Addition of Sodium Chloride on Culture of *Dunaliella tertiolecta* ATCC 30929 : Implication on Intracellular Lipid Content

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ABSTRACT

The effect of addition sodium chloride (NaCl) on the accumulation intracellular of lipid 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 (eq. 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 culture with initial NaCl concentration of 1.0 M further increased the lipid content (70%). Addition of 2.0 M NaCl decreased final cell concentration to almost half of that without addition, although it resulted in very high lipid content of 77%.

***Key words**: algae, salt stress, lipid, addition*

INTRODUCTION

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.

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 Antarctic hypersaline lake (Xing-Xinq Xu and Berdall, 1997), 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 (Peeler et al., 1989). The percentage of saturated fatty acid decreased as the concentration of NaCl increased, while the percentage of highly unsaturated fatty acid increased (Fujii et al., 2001). Increase of salinity decreased eicosapentanoic acid production and increased arachidonic acid production by *Porphyridium cruentum* (Sasson, 1997). *Dunaliella tertiolecta* cells contain large amount of lipid and are highly salt tolerant (Elenkov et al., 1996), which might be appropriate for the large scale outdoor cultivation.

In this study addition of NaCl during cultivation of *Dunaliella* cells and its implication to intracellular accumulation of lipids was investigated.

MATERIALS AND METHODS

Algal strain and media

Dunaliella tertiolecta ATCC 30929 were used in this study as a marine CO_2-fixing microalgal strains and

·own in modified NORO medium which had the fol-·wing composition (per liter): NaCl, 29.22 g; KNO_3, 0 g (9.9 mM); $MgCl_2.H_2O$, 1.5 g; $MgSO_4.7H_2O$, 0.5 ; KCl,0.2 g; $CaCl_2$, 0.2 g; K_2HPO_4, 0.045 g; is(hydroxymethyl)aminomethane, 2.45 g; EDTA.2Na, 89 mg; $ZnSO_4.7H_2O$, 0.087 mg; H_3BO_3, 0.61 mg; $oCl_2.6H_2O$, 0.015 mg; $CuSO_4.5H_2O$, 0.06 mg; $MnCl_2$, .23 mg; $(NH_4)6Mo_7O24.4H_2O$, 0.38 mg; e(III).EDTA, 3.64 mg; the pH was adjusted to 8.0 with N HCl.

:ultivation

Twenty ml of the modified NORO medium in a 100-·l Erlenmeyer flask was inoculated with the cells (OD_{680} .05) and incubated at 28°C with reciprocal shaking (60 pm) for 6 days. The light intensity on the surface of ·e flask was adjusted to 65 mmol×s^{-1}×m^{-2} using fluo-escent lamps. The flask culture was transfer to 500 ml f the same fresh medium having salt concentration 0.5 r 1.0 M in a Roux bottle to obtain OD_{680} of 0.05. The emperature, light intensity, and aeration conditions vere 30°C, 150 mmol×s^{-1}×m^{-2} and 250 ml/min CO_2-en-iched air (3% CO_2), respectively. Concentrated solu-ion of NaCl (5.0 M) was added at log phase, the end of og phase, or stationary phase during cultivation.

)etermination of cell and nitrate concentrations

The cell concentration was determined by measur-ng the OD_{680} and converted to ash-free dry cell weight employing the coefficient of OD_{680} to cell concentra-·ion of 0.01 (g·l-¹·UOD⁻¹). The precipitate obtained by ·entrifugation (3,000 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 vol-ume (0.01 L). The culture supernatant was diluted with the medium without KNO_3 and the nitrate concentra-tion was determined from the difference between its absorbance values at 221.4 and 232.0 nm.

Determination of the contents of lipids

A culture sample containing about 30 mg cell was centrifuged and resulting precipitate was washed with 1% NaCl. After extraction of lipids from the precipi-tate with methanol-chloroform (2:1), chloroform and 1% NaCl solution was added to adjust the ratio of metha-nol, chloroform and water to 2:2:1. All the chloroform layer collected three times were evaporated, dried in a desiccator, and weighed as the total lipid (Ben-Amozt and Tornabene, 1985).

