Two-Stage Cultivation of *Dunaliella tertiolecta* with Glycerol and Triethylamine for Lipid Accumulation: a Viable Way To Alleviate the Inhibitory Effect of Triethylamine on Biomass

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ABSTRACT Microalgae are promising alternatives for sustainable biodiesel production. Previously, it was found that 100 ppm triethylamine greatly enhanced lipid production and lipid content per cell of *Dunaliella tertiolecta* by 20% and 80%, respectively. However, triethylamine notably reduced biomass production and pigment contents. In this study, a two-stage cultivation with glycerol and triethylamine was attempted to improve cell biomass and lipid accumulation. At the first stage with 1.0 g/liter glycerol addition, *D. tertiolecta* cells reached the late log phase in a shorter time due to rapid cell growth, leading to the highest cell biomass (1.296 g/liter) for 16 days. However, the increased glycerol concentrations with glycerol addition decreased the lipid content. At the second-stage cultivation with 100 ppm triethylamine, the highest lipid concentration and lipid weight content were 383.60 mg/liter and 37.7% of dry cell weight (DCW), respectively, in the presence of 1.0 g/liter glycerol, which were 27.36% and 72.51% higher than those of the control group, respectively. Besides, the addition of glycerol alleviated the inhibitory effect of triethylamine on cell morphology, algal growth, and pigment accumulation in *D. tertiolecta*. The results indicated that two-stage cultivation is a viable way to improve lipid yield in microalgae.

IMPORTANCE Microalgae are promising alternatives for sustainable biodiesel production. Two-stage cultivation with glycerol and triethylamine enhanced the lipid productivity of *Dunaliella tertiolecta*, indicating that two-stage cultivation is an efficient strategy for biodiesel production from microalgae. It was found that glycerol significantly enhanced cell biomass of *D. tertiolecta*, and the presence of glycerol alleviated the inhibitory effect of triethylamine on algal growth. Glycerol, the major byproduct from biodiesel production, was used for the biomass accumulation of *D. tertiolecta* at the first stage of cultivation. Triethylamine, as a lipid inducer, was used for lipid accumulation at the second stage of cultivation. Two-stage cultivation with glycerol and triethylamine enhanced lipid productivity and alleviated the inhibitory effect of triethylamine on the algal growth of *D. tertiolecta*, which is an efficient strategy for lipid production from *D. tertiolecta*.

KEYWORDS *Dunaliella tertiolecta*, glycerol, lipid accumulation, triethylamine, two-stage cultivation

Triacylglycerols (TAGs) from oleaginous microalgae have been considered feedstocks for biodiesel production. The advantages of exploiting microalgae as alternative resources for next-generation renewable fuels are mainly their high lipid contents, relatively high photosynthetic efficiency, high rate of carbon dioxide fixation, short life cycle, low labor requirement, and ease of scale-up and because they are less affected by geographical regions (1). However, the production cost of microalgal lipids as

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biodiesel hinders the commercial production of biodiesel. The price of biodiesel is 1.5 to 3 times more than that of the conventional fossil diesel (2). Heterotrophic cultivation of microalgae has several advantages over autotrophic cultivation, such as the elimination of light requirement, the relative simplicity of operations, and the high cell density (3, 4). However, the cost of raw materials of heterotrophic cultivation accounts for 60% to 75% of the total cost of biodiesel production (3). To reduce the production cost of microalgal lipids as biodiesel by heterotrophic cultivation, less-expensive carbon sources should be considered.

