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High phosphorus concentration induces lipid accumulation in *Chlorella pyrenoidosa* under nitrogen deficiency

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ABSTRACT

The objective of this study was to investigate the effect of different phosphorus concentrations on growth, biomass, photosynthetic pigment, and lipid content of green microalgae *Chlorella pyrenoidosa* under nitrogen deficiency. Four types of media including control with sufficient nitrogen and phosphorus (240 mg/L for N, 5.56 mg/L for P) and three various phosphorus concentrations (5.56, 27.8, and 55.6 mg/L) under limited nitrogen (8 mg/L) were applied under photoautotrophic culture for 5 days. The findings demonstrated that high phosphorus concentration had a marginal impact on the growth and biomass concentration. Moreover, the combination of phosphorus repletion and nitrogen deficiency had an unfavorable effect on Chl *a*+*b* and carotenoid. It is interesting to note that lipid content was considerably accumulated by a slight excess of phosphorus (27.8 mg/L) with nitrogen deficiency. This was 53% higher than that of control and 12% higher than that of sufficient phosphorus with nitrogen deficiency. This study indicates that increasing phosphorus concentration up to 27.8 mg/L under nitrogen deficiency is an effective strategy to enhance the lipid content of *C. pyrenoidosa* in autotrophic culture.

Keywords: autotrophic culture, green microalgae, nitrogen deficiency, phosphorus repletion, lipid content.

INTRODUCTION

Microalgae are unicellular organisms that can convert light energy to biochemical molecules, assimilate CO_2, and utilize organic and inorganic, macronutrients and trace minerals (Daneshvar et al., 2018). Microalgae are a great option for producing biodiesel because they have a higher lipid content, grow more robustly than traditional crops, and don't interfere with food production (Chisti, 2007). However, microalgae-based biofuels are still not entirely regarded as commercially viable because microalgal biomass and lipids have lower productivity (Liu et al., 2016). Several investigations indicate that environmental stressors like variations in pH, temperature, and light intensity (Chokshi et al., 2015; George et al., 2014) and nutrient stressors like phosphorus and nitrogen deprivation (Maltsev et al., 2023) may cause microalgae to accumulate lipids. One of the most popular methods for increasing lipid content in autotrophic culture is nitrogen limitation or deficiency, which has been shown in numerous prior research to be successful (Rai et al., 2017; Maltsev et al., 2023). Nevertheless, while prolonged nitrogen deprivation increases the lipid content of microalgae, it also slows down their

growth, which lowers their total productivity (EL-Sheekh et al., 2012). To address this issue, several investigations discovered that a sufficient quantity of phosphorus was crucial in improving the lipid production of microalgae in the presence of nitrogen limitation (Chu et al., 2014; Fu et al., 2017). The metabolic synthesis of algal cell components, such as proteins, DNA, RNA, and intermediate metabolic products, has been proposed to depend on phosphorus (Roopnarain et al., 2014; Zhu et al., 2015). In addition, the surplus phosphorus may be deposited as polyphosphate, which is high in energy stored in phosphoanhydride bonds. This compound may be utilized as an energy source comparable to ATP in anabolic processes during nitrogen starvation (Fu et al., 2017). Furthermore, phosphate influences the activity of ADP-glucose pyrophosphorylase, which regulates carbon partitioning between the starch and lipid synthesis pathways (Zhu et al., 2015). *Chlorella pyrenoidosa* was chosen as the model organism for this investigation because of its fast specific growth rate and high lipid content (11–26% DW) (Nigam et al., 2011). Additionally, this species has been utilized in variety of industries, including pharmaceuticals, biofuel, food supplements, and biofertilizers (Bindra and Kulshrestha, 2019; Rathore and Nema, 2024). To the best of our knowledge, there is no information on the effects of phosphorus repletion under nitrogen stress on the growth and biomass composition of *C. pyrenoidosa*. In the current investigation, we determined the influence of excess phosphorus supply under nitrogen deficiency on *C. pyrenoidosa* growth, biomass, and photosynthetic pigment. More significantly, we assessed whether a high phosphorus supply causes lipids accumulation on *C. pyrenoidosa*.

