

CULTURE CONDITIONS STIMULATING HIGH γ -LINOLENIC ACID ACCUMULATION BY *SPIRULINA PLATENSIS*

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ABSTRACT

Gamma-linolenic acid (GLA) production by *Spirulina platensis* under different stress-inducing conditions was studied. Submerged culture studies showed that low temperature (25°C), strong light intensity (6 klux) and primrose oil supplement (0.8%w/v) induced 13.2 mg/g, 14.6 mg/g and 13.5 mg linolenic acid per gram dry cell weight respectively. A careful observation of fatty acid profile of the cyanobacteria shows that, oleic acid and linoleic acid, in experiments with varying growth temperature and oil supplements respectively, helped in accumulating excess γ -linolenic acid. In addition, cultures grown at increasing light regimes maintained the γ -linolenic acid to the total fatty acid ratio (GLA/TFA) constant, despite any change in γ -linolenic acid content of the cyanobacteria.

Key words: Gamma linolenic acid, Light intensity, Oil supplementation, Temperature.

INTRODUCTION

Production of oils from microalgae is an active area of research during recent years (13). The Fatty acid, Gamma-linolenic acid (GLA) is a precursor for prostaglandin E1, an important biologically-active compound necessary for reducing inflammation and blood pressure. Further, GLA is also known for treating various conditions including rheumatoid arthritis (21), eczema (22) and premenstrual syndrome (20). This fatty acid is found primarily in plant-based oils. Nevertheless, owing to their larger area of requirement for cultivation and unpredictable yields, alternative microbial sources have been targeted (5). Several species of fungi (mucor) are known to produce GLA (3,18). However, due to the presence of other polyunsaturated fatty acids in fungal oils, extensive and laborious purification steps are required before arriving at pure fatty acid. In addition, the cost factors behind production of γ -linolenic acid from fungal source highlight the need for developing alternative routes.

An alternative promising source for GLA production is *Spirulina platensis* (4,6). Unlike fungi, which should be grown

in sophisticated bioreactors, the cyanobacteria can be grown in large open ponds (2,17). The high operating alkaline pH controls the growth of other unwanted microorganisms (9). Added to the advantage, the isomer of GLA (α -linolenic acid) is found to be absent in total fatty acids of the cyanobacterium. The absence of the fatty acid isomer makes purification simple when GLA is the fatty acid of interest.

The blue green alga is capable of accumulating 1% of GLA in the dry cell mass. Under specific environmental conditions *viz.*, high light intensity and low temperature, the GLA-to-total fatty acid ratio (GLA/TFA) could be increased up to 31.7% (7).

Although Harris and James (10) reported the low temperature effect on the fatty acid biosynthesis in plants, information on the role of temperature (10,12) and light intensity (11) on GLA accumulation in the microalga was limited. In addition only a few studies were conducted to evaluate the effect of supplemented oil in the medium on the GLA content of the cyanobacteria. The present work aims to investigate different culture conditions that stimulate the cyanobacteria to produce excess GLA. Also, the work, suggests, oil supplements such as primrose oil as a possible stimulant for excess GLA accumulation by the cyanobacterium.

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MATERIALS AND METHODS

Microorganism and Culture Conditions

Spirulina platensis ARM-740 was obtained from CFTRI, Mysore, India. Medium used for cultivation of cyanobacteria was SOT medium (14) containing (g/l) of NaHCO₃ - 16.8, K₂HPO₄ - 0.5, NaNO₃ - 2.5, K₂SO₄ - 1.0, NaCl - 1.0, MgSO₄·7H₂O - 0.2, CaCl₂·2H₂O - 0.04, FeSO₄·7H₂O - 0.01, ethylene di-amine tetra acetate 0.08 and A-5 trace metal solution 1.0 ml. The A-5 trace metal solution contained (g/l) H₃BO₃ - 2.36, MnCl₂·4H₂O - 1.81, ZnSO₄·7H₂O - 0.222, Na₂MoO₄·2H₂O - 0.039, CuSO₄·5H₂O - 0.079 and Co(NO₃)₂·6H₂O - 0.49. Experiments on the effect of temperature and oil supplements were performed under light intensity of 1.5 klux. Likewise, experiments on the effect of light intensities were carried at different light intensities ranging from 2-6 klux. Light intensities (i.e., 2, 4 and 6 klux) were accomplished by adjusting the light source (4 Nos. Philips 18W light bulbs) near the cultures. All the experiments were carried out at an initial medium pH 9.2 and a growth temperature 33±1°C unless otherwise stated.

