Production Potential of Eicosapentaenoic Acid by *Monodus subterraneus*

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Interest in the polyunsaturated fatty acid eicosapentaenoic acid (EPA) as a therapeutic agent is steadily increasing. The microalga *Monodus subterraneus* produces EPA, which is concentrated mainly in the galactolipid fraction, as its major fatty acid. Nitrogen starvation increased the fatty acid content but reduced the proportion and content of EPA to 19.5% (of fatty acids) and 1.8% (of dry weight), respectively. Cultivation under low light intensity or high biomass concentration enhanced the proportion of EPA up to 36.7% of fatty acids and the content to 4.4% of dry weight. Maximal EPA productivity of 25.7 mg · L^{-1} · d^{-1} was obtained at the biomass concentration that resulted in the highest biomass productivity. *M. subterraneus* is thus one of the most promising candidates for phototrophic production of EPA.

KEY WORDS: Algal oil, eicosapentaenoic acid, *Monodus subterraneus*, PUFA production.

Recent studies have demonstrated the therapeutic potential of ω3 polyunsaturated fatty acids (PUFA) in the treatment of various diseases and disorders, including cardiovascular problems, a variety of cancers and inflammatory diseases (1). Some of the effects of ω3 fatty acids may be explained in terms of their ability to alter the balance of prostaglandin and leukotriene eicosanoids, which mediate inflammatory and immune responses (2).

At present, the sole commercial source of eicosapentaenoic acid (EPA) is marine fish oil. However, satisfactory utilization of this source is hampered by several drawbacks, such as variations in oil quality and the presence of fatty acids with antagonistic properties such as arachidonic acid. The exact composition and content of the ω3 fatty acids in fish oils depend upon the season and the geographic location of harvest sites, as well as on the species of fish and the availability and types of its primary food chain, namely marine microorganisms (3). Furthermore, it has been predicted that when ω3 PUFA come to be widely used as a prophylactic drug, the total annual production of marine fish oil would be insufficient to meet the worldwide demand (4).

Several alternate sources of EPA have been intensively studied, primarily fungi (5) and heterotrophic (6) and phototrophic (7) microalgae. These sources could provide a more concentrated source of EPA whose quality could be controlled and, thus could be fully utilized for production of pharmaceutical-grade EPA. Seto *et al.* (8,9) reported the presence of EPA in *Chlorella minutissima* (later identified as the eustigmatophyte *Nannochloropsis oculata*). The red alga *Porphyridium cruentum* is unique in that maximal EPA content of this alga is coincidental with optimal growth rate (10,11). Recently, Yongmanitchai and Ward (12) reported the optimization of EPA production in the diatom *Phaeodactylum tricornutum*. Nichols and Appleby (13) have shown that the eustigmatophyte *Monodus subterraneus* contains EPA, which is concentrated mainly in monogalactosyl diacylglycerol (MGDG), constituting 62% of its fatty acids. In a preliminary publication, Iwamoto and Sato (14) reported the effect of environmental conditions on lipid content and EPA production in this alga. In this work we describe the optimization of EPA content in *M. subterraneus* by manipulation of environmental conditions. The potential for EPA production of this alga was evaluated in comparison with that of other EPA-producing algae.

MATERIALS AND METHODS

Growth of cells. Monodus subterraneus UTEX 151 was obtained from the University of Texas Culture Collection (Austin, TX) and cultivated on BG-11 medium as described by Iwamoto and Sato (14). *Monodus subterraneus* cultures were grown in Erlenmeyer flasks, placed in a New Brunswick (Edison, NJ) incubator shaker (model G25) and illuminated from above at a light intensity of 115 μE · m^{-2} · s^{-1} under an air/CO_2 (99:1) atmosphere at 25°C (unless otherwise stated). Cultures were grown exponentially (with proper dilution) under the appropriate conditions for at least four days prior to the onset of the experiment. The specific growth rate was estimated by measurement of the chlorophyll concentration (15).

Lipid fractionation. Freeze-dried samples of biomass were extracted by sand grinding with chloroform/methanol/water (2:1:0.8) according to Bligh and Dyer (16). Separation into neutral lipid, galactolipid and phospholipid fractions was performed with a silica gel cartridge (Sep-Pak; Waters Division of Millipore, Milford, MA); and the individual fractions were eluted successively with chloroform, acetone and methanol, respectively.