MATERIALS AND METHODS

Strain and cultivation medium

C. pyrenoidosa was acquired from the Institute of Brackishwater Aquaculture, Jepara, Indonesia. The microalgae stock was maintained in a 250 mL flask with 150 mL working volume under static culture conditions. The temperature and light intensity of cultures were set at 25 °C and 8.00 lux, respectively, under continuous illumination. BG-11 medium was used to cultivate microalgae (Stanier et al., 1971).

Experimental culture conditions

All experiments used BG-11 as the basal medium, which contained 1,500 mg/L $NaNO_3$ (about 240 mg/L for N) and 31.4 mg/L K_2HPO_4 (about 5.56 mg/L for P) for nitrogen and phosphorus, respectively. Four different media types were used in this study by varying the nitrogen and phosphorus concentrations of the BG-11 medium (Table 1), which included a control with sufficient nitrogen and sufficient phosphorus (the same as those in BG-11, N&P), limited nitrogen and sufficient phosphorus (N_{def}&P), limited nitrogen and slight-excessive phosphorus (N_{def}&P_{+}), and limited nitrogen and excessive phosphorus (N_{def}&P_{++}). In this study, we defined nitrogen deficiency and phosphorus excess as 3.3% and up to 1,000% of nitrogen and phosphorus in the original BG11 medium, respectively. *C. pyrenoidosa* was pre-cultivated for 4 days and then centrifuged at 4.000 rpm for 15 min. Cells were suspended at an initial optical density of 680 nm (OD_{680} nm) of 0.123 in each of the four types of media after being washed with sterilized distilled water to get rid of any remaining orthophosphate and nitrate. All treatments were performed in a batch system at a temperature of 25 °C and light intensity of 11.000 lux with constant illumination. Sterilized air was used to continuously aerate the cultures. Algal cells were collected daily for growth analysis. After 5 days of cultivation, cells were harvested by centrifugation and dried in the oven at 65 °C to generate microalgal powder. Finally, dried biomass was stored at -20 °C for further analysis. All experiments were performed in triplicate.

Growth analysis

To evaluate *C. pyrenoidosa* cell growth, optical density at 680 nm (OD_{680}) was measured at 24-hour

Table 1. Initial nitrogen and phosphorus concentrations in the modified BG-11 medium

Element	N&P	N_{def}&P	N_{def}&P_{+}	N_{def}&P_{++}
Nitrogen (NO_3^--N) (mg/L)	240 (100%)	8 (3.3%)	8 (3.3%)	8 (3.3%)
Phosphorus (PO_4^{3-}-P) (mg/L)	5.56 (100%)	5.56 (100%)	27.8 (500%)	55.6 (1000%)

Note: * Values inside parenthesis show the ratio of the changed nitrogen/phosphorous concentrations to those in the original BG-11 medium.

intervals using a spectrophotometer (GENESYS™ 20 UV-Vis, Thermo Scientific, USA). The following formula was used to calculate the specific growth rate (SGR) and doubling time (Td):

$$SGR\ (/day) = \frac{Ln\ (Nt) - Ln(N0)}{\Delta t} \quad (1)$$

$$Td\ (day) = \frac{Ln\ 2}{\mu} \quad (2)$$

where: Nt and $N0$ are the final and initial cell concentration and Δt is the duration of culture.

Determination of biomass

At the end of the experiment, 25 mL microalgae samples were collected to determine the biomass. The suspensions were filtered through a 1.2 µm GF/C filter paper (Whatman, USA) and rinsed twice with distilled water and then dried at 105 °C for 2 hours until they reached a constant weight. The filter paper was cooled, then set on a desiccator for half an hour, and the weight was noted. Finally, the dry weight of microalgae was determined as g/L (Fakhri et al., 2021).

