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Enhanced Production of Fatty Acids via Redirection of Carbon Flux in Marine Microalga *Tetraselmis* sp.

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Lipids in microalgae are energy-rich compounds and considered as an attractive feedstock for biodiesel production. To redirect carbon flux from competing pathways to the fatty acid synthesis pathway of *Tetraselmis* sp., we used three types of chemical inhibitors that can block the starch synthesis pathway or photorespiration, under nitrogen-sufficient and nitrogen-deficient conditions. The starch synthesis pathway in chloroplasts and the cytosol can be inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 1,2-cyclohexane diamine tetraacetic acid (CDTA), respectively. Degradation of glycine into ammonia during photorespiration was blocked by aminooxyacetate (AOA) to maintain biomass concentration. Inhibition of starch synthesis pathways in the cytosol by CDTA increased fatty acid productivity by 27% under nitrogen deficiency, whereas the blocking of photorespiration in mitochondria by AOA was increased by 35% under nitrogen-sufficient conditions. The results of this study indicate that blocking starch or photorespiration pathways may redirect the carbon flux to fatty acid synthesis.

Keywords: *Tetraselmis* sp., fatty acids, carbohydrates, nitrogen starvation, chemical inhibitors

Introduction

Recently, the necessity of developing sustainable and renewable energy sources has been emphasized owing to factors such as increased energy consumption, rapid climate change, and limited reserves of fossil fuels. In order to simultaneously replace depleted fossil fuels and begin resolving issues related to climate change, photosynthesis-driven alternative energy sources such as biodiesel, bioethanol, and biogas have been harnessed and developed. Among these options, microalgae have been receiving substantial attention as one of the most effective producers of biofuel, such as biodiesel. Microalgae can convert carbon dioxide and water into energy-dense organic compounds (*i.e.*, carbohydrates and lipids) using their photosynthetic systems. In addition, unlike terrestrial plants, they have the ability to grow rapidly even under harsh conditions [1].

One distinctive characteristic of microalgae is their neutral lipid content, which can be converted into biodiesel, and can be modified by controlling culture conditions [2]. It is well known that stress or unfavorable environmental conditions, such as nutrient deficiency and high light or temperature shock, can lead to considerable increases in the intracellular lipid content of microalgae [3–6]. For instance, by decreasing the concentration of nitrogen sources in the culture medium, *Scenedesmus* sp. can accumulate lipid to levels as high as 53% of dry weight [7]. However, it should be noted that although such stresses can trigger lipid accumulation, overall lipid productivity might not be directly proportional to increased lipid

content owing to the retardation of cell division [8]. In other words, algal growth will cease or become slower, because these adverse stimuli create an imbalance in algal growth conditions. Eventually, a prolonged period of stress will decrease lipid productivity [9].

In recent years, numerous studies have been reported for enhancing lipid content or productivity via the regulation of algal metabolic pathways [10–12]. Lipids and carbohydrates are important energy storage molecules in microalgae. They are closely linked metabolites that are synthesized from the same major precursor (glycerol-3-phosphate) [4, 13]. Thus, augmented production of algal lipids may be possible via appropriate manipulations of lipid- or carbohydrate-related metabolic pathways.

Methods for switching from starch synthesis to fatty acid (FA) synthesis pathways require careful study, particularly for carbohydrate-accumulating strains, to avoid reducing the growth rates of microalgae. According to a recent noteworthy report, 10-fold increases in triacylglycerol (TAG) were achieved in *Chlamydomonas* by inactivation of ADP-glucose pyrophosphorylase (AGPase), which is involved in carbohydrate synthesis [14]. In addition, Daboussi *et al*. [15] have demonstrated that deletion of UDP-glucose pyrophosphorylase (UGPase) in *Phaeodactylum tricornutum* could promote a 45-fold increase in TAG accumulation when compared with the wild type. These results imply that the lipid content of microalgae can be markedly increased by regulating the pathways that compete with lipid synthesis.

