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Production of Eicosapentaenoic Acid (EPA) by Microalgae

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2. Table of Contents

Executive summary	p. 3
Research objectives	p. 4
Methods and results	p. 5
Impact, relevance and technology transfer	p. 17
Project activities/outputs	p. 17
Project productivity	p. 20
Future work	p. 21

3. Executive Summary

The purpose of this project was the development of the microalgae as a source of the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA). The project aimed primarily at:

1) Screening algal species containing high concentrations of EPA.
2) Determining the optimal conditions foe EPA production.
3) Optimizing cultivation methods for large-scale EPA production.
4) Developing methods for purification of EPA.
5) Operation of a mini pilot plant of EPA production.
6) Expending the know-how for cultivation of microalgae for fine chemical production in LDC.

Since the budget was cut by 20% after submission of the research proposal, we had to abandon the fifth target. We have succeeded in all other targets as detailed in the 13 scientific papers (enclosed) which were either published or submitted for publication.

The selection method developed in this project and the application of optimal cultivation conditions resulted in an enhancement of the EPA content in *P. cruentum* from 1 to 2.2% (of dry weight). Very recently we have isolated several clones with an EPA content of higher than 3%. Furthermore, since the maximal EPA content in this alga coincided with the highest biomass production rate the EPA productivity was as high as 25 mg L^{-1} d^{-1}. In *Monodus subterraneus* the EPA content was even higher reaching 5% but the EPA productivity was similar to that of *P. cruentum*. These figures are the highest EPA productivities reported for any micoralga.

Methods for outdoor cultivation and down-stream processing were developed. As a result we were able to cultivate these algae outdoors, extract the lipid fraction and purify EPA up to 90%.

The weaker side of the project was the collaboration with the co-investigator. In general, output as well as quality of work performed were not as high as could

have been expected. Their tardiness in reporting was a continuous subject of our correspondence. During the visit of the P.I. to the co-investigator's lab, only one technician was actually involved in the work. Furthermore, the co-investigator did not even bother to show up for the visit of one of the Israeli P.I.'s to his own lab. This attitude was discussed in several annual reports of this project.

It is the feeling of the P.I. that in similar projects in the future, a significant portion of the funds should be allocated for training and conducting research with personnel from the co-investigator's group in the P.I.'s lab, ensuring the establishment and continuity of basic scientific methodologies. Such training should be continued for several months of each project year.

4) Research Objectives

The following aims and objectives were detailed in the research proposal:

1) Screening of fast growing algal species which may be sustained in dense cultures throughout the year and which contain high concentrations of EPA.
2) Determining the optimal environmental and nutritional conditions for high net productivity and overall maximal EPA production by the selected species.
3) Optimizing cultivation methods applicable for large-scale production of microalgae containing EPA.
4) Developing simple, low-cost methods for the purification of EPA.
5) Operation of a mini-pilot plant for EPA production in Thailand.
6) Expending the know-how for cultivation of microalgae for fine chemical production in LDC.

Since the budget was cut by 20% after submission of the research proposal, we had to abandon the fifth target.

Our main efforts were concentrated in developing methods for production of EPA from microalgae. Our basic hypothesis was that if we find an inhibitor to the

biosynthesis of EPA which will also inhibit growth, then perhaps by selecting mutants resistant to this inhibitor, we may find some which overcome the inhibition by overproducing EPA. This can be proved by a consistent EPA overproduction by the mutant when cultivated in inhibitor-free medium.

We have indeed isolated such mutants. Following the success of this approach we have managed to isolate similar mutants of the alga *Spirulina* which overproduces eicosapentaenoic acid (GLA). An independent follow-up research is currently conducted in China with local funding.

5) Methods and Results

The results obtained in this project are detailed in 13 papers which were published or submitted to various refereed scientific journals. The following is a synopsis of our main findings. Since there was a total lack of communication with the Thai co-investigator, the P.I. can not take any responsibility for the data produced in that lab and the final report of that group is thus presented separately.

INTRODUCTION

Some polyunsaturated fatty acids (PUFA) were recently shown to have therapeutic effects. Eicosapentaenoic acid (20:5ω6, EPA) was claimed to be effective in preventing blood platelet aggregation (1) and to be useful for blood cholesterol reduction, thus reducing the risks of atherosclerosis (2). Arachidonic acid (20:4ω6, AA), being a component of human mother milk, is of potential value as an ingredient in various formulations of artificial baby food.

EPA is found in oils of many marine fish. However, the low EPA content, variability in quality, as well as the presence of other fatty acids of antagonistic properties have initiated several studies aimed at the production of EPA from other sources such as microalgae, marine bacteria (3), and fungi (4).

Pharmaceutical applications will probably require preparations of considerably higher concentrations of the respective PUFA. There are several

physiological means for increasing oil content in algae, i.e., nitrogen or silicon starvation, and holding cells in the stationary phase of growth for extended periods of time. However, their effectiveness with regard to production rate is limited due to the decrease in growth rate and in many examples also in the PUFA proportion of fatty acids (5).

We have concentrated our research on the marine microalga *Porphyridium cruentum* as a source for both EPA and AA, taking advantage of the desert environment where solar irradiation is abundant throughout the year and brackish water are plentiful. Moreover, the cultivation on high salinity medium may provide an ecological niche, thus aiding the maintenance of a monoalgal culture. Harvest of the algae is less problematic as it has a tendency for autofloculation especially at low pH (6). In considering the large scale production of *Porphyridium* attention is pointed out to the possibility of obtaining other products from the biomass. The presence of sulfated polysaccharides which can be used for enhanced oil recovery from oil wells and of phycoerythrine, a red proteinaceous pigment, should further encourage the outdoor cultivation of *Porphyridium*.

In this report, we describe various strategies for increasing the PUFA content in *P. cruentum* and *Monodus subterraneus* by physiological and genetic means as well as chemical methods for the separation and purification of the various valuable chemicals obtained from these organisms. For the first time, selection of EPA overproducing lines of *P. cruentum* is described.

MATERIALS AND METHODS

Organisms and culture conditions. *P. cruentum* was obtained from the Gottingen Culture Collection and was cultivated on Jones' medium at 25 °C as previously described (6). Stock cultures were maintained and inocula performed according to Vonshak (7). *Monodus subterraneus* UTEX 151 was obtained from the University of Texas Culture Collection and cultivated on BG-11 medium as described by Iwamoto (8). Cultures were grown in Erlenmeyer flasks, placed in a

New Brunswick incubator shaker (model G25) and illuminated from above at a light intensity of 115 mE· m^{-2} ·s^{-1}, under air-CO_2 (99:1) atmosphere at 25 ^{o}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

Outdoor Cultures . Cultures of *P. cruentum* strain 113.80 were cultivated in a 1 m^2 pond containing 120 l of medium stirred by a paddle wheel. During the winter, the ponds were covered with polyethylene to increase the ambient temperature. Daily measurements of temperature, turbidity, oxygen, pH and chlorophyll were performed as previously described (6). The pH of the cultures was maintained at a range of 7.2-7.8 by a stream of CO_2 gas. The nutrient concentration in the outdoor culture was estimated biweekly by analysis of PO_4^{-3} and NO_3^-. Water loss by evaporation was replenished by daily addition of tap water.

Selection of SAN 9785 resistant lines. Solutions of SAN 9785 in DMSO were added to exponentially growing cultures. *P. cruentum* cultures were treated with an initial inhibitor concentration of 0.08 mM which was gradually increased over a 9 month period to 0.16 and 0.32 mM, respectively, finally reaching 0.4 mM. Cell lines of *P. cruentum* resistant to 0.4 mM of the herbicide were obtained by screening of the resistant suspension on agar plates containing 0.4 mM of the inhibitor.

Fatty Acid Analysis. Freeze-dried cells or lipid extracts were trans-methylated with methanol-acetyl chloride, as previously described (9). Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed on a Supelcowax 10 fused silica capillary column (30 m x 0.32 mm) at 195 ^{o}C (FID, injector and flame ionization detector temperatures 230 ^{o}C, split ratio 1:100). Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Co.) and by calculation of the equivalent chain length. Fatty acid contents were determined by comparing each peak areas with that of the internal standard. The data shown represent mean values with a range of less then 5% for major (over

10% of fatty acids) peaks and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

Lipid fractionation. Lipid fractionation and purification were performed as previously reported (10).

RESULTS AND DISCUSSION

Porphyridium cruentum

Under conditions, which provide the fastest exponential growth of *P. cruentum*, the dominant PUFA is EPA (6). In contrast, under growth-limiting conditions such as light limitation (high cell concentration or low light intensity), non-optimal temperature and pH, and increased salinity), AA and 18:2 become dominant, and the magnitude of the effect is maximal at the stationary phase. Thus, under high light intensity, the EPA and AA proportions of an exponential culture reached 36 and 16%, respectively. At the stationary phase, EPA was reduced to 25 while AA increased to 27% (Table 1). The total fatty acid content (% of ash-free dry weight) also increased with decreasing EPA, reaching maximal values at the stationary phase (Table 1). This finding may explain some of the earlier studies reporting high levels of AA and little, if any, EPA in *Porphyridium* (11, 12-14).

Lipid class fractionation showed that AA is concentrated in the NL and PC, while EPA is mainly found in the GL (Table 2). Under culture conditions conducive to fast growth rates, (25 ^{o}C, low cell concentration), the content of neutral lipids was minimal and the major lipids were MGDG and DGDG. Unlike most other EPA producing algae, the proportion of EPA in neutral lipids under optimal conditions was relatively high, reaching 22% (6). Under these conditions, EPA became the dominant PUFA in every lipid fraction analyzed, except for PC. It represented 49% of the fatty acids in MGDG. In suboptimal growth conditions of light and temperature, EPA content decreased sharply while the content of fatty acid of the ω6 family (18:2, 18:3, 20:3 and AA) increased.

The elevation of EPA in *P. cruentum* in response to increased light intensity is analogous to that previously mentioned for 18:3ω3 in plants and algae pointing to its possible similar role in cellular functions (15, 16). Indeed, Kates and Volcani (17) suggested that the EPA in diatoms fulfills a role similar to that of 18:3ω3 in other plants and algae. When growth is retarded, less EPA and GLs are produced. It appears however, that this effect relates to the growth rate in *P. cruentum* rather than directly to photosynthetic activity. That is, it occurs in response to any factor limiting growth such as light, non-optimal pH and salinity (Fig. 1).

The temperature effect on the fatty acid composition of *P. cruentum* is similar to those of other factors affecting growth. The effect of cultivation at 30 °C on fatty acid composition and especially on the reduction of EPA percentage was, however, greater than expected on the basis of growth rate retardation alone. A shift from the optimum temperature of 25 °C to 20 °C or up to 30 °C, at low cell concentration, reduced the EPA proportion from 36 to 30 and 24%, respectively (Table 1) while the growth rate was reduced 46 and 20% respectively. The relatively greater decrease in EPA at 30 °C is apparently a combination of several factors: the reduction in growth rate; the increase in the saturation level at elevated temperatures; and the increase in the proportion of neutral lipids from 17 to 34% at the expense of the EPA-rich GLs which were reduced from 65 to 43%.

When exponentially growing cultures of *P. cruentum* (28 °C) were transferred to a nitrogen-free medium, they displayed a gradual increase in the total fatty acid content from 5.5% to 8.8% (% of ash-free dry weight) after four days, an increase of AA from 1.0% to 2.9% and a decrease in EPA from 1.9% to 0.9% (Table 3). The major effect of nitrogen starvation on the lipids of *P. cruentum* was manifested in the increase in neutral lipids from 21% (of total lipids) in the control culture to 61.0% in the N-starved culture, with a proportional decrease in the GL. Under nitrogen starvation, the content of AA increased in neutral lipids and in PLs, but not in the GL. This is contrary to the

increase of AA in all lipids when growth is retarded by other means such as light, temperature and pH limitations (6). Nitrogen starvation was reported to bring about an increased accumulation of neutral lipids and saturated fatty acids and generally a decrease in PUFAs in many algae (18, 19). Thus, the sharp increase of AA in the lipids of *P. cruentum* under these conditions, especially in the neutral lipids is notable.

Outdoors, the fatty acid composition is primarily dependent on the cell concentration and only to a lesser extent on temperature. This finding is of practical value since the cell concentration is more easily controlled than temperature. Thus, it seems feasible that either AA or EPA-rich biomass can be obtained by a close control of the cell concentration.

P. cruentum cultures were grown outdoors in Israel for over a year and the fluctuation of fatty acid composition and content were assessed. While the dependence of the fatty acid composition on cell concentration was similar to the pattern found indoors (20), that of the temperature was different. At suboptimal temperatures, EPA constituted higher proportions than at the optimal growth temperature (25 ^{o}C). The temperature regime outdoors, however, differed greatly from the constant temperature maintained in the laboratory. Also, the effects of other factors (e.g., day length and periodicity) are not clearly understood and some of these factors may create this seeming discrepancy by affecting the fatty acid composition.

Maintaining *Porphyridium* at low cell concentrations results in an EPA rich fatty acid mixture which is relatively poor in AA. In contrast, a higher content of AA is obtained when cell concentrations are kept high in the summer. The EPA proportion reached a maximum value of 50% (of fatty acids), corresponding to a content of 2.5% (of AFDW) in December, while the concentration of AA peaked in September, reaching 30% of fatty acids and 1.4% of AFDW. However, the EPA output rate was primarily dependent on the biomass

production rate and reached 0.28 $g \cdot m^{-2} \cdot d^{-1}$ in the summer and 0.12 $g \cdot m^{-2} \cdot d^{-1}$ in the winter (Table 4).

If one is to rate the strains of *P. cruentum* in terms of their potential to produce EPA, three factors should be primarily considered: 1) the growth rate under optimal conditions; 2) the EPA content (% of AFDW); and 3) the respective ratios of AA to EPA (R value) at the exponential and the stationary phases. The growth rate and EPA content determine the EPA production rate. The R value in 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 (9). It had the highest content of EPA (2.4% of AFDW) and a low R value (0.33) in the exponential phase, which increased to only 0.90 in the stationary phase (Table 5).

AA Production. AA is produced in high quantities at 30 °C by light limitation (low light or high cell concentration) or nitrogen starvation. The intensities of these effects were found to be maximal in the stationary phase. At this temperature, strain 1380-1a had the highest AA content in the exponential phase (2.0%), yet strains 1e and 113.80 reached an AA content of 2.5% in the stationary phase (Table 6).

Another approach for increasing the content of specific cell components is the use of inhibitors of specific steps in biosynthetic pathways. Generally, these compounds inhibit growth as well. It was shown in higher plants that among lines selected for resistance to the growth inhibition, some over producers of the metabolite in question could be found (21-23). Mutants of *Spirulina* showing elevated production of proline were obtained by selection in the presence of

proline analogs (24). Similar results were obtained in the cyanobacterium *Nostoc* (25) and the alga *Nannochloris bacilaris* (26).

Several herbicides of the substituted pyridazinone family were shown to inhibit fatty acid desaturation. Of these, SAN 9785 is the most effective inhibitor of ω3 desaturation (27) and its effect on reduction of 18:3ω3 levels in the GLs of higher plants and algae has been extensively studied. Although SAN 9785 has a certain inhibitory effect on photosynthesis, it was shown that the effect on fatty acid desaturation is a direct inhibition of the 18:2 desaturase enzyme (28).

Over 90% of the EPA in *P. cruentum* (10) is located in chloroplast GLs, which are considered as structural components of the photosynthetic apparatus (29). Thus, it was reasonable to assume that inhibitors having an effect on desaturation reactions involved in PUFA biosynthesis would also affect growth (i.e. by impairment of photosynthetic efficiency). Thus, SAN 9785 was expected to inhibit the desaturation of 18:2ω6 to 18:3ω3 which is a very likely precursor of EPA in *P. cruentum* (Fig. 2).

Indeed, the herbicide inhibited the growth of this alga. Maximal growth inhibition was observed at 0.8mM. Yet even at 0.4mM, growth was arrested after a few days and the culture collapsed thereafter (Fig. 3).

The inhibitory activity of SAN 9785 in *P. cruentum* resulted in a reduction in the level of EPA (Table 7). An increase in the precursor 18:2 or any of its products in other pathways could be predicted. Yet, except for small increases in 20:2 and 20:3 the only major increase was noted in 16:0. In the presence of 0.16 mM SAN 9785, the proportion of EPA declined from 37% (of total fatty acids) to 31% while that of AA was reduced only at higher inhibitor concentrations. The effect of SAN 9785 on the fatty acids increased with herbicide concentration and was maximal at the highest concentration studied, namely 0.4 mM.

We suggest that the growth inhibition may stem from the effect on the chloroplast membrane fatty acid composition; although we can not rule out the possibility that growth may be adversely affected by a reduction in carbon

assimilation resulting from the direct inhibition of photosynthesis by the herbicide.

Selection of Resistant Clones

We have further hypothesized that one means to achieve resistance to inhibitors of lipid biosynthesis would be to elicit fatty acid overproduction. Thus, obtaining resistance to the growth inhibition by such compounds could be the basis for selection of algal lines capable of EPA overproduction. The growth inhibition rendered by SAN 9785 and the specific inhibitory effects it exerted on the production of EPA, led us to utilize it for the selection of cell lines resistant to its growth inhibiting effect, some of which could be EPA over-producers in *Porphyridium*.

Resistance to SAN 9785 was built up by continuous cultivation in the presence of gradually increasing concentrations of the inhibitor. Sublines resistant to 0.4 mM SAN 9785 were obtained by stepwise increases in the herbicide concentration in the growth medium. Cultures resistant to 0.16 mM SAN had, at this concentration, a much higher growth rate than the wild type in the same conditions (Fig. 4) and one almost as fast as the wild type cultivated in inhibitor-free medium. At concentration of 0.4 mM, the growth of the resistant culture resembled that of the wild type in 0.16 mM SAN 9785 (Fig. 4), while the wild type collapsed after a few days. When transferred back to inhibitor-free medium, the growth rates of the resistant lines returned to control levels. The resistance was maintained even after 50 generations in an inhibitor-free medium, indicating an apparently adaptive change of a genetic nature.

In the resistant culture, the level of EPA in the presence of the inhibitor reached 30.7% (Table 8) while it was 24.8% in the wild type. Moreover, the level of 16:0 was even lower than that of the wild type under inhibitor-free conditions. A more focused view on the effect of SAN 9785 was obtained by comparing the fatty acid composition in the GL fraction: the inhibitor reduced the proportion of

EPA in the wild type from 50.0 to 29.1% (Table 9), but only to 45.4% in the resistant culture.

Resistance obtained could be the result of decreased uptake, reduced affinity or enhanced turnover of the inhibitor, but could also emerge as a result of genetic changes leading to an overproduction of the inhibited fatty acid. It appears that the latter was at least a contributing factor to the resistance since elevated EPA levels were also sustained for at least two weeks after resistant cultures were shifted to herbicide-free medium (40.4% compared to 36.8% in the wild type) (Fig. 5) and the increase in EPA was correlated with a decrease in 16:0. Following agar plating, six colonies were selected, three of which displayed even higher proportions of EPA and higher fatty acid contents, resulting in an overall 29% enhancement of the EPA content (Table 10). Those data support the proposed employment of inhibitors of fatty acid desaturation as means for obtaining fatty acid overproduction. To the best of our knowledge, this is the only report of a fatty acid over-production in either higher or lower plants induced by perturbation of fatty acid metabolism.

It is difficult at present to identify the mechanism(s) of resistance to the herbicide. Murphy et al. (27) have shown that the uptake of SAN 9785 varies in different plants. They have further shown that the herbicide was rapidly metabolized in pea but only gradually in cucumber and ryegrass (27). Other possibilities could be a modification of the target enzyme to reduce its affinity to the inhibitor or the increase in the level of the relevant metabolizing enzyme (30). The increase of EPA in *P. cruentum* may be attributed to one of the last two mechanisms.

Further exploitation of this approach may be hampered by the low specificity of the herbicide. Thus, more specific inhibitors such as transition stage analogs are being sought. We anticipate that further selection of EPA overproducing strains in conjunction with molecular biology techniques will

allow manipulation of the regulation of relevant genes, thus, making algal mass production of these fatty acids feasible.

The establishment of herbicide resistance would also be advantageous for large scale cultivation of *Porphyridium*. The herbicide may be utilized for the maintenance of monoalgal cultures - a major problem in continuous outdoor cultivation.

Our data indicate that the hypothesis suggesting the use of inhibitors of fatty acid desaturation as means for obtaining fatty acid over-production was apparently correct. Further exploitation of this approach could be hampered by the reduced specificity of the herbicide. Thus, more specific inhibitors such as transition stage analogs are being sought. We anticipate that further selection of GLA over-producing strains in conjunction with molecular biology methodology will allow manipulation of the regulation of relevant genes, thus, making algal mass production of these fatty acids feasible.

Monodus subterraneus

The microalga *Monodus subterraneus* produces EPA as its major fatty, which is concentrated mainly in the galactolipid fraction. 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 $mg \cdot L^{-1} \cdot d^{-1}$ was obtained at the biomass concentration resulting in the highest biomass productivity. *M. subterraneus* is thus one of the most promising candidates for phototrophic production of EPA.

Iwamoto and Sato (8) have studied the effect of environmental and nutritional conditions on EPA production in the alga *M. subterraneus* (Eustigmatophyceae), which belongs to an apparently very small group of freshwater species capable of EPA production. EPA is the only major PUFA in this

organism, other predominant fatty acids being 16:0 and 16:1 (Table 11). The highest growth rate (0.77 d^{-1}) in this alga was obtained at 25 °C. Thus, by computing the EPA levels, the total lipid content and the growth rate, it emerges that the highest EPA production rate would be obtained at 25 °C under relatively high light intensity.

EPA purification

At present, algal-derived EPA can not compete economically with fish oil as dietary supplement. However, once EPA can be introduced as a pharmaceutical commodity, the requirements for high content and lack of contaminants will result in preference of sources from which purification would be the least costly. Generally, algae contain EPA mainly in polar lipids and primarily in their GL fraction. Separation of this fraction from the lipid mixture is much easier than separation of EPA from other similar fatty acids. This concept was tested on a *P. cruentum* biomass (9), which was grown under conditions conducive to high EPA content i.e. a temperature of 25 °C, high light intensity and low cell concentration. Under these conditions EPA constituted 40% of total fatty acids and AA 15%. Moreover, ca. 90% of the EPA was restricted to the GL while the AA was primarily contained in the NL and PL fractions. Therefore, in order to purify EPA from other fatty acids and especially from AA, a preliminary separation of the GL fraction from the oil was undertaken (10) by elution on silica gel. In the acetone eluted fraction which included the GL, the proportion of EPA increased to 47.5% while that of AA decreased from 14.6% to 5.4% (Table 12). The GL fraction was transmethylated and treated with urea. By formation of the urea inclusion complexes, almost all of the saturated and monounsaturated fatty acids were effectively removed (10). The EPA and AA contents increased to 81.9% and 6.6%, respectively (Table 12). The same procedure was utilized for the preparation of an AA concentrate. By growing a culture of *P. cruentum* strain 1380-1b to the stationary phase, the AA content increased to 41.7% of fatty acids. After extraction, the resulting oil was

transmethylated and treated with urea as above, resulting in an increase in the AA content to 80.0%.

Reverse phase chromatography is known to separate fatty acids according to chain length and the degree of unsaturation (31). Yet this technique could be prohibitively costly on a large scale. However, preliminary separation of the GL fraction followed by urea treatment will provide an outstanding starting material for chromatography, increasing the output and thus reducing the cost per run. Cohen et al. (10) have demonstrated the potential of this method for purification of EPA from *Porphyridium* oil by successive elution with acetonitrile-water mixtures on C_{18} Sep-pak filters. More then 80% of the EPA was eluted in 3 fractions whose EPA content ranged from 85.4% to 93.2% (Table 13). Moreover, when reverse phase chromatography was preceded by the urea treatment, 85% of the EPA was recovered at a 97.3% purity.

6) Impact, Relevance and Technology Transfer

We believe that should more work be invested towards the isolation of EPA-overproducing strains, mutants with much higher EPA contents will be obtained. Indeed, research along that line is currently continued in China under the guidance of the P.I. Similar cooperation agreements are currently discussed with a few laboratories from several LDC.

The Thai lab has gathered a lot of know-how in various areas which previously did not exist there, such as fatty acid analysis, Selection of mutants and basic biochemical and physiological methodology.

7) Project Activities / Outputs

Meetings

1988 Z. Cohen. Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. 8th International Symposium on Plant Lipids, Budapest Hungary.

1990 Z. Cohen & Y. M. Heimer. Linoleic acid desaturase inhibitors as tools for selection of GLA over-producing lines in *Spirulina*. 9th International Symposium on Plant Lipids, Wye England.

1990 Z. Cohen, Production of polyunsaturated fatty acids by microalgae, Workshop on AID/SCI Funded Research in Agricultural Biotechnology, Kasetsart University, Bangkok Thailand.

1990 Z. Cohen. *Porphyridium cruentum* - an alternative EPA producer. 81st Meeting of the American Oil Chemists' Society, Baltimore MD.

1990 Z. Cohen. Production of EPA by *Porphyridium cruentum*. Annual Meeting of the Phycological Society of America, College Park MD.

1991 Z. Cohen & Y. M. Heimer. Production of polyunsaturated fatty acids (EPA, AA and GLA) by the microalgae *Porphyridium* and *Spirulina*. 83rd Meeting of the American Oil Chemists' Society, Chicago IL.

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1994 I. Khozina, H. A. Norman, D. Shiran and Z. Cohen. The inhibitory effect of cerulenin on linoleic acid desaturation. 11th International Symposium on Plant Lipids, Paris France.

Training

Mr. Sayamrat Panpoom attended the algal biotechnology course held in our lab on 6/91 and stayed there for another 2 months for training in algal cultivation and fatty acid analysis.

