Increase of the Yields of Eicosapentaenoic and Docosahexaenoic Acids by the Microalga *Pavlova lutheri* Following Random Mutagenesis

Luís A. Meireles, A. Catarina Guedes, F. Xavier Malcata

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, P-4200-072 Porto, Portugal; telephone: +351-22-5580004; fax: +351-22-5090351; e-mail: xmalcata@esb.ucp.pt

Keywords: genetics; EPA; DHA; microorganisms; PUFA

Abstract: The high commercial values of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have driven a strain-improvement program, aimed at increasing the content of those fatty acids in the microalga *Pavlova lutheri* (SMBA 60) as parent strain. After a round of mutation using UV-light as mutagenic agent, an isolate strain (tentatively called II#2) was obtained, the EPA and DHA contents of which (in % dry biomass) were 32.8% and 32.9% higher than those of the control, native strain. The final EPA yields, when the cultures were maintained under appropriate conditions, were 17.4 and 23.1 $mg \cdot g^{-1}$ dry biomass, for the wild-type and the II#2 strain, respectively, whereas the final DHA yields were 8.0 and 10.6 $mg \cdot g^{-1}$ dry biomass, respectively. These results suggest that random mutagenesis can successfully be applied to increase the yield of n-3 fatty acids by microalgae.

INTRODUCTION

Microalgae are widely used in the aquaculture industry, mostly to feed fish, crustaceans, and bivalves directly. They are also used indirectly, although to a much lesser extent, via feeding of rotifers, copepods, and brine shrimps, which are in turn used to feed the former (Borowitzka, 1997; Spektorova et al., 1986; Volkman et al., 1989). The final nutritional value and biomass productivity of the products of aquaculture will thus be constrained by the nutritional quality of the nourishment, specifically in terms of several growth-promoting compounds. Fish are one of the most important sources of polyunsaturated fatty acids (PUFA) in human nutrition, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are well known for their beneficial effects on human health (Carvalho and Malcata, 1996; Dyerberg, 1986; Radwan, 1991; Reis et al., 1996). Furthermore, it is believed (de Pauw and Persoone, 1988) that fish do not synthesize EPA and DHA themselves, but instead obtain those compounds strictly from their diet. Therefore, the EPA and DHA contents of the feed of crustacean and fish in aquaculture are of the utmost importance. A few microalgae species are particularly rich in EPA and DHA, and hence appear to be high-quality feed for aquaculture; among them, *Pavlova lutheri* has attracted much attention due to its considerable amounts of PUFA (Spektorova et al., 1986; Thompson et al., 1996; Volkman et al., 1991).

The PUFA content of microalgae can be modulated by culture conditions, and several studies aimed at the optimization of culture parameters are available (Carvalho and Malcata, 2000; Dunstan et al., 1993; Grima et al., 1994a,b). However, use of microalgae as industrial source of PUFA is still not economically feasible (Borowitzka, 1997), mainly because of productivity limitations. Improvement of EPA and DHA contents of microalgal strains, either by genetic engineering or induced mutagenesis, would thus be in order, not only per se for eventual human consumption but also to increase the quality of aquaculture products. At present, induced mutagenesis presents a substantial advantage compared to genetic engineering, as it does not require the wealth of biochemical and genetic information on the organism in stake (Queener and Lively, 1986), which is not currently available for most microalga species (Alonso et al., 1996). Strain improvement by induced mutation is indeed broadly applied in biotechnological industries; e.g., the yield of penicillin was stepwise increased from 0.06 to 26 $mg \cdot mL^{-1}$ via application of this method (Queener and Lively, 1986).

A strain improvement program, aimed at the increase of EPA and/or DHA contents of *P. lutheri* by induced mutagenesis, was duly followed. UV light was chosen as mutagenic agent, because it is considered as one of the simplest

Correspondence to: Dr. F. Xavier Malcata, Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal

ways to obtain a wide variety of mutant strains from bacteria (Carlton and Brown, 1981) and cyanobacteria (Williams et al., 1979); it has also been shown to improve the EPA content of the microalga *Phaeodactylum tricornutum* (Alonso et al., 1996). Experiments with different culture media, solidifying agents, and cultivation conditions were performed, so as to determine the best conditions for growth of *P. lutheri* on solid medium, prior to isolation of colonies of putative mutants; note that difficulties in manipulating species on solid media have hampered genetic studies encompassing other species of microalgae (Williams et al., 1979). After having established the best conditions for clone isolation, a one-step mutation procedure, adapted from Alonso et al. (1996), was carried out in attempts to achieve EPA- and/or DHA-overproducing strains of *P. lutheri*.

