28/29, 877–885
15. Pronina NA, Rogova NB, Furnadzhieva S, Klyachko-Gurvich GL (1998) Effect of CO_2 concentration on the fatty acid composition of lipids in *Chlamydomonas reinhardtii* ω-3, a mutant deficient in CO_2–concentrating mechanism. Russian J Plant Physiol 45, 447–455
16. Reis A, Gouveia L, Veloso V, Fernandes HL, Empis JA, Novais JM (1996) Eicosapentaenoic acid–rich biomass production by the microalga *Phaeodactylum tricornutum* in a continuous-flow reactor. Bior Technol 55, 83–88
17. Robin JH (1995) The importance of n-6 fatty acids in the culture of marine fish larvae. In: *Proceedings of the Symposium in Mass Rearing of Juvenile Fish,* Pittman K, Batty RS, Verreth J, eds. (Norway: Bergen) pp 106–111
18. Sargent JR, Bell JG, Bell MV, Henderson, RJ, and Tocher, DR (1994) Marine organisms as models for essential fatty acid research. In: *Proceedings of the 3rd International Marine Biotechnology Conference*. (Tromsoe, Norway: Tromsoe University, 94)
19. Sato N, Tsuzuki M, Kawaguchi A (2003) Glycerolipid synthesis in *Chlorella kessleri* 11 h, II: effect of the CO_2 concentration during growth. Biochim. Biophys Acta 1633, 35–42
20. Sorgeloos P, Dehasque M, Dhert P, Lavens P (1995) Review of some aspects of marine fish larviculture. In: *Proceedings of the Symposium in Mass Rearing of Juvenile Fish,* Pittman K, Batty RS, Verreth J, eds. (Norway: Bergen) pp 138–142
21. Sukenik A, Carmeli Y (1989) Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. J Phycol 25, 686–692
22. Sukenik A, Yamaguchi Y, Livne A (1993a) Alterations in lipid molecular species of the marine eustigmatophyte *Nannochloropsis* sp. J Phycol 29, 620–626
23. Sukenik A, Zmora O, Carmeli Y (1993b) Biochemical quality of marine unicellular algae with special emphasis on lipid composition, II: *Nannochloropsis* sp. Aquaculture 117, 313–326
24. Tsuzuki M, Ohnuma E, Sato N, Takaku T, Kawaguchi A (1990) Effects of CO_2 concentration during growth on fatty acid composition in microalgae. Plant Physiol 93, 851–856
25. Volkman JK, Jeffrey SW, Nichols PD, Rogers GL, Garland CD (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J Exp Mar Biol Ecol 128, 219–240
26. Volkman JK, Dunstan GA, Jeffrey SW, Kearney PS (1991) Fatty acids from microalgae of the genus *Pavlova*. Phytochem 30, 1855–1859
27. Volkman JK, Dunstan GA, Barret SM, Nichols PD, Jeffrey SW (1992) Essential polyunsaturated fatty acids of microalgae used as feedstocks in aquaculture. In: *Proceedings of the Aquaculture Nutrition Workshop,* Allan GL, Dall W, eds. (Salamander Bay, Australia: NSW Fisheries) pp 180–186
28. Yongmanitchai W, Ward OP (1991) Screening of algae for potential alternative sources of eicosapentaenoic acid. Phytochemistry 30, 2963–2967
29. Yongmanitchai W, Ward OP (1992) Growth and eicosapentaenoic acid production by *Phaeodactylum tricornutum* in batch and continuous culture systems. JAOCS 69, 584–590