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# Evaluation of Fatty Acids in *Dunaliela tertiolecta,* in Various Culture Conditions

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# 배양 조건을 달리한 *Dunaliela tertiolecta*의 조체내 지방산 분석

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## ABSTRACT

Fatty acid contents were measured in the cultures of the flagellate green algae *Dunaliella tertiolecta* Butcher under different conditions of light intensity, duration of light, and temperature. Duration of light and temperature, in particular, affected the growth rate of *D. tertiolecta.* The maximun cell number reached 2.32 x $10^6$ cells/ml. The division rate per day was 1.97 in the exponential phase. The analysis of fatty acids obtained from various conditions showed that the lipid mainly contained C16, C18:3ω3 fatty acids and there was no significant level of polyunsaturated fatty acids such as EPA and DHA. Polyene fatty acid increased with decreasing temperature and light intensity did not influence on fatty acid composition.

The increasing duration of light enhanced the growth of *D. tertiolecta,* whereas polyene(ω3) slightly increased with decreasing the light period.

## 要約

녹조 편모류인 *Dunaliella tertiolecta* Butter를 광도, 광주기 및 온도 조건을 달리하여 배양한 후 조체내의 지방산 조성을 분석하였다. 광주기와 온도 조건은 *D. tertiolecta*의 성장에 뚜렷한 영향을 주었고, 성장기에 최대 세포수는 $2.32\times10^6$, 세포 분열율은 1.97 division/day였다. 여러조건에서 배양한 조체의 지방산을 분석한 결과 C16(Palmitic acid),C18 : 3ω3(*cis*-Linolenic acid)가 주성분이었으며 EPA나 DHA와 같은 고도의 불포화 지방산은 소량 검출되었다. 불포화 지방산(Polyene)은 배양 온도가 낮을수록 증가되었으며, 광도는 불포화 지방산 함량에 별로 영향을 미치지 못했다. 광주기 조건에서, 명주기를

오래할수록 *D. tertiolecta*의 성장은 증가했지만, 반면에 조체내의 불포화 지방산(Polyene)은 감소되는 것으로 나타났다.

# INTRODUCTION

The value of phytoplankton species as food for bivalve and other living organisms(rotifers, *Artemia*, etc.) is important in determining suitable diets for larval and juvenile animals in aquaculture system. Phytoplankton as food must supply both energy and essential nutrients. Although little is known of specific nutrient requirements for juvenile, lipid, carbohydrate and protein remain the major dietary source of energy for growth and development for which phytoplankton dictates biochemical composition and can affect energy and nutrient value(Fabregas *et al.* 1985). Especially, juvenile oysters are to a limted extent able to desaturate and elongate ω3 fatty acids to produce EPA and DHA, which are essential nutrients for their embryonic, larval development and sterol metabolisms in oyster adults. Pillsbery(1985) reported that lipid was accumulated during the larval stage to provide energy for the process of metamorphosis and Mortensen(1988) suggested that the growth potential of the bivalve molluscan larvae was correlated with the amount of lipid in the diets.

Much attention has been given to the algae of the genus *Dunaliella.* One of the merits of using these algae is the absence of the thick cellulose membrane. In addition to its use in aquaculture, *D. tertiolecta* is useful in the production of chemicals, e. g. glycerol, β-carotene and high-protein material as a source of single-cell-protein(SCP) and as source of minerals in fish diets. Here we investigated the response of the fatty acids in the cell of *Dunaliella* sp. to a variety of environmental conditions. The aim of the study was to determine the conditions required for the production of essential fatty acids and to predict the response of the organism, since this variability can affect its nutritive and commercial values when this species is used as food in mariculture, or in the production of chemicals and SCP.

# MATERIALS AND METHODS

## Materials

The marine microalgae *Dunaliella tertiolecta* Butcher, was obtained from the Culture Center for Algae and Protozoa(CCAP), Cambridge, England. It was mass-cultured under axenic conditions in F/2 media (Guillard & Ryther, 1962), harvested by a continuous centrifuge (Sorvall Model SS-A) and washed two or three times with distilled water. After lyophilization, the algal mass was kept below -30℃ until used.