Glycerol, the major by-product from biodiesel production, has become an inexpensive and abundant carbon source. The increasing growth of biodiesel industries resulted in an excess of glycerol that led to a dramatic decrease in crude glycerol prices. Researchers are looking for ways to convert glycerol into high-value-added products (5). Recently, pure or crude glycerol (55% to 90% pure) has been used as a carbon source in the production of microalgal lipids and to lower the cost of microalgal biodiesel. *Chlorella* sp. strain LB1H10 showed a 370% increase in lipid productivity and a 96% increase in biomass compared to those under the photoautotrophic condition (6). With the addition of 0.5% glycerol (by volume fraction), the biomass productivity and lipid productivity of *Chlorella* (*Auxenochlorella*) *pyrenoidosa* were three times and twenty times higher, respectively, than those of the control (7). It was reported that the maximum biomass concentration and lipid productivity of *Chlorella* (*Auxenochlorella*) *protothecoides* UTEX 256 grown on crude glycerol were higher than those of pure glycerol culture in batch mode (8). Both biomass productivities and TAG contents of *Botryococcus braunii*, *Neochloris oleabundans*, and *Dunaliella* sp. were enhanced with the addition of 5 g/liter crude glycerol (9). However, the growth rate and lipid production under mixotrophic growth with glycerol varied greatly with strains (10). It was reported that glycerol was inhibitive to the cell growth of *Chlorella vulgaris* (11). Of the twelve tested strains of *Chlorella*, four strains showed a remarkable increase in growth rates with glycerol addition, while the growth rates of five strains were relatively unaffected in the presence of glycerol and three strains displayed 18% to 49% reductions in growth rates when grown on glycerol (10). Besides, eight strains contained obviously higher lipid contents while some produced only minimal amounts of neutral lipids under mixotrophic conditions with glycerol addition (10). To our knowledge, little is known about the effects of glycerol on biomass and lipid production by *Dunaliella tertiolecta*.

D. tertiolecta is a halotolerant unicellular green alga that is simple to cultivate and not easily polluted and has been considered a potential candidate for biodiesel production. The lipid content of *D. tertiolecta* can reach up to 71% per dry cell weight (DCW) (12). *D. tertiolecta* displays great adaptability to abiotic stress (nutrient deprivation, salt stress, chemical stress, etc.) and accumulates large amounts of lipid (12–14). Like other chemical triggers that induced lipid accumulation in *Chlamydomonas* and *Chlorella* (15, 16), 100 ppm triethylamine increased lipid productivity and lipid content per cell up to 20% and 80%, respectively, in *D. tertiolecta* (13). However, biomass production and pigment contents were greatly decreased by triethylamine treatment in *Dunaliella* cells (13, 17). In fact, for the economic utilization of *D. tertiolecta* for biodiesel production, two factors should be optimized: biomass and lipid content. Here, a two-stage cultivation with glycerol and triethylamine was proposed in *D. tertiolecta* for greater lipid accumulation. For the first stage, cells were grown under an optimal growth condition (with glycerol addition) to achieve maximum biomass productivity. Then, the culture condition was altered to induce chemical stress (with triethylamine treatment) to enhance lipid accumulation in the second stage.

RESULTS AND DISCUSSION

Effect of glycerol on cell growth of *D. tertiolecta*. In this study, the effect of the addition of glycerol on cell growth of *D. tertiolecta* was investigated. Glycerol was added to the algal culture of *D. tertiolecta* at inoculation. Figure 1A shows that glycerol promoted the cell growth of *D. tertiolecta*. With the addition of glycerol, *D. tertiolecta*

FIG 1 Effects of different concentrations of glycerol on cell growth and lipid production of *D. tertiolecta*. (A) Cell growth of *D. tertiolecta* with glycerol addition. (B) Lipid production of *D. tertiolecta* with glycerol addition. au, arbitrary unit; **, $P < 0.01$ compared to the control without glycerol addition. (C) Images of cells stained with BODIPY 505/515 observed with confocal laser scanning microscopy (CLSM) after treatments with different concentrations of glycerol. The green indicates BODIPY-stained lipids. The red represents cell autofluorescence.

cells reached the late log phase in a short time (at day 10) due to rapid cell growth, while the normal growth of algal cells without glycerol addition reached late log phase at day 18. The cell number increased with increased concentrations of glycerol at a range of 1 to 4 g/liter. At a glycerol concentration of 5 g/liter, the cell number no longer increased due to an effect on the photosynthetic efficiency of algal cells. The growth rate under mixotrophic growth conditions with glycerol varied greatly with strains (10). Although 5 g/liter glycerol has been shown to yield the highest biomass concentration for *Chlorella vulgaris*, *Botryococcus braunii*, and *Scenedesmus* sp. (18), other reports found that high concentrations of glycerol inhibit the cell growth of microalgae and yeast (11, 19). Glycerol has become an inexpensive and abundant carbon source due to its generation from biodiesel production as an inevitable by-product.

