**PhD Dissertation**

# Studies on the practical production of docosahexaenoic acid using thraustochytrid microorganisms

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# Abstract

Docosahexaenoic acid (DHA) is one of the essential fatty acids required for the maintenance of good health. It is found in high amounts in the brain and retina as part of the phospholipid component of neurons and therefore is necessary for good functioning of the brain as well as in treating illnesses such as Alzheimer disease and depression. As consumption of fish, which is a major source of DHA in the diet, decreases because of the modern lifestyle, DHA can be consumed directly as a food supplement. Fish oil is the main source of commercial DHA; however, the industry is currently facing some problems such as decreasing fish catch and accumulation of heavy metals because of marine pollution. Microbial oil is a potential alternative to fish oil such as that from marine thraustochytrid microorganisms. These microorganisms normally require seawater for cultivation because of the requirement of NaCl for growth; however, the chloride ion corrodes industrial equipment, thereby resulting in high maintenance costs. This study focuses on the cultivation of thraustochytrid strain 12B without the use of seawater by investigating the impact of major salts present in the seawater to come up with a medium containing low NaCl that can produce cells with high DHA yield. The taxonomic attributes of strain 12B were also studied in relation to other thraustochytrids as well as in relation to DHA production. The possible role of DHA accumulated in the thraustochytrid was also investigated. NaCl in the culture medium was able to be reduced to 0.1% (w/v), and high cell yield was obtained when $MgSO_4$ was also supplemented in the culture medium. The NM medium, which contains 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$, was found to produce a higher cell yield and DHA productivity compared with the conventional seawater containing F medium, and cells grown in the NM medium produced a 18.8 mg/ml cell yield with DHA productivity of 2.4 mg/ml/day, compared with cells grown in the F medium, which produced a 11.4 mg/ml cell yield and 0.7 mg/ml/day DHA, respectively. The low 0.1% (w/v) NaCl content in the culture medium is very favorable, as it does not lead to the corrosion of metal industrial equipment. Using inexpensive industrial-grade peptone, glucose and yeast extract, production cost could be reduced by 30% compared with the F

medium. The DHA yield was also increased in cells grown in the industrial-grade medium (INM) compared with cells grown in the NM medium. Based on the sequence analysis of the mitochondrial cytochrome oxidase subunit II gene and the 18S rRNA gene, together with data from morphological and physiological features, strain 12B is tentatively considered to be a new thraustochytrid species under the genus *Aurantiochytrium*.

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# List of symbols, acronyms and abbreviations

BHT: Butylated hydroxyl toluene

C16:0: Hexadecanoic acid

C15:0: Pentadecanoic acid

C18:0: Octadecanoic acid

C18:1: Oleic acid

C18:3: Lenolenic

C20:5: Docosapentadecanoic acid

C22:6: Docosahexaenoic acid

CoA: Coenzyme A

COX: Cytochrome oxidase

CW: Cell wall

EPA: Eicosapentaenoic acid

DCW: Dry cell weight

DHA: Docosahexaenoic acid

Dob: Dark oil body

FAME: Fatty acid methyl ester

F.a.: Fatty acid

ITS: Internal transcribed spacer

INM: Industrial grade NM medium containing industrial grade glucose, peptone and yeast extract.

INMK: INM medium supplemented with 0.05% (w/v) $K_2SO_4$

Lob: Light oil body

M: Medium supplemented with $MgSO_4$ but deficient in NaCl

mt: Mitochondrium

N: Medium supplemented with NaCl but deficient in $MgSO_4$

n: Cell nucleus

NM: Medium supplemented with 0.1% NaCl and 1% $MgSO_4$

OD: Optical density through absorbance value at 600 nm

PB: Potassium phosphate buffer

PCR: Polymerase chain reaction

PUFA: Polyunsaturated fatty acid

ROS: Reactive oxygen species

r.t.: Room temperature

SEM: Scanning electron microscope

SWP: Seawater containing pollen

TEM: Transmission electron microscope

TG: Triacylglycerol

TLC: Thin layer chromatography

UV: Ultraviolet radiation.

w/v: Weight by volume

v/v: Volume by volume

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# General introduction

## A. Food and diet

Food is one of the essential keys for life by providing energy for growth and for physiological maintenance. Human beings require a balanced diet consisting of 45–65% carbohydrate, 10–35% proteins and less than 30% fats within the total daily calories (NIH, 2001). The amount of food is further determined by factors such as age, gender, and activity (resting or exercising), and body conditions, such as injury, sickness, and pregnancy. The environment, including both geography and climate, can become a factor determining the amount and kind of food consumed, and mental states, temperature, and humidity can affect body physiology. The environment also determines what sources of food will be available in any particular geographic location, which shapes the food culture and is reflected in the differences in the servings within the balanced diet food pyramid published by different nations around the world (Fig. 1). The recommended dietary allowance for Japanese has been determined and published by the Japan Society of Nutritional and Food Science. The estimated requirement for an average adult (18–49-year-old) Japanese man is 2650 kcal/day (Tabata et al., 2013), out of which 20–30% of the total energy should come from fat.

The type of fatty acid can be divided into saturated fatty acids, n-6 and n-3 unsaturated fatty acids, for which an estimated dietary goal has been determined to be 4.5–7%, <10% and <2.2%, respectively, in order to prevent the onset of lifestyle-related diseases (Ezaki et al., 2013). The consumption of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are n-3 fatty acids, are 0.36 and 0.63 g/day, respectively, in Japan, which is the highest in the world (Elmadfa & Kornsteiner, 2009). However, it is still below the maximum recommended daily intake for combined EPA-DHA of 2 g/day (WHO, 2010).

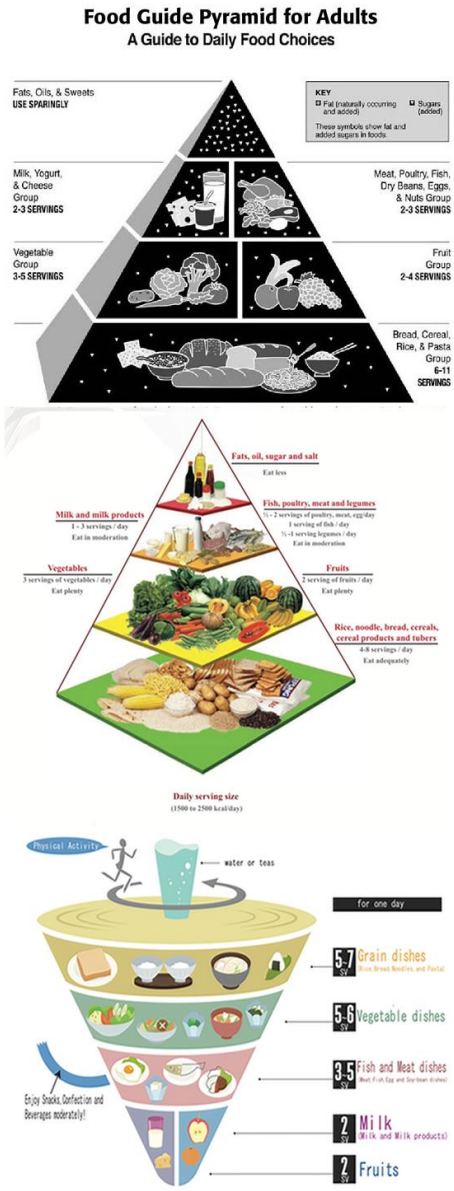


Figure 1. Food pyramid for a balanced diet published by different nations. From top to bottom: U.S. (NIH, 2001), Malaysia (MOH, 2013) and Japan (MAFF, 2013). 'Serving' refers to the portion normally served per sitting. Type of food and serving varies geographically and culturally. Generally, the amount of food from large to small is in the order of carbohydrates, vegetables and proteins. Fats are included in the U.S. and Malaysian schemes but not in the Japanese one probably because of the high intake of fish, which is a good source of oil, among the Japanese population.

## B. Benefit of DHA to health

DHA contributes to good health through its effects on the central nervous system, the immune system, and the cardiovascular system. It is a major component of the brain, retina and neurons, making up 50% of fatty acid in the brain (Makrides et al., 1993, Sinclair, 1997) and more than 60% of the fatty acid in the rods of retina photoreceptors (Giusto et al., 2000). The fact that it is difficult to deplete DHA from the neural membranes further emphasizes its important role in the nervous system (Kim, 2007). DHA can be transformed into neuroprotectin D1 (Hong et al., 2003), which prevents neuronal apoptosis (Kim, 2000, Akbar, 2005) and promotes neuron survival (Akbar et al., 2005) as well as neuronal differentiation (Calderon & Kim, 2004). Besides its structural role in membranes, DHA also plays a role in gene expression, where it was found to act as a ligand to a nuclear receptor, the retinoid X receptor (RXR), which in turn acts as a transcription factor for the transcription of retinol-binding protein type II gene in the brain (Mangelsdorf et al., 1991, Mata de Urquiza et al., 2000, Lenqovist et al., 2004). In the immune system, n-3 fatty acids, DHA and EPA, have been known to reduce inflammation, while eicosanoids from n-6 fatty acids, such as arachidonic acid (ARA), promote inflammation (Sinclair, 1993). The degree of DHA and EPA effects, however, may vary and may also be mutually exclusive. DHA has a stronger effect than EPA, for example, in decreasing proinflammatory cytokines TNF-α, IL-1β and IL-6 expression in THP-1 macrophage (Weldon et al., 2007), and inhibiting cytokine activator NF-kB mediated proinflammatory nitric oxide production in RAW264 macrophages (Komatsu et al., 2003). On the other hand, DHA—but not EPA—was found to decrease amounts of the membrane lipid raft harboring the cytokine IL-2 receptor (Li et al., 2005), which probably contributes to the reduction in inflammation. Similarly, in the cardiovascular system, both DHA and EPA may have overlapping positive effects on health such as protection against arrhythmia (abnormal heartbeat) by binding to sodium channels, resulting in longer recovery (Xiao et al., 1998), by decreasing intracellular calcium release in myocytes, and by reducing the contractile events (Negretti et al., 2000). Studies also found that DHA prevents vascular diseases (Leaf et al., 2008) such as atheroma (thickening of

artery walls) by reducing the expression of vascular and intercellular adhesion molecules VCAM-1 and ICAM-1, respectively (De Caterina et al., 1994, 2000).

Other health benefits of DHA and EPA are their effects of lowering triacylglycerol (TG) level (Mozaffarian & Wu, 2012) and enhancing the viability of insulin-secreting cells (Suresh & Das, 2001). DHA also has antioxidative potential (Okuyama et al., 2008b), lowering the risk to colorectal cancer and breast cancer with an intake of 500 mg/day (WHO, 2010). Furthermore, the bioconverted DHA also has antibacterial (Shin, 2007) as well as antifungal properties (Bajpai et al., 2008). DHA also has been shown to be safe for consumption. At high concentration (2 g per kg body weight per day), a slight increase in liver and spleen weight (extramedullary hematopoiesis) to accommodate the large dietary lipid load without any accompanying histopathological symptoms was observed, and the only significant pathological symptom with very high DHA consumption (7.5% of diet) is lipogranuloma; i.e., the formation of yellow spots in the abdominal adipose tissue (Kroes et al., 2003).

## C. Sources of DHA

There are two major sources for DHA synthesis in the human body: the liver (Scott & Bazan, 1989) and the astrocyte glial cells (Moore et al., 1991); however, DHA can also be directly derived from the diet. DHA (C22:6) is made up of 22 carbon chain fatty acids with six *cis*-unsaturated double bonds (at Δ4, 7, 10, 13, 16, 19). Inside the body, it is synthesized in low quantities from n-3 lenolenic acid (C18:3), which is an essential fatty acid. It can be synthesized through three possible pathways: the 'aerobic Δ4 desaturation-independent', the 'aerobic Δ4 desaturation-dependent' (Sprecher pathway) and the 'anaerobic polyketide synthase' pathways (Qiu, 2003). The Δ4 desaturation-independent pathway occurs in mammals through desaturation and elongation where successive elongation and desaturation from C18:3, to C22:5 occur in the microsome, while C22:5 to C22:6 (DHA) occurs in the peroxisome. The aerobic Δ4 desaturation-dependent pathway, on the other hand, occurs in thraustochytrid microorganisms, where DHA synthesis

only involves the microsome by direct desaturation of C22:5 to C22:6. The anaerobic polyketide synthase pathway slightly resembles the fatty acid synthase cycle. The fatty acid chain is increased by the condensation of acyl-ACP with malonyl-ACP, producing a 3-ketoacyl-ACP, which is then reduced forming 3-hydroxyacyl-ACP followed by dehydration to enoyl-ACP and another reduction to acyl-ACP. For DHA synthesis, however, double bonds are formed by the condensation of enoyl-ACP with malonyl-ACP instead of undergoing another reduction to acyl-ACP.

DHA in the diet is mainly obtained from eating fish and eggs; however, DHA can also be consumed directly in the form of supplements, with the added benefit of purity and information on its concentration. Presently, DHA is mostly extracted from marine fish, which accumulate DHA through feeding on microalgae, as they are unable to synthesize DHA from C18:3 (Sargent et al., 1999). Conversely, freshwater fish are able to synthesize DHA from essential fatty acids using the same pathways as mammals (Bell et al., 2003), and by taking advantage of the possibility of large-scale aquaculture, this is an alternative source that could solve the problem of dwindling marine fish stock. However, fish oil still presents various problems, such as undesirable taste, odor and a complex fatty acid composition, which is costly to purify (Wen et al., 2003). Higher terrestrial plants generally do not produce DHA (Gill & Valivety, 1997). Some bacteria, particularly those from marine habitats, also produce DHA; however, it is incorporated in the cell membrane and is rarely stored in the cell as triacylglycerol (Alvarez & Steinbüchel, 2002); therefore, cellular levels of DHA in bacteria are normally very low. The other main alternative sources of DHA are marine microalgae, such as the golden algae (Grima et al., 1992), green algae (Yongmanitchai & Ward, 1989, Renaud et al., 1999) and diatoms (Yongmanitchai & Ward, 1989, Renaud et al., 1999); however, their DHA content is not very high. The most promising source of DHA is now a heterotrophic dinoflagellate *Cryptocodenium cohnii* (Mendes et al., 2009) and thraustochytrid microorganisms.

# Chapter 1: Cultivation of thraustochytrid strain 12B in media containing low NaCl concentration

## 1.1. Summary

Thraustochytrid strain 12B produces a higher amount of DHA in the NM medium containing 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$ compared with the F medium containing 50% (v/v) seawater, both of which were supplemented with 8% (w/v) glucose, 1% (w/v) peptone and 1% (w/v) yeast extract. The DHA productivities of cells grown in NM and F media were 0.8 and 0.2 mg/ml/day, respectively. Supplementation of NaCl at 0.1% (w/v) and $MgSO_4$ at 1% (w/v), which are the most necessary inorganic compounds, resulted in a high accumulation of large oil bodies inside the cell. The source of DHA in the cell was mostly triacylglycerol (TG), which was approximately 71% of the total DHA when the cells were grown in the F medium and 81% in the NM medium. DHA, however, only makes up 26% of the total fatty acid in the TG in F-cultured cells and 21% in NM-cultured cells. The addition of other salts, $CaCO_3$, $KHSO_4$, $K_2SO_4$, $K_2HPO_4$ and $KH_2PO_4$, in the NM medium did not substantially increase the DHA yield of strain 12B. The cells grown in the NM medium observed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were found to have thin cell walls and sloughing of the cell surface, which indicate prolific growth and cell maturity. The walls of cells grown in the NM medium were as thin as those of cells grown in the F medium, being 0.08 and 0.1 μm thick, respectively, while cells grown in N and M media were thicker, 0.24 and 1.5 μm thick, respectively. Preparation of cells using the TEM cyrofixation technique, which preserves cellular structures better than the standard TEM preparations, shows a much thicker cell wall, 2.14 μm, for cells cultured in N, indicating the fragility of the wall of cells grown in the N medium in the absence of $MgSO_4$. The growth profile based on the optical density showed earlier entry into the stationary phase by cells grown in the

NM medium compared with that of cells grown in the F medium, which may also be one reason for the high lipid accumulation, as cells normally accumulate lipid more during the stationary phase. Besides the simple medium composition, cells grown in the NM medium have higher glucose utilization efficiency, at less than 8% (w/v) glucose compared with previous studies, with high DHA yield obtained starting from 5% (w/v) glucose and highest at 7% (w/v) glucose, being 2.7 mg/ml and 3.1 mg/ml respectively. The NM medium was also applicable to another thraustochytrid species, *Aurantiochytrium limacinum* SR21. NaCl may have a role in cell division, as cells grew abnormally large and have abnormal shapes in NaCl-deficient M medium, which contains 8% (w/v) glucose, 1% (w/v) peptone, 1% (w/v) yeast extract and 1% (w/v) $MgSO_4$. On the other hand, $MgSO_4$ may have a role in osmotic regulation, as some cells appear to shrink in $MgSO_4$-deficient N medium, which contains 8% (w/v) glucose, 1% (w/v) peptone, 1% (w/v) yeast extract and 0.1% (w/v) NaCl. The yield of DHA was increased when strain 12B was cultured in NM medium using industrial-grade glucose, peptone and yeast extract from 2.4 to 4.6 mg/ml during flask cultivation and was further increased through jar cultivation from 4.6 to 11.7 mg/ml. The high DHA yield from the use of the NM medium is therefore very desirable for large-scale industrial application in the cultivation of thraustochytrid microorganisms for DHA production.

# 1.2. Introduction

## 1.2.1. Cultivation of thraustochytrids for DHA production

Thraustochytrid microorganisms are generally referred to as microalgae despite being heterotrophic microorganisms, yet these microorganisms do have some similar features such as being unicellular, having biflagellate zoospores and being taxonomically placed under the same phylum as algae, Heterokonta. A number of thraustochytrid strains with high DHA productivity have been reported, such as *Schizochytrium* sp. SR21 (Yaguchi et al., 1997), *Schizochytrium* sp. (Qu et al., 2013), thraustochytrid ATCC PTA-9695 (Apt et al., 2012) and *Aurantiochytrium* sp. KRS101 (Hong et al., 2013), with DHA productivities of 3.3, 3.4, 4.1 and 5.6 g/l/day, respectively. The strain 12B used in this study has also been reported to have high DHA productivity, as high as 5.4 g/l/day so far under jar cultivation (Okuyama et al., 2008a). The strain has been used to produce not just DHA-rich triacylglycerol (TG) but also phospholipid, particularly DHA-rich phosphotidylserine (Okuyama et al., 2007, Okuyama et al., 2012, Bin Haji Mohd Taha et al., 2012). Parameters such as temperature, salinity, pH, oxygen level, culture mode and age of culture as well as various aspects of the culture composition such as carbon source, nitrogen source, carbon-to-nitrogen ratio, macroelements such as $Na^+$, microelements (trace metals) and vitamins have been known to affect the growth and culture yield of thraustochytrids (Raghukumar, 2008b). Different thraustochytrid species from different genera do not have the same growth rate in any one medium with the same composition (Lewis et al., 1999). In a chemically defined culture medium, some thraustochytrid species have an obligate requirement for vitamin B supplementation (Goldstein, 1973), and even the presence of specific trace metals such as manganese and copper results in a large increase or decrease in dry cell weight (DCW) yield depending on the thraustochytrid species (Nagano et al., 2013).

### 1.2.2. Culture medium and seawater

The conventional cultivation method for thraustochytrids including strain 12B normally uses a medium containing half the concentration of seawater because thraustochytrids are marine microorganisms. In large-scale industrial cultivation, the use of seawater imposes several problems such as the cost of transporting seawater, dependability on sites close to the sea, and corrosion of cultivation facilities because normal tank fermenters (N type SS316) can withstand less than 0.1% chloride concentration at 30°C (Grundfos, 2012).

Seawater is also not very suitable for thraustochytrid cultivation because of the presence of various chemical and heavy metal pollutants. The use of artificial seawater made with distilled water is preferable as it avoids the introduction of heavy metals such as mercury into the biomass as shown in a cultivation involving *S. limacinum* (Pyle et al., 2008), while cultivation using seawater may introduce mercury into oils such as those extracted from fish caught from the sea (Foran et al., 2003). The study of pollution of thraustochytrid microorganisms is very limited, and the only study reported on *Schizochytrium* sp. shows deformation of the cell structure, formation of holes in the cell wall, and shrinkage of cells when 32 ppm zinc and copper heavy metals were added during cultivation, while cell lysis occurred when higher concentration of 256–384 ppm were added (Lin et al., 2010). Despite the decrease of heavy metal pollutants to conform to the minimum environmental quality standard throughout 1971 to 1997, the organic pollutant level of the sea around Japan in the same period did not improve (MOE, 2013). Unfortunately, recent data are unavailable. On the other hand, a survey on chemical substances suspected to have long-term toxicity to humans has detected 357 pollutants out of 1069 chemicals tested in both aquatic and marine surface water around Japan (MOE, 2011), a small number relative to the total 19,247 existing chemical substances registered under the chemical substances control law (NITE, 2013).

A bigger concern nowadays, however, is the presence of radioactive isotopes in the sea surrounding Japan because of the accident at the Fukushima nuclear

power plant, which is thought to have released about $5.5 \times 10^{15}$ becquerel (Bq) of radioactive isotope cesium 137 ($^{137}Cs$) during the first few days of the accident (Estournel et al., 2012). $^{137}Cs$ has a half-life of about 31 years (Unterweger, 2002), and increased concentration from 1.4 to 6.0 (Bq $kg^{-1}$) in the migratory Pacific bluefin tuna *Thunnus orientalis* caught in waters off California has already been detected because of the Fukushima accident (Madigan et al., 2012). There are no studies yet on the effect of radioactive isotopes on thraustochytrids; however, the uptake of $^{137}Cs$ by some phytoplankton species has already been demonstrated (Adam and Garnier-Laplace, 2003, Heldal et al., 2001).

### 1.2.3. Medium with simple composition and low NaCl concentration

Artificial seawater (ASW), with defined chemical components and a concentration of NaCl almost the same as seawater at about 3% (w/v), has been used instead of seawater in some investigations on the cultivation of thraustochytrids (Nagano et al., 2009, Min et al., 2012). The effectiveness of the ASW compared with actual seawater has been the subject of a review paper (Berges and Franklin, 2001), which reported inconsistencies of superiority compared with seawater depending on marine species. The composition of ASW for particular species such as thraustochytrids has probably been tailored for this group of microorganisms and is slightly different from the general ASW mentioned. For thraustochytrid microorganisms, there is a necessity for sodium ions (Raghukumar, 2008b) but not for chloride ions, leading to experimentation in reducing or excluding them completely from the culture medium, as they are also unfavorable for culture equipment.

There are very few papers that have reported chloride-independent medium alternatives such as those made by substituting NaCl with $Na_2SO_4$ (Barclay, 1994); however, the culture yield was found to be very low. Another study that uses $Na_2SO_4$ (Prabu et al., 2012) has also been published; however, the absolute DCW yield data from which the DHA yield was calculated in terms of gram per gram were not disclosed and therefore open to speculation. Mannitol and sucrose are also alternatives; however, similarly, studies have only concentrated on cell growth

based on optical density (Shabala et al., 2009) or percentage fatty acid without disclosing the absolute DHA yield and productivity (Shabala et al., 2013). Apart from this, media with complex ingredients such as M-5 medium, FFM medium (Barclay et al., 1994) and medium 1–6 (Singh and Ward, 1997) with various trace elements as supplementation have commonly been used. There is normally a preference for using an artificial and chemically defined medium over a natural medium for the cultivation of microbes because every component in the chemically defined medium is known. The use of peptone and yeast extract in culture medium may categorize it as a natural medium because of its complex composition, and the exact constituents of some components such as trace elements are not fully quantified by manufacturers. Natural media are considered to have poor reproducibility (Arora, 2013); however, both peptone and yeast extract available nowadays are produced using a standard procedure, and therefore high reproducibility in cell yield during cultivation is normally achieved. The use of natural ingredients consequently makes the supplementation of trace amounts of minerals and vitamins unnecessary. The aim of this study is to compose a simple culture medium that is not corrosive to industrial equipment by having a low NaCl content. This is an important aspect for large-scale industrial DHA production, as it will reduce manufacturing cost and time, as preparation will become uncomplicated, practical and efficient.

