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Review

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

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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

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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 $45 \times 10^3$ 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