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Microalgae *Haemococcus pluvialis*, Cultured in OHM Medium under Stressful Conditions: Phenolic Content and Antioxidant Capacity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Haematococcus pluvialis, a unicellular green microalgae, is able to accumulate the large amounts of astaxathin under stress conditions. Nutritional factors such as nitrate or phosphate and light intensity play an important role in the growth, accumulation of phenolic compounds and antioxidant capacity microalgae H. pluvialis. In this study, *H. pluvialis* was cultured under three conditions of stress, nutrient depletion, denitrification of nitrate and addition of NPK fertilizer, and natural light in OHM medium to investigate the phenolic content and antioxidant capacity of the biomass of H. pluvialis microalgae. The results showed that, under denitrification of nitrate and addition of NPK fertilizer, the phenolic content reached a high value (8.054 µg gallic acid/g) and there was no significant difference with the two conditions of nutrient depletion (4.115 µg gallic acid/g) and

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natural light (4.707 µg gallic acid/g) ($p<0.05$). However, no differences were found between the antioxidant capacity under denitrification of nitrate and addition of NPK fertilizer (51.390%) and natural light (52.301%) ($p=0.552$) that were higher than nutrient depletion (45.257%) ($p<0.05$). Thus, under the stress conditions of nitrate depletion and high light intensity, H. pluvialis has the ability to synthesize and accumulate high amounts of bioactive compounds and microalgae biomass can be applied in dietary supplement and cosmeticeuticals.

Keywords: Haematococcus pluvialis; astaxanthin; phenolic compound; antioxidant capacity.

1. INTRODUCTION

Haematococcus pluvialis, a green microalga, is a rich source of valuable bioactive compounds, such as astaxanthin, carotenoids, proteins, lutein, and fatty acids (FAs). Astaxanthin (3,3′-dihydroxy-ß-carotene-4,4′-dione) is a secondary carotenoid with a brilliant blood-red colour that can be *synthesize high amounts* by applying cellular stressors to *H. pluvialis*. Astaxanthin has a variety of health benefits and is used in the nutraceutical and pharmaceutical industries [1]. Astaxanthin is also widely used in various fields such as food, and aquaculture feed [2]. Astaxanthin plays a crucial role not only as a coloring agent for aquatic animals, particularly salmon and ornamental fish, but also possesses other functions such as antioxidant, immune enhancement, and anticancer properties [3, 4].

The synthesis of astaxanthin in *Haematococcus pluvialis* is linked to the growth process and the transition of cell states from the growth phase to the cyst phase [5]. The accumulation of astaxanthin in *H. pluvialis* can be induced under growth-limiting conditions, such as nutrient deprivation, high light, and/or high salinity [2], [6-9]. According to Li *et al.*, (2008), *H. pluvialis* synthesizes and accumulates a large amount of astaxanthin and carotenoids in algal cells under conditions of salt inhibition or nutrient deprivation [10]. Additionally, high light intensity is one of the critical factors influencing the accumulation of astaxanthin and antioxidant compounds [11]. Therefore, this inhibition may affect the synthesis of astaxanthin and antioxidant compounds in *H. pluvialis* [12, 13].

Under stress conditions such as light, nitrate or phosphate deficiency, all affect growth, astaxanthin accumulation, and antioxidant compounds in *Haematococcus pluvialis* algal cells. Hence, this study aims to investigate the phenolic content and antioxidant capacity of *H. pluvialis* algae cultured under three stress conditions: nutrient deprivation, nitrate reduction supplemented with NPK, and natural light in OHM medium. The research results aim to guide the cultivation process of *H. pluvialis* to achieve biomass accumulation with high levels of carotenoids, phenolic compounds, and antioxidant capacity on a pilot scale.

2. MATERIALS AND METHODS

2.1 *Haematococcus pluvialis* Strain and Cultivation Conditions

The strain of *Haematococcus pluvialis* (UTEX 2505) was maintained in the Laboratory of Biochemistry and Toxicology, Nguyen Tat Thanh University. *H. pluvialis* was cultured in OHM medium with light intensity of 20 µmol photon/m^2/s, 12-hour light-dark cycle, and cultivation temperature of 25℃ ± 2℃.

