



Final Report

Isolation of starchless mutants of the alga *Dunaliella tertiolecta*

for enhanced biodiesel production

By Anchalee Sirikhachornkit

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Researcher

Institute

Anchalee Sirikhachornkit

Kasetsart University

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Abstract

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Investigator : Anchalee Sirikhachornkit

E-mail Address : fscialsk@ku.ac.th

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Abstract:

The production cost of biodiesel from microalgae is still not competitive, compared to that of petroleum fuels. The genetic improvement of microalgal strains to increase triacylglycerol (TAG) accumulation is one way to reduce production costs. One of the most promising approaches is the isolation of starch-deficient mutants, which has been reported to successfully increase TAG yield. To date, such a stable mutant is not available in an oleaginous marine microalga, despite several advantages of using marine species for biodiesel production. Algae in the genus *Dunaliella* are known to tolerate high salt concentration and other environmental stresses. In addition, the cultivation processes for large-scale outdoor commercialization have been well established for this genus. In this study, *Dunaliella tertiolecta* was used to screen for starch-deficient mutants, using an iodine vapor-staining method. Four out of 20,016 UV-mutagenized strains showed a substantial reduction of starch content. A significantly higher TAG content, up to three folds of the wild-type level, was observed in three of the mutants upon induction by nitrogen depletion. The carotenoid production and growth characteristics of these mutants, under both normal and oxidative stress conditions, were not compromised, suggesting that these processes are not necessarily affected by starch deficiency. The results from this work open up new possibilities for exploring *Dunaliella* for biodiesel production.

Keywords : *Dunaliella*; biodiesel; microalgae; starch; triacylglycerol

บทคัดย่อ:

ต้นทุนการผลิตไบโอดีเซลจากสาหร่ายขนาดเล็กยังไม่สามารถแข่งขันกับการผลิตเชื้อเพลิงปิโตรเลียมได้ ดังนั้นการปรับปรุงสายพันธุ์สาหร่ายให้มีปริมาณไตรเอซิลกลีเซอรอลมากขึ้นเป็นทางหนึ่งในการลดต้นทุนการผลิต หนึ่งในวิธีปรับปรุงพันธุ์คือการคัดแยกพันธุ์กลายที่มีแป้งลดลงซึ่งได้มีการรายงานว่าสามารถทำให้ปริมาณไตรเอซิลกลีเซอรอลเพิ่มขึ้นได้แต่ยังไม่เคยมีการรายงานสายพันธุ์ลักษณะดังกล่าวในสาหร่ายน้ำเค็มที่มีการสะสมไขมันได้สูงถึงแม้สาหร่ายน้ำเค็มจะมีข้อได้เปรียบหลายประการในการใช้ผลิตไบโอดีเซล สาหร่ายในจีนัสดูนาเลียเอลลาสามารถทนความเข้มข้นเกลือสูงและสภาวะความเครียดจากสภาวะแวดล้อมต่าง ๆ ได้ อีกทั้งระบบการเพาะเลี้ยงขนาดใหญ่ในเชิงพาณิชย์ก็ได้รับการพัฒนามาแล้วสำหรับสาหร่ายจีนัสนี้ ในงานวิจัยนี้จึงใช้สาหร่าย *Dunaliella tertiolecta* ในการคัดเลือกพันธุ์กลายที่มีแป้งน้อยลงด้วยวิธีการย้อมด้วยไอโอดีน และจากตรวจสอบพันธุ์กลายที่ได้จากการฉายรังสียูวีทั้งหมดจำนวน 20016 สายพันธุ์ พบ 4 สายพันธุ์ที่มีแป้งน้อยลง และพบว่าสามสายพันธุ์มีปริมาณไตรเอซิลกลีเซอรอลมากขึ้นอย่างมีนัยสำคัญถึงสามเท่าเทียบกับปริมาณในสายพันธุ์ปกติเมื่อเซลล์อยู่ในอาหารขาดไนโตรเจน ปริมาณแคโรทีนอยด์และการเจริญในพันธุ์กลายไม่มีความแตกต่างจากสายพันธุ์ปกติไม่ว่าจะเป็นในสภาวะปกติหรือสภาวะที่มีความเครียดออกซิเดทีฟ ซึ่งหมายถึงว่ากระบวนการดังกล่าวไม่ได้รับผลกระทบจากการที่มีแป้งลดลง ผลการทดลองจากงานวิจัยนี้ช่วยเปิดโอกาสการศึกษาเกี่ยวกับสาหร่ายจีนัสนี้เพื่อการผลิตไบโอดีเซล

คำสำคัญ: ดูนาเลียเอลลา ไบโอดีเซล สาหร่ายขนาดเล็ก แป้ง ไตรเอซิลกลีเซอรอล

Executive Summary

Microalgae have emerged as one of the most promising sources of biofuel feedstock, owing mainly to their ease of cultivation, their ability to accumulate high lipid content, and their reduced land area requirement compared to plant-based fuels. Several strains have been found to accumulate neutral lipids, in the form of triacylglycerol (TAG), at up to 50% of dry cell weight [14]. TAG could be extracted from the cells for subsequent production of biodiesel via a transesterification reaction. However, at present, the algal biofuel is still a long way from commercialization, largely due to its high production cost. To help make it possible, a large number of studies have been performed on various aspects of microalgal biodiesel production in recent years [25]. These studies have collectively led to an immense improvement in the cultivation, harvesting, dewatering, and lipid extraction processes [5]. However, there have only been a few reported achievements in the genetic improvement of algal lipid content.

Although many natural oleaginous strains of microalgae have been discovered, most of the strains with exceptional lipid content grow slowly and/or are difficult to cultivate under outdoor conditions, resulting in low biomass and lipid productivity. Therefore, improving the lipid content of robust strains and/or strains with cultivation technology already in place remains a favorable option. The genetic improvement of microalgal strains for biodiesel production has been carried out using several approaches. However, these attempts have resulted in limited success and are restricted to a few strains that are amenable to genetic transformation. For example, the straightforward metabolic engineering approach to induce the overexpression of the *ACC*ase gene—encoding acetyl-CoA carboxylase, which catalyzes the first committed step of fatty acid synthesis—could not sufficiently increase lipid production, probably due to the complex regulation of the pathway [12]. The overexpression of the genes encoding different isoforms of acyl-CoA—diacylglycerol acyltransferase (DGAT), which catalyzes the last step of TAG formation—did not lead to higher TAG production [17]. On the contrary, genetic modifications designed to inhibit pathways that compete with lipid biosynthesis appear to have met with the most success. An RNA interference approach designed to silence the gene that encodes citrate synthase, which mediates the entrance of acetyl-CoA into the tricarboxylic cycle, led to a *Chlamydomonas reinhardtii* strain that showed a near two-fold increase in its TAG content [11]. Targeting TAG catabolism by down-regulating a lipase, via antisense RNA in *Thalassiosira pseudonana*, also led to a higher lipid content with no growth compromise [37].

Starch and TAG are the main storage compounds of plants and algae. Since the synthesis of starch and that of lipids share common precursors, blocking starch synthesis has been hypothesized as a way to improve TAG yield. Various starch-deficient strains of *Chlamydomonas reinhardtii* have

been isolated or constructed to carry a mutation in one of the starch biosynthesis genes [1, 26, 41]. Many of these mutants were shown to accumulate a several-fold increase in TAG content, although it remains unclear whether the TAG overaccumulation in these mutants could be attributed to the starch synthesis deficiency [32]. Several starchless mutants of *Scenedesmus obliquus* were isolated [10], and the TAG content was significantly enhanced, from 45% per dry weight in the wild type to 57% in the most promising strain [6]. The screen used for isolating randomly mutagenized microalgal strains that are deficient in starch accumulation is straightforward and well described, and does not involve genetic transformation, provided that the algal genome is haploid (i.e., not genetically redundant). However, this approach has not yet been commonly applied in order to improve the lipid content of algal strains that are sufficiently robust for large-scale commercial cultivation.