Zvi Cohen (P.I.) visited the co-P.I.'s lab in Thailand on 3-4/88 and on 7-8/90.

Publications

1988 Z. Cohen. Production of eicosapentaenoic and arachidonic acid by the red alga *Porphyridium cruentum*. In *Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry*, T. H. Appelwhite ed, American Oil Chemists' Society, Champaign IL, pp. 285-287.

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8) Project Productivity

The project advanced our know-how in each of the designated areas. We have developed a unique method for selection of EPA overproducing algal mutants. The conditions for maximizing EPA productivities under indoor and outdoor conditions in both *P. cruentum* and *M. subterraneus* were delineated. Down stream processing methodology was developed including extraction, concentration and purification methods as well as analytical methods for quality control. We have made a significant progress towards the elucidation of EPA biosynthesis in microalgae.

While the gain in our knowledge concerning EPA production was enormous, economical feasibility is still not within reach.

A windfall profit of this project is the development of conditions leading to enhanced production of arachidonic acid (AA) in *P. cruentum*. The AA content is the highest reported for any microalga. According to a recent market analysis it the figures we have reached are high enough to allow commercial production.

9) Future Work

This project launched a long range study aimed at gaining the know-how that will allow the manipulation of polyunsaturated fatty acid production in algae and eventually in plants. Future direction will concentrate on the use of the recently developed molecular biology tools that will enable overexpression of the genes in question.

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TABLE 1

Effect of Light Intensity, Cell Concentration and Temperature on the Fatty Acid Composition in *P. cruentum*.

Temp (°C)	Light int.[a]	Cell conc.[b]	Growth rate[c]	Fatty acid composition (%)										
				16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5	R[d]
20	170	2	0.65	35.0	3.7	0.2	3.1	8.3	0.2	0.1	0.2	19.3	30.0	0.6
	170	28	stationary	32.7	1.8	0.5	1.0	8.3	0.3	1.2	1.2	28.8	24.1	1.2
25	170	1.5	1.2	35.0	2.8	0.6	2.0	6.1	0.5	0.3	0.9	15.9	35.8	0.4
	170	12	0.77	36.8	2.3	0.5	0.7	5.8	0.4	0.6	0.6	18.1	34.1	0.5
	170	30	stationary	33.3	1.4	0.3	1.0	9.0	0.2	0.7	1.7	27.3	25.1	1.1
30	170	1.5	0.98	38.9	2.9	1.1	1.5	11.0	1.0	0.5	0.8	18.6	23.7	0.8
	170	8	0.73	36.3	1.2	1.2	2.7	17.2	1.0	0.6	2.3	28.1	9.4	3.0
	30	3	0.30	33.9	0.1	0.1	2.3	12.0	0.1	0.2	1.5	42.1	7.8	5.4
	170	17	stationary	31.0	0.5	1.5	7.7	18.5	0.3	1.6	3.3	32.7	2.9	11

[a]$\mu E/m^2 \cdot s$.
[b]mg chlorophyll/L
[c]doublings/d.
[d]ARA/EPA.

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TABLE 2

Effect of Temperature and Cell Concentration on the Fatty Acid Composition of the Various Lipids in *P. cruentum* (weight percent).[a]

Lipid	Temp. (°C)	Cell conc.[b]	Fatty acid composition (%)										R[c]	% of total fatty acids
			16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5		
Total	25	1.5	35.1	2.8	0.6	2.0	6.1	0.5	0.3	0.9	15.9	35.8	0.4	
	30	1.5	38.9	2.9	1.1	1.5	11.0	1.0	0.5	0.8	18.6	23.7	0.8	
	30	8	36.3	1.2	1.2	2.7	17.2	1.0	0.6	2.3	28.1	9.4	3.0	
Neutral	25	1.5	21.8	5.3	9.2	7.6	15.5	0.6	0.4	0.8	16.9	21.9	0.8	17
	30	1.5	20.8	1.5	8.3	4.8	20.6	1.7	+	3.6	31.3	7.4	4.2	34
	30	8	25.7	1.1	6.0	4.5	22.9	2.1	0.6	3.2	29.8	4.2	7.1	58
MGDG[d]	25	1.5	31.5	3.6	1.0	3.1	8.3	0.2	0.1	0.1	2.9	49.1	0.06	26
	30	1.5	47.3	2.2	2.8	2.1	9.8	+	0.9	+	6.5	28.4	0.2	18
	30	8	51.0	2.9	4.2	4.9	10.2	1.8	2.4	0.4	13.8	8.4	1.6	14
DGDG[e]	25	1.5	46.9	8.6	0.8	2.2	4.2	0.4	0.2	0.1	1.4	35.2	0.04	26
	30	1.5	64.0	3.6	0.9	1.4	4.0	+	0.2	0.3	2.7	22.9	0.1	15
	30	8	69.5	2.6	1.9	2.6	5.2	1.3	0.1	0.1	8.5	8.3	1.0	9
SQ[f]	25	1.5	55.7	3.0	2.9	8.8	4.7	+	1.2	+	2.5	21.2	0.1	13
	30	1.5	65.9	0.6	2.0	3.6	3.7	0.8	2.7	1.2	8.0	11.5	0.7	10
	30	8	68.8	0.8	5.8	5.1	9.1	0.7	2.7	1.0	3.3	2.6	1.3	8
PC[g]	25	1.5	30.9	6.0	2.6	3.6	6.7	2.0	2.3	3.0	38.9	3.9	10	9
	30	1.5	31.7	2.0	2.1	3.1	9.5	0.9	+	2.0	42.3	6.4	6.6	8
	30	8	26.0	+	4.4	5.2	9.2	1.5	0.6	3.5	46.6	2.9	16	6

[a]Cultures were grown at a light intensity of 170 $\mu E/m^2 \cdot s$. [b]mg chlorophyll/L. [c]ARA/EPA. [d]MGDG, monogalactosyldiacylglycerol. [e]DGDG, digalactosyldiacylglycerol. [f]SQ, sulfoquinovosyldiacylglycerol. [g]PC, phosphatidylcholine.

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 113.86, cultivated at 28°C, 170 $\mu E/m^2 \cdot s$.
[b]NL, Neutral lipids.
[c]MGDG, Monogalactosyldiacylglycerol.
[d]DGDG, Digalactosyldiacylglycerol.
[e]SQ, Sulfoquinovosyldiacylglycerol.
[f]PC, Phosphatidylcholine.
[g]+N, Nitrogen sufficient.
[h]−N, three days nitrogen starvation.
[i]Percentage of total fatty acids.
[j]R = ARA/EPA.
[k]Fraction percentage of total fatty acids.

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TABLE 4

Effects of Cell Concentration in Winter and Summer on EPA and ARA yields in *P. Cruentum* Cultured in Open Ponds.

Cell[a] conc.	Fatty acid content (% of AFDW[b])		Output rate (AFDW g/m²·d)			R[c]
	EPA	ARA	Biomass	EPA	ARA	
Winter[d]						
2.5	2.21	0.76	3.6	0.08	0.03	0.34
3.5	2.30	0.75	5.0	0.12	0.04	0.33
4.5	2.18	0.73	2.1	0.05	0.02	0.33
Summer[e]						
3.0	1.28	0.71	19.7	0.25	0.14	0.45
4.5	1.16	1.18	24.0	0.28	0.28	0.69
6.0	1.00	1.31	13.0	0.13	0.17	0.88

[a]mg chlorophyll/L.
[b]Ash-free dry weight (AFDW).
[c]R = ARA/EPA.
[d]Max. daily temp. 16–18°C (Nov.–Jan.).
[e]Max. daily temp. 28–31°C (Jul.–Aug.).

TABLE 5

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

Strain	Growth phase	μ (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[a]	ARA	EPA	R[b]
1380-1a	E[c]	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[d]		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]TFA, weight percent of AFDW.
[b]R = ARA/EPA.
[c]E, exponential phase, 4-5 mg chlorophyll/L.
[d]S, stationary phase, 27-30 mg chlorophyll/L.

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TABLE 6

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

Strain	Growth phase	μ (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	ARA	EPA	R[b]
1380-1a	E	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		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

TABLE 7

Effect of SAN 9785 on the Fatty Acid Composition in *P. cruentum*.

Inhibitor conc. (mM)	Fatty acid composition (% of total fatty acids)										
	16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
0	31.8	5.9	0.5	0.9	1.0	5.3	1.4	0.1	0.5	16.0	36.8
0.08	36.1	5.0	0.8	0.9	1.2	5.0	0.8	0.4	0.7	13.3	35.4
0.16	37.8	5.1	0.8	0.7	1.3	5.0	0.7	0.6	0.9	15.4	31.2
0.40	43.9	5.9	0.8	0.9	1.9	5.5	1.5	0.8	1.3	11.6	25.4

Table 8. Effect of SAN 9785 on the fatty acid composition of wild-type (WT) and SAN 9785-resistant (SRP) cultures of *P. cruentum*

Culture	SAN 9785 concn (mM)	Fatty acid composition (% total fatty acids)										
		16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT*	0	32.9[a]	4.2	0.6	0.5	0.4	5.7[a]	0.7	0.6	0.6	17.3	36.1[a]
SRP	0	28.5[b]	5.4	0.4	0.5	1.0	5.7[a]	0.9	0.5	0.7	15.8	40.4[b]
SRP-6†	0	29.2[b]	4.7	0.6	0.5	0.5	5.3[a]	0.9	0.6	0.4	15.4	41.1[b]
WT	0.4	37.5[c]	3.7	0.7	1.3	2.1	7.9[b]	1.5	0.6	2.0	17.4	24.8[c]
SRP‡	0.4	29.0[b]	3.1	0.6	1.8	2.8	12.2[c]	1.3	1.0	2.7	14.3	30.7[d]

*Wild-type.
†Resistant line selected by agar plating of resistant culture.
‡SAN 9785-resistant culture.
Data shown represent mean values of four independent samples, each analysed in duplicate.

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Table 9. Fatty acid composition of the galactolipid fraction of wild-type (1380-1d) and SAN 9785-resistant (SRP) cultures of *P. cruentum*

Culture	Medium	Fatty acid composition (% total fatty acids)										
		16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT	−*	36.4	1.2[a]	nd†	0.5	1.0[a]	4.3[a]	nd	0.6[a]	nd	5.9[a]	50.0[a]
WT	+*	36.4	8.2[b]	0.5	3.3	5.7[b]	4.1[a]	0.4	1.3[b]	0.2	9.2[b]	29.1[b]
SRP‡	+	36.0	2.0[c]	0.2	1.2	2.0[c]	7.4[b]	0.3	1.2[b]	0.2	3.8[c]	45.4[c]

*(−) Inhibitor-free medium. (+) Medium containing 0.4 mM SAN 9785.
†Not determined.
‡A culture of *P. cruentum* 1380-1d resistant to 0.4 mM SAN 9785.
Data shown represent mean values of four independent samples, each analysed in duplicate.

Table 10. *Fatty Acid Composition of P. cruentum Cell Lines Resistant to SAN 9785*

The values presented are means ± SD ($n = 3$). A t test was used to determine significantly different values of EPA and TFA at $P < 0.05$.

Culture	Fatty Acid Composition											Fatty Acid Content	
	16:0	16:1	16:3	18:0	18:1 ω9	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	EPA	TFA[a]	EPA
	% of total fatty acids											% of dry wt	
WT[b]	30.3 ±0.62	5.20 ±0.34	0.62 ±0.06	0.44 ±0.02	0.53 ±0.04	5.44 ±0.11	0.89 ±0.13	0.54 ±0.15	0.68 ±0.07	16.1 ±0.40	38.2 ±0.75	4.78 ±0.17	1.83 ±0.04
SRP-6[c]	29.2 ±1.5	4.70 ±0.07	0.62 ±0.08	0.47 ±0.07	0.53 ±0.04	5.31 ±0.24	0.92 ±0.15	0.63 ±0.14	0.37 ±0.01	15.4 ±0.10	41.1 ±0.39[d]	5.64 ±0.67[d]	2.32 ±0.39[d]
SRP-7[c]	29.5 ±1.2	4.54 ±0.30	0.60 ±0.06	0.46 ±0.10	0.47 ±0.06	5.34 ±0.23	0.90 ±0.04	0.47 ±0.02	0.59 ±0.16	15.5 ±0.45	40.9 ±1.1[d]	5.62 ±0.67[d]	2.31 ±0.36[d]

[a] Total fatty acids. [b] Wild type. [c] Resistant cell lines selected by agar plating of the resistant culture. [d] Significantly different from wild type.

Table 11. Fatty acid composition of the lipid fractions of *M. subterraneus*.

Fraction	Fatty acid composition (% of total fatty acids)													
	14:0	16:0	16:1 ω11†	16:1 ω7†	16:1 ω5†	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	EPA
Neutral lipids[a]	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[b]	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[c]	0.4	24.3	-	17.3	1.7	1.2	4.4	1.4	2.6	3.2	0.3	0.6	8.5	31.9

† Tentative assignment; [a] Fraction eluted with chloroform; [b] Fraction eluted with acetone; [c] Fraction eluted with methanol.

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TABLE 12

Purification of EPA Methyl Ester from *P. cruentum*.

	Fatty acid composition (% of total fatty acids)									
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	R
Total lipids	30.6	5.3	0.3	0.9	4.8	0.8	2.0	14.6	40.7	0.36
FAME[a]	34.8	0.4	0.8	1.3	9.6	0.1	0.2	5.4	47.5	0.11
FAME after urea adduction	3.1	0.3	--	0.6	7.3	0.1	0.2	6.6	81.0	0.08
FAME after chromatography	0.3	--	--	--	0.0	--	0.2	1.4	97.3	0.01

[a]FAME of glycolipid fraction.

TABLE 13

EPA Purification by Reverse Phase Chromatography.[a]

	Fatty acid composition (% of total fatty acids)									% of total
% CH_3CN[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	EPA[c]
58	14.2	2.0	1.2	1.6	3.2	2.9	1.4	6.1	67.3	2.7
59	1.6	0.1	0.2	0.0	1.7	0.4	0.5	2.3	93.2	29.1
59	3.2	0.2	0.3	0.0	2.8	0.8	0.9	3.0	88.9	27.2
59	2.4	0.3	0.4	0.2	4.0	1.0	1.5	4.8	85.4	25.2
59	19.0	1.0	0.9	0.8	16.8	1.1	0.5	15.2	44.8	7.1
60	32.4	1.1	0.8	1.3	21.3	0.7	1.9	17.1	23.4	4.2
61	46.7	1.2	0.2	1.3	25.1	1.2	1.8	15.1	7.4	1.5
62	60.8	1.2	0.7	1.7	15.9	0.9	3.9	10.1	5.6	0.9
63	63.7	1.3	1.2	2.3	13.1	0.6	3.8	6.7	7.2	0.6
65	68.8	1.5	0.7	3.1	7.6	2.0	1.9	5.3	9.1	0.5
100	74.3	1.7	4.5	2.7	4.6	0.4	3.9	2.4	5.5	0.4
Pre-separation mixture[d]	33.8	0.4	0.8	1.2	9.3	0.1	0.2	8.2	46.1	

[a]Representative example.
[b]Percentage of acetonitrile-water.
[c]The ratio of EPA content in fraction to total EPA eluted.
[d]Composition of FAME mixture loaded on the column.

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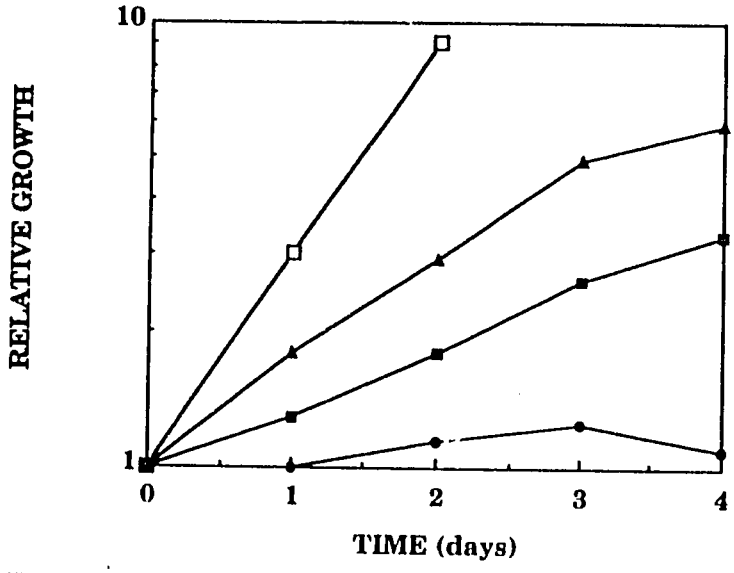

Fig. 1 . Effect of SAN 9785 on the growth rate (expressed as the increase in chlorophyll concentration relative to day zero of *P. cruentum* (□, control; Δ, 0.08mM; ■, 0.16mM; ●, 0.4mM).

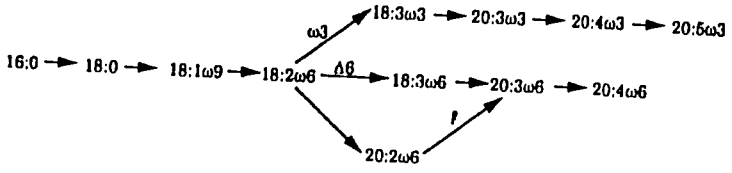

Fig. 2

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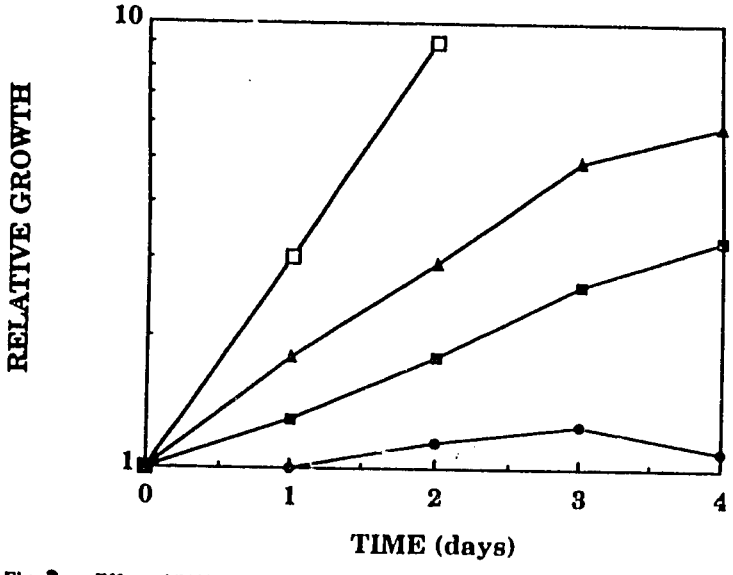

Fig. 3 . Effect of SAN 9785 on the growth rate (expressed as the increase in chlorophyll concentration relative to day zero of *P. cruentum* (□, control; Δ, 0.08mM; ■, 0.16mM; ●, 0.4mM).

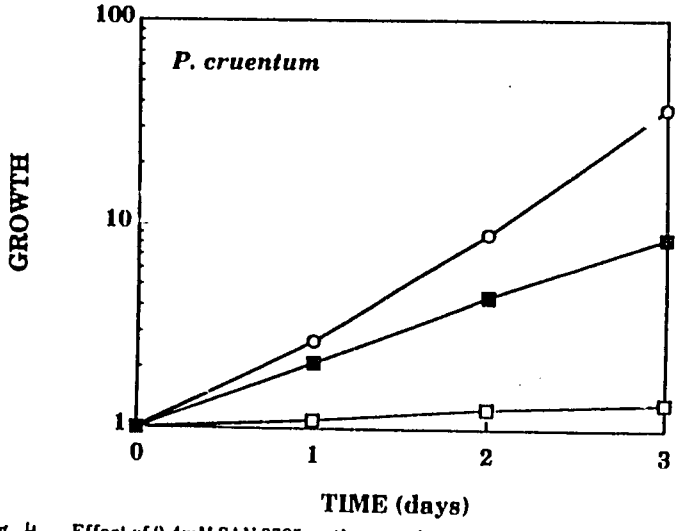

Fig. 4. Effect of 0.4mM SAN 9785 on the growth rate (expressed as the increase in chlorophyll concentration relative to day zero of *P. cruentum* (○, control; □, freshly exposed to the inhibitor; ■, inhibitor resistant culture).

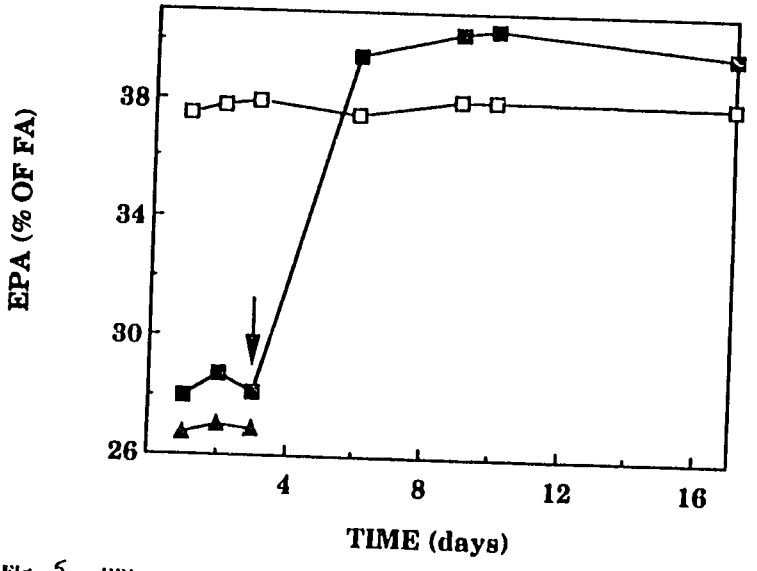

Fig. 5. EPA content (% of fatty acids) of *P. cruentum* wild type, grown with (▲) or without (□) SAN 9785, and of a resistant subline grown continuously in the presence of the herbicide (■) and shifted to a herbicide-free medium (arrow).

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XN-ABU-414-A

FINAL REPORT

AID-CDR PROJECT : DPE-5544-G-SS-8017-00

PRODUCTION OF EPA BY MICROALGAE

BY

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JANUARY, 1994

Abstract

The investigation under the AID-CDR-supported project on " Production of Eicosapentaenoic Acid (EPA) by Microalgae " has been completed. In this report, we summarize the results of the investigation achieved by TISTR's group and evaluate the whole project in terms of its contribution to both TISTR and scientific community, especially in the field of algal biotechnology.

The main task of TISTR as a collaborator to the project was primarily to select an algal species with high EPA production and to study nutritional and environmental conditions affecting EPA production by the selected species. Under the designated research plan, TISTR group selected the green alga Monodus subterraneus as a promising species for EPA production. Subsequently, a series of experimental studies on the optimal conditions for EPA production showed that the alga produced up to 4.5 % of dry weight EPA in the laboratory at 23°C. However, when grown outdoor at higher temperatures the EPA production was drastically decreased. This inherent limitation makes impossible the large-scale algal cultivation for industrial EPA production in Thailand in economic perspective.

Contents

	Abstract	Page i
I.	Introduction	" 1
II.	Overall aim and specific objectives	" 2
III.	Materials and methods	" 2
IV.	Scientific collaboration	" 3
V.	Results and discussion	" 4
VI.	Conclusion and suggestion	" 9
VII.	Contribution of the project	" 10
VIII.	Acknowledgements	" 12
IX.	References	" 12
X.	Tables	" 13

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I. Introduction

Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) have attracted increased attention in recent years due to their remarkable nutritional and pharmaceutical properties. Most importantly, EPA has been shown to be highly effective in reducing blood cholesterol levels and in preventing formation of atherosclerotic plaques in the arteries, thus facilitating prevention and treatment of heart diseases in humans. At present, the main commercial source of EPA is marine fish oil which contain relatively low amount of EPA and the global supply of EPA from fish is unlikely to meet future requirement. Thus, alternative sources of this valuable fatty acid are being sought.

A variety of PUFAs have been detected in microorganisms including bacteria, fungi, algae, mosses, and protozoa. However, EPA was not detected in higher plants and only small number of fungal and algal species are capable of producing significant quantities of EPA. The inadequacy of fish oil as a source of EPA prompted us to study EPA production by mocroalgae which have many advantages over marine fish oil in terms of biomass production as well as the cost of cultivation.

The AID-CDR project on " Production of Eicosapentaenoic Acid (EPA) by Microalgae " represented an initial effort to explore the possibility of utilizing microalgae as a source of chemical products, especially those with economic importance. This project was a collaboration between the International Institute for Desert Research (IDR), the Ben-Gurion University of Israel and Biotechnology Department of Thailand Institute of Scientific and Technological Research (TISTR). The duration of the project was 5 years from January 1, 1988 to December 31, 1993.

II. Overall aim and specific objectives

1. Screening of fast growing algal species which may be sustained in dense cultures throughout the year and which contain high concentration of EPA.

2. Determining the optimal environmental and nutritional conditions for high net productivity and overall maximal EPA production by the selected species.

3. Optimizing cultivation methods applicable for large-scale production of microalgae containing EPA.

4. Developing simple, low-cost methods for the purification of EPA.

5. Operation of a mini-pilot plant for EPA production in Thailand.

6. Expanding the knowhow for cultivation of microalgae for fine chemical production in less-developed country (LDC).

III. Materials and methods

1. Organisms : Algal species used throughout this study was *Monodus subterraneus* obtained from Gottingen University Culture Collection and was maintained at 25°C under continuous light condition.