2.2 Research Methodology

2.2.1 Harvesting *Haematococcus pluvialis* biomass

Centrifuge algal culture in a Falcon tube (50 mL) at 5000 rpm for 15 minutes to collect algal biomass. Dry the harvested algae at 60℃ until a constant weight is achieved. Store the obtained samples at -20℃.

2.2.2 Total phenolic content determination

Take 0.01g of algal powder and add 5 mL of methanol to a capped test tube, vortex for 5 minutes, and then heat in a water bath at 50℃ for 15 minutes. After 15 minutes, centrifuge at 5000 rpm for 10 minutes, collect the extract solution, and then evaporate the extract solution until dry. The pellet continues to be extracted until the solution is clear. The collected extract will be dissolved in 3 mL of methanol.

Take 1.0 mL of the dissolved solution of methanol into a 2 mL Eppendorf tube, add 0.5 mL of Folin Ciocalteu's phenol reagent, vortex, and incubate for 5-10 minutes. Then, slowly add

0.5 mL of 10% Na_2CO_3 solution. Incubate in the dark for 90 minutes at room temperature. Measure the absorbance at 750nm. Phenolic content (mg/L) was determined according to the gallic acid standard curve equation.

Phenolic standard curve: Use a standard gallic acid concentration ranging from 10 to 200 mg/L and determine the total phenolic content in *Haematococcus pluvialis* samples using the equation:

$$y = 30.263x - 0.0638; R^2 = 0.9948$$

where: y is OD and x is concentration of gallic acid

2.2.3 Antioxidant capacity determination

Prepare the DPPH reagent: Take 0.004g of DPPH (1,1-diphenyl-2-picrylhydrazyl) and dissolve it in 100 mL of methanol.

Take 0.01g of algal powder and add 5 mL of ethanol to a capped test tube, vortex for 5 minutes, and then heat in a water bath at 50°C for 15 minutes. After 15 minutes, centrifuge at 5000 rpm for 10 minutes, collect the extract solution, and then evaporate the extract solution until dry. The pellet continues to be extracted until the solution is clear. The collected extract will be dissolved in 10 mL of ethanol.

Take 1.0 mL of the dissolved solution into a 2 mL Eppendorf tube, add 1.0 mL of the DPPH reagent, vortex, and incubate in the dark for 30 minutes at room temperature. Measure the absorbance at 517nm. The antioxidant capacity (I%) is calculated based on the inhibition ability of DPPH free radicals using the following formula [14]:

$$I\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100$$

2.2.4 Experimental design

Preparation of *Haematococcus pluvialis* for the experiment: After 10 days of cultivation, *Haematococcus pluvialis* was cultured in OHM medium reached the growth phase. The experiment was conducted in two stages:

Growth cultivation stage: *Haematococcus pluvialis* was cultured in 1L Erlenmeyer flasks containing OHM medium and algal solution, pH = 7.5, initial cell density about 0.3×10^6 cells/mL, light intensity 20-50 μmol photon/m^2/s (12:12 light-dark cycle), at room temperature 25℃ ± 2℃, and continuous aeration.

Stress cultivation stage: *Haematococcus pluvialis* was transferred to stress cultivation conditions after 18 days of cultivation:

Nutrient depletion (Control): After 18 days of cultivation, *H. pluvialis* continued to be cultured under the initial conditions in 1L Erlenmeyer flasks.

Nitrate reduction and NPK supplementation (-Nitrate + NPK): After 18 days of cultivation, the initial medium was removed by centrifugation at 5000 rpm for 10 minutes, at 15°C, and the OHM medium without nitrate was supplemented with NPK fertilizer MK501 at a concentration of 0.1g/L.

Natural light (NL): After 18 days of cultivation, *H. pluvialis* was transferred to conditions of cultivation under natural light. Natural light intensity measured above 1000 μmol photon/m^2/s at 12 am.