Many algal species in the genus *Dunaliella* are common in marine habitats, with some exhibiting tolerance to high salinity and extreme environments. The large-scale commercial cultivation of *D. salina* is already in operation to produce natural β -carotene [28, 33]. In addition to their rapid growth rate, the ability of *Dunaliella* to thrive in high-salinity culture provides a selective advantage, as high-salt concentration reduces the likelihood of contamination [30]. The present study focuses on *D. tertiolecta*, which is known as a potential candidate for biodiesel feedstock, due to its ability to grow rapidly in various environments, along with its high lipid accumulation [34, 35]. Transcriptome data for *D. tertiolecta* under nitrogen-starvation conditions are also available, to facilitate metabolic engineering [31]. To further improve its TAG content, we explored the consequence of starch synthesis inhibition via random mutagenesis. Starch-deficient mutants were screened from a population of UV-mutagenized *D. tertiolecta* strains, and were characterized by their growth and biochemical content. Results showed that some of the mutants accumulated significantly higher TAG level with no measurable effects on growth, photosynthesis, and carotenoid content, rendering them suitable for further development regarding biodiesel production.

Objective

To increase the triacylglycerol content in *Dunaliella tertiolecta* by screening for starch-deficient mutants

Research Methodology

Strain and growth conditions

The *D. tertiolecta* strain UTEX LB999 (UTEX Culture Collection of Algae, University of Texas at Austin) culture was routinely maintained on a hypersaline medium [36]. Unless otherwise indicated, cultures were grown at 25°C under continuous illumination of approximately 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ cool-white fluorescent light. For the medium without nitrogen, the medium was

prepared by substituting KNO_3 with KCl. Liquid cultures were aerated by bubbling with normal air at an aeration rate of 1 vvm. For phenotypic testing on plates under high light conditions, and in the presence of chemicals, log-phase cells were collected by centrifugation and cell density was adjusted as indicated. Three microliters were spotted onto plates and the plates were kept at the indicated conditions for 10 days.

Mutagenesis and mutant isolation

For UV mutagenesis, *D. tertiolecta* cells—at a density of 5×10^6 cells ml^{-1} in a glass petri dish—were kept in the dark for at least two hours, before being exposed to UV irradiation using a UV transilluminator (Model M-26; Upland, USA) for 22 minutes, to obtain a 1% survival rate. UV-irradiated cells were immediately wrapped in foil and allowed to recover overnight in the dark to prevent light-induced DNA repair. Cells were then spread onto agar plates and grown at $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Single colonies were transferred to fresh agar plates. After approximately two weeks, cells were transferred to agar medium without nitrogen, using a replicator. After approximately three weeks, when cells started to turn yellow, they were stained with iodine vapor. The *C. reinhardtii sta1-1* mutant [1] growing on TAP-N plates was patched onto each plate prior to iodine staining, as a control. Cell patches that exhibited a substantially lighter color than that of the wild-type control on each plate were selected for further analysis.

Starch, lipid and triacylglycerol quantification

For preliminary characterization of the isolated mutants, mid-log phase cells in liquid medium were collected, washed once with medium without nitrogen, and inoculated into fresh medium—with or without nitrogen—for an additional two days. The cells were then harvested, kept at -80°C and lyophilized at -50°C . Twenty to thirty milligrams of each sample were sonicated for 15 minutes and used for starch analysis using the Total Starch (AA/AMG) assay kit from Megazyme (Ireland), according to the manufacturer's protocols for starch samples that also contain D-glucose.

Similar amounts of the lyophilized cells were also used for lipid extraction in chloroform:methanol (1:2 v/v), according to the protocol from Bligh and Dryer [2]. Total lipid was determined on a pre-weighted aluminum foil. Two-hundred micrograms of total lipid were spotted onto a 20 X 20 cm silica gel 60G F254 TLC plate (Merck, Germany). Plates were developed in hexane:diethyl ether:acetic acid (70:30:1) for 45 minutes and stained with iodine vapor. TAG bands were identified using standard soy oil and quantified by a densitometry method using the ImageJ program [29], in comparison to a standard curve generated from varied soy oil concentrations.

For gas chromatography (GC) analysis of the lipids produced by the *sds1*, *sds3*, and *sds4* mutants, the algal strains were inoculated in 500 ml Erlenmeyer flasks containing 450 ml liquid

medium at the initial optical density at 680 nm (OD_{680}) of 0.1 and maintained under continuous illumination at $700 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. After 5 days, the cells were collected, washed once with medium without nitrogen, and transferred into fresh medium without nitrogen for additional 2 days. Final cell density was determined by manual counting under a hemocytometer. Subsequently, the cultures were harvested by centrifugation, washed once with 1.5 M NH_4HCO_3 , and lyophilized. Dry biomass yield was measured gravimetrically, and 40 mg of each sample were used for lipid extraction as described above. One half of the crude lipid extract was weighted to determine total lipid yield, whereas the other half was quantified as fatty acid methyl esters (FAMES) following a published protocol with modification [8]. First, 0.1 mg of glyceryl triheptadecanoate (catalog no. T2151, Sigma) was added as an internal standard. Next, for purification of the TAG constituents, the crude extract was dissolved in 400 μl chloroform, and one half of this amount was loaded into a VertiPak™ Si solid-phase-extraction (SPE) cartridge containing 300 mg silica (Vertical Chromatography, Thailand) and pre-washed with 4 ml hexane. The TAG-enriched neutral lipids were eluted by 2 ml hexane:diethyl ether (83:17, v/v). Purity of the eluate was assessed by TLC in comparison to the crude lipid extract, using a two-phase mobile solvent system consisting of hexane:diethyl ether:acetic acid (70:30:1, v/v/v) followed by chloroform:methanol:water (65:25:4 v/v/v). After solvent evaporation, the purified and non-purified lipids were transesterified using 2 ml of 5% sulfuric acid in methanol under 90 °C for 120 min. After addition of 2 ml of hexane containing 0.01% (w/v) butylated hydroxytoluene (BHT), the reaction was washed with deionized water, and trace water was removed by anhydrous Na_2SO_4 . For GC analysis using Agilent 6890 N (Agilent Technologies, USA) coupled with Agilent 5973 Network Mass Selective Detector (Agilent Technologies, USA), 1 μl of the derivatized FAMES in hexane was injected into a capillary HP-Innowax column (Agilent Technologies, USA; 30 m x 0.25 mm x 0.25 μm). The oven temperature was programmed from 45 °C (1 min hold) to 180 °C at a rate of $50 \text{ }^\circ\text{C min}^{-1}$ and increased to 260 °C at a rate of $2 \text{ }^\circ\text{C min}^{-1}$. Helium was used as a carrier gas with a split ratio of 1:1. The corresponding amount of each FAME was calculated by comparison of the peak area to that of methyl heptadecanoate.

Analysis of growth and photosynthetic oxygen evolution rate

Inoculum cells were cultured in 5 ml liquid medium for two days and then transferred to 50 ml liquid medium to give an initial density at OD_{680} of 0.05. The cultures were continuously illuminated at $700 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and the culture density was monitored over a course of six days. For oxygen evolution measurements, cells were grown at $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ to mid-log phase and the density adjusted to OD_{680} of 0.1. Cells were dark-adapted for 15 minutes prior to measurements with a polarographic, Clark-type oxygen electrode - OX1LP Dissolved Oxygen Package (Qubit

Systems, Canada) at 23°C.

Pigment quantification

Cells were grown in liquid medium at 700 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ to a density of $1\text{--}2 \times 10^6$ cells ml^{-1} . Cells were collected, washed once with medium without nitrogen, and inoculated into fresh medium—with or without nitrogen—for an additional two days. Four milliliters of culture were harvested and used for pigment extraction in 80% acetone, and the analysis of carotenoid content was performed as previously described [24].