## Culture conditions

Cell were cultured in seawater which had been filtered and sterilized under UV lamps and vitamin was injected into medium through Acrodisc(Gelman) after the sterilized medium was cooled. The salinity of the sea water was 30% . *D. tertiolecta* was grown in a sillicon-rubber stopped 3 L flask containing 2.5 L of medium. The flask was shaken 1-2 times/day to prevent cell adherring to the culture vessel wall. Inoculation and sampling of *D. tertiolecta* were axenically done by disposable syri-

nge through a fixed glass tube in the culture flask. The culture conditions were established in order to compare the effects of the different environmental factors; light intensity, duration of light and temperature. All cultures were maintained in three environments; controlled culture-room at 20±2℃ and in a diurnal growth chamber (Forma Scientific) with a diurnal cycle of 20℃ and 10℃. Duration of light were graded with 24L : 0D, 16L : 8D, 12L : 12D, 8L : 16D, at 2500Lux, 20℃ with aluminium foil and 24L : 0D at 1600 Lux. Cell counts were made daily with a hemocytometer after 1 ml sample was taken and fixed with 1 ml of formalin. The growth constants were determined by Stein(1973).

Fatty acid analysis(Fig. 1)

Dried cell were repeatedly extracted with a homogeneous mixture of chloroform : methanol : water (2 : 4 : 1, v/v, 50ml portion) until extracts were free from colour. Combined extracts were filtered through preweighed fused glass-fibre filters. Resultant filtrates were added to a 1 L seperating funnel and seperated into chloroform and aqueous-methanol layers by adding 100ml of chloroform and water. The chloroform layer concentrated in an evaporate (Buchi model 011) to an oil was weighed as the lipid fraction. Extracted lipid was vented with $N_2$ gas. MeOH-KOH(1ml, 0.5M) was added using glass syringe and the contents were stirred with a vortex mixer. The mixture was stirred at 85℃ for 30min. in a water bath and when cooled, haxane(1 ml) was added and stirred. The hexane containing unsaponified materials was withdrawn and discarded. Esterification regrent, $BF_3$-MeOH 2ml, 12% from sealed added and the mixture was stirred at 85℃ for 15min. When the mixture were tepid, hexane(1 ml) and saturated aqueous NaCl(0.5 ml) were added and stirred. The lower layer was withdrawn and the resultant organic layer was washed twice with satureted aqueous $NaHCO_3$. The hexane solution of methylesters was transferred to a clean, tared, screw-hole-cap vial fitted with a teflon-linsed disc and the solvent was evaporated in a vaccum chamber. After venting with $N_2$ gas, the vials was capped tightly and weighed, then hexane, 10μl/mg methylesters, was injected through the disc and the solution was stored at 2℃ until the analysis.

Chromatographic analysis

A Varian model VISTA 401 gas chromatography, fitted with a capillary inlet system, a film ionization and a detector and connected to an FID intergrator, was used for the fatty acid methylester. It was seperated on a Supelcowax 10(30m×0.32mm ID, 0.25μm film) operating 200℃ for 10 min. Hydrogen carrier gas was used and controlled at a linear pressure of 12 psi/min. with a split ratio of 1:30.

## RESULTS AND DISCUSSIONS

Cell growth

Fig.2 shows the growth rates of *Dunaliella tertiolecta* under various experimental conditions. Although light and temperature were major factors on the cell growth, temperature was responsible for the growth of *Dunaliella* sp. followed by light intensity(Chang 1968). Spectorova(1982) reported that the maximum algal density obtainable in closed installation system was 2.5 x $10^6$ cells/ml for *Tetraselmis,* 3 x $10^6$ cells/ml for *Denaliella* and 8 x $10^6$ cells/ml for *Isocrysis,* which in terms of dry biomass did not exceed 0.5g/l. In our experiment, with a full light period at 20℃, the maximum cell number reached 2.32 x $10^6$ cells/ml and the division rates per day was 1.97 in the exponential

