The Effect of Different Concentrations of Nitrogen and Phosphorous on the Production of Lipid Metabolites in *Haematococcus Pluvialis* UTEX 25051

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Research

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1 THE EFFECT OF DIFFERENT CONCENTRATIONS OF NITROGEN AND PHOSPHOROUS
2 ON THE PRODUCTION OF LIPID METABOLITES IN *Haematococcus pluvialis* UTEX 2505¹

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17
18 ABSTRACT

19 Microalgae as *Haematococcus pluvialis* produce lipid metabolites of great interest for different
20 industries as the nutraceutical, pharmaceutical, cosmetic, among other. Currently, one of the main
21 limitations for the massive commercialization of these metabolites are the low yields between cell
22 growth and the production of lipid metabolites. Thus, a better understanding of culture parameters
23 that allow to get a balance between both variables is needed. Herein we evaluated the effect of
24 Bold's Basal Medium (BBM) on the production of lipid metabolites of high biotechnological value
25 in *Haematococcus pluvialis* UTEX 2505. A factorial experimental design of 3² was used whereby
26 the concentrations of the nitrogen and phosphorus source (0.2, 0.6 and 1.0 g / l) in the BBM culture
27 medium were varied. Cell growth was measured, showed the highest concentration (1.148±0.842
28 g/l) for the treatment with the highest nitrate and phosphate content (a concentration of 1.0 g/l for
29 both). The Astaxanthin content showed a maximum value of 76.22 ± 3.02 µg/mL for treatment
30 with low nitrate (0.2 g/L) and medium phosphate (0.6 g/L). The antioxidant capacity was
31 determined by the ORAC method, achieving superior values to those found in the literature for
32 compounds such as vitamins E and C. The results suggested that low concentrations of nutrients,
33 nitrate and phosphate have a positive effect on the concentration of lipid metabolites and affirm
34 that the biomass of *H. pluvialis* UTEX 2505 is an excellent source of antioxidants and rich in
35 essential fatty acids for human nutrition.

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37 Key words: Astaxanthin, *Haematococcus pluvialis*, lipids, antioxidants, cell morphology.

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INTRODUCTION

Haematococcus pluvialis is a unicellular freshwater microalga that can synthesize a red pigment and potent antioxidant known as astaxanthin. In addition, when the conditions for cell growth are favorable, *H. pluvialis* grows as a green cell with two flagella (vegetative stage) that provide mobility. On the other hand, in extreme environmental conditions (i.e., nutrient limitation), the microalga undergoes physiological and morphological changes that lead to the loss of its flagella and the development of red cysts spanning the center of the cell (palmella stage) to its periphery, ultimately reaching the cytoplasm (aplanospore) stage, when there is an increase in lipid biosynthesis and carotenogenesis that allows *H. pluvialis* to survive in a dormant state (Saha *et al.*, 2013). During the aplanospore stage, when the maximum encystment is expected, *H. pluvialis* can produce 1-4% of the dry weight astaxanthin, from which approximately 95% of the carotenoid is esterified (5% is free astaxanthin, 25% di-ester and 70% monoester) with fatty acids. These are stored in cytosolic lipid bodies rich in triacyl-triglycerides (Camacho *et al.*, 2013; Chen *et al.*, 2015); recent studies (Shang *et al.*, 2016) note the existence of biochemical evidence that confirms that astaxanthin and cellular fatty acids have a linear correlation when *H. pluvialis* faces extreme conditions. For example, Chen *et al.* (2015) presents a possible mechanism of coordination for the synthesis of astaxanthin and fatty acids from *H. pluvialis*. However, this process needs to be further investigated for a broader understanding. Nevertheless, lipid metabolites such as astaxanthin have a worldwide market in many industries, such as food, cosmetics, etc. Due to their variety of applications (Orosa *et al.*, 2005) and the benefits they provide to humans (e.g., protection against numerous forms of cancer and UV rays, use in food supplements, amongst others) (Wang *et al.*, 2016; Satoh *et al.*, 2016), interest in efficiently obtaining them has increased in recent years.

Thus, the main biotechnological challenges for the bulk production of lipid metabolites from *H. pluvialis* are how to enhance the cell growth rate, as well as the synthesis and extraction yield of metabolites.

