



Augmenting *Fremyella diplosiphon* Cellular Lipid Content and Unsaturated Fatty Acid Methyl Esters Via Sterol Desaturase Gene Overexpression

Somayeh Gharai Fathabad¹ · AnithaChristy S. Arumanayagam² · Behnam Tabatabai¹ · Huan Chen³ · Jie Lu^{3,4} · Viji Sither¹ 

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Abstract

Cyanobacteria have immense prospective as a platform for renewable energy; however, a major barrier in achieving optimal productivity is the low lipid yield. *Fremyella diplosiphon*, a model cyanobacterium, is an ideal biofuel agent due to its desirable fatty acid methyl esters (FAMES). To enhance lipid content, we overexpressed the sterol desaturase (SD) gene in *F. diplosiphon* B481 wild type by genetic transformation. This effort resulted in a transformant (B481-SD) with a 64-fold increase in the SD gene at the mRNA transcript level, with no loss in growth and pigmentation. The transformant was persistently grown for over 32 generations indicating long-term stability and vitality. We observed 27.3% and 23% increases in total lipid content and unsaturated FAMES respectively in B481-SD transesterified lipids with methyl octadecadienoate as the most abundant unsaturated component. In addition, we detected an 81% increase in FAME composition in the transformant compared with the wild type. Theoretical physical and chemical properties confirmed a FAME profile with very high cetane number (65.972–67.494) and oxidative stability (50.493–18.66 h) in the engineered strain. Results of the study offer a promising approach to augment *F. diplosiphon* total lipid content and unsaturated FAMES, thus paving the way to enhance biofuel capacity of the organism.

Keywords Alkane · Biofuel · Cyanobacteria · Sterol desaturase · Transcript abundance

Introduction

Fossil fuels are a primary source of energy worldwide; however, increasing environmental consequences of their overuse, diminishing oil reserves, and rising costs have

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✉ Viji Sither
viji.sither@morgan.edu

Extended author information available on the last page of the article

driven scientists to develop renewable sources of energy [1]. In recent years, cyanobacteria have gained great importance as third-generation biofuel feedstock due to their rapid biomass production and lipids such as monogalactosyldiacylglycerol (MGDG), glycolipids, and digalactosyldiacylglycerol (DGDG) in their thylakoid membrane [2–5]. In particular, fatty acids are a major class of lipids and essential biomolecules in the cyanobacterial cell membrane which determine important chemical and physical properties such as viscosity and permeability [1, 5].

Fatty acid desaturase enzymes such as acyl-lipid desaturase and sterol desaturase (SD) affect cyanobacterial lipid metabolism by synthesizing MGDG and DGDG, which are two major classes of galactolipids desirable for biofuel production [6–8]. However, insufficient lipid and fatty acid yields are key obstacles that hinder industrial-scale production of cyanobacterium-based biofuel. In addition, the high capital extraction/conversion cost poses a significant challenge and limits energy productivity of these biofuels [9]. Critical engineering breakthroughs to enhance lipid yield per harvest volume, which will reduce the cost of extraction per unit product, are thus crucial to unlock the possibility of selected strains to serve as cost-effective biofuels.

Of the various cyanobacterial species, *Fremyella diplosiphon* has great potential as a commercial biofuel agent due to the generation of desirable fatty acid methyl esters (FAMES) when its lipids are subjected to transesterification [10]. While few studies have been reported to enhance *F. diplosiphon* stress tolerance and photosynthetic efficacy [11–13], there has been no effort to increase its lipid and essential fatty acids. The objective of the present study was to overexpress the SD gene in *F. diplosiphon* via electroporation-mediated transformation. Level of gene overexpression was quantified, and its effect on total lipid content and FAME composition/abundance characterized to confirm the role of the SD gene in fatty acid synthesis.

Materials and Methods

Strain and Culture Conditions

F. diplosiphon strain B481 obtained from the UTEX algal repository was used as the wild type (WT). Cultures were grown in BG11 liquid medium containing 20 mM HEPES at 170 rpm and 28 °C for 9 days under continuous white light adjusted to 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For transformation studies, *Escherichia coli* FB5 α competent cells (Thermo Fisher Scientific) were grown at 37 °C in Luria-Bertani (LB) broth or agar plates supplemented with 80 mg L⁻¹ ampicillin as the selective antibiotic.

RNA Isolation and cDNA Synthesis

F. diplosiphon WT was grown to an optical density of 0.6 at 750 nm (OD₇₅₀) under culture conditions as mentioned above. Total RNA was extracted using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. The purity of extracted RNA was tested on an agarose gel, and A_{260/280} absorbance ratio measured using a Nanodrop 2000 (Thermo Fisher Scientific). Complementary DNA (cDNA) was reverse transcribed using the High Capacity RNA to cDNA kit (Life Technologies) and used for transformation.