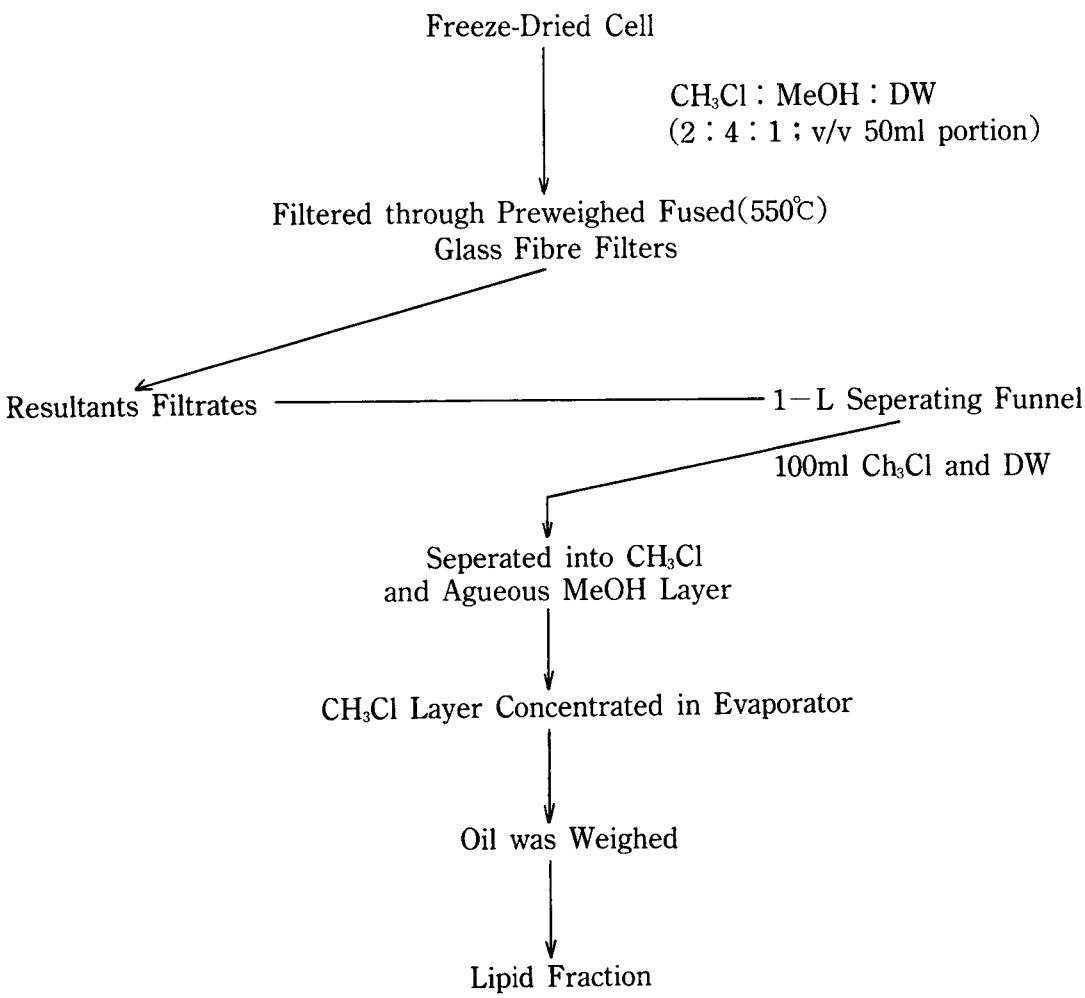


Fig.1 Flow sheet for lipid fraction.

phase and its dry mass was 0.4/gl. At 10*, the growth of *D. tertiolecta* was inhibited for 10 days and showed 0.37 division/day during these period. After increasing the temperature to 20℃ on 11th day, the growth rate rapidly increased up to 1.74 division/day for 5 days. As shown in Table 3, total lipid was increased at 10℃ culture more than at 20℃. It appeared that low-temperature tolerant strain formed the lipid layer in their cell when kept in low temperature condition.

Fatty acid composition

Analysis of fatty acids showed that the lipid was mainly composed of the palmitic acid(C16 : 0) and *cis*-linolenic acid(C18 : 3ω3) which are typical constituents of chloroplasts, and their formations were shown by various author to be enhanced by illumination for the chlorphycean species *Euglena gracilis* and *Chlorella vulgalis*(Pohl and Zurheide 1979). Although, Parsons *et al*(1961) determined that the average lipid content of phytoplankton was 10% of the dry weight of the cells, lipid content of *D. tertiolecta* was over 15% of dry weight in our unpublished data. Shifrin and Chisolm(1981)

