

Methionine Enhances EPA Biosynthesis via Coordinated Metabolic Flux Redistribution and Membrane Lipid Remodeling in *Schizochytrium* sp.

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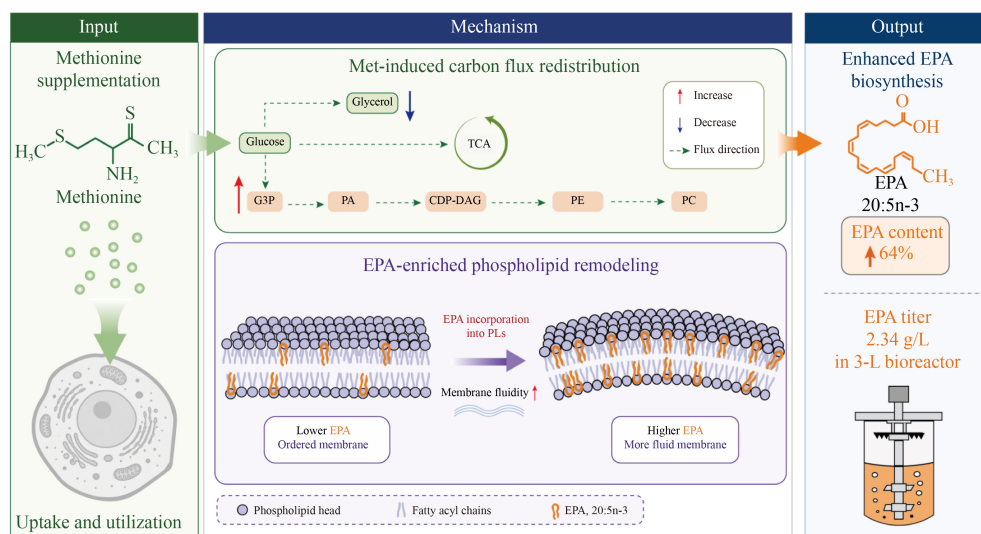
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GRAPHICAL ABSTRACT



HIGHLIGHTS

- Methionine supplementation markedly enhanced EPA yield in the industrially relevant, food-grade microalga *Schizochytrium* without genetic modification.
- An optimal Met supplementation strategy was established through systematic evaluation of fermentation performance.
- Integrated multi-omics analysis demonstrated that Met activated phospholipid biosynthesis pathways and redirected metabolic flux.
- Membrane lipid remodeling increased membrane fluidity, thereby facilitating efficient channeling of EPA into phospholipids.
- Fed-batch fermentation verified the feasibility of scalable and industrially applicable EPA production.

ABSTRACT *Schizochytrium* has emerged as a promising platform for eicosapentaenoic acid (EPA) production because of its high biomass and lipid accumulation capacities. However, the regulatory mechanisms underlying EPA accumulation remain unclear. This study investigated the effects of supplementing methionine (Met), a key precursor of S-adenosylmethionine (SAM), on lipid metabolism and EPA

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biosynthesis. An optimal Met concentration of 5 g/L significantly increased the proportion of EPA in shake flasks from 1.7% to 2.8%. In a 3-L bioreactor, the EPA production reached 2.34 g/L. Metabolomic analysis suggested that Met supplementation was associated with altered central carbon metabolism and a shift towards glycerol-3-phosphate (G3P) accumulation, which is a key precursor of lipid synthesis. Lipid analysis revealed that Met promoted the redistribution of fatty acids from triacylglycerols to phospholipids (PLs), with EPA being preferentially enriched in PLs. This shift was accompanied by enhanced membrane fluidity and upregulation of genes essential for PLs biosynthesis. Overall, these results indicate that Met enhances EPA accumulation through coordinated carbon flux redistribution and phospholipid remodeling, highlighting a potential linkage between G3P metabolism, PLs remodeling, and EPA biosynthesis. This study provides new insights into sulfur-mediated lipid regulation and offers a practical strategy for enhancing EPA production in *Schizochytrium*.

KEYWORDS *Schizochytrium*; Eicosapentaenoic acid; Methionine; Phospholipids; Metabolomics

1 Introduction

Polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), are essential for maintaining cellular structural stability and functional integrity by modulating membrane fluidity, membrane protein conformation, and signal transduction processes [1,2]. Beyond their biological roles, PUFAs confer substantial health benefits, including the mitigation of cardiovascular diseases, inflammation, and neurodegenerative disorders such as Alzheimer’s disease [3]. Furthermore, omega-3 long-chain polyunsaturated fatty acids, particularly EPA and DHA are vital for neonatal neural and visual development [4]. Despite their successful commercialization in food and pharmaceutical sectors [5], the supply of EPA and DHA from their primary source, deep-sea fish oil, is increasingly constrained by concerns over sustainability, heavy metal contamination, and the ecological impact of overfishing [6,7].

Microbial synthesis has emerged as a promising alternative sustainable EPA production. Representative strategies for improving EPA production are summarized in Table 1. Among naturally occurring oleaginous microorganisms, the marine protist *Schizochytrium* has been granted GRAS status as a food-grade DHA producer and is also recognized for its potential in sustainable lipid production [8,9]. This organism offers distinct industrial advantages, including exceptional biomass concentrations (up to 150–200 g/L), high total

lipid accumulation (exceeding 50% of dry cell weight), and a mature large-scale fermentation infrastructure [10]. Although DHA derived from *Schizochytrium* has been successfully commercialized, breakthroughs in enhancing EPA titers in this species remain elusive [11]. This technical bottleneck underscores the urgent need to elucidate the metabolic mechanisms and regulatory networks governing EPA and DHA biosynthesis in *Schizochytrium*.

Another important native EPA producer is *Nannochloropsis*, which naturally accumulates high levels of EPA. However, its industrial application is limited by relatively slow growth under photoautotrophic conditions, high sensitivity to light availability, and high production costs associated with large-scale cultivation and downstream processing [12].

In addition to native EPA-producing microorganisms, heterologous production platforms have also achieved remarkable progress. *Yarrowia lipolytica* has become a robust chassis for the heterologous production of high-value fatty acids. For instance, a recombinant *Y. lipolytica* strain was engineered in which EPA accounted for 56% of total lipids using glucose as the sole carbon source [13]. Subsequently, Huang et al. further improved EPA yields to 27.5 g/L in a 50 L bioreactor by combining a CDS variant calculator and machine learning-driven gene copy number optimization [14]. Nevertheless, the use of yeast-derived synthetic compounds in foodstuffs, particularly infant formula, still lacks official authorization in many countries. Therefore, despite the impressive

Table 1 Representative strategies for enhancing EPA production in microorganisms.

Strategy	Strain	EPA titer	Scale	Ref.
PKS pathway engineering	<i>Aurantiochytrium</i> sp. SD116	2.7 g/L	5 L	[11]
Heterologous metabolic engineering	<i>Yarrowia lipolytica</i>	56% of total fatty acids	Shake flask	[13]
Machine learning-assisted engineering	<i>Yarrowia lipolytica</i>	27.5 g/L	50 L	[14]
PKS AT domain engineering	<i>Schizochytrium</i> sp.	3.79 g/L	5 L	[15]
FAS pathway engineering	<i>Schizochytrium limacinum</i> SR21	1.95 g/L	5 L	[9]
ARTP/NTG mutagenesis	<i>Schizochytrium</i> sp. HX-308	8.26 g/L	5 L	[16]
Methionine supplementation	<i>Schizochytrium</i> sp. HG-20	2.34 g/L	3 L	This study

performance of heterologous systems, developing native food-grade EPA-producing microorganisms, especially *Schizochytrium*, remains an attractive strategy for sustainable EPA production.

Moreover, unlike microorganisms relying on a single biosynthetic route, *Schizochytrium* possesses both the fatty acid synthase (FAS) and polyketide synthase (PKS) pathways for PUFA biosynthesis, conferring remarkable metabolic flexibility for targeted engineering [17–19]. In our previous studies, metabolic network analyses based on genome-scale metabolic modeling and flux simulation suggested that EPA biosynthesis in *Schizochytrium* is closely associated with intracellular carbon flux redistribution, NADPH supply, and fatty acid elongation pathways. These findings imply that metabolic regulators capable of coordinating central carbon metabolism and lipid remodeling may provide an effective strategy for improving EPA accumulation.

