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Total lipid accumulation and fatty acid profiles of microalga *Spirulina* under different nitrogen and phosphorus concentrations

R. N. Bhakar, Brahmdutt & Sunil Pabbi *

Centre for Conservation & Utilisation of Blue Green Algae, IARI, New Delhi India.

Abstract

Nutrient limitation in terms of nitrogen and phosphorus increased lipid accumulation under depleted growth in *Spirulina* strains. Nitrogen limitation was found more effective than phosphorus in accumulating lipid. The fatty acid profile was variable: palmitic (48%), linolenic (21%) and linoleic acids (15%) were the most prevalent types. It was interesting that nitrogen limitation was more effective in increasing total lipid content, whereas phosphorus limitation had more effect on the fatty acid profile. Fatty acid ratios were different under the two conditions of nutrient limitation, with γ -linolenic acid being accumulated in higher quantities under nitrogen than phosphorus limitation.

Keywords: palmitic acid, linoleic acid, γ -linolenic acid.

Introduction

The filamentous cyanobacterium *Spirulina platensis* has been used as a food for centuries by native peoples from Lake Chad in Africa and Lake Texcoco in Mexico (Vonshak 1997; Henrikson 1994). This observation led to the use of *Spirulina* as a food supplement for undernourished people in many parts of the world (Henrikson 1994) due to its high protein content (65%), high digestibility (Henrikson 1994) and specific amino acid content. Apart from use as human food supplement, it can also be incorporated in the feed for a wide variety of animals, as well as being a good source of pigments and natural colours (Chakdar *et al*. 2012).

Among several cellular components contributing to its value for this purpose, the high content of polyunsaturated fatty acids has attracted considerable attention, as a great part of these is formed by γ –linolenic acid (*cis*-6,9,12-octadecatrienoic acid) (Cohen *et al*. 1987). *Spirulina* is a rich source of this rare essential fatty acid (Grattan 1989): 10 g of *Spirulina* contains over 100 mg. It is well known that the conditions of culture and nutrition greatly influence the composition and physiological state of these organisms, and nutrient starvation in particular has been the main strategy for enhancing lipid accumulation. Nitrogen, iron and phosphorus have been reported to cause cell growth cessation and channel metabolic flux to lipid biosynthesis (Goldberg & Cohen 2006; Courchesne *et al*. 2009). Nitrogen limitation, for example, causes three changes: a decrease of the cellular content of thylakoid membrane, activation of acyl hydrolase and stimulation of phospholipid hydrolysis. These changes may increase the intracellular content of fatty acid acyl-CoA. Nitrogen limitation could also activate diacylglycerol acyltransferase, which converts acyl-CoA to triglyceride (Takagi *et al*. 2000). Therefore nitrogen limitation could both increase lipid and triglyceride content in microalgal cells.

Under normal growth conditions, ATP and NADPH produced by photosynthesis are consumed by generating biomass, with ADP and $NADP^+$ eventually being available again as acceptor molecules in photosynthesis. When cell growth and proliferation is impaired due to the lack of nutrients such as phosphorus, the pool of the major electron acceptor for photosynthesis ($NADP^+$) can become depleted. Since photosynthesis is mainly controlled by the abundance of light, and cannot be shut down completely, this can lead to a potentially dangerous situation for the cell, damaging cell components. NADPH is consumed in fatty acid biosynthesis; therefore, increased fatty acid production (which in turn are stored in

* Author for correspondence: tel: +91 9818024130 email : *sunil.pabbi@gmail.com*

triglycerides) replenishes the pool of $NADP^+$ under growth-limiting conditions (Thompson 1996).

The objective of the study presented here in this paper was to evaluate the influence of varying nitrogen and phosphorus concentrations on the fatty acid profile of selected *Spirulina* strains.

Materials & Methods

Three *Spirulina* strains used in this study were procured from the Culture Collection of the Centre for Conservation & Utilisation of Blue Green Algae (IARI, New Delhi, India) and maintained in Zarrouk's medium (Zarrouk 1966). For experimental purposes the *Spirulina* strains were cultivated in 100 mL autoclaved growth medium contained in 250 mL ehrlenmeyer flasks. One ml of 14-day-old culture was inoculated into each flask. The cultures were cultivated in a growth chamber at 25 $\pm$ 2 °C and light intensity of 55-60 µmol photon m^{-2} s^{-1} with light/dark regime of 16:8 h. For varying concentrations of nitrogen and phosphorus, the medium was supplemented with initial $NaNO_3$ concentrations of 1.0, 1.5 and 2.5 (control) g/L and K_2HPO_4 concentrations of 0.2, 0.3 and 0.5 (control) g/L. Both N and P limitation was studied independently and all the experiments were conducted in triplicate.