Effect of glycerol on lipid concentration of *D. tertiolecta*. A lipophilic fluorescent dye, BODIPY 505/515, was used to measure lipid content, which is an efficient and inexpensive analytic method for detecting neutral lipid content and only needs a small sample volume (12). A linear relationship between fluorescence intensity detected with the BODIPY staining method and lipid weight measured with a traditional gravity weighing method was found: $y = 0.00115x + 0.170$ ($R^2 = 0.978$), where y was lipid content (grams per liter), and x was fluorescence intensity. In the following experiments

FIG 2 Effect of glycerol on cell growth and pigment contents of *D. tertiolecta* at the end of the first stage of cultivation. *, $P < 0.05$; **, $P < 0.01$ compared to the control without glycerol addition.

of lipid measurement, the BODIPY staining method and the aforementioned linear equation were used for the calculations of lipid content. The stronger green fluorescence intensity indicated larger lipid droplets and higher lipid accumulation. From Fig. 1B and C, stronger fluorescence intensity was observed without glycerol addition. This indicated that the addition of glycerol caused a decrease in the lipid production of *D. tertiolecta* with higher glycerol concentrations. This may be because the algal cells mainly used nutrients for cell division in the favorable growth environment, which led to the decline of lipid production. The high cell density caused by the addition of glycerol meant more precursors for membrane lipid biosynthesis, such as phosphatidic acid and diacylglycerol, which were also partitioned to the biosynthesis of storage lipids. This may be the reason for the decreased lipid production with glycerol supplementation. In addition, in the presence of glycerol, cells appeared smaller and more rounded than the controls (Fig. 1C).

The lipid production during mixotrophic growth with glycerol varied greatly with strains (10). It was reported that lipid productivities of *Botryococcus braunii*, *Neochloris oleabundans*, and *Dunaliella* sp. are enhanced with the addition of 5 g/liter crude glycerol (9). Of the twelve tested *Chlorella* strains, eight strains showed significantly high lipid contents, while some strains contained small amounts of neutral lipids under mixotrophic conditions with glycerol addition (10).

Biomass accumulation at the first-stage cultivation of *D. tertiolecta* with glycerol. Triethylamine inhibits the cell growth of *Dunaliella* cells, including *D. tertiolecta* and *Dunaliella bardawil* (13, 17), but increases lipid yield and lipid content per cell up to 20% and 80%, respectively, at 100 ppm compared with those of the control (13). We found that glycerol resulted in a high cell density and enhanced biomass of *D. tertiolecta* in this study. Thus, lipid can only be produced in a small amount under optimal growth conditions. Microalgal cells accumulate large amounts of lipids under stress conditions, such as nutrient limitation, salt stress, light stress, and chemical stress (20). Here, a two-stage cultivation with glycerol and triethylamine was proposed in *D. tertiolecta* for greater lipid accumulation. For the first stage, different concentrations of glycerol (0, 0.5, 1.0, and 1.5 g/liter) were added during the initial growth phase for high biomass density. After 16 days of glycerol addition, the first-stage cultivation was finished; the cell growth and pigment contents of *D. tertiolecta* are shown in Fig. 2. The cell biomass was increased by glycerol addition. The addition of glycerol at 1.0 g/liter resulted in the maximum biomass production (1.296 g/liter) for 16-day cultivation. The chlorophyll *a*, chlorophyll *b*, and carotenoid contents were also increased with the addition of glycerol.

FIG 3 Final biomass of *D. tertiolecta* cells treated with different concentrations of glycerol and triethylamine after the second stage of cultivation. *, $P < 0.05$; **, $P < 0.01$ compared to the control without triethylamine treatment.

Lipid accumulation at the second-stage cultivation of *D. tertiolecta* with trimethylamine. After the first-stage cultivation, different concentrations of triethylamine (0, 50, 100, and 150 ppm) were added for three more days at the lipid accumulation stage. It was reported that 100 ppm triethylamine for 3-day treatment on *D. tertiolecta* increases lipid productivity and lipid content per cell up to 20% and 80%, respectively, compared with those of the control (13). Here, the effect of the combined treatment with glycerol and triethylamine on lipid accumulation was compared with that of the single treatment with triethylamine for the same cultivation time. In the two-stage cultivation of glycerol-triethylamine-treated *D. tertiolecta* cells, the inhibitory effect of triethylamine on cell growth was alleviated by glycerol (Fig. 3). With the addition of 1.0 g/liter glycerol and 100 ppm triethylamine, the cell biomass was higher than for the other triethylamine-treated cells. The addition of triethylamine was reported to suppress the growth of *Dunaliella* cells, with a higher reduction in cell biomass with an increased concentration (13, 17).