MATERIALS AND METHODS

Culture Source

The parent culture of *P. lutheri* (strain SMBA60) was kindly supplied by IPIMAR (Instituto Português de Investigação Marítima, Portugal); the liquid culture medium was artificial sea water (ASW) (Borowitzka, 1988).

Several media, solidifying agents, and cultivation conditions were previously tested in order to screen for the best combination for isolation of single colonies of *P. lutheri* on plate. They included both ASW and GPM (without soil extract) (Anonymous, 1986), solidified with agar no. 1 (Lab M, Amersham, UK), agar–gel (Sigma, St. Louis, MO), and gelatin (Lab M). With respect to agar no. 1, the range of concentrations considered was 0.3–1.5% (m/v); for both agar–gel and gelatin, the minimum concentration that allowed the media to remain solid, viz. 0.35% and 2.5% (m/v), respectively, was also tested. Cultivation under all combinations was under normal humidity (<50% RH) and under high humidity (>70% RH) overhead atmosphere, so as to avoid surface drying. As it had the best performance (i.e., fully grown colonies within 1 month), ASW solidified with 0.3% (m/v) agar no. 1 was employed thereafter to proceed with isolations.

Experimental Design

The mutation program used ultraviolet radiation (UV) as mutagenic agent. The UV source was a 254-nm wavelength lamp placed 20 cm above the culture.

The first step of the mutation program was determination of the appropriate times for UV exposure of the parent culture, so as to guarantee a successful strain improvement methodology; for that purpose, death curves under UV light were obtained. Volumes of 50 mL of culture were exposed to UV light for 40 min, with continuous agitation; a sample was removed every 2 min, plated after appropriate dilution, and colonies counted after 3 weeks of incubation.

Preliminary analysis of several death curves indicated that UV doses corresponding to irradiation for 15 and 22 min, with associated killing rates of ca. 50% and 98%, respectively, were appropriate, so they were selected for further consideration. Exposed cultures were then plated after appropriate dilution, and maintained in darkness overnight to avoid photoreactivation (Carlton and Brown, 1981). The resulting colonies were recovered and recultured.

Forty-seven clones have been successfully isolated and recovered in this way. All clones were cultivated in 100-mL Erlenmeyer flasks until the late exponential phase was attained. Afterward, they were cultivated in 250-mL Erlenmeyer flasks up to the stationary phase, under identical conditions of nutrient level, light (8.6 $W \cdot m^{-2}$) provided by cool-light fluorescent lamps (OSRAW 23W/21), and temperature (22°C). Cultures were manually shaken twice a day and continuously supplied with light. Cultures were eventually harvested, spray-dried, and stored at −30°C under nitrogen, for subsequent lipid analyses.

Six colonies not exposed to UV were cultivated as control so that the effect of mutation on fatty acid yield could be statistically assessed.

Stored samples were analyzed by gas chromatography (GC) to determine the qualitative and quantitative fatty acid profile, according to the procedure described below. By the end of the first stage, the cultures with EPA and DHA contents higher than those of the control were selected for the second stage of cultivation. In this second stage, tentative mutants were recultured in quadruplicate using 250-mL Erlenmeyer flasks under similar growth conditions.

After lipid analyses following the second stage, promising cultures bearing a product level statistically above that observed in the control were further used for double checking of results. The strains selected were thus recultured in triplicate in 1-L flasks. Cultures were bubbled with filtered (0.22 μm) atmospheric air; temperature was maintained at 25°C, and light was continuously made available at 17.2 $W \cdot m^{-2}$, thereby providing optimal conditions for growth of biomass and lipid production. When cultures reached the stationary phase, they were harvested, spray-dried, and stored under nitrogen at −30°C, prior to GC analysis.

In all experiments, at several stages, optical density (OD) of the control was used to monitor growth; when the control reached the stationary phase, cultures were duly harvested and processed as mentioned above.

