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Optimization of γ-linolenic acid (GLA) production in *Spirulina platensis*

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Abstract

The cyanobacterium *Spirulina platensis* is one of the most promising sources of the polyunsaturated fatty acid γ-linolenic acid (GLA). The GLA content of *Spirulina* can be enhanced by cultivation under light–dark cycles in the laboratory or outdoors. Thus, in strain BP, the GLA content increased from 1.2 to 1.6% when cultivated under light–dark cycles. Moreover, in the derived mutant Z19, the GLA content reached 2.4% when cultivated outdoors. To the best of our knowledge, this is the highest GLA content ever reported for any alga.

Introduction

The polyunsaturated fatty acid (PUFA) γ-linolenic acid (GLA, 18:3ω6) was shown to be of potential value for lowering low density lipoproteins in hypocholesterolemic patients (Ishikawa *et al.*, 1989), for alleviating the symptoms of pre-menstrual syndrome (Horrobin, 1983), and for treatment of atopic eczema (Biagi *et al.*, 1988). The main commercial sources for GLA are oils of evening primrose and black currant. The GLA content of the former source is only 8–12% of total fatty acids, while the latter contains the undesired and relatively difficult to separate 18:4 acid. Production of GLA using the fungus *Mortierella* was suggested by Suzuki and Toshihiro (1985); however, the technology for large-scale cultivation of these fungi is not yet available. Furthermore, the GLA proportion of the total fatty acids of *Mortierella* is less than 15% (Nakahara *et al.*, 1992). While GLA containing oils are considered as a health food, it is expected that future pharmaceutical applications of GLA will require preparations of considerably higher concentration of this fatty acid.

Nichols and Wood (1968) showed the existence of GLA in the cyanobacterium *Spirulina*, and that it is mainly concentrated in galactolipids (GLs). Later works claimed that the proportion of GLA in the fatty acids of *Spirulina* is rather low (Hudson & Karis, 1974), or that 18:3ω3 rather than GLA is the main PUFA in this alga (Kenyon & Stanier, 1970). Ciferri (1983) was the first to suggest that *Spirulina* can be used as a source of PUFAs, and especially of GLA and it is now clear that *Spirulina* is the richest algal source of GLA. Cohen *et al.*, (1987) evaluated the fatty acid composition of 19 different *Spirulina* strains and studied the effect of temperature, light intensity and growth phase on fatty acid and GLA content. This study showed that the GLA content of *Spirulina* can reach 31.7% of fatty acids and 1.4% of biomass (dry weight). Hirano *et al.* (1990) have shown that a 50% enhancement of the cellular GLA content was achieved by keeping *Spirulina* cultures in the dark for a week. Cohen *et al.* (1993a) demonstrated that the GLA content in the biomass can be enhanced by keeping a higher concentration of biomass, and that GLA of over 90% purity could be obtained via isolation of the galactolipids (GLs) and further separation of the saturated and monounsaturated fatty acids by urea complexes formation. Recently, Cohen *et al.* have shown

(1992, 1993b) that the herbicide SAN 9785 inhibited the biosynthesis of GLA in *Spirulina*, and that some of the mutants resistant to this herbicide are GLA-overproducers.

In this work, we further report on the application of cultivation regimes and culture conditions for continued enhancement of the GLA content of *Spirulina*.

Materials and methods

Cyanobacteria and cultivation

Spirulina platensis LB 2340 was obtained from the University of Texas Culture Collection. Strain BP was isolated from a local pond in Ban Pong, Thailand. Strain P4P is a NaCl resistant mutant isolated from a culture of BP. Strains of the Z series are mutants resistant to the herbicide SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) Pyridazinone) (Cohen *et al.*, 1993b). Cultures 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). The growth rate was estimated by measuring the increase in total chlorophyll content.

Analytical and chemical methods

Lipids were transmethylated by treatment of the freeze-dried biomass with methanol-acetyl chloride. The resulting fatty acid methyl esters were analyzed by gas chromatography, as previously described (Cohen & Cohen, 1991).

