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The Alga *Spirulina* as a Source of γ-Linolenic Acid and Other Valuable Chemicals

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3. Executive Summary

The purpose of this project was the development of the cyanobacterium *Spirulina* as a source of chemicals. This alga contains relatively large concentrations of the pharmaceutically valuable fatty acid γ-linolenic acid (GLA), and of the commercially important pigments phycocyanin and xanthophylls. The project aimed at: a) identifying the nutritional and environmental conditions affecting the biosynthesis of these chemicals in an attempt to optimize their production; b) isolation of GLA-overproducing mutants of *Spirulina*; c) development of an extraction and separation scheme for these chemicals.

We have succeeded in all three targets as detailed in the seven scientific papers (attached) 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 GLA content in one strain from 1 to 1.8% (of dry weight). This is the highest GLA content ever reported for *Spirulina* or any other alga.

The project developed skills and created know-how in the physiology of fatty acid production, fatty acid analysis and algal cultivation. Furthermore, based on the findings of this project and the accumulation of know-how, a commercial plant for *Spirulina* cultivation was established by the co-investigators in Thailand and the owners of a Casawa factory (see attached report of the Thai group). The *Spirulina* ponds takes advantage of the mineral and carbon rich effluents of the Casawa plant. Thus, the ponds serve for both purifying the effluent and production of *Spirulina*. The product is sold as feed for ornamental fish owing to its high content of xanthophylic pigments. Furthermore, the Thai group is independently continuing the research along the lines delineated in this project with funding obtained from local sources.

The weaker side of the project was the collaboration with the co-investigators. In general, output as well as quality of work performed were not as high as could have been expected, partly due to the lack of a basic scientific methodology, primarily in areas such as designing experiments, drawing conclusions and assessment of data.

Their tardiness in reporting was a continuous subject of our correspondence. However, this should not be interpreted as a sign of ill will or lack of cooperation. We have observed a dramatic improvement in their ability for scientific expression and to design and conduct experiments independently during the course of this research.

It is the feeling of this 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**

This project was designed to take advantage of the natural advantage of Thailand as a place for large-scale algal cultivation, primarily *Spirulina*. Thailand is one of the world's main *Spirulina* producers. The *Spirulina* biomass is marketed as health food catering to a limited market. We perceived that it could be produced in a much larger scale if it can be developed into a source of valuable chemicals.

Our main efforts were concentrated in developing methods for production of γ-linolenic acid (GLA) from *Spirulina*. Our basic hypothesis was that if we find an inhibitor to the biosynthesis of GLA which will also inhibit growth, then perhaps by selecting mutants resistant to this inhibitor, we may find some which overcome the inhibition by overproducing GLA. This can be proved by a consistent GLA 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 *Porphyridium* which overproduces eicosapentaenoic acid (EPA). Similar results were since reported in other algae by other scientists (1). As mentioned above an independent follow-up research is currently conducted by the Thai coinvestigators with local funding.

5) Methods and Results

The results obtained in this project are detailed in the enclosed 7 papers which were published or submitted to various refereed scientific journals. One more paper is currently being prepared by our co-investigators. The following is a synopsis of our main findings. More data is provided in the attached report of the Thai group. Unfortunately the late arrival of the latter did not allow for an integration of the data and a unified format.

INTRODUCTION

Recent studies have shown that γ-linolenic acid (18:3ω6, GLA) is of potential value for lowering low density lipoproteins in hypocholesterolemic patients (2), for alleviation the symptoms of the pre-menstrual syndrome (3), and for treatment of atopic eczema (4).

GLA is found in evening primrose, black currant (5) and borage (6), as well as in cyanobacteria (7) and fungi (8). However, large scale cultivation of these fungi is not yet feasible while the GLA content in plants is either low (8-12% of fatty acids in the seed oil) or is accompanied by other fatty acids (e.g., 18:4) of undesired properties from which separation could become very expensive in a large scale.

Spirulina is so far the best algal source for GLA. It is a multicellular, filamentous cyanobacterium. Typical are *S. platensis* and *S. maxima* which profusely populate certain alkaline lakes in Africa and Mexico (9). Several properties make this alga an outstanding candidate for outdoor cultivation. It is both thermophylic, the optimum growth temperature being 35 to 37 °C and alkalophylic, with a pH optimum of about 10. These properties provide an ecological niche which facilitates the maintenance of monoalgal cultures outdoors.

Commercial production of *Spirulina* was attempted in several countries, primarily for health food but also for food, pigments and fish feed. It contains several pigments of economic interest. Phycocyanin, a blue proteinaceous pigment, which constitutes up to 25% of the algal dry weight (10), was introduced in Japan as a natural coloring agent for feed and cosmetics. The alga also contains zeaxanthin, a xanthophylic

pigment which was shown to be effective in enhancing fish and shrimp pigmentation (11).

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, non of these methods were shown to be effective for cyanobacteria and even in eukaryotic algae 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 (12).

In this report, we describe various strategies for increasing the PUFA content in *S. platensis* 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 GLA overproducing lines of *S. platensis* is described.

MATERIALS AND METHODS

Organisms and culture conditions. *S. 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 (13). Stock cultures were maintained and inocula performed according to Vonshak (14).

Selection of SAN 9785 resistant lines. Solutions of SAN 9785 in DMSO were added to exponentially growing cultures. 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. Inhibitor resistant lines of *S. platensis were* obtained by filament dilution. Culture suspension 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.

Fatty Acid Analysis. Freeze dried samples of biomass were trans-methylated with MeOH - Acetyl chloride and analyzed by GC as previously described (Cohen et al., 1987*).

Lipid fractionation, Lipid fractionation and formation of urea inclusion complexes were performed as previously reported (Cohen et al., 1993b).

RESULTS AND DISCUSSION

Influence of environmental factors on GLA content

By proper choice of strains and growth temperature, it was possible to increase the content of GLA in *Spirulina* to 31.7% of total fatty acids and 1.4% of dry weight (48). Increasing the cell concentration of *Spirulina* cultures under either batch or semi-continuous conditions resulted in an enriched content of fatty acids and thus of GLA. In batch culture, the fatty acid content increased from 3.5% to 6.9% (of dry weight) after 7 days while in a daily diluted culture, the fatty acid content did not change significantly and ranged between 3.5% and 3.7%. Since the GLA proportion of fatty acids did not change significantly in the batch, the overall GLA content still increased by about 25%.

Increasing the cell concentration under either batch or semi-continuous growth conditions resulted in enhanced fatty acid and therefore GLA contents. However, the effect of the increase in the fatty acid content on the GLA content was partly negated by a slight decrease in the GLA proportion (Table 1, Cohen et al. 1993b). GLA was primarily concentrated in polar lipids and especially in GLs (92% of total GLA), while neutral lipids contained only very low proportions of cellular GLA (1.5%). The decrease in the proportion of GLA may indicate a larger proportion of NL in newly synthesized lipids than in lipids of exponentially growing cells. However, this increase was not exclusively made of NL since the overall increase in GLA content indicates a net increase in GLs. At higher cell concentrations corresponding to stationary phase conditions the increase in fatty acid content resulted to some extent from an increase in NLs, hence the decrease in both the proportion and the content of GLA. These results

* References cited by name and year are attached.

are in keeping with earlier findings demonstrating an increase of fatty acids and GLA contents of various *Spirulina* strains cultivated under lower light intensities (Cohen et al., 1987). This effect could be related to the reduction in growth rate at higher cell densities or low light intensities, or could directly evolve from the effect of light intensity on cell biochemistry. However, reduction of the growth rate by increasing the salinity did not enhance the fatty acid content which remained unaffected at low salinities (0.1-0.75 g L^{-1}) and even decreased at high salinities (1.5 g L^{-1} and beyond). The cell concentration-dependent increase in fatty acid and GLA contents could be demonstrated in both the wild type strain BP and its GLA-overproducing variant Z19. Thus, it seems that the combination of two methods we have developed for increasing GLA content, namely, the use of GLA-overproducers and cultivation at higher cell concentrations, could be employed for a further enhance of the GLA contents in *Spirulina*.

Increasing the cell density also resulted in higher chlorophyll and phycocyanin contents. The latter more then doubled, from 10.4 under a high dilution rate to 22.4% under a low dilution rate (Table 1, Cohen et al., 1993b). Thus, the increase in cell density resulted in an increase in the content of the two major products of *Spirulina*, GLA and phycocyanin. However, since maximal biomass productivity will be obtained at lower cell densities, optimal productivities of these products will probably be reached at some intermediate value.

The high cost of production of *Spirulina* and the low content of GLA has so far prevented it so far from becoming a source of the latter. However, we believe that a concerted extraction of GLA, phycocyanin and xanthophylls, concurrent with a further decrease in the cost of production will contribute to the emergence of an economically viable process.

Selection of overproducers

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 indeed shown in higher plants that among lines selected

for resistance to the growth inhibition some over-produced the metabolite in question (15-17). Mutants of *Spirulina* showing elevated production of proline were obtained by selection in the presence of proline analogs (18). Similar results were obtained in the cyanobacterium *Nostoc* (19) and the alga *Nannochloris bacilaris* (20).

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 (21) and its effect on reduction of 18:3ω3 levels in the glycolipids of higher plants and algae was widely studied (21). The site of inhibition was proposed to be the desaturation of 18:2 on MGDG (22). 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 (23). We found (24) this herbicide to be an effective inhibitor also for Δ6 desaturation of linoleic acid in *Spirulina*, i.e., the conversion of 18:2 to GLA (Scheme 1, Cohen et al., 1992b).

We have also shown (Cohen and Heimer, 1990) that Norflurazon, another substituted pyridazinone which is known to inhibit carotenoid biosynthesis and chlorophyll accumulation and consequently growth (25), inhibits fatty acid desaturation as well, primarily the Δ6 desaturation. Its effect on the Δ6 desaturase is two orders of magnitude higher than that of SAN 9785. Almost all the GLA in *S. platensis* are located in the glycolipids which are important components of the photosynthetic apparatus. 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 in the systems leading to these fatty acids. Although Norflurazon is a more effective Δ6 inhibitor, we have chosen to employ SAN 9785 for the *Spirulina* study due to the significant effect of the former on pigment accumulation. Attempts to obtain resistance would apparently require a mutation in two independent sites.

Maximal growth inhibition of *S. platensis* by the herbicide SAN 9785 was observed at 0.8 mM. Yet even at 0.4 mM, growth was arrested after a few days and the culture collapsed thereafter (Fig. 1, Cohen et al., 1992b). Exposure of *S. platensis* to SAN 9785 resulted in decreases in 18:3ω6 and 18:2 and in increases in 18:1 and 18:0

(Table 1, Cohen et al., 1993a). In the presence of 0.4 mM SAN 9785, the proportion of 18:3 declined from 19.6% (of total fatty acids) in inhibitor-free medium to 12.4% and that of 18:2 from 25.6% to 22.5%, while 18:1 increased from 2.6% to 11.3% and 18:0 from 1.0% to 3.2%. The fatty acid content was also affected and was reduced from 4.9% (of dry weight) to 2.7%, resulting in a drastic reduction of the content of 18:3 from 0.96% to 0.33%.

It is reasonable to assume that the growth inhibition incurred by this herbicide emanates to a certain extent from its effect on fatty acid desaturation, particularly the transformation of linoleic acid. Linoleic acid is further desaturated to GLA in *S. platensis*. Thus, we hypothesized that over-producers of GLA may be found among SAN 9785-resistant sublines of *S. platensis*.

Clones of these algae which are resistant to the growth inhibition of SAN 9785 could indeed be selected from the wild type (Fig. 1, Cohen et al., 1992b). Sublines resistant to 0.4 mM SAN 9785 were obtained by stepwise increasing the herbicide concentration in the growth medium. In the presence of this concentration the resistant cultures continued to grow, though at a reduced growth rate, while the wild type collapsed after a few days (Fig. 1, Cohen et al., 1992b). When brought back to inhibitor-free medium, their respective growth rates were back to normal. The resistance was maintained even after 50 generations in an inhibitor-free medium indicating an apparently adaptive change of a genetic nature.

Relative to the wild type the resistance in *Spirulina* was associated, as predicted, with elevated levels of GLA both in the presence and in the absence of the inhibitor (Table 1, Cohen et al., 1992a). In inhibitor-free medium GLA increased from 21.2% (of total fatty acids) in the wild type to 23.6% and 22.8% in the resistant isolates SR1 and SR3, respectively. The proportions of 18:0, 18:1 and 18:2 decreased (Table 1, Cohen et al., 1992a). The two isolates, had an improved fatty acid content of 5.55% (of dry weight) and 5.34%, respectively, compared with 4.50% in the wild type. Even more apparent was the increase in GLA content on a dry weight basis, from 0.95% in the wild type to 1.31% and 1.22% in SR1 and SR3, respectively.

It is difficult at present, to point at the mechanism(s) of resistance to the herbicide. Murphy et al. (21) 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 (21). 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 enzyme (22). The increase of GLA in *S. platensis* may be attributed to one of the last two mechanisms. Thus, over-production could be a means for counteracting the effect of the herbicide.

The establishment of herbicide resistance would also be advantageous for large scale cultivation of *Spirulina*. 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.

GLA purification

While saturated and monounsaturated fatty acids could be efficiently removed by the formation of urea adducts, it is the PUFAs 16:3 and 18:2 that are more difficult to eliminate. Therefore, in order to concentrate and separate GLA from other PUFAs, a preliminary fractionation step of the oil was undertaken. Fractionation of the *Spirulina* oil yielded a fraction (GL) which contained the lion share of GLA. Moreover, virtually all the 16:3 and most of the 18:2 were restricted to the NL and PL fractions respectively. Indeed, the GLA content of the GL fraction increased to 90.5% following the urea treatment, while a similar treatment of the total lipids resulted in only 79.7% GLA (Table 3, Cohen et al., 1993b).

The silica gel fractionation process achieved yet another goal, i.e., the separation of the zeaxanthin containing xanthophylls, which were found in the NL fraction. Since very little GLA was present in the NL fraction and the bulk of it was restricted to the GL, the fractionation enabled the practical separation of GLA and zeaxanthin. For obtaining high purity GLA, the GL fraction can be isolated by an acetone wash. For higher yield, the last step can be replaced with a methanol wash, resulting in elution of the GL and PL fractions together which comprised 93% of cellular GLA. Extraction of the third valuable product, phycocyanin, should however take precedence in order to avoid its denaturing by contact with organic solvents. In order to take advantage of the various chemicals in *Spirulina*, a fractionation scheme allowing for optimal recovery and purity was devised (Fig. 3, Cohen et al., 1993b):

6) Impact, Relevance and Technology Transfer

The results of this project were already implemented in the establishment of a Spirulina production plant that utilizes Casawa effluents. The product is used for fish feeding and is valued according to its pigment content. The co-P.I. of this project, Dr. Tanticharoen, is the scientific advisor of this plant. We believe that should more work be invested towards the isolation of GLA-overproducing strains, growing Spirulina on Casawa effluents would be even more economic due to the increase in the value as well as the market of the product. Indeed, research along that line is currently conducted by the co-P.I. with local funding.

The Thai lab has gathered a lot of know-how in various areas which previously did not exist there, such as indoor and outdoor algal cultivation; Selection of mutants and basic biochemical and physiological methodology. Being able to supervise the operation of an algal production site which is by no means an easy accomplishments is an evidence to the successful implementation of these methodologies.

7) Project Activities / Outputs

Meetings

1989 M. Tanticharoen, N. Jeeyashok & B. Bunnag. Pilot plant scale production of *Spirulina* using Tapioca starch waste water. 15th Science and Technology Conference of Thailand, Chiangmai University, October 18-20.

1989 M. Tanticharoen, M. Reungjitchachawali, S. Vetayasuporn & P. Chitnumsub. Effect of environmental conditions on fatty acid composition of *Spirulina* BP-1. 15th Science and Technology Conference of Thailand, Chiangmai University, October 18-20.

1989 M. Tanticharoen, S. Vetayasuporn, P. Chitnumsub & M. Reungjitchachawali. Extraction of fatty acid and pigments from *Spirulina*. 15th Science and Technology Conference of Thailand, Chiangmai University, October 18-20.

1990 Z. Cohen & Y. M. Heimer. Linolenic acid desaturase inhibitors as tools for selection of GLA over-producing lines in *Spirulina*. 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 N. Jeyashoke, B. Bunnag & M. Tanticharoen. Pilot plant scale production of *Spirulina* using Tapioca starch waste water: Effect of supplemented fertilizers on productivity. Paper presented at the 5th International Conference of the Society of Applied Algology. Recent Advances in Algal Biotechnology, Israel, January.

1990 M. Reungjitchachawali, S. Vetayasuporn, P. Chitnumsub & M. Tanticharoen. Study on the fatty acid composition of *Spirulina* BP-1. Paper presented at the 5th International Conference of the Society of Applied Algology. Recent Advances in Algal Biotechnology, Israel, January.

1990 P. Chitnumsub, S. Vetayasuporn, M. Reungjitchachawali, M. Tanticharoen & K. Krisnangkura. Extraction of fatty acids from *Spirulina* BP-1. Paper

presented at the 5th International Conference of the Society of Applied Algology. Recent Advances in Algal Biotechnology, Israel, January.

1990 M. Tanticharoen, et al. Research and development on *Spirulina* production for feeds and chemicals. Paper presented at Seminar on Biology for Agro-Industrial Wastes Management, Bangkok, Thailand, February 5-6.

1990 M. Tanticharoen, et al. The cultivation of *Spirulina* using Tapioca starch waste water. Paper presented at Regional Seminar on Management and Utilization of Agricultural and Industrial Wastes, Kuala Lumpur, Malaysia, March 21-23.

1990 P. Vongtaweesuk, C. Wantawin, M. Tanticharoen, B. Bunnag, N. Ieyachoke & S. Bhuminatana. The use of CO_2 as carbon source for *Spirulina*. 16th Science and Technology Conference of Thailand, Khonkaen University, October 25-27.

1991 P. Vongtaweesuk, B. Bunnag & M. Tanticharoen. Effect of light-dark cycle and temperature on fatty acid composition of *Spirulina platensis* BP. 17th Science and Technology Conference of Thailand, Khonkaen University, October 24-26.

1991 P. Vongtaweesuk, M. Tanticharoen, A. Petiraksakul, S. Bhumiratana, Suwanayen, B. Bunnag & N. Jeyashoke. Industrial scale cultivation of *Spirulina* using Tapioca starch waste water. 17th Science and Technology Conference of Thailand, Khonkaen University, October 24-26.

1991 P. Chitnumsub, M. Tanticharoen, S. Bhumiratana & S. Suwanayuen. Solvent extraction of gamma-linolenic acid from *Spirulina*. 17th Science and Technology Conference of Thailand, Khonkaen University, October 24-26.

1992 W. Siangdung, M. Tanticharoen & B. Bunnag. Effect of Sandoz 9785 on gamma-linolenic acid (GLA) in *Spirulina*. First Asia-Pacific Conference on Algal Biotechnology, University of Malaya, Kuala Lumpur, January 29-30.

1992 L. Chanawongse, M. Tanticharoen & B. Bunnag. Photoinhibition in *Spirulina* and its recovery. First Asia-Pacific Conference on Algal Biotechnology, University of Malaya, Kuala Lumpur, January 29-30.

1992 M. Tanticharoen, B. Bunnag & S. Bhumiratana. Mass cultivation of *Spirulina* in Thailand. First Asia-Pacific Conference on Algal Biotechnology, University of Malaya, Kuala Lumpur, January 29-30.

Training

Marasri Reungjitchachawali attended the algal biotechnology course held in our lab on 5/89.

Morakot Tanticharoen (co-P.I.) visited our lab on 6/89.

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

Wipawan Siangdung attended the algal biotechnology course held in our lab on 5/91. The P.I. is a co-advisor in her M.Sc thesis.

Publications

1987 Z. Cohen, A. Vonshak & A. Richmond. Fatty acid composition in different *Spirulina* strains and under various environmental conditions. *Phytochem.*, **26**, 2255.

1990 Z. Cohen & Y. M. Heimer. Δ6 desaturase inhibition: A novel mode of action of Norflurazon. *Plant Phys.*, **93**, 347.

1991 Z. Cohen & A. Vonshak. The fatty acid composition of *Spirulina*-like cyanobacteria as a tool for chemotaxonomy. *Phytochem.*, **30**, 205.

1992a Z. Cohen, S. Didi & Y. M. Heimer. Over-production of γ-linolenic and eicosapentaenoic acids by algae. *Plant Phys.*, **98**, 569.

1992b Z. Cohen, M. Reungjitchachawali, W. Siangdung, M. Tanticharoen & Y. M. Heimer. Herbicide resistant lines of microalgae: Growth and fatty acid composition. *Phytochem.*, (submitted).

1993a Z. Cohen, H. A. Norman & Y. M. Heimer. Evaluating the potential of substituted pyridazinones for inducing polyunsaturated fatty acid overproduction in algae. *Phytochem.*, **32**, 259.

1993b Z. Cohen, M. Reungjitchachawali, W. Siangdung, & M. Tanticharoen. Production and partial purification of γ-linolenic acid and some pigments from *Spirulina platensis*. *J. Appl. Phyc.* (in press).

1993c M. Tanticharoen, M. Reungjitchachawali, B. Boonag, A. Vonshak & Z. Cohen. Effect of growth and light-dark cycle on fatty acid composition in the cyanobacterium *Spirulina platensis*. (in preparation).

8) Project Productivity

The project advanced our know-how in each of the designated areas. However, more and better results could have been obtained. The workload was so divided that ground breaking work was to be performed in Israel and further developed in Thailand. For instance, the development of methods for isolation of GLA-overproducing mutants and the first isolation of such mutants was done in Israel. In order to get maximal output from this method, several thousand mutants were to be screened. This task is labor -intensive and due to the differences in cost of manpower between Israel and Thailand, the latter was considered to be an ideal place for this study. Representatives of the Thai group spent several months in Israel and brought back the methodology to Thailand. Unfortunately, implementation was not as fast. As a result, at the conclusion of this project only 20 mutants were studied at any depth. This however will be remedied by a follow-up project currently conducted by the Thai group.

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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FINAL REPORT

THE ALGA SPIRULINA AS A SOURCE OF γ-LINOLENIC ACID AND OTHER VALUABLE CHEMICALS

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SUMMARY

This paper reports the work on "The Algal Spirulina as a Source of γ-Linolenic Acid and Other Valuable Chemicals done at King Monkut's Institute of Technology Thonburi. Results were divided into three parts.

1. The effects of environmental conditions and nutritional factors.
2. The use of Sandoz 9785 to select the strains resistance to analog and to study the fatty acid compositions of these strains.
3. The extraction for GLA and phycocyanin.

The percentage of C18:3 in Spirulina BP-1 varied from 20 to 30% of total fatty acids. The composition depended upon growth phase and environmental condition. C18:3 increased during the exponential growth phase then decreased through the stationary phase. Palmitic acid (C16:0) which was the main composition of fatty acids remained constant until the culture started to deteriorate. Its content decreased dramatically in correspondent with the increase of C18:0. Total fatty acid of Spirulina increased with increasing the age of culture. Nevertheless, it reduced after the stationary phase.

Results from the semicontinuous experiment showed that the percentage of C18:3 depended upon the specific growth rate. The higher the specific growth rate, the higher the C18:3 was. Therefore the C18:3 decreased at the dilution rate of 0.6 day^{-1} which was near the critical dilution under the experimental conditions.

Light intensity had no effect on the composition of fatty acid but the total fatty acid. TFA increased with increasing light intensity. Diurnal effect enhanced the desaturation of C18.

Increasing NaCl concentration in Zarouk's media reduced the specific growth rate of Spirulina BP-1 C18:3 decreased a little bit at 4% NaCl with an increase in C18:1. TFA decreased thus reducing the amount of GLA as percent dry weight.

The attempts have been tried to select the salt tolerant strain of Spirulina. The strain which lost gas vacuole grew faster in 4% NACl than the wild type.

The effects of the herbicide pyridazinone [{4-chloro-5-(dimethylamino)-2-phenyl-3(2H) pyridazinone; SANDOZ 9785, BASF 13-338}] on growth, fatty acid composition and photosynthesis were studied in Spirulina. Strains resisted to SAN 9785 were selected and screened for a high gamma-linolenic acid (GLA)

producer. SAN 9785 inhibited growth and caused a change in fatty acid profile in which the desaturation of oleic acid (C18:1) was inhibited resulted in the increase in percentage in oleic acid and decrease in linoleic acid (C18:2) and linolenic acid (C18:3). At 25°C, the synthesis of C18:3 was higher than those at 30 and 37°C. At low temperature, the inhibition of denaturation process was lower in the presence of SAN 9785.

The effect of SAN 9785 on the desaturation process was reversible, the level of C18:3 in washed cell resumed to normal after 10-24 hours incubation. This was in contrast to photosynthetic rate which was also inhibited by SAN 9785 but regained immediately to normal after removal of the inhibitor. It was suggested that GLA-synthesis might not be involved with the photosynthetic electron flow. Reduction in photosynthetic rate decreased the energy yield thus lower growth yield was found in the presence of the inhibitor.

By increasing the concentration of SAN 9785 stepwise, the strains resisted to SAN 9785 were selected. Among these, Z1A, Z1B, Z5B,Z17B, Z19 and Z19/2 could grow in the media containing SAN 9785 up to 1.0 mM. In the presence of the inhibitor, the resistant strains were able to synthesize higher C18:3 than the wild type (BP, P4P). Though, Z19, Z19/2 seemed to produce higher GLA when growing in the absence of the inhibitor under laboratory and outdoor conditions, it was not obvious to indicate that these strains would be better GLA-producers.

