Postprint: Synthesis of Polyunsaturated Fatty Acids via the Fatty Acid Synthase Pathway in Schizochytrium

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Abstract

Schizochytrium, an important DHA-producing microorganism, retains functional enzymes and intermediates of the fatty acid synthase (FAS) pathway, which is common in eukaryotic microorganisms, in addition to the well-characterized polyketide synthase (PKS) pathway. Using genetic engineering approaches, a vector harboring the known Δ12-desaturase expression module from a closely related algal species along with relevant selection markers was constructed and introduced into Schizochytrium sp. ATCC 20888 via electroporation to restore the synthetic capacity of the FAS pathway. Transformants were analyzed by gas chromatography. Following molecular-level confirmation of stable transformation of the target functional gene, the fatty acid composition distribution in cellular lipids was examined. Through long-term screening and validation, two positive transformants were ultimately obtained, which exhibited respective biomass increases of 11.14% and 4.12% over the wild-type strain, and respective increases in DHA content of 19.50% and 14.65% in their lipids compared to the wild-type.

Full Text

Study on the Synthesis of Polyunsaturated Fatty Acids via the Fatty Acid Synthase Pathway in *Schizochytrium* sp.

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Abstract

Schizochytrium sp. possesses two distinct pathways for docosahexaenoic acid (DHA) production: the fatty acid synthase (FAS) pathway and the polyketide synthase (PKS) pathway. However, the FAS pathway plays only a limited role in DHA synthesis due to low activity of one or more key enzymes, restricting its function to short-chain fatty acid production. To enhance DHA synthesis through the FAS pathway and increase DHA content in *Schizochytrium* lipids, we constructed an exogenous Δ12-desaturase expression plasmid with appropriate selection markers for *Schizochytrium* sp. ATCC 20888, derived from a closely related algal species. The plasmid was introduced into *Schizochytrium* via electroporation. Following molecular confirmation of stable integration of the target gene, we analyzed the fatty acid composition of transformant lipids using gas chromatography. Through long-term screening and validation, we successfully obtained two positive transformants. Initial biomass measurements showed increases of 11.14% and 4.12% compared to the wild-type strain, while DHA content in their lipids increased by 19.50% and 14.65%, respectively.

Keywords: Genetic engineering; Fatty acid synthase pathway; *Schizochytrium* sp.; DHA

Introduction

Docosahexaenoic acid (DHA), an essential omega-3 fatty acid, is a critical component of the human brain, cerebral cortex, skin, and retina. Appropriate supplementation can enhance cognitive function, reduce cardiovascular disease risk, and provide anti-inflammatory benefits [1]. Currently, DHA is widely used in nutritional supplements, food additives, and animal feed. With rising living standards, demand for DHA continues to grow. DHA is primarily obtained from two sources: fish oil and microalgal oil. Compared to fish oil, microalgal oil offers greater safety and stability, higher metabolic absorption rates, no unpleasant odor, lower heavy metal content, and is more environmentally sustainable. Consequently, identifying high-yield DHA-producing algal strains is of significant importance [2].

Schizochytrium sp. is one of the most promising industrial DHA production strains due to its rapid growth, short fermentation cycle, simple unsaturated fatty acid composition, high DHA content, excellent product quality, ease of extraction, and non-toxicity to humans and animals [3]. This microalga possesses two distinct fatty acid synthesis pathways: the fatty acid synthase (FAS) pathway and the polyketide synthase (PKS) pathway [4, 10]. The FAS pathway utilizes acetyl-CoA and malonyl-CoA as substrates, generating palmitic

acid through the FAS enzyme complex, which is then elongated to stearic acid. Subsequent desaturation and elongation by a series of enzymes (Δ9-desaturase, Δ12-desaturase, Δ6-desaturase, Δ5-desaturase, Δ4-desaturase, n-3 desaturase, and various elongases) produces a range of unsaturated fatty acids including DHA. In contrast, the PKS pathway employs a PKS multi-enzyme complex that uses acetyl-CoA and malonyl-CoA to generate EPA and DHA through a series of acyl intermediates, typically bypassing the dehydration steps of the FAS cycle and reducing enoyl intermediates. The two pathways generate different intermediate products en route to DHA.

Research has demonstrated that *Schizochytrium* primarily relies on the PKS pathway for DHA synthesis, as the FAS pathway contributes minimally due to low activity of certain functional enzymes, limiting its role to short-chain fatty acid synthesis [4-11]. Specifically, Lippmeier et al. [8] used radiolabeled fatty acid tracing and chromatographic analysis to characterize a PKS-deficient *Schizochytrium* sp. ATCC 20888 mutant, suggesting that the FAS pathway' s impaired function likely resulted from insufficient Δ12-desaturase activity. In 2015, Ren et al. [11] successfully introduced an exogenous -3 desaturase gene into *Schizochytrium* sp. HX-308, demonstrating that this modification shifted fatty acid metabolism toward enhanced DHA synthesis and validating the feasibility of improving DHA content by engineering the FAS pathway.

