The Production Potential of Eicosapentaenoic and Arachidonic Acids by the Red Alga *Porphyridium cruentum*

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The red microalga *Porphyridium cruentum* is a new source for eicosapentanoic acid (EPA) and arachidonic acid (AA) fatty acids of potential pharmaceutical value. The conditions leading to a high content of either fatty acid were investigated. The highest EPA content was obtained under conditions resulting in high growth rate (2.4% of ash free dry weight in Strain 1380-1d). High AA content was obtained under slow growth conditions and was maximal in the stationary phase or under nitrogen starvation (2.9%). Strain 1380-1a had the highest content (1.9%) of arachidonic acid under exponential growth conditions. By imposing nitrogen starvation, it was possible to obtain a lipid mixture which may be separated into AA and EPA rich fractions.

KEY WORDS: Arachidonic acid, eicosapentaenoic acid, fatty acids, lipids, microalga, *Porphyridium*, PUFA.

Eicosapentaenoic acid (EPA, 20:5ω3) and arachidonic acid (AA, 20:4ω6) are rare fatty acids of potential pharmaceutical value. EPA was shown to be effective in preventing blood platelet aggregation (1) and to be useful for blood cholesterol reduction, thus reducing the risks of atherosclerosis (2). AA serves as a starting material for a biosynthetic production of prostaglandin PGE_2 (3). Also, being a component of human mother milk, it is of potential value as an ingredient in various formulations of artificial baby food.

EPA is found in many marine fish oils, but the low EPA content, variability in quality, a well as the presence of other fatty acids of less desired properties have initiated several studies aimed at the production of EPA from microalgae. The marine alga *Chlorella minutissima* (4) and the freshwater alga *Monodus subterraneus* (5) were suggested as potential sources for EPA. AA was shown (6) to be present in *Euglena gracilis*, *Ochromonas danica* and in the red microalga *Porphyridium cruentum*. The latter also was found to contain EPA. *P. cruentum* was also studied as a source for sulfated polysaccharides (7,8) which could be utilized for enhanced oil recovery from oil wells. It also contains phycoerythrine—a red protein pigment used for the immunoflurescent detection of tumors (9).

Cohen *et al.* (10), as well as Lee *et al.* (11) have shown that the fatty acid composition of *P. cruentum* is highly dependent on environmental conditions. Contray to previous reports (12), it was found that in cultures cultivated at optimal temperature and under non-limiting light conditions, EPA was the main polyunsaturated fatty acid (PUFA). When growth was slowed by decreased light intensity, increased cell concentration, non-optimal temperatures, non-optimal pH or salinity, the content of EPA decreased. Simultaneously, the level of AA increased and became the major PUFA. The feasibility of outdoor mass production of *P. cruentum* has already been studied (13,14). Recently, we have shown that environmental conditions could be manipulated outdoors so as to produce high levels of either EPA of AA from *Porphyridium* (15).

The occurrence of relatively high levels of AA in *P. cruentum* is disadvantageous because the properties of AA are antagonistic to that of EPA with respect to lowering blood cholesterol levels (2). In order to become a useful source, growth conditions and strains of the algae producing higher concentrations of EPA of AA must be strictly determined. In this work, we studied various strains of *P. cruentum* as potential sources of EPA and AA. Also, environmental and nutritional conditions which may yield maximum productivity of each of these fatty acids were elucidated.

MATERIALS AND METHODS

Growth of cells, *P. cruentum* strains 1380 1a-f and B113.80 were obtained from the Göttingen Algal Culture collection. Maintenance of stock cultures and inocula preparation were performed according to Vonshak (16). Cultures were grown on Jones' medium (17) in glass tubes incubated in a temperature-regulated water bath, illuminated with four cool-white fluorescent lamps providing 170 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the side of the waterbath. The cultures were mixed by bubbling an air/CO_2 mixture (99:1) through a sintered glass tube placed at the bottom of each culture tube. For the nitrogen starvation experiments, cultures in the exponential phase of growth maintained at 28°C were centrifuged, washed and resuspended in a nitrogen-free medium. The cultures were kept for an additional three days under the same light and temperature conditions. Cultures were grown to the exponential phase and maintained at steady state by daily dilution for at least three days prior to the onset of the experiment. Growth rate (μ) was estimated by measuring chlorophyll and turbidity.