Work on the extraction of phycocyanin and fatty acid was still in the preliminary stage. After phycocyanin separation fatty acid was extracted from cell residue. The recovery was over 80%. GLA was concentrated by urea complexation.

In general, the results were satisfactory. Combining with KMITT previous experience on "Mass cultivation to Spirulina using secondary treated starch wastewater", the use of Spirulina as a source of high value chemical is very promising. Presently, the National Center for Genetic Engineering and Biotechnology has granted us (1992-1994) to conduct further study on the production of high value chemicals from Spirulina. In this study the selected strain containing high percentage of polyunsaturated fatty acid will be cultivated outdoor. The productivity will be compared with the strain normally in use in the commercial production at the Neo Tech. Food Ltd.. Besides this the extraction study will be continued to get more informations necessary for engineering design. If the project is feasible, the pilot plant will be built and the Neo Tech. Food Ltd. will supply the raw material for extraction.

It is worthnoting that the project on the Alga Spirulina as a Source of

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gamma linolenic acid and other valuable chemicals gave us more opportunity to expand our interest on this algae. Cloning of phycocyanin gene and Development of transformation system in Spirulina is being investigated. This is the prerequisite step in using recombinant DNA technology to study the regulation of phycocyanin and for strain improvement.

In terms of human resource development, three people have been trained at Dr. Zvi Cohen's Laboratory during the project. One graduate student was produced from with master thesis entitled "Effect of the herbicide Sandoz 9785 on growth and fatty acid profile in Spirulina spp. Sixteen presentations were counted.

INTRODUCTION

King Mongkut's Institute of Technology Thonburi has started research work on algal biotechnology since 1987. The research is mainly on Spirulina, the filamentous blue green algae. The first project related to Spirulina cultivation was granted by the National Center for Genetic Engineering and Biotechnology. The main objective of the project was to treat and to utilize wastewater from starch factory. Spirulina BP-1 was isolated from the stabilization pond of the starch factory. The occurrence of Spirulina corresponded to the high alkalinity of water in the pond. The result from four 6 x 26 x 0.5m concrete raceway type ponds equipped with paddle wheels showed that wastewater from the stabilization pond could be used to cultivate Spirulina with the addition of nitrogen and phosphorus fertilizer. To prevent the outgrowth of Chlorella, bicarbonate alkalinity was maintained at 2000-2500 mg/l by adding sodium bicarbonate. The sun-dried product was sold to the feed factory at the price ranged from US$ 10-12 during 2 years tested period.

Generally speaking, Spirulina is suitable as health food and feed. However, use as animal feed is prohibited by its high production cost. Our study demonstrated that the production cost was below US$ 6/kg dried Spirulina. The economic study revealed that it was feasible to produce Spirulina for animal feed using the above mentioned technology. Presently the technology was transferred to private sector (Neo Tech Food Ltd.) to produce 50 tons dried annually.

The project entitled "The Alga Spirulina as a Source of γ-Linolenic Acid and Other Valuable Chemicals" was started in June 1987. The aims of the project were:-

a. Development of γ-Linolenic acid (GLA) rich (possible over producing) strains of Spirulina.
b. Identification of the effects of environmental conditions and nutritional factors, to optimize GLA production.
c. Determination of the pigment content under the conditions for optimum GLA production.
d. Development of extraction and purification methods for GLA and the pigments.

This final reports covered the work on " The Algal Spirulina as a Source of γ-Linolenic Acid and Other Valuable Chemicals done at King Mongkut's Institute of Technology Thonburi. Results were divided into three parts.

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i) the effects of environmental conditions and nutritional factors.

ii) the use of inhibitor (herbicide Sandoz 9785) to select the strains resistance to analog and to study the fatty acid compositions of these strains.

iii) the extraction for GLA and phycocyanin.

MATERIALS AND METHODS

1. Organisms

Spirulina platensis strain BP was isolated from a stabilization pond of the tapioca starch factory, Rajburi Province, Thailand. Strain P4P was from the collection at King Mongkut's Institute of Technology Thonburi(KMITT). Strain 2340 was obtained from Dr.Zvi Cohen (Microalgal Biotechnology Laboratory of the Jacob - Blaustein Institute for Desert Research, Israel).Z group strains were selected from wild type (BP,P4P) exposed to SAN 9785. All strains were maintained on Zarrouk's medium agar. Maintenance of stock cultures and inocula preparation were performed according to Vonshak (1).

2. Culture conditions

The strains were cultivated under sterile condition in Zarrouk's medium in Erlenmeyer flasks containing 100 ml of the broth cultures, place in incubator shaker at 120 rpm and continuously illuminated with white fluorescent lamps providing 80 $\mu E.m^{-2}.s^{-1}$ at surface of flask. The temperature was maintained constant at 30°C. Cultures were grown batchwise or in a semi-continuous condition (with proper dilution) under the appropriate conditions depending on the experiment. Cultures were harvested by filtration through Whatman GF/C filter paper, collected and freeze-dried for further analyses.

3. Inhibitor

SAN 9785 (4-chloro-5(dimethylamino)-2-phenyl-3(2H)-pyridazinone; BASF 13-338) was a gift from Dr. Zvi Cohen (Microalgal Biotechnology Laboratory of the Jacob Blaustein Institute for Desert Research, Israel).

Molecular weight of SAN 9785 = 248.5

Stock SAN 9785 solution : Dissolved powder of SAN 9785 in dimethyl sulfoxide (DMSO).

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4. Parameter analysis

4.1 Measurement of growth of Spirulina

4.1.1 Growth was estimated by measuring the absorbance of cell density at 560 nm.

4.1.2 Growth was estimated by chlorophyll-a determination with measuring the absorbance at 665 nm (1).

4.2 Lipid analysis

4.2.1 Lipid extraction and transmethylation

Freeze-dried sample of Spirulina (100 mg) were treated with 2 ml of MeOH-AcCl (19:1) according to Lepage (2). C17:0 was added as an internal standard and the mixture sealed in a vial under Ar atmosphere and heated to 80°C for 1 hr. The vial was then cooled, its contents diluted with 1 ml H_2O and the mixture extracted with 1 ml hexane. The hexane layer was dried (Na_2SO_4, evaporated to dryness) and redissolved in hexane.

4.2.2 Fatty acid analysis

GC analysis was performed on a 3mx3mm. i.d. stainless steel column packed with 15% DEGS on uniport B 80/100 mesh at 190°C, FID injector and FID detector temperature 230°C, attenuation range, $2^4 x 10^2$ mV.V.). Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma Co.). Fatty acid contents were determined by comparing their integrated peak areas with that of the internal standard.

4.3 Measurement of Photosynthesis

Principle: Measurement of photosynthesis in algal cultures using an O_2 electrode. The use of an oxygen electrode to measure change in O_2 evolution rate as an indication of changes in the photosynthetic activity of microalgae.

Procedure: The cultures were diluted to a final concentration of 2 mg chl.l^{-1} with fresh Zarrouk's medium. O_2 evolution was measured using a clark type O_2 electrode. Cell cultures (5 ml) were transferred into a thermoregulated double jacket cylindrical glass vessel with the oxygen electrode immersed in the culture. The culture was bubbled with O_2 free N_2 to lower the O_2 concentration and to avoid O_2 saturation during the measurements. The temperature was kept constant, and illumination was provided by slide

projector lamp at an intensity of 160 $\mu Em^{-2}.S^{-1}$. The signal from the electrode was registered a chartpen recorder and when stable rates were obtained, photosynthesis was calculated as moles O_2 evolved. $hr^{-1}.mg\ chl^{-1}$. The electrode was calibrated to the absolute mode by using O_2 free N_2 and air-saturated water at experiment temperature.

PART I

The Effects of Environmental Conditions and Nutritional Factors.

PART I: The Effects of Environmental Conditions and Nutritional Factors.

RESULTS AND DISCUSSIONS

1. Fatty acid composition of Spirulina BP-1 cultivated outdoor.

Spirulina BP-1 was isolated from stabilization pond of Ban-Pong Starch Factory, Rajburi Province, Thailand. This strain has been using for mass cultivation by Neo Tech Food Ltd. Our initial work on fatty acid was carried out using strain BP-1. Table 1 shows the fatty acid composition of Spirulina BP-1 cultivated in the high rate 6 x 26 x 0.5m algal ponds during March-May 1989. The media was water from the stabilization pond of starch factory with the addition of $NaHCO_3$ and N:P:K (16:16:16). After harvesting the algae was sun dried and samples were analysed for fatty acid composition.

Table 1. Fatty acid composition of outdoor culture Spirulina BP-1

No.	Fatty acid composition (% of total fatty acid)						Fatty acid content as% dry weight(DW)	
	16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA(18:3)
1.	45.2	3.0	2.0	2.5	18.7	28.0	5.9	1.6
2.	45.6	3.0	1.6	1.9	19.4	27.3	6.1	1.7
3.	45.0	3.2	1.8	1.5	19.3	27.7	5.9	1.6
4.	44.2	2.9	1.8	1.9	18.9	28.5	5.7	1.8
5.	43.7	3.0	1.9	1.9	19.3	26.9	5.6	1.5
6.	43.9	2.8	1.9	2.4	19.2	28.6	5.7	1.6
7.	45.0	3.4	1.4	1.7	17.8	28.6	5.6	1.6
8.	44.4	4.1	1.4	1.7	17.6	28.4	5.6	1.6
9.	43.9	4.9	1.4	2.1	17.6	29.0	5.7	1.7
10.	45.3	2.5	1.5	1.3	17.8	27.5	5.9	1.6
11.	48.5	1.6	1.3	1.8	16.9	29.4	5.8	1.7

Samples from Ban Pong Pilot Plant (March - May 1989)
TFA - Total fatty acid
GLA - Gamma Linolenic acid
DW - Dry weight

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2. Effect of environmental conditions and nutritional factors on fatty acid composition

2.1. Growth phase

Nine strains of Spirulina were cultivated in batch culture under continuous light (40 $\mu E/m^2/s$) at 32°C and sampled at various growth phase. Palmitic acid (C16:0) was the predominant fatty acid accounted for almost 50% of total fatty acids. Its percentage did not change much during the growth period but reduced dramatically at the beginning of death phase. The culture turned from dark green to yellowish green and died in the next day.

Change on C18 fatty acids composition was observed from the early exponential through the stationary phases. The linoleic (C18:2) and gamma linolenic acid (GLA, C18:3 ω6) were high in the exponential phase then gradually declined till the stationary phase (Table 2). This was in contrast with the oleic acid (C18:1). Surprisingly, C18:1 increased to 30% of total fatty acid at the latest stage correspondence with the reduction of C16:0. The dramatic change in C16:0 occured before the death phase. (Table 3) It has been suggested that the synthesis of C18:3 occur via the desaturation of C18:1 and C16:0. The function of C18:3 might involve with the membrane integrity. The desaturation of C16:0 to C18:1 and then to C18:3 might be the process to compensate for the need of C18:3 which tended to be low in older culture.

The content of total fatty acids of the culture was low at the early stage of growth. The synthesis of TFA increased to the maximum level during the exponential. The older the culture, the lower TFA content was.

The decrease in C16:0 and the increase in C18:1 when culture ages can probably be best explained by a shift from polar, primarily galactolipids (GLS) to neutral lipids, mainly triglycerides (TGS). While in polar lipids of cyanobacteria there is a 1:1 ratio of C16 and C18 fatty acids (Murata ..), TGS in Spirulina will include lower levels of C16:0, C18:2 and C18:3 than in GLS and higher levels of C16:1, C18:0 and C18:1. A typical neutral lipids mixture of Spirulina strain (2340) contained C16:0 (27%) C16:1 (26%) C18:0 (14%) and C18:1 (17%) (Z. Cohen, unpublished data.)

Unlike green algae, lipid accumulation in the stationary phase could never be demonstrated in cyanobacteria. Thus, an expected increase in neutral lipids in the stationary phase must be accompanied by a concurrent decrease in polar lipids resulting in increases in C16:1, C18:0 and C18:1.

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Table 2. Total fatty acid and fatty acid profile of Spirulina cultures at various growth phase.

Exp.	Strain	Day	Fatty acid composition (% of TFA)						as % Qry weight	
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA
I	BP	0.8	48.2	0.7	1.0	5.4	19.0	24.5	2.6	0.6
		2	50.1	0.5	0.9	7.8	17.0	23.4	4.6	1.1
		3	49.5	0.9	1.1	8.9	14.6	23.6	5.1	1.2
		4	49.8	0.8	1.1	12.7	12.7	21.1	6.3	1.3
		5.4	51.2	0.8	1.3	11.6	11.6	19.8	5.7	1.1
		11	45.9	2.0	1.5	9.7	9.7	18.9	5.7	1.1
II	BP	2	44.8	2.1	1.1	5.6	17.8	25.5	4.3	1.0
		4	44.3	2.5	1.6	7.9	14.6	22.3	5.2	1.2
		7	45.7	2.0	2.0	11.9	12.6	20.6	5.2	1.1
		9	44.1	2.1	2.3	14.9	12.3	18.1	5.2	1.0
		14	45.5	1.7	1.7	14.7	12.1	20.9	2.8	0.6

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Table 3. Total fatty acid and fatty acids profile of Spirulina cultures at various growth phase.

Strain	day	Fatty acid composition (% of total fatty acid)						as % dry weight	
		16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
BP	3	46.9	4.4	0.8	6.5	15.7	24.3	5.3	1.3
	6	43.2	4.3	0.9	11.3	12.7	20.7	5.7	1.2
	8	47.9	3.8	1.0	14.0	19.1	19.1	5.7	1.1
	10	38.9	3.7	1.5	16.8	20.1	20.1	5.9	1.2
	14	23.9	2.3	3.8	30.5	20.4	20.4	5.1	1.0
Z2	0	44.4	3.1	1.1	10.8	13.4	20.1	4.9	1.0
	2	44.1	3.1	1.1	12.6	12.1	19.6	5.6	1.1
	4	44.1	3.2	1.1	14.0	11.6	18.9	5.9	1.1
	6	42.3	3.3	0.9	14.4	12.0	19.3	5.3	1.0
	9	29.4	3.2	2.6	23.9	10.5	22.7	5.2	1.2
	10	29.4	2.9	2.4	23.6	10.3	24.1	4.9	1.2
Z4		45.7	2.6	1.3	10.8	10.8	13.6	4.9	1.0
		48.3	3.2	1.1	13.2	10.3	13.2	5.1	1.1
		44.0	3.3	1.1	12.4	11.9	12.4	5.1	1.0
		31.1	3.2	2.0	11.1	19.5	11.1	4.5	1.2
Z5	0	48.7	3.3	1.0	8.8	13.7	21.8	4.7	1.0
	2	44.2	3.4	0.9	9.1	13.6	21.3	4.6	1.0
	4	47.8	3.6	0.8	10.7	13.1	20.9	5.9	1.2
	10	25.3	2.3	3.2	26.4	11.5	25.1	5.0	1.2
Z6	0	47.7	4.4	1.0	7.6	7.6	14.6	5.3	1.2
	2	46.8	4.3	1.5	6.6	6.6	14.7	5.7	1.3
	4	46.3	4.2	0.8	8.4	8.4	14.8	6.2	1.4
	8	47.4	4.4	0.8	7.3	7.3	14.8	6.3	1.4
	12	31.2	3.3	2.6	22.9	22.9	12.1	5.1	1.3
Z12	8	47.7	3.4	1.1	12.0	13.0	20.6	-	-
	12	34.9	3.9	2.5	18.9	12.2	24.8	-	-
Z14	6	46.9	4.1	1.0	12.1	12.6	20.1	-	-
	12	31.8	3.6	2.5	20.2	11.9	25.8	-	-
	14	29.0	2.2	1.1	29.9	12.5	17.5	-	-
Z17	0	48.6	3.6	0.9	6.5	15.6	22.5	4.6	1.0
	4	49.3	3.4	1.0	10.0	13.3	20.3	5.1	1.0
	8	46.9	3.4	1.2	12.6	12.7	20.5	5.6	1.12
	12	32.8	3.1	2.4	21.5	12.0	25.6	5.1	1.3
P4P	0	48.4	3.1	1.2	11.3	11.3	20.1	5.4	1.1
	2	48.4	3.7	0.7	7.7	7.7	22.2	5.4	1.2
	4	49.1	3.8	0.7	8.9	8.9	21.3	5.8	1.2
	8	47.2	4.7	0.7	8.9	8.9	22.6	6.4	1.4
	12	29.9	2.6	3.0	28.5	28.5	22.7	5.3	1.1

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2.2 Effect of growth rate.

Results from batch experiments showed that culture in the early and exponential phases contained higher GLA than the stationary phase. It might be on the growth rate of the culture. In animal, the percent desaturation of linoleic acid to gamma linolenic acid decreases with the age It was suggested that the very high desaturation found in the very young animals may be correlated to the building of new tissues, considering that polyunsaturated fatty acids are very important components required for the synthesis of phospholipids, and the phospholipids associated to proteins are fundamental constituents of metabolically active membranes.

To study the effect of growth rate on fatty acid profile, Spirulina were cultivated semicontinuously at various dilution rates. GLA increased with

specific growth rate. However, the percentage of GLA decreased at specific growth rate closed to the critical dilution rate (0.6 day^{-1}). The C18:1 reduced at higher specific growth rate. A slight change in C 18:2 was noted. (Table 4,5)

Culture cultivated at high dilution rate contained less chlorophyll-a and phycocyanin (Table 4,5). Increasing light intensity increased these two components and total fatty acid (Table 4). Though there was higher biomass concentration at higher light intensity, fatty acid profile of the culture growing at high and low light intensity was the same at the same specific growth rate (Table 4,5). The effect of shading was ruled out at low specific growth rate with higher biomass concentration. Nevertheless, light intensity affected the fatty acid content.

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Table 4. Effect of specific growth rate on phycocyanin, chlorophyll-a content and fatty acids composition of Spirulina.

	Specific growth rate (day^{-1})							
	0.1[a]	0.2[a]	0.3[a]	0.4[a]	0.5[a]	0.6[a]	0.2[b]	0.4[b]
Chlorophyll a(mg/g)DW	13.4	14.6	14.9	12.7	12.1	9.7	17.0	18.3
Phycocyanin (% DW)	23.3	23.5	23.5	20.5	16.4	11.3	28.1	28.6
Dry weight (g/l)	-	0.4	0.4	0.22	0.16	-	0.87	0.54
(% TFA)[c]								
16:0	45.0	42.8	43.6	44.5	42.0	41.2	45.1	44.3
16:1	3.0	3.6	3.8	4.7	7.1	7.0	3.0	3.3
18:0	1.3	1.3	1.0	0.9	1.1	0.9	0.8	1.2
18:1	12.0	11.0	9.2	7.7	6.2	5.8	12.1	8.6
18:2	13.1	13.7	14.2	15.1	15.6	15.8	13.3	14.8
18:3	20.9	22.9	22.9	24.5	23.5	21.7	21.0	23.4
(% Dryweight)								
18:3	1.0	1.2	1.2	1.2	1.2	1.0	1.3	1.3
TFA	4.7	5.0	5.1	5.0	5.0	4.6	6.1	5.5

a - light intensity 30 $\mu E.\ m^{-2}.s^{-1}$

b - light intensity 48 $\mu E.\ m^{-2}.s^{-1}$

c - Total fatty acid

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Table 5. Effect of dilution rate.

Media Zarrouk's medium, semicontinuous method.
Light intensity ~ 50 µE. m^{-2} s^{-2},
Room temperature - (29-31°C).
Cultivation vessel - Erlenmeyer's flask 1 litre with 500 ml. working volume 2 vessels for each dilution rate.

	Dilution rate (day^{-1})				
	0.01	0.05	0.25	0.40	0.6
Chlorophyll-a(mg/g)DW	13.4	-	13.2	11.9	9.2
Phycocyanin (% DW)	23.3	-	11.9	17.7	10.5
Dry weight (g/l)	1.42	-	9.2	0.66	0.43
(% TFA)[c]					
16:0	-	45.0	44.7	43.7	42.1
16:1	-	3.0	3.5	3.5	5.6
18:0	-	1.3	1.6	1.0	1.0
18:1	-	12.0	9.6	6.0	6.2
18:2	-	13.1	14.7	16.3	16.2
18.3	-	20.9	23.1	24.2	23.6
(% Dryweight)					
18:3	-	0.99	1.04	1.06	0.97
TFA	-	4.71	4.49	4.30	4.07

2.3. Salt concentration

Spirulina BP-1 was cultivated batch in Zarouk's media containing 0.1-4% NaCl. The growth rate decreased in salt media higher than 2%. Table6 compares the specific growth rate of BP-1 strain in various salt concentrations.

Table 6. The specific growth rate (hr^{-1}) Spirulina BP-1 cultivated in Zarouk's media containing 1-4% NaCl.

Exp.	NaCL concentration (%)				
	0.1	1	2	3	4
I	0.032	0.032	0.029	0.021	0.021
II	0.032	0.033	0.031	0.026	0.02
III	0.029	0.028	0.026	0.03	0.013
IV	0.31	-	-	0.028	0.02

Spirulina BP-1 showed a longer lag period at higher salt concentration. An adaptation might be the mechanism for salt tolerance. Attempts have been tried to get salt tolerance strain of BP-1 by transferring the culture to higher salt concentration step by step. The culutre was acclimatized in 4% salt media for a period of time. Table 7 compares the specific growth rate between the normal and the acclimatized culture.

Table 7. Specific growth rate (hr^{-1}) of normal and acclimatized culture in media containing 4% NaCl.

Normal culture		Acclimatized culture	
NaCL 0.1 %	NaCl 4 %	NaCl 0.1 %	NaCl 4 %
0.029	0.016	0.031	0.024

The acclimatized culture was maintained semicontinuously in 4% NaCl. The culture grew quite well but some trichome was broken. The filaments were thicker than the normal BP-1. When the culture was left static,all cell settled within 1 hour. This was in contrast with the original BP-1 which floated on the surface or in suspension. The vacuole of the acclimatized culture might be disrupted from osmotic pressure during the exposure to high salt concentration. The vacuole could not be regenerated in normal media. The event was permanent and the dense culture looked darker than the vacuolated culture due to light reflection.

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The salt adapted cells (non - vacuolated) were well selected during the semicontinuous culture in 4% NaCl. The culture was switched back to normal media for several passages, the ability to grow in 4% NaCl was still better than the vacuolated BP-1. (Table 8)

Table 8. The Specific growth rate (hr^{-1}) of the non-vacuolated culture in salted media after several passages in normal Zarouk's media.

Exp	NaCl concentration %				
	0.1	1	2	3	4
I	0.042	0.042	0.042	0.035	0.03
II	0.03	0.03	0.031	0.025	0.021

Effect of salt concentration on fatty acid composition of non-acclimatized and acclimatized Spirulina cultures are shown in Table 9-13. Most of the results either batch or continuous indicated the reduction of C18:3 (GLA) and the accumulation of 18:1 at increasing salt concentration. The C18:3 was inhibited through out the entire stage of cultivation. TFA depended on the stage of growth (see result 2.1). Due to the longer log phase at increasing salt concentration, total fatty acid during the early stage of growth was much lower than the control. The difference in TFA at the stationary phase between low and high salt concentration was less than the early stage of growth.

Table 9. Fatty acids profile of Spirulina BP-1 (non - acclimatized culture) growingbatch wise in media containing NaCl.

Exp.	Day	% NaCl	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
			16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
I		Control	45.3	2.5	1.2	12.4	12.7	20.6	4.9	1.0
		1	45.2	1.8	1.6	14.2	13.0	19.5	4.4	0.86
		2	44.6	2.4	1.4	13.8	13.3	19.5	4.1	0.80
		3	43.9	2.7	1.5	14.1	13.8	18.8	3.6	0.68
		4	41.2	2.9	2.4	18.8	14.1	17.0	3.6	0.61
II										
	24	Control	44.7	2.8	1.2	14.8	13.1	20.5	5.6	1.15
[I]=70-80 $\mu E.m^{-2}.s^{-1}$		4	44.1	3.7	1.4	18.1	14.4	17.7	4.6	0.82
T = 30°C	7	Control	45.3	3.0	1.3	10.6	14.5	20.6	4.8	1.0
		1	44.4	3.3	1.4	12.8	14.1	19.3	4.3	0.84
		2	44.1	3.2	2.2	13.6	13.8	18.7	4.0	0.75
		3	43.6	3.4	1.6	13.2	13.6	18.1	3.8	0.69
		4	42.9	3.6	2.0	15.4	13.6	18.7	3.3	0.61

FA - Fatty acid

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Table 10. Falty acids profile of Spirulina BP-1 (acclimatized culture) growing batchwise in media containing NaCl.

Experiment I. - 24 days light intensity [I] 70-80 $\mu E.m^{-2}.s^{-1}$, Temp. 30°C inoculum form 4%
- NaCl media.

Experiment II. - 6 days inoculum was culture growing in normal media

Experiment III. - 5.8 days inoculum from normal media.