In this study, we employed genetic engineering techniques to construct an exogenous Δ12-desaturase expression vector for *Schizochytrium* sp. ATCC 20888, using the Δ12-desaturase gene from *Phaeodactylum tricornutum*. The vector was introduced via electroporation [12] to enhance the FAS pathway, with gas chromatography used to verify whether exogenous Δ12-desaturase expression improved DHA levels and to assess its impact on related fatty acid synthesis. Previous efforts to increase DHA production in *Schizochytrium* have focused primarily on optimizing culture media and fermentation conditions or screening high-yield mutants—all essentially aimed at enhancing PKS pathway activity. Our approach of genetically engineering the FAS pathway represents a novel molecular mechanism for DHA improvement, offering more efficient targeted modification while providing valuable insights into DHA synthesis mechanisms in *Schizochytrium*.

Materials and Methods

1.1 Strains, Plasmids, Reagents, and Culture Media Algal Strains: *Schizochytrium* sp. ATCC 20888 and *Phaeodactylum tricornutum* were maintained in our laboratory.

Plasmids: pPICZ A, pBluescriptII SK (+), pcDNA3.1(+), and pUXSHCL were maintained in our laboratory.

Reagents: Total RNA extraction kit (Promega Eastep™), RevertAid™ First

Strand cDNA Synthesis Kit (Thermo), gel extraction kit (Axgen), plasmid extraction kit (Axgen), Xmn I (NEB), PrimeSTAR, Spe I, Sac I, Bam I, Not I (Takara), fungal genomic DNA extraction kit (Solarbio), and others.

Culture Media: YPD medium contained 10 g yeast extract, 20 g glucose, and 20 g peptone dissolved in a 1:1 mixture of sterile natural seawater and distilled water, adjusted to 1 L. For solid medium, 15 g agar was added per liter. LB medium contained 20 g LB nutrient broth per liter of distilled water (liquid) or 40 g LB nutrient agar per liter (solid). All media were sterilized at 121°C for 20 minutes. Except for glucose (purchased from Tianjin Chemical Reagent Third Factory), all media components were from Solarbio.

Instruments: PCR thermal cycler (Eppendorf), electroporator (Bio-Rad), constant temperature shaker (Crystal), climate-controlled incubator (Harbin Electronic Instrument Technology Development Co.), freeze dryer (CHRIST), and water bath (Shanghai Jinghong Experimental Equipment).

1.2 Experimental Procedures 1.2.1 Plasmid Construction

We designed homologous arms for *Schizochytrium* 18S rDNA, an exogenous Δ12-desaturase gene expression cassette from *P. tricornutum*, and a G418 resistance cassette, then mapped the Δ12-desaturase expression vector using Vector NTI. Primers used for plasmid construction are listed in Table 1.

[Figure 1: see original paper] shows the expression vector pBlu2SKP-PhtΔ12. The plasmid consists of: (1) homologous arms LB18S and RB18S; (2) Δ12-desaturase expression cassette (Δ12-desaturase CDS from *P. tricornutum*, ubiquitin promoter, and Upoly terminator); and (3) G418 resistance cassette (PTEF1-PEM7 promoter, neo resistance gene, and CYC1TT terminator).

Fragment Cloning: We cloned the 18S fragment from *Schizochytrium* genomic DNA and verified the sequence via NCBI BLAST. Using the gel-purified 18S as template, we cloned LB18S and RB18S. Total RNA was extracted from *P. tricornutum* and immediately reverse-transcribed to cDNA for cloning the Δ12-desaturase gene (gel-purified product designated Pht12). The ubiquitin promoter was cloned from plasmid pCXUN (product: Ubi), Upoly terminator from pUXSHCL (product: Upoly), neo gene from pcDNA3.1(+) (product: neo), PTEF1-PEM7 promoter from pPICZ A (product: PTE), and CYC1TT terminator from pPICZ A (product: CYC).

