Effect of Salt Concentration on Intracellular Accumulation of Lipids and Triacylglyceride in Marine Microalgae *Dunaliella* Cells

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Running title: SALT STRESS FOR ACCUMULATION OF LIPIDS

In order to get the high liquefaction yield from marine algae cell mass to fuel oil, the effect of salt stress on the accumulation of lipids and triacylglyceride in *Dunaliella* cells was investigated. Although initial NaCl concentration higher than 1.5 M markedly inhibited cell growth, increase of initial NaCl concentration from 0.5 (equal to sea water) to 1.0 M resulted in a higher intracellular lipid content (67%) in comparison with 60% for the salt concentration of 0.5 M. Addition of 0.5 or 1.0 M NaCl at mid-log phase or the end of log phase during cultivation with initial NaCl concentration of 1.0 M further increased the lipid content (70%).

[**Key words**: algae, salt stress, liquefaction, lipid, feeding]

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Since atmospheric CO_2 accumulation has a serious effect on the global environment, the control of total CO_2 emission into the atmosphere is considered to be an important issue related to the biosphere. Marine microalgae are expected to play an important role in resolving this problem because they have a high capability for photosynthesis and grow well in the sea which solubilizes a high amount of CO_2 and which accounts for 70% of the surface area of the earth. There are two major approaches to large-scale CO_2 fixation by marine microalgae.

One is the production of non-carbon energy sources such as hydrogen gas from the algal biomass. The other is the conversion of algal biomass into liquid fuel oil by thermochemical liquefaction reactions at a high temperature and pressure (1). The latter process is expected to stimulate CO_2 circulation on earth, and is considered to be a simpler and more appropriate process for worldwide implementation than the former.

One of the most important criteria in assessing the performance of thermochemical liquefaction is the oil yield from the organic materials in the algal biomass. A report on liquefaction using various model substrates revealed that a high liquefaction yield was obtained from cell components containing hydrophobic compounds such as lipids, fatty acids and fatty acid esters (2). Among marine microalgae species, cells of the genus *Nannochloris* are known to contain a large amount of intracellular lipids (3, 4). It was reported that the intracellular triacylglyceride content increased after nitrate depletion during a cultivation of *Nannochloris* sp. UTEX LB1999 cells

in modified NORO medium containing 9.9 mM KNO_3 even though the intracellular lipid content did not increase and the oil yield following liquefaction of the cells increased in proportion to the intracellular triacylglyceride content (5). When 0.9 mM nitrate was intermittently fed 10 times during the log phase in addition to initial nitrate feed (0.9 mM), the concentration of *Nannochloris* sp. UTEX LB1999 reached almost the same (2.16 g/*l*) and the contents of intracellular lipids and the percentage of triacylglycerides in the lipids were respectively increased from 31.0% to 50.9% and 26.0% to 47.6%, compared with those of cells cultured in a modified NORO medium containing 9.9 mM KNO_3 without additional nitrate feeding (6).

The composition of intracellular lipid of microalgae was reported to change in response to environmental salinity. Increase of NaCl concentration from 0.4 M to 4 M increased saturated and monounsaturated fatty acids in *Dunaliella* cells isolated from an Antractic hypersaline lake (7), while polyunsaturated fatty acid decreased. The fatty acid composition of polar lipid in *Dunaliella salina* Teodoresco was affected significantly by the change in NaCl concentration (8). The percentage of saturated fatty acid decreased as the concentration of NaCl increased, while the percentage of highly unsaturated fatty acid increased (9). *Dunaliella tertiolecta* cells contain large amount of lipid and are highly salt tolerant (10), which might be appropriate for the large scale outdoor cultivation.

In this study, loading of salt stress during cultivation of *Dunaliella* cells for highly intracellular accumulation of lipids and triacylglycerides was

investigated, because the algae cell mass having high contents of lipids and triacylglycerides should result in a high liquefaction yield.

