Bioresource Technology 101 (2010) 3801–3807

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Review

Lipid analysis in *Haematococcus pluvialis* to assess its potential use as a biodiesel feedstock

M. Cecilia Damiani [a], Cecilia A. Popovich [a], Diana Constenla [b], Patricia I. Leonardi [a,c,*]

[a] Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina
[b] Planta Piloto de Ingeniería Química (PLAPIQUI) – UNS – CONICET, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina
[c] Laboratorio de Estudios Básicos y Biotecnológicos en Algas y Hongos (LEBBAH), Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS) – CONICET, Camino La Carrindanga, Km 7, 8000 Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 9 September 2009
Received in revised form 23 December 2009
Accepted 31 December 2009
Available online 1 February 2010

Keywords:
Haematococcus pluvialis
Biodiesel
Neutral lipid
High light intensity
Nitrogen deprivation

ABSTRACT

The lipid content and composition of *Haematococcus pluvialis* exposed to stress conditions were analyzed to assess the potential of this microalga as a biodiesel feedstock. The total lipid content of control cells was 15.61% dw, whereas that of cells exposed to continuous high light intensity with nitrogen-sufficient medium (A-stress condition) or under continuous high light intensity with nitrogen-deprivation medium (B-stress condition) was 34.85% dw and 32.99% dw, respectively. The fatty acid profile was similar under all conditions and indicated that the main components were palmitic, stearic, oleic, linoleic, linolenic and linolelaidic acids. The neutral lipid fraction increased about 2-fold under both stress conditions. The percentage of saturated fatty acids in the neutral lipid fraction was 30.36% and 29.62% in cultures grown under A-stress and B-stress, respectively, and 27.81% under control conditions. The monounsaturated fatty acid content was not significantly different in control and A-stress cultures (20.07% and 19.91%, respectively), but was 18.96% under B-stress. The content of polyunsaturated fatty acids was 47.23% under B-stress and 43.15% under A-stress. Growth-rate was higher under A-stress compared to B-stress. This is the first study of *H. pluvialis* that provides a detailed characterization of its lipid content in relation to bioenergy. The results indicate the potential of this microalga as a biodiesel feedstock; however, culture conditions still have to be improved in order to achieve an adequate energy balance in mass culture.

1. Introduction

Haematococcus pluvialis Flotow is an unicellular biflagellate green microalga that develops resistant cells, called cysts or aplanospores, under stress conditions (Boussiba et al., 1999; Boussiba, 2000), which accumulate astaxanthin and lipids (Grünewald et al., 2001; Damiani et al., 2006; Cerón et al., 2007). The ability of this and other microalgae to grow or survive in a wide range of environmental conditions, coupled with their capacity to efficiently modify lipid metabolism in response to different stress conditions (Roessler, 1990; Guschina and Harwood, 2006; Hu et al., 2008), has made them interesting organisms regarding the synthesis of non-polar triacylglycerols (TAGs). TAGs are the best substrate to produce biodiesel (Xu et al., 2006; Chisti, 2007; Hu et al., 2008; Schenk et al., 2008; Vasudevan and Briggs, 2008; Rodolfi et al., 2009). Biodiesel is obtained by trans-esterification of oil or fat with a monohydric alcohol, yielding the corresponding mono-alkyl esters (Knothe, 2005). Since trans-esterification maintains the relative ratio of fatty acids present in the feedstock (Costa Neto et al., 2000), the profile of the fatty acid ethyl esters is a reflection of the feedstock fatty-acid composition (Lang et al., 2001; Ferrari et al., 2005).

Although, at present, the technology for production and extraction of microalgal oils remains expensive, and therefore, non-profitable for biodiesel production (Sheehan et al., 1998; Hu et al., 2006; Chisti, 2007), the rise in fuel prices together with the gradual depletion of world reserves of fossil fuels, continues to encourage the search of new algal species as a renewable biofuel sources.

The intrinsic ability to produce large quantities of lipid and oil is species- and strain- rather than genus-specific (Hu et al., 2006). Moreover, the lipid content increases when algal cells are subjected to unfavorable culture conditions, such as high salinity (Ching-Piao and Liang-Ping, 2001), nitrogen starvation (Yu et al., 1987; Illman et al., 2000; Zhekisheva et al., 2002), and high light intensity (Gordillo et al., 1998). Besides, the lipid composition in microalgae also depends on the age of the culture and the different life-cycle stages (Siron et al., 1989; Fidalgo et al., 1998).

Hu et al. (2008) compiled information from 60 year-old to current reports about algal species that produce lipids, their fatty

* Corresponding author. Address: Laboratorio de Estudios Básicos y Biotecnológicos en Algas y Hongos (LEBBAH), Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS) – CONICET, Camino La Carrindanga, Km 7, 8000 Bahía Blanca, Argentina. Tel.: +54 291 4861124; fax: +54 291 4862882.
E-mail address: leonardi@uns.edu.ar (P.I. Leonardi).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.12.136

acids and TAG biosynthetic pathways, and also about environmental and biological factors that may affect these pathways. The authors indicated that much of the information on algal fatty-acid composition belongs to a limited number of algal species, and most of the data concern total extracted lipids instead of individual lipid classes.