# 1.3. Materials and methods

## 1.3.1. Microorganisms and cultural media

The thraustochytrid microorganism strain 12B (strain 12B; NITE P-68) was mainly used throughout this work. Strain 12B was previously isolated from a mangrove ecosystem in Okinawa, Japan (Perveen et al., 2006). Another thraustochytrid microorganism, *Aurantiochytrium limacinum* SR21, was isolated from a coral reef area of the Yap Islands, Federated States of Micronesia (Nakahara et al., 1996) and was originally identified as *Schizocytrium limacinum* SR21 (IFO 32693). It was kindly provided by the Institute of Fermentation, Osaka (IFO), with the permission of Dr. Yokochi Toshihiro, National Institute of Advanced Science and Technology (AIST).

## 1.3.2. Cultivation, growth measurement, and harvesting of cells

All media contain the same base organic composition of 1% (w/v) yeast extract (Nacalai Tesque, special reagent grade), 1% (w/v) peptone (Kyokuto, laboratory grade) and 8% (w/v) glucose (Kanto Chemical, guaranteed reagent for JIS grade). The basic culture media for the cultivation of the thraustochytrids were BY+ agar or liquid media as well as F medium. Both BY+ and F media are composed of 50% filtrated seawater. The growth-optimized condition of strain 12B under liquid cultivation was determined by investigating the effect of major target compounds supplemented at different concentrations on the growth of cells. The target inorganic compounds investigated were NaCl at 0, 0.1, 0.2, 0.3 and 0.5% (w/v), $MgSO_4 \bullet 7H_2O$ at 0, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5 and 3% (w/v), $CaCO_3$ at 0, 0.05, 0.1, 0.5% (w/v) and $KHSO_4$ at 0, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5 and 3% (w/v). To assess the influence of types of potassium-containing compounds on the growth of cells, determined as increase in DCW, $K_2SO_4$, $KH_2PO_4$, and $K_2HPO_4$ were used at 0, 0.01, 0.05, 0.1, 0.5, 1, 1.5 and 3% (w/v). The best medium—i.e., the NM medium—was only supplemented with 0.1% NaCl (w/v) and 1% $MgSO_4 \bullet 7H_2O$ (w/v). Cells were

also grown in N medium, which was only supplemented with 0.1% NaCl (w/v) and M medium, which was only supplemented with $MgSO_4 \bullet 7H_2O$ (w/v) to see the effect of the deficiency of either inorganic compound on culture yield and cell morphology.

In order to simulate an industrial cultivation, analytical-grade glucose, yeast extract and peptone were replaced with industrial-grade substitute yeast extract Koucho P-81 (Cosmo Foods Co. Ltd) and peptone Proyield® Soy SE50MK Friesland Campina (Oriental Yeast Co. Ltd). The INM cultures were not supplemented with 0.1% NaCl (w/v) because the industrial-grade yeast extract already contained 15% salt. All cultivations, except for the jar cultivation, were done in 10 ml medium in a 50 ml flask with shaking at 150 rpm in the dark for 3 days at 30°C. Upscaling of the experiment using an industrial NM medium (INM) was carried out in a jar fermenter (MBF, EYELA, Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The inoculum was prepared by inoculating one loop full of 12B cells from plate agar culture into 30 ml F medium in a 300 ml flask and incubated with shaking at 160 rpm in the dark for 3 days at 30°C. The jar was filled with 1.5 l INM medium, and 15 ml liquid inoculum was then added. The jar cultivation was carried out for 5 days under the following conditions: incubation temperature at 30°C, mixing speed at 300 rpm, with air purged into the medium at 1.4–1.6 l/min.

Cells were harvested by centrifugation (RX-200, TOMY SEIKO Co. Ltd, Tokyo, Japan) at 6000 rpm for 10 min, which is equivalent to 10,000 × g, washed with 0.8% NaCl, recentrifuged and freeze-dried for 24 hr at –4°C, –0.092 MPa (Labconco, Kansas, US) with an external vacuum pump (SVP-100S, Sato Vacuum Machinery Industrial Co. Ltd, Tokyo, Japan). The DCW was determined after freeze-drying, and the sample was stored at –80°C until used.

### 1.3.3. Lipid extraction

Lipid was extracted from the dried cells using the Bligh and Dyer method (Bligh and Dyer, 1959) with modification in the ratio of solvent used. Freeze-dried sample was initially mixed with a mixture of water, chloroform and methanol in the

ratio of 4:5:10 instead of 1:1:1. After vortexing for 30 s, water was again added to the mixture in the ratio of 5:19 instead of 1:3. In addition, chloroform was again added to the new mixture in the ratio of 1:6, and the chloroform layer was collected by centrifugation at 2000 rpm (1856 × g) for 5 min (KN-70, Kubota, Osaka, Japan), and chloroform was added two more times by repeating this step.

Extracted lipid was then dried with $N_2$ and redissolved to 50 mg/ml by adding chloroform:methanol (2:1, v/v) solution containing 1% (w/v) butylated hydroxy toluene (BHT). Separation of lipid into triacylglycerol (TG), polar (polar) and other lipid classes was done by thin-layer chromatography (TLC). An aliquot of 10 μl (500 μg) of lipid was pipetted on to a silica glass plate (silica gel 60; Merck, Damstadt, Germany) and allowed to develop in solvent containing hexane, diethyl ether and acetic acid in the ratio of 50:50:1. Separated lipid classes were observed by first spraying the plate with an acetone and water (4:1) solution containing 0.01% (w/v) primulin and then exposing the plate to ultraviolet radiation (UV). Spots of lipid classes were scrapped and collected into screw-capped test tubes and methanolyzed as described below.

### 1.3.4. Preparation and analysis of fatty acid methyl esters

Methanolysis of lipid was carried out by adding 10 μl lipid, 10 μl heneicosanoic acid internal standard (2 mg/ml in benzene) and 1 ml methanol containing 10% acetyl chloride into a screw-capped test tube and heating at 100°C for 1 hr (Dry Block Bath MG-2, Torika Corp., Nagoya, Japan). The resulting fatty acid methyl esters (FAMEs) were extracted with 2 ml of hexane three times. After concentrating the FAME fractions through evaporation under $N_2$, the samples were subjected to gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS).

The FAME fraction was analyzed by gas chromatography (GC) using a GC-353B unit (GL Sciences, Tokyo, Japan) equipped with a capillary column BPX70 (25 m × 0.22 mm i.d., SGE, Yokohama, Japan) and flame ionization detection (FID) with nitrogen as carrier gas. The column temperature was programmed to increase at

5°C/min from 150°C to 240°C and then maintained for 2 min while the injector and detector temperatures were set at 240°C. Samples were also analyzed by a gas chromatograph CP-3800 (Varian, Tokyo, Japan) with an ion-trap mass spectrophotometer Saturn 2200 (Varian, Tokyo, Japan) equipped with the same column. The column temperature was programmed to increase from 80°C to 240°C with a split ratio of 20:1, helium carrier gas with a flow rate of 0.9 ml/min, and the injector temperature set at 240°C. GC-MS was used to confirm the identification of peaks corresponding to specific fatty acids, while gas chromatography–flame ionization detection (GC–FID) was used for routine calculation of fatty acid yield including DHA.

### 1.3.5. Analysis of glucose

The depletion of glucose in culture media was determined by pipetting 10 μl of culture sample onto silica gel, developed by using solvent containing 1-butanol, 2-propanol, $H_2O$, and acetic acid (7:5:4:2, v/v) and then detected by spraying with anisaldehyde (anisaldehyde, conc. $H_2SO_4$, and 95% ethanol, 1:1:18, %v/v) followed by heating in an oven at 120°C for 10 min.

### 1.3.6. Microscopic observation

In order to determine any possible link between culture conditions, lipid accumulation and changes in the external and internal cell structures of strain 12B, SEM and TEM were performed. Cultures were grown in F, NM, N and M media in a 50 ml flask as mentioned above. Sampling of cells was done by centrifuging the cultures at 5000 rpm (8000 × g) for 5 min at 4°C, followed by washing with 0.1 M potassium phosphate buffer (PB) at pH 7.4. The initial fixation and dehydration steps are similar in SEM and TEM.

#### 1.3.6.1. Scanning electron microscopy

Cells were harvested, washed in 0.1 M PB and cut into 1.5 × 1.5 × 0.5 mm sections while submerged in PB. Prefixation was carried out in 5 ml PB containing

2% parafolmaldehyde (Wako Pure Chemical) and 2.5% glutaraldehyde (Nisshin EM), and rotated for 2 hr (RT-50, Taiyo, Musashi, Japan) followed by washing thrice with PB and rotating for 10 min each time. Postfixation was carried out by mixing 2% $OsO_4$ (Nisshin EM) and 0.2 M PB (1:1), and incubating at 4°C for 2 hr. The dehydration of cell samples for SEM was done by increasing ethanol concentration from 70, 80, 90, 95 to 100%, with 15 min at each step, at room temperature (rt), except for 70% ethanol, which was done overnight. Further drying was carried out by exposing the cells to isoamyl acetate (Kanto Chemical) for 10 min and finally with liquid $CO_2$ for 2 hr (Critical point dryer HCP-1, Hitachi, Japan). Dried samples were then fixed onto a metal plate and coated with platinum–palladium (Ion sputter 1030, Hitachi, Tokyo) followed by observation under a scanning electron microscope (S-4000, Hitachi, Tokyo, Japan).

### 1.3.6.2. Transmission electron microscopy

The cell preparations were essentially the same as for SEM until the dehydration stage, after which cells were infiltrated by increasing the concentration of EPON (EPON 812, TAAB Laboratories Equipment Ltd), by immersing the cells in 5 ml of 100% QY-1 solvent, QY-1:EPON (2:1, v/v), and QY-1:EPON (1:2, v/v) successively for 15 min each, and finally in 100% EPON overnight. Prior to casting, the EPON used overnight was discarded and replaced with fresh EPON, and rotated for another 4 hr. Samples were then embedded in casting plates and allowed to harden at 60°C for 2 days. Ultrathin sections of samples were cut by an Ultra Diamond Knife with a 2.5 mm, 35° blade (DiATOME, Nisshin EM Co. Ltd, Tokyo, Japan) and placed on copper grids. Samples were stained with 2% uranyl acetate (Wako Pure Chemical) and then lead citrate (Kanto Chemicals) for 15 min and then 5 min, respectively, before being observed under a transmission electron microscope (JEM 1400, JEOL, Tokyo, Japan).

Another TEM preparation method—i.e., the ultrarapid cryofixation technique—was also tried for samples cultivated in a 50 ml flask for 7 days in 10 ml F, NM and N media. This technique offers better preservation of cellular structures compared with the conventional TEM method. After washing and centrifuging the cell in PB, about 1 μl of cell was transferred onto a copper plate

and covered by another plate. The grid was then dropped into a liquid nitrogen tank (Reichert KF80, Reichert Inc., New York, US) to preserve the cellular structures, followed by freeze substitution using 2% $OsO_4$ in acetone from –80°C, –40°C, –20°C, 4°C to room temperature (rt) for 3 days, 2 hr, 2 hr and 1 hr, respectively. Samples were then washed and infiltrated with increasing QY-2 solvent from 100% acetone to acetone:QY-2 (1:1) to 100% QY-2 for 15 min each. Solvent substitution was then carried out by increasing the concentration of quetol 812 from 10, 30, 50, 70, 80, 90 to 100% for 30, 40, 60, 60 min, overnight, 2 hr and 2 hr, respectively. Samples were then embedded in EPON and allowed to solidify at 60°C for 2 days before being cut and stained for observation under the electron microscope.

### 1.3.7. Protein analysis

The influence of compounds in the culture medium on the cellular metabolism of thraustochytrid strain 12B such as in protein and enzyme expression could probably be seen through differences in its protein expression. Thraustochytrid strain 12B grown in 10 ml F, NM, N and M media was cultivated in a 50 ml flask as mentioned previously, and the cells were centrifuged, washed and stored at –80°C until analyzed. For protein extraction, the cells were resuspended in a small amount of PB and then ground using a sterilized pestle and crucible. The suspension was centrifuged (4°C, 10,000 × g, 15 min) to collect the supernatant containing cellular proteins and was then concentrated by adding 100% acetone at four times the volume of sample. The mixture was then vortexed and incubated at –20°C for 10 min. Precipitated protein was collected by centrifugation (12,000 × g, 5 min), and the acetone supernatant was discarded. The protein was redissolved in 100 μl PB, and the protein concentration was measured and equalized to other samples (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, California, US). Equal volumes of protein sample and SDS sample buffer were mixed, and a volume from this was taken and further treated with 2-mercaptoethanol (1% v/v) and heated for 5 min at 100°C. Samples were run in gel containing 5–20% denaturant (e-Pagel E-R520L, ATTO, Tokyo, Japan) in a tank containing SDS running buffer at 200 V, 20

mA, 4.5 W for 80 min. Observation of the protein banding profile was made by silver staining (silver stain II kit, Wako, Osaka, Japan).

# 1.4. Results

## 1.4.1. Effect of the salt composition of the culture media on growth, and lipid and DHA yields of thraustochytrid strain 12B

Identification of the most relevant compounds and the concentrations leading to the formulation of the NM medium that yields high cell yield, lipid, fatty acid and ultimately high DHA yield was obtained by investigating the effect of NaCl, $MgSO_4$, $CaCO_3$, potassium compounds $KHSO_4$ and $K_2SO_4$, and phosphorus-containing potassium compounds $KH_2PO_4$ and $K_2HPO_4$. The phosphorus-containing compounds were also investigated to see any possible additional effect of phosphorus as a potential limiting compound in the thraustochytrid cultivation.

### 1.4.1.1. Effect of NaCl and $MgSO_4$ concentrations

Strain 12B is normally cultivated in F medium, in which 1% peptone, 1% yeast extract, and 8% glucose are dissolved in 50% seawater. To investigate the necessity of NaCl in the cultivation of strain 12B, modified F media containing various concentrations of NaCl, instead of 50% seawater, were prepared. During 3 days' cultivation, the thraustochytrid strain 12B was unable to grow in the absence of NaCl (Fig. 1.1A). The DCW and lipid yields decreased from 4.3 to 3.1 mg/ml and from 1.3 to 0.6 mg/ml, respectively, with decreasing NaCl concentration from 0.5 to 0.2% (w/v). However, both DCW and lipid yields suddenly increased again to 4.1 and 1.1 mg/ml, respectively, at 0.1% (w/v) NaCl. The modified F medium with 0.1% (w/v) NaCl was then used in the subsequent experiments.

The magnesium ion is the fourth most abundant ion in seawater (Brown et al., 1989). In this experiment, $MgSO_4$ and not $MgCl_2$ was used because of the corrosive nature of $Cl^-$. The addition of a small amount of $MgSO_4$ to 0.1% NaCl-containing modified F medium considerably increased the DCW and lipid yields of strain 12B (Fig. 1.1B). The highest amounts of DCW and lipid at 18.8 and 11.2 mg/ml, respectively, were obtained when 1% (w/v) $MgSO_4$ was added to the

medium. This modified medium that contained 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$ together with the base organic ingredients was then designated as an NM medium. Further addition of $MgSO_4$ yielded more or less the same amounts of DCW and lipid (Fig. 1.1B).

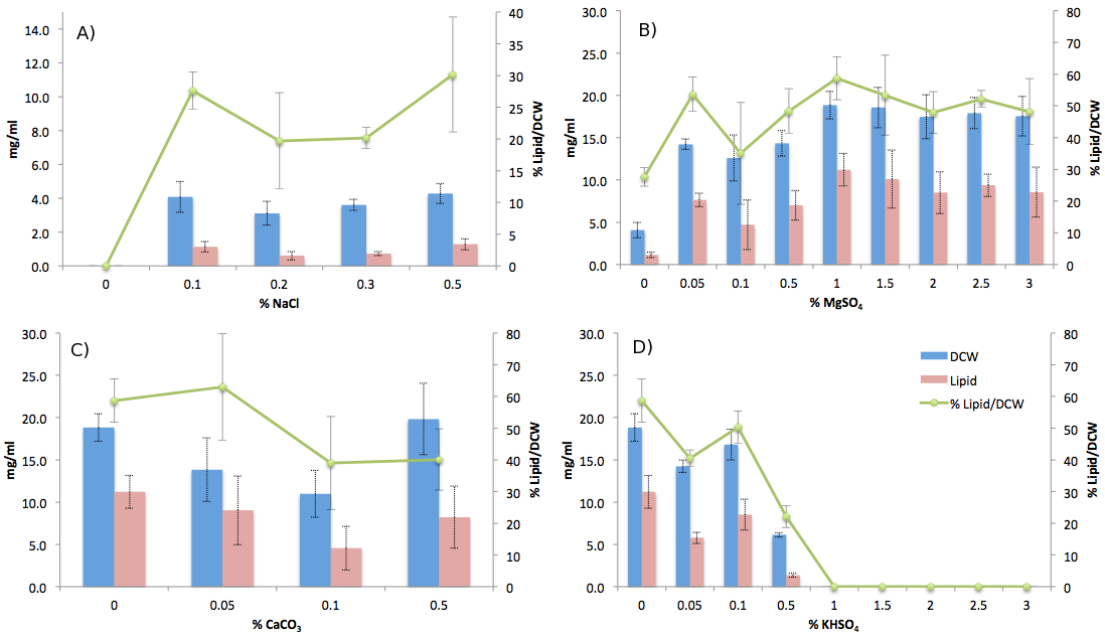


Figure 1.1. Effect of NaCl (A), $MgSO_4$ (B), $CaCO_3$ (C), and $KHSO_4$ (D) concentrations in medium on cell and lipid yields of thraustochytrid strain 12B. (A) Reduction in NaCl concentration from 0.5% to 0.2% (w/v) initially reduced the DCW and lipid yields and the percentage lipid per DCW. All three values increased again at 0.1% NaCl; however, cells were not able to grow at zero NaCl concentration. (B) Supplementing the N medium containing 0.1% NaCl with $MgSO_4$ drastically increased the DCW and lipid yield, with the highest yield being obtained at 1% (w/v), beyond which the yield remained more or less stationary. (C) Supplementing the NM medium containing 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$ with $CaCO_3$ initially decreased both the DCW and lipid yields when $CaCO_3$ concentration was increased from 0 to 0.1% (w/v). An increase of DCW was observed at 0.5% (w/v) $CaCO_3$; however, the % lipid per DCW was low. (D) Supplementation with $KHSO_4$ decreased the DCW and lipid as well as the % lipid per DCW, with yields reaching zero at 1% (w/v) and higher concentrations. All media contained a similar organic base composition of 1% (w/v) yeast extract, 1% (w/v) peptone and 8% (w/v) glucose. A colony from a 2-day BY+ agar plate was used as an inoculum, and all cultures were incubated in a 50 ml flask containing 10 ml culture medium in the dark for 3 days at 30°C and 150 rpm before cells were harvested, freeze-dried and the lipid extracted.

### 1.4.1.2. Effect of $CaCO_3$ and $KHSO_4$ supplementation

The effect of combined addition of calcium carbonate and potassium hydrogen phosphate on the growth, lipid and DHA yields of strain 12B was examined in the NM medium. Although both DCW and lipid yields gradually decreased with increasing concentration of $CaCO_3$ from 0% to 0.1% (w/v), these parameters suddenly increased at 0.5% (w/v) $CaCO_3$. However, the percentage lipid per DCW was almost unchanged (Fig. 1.1C). The decrease in DCW and lipid yields was also seen when potassium hydrogen sulfate was added to the NM medium, and both yields became zero when 1% (w/v) $KHSO_4$ or more was added (Fig. 1.1D). When both $CaCO_3$ and $KHSO_4$ were added together in the absence or presence of 1% (w/v) $MgSO_4$, no difference in yield was obtained, and positive yield was obtained only because of $MgSO_4$ supplementation (data not shown).

### 1.4.1.3. Effect of different molecular forms of potassium compounds on cultivation

The addition of potassium generally decreased the DCW yield with increasing concentration. However, a slight increase in DCW yield was observed when the amount added was below 0.1% (w/v), except for $KHSO_4$ (Fig. 1.2). Each compound specifically increased one type of yield and not the other, with $K_2SO_4$ as the best compound to increase DCW, $K_2HPO_4$ for the overall lipid, and $KH_2PO_4$ for the overall fatty acid, although the difference in DHA yield is not considerably different compared with the NM (data not shown). In terms of percentage fatty acid composition, the addition of potassium compounds also resulted in a higher amount of octadecanoic acid (oleic acid; C18:1) from 0.6% in cells grown in NM medium to more than 20% when potassium compounds were added. The percentage of pentadecanoic acid (C15:0) was low for all potassium-containing media except $KHSO_4$. The percentage composition of DHA (C22:6), on the other hand, appears to be more or less the same with or without the addition of potassium (data not shown).

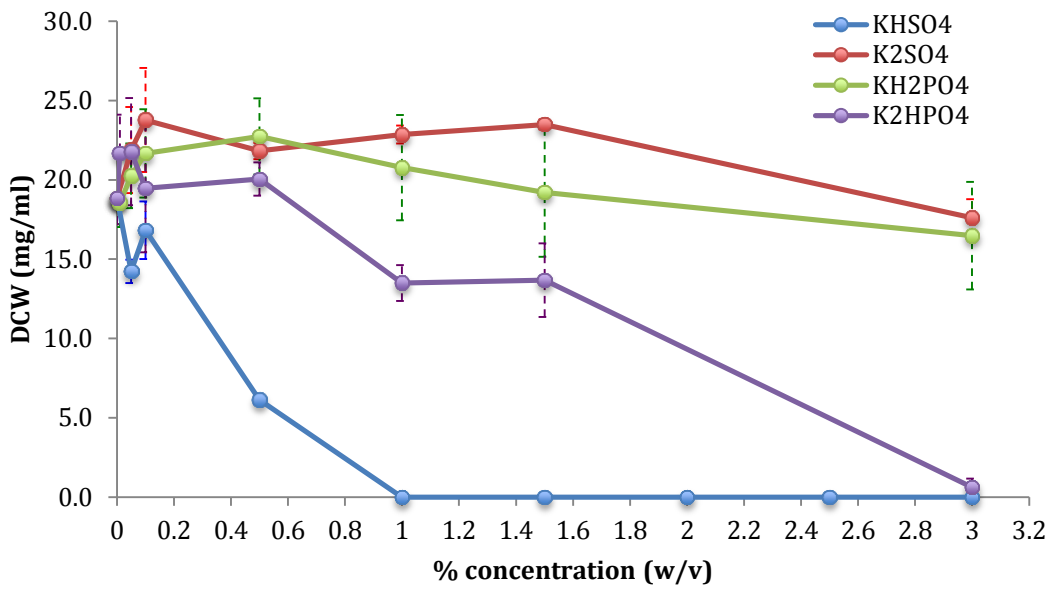


Figure 1.2. Effect of supplementing the NM medium with different forms of potassium compound on the DCW yield of thraustochytrid strain 12B. $KHSO_4$ and $K_2SO_4$ were used as representative potassium compounds without phosphorus, while $KH_2PO_4$ and $K_2HPO_4$ were used as representative potassium compounds containing phosphorus. There was generally a decrease in DCW with increasing concentration of potassium compounds; however, a slight spike in DCW was obtained at very low potassium compound concentrations. All media were inoculated with a colony from a 2-day BY+ agar plate, and incubation was carried out in a 50 ml flask containing 10 ml of culture medium in the dark for 3 days at 30°C.

### 1.4.1.4. Application of NM medium to other thraustochytrid strain

The *A. limacinum* SR21 is a well-known thraustochytrid strain that accumulates one of the highest amounts of DHA during cultivation in artificial seawater containing about 1.5% NaCl (w/v) (Yaguchi et al., 1997). The application of the NM medium to the cultivation of *A. limacinum* SR21 showed an almost similar growth pattern to that of thraustochytrid strain 12B. There was steady growth from zero time onwards, and the stationary phase started at around 3–5 days during cultivation. The number of days for glucose to be depleted from the culture media varied between thraustochytrid strain 12B and *A. limacinum* SR21. Glucose was depleted by thraustochytrid strain 12B after 7 days' cultivation in both F and NM, while for *A. limacinum* SR21, glucose depletion was even faster, at 6 and 5 days, respectively (data not shown). Generally, there were higher DCW, percentage lipid, percentage fatty acid and percentage DHA yields in NM compared with the F culture, especially for thraustochytrid strain 12B after 3 days' cultivation (Table 1.1). The DCW and DHA yields of cells grown in the NM medium after 3 days' cultivation were 18.8 and 2.4 mg/ml, respectively, for strain 12B, while the yields from cells grown in F medium were 10.4 and 0.5 mg/ml, respectively. In the case of *A. limacinum* SR21, the DCW and DHA yields from cells grown in NM and F media were almost the same. Higher DCW and DHA yields were obtained from strain 12B compared with *A. limacinum* SR21 when each was cultivated in NM medium. The DCW of strain 12B was 18.8 mg/ml compared with 12.9 mg/ml for *A. limacinum* SR21, while the DHA yield was also higher for strain 12B at 2.4 mg/ml compared with 1.1 mg/ml for *A. limacinum* SR21.

