ELSEVIER

Journal of Biotechnology 70 (1999) 299–312

JOURNAL OF Biotechnology

Production of eicosapentaenoic acid by *Nannochloropsis* sp. cultures in outdoor tubular photobioreactors

G. Chini Zittelli, F. Lavista, A. Bastianini, L. Rodolfi, M. Vincenzini, M.R. Tredici *

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche dell'Università di Firenze and Centro di Studio dei Microrganismi Autotrofi del CNR, P.le delle Cascine, 27, 50144 Firenze, Italy

Received 3 November 1998; received in revised form 8 December 1998; accepted 22 December 1998

Abstract

Autotrophic microalgae cultures have been proposed as an alternative source of EPA, a nutritionally important polyunsaturated fatty acid that plays a key role in the prevention and treatment of several human diseases and disorders. The technology currently available is however, considered commercially not viable because of the low degree of control of algae cultures in outdoor open ponds. The use of closed reactors could overcome these limitations and bring EPA production by microalgae closer to becoming a reality. In this study, we have demonstrated the feasibility of outdoor cultivation of *Nannochloropsis* sp. in tubular reactors and the potential of this eustigmatophyte as an alternative source of EPA. *Nannochloropsis* sp. was cultivated in NHTRs of different sizes (from 10.2 to 610 l) from spring to autumn under the climatic conditions of central Italy. EPA productivity essentially reflected the productivity of the culture and reached its maximum in May–June (mean monthly value: 32 mg l^{-1} day^{-1}). Although the fatty acid composition of the biomass varied significantly during the cultivation period, EPA content remained rather stable around the value of 4% of dry biomass. The transfer of the cultures from laboratory to outdoor conditions, the exposure to natural light–dark cycles, along with lowering the salt concentration from 33 g l^{-1} (seawater salinity value) to 20 g l^{-1}, factors that caused lasting modifications in the fatty acid content and composition of *Nannochloropsis* sp., did not significantly affect the EPA content of the biomass.

Keywords: Eicosapentaenoic acid; *Nannochloropsis* sp.; Microalgae mass cultivation; Tubular photobioreactors

1. Introduction

PUFAs of the ω-3 series play a key role in the prevention and treatment of a wide range of human diseases and disorders and have been recognized as important dietary compounds. EPA (20:5ω3), in particular, has been shown to have

Abbreviations: AA, arachidonic acid or 20:4ω6; DCMU, 3-(3:4-dichlorophenyl)-1:1-dimethyl urea; DHA, docosahexaenoic acid or 22:6ω3; EPA, eicosapentaenoic acid or 20:5ω3; NHTR: near-horizontal tubular photobioreactor; PUFAs, polyunsaturated fatty acids; PVC, polyvinyl chloride; TFA, total fatty acids.

* Corresponding author. Fax: +39-55-330431.

E-mail address: tredici@csma.fi.cnr.it (M.R. Tredici)

0168-1656/99/$ - see front matter
PII: S0168-1656(99)00082-6

several highly beneficial effects such as preventing atherosclerosis and cardiovascular diseases, lowering plasma cholesterol and triacylglycerol levels, and alleviating inflammatory conditions such as arthritis (Nordoy, 1991; Gill and Valivety, 1997). Recently, the potential of EPA as an antitumor agent has been also documented (Senzaki et al., 1998).

At present, the only commercial source of EPA is marine fish oil, a rather unsatisfactory source because of problems of contamination, taste, odor and stability. In addition, the presence of considerable amounts of other PUFAs in the fish oil complicates the EPA purification process, resulting in high retail prices of the pure product (Yongmanitchai and Ward, 1989; Barclay et al., 1994). These factors have led to investigation of alternative EPA sources (Gill and Valivety, 1997).

Several microorganisms have long been recognized as potential EPA producers (Radwan, 1991). Fungi, especially of the order Mucorales, and bacteria of the genera *Shewanella*, *Alteromonas*, *Flexibacter* and *Vibrio* can accumulate relatively large amounts of EPA (Yongmanitchai and Ward, 1989; Gill and Valivety, 1997; Leman, 1997), and have been indicated as promising sources of this long chain fatty acid. However, the ability of bacterial and fungal fermentations to compete economically with traditional sources of ω-3 fatty acids is limited by low productivities and excessively long fermentation times (Barclay et al., 1994; Leman, 1997). Marine microalgae, such as *Porphyridium cruentum* (Cohen et al., 1988), *Phaeodactylum tricornutum* (Chrismadha and Borowitzka, 1994; Molina Grima et al., 1994a), *Isochrysis galbana* (Molina Grima et al., 1994b) and the eustigmatophytes *Nannochloropsis oculata*, *Nannochloropsis* sp. and *Monodus subterraneus* (Seto et al., 1992; Hu et al., 1997; Sukenik 1998) have also been proposed for commercial production of EPA under autotrophic conditions. Algae-based technologies in outdoor ponds, however, are considered commercially not viable for EPA production because of low cell concentrations, low production rates and a reduced degree of control of growth parameters (Barclay et al., 1994). For this reason, some companies, such as Martek Biosciences, MD, and Omega Tech, CO, have developed processes based on cultivation of heterotrophic microalgae for the commercial production of EPA and DHA (Barclay et al., 1994). Heterotrophic algae production in fermentors has several potential advantages over autotrophic production in open ponds, including a higher degree of process control, easier maintenance of monoalgal cultures, lower costs for harvesting due to the higher cell concentration obtained, and significantly greater volumetric productivities (Barclay et al., 1994).

Nannochloropsis is a marine eustigmatophyte currently cultivated in many aquaculture hatcheries as the basis of an artificial food chain. In Japan, *N. oculata* is the most important cultured feed for the rotifer *Brachionus plicatilis* and concentrated suspensions and frozen biomass of this microalga are commercially available (Okauchi, 1991). Because of its high EPA content this eustigmatophyte is considered a good potential EPA source (Sukenik, 1998). Although the alga is grown extensively, its mass production in outdoor open ponds suffers from sudden culture collapse due to predation by protozoa (e.g. *Paraphysomonas* sp.), contamination by bacteria (e.g. *Cytophaga* sp.) and competition of cyanobacteria and other microalgae (Okauchi, 1991; Sukenik et al., 1993a; Sukenik, 1998). Chlorination and the use of antibiotics do not prevent these problems (Okauchi, 1991; Sukenik 1998). Recently, Gonen-Zurgil et al. (1996) have suggested the use of the herbicide DCMU to maintain the cultures free of contaminating algae, since *Nannochloropsis* shows a high resistance to this compound. Although the addition of DCMU at concentrations higher than 10^{-7} M to the cultures significantly decreases the growth of undesired microalgae, it does not solve the problem of bacteria and protozoa contamination and presents obvious negative implications.