RNA extraction and real time RT-PCR analysis

Cells were grown in liquid medium to mid-log phase before being collected, washed once with medium without nitrogen, and inoculated into fresh medium—with or without nitrogen—at a density of 2×10^6 cells ml^{-1} for additional two days. Total RNA was extracted using GENEzol™ TriRNA Pure Kit (Geneaid, Taiwan) and the concentration was determined spectrophotometrically. Genomic DNA was eliminated by treatment with RQ1 RNase-free DNase (Promega, USA) according to the manufacturer's protocol. One and half microgram of total RNA was used for cDNA synthesis using MMuLv reverse transcriptase (Biotechrabbit, Germany). Specific primers targeting genes involved in starch synthesis and metabolism were designed based on RNA-seq and annotation data [31]. The primer sequences and PCR product sizes are shown in Table 1.

Real-time PCR was performed on a Mastercycler ep realplex 4 (Eppendorf, Germany) using QPCR Green Master Mix (Biotechrabbit, Germany). Each reaction contained 30 ng of total RNA, 5 pmol of each primer, 10 μl of QPCR Green Master Mix, in a final volume of 20 μl . The PCR cycle was 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 69°C for 30 s and 72°C for 30 s. For each sample, the PCR was carried out in triplicate. A melting curve program was employed to confirm the presence of only one specific PCR product. Relative gene expression was calculated by the $2^{-\Delta\Delta\text{CT}}$ method using *RACK1* as a reference gene.

Results

Screening for starch-deficient mutants

Four putative starch-deficient strains (*sds*) were identified from a screen of 20,016 UV-mutagenized *D. tertiolecta* strains. Whereas the parental wild-type strain turned dark purple after iodine-vapor staining, the *sds1* and *sds3* mutants appeared as light brown to purple and the *sds2* and *sds4* mutants remained yellowish (Fig. 1A). To confirm that the mutants had a lower starch accumulation, their starch content was quantified in cells grown in nitrogen-replete (N-replete) and

nitrogen-depleted (N-depleted) liquid medium. In the N-replete medium, there was no significant difference in the starch contents between the mutants and the wild type, as all strains showed around 2-6% starch per dry weight (Fig. 1B). In the N-depleted medium, however, the starch content in the wild type increased to 21% per dry weight while this number ranged from 6 to 16% in the mutants: a significantly lower starch content. The starch contents also correlated well with the color intensity after iodine staining, as the *sds2* and *sds4* mutants exhibited the lowest starch content: 8% and 6% respectively. These results confirm that these *D. tertiolecta* strains were deficient in starch accumulation and the iodine-staining method could be used in rapid screening for such mutants.

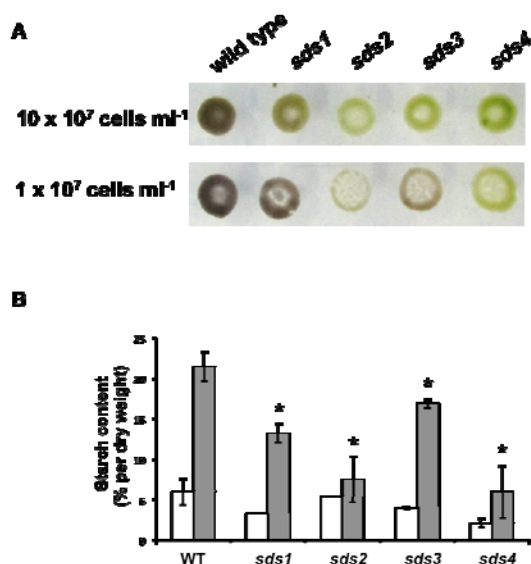


Fig. 1. Mutant screening and starch measurement. (A) Appearance of cells after iodine staining. Different densities of cell cultures were spotted onto agar medium without nitrogen for two weeks before staining. (B) Starch content of each strain. Values are from three biological experiments. Error bars are standard deviations. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($P < 0.05$) between the mutant and the wild type. Light bars, + N; dark bars, - N.

Determination of total lipid, TAG and carotenoid content

To determine whether the *sds* mutants accumulated a higher amount of lipid, the total lipid content was quantified gravimetrically in cells grown under the N-replete and N-depleted conditions. In general, the total lipid contents of all strains were slightly lower when grown in the N-depleted medium than in the N-replete medium (Fig. 2). Under both conditions, however, the lipid contents of

the mutants were not significantly different from those of the wild type, except *sds3* under N-replete condition.

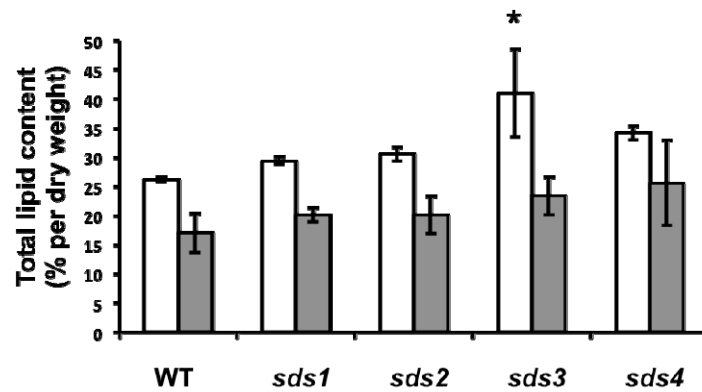


Fig. 2. Lipid content of wild type and mutant *D. tertiolecta* strains under N-replete and N-depleted conditions. Values are from three biological replicates. Error bars are standard deviations. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($P < 0.05$) between the mutant and the wild type. Light bars, + N; dark bars, - N.

Because both starch and TAG serve as the carbon and energy storage compounds in algae, and their biosynthesis pathways share common precursors, the blockade of starch biosynthesis may have a more prominent effect on the TAG content than it does on other classes of lipid. To test this possibility, equal amounts of the lipid extracts were separated in TLC to determine the amount of TAG. From the TLC plate, it was clear that the TAG constituents were enriched to a much greater extent in the lipid extracts from the *sds1*, *sds3* and *sds4* mutants under the N-depleted condition, compared to the wild type (Fig. 3A). However, there was no detectable TAG in the lipid extract from *sds2*. Quantification of the TAG spots confirmed that the three mutants had a significantly improved TAG content, at an increase of approximately 1.5-2 folds compared to the wild type (Fig. 3B).

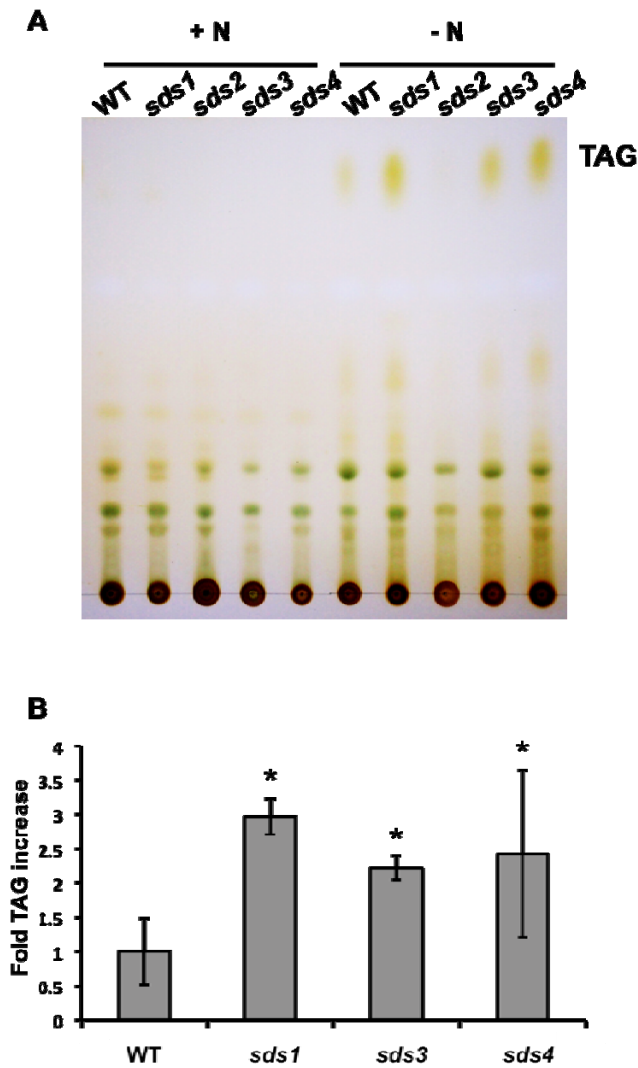


Fig. 3. TAG content of wild type and mutant *D. tertiolecta* strains under N-replete and N-depleted conditions. (A) Thin-layer chromatography (TLC) showing TAG from cells grown under N-replete and N-depleted conditions. (B) TAG content quantified from TLC chromatogram. Values are from three biological replicates. Error bars are standard deviations. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($P < 0.05$) between the mutant and the wild type.

