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Growth, Carotenoid Production, Antioxidant Capacity and Lipid Accumulation of *Haematococcus* sp. Under Different Light Intensities

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Abstract: *Haematococcus* is a genus of unicellular green microalgae and it is known as an important resource of keto-carotenoid: astaxanthin. In this work, a strain of *Haematococcus* sp. grew rapidly when cultivated in BBM medium under different light intensities from 30 to 120 µmol photons.$m^{-2}.s^{-1}$. Production of antioxidant compounds (carotenoid and phenolic compounds) as well as total antioxidant capacity and lipid accumulation of *Haematococcus* sp. increased with increase in light intensity especially after 21 days of cultivation. The results demonstrated that there was significant relation between production of carotenoid, phenolic compound and lipid accumulation in *Haematococcus* microalgae under high light cultural conditions.

Keywords: *Haematococcus*, Carotenoid, Astaxanthin, Antioxidant Capacity

1. Introduction

"Green microalgae" comprise more than 7000 species growing in a variety of habitats. The unicellular green alga *Haematococcus* is a fresh water microalgae, belongs to the class Chlorophyceae, order Volvocales and family Haematococcaseae distributed in many habitats worldwide. *Haematococcus* is considered the best natural source of astaxanthin – a red-colored microalgal carotenoid well known for its high antioxidant capacity [36]. *Haematococcus* species are also the main producer of this commercial product [11], [26], [21].

Culture conditions such as high light, salinity, temperature and C/N ratio affect growth and astaxanthin production of *H. pluvialis*. Light intensity and direction of illumination, as well as effective culture ratio, are among the most important environmental factors determining optimal growth and astaxanthin production of *H. pluvialis* [35], [37], [21]. According to [29], [30], [25] astaxanthin accumulation in *Haematococcus* is induced by a variety of environmental stressors which limit cell growth, e. g.: adverse light conditions, phosphate starvation and salt stress. Under such culture conditions, cell division rate decreased, but astaxanthin content per cell increased. Accumulation of astaxanthin requires nitrogen and was associated with a change in the cell stage from biflagellate vegetative green cells to non-motile and large resting cells [30].

H. pluvialis has antioxidative enzymes in vegetative cells and the antioxidative ketocarotenoid, and astaxanthin, in cyst cells, that protect the alga against environmental oxidative stress [20]. Astaxanthin accumulated in the cyst cells functions as an antioxidant against excessive oxidative stress. Its activities against O_2^- in cyst cells was comparable to superoxide dismutase in vegetative cells. According to [18], *Haematococcus* was one of the favored organisms for the biotechnological production of these antioxidative compounds.

Biosynthesis of astaxanthin in *H. pluvialis* is a complex process that is highly up-regulated in conditions of stress which coincides with the accumulation of triacylglycerols (TAGs). Both compounds are deposited in the cytosolic lipid bodies during the "red" stage of *H. pluvialis* cultivation [21], [18]. Under stress, the unicellular green alga *Haematococcus*

pluvialis accumulates secondary carotenoids, mainly astaxanthin esters, in cytoplasmic lipid vesicles up to 4% of its dry mass. Changes were observed in the lipid composition during the period of induction of secondary carotenoid synthesis in *H. pluvialis*, especially in the fatty acids pool (oleic, palmitic and linoleic acids) [22], [18]. This research aimed to investigate effect of different light intensities on the growth and accumulation of antioxidant compounds such as carotenoid, phenolic compound and lipid in *Haematococcus* sp.

2. Material and Methods

2.1. Haematococcus sp. Strain and Cultural Condition

The unicellular green microalgal *Haematococcus* sp. Strain was preserved and cultivated in the BBM medium [10], [12] at the Laboratory of Biochemistry and Toxicology, Nguyen Tat Thanh University, Viet Nam. The growth medium was adjusted to pH 7.5 prior to autoclaving 121°C for 15 min.

2.2. Experimental Design

Haematococcus sp. cells at exponential phase after 7 days of culture in BBM medium were used as inoculum for experiments under different light intensities. Firstly, cells were grown in the BBM medium with an initial cell density (OD 750nm) of ~ 0.03, under light intensity of 30 µmol photons.m^{-2}.s^{-1}. After 12 days of cultivation, culture was transferred to a high-intensity light condition of either 50, 80 and 120 µmol photons.m^{-2}.s^{-1}. All experiments were performed in triplicate flasks for each light condition and repeated at least twice.