Exp.	Day	% NaCl	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
I [I]=70-80 $\mu E.m^{-2}.s^{-1}$ T=30°C	24	Control	42.7	2.9	1.1	15.1	12.1	21.7	4.4	0.96
		4	41.2	3.7	1.3	18.4	13.7	18.1	4.7	0.84
II	6	Control	44.2	3.3	1.1	11.7	14.1	19.9	5.0	1.0
		1	43.5	3.5	1.1	12.3	13.6	20.6	4.8	0.98
		2	43.2	3.9	1.2	13.4	13.3	19.3	4.0	0.77
		3	42.1	4.0	1.1	17.2	12.8	17.7	4.1	0.72
		4	45.9	3.6	1.6	19.7	13.8	17.8	2.5	0.44
III	5.8	Control	49.1	2.7	1.7	10.9	13.8	20.2	5.3	1.08
		1	50.1	2.1	1.4	10.9	13.7	19.8	4.5	0.89
		2	49.2	2.4	1.5	12.7	13.6	18.6	4.8	0.9
		3	42.2	4.3	2.2	15.0	16.0	19.3	4.0	0.76
		4	45.7	3.7	2.1	13.4	14.9	17.2	3.3	0.57

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Table 11. Fatty acids profile of Spirulina BP-1 (acclimatized culture) growing batchwise in media containing NaCl.

(This acclimatized culture was growing in normal media for several transfers.)

Day	% NaCl	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
		16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
	Control	47.4	4.3	1.0	6.3	15.7	23.3	4.5	1.05
(Exp.I)	4	44.1	4.9	1.3	11.1	17.3	20.1	3.3	0.65
	Control	45.5	2.3	1.1	11.9	14.1	19.9	5.7	1.12
6	1	44.9	2.8	1.1	13.8	13.7	18.5	5.7	1.06
(Exp.II)	2	45.0	2.0	1.2	14.4	13.7	18.4	4.1	0.75
	3	44.7	1.9	1.1	14.7	13.5	17.8	3.6	0.64
	4	42.2	2.9	1.6	18.6	13.6	15.8	3.7	0.59

Table 12. Fatty acids profile of Spirulina BP-1(non-acclimatized) growing semicontinuously in salt media (dilution rate 0.25 day^{-1}, 31°C)

% NaCl	Fatty acid composition (% of total fatty acid)					
	16:0	16:1	18:0	18.1	18:2	18:3
Control	44.1	4.3	0.9	8.9	14.2	22.4
0.75	44.3	4.2	0.9	8.7	15.0	22.4
1.5	43.9	5.4	1.0	11.6	13.5	20.1
3	43.0	5.7	1.1	13.3	13.3	19.1

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Table 13. Fatty acids profile of Spirulina BP-1 (acclimatized) growing semicontinuously in salt media (dilution rate 0.33 day^{-1} light intensity 70-80 $\mu E.m^{-2}.s^{-1}$.

Exp.	% NaCl	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
		16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
I	Control	46.4	2.9	1.0	9.1	14.2	23.5	5.4	1.28
	3	43.6	4.5	1.0	9.4	16.2	22.8	4.3	0.98
II	Control	45.0	3.3	0.8	7.8	15.3	25.3	5.5	1.4
	4	43.0	4.4	1.1	12.2	16.4	20.5	4.4	0.9

Effect of salt on phycocyanin and chlorophyll was also studied in semicontinuous culture at the dilution rate of 0.25 - 0.3 day^{-1} and 30°C. The inoculum was adapted to salt concentration corresponded to the concentration used in the experiment. Table 14 shows that chlorophyll and phycocyanin were affected in the presence of salt. Again, the reduction on C18:3 and TFA was repeated (Table 15)

Table 14. Chlorophyll and Phycocyanin contents of Spriulina BP-1 grown in Zarrouk's medium at various NaCl concentrations.

NaCl concentration g/litre	Chlorophyll a mg/g dry weight	Phycocyanin mg/g dry weight
0.1	15.5	22.3
0.5	17.0	22.0
0.75	16.7	22.3
1.0	13.3	22.3
1.5	15.9	23.9
2.0	13.3	20.5
3.0	11.9	19.0
4.0	11.2	17.9

Table 15. Fatty acids composition of Spirulina BP-1 grown in Zarrouk's medium at various NaCl concentration

NaCl g/litre	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
	16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
0.1	43.2	4.43	1.2	9.5	13.8	22.0	4.9	1.1
0.5	45.0	3.9	1.0	9.9	14.3	22.4	4.8	1.1
0.75	44.0	4.3	0.9	8.9	14.2	22.4	4.6	1.0
1.0	42.8	4.0	0.9	9.1	15.2	22.9	4.7	1.1
1.5	43.9	5.4	1.0	11.6	13.5	20.1	4.0	0.8
2.0	42.2	4.0	1.1	9.4	15.5	21.9	4.4	1.0
3.0	42.2	4.5	1.1	12.8	14.5	20.0	4.1	0.8
4.0	41.7	4.8	1.1	13.7	13.8	18.5	4.1	0.8

2.4 Effect of $NaNO_3$ concentration

Experiments were conducted on a semicontinuous basis. The nitrogen source was $NaNO_3$ whose concentration varied from 0.05 to 2.5 gram/litre. The growing cells were withdrawn daily and the fresh media was added to keep the optical density (560 nm) of the cultre at 0.3. At steady state, cells were filtered and washed with water. After drying, fatty acid, phycocyanin and chlorophyll were assayed. The chlorophyll and phycocyanin content of the harvested cells were constant until nitrate input had been reduced to 0.25 gram/litre. They then declined from 15 $mg.g^{-1}$ of dry weight and 15% of dryweight, to 7.4 $mg.g^{-1}$ and 6.8% at 0.1 gram/litre $NaNO_3$ respectively. A further declined to 0.7 $mg.g^{-1}$ and 3.5% occured at 0.05 gram/litre $NaNO_3$ (Table 16). At these low nitrate concentrations, the cells changed color from dark green to yellow green.

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Table 16. Effect of $NANO_3$ concentration on phycocyanin and chlorophyll of Spirulina BP-1 (Temp 30°C, Light Intensity 50-60 $\mu E.m^{-2}.s^{-1}$).

$NaNO_3$ g/litre	Cell dryweight g/litre	Chlorophyll mg/g	Phycocyanin % dry weight
2.5	0.49	14.5	15.2
1.0	0.49	14.9	15.4
0.5	0.41	14.9	14.9
0.25	0.49	14.5	16.9
0.1	0.50	7.4	6.9
0.05	0.46	0.7	3.5

Total fatty acids (as percent dry weight) decreased sharply when $NaNO_3$ was below 0.25 gram/litre. Reduction in total fatty acid affected the amount of GLA (as percent dry weight Table 17). Though the result was not clearly indicated the change of fatty acid profile at very low $NaNO_3$ concentration,it was very interesting to note the decrease of C18:1 and the increase of C18:3 at 0.05 gram/litre $NaNO_3$.

Table 17. Fatty acids profile of Spirulina BP-1 in Zarrouk's media at various $NANO_3$ concentration.

$NANO_3$ g/litre	Dilution rate day^{-1}	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
		16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
2.5	0.48	43.2	3.6	0.9	5.7	16.2	24.7	5.0	1.2
1.0	0.49	43.9	3.5	0.9	5.7	16.3	24.7	5.2	1.3
0.5	0.46	43.0	3.4	0.95	5.6	16.4	25.1	5.0	1.25
0.25	0.47	44.0	3.6	0.8	5.3	16.4	25.3	4.8	1.25
0.1	0.34	42.8	4.2	1.7	5.1	15.8	24.8	2.9	0.8
0.05	0.29	44.1	3.5	1.2	2.3	17.5	27.5	3.1	1.9

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In another experiment, the dilution rate was kept constant at 0.4 and 0.6 day^{-1}. At each dilution rate, there was no change in fatty acid composition of Spirulina with increasing $NaNO_3$ from 0.25 to 1.0 gram/litre (Table 18).

Table 18. Effect of $NANO_3$ concentration on fatty acid composition of Spirulina BP-1 (Semicontinuous culture at 0.4 and 0.6 day^{-1}, temp 30°C, light intensity 40-80 $\mu E.m^{-2}.s^{-1}$).

$NANO_3$ Concentration g/litre	Dilution rate day^{-1}	Fatty acid composition (% of total fatty acid)						FA content as % Dry weight	
		16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA
0.25	D = 0.4	48.5	2.7	0.72	6.7	15.0	23.4	4.6	1.07
	D = 0.6	47.6	3.7	0.6	5.1	15.7	24.9	4.3	1.08
1.0	D = 0.4	49.0	2.7	0.8	7.0	15.1	23.1	4.8	1.10
	D = 0.6	47.2	2.8	0.7	5.6	16.0	24.8	4.3	1.07

However, chlorophyll reduced by 8-11% when decreasing $NaNO_3$ concentration at both dilution rate. Effect on phycocyanin was obserbed only at dilution rate 0.6 day^{-1} (Table 19).

Table 19. Effect of $NANO_3$ concentration on chlorophyll and phycocyanin of Spirulina BP-1 (Semicontinuous culture at 0.4 and 0.6 day^{-1}, temp 30°C, light intensity 70-80 $\mu E.m^{-2}.s^{-1}$).

$NANO_3$ concentration g/litre	Dilution rate day^{-1}	Chlorophyll a mg/g DW	Phycocyanin % DW	Dry Weight g/litre
0.25	0.4	13.4	23.5	0.44
	0.6	12.08	16.8	0.3
1	0.4	15.1	23.3	0.41
	0.6	13.1	18.1	0.27

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Effect of Nitrogen Limitation

Spirulina BP-1 grown on Zarrouk's medium (various $NaNo_3$ concentrations) working volume 1000 ml. in Erllnmyer flasks 1000 ml. air bubbling. light intensity about 50 $\mu E.m^{-2}.s^{-1}$, temperature 29-31°C. semicontinuous

	$NaNO_3$ concentration (g/litre)					
	0.05	0.1	0.25	0.50	1.0	2.5
Chlorophyll-a (% D)	0.07	0.74	1.45	1.49	1.49	1.45
Phycocyanin(% DW)	3.54	6.86	16.92	14.94	18.40	15.18
Dry weight (g/l)	0.46	0.50*	0.48	0.41	0.49	0.49
Dilution rate (D) or Specific growth rate (μ) day^{-1} (%TFA)	0.29	0.32	0.42	0.42	0.41	0.41
16:0	44.1	43.9	44.0	42.6	44.7	43.7
16:1	3.5	5.1	3.7	3.6	3.3	3.7
18:0	1.2	1.7	0.9	0.9	0.9	0.9
18:1	2.3	4.8	4.6	4.4	4.8	4.0
18:2	17.5	15.6	16.9	17.5	16.9	17.1
18:3	27.5	24.6	25.5	26.4	25.3	25.3
(% DW)						
18:3	0.9	0.8	1.3	1.2	1.3	1.3
TFA	3.1	2.7	5.1	4.6	5.2	5.2

Note: Sample were dried in 60°C oven about 20 hours. single culture and analysis.

* - Culture were yellow green.

2.5 Light - Dark Cycle

Previous studies showed that the C18:3 of outdoor culture was quite high compared to the laboratory cultivation. Due to the uncontrolled environmental conditions of the outdoor, the result may stem from various

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factors. The outdoor culture was cultivated in Zarrouk's media for 7 days at 32°C under the continuous light at 80 $\mu E.m^{-2}.s^{-1}$ intensity. Cell was harvested for fatty acid analysis then compared with outdoor culture of the same inoculum batch. It should be noted that the average light intensity for Thailand is 925 $\mu E.m^{-2}.s^{-1}$ and 6.4 sun shine hour. The temperature is 29.3°C during the day. Due to the high light intensity, the outdoor cell had less phycocyanin and chlorohyll a (Table 20). Light intensity wouldn't be the case for the difference in C18:3 where as TFA was not affected. Nevertheless, this factor should not be completely ruled out. Many reports including our study showed that increasing temperature the percentage of C18:3 decreased. The data excluded the effect of temperature and we intended to study further the light-dark cycle under controlled conditions.

Table 20. A comparison on fatty acid profile, phycocyanin and chlorophyll a between culture grown outdoor and in laboratory.

Culture condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		Phycocyanin	Chorophylla
	16:0	16:1	18:0	18.1	18:2	18:3	TFA	GLA	% DW	mg/g DW
Outdoor	43.9	3.6	1.3	2.3	18.3	29.1	5.6	1.61	21.3	9.42
Labora-tory	44.2	3.0	1.5	7.5	16.3	23.4	5.6	1.3	28.9	14.1

Many strains of Spirulina were cultivated 24 hours light for 3-4 days. Each culture was divided into 2 sets. The first set was incubated further under 24 hours light and the other was at 12 light: 12 dark. Most of the results clearly indicated the effect of light: dark cycle on C18:3. The desaturation process was enhanced by the light: dark condition hence the culture had higher PUFA (see ratio 18:2+18:3/18:1).

Cell exposed to light continuously grew at faster rate than in 12 light: 12 dark thus cell concentration was usually higher after incubation. Table 21 gives the result inwhich the experiment was started with 2 different cell concentration. Regardless any cell densities the light: dark condition was still better in the content of C18:2 and C18:3. A slightly increase in polyunsaturated fatty acid in parallel with a drop in C18:1 was observed at lower cell density.

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Light-dark grown cells showed a marked decrease in C18:1 in contrast to light grown cells in which an increase in C18:1 was observed. The C18:1 in light-dark cells was apparently further desaturation to C18:2 and C18:3. Molecular oxygen is required for desaturation processes (3). Desaturation may be enhanced by the effect of dark respiration which plays a role in oxygen scavenging as in the case of nitrogen fixation in heterocysts (4,5). Further investigations to prove our postulation is needed.

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Table 21. Effect of light-dark condition and growth phase fatty acid composition strain of Spirulina

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		R
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	
BP	d0		46.3	3.2	1.2	14.9	12.8	18.3	4.9	0.9	2.1
	d5	Light	46.4	3.3	1.2	13.9	12.2	20.6	5.5	1.13	2.4
[I]= 60-70	d5	9 L 15D	46.5	2.7	1.0	11.1	13.5	21.9	5.3	1.17	3.2
$\mu E.m^{-2}.s^{-1}$	d7	L	46.2	3.6	1.0	14.4	12.1	20.2	5.2	1.06	2.2
	d7	LD	46.7	2.9	1.1	10.1	14.1	23.1	5.5	1.26	3.7
T=30°C	d9	L	44.9	4.3	1.0	14.6	11.7	19.6	5.2	1.0	2.1
	d9	LD	46.1	2.9	0.9	9.5	14.8	23.3	5.5	1.28	4.0
	d11	L	44.5	5.0	1.0	14.6	11.4	19.7	4.9	0.97	2.1
	d11	LD	45.5	3.3	0.9	8.9	14.9	23.7	5.1	1.2	4.3
	d13	L	43.3	6.2	1.1	14.7	10.6	19.9	4.4	0.87	2.1
	d13	LD	43.9	3.5	1.1	8.7	14.8	24.6	5.6	1.38	4.5
	d15	L	42.4	6.8	1.0	14.7	10.6	19.5	3.8	0.73	2.1
	d15	LD	45.1	3.7	0.7	9.5	14.7	23.1	5.8	1.34	4.0
	d17	LD	44.2	4.1	0.7	8.4	15.2	24.1	5.2	1.27	4.7
BP	d5	L	45.3	3.8	1.0	13.7	12.1	20.2	5.4	1.13	2.4
	d5	9L 15D	46.6	2.9	1.0	10.1	14.6	22.4	5.1	1.15	3.7
[I]= 80-90	d7	L	45.0	3.7	1.1	14.9	12.4	19.8	5.0	0.98	2.2
$\mu E.m^{-2}.s^{-1}$	d7	LD	45.8	3.2	0.9	9.3	15.3	22.5	5.1	1.18	4.1
	d9	L	43.9	4.3	1.0	15.2	12.1	18.7	4.1	0.83	2.0
T=30°C	d9	LD	45.1	3.5	1.1	8.7	15.2	22.8	4.6	1.06	4.4
	d11	L	43.6	5.5	1.0	14.5	12.5	19.4	3.7	0.72	2.2
	d11	LD	43.9	3.1	0.9	8.4	15.6	22.8	4.5	1.03	4.6
	d13	L	43.2	6.4	1.0	14.0	11.8	19.5	3.4	0.61	2.2
	d13	LD	44.4	3.5	0.8	7.7	14.7	23.3	4.9	1.19	4.9
	d15	L	42.5	9.0	0.8	13.2	10.9	18.6	3.0	0.47	2.2
	d15	LD	44.4	3.8	0.7	8.7	16.2	24.1	5.0	1.2	4.6
	d17	LD	43.4	3.9	0.5	9.3	15.4	24.0	4.6	1.11	4.2
BP	d5	L	45.1	3.2	1.1	13.9	12.4	21.0	5.5	1.1	2.4
	d5	L9 15D	46.2	3.0	1.1	10.4	14.4	22.3	5.7	1.28	3.5
[I]=94-100	d7	L	44.8	3.6	1.4	14.8	12.0	19.9	4.9	0.98	2.2
$\mu E.m^{-2}.s^{-1}$	d7	LD	46.1	3.2	1.1	9.3	14.7	23.0	5.6	1.25	4.1
	d9	L	44.0	4.2	1.1	15.1	11.5	20.1	5.3	0.99	2.1
T=30°C	d9	LD	45.0	3.3	1.2	9.5	15.4	23.0	5.4	1.24	4.0
	d11	L	43.4	5.4	1.1	15.6	10.9	19.4	4.8	0.93	1.9
	d11	LD	44.7	3.5	1.2	8.9	15.7	23.1	5.9	1.34	4.4
	d13	L	42.1	6.7	1.3	15.9	10.3	17.7	4.3	0.85	1.8
	d13	LD	46.8	1.1	1.1	9.6	14.7	24.2	5.8	1.36	4.1
	d15	L	39.4	10.9	1.1	14.3	8.8	15.5	3.8	0.71	1.7
	d15	LD	44.2	4.0	0.9	9.3	14.5	24.1	5.3	1.28	4.2
	d17	LD	43.2	4.1	0.8	10.0	14.6	24.2	5.2	1.26	3.9
	d3	L	46.9	4.4	0.7	6.5	15.7	24.3	5.3	1.03	6.2
BP	d3	LD	48.5	3.5	2.6	2.0	16.8	25.0	4.8	1.21	20.7
	d6	L	43.2	4.3	0.9	11.3	12.4	20.7	5.7	1.18	2.9
	d6	LD	47.7	4.0	2.6	2.1	18.1	24.0	5.3	1.28	20.0
T=30°C	d8	L	47.9	3.8	1.0	14.0	11.6	19.1	5.7	1.09	2.2
	d8	LD	47.6	3.4	2.6	2.0	17.3	25.4	5.2	1.33	20.6
	d10	L	38.9	3.7	1.5	16.8	11.4	20.1	5.9	1.19	1.9
	d10	LD	40.8	4.0	1.6	1.9	18.3	25.6	5.8	1.49	22.9
	d11	L	23.9	2.3	3.8	30.5	10.4	20.4	5.1	1.04	1.01
	d11	LD	43.8	3.0	1.1	1.2	16.6	24.7	6.1	1.62	35.6

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Table 21 continued.

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	R
Z1 EXPERIMENT I	d0		43.6	2.7	1.2	9.0	15.8	22.0	-	-	4.2
	d2	L	44.7	3.2	1.0	8.7	14.6	22.6	-	-	4.3
		L	44.8	3.8	0.9	6.6	16.0	23.4	-	-	6.0
		LD	44.8	3.0	0.9	5.9	15.7	24.6	-	-	6.8
		LD	45.1	2.7	0.9	3.9	16.7	25.5	-	-	10.8
	d4	L	43.4	3.5	1.1	9.7	13.9	22.1	-	-	3.7
		L	44.6	3.7	1.0	8.9	14.7	22.4	-	-	4.2
		LD	44.4	3.2	0.9	5.4	16.6	24.3	-	-	7.6
		LD	43.6	3.5	1.0	2.6	18.0	25.5	-	-	16.7
	d7	L	40.7	3.8	1.2	8.8	15.2	24.2	-	-	4.5
		L	42.1	4.0	1.1	10.5	14.0	22.9	-	-	3.5
		LD	42.7	3.4	0.8	3.0	17.7	26.1	-	-	14.6
		LD	44.3	3.5	0.9	1.9	18.4	26.2	-	-	23.5
Z1 EXPERIMENT II	d0		43.7	3.4	0.9	10.4	13.5	20.5	5.07	1.04	3.3
	d2	L	43.4	3.2	1.1	11.8	13.1	19.9	5.12	1.02	2.8
		LD	43.6	3.4	0.8	9.0	14.6	21.8	4.99	1.09	4.0
	d4	L	43.0	3.4	1.1	11.7	13.2	20.2	5.03	1.02	2.9
		LD	43.2	3.5	0.9	8.0	14.3	22.7	5.34	1.21	4.6
		D	43.1	3.4	0.9	9.0	14.7	21.8	-	-	4.0
	d6	L	41.6	3.4	1.3	13.2	13.3	19.9	4.89	0.97	2.5
		LD	43.3	3.4	0.9	6.6	15.1	23.2	5.06	1.18	5.8
Z2	d0		44.4	3.1	1.1	10.8	13.4	20.1	-	-	3.1
	d2	L	44.1	3.1	1.1	12.6	12.1	19.6	-	-	2.5
		LD	48.4	3.3	1.0	9.8	12.7	22.1	-	-	3.6
	d4	L	44.1	3.2	1.1	14.0	11.6	18.9	-	-	2.2
		LD	43.9	3.0	1.0	10.6	12.9	21.1	-	-	3.2
	d6	L	42.3	3.3	0.9	14.4	12.0	19.3	-	-	2.2
		LD	44.0	3.3	0.9	8.9	13.2	22.1	-	-	4.0
Z2A	d0		48.6	2.1	0.9	12.7	12.8	20.7	-	-	2.6
	d2	L	48.7	3.8	0.7	8.6	13.6	21.5	-	-	1.1
		LD	48.0	2.6	0.9	8.7	14.0	23.7	-	-	4.3
	d3	L	49.1	4.0	0.7	9.9	12.9	21.2	-	-	3.5
		LD	48.1	2.8	0.9	6.3	15.3	24.3	-	-	6.3
	d4	L	48.4	4.0	1.0	10.9	12.8	20.4	-	-	3.0
		LD	48.5	4.6	1.4	6.9	14.6	21.0	-	-	5.2
	d6	L	54.3	4.7	1.1	10.5	10.9	17.0	-	-	2.7
		LD	47.5	3.8	0.8	4.9	15.9	24.6	-	-	8.2
	d7	L	43.7	5.2	3.0	12.5	12.0	16.3	-	-	2.3
		LD	48.4	4.0	0.8	4.2	16.0	24.2	-	-	9.6
	d9	L	43.6	3.4	1.6	16.1	11.8	21.0	-	-	2.0
		LD	50.1	4.0	0.6	5.7	15.0	22.2	-	-	6.5

R - C_{18} desaturation ratio = (18:3+18:2)/18:1.
L - Light.
LD - Light Dark.

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Table 21 continued.

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		R
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	
Z3	d0		48.3	3.0	0.4	7.8	14.6	22.2	-	-	4.7
	d2	L	49.5	3.4	0.8	8.4	13.9	21.6	-	-	4.2
		LD	49.4	2.9	0.9	8.0	13.6	23.4	-	-	4.6
	d5	L	48.2	3.6	0.8	10.1	13.3	21.8	-	-	3.5
		LD	49.5	3.2	0.7	4.2	16.5	23.9	-	-	9.6
	d6	L	49.2	3.5	0.9	10.5	13.4	20.2	-	-	3.2
		LD	48.1	3.6	0.8	3.8	16.3	24.6	-	-	10.8
	d7	L	48.2	3.9	1.0	9.8	13.9	20.8	-	-	3.5
		LD	46.9	4.1	1.2	3.4	16.6	24.1	-	-	12.0
	d9	L	40.2	3.7	1.9	15.1	12.6	22.3	-	-	2.3
		LD	41.8	2.8	0.6	4.0	14.6	32.4	-	-	11.8
Z4	d0		45.7	2.6	1.3	10.8	13.6	19.6	4.9	0.97	3.2
	d2	LD	47.8	2.8	1.0	8.2	14.1	23.0	4.9	1.12	4.5
	d4	L	48.3	3.2	1.1	10.6	13.2	20.7	5.1	1.06	3.2
		LD	44.3	2.6	1.1	5.6	15.8	23.9	5.0	1.19	7.0
	d6	L	44.0	3.3	1.1	11.9	12.4	20.0	5.1	1.02	2.7
		LD	44.1	3.0	0.9	4.7	16.6	23.5	5.1	1.19	8.5
	d10	L	31.1	3.2	2.0	19.5	11.1	26.7	4.5	1.20	1.9
		LD	43.4	3.3	1.0	3.7	17.1	22.9	5.3	1.21	10.8
	d12	LD	43.6	3.5	0.9	3.8	17.1	23.4	5	1.33	10.7
	d5	Outdoor	47.4	3.9	0.8	3.6	20.1	21.9	6.6	1.44	11.7
	d7		48.1	3.4	1.1	4.4	20.4	21.6	6.2	1.33	9.6
	d9		44.2	3.9	1.0	5.4	18.7	24.1	5.9	1.42	7.9
	d11		42.7	4.2	1.3	6.0	18.3	25.5	6.4	1.62	7.3
	d5		46.4	4.1	1.3	5.3	18.6	23.0	6.5	1.49	7.9
	d7		47.0	3.8	1.6	4.5	18.7	23.3	4.7	1.09	9.3
	d11		42.0	4.2	1.4	6.1	18.3	25.4	6.2	1.57	7.2
Z5	d0	L	48.7	3.3	1.0	8.8	13.7	21.8	4.7	1.02	4.0
	d2	LD	44.2	3.4	0.9	9.1	13.6	21.3	4.6	0.99	3.8
		L	47.2	3.4	0.9	7.4	14.7	23.3	4.7	1.10	5.1
	d4	LD	47.8	3.6	0.8	10.7	13.1	20.9	5.9	1.23	3.2
		L	46.9	3.7	1.0	6.4	15.3	23.7	5.8	1.37	6.1
	d7	LD	48.0	3.5	0.9	3.6	16.4	24.6	5.5	1.36	11.4
	d8	LD	46.9	3.6	0.9	3.4	17.6	24.6	6.0	1.47	12.4
Z5	d5	Outdoor	47.6	3.4	1.4	4.7	21.6	20.1	3.9	0.78	8.9
	d7		45.4	4.0	1.4	5.0	19.9	22.5	4.7	1.05	8.4
	d9		47.1	4.9	1.0	5.4	16.4	23.9	4 [illegible]	1.05	7.5
	d11		41.3	4.6	1.3	5.8	18.3	28.0	3.4	0.95	8.0
	d13		40.9	4.5	1.8	6.0	18.5	27.3	3.6	0.97	7.6

Table 21 continued.

