Effect of Phosphorus on the Growth, Pigmentation and Lipid Accumulation in Microalgae *Picochlorum* sp.

Phuc Thi Hong Nguyen

Department of Biochemistry and Toxicology, Faculty of Pharmacy, Nguyen Tat Thanh University, VietNam

Peter Cao

Department of Biochemistry and Toxicology, Faculty of Pharmacy, Nguyen Tat Thanh University, VietNam

Trung Vo

Department of Biochemistry and Toxicology, Faculty of Pharmacy, Nguyen Tat Thanh University, VietNam

ABSTRACT:

*Picochlorum* sp. algae are recognized for their abundant bioactive compounds, which include valuable substances such as astaxanthin, carotenoids, fatty acids, and lutein. The significant total lipid content found in *Picochlorum* sp. algae presents diverse opportunities for utilization across the realms of bioenergy, food, and pharmaceuticals. Phosphorus concentrations ranging from 0.02 g/L to 0.16 g/L, the growth of *Picochlorum* sp. did not exhibit any significant differences across these concentrations. However, the chlorophyll and carotenoid contents peaked in the control group and at phosphorus concentrations of 0.02 g/L and 0.04 g/L, respectively, after 12 days of cultivation. In contrast, the lipid content increased in both the control group and at a phosphorus concentration of 0.16 g/L after 9 days of cultivation.

Keywords: *Picochlorum* sp., chlorophyll, lipid, carotenoids, MD4 medium.

INTRODUCTION

*Picochlorum*, a small, single-celled green alga, has drawn attention for its ability to produce abundant intracellular lipids and thrive under challenging cultivation conditions [1],[2],[3]. *Picochlorum oklahomensis* stands out among the *Picochlorum* species due to its high lipid, protein, and unsaturated fatty acid contents, making it a promising candidate for a wide range of biotechnological and biofuel applications. Additionally, *Picochlorum* sp. is well known for its rich carotene, amino acids, and essential fatty lipids, opening doors to functional food and pharmaceutical production [2],[4].

Furthermore, microalgae serve as a source of nutrition for various aquatic organisms, including fish fry, shrimp, and more, contributing to the growth of the aquaculture industry. Microalgae have the potential to treat wastewater and mitigate air emissions, aiding in the reduction of environmental pollution [1],[5],[6]. Consequently, microalgae, such as *Picochlorum* sp., help address the challenges faced by various industries, increasingly asserting their role in domestic industrial development and serving as a sustainable and environmentally safe source of fuel and nourishment for human consumption. The criteria for selecting and propagating microalgae depend on the end-use of the biomass, such as lipid content, protein content, carotene, or high-value products such as antioxidants, antibacterial agents, and anticancer compounds. In Vietnam, numerous microalgal
strains, including *Picochlorum* sp., have been selectively isolated from various water sources, ranging from freshwater to brackish and marine waters, with the aim of producing lipids for the food industry.

Nitrogen and phosphorus sources provide essential nutrients for the growth of algae. The presence of these compounds in the culture medium promotes the synthesis of vital compounds for the development and growth of algae. Additionally, they facilitate the production of chlorophyll, a crucial photosynthetic pigment that enables algae to absorb solar energy for photosynthesis. Nitrogen and phosphorus deficiencies can lead to reduced chlorophyll synthesis, impacting photosynthesis and algal growth. Furthermore, lipids serve as a significant energy source and hold high economic value, particularly in the production of biofuels. The adjustment of nitrogen and phosphorus levels in the culture environment can influence the accumulation of lipids in algae. Therefore, nitrogen and phosphorus sources play pivotal roles in the growth, chlorophyll synthesis, and lipid accumulation of algae [7],[8],[9].

*Picochlorum* sp. robustly grows and accumulates lipids, carotenoids, and chlorophyll under various phosphorus conditions. Thus, the research project, "The Effect of Phosphorus Sources on the Growth, Pigmentation, and Lipid Content of Microalga *Picochlorum* sp.,".

**MATERIALS AND METHODS**

**Picochlorum sp. and Culture Media**

*Picochlorum* sp. was obtained from the Ph.D. Trung Vo, Department of Biochemistry and Toxicology, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam [4].

