Effect of Nitrogen Sources on γ-Linolenic Acid Accumulation in *Spirulina platensis*

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ABSTRACT: The effect of ammonium phosphate on the growth, lipid content, and γ-linolenic acid accumulation was determined in the cyanobacterium *Spirulina platensis*. After 14 d on media containing 0.041 g N/L, γ-linolenic acid concentration of cultured cells increased up to 35.3 ± 0.13%, w/w. In treatments containing $NaNO_3$, NH_4NO_3, and NH_4Cl, γ-linolenic acid concentration increased up to 31.2 ± 0.23%, w/w. After the same period, lipid content in the dry biomass was 12.2 ± 0.03%, w/w, with $(NH_4)_2HPO_4$ compared to about 14.1 ±.0.12%, w/w, in treatments with $NaNO_3$. When $(NH_4)_2HPO_4$ concentration in the medium was increased to 0.082 g N/L, 30.8 ± 0.28%, w/w, γ-linolenic acid had formed after 10 d and the lipid percentage in the dry cell mass was 16.7 ± 0.16%, w/w. However, in treatments with $NaNO_3$, NH_4NO_3, or NH_4Cl, γ-linolenic acid concentration increased up to 30.6 ± 0.23%, w/w, and the lipid content was found to be 18.0 ± 0.17 to 18.9 ± 0.03%, w/w. These data showed that $(NH_4)_2HPO_4$ is a suitable source of nitrogen for growth of *S. platensis,* with increased accumulation of γ-linolenic acid at lower N concentration.

Paper no. J8844 in *JAOCS 76,* 153–156 (January 1999).

KEY WORDS: γ-Linolenic acid, nitrogen sources, *Spirulina platensis.*

γ-Linolenic acid (GLA) is among the various polyunsaturated fatty acids recognized for their food and pharmaceutical value. GLA is a precursor of prostaglandins and also is essential for patients who suffer from diabetes, cancer, aging, and viral infection (1–3).

The conventional and commercial source of GLA is evening primrose (*Oenothera biennis* L.) oil, which contains nearly 8%, w/w, GLA. However, borage (*Borago officinalis*) oil contains as much as 21%, w/w, GLA (4). Algae are another source for GLA and have the intrinsic advantage of requiring less space for growth than the other commercial sources.

The cyanobacterium *Spirulina platensis* typically contains a high amount of GLA. The lipid of *S. platensis* contains nearly 18–20%, w/w, GLA as determined from commercial samples obtained from K.N. Oil Industries, Raipur, M.P. India. *Spirulina platensis* may be an excellent commercial source for GLA if the GLA plus lipid content can be increased. Growth of this alga in presence of different inorganic nitrogen sources may be a way to optimize GLA accumulation in this organism. Manabe *et al*. (5,6) enhanced accumulation of GLA in *S. platensis* by adding alkali metal salts of nitric acid and ammonium salt in the logarithmic growth period. The GLA content reached 15.0 mg/g dried cells 72 h after addition of NH_4Cl. Total fatty acid content also increased after addition of 15 to 50 mM NH_4Cl, and plateaued at 40–48 h after addition of 25 mM NH_4Cl. Content of palmitic and oleic acids initially increased after addition of 25 mM NH_4Cl but then fell after 48 h while GLA content increased continuously during the 72-h incubation.

Hirano *et al*. (7) reported that GLA production was increased by 50% when *S. platensis* was grown in the dark. Light-dark cycles also can enhance production of GLA by 1.2 to 1.6% (8), as may different growth temperatures (9,10). Lipid content can also be increased by addition of a polyether ester (11) at 0.01 to 10% (of the fermentation medium). Again, most of the oleaginous algae respond to nutrient limitations by producing increased levels of triacylglycerol. The most common limiting factor is nitrogen (12).

Several alkali metal salts of nitric acid and ammonium salts have been shown to enhance the growth of *S. platensis* and to increase the accumulation of GLA in its biomass. However, similar experiments with the ammonium salt of phosphoric acid have not been conducted. In this study, the effect of diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, on the production of GLA was compared to treatments with $NaNO_3$, NH_4NO_3, and NH_4Cl.