RESULTS AND DISCUSSION

Effect of initial NaCl concentration on the intracellu-lar lipid content

To confirm the effect of initial NaCl concentration less than 1.0 M on intracellular lipid con·ent, Roux bottle cultures were performed employing two kinds of ini-tial 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. There was no apparent difference in the course of nitrate concentration during these cultures (Fig. 1). The intracellular contents of lipids of cells harvested at the end of cultivation with initial NaCl concentration 1.0 M (67 %) were markedly higher than those in the culture with 0.5 M NaCl (60 %) as shown in Table 1.

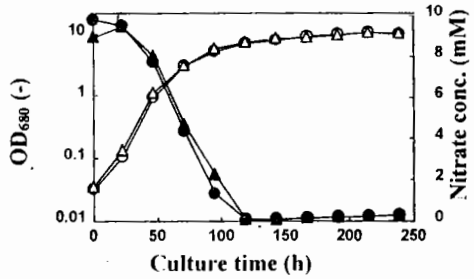

Figure 1. Cell growth with high initial NaCl concen-tration. During cultivations in Roux bottle with initial NaCl concentrations of 0.5 (Ë%) or 1.0 M (¡%), OD_{680} (open symbols) and nitrate concentration (closed symbols) were measured. Average of duplicate cultures was shown.

Table 1. Effect of initial NaCl concentration on intracellular contents of lipid

Initial NaCl (M)	Cell (%)	Lipid (%)
0.5	1.00 ±0.10	60.6 ±0.5
1.0	1.03 ±0.05	67.8 ±0.7

The intracellular contents lipid of cells harvested at the end of Roux bottle cultivations with initial NaCl concentrations of 0.5 and 1.0 M shown in figure 1 were determined. The average of duplicate cultures and deviation were shown.

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, (1) addition of 0.5 or 1.0 M NaCl at the end of log-phase, (2) addition of 0.5 M NaCl at mid-log phase. The intracellular contents of lipid was 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. 2).

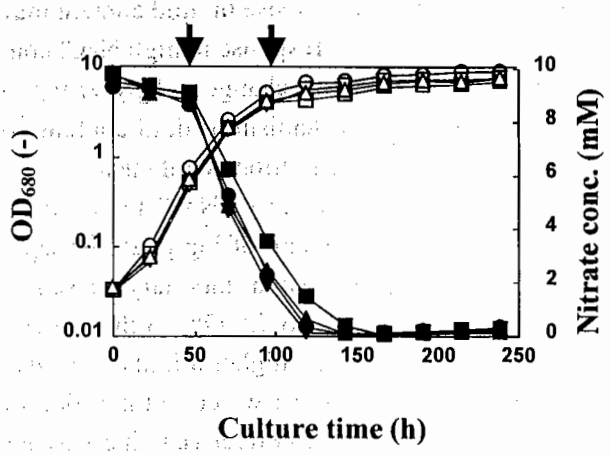

Figure 2. Addition of NaCl during the culture with initial NaCl concentration of 1.0 M. During cultivation in Roux bottle with initial NaCl concentration of 1.0 M, NaCl was added at mid-log phase (³%, 0.5 M) or the end of log phase (½%, 0.5 M; ¡%, 1.0 M). The addition times are indicated by arrows. OD_{680} (open symbols) and nitrate concentration (closed symbols) were measured. Average of duplicate cultures was shown.

The final cell concentration and cell mass were not significantly affected by the NaCl addition during culture. Lipid contents in the culture with NaCl addition (70.6 – 71.4%) were apparently higher than that without addition (63.5%) as shown in Table 2.

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

	Culture NaCl addition		Cell	Vol.	Cell	Lipid
	Amount (M)	Phase	(mg/ml)	(ml)	(mg)	(%)
1	-	-	0.92 ±0.02	360	331	63.5 ±1.0
2	1.0	End of log	0.68 ±0.04	400	272	70.6 ±3.9
3	0.5	Middle of log	0.76 ±0.06	390	296	71.4 ±2.3
4	0.5	End of log	0.72 ±0.05	430	309	70.6 ±1.5
5	-	-	0.90 ±0.04	350	315	67.1 ±2.5
6	1.0	End of log	0.66 ±0.03	410	270	72.7 ±5.4
7	2.0	End of log	0.45 ±0.03	580	260	77.7 ±3.3
8	1.0	End of log and stationary	0.51 ±0.03	440	224	72.0 ±0.7

Several amounts of NaCl were added at several culture phase during the culture with initial NaCl concentration of 1.0 M as shown in Figs. 2 and 3. The intracellular contents of lipid of cells harvested at the end of each culture were determined. The average of duplicate cultures and deviation were shown.

