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Research Note

Effect of *Ascophyllum nodosum* extracts on the growth, yield and antioxidant capacity of *Haematococcus pluvialis* carotenoids

Efecto del uso de extractos de *Ascophyllum nodosum* en el crecimiento, producción y actividad antioxidante de carotenoides de *Haematococcus pluvialis*

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Abstract.- Recent research has shown that the use of biological extracts, which contain a wide variety of nutrients and phytohormones are potentially useful in the production of plants of agricultural importance. Seaweed extracts are particularly rich in minerals and micronutrients necessary for the growth of microalgae. The effect of three commercial extracts of *Ascophyllum nodosum* as plant growth inducers (Stimplex®, Acadian Soils® and Liquid Seaweed Concentrate®), provided by Acadian Sea Plants Ltd., were evaluated in relation to the growth and production of biomass and carotenoids in *Haematococcus pluvialis*. The results were evaluated by one-way ANOVA and post-hoc analysis utilizing Tukey's test with a significance level of 95% (α= 0.05). The reference medium used was the Bold basal medium, to which 250 ppm of each extract was added. The Stimplex® extract had an increase of 22.79% in cell density and 17% in dry matter, higher than the control culture. The content of carotenoids and antioxidant capacity, respectively, with Stimplex® was 30.05% and 141.76% higher than the control culture.

Key words: Antioxidant capacity, *Ascophyllum nodosum*, carotenoids production, *Haematococcus pluvialis*

Introduction

Haematococcus pluvialis is freshwater microalgae which under stress conditions such as nutrient limitation and excessive irradiation or drought conditions accumulates high value carotenoids (Gómez *et al*. 2016, Zhao *et al*. 2019). Thus, *H. pluvialis* is commercially exploited for its high content of metabolites echinenone, canthaxanthin, adonirubin and astaxanthin (Hernández & Labbé 2014). The cost of these metabolites is due in part to their enormous demand in the pharmaceutical and food industries, coupled with their low production of biomass (0.5-5.0 g L^{-1} dry weight) and the poor accumulation of carotenoids (4% of dry weight) (Collins *et al*. 2011, Vandamme *et al.* 2013). This issue has generated the development of a series of research projects seeking to reduce the growth period of *H. pluvialis* and promote a greater production of the metabolites of interest (Panis & Carreon 2016). In this regard (Fábregas *et al*. 2000), the physicochemical conditions of growth, as well as design and operation of photobioreactors for mass production has been highlighted (Martinez *et al*. 2010). Correspondingly, to date, the possibility of using natural growth inducers (*i.e*., bioactives extracts) has been reported in the cultivation of microalgae (Raposo & Morais 2013, Beltrán-Rocha *et al.* 2017). In addition, the use of purified phytohormones such as auxins, cytokinins, abscisic acid and indol acetic acid have been used successfully to promote the growth of their plant counterparts (Lu & Xu 2015, Rowe *et al*. 2016, Kumar *et al*. 2017). For instance, in a research conducted with the microalgae *Scenedesmus obliquus*, it was shown that phytohormones accelerate growth and increase the quantity of the fatty acids (up to 59%) used for biodiesel production (Salama *et al*. 2014). It was also shown that auxins and indole-3-acetic acid benefit the process of photosynthesis and the accumulation of lipids in *Chlorella vulgaris* (Liu *et al*. 2016). Similarly, it was found that the

gibberellins used in a culture of *Aurantiochytrium* sp. at a concentration of 4% significantly increased its biomass, lipid content and docosahexaenoic acid levels (Yu *et al*. 2016). The marine macroalga *Ascophyllum nodosum* belonging to the Phaeophytes family provided minerals, macronutrients and growth hormones that significantly promote root and foliar development in plants (Rahmann *et al*. 2017). This is comparable to green microalgae because of their photosynthetic and their physiological similarity, as well as their chemical nature; there is the possibility that such stimulating effects on plant growth are also reflected in microalgae such as *H. pluvialis*. For this reason, the exploration of the potential stimulation of the commercial market for products made from extracts of *A. nodosum* on the production of biomass and carotenoids, as well as their antioxidant activity on *H. pluvialis*, has been proposed in this research.

Table 1. Composition of the extracts *A. nodosum* (provided by Acadian Sea Plants Ltd.) / Composición de los extractos de *A. nodosum* (proporcionada por Acadian Sea Plants Ltd.)

