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Ultrastructural study and lipid formation of *Isochrysis* sp. CCMP1324

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Abstract. This study investigates methods for extracting lipids from microalgae and analyzes the effects of culture media as well as culture conditions on PUFA yields and total fatty acid contents. Experimental results of an optimal culturing of *Isochrysis* spp. were based on a 3.2% salinity culture medium. These microalgae were cultured in a 1-2 L Roux's flat-flask and a 5 L jar fermentor. The optimum culture temperature and initial pH for DHA production were 25°C and 8.0, respectively. Pigments included chlorophylls a and c. The DHA yield increased with cultivation time until the eighth day. Optimum DHA amounts in the cells were reached under aeration with 10% CO_2 and with continuous illumination of 10 klux. The biomass dry weight reached 4 g per liter of culture, and the DHA production reached 16 mg per liter of culture. Lipid bodies in *Isochrysis* spp. and related genera were observed during culture by light and transmission electron microscopy; 0.5~3.0 μm sized lipid bodies were confirmed by staining with Sudan Black B in cells from log stage to stationary stage cultures. These results demonstrated that DHA-containing lipid bodies in cells can be produced and accumulated in marine *Isochrysis* spp.

Keywords: Docosahexaenoic acid; *Isochrysis* sp.; Lipid formation; Polyunsaturated fatty acids (PUFA); Ultrastructure.

Introduction

Marine microalgae such as *Isochrysis* have received increasing interest because of their ability to produce the polyunsaturated fatty acid docosahexaenoic acid (DHA), one of the n-3 fatty acids believed to provide health benefits associated with the consumption of certain marine fish and their oils. DHA, a C_{22}-polyunsaturated fatty acid, and its derivatives help prevent and treat pathologies such as coronary heart disease and atherosclerosis (Norday and Hansen, 1994), inflammatory problems, and some cancers, and are believed to play a role in infant nutrition (Conner and Neuringer, 1987). DHA accumulates in the membranes of nervous, visual, and reproductive tissues (Dratz and Deese, 1986). Polyunsaturated fatty acids are especially helpful in preventing heart and circulatory disease and facilitating brain development in infants (Yongmanitchai and Ward, 1991). Fish oils may not be an ideal source of n-3 PUFAs due to their scarcity and odor, as well as geographical and seasonal variations in quality (Varela et al., 1990).

Isochrysis has been widely used as a mariculture feed due to its high content of long chain polyunsaturated fatty acids (PUFAs) (Jeffrey et al., 1994). However, the lipid class and fatty acid compositions of microalgal cells at different growth phases can differ significantly (Emdadi and Berland, 1989), and can change with variations in culture conditions e.g. nutrient status, temperature, salinity, pH, photoperiod, light intensity and light quality (reviewed by Yongmanitchai and Ward, 1989; Roessler, 1990). The cell structure of *Isochrysis* has attracted the attention of many investigators. Earlier studies (Green and Pienaar, 1977; Hori and Green, 1985; 1991) on *Isochrysis galbana* have mainly focused on its flagellar root system.

In this study, EM technologies were employed to survey this alga since previous papers have lacked detailed investigations of lipid formation in marine *Isochrysis*. This work also examines marine microalgae *Isochrysis* spp. as an alternative source of PUFAs and analyzes the culture medium and culture conditions that affect yields of PUFAs and their content in the total fatty acids. Lipid bodies in *Isochrysis* spp. and related genera are observed by light and transmission electron microscopy. Lipid granules are confirmed by staining with Sudan Black B in cells from the stationary cultures. The results suggest that DHA-containing lipid bodies in cells can be produced by marine *Isochrysis*. The possible commercial production of biomass and DHA-rich oil for use as food and feed ingredients is also predicted.

Materials and Methods

Cell Growth

Microalgal strains CCMP 463, 1324, 1325 and *Pavlova salina* were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West Boothbay Harbor, Maine USA). *Isochrysis galbana* TK1, TK2, were originally isolated by the Tungkang Marine Laboratory (Pingstung, Taiwan). *Nannochloropsis oculata* and *Chlo-*

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rella minutussima UTEX 2341 were obtained from our laboratory's previously collected strains. The cultures were grown under constant illumination in f/2 medium (Guillard and Ryther, 1962) at 25°C, pH 8, and an air-specific supply rate of 250 mL min^{-1}. Artificial seawater was sterilized in an autoclave at 120°C for twenty minutes. Microalgae were cultured in 1-2 L Roux's flat-flasks and 5 L jar fermentors. The different salinities (NaCl concentrations) examined were 0.8, 1.6, 2.4 and 3.2%. Sodium acetate 10~50 mM was applied to the mixotrophic culture of microalgae. All cultures were harvested by centrifugation during the stationary growth phase for subsequent analysis. The algal biomass was lyophilized and stored at -30°C, and the lyophilized cells were utilized for analysis within two weeks. A lipid analysis was performed after the saponification and methylation. The extracted pigment concentrations of chl a and chl c were estimated by spectrophotometry (Jeffrey and Humphrey, 1975).