Chemical inhibitors can be important tools for understanding carbon allocation and metabolic pathways in microalgae. Eukaryotic microalgae have two distinctive pathways for carbohydrate synthesis by AGPase and UGPase, which originated differently from prokaryotic (*i.e*., cyanobacteria) and eukaryotic hosts, respectively [16, 17]. AGPase is involved in pyrenoid starch synthesis and inhibited by dichlorophenyl dimethylurea (DCMU) [18]. UGPase plays a key enzyme in carbohydrate metabolism for sucrose or chrysolaminarin as storage polysaccharides in the cytosol [13]. Cyclohexane diamine tetraacetic acid (CDTA) is known as a chelating agent, which could inhibit UGPase activity by quenching the magnesium-dependent prosthetic groups [19]. Similarly, aminooxyacetate (AOA) is able to block the degradation of glycine to ammonia during photorespiration, which is known to lower photosynthetic efficiency [20].

In the present study, we used these three chemical inhibitors to investigate metabolic regulation and lipid productivity in the marine green microalga *Tetraselmis* sp. KCTC12432BP, cultured in the Korean Ocean, for the production of biodiesel [21]. Experiments were conducted under nitrogen-sufficient and nitrogen-deficient conditions to compare FA accumulation patterns.

Materials and Methods

Algal Strain and Growth Conditions

The green microalga *Tetraselmis* sp. KCTC12432BP was locally isolated from coastal seawater in Young-Heung Island (Korea). *Tetraselmis* sp. was cultivated in a 0.5-L bubble column photobioreactor containing 400 ml of 3-fold-fortified f/2-Si medium at 20 ± 1°C under continuous illumination from fluorescent lamps at a constant intensity of 100 ± 5 $\mu E \cdot m^{-2} \cdot s^{-1}$, and with aeration by 2% CO_2 balanced with air at 0.1 vvm. Nitrogen-stress conditions were applied when cultures reached the stationary phase of growth. After centrifuging the culture to harvest cells, the cell pellets were collected and then washed twice with distilled water (DW) to remove residual medium. Collected cells were resuspended in nitrogen-free culture medium. The experiments were performed in duplicate.

Measurement of Cell and Nitrate Concentrations

The cell concentration, average cell size, and cell size distribution were measured using a Coulter Counter (Multisizer 4; Beckman Coulter Inc., USA). Fresh cell weight (FCW) was determined by the data from the Coulter Counter [22]. The FCW was converted to dry cell weight (DCW) using the ratio of FCW to DCW (3.34 ± 0.05) for microalgal growth analysis, as previously described [23]. The DCW for calculation of FAs and carbohydrate content in the cell was gravimetrically determined after washing and lyophilization. The nitrate concentration was spectrophotometrically measured using 1 N HCl, as previously described [24]. All measurements of cell and nitrate concentrations, FAs, and total carbohydrate (TC) analysis were performed in triplicate.

Fatty Acid Analysis

Harvested cells were centrifuged at 1,900 ×*g* for 5 min and then washed twice with DW. Prior to FA analysis, the cells were freeze-dried. FAs extracted in 0.9 ml of methanol containing 5% (v/v) acetyl chloride and 0.1 ml of hexane containing internal standard with methyl nonadecanoate were added to a 2-ml O-ring seal screw-capped tube. The sample caps were maintained at 80°C for 50 min and then 1 ml of hexane was added to each tube. The FAs were analyzed using a gas chromatograph (YL6500GC; Young Lin Inc., Korea) equipped with a flame ionization detector and an HP-INNOWAX capillary column (30 m in length and 0.53 mm in internal diameter). Helium was used as the carrier gas at a flow rate of 20 ml/min. Air and hydrogen flow rates were 300 ml/min and 30 ml/min, respectively. Analytic oven conditions were as follows: 140°C for 5 min, 5°C/min to 240°C, held at 240°C for 10 min.

Total Carbohydrate Analysis

The TC content was determined using a modified phenol-sulfuric acid method [25]. Equal volumes of the sample and 5% phenol were added to glass vials covered with aluminum foil to exclude light. Sulfuric acid at 2.5 times the volume of the initial mixture was then added to the glass vials. The vials were vigorously stirred for 30 sec and maintained at 80°C for 30 min. Sample absorbance was determined at 492 nm using a UV/VIS spectrophotometer (UV-1800; Shimadzu Co., Japan) [26].

