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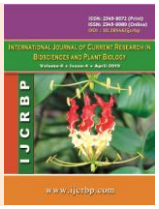

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The effect of nutrients on the growth of microalgae *Haematococcus lacustris* (Girod-chantrans) Rostafinski 1875

Dang Mau TRINH[1]*, Thi Trang NGUYEN[1], Phuoc Kha NGUYEN[1], Nguyen Quynh Anh TRAN[2] and Chau Tuan VO[1]

[1]Faculty of Biology and Environmental Sciences, The University of Danang - University of Education, Danang, Vietnam
[2]Faculty of Chemistry, The University of Danang - University of Education, Danang, Vietnam

*Corresponding author; e-mail: tdmau@ued.udn.vn, trinhdangmau@gmail.com

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ABSTRACT

Haematococcus lacustris is capable to synthesize high content of astaxanthin. Moreover, astaxanthin extracted from *H.lacustris* has been shown to have the highest anti-oxidativeactivity. While the demand for astaxanthin in the world is increasing, the production of astaxanthin from *H. lacustris* is still not easy since they have low growth rate and are very sensitive to changes in culture conditions. This study aimed at investigating the effect of nutritional factors on the growth rate of *H. lacustris* in order to determine the optimal medium for the biomass growth and astaxanthin synthesis of this specy. The results showed that *H.lacustris* grew better in RM culture medium (2.62 ± 0.17 × 10^5 cells/ml) than in BBM culture medium (2.44 ± 0.10 × 10^5 cells/ml). The highest cell density of *H. lacustris* was achieved in the RM medium with $NaNO_3$ at 1200 mg/l (3.72 ± 0.022 × 10^5 cells/ml), and in the RM medium with K_2HPO_4 at 320 mg/l (3.42 ± 0.05 × 10^5 cells/ml).

Introduction

Astaxanthin (3, 3'- dihydroxy ß, ß' carotene - 4,4 - dione) is a keto-carotenoid found in a variety of seafoods such as salmon, shrimp, crabs, fish eggs (Hagen et al., 2002). Its antioxidant activity is 10 times higher than other carotenoids such as ß-carotene, zeaxanthin, lutein, canthaxanthin and even 500 times higher than α-tocopherol (Olaizola, 2000). Due to its capacity of blocking certain types of cancer and stimulating the immune system to be superior to ß-carotene and α-tocopherol, astaxanthin is increasingly applied in the field of medicine and pharmacy (Nguyen and Nguyen, 2014; Dang et al., 2010). Furthermore, astaxanthin is widely used in the food industry and aquaculture. For example, it is used as a type of food for salmon to enhance the color of fish meat - an important quality parameter for consumer's choice (Gouveia et al., 2003; Gouveia and Rema, 2005; Torrissen, 2000).

Currently, the demand for astaxanthin in the world is increasing. However, astaxanthin supplies are mainly from chemical synthesis (accounting for 95%). This source of astaxanthin is very expensive, but its biological activity is rather low. *Haematococcus lacustris*, a species of green algae (Chlorophyta), has a high astaxanthin

accumulation capacity, reaching 5-6% its dry weight (Bubrick, 1991; Cifuentes et al., 2003). Moreover, 100% of astaxanthin extracted from *H. lacustris* is in the form of 3S-3'S isomers, which provides the highest levels of antioxidant activity (Lorenz and Cysewsko, 2000). Therefore, the production of astaxanthin from *H. lacustris* has attracted much research interest.

Meanwhile, the production of astaxanthin from *H. lacustris* is still not easy since they have a low growth rate and are susceptible to changes in culture conditions. When cultured under appropriate conditions, most of the microalgal cells remain in vegetative state, which does not accumulate astaxanthin or accumulate with a low content. Under stress conditions, the algae cell will convert to a non-moving cyst, and they can accumulate large amounts of astaxanthin when appropriately stimulated. Therefore, the conditions for algae cell to grow and synthesize astaxanthin are very different. This study aimed at investigating the effect of nutritional factors on the growth rate of *H. lacustris* in order to determine the optimal medium for the biomass growth and astaxanthin synthesis of this species.

Materials and methods

H. lacustris was acquired from the Faculty of Biotechnology, Vietnam National University of Agriculture. The stock culture and the inoculum were grown in the Bold Basal Medium (BBM) (Andersen, 2005). The inoculum was grown aseptically in a 700 ml flasks containing 400 ml of BBM medium with a stable temperature of 25 ^{o}C, 16 h light:8 h dark cycle of 30 µmol photon m^{-2} s^{-1} white fluorescent light.