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		R
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	
Z6											
	d0		47.7	4.4	1.0	7.6	14.6	22.4	5.3	1.17	4.9
[I] = 40	d2	L	46.8	4.3	1.5	6.6	14.7	22.4	5.7	1.28	5.6
$\mu E.m^{-2}.s^{-1}$	d2	LD	47.8	4.2	1.2	6.7	14.8	23.0	5.2	1.20	5.6
	d4	L	46.3	4.2	0.8	8.4	14.8	22.6	6.2	1.39	4.5
T= 30°C	d4	LD	47.0	4.2	0.9	3.9	16.3	25.1	5.9	1.48	10.6
	d6	LD	47.2	4.6	1.4	4.1	17.0	23.5	6.0	1.40	9.9
	d8	L	47.4	4.4	0.8	7.3	14.7	22.3	6.3	1.41	5.1
	d8	LD	46.9	4.1	0.8	3.2	17.7	24.2	6.4	1.55	13.1
	d10	LD	47.0	4.3	0.9	1.9	19.0	25.0	4.8	1.20	23.2
	d12	L	31.2	3.3	2.6	22.9	12.1	25.6	5.1	1.29	1.6
	d12	LD	46.2	4.4	0.8	2.5	18.0	25.0	5.7	1.42	17.2
Z9	d6	L	48.4	3.7	0.8	8.2	14.6	21.7	-	-	4.4
	d6	LD	48.2	2.5	1.0	4.3	16.7	25.6	-	-	9.3
	d8	L	49.0	3.5	0.9	10.6	13.3	20.3	-	-	3.2
	d8	LD	48.6	3.2	0.7	2.3	17.3	25.4	-	-	18.6
	d10	L	48.0	3.5	1.0	11.2	13.1	20.5	-	-	3.0
	d10	LD	48.7	3.8	0.8	1.5	17.2	25.4	-	-	28.4
	d12	L	46.0	3.6	1.1	13.1	12.5	21.4	-	-	2.6
	d12	LD	43.3	4.1	0.7	0.9	18.5	25.1	-	-	48.4
Z10	d0		45.5	2.4	1.6	8.4	13.4	22.1	-	-	4.2
	d2	L	44.9	2.7	0.7	7.3	13.2	21.7	-	-	4.8
	d2	LD	45.7	2.9	0.8	5.3	13.8	22.4	-	-	6.8
	d4	L	43.2	0.6	0.8	8.9	13.4	21.2	-	-	3.9
	d4	LD	45.7	2.6	0.6	2.9	15.3	24.5	-	-	13.6
	d7	L	41.3	2.6	1.1	10.7	13.2	20.7	-	-	3.2
	d7	LD	43.3	2.5	0.7	2.4	17.1	25.3	-	-	17.5
Z11	d0		46.2	3.3	0.6	7.9	15.4	25.5	-	-	-
	d2	L	42.5	2.8	0.9	10.2	13.7	21.5	-	-	3.5
	d2	LD	50.7	3.1	1.5	5.6	15.6	22.0	-	-	6.8
	d7	L	48.6	2.7	1.6	11.6	14.0	20.3	-	-	3.0
	d7	LD	50.7	3.1	1.0	4.8	17.5	21.5	-	-	8.1
	d11	L	50.8	2.5	1.6	12.2	12.8	19.4	-	-	2.6
	d11	LD	48.3	3.0	1.1	6.4	17.1	22.2	-	-	6.2
	d14	L	46.2	3.4	1.2	12.1	14.3	21.6	-	-	3.0
	d14	LD	46.9	3.3	0.7	4.5	18.1	24.9	-	-	9.6
Z12	d2	LD	45.5	4.1	0.6	2.9	18.1	31.5	6.1	1.91	17.1
	d4	LD	47.6	3.9	1.2	2.8	17.8	26.0	4.9	1.27	6.6
[I] = 40	d4	L	46.9	3.3	1.1	10.1	14.2	22.2	4.9	1.08	3.6
$\mu E.m^{-2}.s^{-1}$	d6	LD	45.1	3.4	0.7	2.5	18.7	26.4	5.0	1.30	47.6
	d8	LD	47.7	3.9	0.7	2.6	19.3	27.2	5.9	1.60	17.9
T= 30°C	d8	L	47.2	3.4	1.1	12.0	13.0	20.6	5.7	1.17	2.8
	d10	LD	47.6	3.9	1.2	2.8	18.7	23.6	5.1	1.20	15.1
	d12	LD	41.7	3.5	0.7	3.0	18.8	24.5	5.2	1.28	14.4
	d12	L	34.9	3.9	2.5	18.9	12.2	24.8	5.0	1.23	1.96

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Table 21 continued.

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		R
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	
Z12											
[I]= 40	d5	Outdoor	46.2	3.7	1.2	5.6	18.3	23.9	7.1	1.7	7.5
$\mu E.m^{-2}.s^{-1}$	d7		47.2	3.9	1.4	4.7	19.0	23.0	4.6	1.05	8.9
T= 30°C											
Z13	d2	L	48.1	5.8	0.9	4.7	16.1	22.3	4.3	1.02	8.2
	d2	LD	49.7	3.8	0.9	2.8	17.8	22.6	4.1	0.9	14.4
	d4	L	48.2	4.2	0.6	6.9	15.3	22.8	5.2	1.18	5.5
	d4	LD	52.0	3.5	0.7	1.2	18.0	22.7	4.4	1.01	33.9
	d6	L	51.1	3.7	0.9	9.3	13.4	20.5	4.2	0.86	3.7
	d6	LD	52.7	3.2	1.4	0.4	18.6	21.2	4.3	0.91	100.0
	d8	L	50.1	3.5	0.7	10.9	13.2	19.6	5.0	0.97	3.0
	d8	LD	52.1	3.8	0.5	1.9	18.1	22.3	4.5	0.99	21.3
	d10	L	51.5	3.7	0.5	12.3	11.8	18.5	5.4	0.99	2.5
	d10	LD	50.2	4.0	1.8	0.2	17.8	23.7	4.9	1.16	207.5
Z14	d0		44.9	4.49	0.9	8.1	15.5	22.5	5.3	1.18	4.7
	d2	L	45.7	4.8	1.5	5.5	15.9	23.0	5.2	1.2	7.1
	d4	LD	46.9	4.5	1.2	10.7	13.2	20.3	5.3	1.1	3.1
	d4	L	47.4	4.8	1.0	3.9	16.4	23.5	5.0	1.2	10.2
	d6	LD	46.9	4.1	1.0	12.1	12.6	20.1	5.3	1.1	2.7
	d6	L	46.9	4.4	0.9	3.3	17.4	24.5	5.3	1.3	12.7
	d8	LD	46.7	4.2	0.9	3.8	17.2	24.3	6.2	1.52	10.9
	d10	LD	47.1	4.1	0.8	3.7	17.4	24.3	5.7	1.4	11.3
	d12	L	31.8	3.6	2.5	20.2	11.9	25.8	5.0	1.3	1.9
	d12	LD	47.0	3.9	0.9	3.2	17.5	24.8	5.8	1.5	13.2
	d14	L	29.0	2.2	1.1	29.9	12.5	17.5	4.7	0.82	1.0
	d14	LD	46.7	4.1	0.95	3.2	17.3	24.9	6.0	1.5	13.2
Z17	d0		48.6	3.6	0.9	6.5	15.6	22.5	4.6	1.03	5.9
	d2	L	48.5	3.7	0.8	3.6	16.6	24.2	5.2	0.25	11.3
	d4	LD	49.3	3.4	1.0	10.0	13.3	20.3	5.1	1.03	3.4
[I] = 40	d4	L	48.1	3.4	0.8	2.9	17.3	25.0	4.9	1.23	14.6
$\mu E.m^{-2}.s^{-1}$	d6	LD	48.3	3.5	0.8	2.4	18.3	24.9	5.0	1.25	18.0
	d8	L	46.9	3.4	1.2	12.6	12.7	20.5	5.6	1.15	2.6
T= 30°C	d8	LD	48.5	3.6	0.7	2.3	18.3	24.0	6.2	1.49	18.4
	d10	L	48.4	3.8	0.9	2.2	18.5	23.8	5.3	1.25	19.2
	d12	LD	32.8	3.1	2.4	21.5	12.0	25.6	5.1	1.30	1.8
	d12		49.3	4.6	0.6	2.5	17.5	22.7	5.8	1.32	16.1
Z17	d5	Outdoor	45.5	4.4	0.9	4.4	19.0	24.0	5.5	1.33	9.8
	d6		47.0	3.4	1.4	6.6	17.0	22.7	6.3	1.44	6.0
	d7		47.0	3.8	0.9	5.7	16.1	24.9	4.2	1.05	7.2
	d8		47.5	3.4	1.3	5.8	14.9	26.1	4.0	1.05	7.1
	d9		44.0	4.1	1.9	7.2	15.2	25.3	3.8	0.96	5.6
	d10		43.6	3.6	1.6	7.0	16.3	26.4	3.5	0.93	6.1

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Table 21 continued.

Strain	Day	Condition	Fatty acid composition (% of total fatty acid)						as % Dry weight		R
			16:0	16:1	18:0	18:1	18:2	18:3	TFA	GLA	
P4P											
	d0		48.4	3.1	1.2	11.3	13.0	20.1	5.4	1.08	2.9
I I=40	d2	L	48.8	3.7	0.7	7.7	14.3	22.2	5.4	1.20	4.8
$\mu E.m^{-2}.s^{-1}$	d2	LD	48.9	3.3	0.9	9.8	13.3	21.1	5.1	1.08	3.5
	d4	L	49.1	3.8	0.7	8.9	14.0	21.3	5.8	1.23	3.4
T=30°C	d4	LD	49.0	3.4	0.9	6.0	15.1	23.6	6.1	1.44	6.5
	d6	LD	48.1	3.8	1.1	4.4	16.4	23.5	5.9	1.39	9.1
	d8	L	47.2	4.7	0.7	8.9	13.2	22.6	6.4	1.44	4.0
	d8	LD	47.3	4.0	0.8	3.8	17.1	24.1	6.0	1.45	10.9
	d10	LD	48.7	3.8	0.8	3.4	17.5	23.6	6.3	1.47	12.0
	d12	L	29.9	2.6	3.0	28.5	10.7	22.7	5.3	1.12	1.2
	d12	LD	47.6	4.3	0.7	4.1	16.7	23.8	6.0	1.44	9.9

PART II

The use of inhibitor (herbicide Sandoz 9785) to select the strains resistance to analog and to study the fatty acid compositions of these strains.

PART II: The use of inhibitor (herbicide Sandoz 9785) to select the strains resistance to analog and to study the fatty acid compositions of these strains.

RESULTS AND DISCUSSIONS

1. Effect of SAN 9785 on S. platensis wild type (BP) on growth and fatty acid composition

The effects of the herbicide SAN 9785 on the growth rate and fatty acid composition and content of the wild type (BP) strain were studied. The cultures were grown on media with and without 0.4 mM of SAN 9785. The biomass was harvested after 5 days cultivation for fatty acid analysis. In the presence of inhibitor, the culture grew, at a slower growth rate than in the inhibitor-free medium (Table 1). The growth rate reduced from 0.62 day^{-1} to 0.14 day^{-1} or doubling time increased to 118.8 hrs from 26.9 hrs. SAN 9785 had a notable inhibitory effect on the fatty acid profile of S. platensis. Exposure of the culture to SAN 9785 resulted in the decrease in proportion of C18:3w6 and C18:2 and the increase of C18:1 and C18:0 (Table 2). During incubation in medium with 0.4 mM SAN 9785 the proportion of C18:3w6 declined from 26.6% (of total fatty acids) in inhibitor-free medium to 15.0% and C18:2 from 18.2 to 9.3%, while C18:1 increased from 5.5 to 20.2% and C18:0 from 1.3 to 3.0%. Relatively small changes were also observed in the C16 fatty acid series. The GLA content reduced from 1.10% (of dry weight) to 0.74%. Therefore, TFA content was often found to be higher in the presence of SAN 9785 indicating that TFA synthesis might not be inhibited by this inhibitor. SAN 9785 showed an effect on fatty acid desaturation, particularly the transformation of oleic acid (C18:1) to linoleic acid (C18:2). Linoleic acid is further desaturated to GLA in S. platensis. Due to its inhibitory effect, we chose SAN 9785 as an inhibitor for the selection of GLA over-producing strains. The over-producers of GLA might be found among SAN 9785-resistant strains of S. platensis.

Table 1. Effect of SAN 9785 (0.4 mM) on the growth rate of Spirulina platensis in the wild type strain (BP)

Culture condition	Specific growth rate (μ) (day^{-1})	Doubling time (hour)
No inhibitor	0.62	26.9
0.4 mM SAN 9785	0.14	118.8

Table 2. Effect of SAN 9785 (0.4 mM) on the fatty acid composition and content of Spirulina platensis in the wild type strain (BP)

Culture condition	Fatty acid composition (% of total fatty acid)							Fatty acid content (as % Dry weight)	
	16:0	16:1	16:3	18:0	18:1	18:2	18:3	18:3	TFA
No inhibitor	42.5	4.4	1.5	1.3	5.5	18.2	26.6	1.10	4.14
0.4 mM SAN 9785	49.5	2.4	1.5	3.0	20.2	9.3	15.0	0.74	4.93

2. Effect of SAN 9785 on photosynthesis

It has been known that there are at least four types of mechanism of action of the pyridazinones in plants (6). Relative influence of the pyridazinones on the fatty acids, pigments and the inhibition of the Hill's reaction depends on the structure of compound. The experiment Spirulina showed the influence of SAN 9785 strongly effect on decreasing in linolenic acid accompanied by an increase of oleic acid (see Table 2). In this experiment, effect on photosynthesis was studied in Spirulina Z19/2. The 4 days old culture grew in media with and without 0.4 mM SAN 9785 were used for photosynthetic measurement. The cells were filtered, washed and resuspended in fresh media to a concentration of 2 mg chlorophyll/litre. The photosynthesis was measured in the presence andabsence of SAN 9785. Oxygen

evolution was measured at the light intensity of 160 μE $m^{-2}.s^{-1}$. Table 3 shows that photosynthesis in Spirulina Z19/2 decreased almost 79% in the presence of SAN 9785. In an other experiment we studied with the Z19/1 strain, both cultures that previously grown in the presence and absence of SAN 9785 showed the 85% reduction in photosynthetic rate when exposed to the inhibitor,therefore, the effect was reversible. The photosynthetic activity regained to normal almost immediately by after the removal of the inhibitor. No recovery period was required in contrast to the effect on linolenic acid in which protein synthesis (desaturase enzyme) may be inhibited. The result suggested the role of GLA which may not be involved in photosynthesis. In separated experiments, cells grown in the presence of the inhibitor contained not the chlorophyll a and TFA (% of dry weight) less than the control. SAN 9785 did not directly affect on pigment formation and TFA in Spirulina. This is in agreement with that reported by Eder [7]. Its effect seemed to be on the light harvesting reaction center thus less energy was generated and growth rate was reduced.

Table 3. Effect of SAN 9785 on photosynthesis of Spirulina.

Strain	Growth condition	Measuring condition	O_2 evolution mole O_2 $hr^{-1}.mg$ ch^{-1}
Z19/2	Without SAN 9785	Without SAN 9785	568
		With SAN 9785	Not determined.
	With SAN 9785	Whithout SAN 9785	642
		With SAN 9785	135
Z19/1	Without SAN 9785	Without SAN 9785	789
		With SAN 9785	116
	With SAN 9785	Without SAN 9785	796
		With SAN 9785	122

In the other experiment, we studied the effect of SAN 9785 on photosynthesis at different temperatures. The cultures that grown in normal Zarrouk's medium was measured for their photosynthetic rate in the absence and presence of 0.4 mM SAN 9785 at 27 and 35°C. At 35°C, the photosynthetic rate was higher than that measured at 27°C, both in the presence and the absence of the inhibitor (Table 4). SAN 9785 inhibited about 85% of the photosynthetic rate both at 27°C and 35°C. It seemed that the inhibition of SAN 9785 was not depended on the temperature.

Table 4. The effect of SAN 9785 on the photosynthesis as measure by O_2 evolution; mole O_2 $hr^{-1}.mgch^{-1}$) at different temperatures

Measuring condition	Measuring temperature (°C)	
	27	35
Without SAN 9785	384	706
With SAN 9785	73.8	137

3. <u>Recovery of cell from the effect of SAN 9785</u>

To see whether or not the cells can recover after exposure to the herbicide, the cells cultivated in the presence of 0.4 mM SAN 9785 for 5 days were harvested and washed with fresh medium for several times to remove the residue of SAN 9785. The washed cells were resuspended in the inhibitor-free medium and incubated at 10 and 24 hours before being harvested for fatty acid analysis.

Figure 1 shows that growth rate of treated cultures was resumed to normal when the inhibitor was removed from the medium. Its chlorophyll content increased from 5.6 to 9.95 mg/l after 24 hours compared with 6.8 mg/l in the presence of SAN 9785. Increase in growth rate corresponded with changes in fatty acid composition. The level of C18:1 decreased from 22% to 17% and 7% after 10 and 24 hours, respectively. A decreased in C18:1 was paralleled with an increase in C18:2 and C18:3 (Table 5).

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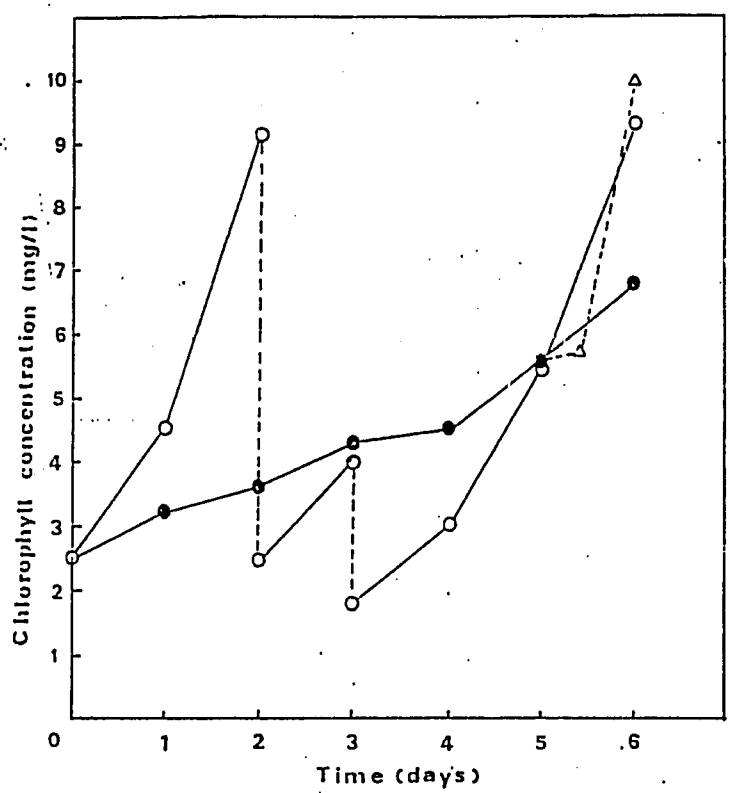

Figure 1. Effect of SAN 9785 and the recovery of cells in the inhibitor-free medium on growth rate of Spirulina BP.
(o, inhibitor-free medium; ●, 0.4 mM SAN 9785; ●-Δ, transferred to inhibitor-fee medium).

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The study revealed that the synthesis of C18:3 was in connection with desaturation of C18:1 to C18:2 and then to C18:3. While SAN 9785 caused the accumulation of C18:1 in Spirulina, the accumulation of C18:2 instead, was reported in some higher plants [8].

Table 5. Fatty acid composition of cells recovered from media containing inhibitor to inhibition-free medium of Spirulina BP strain.

Medium	Time	Fatty acid composition (% of total fatty acid)							Fatty acid content as % Dry weight	
		16:0	16:1	16:3	18:0	18:1	18:2	18:3	18:3	TFA
-[a]	5d	40.8	6.4	2.3	1.1	4.8	17.3	27.2	1.01	4.2
+[a]	5d	44.8	2.7	1.5	3.9	21.1	11.2	15.1	0.63	4.1
+	6d	45.1	3.5	0.8	4.2	22.1	10.9	13.4	0.58	4.3
+,-[b]	10hrs	44.5	3.8	1.1	3.5	16.7	14.8	15.6	0.66	4.2
+,-	24hrs	42.8	5.8	1.8	2.0	6.7	16.0	24.9	1.03	4.1

a -- (-) inhibitor-free medium, (+) medium containing 0.4 mM SAN 9785;
b - (+,-) transferred culture back to inhibitor-free medium.

4. Effect of SAN 9785 and temperature on fatty acid profiles

Effect of temperature on fatty acid composition has been reportedin many cyanobacteria (9,10,11,7). The study by Cohen (9) and this study showed that Spirulina growing at temperatures below its optimum (35°C) contained higher percentage of C18:3. The role of C18:3 in Spirulina might be associated with chilling tolerance as that as described in Synechocystis by Wada and Murata (12). The inhibition of SAN 9785 on synthesis of C18:3 was studied at 25°C, 30°C and 37°C in wild type and resistant strains. The study showed that C18:3 was more inhibited at 37°C than 30°C and 25°C, in descending order (Table 6). In addition to the reduction of C18:3, the decrease in C18:2 was observed at 30°C and 37°C in concomitant to the accumulation of C18:1. At 25°C, only slightly change of C18:2 and less accumulation of C18:1 was observed. A reduction of C16:1 was noted at this low temperature both in BP and Z19/2 strains. The difference in fatty acid profiles of Spirulina at these three temperatures inthe presence of SAN 9785 indicated that the desaturation of C18:3 may be regulated differently at low and high temperature.

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Table 6. Effect of temperature on the fatty acid composition in the presence and the absence of SAN 9785.

Strain	Temp	Medium	Fatty acid composition (% of total fatty acid)						Fatty acid content as % dry weight	
			16:0	16:1	18:0	18.1	18:2	18:3	18:3	GLA
BP	25	-[a]	47.7	5.5	1.9	4.6	17.6	27.7	1.2	4.3
		+[a]	49.7	0.42	1.7	7.6	17.4	23.2	0.6	2.4
	30	-	45.8	6.4	2.1	6.4	14.2	25.1	1.0	4.0
		+	50.3	6.2	3.5	18.1	8.4	13.5	0.5	3.6
	37	-	49.4	3.8	0.1	10.0	13.9	22.8	0.9	4.1
		+	52.2	3.0	2.6	24.0	7.4	10.9	0.4	3.5
Z19/2	25	-	44.2	3.3	1.5	3.2	18.9	28.9	1.2	4.2
		+	49.2	2.5	1.1	5.9	17.8	23.5	0.8	3.4
	30	-	46.0	4.8	1.8	5.3	15.6	26.5	1.3	4.8
		+	49.9	6.3	2.5	15.8	9.7	15.8	0.7	4.4
	37	Not determined								

[a] - (-) inhibitor free medium, (+) medium containing 0.4 mM SAN 9785.

5. **Selection of strains resisted to SAN 9785 and the analysis of fatty acid composition of the selected strains.**

Previous studies in our laboratory shown that Spirulina strains could grow in media containing upto 0.4 mM SAN 9785. In orderto get strains resisted to SAN 9785, Spirulina BP and P4P were inoculated into 3 ml Zarrouk's media containing 0.4-1.2 mM SAN 9785. The cultures were incubated under fluorescent light for 30 days. The survived cultures were picked up for isolation using dilution method in Zarrouk's media with and without inhibitor (obtaining colonies derived from single cells on agar plate was considered as a difficult task due to its filamentous form). Cell from the last tube showing growth was kept on agar slant. To enhance the possibility of getting high resistant strain. These selected cells were exposed to SAN 9785 once again and reisolated. Morethan 50 cultures were selected and 19 strains were used for further testing.