2. Growth conditions : The culture was grown for 7 days in BG-11 medium supplemented with three vitamins (B_1, B_{12} and Biotin at $1x10^{-5}$ g/L) in glass tubes incubated in a temperature regulated water bath at 25°C illuminated with three cool-white fluorescent lamps providing approximately 75 $\mu E.m^{-2}.s^{-1}$ (unless otherwise stated) at the side of

the water bath. The culture was mixed by bubbling an air through a glass tube connected to the bottom of each culture tube. Specific growth rate was estimated by measurment of cell concentration at OD-970 nm.

3. Lipid transmethylation : Freeze-dried samples of algal biomass (100 mg) was transmethylated with 2 ml of methanol/acetyl chloride (95/5) as described by Cohen et. al. (1). Heptadecanoic acid was added as internal standard, and the mixture was sealed in a light protected Teflon-lined vial and heated at 90°C for 1 h. The vial contents were then cooled, diluted with 1 ml hexane. The hexane layer was dried over Na_2SO_4, evaporated to dryness under nitrogen atmosphere, and redissolved in hexane.

4. Fatty acid analysis : Gas chromatographic analysis was performed with a SP 2330 fused silica capillary column (30 m x 0.2 mm.) at 195°C (injector and flame ionization detector temparature 250°C, split ratio 1:100). The results were formulated with a Water 720 integrator. Fatty acid methyl esters were identified by co-chromatography with authentic standard (Sigma). 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.

IV. Scientific Collaboration

According to the agreement in the proposal of the project, TISTR's group will study the environmental and nutritional effects on EPA content and purification methods will be studied in parallel. Once a method for selection of EPA over producing strains is developed by Israeli group, the Thai group will attempt to isolate such strain. During the last two

years of the project a pilot plant for algal cultivation and EPA extraction are planned to be established in Thailand.

During the operation of the project a researcher from TISTR made one visit to Israel during May 12 - August 24, 1991 to attend a training course on Photosynthesis and Algal Biotechnology organized by BGU's International Institute for Desert Research (IDR) and to conduct a joint research on EPA production by microalgae.

Meanwhile, the principal investigator from Israel made two visits to TISTR during the first and fourth year of the project.

The visit of researcher from TISTR to IDR was judged as successful in terms of scientific collaboration, especially in improving the techniques of algal cultivation and determination of fatty acid composition by gas chromatography.

V. Results and discussion

The results of investigation achieved by TISTR's group can be summarized as follows :

1. Species selection

During the first year of the project, we attempted to select potential EPA producing algal species from Marine Bacillariophyceae which included *Navicula* sp.1, *Thalassionsira pseudonana*, *Tetraselmis chuii*, *Isochrysis* sp.1, *Skeletonema costatum* and *Chlorella* sp. 1. Among these species, *Navicula* sp.1 was found to be the fastest growing species and contain the highest content of EPA. However, since the amount of EPA produced by *Navicula* sp.1 was still ralatively low, we had to look for

other species with higher EPA content and eventually we found that the freshwater green alga Monodus subterraneus was the most suitable species for further investigation. We therefore selected and subsequently used the species in all studies conducted during the second to fourth year.

2. Study on the effects of nutritional and environmental condition on EPA production

2.1 Types of media

In our preliminary studies, we found that the most suitable medium for the diatom Navicula sp.1 and M. subterraneus were Guillard's medium (Table 1) and BG-11 medium, respectively.

2.2 Media composition

In many investigations,we found that varying various media compositions and addition of certain vitamins led to different proportion of fatty acids in algal cells.

In M. subterraneus, varying nitrate concentration from 0.25 to 5.0 g/L resulted in a remarkable increase in total fatty acid production, but did not affect growth and fatty acid composition (Table 2). An overall EPA production was markedly increased as a result of elevating nitrate concentration, by which an EPA content of up to 4.56% of cell dry weight was achieved.

A double increase in growth rate and more than 50% increase in the proportion of EPA in M. subterraneus was observed when the culture was grown in BG-11 medium supplemented with three vitamins, namely Vitamin B_1, Vitamin B_{12} and Biotin at the concentration of 1×10^{-5} g/L. (Table 3)

A concomitant change in the content and composition of other fatty acids resulting from the supplementation was also observed.

2.3 Temperature and light intensity

In a number of investigations, we found that temperature exerted a pronounced effect on growth and composition of fatty acid in both Navicula sp.1 (Table 4) and M. subterraneus (Table 5). For Navicula sp. 1, the optimum growth temperature was observed at 20°C. An increase in temperature from 20°C to 40°C resulted in a reduced growrh rate as well as a remarkable change in fatty acid composition. At higher growth temperature, the saturation of fatty acids was observed with arachidonic acid (AA, 20:4) becoming the dominant PUFA, whereas at lower temperatures, the desaturation of fatty acids took place resulting in a rise in the proportion of EPA .

In M. subterraneus, similar results were obtained. An increase in growth temperature from 23°C to 32°C resulted in a drastic reduction in EPA synthesis and a lowered biomass production. An approximated 70% decrease in the output rate of EPA was accordingly obtained. Similar results were observed when light intensity was increased from 75 $\mu M.m^{-2}.s^{-1}$ to 170 $\mu M.m^{-2}.s^{-1}$. This inherent limitation of EPA production by temperature and light intensity constituted a major difficulty in achieving EPA production at outdoor temperature and thus make impossible the industrial scaling up for EPA production in Thailand where temperature is relatively high.

2.4 CO_2 supplementation

CO_2 content in the atmosphere is about 0.03% while most green plants need up to 0.3% for their saturated photosynthesis. CO_2 supply

to the plants at concentrations higher than 0.03% therefore usually enhances growth and productivity. In our experiment with M. subterraneus (conducted in Israel), we found that maximum biomass and EPA production per unit volume of culture was observed when the air was supplemented with 1% (vol/vol) CO_2 (data not shown). The doubling time of M. subterraneus grown with and without the supply of 1% CO_2 were 0.85 and 1.30 days, repectively. This clearly shows that supplementation of CO_2 to the culture apparently help increase the growth rate of the alga about 1.5 times.

3. Study on induced lipogenesis

One possible way to achieve a high net production of EPA in algae is through the addition of exogenous EPA precursors such as oleic acid, linoleic acid and α-linolenic acid. In a process called "induced lipogenesis" algal cells convert the C-18 fatty acids into C-20 fatty acids including EPA. In our study, induced lipogenesis was attempted by adding oleic acid (cis-9-octadecenoic) into the culture of M. subterraneus. Oleic acid at all concentration tested (0.1-0.5%) was found to increase total fatty acid production when calculated on a cell dry weight basis (Table 6). However, the addition of oleic acid into the culture of the alga apparently led to a reduction in biomass production, resulting in a lower net production of both total fatty acid and EPA. The study showed that exogenously applied EPA precursor such as oleic acid did not significantly improve the net production of EPA.

In a related experiment, we used linseed oil which contains up to 60% α-linolenic acid as an exogenous EPA precursor. Linseed oil at all concentrations tested (0.1 -0.5% v/v) caused a severe damage to the cultures. All cultures died out in a few days of cultivation. This study

suggests that more suitable and effective exogenous EPA precursors may have to be explored if a more desirable net production of EPA by algae is to be achieved.

4. Study on the output rate (productivity) of EPA

The overall maximal productivity of EPA by *M. subterraneus* grown under optimum condition in the laboratory; that is at the temperature of 23-25°C, light intensity of 75 $\mu M.m^{-2}.s^{-1}$ and dark light cycle of 12:12 h., was estimated at 6.0 mg/liter/day.

In comparison with EPA productivity by other EPA producers (Table 7), EPA productivity by *M. subterraneus* was still very low, especially when compared to the figure of 135 mg/liter/day achieved by the fungus *Mortierella alpina*. Therefore, it is unlikely that EPA production by microalgae can compete with the fungus as far as the cost of production is concerned.

5. Preliminary study on outdoor cultivation

A preliminary study on the outdoor cultivation of *M. subterraneus* in 300-L fiber-glass tanks was attempted. It was found that under outdoor conditions (temperature 28-35°C, full sunlight), biomass and EPA production were approximately 2.5 times lower than those obtained in the laboratory. That is EPA productivity was about 2.4 mg/l/day. This figure suggests that scaling up for EPA production under outdoor condition such as in the open-raceway ponds is totally impossible in economic perspective.

The inherent limitation on EPA production by temperature was a major obstacle that made impossible further investigation on a large-scale production of EPA using 10,000 Liter open-raceway ponds as

indicated in the research program.

VI. Conclusion and suggestion

The alga *M. subterraneus* investigated by our group has been so far the only species that accumulates the highest EPA content of 4.5-6% of dry weight under optimum condition. However, it has one important disadvantage i.e. when grown at high temperature it produces much lower EPA content than at low temperature. From a biotechnological viewpoint, it is not worthwhile to produce EPA at low temperature since the cost of temperature control is high. Considering climatic condition of Thailand, which is a subtropical country with intensed sunlight and high temperature, it looks obvious that large-scale commercial production of EPA may not be possible in economic perspective.

The inherent limitation on EPA production by temperature implies that the dream of establishing a factory for industrial EPA production in Thailand may not come true in the coming years. The situation is even worst when the recent study by Japanese researchers showed that substantial amount of EPA can be obtained more easily from the fungus *Mortierella alpina* whose EPA productivity at 28°C was as high as 135 mg/l/day. This indicates that more readily available and cheaper source of EPA at present and in the near future is fungi not microalgae. Nevertheless, there is still a hope to exploit microalgae as a good soure of EPA, possibly through the use of genetic manipulation.

Theoretically, we can identify , characterize and even isolate EPA-synthesizing gene(s) from EPA-producing eukaryotes such as microalgae and eventually introduce the gene(s) into other lipid containing organisms including agricultural crops, especially oilseed plants in order to create

transgenic organism capable of producing their own EPA. If successful, this would greatly help increase the global supply of EPA in the future.

However, the ultimate goal of expanding EPA sources by the application of gene technology may not be achieved without a prerequisite understanding on the molecular nature of EPA biosynthetic pathway in EPA producing organisms as well as lipid metabolism in the transformants. To date, very little has been known about the nature of EPA biosynthesis in algae. It is therefore an obviously important area for an urgent and extensive investigation.

VII. Contribution of the project

1. Contribution to TISTR

The AID-CDR project on "EPA production by microalgae" was particularly important in itself since it represents the effort to exploit microalgae as a source of useful products by using the state-of-the-art technology in microalgal cultivation. Likewise, since TISTR has been trying to establish itself as a leader in the field of algal biotechnology in Thailand, it should directly benefit from this project. In fact, the contribution of the project to TISTR was plentiful. First of all, in terms of funding, TISTR received quite large amount of fund to operate the project in one way, and to buy various laboratory equipments in another. Secondly, the project provided a good opportunity for TISTR's researchers to become involved and gain vast experience in more advanced research in the novel field of algal biotechnology. Thirdly, it helped TISTR to be more open to outside community, especially in strengthening and tightening its cooperation in R&D activities with foreign counterparts, especially in the top priority area of biotechnology.

2. Contribution to scientific community

Since biotechnology has recently been designated by the government as one of the three priority areas of R&D in Thailand, an increasing number of R&D projects in biotechnology, especially microbial and plant biotechnology have been substantially augmented. Algal biotechnology which is an emerging field in biotechnology has also attracted increased attention in recent years. As a result of the progress and the popularity of the field, a number of scientific meetings on algal biotechnology have been regularly organized over the past 5 years.

In many occasions of the meetings, researchers from TISTR were cordially invited to attend and to present scientific papers or posters on EPA production. These included :

1. NRCT-JSPS Joint Seminar on "Current Advances in Biotechnology" ; Prince of Songkhla University, Thailand; December 21-23, 1990.

2. The Third Annual Meeting of the Thai Society for Biotechnology on "Current Advances in Biotechnology : Industry and Environment" Chulalongkorn University, Thailand; October 30 - November 1, 1991.

3. UNESCO-Sponsored Research Seminar and Workshop on "Mass Culture of Microalgae" Silapakorn University, Thailand, November 18-23, 1991.

4. USAID-Sponsored Networking Meeting on "Algal Biotechnology" Singapore; September 17-24, 1992.

The participations of the researchers from TISTR in these meetings were all successful. This means at least the results of the study in the project has been widely publicized and more or less contributed to scientific society, especially in the field of algal biotechnology.

VIII. Acknowledgements

On behalf of TISTR, we would like to express our deep gratitude and sincere appreciation to USAID for kindly granting us the prestigious fund. We would also like to sincerely thank our collaborators in Israel, especially Dr. Zvi Cohen, for their full collaboration and kind assistance throughout the project.

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Table 1 Growth and fatty acids composition of *Navicula* sp.1 cultivated in different media

Medium	K (d-1)	td (day)	total fatty acid (% of AFDW)	Fatty acids content (% of total fatty acid)							
				16:0	16:1	18:1	18:2	18:3	20:1	20:4	20:5
Guillard's	0.394	0.764	1.15	28.73	-	27.07	-	-	0.51	17.51	26.18
Sato	0.213	1.413	1.21	28.75	23.53	13.12	3.20	3.09	0.98	11.13	17.21
Watanabe	0.261	1.153	0.98	33.58	23.87	16.98	2.63	3.89	0.60	12.84	18.98
Enriched sea water	0.308	0.977	0.82	47.92	38.22	-	-	-	0.79	4.89	8.18

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Table 2 Effect of nitrate concentration on fatty acids and EPA production by *M. subterraneus*

Nitrogen conc. (g/liter)	Fatty acid production (% of total fatty acids)							Total fatty acid production (% of cell dry wt)	EPA production	
	16:1 (w-11)	16:0	16:1 (w-7)	18:1	18:2	20:4	20:5		% of cell (dry wt)	mg/g of cell (dry wt)
0.25	3.23	19.13	22.88	9.35	5.61	4.49	35.25	8.75	3.08	30.8
0.5	3.59	18.32	23.84	7.46	4.47	5.62	36.61	9.18	3.36	33.6
1.0	3.19	19.20	23.18	9.22	5.03	5.34	35.80	9.48	3.39	33.9
1.5	3.98	18.90	24.13	6.76	4.02	5.51	36.67	9.57	3.51	35.1
2.0	3.79	19.30	23.97	8.94	5.79	5.10	35.08	10.04	3.52	35.2
3.0	4.17	20.60	25.22	5.06	3.64	5.27	36.00	12.51	4.50	45.0
4.0	4.42	21.94	26.51	5.21	4.01	5.70	34.17	13.23	4.52	45.2
5.0	4.56	20.61	25.95	5.83	2.97	5.33	34.65	13.16	4.56	45.6

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Table 3 Fatty acid conposition of *M. subterraneus* cultivated on BG-11 medium and BG-11 medium supplemented with three vitamins

Medium	Fatty acid composition (%)							
	16:1 (w-11)	16:0	16:1 (w-7)	18:1	18:2	20:4	20:5	TFA
BG-11	4.41	6.10	23.33	9.30	5.33	6.33	28.12	3.52
BG-11 + vitamins	5.10	18.84	26.52	5.21	1.86	5.26	38.61	3.32

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48

Table 4 Fatty acid composition of Navicula sp.1 grown at different temperature (Exp = Exponential phase, STA = stationary phase)

Temp. (°C)	Growth phase	Fatty acid composition (% of total fatty acid)							
		16:0	16:1n11	16:1n7	18:1n7	18:2	20:4	20:5	others
20	EXP	17.80	6.49	29.48	0.91	1.30	3.60	7.79	43.57
	STA	10.03	5.22	21.74	1.10	1.32	8.96	22.43	29.20
25	EXP	15.18	7.15	30.18	1.07	1.00	5.71	9.06	30.65
	STA	11.31	6.36	22.71	1.19	2.47	10.67	16.96	28.33
30	EXP	13.66	6.89	27.86	1.55	1.35	11.83	9.65	27.21
	STA	11.25	6.95	23.30	0.96	2.68	11.66	14.69	28.51
35	EXP	12.35	5.30	26.04	-	8.74	15.03	6.50	26.04
	STA	11.04	5.76	23.54	-	1.22	21.65	10.19	26.60
40	EXP	-	-	-	-	-	-	-	-
	STA	28.09	2.68	29.97	-	-	7.22	4.40	27.64

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Table 5 Effect of temperature and light intensity on EPA production and output rate by *M. subterraneus*

Temperature (°C)	Light intensity (uM. $m^{-2}.s^{-1}$)	Cell dry weight (g/L)	Fatty acid production (% of total fatty acids)								Total fatty acid production		EPA production		EPA productivity (mg/L/day)
			16:1 (w-11)	16:0	16:1 (w-7)	18:1	18:2	20:4	20:5	others	mg/g of cell dry wt.	mg/L of culture broth	mg/g of cell dry wt.	mg/L of culture broth	
23	75	0.91	3.23	20.16	25.95	5.83	2.97	5.33	34.65	1.88	131.6	119.8	45.6	41.5	6.0
	170	1.20	3.55	21.13	24.44	6.57	3.07	6.75	34.01	0.48	100.5	120.6	25.7	30.8	4.4
28	75	0.76	3.94	21.85	23.17	6.81	3.14	6.12	32.61	2.36	75.7	57.5	34.0	25.8	3.7
	170	0.84	3.79	22.07	22.98	6.77	3.45	7.04	31.48	2.42	71.9	60.4	18.1	15.2	2.2
32	75	0.47	4.43	22.46	22.65	7.10	3.58	7.80	30.80	1.18	52.3	24.6	26.2	12.3	1.8
	170	0.66	5.58	23.06	22.25	8.21	3.63	10.40	25.46	1.41	50.2	33.1	10.3	6.8	1.0

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Table 6 Effect of oleic acid on biomass production and fatty acid composition in *M. subterraneus*

Oleic acid concentration (% weight of culture liquid)	Cell dry weight (g/L)	Fatty acid production (% of total fatty acid)								Total fatty acid production		EPA production	
		16:1 (w-11)	16:0	16:1 (w-7)	18:1	18:2	20:4	20:5	others	mg/g of cell dry weight	mg/L of culture broth	mg/g of cell dry weight	mg/L of culture broth
0	0.95	3.50	20.41	25.83	5.91	3.12	5.66	34.41	1.16	129.81	123.31	44.66	42.43
0.1	0.92	4.51	22.11	24.68	5.96	2.04	5.71	33.84	1.15	139.26	128.11	47.12	43.35
0.2	0.84	4.48	22.80	23.71	6.45	2.77	4.43	34.11	1.25	137.55	115.54	46.91	39.40
0.3	0.75	3.96	21.94	26.28	7.94	3.12	4.71	30.96	1.09	165.41	124.05	51.21	38.40
0.5	0.51	4.91	22.41	26.44	11.14	4.86	3.06	25.62	1.56	167.31	85.32	42.86	21.86

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Table 7 Comparison of EPA production and output rate of the cultures of *M. subterraneus*, *Chlorella minutissima*, the fungus *Mortierella alpina* and *Phaeodactylum tricornutum*

Culture of	Culture Temperature (°C)	Culture Days	Dry Cell Concentration (g/liter)	Total fatty acids (mg/g of cell dry weight)	EPA Content (% of total fatty acids)	EPA Yield (mg/liter of culture broth)	EPA productivity (mg/liter/day)
M. subterraneus	23	7	0.9	131.6	34.7	41.5	6.0
Chlorella minutissima (2)	19	4	0.38	79.7	39.9	12.05	3.01
Mortierella alpina (3)	28	10	32.5	-	13.5	1,350.00	135.0
Phaeodactylum tricornutum (4)	23	7	4.0	118.3	28.0	132.9	19.0

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THE EFFECT OF TEMPERATURE AND CELL CONCENTRATION ON THE FATTY ACID COMPOSITION OF OUTDOOR CULTURES OF PORPHYRIDIUM CRUENTUM

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ABSTRACT

The red microalga Porphyridium cruentum is a potential source for pharmaceutically valuable fatty acids eicosapentaenoic acid and arachidonic acid. The conditions promoting a high content of either fatty acid were investigated. The fatty acid composition was effected by cell concentration. In outdoor cultures maintained at a relatively low cell concentration, eicosapentaenoic was the main polyunsaturated fatty acid. At a higher cell concentration, arachidonic acid was dominant. The maximal daily temperature exerted a similar (although less intensive) effect on fatty acid composition. In winter, eicosapentaenoic acid constituted up to 50% of the fatty acids and up to 2.5% of the biomass dry weight but its output rate was only 0.12 $g \cdot m^{-2} \cdot d^{-1}$. In summer, the content of eicosapentaenoic acid was only 1.3% but the output rate increased to 0.28 $g \cdot m^{-2} \cdot d^{-1}$ resulting from the elevated biomass yield which increased to 24 $g \cdot m^{-2} \cdot d^{-1}$ (AFDW) . The highest arachidonic acid content was obtained in summer at high temperatures and cell concentrations reaching 30% of the fatty acids and up to 1.4% of the dry weight.

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INTRODUCTION

The red microalga P. cruentum is an excellent source for the two rare polyunsaturated fatty acids (PUFA) arachidonic acid (AA) and eicosapentaenoic acid (EPA) [1,2]. Arachidonic acid can be utilized for the production of prostaglandins [3] and was claimed to protect gastric and duodenal mucosa against ethanol induced damages [4]. EPA was shown to be effective in reducing blood cholesterol [5]. Manipulation and control of the fatty acid composition of the biomass resulting in a lipid mixture rich in one of the desired fatty acids and poor in the other is a prerequisite for a biotechnological process of EPA or AA production from Porphyridium biomass, since equimolar concentrations of both fatty acids would mandate a prohibitively expensive separation process. In a recent study [2] we have shown that in laboratory grown P. cruentum, arachidonic acid prevails under conditions that result in reduced growth rate such as high temperature (30 °C), high cell concentration or low light intensity. In contrast, when the growth rate is maximal, EPA is the dominant fatty acid.

As previously reported [6-8], P. cruentum can be grown outdoors and sustain good production rates. The aim of this work was to further study the effects of cell concentration and temperature on the fatty acid composition under outdoor conditions.

MATERIALS AND METHODS

Outdoor Cultures P. cruentum strain 113.80 was obtained from the Gottingen algal culture collection and was cultivated on Jones' medium [9] in a 1 m^2 pond containing 120 l of medium stirred by a paddle wheel . During the winter, the ponds were covered with polyethylene lining to increase the ambient temperature.

Daily measurements of temperature, turbidity, oxygen, pH and chlorophyll were performed as previously described [11]. The pH of the cultures was maintained in a range of 7.2-7.8 by a stream of CO_2 gas. The nutrient concentration in the outdoor culture was estimated biweekly by analysis of PO_4^- and NO_3^-. Water loss by evaporation was replenished by daily addition of tap water.

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Fatty Acid Analysis Freeze-dried samples of Porphyridium were treated with methanol-acetyl chloride (95:5) according to Lepage and Roy (12). Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed on a SP-2330 fused silica capillary column (30 m, 0.2 mm) at 200°C (FID, injector and flame ionization detector temperatures 230°C, split ratio 1:100) and integrated with an HP 3390A integrator. Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma Co.) and by gas chromatography -mass spectrometry (GC-MS) performed in a Finnigan 500 mass spectrometer on a carbowax capillary column (30 m). Chemical ionization spectra were obtained at 250 ev with isobutane as reactant gas. Fatty acid contents were determined by comparing their peak areas with that of the internal standard. The data shown represent mean values of at least two independent samples, each analyzed in duplicate.

RESULTS AND DISCUSSION

The fatty acid composition of P. cruentum culture maintained outdoors for over a year was analyzed and was found to be modified both daily and seasonally, in relation to changes in cell concentration and temperature .

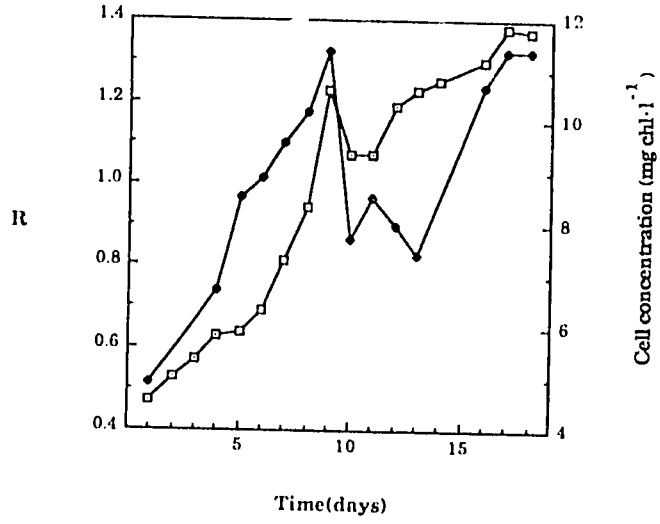

Fig. 1. Variations of R (A /EPA, ▫) value and cell concentration (◆) in a P. cruentum pond (July-August). Maximal daily temperature 28-31°c.

In the summer (July-September, maximum daily temperature range 28-31 °C) the culture was allowed to reach high cell concentrations (from 3 to 14 mg chl·l^{-1}) resulting mainly in an increase in AA and a decrease in EPA (Figure 1). The AA to EPA ratio (R) increased over this range from 0.47 to 1.64. Due to the limited variability in the maximal daily temperatures, the cell concentration effect on the R value could be resolved and were found to vary greatly. Along the course of a year, similar temperature and chlorophyll concentrations resulted at times in different R values. We believe the reason for this apparent irregularity may stem from the fact that the fatty acid composition reflects the environmental conditions of preceding days. Such a "memory effect" was demonstrated when dense cultures diluted to a low cell concentration displayed reduced R values only after a few days of adjusting. Similarly, when exponentially growing Porphyridium cultures were sampled along the growth curve, fatty acid composition was not dependent on cell concentration, and an increased R value was observed only towards the end of the exponential growth phase. Following a two days adjusting time , however, an effect of cell concentration could be readily observed.