Among the potential regulatory networks involved in these processes, sulfur metabolism has attracted increasing attention due to its multifaceted roles in cellular metabolism and lipid biosynthesis. Sulfur is an essential constituent of key cofactors and metabolites, including Fe–S clusters, thiamine, S-adenosylmethionine (SAM), and the amino acids cysteine (Cys) and methionine (Met) [20,21]. Sulfur-containing compounds participate in multiple metabolic processes, including substrate activation, enzyme function and cellular redox regulation, all of which are closely associated with fatty acid biosynthesis [22]. Glutathione (GSH), a major sulfur-containing antioxidant, plays a central role in maintaining intracellular redox homeostasis, which is essential for efficient lipid synthesis [23–25].

Beyond its direct involvement in coenzyme biological reactions and antioxidant systems, sulfur is also essential for cellular methylation processes. S-adenosylmethionine (SAM), derived from Met, serves as the primary methyl donor in the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) [26]. PC is a central intermediate in lipid metabolism and plays a key role in determining fatty acid distribution among lipid classes. Since phospholipid metabolism is closely associated with EPA distribution, sulfur metabolism has been proposed to influence EPA dynamics through SAM-dependent methylation reactions [27,28]. However, whether this mechanism operates in *Schizochytrium* remains largely unknown.

In this study, exogenous additive Met was added to evaluate its effect on EPA accumulation in *Schizochytrium*. Metabolic mechanism was explored with discussions on phospholipid synthesis along with cell membrane fluidity. This study demonstrates that metabolic regulation can selectively redirect carbon flux

toward EPA synthesis, enabling commercially relevant EPA production while leveraging the existing industrial infrastructure of *Schizochytrium*, thereby avoiding the need to establish new production systems.

2 Methods

2.1 Strain, culture and additive treatment

The *Schizochytrium* sp. HG-20 strain used in this study was obtained by domestication of *Schizochytrium* ATCC 1381 in our laboratory under high sugar conditions. After two generations of cultivation, the seed culture (5%, v/v) was then transferred to fermentation broth and incubated at 28 °C with agitation at 200 r/min for 144 h or longer.

The cysteine (Cys) and methionine (Met) used in this study were of analytical purity. They were dissolved in sterile water. All solutions were sterilized using 0.22 µm polyvinylidene fluoride (PVDF) or polyethersulfone (PES) syringe filters (Millipore, MA, USA).

2.2 Lipid extraction and fatty acids composition analysis

A total of 3 mL of fermentation broth was mixed with 4 mL of concentrated hydrochloric acid and incubated in a water bath at 65 °C for 1 h. Total lipids from the mixture were extracted three times with 4 mL of n-hexane:ethanol (1:2, v/v). The collected extracts were then combined and purified using the nitrogen blowing method at room temperature.

Fatty acid methyl esters (FAMES) were prepared following the methods of Chang et al. [29] with modifications. Heptadecanoic acid methyl (16 g/L, Sigma, USA) was added as an internal standard to the tubes and mixed by vortexing for 1 min. The upper phase containing FAMES was then analyzed using a gas chromatograph (Agilent GC 7890, USA) equipped with a 100 m × 0.25 mm capillary column (SPTM-2560, USA). Peaks were identified using authentic standards of FAMES (Sigma, USA).

2.3 Isolation and identification of triglycerides (TAGs) and phospholipids (PLs)

The extracted total lipid fraction was partitioned into TAGs and PLs. First, an amino solid-phase extraction (SPE) column was activated with 5 mL of a prepared organic solvent mixture (chloroform and acetone in a 2:1 v/v). Each 50 mg sample of oil was dissolved with 1 mL of the organic solvent mixture, and the resulting solution was added to the activated SPE column to adsorb the oil.

TAGs were eluted with 10 mL the organic solvent mixture into pre-weighed, oven-dried 50 mL polypropylene tubes. PLs were subsequently recovered using 5 mL methanol into equivalently prepared collection tubes. The collected eluents were dried by nitrogen blowing, and the weight difference of the centrifuge tube before and after drying represented the weights of the two components.

2.4 Extraction of biofilm and determination of protein concentration

Take 5 mL of bacterial solution and transfer it into a 50 mL centrifuge tube, centrifuge at 4 °C at 5000×g for 10 min to collect the bacteria. Wash the bacteria with PBS buffer solution for 2–3 times, then resuspend them in PBS buffer containing 1 mM DTT and 0.05% Tween 20. Disrupt the bacterial suspension using an ultrasonic cell crusher in an ice bath for 15 min. Following this, centrifuge the mixture at 10,000×g for 20 min at 4 °C, discarding the precipitate. The supernatant containing the biofilm and protein suspension should be stored at –80°C for future use. The protein concentration in the supernatant was determined using a detergent-compatible Bradford assay Kit.

2.5 Analysis of cell membrane fluidity

Membrane fluidity quantification was performed via 8-aniline-1-naphthalenesulfonic acid (ANS) fluorescence staining method. ANS stock solution (5 mM in Tris-HCl buffer, pH 7.4) was diluted to working concentration (0.1 mM) immediately prior to experimentation. The ANS solution was then mixed with the extracted biofilm and protein suspension to achieve a final ANS concentration of 0.25 mM, with the final protein concentration in the suspension set at 100 mg/L. The samples were incubated in the dark at room temperature for 20 min. Following this incubation, the samples were transferred to opaque enzyme plate, and the microplate reader was set to an excitation wavelength of 385 nm and emission wavelength of 480 nm to measure the absorbance values.

2.6 Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit. cDNA was synthesized using the EasyScript First Strand cDNA Synthesis SuperMix (TransGen Biotech, China) for reverse transcription. qPCR was performed using the Novozan ChamQ Universal SYBR qPCR Master Mix kit. A reaction system was prepared in qPCR tubes using ACT as the internal reference gene, and three parallel runs were conducted for all samples.

2.7 Preparation of metabolomic samples

Cell aliquots (5 mL) were aseptically harvested at 144 h of batch fermentation. The collected cells were quenched, and cellular contents were extracted according to our previous study with moderate modifications. The sample was rapidly mixed with 60% cold methanol (–40 °C, v/v) to instantly capture cellular metabolism, followed by centrifugation (5000×g, 4 °C, 5 min). The cell pellet was washed twice with cold physiological saline and stored at –80 °C until further use. Samples were ground into a fine powder under liquid nitrogen. Cell powder (0.1 g) was homogenized with 0.5 mL ice-cold methanol (–40 °C) using vortex mixer (30 s). The mixture was centrifuged at 8000×g and 4 °C for 5 min, and the supernatant was collected. An additional 0.5 mL of pre-chilled methanol (–40 °C) was added to the cell pellet, which was shaken vigorously for 30 s, followed by centrifugation at 8000×g and 4 °C for 5 min. The supernatants from both centrifugation steps were pooled together, dried in a vacuum freeze dryer, and stored at –80 °C for further analysis.

2.8 Derivatization and GC–MS analysis

Before gas chromatography-mass spectrometry (GC–MS) analysis, sample derivatization was performed according to the method established in our previous study [30], with moderate modifications. The samples obtained as described above were mixed with 50 µL of internal standard (myristic acid) and then dried in a vacuum freeze dryer. To the dried sample, 50 µL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added and incubated at 37 °C for 2 h. Next, the sample was silylated by adding 60 µL of N-methyl-N-(trimethylsilyl) trifluoro-acetamide (MSTFA, Sigma, Burlington, USA) and vortex mixing for 30 s, followed by a further incubation at 37°C for 2 h.

A 1 µL sample was injected using a splitless injection into the Agilent 7890-5975C GC–MS, which was equipped with a fused silica capillary column (30 m × 0.25 mm × 0.25 µm, DB-5MS, Agilent). The column and ion source temperatures were set according to our previous study. The mass scan range was 50–600 m/z.

