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Phosphomolybdic acid boosts polyunsaturated fatty acid and neutral lipid production in *Phaeodactylum tricornutum*

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Phaeodactylum tricornutum is considered a potential lipid production platform due to its high growth rates and elevated natural neutral lipid and polyunsaturated fatty acid (PUFA) contents. Furthermore, microalgae are emerging as promising sources of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). In this study, phosphomolybdic acid (PMo_{12}), as a photocatalyst, can enhance the synthesis of neutral lipids and PUFAs by influencing the expression of lipid metabolism-related genes and photosynthesis in *P. tricornutum*. We also observed the contents of EPA and PUFA in engineered microalgae nearly doubled compared to the wild type, while neutral lipid content showed a significant increase of 69.7% in engineered microalgae. Notably, the growth rate of engineered microalgae remained comparable to that of the wild type. This work presents an effective approach to enhance the production of microalgal bioproducts, suggesting that photocatalysts such as PMo_{12} could serve as viable alternatives to genetic engineering technology, facilitating the commercialization of microalgal biodiesel.

KEYWORDS

phosphomolybdic acid, microalgae, lipid, polyunsaturated fatty acid, EPA, DHA

1 Introduction

The need for secure, low-cost, and renewable energy sources has become increasingly urgent due to the depletion of fossil fuels and global geopolitical tensions (Lv et al., 2019). Biofuels have emerged as a critical renewable energy source, with each generation of biofuels building on the limitations of the previous one. First-generation biofuels rely on

feedstocks like corn and soybeans, while second-generation biofuels utilize non-food sources such as switchgrass and food waste. The third and fourth generations have shifted to microalgae as a feedstock, with the latter focusing on genetically modified strains (Wang et al., 2024).

Microalgae are gaining attention for their rapid growth, high photosynthetic efficiency, minimal land use, and ability to produce a wide range of biofuel products (Maliha and Abu-Hijleh, 2022). Moreover, they offer significant environmental benefits, including a carbon sequestration capacity that is 10–50 times greater than that of terrestrial plants (Wei et al., 2020). High value-added bioproducts produced by microalgae can provide antioxidants, carotenoids, proteins, and essential vitamins, which are of great benefit to human health (Del Mondo et al., 2020; Singh et al., 2020). Additionally, microalgae have potential applications in vaccine development against various infectious diseases (Ramos-Vega et al., 2021). Historically, algae have been an essential nutrient-rich food source, often surpassing traditional crops in nutritional value. The expanding toolkit for improving algae varieties offers a viable solution to the global food shortage challenges of the twenty-first century (Torres-Tiji et al., 2020).

Microalgae research gained momentum with the US Department of Energy's Aquatic Species Program, which screened over 3,000 microalgae species for biofuel potential between 1978 and 1996 (Sheehan et al., 1998). In recent years, species such as *Chlorella vulgaris*, *Nannochloropsis oceanica*, and *Dunaliella salina* have been identified as suitable candidates for biodiesel production (Fu et al., 2019; Gui et al., 2021). Additionally, various abiotic stresses, such as heat and light intensity, have been shown to stimulate the production of bioactive compounds in algae (Liu et al., 2020; Fu et al., 2021; Huang et al., 2021). Among these compounds, PUFAs like EPA and DHA have attracted attention due to their health benefits, including their role in mitigating neurological disorders, inflammatory diseases, and even certain cancers (Ghasemi Fard et al., 2018; Kapoor et al., 2021). As the demand for sustainable sources of PUFAs grows, marine microalgae are emerging as a viable alternative to overexploited deep-sea fish (Colombo et al., 2019; Kumari et al., 2023).

Research has demonstrated that optimizing environmental factors such as temperature, nutrients, and light can enhance lipid and PUFA production in microalgae. For example, the DHA content in *Tisochrysis lutea* increased significantly under low-temperature conditions, and similar results were observed for EPA in *Nannochloropsis oculata* (Gao et al., 2022; Sousa et al., 2022). However, these methods often reduce biomass production and are not practical for large-scale applications.