Light and Electron Microscopies

The lipid bodies in cells from the stationary cultures were stained with Sudan Black B (Weete et al., 1997) and observed under a Normarski differential interference contrast light microscope (LM). The algal cells were collected by centrifugation at a higher concentration for viewing by a Normarski DIC on a Nikon E-600 microscope.

The algal cells for electron microscopy (EM) were collected by centrifugation at 3,000 *g*, and fixed with 2.5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2. The cells were postfixed for two hours with 1% osmium tetroxide in a cacodylate buffer. The fixed material was washed once in a cacodylate buffer prior to dehydration. The samples were dehydrated in a series of 30, 50, 70, 85, 90, 95 and 100% (v/v) acetone solutions for ten minutes each. The dehydrated cells were suspended in a 50:50 mixture of Spurr's resin and acetone for one hour and then embedded in 100% Spurr's resin. The embedded samples were polymerized at 65°C for twenty-four hours and sectioned using an LKB ultramicrotome. Thin sections were picked up on 300-mesh copper grids and post-stained with uranyl acetate for thirty minutes. After rinsing with distilled water, the ultra-sections were post-stained with lead citrate for four minutes and finally rinsed with distilled water. The sections were examined under a transmission electron microscope (JEOL JEM 1200 EXII) at an accelerating voltage of 80 kV.

Total Lipid and Fatty Acid Analysis

The total lipid was extracted from dry cells (ca. 20 mg) using the Bligh and Dyer (1959) procedure. The dry cells were re-extracted two or three times with small portions of $CHCl_3$-MeOH (2:1, v/v) and purified by removing nonlipid contaminants (Folch et al., 1957). The total amount of lipid was calculated from the gas chromatographic data. The methyl ester derivatives of the fatty acid methyl esters were directly prepared from the cells, after adding pentadecanoic acid FAME (fatty acid methyl ester) as an internal standard. All FAME were analyzed by FID-GC, using a capillary column (RTX 225, 30 m length, 0.32 mm in diameter) in a Hewlett Packard 5890 gas chromatograph. The initial oven temperature was set at 150°C, followed by a temperature program of 4°C min^{-1} to a final oven temperature of 210°C. The injector and detector temperature were set at 230°C, and the flow rates for hydrogen and air were 30 and 400 mL min^{-1}, respectively. Fatty acid contents were determined by comparing their peak areas with that of the internal standard.

Results

The growth and biomass production of *Isochrysis* spp. (in Roux's flat flask cultures) were examined after eight days of growth, the cells were harvested and the solvent extractable lipid content of cells was determined. The DHA

Table 1. The fatty acid composition of several marine microalgae (% total fatty acids).

Fatty acid	*Pavlova salina*	*Isochrysis* sp. CCMP 463	*Isochrysis* sp. CCMP 1324	*Isochrysis galbana*	*Isochrysis galbana* TK1	*Pavlova lutheri* CCMP 1325 TK2	*Nannochloropsis oculata*	*Chlorella minutussima* UTEX2341
14:0	10.1±0.2	10.4±0.9	10.2±0.5	17.5±1.0	16.3±0.9	10.3±0.5	5.1±0.4	4.5±0.4
16:0	23.4±0.4	14.9±0.7	17.6±0.6	14.3±0.4	12.9±0.7	20.8±1.2	32.1±1.4	33.9±1.6
16:1n-7	6.2±0.1	4.5±0.2	3.9±0.1	6.3±0.5	4.0±0.1	18.4±0.4	24.9±1.7	23.2±1.0
18:0	0.9±0.1	N.D.	N.D.	N.D.	N.D.	0.4±0.1	2.7±0.2	2.9±0.1
18:1n-9	16.9±0.3	29.8±1.2	32.0±1.4	15.1±0.7	28.1±1.0	3.3±0.5	16.5±0.9	20.4±1.1
18:2n-6	7.8±0.4	5.7±0.4	4.1±0.3	8.8±0.4	3.0±0.1	1.9±0.1	1.9±0.3	3.4±0.1
18:3n-3	3.1±0.1	6.4±0.5	6.4±0.3	8.2±0.3	5.5±0.2	1.5±0.2	N.D.	N.D.
18:4n-3	5.9±0.1	17.5±0.9	15.1±0.6	24.9±1.4	18.9±0.3	6.8±0.5	N.D.	N.D.
20:4n-6	1.6±0.1	N.D.	N.D.	N.D.	N.D.	N.D.	2.8±0.2	1.9±0.1
20:5n-3	11.8±0.3	N.D.	N.D.	N.D.	N.D.	21.0±0.5	9.4±0.7	8.7±0.2
22:6n-3	4.4±0.4	10.7±0.5	10.9±0.3	8.2±0.6	11.1±0.4	6.2±0.3	N.D.	N.D.
ΣUn-3	25.2	34.6	32.3	41.4	31.8	35.5	12.2	8.7
ΣUn-6	9.4	5.7	4.1	8.8	3.0	1.9	4.7	5.2
n-3/n-6	2.7	6.1	7.9	4.7	10.6	18.7	2.6	1.7