2.9 Fed-batch fermentation

The second-generation seeds were inoculated into the fermentation tank with a 10% inoculum using the flame inoculation method. The initial dissolved oxygen was calibrated at 100%, and the stirring speed was set to 250 r/min. The stirring speed was adjusted based on the dissolved oxygen levels during fermentation, reaching up to 750 r/min. Aeration rate was maintained at 2 L·L^{–1}·min^{–1}, and the pH was controlled at 6.5 using ammonia

and citric acid. Approximately 10 mL of fermentation broth was sampled every 12 h to monitoring residual sugar concentration and oil content. When the residual sugar concentration fell below 20 g/L, a carbon source was added in one dose to achieve a final concentration of 60 g/L. To maintain micronutrient availability, inorganic salt components were also supplemented during fermentation process.

3 Results and Discussion

3.1 Effects of Met on EPA biosynthesis in *Schizochytrium* sp. HG-20

Different concentrations of Met were evaluated for their effects on EPA production in HG-20. The results showed that biomass and oil content decreased with increasing

Met concentration (Fig. 1A). Regarding fatty acid composition, the proportions of C14:0 and C16:0 decreased with increasing Met supplementation, whereas C18:0, C20:4 and EPA exhibited an increasing trend.

The proportion of EPA in total fatty acids increased from 1.7% to a maximum of 5.2% at 10 g/L of Met (Fig. 1B and Table S1). The highest EPA yield (430.6 mg/L) was achieved at 5 g/L Met, representing a 24.3% increase compared to the control. Overall, the proportion of EPA increased by 67% compared to the control (Fig. 1A). This distinct shift in the fatty acid profile suggests that Met supplementation alters fatty acid metabolism, potentially promoting the conversion of SFAs to PUFAs. This effect may be associated with SAM-dependent regulation of lipid metabolic processes [31].

Although the highest EPA yield was achieved at 5 g/L Met, the total oil content decreased by 25.6% from 20.4 g/L to 15.2 g/L. This trade-off between EPA yield and total lipid accumulation suggests that optimizing the

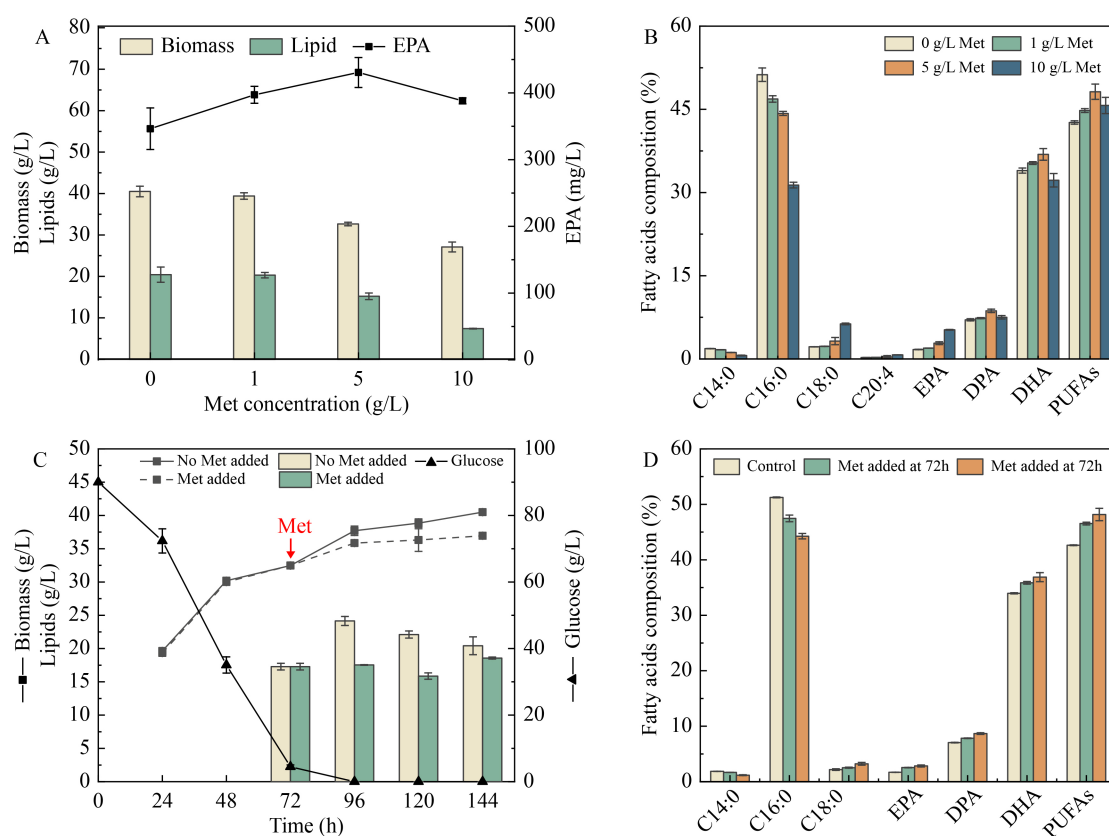


Fig. 1 Effects of Met on Biomass, lipid accumulation, and fatty acid composition in *Schizochytrium*. (A) Effects of different Met concentrations on *Schizochytrium* biomass (light beige column), total lipid yield (sage green column), and EPA yield (black square line) after fermentation; (B) Fatty acid composition (relative proportion, %) of *Schizochytrium* cultured with different Met concentrations. The tested fatty acids include C14:0, C16:0, C18:0, C20:4, EPA, DPA, DHA, and total PUFAs; (C) Time-course profiles of biomass (square lines), lipid yield (columns), and residual glucose concentration (black triangle line) in *Schizochytrium* cultures. Arrows indicate the time point (72 h) of Met addition; (D) Fatty acid composition (relative proportion, %) of *Schizochytrium* under three conditions: Control (no Met), Met added at 72 h, and Met added at 0 h. Error bars represent the standard deviation of three biological replicates.

timing of Met supplementation is critical. Therefore, it is proposed that the supplementation of Met at the late logarithmic growth phase, a stage characterized by near-complete glucose depletion, represents the optimal strategy.

Met supplementation at 72 h post-inoculation resulted in a slower biomass accumulation rate. The lipid content initially declined but gradually recovered, reaching 18.6 g/L at 144 h, compared to 20.4 g/L in the control (Fig. 1C). Similarly, biomass reached 37.0 g/L, slightly lower than the control (40.5 g/L). Notably, both biomass and lipid content exhibited partial recovery over time, indicating that the inhibitory effect of Met was transient rather than permanent. Compared with the control, supplementation at 72 h increase in EPA proportion by 50% and EPA yield by 36.4%. The EPA content per unit biomass also increased by 49.5% (Fig. 1C and Table S2).

Further analysis showed a decrease in SFAs accompanied by a significant increase in EPA, while DHA and

DPA levels remained largely unchanged. This pattern suggests that Met supplementation may promote the conversion of SFAs toward EPA biosynthesis, whereas no significant changes were observed in the accumulation of downstream PUFAs under the tested conditions.

3.2 Effects of Met on PLs and membrane fluidity in *Schizochytrium* sp. HG-20

Lipids in *Schizochytrium* sp. HG-20 are predominantly stored as TAGs, with PLs accounting for a smaller fraction. As shown in Fig. 2A, the TAG to PL ratio (RV) in the control group progressively increased from 96 h to 144 h, indicating a continuous accumulation of TAGs over time. In contrast, Met supplementation led to a marked reduction in RV compared with the control. At 144 h, the RV decreased by 84.2% relative, indicating that PLs in total lipids significantly increased with the addition of Met.