The latter two challenges have been explored in various ways to improve them by means of stress induction in microalgae. For instance, (Orosa *et al.*, 2005) found that different concentrations of NaNO₃ had a significant effect on cell growth and astaxanthin accumulation. Similarly, Choi *et al.* (2011) studied the impact of differing light intensities on *H. pluvialis* and determined that this can also affect astaxanthin concentration. Therefore, the present study aims to investigate the effect of low concentrations of nitrogen and phosphorus source on the morphology of *H. pluvialis* UTEX 2505, as well as other important parameters like specific growth rate, lipid content, the amount of astaxanthin and the antioxidant capacity, which will deepen the understanding of the incidence of the culture media to produce lipid metabolites in the microalgae.

MATERIALS AND METHODS

Microorganism

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The strain *Haematococcus pluvialis* UTEX 2505 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX) and the corresponding experimentation was carried out at the Laboratory of Biotechnology of the Argos Center for Innovation at EAFIT University in Medellín, Colombia.

Culture conditions

Initially, the cryogenized strain was thawed at 35 °C in a serological water bath (Mettler, WNB 7-45 Germany). Then, it was pre-inoculated in a solid culture Bold's Basal Medium (BBM), composed of (g/l): 25.0 NaNO₃; 2.5 CaCl₂·2H₂O; 7.5 MgSO₄·7H₂O; 7.5 K₂HPO₄; 17.5 KH₂PO₄; 2.5 NaCl; 10.0 EDTA Na₂; 4.9 FeSO₄; 11.5 H₃BO₄; 8.8 ZnSO₄; 1.4 MnCl₂·4H₂O; 0.7 MoO₃; 1.5 CuSO₄·5H₂O; 0.5 (NO₃)₂Co·6H₂O (Cifuentes *et al.*, 2003).

Subsequently, the biomass from the Petri dishes was inoculated in 40 ml of culture medium contained in a 50 ml Erlenmeyer flask and used as a pre-inoculum. Once sufficient biomass was obtained, 860 ml of sterile BBM culture medium was inoculated with 100 ml of algal suspension. The growing conditions were 1000 ml of work volume at a temperature 27 °C ± 3, artificial light (LED) with an intensity of 55 ± 4 μmol / m²s, enriched air with CO₂ at 3% and a pH of between 6.4 and 6.6.

Morphological evaluation

Microscopy

The structure of the cells of *H. pluvialis* UTEX 2505 was observed in an optical microscope Zeiss, Axio Scope A1 incorporating an Axiocam ICc 5 digital camera. Moreover, a Phenom G2 ProDesktop scanning electron microscope (SEM) was used to study the variation in cell surface morphology. For that purpose, the cells were concentrated by centrifugation at 4000 rpm for 10 minutes. The samples were fixed with 2.5% glutaraldehyde in 0.1 Molar of Cocodilatos Buffer at pH 7.4, then washed three times with a solution of PSB at 0.1M. Subsequently, the samples were gradually dehydrated with 20, 40, 60, 70 and 100% of ethanol. Finally, using supercritical fluids, they were dried using a Samdri - PVT - 3D, Tousimis. Furthermore, for the photographic shots in the SEM, a Gold-Palladium coating was made using an SC7620 Mini Sputter Coater (Gao *et al.*, 2012). The morphological evaluation of *H. pluvialis* UTEX 2505 was performed in two separated groups: a) based on different stages of growth; and b) based on the effect of the different concentrations of phosphate and nitrate on the last stage of growth (aplanospore).

Biomass quantification

The biomass of *H. pluvialis* UTEX 2505 was quantified by dry weight, with the samples taken every 24 hours until the stationary phase was reached (Castillo *et al.*, 2017). A three-branch manifold filtration vacuum was used, with sterile cellulose ester filters (*Advantec MFS, Inc*) with a diameter of 47 mm, pore size of 0.4 μm and moisture scales of Sartorius Mark 3.

Kinetic modeling

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The kinetic parameters of the growth of *H. pluvialis* UTEX 2505 were evaluated with the logistic model, using the following equation:

$$X = (X_{max}X_0 e^{\mu t})/(X_{max} + X_0 (e^{\mu t} - 1))$$

where X is the biomass, X_{max} the maximum biomass obtained, μ the specific growth rate and t the cultivation time in days.