Several studies on content, synthesis and biological activity of fatty acids and astaxanthin from *H. pluvialis* have already been performed (Yuan and Chen, 2000; Cifuentes et al., 2003; Zhekisheva et al., 2002; Zhekisheva et al., 2005; García-Malea López et al., 2005; Rosa et al., 2005; Cerón et al., 2007). *H. pluvialis* vegetative cells grow optimally under low irradiation, i.e., below 100 μmol photon $m^{-2}s^{-1}$ (Fan et al., 1994; Harker et al., 1995), and saturated nutrient conditions (Cifuentes et al., 2003). In contrast, lipid accumulation, which occurs during cyst formation, is induced by stress factors, such as nutrient limitation and high light intensity (Zhekisheva et al., 2002, 2005). These conditions are also conducive to enhancing astaxanthin synthesis (Boussiba et al., 1999; Boussiba, 2000; Cifuentes et al., 2003; Cerón et al., 2007). Zhekisheva et al. (2002) observed that the accumulation of oleic acid was correlated with an increase in astaxanthin esters when *H. pluvialis* was grown under nitrogen starvation or high light intensity conditions. In addition, a strong connection between the synthesis of TAGs and carotenoids has previously been shown in the microalgae *Dunaliella bardawil* (Rabbani et al., 1998) and *Dunaliella salina* (Mendoza et al., 1999).

The aims of this study were: (a) to increase knowledge on lipid content and fatty-acid composition of an Argentinian strain of *H. pluvialis* grown under optimal and stress conditions in the laboratory, and (b) to analyze the potential of this microalga's oil as a biodiesel feedstock.

2. Methods

2.1. Algal strain and culture conditions

H. pluvialis samples were collected from rainwater in Bahía Blanca (38°50′S, 63°30′W), Buenos Aires Province, Argentina. Unialgal cultures were obtained by means of serial dilutions (Stein, 1973). Biflagellate cells were cultured in Bold's Basal Medium (BBM), containing 3.4 mM of sodium nitrate (Stein, 1973). The cells were kept at 24 °C with continuous bubbling of air (500–700 cm^3/min) containing 0.30 cm^3/min of CO_2. A 12/12 h light/dark photoperiod and cool-white fluorescent lamps, which provide 90 μmol photons $m^{-2}s^{-1}$, were used. The pH was adjusted to 7.0 with NaOH before autoclaving. An inoculum of 45×10^3 biflagellate cells/ml was resuspended for a two-week period in one liter of: (i) full medium (MBB), under the same conditions as those indicated above (control). (ii) full medium, under 300 μmol photons $m^{-2}s^{-1}$ of continuous light and without aeration (A-stress condition) and (iii) nitrogen-free medium, under 300 μmol photons $m^{-2}s^{-1}$ of continuous light and without aeration (B-stress condition). In both stress conditions the samples were manually agitated twice daily and the first cysts appeared after four days of culture stress. Three replicates of the cultures were done.

2.2. Growth measurements

The cell concentration was determined by counting three replicate samples by means of Sedgwick–Rafter chambers. Growth-rate (k) was estimated during the period of exponential growth by least squares fit of a straight line of the data logarithmically transformed (Guillard, 1973). Doubling time was also calculated as DT: ln 2/k.

2.3. Transmission electron microscopy (TEM)

Cysts of *H. pluvialis* were fixed at 5 °C in 3% glutaraldehyde and 1.5% paraformaldehyde in 0.05 M Na-cacodylate buffer (pH 7.4). The samples were postfixed in 2% OsO_4, dehydrated in acetone series, and embedded in low-viscosity Spurr's resin. Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed using a Jeol 100 CX-II electron microscope.

2.4. Nile Red staining

Cysts of *H. pluvialis* were frozen at −40 °C and hand-ground for 10 min (Wiltshire et al., 2000). Five microliters of Nile Red (9-diethylamino-5H-benzo[α] phenoxazine-5-one, Sigma) in acetone (1 mg/L) was added to 5 mL of algal suspension (Elsey et al., 2007). The mixture was vigorously agitated in a vortex mixer. Fluorescence was detected 5 min after staining by using a Leica DMIRE2 Conphocal TCS SP2 SE microscope with a 475 nm band-excitation filter and a 580 nm band-emission filter.

2.5. Lipid extraction

Methanol extraction was performed according to Zhekisheva et al. (2002). For the three culture conditions, *H. pluvialis* biomass was harvested on day 14 by centrifugation at 3000*g* and lyophilized. Freeze-dried samples of 50 mg of biomass were treated with 1 mL DMSO for 5 min at 70 °C and further extracted with 5 mL of methanol at 4 °C during 1 h. The mixture was centrifuged at 3000*g*, the supernatant collected and the pellet re-extracted with methanol at 4 °C for 15 min. Peroxide-free diethyl ether, *n*-hexane and water were added to the methanol extract up to a final ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken in a separatory funnel, centrifuged, and the upper phase collected. The lower phase was acidified with acetic acid to pH 3–4 and re-extracted with a mixture of diethyl ether:hexane (1:1, v/v). The combined upper phases were evaporated to dryness under nitrogen and kept at −20 °C. Two replicates for each extraction were done. All chemicals used were analytical grade.

2.6. Lipid fractionation

Fractionation of lipids into neutral, glycolipids and phospholipids was performed using a silica cartridge Sep-Pack (SP) of 1000 mg, according to Berger et al. (1995). Briefly, this procedure included: (i) adsorbent conditioning with 30 mL of chloroform, (ii) sample loading, 1 mL of chloroform/oil solution containing 20 mg of oil; (iii) elution of neutral lipids from the adsorbent bed with 15 mL of chloroform: acetic acid (9:1, v:v); (iv) glycolipids recovery by elution with 20 mL acetone:methanol (9:1, v:v) and (v) phospholipids recovery by elution with 20 mL of methanol. Each fraction was collected into a conical vial and evaporated to dryness under nitrogen.

The efficiency of SP separation was verified by thin-layer chromatography (Silicagel G 60 70-230 mesh, Merck). New plates were pre-run in a tank containing chloroform: methanol (50:50, v:v) to remove contaminants from the silica gel. Concentrated solutions of each fraction in chloroform (10 mg/mL) were applied to the bottom of the plates and the plates were developed with chloroform: methanol (2:1, v:v). After evaporation of the solvent, the plates were sprayed with phosphomolybdic acid and heated at 120–130 °C.