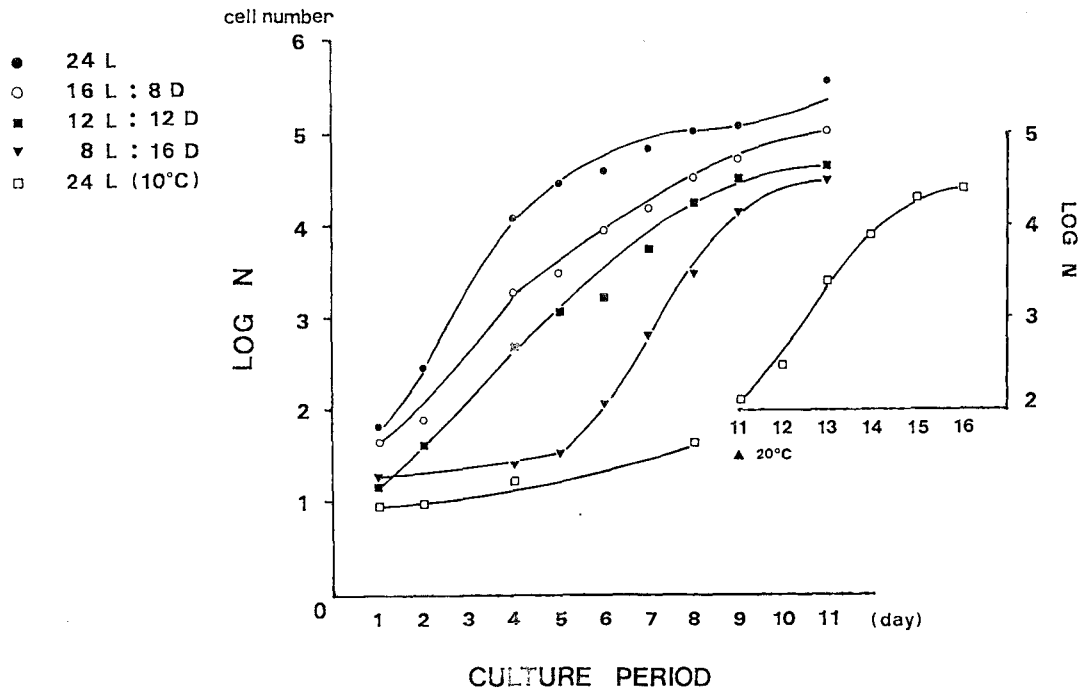


Fig.2 Growth rates of *Dunaliella tertiolecta* under various experimental condition.

reported that stationary phase phytoplankton cultures have lipid levels which are significantly higher than that of exponential phase culture. The same result was observed in our experiment. In recent years, the levels of poly unsaturated fatty acids(PUFA) ω3 have been subject to numerous investigation for their importance in fish and shellfish nutrients(Trider and Castell 1980a ; Chu and Webb 1984 ; Enright *et al.* 1986). In these experiments, however, no significant level of PUFA such as EPA and DHA was found.

Table 1 shows the fatty acid composition at different temperatures ; 20℃ and 10℃.. Polyene(include ω3) fatty acid increased at lower temperature and these results were in accordance with the reports of Mentensen(1988) which measured the ration of unsaturated to saturated and PUFA to monoene was showed highest at the lowest temperature. These results were in agreement with the fact that fatty acids with a high degree of unsaturation are required at low temperatures to maintain the flexibility and permeability of membrane phosholipid layers(Castell 1984). It has been shown that low temperature favors the formation of polyunsaturated fatty acids in marine microalgae.

Light intensity influence on fatty acid composition were examined from batch cultures grown at 20℃ under light intensities of 2500 Lux, 1600 Lux and 1000 Lux. Jones(1979) reported that there was no significant effect of light quality on the growth rate of *D. tertiolecta* at light saturation.However, when light intensity was below saturation, as in the low-intensity cultures, blue light enhanced growth relative to white light at equal quantum flux. Table 2 shows the polyene(include ω3) fatty acids in these culture were quite uniform, although fatty acids proportion is slightly increased with 2500 Lux. However, Mentensen *et al.*(1988) reported that the levels of PUFA(ω3) increased with

Table 1. Fatty acid composition of *Dunaliella tertiolecta* cultured

(unit, %)

| Fatty Acid | 20℃ 1600 Lux, 24L | 10℃ 1600 Lux, 24L |
|---|---|---|
| C 16 : 0 | 15.7 | 16.8 |
| C 16 : 1ω9 | 4.3 | 2.4 |
| C 18 : 0 | 0.3 | 0.3 |
| C 18 : 1ω9 | 3.5 | 0.3 |
| C 18 : 1ω7 | 0.6 | 0.9 |
| C 18 : 2ω6 | 5.2 | 5.6 |
| C 18 : 3ω3 | 34.1 | 41.4 |
| C 18 : 4ω3 | 0.8 | 0.8 |
| GLA | 3.4 | 3.2 |
| Total | 67.9 | 78.6 |
| Saturated | 16.0 | 17.1 |
| Monoene | 8.4 | 10.5 |
| Polyene | 43.5 | 51.0 |
| (ω3) | 34.9 | 42.2 |

Table 2. Fatty acid composition of *D. tertiolecta* cultured at different light intensity at 20℃