Carotenoids are high-value products of microalgae that have a close relationship with TAG formation [18]. The synthesis of TAG and that of carotenoids are coupled, since carotenoids are esterified with TAG and sequestered in lipid bodies, which are distributed to the cell periphery to protect cells from oxidative damage caused by excess light [14]. In *D. bardawil*, which overproduces β -carotene under stress conditions, the inhibition of lipid synthesis also blocked the overproduction

of β -carotene, indicating that the lipid droplet is used as a sink for carotenoids [27]. Furthermore, the starchless mutant of *S. obliquus* has also been suggested to contain a higher carotenoid level than the wild type in nitrogen-depleted conditions [6]. Therefore, it might be possible that high TAG content in starchless mutants may also lead to carotenoid overaccumulation. To investigate the effect of high TAG content on carotenoid level, cells grown at $700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ under both N-replete and two days of N-depleted conditions were used for carotenoid measurements. The results showed that all starch deficient strains exhibited similar levels of carotenoids as the wild type (Fig. 4). Moreover, nitrogen depletion did not cause a significant change in total carotenoid level, except in *sds4* whose carotenoid level slightly decreased.

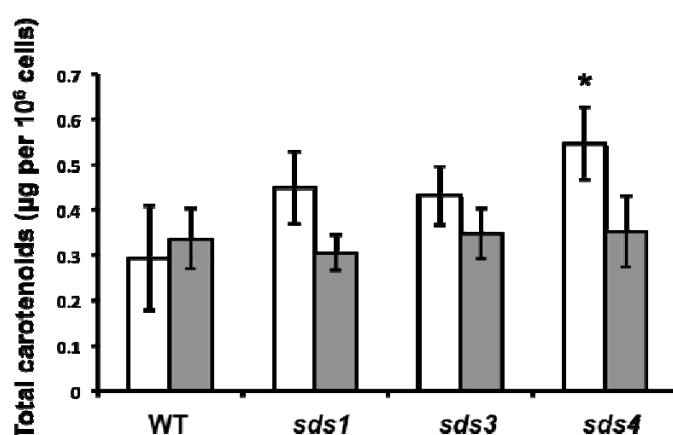


Fig. 4. Total carotenoid content of wild type and mutant *D. tertiolecta* strains under N-replete and N-depleted conditions. Values are from three biological replicates. Error bars are standard deviations. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($P < 0.05$) between the mutant and the wild type. Light bars, + N; dark bars, - N.

Growth and photosynthesis

Some of the algal starch-deficient strains, such as the *C. reinhardtii* *sta6* mutant, are reported to have growth impairments and an increased susceptibility to photoinhibition [22, 23]. Reduced growth and photosynthesis efficiency are important factors to consider in commercial outdoor cultivation. To evaluate whether the mutations in these *D. tertiolecta* starch-deficient mutants might lead to the impairment of growth and photosynthetic characteristics, growth performance was monitored over the course of six-days' cultivation under moderate light intensity, at $700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Because the *sds2* mutant did not show an improved TAG content, it was not included in this and further studies. On the first day, the *sds1* and *sds3* exhibited slightly slower growth when compared to the wild type (Fig. 5A). From the second day onward, only *sds3* showed a lower biomass

compared to other strains. Nevertheless, all strains entered a stationary phase with no significant difference among them, suggesting that the mutations did not cause significant aberrations to the growth of these algal strains in this condition. Additionally, the photosynthetic performance—as determined by light-saturation curves of photosynthesis—followed a similar trend to the growth kinetics (Fig. 5B). Although the mutants seemed to have lower average values for each data point, there were no statistically significant differences between any of the strains at any of the tested light intensities.

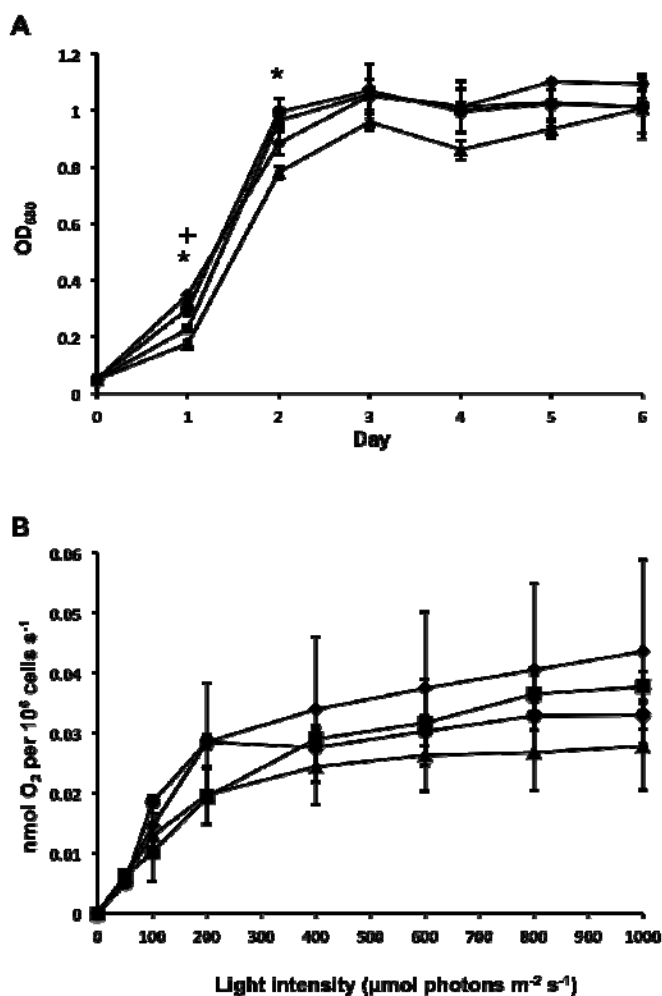


Fig. 5. Growth under 700 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (A) and oxygen evolution rates (B) of wild type and mutant *D. tertiolecta* strains. Values are from three biological replicates. Error bars in (A) are standard deviations. Error bars in (B) are standard errors. A plus sign (+) and an asterisk (*) indicate a significant difference calculated by one-way ANOVA ($P < 0.05$) between the *sds1* and the wild type, and between the *sds3* and the wild type, respectively. Diamonds, wild type; squares, *sds1*; triangles: *sds3*; circles: *sds4*.

Under adverse conditions, both starch and TAG are believed to accumulate as sinks for excess electrons, which could lead to harmful ROS formation. For example, the lack of starch in the *C. reinhardtii* *sta6* mutant resulted in a higher sensitivity to photoinhibition [16]. Similarly, the *pgd1* mutant of *C. reinhardtii*, which failed to accumulate the normal amount of TAG, also accumulated more TBARS than the wild type under nitrogen-starvation conditions, implying a higher amount of ROS was produced [21]. In outdoor cultivation, oxidative stress is unavoidable due to uncontrolled light intensity. Nitrogen starvation has also been shown to induce oxidative stress upon illumination [42]. Therefore, to examine the effect of reduced starch synthesis on growth under oxidative stress conditions, cells were subjected to high light intensity at $1,200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ or to low light intensity at $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, in the presence of 5 mM H_2O_2 or the presence of 5 μM Rose Bengal: a singlet oxygen generator. High light intensity did promote the growth of all strains, except *sds3*, which proved very sensitive to high light, as it failed to grow at a lower cell density (Fig. 6). Under the presence of H_2O_2 , *sds4* seemed to grow slightly slower than the other strains. On the other hand, all stains grew as well as the wild type in the presence of Rose Bengal.