N.D. = None detected.

yield increased with cultivation time until the eighth day (data not shown). Eight species of marine microalgae were examined, including *Pavlova lutheri* CCMP1325, *Pavlova salina*, *Isochrysis galbana* TK1, *Isochrysis galbana* TK2, *Isochrysis* sp. CCMP1324, *Isochrysis* sp. CCMP463, *Nannochloropsis oculata*, and *Chlorella minutussima* UTEX 2341. Each species was cultured and analyzed for its fatty acid composition (Table 1). The fatty acid distribution of *Isochrysis* spp. and *P. lutheri* (Haptophyceae) revealed the dominance of 14:0, 16:0, 16:1n-9 or 18:1n-9 and 22:6n-3. In addition, *Isochrysis* spp. had a higher content of 18:1n-9 and 18:4n-3, whereas *P. lutheri* had a higher level of 20:5n-3. The abundance of PUFAs demonstrated a pronounced variation between algal species and classes. A similar finding was reported previously (Kjell et al., 1994). According to Table 1, 22:6 n-3 composition of *Isochrysis* sp. CCMP1324 and *Isochrysis* sp. CCMP463 were 10.9% and 10.7%, respectively, found to be higher in 22:6 contents and 20:5n-3 was absent. They were then subjected to further culture tests, and to test similarities in growth rates. Typical growth curves showed a lag phase of 4 days, a log phase of 4 to 8 days, and 8 to 14 days in a stationary phase under these conditions.

The extracted pigment concentration of chl a and chl c were estimated by spectrophotometry; chl a and c content were 2.8 µg/mL and 1.4 µg/mL in the culture periods (data not shown). Optical and electron microscopic photographs, confirmed the presence of lipid bodies (or osmiophilic droplets) in *Isochrysis* sp. CCMP 1324 (Figures 1-3). Many oil droplets accumulated in the late phase of culture, and the lipid granules in cells from the stationary cultures were confirmed by staining with Sudan Black B (Figure 1). The TEM photomicrographs demonstrate the lipid body formation occurs in the thylakoid space of the chloroplast structure. Their sizes vary with growth phase stage, and they finally form a rounded shape (Figure 2). The accumulated dense lipid granules were partially dissolved and diffused into cytosol and form less dense, large lipid globules. Lipid bodies from 0.5 to 3.0 µm were detected in samples harvested from the four to eleven day culture. The oil droplet accumulation, surveyed at each growth stage and illustrated in the EM photographs (Figure 3), was similar to that in a previous report (Weete et al., 1997). The *Isochrysis* sp. cells have no distinct cell wall, as confirmed by Zhu et al. (1997), and only possess a plasma membrane covering. Cells are generally solitary, motile, 5-6 µm long, 2-4 µm wide, and 2.5-3 µm thick in ellipsoid forms. There are two flagella, more or less equal, smooth, approximately 7 µm long, cells inserted with abbreviated haptonema; normally plastid usually single, parietal, yellow-brown with an immersed fusiform pyrenoid, the latter traversed by a pair of thylakoids, resembled that described for *Isochrysis galbana* previously (Green and Pienaar, 1977). The cells were fragile, and plasmolysis occurred when the naked cells were exposed to a sudden change of osmotic pressure. Some of the TEM micrographs are similar to those in a previous report (Goldman and Dennet, 1985). The ultrastructural morphological