These limitations of open ponds could be overcome by use of closed reactors (photobioreactors). In closed systems, protection against contaminating microorganisms is much more easily obtained, and much higher cell densities and more efficient control of principal culture

parameters can be achieved so as to ensure a sustainable cultivation process (Tredici and Chini Zittelli, 1998).

The aims of the present study were to evaluate the feasibility of outdoor cultivation of *Nannochloropsis* sp. in tubular photobioreactors and to assess the potential of this microalga as an alternative source of EPA.

2. Materials and methods

2.1. Description of the photobioreactors

Single-tube, three-tube and eight-tube NHTRs (Tredici and Chini Zittelli, 1998) installed at the experimental station of Scandicci (Florence; latitude 43° 46′ N, longitude 11° 15′ E) were used for the outdoor cultivation of *Nannochloropsis* sp. The reactors consisted of flexible plastic tubes (length: 6.4 m; diameter: 43 mm; wall thickness: 0.15 mm) connected by two PVC manifolds. The upper, larger manifold acted as a degasser. The reactors were laid on a platform facing south and tilted to form an angle of 5° with the horizontal. The platform was covered with a white corrugated plastic sheeting, which kept the tubes of the reactor well-aligned and at the proper inclination. Mixing and degassing of the culture suspension were achieved by continuously injecting air into each tube by means of a perforated pipe inserted into the lower manifold. In the eight-tube reactors two tubes were not aerated in order to increase the circulation speed of the culture suspension and to obtain a better mass transfer. The total volume of each system (including degasser and connecting tubing) was 10.2, 36.6 and 97.9 l for the single-tube, three-tube and eight-tube reactors, respectively. Electrodes and sensors were inserted into the degassers. In September and October 1998, two larger eight-tube NHTR units (lengths: 11 and 44 m; volumes: 155 and 610 l) were installed at Azienda Agricola Montepaldi (San Casciano Val di Pesa, Florence, Italy; Fig. 1). For both reactors, the inclination of the platform was 7°. For the laboratory experiments a 1.5-m^2 alveolar panel was used (Tredici et al. 1991).

2.2. Organism and culture conditions

The marine eustigmatophyte *Nannochloropsis* sp. was obtained from Dr Zmora of the National Center for Mariculture, Israel Oceanographic and Limnological Research (Eilat, Israel). Cultures were grown in artificial seawater (Tropic Marine Salt, Euraquarium, Bologna, Italy), enriched with f/2 medium nutrients (Guillard and Ryther, 1962) at either 33 or 20 g l^{-1} salinity. $NaNO_3$ and NaH_2PO_4 were added regularly to the cultures in order to prevent nutrient limitation. For laboratory experiments artificial seawater was autoclaved at 120°C for 20 min, whereas for outdoor experiments it was filtered through 10- and 1.5-μm polypropylene filters (Domnick Hunter, UK). Sterile nutrient solutions were then added. The air injected into the photobioreactors was filtered through 1-μm Polycap HD encapsulated filters (Arbor Tech, USA). Air-flow rate was maintained at 0.17 l l^{-1} min^{-1}, and gas hold-up was about

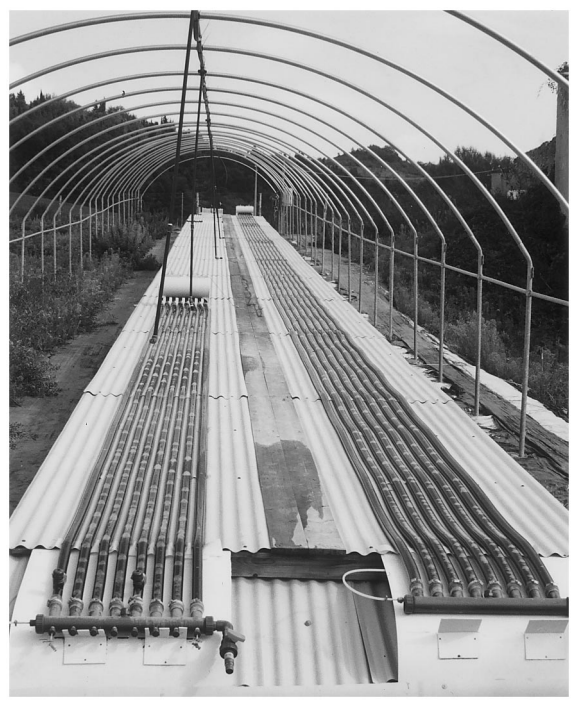

Fig. 1. Pilot eight-tube NHTR units installed at Azienda Agricola Montepaldi (San Casciano Val di Pesa, Florence, Italy).

5.5% in all the NHTRs but the pilot eight-tube units. In these reactors air-flow rate was maintained at 0.02 l l^{-1} min^{-1}, and gas hold-up was about 4%. In the alveolar panel air-flow rate was 0.4 l l^{-1} min^{-1}, and gas hold-up was about 2%.

A control unit including a thermostat provided temperature regulation of the outdoor units by automatically activating water spraying onto the reactors, when the culture temperature exceeded the preset value of 25°C (optimum growth temperature of *Nannochloropsis* sp.). This cooling system was inadequate to maintain the culture temperature at the optimum value during the hours of strongest irradiation, particularly during the summer. During the night, the culture temperature was allowed to equilibrate to ambient. Under laboratory conditions, cultures were kept at a constant temperature of 25 ± 0.2°C, by circulating thermostating fluid from a water bath through a stainless-steel tube placed inside the reactor. Continuous illumination (100 μmol photons m^{-2} s^{-1}) was provided on the reactor surface by banks of 58-W daylight fluorescent tubes. For both outdoor and laboratory experiments, culture pH was maintained at 7.8 ± 0.2 by addition of pure CO_2 to the air stream using a pH-stat system. A semi-continuous daily harvesting regimen was adopted in order to maintain a mean cell concentration of about 3.5 and 5.0 g l^{-1} in the laboratory and outdoor cultures, respectively. The experiments started when quasi-steady state conditions were achieved. The biomass produced was harvested by centrifugation at $8000 \times g$ using a Westfalia separator (model TA05-00-105, Westfalia Separator AG, Olde, Germany) and lyophilized for biochemical analysis.