Determination of pigment

To determine the amount of carotenoid and chlorophylls (Chl *a*+*b*) in *C. pyrenoidosa* at the end of the cultivation period, the pigments were extracted using 90% methanol. A 2 mL sample of algal cultures was centrifuged for 15 min at 4.000 rpm, and then the sample was washed with distilled water. Two mL of 90% (v/v) methanol solution were used to extract the pellet, and it was sonicated for 10 min. The mixtures were heated in a water bath for 10 min at 70 °C, and then centrifuged for 15 min at 4.000 rpm. Using a spectrophotometer (GENESYS™ 20 UV-Vis, Thermo Scientific, USA), the absorbance of the supernatant was determined. The following formula was used to calculate the content of chlorophyll *a*+*b* (Ritchie, 2006) and carotenoid (Kim et al., 2014):

$$Chl\ a\ (\mu g/mL) = 16.5169 \times \times A_{665} - 8.0962 \times A_{652} \quad (3)$$

$$Chl\ b\ (\mu g/mL) = 27.4405 \times \times A_{652} - 12.1688 \times A_{665} \quad (4)$$

$$Carotenoid\ (\mu g/mL) = 4 \times A_{480} \quad (5)$$

Determination of lipid

Lipids were extracted from dried biomass using a modified version of the Bligh and Dyer (1959) method. A pestle and mortar were used to grind the dry sample into a fine powder. First, 30 mg of the powder (M1) was combined with 3 mL of chloroform/methanol (2:1, v/v). The mixture was then subjected to phase separation by adding 0.8 mL of the 0.73% NaCl solution. After that, the mixture was centrifuged for 2 min at 2,000 rpm. The top layer was removed, and the lower layer was collected and put in a glass tube that had been previously weighed (M2). The extraction procedure was carried out once again to improve the recovery rate. After using N_2 to dry the collected supernatant, they were further dried in an oven set at 65 °C. Furthermore, the residue and glass tube were desiccated until their weight remained constant (M3). Lipid content was reported as a percentage of dry weight (%DW) and computed as follows (Zhao et al., 2016):

$$Lipid\ content\ (\%DW) = \frac{M3 - M2}{M1} \times 100\% \quad (6)$$

Data analysis

Every treatment was carried out in triplicate. One-way analysis of variance (ANOVA) and Duncan's statistically significant differences test were used to assess the data. The data were displayed as mean±standard deviation, with a p-value of < 0.05 was considered significant. All statistical data were evaluated using IBM SPSS version 27.

RESULTS AND DISCUSSION

Effect of excessive phosphorus on growth and biomass of *C. pyrenoidosa* under nitrogen deficiency

Growth profiles of *C. pyrenoidosa* under four different media types are presented in Figure 1. The optical density of all treatments increased in a similar pattern until day 2 of cultivation. After that, growth under N&P conditions was significantly higher than that of other media types until day five. N&P produced the highest OD_{680} of 2.13, whereas N_{def}&P produced the lowest OD_{680} of 1.29. Table 2 shows specific growth rates and doubling times of *C. pyrenoidosa* in each type of media. The fastest growth rate (0.569/day) and doubling time (1.217/day) were recorded by N&P media. On the other hand, N_{def}&P had the slowest growth rate of 0.469/day and a doubling time of 1.478 days. This result is in accordance with Shen et al. (2016), who found

