CHAPTER 3

Physiological role of DHA production
3.1. Introduction

Lipids in a cell can be classified as complex or simple lipids (Lehninger, 1978). Complex lipids essentially contain fatty acids as a component and include acylglycerol, phosphoglycerides, spingolipids and waxes. Simple lipids do not contain fatty acids, but consist of steroids and terpenes. Phosphoglycerides, or phospholipids are polar lipids, where one of the primary hydroxyl groups of glycerol is esterified to phosphoric acid, forming the polar head and the other hydroxyl groups are esterified with fatty acids, forming the acyl tails. Phosphoglycerides or phospholipids are predominantly present as membrane lipids (Lehninger, 1978; Parrish et al., 2000).

The most abundant form of lipids are triacylglycerols, which are the major components of storage lipids, and are largely found as storage bodies in the cell (Parrish et al., 2000; Ashford et al., 2000; Anderson and Wynn, 2001). Triacylglycerols have high free energy content and a tendency to form aggregates in water, which allows for compact unhydrated intracellular packing (Stryer, 1988). Triacylglycerols are neutral lipids where all the three –OH groups of glycerol are esterified with fatty acids (Fig. 3.1).

DHA, when present in the form of phospholipids in the membranes, increases the fluidity of the membrane (Kendrick and Ratledge, 1992; Jump, 2001; Stillwell and Wassall, 2003). Membrane fluidity is essential for transport mechanisms, as well as endo- and exocytotic processes. The presence of a double bond in the lipid bilayer introduces a ‘kink’ and thus the acyl chains are not packed compactly, making the membrane more fluid. More over the melting
point of the acyl chains increases with increase in the carbon atoms and the presences of double bonds (Lehninger, 1978; Anderson and Wynn, 2001). Organisms growing at low temperatures have a higher concentration of Long chain PUFAs (LCPUFAs) in their membrane phospholipids as saturated fatty acids and other small chain unsaturated fatty acids will become solids and thus decreasing the membrane fluidity (Parrish et al., 2000; Nichols, 2003; Stillwell and Wassall, 2003).

Thraustochytrids accumulate up to 50 % of their dry weight as lipids (Bajpai et al., 1991 a; Yaguchi et al., 1997; Yokochi et al., 1998). Bulk of their total lipids can occur as either triacylglycerols or as phospholipids. The ratio of triacylglycerols to phospholipids, as well as their fatty acid profiles in thraustochytrids depend on culture conditions (Ashford et al., 2000; Lewis et al., 2001). Yaguchi et al. (1997) and Volkman et al. (1998) have shown that in *Schizochytrium* species strain SR21 and microalgae, neutral and polar lipids were produced in equal amounts in the early growth phase, but on further cell growth only the neutral lipids increased. Generally, 70 – 98 % of lipids in thraustochytrids are present as triacylglycerols and up to 5 % as phospholipids (Nakahara et al., 1996; Yaguchi et al., 1997; Ashford et al, 2000).

Docosahexaenoic acid (DHA), the signature fatty acid in thraustochytrids can amount up to 77 % of the total fatty acids (Ellenbogen et al., 1969; Nakahara et al., 1996). DHA in these organisms is present both in phospholipids and triacylglycerols (Singh and Ward, 1997). In a strain of *Schizochytrium* designated ‘SR21’, neutral lipids contained 35 % DHA (Yaguchi et al., 1997). DHA
constituted 63.5% of the phospholipids. However, phospholipids formed only a small fraction of the total lipids in this species.