Results and discussion

Effect of growth phase

Cultures of *Spirulina* strain BP and two of its derived mutants, P4P and Z6, were cultivated in a batch mode. In keeping with previous findings (Cohen, 1993a), the fatty acid content of the biomass increased concurrently with the increase in cell concentration. Thus, in strain BP the content increased from 2.6 (% of dry weight) to 6.3% after 4 days (Table 1). Similar increases, yet of lower magnitude, were noted in the P4P and Z6 mutants, whose initial fatty acid contents were much higher than in strain BP. The major effect noted on fatty acid composition was in a continuous decrease in the level of desaturation of C_{18} fatty acids. In strain BP, the proportion of GLA and 18:2 decreased from 24.5 (% fatty acids) and 19%, respectively in the early log phase to 20.4 and 10.4%, respectively, in the stationary phase. Concurrently, the proportions of 18:0 and 18:1, increased from 1.0 and 5.4% to 3.8 and 30.5%, respectively. The level of 16:0, which was stable at 48–50% during the early logarithmic phase, declined in the late logarithmic phase and even further so in the stationary phase, to finally reach 23.9%. The fatty acid compositions of the two mutants P4P and Z6 were less affected by changes in growth phase; the level of GLA was stable and didn't change significantly.

Alterations in fatty acid composition at the stationary phase can be best explained by a shift in the fatty acid flux from production of polar to neutral lipids (Cohen *et al.*, 1993a). Polar lipids are membrane components with an active role in cell function. Thus, under conditions resulting in intensive growth, the proportion of polar lipids and primarily that of GLs can be expected to reach its maximum. In the stationary phase, however, accumulation of neutral lipids may take place, perhaps as reserve material. While GLA and 18:2 are primarily present in GLs and phospholipids (PLs), respectively, 18:0 and 18:1 are mainly found in neutral lipids (Cohen *et al.*, 1993a). Also, in cyanobacteria, position 2 of the GL glycerol backbone is predominantly occupied by 16:0 (Murata & Isihida, 1987). Therefore, a shift in production of GLs to formation of neutral lipids will be manifested by an increase in 18:1 and 18:0 and a decrease in 16:0, 18:2 and GLA.

The increase in fatty acid content during the growth cycle was more intense than the comparatively moderate decrease in the proportion of GLA. This resulted in a gradual increase in the GLA content of *Spirulina* BP from 0.6% (of dry weight) at the early log phase to 1.2–1.3 in the log phase. This was followed by a decrease to a level of 1.0% at the stationary phase (Table 1). While the GLA content showed a smaller increase in P4P and Z6, these mutants achieved a higher value of 1.4% (Table 1).

Light-dark cycles

Cultures of *Spirulina* BP and derived mutants were batch cultivated under either continuous light or light–dark (L–D, 12 h/12 h) cycles. On transfer to a L–D cycle the cultures displayed a significant increase in C_{18} desaturation. When cultures of similar biomass concentration were compared (Table 2), the propor-

Table 1. Fatty acid content and distribution profile of *Spirulina* strains at various growth phases (32 °C)

Strain	Day	Growth phase[a]	Fatty acid composition (% total fatty acids)						FA[b] content (% dry wt)	
			16:0	16:1	18:0	18:1	18:2	GLA	TFA[c]	GLA
BP	0.8	EL	48.2	0.7	1.0	5.4	19.0	24.5	2.6	0.6
BP	2	L	50.1	0.8	0.9	7.8	17.0	23.4	4.6	1.1
BP	3	L	49.5	0.9	1.1	8.9	14.6	23.6	5.1	1.2
BP	4	L	49.8	0.8	1.1	12.7	12.7	21.1	6.3	1.3
BP	6	LL	43.2	4.3	0.9	11.3	12.4	20.7	5.7	1.2
BP	14	S	23.9	2.3	3.8	30.5	10.4	20.4	5.1	1.0
P4P	1	EL	48.4	3.1	1.2	11.3	13.0	20.1	5.4	1.1
P4P	2	L	48.8	3.7	0.7	7.7	14.3	22.2	5.4	1.2
P4P	4	L	49.1	3.8	0.7	8.9	14.0	21.3	5.8	1.2
P4P	8	LL	47.2	4.7	0.7	8.9	13.2	22.6	6.4	1.4
P4P	12	S	29.9	2.6	3.0	28.5	10.7	22.7	5.3	1.1
Z6	1	EL	47.7	4.4	1.0	7.6	14.6	22.4	5.3	1.2
Z6	2	L	46.8	4.3	1.5	6.6	14.7	22.4	5.7	1.3
Z6	4	L	46.3	4.2	0.8	8.4	14.8	22.6	6.2	1.4
Z6	8	LL	47.4	4.4	0.8	7.3	14.8	22.3	6.3	1.4
Z6	12	S	31.2	3.3	2.6	22.3	12.1	25.5	5.1	1.3

