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EPA from *Isochrysis galbana*. Growth Conditions and Productivity

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The marine microalga Isochrysis galbana *Parke is presented as an alternative source of eicosapentaenoic acid (EPA). The effects of growth conditions on polyunsaturated fatty acid productivity with special attention to EPA and docosahexaenoic (DHA) acids were studied. The variables tested were: aeration flow rate, nitrate concentration, temperature, light intensity, micronutrient and vitamin concentration, and pH.*

Growth conditions producing the greatest EPA yields of over 200 μg/litre/h, are indicated as a starting point for a large scale mass culture system which would develop I. galbana's *potential.*

INTRODUCTION

Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are becoming of more and more interest to researchers due to their importance in the prevention of heart and circulatory diseases, a major health problem in industrialized countries.[1,2]

The commercial source of these fatty acids is marine fish and their oils, which composition is determined by the availability of plankton, mainly microalgae, the first link of the marine food chain. This production fills only 60% of world demand for these substances.[3]

The marine microalga *Isochrysis galbana*, widely used in aquaculture,[4] is a species rich in lipids. The fatty acid composition in 59 clones isolated from the original culture of *I. galbana* Parke used in the present study, was recently analysed. The major fatty acid was EPA with the DHA content also being high, making it a potential alternative source of these two substances.[5]

There are two possible ways of producing EPA/DHA from microalga. The first of these is to obtain the maximum biomass concentration possible in the production system while alternatively the biomass composition may be influenced by varying growth conditions to increase the total lipid content and/or the composition of the fatty acids.

The lipid content of microalgae tends to be inversely proportional to growth rate,[6] although when the results from different authors are compared, different culture conditions, analytical methods, state of growth and even the variety of species should be borne in mind. The importance of the latter is commonly observed in papers in which *Isochrysis galbana* Parke or *Isochrysis* aff. *galbana*

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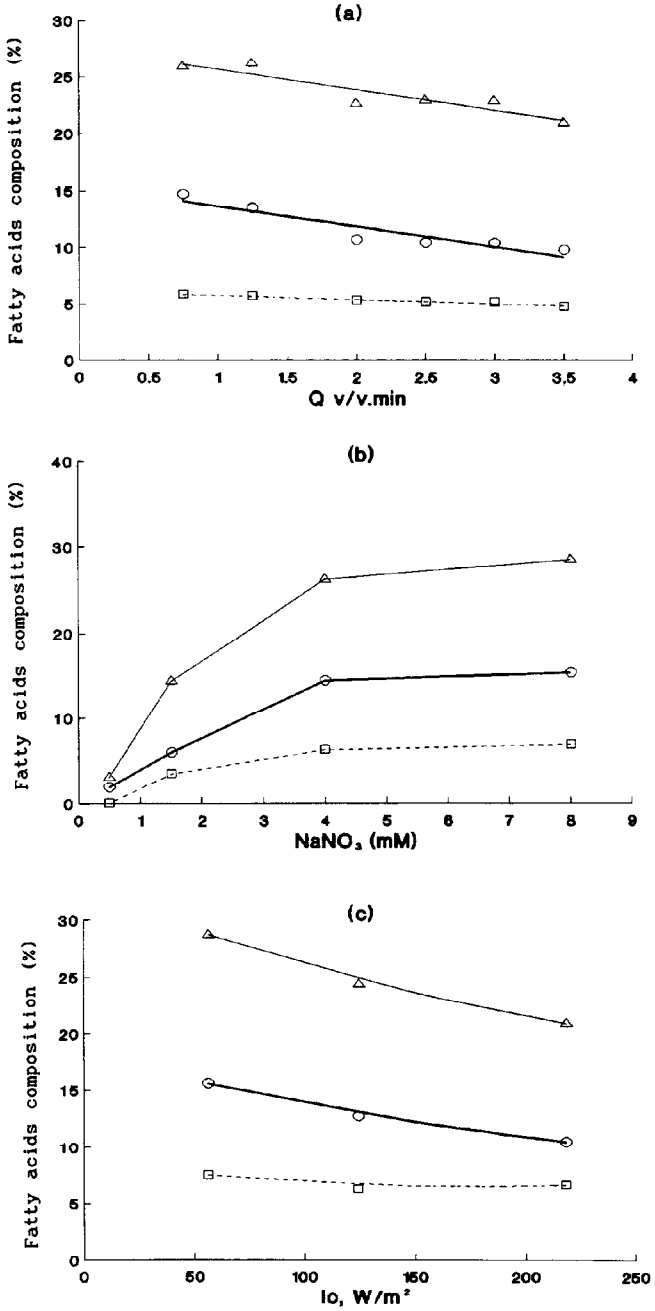