Identification, Cloning, and Expression of *F. diplosiphon* Sterol Desaturase Gene

Homologs of the SD gene were amplified from 50 ng *F. diplosiphon* WT cDNA using specific primers designed according to the gene sequence (JGI/IMG # 2501541024) and contained a *HindIII* restriction site added to the 5' end and *BamHI* to the 3' end (Table 1). Amplifications were performed using a C1000 Touch Thermocycler (Bio-Rad) under the following conditions: 95 °C for 2 min, 40 cycles at 95 °C for 30 s and an annealing temperature of 59 °C for 30 s, followed by a final elongation step at 72 °C for 45 s. Amplified products were electrophoresed on a 1.2% agarose gel, bands excised at the expected size ranges, and cDNA extracted using the Gel Recovery kit (Zymo Research). Amplified gene products were double digested with *HindIII* and *BamHI*, and pGEM-7Zf (+) vector triple digested with *HindIII*, *BamHI*, and *ScaI* restriction enzymes (Promega). Purified inserts were ligated into the vector at the *HindIII* and *BamHI* restriction sites with T4 DNA ligase (New England BioLabs), and the pGEM-7Zf (+)-SD expression plasmid constructed to overexpress the sterol desaturase gene. The plasmids were then cloned into *E. coli* FB5 α competent cells via heat shock at 42 °C for 20 s. Transformed competent cells were plated on LB agar containing 80 mg L⁻¹ ampicillin and incubated for 16 h at 37 °C. Resistant single colonies were selected, confirmed with X-gal staining for detection of β -galactosidase, and grown overnight in liquid LB medium containing 80 mg L⁻¹ ampicillin. Ten random colonies were selected and plasmids extracted using the Zippy Plasmid Miniprep kit (Zymo Research). Of these extracted plasmids, two were randomly selected and used for electroporation-mediated transformation. The presence of the insert was checked by PCR and further confirmed by sequencing.

Electroporation-Mediated Transformation

Expression plasmids containing the SD gene were transformed into *F. diplosiphon* WT according to electroporation parameters described by Kehoe and Grossman [14]. The transformed strain was grown in 10 mL BG11/HEPES liquid medium for 16 h and transferred to BG11/HEPES agar containing 80 mg L⁻¹ ampicillin. To verify the insertion of plasmid-contained SD gene, PCR was performed using gene-specific primers as mentioned above (Table 1), and products visualized on a 1.2% agarose gel with a GeneRuler 100 bp Plus DNA marker (Thermo Scientific).

Quantification of *F. diplosiphon* Gene Expression Levels Using RT-qPCR

Total RNA in WT and transformant were extracted, cDNA synthesized as mentioned above, and RT-qPCR performed to quantify gene overexpression. Gene-specific primers were designed (Table 1) according to the gene sequence and real-time amplifications performed using

Table 1 Primers for the sterol desaturase gene used in plasmid construction and reverse transcription–quantitative PCR assay

Primer	Sequence (5-3)
Sterol desaturase (forward)	ATCAAGCTTGGATGTTGACTTTTGACTTCTTCATAGCGGG
Sterol desaturase (reverse)	GCGGGATCCGTTAGTTCAAAGATTGAACCTTTTIA
Sterol desaturase (qPCR forward)	CGGGACTAGCGCTGATAAAA
Sterol desaturase (qPCR reverse)	TTTACCAAGCGAGGGGACAT

SYBR green master mix (Applied Biosystems) in a Thermal Cycler CFX96 Real-Time machine (Bio-Rad). The reactions were performed in 20- μ L volume containing 10 μ L SYBR green Master Mix and 10 ng cDNA template. Amplifications were performed under the following conditions: 95 °C for 20 s, 95 °C for 20 s, and 40 cycles at 50.9 °C for 30 s. Four replicates were maintained for each treatment type. Relative quantification (RQ) data of the transformant was analyzed using the Δ Ct method with CFX Manager 3.1 (Bio-Rad) with the WT serving as the calibrator (RQ set to 1).

Evaluation of Transformant Growth

Stability of the transformant was tested on liquid BG11/HEPES media containing 80 mg L⁻¹ ampicillin over a 10-day period under culture conditions described above. Wild type strain grown in the absence of ampicillin served as control. Growth (OD₇₅₀) was measured every 24 h, and stability of the strain confirmed after 32 generations by RNA extraction, cDNA synthesis, and PCR as mentioned above.