Chemical Analysis

Fatty acid methyl esters were obtained by direct transesterification of duplicated spray-dried samples, according to the acidic method described by Lepage and Roy (1984), after the modifications introduced by Cohen et al. (1988), using heptadecanoic acid as internal standard and acetyl chloride as catalyst. Those esters were analyzed in a Perkin-Elmer gas chromatograph (Norwalk, CT) with a flame ionization detector. The column was a 60-m fused silica, capillary column Supelcowax-10 from Supelco (St. Louis, MO). Helium was used as carrier gas in splitless mode; the injector

and detector were maintained at 250 and 270°C, respectively; and the oven heating program increased the column temperature linearly from 170 to 250°C, at a rate of 1°C min^{-1}. Pure standards of fatty acids from Sigma were used for tentative identification based on comparison of retention times.

Statistical Analysis

Following the second experiment, a Fisher's *F*-test was used when searching for possible differences in the variances of EPA and DHA contents between individual mutants and the control. All those variances proved to be statistically similar ($P < 0.01$); hence, a Student's *t*-test assuming equal variances was used, so as to pinpoint tentative differences in EPA and DHA contents between individual mutants and the control.

Following the third experiment, statistical analysis of variance (ANOVA) was applied to identify inter-strain differences regarding contents of each individual fatty acid, including both the control and all mutants.

In the various statistical analyses, the average of duplicate results of the lipid chemical assays was used as a datum point.

RESULTS

When several cultivation media and conditions were tested, we realized that a strong inoculum would lead to growth in all cases, except media solidified with gelatin and with more than 0.8% (m/v) agar no. 1; however, when small inocula were used, growth was observed only in ASW solidified with less than 0.8% (m/v) agar no. 1. Finally, when plates were inoculated with diluted cultures in attempts to isolate colonies, no growth was observed but in ASW with 0.3% and 0.5% (m/v) agar no. 1, under high relative humidity. The minimum cultivation time required by formation of colonies with adequate size was at least 25 d, with 0.3% (m/v) agar no. 1.

As mentioned before, 47 strains were isolated and successfully recovered following exposure to UV light of the parent culture. A cultivation experiment was then performed with all 47 strains (first stage). By 22 d the control entered the stationary phase, so samples were harvested. The average results of EPA and DHA contents, together with range and coefficient of variation (CV) among wild type and putative mutants, are shown in Table I. The CVs of EPA and DHA among putative mutants were markedly higher than those found among the wild type. Comparing the 47 putative mutants with the wild type, three strains could easily be selected that exhibited a higher EPA yield (viz. I#3, I#14, and I#25), and another two that exhibited both higher EPA and higher DHA yields (viz. II#2 and II#25). The aforementioned five strains were subjected to a second experiment (second stage) with quadruplicate cultures, in order to statistically analyze tentative differences in EPA and DHA yields. This experiment ran for 25 d, until the control reached the stationary phase. The average results for the major fatty acids (including EPA and DHA), and associated standard deviations, are included in Table II. Total fatty acid (TFA) content was higher than that of the control for all mutants but I#25. For all strains selected, the content of each individual fatty acid was also higher than that found in the wild type; as expected, this difference was not so apparent for those fatty acids present is small

Table I. Experimental results of the first-stage experiments, encompassing EPA and DHA average yield, coefficient of variation (CV) from wild-type ($n = 6$ cultures) and isolated cultures ($n = 47$ cultures), and range of values (min–max).

		Fatty acid composition (mg · g^{-1} dry biomass)		
Strain		EPA	DHA	TFA
Wild-type	Max	6.32	4.25	24.68
	Min	4.97	2.83	17.66
	Average	5.94	3.51	20.34
	CV	10.90	17.10	18.60
Mutants	Max	9.50	4.38	35.53
	Min	0.97	0.01	13.14
	Average	4.10	2.30	21.98
	CV	44.40	40.90	32.20
Selected strains	I#3	6.93	2.75	26.29
	I#14	7.20	3.83	26.55
	I#25	6.50	3.45	26.13
	II#2	9.50	4.38	35.53
	II#5	7.75	4.30	32.63