Inhibition of Carbohydrate Synthesis and Photorespiration

Three types of inhibitors were used for redirection of the carbon flux from carbohydrate synthesis and photorespiration pathways toward FA synthesis pathways. To inhibit carbohydrate synthesis pathways in chloroplasts and the cytosol, the same amounts of DCMU (Sigma-Aldrich, USA) and CDTA (Sigma-Aldrich) were added to *Tetraselmis* sp. cultures, respectively. To block carbon flux during photorespiration in mitochondria, AOA (Sigma-Aldrich) was added to the cultures. All inhibitors were added to the *Tetraselmis* sp. cultures at the stationary phase. The nitrogen-sufficient condition was maintained by periodical supply of nitrate to the cultures when the level had decreased to a determined level (50 mg/l), and the nitrogen-deficient condition was produced by supplying nitrogen-free fresh culture medium prior to applying inhibitors. DCMU, CDTA, and AOA were added to the medium at final concentrations of 1 μM, 1 mM, and 1 mM, respectively, which were conventionally used for other microalgae or plants [27–29].

Statistical Analysis

Data are expressed as the mean ± standard deviation of three measurements. The total fatty acid (TFA) content with DCMU was analyzed using two-way analysis of variance (ANOVA) with replication using Microsoft Office Excel 365 (Microsoft, USA). A significant difference was considered at the level of $p < 0.05$.

Results and Discussion

Biochemical Responses of *Tetraselmis* sp. under Nitrogen Deficiency

To investigate the biochemical responses to nitrogen starvation, cells were cultivated under nitrogen-deficient conditions for 84 h. The time courses of *Tetraselmis* sp. culture growth, TC content variation, and TFA content variation are plotted in Fig. 1. Cultures grown under nitrogen-deficient conditions (N–) showed slow growth and attained a DCW of 0.1 g/l by the end of the cultivation period, as shown in Fig. 1A. The TC content under N– was increased by 514% (Fig. 1B) and the TFA content of cultures grown under nitrogen-deficient conditions was 21% greater after 84 h than in cultures grown under nitrogen-sufficient conditions (N+, Fig. 1C). Consequentially, *Tetraselmis* sp. KCTC12432BP showed low growth rates under N– conditions at the initial growth stage and responded by accumulating carbohydrate rather than FAs under N– conditions. Although the N– cells showed higher TC and TFA contents, the TC and TFA productivities obtained were only 34% and 7%