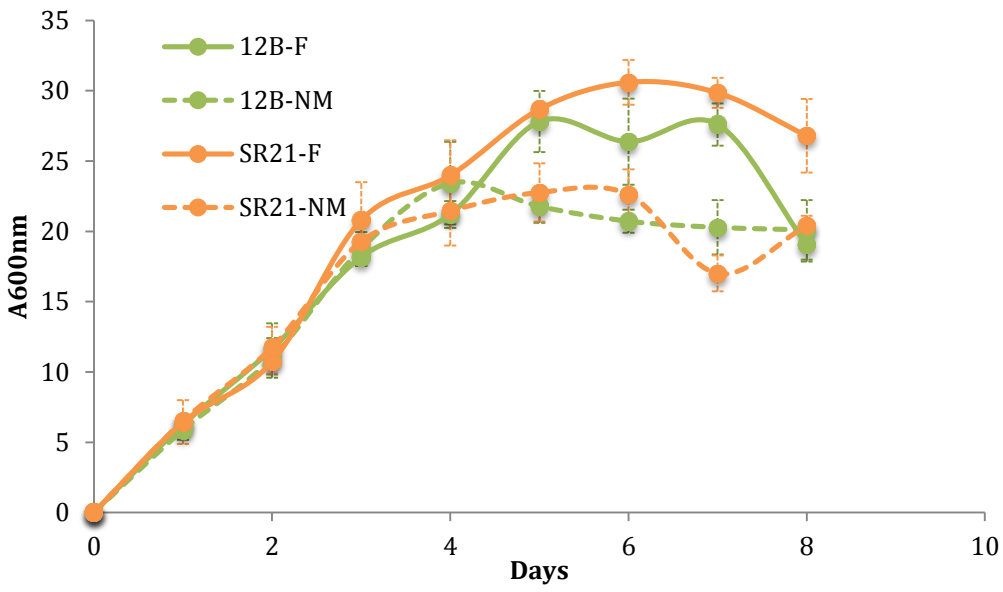


Figure 1.3. Growth curves of thraustochytrid strain 12B and *A. limacinum* SR21 in F and NM media. Growth patterns were more or less the same for both strains 12B and SR21 in the F and NM media for up to 3 days' cultivation. Longer cultivation resulted in the stationary phase for the strains in the NM culture, while growth appeared to continue up to the 5$^{th}$ day in the F medium before entering the stationary phase. Both F and NM media contain the same organic composition consisting of 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (peptone). F medium is also composed of 50% seawater, while NM medium was made by using distilled water supplemented with 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4 \bullet 7H_2O$. A colony from a 2-day BY+ agar plate was used as an inoculum, and incubation was carried out in a 50 ml flask containing 10 ml culture medium incubated in the dark at 30°C for up to 8 days. Triplicate sampling was taken from a 10 ml culture each day.

Table 1.1. Effect of F and NM culture media on DCW and DHA yields (mg/ml) and percentage composition of lipid, fatty acid and DHA of strain 12B compared with *A. limacinum* SR21.*

| Strain | Medium | DCW | % lipid/DCW | % f.a./lipid | % DHA/f.a. | DHA |
|---|---|---|---|---|---|---|
| 12B | F | 10.4 ± 0.8 | 19.8 ± 2.0 | 72.7 ± 3.0 | 30.6 ± 2.3 | 0.5 ± 0.1 |
| | NM | 18.8 ± 1.6 | 59.5 ± 2.7 | 75 ± 4.9 | 31.2 ± 0.6 | 2.4 ± 0.4 |
| SR21 | F | 13 ± 4.1 | 32.9 ± 7.6 | 66 ± 2.5 | 32.9 ± 0.6 | 1 ± 0.4 |
| | NM | 12.9 ± 3.2 | 41.5 ± 7.0 | 59.7 ± 5.9 | 33.5 ± 1.7 | 1.1 ± 0.3 |

A higher yield was obtained after 3 days' cultivation in NM compared with the F medium. *A. limacinum* SR21 yields are generally higher compared with strain 12B grown in the F medium, and the opposite result was obtained when the strains were grown in the NM medium. A colony from 12B or SR21 cells grown for 2 days on a BY+ agar plate was used to inoculate a 10 ml culture in a 50 ml flask and incubated at 30°C for 3 days.

*Average ± standard deviation from three independent cultures

### 1.4.1.5. Distribution of DHA in lipid classes

Fatty acids including DHA were mostly stored as TG in the thraustochytrid strain 12B (Okuyama et al., 2007). In this study, the composition of TG, polar lipid, and other lipid classes was expressed as the amount of their constituent fatty acids after TLC separation. The percentage of TG from cells grown 3 days in the F medium was 81.4%, while that in the NM medium was 90.2% (Table 1.2). The amount of DHA constituting the fatty acid in TG was 26% in F and 21% in NM media-cultured cells; however, the DHA from TG contributed to 71% of the total DHA in cells from the F culture and 91% in cells grown from the NM culture. About 20% of the fatty acid was lost through TLC analysis compared with lipid that was directly methanolyzed (data not shown).

### 1.4.1.6. Application of industrial-grade ingredients and upscaling to jar cultivation to increase DHA yield

To examine the usefulness of industrial-grade ingredients in the productivity of DHA, strain 12B was cultivated in a flask and jar fermenter using NM medium that had been prepared using industrial-grade organic ingredients (yeast extract, peptone, and glucose), then called INM medium. There is a gradual increase of DHA yield from 0.7 to 2.4 mg/ml between cells grown in F and NM media during flask cultivation. A further increase was seen in the DHA yield from 2.4 to 4.6 mg/ml between analytical-grade NM medium and industrial-grade INM medium. The DHA yield was again increased from 4.6 to 11.7 mg/ml between flask and jar cultivation in INM medium (Table 1.3).

Table 1.2. Effect of medium on lipid and DHA contents of lipid groups of thraustochytrid strain 12B*.

| Medium | Lipid class | Content, mg/ml (recovery, % total)** | |
|---|---|---|---|
| | | Lipid | DHA |
| F | TG | 1.6 ± 0.58 (81.4) | 0.43 ± 0.15 (71.4) |
| | Polar | 0.3 ± 0.05 (12.9) | 0.12 ± 0.02 (19.6) |
| | Others | 0.1 ± 0.02 (4.0) | 0.02 ± 0.01 (3.3) |
| NM | TG | 6.5 ± 0.85 (90.2) | 1.37 ± 0.17 (91.0) |
| | Polar | 0.3 ± 0.02 (4.3) | 0.12 ± 0.01 (7.7) |
| | Others | 0.4 ± 0.09 (5.2) | 0.05 ± 0.01 (3.3) |

The amount of fatty acid and DHA was highest in the TG group followed by the Polar and Other lipid groups. Fatty acid and DHA were mostly distributed in the TG group as shown by the high percentage of fatty acid and DHA per total fatty acid or total DHA. The distribution of the cells' total fatty acid and DHA in the TG group was higher in cells grown in the NM medium than in cells grown in the F medium.

* The amounts of individual lipids are expressed as constituent fatty acids per culture.

** Average ± standard deviation from three independent cultures.

Table 1.3. Effect of culture medium and cultivation method on the DCW, lipid, fatty acid and DHA yields of thraustochytrid strain 12B.*

| Culture | Cultivation method | Content, mg/ml | | | |
|---|---|---|---|---|---|
| | | DCW | Lipid (% lipid/DCW) | Fatty acid | DHA (per day**) |
| F | 50ml flask | 11.3 ± 2.7 | 2.6 ± 1.1 (24.2) | 2.2 ± 0.6 | 0.7 ± 0.2 (0.2) |
| NM | 50ml flask | 18.8 ± 1.6 | 11.2 ± 1.9 (58.7) | 7.8 ± 1.3 | 2.4 ± 0.4 (0.8) |
| INM | 50ml flask | 22.5 ± 1.8 | 16.1 ± 1.5 (71.6) | 13.7 ± 1.3 | 4.6 ± 0.5 (1.5) |
| INMK | 50ml flask | 22.4 ± 1.0 | 16.3 ± 0.1 (72.8) | 14.2 ± 1.4 | 4.9 ± 0.6 (1.6) |
| INM | 2l jar | 39.4 ± 0.2 | 29 ± 0.4 (73.6) | 31.2 ± 0.4 | 11.7 ± 0.2 (2.3) |

The DCW, lipid, fatty acid and DHA yields increase successively from F to NM and industrial-grade NM (INM) through flask cultivation. The yields were further increased through jar cultivation. The DCW, lipid, fatty acid and DHA yields were more or less the same with the supplementation of 0.05% (w/v) $K_2SO_4$ in the INM medium (INMK).

* Average ± standard deviation from three independent cultures.

** Productivity calculated by dividing the yield by the number of cultivation days, which was 3 days, except for jar cultivation, which was 5 days.

1.4.1.7. Effect of the concentration of glucose in NM medium on the growth and DHA yield in strain 12B

In order to see the influence of glucose concentration on the DCW, lipid, fatty acid and DHA yields in the NM medium, strain 12B was grown in 10 ml medium in a 50 ml flask, supplemented with 0.1, 1, 2, 3, 4, 5, 6, 7 and 8% (w/v) glucose, and incubated in the dark at 30°C and 150 rpm for 3 days. The efficiency of thraustochytrid strain 12B in converting glucose to biomass (DCW) in the NM medium was found to be highest when the glucose concentration in the medium was 1 to 5% (w/v). Within this range of concentration, the increase of DCW was found to be proportional to the glucose concentration, while lipid and DHA yields increased exponentially. The highest DCW yield at 5% (w/v) glucose was 19.6 mg/ml, while the highest lipid and DHA yields were 11.6 and 3.1 mg/ml, respectively. Cell optical density, however, reached a maximum at 3% (w/v) NaCl without further increase at higher glucose concentrations, and the highest amount of DHA (3.1 mg/ml) was obtained when the glucose concentration was 7% (w/v) (Fig. 1.4).

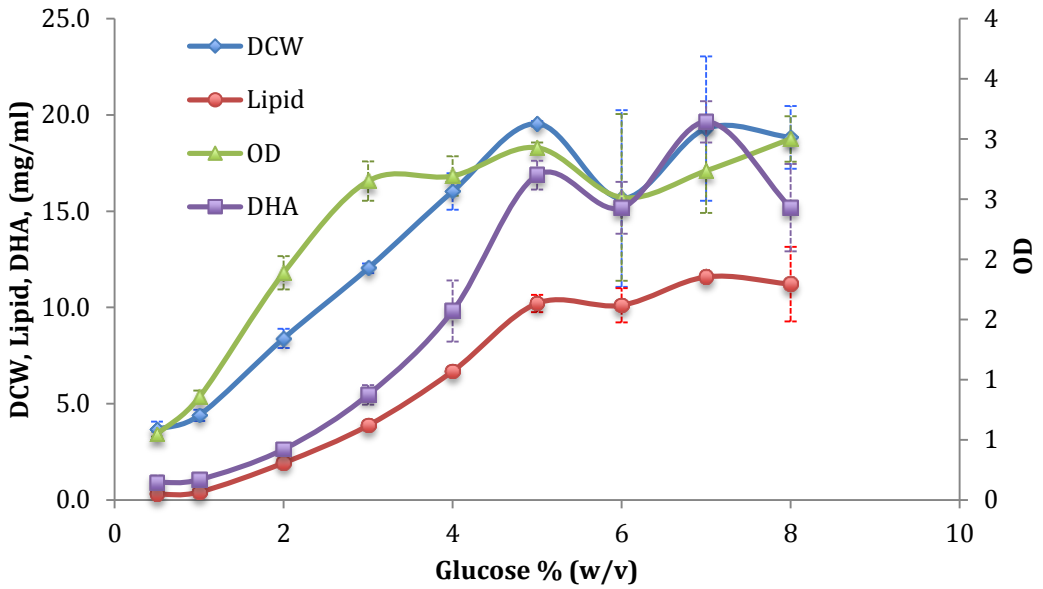


Figure 1.4. Effect of different glucose concentration on the DCW, lipid and DHA yields (mg/ml) and culture optical density in the NM culture medium. Growth based on the optical density of cells grown in the NM medium reaches a maximum at a minimum of 3% (w/v) glucose concentration and does not increase with higher concentrations. DCW increases almost proportionally with increasing glucose concentration up to 5% (w/v) glucose, while lipid and DHA appear to increase exponentially up to 5% (w/v) glucose concentration. The highest amount of DHA, 3.1 mg/ml, was obtained at 7% (w/v) glucose concentration. Cultivation was carried out for 3 days at 30°C. Values were averages from triplicate samples.

## 1.4.2. Microscopic observation

### 1.4.2.1. Scanning electron micrography

Observation of cells grown in F and NM media did not show any visible differences when seen through the light microscope; therefore, observation through a scanning electron microscope was carried out to look for possible

differences in cell morphologies, which may be related to the high accumulation of lipid and thus DHA inside the cell. One of the first differences observed under SEM was the presence of two forms of the sporangium, which appears as either smooth or with a bumpy surface. The percentage of smooth cells from the F medium was 28.8%, while the percentage of cells with a bumpy surface was 71.3%. This is almost the same for cells grown in the NM medium as well, which has 27.9% smooth cells and 72.1% cells with a bumpy surface. In the case of cells grown in N and M media, almost all of the cells appear to be smooth, without bumpy surfaces.

Some cells grown in N medium in particular seemed to exhibit 'shrinkage', unlike other cells, which appeared round (Fig. 1.8). On the other hand, the cells cultivated in medium containing $MgSO_4$ appeared to be fragile compared with cells grown in $MgSO_4$-deficient F and N media (Fig. 1.5). Cells grown in NM medium have the most brittle-looking surface, followed by cells grown in the M medium. Another striking feature observed under SEM was differences in sporangium cell size depending on the culture medium. Cells grown in $MgSO_4$-deficient (N) medium have the smallest average cell diameter of 6.1 μm, and cells grown in NaCl-deficient (M) medium have the largest cell diameter of 14.5 μm, while cells grown in F and NM media had diameters of 9.2 and 11.6 μm, respectively (Fig. 1.6). The large size of cells grown in M medium is also accompanied by the presence of various abnormally shaped cells commonly seen with this medium (Fig. 1.7).

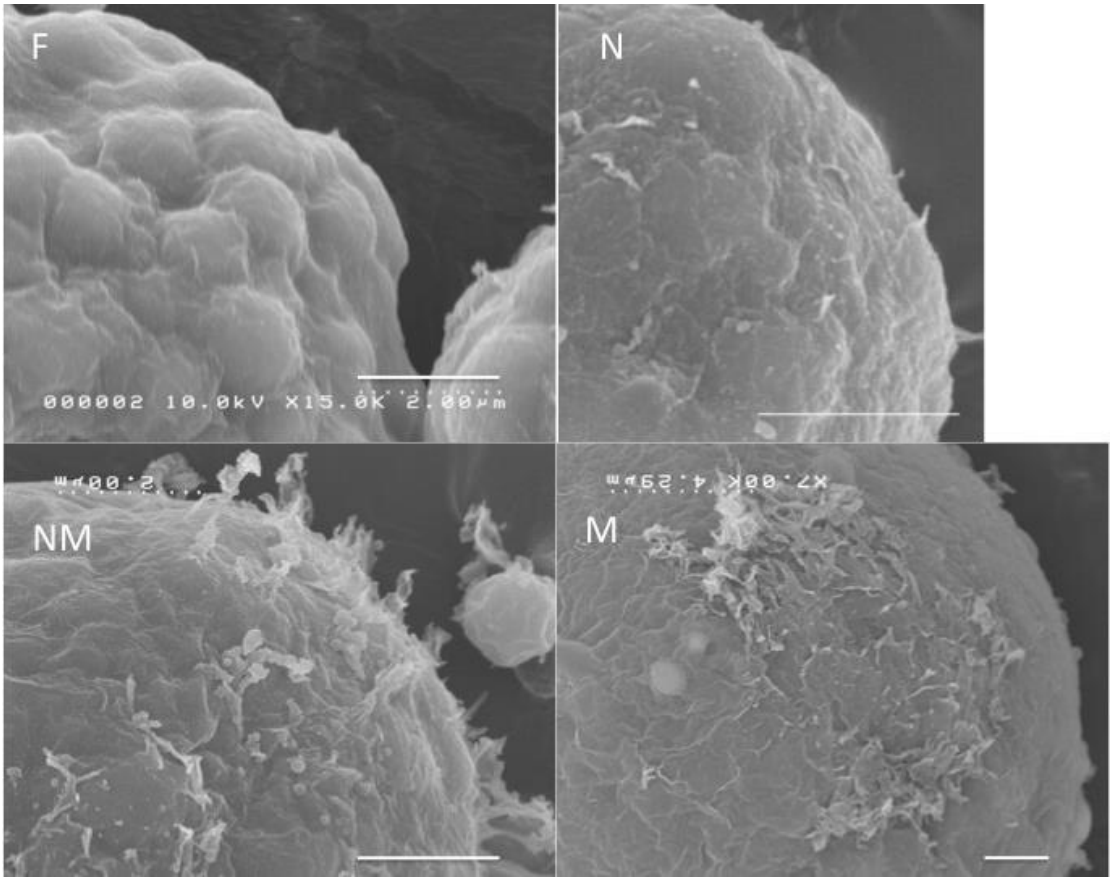


Figure 1.5. Observation of the cell surface of thraustochytrid strain 12B grown in F, NM, N and M media under SEM. Sloughing was very pronounced in $MgSO_4$-containing culture, especially in the NM medium. A colony from a 2-day BY+ agar plate was used as an inoculum, and incubation was carried out in a 50 ml flask containing 10 ml culture medium. Cell samples for SEM were collected after 3 days' cultivation at 30°C. All media contain 8% (w/v) glucose, 1% (w/v) peptone and 1% (w/v) yeast extract. In F medium, 50% (v/v) seawater was used, while NM, N and M media used distilled water. NM medium was supplemented with 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$. N medium was only supplemented with 0.1% (w/v) NaCl, while M medium was only supplemented with 1% (w/v) $MgSO_4$. The straight white bars represent 2 μm.

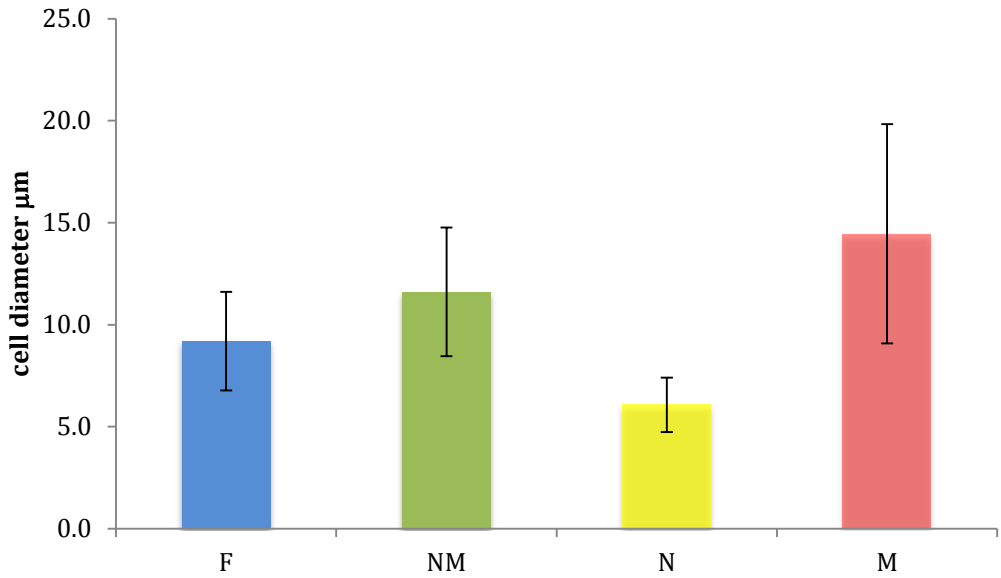


Figure 1.6. Effect of medium composition on the size of sporangium in thraustochytrid strain 12B. Averages were calculated from a total of 67, 105, 109 and 158 cells grown in F, NM, N and M media, respectively, seen under SEM. Cells grown in the M medium are largest in diameter compared with the cells grown in F, NM and N media. All cultures contain the same organic base of 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (w/v) peptone. F medium contains 50% seawater, N medium contains 0.1% (w/v) NaCl without $MgSO_4 \cdot 7H_20$ supplementation, and M medium contains 1% $MgSO_4 \bullet 7H_2O$ without NaCl supplementation, while NM contains both 0.1% NaCl and 1% $MgSO_4.7H_2O$. A colony from a 2-day BY+ agar plate was used as an inoculum, and incubation was carried out in a 50 ml flask containing 10 ml culture medium incubated in the dark for 3 days at 30°C.

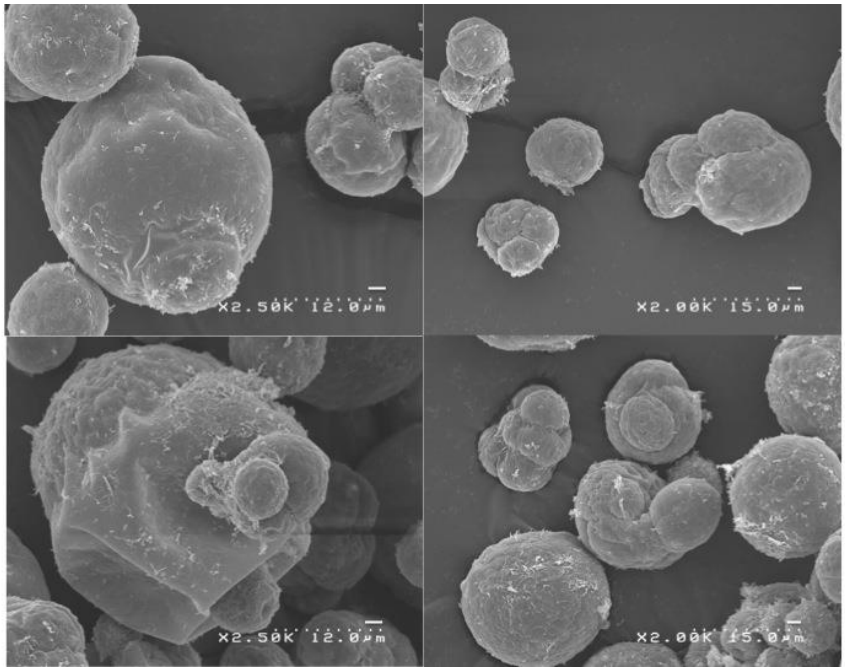


Figure 1.7. Abnormal cell shapes seen from thraustochytrid strain 12B cells grown in M medium. Some cells from the M culture show irregular cell shapes from the normal round sporangium seen in F and NM culture. The M medium is composed of 1% (w/v) $MgSO_4.7H_2O$ without NaCl supplementation but contains an organic base of 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (w/v) peptone. The culture was carried in a 50 ml flask by using 10 ml culture solution. The culture was grown for 3 days at 30°C in the dark. The straight white bars represent 2 μm.

### 1.4.2.2. Transmission electron micrography

Observation of the thraustochytrid strains under TEM helps to provide some information regarding the intracellular cell structures related to lipid accumulation such as oil bodies and therefore DHA. Cells grown in F and NM media were found to be filled with many large light and dark oil bodies (Lob and Dob, respectively), while a very limited number of smaller oil bodies were present in cells grown in N and M media (Fig. 1.8). Cells containing zoospores were also present, particularly in cells grown in F and NM media (data not shown). There were a total of 44 and 42 large oil bodies in the cells grown in F and NM media, respectively, while there were two large and seven small oil bodies in the N-cultured-cell cross-section micrograph and 112 small oil bodies in the M-cultured cell (Fig. 1.8). The distribution and density of mitochondria (mt) was also found to be different. Cells from F medium have randomly distributed mitochondria in the cytoplasm, while cells grown in NM medium had their mitochondria mostly at the periphery of the cell. Relatively large vacuoles were also seen, particularly in F medium- and NM medium-cultured cells. The cell nucleus was clearly seen in the TEM micrograph of the cells cultured in the NM medium

The thickness of the walls of cells from NM, F, N and M media was 0.08, 0.1, 0.24 and 1.5 μm, respectively (Fig. 1.8). However, the walls of cells grown in N medium seemed to be exfoliated easily through the conventional TEM preparation. The actual thickness of the walls of cells cultured in the N medium was greater, 2.14 μm, when the samples were prepared using the ultrarapid cryofixation technique because of its ability to preserve cellular structures during TEM preparation (Fig. 1.9). In either case, the walls of N medium- and M medium-cultured cells were found to be thicker compared with the productive cells from the F and NM media.