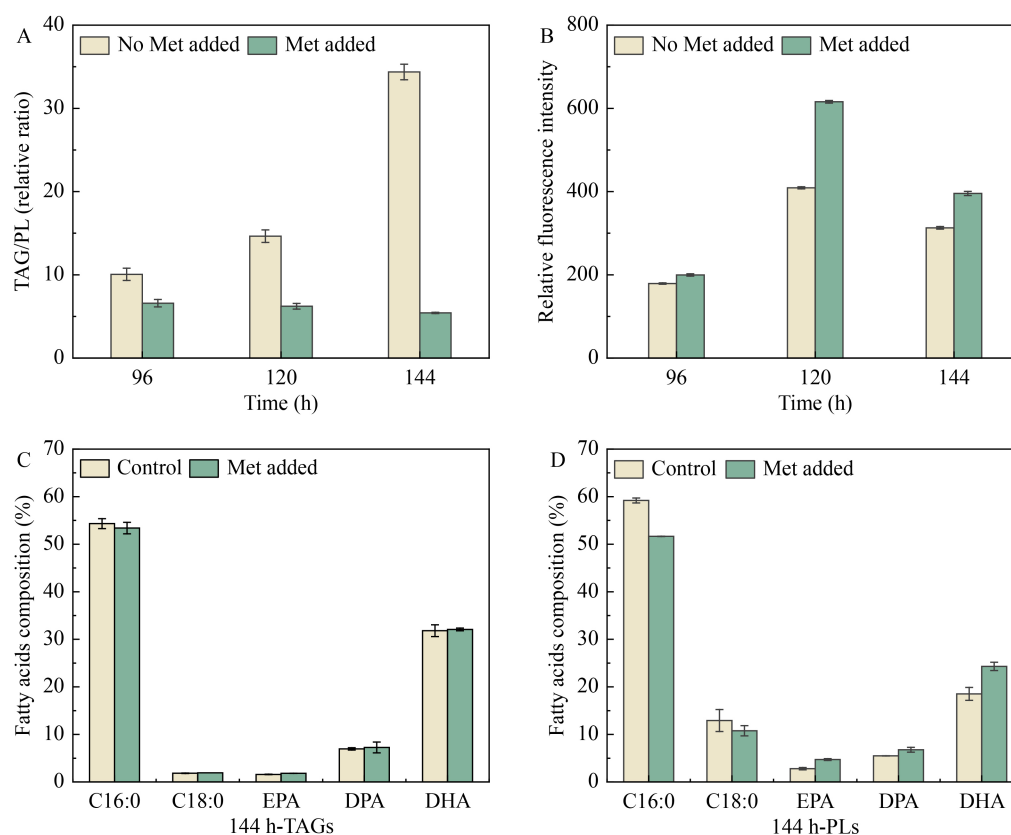


Fig. 2 Effects of Met addition on lipid partitioning, membrane fluidity, and fatty acid composition in *Schizochytrium*. (A) Changes in the relative ratio of TAG to PL (TAG/PL ratio) in *Schizochytrium* cultured at 96 h, 120 h, and 144 h of fermentation; (B) Relative fluorescence intensity (indicating membrane fluidity) of *Schizochytrium* cultured at 96 h, 120 h, and 144 h of fermentation. Higher fluorescence intensity corresponds to greater membrane fluidity; (C) Fatty acid composition (relative proportion, %) of TAGs in *Schizochytrium* at 144 h of fermentation. Detected fatty acids include C16:0, C18:0, EPA, DPA, and DHA; (D) Fatty acid composition (relative proportion, %) of PLs in *Schizochytrium* at 144 h of fermentation. Error bars represent the standard deviation of three biological replicates.

To further dissect the impact of Met on lipid composition, the fatty acid profiles of TAGs and PLs were analyzed separately (Figs. 2C and 2D). Consistent with previous observations, Met supplementation reduced the proportion of SFAs in TAGs, accompanied by a modest increase in total PUFAs. In contrast, PLs exhibited more pronounced compositional changes. At 144 h, the SFAs (C16:0 and C18:0) decreased from 72.1% to 62.4%, while total PUFA increased to 35.8%. Major PUFAs, including EPA (4.7%), DPA (6.8%), and DHA (24.3%), contributed to this shift.

Notably, the increase in EPA was substantially higher in PLs (68.6%) than in TAGs (14.5%), suggesting a preferential incorporation of EPA into membrane lipids rather than storage lipids. This observation is consistent with previous findings by Xu et al. [32], who reported that EPA was primarily incorporated into TAGs during the logarithmic phase, whereas in the stationary phase, it was mainly integrated into PC, indicating a dynamic redistribution of EPA between lipid pools. This difference likely arises from the distinct physiological roles of these lipid classes, where TAGs mainly function in energy storage, whereas PLs are essential components of cellular membranes.

Given that PLs are the primary structural components of cell membranes, their fatty acid compositions directly influence the membrane fluidity. Increase PUFAs content enhances membrane fluidity, whereas higher SFA levels have the opposite effect [33]. Cells can maintain membrane homeostasis by adjusting PUFAs synthesis in response to environmental or metabolic changes [34]. As shown in Fig. 2B, Met supplementation enhanced membrane fluidity by 50.6% at 120 h compared with the control, although fluidity decreased in both groups during the late fermentation stage. This increase in fluidity is likely associated with the elevated PUFA content in PLs, particularly the enrichment of EPA. Consistent with the finding that membrane fluidity is positively correlated with ω -3/ ω -6 PUFA levels [35,36].

Collectively, the results further demonstrate that Met promotes PUFA enrichment in PLs, which in turn enhances membrane fluidity and may contribute to improved cellular performance.

3.3 Metabolomics analysis of Met-mediated lipid metabolic reprogramming in *Schizochytrium* sp. HG-20

GC–MS-based metabolic profiling combined with multivariate analysis was performed to characterize changes in intracellular metabolites induced by Met. Principal component analysis (PCA) revealed a clear separation between the Met-added and control group (Fig. 3B), indicating distinct metabolic profiles. A total of

21 metabolites with variable importance in projection (VIP) > 1 were identified as key contributors to this variation (Fig. 3A).

As shown in Fig. 3C, Met supplementation led to a significant decrease in glycerol levels and was accompanied by a marked increase in glycerol-3-phosphate (G3P), indicating enhanced conversion of glycerol to G3P. As a key intermediate linking glycerol metabolism to glycerolipid biosynthesis, G3P serves as a direct precursor for both TAGs and PLs. Considering the observed increase in PLs content without a corresponding rise in total lipid levels, it is suggested that Met may influence lipid metabolism by modulating G3P availability and its allocation, thereby contributing to a redistribution of carbon among different lipid fractions. Previous studies have reported divergent effects of G3P accumulation on lipid synthesis across different organisms. For example, elevated G3P levels promoted lipid accumulation in *Yarrowia lipolytica* [37], whereas no significant change in lipid content was observed in *Saccharomyces cerevisiae* despite a substantial increase in G3P levels. In the present study, elevated G3P levels were associated with enhanced PL accumulation without a corresponding increase in total lipid content, suggesting that Met supplementation primarily altered the partitioning of glycerolipid precursors rather than simply promoting overall lipid biosynthesis. As G3P serves as the common precursor for both TAG and PL synthesis, these results indicate a preferential redirection of carbon flux toward phospholipid biosynthesis under Met supplementation.

Metabolic profiling showed that pyroglutamic acid and γ -aminobutyric acid (GABA) were significantly reduced in Met-supplemented cultures. Pyroglutamic acid is associated with glutathione (GSH) metabolism and cellular redox regulation, while GABA has been reported to contribute to ROS scavenging and lipid synthesis in microalgae [38]. The decreased levels of pyroglutamic acid and GABA may reflect alterations in cellular redox homeostasis under Met supplementation, although direct measurements of intracellular ROS or glutathione status were not performed [39]. In addition, the reduced levels of glyoxylic acid and malate may reflect changes in central carbon metabolism and energy metabolism [40,41]. As key intermediates connecting the TCA cycle, glyoxylate cycle, and lipid precursor metabolism, the reduced abundance of malate and glyoxylic acid may indicate a redistribution of carbon skeletons from energy metabolism toward biosynthetic processes. These metabolic alterations may be associated with the observed shift toward PL and PUFA accumulation under Met supplementation. However, further studies are required to determine whether these metabolites directly contribute to lipid remodeling.