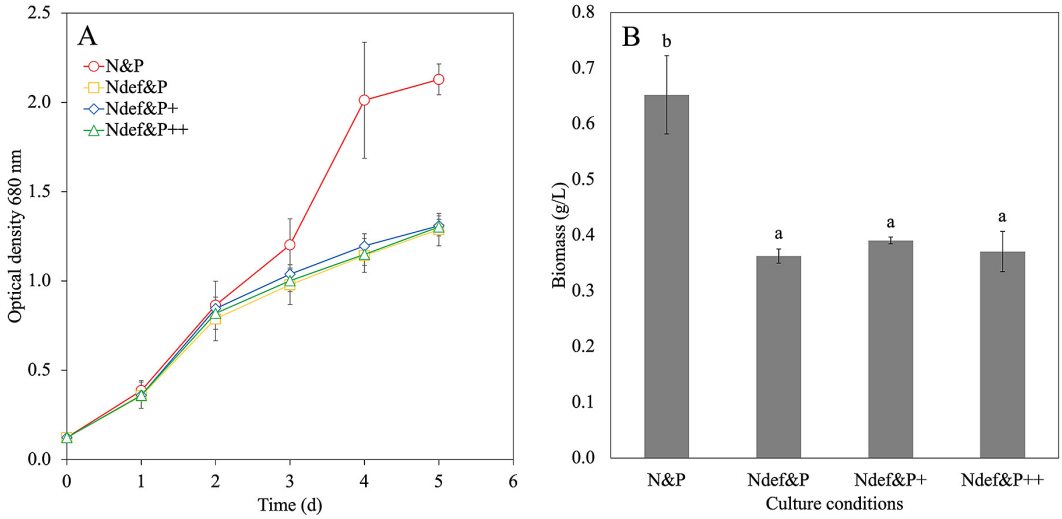

Figure 1. Growth of the *Chlorella pyrenoidosa* cells: A) changes in optical density at 680 nm (OD680); and B) bbiomass concentration of *C. pyrenoidosa* grown in different media combinations. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). At a 95% confidence level, different letters show that there was a remarkable difference between treatments

Table 2. Specific growth rate (SGR), doubling time, and nutrient assimilation of *C. pyrenoidosa* under various media types

Media types	SGR (/day)	Doubling time (days)
N&P	0.569 ± 0.015^{b}	1.217 ± 0.033^{b}
N_{def}&P	0.469 ± 0.009^{a}	1.478 ± 0.031^{a}
N_{def}&P_{+}	0.472 ± 0.005^{a}	1.468 ± 0.017^{a}
N_{def}&P_{++}	0.471 ± 0.009^{a}	1.471 ± 0.030^{a}

Note: Values shown are averages of triplicates ± standard deviation. Same letters show that there was no significant difference between treatments.

that nitrogen deficiency stress caused a significant decrease in *C. vulgaris* growth rate.

The growth rate of *C. pyrenoidosa* was slightly accelerated by increasing the phosphorus concentration under nitrogen limitation; nevertheless, there was no significant difference ($p > 0.05$) between adequate phosphorus (N_{def}&P) and excessive phosphorus (N_{def}&P_{+} and N_{def}&P_{++}). This finding suggests that when nitrogen was adequate, the impact of phosphorus content on growth rate was substantial. Similarly, Chu et al. (2014) found that *Scenedesmus obliquus* did not significantly enhance its specific growth rate when exposed to high phosphorus concentrations under nitrogen limitation.

The biomass concentration of *C. pyrenoidosa* under different nitrogen and phosphorus supplies was shown in Figure 1B. Nitrogen deficiency stress (N_{def}&P) caused the biomass production to drop dramatically to 0.363 g/L from the maximum biomass yield of 0.652 g/L under N&P conditions. El-Kassas (2013) reported a similar finding, stating that microalgae biomass was considerably reduced by nitrogen deprivation. *C. pyrenoidosa* biomass slightly increased in response to an increase in phosphorus concentration. Similarly, Chu et al. (2014) found that adding a high phosphorus concentration to a medium with limited nitrogen did not significantly increase the biomass of *S. obliquus*.

Effect of phosphorus repletion on Chl a+b and carotenoid of *C. pyrenoidosa* under nitrogen deficiency

The Chl *a+b* and carotenoid content of *C. pyrenoidosa* after 5 days of cultivation in various media types are shown in Fig. 2A. The highest Chl *a+b* level, 27.85 µg/mL, was produced by N&P. The amount of chlorophyll in the N_{def}&P media decreased to 9.65 µg/mL, which was 2.9 times lower than in the N&P media.