Most of the DHA in thraustochytrids is present as triacylglycerols in oil bodies of the cell (Ashford et al., 2000). However, in other photosynthetic microalgae, belonging to the classes, Dinophyceae, Haptophyceae Cryptophyceae and others, DHA is present mainly as phospholipids, located in the thylakoid membranes (Volkman et al., 1989; Sijtsma and Swaaf, 2004). Algal triacylglycerols, present as membrane-bound oil droplets in cytoplasm, generally contain lower concentrations of ω-3 PUFAs and higher concentrations of saturated and monounsaturated fatty acids (Singh and Ward, 1997). Nakahara et al. (1996) and Ashford et al. (2000) have shown that the triacylglycerols in thraustochytrids are not simple triacylglycerols containing the same fatty acid at all 3 carbon positions of glycerol. Instead, they are composed of DHA, palmitic acid, myristic acid, palmitoleic acid and DPA. DHA is preferentially (up to 75%) esterified at sn-2 position of the glycerol. The triacylglycerols are organized in the semicrystalline state by the segregation of the different fatty acid type into separate layers (Ashford et al., 2000).

Although the role of PUFAs as membrane fatty acids has been fairly well studied, many questions regarding the biological and ecological role of lipids and fatty acids in thraustochytrids have not been examined. An attempt has been made to address a few questions in this chapter;

- Why do thraustochytrids generally accumulate high amounts of lipids?
- Why are the triacylglycerols generally rich in DHA?
- Why does DHA increase during refrigeration and when the medium viscosity is increased?

Fig. 3.1. Schematic representation of triacylglycerol, \( R_1 - CO, R_2 - CO \) and \( R_3 - CO \) are the fatty acyl groups.
3.2. Materials and Methods

3.2.1. Fate of lipid bodies during development

The culture NIOS-1 was grown in M4 medium as described in Chapter 2. Cells from 3 day old cultures were centrifuged at 7500 rpm for 15 min, washed thrice with sterile seawater and stained with 0.5 % w/v nile blue in seawater. Nile blue, a phenoixazine cationic dye, is a vital stain for lipids, fluorescing golden yellow when observed under an epifluorescence microscope using a green excitation filter. The staining reaction of the dye is attributed to the presence of oxazine and oxazone (Dunningan, 1968 a and b; Vijayalakshmi et al., 2003). The cells were then spread on a thin layer of MV agar on a sterile slide. A coverslip was gently placed on it and the slide was observed under oil immersion objective (100X) using an Olympus BX60 epifluorescence microscope. A dichroic mirror system DM 570 with excitation filter of 510 – 550 nm wavelength and emission filter of 570 nm wavelength were used. The slide was transferred to a sterile moist chamber during observations to avoid drying of the agar, and examined periodically for up to 2 days.

3.2.2. Role of DHA as a storage reserve

The culture NIOS-1 was grown in M4 medium at room temperature under shaken conditions for 5 days as described in the previous chapter. The cells were harvested by centrifugation at 7500 rpm for 15 min under sterile conditions, rinsed thrice with sterile, 0.22 µm filtered seawater (FSW) and finally suspended in a small amount of FSW to form a thick slurry of cells. This was divided into two
sets. One of these was first refrigerated at 10 °C for 48 h to increase the DHA content, as stated in Chapter 2, while the other was immediately subjected to the experiment. For the experiment, the two sets of slurry of cells in seawater were incubated without addition of nutrients for various time intervals, in order to starve them. One milliliter of the slurry of each set was sampled at 12 h, 24 h, 48 h, 4 d, 8 d, 12 d, 16 d, 20 d, 24 d and 28 d. The samples were tested for viability by inoculating in sterile seawater and baiting with pine pollen. Presence or absence of growth was examined after 3 days. Each starved set of sample was analyzed for total lipids and fatty acid profiles, following methods given in Chapter 2.

3.2.3. Relation between DHA and other PUFAs and specific gravity of cells

Experiments were carried out using 4 isolates – NIOS-1, NIOS-2, NIOS-4 and NIOS-10, grown in M4 medium at room temperature of ~ 28 °C under shaken conditions for 5 days. The cells were harvested by centrifugation at 7500 rpm (5200 g). The biomass of each culture was divided into two sets. The specific gravity of one set was estimated immediately, while that of the other set was estimated following refrigeration at 4 °C for 48 h to increase DHA content, as in chapter 2.

