Polyunsaturated fatty acids and extracellular polymeric substances from thraustochytrid protists

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BY

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UNDER THE GUIDANCE
OF

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CERTIFICATE

This is to certify that the thesis entitled **"Polyunsaturated fatty acids and extracellular polymeric substances from thraustochytrid protists "** submitted by **Ms. Ruchi Jain** for the award of the degree of Doctor of Philosophy in Department of Marine Science is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any university or institution.

S. Raghu Kumar

Place: Dona Paula
Date: 24.2.05

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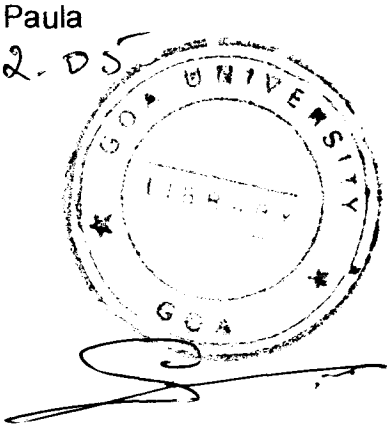

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(S. B. SULLIA)
External examiner

Statement

As required under the University Ordinance 0.19.8(vi), I state that the present thesis entitled **"Polyunsaturated fatty acids and extracellular polymeric substances from thraustochytrid protists** " is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the fist comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilitates and suggestions have been availed of.

Ruchi Jain
17/03/05

(Ruchi Jain)

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Ruchi Jain

Ruchi Jain

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Dedicated to my

Dadi aur Baba,
Nani aur Nana

CHAPTER 1

General Introduction

Thraustochytrids are a group of marine protists, which have attracted much attention in recent years owing to their ubiquitous and abundant occurrence in the marine ecosystem and their commercial importance in the production of the polyunsaturated fatty acid or PUFA, the docosahexaenoic acid (DHA) (Bahnweg and Sparrow, 1974 a and b; Gaertner, 1982; Raghukumar, 1985; Porter, 1990; Naganuma *et al.*, 1998; Lewis *et al.*, 1999; Kimura *et al.*, 1999; Raghukumar 2002). Following are the essential characteristics of these organisms (Goldstein, 1963; Porter, 1990; Raghukumar, 2002).

- Eukaryotic;
- Single celled;
- Obligatly marine;
- Heterotrophic, chemoorganotrophic mode of nutrition;
- Reproduction by biflagellated zoospores;
- Production of ectoplasmic net elements.

A general review of thraustochytrids is given below.

1.1. General characteristics of thraustochytrids

The typical life cycle of a thraustochytrid is shown in Fig 1.1 (Porter, 1990). Motile biflagellate zoospores lose their flagella and settle on a suitable substratum and grow to become vegetative cells. Whether the flagella are shed, lost or absorbed is not known. The nucleus and the cytoplasmic contents of the mature vegetative cell, the zoosporangium, divide to form zoospores, which initiate the life cycle once again upon release (Goldstein and Belsky, 1964;

Sparrow, 1969; Chamberlain, 1980). Newly rounded-off zoospores often have a thin cell wall. As the thallus develops the wall thickens due to apposition of wall scales and fibrillar matrix (Chamberlain 1980).

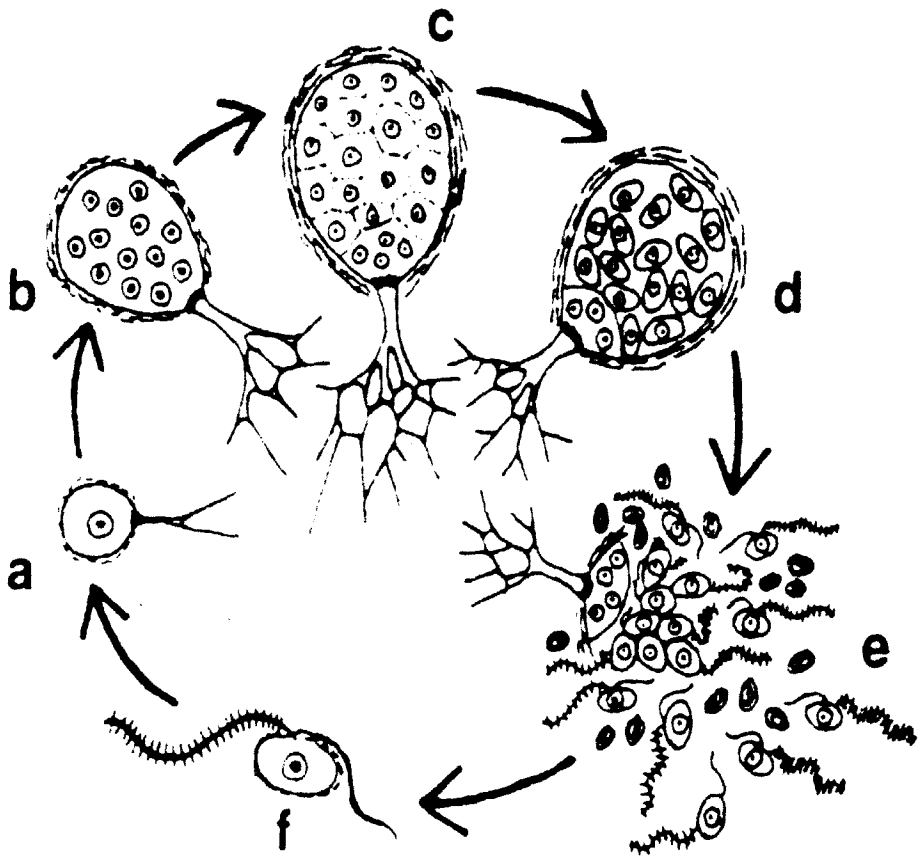


Fig. 1.1: A young thallus (a) grows to a mature cell, the zoosporangium, followed by nuclear divisions (b). Progressive cleavage of the protoplast (c) occurs in the zoosporangium (d), which releases zoospores (e and f) that settle down and grow to form a new thallus (From Porter, 1990)

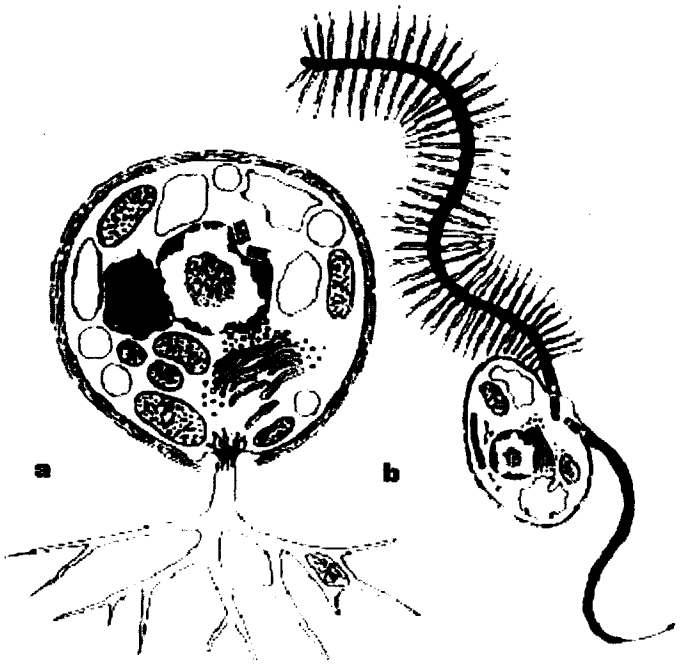


Fig. 1.2: Schematic representation of the ultrastructure of thraustochytrid cell (a) and zoospore (b) from Leander *et al*, 2004.

The zoospores of thraustochytrids possess two unequal flagella (heterokont), with one long anterior and a short posterior flagellum. The anterior flagellum is characterized by a row of hairs, the tinsel, each ending in a tripartite branch. The posterior whiplash flagellum is smooth (Porter, 1974; Kazama, 1974; Perkins, 1976; Raghukumar, 1981; Chamberlain and Moss, 1988).

The vegetative cells of thraustochytrids posses a characteristic ultrastucture (Moss, 1986). The cell wall is made up of circular scales, which are transported fully formed in the Golgi vesicles of the dictosomes (Darley *et al.*, 1973; Chamberlain, 1980; Raghukumar, 1981; Moss, 1985). Mitochondria typically posses tubular cristae (Kazama 1974; Porter, 1990). The plasma membrane extends through an opening in the cell wall and forms a branched

network called the ectoplasmic net (EN), the individual elements of which are called the ectoplasmic net elements. A peculiar organelle called the sagenogenetosome, sagenogen or bothrosome is typically associated with the EN at its point of origin within the cell (Perkins, 1972; Perkins, 1973; Perkins, 1976; Moss, 1980). The cell wall typically contains proteins and sulfated polysaccharides in almost equal proportions, the polysaccharide being predominantly made up of galactose (Porter, 1974; Chamberlain, 1980; Bahnweg and Jäckle, 1986)

Different species of thraustochytrids are characterized by variations in the basic pattern of the life cycle mentioned above (Porter, 1990). These are dealt with in Chapter 6.

1.1.2. Phylogeny of thraustochytrids

The first thraustochytrid was described in 1936 by the famous mycologist Fredrick K Sparrow Jr. They were originally placed under the Class Phycomycetes of fungi based on the morphology of the zoosporangia (Sparrow 1936). The presence of the biflagellated zoospores prompted a transfer of thraustochytrids to the class Oomycetes of fungi (Sparrow 1973). However numerous ultrastructure and cell wall chemistry studies in the 1960s - 80s revealed that thraustochytrids were different from fungi in many ways (Perkins, 1972; Perkins, 1973; Bahnweg and Jäckle 1986; Moss, 1986; Chamberlain and Moss, 1988). The cell wall lacks chitin and is not microfibrillar (Banhweg and Jäckle. 1986; Porter, 1974; Chamberlain, 1980). The EN is not homologous to

the rhizoids of chytrids, being mere extensions of plasma membrane (Perkins 1972; Perkins 1973; Coleman and Vestal, 1987). The mitochondria contain tubular cristae and not flat as in fungi (Kazama, 1974; Porter 1990; Dick, 2001). Molecular studies in the 1980s and later have shown that thraustochytrids as well as the oomyceten fungi are more closely related to single celled algae such as diatoms, chyrsophyceans, xanthophyceans and brown algae (Chamberlain and Moss, 1988; Patterson, 1989; Krishna, 1990; Cavalier-Smith, 1993; Cavalier-Smith, 1994; Leipe *et al.*, 1994; Leipe *et al.*, 1996). Thraustochytrids are classified under the Kingdom Straminipila (Dick 2001) or the Kingdom Chromista (sensu Cavalier Smith *et al.,* 1994). The position of this Kingdom within the eukaryote crown of trees is represented in Fig. 1.3. The closest relatives of thraustochytrids are the aplanochytirds and the labyrinthulids (Leander and Porter, 2000; Leander and Porter, 2001. The former produce cells that glide using EN, while the labyrinthulids produce spindle shaped cells that glide through the EN. The taxonomic position of thraustochytrids is indicated below.

	(Dick, 2001)		(Cavalier-Smith, 1993)
Kingdom	Straminipila	Kingdom	Chromista
Phylum	Heterokonta	Phylum	Heterokonta
Class	Labyrinthista	Subphylum	Labyrinthista
Order	Thraustochytriales	Class	Labyrinthulea
Family	Thraustochytriaceae.	Subclass	Thraustochytridae

1.1.3. Thraustochytrids in the marine ecosystem

In the nearly 70 years since the first thraustochytrid was described, these protists have been discovered to occur in most of the conceivable habitats of the sea. They appear to play a major role as saprotrophs and are found abundantly in both costal and offshore regimes (Porter, 1990; Raghukumar, 2002). Most of the species of thraustochytrids have an optimum temperature for growth at around 23-28 °C (Goldstein, 1963; Alderman and Jones, 1971; Goldstein, 1973; Bremer 1974). Some psychrophilic thraustochytrids have also been reported (Bahnweg and Sparrow, 1974 a and b; Riemann and Schaumann, 1993; Rieman and Schrage, 1983). *Thraustochytrium antarcticum* grew well at 0 °C, had an optimum at 4 °C and did not grow beyond 17 °C (Bahnweg and Sparrow, 1974b). Thermophilic thraustochytrids have not yet been reported (Porter, 1990). Thraustochytrids are not found in salinities less than 10 ppt, but above these salinities they may be eury – or stenohaline depending on the species (Goldstein, 1963; and Goldstein, 1973; Bremer, 1974; Amon, 1978, Wethered and Jennings, 1985). In coastal regions, they are extremely common in decaying algae, mangrove leaves and sediments (Miller and Jones, 1983; Raghukumar, 1985; Raghukumar, 1986; Ulken, 1986; Raghukumar, 1987a; Raghukumar, 1988b; Ulken *et al.*, 1990; Raghukumar and Raghukumar, 1992; Naganuma *et al.*, 1998; Chien and Lo, 1999; Kimura *et al*,. 1999; Santangelo *et al.*, 2000; Bongiorni and Dini, 2002). They often attain substantial biomass in such habitats (Sathe *et al.*, 1993; Raghukumar *et al.*, 1994; Raghukumar *et al.*, 1995). Thraustochytrids utilize a wide variety of organic carbon sources and elaborate several

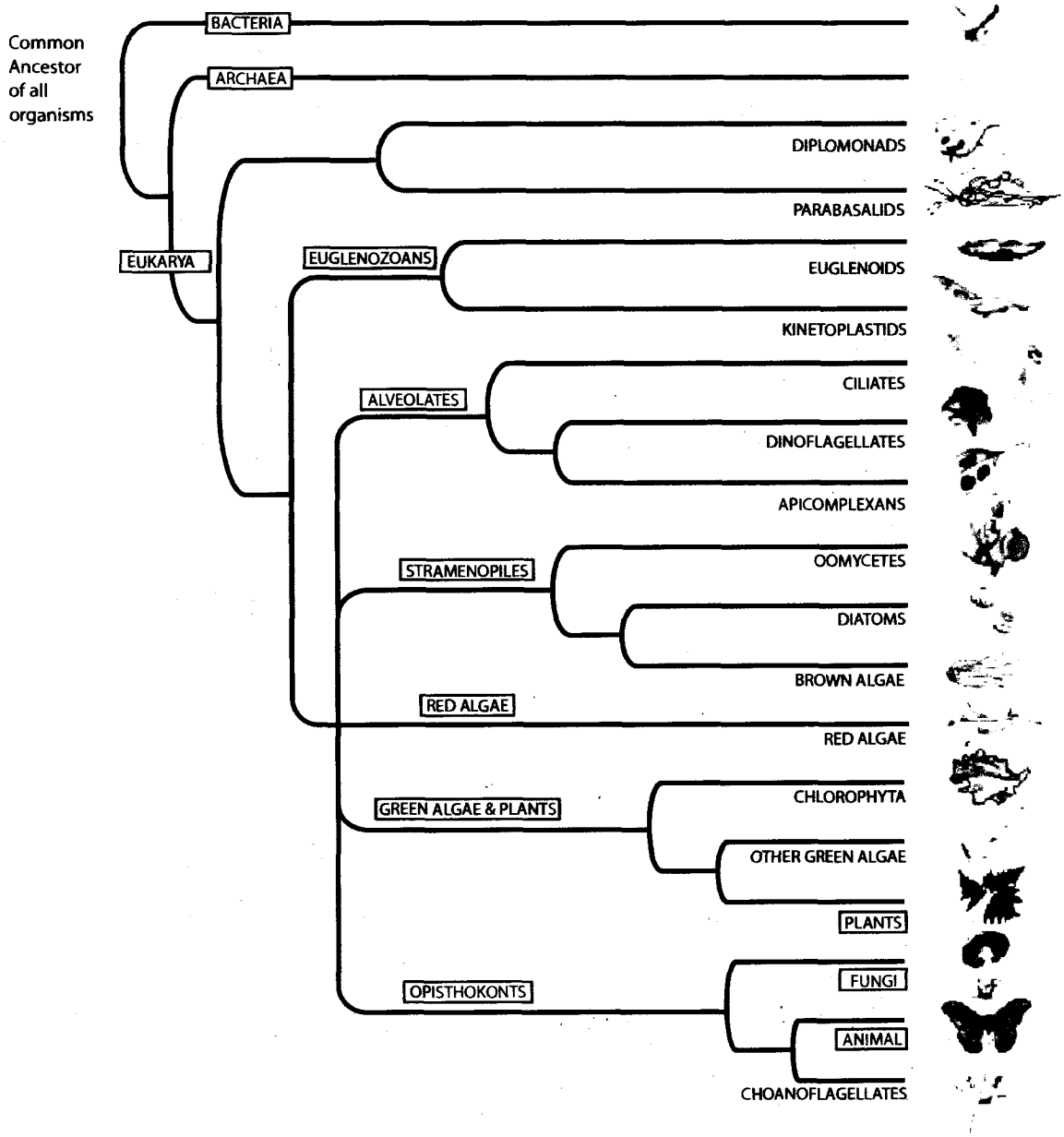


Fig. 1.3: Position of Kingdom Straminipila within the eukaryote crown. (Purves *et al.*, 2004)

extracellular enzymes (Goldstein, 1963; Goldstein, 1973; Bahnweg, 1979a and b; Sharma *et al* .,1994).

Several pathogens have also been reported, particularly in molluscan hosts (McLean and Porter, 1982; Bower, 1987; Kleinsohuster *et al.*, 1998; Ragonecalvo *et al.*, 1998; Brothers *et al.*, 2000; Ragan *et al.*, 2000). These protists are also abundant in the oceanic water column (Bahnweg and Sparrow, 1974b; Gaertner and Raghukumar, 1980; Gaertner, 1982; Raghukumar, 1985; Raghukumar, 1990; Raghukumar *et al.*, 2001.). Thraustochytrid populations in the water column attain densities of more than 1.0×10^2 cells L^{-1} to 6.57×10^3 cells L^{-1}, as estimated by the by pine pollen baiting technique (Banhweg and Sparrow, 1974b; Raghukumar and Gaertner, 1980; Ulken, 1986). However, these values obtained by culture techniques may be underestimates, since the numbers are much higher using the acriflavine direct detection (AfDD) technique, described by Raghukumar and Schaumann, 1993). Direct detection yielded values ranging from 5.6×10^4 to 1.83×10^5 cells L^{-1} in the North Sea, Arabian Sea and the Japan Sea (Raghukumar and Schaumann, 1993; Raghukumar *et al.*, Kimura *et al.*, 1999; Kimura and Naganuma, 2001). Thraustochytrid densities may be much higher in the sediments (Raghukumar and Gaertner, 1980). Decaying phytoplankton aggregates have been reported to harbour thraustochytrid biomass often exceeding that of the bacteria (Raghukumar, 1993; Riemann and Schaumann, 1993; Raghukumar, 1997; Raghukumar *et al.*, 2001; Kimura and Naganuma, 2001). Dense populations of thraustochytrids have been isolated in fecal pellets of the salp *Pegea confoederata* in the Arabian Sea (Raghukumar and Raghukumar, 1999). These marine protists have also been

isolated from healthy sponges (Ilan *et al.*, 1996). Thraustochytrids have also been reported up to depths of 4000 m in the sea (Gaertner, 1973).

It has been suggested that thraustochytrids make a substantial contribution to the organic carbon transfer compared to bacteria (Kimura *et al.*, 1999). The microbial food chain in the sea is a pathway of particle-sized increments: from micrometer–sized bacteria to flagellates, to ciliates and to mesozooplankton above the size range of 200 μm, thus involving 4 trophic levels. When the chain starts with 10 μm – sized thraustochytrids, only three trophic levels, namely thraustochytrids, ciliates and mesozooplankton, are involved and thus the whole transfer efficiency would be ten times higher than the chain starting with bacterioplankton, considering that the energy transfer efficiency between each trophic level is only 10 % (Naganuma *et al.*, 1998; Kimura *et al.*, 1999; Kimura and Naganuma, 2001, Nagao *et al.*, 2002).

Thraustochytrids possess several polyunsaturated fatty acids (PUFAs), the most abundant being docosahexaenoic acid (DHA) (Lewis *et al.*, 1999). DHA is an essential nutrient for growth and reproduction in crustaceans such as zooplankton, which are abundant in the marine ecosystem (Harrison, 1990). Therefore, they may play an important role in the food web. These aspects are discussed further.

1.2. Polyunsaturated fatty acids (PUFAs) in thraustochytrids

Thraustochytrids are a rich source of PUFAs, particularly docosahexaenoic acid (DHA) (Barclay 1994 a and b,; Lewis *et al.*, 1999;

Ratledge and Wynn, 2002; Sijtsma and Swaaf, 2004). PUFAs are fatty acids with 18 or more carbon atoms containing more than two double bonds. The first double bond may be at the 3rd, 6th or 9th positions from the methyl end. Such fatty acids are designated as omega –3 (ω-3 or n-3), omega –6 or omega – 9 fatty acids respectively. Fatty aids are designated by the number of carbon atoms: number of double bond: the position of the first double bond from the methyl end (Parrish *et al.,* 2000). Thus DHA is designated as 22:6:ω-3. Some commonly occurring fatty acids are listed in Table 1.1. Representative structure of DHA is depicted in Fig.1.4. The metabolic pathway of some of the essential PUFAs is presented in Fig. 1.5 and 1.6. Omega-6 fatty acids are predominantly found in plants and animals, while ω-3 PUFAs are more common in marine animals and phytoplankton (Singh and Ward, 1997). Linoleic acid and α-linolenic acid are essential fatty acids and are precursors for a number of long chain PUFAs as shown in Fig. 1.6 (Fidler *et al.,* 1999; Wallis *et al.,* 2002).

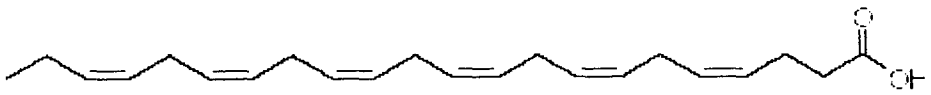


Fig. 1.4.: Schematic representation of docosahexaenoic acid (DHA; 22:6 ω-3)

1.2.1. Importance of PUFAs in animal and human nutrition

The omega-6 PUFA, arachidonic acid (AA, 20:4:ω6), and the omega –3 PUFAs such as α-linolenic acid (18:3:ω3); eicosapentanoic acid (EPA, 20:5 ω3) and docosahexenoic acid (DHA, 22:6 ω3) are essential dietary components of

humans and animals (Harrison, 1990; Bajpai and Bajpai, 1993). They play an important role in biosynthesis of prostaglandins and eicosanoids. Among these the role of DHA is considered very crucial in health. DHA is the primary structural fatty acid of the highly active neural tissue and makes up to about 60 % of the structural lipid in the gray matter of the human brain. DHA is essential for normal development of neural tissues in infants, especially in the eye and brain. They are necessary constituents of cell membranes, are involved in many cell-signaling systems and are major precursors of many prostaglandins and eicosanoids. They are known to decrease the incidence of coronary heart disease, strokes and rheumatoid arthritis (Bajpai and Bajpai, 1993; Connor, 2000; Sprecher, 2000; Jump, 2001; Stillwell and Wassall, 2003). Mammals lack the $\Delta 12$ and $\Delta 15$ desaturase enzymes required for the production of the DHA precursor, α-linolenic acid (Fidley *et al.*, 1999; Wallis *et al.*, 2002). This is further elongated and desaturated to DHA (Qiu, 2003). Even if α-linolenic is present in the diet, it is believed that the conversion of α-linolenic to DHA is very slow in mammals, since α-linolenic acid and linoleic acid (the precursor of ω-6 fatty acids) compete for the same substrate (Singh and Ward, 1997). Therefore, supplementation of nutrition with DHA is considered the surest way of incorporating this important fatty acid in humans.

Marine animals require DHA for growth and reproduction. Many of these, including the commercially important fishes and prawns, cannot synthesize their own DHA and depend upon the food sources such as phytoplankton for procuring these fatty acids through biomagnification. Therefore DHA plays a

crucial role both in the marine food web as well as aquaculture industries (Rainuzzo *et al.*, 1997; Mullar-Navarr *et al.*, 2000; Mullar-Navarr *et al.*, 2003; Phleger *et al.*, 2001; Saito *et al.*, 2002; Carter *et al.*, 2003; Park *et al.*, 2003).

1.2.2. Commercial sources of DHA

The demand for ω-3 fatty acids is rapidly increasing in aquaculture and nutraceutical industries (Fidler *et al.*, 1999; Lewis *et al.*, 1999; Ratledge and Wynn, 2002; Carter *et al.*, 2003; Ratledge, 2004; Sijsma and Swaaf, 2004). DHA has been reported in psychrophilic bacteria and lower straminipilan fungi. However, psychroplilic bacteria require cold conditions and high pressure to produce high DHA concentrations and therefore production is not cost effective (Jøstensen and Landfald, 1996; Nicholas and McMeekin, 2002, Nicholas, 2003). Oomycetan fungi have very low concentrations of DHA and therefore cannot be a commercial source of DHA (Singh and Ward, 1997; Ratledge and Wynn, 2002; Saito *et al.*, 2002). The potential commercial sources of DHA are tabulated in Table 1.2. Apart from fish oils, DHA is commercially produced from;

1. *Crypthecodinium cohnii*, a heterotrophic marine dinoflagellate: This can produce high percentages of DHA (25-60 %), whereas other PUFAs represent less than 1 % of the derived oil. In early 1990's MartekBiosciences Corp. commercially produced oil rich in DHA from a strain of this organism (Sijtsma and Swaaf, 2004). This product is called DHASCO and 180 tons were produced in the year 2002 (Ratledge, 2004).

Common name	Systemic name*	Short name
Saturated fatty acids		
Lauric acid	Dodecanoic acid	12:0
Myristic acid	Tetradecanoic acid	14:0
Palmitic acid	Hexadecanoic acid	16:0
Stearic acid	Octadecanoic acid	18:0
Monounsaturated fatty acids		
Palmitoleic acid	Δ9-Hexadecenoic acid	16:1
Oleic acid	Δ9-Octadecenoic acid	18:1
ω-6 Polyunsaturated fatty acids		
Linoleic acid (LA)	Δ9, Δ12-Octadecadienoic acid	ω-6 18:2
γ-Linolenic acid (GLA)	Δ6, Δ9, Δ12-Octadecatrienoic acid	ω-6 18:3
Arachidonic acid (ARA)	Δ5, Δ8, Δ11, Δ14-Eicosatetraenoic acid	ω-6 20:4
ω-3 Polyunsaturated fatty acids		
α-Linolenic acid (LNA)	Δ9, Δ12, Δ15-Octadecatrienoic acid	ω-3 18:3
Eicosapentaenoic acid (EPA)	Δ5, Δ8, Δ11, Δ14, Δ17-Eicosapentaenoic acid	ω-3 20:5
Docosahexaenoic acid (DHA)	Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-Docosahexaenoic acid	ω-3 22:6

*All double bonds are in *cis*-configuration

Table 1.1: Some naturally occurring fatty acids.

2. The thraustochytrid, *Schizochytrium* sp., produces about 40 % of their biomass as oil with their major PUFA being DHA. About a decade ago, Barclay of Omega Tech, (recently acquired by Martek Biosciences, Columbia, Md., USA) developed a process in which, it was used as aquaculture feed, fortified eggs and poultry feed with DHA and as nutraceuticals (Barclay, 1994a; Barclay, 1997; Ratledge, 2004).

1.2.3. Biosynthesis of DHA

Two key enzymes, ATP: citrate lyase and malic enzyme are involved in lipid accumulation in oleaginous fungi (Ratledge and Wynn, 2002; Ratledge, 2004). Malic enzyme generates the NADPH by which the acetyl units can be reduced and used as the backbone of the fatty acids. The site of biosynthesis of fatty acids is mainly in the cytoplasm outside the mitochondria. However most of the acety-CoA is derived from the oxidation of pyruvate in mitochondria and mitochondrial membrane is relatively permeable to acetyl-CoA. Therefore oleaginous eukaryotes, under nitrogen limitation accumulate citrate in the mitochondria, which is then transported into the cytoplasm and cleaved there by ATP-citrate lyase (Song *et al.*, 2001; Wynn *et al.* a and b, 1999; Wynn *et al* ., 2000; Wynn *et al.*, 2001).

Fatty acids are synthesized from acetyl-CoA by concerted action of two complex enzyme systems: acetyl-CoA carboxylase and fatty acid synthetase. This involves a series of condensation-reduction-dehydration-reduction reactions that results in lengthening of the acyl chain by 2 carbon atom and the formation

of even numbered fatty acids. Malonyl CoA supplies all the carbon atoms of the long chain with exception of the two methyl terminal carbons, which are supplied by acetyl CoA (Singh and Ward, 1997; Fidley *et al.*, 1999; Ratledge and Wynn, 2002).

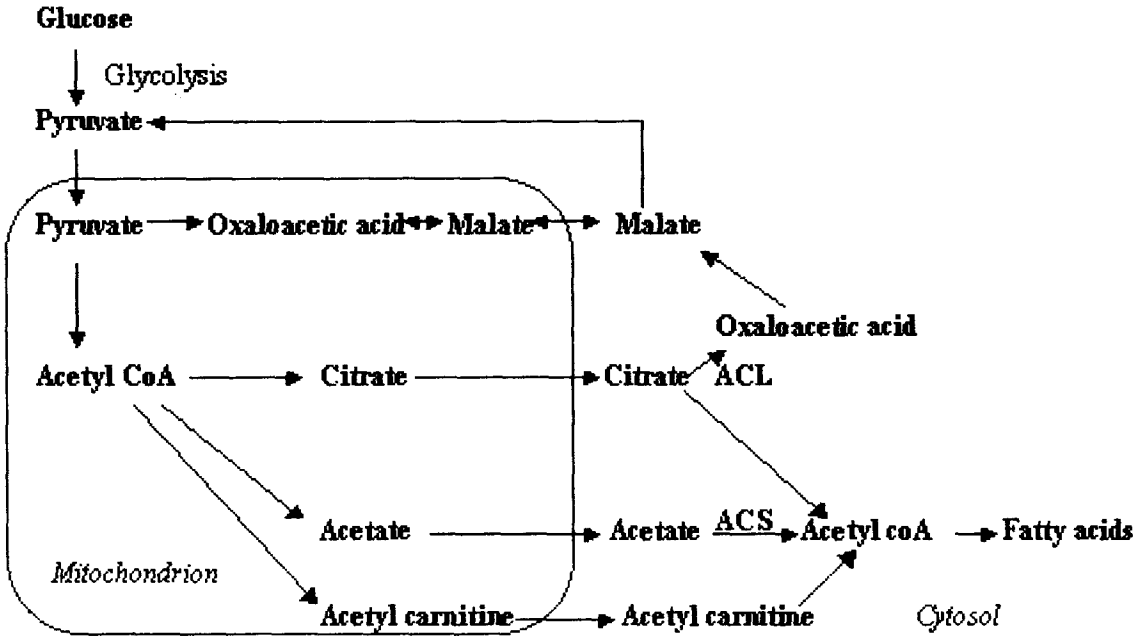


Fig. 1.5.: Acetyl-CoA metabolism in oleaginous yeasts (modified from Ratledge and Evans 1989). ACL=ATP:citrate lyase, ACS=acetyl-CoA synthetase

Sources	Advantages	Disadvantages
Fish oils from fishes such as herring, mackerel, sardine, salmon and tuna.	Contain up to 30 % of PUFA	Oil quality variable and depends on species, geographical location and season. Have strong fishy odour and taste. Contain up to 80 % of saturated fatty acids. Complex fatty acid profile and therefore, expensive DHA purification. Oil could be contaminated by environmental pollutants. ω- 3 fatty acids lost during processing Have high levels of fat soluble vitamins, which are retained in the human body and can lead to kidney problems.
Micro algae belonging to Dinophyceae, Haptophyceae, Cryptophyceae, Chrysophyceae, Xanthophyceae, Eustigmatophyceae, Chlorophyceae, Rhodophyceae, Bacillariophyceae and Prasinophyceae	Contain up to 35 % of PUFA	Phototropic and traditionally cultivated in open ponds, and therefore, production systems are not axenic. DHA is mostly as phospholipids. Triacyglycerols contain lower concentrations of ω- 3 PUFAs. Economically not viable since, require large surface areas. Complex fatty acid profile and therefore high cost of purification. Light penetration, a problem in large photobioreactors.
Thraustochytrids	Accumulate up to 70% of weight as lipids, of which 50 % can be DHA Over 90 % of DHA is present as neutral lipids Optimal and axenic conditions can be maintained Oil production can be carried out throughout the year. High cell densities can be achieved	Could contain other fatty acids, effects of which on human health are not known.

Table 1.2: Potential commercial sources of DHA.

Unsaturated fatty acids are synthesized by two mechanisms as shown in Fig. 1.5.

a) Aerobic Pathway: This pathway requires molecular oxygen as a cofactor and occurs in animals and some lower eukaryotes such as thraustochytrids. Thraustochytrids contain Δ4 desaturases, unlike mammals, which introduces a Δ4 double bond into 22:5, resulting in the production of DHA (Singh and ward, 1997; Wallis *et al.*, 2002; Metz *et al.*, 2001).

b) Anaerobic polyketide synthase pathway: This pathway occurs in microorganisms including bacteria and some thraustochytrid species, such as those of *Schizochytrium*. This pathway differs from the elongation / desaturation pathway in that it does not require aerobic desaturation for introducing double bonds into the existing acyl chain. Instead the double bonds are introduced during the process of fatty acid synthesis and therefore is anaerobic in nature. However, it can also take place under aerobic conditions. Polyketides are secondary metabolites containing multiple building blocks of ketide group ($-CH_2-CO-$) and are synthesized by polyketide synthase (PKS). It uses acyl carrier protein (ACP) as a covalent attachment for chain synthesis proceeding with reiterative cycles. Although the exact mechanisms underlying DHA biosynthesis by this new pathway is not clear, the likely key features include the condensation and isomerisation step. Ketosynthase, the key enzyme in the pathway, can catalyze the condensation of a wide range of substrates including unsaturated fatty acids. Thus, selective condensation of enoyl-ACP with malonyl-ACP would result in fatty

acids with double bonds. An isomerase activity, another key enzyme for DHA biosynthesis occurring either associated with dehydratase or separately would warrant precise location and stereospecific configuration of double bonds in the PUFAs (Metz, 2001; Wallis *et al.*, 2002; Qui, 2003).

Organic carbon nutrients are the likely sources of reserve lipids in thraustochytrids, as in any other heterotroph. However such nutrients may also be shunted into other metabolic pathways leading to the formation of other compounds such as extracellular polysaccharides (EPS). Therefore, any study on the production of lipids should also address other possible utilization of organic carbon. Although EPS have not been reported in thraustochytrids, it was considered important to address this aspect in view of the above and also since EPS are biotechnologically important.

1.3. Extracellular polymeric substances

Extracellular polysaccharides, more appropriately called extracellular polymeric substances in recent years, are organic, hydrated, extracellular polymers produced by numerous organisms (Gutierrez *et al.*, 1996). The production of EPS is a general property of microorganisms in natural environments and occurs both in prokaryotes (Archeabacteria and Eubacteria) and in eukaryotes, such as algae, fungi, phytoplankton and macroalgae (Phillips and Vincenzini, 1998). Microbial EPS may occur as capsules, which are firmly associated with the cell surface or as slime, which are loosely bound to the cells

and are easily dissociated in the surrounding medium (Sutherland, 1982; Decho, 1990). Capsules form discrete extracellular structures and are bound to the cell wall by linkages, whereas slime layers are less organized structures. Sheaths are a form of capsular EPS, which envelops a group of cells (Whitefield, 1988). The extracellular localization of EPS and their composition may be due to active secretion, shedding of the cell surface material, cell lysis or adsorption from the environment (Wingender *et al.*, 1999). Most EPS have high molecular weights and can range from 10^3 Da to 10^8 Da. The sizes of these polysaccharides can vary greatly between and within strains. Some strains produce different polysaccharides in different phases of growth (Sutherland, 1985).

1.3.1. Chemistry of EPS

Microbial EPS consist of a polysaccharide backbone. Some examples of EPS produced by different groups of organisms are presented in Table 1.3. Depending on the monomeric sugar composition, EPS can be classified into two groups. The homopolysaccharides are composed of a single type of sugar, while the heteropolysaccharides are composed of repeating units of different sugars. Examples of homopolymers are bacterial cellulose, levans, dextrans or some glucans. Heteropolymers like alginates, carragenanes and agar, produced by macroalgae, are more common under natural conditions (Sutherland, 1982; Whitefield, 1988). However EPS can often contain a wide variety of non-carbohydrate components such as amino acids, amino sugars, proteins, lipids, uronic acids, sulphates, pyruvates, acyl groups. These components are mostly in

the form of residues and side groups. The presence of these substituents greatly alters the tertiary structure, physiological and biological properties of these biopolymers (Majumdar *et al.*, 1999).

1.3.2. Importance of EPS to the cell

The production of EPS involves a significant investment of carbon and energy by microorganisms. Nature has a tendency to conserve rather than to waste energy. Therefore, this expenditure of energy is likely to be beneficial to the producer of EPS or organisms associated with it (Wolfaardt *et al.*, 1999). The physiological and ecological importance of EPS for organisms that produce them has been reviewed by Decho (1990). Production of EPS under natural conditions helps them in better survival (Costerton, 1974). They create a microenvironment around the cell, which allows it to metabolize, grow and reproduce more efficiently. Their presence renders protection to the cells against quick ionic and environmental changes such as pH, salinity, desiccation, or nutrient regimes. These secretions help in the attachment of the microbial cells to surfaces. Exopolymers sequester and concentrate nutrients and are believed to maintain the activity of exoenzymes. They also provide protection against heavy metals and other toxins (Decho, 1990).

1.3.3. Importance of EPS to the ecosystem

EPS produced by marine organisms also have a role in ecologic

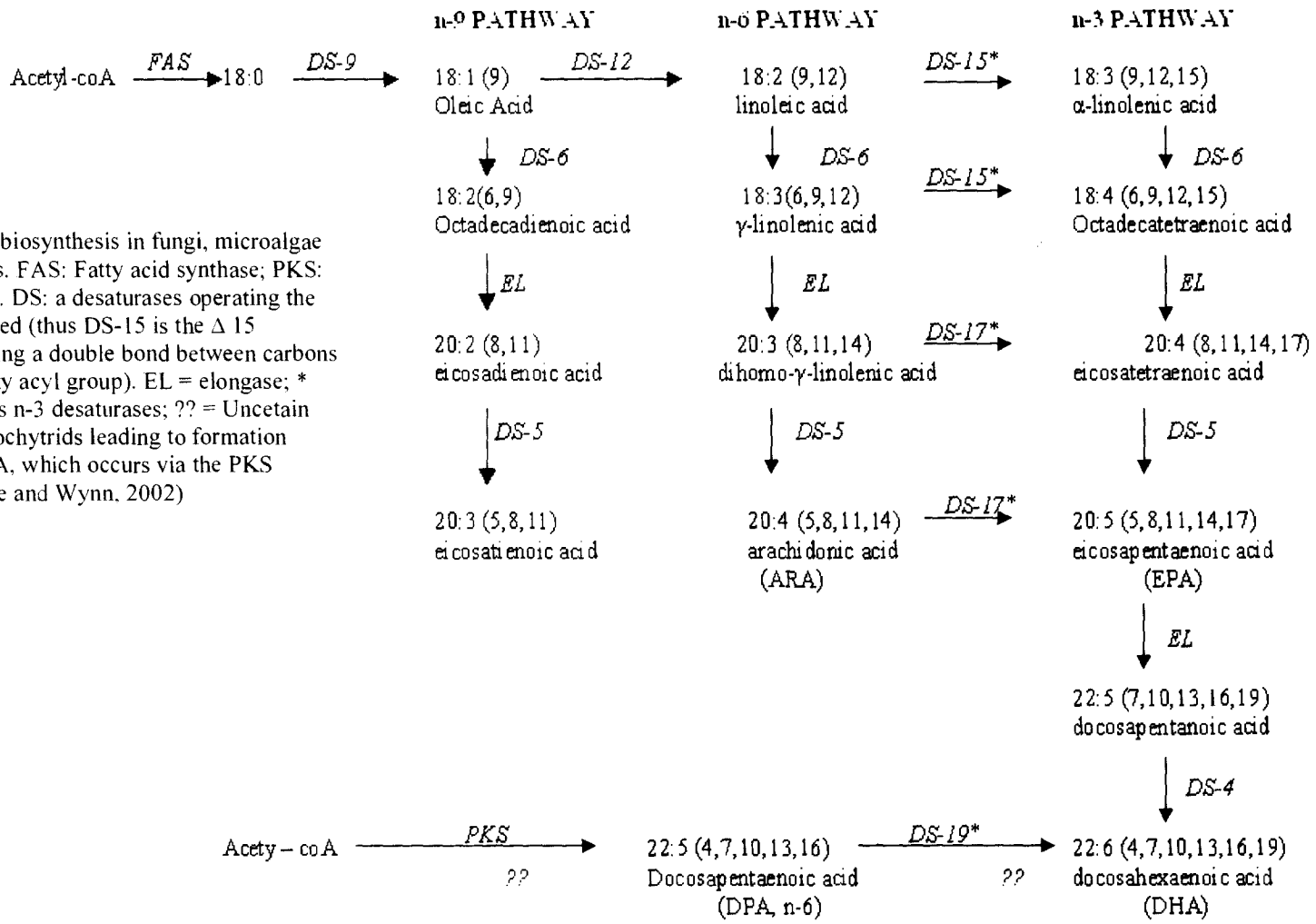


Fig. 1.6.: Routes of PUFA biosynthesis in fungi, microalgae and thraustochytrids. FAS: Fatty acid synthase; PKS: polyketide synthase. DS: a desaturases operating the carbon atom indicated (thus DS-15 is the Δ 15 desaturase introducing a double bond between carbons 15 and 16 in the fatty acyl group). EL = elongase; * could be regarded as n-3 desaturases; ?? = Uncetain sequence in thraustochytrids leading to formation DPA (n-6) and DHA, which occurs via the PKS pathway. (Rathledge and Wynn, 2002)

processes (Decho, 1990). In the pelagic water column, EPS from phytoplankton help in sedimenting phytoplankton blooms and other types of marine snow and play a major role in aggregate formation. These aggregates help the organisms to gather and concentrate nutrients. They also influence the transfer of heavy metals and other toxic compounds from water column to sediments through the food web. Extracellular polysaccharides play a major role in biofilm formation and biofouling and in the localization of microbiogeochemical processes within aggregates and sediments. Exopolymers play an important role in mediating adhesion of bacteria and other microorganisms to surfaces. In biofilm formation, initial attachment may occur without the requirement for the production of new EPS. EPS are also involved in maintaining the structural integrity of the biofilm (Costerton *et al.*, 1981). In the benthic system, they help in sediment binding and stabilization and regulate the attachment of certain invertebrate larvae to surfaces. Settlement and metamorphosis of certain marine larvae are closely linked to specific biofilms. These larvae bind to the exopolymers using highly specific lectin binding mechanisms. Lectins are proteins which bind only to a specific arrangement of sugars within a polysaccharide (Sharon, 1977). Once the larvae have settled, secretion of proteinaceous cement by the larva occurs. They also serve as food for meiofauna, such as harpacticoid copepods, nematodes, turbellarians and nauplii of various animals (Bhosle *et al.*, 1995, Decho., 1990).