Table II. Experimental results of fatty acid profile of wild-type, WT ($n = 4$ cultures) and five selected strains ($n = 4$ cultures), in the second-stage experiments, in terms of average and standard deviation.[a]

	Fatty acid composition (mg · g^{-1} dry biomass)								
Strain	14:0	16:0	16:1(*n*-7)	18:1(*n*-7)	18:3(*n*-3)	18:4(*n*-3)	20:5(*n*-3)	22:6(*n*-3)	TFA
WT	4.45 ± 0.88	3.55 ± 0.84	3.20 ± 0.79	1.04 ± 0.21	0.29 ± 0.10	1.21 ± 0.24	3.76 ± 0.72	2.38 ± 0.55	31.71 ± 5.22
I#3	5.02 ± 0.23	3.76 ± 0.73	3.37 ± 0.68	1.07 ± 0.11	0.41 ± 0.05	1.42 ± 0.21	4.75^{s} ± 0.73	2.01^{ns} ± 0.17	36.18 ± 1.60
I#14	5.11 ± 1.19	4.65 ± 0.92	5.68 ± 0.83	1.20 ± 0.12	0.33 ± 0.03	1.59 ± 0.30	5.02^{s} ± 0.80	2.95^{ns} ± 1.05	34.32 ± 5.84
I#25	5.15 ± 0.50	3.60 ± 0.44	3.60 ± 0.09	1.22 ± 0.32	0.33 ± 0.03	1.41 ± 0.13	5.11^{ns} ± 0.56	2.38^{ns} ± 0.29	30.89 ± 3.88
II#2	5.99 ± 0.71	4.08 ± 0.54	3.89 ± 0.25	1.39 ± 0.19	0.44 ± 0.12	1.71 ± 0.45	5.36^{s} ± 0.16	2.81^{s} ± 0.23	34.46 ± 2.27
II#5	5.84 ± 2.69	4.07 ± 2.11	4.15 ± 1.92	1.47 ± 0.51	0.34 ± 0.30	1.77 ± 1.13	5.81^{ns} ± 3.86	2.50^{ns} ± 1.54	35.68 ± 3.90

[a]s, significantly different ($P < 0.05$); ns, not significantly different, for strain relative to WT.

amounts, viz. 18:1(n-7), 18:3(n-3), and 18:4(n-3), although there was still an increasing tendency. In terms of overall fatty acid profile, there were no differences in the fraction of the major fatty acids between wild type and mutants, except for 18:4(n-3) and EPA, for which this fraction was always more concentrated in the mutants. The statistical analyses, encompassing differences in EPA and DHA contents between individual mutants and the control, indicated that strains I#3 and I#14 produced significantly more EPA ($P < 0.05$), and that strain II#2 produced significantly more EPA and DHA ($P < 0.05$) than the control.

The third and final experiment performed with strains I#14 and II#2 was aimed at further confirming that the EPA and DHA yields were statistically higher than the control, under particularly appropriate cultivation conditions for growth and lipid production; these cultures thus presented higher growth rates, and the control reached the stationary phase within only 16 d. The fatty acid profile of the wild type and of the mutants selected is presented in Table III. As in the second stage, the TFA was higher in the mutants, viz. 134.89, 148.39, and 162.75 $mg \cdot g^{-1}$ dry biomass, in the wild type, the strain I#14 and the strain II#2, respectively. The individual fatty acid content was also higher in the mutants, except for 14:0; however, the fraction of EPA in TFA was not markedly higher in the mutants as it was in the second stage, with figures of 12.7%, 12.9%, and 14.2% of TFA in the wild type, strain I#14, and strain I#2, respectively. ANOVA was used to compare inter-strain differences in terms of each individual fatty acid, and it was apparent that there were differences in the fatty acid content between the cultures: not only were the contents of EPA and DHA significantly different, but the contents of 18:3(n-3), 18:4(n-3), and TFA were also significantly different between the modified strains and their native counterparts.

The average results pertaining to EPA and DHA productivities of the final experiment are presented in Table IV. As there were no significant differences in growth rate, and consequently in final biomass, of both wild type and mutants, the EPA productivity was higher in both mutants, and the DHA productivity was higher in strain II#2.