Fig. 1. Effect of (a) aeration flow rate (Q), (b) initial nitrate concentration and (c) light intensity (I_o) on the content of PUFAs (△), EPA (○) and DHA (□) as a percentage of total fatty acids.

clone T-ISO (also 'Tahiti *Isochrysis*'), are often referred to simply as *I. galbana*, whereas these two species are quite different. T-ISO is capable of adapting to a much broader range of temperature/light conditions with an optimum growth temperature of 27·5 °C (specific growth rate $\mu = 0{\cdot}082\ h^{-1}$) while *I. galbana* Parke's optimum growth temperature is near 20 °C and no growth at all was observed at 27·5 °C,[7] which agrees with the results reported by Kain & Fogg[8] for *I. galbana* Parke. With regard to their biochemical composition, the total lipid content of *I. galbana* Parke is similar to that of T-ISO.[9,10] Whyte[9] reported 19·92% and 22·07 (% dry weight (DW)) of lipids for *I. galbana* Parke in the exponential and stationary phases of growth respectively, while for T-ISO, the percentages were found to be 23·54 and 23·27 (% DW) in the same growth phases respectively. The paper referred to above,[10] presents a comparison of the fatty acid profiles in both species which shows that T-ISO lacks PUFA 20:5n3 while 22:6n3 is present in both.

In this paper, those culture conditions most favourable to the industrial production of EPA and DHA in *I. galbana* were investigated.

MATERIALS AND METHODS

Isochrysis galbana Parke was kindly provided by Dr Jaime Fábregas (University of Santiago de Compostela, Spain) through Pescanova's Cabo de Gata factory in Almeria, Spain. This culture was originally obtained from the Culture Centre for Algae and Protozoa, Cambridge, UK by Dr Fábregas.

Cylindrical culture flasks (1 litre) with an outer sleeve permitting the circulation of water for temperature control were used for algal growth. The culture medium was sea water enriched with $NaNO_3$, 2 mM; NaH_2PO_4, 100 μM; $ZnCl_2$, 1 μM; $MnCl_2$, 1 μM; Na_2MoO_4, 1 μM; $CoCl_2$, 0·1 μM; $CuSO_4$, 0·1 μM; ferric citrate, 20 μM; thiamine, 35 μg/litre; biotin, 5 μg/litre; B_{12}, 3 μg/litre; EDTA, 26·4 mM; Tris-HCl, 5 mM; pH, 8·0. Sterilization processes were as in Molina *et al.*[11]

The cultures were constantly illuminated with four Phillips TLD W/54 fluorescent lamps. Cell concentration was determined by absorbance at 530 nm. All growth experiments were carried out in batch cultures at an initial concentration of 2 mM $NaNO_3$, 20 °C, pH 8, and a light intensity of 110 W/m², except those in which the variable was studied, in which case, only this variable was altered. The range of variations used were: Air flow rate, 0·75–3·50 v/v/min; light intensity, 56–218 W/m²; $NaNO_3$ concentration, 0·5–8 mM; temperature,

10–25 °C; micronutrient concentration, 53–1070 mg/litre; vitamin concentration, 4·3–86 μg/litre, and pH 6–8.