2.7. Methyl ester derivation and fatty acid (FA) analysis

An aliquot of the lipids (about 25–30 mg) was weighed in a hermetic flask and 2 mL of 10% KOH methanolic solution was added

while shaking vigorously. The flask was sealed hermetically, the air space was purged with a stream of nitrogen, and the flask was heated for 45 min in a water bath at 80 °C. The unsaponifiable material was extracted using petroleum ether. The lower phase was treated with concentrated HCl and the liberated fatty acids were extracted with petroleum ether. The ether extract was dried under a nitrogen stream, 1.5 mL of BF_3 solution (10% in methanol) was added, and the sample was incubated at 80 °C for 30 min. Finally, fatty acid methyl esters (FAME) were extracted with petroleum ether, evaporated to dryness under nitrogen and chromatography-grade hexane was added to a final volume of 100 μL.

FAMEs were analyzed by gas chromatography with an HP Agilent 4890D gas chromatograph, equipped with a flame-ionization detector at a temperature of 260 °C, a split/splitless injector and a capillary column SP – 2560 (100 m, 0.25 mm and 0.2 μm) (Supelco Inc., Bellefonte, PA). The carrier gas was high purity hydrogen at 18 cm/s. The GC oven was initially held at 140 °C for 5 min; then, the temperature was increased at 4 °C/min to 240 °C and held isothermal for 15 min. The detection limit of the chromatographic method was set to 0.01%. The HP 3398A GC Chemstation Software (Hewlett Packard, 1998) was used for chromatographic analysis. FAMEs identification was performed by comparison with standard certificate material, Supelco FAME 10 mix 37 (Bellefonte, PA, USA), according to AOCS Official Method Ce 1b-89. Two replicates of each FAME analysis were done.

2.8. Calculated Iodine value

The iodine value of algal oils was calculated according to AOCS recommended practice Cd 1c-85. This method estimates the grams of halogen absorbed by 100 g of the fat.

2.9. Statistical analysis

The differences in mean values ($n = 4$) as well as between mean percentages ($n = 4$) of fatty acids were assessed with ANOVA and Student's t-test, being statistically different at a significance level of 5%.

3. Results

3.1. Growth measurements

Cultures stressed with high light intensity (condition A) and control cultures behaved in a similar way until the third day (Fig. 1). In control cultures, the maximum cell concentration of 1.1×10^6 cells ml^{-1} (0.79 div d^{-1} and doubling time of 21 h) occurred after 12 d. In A-stress cultures, the highest cell concentration of 5.2×10^5 cells ml^{-1} (0.56 div d^{-1} and doubling time of 30 h) was measured after 10 d. Cultures stressed with high light intensity and nitrogen-free medium (condition B) showed lower cell concentrations (1.8×10^5 cells ml^{-1} after 10 d) than A-stress cultures and did not exhibit an exponential phase.

3.2. Lipid droplets characterization and neutral lipid identification

TEM observations indicated the presence of cytoplasmatic oil droplets in *H. pluvialis* cysts (Fig. 2). Before the lipid extraction, the presence of neutral lipids was ascertained by observation of yellow-gold fluorescence droplets after Nile Red staining (Fig. 3).

3.3. Lipid composition

Under A- and B-stress conditions, the total lipid content (percentage dry weight = % dw) was 34.85%$^{(c)}$ and 32.99%$^{(c)}$, respectively, which was considerably higher than that observed under control conditions (15.61%$^{(b)}$, $\alpha = 0.05$) (Table 1). The neutral lipids were the majority of lipids under all culture conditions tested; however, the neutral lipid content increased significantly under both stress conditions, compared to control cultures. The phospholipid content was also higher in stressed than in control cultures. There was no significant difference in glycolipid content between control and stressed cultures. Moreover, no significant differences were found between glycolipids and phospholipids under the two stress conditions.

3.4. Fatty-acid composition

The fatty acid profile in *H. pluvialis* was similar under control and stress conditions and revealed that palmitic, estearic, oleic, linoleic, linolenic and linolelaidic acids were the major components (Table 2).

The percentage of saturated fatty acids (SFA) in the neutral lipid fraction was significantly higher in cultures grown under A- (30.36%) and B-stress (29.62%) conditions compared to the control (27.81%). No significant differences ($\alpha = 0.05$) were found between stressed cultures (Table 2). The palmitic acid content (C16: 0) declined under stress conditions, particularly in A-stress condition (Fig. 3). In contrast, the relative content of stearic acid (C18: 0) increased in both stress conditions.

The monounsaturated fatty acid content (MUFA) present in the neutral lipid fraction showed no significant differences between control and A-stress cultures; however, there was a lower MUFA percentage in algae grown under B-stress condition ($\alpha = 0.05$) (Table 2). The best-represented acid was the oleic acid.

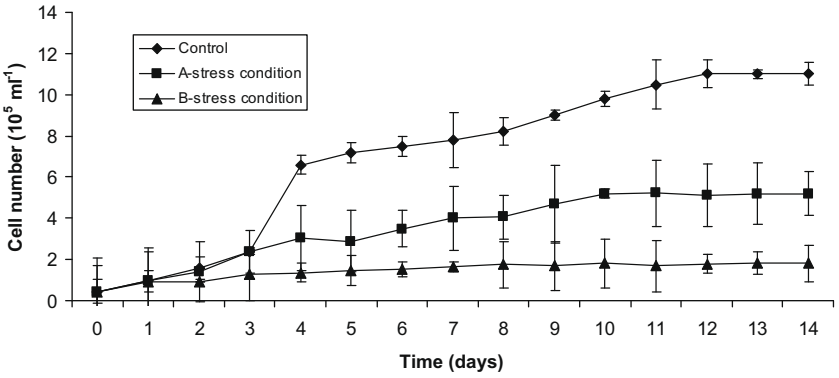

Fig. 1. Growth of *Haematococcus pluvialis* in control culture, in A-stress condition (continuous light with high intensity) and in B-stress condition (continuous light with high intensity and without nitrogen). Error bars denote standard deviations among replicates.