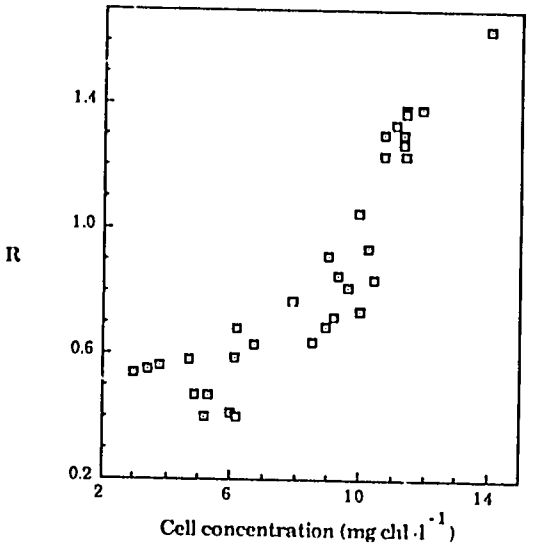

Figure 2. Correlation of R value and cell concetration in a P. cruentum pond. Maximal daily temperature 28-31°c. The data pertaining to harvest time as well as the following 2 days were excluded .

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To test the correlation of R to cell concentration, these parameters were plotted against each other, excluding the data evolved from the first two days following harvest (Figure 2). In this way, high R values were highly correlated with increased cell concentrations.

In the winter (December-February), daily maximum temperatures were 10-15°C, and cell concentration did not exceed 7 mg chl·l^{-1}. Variability in fatty acid composition was relatively low resulting in a low and narrow range of R values (0.25-0.39). Three cultures maintained at cell concentrations of 2.5 , 3.5 and 4.5 mg chl·l^{-1} displayed the same R value (Table 1).

The EPA content reached a maximum value of 50% of the fatty acids corresponding to 2.5% of ash free dry weight (AFDW) in December, while the concentration of arachidonic acid peaked in September reaching 30% of the fatty acids and 1.4% of AFDW.

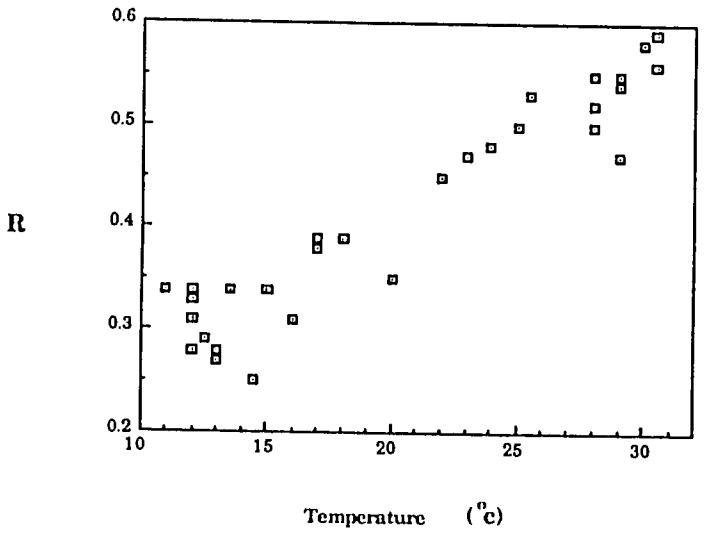

Figure 3 . Correlation of R value and maximal daily temperature in a P. cruentum pond .Data depicts cell concentrations corresponding to less then 5 mg chl ·l^{-1} sampled at least 3 days after harvest.

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The R value was also temperature-dependent although temperature exerted a lesser effect than the cell concentration. Since the dependence of R value on cell concentration is exponential , the variability in the R value at low cell concentration (1-5 mg chl·l^{-1}) is minimal. By plotting R values corresponding to low cell concentrations only (Figure 3) against maximum daily temperature, a temperature effect could be demonstrated.

In order to further probe the effect of temperature, two outdoor cultures were cultivated at winter maintaining identical cell concentrations. One culture was placed in a greenhouse (maximal daily temperature 25°C) while the other was not (17°C). The difference in the resulting R values for the two cultures was ca. 0.15 (Figure 4).

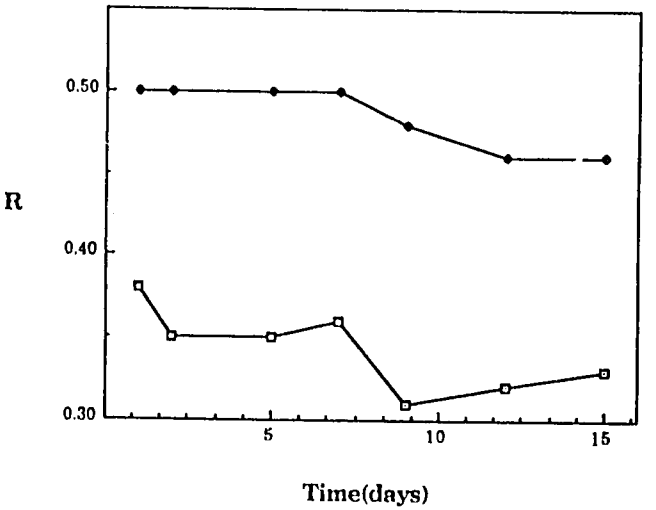

Figure 4. Effect of pond temperature on R values in P. cruentum Cultures were cultivated in a greenhouse (max. daily temperature 25±1°c) and in an uncovered pond (17±1 °c) , maintaining a ΔT of 8 °c .

An interesting observation resulting from these data is that the effects of temperature on outdoor cultures is different from what was found in the laboratory. While under controlled condition, the lowest R values were obtained at the optimal temperature for growth (25°C), the R value outdoors

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was found to be directly related to the maximum daily temperature the lowest R values being obtained at the lowest daily temperatures (12-15°C). The temperature regime outdoors however, is quite complex greatly differing from the constant temperature maintained in the laboratory. Also, the effects of other factors e.g. - day length and periodicity are not clearly understood , some of which may create this seeming discrepancy by affecting the fatty acid composition.

The R value and the fatty acid composition are primarily dependent on cell concentration and only to a lesser extent on temperature. This finding is of significant practical value as cell concentration is easier controlled than temperature.

In an attempt to estimate the EPA and AA output rate, three cultures of P. cruentum were maintained outdoors by daily dilution to cell concentrations equivalent to 3, 4.5 and 6 mg chl·l^{-1} for 14 days (Table 1). The highest EPA

TABLE 1
Effects of the season and the cell concentration on EPA and AA yields in open ponds of P. cruentum .

Cell[a] conc.	Fatty acid content (% of AFDW)		Output rate (AFDW g·m^{-2}·d^{-1})			
	EPA	A A	Biomass	EPA	A A	R[b]
WINTER[c]						
2.5	2.21	0.76	3.6	0.08	0.03	0.34
3.5	2.30	0.75	5.0	0.12	0.04	0.33
4.5	2.18	0.73	2.1	0.05	0.02	0.33
SUMMER[d]						
3.0	1.28	0.71	19.7	0.25	0.14	0.45
4.5	1.16	1.18	24.0	0.28	0.28	0.69
6.0	1.00	1.31	13.0	0.13	0.17	0.88

a. mg chl·l^{-1} ; b. AA/EPA ; c. max. daily temp. 16-18°c (Nov-Jan) ; d. max. daily temp. 28-31°c (Jul-Aug) .

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output rate (0.28 $g \cdot m^{-2} \cdot d^{-1}$) coincided with the higher biomass output rate. This was observed at a cell concentration corresponding to 4.5 mg $chl \cdot l^{-1}$. Cell concentration corresponding to 3 mg $chl \cdot l^{-1}$ was nevertheless considered superior since its EPA output rate was only slightly lower (0.25) where as its R value was much lower. R value reflects the extent of AA "contamination" and hence the relative difficulty in EPA purification . Clearly, the smaller the R the easier the separation from AA.

In a similar experiment conducted in the winter (cell concentration of 2.5, 3.5 and 4.5 mg $chl \cdot l^{-1}$), as the cell concentration effect on the R value was not observed at low cell concentrations ,the highest EPA output rate (0.12 $g \cdot m^{-2} \cdot d^{-1}$) was obtained in a culture with the highest output rate of biomass .

CONCLUSIONS

The data presented here demonstrates that the fatty acid composition of P. cruentum cultures can be manipulated outdoors as well as under controlled conditions. Keeping a low cell concentration in Porphyridium, results in an EPA rich fatty acid mixture which is relatively poor in AA. In contrast, a higher content of AA is obtained when cell concentrations is kept high in the summer. It thus seems feasible that a close control of cell concentration will result in either AA- or EPA-rich biomass.

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To Ms. Shoshana Didi and Ms. Rosa Milkis for algal cultivation and chemical analyses. This study was supported by the Israeli National Council for Research and Development.

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61

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EFFECT OF ENVIRONMENTAL CONDITIONS ON FATTY ACID COMPOSITION OF THE RED ALGA *PORPHYRIDIUM CRUENTUM*: CORRELATION TO GROWTH RATE[1]

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ABSTRACT

The lipid and fatty acid composition of Porphyridium cruentum *was determined as a function of light intensity, temperature, pH, and salinity. In cultures cultivated at the optimal temperature under non-limiting light conditions, eicosapentaenoic acid was the main polyunsaturated fatty acid. When growth rate was reduced by decreased light intensity, increased cell concentration, suboptimal temperature, suboptimal pH, or increased salinity, the content of eicosapentaenoic acid decreased and that of arachidonic acid increased, the latter becoming the major polyunsaturated fatty acid.*

Key index words: arachidonic acid; eicosapentaenoic acid; environment effects, microalgal mass cultivation; Porphyridium cruentum

The unicellular red marine alga *Porphyridium cruentum* contains arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3) (Pohl and Wagner 1958, Nichols and Appelby 1969, Nyberg 1985), both of which are relatively rare polyunsaturated fatty acids (PUFA). In preliminary experiments, we found that the fatty acid composition of *P. cruentum* changed drastically with temperature and culture density (Cohen et al. 1986). This result was consistent with that reported by Kost et al. (1984) who found variations of 50–100% in the fatty acid composition of this alga and with the recent report of Klyachko-Gurvich et al. (1985) of increased arachidonic acid accumulation in this alga, when grown under extreme conditions.

The effect of environmental conditions on the lipids and fatty acids of algae is not fully understood. Similar environmental factors have been shown to exert opposite effects. In *Nitzschia closterium* the content of 20:5 acid decreased when light intensity was increased (Orcutt and Patterson 1974), whereas in *N. palea* it increased (Opute 1973). In higher plants, an increase in irradiance causes an increase in the glycolipids that characterize chloroplast membranes. It is the 18:3 (n-3) acid that generally increases in the glycolipids (Harwood and Russell 1984), but in species lacking this acid, the concentration of other PUFA increases in response to increased light intensity. In *Euglena gracilis*, Pohl and Wagner (1972) found that, when photosynthesis was inhibited by DCMU, the total quantity of glycolipids and their 16:4 acid was drastically decreased. Other studies have also shown that the content of several unsaturated C-16 and C-18 fatty acids, which are prevalent in glycolipids, increased upon illumination (Rosenberg and Pecker 1964, Constantopoulos and Bloch 1967). The results of many studies on the effect of temperature on fatty acid composition (see review by Harwood and Russell 1984) clearly indicate that decreased temperatures result in increased fatty acid unsaturation.

Some PUFA have recently been suggested as having therapeutic value—the 20:5 acid for reducing blood cholesterol (Dyerberg 1986) and the 20:4 acid as a starting material for the synthesis of prostaglandin PGE_2 (Ahern 1984).

The feasibility of outdoor mass production of *P. cruentum* has already been studied (Golueke and Oswald 1962, Vonshak et al. 1985). In this work we studied the effect of light intensity, cell concentration, temperature, pH, and salinity on the fatty acid composition of *P. cruentum*. A clear correlation between growth rate and fatty acid composition was observed.

MATERIALS AND METHODS

Organism. P. cruentum strains 1380 1a–f and B113,80 were obtained from the Gottingen Algal Culture Collection. Stock cultures were maintained according to Vonshak (1986).

Growth conditions. Cultures were grown on Jones' medium (Jones et al. 1963) in glass tubes (80 × 5 cm), incubated in a temperature-regulated water bath (±1° C), illuminated (unless otherwise stated) with four cool-white fluorescent lamps providing 170 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the side of the water bath. The culture was mixed by bubbling an air-CO_2 mixture (99:1) through a sintered glass tube placed at the bottom of each culture tube. Cultures were also grown in Erlenmeyer flasks placed in a New Brunswick incubator shaker model G25 and illuminated from above at a light intensity of 115 $\mu E \cdot m^{-2} \cdot s^{-1}$, under air-$CO_2$ (99:1) atmosphere. For at least three days prior to sampling, the cultures were kept in the exponential stage by daily dilution. The specific growth rate was estimated by measurements of chlorophyll concentration and of turbidity (Vonshak 1986). The pH of the medium was kept constant with a buffer solution (20 mM Tris HCl + 10 mM 2(N-Morpholino)ethanesulfonic Acid (MES) for pHs 7.0–8.5 and 10 mM Tris-HCl + 20 mM MES for pH 5.5–6.5). Final pH was adjusted daily with 1 N NaOH.

Lipid extraction and fractionation. Freeze-dried samples of biomass were extracted with chloroform-methanol-water according to Bligh and Dyer (1959). Lipids were separated by TLC on 20 × 20 cm glass plates coated with silica gel-60 (Merck). Chromatography was carried out in light-protected Teflon-lined jars under an Ar atmosphere. Lipids were eluted with chloroform/ace-

[1] *Accepted: 4 April 1988.*
[2] Address for reprint requests.

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62

TABLE 1. *Effect of light intensity, cell concentration and temperature on fatty acid composition in* P. cruentum.

Temp (°C)	Light (μE m⁻² s⁻¹)	Cell conc. (mg chl L⁻¹)	Growth rate (day⁻¹)	Fatty acid composition (%) 16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5	R[a]
20	170	2	0.65	35.0	3.7	0.2	3.1	8.3	0.2	0.1	0.2	19.3	30.0	0.6
	170	28	S[b]	32.7	1.8	0.5	1.0	8.3	0.3	1.2	1.2	28.8	24.1	1.2
25	170	1.5	1.2	35.0	2.8	0.6	2.0	6.1	0.5	0.3	0.9	15.9	35.8	0.4
	170	12	0.77	36.8	2.3	0.5	0.7	5.8	0.4	0.6	0.6	18.1	34.1	0.5
	170	30	S[b]	33.3	1.4	0.3	1.0	9.0	0.2	0.7	1.7	27.3	25.1	1.1
30	170	1.5	0.98	38.9	2.9	1.1	1.5	11.0	1.0	0.5	0.8	18.6	23.7	0.8
	170	8	0.73	36.3	1.2	1.2	2.7	17.2	1.0	0.6	2.3	28.1	9.4	3.0
	30	3	0.30	33.9	0.1	0.1	2.3	12.0	0.1	0.2	1.5	42.1	7.8	5.4
	170	17	S[b]	31.0	0.5	1.5	7.7	18.5	0.3	1.6	3.3	32.7	2.9	11.

[a] Ratio of % 20:4 to % 20:5.
[b] Stationary culture.

tone/methanol/acetic acid/water (100:40:20:20:10) as the developing solvent (Kates 1972). 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. (1987). For analytical purposes, the bands were extracted with chloroform–methanol (9:1), and 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-naphthol for glycolipids and molybdate for phospholipids (Kates 1972). The neutral lipids fraction consisted of all the components of *Rf* values higher than 0.9 and contained tri-, di- and mono-glycerides, free fatty acids, and pigments.

Lipid transmethylation. Freeze-dried samples of *P. cruentum* (100 mg) were transmethylated with 2 mL of methanol/acetyl chloride (95:5), as previously described (Cohen et al. 1987). 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 at 80° C for 1 h. The vial contents were then cooled, diluted with 1 mL water, and extracted with 1 mL of hexane containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant. The hexane layer was dried over Na_2SO_4, evaporated to dryness under vacuum, and redissolved in hexane.

Fatty acid analysis. Gas chromatographic analysis was performed with a SP-2330 fused silica capillary column (30 m × 0.2 mm) at 200° C (injector and flame ionization detector temperatures 230° C, split ratio 1:100). The results were formulated with an HP 3390A integrator. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma) and by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed with a Finnigan 500 mass spectrometer 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, analyzed in duplicate.

RESULTS

The major fatty acids of *P. cruentum* were found to be 16:0, 18:2, 20:4 (n-6) and 20:5 (n-3) under all conditions tested.

An increase in cell concentration resulted in an increase in the proportions of the 18:2 and 20:4 fatty acids accompanied by a decrease in the 20:5 acid (Table 1). Under high light intensity, the calculated ratio R of the % of 20:4 to the % of 20:5 acid of a culture grown at a low cell concentration was 0.78, whereas the R for a culture maintained at a high cell concentration was 3.0. Cultivation under low light intensity produced similar results. The increase in the R value resulted mainly from the decrease in the 20:5 acid and to a lesser extent from the increase in the 20:4 acid (Table 1).

The change in temperature had a more complex effect than light. A temperature shift from the optimum of 25° C (Vonshak et al. 1985) to 20° C or to 30° C at low cell concentration resulted in a predictable reduction in the growth rate (Table 1). The effect of these temperatures on the fatty acids was an increase in R from 0.44 at 25° C to 0.64 at 20° C and to 0.78 at 30° C. The R value correlated with a reduction of about 20% and 46% of the growth rates at 30° C and 20° C, respectively, as compared with the growth rate at 25° C. The largest differences in R were observed at the stationary phase relative to the early exponential phase, i.e. a 14-fold increase at 30° C compared with a 2.5-fold increase at 25° C.

Cultivation of *P. cruentum* at pH values of 5-8.5 resulted in altered growth ratse. Cultivation at suboptimal pH effected an increase in the 20:4 and 18:2 acids and a decrease in the 20:5 acid (Fig. 1). The R value over this pH range increased from 0.64 at pH 7.6 to as high as 1.31 at pH 5.5. In a comparable experiment conducted under lower light intensity, the effect was even greater: at pH 8.5 and 5.0, R was 1.67 and 1.52, respectively. These values were even higher than the corresponding values obtained in a stationary culture at the same temperature and under optimum pH.

Increasing the NaCl concentration in the growth medium from 1M to 2M resulted in retarded growth, reduced fatty acid content and an increased R. NaCl concentrations lower than in the control medium had no effect on the growth rate or on the R value (Table 2).

Lipid fractionation (Table 3) showed the presence of the neutral and polar lipids, monogalactosylacyldiglyceride (MGDG), digalactosylacyldiglyceride (DGDG), and sulfoquinovosylacyldiglyceride (SQ). The phospholipids were phosphatidylcholine (PC), phosphatidylglycerol, phosphatidylethanolamine,

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63

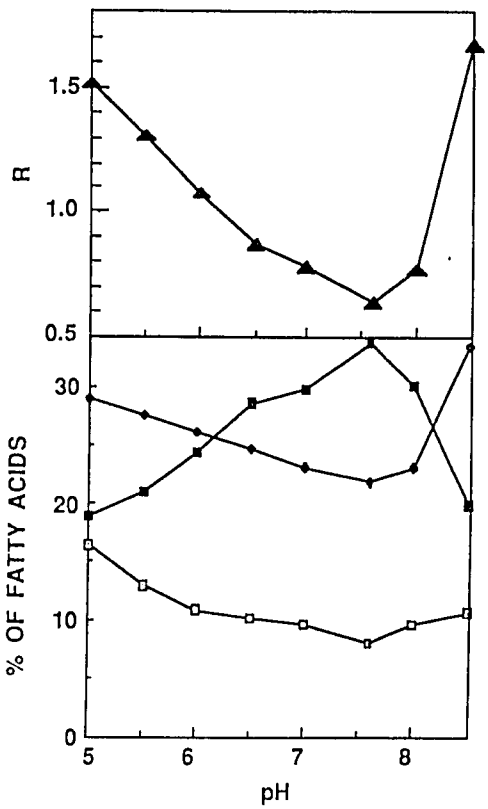

FIG. 1. Effect of culture pH on fatty acid composition of *P. cruentum* and the 20:4/20:5 ratio (R) (▲): 18:2 (□); 20:4 (◆); 20:5 (■). Cultures were cultivated in glass tubes (170 μE·m⁻²·s⁻¹).

and phosphatidylinositol. The latter three together comprised only 5–8% of the total lipid content and were not analyzed further. Fatty acid analysis of each lipid class showed that the 20:4 acid is concentrated in the neutral lipids and PC, whereas the 20:5 acid is found mainly in the glycolipids (Table 3).

In cells grown at the optimal temperature (25° C) and low cell concentration, the content of neutral lipids was minimal, and the major lipids were MGDG and DGDG. Increasing the temperature to 30° C resulted in an increase in the neutral lipid content and a decrease in the other lipids at low cell concentration and even more so at high cell concentrations. In the latter case, 58% of total lipids were neutral lipids. A high content of neutral lipids was also found in cultures cultivated at 28° or 30° C under low light intensity (data for 28° C not shown).

Under growth conditions conducive to fast growth rates, (25° C, low cell concentration), the 20:5 acid became the dominant PUFA in every lipid fraction analyzed but PC. In MGDG it represented 49.1% of the fatty acids. Under suboptimal growth conditions of light and temperature, the 20:5 acid content decreased sharply whereas the contents of the 18:2, 18:3, 20:3 and 20:4 acids increased. These effects were maximal during the stationary phase. Similar results were obtained for cultures grown at 28° C (data not shown).

The most significant effect of light and temperature stresses on the fatty acid composition was observed in the lipid composition of cells cultured at 30° C. Increasing the temperature to 30° C resulted in a marked increase of the 16:0 and 18:0 acids in both glycolipids and neutral lipid fractions. This effect was barely observable in the total lipid mixture due to the increase in the proportion of neutral lipids, which were relatively poor in the 16:0 acid.

DISCUSSION

The fatty acid composition of *P. cruentum* was influenced by environmental conditions. Under optimal conditions, which provide the fastest exponential growth, the dominant PUFA was the 20:5 acid. In contrast, under growth-limiting conditions (high cell concentration, low light intensity, nonoptimal temperature and pH, and increased salinity), the 20:4 and 18:2 acids became dominant. The level of this effect was maximal at the stationary phase. The relationships we found between fatty acid composition, environmental conditions and growth rates may explain the quantitative discrepancy between our results and those of Pohl and Wagner (1968), Nichols and Appelby (1969) and Ahern et al. (1983).

The results suggest that the previous reports were based on work conducted with either stationary or

TABLE 2. *Effect of NaCl concentration on growth rate and fatty acid content and composition in* P. cruentum. *Cultures were cultivated (25°C, 115 μE·m⁻²·s⁻¹) on an Erlenmeyer shaker.*

NaCl conc (M)	Specific growth rate (d⁻¹)	Total fatty acids (% of AFDW)[a]	Fatty acid composition (%)											R[c]
			16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5		
0.25	1.2	4.4	33.5	3.0	0.8	0.7	5.7	0.2	0.6	0.6	17.3	37.5		0.5
0.5	1.2	3.7	33.1	3.9	1.0	1.2	5.9	0.2	0.6	0.5	17.6	36.0		0.5
1	0.88	3.5	31.7	3.9	0.7	0.8	7.5	0.4	0.8	1.0	17.7	35.3		0.5
1.5	0.52	3.0	27.6	2.9	1.0	1.6	13.4	1.1	2.4	1.2	22.6	26.3		0.9
2	0.34	1.2	30.0	4.3	2.2	4.5	15.3	1.2	–[b]	1.4	22.5	18.9		1.2

[a] AFDW = ash-free dry weight.
[b] + = trace amount.
[c] Ratio of % 20:4 to % 20:5.

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64

TABLE 3. *Effect of temperature and cell concentration on fatty acid composition of various lipids[a] in* P. cruentum. *Cultures were cultivated at a light intensity of 170 μE·m⁻²·s⁻¹.*

Lip.	Temp (°C)	Cell conc. (mg chl L⁻¹)	Fatty acid composition (%)											% of total fatty acids
			16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5	R[b]	
Total	25	1.5	35.1	2.8	0.6	2.0	6.1	0.5	0.3	0.9	15.9	35.8	0.4	
	30	1.5	38.9	2.9	1.1	1.5	11.0	1.0	0.5	0.8	18.6	23.7	0.8	
	30	8	36.3	1.2	1.2	2.7	17.2	1.0	0.6	2.3	28.1	9.4	3.0	
Neutral	25	1.5	21.8	5.3	9.2	7.6	15.5	0.6	0.4	0.8	16.9	21.9	0.8	17
	30	1.5	20.8	1.5	8.3	4.8	20.6	1.7	+[c]	3.6	31.3	7.4	4.2	34
	30	8	25.7	1.1	6.0	4.5	22.9	2.1	0.6	3.2	29.8	4.2	7.1	58
MGDG	25	1.5	31.5	3.6	1.0	3.1	8.3	0.2	0.1	0.1	2.9	49.1	0.06	26
	30	1.5	47.3	2.2	2.8	2.1	9.8	+	0.9	+	6.5	28.4	0.2	18
	30	8	51.0	2.9	4.2	4.9	10.2	1.8	2.4	0.4	13.8	8.4	1.6	14
DGDG	25	1.5	46.9	8.6	0.8	2.2	4.2	0.4	0.2	0.1	1.4	35.2	0.04	26
	30	1.5	64.0	3.6	0.9	1.4	4.0	+	0.2	0.3	2.7	22.9	0.1	15
	30	8	69.5	2.6	1.9	2.6	5.2	1.3	0.1	0.1	8.5	8.3	1.0	9
SQ	25	1.5	55.7	3.0	2.9	8.8	4.7	+	1.2	+	2.5	21.2	0.1	13
	30	1.5	65.9	0.6	2.0	3.6	3.7	0.8	2.7	1.2	8.0	11.5	0.7	10
	30	8	68.8	0.8	5.8	5.1	9.1	0.7	2.7	1.0	3.3	2.6	1.3	8
PC	25	1.5	30.9	6.0	2.6	3.6	6.7	2.0	2.3	3.0	38.9	3.9	10	9
	30	1.5	34.7	2.0	2.1	3.1	9.5	0.9	+	2.0	42.3	6.4	6.6	8
	30	8	26.0	+	4.4	5.2	9.2	1.5	0.6	3.5	46.6	2.9	16	6

[a] MGDG, monogalactosylacyldiglyceride; DGDG, digalactosylacyldiglyceride; SQ, sulfoquinovosylacyldiglyceride; PC, phosphatidylcholine.
[b] Ratio of % 20:4 to % 20:5.
[c] + = trace amount.