Investigate the effect of Sodium nitrate concentration on the growth of *H. lacustris*

The experiment was carried out with an initial algae density of 1.07 × 10^4 cells / ml inserted into a 50ml BBM and RM culture media, a luminous intensity of 30 µmol photon m^{-2} s^{-1}, at a stable temperature of 25 ± 0.5^{o}C on a 16:8 dark-light cycle. The growth of *H. lacustris* was tested on two mediums BBM and RM, which had different concentrations of Sodium nitrate (Table 1). The growth rate of *H. lacustris* was assessed at a frequency of 2 days.

Table 1. Experimental design to investigate the effect of sodium nitrate concentration on the growth of *H. lacustris*.

RM culture medium		BBM culture medium	
$NaNO_3$ concentration	Number of sample	$NaNO_3$ concentration	Number of sample
300 mg/l	3 bottles	250 mg/l	3 bottles
600 mg/l	3 bottles	500 mg/l	3 bottles
900 mg/l	3 bottles	750 mg/l	3 bottles
1200 mg/l	3 bottles	1000 mg/l	3 bottles
1500 mg/l	3 bottles	1250 mg/l	3 bottles
1800 mg/l	3 bottles	1500 mg/l	3 bottles

Investigate the effect of dipotassium phosphate concentration on the growth of *H. lacustris*

Experimental conditions to assess the effect of dipotassium phosphate concentration on the growth of *H. lacustris* were designed the same as those for sodium nitrate. The only difference was that the BBM and RM media had different concentrations of dipotassium phosphate).

Table 2. Experimental design to investigate the effect of dipotassium phosphate concentration on the growth of *H. lacustris*.

RM culture medium		BBM culture medium	
K_2HPO_4 concentration	Number of sample	K_2HPO_4 concentration	Number of sample
80 mg/l	3 bottles	100 mg/l	3 bottles
160 mg/l	3 bottles	200 mg/l	3 bottles
240 mg/l	3 bottles	300 mg/l	3 bottles
320 mg/l	3 bottles	400 mg/l	3 bottles
400 mg/l	3 bottles	500 mg/l	3 bottles
480 mg/l	3 bottles	600 mg/l	3 bottles

Determination of growth rate

Cell density was counted under the microscope by the Neubauer counting chamber, and calculated according to the following formula:

$$D = \frac{A}{X} \times 10^4$$

Where:
D: Cell concentration (cell/ml),
A: Total number of cells counted (cell),
X: Number of squares.

Statistical analyses

Data analysis was performed on the Microsoft Excel 2013 software. The statistical significance of the differences between groups was assessed by one-factor analysis of variance (Anova One way).

Results and discussion

The effect of Sodium nitrate concentration on the growth of *H. lacustris*

Nitrate concentration was identified as the best source of nitrogen for the growth of microalgae cell (Yuan and Chen, 2001). Furthermore, it has the ability to prolong the vegetative state of microalgae (Ranjbar et al., 2008). That is the reason why we conducted the experiments to investigate the effect of nitrate concentration on the growth rate of *H. lacustris* in BBM and RM media. In RM medium, the results showed that at a concentration of 1200 mg/L $NaNO_3$, the highest cell density was recorded after 18 days culture with 3.72 ± 0.022 × 10^5 cells/mL, with a peak growth rate of 0.2 µ. The highest cell densities at $NaNO_3$ concentrations of 1800 mg/L, 1500 mg/L, 900 mg/L, 600 mg/L, and 300 mg/L were 3.48 ± 0.051 × 10^5, 3.27 ± 0.083 × 10^5, 2.86 ± 0.230 × 10^5, 2.70 ± 0.055 × 10^5, and 2.62 ± 0.017 × 10^5 cells/mL, respectively (Fig. 1). According to Thom et al. (2013), the algae cell reached at the highest density (1.74 × 10^6 cells/mL) at the $NaNO_3$ concentration of 876 mg/L. However, in Thom's study, the time for the algae to reach the highest density was 36 days and the peak growth rate was 0.03 µ. The highest density in this study is lower than in other studies due to the initial algal cell density (0.1 × 10^5 cell/mL in this study compared to 0.5×10^5 cell/mL to 1.74 × 10^6 cell/mL in other studies). However, the growth rate peaked in the survey is higher (0.2 µ compared with 0.03 µ compared with other studies). Furthermore, the initial stocking density may be one of the factors affecting the growth of *H. lacustris*.