The 0.5 M MD4 medium included seawater stored in can and the following ingredients: MgSO₄·7H₂O (3.813 g/L), EDTA·2H₂O (0.4849 g/50 ml), FeCl₃·6H₂O (0.0408 g/50 ml), and MnCl₂·4H₂O (0.1485 g/50 ml, pH 7.5) [10], with continuous aeration and illumination at 50 μmol photons/m/s and a frequency of 12:12 light/dark cycles.

**The Experimental Design**

*Picochlorum* sp. was cultivated in 0.5 M MD4 medium in a 50 ml Erlenmeyer flask with continuous aeration and illumination at 50 μmol photons/m/s with a 12:12 light/dark cycle. After 18 days, when the algae reached the exponential growth phase, they were subjected to various experiments.

After 18 days of cultivation, *Picochlorum* sp. was transferred to MD4 media containing different phosphorus concentrations (KH₂PO₄): 0.02 g/L, 0.04 g/L, 0.08 g/L, and 0.16 g/L and stock KNO₃, with 0 g/L KH₂PO₄ as a control sample. Cultivation of *Picochlorum* sp. in 250 mL Erlenmeyer flasks with 100 mL of MD4 medium at an initial cell density of ≈ 10⁶ cells/ml, with continuous aeration and illumination at 50 μmol photons/m²/s with a frequency of 12:12 light/dark cycle.

Estermination of cell density, growth rate, total carotenoid, chlorophyll, and total lipid content of *Picochlorum* sp. after each 3-day cultivation. The results are expressed as units per volume (cell/ml, μg/ml) and per cell (pg/cell).

**Analysis Methods**

**Cell Density**

The cell density of the algae was directly quantified using a chamber. A 100 μL aliquot of the algae sample was fixed with 10 μL of Lugol's solution (5% iodine and 10% KI). The cell count was performed using a hemocytometer chamber with a depth of 0.1 mm and a square grid area of 1 mm². The cell density in 1 mL was calculated using the following formula [11]:

\[
D = \frac{n}{i} \times 10^4 \times \text{dilution factor}
\]

\[n: \text{total number of counted cells}\]
i: Counting area  
D: cell density (cell/ml)

**Cell Density Standard Curve**

Step 1: Cultivate *Picochlorum* sp from test tubes into 50 ml Erlenmeyer flasks with 0.5M MD4 medium, continuous aeration and illumination at 50 μmol photons/m²/s with a frequency of 12:12 light/dark cycle. The Erlenmeyer flasks were cultivated until the 18th day, when *Picochlorum* sp. reached its maximum growth phase.

Step 2: Several Erlenmeyer flasks containing 90 ml of sterile deionized water, test tubes with 9 ml of sterile deionized water, and sterile test tubes (1 mL) were prepared. The samples were diluted to 100%, 80%, 60%, 40%, 20%, and 10%.

Step 3: Cell growth in the culture solution was determined by measuring the optical density (OD) at a wavelength of 750 nm after dilution using a spectrophotometer. Concentrations of 10%, 20% and 40% were chosen for cell counting using the Neubauer Thomas hemocytometer method. After determining the OD of the diluted samples and counting the number of cells in the diluted samples, we established a correlation equation between the number of cells and the OD as follows: $Y = ax + b$

where $Y$ is the OD value and $x$ is the cell density (cell/ml.10⁶).

**Specific Growth Rate**

The cell density at two different time points during the growth process of the sample was used to calculate the specific growth rate ($\mu$: cell/ml/day) over that time interval according to the formula [12]:

$$\mu = \frac{\ln N_t - \ln N_0}{\Delta t}$$

(2)

where $N_t$ is cell density at time $t$ and $N_0$ is cell density at initial day, $t$ is the length of the time interval ($t_t - t_0$).

**Determination of Total Chlorophyll and Carotenoid Content**

**Step 1:** The reagents were prepared at a ratio of ethanol:hexane = 2:1. Specifically, 100 mL of ethanol was mixed with 50 mL of hexane.