After the two-stage cultivation, the pigment contents of *D. tertiolecta* cells supplemented with different concentrations of glycerol and triethylamine were measured (Fig. 4). With the single treatment of triethylamine, the pigment contents decreased dramatically, which was in agreement with another report (13). Pigment contents after treatment with 50 ppm triethylamine were higher than those with 100 to 150 ppm triethylamine. Triethylamine, as a lycopene cyclase inhibitor, enhances lycopene metabolic flow but significantly decreases the production of β -carotene and total carotenoids (17). With the combination of glycerol and triethylamine, cell growth and pigment contents increased with increasing glycerol concentrations when triethylamine was added at a certain amount (Fig. 3 and 4). These results indicated that glycerol reduced the inhibitory effect of triethylamine on cell growth and pigment accumulation in *D. tertiolecta*.

The effects of the combination of glycerol and triethylamine on lipid accumulation are shown in Fig. 5. Lipid weight contents were the highest with the single treatment of 50 to 150 ppm triethylamine, while 150 ppm triethylamine decreased the lipid productivity, which may be due to the low biomass caused by a high concentration of triethylamine. As for the treatment with the combination of glycerol and triethylamine, when glycerol addition was 1.0 g/liter and triethylamine was 100 ppm (experiment 11), the highest lipid concentration and lipid productivity were 383.60 mg/liter and 20.19 mg/liter/day in the presence of 1.0 g/liter glycerol, which were 27.36% higher than those of the control group. In contrast, the lipid weight content in experiment 11

FIG 4 Pigment contents of *D. tertiolecta* cells treated with different concentrations of glycerol and triethylamine after the second stage of cultivation. (A) chlorophyll a; (B) chlorophyll b; (C) chlorophyll a and b; (D) carotenoids. *, $P < 0.05$; **, $P < 0.01$ compared to the corresponding control without triethylamine treatment.

(0.377 mg lipid/mg cells) was lower than that with the single treatment of 100 ppm triethylamine (experiment 3). Without the addition of triethylamine, lipid productivities decreased with increasing concentrations of glycerol. All in all, lipid concentration and lipid productivity were increased by two-stage cultivation with 1.0 g/liter glycerol and 100 ppm triethylamine, which also provided a viable way to alleviate the inhibitory effect of triethylamine on algal growth and enhance lipid accumulation from *D. tertiolecta*.

The facilitation of lipid accumulation in *D. tertiolecta* by triethylamine may be due to the inhibition of the synthesis of β -carotene and the decrease of carotenoids. The carbon precursors for the synthesis of β -carotene may be used for lipid storage. Thus, triethylamine, as a lycopene cyclase inhibitor, can also act as a lipid inducer (13).

The two-stage cultivation of microalgae for lipid production is a widely used and efficient strategy. Auxin addition during the first stage enhances the biomass production of *D. tertiolecta* by 40%, and salt stress in the second stage results in lipid increases from 24% to 69.6% (14). When *C. vulgaris* is cultivated under a nutrient-sufficient condition at the first stage for high cell density, maximum lipid productivity is favored by nitrogen starvation conditions for 4 more days at the second stage (21). In the heterotrophic (with 10 g/liter glucose for high biomass) photoautotrophic (for high lipid content) two-stage cultivation, 25 mg/liter fulvic acid increases the lipid content of *Monoraphidium* sp. FXY-10 from 30.78% to 54.65% (22). The two-stage strategy has been also widely applied to increase the productivity of other important bioproducts, such as astaxanthin (23), eicosapentaenoic acid (24), carbohydrate, and starch (25).

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Glycerol (g/L)	0	0	0	0	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5
Triethylamine (ppm)	0	50	100	150	0	50	100	150	0	50	100	150	0	50	100	150

FIG 5 Lipid concentration and lipid weight content of *D. tertiolecta* cells treated with different concentrations of glycerol and triethylamine after the second stage of cultivation. *, $P < 0.05$; **, $P < 0.01$ compared to the control without glycerol or triethylamine treatment.

Effect of glycerol and triethylamine on cell morphology of *D. tertiolecta*. Figure 6 shows that 100 to 150 ppm triethylamine altered the cell morphology of *D. tertiolecta*, resulting in damage that was time dependent. Higher triethylamine concentrations and longer times of treatment led to more serious cell damage and toxicity to cells, while 50 ppm triethylamine did not cause an observable change in cell morphology. The addition of 0.5 to 1.5 g/liter glycerol alleviated the cell damage induced by 100 to 150 ppm triethylamine, suggesting that the two-stage cultivation with the combination of glycerol and triethylamine is a feasible way to alleviate the inhibitory effect of triethylamine on algal growth of *D. tertiolecta*.