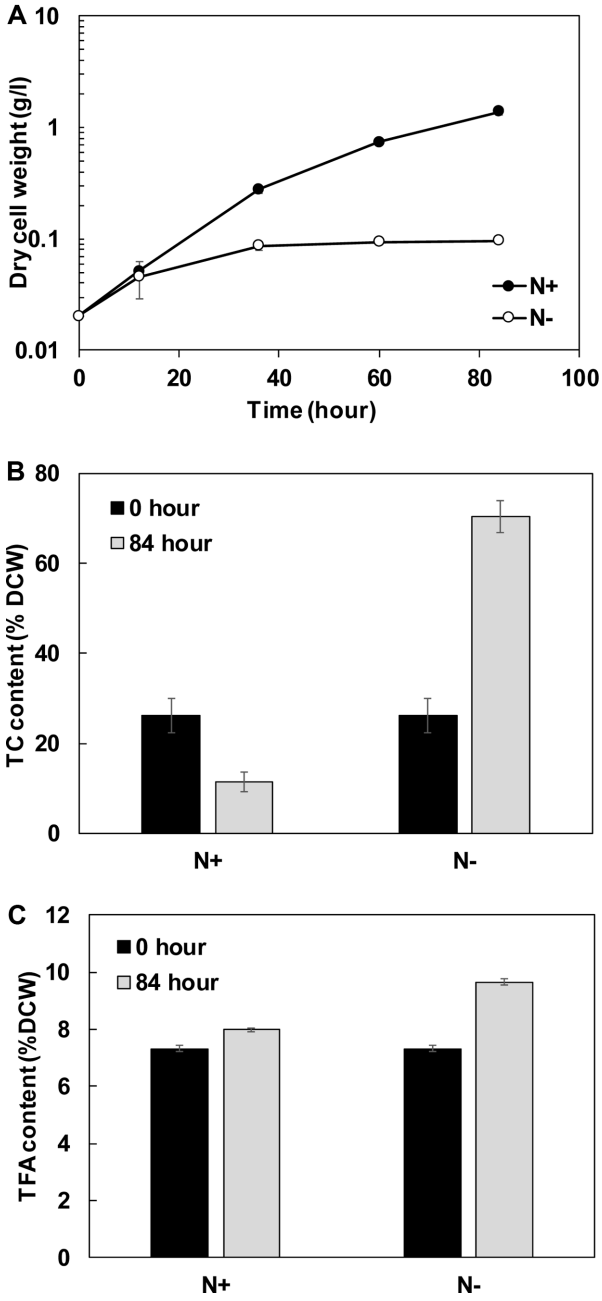

Fig. 1. Effects of nitrate sufficiency (N+) and deficiency (N–) on (**A**) dry cell weight (g/l), (**B**) total carbohydrate content (% dry cell weight (DCW)), and (**C**) total fatty acid content (% DCW) of *Tetraselmis* sp.

Table 1. Total carbohydrate (TC) and total fatty acid (TFA) productivity of *Tetraselmis* sp. under nitrogen-sufficient (N+) and nitrogen-deficient (N–) conditions.

	N+	N–
TC productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	44.4 ± 9.6	15.2 ± 1.2
TFA productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	31.2 ± 2.2	2.1 ± 0.0

over those under N+ conditions, respectively, due to lack of the essential nutrient for algal cellular division (Table 1).

Nutrient deficiency leads to changes in cellular metabolism in microalgae [7, 30, 31]. Understanding the physiological and biochemical changes under various types of abiotic stress, such as nutrient deficiency and exposure to high light and temperature, can serve as a key strategy to enhance the production of useful microalgal compounds. In particular, nitrogen-deficient culture conditions have been widely used to increase the lipid content within the cell body, because lipid biosynthesis genes such as *DGTT3*, *DGTT4*, and *DGAT* are expressed at significantly high levels under nitrogen deprivation [32]. However, the responses of cells to nitrogen deficiency have been shown to be species-specific. For instance, under nitrogen-deficient conditions, *Chlamydomonas reinhardtii* can accumulate carbohydrates from 5% to 45% of DCW, whereas *Nannochloropsis* sp. is known to increase TAG up to 60% of DCW [14, 26]. Furthermore, under conditions of nitrogen deprivation, *Tetraselmis suecica* F&M-M33 does not induce lipid accumulation, but instead stimulates carbohydrate synthesis in outdoor cultivation [33]. Therefore, in this study, we attempted to investigate whether the addition of inhibitors to *Tetraselmis* sp. cultures can redirect the carbon flux from carbohydrate synthesis to FA synthesis pathways, thereby enhancing FA production.

Inhibition of the Starch Synthesis Pathway by DCMU and CDTA

In order to avoid biomass loss attributable to nitrogen-deficiency, DCMU was applied to *Tetraselmis* cultures at the starting point of exposure to nitrogen-deficient conditions, when the cells reached the stationary phase. No differences in the DCW of all cultures and TC content under nitrogen-sufficient conditions with/without DCMU (N+D+, N+D–) were observed for 84 h (Fig. 2A). The TC content under nitrogen-deficient conditions with DCMU (N– D+) decreased from 48% DCW to 34% DCW compared with nitrogen deficiency without DCMU (N– D–). The TFA contents in N+D+ and N– D+ cells increased to 9% DCW and 12% DCW, respectively, showing higher TFA content than those in N+D– and N– D– cells. According to ANOVA, the TFA content was significantly affected by nitrogen deficiency ($p < 0.01$) and DCMU ($p < 0.1$). However, the TC content was not affected by DCMU under N+ conditions. These results showed that DCMU led to a decline in TC and TFA contents in *Tetraselmis* cells only under N– conditions. DCMU is known as a chemical inhibitor that blocks the electron transfer chain in the thylakoid membranes [34, 35]. Among the target enzymes blocked by DCMU is AGPase, which is a light-dependent enzyme of starch synthesis in the chloroplasts [36]. Therefore, the TC content in N– D+ cells was markedly decreased by inhibition of the light-dependent starch pathway induced under stressful conditions. However, the carbon flux was partially redirected to the FA synthesis pathway, because the TFA content in N– D+ cells increased slightly, whereas the TC content decreased.

