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Production of Eicosapentaenoic Acid (EPA) by Microalgae

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Executive Summary

This research aimed at enhancing the production of the polyunsaturated fatty acid (PUFA) EPA in microalgae, using physiological biochemical and genetic means.

We hypothesized that using inhibitors for fatty acid desaturation it would be possible to reduce the level of EPA in the cell, resulting in a severe inhibition of growth. Indeed, such inhibitors brought about the expected results. In the next stage we induced resistance to one of theses inhibitors and in the third stage we selected inhibitor-resistant mutants, hoping that at least in some of them the resistance would evolve from EPA overproduction. Being able to produce more EPA in the presence of the inhibitor, the growth of such cells would be less affected by the inhibitor. Once again, our hypothesis proved to be right and such EPA over-producing mutants were indeed isolated. The change that caused the overproduction was permanent and the EPA overproduction was demonstrated also when cultivated in inhibitor-free medium.

Collaboration with the Thai co-investigator is minimal, most of our letters remain unanswered.

Section I

The scientific accomplishments obtained in the last reporting period were summarized and submitted as a paper to the journal Phytochemistry (attached).

Scientific Impact of Collaboration

Unfortunately, the collaboration with the Thai co-investigator is minimal (see further in Section II).

Description of Project Impact

While large strides have been made in the direction of EPA production from microalgae, it is still premature to expect any results.

Future Work

Originally, it was planned that once the concept of EPA overproduction would be proven, the task of mutant selection would be performed by the Thai co-investigator. However, due to the lack of cooperation of that lab, we will concentrate our efforts in the last year of this project in studies aimed at the understanding the biosynthesis of EPA. This will aid in future work directed at enhancement of EPA production by genetic manipulations.

Section II

Managerial Issues

As already mentioned in earlier reports, communication with the Thai co-investigator is mostly unidirectional. A personal letter received from one of the employees of this group indicate the lack of interest of the group head in all but the financial aspects of this project (the writer of the letter requested that the content of the letter or even its mere existence would not be revealed to his group leader for obvious reasons). Our last letters to him remained unanswered.

Collaboration, Travel, Training and Publications

One of the P.I.s (Z.C.) will be in Thailand this summer in an attempt to salvage as much as possible from the cooperation with the Thai group. Our lab will hold an

international training course on Algal Biotechnology. Most of its participants will be from less developed countries. The methodologies developed in this project will be taught and discussed.

The scientific part of this report was submitted to the journal Phytochemistry. Another paper published in Plant Physiology was attached to the semi-annual report.

Request of AID Actions

The help of AID or BOSTID authorities is urgently called upon in light of the above mentioned problems.

Phytochem. (in press)

HERBICIDE RESISTANT LINES OF MICROALGAE: GROWTH AND FATTY ACID COMPOSITION

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

Running title - Resistance of algal cultures to SAN 9785

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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 growth independent overproduction of lipids in general and fatty acids in particular.

In this work, we further elaborate the effect of SAN 9785 on the fatty acid composition in both the wild type and resistant cultures of these algae 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 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 (Tab. 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 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 increased from 2.7 in the wild type to 4.3 (% of dry weight), 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%) (Tab. 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 *Spirulina* BP (wild type) was reduced from 36.8 to 22.2% (Tab. 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 (Tab. 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 (Tab. 2).

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

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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% (Tab. 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% (Tab. 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 (Tab. 3).

GLA overproduction

S. platensis is a filamentous organism. Obtaining colonies derived from single cells was considered as a difficult task. Consequently, we have chosen to select colonies from single filaments. 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 (Tab. 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. Linoleic acid is further desaturated to GLA in *S. platensis* (Scheme 1) and in *P. cruentum* to α linolenic acid which is in all likelihood a precursor of EPA (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 the cyanobacterium *Spirulina*, 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.