2.3. Analytical procedures

The productivity of the cultures was estimated by daily measurement of the dry biomass concentration. For dry weight determination, triplicate culture samples (2–5 ml) were diluted (1:10) with distilled water and filtered through pre-weighed 1.2-μm membrane filters (Sartorius, Goettingen, Germany). Filtered cells were then quickly washed with 25 ml of distilled water and dried to a constant weight at 105°C. Solar irradiance was measured with a pyranometric sensor (Kipp and Zonen, model CM 6, Delft, Holland) coupled with a Micros solarimeter (Model IS, Treviso, Italy). The photosynthetic photon flux density (PPFD) was measured using a Li-Cor quantum sensor (Model LI-190 SB, Li-Cor, Lincoln, NE) connected to a quantum/radiometer/photometer (Model LI-185 B).

Fatty acid analyses of outdoor *Nannochloropsis* sp. cultures were made on samples collected at the end of the dark period, except that when otherwise stated. For fatty acid determination cells were harvested by centrifugation. Aliquots of the paste obtained (75% moisture) were lyophilized and the dried biomass (3% moisture) immediately analyzed. Fatty acids were extracted and methylated according to Bousfield et al. (1983). The methyl esters were analyzed with a gas chromatograph (GC 8380 Fisons Instruments) equipped with a hydrogen flame ionization detector and a SP 2380 column (30 m × 0.20 μm film thickness). Injections were made in split mode (split ratio 1:70, sample size 1.0 μl). Injector and detector were maintained at 250°C; the oven temperature was programmed from 90 to 220°C at a rate of 3°C min^{-1}. Carrier gas was helium at 1.5 ml min^{-1}. Compounds were identified by comparing retention times of known standards. Quantification was based on the internal standard method.

3. Results

3.1. Transfer of Nannochloropsis sp. cultures from laboratory to outdoor conditions and its influence on fatty acid profile and content

Laboratory *Nannochloropsis* sp. cultures were grown in a 1.5-m^2 alveolar panel (Tredici et al., 1991) under continuous illumination (100 μmol photons m^{-2} s^{-1}) and at the optimal growth temperature of 25 ± 0.2°C. A mean dry biomass productivity of 0.84 ± 0.16 g l^{-1} 24 h^{-1} and a mean EPA productivity of 36 ± 4 mg l^{-1} 24 h^{-1} were attained. The total fatty acid content of the biomass was 13 ± 0.4%, whereas the EPA content was 4.3 ± 0.2%. At the end of August 1996, out-

door cultures were set up in single-tube NHTR systems (1NHTRs) using the laboratory culture as inoculum. The outdoor cultures, which were operated throughout September, achieved a mean productivity of 0.73 ± 0.37 g l^{-1} day^{-1}. The transfer from laboratory to natural growth conditions caused significant and lasting modifications in the fatty acid composition of the biomass (Table 1). During the first 2 weeks of outdoor cultivation, the total fatty acid content of the biomass increased from 13%, typical of laboratory cultures, to 21%. This increase was essentially due to the increase of three fatty acids: 16:0 (from 3.2 to 6.7%), 16:1ω7 (from 3.1 to 5.4%) and 18:1ω9 (from 0.3 to 2%). In the second half of September the opposite trend was observed: the content of these three fatty acids, as well as that of total fatty acids, decreased steadily to the values typical of laboratory conditions. Among the PUFAs, 20:4ω6 followed the general trend, first increasing from 0.7 to 1.2%, then decreasing to 0.8%, while EPA showed the opposite behavior although its content did not vary significantly from an average value of 4%. The considerable increase of saturated and monounsaturated fatty acids observed during the first 2 weeks of outdoor cultivation can be ascribed to the sudden exposure of laboratory cultures (cultures kept at low irradiance and optimal temperature) to high solar irradiances during the day and to low temperatures during the night and early morning (Fig. 2B,C). The opposite trend observed during the second half of the month can be related both to acclimation of the culture to outdoor conditions, and to lower solar irradiances and higher minimum temperatures during this period (irradiance decreased from about 20 to about 5 MJ m^{-2} day^{-1}, whereas the minimum culture temperature increased from 2 to 5 to 10°C). It is worth noting that the highest total fatty acid content (about 21%) was reached between 9 and 11 September, i.e. after a period of 4–5 consecutive days characterized by relatively high diurnal irradiances (17–19 MJ m^{-2} day^{-1}) and low night temperatures (2–5°C). It is difficult to establish the separate influence of each of these two environmental factors, since in autumn both high diurnal irradiances and low night temperatures occur under clear sky conditions. The EPA

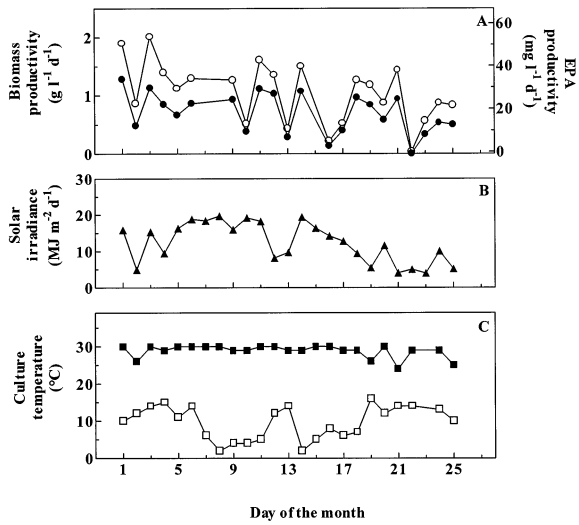

Fig. 2. Biomass (●) and EPA (○) productivity of *Nannochloropsis* sp. cultures grown outdoors in 1NHTRs in September 1996 (A). The total daily solar irradiance (B) and the minimum (□) and maximum (■) temperatures (C) of the cultures are also shown.

content of the biomass was not significantly affected by environmental conditions, although an initial decrease and a subsequent increase are evident. Consequently, the productivity of EPA depended essentially on the productivity of the culture, reaching a peak of 54 mg l^{-1} day^{-1} after 1 week of cultivation and declining to about 20 mg l^{-1} day^{-1} towards the end of the month (Fig. 2A).