Specific gravity was estimated as follows. The biomass was made into a thick slurry using sterile seawater. A series of sucrose solutions in sterile seawater, with 0, 0.2, 0.4, 0.6, 0.8 and 1.0, 1.2 and 1.4 % concentrations was prepared and 10 ml was distributed in test tubes. The specific gravity of each of
the solutions was measured using a refractometer (Atago S/Mill, Japan). A small amount of the biomass was picked up at the tip of a Pasteur pipette and suspended in the middle of the sucrose solution in the test tube. Sinking of the biomass to the bottom of the test tube indicated negative buoyancy and specific gravity greater than that of the sucrose solution and floatation indicated positive buoyancy and lesser specific gravity. The specific gravity of the sucrose concentration where the biomass remained suspended was considered as representing the specific gravity of the cells, having neutral buoyancy.
3.3. Results

3.3.1. Fate of lipid bodies during development

Lipid bodies in the culture NIOS-1 stained bright golden yellow in colour under an epifluorescence microscope, following nile blue staining. After 4-6 h, some of the cells became amoeboid in shape and showed slow movements. Such amoeboid cells had less number of fluorescent lipid bodies (Fig 3.3.1). Older amoeboid cells, which had moved farther away from the colonies showed granular contents. As the lipid bodies reduced in number, the fluorescence also reduced and the granular amoebae showed no fluorescence (Fig. 3.3.1). After 36 h, the cells produced an extensive ectoplasmic network. The ectoplasmic net elements also showed faint fluorescence (Fig. 3.3.1).

3.3.2. Role of DHA as a storage reserve

Unrefrigerated cells remained viable up to 16 d, whereas the cells in which DHA was increased by cold shock remained viable up to 24 d. The major fatty acids in both the sets were palmitic acid and DHA. Cells stored at 10°C had higher percentage of total lipids than unrefrigerated ones (Fig. 3.3.2). Total lipids decreased with increasing days of starvation. Total and percentage values of palmitic acid in refrigerated cells and non-refrigerated cells was almost the same. (Fig. 3.3.3 a and b). However, total DHA showed increased amounts in refrigerated cells although their percentage in total lipids remained same (Fig 3.3.3 a and b). No significant trends in changes of fatty acid were noticed till 6 – 8 days in both refrigerated, and non-refrigerated treatments. However, palmitic acid
and DHA showed changes upon starvation after about 8 days. While percentage levels of palmitic acid showed a distinct increase from ~60 to 80% from 8 to 20 days in both refrigerated and non-refrigerated treatments, their absolute values increased only marginally or showed no clear trends (Fig. 3.3.3 a and b.). Both percentage, as well as absolute values of DHA showed a clear decline from about 6 to 20 days of starvation in both treatments. No distinct trends were noticed with other fatty acids, which were much lower in concentrations.

### 3.3.3. Relation between DHA and other PUFAs and specific gravity of cells

The specific gravity of the cells prior to refrigeration ranged from a low of 1.02 in NIOS-10 to 1.028 in NIOS-1 cells respectively (Fig. 3.3.4). The specific gravity increased in all four isolates upon refrigeration. The highest specific gravity after refrigeration was seen in NIOS-1, corresponding to a value of 1.031. Fatty acid profiles of the four isolates before and after the cold shock are presented in Table 3.3.1. It was observed that lower the lipid content, lower was the specific gravity. More over as total lipid increased on a cold shock the specific gravity also increased (Table 3.3.1). Cells with higher specific gravity, resulting from refrigeration contained slightly lower percentage of palmitic acid. However, their percent DHA levels increased in such cells. This change was most noticeable in NIOS-4 and NIOS-10. In these cultures, the cells with higher specific gravity contained nearly 55% more of DHA than in those with lower specific gravity (Table 3.3.1).
Fig. 3.3.1: Nile blue staining of isolate NIOS-1. A and B: Vegetative and amoeboid cells 2 h after spreading on MV agar. A: Bright field photomicrograph. B: Epifluorescence photomicrograph showing fluorescent lipid bodies. Note their presence in round cells and absence in the amoeboid cell (arrow). C and D: Vegetative cells and ectoplasmic net elements 36 h after spreading on MV agar plate. C: Bright field photomicrograph. D: Epifluorescence photomicrograph showing fluorescent lipid bodies and ectoplasmic net elements (arrow). Bar represents 10 μm.
Fig 3.3.2: Total lipid profile of cells during starvation.
Fig 3.3.3a: Total amounts of fatty acids in refrigerated (----) and non-refrigerated (- - -) cells present per gram of dry weight biomass on starvation of isolate NIOS-1 at room temperature.