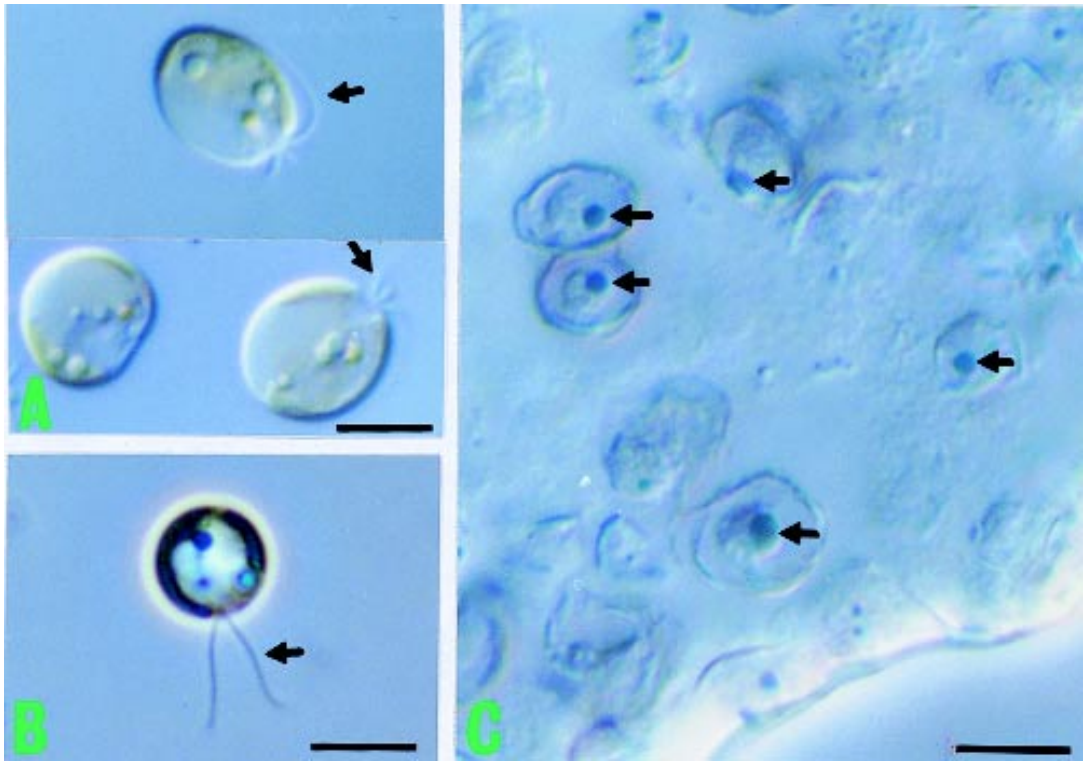

Figure 1. Nomarski differential interference contrast microscopic photograph of *Isochrysis* sp., cells, showing two flagella at one end of a cell (A) (arrows). The photograph of phase contrast microscope, was shown in (B). After staining with Sudan Black B, showing the lipid accumulation inside the cells (C) (arrows). Scale bar = 5 µm.