MATERIALS AND METHODS

Growth of cells. The culture of *S. platensis* was a gift from School of Environmental Sciences, University of Kalyani, Nadia, W.B., India. Fresh algal cultures of *S. platensis* were grown on autoclave-sterilized Zarrouk's medium (13) with variation of $NaHCO_3$, nitrogen content, and the nitrogen source [$NaNO_3$, NH_4NO_3, $(NH_4)_2HPO_4$, and NH_4Cl]. Constituents were as follows (g/L): $NaHCO_3$, 8.0; K_2HPO_4, 0.5; K_2SO_4, 1.0; NaCl, 1.0; $MgSO_4$, 0.2; $CaCl_2$, 0.04; $FeSO_4$, 0.01; pH, 8–9; and nitrogen source, either 0.041 (Medium 1) or 0.082 (Medium 2). Into 500-mL conical flasks containing

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200 mL of treatment media was introduced 15 mL of algal inoculum (containing 35–40 mg dry biomass). Algal cultures were incubated in a water bath, 35 ± 1°C and illuminated with normal diffused sunlight providing 3000–4000 micro Joule/m^2·s. The cultures were shaken for 5 to 10 s at an interval of 10 min. A photoperiod of 12 h light–12 h dark was used throughout the experiment.

For treatments containing 0.041 g N/L, 24 conical flasks containing 200 mL of media were inoculated with *S. platensis* culture. These 24 flasks comprised of six sets of four conical flasks containing $NaNO_3$, NH_4NO_3, $(NH_4)_2HPO_4$, or NH_4Cl as the N-source. Four flasks from each N-source treatment were harvested after 8 d. This was repeated after 9, 10, 11, 12, and 14 d.

Five sets of four flasks containing $NaNO_3$, NH_4NO_3, $(NH_4)_2HPO_4$, or NH_4Cl were used in the second set of treatments. Each flask contained 0.082 g N/L. These treatments were harvested sequentially after 7, 9, 10, 12, and 14 d.

Determination of growth. After the desired period of growth, the algal mass was harvested by filtration over Whatman 41 filter paper (Maidstone, England) and washed twice with saline water. The amount of growth was determined by drying a known quantity of fresh algal mass at 105°C for 70 min.

Lipid extraction. Vacuum-dried algal biomass was extracted with chloroform/methanol/water (2:1:0.8, vol/vol/vol) according to Bligh and Dyer (14). Lipid content was expressed as a percentage of dry basis.

Fatty acid composition. Fatty acid composition of the lipid was monitored under batch culture conditions and was determined by gas–liquid chromatography (15). A Hewlett-Packard Model 5890 gas chromatograph was equipped with a 10% DEGS stainless steel column (i.d. 3/8″, length 6′) and a flame-ionization detector. The oven, injection, and detector temperatures were maintained at 190, 230, and 240°C, respectively. Peaks were identified by comparison with standard fatty acid methyl esters. GLA methyl ester was identified by comparing the relative retention time with that of the triunsaturated band from argentation thin-layer chromatography (TLC) of methyl esters derived from evening primrose oil, as conducted on thin-layer plates (20 × 20 cm) coated with a 0.5-mm thick layer of silica gel and impregnated with 7.5%, w/w, silver nitrate (16). After air drying and activation for 1 h at 110°C (followed by cooling to room temperature, 30°C), the fatty acid methyl ester was applied and the plate was developed with two solvent systems: light petroleum ether (b.p. 40–60°C)/diethyl ether/methanol/acetic acid (40:10:1:1, by vol), followed by light petroleum ether/diethyl ether/acetic acid (97:3:1, vol/vol/vol). The band of triene fatty acid methyl ester was extracted with petroleum ether (b.p. 40–60°C). After evaporation of the solvent, the purity of this methyl ester was confirmed by gas chromatographic analysis.

RESULTS AND DISCUSSION

Nitrogen source treatments for the growth of *S. platensis* were established on Zarrouk's medium (13) with the following modifications: $NaHCO_3$, 8.0 g/L; N-concentration, 0.041 or 0.082 g N/L and pH 8–9 in Medium 1 and Medium 2. The variation for nitrogen content was either ten- or fivefold lower than the original Zarrouk's medium. Most oleaginous microalgae respond to nutrient limitations by producing an increased amount of storage triacylglycerol. Nitrogen is the most common limiting factor (12).

Table 1 shows GLA concentration as affected by time and N-source. Under identical growth conditions with $NaNO_3$, NH_4NO_3, or NH_4Cl, *S. platensis* produced 29.8 ± 0.21 to 31.2 ± 0.23% GLA after 14 d in the lower N-concentration treatments. However, growth on $(NH_4)_2HPO_4$ under identical conditions increased GLA concentration to as high as 35.3 ± 0.13% after 14 d. From Table 1 it is also evident that GLA concentration plateaued between 12 and 14 days. Thus, cell harvest may be economical even after 12 d.