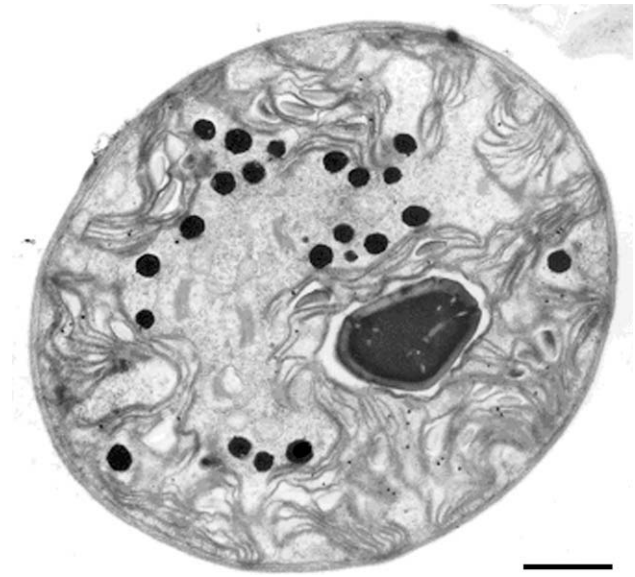

Fig. 2. Transmission electron micrograph of a *Haematococcus pluvialis* cyst. Cytoplasmatic lipid droplets in A-stress condition after 48-h growth are shown. Scale bar = 0.5 μm.

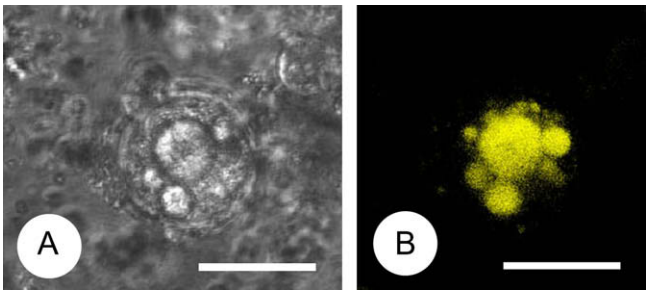

Fig. 3. Light micrographs of a *Haematococcus pluvialis* cyst. (A) Phase contrast microscopy. (B) Epifluorescent microscopy. Neutral lipid droplets in A-stress condition after 11-day growth are shown. Scale bars = 20 μm.

Regarding polyunsaturated fatty acids (PUFA) present in the neutral lipid fraction, there was a significant increase in the B-stress condition compared to the control and A-stress ones (Table 2). This increase may be attributed to higher proportions of linoleic (C18:2*n*6cis), linolelaidic (C18:2*n*6trans), and linolenic acids (C18:3*n*3).

The iodine value of *H. pluvialis* oil was 110.95 g I_2/100 g, 99.64 g I_2/100 g and 123.56 g I_2/100 g under control, A-stress and B-stress conditions, respectively.

4. Discussion

Nitrogen (Roessler, 1990; Rodolfi et al., 2009) and phosphorus deficiency (Siron et al., 1989) as well as extreme environmental conditions, e.g. high salinity (Roessler, 1990), high light intensity (Zhukova and Titlyanov, 2006) or extreme temperatures (Norman and Thompson, 1985) induce the accumulation of lipids in several microalgae. Although the accumulation of fatty acids under nitrogen starvation is a widely known phenomenon (Ben-Amotz et al., 1985; Yu et al., 1987; Roessler, 1990; Rodolfi et al., 2009), the effect of high light intensity on fatty acid content has not been studied extensively (Zhekisheva et al., 2002; Cerón et al., 2007). Thus, we were particularly interested in examining the relationship between lipid accumulation and continuous high light intensity in *H. pluvialis* cultures with or without nitrogen. The two stress conditions induced an increase of about 2-fold in the total lipid content; however, in cultures with high light intensity and without nitrogen (B-stress) the growth-rate was extremely low, compared to cultures with high light intensity (A-stress). The highest fatty acid content was obtained by Cerón et al. (2007) in *H. pluvialis* also growing under continuous high illumination, but under moderate limitation of nitrate availability. It is interesting to remark that the presence of some nitrogen source is necessary for the growth.

The fatty acid profile of the Argentinian strain of *H. pluvialis* was similar in control cultures and under both stress conditions. Zhekisheva et al. (2002) also found no differences in the fatty-acid composition in cultures of the German strain of *H. pluvialis* subjected to either high light intensity or nitrogen deprivation. Under both stress conditions, the oleic acid content decreases slightly in the neutral lipid fraction, but doubled and tripled in the glycolipid and phospholipid fractions, respectively. Zhekisheva et al. (2002) reported an increase in oleic acid content of the triglyceride fraction in *H. pluvialis*, which was reflected in the increased total lipid content. Cerón et al. (2007) also showed that the oleic acid was the major fatty acid present in cysts. The differences in oleic acid content observed in this study could be attributed to differences in growing conditions since the Argentinian strain cultures were not supplemented with CO_2.

The presence of linolelaidic acid (C18:2*n*6trans) in *H. pluvialis* had not been reported previously for other strains of *Haematococcus* (Zhekisheva et al., 2002; Rosa et al., 2005; Cerón et al., 2007). According to Khotimchenko and Yakovleva (2005), the increase in the relative content of trans fatty acids would be related to exposure to high light intensities; however, equal or even higher trans fatty acids contents were observed in neutral and polar lipid fractions in control cultures of *H. pluvialis*.