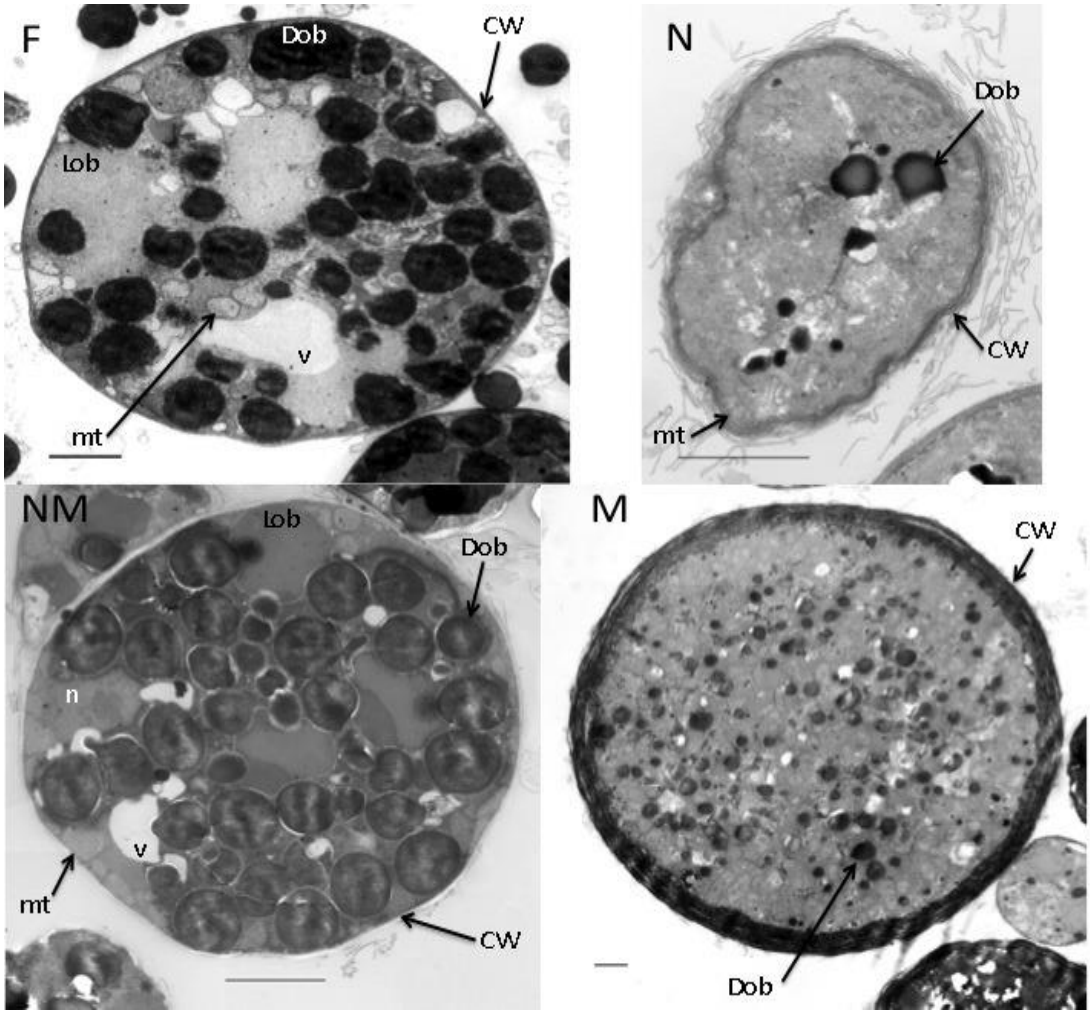


Figure 1.8. Cross-sectional observation of thraustochytrid strain 12B grown in F, NM, N and M media under TEM. Light oil bodies (Lob) contain saturated fatty acids, while dark oil bodies (Dob) contain high amounts of polyunsaturated fatty acid (PUFA). Higher amounts of oil bodies were found in F- and NM-cultured cells. Mitochondria (mt) are mostly found at the periphery of the cells cultured in the NM medium, while the mitochondria of cells grown in the F medium are more freely distributed throughout the cytoplasm. On the other hand, very few mitochondria were seen in the N- and M-cultured cells. Vacuoles (v) can be seen clearly in the F- and NM-cultured cells. The cell wall (CW) appears to be thicker in the N and M cultures compared with the F and NM cultures. The nucleus (n) is visible in the NM-cultured cell. The straight black bars represent 2 μm.

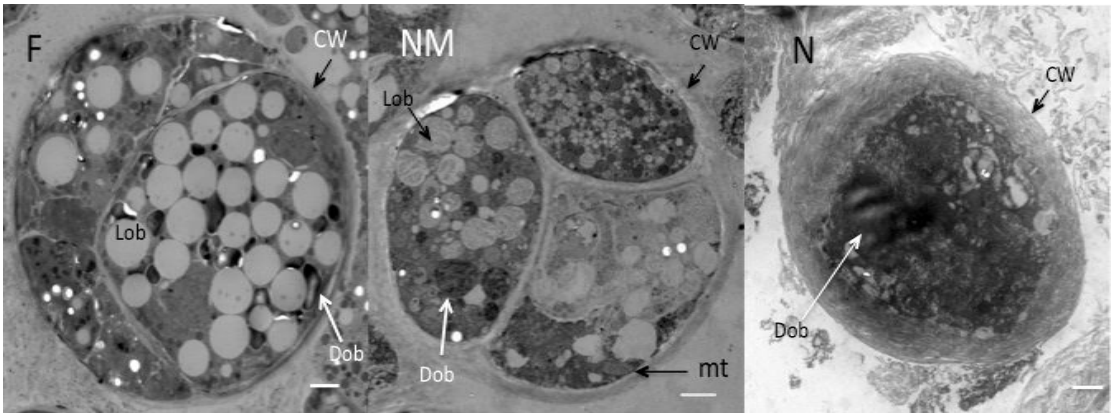


Figure 1.9. TEM image of thraustochytrid strain 12B in F, NM and N media sampled using the ultrarapid cryofixation technique. Many oil bodies were found in F- and NM-cultured cells. Light oil bodies (Lob) indicate higher amounts of saturated fatty acids, while dark oil bodies (Dob) indicate a higher composition of unsaturated fatty acids. Peripheral distribution of mitochondria (mt) for cells grown in NM is indicated. The original thick cell wall (CW) was preserved in the N-cultured cell by using the ultrarapid cryofixation technique. The walls of cells grown in F and NM media, however, remained thin and similar to when the cells were prepared using the standard noncryofixation TEM method. The strain 12B observed here was cultured in media containing the same organic base of 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (w/v) peptone. Only the F culture is composed of 50% (v/v) seawater, while the N medium contains 0.1% (w/v) NaCl without $MgSO_4 \cdot 7H_20$ supplementation, the M medium contains 1% $MgSO_4 \bullet 7H_2O$ without NaCl supplementation, and the NM medium contains both 0.1% NaCl and 1% $MgSO_4 \bullet 7H_2O$. A colony from a 2-day BY+ agar plate was used as an inoculum, and incubation was carried out in a 50 ml flask containing 10 ml culture medium incubated in the dark for 3 days at 30°C. The white bars represent 2 μm.

# 1.5. Discussion

## 1.5.1. NaCl and cell productivity

Thraustochytrids are marine microorganisms and require minerals that exist in seawater for growth. The major inorganic compounds present in seawater are ions of chloride ($Cl^-$), sodium ($Na^+$), sulfate ($SO_4^{2-}$), magnesium ($Mg^{2+}$), calcium ($Ca^{2+}$), potassium ($K^+$) and bicarbonate ($HCO^{3-}$) at concentrations of 19.0, 10.6, 2.6, 1.3, 0.4, 0.4 and 0.1 mg/ml, respectively (Brown et al., 1989). It is considered that one or more combinations of these ions could be necessary for optimal growth and high DHA production by microorganisms including thraustochytrids, and for this reason, NaCl, $MgSO_4$, $KHSO_4$, and $CaCO_3$ were used as representative compounds to screen for the most essential inorganic supplement using thraustochytrid strain 12B. To assess this, initially the amount of lipid was used as an indirect indicator for DHA production, as higher lipid production would also lead to an increase in the absolute amount of DHA (data not shown).

As shown in Figure 1.1A, strain 12B exhibited no growth in a NaCl-deficient medium, indicating an obligate requirement of NaCl for growth. This is in agreement with another report on the obligate requirement for NaCl in *T. aureum* (Garrill et al., 1992). This is probably because both the sodium cation and the chloride anion in thraustochytrids and other marine microorganisms have an important role in osmotic regulation (Garrill et al., 1992, Wethered & Jennings, 1985). According to Perveen et al. (2006), strain 12B grew in a medium in which 50% (v/v) seawater was replaced with water containing only glucose, peptone and yeast extract without NaCl. The growth of strain 12B observed in the absence of NaCl in that experiment was probably due to the transfer of 0.1 ml inoculum from a 1 day preculture of strain 12B in F medium, which contains 50% (v/v) seawater, thus giving rise to approximately 0.02% (w/v) NaCl in the culture medium. The use of a cell colony directly for inoculation makes the present study more accurate, as no carry-over of salts occurred, and strain 12B was indeed unable to grow in the absence of NaCl. However, the fact that the strain was able to grow in medium

containing as low as 0.02% (w/v) NaCl may indicate that the lower limit of NaCl concentration may not be 0.1% (w/v), which was the minimum concentration used in this study, and the actual minimum may still need to be determined. However, growth was still observed in the 0% (w/v) NaCl M medium when only 1% (w/v) $MgSO_4$ was supplemented, giving rise to 1.1 mg/ml DCW, although the amount of lipid was negligible (data not shown), which will be discussed later.

NaCl also appears to have a role in cell division, as cells grown in NaCl-deficient M medium have irregular cell morphology as observed under SEM (Fig. 1.7), in contrast to the generally round cells from the F and NM cultures, which also probably led to the formation of abnormally large cells about twice the size of other cells (Fig. 1.6). The dry cell and lipid yields of strain 12B in N medium at 0.1% (w/v) NaCl concentration without $MgSO_4$ supplementation were 4.1 and 1.1 mg/ml, respectively. Corresponding values were 11.8 mg/ml and 3 mg/ml (data not shown), respectively, for cells grown in the F medium containing 50% seawater, indicating that decreasing salinity has a direct effect on cell and lipid yields. This appears to be a common trend in thraustochytrid species such as *A. limacinum* (Nagano et al., 2009), *Thraustochytrium aureum* (Iida et al., 1996), *A. limacinum* 0UC88 (Zhu et al., 2008), *A. mangrove*i BL10 (Chaung et al., 2012), *A. mangrovei* KF-2/KF-7/KF-12, *T. striatum* KF-9 and *Ulkenia* sp. KF-13 (Fan et al., 2002). In a previous study, thraustochytrid strain 12B also showed a reduction in DCW, total fatty acid and DHA yield when seawater concentration was less than 50% (Perveen et al., 2006). Although studies that have investigated the effect of salinity on thraustochytrids by using pure NaCl without the use of seawater are very limited, apart from the present study, two other studies (Min et al., 2012 and Shabala et al., 2013) also showed a decrease in DCW when NaCl concentration was decreased. However, cell growth and cell count were not affected when the osmolality of the culture medium was maintained by replacing NaCl with a compatible osmolyte such as mannitol; thus, it was indirectly concluded by the author that besides its role in osmotic regulation, sodium is not involved in cell metabolism (Shabala et al., 2009). Yet it is known that sodium plays various metabolic roles inside the cell such as in cellular pH regulation through the $Na^+/K^+$ antiport (Krulwich, 1983), in

ATP synthesis by F-type ATPase on the mitochondrial inner membrane (Junge et al., 1997, Mulkidjanian et al., 2008) and in the transport of amino acids and nucleosides into the cell nucleus for nuclear protein synthesis (Allfrey et al., 1961). Although the conclusion of Shabala et al. (2009) regarding sodium being unnecessary for cell metabolism is obviously misleading, it gives an impression that the main role of sodium is indeed for osmotic regulation; however, a minimal amount of sodium for cell metabolic processes must be present. This is proven by the subsequent paper published by the same author (Shabala et al., 2013), which supplemented all of the culture media with 1 mM NaCl, and an implicit carry-over of NaCl from the inoculum appears to be present as well, which, based on calculation, caused the culture medium in that experiment to contain 0.6% (w/v) NaCl.

In other marine organisms, the relationship between salinity and lipid yield, however, is less consistent. *Isochrysis* sp. and *Nannochloropsis oculata* microalgae (Renaud & Parry, 1994) showed a decrease in lipid content with decreasing NaCl concentration, while in other algal species, such as *Cladophora vagabunda* (Elenkov et al., 1996), *Condria tenuissima* (Stefanov et al., 1994) and *Dunaliella salina* (Al-hasan et al., 1987), there is an increase in lipid content with decreasing salinity. This may indicate that thraustochytrids are a proper group of microorganisms, while microalgae, which are a large assembly of various species from different taxonomic classes and orders, may respond differently to changes in the environment.

### 1.5.2. Effect of magnesium and other compounds on cell productivity

Apart from reduced NaCl concentration, the absence of other inorganic nutrients was also a contributing factor to the lower DCW, lipid, fatty acid and DHA yields. The significance of other inorganic nutrients was seen when $Mg^{2+}$ and $SO_4^{2-}$, which are the second and third most abundant ions, respectively, were added as $MgSO_4$. When 1% (w/v) $MgSO_4$ was added to medium that contained 0.1% NaCl, DCW and lipid yields increased to 18.8 mg/ml and 11.2 mg/ml, respectively (Fig. 1.1B), which are about four- and 11-fold increases. One percent (w/v) of $MgSO_4$

seems to be saturated for strain 12B because further addition of this salt has no further effects on the cell yield in the flask cultivation. The importance of magnesium lies in its various roles in cell proliferation, apoptosis and protein synthesis (Wolf & Cittadini, 1999) as well as in diverse enzyme reactions (Jahnen-Dechnet & Ketteler, 2012). In the presence of a low NaCl concentration of 0.1% (w/v), the addition of magnesium increased both DCW and lipid yields; however, in the absence of NaCl, a negligible amount of lipid was extracted (data not shown). The dependence on sodium is probably because it is involved in the transport of magnesium into the cell through the sodium–magnesium antiport mechanism (Flatman, 1991). This has been observed in *Schizochytrium mangrovei*, in which a decrease in intracellular sodium concentration was seen together with an increase in magnesium concentration (Unagul et al., 2006). However, as mentioned above, thraustochytrids, including strain 12B, have an obligate requirement for sodium, resulting in a slight growth of 1.1 mg/ml DCW from cells cultivated in the M medium containing 1% (w/v) $MgSO_4$ and no NaCl supplementation. Based on the cell morphology from SEM and TEM observation with low 0.1% (w/v) NaCl and the absence of $MgSO_4$, some cells exhibit shrinkage (Fig. 1.8), suggesting that $MgSO_4$ may have some role in osmotic regulation. Because one of the important roles of NaCl is in osmotic adjustment (Shaballa et al., 2009), $MgSO_4$ may have partly fulfilled this role during NaCl deficiency. Another structural feature shown by strain 12B cells grown in the absence of either NaCl or $MgSO_4$ was the presence of thicker cell walls as seen under TEM (Fig. 1.8). Thick-walled thraustochytrids have been associated with low cell proliferation (Alderman et al., 1974) and therefore poor growth during cultivation. This is reflected by the low amounts of DCW from cells grown in NaCl-deficient M medium and $MgSO_4$-deficient N medium, which are 4.1 and 1.1 mg/ml, respectively, as well as by the low lipid yields, which are 1.1 mg/ml and negligible, respectively.Thraustochytrid microorganisms normally grow well in a medium containing 50% (v/v) seawater, which contains about 1.5% (w/v) NaCl. When the cells are grown in medium containing no NaCl, such as the M medium, or a low NaCl concentration, such as the N medium (0.1% NaCl, w/v), the hydrostatic pressure inside the cell is expected to be lower than outside the cell. This would result in an osmotic influx of water into the cell through the cell

membrane, which is somehow permeable to water and consequently could cause the cell to burst (Lodish et al., 2000). For eukaryotic cells that have a cell wall such as the thraustochytrids, an increase in cell volume because of osmotic inflow of water is normally prevented, and an increase in intracellular pressure occurs instead. However, unlike plant cells, which have a rigid cell wall, the cell wall of thraustochytrids is flexible and lacks any rigid fibrillar structural components (Darley et al., 1973). One way to increase the tensile strength of the cell wall is therefore by increasing its thickness, as in the case of algae that have high turgor pressure (Bisson, 1995). In addition, the thraustochytrid cell wall is also known to be hydrophobic to some extent (Raghukumar et al., 2000); therefore, a thicker cell wall would increase cell hydrophobicity, which therefore might restrict the osmotic inflow of water into the cell. Thraustochytrid cells that were grown in a medium with optimal osmolality would therefore have thinner cell walls, as seen in the high-DHA-productive cells grown in the F and NM media.

### 1.5.3. Possible effect of limiting compounds

Apart from nitrogen, phosphorus is also considered to be a limiting nutrient in seawater (Downing, 1997). Because $KHSO_4$ has a corrosive property, the effect of both noncorrosive phosphorus and non-phosphorus-containing potassium compounds $KH_2PO_4$, $K_2HPO_4$ and $K_2SO_4$ was also investigated. The chemical nature of the potassium-containing compounds varies as the pH of the culture medium changes when the NM medium is supplemented by individual compounds. The pH of $K_2HPO_4$ tends to increase, and that of $KHSO_4$ and $KH_2PO_4$ tends to decrease, while the pH of $K_2SO_4$ remains stable with increasing concentration (data not shown). A slight increase in DCW was obtained initially at very low potassium compound concentrations, but in general, the addition of potassium compounds decreases DCW yield with increasing concentration (Fig. 1.2). Further analysis was carried out at low potassium compound concentrations, and a slight increase in the lipid, fatty acid and DHA absolute yields was obtained (data not shown). When the culture medium was only composed of NaCl and $K_2SO_4$ as inorganic nutrients, the DCW yield was only one-third that of NM medium, while the lipid yield was

approximately 19 times far lower than that of NM medium. Furthermore, the effect on the yields was almost the same regardless of the absence (INM medium) or presence (INMK medium) of $K_2SO_4$ in the medium with DHA productivity of 1.5 and 1.6 mg/ml/day, respectively (Table 1.3). This indicates that the magnesium ion, not the sulfate ion, is the most essential ion in $MgSO_4$ contrary to the previous suggestion by Nagano et al. (2009). The cell wall is thought to be made up of circular scales synthesized by the Golgi apparatus, which are transferred to the cell surface, resulting in the laminated structure of the cell wall as seen in thraustochytrid species *T. aggregatum* (Darley et al., 1973) and *Althornia crouchii* (Alderman et al., 1974). In *Aplochytrium* sp. 2400/57, the cell wall is composed of protein, carbohydrate and sulfate at 27.4%, 20% and 5.3%, respectively, of the total cell wall matter (Ulken et al., 1985), while in *Thraustochytrium* sp., this varies from 30% to 43% protein and from 21% to 36% carbohydrate. The other half of the cell wall composition is not yet known (Ulken et al., 1985). Because sulfate makes up 5.3% of the cell wall (Ulken et al., 1985), it is therefore still a necessary inorganic component in the culture media.

The exclusion of potassium compounds from the culture medium can be considered as advantageous as it will help to reduce industrial cost with simple medium composition. Analysis of the fatty acid composition also shows that the percentage of DHA per total fatty acid is generally higher in the absence of potassium compound (data not shown). The percentage composition of C18:1 is also lower, at 1.4% instead of about 29%, when the medium is not supplemented with potassium; therefore, the extracted oil has higher quality and lower complexity for DHA purification when potassium compounds are not added to the culture medium. On the other hand, the addition of calcium and carbonate ions also tended to reduce the DCW and lipid yields (Fig. 1.1C, 1.1D), proving it to be unnecessary for strain 12B and possibly for other thraustochytrids as well. The concentration needed for these nutrients is probably low and could be obtained from the organic nutrients such as the yeast extract.

The characteristic genus-specific, fatty acid composition of strain 12B additionally was found not to be affected by the culture medium. The ARA

percentage composition was unaffected at less than 3%, which can be used to distinguish it from the genus *Sicyoidochytrium*, which normally has no AA, and this also is the case for the genus *Aplanochytrium* (Yang et al., 2010), while *Schizochyrium*, *Ulkenia*, botryochytrium and parietichytrium all have AA at more than 3% per total fatty acid (Laby Base, 2009a). The strain 12B n-6 docosapentaenoic acid (DPA) was also unaffected, and the absence of n-3 (DPA) eliminates the probability of it being under the genus *Oblongichytrium* (Laby Base, 2009b). Apart from the lack of lipid information for the genera *Althornia* and *Japonochytrium*, and the absence of a unifying fatty acid characteristic for *Thraustochytrium*, it is therefore most likely that thraustochytrid strain 12B belongs to the genus *Aurantiochytrium*, based on the information of its fatty acid composition.

### 1.5.4. Applicability of the NM medium

Apart from being noncorrosive to culture equipment at low NaCl concentration, the NM medium's DHA yield is also about five times higher than that of the F medium after 3 days' cultivation, hence exhibiting a faster lipid accumulation (Table 1.1). DHA was mostly stored in the form of triacylglycerol (TG). The NM medium was also applicable to other thraustochytrid strains such as the *Aurantiochytrium* sp. SR 21 strain, which has a higher efficiency of glucose utilization in the NM medium compared with the F medium, which becomes depleted after 5 days instead of 6 days (data not shown). The NM medium also requires less than 8% (w/v) glucose (Fig. 1.4) as opposed to the optimal concentration previously reported (Perveen et al., 2006). The faster lipid accumulation is probably related to growth as both the 12B and the SR21 thraustochytrid strains enter the stationary phase earlier during cultivation in the NM medium (Fig. 1.3). This is an important feature because cells in the stationary phase tend to have a high amount of triacylglycerol (Ratha & Prasanna, 2012). Cells grown in medium containing $MgSO_4$, especially in NM medium, show sloughing of the cell surface (Fig. 1.5), which is a characteristic feature of mature cells (Harrison & Jones, 1974) and might be related to the early stationary growth (Fig. 1.3).

The thraustochytrid strain 12B cells appeared to have a surface that was either relatively smooth or bumpy, with the latter being most common, especially in cells grown in F and NM media. The bumpy surface is most likely due to the presence of oil bodies inside the cell, suggesting a soft or flexible nature of the cell wall, and therefore can be used as an indirect indication of high lipid production. The flexible nature of the cell wall has been reported by Darley et al. (1973) in *T. aggregatum,* whereby the cell wall obtained from sonication was easily folded or wrinkled during whole mount preparation because of the absence of rigid fibrillar structural components. Oil bodies appear as light, grayish or dark regions inside the cells, which occurs through the reduction of EM reagent $OsO_4$ to the black colored $OsO_2$, especially in lipid (Hayes et al., 1963), particularly in fatty acids with *cis*-unsaturated bonds (Adams et al., 1967) with increased in the intensity with the increase in the number of double bonds as has been demonstrated previously using C18:1, C18:2 and DHA (Cheng et al., 2009). Some oil bodies also have gradation of intensity, which is probably due to the formation of a semicrystalline triacylglycerol state, forming alternate dark PUFA layers and light saturated-monounsaturated fatty acid layers (Ashford et al., 2000). Hollow membrane-like structures can also be seen inside some of the smaller oil bodies (data not shown), which is thought to be associated with lipid synthesis, which become internalized during lipid deposition (Weete et al., 1997) and disappear in larger oil bodies (Morita et al., 2006).

Compared with previous studies, the 0.2 g/l/day DHA productivity from the flask cultivation in F medium from this experiment was lower than that in earlier experiments, which reported a productivity of 2.8 g/l/day (Perveen et al., 2006) and 3.1 g/l/day (Okuyama et al., 2008a), which is probably due to differences in inoculation. The DHA productivity of strain 12B in the NM medium increased successively from cultivation using laboratory-grade to industrial-grade ingredients and from flask to jar fermentation (Table 1.3). However, when the industrial-grade medium (IN) was not supplemented with magnesium, productivity was comparatively very low, with DCW and lipid yields being 4.6 and 2.7 mg/ml, respectively; therefore, the presence of possible contaminating

compounds is still not as crucial as magnesium supplementation (data not shown). Alternatively, the strain probably favors plant-based nitrogen sources, as the laboratory-grade peptone used was animal based (casein pancreatin digest), while the industrial-grade peptone was plant based (made from soy).