The carotenoid content under various nitrogen and phosphorus media showed a similar pattern. Under N&P, the highest carotenoid level of 4.15 µg/mL was recorded. Expectedly, the carotenoid content was 76% lower than in N&P media due to nitrogen deficiency. These results suggest that the pigment concentration of *C. pyrenoidosa* is negatively impacted by limited nitrogen in the medium. This result was in line with Zarrinmehr et al. (2020), who found that carotenoid and chlorophyll levels of *Isochrysis galbana* decreased because of nitrogen deficiency. Nitrogen is an essential ingredient and a component of high-value biological macromolecules like protein and chlorophylls, which are crucial for microalgal production (Chokshi et al., 2017). Regarding the effect of phosphorus, no positive influence in all photosynthetic pigments was observed after a high phosphorus concentration was supplied in the media. Our study suggests that a high phosphorus supply did not promote the photosynthetic pigment content in *C. pyrenoidosa* under nitrogen deficiency. We also found that nitrogen stress affected the color of microalgae. As shown in Figure 2B, the color of *C. pyrenoidosa* cultures was dark green when cultured in N&P condition but changed to pale green under all nitrogen deficiency treatments. A similar culture color was also observed in high phosphorus supply (N_{def}&P_{+} and N_{def}&P_{++}). The color of culture media indicates the change in pigment content in microalgae (Mirzaie et al., 2016).

Lipid accumulation enhancement was induced by the phosphorus supply

The lipid content of *C. pyrenoidosa* under various nitrogen and phosphorus conditions is shown in Figure 3. All limited nitrogen treatments induced the lipid accumulation of *C. pyrenoidosa*. The lipid content of N_{def}&P (20.33% DW) was significantly higher ($p < 0.05$) than that of N&P (14.8% DW). Similarly, Shen et al. (2016) found that *C. vulgaris* accumulated lipids as a result of nitrogen deficiency. According to previous research, this phenomenon can be explained by two different mechanisms: (1) nitrogen stress leads to an increase in the activity of the malic enzyme, which produces NADPH and causes lipid accumulation (Li et al., 2013) and (2) increased glutamate dehydrogenase promotes the Krebs cycle, providing substrates and energy for lipid biosynthesis (Liu et al., 2016). Interestingly, when the concentration of phosphorus was increased up to 27.8 mg/L (N_{def}&P_{+}), the lipid content of *C. pyrenoidosa* remarkably increased up to 22.78% DW which was 53% and 12% greater than that of N&P and N_{def}&P, respectively. These findings suggest that under conditions of nitrogen deficiency, phosphorus repletion stimulated the lipid synthesis in *C. pyrenoidosa*. These results were consistent with Fu et al. (2017) who reported that high phosphorus concentration (45 mg/L) under limited nitrogen enhanced the lipid content of *C. regularis*. Zhu et al. (2015) explained that an abundance of phosphorus led to a decrease in the

