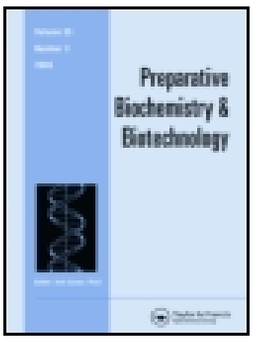

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Enhancement of astaxanthin production from *Haematococcus pluvialis* under autotrophic growth conditions by a sequential stress strategy

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ABSTRACT

The study of microalgal culture has been growing in recent decades, because the cellular structure of microalgae has diverse highly valuable metabolites that have attract attention of numerous companies and research groups. The pigment astaxanthin is considered one of the most powerful antioxidants in nature. The microalga *Haematococcus pluvialis* was proposed as one of the best natural astaxanthin sources, because it can accumulate high amount of the pigment. In this work, we studied different stress treatments on *H. pluvialis* growth cultures as well as astaxanthin production under autotrophic growth conditions. The results showed that extending nitrogen starvation before increasing radiation intensity up to 110 μmol photons m^{-2} s^{-1} during late the palmella cell phase incremented the astaxanthin concentration up to 2.7% of dry biomass with an efficient light energy utilization during the stress stage.

KEYWORDS

Astaxanthin; autotrophic growth; *Haematococcus pluvialis*; microalgae; radiation intensity

Introduction

Culture of microalgae as potential candidates in biorefinery processes has become an important research subject over the past decades, given their capacity to synthesize diverse valuable products.[1] The metabolites of interest and possible uses of microalgae are lipid production as feedstock for the production of biodiesel;[2] the extraction of carbohydrates as a carbon source in fermentation industries replacing conventional carbohydrate sources;[3] the extraction of carotenes and other pigments to be used in the nutraceutical industry;[4] biomass itself as food for humans and animals;[5] bioactive compounds production,[6] among others. Currently, commercial production of microalgae is carried out mainly using open culture systems, but this method entails problems associated with contamination. Accordingly, an interesting alternative is the use of closed reactors, commonly called photo-bioreactors (PBRs). However, their construction and operation costs make the process more expensive than using open reactors. Therefore, large-scale cultures using PBRs is almost exclusive to high-added value metabolite production, accounting for a greater investment.[7]

Astaxanthin, a pigment from the xanthophylls group, is considered one of the most powerful antioxidants in nature. The molecule structure is singular because of the presence of hydroxyl and keto moieties on both ends; conferring some unique features such as the ability to be esterified, a higher anti-oxidant activity and a more polar configuration than other carotenoids.[8] Moreover, some epidemiological studies have shown that a high intake of this carotenoid has resulted in a reduction of heart diseases and cancer occurrence and in an increment of body defense to different types of infections. As a dietary supplement, it has effects of anti-aging, anti-inflammatory, and sun proofing.[9] Finally, astaxanthin is responsible for the reddish or pinkish color of fish, crustaceans, and birds (e.g. salmon and flamingoes). Hence, in last years, the production of astaxanthin for further incorporation into new products has caught the interest of the pharmaceutical, cosmetic, and food industries. Astaxanthin is commercially available either from chemical synthesis or natural resources such as microalgae, yeast, and crustacean byproducts.[10] Nowadays, microalgal astaxanthin represents less than 1% of the global market, since the synthetic alternative involves lower production costs.[11] Accordingly, the global market for natural astaxanthin is estimated to exceed US$1.5billion for 2020.[12]

The microalga *Haematococcus pluvialis* was proposed as one of the best sources of natural astaxanthin, since it could accumulate more than 3% dry weight of the metabolite.[13] This species has a complex life cycle, presenting three well differentiated (physiologically and morphologically) growth phases: a motile flagellate, a nonmotile palmella, and a resting aplanospore phase.[14] Under favorable culture conditions, the cells are in their flagellate or palmella stage.

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In adverse conditions, when the aplanospore stage occurs, the highest synthesis of astaxanthin takes place.