MATERIALS AND METHODS

Algal strain and media *Dunaliella tertiolecta* ATCC 30929 were used in this study as a marine CO_2-fixing microalgae strains and grown in modified NORO medium which had the following composition (per liter): NaCl, 29.2 g; KNO_3, 1.0 g (9.9 mM); $MgCl_2 \cdot H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.2 g; $CaCl_2$, 0.2 g; K_2HPO_4, 0.045 g; tris(hydroxymethyl)aminomethane, 2.45 g; EDTA · 2Na, 1.89 mg; $ZnSO_4 \cdot 7H_2O$, 0.087 mg; H_3BO_3, 0.61 mg; $CoCl_2 \cdot 6H_2O$, 0.015 mg; $CuSO_4 \cdot 5H_2O$, 0.06 mg; $MnCl_2$, 0.23 mg; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.38 mg; Fe(III) · EDTA, 3.64 mg; the pH was adjusted to 8.0 with 1 N HCl.

Cultivation Twenty ml of the modified NORO medium in a 100-ml Erlenmeyer flask was inoculated with the cells (OD_{680} 0.05) and incubated at 28°C with reciprocal shaking (60 spm) for 6 d. The light intensity on the surface of the flask was adjusted to 65 $\mu mol \cdot s^{-1} \cdot m^{-2}$ using fluorescent lamps. The flask cultivation was transfer to 500 ml of the same fresh medium having salt concentration 0.5 or 1.0 M in a Roux bottle to obtain OD_{680} of 0.05. The temperature, light intensity, and aeration conditions were 30°C, 150 $\mu mol \cdot s^{-1} \cdot m^{-2}$ and 250 ml/min CO_2-enriched air (3% CO_2), respectively.

Concentrated solution of NaCl (5.0 M) was fed at log phase, the end of log phase, or stationary phase during cultivation. Besides, test-tube static cultivation (10 ml) was performed.

Determination of cell and nitrate concentrations The cell concentration was determined by measuring OD_{680} and converted to ash-free dry cell weight employing the coefficient of OD_{680} to cell concentration of 0.01 ($g \cdot l^{-1} \cdot UOD^{-1}$). The precipitate obtained by centrifugation (3000 rpm, 15 min) of a 10-ml culture was heated overnight at 105 °C and weighed (w1 g). After carbonization of the precipitate for 3 min on a Bunsen burner, it was weighed again (w2 g). The ash-free dry cell weight (g/*l*) was determined by dividing the difference between w1 and w2 by the culture volume (0.01 *l*). The culture supernatant was diluted with the medium without KNO_3 and the nitrate concentration was determined from the difference between its absorbance values at 221.4 and 232.0 nm.

Determination of osmolarity The osmolarity of culture supernatant was determined using an osmometer (model OM-801; Vogel, Berlin, Germany).

Determination of the contents of lipids and triacylglycerides A culture sample containing about 30 mg cell was centrifuged and the resulting precipitate was washed with 1% NaCl. After extraction of lipids from the precipitate with methanol-chloroform (2 : 1), chloroform and 1% NaCl

solution was added to adjust the ratio of methanol, chloroform and water to 2 : 2 : 1. All the chloroform layer collected three times were evaporated, dried in a desiccators, and weighed as the total lipid (3). The percentage of triacylglyceride (TG) in the total lipids solubilized in isopropyl alcohol was determined by the glycerol-3-phosphate oxidase-p- chlorophenol method (triacylglyceride G-test Wako; Wako Pure Chemical, Osaka), with triolein as the standard.

RESULTS

Effect of initial NaCl concentration on the cell growth To clarify the effect of initial NaCl concentration on cell growth of *D. tertiolecta* ATCC 30929, test tube cultivations were performed with several initial NaCl concentrations (Fig. 1). Cell concentration decreased as NaCl concentration increased from 1.0 M to 2.0 M, while there was no decrease in the range of NaCl concentration less than 1.0 M. There should be marked growth inhibition by NaCl concentration higher than 1.0 M. Consequently, initial NaCl concentration less than 1.0 M was considered to be appropriate to achieve high cell concentration.