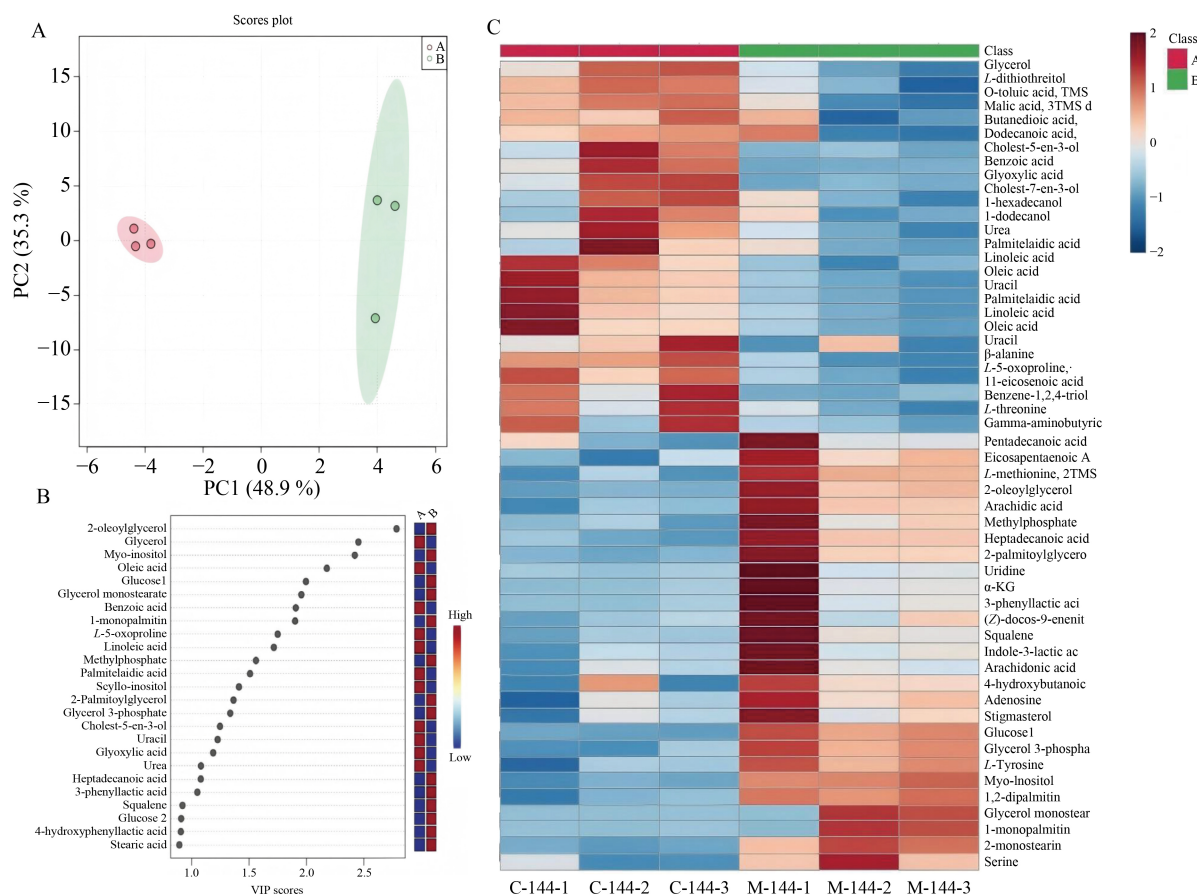


Fig. 3 Integrated metabolomic analysis of *Schizochytrium* under Met regulation. (A) PCA scores plot showing the metabolic discrimination between Group A (pink) and Group B (green); (B) VIP scores plot illustrating the contribution of metabolites to the separation between Group A and Group B (VIP score > 1 indicates metabolites with significant discriminatory power). The color gradient represents the magnitude of VIP scores; (C) Heatmap cluster analysis of differential metabolites between Group A and Group B, depicting the relative abundance of metabolites (C: Control group; M: Met-supplemented group; suffixes 1–3 = biological replicates). Red = high abundance, blue = low abundance.

Collectively, these metabolic changes suggest that Met supplementation induces coordinated metabolic reprogramming rather than isolated alterations of individual metabolites. The simultaneous increase in G3P and decrease in malate and glyoxylic acid support a metabolic framework in which carbon flux is redistributed from central carbon metabolism toward phospholipid biosynthesis. This interpretation is consistent with the increased PL content observed in lipidomic analysis despite the absence of a significant increase in total lipid content, suggesting that Met primarily affects carbon allocation among lipid classes rather than overall lipid synthesis.

Further metabolic analysis suggested that the decrease in glycerol and increase in G3P were associated with enhanced PLs biosynthesis (Fig. 4). Myo-inositol, a sugar alcohol involved in carbon storage and intracellular redox homeostasis [42], was significantly upregulated by 4.13-fold (Fig. 4) in Met-treated cultures. Previous studies

have shown that myo-inositol can promote lipid synthesis and increase the proportion of unsaturated fatty acids (PUFAs) through regulation of central carbon metabolism and fatty acid desaturation pathways [43]. The elevated level of myo-inositol observed in this study may therefore contribute to phosphatidylinositol (PI) synthesis, an important component of PLs. Together with the elevated G3P level, increased myo-inositol may further enhance the availability of phospholipid precursors, thereby supporting phospholipid remodeling under Met supplementation. In addition, SAM derived from Met metabolism, may be involved in phospholipid remodeling through methylation-related processes [41,44], potentially influencing membrane lipid composition. This is consistent with the observed preferential enrichment of EPA in phospholipids compared with TAGs.

Changes in fatty acid composition further supported the effect of Met on lipid metabolism. The relative

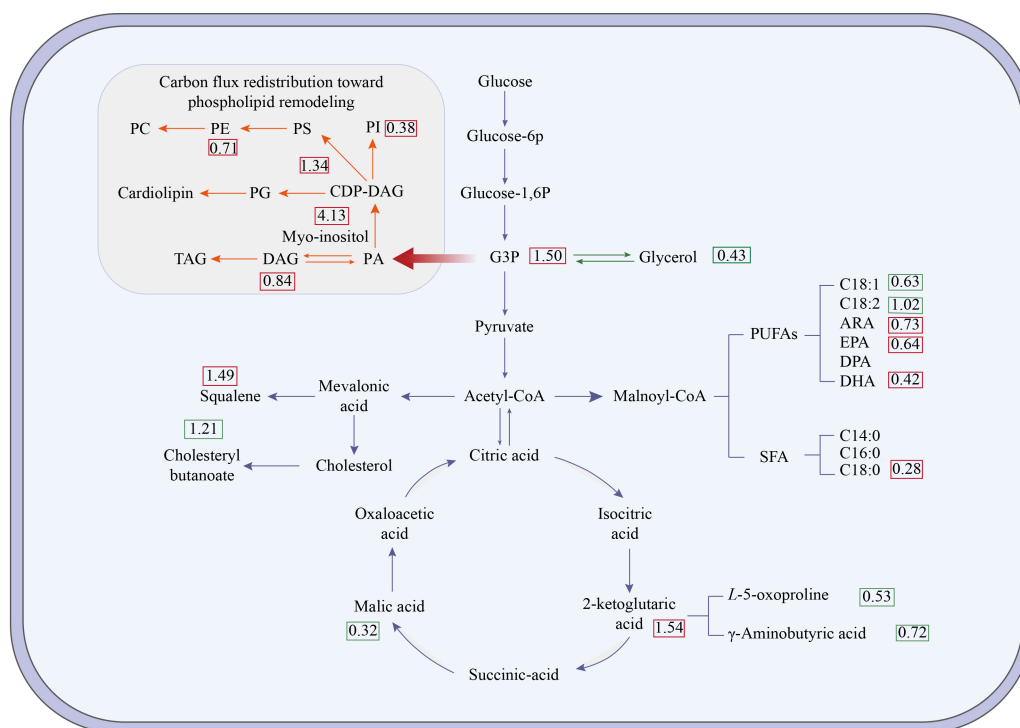


Fig. 4 Proposed metabolic remodeling pathway in *Schizochytrium* sp. under Met supplementation. Red boxes indicate significantly increased metabolites ($P < 0.05$), and green boxes indicate significantly decreased metabolites ($P < 0.05$) compared with the control group. The values shown in the colored boxes represent fold changes (Met-treated/Control) of the corresponding metabolites based on metabolomic analysis.

proportions of C18:1 and C18:2 decreased, whereas those of C18:0 and long-chain PUFAs, including DHA, ARA, and EPA, increased. These shifts suggest enhanced elongation and desaturation processes, leading to the accumulation of long-chain PUFAs. This observation is consistent with previous results (Table S3), indicating that Met promotes PUFAs biosynthesis. Combined with the observed increase of PUFAs in phospholipids, these results indicate that newly synthesized PUFAs are preferentially incorporated into membrane lipids rather than neutral storage lipids. Together with the metabolomic results, these findings support a model in which Met supplementation reprograms central carbon metabolism and carbon allocation, thereby favoring phospholipid remodeling and the preferential incorporation of EPA into membrane phospholipids.