Comparison of Wild Type and Transformant Growth and Photosynthetic Pigment Levels

We compared WT and transformant growth over 10 days to determine the effect of SD gene overexpression. Culture conditions were maintained as described above with three biological replicates, and growth rate calculated as doublings per day using the least fitting squares method. The mean growth (OD₇₅₀) and growth rate were calculated, and statistical significance determined using one-way analysis of variance (ANOVA) and Tukey's honest significant differences post hoc test at 95% confidence intervals ($P < 0.05$). The single factor, fixed-effect ANOVA model, $Y_{ij} = \mu + \alpha G_i + \varepsilon_{ij}$, was used where Y is the growth (or growth rate) of strain i and biological replicate j . The μ represents mean growth with adjustments from the effects of strain (αG), and ε_{ij} is the experimental error from strain i and biological replicate j .

We assessed photosynthetic efficacy of the transformant by extracting and quantifying chlorophyll a (chl a), carotenoids, and phycobiliprotein (phycocerythrin (PE), phycocyanin (PC), and allophycocyanin (AP)) pigments. Carotenoid and chl a absorption spectra were measured at A₄₇₀ and A₆₆₅ respectively, while phycobiliproteins were quantified at A₅₆₅, A₆₂₀, and A₆₅₀ to estimate the photosynthetic capacity of the transformed strain [15–17].

Extraction and Quantification of Total Lipids in *F. diplosiphon* WT and Transformant

We compared total lipid content in WT and transformant using chloroform: methanol extraction method and gravimetric analysis based on Folch et al. [18] reported in Wahlen et al. [19]. Three biological replicates were maintained, and the experiment repeated once. Significance among cumulative treatment means was determined using ANOVA and Tukey's honest significant differences post hoc test at 95% confidence intervals ($P < 0.05$). The single factor, fixed-effect ANOVA model, $Y_{ij} = \mu + \alpha S_i + \varepsilon_{ij}$, was used where Y is the total lipid content in strain i and biological replicate j . The μ represents overall total lipid content mean with adjustments from the effects of strain (αS), and ε_{ij} is the experimental error from strain i and biological replicate j .

Gas Chromatography–Mass Spectrometry of Transesterified Product for FAME Analysis

Total lipids in *F. diplosiphon* WT and transformant were extracted and converted to FAMES via direct transesterification, a reaction that combines extraction and in situ biofuel production as described by Wahlen et al. [19] and modified by Tabatabai et al. [10]. We determined FAME composition of transesterified material using a Shimadzu GC17A/QP5050A GC-MS combination (Shimadzu Instruments) at the Mass Spectrometry Facility (Johns Hopkins University, Baltimore, MD) as previously described [5, 20]. GC/MS peaks were identified by comparing mass spectra with the Lipid Web Archive of FAME Mass Spectra. Three biological replicates of each treatment were maintained, and the experiment repeated once. Theoretical chemical and physical properties of the FAME profiles (w%) were calculated using the BiodieselAnalyzer© software Version 2.2 [21].

GC × GC-TOFMS Analysis of Total Transesterified Lipids

High-resolution two-dimensional gas chromatography–time of flight mass spectrometry (GC × GC-TOFMS) was used to identify FAME components. Total lipids from the WT and transformant strains were extracted, subjected to direct transesterification as mentioned above [19], and GC × GC-TOFMS (LECO) carried out as described by Tabatabai et al. [10].

Results

Identification of Sterol Desaturase Homologs in *Fremyella diplosiphon*

Sterol desaturase gene-specific primer amplification revealed a single discrete band at the expected size of 1314 base pairs (bp). PCR product subjected to Sanger sequencing and NCBI BLAST confirmed identity of the SD gene with an open reading frame of 1314 bp and its encoded proteins with 437 amino acids. Sequence alignment identified a 94% match to the SD gene in *F. diplosiphon*, thus confirming the identity of the gene. The SD amino acid sequence revealed 93%, 86%, 77%, and 77% identities to *Nostoc carneum* NIES-2107, *Calothrix* sp. NIES-2100, *Nodularia* sp. NIES-3585, and *Fortiea contorta*, respectively. The SD gene sequence was deposited at NCBI Genbank with an accession number of MH329183.

Cloning and Transformation of Sterol Desaturase Gene in *F. diplosiphon*

Amplified SD gene and pGEM-7Zf (+) vector were successfully double and triple digested, followed by ligation of purified inserts at the corresponding restriction sites. The plasmid containing the SD gene (pGEM-7Zf-SD) was successfully transformed into WT via electroporation and gene insertion confirmed by PCR and Sanger sequencing.

RT-qPCR Analysis Confirms Overexpression of Sterol Desaturase Gene in B481-SD

We observed a significant increase in transcript abundance in the transformant. Relative quantification values revealed a 64-fold increase in SD gene expression of the transformant compared with WT (Fig. 1). The sterol desaturase–overexpressing *F. diplosiphon* strain was designated as B481-SD.