Effect of initial NaCl concentration on the intracellular lipid content

To confirm the effect of initial NaCl concentration less than 1.0 M on

intracellular lipid content, Roux bottle cultivations were performed employing two kinds of initial NaCl concentrations (0.5 and 1.0 M). Cell growth with initial NaCl concentration of 1.0 M was almost similar to the culture with initial NaCl concentration of 0.5 M, which was equivalent to that of sea water (Fig. 2). There was no apparent difference in the course of nitrate concentration during these cultures. The intracellular contents of lipids and triacylglyceride in lipid of cells harvested at the end of cultivation with initial NaCl concentration 1.0 M (67% and 56%) were markedly higher than those in the culture with 0.5 M NaCl (60% and 40%) as shown in Table 1.

Effect of NaCl addition during culture on cell growth and lipid content To investigate the effect of NaCl addition on cell growth and lipid content, several modes of NaCl addition were performed during the culture with initial NaCl concentration of 1.0 M. Namely, (i) addition of 0.5 or 1.0 M NaCl at the end of log-phase, (ii) addition of 0.5 M NaCl at mid-log phase. The intracellular contents of lipid and TG in lipids were measured at the end of cultivation. There was not marked difference in the course of OD_{680} and nitrate concentration except for the short time lag of growth after the addition 1.0 M NaCl at the end of log phase (Fig. 3). The final volume of culture with the addition was higher than that without the addition because of the addition. Although the final cell concentration and cell mass decreased by the NaCl addition during culture, the influence was smaller than the marked inhibition by initial high concentration of NaCl shown in Fig. 1.

Lipid contents in the culture with NaCl addition (70.6 – 71.4%, Table 2) were apparently higher than that without addition (63.5%). Consequently, the addition of NaCl (0.5 or 1.0 M) during culture with initial NaCl concentration of 1.0 M could increase lipid content with a slight decrease in cell mass.

DISCUSSION

Increase of initial NaCl concentration from 0.5 M (equal to Sea water) to 1.0 M did not decrease cell concentration, which might due to salt tolerant character of *Dunalilella* cells (8, 11). However, initial NaCl concentration higher than 1.0 M markedly inhibit cell growth. Consequently, initial NaCl concentration less than 1.0 M was considered to be appropriate to achieve high cell concentration.

Although the cultivations with initial NaCl concentration of 0.5 and 1.0 M showed similar time course of cell and nitrate concentrations, lipid content of cells cultivated with 1.0 M initial NaCl (67%) was higher than that with 0.5 M NaCl (60%) as shown in Table 1. Consequently, the reason for higher lipid content might be not nitrate limitation but high NaCl concentration. However, further increase in initial NaCl concentration should not be good strategy, because marked decrease in cell concentration by further increase in initial NaCl concentration was anticipated from the results of Fig. 1.

Addition of 0.5 and 1.0 M NaCl at mid-log phase or the end of log phase during cultivation with initial NaCl concentration of 1.0 M gave the

significant increase in lipid content (Fig. 3). Increase of initial NaCl concentration from 1.0 M to 1.5 and 2.0 M resulted in the 42% and 86% decrease in cell concentration (Fig. 1). However, the decrease in final cell mass by the addition of 0.5 and 1.0 M NaCl were only 6-10% and 17%, respectively (Fig. 3), although cell concentration at the time of NaCl addition was less than half of final cell concentration. Consequently, there might be the possibility of cell adaptation to high NaCl concentration by gradual increase in NaCl concentration, while mechanism is not clear.

The reversible change of cell size in response to water potential was reported (12). Microscopic observation in this study revealed that the size of most algae cells decrease after the addition of NaCl (data not shown). Ratio of smaller cells after 2.0 M addition was apparently higher that that with 1.0 M addition. Cell size recovered within 48 h after the additions. *Dunaliella* cells was reported to secrete glycerol in response to increase of NaCl concentration (13). The increase in lipid content may correlate with the adaptive response to high NaCl concentration such as cell volume change and glycerol production. However, the mechanism of lipid content increase by high NaCl concentration is not clear.