In *P. cruentum*, the resistance was demonstrated by a higher growth rate in the presence of the inhibitor and in higher proportions of EPA. 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 while in others, e.g. *Monodus subterraneus* [10], *Chroomonas salina* and *Nannochloropsis oculata* [17], it does not. We hypothesized [18] that in *Porphyridium*, EPA is the product of elongation and further desaturation of GL bound 18:3ω3. The strong inhibitory effect of the herbicide on EPA in GL of freshly exposed cells and the overproduction of EPA in the GL of resistant cells (Tab. 4) support this hypothesis. The resistant lines will undoubtedly be of 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. [19] have shown that this herbicide causes a direct inhibition of photosynthetic electron transport. We have recently shown [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 (Tab. 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 (Tab. 2). 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

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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. S. platensis 2340 was obtained from the University of Texas Culture Collection. Strain BP was isolated from a local pond in Ban Pong, Thailand. *P. 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 a New Brunswick incubator shaker (model G25) and illuminated from above at a light intensity of 115 $\mu E \cdot m^{-2} \cdot s^{-1}$, under air-$CO_2$ (99:1) atmosphere at 25°. Cultures were grown exponentially (with proper diln) under the appropriate conditions for at least 4 days prior to the onset of the experiment. The specific growth rate was estimated by measurements of chlorophyll concentration.

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 0.4mM and the culture was cultivated as above for several months. *P. cruentum* cultures were similarly treated with an initial inhibitor concentration of 0.08mM which was gradually increased over a 9 month period to 0.16 and 0.32mM, respectively, finally reaching 0.4mM. Inhibitor resistant sublines of *S. platensis were* obtained by filament dilution. Culture suspension resistant to 0.4mM 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 0.4mM of the herbicide were obtained by screening of the resistant suspension on agar plates containing 0.4mM of the inhibitor.

Fatty Acid Analysis. Fatty acid composition and content were measured following 3 days exposure to the inhibitor. Freeze dried cells were trans-methylated with MeOH - acetyl chloride as previously described [22]. Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed on a Supelcowax 10 fused silica capillary column (30 m x 0.32 mm) at 200° (FID, injector and flame ionization detector temperatures 230°, split ratio 1:100). Peak areas were obtained using an HP 3396A integrator. Fatty acid methyl 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 internal standard. The data shown represent mean values with a range of less then 3% for major (over 10% of fatty acids) peaks and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