Cells were analysed after 14 days of incubation. Algal biomass was harvested by centrifugation at 4000 g, and the dry weight determined (Richmond & Gobbelaar 1986). The total lipids were extracted with a chloroform/methanol solution (2/1 v/v) and quantified colorimetrically (Snyder & Stephens 1959).

Fatty acid methyl esters were prepared from the lipid samples using the method of Metcalfe *et al.* (1966). The fatty acid profile for each of the nutrient conditions was determined by gas chromatography. 2.5 µl of esterified sample was injected into a Shimadzu GC-2010 and analysed through FID (detector temperature, 280 °C; injector temperature, 270 °C; carrier gas, nitrogen at a flow rate of 1.21 ml/min) fitted with a 100 m long, 0.25 mm i.d. SP-2560 column with a 0.20 mm film thickness and a polyethylene glycol modified nitroterephthalic acid stationary phase. Chromatography conditions were: initial column temperature 140 °C with equilibration time of 0.5 min which was held for 5 min, with 4 °C/min rise to 240 °C, held for 15 min (total 45 min). Fatty acids were identified by comparing the retention times with standards (Sigma) and quantified by normalization of the area under relevant peaks using Varian Star software version 4.51.

Strains	Culture conditions (g/L)		Nitrogen			Phosphorus		
	$NaNO_3$	K_2HPO_4	Lipid (mg/ml)	Biomass (mg/ml)	Lipid %	Lipid (mg/ml)	Biomass (mg/ml)	Lipid %
Spirulina platensis	1.0	0.2	0.67 ± 0.02^{b}	2.7 ± 0.1^{e}	24.9 ± 0.3^{a}	0.61 ± 0.03^{c}	3.1 ± 0.2^{g}	20.1 ± 0.6^{a}
	1.5	0.3	0.45 ± 0.03^{e}	3.3 ± 0.2^{d}	13.4 ± 1.2^{d}	0.44 ± 0.02^{e}	3.4 ± 0.2^{f}	12.9 ± 0.6^{c}
	2.5	0.5	0.34 ± 0.03^{g}	4.7 ± 0.3^{c}	7.2 ± 0.7^{g}	0.34 ± 0.01^{f}	4.3 ± 0.2^{d}	7.9 ± 0.2^{de}
Spirulina maxima	1.0	0.2	0.82 ± 0.03^{a}	4.5 ± 0.3^{c}	18.1 ± 1.3^{c}	0.78 ± 0.01^{a}	4.9 ± 0.2^{c}	16.1 ± 0.6^{b}
	1.5	0.3	0.49 ± 0.02^{d}	5.8 ± 0.5^{b}	8.6 ± 0.8^{f}	0.50 ± 0.03^{d}	5.9 ± 0.3^{b}	8.5 ± 0.2^{d}
	2.5	0.5	0.34 ± 0.02^{g}	8.3 ± 0.3^{a}	4.2 ± 0.2^{h}	0.34 ± 0.02^{f}	8.3 ± 0.2^{a}	4.1 ± 0.2^{f}
Spirulina sp	1.0	0.2	0.57 ± 0.03^{c}	2.8 ± 0.1^{e}	20.4 ± 0.9^{b}	0.68 ± 0.03^{b}	3.4 ± 0.2^{f}	19.9 ± 1.6^{a}
	1.5	0.3	0.41 ± 0.03^{f}	3.6 ± 0.1^{d}	11.6 ± 0.4^{e}	0.45 ± 0.03^{e}	3.7 ± 0.1^{e}	12.0 ± 0.2^{c}
	2.5	0.5	0.35 ± 0.01^{g}	3.7 ± 0.1^{d}	9.6 ± 0.3^{f}	0.34 ± 0.02^{f}	4.8 ± 0.1^{c}	7.2 ± 0.1^{e}

Table 1: Total lipid production by *Spirulina* strains under varied N ($NaNO_3$) and P (K_2HPO_4) concentrations. Values are given as mean ± SD of three replicates. Means in columns with different letters are significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Results

Growth and lipid accumulation under nitrogen and phosphorus limitation is shown in Table 1. All the three *Spirulina* strains showed increase in total lipid accumulation under both nitrogen and phosphorus limitation. *Spirulina platensis* and *Spirulina* sp. recorded more lipid accumulation under both nitrogen and phosphorus limitation than *Spirulina maxima*. There was growth reduction in all the three *Spirulina* strains under nutrient limitation, with nitrogen having more effect on biomass reduction than phosphorus.