Whereas the fatty acid profile was qualitatively similar in the two stress conditions tested in *H. pluvialis*, some quantitative differences can be highlighted: (1) a significant increase of MUFAs and a decline of PUFAs' content ($\alpha = 0.05$) were observed in the neutral lipid fraction of algae cultured with high light intensity and full medium (A-stress). SFAs presented no significant differences in this fraction in both stress conditions. (2) The percentage of linolenic acid (C18:3*n*3) was higher in cultures growing under both high light intensity and nitrogen starvation (B-stress condition).

Not all algal oils are satisfactory or compatible with the engines used today. For example, hydrocarbons produced by *Botryococcus braunii* have a chain length greater than C30 (Banerjee et al.,

Table 1
Lipid content (percentage of dry weight biomass = % dw) and classes (neutral, glycolipid and phospholipid) in *Haematococcus pluvialis* under different culture conditions. Values are means ± standard deviations of three replicates. Identical superscripts indicate non-significant ($\alpha = 0.05$) differences.

H. pluvialis	Maximum cells (m^{-1})	k (Div. d^{-1})	Doubling time (h)	Total lipids (% dw)	Neutral lipids (% dw)	Glycolipids (% dw)	Phospholipids (% dw)
Control	1.1×10^6	0.79	21	$15.61 \pm 1.46^{(b)}$	$9.20 \pm 0.67^{(1)}$	$3.70 \pm 0.38^{(3)}$	$1.87 \pm 0.05^{(4)}$
A-stress condition	5.2×10^5	0.56	30	$34.85 \pm 0.78^{(c)}$	$19.80 \pm 0.14^{(2)}$	$7.85 \pm 1.77^{(3)}$	$9.50 \pm 0.00^{(3)}$
B-stress condition	1.8×10^5	0.07	246	$32.99 \pm 2.77^{(c)}$	$16.60 \pm 1.41^{(2)}$	$6.67 \pm 1.65^{(3)}$	$9.80 \pm 1.84^{(3)}$

Table 2
Fatty acid profile (percentage of total fatty acids) in neutral, glycolipid and phospholipid fractions in control and A- and B-stress culture conditions. Identical superscripts indicate non-significant differences in the values ($\alpha = 0.05$). Values are means of four replicates. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. tr: trace, nd: no detected.

	Neutral lipids			Glycolipids			Phospholipids		
Fatty acids	Control	A-stress	B-stress	Control	A-stress	B-stress	Control	A-stress	B-stress
C6:0	nd	1.33	0.25	nd	3.99	2.65	tr	2.50	0.93
C8:0	nd	0.27	0.13	nd	0.40	0.35	tr	0.40	tr
C10:0	tr	0.23	tr	nd	0.35	0.28	nd	0.48	0.25
C12:0	0.21	0.30	0.44	nd	tr	0.36	nd	nd	tr
C13:0	nd	nd	0.16	nd	nd	0.25	nd	nd	tr
C14:0	1.25	1.35	1.35	1.75	1.81	1.55	2.01	1.82	2.22
C14:1	tr	nd	nd	3.47	tr	tr	5.01	tr	tr
C15:0	0.19	0.27	0.44	3.86	0.79	0.47	5.54	0.79	0.44
C15:1	nd	nd	nd	5.19	tr	nd	7.27	nd	nd
C16:0	22.49	18.87	21.29	25.12	18.94	20.32	23.27	25.67	19.10
C16:1	0.64	0.58	0.83	0.59	0.88	1.47	0.51	0.82	0.76
C17:0	0.19	0.32	0.32	tr	0.43	0.47	0.47	0.51	0.47
C17:1	tr	tr	tr	tr	nd	tr	tr	tr	0.06
C18:0	3.15	7.07	5.69	3.98	9.62	10.80	4.38	7.45	5.68
C18:1*n*9t	tr	0.67	tr	tr	1.73	1.05	0.88	1.91	0.20
C18:1*n*9c	19.36	18.25	18.35	8.09	14.71	16.11	7.96	21.56	20.11
C18:2*n*6t	6.67	5.37	7.57	6.56	4.72	5.09	3.50	0.51	0.41
C18:2*n*6c	20.23	22.06	22.9	9.65	15.49	14.06	8.11	19.16	31.78
C20:0	0.2	0.32	0.27	tr	0.33	0.49	0.4	0.36	0.52
C18:3*n*6	0.86	1.02	0.95	0.60	0.37	0.30	0.8	1.50	0.70
C20:1	0.13	0.23	0.17	tr	tr	tr	nd	0.20	0.16
C18:3*n*3	16.18	12.01	18.69	20.51	12.45	11.80	10.67	6.01	7.78
C21:0	tr	tr	tr	tr	tr	tr	nd	nd	0.14
C20:2	0.32	1.15	1.59	0.71	tr	tr	0.97	0.35	1.07
C22:0	0.18	0.31	0.25	tr	0.37	0.73	0.4	0.35	0.44
C22:1*n*9	tr	0.17	0.16	nd	nd	2.76	nd	nd	nd
C20:4*n*6	0.89	1.21	0.84	0.26	0.68	0.84	0.55	0.58	0.62
C24:0	tr	0.20	tr	tr	0.11	0.37	0.42	tr	0.13
C20:5*n*3	0.57	0.48	0.49	tr	0.38	0.18	0.21	0.39	0.81
C22:5*n*3	nd	nd	nd	nd	nd	nd	0.15	0.19	0.31
SFA (%)	$27.81 \pm 0.42^{(a)}$	$30.36 \pm 1.19^{(b)}$	$29.62 \pm 0.73^{(b)}$	$34.71 \pm 4.32^{(d)}$	$38.06 \pm 5.97^{(d)}$	$40.23 \pm 8.34^{(d)}$	37.08 ± 1.38(e)	$46.91 \pm 3.19^{(f)}$	$30.14 \pm 9.31^{(e,o)}$
MUFA (%)	$20.07 \pm 0.06^{(g)}$	$19.91 \pm 0.12^{(g)}$	$18.96 \pm 0.65^{(h)}$	$17.34 \pm 2.02^{(1)}$	$18.14 \pm 6.09^{(1)}$	$17.78 \pm 1.73^{(1)}$	$19.69 \pm 3.01^{(j)}$	$24.49 \pm 3.81^{(j)}$	$21.08 \pm 0.73^{(j)}$
PUFA (%)	$45.80 \pm 0.18^{(k)}$	$43.15 \pm 0.68^{(l)}$	$47.23 \pm 0.56^{(m)}$	$38.28 \pm 1.95^{(d)}$	$35.01 \pm 2.63^{(d)}$	$36.35 \pm 2.55^{(d)}$	$24.97 \pm 4.38^{(j)}$	$29.51 \pm 5.58^{(j)}$	$43.07 \pm 14.06^{(o)}$