In the past decade, genetic engineering has emerged as a powerful tool for enhancing lipid accumulation in microalgae, particularly in species with sequenced genomes (Bhattacharjya et al., 2021). Previous research has identified Δ4-FAD, Δ5-FAD, and GPAT as critical enzymes for DHA, EPA, and TAG synthesis in microalgae (Kumari et al., 2023). The overexpression of the native Δ5-FAD gene in *P. tricornutum* has been shown to increase EPA production by up to 58% (Peng et al., 2014). Genetic modifications, such as the deletion of specific genes or the overexpression of key enzymes, have led to significant increases in lipid and PUFA yields (Han et al., 2020; Wang et al., 2021; Han et al., 2022). Advanced genome editing techniques like CRISPR/Cas9 and TALEN have further refined these capabilities (Fayyaz et al., 2020). However, the outdoor cultivation of genetically modified microalgae remains limited due to high costs and environmental concerns (M. U et al., 2019). Thus, there is a pressing need for methods that could enhance lipid production without compromising growth or economic viability.

Polyoxometalates (POMs), particularly phosphomolybdic acid (PMo_{12}), have emerged as promising photocatalysts due to their low cost, high efficiency, and recyclability (Ilbeygi and Jaafar, 2024). POMs are known for their redox properties and strong Brønsted/Lewis acidity, making them useful in various applications, including microbial fuel cells, rechargeable batteries, and the conversion of biomass into biofuels (Bijelic et al., 2018; Huang et al., 2020; Zhong et al., 2021). Notably, PMo_{12} has been shown to catalyze the conversion of CO_2 into high-value fuels with low energy requirements (Yang et al., 2019), and it has also been used in the production of biodiesel from waste cooking oil (Helmi et al., 2022).

In this study, we explored the potential of PMo_{12} to enhance lipid and PUFA accumulation in *P. tricornutum*. By incorporating PMo_{12} into the growth medium, we aimed to identify a strategy that boosts lipid production without adversely affecting growth while remaining economically feasible. The results of this research could pave the way for microalgae to serve as an alternative source of deep-sea fish oil, with significant implications for industrial applications.

2 Materials and methods

2.2 *P. tricornutum* cultures

P. tricornutum Bohlin (CCMP2561) was prepared in this research. Microalga strain was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, CAS, China (No. FACHB-863). Microalgae were cultured in f/2 si^- medium, which contains $Na_2MoO_4 \cdot 2H_2O$ and filtered using 0.22-μm filter membranes (Millipore, Billerica, MA, USA). PMo_{12} (1 mol/L), which was purchased from TCI America, with purity of 99%, was added in the medium as a photocatalyst, and the culture was maintained at 21 ± 0.5°C under a light/dark cycle of 15 h and 9 h, respectively. The light intensity is 4,000 lx.

2.2 The growth curve of *P. tricornutum*

The growth of the algae was monitored daily for 10 consecutive days using a hemocytometer and microscope. The number of algal cells was counted under an inverted microscope with a blood cell counting plate at the same time each day during the growth cycle. The growth status of the algal cells was calculated using the formula: cell density = (total number of 80 small square cells/80) × 4 × 10^6 × dilution times, and the growth curve was generated.

2.3 Observation of the morphological changes in the *P. tricornutum*

The morphology of engineered microalgae cells was observed under laser scanning confocal microscope LSM 510 META (Zeiss, Oberkochen, Germany). After culture to the plateau stage (7 days), 1 mL algal solution was taken into 1.5 mL EP tube, and 10 μL Nile red solution (0.1mg/mL, soluble in acetone) was added to the algal solution, then incubated for 30 min under 37℃ in the dark. The fluorescence detection was 488 nm for excitation and 505–550 nm for emission.