**Step 2:** One milliliter of the algal culture was taken and centrifuged at 13,000 rpm for 5 minutes. The settled algal pellet at the bottom was then extracted with 3 mL of ethanol:hexane (2:1) in a 15 mL Falcon tube and thoroughly mixed. Subsequently, 2 mL of distilled water and 4 mL of hexane were...
added to the mixture, followed by vigorous shaking. This extraction mixture was then centrifuged at 3,000 rpm for 5 minutes, resulting in phase separation into two layers. The pigment layer with hexane on top was measured at wavelengths of 450 nm, 662 nm, and 645 nm. The blank sample consisted of 4 mL of hexane. The total carotenoid content was determined using the following formula [13]:

\[
\text{Carotenoid (μg/ml)} = A_{450} \times 25.2
\]  

(3)

The levels of chlorophyll a and b were determined as follows [14]:

\[
\text{Chlorophyll a (μg/ml)} = 11.75 \ (A_{662}) - 2.35 \ (A_{645})
\]

\[
\text{Chlorophyll b (μg/ml)} = 18.61 \ (A_{645}) - 3.96 \ (A_{662})
\]

Total chlorophyll (μg/ml) = Chlorophyll a ± Chlorophyll b (is the absorbance at 645 nm, 662 nm)

**Determining the Total Lipid Content Using the Sulfo-Phospho-Vanillin Method**

**Stage 1: Preparation of the reagent**

The phosphovanillin reagent was prepared by dissolving 0.12 g of vanillin in 4 mL of absolute ethanol. Then, 16 mL of distilled water was added, and 80 mL of concentrated phosphoric acid solution was added to the mixture. The reagents were stored in the dark throughout the analysis.

**Stage 2: Determination of lipid content**

One milliliter of the algal solution was centrifuged at 13,000 rpm for 5 minutes. The cell pellet was extracted with 2 mL of concentrated sulfuric acid in a glass test tube with a lid. The test tube was heated in a water bath at 100°C for 10 minutes and then cooled in an ice-water bath. Then, 5 mL of the phosphovanillin reagent was added, and the mixture was incubated at room temperature for 15 minutes while continuously shaking. The sample was measured at a wavelength of 530 nm [15].

Lipid standard curve: A standard lipid curve was prepared by concentration ranges of 0 – 150 μg/ml using Tuong An sesame oil (100% lipid) with steps 1 and 2.

The standard lipid curve formula was 
\[
y = 0.0057x - 0.0082 \quad (R^2 = 0.9992)
\]

y: OD value; x: lipid content (μg/ml)

![Figure 2. Standard Curve of Lipid](image)

**Data Analysis**

The data were processed using Microsoft Excel 2019 software and SPSS 20.0 software for one-way analysis of variance (ANOVA). Whether or not an observed correlation was statistically significant was evaluated by P values (significant when p ≤ 0.05). The data are presented as the mean ± standard error of the mean (SEM). All treatments were evaluated three times.
RESULTS AND DISCUSSION

The Growth of *Picochlorum* sp.

The *Picochlorum* sp. algae were cultivated in 0.5 M MD4 media supplemented with varying concentrations of phosphorus, including a control group and four different phosphorus concentrations (KH$_2$PO$_4$): 0.02 g/L, 0.04 g/L, 0.08 g/L, and 0.16 g/L. Among all the control and phosphorus-supplemented groups, the group with 0.16 g/L phosphorus displayed the highest cell density from days 18 to 24 (p>0.05) (Figure 3a). As Figure 3b illustrates, the addition of different phosphorus sources to 0.5 M MD4 media did not significantly impact the specific growth rate of *Picochlorum* sp. The control group and the phosphorus-supplemented group exhibited similar specific growth rates, with no statistically significant differences (p=0.571).

These findings align with the research conducted by Lovio (2019) on *Picochlorum oklahomensis*, which demonstrated the significant influence of phosphorus on vital nutrients to support algal growth, which is essential for producing a large quantity of algae for use in biofuel production. Ongoing research aims to explore how different concentrations of Na$_3$PO$_4$ affect algal development. The increasing presence of Na$_3$PO$_4$ enhances algal growth by providing an essential nutrient. The results indicate that Na$_3$PO$_4$ maximizes algal growth and is beneficial for biofuel production. These results are consistent with findings from other studies investigating the influence of individual nutrients on algal development [16].

The Total Chlorophyll Content of *Picochlorum* sp.

The total chlorophyll content per unit volume in the 0.5 M MD4 culture medium supplemented with phosphorus tended to increase beginning on day 9 and peaked on day 12, followed by a decreasing trend from day 15 onward, after which it stabilized (p≤0.05) (Figure 4a). Similarly, as shown in Figure 4b, the total chlorophyll content per cell exhibited an increasing trend from day 9, reaching its peak on day 12 during the 24-day cultivation period. There was a decreasing trend from day 15 onward.