Oil Sources

Refined oils of Sunflower and Peanut were obtained from Godrej Industries and are used for Fatty acid analysis. Evening Primrose oil (Primosa) was procured as a 100 mg capsule and was used in oil supplementation experiments.

Estimation of Biomass

In all the experiments, biomass was reported in terms of cell concentration, which was calculated using dry cell weight. For estimation of dry cell weight, duplicate samples of cyanobacterial cell suspensions were filtered through pre-weighed microfibre filters (Whatman GF/C), washed with 0.5N HCl, dried in an oven at 105°C for 4h and its final weight recorded.

Lipid Extraction and Transmethylation

Extraction and lipid trans-methylation (1,7) were carried out on freeze dried samples of *Spirulina platensis* (100 mg) in a light protected Teflon-lined vial and were further analyzed for fatty acid methylation.

Fatty Acid Methyl Ester (FAME) Analysis

Gas Chromatography (Chemito 8510 HR) analysis was done on the extracted fatty acid methyl esters by using an EGSS-X column (14). Components of fatty acid esters were identified by comparing the retention times of authentic fatty acid standards (Sigma & Co). Quantification of the fatty acids was done using internal standard method with heptadecanoic acid as internal standard. The total fatty acid content was quantified based on the sum of percentages of the evaluated individual fatty acids. The data shown for cell concentration and fatty acid content were mean values of at least two independent samples analyzed in duplicate.

RESULTS AND DISCUSSION

Effect of Temperature

Low temperature plays a key role in increasing un-saturation in the fatty acids of the cyanobacteria. Physical and biochemical characteristics of lipid membrane depend on the un-saturation level of the fatty acids (15). Increase in un-saturation can probably be a pre-cautionary measure adopted by microalgae to increase membrane fluidity of the cell (16). The effect of temperature on cyanobacterial fatty acid production was shown in Fig. 1, depicting the GLA content to be increasing with decreasing temperature. The cyanobacteria showed highest GLA (13.2mg/g dry biomass) at 25°C as against 10.8 mg/g at 33°C. Although GLA content was high at 25°C, the GLA on volume basis was relatively more at 33°C because of higher biomass. Fig. 2 shows the variation of fatty acid composition of the cyanobacteria with temperature. GLA and linoleic acid showed a progressive decrease in fatty acid composition with increase in temperature, while oleic and palmitic acids showed increasing trend with increase in temperature. Clearly, a distinct inverse relationship was established between oleic acid and GLA. The results showed that oleic is major precursor among unsaturated fatty acids for GLA accumulation; GLA increased predominantly at the expense of oleic acid.

Effect of Light Intensity

Higher light intensities not only increase biomass, but also stimulate cyanobacteria to accumulate larger amount of unsaturated fatty acids. Earlier studies (8) have shown that

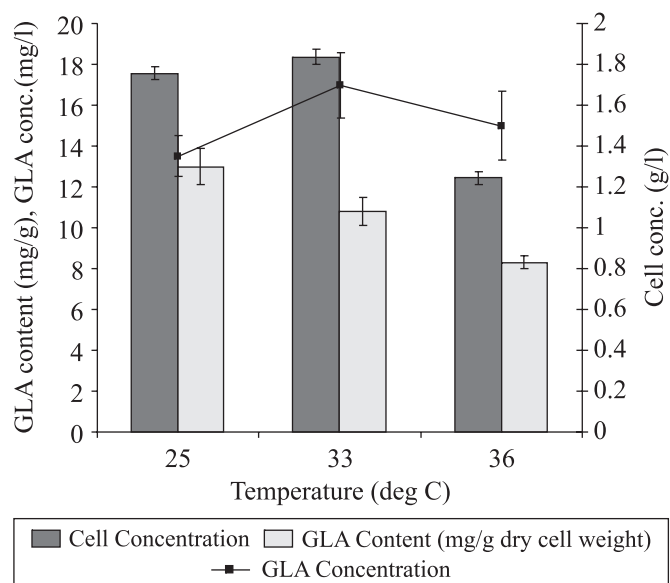


Figure 1. Effect of growth temperature on GLA production by *S. platensis*.