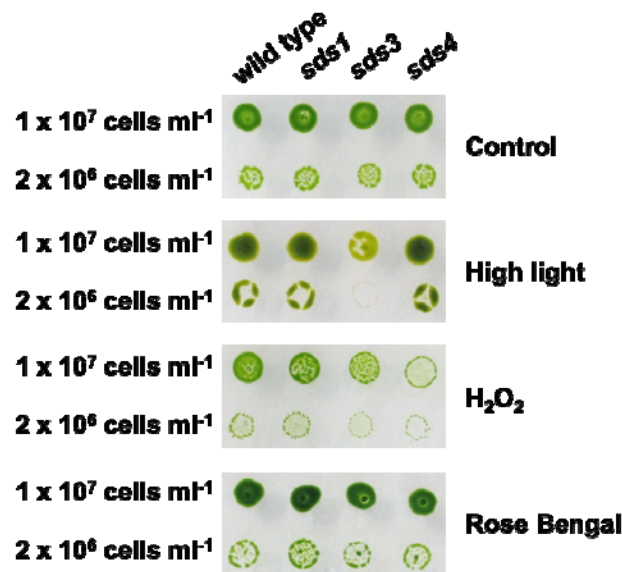


Fig. 6. Growth phenotype of wild type and mutant *D. tertiolecta* strains under high light ($1,200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), 5 mM H_2O_2 , and 5 μM Rose Bengal.

Expression profile of starch synthesis genes

To evaluate whether the mutations in the *sds* mutants affect the expression of genes involved in starch biosynthesis and metabolism, quantitative RT-PCR was employed to monitor the transcript abundances of the genes involved. Genes encoding phosphoglucomutase (PGM), small and large subunits of ADPglucose pyrophosphorylase, several starch synthases, branching enzymes,

isoamylase, and starch phosphorylase were identified in the *D. tertiolecta* RNA-seq and annotation data [31]. Gene expression profiling of all genes would provide a better understanding of the nature of these mutants. Nevertheless, partly due to short sequence length and the presence of multiple members in each gene family, no suitable primers for RT-PCR analysis of some genes were obtained despite several attempts. In the end, five genes were successfully analyzed by quantitative RT-PCR (Fig. 7A and 7B). The steps where these genes may be involved in the starch biosynthesis and metabolism are shown in Fig. 7C. Generally, expression levels of the genes tested were either unchanged or downregulated in the mutants compared to the wild type. The *sds1* mutant showed a reduced level of transcripts encoding starch phosphorylase and starch synthase in N-replete and N-depleted condition, respectively. Similarly, the *sds3* also exhibited a decrease in expression of the gene encoding starch phosphorylase in N-replete cultivation. Under nitrogen starvation, however, this mutant had a reduced abundance of transcripts encoding the large subunit of ADPglucose pyrophosphorylase and a branching enzyme. In both growth conditions, the *sds4* exhibited differential expression of transcripts encoding the large subunit of ADPglucose pyrophosphorylase, starch synthase, and a branching enzyme. There was no differential expression detected in the one of the branching enzymes (BE-1) in any of the mutants compared to that of the wild type both in N-replete and N-depleted conditions.

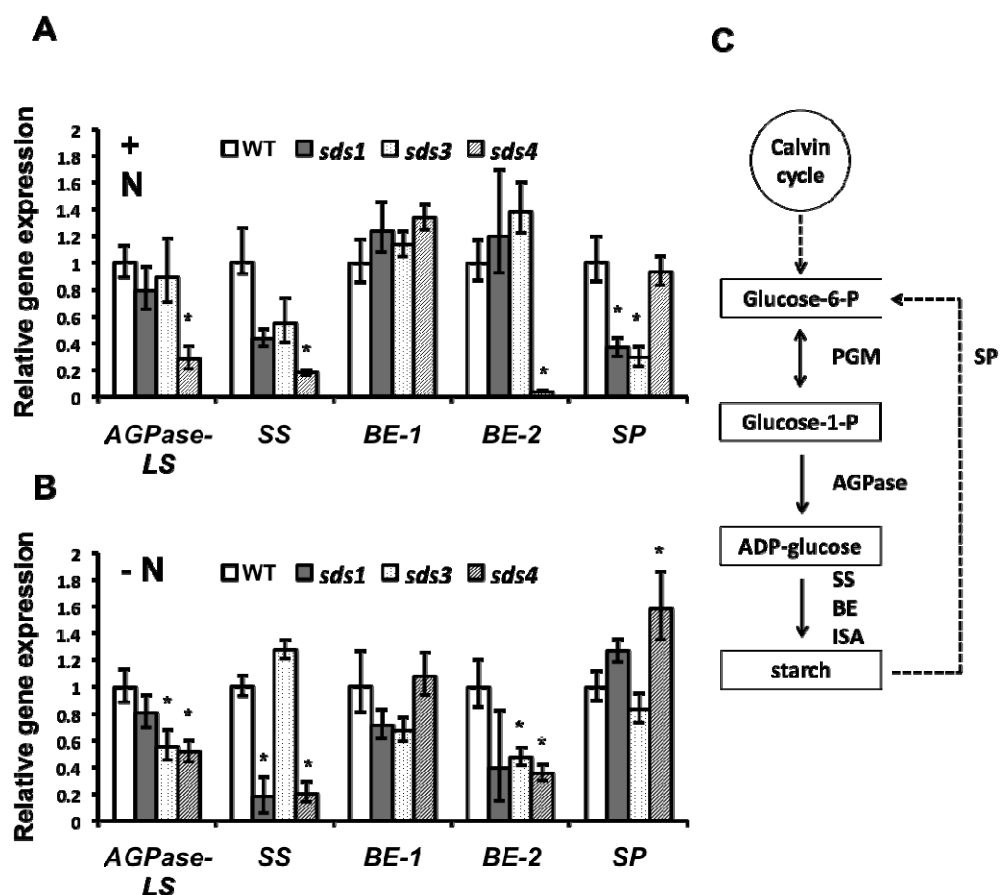


Fig. 7. Relative transcript levels of genes involved in starch biosynthesis and metabolism in *D. tertiolecta* cells under N-replete condition (A) and N-depleted condition (B). Error bars are standard errors (n = 3). An asterisk (*) indicates a significant difference calculated by one-tailed *t*-test ($P < 0.05$) between the mutant and the wild type. *AGPase-LS*, ADPglucose pyrophosphorylase-large subunit; *SS*, soluble starch synthase; *BE-1*, branching enzyme-1; *BE-2*, branching enzyme-2; *SP*, starch phosphorylase. (C) Simplified pathway of starch synthesis. Glucose-6-P, glucose-6-phosphate; Glucose-1-P, glucose-1-phosphate; ADP-glucose, adenosine diphosphate-glucose; PGM, phosphoglucomutase; *AGPase*, ADP-glucose pyrophosphorylase; *SS*, starch synthase; *BE*, branching enzyme; *ISA*, isoamylase; *SP*, starch phosphorylase. Dashed arrows indicate steps where several enzymes and intermediates are involved.

Table 1. List of primers used in this study.