2002), while vegetable oils currently used for biodiesel are mainly C16 and C18 (Durrett et al., 2008). In fact, biodiesels need to comply with existing standards. The most important properties of biofuel, cetane number (ignition quality), cold-flow properties, oxidative stability, and iodine value, are determined by the structure of fatty esters, which are part of it (Knothe, 2005; Chisti, 2007). In turn, properties of fatty esters are determined by the characteristics of fatty acids; i.e., carbon chain length and degree of unsaturation, and the alcohol content (Knothe, 2005). Most microalgal oils differ from plant oils because they are rich in polyunsaturated fatty acids with four or more double bonds (Belarbi et al., 2000; Chini Zittelli et al., 2006; Rodolfi et al., 2009). This feature limits the algal species that can be used. According to the fatty acid profile observed in *H. pluvialis*, we could infer some of the features of the biodiesel that we would obtain from this alga. With regard to PUFAs, the maximum degree of unsaturation of the chains is three and the length of the fatty acid is intermediate with a maximum of 18 carbons. The iodine value is a measurement of the oil unsaturation (Kyriakidis and Katsiloulis, 2000; Knothe, 2002). The calculated iodine value of *H. pluvialis* oil obtained under A-stress condition (99.64 g I_2/100 g) was well below the European allowed biodiesel standards (120 g I_2/100 g) (Mittelbach, 1996; Chisti, 2007). In comparison, the B-stress condition presented a slightly higher iodine value (123.56 g I_2/100 g). However, it was lower than the registered value for other biodiesel feedstocks, such as the soybean oil (129.50%) and sunflower oil (125%) (Ferrari et al., 2005; Agba and Abanga, 2008).

H. pluvialis is considered a robust species that is easy to culture under optimal conditions (Vonshak and Richmond, 1985; Cohen, 1999); however, the conditions to induce lipid synthesis reduced their growth, just as reported for other microalgae (Siron et al., 1989; Illman et al., 2000; Zhekisheva et al., 2002; Rodolfi et al., 2009; Griffiths and Harrison, 2009). The lipid content was high under both stress conditions tested (34.85% and 32.99% weight of dry biomass), but not high enough to meet the requirements for commercial biodiesel production. Therefore, it will be necessary to further optimize culture conditions for this organism so that it becomes a viable source of biofuel. For example, it is known that CO_2 supplementation can increase lipid content, especially the triglyceride fraction (Gordillo et al., 1998; Huntley and Redalje, 2007). Furthermore, the strain must be tested under outdoor conditions to evaluate lipid production under a natural light source (Rodolfi et al., 2009). To increase biomass, a two-phase culture strategy could be implemented including an initial phase in nutrient-sufficient medium and low light intensity aimed at obtaining high biomass productivity. This phase should be followed by a lipid induction phase under high natural light intensity in open ponds (Borowitzka et al., 1991, 2005; Olaizola, 2000; García-Malea López et al., 2005, 2006; Masojídek et al., 2009). Northern Argentina has some of the best conditions, such as a warm climate, high solar irradiation and large expanses of land unfavorable to agriculture for testing small-scale outdoor cultures of *H. pluvialis* in open ponds.

Selection of fast-growing, productive strains, optimized for the local climatic conditions are of fundamental importance to the success of any algal mass culture and particularly for low-value products such as biodiesel (Griffiths and Harrison, 2009). According to Borowitzka (1997), it is also important to evaluate harvesting costs at the time of choosing the species. Low-cost harvesting requires large cell size, high specific gravity compared to the medium and

reliable autofloculation. With respect to *H. pluvialis*, the following features make it a potential resource of oil feedstock for biodiesel production: (1) its capacity to accumulate lipids in response to high light intensity or nitrogen deprivation, (2) neutral lipids as the main fraction, which is the preferred substrate for biodiesel production by trans-esterification, (3) hydrocarbon chain length shorter than C18, (4) polyunsaturated fatty acids with less than three unsaturated double bonds, (5) possibility of a combined production of biofuel and astaxanthin, which is a high-value co-product (Huntley and Redalje, 2007), (6) ease of cultivating in photobioreactors and at large scale in open ponds (Borowitzka et al., 1991; Borowitzka, 2005; Olaizola, 2000; García-Malea López et al., 2006; Huntley and Redalje, 2007; Masojídek et al., 2009), (7) big cell size that makes harvesting very easy and economic (Mata et al., 2010), and (8) the application of industrial mechanical methods conventionally used for cyst drying and astaxanthin extraction (Olaizola, 2000; Cysewski and Todd Lorenz, 2004).

A drawback of this species is the need for freshwater conditions, although other freshwater species have been tested (Rodolfi et al., 2009) and proposed for the purpose of biodiesel production (Gouveira and Oliveira, 2009).