Consequently, the addition of NaCl (0.5 or 1.0 M) during culture with initial NaCl concentration of 1.0 M could markedly increase lipid content without significant decrease in cell mass.

To understand the effect of addition of higher amount NaCl than 1.0 M on cell growth and lipid content, 1.0 or 2.0 M NaCl was added during cultures with initial NaCl

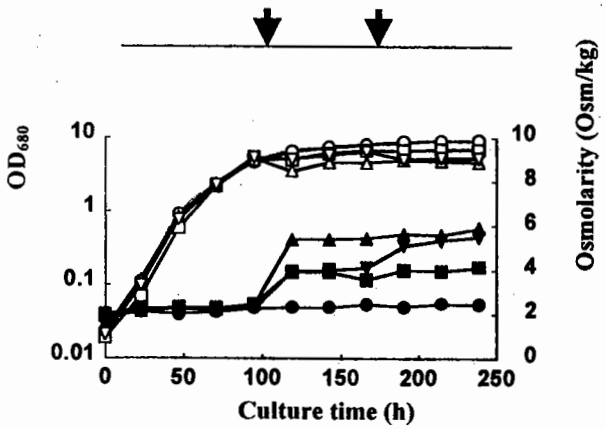

Figure 3. Addition of 2.0 M NaCl in the culture with initial NaCl concentration of 1.0 M. During cultivation in Roux bottle with initial NaCl concentration of 1.0 M, NaCl was added at the end of log phase (¡%, 1.0 M; ³%, 2.0 M) or at both of the end of log phase and stationary phase (½%, 1.0 M for each times). The addition times are indicated by arrows. OD_{680} (open symbols) and nitrate concentration (closed symbols) were measured. Average of duplicate culture was shown.

Contents of lipid and triglyceride in lipid were determined at the end of cultures. Namely, 1.0 or 2.0 M NaCl addition at the end of log phase, (2) two times addition of 1.0 M NaCl at the end of log phase and stationary phase (Fig. 3).

Addition of 1.0 M NaCl at the end of log phase during the culture with initial NaCl concentration of 1.0 M increased lipid content from 67 to 72% together with slight decrease in final cell concentration and cell mass. Lipid contents in the culture with 2.0 M NaCl addition at the end of log phase (77%) were apparently higher than that with 1.0 M addition (72%) although cell mass was slightly reduced (Table 2).

Increase of initial NaCl concentration from 0.5 M (eq. Sea water) to 1.0 M did not decrease cell concentration, which might due to salt tolerant character of *Dunalilella* cells (Peeler et., 1989; Borowitzka et al., 1977). 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 cultures 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.

The reversible change of cell size in response to water potential was reported (Galisnski, 1995). 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 with 1.0 M addition. Cell size recovered within 48 h after the additions. *Dunaliella* cells were reported to secrete glycerol in response to increase of NaCl concentration (Sadka et al., 1989). 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.

Addition of higher amount of NaCl than 1.0 M, namely 2.0 M, once at the end of log phase or separately at the end of log phase and stationary phase further increased lipid content to 72-77%, although cell mass was reduced. However, higher initial concentration compared with that of sea water and addition of NaCl during culture were very effective to increase intracellular lipid content and TG content in lipid (Tables 1 and 2). This change in cell composition might in-

crease the oil yield from algae cell mass as previously reported (Yamaberi et al., 1998). On the other hand, the amount of cell mass harvested from culture may decrease somewhat by NaCl addition during culture as shown in Table 2. Final objective of this process is achievement of higher oil productivity (g-oil/L-culture), which is the product of cell concentration and the oil yield. So, the quantitative correlation between oil yield and cell composition of lipid content and TG content in lipid should be studied in future, in order to decide 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 to 2.0 M NaCl during culture resulted in high intracellular lipid content and high percentage of triglyceride 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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