The preference for plant-based nitrogen sources may be related to the ecology of thraustochytrids, as these microorganisms generally live on plant detritus in nature (Raghukumar, 2002). In particular, one of the major amino acids produced from metabolism of ammonia in plant leaves is glutamic acid (Miflin & Lea, 1982). This is a major amino acid component of the cell wall, accounting for 28.5% of the total amino acids in *T. aggregatum* (Darley et al., 1973). It is also among the most efficient nitrogen sources for thraustochytrid growth (Singh & Ward, 1997) and has been reported to elicit strong attraction responses in *S. mangrovei* zoospores (Fan et al., 2002). Although the information on the amount of glutamic acid in the peptone was not disclosed by the manufacturer, a high amount of glutamic acid has been known to be present in soybean (Wolf et al., 1982). The use of plant-based ingredients is also advantageous for industrial application as it will be able to comply with the halal food industry requirements. The increase in DCW, lipid, fatty acid and DHA yields when the cultivation method was changed from flask to jar is thought to be due to the better aeration and mixing in the jar fermenter. The highest DHA productivity from the jar fermenter for strain 12B reported so far is 5.4 g/l/day (Okuyama, 2008a); therefore, further optimization of large-scale cultivation parameters is possible when using the NM medium to increase the DHA yield from the current 2.3 g/l/day productivity.

## 1.6. Conclusions

A noncorrosive NM medium consisting of 8% glucose, 1%(w/v) yeast extract and 1% (w/v) peptone supplemented with 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$, resulted in high DCW, lipid and DHA yields of 18.8, 11.2 and 2.4 mg/ml, respectively, after 3 days' cultivation, which is therefore superior to the F medium containing 50% seawater. The DHA yield was further increased through jar cultivation using industrial-grade chemicals producing 11.7 mg/ml DHA or a productivity of 2.3 mg/ml/day. DHA in TG contributed to 71% of total DHA when strain 12B was grown in F medium and 81% in the NM medium. NaCl and $MgSO_4$ are the most important inorganic compounds for the cultivation of thraustochytrid strain 12B, while other compounds—$CaCO_3$, phosphorous and non-phosphorous-containing potassium compounds $KHSO_4$, $K_2SO_4$, $KH_2PO_4$ and $K_2HPO_4$—either reduce the DHA yield or decrease the DHA purity by increasing the percentage composition of C18:1. Cells grown in the NM medium have high amounts of oil bodies and exhibit thin cell walls and sloughing of the cell surface, indicating prolific growth and rapid cell maturity. This is supported by the growth profile, which shows cells from the NM culture entering the stationary phase earlier than cells from the F culture. Cells from the NM medium also have thin cell walls, which indicate prolific growth compared with the case when either NaCl or $MgSO_4$ is deficient (N and M media). The absence of NaCl results in abnormally large cells and unusual cell morphology, which may indicate the role of NaCl in cellular division. The absence of $MgSO_4$, on the other hand, results in shrinking of some cells, indicating its possible role in osmotic regulation. The NM medium also has higher glucose utilization efficiency, with glucose being depleted faster than in F medium, and less than 8% (w/v) glucose is required for high DCW, lipid and DHA yields. The NM medium was also applicable to the thraustochytrid species *Aurantiochytrium limacinum* SR21 and possibly to other thraustochytrid species as well.

# Chapter 2: Tentative identification of thraustochytrid strain 12B

## 2.1 Summary

Molecular and morphological taxonomic data indicated that the thraustochytrid strain 12B belongs to the genus *Aurantiochytrium*. Strain 12B exhibited the presence of an amoebic stage, the disintegration of the cell wall during zoospore release, a lack of naked protoplasm and the rare presence of an ectoplasmic network, all indicating that it belongs to the genus *Aurantiochytrium*. Moreover, phylogenetic analysis using the 18S rRNA gene sequence showed that strain 12B groups together well with other *Aurantiochytrium* species. This is further supported by mitochondrial DNA analysis, which shows a very high sequence similarity to *Aurantiochytrium limacinum* SR21 compared with a strain from another genus, *Thraustochytrium aureum*. The cytochrome oxidase II (COXII) gene probably has a high taxonomic value at the genus level for thraustochytrid microorganisms. The proposed cutoff value for the percentage 18S rRNA sequence similarity of 98.8% for a species was higher than the 98.5% similarity found between strain 12B and *A. limacinum*, which suggests that strain 12B and *A. limacinum* may be closely related but are two different species. This is further supported by the differences in physiology and phenotype between strain 12B and *A. limacinum* SR21, which showed differences seen in terms of coloration on agar culture, rate of glucose utilization, differences in DCW, lipid, fatty acid and DHA yields during cultivation as well as differences in the zoospore flagellum structure. It has also been reported previously that fusion between cells was observed in strain 12B, which has never been mentioned in other thraustochytrid species. The presence of multiple 18S rRNA gene copy in the previous study of strain 12B is also almost unique among thraustochytrid microorganisms. Comparison of the structure of tubular hair on the anterior flagellum between strain 12B and SR21

also indicates an apparent difference, providing further support of their status as separate species. In addition, from the life-cycle study, when grown in the NM medium, strain 12B was observed to have shorter motile stages and longer immotile stages compared with cells grown in the F medium containing 50% seawater, which could explain why cells grown in the NM medium accumulated lipid faster and more through conservation of oil bodies than those grown in the F medium.

## 2.2. Introduction

### 2.2.1. Taxonomic overview of thraustochytrid microorganisms

Thraustochytrid microorganisms have been grouped together with brown algae (Phaeophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae) and diatoms (Bacillariophyta) under the same phylum, Heterokonta (Cavalier-smith, 1994, NCBI taxonomy, 2013). Some authors use the term 'thraustochytrids' to refer to genera or species from both families of thraustochytriaceae and labyrinthulaceae (Gupta et al., 2012, Metz et al., 2010), while others use the term specifically for genera or species under the thraustochytriaceae family only (Raghukumar, 2008b, Tsui and Vrijmoed, 2012). In this study, the meaning of 'thraustochytrids' used by Raghukumar (2008b) and Tsui and Vrijmoed (2012) is followed. Various taxonomic changes were made for the thraustochytrid group, with the latest taxonomic classification being domain Eukaryota, kingdom Chromalveolata, phylum Heterokonta/Stramenopiles, class Labyrinthulomycetes, order Labyrinthulales and under the family Thraustochytriaceae, while Labyrinthulids, on the other hand, belong to the same class but under the family Labyrinthulaceae (Honda, 2009, Gupta et al., 2012, NCBI, 2013). Thraustochytrid strains form 11 genera: *Althornia* (1), *Aplanochytrium* (8), *Aurantiochytrium* (2), *Botryochytrium* (1), *Japonochytrium* (1), *Oblongichytrium* (3), *Parietichytrium* (1), *Schizochytrium* (1), *Sicyoidochytrium* (1), *Thraustochytrium* (17) and *Ulkenia* (3) species, with the number of known species in brackets (Laby Base, 2009c). Most thraustochytrid species are considered to have proper scientific names; however, there are hundreds of thraustochytrid strains that have been isolated but are still without proper species names and are waiting to be studied.

### 2.2.2. Characterization of thraustochytrid microorganisms

The unique characteristic of thraustochytrid microorganisms is that they all have PUFAs, particularly DHA, in high quantity, and the percentage per total fatty

acid varies depending on genus (Huang et al., 2003, Yokoyama et al., 2007, Yokoyama & Honda, 2007, Yang et al., 2010). The thraustochytrid strain 12B, for example, was reported to contain DHA at 43% of total fatty acid, producing 2.8 mg/ml/day DHA (Perveen et al., 2006). The labyrinthulid species too may contain DHA (Sakata et al., 2000, Kumon et al., 2002, 2006) but probably not in all species (Kumon et al., 2003). Fatty acid composition has been used as a taxonomic tool for differentiation of thraustochytrids at the genus level (Laby Base, 2009a, b, Yang et al., 2010) as mentioned in Chapter 1. Taxonomic studies on strain 12B have been carried out in the past. The zoospore of strain 12B has previously been observed to have two different laterally inserted flagella, with the anterior flagellum being lined with tubular hairs (Perveen et al., 2006). The presence of a multilayered cellulosic cell wall, mitochondria with tubular cristae, an ectoplasmic network and the presence of the amoebic form have been documented by Ando (2005) and were also seen in this study, some of which are unique characteristics of thraustochytrid microorganisms. Phagocytosis or fusion among thraustochytrid cells has never been reported in the literature but was observed in strain 12B (Perveen and Okuyama, unpublished data). Molecular characterization by using the 18S rRNA gene was also undertaken for strain 12B, and direct sequencing of the PCR product from the 18S rRNA amplification was not possible unless the product was cloned into *Escherichia coli* first. It was found that as many as 32 multiple sequences for the 1727 base pair (bp) 18S rRNA gene existed for strain 12B (Ando, 2005). Although the presence of several 18S rDNA units in tandem is common in eukaryotes (Long and Bawid, 1980), reports of this in thraustochytrids appears to be limited to only one paper, regarding *Schizochytrium* sp. TIO01 (Cheng et al., 2011); therefore, this might be a unique characteristic only for some thraustochytrid species including strain 12B. Definitive taxonomic identification, however, has not yet been made for strain 12B because of the confusion in the taxonomic classification at that time. Major strides in improving the taxonomic classification of thraustochytrids have been made and are still going on; therefore, reprocessing of the accumulated information on strain 12B to cope with the changes in taxonomy, and further studies, are still necessary for its identification.

Traditional taxonomic studies on thraustochytrids normally focus on phenotypic characterization and life cycle for strain identification (Harrison & Jones, 1974, Alderman et al., 1974), and these are still used today. A biochemical method using acriflavine and ThrFL1 probe has been available to identify thraustochytrids from other protists through florescence (Gupta et al., 2012). For intergenus taxonomic characterization, apart from morphology, fatty acid composition analysis (Huang et al., 2003, Yokoyama et al., 2007a, Yokoyama & Honda, 2007, Yang et al., 2010) and carotenoid composition (Yokoyama et al., 2007, Yokoyama & Honda, 2007) have already been used. Molecular methods are increasingly used together with morphological information to reinforce the species taxonomic identification. The first attempt to use the molecular method in the phylogenetic study of thraustochytrids was probably by Porter and Kochert (1978) using rRNA molecular weight, and over the past decade, an increasing number of studies have incorporated the molecular approach. Molecular markers that have been used in the phylogenetic study of thraustochytrids include genes of 5S rRNA (Mannella et al., 1987), 18S rRNA (Cavalier-Smith et al., 1994, Leipe et al., 1996, Honda et al., 1999, Mo et al., 2002, Yokoyama et al., 2007, Yokoyama & Honda, 2007, Tsui et al., 2009), SSU rRNA, 5.8S rRNA (Stokes et al., 2002, Qian et al., 2007), internal transcribed spacers 1 and 2 (ITS 1 & 2, Qian et al., 2007), actin, tubulin and elongation factor 1-alpha (Tsui et al., 2009), as well as DNAs amplified by the randomly amplified polymorphic DNA (RAPD) method (Oclarit & Hepowit, 2007). Mitochondrial DNA is also a useful molecular marker and may help to reinforce the phylogeny data from the 18S rRNA gene, which is the most widely used molecular marker for phylogenetic relationship in eukaryotes. Mitochondrial genes such as cytochrome oxidase I (COXI) and NADH dehydrogenase subunit 5 (ND5 have been used for phylogenetic studies of various organisms including, but not restricted to, flies (Bajpai & Tewari, 2010), cytochrome oxidase subunit II (COXII) and cytochrome b in butterflies (Torres et al., 2001) and mammals (Honeycutt et al., 1995, Piaggio & Spicer, 2001), cytochrome oxidase III (COXIII) in protozoa (Tian et al., 2013), and ND2 and ATPase6 in fish (Mu et al., 2012). Phylogenetic analysis using mitochondrial DNA COXI has also been used on thraustochytrids (Qian et al., 2007).

Mitochondrial COXI DNA sequence has become a standard molecular tool for species identification in eukaryotic organisms (Ratnasingham and Hebert, 2007). Cytochrome oxidase functions as the final enzyme in the respiratory chain (Denis, 1985) and also as a proton pump that generates transmembrane electrochemical gradient for ATP synthesis (Richter and Ludwig, 2003). In the mature enzyme complex, the center is made up of subunits I, II and III, which are encoded by the mitochondrion (Richter and Ludwig, 2003). Like the commonly used nuclear DNA molecular tool, the 18S rRNA gene, the important role of COX in the fundamental functioning of the cell results in the conservation of the DNA sequence. However, compared with the 18S rRNA gene, the COX gene sequence is more variable because of its location in the mitochondria, as it is predisposed to damage from reactive oxygen species produced during mitochondrial oxidative phosphorylation (Yakes and Houten, 1997) and because there are fewer DNA repair mechanisms in the mitochondria compared with the nucleus (Larsen et al., 2005). Compared with nuclear DNA, mitochondrial DNA is also a favorable molecular tool for several reasons including the presence of multiple copies allowing easy amplification and the general absence of introns (Galtier et al., 2009). Moreover, the short length of about 600 bp pairs of the COX gene facilitates rapid sequencing but contains adequate nucleotide variation for species identification (IBOL, 2014). The phylogenetic analysis of the COXII gene in the pseudofungus *Pythium* species under the class Oomycete, which was the same class to which thraustochytrids previously belonged (Sparrow, 1960), has shown good resolution of species and forms groupings that agree with 28S rRNA gene analysis (Martin, 2000) or in some cases the phylogenetic analysis of the COXII gene is better than that of the ribosomal internal transcribed spacer (Robideau et al., 2011). The interspecies resolution of COXII also helped to differentiate between two morphologically similar *Pythium* species, *P. oligandrum* and *P. hydrosporum* (Villa, 2006), while COXI was also used to identify new *Pythium* species (Long et al., 2013). The use of COXI in microalgae (Evans and Mann, 2009) and algae (Robba et al., 2006, Boo et al., 2011) has also been reported.

The aim of this chapter is to investigate the taxonomic status of strain 12B, which previously has been poorly characterized, and to apply the findings in life-cycle and other characterization studies to help explain further the accumulation of lipid and DHA in the cells grown in NM medium that contain 0.1% NaCl and 1.0% $MgSO_4$ but not seawater (see Chapter 1).

## 2.3. Materials and methods

### 2.3.1. Microorganisms and their cultivation

Two thraustochytrid microorganisms were cultivated, the thraustochytrid strain 12B (strain 12B; NITE P-68) and *Aurantiochytrium limacinum* SR21. Strain 12B was previously isolated from a mangrove ecosystem in Okinawa, Japan (Perveen et al., 2006), while *A. limacinum* SR21, isolated from a coral reef area of the Yap Islands, Federated States of Micronesia and originally identified as *Schizocytrium limacinum* SR21 (IFO 32693) (Nakahara et al., 1996), was kindly provided by the IFO, with permission of Dr. Yokochi Toshihiro, National Institute of Advanced Science and Technology (AIST). For DNA extraction, both strains were cultivated in NM medium containing 8% (w/v) glucose, 1% (w/v) yeast extract, 1% (peptone), 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4$ for 3 days at 30°C. F medium was also used for cultivation. The composition of F medium is almost the same as NM medium except that in place of NaCl and $MgSO_4$, 50% (v/v) seawater was used.

### 2.3.2. DNA extraction, PCR, and sequencing the mitochondrial COX gene

In this study, the 18S rRNA gene sequence, which had been determined by Ando (2005), was reanalyzed with other thraustochytrid sequences from GenBank by choosing representative species from every thraustochytrid genus based on the latest taxonomic classification. In order to know the phylogenetic relationship between strain 12B and *Schizochytrium* (*Aurantiochytrium*) *limacinum* SR21, which is considered to be the closest organism to strain 12B Ando (2005), mitochondrial DNA COXI, COXII, and COXII including ITS (COXII+ITS) gene sequences of these two strains were determined.

Fresh genomic DNA was extracted from strain 12B and *A. limacinum* SR21 cells using the DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Three sets of primers were used for the amplification of COXI, COXII, and COXII+ITS. Primers FM55 and FM52R (Long et al., 2013) were used to amplify the COXI gene, and primers FM58

and FM66 (Villa et al., 2006) were used to amplify the COXII gene, while primers FM35 and FMPhy-10b (Martin, 2000) were used to amplify the COXII+ITS gene (Table 2.1). The PCR mix is composed of 21.75 μl $H_2O$, 25 μl PCR buffer, 1 μl forward and reverse primers (6.4 pmol), 0.25 μl MightyAmp DNA polymerase and 1 μl template DNA (10 ng/μl). The PCR temperature program for COXI was: 95°C for 3 min initial denaturation, 35 set cycles of 95°C for 30 sec denaturation, 54°C for 30 sec annealing, 72°C for 30 sec extension, and a final 72°C for 5 min extension step, while the annealing temperature for COXII and COXII+ITS was set to 56°C (GeneAmp 2720 Thermal Cycler, Applied Biosystems, Tokyo, Japan). The amplified gene segment was confirmed by electrophoresis (Mupid, Advance Co. Ltd, Tokyo, Japan) against a known 100 bp molecular marker (Takara, Shiga, Japan). PCR products were purified for sequencing using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Tokyo, Japan). Samples for sequencing were prepared by mixing 1 μl PCR product, 1 μl primer (6.4 pmol) and 12 μl distilled water. A total of six reaction mixtures per sample, one for each primer (Table 2.1), were sent to Operon Biotechnology Corp. and sequenced (ABI 3730XL, Applied Biosystems, Tokyo, Japan). Sequences obtained were then aligned, edited and run in the NCBI BLAST database search (Discontiguous MegaBLAST) for cross-species sequence similarity analysis. For sequence identity/similarity analysis of multiple sequences, the sequences were submitted online to http://www.ebi.ac.uk/Tools/msa/clustalw2/ (European Bioinformatics Institute, Cambridge, UK) for pairwise alignment and DNA weight matrix analysis with ClustalW. The short length of the sequence at the start and end containing the 'N' unknown nucleotides, which occurred because of sequencing error, were not included in the sequence similarity calculation.

Table 2.1. Primer sequence used for the amplification of COX genes.

| Primers | Sequence 5'-3' | Target gene | References |
|---|---|---|---|
| FM55 | GGCATACCAGCTAAACCTAA | COXI | Long et al. (2013) |
| FM52R | TTAGAATGGAATTAGCACAAC | COXI | Long et al. (2013) |
| FM58 | CCACAAATTTCACTACATTGA | COXII | Villa et al. (2006) |
| FM66 | TAGGATTTCAAGATCCTGC | COXII | Villa et al. (2006) |
| FM35 | CAGAACCTTGGCAATTAGG | COXII+ITS | Martin (2000) |
| FMPhy-10b | GCAAAAGCACTAAAAATTAAATATAA | COXII+ITS | Martin (2000) |

### 2.3.3. Observation of a life cycle of strain 12B

For the direct observation of life cycle, a small amount of cells was transferred from a 2-day agar culture, by touching the colony with the tip of a sterilized toothpick, into an Eppendorf tube containing 1 ml F medium, NM medium or seawater. About 5–20 μl of the suspension was then pipetted into the well of a sterilized glass slide and covered with a coverslip. The well on the glass slide was made by using vinyl tape (Nichiban, Tokyo, Japan), with one part of the tape being discontinuous to provide ventilation. Observation of the life cycle of thraustochytrid strain 12B was carried out using a light microscope (BX 51TF, Olympus, Tokyo, Japan) under a UPlanf1 40×/0.75 objective, with the light set to between 4 and 6. An eyepiece camera (MacWin MW130, Science-eye, Niiza, Japan) was inserted in the microscope observation tube and connected directly to a computer, which recorded a continuous or snapshot video of 1 frame every 15, 30 or 60 sec (DinoCapture 1.0 software, AnMo Electronics Corporation, Taipei, Taiwan).

# 2.4. Results

## 2.4.1. Phenotypic and life-cycle study of strain 12B

The phenotypic characteristics observed in this study are shown in Table 2.2. From the SEM observation in Chapter 1, the size of the round sporangium varied depending on the culture medium, and it increased in the order of N (6.1 μm in diameter), F (9.2 μm), NM (11.6 μm) and M (14.5 μm) media. Cell size appeared to be bigger when observed through the light microscope compared with when the size was determined under SEM (Table 2.2), which was also the case in the work by Ando (2005). Most of the phenotypic characteristics, including cell size, shape of sporangium and zoospore, disintegration of cell wall after zoospore release, type of cell division and resultant number of cells produced after cell division, were almost the same among the three species, strain 12B, *A. limacinum* SR21 and *A. mangrovei* (Table 2.2). The most interesting morphological feature is probably related to the structure of the zoospore anterior flagellum and the presence of pseudopodia during formation of the amoebic cells, which can be seen in *A. limacinum*. *A. limacinum* has zoospores, which have hairs with a tapered base and a straight shaft. This was not observed in the zoospore of strain 12B, while the type of flagellum in *A. mangrovei* is still unknown. Because of the lower resolution of the light microscope, the presence of pseudopodia was not clearly seen in strain 12B; however, they do appear to be present, although not as much as in *A. limacinum* (data not shown). The presence of pseudopodia in the amoebic cells of *A. mangrovei*, on the other hand, has never been reported. On agar culture, the colonies of strain 12B were cream white in color, and they formed a round, entire margin with a smooth surface, while *A. limacinum* appeared slightly pinkish in color during dense growth on BY+ agar culture. Growth seen through an increase in optical density for thraustochytrid strain 12B cultivation in nutrient-rich F and NM media containing 1% yeast extract, 1% peptone and 8% glucose was followed for 8 days. The stationary phase began to appear around 3–5 days, while the death phase was very pronounced after 7 days in the F medium (Fig. 2.1)

Table 2.2. Morphological features of strain 12B and other *Aurantiochytrium* species

| Cell features | | *Aurantiochytrium* species | | |
|---|---|---|---|---|
| | | Strain 12B[+] | *A. limacinum* (Honda et al, 1998)[*] | *A. mangrovei* (Raghukumar, 1988)[#] |
| Vegetative cell | size (μm) | 24.3 | 7–15 | 6.3–9.2 |
| Ectoplasmic network | type | may be monoaxial[$] | monoaxial | monoaxial |
| Sporangium | size (μm) | 9.2–24.3 | 12–24 | nd[&] |
| | times of binary division | 2–5 | 4–5 | 4–5 in SWP |
| | cell no. in a sporangium | 4–32 | 16–32 | 12 in SWP; 4, 8, 16 or 32 in N |
| Sporangium | shape | ovoid | ovoid | elliptical |
| zoospore | length (μm) | 10 | 6–8.5 | 4.1–4.8 |
| | width (μm) | 5 | 5–7 | 2.6–3.3 |
| | cell wall after zoospore released | disintegrated | disintegrated | disintegrated |
| Flagellum | base | nd | tapered | nd |
| | shaft | not straight | straight | nd |
| Amoeba | length (μm) | 16.8–121.3 | 12–20 | nd |
| | width (μm) | 7.5–16.8 | 3.5–5 | nd |
| | pseudopodia | may be present but not dominant | present | nd |
| | no. of nucleus | nd | 2 | nd |
| Settled amoeba | diameter (μm) | 13.1 | 9.5–10.5 | nd |
| Amoeba zoospore | no. of zoospore | 4 | 8 | 2, 4, 8, 16 or 32 |
| | length (μm) | 9.3 | 4.5–6 | nd |
| | width (μm) | 8.4 | 3.5–5 | nd |

*A. limacinum* and *A. mangrovei* were the only two described species under the genus *Aurantiochytrium* with morphologies based on the size of the vegetative cell, the sporangium, the zoospore and the amoebic form. Binary cell division, disintegration of the cell wall after zoospore release and monoaxial location of an ectoplasmic net are shared by all *Aurantiochytrium* species. The number of cells after cell division or zoospore formation generally depends on the size of the original cell, which in turn is affected by the culture medium, for example. The morphology of the zoospore anterior flagellum and the presence of pseudopodia during the amoebic stage may potentially be strong distinguishing features among the *Aurantiochytrium* species. All data except for flagellum were based on light microscopic observation.

[+] Strain 12B was grown in nutrient F or NM media except for the vegetative cells, which were grown in seawater.

[*] All observations were from cells grown in nutrient (N) media prepared using seawater.