2.3. The Growth Measurement

Optical density of cell suspensions were measured at A750nm every 2 days during the first 12 days and every 3 days from the 12^{th} day to 24^{th} day of cultivation. Measurements were performed with UV-Vis spectrophotometer.

2.4. Total Carotenoid and Phenolic Contents

Total carotenoid measurement: One mL of algal suspension was centrifuged at 5000×g for 5 min and the pellet was extracted with 3 mL of ethanol: hexane 2: 1 (v/v). Two mL of water and 4 mL of hexane were added and the mixture was vigorously shaken and centrifuged again at 1000×g for 5 min. After phase separation was observed, absorbance of the hexane layer was determined at 450 nm. Total carotene was calculated as A450 × 25.2, equivalent to the micrograms of carotene in 1 mL of sample [2], [1].

Total phenolic determination: Total phenolic was determined using Folin Ciocalteu reagent [32], [19], [17]. For each sample, one milliliter algal suspension was centrifuged at 5000 rpm for 5 mins. The pellet was extracted with 1 mL absolute Methanol. 0.5 mL extract was mixed with 0.5 mL of Folin–Ciocalteu reagent and allowed to stand at room temperature for 5 mins before addition of 0.5 mL of sodium bicarbonate solution (10% Na_2CO_3). After 90-minute incubation at room temperature, the absorbance was measured at 750 nm. The same procedure was performed on the standard solution of gallic acid to derive a calibration line. Phenolic content is expressed as µg Gallic acid equivalent per volume.

2.5. Antioxidant Capacity

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) solution was prepared by dissolving 0.004 g of DPPH in 100 ml of methanol. One milliliter of algae suspension was centrifuged in 10000 rpm at 4°C for 15 mins and pellet was extracted with 1 mL ethanol absolute and gently vortex. The extract was incubated at 4°C for 4 hours and 2 mL DPPH solution was subsequently added. The mixture was incubated for 30 mins in the dark at room temperature. A blank sample (absolute ethanol) was also taken as a control. Absorbance at 517 nm of the extract was determined spectrophotometrically. Antioxidant activity was calculated based on the inhibition of free radical DPPH in percentage according to the formula: $I\% = (A_{blank} - A_{sample})/A_{blank} \times 100$ [34], [28], [7].

2.6. Lipid Accumulation by Sulfo-Phospho-Vanillin Assay

For each sample, one mL of algal suspension was centrifuged at 5000 rpm for 5 mins. The pellet was treated with 2 mL of concentrated (98%) sulfuric acid, heated for 10 mins at 100°C and subsequently cooled for 5 mins in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 mins at 37°C incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid content of the sample [31].

2.7. Data Analysis

Data was processed in Excel 2013 and analyzed by one way ANOVA using SPSS software version 20.0. All significant levels were set at $p < 0.05$.

3. Results and Discussion

3.1. The Growth of Haematococcus sp.

Haematococcus sp. cells grown in 250 mL flasks each containing 125 mL BBM medium with initial cell density (A750 nm) of 0.033. The light intensity was 30 µmol photons.m^{-2}.s^{-1} and the temperature was 25°C. Growth of *Haematococcus* sp. rapidly increased and reached high optical density at 12^{th} day (OD=0.169). *Haematococcus* suspension after 12 days of cultivation was transferred to higher light intensities, 50, 80 and 120 µmol photons.m^{-2}.s^{-1}. In these light conditions, the growth of *Haematococcus* cells continuously increased and there was no significant difference in growth rate compared to that of the first 12 days

(p=0.281). The results showed that *Haematococcus* sp. cells can grow well with high growth rate when they cultivated under high light intensity of 120 µmol photons.$m^{-2}.s^{-1}$ (figure 1). [24]. This agrees with several other studies on the growth curve of *Haematococcus* sp. According to [9], productivity of *H. pluvialis* increased with increasing irradiance when exposed to irradiances ranging from 50 to 200 µmol photons.$m^{-2}.s^{-1}$. Maximal growth of *H. pluvialis* (green cells) was obtained in the BBM medium under continuous illumination (177 µmol photons $m^{-2}s^{-1}$) with continuous aeration (1.5 vvm) [3]. In *H. pluvialis* Flotow, the growth was significantly higher in 75 and 150 µmol photon m^{-2} s^{-1} illuminations compared to 20 and 40 µmol photon m^{-2} s^{-1} [33].