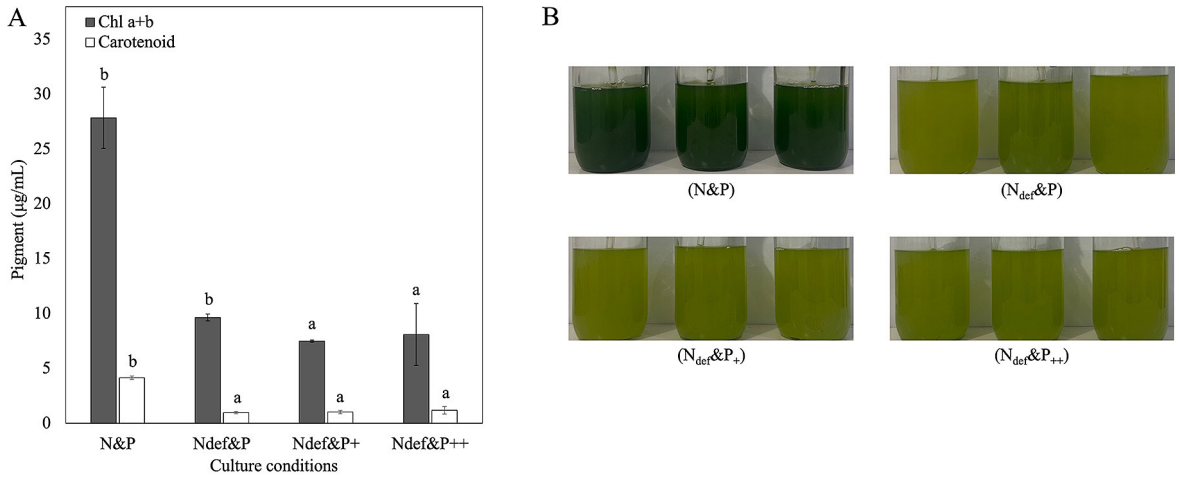

Figure 2. Chl a+b and carotenoid of *Chlorella pyrenoidosa* cultivated in different media types (A). Color changes of microalgae cells at the end of the experiment (B). Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). At a 95% confidence level, different letters show that there was a remarkable difference between treatments

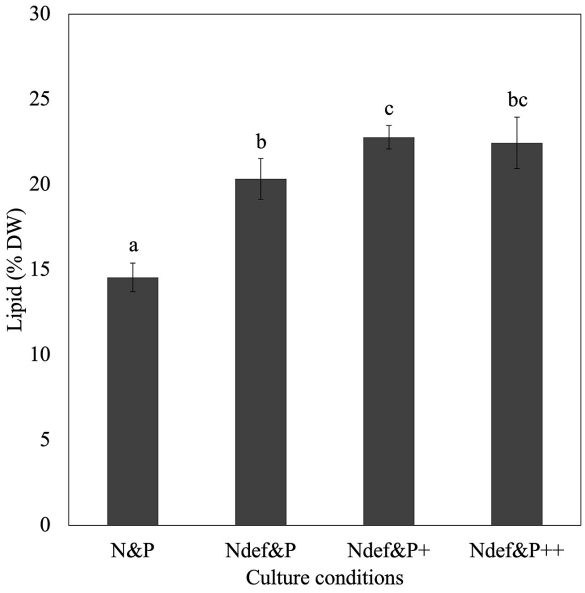

Figure 3. Lipid content of *Chlorella pyrenoidosa* under different phosphorus and nitrogen supply. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). At a 95% confidence level, different letters show that there was a remarkable difference between treatments

activity of *Chlorella* ADP-glucose pyrophosphorylase activity, which inhibits starch production and controls carbon assimilation to synthesize the lipid. However, further increases in phosphorus concentration up to 55.6 mg/L (N_{def}&P_{++}) did not increase the lipid content of *C. pyrenoidosa*. These findings suggest that the lipid production of *C. pyrenoidosa* was not stimulated by extremely high phosphorus concentrations.

CONCLUSIONS

This finding showed that nitrogen deficiency significantly decreased the growth, biomass, and photosynthetic pigment of *C. pyrenoidosa*. A high concentration of phosphorus supply under limited nitrogen did not significantly increase the growth, biomass, Chl *a*+*b*, and carotenoid content in *C. pyrenoidosa*. Lipid content was remarkably accumulated under slightly excessive phosphorus supply (27.8 mg/L) and nitrogen deficiency conditions (N_{def}&P_{+}). This study suggested that *C. pyrenoidosa* can be effectively stimulated to produce more lipids by combining slightly excessive phosphorus with a limited amount of nitrogen. Future investigation on optimizing culture conditions in mixotrophic systems is needed to improve the lipid content of *C. pyrenoidosa* under excessive phosphorus and nitrogen deficiency.