The fatty acid composition revealed that the fatty acid profiles varied among all three *Spirulina* strains with respect to nutrient limitation (Tables 2 & 3). Palmitic, linolenic and linoleic acids (in order of abundance) were the most prevalent. γ-linolenic acid increased under nitrogen limitation in *Spirulina platensis* (21%) and *Spirulina* sp. (21%), but decreased in *Spirulina maxima* (18.5%). The percentage of linoleic acids reduced but palmitic acid increased in all three strains under nitrogen limitation. Phosphorus limitation resulted in a decrease in γ-linolenic acid content in all three strains, while linoleic acids varied, increasing in percentage in *Spirulina maxima* (17%) but reducing in *Spirulina* sp. In *Spirulina platensis* there was a reduction at lower concentration followed by an increase and then a decrease at maximum nutrient concentrations of phosphorus. Palmitic acid increased in *Spirulina platensis* and *Spirulina* sp., but decreased in *Spirulina maxima* under phosphorus limitation.

Strains	$NaNO_3$ conditions (g/L)	Fatty acid concentration (%)							
		C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Spirulina platensis	1.0	0.86	1.64	48.31	2.73	1.86	8.56	12.29	20.71
	1.5	0.72	1.53	45.87	2.36	1.57	9.19	12.64	20.38
	2.5	0.95	1.95	44.95	2.02	1.24	7.90	13.01	19.89
Spirulina maxima	1.0	1.97	1.41	45.62	2.81	2.57	8.34	12.34	18.54
	1.5	2.05	1.63	45.13	2.24	2.24	8.02	14.51	19.43
	2.5	1.46	1.56	44.68	2.04	2.09	7.51	14.63	19.71
Spirulina sp	1.0	1.65	1.70	45.74	2.71	1.89	7.88	11.85	20.33
	1.5	1.36	2.16	45.26	2.51	1.95	8.67	11.76	19.80
	2.5	0.87	1.01	44.91	1.64	1.90	6.94	12.21	20.08

Table 2: Fatty acid composition of *Spirulina* strains under varied N ($NaNO_3$) concentrations. C14:0 = myristic acid; C14:1 = myristoleic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C18:0 = stearic acid; C18:1 = oleic acid; C18:2 = linoleic acid; C18:3 = gamma-linolenic acid (nomenclature based on Sigma catalogue).

Strains	K_2HPO_4 conditions (g/L)	Fatty acid concentration (%)							
		C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Spirulina platensis	0.2	0.86	1.78	28.61	1.87	2.20	22.22	17.60	16.05
	0.3	1.25	1.69	26.90	2.79	2.81	21.22	15.88	14.65
	0.5	0.99	2.03	27.11	1.82	2.02	21.93	17.67	17.92
Spirulina maxima	0.2	0.90	1.77	26.17	3.89	2.04	24.12	16.64	16.40
	0.3	0.90	1.84	26.52	4.04	4.25	20.74	15.23	16.16
	0.5	0.93	2.04	33.71	4.69	3.45	15.84	12.79	17.09
Spirulina sp	0.2	2.53	2.24	38.31	1.78	3.23	13.40	14.77	11.20
	0.3	1.24	2.64	40.16	2.04	3.64	15.30	11.42	13.51
	0.5	1.00	1.39	28.26	2.96	2.88	19.57	16.28	17.78

Table 3: Fatty acid composition of *Spirulina* strains under varied P (K_2PO_4) concentrations. (names as in Table 2).

The percentage ratio of γ-linolenic acid to total fatty acids, unsaturated to total fatty acids, and γ-linolenic acid to oleic plus linoleic acids varied greatly under nitrogen (Table 4) and phosphorus limitation (Table 5).

Strains	$NaNO_3$ conditions (g/L)	Fatty acid ratio (%)		
		GLA/TFA	UFA/TFA	GLA/O+L
Spirulina platensis	1.0	21.36	47.37	0.99
	1.5	21.62	48.91	0.93
	2.5	21.64	48.71	0.95
Spirulina maxima	1.0	19.81	46.41	0.90
	1.5	20.40	48.12	0.86
	2.5	21.04	48.52	0.89
Spirulina sp	1.0	21.69	47.43	1.03
	1.5	21.18	48.04	0.97
	2.5	22.42	46.76	1.05

Table 4: Percentage ratio of fatty acids in *Spirulina* strains under varied N ($NaNO_3$) concentrations. GLA = gamma-linolenic acid; TFA = total fatty acids; UFA = unsaturated fatty acids; O+L = oleic + linoleic acid.