Microalgal biomass harvesting: After 7 days of stress cultivation, *Haematococcus pluvialis* cells was harvested by centrifugation at 5000 rpm for 10 minutes, at 4°C, and the biomass was stored at -20℃. The analysis of total phenolic content and antioxidant capacity of *H. pluvialis* was performed on the experimental conditions. The experiments were repeated three times.

2.3 Data Analysis

The data were processed using Microsoft Office Excel software and analyzed by one-way ANOVA using SPSS 20.0 software with a significance level of $p \leq 0.05$. All data in the experiment are presented as Mean ± Standard Error (SE).

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content

In the OHM medium, the phenolic content of *H. pluvialis* reached the highest value under the stress condition -Nitrate + NPK (8.054 μg gallic acid/g) and there was a significant difference compared to two other cultivation conditions ($p < 0.05$) (Figure 1, Table 1). Under the restrictive conditions NL, the phenolic content in *H. pluvialis* (4.707 μg gallic acid/g) was higher than the nutrient depletion cultivation (control) (4.115 μg

gallic acid/g) ($p < 0.05$). Thus, under the -Nitrate + NPK and NL stress conditions, *H. pluvialis* cells were strongly inhibited, leading to the formation of free radicals. Therefore, microalgae could synthesize a large amount of phenolic compounds compared to the nutrient depletion (control) stress.

Phenolic compounds are considered natural antioxidants that provide numerous health benefits. Complex polyphenolic compounds, specifically complex polyphenol groups, can inhibit lipid oxidation by directly eliminating •OH, HOCl, singlet oxygen, and peroxyl radicals, as well as by chelating metals and inhibiting lipoxygenase. However, there is limited information about the presence of phenolic compounds in algae [15]. According to Klejdus *et al*., (2010), flavonoid groups such as isoflavones, flavanones, flavonols, and dihydrochalcones have been found in algae and cyanobacteria [16].

The concentration of nitrate in the cultural media is a determining factor for the growth and development of algae [6, 7]. Depleting nitrate from the environment restricts the development of *H. pluvialis* algae but may lead to the accumulation of phenolic compounds. Subsequently, +NPK stimulates the proliferation of algae once again. Therefore, phenolic compounds are synthesized in large quantities under these inhibitory conditions. In addition, light is a crucial factor influencing the biomass growth of algae. Under natural light conditions, igh light intensity causes the algal cells to transition from green vegetative form to red cyst form, contributing to the accumulation of phenolic content in *H. pluvialis* algae [6].

3.2 Antioxidant Capacity

The evaluation of the antioxidant capacity of *H. pluvialis* in the OHM medium under different conditions reveals notable findings. Under -Nitrate + NPK and NL stress conditions, the antioxidant capacity exhibited comparable high values of 51.390% and 52.301%, respectively, with no statistically significant difference ($p=0.552$). These values surpass the antioxidant capacity observed under the control condition (45.257%), showing a statistically significant difference ($p<0.05$) (Figure 2, Table 1).

The heightened antioxidant capacity of *H. pluvialis* under -Nitrate + NPK and NL conditions can be attributed to the presence and activity of various antioxidants, including phenolic compounds, astaxanthin, lutein, and ketocarotenoids. *Haematococcus pluvialis* is known to possess two primary antioxidant systems, namely antioxidant compounds such as ketocarotenoids and astaxanthin found in cysts, and antioxidant enzymes present in vegetative cells [17, 18].

Under inhibitory conditions, such as nitrate deficiency, intense light exposure, or salt inhibition, there is an accumulation of astaxanthin, which acts as a potent antioxidant against oxidative stress [6]. Despite a relatively high cell death rate under NL conditions, surviving cells exhibit a remarkable accumulation of astaxanthin [8]. Furthermore, the reduction of nitrate and NPK supplementation, applied after a few days, promotes the growth of *H. pluvialis*, resulting in a higher accumulation of astaxanthin. Consequently, under inhibitory conditions -Nitrate + NPK and NL, the antioxidant capacity demonstrates a significant elevation compared to the nutrient depletion condition.