Another starch synthesis inhibitor, CDTA, was added to *Tetraselmis* cultures under N+ conditions with and without CDTA (N+ C+, N+ C–) and nitrogen-deficient conditions with and without CDTA (N– C+, N– C–) at the stationary phase. In all cultures, the TFA content was unaffected by CDTA, whereas the TC contents in the N+ C+ and N– C+ cells were lower, at 17% DCW and 21% DCW, respectively (Fig. 2B), than those in the N+ C– and N– C– cells. The FA productivity of N– C+ cells was 24.9 ± 1.7 $mg \cdot l^{-1} \cdot day^{-1}$, which was 14% higher than that of N– D+ cells (Table 2). CDTA inhibits UGPase, which is involved in starch synthesis in the cytosol [19]. Therefore, decreases in the TC content in N+ C+ cells indicated that *Tetraselmis* sp. KCTC12432BP accumulates carbohydrate in the cytosol under nitrogen-sufficient conditions. The TFA content in N+ C+ cells was not affected by CDTA, because FA synthesis occurs in the chloroplast.

It is well known that the starch and FA synthesis pathways are closely associated, because they are derived from the same precursor. For that reason, redirection of the carbon flux from the starch synthesis pathway to the FA pathway has recently been suggested as a promising strategy to increase the FA content in *Chlamydomonas reinhardtii* [14]. However, the mechanisms whereby carbohydrate metabolism and FA metabolism interact to enhance FA production is still poorly understood in almost all microalgae [37]. FAs are derived from the same precursor, glycerol-3-phosphate (G3P), from the Calvin cycle [4, 38]. G3P is involved in the synthesis of ADP-glucose in chloroplasts and transferred to the cytosol using C3 transport. In the cytosol, UDP-glucose can be produced from G3P and converted to starch [13]. G3P is also involved as a precursor in the FA synthesis pathway. Thus, many researchers have investigated the