Composition (%)	Stimplex®	Liquid seaweed concentrate®	Acadian soils®
Total nitrogen	0.3	0.1	0.34
Phosphorous (P_2O_5)	1	-	-
Potassium (K_2O)	4	5	6.8
Sulfur (S)	0.2	8.1	1.58
Calcium (Ca)	2	1	0.12
Boron (B)	0.15	0.25	0.65
Magnesium (Mg)	0.03	0.05	-
Manganese (Mn)	-	0.09	0.04
Zinc (Zn)	0.05	0.1	0.07
Organic matter	8	5	14.16
Cytokinins	0.01	-	-
pH	8	5	8

Materials and methods

Ascophyllum nodosum extracts

Three extracts were obtained by Acadian Sea Plants Ltd. and included the following: Stimplex® (S), Acadian Soils® (F) and Liquid Seaweed Concentrate® (AE). The main components are shown in Table 1.

Culture of *Haematococcus pluvialis*

H. pluvialis CIB 68, which was acquired from the microalgae collection of the Northwest Biological Research Center S.C., Baja California Sur, Mexico (CIBNOR, Spanish abbreviation), was cultured in a 3 L type air lift photobioreactor (Ranjbar *et al*. 2008), with constant light (24 h) of 40 µmol photon m^{-2} s^{-1}, at a temperature of 25 °C, and with an air flow of 1 L-min^{-1}. At the beginning of the experiment each photobioreactor was inoculated with 1 x 10^4 cell-mL^{-1} in the haematocystic phase. The initial volume on day 0 was 3 L. The Bold basal medium (BBM) was used as control. Medium was prepared as follows: $NaNO_3$ 2.94 mM, NaCl 0.43 mM KH_2PO_4 1.29 mM, K_2HPO_4 0.43 mM, $MgSO_4 \cdot 7H_2O$ 0.3 mM, H_3BO_3 18.5 mM, $MnCl_2 \cdot 4H_2O$ 0.71 mM, $CaCl_2.2H_2O$ 0.17 mM, $ZnSO_4.7H_2O$ 1.44 mM, $CuSO_4 \cdot 5H_2O$ 320 mM**,** MoO_3 1.57 mM, $FeSO_4 \cdot 7H_2O$ 0.179 mM and Na_2.EDTA 55.3 mM (Stein 1973). The experiment consisted of three treatments, in which 250 ppm extract of *A. nodosum* was added to the BBM as follows: In the first treatment 250 ppm of S (BBM-S) was added, in the second treatment 250 ppm of F (BBM-F) was added and in the third treatment, 250 ppm of AE (BBM-AE) was added. At the same time, a control without an extract was carried out (BBM). All treatments were performed in triplicate. The cultivation time was 17 days (stationary phase). Subsequently the cultures were stressed for 10 days to induce carotenogenesis (as will be described below). To later evaluate cell growth, total carotenoids and antioxidant capacity. Finally, the results were subject to one-way ANOVA and Tukey tests with a significance level of 95% (α= 0.05).

Determination of cell growth

Cell density was measured using a Neubauer hematocytometer. To evaluate the production of biomass in dry weight (dw) of *H. pluvialis*, a 15 mL sample culture was taken and filtered on glass fiber filter Whatman GF/F, 47 mm (w_1) with pore size 0.7 µm, the vacuum pressure differential was maintained at 35-55 mm Hg and dried at 70 °C for 48 h (w_2) (Zhu & Lee 1997). The value was obtained using the following formula:

$$\text{Biomass (\%dw)} = \left(\frac{w_2 - w_1}{w_1} \right) \times 100 \times DF \quad \text{(Eq. 1)}$$

Where *DF* is the dilution factor.

Stress induction in *H. pluvialis*

H. pluvialis was induced to stress by nitrogen depletion. The light intensity was 60 µmol m^{-2} s^{-1}, and the adjustment of sodium chloride to 17.1 mM and sodium acetate to 4.4 mM (Vidhyavathi *et al.* 2008) for a period of 10 days.

Total carotenoids and antioxidant capacity

The carotenoids were extracted in the dark using a chloroform-methanol-water ratio of 2:2:1.8 ratio (Bligh & Dyer 1959). The quantification of total carotenoids was performed through UV-Vis spectrophotometry Beckman DU-650 (Vo *et al*. 2017). The chloroform was evaporated in a rotary evaporator and the residue was dissolved in methanol and adjusted to 100 mL. Antioxidant activity of the carotenoids extract of each treatment was determined by the 2.2 diphenyl-1-picrylhydrazyl (DPPH) method (Marxen *et al*. 2007). This analysis was a measure of TEAC (trolox equivalent antioxidant capacity).