Lipid separation. Freeze dried samples of biomass were extracted with chloroform/methanol according to Bligh and Dyer (18). Lipids were separated by TLC on 20 × 20 cm glass plates coated with silica gel-60 (Merck, Darmstadt, Germany). Chromatography was carried out in light-protected jars under an Ar atmosphere. Lipids were eluted with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1) as the developing solvent (19). The components were visualized by a brief exposure to I_2 vapors. The lipid-containing bands were scraped off and immediately treated with a methanol/acetyl chloride mixture as described by Cohen *et al.* (20). For analytical purposes, the bands were extrated with chloroform/methanol (9:1), and the identity and purity of the lipids were determined by comparison with standard lipids in three different solvent systems and by characteristic color reactions, i.e., α-naphtol for glycolipids and molybdate for phospholipids (19). The neutral lipids fraction

TABLE 1

Fatty Acid Composition of *P. cruentum* Strains Grown at 25°C

Strain	Growth phase	μ^a (d^{-1})	Fatty acid composition (% of total fatty acids)										Fatty acid content (% of AFDW)			
			16:0	16:1	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3	TFA[b]	AA	EPA	R[c]
1380-1a	E[d]	1.10	30.8	3.4	0.2	0.5	5.9	0.1	0.6	1.0	22.2	35.3	5.3	0.8	1.9	0.63
	S[e]		30.8	2.1	0.6	1.1	9.6	—	1.4	1.5	33.5	19.3	5.8	1.9	1.1	1.7
1380-1b	E	1.13	30.3	3.0	0.3	0.4	5.2	0.7	0.8	0.5	15.2	43.5	5.6	0.9	2.1	0.43
	S		32.4	2.3	0.5	1.2	7.6	0.3	0.8	2.0	29.2	23.2	5.4	1.6	1.2	1.3
1380-1c	E	1.13	32.0	3.2	0.4	0.4	5.7	0.7	0.5	0.5	16.9	39.5	5.4	0.9	2.1	0.43
	S		33.4	2.3	0.8	1.2	7.6	0.2	0.7	2.2	23.8	27.8	5.4	1.3	1.5	0.86
1380-1d	E	1.15	30.6	3.5	0.1	0.3	4.9	0.6	0.6	0.4	14.7	44.1	5.5	0.8	2.4	0.33
	S		32.1	2.1	0.5	1.6	7.9	0.2	1.0	2.5	24.7	27.4	5.1	1.2	1.4	0.90
1380-1e	E	1.39	29.3	2.9	0.1	0.3	6.9	1.2	0.7	0.7	18.4	39.3	5.3	1.0	2.1	0.47
	S		29.7	1.8	0.7	2.3	11.4	0.6	0.7	2.9	33.5	16.3	5.7	1.9	0.9	2.1
1380-1f	E	1.10	29.7	2.9	0.1	0.7	6.2	1.0	0.7	0.6	17.8	40.1	5.2	0.9	2.1	0.44
	S		31.6	2.0	0.5	1.4	9.2	0.4	0.7	2.3	33.4	18.4	5.1	1.7	0.9	1.8
113.80	E	0.98	28.6	2.9	0.2	0.2	6.4	0.6	0.5	0.6	17.9	41.6	4.4	0.8	1.9	0.43
	S		33.3	1.4	0.4	1.0	9.0	0.3	0.7	1.7	27.3	25.1	5.1	1.4	1.3	1.1

[a]Growth rate.
[b]Weight percent of AFDW.
[c]R = AA/EPA.
[d]E = exponential phase, 4–5 mg chl·l^{-1}.
[e]S = stationary phase, 27–30 mg chl·l^{-1}.