1.3.4. Biotechnological implications of EPS

EPS produced by different organisms have been explored for various biotechnological applications (Table 1.3.), such as antitumor agents, anticoagulant agents (heparin analogues), wound dressings and for eye and joint surgery. Apart from medical applications, EPS are also important as emulsion stabilizers (in food and thixotrophic paints), flocculants (in water clarification and ore extraction), foam stabilizers (in beer and fire fighting fluids), gelling agents (in cell and enzyme technology and foods), hydrating agents (in cosmetics and pharmaceuticals) and as inhibitors of crystal formation in frozen foods and sugar syrups (Colwell *et al.*, 1986, Sogawa *et al.*, 1998, Sutherland, 1998). It is likely that hitherto unexplored groups of marine microorganisms produce novel and useful EPS.

Properties	Organism	Polymer	Use
Biological	*Acetobacter xylinum*	B-D-Glucans	Anitumor agents
	Streptococcus equi, S. zooepidemicus	Hyaluronic acid	Eye and joint surgery
	Escherichia coli K5	Desulfatoheparin	Heparin analogues
	Acetobacter xylinum	Bacterial cellulose	Wound dressing
Chemical			
	Escherichia coli K5, *E.coli* K4	Escherichia coli K4 & K5 EPS	Enzyme substrates
	Agrobacterium, Aureobasidium pullulans, Sclerotium rolfsii	Curdlan, pullulan, scleroglucan	Oligosaccharide preparation
Physical			
Emulsion stabilization	*Xanthomonas campestris*	Xanthan	Foods, thixotropic paints
Fibre strength	*A. xylinum*	Bacterial cellulose	Acoustic membranes
Film formation	*A.pullulans*	Pullulan	Food coating
Flocculant		Various	Water clarification, ore extraction
Foam stabilization	*X. campestris*	Xanthan	Beer, fire fighting fluids
Gelling agents	*Sphingomonas paucimobilis*	Gellan	Cell and enzyme technology
Hydrating agents	Agrobacterium, *S.paucimobilis*	Curdlan, gellan	Foods
Inhibitor of crystal formation	*Agrobacterium, X.campestris*	Curdlan, xanthan	Oil recovery (blockage of permeable zones)
Shear thinning and viscosity control	*S. equi, S. zooepidemicus*	Hyaluronic acid	Cosmetics, pharmaceuticals
Suspending agent	*X.campestris*	Xanthan	Frozen foods, pastilles and sugar syrups
Viscosity control	*X.campestris*	Xanthan	Oil-drilling 'muds'
	X.campestris	Xanthan	Food
	X.campestris	Xanthan	Agrochemical pesticides and sprays
	X.campestris	Xanthan	Jet printing

Table1. 3. Established applications of microbial exopolysaccharides

Studies on EPS produced by thraustochytrids assume importance in view of the above. The following objectives were set for this thesis in view of the above,

1.4. Objectives of the study

- Screen thraustochytrids for biomass, lipid and PUFA production, optimize media and develop methods for enhancing DHA production.
- Carry out experimental studies to understand the physiological role of DHA production.
- Estimate production of EPS in thraustochytrids and carry out preliminary chemical characterization of the EPS.
- Elucidate the ecological significance of EPS production to thraustochytrids.
- Identify some of the isolates using morphological and molecular methods.

CHAPTER 2

Screening, media optimization and enhancement of PUFA production by thraustochytrids

2.1 Introduction

The present sources of polyunsaturated fatty acids (PUFAs) are the evening primrose plant *Oenothera biennis*, and the borage plant *Hippobroma longiflora* for γ-linolenic acid (GLA) and fish oil for docosahexaenoic acid (DHA) (Ratledge, 2004). It is expected that the production of PUFAs from the current sources will become inadequate for supplying the expanding markets of aquaculture and nutraceutical industries (Lewis *et al.*, 1999). Microbial oils or single cell oils (SCO) may be increasingly used in the near future for several reasons. Microbial oils may have a high PUFA content with a high oxidative stability. They may be produced from sustainable raw materials. Further, knowledge on the biochemical pathways and genetics may provide tools for the development of new, interesting production systems or products. (Ratledge, 2004; Sijtsma and Swaaf, 2004).

The present microbial sources of individual PUFAs are *Mortierella alpina* for arachidonic acid (ARA) and *Crypthecodinium cohnii* and *Schizochytrium* sp. for DHA. Individual PUFAs such as DHA from microbial sources as food supplements might be expected to have a large demand in future. In order to develop commercially viable PUFA production, proper selection of the candidate organism and controlled production methods are essential (Sijsima and Swaaf, 2004).

Thraustochytrids contain many PUFAs, including ARA, EPA, DPA and DHA. However, DHA concentrations far exceed that of others in thraustochytrids. As DHA is important in nutraceuticals and pharmaceuticals, thraustochytrids

have attracted a lot of attention in the past decade or so (Gunstone, 1999; Lewis *et al.*, 1999; Sijtsma and Swaaf, 2004). Thraustochytrids are now increasingly becoming the organisms of choice for DHA production. There are two ways of achieving commercial production of useful compounds. One is to obtain superior and mutant strains. The second method is to optimize nutritional conditions and the physical environment in which the organism grows (Greasham and Inamine, 1986). In order to develop culture media for economic production of useful compounds it is important to select suitable sources of carbon, nitrogen, minerals and vitamins. It is also crucial to optimize other growth parameters such as pH and temperature. In the case of marine organisms such as thraustochytrids it is also necessary to optimize salinity conditions.

The important parameters to be considered to select appropriate strains of thraustochytrids for DHA production include the growth rate, biomass production under optimal culture condition, total lipid content and DHA proportion of the lipid (Lewis *et al.*, 1999). Furthermore, for downstream processing, it is important to know whether DHA is present as part of the membrane structure (phospholipids), or as part of triacylglycerols in the cytosol and whether or not other PUFAs are present (Ratledge, 2004). For maximum production of DHA production by thraustochytrids suitable media have to be designed, which can be based upon available literature on thraustochytrid nutrition and physiology.

Growth and physiology of thraustochytrids were first studied in detail by Goldstein (1973). Further, exhaustive work was carried out by Bahnweg (1979 a and b). These studied were purely based on growth and physiology. The

presence of DHA in thraustochytrids was discovered in the 1969 by Ellenbogen *et al.* Findlay *et al.* (1986) used this PUFA as a signature molecule in understanding the role of thraustochytrids in decomposition of mangrove detritus. DHA production in thraustochytrids was not studied from a biotechnology perspective till 1994, when Barclay (1994 b) carried out the first study on growth in relation to DHA production.

2.1.1. Factors influencing growth and DHA production

2.1.1.1. Carbon source

Thraustochytrids are known to utilize a wide variety of carbon sources including pentoses, hexoses, di- and oligosaccharides, polysaccharides, sugar alcohol and glycosides, alcohols and organic acids (Bahnweg, 1979 a). Goldstein (1973) showed that D-glucose and its di-, oligo and polysaccharides support good growth. Bahnweg (1979 a) concluded that apart from glucose and its polymers, glycerol and laminarin in general served as good cabon sources. Other sugars, glycosides and organic acids were utilized to a lesser extent. Barclay (1994 b) also showed that glucose was comparatively a good substrate for DHA production. Yokochi *et al.* (1998) showed that the *Schizochytrium* strain SR21 grew well on glucose, fructose and glycerol.

2.1.1.2. Nitrogen Source

Organic sources of nitrogen like peptone, yeast extract, sodium glutamate support good growth of thraustochytrids (Bahnweg, 1979 b). Thraustochytrids are

not denitrifiers and cannot readily utilize inorganic nitrogen compounds such as KNO_3, $NaNO_3$ and urea. Goldstein (1973) demonstrated that, in general, thraustochytrids were not able to readily utilize ammonium salts as sole source of nitrogen but grew well on organic sources of nitrogen, particularly glutamate. Bahnweg (1979 b) in his studies showed that amino acids such as proline, arginine, leucine, isoleucine, glutamine, and aspartate could be readily utilised by most thraustochytrids. Yokochi *et al.* (1998) showed that corn steep liquor was a better nitrogen source for DHA production than yeast extract.

2.1.1.3. Minerals, Vitamins and Phosphates

Adair and Vishnaic (1958) demonstrated for the first time the requirement of vitamins by thraustochytrids. Vitamins such as B_1, B_2, B_6 and B_{12} are known to be essential for the growth of thraustochytrids. Goldstein (1973) and Bahnweg (1979 b) showed the requirement of trace metals and phosphate for growth of these organisms. Additional supplementation of media with various minerals, vitamins and phosphates are known to stimulate growth and DHA content of thraustochytrids (Barclay, 1994 b; Singh and Ward, 1997). Various minerals serve as cofactors in the DHA synthesis, either activating the enzyme or increasing its activity. Phosphate has also been shown to increase DHA content (Barclay, 1994 b).

2.1.1.4. Salinity

Seawater has an average salinity of about 34 ppt (Lalli and Parsons, 1997). Microorganisms isolated from a marine environment are frequently characterized by salinity optima that approximate the conditions of their natural habitats. Thus, natural seawater is necessary in cultivation of marine organisms, unless the basal medium contains sufficient amount of metal salts. Thraustochytrids are not found in salinities less than 10 ppt but above these values of salinity they are eury- or stenohaline depending on the species (Jones and Harrison, 1976). Thraustochytrids have an essential requirement for Na^+ ions, which cannot be substituted by either K^+ ions or mannitol. (Goldstein, 1973; Amon, 1978; Wethered and Jennings; 1985). However, optimal salinity levels may vary for individual thraustochytrids

2.1.1.5. pH

Seawater has an average pH of 8.2 to 8.5 (Lalli and Parsons; 1997). Thraustochytrids can grow and multiply in a wide range of pH from 4 – 9 (Jones and Harrison, 1976). However, high biomass is normally achieved in the pH the range of 6-8 (Singh and Ward, 1997). Iida *et al.* (1996) have shown that DHA production also occurs at pH 5.5.

2.1.1.6. Temperature

In general thraustochytrids grow slowly at 4 °C, optimally between, 24 –28 °C and reduced growth occurs at temperatures above 30 °C (Goldstein, 1973).

However, psychrophilic thraustochytrids have been described in literature. Bahnweg and Sparrow (1974 b) described *Thraustochytrium antarcticum* from Antarctic waters, which had a temperature optimum of 4 °C DHA production is known to be higher at lower temperatures (Barclay, 1994 b). Bowles *et al.* (1999) concluded that tropical strains of thraustochytrids produced more biomass (at 25 °C) but have a lower DHA content, whereas cold water isolates produced lesser biomass under same conditions but high DHA content.

2.1.2. Use of Response Surface Methodology (RSM) for media optimization

Two methods are used for optimization of media – the conventional method and the Response Surface Methodology (RSM). The conventional method of optimization uses the 'one factor at a time' approach (OFAT), wherein each growth parameter is optimized separately and a final combination of optimal levels of the various parameters is used. A major drawback of this method is that it may not consider the possible interaction between the input variables. An alternative approach, the RSM, is a set of statistical techniques that allows us to estimate interaction and even quadratic effects (second order), and therefore find optimal process settings (Montgomery, 1977; www.itl.nist.gov/div898/handbook/pri/section3/pri3362.htm). Response Surface Methodology was described about 5 decades ago by Box and Wilson (1951). When the experiment design consists of 2-4 factors, an experimental design called the Box and Wilson central composite design is used.

When three growth variables are used, the three factors in the composite design can be presented as the 3 axis of a cube (X_1, X_2 and X_3) (Fig. 2.1.1). The optimal levels of the three factors for an independent variable, for example growth, will lie anywhere within the cube. One end of each axis represents the high level (+1), other as low level (-1) and the center of the axis is presented as central level (0) of variable. According to the central composite design, complete randomization can be achieved by assuming all the levels as equally important and the central value as the optimum for maximum and minimum of response. Boundary limits of the design, in which a sphere is fitted over the cube, are considered as 2 limits ($-\alpha$ and $+\alpha$). All the 5 limits of the three variables are presented in Table 2.1.1. The value of alpha (distance from the edge of the cube to the border of the sphere) for a 3 variable CCD design is 0.68. This requires the minimum of 8 experiments described as 8 corner points (high – low level combination), 6 face / star points (boundary limits) and one center point (optimum level, 5 replicates) of the cube. Thus, a total of 15 experiments are performed (Table 2.1.2). To achieve the goal of regression and estimation of error within experiments the center point is repeated 5 times to give a total of 20 trials. A quadratic model assumed to describe, the relationship between the response (Y) and the experimental factors $(X_1 + X_2 + X_3)^2$ is

$$= \beta o$$

$$+ \beta_1 * X_1$$

$$+ \beta_2 * X_2$$

$$+ \beta_3 * X_3$$

$+\beta_{11} * X_1^2$

$+\beta_{22} * X_2^2$

$+\beta_{33} * X_3^2$

$+\beta_{12} * X_1 * X_2$

$+\beta_{13} * X_1 * X_3$

$+\beta_{23} * X_2 * X_3$

where βo = constant co-efficient

$\beta_1, \beta_2, \beta_3$ = linear co-efficients

$\beta_{11}, \beta_{22}, \beta_{33}$ = quadratic co-efficients

$\beta_{12}, \beta_{13}, \beta_{23}$ = second order interaction co-efficients.

Variable	Levels				
	Star	Low	Center	High	Star
X_1	-α	-1	0	+1	+α
X_2	-α	-1	0	+1	+α
X_3	-α	-1	0	+1	+α

Table 2.1.1: Different levels of three variables used for full factorial composite design

Experiment No.	Variable		
	X1	X2	X3
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	-1	+1	+1
7	+1	-1	+1
8	+1	+1	+1
9	-α	0	0
10	+α	0	0
11	0	-α	0
12	0	+α	0
13	0	0	-α
14	0	0	+α
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table 2.1.2: Full factorial design for 15 experiments plus 5 experiments of repetition of central points.

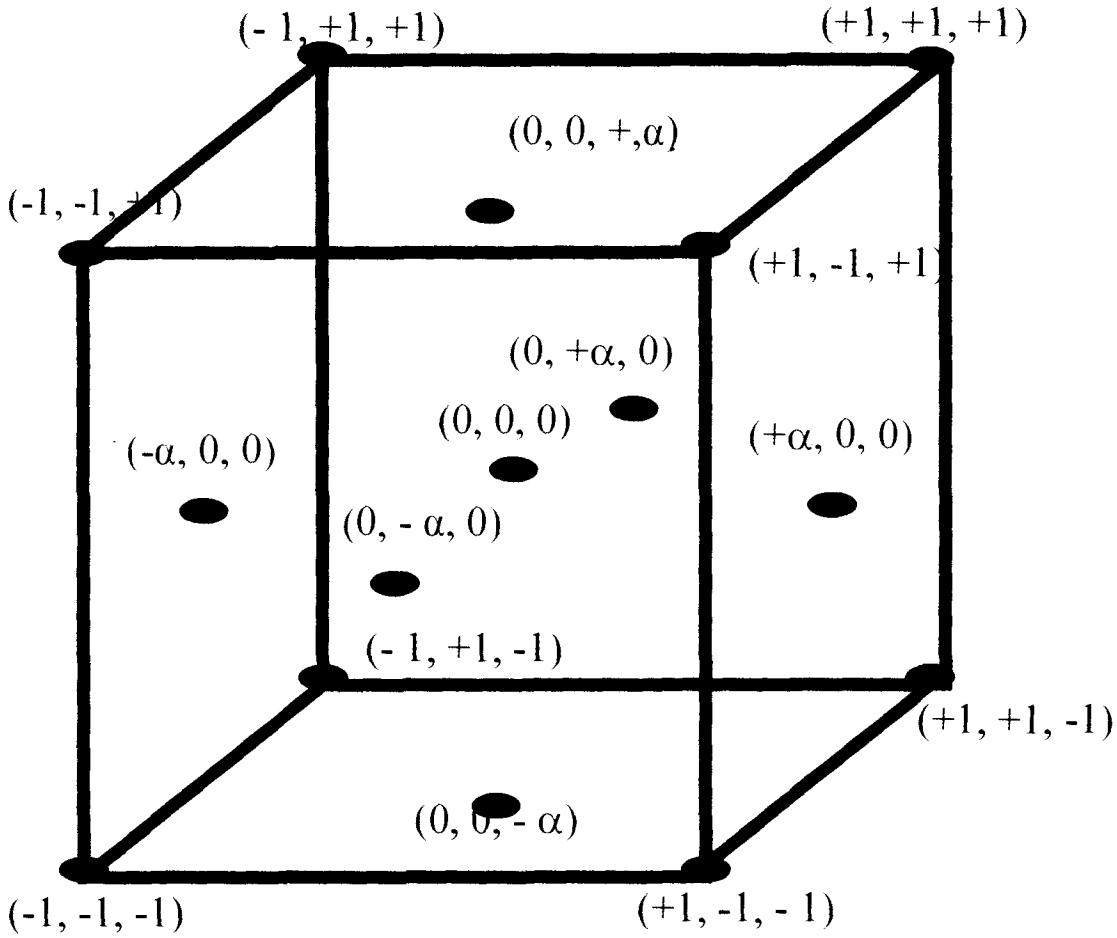


Fig. 2.1.1: Central composite design with three variables.

2.2. Materials and Methods

2.2.1. Cultures and maintenance

A total of 12 cultures were used in the present study. The sources of isolation are given in Table 2.2.1 and the method of isolation in Fig. 2.2.1 The method of pine pollen baiting was used for isolation of thraustochytrids and these were brought into axenic cultures using antibiotics, following the methods given by Porter (1990) and Raghukumar (1988) (Fig. 2.2.1). The composition of the MV medium used for isolation and maintenance of cultures is given in Appendix 1 (Porter, 1990).

2.2.2. Preparation of inocula for experiments and estimation of biomass production, total lipids and PUFA contents

Inocula for experiments were raised as follows. Cultures from a stock culture tube were inoculated onto M3 medium broth (Appendix 1) at a room temperature of ~ 28 °C on a shaker at 150 rpm. After 5 days, a 5 % inoculum was transferred to 100 ml of M3 broth. After a further 2 days, a 5 % of this was transferred similarly. A two day old culture of this was used as inoculum. A 5 % inoculum was used and the experimental flasks were incubated at a room temperature of ~28 °C for the required number of days under shaken conditions at 150 rpm.

Biomass of thraustochytrids produced under various experimental conditions was estimated either as optical density (OD) at 660nm, wet weight or dry weight. For gravimetric estimation of biomass, cells were harvested from the

medium by centrifugation at 7500 rpm (5200 g) for 15 min in pre-weighed 50 ml tubes. The wet weight was then estimated gravimetrically. Dry weight was estimated either by drying the wet biomass at 50 °C for 5 d or by freeze drying, using a Thermo Savant 'Micro Modulyo' lyophilizer. Wherever the samples had to be subjected to fatty acid analyses, drying was carried out using lyophilization, after acidification by the addition of 0.5 M ascorbic acid as an antioxidant (typically, 250 µl to ~ 2 g wet biomass) (Barclay, 1994 b).

Total lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959; Parsons *et al.,* 1984). A known amount of dry biomass (oven-dried or lyophilized) was taken in a pre-weighed tube, to which 0.8 ml of distilled water was added and ultrasonicated in an ice bath for a pulse of 40 sec at 50 W with a pause of 20 sec, using a Vibra cell, USA, ultrasonicator. Ten such pulses were given. Two milliliter of methanol and 1 ml of chloroform were then added and vortexed for 5 min. Further 2 ml of chloroform was added and the lower chloroform layer was collected in a preweighed tube. The chloroform was evaporated under a jet of nitrogen and the percent lipid was estimated gravimetrically.

PUFA analyses were carried out on acidified, lyophilized dry weight biomass as above, following the method of Morrison and Smith (1964). Lipids were extracted from the biomass as given above. A volume of 20 µl of arachidic acid (100 µg ml^{-1}) was added as an internal standard to the total extracted lipids prior to analyses. The lipids were then saponified with 1 ml, 0.5 M KOH in methanol at 65 °C for 1 h. The unsaponified lipids were removed by adding 2 ml

ml of hexane. The lower aqueous phase was discarded and 0.7 M HCl in methanol was added to the lower layer and mixed. To this 2 ml hexane was added and centrifuged at 1000 rpm for 10 min. This was repeated thrice. All the hexane phases were pooled together and the hexane was evaporated under a jet of nitrogen. Benzene (0.2 ml) and methanolic boron trifluoride (0.5 ml) were added and incubated at 65 °C for 45 min. The solution was cooled and 1 ml of distilled water was added to it. This was extracted twice with hexane. Hexane was again evaporated under a jet of nitrogen and the fatty acid methyl esters were reconstituted in 100 µl of benzene.

Analysis of fatty acid methyl esters was carried out using a gas chromatograph (Fisons instrument, GC 8000 series) fitted with flame ionization detector. A BP-21 (SGE, Australia) fused silica capillary column (25 m X 0.25 mm) was used for analysis. The injector and detector port temperature were maintained at 220, 230 and 250 °C respectively. Nitrogen was used as the carrier gas at the flow rate of 1.0 ml min^{-1}. Fatty acids were identified by comparing retention time of standards and were quantified by online Chromapack CR 6A integrator. Some of the analyses were carried out commercially by Microbial ID, Inc, Newark, USA, while some were kindly carried out by Dr. Toro Nakahara, National institute of Bioscience and Human Technology, Tsukuba, Japan.

2.2.2. Screening of thraustochytrids for biomass, lipids and PUFA production

Isolates NIOS-1, NIOS-2, NIOS-4, NIOS-6, NIOS-7 and NIOS-8 were screened using a medium designated as M3 in this study, the composition of which is given in Appendix 1. Inocula were prepared as given above. Cultures were grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28 °C. Cells were harvested and the wet and dry weight biomass, total lipids and PUFA profiles analyzed as given earlier.

2.2.4. Initial selection of a culture medium for biomass production by NIOS-1

Five different media from literature were tested for biomass production by NIOS-1 (Table 2.2.2.). The experiments were setup in triplicates. Inoculum preparation was carried as mentioned earlier. Cultures were grown at a room temperature of ~ 28 °C on a shaker at 150 rpm. Wet weight biomass produced using the different media was estimated.

2.2.5. Effect of vitamins and trace metals, carbon sources, pH, salinity and exogenous oils on biomass production by NIOS-1

The method followed for inoculum preparation and wet weight biomass estimation is given above. All experiments were set up in triplicates. M3 medium was used for experiments on vitamins and trace metals. The culture was grown

with various combinations of trace metals and vitamins. on a shaker at 150 rpm for 5 days at a room temperature of ~ 28 °C.

Experiments on pH were carried out using M4 medium (M3 medium without vitamins and trace metal solution) at pH 4, 5, 6, 7, 8 and 9. The pH of the autoclaved medium was 6.0. This was adjusted to the different pH values using 1 N NaOH or 1 N HCl. The culture was grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28 °C. The cells were harvested and the biomass was assessed in terms of dry weight and percent lipids as above.

Eight different salinities, namely 15, 20, 25, 30, 35, 40, 45 and 50 ppt were tested to see their effect on growth of NIOS-1. Water of different salinities was constituted by dissolving the appropriate amounts of crude, crystalline sea salt to distilled water, followed by filtration. Media ingredients were added to this. Cultures were grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28 °C. The cells were harvested as above and the biomass content was assessed in terms of wet weight.

The culture NIOS-1 was grown in M4 medium containing 10 different carbon sources at 2 % concentration. The carbon sources were: glucose, fructose, arabinose, galactose, xylose, cellobiose, sorbitol, glycerol, rhamnose and maltose. Cultures were grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28°C. The biomass was assessed in terms of OD at 660nm.

The effect of coconut and sunflower oils on growth and DHA production was studied by addition of 0.5 % oil directly to the medium. Cultures were grown

on a shaker at 150 rpm for 5 days at a room temperature of ~ 28°C. The biomass was assessed in terms of wet weight.

2.2.6. Optimization of concentrations of media ingredients in medium M4 for growth of NIOS-1

The effect of concentrations of the three major ingredients of the M4 medium, peptone, yeast extract and glucose was studied. Cultures were grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28 °C. Growth and glucose consumption were estimated every 24 h. At the end of the experiment, growth, wet weight biomass, dry weight biomass and lipids were estimated for all the experiments.

Growth was estimated based on optical densities at 660 nm and glucose by the dinitrosalicylic acid (DNSA) method (Plummer, 1994). Wet weight, dry weight biomass and percent lipids were estimated as given earlier.

The experiments were carried out as follows. Initially, peptone concentration was optimized between the range of 0.01 % to 0.5 %, keeping all other ingredients at the same level as given for medium M4 (see Appendix 1). Next, the best peptone level was retained and the yeast extract concentration was optimized between the range 0.01 to 1.5 %, keeping all other ingredients, the same as given for medium M4. Finally, the optimum peptone and yeast extract concentrations were retained and glucose concentration was optimized between the range 0.5 to 5 %, retaining the phosphate levels as in medium M4.

2.2.7. Response surface methodology to study growth and DHA production in NIOS-1

A full factorial central composite design (CCD) (Greasham and Inamine, 1986) was used to study the interaction between different levels of peptone, yeast extract and glucose in M4 medium on biomass accumulation, lipids and DHA production (Table 2.2.3.) as detailed under 'Introduction' (2.1), This uses 5 levels of each variable and the central level is assumed to be the optimum level (Table 2.2.3.). A minimum number of experiments for 3 factors are 15 and 5 replicates for the 15^{th} trial for central level was designed as in Table 2.2.4.

Inoculum preparation was carried out as given above. Cultures were grown on a shaker at 150 rpm for 5 days at a room temperature of ~ 28°C at the end of which cells were harvested and dry weight biomass, total lipids and DHA were estimated following methods given earlier. The results were subjected to a multiple regression analysis using the software Statistica version 5.0 to derive β values and statistically significant variables. Surface plots were generated using the same program for combination of factors.

2.2.8. Fed Batch culturing

A fed batch regimen was followed to increase the biomass content. Modified M4 medium (3 % glucose, 0.225 % peptone, 0.15 % yeast extract and 0.0375 % KH_2PO_4), 100 ml in a 250 ml Erlenmeyer flask was used. The fed batch was given at intervals of 48 and 96 h. The first addition was carried out by adding 2 ml of distilled water containing 50 % the entire medium ingredients and

second addition after 96 h, a total of 5 ml aliquot containing 150 % of the ingredients was added. The flasks were harvested at the end of 168 h. Biomass was estimated as wet weight and the DHA content was estimated commercially by MIDI as given earlier.

2.2.9. Effect of storing biomass at low temperatures on PUFA contents of thraustochytrids

M4 medium with a fed batch regimen as given in 2.2.8 was used for all the experiments. Cultures were grown on a shaker at 150 rpm for 7 days at a room temperature of ~ 28 °C. Inoculum preparation, harvesting of cells, and estimation of the PUFAs, EPA and DHA were carried out as given in 2.2.2.

2.2.9.1. Refrigeration of cell biomass

Experiments were carried out using the isolates NIOS-1, NIOS-2, NIO-4 and NIOS-9. The wet biomass obtained as above was estimated and then refrigerated at 10 °C for 48 h prior to estimation of total lipids, EPA and DHA. Biomass frozen immediately at – 20 °C in place of refrigeration served as controls.

2.2.9.2. Refrigeration of cell biomass at different temperatures

The isolate NIOS-1 was grown as above for 7 days. The total harvested wet biomass was estimated and then refrigerated at 5, 10, 15, or 20 °C for 48 h,

prior to estimation of total lipids and EPA and DHA. Biomass frozen at immediately – 20 °C in place of refrigeration served as controls.

2.2.9.3. Refrigeration of cell biomass for various time intervals

The isolate NIOS-1 was grown as above for 7 days. The total harvested wet biomass was estimated and then refrigerated at 10 °C for 6, 12, 24, 48 or 72 h, prior to estimation of total lipids and EPA and DHA. Biomass frozen at immediately – 20 °C in place of refrigeration served as controls.

2.2.10 Effect of increased viscosity of culture medium on PUFA contents of thraustochytrids

The effect of increased viscosity of the culture medium on EPA and DHA production was studied by incorporating polyvinyl pyrrolidone (PVP) in the medium. PVP is a water-soluble dextran of basic nature commonly used to increase fluid viscosity (Stecchini *et al.*, 2004).

Two experiments were carried out. (1) Isolate NIOS-1 was grown as batch cultures in M4 medium, containing additions of 0.1, 0.5 and 1.0 % PVP prior to various analyses of the biomass. (2) The isolates NIOS-1, NIOS-2, NIO-4 and NIOS-9 were grown in M4 medium with 0.5 % PVP as fed batch cultures as given above, prior to the analyses.

Cultures without PVP served as controls in both the experiments. Total harvested wet biomass was estimated at the end of 7 days, prior to estimation of total lipids, EPA and DHA.

2.2.11. The combined effect of increased viscosity of culture medium and storage of biomass at low temperatures on PUFA contents of thraustochytrids

The experiments were carried out using isolates NIOS-1, NIOS-2, NIO-4 and NIOS-9. The organisms were grown in M4 medium with 0.5 % PVP as fed batch cultures, as described in 2.2.8. Cultures were grown on a shaker at 150 rpm for 7 days at a room temperature of ~ 28°C. Inoculum preparation, harvesting of cells, and estimation of the PUFAs, EPA and were carried out as given in 2.2.2. The cells were harvested and further refrigerated for 48 h at 10 °C. The biomass thus obtained was analyzed for DHA and EPA content. Cultures without PVP and frozen immediately at – 20 °C, served as controls.

Isolate	Source	Identification
NIOS-1	Sediment samples from Cidade de Goa, Dona Paula, Goa, India	*Schizochytrium* sp.
NIOS-2	Provided by Dr. Lucia Bongiorni, Italy	*Thrasutochytrium* sp.
NIOS-4	Water sample from Dona Paula, jetty, Goa, India	*Schizochytrium* sp.
NIOS-6	Water sample from Chorao islands, Goa, India	*Thraustochytrium* sp.
NIOS-7	Water samples from Anjuna, Goa, India	*Thraustochytrium* sp.
NIOS-8	Fronds of the brown alga *Padina* from Cidade de Goa, Dona Paula, Goa, India	*Schizochytium* sp.
NIOS-9	Fronds of the brown alga, *Sargassum* alga from Dias beach, Dona Paula, Goa, India	*Aplanochytrium* sp.
NIOS-10	Water sample, Central Indian Ocean	*Thraustochytrium* sp.
NIOS-11	Sediment sample, Mangrove, Goa, India	*Ulkenia* sp.
NIOS-12	Provided by Dr. Lucia Bongiorni, Italy	*Thraustochytrium* sp.

Table 2.2.1: Isolates used in the study

Media Components	Source				
	M3	**Lida *et al*, (1996) M2**	**Yokochi *et al*, (1998) M4**	**Bajpai *et al*, (1991) M5**	**Barclay, (1994) M6**
Glucose	20 g	10 g	60g	20 g	5 g
Peptone	1.5 g	1 g	-	-	-
Yeast extract	1 g	1 g	2g	-	1 g
KH_2PO_4	0.25 g	-	4g	0.2 g	1 g
Vitamins	1 ml	1 ml	-	1 ml	1 ml
Trace elements	1 ml	1 ml	-	-	5 ml
Starch	-	-	-	-	-
$(NH_4)_2SO_4$	-	-	-	0.2 g	-
Na Glutamate	-	25 g	-	2 g	5 g
Distilled water	-	500 ml	500 ml	-	
Sea water	1000 ml	500 ml	500 ml	1000 ml	1000 ml
pH	7	5.5	7	7	7

Table 2.2.2: Media screened for high biomass production by NIOS-1

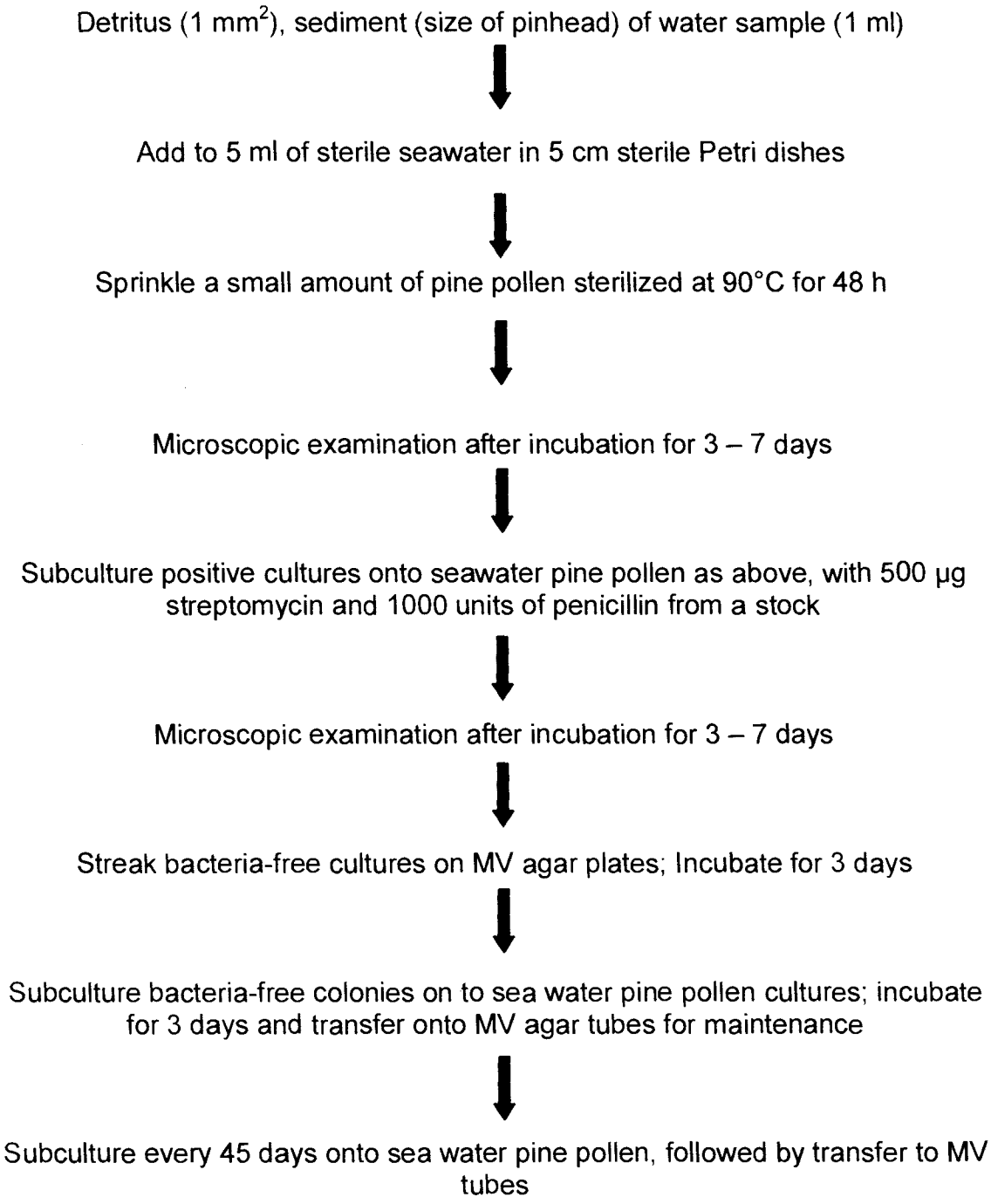


Fig 2.2.1: Isolation method for thraustochytrids

Variable	Concentrations (%)				
	-α	-1	0	+1	+α
Glucose	0.38	1	2	3	3.62
Peptone	0.057	0.15	0.3	0.45	0.543
Yeast Extract	0.007	0.1	0.25	0.4	0.493

Table 2.2.3: Variables and their different concentrations used to optimize the medium

Experiment No.	Variable					
	Conc	%	**Conc**	%	**Conc**	%
1	**-1**	1	**-1**	0.15	**-1**	0.1
2	**+1**	3	**-1**	0.15	**-1**	0.1
3	**-1**	1	**+1**	0.45	**-1**	0.1
4	**+1**	3	**+1**	0.45	**-1**	0.1
5	**-1**	1	**-1**	0.15	**+1**	0.4
6	**-1**	1	**+1**	0.45	**+1**	0.4
7	**+1**	3	**-1**	0.15	**+1**	0.4
8	**+1**	3	**+1**	0.45	**+1**	0.4
9	**-α**	0.38	**0**	0.3	**0**	0.25
10	**+α**	3.62	**0**	0.3	**0**	0.25
11	**0**	2	**-α**	0.057	**0**	0.25
12	**0**	2	**+α**	0.543	**0**	0.25
13	**0**	2	**0**	0.3	**-α**	0.088
14	**0**	2	**0**	0.3	**+α**	0.493
15	**0**	2	**0**	0.3	**0**	0.25
16	**0**	2	**0**	0.3	**0**	0.25
17	**0**	2	**0**	0.3	**0**	0.25
18	**0**	2	**0**	0.3	**0**	0.25
19	**0**	2	**0**	0.36	**0**	0.25
20	**0**	2	**0**	0.3	**0**	0.25

Table 2.2.4: Set of 20 trials with actual values of three variables in each experiment.

2.3. Results

2.3.1. Comparison of biomass production and fatty acid profiles in thraustochytrids

Six isolates, as described under Materials and Methods were used to screen for biomass production in this study (Fig. 2.3.1). The isolate NIOS-1 produced the maximum wet weight and dry weight biomass of 19.12 g L^{-1} and 6.58 g L^{-1} respectively. NIOS-8 produced the least wet weight biomass (3.66 g L^{-1}) and dry weight biomass (0.8 g L^{-1}). Dry weight biomass amounted to more than 34.4 % of the wet weight biomass in NIOS-1. Dry weight constituted 18.6 to 26.4 % of the total wet weight in the other isolates. The total lipid concentration in the dry weight biomass ranged from a maximum of 36 % in NIOS-1 to 7.5 % in NIOS-2 (Fig. 2.3.1).

The common saturated fatty acids seen in all the six isolates were myristic acid (14:0) and palmitic acid (16:0) (Table 2.3.1). Among the PUFAs, EPA and DHA were found in all the isolates. Another PUFA, the docosapentaenoic acid (22:5 ω3) occurred in 4 of the 6 isolates examined. Among all the fatty acids, palmitic acid had the highest concentration in 5 of the 6 isolates (25 – 60 %). The isolate NIOS-6 was an exception, where lauric acid (12:0) contributed up to 81 % of the total fatty acids. Percentage DHA among total fatty acids ranged from 2.397 % in NIOS-6 to 22.6 % in NIOS-1 (Table 2.3.1).

2.3.2. Effect of different media on growth of isolate NIOS-1

Since the isolate NIOS-1 produced the highest biomass among all those screened, this isolate was chosen for further studies on media optimization for growth (Fig. 2.3.2). The media compositions are give in Table 2.2.2 in Materials and Methods. Of the 5 media tested, the medium M3, containing 2 % glucose, 0.15 % peptone, 0.1 % yeast extract, 0.025 % KH_2PO_4, 0.1 % vitamin and trace metal solution, yielded the highest dry weight biomass of 7.25 g L^{-1}. The lowest biomass of 3.8 g L^{-1} was obtained with M6 medium containing 0.5 % glucose, 0.5 % sodium glutamate, 0.1 % yeast extract, 0.1 % KH_2PO_4, 0.5 % trace metal solution and 0.1 % vitamin solution. The medium M3 was used for further optimization.