(unit, %)

| Fatty acid | 2500 Lux | 1600 Lux | 1000 Lux |
|---|---|---|---|
| C 16 : 0 | 22.2 | 15.7 | 12.8 |
| C 16 : 1ω9 | 1.6 | 4.3 | 6.8 |
| C 18 : 0 | 0.4 | 0.3 | 0.2 |
| C 18 : 1ω9 | 6.0 | 3.5 | 2.4 |
| C 18 : 1ω7 | 1.0 | 0.6 | 0.5 |
| C 18 : 2ω6 | 5.9 | 5.2 | 6.0 |
| C 18 : 3ω3 | 38.3 | 34.1 | 38.1 |
| C 18 : 4ω3 | 0.7 | 0.8 | 0.7 |
| GLA | 3.3 | 3.4 | 3.0 |
| Total | 79.4 | 67.9 | 70.5 |
| Saturated | 22.4 | 16.0 | 13.0 |
| Monoene | 8.6 | 8.4 | 9.7 |
| Polyene | 48.2 | 43.5 | 47.5 |
| (ω3) | 39.0 | 34.9 | 38.9 |

the increasing light intensity in the case of *Cbaetoceros gracilis* and suggested that there was a relationship between the light intensity and the level ω3 fatty acids present.

Duration of light affected on the growth of *D. tertiolecta* in these experiments. However, Table 3 showes that the amount of polyene (include ω3) fatty acids was slightly increasing with the decreasing light periods. These results are in accordance with Ressel *et al.*(1084) report. They demonstrated that with *D. tertiolecta* the rates of net protein synthesis at night equaled to those during the day. In our experiments, we found a negative correlation between polyene (include ω3) fatty acids contents and the growth rates of *D. tertiolecta.*

Even if the percent composition of certain long-chained poly unsaturated fatty acis (PUFA) is high, and if total lipid content is low, additional work is needed to determine the potential interaction between fatty acids and total lipid content, and their infleunce on food value. And Sournia (1982) reported that the reason why the genus *Dunalilla* has attracted the attention of inversigations are as follows ; 1) The cells have no thick cellulose membrane and are surrounded only by a thin elastic protoplasmic membrane which facilitated digestibility and raises assimilability by organisms of the next trophic link. 2) Flagellated cells do not settle and so are suitable for cultivation in tanks. 3) Can be adapted to extreme living conditions and to a chemical culture regime, for example, with their high carotenoid content, some *Dunaliella* species are used as raw material for the vitamin manufactoring industry (Masuk 1972). It may be also possible to produce glycerin from *D. tertiolecta* cells grown in a medium with high NaCl concentration (Frank and Wegmann 1974). As food for organi-

Table 3. Fatty acid composition of *D. . tertiolecta* at various duration of light at 20℃ under 2500lux

(unit, %)

| Fatty acid | 24L : 0D | 16L : 8D | 12L : 12D | 8L : 16D |
|---|---|---|---|---|
| C 16 : 0 | 22.2 | 17.3 | 22.6 | 20.9 |
| C 16 : 1ω9 | 1.6 | 3.5 | 3.7 | 1.4 |
| C 18 : 0 | 0.4 | — | 0.1 | 0.1 |
| C 18 : 1ω9 | 6.0 | 2.6 | 3.0 | 3.1 |
| C 18 : 1ω7 | 1.0 | — | 1.0 | 0.6 |
| C 18 : 2ω6 | 5.9 | 5.4 | 5.6 | 4.7 |
| C 18 : 3ω3 | 38.3 | 41.2 | 39.4 | 43.3 |
| C 18 : 4ω3 | 0.7 | 0.8 | 0.8 | 0.8 |
| GLA | 3.3 | 3.5 | 3.0 | 3.3 |
| Total | 79.4 | 74.3 | 79.2 | 78.2 |
| Saturated | 22.6 | 17.3 | 22.7 | 21.0 |
| Monoene | 8.6 | 6.1 | 7.7 | 5.1 |
| Polyene | 48.2 | 50.9 | 48.8 | 52.1 |
| (ω3) | 39.0 | 42.0 | 40.2 | 44.1 |

sms of the next trophic link, *D. tertiolecta* has been used, for instance, for feeding Harpacticoida, Artemia and rotifers in laboratories in Israel, the U.S.A and Great Britain (Mason 1963 ; Scott and Baynes 1979). *D. tertiolecta* has been cultured in the rearing of marine fish (Howell 1973 ; Spectorova and Doroshev 1979), although there is evidence that its use in the reasing of flatfish is limited (Howell 1979 ; Scott and Middleton 1979). *D. tertiolecta* is also used in rearing bivalve molluscs. For instance, in Australia it has been cultured as food for oysters in 400-l fuel barrels with an internal source of light, in Erdshreiber's medium without thermo-statting.

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