Results and discussion

The logarithmic growth phase occurred in all treatments after 5 days. After 17 days of cultivation, the cell density reached the stationary phase, as can be seen in (Fig. 1). The maximum growth with respect to the control was obtained with the BBM-S.

In the present work, different commercial extracts of *A. nodosum* were used in the effort to stimulate different parameters of growth and productivity of *H. pluvialis* when cultured in a photobiorector using BBM as a growth substrate. The significant effect was shown by the S extract with respect to the other 2 extracts (F and AE), allowing us to infer that the formulation of the first contains some components that may be stimulating this effect.

The BBM-F and BBM-AE obtained the lowest values (Fig. 2). Regarding the production of biomass in dry weight, the trend remained the same with a maximum value of 5.85% in the BBM-S and the lowest value was observed in the BBM-EA treatment, with 4.40%. At the end of the experiment (17 days), carotenoid production was significantly high in the BBM-S treatment, while BBM-F and BBM-AE treatments remained statistically equal. After the BBM-S treatment, the highest concentration of carotenoids was found in the BBM (control) (Fig. 3). The antioxidant capacity (DPPH method) of the carotenoids produced after 17 days of culture indicates that the BBM treatment was the one displaying a lower antioxidant capacity. The BBM-F and BBM-AE treatments yielded the same antioxidant capacity (Fig. 4).

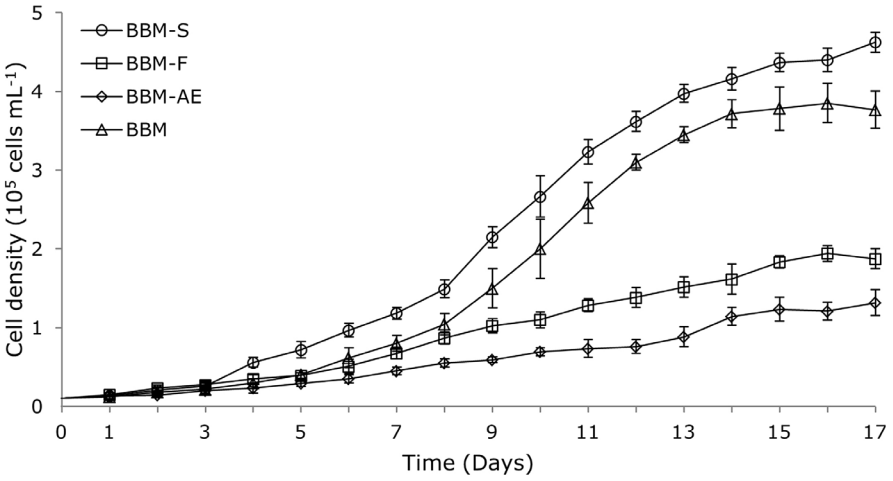

Figure 1. Growth kinetics of *H pluvialis* stimulated the different extracts of *A. nodosum* (n= 3) / Growth kinetics of *H. pluvialis* with different extracts of *A. nodosum* (n= 3)

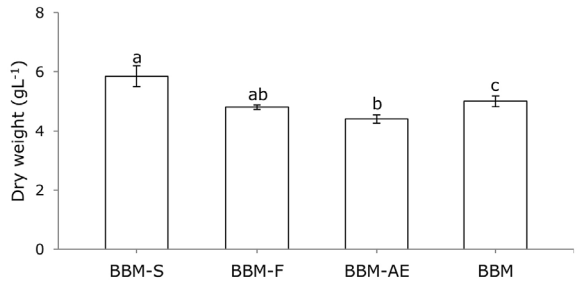

Figure 2. Dry weight of *H. pluvialis* obtained with different extracts of *A. nodosum* at 17 days of culture. One-way ANOVA. Different letters indicate significant difference (Tukey, α= 0.05) / Concentración de biomasa (peso seco) de *H. pluvialis* obtenida con diferentes extractos de *A. nodosum* a 17 días de cultivo. ANOVA de una vía. Letras diferentes indican diferencias significativas (Tukey, α= 0,05)