2.3.3. Effect of vitamins, trace elements, carbon sources, pH and salinity on growth of NIOS-1

The requirement of vitamins, in addition to yeast extract, and trace elements was examined (Fig. 2.3.3). The composition of vitamins and trace elements solutions are provided in Appendix 1. Presence or absence of additional vitamins and trace elements in M3 medium did not affect the growth substantially. The wet weight biomass ranged from 21.68 g L^{-1} in the presence of trace metal and vitamin solution to 21.12 g L^{-1} in M3 medium without trace metal and vitamin solution. Since there was no significant effect of additional vitamins and trace metals on the growth of NIOS-1, M3 medium without trace

metal and vitamins was used for further studied and was designated as 'M4 medium'.

The salinity of M4 medium was optimized in the range of 15 to 50 ppt (Fig. 2.3.4). The wet weight biomass yield ranged from a low of 15.7 g L^{-1} at 15 ppt to 22.1 g L^{-1} at 35 ppt. Therefore, M4 medium with 35 ppt salinity was used for further studies.

The pH of M4 medium was optimized in the range of 4 to 9. OD values for growth remained almost the same at the end of 5 days (Fig. 2.3.5). A wet weight biomass of 19.45 g L^{-1} was obtained at pH 4. Growth, as observed visually, was much better at pH values above this. However, cells accumulated high amounts of lipids, leading to floatation of cells. Therefore, complete harvesting of the cells through centrifugation was not possible, leading to values that were underestimates of actual growth. However, the percentage lipid content was the highest at pH 7 and attained the value, 53.9 % (Fig. 2.3.6). Hence pH 7 was used for further studies.

Ten different carbon sources were examined for their effect on growth of NIOS-1 (Fig. 2.3.7). The best growth densities were obtained with glucose as the carbon source, followed by fructose and glycerol. The pentoses - arabinose, rhamnose and xylose gave very poor growth. Hence glucose was used for further studies.

The effect of exogenous oil was also studied to optimize the biomass (Fig. 2.3.8). The wet weight biomass values increased from 25 g L^{-1} in control to 32 g L^{-1} when the medium was supplemented with coconut oil. The lipid concentration

increased from 36.4 % in control to 46.7 %. However, total DHA contents decreased in the presence of coconut oil (relative GC units of 114177) as compared to the controls (relative GC units of 264504)

2.3.4. Further optimization of M4 medium for growth of NIOS-1

In a further experiment, the effect of different levels of peptone, glucose and yeast extract on biomass production, growth, glucose consumption and lipid concentration was examined over a period of 4 days (Figs. 2.3.9 – 2.3.18), in order to optimize the levels of the three media ingredients, as given in 2.2.6.

Growth rates were very low at peptone concentrations of 0.01 % and 0.05 % (Fig. 2.3.9). Growth rates were high and almost similar at the higher peptone concentrations of 0.2 to 0.5 %, declining after 72h. Except for the peptone concentrations of 0.01 and 0.05 %, glucose was consumed rapidly from 24 to 72 h (Fig. 2.3.10). The wet weight biomass obtained ranged from 3.5 g L^{-1} at 0.01 % peptone concentration and 16 to 18 g above 0.15 % (Fig. 2.3.11). The percentage of total lipids ranged from 20.2 % to 45.38 % at peptone concentrations of 0.01 % and 0.4 % respectively.

After optimizing peptone concentrations the effect of various concentrations of yeast extract was optimized (Fig. 2.3.12). Growth rate declined sharply after 24 h at lower yeast extract concentrations of 0.01 to 0.5%, whereas at the higher concentrations up to 1.5 %, growth rates increased steadily up to 2 days, leveling off subsequently. Glucose was consumed very rapidly by 48h at the lower yeast concentrations of 0.01 to 0.5 %, while glucose was consumed

more steadily till 3 days at higher concentrations (Fig. 2.3.13). The maximum wet weight biomass values of 34.6 g L^{-1} was obtained at the highest yeast extract concentration of 1.5 %. Dry weight biomass and total lipid concentration also steadily increased up to 1.5 % yeast extract (Fig. 2.3.14) The percentage of total lipids ranged from 24.0 % to 40.49 % at yeast extract concentrations of 0.01 % and 0.5 % respectively.

After optimizing concentrations of peptone and yeast extract, glucose was optimized. Results on the effect of various concentrations of glucose on growth rate, glucose consumption, biomass and lipids are presented in Fig. 2.3.15 to 2.3.17. Growth rates at lower glucose concentrations of 0.5 and 1 % increased up to 48h, after which they declined more sharply than at higher concentrations up to 5 % (Fig. 2.3.15). The rate of glucose consumption was almost the same at glucose concentrations of 2 to 5 %. Glucose was used up even by 48 h at the two lower glucose concentrations, while excess glucose remained in culture even by 4 days at concentrations of 4 and 5 %. When 3 % glucose was used, no glucose remained at the end of 4 days (Fig.2.3.16). The maximum biomass was obtained at 3.0 % and glucose concentrations above this did not make any difference in terms of growth, dry weight biomass and concentration of total lipids (Fig. 2.3.17) The percentage of total lipids ranged from 10.18 to 34.14 % at glucose concentrations of 0.5 % and 4. 0 % respectively.

The overall medium, contained 3 % glucose, 0.15 % peptone and 1.5 % yeast extract. This medium yielded, 37.76 g L^{-1} wet weight biomass, 13.12 g L^{-1}

dry weight biomass of which 32.94 % was lipids. The percentage break-up is given in Fig. 2.3.18.

2.3.5. Response Surface Method (RSM) to optimize optimal levels of medium ingredients

The interaction of the three key components of the medium M4 was studied using RSM in order to achieve the optimum biomass, lipid and DHA production. Results obtained on biomass, total lipids and DHA for the 20 trials carried out under RSM, as given in Materials and Methods, are presented in Table 2.3.2. Multiple regression analysis on data obtained for each of the above, namely biomass, total lipids and DHA contents are presented in Table 2.3.3.

The dry weight biomass ranged from 3.81 - 9.4 g L^{-1}, obtained in Trial No. 9 and Trial No. 8 respectively. The medium composition of Trial No. 9 was 0.38 % glucose, 0.3 % peptone and 0.25 % yeast extract and of Trial no. 8 was 3 % glucose, 0.45 % peptone and 4 % yeast extract. Biomass production was significantly affected by glucose ($p = 0.013$), but not by yeast extract and peptone (Table 2.3.3). Surface plots indicated that more than 4.0 % glucose produced high biomass (Figs. 2.3.19. – 2.3.20).

The percentage of total lipids, ranged from 9.6 % to 57.6 % in Trial No. 6 and Trial No. 13 respectively (Table 2.3.2). The highest percentage of total lipids ranging from 49 to 57 % were obtained in Trials 7, 10 and 13, in which the medium contained 3 %, 3.62 % and 2 % glucose; 0.15%, 0.3 %, 0.3 % peptone and 0.4 %, 0.25 %, 0.088 % yeast extract respectively. Peptone was statistically

significant (p= 0.01) in affecting the levels of lipids (Table 2.3.3). Surface plots indicated that peptone at a concentration of about 0.4 % in combination with glucose gave the best percentage of lipids (Fig. 2.3.21). However with increasing concentrations of yeast extract the optimal peptone concentration came down to about 0.15 % (Fig. 2.3.22).

The percentage of DHA, ranged from 26.16 % to 38.13 % in Trial No. 2 and Trial No. 5 respectively (Table 2.3.2). High percentage DHA of total lipids, corresponding to ~ 36 % of total fatty acids was obtained in Trials 3, 6 and 9, containing 1 % or lesser levels of glucose, and a combined protein and yeast extract values of 0.46 to 0.59 %. The beta values obtained showed that none of the three medium ingredients had a statistically significant effect (Table 2.3.3). A combination of about 0.4 % peptone and 1.0 % glucose levels, or 0.1 % peptone and 0.55 % yeast extract were optimal for DHA production. Surface plots indicated that glucose at concentration of about 2.5 % and peptone at 0.4 % yielded the best percentage of DHA (Fig. 2.3.23). The concentration of yeast extract in combination with peptone did not affect the DHA percentage in total lipids (Fig 2.3.24).

The highest total yield of DHA was obtained in Trial 10, containing 3.62 % glucose, 0.3 % peptone and 0.25 % yeast extract (Table 2.3.2.). The lowest total yield was seen in Trial 9, containing 0.38 % glucose, 0.3 % peptone and 0.25 % yeast extract. Beta values were significant for peptone (p = 0.049) (Table 2.3.3). A combination of about 0.5 % peptone and yeast extract of 0.45 %, or a combination of 0.3 % peptone and 3.62 % glucose yielded the best values

(Table. 2.3.2). Surface plots showed that the highest concentration of peptone used (0.5%) together with about 3.5 % glucose was optimal for total DHA yield (Fig. 2.3.25). Likewise the highest amount of yeast extract used (0.5 %) was optimal (Fig. 2.3.26).

2.3.6. Effect of low temperature and increased density / viscosity in enhancing levels of DHA in thraustochytrids

Refrigeration of harvested cell biomass resulted in a distinct increase in absolute values of DHA by a factor of about 1.5 to 2 in the isolates NIOS-2 and NIOS-1 (Fig. 2.3.27). However, neither of these isolates showed an increase in the percentage of DHA with respect to the total fatty acids. The cell biomass of NIOS-9 yielded lower DHA when refrigerated. No distinct difference in DHA contents was observed in NIOS-4 cell biomass after refrigeration. EPA was negligible in NIOS-2, NIOS-9 and NIOS-1. EPA did not increase with cell biomass refrigeration in the first two, while NIOS-1 yielded a slight increase.

Storage of biomass at different temperatures showed that total fatty acids increased at 5 – 20°C compared to the control (Fig. 2.3.28). Total DHA yield was best when refrigerated at 10°C. Percentage DHA did not vary much.

Total fatty acids, as well as DHA contents in the cell biomass of the isolate NIOS-1 stored at 10 °C for 12 to 48 h increased gradually, declining at 72 h (Fig. 2.3.29). EPA, which was present in low amounts in this isolate, also showed a similar increase (not represented in Fig. 2.3.29). Since the contents of other fatty

acids also increased simultaneously, the percentages of EPA and DHA did not reflect the increase in terms of absolute values.

Total amounts of EPA and DHA in the isolate NIOS-11, belonging to the genus *Ulkenia*, increased nearly 2 to 3 times in media containing 0.5 and 1.0 % PVP (Fig. 2.3.30). Other fatty acids also increased at 1.0 % PVP concentration. Percentages of EPA and DHA were considerably higher when 0.5 % PVP was added (14.2 and 21.7 % respectively) compared to the control (6.7 and 13.2 %, respectively). Absolute values of EPA, but not DHA showed an increase at the lower PVP concentration of 0.1 %, while the total fatty acids decreased.

Four isolates were further analyzed for DHA contents in the presence and absence of 0.5 % PVP (Fig. 2.3.31). With the exception of isolate NIOS-4, all others showed an increase in total DHA values of 1.5 to 4 times in the presence of PVP. Quantities of total fatty acids also increased with PVP. Although the absolute values of DHA increased with PVP, percentage values changed only marginally.

The cell biomass of the 4 isolates grown in PVP was additionally subjected to refrigeration at 10 °C as earlier. A comparison of results obtained by refrigeration, PVP addition and PVP addition plus refrigeration is given in Fig. 2.3.32. Although all 3 treatments resulted in higher DHA and fatty acid contents compared to the control, refrigeration alone was marginally better than the other two treatments. A combination of PVP and refrigeration was least favorable compared to plain PVP or refrigeration treatments. Refrigeration was detrimental to DHA and fatty acid components in the isolate NIOS-9.

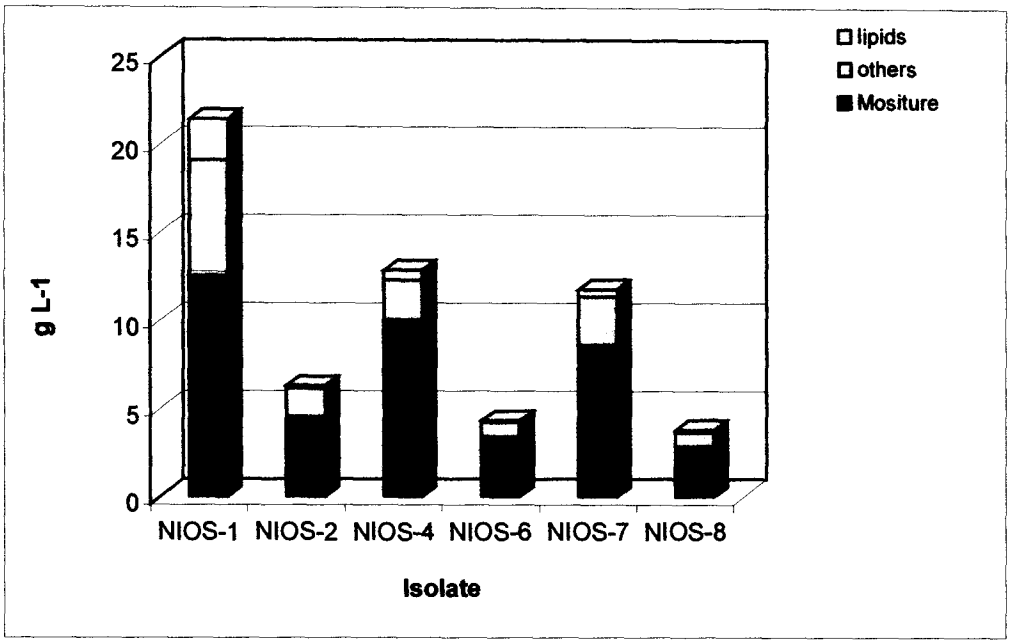


Fig. 2.3.1: Biomass production by various isolates of thraustochytrids. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.0007 to 0.116.

Isolate	Percent Fatty Acid								
	12:0	**14:0**	**16:0**	**16:1**	**18:0**	**18:1**	**20:5**	**22:5**	**22:6**
NIOS-1	-	3.79	59.59	-	1.82	-	7.54	0.61	22.67
NIOS-2	-	1.57	25.02	-	4.10	15.33	5.10	0.57	7.70
NIOS-4	-	12.46	42.69	3.28	3.77	-	5.96	0.98	15.53
NIOS-6	81.48	1.81	9.58	-	-	-	1.10	-	2.40
NIOS-7	-	5.63	70.64	1.02	1.84	1.87	2.35	-	10.23
NIOS-8	-	2.50	33.51	-	2.04	4.91	3.61	0.40	8.61

Table. 2.3.1: Fatty acid profile of various isolate screened for PUFA production.

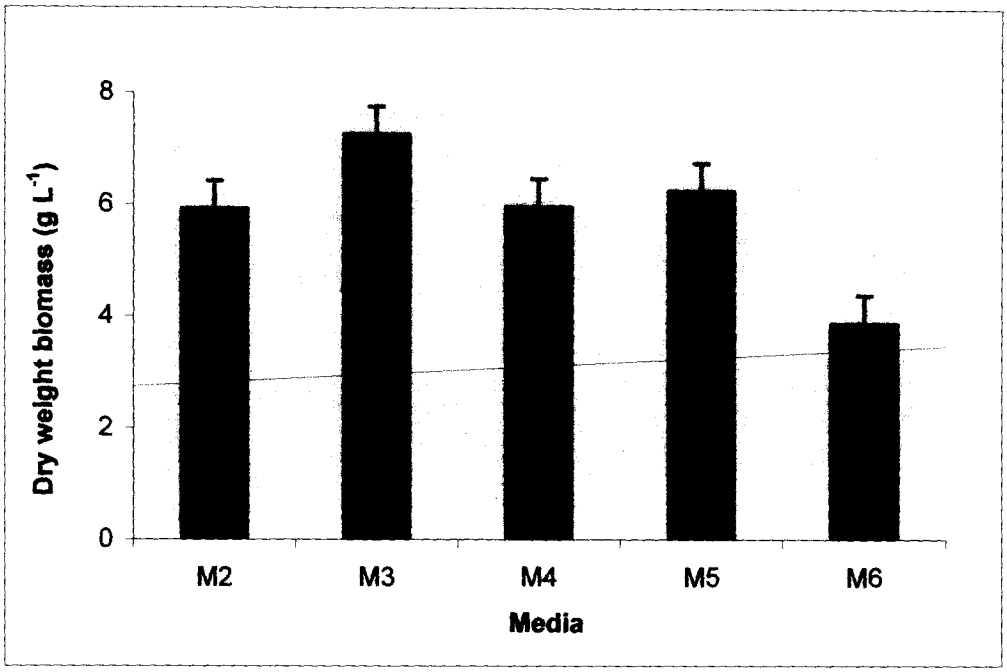


Fig 2.3.2. Effect of various media, from literature tested for biomass production of NIOS-1. The error bars indicate SD.

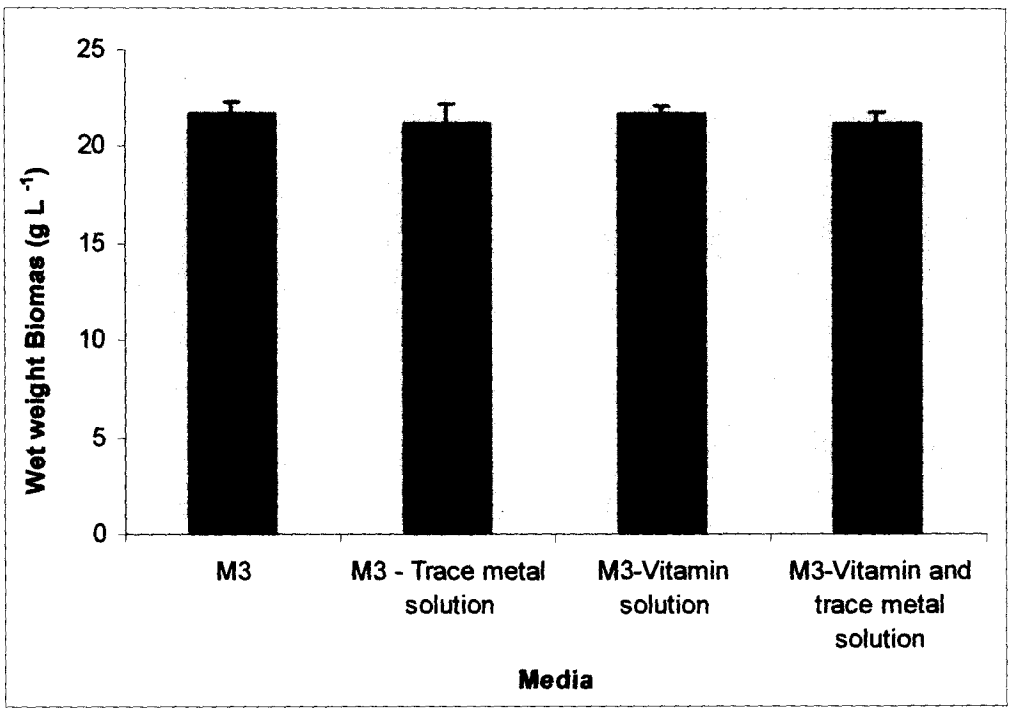


Fig. 2.3.3: Effect of vitamins and trace metals on biomass production of NIOS-1. The error bars indicate SD.

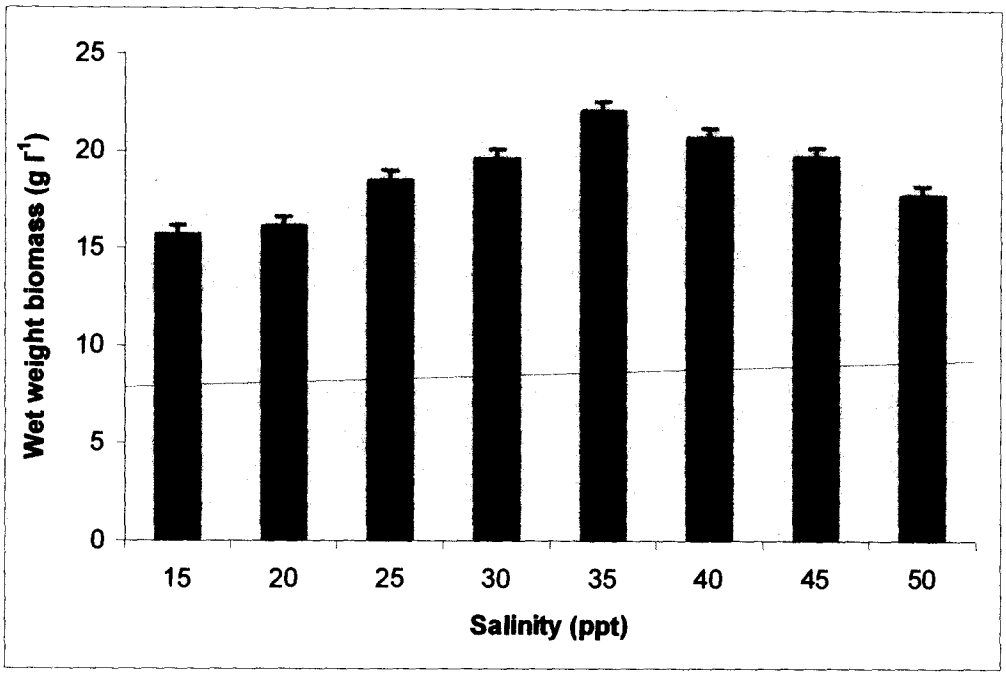


Fig. 2.3.4: Effect of salinity on biomass production of NIOS-1. The error bars indicate SD.

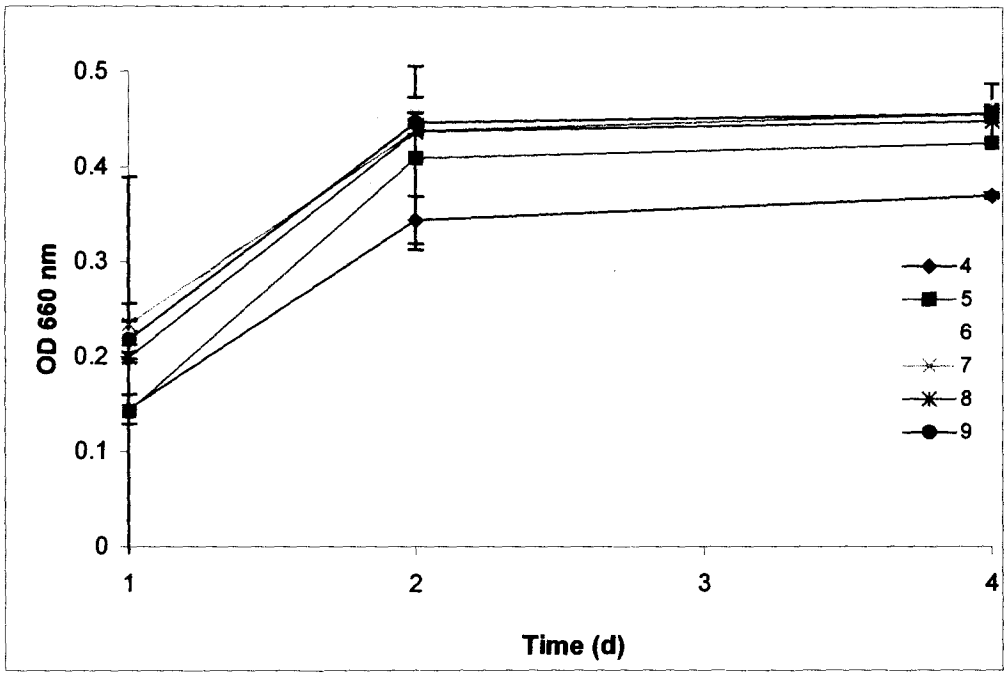


Fig. 2.3.5: Effect of pH on growth of NIOS-1. The error bars indicate SD.

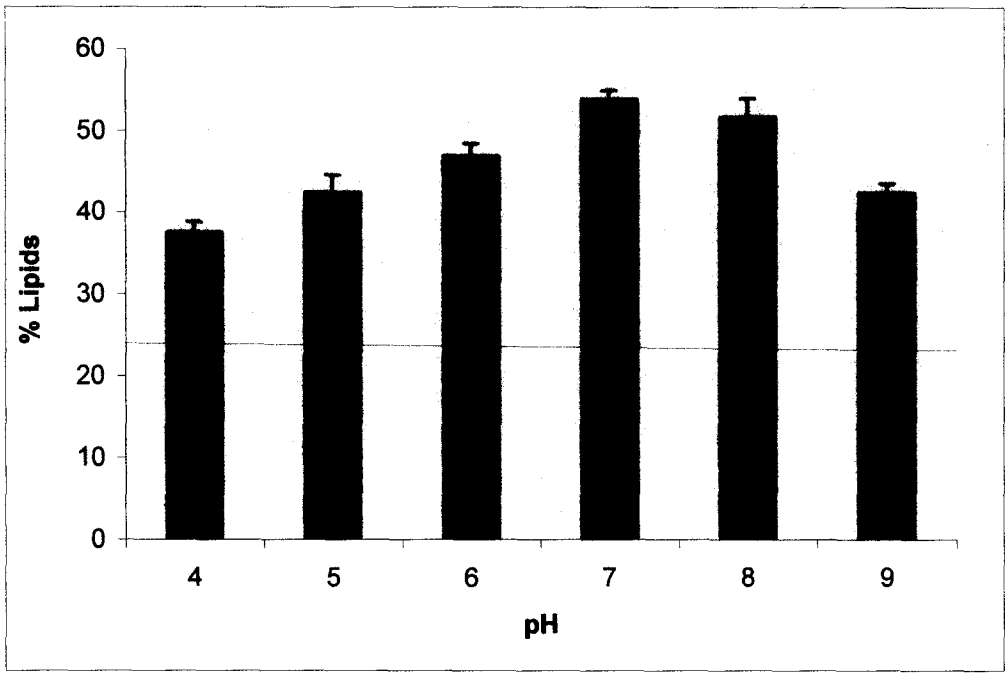


Fig. 2.3.6: Effect of pH on lipid production of NIOS-1. The error bars indicate SD.

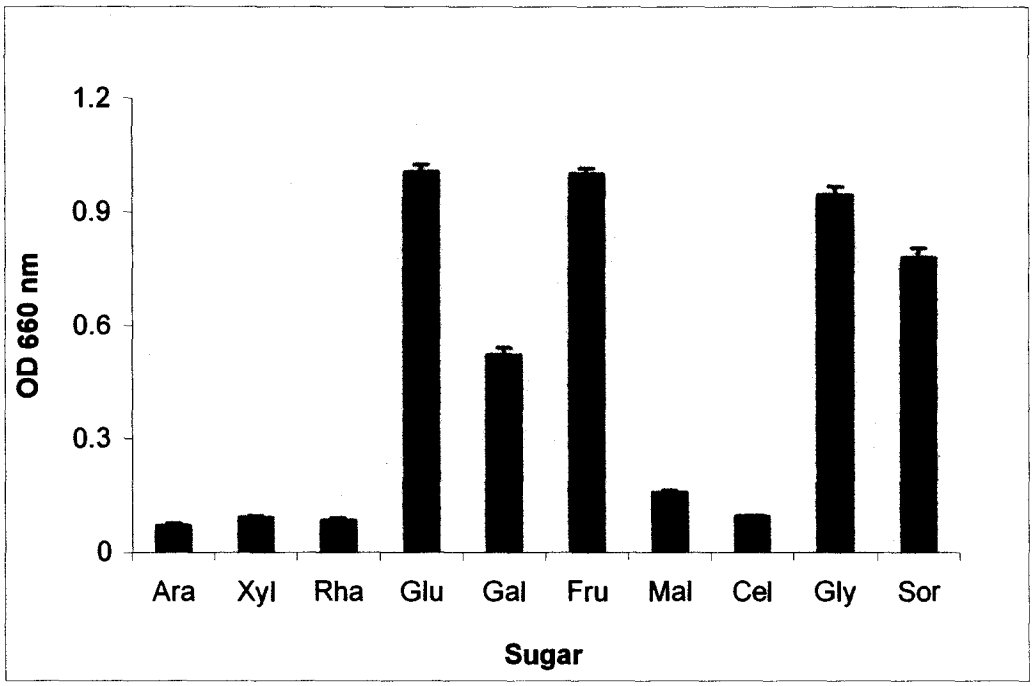


Fig. 2.3.7: Effect of sugars on growth of NIOS-1. The error bars indicate SD.

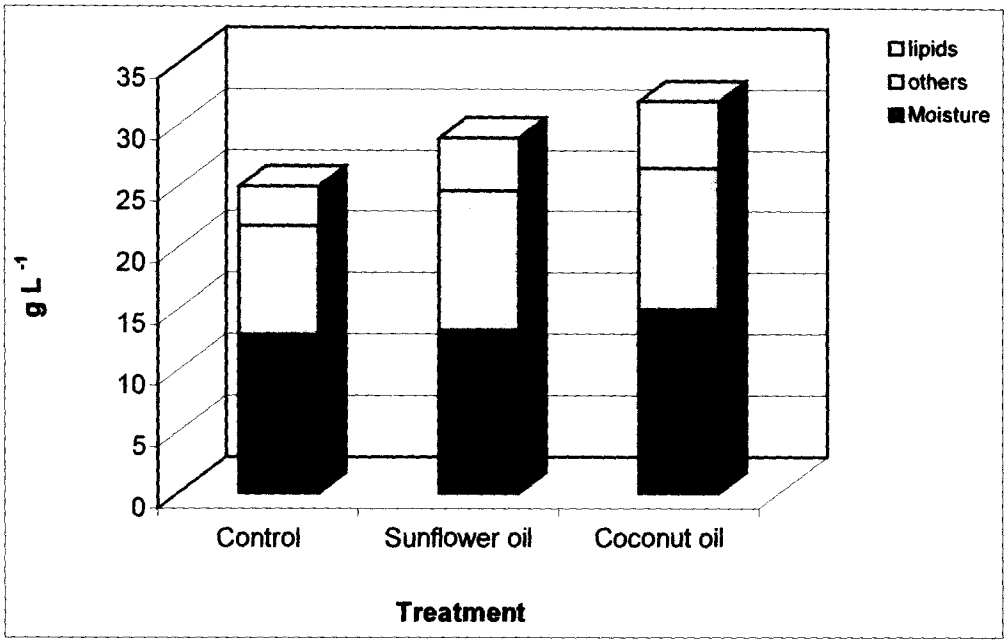


Fig. 2.3.8: Effect of exogenous oil on biomass and lipid production of the isolate NIOS-1. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.09 to 0.24.

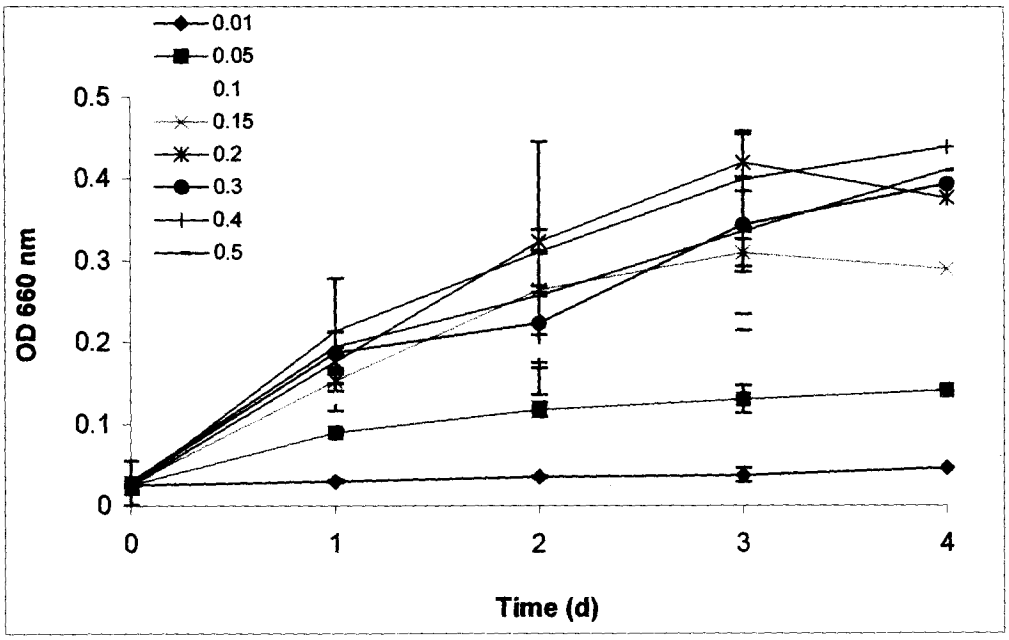


Fig. 2.3.9: Effect of peptone concentration on growth of NIOS-1. The error bars indicate SD.

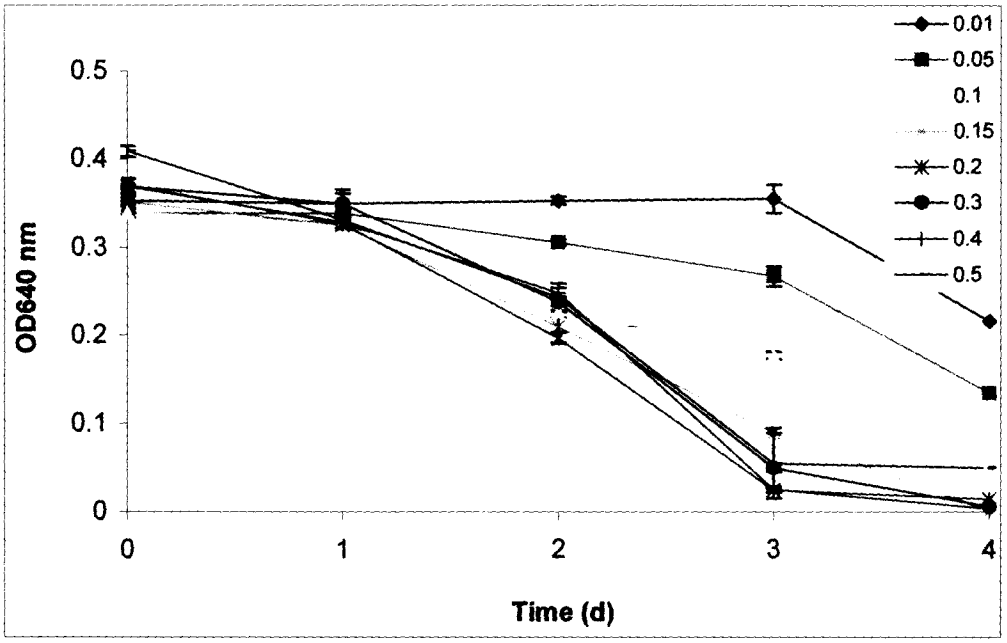


Fig. 2.3.10: Effect of peptone concentration on glucose consumption of NIOS-1. The error bars indicate SD.

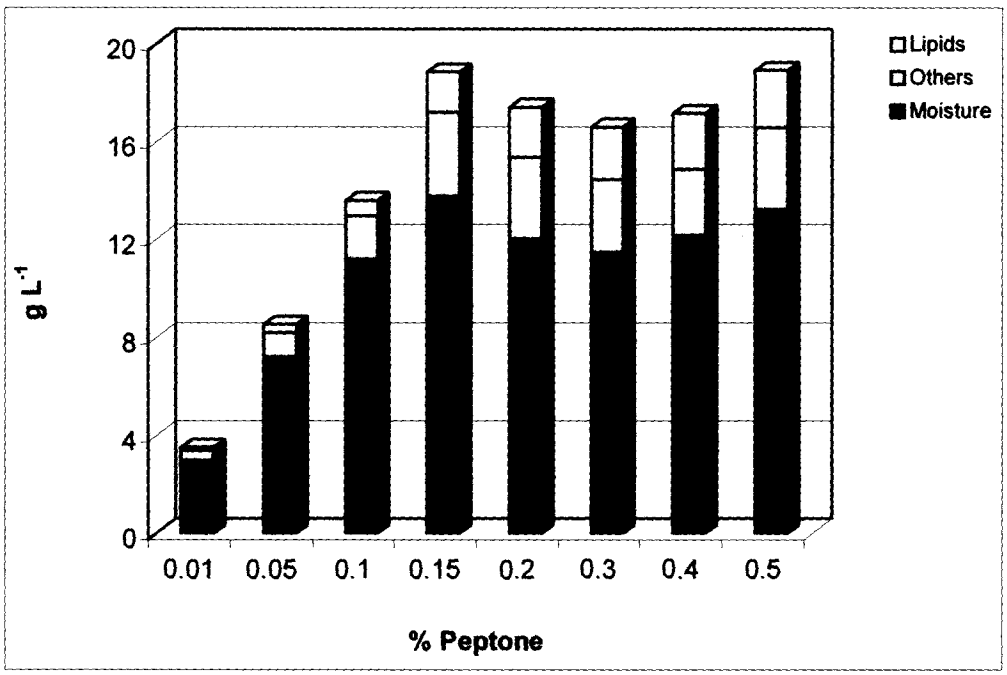


Fig. 2.3.11: Effect of peptone concentration on biomass and lipid production by NIOS-1. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.02 to 3.16.

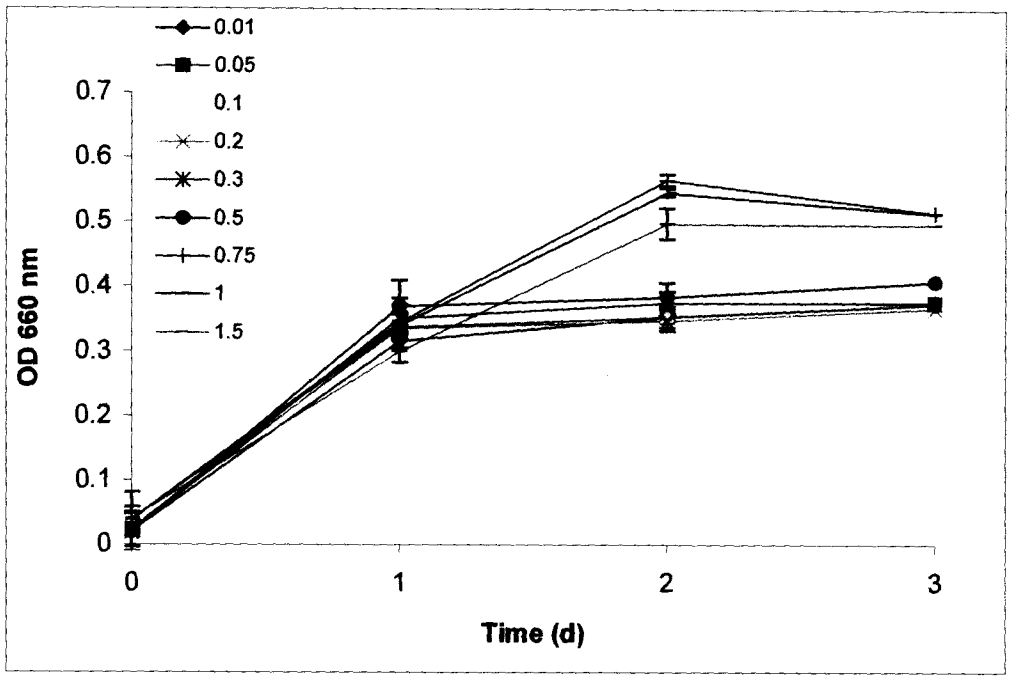


Fig. 2.3.12: Effect of yeast extract concentration on growth of NIOS-1. The error bars indicate SD.

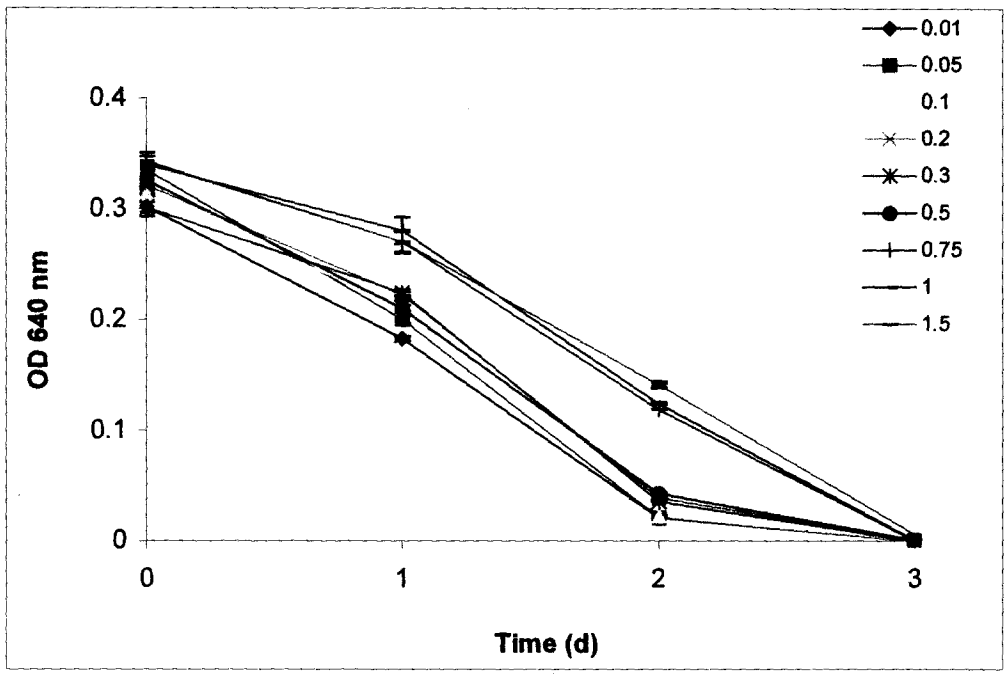


Fig. 2.3.13: Effect of yeast extract concentration on glucose consumption of NIOS-1. The error bars indicate SD.