The amount of cell mass harvested from culture may decrease somewhat by NaCl addition during culture as shown in Table 2. The final objective of this process is achievement of not higher lipid and TG productivity's but higher oil productivity from cell mass by liquefaction reaction, which is the product of cell concentration and the oil yield. Therefore, the quantitative correlation between oil yield and cell composition such as lipid content and

TG content in lipid should be studied in future, in order to find the optimum operation condition about the time and amount of NaCl addition to the culture with initial NaCl concentration of 1.0 M.

In conclusion, the cultivation of *Dunaliella* cells with the high initial NaCl concentration of 1.0 M and the addition of 1.0 M NaCl during culture resulted in high intracellular lipid content and high percentage of triacylglyceride in the lipid. This cultivation technique should contribute to the increase of fuel oil productivity in the combined process of marine microalgae culture and thermo-chemical liquefaction.

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TABLE 1. Effect of initial NaCl concentration on intracellular contents of lipid an TG in lipid

Initial NaCl (M)	Osmolarity (Osm/kg)	Cell (g/*l*)	Lipid (%)	TG in lipid (%)
0.5	1.10 ±0.05	1.00 ±0.10	60.6 ±0.5	40.8 ±2.9
1.0	1.87 ±0.15	1.03 ±0.05	67.8 ±0.7	56.6 ±1.8

The intracellular content of lipid and TG content in lipid of cells harvested at the end of Roux bottle cultivations with initial NaCl concentrations of 0.5 and 1.0 M shown in Fig. 2 were determined. Osmolarity of culture supernatant was determined at the end of cultures. The average of duplicate cultures and the deviation were shown.

TABLE 2. Effect of NaCl addition on the contents of lipid and TG in lipid

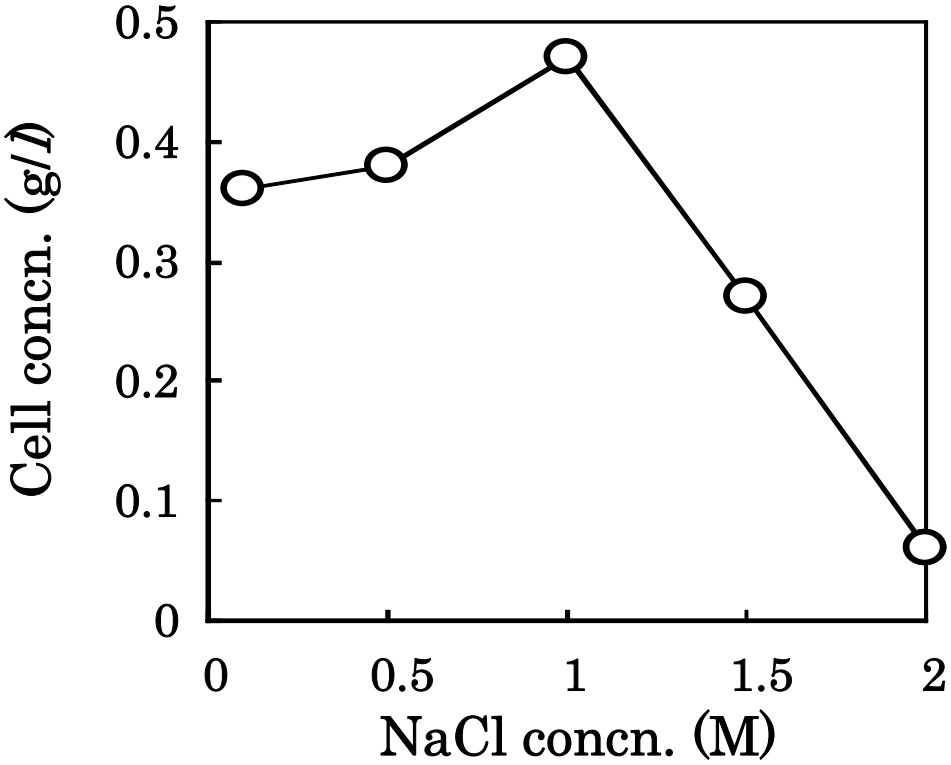
Culture	NaCl addition		Cell (mg/ml)	Volume (ml)	Cell (mg)	Lipid (%)	TG in lipid (%)
	Amount (M)	Phase					
1	-	-	0.92 ±0.02	360	331	63.5 ±1.0	30.4 ±3.5
2	1.0	End of log	0.68 ±0.04	400	272	70.6 ±3.9	34.5 ±2.2
3	0.5	Middle of log	0.76 ±0.06	390	296	71.4 ±2.3	34.3 ±3.7
4	0.5	End of log	0.72 ±0.05	430	309	70.6 ±1.5	31.8 ±6.0