Strains	K_2HPO_4 conditions (g/L)	Fatty acid ratio (%)		
		GLA/TFA	UFA/TFA	GLA/O+L
Spirulina platensis	0.2	17.60	65.27	0.40
	0.3	16.80	64.49	0.39
	0.5	19.59	67.08	0.45
Spirulina maxima	0.2	17.84	68.33	0.40
	0.3	18.02	64.69	0.45
	0.5	18.88	57.93	0.60
Spirulina sp	0.2	12.81	49.61	0.40
	0.3	15.02	49.93	0.51
	0.5	19.73	64.34	0.50

Table 5: Percentage ratio of fatty acids in *Spirulina* strains under varied P (K_2PO_4) concentrations. GLA = gamma-linolenic acid; TFA = total fatty acids; UFA = unsaturated fatty acids; O+L = oleic + linoleic acid.

Discussion

Nutrient limitation is an efficient trigger to increase lipid content per unit algal biomass, as reported by many other researchers (Goldberg & Cohen 2006; Li *et al*. 2010; Leyla *et al*. 2011; Hifney *et al*. 2013). Our results have clearly shown that lipid accumulation increases under nitrogen and phosphorus limitation. Generally, microalgae accumulate lipid under nutrient limitation when an energy (light) and a carbon source (CO_2) are available and when the cellular mechanisms for photosynthesis are active (Courchesne *et al*. 2009). The relationship between the concentration of the nitrogen source in the culture medium and the fatty acid content of *Spirulina* was studied by Rijn & Shilo (1986), showing that reserve compounds accumulate during nitrogen depletion. Under phosphorus limitation, the total cellular lipid content of starved cells increased, mainly due to the dramatic increase in triglyceride levels from 6% to 39% of total lipids (Goldberg & Cohen 2006). In our study at lower initial concentrations of nitrogen or phosphorus, all the three *Spirulina* strains accumulated high lipid

content per unit algal biomass. This may be due to the fact that the whole nutrient concentration, which was already low, would have been exhausted earlier at the initial stages of growth with low cell density; all three *Spirulina* strains started to accumulate lipid when individual cells were exposed to a large quantity of light energy, resulting in more metabolic flux generated from photosynthesis to be channelled to lipid accumulation on an unit biomass basis (Courchesne *et al*. 2009). In the USA Aquatic Species Program, a similar conclusion was also drawn, stating that under environmental stress (such as nutrient limitation), cell division may cease, but lipid productivity still seems to remain high, leading to an accumulation of lipids in the cells: this is what we found. On the other hand, there are reports that state that increased oil content does not always lead to increased lipid productivity because the overall rates of lipid productivity are lower during periods of nutrient limitation. Higher levels of oil in the cells are more than offset by lower rates of cell growth (Sheehan *et al*. 1998).

The percentages of the major fatty acid methyl esters recorded in this study were in accordance with previous works by other authors (Olguín *et al.* 2001, Quoc *et al.* 1994; Cohen *et al.* 1987). The principal fatty acids present were palmitic, γ-linolenic and linoleic acid. It may be possible to increase the content of γ-linolenic acid because Olguín *et al.* (2001) obtained 26-31% of γ-linolenic acid (C18:3) under low light flux and nitrogen deficiency. Here also, γ-linolenic acid increased in two strains (*Spirulina platensis* and *Spirulina* sp.) under nitrogen limitation, but under phosphorus limitation showed a reduction in all three strains. Piorreck *et al.* (1984) showed that such reserve compound accumulation occurs mainly in the metabolism of green algae, while in the cyanobacterium *Spirulina* the fatty acids of the lipid polar fraction remain constant for potassium nitrate concentrations between 0.001% and 0.1%.

The GLA/TFA ratio varied from 19.8 - 22.4 % and 12.8 -19.6 % under nitrogen and phosphorus limitation respectively. Nitrogen limitation was superior because greater GFA/TFA and GLA/(O+L) ratios were found in the samples. This further corroborates the view of other researchers (Alonso & Maroto 2000; Quoc *et al.* 1994; Cohen *et al.* 1987) that *Spirulina* is a potential source of this fatty acid.

Spirulina is used as a food supplement, and mostly is consumed in the form of a capsule or a tablet - which appears to be the preferred form at present. Since the costs of extraction of poly-unsaturated fatty acids (especially GLA) from *Spirulina* is very high, the best way to use *Spirulina* is by its direct consumption as a nutritional supplement, especially because in this way the consumer will benefit not only from the beneficial properties ascribed to GLA but also to the other nutritionally active components. Our results have shown that different *Spirulina* strains have different fatty acid profiles, and the *Spirulina* strains cultivated by us demonstrate that palmitic acid is the most abundant followed by linolenic and linoleic acids. Nitrogen limitation was more important than phosphorus limitation, with greater amounts of GLA being obtained at 1.0 g/L of $NaNO_3$ supplementation. It seems that *Spirulina* produced under the culture conditions described in this paper is a potential source of GLA for use as a food additive or a nutritional supplement.

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