Name of protein	Transcript	Primer sequence	Product size (bp)
AGPase-large subunit	25664_c1_seq1	F: 5'-CACCTCTTTACCCTTAACCCC-3' R: 5'-AGAGTGCTGCTGAAGTTGGTAA- 3'	106
Starch synthase	29472_c0_seq14	F: 5'-CTCGTGAAACTTCCGGGTGA-3' R: 5'-TGGGAGCATACGAGGAGGAA-3'	168
Branching enzyme-1	17945_c0_seq1	F: 5'-CTTGATGCCTTTGGTCACCTTG-3' R: 5'-GTGCTTTGACATGCTTGGATGT-3'	188
Branching enzyme-2	27769_c2_seq4	F: 5'-TTCAAACACTCCGCGTAGTTGTGA-3' R: 5'-GCATGAAGGTGGACTAGGCTTTGA-3'	198
Starch phosphorylase	483677_c0_seq1	F: 5'-GTTCTGCTGGCCAATGATTTT-3' R: 5'-GCAGTGATACATGATTGACCTCCT-3'	108
Receptor of activated protein kinase C 1 (RACK1)	31670_c0_seq1	F: 5'-CCACATGAACCAACTGACAACC-3' R: 5'-GTGACCTCCATCTAAGTGTGCA-3'	165

Lipid production potential and fatty acid profile

Since the previous results showed that the *sds1*, *sds3* and *sds4* mutants could tolerate light intensity up to $700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and their TAG yield increased in response to nitrogen depletion, the lipid production potential and fatty acid composition of the algal strains grown under this condition were reevaluated and quantified in more details. The results showed that growth performance of the wild type and the mutants were similar in terms of final biomass concentration and cell density (Fig. 8A). Gravimetric quantification of the total lipid extracted from the wild type and the mutant also showed no significant difference, ranging from 20.0 to 23.2% (w/w) lipid per dry algal biomass or 2.3 to $3.0 \mu\text{g lipid per } 10^6 \text{ cells}$ (Fig. 8B). Since the crude lipid extract from algae usually contains several classes of non-acyl lipid, e.g. carotenoids, which cannot be converted into fatty acid esters, the total acyl lipid content was further determined by transesterification of the total lipid with methanol. GC analysis of fatty acid methyl esters (FAMES) showed that the wild type produced 2.3% (w/w) acyl lipid per dry algal biomass or $0.27 \mu\text{g acyl lipid per } 10^6 \text{ cells}$. In contrast, the amounts of acyl lipid produced by the mutants were increased by 39-52% per dry weight, or 48-63% per cell (Fig. 8C). To quantify the TAG content, non-polar lipids were further purified from the crude lipid extracts using a silica-SPE cartridge. Preliminary experiments were conducted to determine an appropriate volume of the eluent (Fig. S1) and assess purity of the eluates (Fig. S2). The SPE-purified non-polar lipids were subsequently transesterified into FAMES. GC analysis of FAMES from TAG showed that the wild type produced 1.2% (w/w) TAG per dry algal biomass or $0.14 \mu\text{g TAG per } 10^6 \text{ cells}$. In contrast, the amounts of TAG produced by the mutants were increased by 42-92% per dry weight, or 43-100% per cell (Fig. 8D).

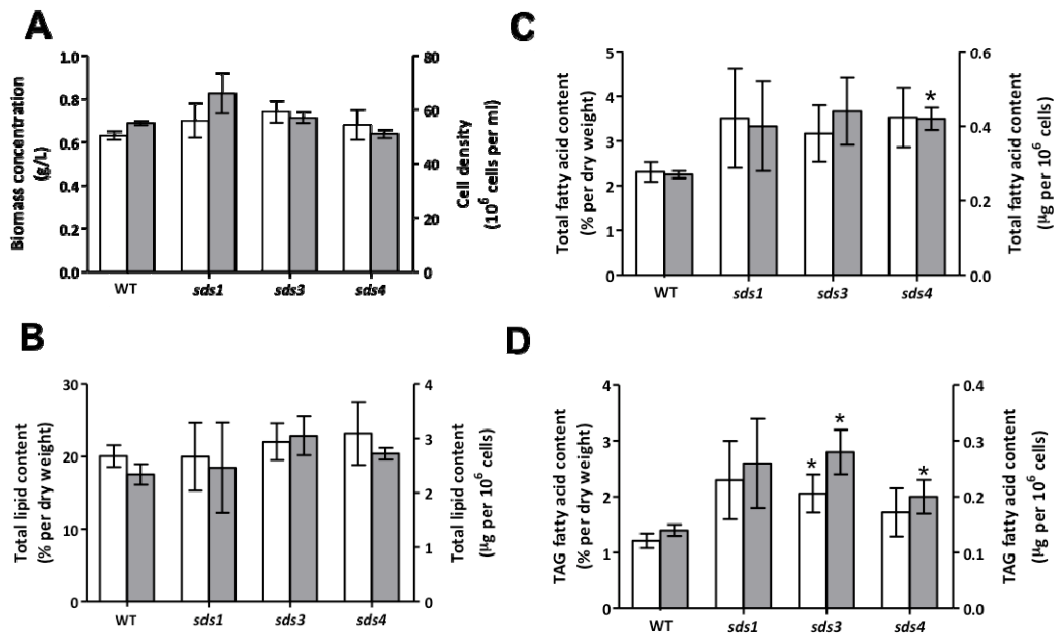


Fig. 8. Growth and lipid production potential of the *D. tertiolecta* wild type and starch-deficient mutants. The algal strains were cultured under $700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in N-replete medium for 5 days and transferred to N-depleted medium for 2 days before the harvested cells were analyzed for growth yield (A), total lipid content (B), total fatty acid content (C), and fatty acid content of TAG (D). Values are from three biological replicates. Error bars are standard errors. An asterisk (*) indicates a significant difference calculated by one-tailed *t*-test ($P < 0.05$) between the mutant and the wild type. Light bars, biomass concentration or calculated per biomass; dark bars, cell density or calculated per cell.

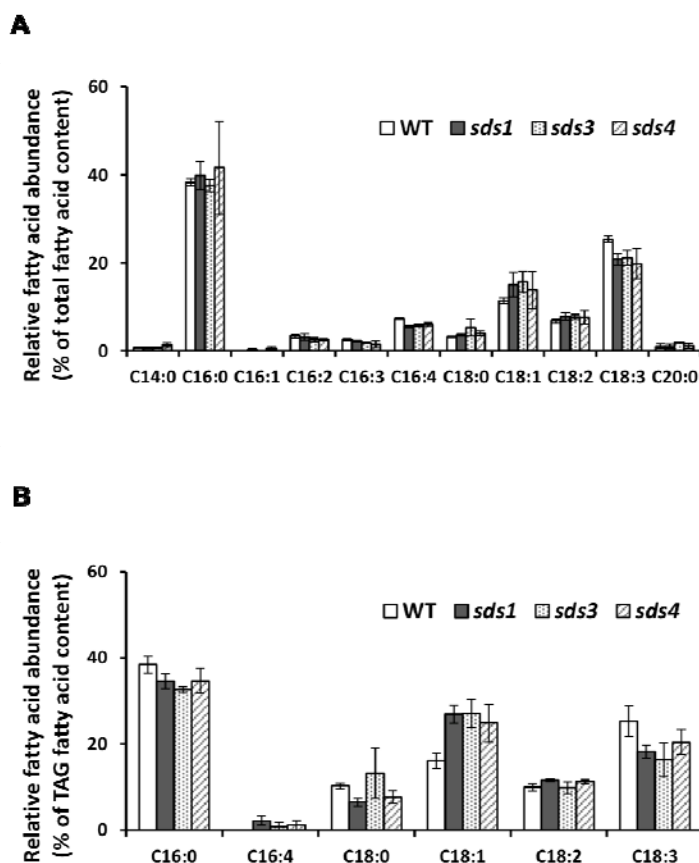


Fig. 9. Fatty acid profile of total lipid (A) or TAG (B) from the *D. tertiolecta* wild type and starch-deficient mutants. The algal strains were cultured under $700 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in N-replete medium for 5 days and transferred to N-depleted medium for 2 days before lipid extraction. Relative amounts of major fatty acids are shown. Values are from three biological replicates. Error bars are standard errors.

Comparison of fatty acid constituents of total lipid (Fig. 9A) and TAG (Fig. 9B) of the wild type and the mutants showed that there was no substantial difference, except for a slight increase of oleic acid (C18:1) and a decrease in linolenic acid (C18:3) in the mutants compared to the wild type.