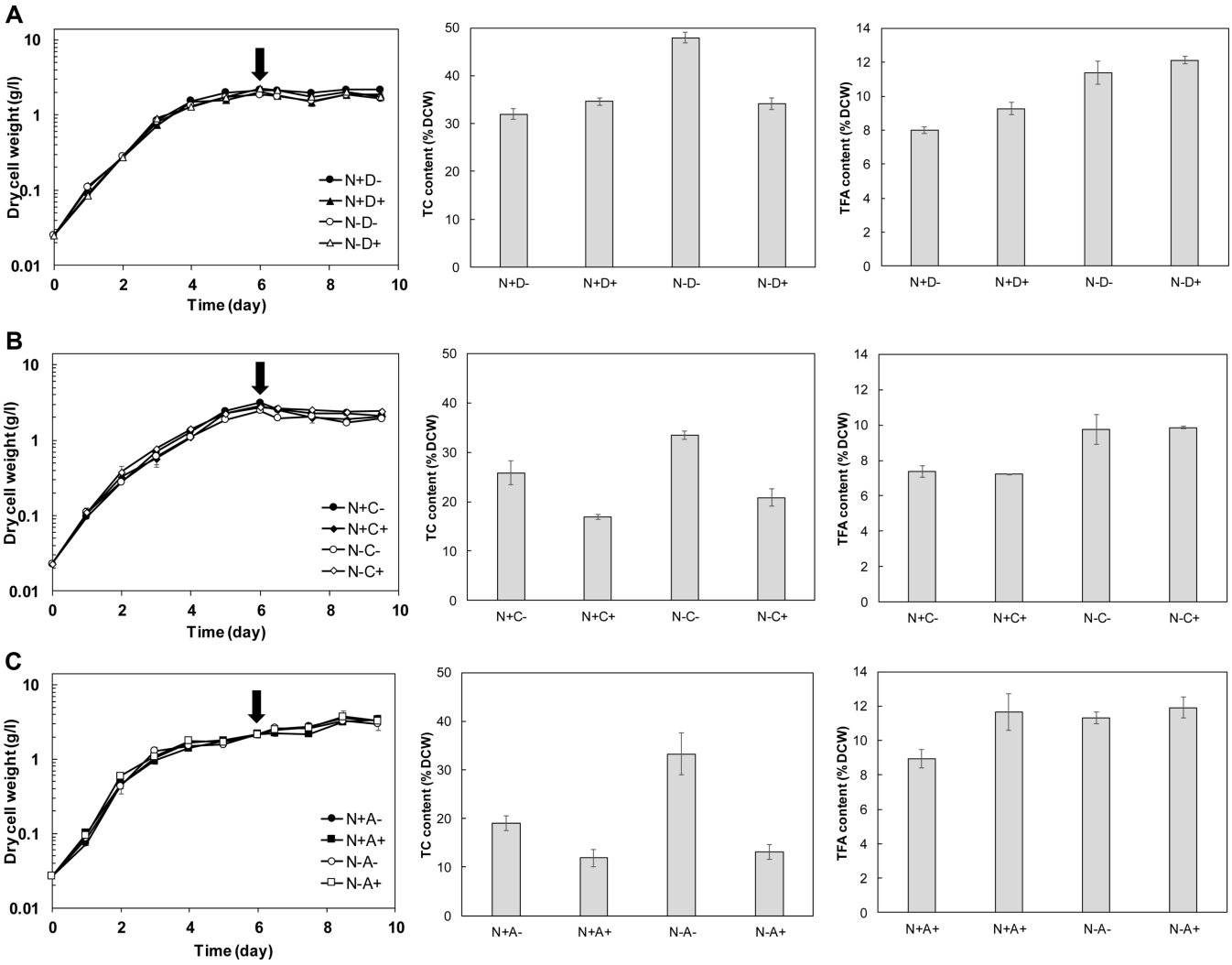

Fig. 2. Effects of chemical inhibitors on dry cell weight, total carbohydrate (TC) content, and total fatty acid (TFA) content in *Tetraselmis* sp.

(**A**) Dichlorophenyl dimethylurea (N+ D– : nitrogen sufficiency without DCMU; N+ D+: nitrogen sufficiency with DCMU; N– D– : nitrogen deficiency without DCMU; N– D+: nitrogen deficiency with DCMU). (**B**) Cyclohexane diamine tetraacetic acid (N+ C–: nitrogen sufficiency without CDTA; N+ C+: nitrogen sufficiency with CDTA; N– C–: nitrogen deficiency without CDTA; N– C+: nitrogen deficiency with CDTA). (**C**) Aminooxyacetate (N+ A–: nitrogen sufficiency without AOA; N+ A+: nitrogen sufficiency with AOA; N– A–: nitrogen deficiency without AOA; N– A+: nitrogen deficiency with AOA). Arrows indicate the starting point of nitrogen deficiency.

enhancement of FAs by inhibiting starch pathways [39]. In particular, AGPase and UGPase are known as key enzymes in carbohydrate metabolism [40]. For example, TAG in *Chlamydomonas reinhardtii* is accumulated within the cells by inactivation of AGPase [41]. In *P. tricornutum*, a 45-fold accumulation of TAG can be induced by deactivating UGPase using transcription activator-like effector nuclease [15]. Our results indicate that in *Tetraselmis* sp., there is an interrelationship between the starch and FA pathways in the chloroplasts. Thus, inhibiting AGPase could be a promising approach for enhancing TFA content and production.