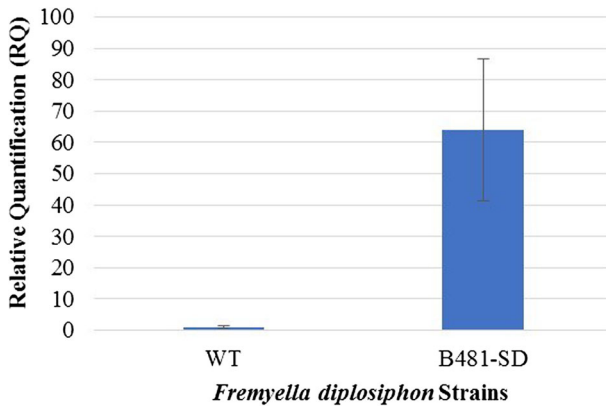


Fig. 1 Reverse transcription–quantitative polymerase chain reaction relative quantification (RQ) of sterol desaturase transcript levels in *Fremyella diplosiphon* wild type (WT) and transformant (B481-SD). Error bars represent the standard error of ΔC_t values at a 95% confidence interval across four replicates

Evaluation of Growth and Photosynthetic Pigment Accumulation in the Transformant

We did not observe significant differences ($P > 0.05$) in growth and growth rate of B481-SD grown in liquid BG11/HEPES medium compared with WT (Figs. 2 and 3). No growth was observed in WT exposed to 80 mg L⁻¹ ampicillin. Concurrent analysis of photosynthetic pigments (Fig. S1 and S2) and phycobiliproteins (Fig. S3) revealed no significant differences ($P > 0.05$) between WT and transformant. We were able to grow the transformant persistently in BG11/HEPES solid media containing 80 mg L⁻¹ ampicillin for 32 generations. Results of PCR revealed a single discrete band at the expected size of 1314 bp for B481-WT as well as B481-SD (Fig. 4).

Comparison of Total Lipid Content in B481-SD and WT by Gravimetric Analysis

We identified a significant increase ($P < 0.05$) in B481-SD total lipid content when compared with the WT (Fig. 5). While 23.6% total lipid content of cellular dry weight was detected in

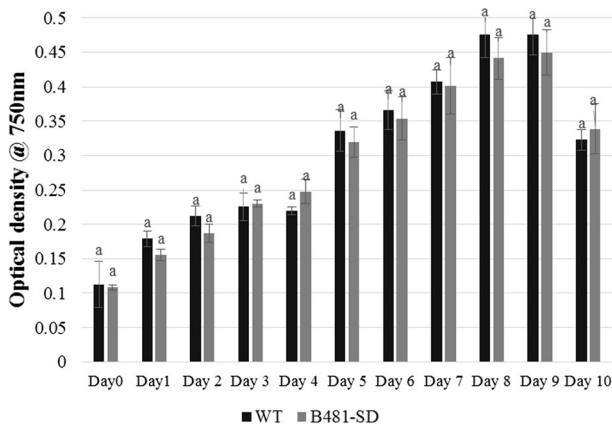


Fig. 2 Growth of *Fremyella diplosiphon* wild type (WT) and transformant (B481-SD) strains in BG11/HEPES medium. Average optical density at 750 nm (\pm standard error) for three biological replicates is shown for each time point. Different letters above final time point indicate significance among treatment means ($P < 0.05$)

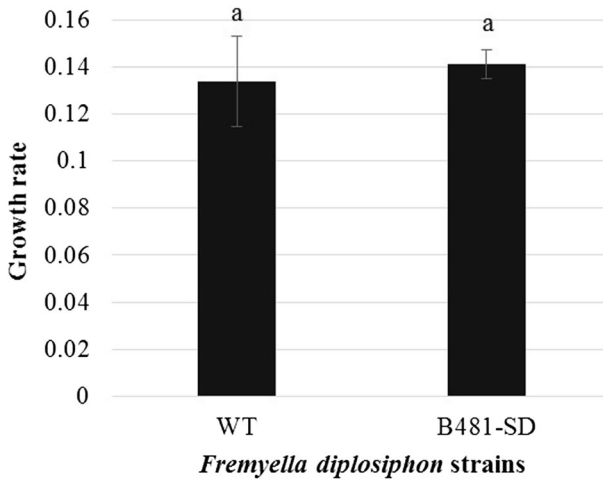


Fig. 3 Effect of sterol desaturase gene overexpression on *Fremyella diplosiphon* wild type (WT) and transformant (B481-SD) growth rate in liquid BG11/HEPES medium over a 9-day period. Growth rate was denoted as doublings per day using the least fitting squares method calculator

B481-SD, 18.5% lipid content was recovered in WT. Thus, a 27.2% increase in B481-SD total lipid content relative to WT was observed.