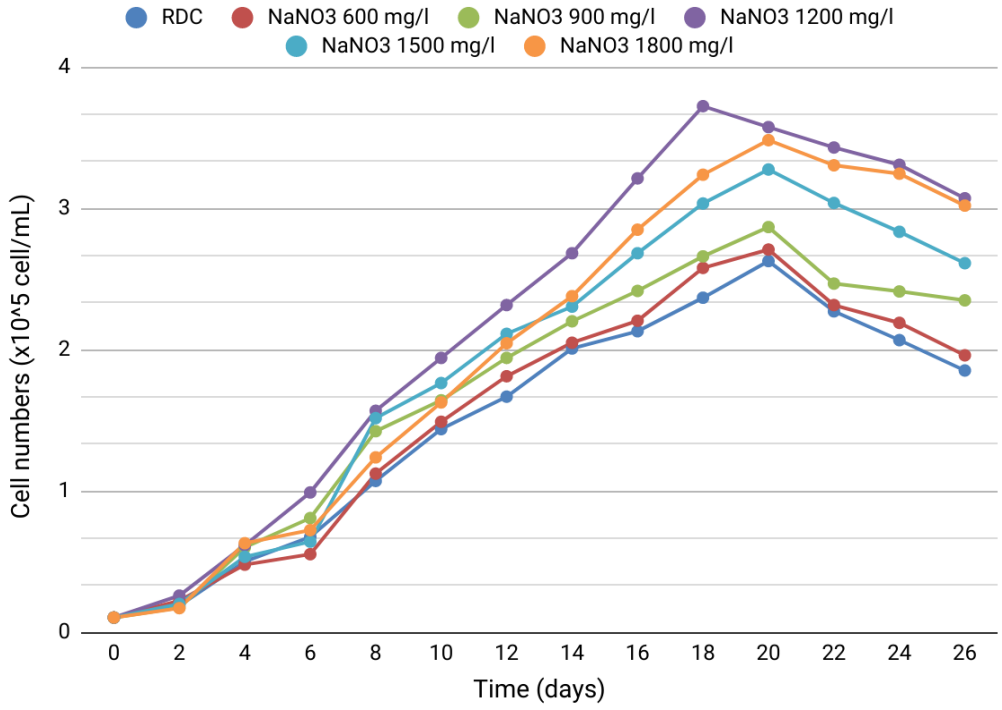

Fig. 1: The cell density of *H. lacustris* cultured in RM medium with different concentrations of $NaNO_3$.

In BBM medium, the highest cell density of the algae was obtained at $NaNO_3$ concentration of 1000 mg/L with 3.46 ± 0.030 ×10^5 cells/mL at day 20th, with the peak growth rate of 0,168 µ. At $NaNO_3$ concentrations of 1500 mg/L, 1250 mg/L, 750 mg/L, 500g/ml and 250 mg/L (control medium), the highest density of microalgae cells were 3.31 ± 0.07 × 10^5, 3.14 ± 0.04 × 10^5, 2.75 ± 0.2 × 10^5, 2.59 ± 0.06 × 10^5, 2.44 ± 0.1 × 10^5 cells/mL (Fig. 2).