Inhibition of Photorespiration by AOA

To enhance FA productivity by increasing *Tetraselmis* biomass, AOA was applied to inhibit photorespiration. When AOA was added to cultures at the stationary phase, the biomass at the end of the culture period under nitrogen-sufficient conditions (N+ A+) and nitrogen-deficient

Table 2. Total carbohydrate (TC) and total fatty acid (TFA) productivity of *Tetraselmis* sp. with or without chemical inhibitors under nitrogen-deficient and -sufficient conditions.

	N+ D–	N+ D+	N– D–	N– D+
TC productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	72.6 ± 0.7	66.6 ± 1.3	82.1 ± 4.2	61.7 ± 0.6
TFA productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	18.2 ± 1.4	17.9 ± 1.9	19.6 ± 3.3	22.0 ± 1.8
	N+ C–	N+ C+	N– C–	N– C+
TC productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	57.3 ± 7.8	36.3 ± 1.8	67.5 ± 3.6	52.4 ± 3.1
TFA productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	16.3 ± 1.0	15.5 ± 0.1	19.6 ± 2.0	24.9 ± 1.7
	N+ A–	N+ A+	N– A–	N– A+
TC productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	66.4 ± 10.1	41.8 ± 2.6	102.3 ±0.2	44.3 ± 3.8
TFA productivity ($mg \cdot l^{-1} \cdot day^{-1}$)	31.2 ± 3.8	42.0 ± 11.7	35.5 ± 8.0	40.7 ± 6.2

D, Dichlorophenyl dimethylurea; C, cyclohexane diamine tetraacetic acid; A, aminooxyacetate.

conditions (N– A+) was 3.4 g/l and 3.3 g/l, respectively (Fig. 2A). These values were similar to those obtained under nitrogen-sufficient conditions without AOA (N+ A–) and nitrogen-deficient conditions with AOA (N– A+). The TFA content in all the experimental groups was approximately 20% higher than that of N+ A– cells. This indicates that AOA does not affect biomass, but does influence FA synthesis in *Tetraselmis* cells. Consequently, the TFA productivities in N+ A+ and N– A+ cells were 42.0 $mg \cdot l^{-1} \cdot day^{-1}$ and 40.7 $mg \cdot l^{-1} \cdot day^{-1}$, 35% and 15% higher than those in N+ A– and N– A– cells, respectively (Table 2).

Photorespiration is in competition with the Calvin–Benson cycle for uptake of O_2 and release of CO_2 [42]. Many algae have been reported to produce glycolate, which is an intermediate between photorespiration and the Calvin-Benson cycle [43, 44]. Glycolate is oxidized to glyoxylate by glycolate oxidase in the peroxisomes, and this glyoxylate is transaminated to glycine, which is subsequently exported to the mitochondria. AOA blocks 4-aminobutyrate aminotransferase, which converts glycine to serine in the mitochondria [45]. This reaction is also involved in nitrate assimilation for synthesis of all nitrogen-containing metabolites in the cell, which is associated with photorespiration and photosynthesis [46, 47]. In this experiment, AOA treatment could not increase the biomass despite inhibition of photorespiration. However, the TFA content under nitrogen-sufficient condition with AOA showed a significant increase to the TFA content under nitrogen-deficient conditions. These results indicate that an inhibition in nitrogen assimilation could be used for increasing the TFA content without nitrogen-deficient condition.

By using chemical inhibitors as regulators of target metabolic reactions, this study identified AGPase and 4-aminobutyrate aminotransferase as target enzymes for the enhancement of FA production in *Tetraselmis* sp. KCTC12432BP. Inhibition of AGPase by CDTA led to increases in FA productivity of up to 27% by redirecting the carbon flux in chloroplasts under nitrogen-deficient conditions. Additionally, the TFA productivity was increased by up to 35% via inhibition of nitrate assimilation by AOA under nitrogen-sufficient conditions. These results show not only an increase in FA productivity but also the potential for chemical inhibitors to confirm the effects of targeted gene knockout. Based on the results of this study, we established the possibility of diverting the carbon flux from carbohydrate synthesis to FA synthesis and of enhancing photosynthetic efficiency by blocking nitrate assimilation using chemical inhibitors. These enhancements could suggest a viable strategy for the effective production of microalgal biofuels as well as for identification of potential target genes in microalgae.

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