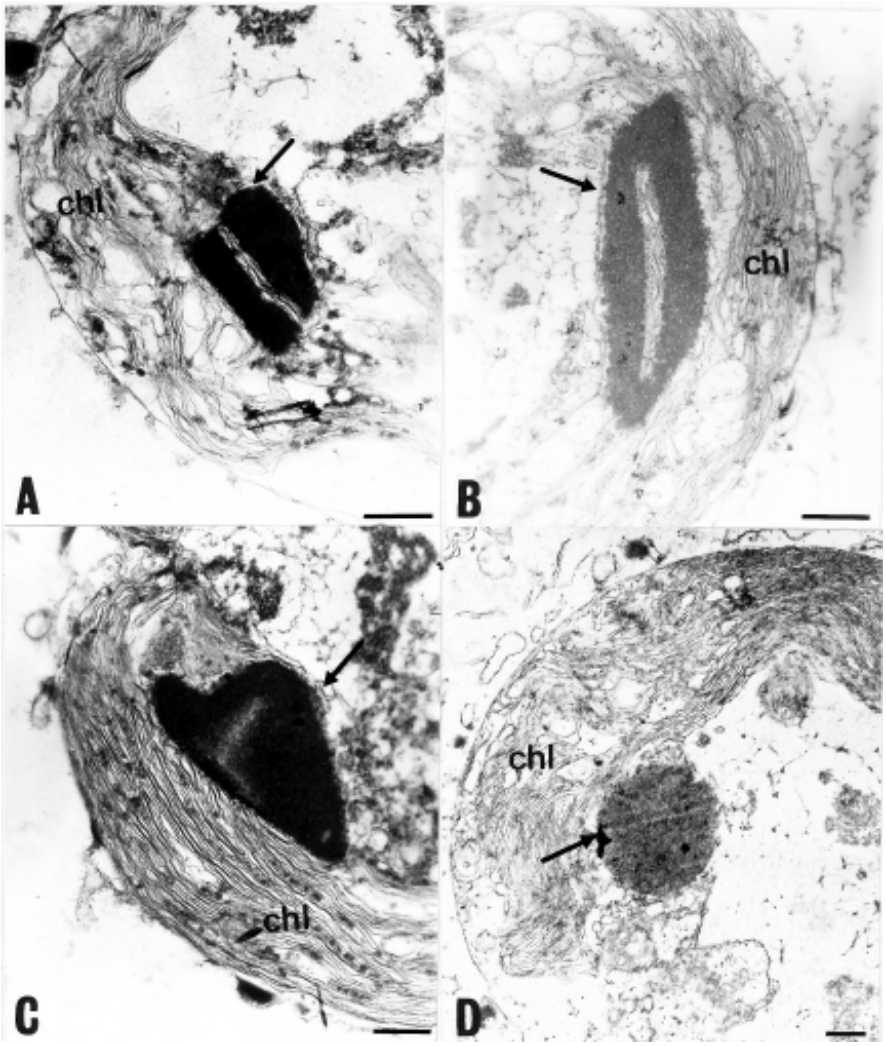

Figure 2. Transmission electron micrographs of *Isochrysis* sp. CCMP 1324, showing the lipid body (arrow) formation in chloroplast (A~C), their size from small to large and finally rounded in spherical form (D) at early log phase. Scale bar = 200 nm.

Table 2. Influence of different salinities on the fatty acid composition of *Isochrysis* sp. CCMP 1324 (% total fatty acids).

Fatty acids	NaCl conc			
	0.8%	1.6%	2.4%	3.2%
14:0	18.4±0.9	18.6±0.9	16.9±1.2	16.3±0.6
16:0	15.5±0.7	13.4±0.7	13.4±0.7	12.9±0.4
16:1n-7	5.6±0.3	5.5±0.4	4.3±0.2	4.0±0.2
18:0	N.D.	N.D.	N.D.	N.D.
18:1n-9	27.7±1.1	27.9±1.7	28.0±1.4	28.1±1.5
18:2n-6	4.3±0.2	6.2±0.7	5.0±0.4	3.0±0.3
18:3n-3	5.1±0.2	4.8±0.2	4.9±0.3	5.5±0.1
18:4n-3	14.9±0.9	14.6±0.7	17.1±0.8	18.9±1.1
20:5n-3	N.D.	N.D.	N.D.	N.D.
22:6n-3	9.4±0.9	8.9±0.7	10.5±0.9	11.2±0.9
Total n-3	29.4	28.4	32.4	35.6
PUFA	33.6	34.9	37.4	38.6

N.D. = None detected.