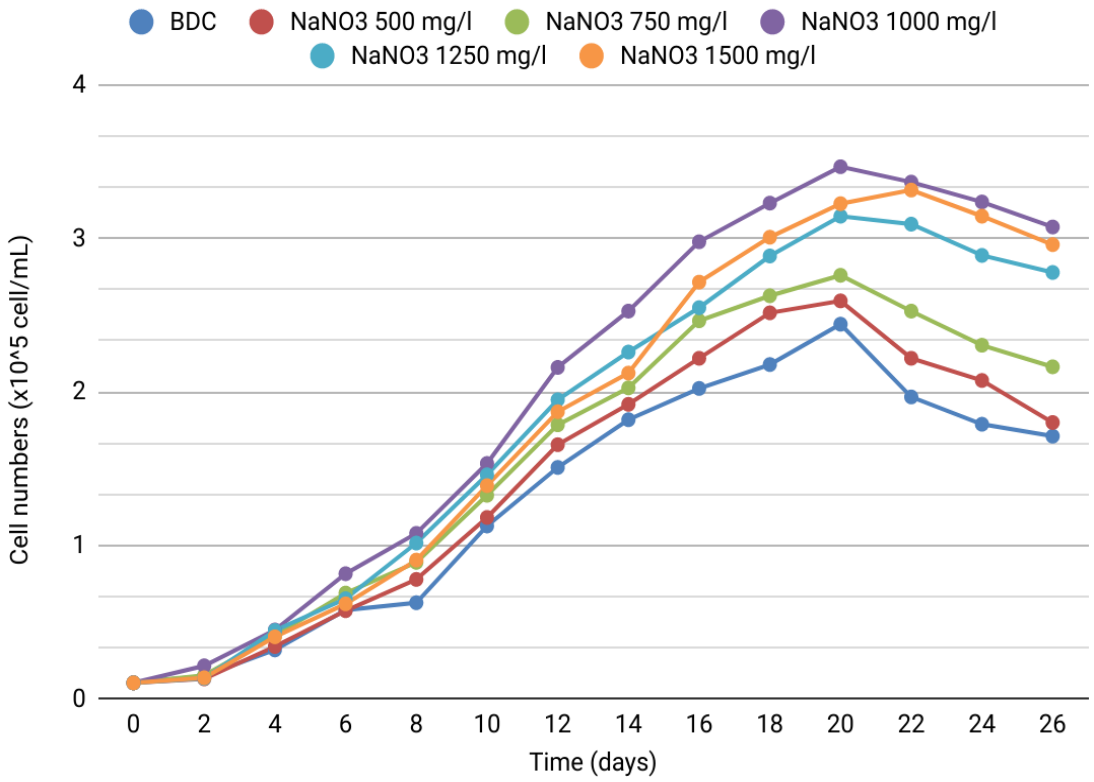

Fig. 2: The cell density of *H. lacustris* cultured in BBM medium with different concentrations of $NaNO_3$.

All treatmentsin both BBM and RM media had a positive effect on the increase of *H. lacustris* cell density in comparison to the control medium. However, it should be noted that algae cell density did not increase linearly with the increase of $NaNO_3$ concentration. The highest cell density in RM media at $NaNO_3$ concentrations of 1400 mg/L and 1800 mg/L (3.27 ± 0.083 × 10^5 cells/mL and 3.48 ± 0.051 × 10^5 cells/mL, respectively) were lower than that at $NaNO_3$ concentrations of 1200 mg/L (3.72 ± 0.022 × 10^5 cells/mL). Similarly, in BBM medium, the highest cell density (3.46 ± 0.03 × 10^5 cells/mL) was obtained at 1000 mg/L of $NaNO_3$ concentration, not at the higher concentrations of $NaNO_3$ (1250 mg/L or 1500 mg/L). Moreover, the results also showed that increasing nitrogen concentration in the RM medium gave better results than in the BBM medium, with the growth rate of 0.2 µ (RM) in comparison to 0.168 µ (BBM). This might be explained by the difference in the nitrogen source in the two media. The RM medium contained two nitrogen sources for microalgae growth, that are NO_3^- and N-NH_4^+; while the BBM medium had only one nitrogen supply, which is NO_3^-.

The effect of dipotassium phosphate concentration on the growth of *H. lacustris*

Phosphorus is one of the main sources of nutrients for the growth and development of microalgae. It is necessary to investigate exactly the content of phosphorus giving the best growth and development for microalgae. This experiment examined the growth of *H. lacustris* in RM and BBM media at different K_2HPO_4

concentration. In RM medium, the best result was obtained at the K_2HPO_4 concentration of 320 mg/L. At this level of concentration, cell density of the microalgae was highest with 3.42 ± 0.05 × 10^5 cells/mL at day 22nd, and the peak growth rate was 0.15 µ. At K_2HPO_4 concentrations of 480 mg/L and 400 mg/L, the highest densities were 3.12 ± 0.02 × 10^5 and 2.94 ± 0.04 × 10^5 cells/mL, respectively, at day 22nd. For the remaining concentrations [240mg/l, 160 mg/l and control medium (RDC)], the highest cell densities were 2.86 ± 0.13 × 10^5, 2.76 ± 0.03 × 10^5, 2.62 ± 0.02 × 10^5 cells/mL, and recorded on day 20th (Fig. 3).

