Eicosapentaenoic Acid and Docosahexaenoic Acid Production Potential of Microalgae and Their Heterotrophic Growth

Rema Vazhappilly and Feng Chen*

Department of Botany, The University of Hong Kong, Hong Kong

ABSTRACT: Twenty microalgal strains were investigated in photoautotrophic flask cultures for their potential for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) production. The highest EPA proportion (% of total fatty acids) was produced by *Monodus subterraneus* UTEX 151 (34.2%), followed by *Chlorella minutissima* UTEX 2341 (31.3%) and *Phaeodactylum tricornutum* UTEX 642 (21.4%). The highest DHA proportion (% of total fatty acids) was obtained in *Crypthecodinium cohnii* UTEX L1649 (19.9%), followed by *Amphidinium carterae* UTEX LB 1002 (17.0%) and *Thraustochytrium aureum* ATCC 28211 (16.1%). Among the 20 strains screened, the EPA yield was high in *M. subterraneus* UTEX 151 (96.3 mg/L), *P. tricornutum* UTEX 642 (43.4 mg/L), *Chl. minutissima* UTEX 2341 (36.7 mg/L), and *Por. cruentum* UTEX 161 (17.9 mg/L) owing to their relatively high biomass concentrations. The DHA yield was high in *C. cohnii* UTEX L1649 (19.5 mg/L) and *A. carterae* UTEX LB 1002 (8.6 mg/L). Heterotrophic growth of these 20 microalgae was also tested on two different carbon sources, acetate and glucose. All microalgae except *Nannochloropsis oculata* UTEX LB 2164 showed growth on glucose (5 g/L) under heterotrophic conditions. Twelve of them could grow heterotrophically when acetate (1 g/L) was used as their sole carbon and energy source.

JAOCS 75, 393–397 (1998).

KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, n-3 fatty acids, heterotrophic, microalgae, photoautotrophic.

Recent clinical and epidemiological studies have indicated that long-chain n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis, and diseases of old age, such as Alzheimer's disease and age-related macular degeneration (1,2). The eicosanoids, such as prostaglandin, prostacyclin and leukotriene, derived from n-3 polyunsaturated fatty acids are also important in infant development, modulatory vascular resistance, and wound healing (2,3).

Fish oil is the richest source of n-3 fatty acids and contains up to 30% of DHA and EPA (4). At present, fish oil is used for the commercial production of EPA and DHA. However, there are some limitations to using fish oil as a source of n-3 fatty acids, such as peculiar taste, odor and stability problems. Purification costs of these fatty acids from fish oil are also high. Because fish obtain n-3 fatty acids from zooplankton, which consume algae, research has become focused on developing a commercially feasible technology to produce n-3 fatty acids directly from microalgae (5). Several attempts have been made recently to develop a commercial heterotrophic process for n-3 fatty acid production because photoautotrophic production is limited by the cells' mutual shading and the consequent increase in downstream processing cost (6). Heterotrophic production of DHA by the dinoflagellate *Crypthecodinium cohnii,* grown in fermentors, has just been commercialized by Martek Biosciences in Maryland (7). The advantages and limitations of heterotrophic growth of microalgae have been recently reviewed (8).

Although the fatty acid composition and growth of microalgae may be influenced by nutrients and environmental conditions, the fatty acid production potential of microalgae depends highly on primary strain selection for maximal fatty acid production. The industrial heterotrophic production potential of microalgae mainly depends on the ability of the selected strains to grow heterotrophically with good fatty acid compositions and yields. The aim of the present study was to screen and identify microalgal strains with high EPA and DHA yields and to test their heterotrophic growth potential.

MATERIALS AND METHODS

Organisms. The microalgae were obtained from culture collections (i.e., UTEX, ATCC, and CSIRO) as shown in Table 1.