TABLE 2

Fatty Acid Compositions of *P. cruentum* Strains Grown at 30°C

Strain	Growth phase	μ^a (d^{-1})	Fatty acid composition (% of total fatty acids)										Fatty acid content (% of AFDW)			
			16:0	16:1	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3	TFA[b]	AA	EPA	R[c]
1380-1a	E[d]	0.67	34.1	1.1	0.9	1.6	11.5	0.5	0.8	2.4	40.3	6.6	4.8	2.0	0.3	6.1
	S[e]		32.9	0.6	0.5	2.7	15.1	0.4	1.1	1.4	35.0	9.5	6.2	2.2	0.6	3.7
1380-1b	E	0.79	36.1	1.5	0.6	0.7	5.6	—	0.8	0.9	39.3	14.2	3.8	1.5	0.5	2.8
	S		31.8	1.9	1.6	0.5	1.0	0.2	1.9	1.5	41.7	6.5	4.7	2.0	0.3	6.4
1380-1c	E	0.80	35.2	1.5	0.7	0.9	7.1	0.1	1.0	0.9	40.5	11.9	4.4	1.8	0.5	3.4
	S		34.5	1.6	0.5	1.0	6.6	0.1	1.1	1.2	37.1	15.9	5.4	2.0	0.8	2.3
1380-1d	E	0.70	33.0	1.8	0.6	0.8	8.8	0.3	1.4	1.2	32.8	19.0	5.4	1.8	1.0	1.7
	S		31.5	0.5	0.6	3.3	13.2	0.4	1.4	2.0	38.4	8.6	4.7	1.8	0.4	4.5
1380-1e	E	0.76	34.2	1.5	0.6	1.2	5.9	—	1.1	1.0	41.4	12.9	4.3	1.8	0.6	3.2
	S		33.4	0.5	0.6	3.9	15.3	0.8	0.6	3.1	37.7	4.0	6.5	2.5	0.3	9.4
1380-1f	E	0.49	35.6	1.4	0.5	0.8	5.7	0.1	0.8	0.9	41.4	12.4	4.5	1.9	0.6	3.3
	S		32.9	0.6	0.6	2.8	13.0	0.7	1.2	3.0	40.0	5.1	5.5	2.2	0.3	7.8
113.80	E	0.62	32.5	0.7	0.7	2.7	14.1	0.4	0.9	1.6	38.7	7.4	4.2	1.6	0.3	5.2
	S		30.9	0.5	1.5	7.7	18.4	0.3	1.6	3.3	32.3	2.8	7.7	2.5	0.2	11.5

[a]Growth rate.
[b]Weight percent of AFDW.
[c]R = AA/EPA.
[d]E = exponential phase, 4–5 mg chl·l^{-1}.
[e]S = stationary phase, 27–30 mg chl·l^{-1}.

consisted of all the components of Rf values higher then 0.9 and contained tri-, di- and monoglycerides, free fatty acids, and pigments.

Lipid transmethylation. Freeze-dried samples of *Porphyridium* (100 mg) were treated with 2 mL of methanol/acetyl chloride (95:5) as previously described (20). Heptadecanoic acid was added as an internal standard and the mixture was sealed in a light-protected Teflon-lined vial under an Ar atmosphere and heated to 80°C for 1 hr. The vial contents were then cooled, diluted with

1 mL water and extracted with 1 mL of hexane containing 0.01% buylated hydroxytoluene (BHT). The hexane layer was dried over Na_2SO_4, evaporated to dryness and redissolved in hexane.

Fatty acid analysis. Gas chromatographic analysis was performed with a SP-2230 fused silica capillary column (30 m × 0.2 mm) at 200°C (injector and flame ionizaiotn detector temperatures 230°C, split ration 1:100). The results were formulated with an HP 3390A integrator. Fatty acid methyl ester were identified by cochromatography with authentic standards (Sigma Chemical Co., St. Louis, MO) and by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed with a Finnigan 500 mass spectrometer (Finnigan Corp., Sunnyvale, CA) equipped with a carbowax capillary column (30 m × 0.25 mm). Chemical ionization spectra were obtained at 250 ev with isobutane as the reactant gas. The quantity of the fatty acids was determined by comparing their peak areas with that of the internal standard. The data shown are mean values of at least two independent samples, each analyzed in duplicate.