Fig 3.3.3b: Percentage fatty acids in refrigerated (-----) and non-refrigerated (---) cells on starvation of isolate NIOS-1 at room temperature.
Fig 3.3.4: Specific gravity of cells before and after refrigeration.
<table>
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<tr>
<th>Isolate</th>
<th>Treatment</th>
<th>Fatty acid (mg) DCW (g)^{-1}</th>
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<td></td>
<td></td>
<td></td>
<td>14:0</td>
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<td>NIOS-1</td>
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<td></td>
<td>Refrigerated</td>
<td>501.4</td>
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<tr>
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<td></td>
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<td></td>
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<td>368.45</td>
<td>4.65</td>
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*Table 3.3.1: Fatty acid profile of percent fatty acids in refrigerated and non-refrigeration cells.*
3.4. Discussion

Storage lipids in thraustochytrids may have various roles in the biochemistry of the cell.

a) Mature vegetative cells in thraustochytrids contain high amounts of lipids. Such cells are subsequently transformed into zoosporangia, which reproduce by means of zoospores (see Chapter 1). The reproductive process will require heavy investment in energy, particularly for various metabolic functions related to the process, as well as the formation of various special structures, such as flagella of zoospores. This could be one of the roles of storage lipids in thraustochytrids. Lipids are known to be used as energy reserves in higher organisms.

b) Cell motility requires a rapid expenditure of energy. The energy source for this might be provided by lipids. In the present study, it was observed that while freshly formed amoebae were fairly rich in lipid bodies, those that had moved away a certain distance had less, or no detectable lipids (Fig. 3.3.1).

c) Lipids might serve as a reserve of fatty acids for a rapid buildup of membranes. Cohen et al., (2000) have suggested that lipids might serve as sources of fatty acids in cells that undergo rapid changes in the environment that necessitates the production of membranes. These authors have further shown that in cells of the red alga Porphyridium cruentum and a green alga, the PUFAs, eicosapentanoic acid and arachidonic acid served as sources of chloroplast membrane lipids.
Thraustochytrids grown in nutrient rich media typically produce cells, which lack ectoplasmic net elements (EN) (Fig. 3.3.1). However, such cells produce enormous amounts of EN under nutrient poor conditions, the EN enabling them take up nutrients from the surrounding medium (Coleman and Vestal, 1987). Storage lipids might be rapidly converted into membrane lipids under such exigencies. Lipids stained with nile blue appeared to be rapidly disseminated into the membrane system of EN (Fig. 3.3.1). Membranes are typically high in phospholipids, when compared to the storage lipids, which are typically triacylglycerols. An interesting possibility is that triacylglycerols in cells grown under conditions of abundant nutrition are converted into membrane phospholipids in thraustochytrids under circumstances that require the production of EN.

d) Lipids might help overcome starvation of cells. In the present study, it was observed that the total lipids decreased with increasing periods of starvation. Besides, it was also observed that DHA was the only fatty acid, which showed a significant decrease in concentration. Similar results were found by Coutteau and Mourente (1997), who showed that the ω-3 PUFAs, 18:3 (α-linolenic acid), 22:5 (docosapentaenoic acid) and 22:6 (docosahexaenoic acid) were specifically catabolized by Artemia during starvation. It is likely that DHA is be converted to other fatty acids in thraustochytrids, before being fully. Palmitic acid content may remain more or less constant during the entire period of starvation (Fig. 3.3.3).
DHA contents increased when cells were refrigerated, or when polyvinylpyrrolidone (PVP) was added to the growth medium (see Chapter 2). The reasons for this are not clear. It was earlier presumed that the increase in viscosity of the medium by addition of PVP might have caused a DHA increase (Jain and Raghukumar, 2005). Addition of PVP also has the effect of increasing the density and specific gravity of the medium. Therefore, the relation between the specific gravity of thraustochytrid cells and their DHA contents were examined (Figs. 3.3.4 and Table 3.3.1).