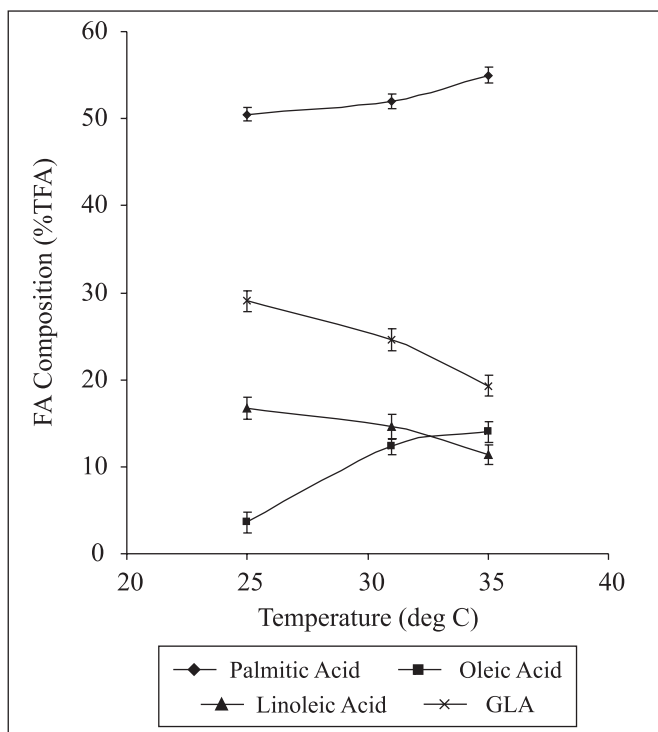


Figure 2. Effect of temperature on fatty acid (FA) profile of *S. platensis*.

light intensities alter the lipid composition in photosynthetic organisms. Light treatments on the expression level of the four desaturase genes of *Synechocystis* PCC 6803 (15) proved that light intensities show effect on expression level of desaturase genes which control production of unsaturated fatty acids.

In the present work different light intensities, 2, 4 and 6 klux, were considered for studying their effect on GLA production. Fig. 3 shows the effect of light intensity on algal growth (biomass) and GLA production. Although light at 2 and 4 klux stimulated algal growth, only a slight variation in GLA content was observed at these medium light regimes, which is equivalent to the optimal GLA content (10 mg/g) of the cultures being grown under normal day light conditions. On the other hand, cultures exposed to higher light intensity (6 klux) showed a slight reduction in biomass with concomitant increase in GLA content (14.6 mg/g dry cell weight). The reduction in cell concentration can be attributed to the photo-oxidation of the pigments around this light intensity (6 klux). Within the range of tested light intensities, GLA concentration increased linearly. The increase in GLA concentration at higher light intensity is largely influenced by GLA content (w/w) rather than biomass. At all light intensities, the GLA/TFA ratio was constant despite any change in GLA content. It can be hypothesized that the

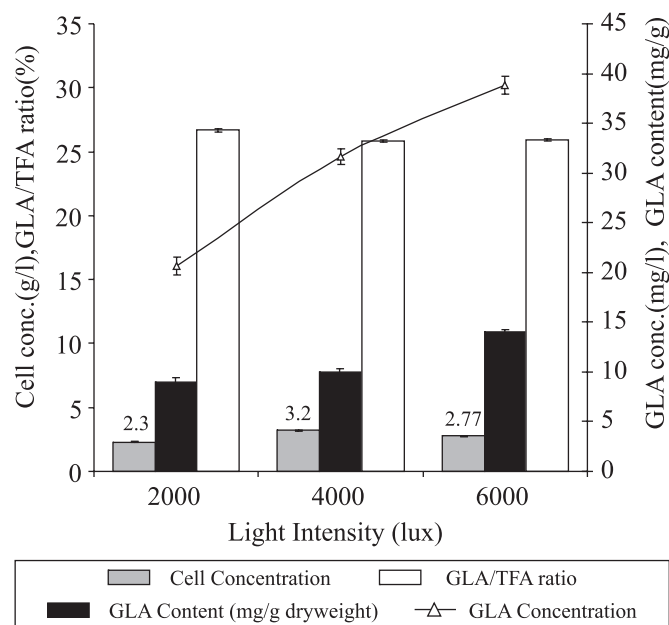


Figure 3. Effect of light intensity on cell concentration, GLA production and GLA/TFA ratio.

alga tries to accumulate more total fatty acids than normal at high light intensities to balance GLA/TFA ratio, which is essential for maintenance of membrane fluidity of the cell. However, restrictions apply on light induced increase in GLA as higher light intensities beyond 6 klux would result in heavy loss in biomass and thus GLA due to permanent damage caused to the cells as a result of photo-oxidation of pigments. Light-dependent changes in fatty acid composition are probably due to the association of activity of photosystems with processes of production and desaturation of fatty acids.