5. Conclusions

This study provides baseline information for future investigations on improving growth rates lipid production features of *H. pluvialis*. The analysis of the lipids in *H. pluvialis* indicated the potential of this microalga as a biodiesel feedstock; however, culture conditions still have to be improved in order to achieve an adequate energy balance in mass culture.

Acknowledgements

Funds were provided by Secretaría de Ciencia, Tecnología e Innovación Productiva (SeCyT) de la República Argentina, Estudio Exploratorio No. E658/07PET29 and Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) PIP 112-200801-00234. We thank Mario Tredici and the Associate Editor Rolf Joerger for their valuable suggestions. P.I.L. is Research Member of CONICET.

References

Agba, E.H., Abanga, E.J., 2008. Effects of X-rays on the physico-chemical properties of soybean oil. Pac. J. Sci. Technol. 9, 665–667.

AOCS, 2001. In: Firestone, D.E. (Ed.), Official Methods and Recommended Practices of AOCS, fourth ed. The American Oil Chemistś Society, AOCS Press, Illinois.

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U.C., 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol. 22, 245–279.

Belarbi, E.H., Molina Grima, E., Chisti, Y.A., 2000. A process for high yield and scalable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. Enzyme Microb. Technol. 26, 516–529.

Ben-Amotz, A., Tornabene, T., William, T., 1985. Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. 21, 72–81.

Berger, J.P., Gouygou, J.P., Dubacq, J.P., Durand, P., 1995. Reassessment of lipid composition of the diatom *Skeletonema costatum*. Phytochemistry 39, 1017–1021.

Borowitzka, M.A., 1997. Microalgae for aquaculture: opportunities and constraints. J. Appl. Phycol. 9, 393–401.

Borowitzka, M.A., 2005. Culturing microalgae in outdoor ponds. In: Anderson, R.A. (Ed.), Algal Culturing Techniques. Elsevier, Burlington, USA, pp. 205–219.

Borowitzka, M.A., Huisman, J.M., Osborn, A., 1991. Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. J. Appl. Phycol. 3, 295–304.

Boussiba, S., 2000. Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol. Plant. 108, 111–117.

Boussiba, S., Bing, W., Yuan, J.P., Zarka, A., Chen, F., 1999. Changes in pigments profile in the green alga *Haematococcus pluvialis* exposed to environmental stress. Biotech. Lett. 21, 601–604.

Cerón, M.C., García-Malea, M.C., Rivas, J., Acien, F.G., Fernández, J.M., Del Río, E., Guerrero, M.G., Molina, E., 2007. Antioxidant activity of *Haematococcus pluvialis* cells grown in continuous culture as a function of their carotenoid and fatty acid content. Appl. Microb. Cell Physiol. 74, 1112–1119.

Ching-Piao, L., Liang-Ping, L., 2001. Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP1324. Bot. Bull. Acad Sin. 42, 207–214.

Chini Zittelli, G., Rodolfo, L., Biondi, N., Tredici, M.R., 2006. Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. Aquaculture 261, 932–943.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Cifuentes, A.S., González, M.A., Vargas, S., Hoeneisen, M., González, N., 2003. Optimization of biomasa, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions. Biol. Res. 36, 343–357.

Cohen, Z., 1999. Chemicals from Microalgae. Taylor and Francis, London. p. 419.

Costa Neto, P.R., Rossi, L.F.S., Zagonel, G.F., Ramos, L.P., 2000. Produção de biocombustivel alternativo ao óleo diesel a través da transesterificação de óleo de soja usado em frituras. Quim. Nova 23, 531–537.

Cysewski, G.R., Todd Lorenz, R., 2004. Industrial production of microalgal cell-mass and secondary products – species of high potential. In: Richmond, A. (Ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Publisher Ltd., pp. 281–288.

Damiani, M.C., Leonardi, P.I., Pieroni, O., Cáceres, E.J., 2006. Ultrastructure of the cyst wall of *Haematococcus pluvialis* (*Chlorophyceae*): wall development and behaviour during cyst germination. Phycologia 45, 616–623.

Durrett, T., Benning, C., Ohlrogge, J., 2008. Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. 54, 593–607.

Elsey, D., Jameson, D., Raleigh, B., Cooney, M.J., 2007. Fluorescent measurement of microalgal neutral lipids. J. Microbiol. Method 68, 639–642.

Fan, L., Vonshak, A., Boussiba, S., 1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis*. J. Phycol. 30, 829–833.

Ferrari, R.A., Oliveira, V.S., Scabio, A., 2005. Oxidative stability of biodiesel from soybean oil fatty acid ethyl esters. Sci. Agricola 62, 291–295.

Fidalgo, J.P., Cid, A., Torres, E., Sukenik, A., Herrero, C., 1998. Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. Aquaculture 166, 105–116.

García-Malea López, M.C., Brindley, C., Del Río, E., Acién, F.G., Fernández, J.M., Molina, E., 2005. Modeling of growth and accumulation of carotenoids in *Haematococcus pluvialis* as a function of irradiance and nutrients supply. Biochem. Eng. J. 26, 107–114.

García-Malea López, M.C., Del Río Sánchez, E., Casas López, J.L., Acién Fernández, F.G., Fernández Sevilla, J.M., Rivas, J., Guerrero, M.G., Molina, E., 2006. Comparative analysis of the outdoor culture of *Haematococcus pluvialis* in tubular and bubble column photobioreactors. J. Biotechnol. 123, 329–342.

Gordillo, F.J., Goutx, M., Figueroa, F.L., Niell, F.X., 1998. Effects of light intensity, CO_2 and nitrogen supply on lipid class composition of *Dunaliella viridis*. J. Appl. Phycol. 10, 135–144.