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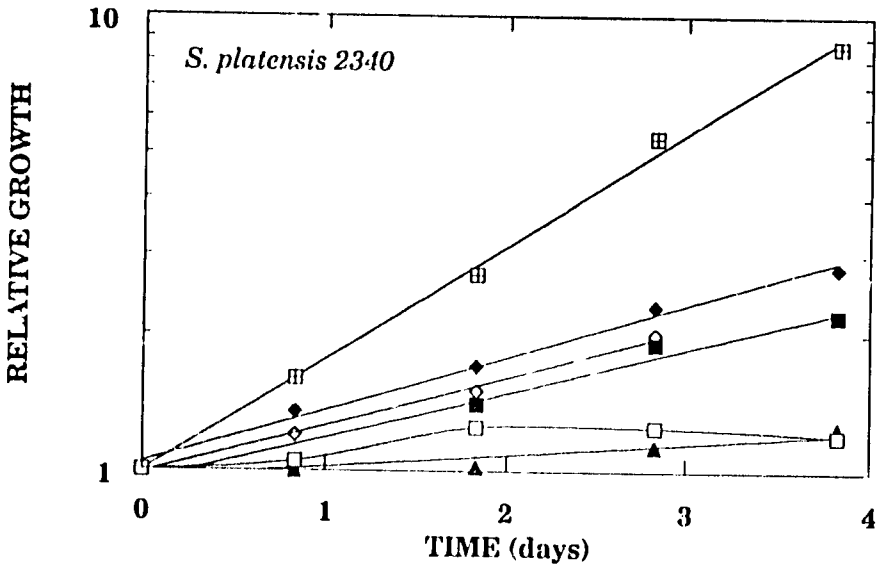
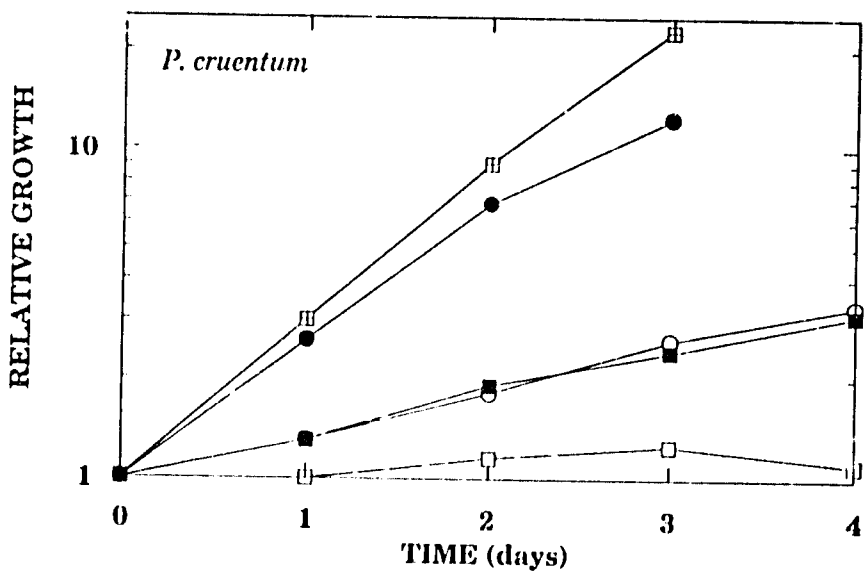
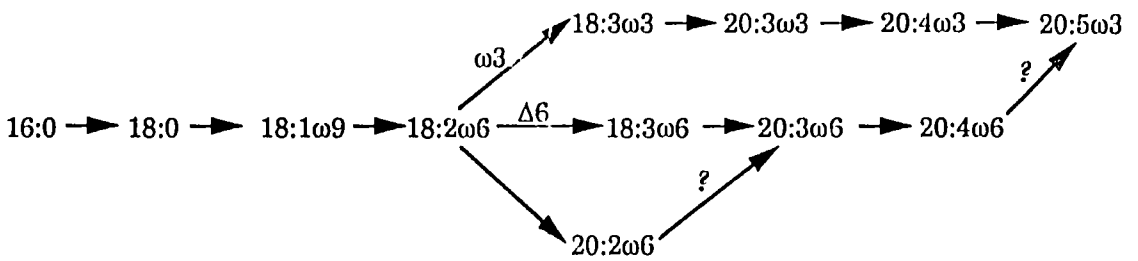
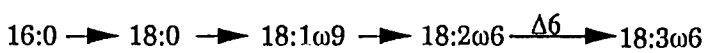
FIG. 1 Effect of SAN 9785 on the growth rate (expressed as the increase in chloropyll concentration relative to day 0) of *S. platensis* 2340 and *P. cruentum*, (▥ -control;● -0.16 mM SAN 9785; ◆ -0.2 mM; ■ -0.4 mM; ▲ -0.8 mM). Empty symbols denote wild type cultures freshly exposed to the herbicide.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


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


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



Scheme 1. Possible pathways of PUFA biosynthesis in *P. cruentum*.



Scheme 2. Suggested pathway of fatty acid biosynthesis in *Spirulina*

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	Inhibitor conc. (mM)	Fatty acid composition (% of total fatty acids)								FA content (% of dry wt)		R[b]
		14:0	16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	TFA[a]	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)[c]	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

a - Total fatty acids; b - R: (18:3 + 18:2)/18:1; c - Culture resistant to a concentration of SAN 9785 whose level is indicated in parenthesis. Fatty acid analysis were taken following 4 days exposure to the indicated concentration of the inhibitor. Traces (< 0.3%) of 16:4 were also present.