Light condition is one of the important environmental factors affecting the growth and bioactive compound synthesis in microalgae, especially *Haematococcus*, *Dunaliella salina*, *Chlorella*; however very high light intensities can lead to photoinhibition and biosynthesis of secondary compounds. The growth of *H. pluvialis* was saturated at 200 µmol photons.$m^{-2}.s^{-1}$; growth rate was observed to be lower in the 200–400 µmol photons.$m^{-2}.s^{-1}$ irradiance range [9]. Furthermore, the effect of light intensity was dependent on the nutritional state of the cultures [15] and thus might intensify with growth stages. Under optimal growth conditions, photosynthesis pigments absorbed light and converted to chemical energy forming ATP and NADPH through a photosynthetic electron transport chain. This chemical energy is finally stored in starch by fixing CO_2 through the Calvin cycle [30]. Therefore, the light intensities ranging from 30 to 120 µmol photons.$m^{-2}.s^{-1}$ in this experiment did not cause growth inhibition in the culture and was able to induce significant amount of secondary carotenoid, especially astaxanthin in *Haematococcus* sp.

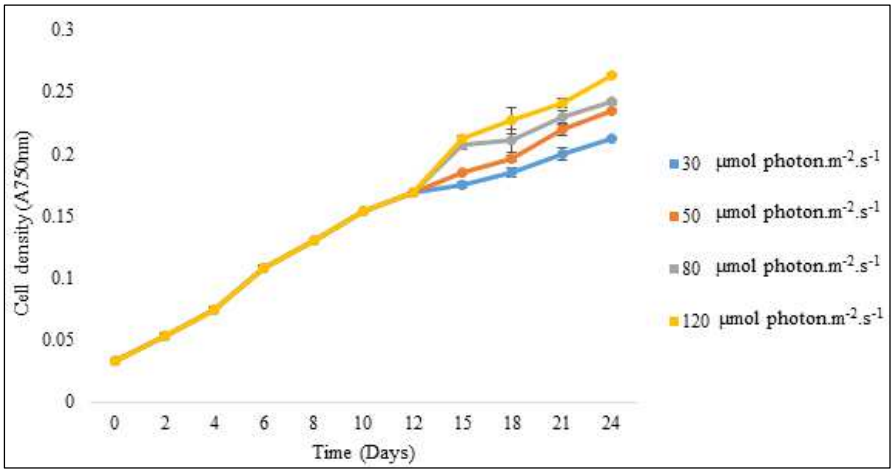

Figure 1. *Effect of different light intensities on the growth of Haematococcus sp.*

3.2. Total Carotenoid and Phenolic Content of Haematococcus sp.

Total carotenoid concentration per volume of *Haematococcus* cells increased after shifting from 30 µmol photons.$m^{-2}.s^{-1}$ to higher light intensities (50, 80 and 120 µmol photons.$m^{-2}.s^{-1}$). Light intensity of 120 µmol photons.$m^{-2}.s^{-1}$ promotes the highest total carotenoid per biomass after 6 days of stressed culture (figure 2A). Increase in the carotenoid concentration might have also resulted in increase in optical cell density (figure 1). In addition, an increase in light intensity after 12 days of the culture resulted in synthesis of carotenoid, especially astaxanthin in *Haematococcus* sp. cells.

Carotenoids are integral and essential components of the photosynthetic membranes in all plants. Primary carotenoids function within the photosynthetic machinery. In contrast, secondary carotenoids are not required for photosynthesis and are not localized in the thylakoid membranes of the chloroplast. In the unicellular green alga *H. pluvialis*, carotenoids are accumulated large amounts of carotenoids in lipid vesicles outside the plastid [30], [14].

Carotenoids content from day 21 to day 24 reached a higher value compared to initial period of stress (from day 12 to day 18). This increase in carotenoid content can be explained by synthesis of secondary carotenoids when nutrients become limited and cells were also stressed by continuous high irradiance. According to [27], major carotenoid constituents of green motile *Haematococcus* cells in the initial period of stress induction was lutein and β-carotene, followed by neoxanthin and violaxanthin (in trace amount). Secondary carotenoids were absent. However, carotenoid content increased with astaxanthin production while chlorophyll content decreased after 9 days of nutrient limitation and high light intensity.