Due to the characteristics of *H. pluvialis* growth, a two-stage process has been proposed for its culture to improve astaxanthin production. During the first stage, the optimum conditions for microalgal growth are provided to achieve massive cell propagation. In the second stage, the growth conditions are modified to cause cell stress and stimulate the synthesis and accumulation of astaxanthin.[15]

In general, the highest productions of astaxanthin reported are achieved using high radiation intensities (above 300 µmol photons m^{-2} s^{-1}), and nitrogen starvation as stress factors, under mixotrophic growth conditions.[16] However, the use of high radiation intensities to induce carotenogenesis may impose a limitation in the scaling-up process, given the energy required to maintain these lighting conditions for a long period, either using a natural (sunlight) or an artificial radiation source. Furthermore, due to the low growth rate of the microalga,[17] the addition of an organic carbon source to the mixotrophic culture medium formulation significantly increases the risk of contamination by other microalgal species and other microorganisms as well.[18]

In this study, we focused on the analysis of different stress strategies on *H. pluvialis* culture development and astaxanthin synthesis under autotrophic growth conditions, with the aim of improving the pigment production and reducing the energy demand and contamination risks associated with either high irradiation intensities or mixotrophic growth conditions.

Experimental

Strain and culture medium

The local strain *H. pluvialis* FAUBA 57 isolated in Buenos Aires was acquired from Culture Collection of Algae at University of Buenos Aires, Argentina. The culture medium used in this study was a modified BBM,[19] which contained: $NaNO_3$ (2.94 mM); $CaCl_2{\cdot}2H_2O$ (0.17 mM); $MgSO_4{\cdot}7H_2O$ (0.304 mM); $K_2HPO_4{\cdot}3H_2O$ (0.329 mM); KH_2PO_4 (1.29 mM); NaCl (0.428 mM); $FeCl_3{\cdot}6H_2O$ (3.59×10^{-3} mM); $MnCl_2{\cdot}4H_2O$ (1.24×10^{-3} mM); $ZnCl_2$ (2.20×10^{-4} mM); $COCl_2{\cdot}6H_2O$ (5.04×10^{-5} mM); $Na_2Mo_4{\cdot}2H_2O$ (9.92×10^{-5} mM); EDTA (1.54×10^{-2} mM). The inocula were cultivated in 200 mL of the modified BBM medium in 1 L Erlenmeyer flasks shaken manually once a day, in a culture chamber continuously irradiated under 70 µmol photons m^{-2} s^{-1} at ambient temperature (between 24 and 28 °C). After 3 weeks, the cells (at aplanospore phase) were centrifuged and used to inoculate the Erlenmeyer flasks for the different assays.

Biomass dry weight determination

The algal biomass concentration was determined according to previous studies.[20] A culture sample of 15 mL was centrifuged at 5000 rpm for 10 min. Then, the pellet was washed once with distilled water and dried at 80 °C overnight.

Sodium nitrate quantification

Sodium nitrate ($NaNO_3$) was determined using UV spectrophotometry based on Collos et al.[21] A culture sample was centrifuged at 5000 rpm for 10 min. The absorbance of supernatant dilution was measured at 220 nm. A standard curve was determined from the originally modified BBM medium dilutions.

Astaxanthin determination

Astaxanthin was determined using the first-order derivative spectrophotometric method. These methods are useful for solving the spectral overlapping when pigments are present such as chlorophyll and β-carotene in the same extract.[22] The protocol involves the extraction of intracellular astaxanthin using a mixture of ethyl acetate and ethanol (1:1 v/v), saponification of the astaxanthin extracts for obtaining the pigment in the free form and determination of free astaxanthin content by performing the first-order derivative spectrum of the extract absorption profile in the range of 400–700 nm. A calibration curve was determined previously from a standard reagent of astaxanthin (DRE-CA10307000), obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Culture conditions

Five different culture conditions were used to evaluate the effects of nitrogen starvation and incident radiation on cell development and astaxanthin production. Cultures were prepared in 500 mL Erlenmeyer flasks containing 450 mL (including the inoculum volume) of modified BBM medium with initial $NaNO_3$ concentration of 5.88 mM, stirred with a magnetic bar and aerated with diaphragm pumps providing 1.4 gas volume flow per unit of liquid volume per minute (vvm) of pure air filtered with 0.20 µm nylon filters. The whole system was previously autoclaved. After 72 hr since inoculation (until cells turn from red aplanospores to green motile cells), samples (20 mL) were taken three times a week and replaced by fresh culture medium.

The assays were divided in two different stages (green phase and stress phase), including changes in culture conditions in order to evaluate the influence of the proposed strategies on cell growth and astaxanthin production. Table 1 provides the growth conditions of each stage for the different cultures.