2.4 Neutral lipid content of *P. tricornutum*

Nile red as a fluorescent marker has been widely used in the determination of neutral lipid content in cells. Consequently, Nile red was employed as a fluorescent probe to assess the neutral lipid content in *P. tricornutum* cells (Yang et al., 2013). Algal cells were initially collected using a refrigerated centrifuge, followed by treatment with 20% DMSO for 20 min at room temperature. A total of 30 μL of Nile red, dissolved in 0.1 mg/mL acetone, was added to a 3-mL aliquot of pretreated cell culture in triplicates. The mixture was inverted rapidly and shielded with tin foil for 20 min at room temperature. Subsequently, the *P. tricornutum* cell cultures were transferred to a 96-well plate for fluorescence intensity determination using a Hitachi F4600 microplate reader (Hitachi, Japan). Fluorescence intensity was measured at 580 nm under 530 nm light excitation, with the intensity of the unstained algal liquid at this wavelength subtracted from the readings. Concurrently, the density of algal cells was assessed using a hemocytometer and microscope, enabling the calculation of neutral lipid content per algal cell.

2.5 Fatty acid composition of *P. tricornutum*

Fatty acid composition of microalgae was detected by the previous research (Yang et al., 2013). The fatty acid extraction steps are as follows: first, diatom cells were harvested by centrifugation at 3,000×*g* for 10 min at 4°C. Then, 5 mL KOH–CH_3OH(2 mol/L) was added to the cell culture. After ultrasonic breaking in an ice bath, the cell culture was filled with nitrogen for 1 min. The crushed algal cell culture was shaken with a turbine shaker and then reacted in a water bath at 75°C for 10 min. The layering is left standing at room temperature, and the supernatant is transferred to a new centrifuge tube. The above steps were repeated twice, and all the supernatant was collected in a centrifuge tube. Then, 4 mL of n-hexane was added to the centrifuge tube, and the upper extract was transferred to a new centrifuge tube after standing for stratification. Total lipids of microalgae were detected by gas chromatography–mass spectrometry according to Yang et al. (GC-MS) (Finnigan TRACE DSQ; Thermo Fisher, Waltham, MA, USA) at the Guangdong University of Technology. To calculate the content of EPA and DHA at dry cell weight (DCW), 150 μL of N-nonadecyl ester (1 mg/mL) was added as an internal standard to the samples for analysis of fatty acid composition by gas chromatography–mass spectrometry (GC–MS). The concentration of fatty acids (C_{FA}, mg/g) was determined by comparing the peak area of fatty acid in the sample with the peak area of internal standard, according to the following equation: $C_{FA} = A_S/A_{IS} \times C_{IS}/W_S$, where A indicates the peak area; C is the concentration; W is the weight; IS is the internal standard; S is the sample (Abdulkadir and Tsuchiya, 2008).

2.6 Molecular analysis of the *P. tricornutum* by quantitative PCR

To investigate the influence of PMo_{12} on regulatory genes associated with lipid metabolism in *Phaeodactylum tricornutum*, several molecular biological experiments were conducted. For the detection of mRNA expression levels in *P. tricornutum*, quantitative real-time PCR (qPCR) was performed. The target gene are Δ5 fatty acid desaturase gene (PTD5b), Δ4 fatty acid desaturase gene (PTD4), and glycerol-3-phosphate acyltransferase (PTGPAT). Primers used for qPCR were as follows: Δ5 fatty acid desaturase gene PTD5b (forward primer, CATCACGGACCCAATCAATAC; reverse primer, CGACGGACAATCTGGAAGAC), PTD4 (forward primer, GCGAC GATTGGGCTTGACCT; reverse primer, TCCG TGGAT GATG CTTTGATTTCT), PTGPAT (forward primer, ACGATTCGGACGAAGATCAG; reverse primer, CCA TGCAACAATCGTAGTGG), and β-actin (forward primer, AGGCAAAGCGTGGTGTT CTTA; reverse primer, TCTGGGGAGCCTCAGTCAATA). In *P. tricornutum* genome, putative β-actin (ACT1, Phatrdraft_51157) was used as a housekeeping marker. The relative expression level of PTD5b, PTD4, and PTGPAT gene was calculated by normalization to β-actin expression.