Total phenolic content of *Haematococcus* sp. slowly increased when cells were shifted to higher light intensities (50, 80 and 120 µmol photons.$m^{-2}.s^{-1}$). Total phenolic content reached high value after stress 9 days (day 21, 24) under high light intensities. There was no significant difference in total phenolic between the light conditions from day 12 to day 18 of the cultivation (p= 0.642) (figure 2B). Phenolic compounds are recognized as important natural antioxidants. Polyphenols act as antioxidant through single electron transfer and through hydrogen atom transfer. [17], [13]. The studies demonstrated that microalgae were able to produce

more complex phenolic compounds, especially novel phenolic compounds [8], [13]. The fact that content of phenolic substances in microalgal biomass increases upon exposure to UV-light suggests that these compounds indeed play a role in the antioxidative response to this type of stress [4], [16], [17].

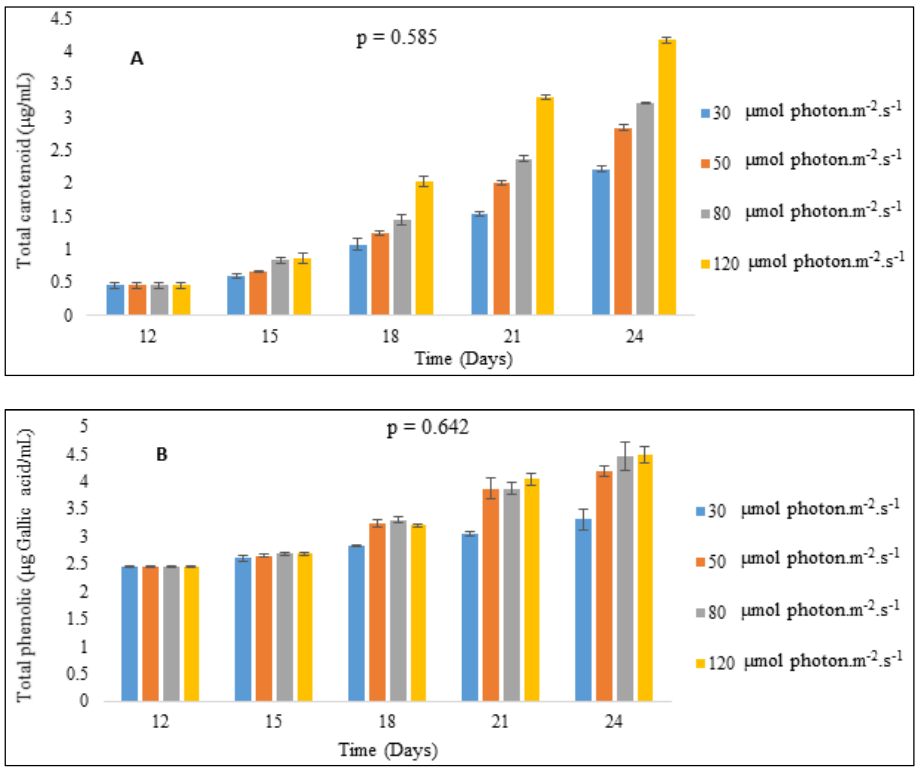

Figure 2. *Total carotenoid (A) and phenolic (B) contents of Haematococcus sp. under different light intensities.*

3.3. Antioxidant Capacity of Haematococcus sp.

Figure 3 showed antioxidant capacity of *Haematococcus* sp. after shifting from light of 30 µmol photons.$m^{-2}.s^{-1}$ to high light intensities of 50, 80 and 120 µmol photons.$m^{-2}.s^{-1}$. High antioxidant activities were observed after 9 days of stress process (day 21 and 24 of cultivation). There was no significant difference of antioxidant capacity from day 12 to day 18 of the cultivation. The high antioxidant capacity of *Haematococcus* sp. corresponded with accumulation of high carotenoid and phenolic contents after 9 days of stress (day 21) (figure 2, 3). Therefore, the results showed that there was a relationship between antioxidant capacity and carotenoid and phenolic contents of *Haematococcus* sp. under stress conditions. Under exposure to light and oxygen, free radicals and other strong oxidizing agents are induced in microalgae and higher plant cells [13]. Therefore, such photosynthetic organisms have two antioxidative systems to counter environmental oxidative stresses: antioxidative enzymes and antioxidative compounds such as carotenoid (astaxanthin for *Haematococcus*, β-carotene for *Dunaliella salina,…*) and phenolic compounds [20]. *H. pluvialis* is known as a potent producer of astaxanthin [23], [29], [20]. Under deficiency of a nutrient such as nitrogen and other stress conditions, *Haematococcus* cells induced encystment and enhanced carotenoid synthesis in the cyst cells [20]. Exposure of *Haematococcus* to high light intensity caused the accumulation of excess energy which leading to ROS formation. Synthesis of antioxidant carotenoids, especially astaxanthin, protects the cells from photodamage [5].