Photoautotrophic growth. The media used for culturing these microalgae were prepared according to recommendations by the culture collections (Table 1). All media were inoculated with a 5% inoculum of exponentially growing cells. The microalgae were grown in 250-mL flasks that contained 150 mL medium in batch mode at 25°C with intermittent shaking and continuous illumination (60 $\mu E/m^2$ s).

Heterotrophic growth. The same media recommended by the culture collections were used. An inoculum (5%) of exponentially growing cells was used for inoculation. All microalgae were grown separately on glucose (5 g/L) and acetate (1 g/L) in 250-mL flasks with 150 mL medium in batch mode in

*To whom correspondence should be addressed at Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong.
E-mail: sfchen@hkusua.hku.hk.

TABLE 1
Growth of Microalgae Under Photoautotrophic and Heterotrophic Conditions[a]

Microalgae	Medium[b,c]	Culture time[d] (d)	Biomass concentration[d] (mg/L)	On glucose[e] (5 g/L)	On acetate[e] (1 g/L)
A. carterae UTEX LB 1002	Erd-Schreiber	23	499	+ + +	–
Amphidinium sp. CSIRO CS-259	G2	23	330	+ + +	+ +
Chaetoceros calcitrans CSIRO CS-178	f/2	20	409	+	+
Chlorella minutissima UTEX 2219	Proteose	10	894	+	+
Chl. minutissima UTEX 2341	Artificial seawater	10	995	+	+
Chröomonas salina CSIRO CS-174	fE	23	157	+	–
Crypthecodinium cohnii UTEX L1649	Porphyridium	10	1510	+ + + +	+ + +
Cryptomonas sp. UTEX LB 2423	ES-Enriched seawater	25	316	+ +	–
Isochrysis galbana UTEX LB 987	Soil + seawater	20	871	+	+
Monodus subterraneus UTEX 151	Proteose	10	2566	+	+
Nannochloropsis oculata UTEX LB 2164	Erd-Schreiber	25	345	-	–
Pavlova lutheri UTEX LB 1293	Erd-Schreiber	25	235	+	–
P. lutheri ATCC 50092	Hesnw	25	368	+	–
Phaeodactylum tricornutum UTEX 642	LDM	10	1934	+ + +	+
Porphyridium cruentum UTEX 161	Porphyridium	10	964	+ +	+
Por. purpureum CSIRO CS-25	f/2	23	456	+ +	–
Prorocentrum minimum UTEX LB 1995	Erd-Schreiber	20	398	+	–
Schizochytrium aggregatum ATCC 28209	By+	20	488	+ + +	+ +
Thalassiosira pseudonana CSIRO CS-173	G2	25	153	+ +	+
Thraustochytrium aureum ATCC 28211	By+	20	101	+ + +	+ +

[a]Culture collections: UTEX—The Culture Collection of Algae at The University of Texas at Austin, Texas; ATCC—American Type Culture Collection, Rockville, Maryland; CSIRO—CSIRO Marine Laboratories, Hobart, Tasmania, Australia.
[b]Medium : Erd-Schreiber, proteose, artificial seawater, porphyridium, ES-enriched seawater, soil + seawater and LDM (9); Hesnw and By+ (By+ medium was prepared without glucose) (10); f/2, G2 and fE medium compositions were provided by CSIRO Marine Laboratories.
[c]Artificial seawater was used instead of natural seawater in all media.
[d]Photoautotrophic growth.
[e]Heterotrophic growth: + poor growth; + + moderate growth; + + + good growth; + + + + excellent growth.

darkness. The culture temperature was maintained at 25°C with continuous shaking at 200 rpm in an orbital shaker.

Determination of biomass. The cell dry weight concentration was determined according to the method previously reported by Chen and Johns (11).