Table IV. Volumetric productivity ($mg \cdot g^{-1} \cdot L^{-1}$) of EPA and DHA in wild-type (WT) and two selected strains, in the third-stage experiments (run for 16 d), in terms of average.

Strain	EPA	DHA
WT	2.90	1.33
I#14	3.15	1.32
II#2	3.85	1.77

DISCUSSION

Random mutagenesis is a simple way to enhance metabolite productivity by microorganisms; however, it requires screening of the highest possible number of putative mutants because its accuracy is rather low (Carlton and Brown, 1981). Although the random screening protocol followed does not conform to the standard of Queener and Lively (1986), note that it was based on the modified protocol initially proposed by Alonso et al. (1996); moreover, the second round of mutation in the latter presented a very small efficiency, so a single round of mutation was done. In microorganisms that grow and form colonies on solid media (e.g., yeasts, bacteria, and a few microalgae), it is relatively easy to attain a high number of clones using current bacteriological techniques. However, cultivation of flagellate and filamentous organisms on solid medium plates, as is the case of other microalgae and cyanobacteria, may be cumbersome and laborious; this realization has indeed restricted genetic studies to only a few species (Williams et al., 1979). *Pavlova lutheri* is one such flagellate microalga, which displays poor grow rate on solid plate, mainly due to drying out of medium (Droop, 1954); previous establishment of the best conditions to cultivate and grow colonies of this microalga on plate was then a crucial step of our research effort. The semisolid medium, containing 0.3% (m/v) agar no. 1, cultivated under high relative humidity (>70%) proved to be an effective method to produce distinct colonies of this microalga, probably owing to the high amounts of water present in both medium surface and overhead atmosphere.

The reproduction of marine flagellates is usually asexual, via binary fission (Throndsen, 1997); however, some atypical cases of sexual reproduction in both iso- and anisogamy have been described. Single cells were successfully isolated under the light microscope in our laboratory; they could be grown on liquid media (unpublished results), hence proving their ability to reproduce asexually. This detail, coupled with the fact that several replicates of all cultures, cultivated

Table III. Experimental results of fatty acid profile of wild-type, WT ($n = 3$ cultures) and two selected strains ($n = 3$ cultures), in the third-stage experiments, in terms of average and standard deviation, as well as ANOVA F-ratio for inter-strain comparison.[a]

	Fatty acid composition ($mg \cdot g^{-1}$ dry biomass)								
Strain	14:0	16:0	16:1(*n*-7)	18:1(*n*-7)	18:3(*n*-3)	18:4(*n*-3)	20:5(*n*-3)	22:6(*n*-3)	TFA
WT	16.42 ± 7.88	15.50 ± 1.85	16.54 ± 2.73	3.47 ± 0.59	1.01 ± 0.34	6.61 ± 1.31	17.41 ± 1.82	7.99 ± 2.90	134.89 ± 9.01
I#14	15.74 ± 8.05	17.54 ± 7.31	18.88 ± 10.01	3.24 ± 0.93	1.36 ± 0.64	7.26 ± 0.52	18.87 ± 2.28	7.91 ± 2.69	148.39 ± 36.35
II#2	15.47 ± 4.37	18.35 ± 2.85	21.29 ± 6.86	3.86 ± 0.41	1.60 ± 0.55	7.97 ± 1.33	23.12 ± 0.23	10.62 ± 1.93	162.75 ± 18.53
ANOVA *F*-ratio	0.10^{ns}	1.48^{ns}	2.03^{ns}	3.99^{ns}	6.04^{s}	6.79^{s}	7.68^{s}	6.79^{s}	6.17^{s}

[a]s, significantly different ($P < 0.05$); ns, not significantly different, for strain relative to WT.

many times (for almost 2 years), still maintained the same characteristics in terms of growth and lipid production, supported the assumption of clonal cultures rather than sexually reproduced cultures.

The mutagenic agent used (UV light) is a practical, but not very efficient way to induce mutagenesis; hence, it is preferable to use long exposures, with concomitant high killing rates to increase the probability of mutation (Carlton and Brown, 1981). However, when working with microalgae, it is difficult to recover clones; as the highest possible number is essential, two different times of UV exposure were chosen, with corresponding killing rates of 50% and 98%. These times of exposure either facilitate recovery of a high number of clones (as the former), or increase the mutation probability (as the latter).