Quantification of astaxanthin

A visible ultraviolet spectrophotometer Halo RB-10 was used to quantify astaxanthin with a wavelength of 472 nm and an extraction methodology suggested by Zhang *et al.* (2009). Then, 10 ml of 100% dimethylsulfoxide (DMSO) were added to the concentrated biomass, and this solution placed in an ultrasound VWR at 4 °C for 60 minutes. After 16 hours, the solution was transferred to a serological bath for 40 minutes at 85 °C. The equation used for the quantification of the astaxanthin was:

$$Astaxanthin(\mu g/ml) = (A_{492} * 10^6 * V)/(E_{1cm}^{1\%}) * 100$$

where A_{492} is the absorbance calculated at 492 nm, 10^6 the factor for expressing carotenoids, V the volume of the solvent used in ml, $E_{1cm}^{1\%}$ the specific extraction coefficient for astaxanthin in $\mu g/ml$, with a coefficient to cancel the percentage of 100.

Determination of total lipids

The extraction of the lipids was performed by immersion or simple reflux in Soxhlet (Luque de Castro *et al.*, 2010), using petroleum ether as the drag solvent at a boiling temperature of 57 °C. The extract with the lipid content (oil extract) was concentrated to reduced pressure, after which the methyl esters of the fatty acids were obtained through extraction from *H. pluvialis* UTEX 2505 by means of direct transesterification, as was suggested by (Dai *et al.*, 2014) using 7% of KOH of the weight of the oil extract. Next, it was mixed with methanol at a molar ratio of 1:6 for 20 min at 400 rpm and 60 °C.

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Table 1. Treatments of the factorial experimental design 3^2 for the study of the culture medium of *H. pluvialis* UTEX 2505.

Levels	Nomenclature	Nitrate	Phosphate	Nitrate (g/l)	Phosphate (g/l)
1	1	NaPa	High	High	1.0
0	0	NmPm	Medium	Medium	0.6
-1	-1	NbPb	Low	Low	0.2
1	0	NaPm	High	Medium	0.6
1	-1	NaPb	High	Low	0.2
0	1	NmPa	Medium	High	1.0
0	-1	NmPb	Medium	Low	0.2
-1	1	NbPa	Low	High	1.0
-1	0	NbPm	Low	Medium	0.6

The mixture was kept at room temperature for 18 h to separate the phase containing the esters from the glycerin. Finally, a gas chromatograph Shimadzu, GC 2014, was used. The volume of the sample injected was 1µl, with injection mode Split and a Column RT-2560 with a film thickness of 0.20µm, column length of 100m and an internal diameter of 0.25mm.

Determination of antioxidant capacity

The determination of the antioxidant capacity was carried out by the Fluorometric method known as the Oxygen Radical Absorbance Capacity (ORAC) assay (Zapata *et al.*, 2014) using TROLOX® as the standard solution. The runs were performed at a length of excitation and emission of 493 nm and 515 nm, respectively, according to the following equation:

$ORAC = ((AUC - AUC^{\circ}) / (AUC_{TROLOX} - AUC))f(TROLOX)$. The ORAC antioxidant capacity was expressed in micromoles of Trolox per 100g of sample. Whereas AUC is the area under the curve for the sample, AUC° is the area under the curve for control, while AUC TROLOX® is the area under the curve for TROLOX® and f the dilution factor of the extracts (Rautenbach *et al.*, 2010; Naranjo *et al.*, 2011).

Statistical experimental design

To study the effect of the variation of the concentration of nutrients on the production of lipid metabolites of *H. pluvialis* UTEX 2505, a factorial experimental design 3^2 was used, where

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macronutrients such as phosphates and nitrates were the factors and the decrease in concentration (with respect to the BBM) corresponds to the levels of 0.2, 0.6 and 1.0 g/l. The treatments were carried out in triplicate, and therefore there were a total of 27 experimental units.

The response variables were the cell growth, accumulation of astaxanthin and total lipids, to which an analysis of variance (ANOVA) was applied with a confidence level of 95%. The statistical analysis of the results was carried out using version XVI of the Statgraphics Centurión software.

RESULTS

Morphological evaluation

Figure 1.A corresponds to the morphological changes of *H. pluvialis* UTEX 2505 during its growth. Also, in Figure 1.B there was a transition from the green color (vegetative stage) to orange/brown (palmella stage) with the formation of small red cysts inside the cell and a gradual increase in cell diameter from 10 up to 40µm. Subsequently, from the palmella to aplanospore stage, the synthesis of astaxanthin increased and the culture went from being brown to red, along with the cells reaching up to 60µm in diameter. Meanwhile, Figure 1.C indicates the fact that there was a formation of roughness on the surface of the cell in the aplanospore stage, unlike the smooth surface of the vegetative stage. Regarding the effect of the factors on the aplanospore growth stage, no changes were observed on the surface of the *H. pluvialis* UTEX 2505 cell (Figure 2).