Lipids were extracted by the Kochert method.[12] Fatty acid methylation was by direct transesterification with acetyl chloride: methanol (1:20).[13] The analysis of methyl esters was carried out by gas chromatography using a 30 m capillary column of fused silica (SP2330, Supelco, Bellefonte, Pa, USA), internal diameter of 0·25 mm, 0·20 mm standard film, split retention 100:1, and a flame ionization detector. SIGMA Lipid Standard 189–15, Supelco Rapeseed oil mixture and Supelco PUFA-1 patterns were used for the determination. The pigment content was subtracted from the total lipid to calculate the fatty acid content.

RESULTS AND DISCUSSION

Aeration flow rate

When aeration was increased from 0·75 to 3·50 v/v/min, there was no variation in the lipid content of the biomass harvested, which averaged 35·36% of dry weight, providing lipid concentrations of around 210 mg/litre when cultures reached the stationary phase of growth. However, the proportion of polyunsaturated fatty acids (PUFAs) in the total fatty acids tended to decrease when aeration was increased, as reflected mainly in the EPA content (Fig. 1(a)).

Furthermore, fatty acid productivity also depended on the microalgal growth kinetics. Thus, for aeration rates of about 1 v/v/min, at which the EPA content was greater than at higher rates (approx. 14% of total fatty acids), the cells began to grow at the same specific growth rate of 0·032 h^{-1}, but when concentration rose to over 300 mg/litre the growth rate slowed down due to limitation produced by CO_2 transfer. This transport limitation caused the pH to increase as the internal carbonate buffer system was disturbed as $CO_{2(aq)}$ was consumed by the cells.[14] Growth then changed from exponential to linear (Fig. 2(a)). However, for aeration flow rates above 2·50 v/v/min, the exponential growth phase was maintained up to higher concentrations, and the linear growth phase due to CO_2 limitation was short, attaining the stationary phase in a shorter time and with a consequent increase in productivity.

The excessive turbulence produced at high aeration rates may produce some cell damage,[15] especially to those naked flagellate species such as *I. galbana*. This could be the reason why the greatest biomass concentration was attained at 2·50 v/v/min.

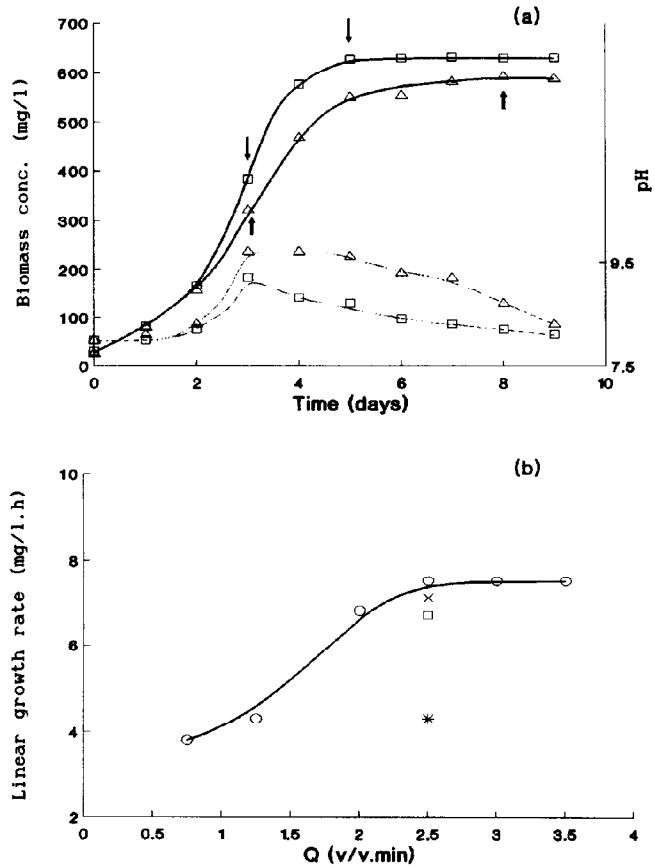

Fig. 2. Effect of aeration flow rate on growth. (a) Biomass concentration (solid lines) and pH (dashed lines) versus culture time at 0·75 (△) and 2·50 (□) v/v/min. Linear growth phase is marked by arrows. (b) Linear growth phase rate versus aeration flow rate (Q). Values obtained at different light intensities are given for comparison. [110 W/m²(○), 56 W/m²(*), 124 W/m²(□), 218 W/m²(×)].