Assembly of Plasmid Components: The G418 resistance cassette and RB18S homologous arm were assembled with the Δ12-desaturase terminator Upoly and pBluescript II SK (+) backbone. Overlap PCR connected RB18S, CYC, and neo fragments (product: neo-CYC-RB18S). Upoly and PET were similarly joined (product: Upoly-PET). A final overlap PCR combined neo-CYC-RB18S and Upoly-PET (product: Upoly-PET-neo-CYC-RB18S). Both this fragment and pBluescript II SK (+) were digested with Spe I and BamH I, gel-purified, ligated with T4 ligase, and transformed into *E. coli* DH5 . Positive

clones were sequenced; a correct clone was designated Ecoli-pSKII-pht-1, with plasmid pBlu2SKP-PhtΔ12-1.

For the second step, LB18S and Ubi were connected to pBlu2SKP-PhtΔ12-1. Overlap PCR joined LB18S and Ubi (product: LB18S-Ubi). Both pSKII-pht1 and LB18S-Ubi were digested with Sac I and Not I, gel-purified, ligated, and transformed into DH5 . A verified clone was designated Ecoli-pSKII-pht-2, with plasmid pBlu2SKP-PhtΔ12-2.

Finally, Pht12 was inserted into pBlu2SKP-PhtΔ12-2. Both pBlu2SKP-PhtΔ12-2 and Pht12 were digested with Spe I and Not I, gel-purified, ligated, and transformed into DH5 . A verified clone was designated Ecoli-pBlu2SKP-PhtΔ12, with the final plasmid pBlu2SKP-PhtΔ12.

1.2.2 Electroporation of *Schizochytrium*

Resistance Gradient Assay: G418 concentration gradients of 100, 200, 300, and 400 mg/L were tested on YPD solid plates inoculated with *Schizochytrium* to determine the minimum inhibitory concentration.

Plasmid Linearization: Plasmid pBlu2SKP-PhtΔ12 was extracted from overnight culture of Ecoli-pBlu2SKP-PhtΔ12, linearized with Xmn I, and purified using a gel extraction kit.

Electroporation: Exponential-phase *Schizochytrium* cells were prepared for electroporation. Ten microliters of linearized plasmid were mixed with 80 L competent cells, incubated for 30 minutes, and electroporated at 2.1 kV, 200 Ω. Post-electroporation, cells were resuspended in YPD medium to 1 mL, transferred to 1.5 mL tubes, and incubated at 28°C, 180 rpm for 2-3 hours before plating on YPD solid medium containing 350 mg/L G418 and incubating at 28°C in darkness [13].

Serial Selection: Colonies on antibiotic plates were streaked to fresh plates every 3-5 days for three passages, then cultured once in 250 mL flasks containing 200 mL YPD without antibiotics for seven days, followed by another passage on antibiotic plates. This two-round cycle yielded preliminary transformants (during which low-biomass strains were eliminated despite good antibiotic resistance).

1.2.3 DNA-Level Verification of Transformants

Genomic DNA was extracted from putative transformants and used as template for PCR with primers Jh-F (CCAAAGCAAGGAACAGGTATG-GACTTCGTC) and Jh-R (GCACACCCTCGTTTGACTCCACATAGT-GACAG) to amplify the introduced Δ12-desaturase fragment (~1.2 kb).

1.2.4 RT-PCR Verification of Transformants

Total RNA was extracted from transformants, reverse-transcribed to cDNA, and amplified with Jh-F/R primers to verify expression at the RNA level, with 18S rRNA as a reference to estimate expression levels.

1.2.5 Biomass Determination

Seed cultures of transformants and wild-type strain (~5×10 cells/mL) were inoculated at 1:20 ratio into 200 mL YPD medium in 250 mL flasks (three replicates). Cultures were grown at 28°C, 180 rpm for 4 days, then at 23°C, 180 rpm for 3 days. Cells were harvested, washed twice with distilled water, lyophilized, and weighed.

1.2.6 Gas Chromatographic Analysis of Lipid Content

Lipids were extracted using the Bligh-Dyer method [14] and analyzed by gas chromatography (Agilent 7693A, USA) with a flame ionization detector and SUPELCO SP™-2560 column. A 37-component fatty acid methyl ester mix served as standard. Detector temperature was 280°C, injection volume 1 L, split ratio 1:3, and column flow 1 mL/min. Each sample was analyzed in duplicate.

Results

2.1 Plasmid Construction After cloning all required fragments, plasmid assembly proceeded in three stages. First, pBluescript II SK (+) was ligated with Upoly, PET, neo, CYC, and RB18S fragments to generate pBlu2SKP-PhtΔ12-1. Second, LB18S and Ubi fragments were inserted into pBlu2SKP-PhtΔ12-1 to yield pBlu2SKP-PhtΔ12-2. Finally, the Δ12-desaturase gene (Pht12) was cloned into pBlu2SKP-PhtΔ12-2, producing the final plasmid pBlu2SKP-PhtΔ12. Each intermediate construct was sequenced before proceeding to ensure final plasmid accuracy.