2.7 Product analysis of *P. tricornutum* biochemical reaction

Cultured solution of *P. tricornutum* after a culture cycle was dried at 95℃, then resolved in D_2O for ^{31}P NMR; the spectral data were collected on a Bruker Avance/DMX 400MHz NMR spectrometer with an 8-s pulse delay, and the internal standard is triphenyl phosphate (TPP).

2.8 Statistical methods

SPSS software was used for statistical analysis. In the study, we used t-test to analyze whether there were significant differences between the experimental algae strains and wild strains. $p<0.05$ indicates a significant difference, $p < 0.01$ indicates an extremely significant difference.

3 Result and discussion

3.1 The growth analysis of transgenic and PMo_{12}-treated *P. tricornutum*

As shown in the growth curves, the overexpression of Δ5 fatty acid desaturase gene in *P. tricornutum* did not adversely affect the growth of the algae (Figure 1A). Moreover, the growth rate of the microalgae with PMo_{12} (1mol/L) added to the medium was slightly slower compared to both the wild-type and transgenic strains. Notably, during the late exponential growth phase, the algae with PMo_{12} supplementation exhibited a significantly lower growth rate than the wild type, and the inclusion of PMo_{12} caused *P. tricornutum* to enter the decline phase more rapidly. The slower growth rate observed in *P. tricornutum* supplemented with PMo_{12} may be attributed to the photocatalytic properties of PMo_{12}. As a photocatalyst, PMo_{12} can oxidize and degrade photosynthetic products under light conditions. These products, including natural antioxidants, carotenoids, proteins, polysaccharides, PUFAs, triacylglycerols (TAGs), sterols, and vitamins, are crucial for the growth and survival of *P. tricornutum*. The degradation of these essential compounds likely contributes to the observed reduction in growth rate.

3.2 The lipid content of transgenic and PMo_{12}-treated *P. tricornutum*

Observations from confocal laser scanning microscopy indicated that the morphology and size of the engineered microalgae cells were comparable to those of the wild type. However, the volume of organelles (oil bodies) storing triacylglycerol (TAG) in the engineered microalgae strains was significantly larger, and their number was also slightly increased (Figure 2). Furthermore, when compared to transgenic algae, the volume and number of organelles (oil bodies) storing TAG in engineered algae strains treated with PMo_{12} (1 mol/L) showed a slight increase. The neutral lipid content in microalgae supplemented with PMo_{12} (1mol/L) demonstrated a notable increase of 69.7% and 25.7% compared to the wild-type and transgenic microalgae, respectively (Figure 1B). These findings suggest that PMo_{12} more effectively promotes neutral lipid accumulation in *P. tricornutum* than the overexpression of the PtD5b gene. Monoacylglycerols (MAGLs), diacylglycerols (DAGs), and triacylglycerols (TAGs) are the most abundant neutral lipids found in the microalgae (Muñoz et al., 2021). A substantial accumulation of TAG has been observed in the microalgae *Nannochloropsis gaditana* under nitrogen starvation conditions (Janssen et al., 2018). The first step of TAG synthesis is catalyzed by glycerol 3-phosphate acyltransferase (GPAT), which is considered a key regulator in this process (Yu et al., 2018). The overexpression of endogenous GPAT in the *P. tricornutum* resulted in a significant increase in neutral lipid accumulation compared to the wild type, without any growth inhibition (Niu et al., 2016; Balamurugan et al., 2017; Wang et al., 2020). The subsequent enzyme in TAG synthesis, lysophosphatidic acid acyltransferase (LPAT), has also been overexpressed in *C. reinhardtii*, leading to an increase in lipid content (Yamaoka et al., 2016). Diacylglycerol acyltransferase (DGAT) is the final enzyme involved in triacylglycerol (TAG) synthesis, and the overexpression of genes encoding DGAT has emerged as a promising strategy for enhancing TAG content in microalgae, including *C. reinhardtii*,