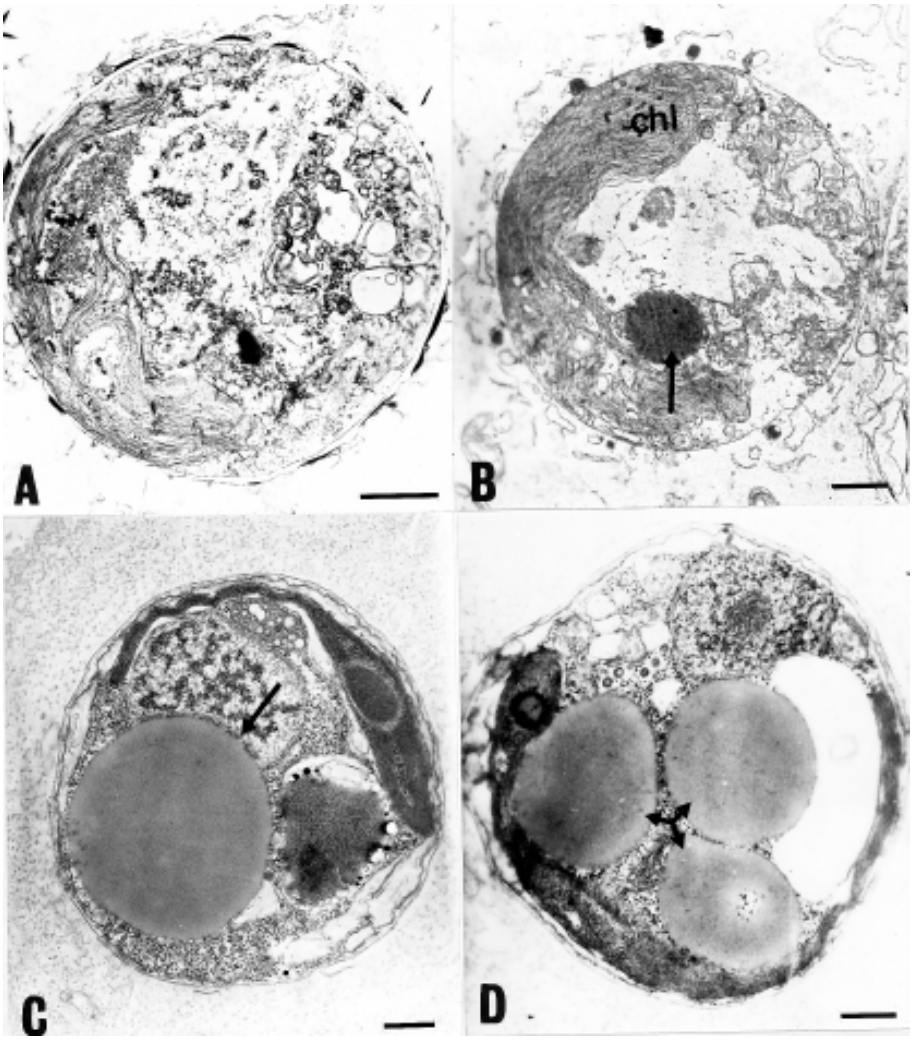

Figure 3. Transmission electron micrographs of *Isochrysis* sp. CCMP 1324 vegetative cell. After the fourth day of growth, no oil drop was observed (A), but oil droplets (arrows) could be observed in the stationary phase (6~11th day) (B~D). Scale bar = 500 nm.

Table 3. Variation in fatty acid composition of *Isochrysis* sp. CCMP 1324 in different sodium acetate concentration (% total fatty acids).

Fatty acids	CH_3COONa				
	10 mM	20 mM	30 mM	40 mM	50 mM
14:0	17.8±0.9	16.5±0. 8	16.4±0.4	15.9±0.7	16.9±0.5
16:0	12.8±0.6	11.9±0.5	12.0±0.4	11.7±0.4	12.7±0.4
16:1n-7	4.7±0.2	4.5±0.2	3.7±0.1	4.6±0.1	4.8±0.1
18:0	N.D.	N.D.	N.D.	N.D.	N.D.
18:1n-9	30.7±1.2	31.8±1.4	32.9±1.2	32.4±1.5	33.5±1.0
18:2n-6	5.7±0.2	4.8±0.1	4.7±0.2	4.9±0.2	3.8±0.1
18:3n-3	5.1±0.3	4.4±0.2	4.7±0.1	4.7±0.4	3.9±0.1
18:4n-3	12.8±0.5	13.1±0.4	13.6±0.7	13.0±0.5	12.7±0.3
20:5n-3	N.D.	N.D.	N.D.	N.D.	N.D.
22:6n-3	10.3±0.6	13.1±0.5	11.9±0.5	12.7±0.8	12.1±0.1
Total n-3	28.3	30.8	30.2	30.4	28.7
PUFA	34.0	35.4	34.9	35.3	32.2

N.D. = None detected.

changes appear to be associated with lipid synthesis in these microalgae.

The fatty acid analysis, from the GC profile of *Isochrysis* sp. CCMP1324, indicated that 22:6n-3 ranged from approximately 9-11%. The acid 18:1n-9, was the dominant fatty acid, and 20:5n-3 was below detection. The fatty acid composition of microalgal cells can vary in different growth phases and with changes in culture conditions such as nutrient status, temperature, salinity, pH, light intensity, and aeration rate (Yongmanitchai and Ward, 1989). In spite of these variations 18:1n-9 was the main fatty acid at all growth rates. Environmental factors were altered to enhance PUFA production, especially the DHA content. The optimum culture temperature and initial pH for DHA production were 25°C and 8.0, respectively. Cells did not grow well when deprived of suitable illumination, but optimum growth was achieved by continuous illumination at 10 klux light.