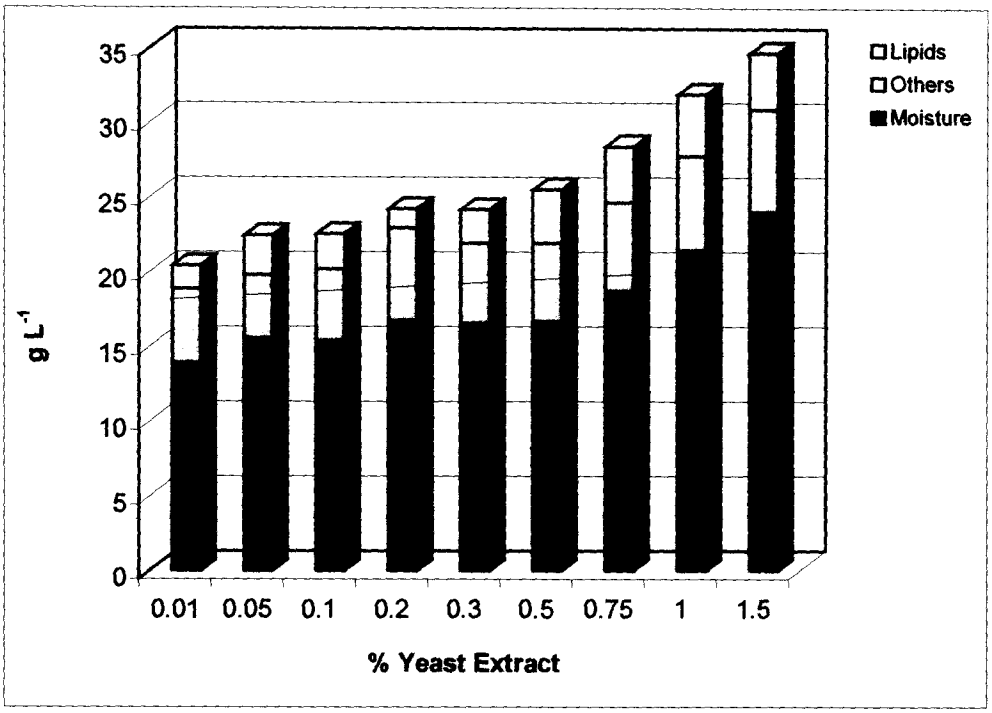


Fig. 2.3.14: Effect of yeast extract concentration on biomass and lipid production by NIOS-1. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.02 to 3.25.

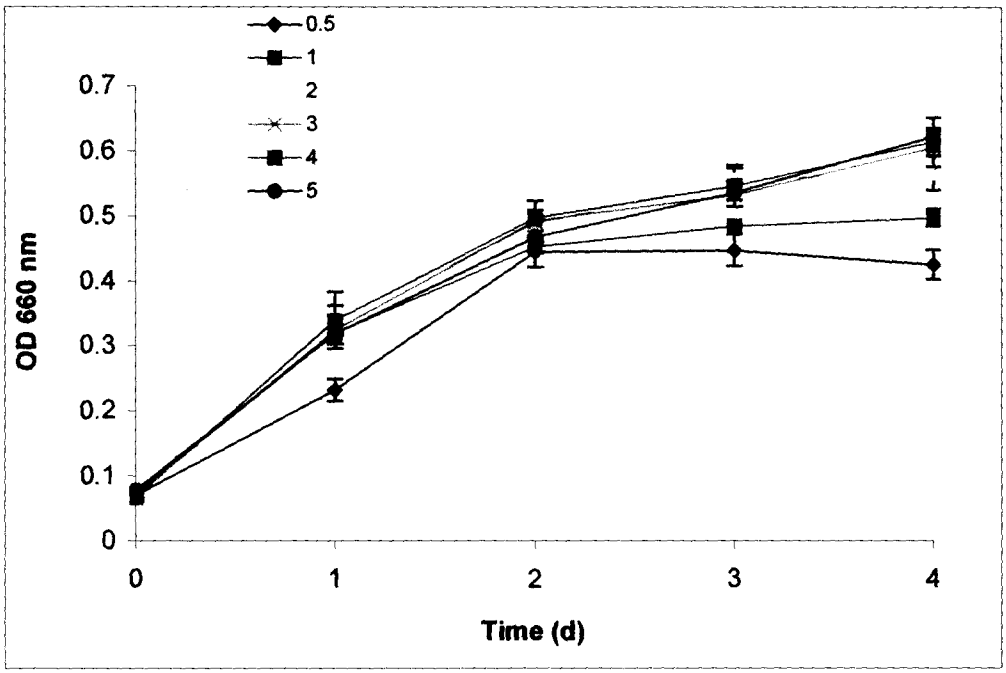


Fig. 2.3.15: Effect of glucose concentration on growth of NIOS-1. The error bars indicate SD.

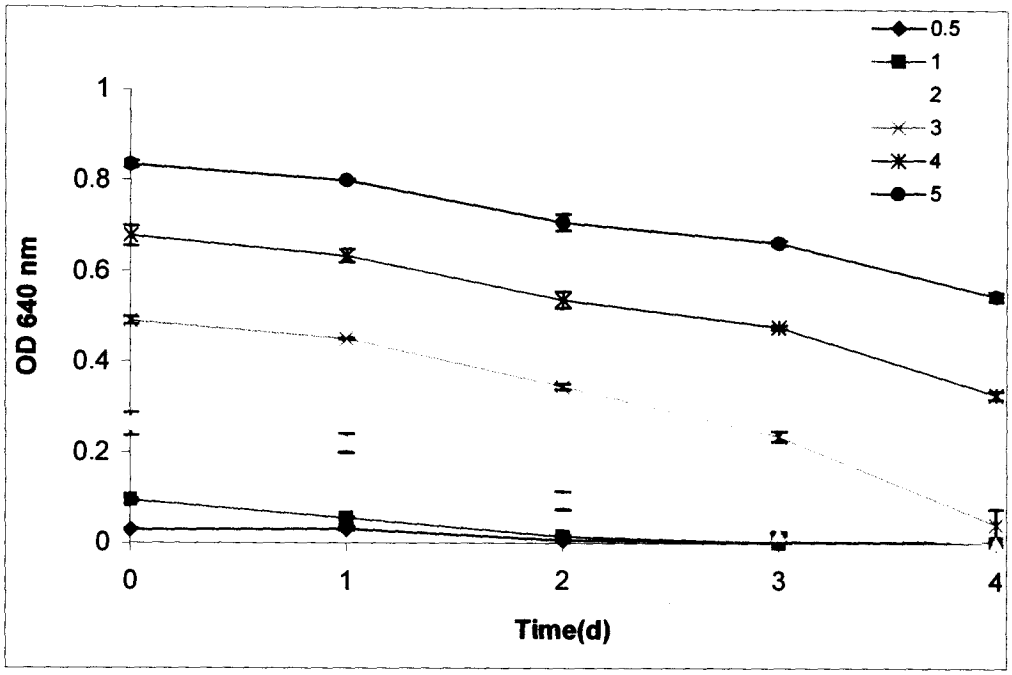


Fig. 2.3.16: Effect of glucose concentration on glucose consumption of NIOS-1. The error bars indicate SD.

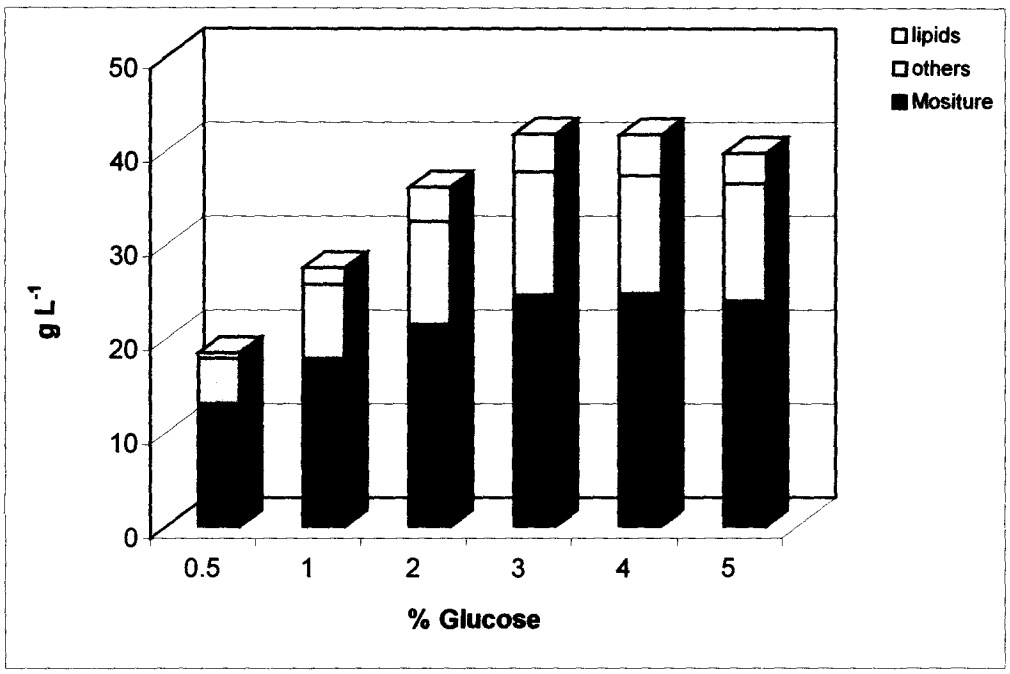


Fig. 2.3.17: Effect of glucose concentration on biomass and lipid production by NIOS-1. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.02 to 3.65.

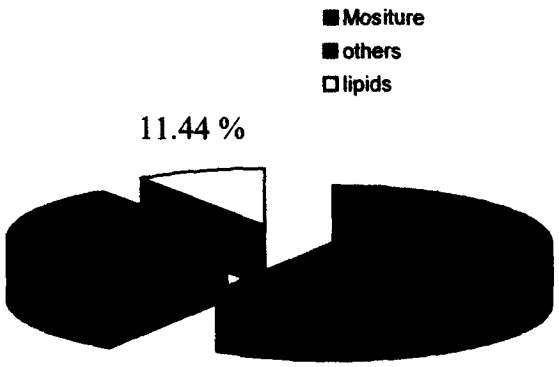


Fig. 2.3.18: Total production of lipids and dry weight biomass by the optimized medium (3 % glucose, 0.15 % peptone and 1.5 % yeast extract) using the OFAT method. Each values is a mean of triplicate sample and the standard deviation (SD) ranged from 0.09 to 3.44.

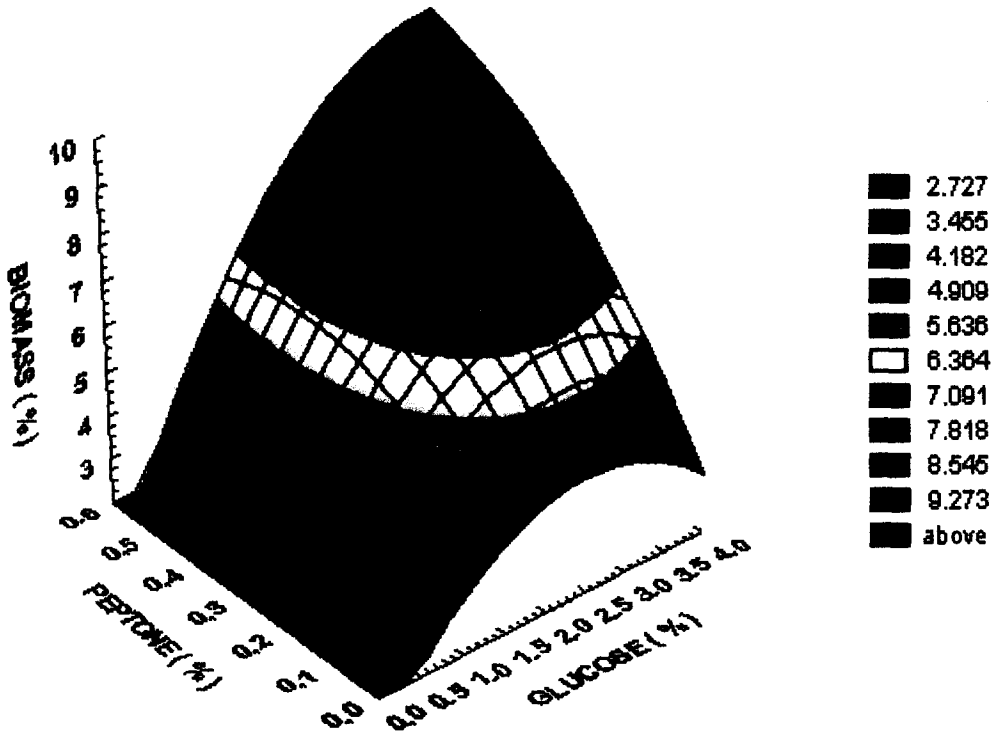


Fig. 2.3.19: Effect of various concentrations of glucose and peptone on biomass of isolate NIOS-1 by RSM.

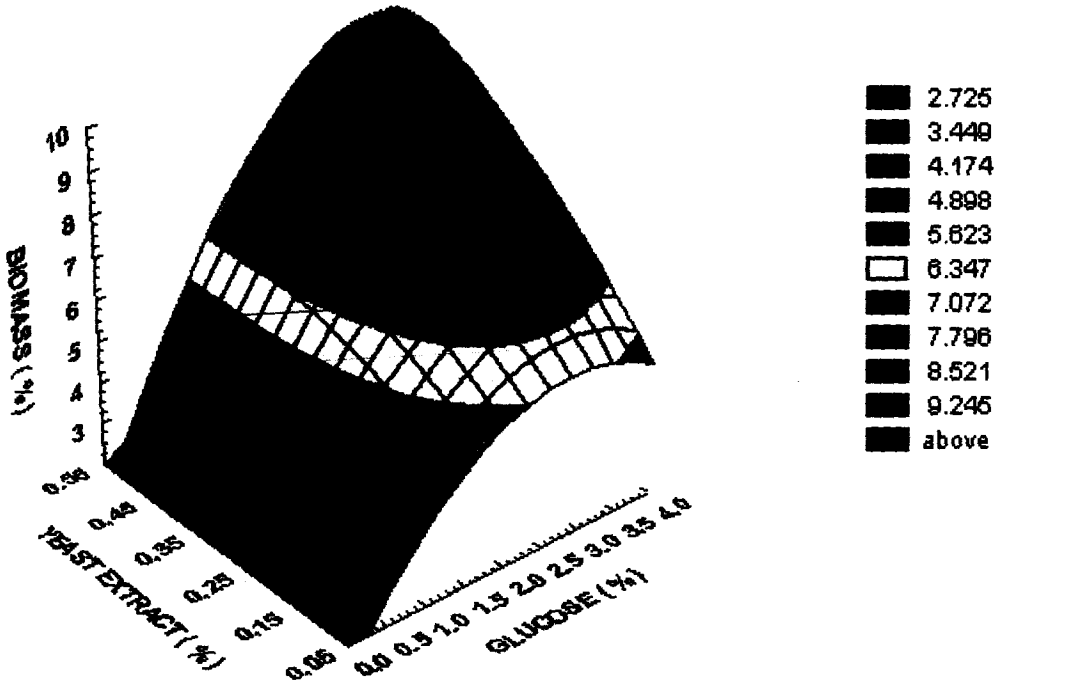


Fig. 2.3.20: Effect of various concentrations of glucose and Yeast Extract on biomass of isolate NIOS-1 by RSM.

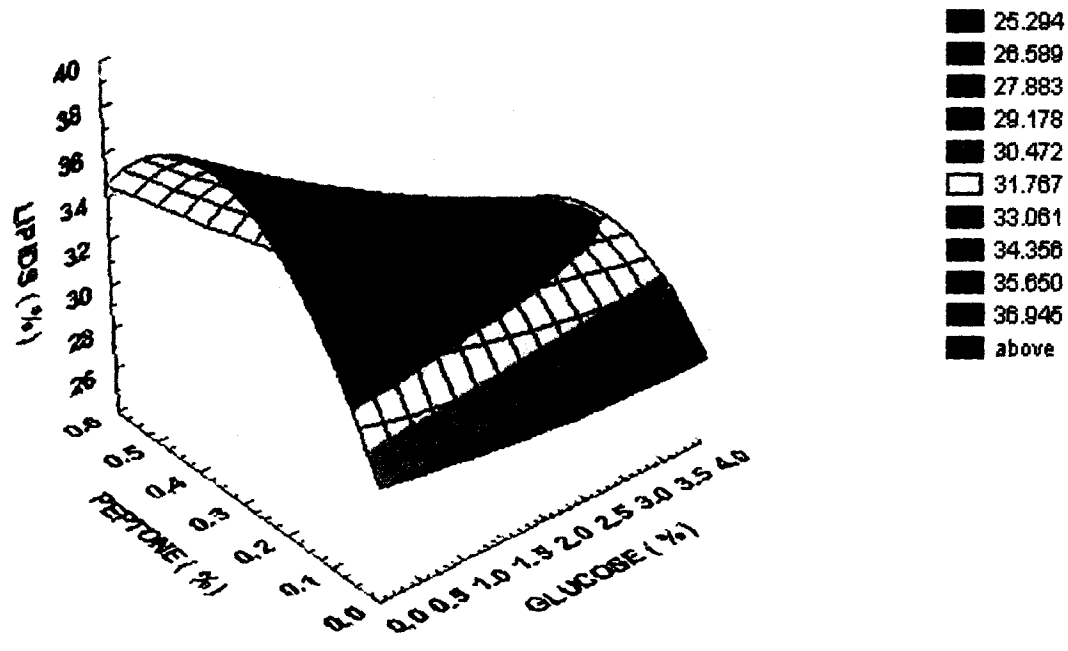


Fig. 2.3.21: Effect of various concentrations of glucose and peptone on percentage lipids of isolate NIOS-1 by RSM.

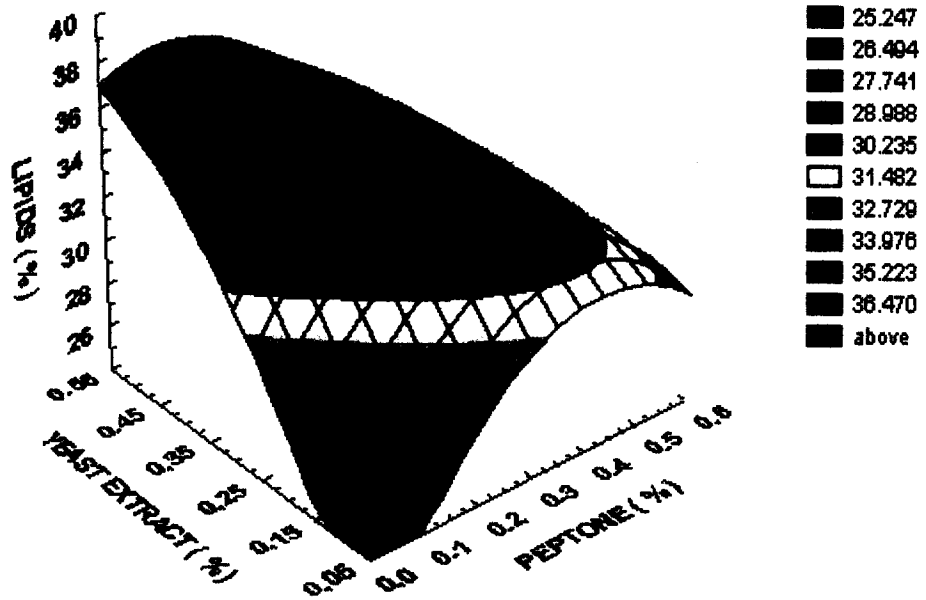


Fig. 2.3.22: Effect of various concentrations of yeast extract and peptone on percentage lipids of isolate NIOS-1 by RSM.

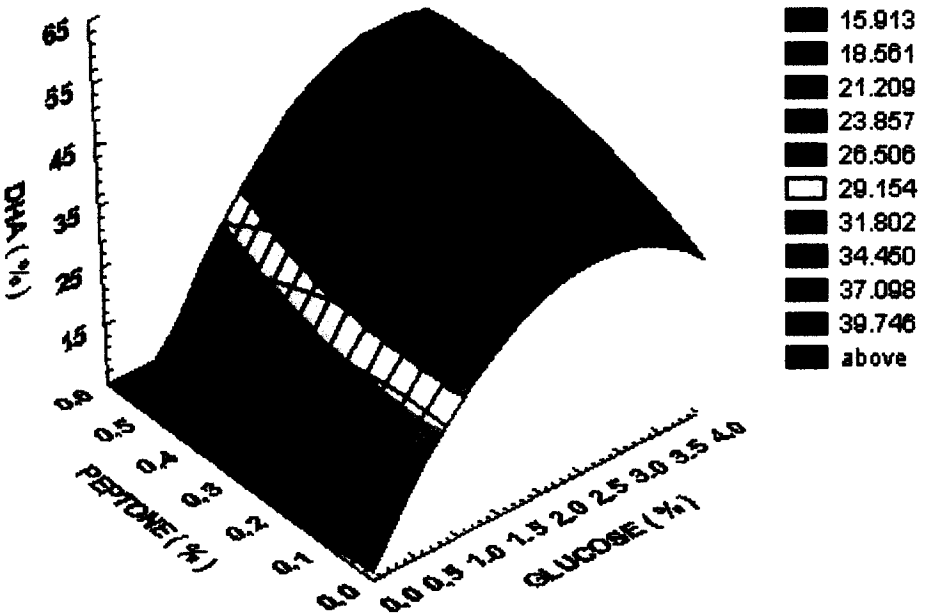


Fig. 2.3.23: Effect of various concentrations of glucose and peptone on DHA concentration of isolate NIOS-1 by RSM.

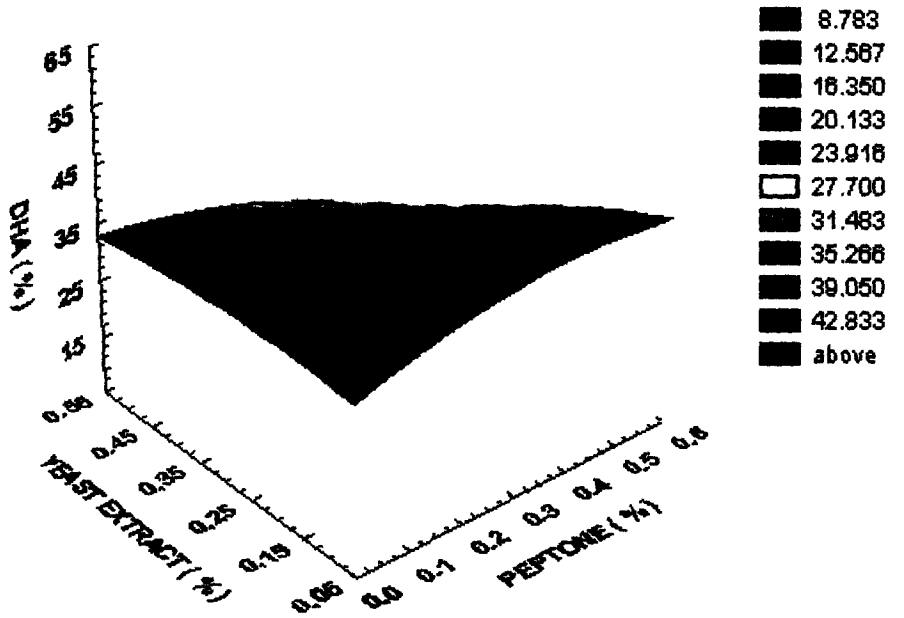


Fig. 2.3.24: Effect of various concentrations of yeast extract and peptone on DHA concentration of isolate NIOS-1 by RSM.

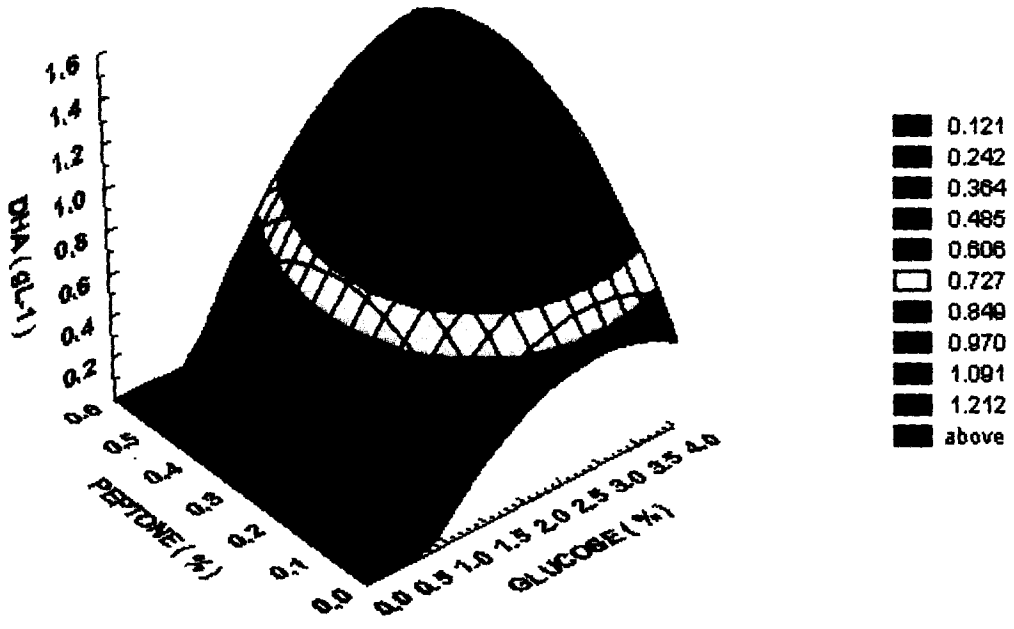


Fig. 2.3.25: Effect of various concentrations of glucose and peptone on absolute DHA content of isolate NIOS-1 by RSM.

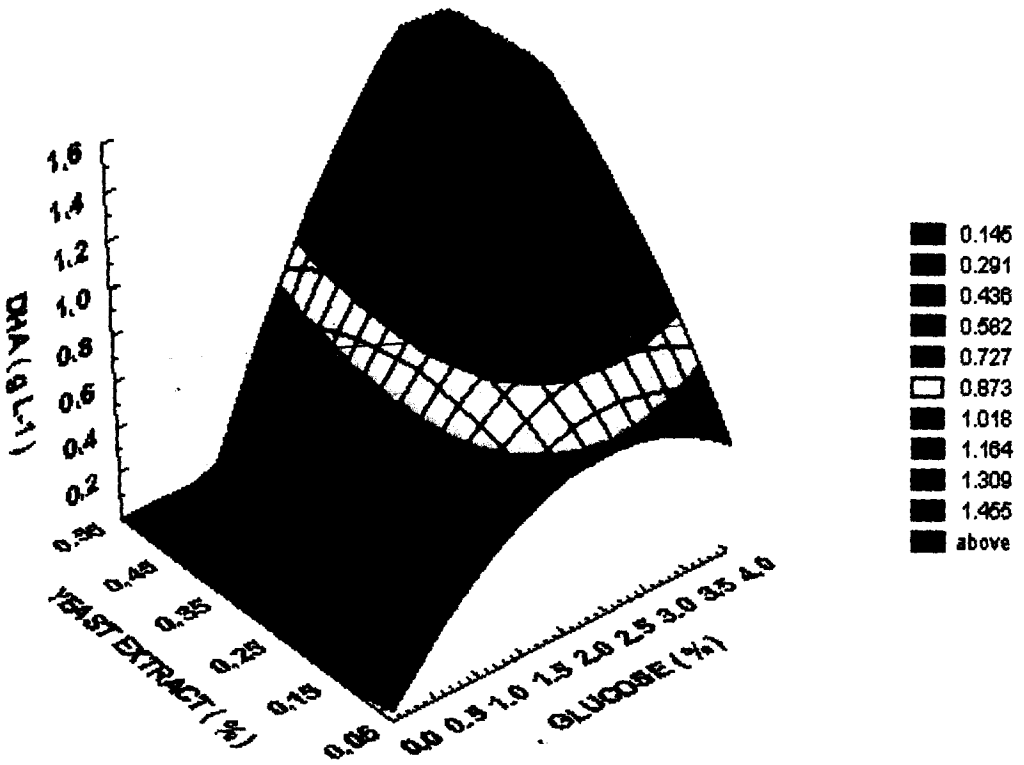


Fig. 2.3.26: Effect of various concentrations of glucose and yeast extract on absolute DHA content of isolate NIOS-1 by RSM.

Trial No.	Percent			$g\ L^{-1}$	Percent		$g\ L^{-1}$
	Glucose	Peptone	Yeast extract	Biomass	Lipids	DHA	DHA
1	1	0.15	0.1	3.81	35.97	27.81	0.381
2	3	0.15	0.1	4.39	33.60	26.16	0.386
3	1	0.45	0.1	4.54	26.40	36.69	0.440
4	3	0.45	0.1	8.06	43.20	29.61	1.031
5	1	0.15	0.4	4.84	16.80	38.13	0.310
6	1	0.45	0.4	5.38	9.60	36.79	0.190
7	3	0.15	0.4	8.20	50.40	33.27	1.376
8	3	0.45	0.4	9.39	42.00	32.11	1.267
9	0.38	0.3	0.25	3.08	12.00	36.12	0.133
10	3.62	0.3	0.25	8.44	49.20	34.01	1.412
11	2	0.057	0.25	5.33	39.60	33.82	0.714
12	2	0.543	0.25	7.82	40.80	27.81	0.888
13	2	0.3	0.088	5.95	57.60	31.07	1.064
14	2	0.3	0.493	8.21	32.40	35.36	0.941
15	2	0.3	0.25	6.58	38.40	33.39	0.844
16	2	0.3	0.25	7.14	37.20	35.63	0.946
17	2	0.3	0.25	7.96	43.20	33.52	1.153
18	2	0.3	0.25	7.06	38.40	36.08	0.978
19	2	0.3	0.25	7.58	42.00	33.91	1.080
20	2	0.3	0.25	6.56	34.80	36.17	0.825

Table 2.3.2: Biomass, total lipids and DHA for the 20 trials carried out under RSM.

	Biomass (g L^{-1})	% Lipid	% DHA	DHA (g L^{-1})
β	-0.7415	16.9018	27.1542	-0.48183
Glu (β_1)	**1.03867 (P = 0.0125)**	-0.22289 (P = 0.7646)	1.01427 (P = 0.1365)	0.714706 (P = 0.1696)
Pep (β_2)	0.68019 (P = 0.0744)	**2.52994 (P = 0.0125)**	0.17348 (P = 0.7833)	**1.076266 (P = 0.0499)**
YE (β_3)	0.65534 (P = 0.0906)	2.32952 (P = 0.212)	-0.77159 (P = 0.2562)	0.390708 (P = 0.4476)
(Glu-Glu) β_1^2	-1.09806	0.18968	-1.37044	-0.976351
(Pep-Pep) β_2^2	-0.49152	-1.47273	-0.33835	-0.882225
(YE-YE) β_3^2	-0.35073	-0.93068	-0.09484	-0.586507
(Glu-Pep) β_1 β_2	0.62207	-0.48036	0.45105	0.470611
(Glu-YE) β_1 β_3	0.51120	-0.06643	1.16242	1.094311
(Pep-YE) β_2 β_3	-0.41644	-1.21829	-0.35240	-0.660353

Table 2.3.3: Beta and p-values of biomass, total lipids and DHA for the 20 trials carried out under RSM. Significant values are given in bold. Glu = glucose, Pep = peptone and YE = yeast extract.

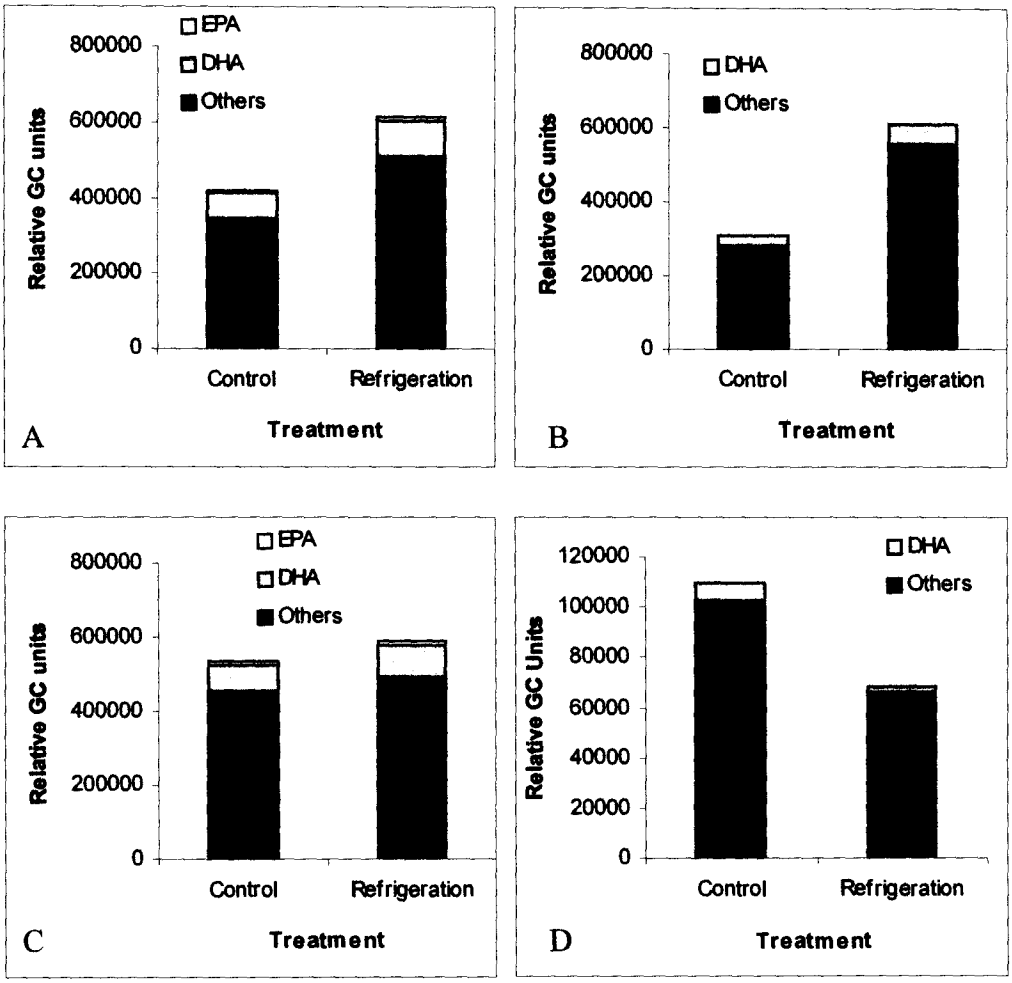


Fig.2.3.27: Effect of refrigeration on total fatty acids, EPA and DHA contents of the isolate NIOS-1 (A), NIOS- 2 (B), NIOS-4 (C) and NIOS-9 (D) following growth at room temperature.

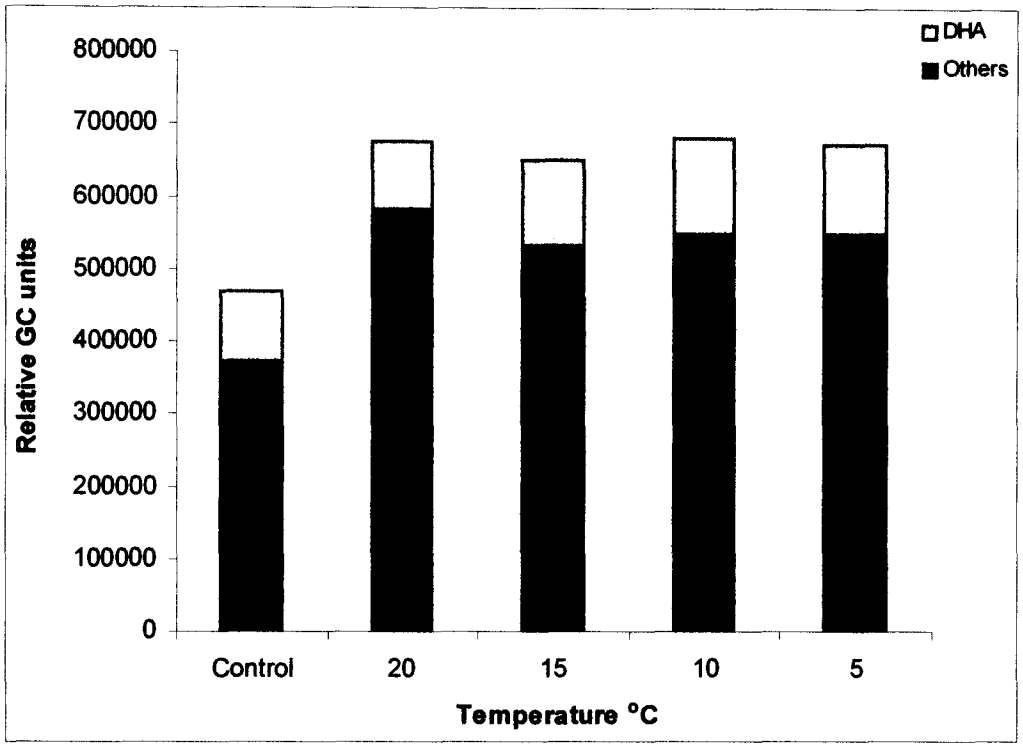


Fig. 2.3.28: Effect of refrigeration temperature following growth at room temperature on total fatty acids and DHA contents of the isolate NIOS-1.

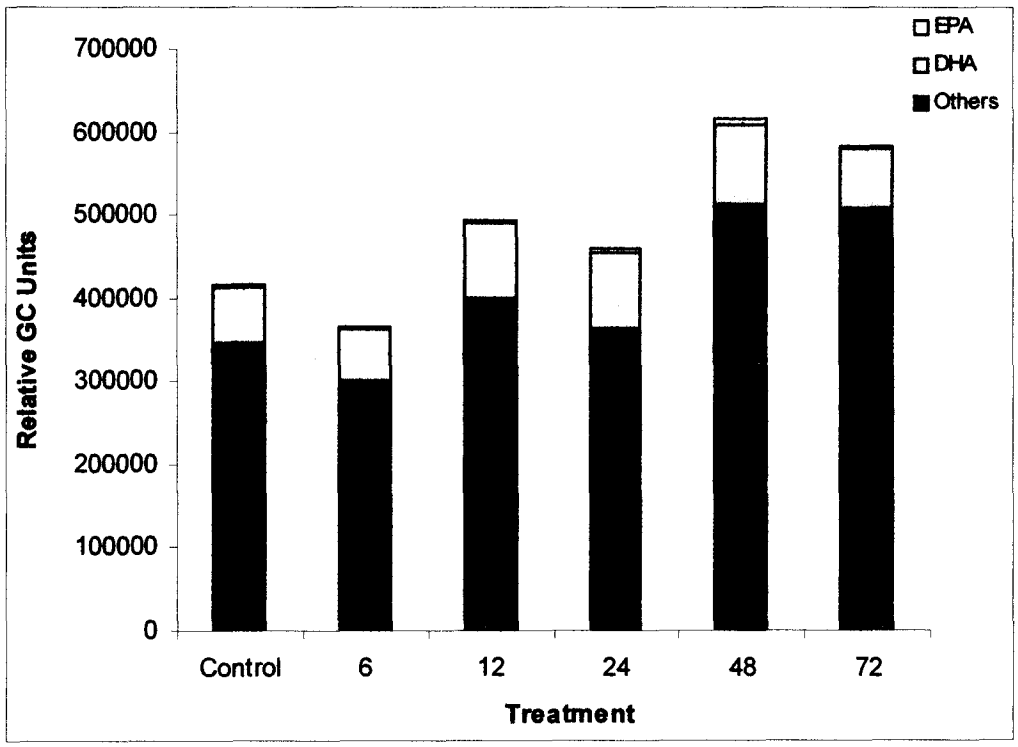


Fig. 2.3.29: Effect of refrigeration time at 10 °C following growth at room temperature on total fatty acids, DHA and EPA contents of the isolate NIOS-1.