When the linear phase growth rate was plotted against the aeration rate, a proportional increase was observed up to a maximum value of 7·5 mg/litre/h at 2·50 v/v min (Fig. 2(b)). This demonstrated that at aeration rates below 2·50 v/v min, growth was limited by the CO_2 available to the cells, and at higher flow rates the air to culture medium transfer was maximum in the system used here. Therefore, when the aeration flow rate rose to over 2·50 v/v min, $CO_{2(gas)}$ to $CO_{2(aq)}$ transport was not increased. It may be that the change of slope observed could be caused by the other continuously supplied 'nutrient', light intensity. However, in cultures grown with varying

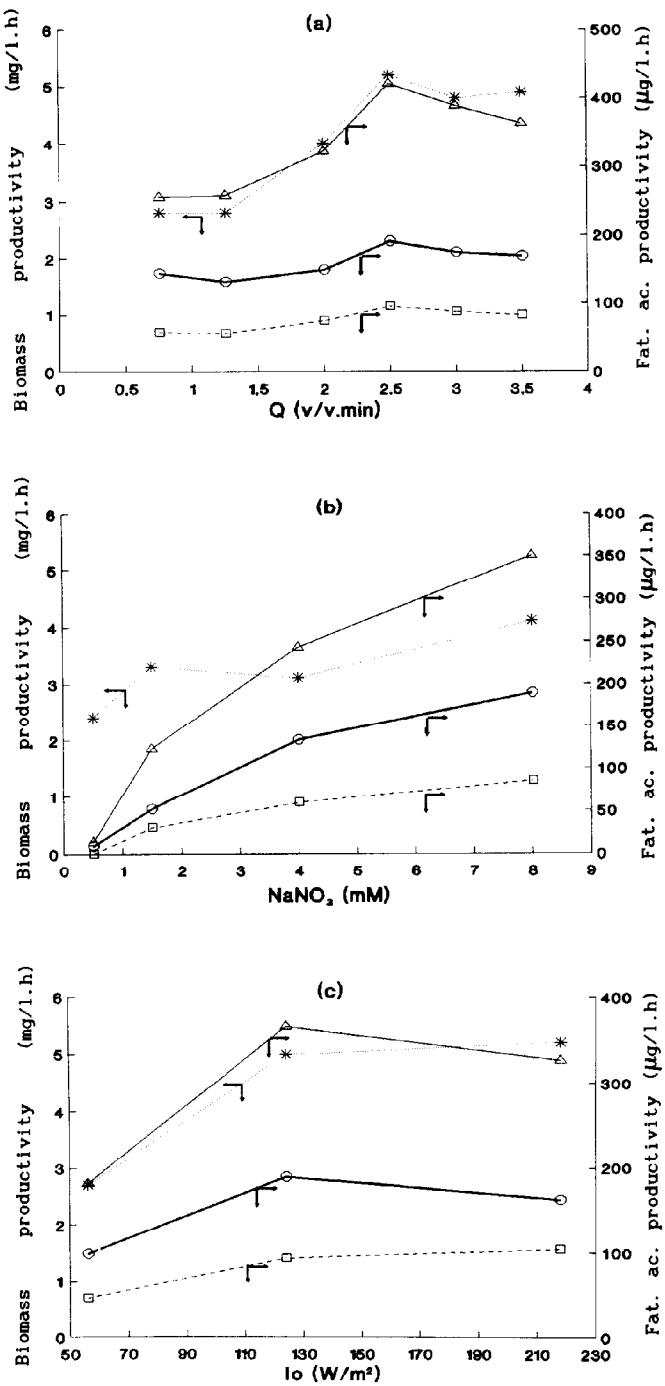

Fig. 3. Influence of (a) aeration flow rate (Q), (b) initial nitrate concentration and (c) light intensity (I_o) on the productivity of biomass (*), PUFAs (△), EPA (○) and DHA (□).

incident light intensities the linear growth rate did not increase over 110 W/m², so that this possibility may be discarded (Fig. 2(b)).