Radiation intensity used on cultures 1, 2, 4, and 5, during the stage 1, was 70 µmol photons m^{-2} s^{-1} (low intensity culture chamber). After nitrogen source depletion, cultures 1, 4, and 5 were exposed to different growth conditions on stage 2. Culture 1 was maintained in the low intensity culture chamber until the end of the assay. In the case of culture 4, it was moved immediately to the moderated intensity culture chamber (110 µmol photons m^{-2} s^{-1}); whereas, culture 5 remained 6 d in the low light intensity culture chamber. Afterward, it was moved to the moderated light intensity culture chamber to extend the nitrogen starvation condition until the radiation intensity increment. Likewise, culture 2 was transferred to the moderate intensity culture chamber, before the nitrogen source depletion was achieved, to evaluate the individual effect of the radiation intensity increment.

Culture 3 was maintained in the moderated intensity culture chamber during the entire culture period in order to evaluate the nitrogen starvation effect over the astaxanthin production independently of a radiation increment.

Astaxanthin production yield

To evaluate the efficiency of the light energy consumed during the stress stage for the astaxanthin accumulation, an astaxanthin production yield (q_{astax}) was calculated according to Eq. (1):

where ΔC_{astax} is the astaxanthin accumulation during the stress phase; Δt is the stress phase duration; h is the Planck constant; and v is the photon frequency. The photon energy ($h * v$), used for the calculation of the light energy consumed, was the average value for 400–700 nm region (3.60 10^{-19} J phot^{-1}).[23]

$$q_{astex}\left[\frac{mg}{L\ day}\frac{m^2}{J}\right] = \frac{\Delta C_{astax}[mg\ L^{-1}]}{\Delta t[day]}\left(\frac{1}{photon\ flux\ density\ [\mu\ mol\ phot\ m^{-2}s^{-1}] * \Delta t[s] * h[J\ s\ phot^{-1}] * \upsilon[s^{-1}]}\right) \quad (1)$$

Results and discussion

High salinity, nutrient starvation (nitrogen and phosphorus), and high radiation intensities are the usual carotenogenesis inductors reported. Different strategies for astaxanthin production, from *H. pluvialis* cultures under autotrophic conditions, are reported in literature using these stressors (Table 2).

Usually, astaxanthin concentrations above 2% under autotrophic growth conditions are obtained using high radiation intensities. Kang et al.[32] achieved high astaxanthin

Table 1. Growth conditions for the different phases of *H. pluvialis* cultures.

Culture	Culture condition during green phase	Sample code
1	Available nitrogen source under low radiation intensity[a]	C1S1
2	Available nitrogen source under low radiation intensity[a]	C2S1
3	Available nitrogen source under moderated radiation intensity[b]	C3S1
4	Available nitrogen source under low radiation intensity[a]	C4S1
5	Available nitrogen source under low radiation intensity[a]	C5S1
Culture	Culture condition during stress phase	Sample code
1	Nitrogen starvation under low radiation intensity[a] during late palmella phase development	C1S2
2	Moderated increment of radiation intensity[b] during palmella phase development	C2S2
3	Nitrogen starvation under moderated radiation intensity[b] during late palmella phase development	C3S2
4	Simultaneous moderated increment of radiation intensity[b] and nitrogen starvation during late palmella phase development	C4S2
5	Six days of nitrogen starvation followed by moderated increment of radiation intensity[b] during late palmella phase development	C5S2

[a]Low light intensity is 70 µmol photons m^{-2} s^{-1}.
[b]Moderated light intensity is 110 µmol photons m^{-2} s^{-1}.

Table 2. Astaxanthin concentration values reported and astaxanthin productions yield calculated under diverse stress strategies for *H. pluvialis* cultures using autotrophic growth conditions.