Fatty acid analysis. Freeze-dried cells or lipid extracts were transmethylated with methanol–acetyl chloride, as previously described (17). Heptadecanoic acid was added as an internal standard. Gas-chromatographic analysis was performed on a Supelcowax (Supelco, Bellefonte, PA) 10 fused-silica capillary column (30 m × 0.32 mm) at 195°C (injector and flame-ionization detector temperatures, 230°C, split ratio, 1:100). Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Co., St. Louis, MO) and by calculation of the equivalent chainlength (18). Fatty acid contents were determined by comparing each peak area with that of the internal standard. The data shown represent mean values with a range of less than 5% for major (over 10% of total fatty acids) peaks and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

RESULTS

Fatty acid composition. The fatty acid analysis of *M. subterraneus* revealed that, in keeping with previous reports (13,14), EPA was generally the major fatty acid. Depending on the culture conditions, its share of total fatty acids ranged from 20 to 37%. Other major fatty acids were (in decreasing order) 16:1ω7, 16:0 and 18:1ω9. Fractionation of the lipids on silica gel cartridges into neutral lipids, galactolipids and phospholipids demonstrated that EPA was located primarily in the galactolipid fraction,

TABLE 1

Fatty Acid Composition of the Lipid Fractions of *Monodus subterraneus*

Fraction	Fatty acid composition (% of total fatty acids)													
	14:0	16:0	16:1 ω11[a]	16:1 ω7[a]	16:1 ω5[a]	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	EPA[b]
Neutral lipids[c]	4.4	27.2	0.9	32.6	0.4	1.3	15.9	1.1	0.9	0.8	0.4	1.2	2.0	8.8
Galactolipids[d]	0.7	19.0	—	23.9	0.2	0.5	2.3	0.5	0.4	0.3	0.3	0.2	1.5	49.3
Phospholipids[e]	0.4	24.3	—	17.8	1.7	1.2	4.4	1.4	2.6	3.2	0.3	0.6	8.5	31.9

[a]Tentative assignment.
[b]EPA, eicosapentaenoic acid.
[c]Fraction eluted with chloroform.
[d]Fraction eluted with acetone.
[e]Fraction eluted with methanol.

yet it also constituted a significant proportion of the phospholipids and, to a smaller extent, of the neutral lipids (Table 1). The other three major fatty acids contributed significantly to all the lipid groups but were concentrated mainly in the neutral lipids. This finding was especially true for 18:1, whose concentration in the neutral lipids (15.9%) was four to five times higher than that in the other fractions (2–4%). As was the case for other algae (7), most of the fatty acids of the ω6 family (18:2ω6, 18:3ω6 and 20:4ω6) were concentrated in the phospholipid fraction.

Effect of light intensity. When cultures of *M. subterraneus* were batch-cultivated at different light intensities, the fatty acid content increased with decreasing light intensity (Table 2). At a light intensity of 170 $\mu E \cdot m^{-2} \cdot s^{-1}$, the proportion (% of fatty acids) of EPA reached 29.6%, and the fatty acid content (% of dry weight) was 10.4%. Reducing the light intensity to 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ brought about an increase in the two parameters to 35.7 and 12.2%, respectively. As a result, the EPA content increased from 3.1 to 4.4%.

When semicontinuous (daily dilution) and batch cultures were compared (Table 3), the proportion of EPA and the content of fatty acids in the batch culture were higher (36.7 vs. 26% and 12.1 vs. 10.7%, respectively). As a result, the overall amount of EPA was higher in the batch mode (4.4 vs. 2.8%). No significant changes were noted thereafter, even though cell concentration was allowed to reach 65 mg chl $\cdot L^{-1}$ (data not shown).