Several amounts of NaCl was added at several culture phase during the culture with initial NaCl concentration of 1.0 M as shown in Fig. 3. The intracellular contents of lipid and TG in lipid of cells harvested at the end of each culture were determined. The average of duplicate cultures and deviation were shown.

FIG. 1. Effect of initial NaCl concentration on cell growth. Test tube cultivations were performed with several initial NaCl concentrations. The average of duplicate cultivations was shown.

FIG. 2. Cell growth with high initial NaCl concentration. During cultivations in Roux bottle with initial NaCl concentrations of 0.5 (circles) or 1.0 M (triangles), OD_{680} (open symbols) and nitrate concentration (closed symbols) were measured. The average of duplicate cultures was shown.

FIG. 3. Addition of NaCl during the culture with initial NaCl concentration of 1.0 M. During cultivation in Roux bottle with initial NaCl concentration of 1.0 M, NaCl was added at mid-log phase (triangles, 0.5 M) or the end of log phase (reverse triangles, 0.5 M; squares, 1.0 M). The data without NaCl addition during the culture was shown with circles. The addition times are indicated by arrows. OD_{680} (open symbols) and nitrate concentration (closed symbols) were measured. The average of duplicate cultures was shown.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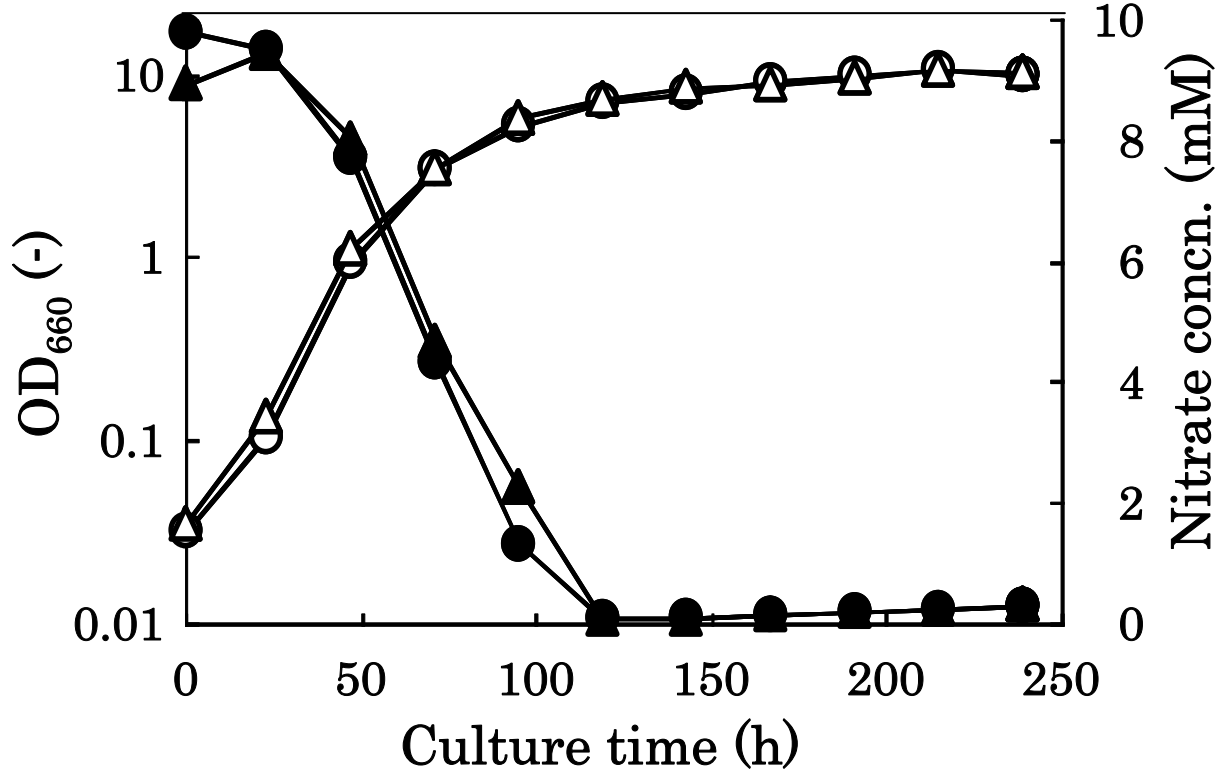
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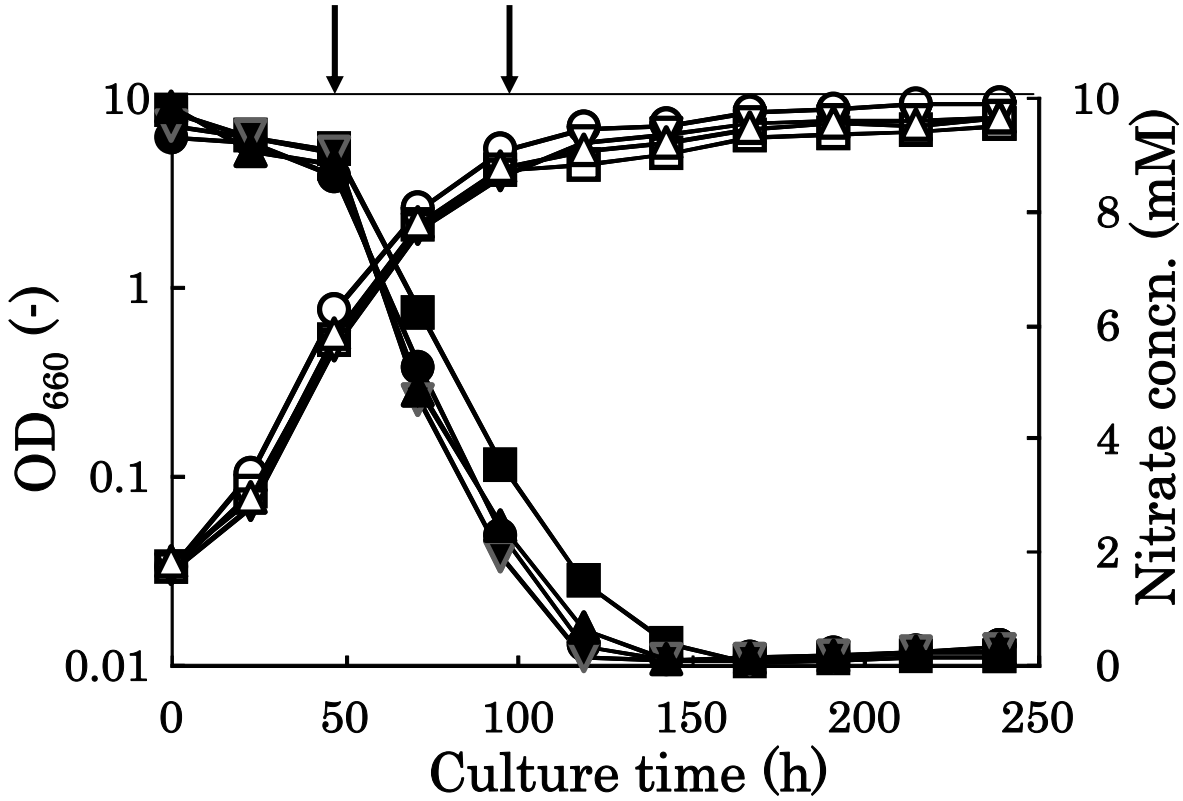
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