When analyzing the CV of the wild type in the first stage, values of 10.9% and 17.1% were obtained for EPA and DHA, respectively. These relatively high variance levels are accounted for by the intrinsic variability among strains, and are consistent with those previously reported for other species (Alonso et al., 1992, 1994). The CV figures pertaining to EPA and DHA in the 47 mutants were almost twice those found in the literature for EPA in *Phaeodactylum tricornutum* mutants (Alonso et al., 1996). Considering the ultimate goal of this work—to increase EPA and DHA contents—the efficiency was better for EPA as the number of hyperproducers was higher, but the CV among mutants was very similar for the two fatty acids, so the higher efficiency with EPA may have been the result of random selection of mutants.

Five strains were selected by the end of the first stage, as discussed before; however, this selection was based on analysis of a single culture, which does not take into account the intrinsic variation in performance of individual strains. This procedure may lead to errors, yet note that the major goal of the first stage was to isolate the highest possible number of putative mutants. A second stage, with quadruplicate cultures, was then implemented, aimed at assessing tentative differences in fatty acid contents according to a statistically validated procedure.

From the five strains selected in the second stage, only three presented significant differences in EPA content, which unfolds the limitation of the single culture analysis. Under such stricter validation conditions, two cultures with significantly higher ($P < 0.05$) EPA content (I#3 and I#14), and one with significantly higher ($P < 0.05$) EPA and DHA contents (I#2) than the wild-type were selected.

Our results appear promising, as a single round of mutation permitted a 42.6% increase in EPA content and an 18.1% increase in DHA content for the same strain (II#2); however, the cultivation conditions were not at an optimum for growth and lipid production. It is well known that the culture parameters may, within certain limits, modulate growth and fatty acid production (Emdadi and Berland, 1989; Grima et al., 1993; Thompson et al., 1996); hence, the two most promising strains (II#2 and I#14) were subjected to a further cultivation step (third stage), so differences already found in EPA and DHA yields were re-evaluated under particularly favorable conditions for growth and lipid production. The statistical differences based on ANOVA, encompassing both strains and the wild type, indicated that the production of EPA and DHA by the three strains was different ($P < 0.05$), as well as the production of TFA and of other n-3 fatty acids. The yield of EPA in strains I#14 and II#2 was 7.4% and 32.8% higher, respectively, than in the wild type, whereas the DHA yield in II#2 was 32.9% higher than in the wild type. The difference in the total amounts of fatty acids between the second and the third stages arises from the different cultivation conditions: the final stage was performed in aerated flasks, which promote growth and thus metabolite production.

The results for EPA and DHA productivity, presented in Table IV, indicate that it is possible to increase the volumetric productivity of both fatty acids using this method. It is important to note that the major scope of this work was to increase the EPA and DHA yields in this microalga, so as to obtain a product for aquaculture. As previously mentioned, this species is currently used in aquaculture, so an increase in the yield of such important fatty acids will automatically lead to enhancement of product quality.

Several authors (Alonso et al., 1994; Carvalho and Malcata, 2001) have in fact reported great increases in growth and fatty acid production when cultures were provided with CO_2; since our cultures were aerated with atmospheric air with no further addition of CO_2, then total EPA and DHA contents may undergo further enhancement if culture conditions are duly engineered (which is, nevertheless, beyond the scope of this communication).

References

Alonso DL, Castillo CIS, García-Sánchez JL, Sánchez-Pérez JA, Camacho FG. 1994. Quantitative genetics of fatty acid variation in *Isochrysis galbana* (Prymnesiophyceae) and *Phaeodactylum tricornutum* (Bacillariophyceae). J Phycol 30:553–558.

Alonso DL, Castillo CIS, Grima EM, Cohen Z. 1996. First insights into improvement of eicosapentaenoic acid content in *Phaeodactylum tricornutum* (Bacillariophyceae) by induced mutagenesis. J Phycol 32: 339–345.

Alonso DL, Grima EM, Sánchez-Pérez JA, García-Sánchez JL, Camacho FG. 1992. Isolation of clones if *Isochrysis galbana* rich in eicosapentaenoic acid. Aquaculture 102:363–371.