Characterization of FAMES in WT and B481-SD by GC-MS

We identified methyl palmitate as the dominant FAME component, which accounted for 76.35% and 65.93% of total FAMES produced from WT and B481-SD total lipids respectively (Table 2). In addition, we identified the following FAME species: methyl tetradecanoate (C14:1), methyl hexadecenoate (C16:1), methyl octadecanoate (C18:0), methyl octadecenoate

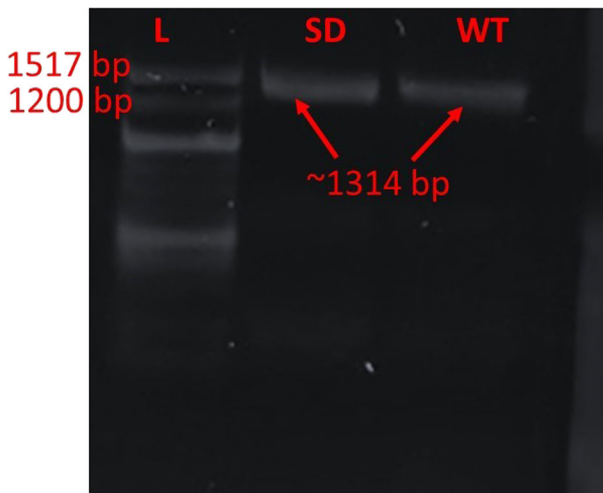


Fig. 4 PCR amplification of sterol desaturase gene in *Fremyella diplosiphon* B481-wild type (WT) and B481-SD after 32 generations with a 100-bp ladder (L)

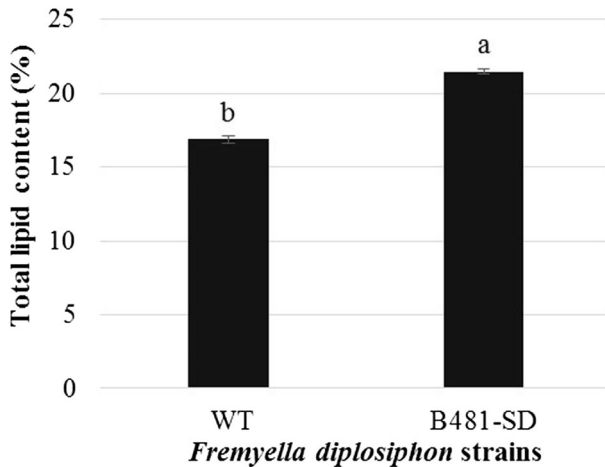


Fig. 5 Comparison of total lipid content in wild type (WT) and transformant (B481-SD) *Fremyella diplosiphon*. Average percentage lipid content of cell dry weight (% w/cdw) (\pm standard error) of three biological replicates for each strain is shown. Different letters above bars indicate significance among treatment means ($P < 0.05$)

(C18:1), and methyl octadecadienoate (C18:2) (Table 3). Results of the study also revealed significant increases ($P < 0.05$) in methyl octadecenoate (C18:1), and methyl octadecadienoate (C18:2) levels in B481-SD transesterified lipids, while no significant differences ($P > 0.05$) were observed in other FAME components (Figs. 6 and 7). Calculation of theoretical chemical and physical properties revealed high cetane number (65.97–67.49) and oxidative stability (50.49–18.67 h) in the FAME produced from B481-SD. In addition, calculated values for density (0.867–0.868 g/cm³), viscosity (3.781–3.817 mm²/s), and iodine (17.723–25.590 g I₂/100 g) (Table 4) were higher than minimum American and European fuel standards [22].

Lipid Characterization in B481-SD by GC \times GC-TOFMS

In addition to conventional GC-MS, we used high-resolution GC \times GC-TOFMS to further separate polar, aromatic compounds. GC \times GC-TOFMS analysis revealed the presence of FAMES with carbon number from 12 to 18, as well as alkanes from C11 to C34. We observed FAME composition in B481-SD (80.92% transesterified lipids) to be significantly higher ($P < 0.05$) than WT (77.92% transesterified lipids) (Figs. 8 and 9). FAME components such as C12:0, C15:0, C18:3, and C18:4 not detected in conventional GC-MS were identified by GC \times GC-TOFMS.