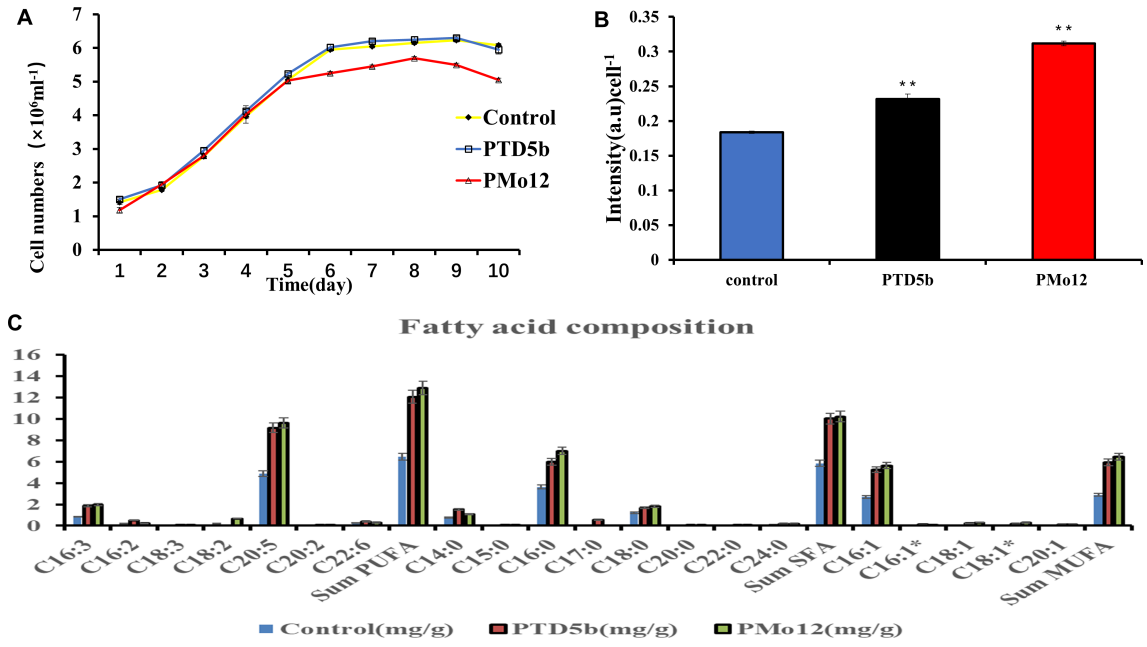

FIGURE 1
Growth and lipid analysis in diatom cells. **(A)** Growth curves of *P. tricornutum*. **(B)** Neutral lipid content of *P. tricornutum*. ** indicate extremely significant difference (p<0.01). **(C)** Fatty acid composition of *P. tricornutum*.