[Figure 2: see original paper] illustrates construction of pBlu2SKP-PhtΔ12-1. [Figure 3: see original paper] shows assembly of pBlu2SKP-PhtΔ12-2. [Figure 4: see original paper] depicts final construction of pBlu2SKP-PhtΔ12.

2.2 Electroporation Results The resistance gradient assay ([Figure 5: see original paper]) revealed that G418 concentrations below 300 mg/L failed to inhibit *Schizochytrium* growth after 8 days. At 300 mg/L, a few colonies emerged, while only one colony grew at 400 mg/L. To balance transformant recovery rate and survival, 350 mg/L was selected as the initial screening concentration.

Two days post-electroporation, small colonies appeared on selective plates. After the first passage at day 4, larger colonies were selected for further screening, ultimately yielding 15 transformants. Notably, we observed that the growth stage of *Schizochytrium* significantly affected electroporation efficiency, with exponential-phase cells showing markedly higher transformation rates than stationary-phase cells.

2.3 DNA Verification Results PCR amplification with Jh-F/R primers identified two positive transformants from the genomic DNA, indicating a low

transformation efficiency. This suggests that *Schizochytrium* has a strong tendency to eliminate foreign DNA, as only two strains remained stable after continuous antibiotic selection.

[Figure 6: see original paper] shows the DNA-level screening results, with positive bands at ~1.2 kb representing the functional pht12 gene fragment.

2.4 RT-PCR Verification Results Using 18S rRNA as a reference, both positive transformants showed expression at the RNA level, with transformant 1 exhibiting higher expression than transformant 2. The qPCR primers matched those used for DNA verification for convenience and specificity. Although the native Δ12-desaturase in *Schizochytrium* has low activity, its gene may still be expressed and could share homology with the *P. tricornutum* enzyme. These primers were validated in DNA screening to specifically amplify the introduced *P. tricornutum* Δ12-desaturase fragment.

[Figure 7: see original paper] presents the RT-PCR results, showing amplification of the functional gene fragment (H) in both transformants.

2.5 Biomass Measurements Preliminary biomass measurements demonstrated that both transformants outperformed the wild-type strain under our experimental conditions. Transformant 1 showed an 11.14% increase in biomass, while transformant 2 showed a 4.12% increase.

compares biomass between wild-type and transformants.

2.6 Gas Chromatography Results Representative gas chromatograms clearly showed that transformants had smaller palmitic acid peaks and significantly larger DHA peaks compared to the wild-type strain.

[Figure 8: see original paper] displays the gas chromatograms of transformants and wild-type. summarizes the percentage content of key fatty acids. The data, combined with [Figure 9: see original paper], reveal that in the FAS pathway, the substrate oleic acid (C18:1) decreased while the product linoleic acid (C18:2) increased. Upstream fatty acids palmitic acid (C16:0) and stearic acid (C18:0) also decreased, with palmitic acid showing substantial reductions of 17.51% and 25.89% in transformants 1 and 2, respectively. Downstream fatty acids - linolenic acid and EPA showed minimal changes, while DHA content increased markedly by 19.50% and 14.65% in transformants 1 and 2, respectively. These results confirm that the introduced Δ12-desaturase was functional, enhanced its catalytic activity, and increased the final DHA product of the FAS pathway.

[Figure 9: see original paper] illustrates the FAS pathway for polyunsaturated fatty acid synthesis [5].

Discussion

Through plasmid construction, electroporation, and verification at both DNA and RNA levels, we successfully obtained two stable transformants. Both exhibited superior biomass and DHA content compared to the wild-type strain, with transformant 1 outperforming transformant 2 in all metrics. The higher RNA expression level in transformant 1 suggests that differences in transgene copy number may account for the observed variation, though integration site effects could also contribute [13].

Schizochytrium possesses two fatty acid synthesis pathways, but the FAS pathway' s capacity for unsaturated fatty acid production is limited by low activity of certain enzymes. We hypothesized that enabling both pathways to function efficiently would increase unsaturated fatty acid content and potentially boost DHA levels. This study confirms that enhancing Δ12-desaturase expression in *Schizochytrium* sp. ATCC 20888 indeed increases the percentage of DHA in its lipids, validating our hypothesis and providing both theoretical and technical support for improving DHA production by activating the FAS pathway.

While we obtained two high-quality transformants, biomass accumulation does not always correlate with lipid accumulation. Industrial applications require high-density fermentation and further optimization of culture media and conditions to maximize DHA yield while minimizing production costs.

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Note: Figure translations are in progress. See original paper for figures.