Table 2 Saturated and unsaturated fatty acid methyl ester (FAME) proportions in wild type (WT) and transformant (B481-SD) *Fremyella diplosiphon*

Strain	FAME type (%)		Ratio of FAME
	Saturated	Unsaturated	Saturated/Unsaturated
WT	80.99	19.01	4.26
B481-SD	76.62	23.38	3.27

Table 3 Quantitative composition of fatty acid methyl ester in transesterified lipids of *Fremyella diplosiphon* wild type (WT) and transformant (B481-SD) strains

	WT				B481-SD			
	:0 [†]	:1	:2	Sum	:0	:1	:2	Sum
C14*	–	3.07	–	3.07	–	3.21	–	3.21
C16	72.39	11.66	–	84.05	65.67	7.67	–	73.34
C18	8.60	1.79	2.49	12.88	10.95	5.16	7.34	23.45
Sum	80.99	16.52	2.49	100	76.62	16.04	7.34	100

*Column represents length of the carbon chain

[†] Row represents degree of saturation (number of double bonds in chain)

Discussion

Although there is a great potential of cyanobacterial-based biofuels, major hurdles in terms of strain improvement and oil productivity exist before these fuels can be deployed into use. Efforts to overcome these challenges will significantly reduce the cost and progress the use of biofuel from promise to reality [9, 11, 12]. In the present study, overexpression of the SD gene in *F. diplosiphon* via homologous transformation resulted in a significant increase in total lipid content and essential unsaturated fatty acid (FA) composition. Homology of the cDNA-encoding gene exhibited high similarities (93% and 86%) to published sequences of these proteins in other cyanobacterial species, confirming their functional importance in lipid biosynthesis pathways. Quantification of gene expression in the transformant revealed a 64-fold increase in mRNA transcript levels (Fig. 1), suggesting successful integration of the pGEM-7Zf-SD plasmid in *F. diplosiphon*, thereby enhancing the robustness of the organism to produce lipids. A patent application describing this approach to enhance total lipid content and essential unsaturated fatty acid (FA) composition has been filed (US Patent Application No.: 16/123,484; September 6, 2018).

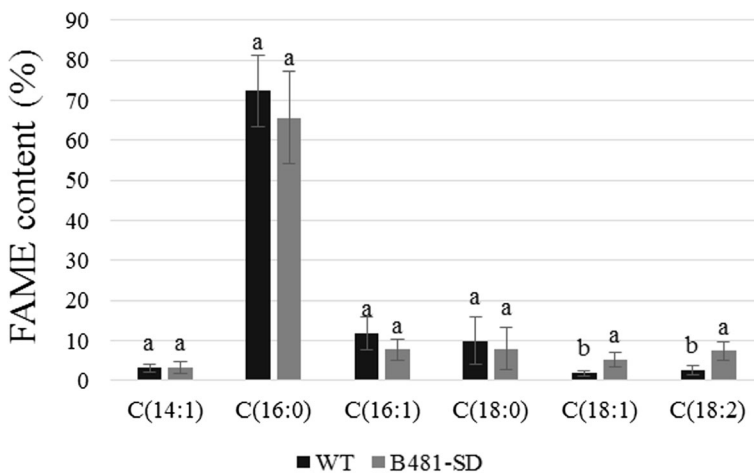


Fig. 6 Comparison of fatty acid methyl ester (FAME) composition of wild type (WT) and transformant (B481-SD) *Fremyella diplosiphon* total lipids subjected to direct transesterification. Average percent FAME content (\pm standard error) for three biological replicates of each strain is shown. Different letters above bars indicate significance among treatment means ($P < 0.05$)