Reference	Astaxanthin concentration (%)	Astaxanthin production yield $1 \times 10^9 \left[\frac{astax\ mg}{L\ day}\ \frac{m^2}{Joule}\right]$	Stress condition	Gas atmosphere used
[24]	1.60	36.2	Nitrogen and phosphorous limitation under low radiation intensity (60 µmol photons m^{-2} s^{-1})	Pure air
[25]	1.77	n.d.	Salinity stress under low radiation intensity (50 µmol photons m^{-2} s^{-1})	Pure air
[17]	1.95	101.1	Moderated radiation intensity (108 µmol photons m^{-2} s^{-1})	6% CO_2 enriched air
[26]	2.00	76.3	Nitrogen limitation under moderated radiation intensity (150 µmol photons m^{-2} s^{-1})	Pure air
[27]	2.00	n.d.	Nitrogen limitation under moderated radiation intensity (150 µmol photons m^{-2} s^{-1})	Pure air
[28]	2.30	30.5	Nitrogen starvation under high radiation intensity (350 µmol photons m^{-2} s^{-1}) at pH = 4	Pure air
[29]	2.55	17.1	Phosphorous starvation, nitrogen limitation and high radiation intensity (240 µmol photons m^{-2} s^{-1})	Pure air
[30]	2.90	n.d.	Nitrogen starvation under high radiation intensity (546 µmol photons m^{-2} s^{-1})	1.5% CO_2 enriched air
Present work	2.70	183.6	Prolonged nitrogen starvation followed by moderated radiation increment (up to 110 µmol photons m^{-2} s^{-1})	Pure air
[31]	3.50	93.4	Nitrogen starvation under high radiation intensity (250 µmol photons m^{-2} s^{-1}) at 28 °C	Pure air
[32]	4.70	207.6	Nitrogen and phosphorous starvation under high radiation intensity (400 µmol photons m^{-2} s^{-1})	5.0% CO_2 enriched air

n.d.: not determined (the information necessary to determine q_{astax} was not available).