3.4 Differential expression of key PLs synthesis genes mediated by Met in *Schizochytrium* sp. HG-20

The transcription levels of key genes involved in PLs biosynthesis were analyzed to evaluate the effect of Met supplementation. As shown in Fig. 5B, the expression levels of CDS, CDIP and PSS were significantly upregulated. These genes encode enzymes involved in the conversion of phosphatidic acid (PA) to CDP-

diacylglycerol (CDP-DAG), a central precursor for the *de novo* synthesis of structural PLs [45]. The upregulation of these genes suggests an enhanced metabolic capacity for PL biosynthesis, particularly for PI and phosphatidylserine (PS) (Fig. 5A). Notably, PI and PS are critical components of membranes phospholipids, their increased synthesis may contribute to membrane remodeling and altered fluidity under Met supplementation [46,47]. In contrast, the expression levels of PSD and PGP were relatively decreased. Notably, a significant reduction in PAP expression was observed, which may limit the conversion of PA to diacylglycerol (DAG), thereby reducing TAG biosynthesis.

Met-induced alterations in membrane lipid composition suggest a coordinated regulation between PLs biosynthesis and cellular metabolism. Changes in PLs composition have been reported to influence membrane properties and cellular functions [48,49]. Together with the increased G3P level identified in the metabolomic analysis, these transcriptional changes suggest that Met supplementation coordinately regulates phospholipid metabolism at both the metabolic precursor and transcriptional levels, thereby promoting phospholipid biosynthesis over neutral lipid formation.

The selective enrichment of EPA in phospholipids is likely associated not only with enhanced phospholipid

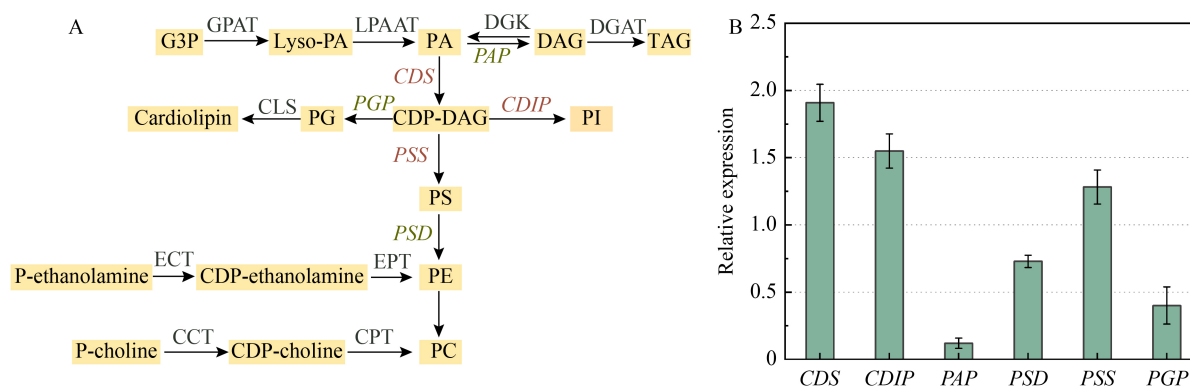


Fig. 5 Key gene expression of pLs biosynthesis in *Schizochytrium* regulated by Met. (A) Schematic representation of the PLs biosynthesis pathway in *Schizochytrium*, illustrating the conversion of core precursors (e.g., G3P, PA) to major PLs components (e.g., PI, PE, PC) and TAG. (B) Relative expression levels of key genes involved in the PLs biosynthesis pathway of *Schizochytrium* (after Met supplementation). Data are the mean $\pm$ standard deviation from three biological replicates, normalized to the control group.

biosynthesis but also with phospholipid remodeling processes that govern fatty acid incorporation into membrane lipids. Previous studies have suggested that enzymes involved in phospholipid remodeling, together with intracellular fatty acid trafficking, play important roles in determining the distribution of PUFAs among different lipid classes [50,51]. Although these processes were not directly examined in the present study, the coordinated changes observed in phospholipid biosynthetic genes, lipid composition, and EPA distribution are consistent with enhanced phospholipid remodeling under Met supplementation. Among the mechanisms proposed in previous studies, SAM-mediated PE-to-PC methylation has been suggested to participate in phospholipid remodeling. However, its involvement in Met-induced lipid remodeling could not be confirmed in the present study.

Collectively, these findings suggest that Met supplementation may promote the preferential incorporation of EPA into membrane phospholipids through coordinated regulation of central carbon metabolism, phospholipid biosynthesis and lipid remodeling, thereby altering carbon allocation between structural and storage lipids. These findings provide new insights into the role of Met in lipid metabolic regulation and may offer potential strategies for optimizing PUFAs production in microbial systems.

3.5 Fed-batch fermentation of EPA in a 3-litre fermenter

A batch-fed fermentation was conducted in a 3 L fermenter to explore the potential of EPA production at a larger scale. The fermentation results are presented in Fig. 6.

During the first 72 h, lipid accumulation in *Schizochytrium* remained at a relatively low level (6.3

g/L at 72 h), which was consistent with the slow rate of glucose consumption during this period. This observation indicated that cellular growth was prioritized over lipid accumulation in the initial growth phase, with carbon resources mainly supporting biomass formation rather than lipid biosynthesis. Meanwhile, EPA accounted for 2.4% of total lipids at 72 h, suggesting that EPA synthesis had already been initiated but was limited by the low overall content.

After 72 h, lipid content increased sharply, peaking at 68.3 g/L at 120 h, accompanied by notable changes in fatty acid composition. Specifically, the proportion of EPA decreased to 1.87%, while C16:0 increased from 35.04% to 50.49%. This shift may be associated with reduced desaturation activity under nitrogen-limited conditions, favoring the accumulation of saturated fatty acids over PUFAs accumulation [52,53].

To minimize the potential interference with cell growth and initial lipid accumulation, 5 g/L of Met was supplemented at 120 h, corresponding to the lipid accumulation phase. This supplementation strategy was designed to minimize the influence of Met on biomass formation while allowing its regulatory effects to be exerted during the later stage of lipid metabolism. Following Met addition, a transient decrease in total lipid content was observed, suggesting a temporary redistribution of metabolic resources. Subsequently, lipid accumulation resumed, accompanied by a gradual increase in EPA proportion. These results suggest that the primary effect of Met at this stage may be associated with lipid remodeling and EPA enrichment rather than promoting additional bulk lipid accumulation.