Effect of Oil Supplement on GLA Production

Supplementation of algal growth media with intermediate fatty acids in the form of oil precursors and their subsequent conversion to desired long chain fatty acids offers a better approach for studying their effect on desired fatty acid biosynthesis than having to test the effect of conventional carbon sources on synthesis of fatty acids *de novo*. As linoleic acid can be converted to γ -linolenic acid, the uptake of the oil and subsequent conversion of linoleic acid to GLA can be tested. Different plant oils having high linoleic content, *viz.*, peanut, sunflower and primrose oil were analyzed using gas chromatography (Table 1). The results showed that primrose oil has maximum linoleic acid (82.7% of TFA) with little GLA (3.4% of TFA). The low GLA content supports the assumption that during microalgal cultivation in medium with primrose any increase in GLA in the cyanobacterial biomass beyond normal

Table 1. Gas Chromatography analysis of different oils.

% Fatty acid in Total fatty acids	Sunflower	Peanut	Evening Primrose
Palmitic(C16:0)	7.0±0.6	15.5±0.9	6.90±0.6
Stearic acid(18:0)	0.2±0.8	2.8±0.6	1.36±0.3
Oleic acid(C18:1)	33.0±1.1	45.5±1.3	5.54±0.8
Linoleic(C18:2)	59.0±1.4	36.0±0.6	*82.70±0.4
GLA(C18:3)	-	-	3.40±0.3
% Total unsaturation	92.0	81.5	91.64

* Linoleic acid as major fatty acid in primrose oil; (-) Undetected.

was not a possible cross contamination of the oil with the biomass. Also during fatty acid analysis, a supplementary precaution was taken by washing biomass with alcohol and hexane before freeze-drying, to avoid cross contamination of GLA in biomass with external GLA if present in the oil.

With the best suited oil of Primrose as a source for linoleic acid (C18: 2), a series of experiments was conducted to observe its effect on algal growth and fatty acid production.

As shown in Fig. 4a cyanobacteria grown in primrose oil at 0.8%(w/v) accumulated maximum GLA (13.5 mg/g dry cell

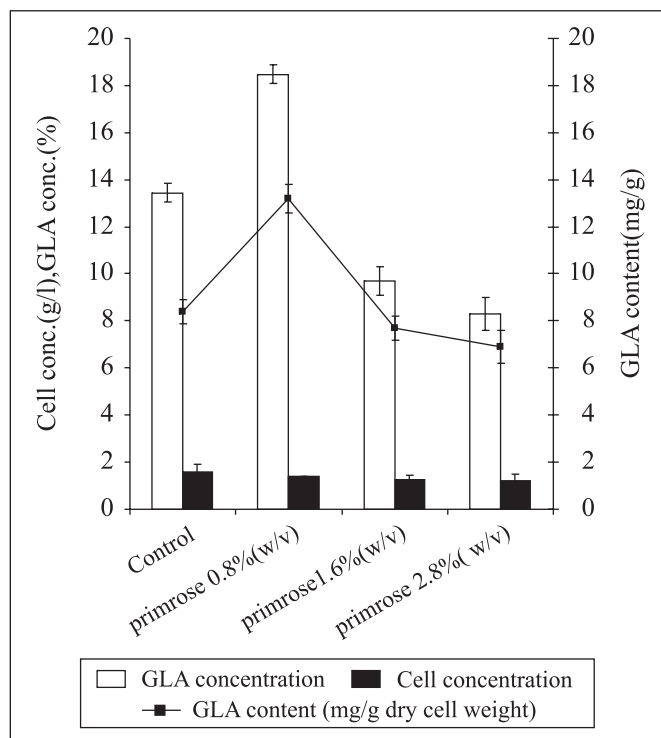


Figure 4a. GLA production in various supplemented concentrations of primrose oil in submerged cultures.