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

Lipid fraction	Strain	Medium	Fatty acid compsition (% of total fatty acids)									R[a]
			16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2 ω6	18:3 ω6	
GL	BP	-[b]	41.2	4.5	nd[c]	nd	2.1	1.6	4.0	9.8	36.8	11.7
	Z19/2[d]	-	41.9	5.2	nd	nd	nd	0.8	2.7	11.1	38.3	18.5
	BP	+[b]	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[d]	-	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

a - R: (18:3 + 18:2)/18:1; b - (-) inhibitor free medium, (+) medium containing 0.4 mM SAN 9785; c - nd: not determined; d - Z19/2 and SRS-1; cultures resistant to 0.4 mM SAN 9785 deriving from *S. platensis* strains BP and 2340, respectively.

Table 3. Effect of SAN 9785 on the fatty acid composition of wild type and SAN 9785 resistant cultures of *P. cruentum* 1380-1d.

Culture	Inhibitor conc. (mM)	Fatty acid composition (% of total fatty acids)										
		16:0	16:1	16:3	18:0	18:1	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
WT[a]	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[b]	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[c]	0	29.2	4.7	0.6	0.5	0.5	5.3	0.9	0.6	0.4	15.4	41.1

a - WT: Wild type; b - SAN 9785 resistant culture; c - Resistant line selected by agar plating of the resistant culture.

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	+	36.4	8.2	0.5	3.3	5.7	4.1	0.4	1.3	0.2	9.2	29.1
SRP[c]	+	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 mediun , + medium containing 0.4 mM SAN 9785; b - nd: not determined; c - A culture of *P. cruentum* 1380-1d resistant to 0.4 mM SAN 9785.

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Production of EPA by Microalgae

Report No.6

Effect of Temperature and Light Intensity on Production and Output Rate of Eicosapentaenoic Acid (EPA) by *Monodus subterraneus*

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Effect of Temperature and Light Intensity on Production and Output Rate of Eicosapentaenoic Acid (EPA) by *Monodus subterraneus*

Abstract

An increase in growth temperature from 23°C to 32°C resulted in a drastic reduction in EPA synthesis and a lowered biomass production by *M. subterraneus*. An approximated 70% decrease in the output rate of EPA was accordingly obtained. Similar results were observed when light intensity was increased from 75 $\mu M.m^{-2}.s^{-1}$ to 170 $\mu M.m^{-2}.s^{-1}$. The inherent limitation of EPA production by temperature and light intensity was purposely decribed as disadvantageous from a processing perspective. An alternative strategy to overcome the difficulty in achieving significant production of EPA at outdoor temperature was subsequently proposed and discussed.

Introduction

Temperature and light intensity exert pronounced effects on the composition of lipid and fatty acids in micro-organisms including algae. It has been found that psychrophilic micro-organisms with optimum temperature below 20°C contain more highly unsaturated fatty acids than mesophiles (1,2,3). Similarly, thermophiles contain negligible amount of polyunsaturated fatty acids (PUFAs). Increased synthesis of PUFAs, particularly of EPA at lower temperatures has been widely observed in most PUFAs synthesizing micro-organisms. It has been postulated (4) that because of increased solubility of oxygen at low temperatures, a greater amount of intracellular molecular oxygen was available which would be required by oxygen-dependent enzymes catalysing the desaturation of long-chain unsaturated fatty acids. On the other hand, it has been suggested (5) that the temperature-associated production of PUFAs in micro-organisms is a means of adaptation to the environment. Shinmen and his colleagues (6) found that several species of the fungus *Mortierella* produced large amounts of EPA only at low temperature (12°C). Seto et. al. (7) also observed this phenomenon in *Chlorella minutissima*. They concluded that the activities of enzymes involved in desaturation and chain elongation in the biosynthetic pathways of PUFAs might be extremely sensitive to temperature changes because at higher temperature there was an accumulation of intermediates in the biosynthetic route of EPA.

In general, low light intensity enhances formation and accumulation of PUFAs in many diatoms and euglenids, such as *Cyclotella menaghiniana* (8),

Nitzschia closterium (9), and Euglena gracilis (10). However, for the green alga, Chlorella minutissima (7) and the red alga, Porphyridium cruentum (11), the effect of light intensity was reversed.

This study was primarily intended to determine the extent by which the increase in growth temperature and light intensity from the relatively low and optimal levels to the higher outdoor conditions reduces the production and the output rate of EPA by M. subterraneus before any attempt to cultivate the alga under outdoor conditions can be made.