Gouveira, L., Oliveira, A.C., 2009. Microalgae as a raw material for biofuels production. J. Ind. Microbiol. Biotechnol. 36, 269–274.

Griffiths, M.J., Harrison, S.T.L., 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J. Appl. Phycol. 276, 23–25. 10.1007/s10811-008-9392-7.

Grünewald, K., Hirschberg, J., Hagen, C., 2001. Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. J. Biol. Chem. 276, 6023–6029.

Guillard, R.R.L., 1973. Division rates. In: Stein, J.R. (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, pp. 289–311.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45, 160–186.

Harker, M., Tsavalos, A.J., Young, A.J., 1995. Use of response surface methodology to optimise carotenogenesis in the microalga *Haematococcus pluvialis*. J. Appl. Phycol. 7, 399–406.

Hu, Q., Zhang, C.W., Sommerfeld, M., 2006. Biodiesel from algae: lessons learned over the past 60 years and future perspectives. J. Phycol. 42, 12.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621–639.

Huntley, M.E., Redalje, D.G., 2007. CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. Mitigat. Adapt. Strategies Global Change 12, 573–608.

Illman, A.M., Scragg, A.H., Shales, S.W., 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27, 631–635.

Khotimchenko, S.V., Yakovleva, I.M., 2005. Lipid composition of the red alga *Tichocarpus crinitus* exponed to different levels of photon irradiance. Phytochemistry 66, 73–79.

Knothe, G., 2002. Structure indices in FA chemistry. How relevant is the iodine value? J. Am. Oil Chem. Soc. 9, 847–853.

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process. Technol. 86, 1059–1070.

Kyriakidis, N.B., Katsiloulis, T., 2000. Calculation of iodine value from measurements of fatty acid methyl esters of some oils: comparison with the relevant American oil chemist's society method. J. Am. Chem. Soc. 77, 1235–1238.

Lang, X., Dalai, A.K., Bakhshi, N.N., Reaney, M.J., Hertz, P.B., 2001. Preparation and characterization of bio-diesels from various bio-oils. Bioresour. Technol. 80, 53–62.

Masojídek, J., Sergejevová, M., Rottnerová, K., Jirka, V., Korečko, J., Kopecký, J., Zaťková, I., Torzillo, G., Štys, D., 2009. A two-stage solar photobioreactor for cultivation of microalgae based on solar concentrators. J. Appl. Phycol. 21, 55–63.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renewable Sustainable Energy Rev. 14, 217–232.

Mendoza, H., Martel, A.M., Jiménez del Río, M., García Reina, G., 1999. Oleic acid is the main fatty acid related with carotenogenesis in *Dunaliella salina*. J. Appl. Phycol. 11, 15–19.

Mittelbach, M., 1996. Diesel fuel derived from vegetable oils, VI: specifications and quality control of biodiesel. Bioresour. Technol. 56, 7–11.

Norman, H.A., Thompson Jr., G.A., 1985. Effects of low-temperature stress on the metabolism of phosphatidylglycerol molecular species in *Dunaliella salina*. Plant Physiol. Biochem. 242, 168–175.

Olaizola, M., 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. J. Appl. Phycol. 12, 499–506.

Rabbani, S., Beyer, P., Lonting, J.V., Hugueney, P., Kleining, H., 1998. Induced β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. Plant Physiol. 116, 1239–1248.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100–112.

Roessler, P.G., 1990. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26, 393–399.

Rosa, A., Deidda, D., Serra, A., Deiana, M., Assunta Dessi, M., Pompei, R., 2005. Omega-3 fatty acid composition and biological activity of three microalgae species. J. Food Agric. Environ. 3, 120–124.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Markx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenerg. Res. 1, 20–43.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A Look Back at the US Department of Energy's Aquatic Species Program – Biodiesel from Algae. National Renewable Energy Laboratory, Golden, Colorado.

Siron, R., Giusti, G., Berland, B., 1989. Changes in the fatty acid composition of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* during growth and under phosphorus deficiency. Mar. Ecol. Prog. Ser. 55, 95–100.

Stein, J.R., 1973. Handbook of Phycological Methods. Culture methods and growth measurements, Cambridge University Press.

Vasudevan, P.T., Briggs, M., 2008. Biodiesel production current state of the art and challenges. J. Ind. Microbiol. Biotechnol. 35, 421–430.

Vonshak, A., Richmond, A., 1985. Problems in developing the biotechnology of algal biomass production. Plant Soil 89, 129–135.

Wiltshire, K.H., Boersma, M., Möller, A., Buhtz, H., 2000. Extraction of pigments and fatty acids from the green alga *Scenedesmus obliquus* (Chlorophyceae). Aquat. Ecol. 34, 119–126.

Xu, H., Miao, X., Wu, Q., 2006. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J. Biotechnol. 126, 499–507.

Yu, S., Hubbard, J.S., Holzer, G., Tornabene, T.G., 1987. Total lipid production of the green alga *Nannochloropsis* sp. QII under different nitrogen regimes. J. Phycol. 23, 289–296.

Yuan, J.P., Chen, F., 2000. Purification of trans-astaxanthin from a high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis*. Food Chem. 68, 443–448.

Zhekisheva, M., Boussiba, S., Khozin-Goldberg, I., Zarka, A., Cohen, Z., 2002. Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. J. Phycol. 38, 325–331.

Zhekisheva, M., Zarka, A., Khozin-Goldberg, I., Cohen, Z., Boussiba, S., 2005. Inhibition of astaxanthin synthesis under high irradiance does not abolish triacylglycerol accumulation in the green alga *Haematococcus pluvialis* (Chlorophyceae). J. Phycol. 41, 819–826.

Zhukova, N., Titlyanov, E., 2006. Effect of light intensity on the fatty acid composition of dinoflagellates symbiotic with hermatypic corals. Bot. Mar. 49, 339–346.