Extraction and purification of lipids. Lipids were extracted by modified procedures of Bligh and Dyer (12) and Chen and Johns (11). The cells were first homogenized with chloroform/methanol/water (1:2:0.8, vol/vol/vol) for 2 min. An equal volume of chloroform and water was then added, to bring the final ratio of the mixture to 2:2:1.8, and the mixture was then homogenized for 1 min. The chloroform layer (lower) with the lipids was then separated, and the alcoholic layer (upper), which contained the residues, was reextracted twice with methanol/chloroform (2:1, vol/vol).

The chloroform layers were combined and subjected to a "Floch wash" to remove all nonlipid contaminants (13). The mixture was washed with one-fourth of the volume of 0.88% (wt/vol) potassium chloride, followed by methanol/saline solution (1:1, vol/vol). The purified chloroform layer was then evaporated to dryness under N_2 and weighed to get the total lipid content.

Fatty acid analysis. The lipids were then transesterified by alkali catalysis (13). The lipid samples were dissolved in benzene (up to 100 mg in 2 mL). An appropriate amount of internal standard, C17:0 (heptadecaenoic acid), was added at this stage. Sodium methoxide (0.5 M) in anhydrous methanol (2 mL) was added, and the mixture was incubated at 50°C for 10 min. Glacial acetic acid (0.1 mL), followed by water (5 mL), was added, the esters were extracted with hexane (10 mL), and the hexane layers were then concentrated under N_2.

The purity of fatty acid methyl esters was checked by thin-layer chromatography (13). The algal fatty acid methyl esters were run along with standard fatty acid methyl esters (Sigma Chemical Co., St. Louis, MO) in hexane/diethyl ether (9:1, vol/vol) as the mobile phase. The fatty acid methyl esters were then used for gas-chromatographic analysis in an HP-6890 GC (Hewlett-Packard, Palo Alto, CA), equipped with a flame-ionization detector and a Supelco (Bellefonte, PA) Omegawax™ 250 capillary column (30 m × 0.25 mm). The column and detector temperatures were kept at 210 and 260°C, respectively. Nitrogen was used as the carrier gas, and the flow rate was kept at 2 mL/min. The injector was kept at 250°C, with an injection volume of 3 μL under splitless injection mode. Authentic standards (Sigma Chemical Co.) were employed for the identification of fatty acids. The fatty acids were identified by comparison of relative retention times and by calculation of equivalent chainlengths. The fatty acid contents were determined by comparing their peak areas with that of the internal standard (C17:0).

RESULTS AND DISCUSSION

Among the 20 microalgae tested under photoautotrophic con-

ditions, six showed good growth after 10 d, while the rest required 20–25 d to produce significant biomass. The growth times, media, and biomass concentrations are shown in Table 1. The fatty acid composition and the EPA and DHA yields, obtained from each species, are shown in Tables 2 and 3, respectively.

Monodus subterraneus UTEX 151, which is a freshwater strain, produced the highest cell dry weight concentration (Table 1). This alga also had the highest EPA proportion, EPA content, and EPA yield (Tables 2 and 3). Increased EPA proportion and content at a higher light intensity (90 $\mu E/m^2$ s) and at a lower temperature (20°C) were previously reported in *M. subterraneus* (14). The slight reduction in EPA proportion and content may be due to the higher temperature (25°C) and different culture conditions (e.g., light intensity) used. The inverse relation of temperature to EPA content in *M. subterraneus* was also reported by Iwamoto and Sato (15). *Chlorella minutissima* UTEX 2341 also had a high EPA proportion, content, and yield (Tables 2 and 3), although they were lower than for *M. subterraneus*. Even though *Phaeodactylum tricornutum* UTEX 642 had lower EPA proportion and content than *Chl. minutissima* UTEX 2341, the EPA yield was higher owing to higher biomass concentrations achieved. *Porphyridium cruentum* UTEX 161 also had high EPA proportion, content, and yield.