To assess the EPA productivity of *M. subterraneus*, the biomass production and EPA content of batch cultures were determined, and the EPA productivities were calculated (Fig. 1). Cultivation of *M. subterraneus* at low biomass concentration (200–300 mg $\cdot L^{-1}$) and maximal growth rate yielded a relatively low EPA content of 2.5%. Furthermore, because the biomass productivity was also low (less than 100 mg $\cdot L^{-1} \cdot d^{-1}$), EPA productivity was only 2–3 mg $\cdot L^{-1} \cdot d^{-1}$. At a high biomass concentration

TABLE 2

Effect of Light Intensity on Fatty Acid Composition of *Monodus subterraneus*[a]

Light intensity ($\mu E\ m^{-2}\ s^{-1}$)	Fatty acid composition (% of total fatty acids)														FA content (% of dry wt)	
	14:0	16:0	16:1 ω11[b]	16:1 ω7[b]	16:1 ω5[b]	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	EPA	TFA	EPA
170	2.5	20.2	3.8	23.1	1.0	0.6	8.1	0.7	2.3	0.8	0.3	0.5	5.3	29.6	10.4	3.09
90	2.6	19.0	6.4	21.8	1.3	0.6	3.3	0.6	1.9	1.0	0.5	0.4	4.2	35.7	12.2	4.36

[a]Cultures were batch-cultivated and harvested at the late exponential phase. TFA, total fatty acids; EPA, eicosapentaenoic acids; FA, fatty acids.
[b]Tentative assignment.

TABLE 3

Effect of the Mode of Cultivation on Fatty Acid Composition and Content of *Monodus subterraneus*[a]

Cultivation method	Fatty acid composition (% of total fatty acids)														FA content (% of dry wt)	
	14:0	16:0	16:1 ω11[b]	16:1 ω7[b]	16:1 ω5[b]	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	EPA	TFA	EPA
Semicontinuous	2.7	22.7	2.7	24.4	1.0	0.8	9.3	1.0	1.7	1.1	0.4	0.5	4.6	26.0	10.7	2.77
Batch	2.3	19.9	6.0	19.2	1.0	0.5	3.3	0.8	2.4	1.2	0.5	0.3	4.0	36.7	12.1	4.42

[a]Cultures were cultivated at a light intensity of 170 $\mu E\ m^{-2}\ s^{-1}$ for three days in batch or by daily dilution to the initial biomass concentration. Abbreviations as in Table 2.
[b]Tentative assignment.

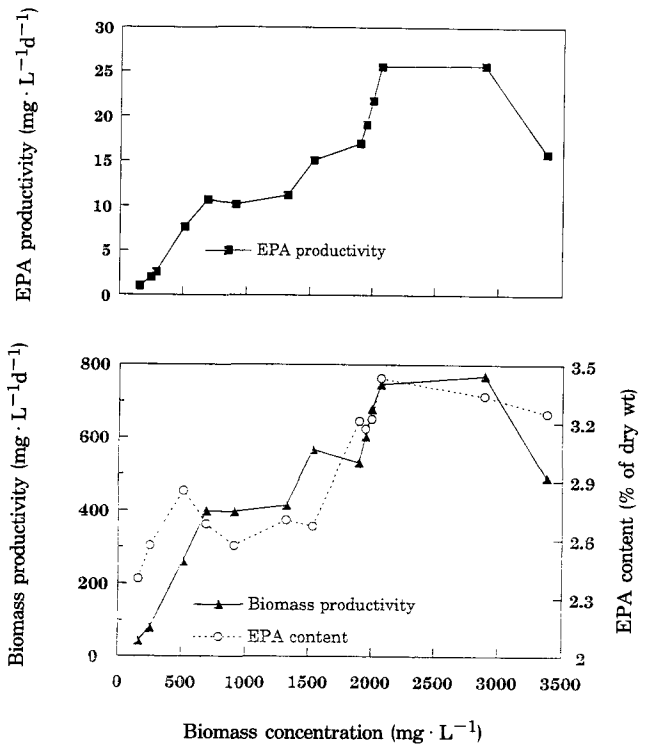

FIG. 1. Changes in biomass productivity, eicosapentaenoic acid (EPA) content and EPA productivity during growth of *Monodus subterraneus* in batch mode. Data were accumulated from several experiments.

of over 2000 mg · L^{-1}, the growth rate decreased but the biomass productivity increased up to 700 mg · L^{-1} · d^{-1}. Simultaneously, the EPA content increased to 3.5%, resulting in an EPA productivity of 25.7 mg · L^{-1} · d^{-1}.