To test for the degree of resistance, growth of each selected strain was followed in Zarrouk's media containing various concentration of SAN 9785. The

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growth conditions were 30°C, 80 $\mu E.m^{-2}.s^{-1}$ light intensity and 150 rpm in shaking incubator. If cell was able to grow and if the concentration was too high, the culture was diluted by half with fresh media containing appropriate SAN 9785 concentraion. The concentration of SAN 9785 in the culture was kept constant through out the experiment. Figure 2 shows the growth curve of BP, 1B, 17B and Z19/2 in the media with and without SAN 9785. Generally it could be concluded that most of the culture could grow in the presence of low concentration of SAN 9785 (0.32-0.4 mM) but at different growth rate. Increasing inhibitor concentration resulted in the decrease in the growth rate and cell bleaching. Cell eventually died. The survival period at each inhibitor concentration and the lethal concentration varied among the strains.

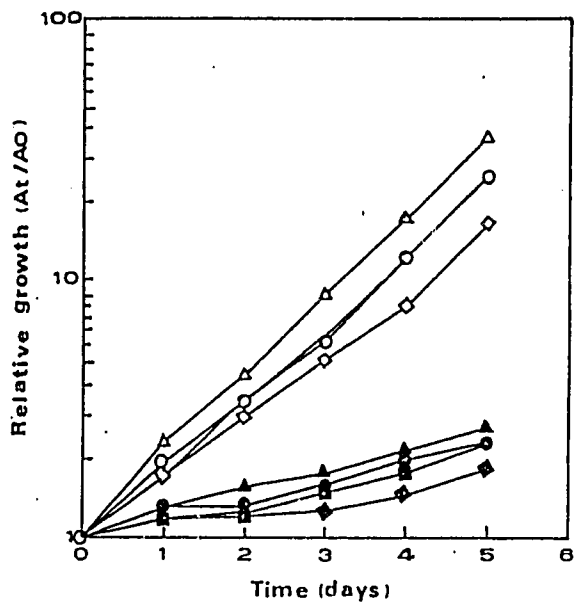

Figure 2. Effecct of SAN 9785 on growth rate (chlorophyll absorption divided by initial chlorophyll absorption) of Spirulina cultures.

(○, BP; △, Z19/2; □, 17B; ◇, Z1B; open symbol represents the culture grown in the inhibitor free medium; filled symbol represents grown in medium with 0.4 mM SAN 9785).

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Degree of tolerance was compared by dividing the strain into 2 groups. The first group included BP, P4P, India and 2340 strain. All never been exposed to SAN 9785. The rest called the selected strains which had been exposed to inhibitor at various concentrations. Except 2340, all strains from group one grew well at media with 0.4 mM SAN 9785. In the case of 2340, we found that growth was better at 0.2 and 0.32 mM SAN 9785 but eventually cell died afer 28 days. The least resistant of this strain might be related to the very low percentage of GLA content. Cohen et al. (9) reported that among 19 strains of Spirulina, GLA content of 2340 strain was only 8% while the rest composed of 19.3 to 31.7%. This was in agreement with our test inwhich GLA was only 13.2% while the linoleic acid was 28.3%. At high SAN 9785 concentration, BP was the most sensitive to inhibitor. At 0.6 mM, cell died after 19 days. P4P showed slightly growth at 0.6 mM but could tolerate up to 30 days. Strain India was the most resistant strain of group I. Morphologically, its filament was a straight chain rather than spiral.

Since the culturesin group II were selected from BP and P4P after exposure to SAN 9785, the tolerance of the group II culture was compared with BP and P4P. Several strains of the Group II were foundto be more resistant than BP and P4P. These included Z1A, Z1B, Z5B, Z17B, Z19 and Z19/2 strains. Table 8 shows that the cultures grew quite well in 0.8and 1.0 mM SAN 9785 and survived more than 26 and 21 days, respectively.

Table 8. Growth of wild type and selected strains in the presence of SAN 9785.

Group	Strain	SAN 3785 concentration (mM)			
		0.80		1.00	
		Day	Optical density (560 nm)	Day	Optical density (560 nm)
I	BP	8	0.11	8	0.09
		14	0.22	14	0.23
		19	died	19	died
		7	0.16	7	0.16
	P4P				
		14	0.50	14	0.21
		32	died	30	died
II	Z1A	7	0.15	7	0.12
		11	0.27	11	nd.
		14	0.41	14	0.26
		19	0.67	19	nd.
		21	0.75	21	0.42
		28	0.52	28	nd.
		31	died	29	died
	Z1B	10	0.54	7	0.14
		13	0.95	11	0.27
		21	0.72	20	0.35
		31	0.62	21	0.460
		39	died	30	0.52
				36	died
		9	0.26	15	0.47
		22	0.45	25	0.60,dil.
		29	0.53,dil	33	0.25
		44	died	36	0.39
					bleached
		nd	nd	14	0.36
				21	0.60
				28	0.90
				32	diluted
				37	died

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Table 8 continued.

Group	Strain	SAN 9785 concentration (mM)			
		0.80		1.00	
		Day	Optical density (560 nm)	Day	Optical density (560 nm)
II	Z19	35	flake	7	0.10
		45	died	11	0.18
				17	0.36
				21	0.48
				29	died
	Z19/2	6	0.51	6	0.12
		9	0.64-diluted	-	-
		16	0.42	14	0.41
		19	0.43	20	0.75
		26	0.50	23	0.93-diluted
		32	died	26	died

Group I - The wild type, never been exposed to SAN 9785.
Group II - The selected strain from SAN 9785 resistant.
diluted - Diluted by half with fresh medium with coresponding SAN 9785 concentration.
nd - Not determined.

The selected strains might be categorized into 3 groups according to their ability to grow and survive in the presence of SAN 9785 (Table 9). First group included Z6, Z6/310, Z7, and Z13 which grew well at 0.2-0.4mM SAN 9785. Z1, Z1A/1, Z1C, Z1D, Z2, Z6A, Z10, Z18 and Z20 were in the second group that grew at 0.6-0.8 mM SAN 9785 and the last group included Z1A Z1B, Z5B, Z17B, Z19 and Z19/2 that grew well at 0.8-1.0 mM SAN 9785. It should be noted that in each group, cells could survive at higher SAN 9785 concentration reported but no growth was observed.

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Table 9. The selected strains categorized by the ability to grow in various SAN 9785 concentration .

	SAN 9785 concentration (mM)		
	0.2 - 0.4	0.6 - 0.8	0.8 - 1.0
Strain	Z6,Z6/310,Z7,Z13	Z1,Z1A/1,Z1C,Z1D,Z2,Z6A,Z10, Z18,Z20	Z1A,Z1B,Z5B,Z17B,Z19, Z19/2

To determine whether the strains resistant to SAN 9785 would produce higher GLA than the wild type, the cultures were cultivated in normal Zarrouk'smedia under the laboratory and outdoor conditions. Fatty acid analysis of these cultures were performed. Data are shown in Table 10. Each figure represented the average of many experiments. Fattyacid composition of Spirulina varies during the growth phase and depending upon the growth rate (9). On the average basis, however, each culture did not show much difference on fatty acid profiles among the laboratory experiments particularly of PUFA (C18:2 and C18:3). Nevertheless, TFA varied depending on growth conditions. Moreover, PUFA of the outdoor cultures were higher than those of laboratory cultures. Eventhough the percentage of C18:3 was higher in Z19 and Z19/2 than that of the wild type (in the absence of SAN 9785), we could not indicate from the data the relationship between the resistance to SAN 9785 and the content of C18:3 except in the case of strain 2340 stated earlier.

Table 10. The average value of fatty acid composition and content of Spirulina cultivated under laboratory and outdoor conditions.

Exp.	Strain	Condition	Fatty acid composition (% of total fatty acid)						Fatty acid content as % Dry weight	
			16:0	16:1	18:0	18:1	18:2	18:3	18:3	TFA
I	BP	Lab (3)	48.0	3.3	1.6	6.0	16.3	23.1	1.2	5.1
		Out (1)	46.5	3.6	0.9	4.6	18.4	24.3	1.5	6.3
		Lab (2)	49.7	3.5	1.7	4.0	15.7	22.5	1.1	4.8
		Out (1)	46.2	3.8	1.0	4.2	17.6	25.8	1.6	6.1
		Lab (1)	45.5	3.5	4.4	9.7	14.9	24.8	1.6	6.5
		Lab (2)	18.3	2.8	1.4	4.0	28.3	13.2	0.7	4.5
II	Z1	Lab (5)	46.2	5.1	1.8	6.9	15.4	21.9	1.2	5.2
		Out (4)	46.1	3.8	1.1	5.0	17.2	24.4	1.4	5.4
	Z1A	Lab (9)	42.5	1.7	1.3	5.3	15.9	22.8	1.3	5.6
		Out (4)	44.2	3.3	1.6	6.0	18.9	24.6	1.2	4.8
	Z1A/1	Lab (2)	49.7	3.6	1.6	8.9	15.1	18.8	1.1	6.6
		Out (1)	46.2	4.0	1.7	8.9	15.3	22.8	1.0	4.5
	Z1B	Lab (8)	45.0	5.4	1.1	5.4	16.2	24.6	1.3	5.3
		Out (4)	42.9	5.5	1.7	6.7	17.3	24.5	1.3	5.2
	Z1C	Lab (3)	44.1	8.3	1.5	5.2	15.5	22.3	1.0	4.6
		Out (4)	46.7	2.2	1.6	6.2	18.2	22.2	1.1	4.6
	Z1D	Lab (2)	51.0	3.4	2.0	8.6	19.6	19.6	0.9	4.8
		Out (3)	45.5	5.9	1.1	8.0	22.3	22.3	1.1	5.0
	Z2	Lab (2)	47.4	3.1	1.5	11.5	19.4	19.4	1.0	5.1
		Out (2)	45.3	3.9	1.5	6.8	24.8	24.8	1.2	5.3
	Z5B	Lab (3)	49.9	3.5	1.7	7.2	20.9	20.9	1.1	5.1
		Out (7)	44.4	3.5	1.6	7.3	24.0	24.0	1.3	5.3
	Z6	Lab (6)	46.6	5.3	1.4	5.6	23.1	23.1	1.3	5.7
		Out (5)	43.8	4.7	1.5	6.4	23.7	23.7	1.3	5.3
	Z6A	Lab (6)	46.7	6.7	1.8	5.4	21.8	21.8	1.1	4.8
		Out (2)	43.6	6.7	1.3	6.3	23.5	23.5	1.3	5.6
	Z6/310	Lab (2)	51.1	1.5	1.8	6.6	22.1	22.1	1.1	5.1
		Out (1)	48.5	2.9	nd.	5.7	26.6	26.6	1.4	5.4
	Z7	Lab (2)	46.7	3.0	1.5	3.6	23.4	23.4	1.1	4.7
		Out (3)	49.0	2.3	1.6	1.0	23.6	23.6	1.0	4.4
	Z13	Lab (3)	49.5	3.8	1.0	6.3	21.7	21.7	1.0	4.8
		Out (1)	48.9	3.8	1.5	3.9	21.4	21.4	1.0	4.7

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Table 10 continued.

Exp.	Strain	Condition	Fatty acid composition (% of total fatty acid)						Fatty acid content as % Dry weight	
			16:0	16:1	18:0	18:1	18:2	18:3	18:3	TFA
II	Z17	Lab (4)	18.4	3.7	1.5	2.8	17.2	24.7	1.4	5.7
		Out (3)	13.7	4.4	1.1	6.8	18.8	23.9	1.3	5.5
	Z18	Lab (3)	18.3	2.4	1.3	8.5	15.2	22.5	1.0	4.1
		Out (1)	15.1	3.9	1.2	6.8	17.0	25.3	1.4	5.4
	Z19	Lab (2)	15.2	2.9	1.0	2.4	17.5	26.2	1.3	5.1
		Out (1)	11.1	4.9	0.9	1.4	19.4	28.7	1.8	6.3
	Z19/2	Lab (1)	14.6	3.9	1.1	3.5	16.0	22.3	1.3	6.0
		Lab (2)	12.3	4.4	1.1	5.2	16.9	27.9	1.2	4.4
	Z20	Lab (5)	15.5	3.2	1.1	3.9	17.0	25.7	1.3	5.0
		Out (5)	15.0	3.5	1.1	6.2	17.0	23.4	1.3	5.4

Lab - Laboratoryexperiment;
Out - Outdoor experiment;

Numbers in parenthesis are repetition of the experiments.

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Not only the genetics, environmental conditions affect the GLA content of Spirulina. In other experiments, 4 selected strains and a wild type BP were cultivated under the same conditions (30°C, -90 $\mu E.m^{-2}.s^{-1}$). Their chlorophyll concentration were kept constant at 4 mg/l. Fatty acid profiles of these strains are shown in Table 11. Again we did not see much the difference of C18:3 among the resistance and the wild type. The physiology of each resistant strain might be changed thus the conditions for the maximum GLA production might be varied.

Table 11. The comparison of fatty acid composition among BP and the selected strains cultivated under the same condition (30°C, 90 $\mu E.m^{-9}.s^{-1}$ light intensity; semi-continuous condition)

Culture	Fatty acid composition (% of total fatty acid)						Fatty acid content as % dry weight	
	16:0	16:1	18:0	18.1	18:2	18:3	18:3	GLA
BP	45.9	1.6	1.9	6.7	16.9	26.3	1.1	4.2
Z1C	50.5	3.5	nd.	9.1	12.6	22.0	1.0	4.6
Z7	51.4	0.1	0.3	5.6	15.9	26.3	1.1	4.1
Z18	46.8	3.7	0.7	6.5	13.9	28.1	1.1	3.9
Z19/2	42.4	5.2	0.8	4.2	18.7	27.7	1.3	4.7

nd. - Not determined.

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The data from batch experiments of the selected resistant strains indicated that the high GLA-producing strains might be found (Table 12). Studies onthe physiology of each strain and more experiment on cultivation conditions in outdoor cultures is needed prior to the decision on suitable strains for GLA-producer in large scale.

Table 12 The fatty acid composition in the high GLA-producing strain (batch condition)

Culture	Condition	Day	Fatty acid composition (% of total fatty acid)						Fatty acid content as % Dry weight	
			16:0	16:1	18:0	18:1	18:2	18:3	18:3	TFA
Z1B	Lab (LD)	2	42.7	4.4	0.7	1.9	19.3	30.0	1.3	4.2
		3	41.1	4.8	0.5	1.0	21.0	30.7	1.2	3.8
		4	40.3	5.0	0.6	1.0	20.7	31.9	1.6	4.9
		7	38.9	5.0	0.5	1.9	20.1	32.9	1.6	4.9
		5	42.2	4.8	0.5	1.7	19.9	29.7	1.7	5.4
		9	40.1	4.5	0.4	2.1	19.9	31.2	1.4	4.6
		11	43.0	4.7	0.4	2.3	18.8	29.9	1.4	4.6
	Outdoor	2	44.4	3.7	0.7	4.1	20.4	24.5	1.5	5.4
		3	47.0	5.1	2.7	0.5	17.1	27.6	1.8	6.7
		4	44.4	4.6	1.2	4.0	17.5	25.8	1.7	6.5
		5	41.8	4.4	1.1	7.3	17.8	25.2	1.5	6.1
		6	38.8	4.5	2.1	9.2	16.1	27.2	1.5	5.6
Z19	Lab (LD)	3	40.5	2.5	0.8	2.0	18.9	31.0	1.5	4.9
		4	41.2	3.1	0.6	2.2	18.8	28.9	1.7	5.9
		5	41.3	3.2	0.6	2.3	18.8	29.9	1.6	5.4
		6	42.3	2.8	0.7	1.4	18.5	29.5	1.6	5.4
	Outdoor	2	44.1	5.7	0.4	3.0	19.7	26.0	1.8	6.8
		3	43.2	5.3	0.5	2.8	18.6	27.6	2.2	8.0
		4	40.9	6.3	0.9	2.8	17.9	29.7	2.4	8.2
		5	40.7	6.0	0.5	4.6	17.0	29.7	2.3	7.6
		6	39.0	4.6	0.3	2.7	19.4	31.0	2.4	7.7
		7	39.4	5.1	1.4	6.8	17.3	28.9	2.0	7.0

(LD) - Light 12 hrs. Dark 12 hrs.

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6. Degree of SAN 9785 inhibition to the wild type and the selected strain

The previous data did not clearly shown that the selected strains growingin normal Zarrouk's medium could produce hogher GLA than the wild type. In this study, we chose Z19/2 to study extensively on the inhibitory effect of SAN 9785 in comparison with BP strain.

6.1 Growth inhibition

Attempts have been made to grow both strains in media containing various levels of the herbicide in order to demonstrate that the Z19/2 strain was more resistant than the BP strain in the presence of the inhibitor.

The strains were grownin media containing different concentrations of SAN 9785 i.e., 0, 0.4, 0.6 and 0.8 mM. The biomass was harvested after 4 days. SAN 9785 inhibited growth of both the wildtype andthe resistant culture. Reduction in growth rate of both cultures was evident at higher herbicide concentration (Table 13). Growth rate of BP strain was inhibited 78 and 89% in the presence of SAN 9785 0.4 and 0.8 mM, respectively, while Z19/2 was 73 and 86%, respectively.

Table 13. Effect of SAN 9785 on the growth rate of S. platensis in the wild type (BP) and the resistant strain (Z19/2).

Strain	Inhibitor conc. (mM)	Specific growth rate (μ) (day^{-1})	Doubling time (hour)	% Inhibition
BP	0	0.63	26.4	
Z19/2	0	0.69	23.8	
BP	0.4	0.14	118.8	78
Z19/2	0.4	0.19	87.6	73
BP	0.6	0.11	151.2	83
Z19/2	0.6	0.13	127.9	81
BP	0.8	0.07	237.6	89
Z19/2	0.8	0.10	166.3	86

6.2 Fatty acid inhibition.

SAN 9785 had a notable inhibitory effect on the fatty acidprofiles of S. platensis. Exposure of BP and Z19/2 to SAN 9785 resulted in decrease in the proportions of C18:3w6, C18:2 ω6 and increase in that ofC18:1 and C18:0 (Table 14). In the presence of 0.8 mM SAN 9785, the proportion of C18:3 ω6 in the BP declined from 27.9% (of total fatty acids) in inhibitor-free medium to 12.4% while thelevel of C18:3w6 in the Z19/2 strain decreased from 28.5 to 16.4%. The levelof C18:2 in the wild type decreased from 17.6 to 9.7%, but only from 18.9 to 11.8% in the resistant. The reduction of C18:3 ω6 and C18:2 accompanied by an increase in the level of C18:1, which increased from 5.6to 18.4% and 3.8 to 14.7%in the wild type and the resistant strain, respectively (Fig. 3). The GLA content was also reduced from 1.3% (of dry weight) to 0.49% and from 1.4 to 0.72% in BP and Z19/2, respectively. The resistant strain had higher content of GLA in all herbicide concentrations studied. The percentage of C18:3w6 and C18:2 were always higher, while that of C18:1 was always lower in the resistant strain. Invoking the R value [C18 desaturtion ratio = (C18:3 + C18:2) / C18:1], the R value in the resistant strain are always higherthan in the wild type strain. The study revealed that Z19/2 which was more resistant to SAN 9785 could grow faster and had higher fatty acid contentin the presence of inhibitor than the wild type BP.

It is difficult at present, to point at the mechanism(s) of resistance to the herbicide. Murphy et al. (8) have shown that the uptake of SAN 9785 varies in various plants. They have further shown that the herbicde was rapidly metabolized in pea but only gradually in cucumber and ryegrass (8). Other possibilities could involve the modification of the target enzyme to reduce its affinity to the inhibitor oran increase in the level of the relevant enzyme (12). The increaseof GLA in S. platensis and EPA in P. cruentum may be attributed to one of the last two mechanisms (13). Thus, over-production could be a means for counteracting the effect of the herbicide (13). Since the inhibitorinhibits the synthesis of GLA, the ability to resist to inhibitor might relate to the ability to synthesize higher GLA than the less resistance one.

Table 14 Effect of SAN 9785 on fatty acid composition and content of the wild type (BP) and SAN 9785 resistant strain - (Z19/2)

Strain	SAN 9785 conc. (mM)	Fatty acid composition (% of total fatty acid)						Fatty acid content as % Dry weight		R
		16:0	16:1	18:0	18:1	18:2	18:3	18:3	TFA	
BP	0	41.5	5.2	1.1	5.6	17.6	27.9	1.30	4.66	8.1
Z19/2	0	40.2	6.7	0.8	3.8	18.9	28.5	1.40	4.91	12.5
BP	0.4	46.4	6.6	2.2	16.9	11.4	15.7	0.72	4.56	1.6
Z19/2	0.4	47.0	5.5	2.2	13.7	12.6	17.8	0.82	4.61	2.2
BP	0.6	51.1	2.8	3.2	17.6	10.1	13.5	0.54	4.00	1.3
Z19/2	0.6	46.6	6.3	2.5	14.4	12.0	17.1	0.80	4.68	2.0
BP	0.8	52.5	1.1	3.9	18.4	9.7	12.4	0.49	3.95	1.2
Z19/2	0.8	47.8	5.4	2.8	14.7	11.8	16.4	0.72	4.39	1.9

R:C18 desaturation ratio = (18:3+18:2)/18:1.

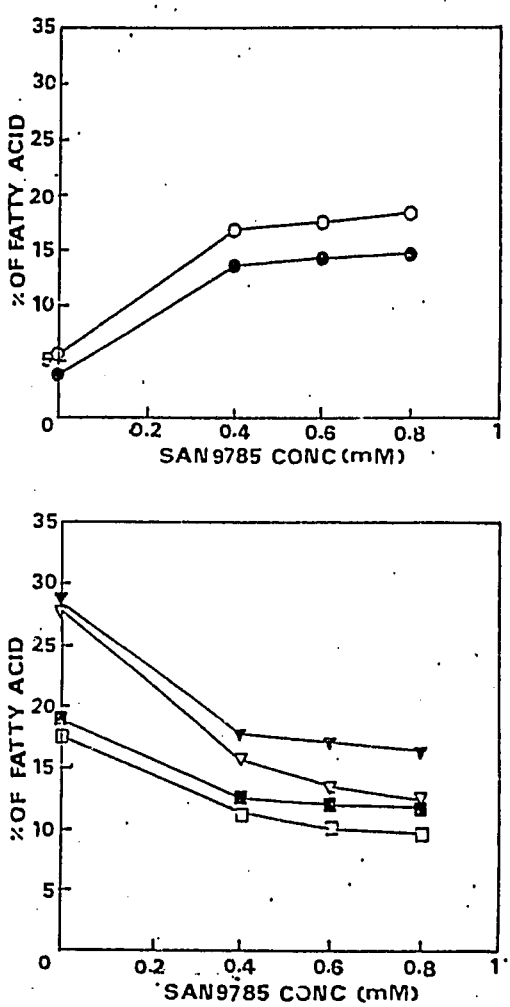

Figure 3. Effect of SAN 9785 concentration on C18:1, C18:2 and C18:3.

(O, C18:1; □, C18:2; ▽, C18:3; open symbol represents BP strain; filled symbol represents Z19/2 strain).

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PART III

The extraction for Gamma-Linolenic Acid (GLA)

PART III: The extraction for Gamma-Linolenic Acid (GLA)

RESULTS AND DISCUSSIONS

Extraction of Gamma-linolenic acid from Spirulina platensis BP-1

1. Use of organic solvent in the extraction of fatty acid

Several organic solvents were used to extract fatty acid from Spirulina. Table 1 shows dielectric constant of the used solvents. Percent recovery in Table 2 reveals that the extraction of total fatty acid and GLA depended on the polarity of the solvent. Ethanol which has the highest polarity gave the best result with hexane was the least.

Table 1. Dielectric constant of various organic solvents at 25°C.

Organic solvent	Dielectric constant
Hexane	1.90 (lowest polarity)
Ethylacetate	6.02
Acetone	20.70
Ethanol	24.30

Table 2. Percent recovery of fatty acid from 1 g dried algae cell with various organic solvents

Organic solvent	% Recovery	
	TFA	GLA
Ethanol	63	52
Acetone	22	21
Ethylacetate	15	18
Hexane	10	10

TFA and GLA from direct transmethylation of dried algae cell is 5.31 and 1.35% dry weight respectively.

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2. Comparison between dried and wet algae cell as substrate in the extraction of fatty acid

The extraction of total fatty acid and GLA from wet algae (90% moisture) gave a higher yield than from the dried cell in every case of organic solvent used as shown in Table 3. Sixty two and fifty three percent of TFA and GLA, respectively were extracted with ethanol from the sample of wet algae compared with 47 and 38% from the dried sample (Table 3). Acetone or hexane gave similar result but the percent of recovery decreased in the following order, ethanol, acetone and hexane, respectively. It was observed that dried cell could not disperse well thus reducing the efficiency of extraction.

Table 3. Comparison of dried and wet algae cell using various organic solvents for fatty acid extraction

Organic Solvent	% Recovery	
	TFA	GLA
Wet cell (90% moisture)		
Ethanol	62	53
Acetone	56	46
Hexane	21	27
Dried cell		
Ethanol	47	38
Acetone	21	19
Hexane	10	10

TFA and GLA from direct transmethylation of dried cell is 5.31 and 1.35% dry weight respectively.

3. Extraction of fatty acid with the mixture of organic solvent

Since the fatty acid could be extracted easier from the wet cell than the dried cell. The wet samples were used in the following experiments. In this experiment, the mixture of two organic solvents was used in the extraction.

Table 4 shows that ethanol and hexane in the ratio of 75:25 gave the best result with 81 and 68% recovery of TFA and GLA, respectively.