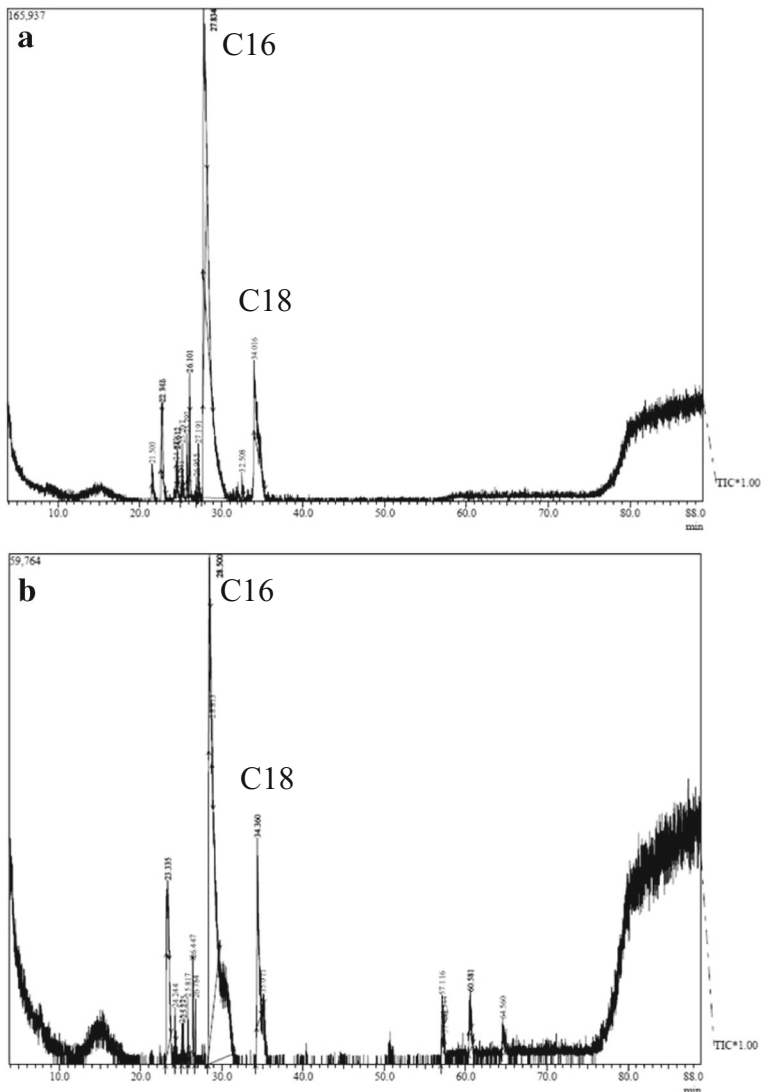


Fig. 7 Representative one-dimensional gas chromatogram of *Fremyella diplosiphon* strains **a** wild type (WT) and **b** transformant (B481-SD) total lipids subjected to direct transesterification

Comparison of growth and growth rate of B481-SD and WT did not reveal significant differences indicating that overexpression of the SD gene was not detrimental to the organism (Figs. 2 and 3). In addition, unaltered growth of the transformant and a clear single band at the expected size of 1314 bp after 32 generations confirmed stable integration of the gene in the pGEM-7Zf-SD plasmid. Further, we did not observe differences in pigmentation levels, confirming that photosynthetic activity of the transformant was unaltered by the transformation process (Fig. S1, S2, and S3). It is known that lipid production and fatty acid composition are impacted by cellular photosynthetic pathways in cyanobacteria [23], which indicate a direct correlation between cyanobacterial lipid and photosynthetic pigments. This antioxidative

Table 4 Theoretical biodiesel properties of *Fremyella diplosiphon* wild type (WT) and transformant (B481-SD) transesterified lipids

Biodiesel properties	WT	B481-SD
Saponification value (mg KOH/g fat)	216.743	214.627
Iodine value (g I ₂ /100 g)	17.723	25.590
Cetane number	67.494	65.972
Long-chain saturated factor	11.540	12.044
Cold filter plugging point (°C)	19.771	21.361
Cloud point (°C)	33.087	29.550
Pour point (°C)	29.097	25.258
Allylic position equivalent	6.722	19.836
Bis-allylic position equivalent	2.462	7.336
Oxidation stability (h)	50.493	18.666
Higher heating value (MJ/kg)	39.208	39.259
Kinematic viscosity (mm ² /s)	3.781	3.817
Density (g/cm ³)	0.867	0.868

property of photosynthetic pigments is known to assist in a cellular protective response to oxidative stress induced by SD gene overexpression [7, 8, 24]. This protective mechanism has been known to significantly reduce reactive oxidative stress up to 82% in the cyanobacterium *Spirulina platensis* [24].

In cyanobacteria, sterol desaturase enzymes assist in biochemical processes such as reduction of NADP⁺ to NADPH [8] and lipid biosynthetic pathways inducing oxidative stress leading to increased lipids, specifically unsaturated fatty acids [25]. There were no significant differences in saturated FAs such as palmitic acid (C16:0) and octadecanoic acid (C18:0) between the WT and B481-SD; however, 8% and 65%

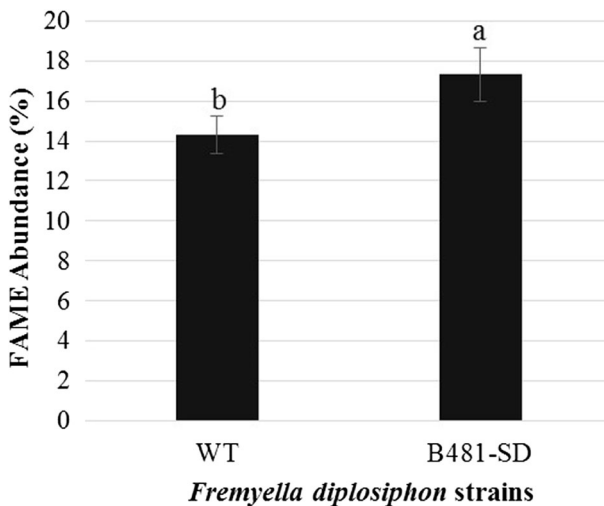


Fig. 8 Fatty acid methyl ester (FAME) composition in transesterified extractable lipids of wild type (WT) and transformant (B481-SD) *Fremyella diplosiphon* strains determined using GC × GC-TOFMS. Average percent FAME content (± coefficient of variation) of each strain for three biological replicates is shown. Different letters above bars indicate significance among treatment means ($P < 0.05$)