[#] Both seawater/pine pollen (SWP) and nutrient (N) media were used to observe cell development.

[$] Evaluated from Ando (2005)

[&] Not determined because of a lack of information.

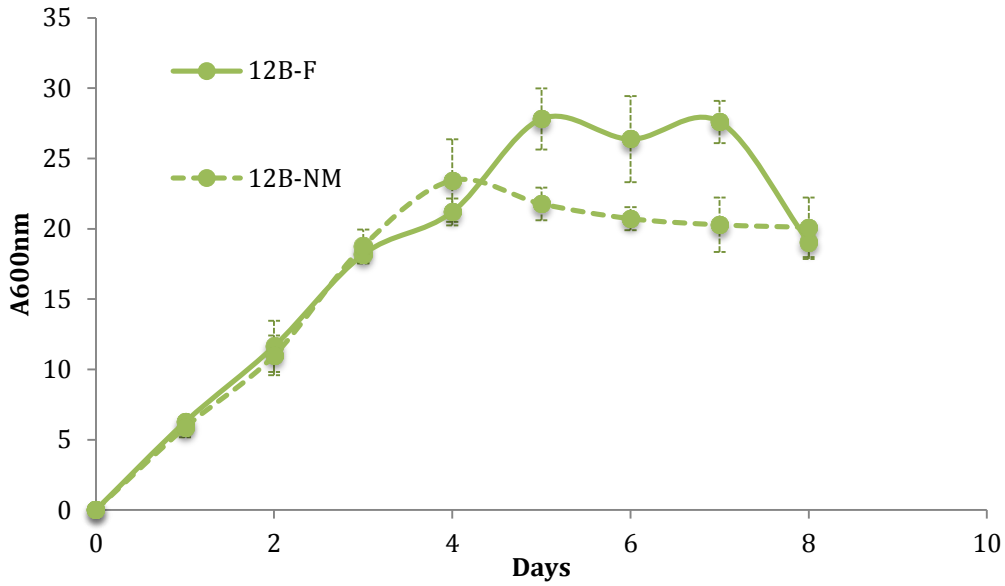


Figure 2.1. Growth profile of thraustochytrid strain 12B in F and NM media. The 12B cells grown in the NM medium appeared to enter the stationary phase earlier than in the F medium, at 4 days and 5 days respectively. Cells were cultivated in a 50 ml flask containing 10 ml liquid medium and incubated at 30°C and 150 rpm. Optical densities of cultures were measured photometrically at 600 nm. Triplicate sampling was performed every day from new 10 ml culture up to 8 days. Both the F and NM media contained the same organic composition, consisting of 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (peptone). F medium was also composed of 50% seawater, while NM medium was made using distilled water and supplemented with 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4 \bullet 7H_2O$, and a colony from a 2-day BY+ agar plate was used as an inoculum.

The life cycle of thraustochytrid strain 12B was observed through a light microscope, and a continuous video was recorded for up to 72 hours. The complex life cycle of strain 12B is shown in Figure 2.2 and could probably be generalized into three main stages: (1) the sporangium stage, (2) the amoebic stage, and (3) the zoospore stage, with various transition stages in between the main stages. The characteristic of the sporangium stage was its rounded shape and immovability. The amoebic cell had an elongated and flexible body and could move around slowly. The zoospore, on the other hand, moved very quickly, appearing to be ovoid under

the light microscope, and was the smallest compared with the other stages. Settled zoospores were chosen as the starting cell for the observation. Initially, the zoospore increased in size to become a sporangium, and a total of 8.4 and 22 hr was taken for the initial fission stage to complete in F and NM media, respectively. This was followed by the settling stage, a period when no observable changes occurred, which lasted for 1.4 and 1.6 hr in F and NM media, respectively, followed by a transition to the amoebic stage for a period of 2.3 and 1 hr, respectively, before settling again to form a rounded cell for 1 and 1.7 hr, respectively. The cell then divided, forming zoospores, which moved actively for 0.1 and 0.2 hr, respectively, before settling down and either reforming into the amoebic form or back into a sporangium. The zoospore was the smallest and fastest-moving stage and was exceptionally fast when suspended in seawater deficient in nutrients. The vegetative cell is characteristic, with its ectoplasmic network seen as white streaks radiating out from the cell. The amoebic form could exist at any time during the life cycle, as shown by the arrows radiating out of the core cycle. The final fate of the cell, shown as B, C and D, was not yet determined. Point A may have come from D or directly from the zoospore (3). The sporangium and the vegetative cell may be linked through pathway E.

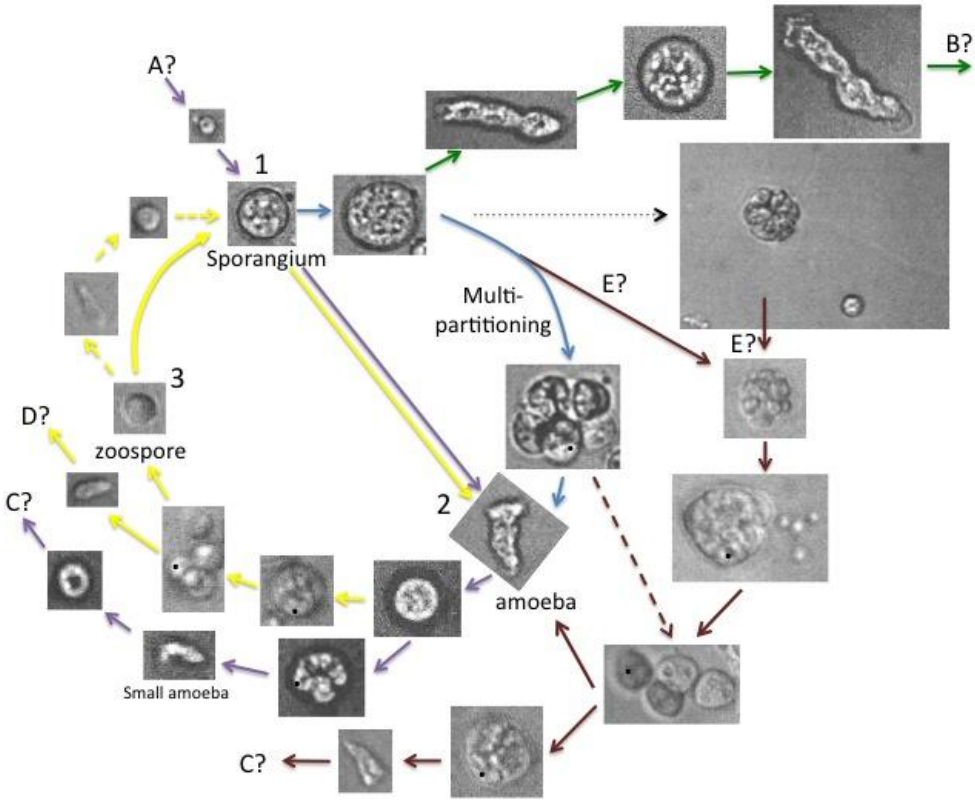


Figure 2.2. Generalized life cycle of thraustochytrid strain 12B observed in cells grown in F medium, NM medium and actual seawater. Cells from a 2-day BY+ agar plate were used to make a cell suspension in an Eppendorf tube containing 1 ml culture medium before transferring it to a microscope slide. The cell development was followed for up to 3 days by continuous video recording using DinoCapture 1.0 software and analyzed. A?, B?, C? D? and E? indicate an unknown origin or end. Development shown by the black arrow is deduced from Laby Base life-cycle data, and full lines were based on observation in F medium, while additional developments seen in NM medium are indicated by broken lines. Lines with the same color indicate stages that were actually followed consecutively. Black dots represent the cell from which the whole subsequent cells originally came. Three main types of cell type were seen: 1) the sporangium, 2) amoebic form, 3) zoospore. The amoebic form and the zoospore are the motile forms of strain 12B, while the sporangium and vegetative cell are stationary. The vegetative cell was found in the nutrient-deficient seawater and was seen to produce an ectoplasmic network.

Table 2.3. Duration of major life stages observed in the thraustochytrid strain 12B in NM medium, F medium and seawater.*

| | Duration (hr) of cell stage | | |
|---|---|---|---|
| Cell stage | NM | F | SW |
| first zoospore skid seen | 22.5 | 10.5 | 2.4 |
| fission stage | 22.3 ± 11.2 | 8.4 ± 1.3 | - |
| fission settle | 1.6 ± 0.4 | 1.4 + 0.5 | - |
| big amoeba stage | 1.0 ± 0.2 | 2.3 + 1.7 | - |
| big amoeba settle | 1.7 | 1.0 | - |
| zoospore stage | 0.2 | 0.1 | - |
| zoospore settle | 1.9 ± 1.2 | 2.0 | - |

*Values are averages ± standard deviation. A dash indicates that no data are available.

Stages without standard deviation are due to difficulty in following cells at a particular stage, as the microscope's field of view becomes crowded with cells, with an increase in observation time. Right after suspending the cells from the agar culture, the period of the nonmotile cell before releasing zoospores was determined by the time taken for the first released zoospore to skid away. This was found to take a longer time for cells growing in the NM medium compared with the F medium. The fission stage of the sporangia and the time taken for the settled amoeba to release a zoospore was also longer in the NM medium compared with the F medium. Conversely, the motile amoebic stage was longer in the F medium than in the NM medium; however, the motile zoospore stage is almost the same in both F and NM media. Cells were found to divide immediately to produce zoospores when exposed to seawater, particularly 2 hr after inoculation.

### 2.4.2. Phylogenetic study based on the 18S rRNA gene sequence

In the previous phylogenetic study on thraustochytrid strain 12B based on the 18S rRNA gene, both *Schizochytrium* (*Aurantiochytrium*) *limacinum* SR21 and *Thraustochytriidae* sp. A5-20 were found to be very closely positioned on the phylogenetic tree (Ando, 2005). However, many genera were not monophyletic, particularly in *Thraustochytrium*, *Schizochytrium* and *Ulkenia*. Various taxonomic changes have also occurred ever since the study by Ando (2005), giving rise to the new genera *Aurantiochytrium*, *Oblongichytrium*, *Botryochytrium*, *Parietichytrium* and *Sicyoidochytrium* (Yokoyama and Honda, 2007, Yokoyama et al., 2007). By comparing sequences from nine representative thraustochytrid genera from the latest taxonomic classification, strain 12B was found to be grouped well in the genus *Aurantiochytrium*, with high bootstrap values (Fig. 2.3). The 18S rRNA gene sequence similarity decreases from the species level to the genus level and the family level, with a percentage similarity of 98.6%, 83.4% and 78.6%, respectively (data not shown). Strain 12B had a higher sequence similarity to *A. limacinum* (98.5%) than to *A. mangrovei* (96.6%). The average sequence similarity of the 18S rRNA gene between two species of the 17 *A. limacinum* isolates as a sample organism was calculated to be 98.8% (data not shown), and this value of similarity could probably be used as the cutoff value for species identification of *Aurantiochytrium* species in thraustochytrid microorganisms.

### 2.4.3. Phylogenetic study based on mitochondrial COXII gene

COXII gene sequences of strain 12B and *A. limacinum* SR21 were aligned together to analyze their sequence similarity/identity to each other. At the same time, COXII sequences from *Thraustochytrium aureum* were also analyzed to provide a comparison between different thraustochytrids at the genus level (Fig. 2.4). There was a high percentage identity or similarity of 98.6% between strain 12B and SR21, while a very low sequence similarity of about 59% was obtained for both strain 12B and SR21 compared with *T. aureum.*

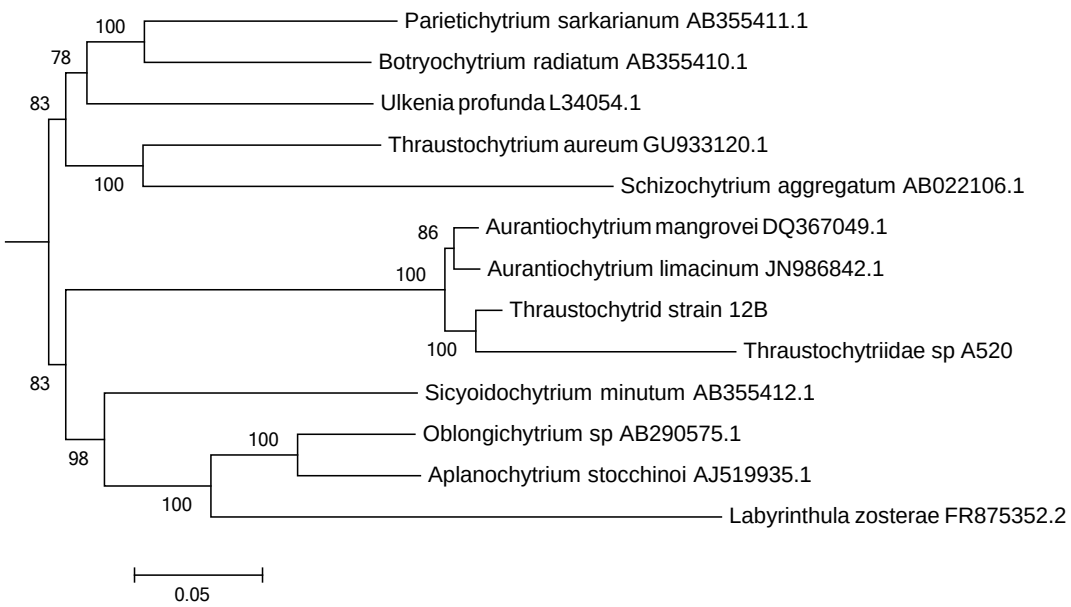


Figure 2.3. Phylogeny of thraustochytrid strain 12B to other thraustochytrid species based on 18S rRNA gene analysis. The thraustochytrid strain 12B grouped well together with other *Aurantiochytrium* species, with high bootstrap values. The species name is followed by the sequence's accession number in NCBI. Bootstrap values are also shown on the branches. Sequences from one representative species from every thraustochytrid genus were available from NCBI, except for *Althornia* and *Japonochytrium*, and analyzed together with strain 12B using the CLC Sequence Viewer software ver. 6.5.4. (QIAGEN, Tokyo, Japan). The *Labyrinthula zosterae* was used as an out-group.

```
                                20                      40                      60
                                 |                       |                       |
     12B CC-ACAAANT TNNNNNNCAT TGACCATAAA AAACACCAGG ACGTTGAATA AACAAACTAG 59
    SR21 ..-.....TN N....TA... .......... .......... ......C... .......... 59
T_aureum TTTG.T..CG CAAAAAGA.. CAC.TGA... GT.T.A.T.C .AAG.TG.A. TT....GG.A 60
                                80                      100                     120
                                 |                       |                       |
     12B CTTCGTTAAG ACGACCAGGG C-----AAGC ATCCATTTTA ACTCCAAAAG ACGGTACAGT 114
    SR21 ....A..... .......... .-----.... .......... .......... .......... 114
T_aureum T.GACGGG.. CTA.TT.AA. GCAGTGG.A. ..GTTGA... .T..G.C..T ...CG.A.A. 120
                                140                     160                     180
                                 |                       |                       |
     12B CCAAG-AATG TAGCACATCA GCACTGGT-- -TACAATAAG ACGAACATGA GTATCAACAG 170
    SR21 .....-.... .......... ........-- -......... .......... .......... 170
T_aureum .TT.CC...T CTAG...GT. ...T.TT.AT G.TA..C.G. TGTTG....G T.G..GT... 180
                                200                     220                     240
                                 |                       |                       |
     12B GCAATACT-- -ACACGGTTA TCTA---CTT CCAACATTCG CAAAT-CTCC CAAAGACAAT 223
    SR21 .........-- -......... ....---... .......... .....-.... .......... 223
T_aureum TTCG.GT.TT G.A.T.T..G GT.TAGT.C. G....GAA.. ...C.T..A. T.TTT.AT.. 240
                                260                     280                     300
                                 |                       |                       |
     12B TCATCATCTG GCAACAT--- --------GT AAGAATCGAA AACTAGATTT TCTCCA-TCT 271
    SR21 ........A. .......--- --------.. .......... .......... .....-.... 271
T_aureum .TT.T..TAA AT..T..CGC TTGATATAA. C.AG..GA.. G.GG.A..GA CG..A.A... 300
                                320                     340                     360
                                 |                       |                       |
     12B TCATTTTCAT AGTCAGAGTA TTCATATGAC CAATACCATT GAT------G CCCAATAACT 325
    SR21 .......... .......... ...G...... .......... ...------. .......... 325
T_aureum ....GAC.C. TA.G.ATT.G GG.T.CAA.. GTG.TA.... ...AAAGACA A.T.GA.G.. 360
                                380                     400                     420
                                 |                       |                       |
     12B TTCAAAGTAA --CAGAGGGA ---TCTACTA CTTCATCT-- ------ACAG AGTATAGAAG 372
    SR21 .......... --........ --........ ........-- ------.... .......... 372
T_aureum .ATG...... TT.....CA. GTC.A..AA. ...T..T.TT GTTCGG.TTA ..GT.T...A 420
                                440                     460                     480
                                 |                       |                       |
     12B AGCAA----- --AAGAAGGA ACTGC-AATA ATCATAAGAA GAACTGCAGG AATCAAAGTC 424
    SR21 .....----- --........ .....-.... .......... .......T.. .......... 424
T_aureum CT...CCTTA TG...TT... .T...T.G.. ...G.G.AT. AGCAC.TCAC GG.G...T.T 480
                                500                     520                     540
                                 |                       |                       |
     12B CAAATAATTT CAATA-GTA- ---------- GTTCCGTGAA CTA----CAC CTGAAGGGTT 468
    SR21 .......... .....-...- ---------- .......... ...----... .......... 468
T_aureum T..C.T.GC. TTG..CA..C CGCCCGTCAC A..AT.G... T..AAAT.GT TC...AAAG. 540
                                560                     580                     600
                                 |                       |                       |
     12B AGGGTTGGTT TTCACGTTGA AATGCATTAC GATA-CGAGT AAGCATCCAA AAAACAAAAA 527
    SR21 .......... ..T....... .......... ....-..... .......... .......... 527
T_aureum ..AAAATAC. ....AAA... G..TTT.A.. TG..AT..AG TC.T.A.A.G GT.G.CGT.. 600
                                620                     640                     660
                                 |                       |                       |
     12B ------CTAC AACAAAAACC ATAATAAACA TCAAATCGTG ATGAAATCGA ATAATACCTT 581
    SR21 ------.... .......... .......... .......... .......... .......... 581
T_aureum GTGAACT.GT GG.TG....T .......... .--.T.TT.A ..TG..A.AC ..TCCGAAC. 658
                                680                     700
                                 |                       |
     12B CTGCAATAGG AGTAGCAGGA TCTTGAAAAT -CCT-----A A 616
    SR21 .......... .........N NNNNN....N N..------. . 617
T_aureum TCAA....AA .AATAA.CTT .T....T.TA TTT.TATGAG . 699
```

Figure 2.4. Sequence similarity of COXII mitochondrial DNA between thraustochytrid strain 12B, *Aurantiochytrium limacinum* SR21 and *Thraustochytrium aureum*. The total number of bases is shown on the 3′ end of the sequence. The amplified COXII mitochondrial sequence using the FM58 and FM66 primers is approximately 620 bases for strain 12B and *A. limacinum* SR21. Identical nucleotides in the sequence are indicated as dots, and sequence gaps are shown as dashes, while differences in nucleotide sequence between strain 12B and *A. limacinum* SR21 are marked by red circles. Most of the COXII sequences between strain 12B and *A. limacinum* SR21 are similar, in contrast to that of *T. aureum.* The sequence percentage similarity obtained from ClustalW pairwise alignment analysis shows 98.6% similarity between strain 12B and SR21, while the sequence similarity for both these strains compared with *T. aureum* was only 58.5 and 59.4% respectively.

## 2.5. Discussion

### 2.5.1. Phenotypic characteristics and life cycle of strain 12B

Microscopic observations under light and electron microscopes were carried out to see the effect of culture medium on cell structure and life cycle in order to provide some explanation for the increase of cell yield and DHA productivity in the NM medium. Changes brought about in the cell may affect the taxonomic value of the features observed; however, at the same time, they are also considered to be important (Goldstein, 1973). All thraustochytrids share some morphological features that are characteristic of species belonging to Thraustochytriaceae, such as the presence of a noncellulosic scaly cell wall—the sagenogenetosome—an intracellular organelle that produces an ectoplasmic net (Huang et al., 2003), and the formation of biflagellate zoospores, one smooth and another with tripartite tubular hairs (Metz et al., 2010). The flagella on the strain 12B zoospore have already been shown in a previous study (Perveen et al., 2006), and the characteristic cell wall of strain 12B observed under TEM is shown in Figure 1.8.

There are various differences seen between strain 12B and SR21 in terms of phenotype and physiology, such as colony morphology and rate of glucose utilization (data not shown), and in terms of DCW, lipid, fatty acid and DHA yields during cultivation in F and NM media (Table 1.1). The colony morphology of 12B and SR21 appeared to be almost the same, with a round, smooth margin and a surface with a cream white color, although that of SR21 was slightly pinkish in color in a dense plate culture. As mentioned in Chapter 1, the number of days for the strains to deplete glucose in the culture medium varied. The SR21 culture appeared to deplete glucose faster, at 6 days in F culture and 5 days in NM culture, while strain 12B took 7 days in both F and NM (data not shown). The DCW, lipid, fatty acid and yields were also different between the two strains (Table 1.1). After 3 days' cultivation, the DCW yield in NM medium for strain 12B and SR21 was 18.7 and 12.9 mg/ml, respectively; the percentage lipid per DCW was 55.5 and 41.5%,

respectively; the percentage fatty acid per total lipid was 75% and 59.7%, respectively, and there was a slight difference in percentage DHA per total fatty acid of 31.2 and 33.5%, respectively. There were also differences in the fatty acid composition between the two, particularly when they were cultured in the F medium; however, the fatty acid composition was almost the same when they were grown in the NM medium (data not shown). The structure of the tubular hair on the anterior flagella, which does not appear to have a tapered base and a smooth shaft (Perveen et al., 2006), both of which are observable in *A. limacinum* (Honda et al., 1998), further supports the notion that they are probably two different species. Perveen et al. (2006) has also observed fusion among the strain 12B cells. Phagocytosis of bacteria by thraustochytrid microorganisms has been documented (Raghukumar, 1992, Yokochi et al., 2001); however, no fusion or phagocytosis between thraustochytrid cells has ever been reported in the literature, which therefore makes this a unique feature for strain 12B.

Observation of the life cycle through a light microscope shows that thraustochytrid strain 12B is generally made up of three main stages, the sporangium, amoebic and zoospore stages, with a period of 'settlement' in between transformations. However, there are still many questions regarding the transformation preference of strain 12B, as it was seen to be able to transform into the amoebic form directly from the sporangium state (course B in Fig. 2.2), or to transform into an even smaller amoebic form after the settling of the big amoebic stage (course C), or to transform from an undeveloped zoospore (course D) or from a developed zoospore stage (Fig. 2.2). The eventual outcome from all these courses, however, might be the reformation of the zoospore or the sporangium. The presence of the amoebic stage is a characteristic feature of the genera *Ulkenia*, *Parietichytrium*, *Sicyoidochytrium* and *Botryochytrium* (Yokoyama, 2007a); however, there are also exceptions for some species of *Aurantiochytrium*, in particular *A. limacinum* and *A. mangrovei*, which were also found to have an amoebic stage (Yokoyama & Honda, 2007). The characteristic feature is a naked protoplasm where the cell suddenly becomes 'invisible' in *Ulkenia*, *Parietichytrium*, *Sicyoidochytrium* and *Botryochytrium*; however, such a stage was not seen in strain

12B. An ectoplasmic network was also very rarely seen among strain 12B cells, and together with the disintegration of the cell wall during the release of the zoospore, this further emphasizes that strain 12B belongs to the genus *Aurantiochytrium*.

Thraustochytrid strain 12B grew to abnormal sizes in the M culture (Fig. 1.7), as seen in Chapter 1. The repeating sporangium–amoeba transformations (course B in Fig. 2.2) might be how the strain 12B cells in NaCl-deficient M medium acquire a bigger cell size. The possible effect of NaCl deficiency in the disruption of the cell division mechanism is probably linked with zoospore formation. In this experiment, the zoospore was seen earliest in seawater containing no nutrients, followed by cells grown in glucose-rich F medium containing 50% seawater, and latest in 0.1% NaCl glucose-rich NM medium (Table 2.3). Assuming that F medium contains 1.5% NaCl, while the seawater contains 3% NaCl, zoospore release seems to be related to increasing salinity for strain 12B. The thraustochytrid strain 12B may have to undergo transformation through course B (Fig. 2.2), probably because it cannot form zoospores in the absence of NaCl. In a different study, the opposite was seen, whereby decreasing salinity from 2.3% to 0.75% increased zoospore production in *Ulkenia* sp. and *Aurantiochytrium mangrovei* (Tsui et al., 2012). The study did not include lowering the NaCl concentration to 0% salinity, and therefore the high zoospore production at 0.75% salinity probably indicates the optimum concentration for these two strains. This is because a normal distribution pattern in zoospore production with salinity was evident for the *Schizochytrium* sp. KF-1 at 30°C, which shows the presence of an optimal concentration for salinity (Fan et al., 2002).