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severely light limited cultures. The R values which we calculated for their data were in the range of 2.1 to 2.9, and the proportion of the 18:2 acid was 15–16% of total fatty acids. The rise in the content of the 18:2 acid was essentially a marker of the proportion of neutral lipids, since large quantities of the 18:2 acid were found only in this lipid class. High levels of the 18:2 acid can thus be correlated with high levels (>50%) of neutral lipids, which accumulate only under slow growth conditions. The relationship of fatty acid composition to growth rate also explains the variations of 50 to 100% in the fatty acid composition found by Kost et al. (1984) for *P. cruentum* cultures grown under apparently identical conditions. We found that the fatty acid composition changed as batch cultures became dense and light became growth limiting. In contrast, in a chemostat culture in which an exponential growth rate was kept constant, the fatty acid composition also remained constant (data not shown).

It appears that the relative variations described here in the proportions of the 20:4 and 20:5 acids in response to environmental factors are not limited to *P. cruentum*. An analysis of the data of Pohl and Zurheide (1982) and of Kayama et al. (1986) reveals similar variations in the R value of various algae in response to changes in light or temperature. A controversy exists regarding the fatty acid composition of the red macroalga *Gracilaria confervoides*. Pohl and Wagner (1968) and Araki et al. (1986) reported that the 20:4 acid was the main PUFA, whereas Sato (1975) and Hayashi et al. (1974) claimed the 20:5 acid to be the main PUFA. We suggest that this disagreement could possibly stem from the different environmental conditions that govern the growth rate of the alga.

The increase of the 20:5 acid in response to increased light intensity is analogous with the previously mentioned increase in the 18:3 (n-3) acid in plants and algae in response to enhanced illumination (Rosenberg and Pecker 1964, Constantopoulos and Bloch 1967). Kates and Volcani (1966) suggested that in diatoms the 20:5 acid fulfills a role in photosynthesis similar to that of the 18:3 acid (n-3) in other plants and algae. *P. cruentum* does not contain a significant quantity of the 18:3 (n-3) acid, and the 20:5 acid may have an analogous role to that of the 18:3 acid. When growth is retarded, less 20:5 acid and glycolipids are produced. It appears, however, that in *P. cruentum* this effect relates to the growth rate rather than directly to the light intensity, since it occurs not only as a result of light limitation but also in response to any factor limiting growth, such as nonoptimal pH and salinity (Fig. 2), which affect an overlapping R curve.

The temperature effect on the fatty acid composition of *P. cruentum* is similar to the effects of other factors affecting growth. The effect of cultivation at 30° C on fatty acid composition in terms of increased R and 18:2 acid was, however, more intense than could be expected on the basis of growth rate retardation alone. The increase in unsaturation at the lower growth temperatures was explained by the need to compensate for the decrease in membrane fluidity at low temperatures (Marr 1962). The influence of temperature on the content of the 20:5

65

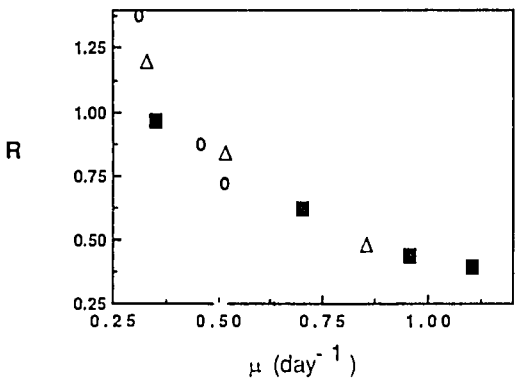

FIG. 2. Correlation between the specific growth rate (μ) and the 20:4/20:5 ratio (R) in *P. cruentum* as affected by the environmental variables, cell concentration (■), salinity (△) and pH (○).

acid seems to be more intense than on that of the 20:4 acid, since most of the 20:5 acid was found in glycolipids, which are the main membrane lipids. This may result in preferential desaturation towards the 20:5 acid. We suggest that temperature exerts a two fold effect on the composition and content of the fatty acids. First, any deviation from the optimum leads to reduction in the growth rate and to an accumulation of the 18:2 and the 20:4 acids as well as a decrease in the 20:5 acid. Second, the direct effect of high temperature (30° C) is expressed in *P. cruentum* by a rise in saturated fatty acids (16:0 and 18:0) and in a reduction in the 20:5 acid. Thus, at supraoptimal temperatures, the two effects will be similar, whereas at suboptimal temperatures the contents of the 16:0 and 18:0 acids are reduced and that of the 20:5 acid increases. The direct effect of temperature on fatty acid composition and the temperature effect on the growth rate and thus on the fatty acids oppose each other and as a result the increases in the 20:4 acid and in the value of R at 20° C are rather modest.

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66

Production of Eicosapentaenoic and Arachidonic acids by the Red Alga *Porphyridium Cruentum*

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The red microalga *P. cruentum* is a potential source for the pharmaceutically valuable fatty acids eicosapentaenoic acid and arachidonic acid. The conditions leading to a high content of either fatty acid were investigated. Strain 1380-1d produced high eicosapentaenoic acid content (2.4%, w/w) under conditions resulting in a high-growth rate. High arachidonic acid content (2.9%) was obtained under slow growth conditions in the stationary phase or under nitrogen starvation. Strain 1380-1a produced 1.9% w/w arachidonic acid under exponential conditions. By imposing nitrogen starvation, it was possible to obtain a lipid mixture enriched with arachidonic acid and eicosapentaenoic acid.

Arachidonic acid (AA, 20:4w6) and eicosapentaenoic acid (EPA, 20:5w3) are rare fatty acids of potential pharmaceutical value. AA was suggested as a precursor for biosynthetic production of prostaglandin PGE_2 (1). EPA was shown to be effective in preventing blood platelet aggregation (2) and is claimed to be useful for blood cholesterol reduction (3).

EPA is found in marine fish oils, yet the low EPA content and 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) as present in *Euglena gracilis*, *Ochromonas danica* and in the red microalga *Porphyridium cruentum*, which also contained EPA.

P. cruentum was studied as a source for sulfated polysaccharides (7,8), which could be utilized for enhanced oil recovery from oil wells. It also contained phycoerythrine, a red protein pigment used for immunofluorescent detection of tumors.

We have found that the fatty acid composition of *P. cruentum* was highly dependent on environmental conditions. Contrary to previous reports, EPA was the main polyunsaturated fatty acid (PUFA) found in cultures cultivated at optimal temperature and nonlimiting light conditions. When growth was slowed by increased light intensity, increased cell concentration, nonoptimal temperature, pH or salinity, the content of EPA decreased, and AA became the major PUFA. This work reports the potential of various strains of *P. cruentum* as a source of EPA and AA, and the growth conditions under which maximum productivity of each of these fatty acids can be obtained.

MATERIALS AND METHODS

P. cruentum strains 1380 1a to f and B113.80 were obtained from the Göttingen Algal Culture collection. Maintenance of stock cultures and inocula preparation were performed according to Vonshak (9). Cultures were grown on Jones' medium (10) in a temperature-regulated water bath illuminated with fluorescent lamps providing 170 $\mu mE.m^{-2}.s^{-1}$ at the side of the bath. The cultures were mixed by bubbling an air-CO_2 mixture (99:1, v/v) through a sintered glass tube placed in the bottom of each culture tube. For the nitrogen starvation experiments, cultures in the exponential phase of growth (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 stage and maintained at steady state by daily dilution for at least three days before the onset of the experiment. Growth rate was estimated by chlorophyll and turbidity measurements.

Fatty Acid Analysis

Gas chromatographic analysis was performed on a SP-2330 fused silica capillary column. Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma Chemical Co., St. Louis, MO) and by gas chromatography-mass spectrometry (GC-MS). Fatty acid contents were 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

Under optimal conditions, the seven strains of *P. cruentum* had similar growth rates, ca. 1.1 d^{-1}, (Table 1). The EPA content ranged from 1.85% to 2.44% of ash-free dry weight (AFDW). The range of AA/EPA values in the exponential phase (0.35-0.47) was lower than the range at the stationary phase (0.9-2.1) because AA increased, and the quantity of EPA decreased. The highest EPA content was found in strains 1380-1b and 1d. At 30 C, exponentially cultivated strains contained ca. 39.2% AA and 12.1% EPA on average (Table 2). However, total fatty acid content was in most cases the highest in the stationary phase at 30 C. In strain 113.80, total fatty acid content increased from 4.2% in the exponential phase to 7.7% (AFDW) in the stationary phase. The R-value (11.5) at the stationary phase in strain 113.80 was the highest value observed.

Nitrogen Starvation

When the exponentially cultivated *P. cruentum* strain 113.80 was transferred to a nitrogen-free medium, the total fatty acid content increased to 8.8% (AFDW) after four days. The fatty acid composition resembled that observed at the stationary phase. The content of AA increased to 2.9% and that of EPA decreased to 0.9% (AFDW).

The major effect of the nitrogen starvation on the lipid concentration of *P. cruentum* strain 113.80 was the increase in neutral lipids from 20.6% in the control cul-

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67

TABLE 1

Fatty Acid Composition and Content in *P. cruentum* Strains Cultivated at 25 C

Strain	Growth phase[1]	μ[2] (d^{-1})	Fatty acid content: Total	AA	EPA	AA/EPA
		d^{-1}	% w/w ash-free dry wt.			
1380-1a	E	1.05	5.34	0.84	1.89	0.6
	S	—	5.83	1.91	1.12	1.7
1380-1b	E	1.13	5.61	0.86	2.44	0.4
	S	—	5.36	1.58	1.24	1.3
1380-1c	E	1.13	5.38	0.91	2.13	0.4
	S	—	5.35	1.27	1.48	0.9
1380-1d	E	1.15	5.47	0.81	2.41	0.3
	S	—	5.05	1.24	1.36	0.9
1380-1e	E	1.39	5.34	0.98	2.10	0.5
	S	—	5.65	1.89	0.92	2.1
1380-1f	E	1.10	5.15	0.91	2.06	0.4
	S	—	5.10	1.69	0.91	1.8
113.80	E	0.98	4.44	0.80	1.85	0.4
	S	—	5.10	1.39	1.30	1.1

[1]E, exponential phase, 4-5 mg l^{-1} chlorophyll; s, stationary phase, 27-30 mg l^{-1} chlorophyll.
[2]Growth rate, turbidity.

TABLE 2

Fatty Acid Composition and Content in *P. cruentum* Strains Cultivated at 30 C

Strain	Growth phase	μ	Fatty acid content: Total	AA	EPA	AA/EPA
		d^{-1}	% w/w ash-free dry wt.			
1380-1a	E	0.67	4.84	1.95	0.32	6.1
	S	—	6.23	2.18	0.60	3.7
1380-1b	E	0.79	3.78	1.49	0.54	2.8
	S	—	4.67	1.95	0.31	6.4
1380-1c	E	0.80	4.42	1.80	0.52	3.4
	S	—	5.42	2.01	0.82	2.3
1380-1d	E	0.70	5.39	1.78	1.02	1.7
	S	—	4.74	1.84	0.40	4.5
1380-1e	E	0.76	4.30	1.78	0.55	3.2
	S	—	6.48	2.45	0.26	9.4
1380-1f	E	0.49	4.46	1.85	0.55	3.3
	S	—	5.53	2.23	0.29	7.8
113.80	E	0.62	4.19	1.62	0.30	5.2
	S	—	7.67	2.48	0.22	11.5

ture to 61.0% in the N-starved culture with a concurrent decrease in the glycolipids. A major increase in AA was noted in the neutral lipids and in phosphatidylcholine. Both AA and EPA decreased proportionally in the glycolipids. The combined fraction containing neutral and phospholipids was rich in AA (33.0% of total fatty acid). The glycolipids remained rich in EPA and low in AA.

TABLE 3

Effect of Nitrogen Starvation on the Fatty Acid Composition in *P. cruentum* Strains T13.80[1]

Statistic	Lipid: NL[2] +N[5]	NL[2] -N[6]	GL[3] +N	GL[3] -N	PC[4] +N	PC[4] -N
EE/APA	2.4	7.6	0.1	0.2	2.7	21.4
% of Total	20.6	61.0	66.6	21.8	7.7	8.7

[1]Cultivated at 28 C, 170 $\mu E\ m\ s^{-1}$.
[2]Neutral lipids.
[3]Glycolipids monogalactosyldiacylglycerol, digalactosyldiacylglycerol plus sulfolipid.
[4]Phosphatidylcholine.
[5]Nitrogen sufficient.
[6]Three day nitrogen starvation.

DISCUSSION

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 and the R-values at the exponential and at the stationary phases. The combination of growth rate and EPA content yields the EPA production rate. In an unpublished study, it was 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, the easier the separation of EPA from AA. The differences in the corresponding R-values for the exponential and stationary phases in the various strains could be utilized as an indicator of EPA contents under other growth conditions.

On the basis of these data, strain 1380-1d was judged to be the most promising as far as EPA production and relative purity are concerned. 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

High AA contents were obtained by inducing light limitation (low light or high cell concentration) and under increased temperature, reduced pH or increased salinity (Z. Cohen, A. Vonshak and A. Richmond, unpublished data). The intensities of these effects was found to be maximal at the stationary phase. Cultivation under low pH or high salinity resulted however in sharp reductions in the total fatty acid content.

For large-scale production of AA in outdoor ponds of *Porphyridium*, cultivation at 30 C presents a favorable option, as one stage exponential cultivation is possible. Under these conditions, strain 1380.1a had the highest AA content (1.95%). However, cultivation at this temperature outdoors could be prohibitively expensive in the winter.

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AA enrichment also could be achieved by nitrogen starvation. In N-starved *P. cruentum* cultures, the content of EPA in the various lipids decreases, while that of AA increases in neutral lipids and in phospholipids but not in glycolipids. This is contrary to the situation found when growth is retarded by other means (i.e light, temperature, pH) and where the content of AA increases in all lipids. The practical outcome of this phenomenon is that while the increases in R-values in the neutral lipids and in PC result in a relatively pure AA in these lipids, the glycolipids are still low in AA and could be separated and used as a source of a high-quality EPA.

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 monoalgal culture. Harvest is less problematic because the alga have a tendency for autofloculation, which could be enhanced by pH reduction (11). Another important factor in the assessment of the feasibility of large-scale *Porphyridium* production is the possibility to obtain several products from the biomass. The presence of sulfated polysaccharides and phycoerythrine also should encourage outdoor cultivation of *Porphyridium*.

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69

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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 immunofluorescent 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

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70

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	7.5	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	1.5	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	32.6	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	38.1	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	41.1	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

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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 co-chromatography 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 digalactosyldiglyceride (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		NL[b]		Lipid fraction: 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 $\mu E\ m^{-2}\ s^{-1}$.
[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.

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72

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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173

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Communication

Δ6 Desaturase Inhibition[1]

A Novel Mode of Action of Norflurazone

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ABSTRACT

The herbicide Norflurazone was shown to be an inhibitor of fatty acid of the Δ6 desaturation system. Treating cultures of the microalgae *Spirulina platensis* or *Monodus subterraneus* with this herbicide brought about a reduction in the level of γ-linolenic acid and in an increase in the level of linoleic acid. In *Monodus*, the increase in linoleic acid made it more available for ω3 desaturation, resulting in an increase in the proportion of eicosapentaenoic acid. The proportion of arachidonic acid did not decrease albeit the drastic decrease in its precursor γ-linolenic acid.

The interest in fatty acids of the ω6 family derives from the fact that they are precursors for prostaglandins, thromboxanes, and leukotrienes (5). GLA[2] (18:3ω6) was also shown to alleviate the symptoms of the premenstrual syndrome (6). Various plant species contain high levels of fatty acids of this group. Thus, GLA is found in evening primrose (14), black currant (13), and in a few cyanobacteria, *e.g. Spirulina* (8). AA (20:4ω6) is found as a major fatty acid component in a few algal species, *e.g. Porphyridium* and *Ochromonas* (9). Our interest in polyunsaturated fatty acids production by microalgae prompted us to search for potential fatty acid desaturase inhibitors.

Several substituted pyridazinones were shown to be potent herbicides (4). They were grouped by St. John *et al.* (12) according to their structural characteristics and activities in inhibiting growth, Hill reaction, chloroplast ribosome formation, and the formation of linolenic acid (18:3ω3) via inhibition of the Δ15 desaturation of linolenic acid (18:2ω6). Some of these herbicides were also shown to inhibit carotene biosynthesis and Chl accumulation (3). The pyridazinone San 9785 is the most effective inhibitor of Δ15 desaturation and its effect on reduction of 18:3ω3 levels in the glycolipids of higher plants and algae was widely studied (7). The pyridazinone Norflurazone was shown to be the most effective inhibitor of carotenoid biosynthesis and Chl accumulation. Its inhibitory activity on Δ15 desaturation is low, relative to other members of its group (12).

In this work, we suggest that Norflurazone is capable of yet another mode of action, namely, the inhibition of Δ6 desaturation. To the best of our knowledge, this effect has not yet been reported. The rarity of GLA and AA, which are the eventual products of the Δ6 desaturation in algae, could be the reason for overlooking this effect.

MATERIALS AND METHODS

Algal strains were obtained from the University of Texas culture collection. *Spirulina platensis* strain 2340 was grown on Zarrouk's medium at 30°C as previously described (1). *Monodus subterraneus* was cultivated on BG-11 medium at 25°C. A solution of Norflurazone in DMSO was added to exponentially growing cultures. The final concentration of DMSO did not exceed 1%. The biomass was harvested after 4 d, freeze dried, and transmethylated with methanol-acetyl chloride and the fatty acid composition was analyzed by gas chromatography (10). The data shown are mean values of at least two independent samples, analyzed in duplicate.

RESULTS AND DISCUSSION

Norflurazone reduced markedly the growth of *Spirulina platensis* and *Monodus subterraneus* and had a notable inhibitory effect on pigment accumulation (data not shown) and total fatty acid content. In the presence of 15 μM Norflurazone in *S. platensis* cultures, the total fatty acid content was reduced from 3.5% (of dry weight) to 1.6%. Furthermore, the profile of the fatty acids changed markedly, resulting in a decrease of 18:3ω6 from 21.7% (percent of total fatty acids) to 12.7% (Table I) and in an increase of 18:2ω6 from 23.0% to 25.7%. The levels of 18:0 and 18:1ω9 too increased from 1.6% to 4.7% and 0.80% to 2.5%, respectively. The reduction in 18:3ω6 was even more evident at higher herbicide concentrations (Table I). The 18:2/18:3 ratio rose from 1.06 to 3.05 in the presence of 150 μM Norflurazone. A possible explanation for the decrease in 18:3ω6 and the accumulation of its precursors may be that Norflurazone inhibits the Δ6 desaturation of 18:2ω6. However, since Norflurazone was shown to have some inhibitory activity on ω3 desaturation (12), its specificity

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[2] Abbreviations: GLA, γ-linolenic acid; x:y, fatty acyl group containing x carbons with y double bonds; AA, arachidonic acid.

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75

for the Δ6 desaturase enzyme was further studied on the microalga *M. subterraneus*. In this alga, further desaturation of 18:2ω6 is conducted either by the Δ6 desaturase or Δ15 (ω3) desaturase enzyme systems resulting in 18:3ω6 or 18:3ω3, respectively, ultimately leading to 20:4ω6 and 20:5ω3 (Fig. 1). *M. subterraneus* cultures were treated with concentrations of the herbicide ranging from 3 to 150 μM (Table II). At 3 μM, the level of 18:3ω6 decreased significantly from 1.5 to 0.15%, while that of 18:2ω6 increased from 2.0 to 2.9%, resulting in an increase of the 18:2/18:3 ratio from 1.4 to 19.5. The level of 20:5ω3 rose from 31.4 to 35.4% and that of 16:0 decreased from 24.4 to 20.0%. At the highest Norflurazone concentration tested, 20:5ω3 reached levels as high as 39.7%.

The inhibition of Δ6-desaturation, which is manifested in the increase of 18:2ω6 and the decrease of 18:3ω6, is quite evident. However, one would expect the level of 20:4ω6, which is the ultimate product of the ω6 pathway, to be reduced as well. Yet, its level slightly increased. This discrepancy needs further clarification. Phospholipids are important membrane constituents and are necessary for its integrity. It may be suggested that 20:4ω6 fulfills an important role in this respect which may be crucial for the survival of the organism. Indeed, in *M. subterraneus* as well as in *Ochromonas danica* 20:4ω6 is localized in phospholipids, mainly in phosphatidyl ethanolamine while in *P. cruentum* it is mainly found in phosphatidyl choline (9). Furthermore, Cohen *et al.* (1) have recently shown that in *P. cruentum*, when growth rate was slowed down the level of 20:4ω6 increased significantly. Thus, in *M. subterraneus*, when the availability of its precursor 18:3ω6 is limited due to the inhibition of Δ6-desaturation and at the same time the total fatty acid content is reduced there is still a demand for 20:4ω6 production. This demand may be answered by an increase in the fatty acid influx 16:0 toward 18:2ω6. As a result, the level of 20:4ω6 is maintained or even slightly increased. Moreover, if Norflurazone inhibits the Δ6 desaturation of 18:2ω6, then more of it will be available for Δ15 desaturation, resulting in an increased production of 18:3ω3 that is subsequently converted to 20:5ω3, which is indeed the case (Table II). The increased level of 20:5ω3 does not necessitate an increase in the steady-state level of 18:3ω3. Its level is low even under normal conditions when the level of 20:5ω3 is already high. This may suggest a rapid turnover of 18:3ω3.

It was shown that there are two sites for ω3 desaturation of 18:2ω6, one which affects galactolipids while the other affects phospholipids (11). San 9785 inhibits only the desaturation step associated with galactolipids. There may also exist two distinct Δ6 desaturation mechanisms only one of which is associated with galactolipids and is inhibited by Norflurazone. The noninhibited Δ6 desaturation pathway may be responsi-

Table I. *Effect of Norflurazone on the Fatty Acid Composition in Spirulina*

Cultures of *Spirulina* (strain 2340) were cultivated at 30°C as previously described (1). A solution of Norflurazone in DMSO was added to exponentially growing cultures to a final concentration of 1% DMSO. The filaments were harvested after 4 d, freeze dried, and transmethylated with methanol-acetyl chloride and the fatty acid composition was analyzed by GC (12). The data shown are mean value of at least two independent samples, analyzed in duplicate.

Fatty Acid	Fatty Acid Composition			
	Norfluorazone concentration (μM)			
	0	3	15	150
	%			
14:0	0.9	1.7	1.4	2.1
16:0	44.8	41.6	45.3	45.5
16:1	6.6	7.6	5.1	3.4
16:3ω4	0.4	0.5	0.2	0.4
16:4ω1	0.3	0.2	0.2	0.2
18:0	0.7	1.8	2.2	2.5
18:1ω9	1.6	1.9	2.4	6.3
18:1ω7	0.2	4.3	4.7	4.1
18:2ω6	22.9	24.1	25.7	26.8
18:3ω6	21.7	16.3	12.7	8.8
TFA[a]	3.5	1.2	1.6	1.7

[a] Total fatty acid, % of dry weight.

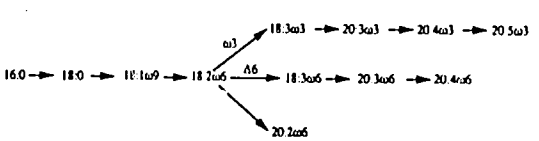

Figure 1. Scheme of major pathways of fatty acid biosynthesis in *M. subterraneus*.

Table II. *Effect of Norflurazone on the Fatty Acid Composition in M. subterraneus*

M. subterraneus cells were grown on BG-11 medium at 25°C. For inhibitor introduction and fatty acid analysis, see legend of Table I.

Fatty acid	Fatty Acid Composition		
	Norflurazone concentration (μM)		
	0 (0.82)[a]	3 (0.17)	150 (0)
	%		
14:0	2.1	1.8	2.3
16:0	24.4	20.0	19.8
16:1ω7	21.6	19.0	17.2
16:1tω13	0.5	0.6	0.9
16:2ω6	0.4	0.5	0.4
16:3ω4	0.3	0.5	0.6
16:3ω1	0.2	0.2	0.3
18:0	0.8	1.4	1.1
18:1ω9	7.0	6.8	6.1
18:1ω7	0.7	0.8	1.3
18:2ω6	2.0	2.9	2.7
18:3ω6	1.5	0.2	0.1
18:3ω3	0.4	0.4	0.4
18:4	0.3	0.4	0.5
20:0	0.2	0.1	0.1
20:2ω6	t	0.1	0.1
20:3ω6	0.6	0.2	0.1
20:4ω6	5.6	6.4	6.4
20:5ω3	31.4	35.4	39.7
TFA	11.4	6.5	5.8

[a] Value in parentheses is specific growth rate (d^{-1}). [b] Trace amount. [c] Total fatty acid, % of dry weight.