Discussion

Several microalgal species have established themselves as candidates for biodiesel feedstock. In addition to rapid growth rate and high oil content, ease of large-scale cultivation and co-production of high-value products are among major considerations. These latter attributes are particularly important characteristics of *Dunaliella*. In fact, *D. salina* has been cultivated at a commercial scale to produce β -carotene [34]. Although the TAG content of *Dunaliella* is relatively low compared to other algal biodiesel candidates [8], our work has demonstrated that it can be genetically improved. This

technique may also be applicable to other algal species that have all the important characteristics but possess relatively low oil content.

Starch accumulation has been shown as an initial response to nitrogen starvation in a number of algal species, including *Dunaliella* [22, 38, 40, 43], as it is believed to operate as a short-term energy storage mechanism under adverse conditions. The degradation of starch, which usually occurs when the duration of nitrogen starvation is prolonged, has the possible function of supporting TAG synthesis as a long-term energy storage mechanism [6, 13, 20, 43]. Impediment in starch synthesis would redirect carbon metabolites toward TAG synthesis, at least during the early response to nitrogen starvation. However, this relationship between the starch and TAG metabolic pathways had previously been shown only in a number of *C. reinhardtii* starchless mutants and in a few algal species. The results shown in this study further supported this hypothesis and provided another clear demonstration that such mutants could be quite easily obtained through random mutagenesis and mutant screen – which do not involve transgenic modification. All of the mutants presented in this study were confirmed to have significant reduction in starch content, but some starch remained present – hence, we referred to these mutants as starch-deficient strains. The reduction in starch content could be observed only under N-depleted condition (Fig. 1B) possibly because the basal starch content under N-replete condition was too low to detect a further reduction, or because the genes affected in these mutants may have a role in starch metabolism predominantly under the N-depleted condition. Consistently, the difference in TAG content between the wild type and the mutants could not be observed when the cells were grown in N-replete medium, as neither the wild type nor the mutants accumulated TAG (Fig. 3A). Under the N-depleted condition, three of the isolated mutants showed a significant and/or substantial increase in TAG content, ranging from 0.4- to 2-fold elevation in TAG accumulation (Fig. 3B and 8D).

The gravimetric quantification of crude lipid extracts (Fig. 2 and 8B) showed little or no difference between the wild type and the mutants, whereas the GC analysis of derivatized FAMES (Fig. 8C) showed the mutants had a higher lipid content. This may be explained by impurity of the crude lipid extracted using Bligh and Dryer's solvent, which typically includes additional cellular components, such as pigments, that out-weighted the acyl lipid content and masked the differences among the algal strains. Derivatization of the lipid into FAMES allows more specific quantification of the acyl lipid, which is the primary substrate for biodiesel production [7]. SPE purification of the crude lipid extract followed by FAME derivatization allows assessment of TAG (Fig. S2). Based on the GC analysis of FAMES, the total acyl lipid and TAG content of wild-type *D. tertiolecta* cell are slightly lower than, those reported by Breuer et al. [8]. The lower lipid yields in our study may be caused by discrepancies in culture conditions, e.g. no additional CO₂ supply and shorter duration of nitrogen starvation. Based on mass balance, the elevation in total acyl lipid content of the *sds*

mutants over the wild type is mostly due to their increased TAG pool, suggesting that the metabolic redirection from reduced starch biosynthesis is used toward TAG production rather than synthesis of other classes for acyl lipid. Comparative profiling of FAMES derived from the total acyl lipid of *D. tertiolecta* grown under N-depleted condition showed that the most abundant fatty acid was C16:0, followed by C18:3, C18:1, C18:2, C16:4, and C18:0, respectively (Fig. 9A). Similar results were also reported by Breuer et al. [8] and Lee et al. [19], although in these studies C18:3 appeared more abundant than C16:0. These major fatty acids were also the main constituents of TAG (Fig. 9B). Interestingly, all three mutants possessed a higher content of C18:1 in TAG than the wild type. Thus, it might be possible that C18:1 is preferentially involved in TAG synthesis, e.g. as a main acyl substrate, during the metabolic redirection in the *sds* mutants. Consistent with this hypothesis, a lipidomic study of *D. tertiolecta* grown under nitrogen starvation showed a marked increase of diacylglyceryltrimethylhomoserine carrying C18:1, which was further hypothesized to function as an acyl donor in the biosynthesis of TAG under this condition [15]. However, this hypothesis remains to be tested.

The four isolated starch-deficient mutants exhibited varying degrees of starch deficiency and elevation of lipid content. In addition, the gain in TAG in each *sds* mutant did not strictly correlate with the loss of starch content, implying that the characteristics of starch synthesis inhibition—and consequently, the metabolic flux modifications—in these mutants are distinct. The fact that no TAG was detected in *sds2* suggested that the mutation might affect an upstream metabolic or regulatory process, leading to the inhibition of both starch and TAG synthesis. Real time RT-PCR analysis showed that different sets of genes exhibited altered expression in the three *sds* mutants. One explanation is that these mutants carried different mutations and thus were affected at different steps of starch synthesis and metabolism, resulting in varying degrees of starch deficiency and accumulation of different metabolic intermediates. Some intermediates might also function as signaling molecules that could affect expression of multiple genes in the pathway. In fact, it has been suggested that in the *C. reinhardtii* *sta6* mutant, which cannot convert glucose-1-phosphate to ADP-glucose, glucose-1-phosphate might be converted to trehalose, a molecule that might have a role in signaling [3, 4]. In addition, the recently described starch-deficient mutants of *Chlorella sorokiniana* did not show increased accumulation of TAG [39]. These mutants were defective in isoamylase and starch phosphorylase, which are involved in the formation of starch granule from glucan chains [39]. It was suggested that the regulation of carbon partitioning between starch and TAG formation might actually be linked to glucan synthesis rather than the availability of carbon precursors [39]. Therefore, the steps where the starch synthesis is blocked might largely contribute to differences in phenotypes observed in starch-deficient mutants.

It has been reported that the lack of starch in the *C. reinhardtii* BAFJ5 mutant causes an

immense reduction in photosynthesis efficiency, leading to susceptibility to high light intensity and growth retardation, particularly under N-depleted condition [16, 22]. Metabolomic study revealed that the loss of starch biosynthesis in this mutant led to a slower rate of NADPH re-oxidation, resulting in redox imbalance and ultimately photoinhibition [16]. In contrast to the reported high-light sensitivity of the *C. reinhardtii* starch-deficient mutant, our study showed that only the *sds3* among the isolated *D. tertiolecta* mutants was severely sensitive to high light intensity at $1,200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, suggesting that a decrease of starch content is not sufficient to cause this sensitivity. One possible explanation for this contradiction is that our mutants retained a substantial level of starch synthesis capability, which might still be enough to maintain a normal cellular redox state. However, the *S. obliquus slm1*, with only 1.2% starch remained, also did not show adverse effects on photosynthesis compared to the wild type, even under N-depleted condition [6], implying that there might be other mechanisms that could compensate for the lack of starch as an energy carbon sink. An accumulation of TAG under stress conditions is also suggested to be a protective mechanism, protecting the cells from the damage caused by reactive oxygen species [14, 21]. The relative importance of these storage compounds in alleviating cellular damage from oxidative stress should be further explored. Nevertheless, our work and the work on *S. obliquus* demonstrate the feasibility that a starch-deficient mutant can be isolated without associated growth retardation or high light sensitivity.