3.2. Influence of the natural light–dark cycle on the fatty acid composition of outdoor Nannochloropsis sp. cultures

Table 2 shows the variation of the fatty acid composition of a *Nannochloropsis* sp. culture grown outdoors in a 1NHTR unit during a period of 7 consecutive days in the second half of September 1996. Besides the decrease of saturated and monounsaturated fatty acid content that characterized this period (described in the previous paragraph) we could observe the influence of the natural light–dark cycle on the fatty acid composition of the biomass. The content of saturated and monounsaturated fatty acids decreased

Table 1
Variation of fatty acid content and profile (% of dry weight) of *Nannochloropsis* sp. cultures after transfer from laboratory to outdoor conditions during August–September 1996[a]

Fatty acid	Day of the month										
	28 August	31 August	3 Sept.	6 Sept.	9 Sept.	11 Sept.	14 Sept.	17 Sept.	20 Sept.	23 Sept.	25 Sept.
14:0	0.72	0.79	0.77	0.90	1.13	1.05	0.83	0.76	0.63	0.49	0.51
16:0	3.16	3.52	3.79	4.97	6.74	6.70	5.56	4.49	3.45	2.72	2.79
16:1ω7	3.09	3.27	3.52	3.96	5.10	5.40	4.70	4.43	3.82	3.29	3.30
18:1ω9	0.33	0.53	0.63	0.82	1.93	1.99	1.46	1.09	0.76	0.66	0.54
18:2ω6	0.61	0.38	0.43	0.36	0.49	0.50	0.38	0.36	0.32	0.32	0.32
20:4ω6	0.65	0.99	1.16	1.03	1.13	1.16	1.03	0.91	0.89	0.83	0.83
20:5ω3	4.29	3.96	4.26	3.96	3.59	3.84	3.73	3.82	3.85	4.20	4.31
TFA	13.0	13.7	14.8	16.3	20.5	21.0	18.0	16.1	13.9	12.7	12.8

[a] Only the major fatty acids are shown (>0.3%). The values of August 28 are those of the laboratory culture used as inoculum. The analyses were done at the end of the light period.

Table 2
Variation of fatty acid content and profile (% of dry weight) of *Nannochloropsis* sp. cultures grown under natural light–dark cycle conditions in September 1996[a]

Fatty acid	Day of the month													
	19 September		20 September		21 September		22 September		23 September		24 September		25 September	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D
14:0	0.71	0.63	0.63	0.57	0.59	0.57	0.58	0.47	0.49	0.47	0.56	0.50	0.51	0.52
16:0	3.71	2.94	3.45	3.02	2.98	2.50	2.78	2.24	2.72	2.35	3.43	2.80	2.79	2.43
16:1ω7	4.20	3.82	3.82	3.67	3.73	3.53	3.52	3.13	3.29	3.15	3.43	3.29	3.30	3.32
18:1ω9	0.97	0.94	0.76	0.77	0.80	0.70	0.64	0.61	0.66	0.61	0.60	0.47	0.54	0.49
18:2ω6	0.36	0.44	0.32	0.42	0.34	0.41	0.33	0.39	0.32	0.41	0.31	0.38	0.32	0.41
20:4ω6	0.87	0.88	0.89	0.81	0.85	0.79	0.78	0.80	0.83	0.81	0.86	0.74	0.83	0.83
20:5ω3	3.48	3.82	3.85	4.09	4.05	4.09	4.02	4.32	4.20	4.54	4.20	4.35	4.31	4.73
TFA	14.5	13.6	13.9	13.5	13.5	12.7	12.6	12.1	12.7	12.5	13.6	12.8	12.8	12.9

[a] Only the major fatty acids are shown (>0.3%). The analyses were done at the end of the light period (L) and at the end of the dark period (D).

significantly during the night (on average, 14:0 by 8.3%, 16:0 by more than 16%, 16:1ω7 by 5.4% and 18:1ω9 by 8%). Among the PUFAs, 20:4ω6 did not change significantly, while 18:2ω6 and EPA increased by more than 24% and by 7%, respectively. This increase of both EPA and 18:2ω6 was relative, since it was due to the night consumption of storage material (carbohydrates, and saturated and monounsaturated fatty acids) and not due to new synthesis (data not shown).

3.3. Prolonged outdoor cultivation of Nannochloropsis sp. in NHTR reactors under the climatic conditions of central Italy: biomass and EPA productivity

Nannochloropsis sp. was grown outdoors in different types of NHTR systems uninterruptedly from March to September 1997 under the climatic conditions of central Italy (Florence). From March to June and in September, 1 NHTR units were used; during the summer three-tube (3 NHTRs) and eight-tube reactors (8 NHTRs) were used. Table 3 shows the mean monthly productivities attained, together with the mean minimum and maximum culture temperatures, and the mean solar irradiance values recorded. The highest volumetric productivities (0.7–0.8 g l^{-1} day^{-1}) were obtained in late spring. The slightly lower productivities (0.5–0.6 g l^{-1} day^{-1}) obtained in March–April might be due either to the lower solar irradiance available to the cultures (13.5 MJ m^{-2} day^{-1} in March and 15.3 MJ m^{-2} day^{-1} in April) compared to the May–June period (17–19 MJ m^{-2} day^{-1}) or to the excessively low temperatures during the night and the early morning (mean minimum temperatures of 2–3°C were recorded in the cultures during the spring). Since a mean monthly productivity of 0.73 g l^{-1} day^{-1} had been obtained in the previous September, with a mean minimum temperature of 10°C and at irradiance levels lower than 13 MJ m^{-2} day^{-1}, it is likely that the lower performance of early spring cultures was due to the low night temperatures. Similar productivities obtained in May 1997 with a mean irradiance of 19 MJ m^{-2} day^{-1}, and in September 1996 with only 13 MJ m^{-2} day^{-1}, also indicate that solar irradiances higher than 13 MJ m^{-2} day^{-1} do not significantly improve the productivity of *Nannochloropsis* sp. cultures under the conditions adopted.