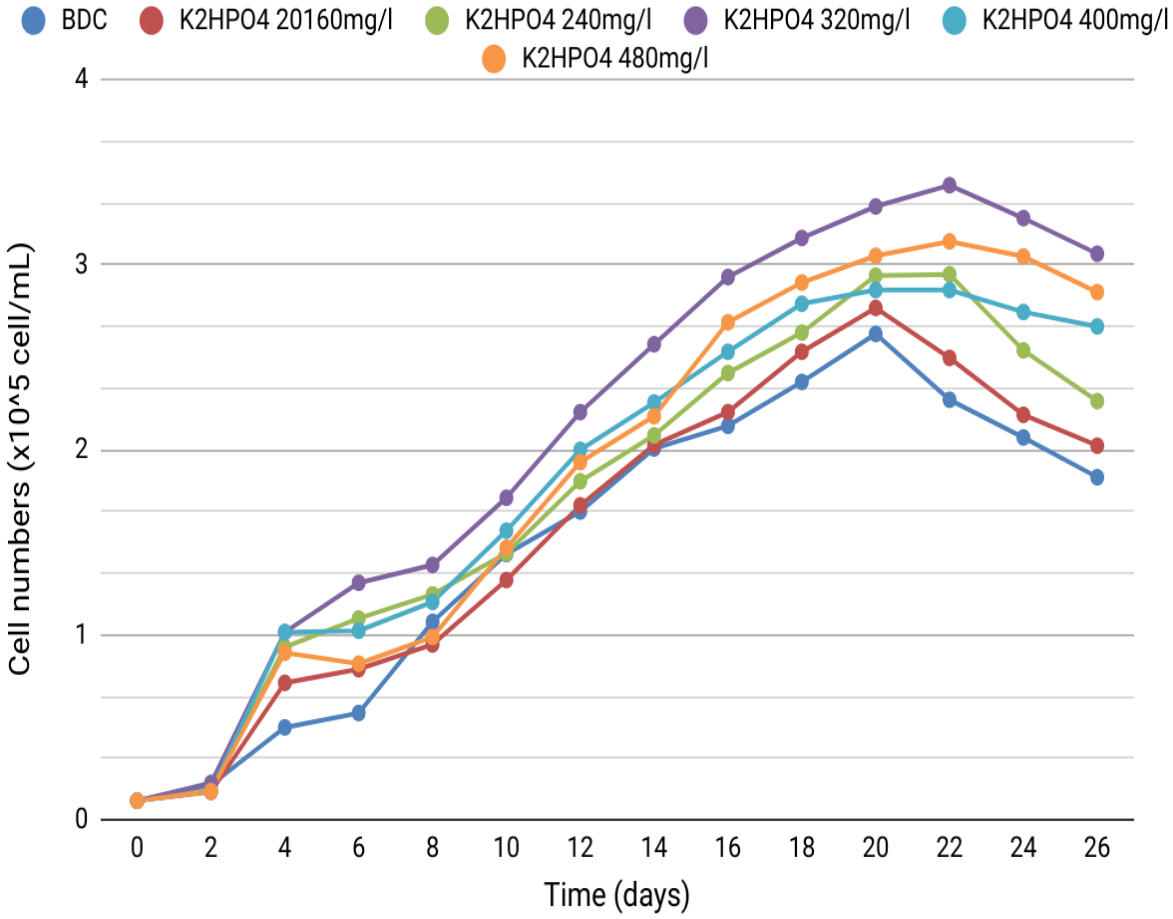

Fig. 3: The cell density of *H. lacustris* cultured in RM medium with different concentrations of K_2HPO_4.

Experimental results on BBM medium showed that concentration of K_2HPO_4 at 400 mg/L gave the highest increase in *H. lacustris* cell density compared with other concentrations. At this concentration level, *H. lacustris* had the highest cell density of 3.11 ± 0.4 × 10^5 cells/mL on day 22nd, with the peak growth rate of 0.14 µ. This cell density level was 27% higher than that in the control medium (2.44 ± 0.14 × 10^5 cells/mL), and also higher than that (2.54 ± 0.023 × 10^5, 2.59 ± 0.08 × 10^5, 2.77 ± 0.06 × 10^5, 2.91 ± 0.03 × 10^5 cells/mL) in other K_2HPO_4 concentrations (375 mg/L, 475 mg/L, 675 mg/L, and 775 mg/L) (Fig. 4).