Table 4. Effect of the mixture of organic solvent in extraction of fatty acid and GLA

Organic Solvent	% Recovery	
	TFA	GLA
Ethano' 95%	73	60
Ethanol 75%	64	49
Ethanol 50%	46	38
Ethanol 25%	33	28
Ethanol:Hexane		
25:75	67	56
50:50	77	65
75:25	81	68
Ethanol:Ethylacetate		
50:50	69	59
Acetone	56	46
Ethanol:Acetone		
50:50	69	56

TFA and GLA from direct transmethylation of dried algae cell is 5.31 and 1.35% dry weight respectively.

4. Effect of the amount of algae cell in the extraction of fatty acid

The recovery of fatty acid depended on the amount of algae cell as shown in Table 5. It was observed that increasing amount of algae cell decreased in percentage recovery of total fatty acid and GLA. The amount of algae cells which was suitable for the recovery of total fatty acid and GLA should not exceed 1.50 g/100 ml ethanol.

Table 5. Effect of the amount of algae cell in the extraction of fatty acid using 100 ml of 95% ethanol

Amount of algae cell (g dried cell)	% Recovery	
	TFA	GLA
1.00	83	69
1.50	80	65
1.70	78	63
2.00	70	57

5. Preliminary study on thermodynamic equilibrium diagrams

Overall stage efficiency depends on the contact time between solid and liquid in each stage. To determine the number of actual stage it is necessary to know the 3 phase equilibrium among Spirulina, fatty acid and organic solvent. The thermodynamic equilibrium diagrams on the extraction of fatty acid from Spirulina was studied. The extraction was carried out in the reactor at 40°C, 300 rpm using 95% ethanol (ratio of cell: ethanol 1:9.4 by weight). At different time interval, the concentration of TFA in solvent (overflow) and the residue were determined. Part of TFA in solvent bound to the cell surface was removed by rinsing with ethanol (underflow). Fig.1 shows the TFA in overflow, underflow and cell residue at 1,2 and 3 hours of extraction. The extraction was compeleted in 3 hours and the extract contained 70% lipid, 24% protein and 5% sugar.

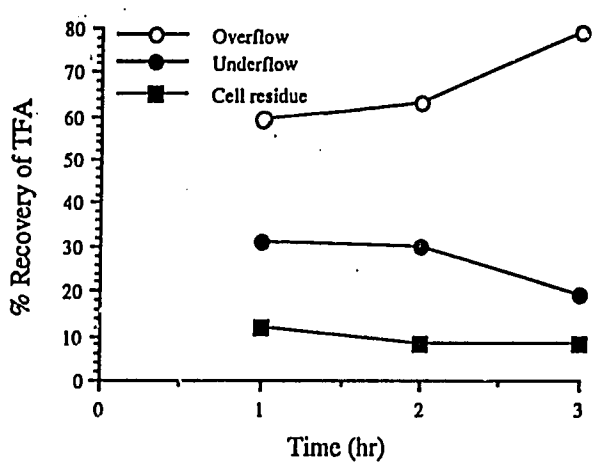

Fig. 1 Extraction of fatty acid using wet algae cell (84% moisture) in the reactor at 300 rpm, 40°C (ratio cell: ethanol 1:9.4 by weight) in total volumn 2200 ml

6. Effect of temperature on the extraction of fatty acids.

In this experiment, fatty acid extraction was done at temperature aried from 40 to 60°C. Table 6 shows that increasing temperature up to 60°C has no effect on the yield of TFA and GLA.

Table 6. Effect of temperature on the extraction of fatty acid using 95% ethanol as organic solvent

Temperature (°C)	Efficiency of extraction (%) (2 hours)	
	TFA	GLA
60	87	77
50	85	76
40	83	77

7. Purification of GLA

After solvent extraction, GLA was purified. Three methods were compared namely, urea complexation, acetone precipitation and low temperature. The results demonstrated that palmitic acid (16:0) whcih i' the main component of total fatty acids could be removed by 67% by urea complexation. Thus the percentage of GLA increased from 25 to 75% (Table 7). About 50% of C16:0 was eliminated by chilling at 2-3°C. However, the method could not separate other unsaturated C18 (C18:1, 18:2) from C 18:3. After crystallisation, C 18:2 increased from 12 to 25% while C18:3 increased from 22 to 32% (Table 8).

Purification of fatty acid using acetone precipitation contained 2 fractions of acetone - insoluble and acetone soluble fractions. Each fraction was further separated by TLC on 20 x 20 cm glass plated coated with silica gel II. The solvent was chloroform, methanol, ammonium hydroxide and n-butylamine (130:70:10:1). The results were shown in Fig 2.

The insoluble fraction was separated into 2 spots (A and B). However, 4 spots were develop after spraying with α-naphthol. The soluble fraction contained totally 4 spost. (C, D, E and F). The lipid - containing bands (A to F) were scraped off and immediately treated with 5% HCL in methanol and transmethylated. Table 9 shows that band A contained the most C18:3 and the least C16:0. Though the C16:0 remained in the soluble fraction, but C18:3 still retained in this fraction in appreciable amount.

Table 7. Comparison of fatty acid before and after urea complexation

Sample	Fatty acid composition (% of total fatty acid)					
	16:0	16:1	18:0	18.1	18:2	18:3
Before urea complexation	48.5	3.7	1.5	4.0	17.2	25.1
Solution after urea complexation	16.2	-	-	-	2.3	75.4

Table 8. Comparison of percentage of palmitic (C16:0) and gamma-linolenic acid (C18:3) before and after crystalisation at low temperature of 3-4°C

Sample	Fatty acid composition (% of total fatty acid)					
	16:0	16:1	18:0	18.1	18:2	18:3
Before urea complexation	47.2	7.1	1.4	6.0	12.2	21.6
After crystallisation	23.6	8.7	1.3	8.6	24.6	31.9

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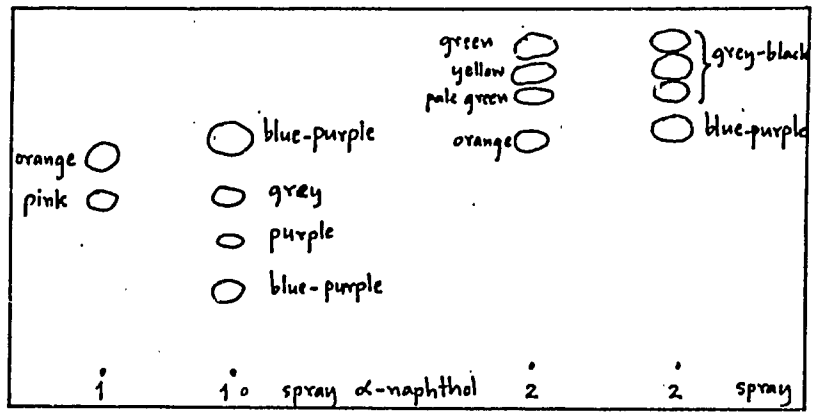

Fig 2. Insoluble (1) and soluble-acetone (2) fractions from TLC on 20 x 20 cm glass plates coated with silica gel H
(eluted with chloroform/methanol/ammonium hydroxide/n-butylamine (130:70:10:1))

Table 9. Composition of fatty acid from the lipid-containing bands (A-F) after transmethylation.

Fractions	Fatty acid composition (% of total fatty acid)						Kind of lipid
	16:0	16:1	18:0	18.1	18:2	18:3	
A	6.1	-	4.7	5.6	74.3	74.3	MGDG
B	52.9	-	-	6.7	3.2	3.2	PG
C	43.6	5.5	-	3.4	47.0	47.0	
D	47.7	5.8	-	10.5	31.5	31.5	MGDG
E,F	47.2	-	10.4	7.4	6.7	6.7	

MGDG - (Monogalactosyl diglycerol)
PG - (Phosphoglycerol)

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8. Conclusion

Preliminary studies on the extraction and purification of polyunsaturated fatty acids particularly GLA from Spirulina platensis BP-1 have been investigated in the laboratory scale. The results shown that ethanol was the most suitable organic solvent in the first step of GLA extraction. It gave not only a better yield but also the cost and safty reason when the commercial application is concerned. Presently, aqueous organic 2 phase is being tried to remove the contamination in particular the saturated fatty acid.

Work on the extraction and purification is still at the preliminary stage. More studies are being conducted especially on the informations for engineering design for larger scale extraction. The recovery of organic solvent is included in the design to reduce the cost of extraction. Regarding the purity of the product, market survey is needed to determine the step of purification to suit the market need.

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FATTY ACID COMPOSITION OF *SPIRULINA* STRAINS GROWN UNDER VARIOUS ENVIRONMENTAL CONDITIONS

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Key Word Index—*Spirulina* species; cyanobacteria; fatty acids; γ-linolenic acid; environment.

Abstract—The fatty acid distribution in 19 strains of *Spirulina* was studied. All but one contained γ-linolenic acid (GLA). No GLA was found in *S. subsalsa*, which had a very high content of palmitoleic acid. The fatty acid content of all but one of the tested strains increased with cultivation temperature and the relative amount of polyunsaturated fatty acid decreased. The highest content of GLA was found at 30–35° for most strains. High light intensities at a high temperature (38°), while not affecting the fatty acid composition, had a drastic effect on the fatty acid content, reducing it by as much as 46%.

INTRODUCTION

The cyanobacterium *Spirulina* is cultivated commercially in several places the world over, mainly as a health food. This alga is protein rich and easily digestable, and it is much larger than unicellular algae, which simplifies harvesting [1].

Spirulina was shown to contain the rare fatty acid, γ-linolenic acid (GLA) 18:3 (6, 9, 12), as early as 1968 [2–5]. This acid was shown to have many therapeutic properties. It is *ca* 170-fold more effective in lowering the plasma cholesterol level than linoleic acid [6], the major constituent of most polyunsaturated oils. In addition, tests on children have shown that GLA is of benefit in treating atopic eczema [7], while in women it appears to reduce the severity of premenstrual syndrome [8]. It was also claimed to have a positive effect in heart diseases, Parkinson's disease, and multiple sclerosis. Direct provision of GLA could thus have an important role in human nutrition.

Since GLA is quite rare and is not found in foods other than human milk, other sources for this fatty acid were sought. The oil of evening primrose was found to contain 8–12% GLA and is presently its commercial source. Wolf *et al.* [9] showed that GLA exists in some other plants as well, and recently it was reported that GLA can be extracted from the residues of blackcurrant remaining after manufacture of jam and juice products [10].

In this paper, we report on the fatty acid content and composition of various strains of *Spirulina* which can be modified by temperature, light intensity and growth phase. The possibility of selecting high GLA strains of *Spirulina* is currently under investigation.

RESULTS AND DISCUSSION

Fatty acid composition of Spirulina *strains*

Nineteen strains of *Spirulina* were cultivated under the same conditions. All the tested strains except *S. subsalsa* contained the same fatty acids (Table 1). The predominant fatty acids were palmitic acid (16:0), GLA, linoleic acid (18:2) and oleic acid (18:1), as already reported [4]. A great diversity was found in the distribution of fatty acids in the various *Spirulina* strains, which is greater for the unsaturated acids. The proportion of 16:0 was consistent, ranging between 44.6 and 54.1% of the total fatty acids. The percentage of C_{18} acids varied greatly: 18:1 ranged from 1.0% to 15.5%; 18:2 between 10.8% and 30.7% and GLA between 8.0% and 31.7%. The lowest fatty acid content (% of ash free dry weight) among freshwater strains of *Spirulina* was found in strain L2 (3.0%); L1 had the highest content (5.6%).

In fourteen strains, high levels of GLA were accompanied by low levels of 18:2 and vice versa (strains 1928, L_1, B_3 and Eth being, however, exceptions to this pattern). For example, in strain Mad the percentages of 18:2 and 18:3 (with respect to total fatty acids) were 10.8% and 31.7%, respectively, while in strain 2340 the percentages of these fatty acids were 30.7% and 8.0%, respectively. Strains SB, Mad and Cat had the highest GLA contents, while strains 2340 and 2342 had the lowest. The tested strains are arranged in Table 1 in decreasing order of GLA content.

A strain which was tentatively identified as *S. subsalsa*, a marine *Spirulina*, was the only one to deviate from the above patterns of fatty acid composition and was significantly different from all other tested strains. It had a very low fatty acid content and contained no GLA. However, it was very high in 16:1. Wood [11] questioned the validity of the classification system of the various *Spirulina* strains as Nichols and Wood [2] detected GLA in *Spirulina* while Kenyon *et al.* [12] claimed the existence of two *Spirulina* strains, one containing α-linolenic acid [18:3 (9, 12, 15)] and the other devoid of both acids. Except for the latter, other studies [3–5, 13] reported the occurrence of linolenic acid (in most cases the γ-form) in *Spirulina*. Similarly, the fatty acid composition of *S. subsalsa* makes it doubtful that it is a genuine *Spirulina*.

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Table 1. Fatty acid content* of *Spirulina* strains†

Strain	Fatty acids							
	16:0	16:1	18:0	18:1	18:2	GLA	Total‡	GLA‡
SB	44.6	4.4	0.5	6.4	17.1	27.0	5.2	1.4
Mad	47.0	0.5	0.7	9.3	10.8	31.7	4.2	1.3
Cat	47.6	2.5	1.0	8.0	15.3	25.6	5.1	1.3
Art. B	46.1	1.0	1.6	10.9	13.6	26.8	4.7	1.3
1928	47.3	2.0	1.0	2.9	18.1	28.7	4.3	1.2
L1	45.0	1.4	1.0	15.5	16.4	20.7	5.6	1.2
AR	49.1	2.2	1.0	6.4	15.7	25.6	4.3	1.1
B4	49.6	2.1	0.7	5.0	16.5	26.1	3.9	1.0
B2	47.3	3.4	0.8	5.8	20.7	20.7	3.8	1.0
G	49.2	2.9	0.9	8.0	15.7	23.3	4.0	0.9
PC	52.5	2.4	0.8	7.2	14.0	23.2	4.0	0.9
B3	52.9	2.2	1.1	7.6	13.7	22.5	4.1	0.9
Art. A	48.5	2.4	1.3	6.0	15.8	26.0	3.4	0.9
Eth	54.1	2.6	1.0	7.7	13.5	21.3	4.1	0.9
L2	50.7	1.1	0.8	7.3	14.3	25.8	3.0	0.8
Minor	46.8	1.2	1.5	12.0	18.4	20.1	3.6	0.7
2342	47.5	1.6	0.5	9.3	21.8	19.3	3.8	0.7
2340	49.3	2.2	1.2	8.6	30.7	8.0	3.2	0.3
Subsalsa	49.2	35.0	1.7	1.0	13.1	—	1.6	—

* Wt percent of total fatty acids.
† Cultures were grown at 35°.
‡ Weight as percentage of biomass (ash-free dry wt-AFDW).

Effect of temperature

The fatty acid content increases with increasing temperature in many algal species [14], and the composition generally changes so that the proportion of polyunsaturated fatty acids (PUFA) decreases. The latter phenomenon has been explained by the role of PUFA in increasing membrane fluidity at low temperatures [15]. Another explanation is that at lower temperatures more oxygen is dissolved in water and thus more is available for the oxygen-dependent desaturase enzymes [16]. The effects of temperature on the fatty acids of *Spirulina* were studied in eight strains. Several examples are presented in Table 2. Strains Art. B, G and 2340 were studied as they are used in commercial *Spirulina* cultivation at various production sites. *S. minor* was chosen because of the exceptional response of its fatty acids to temperature.

The fatty acid content of the tested strains increased with increasing cultivation temperature, reaching the highest value for most strains at 30–35° and decreasing at higher temperatures. In only one strain (Minor), the maximum content was reached at a lower temperature (25°), and no *Spirulina* strain of those tested exhibited a fatty acid maximum at a temperature higher than 35°. Strains SB, Mad, Cat, and 2342 displayed similar patterns to that of strain Art. B (data not shown).

The fatty acids were less desaturated at higher temperatures; two patterns were observed. In the first, the percentage of GLA decreased while those of 18:2 or 18:1 increased. In the other pattern, an increase of 16:0 at the expense of 16:1 was observed. As a result, in most of the tested strains the percentage of GLA decreased only slightly with increasing temperature (up to 30–35°) while the total fatty acid content increased significantly. The net result was an increase in GLA content with increasing temperature, reaching a maximum for most strains at 30° or 35°, the optimum temperature for growth of most strains being 35°. This is of importance for GLA production from *Spirulina* as higher GLA production rates can be envisaged at this temperature.

Effect of light intensity

The effects of three different light intensities on the fatty acid compositions of two strains of Spirulina cultivated at 32, 35 and 38° were studied (Table 3). The distribution of fatty acids did not change much with light intensity (data not shown), the fatty acid content nevertheless being light-dependent. The content of fatty acids at 300 $\mu E/m^2/sec$ was much lower than at 150 $\mu E/m^2/sec$. This light effect was most pronounced at 38°. A 28–29% decrease in fatty acid content was recorded at 32° and a 22% decrease at 35°. Comparing strains minor and G at these light intensities at 38° showed 46% and 71% decreases, respectively. A slight increase in the percentage of GLA was noted at the high light intensity. However, due to the decreased fatty acid content, the overall effect was a reduction in GLA content which was most strongly demonstrated at 38°. Decreasing the light intensity to 75 $\mu E/m^2/s$ did not cause a further change in the fatty acid content.

The effect of light intensity on the fatty acid content and on the degree of fatty acid unsaturation in algae cannot be generalized, and conflicting data have been reported for different species. *Porphyridium cruentum* grown at 1700 and 8000 lux had fatty acid contents of *ca* 9% and 4%, respectively [17]. A similar effect was observed in *Anacystis nidulans* [18], where the fatty acid content doubled at low light intensity and the degree of unsatur-

Table 2. Fatty acid content of *Spirulina* strains grown at various temperatures

Strain	Growth Temperature (o)	Fatty acid Composition* 16:0	16:1	18:1	18:2	GLA	Content† FA	GLA
Art. B	25	41.2	7.1	3.6	17.6	28.2	2.7	0.8
	30	44.5	5.1	7.2	14.5	29.7	3.4	1.0
	35	46.1	1.0	10.9	13.6	26.8	4.7	1.3
	38	53.7	2.0	8.2	13.2	21.3	3.9	0.8
G	20	39.5	9.6	1.8	16.4	32.1	2.7	0.9
	25	40.6	8.6	2.2	16.0	31.7	3.2	1.0
	30	44.6	3.1	5.1	17.9	29.0	4.0	1.1
	35	49.2	2.9	6.3	15.7	23.3	4.0	0.9
	38	47.8	0.2	9.3	17.0	25.0	3.3	0.8
Minor	25	43.2	6.9	4.8	20.5	22.2	4.8	1.1
	30	46.2	4.6	9.0	15.0	24.5	3.3	0.8
	35	46.8	1.2	12.0	18.4	20.1	3.6	0.7
	38	47.7	1.1	9.9	22.4	18.2	3.2	0.6
2340	25	43.5	7.9	4.3	21.4	22.9	2.4	0.6
	30	44.4	5.5	4.2	28.6	16.9	4.2	0.7
	35	49.3	2.2	8.6	30.7	8.0	3.2	0.3
	38	50.0	2.0	7.1	32.3	6.0	3.3	0.2

* Percentage of total fatty acids.
† Percentage of AFDW.

Table 3. The effect of light intensity on fatty acid content in *Spirulina*

Strain	Light intensity uE/m/s	Fatty acid content* 32° FA†	18:3	35° FA	18:3	38° FA	18:3
G	75	3.26	1.02	–	–	3.28	0.86
	150	3.43	0.90	3.82	0.95	3.29	0.79
	300	2.45	0.70	2.99	0.90	0.95	0.26
Minor	75	3.50	0.94	–	–	3.32	0.51
	150	3.27	0.80	3.63	0.73	3.27	0.60
	300	2.36	0.65	2.89	0.70	1.78	0.28

* Percentage of AFDW.
†Fa, Fatty acids.

ation increased. In *Chlorella minutissima* [19], low light intensity had the opposite effect, both on fatty acid content (15% reduction) and on percent PUFA (10% reduction). Similar results were reported for *Nitzschia closterium* [20].

The reasons for the inhibitory effect of high light intensities on fatty acid content described herein are as yet unknown.

Growth Phase

Many algal species are known to accumulate lipids during the stationary phase [21], but no such effect has ever been found in cyanobacteria. Strain Mad cultures were sampled at the mid-exponential phase and at the stationary phase (Table 4). The total fatty acid content was reduced in the stationary phase and the relative amount of PUFA was also decreased. The content of GLA was reduced by *ca* 50%.

The data presented here suggests that *Spirulina* could be utilized as a source for the valuable fatty acid GLA. Since the maximal GLA content was found at the optimal growth temperature, a relatively high GLA production rate may be expected. Also, in many strains GLA is a major fatty acid comprising more than 25% of total fatty acids. The introduction of GLA as a drug will necessitate its purification, the costs of which will be lower, the higher the initial GLA concentration in the fatty acid mixture.

EXPERIMENTAL

Organisms. A culture collection of *Spirulina* strains was established in the Algal Biotechnology Laboratory of the Desert Research Institute at Sede-Boqer. Strains No. 1928, 2340 and 2342 were obtained from the UTEX culture collection (Austin, Texas, USA), strain Art A from Carolina Biological Supplies (Burlington, North Carolina, USA), strain G from Prof. C. Soeder (F.R.G.) (originally isolated from Lake Chad) and strains

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Table 4. Distribution and content of *Spirulina* (S. mad) fatty acids in different growth phases*

Growth phase	Fatty acids							
	16:0†	16:1†	18:0†	18:1†	18:2†	GLA†	Total‡	GLA‡
Exponential	48.9	2.3	0.8	7.3	12.1	30.5	4.3	1.3
Stationary	52.6	2.7	2.1	12.3	11.1	18.6	3.1	0.6

*At 35°.
† Percentage of total fatty acids.
‡ Percentage of AFDW.

Mad and Cat from Dr. Venkataraman (India). Strains PC, B_2, B_3 and B_4 were isolated by the authors from a flood-irrigated field in Thailand. Strains SB, L_1, L_2, Art. B and AR were also isolated by the authors from water reservoirs in Israel. Strain Eth was isolated from a lake in Ethiopia, and *S. subsalsa* was obtained from Dr. E. Tel-Or (Hebrew University of Jerusalem). Stock cultures were held in flasks under constant illumination with frequent dilutions. Isolation procedures and inocula prepn were performed according to ref. [22].

Culture conditions. Cultures (500 ml) were grown on Zarrouk's medium [23] in flat-bottomed 1l flasks placed in a transparent water bath illuminated from below with four cool-white fluorescent lamps providing 150 $\mu E/m^2/sec$ at the surface of the bath, unless otherwise stated. Mixing was achieved with an air-CO_2 mixture (99:1) bubbled through a sintered glass tube placed in the bottom of the culture flask. The temp was maintained constant within $\pm 1°$. The marine strain *S. subsalsa* was cultivated similarly in Abeliovich's medium [24]. Cultures (mid log phase unless otherwise stated) were harvested by filtration.

Cultures were grown exponentially (with proper diln) under the experimental conditions for at least six days (depending on the individual culture's growth rate). Although cultures used were not bacteria free, they were cultivated on sterile medium (bacterial counts not exceeding 100 colonies/ml). Ash and chlorophyll content were determined according to ref. [22].

Lipid extraction and transmethylation. Freeze-dried samples of *Spirulina* (100 mg) were treated with 2 ml of MeOH-AcCl (19:1) according to ref. [25]. 17:0 (its absence in the sample was previously checked) was added as an int. standard at 1 the mixt sealed in a vial under an Ar atmospbere and heated to 80° for 1 hr. The vial was then cooled, its contents dil. with 1 ml H_2O and the mixt. extd with 1 ml hexane. The hexane layer was dried (Na_2SO_4), evapd to dryness and redissolved in hexane.

Fatty acid analysis. GC analysis was performed on a SP-2330 fused silica capillary column (30 m, 0.2 mm) at 200° (FID, injector and FID detector temps 230°, split ratio 1:100). Fatty acid Me esters were identified by cochromatography with authentic standards (Sigma Co.) and by GC/MS using a Carbowax capillary column (30 m). CI spectra were obtained at 250 eV with *isobutane* as reactant gas. Fatty acid contents were determined by comparing their integrated peak areas with that of the int. standard. The data shown are mean values of at least two independent samples, each analysed in duplicate.

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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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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.

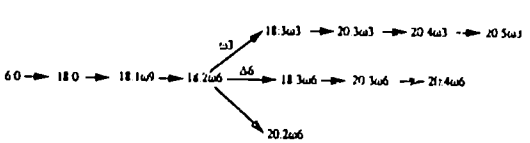

Figure 1. Scheme of major pathways of fatty acid biosynthesis in *M. subterraneus*.

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.[illegible]
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.

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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FATTY ACID COMPOSITION OF *SPIRULINA* AND *SPIRULINA*-LIKE CYANOBACTERIA IN RELATION TO THEIR CHEMOTAXONOMY

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Key Word Index *Spirulina* species; cyanobacteria; fatty acids; chemotaxonomy.

Abstract—The fatty acid composition of several *Spirulina*-like cyanobacteria strains was investigated. While, on the basis of morphology alone, these strains could not be distinguished from *Spirulina*, their fatty acid composition demonstrated a different pattern having large amounts of 18:3 (9, 12, 15), 16:1 or 16:2, but small quantitites of γ-linolenic acid. On the basis of these findings, it is suggested that fatty acid composition might be utilized for the classification of *Spirulina* strains.