In the summer the productivity of cultures in the 3 NHTR and 8 NHTR units declined regularly to a minimum of 0.45 g l^{-1} day^{-1}. The

Table 3
Productivity of *Nannochloropsis* sp. cultures grown outdoors in NHTRs from March to September 1997 under the climatic conditions of central Italy (Florence)[a]

Month	Reactor type	Mean volumetric productivity (g l^{-1} day^{-1})	Culture temperature (°C)		Solar irradiance (MJ m^{-2} day^{-1})
			Min.	Max.	
March	1 NHTR	0.51 ± 0.36	2.0 ± 3.3	26.2 ± 0.6	13.5 ± 4.1
April	1 NHTR	0.60 ± 0.31	3.0 ± 4.0	25.2 ± 3.8	15.3 ± 6.1
May	1 NHTR	0.76 ± 0.39	10.6 ± 3.8	28.1 ± 1.3	18.9 ± 4.4
June	1 NHTR	0.69 ± 0.30	15.3 ± 2.2	28.9 ± 1.6	17.4 ± 5.7
	3 NHTR	0.56 ± 0.30	14.5 ± 2.5	29.7 ± 1.5	
July	3 NHTR	0.60 ± 0.23	13.9 ± 3.0	28.3 ± 1.1	21.0 ± 2.5
	8 NHTR	0.56 ± 0.26	13.6 ± 3.0	28.9 ± 1.1	
August	3 NHTR	0.45 ± 0.17	14.2 ± 2.0	28.3 ± 0.5	17.6 ± 3.3
	3 NHTR shaded	0.41 ± 0.24	15.3 ± 2.0	27.6 ± 1.0	
September	1 NHTR	0.57 ± 0.20	10.5 ± 3.6	27.3 ± 1.3	15.9 ± 3.2
September 1996	1 NHTR	0.73 ± 0.37	10.2 ± 4.2	28.8 ± 1.4	12.7 ± 5.1

[a] The minimum and the maximum culture temperatures and the solar irradiance are also reported. Data of September 1996 is shown for comparison. Mean monthly values ± S.D. are shown.

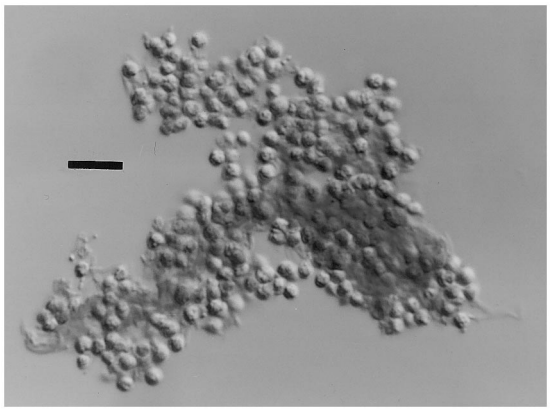

Fig. 3. A cluster of *Nannochloropsis* sp. cells. The exuded polysaccharide matrix was stained with alcian-blue (Crayton, 1982). Bar: 10 μm. Photomicrograph by C. Sili.

reason for this is not yet clear. Signs of stress, such as abundant exudation of polysaccharide and presence of numerous and very large cell clusters (Fig. 3), possibly due to high light intensity or to prolonged exposure to higher than optimal diurnal temperatures, were observed in summer cultures. An experiment carried out to compare shaded versus unshaded cultures did not give a clear response. The shaded cultures showed a better appearance (lower degree of aggregation and higher pigment content), but attained a lower productivity. In September, the productivity of *Nannochloropsis* sp. rose again to about 0.6 g l^{-1} day^{-1}. The fact that the productivity values of the previous September were not reached despite similar climatic conditions, suggests that the cultures had not yet fully recovered from the stress of the summer.

The mean fatty acid content of the biomass and the mean monthly productivity of EPA for the entire cultivation season (March–September 1997) are shown in Table 4. The culture used as inoculum, which had been grown in the laboratory as previously described, had a total fatty acid content of 12% and an EPA content of 3.6%. As observed in the previous autumn, the transfer from laboratory to outdoor conditions caused a significant increase of the total fatty acid content with a maximum of 26% in mid-April. The increase mainly involved 16:0, 16:1ω7 and 18:1ω9. EPA content, except for a peak of 4.6% in June, was rather stable around 4% throughout the cultivation period. Exposure to low temperatures during the night and in the early morning (Table 3), after growth at optimal temperature in the laboratory, can again be considered the main cause of the phenomenon. In May, when minimum temperatures rose above 10°C, the fatty acid profile and content showed values similar to those observed in the laboratory. EPA productivity essentially reflected culture productivity. A maximum of 32 mg l^{-1} day^{-1} and a minimum of 18 mg l^{-1} day^{-1} were achieved in May–June and August, respectively.

Table 4
Fatty acid composition (% of dry weight) and EPA productivity (mg l^{-1} day^{-1}) of *Nannochloropsis* sp. cultures grown outdoors in NHTRs from March to September 1997 under the climatic conditions of central Italy (Florence)[a]

	March	April	May	June	July	August	September
Fatty acid							
14:0	0.75	1.06	0.91	0.82	1.03	0.90	1.20
16:0	3.53	7.46	5.10	4.71	4.12	3.83	4.63
16:1ω7	3.97	8.05	5.31	5.01	4.32	3.44	4.63
18:1ω9	1.31	3.38	1.29	1.22	0.61	0.54	0.70
18:2ω6	0.58	0.52	0.38	0.32	0.70	0.56	0.74
20:4ω6	0.86	0.85	0.60	0.68	1.11	0.90	0.89
20:5ω3	3.90	3.95	4.22	4.64	4.18	3.96	3.98
TFA	15.3	25.8	18.2	17.9	16.6	14.5	17.1
EPA productivity	19.9	23.8	31.5	32.0	23.4	17.8	22.0

[a] Only the major fatty acids are shown (>0.3%).