Crypthecodinium cohnii UTEX L 1649 had the highest DHA proportion and yield among all 20 microalgal strains. *Amphidinium carterae* UTEX LB 1002 also had a high DHA proportion and yield. Although the DHA proportion and content of *A. carterae* UTEX LB 1002 were comparable to *Cr. cohnii* UTEX L 1649, the yield was much lower due to lower attainable biomass concentration (Table 1). *Thraustochytrium aureum* ATCC 28211 also had a relatively high DHA proportion and content, but the yield was low owing to poor biomass concentration (only 0.1 g/L after 23 d of cultivation). The DHA proportion and content of *Thr. aureum* ATCC 28211 were both lower than those of another *Thr. aureum* strain (ATCC 34304) reported by Bajpai *et al.* (16).

Although the EPA yield (17.9 mg/L) was low in *Por. cruentum* UTEX 161, the EPA yield (Table 3) obtained from *Chl. minutissima* UTEX 2341 and *Ph. tricornutum* UTEX 642 were high compared to the screening results obtained from the same strains by Yongmanitchai and Ward (17) owing to higher biomass concentrations. This is probably due to the different growth conditions used. The EPA contents (% of biomass) of *Chl. minutissima* UTEX 2341 and *Por. cruentum* UTEX 161 were low compared to the results of Yongmanitchai and Ward (17). This may be due to the effects of culture conditions, especially temperature and aeration. In their experiments, a lower temperature (20°C) and higher aeration rate (75 mL/min) were employed. Among the 20 microalgae tested, *Amphidinium* sp. CSIRO CS-174, *Chaetoceros calcitrans* CSIRO CS-178, *Cryptomonas* sp. UTEX LB 2423, *Isochrysis galbana* UTEX LB 987, *Pavlova lutheri* ATCC 50092, *Schizochytrium aggregatum* ATCC 28209, and *Thalassiosira pseudonana* CSIRO CS-173 also contained significant proportions of EPA (Table 2). The EPA and DHA contents were not reported in the specific strains of microalgae used in this experiment except in *A. carterae* UTEX LB 1002, *Chl. minutissima* UTEX 2219, *Chl. minutissima* UTEX 2341, *M. subterraneus* UTEX 151, *Ph. tricornutum* UTEX 642, and *Por. cruentum* UTEX 161 (Tables 2 and 3).

The high accumulation of arachidonic acid (ARA, C20:4n-6) with EPA or DHA is disadvantageous because ARA can cause deleterious health effects and problems in EPA recovery (17–19). The ARA content was relatively low

TABLE 2
Fatty Acid Compositions of Microalgae (% of total fatty acids)[a]