When *Spirulina* was grown in a medium having 0.082 g N/L, only 9 d of growth were required to achieve 29.0 ± 0.15 to 31.2 ± 0.15% GLA, regardless of the N-source. There was no further increase in GLA concentration even after 14 d.

TABLE 1
γ-Linolenic Acid (GLA) Concentration[a] in *Spirulina platensis* as Affected by Time and Nitrogen Source

Nitrogen in medium (g N/L)	Time (d)	GLA% (w/w) (per 100 mL)			
		$NaNO_3$	NH_4NO_3	$(NH_4)_2HPO_4$	NH_4Cl
0.041	8	17.6 ± 0.05	19.2 ± 0.23	19.2 ± 0.56	18.0 ± 0.29
	9	20.1 ± 0.28	24.3 ± 0.31	29.2 ± 0.14	28.4 ± 0.11
	10	22.2 ± 0.25	28.4 ± 0.25	29.9 ± 0.26	29.7 ± 0.12
	11	28.0 ± 0.26	29.8 ± 0.30	31.6 ± 0.28	30.0 ± 0.12
	12	29.6 ± 0.18	31.1 ± 0.21	34.6 ± 0.05	30.0 ± 0.12
	14	31.2 ± 0.23	30.4 ± 0.11	35.3 ± 0.13	29.8 ± 0.21
0.082	7	23.3 ± 0.13	20.1 ± 0.24	24.9 ± 0.33	26.8 ± 0.18
	9	31.2 ± 0.15	29.0 ± 0.15	29.7 ± 0.09	30.3 ± 0.33
	10	30.6 ± 0.23	30.1 ± 0.18	30.8 ± 0.28	30.2 ± 0.05
	12	30.8 ± 0.38	29.7 ± 0.11	30.5 ± 0.06	29.5 ± 0.23
	14	31.2 ± 0.19	30.2 ± 0.09	30.5 ± 0.28	30.0 ± 0.32

[a]Mean ± standard error of three replicates.

TABLE 2
Growth[a] of *S. platensis* and Oil Content[a] in the Cell Mass During Different Growth Periods (per 100 mL medium)

Nitrogen in medium (g N/L)	Time (d)	$NaNO_3$ Mass (mg)	$NaNO_3$ Oil (%w/w)	NH_4NO_3 Mass (mg)	NH_4NO_3 Oil (%w/w)	$(NH_4)_2HPO_4$ Mass (mg)	$(NH_4)_2HPO_4$ Oil (%w/w)	NH_4Cl Mass (mg)	NH_4Cl Oil (%w/w)
0.041	0	35.9 ± 0.16	n.d.	37.7 ± 0.21	n.d.	35.6 ± 0.13	n.d.	36.9 ± 0.09	n.d.
	8	60.1 ± 0.73	29.5 ± 0.13	64.5 ± 0.63	30.1 ± 0.07	68.9 ± 0.42	27.4 ± 0.09	67.7 ± 0.59	28.6 ± 0.13
	10	85.9 ± 1.27	19.0 ± 0.06	87.6 ± 0.98	18.5 ± 0.22	87.0 ± 0.81	17.7 ± 0.17	87.0 ± 1.00	19.7 ± 0.06
	12	105.1 ± 1.19	16.2 ± 0.03	105.2 ± 1.06	15.8 ± 0.16	104.6 ± 1.03	15.1 ± 0.19	109.6 ± 0.82	16.2 ± 0.19
	14	138.9 ± 1.11	14.1 ± 0.12	145.9 ± 1.46	13.1 ± 0.03	136.2 ± 1.51	12.2 ± 0.03	139.3 ± 1.09	14.0 ± 0.21
0.082	0	34.9 ± 0.18	n.d.	37.9 ± 0.12	n.d.	36.6 ± 0.13	n.d.	36.5 ± 0.09	n.d.
	7	64.3 ± 0.23	28.1 ± 0.28	60.9 ± 0.26	29.8 ± 0.31	66.0 ± 0.51	27.4 ± 0.37	60.7 ± 0.83	29.2 ± 0.16
	9	89.1 ± 0.88	18.4 ± 0.16	97.2 ± 0.62	19.0 ± 0.12	81.3 ± 0.63	17.0 ± 0.03	79.9 ± 0.79	18.4 ± 0.22
	10	123.9 ± 1.23	18.0 ± 0.17	133.9 ± 0.92	18.9 ± 0.03	135.1 ± 0.89	16.7 ± 0.16	124.6 ± 1.26	18.1 ± 0.13
	12	151.5 ± 0.98	13.6 ± 0.07	156.2 ± 1.06	13.5 ± 0.13	143.6 ± 0.99	11.1 ± 0.09	141.2 ± 1.19	13.0 ± 0.27

[a]Mean ± standard error of three replicates. n.d., not determined. For other abbreviation see Table 1.