In September and October 1998, *Nannochloropsis* sp. was grown outdoors in pilot 8 NHTR units (Fig. 1). The mean productivity was 0.4 g l^{-1} day^{-1} and the EPA content of the biomass was 4%, with a mean EPA productivity of 16 mg l^{-1} day^{-1}. This experiment is still in progress.

Contamination by protozoa or other microalgae was not a major problem in our NHTRs once efficient filtration systems for both the air and for the culture medium were adopted. Several bacterial species were observed in *Nannochloropsis* sp. cultures, but in very low concentrations. Moreover, the composition of the bacterial population associated with *Nannochloropsis* sp. cultures was rather stable and did not change significantly even after transfer from laboratory to outdoor conditions (Pastorelli et al., 1998). A noticeable exception occurred in the summer when, along with the abundant exudation of polysaccharidic material and massive formation of cell clusters, the bacterial load of the cultures increased by two orders of magnitude (data not shown).

3.4. Effect of salinity on productivity and EPA content of Nannochloropsis sp. cultures

To evaluate the effect of a salinity value lower than that of seawater on culture productivity and fatty acid composition, *Nannochloropsis* sp. was grown outdoors in two 1 NHTR units at salt concentrations of 20 and 33 g l^{-1}. Neither the productivity (the mean monthly productivities were 0.69 ± 0.4 at 20 g l^{-1} and 0.71 ± 0.4 g l^{-1} day^{-1} at 33 g l^{-1} salinity), nor the total fatty acid content of the biomass (about 16.8% at both salt concentrations) was influenced by salinity in the range tested, although the fatty acid profile was modified (Table 5). At a salinity of 20 g l^{-1} saturated fatty acids decreased by about 4% and monounsaturated fatty acids by about 23%, while PUFAs increased by more than 13%. EPA content rose from 4.3 to 4.8% of the dry biomass. Since the productivity of the culture was the same at both salinity values, EPA productivity was slightly higher at 20 g l^{-1} (33.1 mg l^{-1} day^{-1}) than at 33 g l^{-1} (30.5 mg l^{-1} day^{-1}).

Table 5
Influence of salinity on fatty acid content and profile (% of dry weight) of *Nannochloropsis* sp. cultures grown outdoors in 1 NHTR units in May 1997[a]

Fatty acid	Salinity (g l^{-1})	
	20	33
14:0	0.81	0.81
16:0	4.26	4.45
16:1ω7	4.42	4.82
18:1ω9	0.80	1.15
18:2ω6	0.47	0.37
20:4ω6	0.84	0.65
20:5ω3	4.77	4.32
TFA	16.70	16.90

[a] Only the major fatty acids are shown (>0.3%).

4. Discussion

Due to its high EPA content, *Nannochloropsis* has been proposed as a potential source of EPA for human consumption (Seto et al., 1984; Sukenik, 1991, 1998). Although in Japan *Nannochloropsis* is currently cultivated year-round in large outdoor tanks to provide a food chain for the production of fish larvae (Okauchi, 1991), data from outdoor mass cultivation of these microalgae is scanty. Boussiba et al. (1987) cultivated *Nannochloropsis salina* outdoors in 1- and 2.5-m^2 raceway-type ponds; prevention of contamination by diatoms and of predation by ciliates appeared to be the crucial factors for the successful operation of the cultures. Outdoor cultivation of *Nannochloropsis* sp. in raceway ponds with surface area ranging from a few square meters to 3000 m^2 was carried out at the National Center for Mariculture, Israel Oceanographic and Limnological Research, and at the Nature Beta Technology algae production plant, both located in Eilat, Israel (Sukenik et al. 1993a; Sukenik, 1998). The work done by Sukenik and co-workers at Eilat facilities has elucidated some fundamental aspects of the mass cultivation of this microalga and has confirmed the difficulty of maintaining monoalgal cultures outdoors for prolonged periods (Sukenik, 1998).

The present work shows that photobioreactors have the potential to overcome the main limita-

tions encountered in open ponds and may allow long lasting outdoor cultivation of this eustigmatophyte achieving a relatively high EPA productivity compared with other autotrophic systems. In fact, contamination by bacteria, protozoa or other microalgae has not been a major problem in the NHTR units used in our experiments. Cultivation of microalgae in photobioreactors is hampered by some problems, including overheating, fouling and accumulation of oxygen to toxic levels (Tredici and Materassi, 1992). Temperatures of up to 5°C over the optimal value (25°C) were measured in our *Nannochloropsis* cultures during the hours of strongest irradiation, particularly in the summer. Prolonged exposure to supraoptimal diurnal temperatures, together with high irradiances at midday, were considered the main cause of the low performance of the cultures in July and August. Fouling and accumulation of oxygen to toxic levels have not been a particularly serious problem in the NHTR design. It must be pointed out, however, that during the summer, when maximum productivities were expected, the cultures performed poorly. Hence, it was not possible to demonstrate the ability of NHTR systems in avoiding build-up of toxic oxygen tensions during periods of high production rates for *Nannochloropsis* cultures.

EPA productivity by *Nannochloropsis* sp. grown in 16 mm thick alveolar panels under laboratory conditions averaged 36 mg l^{-1} 24 h^{-1}. Outdoors, in the NHTRs, mean monthly EPA productivity varied between 18 and 32 mg l^{-1} day^{-1} essentially following the trend of biomass productivity which in turn was dependent upon the season. This data is comparable to that obtained with the diatom *P. tricornutum* (Molina Grima et al., 1994a) and the freshwater eustigmatophyte *M. subterraneus* (Hu et al., 1997). These two microalgae are considered among the most promising EPA producers under autotrophic conditions and are presently under investigation as part of the EC Brite–Euram project 'An integrated production system of highly purified eicosapentaenoic acid from microalgae' coordinated by Professor Molina Grima.