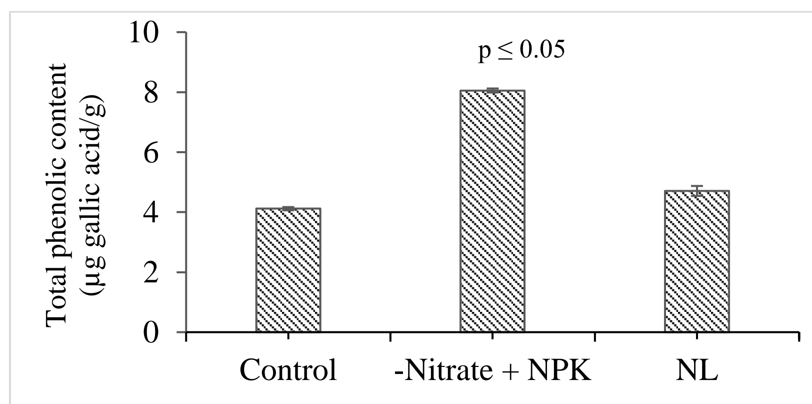

Fig. 1. The phenolic content of Haematococcus pluvialis cultivated under different stress conditions

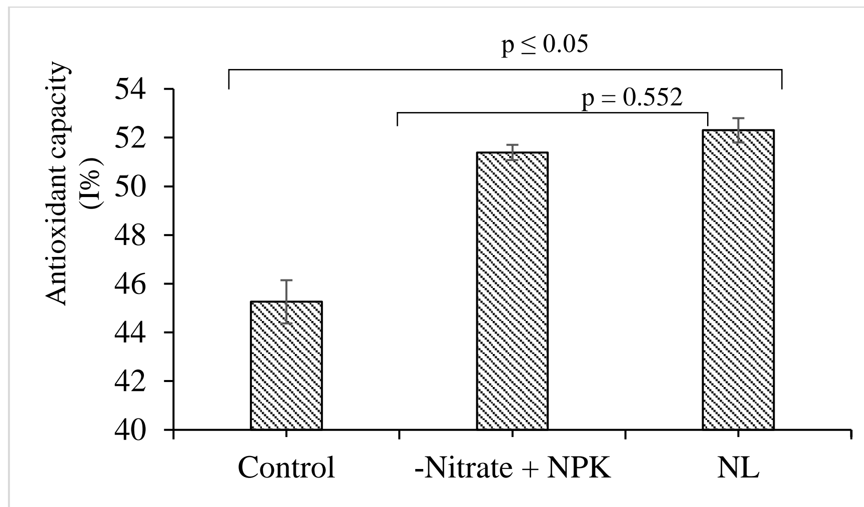

Fig. 2. The antioxidant capacity of *Haematococcus pluvialis* cultivated under different conditions

Table 1. Phenolic content and antioxidant capacity of Haematococcus pluvialis cultivated in OHM medium under different stress conditions

	Nutrient depletion (Control)	**-Nitrate + NPK**	**Natural light (NL)**
Phenolic content (µg acid galic/g)	4,115 ± 0,054^{a}	8,054 ± 0,068^{c}	4,707 ± 0,166^{b}
Antioxidant capacity (I%)	45,257 ± 0,886^{a}	51,390 ± 0,313^{b}	52,301 ± 0,498^{b}

4. CONCLUSION

Haematococcus pluvialis algae exhibit a notable accumulation of phenolic compounds and high antioxidant capacity under stressful cultivation conditions, specifically, nitrate deficiency supplemented with NPK and natural sunlight. Phenolic compounds as antioxidant were synthesized with a large amount in *H. pluvialis* cells cultivated in nitrate deficiency. The antioxidant capacity in *H. pluvialis* reached the highest value under nitrate deficiency and natural light stress. The results showed that there was a positive correlation between accumulation of phenolic compounds and antioxidant capacity in the microalgae. Microalgae *H. pluvialis* play an important role in providing biomass sources for applications in the food, pharmaceutical and functional food industries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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