The lipid contents of *Isochrysis* sp. increased with an increase of salinity. Lipid content was higher (DHA and PUFA were 11.2% and 38.6%, respectively) at 3.2% salinity (Table 2). The biomass dry weight reached 0.23 g per liter of culture, and the DHA production reached 4.6 mg per liter of culture (Figure 4). From the data, the microalgae growth well in higher salinity, and attempt to reduce the salinity, the cell growth and lipid composition were not good than in higher salinity. For mixotrophic culture, the DHA in the cells increased when sodium acetate was added to the *Isochrysis* sp. CCMP1324 culture. The appropriate concentration of sodium acetate for mixotrophic culture of *Isochrysis* sp. was 10 mM (Figure 5) and the DHA content was 20 mM (Table 3). Optimum DHA amounts in the cells were reached under aeration with 10% CO_2, and continuous illumination at 10 klux in our study. The dry weight of cells reached 4 g per liter of culture, and the DHA production reached 16 mg per liter of culture in a fermentor experiment (data not shown).

Discussion

Isochrysis is a commonly used marine algal feed for aquaculture. The main pigments are chlorophyll a, c_1, and c_2, but c_3 is not present (Zapata and Garrido, 1997), and there is more chl a than chl c. *Isochrysis galbana* Parke and clone T-ISO strains, both members of the Haptophyceae, are often referred to simply as *Isochrysis galbana* despite some obvious differences (Whyte, 1987). While DHA is present in both, the *Isochrysis galbana* clone T-ISO lacks EPA. In addition, the optimum growth temperature of T-ISO is 27.5°C, while for *Isochrysis galbana* Parke it is 20°C (Molina et al., 1994). The strains employed herein were *Isochrysis* spp. CCMP 463 and 1324. These strains also lack EPA, which correlates with data in a report by Jeffrey et al. (1994), and we believe that they belong to *Isochrysis galbana* clone T-ISO and the strain CCMP1324, which both originate from Tahiti. The original CCMP report also notes this *Isochrysis* sp. is the clone synonym : TISO : NEPCC 601. Thus, *Isochrysis* sp. CCMP 1324 species is termed herein as *Isochrysis galbana* clone T-ISO. Since the strain grows at relatively high temperature (30°C in the present study) and contains DHA, it could be cultivated in the tropics as planktonic feed for mariculture.

Fatty acid synthesis may be qualitatively and quantitatively affected by environmental conditions such as media composition, temperature, light intensity, and the age of the culture (Bajpai and Bajpai, 1993). For *Isochrysis* sp. CCMP 1324, this study demonstrated that the highest total DHA amounts in cells were attained under aeration with 10% CO_2. The optimum illumination was achieved by continuous illumination at 10 klux light. The GC profile revealed that *Isochrysis* sp. CCMP 1324 had DHA as a major, and 18:1n-9 as the dominant, fatty acid. Previous reports by Saoudi-Helis et al. (1994) and Kjell et al. (1994) have suggested that oleic acid (18:1n-9) prevents heart and cir-

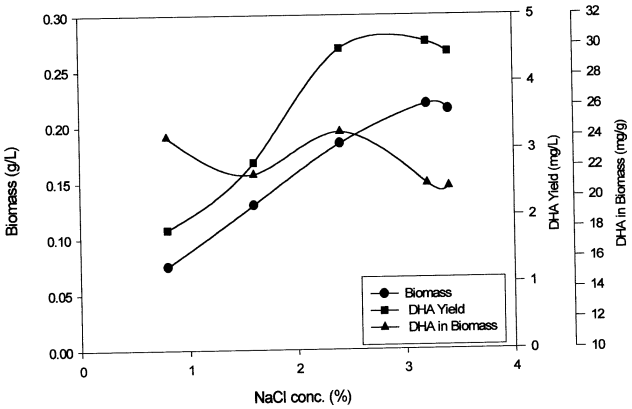

Figure 4. The biomass and DHA yield of *Isochrysis* sp. CCMP 1324 in each liter from cultures grown at different NaCl concentrations.