76

ble for the fact that the level of 20:4ω6 in *Monodus* was kept unchanged. In cyanobacteria, there is only one mechanism for ω3 desaturation of 18:2ω6 and it involves galactolipids. Indeed, in Norflurazone treated *Spirulina* cultures, the level of 18:3ω6 is decreased as there is no way to overcome this inhibition.

Norflurazone is known for its inhibitory effect on pigment accumulation. One may argue, therefore, that the inhibition of fatty acid desaturation is but a consequence of its effect on the pigments. However, St. John (14) pointed out that the effects of substituted pyridazinones on pigment and 18:3ω3 accumulation are related to different activities: the inhibition on pigment accumulation is associated with the trifluoromethyl substitution on the phenyl ring, while the effect on ω3 desaturation of 18:2ω6 is associated with other substituents of the pyridazinone structure.

Other possible interpretations for the effect of Norflurazone might include a shunt in the fatty acid flux via 20:2ω6 to 20:4ω6 (Fig. 1), a process which does not occur under normal conditions (9), a redistribution of 20:4ω6 in various lipid species, and/or a slow down of phospholipid turnover rate. Although these pathways cannot be negated, the above outlined hypothesis seems to be the most attractive at present.

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77

Preparation of Eicosapentaenoic Acid (EPA) Concentrate from *Porphyridium cruentum*

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The polyunsaturated fatty acid eicosapentaenoic acid (EPA) has attracted increased attention due to its pharmaceutical properties. The main source is marine fish oil which contains approximately 15% EPA. However, pharmaceutical applications of EPA will probably require higher concentrations, perhaps as high as 90%. The red microalga *Porphyridium cruentum* is a potential source, because its EPA content approaches 44.1% of the total fatty acids. Three methods were attempted for EPA concentration and arachidonic acid (AA) removal from the oil of this alga. Separation of the glycolipids, formation of a urea inclusion complex and reverse phase chromatography on C-18 Sep-Pak filters resulted in an EPA concentrate of 97% purity. Similar methods resulted in an AA concentrate of 80% purity.

KEY WORDS: AA, algal oil, EPA, *Porphyridium*, PUFA purification.

The polyunsaturated fatty acids eicosapentaenoic acid (EPA) and arachidonic acid (AA) have attracted increased attention in recent years. EPA was shown to reduce blood pressure in rats (1,2) as well as in humans (3,4). It has been suggested that EPA competitively inhibits the conversion of n-6 fatty acid precursors to AA, suppresses the production of eicosanoids of the 2 series (5) and increases the formation of eicosanoids of the 3 series, some of which are hypotensive (6). EPA was also found to reduce blood cholesterol levels (7). AA serves as a starting material for a biosynthetic production of prostaglandin PGE_2. Also, being a component of human mother's milk, it is of potential value as an ingredient in various formulations of artificial baby food.

The main source of EPA is marine fish oil which contains approximately 15% EPA. However, it contains also significant amounts of cholesterol. Daily consumption of marine fish oil eventually results in an increased cholesterol uptake which could be counterproductive for reducing blood lipid level. Pharmaceutical applications of EPA will probably require preparations of considerably higher EPA concentrations. An n-3 fatty acid concentrate was made from cod liver oil by urea inclusion (8). Although the product contained 85% n-3 fatty acids, only 28% consisted of EPA.

The red microalga *Porphyridium cruentum* is a potential source for the pharmaceutically valuable fatty acids EPA and AA (9). In previous studies we elucidated the environmental conditions leading to high contents of either EPA or AA (9–11). In this paper, methods are described for the fractionation of *P. cruentum* oil resulting in EPA and AA concentrates.

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MATERIALS AND METHODS

Growth of cells: P. cruentum strain 1380.1d was obtained from the Göettingen Algal Culture collection (Göettingen, Germany). Cultures were grown on Jones' medium (12) in glass tubes that were incubated in a temperature-regulated water bath and illuminated with four cool-white fluorescent lamps providing 170 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the side of the water bath. The cultures were mixed by bubbling an air — CO_2 mixture (99:1) through a sintered glass tube placed at the bottom of each tube.

Lipids fractionation. Freeze-dried samples of biomass were extracted with chloroform-methanol-water (2:1:0.8) according to Bligh-Dyer (13). Separation into neutral lipid, glycolipid (GL) and phospholipid classes was achieved on a silica gel column. The individual classes were successively eluted with chloroform, acetone and methanol, respectively.

Lipid transmethylation. The GL fraction (0.65 g) was treated with 13 mL of methanol-acetyl chloride (95:5) at 0°C. The mixture was sealed in a light-protected Teflon-lined vial under an Argon atmosphere and heated to 80°C for 1 hr. The vial contents were then cooled, diluted with 5 mL water and extracted with 5 mL of hexane containing 0.01% butylated hydroxytoluene. 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 Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200°C (injector and flame ionization detector temperatures 230°C, split ratio 1:100). The results were formulated with an HP 3390A integrator. For quantitative analyses, 17:0 methyl ester was added as an internal standard. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma, St. Louis, MO) and by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed with a Finnigan 500 mass spectrometer 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 data shown are mean values of at least two independent samples, each analyzed in duplicate.

Fractionation with urea. One gram of fatty acid methyl esters (FAME) was dissolved in 10 mL methanol containing 4 gram urea (E. Merck, Darmstadt, Germany) by heating the mixture to 65°C until clear. The urea and the urea inclusion complexes were allowed to crystallize at room temperature and then were refrigerated overnight at 4°C and then at −15°C. The mother liquor was separated by vacuum filtration and was extracted with 3 mL methylene chloride after addition of 6 mL water and 3 mL concentrated hydrochloric acid. The lower phase was separated and the aqueous

78

layer was reextracted with methylene chloride. The combined extracts were evaporated to dryness and dissolved in methanol.

Reverse phase chromatography. C-18 Sep pak filters (Waters Associates, Milford, MA) were preconditioned with 5 mL each of acetonitrile, water and 25% acetonitrile-water. A solution of 1 mg of the FAME mixture in 100 μL methanol was loaded onto the filter and eluted by washing the column with 10 mL vol of acetonitrile-water mixtures of increasing acetonitrile concentrations (58%–100%), at a flow rate of approximately 5 mL/min. Heptadecanoic acid methyl ester was added as an internal standard. Subsequently, each fraction was extracted twice with methylene chloride and water. The combined organic phases were evaporated to dryness, reconstituted in hexane and analyzed by GC.

RESULTS AND DISCUSSION

A preliminary evaluation of several strains of *P. cruentum* indicated that strain 1380.1d was the most promising EPA producer (11). It had the highest content of EPA, i.e., 2.4% of ash-free dry weight. This strain had also a relatively low AA content. The AA to EPA ratio (R) reflects the degree of difficulty in the purification of EPA. The lower the R value, the easier the separation from AA.

In previous studies we have demonstrated that by environmental and nutritional control it is possible to either increase or decrease the EPA and AA content and thus the R value (9,10). Under optimal growth conditions EPA was found to be the major fatty acid, while under reduced growth rate AA becomes the main fatty acid.

P. cruentum strain 1380.1d was cultivated under conditions leading to a high EPA content, i.e. a temperature of 25°C, high light intensity and low cell concentration. Under these conditions, the extracted oil contained 40.7% EPA (% of total fatty acids) and 14.6% AA. Moreover, approximately 90% of the EPA was restricted to the GL while the AA was primarily contained in the neutral lipid and the phospholipid fractions. Therefore, in order to purify EPA from other fatty acids and especially from AA, a preliminary fractionation of the GL from the oil was undertaken. In the acetone fraction which included the GL, the proportion of EPA increased to 47.5% while that of AA decreased from 14.6% to 5.4% (Table 1).

EPA enrichment by urea fractionation. The GL fraction was transmethylated and, by forming urea inclusion complexes, almost all of the saturated and monounsaturated fatty acids were effectively removed. The EPA and AA contents increased to 81.9% and 6.6% respectively. It is interesting to note that the removal was more efficient for C-18 FAME than for C-16 FAME (Table 2). Also, while the urea inclusion complexes are primarily formed with saturated and monounsaturated fatty acids, the inclusion complexes contained some linoleic acid and AA, while EPA was completely excluded. Thus, not only was EPA purified from saturated and monounsaturated FAME, but a significant purification from AA was also achieved, as is evidenced in the R values which decreased from 0.11 to 0.08 (Table 2). Similarly, Traitler *et al.* (14) have shown that by urea fractionation of black currant seed oil, it was possible to selectively enrich γ-linolenic acid over α-linolenic acid. It appears therefore that the urea fractionation method could be utilized for more than just the removal of saturated and monounsaturated

TABLE 1

EPA Enrichment by Silica Gel Fractionation of *P. cruentum* Lipids

	Fatty acid composition (% of total fatty acids)									
	16:0	16:1	18:0	18:1	18:2	20:2	20:3	20:4	20:5	R
Total lipids	30.6	5.3	0.3	0.9	4.8	0.8	2.0	14.6	40.7	0.36
Glycolipids fraction	34.8	0.4	0.8	1.3	9.6	0.1	0.2	5.4	47.5	0.11

TABLE 2

EPA and AA Concentration by Urea Treatment

	Fatty acid composition (% of total fatty acids)									
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	R
FAME[a]	34.8	0.4	0.8	1.3	9.6	0.1	0.2	5.4	47.5	0.11
FAME after urea treatment	3.1	0.3	—	0.6	7.3	0.1	0.2	6.6	81.9	0.08
FAME after chromatography	0.3	—	—	—	0.9	—	0.2	1.4	97.3	0.01

[a]FAME of glycolipid fraction.

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79

TABLE 3

EPA Purification by Reverse Phase Chromatography[a]

% CH_3CH[b]	Fatty acid composition (% of total fatty acids)									% of total EPA[c]
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	
58	14.2	2.0	1.2	1.6	3.2	2.9	1.4	6.1	67.3	2.7
59	1.6	0.1	0.2	0.0	1.7	0.4	0.5	2.3	93.2	29.1
59	3.2	0.2	0.3	0.0	2.8	0.8	0.9	3.0	88.9	27.2
59	2.4	0.3	0.4	0.2	4.0	1.0	1.5	4.8	85.4	25.2
59	19.0	1.0	0.9	0.8	16.8	1.1	0.5	15.2	44.8	7.1
60	32.4	1.1	0.8	1.3	21.3	0.7	1.9	17.1	23.4	4.2
61	46.7	1.2	0.2	1.3	25.1	1.2	1.8	15.1	7.4	1.5
62	60.8	1.2	0.7	1.7	15.9	0.0	3.9	10.1	5.6	0.9
63	63.7	1.3	1.2	2.3	13.1	0.6	3.8	6.7	7.2	0.6
65	68.8	1.5	0.7	3.1	7.6	2.0	1.9	5.3	9.1	0.5
100	74.3	1.[illegible]	4.5	2.7	4.6	0.4	3.9	2.4	5.5	0.4
Pre-separation mixture[d]	33.8	0.4	0.8	1.2	9.3	0.1	0.2	8.2	46.1	

[a]Representative example.
[b]Percent of acetonitrile in acetonitrile-water.
[c]Percent of total EPA — the ratio of EPA content in fraction to total EPA eluted.
[d]Composition of FAME mixture loaded on the column.

fatty acids. The inclusion complex formation reported here was not optimized, and it is quite possible that optimization could result in an even more efficient removal of AA.

The same procedure was utilized for the preparation of an AA concentrate. *P. cruentum* strain 1380.1b was previously identified as a rich source of AA (11). At the stationary phase, the AA content increased to 41.7% of total fatty acids. The oil was transmethylated and treated with urea as above resulting in the AA content increasing to 80.0% (data not shown).

EPA purification by reverse phase chromatography. Reverse phase chromatography is known to separate fatty acids according to chain length and by degree of unsaturation (15). However, separation is generally achieved by high-performance liquid chromatography (HPLC) which is quite expensive. In this work, we attempted to concentrate EPA by open-bed reverse phase chromatography. C-18 Sep pak filters were utilized as a model. Acetonitrile-water mixtures proved to be more selective than methanol-water in the separation of EPA from AA. Equal volumes of increasing concentrations of acetonitrile in water were utilized to elute the FAME mixtures (Table 3). The first two fractions (40% and 55% acetonitrile) consisted of unretained FAME. However, the percent of total EPA found in these fractions combined was less than 0.7%. EPA started to elute in the third fraction (58% acetonitrile) which still contained some unretained material. No attempts were made to improve the separation at this point, as this fraction contained only 2.7% of total EPA. The bulk of the EPA was eluted in the next 3 fractions (59% acetonitrile) which contained 81.5% of total EPA. The EPA content in each fraction ranged from 85.4% to 93.2%, while AA did not exceed 4.8%. The AA and 18:2 contents peaked at 60% and 61% acetonitrile respectively. While this procedure is effective for removal of saturated and monounsaturated FAME, it is envisaged that on a large scale it would be advantageous to pretreat the mixture with urea. Indeed, when reverse phase chromatography was preceded by urea treatment, 85% of the EPA was recovered at a 97.3% purity.

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80

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61

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Overproduction of γ-Linolenic and Eicosapentaenoic Acids by Algae[1]

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ABSTRACT

The pharmaceutical interest and limited availability of γ-linolenic acid (GLA) and eicosapentaenoic acid (EPA) prompted the search for genetic means for increasing the production of these fatty acids from algal sources. Cell lines of *Spirulina platensis* and *Porphyridium cruentum* resistant to the growth inhibition of the herbicide Sandoz 9785 were selected by serial transfers of the culture in the presence of increasing concentrations of the herbicide. The resistant cell lines of *S. platensis* overproduced GLA and those of *P. cruentum* overproduced EPA and were stable for at least 50 generations in the absence of the inhibitor.

The recent pharmaceutical interest in the polyunsaturated fatty acids, EPA[2] and GLA (7, 17) and the limitations of their current availability (2, 5) triggered the search for potential new sources for these fatty acids. We (1, 5) have previously demonstrated that the marine red microalga *Porphyridium cruentum* and the cyanobacterium *Spirulina platensis* are among the best producers of EPA and GLA, respectively. Strain selection and manipulation of physiological and environmental conditions brought about an increased content of these polyunsaturated fatty acids (1, 2, 4–6). Yet, the inherent limitation of these approaches prompted us to look for genetic means that could result in even higher contents of EPA and GLA.

A possible approach for increasing the content of particular cell metabolites is the use of inhibitors of specific steps in biosynthetic pathways or analogs of specific products. Generally, such inhibitors and analogs inhibit growth as well. Thus, resistance to the inhibitor could be achieved by overproduction of the inhibited metabolites. It was indeed shown in higher plants that some lines selected for resistance to the growth inhibition are overproducers of the metabolite in question (8, 10, 15). Mutants of *Spirulina* showing elevated production of proline were obtained by selection in the presence of proline analogs (12). Similar results were obtained in the cyanobacterium *Nostoc* (9) and the alga *Nannochloris bacilaris* (13).

Several herbicides of the substituted pyridazinone family were shown to inhibit fatty acid desaturation. Of these, SAN 9785 is the most effective inhibitor of ω3 desaturation (11), and its effect on reduction of 18:3ω3 levels in the glycolipids of higher plants and algae was widely studied. Recently, we (1) found this herbicide to be an effective inhibitor also for Δ6 desaturation of linoleic acid in *Spirulina*. Although SAN 9785 has a certain inhibitory effect on photosynthesis, it was shown that the effect on fatty acid desaturation is a direct inhibition of the desaturase (16).

In the present paper, we describe the successful selection of *P. cruentum* and *S. platensis* cell lines that display stable resistance to the growth inhibition of SAN 9785. These cell lines over produced EPA and GLA, respectively. To the best of our knowledge, this is the first report of a fatty acid overproduction in either higher or lower plants induced by perturbation of fatty acid metabolism.

MATERIALS AND METHODS

Organisms and Culture Conditions

Spirulina platensis strain 2340 was obtained from the University of Texas Culture Collection and was cultivated on Zarrouk's medium at 30°C as previously described (5). *Porphyridium cruentum* strain 1380 1d was obtained from the Goettingen Algal Culture Collection (Goettingen, Germany). Cultures were grown on Jones's medium as previously described (6). Cultures were grown exponentially (with proper dilution) under the appropriate conditions for at least 4 d prior to the onset of the experiment. The specific growth rate was estimated by measurements of Chl concentration and turbidity. Solutions of the herbicides in DMSO were added to exponentially growing cultures. The final concentration of DMSO did not exceed 1%. Cultures of *S. platensis* were cultivated in the presence of 0.2 mM SAN 9785, which inhibited growth by about 80%. After several weeks of growth in the presence of the inhibitor (with occasional dilution with fresh medium containing the required concentration of the herbicide), the growth rate gradually increased, approaching that of the control cells. The concentration of the inhibitor was increased to 0.4 mM and the culture was cultivated as above for several months. *P. cruentum* cultures were similarly treated with an initial inhibitor concentration of 0.08 mM,

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[2] Abbreviations: EPA, eicosapentaenoic acid, 20:5ω3; GLA, 18:3ω6, γ-linolenic acid; SAN 9785, BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone; SRS-1–3, SAN-resistant lines of *Spirulina*; SRP-6–7, SAN-resistant lines of *Porphyridium*.

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62

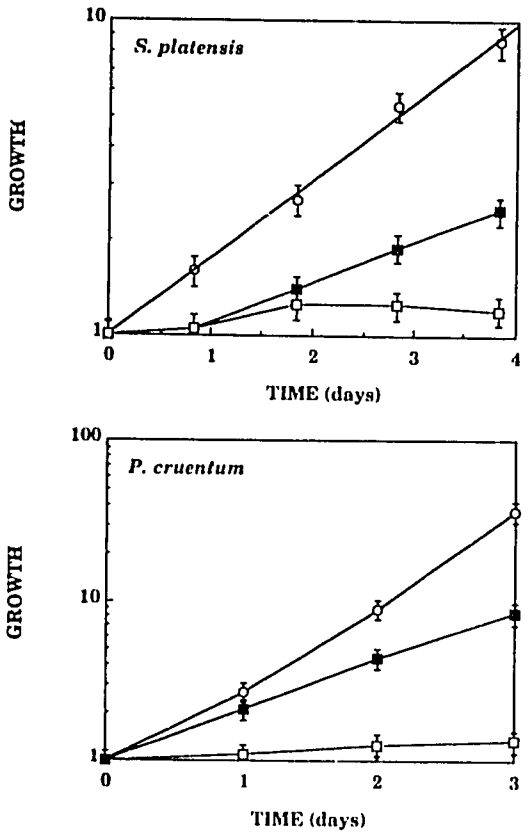

Figure 1. Effect of 0.4 mM SAN 9785 on the growth rate (expressed as the increase in Chl concentration relative to day 0) of *S. platensis* and *P. cruentum* (○, control; □, freshly exposed to the inhibitor; ■, inhibitor resistant culture).

which was gradually increased over a 9-month period, finally reaching 0.4 mM.

Selection of Resistant Lines

S. platensis culture resistant to 0.4 mM herbicide was diluted with inhibitor-containing medium and distributed to 50 test tubes having on the average half a filament per test tube. The test tubes were incubated for a period of several weeks. The test tubes in which growth occurred were rescued. Cell lines of *P. cruentum* resistant to 0.4 mM of the herbicide were obtained by screening of the resistant suspension on agar plate containing 0.4 mM of the inhibitor.

Fatty Acid Analysis

Freeze-dried samples of biomass were transmethylated with MeOH-acetyl chloride as previously described (2). Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed on a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200°C (injector and flame ionization detector temperatures 230°C, split ratio 1:100) and integrated with an HP 3396A integrator. Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma) and by GC-MS. Fatty acid contents were determined by comparing their peak areas with that of the internal standard.

RESULTS AND DISCUSSION

It is reasonable to assume that the growth inhibition incurred by these herbicides emanates to a certain extent from their effect on fatty acid desaturation, particularly the transformation of linoleic acid. Linoleic acid is further desaturated in *S. platensis* to GLA and in *P. cruentum* to α-linolenic acid, which is in all likelihood a precursor of EPA. Thus, we hypothesized that overproducers of GLA and EPA may be found among SAN 9785-resistant cell lines of *S. platensis* and *P. cruentum*, respectively. Clones of these algae that are re-

Table I. *Fatty Acid Composition of S. platensis Cell Lines Resistant to SAN 9785*

The values presented are means ± SD (n = 4). A t test was used to determine significantly different values of 18:3 and TFA at $P < 0.025$.

Culture	Fatty Acid Composition						Fatty Acid Content	
	16:0	16:1	18:0	18:1	18:2 ω6	18:3 ω6	TFA[a]	18:3
	% of total fatty acids						% of dry wt	
WT[b]	42.11 ± 0.82	5.66 ± 0.91	0.90 ± 0.05	2.06 ± 0.45	25.52 ± 0.46	21.57 ± 0.47	4.09 ± 0.42	0.88 ± 0.07
WT[c]	42.16 ± 0.93	6.29 ± 0.16	0.85 ± 0.08	2.77 ± 0.10	25.91 ± 0.47	20.21 ± 0.41	4.30 ± 0.14	0.87 ± 0.05
SRS-1[d]	43.34 ± 1.29	4.44 ± 1.89	0.70 ± 0.07	2.39 ± 0.87	24.48 ± 0.76	23.13 ± 1.18[e]	5.55 ± 0.35[e]	1.28 ± 0.13[e]
SRS-3	42.80 ± 1.34	5.16 ± 0.22	0.84 ± 0.34	2.66 ± 1.11	24.07 ± 0.27	23.36 ± 1.21[e]	4.93 ± 0.24[e]	1.15 ± 0.09[e]
SRS-1h	40.69 ± 1.67	4.95 ± 0.39	0.67 ± 0.04	2.58 ± 0.42	25.09 ± 0.53	23.57 ± 0.52[e]	6.07 ± 0.18[e]	1.43 ± 0.05[e]

[a] Total fatty acids. [b] Wild type. [c] Wild type culture subjected to 0.4 mM SAN 9785 for 4 d and brought back to inhibitor-free medium, similar to the resistant culture. [d] Resistant cell lines selected by filament isolation from the resistant culture and cultivated on inhibitor-free medium for 11 d. [e] Significantly different from wild type. Lipid transmethylation and fatty acid analyses were performed as previously reported (3).

83

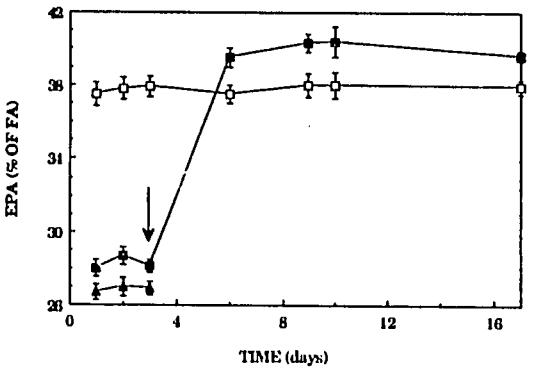

Figure 2. EPA content (% of fatty acids) of *P. cruentum* wild type grown with (▲) or without (□) the herbicide SAN 9785 and of a resistant cell line grown continuously in the presence of the herbicide (■) and shifted to a herbicide-free medium (arrow). Due to dependence of the fatty acid composition on the Chl level (5), the fatty acid composition was measured at a cell concentration corresponding to 4 mg/L Chl.

sistant to the growth inhibition of SAN 9785 were indeed selected from the wild type (Fig. 1).

Cell lines resistant to 0.4 mM SAN 9785 were obtained by a stepwise increase of the herbicide concentration in the growth medium. When cultivated in the presence of this concentration, the resistant cultures could grow, although at a reduced growth rate, whereas the wild type collapsed after a few days (Fig. 1). When brought back to inhibitor-free medium, their respective growth rates were back to normal (data not shown). The resistance was maintained even after 50 generations in an inhibitor-free medium, indicating a genetic change.

Relative to the wild type, the resistance in *Spirulina* was associated, as predicted, with elevated levels of GLA both in the presence (data not shown) and in the absence (Table I) of the inhibitor. In inhibitor-free medium, GLA increased from 21.6% (of total fatty acids) in the wild type to 23.1, 23.4, and 23.6% in the resistant isolates SRS-1, SRS-3, and SRS-1h, respectively. The proportions of 18:0, 18:1, and 18:2 decreased compared with the wild type (Table I). The three isolates had an improved fatty acid content (percent of dry weight) of 5.55, 4.93, and 6.07%, respectively, compared with 4.09% in the wild type. The increase in GLA content on a dry weight basis from 0.88% in the wild type to 1.28, 1.15, and 1.43% in SRS-1, SRS-3, and SRS-1h, respectively, became even more apparent. It was possible that the GLA overproduction obtained by transferring to inhibitor-free medium was merely an overshoot resulting from the absence of the inhibition. To overrule such a possibility, a wild-type culture was subjected to the inhibitor for 4 d and then brought back to inhibitor-free medium. Both its fatty acid composition and content were not significantly different from that of a wild type cultivated on control medium (Table I).

The culture of *P. cruentum* resistant to SAN 9785 displayed, in the presence of the inhibitor, EPA levels higher than those of freshly exposed cultures of the wild type (28.0 and 26.5% of fatty acids, respectively) (Fig. 2). Furthermore, elevated EPA levels were obtained when the resistant cultures were shifted to herbicide-free medium, where it reached 40.4% as compared with 36.8% in the wild type. The increase in EPA resulted primarily from a decrease in 16:0. The enhanced EPA level under inhibitor-free conditions was sustained for at least 2 weeks (Fig. 2). By plating on agar, six colonies were selected, three of which displayed even higher proportions of EPA and a higher fatty acid content, resulting in a 27% enhancement of the EPA content. Strains SPR-6 and SPR-7 showed the highest EPA proportions, each attaining a level of ~41% (of total fatty acids). On a dry weight basis, the fatty acid content of each of these lines increased to 5.6% (of dry weight) compared with 4.8% in the wild type, resulting in an EPA content of 2.3% as compared with 1.8% in the wild type (Table II).