Glycerol itself had no significant effect on the cell morphology of *D. tertiolecta* (Fig. 6). Glycerol enters cells simply by diffusion via a glycerol gradient (26, 27), which explains the high utilization of glycerol in *Dunaliella* cells. When glycerol was added to algal cells at inoculation, the cell growth of *D. tertiolecta* was boosted rapidly (Fig. 1A); however, the lipid content was decreased (Fig. 1B). These results indicated that glycerol promoted cell growth and decreased lipid production of *D. tertiolecta*. The glycerol added to *D. tertiolecta* was probably converted to glycerol-3-phosphate (G3P) by glycerol kinase, which may contribute to a larger amount of energy via glycolysis instead of precursors for lipid biosynthesis (28). Triethylamine treatment may result in a very high maintenance energy that can be (at least partially) alleviated by glycerol oxidation. Furthermore, glycerol is an important compatible solute in *D. tertiolecta*, which can accumulate large amounts of glycerol, especially under high salt stress (27). The addition of glycerol may reduce the precursors and energies for glycerol biosynthesis in *D. tertiolecta* to maintain its intracellular osmotic pressure. For further study, a dynamic analysis of glycerol in this two-step cultivation may be performed, which will be helpful for accurately monitoring the culture system for control purposes. Mathematical modeling of the dynamics of nitrogen and glucose indicates that there is good management of the N/C ratio for the optimization of lipid production in the oleaginous yeast *Yarrowia lipolytica* (29). Triethylamine greatly enhances lipid production and also affects the fatty acid profile, with an increase of $C_{16:0}$ and decrease of $C_{18:2}$ and $C_{18:3}$, which is beneficial for the use of *D. tertiolecta* as biodiesel feedstock (13). Since triethylamine suppressed cell growth and degraded chlorophylls and carotenoids, the incubation time with triethylamine should not be so long. The two-stage cultivation with the combination of glycerol and triethylamine is a feasible way to further improve

FIG 6 Effect of glycerol and triethylamine on cell morphology of *D. tertiolecta*. (A) Effect of glycerol and triethylamine treatment for 24 h on cell morphology of *D. tertiolecta*. (B) Effect of glycerol and triethylamine treatment for 72 h on cell morphology of *D. tertiolecta*.

lipid production and alleviate the inhibitory effect of triethylamine on algal growth of *D. tertiolecta*.

Conclusions. In conclusion, two-stage cultivation of *D. tertiolecta* with glycerol and triethylamine is a viable way to improve lipid productivity and alleviate the inhibitory effect of triethylamine on biomass production. Although glycerol decreased the lipid content, the late log phase was reached in a shorter time, with a high cell density due to the rapid cell growth of *D. tertiolecta* after the addition of glycerol. At the first stage, the maximum biomass production of *D. tertiolecta* was observed in the cultivation with 1.0 g/liter glycerol. At the second stage, triethylamine was added to induce chemical

stress to enhance lipid accumulation. With 100 ppm triethylamine and 1.0 g/liter glycerol for three more days, the highest lipid concentration and lipid productivity were obtained, which were 27.36% higher than those of the control group.

MATERIALS AND METHODS

Algal strains and biomass analysis. *Dunaliella tertiolecta* FACHB-821 was obtained from the Collection of Freshwater Algae Culture at the Institute of Hydrobiology, FACHB collection, Chinese Academy of Sciences. *D. tertiolecta* cells were grown in a *Dunaliella* medium containing 0.420 g/liter NaNO_3 , 0.015 g/liter $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.840 g/liter NaHCO_3 , 1.230 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.074 g/liter KCl, 0.044 g/liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 ml/liter Fe-EDTA solution (containing 1.804 g/liter Na_2EDTA and 0.483 g/liter $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), 1 ml/liter A5 trace elements solution [2.860 g/liter H_3BO_3 , 1.810 g/liter $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.220 g/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.079 g/liter $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.039 g/liter $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$], and 2.0 M NaCl at 26°C under a 14/10-h dark/light cycle, with 8,000 lx provided by cool-white fluorescent lamps, for approximately 24 days (30). The optical density of the algal cultures was read at 630 nm (OD_{630}) in a spectrophotometer (Agilent, USA). The dry cell weight (DCW) of algal biomass was determined from the OD_{630} value: $y = 993.21x - 16.359$, $R^2 = 0.9988$, where y is DCW (mg/liter) and x is the OD_{630} value ($0.05 < \text{OD}_{630} < 0.9$) (12). The algal cell number was also obtained from the OD_{630} value: $y = 3,418.3x + 226.33$, $R^2 = 0.9908$, where y is the cell number ($\times 10^4$) and x is the OD_{630} value (12).