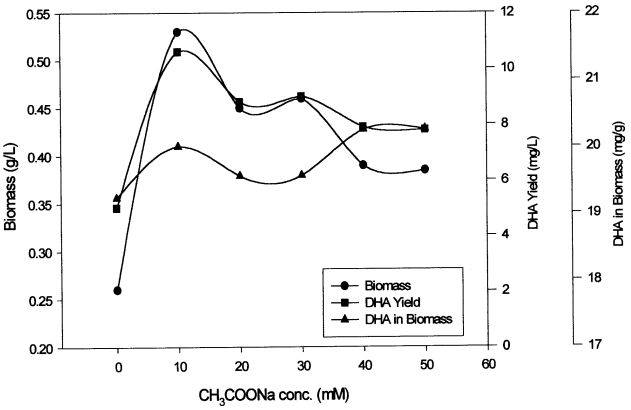

Figure 5. The biomass and DHA yield of *Isochrysis* sp. CCMP 1324 in each liter from different sodium acetate concentration in cultures.

culatory diseases, and also has general health benefits. However, *Isochrysis* sp. (clone T-ISO) contained the unusual very-long-chain unsaturated methyl and ethyl alkenones, hydrocarbons, and methyl and ethyl esters of a 36:2 fatty acid (Volkman et al., 1980; Marlowe et al., 1984). The role of these compounds in *Isochrysis* sp. is not known, but it seems likely that they are associated with membrane structure (Dunstan et al., 1993). These alkenones are significant components in stationary phase cultures of *Isochrysis* (25.6% of total lipid), a fact which must be taken into account when calculating fatty acid content from total lipid (Lopez Alonso et al., 1992). It may also affect the lipid formation in this microalga, especially for the distribution of lipid body's density.

The EM photographs illustrate that several oil drops accumulated during the late phase of growth. The lipid body formed in the inner thylakoid spaces of the chloroplast structure. Their sizes ranged from small to large in different growth phase stages, and they finally formed a rounded shape. The accumulated dense lipid granules were partially dissolved and diffused into cytosol and formed less dense large lipid globules. The oil droplet accumulation was surveyed at each growth stage and is illustrated in the EM photographs (Figure 3). These show prominent lipid bodies similar to those in a previous report (Weete et al., 1997). Lipid bodies of 0.5 to 3.0 μm existed in cells containing 3~7 granules. These structures seem to be closely associated with lipid synthesis. The concentrations of polyunsaturated fatty acids increased significantly as the culture reached stationary stage, which correlates with data in a report by Moreton (1987). LM and EM technologies were employed to survey this alga since no investigations of the marine *Isochrysis* sp. CCMP 1324 lipid formation have previously been done. The DHA in algal oil exhibited a greater degree of oxidative stability than that in fish oil. It also lacks the fishy odor or taste present in fish oils (Varela et al., 1990). The oil from *Isochrysis* sp. CCMP 1324, which contains various polyunsaturated fatty acids, has an advantage in the DHA purification process. The haptophyte *Isochrysis* sp. is a common marine unicellular algae for aquaculture (Sukenik and Wahnon, 1991). The developed strain can be cultivated in the tropics as a planktonic feed for mariculture since it can grow at relatively high temperatures and contains a high quantity of n-3 PUFAs (Zhu and Lee, 1997). Haptophytes are also rich in B, C, D, and K vitamins. The cells are easily assimilated by larval animals because of their small size and absence of a tough cell wall. Other attributes include fast growth rates, easy mass-culture, wide temperature and salinity tolerance, and absence of toxins (Jeffrey et al., 1994). Therefore, *Isochrysis* spp. are a potential source of DHA where marine microalgae are used. *Isochrysis* sp. CCMP 1324 appears be an another promising source for microbial DHA production since it has a simple polyunsaturated fatty acid profile and is quite productive. We believe that DHA-containing lipid bodies in cells can be produced and accumulated in sufficient quantity for mass culturing and DHA production.

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等鞭金藻的脂質形成過程與超微結構之研究

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利用光學及穿透式電子顯微鏡技術，觀察海洋微藻 *Isochrysis* sp. CCMP 1324 其形態與脂肪形成之過程，發現其脂肪含有多元不飽和脂肪酸，尤其是廿二碳六烯酸 (DHA)，為時下流行之健康食品，對於人體健康維護及水產養殖之飼料，提供相當大之助益。在 10% CO_2 之通氣及 10 klux 之連續照明下，細胞中廿二碳六烯酸之含量繼續增加至八天之培養，最終含量為每升含16毫克。實驗中並探討其脂肪在生長過程之形成與變化，為首次利用電顯探討等鞭金藻油脂形成之研究報告。

關鍵詞：廿二碳六烯酸；等鞭金藻；油脂形成；多元不飽和脂肪酸；超微結構。