[a] EL = Early log phase; L = log; LL = Late log phase; S = Stationary phase.
[b] Fatty acids.
[c] Total fatty acids.

tions of 18:3 and 18:2 in the cycled cultures were higher while that of 18:1 was much lower. In the wild type culture, BP, the proportions of 18:3 and 18:2 under light–dark conditions, reached 24.7 and 16.6%, respectively as compared with only 20.7 and 12.4%, respectively under continuous light, while that of 18:1 was as low as 1.2 in comparison with 11.3% under continuous light. Similar results were obtained with 10 other mutants of strain BP (data pertaining to 4 mutants is shown in Table 2).

During the dark period, some of the biomass is consumed by respiration (Vonshak, 1986) and this apparently consists of reserve materials such as carbohydrates and neutral lipids. A decrease in the former will result in a relative increase in the fatty acid content, while a decrease in the latter will reduce the proportion of typical triacylglycerol fatty acids such as 16:1 and 18:1, while increasing the share of the major GL fatty acids, i.e., 18:3, 16:0 and 18:2. Indeed, by cultivation under L–D cycles the contribution of TGs in the alga *Nannochloropsis oculata* was shown to decrease at the beginning of the light period (Sukenik & Carmeli, 1990). Furthermore, Hirano *et al.* (1990) have shown that by keeping *Spirulina* cultures in the dark for a week, preferential consumption of sugars over fatty acids during the dark period resulted in a relative increase in the fatty acid content including that of GLA by about 50%. Thus the extent at which net production of fatty acids or GLA occurs, is not clear.

It appears that cultivation under light–dark conditions which, currently, is the only practical means for outdoor cultivation, is preferential to cultivation under continuous light. Moreover, it is conceivable that highest GLA contents will be obtained by harvesting at the end of the dark period.

Outdoor cultivation

The fatty acid composition of cultures grown indoors and outdoors was compared. The major difference was noted in the total fatty acid content, which in mutant Z19 reached 8.2 as compared with 5.9% under laboratory conditions. The GLA content similarly increased from 1.7 to 2.4% (Table 3). The latter value represents

Table 2. Comparison of fatty acid composition and GLA content in various *Spirulina* mutants cultivated under continuous light or light-dark conditions.

Mutant	Light regime	Fatty acid composition (% total fatty acids)						Fatty acid content (% dry wt.)	
		16:0	16:1	18:0	18:1	18:2 ω6	18:3 ω6	TFA*	GLA
BP	CL†	43.2	4.3	0.9	11.3	12.4	20.7	5.7	1.18
BP	L-D♦	43.8	3.0	1.1	1.2	16.6	24.7	6.1	1.62
Z5	CL	44.2	3.4	0.9	9.1	13.6	21.3	4.6	0.99
Z5	L-D	46.9	3.6	0.9	3.4	17.6	24.6	6.0	1.47
Z12	CL	47.6	3.3	1.1	10.1	14.2	22.2	4.9	1.08
Z12	L-D	45.1	3.9	0.7	2.6	19.3	27.2	5.9	1.60
Z14	CL	46.9	4.5	1.2	10.7	13.2	20.3	5.3	1.10
Z14	L-D	47.0	3.9	0.9	3.2	17.5	24.8	5.8	1.50
Z17	CL	49.3	3.4	1.0	10.0	13.3	20.3	5.1	1.03
Z17	L-D	48.5	3.6	0.7	2.3	18.3	24.0	6.2	1.49

† Continuous light. ♦ Light-dark (12h/12h) cycles.
Compared cultures were of similar biomass concentration.

the highest GLA content ever reported for *Spirulina* or any other algal species. Similar results were obtained in several other *Spirulina* mutants as well as in the wild type strain BP (Table 3). Only small differences were observed in the fatty acid composition. Thus, the level of 18:1 and 16:1 increased while that of 16:0 and 18:2 decreased (data not shown). As mentioned above, these changes apparently reflect some increase in the share of NL associated with increased fatty acid content.