The net improvement of TAG content in starch-deficient mutants varies from species to species. For example, the starchless *C. reinhardtii* BAFJ5 mutant was reported to have 10-fold elevation of TAG [22]. On the other hand, the *S. obliquus slm1* mutant only increased its TAG content from 45% in the wild type to 57% in the mutant [6]. These variations in the fold increase of TAG might partly be explained by the differences in basal starch and TAG contents produced by the wild type strains and the increased level of starch synthesis that normally occurs upon stress conditions. However, the degree of TAG improvement may be compounded by additional factors. Both *C. reinhardtii* and *D. tertiolecta* favor starch as their main storage compound [8, 22, 31]. In case of *C. reinhardtii*, the starch content increases from 2-6% to over 40% during its initial response to nitrogen starvation, thus the blockade in starch synthesis resulted in large TAG improvement as in the BAFJ5 mutant [1, 22]. In contrast, our study showed that the starch content of wild-type *D. tertiolecta* increased approximately 3 folds, from 5% to 21%, yet the TAG content of the *sds* mutants increased by only 1-2 folds. The difference may be partly explained by the substantial amounts of starch remained in our mutants. Nevertheless, it is also possible that the starch and TAG metabolic pathways are not as connected in *D. tertiolecta* as in *C. reinhardtii*. In fact, Breuer et al. [8] showed that unlike the other algae tested, *D. tertiolecta* did not degrade their starch under prolonged exposure to nitrogen starvation, implying that *D. tertiolecta* may not depend on the stored starch for TAG biosynthesis.

Furthermore, in *D. tertiolecta* subjected to nitrogen starvation, while TAG is accumulated, the total acyl lipid content did not increase – suggesting that its TAG biosynthesis is coupled with the breakdown of preformed acyl lipid [8]. Consistent with this hypothesis, using ^{14}C isotope labeling, Davidi et al. [9] showed that in *D. bardawil* the TAG pool stored in cytoplasmic lipid bodies is synthesized mostly through *de novo* fatty acid synthesis, which occurs for a brief period of time following nitrogen starvation, whereas TAG in the chloroplastic plastoglobuli continues to be produced using the precursors from membrane lipid and cytoplasmic TAG.

Carotenoid levels have been found to increase in response to nitrogen starvation in carotenogenic species, such as *D. salina* and *D. bardawil* [9, 18]. In *D. tertiolecta*, carotenoid overaccumulation is typically not substantial in cells under nitrogen starvation condition, although it has been observed in cells grown in an N-replete medium at stationary phase [15]. In contrast to the *S. obliquus* starch-deficient *slm1*, which contained more carotenoid than the wild type [6], our results showed that the inhibition of starch synthesis in *D. tertiolecta* did not result in carotenoid overaccumulation. As carotenoids have a high commercial value, the fact that carotenoid content in starch-deficient mutants did not decrease under TAG-accumulating conditions indicates that carotenoids could be used as potential co-products for reducing the cost of biodiesel production from this algal species.

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Appendix

Supplementary figures

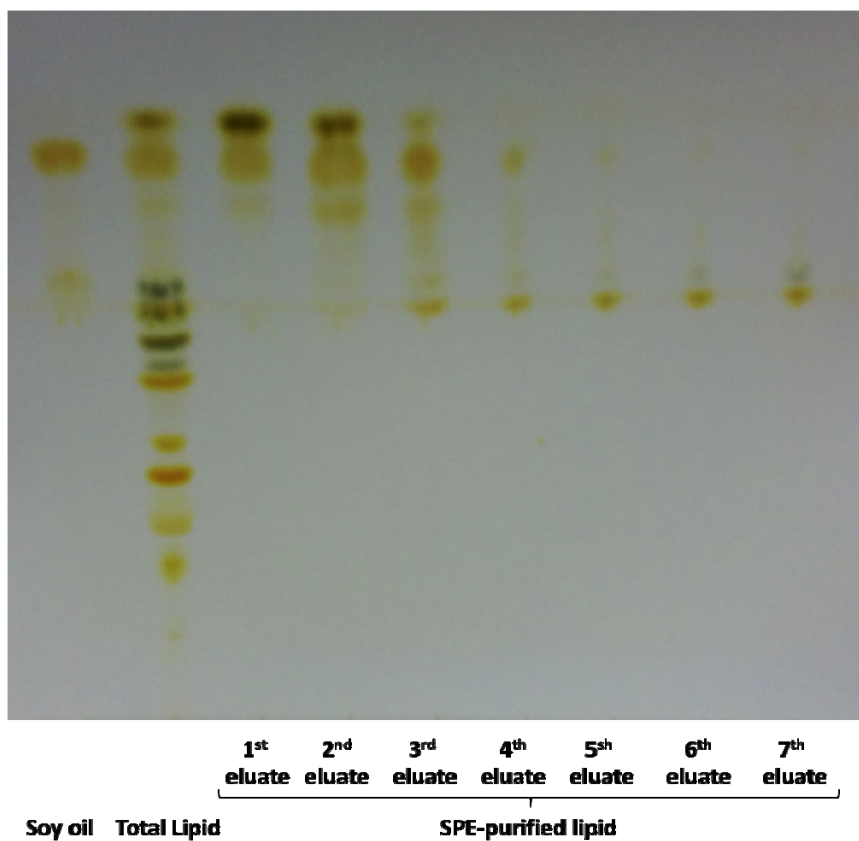


Fig. S1. Preliminary analysis of lipid fractions obtained from serial elution of an SPE cartridge loaded with total lipid extracted from 10 mg of wild-type dry biomass. Each eluate was obtained by 1 ml elution with hexane:diethyl ether (83:17, v/v), and 250 μ l of the eluate was spotted in each TLC lane. The total lipid lane corresponded to a non-purified extract from 1 mg of wild-type dry biomass. Soybean oil, 50 μ g, was used as a TAG standard.

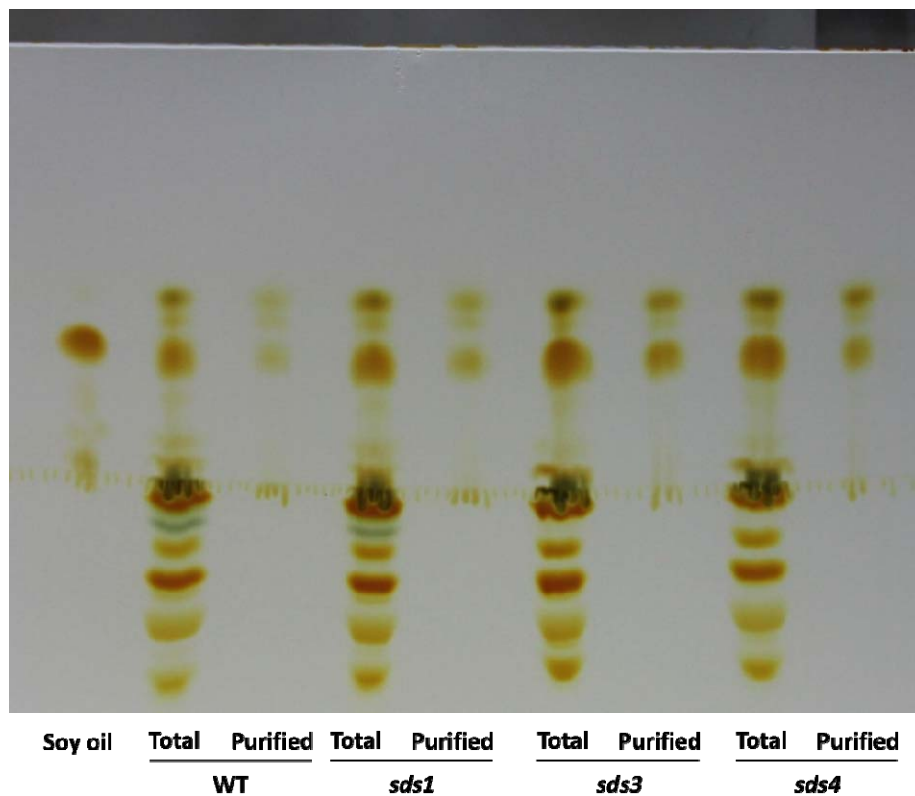


Fig. S2. TLC analysis of SPE-purified lipid from the wild type and starch-deficient mutant strains. The crude lipid extract was obtained from 40 mg dry algal biomass, and one fourth of this amount was purified using a silica SPE cartridge, as described in materials and methods. Total lipid and purified lipid (using 2 ml eluent) equivalent to the amount extracted from 1 mg of dry biomass were spotted in each TLC lane. Soybean oil, 50 μ g, was used as a TAG standard.

Output

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