RESULTS

A comparison of the seven tested strains of *P. cruentum* showed that under optimal growth conditions at 25°C, the strains exhibited similar growth rates (ca. 1.1 d^{-1}). Under these conditions, EPA was the major fatty acid. All but one strain had more than 39% of their fatty acids as EPA and only 14.7-17.9% of AA. The highest proportion of EPA was found in strains 1380-1b and 1d, which contained 43.5% and 44.1% EPA, respectively. On an ash free dry weight basis (AFDW) the EPA content ranged between 1.9% and 2.4% (Table 1). In the exponential phase the range of R (AA/EPA) values was 0.33-0.63. However, R increased to 0.86-2.1 at the stationary phase as AA increased, and in most strains became the leading PUFA, while the level of EPA decreased drastically. At 30°C, when exponetially cultivated under the same light intensity and cell concentration, most strains had more than 39% of their fatty acids as AA and, except for one strain, only 6.6-14.2% as EPA (Table 2). In most cases the fatty acid content was the highest at the stationary phase at 30°C. In strain 113.80, the fatty acid content increased from 4.2% in the exponential phase to 7.7% (AFDW) in the stationary phase, resulting in an AA content of 2.5%. The R values at the stationary phase were also rather high, the maximum value observed being 11.5 in strain 113.80.

When exponentially cultivated (28°C) *P. cruentum* cultures were transferred to a nitrogen free medium, a gradual change in the fatty acid content and composition was observed (table 3). The fatty acid content increased to 8.8% (AFDW) after four days. The changes in fatty acid composition resembled those observed at the stationary phase, however, the effect was more pronounced. The content of AA increased to 2.9% and that of EPA decreased to 0.9%.

The major effect of the nitrogen starvation on the lipids of *P. cruetum* was an increase in neutral lipids from 20.6% in the control culture to 61.0% in the N-starved culture, with a concurrent decrease in the glycolipids. The percent of monogalactosyldiglyceride (MGDG) and digalactocyldiglyceride (DGDG) decreased from 36.5% (% of total lipids) and 17.7% respectively, to ca. 8.5% each, and that of sulfoquinovosyl diglyceride (SQ) from 12.4% to 4.7% The fatty acids 18:0, 18:2, 20:3 and AA increased while EPA decreased. An increase in AA was noted in the neutral

TABLE 3

Effect of Nitrogen Starvation on the Fatty Acid Composition in *P. cruentum*[a]

Fatty acid	Total lipids		Lipid fraction									
			NL[b]		MGDG[c]		DGDG[d]		SQ[e]		PC[f]	
	+N[g]	-N[h]	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N
16:0	34.8[i]	28.4	25.7	23.3	33.5	37.4	40.1	46.1	55.2	66.6	28.7	15.4
16:1	2.9	1.6	1.1	1.4	—	1.3	7.1	6.9	0.7	—	—	—
18:0	1.2	2.1	11.1	5.0	0.5	5.6	0.6	5.3	1.9	5.9	3.6	1.7
18:1	0.8	2.6	3.5	5.2	1.0	7.4	1.2	4.0	2.2	5.2	1.0	2.0
18:2	10.6	21.3	16.5	27.4	11.1	26.8	4.3	7.1	2.9	7.9	3.9	6.3
20:3	0.7	3.2	2.4	2.9	0.7	1.2	—	—	—	—	—	6.9
20:4	20.7	30.1	19.9	30.7	4.5	3.3	5.6	4.2	3.4	1.6	46.3	61.2
20:5	28.7	10.2	8.3	4.1	43.2	16.9	35.0	26.4	29.4	8.6	17.4	2.9
R[j]	0.7	3.0	2.4	7.6	0.1	0.2	0.2	0.2	0.1	0.2	2.7	21.4
% of total[k]			20.6	61.0	36.5	8.5	17.7	8.6	12.4	4.7	7.7	8.7

[a]Strain 113.80, cultivated at 28°C,170 $\mu E \cdot m \cdot s^{-1}$.
[b]Neutral lipids.
[c]Monogalactosyl-monoglyceride.
[d]Digalactosyldiglyceride.
[e]Sulfoquinovosyldiglyceride.
[f]Phosphatidylcholine.
[g]Nitrogen sufficient.
[h]Three days nitrogen starvation.
[i]Percent of total fatty acids.
[j]R = AA/EPA.
[k]Fraction % of total fatty acids.

lipids and mainly in phosphatidylcholine (from 46.3% to 61.0%), but not in MGDG and DGDG, where a small decrease was observed.