Nutritional factors. Nitrogen starvation has been reported to enhance the fatty acid content in many species of algae (19). Generally, however, it is accompanied by a sharp decrease in the proportion of PUFA (7). A Japanese patent has claimed that when *M. subterraneus* was cultivated under a high CO_2 concentration (5% in air) for eight days in complete medium, and then harvested and resuspended in nitrogen-free medium for another 12 d, the resulting biomass contained 36% fatty acids with an EPA proportion of 31%, amounting to an EPA content of 11% (20). I thus attempted to study each one of these effects separately. Increasing the CO_2 concentration did not affect the fatty acid content but reduced the proportion of EPA to a mere 19.5%. As a result, the EPA content was reduced from 2.4 to 1.8% (Table 4). Nitrogen starvation was effected by cultivation in complete medium during the exponential phase, centrifugation and resuspension in nitrogen-free medium and further cultivation for another 12 d. Although the fatty acid content increased to a record high value of 16.6%, the proportion of EPA plummeted to just 11.2%. Consequently, the EPA content was reduced to 1.9% (Table 5).

DISCUSSION

EPA is the major fatty acid in the eustigmatophyte *M. subterraneus*. As is the case for most other EPA-containing microalgae, EPA is concentrated mainly in the galactolipids. Reverse-phase high-performance liquid chromatography analysis of MGDG, the major galactolipid, showed the major molecular species to be 20:5/20:5, 20:5/16:1 and 20:5/16:0 (data not shown). Increases in EPA were accompanied by decreases in the proportions of 16:1ω7, 16:0 and 18:1ω9 fatty acids. As these are the predominant components of neutral lipids, this shift is indicative of a change in the ratio of galactolipids to neutral lipids.

TABLE 4

Effect of CO_2 on the Fatty Acid Composition of *Monodus subterraneus*[a]

	Fatty acid composition (% of total fatty acids)														FA content (% of dry wt)	
CO_2 concentration (%)	14:0	16:0	16:1 ω11[b]	16:1 ω7[b]	16:1 ω5[b]	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	EPA	TFA	EPA
1	3.5	23.1	2.1	26.7	1.2	0.7	7.8	0.9	1.6	0.9	0.3	0.8	4.3	25.7	9.49	2.43
5	3.7	26.7	0.7	28.0	0.9	0.6	9.1	1.4	3.6	0.4	0.2	0.3	3.9	19.5	9.43	1.84

[a]Cultures were cultivated at 25°C at a light intensity of 170 μE m^{-2} s^{-1}. Abbreviations as in Table 2.
[b]Tentative assignment.

TABLE 5

Effect of Nitrogen Starvation on the Fatty Acid Composition and Content of *Monodus subterraneus*[a]

	Fatty acid composition (% of total fatty acids)										Fatty acid content (% of dry wt)	
Culture	14:0	16:0	16:1[b]	18:0	18:1[b]	18:2 ω6	18:3[b]	20:3 ω6	20:4 ω6	EPA	TFA	EPA
Control	3.9	25.9	28.2	0.8	6.9	3.2	0.6	0.2	4.6	25.5	13.3	3.38
N-starved	3.6	28.5	30.8	2.0	13.8	3.1	0.7	0.2	5.8	11.2	16.6	1.86

[a]Cultures were grown in columns at 27°C. Exponential cultures were harvested, centrifuged and resuspended in N-free medium. Cultivation was continued for an additional 12 d. Abbreviations as in Table 2.
[b]Total isomers.

TABLE 6

Comparison of Eicosapentaenoic Acid (EPA) Content and Production Rate in Various Species of Microalgae

Algal species	Cultivation conditions	EPA content (% of dw)	EPA productivity (mg • L^{-1} • d^{-1})	Reference
Nannochloropsis oculata	Batch, low light	—[a]	3.7	(21)
N. oculata	Batch, high light	—[a]	5.5	(21)
Phaeodactylum tricornutom	Batch, light-dark cycle	3.3	19	(12)
Monodus subterraneus	SC[b], high, continuous light, high cell concentration	3.4	25.7	This work

[a]Data not available.
[b]SC, semicontinuous cultivation (daily dilution).