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REFERENCES

1. Bindra, S., & Kulshrestha, S. (2019). Converting waste to energy: Production and characterization of biodiesel from *Chlorella pyrenoidosa* grown in a medium designed from waste. *Renewable Energy*, *142*, 415–425. https://doi.org/10.1016/j.renene.2019.04.104
2. Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099
3. Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, *25*(3), 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001
4. Chokshi, K., Pancha, I., Ghosh, A., & Mishra, S. (2017). Nitrogen starvation-induced cellular crosstalk of ROS-scavenging antioxidants and phytohormone enhanced the biofuel potential of green microalga *Acutodesmus dimorphus*. *Biotechnology for Biofuels*, *10*(1), 1–12. https://doi.org/10.1186/s13068-017-0747-7
5. Chokshi, K., Pancha, I., Trivedi, K., George, B., Maurya, R., Ghosh, A., & Mishra, S. (2015). Biofuel potential of the newly isolated microalgae *Acutodesmus dimorphus* under temperature induced oxidative stress conditions. *Bioresource Technology*, *180*, 162–171. https://doi.org/10.1016/j.biortech.2014.12.102
6. Chu, F.F., Chu, P.N., Shen, X.F., Lam, P.K.S., & Zeng, R.J. (2014). Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. *Bioresource Technology*, *152*, 241–246. https://doi.org/10.1016/j.biortech.2013.11.013
7. Daneshvar, E., Antikainen, L., Koutra, E., Kornaros, M., & Bhatnagar, A. (2018). Investigation on the feasibility of *Chlorella vulgaris* cultivation in a mixture of pulp and aquaculture effluents: Treatment of wastewater and lipid extraction. *Bioresource Technology*, *255*, 104–110. https://doi.org/10.1016/j.biortech.2018.01.101
8. El-Kassas, H.Y. (2013). Growth and fatty acid profile of the marine microalga *Picochlorum* sp. grown under nutrient stress conditions. *Egyptian Journal of Aquatic Research*, *39*(4), 233–239. https://doi.org/10.1016/j.ejar.2013.12.007
9. EL-Sheekh, M.M., Bedaiwy, M.Y., Osman, M.E.,

& Ismail, M.M. (2012). Mixotrophic and heterotrophic growth of some microalgae using extract of fungal-treated wheat bran. *International Journal of Recycling of Organic Waste in Agriculture*, *1*(1), 1–9. https://doi.org/10.1186/2251-7715-1-12