DISCUSSION

In order for *P. cruentum* to be used as a source for either fatty acid, it must produce them as free as possible from other similar C-20 PUFA. In this case, AA and EPA are the main two PUFAs in the organism. Although 18:2 is also present, its removal is relatively easy.

The fatty acid composition of *P. cruetum* was found to be closely related to its growth rate. Cultures cultivated under optimal conditions resulting in high growth rated were high in EPA but low in AA. The opposite was true when growth was slowed due to light limitation, non-optimal temperature or pH, increased salinity, nutrient starvation (N,C) and presence of inhibitors. Under these conditions, the level of the glycolipids (where most of the EPA was found) decreased, while neutral lipids increased and as a result the contents of AA and 18:2 increased (10).

EPA production. If one is to rate the strains of *P. cruentum* in terms of their potential to produce EPA, three factors should be primarily considered: the growth rate under optimal conditions, the EPA content (as % of AFDW) and the R values at the exponential and at the stationary phases. The growth rate and EPA content determine the EPA production rate. In a previous study, Cohen *et al.* (10) have shown that under optimal growth conditions, both the EPA content and the growth rate are maximal leading to the highest EPA production rate. The R value at the exponential phase reflects the extent of AA "contamination" and the degree of difficulty in EPA purification. The lower the R value, the easier the separation from AA. The differences in the corresponding R values for the exponential and stationary phases in the various strains could be used as indicators of the expected variability in EPA contents under outdoor conditions, as a result of light and cell concentration changes. On this basis, strain 1380-1d was judged to be the most promising from the standpoint of EPA production and relative purity. It had the highest content of EPA (2.4% of AFDW) and a low R value (0.33) at the exponential phase, which increased to only 0.90 in the stationary phase.

AA production. AA is produced in high quantities only when growth is slowed. Diminished growth could be achieved by light limitation (low light or high cell concentration), increased temperature, increased temperature, increased salinity or reduced pH (10). The intensities of these effects were found to maximal at the stationary phase. Cultivation under low pH or high salinity resulted in a sharp reduction of total fatty acid content. For large scale production of AA in outdoor ponds of *Porphyridium* cultivation at 30°C presents a favorable option, as a one stage exponential cultivation is possible. Under these conditions, strain 1380.1a had the highest AA content (2.0%). However, cultivation at this temperature in open outdoor ponds could be prohibitively expensive in the winter.

Nitrogen starvation was reported to bring about an increase accumulation of neutral lipids and saturated fatty acids (21,22) in many algae. In most studies, PUFA decreased under N-starvation (21,22). The sharp increase of AA in the lipids of *P. cruentum* under these conditions, especially in the neutral lipids, is thus notable. Under nitrogen starvation, the content of AA increases in neutral lipids and in phospholipids but not in the glycolipids. This is contrary to the situation found when growth is retarded by other means (i.e., light, temperature, pH) in which case the content of AA increases in all lipids (10). The practical outcome of this phenomenon is that while the increases in R values in the neutral lipids and in phosphatidylcholine result in relatively pure AA, the glycolipids remain low in AA and can be separated and used as a source of EPA of high purity. Indeed, by class separation of lipids, the combined fraction containing neutral and phospholipids was rich in AA (33.0%) and had a high R value (7.9). The other fraction contained mainly glycolipids and was rich in EPA and low in AA with a R value as low as 0.18 (23).

The cultivation of *P. cruentum* as a source for EPA or AA offers several advantages over other algal alternatives. The alga is cultivated on a marine medium which is especially advantageous where freshwater is scarce. Moreover, the cultivation on this high salinity medium may provide an ecological niche, thus aiding the maintenance of a monoalgal culture. Harvest is less problematico as the alga has a tendency for autoflocculation which could be enhanced by pH reduction (14). Another important factor in assessing the feasibility of large scale *Porphyridium* production concerns the possibilty to obtain several products from the biomass. The presence of sulfated polysaccharides and phycoerythrine should further encourage the outdoor cultivation of *Porphyridium.*

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