At 216 h, the proportion of EPA in total lipids reached a maximum of 3.46%, representing a 1.12-fold increase compared to the control group (1.63%). The highest yield of EPA reached 2.34 g/L at 228 h, approximately 1.03

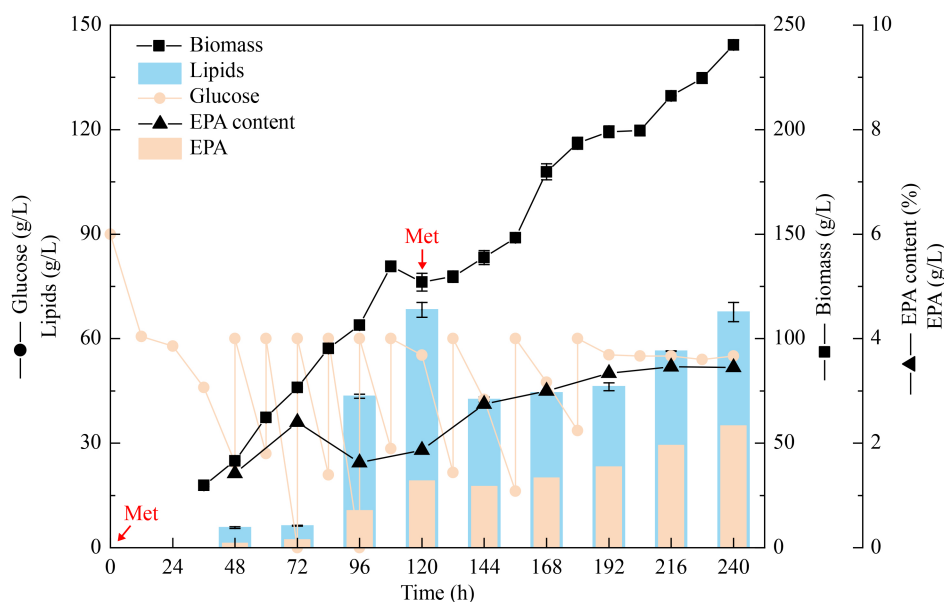


Fig. 6 Fed-batch fermentation of *Schizochytrium* sp. HG-20 in a 3 L fermenter. Time-course profiles of biomass, lipids, glucose consumption, and EPA content in *Schizochytrium* sp. HG-20. Met supplementation is indicated by the red arrow. The black lines and symbols represent biomass (squares), EPA content (triangles). The yellow lines and circular symbols represent the residual glucose content. The bar graphs show the corresponding EPA content (orange bars) and lipids (blue bars) at different time points. Error bars represent the standard deviation of triplicate experiments.

times higher than that of the control group (1.15 g/L). Notably, the temporal difference between the peak of total lipid content (120 h) and the peak of EPA yield (228 h) suggests that bulk lipid accumulation and subsequent fatty acid modification may occur during distinct metabolic phases in *Schizochytrium*. This phase-dependent response is consistent with the regulatory role of Met proposed from our multi-omics analyses, suggesting that the beneficial effect of Met is primarily associated with metabolic regulation during the lipid accumulation stage rather than stimulation of biomass growth or overall lipid biosynthesis. From an industrial perspective, these findings indicate that stage-specific Met supplementation may represent a practical strategy for improving EPA production.

4 Conclusions

The addition of Met effectively enhanced EPA biosynthesis in *Schizochytrium*, increasing the proportion of PUFAs and EPA in total lipid. In a 3 L fermenter, Met supplementation elevated the EPA content in total oil to 3.42%, with a final EPA yield of 2.34 g/L, demonstrating its practical potential for industrial application. Integrative metabolomics and qPCR analyses revealed that Met reprogrammed central carbon metabolism by reducing intracellular levels of glyoxylic acid and malate, thereby suppressing the TCA cycle and associated energy

metabolism. Meanwhile, Met redirected glycerol metabolism toward G3P accumulation and upregulated key genes involved in PLs biosynthesis. This coordinated regulation promoted phospholipid remodeling and facilitated the preferential incorporation of PUFAs, particularly EPA, into membrane lipids.

As a representative sulfur-containing compound, Met exerted a pronounced stimulatory effect on EPA accumulation in *Schizochytrium*, highlighting the critical role of sulfur metabolism in PUFA metabolism in oleaginous microorganisms. Notably, this study demonstrates that Met enhances EPA production through coordinated carbon flux redistribution and phospholipid remodeling, providing new insights into lipid metabolic regulation in oleaginous microorganisms. Moreover, the mechanistic insights and scalable fermentation performance reported here provide a solid foundation for the development of cost-effective and robust industrial fermentation strategies, supporting the sustainable production of EPA for nutraceutical and pharmaceutical applications.

Supporting Information Supporting Information is available in the online version of this article at <https://doi.org/10.2738/ENGF.2026.0019> and is accessible for authorized users.

Data availability No datasets were generated or analyzed for this work.

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authors used GPT-4 as a language-editing tool to improve grammar, clarity, and readability. The AI tool was not used for data analysis, data generation, image processing, or scientific content creation. All outputs were carefully reviewed and verified by the authors, who take full responsibility for the final content.

Competing interests The authors declare no conflicts of interest.

References

- Wang Z T, Zhao J J, Wang Y D, et al. Advances in EPA-GPLs: structural features, mechanisms of nutritional functions and sources. *Trends in Food Science & Technology*, 2021, 114: 521–529
- Wu J S, Barbey A K. Nutrition and mental health: advances in nutritional cognitive neuroscience. *Annual Review of Food Science and Technology*, 2026, 17: 1–24
- Wen J, Satyanarayanan S K, Li A, et al. Unraveling the impact of Omega-3 polyunsaturated fatty acids on blood-brain barrier (BBB) integrity and glymphatic function. *Brain, Behavior, and Immunity*, 2024, 115: 335–355
- Rao A S, Nair A, Nivetha K, et al. Impacts of Omega-3 fatty acids, natural elixirs for neuronal health, on brain development and functions. In: Ray S K, ed. *Neuroprotection: Method and Protocols*. New York: Humana, 2024, 209–229
- Gupta A, Barrow C J, Puri M. Multiproduct biorefinery from marine thraustochytrids towards a circular bioeconomy. *Trends in Biotechnology*, 2022, 40(4): 448–462
- Gong Y M, Wan X, Jiang M L, et al. Metabolic engineering of microorganisms to produce omega-3 very long-chain polyunsaturated fatty acids. *Progress in Lipid Research*, 2014, 56: 19–35
- Harlina P W, Nasution A Y, Musfiroh I, et al. A sustainable source of freshwater fish oil: nutritional and functional insights through lipidomics and metabolomics. *LWT*, 2026, 241: 119046
- Ratlidge C. Omega-3 biotechnology: errors and omissions. *Biotechnology Advances*, 2012, 30(6): 1746–1747
- Duan Y, Chen L M, Ma L X, et al. CRISPR/Cas9-mediated metabolic engineering for enhanced PUFA production in *Schizochytrium limacinum*. *Chemical Engineering Journal*, 2025, 517: 164320
- Chi G X, Xu Y Y, Cao X Y, et al. Production of polyunsaturated fatty acids by *Schizochytrium (Aurantiochytrium)* spp. *Biotechnology Advances*, 2022, 55: 107897
- Wang S, Lan C Z, Wang Z J, et al. Optimizing eicosapentaenoic acid production by grafting a heterologous polyketide synthase pathway in the *Thraustochytrid*. *Journal of Agricultural and Food Chemistry*, 2020, 68(40): 11253–11260
- Barta D G, Coman V, Vodnar D C. Microalgae as sources of omega-3 polyunsaturated fatty acids: biotechnological aspects. *Algal Research*, 2021, 58: 102410
- Xue Z X, Sharpe P L, Hong S P, et al. Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. *Nature Biotechnology*, 2013, 31(8): 734–740
- Du F, Li Z J, Li X, et al. Optimizing multicopy chromosomal integration for stable high-performing strains. *Nature Chemical Biology*, 2024, 20(12): 1670–1679
- Zhang Z X, Wu H X, Lin Y C, et al. Polyketide synthase acyltransferase domain swapping for enhanced EPA recognition and efficient coproduction of EPA and DHA in *Schizochytrium* sp. *Journal of Agricultural and Food Chemistry*, 2025, 73(4): 2461–2470
- Liu K X, Chen Q, Chen Z L, et al. Development of combined mutagenesis strategy for enhanced eicosapentaenoic acid production in *Schizochytrium* sp. *Bioresource Technology*, 2026, 454: 134743
- Jia Y L, Du F, Nong F T, et al. Function of the polyketide synthase domains of *Schizochytrium* sp. on fatty acid synthesis in *Yarrowia lipolytica*. *Journal of Agricultural and Food Chemistry*, 2023, 71(5): 2446–2454
- Ma W, Liu M Z, Zhang Z X, et al. Efficient co-production of EPA and DHA by *Schizochytrium* sp. via regulation of the polyketide synthase pathway. *Communications Biology*, 2022, 5(1): 1356
- Ou Y, Qin Y, Wang Y Y, et al. Co-utilization of corn cob hydrolysate and fermentation wastewater for eicosapentaenoic acid production by *Schizochytrium* sp. *Biotechnology for Biofuels and Bioproducts*, 2025, 18(1): 94
- Droux M. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. *Photosynthesis Research*, 2004, 79(3): 331–348
- Ouyang Y, Wu Q, Li J J, et al. S-adenosylmethionine: a metabolite critical to the regulation of autophagy. *Cell Proliferation*, 2020, 53(11): e12891
- Koprivova A, Kopriva S. Molecular mechanisms of regulation of sulfate assimilation: first steps on a long road. *Frontiers in Plant Science*, 2014, 5: 589
- Noctor G, Mhamdi A, Chaouch S, et al. Glutathione in plants: an integrated overview. *Plant, Cell & Environment*, 2012, 35(2): 454–484
- Chai Y C, Mieyal J J. Glutathione and Glutaredoxin—key players in cellular redox homeostasis and signaling. *Antioxidants*, 2023, 12(8): 1553
- Xiao B P, Li Y Y, Lin Y Q, et al. Eicosapentaenoic acid (EPA) exhibits antioxidant activity via mitochondrial modulation. *Food Chemistry*, 2022, 373: 131389
- Ye C Q, Sutter B M, Wang Y, et al. A metabolic function for phospholipid and histone methylation. *Molecular Cell*, 2017, 66(2): 180–193. e8
- Rajakumar S, Suriyagandhi V, Nachiappan V. Impairment of MET transcriptional activators, *MET4* and *MET31* induced lipid accumulation in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 2020, 20(5): foaa039
- Cui X, Chen T, Meng Y, et al. Determining metabolic mechanism linking phospholipids and docosahexaenoic acid through phosphatidylcholine synthesis by phosphocholine cytidyltransferase (CCT) overexpression in *Schizochytrium* sp. *Microbial Cell Factories*, 2025, 24(1): 81
- Lee Chang K J, Dumsday G, Nichols P D, et al. High cell density