INTRODUCTION

The cyanobacterium *Spirulina* is one of the few commercially cultivated microalgae used primarily as a health food. *Spirulina* is also one of the few organisms known to have a high content of γ-linolenic acid [GLA, 18:3 (6, 9, 12)] [1–4]. This fatty acid is known to be of value in the treatment of various diseases [5–9].

Although a few commercial sites for production of *Spirulina* are operated, very little is known about the strains used for commercial production. Because the classification system used for *Spirulina* is essentially morphological and mainly based on its spiral shape, it is difficult to distinguish between any strains used. The morphology of the filaments is highly dependent on growth conditions and environmental factors [10] and under certain conditions the filaments may even lose their spiral shape [11]. Fatty acid distribution is one of the tools available for taxonomic classification of microalgae but its use is generally limited to the level of classes and orders but not for a genus. Kenyon *et al.* used fatty acid composition as a criterion for dividing cyanobacteria into several subgroups [12] and claimed the existence of two strains of *Spirulina*, one containing α-linolenic acid [18:3(9, 12, 15), ALA] and no GLA, the other one being devoid of both fatty acids. Based on these data, the validity of the commonly used classification system has already been questioned by Wood [13].

In a previous study, we have shown [14] that 18 strains of *Spirulina* obtained from culture collections, or isolated from their natural habitat, had the same fatty acid composition, the major fatty acids being 16:0, 16:1, 18:0, 18:1, 18:2 and GLA. The proportion of GLA is quite high, generally ranging in % of total fatty acids between 19.3 and 31.7% at 35°. Only one strain (2340) had a significantly lower proportion (8%) which increased however to 23% at lower temperature (25°). All of these strains were cultivated on the standard alkaline *Spirulina* medium (Zarrouk's medium, pH 9.8). These findings were in agreement with those described in most other reports [1, 15–17]. The great commercial interest in *Spirulina* and the need for applying modern techniques of strain selection makes it important to set standards for classification other than those based on morphology. In this paper, we provide evidence suggesting that the fatty acid composition of *Spirulina* may be used as a basis for characterization.

RESULTS AND DISCUSSION

We studied the fatty acid composition of six strains, originally characterized as *Spirulina*, based on their morphological appearance and pigment composition, yet which did not grow on alkaline medium. These strains were spiral shaped, filamentous with no heterocystous cells, similar to the *Spirulina* strains previously studied. Four strains (Th 11, Th 21, Th 30, Sh) displayed similar characteristics in their fatty acid composition (Table 1). Although no GLA was found in these strains they contained high proportions of ALA. Also, the proportion of palmitoleic acid 16:1 was unusually high compared with GLA containing *Spirulina* strains ranging from 13.8 to 32.3%. The amount of 16:1 in alkaline strains was in the range 0.5–4.4% (% total fatty acids).

Strain LB 2179 had a higher proportion of ALA (35.3%) and contained a significant proportion of another fatty acid (16:2) found only in trace amounts in alkaline strains. Only one freshwater strain (N 27), contained GLA (2.6%). However, this strain is unique in its high 16:1 and 16:2 contents, 18.6 and 19.2%, respectively.

In a previous study we found [14] another strain, *S. subsalsa*, which did not grow on the alkaline medium but could be cultivated on an artificial sea water medium. Its fatty acid composition was significantly different. No trace of GLA was found and the proportion of 16:1 was 35.0% as compared with 0.5–4.4% in the other strains.

It appears that several cyanobacteria, although morphologically indistinguishable from *Spirulina*, display

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Table 1. Fatty acid composition of *Spirulina*-like strains

Strain	Medium	Fatty acid composition*									
		14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	ALA	GLA
Th 11	BG 11	1.4	41.2	13.8		1.7	2.6	8.9	18.1	12.3	—
Th 21	BG 11	1.8	40.6	21.2	—	0.3	1.0	3.1	13.2	18.8	—
Th 30	BG 11	2.5	40.4	22.3	—	0.5	1.0	3.4	13.7	16.2	—
Sh	BG 11	0.6	33.1	16.8	0.2	1.2	1.4	5.8	18.7	20.5	—
LB 2179	BG 11	2.2	31.2	7.6	9.5	0.8	1.7	4.9	6.9	35.3	—
N 27	BG 11	2.9	47.0	18.6	19.2	2.3	0.6	1.8	5.0	—	2.6

*Wt per cent of total fatty acids.
ALA = α-linolenic acid.
GLA = γ-linolenic acid.

patterns of fatty acid distribution which are different from *Spirulina* both qualitatively and quantitatively. These dissimilarities appear to be significant enough as to exclude from classification in the genus *Spirulina*. This argument is further strengthened by the finding that these strains will generally not grow in the typical alkaline *Spirulina* medium. One may argue that the differences in fatty acid compositions are just manifestations of the different cultivation conditions resulting from the differences of pH and salinity between the various media. However, the fact that the freshwater strain N 27 contains GLA and not ALA suggests otherwise. Moreover, the differences between the enzymatic systems necessary to generate GLA and ALA are considerable and only rarely can both GLA and ALA be found in the same organism. These results suggest that cyanobacteria unable to grow in an alkaline medium are probably unrelated to *Spirulina*. We propose that in addition to the already existing morphological criteria for *Spirulina*, the fatty acid composition should be used as an additional criterion for the characterization of the genus. Authentic *spirulina* strains display a significant proportion of GLA, contain no ALA, and probably also contain no more than a low content of 16:1 (< 10%) and a very low content of 16:2 (< 0.1%).

EXPERIMENTAL

Organisms. Cyanobacterial strains Th-11, Th-21 and Th-30 were isolated from standing water during the dry season in northeast Thailand. Strain Sh was isolated from fish ponds in the north of Israel by Prof. M. Shilo and coworkers (Hebrew University, Jerusalem, Israel). Strains LB 2179 was obtained from the University of Texas Culture Collection and strain N 27 from the Microbial Culture, The National Institute for Environmental Studies, Japan. These strains were grown on BG-11 medium. Algal cultures were unialgal. Bacterial counts did not exceed 50 viable counts of bacteria per ml, as measured by plating samples of the cultures on nutrient agar containing *Spirulina* medium.

Culture conditions. Cultures were cultivated at 35° as previously described [14].

Fatty acid analysis. Freeze-dried samples of algal biomass were transmethylated with MeOH-AcCl as previously described [18]. Heptadecanoic acid was added as int. standard. Gas chromatographic analysis was performed on a Supelcowax 10 fused silica capillary column (30 m, 0.32 mm) at 200° (FID, inj. and flame ionization detector temps 230°, split ratio 1:100). Fatty acid Me esters were identified by co-chromatography with authentic standards (Sigma) and by GC-MS using a Carbowax capillary column (30 m). CI spectra were obtained at 250 eV with *iso*butane as reactant gas. Fatty acid contents were determined by comparing their peak areas after integration with that of the int. standard. Data shown represent mean values (with a range of less than 3% for major peaks and 8% for minor peaks) of at least two independent samples, each analysed in duplicate.

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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 cell. 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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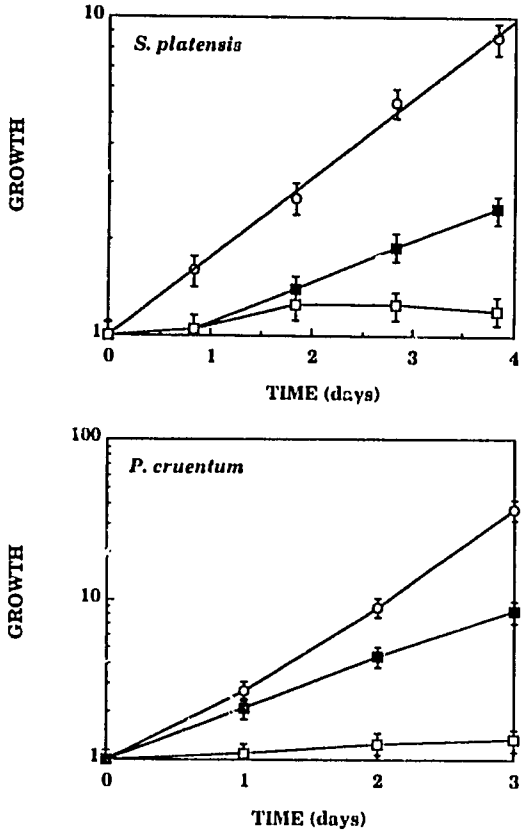

Figure 1. Effect of 0.4 mM SAN 9785 on the growth rate (expressed as the increase in Chl concentration relative to day 0) of *S. platensis* and *P. cruentum* (O, control; □, freshly exposed to the inhibitor; ■, inhibitor resistant culture).

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	16:1	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]	42.10 ± 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*	5.55 ± 0.35*	1.28 ± 0.13*
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*	4.93 ± 0.24*	1.15 ± 0.09*
SRS-11	40.69 ± 1.67	4.95 ± 0.39	0.67 ± 0.04	2.58 ± 0.42	25.09 ± 0.53	23.57 ± 0.52*	6.07 ± 0.18*	1.43 ± 0.05*

[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. * Significantly different from wild type. Lipid transmethylation and fatty acid analyses were performed as previously reported (3).

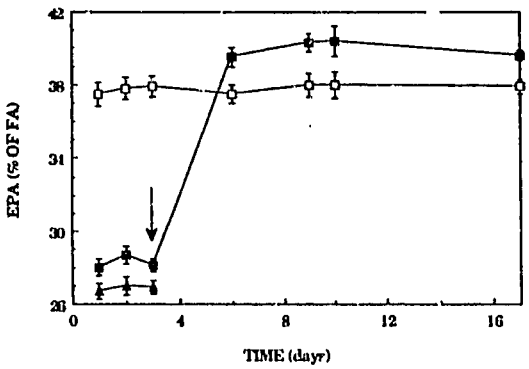

Figure 2. EPA content (% of fatty acids) of *P. cruentum* wild type grown with (▲) or without (□) the herbicide SAN 9785 and of a resistant cell line grown continuously in the presence of the herbicide (■) and shifted to a herbicide-free medium (arrow). Due to dependence of the fatty acid composition 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 nM 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	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.

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

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Running title - Resistance of algal cultures to SAN 9785

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Abstract—Cell lines of *Spirulina platensis* and *Porphyridium cruentum* resistant to the growth inhibition of the herbicide SAN 9785 had a siginicantly higher growth rate than their respective wiid type strains. These lines were also shown to overprodue γ-linolenic acid (GLA) and eicosapentaenoic acid (EPA), respectively, in the presence and in the absence of the inhibitor. The effect was most conspicuous in polar lipids. Thus, the proportion of GLA in the galactolipid (GL) fraction of the SAN 9785 resistant strain *S. platensis* SRS-1 increased in the absence of the inhibitor from 33.3 in the wild type to 39.0%. Similarly, the EPA proportion of the GL fraction of the resistant strain *P. cruentum* SRP increased in the presence of the inhibitor from 29.1 to 45%.

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] 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 acid in question. The substituted pyridazinone SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone) was widely used for inhibition of 18:3ω3 levels in the glycolipids of higher plants and algae [11] and was shown to directly inhibit *in vitro* desaturation of 18:2 in MGDG [12]. Recently, we have shown [10] that the herbicide 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 suitable for 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 was 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 this work, we further elaborate 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 the growth inhibition of SAN 9785

The herbicide SAN 9785 inhibited the growth of *S. platensis* and *P. cruentum* cultures. Growth was arrested after 2 and 4 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 the 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, at this concentration, a much higher growth rate than the wild type in 0.16 mM (Fig. 1) and almost as fast as the wild type cultivated in 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).

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 the 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 of this herbicide on the fatty acids of wild type *Spirulina* is primarily characterized by a decrease in the level of GLA and in an increase in that of 18:1ω9 [10]. Yet, the intensity of the 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 the inhibitor, the resistance was 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. Yet, in the resistant culture it was reduced to only 7.8. The fatty acid content 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 which was still lower than that of the wild type in the absence of the inhibitor (0.9%) (Table 1).

The effect of the inhibitor on both the wild type and the resistant cultures was even more conspicuous in the polar lipids. When exposed to 0.4 mM SAN 9785, the proportion of GLA in the glycolipids (GL) of another *Spirulina* strain - BP was reduced from 36.8 to 22.2% (Table 2). A slight decrease was noted in the level of 18:2ω6 while the level of 18:1ω9 increased from 4.0 to 19.2%. Consequently, the R value dropped from 11.7 to 1.7. In culture Z19/2 (derived from *Spirulina* BP), which was resistant to 0.4 mM SAN 9785, the level 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 level of GLA from 5.8 to 3.4% and that of 18:2ω6 from 34.5 to 18.2%. The 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 36.8 to 25.4 (% of fatty acids) while

16:0 increased from 31.8 to 43.9%. In the resistant culture, however, the level 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 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 level of GLA which reached 39.0 in comparison with 33.3% in the wild type (Table 2). The level 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 a 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 incurred 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 2). 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 rendered 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*.

In keeping with Murata et al. [16], it can be deduced that in *Spirulina*, being a cyanobacterium, only saturated and monounsaturated C_{18} acyl groups are incorporated to 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 the 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.

Apparently, there are several possible routes for the synthesis of EPA in microalgae. This conclusion was drawn from the findings that in some algae such as *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 this herbicide causes a direct inhibition of photosynthetic electron transport. We have recently demonstrated [10] that SAN 9785 which was 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 this herbicide. Yet another example for the lack of specificity is the observation that this herbicide affect also 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 level was already very low (Table 2). In plants, it was already 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 analogs are being sought.

The selected strain, *S. platensis* 2340, was previously shown to be very low in GLA content [5]. However, it was used as an experimental model with the assumption that a further reduction in the already low GLA level 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]; *P. cruentum* was grown on Jones' medium [20]. Stock cultures were maintained and inocula performed according to Vonshak [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 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 conc.

Selection of SAN 9785 resistant lines Solutions of SAN 9785 in DMSO were added to exponentially growing cultures. The final concentration 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 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 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 suspension 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 plates containing 0.4 mM of the inhibitor.

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 [22]. Heptadecanoic acid was added as an int. std. GC analysis was performed on a Supelcowax 10 fused silica capillary column (30m x 0.32 mm) at 200° (FID, inj. and detector 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 Co.) and by comparison of their equivalent chain length (ECL). Fatty acid contents were determined by comparing their peak areas with that of the int. std. 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 four independent samples, each analyzed in duplicate.

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

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

†Total fatty acids.

◆Wild type.

•R: (18:3 + 18:2)/18:1.

*Culture resistant to a concentration of SAN 9785 whose level is indicated in parenthesis. Cultures were analyzed 4 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 analyzed in duplicate.

Table 2. Effect of SAN 9785 on the fatty acid composition of the polar lipids of wild type and SAN 9785 resistant cultures of *Spirulina*.

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

†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 analyzed in duplicate.

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

Culture	SAN 9785 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
WT†	0	31.8	5.9	0.5	0.9	1.0	5.3	1.4	0.3	0.5	16.0	36.8
WT	0.16	37.8	5.1	0.8	0.7	1.3	5.0	0.7	0.6	0.9	15.4	31.2
WT	0.4	43.9	5.9	0.8	0.9	1.9	5.5	1.5	0.8	1.3	11.6	25.4
SRP•	0.4	29.0	3.1	0.6	1.8	2.8	12.2	1.3	1.0	2.7	14.3	30.7
SRP	0	28.5	5.4	0.4	0.5	1.0	5.7	0.9	0.5	0.7	15.8	40.4
SRP-6*	0	29.2	4.7	0.6	0.5	0.5	5.3	0.9	0.6	0.4	15.4	41.1

†Wild type.

•SAN 9785 resistant culture.

*Resistant line selected by agar plating of the resistant culture.

Data shown represent mean values of four independent samples, each analyzed in duplicate.

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

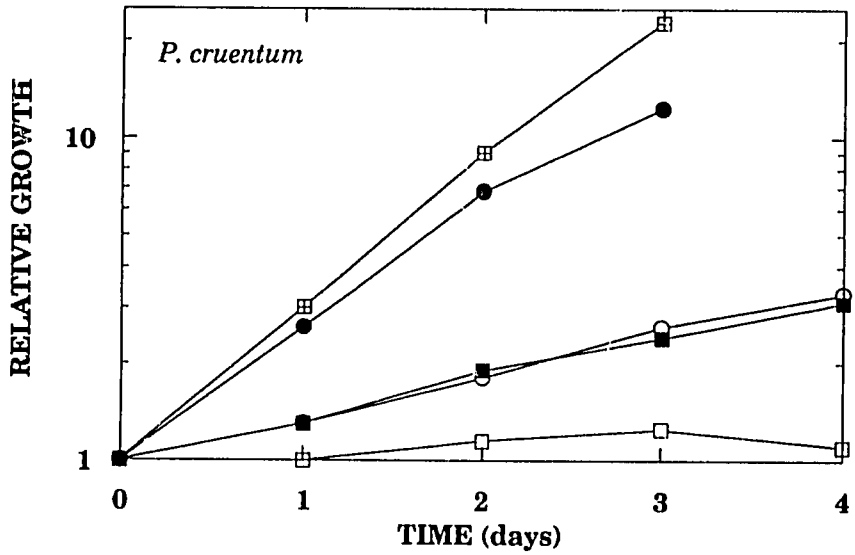
Culture	Medium	Fatty acid composition (% of total fatty acids)										
		16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT	-[a]	36.4	1.2	nd[b]	0.5	1.0	4.3	nd	0.6	nd	5.9	50.0
WT	+[a]	36.4	8.2	0.5	3.3	5.7	4.1	0.4	1.3	0.2	9.2	29.1
SRP*	+	36.0	2.0	0.2	1.2	2.0	7.4	0.3	1.2	0.2	3.8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

[a](-) Inhibitor free medium; (+) medium containing 0.4 mM SAN 9785.

[b]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 analyzed in duplicate.


P. cruentum
RELATIVE GROWTH
10
1
0
1
2
3
4
TIME (days)

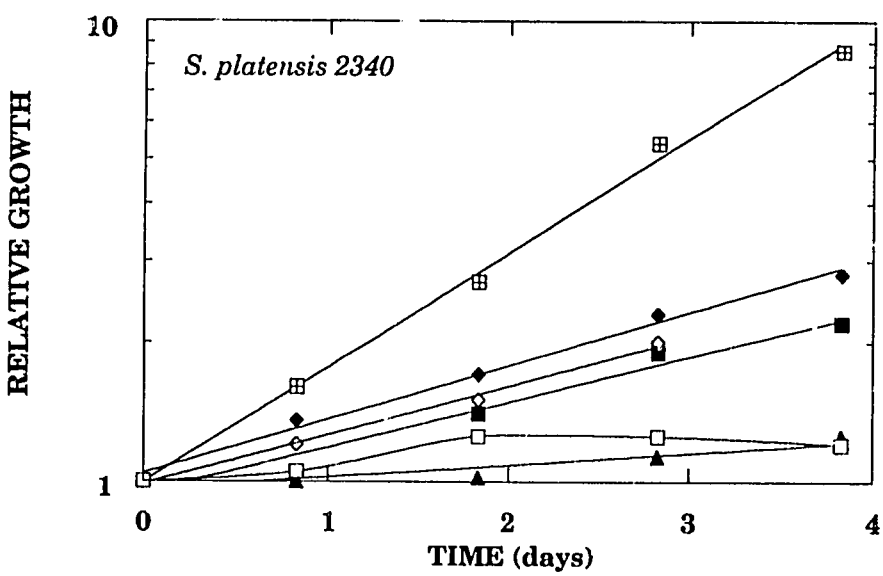
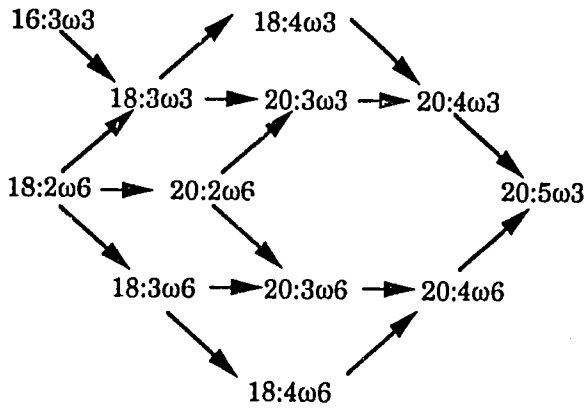

S. platensis 2340
RELATIVE GROWTH
10
1
0
1
2
3
4
TIME (days)

Fig. 1

Fig. 1 Effect of SAN 9785 on the growth rate (expressed as the increase in chloropyll concentration relative to day 0) of *S. platensis* 2340 and *P. cruentum*, (⊞ -control;● -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.

Scheme 1. Suggested pathway of fatty acid biosynthesis in *Spirulina*.

Scheme 2. Possible pathways of PUFA biosynthesis in *P. cruentum*.


16:3ω3
18:4ω3
18:3ω3
20:3ω3
20:4ω3
18:2ω6
20:2ω6
20:5ω3
18:3ω6
20:3ω6
20:4ω6
18:4ω6

Scheme 1

$$16{:}0 \rightarrow 18{:}0 \rightarrow 18{:}1\omega9 \rightarrow 18{:}2\omega6 \xrightarrow{\Delta 6} 18{:}3\omega6$$

Schème 2.

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EFFECTS OF SUBSTITUTED PYRIDAZINONES ON FATTY ACID PRODUCTION IN SOME 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 contact of 20:5ω3 indicating a possible 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 phosphati-

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dylglycerol (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.

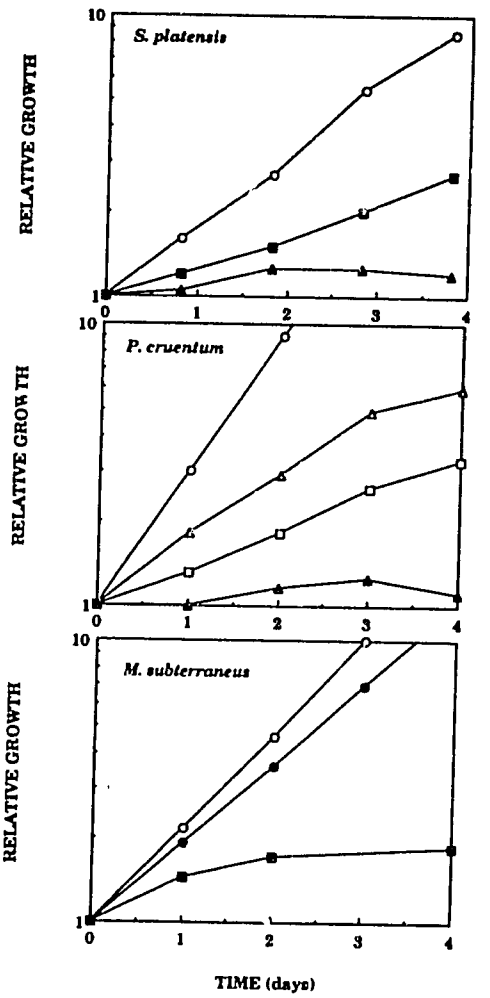

Fig. 1. Effect of SAN 9785 on growth (expressed as the increase in chlorophyll concentration relative to day 0) of *S. platensis*, *P. cruentum* and *M. subterraneus* (○, control; ●, 0.04 mM; △, 0.08 mM; □, 0.16 mM; ■, 0.2 mM; ▲, 0.4 mM).

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).

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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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 the amounts 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

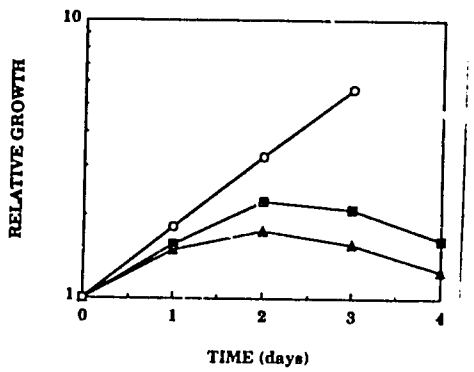


Fig. 2. Effect of norflurazon on growth rate (expressed as the increase in chlorophyll concentration relative to day 0) of *S. platensis* (o, control; ■, 3 μM; ▲, 15 μM).

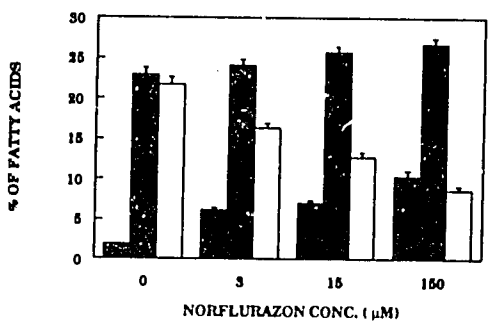


Fig. 3. Effect of norflurazon on 18:3 (□), 18:2 (□) and 18:1 (■) contents in *S. platensis*.

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	29:2 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

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

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.

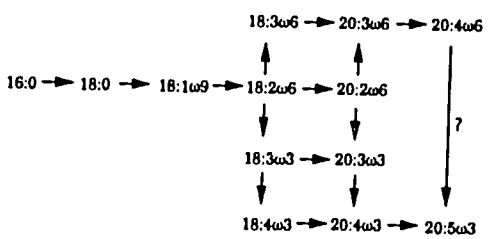
Cultures were analysed four days after exposure to the herbicide. Data shown represent mean values of two independent samples, each analysed in duplicate.