Effect of low concentrations of nitrate and phosphate on cell growth

The effect of reducing the concentrations of nutrients like the phosphates and nitrates was negative with respect to the cell growth variable. The NaPa treatment showed greater cellular growth, of 1.14g/l, while the treatments with low nitrates NbPm and NbPb showed the lowest cellular concentrations, 0.54 and 0.58g/l, respectively (Figure 3).

The cell growth (μ) of the treatments ranged from 0.13 to 0.36 days⁻¹, as shown in Table 1, while correspondingly the values of R² ranged from 0.84 to 0.99, which indicates a strong linear relationship amongst the regression variables. With the ANOVA, it was determined that there are no significant differences between the treatments with respect to μ .

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Figure 1. Micrographs of *H. pluvialis* UTEX 2505 in its main three growth stages: A. Microalga in suspension B; state of the cells taken in the optical microscope C; cell surface morphology taken with SEM.

Figure 2. Morphology of the cell surface in the last stage of cell growth (aplanospora), taken with SEM.

The ANOVA presented a significant difference amongst the treatments for the response variable of productivity, where NaPa reached values of up to 0.05g/l day.

Extraction of astaxanthin

Figure 4 displays the results obtained for the effect of the factors on the production of the astaxanthin of *H. pluvialis* UTEX 2505 and it was found with the ANOVA that there are statistically significant differences amongst the treatments, with a P-value of 0.000. The treatment N_bP_m showed greater accumulation of the astaxanthin, with 76.22 µg/ml, while the lowest accumulation of the carotenoid was 39,27µg/ml on average with the treatment N_aP_a.

The equation that best describes the behavior of astaxanthin with respect to the factors is shown below, with an adjusted R^2 of 73.6%.

$$\begin{aligned} \text{Astaxanthin} = & 32.4163 - 20.9699 * \text{Nitrate} - 7.07806 * \text{Phosphate} + 9.83783 \\ & * \text{Nitrate}^2 - 12.1868 * \text{Nitrate} * \text{Phosphate} + 0.4385 * \text{Phosphate} \end{aligned}$$

Lipid extraction

The results for the total lipids, saturated and unsaturated fatty acids, are shown in Figure 5. The highest lipid accumulation was of 6.1g /100g and was obtained with NmPb, while in contrast the lowest accumulation was 3.46g/100g with NbPb. The ANOVA showed significant differences amongst the treatments, with a P-value of 0.0023.

Table 2. Kinetic parameters obtained from the logistic model with *H. pluvialis* UTEX 2505.

Treatment	μ (day ⁻¹)	Xmax (g/l)	Productivity (g/l days)	R ²
NaPa	0.15 ± 0.085	1.15 ± 0.057	0.053 ± 0.002	0.89
NaPm	0.18 ± 0.023	1.02 ± 0.051	0.046 ± 0.002	0.88
NaPb	0.14 ± 0.048	0.90 ± 0.045	0.044 ± 0.002	0.99
NmPa	0.36 ± 0.503	0.77 ± 0.038	0.035 ± 0.001	0.91
NmPm	0.24 ± 0.413	0.77 ± 0.038	0.032 ± 0.001	0.95
NmPb	0.29 ± 0.223	0.71 ± 0.036	0.043 ± 0.002	0.95
NbPa	0.29 ± 0.212	0.69 ± 0.034	0.031 ± 0.001	0.93
NbPm	0.26 ± 0.826	0.54 ± 0.027	0.022 ± 0.001	0.84

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NbPb	0.23 ± 0.346	0.58 ± 0.029	0.024 ± 0.002	0.96
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Figure 3. Growth kinetics for the treatments, where X is the biomass in g/l in a time range of 18 days.

Figure 4. Relationship between the concentration of astaxanthin in µg/ml of *H. pluvialis* UTEX 2505 and the maximum biomass for all the treatments. Equal letters correspond to statistically homogeneous groups according to Fisher's minimum significant difference test (LSD), with a confidence level of 95%.

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Figure 5. A. Lipid concentration (g/100g of biomass) of *H. pluvialis* UTEX 2505 for the different treatments. B. Percentage of the main fatty acids synthetized with each treatment.

Figure 6. Antioxidant capacity, measured in μ moles, of the TE/100g sample.