The major fatty acids in *Nannochloropsis* are 14:0, 16:0, 16:1ω7 and EPA, whereas C18 fatty acids and 20:4ω6 are present in lower quantities (Teshima et al., 1983; Sukenik et al., 1989; Volkman et al., 1993). Fractionation of lipid extracts from *Nannochloropsis* sp. indicated that EPA is mainly associated with galactolipids (the major lipid constituents of the thylakoid membranes) and phosphatidyl glycerol, whereas the storage lipid triacylglycerol contains primarily 14:0, 16:0 and 16:1ω7 (Sukenik et al., 1989, 1993b). The effect of environmental factors on lipid content and fatty acid composition has been investigated in various species of *Nannochloropsis* with the majority of the work conducted under laboratory conditions (Teshima et al., 1983; Seto et al., 1984; Sukenik et al., 1989, 1993a,b; Sukenik and Carmeli, 1990; Renaud et al., 1991; Sukenik, 1991). These studies have shown that light intensity and temperature, as well as nitrogen starvation, play a key role in regulation of the relative abundance of fatty acids and of the absolute quantity of EPA.

The effect of temperature on EPA biosynthesis appears to be complex and has not yet been clarified. Seto et al. (1992) found that cells grown at 20°C contained 60% more EPA than cells grown at the optimal growth temperature of 25°C. Similarly, Sukenik et al. (1993b) showed that cultures grown at a low temperature (20°C) were characterized by a high rate of galactolipid synthesis and hence by a relatively high level of EPA, as compared with cells grown at a high temperature (30°C). In contrast with these authors, Teshima et al. (1983) found maximal EPA synthesis at the optimal growth temperature of 25°C. Although the effect of temperature is not easy to establish in outdoor cultures, where both temperature and irradiance vary during the day, the present work would suggest that minimum night temperatures play a key role in influencing the fatty acid level and composition. The main outcome of our work, in partial disagreement with conclusions drawn from most other studies, is that even when the fatty acid profile is deeply modified in response to an environmental stimulus (in our case temperature), the total content of EPA in the biomass is not significantly affected.

Sukenik et al. (1989, 1993b) studied the influence of light intensity on lipid content and com-

position in *Nannochloropsis* sp. grown in the laboratory. Under light-limiting growth conditions, the cells were characterized by a high density of thylakoids with most of the lipid carbon allocated in the EPA-rich galactolipids. In contrast, cells grown under saturating light conditions accumulated carbohydrate and triacylglycerols as storage materials, resulting in a high content of 16:0 and 16:1ω7 and a reduction in the cellular EPA content of more than 50%. Similarly, Renaud et al. (1991) showed increasing saturation of the fatty acids of *N. oculata* with increasing irradiance using large-scale outdoor cultures. In the same organism, Seto et al. (1992) found no effect of high irradiances on EPA distribution and content. In our experiments, light intensity seems to play a minor role in fatty acid composition with respect to temperature. However, high irradiances might have been involved in causing excessive exudation and cell aggregation in *Nannochloropsis* sp. cultures.

Sukenik and Carmeli (1990) investigated the synthesis of fatty acids in *Nannochloropsis* sp. grown in the laboratory under a 12:12-h light–dark cycle. These authors showed that storage triacylglycerol lipids are synthesized and accumulated during the light period and are rapidly consumed for cellular maintenance along with carbohydrates during the dark period. Galactolipids are also synthesized during the light period, but not turned over in the dark. Thus, the dark period is characterized by an increase of the relative proportion of EPA which is associated with the galactolipids. Our experiments confirm that *Nannochloropsis* sp. also uses fatty acids associated with storage lipid (mainly 14:0 and 16:0) during the dark period when cultivated under natural light–dark cycle conditions. Thus, as suggested by Sukenik and Carmeli (1990), the early morning is the preferred time for harvesting *Nannochloropsis* sp. biomass to obtain a high EPA content.

Teshima et al. (1983) found no significant variation of the EPA content of *N. oculata* biomass with salinities between 4 and 30 g l^{-1}. Similarly, Renaud and Parry (1994) found that EPA content did not vary significantly with salinity in the range of 10–35 g l^{-1} although salinities from 20 to 25 g l^{-1} were optimal for growth and production of EPA. Our results show that lowering salinity from 33 to 20 g l^{-1} slightly stimulates EPA accumulation and productivity.

The effect of seasonal variations in temperature and light availability on fatty acid distribution was studied in outdoor mass cultures of *Nannochloropsis* sp. by Sukenik et al. (1993a). The highest cellular EPA content (3.8%) was observed during winter with water temperatures between 8 and 16°C and under low irradiances, i.e. in conditions of very low biomass productivity. In summer, when biomass productivity was highest, EPA content decreased to less than half the winter value. Maximum EPA productivity of our cultures was attained in late spring and early autumn, i.e. under relatively low irradiances and when prolonged exposure at supraoptimal diurnal temperatures could be prevented.

The primary goals of algal biotechnology for production of EPA are high EPA productivity and high EPA content of the biomass. Most previous studies conducted with *Nannochloropsis* have shown that maximum EPA content is obtained under low light conditions and at temperature values slightly lower than the optimal temperature for growth, and have concluded that the conditions required to maximize EPA productivity are different from those needed to maximize EPA content (Sukenik, 1991). Our work has led us to a different conclusion: since the main environmental factors, such as temperature and irradiance, do not significantly influence the EPA content of the biomass in our photobioreactors, conditions for maximum EPA productivity are those required to maximize biomass production. Moreover, for establishing a reliable system for EPA production based on cultivation of *Nannochloropsis* in photobioreactors, high irradiances are not ideal or can even be detrimental, as is prolonged exposure to supraoptimal temperatures that cause a serious imbalance of algal growth. The potential of *Nannochloropsis* cultivation in closed systems would therefore be best realized in temperate countries.

Acknowledgements

We are deeply indebted to Drs Susan Blackburn and Laura Barsanti for critically reviewing the manuscript. This work was partly supported by EXENIA S.r.l. (Milano, Italy).

References

Barclay, W.R., Meager, K.M., Abril, J.R., 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J. Appl. Phycol. 6, 123–129.

Bousfield, I.J., Smith, G.L., Dando, T.R., Hobbs, G., 1983. Numerical analysis of total fatty acid profiles in the identification of coryneform, nocardioform and some other bacteria. J. Gen. Microbiol. 129, 375–394.

Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y., Richmond, A., 1987. Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. Biomass 12, 37–47.

Chrismadha, T., Borowitzka, M.A., 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.

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.

Crayton, M.A., 1982. A comparative cytochemical study of Volvocacean matrix polysaccharides. J. Phycol. 18, 336–344.