Microalgae	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:5	22:6
A. carterae	5.4	2.1	30.9	7.1	10.5	0.3	5.6	3.1	1.6	15.1	1.3	17.0
Amphidinium sp.	7.8	0.6	28.0	23.7	6.7	8.5	4.7	0.8	1.1	16.3	1.8	0.0
Cha. calcitrans	7.0	1.4	27.5	26.5	7.2	0.1	4.9	1.0	1.1	18.8	4.5	0.0
Chl. minutissima (UTEX 2219)	11.4	2.7	30.5	26.8	4.9	3.3	0.3	10.3	2.6	3.3	3.9	0.0
Chl. minutissima (UTEX 2341)	0.7	4.7	11.9	15.0	7.8	12.0	6.3	4.6	5.6	31.3	0.1	0.0
Chr. salina	12.0	5.1	22.6	21.9	2.0	0.3	19.7	3.1	4.1	8.2	1.0	0.0
Crypth. cohnii	4.4	16.9	20.6	22.6	9.0	0.3	2.3	1.1	0.9	0.0	2.0	19.9
Cryptomonas sp.	7.9	1.0	21.7	17.1	15.6	3.2	1.4	1.8	2.8	16.6	0.7	10.2
I. galbana	3.5	2.7	28.2	19.3	6.7	0.3	4.0	12.9	1.2	16.6	1.0	3.6
M. subterraneus	0.1	12.7	18.7	10.1	0.9	5.4	2.4	0.4	13.7	34.2	1.4	0.0
N. oculata	3.0	3.2	28.8	14.5	4.1	3.3	10.0	14.1	5.1	5.5	1.3	7.1
Pa. lutheri (UTEX LB1293)	8.6	7.6	22.8	4.6	11.5	0.6	20.3	1.4	6.8	3.9	4.2	7.7
Pa. lutheri (ATCC 50092)	13.4	3.0	10.7	3.2	12.6	7.5	9.2	9.0	5.4	14.3	5.9	5.8
Ph. tricornutum	6.6	2.7	14.0	33.9	0.0	7.2	4.0	0.7	3.2	21.4	5.8	0.5
Por. cruentum	0.3	17.5	35.5	8.5	0.0	0.0	1.3	0.0	17.2	19.7	0.0	0.0
Por. purpureum	0.1	2.8	37.9	1.4	13.7	4.4	8.1	8.6	5.8	6.7	10.5	0.0
Pro. minimum	6.3	2.9	33.2	2.8	17.8	0.3	6.1	10.6	2.7	8.7	2.7	5.9
S. aggregatum	2.6	2.7	15.3	18.6	8.0	14.7	15.1	0.6	7.2	15.7	0.0	0.0
Tha. pseudonana	5.8	0.8	31.7	17.6	2.0	5.4	2.9	0.6	9.2	10.8	6.7	6.5
Thr. aureum	0.3	8.9	8.5	5.4	6.7	7.8	10.5	15.2	12.1	4.5	4.0	16.1

[a]See Table 1 for abbreviations.

TABLE 3
Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Yields of Microalgae[a]

Microalgae	EPA of biomass (% w/w)	DHA of biomass (% w/w)	EPA yield (mg/L)	DHA yield (mg/L)
A. carterae UTEX LB 1002	1.5	1.7	7.7	8.6
Amphidinium sp. CSIRO CS-259	1.2	0.0	4.0	0.0
Cha. calcitrans CSIRO CS-178	1.0	0.0	4.2	0.0
Chl. minutissima UTEX 2219	0.3	0.0	2.7	0.0
Chl. minutissima UTEX 2341	3.7	0.0	36.7	0.0
Chr. salina CSIRO CS-174	0.4	0.0	0.6	0.0
Crypth. cohnii UTEX L1649	0.0	1.3	0.0	19.5
Cryptomonas sp. UTEX LB 2423	1.0	0.6	3.2	2.0
I. galbana UTEX LB 987	0.9	0.2	8.0	1.7
M. subterraneus UTEX 151	3.8	0.0	96.3	0.0
N. oculata UTEX LB 2164	0.6	0.7	2.0	2.6
Pa. lutheri UTEX LB 1293	0.2	0.5	0.6	1.1
Pa. lutheri ATCC 50092	0.8	0.3	3.0	1.2
Pha. tricornutum UTEX 642	2.2	0.06	43.4	1.08
Por. cruentum UTEX 161	1.9	0.0	17.9	0.0
Por. purpureum CSIRO CS-25	0.8	0.0	3.7	0.0
Pro. minimum UTEX LB 1995	0.6	0.4	2.3	1.5
S. aggregatum ATCC 28209	1.2	0.0	6.1	0.0
Tha. pseudonana CSIRO CS-173	0.5	0.3	0.8	0.5
Thr. aureum ATCC 28211	0.3	1.0	0.3	1.0

[a]See Table 1 for abbreviations.

in all 20 microalgae except in *Por. cruentum* UTEX 161, which is a well-known species for ARA or EPA production, depending on the environmental conditions used (19–21). Optimization of growth conditions, such as temperature and salinity, can reduce ARA content and enhance EPA accumulation in *Por. cruentum* (20,21).