Materials and Methods

Organisms: M. subterraneus was obtained from Gottingen University Culture Collection and was maintained at 25°C under continuous light condition.

Growth conditions: The culture was grown for 7 days in BG-11 medium supplemented with three vitamins (B_1,B_{12}, and Biotin at 1 x 10^{-5}g/L) in glass tubes, incubated in a temperature-regulated water bath, illuminated with cool-white fluorescent lamps at the side of the water bath. The culture was mixed by bubbling an air through a glass tube connected to the bottom of each culture tube. Specific growth rate was estimated by measurement of cell concentration at OD-970 nm.

Lipid transmethylation: Freeze-dried samples of algal biomass (100 mg) was transmethylated with 2 ml of methanol/acetyl chloride (95/5) as described by Cohen et. al.(12). Heptadecanoic acid was added as internal standard, and the mixture was sealed in a light-protected Teflon-lined vial and heated at 90°C for 1 h. The vial contents were then cooled, diluted with 1 ml hexane. The hexane layer was dried over Na_2SO_4, evaporated to dryness under nitrogen atmosphere, and redissolved in hexane.

Fatty acid analysis : Gas chromatographic analysis was performed with a SP 2330 fused silica capillary column (30m x 0.2 mm) at 195°C (injector and flame ionization detector temperature 250°C, split ratio 1:100). The results were formulated with a Water 720 integrator. Fatty acid methyl esters were indentified by co-chromatography with authentic standard (Sigma). The quantity of the fatty acids was determined by comparing their peak areas with that of the internal standard. The data shown are mean values of at least two independent samples, analyzed in duplicate.

Results and Discussion

As expected, an increase in growth temperature from 23°C to 32°C led to a drastic decrease in both biomass and EPA production which further resulted in a 70% reduction in the output rate (Table 1). In concomitant with the decreased biosynthesis of EPA, the contents of other fatty acids variably increased with those of 18:1 and 20:4 acids becoming more prominent. These results once again confirm early observations that EPA production favorably occurs at low temperature.

In addition to the pronounced effect of temperature, light intensity also exerted a similar influence. An increase in light intensity from low and optimal (ca. 75 $\mu M.m^{-2}.s^{-1}$) to relatively high level (ca. 170 $\mu M.m^{-2}.s^{-1}$) resulted in a 2-fold decrease in EPA production (Table 1). Although the elevation of light intensity appeared to promote growth and subsequently led to a higher biomass production, the net productivity of EPA was still lower.

The findings of this study clearly showed the preferrence of Monodus to grow and accumulate significant amount of EPA at lower temperatures and under low irradiance. This obvious nature of Monodus is seen as extremely disadvantageous from a process productivity point of view, especially when the energy cost for temperature control is concerned.

The inherent limitation of EPA production by temperature implies a serious problem of achieving mass cultivation for EPA production at outdoor temperature in a tropical country like Thailand where average annual temperature is usually higher than 28°C.

From this perspective, it looks like that scaling-up for EPA production in Thailand may not come into reality in the coming years. Accordingly, tremendous efforts must be put towards intensive investigations aimed at overcoming the obstacles. One possible solution to the problem of low temperature requirement for EPA production has recently been suggested by a Japanese group (13). Using α-linolenic acid (ALA) from linseed oil as an exogenous precursor of EPA for the culture of the fungus Mortierella alpina, the group achieved a relatively high content of EPA in the culture grown at normal growth temperatures (up to 30°C) with no requirement of low temperature. This resulted from the ability of the fungal cells to convert added ALA to EPA via the biosynthetic pathway of n-3 family (Figure 1) which does not require low temperature. Interestingly, EPA production obtained from the addition of ALA at normal growth temperature was 2.8-fold higher than that achieved at low temperature conditions. This process, known as " induced lipogenesis " has been earlier claimed to appear in the red alga Porphyridium cruentum (11). Thus, this marvellous phenomenon may occur in all micro-organisms capable of synthesizing EPA. However, the extent of its occurrence in microalgae remains to be investigated. Further investigation, therefore, should be concentrated on the use of the induced lipogenesis to overcome the requirement for low temperature of EPA production.