- cultivation of a novel *Aurantiochytrium* sp. strain TC 20 in a fed-batch system using glycerol to produce feedstock for biodiesel and omega-3 oils. *Applied Microbiology and Biotechnology*, 2013, 97(15): 6907–6918
30. Li Z P, Meng T, Ling X P, et al. Overexpression of malonyl-CoA: ACP transacylase in *Schizochytrium* sp. to improve polyunsaturated fatty acid production. *Journal of Agricultural and Food Chemistry*, 2018, 66(21): 5382–5391
 31. Gong Y H, Wang Q T, Wei L, et al. Genome-wide adenine N6-methylation map reveals epigenomic regulation of lipid accumulation in *Nannochloropsis*. *Plant Communications*, 2024, 5(3): 100773
 32. Xu Y Q, Zhang Z, Bian Y Q, et al. Regulatory mechanisms of EPA and DHA proportions in a PUFA-producing microalga, *Schizochytrium* sp. ATCC 20888: from the biosynthesis and storage distribution aspects. *Journal of Agricultural and Food Chemistry*, 2025, 73(7): 4137–4151
 33. Harayama T, Riezman H. Understanding the diversity of membrane lipid composition. *Nature Reviews Molecular Cell Biology*, 2018, 19(5): 281–296
 34. Almeyda M D, Bilbao P G S, Popovich C A, et al. Enhancement of polyunsaturated fatty acid production under low-temperature stress in *Cylindrotheca closterium*. *Journal of Applied Phycology*, 2020, 32(2): 989–1001
 35. Lu L, Okada N, Nakatani S, et al. Eicosapentaenoic acid-induced changes in membrane fluidity and cell adhesion molecules in cultured human keratinocytes. *British Journal of Dermatology*, 1995, 133(2): 217–222
 36. Alagawany M, Elnesr S S, Farag M R, et al. Nutritional significance and health benefits of omega-3, -6 and -9 fatty acids in animals. *Animal Biotechnology*, 2021, 33(7): 1678–1690
 37. Silverman A M, Qiao K J, Xu P, et al. Functional overexpression and characterization of lipogenesis-related genes in the oleaginous yeast *Yarrowia lipolytica*. *Applied Microbiology and Biotechnology*, 2016, 100(8): 3781–3798
 38. Li Q Q, Zhao Y T, Ding W, et al. Gamma-aminobutyric acid facilitates the simultaneous production of biomass, astaxanthin and lipids in *Haematococcus pluvialis* under salinity and high-light stress conditions. *Bioresource Technology*, 2021, 320: 124418
 39. Zhang Y Y, Jelleschitz J, Grune T, et al. Methionine restriction-association with redox homeostasis and implications on aging and diseases. *Redox Biology*, 2022, 57: 102464
 40. Jung H M, Han J H, Oh M K. Improved production of 2,3-butanediol and isobutanol by engineering electron transport chain in *Escherichia coli*. *Microbial Biotechnology*, 2021, 14(1): 213–226
 41. Liu Z X, You S, Tang B P, et al. Inositol as a new enhancer for improving lipid production and accumulation in *Schizochytrium* sp. SR21. *Environmental Science and Pollution Research*, 2019, 26(35): 35497–35508
 42. Su X B, Ko A L A, Saiardi A. Regulations of *myo*-inositol homeostasis: mechanisms, implications, and perspectives. *Advances in Biological Regulation*, 2023, 87: 100921
 43. Qiao T S, Zhao Y T, Han B Y, et al. *Myo*-inositol promotes lipid production and nutrients removal by microalga under molasses wastewater. *Renewable Energy*, 2021, 172: 327–335
 44. Li J Y, Xin Y G, Li J Y, et al. Phosphatidylethanolamine N-methyltransferase: from Functions to Diseases. *Aging and Disease*, 2023, 14(3): 879–891
 45. Mak H Y, Ouyang Q, Tumanov S, et al. AGPAT2 interaction with CDP-diacylglycerol synthases promotes the flux of fatty acids through the CDP-diacylglycerol pathway. *Nature Communications*, 2021, 12(1): 6877
 46. Ikhlef S, Lipp N F, Delfosse V, et al. Functional analyses of phosphatidylserine/PI(4)P exchangers with diverse lipid species and membrane contexts reveal unanticipated rules on lipid transfer. *BMC Biology*, 2021, 19(1): 248
 47. Long T, Li D Y, Vale G, et al. Molecular insights into human phosphatidylserine synthase 1 reveal its inhibition promotes LDL uptake. *Cell*, 2024, 187(20): 5665–5678. e18
 48. Ebner M, Puchkov D, López-Ortega O, et al. Nutrient-regulated control of lysosome function by signaling lipid conversion. *Cell*, 2023, 186(24): 5328–5346. e26
 49. Xu J K, He Y L, Wu X F, et al. Conformational changes of a phosphatidylcholine flippase in lipid membranes. *Cell Reports*, 2022, 38(11): 110518
 50. Li-Beisson Y, Thelen J J, Fedosejevs E, et al. The lipid biochemistry of eukaryotic algae. *Progress in Lipid Research*, 2019, 74: 31–68
 51. Guschina I A, Harwood J L. Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 2006, 45(2): 160–186
 52. Rismani-Yazdi H, Haznedaroglu B Z, Hsin C, et al. Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation. *Biotechnology for Biofuels*, 2012, 5(1): 74
 53. Qiao K, Meng C F, Huang Y J, et al. Transcriptomic analysis of the mechanism of nitrogen and phosphorus limitation on improving the yield of DHA by *Schizochytrium* sp. *Food Research International*, 2026, 231: 118623