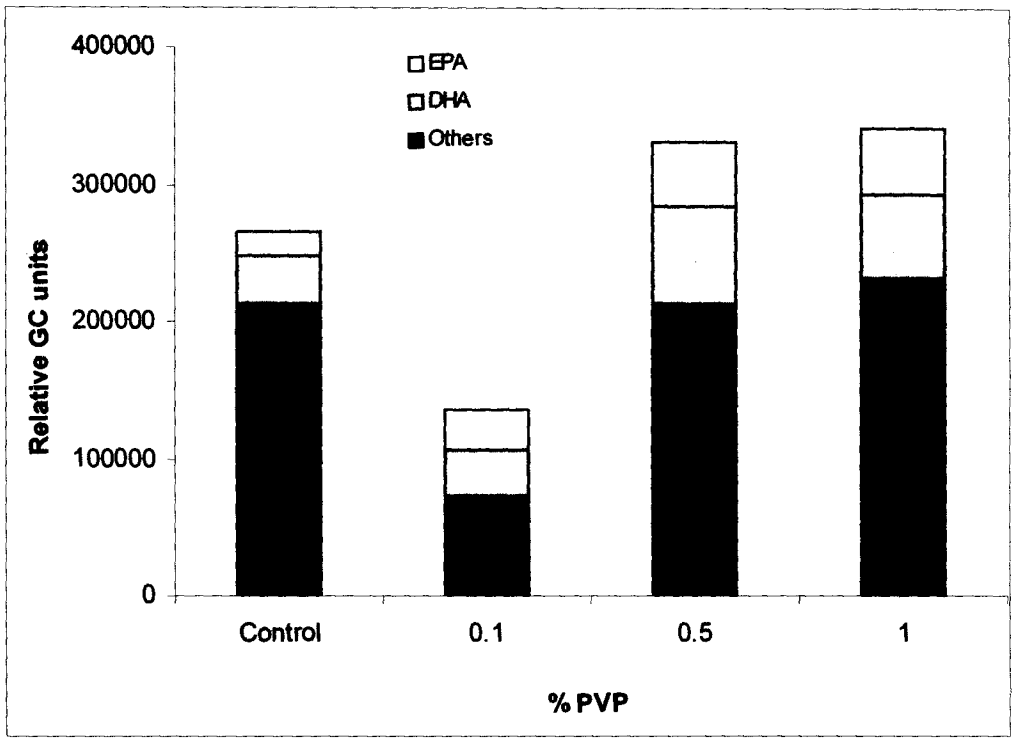


Fig 2.3.30: Effect of percent PVP on total fatty acids, DHA and EPA contents of the isolate NIOS-11.

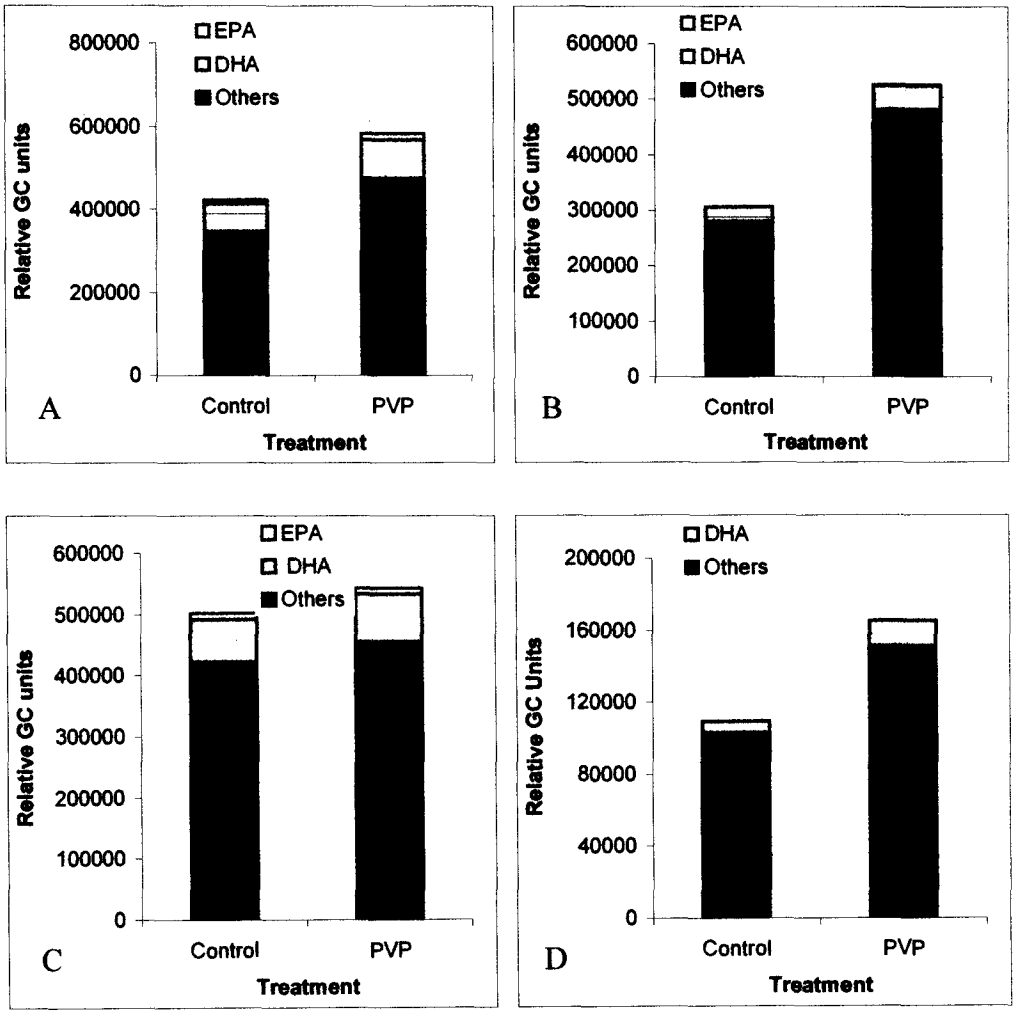


Fig.2.3.31: Effect of increased viscosity of culture medium on total fatty acids, EPA and DHA contents of the isolate NIOS-1 (A), NIOS- 2 (B), NIOS-4 (C) and NIOS-9 (D).

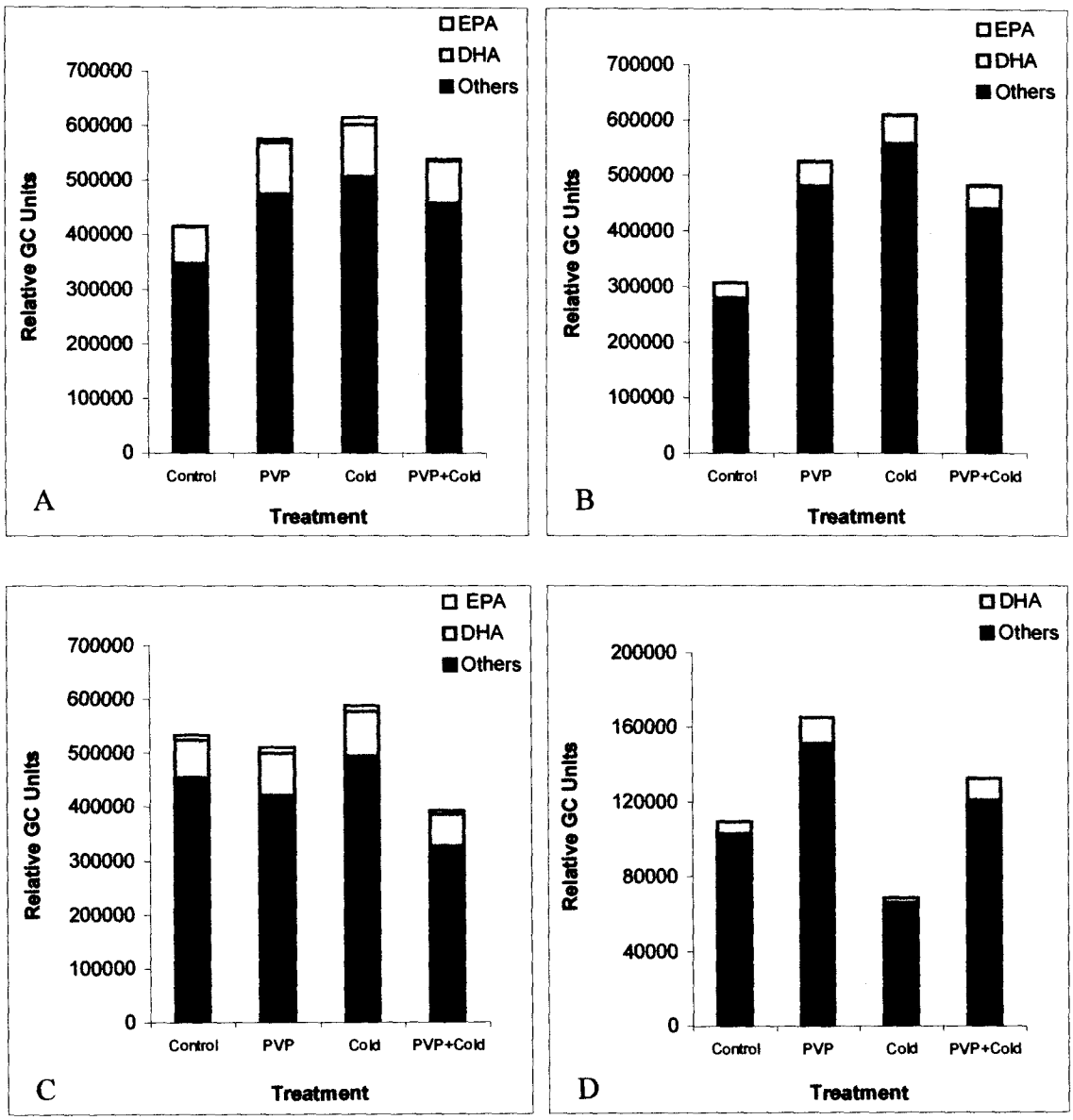


Fig.2.3.32: Effect of refrigeration and increased medium viscosity on total fatty acids, EPA and DHA contents of the isolate NIOS-1 (A), NIOS- 2 (B), NIOS-4 (C) and NIOS-9 (D) following growth at room temperature.

2.4. Discussion

Glucose was found to be the best carbon source for growth in all isolates of thraustochytrids examined in this study (Fig. 2.3.7). Although thraustochytrids are capable of utilizing a wide range of organic carbon sources (Bahnweg, 1979 a), glucose, maltose, cellobiose and starch in general appear to be the best for their growth (Goldstein, 1973; Bahnweg, 1979 a). Moreover, glucose can be efficiently converted into lipids by a number of fungi and thraustochytrids (Singh and Ward, 1997). Glycerol is yet another good organic carbon source (Bahnweg, 1979 a). An exception is the genus *Althornia*, which grows best with malate (Goldstein, 1973). Glucose and polymers of glucose are generally the most ideal source of organic carbon for biomass production in thraustochytrids (Yokochi *et al.*, 1998; Huang *et al.*, 2001). The isolate *T. aureum* ATCC 34304 (Bajpai *et al*, 1991 b) and *T. roseum* ATCC 28210 (Li and Ward, 1994) produced maximum DHA yield with starch. Fructose and glycerol also yielded good growth. Yokochi *et al.* (1998) also have shown that fructose and glycerol supported the best biomass production in the isolate of *Schizochytrium limacium* SR 21. The isolate *T. aureum* ATCC 34304 (Bajpai *et al*, 1991 b) and *T. roseum* ATCC 28210 (Li and Ward, 1994) grew poorly on fructose and also produced less lipids. This suggests that conversion of carbohydrates to lipids is low when, carbon source substantially limits cell growth.

Inorganic nitrogen sources, such as nitrate, nitrite and ammonia are poorly utilized by these organisms (Goldstein, 1973; Bahnweg, 1979 b.). Therefore, organic sources need to be incorporated in the medium. While thraustochytrids

are capable of growing in a minimal medium using sodium glutamate alone (Goldstein, 1973; Bahnweg, 1979 b), peptone or corn steep liquor has generally been used for biomass production (Bajpai *et al.*, 1991 a; Barclay *et al.,* 1994 b; Yokochi *et al.*, 1998; Bowles *et al.*, 1999; Huang *et al.*, 2001; Fan *et al.*, 2002; Kamlangdee and Fan, 2003). Therefore, peptone was used as the nitrogen source in this study.

Various species of thraustochytrids require either thiamine, biotin, or cyanocobalamine, or combinations of these for growth (Goldstein, 1973; Bahnweg, 1979 b). Many authors have incorporated a vitamin supplement in the growth medium, in addition to yeast extract (Bajpai *et al.*, 1991 b; Barclay *et al.,* b 1994). Additional trace elements also have been used in growth media for thraustochytrids (Bajpai *et al.*, 1991 a; Barclay *et al.,* 1994 b). However, NIOS-1 grew equally well both in a medium with only yeast extract, as well as one where additional vitamins were provided (Fig. 2.3.3). Metal ions such as Mn^{+2}, Co^{+2}, Zn^{+2} Mg^{+2}, promote synthesis of lipids and PUFAs (DHA, ARA, GLA) in microorganisms (Singh and Ward, 1997). Acetyl-CoA carboxylase catalyzes the initial step of fatty acid synthesis and requires Co^{+2} or Mg^{+2} as cofactors. Similar results were obtained by Singh *et al.* (1996) where the production of DHA significantly increased with supplementation of trace metal solution containing Co^{+2} and Mg^{+2}. However, no significant effect of trace metal supplementation was found in cultures of *Thraustochytrium* sp. ATCC 20891 (Barclay *et al*, 1994 b). This may be because that the trace metal requirements of these isolates was

low, being fulfilled by the metal ions present in seawater. Extra addition of trace elements was not found necessary for growth of NIOS-1 (Fig. 2.3.3).

The pH of seawater is approximately 8 to 8.4. Most of the isolates studied by Bahnweg (1979 b) had a pH optimum between 6.0 and 8.0, few growing below a pH of 5.0 or above 11.0. However, the pH optimum may vary from 4 to 7, depending upon the species (Nakahara *et al.*, 1996; Fan *et al.,* 2002). A pH value of 7.0 was preferred in this study, because growth and percentage lipids were highest at this pH (Fig. 2.3.5 and 2.3.6) Since thraustochytrids are obligate marine organisms, a salinity value corresponding to that of seawater, which is approximately 34 ppt might be expected to be ideal. Although thraustochytrids are euryhaline and grow at salinities ranging from approximately 4 ppt to that of 70 ppt, optimal growth is often achieved best at salinities approximating that of seawater (Jones and Harrison, 1976). Mangrove isolates, however, might have a lower optimal range of salinity, ranging from 7.5 to 30 ppt (Fan *et al.*, 2002). A high DHA-yielding strain of *Schizochytrium* grew best between 50 and 200 % seawater (Yokochi *et al.*, 1998). Sodium chloride concentrations of 1 to 3 % are optimal, few growing at a concentration of 0.5 % (Goldstein, 1973; Bahnweg, 1979 b). NIOS-1 grew best at the normal seawater salinity of 35 ppt. Growth was very poor below 15 ppt, indicating its obligate marine nature (Fig. 2.3.4).

Biotechnologically meaningful production of DHA depends on optimal biomass, lipid and DHA production. Representative values of biomass, lipid and DHA production by thraustochytrids are shown in Table 2.4.1. The four isolates used in the present study produced 3.6 g L^{-1} to 19.12 g L^{-1} wet weight biomass.

The isolate NIOS-1 produced a biomass of 19.12 g L^{-1}, suggesting that it was an ideal strains to work with (Fig. 2.3.1). Numerous factors influence biomass production, particularly the carbon and nitrogen content in the medium (Kendrick and Ratledge, 1992; Barclay, 1994 b; Nakahara *et al.*, 1996; Singh and Ward, 1997). Experiments using the conventional 'one factor at a time' (OFAT) approach (Fig. 2.3.9 to 2.3.17) suggested that the following medium composition was the best for biomass production in isolate NIOS-1: Glucose: 3 %; Peptone: 0.15 % and Yeast extract: 1.5 %. This combination yielded 34.6 g L^{-1} biomass in NIOS-1 (Fig. 2.2.18). However, media optimization using RSM suggested that, while glucose had a significant effect on biomass production, peptone and yeast extract did not. Surface plots for growth indicated that even higher biomass production could have been reached using glucose concentrations in excess of 3 % (Fig. 2.3.19).

Lipid concentration in thraustochytrids amount 11.93 % to 65.0 % of their biomass (Table 2.4.1). The six isolates examined in this study produced 7.5 % to 36 % lipids in their biomass (Fig. 2.3.1.). Once again, NIOS-1 produced the highest amounts of lipids of 36 %. Lipid concentration in biomass appears to be characteristic of individual thraustochytrid species (Singh and Ward, 1997). While optimizing the production of lipids using the OFAT method, it was seen that total lipids was related to biomass, thus being probably related to glucose. (Figs. 2.3.11, 2.3.14, 2.3.17,). Bajpai *et al.* (1991 a) have also found that lipids were positively related to glucose concentrations in *Thraustochytrium aureum*. In oleaginous microorganisms, cells continue to assimilate carbon, but can no

longer undergo cell division when nitrogen becomes limiting, preventing both protein and nucleic acid synthesis. This leads to sugar getting converted into storage oil (Ratledge, 2004). Yokochi *et al.* (1998) have shown that total fatty acid content was negatively influenced by total nitrogen, while RSM indicated that lipid production was positively related to peptone concentration. However, this was possibly more the effect of C:N ratios, since high C:N values were typically associated with enhanced lipid contents (Table 2.3.2).

It was found in the present study that one way to increase lipid contents in the cell was either to refrigerate cells, or to use a thickener to the medium, namely PVP, which increases the density and viscosity of the medium (Figs. 2.3.27 and 2.3.30). It is not clear as to how this led to a lipid increase. However, such increases were significantly higher, compared to controls that were not either refrigerated or did not contain PVP in the medium.

The kinds of PUFAs contained in lipids depend upon the species, both among the thraustochytrids (Fan *et al.*, 2001), as well as among different groups that produce lipids. Kendrick and Ratledge (1992) have compared the lipid composition of organisms belonging to thraustochytrids, oomycetes and fungi. Huang *et al.* (2003) have discussed the fatty acid profiles of different thraustochytrids and have suggested that this character, based on DHA, DPA, EPA and AA may even be applied to study the taxonomy of these organisms. Generally, however, most thraustochytrids contain a high amount of palmitic acid (16:0), ranging from 10.0 to 40.5 (Bajpai *et al.*, 1991; Barclay, 1994 b; Nakahara *et al.*, 1996; Yokochi *et al.*, 1998). Among the commercially useful PUFAs,

thraustochytrids produce EPA, ARA, and DPA, besides DHA (Yokochi *et al.*, 1998; Huang *et al.*, 2001). However, the amounts of the first 3 fatty acids are much lower than those known for commercially useful organisms. Thus, EPA and ARA are produced by fungi belonging to the mucoraceous genus *Mortierella*. The role of DPA is not yet clear.

DHA is the most valuable PUFA from thraustochytrids, ranging from 18.8 % to 52.1 % of lipids (Table 2.4.1; Bowles *et al*, 1999; Huang *et al.*, 2001). Percentage DHA in lipids varied from 2.4 in NIOS-6 to 22.7 in NIOS-1 among the 6 isolates studied (Table 2.3.1.). DHA concentrations are also characteristic of individual species, ranging from 30.5 % to 49%. (Table 2.4.1). A study by Bowles *et al.* (1999) has shown a considerable variation in DHA contents among different thraustochytrid strains. Those from a sub-tropical environment produced high biomass, but less percentage DHA in lipids, while temperate isolates harboured higher percentages of DHA, even if their biomass yield was lower. The isolate NIOS-1 contained fairly high amounts of DHA in the lipids. As with lipids, DHA levels can be improved in each species or isolate within a range that it is capable of. Addition of coconut oil to M4 medium increased the biomass and total lipids of isolate NIOS-1, but the DHA content was lower than the control. Similar results were reported by Bajpai *et al* (1991 a) and Li and Ward (1994) using exogenous oils. When the isolate *S. limacinum* SR21 was grown on oleic acid and linseed oil, the DHA content reduced from 32.5 % to 6.1 % (Yokochi *et al.*, 1998). Fatty acids added to the growth medium can be elongated to larger saturated fatty

acids and can be converted to 18:1 but not to 22:6 (Henderson and Mackinlay, 1991; Swaaf *et al.*, 2003).

RSM experiments showed that % DHA in lipids was not significantly affected by glucose, peptone or yeast extract, confirming similar observations made by Bajpai *et al.* (1991 a). On the contrary, absolute values of DHA were positively influenced by high peptone concentrations. However, a study of the results presented in Table 2.3.2., suggests that high peptone concentrations accompanied by high glucose concentrations yielded the best DHA yields. *Schizochytrium limacinum* SR21 produced higher DHA when glucose levels were increased (Yokochi *et al*, 1998). DHA yields were low even when peptone levels were high, if glucose concentrations were kept low. Therefore, as with lipids, elevated peptone concentrations might be favorable if accompanied by high glucose concentrations, resulting in high C:N ratios of the medium. Bowles *et al.* (1999) came to a similar conclusion while working with a thraustochytrid strain. If % DHA in lipids remains the same, absolute values of DHA can be enhanced by increasing the total lipids in the cell. This was achieved in the present study both by refrigerating the cell biomass, as well as by addition of PVP to the medium (Fig. 2.3.27. to 2.3.30).

The cause for increase in lipids, and therefore DHA by refrigerating cells or by PVP addition is not clear. However, it is possible that, since the solubility of oxygen at low temperatures increases, a greater amount of intracellular molecular oxygen is available under such conditions. This will favour oxygen-dependent enzymes catalyzing the desaturation of PUFAs (Singh and Ward,

1997). Lowering the temperature also induces the production of unsaturated fatty acids to increase membrane fluidity, as an adaptation to lower temperatures. DHA is also reported from many deep-sea bacteria as an adaptation to the ecological niche. (Nichols and McMeekin, 2002; Nicholas, 2003) Interestingly, the temperature for refrigeration and percentage of PVP in the medium appear to play an important role. While lower temperatures (15 and 10 °C), as well as higher PVP concentrations (0.5 % and 1.0 %) increased lipid and DHA levels, a temperature of 20 °C or a concentration of 0.1 % PVP were detrimental.

Organisms	Biomass g L^{-1}	Total lipids %dry weight	% DHA	DHA yield (mg L^{-1})	Reference
Schizochytrium sp. SR21	21	50	35	4700	Nakahara *et al.*, 1996
S. limacinium SR21	38	37	33	4200	Yokochi *et al.*, 1998
T. aureum ATCC 34304	3.8	16.5	49	270	Bajpai *et al.*, 1991
T. roseum ATCC 28210	17.1	25	49	2100	Singh and Ward, 1996
G13	2.93	65	30.2	57.5	Bowles *et al.*, 1999
Schizochytrium sp KF6	13.5	ND	41.1	2762	Fan *et al.*, 2001
Thraustochytrium sp KK17-3	1.5	11.93	30.5	54.6	Huang *et al.*, 2001

Table 2.4.1: Production of biomass, total lipids and DHA by thraustochytrids. ND= not determined.

2.5. Conclusion

Six isolates were screened for biomass, total lipids and DHA concentration. The unsaturated fatty acid, palmitic acid was generally the most abundant of all fatty acids. All isolates produced the polyunsaturated fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). DHA was the most abundant PUFA found. The isolate NIOS-1 produced the best wet weight biomass (19.1 g L^{-1}) with the highest lipid concentration (36 % of dry weight) and maximum yield of DHA (22.6 % of total lipids) from among the six. A medium designated 'M3' yielded the highest dry weight biomass. NIOS-1 had no additional requirements of vitamins and trace metals. The optimum pH and salinity for biomass production was 7 and 35 ppt respectively. Glucose was the best carbon source among a total of 10 examined. Exogenous oils did not promote DHA production. This medium, designated 'M4' medium, was further optimized for maximum biomass production and lipid content by two methods – One factor at a time (OFAT) and Response Surface Methodology (RSM). According to the OFAT method, 3 % glucose, 0.15 % peptone and 1.5 % yeast extract gave the best biomass with optimum percent lipids. According to the RSM method, glucose had a significant effect on biomass production. Percentage of total lipids was increased by high C:N ratio. Percentage DHA was not affected by glucose, peptone or yeast extract. However the total DHA yield was positively related to glucose concentration. The lipid content as well as the PUFA – DHA and EPA contents of thraustochytrids could be enhanced by harvesting the cells and storing them at 10 °C for 48 h. Similar

increases could also be achieved by increasing the medium viscosity by addition of 0.5 % polyvinylpyrrolidone.. Although both treatments increased the total yields of DHA, the percentage of DHA remained constant. Addition of 0.5 % PVP to the growth medium and storage of harvested cells at 10 °C resulted in reduction of total lipids as well as the DHA yield.

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 Crptophyceae 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 c lipids?

- Why are the triacylglycerols generally rich in DHA ?
- Why does DHA increase during refrigeration and when the medium viscosity is increased?

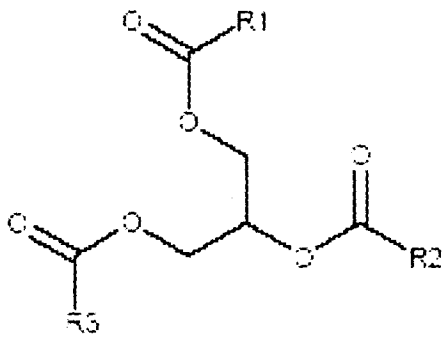


Fig. 3.1. Schematic representation of triacylglycerol, R_1 – CO, R_2 – CO and R_3 - CO are the fatty acyl groups.

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 phenoxazine 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).

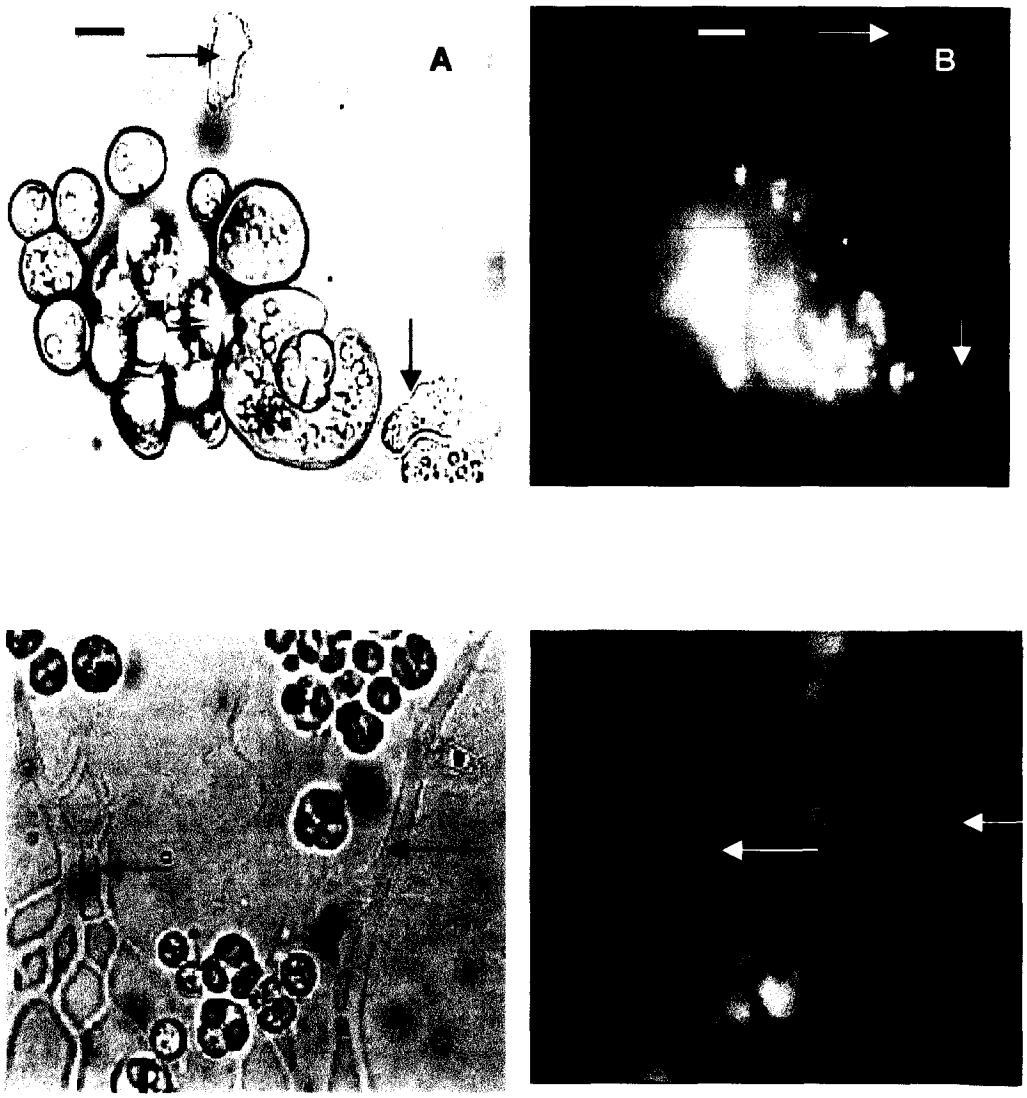


Fig. 3.3.1: Nile blue staining of isolate NIOS-1. A and B: Vegetative and amoeboid cells 2 h after spreading on MV agar. A: Bright field photomicrograph. B: Epifluorescence photomicrograph showing fluorescent lipid bodies. Note their presence in round cells and absence in the amoeboid cell (arrow). C 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.

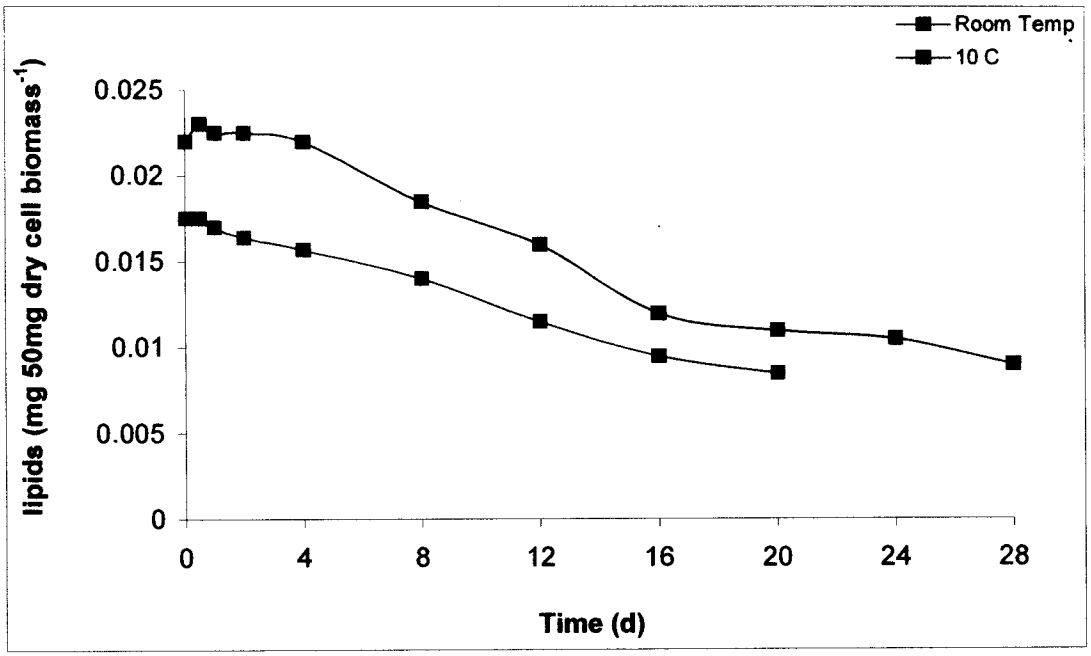


Fig 3.3.2: Total lipid profile of cells during starvation.

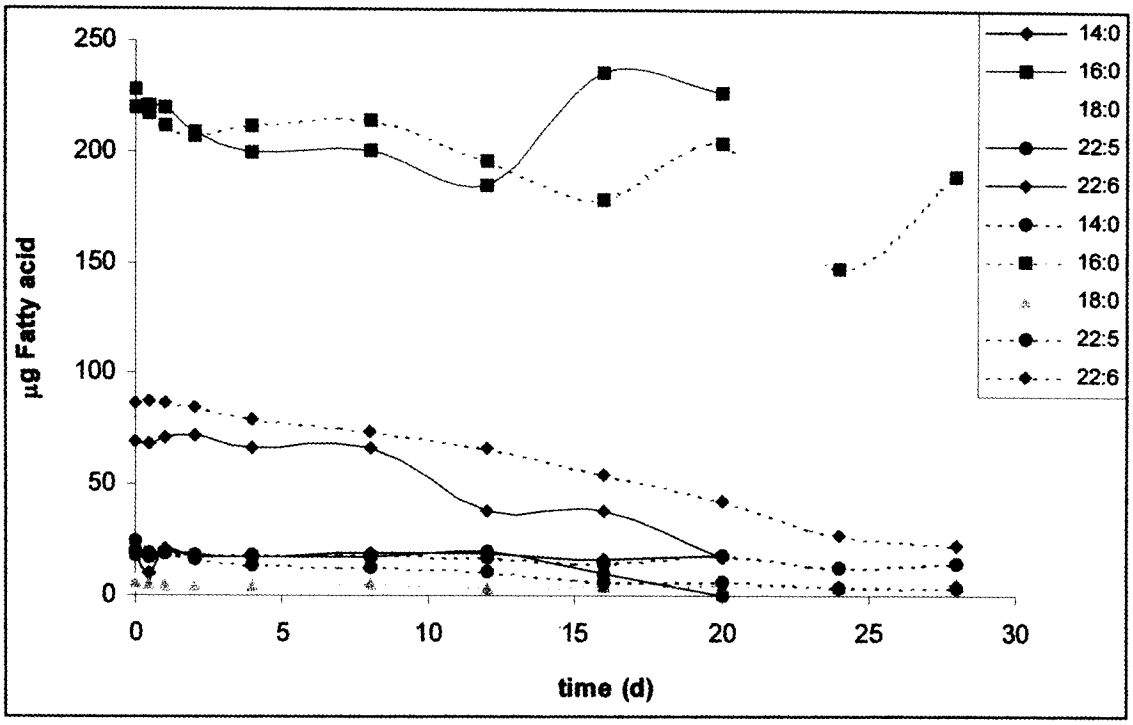


Fig 3.3.3a: Total amounts of fatty acids in refrigerated (-----) and non-refrigerated (——) cells present per gram of dry weight biomass on starvation of isolate NIOS-1 at room temperature.

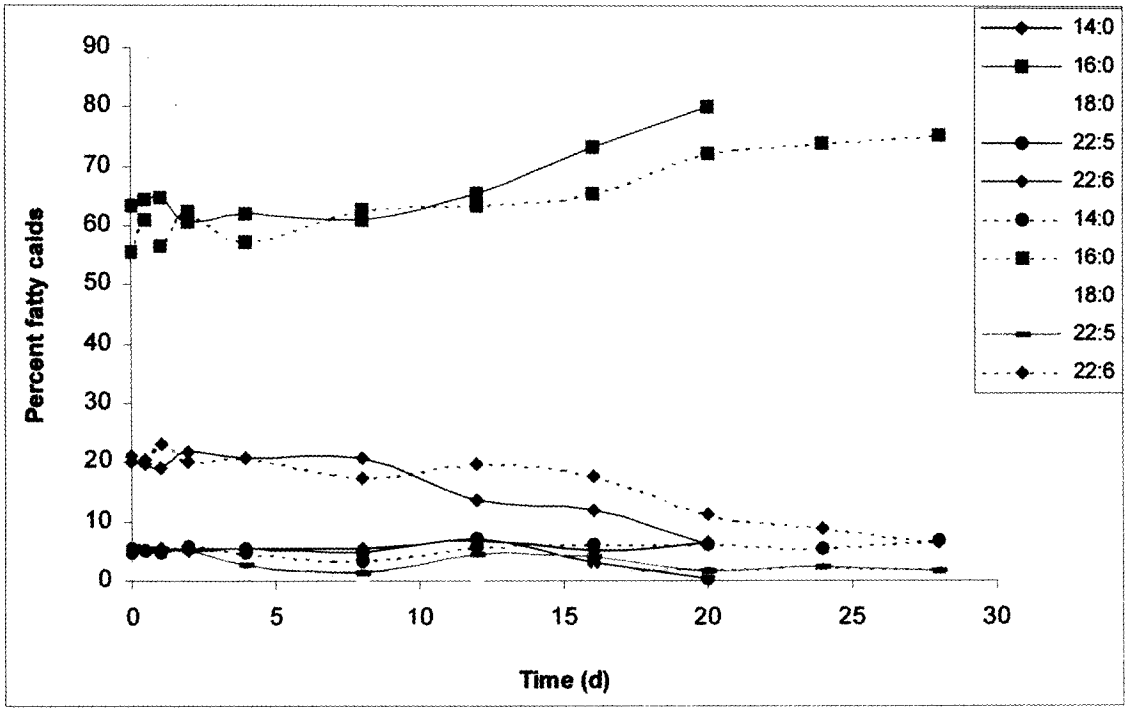


Fig 3.3.3b: Percentage fatty acids in refrigerated (-----) and non-refrigerated (—)cells on starvation of isolate NIOS-1 at room temperature.

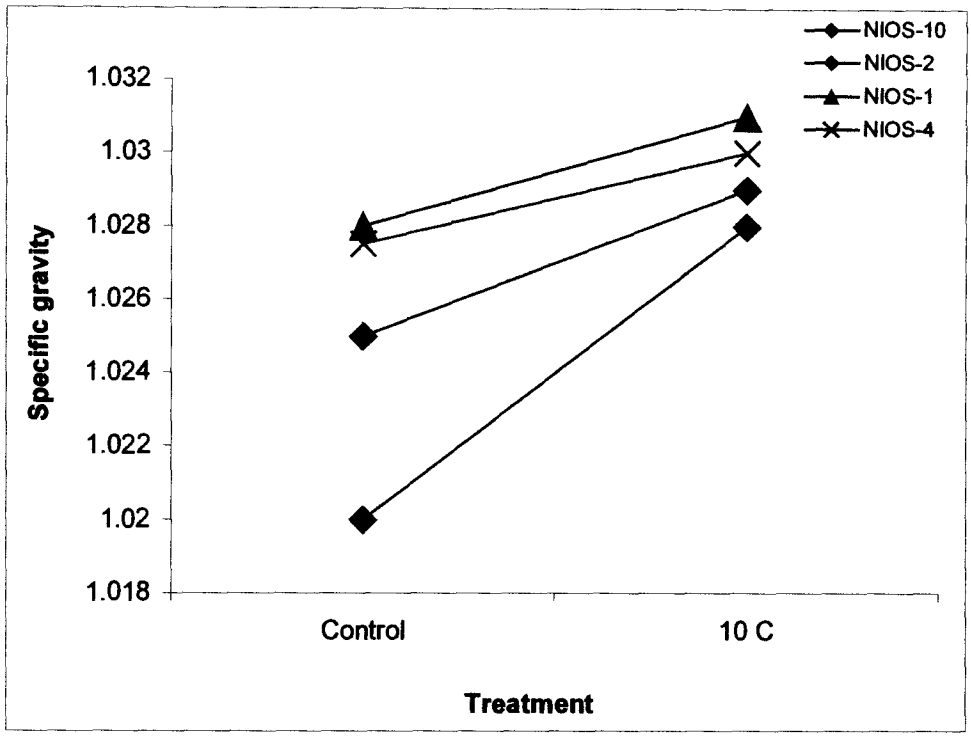


Fig 3.3.4: Specific gravity of cells before and after refrigeration.

Isolate	Treatment	Fatty acid (mg) DCW (g) $^{-1}$	Fatty Acids								
			14:0	16:0	16:1	18:0	18:1	18:2	20:4 n 6	22:5 n 6	22:6 n 3
NIOS-1	Non-refrigerated	258.3	5.18	60.73	0.00	1.43	0.00	0.00	0.03	5.14	23.25
	Refrigerated	501.4	5.23	58.09	0.00	1.35	0.00	0.00	0.03	5.51	25.20
NIOS-2	Non-refrigerated	15.7	0.61	29.75	0.00	2.39	11.95	0.00	5.94	11.51	19.32
	Refrigerated	27.2	0.00	26.70	0.00	3.60	18.15	0.00	5.00	7.40	21.20
NIOS-4	Non-refrigerated	40.35	23.48	52.15	0.67	1.49	0.84	0.00	0.00	3.63	9.57
	Refrigerated	44.45	19.95	45.30	0.00	1.54	0.84	0.00	0.00	5.74	16.94
NIOS10	Non-refrigerated	205.2	5.66	36.88	8.14	2.49	20.06	13.47	2.78	2.25	1.26
	Refrigerated	368.45	4.65	30.65	6.01	3.93	18.05	13.86	3.26	2.55	2.34

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-} 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 Jònasdòttir, 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.

CHAPTER 4

Production and biochemistry of extracellular polymeric substances by thraustochytrid protists

4.1. Introduction

Extracellular polymeric substances (EPS) are organic macromolecules that are formed by polymerization of similar building blocks, which may be arranged as repeating units within the polymer molecule (Wingender *et al.*, 1999). Microbial EPS are studied for several reasons (Neu and Lawrence, 1999).

1. EPS confer several ecological advantages to the cells producing them and also play a major role in biogeochemical cycles in the ecosystem.
2. EPS often represent a structural feature of microbial cell and is, therefore, investigated for pure and basic research.
3. EPS are recognized as antigen determinants of the microbial cell surface. The knowledge of their structure is of great significance in medical microbiology.
4. EPS are polymers with unique properties and so are important in biotechnological applications.

Most marine microorganisms produce EPS in their ecosystem (Decho, 1990).

The amount of EPS produced by various organisms may range from low levels, such as 0.24 g L^{-1}, as in a *Pseudomonas* species, to very high amounts of 33 g L^{-1} as in a *Xanthomonas* species after optimization (Christensen *et al.*, 1985; Sutherland, 1998).