As shown in Table 2, a comparison of data for optimal production of EPA among promising micro-organisms investigated thus far reveals a superiority of the fungus Mortierella alpina over other microalgal species in terms of their EPA productivity. It is reasonable to state that it is obviously impossible for

the microalgae to compete with the fungus as far as commercial production of EPA is concerned. Nevertheless, if the induced lipogenesis proved successful in microalgae which could lead to a higher EPA production comparable to that of the fungus, together with a careful manipulation of the production cost, the ultimate goal of large-scale production as well as commercialization of algae-derived EPA products might be achieved.

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Table 1: Effect of temperature and light intensity on EPA production and output rate by *Monodus subterraneus*

Temperature (°C)	Light intensity ($\mu M.m^{-2}.s^{-1}$)	Cell dry weight (g/L)	Fatty acid production (% of total fatty acids)								Total fatty acid production		EPA production		EPA Productivity (mg/L/day)
			14:0	16:0 (w-11)	16:1 (w-11)	18:1	18:2	20:4	20:5	others	mg/g of cell dry wt.	mg/L of culture broth	mg/g of cell dry wt.	mg/L of culture broth	
23	75	0.91	3.23	20.16	25.95	5.83	2.97	5.33	34.65	1.88	131.6	119.8	45.6	41.5	6.0
	170	1.20	3.55	21.13	24.44	6.57	3.07	6.75	34.01	0.48	100.5	120.6	25.7	30 8	4.4
28	75	0.76	3.94	21.85	23.17	6.81	3.14	6.12	32.61	2.36	75.7	57.5	34.0	25.8	3.7
	170	0.84	3.79	22.07	22.98	6.77	3.45	7.04	31.48	2.42	71.9	60.4	18.1	15.2	2.2
32	75	0.47	4.43	22.46	22.65	7.10	3.58	7.80	30.80	1.18	52.3	24.6	26.2	12.3	1.8
	170	0.66	5.58	23.06	22.25	8.21	3.63	10.40	25.46	1.41	50.2	33.1	10.3	6.8	1.0

Table 2: Comparison of EPA production and output rate of the cultures of *Monodus subterraneus*, *Chlorella minutissima*, and the fungus *Mortierella alpina*

Cultures of	Culture Temperature (°C)	Culture Days	Dry Cell Concentration (g/liter)	Total fatty acids (mg/g of cell dry weight)	EPA Content (% of total fatty acids)	EPA Yield (mg/liter of culture broth)	EPA Productivity (mg/liter/day)
Monodus subterraneus	23	7	0.9	131.6	34.7	41.5	6.0
Chlorella minutissima (7)	19	4	0.38	79.7	39.9	12.05	3.01
Mortierella alpina (13)	28	10	32.5	-	13.5	1350	135.0

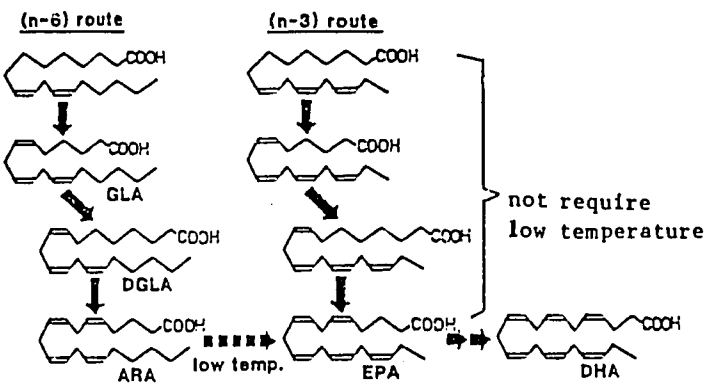

Figure 1: Proposed pathways for the biosynthesis of PUFAs of the n-6 (linoleic) and n-3 (alpha-linolenic) families (13)