Iwamoto and Sato (14) showed that the EPA content of *M. subterraneus* was inversely related to temperature and that the highest content was obtained at 20°C. They found, however, that EPA productivity was maximal at 25°C, because the growth rate was optimal at this temperature. Preliminary experiments (data not shown) confirmed these findings, and thus most of the experiments were conducted at that temperature.

Cultivation conditions had a profound effect on both composition and content of the fatty acids in *M. subterraneus*. The fatty acid composition was influenced by the amount of light available to the cells. Under conditions conducive to a high growth rate, the fatty acid content was low, as was the proportion of EPA. Therefore, EPA content in *M. subterraneus* may be increased by cultivation at low light intensity or high biomass concentration. However, under these conditions the growth rate is reduced, and therefore the biomass and, consequently, the EPA productivities are also reduced. This finding is in keeping with previous reports concerning *N. oculata* (8,21) and *M. subterraneus* (14). The red microalga *P. cruentum* is, however, exceptional in that the proportion of EPA is directly related to the growth rate (10).

The highest EPA content was obtained toward the end of the exponential phase of growth (Fig. 1)—although growth rate is already reduced at this stage, biomass productivity is at its highest and EPA content is close to its maximum. Thus, high EPA productivities could be maintained by semicontinuous cultivation, keeping the highest cell concentration that will still sustain the highest biomass productivity.

Yongmanitchai and Ward (12) evaluated several algal species with respect to their capacity to produce EPA under a particular set of conditions, namely, batch cultivation at 20°C. They concluded that *P. tricornutum* UTEX 640 was the most promising EPA producer. However, light and temperature optima for growth rate and EPA production may vary significantly from one alga to another. Thus, a more valid evaluation may be obtained by comparing individually optimized EPA productivities of the respective algal species (Table 6).

EPA productivity (P_{EPA}), as expressed in Equation 1, is a function of four parameters—the EPA proportion of fatty acids ($\%_{EPA}$); the fatty acid content (C_{FA}); the specific growth rate (μ); and the biomass concentration (X) at harvest. The product of $\%_{EPA}$ and the C_{FA} gives the EPA content (C_{EPA}), and the product $\mu \cdot X$ gives the biomass productivity (P_B) (Equations 2 and 3, respectively):

$$P_{EPA} = C_{EPA} \cdot P_B \quad [1]$$

where

$$C_{EPA} = \%_{EPA} \cdot C_{FA} \quad [2]$$

and

$$P_B = \mu \cdot X \quad [3]$$

Nannochloropsis oculata (21) had the highest proportion of EPA, which reached 44% at 20°C. However, the fatty acid content and the growth rate were depressed, because at this temperature both C_{EPA} and P_B were low, and EPA productivity was only 3.7 mg • L^{-1} • d^{-1}. At 25°C the EPA proportion was lower and the growth rate was much higher. Consequently, EPA productivity increased to 5.5 mg • L^{-1} • d^{-1}. Similar findings were obtained for *P. tricornutum* (12). The EPA content decreased from 5% at 20°C to 3.3% at 25°C, but the growth rate increased, enhancing EPA productivity to 19 mg • L^{-1} • d^{-1}. *Porphyridium cruentum* is unique in that its EPA proportion was maximal under conditions resulting in the highest growth rate (10). However, maximal biomass productivity was obtained at a biomass concentration that was higher than that responsible for the highest growth rate. Increasing the biomass concentration decreased the EPA proportion and content, counterbalancing the increase in biomass productivity. In *M. subterraneus*, the EPA proportion as well as the fatty acid content were high, if not maximal, at a relatively high biomass concentration. Consequent to the high biomass productivity and high EPA content obtained, the EPA productivity was as high as 25.7 mg • L^{-1} • d^{-1}. This value was not optimized, and it is likely that even higher EPA productivities could be obtained.

Outdoor studies are needed to identify the most promising EPA producer. Outdoor conditions are significantly different from laboratory conditions with respect to temperature, light intensity and light/dark regimes. For example, Veloso *et al.* (22) recently reported an outdoor EPA productivity of 0.15 g • m^{-2} • d^{-1} (corresponding to as little as 1.5 mg • L^{-1} • d^{-1}) in *P. tricornutum*. The cost of production of biomass and the cost of EPA obtained will determine the economic potential of these algae because EPA productivity is but one of the criteria required for this evaluation.

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