This limitation by CO_2 transport caused the pH to rise and remain constant during the linear growth phase, when the gas–liquid transfer was equalled by cell consumption (see Fig. 2(a)). Therefore, to maintain growth in the exponential phase, it was necessary to control the pH by means of the carbon supply in order to stabilize the carbonate buffer system and assure the $CO_{2(aq)}$ supply to the cells.

As mentioned above, the greatest EPA concentrations were found at aeration rates of around 1 v/v min (Fig. 1(a)), providing a stationary phase productivity of 140 μg/litre/h for 9-day cultures (Fig. 3(a)), and a biomass productivity of 2·8 mg/litre/h for the same period of time, although it is at 2·50 v/v/min that the maximum EPA productivity was obtained, in spite of its lower content. This was due to the very short linear phase produced at this flow rate. Culture reached the stationary phase by the 5th day (Fig. 2(a)), with productivities of 191 μg/litre/h and 5·2 mg/litre/h for EPA and biomass respectively (Fig. 3(a)). At 3·50 v/v/min, the stationary phase was again reached on the 5th day, the lower biomass concentration causing the productivity to be lower than that obtained at 2·50 v/v/min, 169 μg/litre/h and 4·9 mg/litre/h for EPA and biomass respectively (Fig. 3(a)).

Nitrate concentration

Nutrient concentration often has a dramatic affect on the biochemical profile of microalgae with variations dependent on the species and the nutrients provided.[16,17] Nitrate plays an important role in determining the lipid content of *I. galbana* Parke. Utting[18] reported a decrease in lipid content from 31% to 25% of organic weight (equivalent to 22% and 16·9% DW),[10] when the nitrogen concentration was increased from 0·613 mgN/litre (0·04 mM) to 9·8 mgN/litre (0·7 mM). Our results indicate that for concentrations below 2 mM $NaNO_3$, the lipid content decreased markedly from 30% ([$NaNO_3$] > 2 mM) to 19% of dry weight at 0·5 mM $NaNO_3$, while the PUFA and EPA content of the total fatty acids increased with increasing $NaNO_3$ concentration (Fig. 1(b)). This apparent variance from Utting's results could be due to the very low concentrations used, in which case growth was limited not only in the stationary phase (nitrogen starvation), but in the log phase, producing lower specific growth rates, (as is the case at 0·5 mM $NaNO_3$ in the present paper). On the other hand, an effect similar to Utting's was reported by Ben-Amotz *et al.*[19] with a strain of *Isochrysis* sp. UTEX 2307. An increase in lipid content (7·10 to

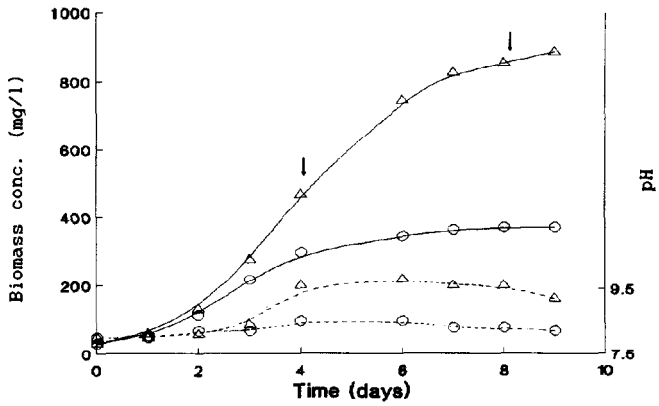

Fig. 4. Biomass concentration (solid lines) and pH (dashed lines) versus culture time at 1·5 mM (○) and 8 mM (△) nitrate concentrations. Linear growth phase is marked by arrows. There is no linear phase at 1·5 mM $NaNO_3$.