Determination of antioxidant capacity

The results obtained for the antioxidant capacity by means of the ORAC method are presented in Figure 6, where the greater antioxidant capacity was for the treated NmPb with a value of 3774.59 $\mu\text{mol TE/100g}$ sample and standard deviation of ± 37.92 . On the other hand, the lowest capacity was 2534.68 ± 98.9 $\mu\text{mol TE/100g}$ sample, obtained with NaPa.

DISCUSSION

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Morphological changes were detected in the different stages of growth of *H. pluvialis* UTEX 2505, indicating that the limitation of nutrients generates stress in the cells. In the same manner, the transition of the color (Figure 1.A.) during the growth stages suggests that in the maturation of the cell, changes in the photosynthetic pigments take place, while the accumulation of the antioxidant generates an alteration in photosynthesis that moves from an active state to one of rest. Lv *et al.* (2016) reported that the content of chlorophyll *a* decrease as it goes through the vegetative, palmella, aplanospore stage to reach 17.3, 6.2 and 100 mg/g, respectively. Furthermore, chlorophyll *b* presents a similarly decreasing tendency (16.7 to 0.9mg/g) throughout the cultivation time.

With respect to the morphological change during the vegetative and aplanospore stages, it is apparent that an increase in the synthesis of astaxanthin and total carotenoids can be increased by up to 14.6 and 8.5 times, respectively (Figure 1), as previously suggested (Lv *et al.*, 2016).

In Figure 1.C, the cell in the aplanospore stage (right) presents some irregularities on the surface, in contrast to the vegetative stage (left) where it is smoother or less rough, indicating that the surface of the cell undergoes slight modifications when there is an accumulation of astaxanthin.

Despite the effect of the factors (nitrogen and the phosphorus source) on the surface of the microalga during its last stage of growth, no representative morphological changes were observed between the cells, although authors such as Miranda *et al.* (2019) suggest that the limitation of nutrients such as nitrogen and phosphorus decrease the rigidity in the cell wall of the microalga, which could be associated with the accumulation of astaxanthin.

The results of the present study in relation to cell growth are comparable to those reported by Nagaraj *et al.*, (2017), where varying stress conditions on *H. pluvialis*, especially macronutrient limitations such as phosphorus and nitrogen, negatively affected the cell growth.

It is possible that this can be explained from the perspective of cellular metabolism because the deficiency of nitrogen reduces the division of the cell, while the limitation of phosphorus hinders the transfer of energy and the formation of membranes, amongst other biological functions (Harker *et al.*, 1996; Nagaraj *et al.*, 2012).

The biomass productivity of *H. pluvialis* UTEX 2505 is comparable to that reported in the literature, of 0.06 g/l day (García-Malea *et al.*, 2005) and 0.052 g/l day (Olaizola, 2000). Pan-utai *et al.* (2017) and Li *et al.* (2017) increased biomass productivity by modifying conditions such as CO₂ injection, light intensity, and the design of the photobioreactor to obtain values of 0.35 g/l day and 0.66 g/l.

As is shown in Figure 4, the NbPm treatment generated the maximum carotenoid accumulation of 76.216 µg/ml in comparison to the others. It indicates that low concentrations of nutrients such as nitrates and phosphates favor the synthesis of antioxidants, while it also maintains the cells of *H. pluvialis* UTEX 2505 in vegetative growth for longer time periods alongside the cultivation, thus reaching good cell density. It is important to highlight that one of the main bottlenecks to the scaling of this process to industrial production is the low productivity of biomass and high carotenoid synthesis (Panis & Carreon, 2016).

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However, Nahidian *et al.* (2018) note that this behavior could be due to the role that phosphorus plays in the synthesis of chlorophyll through the increased production of ATP and polyphosphate accumulation. On the other hand, Scibilia *et al.* (2015) underline that the starvation of nitrogen promotes the biosynthesis and accumulation of astaxanthin, as the cells of *H. pluvialis* redirect their metabolism to the accumulation of lipids (monomeric or oligomeric acids) and carotenoids.

Thompson (1996) reports that, when reducing cell growth due to nutrient deficiency, the fatty acids that are still produced are no longer used for the construction of new membranes but are stored in the form of triglycerides to be used as energy when the conditions for growth improve. This is the reason why the lipid content under stress conditions can triple the percentage of lipids compared to a culture under more favorable conditions (Lei *et al.*, 2012). These results are comparable to those obtained in the present study, where the lipid content increased as the concentration of the macronutrients (nitrogen and phosphorus) decreased.