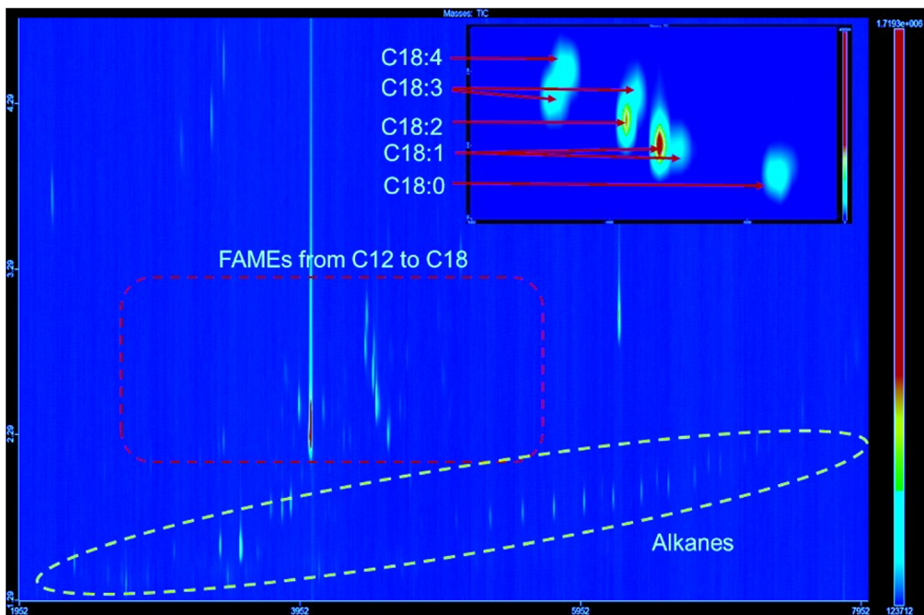


Fig. 9 Representative GC \times GC-TOFMS chromatogram of *Fremyella diplosiphon* transformant (B481-SD) fatty acid methyl esters (FAMES), alkanes, and other components. Compounds were separated by volatility which correlates to molecular weight (carbon number) on the first dimension and polarity which correlates to the degree of unsaturation (number of double bonds) on the second dimension

reduction of these components was observed in the transformant suggesting that SD gene overexpression could be attributed to the desaturation of these components [26]. In addition, we detected methyl octadecadienoate (C18:1) and methyl octadecadienoate (C18:2), which are two most abundant desaturated FAME components known to yield high-quality biofuel. It has been reported that overexpression of the SD gene could increase unsaturated fatty acid levels such as oleic acid (C18:1) in *Saccharomyces cerevisiae* and *Nicotiana tabacum* BY-2 cells [7, 27]. We observed various high-value components such as FAMES and alkanes using high-resolution GC \times GC-TOFMS that were not identified by GC-MS, confirming additional benefits of *F. diplosiphon*-derived biodiesel.

Conclusion

This is the first report of SD overexpression resulting in enhanced lipid content and essential unsaturated fatty acids such as oleic and linoleic acids in *F. diplosiphon*, indicating that this approach is ideal to maximize biofuel production capacity of the organism. These findings offer a novel pathway for a highly efficient and cost-effective renewable fuel production system and pave the way for *F. diplosiphon* as a more efficient production-level agent. Future efforts will be aimed towards optimizing large-scale cultivation and lipid extraction/conversion to determine the viability of *F. diplosiphon* B481-SD for efficient and economical biofuel production.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Affiliations

Somayeh Gharai Fathabad¹ · AnithaChristy S. Arumanayagam² · Behnam Tabatabai¹ · Huan Chen³ · Jie Lu^{3,4} · Viji Sither¹

¹ Department of Biology, Morgan State University, 1700 East Cold Spring Lane, Baltimore, MD 21251, USA

² Department of Pathology, Methodist Hospital Research Institute, 6670 Bertner Ave, Houston, TX 77030, USA

³ National High Magnetic Field Laboratory, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310, USA

⁴ Future Fuels Institute, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310, USA