weight) when compared to either the control or other oil-supplemented cultures. Although, growth was hardly affected in all the oil-grown cultures, which was reflected in their final cell concentrations, GLA production (on volume basis) increased in the culture containing 0.8% (w/v) primrose at the cost of increased GLA content (GLA per unit dry biomass). The decline in GLA content at higher oil concentration can be attributed to the increased porosity and subsequent cell lysis due to extraction of membrane proteins. However, in case of 0.8 % (w/v) primrose supplemented culture, the rise in GLA content can be attributed to the controlled intake of oil supplement and subsequent utilization of precursor component (linoleic acid) for GLA synthesis. The fatty acid profiles (Fig. 4b) of the cyanobacteria grown as control (without oil) and culture grown in supplemented primrose oil show that the alga accumulated higher GLA as well as linoleic acid in 0.8% (w/v) primrose supplemented culture supporting the suggested method of oil-intake. The results revealed that linoleic acid could act as a precursor for excess GLA synthesis in oil-supplemented cultures. Data in Table 2 gives a comparison of the GLA content of the microalgal strain under different culture conditions. Although experiments with temperature and oil supplementation showed high GLA accumulation in the cyanobacteria, light induced GLA from this study was the highest and is comparable to results from the other reported studies with different conditions as tabulated herein.

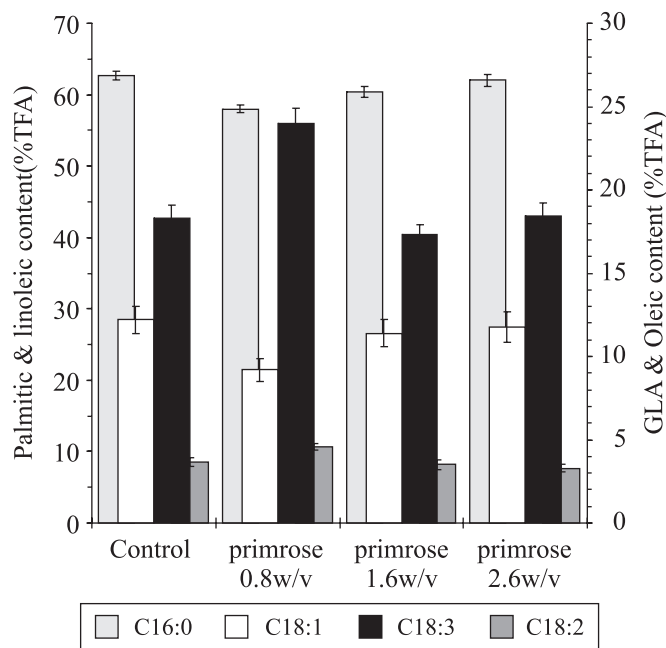


Figure 4b. Fatty acid profile of *S. platensis* grown in different supplemented oil concentrations.

Table 2. Comparison of GLA content in *Spirulina platensis* under different cultivation conditions.

Conditions Tested	GLA Content (mg/g)	Reference
Effect of Herbicide	14.0	(4)
Effect of Urea	13.5	(14)
Effect of Light and Dark Cycles	16.0	(19)
Effect of Light Intensity	14.6	
Effect of Primrose Oil	13.5	Present Work
Effect of Temperature	13.2	

RESUMO

Condições de cultura simulando o levado acúmulo de ácido γ -linolênico por *Spirulina platensis*

Estudou-se a produção de ácido γ -linolênico por *Spirulina platensis* em diferentes condições de estresse. Culturas submersas indicaram que temperatura baixa (25°C), forte intensidade de luz (6 klux) e suplementação com óleo de primula (0,8% p/v) induziram a produção de ácido linolênico de 13,2 mg/g, 14,6 mg/g e 13,5 mg/g peso seco, respectivamente. Uma observação cuidadosa do perfil de ácidos graxos da cianobactéria indica que os ácidos oléico e linoléico, em experimentos com diferentes temperaturas de crescimento e suplementos de óleo, auxiliaram no acúmulo de excesso de ácido γ -linolênico. Além disso, as culturas obtidas em intensidades crescentes de luz mantiveram a relação ácido γ -linolênico/ácidos graxos totais constante, independentemente de qualquer mudança no conteúdo de ácido γ -linolênico da cianobactéria.

Palavras-chave: ácido γ -linolênico, intensidade de luz, suplementação com óleo, temperatura

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