It is difficult at present to point at the mechanism(s) of resistance to the herbicide. Murphy *et al.* (11) have shown that the uptake of SAN 9785 varies in various plants. They have further shown that the herbicide was rapidly metabolized in pea but only gradually in cucumber and ryegrass (11). Other possibilities could involve the modification of the target enzyme to reduce its affinity to the inhibitor, or an increase in the level of the relevant enzyme (14). The increase of GLA in *S. platensis* and EPA in *P. cruentum* may be attributed to

Table II. *Fatty Acid Composition of P. cruentum Cell Lines Resistant to SAN 9785*

The values presented are means ± SD (n = 3). A t test was used to determine significantly different values of EPA and TFA at $P < 0.05$.

Culture	Fatty Acid Composition											Fatty Acid Content	
	16:0	16:1	16:3	18:0	18:1 ω9	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	EPA	TFA[a]	EPA
	% of total fatty acids											% of dry wt	
WT[b]	30.3 ±0.62	5.20 ±0.34	0.62 ±0.06	0.44 ±0.02	0.53 ±0.04	5.44 ±0.11	0.89 ±0.13	0.54 ±0.15	0.68 ±0.07	16.1 ±0.40	33.2 ±0.75	4.78 ±0.17	1.83 ±0.04
SRP-6[c]	29.2 ±1.5	4.70 ±0.07	0.62 ±0.08	0.47 ±0.07	0.53 ±0.04	5.31 ±0.24	0.92 ±0.15	0.63 ±0.14	0.37 ±0.01	15.4 ±0.10	41.1 ±0.39[d]	5.64 ±0.67[d]	2.32 ±0.39[d]
SRP-7[c]	29.5 ±1.2	4.54 ±0.30	0.60 ±0.06	0.46 ±0.10	0.47 ±0.06	5.34 ±0.23	0.90 ±0.04	0.47 ±0.02	0.59 ±0.16	15.5 ±0.45	40.9 ±1.1[d]	5.62 ±0.67[d]	2.31 ±0.36[d]

[a] Total fatty acids. [b] Wild type. [c] Resistant cell lines selected by agar plating of the resistant culture. [d] Significantly different from wild type.

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84

one of the last two mechanisms. Thus, overproduction could be a means for counteracting the effect of the herbicide.

The data presented in this communication indicate that the hypothesis suggesting the use of inhibitors of fatty acid desaturation as means for obtaining fatty acid overproduction was apparently correct. We are aware that further exploitation of this approach could be hampered by the reduced specificity of the herbicide. Thus, more specific inhibitors, such as transition stage analogs, are being sought. We anticipate that further selection of GLA and EPA overproducing strains would make the production of these fatty acids from algal sources feasible.

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90

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POTENTIAL USE OF SUBSTITUTED PYRIDAZINONES FOR SELECTING POLYUNSATURATED FATTY ACID OVERPRODUCING CELL LINES OF ALGAE

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Key Word Index—*Spirulina platensis*; *Porphyridium cruentum*; *Monodus subterraneus*; algae; γ-linolenic acid; eicosapentaenoic acid; fatty acid overproduction; herbicides; SAN 9785; norflurazon.

Abstract—The effects of the herbicides SAN 9785 and norflurazon on growth and fatty acid composition of the cyanobacterium *Spirulina platensis*, the marine microalga *Porphyridium cruentum* and the freshwater alga *Monodus subterraneus* were studied. Norflurazon was more effective than SAN 9785 in causing growth inhibition of *S. platensis* cultures; changes in fatty acid composition confirmed that this compound is a potent inhibitor of Δ6-desaturation. SAN 9785 inhibited the growth of *S. platensis* concomitantly causing a reduction in the amount of the fatty acids 18:3ω6 and 18:2ω6 and an increase in 18:1 and 18:0, thus indicating an inhibitory effect of this herbicide on the Δ6-desaturation system. Similar growth inhibition was observed in *P. cruentum*. In this alga, the herbicide also inhibited the formation of 20:5ω3, apparently by blocking the desaturation of 18:2 to 18:3ω3 in one pathway of eicosapentaenoic acid biosynthesis. However, in *M. subterraneus* SAN 9785 reduced growth, but caused an increase in the proportion of 20:5ω3 indicating a possilbe alternative pathway for the formation of 20:5ω3 in this alga. SAN 9785 was therefore considered to be useful for the selection of cell lines which overproduce the valuable fatty acids 18:3ω6 and 20:5ω3 in *S. platensis* and *P. cruentum*, respectively.

INTRODUCTION

Recent increasing interest in polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5ω3) and γ-linolenic acid (GLA, 18:3ω6) as potential pharmaceuticals [1–4] has triggered the search for new sources of these fatty acids. The current source of EPA is marine fish oil. However, its relatively low EPA content (7–15% of total fatty acids) and the presence of cholesterol in fish oil have prompted several studies aimed at the development of alternative EPA sources. Although fungal production of EPA has been suggested [5], large scale cultivation of a fungal source is not yet feasible. The commercial potential of EPA-containing algae has also been investigated [6–8]. We have previously demonstrated that the marine red microalga *Porphyridium cruentum* is one of the best algal EPA sources [9] because outdoor cultivation is possible [10] and elevated EPA contents can be achieved by the combination of strain selection [11], environmental conditions [9-11] and efficient purification methods [12].

GLA is found in evening primrose, blackcurrant [13] and borage [14], as well as fungi [15] and cyanobacteria [16]. We have shown that by choice of the appropriate strains and environmental conditions *Spirulina* oil can become one of the richest sources of GLA [17].

There are several physiological means for increasing oil content in eukaryotic algae including nitrogen or silicon starvation, and keeping cells in the stationary phase of growth. However, none of these methods was shown to be effective for cyanobacteria and even in eukaryotic algae, their benefits are limited with regard to EPA production because of decreases in growth rate and PUFA content which are generally associated with an increase in oil content [18, 19]. We have hypothesized that PUFA overproduction could be obtained using specific inhibitors which perturb lipid biosynthetic routes. In higher plants, it was indeed shown that lines selected for resistance to certain inhibitors which affect the formation of various essential metabolites, overproduce the metabolite in question [20–22]. Mutants of *Spirulina* showing elevated production of proline were obtained by selection for growth in the presence of proline analogues [23]. Similar phenomena were observed in the cyanobacterium *Nostoc* [24] and the alga *Nannochloris bacilaris* [25].

Most of the information concerning fatty acid biosynthesis has been obtained from studies on higher plants. Increasing evidence [26] suggests that 18:2 and 18:3 are formed by sequential desaturation of 18:1 which is already esterified to a specific glycerolipid (rather than desaturation of CoA thioesters) and that 16:0 may be desaturated when it is esterified at the *sn*-2 position of monogalactosyldiacylglycerol (MGDG) or phosphatidyl-

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91

glycerol (PG) [26]. While these pathways are similar in algae and higher plants, very little is known about the synthesis of 20:5ω3 which is found in several algae but not in higher plants. It is presumed that 20:5 is formed by a series of desaturations and an elongation of 18:2ω6, possibly via 18:3ω3 [27].

Several herbicides have been shown to inhibit fatty acid desaturation. The substituted pyridazinone SAN 9785 [BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone] is the most effective known inhibitor of ω3 desaturation and its effect of reducing 18:3ω3 concentrations in the glycolipids of higher plants and algae has been widely studied [28]. Recently this herbicide was shown to directly inhibit *in vitro* desaturation of 18:2 in MGDG [29]. Although SAN 9785 partially inhibits photosynthesis, it was shown that its effect on fatty acid desaturation is the result of a direct inhibition of the desaturase enzyme, independent of its effect on photosynthesis [30]. In an independent study [31], we have shown that the structurally-related herbicide norflurazon (SAN 9789), a known inhibitor of carotenoid biosynthesis and chlorophyll accumulation, is a very effective inhibitor of the Δ6-desaturation system.

In the present work we tested the effect of SAN 9785 and norflurazon on the growth and fatty acid composition of the cyanobacterium *S. platensis*, the red microalga *P. cruentum* and the freshwater microalga *Monodus subterraneus*. We further evaluated their potential for selecting cell lines which exhibit overproduction of GLA in *S. platensis* and of EPA in *P. cruentum* and *M. subterraneus*.

RESULTS

Effect of SAN 9785 and norflurazon on growth

The effects of various concentrations of the two herbicides on the growth rate of *S. platensis*, *P. cruentum* and *M. subterraneus* were studied. SAN 9785 inhibited the growth of both *S. platensis* and *P. cruentum* cultures. Maximal inhibition was observed at 0.8 mM, yet even at 0.4 mM growth was arrested after a few days (Fig. 1). Cell growth of *M. subterraneus* was almost completely inhibited at 0.2 mM (Fig. 1). Norflurazon was much more effective than SAN 9785 in suppressing growth of *S. platensis*, which was completely inhibited two days in the presence of 3 μM (Fig. 2). However, cells were still viable at a herbicide concentration as high as 15 μM, judging by their ability to grow in inhibitor-free medium.

Effect of SAN 9785 and norflurazon on fatty acid composition

Norflurazon markedly altered the fatty acid profile of *S. platensis*. In the presence of 15 μM, the proportion of 18:3ω6 in the total fatty acids decreased from 21.7% to 12.7% with concurrent increases in 18:2 and 18:1 (Fig. 3). Pigment accumulation (data not shown) and total fatty acid content were also affected. The latter was reduced from 3.5 to 1.6% (of dry weight).

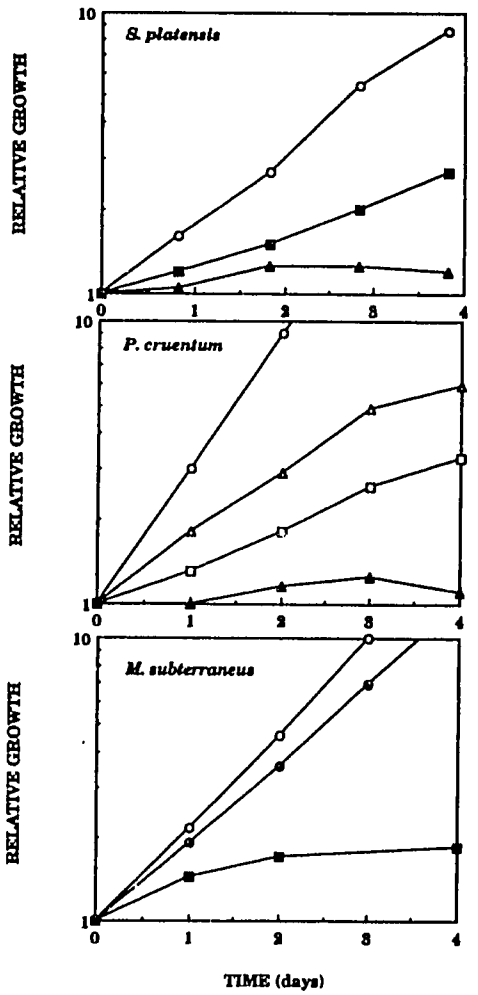

Fig. 1. Effect of SAN 9785 on growth (expressed as the increase in chlorophyll concentration relative to day 0) of *S. platensis*, *P. cruentum* and *M. subterraneus* (○, control; ●, 0.04 mM; △, 0.08 mM; □, 0.16 mM; ■, 0.2 mM; ▲, 0.4 mM).

Exposure of *S. platensis* to SAN 9785 resulted in decreases in the proportion of 18:3ω6 and 18:2ω6 and in increases in those of 18:1 and 18:0 (Table 1). During incubation with 0.4 mM SAN 9785, the proportion of 18:3ω6 declined from 19.6% (of total fatty acids) in inhibitor-free medium to 12.3% and that of 18:2 from 25.6% to 22.4%, while 18:1 and 18:0 increased. Relatively small changes were also observed in the C_{16} fatty acid series. The total fatty acid content was also reduced from 4.90 to 2.66% (of dry weight), resulting in a significant reduction of the content of 18:3 from 0.96 to 0.33%.

The major effect of SAN 9785 on the fatty acid composition of *P. cruentum* was the reduction in 20:5ω3

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92

and the concomitant increases in 20:2ω6, 20:3ω6 and 16:0 (Table 2). In the presence of 0.16 mM SAN 9785, the proportion of 20:5 declined from 36.8% (of total fatty acids) to 31.2% while that of 20:4ω6 was reduced only at higher inhibitor concentrations. The relative amounts of 20:2, 20:3 and 16:0 increased. The effect of SAN 9785 on fatty acids increased with herbicide concentration and was maximal at the highest concentration studied, namely 0.4 mM.

As noted above, SAN 9785 also inhibited the growth of *M. subterraneus* (Fig. 1). However, its concurrent effect on cell PUFA composition was unlike that observed above for the other two algae (Table 3). The proportion of EPA was found to increase significantly from 22.4 to 34.8% while that of 16:0 and 16:1ω7 decreased (Table 3). However, the absolute content of EPA did not change significantly due to the decrease in total fatty acid content which dropped from 12.3 to 7.34% (% of dry weight). The

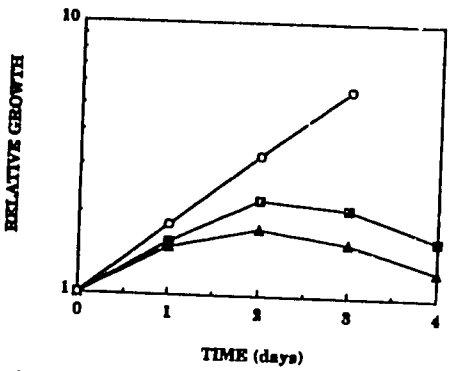


Fig. 2. Effect of norflurazon on growth rate (expressed as the increase in chlorophyll concentration relative to day 0) of *S. platensis* (o, control; ■, 3 μM; ▲, 15 μM).

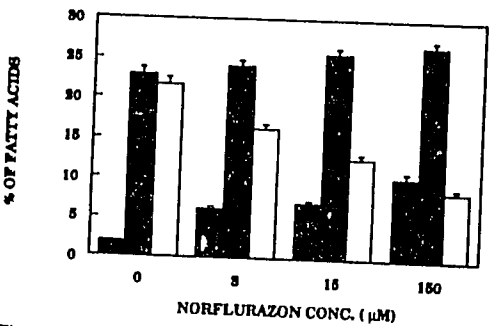


Fig. 3. Effect of norflurazon on 18:3 (□), 18:2 (▨) and 18:1 (■) contents in *S. platensis*.

Table 1. Effect of SAN 9785 on fatty acid composition and content of *S. platensis*.

SAN 9785 concn (mM)	Fatty acid composition (% of total fatty acids)									Fatty acid content (% of dry wt)	
	14:0	16:0	16:1	16:3	16:4	18:0	18:1	18:2 ω6	18:3 ω6	TFA*	18:3 ω6
0	1.6	44.2	4.7	0.5	0.2	1.0	2.6	25.6	19.6	4.90	0.96
0.2	1.9	46.7	3.6	0.7	—†	1.9	5.0	24.3	15.9	3.90	0.62
0.4	0.9	45.0	4.0	1.0	—	3.2	11.2	22.4	12.3	2.66	0.33

*Total fatty acids.
†Trace amount.
Cultures were analysed four days after exposure to the herbicide. Data shown represent mean values of four independent samples, each analysed in duplicate.

Table 2. Effect of SAN 9785 on fatty acid composition of *P. cruentum*

SAN 9785 concn (mM)	Fatty acid composition (% of total fatty acids)										
	16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
0	31.8	5.9	0.5	0.9	1.0	5.3	1.4	0.1	0.5	16.0	36.8
0.08	36.1	5.0	0.8	0.9	1.2	5.0	0.8	0.4	0.7	13.3	35.4
0.16	37.8	5.1	0.8	0.7	1.3	5.0	0.7	0.6	0.9	15.4	31.2
0.40	43.9	5.9	0.8	0.9	1.9	5.5	1.5	0.6	1.3	11.6	25.4

Cultures were analysed four days after exposure to the herbicide. Data shown represent mean values of four independent samples, each analysed in duplicate.

93

Table 3. Effect of SAN 9785 on fatty acid composition of *M. subterraneus*

SAN 9785 concn (mM)	Fatty acid composition (% of total fatty acids)										Fatty acid content (% of dry wt)	
	14:0	16:0	16:1	18:0	18:1 ω9	18:2 ω6	18:3 ω6	20:3 ω6	20:4 ω6	20:5 ω3	TFA*	20:5 ω3
0	4.2	24.6	28.0	0.9	10.0	1.2	1.4	1.0	5.0	22.5	12.30	2.76
0.2	4.0	21.0	23.1	1.4	7.1	2.4	—	—	6.2	34.8	7.34	2.56

*Total fatty acids.

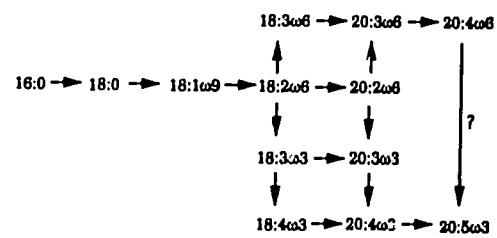
Cultures were analysed four days after exposure to the herbicide. Data shown represent mean values of two independent samples, each analysed in duplicate.

very low concentration of 18:3ω6 present in control cells disappeared during herbicide treatment, and there was a small increase in 18:2ω6 and 20:4ω6.

DISCUSSION

Eighty-five per cent of the GLA in *S. platensis* (Cohen, unpublished data) and over 90% of the EPA in *P. cruentum* [12] are located in chloroplast galactolipids (GL) which are considered to be structural components of the photosynthetic apparatus [32]. Thus, it was reasonable to assume that inhibitors having an effect on desaturation reactions involved in PUFA biosynthesis would also affect growth (i.e., by impairment of photosynthetic efficiency). Elongation and desaturation reactions in proposed pathways of PUFA biosynthesis in microalgae are presented in Scheme 1. As previously mentioned various substituted pyridazinones, such as SAN 9785, are known to inhibit the desaturation of 18:2ω6 to 18:3ω3 [28] which is a possible precursor of 20:5ω3 in both the marine red microalga *P. cruentum* and the freshwater chrysophyte *M. subterraneus* (Scheme 1). The data presented here (Fig. 1 and Table 1) show that in *S. platensis*, which contains 18:3ω6 as a major component of GL, SAN 9785 inhibits both cell growth and synthesis of 18:3ω6. We suggest that growth inhibition may stem from the effect on the chloroplast membrane fatty acid composition, although we cannot rule out the possibility that growth may be adversely affected by a reduction in carbon assimilation resulting from the direct inhibition of photosynthesis by the herbicide. Recently, we have shown [31] that norflurazon, a structurally related pyridazinone known to inhibit carotenoid biosynthesis, chlorophyll accumulation, and consequently growth [33], also effectively inhibits fatty acid desaturation, primarily Δ6-desaturation. The effect of norflurazon on the Δ6-desaturase in *S. platensis* was shown to be two orders of magnitude higher than that of SAN 9785 (Table 1, Fig. 3). Thus, 3 μM norflurazon and 0.4 mM SAN 9785 reduced the amount of 18:3ω6 by 25% and 29%, respectively.

Obtaining resistance to the inhibitory effect of norflurazon on fatty acid desaturation would be complicated due to its dual mode of action. We have previously studied the effect of this herbicide on the fatty acid



Scheme 1. Possible pathways of PUFA biosynthesis in microalgae.

composition of *M. subterraneus* [31] and *P. cruentum* (data not shown). Although this compound inhibited cell growth, no reduction in EPA was observed. On the contrary, its proportion increased significantly.

The inhibitory activity of SAN 9785 in *P. cruentum* resulted in a reduction in the contact of EPA (Table. 2), primarily in the GL fraction (data not shown). An increase in the precursor 18:2, or any of its products in other pathways, could be predicted. Yet, except for small increases in 20:2 and 20:3 the only major increase was noted in 16:0.

In *M. subterraneus*, SAN 9785, while reducing growth and decreasing fatty acid content, surprisingly caused an increase in the proportion of 20:5ω3. Since this fatty acid is primarily located in GL in both *M. subterraneus* and *P. cruentum* (Cohen, unpublished data), this contradictory effect of SAN 9785 on *M. subterraneus* EPA suggests the existance of an alternative pathway for 20:5ω3 biosynthesis in this alga. In this scheme, elongation of 18:2ω6 to 20:2ω6 would precede desaturation of the latter to 20:3ω3 (Scheme 1) which would then be further desaturated to form 20:5ω3. Based on the data presented here, our hypothesis states that these desaturations of C_{20} fatty acids are much less sensitive to the inhibitor. Another possibility is that in this alga the desaturation of 18:2 to 18:3ω3 takes place on extrachloroplastic PC prior to PUFA incorporation into GL. It was suggested that in the 'eukaryotic pathway' of GL synthesis in higher plants, 18:2/18:2-PC [26] or 18:2/18:3-PC [34] are the molecular species which are transported back to the

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chloroplasts for subsequent galactosylation of their glycerol moieties and further fatty acid desaturation. The desaturation of 18:2 occuring on PC was shown in *Arabidopsis* to be less sensitive to SAN 9785 inhibition then chloroplast desaturation reactions [35], and similar mechanisms could be operative in *M. subterraneus*. Henderson *et al.* [36], who studied the effect of this herbicide in the marine microalgae *Chroomonas salina* and *Nannochloropsis oculata*, also obtained data indicating the possible existence of a different biosynthetic pathway for 20:5. The unexpected effect of SAN 9785 on *M. subterraneus* may be helpful in elucidating the biosynthetic pathway of EPA in this alga. Further studies in this direction are currently under way.

The herbicide SAN 9785 inhibited growth in all three algal species studied. We suggest that in *S. platensis* and *P. cruentum* growth inhibition could be related to the effect of the inhibitor on the production of GLA and EPA, respectively, and to a lower extent also to the reduction in total fatty acid content. In *M. subterraneus*, while no such inhibitory effect could be demonstrated, the reduction in total fatty acid content by more then 40% could perhaps be related to inhibition of growth.

We have hypothesized that one means to achieve resistance to inhibitors of lipid biosynthesis would be to elicit fatty acid overproduction. Thus, obtaining resistance to the growth inhibition by such compounds could be the basis for selection of algal lines capable of GLA or EPA overproduction. The growth inhibition rendered by SAN 9785 and the specific inhibitory effects it exerted on the production of EPA and GLA led us to utilize it for the selection of cell lines resistant to its growth inhibiting effect, some of which could be EPA and GLA over producers in *Spirulina* and *Porphyridium*, respectively. Recently we [37] succeeded in achieving resistance to this inhibitor in cultures of both *Spirulina* and *Porphyridium* including cell lines which demonstrated overproduction of GLA and EPA, respectively, in these species.

EXPERIMENTAL

Organisms and culture conditions. S. spirulina platensis strain 2340 and *Monodus subterraneus* UTEX 151 were obtained from the University of Texas Culture Collection. *Porphyridium cruentum* strain 1380 1d was obtained from the Göettingen Algal Culture collection (Göettingen, Germany). *Spirulina platensis* was cultivated on Zarrouk's medium at 30° as previously described [17]; *P. cruentum* was grown on Jones' medium [38]; *M. subterraneus* was cultivated on the BG-11 medium as described by Iwamoto [8]. Stock cultures were maintained and inocula performed according to Vonshak [39]. *Porphyridium* and *Monodus* cultures were grown in Erlenmeyer flasks, placed in an incubator shaker and illuminated from above at a light intensity of 115 $\mu E\ m^{-2} sec^{-1}$, under an air-CO_2 (99:1) atmosphere at 25°. Cultures were grown exponentially (with proper diln) under the appropriate conditions for at least 4 days prior to the onset of the expt. The specific growth rate was estimated by measurements of chlorophyll concn [39]. Solns of the herbicides in DMSO were added to exponentially growing cultures. The final concn of DMSO in treated and control cultures did not exceed 1%.

Fatty acid analysis. Fatty acid composition and content were measured 3 days after exposure to the inhibitor. Freeze-dried cells were transmethylated with MeOH–AcOCl as previously described [12]. Heptadecanoic acid was added as int. standard. GC analysis was performed on a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200° (FID, inj. and detector temps 230°, split ratio 1:100). Peak areas were measured using an integrator. Fatty acid Me esters were identified by co-chromatography with authentic standards and by GC-MS. Fatty acid contents were determined by comparing their peak areas with that of the int. standard. The data shown represent mean values with a range of less than 5% for major (over 10% of fatty acids) peaks and 10% for minor peaks, of at least two independent samples, each analysed in duplicate.

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96

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HERBICIDE-RESISTANT LINES OF MICROALGAE: GROWTH AND FATTY ACID COMPOSITION

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Key Word Index—*Spirulina platensis*; *Porphyridium cruentum*; microalgae; γ-linolenic acid; eicosapentaenoic acid; fatty acid overproduction; herbicide-resistance; SAN 9785.

Abstract—Cell lines of *Spirulina platensis* and *Porphyridium cruentum* resistant to growth inhibition by the herbicide SAN 9785 had a significantly higher growth rate than their respective wild-type strains. These lines were also shown to overproduce γ-linolenic acid (GLA) and eicosapentaenoic acid (EPA), respectively, in the presence and absence of the inhibitor, as compared with wild-type cultures under similar conditions. The effect was most conspicuous in polar lipids. Thus, the proportion of GLA in the galactolipid (GL) fraction of the SAN 9785-resistant strain of *S. platensis*, SRS-1, increased in the absence of the inhibitor from 33.3% in the wild-type to 39.0%. Similarly, the proportion of EPA in the GL fraction of the resistant strain of *P. cruentum*, SRP, increased in the presence of the inhibitor from 29.1 to 45.4%.