In conclusion, we have shown that the fatty acid composition and content of *Spirulina* can be manipulated so as to increase the GLA proportion as well as its content in the biomass. Thus, the GLA content of BP cells maintained at a low biomass concentration was only 0.85% (Table 4). Selection of GLA-overproducing strains yielded the mutant Z19, whose GLA content under similar conditions was 1.15%. Cultivation of this mutant at high biomass concentration resulted in a GLA content of 1.35% which further increased to 1.7% by cultivation under L–D cycles. Finally, by outdoor cultivation the GLA content was enhanced to 2.4%. This value is, to the best of our knowledge, the highest GLA content ever reported for any alga.

Table 3. Comparison of fatty acid and GLA content under indoor and outdoor conditions in various *Spirulina* mutants.

Mutant	GLA (% TFA)		TFA (% dry wt)		GLA (% dry wt)	
	I†	O♦	I	O	I	O
BP (wt)	22.9[a]	28.2	5.4	5.8	1.3	1.6
P4P	22.2[a]	25.8	4.8	6.1	1.1	1.6
Z1	21.9[a]	24.4	5.2	5.4	1.1	1.3
Z18	22.5[a]	25.3	4.1	5.4	0.92	1.4
Z19	28.8[b]	29.2	5.9	8.2	1.7	2.4

† Indoors. ♦ Outdoors. [a] Continuous light, high cell concentration. [b] L-D cycles.

Economic evaluation

Roughan (1989) analyzed the cost effectiveness of *Spirulina* as a source of GLA, and reached the conclusion that GLA from *Spirulina* would be 4–6 times more expensive than GLA from evening primrose. However, it appears that the samples used for that study were far from being characteristic of *Spirulina* biomass. Both their fatty acid content and GLA proportion were low,

Table 4. Increasing the GLA content in *Spirulina* via enviromental changes and selection of mutants.

Strain	Growth conditions	GLA (% TFA)	TFA (% dry wt)	GLA (% dry wt)
BP (wild type)	Low cell conc.	23.6	3.6	0.85
Z19†	Low cell conc.	27.4	4.2	1.15
Z19	High cell conc.	28.1	4.8	1.35
Z19	L-D cycle♦	28.8	5.9	1.70
Z19	Outdoor culture	29.2	8.2	2.40

† SAN 9785-resistant mutant. ♦ Light-dark (12h/12h) cycle.

with the highest GLA content found being 0.6%. In comparison, all but one of the 19 *Spirulina* strains studied by Cohen *et al.*, (1987) registered higher GLA contents of up to 1.4%. Several studies (Cohen *et al.*, 1987; Cohen *et al.*, 1993a; Hirano *et al.*, 1990) have shown that the composition, as well as the content of fatty acids in *Spirulina*, can be manipulated so as to significantly enhance the GLA content. In this work we have further increased the GLA content to a level of 2.4%. Basing the conclusion that *Spirulina* cannot be an economic source of GLA on the analysis of randomly chosen samples, none of which were optimized for GLA production, is therefore groundless.

The occurrence of several other valuable chemicals in *Spirulina* further increases the economic competitiveness of GLA from this biomass. The blue pigment phycocyanin has already been commercialized in Japan as a food pigment, while the xanthophylic pigment zeaxanthin, was shown to increase fish and shrimp pigmentation (Mori *et al.*, 1987). *Spirulina* biomass cultivated on tapioca starch processing effluents was recently produced in Thailand and used as a source of pigments for fish and shrimp (Tanticharoen, 1993). The ability to grow the biomass on the tapioca effluents resulted in a reduction of the cost of production of this biomass down to 6–8 $/Kg.

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