Out of the 20 microalgae screened, *M. subterraneus* UTEX 151, *Ph. tricornutum* UTEX 642, *Chl. minutissima* UTEX 2341, *Por. cruentum* UTEX 161, and *Crypth. cohnii* UTEX L1649 yielded significant amounts of EPA or DHA (18–96 mg/L) (Table 3). When considering the individual production of EPA and DHA attributable to their different nutritional and pharmacological importance, it is desirable to select strains that contain less EPA for DHA production or vice versa, to minimize problems associated with downstream processing (17). For example, *Crypth. cohnii* UTEX L 1649 was the best potential strain for DHA production, which yielded more than 19 mg/L of DHA, without production of EPA (Table 3). In contrast, *A. carterae* UTEX LB 1002 yielded about equal amounts of EPA (7.7 mg/L) and DHA (8.6 mg/L).

The screening results indicated that *M. subterraneus* UTEX 151 was the alga with the best potential for EPA production, with high biomass, high growth rate, high EPA, and absence of DHA. *Crypthecodinium cohnii* showed characteristics for good DHA production, such as high biomass, high growth rate, high DHA content, and complete absence of EPA.

The EPA and DHA proportions and contents of some strains obtained in this study are lower than those reported by other investigators. This is probably due to the effects of culture conditions, such as temperature and aeration. The extreme sensitivity of unsaturated fatty acid composition to growth temperature, to maintain membrane fluidity, is reported by many researchers (19,22,23). Fatty acid unsaturation due to changes in growth temperature is associated with dissolved oxygen concentration in batch culture (24) and also depends on the availability of intracellular molecular oxygen (25). In this screening study, aeration was not provided (only intermittent shaking), and because the optimal temperature for maximal fatty acid unsaturation may vary in each species, the temperature used (25°C) might not be optimal.

The growth of microalgae on glucose and acetate under heterotrophic conditions was also investigated (Table 1). All microalgae except *Nannochloropsis oculata* UTEX LB 2164 showed heterotrophic growth when glucose was used as the sole carbon and energy source. Twelve of them could grow heterotrophically on acetate.

Out of the 20 microalgae tested (Table 1), *Crypth. cohnii* UTEX L1649 showed excellent heterotrophic growth on glucose. *Amphidinium carterae* UTEX LB 1002, *Amphidinium* sp. CSIRO CS-259, *Ph. tricornutum* UTEX 642, *S. aggregatum* ATCC 28209, and *Thr. aureum* ATCC 28211 also showed good heterotrophic growth on glucose.

When acetate was used as the sole carbon and energy source, only *Crypth. cohnii* UTEX L 1649 showed good heterotrophic growth, while several other strains, *Amphidinium* sp. CSIRO CS-259, *S. aggregatum* ATCC 28209 and *Thr. aureum* ATCC 28211, showed moderate growth on acetate. Table 1 shows that the preferred organic substrate for heterotrophic growth is glucose for all strains tested. We are not sure whether this difference is due to the high concentration of acetate (1 g/L) used because acetate might be inhibitory to microalgae above certain concentrations (26,27). An acetate concentration of 0.4 g/L or more was found to be toxic to a green microalga, *Chlamydomonas reinhardtii*, grown heterotrophically.

Although a decrease in fatty acid unsaturation was reported in heterotrophic cultures of microalgae owing to the accumulation of triglycerides, some recent studies indicated that fatty acid unsaturation might increase or decrease under heterotrophic conditions, depending on the algal strains employed (28,29). Studies are going on in this laboratory to further identify potential heterotrophic EPA- and DHA-producing microalgae and to develop a heterotrophic high cell density process for the production of n-3 polyunsaturated fatty acids.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of Hong Kong Research Grants Council, the Industry Department of the Hong Kong Government, and the University of Hong Kong Committee on Research and Conference Grants for this work. The authors also thank Professor Z. Cohen, Dr. L. Ramsden, and Dr. M.K. Pasha for their helpful discussions on gas chromatographic analysis.

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[Received April 28, 1977; accepted September 1, 1997]