INTRODUCTION

The pharmaceutical interest [1-4] and limited availability of γ-linolenic acid (GLA, 18:3ω6) [5] and eicosapentaenoic acid (EPA, 20:5ω3) [6-8] has prompted the search for genetic means for increasing the production of these fatty acids from algal sources. We postulated [9, 10] that among cells resistant to the growth inhibition of an inhibitor that affect fatty acid desaturation, some may overproduce the fatty acids in question. The substituted pyridazinone SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone) inhibits 18:3ω3 in the galactolipids (GL) of higher plants and algae [11], and was shown to directly inhibit *in vitro* desaturation of 18:2 in monogalactosyldiacylglycerols in ref. [12]. Recently, we have shown [10] that SAN 9785 inhibited both the growth and the production of EPA and GLA in the red microalga *Porphyridium cruentum* and in the cyanobacterium *Spirulina platensis*, respectively. This herbicide was considered therefore as being suitable for the introduction of a selection pressure for inducing overproduction of GLA and EPA in these algae. Indeed, we obtained strains of *S. platensis* and *P. cruentum* resistant to the growth inhibition of this herbicide which demonstrated overproduction of GLA and EPA, respectively [9]. While the phenomenon of emergence of overproducing mutants has been widely reported in various systems [13–15], to the best of our knowledge, there are no similar reports regarding overproduction of lipids in general and fatty acids in particular.

In the present work, we elaborate further on the effect of SAN 9785 on the fatty acid composition in both the wild-type and resistant cultures of *S. platensis* and *P. cruentum* and characterize the resistant cultures with respect to their fatty acid composition.

RESULTS

Resistance to growth inhibition by SAN 9785

The herbicide SAN 9785 inhibited the growth of *S. platensis* and *P. cruentum* cultures. Growth was arrested after two and four days, respectively, in the presence of 0.4 mM herbicide (Fig. 1). Resistance to the inhibitor was built up by continuous cultivation in the presence of gradually increasing concentrations of inhibitor. Thus, by this method we obtained a culture of *S. platensis*, 2340, which, in the presence of 0.4 mM herbicide, had a growth rate similar to that of a wild-type culture which was freshly exposed to only 0.2 mM herbicide. Eventually, the resistance was increased to withstand a concentration as high as 0.8 mM (Fig. 1).

Similar treatment of *P. cruentum* also yielded SAN 9785-resistant cultures. The culture resistant to 0.16 mM SAN had a much higher growth rate than the wild-type (Fig. 1), almost as fast as the wild-type cultivated in

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97

inhibitor-free medium. At 0.4 mM, the growth of the resistant culture resembled that of the wild-type at 0.16 mM (Fig. 1).

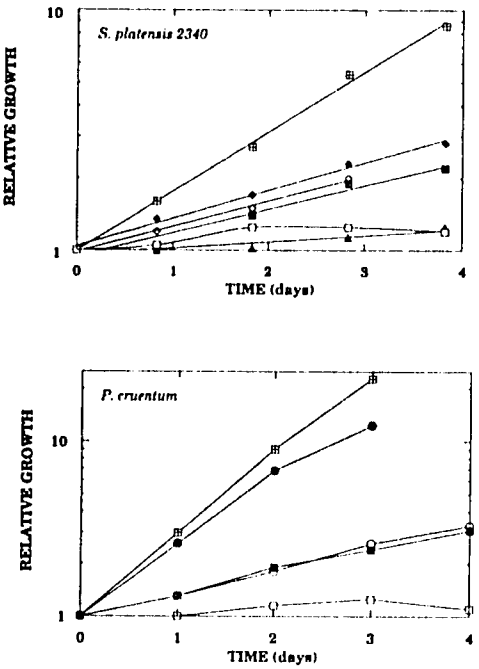

Fig. 1. Effect of SAN 9785 on growth rate (expressed as increase in chlorophyll concentration relative to day 0) of *S. platensis* 2340 and *P. cruentum*, [⊞ control (wild-type, inhibitor-free); ● 0.16 mM SAN 9785; ◆ 0.2 mM; ■ 0.4 mM; ▲ 0.8 mM]. Empty symbols denote wild-type cultures freshly exposed to the herbicide. Filled symbols denote cultures resistant to the indicated concentration of SAN 9785.

In both *S. platensis* and *P. cruentum*, when brought back to inhibitor-free medium, the growth rate of the resistant culture was similar to that of the wild-type. The resistance was maintained even after 50 generations in an inhibitor-free medium.

Effect of SAN 9785 on fatty acid composition of wild-type and SAN 9785-resistant cultures of Spirulina

The resistance to the inhibitor was manifested also in the fatty acid composition. The effect on wild-type *Spirulina* is primarily characterized by a decrease in the level of GLA and in an increase in that of 18:1ω9 [10]. However, the intensity of this effect was considerably lower in the resistant cultures. Small differences were already observed in a culture of *S. platensis* 2340 resistant to 0.2 mM SAN 9785 (Table 1). By adaptation to a higher concentration of inhibitor, the resistance became more salient. In the culture resistant to 0.4 mM SAN 9785, the GLA proportion in the presence of the inhibitor reached 16.5 as compared with only 12.3% in the freshly exposed wild-type culture, representing an overproduction of this fatty acid. Concomitantly, the proportion of 18:1 was lower, attaining only 5.4% while reaching 11.2% in the wild-type. The ratio of 18:3 and 18:2 to 18:1 (R), was reduced in the wild-type, in the presence of 0.4 mM SAN 9785, from 17.4 to 3.1. In the resistant culture it was reduced to only 7.8. The fatty acid content was also higher, reaching 4.3% compared with 2.7% (of dry weight) in the wild-type, resulting in an increase in the GLA content from 0.38 to 0.71% in the resistant culture; this was still lower than that of the wild-type in the absence of inhibitor (0.96%, Table 1).

The effect of the inhibitor on both wild-type and resistant cultures was even more conspicuous in the polar lipids. When exposed to 0.4 mM SAN 9785, the proportion of GLA in the GL of another *Spirulina* strain, BP, was reduced from 36.8 to 22.2% (Table 2). A slight

Table 1. Effect of SAN 9785 on fatty acid composition and content of wild type (2340) and SAN 9785 resistant cultures (SRS) of *S. platensis*

Culture	SAN 9785 concn (mM)	Fatty acid composition (% total fatty acids) 14:0	16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	FA content (% of dry wt) TFA†	18:3 ω6	R*
WT‡	0	1.6	44.2	4.7	0.5	1.0[a]	2.6[a]	25.6[a]	19.6[a]	4.9[a]	0.96[a]	17.4[a]
WT	0.2	1.9	46.7	3.6	0.7	1.9[b]	5.0[b]	24.3[b]	15.9[b]	3.9[b]	0.62[b]	8.0[b]
SRS (0.2)§	0.2	1.5	48.0	2.5	0.6	2.0[b]	4.8[b]	23.6[b]	16.9[b]	4.1[b]	0.69[b]	8.4[b]
WT	0.4	0.9	45.0	4.0	1.0	3.2[c]	11.2[c]	22.4[c]	12.3[c]	2.7[c]	0.38[c]	3.1[c]
SRS (0.4)	0.4	1.3	45.9	2.6	0.8	1.9[b]	5.4[b]	25.4[a]	16.5[b]	4.3[b]	0.71[b]	7.8[b]

*R: (18:3 + 18:2)/18:1.
†Total fatty acids.
‡Wild-type.
§Culture resistant to a concentration of SAN 9785 indicated in parenthesis. Cultures were analysed four days after exposure to the indicated concentration of the inhibitor. Traces (<0.3%) of 16:4 were also present. Data shown represent mean values of four independent samples, each analysed in duplicate. Within each column, numbers marked with different letters are significantly different.

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96

decrease was noted in the amount of 18:2ω6 while that of 18:1ω9 increased from 4.0 to 19.2%. Consequently, the R value dropped from 11.7 to 1.6. In culture Z19/2 (derived from *Spirulina* BP), which was resistant to 0.4 mM SAN 9785, the amount of GLA in GL was higher and that of 18:1ω9 lower than that of the wild-type in the presence of the inhibitor, reaching 26.4 and 15.4%, respectively (Table 2). In the phospholipid (PL) fraction of the wild-type lipids, the inhibitor caused a reduction in the content of GLA from 5.8 to 3.4% and that of 18:2ω6 from 34.5 to 18.2%. A major increase was noted in the proportion of 18:1ω9 which increased to 28.9% from 10.7% in the absence of the inhibitor (Table 2).

Effect of SAN 9785 on fatty acid composition of wild-type and resistant cultures of P. cruentum

The main effect of SAN 9785 on the fatty acid composition of wild-type *P. cruentum* cultures was expressed in the decrease in EPA and the increase in 16:0. At 0.4 mM SAN 9785, EPA decreased from 36.1 to 24.8 (% of fatty acids) while 16:0 increased from 32.9 to 37.5%. In the resistant culture, however, the amount of EPA reached 30.7% (Table 3), while that of 16:0 was even lower than that of the wild-type under inhibitor-free conditions.

A more focused view on the effect of the inhibitor was obtained by comparing the fatty acid composition in the

Table 2. Effect of SAN 9785 on the fatty acid composition of the polar lipids of wild-type (BP and 2340) and SAN 9785-resistant cultures (Z19/2 and SRS-1) of *Spirulina*

Lipid fraction	Strain	Medium	Fatty acid composition (% total fatty acids) 16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2 ω6	18:3 ω6	R*
GL	BP	−†	41.2	4.5	nd‡	nd	2.1	1.6	4.0[a]	9.8[a]	36.8[a]	11.7[a]
	Z19/2§	−	41.9	5.2	nd	nd	nd	0.8	2.7[b]	11.1[b]	38.3[a]	18.5[b]
	BP	+†	44.3	1.8	nd	nd	0.7	2.5	19.2[c]	9.4[a]	22.2[b]	1.6[c]
	Z19/2	+	42.4	3.2	0.3	0.6	0.4	2.0	15.4[d]	9.3[a]	26.4[c]	2.3[d]
	2340	−	40.6	6.8	0.3	0.3	0.1	0.5	1.9[bc]	16.5[c]	33.3[d]	26.2[e]
	SRS-1§	−	38.4	8.4	0.4	0.5	nd	0.5	1.2[e]	11.6[d]	39.0[a]	42.2[f]
PL	BP	−	41.9	nd	nd	2.5	nd	4.7	10.7[a]	34.5[a]	5.8[a]	3.8[a]
	Z19/2	−	41.3	3.9	0.9	0.8	1.7	2.2	7.1[b]	34.7[a]	7.4[b]	5.9[b]
	BP	+	43.6	1.0	0.7	nd	0.9	3.4	28.9[c]	18.2[b]	3.4[c]	0.75[c]
	Z19/2	+	41.3	4.0	0.4	1.4	0.3	2.8	24.5[d]	19.1[b]	5.5[a]	1.0[d]

*R: (18:3 + 18:2)/18:1.
†(−)Inhibitor-free medium. (+) Medium containing 0.4 mM SAN 9785.
‡Not determined.
§Z19/2 and SRS-1-cultures resistant to 0.4 mM SAN 9785 deriving from *S. platensis* strains BP and 2340, respectively.
Data shown represent mean values of four independent samples, each analysed in duplicate.

Table 3. Effect of SAN 9785 on the fatty acid composition of wild-type (WT) and SAN 9785-resistant (SRP) cultures of *P. cruentum*

Culture	SAN 9785 conc (mM)	Fatty acid composition (% total fatty acids) 16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT*	0	32.9[a]	4.2	0.6	0.5	0.4	5.7[a]	0.7	0.6	0.6	17.3	36.1[a]
SRP	0	28.5[b]	5.4	0.4	0.5	1.0	5.7[a]	0.9	0.5	0.7	15.8	40.4[b]
SRP-6†	0	29.2[b]	4.7	0.6	0.5	0.5	5.3[a]	0.9	0.6	0.4	15.4	41.1[b]
WT	0.4	37.5[c]	3.7	0.7	1.3	2.1	7.9[b]	1.5	0.6	2.0	17.4	24.8[c]
SRP‡	0.4	29.0[b]	3.1	0.6	1.8	2.8	12.2[c]	1.3	1.0	2.7	14.3	30.7[d]

*Wild-type.
†Resistant line selected by agar plating of resistant culture.
‡SAN 9785-resistant culture.
Data shown represent mean values of four independent samples, each analysed in duplicate.

99

Table 4. Fatty acid composition of the galactolipid fraction of wild-type (1380-1d) and SAN 9785-resistant (SRP) cultures of *P. cruentum*

Culture	Medium	Fatty acid composition (% total fatty acids)										
		16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT	–*	36.4	1.2*	nd†	0.5	1.0*	4.3*	nd	0.6*	nd	5.9*	50.0*
WT	+*	36.4	8.2*	0.5	3.3	5.7*	4.1*	0.4	1.3*	0.2	9.2*	29.1*
SRP‡	+	36.0	2.0*	0.2	1.2	2.0*	7.4*	0.3	1.2*	0.2	3.8*	45.4*

*(–) Inhibitor-free medium (+) Medium containing 0.4 mM SAN 9785.
†Not determined
‡A culture of *P. cruentum* 1380-1d resistant to 0.4 mM SAN 9785
Data shown represent mean values of four independent samples, each analysed in duplicate.

GL. Thus, in this lipid fraction, the inhibitor reduced the proportion of EPA in the wild-type from 50.0 to 29.1% (Table 4), but only to 45.4% in the resistant culture.

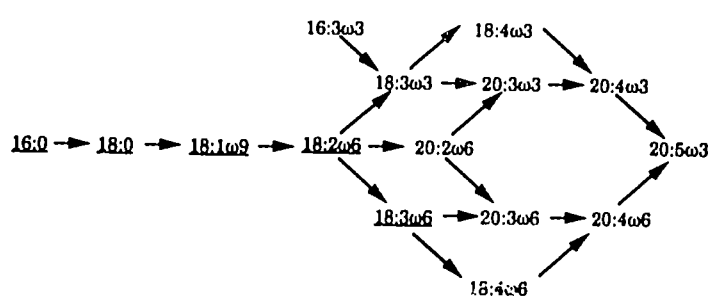
In the absence of the inhibitor, the EPA proportion in the resistant culture reached 40.4% while in a subculture (SRP-6), isolated by agar plating, an even higher proportion of 41.1% was reached (Table 3).

GLA overproduction

In inhibitor-free medium, the GL fraction of one of the SAN 9785-resistant cultures, SRS-1 [9] of *S. platensis* 2340, had a high content of GLA which reached 39.0 in comparison with 33.3% in the wild-type (Table 2). The amount of 18:2 was reduced to 11.6 from 16.5% in the wild-type, resulting in a significant increase in R from 26.2 to 42.2. The level of unsaturation of C_{16} fatty acids also increased.

DISCUSSION

Algal cultures continuously exposed to an agent that inhibits fatty acid desaturation thus causing growth inhibition, may develop resistance to the growth inhibition of that inhibitor. Such resistance could be the result of decreased uptake, reduced affinity or enhanced turnover of the inhibitor, but could also emerge as a result of genetic changes leading to an overproduction of the inhibited fatty acid. Recently, we [10] have shown that the herbicide SAN 9785 inhibited the biosynthesis of both GLA and EPA in *S. platensis* and *P. cruentum*, respectively. It is reasonable to assume that the growth inhibition caused by SAN 9785 emanates to a certain extent from its effect on fatty acid desaturation, particularly the desaturation of linoleic acid. The latter is further desaturated to GLA in *S. platensis* (Scheme 1) and in *P. cruentum* to GLA and perhaps to α-linolenic acid, both of which are possible EPA precursors (Scheme 1). Thus, we hypothesized that GLA and EPA overproducers may be found among SAN 9785-resistant cell lines of *S. platensis* and *P. cruentum*, respectively, and suggested that this inhibitor could be utilized for inducing overproduction of these fatty acids. Indeed, in the presence of the inhibitor, in both *S. platensis* and *P. cruentum*, the growth rate of the resistant cultures exceeded that of the wild-type, and the fatty acid composition was less affected [9]. Actually, the perturbation caused by the inhibitor to the fatty acid composition and content of the culture resistant to 0.4 mM was less than that suffered by a culture freshly exposed to only 0.16 mM in *P. cruentum* and 0.2 mM inhibitor in *S. platensis*.



Scheme 1. Possible pathways of polyunsaturated fatty acid biosynthesis in *S. platensis* (underlined) and *P. cruentum*.

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In keeping with the findings of Murata and Nishida [16], it can be deduced that in *Spirulina*, being a cyanobacterium, only saturated and mono-unsaturated C_{18} acyl groups are incorporated into GLs and esterified to the glycerol backbone at the sn-2 position; hence, any further desaturation is accomplished on GL-bound molecular species. Invoking the R value is thus justified, being a measure of GL-related C_{18} desaturations, reactions which are most likely to be affected by SAN 9785. Indeed, the R value in the GL of the resistant culture SRS-1 increased to 42.2 as compared with 26.2 in the wild-type.

In *P. cruentum* biomass, the differences in EPA proportions between the wild-type and the resistant culture were not that great. However, even when growth was completely arrested EPA proportion did not go down below 24.8% (Table 3). This reflects the fact that the herbicide is known to inhibit desaturation of 18:2-containing GL-bound molecular species formed in the prokaryotic pathway, but not that of 18:2-containing PLs produced in the eukaryotic pathway [12]. Indeed, by comparison of the respective GL fractions the resistance to the herbicide was demonstrated by an increase in EPA proportion to 45.4% from 29.1% in the wild-type in the presence of the inhibitor. The former value almost reached that of the wild-type under inhibitor-free conditions (50%).

Apparently, there are several possible routes for the synthesis of EPA in microalgae. This conclusion was drawn from the findings that in some algae e.g. *P. cruentum*, SAN 9785 inhibits the formation of EPA in GL [10], while in others e.g. *Monodus subterraneus* [10], *Chroomonas salina* and *Nannochloropsis oculata* [17], it does not. We hypothesized that in *Porphyridium*, EPA is the product of elongation and further desaturation of GL-bound 18:3. The increase in the proportion of 20:2 in GL in the presence of the inhibitor, as well as the overproduction of EPA in the GL of resistant cells (Table 4), support this hypothesis. The resistant lines will undoubtedly be of further aid in elucidating the biosynthetic pathways of EPA in *P. cruentum*.

The growth rate of the resistant cultures in the presence of the inhibitor, although higher than that of a freshly exposed culture was still lower than that of the control culture. This may reflect the fact that the herbicide has more than one site of action. Hilton *et al.* [18] have shown that SAN 9785 causes direct inhibition of photosynthetic electron transport. We have recently demonstrated [10] that SAN 9785, which is known to be an inhibitor of the Δ15 system (18:2ω6 to 18:3ω3) in chloroplast lipids [11], was also capable of inhibiting the Δ6 system (18:2ω6 to 18:3ω6), further demonstrating the wide range of activity of the compound. Yet another example for the lack of specificity is the observation that SAN 9785 also affects the desaturation of 18:1ω9 to 18:2ω6 in *Spirulina*, which is indicated by the accumulation of the former (Table 1). This was conspicuously evident in the PL fraction, which, in cyanobacteria, consists of phosphatidyl glycerol (PG) only. The large increase of 18:1 in this lipid fraction resulted primarily from the decrease in 18:2 and not from the decrease in GLA whose content was already very low (Table 2). In higher plants, it has already been shown [19] that the enzymes responsible for the desaturations of 18:1 to 18:2 (Δ12) and from 18:2 to 18:3 in GL and PG are the same. Thus, it follows that there is a direct effect of SAN 9785 on the Δ12 system in both PG and GL which may be independent of its effect on the Δ6 system. We are aware that further exploitation of this approach could be hampered by the reduced specificity of the herbicide. Thus, more specific inhibitors such as transition-stage analogues are being sought.

The selected strain, *S. platensis* 2340, was previously shown to be very low in GLA [5]. However, it was used as an experimental model with the assumption that a further reduction in the already low GLA in this strain would be more detrimental, resulting in a stronger selection pressure for the appearance of GLA over-producing lines. Currently, we are conducting similar selections with strains of high GLA content. We expect that further selection of GLA over-producing *Spirulina* strains would make the production of algal GLA economically feasible.

EXPERIMENTAL

Organisms and culture conditions. *Spirulina platensis* 2340 was obtained from the University of Texas Culture Collection; strain BP was isolated from a local pond in Ban Pong, Thailand. *Porphyridium cruentum*, strain 1380 1d, was obtained from the Goettingen Algal Culture collection (Goettingen, Germany). *Spirulina* cultures were cultivated on Zarrouk's medium at 30° as previously described [5]; *Porphyridium cruentum* was grown on Jones' medium [20]. Stock cultures were maintained and inocula performed according to ref. [21]. *Porphyridium* cultures were grown in Erlenmeyer flasks, placed in an incubator shaker and illuminated from above at a light intensity of 115 $\mu E\ m^{-2}\ sec^{-1}$ under an air CO_2 (99:1) atmosphere at 25°. Cultures were grown exponentially (with proper dilution) under the appropriate conditions for at least 4 days prior to the onset of the expt. The specific growth rate was estimated by measurements of chlorophyll concn.

Selection of SAN 9785-resistant lines. Solns of SAN 9785 in DMSO were added to exponentially-growing cultures. The final concn of DMSO in treated and control cultures did not exceed 1%. Cultures of *S. platensis* were cultivated in the presence of 0.2 mM SAN 9785 which inhibited growth by *ca* 80%. After several weeks of growth in the presence of inhibitor (with occasional dilution with fresh medium containing the required concn of herbicide) the growth rate gradually increased to approach that of control cells. The concn of inhibitor was increased to 0.4 mM and the culture cultivated as described above for several months. *Porphyridium cruentum* cultures were similarly treated with an initial inhibitor concn of 0.08 mM which was gradually increased over a 9-month period to 0.16 and 0.32 mM, respectively, finally reaching 0.4 mM. Inhibitor-resistant sublines of *S. platensis* were obtained by filament dilution. Culture suspensions resistant to 0.4 mM herbicide were diluted with inhibitor containing medium and distributed to 50 test-

101

tubes having on average half a filament per test-tube. Tubes were incubated for a period of several weeks and those in which growth occurred were rescued. Cell lines of *P. cruentum* resistant to 0.4 mM of herbicide were obtained by screening of the resistant suspension on agar plates containing 0.4 mM of inhibitor.

Fatty acid analysis. Fatty acid composition and content were measured 3 days after exposure to inhibitor. Freeze-dried cells were transmethylated with MeOH–AcOCl as previously described [22]. Heptadecanoic acid was added as an int. standard. GC analysis was performed on a Supelcowax 10 fused-silica capillary column (30 m × 0.32 mm) at 200° (FID, inj. and det. temp. 230°, split-ratio 1:100). Peak areas were measured using an integrator. Fatty acid Me esters were identified by co-chromatography with authentic standards (Sigma) and by comparison of their equivalent chain length values. Fatty acid contents were determined by ref. to the int. standard. The data shown represent mean values with a range of less than 3% for major (over 10% of fatty acids) peaks and 10% for minor peaks, of 4 independent samples, each analysed in duplicate.

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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: Agal 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, such as arachidonic acid with antagonistic properties. 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 micro-organisms (3). Furthermore, it has been predicted that when ω3 PUFA come to be widely used as a proplylactic 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 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, MS); 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,

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103

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 semi-continuous (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[c]	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[c]	EPA
Semi-continuous	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.

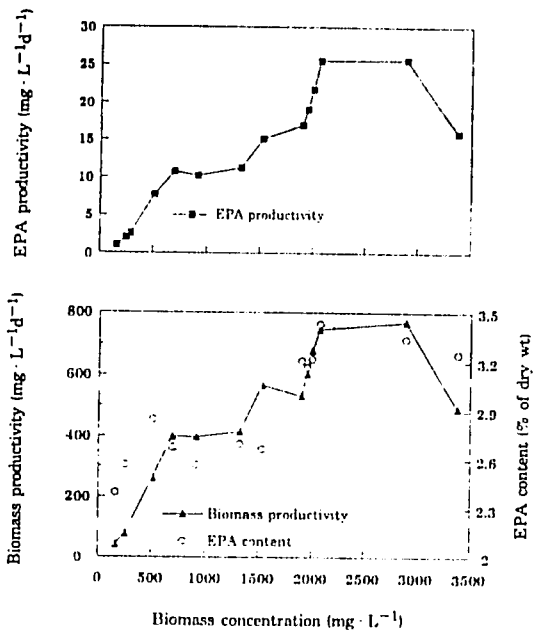

FIG. 1. Changes in biomass productivity, eicosapentaenoic acid (EPA) content and EPA productivity during growth of *Monodus subterraneus* in batch mode. Data were accumulated from several experiments.

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]

CO_2 concentration (%)	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
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]

Culture	Fatty acid composition (% of total fatty acids)									Fatty acid content (% of dry wt)	
	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.

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105

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 ($mg \cdot L^{-1} \cdot d^{-1}$)	Reference
Mannochloropsis oculata	Batch, low light	—[a]	3.7	(21)
N. oculata	Batch, high light	—[a]	5.5	(21)
Porphyridium cruentum	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, semi-continuous 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 semi-continuous 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

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

N. 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 $mg \cdot L^{-1} \cdot d^{-1}$. At 25°C the EPA proportion was lower and the growth rate was much higher. Consequently, EPA productivity increased to 5.5 $mg \cdot L^{-1} \cdot d^{-1}$. 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 $mg \cdot L^{-1} \cdot d^{-1}$. *P. 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 $mg \cdot L^{-1} \cdot d^{-1}$. 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 $g \cdot m^{-2} \cdot d^{-1}$ (corresponding to as little as 1.5 $mg \cdot L^{-1} \cdot d^{-1}$) 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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106

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