EPS is mostly composed of polymer chains with high molecular weight ranging from 70 – 2800 kDa (Philippis and Vincenzini, 1998). The composition and production of EPS is very variable and is dependent on (Decho, 1990):

a) The physiological state or growth stage of the cell. It has been observed that production of EPS starts in the exponential phase of growth but maximum production occurs during the stationary phase in most organisms (Whitefield, 1988). Some strains are known to produce different polymers at different growth stages. (Sutherland, 1997).

b) The composition of the nutrient media in which the culture is grown. Many microorganisms in laboratory culture can be induced to yield large quantities of EPS when grown in a high-carbon: low-nitrogen growth media (Williams and Wimpenny, 1978). Although excess carbon in media increases EPS production, it is not a necessity for its production. EPS production has also been observed when the cultures were grown only on amino acids but the production was very low (Sutherland, 1979; Sutherland, 1982; Wicken, 1985).

c) The ionic and physical condition of the media. Temperature and pH are also known to affect the composition of the polymer.

The ability of microorganisms to produce exopolymers under varying conditions is thought to reflect the important function of these secretions under fluctuating nutrient and environmental conditions.

4.1.1. Structure and Composition of EPS

Backbone polymer: The backbone polymer in EPS is usually a polysaccharide, although some organisms are known to secrete a proteinaceous polymer also (Platt *et al.*, 1985; Frølund *et al.*, 1996). Polysaccharides form a large portion of

EPS. Heteropolysaccharides are more common in the marine ecosystem, being made up of repeating units of 6 or fewer monosaccharides (Anton *et al.,* 1998). The commonly occurring hexoses are glucose, galactose and mannose, while xylose, arabinose and ribose are common among the pentoses. Deoxyhexoses are mostly represented by fucose and rhamnose (Decho, 1990; Philippis and Vincenzini, 1998)

A variety of organic and inorganic substituents are found associated with EPS. These non-carbohydrate components make up a relatively smaller portion of the EPS on a per weight basis but are extremely important to the tertiary structure, as well as physicochemical and biological properties of the EPS. These components are mostly in the form of residues and side groups on the polysaccharide chain (Philippis and Vincenzini, 1998; Majumdar *et al.*, 1999).

Organic substituents: These include amino acids, amino sugars, proteins, uronic acids, acyl, ketals and pyruvates. Proteins can also be glycosylated with oligosaccharides to form glycoproteins or can be substituted with fatty acids to form lipoproteins (Sutherland, 1980; Orr *et al.*, 1982). Proteinaceous material, in the form of exoenzymes is also found to be associated with EPS. However, the concentration of nitrogenous material is generally less than 10 % in purified polymer. Removal of the proteinaceous moiety drastically reduces the viscosity of the aqueous solution of the polysaccharides (Arad *et al.*, 1993).

Uronic acids are carboxylated forms of sugars and the commonly found uronic acids are glucuronic and galacturonic acids. Their presence confers an

overall negative charge and acidic property to the polymer. The absolute amounts of uronic acids increase in the EPS with increase in age and metabolic stress. It has been postulated that the presence of acyl groups such as O-acetyl and pyruvate groups are essential to protect uronic acids from epimerisation (Decho, 1990).

Presence of acetyl groups and the deoxy sugars, fucose and rhamnose confer a lipophilic character to EPS (Shepherd *et al.*, 1995). Presence of acetyl groups hinders cation binding (Geddie and Sutherland, 1994). Moreover acetyl groups may contribute to the stabilization of the ordered form of the polymer, as has been reported in xanthan structure (Sutherland, 1994).

Pyruvates are often ketal linked and contribute to the water binding properties of the EPS (Sutherland, 1979).

Inorganic substitutents: These include phosphate and sulfate groups, and are very common in the marine ecosystem. These groups confer a negative charge to the polymer (Decho, 2000). Polysaccharides having concentrations of charged components usually form stable gels in the presence of metallic ions. However, mere determination of the quantity of the charged groups is not enough for predicting the metal binding capacity of the polymer, as the accessibility of charged groups to ions depends on the conformational structure of the molecule (Philippis and Vincenzini, 1998).

The objectives of the experiments carried out under this chapter are:

1. Microscopic confirmation of the presence of EPS;

2. Kinetics of EPS production and the effect of media ingredients on EPS production;
3. Gross biochemical characterization of the EPS;
4. Preliminary structural elucidation of the EPS.

4.2. Materials and Methods

4.2.1. Cultures and Growth conditions

Five isolates of thraustochytrids - NIOS-1, NIOS-2, NIOS-4, NIOS-7, and NIOS-12 were used to study production of EPS. The protocol given in Chapter 2 was followed for inoculum preparation and growing the cultures to study the EPS production. M4 medium was used for all the studies. The experiments were set up in triplicates and the cultures were grown for 7 day.

4.2.2. Light and scanning electron microscopy

Microscopic mounts of cells from seven-day old cultures grown in M4 broth were examined by phase contrast microscopy for the presence of EPS. The following methods were used for further microscopic examination using light and scanning electron microscopy. Sterile cut pieces of microscope slides, measuring approximately 25 mm^2 (5 mm X 5 mm) were placed in sterile 5 cm Petri dishes and were covered with M4 medium (see Chapter 2). A loopful of culture grown in M4 broth was inoculated into these plates. Following growth for 10 days, some of the glass pieces were stained with 1.0 % alcian blue in 3.0 % acetic acid, pH 2.5 to test for acidic polysaccharides (Passow and Alldredge, 1994). Standard procedures were followed for scanning electron microscopy (Heywood, 1971). Cells grown on glass pieces as above were dehydrated in 10, 20, 50, 80, 90, and 100 % acetone for 15 min each, followed by dehydration in 100 % acetone for 2 rounds of 15 min each. The cells growing on the glass pieces were immediately critical point dried using a SPI supplies, US, make

instrument. This was followed by sputter-coating with gold-palladium for 45 s. Following gold palladium sputter coating, the pieces were examined under a Jeol JSM- 5800LV scanning electron microscope.

4.2.3. Extraction and estimation of exopolysaccharides

The isolates NIOS-1, NIOS-4, NIOS-7 and NIOS-12 were grown in M4 medium as described above. The EPS was extracted as follows. Cultures were transferred to 50 ml centrifuge tubes and centrifuged at 7500 rpm (5200 g) for 15 min. The pellet was discarded and the supernatant was first filtered through Whatman GF/F filter and then through 0.45 µm filters. The filtrate was then concentrated 10 X by ultrafiltration using an Amicon make ultrafilter having a molecular cut off of 10 kDa and dialyzed against distilled water. Three volumes of cold absolute ethanol was added to the retained solution and the mixture was left overnight at 4 °C. The precipitated EPS was centrifuged as above and lyophilized using a Thermo Savant Micro Modulyo freeze drier prior to storage at -20 °C. Total EPS produced was estimated gravimetrically. This EPS was used for all further analyses.

4.2.4. Production of EPS

4.2.4.1. Kinetics of EPS production with respect to growth in isolates NIOS-1 and NIOS-4

The growth and EPS production by isolates NIOS-1 and NIOS-4 in M4 medium were monitored. Three-milliliter aliquots were removed for turbidity measurements (OD 660 nm) and EPS production at regular intervals. In order to

estimate EPS, samples were centrifuged (5200 g, for 15 min) and serially filtered through a Whatman GF/F glass fiber filter and 0.45 µm membrane filters. The filtrate was collected and dialyzed. EPS was extracted as above. EPS was estimated as total carbohydrates using the phenol-sulfuric acid method (Dubois *et al.*, 1956). The experiment was carried out until the culture reached the stationary phase.

Assimilation of organic carbon into EPS in NIOS-4 was studied following the basic protocols provided by Deming (1993). Twenty-five milliliters of M4 medium in a 100 ml Erlenmeyer flask was inoculated with a 2-day culture as above. Radiolabelled glucose (^{14}C glucose, 0.05 mCi ml^{-1}), obtained from Bhabha Atomic Research Centre, Trombay, India, was used for the experiments. An end concentration of 20 µCi of ^{14}C glucose was added to the 25 ml culture. A sterile Eppendorf tube containing a filter paper soaked in phenylethylamine was suspended in the flask using a string to measure the amount of CO_2 respired from the glucose. The flasks were stoppered tightly using rubber stoppers. The culture was incubated for 24 h at room temperature on a shaker at 150 rpm. The cells were then harvested by vacuum filtration through 0.22 µm filter paper. Labeled EPS in the culture filtrate was precipitated by ethanol (70 % final concentration) and its radioactivity was recorded by using Perkin Elmer Wallac 1409DSA liquid scintillation counter. The radioactivity in the respired CO_2, in the cells and unutilized glucose (culture filtrate after EPS precipitation and removal) was also measured.

4.2.4.2. Effect of media ingredients on the production of EPS

The same set of cultures used for optimizing DHA production by the isolate NIOS-1 using the Response Surface Methodology (RSM), as given in Chapter 2, was also used to study the relationship between media ingredients, DHA and EPS production. After harvesting the cells (RSM experiment) the remaining culture filtrate was used for EPS estimations.

4.2.5. Biochemical composition of the EPS

4.2.5.1. Total Carbohydrates

The total carbohydrates were estimated according to the protocol of Dubois *et al.* (1956). To 0.5 ml sample, 0.3 ml 5 % phenol and 1.8 ml concentrated sulfuric acid (sp.gr. 1.84) were added and mixed thoroughly. After 20 min incubation, the OD was read at 480 nm. Sugar content was estimated by referring to a standard graph prepared by using D-galactose (5-50 μg / 0.5 ml).

4.2.5.2. Protein estimation

Composition of the various reagents is given in Appendix 2. A modified Lowry's method was followed to estimate the total protein (Peterson, 1977). EPS at a concentration of 1 mg ml^{-1} in distilled water was taken, to which 0.1 ml of 0.15 % deoxycholate was added. The solution was incubated for 10 min at room temperature, to which 0.1 ml 72 % TCA was added. The mixture was centrifuged for 7500 rpm for 15 min. The pellet was dissolved in 1 ml of distilled water to which 1 ml Reagent A was added (Appendix 2), mixed and the mixture was

incubated at room temperature for 10 min. This was followed by an addition of 0.5 ml of Reagent B (Appendix 2) and the OD was read at 750 nm after 30 min. The concentration of protein was estimated by referring to a standard curve prepared using bovine serum albumin (0 – 100 μg ml^{-1}).

4.2.5.3. Uronic acids

Knutson and Jeanes' (1968) method was followed to estimate the total uronic acid content of the EPS. To 0.5 ml sample, at ice-cold temperature, 3 ml of concentrated sulfuric acid was added drop wise and mixed thoroughly. This solution was boiled in a boiling water bath for 20 min, cooled and 0.1 ml carbazole solution was added (0.1 ml, 0.1 % prepared by dissolving recrystallized carbazole in alcohol. Carbazole was thrice recrystallized by using benzene in order to remove contaminants in the commercially available sample). The tubes were incubated in dark for 2 h and the OD was read at 530 nm. Uronic acids content was determined by referring to the standard graph prepared by using D-galacturonic acid (10-50 μg 0.5 ml^{-1}).

4.2.5.4. Sulfate estimation

Total sufates were estimated according to the procedure of Dodgson (1961). Twenty milligrams of sample was hydrolyzed with 3 ml of 60 % formic acid at 100 °C for 8 h. The hydrolysate was evaporated to dryness and reconstituted with 2 ml distilled water. To 0.5 ml hydrolysate, 3.8 ml of 4 % TCA and 1 ml of 5 % gelatin in 50 % barium chloride were added and incubated at

room temperature for 10 min. The OD was read at 500 nm and the sulfate content was determined by referring to a standard curve prepared by using potassium sulfate (0 – 50 μg)

4.2.5.5. Reducing Sugars

The method of Imoto and Yagishita (1971) was followed to estimate the total reducing sugars. To 1 ml of sample, 2 ml of potassium ferricyanide reagent (0.05 % potassium ferricyanide in 0.5 M sodium carbonate) was added and boiled for 15 min. The samples were cooled and OD was read at 420 nm. Reducing sugar content was estimated by referring to a standard curve prepared by using D-glucose (0-100 μg).

4.2.6.6. Solubility and Viscosity measurements

Solubility of the polymers was studied in 0.1 M EDTA, 0.5 M EDTA, 0.1 N NaOH, 1 N NaOH, 10 % SDS, 0.1 N H_2SO_4, 1 N H_2SO_4, 1 N HCl, DMSO, hexane and distilled water. EPS was dispersed at a concentration of 1 mg ml^{-1} in each of above for 4 h. The insolubles were removed by centrifugation at 7500 rpm for 15 min. The solubility was determined by estimating the concentration of total sugar by phenol-sulfuric acid method in the supernatant.

The polysaccharide (50 mg) was suspended in 10 ml distilled water and stirred for 4 h at room temperature. This solution was centrifuged and the pellet was air dried at room temperature for 5 d, thereby evaluating the solubility of the polymer. For viscosity measurements, 0.6 % EPS in distilled water was analyzed

using a Brookfield Model DV-III programmable rheometer. The values are expressed as centipoises. The standard used was distilled water, with a viscosity of 0.89 cp.

The polymer had maximum solubility in distilled water. Therefore, the water-soluble fractions of both the polymers were lyophilized and stored at –20 °C for further use. The sugar, protein, lipid, uronic acid and sulface contents were estimated for the total, native polymer as well as the soluble fraction.

4.2.6. Molecular weight determination

4.2.6.1. Ion exchange chromatography

The purity of the lyophilized EPS was confirmed by ion exchange chromatography, using DEAE-cellulose (HiMedia, India) (Khandeparkar and Bhosle, 2001; Jayaraman, 2001). The lyophilized EPS was suspended in 200 mM phosphate buffer containing 0.1 M NaCl (pH 7.5), and applied to a column (12.5 cm X 1 cm) of DEAE-cellulose. Samples were eluted with a linear gradient of 0.1 M to 1.0 M NaCl (pH 7.5) in phosphate buffer at a flow rate of 8 ml h^{-1}. One-milliliter fractions were collected and analyzed for total sugars by phenol - sulphuric acid method.

4.2.6.2. Gel permeation chromatography

Molecular weight of the EPS was determined by gel permeation chromatography (GPC) according to the procedure of Brown and Volence (1989). GPC was performed on Sepharose CL – 2B (1.6 cm X 92 cm). One

milliliter of sample (5 mg ml^{-1}) was loaded to the column and was eluted with degassed triple distilled water at a constant flow rate of 1.6 ml min^{-1}. Fractions (1.6 ml) were collected and analyzed for the presence of total sugars. The column was calibrated with T-series dextran standards (T-70, T-150, T-500, T-2000, Pharmacia). A calibration curve was prepared by plotting elution volume (Ve)/ Void volume (Vo) versus log molecular weight and the molecular weight of the unknown sample was determined (Plummer, 1994).

4.2.6.3. High performance size exclusion chromatography (HPSEC)

The molecular weight of the EPS was confirmed by HPSEC. HPSEC was carried out using Shimadzu HPLC system (LC-8A model), equipped with refractive index (RI) detector and CR 4A recorder. E-Linear (7.8 mm X 30 cm) and E-1000 (3.9 mm X 30 cm) columns connected in series, were used for analysis. The EPS sample (5 mg ml^{-1}) were dissolved in tripled distilled water, 10 µl was loaded and eluted with triple distilled water at a flow rate of 0.6 ml min^{-1}. Similarly standards (T-70, T-150, T-500 and T-2000) were loaded individually. The elution was monitored by using RI detector set at 8 $X10^{-6}$ RIU (Kobayashi *et al*, 1985).

4.2.7. Structural elucidation of EPS

4.2.7.1. Determination of neutral sugar composition by GLC

The neutral sugar composition was determined according to the procedure of Sawardekar *et al.* (1967). The EPS (10 mg) was suspended in water and was

completely hydrolyzed by prior solubilizaton with 72 % sulfuric acid at ice-cold temperature followed by diluting to 8 % acid and heating at 100 °C for 10-12 h. The above mixture was neutralized with solid barium carbonate, filtered, deionized with Ambrelite IR-120-H+ resin and concentrated. Ten milligram of inositol was added as an internal standard. The monosaccharides were reduced by adding 20 mg of sodium borohydride at room temperature for overnight. Excess borohydride was decomposed by adding 2 N acetic acid, dropwise till effervescence of hydrogen stopped. The boric acid formed was removed by co-distilling with methanol (2 ml X 3). The dry glycitols were acetylated with acetic anhydride and pyridine (1 ml each). The mixture was kept at 100 °C for 2 h. Excess reagent was removed by co-distilling with water and toluene. The alditol acetates were extracted with chloroform, filtered through glass wool and dried under a jet of nitrogen. The derivatives were reconstituted in a known volume of chloroform and injected into GLC for quantitative and qualitative analyses.

Analysis of alditol acetates was carried out using Shimadzu GLC system (GC-15A), fitted with flame ionization detector and CR4 –A monitor. OV-225 (3 %) stainless steel column (8 feet X 1/8 inch internal diameter) was used for analysis with column, injector and detector port temperatures maintained at 200, 250 and 250 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 35 ml min^{-1}. Fucose, rhamnose, xylose, mannose, galactose and glucose were used as standards.

4.2.8. Linkage studies

4.2.8.1. Periodate Oxidation

Periodate oxidation helps in the determination of the type of linkages and the substituent group arrangements. When α, β and γ-triols are treated with periodate, one molecule proportion of formic acid is produced. Therefore, (1→6) linked hexopyranose units yield formic acid, whereas (1→2), (1→3) and (1→4) linked units do not (Fred, 1966).

To a 5 ml aqueous solution of 0.1 % polysaccharide 5 ml of 20 mM sodium meta periodate solution was added, mixed thoroughly and kept at 4 °C in dark. Aliquots (0.5 ml) of sample were withdrawn at regular intervals of 4 h, mixed with a solution containing 8 ml distilled water, 4 ml 20 % potassium iodide and 0.5 N, 0.6 ml sulfuric acid. The liberated iodine was immediately titrated with 0.1 N sodium thiosulfate using starch as an indicator. The consumption of periodate was calculated using the formula;

$$\text{Periodate consumption} = E - \frac{(V1-V2)}{1000} \times C \times \frac{M}{G}$$

Where; E = Actual moles of periodate taken

V1 = Titre value of blank

V2 = Tire value of sample

C = Concentration of sodium thiosulfate solution

M = Molecular weight of sugar

G = Weight of sample taken in grams

4.2.8.2. Formic acid liberation

Formic acid liberation was studied according to the procedure of Fred (1966). To 0.5 ml of above reaction mixture, after periodate consumption became constant, 2 ml of 50 % ethylene glycol and 2 - 3 drops of 0.02 % methylene red were add as an indicator. This mixture was titrated against 0.01 N NaOH. A reagent blank was prepared in the same way and the difference in the acidity between the blank and the sample represented the formic acid liberated from the polysaccharide and it was calculated by the formula

$$\text{Formic acid liberated} = \frac{V1 - V2\ XN_{NaOH}\ X\ MW}{0.5}$$

V1 = sample titer value

V2 = Blank titer value

N_{NaOH} =Normality of NaOH

MW = Molecular weight of the sugar

0.5 = Volume of sample taken for titration

4.2.8.3. Optical Rotation

Aqueous solution of the polysaccharide (1.0 %) was used to measure the optical rotation in a Perkin Elmer (model 243) polarimeter. Optical rotation is calculated using the formula

$$\text{Optical Rotation } (\alpha)_D = \frac{100\ \theta}{1C}$$

Where, θ = Angle of rotation of plane polarized light

1 = Path length (1 cm)

C = Concentration (%) of the polysaccharide solution

4.2.8.4. Infra-Red Spectroscopy

Polysaccharide (~2.5 mg) was blended thoroughly with solid KBr. IR spectra were obtained with Perkin Elmer Spectrophotometer (2000 system GC-IR) operating at 4 cm^{-1} resolution. Spectra were recorded between 400 – 4000 cm^{-1} (Lijour *et al.*, 1994).

4.2.9. Enzymatic method to deduce the structure of EPS

4.2.9.1. Screening enzymes, hydrolyzing the polysaccharide

Fifty microlitres of 1 mg ml^{-1} enzyme, pectinase (*Rhizopus* sp.), cellulase, α-galactosidase , α-amylase, amyloglucosidase, pullulanase and isoamylase (all from Sigma) were added to 0.5 ml of 5 mg ml^{-1} polysaccharide and incubated for 1 h at room temperature. Two volume of absolute ethanol was added to precipitate the polysaccharide and the mixture was kept at 4 °C for 2 h. This solution was centrifuged at 7500 rpm for 15 min and the supernatant was flash-evaporated to dryness. The resultant oligomers were reconstituted in 0.5 ml distilled water and the reducing sugars were estimated according to the method of Imoto and Yagishita (1971).

4.2.9.2. Separation and molecular weight detemination of oligomers by Gel permeation chromatography

Gel permeation chromatography was performed on Biogel P2 (0.9 cm X 91 cm) according to the procedure of Marry *et al.* (2003). One milliliter of sample (enzyme hydrolysed polysaccharide) was loaded onto it and was eluted with degassed triple distilled water at a constant flow rate of 1.6 ml min^{-1}. Fractions (1.6 ml) were collected and analyzed for the presence of reducing sugars. The void volume was determined using blue dextran and the column was calibrated with maltose, raffinose, maltotetraose and maltopentaose.

4.2.9.3. Identification of hydrolytic products by HPLC

The enzyme hydrolytic oligomers were identified using a Shimadzu HPLC system (LC-8A model), equipped with RI detector and CR 4A recorder. An aminopropyl column was used for analysis and 10 µl sample was loaded and eluted with acetonitrile : water (80 : 20) at a flow rate of 1.0 ml min^{-1}. Similarly standards (galactose, maltose, raffinose, maltotetraose and maltopentaose) were loaded individually. The elution was monitored by using RI detector set at 8 $X10^{-6}$ RIU (Suzuki and Honda, 2001).

4.3. Results

4.3.1. Light and scanning electron microscopy

Phase contrast microscopic examination of all the cultures grown for 10 days on M4 medium showed the production of particulate EPS. The EPS was produced as amorphous particles or fibrillar material surrounding the cells (Fig. 4.3.1 a and b). The EPS was present as extensive sheaths, surrounding and embedding the cells of thraustochytrids. Scanning electron microscopy of thraustochytrids grown on glass pieces confirmed the production of EPS sheaths (Fig. 4.3.1 c an d). Extensive sheaths were particularly noticeable in culture NIOS-4. Further, EPS, which was secreted over the entire surface of the glass piece, stained positively with alcian blue (Fig. 4.3.2).

4.3.2. Estimation of EPS production

Quantitative estimation of EPS in culture filtrates confirmed that all four thraustochytrid isolates produced them (Fig.4.3.3). The isolate NIOS-4 produced the maximum EPS, amounting to 1.1 g L^{-1} (dry weight). Isolate NIOS-12 produced the least amount, corresponding to 0.3 g L^{-1} dry weight. Since NIOS-1 and NIOS-4 produced the maximum quantity of EPS, these cultures were used for further studies. The production of EPS during different phases of growth was studied for the two isolates, under batch culture conditions (Fig.4.3.4 a and b). The cultures showed characteristic sigmoidal growth curves with a lag phase of about 18 h for NIOS-4 and 9 h for NIOS-1 and reached the stationary phase after 46 h and 34 h, respectively. EPS production was observed at all stages, but

lagged behind growth. The concentration increased with age, reaching the highest values during the stationary phase. The concentration of the polymer did not show any decline during 73 h of growth.

Carbon assimilation studies during the late exponential growth phase showed that most of the carbon was assimilated into the cell biomass. About 7.0 % of assimilated carbon was converted to EPS (Fig. 4.3.5).

4.3.3. EPS with relation to media and DHA production

EPS production by the isolate NIOS-1 was studied simultaneously during the Response Surface Methodology (RSM) experiment designed to optimize the media for biomass, total lipids and DHA production (Chapter 2). EPS was estimated for the 20 trials used for this RSM experiments, the results of which are shown in Table 4.3.1. The highest EPS production was seen in Trial 10 containing 3.62 % glucose, 0.3 % peptone and 0.25 % yeast extract. The minimum EPS production was seen in Trial 1 containing 1.0 % glucose, 0.15 % peptone and 0.1 % yeast extract (Table 4.3.1). Correlation coefficient values (r) were estimated to examine the relationship between EPS on one hand and biomass, lipids and DHA on the other (Table 4.3.2) It was observed that DHA production was positively related to biomass and EPS production (Table 4.3.1). In general EPS production was positively influenced by glucose.

4.3.4. Biochemical characterization of EPS

The solubility of EPS produced by NIOS-1 and NIOS-4 in various solvents is shown in Table 4.3.3. The EPS produced by both the isolates dissolved best in distilled water. The EPS of isolate NIOS –1 was 99.4 % soluble whereas that of NIOS-4 was 72.4 % soluble in distilled water.

The total as well as the soluble fraction of EPS produced by both the isolates contained sugars, proteins, lipids and uronic acids (Table 4.3.4 and 4.3.5). Sugars were the most abundant constituent of the EPS in both the isolates, comprising 53 and 39 % in NIOS-1 and NIOS-4, respectively. However, the soluble fraction of EPS in NIOS-1 contained less sugars, while that of NIOS-4 contained much higher levels. Sulfate contents were higher in the soluble fractions of both EPS. Proteins were much less in the soluble fractions of NIOS-1 and NIOS-4, compared to the total EPS. Protein and uronic acid contents were almost the same. EPS of both isolates were viscous in nature, that of NIOS-1 being more so (Table 4.3.4.). The IR spectra of the EPS produced by both NIOS-1 and NIOS-4 were similar. The broad peaks around 3500 cm^{-1} and 1050 cm^{-1} for OH^{-1} and C-O(H) respectively showed the presence of sugars. The broad peak at around 1650 cm^{-1} confirmed the presence of uronic acids in the EPS. The peak at 890 cm^{-1} and 1240 cm^{-1} indicated the presence of SO_4^{-2} (Fig. 4.3.6 a and b).

The EPS of both NIOS-1 and NIOS-4 were retained by 10 kDa ultrafilters. The extracted EPS from both gave a single peak when subjected to ion exchange chromatography and gel permeation chromatography (Figs. 4.3.7 and 4.3.8 a and b) showing that they produced only one type of EPS. Gel permeation

chromatography, as well as HPSEC indicated that the molecular weights of the EPS produced by NIOS-1 and NIOS-4 were more than 2000 kDa (Fig. 4.3.8 a and b). HPSEC also showed that polysaccharide extracted from both NIOS-1 and NIOS-4 was more than 95 % pure in both the cases (Fig. 4.3.9 a and b).

Gas chromatography analysis showed the presence of galactose, mannose, arabinose and fucose or rhamnose in the polymers produced by both NIOS-1 and NIOS-4 (Fig 4.3.10). Galactose formed the major component of the polysaccharide (65 % in NIOS-1 and 89 % in NIOS-4). Mannose and arabinose were present as minor constituents (Table 4.3.6).

Periodate consumption by the EPS of NIOS-1 and NIOS-4 was 0.071 moles and 0.084 moles respectively. No formic acid was liberated from EPS of NIOS-4, while 72 moles were liberated from that of NIOS-1. The optical rotation was –0.140 and +0.060 for NIOS-1 and NIOS-4 polymers (Table 4.3.7).

The elucidation of the structure of EPS was also carried out enzymatically. The various enzymes tested for hydrolysis are listed in Table. 4.3.8. The activity of various enzymes was similar on the EPS produced by both NIOS-1 and NIOS-4. The maximum hydrolysis was by pectinase whereas α-galactosidase, pullulanase, isoamylase and amyloglucosidase had very little or no activity on the polymer. Cellulase and amylase had some activity on the polymer.

Pectinase cleaved the EPS produced by NIOS-4 into 2 types of oligomers (Fig. 4.3.11a) and that of NIOS-1 was cleaved into one (Fig. 4.3.11b) as shown by GPC and HPLC. The smaller oligomer was a monosaccharide, identified as

galactose in NIOS-4 by HPLC and the larger oligomer was a disaccharide from both NIOS-1 and NIOS-4 (Fig. 4.3.12 a and b).

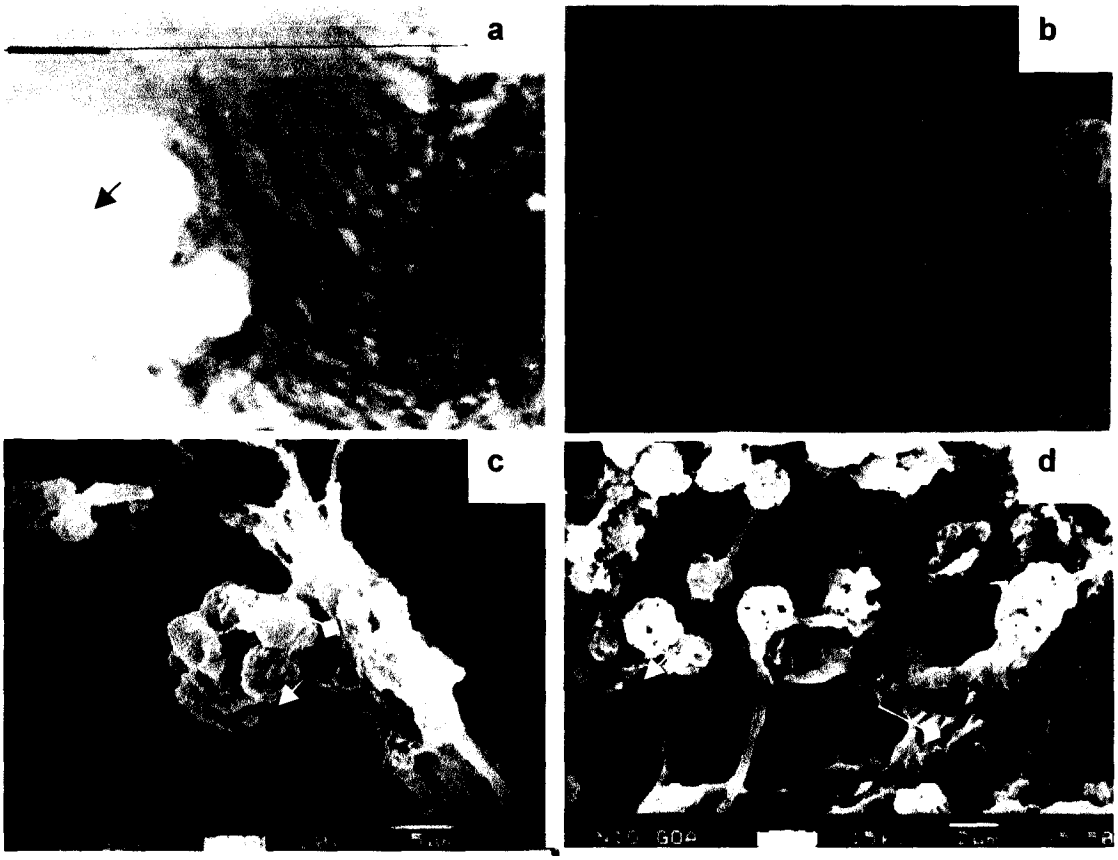


Fig. 4.3.1: a. Phase contrast photomicrograph of EPS matrix produced by the isolate NIOS-2. b. Phase contrast photomicrograph of EPS sheath produced by the isolate NIOS-1. Bar represents 10 µm. c. SEM photograph of EPS sheath produced by the isolate NIOS-1. d. SEM photograph of EPS matrix produced by the isolate NIOS-4. Arrow represents the cells and the diamond arrow, the EPS.

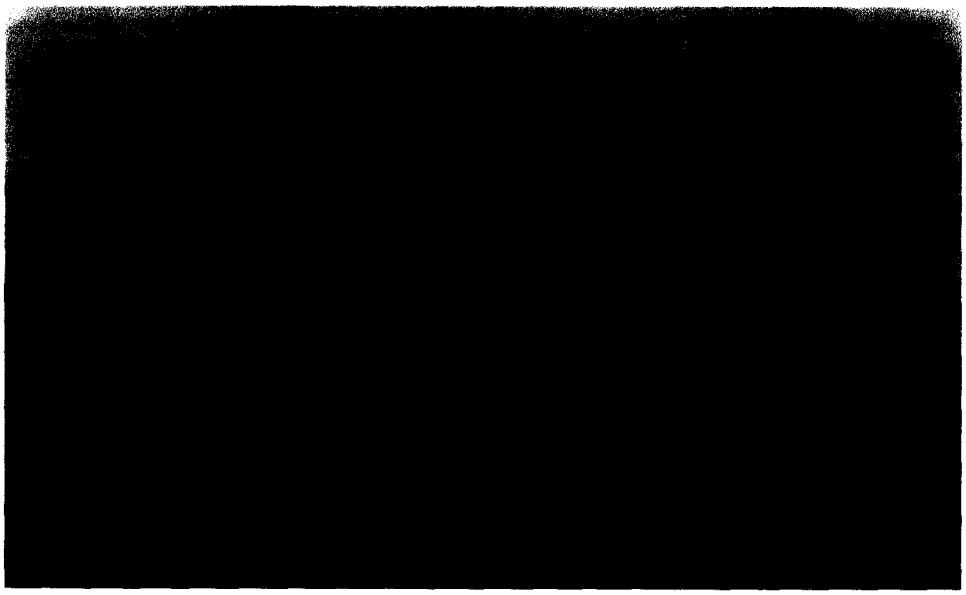


Fig. 4.3.2: Positive staining of EPS by alcian blue (arrow) in isolate NIOS-1. Bar represents 10 µm.

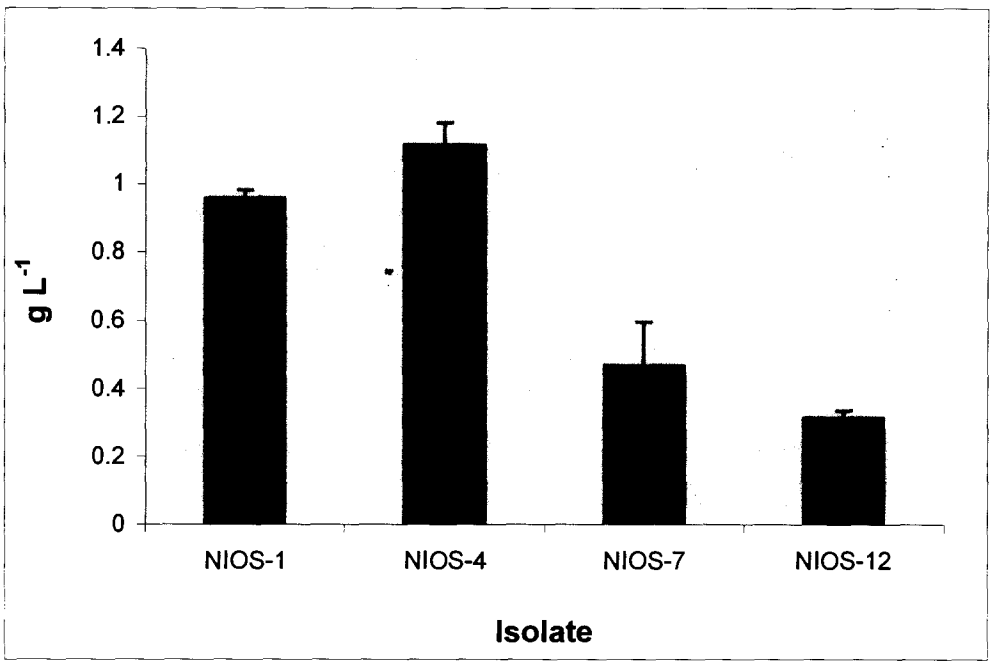


Fig. 4.3.3: Production of EPS by 4 isolates of thraustochytrids. The error bars indicate SD.

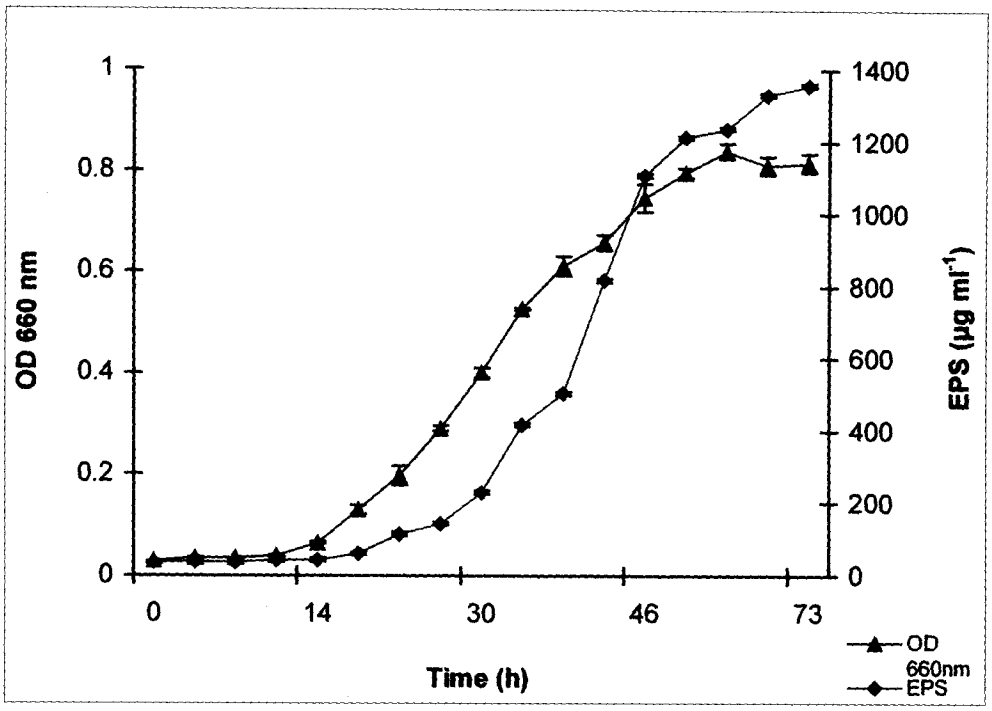


Fig. 4.3.4 a: Kinetics of EPS production with respect to growth in isolates NIOS-1. The error bars indicate SD.

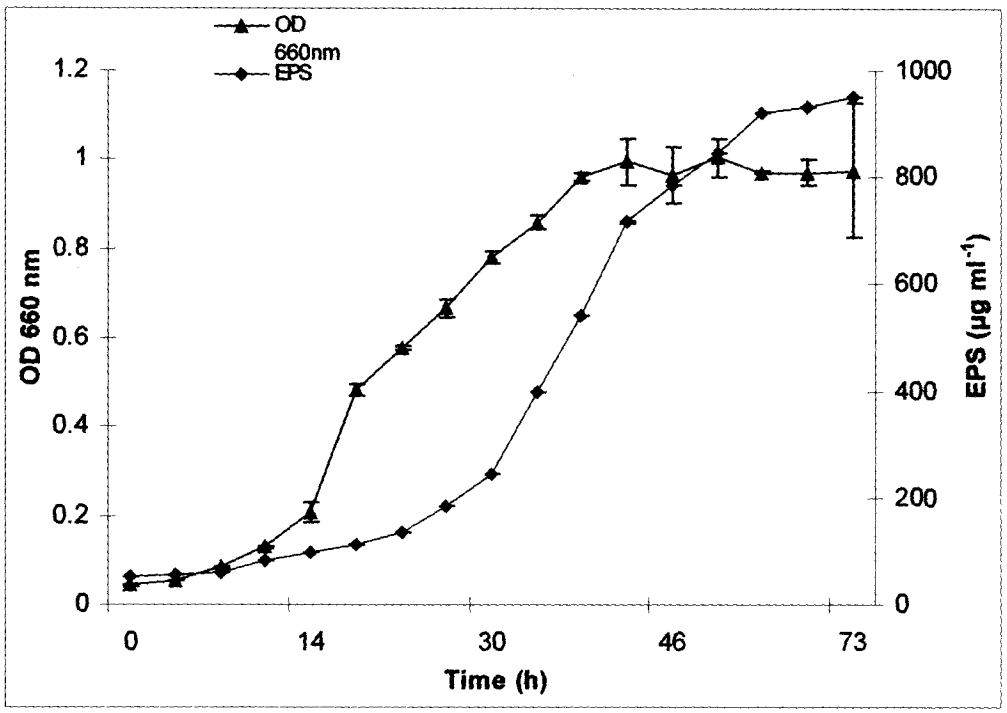


Fig. 4.3.4 b: Kinetics of EPS production with respect to growth in isolates NIOS-4. The error bars indicate SD.

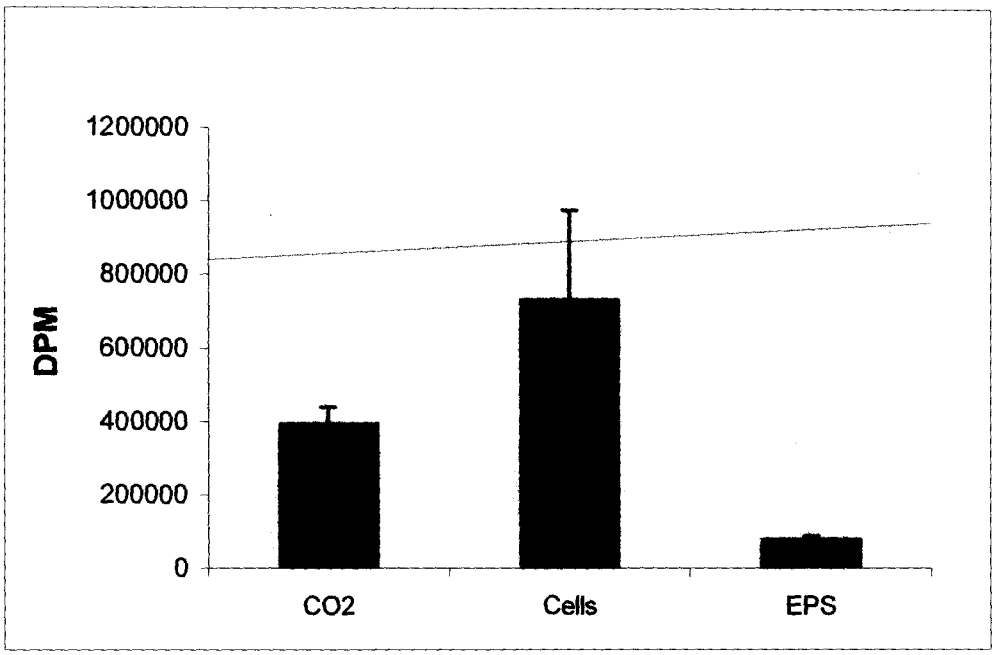


Fig. 4.3.5: Assimilation of ^{14}C carbon into EPS in isolate NIOS-4. The error bars indicate SD.