Anonymous. 1986. Microalgae Culture Collection 1985–1986, Golden CO: Solar Energy Research Institute. p 85–96.

Borowitzka MA. 1988. Algal growth media and sources of algal cultures. In: Borowitzka MA, Borowitzka JL, editors. Micro-algal biotechnology. Cambridge, MA: Cambridge University Press. p 456–465.

Borowitzka MA. 1997. Microalgae for aquaculture: opportunities and constraints. J Appl Phycol 9:393–401.

Carlton BC, Brown BJ. 1981. Gene mutation. In: Gerhardt P. editor. Manual of methods for general bacteriology. Washington DC: American Society for Microbiology. p 222–242.

Carvalho AP, Malcata FX. 1996. Polyunsaturated fatty acids and functional foods. World Ingred March/April:22–26.

Carvalho AP, Malcata FX. 2000. Effect of culture media on production of polyunsaturated fatty acids by *Pavlova lutheri*. Cryptogam Algol 21: 59–71.

Carvalho AP, Malcata FX. 2001. Transfer of carbon dioxide within cul-

tures of microalgae: plain bubbling versus hollow-fiber modules. Biotechnol Prog 17:265–272.

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.

de Pauw N, Persoone G. 1988. Micro-algae for aquaculture. In: Borowitzka MA, Borowitzka JL, editors. Micro-algal biotechnology. Cambridge, MA: Cambridge University Press. p 197.

Droop MR. 1954. A note on the isolation of small marine algae and flagellates for pure cultures. J Mar Biol Assoc 33:511–514.

Dunstan GA, Volkman JK, Barrett 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.

Dyerberg J. 1986. Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr Rev 44:125–134.

Emdadi D, Berland B. 1989. Variation in lipid class composition during batch growth of *Nannochloropsis salina* and *Pavlova lutheri*. Mar Chem 26:215–225.

Grima EM, Pérez JAS, Camacho FG, Sánchez JLG, Alonso DL. 1993. n-3 PUFA productivity in chemostat cultures of microalgae. Appl Microbiol Biotechnol 38:599–605.

Grima EM, Sánchez-Pérez JA, Camacho FG, Fernández FGA, Sevilla JMF, Sanz FV. 1994a. Effect of dilution rate on eicosapentaenoic acid productivity of *Phaeodactylum tricornutum* UTEX 640 in outdoor chemostat culture. Biotechnol Lett 16:1035–1040.

Grima EM, Sánchez-Pérez JA, Camacho FG, Sevilla JMF, Fernández FGA. 1994b. Effect of growth rate on the eicosapentaenoic acid and docosahexaenoic acid content of *Isochrysis galbana* in chemostat culture. Appl Microbiol Biotechnol 41:23–17.

Lepage G, Roy C. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25:1391–1396.

Queener SW, Lively DH. 1986. Screening and selection for strain improvement. In: Demain AL, Solomon NA, editors. Manual of industrial microbiology and biotechnology. Washington, DC: American Society for Microbiology. p 155–169.

Radwan SS. 1991. Sources of C_{20}-polyunsaturated fatty acids for biotechnological use. Appl Microbiol Biotechnol 35:421–430.

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. Bioresour Technol 55:83–88.

Spektorova, LV, Goronkova OI, Nosova LP, Albitskaya ON, Danilova G. 1986. High-density culture of marine microalgae: promising items for mariculture. III. Mass culture of *Monochrysis lutheri* Droop. Aquaculture 55:231–240.

Thompson PA, Guo MX, Harrison PJ. 1996. Nutritional value of diets that vary in fatty acid composition for larval Pacific oysters. Aquaculture 143:379–391.

Throndsen J. 1997. The planktonic marine flagellates. In: Tomas CR, editor, Identifying marine phytoplankton. San Diego: Academic Press. p 591–729.

Volkman JK, Dunstan GA, Jeffrey SW, Kearney PS. 1991. Fatty acids from microalgae of the genus *Pavlova*. Phytochem 30:1855–1859.

Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD. 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J Exp Mar Biol Ecol 128:219–240.

Williams E, Lambert J, O'Brien P, Houghton JA. 1979. Evidence for dark repair of far ultraviolet light damage in the blue-green alga *Gleocapsa alpicola*. Photochem Photobiol 29:543–547.