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 *M. monodus subterraneus* UTEX 151 were obtained from the University of Texas Culture Collection. *P. orphyridium cruentum* strain 1380 1d was obtained from the Goettingen Algal Culture collection (Goettingen, Germany). *S. pirulino 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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Production and Partial Purification of γ-Linolenic Acid and Some Pigments from *Spirulina platensis*

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Key words: *Spirulina platensis*, cyanobacteria, fatty acids, γ-linolenic acid, mutants, purification.

Abstract

The polyunsaturated fatty acid γ-linolenic acid (GLA) is of potential pharmaceutical value. The cyanobacterium *Spirulina platensis* could become an excellent source for this fatty acid, provided that GLA content could be increased and a GLA concentrate could be obtained at a low cost. Increasing the cell concentration in *Spirulina platensis* enhanced the fatty acid content and thus the GLA content. This effect was used to further enhance the GLA content of GLA-overproducing strains. Separation of the galactolipids and their purification via urea complexes formation, resulted in a GLA concentrate of over 90% purity.

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Introduction

Recent studies have shown that the polyunsaturated fatty acid γ-linolenic acid (GLA, 18:3ω6) is of potential pharmaceutical value for lowering low density lipoprotein in hypocholesterolemic patients (Ishikawa *et al.*, 1989), for alleviation the symptoms of the pre-menstrual syndrome (Horrobin, 1983), and for treatment of atopic eczema (Biagi *et al.*, 1988).

GLA is found in evening primrose, black currant (Traitler *et al.*, 1984) and borage (Wolf *et al.*, 1983), as well as in fungi (Shimizu *et al.*, 1988). However, large scale cultivation of these fungi is not yet feasible and the GLA content in plants is either low (8-12%), or is accompanied with other fatty acids (e.g., 18:4) of undesired properties from which separation could be very expensive in a large scale. The cyanobacteria *Spirulina* was shown to be an alternative source of GLA (Nichols & Wood, 1968). *Spirulina* is commercially cultivated in a large scale, primarily as a health food. It contains in addition to GLA, also several other pigments of economic interest. Phycocyanin, a blue proteinaceous pigment, which constitutes up to 25% of the algal dry weight (Tel-Or *et al.*, 1980), was introduced in Japan as a natural coloring agent for feed and cosmetics and is produced at a rate of 600 Kg/month. Phycocyanin purification by treatment with active carbon was recently described by Herrera *et al.* (1989). The alga also contains zeaxanthin, a xanthophylic pigment which was shown to be effective in enhancing fish and shrimp pigmentation (Mori *et al.*, 1987).

By choice of strains and environmental conditions, it was possible to increase the content of GLA in *Spirulina* to 31.7% of fatty acids and 1.4% of dry weight (Cohen *et al.*, 1987). Still, in order to reach economical viability, the GLA content of *Spirulina* must be further significantly enhanced. There are several physiological means for increasing oil content in eukaryotic algae such as nitrogen or silicon starvation, or holding cells in the stationary phase of growth

for extended period of time. However, none of these methods were effective with cyanobacteria. Recently, we (Cohen *et al.*, 92) have isolated herbicide-resistant lines of *Spirulina* which demonstrated GLA overproduction.

Pharmaceutical applications will probably require preparations of considerably higher concentrations of GLA. The most efficient method for purification of polyunsaturated fatty acids (PUFA) is the formation of urea inclusion complexes which enabled the preparation of a GLA concentrate from blackcurrant seed oil (Traitler *et al.*, 1988) and eicosapentaenoic acid concentrates from cod liver oil (Haagsma *et al.*, 1982) or *Porphyridium* oil (Cohen & Cohen, 1991). In this report, we describe methods for enhancing the fatty acid and GLA content in *Spirulina* and an outline for separation and purification of GLA and other valuable chemicals from this biomass.

Materials and methods

Cyanobacteria and cultivation

Spirulina platensis 2340 was obtained from the University of Texas Culture Collection. Strain BP was isolated from a local pond in Ban Pong, Thailand. Both strains were cultivated on Zarrouk's medium at 30 °C as previously described (Cohen *et al.*, 1987). Stock cultures were maintained and inocula transferred according to Vonshak (1986). Growth rate was estimated by chlorophyll measurement.

Analytical and Chemical Methods

Lipid transmethylation.

Freeze-dried samples of *Spirulina* biomass (100 mg) were treated with 2 mL of methanol-acetyl chloride (95:5) as previously described (Cohen & Cohen, 1991). Heptadecanoic acid was added as an internal standard and the mixture was

sealed in a light-protected Teflon-lined vial under an Ar atmosphere and heated to 80 °C for 1 hr. The vial contents were then cooled, diluted with 1 mL water and extracted with 1 mL of hexane containing 0.01% butylated 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 on a Supelcowax 10 fused silica capillary column (30 m x 0.32 mm) at 200 °C (FID, 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 co-chromatography with authentic standards (Sigma Co.) and by calculation of the equivalent chain length (ECL). Fatty acid contents were determined by comparing their peak areas with that of the internal standard. The data shown represent mean values (with a range of less then 5% for major peaks and 10% for minor peaks) of at least two independent samples, each analyzed in duplicate.

Lipid fractionation

Freeze dried samples of biomass were extracted with chloroform-methanol-water (2:1:0.8) according to Bligh and Dyer (1959). Separation into neutral lipid, galactolipid (GL) and phospholipid (PL) classes was achieved by using silica gel cartridges (Sep-pak, Waters division of Millipore, Milford Mass). The individual classes were successively eluted with chloroform, acetone and methanol, respectively.

Urea inclusion complexes formation

One g of fatty acid methyl esters (FAME) was dissolved in 10 mL methanol containing 4 g urea (E. Merck, Darmstadt, Germany) by heating 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, 6 mL water and 3 mL concentrated hydrochloric acid. The lower phase was separated and the aqueous layer was reextracted with methylene chloride. The combined extracts were evaporated to dryness and dissolved in methanol.

Results

Effect of cell concentration

During batch cultivation of *Spirulina* cultures we observed an increase in the fatty acid content concomitant to the increase in cell concentration. Thus, the fatty acid content of *S. platensis* 2340 increased daily from 3.5 to 6.9% (% of dry weight) after 7 days (Fig. 1) while in a daily diluted culture, the fatty acid content did not change significantly and ranged from 3.5 to 3.7% (Fig. 1). The fatty acid composition changed concurrently, resulting in a decrease in the proportion of GLA (% of fatty acids) from 22.9 to 16.1% (Fig. 1) and an increase in that of 18:2 and 16:0 (data not shown). Nevertheless, the total GLA content increased from 0.81 to 1.12%. In another experiment, *Spirulina* cultures were kept for 4 days at various chlorophyll concentration by daily dilutions to the original cell concentration . Once again, denser cultures had higher fatty acid contents. At a relatively low cell concentration (4 mg Chlorophyll L^{-1}) the fatty acid content was 3.6, increasing to 5.2% in the densest culture (19 mg Chl. L^{-1}) (Table 1). With increasing cell density, the proportion of GLA slightly declined resulting in an overall increase in GLA content from 0.86 to 1.09% (Table 1). Cultivation at higher biomass densities resulted also in an enhanced phycocyanin content which increased from 10.4% at 4 mg chl. L^{-1} to 22.4% at 19 mg chl. L^{-1} (Table 1). The fatty acid as well as the GLA contents of the wild type

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strain BP and its GLA-overproducing variant Z19 were similarly affected by even small changes in cell concentration (Fig. 2).

GLA purification

Spirulina biomass was extracted and the resulting lipids were fractionated by selective washes on silica gel cartridges to three major fractions: neutral lipid (NL), galactolipids (GL) and phospholipids (PL). Most of the GLA (92%) was restricted to the GL fraction. The separation of this fraction resulted in an increase in the GLA content from 21.5 to 39.0% (Table 2). The GL fraction was converted into fatty acid methyl esters (FAME) by transmethylation and treated with urea in methanol. By formation of the urea inclusion complexes, virtually all the saturated and monounsaturated fatty acids were effectively removed, increasing the GLA content from 39.0 to 90.5% (Table 3). A similar treatment of the total fatty acids also increased the GLA content but only to 79.7% (Table 3).

Discussion

Increasing the cell concentration under either batch or semi-continuous growth conditions resulted in enhanced fatty acid and therefore GLA contents. However, the effect of the increase in the fatty acid content on the GLA content was partly negated by a slight decrease in the GLA proportion (Table 1). GLA was primarily concentrated in polar lipids and especially in GLs (92% of total GLA), while neutral lipids contained only very low proportions of cellular GLA (1.5%). The decrease in the proportion of GLA may indicate a larger proportion of NL in newly synthesized lipids than in preexisting lipids. However, this increase was not exclusively made of NL since the overall increase in GLA content indicates a net increase in GLs. At higher cell concentrations, the culture already reached the stationary phase and the increase in fatty acid content apparently resulted primarily from an increase in NLs, hence the decrease in both the proportion and the content of GLA. These results are in

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keeping with earlier findings demonstrating an increase of fatty acids and GLA contents of various *Spirulina* strains cultivated under lower light intensities (Cohen *et al.*, 1987). This effect could be related to the reduction in growth rate at higher cell densities or low light intensities, or could directly evolve from the effect of light intensity on cell biochemistry. However, reduction of the growth rate by increasing the salinity ( ) did not enhance the fatty acid content which remained unaffected at low salinities (0.1-0.75 g L^{-1}) and even decreased at high salinities (1.5 g L^{-1} and beyond) (data not shown). The cell concentration-dependent increase in fatty acid and GLA contents could be demonstrated in both the wild type strain BP and its GLA-overproducing variant Z19. Thus, it seems that the combination of two methods we have developed for increasing GLA content, namely, the use of GLA-overproducers and cultivation at higher cell concentrations, could be employed for a further enhance of the GLA contents in *Spirulina*.

Increasing the cell density also resulted in higher chlorophyll and phycocyanin contents. The latter more then doubled, from 10.4 under a high dilution rate to 22.4% under a low dilution rate (Table 1). Thus, the increase in cell density resulted in an increase in the content of the two major products of *Spirulina*, GLA and phycocyanin. However, since maximal biomass productivity will be obtained at lower cell densities, optimal productivities of these products will probably be reached at some intermediate value.

While saturated and monounsaturated fatty acids could be efficiently removed by the formation of urea adducts, it is the PUFAs 16:3 and 18:2 that are more difficult to eliminate. Therefore, in order to concentrate and separate GLA from other PUFAs, a preliminary fractionation step of the oil was undertaken. Fractionation of the *Spirulina* oil yielded a fraction (GL) which contained the lion share of GLA. Moreover, virtually all the 16:3 and most of the 18:2 were restricted to the NL and PL fractions respectively. Indeed, the GLA

content of the GL fraction increased to 90.5% following the urea treatment, while a similar treatment of the total lipids resulted in only 79.7% GLA (Table 3).

The silica gel fractionation process achieved yet another goal, i.e., the separation of the zeaxanthin containing xanthophylls. which were were found in the NL fraction. Since very little GLA was present in the NL fraction and the bulk of it was restricted to the GL, the fractionation enabled the practical separation of GLA and zeaxanthin. For obtaining high purity GLA, the GL fraction can be isolated by an acetone wash. For higher yield, the last step can be replaced with a methanol wash, resulting in elution of the GL and PL fractions together which comprised 93% of cellular GLA. Extraction of the third valuable product, phycocyanin, should however take precedence in order to avoid its denaturing by contact with organic solvents. An outline for an all inclusive extraction scheme of the valuable products in *Spirulina* is described in Scheme 1.

The high cost of production of *Spirulina* and the low content of GLA prevented it so far from becoming a source of the latter. However, we believe that a concerted extraction of GLA, phycocyanin and xanthophylls, concurrent with a further decrease in the cost of production will contribute to the emergence of an economically viable process.

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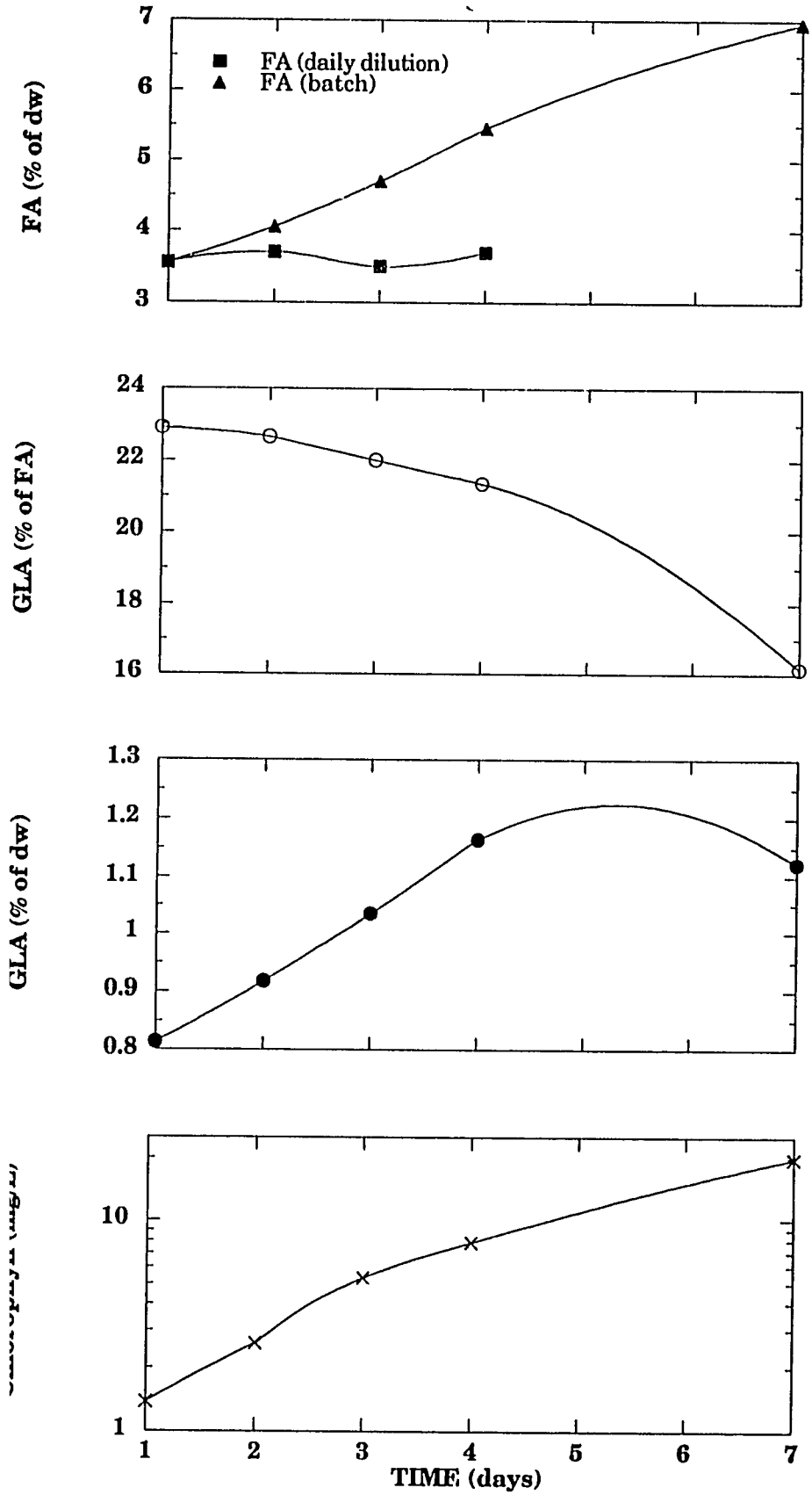
Legend:

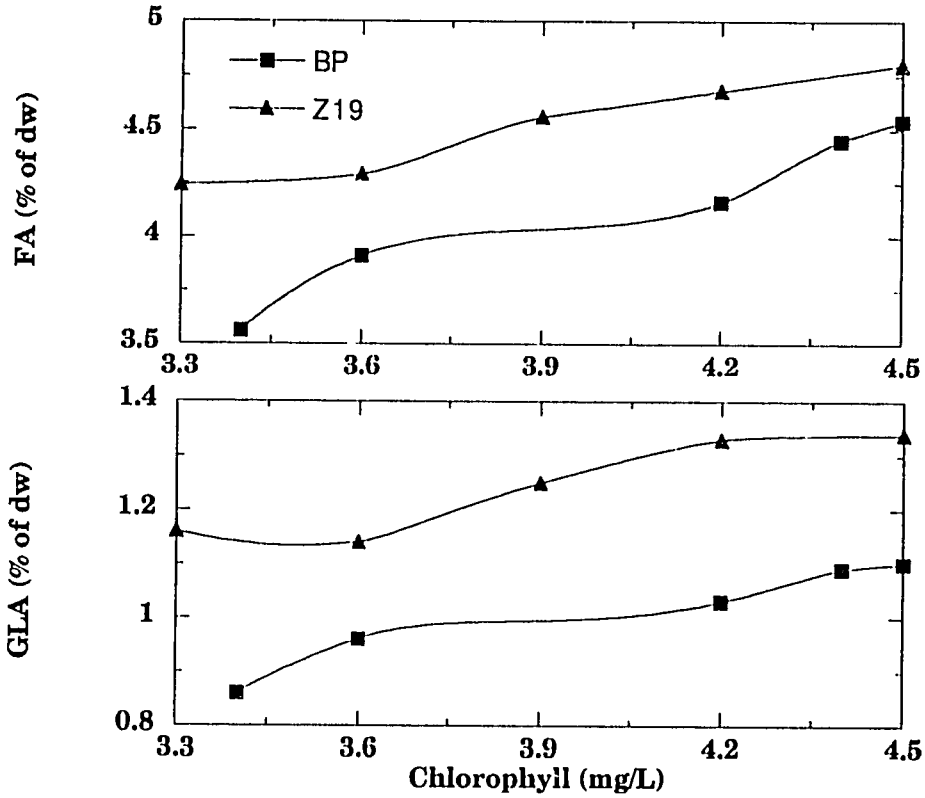
Figure 1. Effect of cell concentration on fatty acid content in *Spirulina* cultures. Fatty acid content was determined in batch cultures (□) and in cultures subjected to daily dilution (■). Cell density in batch cultures (○) is expressed as Chlorophyll concentration (mg/L). In the daily diluted culture the Chlorophyll concentration was kept at about 2 mg/L.

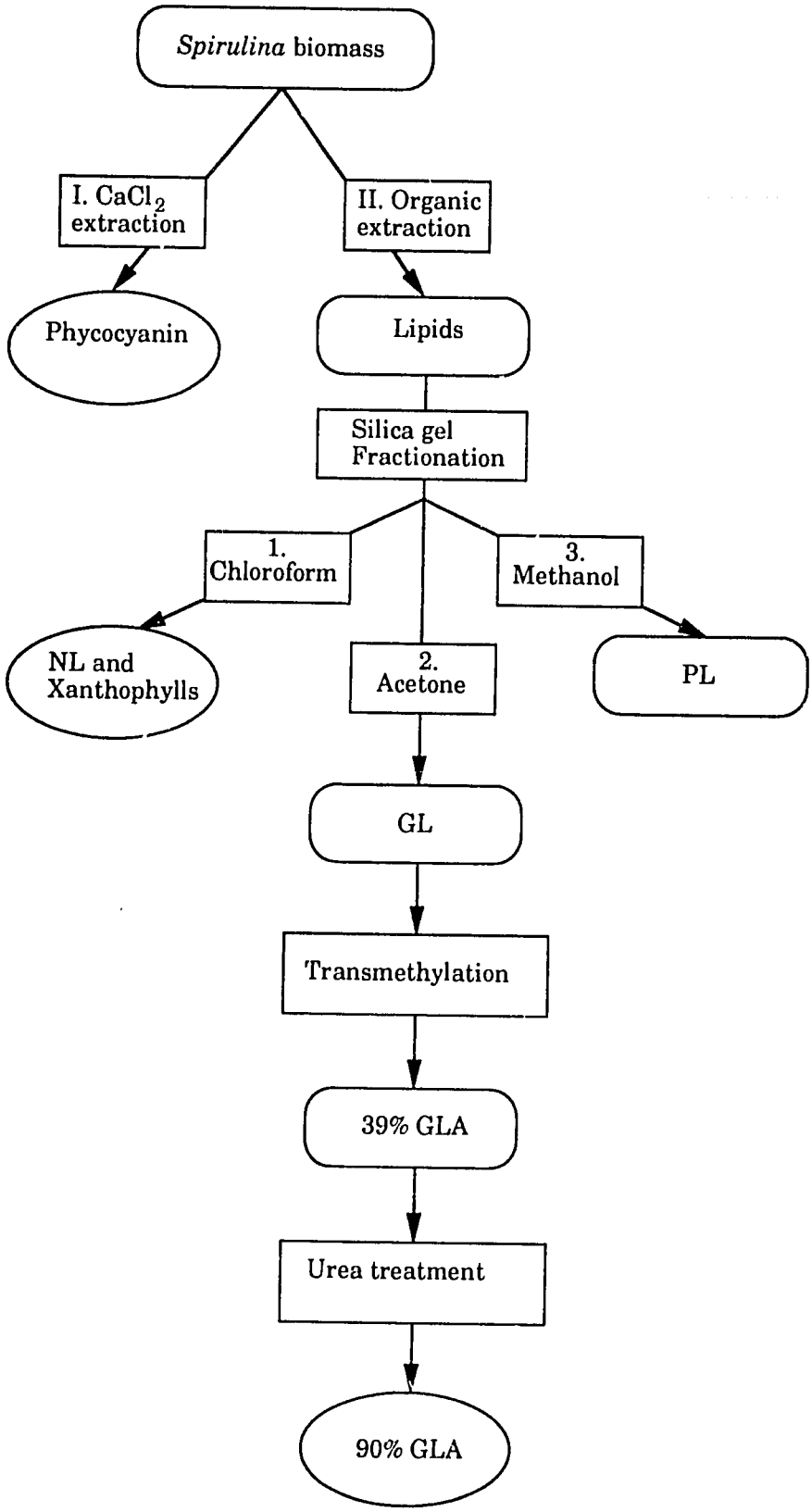
Figure 2. Fatty acid and GLA contents of daily diluted cultures of *Spirulina* BP (■) and Z19 (▲) at various chlorophyll concentrations.

Scheme 1. Outline for extraction and separation of valuable products from *Spirulina* biomass.


FA (% of dw)
FA (daily dilution)
FA (batch)
7
6
5
4
3
GLA (% of FA)
24
22
20
18
16
GLA (% of dw)
1.3
1.2
1.1
1
0.9
0.8
10
1
1
2
3
4
5
6
7
TIME (days)

135


FA (% of dw)
5
4.5
4
3.5
BP
Z19
3.3
3.6
3.9
4.2
4.5
GLA (% of dw)
1.4
1.2
1
0.8
3.3
3.6
3.9
4.2
4.5
Chlorophyll (mg/L)


Spirulina biomass
I. CaCl2 extraction
II. Organic extraction
Phycocyanin
Lipids
Silica gel Fractionation
1. Chloroform
2. Acetone
3. Methanol
NL and Xanthophylls
PL
GL
Transmethylation
39% GLA
Urea treatment
90% GLA

Schema 1

Table 1. Effect of cell concentration on fatty acids, chlorophyll and phycocyanin content in *S. platensis* BP.

Cell conc.[a]	Fatty acids (% dry wt)	GLA % of fatty acids	GLA % dry wt	Chlorophyll (% dry wt)	Phycocyanin (% dry wt)
4	3.6	24.0	0.86	0.92	10.4
8	4.2	23.3	0.98	1.02	12.0
13	4.7	22.0	1.03	1.33	18.7
19	5.2	21.0	1.09	1.44	22.4

[a] Cell concentration expressed in mg chlorophyll L^{-1}.

Table 2. Fatty acid composition of lipid fractions of *S. platensis* 2340.

Fraction	Fatty acid composition (% of total fatty acids) 14:0	16:0	16:1	16:3	18:0	18:1	18:2	18:3	%[a]
Total lipids	1.1	39.8	7.0	0.7	1.0	2.7	25.9	21.5	
Neutral lipids[b]	13.6	25.7	30.8	16.1	10.9	6.6	4.5	5.5	1.5
Galactolipids[c]	0.2	38.4	8.5	0.4	0.5	1.6	11.6	39.0	92.0
Phospholipids[d]	0.7	43.3	4.3	0.5	1.0	4.2	37.2	8.6	6.5

[a] Percent of total GLA in given fraction; [b] Fraction eluted with chloroform; [c] Fraction eluted with acetone; [d] Fraction eluted with methanol.

Table 3. GLA concentration by lipid fractionation and urea adduct formation of *S. platensis* 2340 oil.

	Fatty acid composition (% of total fatty acids)							
Fraction	14:0	16:0	16:1	16:3	18:0	18:1	18:2	18:3
Total lipid extract (TL)	1.1	39.8	7.0	0.7	1.0	2.7	25.9	21.5
Galactolipids fraction (GL)	0.2	38.4	8.5	0.4	0.5	1.6	11.6	39.0
TL after urea treatment[a]	-	0.2	0.1	1.8	-	0.2	18.0	79.7
GL after urea treatment[a]	-	0.2	0.2	1.0	-	0.1	8.0	90.5

a Fatty acids methyl esters resulting from transmethylation and urea treatment of the corresponding lipid.