Cultivation with glycerol and triethylamine in *D. tertiolecta*. To evaluate the effects of glycerol on cell growth and lipid production of *D. tertiolecta*, different concentrations (0, 1, 2, 3, 4, and 5 g/liter) of glycerol were added to the algal culture at inoculation. For two-stage cultivation with glycerol and triethylamine, different concentrations (0, 0.5, 1.0, and 1.5 g/liter) of glycerol were added to the algal culture at inoculation for the first-stage cultivation until the algal cells reached the late log phase (16 days). Then, the resulting algal cells were treated with different concentrations (0, 50, 100, and 150 ppm) of triethylamine for the second-stage cultivation for 3 days. Since the experimental design involves two variables at four levels, the 4^2 full factorial was applied. Each experiment was done triplicates. Glycerol concentrations (0, 0.5, 1.0, and 1.5 g/liter) and triethylamine concentrations (0, 50, 100, and 150 ppm) were selected as two independent variables.

Extraction and estimation of pigments from *D. tertiolecta*. The pigments from *D. tertiolecta* were extracted with 100% methanol, and the procedure was according to that described in our previous report (12). The contents of chlorophyll *a*, chlorophyll *b*, and total carotenoids were then calculated using the following equations (31):

$$\text{chlorophyll } a \text{ } (\mu\text{g/ml}) = 16.72 \times A_{665.2} - 9.16 \times A_{652.4}$$

$$\text{chlorophyll } b \text{ } (\mu\text{g/ml}) = 34.09 \times A_{652.4} - 15.28 \times A_{665.2}$$

$$\text{chlorophylls } a \text{ and } b \text{ } (\mu\text{g/ml}) = 1.44 \times A_{665.2} + 24.93 \times A_{652.4}$$

$$\text{carotenoids } (\mu\text{g/ml}) = (1,000 \times A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b) / 221$$

Lipid extraction from *D. tertiolecta* and measurement by the gravimetric method. Sixty milliliters of algal sample at the log phase was collected by centrifugation at 8,000 rpm for 5 min. Then, the algal pellet was washed twice with 1% NaCl solution, resuspended in 3 ml of chloroform-methanol (1:1 [vol/vol]), and disrupted by ultrasonication (300 W, 3 min) in ice water. Water was added to obtain chloroform-methanol-water (1:1:0.9 [vol/vol/vol]), and the solution was fully mixed and incubated for 10 min. The mixture was centrifuged at $10,000 \times g$ at 4°C for 10 min for phase separation. The upper layer (methanol-water layer) was removed, and the chloroform layer containing the lipids was separated out. The chloroform layer was collected, evaporated, and weighed as the total lipid.

Lipid measurement by the BODIPY 505/515 staining method. A lipophilic fluorescent dye, BODIPY 505/515 (Invitrogen, USA), was used to measure lipid content. Briefly, 2 μl of 1.0 mM BODIPY 505/515 solution in 0.1% dimethyl sulfoxide was added to the dilute algal sample and incubated in the dark at 35°C for 10 min. Then, the fluorescence intensity of the sample was determined by a fluorescence spectrophotometer (F-7000; Hitachi, Japan) with the excitation and emission wavelengths of 488 nm and 490 to 530 nm, respectively.

Confocal laser scanning microscopy observation of *D. tertiolecta* cell morphology. Microscopy analysis of algal cells stained with BODIPY 505/515 was performed using a confocal laser scanning microscope (Zeiss LSM 710 NLO). The BODIPY fluorescence (green) was excited with an argon laser (488 nm) and detected at 505 to 515 nm. The autofluorescence (red) of the algal chloroplasts was detected simultaneously at 650 to 700 nm.

Statistical analysis. All experiments were performed in triplicates. Values are expressed as the means and standard deviations (SDs) from three parallel measurements. The significance of differences between groups was assessed by a one-way analysis of variance (ANOVA). A P value of <0.05 indicated the presence of a statistically significant difference, and a P value of <0.01 was considered highly significant.

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