There was long settling stage for the sporangium and amoebic form of cells grown in the NM culture compared with the F culture. This might lead to better accumulation of lipid and thus DHA inside the cell, and reduced utilization of storage TG as an energy source. The time taken for a sporangium to divide (fission stage) right after inoculation probably takes a long time only at the beginning because of adaptation to the culture medium. However, cells appear to be growing almost immediately as shown in Figure 2.1. Further subsequent fission stages, however, were observed to have a shorter duration (data not shown). Unlike the

observation in F and NM media, growth in nutrient-deficient seawater was not so dynamic, except for the numerous zoospores skidding at extremely high speed within the medium. The size of the zoospore in seawater was also relatively smaller compared with zoospores found in F and NM cultures (data not shown).

### 2.5.2. Molecular phylogenetic studies of 12B

Previous phylogenetic study (Ando, 2005) has shown that many thraustochytrid genera were not monophyletic; however, various taxonomic changes have occurred, and currently only the genus *Thraustochytrium* is still found scattered throughout the phylogenetic tree, while other thraustochytrid genera have become monophyletic (Yokoyama et al., 2007, Yokoyama & Honda, 2007b). Strain 12B is closely related to *A. limacinum* SR21, Thraustochytrid sp. A5-20 and *Schizochytrium mangrovei*, based on phylogenetic analysis of the 18S rRNA gene (Fig. 2.3). In terms of percentage sequence similarity, out of the only two described *Aurantiochytrium* species, *A. mangrovei* and *A. limacinum*, strain 12B has a higher similarity with *A. limacium* at 98.5%. However, considering the percentage sequence similarity cutoff value for species at 98.8%, strain 12B could probably be considered to be a closely related, but different, species. The assignment of strain 12B to a new species was further supported by the physiological and phenotypic differences mentioned above.

Amplification of the mitochondrial DNA was performed using primers that have been developed for the *Pythium* genus, which is also under the phylum Heterokonta/Stramenopiles (Long et al., 2013), with some species under this genus also producing PUFAs such as EPA by *P. irregular* (Wu et al., 2013). Amplification of COXI and COXII+ITS was not successful, resulting in poor sequence quality, indicating unsuitability of the PCR primers (data not shown). There is also a probability that many introns exist in both the COXI and COXII+ITS genes, as this rare occurrence has been associated with low amplification success in fungi (Seifert, 2009). However, the amplification of the COXII gene from the thraustochytrid strain 12B and SR21 was successful, resulting in the production of 620 base amplicons. There were some differences in the COXII sequence between 12B and

SR21, with a sequence similarity of 98.6%. A higher sequence variation was observed when the sequences of both strain 12B and *A. limacinum* SR21 were compared with that of *Thraustochytrium aureum*, with a sequence similarity of 58.5 and 59.4%, respectively (Fig. 2.4, data not shown). This indicates that both strain 12B and *A. limacinum* SR21 are closely related, and the COXII sequence is probably conserved for species from the same genus. The conservative nature of the mitochondrial DNA sequence between closely related thraustochytrid species has also been seen in QPX isolates (Qian et al., 2007), which exhibited a higher variation when the nuclear ITS of SSU was used, and therefore is useful for intraspecies differentiation (Qian et al., 2007). The number of base pair differences has been documented for intraspecies and interspecies variation. A total of 0 to 4 base pair differences in the 539 bp COXI sequence in algae are normally present as intraspecies variation, while 28 to 148 bp differences were seen to be present between species (Robba et al., 2006). There might be some significance in the 6 bp difference in the COXII gene sequence between strain 12B and *A. limacinum* SR21; however, more comparison is necessary, such as by investigating the intraspecies COXII sequence variation, such as by using *A. limacinum* isolates as a possible model organism.

## 2.6. Conclusions

Thraustochytrid strain 12B belongs to the genus *Aurantiochytrium*, based on morphology through the absence of naked protoplasm, the rarity of an ectoplasmic network produced by the cell, and the disintegration of the cell wall during zoospore release as well as the 18S rRNA gene phylogenetic grouping. There is a high 98.6% sequence similarity in the mitochondrial COXII gene sequence between the two *Aurantiochytrium* species, strain 12B and *A. limacinum* SR21, and a very low sequence similarity of less than 60% when both strains are compared with another thraustochytrid genus, *Thraustochytrium aureum*. The mitochondrial COXII gene therefore may have a high taxonomic value for differentiating the genus level for thraustochytrids. Based on the 18S rRNA analysis, the lower sequence similarity of 98.5% to the proposed 98.8% cutoff value between strain 12B and *A. limacinum* sequences may indicate that strain 12B is a different species from *A. limacinum*. This is further supported by the physiological and phenotypic differences seen in terms of coloration of algal culture, rate of glucose utilization, and differences in DCW, lipid, fatty acid and DHA yields during cultivation, as well as differences in the zoospore flagellum structure. Fusion among thraustochytrid cells and the presence of multiple 18S rRNA gene copy in the previous study of strain 12B is also not commonly found or reported in other thraustochytrid microorganisms, making it a unique strain. Additionally, strain 12B has a complex life cycle in nutrient-rich F and NM media. In the life-cycle study, the stationary phases were relatively longer, while the duration for the mobile amoebic form phase was shorter when strain 12B was cultured in NM medium compared with F medium. This probably contributes to the conservation of oil bodies inside the cell, resulting in higher cell, lipid, and DHA yields in the NM medium.

# Chapter 3: Possible functions of DHA and its accumulation in thraustochytrid microorganisms

## 3.1. Summary

One of the possible reasons for the high accumulation of lipid and particularly DHA in thraustochytrid strain 12B is its role in energy antioxidation. During starvation, the percentage composition of pentadecanoic acid (C15:0) and hexadecanoic acid (C16:0) decreased from 31% to 15% and from 22% to 14%, respectively, while the percentage composition of DHA increased from 25% to 42% after 7 days' cultivation in nutrient-deficient 1.5% (w/v) NaCl solution. The preferential use of C15:0 was also seen in the absolute yield data for C15:0, which shows a continuous decrease with time during starvation. The absolute amount of DHA, on the other hand, was the highest among all of the major fatty acids throughout the experiment. This shows that other fatty acids such as the saturated fatty acid C15:0 was preferentially used as a source of energy over DHA, which suggests that DHA may have other important functions apart from being an energy source. During exposure to UV-C, the strain 12B cells' growth profile was more or less similar compared with cultivation in the dark. The percentage DHA composition per total fatty acid was slightly decreased; however, the absolute cell, lipid and DHA yields were higher when strain 12B was cultured under UV, increasing from 0.5 to 3.4 mg/ml. DHA may have been involved in the intracellular antioxidative mechanisms, allowing the cell to survive for 3 days' cultivation under UV. At the same time, the exposure to UV is thought to affect the zoospores' flagella development, resulting in lower motility and hence to contribute to further preservation of intracellular lipid reserves. This approach may have an important application in industrial production of DHA.

## 3.2. Introduction

### 3.2.1. Role of PUFA in adaptation

Microorganisms are considered to synthesize PUFAs to adapt to the environment, where PUFAs such as EPA and DHA play a certain role in their survival. One of the functions of PUFA is believed to be its contribution to providing fluidity to the cell membrane at low temperature or high pressure (Hazel & Williams, 1990), referred to as homeoviscous adaptation. This is defined as a homeostatic process that regulates the viscosity of membrane lipids to achieve a constant fluidity at temperature of growth by varying the fatty acid composition of phospholipids (Sinensky, 1974). Homeoviscous adaptation is important for maintaining the function of the membrane as a barrier and for maintaining the function of proteins in the membrane involved in various enzyme reactions for energy, signaling and intra- or extracellular transport of molecules. The activity of membrane proteins is highly affected by the membrane structure (Jensen & Mouritsen, 2004, Andersen & Koeppe, 2007), which is influenced by thickness, curvature and membrane elasticity (Phillips et al., 2009). Although PUFAs could act as a substitute for monounsaturated fatty acids (Allen et al., 1999), it is actually the monounsaturated fatty acids that are more affected by changes in temperature or pressure compared with PUFAs (Hazel & Williams, 1990). The accumulation of PUFAs such as DHA therefore probably serves other important functions inside the cell.

### 3.2.2. Possible role of DHA as energy source

Triacylglycerol (TG) is a common form of energy and carbon storage in eukaryotes such as plants, yeast, and animals (Coleman, 2004), and even in some bacteria under the group actinomycetes (Alvarez, 2002). DHA is probably used as a source of energy in DHA-producing thraustochytrid microorganisms, because these microorganisms also accumulate a high amount of DHA as TG (Okuyama et al.,

2007, Morita et al., 2006). This was shown earlier in Chapter 1, whereby strain 12B was able to be grown in a low-NaCl-concentration NM medium, resulting in a high DHA yield of up to 11.7 mg/ml after 5 days in jar cultivation (Table 1.3). The TG of the thraustochytrid strain 12B grown in the NM medium in flasks was found to make up to 47% of the total DCW in which up to 21% of the TG fatty acid is composed of DHA (data not shown). However, cells grown in F and NM media have almost the same percentage lipid per DCW, 25.8 and 27.8% respectively (data not shown). The presence of DHA in TG may predispose it to be a feedstock for respiration during periods of nutrient deficiency (of organic carbon and nitrogen), as in the case of the thraustochytrid strain NIOS-1 (Jain et al., 2007). TG was accumulated by the NIOS-1 strain and was used as an energy source in that strain, as the amount of oil bodies or storage lipid, which are typically TG, was found to decrease when the cell changed to motile amoebic forms as seen directly by Nile blue staining. Hexadecanoic acid (C16:0) and DHA are the main fatty acids in the strain, and the analysis of fatty acids after transferring cells that have accumulated a high amount of TG into nutrient-deficient medium shows a reduction in absolute DHA yield and also a decrease in percentage DHA composition, while the percentage of C16:0 increases during starvation (Jain et al., 2007). On the other hand, TG is also known to be a feedstock for phospholipid production (Coleman, 2004), and the conversion of DHA-rich TG into DHA-rich phospholipid was previously achieved by cultivating a thraustochytrid strain in a nitrogen-rich medium that was devoid of glucose as a carbon source (Okuyama et al., 2007). The use of TG as an energy source in thraustochytrid strain 12B grown in glucose-deficient medium was supported by the presence of numerous mitochondria in the cytoplasm (Bin Haji Mohd Taha et al., 2012). From this, it was previously hypothesized that DHA was channeled to the production of DHA-rich phospholipid inside mitochondrial membranes as many mitochondria were synthesized. It is therefore still not clear whether the accumulation of DHA is to serve as an energy source preferentially over other fatty acids or the opposite. However, it is more reasonable for the thraustochytrid microorganisms to utilize the saturated fatty acid preferentially because the beta-oxidation process for saturated fatty acids is shorter, bypassing two reactions involving enoyl-CoA isomerase and 2, 4-dienoyl-

CoA reductase (D'Andrea, 1994), and the synthesis of PUFA is more costly. The preferential use and degradation of saturated fatty acids as an energy source would consequently be one of the possible reasons for DHA accumulation in thraustochytrid microorganisms.

### 3.2.3. Role of DHA in antioxidative stress

DHA also has a direct and indirect antioxidative role in cells. In bacteria, DHA is thought to increase the hydrophobicity of the cell membrane, preventing the entry of hydrophilic extracellular reactive oxygen species (ROS) such as hydrogen peroxide into the cell (Okuyama et al., 2008b). The hypothesis is probably also relevant to eukaryotes, as the general structure of all biological membranes is shared (Alberts et al., 2002). Most DHA-producing organisms including protozoa, algae and bacteria are marine in origin and are rarely terrestrial or freshwater organisms (Okuyama et al., 2008b). Human beings are also able to produce DHA in the astrocyte; however, they are incapable of producing it in significant amounts (Moore et al., 1991). The production of DHA by marine organisms may have arisen as a protective strategy against $H_2O_2$, which is present in a stable concentration, has a long lifetime in seawater and is photochemically produced through UV absorption by organic matter in the seawater or by hydrothermal vents (Lesser, 2006).

Thraustochytrid microorganisms are naturally 'marine' (Raghukumar, 2008a), while those with a terrestrial habitat normally are brackish or saline in nature (Raghukumar, 2008b). The thraustochytrid strain 12B is also from a marine source, originally isolated from a mangrove river in Okinawa (Perveen et al., 2006). Unlike bacteria, most DHA are stored in TG in thraustochytrids (Okuyama et al., 2007, Morita et al., 2006), including strain 12B, and it was suggested that one of the reasons for this is its function as an intracellular antioxidant (Jain et al., 2007). Apart from exogenous sources, ROS could also be intracellularly produced by the exposure of cells to UV, resulting in peroxidation of PUFA (Catalá, 2009). 'Photosensitizers' in the cell could generate ROS by absorbing energy from UV (Bossi, 2008). The damaging effect of UV to key enzymes in the respiration

pathway may also promote ROS formation (He, 2002). The four types of UV (UV-V, UV-C, UV-B, and UV-A) are defined by their wavelength range of 100–200 nm, 200–280 nm, 280–315 nm and 315–400 nm, respectively (Koutchma, 2009). The solar UV on earth is made up of 95% UV-A and 5% UV-B, while UV-C does not penetrate the earth's atmosphere (Santos et al., 2013). However, there is less UV in marine than in terrestrial environments. The amount of UV-B that penetrates water bodies is 10% of the amount at the water surface at depths ranging from 0.1 m to 40 m, depending on the water column properties, the air–water interface properties and the incident irradiance (Booth, 1997). DHA in a mixture with other PUFAs, as it would be in nature, has a high tendency to be oxidized (Miyashita et al., 1983). The low UV penetration coupled with the presence of $H_2O_2$ may have produced a natural selective pressure for DHA-producing microorganisms in marine environments.

The objective of this chapter is to investigate the function of DHA in PUFA-producing thraustochytrid strain 12B to understand further the factors affecting DHA production for possible application in industrial practices.

## 3.3. Materials and methods

### 3.3.1. Microorganisms

Strain 12B (NITE P-68), which is a high-DHA-accumulating thraustochytrid eukaryotic microorganism, was isolated from a mangrove in Okinawa, Japan (Perveen et al., 2006) and was used for both starvation and UV–antioxidation experiments.

### 3.3.2. Culture media and cultivation conditions

The strain 12B working cultures were maintained on BY+ agar plates, and agar cultures used as inoculum were incubated in the dark at 30°C for 2 days. The BY+ agar was prepared by dissolving glucose in seawater, while yeast extract, peptone and agar were dissolved in distilled water to a final concentration of 0.5, 0.1, 0.1 and 1.5% (w/v), respectively. The solutions were autoclaved separately to avoid precipitation and browning of glucose, and were mixed afterwards, resulting in a 50% seawater solution. The preparation of the F liquid medium is similar to that of the BY+ medium except that the concentrations of glucose, yeast extract and peptone were 8%, 1% and 1% (w/v), respectively, without the addition of agar. The NM medium is composed of 8% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) peptone, 0.1% (w/v) NaCl and 1% (w/v) $MgSO_4 \bullet 7H_2O$ dissolved in distilled water.

#### 3.3.2.1. Culture condition during nutrient starvation

TG rich cells for the starvation experiment were prepared by culturing strain 12B in the dark for 3 days at 30°C and 150 rpm, by inoculating a colony from a 2-day BY+ agar culture into a 10 ml F medium in a 50 ml flask. To investigate the potential use of DHA as a preferential source of energy, strain 12B cells, which had accumulated DHA-rich TG, were gathered by centrifugation (6000 rpm, 4°C, 10 min), washed aseptically with 1.5% NaCl and resuspended in 10 ml of the same

solution in a new 50 ml flask. Incubation was then resumed in the dark at 30°C and 150 rpm for 0, 1, 5, and 7 days.

#### 3.3.2.2. Culture condition for UV exposure

To investigate the effect of UV exposure on fatty acid composition of strain 12B, 50 ml flasks containing 10 ml of F or NM medium was inoculated with a colony from a BY+ agar culture, which had been incubated for 2 days at 30°C, and the subsequent liquid cultures were cultivated under UV-C (254 nm, 10 watt, GL 10, Mitsubishi/Osram, Shizuoka, Japan) for 3 days at 30°C and 150 rpm. The distance from the bottom of the flask to the light source was 28.5 cm, and for the control experiment, cultures were incubated in the same incubator in the dark by wrapping the flasks with aluminum foil.

### 3.3.3. Sampling and fatty acid analysis

Starved cells were harvested for DCW determination and fatty acid composition analysis at 0, 1, 5 and 7 days by centrifugation (6000 rpm, 4°C, 10 min). DCW were obtained by washing cells with 0.8% NaCl and were recentrifuged (6000 rpm, 4°C, 10 min), followed by freeze-drying for 24 hr at –40°C, –0.092 MPa (Labconco, Kansas, US) with an external vacuum pump (SVP-100S, Sato Vacuum Machinery Industrial Co. Ltd, Tokyo, Japan). For the fatty acid composition analysis, a wet cell was taken by a thin spatula (equivalent to 2.5 mg DCW) and was directly methanolyzed by suspending the cells into 1 ml methanol containing 10% acetyl chloride with 10 of internal standard (heneicosanoic acid, 2 mg/ml in benzene). Methanolyzed samples were extracted with hexane and analyzed by GC–FID as mentioned below.

Cells cultured under UV exposure were harvested after 3 days' cultivation and similarly washed and freeze-dried for lipid extraction. Lipid was extracted from the dried cell using a modified Bligh and Dyer method (Bligh and Dyer, 1959) as mentioned in Chapter 1, dried with $N_2$ and redissolved to 50 mg/ml by adding chloroform:methanol (2:1, v/v) solution containing 1% (w/v) BHT. Methanolysis of lipid was carried out by adding 10 μl lipid, 10 μl heneicosanoic acid internal

standard (2 mg/ml in benzene) and 1 ml methanol containing 10% acetyl chloride into a screw-capped test tube and heated at 100°C for 1 hr (Dry Block Bath MG-2, Torika corp., Nagoya, Japan). The resulting FAMEs were extracted with 2 ml of hexane thrice. After concentrating the FAME fractions through evaporation under $N_2$, the samples were subjected to GLC and GC–MS. The FAME fraction was analyzed with a gas chromatograph GC-353B (GL sciences, Tokyo, Japan) equipped with a capillary column BPX70 (25 m × 0.22 mm i.d., SGE, Yokohama, Japan) and FID with nitrogen as carrier gas. The column temperature was programmed to increase at 5°C/min from 150°C to 240°C and then maintained for 2 min while the injector and detector temperatures were set at 240°C. Samples were also analyzed with an ion-trap gas chromatograph CP-3800 (Varian, Tokyo, Japan) and a mass spectrophotometer Saturn 2200 (Varian, Tokyo, Japan) equipped with a capillary column TC-70 (30 m × 0.25 mm i.d., GL Sciences Inc., Tokyo, Japan). The column temperature was programmed to increase from 80°C to 240°C, with a split ratio of 20:1, helium carrier gas with a flow rate of 0.9 ml/min and the injector temperature set at 240°C. GC-MS was used to confirm the identity of peak components corresponding to specific fatty acids, while GC–FID was used for routine calculation of fatty acid yield including DHA.

# 3.4. Results

## 3.4.1. Effect of starvation of strain 12B cells on its DCW, fatty acid amount and composition

Cultivation of the thraustochytrid strain 12B under nutrient starvation resulted in a decrease in DCW, with a marked decrease from 30 to 8 mg/ml after 3 days' starvation (Fig. 3.1). In the same period, drastic changes in the absolute amount of major fatty acid in 12B were also found, particularly for DHA, which increased from 69.3 to 128.7 mg/g DCW (Fig. 3.2). The yield of fatty acids of C16:0, heptadecanoic acid (C17:0) and DPA during the 7 days' starvation experiment was almost the same as that of DHA, which initially increased after 3 days' incubation then suddenly decreased after the 5$^{th}$ day and again increased slightly after 7 days' incubation in nutrient-deficient solution. Only the yield for pentadecanoic acid (C15:0) showed a continuous gradual decrease from 57.5 to 23.4 mg/g DCW after 7 days' starvation. Changes in fatty acid composition, however, generally showed a decrease in the composition of short-chain fatty acids from C14 to C18:1 and an increase in longer-chain fatty acids from C19:0 to DHA (Table 3.1). A major decrease was seen especially in C15:0 and C16:0 fatty acids, from 31% to 12% and from 22% to 16%, respectively, while a major increase was seen in DPA and DHA, from 6% to 10% and from 25% to 43%, respectively. Both the major increase and decrease occurred during the first 72 hr of incubation.

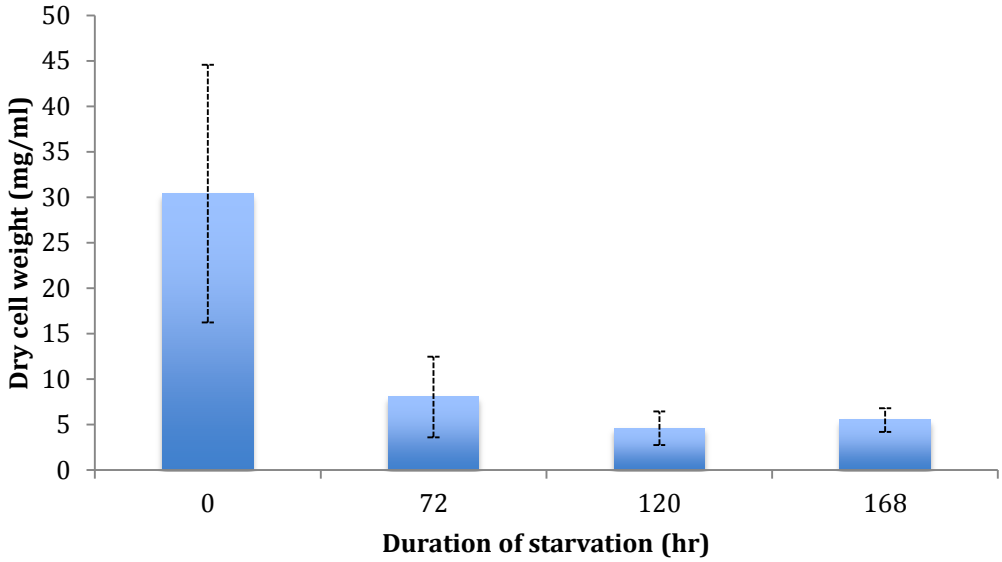


Figure 3.1. Amount of DCW obtained from cultivation of thraustochytrid strain 12B in 1.5% (w/v) NaCl medium without glucose, peptone and yeast extract supplementation. There was a marked decrease in DCW during the first 3 days' incubation, which appears more or less the same afterwards. Strain 12B cells were initially grown in the F medium containing 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (w/v) peptone, to allow the cell to accumulate lipid and DHA for 3 days. The cells were then washed and resuspended in an equal volume of 10 ml 1.5% (w/v) NaCl solution and incubated at 30°C and 150 rpm. For DCW determination, cells were harvested after 3, 5 and 7 days' incubation.