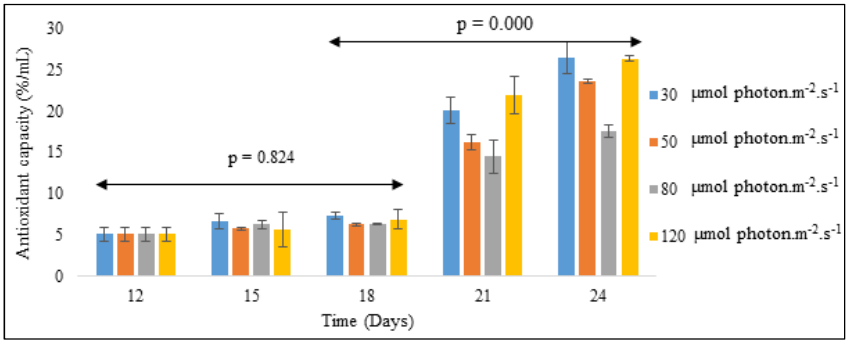

Figure 3. *Antioxidant capacity of Haematococcus sp. under different light intensities.*

3.4. Lipid Accumulation of Haematococcus sp.

Lipid accumulation of *Haematococcus* sp. under different light intensities rapidly increased after shifting to high light intensities. Light condition of 120 µmol photons.$m^{-2}.s^{-1}$ lipid induced highest lipid concentration at day 21 and 24 of culture (figure 4). The results showed that lipid accumulation of *Haematococcus* sp. increased concomitantly to increase in carotenoid concentration under high light intensities (figure 4, 2B). Increase in lipid accumulation, carotenoid and phenolic contents and antioxidant capacity per volume of *Haematococcus* sp. can explained by increase in cell density or higher irradiation and nutrient starvation after 12 days of the cultivation. Stress conditions such as nutrient limitation and high light intensity induced lipid accumulation and astaxanthin synthesis during cyst formation in *H. pluvialis* [22], [6]. Under these stress conditions, production of astaxanthin was accompanied by that of fatty acids in *Haematococcus*. In both nutrient limitation and high light intensity, the newly formed fatty acids, consisting mostly of oleic (up to 34% of fatty acids), palmitic and linoleic acids were mostly in the form of triacylglycerols. Acumulation of oleic acid was correlated with that of astaxanthin stored in lipid globules made of triacylglycerols [22].

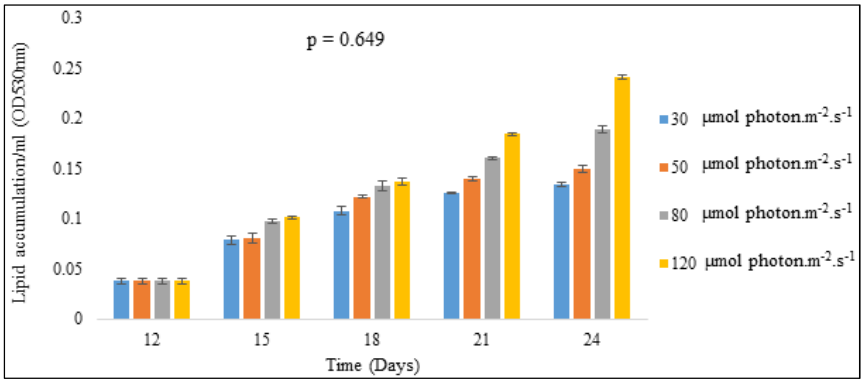

Figure 4. *Lipid accumulation per volume of Haematococcus sp. under different light intensities.*

4. Conclusion

Haematococcus sp. grown rapidly under light intensities from 30 to 120 µmol photons.$m^{-2}.s^{-1}$ with high optical density of *Haematococcus* cell during the culture. In addition, increase in total carotenoid and phenolic contents, and lipid accumulation obtained with high value when cultural suspension transferred to higher light intensities. However, in order to obtain higher level of antioxidant compounds such as carotenoid, phenolic compound and lipid, *Haematococcus* must be induced by higher light intensity (above 120 µmol photons.$m^{-2}.s^{-1}$), nutrient stavartion, temperature or combination of these stress factors to form cyst cells.

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