26% OW) with decreasing nitrate concentrations (5 to 0·5 mM NO_3^-) was observed. This could be due to the difference in the day of harvest, the end of the logarithmic phase at 5 mM NO_3^- and 10 more days of incubation in the stationary phase at 0·5 mM NO_3^-. Several authors[20] have reported that the lipid content increases as cultures age. This must be borne in mind as at very low nitrate concentrations, starvation occurs at different times of incubation, depending on the concentration of nitrogen supplied. Nitrate also has a significant effect on growth. The specific growth rate is limited at initial concentrations below 0·5 mM $NaNO_3$. Above this concentration it was constant at 0·032 h^{-1}. As the nitrate availability increased, the final concentration of cells in the stationary phase increased, reaching 890 mg/litre at a concentration of 8 mM $NaNO_3$ (Fig. 4). Due to limitation by CO_2 transfer, this biomass increase occurred during the linear phase, directly affecting productivity. Thus, at the highest $NaNO_3$ concentration tested the culture productivity in stationary phase at 9 days was 189 μg/litre/h EPA and 4·1 mg/litre/h biomass (Fig. 3(b)), which is similar to the productivity obtained at 2 mM $NaNO_3$ at 2·50 v/v/min, as discussed above (Fig. 3(a)). This indicates that the increase of biomass concentration without increasing the CO_2 supply to maintain exponential growth, and thus avoid linear growth, is not a feasible method of improving productivity.

Temperature

In Fig. 5(a), specific growth rates (μ) are quoted for sea water temperatures in the range of Almería Bay, in Spain (10–25 °C). It may be seen here how up to 20 °C there is a linear increase of μ with a more marked descent when these temperatures are surpassed, an effect common in the majority of microorganisms above their optimum growth temperature.[21] These findings are in agreement with those obtained by both Kain & Fogg[8] and Ewart & Pruder,[7] although the light intensities reported were different in all three papers (present paper: 110 W/m^2; Kain & Fogg: 26 W/m^2; Ewart & Pruder: 7·5 W/m^2). The highest specific growth rate and biomass productivity, reached at 20 °C, combined with a decrease in lipid content found when the temperature was lowered (19% and 16·5% of dry weight at 15° and 10 °C respectively), resulted in higher EPA and DHA production at 20 °C than at the other temperatures tested. Fatty acid biosynthesis was not affected above 15 °C, while at 10 °C a decrease of EPA content occurred (Fig. 5(b)).

Light

According to Zlotnik & Dubinsky[22] carbon assimilation by *Isochrysis galbana* increased linearly with increased light intensity up to 113 W/m^2, followed by a saturation effect up to 376 W/m^2, while higher intensities inhibited photosynthesis. The results of the present study basically agree with those of these authors with a specific growth rate of 0·021 h^{-1} at 56 W/m^2. This is less than the maximum of 0·032 h^{-1} obtained at 124 and 218 W/m^2, within the range they proposed as saturating. The lipid content was 30% in the range of light saturation and decreased to 23·5% at limiting intensity. The EPA content was inversely related to incident light (Fig. 1(c)), as observed with other species of marine phytoplankton.[23] No effect on DHA was observed, but maximum EPA productivity was obtained at 124 W/m^2 (Fig. 3(c)).

pH

Other environmental factors such as micronutrients, vitamins and pH may influence algal growth and physiology. The response of *I. galbana* to these complementary variables were studied at three levels. As growth inhibition occurred at an initial pH greater than 9[8,17] more acid pH values were tested. As reported earlier the specific growth rate decreased below pH 8 with adaptation phases becoming longer as the pH was lowered (pH = 8, μ = 0·032 h^{-1}; pH = 7, μ = 0·019 h^{-1}; pH = 6, μ = 0·014 h^{-1}). The total lipid content was not altered although the percentage of EPA decreased with an increase in pH (Table 1). Thus, pH 8 with the