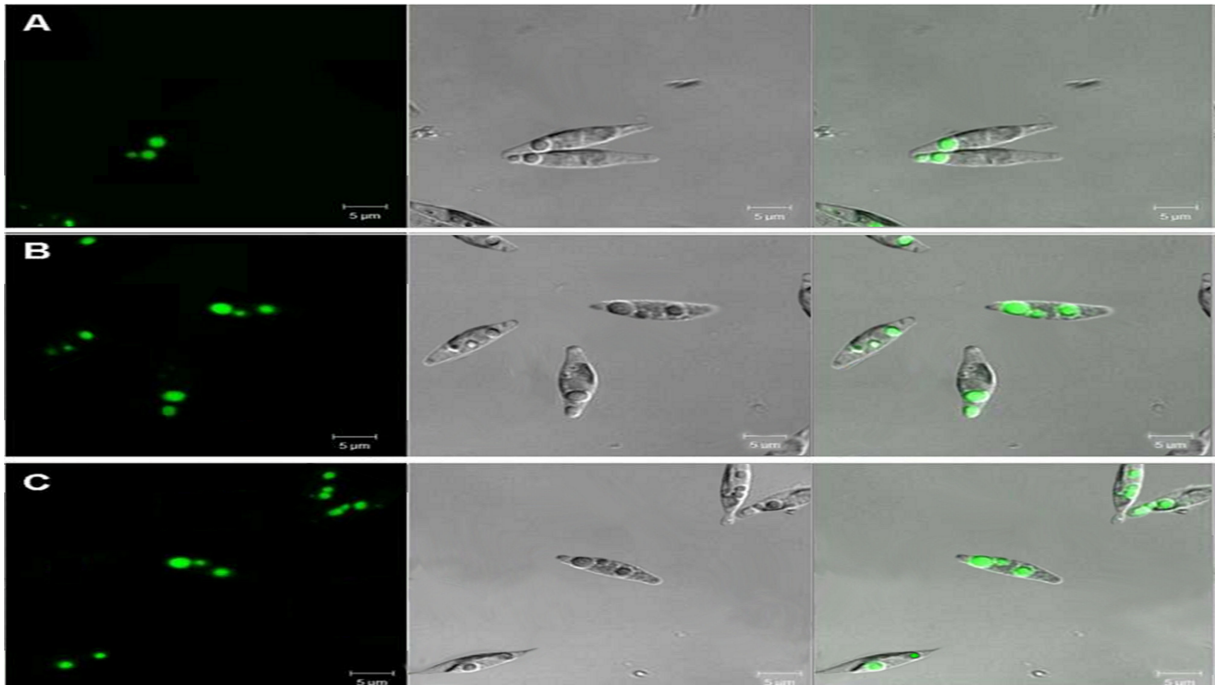

FIGURE 2
The confocal observation of Nile red-stained *P. tricornutum* cells. Part **(A)** indicates wild-type cells, part **(B)** indicates transgenic microalgae, part **(C)** indicates PMo_{12} (1 mol/L) added in the medium of microalgae. Left, red fluorescence of oil bodies; middle, differential interference contrast (DIC); right, overlay image. Bars = 5 μm.

Nannochloropsis, and *Phaeodactylum* (La Russa et al., 2012; Ahmad et al., 2014; Zienkiewicz et al., 2017). In this context, PMo_{12} may be considered a putative positive regulator of genes related to GPAT or DGAT.

3.3 The fatty acid composition of transgenic and PMo_{12}-treated *P. tricornutum*

The results indicate that the contents of polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA), and saturated fatty acid (SFA) in *P. tricornutum* have significantly increased due to PMo_{12} (Figure 1C). Specifically, the contents of EPA and overall PUFA nearly doubled in *P. tricornutum* with PMo_{12} treatment, while both MUFA and SFA also exhibited significant increases compared to the wild type. Similar results were observed in transgenic microalgae. However, it is noteworthy that the content of DHA in *P. tricornutum* decreased by 27.2% with PMo_{12} compared to the transgenic microalgae. The fatty acid desaturases (FADs) and elongases (ELOs) are critical enzymes in PUFA synthesis, and regulating the expression of these genes through genetic engineering is a common approach to enhance PUFA accumulation in *P. tricornutum*. The results suggest that PMo_{12} may stimulate the expression of genes related to fatty acid desaturases and elongases, thereby promoting PUFA and EPA synthesis in *P. tricornutum*. In contrast, the key enzymes involved in DHA synthesis in *P. tricornutum* exhibited lower activity than the overexpression of PtD5b. Previous research has identified Δ4-FAD as a critical enzyme for DHA synthesis in microalgae. For instance, overexpression of Δ4-FAD in *C. reinhardtii* resulted in a 66.7% increase in total monogalactosyldiacylglycerol (MGDG) content (Zäuner et al., 2012). Future studies should focus on the key enzymes involved in DHA synthesis in microalgae to further understand and optimize DHA production.