Trial No.	Percent			g L^{-1}	Percent		g L^{-1}	
	Glucose	Peptone	Yeast extract	Biomass	Lipids	DHA	DHA	EPS
1	1	0.15	0.1	3.81	35.97	27.81	0.381	**0.281**
2	3	0.15	0.1	4.39	33.60	26.16	0.386	**0.421**
3	1	0.45	0.1	4.54	26.40	36.69	0.440	**0.576**
4	3	0.45	0.1	8.06	43.20	29.61	1.031	**0.866**
5	1	0.15	0.4	4.84	16.80	38.13	0.310	**0.561**
6	1	0.45	0.4	5.38	9.60	36.79	0.190	**0.392**
7	3	0.15	0.4	8.20	50.40	33.27	1.376	**0.804**
8	3	0.45	0.4	9.39	42.00	32.11	1.267	**0.741**
9	0.38	0.3	0.25	3.08	12.00	36.12	0.133	**0.435**
10	3.62	0.3	0.25	8.44	49.20	34.01	1.412	**1.02**
11	2	0.057	0.25	5.33	39.60	33.82	0.714	**0.845**
12	2	0.543	0.25	7.82	40.80	27.81	0.888	**0.808**
13	2	0.3	0.088	5.95	57.60	31.07	1.064	**0.786**
14	2	0.3	0.493	8.21	32.40	35.36	0.941	**0.971**
15	2	0.3	0.25	6.58	38.40	33.39	0.844	**0.425**
16	2	0.3	0.25	7.14	37.20	35.63	0.946	**0.373**
17	2	0.3	0.25	7.96	43.20	33.52	1.153	**0.502**
18	2	0.3	0.25	7.06	38.40	36.08	0.978	**0.374**
19	2	0.3	0.25	7.58	42.00	33.91	1.080	**0.379**
20	2	0.3	0.25	6.56	34.80	36.17	0.825	**0.459**

Table 4.3.1: Biomass, total lipids and DHA for the 20 trials carried out under RSM

Variables	Biomass (g L^{-1})	Lipids %	DHA %	DHA (g L^{-1})	EPS (g L^{-1})
Biomass (g L^{-1})	1.00	-0.05	**0.61**	**0 .90**	**0.52**
Lipids %	-0.05	1.00	**- 0.47**	-0.12	-0.12
DHA %	**0.61**	**- 0.47**	1.00	**0.85**	0.44
DHA (g L^{-1})	**0.90**	-0.12	**0.85**	1.00	**0.53**
EPS (g L^{-1})	**0.52**	-0.12	0.44	**0.53**	1.00

Table 4.3.2: A correlation matrix of biomass, total lipids, percent DHA and absolute values of DHA. Values in bold are significant at $p < .05000$, N=20.

No.	Solvent	OD 480 NIOS-4	OD 480 NIOS-1
1	0.1 M EDTA	0.256	0.239
2	0.5 M EDTA	0.278	0.224
3	0.1 N NaOH	0.105	0.113
4	1 N NaOH	0.125	0.165
5	10 % SDS	0.098	0.106
6	0.1 N H_2SO_4	0.253	0.236
7	1 N H_2SO_4	0.165	0.169
8	1 N HCl	0.085	0.063
9	DMSO	0.035	0.048
10	Hexane	0.046	0.026
11	Chloroform	0.071	0.052
12	Distilled water	0.265	0.248

Table 4.3.3: Solubility of EPS produced by NIOS-1 and NIOS-4 in various solvents.

Characteristices	**Isolate**	
	NIOS-1	**NIOS-4**
Sugars	53.27 %	39.09 %
Proteins	23.73 %	18.15 %
Lipids	14.17 %	23.55 %
Sulfates	10.95 %	13.08 %
Uronic acid	4.07 %	1.07 %
Viscosity (6 mg in 1 ml)	2.47 cp	1.32 cp

Table 4.3.4: Physical and chemical characteristics of the total EPS produced by the two isolates.

Characteristices	**Isolate**	
	NIOS-1	**NIOS-4**
Sugars	48 %	72 %
Proteins	2.56 %	2.88 %
Sulfates	12.7 %	8.4 %
Uronic acid	4.8 %	4.4 %

Table 4.3.5: Chemical characteristics of the soluble fraction of EPS produced by the two isolates.

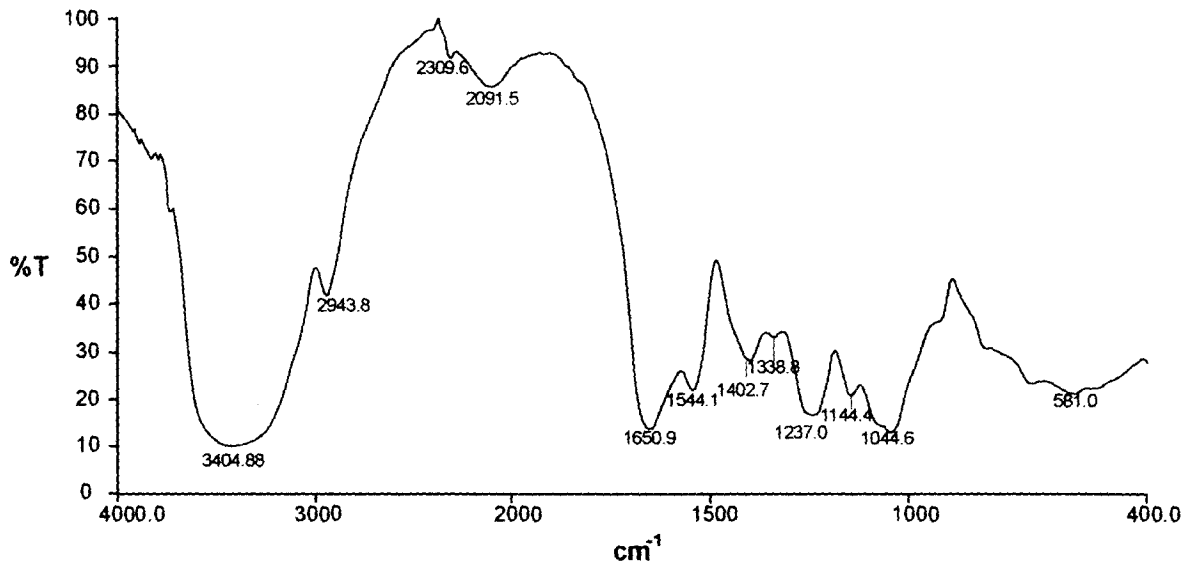


Fig. 4.3.6 a: IR spectrum of the soluble portion of the EPS produced by NIOS-1

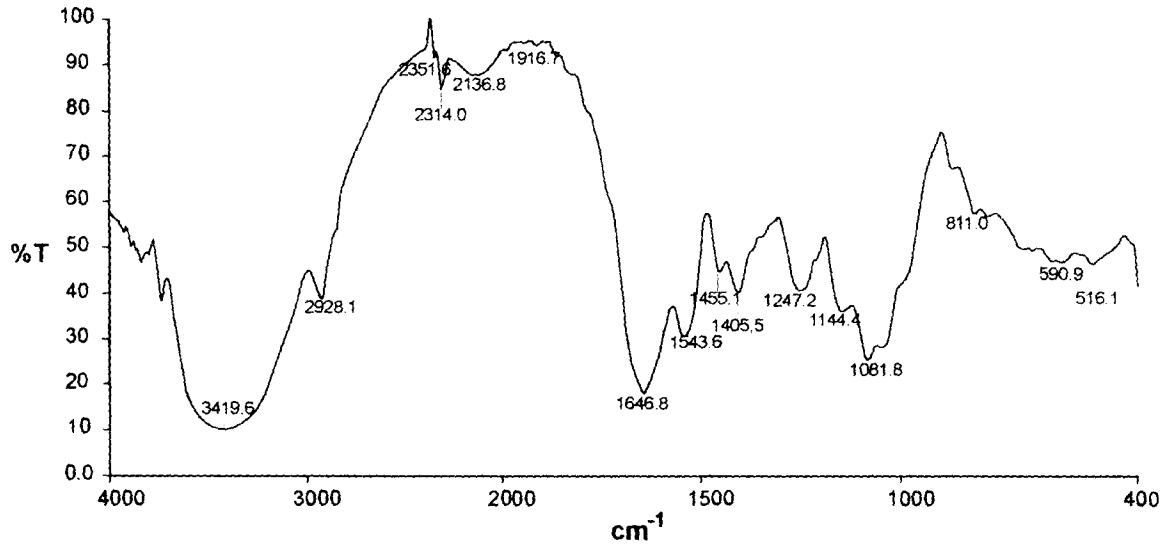
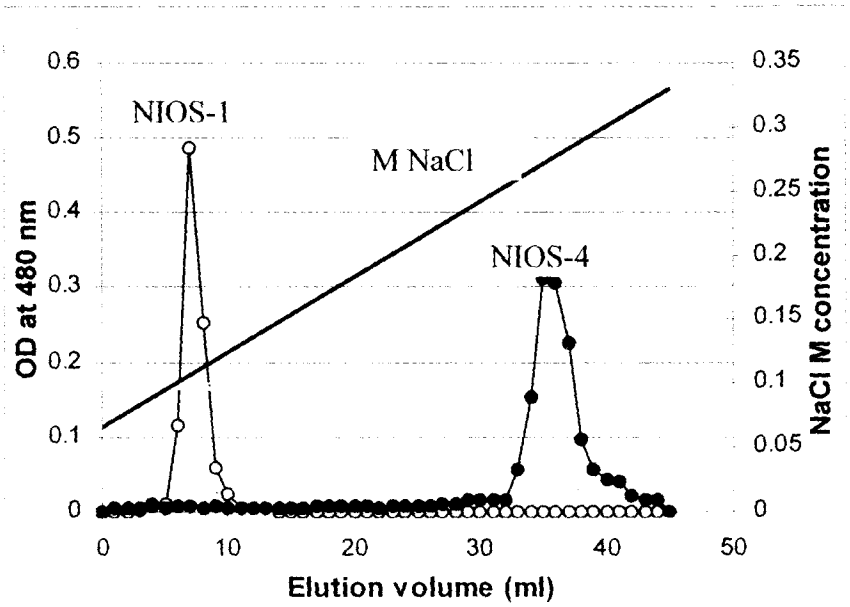
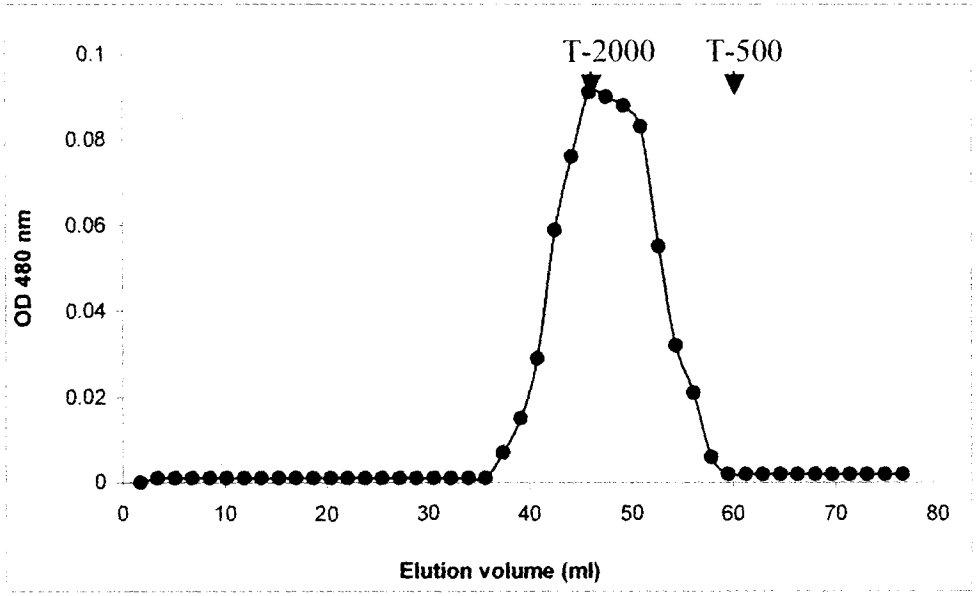


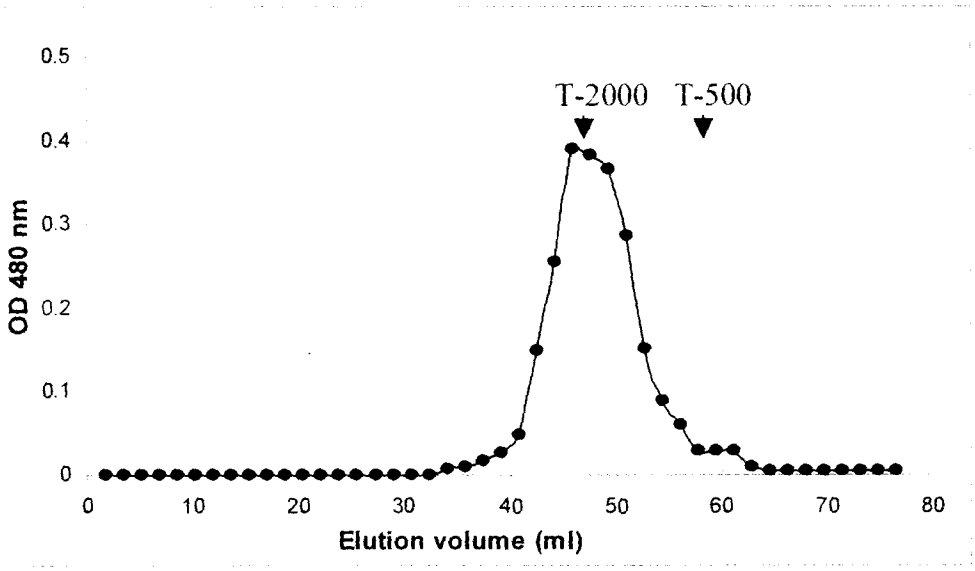
Fig. 4.3.6 b: IR spectrum of the soluble portion of the EPS produced by NIOS-4



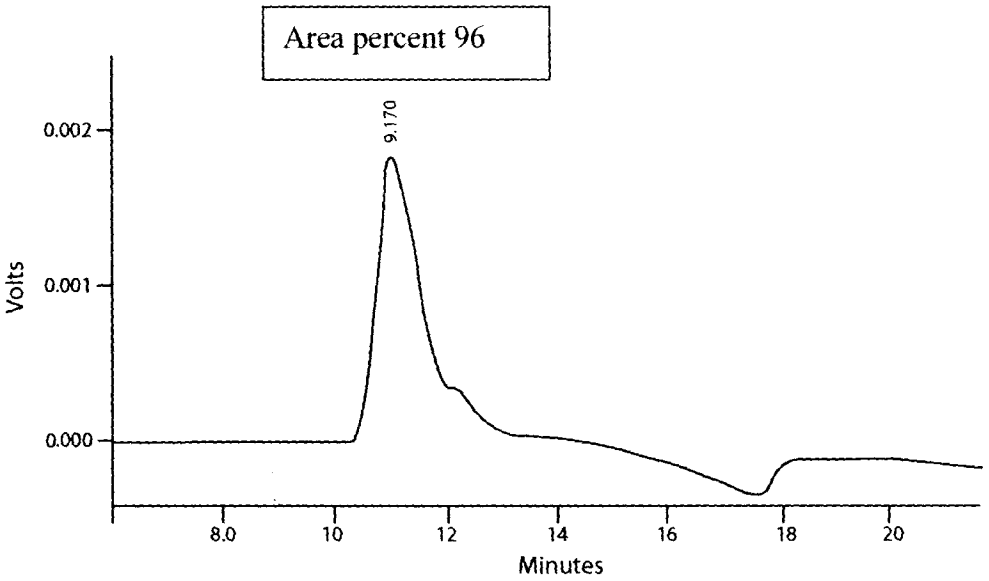
4.3.7: Elution profile of the EPS produced by NIOS-1 and NIOS-4 in an ion exchange column.



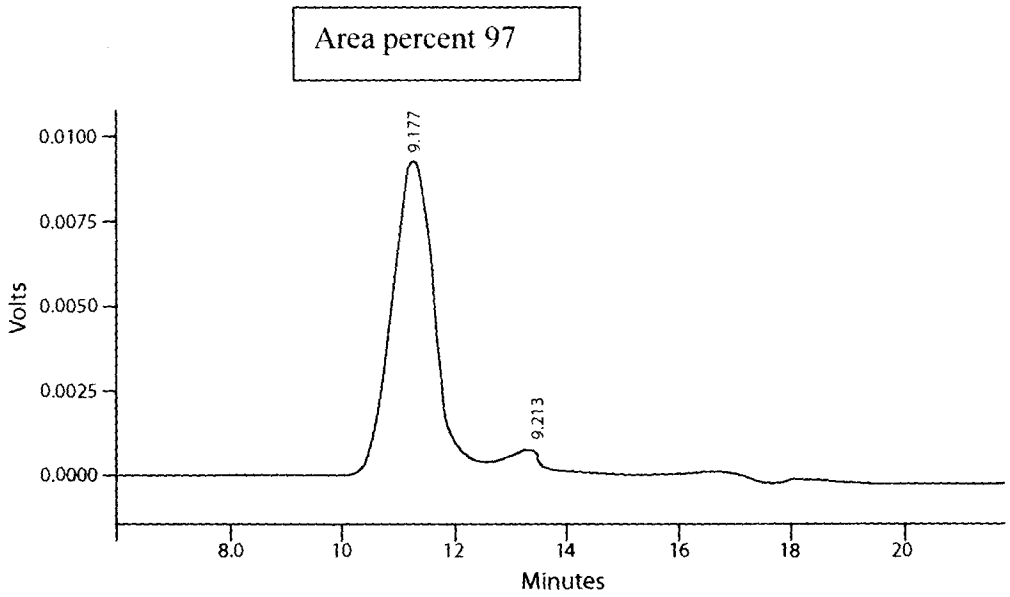
4.3.8 a: Elution profile of the EPS produced by NIOS-1through Sepharose CL – 2B column .



4.3.8 b: Elution profile of the EPS produced by NIOS-4 through Sepharose CL – 2B column.



4.3.9 a: Elution profile of the EPS produced by NIOS-1 through E-linear E-1000 columns.



4.3.9 b: Elution profile of the EPS produced by NIOS-4 through E-linear E-1000 columns.

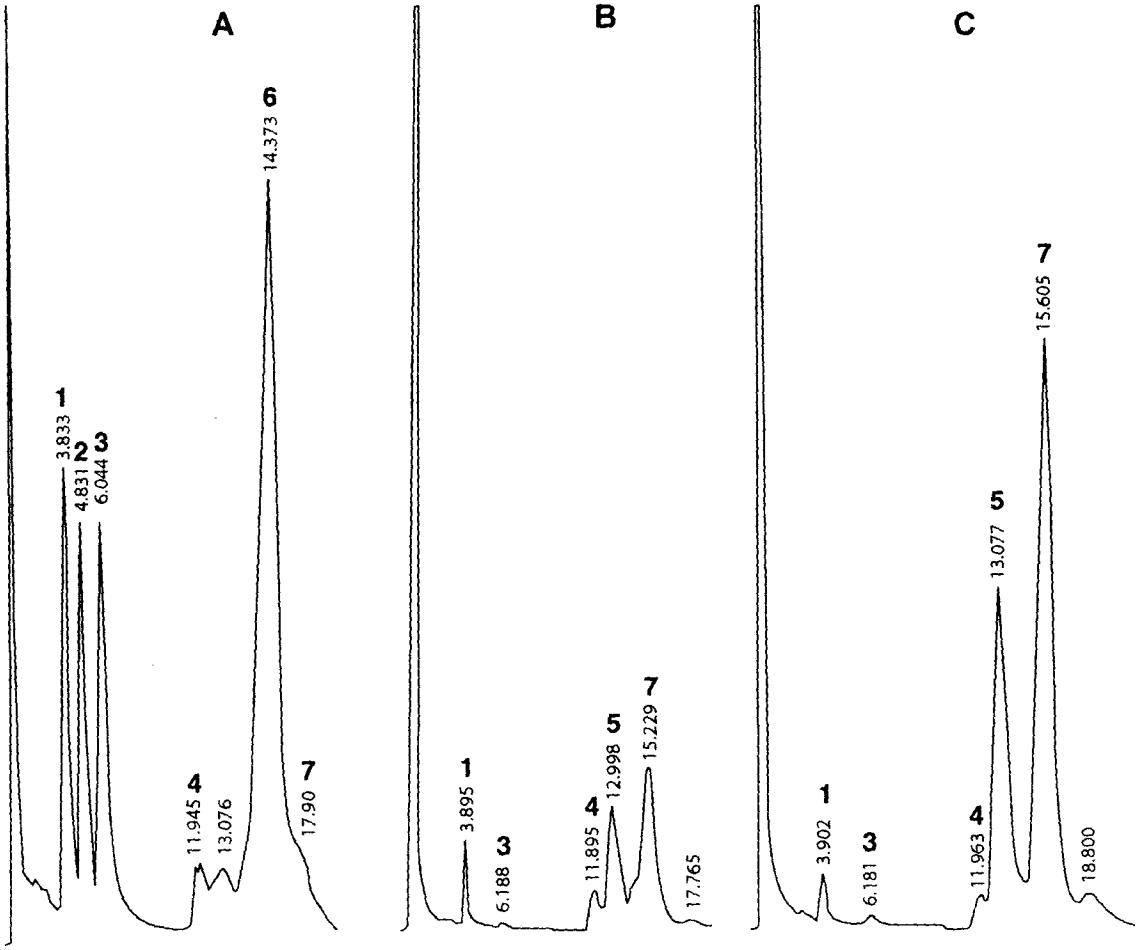


Fig. 4.3.10: GC profiles of component sugars in EPS of NIOS-1 (B) and NIOS-4 (C). 'A' represents standards. Numbered peaks are: 1 = rhamnose / fucose, 2 = xylose, 3 = arabinose, 4 = mannose, 5 = galactose, 6 = glucose, 7 = inositol (internal standard).

Monosaccharide	Isolate	
	NIOS-1	NIOS-4
Fucose/Rhamnose	16.29	3.63
Xylose	-	-
Arabinose	1.43	1.10
Mannose	16.89	5.67
Galactose	65.37	89.58
Glucose	-	-

Table 4.3.6: Monosaccharide composition (mole %) of EPS produced by the isolate NIOS-1 and NIOS-4

Linkage analysis	Isolate	
	NIOS-1	NIOS-4
Periodate consumption	0.071 moles	0.084 moles
Formic acid liberation	72 moles	0
Optical rotation	-0.140	+0.060

4.3.7: Linkage analysis of EPS produced by isolate NIOS-1 & NIOS-4

Enzyme	OD 420 nm	
	Isolate	
	NIOS-1	NIOS-4
Pullulanase	0.096	0.071
α-Galactosidase	0.096	0.108
Pectinase	0.567	0.567
Isoamylase	0.083	0.072
α-Amylase	0.173	0.174
Cellulase	0.117	0.053
Amyloglucosidase	0.069	0.055

4.3.8: Enzyme hydrolysis of EPS produced by isolate NIOS-1 & NIOS-4. The amount of reducing sugar released was estimated by potassium ferricyanide method.

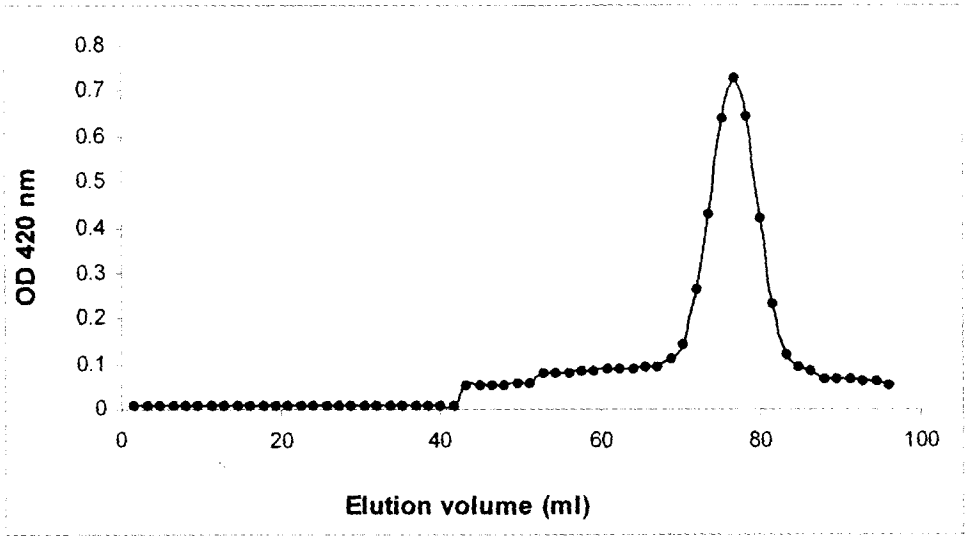


Fig. 4.3.11 a: Elution profile of the Pectinase digest of the EPS produced by NIOS-1through Biogel P-2 column.

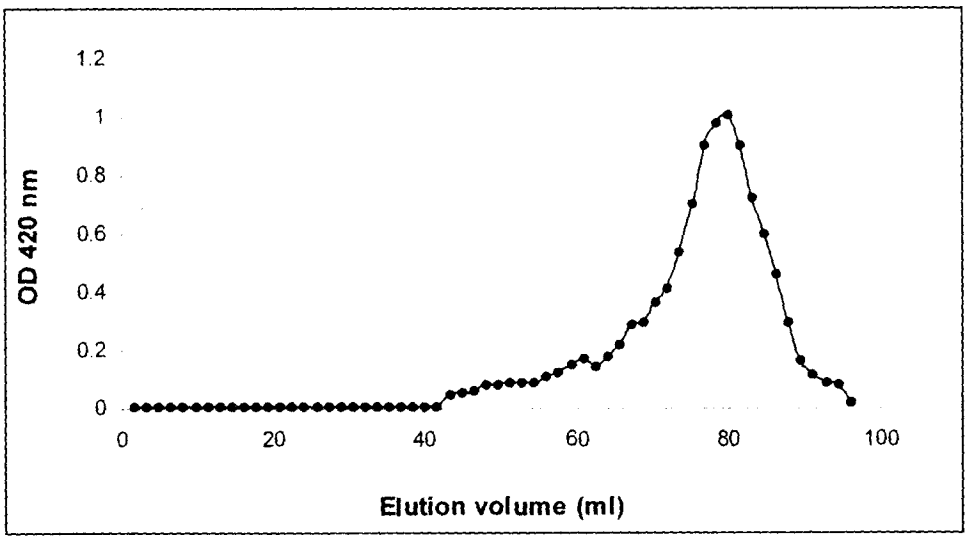
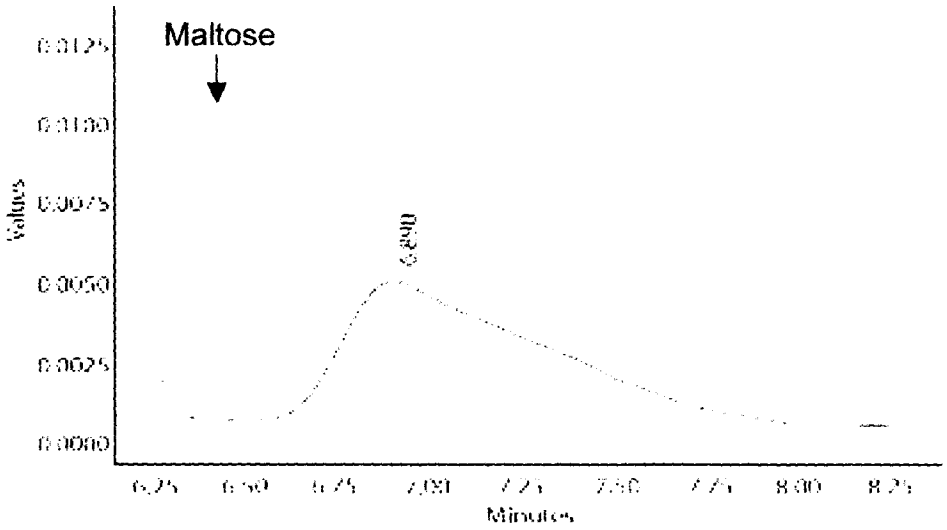
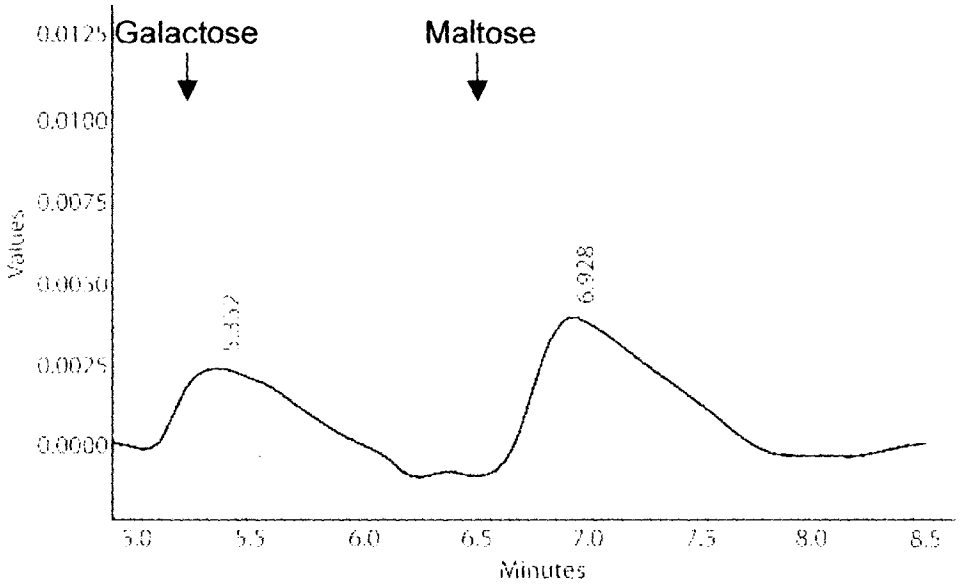


Fig. 4.3.11 b: Elution profile of the Pectinase digest of the EPS produced by NIOS-4 through Biogel P-2 column.



4.3.12 a: Elution profile of the Pectinase digest of the EPS produced by NIOS-1 through aminopropyl column using HPLC.



4.3.12 b: Elution profile of the Pectinase digest of the EPS produced by NIOS-4 through aminopropyl column using HPLC.

4.4. Discussion

This study presents the first conclusive evidence for the production of extracellular polymeric substances (EPS) in thraustochytrids (Jain *et al.* 2005). All 4 species of thraustochytrids examined in this study produced noticeable amounts of EPS (Fig. 4.3.1, 4.3.2 and 4.3.3). An earlier scanning electron microscopic study by Bremer (1976) indicated the presence of adhesive pad like material in *Thraustochytrium kinnei* growing on the surface of the larvae of the brine shrimp, *Artemia.* Raghukumar *et al.* (2000) did not notice any adhesive EPS by thraustochytrids cells growing in biofilms on solid substrata immersed in seawater. It is likely that growth on high nutrient substrates promotes the production of EPS. Several earlier studies have shown that high concentrations of glucose in the culture medium promote EPS production (Sutherland, 1994). EPS was always produced in the 4 thraustochytrids cultures when grown in M4 medium containing 2 % glucose, 0.15 % peptone, 0.1 % yeast extract, 0.025 % KH_2PO_4 in sea water.

Both cultures NIOS-1 and NIOS-4 produced EPS throughout their exponential growth phase and this continued even during the stationary phase (Fig 4.3.4 a. and b). Up to 73 % conversion efficiency of glucose into EPS has been observed for some bacteria during the stationary phase (Williams and Wimpenny, 1978). Although only 7 % of organic carbon appeared to be secreted as EPS in the thraustochytrid NIOS-4 (Fig. 4.3.5), the total amount of carbon that goes into the production of EPS over a period of time would be substantial. Moreover, it is known for several bacteria that despite sugar limitation, EPS may

still be produced through utilization of amino acids as carbon sources (Sutherland, 1982). Therefore, it might be expected that EPS will accumulate with increasing age of cultures. Noticeable EPS was evident in 7 d old cultures of thraustochytrids. EPS in microorganisms is generally known to be produced during the stationary phase of growth. Several diatoms such as species of *Amphora, Navicula* and *Melosira*, and the brown tide alga, *Aureoumbra lagunensis* produced EPS throughout the growth phase but more particularly during the stationary growth phase when nutrients were depleted (Bhosle *et al.*, 1995; Liu and Buskey, 2000; Khandeparkar and Bhosle, 2001; Leandro *et al.*, 2003). A similar observation is seen in most marine bacteria (Decho, 1990).

The four isolates studied produced 0.3 to 1.1 g L^{-1} EPS in M4 medium (Fig. 4.3.3). Bacteria produce EPS ranging from low amounts of about 0.24 g L^{-1} as in a *Pseudomonas* species, 0.35 g L^{-1} *Lactobacillus* species, to very high amounts of about 33 g L^{-1} as in a *Xanthomonas* species after optimization (Christenson *et al.*, 1985; Kimmel *et al.*, 1998; Sutherland *et al.*, 1998). It is likely that under optimal conditions thraustochytrids would produce larger amounts of EPS then those attained in the present experiments.

One of the main aims of this study was to examine the relationship between EPS and DHA production. It is important to understand this since DHA is a commercially important compound and it is necessary to understand how the energy provided to the cell is shunted to the production of other compounds. Likewise, if the EPS of thraustochytrids are found to be biotechnologically useful, it will become relevant if conditions favouring DHA production would suppress

EPS formation. Comparison of EPS production with the results obtained for RSM studies on biomass, lipids and DHA production clearly indicated that EPS production was not detrimental to DHA accumulation (Table 4.3.1.). Indeed, absolute DHA contents in cells and EPS production were positively related (Table 4.3.2). The present study also indicated that EPS production might be positively related to glucose concentration and total biomass. In general, optimal yields of EPS are obtained in the presence of high carbohydrate substrates. Many microorganisms in culture can be induced to yield large quantities of EPS when grown in a high carbon:low-nitrogen growth media (Sutherland, 1994). For many marine bacteria a glucose concentration of 1-2 % yields the highest EPS production, with up to 73 % conversion efficiency of glucose into EPS during the stationary phase (Williams and Wimpenny, 1978). Although EPS is not necessarily induced by high levels of carbon, elevated carbon levels in media promote EPS production. Some marine bacteria can produce EPS merely with ambient levels of nutrients in seawater (Decho, 1990). DHA is a signature compound of thraustochytrids (Ellenbogan *et al.*, 1969). Likewise, EPS also appear to be a signature compound of these protists.

Thraustochytrids did not produce capsular polysaccharides as in the case of *Nostoc* species (Phillips and Vincenzini, 1998). In stationary cultures the EPS in the thraustochytrids was in the form of a matrix or sheaths around the cells (Fig 4.3.1). This is similar to those produced by many biofilm-forming bacteria, cyanobacteria and diatoms (Bhosle *et al.*, 1995; Phillips and Vincenzini, 1998; Khandeparkar and Bhosle, 2001). However, little such particulate EPS was

observed in shake cultures of two isolates, where most of the EPS was in a soluble form. Both isolates studied produced up to 26 % of insoluble EPS. The sheath like EPS seen around the thraustochytrids cells might constitute insoluble EPS as well as the soluble portion, which had formed sheaths under stationary conditions. Likewise, the diatom *Amphora rostrata* produced both soluble, as well as ' biofilm' EPS (Khandeparkar and Bhosle, 2001).

NIOS-1 and NIOS-4 produced high molecular weight EPS of about 2000 kDa (Figs 4.3.9 a and b). High molecular weight EPS are found in many microorganisms such as the bacteria *Pseudomonas caryopholli* (Sudhamani *et al.*, 2004), the marine periphytic bacterium *Pseudomonas* species (Christensen *et al.*, 1985) and the yeast *Aureobasidium pullulans* (Lee *et* al., 1999). Many cyanobacteria are also reported to produce high molecular weight EPS in the range of 1400 - 2800 kDa. (Phillips and Vincenzini, 1998). Most water-soluble polymers with high molecular weight readily forms gels in high concentration (Phillips and Vincenzini, 1998).

The EPS of NIOS-1 and NIOS-4 were acidic polysacharides, staining positively with Alcian blue, indicating the anionic (acidic) nature of the EPS (Fig 4.3.2.) Alcian blue is a copper containing cationic phthalocyanin dye which binds to anionic molecules. Anionic polysaccharides are commonly produced by marine organisms including water column and hydrothermal vent bacteria (Lijour *et a.l*, 1994), cyanobacteria, and many phytoplankton species, including diatoms (Christensens *et al.*, 1985; Bhosle *et al.*, 1995; Phillips and Vincenzini, 1998; Liu and Buskey, 2000; Khandeparkar and Bhosle, 2001). Anionic nature is generally

conferred by uronic acids, sulfates and pyruvates (Decho and Lopez, 1993; Leandro *et al.*, 2003). Uronic acids and sulfates were found to be important constituents of the EPS of the two thraustochytrids studied in detail (Table 4.3.4 and Table 4.3.5). In eukaryotic cells, sulfation of EPS occurs in the Golgi apparatus (Ramus and Robins, 1975). Charged groups such as sulfates, pyruvates and uronic acids significantly contribute to the solubility of the polysacharide in water, thus improving the ability of the EPS to bind water molecules (Sutherland, 1994). Kennedy and Sutherland (1987) showed that bacterial EPS typically contain 20 – 50 % uronic acids. Uronic acids in the EPS increase with increase in age and metabolic stress (Uhlinger and White, 1983). Pyruvates were not estimated in the present study but acyl groups, such as pyruvates and succinates protect uronic acids from epimerisation and thus assure a high uronic acid content in the final polymer.

Sulfated polysaccharides are of much biotechnological importance and may have applications as blood anticoagulants and antiviral compounds (Sogawa *et al.*, 1998; Alban *et al.*, 2002). The importance of sulphated cell wall polysaccharides in macroalgae is well known (Murano, 1998). In addition, several recent papers describe the importance of extracellular sulfated polysaccharides in organisms such as the cyanobacterium, *Aphanocapsa halophytia* and the red alga *Porphyridium* species (Geresh *et al.*, 1991; Matsunaga, 1996). This study demonstrates that thraustochytrids are also characterized by EPS with sulfated polysaccharides, whose biotechnological applications will be worth studying.

EPS with high concentration of charged components usually form stable gels in the presence of metallic ions and are therefore a promising option for the removal of toxic metals from polluted waters (Bender *et al*, 1994). The accessibility of the ions to the EPS depends upon its conformational status. In the absence of a suitable conformation, some of the charged groups of the EPS will not be accessible for the ions. Therefore, the mere determination of the quantity of charged groups is not enough for anticipating the actual binding capability of the polymer (Sutherland, 1994). Further conformational studies on the EPS of thraustochytrids are necessary to understand their potential in biotechnology.

The enormous structural diversity of the EPS arises from composition of the EPS, broad arrangement of monosaccharides and the additional non-carbohydrate constituents (Decho, 1990). Taxonomic groups of organisms often have certain characteristic sugar composition in their EPS. Most common monomers present in EPS of marine bacteria are glucose, galactose, mannose, rhamnose and fucose. Pentoses are less common (Sutherland, 1977; Powell, 1979) but are a common component in cyanobacteria (Phillips and Vincenzini, 1998). Cyanobacterial EPS are more complex in structure than bacterial EPS as the former usually contain 6 or more monosaccharides, while bacterial and macroalgal EPS usually contain less than 4 (Decho, 1990; Phillips and Vincenzini, 1998). The EPS of the two thraustochytrids studied in detail show that galactose was the predominant sugar in EPS of both the thraustochytrids, in addition to smaller quantities of mannose, arabinose and rhamonse or fucose

(Fig 4.3.10; Table 4.3.6). The EPS composition of these indicates some similarities to the general cell wall composition of these protists. The studies of Darley *et al.* (1973), Ulken *et al.*, (1985), Bahnweg and Jackle (1986) and Chambelain and Moss (1988) have shown that thraustochytrid cell walls are comprised of sulfated galactans and proteins. A minor difference between the EPS and cell wall was the absence of xylose in the latter. Galactose is one of the predominant sugars in diatoms, such as *Amphora rostrata* (Khandeparkar and Bhosle, 2001). Diatoms are also members of the Kingdom Straminipila to which thraustochytrids belong. Sulfated galactans are characteristic of the polysaccharides of the red alga *Gracilaria corticata* (Mazumdar *et al.*, 2002).