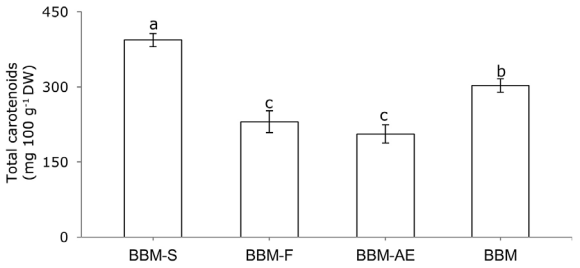

Figure 3. Total carotenoids analyzed in dry matter of each treatment at 17 days of culture. One-way ANOVA. Different letters indicate significant difference (Tukey, α= 0.05) / Concentración de carotenoides totales (base seca) de cada tratamiento a 17 días de cultivo. ANOVA de una vía. Letras diferentes indican diferencias significativas (Tukey, α= 0,05)

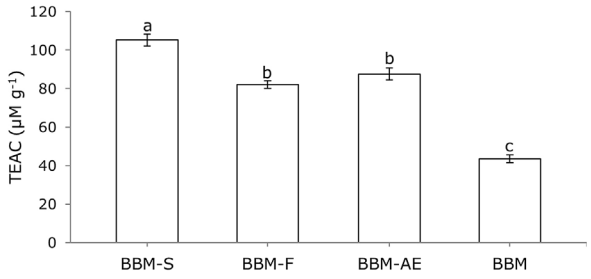

Figure 4. Trolox equivalent antioxidant capacity analyzed in 100 g dry weight of each treatment at 17 days of culture. One-way ANOVA. Different letters indicate significant difference (Tukey, α= 0.05) / Capacidad antioxidante equivalente de Trolox analizada en 100 g (base seca) para cada tratamiento a 17 días de cultivo. ANOVA de una vía. Las diferentes letras indican una diferencia significativa (Tukey, α= 0,05)

The increasing effect of the biomass production with the BBM-S treatment was probably due to the content of cytokinins present in the extract, which stimulates carbon fixation in plants through an increased activity of the enzymes involved in photosynthesis (Chernyad'ev 2009, Renuka *et al.* 2017).

According to the manufacturer data sheet, the main difference between the 3 commercial products tested is the content of cytokinins in S extract, whose proportion is 0.01% (0.0025 ppm in BBM-S). In a research conducted by Kurepa *et al.* (2018), a significant effect on the growth of *Lemna gibba* was demonstrated from a dose of synthetic cytokinins of 0.00225 ppm, reaching its maximum value at 0.225 ppm; while in a trial with *Spirodela polyrhiza*, a significant effect on growth at 0.225 ppm of cytokinins was observed. Therefore, it is likely that these plant growth stimulant molecules are also manifesting a stimulatory effect on the microalgae *H. pluvialis* by making it grow under our experimental conditions. The positive effect of commercial extracts of *A. nodosum* has been previously documented by Khan *et al.* (2011) who supplied the Stimplex product to *Arabidopsis thaliana* cultures with the intention of growth response detection. In this case, a stimulatory effect was observed that was attributed to the cytokinins present in such commercial product. On the other hand, in the study conducted by Morais *et al.* (2013), it was found that adding 1 mg of kinetin and 1 mg of auxin to the culture of *H. pluvialis* could increase the cellular growth of this microalga up to 290% with respect to the control crop.

According to Foo *et al.* (2017), microalgae contain bioactive compounds, mainly carotenoids, which are the main contributors of antioxidant capacity in microalgae. The BBM-S was producing the carotenoids mixture with greatest antioxidant capacity (Fig. 4). Compared to BBM-S, the antioxidant capacity of the carotenoids obtained from BBM-F and BBM-AE treatments were lower. This probably indicated that these extracts increased tolerance to stress, which may explain the lower synthesis of carotenoids, as well as their lower antioxidant capacity. The above correlates with a study carried out by Santaniello *et al.* (2017), in which was shown that tolerance to dehydration and stress increased when treating plants of *Arabidiopsis* sp. with an extract of *A. nodosum*. This may explain the low production of carotenoids induced by BBM-F and BBM-AE. On the other hand, Zhao *et al.* (2018) reports in *H. pluvialis* a significant increase in the

concentration of carotenoid astaxanthin in response to stress conditions due to nitrogen-deficiency. Observing that BBM-S increases the productivity of biomass in *H. pluvialis*, it is necessary to try new strategies for the induction of stress in *H. pluvialis* after the use of this product.

Treatment BBM-S induces a higher production of biomass, carotenoids, and antioxidant capacity in *H. pluvialis* in autotrophic conditions correlated to the cultivation in BBM. BBM-F and BBM-EA, which have a lesser effect on the production of biomass, carotenoids and antioxidant activity than BBM-S, but greater than the BBM.

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