3.4 The quantitative PCR of transgenic and PMo_{12}-treated *P. tricornutum*

The transcript abundance of PTD5b in *P. tricornutum* was quantified using qPCR (Figure 3A), revealing a similar increase of 3.1-fold in both transgenic and PMo_{12} compared to the wild type. Δ5-FAD is the key enzyme responsible for EPA synthesis in *P. tricornutum*. The results indicate that PMo_{12} exhibits effects comparable to those of genes associated with the overexpression of Δ5 fatty acid desaturase in *P. tricornutum*, leading to a greater accumulation of EPA relative to the wild type. In contrast, Δ4 fatty acid desaturase (Δ4-FAD) serves as the key regulator of DHA synthesis in microalgae. The mRNA expression of the PTD4 gene was assessed through qPCR (Figure 3B), showing a relative expression level that increased by 2.3-fold in transgenic microalgae compared to the wild type. However, the mRNA expression of the PTD4 gene in PMo_{12} was similar to that of the wild type, suggesting that PMo_{12} negatively affects the expression of Δ4 fatty acid desaturase-related genes in *P. tricornutum*. Additionally, the transcript abundance of PTGPAT was also measured using qPCR (Figure 3C), revealing significant increases

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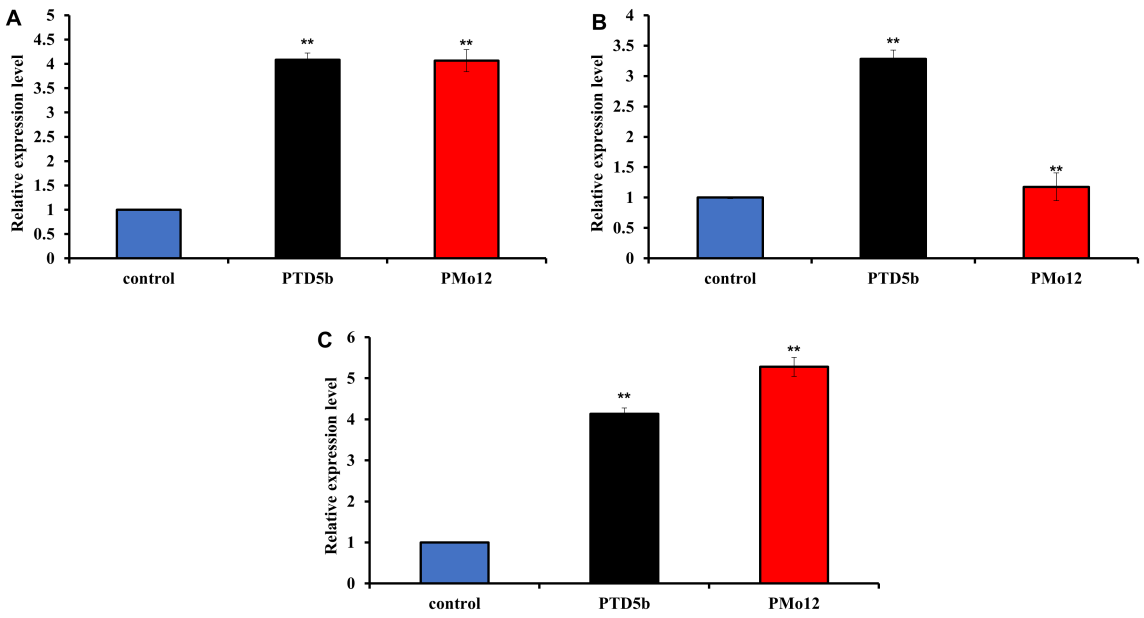

FIGURE 3
Analysis of lipid synthesis-related gene expression in diatom cells. **(A)** Relative mRNA level of PTD5b in *P. tricornutum*. **(B)** Relative mRNA level of PTD4 in *P. tricornutum*. **(C)** Relative mRNA level of PTGPAT in *P. tricornutum*. ** indicate extremely significant difference ($p<0.01$).

of 3.1- and 4.3-fold in transgenic microalgae and PMo_{12}, respectively, compared to the wild type. Thus, PMo_{12} promotes the expression of the GPAT gene in *P. tricornutum* more effectively than in transgenic algae. Overall, these results demonstrate that PMo_{12} enhances the accumulation of neutral lipids in *P. tricornutum* more effectively than genetic engineering approaches involving transgenic microalgae.