Therefore, use of nitrogen at the level of 0.082 g N/L led to earlier attainment of high-GLA concentration. It also was shown that 24.9 ± 0.33 and 26.8 ± 0.18% GLA concentration may be achieved after only 7 d by using $(NH_4)_2HPO_4$ and NH_4Cl, respectively.

Growth and oil production by *S. platensis* as a function of nitrogen species are presented in Table 2. When $(NH_4)_2HPO_4$ was used, growth and lipid concentration in the cell mass were comparable to those achieved with the other N-sources. Biomass increased from 68.9 ± 0.42 mg/100 mL on the eighth day of culture to 136.2 ± 1.51 mg/100 mL on the fourteenth day in the presence of 0.041 g N/L ammonium phosphate. However, oil concentration decreased from 27.4 ± 0.09 to 12.2 ± 0.03% during the same period. After 14 d, algal biomass in the treatments with lower N-concentrations using $NaNO_3$, NH_4NO_3 or NH_4Cl, was 138.9 ± 1.11 to 145.9 ± 1.46 mg/100 mL, and oil concentration in the cells ranged from 13.1 ± 0.03 to 14.1 ± 0.12%. Similar results were obtained in treatments with 0.082 g N/L. Depending on nitrogen source, biomass increased from a range of 60.7 ± 0.83 to 66.0 ± 0.51 mg/100 mL on the seventh day of culture to a range of 141.2 ± 1.19 to 156.2 ± 1.06 mg/100 mL on the twelfth day. Among these treatments, *S. platensis* grown on $(NH_4)_2HPO_4$ produced slightly lower biomass and oil concentration after culture for 12 d.

GLA content in the cell mass is shown in Table 3. GLA content was comparable among all the nitrogen sources when the medium N-content was 0.041 g N/L. When $(NH_4)_2HPO_4$ was used as the N-source, GLA content reached 5.5 ± 0.14 mg/100 mL after 12 d. This level of GLA production was slightly higher than for cells grown on media with $NaNO_3$ or NH_4NO_3. However, at 0.082 g N/L, the GLA content was less (4.9 ± 0.17 mg/100 mL) than that attained in the lower N-content medium.

Apparently $(NH_4)_2HPO_4$ is a suitable N-source in modified Zarrouk's medium for increased production of GLA in *S. platensis.* On media with lower N-content, $(NH_4)_2HPO_4$ also produced a greater GLA concentration than cultures grown with $NaNO_3$ and other nitrogen sources.

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TABLE 3
GLA Content[a] (mg/100 mL medium) in the Cell Mass of *S. platensis* as Affected by Time and Nitrogen Source

Nitrogen in medium (g N/L)	Time (d)	GLA formed: $NaNO_3$	NH_4NO_3	$(NH_4)_2HPO_4$	NH_4Cl
0.041	8	3.1 ± 0.03	3.7 ± 0.07	3.6 ± 0.12	3.5 ± 0.07
	10	3.6 ± 0.09	4.6 ± 0.12	4.6 ± 0.03	5.1 ± 0.06
	12	5.0 ± 0.14	5.2 ± 0.12	5.5 ± 0.14	5.3 ± 0.17
	14	6.1 ± 0.12	5.8 ± 0.07	5.9 ± 0.12	5.8 ± 0.03
0.082	7	4.2 ± 0.07	3.6 ± 0.03	4.5 ± 0.17	4.8 ± 0.12
	9	5.1 ± 0.07	5.4 ± 0.14	4.1 ± 0.03	4.5 ± 0.17
	10	6.8 ± 0.23	7.6 ± 0.14	6.9 ± 0.03	6.8 ± 0.03
	12	6.3 ± 0.07	6.3 ± 0.09	4.9 ± 0.17	5.4 ± 0.13

[a]Mean ± standard error of three replicates. For abbreviations see Table 1.

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[Received April 9, 1998; accepted August 7, 1998]