The initial cell density at different concentrations of K_2HPO_4 on different media affected the growth of microalgae *H. lacustris*. However, cell density did not increase linearly with the increase of K_2HPO_4 concentrations in the media. The growth of microalgae *H. lacustris* was restricted when K_2HPO_4 concentration was higher than 320 mg/L in RM medium, and 400 mg/L in BBM medium. Besides, the growth of *H. lacustris* in different K_2HPO_4 concentration in RM medium was better than in BBM medium (the peak growth rate 0.15 µ in comparison with 0.14 µ).

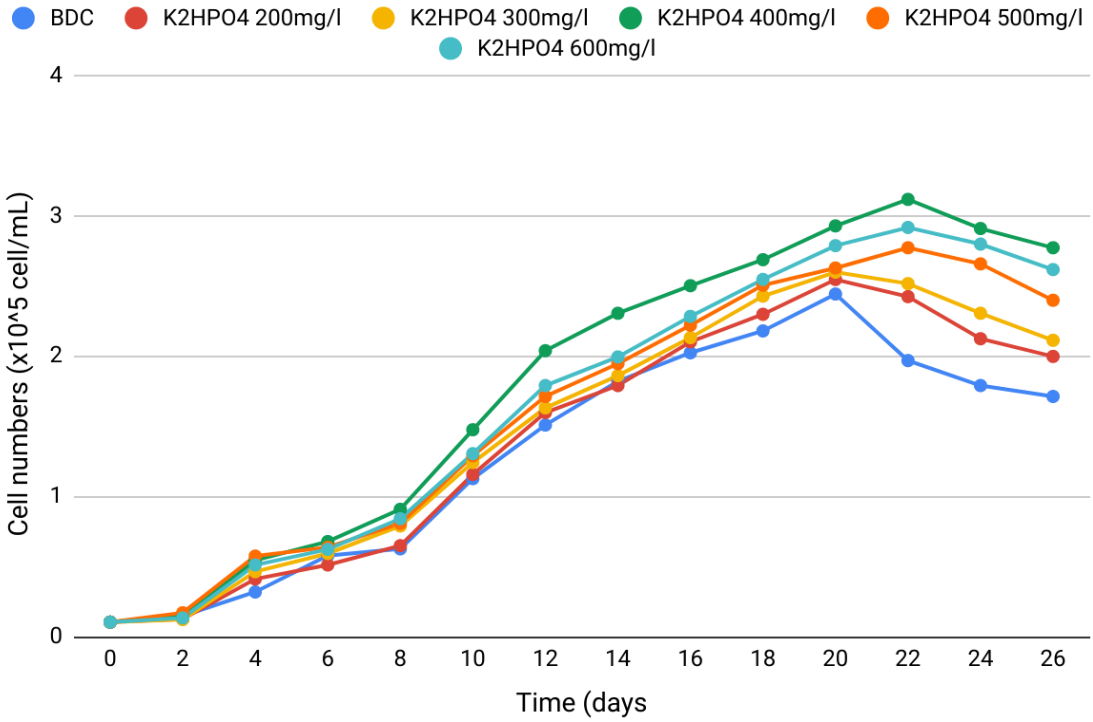

Fig. 4: The cell density of *H. lacustris* cultured in BBM medium with different concentrations of K_2HPO_4.

Conclusion

The results showed that RM culture media was more suitable than BBM media for the growth and development of the microalgae *H. lacustris*. In the RM media, *H. lacustris* gave the best growth at $NaNO_3$ concentration 1200 mg/L, with cell density up to 3.72×10^5 cells/mL, which was 4.2 times higher than the control medium (2.62×10^5 cells/mL). The concentration of K_2HPO_4 at 320 mg/L was the best for the growth of the microalgae was best. At this level of concentration, the highest density of *H. lacustris* reached $3.42 \pm 0.05 \times 10^5$ cells/mL. However, in this study, we only assessed the independent effects of nitrogen and phosphorus on the growth of *H. lacustris*. In fact, the appropriate ratio of nitrogen and phosphorus is an important factor influencing the growth and development of *H. lacustris*. Therefore, more detailed surveys on the effect of nitrogen and phosphorus ratios in culture media on the growth and development of *H. lacustris* should be implemented in near future.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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