Gill, I., Valivety, R., 1997. Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. TIBTECH 15, 401–409.

Gonen-Zurgil, Y., Carmeli-Schwartz, Y., Sukenik, A., 1996. Selective effect of the herbicide DCMU on unicellular algae—a potential tool to maintain monoalgal mass culture of *Nannochloropsis*. J. Appl. Phycol. 8, 415–419.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* (Hustedt) and *Detonula confervacea* (Cleve). Can. J. Microbiol. 8, 229–239.

Hu, Q., Hu, Z., Cohen, Z., Richmond, A., 1997. Enhancement of eicosapentaenoic acid (EPA) and γ-linolenic acid (GLA) production by manipulating algal density of outdoor cultures of *Monodus subterraneus* (Eustigmatophyta) and *Spirulina platensis* (Cyanobacteria). Eur. J. Phycol. 32, 81–86.

Leman, J., 1997. Oleaginous microorganisms: an assessment of the potential. Adv. Appl. Microbiol. 43, 195–243.

Molina Grima, E., Sanchez Perez, J.A., Garcia Camacho, F., Acien Fernandez, F.G., Fernadez Sevilla, J.M., Valdez Sanz, F., 1994a. Effect of dilution rate on eicosapentaenoic acid productivity of *Phaeodactylum tricornutum* UTEX 640 in outdoor chemostat culture. Biotechnol. Lett. 16, 1035–1040.

Molina Grima, E., Sanchez Perez, J.A., Garcia Camacho, F., Fernadez Sevilla, J.M., Acien Fernandez, F.G., 1994b. Effect of growth rate on the eicosapentaenoic acid and docosahexaenoic acid content of *Isochrysis galbana* in chemostat culture. Appl. Microbiol. Biotechnol. 41, 23–27.

Nordoy, A., 1991. Is there a rational use for n-3 fatty acids (fish oils) in clinical medicine? Drugs 42, 331–342.

Okauchi, M., 1991. The status of phytoplankton production in Japan. In: Fulks, W., Main, K.L. (Eds.), Rotifers and Microalgae Culture Systems. Proceedings of a US–Asia Workshop. The Oceanic Institute, Honolulu, HI, pp. 247–256.

Pastorelli, R., Chini Zittelli, G., Tredici, M.R., 1998. Bacterial population associated with *Nannochloropsis* sp. mass cultures. Abstracts of the International Symposium 'Marine Bioprocess Engineering'. Noordwijkerhout, The Netherlands. November 8–11, 1998.

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

Renaud, S.M., Parry, D.L., 1994. Microalgae for use in tropical aquaculture II: Effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. J. Appl. Phycol. 6, 347–356.

Renaud, S.M., Parry, D.L., Luong-Van, T., Kuo, C., Padovan, A., Sammy, N., 1991. Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. J. Appl. Phycol. 3, 43–53.

Senzaki, H., Iwamoto, S., Ogura, E., Kiyozuka, Y., Arita, S., Kurebayashi, J., Takada, H., Hioki, K., Tsubura, A., 1998. Dietary effects of fatty acids on growth and metastasis of KPL-1 human breast cancer cells in vivo and in vitro. Anticancer Res. 18, 1621–1627.

Seto, A., Wang, H.L., Hesseltine, C.W., 1984. Culture conditions affect eicosapentaenoic acid content of *Chlorella minutissima*. J. Am. Oil. Chem. Soc. 61, 892–894.

Seto, A., Kumasaka, K., Hosaka, M., Kojima, E., Kashiwakura, M., Kato, T., 1992. Production of eicosapentaenoic acid by marine microalgae and its commercial utilization for aquaculture. In: Kyle, D.J., Ratledge, C. (Eds.), Industrial Application of Single Cell Oils. American Oil Chemists' Society, Champaign, IL, pp. 219–234.

Sukenik, A., 1991. Ecophysiological considerations in the optimization of eicosapentaenoic acid production by *Nannochloropsis* sp. (Eustigmatophyceae). Bioresour. Technol. 35, 263–269.

Sukenik, A., Carmeli, Y., 1990. Lipid synthesis and fatty acid composition in *Nannochloropsis* sp. (Eustigmatophyceae) grown in a light–dark cycle. J. Phycol. 26, 463–469.

Sukenik, A., Carmeli, Y., Berner, T., 1989. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. J. Phycol. 25, 686–692.

Sukenik, A., Zmora, O., Carmeli, Y., 1993a. Biochemical quality of marine unicellular algae with special emphasis

on lipid composition. II. *Nannochloropsis* sp. Aquaculture 117, 313–326.

Sukenik, A., Yamaguchi, Y., Livne, A., 1993b. Alterations in lipid molecular species of the marine eustigmatophyte *Nannochloropsis* sp. J. Phycol. 29, 620–626.

Sukenik, A., 1998. Production of eicosapentaenoic acid by the marine eustigmatophyte *Nannochloropsis* sp. In: Cohen, Z. (Ed.), Chemicals from Microalgae. Taylor and Francis, London, in press.

Teshima, S., Yamasaki, S., Kanazawa, A., Hirata, H., 1983. Effects of water temperature and salinity on eicosapenatenoic acid level of marine *Chlorella*. Bull. Jpn. Soc. Sci. Fisheries 49, 805.

Tredici, M.R., Chini Zittelli, G., 1998. Efficiency of sunlight utilization: tubular versus flat photobioreactors. Biotechnol. Bioeng. 57, 187–197.

Tredici, M.R., Materassi, R., 1992. From open ponds to vertical alveolar panels: the Italian experience in the development of reactors for the mass cultivation of phototrophic microorganisms. Appl. Phycol. 4, 221–231.

Tredici, M.R., Carlozzi, P., Chini Zittelli, G., Materassi, R., 1991. A vertical alveolar panel (VAP) for outdoor mass cultivation of microalgae and cyanobacteria. Bioresour. Technol. 38, 153–159.

Volkman, J.K., Malcom, R.B., Dunstan, G.A., Jeffrey, S.W., 1993. The biochemical composition of marine microalgae from the class eustigmatophiceae. J. Phycol. 29, 69–78.

Yongmanitchai, W., Ward, O.P., 1989. Omega-3 fatty acids: alternative sources of production. Process Biochem. 24, 117–125.