It should be noted that the treatments with the lowest concentration (0.2g/l) of nitrate underwent a gradual decreasing of the lipid content as the phosphorus also decreased. The fatty acids in *H. pluvialis* UTEX 2505 can be contrasted with those reported by Sanchez *et al.* (2012) and Damiani *et al.* (2010) for the same microalgae strain, being the linoleic acid (also known as omega 3), which is obtained in greater quantities and stands out for its high commercial value.

From the higher values of the antioxidant capacity of all of the treatments (ORAC), it is possible to affirm that the biomass of *H. pluvialis* UTEX 2505 is an excellent source of natural antioxidants, as they feature superior values to fruits and extracts considered with high-capacity antioxidants, such as the strawberry (540 $\mu\text{mol TE}/100\text{ g}$; see Ahmed *et al.*, 2014), blueberries (46 $\mu\text{mol TE}/100\text{ g}$; see Prior *et al.*, 2000; and Huang *et al.*, 2002) and cinnamon (1256 $\mu\text{mol TE}/100\text{ g}$; see Su *et al.*, 2007). The N_mP_b treatment showed greater antioxidant capacity (3774. 59 $\mu\text{mol TE} / 100\text{ g}$) against the other treatments, and therefore it has a high power of absorption of free radicals and can serve as a nutritional supplement to counteract the oxidative stress.

CONCLUSIONS

According to the results obtained, the samples with medium concentrations of nitrogen and low concentrations of phosphorous had a positive effect on the accumulation of lipid metabolites in *H. pluvialis* UTEX 2505. Also, with this concentration it was achieved a maximum peak of cell growth of 0.7 g/L during the 12 day of culture and 55 ug/ml of astaxanthin. There were morphological changes, but they were not significative.

Likewise, this treatment showed a high antioxidant capacity of 3774. 59 $\mu\text{mol TE} / 100\text{ g}$ compared to the other treatments, obtaining the property of counteracting the oxidative stress. That is why biomass obtained is postulated as a potential product for the nutraceutical industry due to the accumulation of astaxanthin, its significative lipid content and superior antioxidant value over classical compounds such as vitamins E and C.

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336 COMPETING INTERESTS

337 The authors declare have no conflicts of interest to disclose.

338 AVAILABILITY OF DATA AND MATERIALS

339 The data that support the findings of this study are available from Argos S.A.S. and EAFIT
340 University, but restrictions apply to the availability of these data, which were used under license
341 for the current study, and so are not publicly available. Data are however available from the authors
342 upon reasonable request and with permission of Argos S.A.S. and EAFIT University.

343 ETHICS APPROVAL AND CONSENT TO PARTICIPATE

344 Not applicable

345 CONSENT FOR PUBLICATION

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349 AUTHORS' CONTRIBUTIONS

350 Brenda Hoyos, Alejandra Miranda, Diana Meneses, Gabriel Vargas and Alex Sáez contributed to
351 the design and implementation of the research, to the analysis of the results and to the writing of the
352 manuscript.

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SUMMARY OF ABBREVIATIONS

Abbreviations	
University of Texas at Austin	UTEX
Scanning electron microscope	SEM
Dimethylsulfoxide	DMSO
Oxygen radical absorbance capacity	ORAC
Analysis of variance	ANOVA
Cell growth	μ
Maximum biomass	Xmax

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Figures

Figure 1

Micrographs of *H. pluvialis* UTEX 2505 in its main three growth stages: A. Microalga in suspension B; state of the cells taken in the optical microscope C; cell surface morphology taken with SEM.

Figure 2

Morphology of the cell surface in the last stage of cell growth (aplanospora), taken with SEM.

Figure 3

Growth kinetics for the treatments, where X is the biomass in g/l in a time range of 18 days.

Figure 4

Relationship between the concentration of astaxanthin in $\mu\text{g/ml}$ of *H. pluvialis* UTEX 2505 and the maximum biomass for all the treatments. Equal letters correspond to statistically homogeneous groups according to Fisher's minimum significant difference test (LSD), with a confidence level of 95%.

Figure 5

A. Lipid concentration (g/100g of biomass) of *H. pluvialis* UTEX 2505 for the different treatments. B. Percentage of the main fatty acids synthesized with each treatment.

Figure 6

Antioxidant capacity, measured in μ moles, of the TE/100g sample.

Supplementary Files

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