The specific gravity of the cell in an aquatic organism has implications in the buoyancy of the cell that determines the depth at which it resides. There are a number of strategies that organisms have evolved to alter their buoyancy and become positively, neutrally buoyant or negatively buoyant. Diatoms produce EPS during their stationary phase. This EPS makes the cells heavy and thus facilitate in sinking of cells, during collapsing of a phytoplankton bloom (Decho, 1990). Some marine fishes and copepods of the genus Tigriopus, maintain negative buoyancy by altering their osmotic balance (McAllen et al., 1998). Another strategy seen in crustaceans is the selectively choice of heavier ions (for example, SO$_4^{2-}$ or Mg$^{2+}$) or lighter ions (for example Na$^+$, Cl$^-$ or NH$_4^+$) or ions with a higher partial molal volume (for example trimethyl amine) (Sanders and Childress, 1988; Newton and Pots, 1993) as the need may be. Gas filled in swim bladders of some fishes and invertebrates and storage of low-density materials; such as lipids are other mechanisms to maintain buoyancy.
High specific gravities and negative buoyancy are possible even with high lipid contents. Although increasing levels of lipids are important mechanisms to achieve positive buoyancy, it was observed that in eggs of the Japanese eel, *Anguilla japonica*, both buoyant and non-buoyant eggs had the same levels of lipids (Seoka et al., 2003). Similarly copepods collected at 2000 m depth showed no significant differences in total lipids or wax ester content from copepods collected at 1000 m depth (Lee et al., 1972). These authors also observed that copepods from depths below 750 m invariably contain large amounts of wax esters and lipids, while those near the surface (0-250 m) contained less lipids and wax esters. The copepods, *Calanus finmarchicus*, during its diapause phase has a high lipid content, up to 76% of dry weight, mostly in form of wax esters, although residing below 750 m depth (Visser and Jónasdóttir, 1999).

While discussing the role of lipids and wax esters in maintaining buoyancy, Yayanos et al. (1978) suggest that this is possible because lipids and wax esters have higher compressibility and higher thermal expansion, being 6-10 times that of seawater. Therefore the potential energy barrier of downward migration becomes smaller rather than greater with depth. Thus, lipids may actually act to facilitate the vertical migration of animals. Therefore, despite possessing high amounts of lipids, thraustochytrids may be able to alter their buoyancies. Thraustochytrid cells with higher DHA contents in storage lipids had a higher specific gravity (Figs. 3.3.4 and Table 3.3.1). However, role of DHA in this is not clear. Increased DHA levels might have been the consequence of increased specific gravity caused by some unexplained mechanism.
Alternatively, DHA levels might determine the buoyancy and specific gravity of the cells. In view of the above, it will be interesting to examine the role of fatty acid composition on compressibility and thermal expansion of lipids, which might have implications in buoyancy alterations.
3.5. Conclusions

The abundant lipid bodies in the mature vegetative cell of the isolate NIOS-1 fluoresced brightly with nile blue under an epifluorescence microscope. These gradually disappeared in motile amoeboid cells, suggesting their use as energy reservoirs. They also became distributed in the ectoplasmic net elements, indicating their incorporation in the plasma membrane. Total lipids gradually decreased in starved cells of NIOS-1. Among the fatty acids, DHA showed decrease in total, as well as percentage values after starvation for 6 days, while palmitic acid contents did not show distinct changes. Cells containing higher levels of DHA following refrigeration also showed greater specific gravity when compared to controls with lower DHA values. This indicates a relation between the buoyancy of the cell and DHA content, which requires further study.