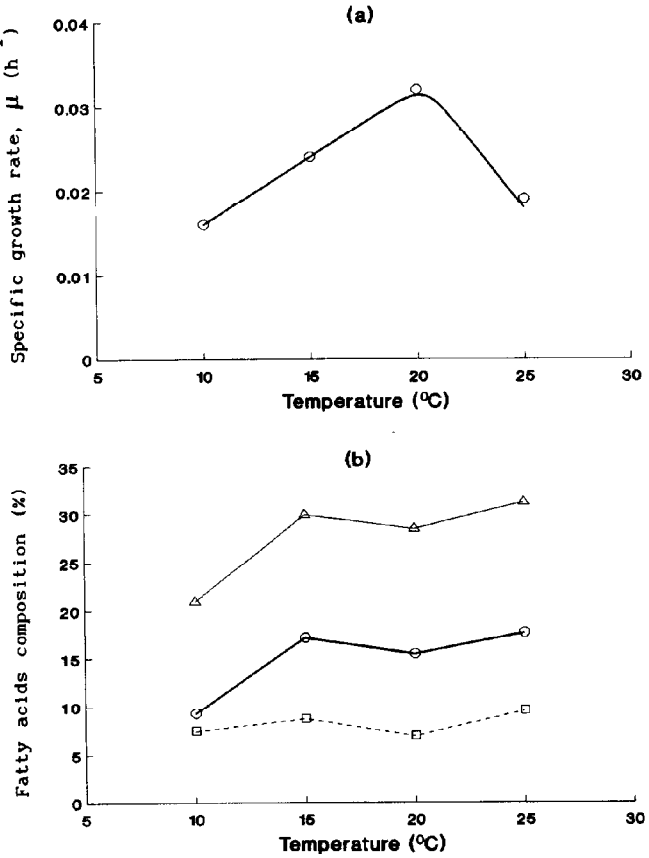

Fig. 5. Effect of temperature on (a) specific growth rate (μ) and (b) PUFAs (△), EPA (○) and DHA (□) content as a percentage of total fatty acids. (Initial nitrate concentration was 8 mM.)

highest specific growth rate and a shorter adaptation phase was the best pH for EPA production.

Micronutrients and vitamins

Micronutrients and vitamins were each varied as a group (without specifying any one component in particular) by multiplying the concentration of the medium normally used by 0·1, 0·5 and 2. No variation in specific growth rate and biomass concentration was observed in any of the nutrient groups once the cultures reached the stationary phase on the 5th day. The lipid content did vary, however, increasing at the lowest concentration of nutrients and vitamins tested. The degree of fatty acid unsaturation was also greatest at the lowest concentration. This suggests that biomass productivities remained constant when the micronutrient and vitamin concentrations were reduced, while EPA productivity increased to 231 μg/litre/h and 243 μg/litre/h at 53 mg/litre micronutrients and 4·3 μg/litre vitamins respectively. As there was no way of attributing the effects observed to a given element or vitamin, more research is necessary to distinguish which are factors affecting *I. galbana* lipid composition. A multifactorial design has been developed in our laboratory, and results will be reported shortly.

CONCLUSIONS

From the aeration flow rate, pH and light intensity experiments described in this paper, an inverse relationship was observed between the most growth-favourable environmental conditions and the biosynthesis of high energy content molecules such as PUFAs, especially EPA.

The operating conditions most favourable for EPA productivity over 200 μg/litre/h were found to be: 20 °C and incident light intensity of 100–120 W/m^2 with gentle agitation and aeration of about 1 v/v/min; pH simultaneously kept near 8·0 through gaseous phase CO_2 partial pressure control; and a culture medium rich in nitrate, without excessive micronutrient or vitamin contents.

Table 1. Effect of pH, micronutrients and vitamin concentrations on lipid (% DW), PUFAs, EPA and DHA (% of total fatty acids) content

	pH			*Micronutrients (mg/litre)*			*Vitamins (μg/litre)*		
	6	*7*	*8*	*53*	*267*	*1070*	*4·3*	*21·5*	*86·0*
Lipid	33·3	32·6	34·5	39·0	30·4	26·4	36·6	24·7	27·6
PUFAs	24·91	21·31	14·77	23·24	18·16	17·66	25·12	23·72	20·64
EPA	10·87	9·96	7·93	11·28	8·43	8·27	12·79	11·95	9·87
DHA	6·37	5·58	3·59	5·53	3·88	3·71	5·97	5·34	5·19

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