3.5 Analysis of biochemical reaction products in *P. tricornutum* by ^{31}P NMR

The ^{31}P NMR results indicate peak species at −3.75 ppm and −5.36 ppm (Figure 4). According to the NMR spectrum, PMo_{12} and its reduced form are present independently in the microalgal culture medium after one culture cycle (Ishikawa and Yamase, 2000).

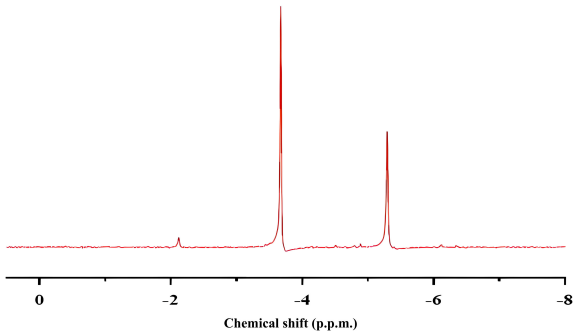

FIGURE 4
^{31}P NMR spectrum of cultured solution of *P. tricornutum*.

Previous studies have shown that PMo_{12} acts as an oxidizing agent capable of catalyzing biomass degradation and functioning as an electron donor. The redox reaction cannot occur at room temperature, and PMo_{12} is only effective when exposed to light or elevated temperatures. In this study, carbohydrates produced through algal photosynthesis, such as starch, are degraded by PMo_{12} under illuminated conditions, leading to the generation of CO_2. Subsequently, the reduced form of phosphomolybdate is reoxidized to its original state by oxygen produced during photosynthesis. This principle can be illustrated by the following reactions (Liu et al., 2016):

$$\text{Biomass} + H_2O + POM_{(OX)} \xrightarrow{\Delta \text{ or } h\nu} \text{Degradation products} + CO_2 + H - POM_{(Red)} \quad (1)$$

$$H - POM_{(Red)} \rightarrow POM_{(OX)} + H^+ + e^- \quad (2)$$

$$H^+ + e^- + O_2 \rightarrow H_2O \quad (3)$$

Consequently, PMo_{12} has been demonstrated to enhance the accumulation of neutral lipids and PUFAs in *P. tricornutum* by modulating the algal photosynthetic processes.

4 Conclusion

In conclusion, the application of PMo_{12} as a photocatalyst in *P. tricornutum* has proven to be a highly effective strategy for enhancing lipid and polyunsaturated fatty acid (PUFA) production without compromising the growth of the microalgae. The significant increase in EPA, PUFA, and neutral lipid contents observed in the engineered

strains underscores the potential of PMo_{12} to modulate lipid metabolism and photosynthesis-related pathways. This study not only highlights the capability of photocatalysts like PMo_{12} to improve the yields of valuable microalgal bioproducts but also suggests a promising alternative to traditional genetic engineering approaches. By providing a non-genetically modified route to enhance lipid production, this method could facilitate the commercial viability of microalgal biodiesel, thereby contributing to the development of sustainable and renewable energy sources. In future studies, we will explore whether PMo_{12} provides nutrients for the growth of *P. tricornutum* and considers whether PMo_{12} interfere with Moco synthesis in microalgae.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

KP: Formal analysis, Resources, Software, Writing – original draft, Writing – review & editing. BX: Software, Supervision, Writing – review & editing. GC: Formal analysis, Funding acquisition, Project administration, Writing – review & editing. YZ: Methodology, Resources, Writing – review & editing. LY: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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