10. Fakhri, M., Riyani, E., Ekawati, A.W., Arifin, N.B., Yuniarti, A., Widyawati, Y., Saputra, I.K., Samuel, P.D., Arif, M.Z., & Hariati, A.M. (2021). Biomass, pigment production, and nutrient uptake of *Chlorella* sp. under different photoperiods. *Biodiversitas*, *22*(12), 5344–5349. https://doi.org/10.13057/biodiv/d221215
11. Fu, L., Cui, X., Li, Y., Xu, L., Zhang, C., Xiong, R., Zhou, D., & Crittenden, J.C. (2017). Excessive phosphorus enhances *Chlorella regularis* lipid production under nitrogen starvation stress during glucose heterotrophic cultivation. *Chemical Engineering Journal*, *330*, 566–572. https://doi.org/10.1016/j.cej.2017.07.182
12. George, B., Pancha, I., Desai, C., Chokshi, K., Paliwal, C., Ghosh, T., & Mishra, S. (2014). Effects of different media composition, light intensity and photoperiod on morphology and physiology of freshwater microalgae *Ankistrodesmus falcatus* - A potential strain for bio-fuel production. *Bioresource Technology*, *171*, 367–374. https://doi.org/10.1016/j.biortech.2014.08.086
13. Kim, C.W., Sung, M.G., Nam, K., Moon, M., Kwon, J.H., & Yang, J.W. (2014). Effect of monochromatic illumination on lipid accumulation of *Nannochloropsis gaditana* under continuous cultivation. *Bioresource Technology*, *159*, 30–35. https://doi.org/10.1016/j.biortech.2014.02.024
14. Li, Y., Yuan, Z., Mu, J., Chen, D., & Feng, B. (2013). Proteomic analysis of lipid accumulation in *Chlorella protothecoides* cells by heterotrophic N deprivation coupling cultivation. *Energy and Fuels*, *27*(7), 4031–4040. https://doi.org/10.1021/ef4000177
15. Liu, J., Mao, X., Zhou, W., & Guarnieri, M.T. (2016). Simultaneous production of triacylglycerol and high-value carotenoids by the astaxanthin-producing oleaginous green microalga *Chlorella zofingiensis*. *Bioresource Technology*, *214*, 319–327. https://doi.org/10.1016/j.biortech.2016.04.112
16. Maltsev, Y., Kulikovskiy, M., & Maltseva, S. (2023). Nitrogen and phosphorus stress as a tool to induce lipid production in microalgae. *Microbial Cell Factories*, *22*(1), 1–22. https://doi.org/10.1186/s12934-023-02244-6
17. Mirzaie, M.A., Kalbasi, M., Mousavi, S.M., & Ghobadian, B. (2016). Investigation of mixotrophic, heterotrophic, and autotrophic growth of *Chlorella vulgaris* under agricultural waste medium. *Preparative Biochemistry and Biotechnology*, *46*(2), 150–156. https://doi.org/10.1080/10826068.2014.995812
18. Nigam, S., Rai, M.P., & Sharma, R. (2011). Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. *American Journal of Biochemistry and Biotechnology*, *7*(3), 126–131. https://doi.org/10.3844/ajbbsp.2011.126.131
19. Rai, V., Muthuraj, M., Gandhi, M.N., Das, D., & Srivastava, S. (2017). Real-time iTRAQ-based proteome profiling revealed the central metabolism involved in nitrogen starvation induced lipid accumulation in microalgae. *Scientific Reports*, *7*, 1–16. https://doi.org/10.1038/srep45732
20. Rathore, V., & Nema, S.K. (2024). Investigating the role of plasma-activated water on the growth of freshwater algae *Chlorella pyrenoidosa* and *Chlorella sorokiniana*. *Plasma Chemistry and Plasma Processing*, *44*(1), 367–391. https://doi.org/10.1007/s11090-023-10372-5
21. Ritchie, R.J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research*, *89*(1), 27–41. https://doi.org/10.1007/s11120-006-9065-9
22. Roopnarain, A., Gray, V.M., & Sym, S.D. (2014). Phosphorus limitation and starvation effects on cell growth and lipid accumulation in *Isochrysis galbana* U4 for biodiesel production. *Bioresource Technology*, *156*, 408–411. https://doi.org/10.1016/j.biortech.2014.01.092
23. Shen, X., Liu, J., Chauhan, A.S., Hu, H., Ma, L., Lam, P.K.S., & Zeng, R.J. (2016). Combining nitrogen starvation with sufficient phosphorus supply for enhanced biodiesel productivity of *Chlorella vulgaris* fed on acetate. *Algal Research*, *17*, 261–267. https://doi.org/10.1016/j.algal.2016.05.018
24. Stanier, R.Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*, *35*(2), 171–205. https://doi.org/10.1128/mmbr.35.2.171-205.1971
25. Zarrinmehr, M.J., Farhadian, O., Heyrati, F.P., Keramat, J., Koutra, E., Kornaros, M., & Daneshvar, E. (2020). Effect of nitrogen concentration on the growth rate and biochemical composition of the microalga, *Isochrysis galbana*. *Egyptian Journal of Aquatic Research*, *46*(2), 153–158. https://doi.org/10.1016/j.ejar.2019.11.003
26. Zhao, Y., Li, D., Ding, K., Che, R., Xu, J.W., Zhao, P., Li, T., Ma, H., & Yu, X. (2016). Production of biomass and lipids by the oleaginous microalgae *Monoraphidium* sp. QLY-1 through heterotrophic cultivation and photo-chemical modulator induction. *Bioresource Technology*, *211*, 669–676. https://doi.org/10.1016/j.biortech.2016.03.160
27. Zhu, S., Wang, Y., Xu, J., Shang, C., Wang, Z., Xu, J., & Yuan, Z. (2015). Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp. under nitrogen depletion. *Bioresource Technology*, *198*, 165–171. https://doi.org/10.1016/j.biortech.2015.08.142