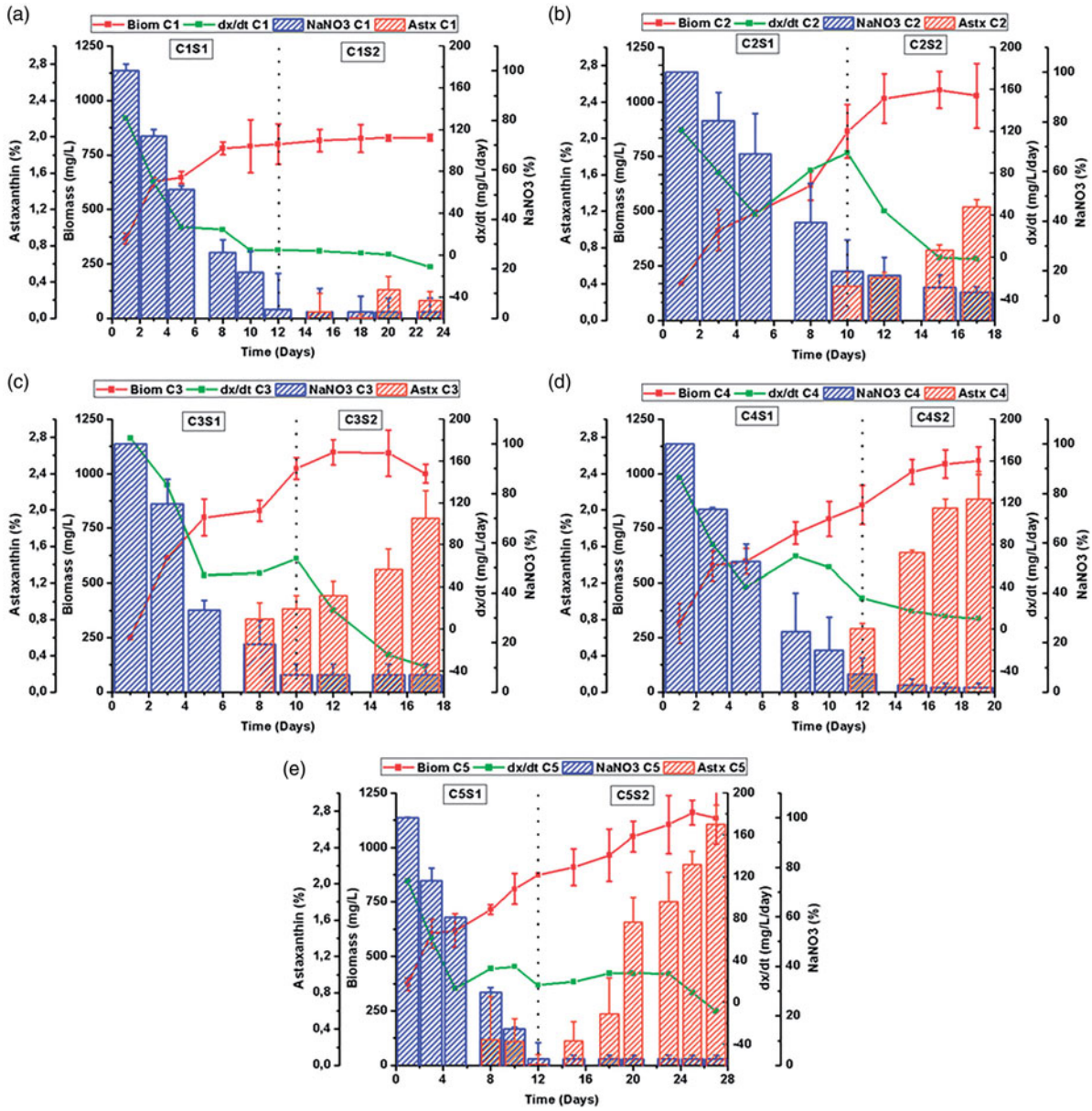

Figure 1. Evolution of biomass (mg L^{-1}), sodium nitrate (percentage of the initial concentration), astaxanthin concentration (percentage of biomass dry weight), and biomass growth rate (mg $L^{-1} d^{-1}$) of the different *H. pluvialis* cultures: (a) C1; (b) C2; (c) C3; (d) C4; and (e) C5.

concentration of 4.7% of biomass dry weight, under nitrogen and phosphorous starvation, increasing the radiation intensity to 400 µmol photons m^{-2} s^{-1} with 5% CO_2 enriched air. However, Hagen et al.[26] obtained one of the highest astaxanthin content reported under autotrophic growth conditions, using moderate radiation intensities. They proposed simultaneous nitrogen starvation with an increase in cultivation irradiance to 150 µmol photons m^{-2} s^{-1} as stress strategy, achieving a pigment concentration of 2% of biomass dry weight.

H. pluvialis cultures in Table 1 present different stress strategies employing nitrogen starvation and moderate radiation intensity as stress factors for carotenogenesis induction. The results of biomass (mg L^{-1}), sodium nitrate (remaining percentage of the initial concentration), astaxanthin (percentage of biomass dry weight), and biomass growth rate (mg $L^{-1} day^{-1}$) for the different cultures are shown in Figure 1. The reported data are the average ± SD of two independent culture replicates.

During *H. pluvialis* cultures, the population consists of a mixture of cells at different phases of the life cycle. Thus, samples taken from microalgal cultures were observed under light microscope to analyze and identify morphological changes during different growth stages (Figure 2).

Throughout the green phase, growth conditions are favorable for cell development, for this reason *H. pluvialis* flagellate and palmella cells are observed. Contrarily, during the stress phase, the growth conditions are adverse to induce the cellular transition from palmella to aplanospore phase.

Effects observed throughout the green phase

During the green phase, a raised in the radiation intensity given during C3S1 caused a rapid increase in biomass production that almost doubled the value reached at a lower radiation intensity (C1S1, C2S1, C4S1, and C5S1).

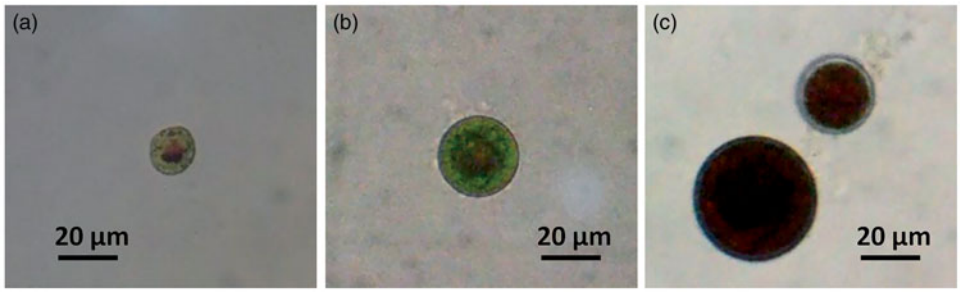

Figure 2. Photographs from *H. pluvialis* cultures on the different growth phases: (a) flagellated cell; (b) palmella; and (c) aplanospore.