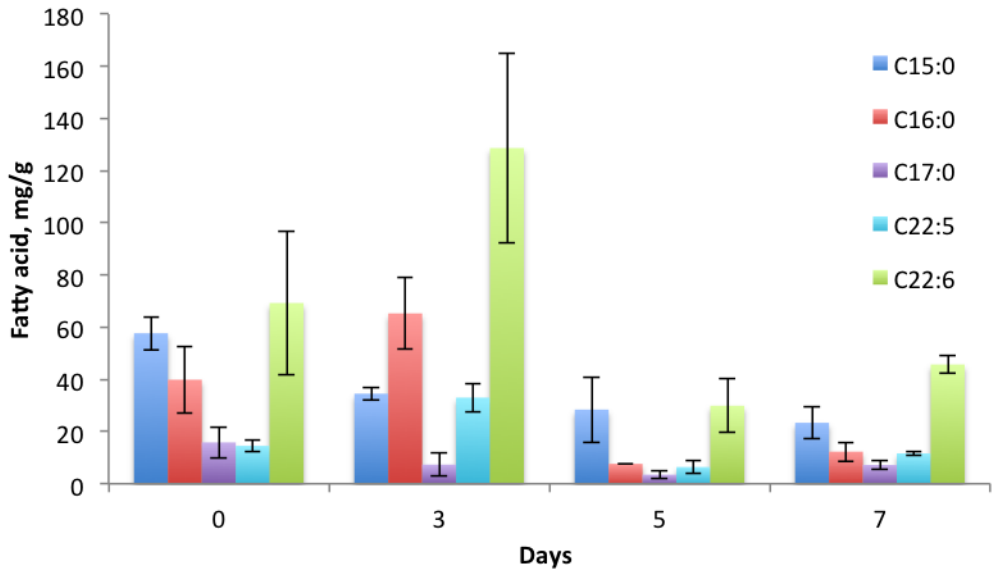


Figure 3.2. Effect of starvation on the absolute amount of major fatty acid component of strain 12B. Two patterns of fatty acid change can be seen with increase in time. A gradual decrease from 0 to 7 days was shown by C15:0, while other fatty acids initially increased during the first 3 days, followed by a sudden decrease and another increase. The amount of DHA is the highest in the cell throughout the starvation experiment. Cells were initially cultivated in F medium for 3 days at 30°C, to allow the cells to accumulate lipid. Cells were than washed in 1.5% (w/v) NaCl and resuspended in the same solution without any glucose or nitrogenous supplementation, and incubation was resumed for 7 days.

Table 3.1. Percentage composition of fatty acid of strain 12B grown in 1.5% (w/v) NaCl.*

| | | Fatty acid composition (% f.a. w/w) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling time | | C14:0 | C15:0 | C16:0 | C17:0 | C18:0 | C18:1 | C19:0 | C20:0 | C20:4 | C20:5 | C22:5 | C22:6 | others |
| 0 time | Av | 1.5 | 30.9 | 21.5 | 5.4 | 1.3 | 0.7 | 0.6 | 0.2 | 0.9 | 0.6 | 5.5 | 24.6 | 6.1 |
| | (sd) | 0.7 | 11.2 | 15.5 | 1.2 | 1.0 | 0.3 | 0.2 | 0.1 | 0.3 | 0.3 | 1.3 | 5.7 | 1.6 |
| 72 hr | Av | 1.2 | 12.4 | 16.0 | 3.6 | 1.3 | 0.3 | 0.6 | 0.3 | 1.2 | 2.1 | 10.2 | 43.2 | 7.7 |
| | (sd) | 0.6 | 2.8 | 8.3 | 1.0 | 0.4 | 0.1 | 0.1 | 0.1 | 0.3 | 1.0 | 0.5 | 5.3 | 1.1 |
| 120 hr | Av | 1.1 | 15.9 | 15.6 | 4.4 | 1.5 | 0.4 | 0.6 | 0.2 | 1.4 | 2.3 | 10.5 | 35.6 | 10.6 |
| | (sd) | 0.2 | 4.2 | 8.5 | 0.5 | 0.7 | 0.2 | 0.1 | 0.2 | 0.2 | 0.7 | 3.4 | 8.0 | 1.4 |
| 168 hr | Av | 0.8 | 15.4 | 13.5 | 4.9 | 0.8 | 0.2 | 0.9 | 0.2 | 1.1 | 2.1 | 10.6 | 41.8 | 7.6 |
| | (sd) | 0.3 | 5.9 | 5.2 | 2.3 | 0.1 | 0.1 | 0.5 | 0.1 | 0.2 | 0.4 | 0.8 | 3.6 | 0.7 |

The percentage composition of DHA (C22:6) was found to increase, while the percentage composition of C15:0 and C16:0 was decreased with time during starvation. Strain 12B cells were initially grown in the NM medium containing 8% (w/v) glucose, 1% (w/v) yeast extract and 1% (w/v) peptone, to allow the cell to accumulate lipid and DHA for 3 days. Zero time was when washed inoculum from 3 days' cultivation in F culture was added to an equal volume of 10 ml 1.5% (w/v) NaCl solution. Incubation was carried out at 30°C and 150 rpm, while sampling was carried out at 0, 3, 5 and 7 days.

* Values are averages (Av) with standard deviation (sd) from three independent samples.

### 3.4.2. Effect of UV on the growth and fatty acid composition of strain 12B

The effect of UV on the growth pattern of 12B appeared to be almost identical when the strain was cultivated in both F and NM media in the absence of UV (Fig. 3.3). When the cultures of strain 12B were exposed to UV, glucose in the NM media was depleted faster than in the F medium. In the F medium, the time for glucose depletion was reduced from 7 to 6 days, and from 7 to 4 days in the NM medium (data not shown). Exposure of strain 12B cultures to UV led to changes in cellular fatty acid composition. After 3 days' cultivation, an increase in percentage composition when exposed to UV was seen for C16:0, octadecanoic acid (C18:0), EPA (C20:5) and DPA (C22:5), and large differences were seen for C16:0, which increased from 17% to 35% in F medium but less in NM medium, with only a slight increase from 42% to 45%. On the other hand, a decrease was found in C15:0, C17:0, C18:1 and C22:6, especially in C15:0, with a marked decrease from 31% to 16% in the F medium and a smaller decrease from 10% to 6% in the M culture after being exposed to UV for 3 days (Table 3.2). The exposure of thraustochytrid strain 12B to UV unexpectedly resulted in an increase in the absolute amount of DCW, lipid, fatty acid and DHA, from 13.6, 4.7, 1.7, 0.5 mg/ml to 24, 16.1, 12.2, 3.4 mg/ml, respectively, compared with when 12B was cultivated in the dark after 3 days' cultivation in F medium, while in the NM medium, the increase was from 18.7, 10.4, 7.8 and 2.4 mg/ml to 24.7, 12.9, 10.2, 3.8 mg/ml, respectively (Table 3.3).

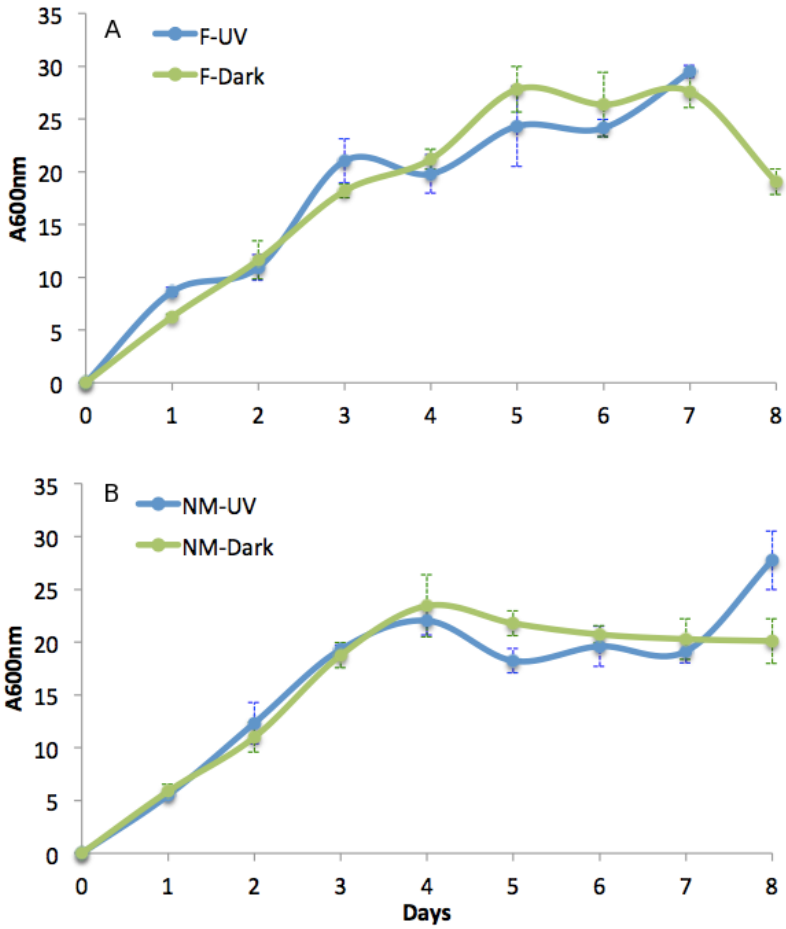


Figure 3.3. Growth curve of thraustochytrid strain 12B cells in (A) F medium and (B) NM medium, in the presence or absence of UV exposure. The growth of thraustochytrid strain 12B was not affected by exposure to UV during cultivation and is almost the same as the growth in the absence of UV (dark). An almost linear growth was seen for cells grown in NM medium from 0 to 4 days, after which the stationary phase followed. Cells grown in F medium seem to continue growing until the 7$^{th}$ day when exposed to UV, while unexposed cells that were cultivated in the dark appear to enter the stationary phase at the 5$^{th}$ day of cultivation.

Table 3.2. Percentage fatty acid composition of cells grown in F and N media in the absence or presence of UV.*

| Medium | Condition | | Fatty acid composition (% w/w) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C14:0 | C15:0 | C16:0 | C17:0 | C18:0 | C18:1 | C19:0 | C20:0 | C20:4 | C20:5 | C22:5 | C22:6 | others |
| F | dark | Av | 1.1 | 30.6 | 17.1 | 5.8 | 0.9 | 0.5 | 0.3 | 0.2 | 0.9 | 0.6 | 5.6 | 31.8 | 4.6 |
| | | (sd) | 0.6 | 10.7 | 12.8 | 1.8 | 0.3 | 0.2 | 0.2 | 0.1 | 0.3 | 0.2 | 0.6 | 2.6 | 1.7 |
| | uv | | 1.8 | 15.7 | 34.7 | 3.5 | 1.0 | 0.4 | 0.2 | 0.2 | 0.6 | 1.5 | 6.6 | 30.9 | 3.0 |
| | | (sd) | 0.6 | 8.8 | 10.2 | 1.3 | 0.7 | 0.1 | 0.0 | 0.2 | 0.4 | 2.5 | 1.1 | 3.3 | 0.8 |
| NM | dark | | 2.7 | 9.9 | 42.2 | 1.8 | 1.4 | 0.6 | 0.2 | 0.2 | 0.6 | 0.3 | 6.1 | 31.2 | 3.0 |
| | | (sd) | 0.1 | 1.9 | 2.4 | 0.3 | 0.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.6 | 0.7 |
| | uv | | 2.4 | 5.9 | 45.9 | 1.5 | 1.6 | 0.3 | 0.2 | 0.2 | 0.6 | 0.5 | 7.1 | 30.1 | 3.6 |
| | | (sd) | 0.0 | 0.4 | 0.4 | 0.2 | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.2 | 1.0 | 0.2 |

A large increase in the saturated fatty acid composition of C16:0 occurred when the cells grown in F and NM media were exposed to UV. On the other hand, the opposite effect occurred with the saturated fatty acid C15:0, which showed a large decrease in fatty acid composition. DHA showed a slight decrease when the cells were exposed to UV.

*Values are averages (Av) with standard deviation (sd) from three independent samples.

Table 3.3. Effect of UV exposure and nonexposure on the DCW, lipid, fatty acid and DHA yields of strain 12B cultivated in F and NM media.*

| Medium | Condition | DCW | Lipid | Fatty acid | DHA (% lipid/DCW) |
|---|---|---|---|---|---|
| F | dark | 13.6 ± 5.4 | 4.7 ± 4.3 | 1.7 ± 0.2 | 0.5 ± 0.2 (34.6) |
| | uv | 24.0 ± 0.3 | 16.1 ± 0.8 | 12.2 ± 0.4 | 3.4 ± 0.3 (66.9) |
| NM | dark | 18.7 ± 1.4 | 10.4 ± 1.2 | 7.8 ± 1.3 | 2.4 ± 0.4 (55.6) |
| | uv | 24.7 ± 0.3 | 12.9 ± 0.2 | 10.2 ± 0.3 | 3.8 ± 0.1 (52.3) |

Exposure of cells to UV during cultivation increases the DCW, lipid, fatty acid and DHA yields of strain 12B compared with when cultures were grown in the dark. The increase in yield was seen in both F and NM cultivation.

*Values are averages (mg/ml) ± standard deviation.

## 3.5. Discussion

### 3.5.1. Role of DHA as an energy source

During the period of nutrient deprivation, the TG reserve can be metabolized as a source of energy. This will be done by the hydrolysis of TG into free fatty acids and diacylglycerol, monoacylglycerol and glycerol successively by the action of lipase. Glycerol will be converted into dihydroxyacetone phosphate and will enter the glycolysis pathway, while the free fatty acids will be esterified to coenzyme A (CoA). In eukaryotic cells, fatty acids are transported into the mitochondria and then oxidized via the β-oxidation system. From one cycle of the β-oxidation of acyl-CoA, two carbons are sequentially removed, forming acetyl-CoA, NADH, and $FADH_2$. The former acetyl-CoA and the latter NADH and $FADH_2$ are used through the TCA cycle and electron transport chain, respectively, to synthesize ATP (Garret and Grisham, 2007).

The thraustochytrid strain 12B cells grown in the F medium for 3 days contained about 16% TG per DCW (data not shown), and therefore full utilization of TG is expected to result in a loss of about 16% of the DCW. However, during the first 72 hr of incubation, about 70% of DCW was lost (Fig. 3.1). This could imply that one of the reasons for the loss of DCW is probably through cell death, leaving only the persistent vegetative form to survive. Clumping of cells was observed in the culture, which further supports the presence of the vegetative form, which is known to have ectoplasmic networks allowing cells to aggregate (data not shown). Major changes in the absolute yield of major fatty acids also occurred during the first 3 days of incubation (Fig. 3.2). During a few days of cultivation in glucose-deficient medium, changes in intracellular structures have been reported for strain 12B whereby a small amount of oil bodies were found and a large number of mitochondria were seen inside the cell (Bin Haji Mohd Taha, 2012). Considering all the facts—that is, the medium was nutrient deficient, large amounts of DCW were lost, and cell activity was assumed to be high—there might be a possibility that the

high amount of DHA (mg DHA/g DCW) was obtained through phagocytosis or cell fusion. This behavior has been observed previously (unpublished), as mentioned in Chapter 2. Based on the absolute yield data, only C15:0 seems to be preferentially used as an energy source throughout the starvation period, while other major fatty acids were also used, particularly between the 3$^{rd}$ and 5$^{th}$ day of the incubation. DHA was not preferentially used as an energy source but instead was accumulated from the 5$^{th}$ to the 7$^{th}$ day of incubation. The amount of DHA was also highest among all the major fatty acids throughout the starvation experiment. This implies that DHA may play some other important role inside the cell.

In terms of fatty acid composition, DHA and DPA generally were found to increase with incubation time, with the highest increase during the first 72 hr from 6% to 10% and from 25% to 43%, respectively (Table 3.1). A reduction was instead seen in the other two major fatty acids C15:0 and C16:0, which further supports the absolute yield data; that is, saturated fatty acids were preferentially used as an energy source. This supports the previous study by Okuyama et al. (2007), whereby the percentage DHA per total fatty acid was found to increase in glucose-deficient and nitrogen-rich medium, where DHA-rich TG was converted to DHA-rich phospholipid, and the absolute amount of lipid and thus TG decreased, indicating starvation. The opposite was reported for the thraustochytrid strain NIOS-1, which shows a reduction in the percentage DHA and an increased percentage of C16:0 during starvation (Jain et al., 2007), with the absolute DHA decreasing from about 90 to 25 μg/g DCW, while C16:0 remains more or less at about 225 μg/g DCW after 20 days' starvation, indicating a possible species-specific preference.

### 3.5.2. Effect of UV on DHA-producing microorganism

In nature, organisms are frequently exposed to various forms of stress including UV, which is part of sunlight. UV has been reported to trigger stress in various ways. UV-A, for example, has been reported to effect membrane transport and tRNA function by producing an adduct at the 13$^{th}$ position in the tRNA, which

consequently alters its ability to load amino acids (Jagger, 1981). UV-B, on the other hand, was reported to reduce the amount of ATP by 75% in marine planktonic assemblages, indicating the effect of UV by reducing metabolic activity (Vosjan et al., 1990), while UV-C has a direct effect on DNA through the formation of cyclobutane dimers and breaks in the sugar phosphate backbone (Santos et al., 2013). The effect of UV on the structure and number of intracellular organelles has also been reported (Meindl and Lütz, 1996, Holzinger and Lütz, 2006, Steinhoff et al., 2007), for which one of the many reasons is the formation of disulfide bonds between tubulin dimers preventing proper microtubule polymerization and attainment of the correct conformation, which in turn affects the functioning of the cell cytoskeleton (Meindl and Lütz, 1996). The deleterious effect of UV on microorganisms led to the widespread use of UV-C in food and medical industry for sterilization, as effective doses have been published for different microorganisms including viruses, bacteria, yeast, fungi, protozoa and algae (Koutchma, 2009, Chang, 1985).

The typical characteristics of stress on cell growth could normally be detected through the presence of an extended lag phase and a reduced maximum cell amount at the exponential phase as well as a rapid death phase. All these features, however, were not seen from the growth profile of the thraustochytrid strain grown in the presence of UV-C compared with the control in the dark, in both F and NM media (Fig. 3.3). The exposure of strain 12B cells cultivated in F and NM media to UV showed a similar growth profile without showing any sign of 'typical' stress. Absorption of 50% of UV-A and UV-B by the culture medium has been reported in zoospore cultivation of brown algae *Alaria esculenta*, which prevents overburdening of the cell repair mechanism (Wiencke et al., 2007). Similarly, the cultivation medium may have absorbed UV to some extent allowing a lesser amount to reach the cell.

In terms of fatty acid composition, an increase or decrease in some fatty acids was observed from both saturated and unsaturated fatty acids (Table 3.2). The major saturated fatty acid C15:0 was found to decrease from 31% to 16% in F culture and from 10% to 6% in cells grown in NM medium, while C16:0 increased

from 17% to 35% in cells grown in F medium and from 42% to 46% in cells grown in NM medium when the culture was exposed to UV compared with cultivation in the dark. On the other hand, only a slight increase in PUFAs of C20:5 and C22:5 was found in cells grown in both F and NM media because of UV exposure compared with the dark, while a slight decrease in percentage was found for C22:6 (DHA) after 3 days' cultivation with less than 1% difference. There are probably no studies to date regarding the effect of UV on fatty acid composition of thraustochytrid microorganisms. Most studies have investigated the effect of UV-B exposure to phytoplankton such as algae, higher plants and also bacteria. The high increase in C16:0 saturated fatty acid composition was also seen together with an increase of 18:1 in the phytoplankton *Tetraselmis sp.* (Goes et al., 1994), microalga *Isochrysis galbana* and dinoflagellate *Prorocentrum micans* (Wang and Chai, 1994), and in Haptophyte alga *Phaeocystis antarctica* (Skerratt et al., 1998). However, unlike the slight decrease in the percentage composition of DHA seen in strain 12B, the *Tetraselmis* sp, *I. galbana* and *P. micans* all showed a large decrease in PUFAs C18:3 and C20:5, C18:4 and C22:6, and C22:6, respectively. Conversely, no observable trend in fatty acid change was observed when the diatoms *Odontella weissflogii* and *Chaetoceros simplex* were exposed to UV-B, which seems to be unaffected. There is therefore some species-specific response to UV depending on the organism; however, the differences in the type of UV used may also be another factor for the more or less constant DHA percentage composition in strain 12B, although the mechanism is still not clear.

Despite the slight decrease in percentage DHA composition in thraustochytrid strain 12B when exposed to UV, the absolute amount of DHA was increased because of the increase in DCW and lipid yields. One of the possible reasons that there was an increase in DCW and lipid yields is the reduction in the duration of the amoebic and zoospore stages and an increase in the settling stage because of UV exposure, allowing lipid to be accumulated, as it is known that motile thraustochytrid strain NIOS-1 would utilize its lipid store during the motile amoebic stage (Jain et al., 2007). This speculation can be supported by the finding that the shorter the duration of the amoebic stage of strain 12B cells grown in NM

medium compared with that of the amoebic-stage cells in F medium, the higher the amount of lipid accumulated in the cells (see Table 2.3 and 1.1). UV has been reported to result in the loss of flagella in the green algae *Chlamydomonas reinhardtii* (Donk and Hessen, 1995) and also in the loss of pili in bacteria *Proteus mirabilis*, *Escherichia coli* and *Neisseria gonorrhoeae* (Silverblatt, 1979). The same effect may possibly occur in the flagella of thraustochytrids, affecting the motility of the zoospores and allowing lipid to be further accumulated in the cell, contrarily to actively swimming zoospores such as those of *Pterygophora californica* algae, which consequently utilize a high amount of their intracellular lipid reserve (Reed et al., 1999).

The relationship between the optical density (OD) and cell number (in CFU) of strain 12B is governed by the equation: OD = (0.0082 × CFU) + 0.3878. The OD at A600 nm increases proportionally with cell number (CFU), with a high correlation $R^2$ value of 0.998, given that the OD is less than 2.0. Equal cell number, however, does not necessarily translate to equal biomass, as seen in this experiment where higher DCW was obtained from UV-exposed culture compared with cells cultivated in the dark, although the growth profile was almost the same (Fig. 16). This is probably due to the accumulation of lipid inside the cell, because a similar observation was also reported in a microalgae *Chlorella sp.* (Hsieh and Wu, 2009), where an increase in biomass due to lipid accumulation was recorded, although the number of cells was found to be constant. As the strain 12B samples were freeze-dried and their weight was measured in this experiment, cells that accumulate lipid and those that do not may have a different biomass because the water in the cytoplasm of the latter was thought to evaporate, leaving lighter cells.

DHA was found to have the highest oxidative stability in vivo among all PUFAs, (Miyashita et al., 1983); however, the tendency for DHA to be oxidized depends on the amount of DHA bonded to the positions in TG. Only the free fatty acid form of DHA, or DHA bound to all three fatty acid positions in TG, results in a high tendency for oxidation (Lyberg et al., 2005). The fact that DHA made up 26% of the fatty acid of TG in thraustochytrid strain 12B cell grown in F medium in the dark for 3 days (data not shown) indicates that not all the OH groups of TG are

being filled by DHA, hence reducing the tendency for DHA to be oxidized. This may allow DHA to play an important role in antioxidation by indirectly interacting with specific or nonspecific ligands instead, which would bind to related transcription factors, increasing the transcription of antioxidant enzymes CuZn-superoxide dismutase and Mn-superoxide dismutase (Huangfu et al., 2013). DHA was also found to suppress indirectly the formation of enzymatically derived intracellular ROS from cyclooxygenase-2 and xanthine oxidase by increasing enzymatic antioxidant such as the reduced glutathione and others (Kim & Chung., 2007). DHA therefore may have indirectly protected strain 12B from oxidative DNA damage from UV-generated ROS during the 3 days' cultivation period. In the case of the zoospores of non-DHA-producing brown algae *Alaria esculenta* (Wiencke et al., 2007), the cellular repair mechanism was much shorter, at less than 16 hr of exposure to UV. The protective antioxidative function of DHA, together with the side effect of UV in reducing the duration of the mobile amoebic and zoospore stage, consequently increases lipid accumulation and ultimately increases the absolute amount of DHA in thraustochytrid strain 12B, which may have industrial applications.

## 3.6. Conclusion

The accumulation of lipid and DHA in thraustochytrid strain 12B may serve as a source of energy and more importantly as a factor in its role in antioxidation. The thraustochytrid strain 12B appears to prefer the use of saturated fatty acid over DHA as a source of energy, as the absolute amount of C15:0 was found to decrease as starvation continued, while the absolute amount of DHA remained high compared with the other major fatty acids throughout the experiment. The percentage composition of saturated fatty acids pentadecanoic acid (C15:0) and hexadecanoic acid (C16:0) was also found to decrease from 31% to 15% and from 22% to 14%, respectively, during starvation, while the percentage composition of DHA was found to increase from 25% to 42% after 7 days. Other fatty acids were therefore preferentially used over DHA as a source of energy, and DHA may serve other important roles in the cell. During exposure to UV, DHA may have allowed the strain 12B cell to survive during the 3 days' cultivation under UV, as the percentage composition of DHA was found to decrease slightly. UV exposure is considered to affect the motility of zoospores of strain 12B, resulting in the conservation of the intracellular lipid store, which may explain the increase from 13.6, 4.7, 1.7, 0.5 mg/ml to 24, 16.1, 12.2, 3.4 mg/ml in the absolute DCW, lipid, fatty acid and DHA yields, respectively.

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