Furthermore, research conducted by Gonzalez-Esquer et al. (2018) on *Picochlorum celeri* revealed that chlorophyll production remained low when cultivated in phosphorus-supplemented media. This study also suggested limitations in enhancing the chlorophyll content during the experiment, resulting in low levels of beta-carotene, indicating the need for further investigation. Additionally, experiments have indicated that beta-carotene accumulation remains low when plants are cultured under both high- and low-light conditions [2].
The Total Carotenoid Content of *Picochlorum* sp.

*Picochlorum* sp. algae were cultivated in 0.5 M MD4 media supplemented with phosphorus nutrients at four concentrations and in the control group. Notably, the cells in the control group accumulated more carotenoids than did those in the other treatment groups during development, and the carotenoid content decreased significantly on the following days ($p \leq 0.05$) (Figure 5a). Additionally, the carotenoid content per cell yielded similar results, reaching its peak on day 9 and decreasing gradually after day 12 ($p \leq 0.05$). However, the carotenoid content per cell of *Picochlorum* algae from day 18 to day 24 tended to stabilize during the equilibrium phase (Figure 5b). Furthermore, Figure 5c shows that the carotenoid-to-total chlorophyll ratio tended to remain stable from day 9 to day 24 in both the control group and the four phosphorus-supplemented groups ($p > 0.05$).

In summary, this study revealed that carotenoid levels in *Picochlorum* sp. remained relatively low when exposed to phosphorus nutrient supplementation, corroborating the findings of previous research conducted by Cano (2021) on *Picochlorum celeri*. However, it is important to consider that external factors may influence stress responses in *Picochlorum* sp. during its growth phase, potentially contributing to the observed equilibrium in carotenoid levels. This also suggested that after the 15th day of cultivation, nutrient levels in the 0.5 M MD4 medium became depleted [17],[18].
**Total Lipid Content of *Picochlorum* sp.**

The lipid content in *Picochlorum* sp. generally increased as the cultivation period progressed. It exhibited the highest lipid content compared to the other conditions from days 18 to 24. Additionally, there were significant differences in lipid content among the conditions over the 24-day cultivation period, with notable differences from day 9 to day 24 ($p \leq 0.05$) (Figure 6a). The lipid content per cell reached its highest level on days 18 to 21 of cultivation and subsequently declined in the following days. Interestingly, the control condition and 0.16 g/L consistently had the highest lipid content from days 18 to 24 when compared to the conditions supplemented with various phosphorus levels ($p \leq 0.05$) (Figure 6b). Figure 6c visually depicts another crucial finding. The lipid content relative to the total chlorophyll content was highest on days 21 to 24. The lipid content in the control group was greater than that in the phosphorus-supplemented group during the cultivation period ($p \leq 0.05$).

The study's results suggest that the addition of phosphorus to the culture medium enhances lipid accumulation in *Picochlorum* sp. This observation aligns with the findings from Cano’s research (2021), which showed that the lipid content in the strain *Picochlorum celeri* increased when the culture medium was supplemented with phosphorus, surpassing the levels of carotenoids and chlorophyll. These insights shed light on the factors influencing lipid accumulation in this particular algal species and may have implications for various applications in the future [19].

![Figure 6. Total Lipid Content per Volume (a), Total Lipid Content per Cell (b) and Total Lipid/Total Chlorophyll Ratio (c) in 0.5 M MD4 Medium Supplemented with Different Concentrations of Phosphorus](image)

**CONCLUSION**

Microalgae *Picochlorum* sp. was small size species, able to accumulate large amount of lipid, essential fatty acids. *Picochlorum* sp. grew well in MD4 media supplement of phosphorus from 0.02 to 0.16 g/L concentration. The chlorophyll and carotenoid contents peaked in the control group and at phosphorus concentrations of 0.02 g/L and 0.04 g/L, respectively, after 12 days of cultivation. In contrast, the lipid content increased in both the control group and at a phosphorus concentration of 0.16 g/L after 9 days of cultivation.

**ACKNOWLEDGEMENT**

I would like to express my sincere gratitude to Dr. Trung Vo for his invaluable guidance and insightful suggestions throughout the course of this research.
CONFLICT OF INTERESTS
No conflict of interest.

REFERENCES