Carotenoid accumulation was observed in C3S1, growing under moderate radiation intensity condition during the entire culture period starting from flagellated phase. This culture condition probably induced an early onset of stress response mechanisms to high radiation.[33]

Effects observed throughout the stress phase

Stress strategies studied generate changes in aplanospore phase development of *H. pluvialis*, achieving different astaxanthin production values in each case: 0.2 ± 0.1%, 1.2 ± 0.1%, 1.9 ± 0.2%, 2.1 ± 0.2%, and 2.7 ± 0.1% for C1S2, C2S2, C3S2, C4S2, and C5S2, respectively. Responses to increasing radiation intensity differed with the life cycle phase of the culture and the nitrogen source availability. Cultures grown under low radiation intensity conditions starting from flagellated phase (C2S2, C4S2, and C5S2) turned to aplanospore phase by increasing radiation intensity during late palmella phase (C2S2, C4S2, and C5S2). Moreover, cultures exposed to moderate radiation intensity condition during the entire culture period starting from flagellated phase (C3S2) were able to achieve aplanospore phase once nitrogen source was depleted. However, culture maintained under low radiation intensity conditions (C1S2) remained in palmella phase without reaching the aplanospore phase, despite nitrogen starvation. Thus, a minimal radiation intensity value would be necessary to induce the transition to the aplanospore phase when cells are grown under autotrophic conditions. By contrast, Kobayashi et al.[14] indicated that in the presence of sodium acetate *H. pluvialis* can produce the transition to aplanospore phase, even under dark conditions.

Interestingly, the highest astaxanthin production occurred in C4S2 and C5S2, under culture conditions combining radiation intensity increase and nitrogen starvation. Therefore, an early onset of stress response mechanisms to higher radiation intensities starting from flagellated phase in C3S1 may help cells develop a photo-adaptation process to this lighting condition.[34] As a consequence, the stress response degree in C3S2 was altered, decreasing the astaxanthin production.

Results show that extending nitrogen starvation condition before incrementing the radiation intensity in C5S2 improved pigment accumulation by over a 25% compared with simultaneous radiation increase and nitrogen starvation in C4S2 (2.7% and 2.1%, respectively).

High radiation intensities in *H. pluvialis* cultures stimulate the synthesis of astaxanthin, which reduces the oxidative stress caused by the accumulation of reactive oxygen species in the microalgal cells.[35] Besides, nitrogen depletion in the medium causes the activation of several enzymatic pathways, tending to increase the production of triglycerides necessary to promote astaxanthin accumulation. Pigment concentration achieved under different culture conditions, suggested a possible interaction between both defense mechanisms. Moreover, the response degree to each stressor would be related to the cell phase occurring in the culture at the moment in which stress conditions are induced.

Astaxanthin concentration achieved during C5S2 was comparable to productions obtained using 2–5 times higher radiation intensities (Table 2). Moreover, the astaxanthin production yield value achieved showed an efficient conversion of the light energy consumed compared with the different yields calculated in Table 2 (the information necessary for the calculation of q_{astax} was not available in references[25,27,30] and thus the q_{astax} in those cases was not reported). The q_{astax} reported in the present work was only slightly surpassed by Kang et al.,[32] which highlights the potential of the proposed methodology.

Conclusion

In this study, we analyzed the effects of nitrogen availability and radiation conditions on autotrophic development of *H. pluvialis* FAUBA 57. The sunlight employment as an energy source exhibits several drawbacks such as: variations in weather conditions, day/night cycles, and seasonal changes. However, although artificial lighting will not only increase productivity, but it will also increase costs associated with microalgae cultivation. Thus, radiation intensity selection is an important parameter to achieve a positive energy balance for high-added value metabolite production using PBRs. Variations in microalgal growth development and the different astaxanthin production values achieved under the stress strategies studied, revealed an interaction between the defense mechanisms triggered by nitrogen starvation and incident radiation. Extending the nitrogen starvation situation before moderate radiation intensity increment during late palmella phase produced a 25% increment of astaxanthin concentration when compared with simultaneous radiation increase and nitrogen starvation, and an efficient light energy use for the pigment biosynthesis compared with other stress conditions reported in literature. The sequential stress strategy proposed can be used to improve astaxanthin production from *H. pluvialis* autotrophic cultures as an

alternative to higher radiation intensities, augmenting the efficiency of the energy demand of the process.

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