Monosaccharide composition of the EPS can have a marked effect on the physical properties of the EPS. Bacterial and algal alginates, which differed only in the monosaccharide composition and have similar polyanionic composition of L-guluronic acid and D-mannuronic acid, had different physical characteristics. Rhamnose or fucose, deoxy sugars present in thraustochytrids may impart a lipophilic character to the EPS.

Generally, proteins get adsorbed on the EPS but are also are present as an integral component of the EPS. It was shown by Arad *et al.* (1993), that removal of the proteinaceous moiety of the EPS from *Porphyridium* species drastically reduces the viscosity of its aqueous solution. Similarly in a strain of *Nostoc*, 2S9B removal of proteins significantly reduced the adhesive capacity of the polysaccharide to the roots of *Triticum vulgar* L (Gantar *et al.*, 1995). The

proteins present in the EPS of thraustochytrids might be an integral part of it and might have an important role in its physical characteristics.

The EPS of marine organisms usually possess a polysaccharide backbone, which predominantly contains either 1,3 or 1,4 linkage in either α or more commonly β-configuration (Wingender, 1999). The structural characteristics of the thraustochytrid EPS were determined using periodate oxidation, optical rotation, enzymatic cleavage and IR (Table 4.3.7, Table 4.3.8, Fig 4.3.6 a and b.). IR spectroscopy and ^{13}C NMR are powerful tools, which have been used to characterize polysaccharides from marine organisms such as the carrageenana producing red algae (Turquois *et al.*, 1996). EPS produced by both the isolates, NIOS-1 and NIOS-4 showed high periodate consumption indicating either a high frequency of adjacent hydroxyl groups or the presence of few substituents on the sugar ring (Table 4.3.7.). However, substantial amounts of substituents such as sulphates and uronic acids were detected in thraustochytrid EPS (Table 4.3.4 and 4.3.5). Besides, the EPS of isolate NIOS-4 showed no formic acid liberation indicating high substitution in form of either uronic acids or sulfates. Therefore, the high periodate consumption might actually indicate the preponderance of 1,4 linkages. Polymers produced by both the isolates were substantially cleaved by α-amylase, suggesting that the linkage was α-1,4. Cellulase also showed a higher activity on the EPS produced by NIOS-1. Therefore the linkage in NIOS-4 is most probably α-1,4.

Marine microorganisms are potential sources of biotechnologically useful EPS. Various groups, including water column bacteria, deep sea hydrothermal

vent bacteria and cyanobacteria have been the objects of research for a variety of applications that include bioleaching, pharmaceuticals, food, cosmetics, microencapsulation, and industries (Rougeaux *et al.*, 1996; Gehre *et al.*, 1998; Murano, 1998; Sutherland, 1998). Thraustochytrids possess sulfated galactans. Sulfated galactans from the red alga *Gracilaria corticata* are known to be antiviral (Mazumdar *et al.*, 2002). Other interesting applications are the induction of apoptosis of human leukamia cells by the EPS of the dinoflagellate, *Gymnodium* sp. (Sogawa *et al.*, 1998). In a quest for polysaccharides with new or improved biotechnological applications, it is important to study different groups of organisms. In this context, future studies on thraustohytrid EPS are likely to produce interesting results.

4.5. Conclusion

EPS were produced in all the four thraustochytrids grown in high nutrient media. EPS was produced throughout the growth phase and continued in stationary phase. EPS production was positively related to DHA production, biomass and glucose concentration. Thraustochytrid EPS was produced both as soluble as well as a matrix like insoluble form. The EPS of the two thraustochytrids had a molecular weight in excess of 2000 kDa. The EPS were acidic polysaccharides containing uronic acids and sulfates. Galactose was the predominant sugar of EPS. Sulfates also formed an important component of the EPS. The EPS appeared to possess α-1,4- linkages. The biotechnological applications of the sulfated polysaccharide of thraustochytrids need to be studied further.

CHAPTER 5

Ecological significance of EPS production by thraustochytrids

5.1. Introduction

Extracellular polymeric substances (EPS) are useful to the organisms that produce them. They are also known to have a large number of biotechnological and ecological functions, as described in the first chapter. Their ecological significance can be both to the cell per se and also to the biotic and abiotic factors surrounding the cell (Decho, 1990). The impact of EPS depends on its chemical make up and the extent to which it can bind to the various substances such as water, organic and inorganic nutrients, toxic compounds (Wingender *et al.*, 1999).

In the marine ecosystem, EPS surrounding the cells plays a major role in access and utilization of nutrients, especially large molecular weight dissolved organic matter (DOM) by the cell (Decho, 2000). Microorganisms secrete a wide variety of extracellular enzymes, which hydrolyse larger molecular weight macromolecules into smaller molecules such as small peptides, aminoacids, and oligosaccharides (Meyer-Reil, 1987). These small molecules can be quickly taken up by the cell and once in the cell can be efficiently metabolized for energy and biomass production. Extracellular enzymes bind to the EPS, the mechanisms of which are not very well studied. However it is hypothesized that EPS acts as a cation exchange matrix and the exoenzymes bind to it by displacing the hydroxyl groups of the bound water molecule (Rees, 1976; Rendelman, 1978). EPS being hydrophilic in nature, not only binds to the extracellular enzymes but also helps in maintaining its activity during local osmotic changes (Darbyshire, 1974). The activity of these enzymes can be

studied by employing substrates attached to fluorescent compounds such as methyl umbelliferon (Hoppé *et al.*, 1988).

EPS not only binds to the extracellular enzymes but also to, many dissolved compounds such as inorganic ions and high molecular weight compounds which can be readily utilized as a food source (Decho, 1990). The physical structure of the EPS resembles a highly dispersed matrix of fibrils, thus increasing the surface area (Sutherland, 1977). The polysaccharide moieties of the EPS are especially important in binding reactions, as they possess abundant carboxyl and hydroxyl groups (Aspinall, 1982). Hydroxyl groups of exopolymer polysaccharide form relatively weak associations, whereas carboxyl groups on carboxylated polysaccharide like pyruvates, ketal groups, acetates, uronic acids form very strong bonds (Kaplan *et al.*, 1987).

EPS also act as a food reserve, but little work has been done to determine the extent to which nutrients in EPS can be utilized, either by the organism by it self or by different organisms (Wolffaardt *et al.*, 1999). Although some reports do suggest that organisms utilize their own EPS under nutrient deficient conditions (Patel and Gerson, 1974), a majority of the EPS producing organisms are unable to utilize their own EPS as a carbon source (Dudman, 1977). Moreover, a whole complex of enzymes, produced by different organisms is required to totaly degrade the EPS, since it is chemically complex (Pirog *et al.*, 1997).

EPS also helps in prevention of desiccation of cells in the interdital zone. EPS are hydrated molecules and 99 % of it is water thus helping the organisms to survive dry conditions (Decho, 1990).

EPS produced by thraustochytrids is likely to play an important role in marine ecological processes. In this chapter we have tried to study the following functions of EPS;

1. The role of EPS in preventing desiccation of cells;
2. The role of EPS as a nutrient reserves to the cell;
3. The role of EPS in binding extracellular enzymes.

5.2. Materials and Methods

5.2.1. Role of EPS in desiccation

Generally, EPS accumulates with age of culture in microorganisms. Therefore, in order to study the role of EPS in desiccation, 2 day old cultures with less EPS and 7 day cultures with more of these were compared. Isolates NIOS-1, NIOS-2, NIOS-4 and NIOS-10 were used for the experiments. The thraustochytrids were grown in M4 medium at a room temperature of ~ 28 °C under shaken conditions. Details of culturing are described in Chapter 4. The cells were centrifuged at 7500 rpm (5200 g) for 15 min and were washed thrice with seawater filtered through a 0.22 μm membrane filter (FSW). The cell pellet was resuspended in FSW to form thick slurry. A drop was placed in a sterile Petri dish and dried for different periods of 0, 12, 24, 72, 96, 120, and 168 h. Viability of the dried cells was checked by adding sterile seawater and pine pollen to the plates containing the cells and by microscopically examining for growth on pine pollen after 4 days. Experiments were carried out in triplicates.

5.2.2. Role of EPS in binding exoenzymes

5.2.2.1. Microscopy methods to detect presence of enzymes in EPS

Experiments were carried out using the 4 isolates, NIOS-1, NIOS-2, NIOS-4 and NIOS-10. Cultures were grown for 7 d on autoclaved, 25 mm^2 glass pieces placed in Petri dishes, covered with M4 medium. They were incubated at a room temperature of ~ 28 °C under static conditions, in order to allow the organisms to

produce EPS. Two methods were used to detect the presence of enzymes in EPS.

1. Fluorescein diacetate (FDA) Staining: FDA is a non-fluorescent stain, which is easily taken up by cells. Within the cell, it is cleaved by non-specific esterases and fluorescein; the cleaved product is fluorescent (Coleman and Vestal, 1987). This method is commonly used for testing viability of cells. The glass pieces containing thraustochytrid cells with EPS were stained with 10 µl of 20 mg ml^{-1} FDA solution and incubated for 15 min at room temperature. These were then observed under an epifluorescence microscope using a green excitation filter. Fluorescence in the red range indicates cleavage of fluorescein diacetate, the presence of enzymes and therefore, viability of cells.
2. Staining for proteolytic, lipolytic and glycosidase activity in EPS: Presence of proteases, lipase and glycosidase were tested using methyl umbelliferyl substrates (MUFs). These substrates contain an artificial fluorescent molecule (MUF) linked to an enzyme substrate by a specific bond. The exoenzyme acts on this specific bond releasing the flurochrome and therefore fluorescence is observed. (Hoppé, 1993). Glass pieces as above, containing growth of thraustochytrids together with their EPS were treated with 100 µl of the following solutions and incubated for 30 min at a room temperature of ~28 °C.

- Proteolytic activity: One milliliter of 0.5 µM L-leucine-4-methyl coumarinyl-7-amide, 1.5 ml of Tris HCl buffer (pH7.4) and 1.5 ml distilled water.
- Lipolytic activity: One milliliter of 0.5 µM 4-methy umbelliferyl butyrate and 100 µl of 0.2 M Tris HCl buffer (pH 7.4).
- Glycosidase activity: One milliliter 0.5 µM 4-methy umbelliferyl-β-D-glucopyranoside, 2 ml of 0.22 µ FSW and 250 µl borate buffer, pH 10.

The methylumbelliferyl substrates were purchased from Sigma Chemicals.

After incubation with the substrates, the cultures were observed under an epifluorescence microscope using a green excitation filter. Fluorescence in the red range indicates the presence of the enzyme.

5.2.2.2.Quantitative analysis of enzyme activity

Experiments were carried out using 4 isolates – NIOS-1, NIOS-2, NIOS-4 and NIOS-10, grown in M4 medium at room temperature under shaken conditions, as described in Chapter 2. EPS was extracted as described in Chapter 4. Methylumbelliferyl substrates, as described above were used for quantitative estimations of lipolytic, proteolytic and glycosidase activity of exoenzymes bound to the EPS, using a modification of procedure of Hoppe' (1993).

1. Protoelytic activity: Five milligram of EPS was taken to which 1 ml of 0.5 µM L-leucine-4-methyl coumarinyl-7-amide, 1.5 ml of Tris HCl buffer (pH 7.4) and 1.5 ml distilled water was added and incubated for 5 h at room

temperature in dark. Fluorescence reading was taken at 380 nm excitation and 440 nm emission at 0 h and 5 h intervals.

2. Lipolytic activity: Five milligrams of EPS was taken to which 400 µl of 0.5 µM 4-methy umbelliferyl butyrate was added and incubated for 30 min at room temperature in dark. The tubes were then placed in boiling water bath for 5 min, cooled immediately and centrifuged at 5000 rpm for 10 mins. One milliliter of supernatant was taken to which 100 µl of 0.2 M Tris HCl buffer (pH 7.4) was added. Fluorescence reading was taken at 364 nm excitation and 445 nm emission at 0 min and 30 min intervals.
3. Glycosidase activity: Five milligrams of EPS was taken to which 6 ml of 0.5 µM 4-methy umbelliferyl-β-D-glucopyranoside was added and incubated for 2 h at room temperature in dark. The tubes were then placed in boiling water bath for 5 min, cooled immediately and centrifuged at 5000 rpm for 10 min. To 1ml of supernatant 2 ml of 0.22 µm filtered seawater and 250 µl borate buffer pH 10 was added and centrifuged at 5000 rpm for 10 min. Fluorescence reading was taken at 365 nm excitation and 455 nm emission at 0 h and 2 h intervals.

Calibration of fluorochrme was carried out using increasing concentration of 4-methy umbelliferon (MUF) and 4-methyl coumarinyl-7-amide (MCA) standards (2-20 nM). A factor was calculated by plotting the fluorescence intensity against the concentration and was used in calculating the enzyme activity. The enzyme activity was further calculated as concentration of MUF or MCA released per gram EPS per hour (mM h^{-1} g^{-1}).

5.2.3. Role of EPS in starvation of cells

The isolates, NIOS-1, NIOS-2, NIOS-4 and NIOS-10 were grown for 2 day in M4 medium as described above. The cells were then washed thrice with 0.22 µm FSW. An inoculum of 5 µl was added to 15 ml screwcapped tubes containing 5 ml of FSW. Experimental tubes contained in addition 0.1 % EPS produced by the respective isolates at room temperature under shaken conditions. The EPS used for these experiments had earlier been extracted and stored at –20°C, as described in Chapter 4. Control tubes lacked EPS. Triplicates were maintained. The tubes were incubated for 6 days at a room temperature of ca. 28 °C. Growth was measured every 2 days by reading the OD at 660 nm. In another experiment, growth was estimated by counting the colony forming units after every 2 days by plating 5 µl on MV agar and counting the colonies after incubation for 3 days at room temperature as above.

5.3. Results

5.3.1. Role of EPS in desiccation

The isolates – NIOS-1, NIOS-2, NIOS-4 and NIOS-10 were dried for various periods, up to 168 h. Cells from seven day cultures survived longer duration of desiccation than that from 2 d cultures. Cultures of NIOS-1 and NIOS-4 survived longer than NIOS-10 and NIOS-2. Cells from seven-day culture of NIOS-1 and NIO-4 survived desiccation up to 120 h. Isolate While NIOS-2 and NIOS10 could tolerate desiccation only for 72 h (Fig. 5.3.1).

5.3.2. Role of EPS in binding exoenzyme

EPS of all four isolates showed fluorescence when stained by FDA and viewed under an epiflurorescence microscope with a green excitation filter (Fig. 5.3.2). This indicated exoenzyme activity in the EPS. Similarly fluorescence was seen when the EPS from all the four isolates were stained by L-leucine-4-methyl coumarinyl-7-amide, 4-methy umbelliferyl butyrate and 4-methy umbelliferyl-β-D-glucopyranoside substrates for protease, lipase and glycosidase activity respectively, demonstrating the presence of these enzymes.

Thraustochytrids differed in the kind and amounts of enzymes bound to their EPS. Thus, protease activity, as estimated using the MUF-substrate was the maximum in isolate NIOS-2 (112.05 mM h^{-1} g^{-1}) and was minimum in NIOS-1 (16.85 mM h^{-1} g^{-1}) (Fig 5.3.3). Glycosidase activity was highest in NIOS-10, with a value of 30.8 mM h^{-1} $g^{-1}$and lowest in NIOS-4, with a value of 0.018 mM h^{-1} g^{-1}. No glycosidase activity was detected in NIOS-2. Lipolytic activity was maximum

in NIOS-4 and minimum was seen in NIOS-10, with values of 2.83 mM h^{-1} $g^{-1}$and 0.14 mM h^{-1} g^{-1}respectively.

5.3.3. Role of EPS in starvation of cells

Growth of only NIOS-1, as judged by changes in OD at 660 nm, increased over a period of time when supplied their own EPS as sole source of nutrients, as compared to their growth without the EPS (Table 5.3.1). NIOS-4 did not show any increase in growth. In the isolate NIOS-1 colony counts increased from 1.6 X 10^3 to 3.9 X 10^3 cells per ml by 6 days when grown with EPS as the sole nutrient source (Fig 5.3.4). However, isolate NIOS-4 and NIOS-2 showed only a slight increase in the CFUs with EPS as a sole source of nutrients.

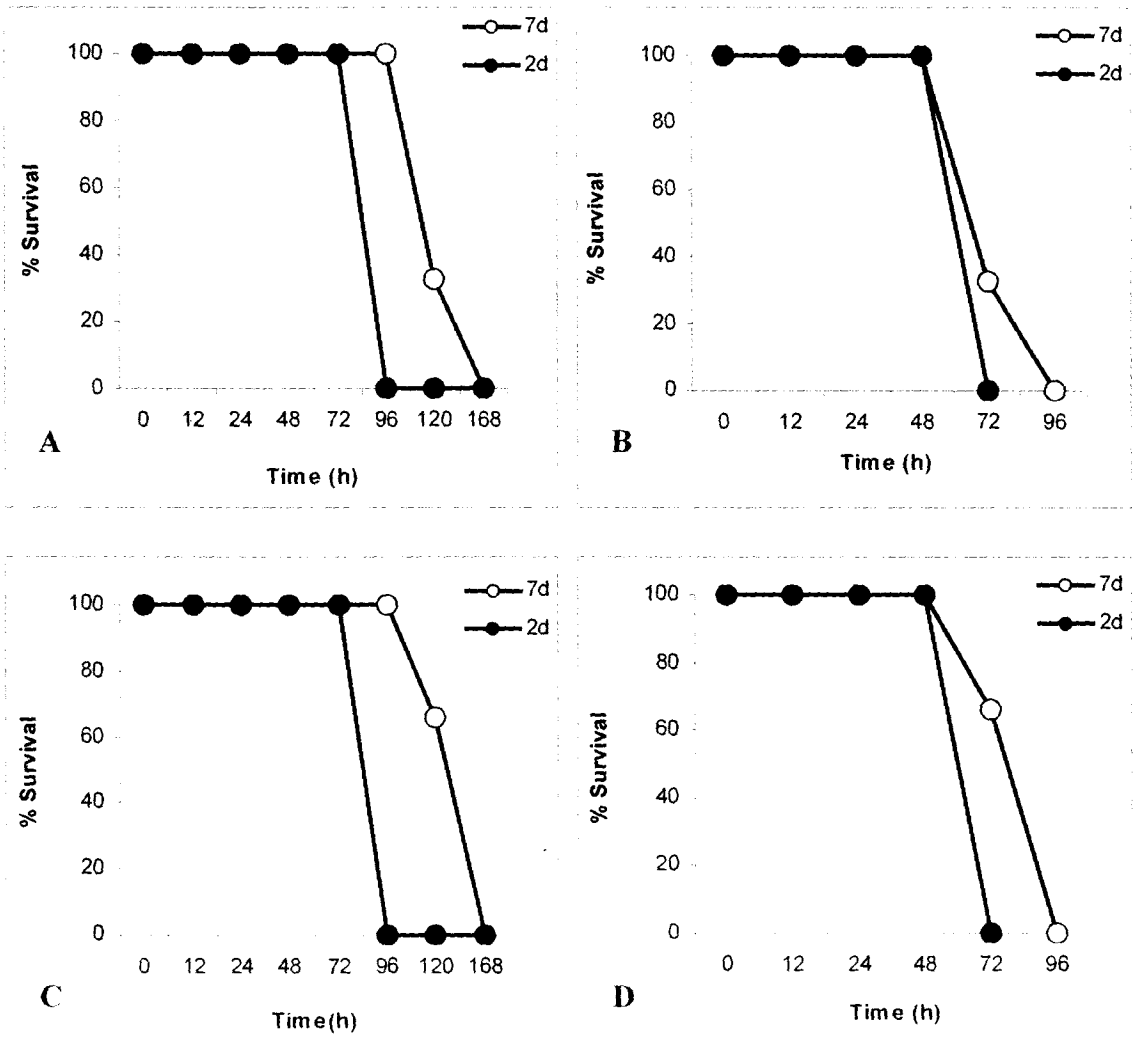


Fig 5.3.1: Percent survival of NIOS-1 (a), NIOS-2 (b), NIOS-4 (c) and NIOS-10 (d) on air drying the cells.

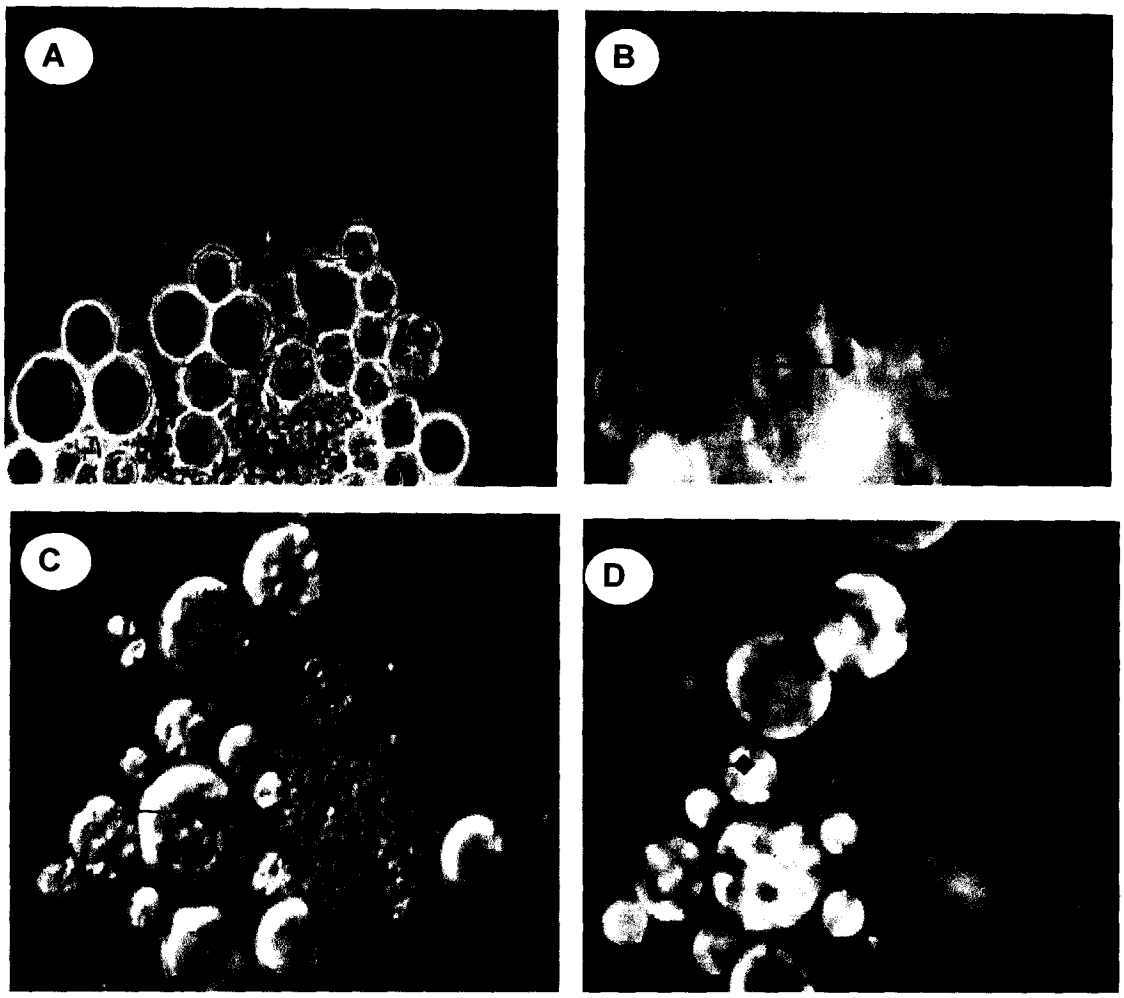


Fig. 5.3.2: Thraustochytrids stained with fluorescein diacetate. A and B: Cells of NIOS-1; A: Phase contrast photomicrograph showing EPS. B: Epifluorescence photomicrograph showing fluorescence of EPS. C and D: Cells of NIOS-2. C: Differential Interference Contrast photomicrograph showing EPS. D: Epifluorescence photomicrograph showing fluorescence of EPS Arrow represents the cells and the diamond arrow, the EPS. Bar represents 10 µm.

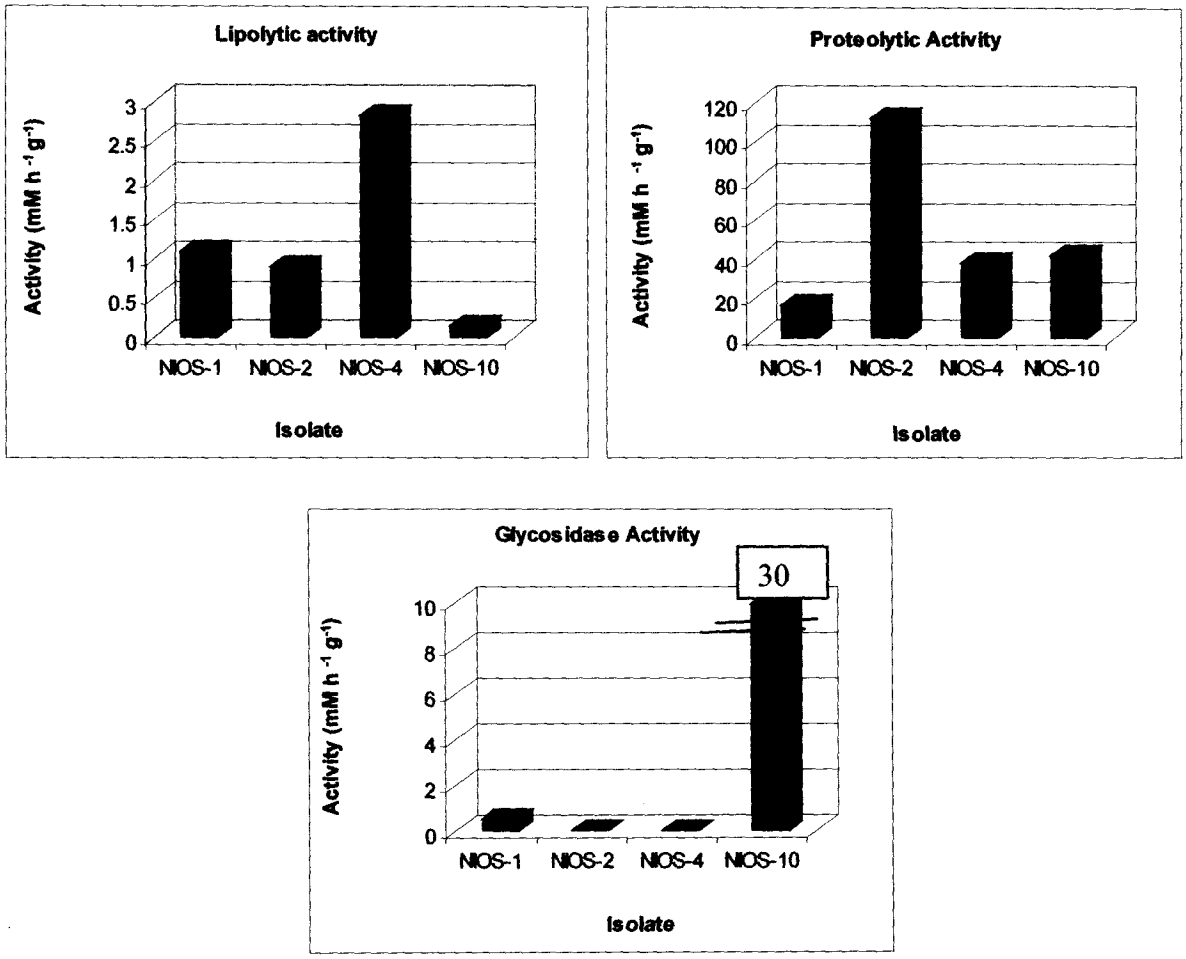


Fig 5.3.3: Enzyme activity of the EPS bound enzyme. A: Lipase, B: Protease and C: glycosidase activity. Each bar is a mean of triplicate sample and the standard deviation (SD) ranged from 0.003 to 2.88.

Isolate	OD 660 nm of 0 day culture		OD 660 nm of 6 day culture	
	Cells	Cells + EPS	**Cells**	Cells + EPS
NIOS-1	**0.192**	0.193	**0.198**	0.314
NIOS-2	**0.021**	0.049	**0.016**	0.064
NIOS-4	**0.114**	0.149	**0.113**	0.149
NIOS-10	**0.036**	0.080	**0.036**	0.126

Table 5.3.1: Growth of 4 isolates on EPS produced by the same organism as a sole source of carbon for Each value is a mean of triplicate sample and the standard deviation (SD) ranged from 0.0007 to 0.116.

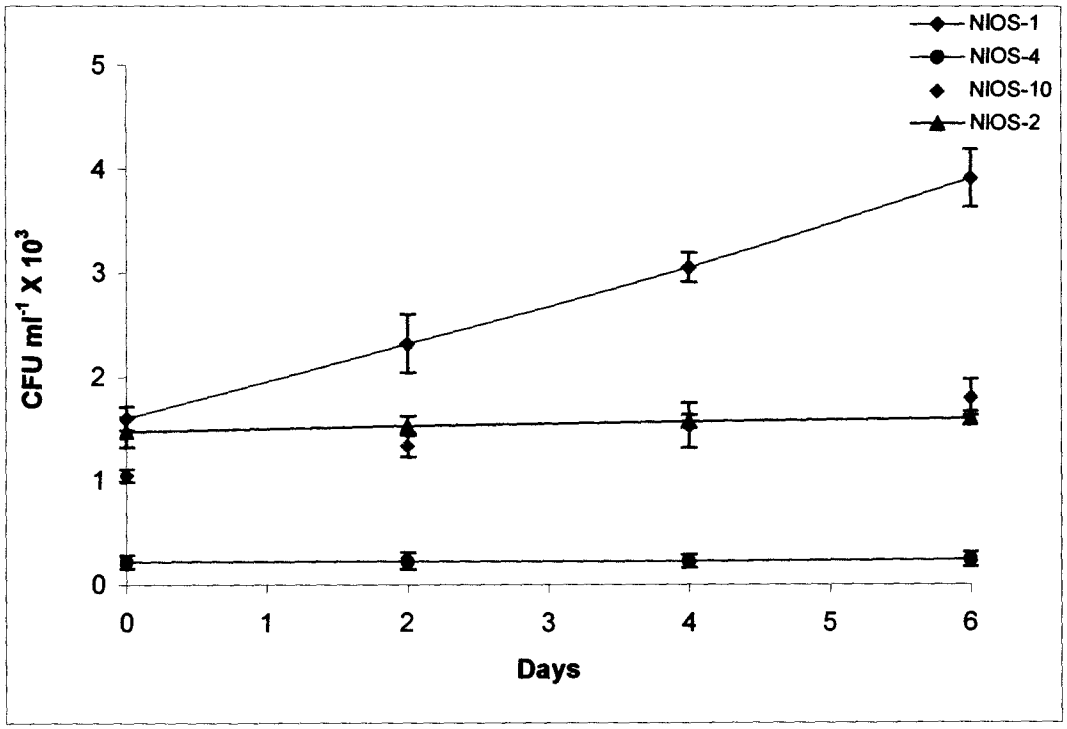


Fig. 5.3.5: Growth profile of 4 isolates on EPS produced by the same organism as a sole source of carbon for a period of 6 days. The error bars indicate SD.

5.4. Discussion

Since EPS production had not been reported earlier in thraustochytrids, their ecological role also has not been studied. Most organisms under natural conditions produce extracellular polymeric substances. EPS confer many ecological benefits to the organism, which are described in Chapter 1. This chapter examines the ecological functions of the EPS produced by thraustochytrids.

One of the functions of EPS could be, to enable adhesion of cells to surfaces. Cells of isolates NIOS-1 and NIOS-4 produced increasing amounts of EPS with age, enabling their adherence to glass pieces when grown on them in stationary cultures (Fig. 4.3.1). Thraustochytrids produced anionic polysaccharides containing a protein moiety. The acidic nature of the polymers is known to be very important in adhesion of cells. Permanent adhesion of marine periphytic bacteria to sediment surfaces involves acidic EPS (Dade *et al.*, 1990). Proteins are known to modulate hydrophobicity of EPS, thus regulating adhesion.

Most of the isolates used in this study were either from the mangrove ecosystem or intertidal region, which are characterized by frequent fluctuations in temperature, salinity, ion concentrations, desiccation, UV-irradiation and wave actions. These fluctuations pose challenges for organisms inhabiting such habitats (Giller *et al*, 1994) EPS may play a major role in preventing desiccation of cells. The results in this studies showed that isolates, which produced more EPS, survived longer periods of desiccation (Fig. 5.3.1). Microbial EPS is a highly hydrated polymer matrix, containing approximately 99 % water. The EPS matrix

maintains a high degree of hydration in the immediate vicinity of the microbial cells and may have a protective function. Ophir and Gutnick (1994) have also shown that EPS substantially increased bacterial resistance to desiccation. Hill *et al.* (1997) proposed that the secreted EPS provides a repository for water. Experimental studies carried out by Roberson and Firestone (1992) indicated that EPS have a high affinity for water over a wide range of water potential and can hold up to five times its weight as water. They also concluded that the EPS matrix might slow the rate at which cells equilibrate with their surroundings. This retardation in the drying rate within the microenvironment allows the cells to accustom to the changing environment.

Charged groups such as sulfates and uronic acids, as is found in thraustochytrid EPS significantly contribute to the solubility of EPS in water, thus improving the binding capacity of EPS to water molecules (Sutherland, 1994). Moreover, heavily cross-linked gels tend to retain water better (Potts, 1994).

Hill *et al.* (1997) reported that exopolysaccharides prevented membrane fusion. In a strain of *Nostoc commune,* the main damaging process that occurs when desiccated cells are rehydrated. Such a role of EPS might have helped in survival of a species of thraustochytrid even on a dried herbarium specimen, from which it was isolated (Porter, 1990).

Microorganisms lie at the base of the food web and are very important in recycling of organic matter. Passive transport through microbial membranes is restricted to very small and chemically simple compounds. Albertson *et al.* (1990) estimated that 5-10 % of the total organic matter comprises amino acids and

peptides. Therefore microorganisms produce extracellular enzymes, which hydrolyze large molecular weight DOM to smaller oligomers, which are then taken up by the cells. An important rate-limiting step in transformation of organic matter is the extracellular hydrolysis of large molecular weight organic matter by microorganisms (Hoppé, 1991). Extracellular enzymes may diffuse out in an aqueous medium, unless retained close to the cell. In thraustochytrids, which are osmotrophic and elaborate a number of extracellular enzymes (Bahnweg, 1979 a and b), the EPS that is produced appear to be responsible for binding them, thus preventing their loss to the surroundings (Fig. 5.3.2). In general EPS are known to bind extracellular enzymes, nutrients and metal ions (Christensen and Characklis, 1990) moreover, Decho and Lopez (1993) showed that EPS can even adsorb and concentrate dissolved organic matter. The mechanisms by which the extracellular enzymes, nutrients and metal ions bind to the EPS are not very well studied. The physical structure of the EPS resembles a highly dispersed matrix of fibrils, thus increasing the surface area (Sutherland, 1977). However it is hypothesized that EPS acts as a cation exchange matrix and the nutrients, metal ions and exoenzymes bind to it by displacing the hydroxyl groups of the bound water molecule (Rees, 1976; Rendelman, 1978). The polysaccharide moieties of the EPS are especially important in binding reactions, as they possess abundant carboxyl and hydroxyl groups (Aspinall, 1982). Hydroxyl groups of exopolymer polysaccharide form relatively weak associations, whereas, carboxyl groups on carboxylated polysaccharide like pyruvates, ketal groups, acetates, uronic acids form very strong bonds (Kaplan *et al.,* 1987). The

EPS of the two thraustochytrids studied in detail consist of uronic acids and sulfates. Therefore these exopolysaccharides could have high binding capacities. However the binding capacity of the EPS cannot be directly related to the amount of charged groups present, but also depends on the conformation attained by the polymer in aqueous solutions. The charged groups if present in the interior of the polymer will not be available to bind to the extracellular enzymes, nutrients or metal ions.

The extracellular enzymes bound to the EPS of thraustochytrids were appeared to be in an active state (Fig. 5.3.3). It is known that EPS, being hydrophilic in nature, not only binds to the extracellular enzymes but also helps in maintaining their activity during local osmotic changes (Darbyshire, 1974).

The hygroscopic properties of the EPS and the high water content not only helps in binding and maintaining the activity of enzymes but also in diffusion of nutrients in the close proximity to the enzyme and also diffusion of the oligomers into the cell. These oligomers if not bound to the EPS could be easily lost in the water column by diffusion. Chenu and Robertson (1996) showed that over a range of water potential values, the diffusion rates of glucose into the cell were higher in EPS and EPS-amended clay than in pure clay.

The variety of enzymes bound by the EPS may differ, depending on the species (Fig. 5.3.3). This could be the characteristic of the niche from which the organism was isolated or its physiological growth stage. Chumak *et al.* (1995) demonstrated the ratio of enzyme to EPS varied even in different growth phases during growth in batch culture.

The number of planktonic thraustochytrids is also high in the water column (Raghukumar, 2002). The EPS production by these cells would facilitate the binding of suspended POM and other organisms thus facilitating the clearing of the water column.

EPS may act as a food reserve but little work has been done to determine the extent to which nutrients in EPS can be utilized, either by the organism itself or by different organisms (Wolffaardt *et al*, 1999). Although some reports do suggest that organisms utilize their own EPS under nutrient deficient conditions (Patel and Gerson, 1974), a majority of the EPS producing organisms is unable to utilize their own EPS as a carbon source (Dudman, 1977). Obayashi and Gaudy (1973) demonstrated that some bacteria could utilize the polymers produced by other organisms. De Brouwer *et al.* (2002) and De Brouwer and Stal (2002) have observed that diatoms utilized the EPS produced during the light phase in the dark phase

In the present study, isolate NIOS-1 and to some extent NIOS-10 could utilize its own EPS as a food source. The other two isolates, NIOS-2, and NIOS-4 could either not utilize or grew poorly when provided with EPS as a sole nutrient source (Table 5.3.1 and Fig. 5.3.4). A whole complex of enzymes, produced by different organisms is required to totaly degrade the EPS, since it is chemically complex (Pirog *et al.*, 1997). NIOS-1 utilized its EPS better, probably because it had an arsenal of all the three enzymes tested (Fig. 5.3.3.).

Thraustochytrids appear to be an important component of the marine ecosystem for two known reasons; firstly, they produce DHA, which is an

essential nutrient in the life cycle of many crustaceans (Harrison, 1990). Secondly they occur in abundance and could be of importance as mineralizers in the ecosystem (Raghukumar, 2002). This study suggests that there could be yet another important role that thraustochytrids play in the ecosystem. The dissolved EPS produced by thraustochytrids may be a source of nutrients to bacteria and the particulate EPS of importance as a food source for zooplankton the water column.

5.5. Conclusion

This is the first report of the ecological significance of the extracellular polymeric substances produced by thraustochytrids. EPS of thraustochytrids may confer benefits to thraustochytrids and are also important to the ecosystem. EPS may help cells to adhere to various substrata. They may prevent desiccation of thraustochytrid cells that inhabit the intertidal region. They may prevent the loss of extracellular enzymes by binding them, retaining them close to cell and maintaining them in active form so that they are useful to the cell. EPS may serve as a serve food source at least for some thraustochytrids that have an enzyme arsenal to break them down. Dissolved and particulate EPS may serve as a nutrient and food source to bacteria and zooplankton in the water column.

CHAPTER 6

Morphology and molecular taxonomy of thraustochytrids

6.1. Introduction

The taxonomy of thaustochytrids has generally been based on their morphology and developmental patterns of their life cycles (Porter, 1990). Characters which are used in the taxonomy of thraustochytrids are:

- Presence or absence of repeated binary divisions;
- Transformation of the mature cell into an amoeboid cell;
- Presence or absence of ectoplasmic net elements;
- Presence or absence of proliferation bodies.

A total of six genera and about 35 species have been described so far among thraustochytrids (Sparrow, 1960; Kobayashi and Ookubo, 1953; Goldstein and Belsky, 1964; Jones and Alderman, 1971; Gaertner, 1977; Dick, 2001). Brief characteristics of these genera are presented in Table 6.1. In addition to the characters given in Table 6.1.1, the size and shape of the zoosporangia, the shape of the zoospores and other characters have been used to delineate species within the genera.

Taxonomy based on morphology and development is often inherently problematic if the characters are variable depending upon the culture medium and other environmental parameters. Thus, Booth (1969) found a high degree of variability in the thraustochytrids that they studied. Characters may often overlap, posing further problems in a proper identification of the species. Such problems can be overcome if molecular techniques are employed. However, molecular taxonomy is usually based on

Characters	Genera						
	Thraustochytrium	*Japanochytrium*	*Schizochytrium*	*Althornia*	*Ulkenia*	*Diplophrys*	*QPX parasite*
Apophysis	-	+	-	-	-	-	-
Binary Division	-	-	+	-	-	-	-
Proliferation body	+/-	-	-	-	-	-	-
Amoeboid Stage	-	-	+/-	-	+	-	-
Ectoplasmic nets	+	+	+	-	+	+	-
Zoospores	+	+	+	+	+	-	+
Sagenogen	+	+	+	-	+	-	+
No. of Species	16	1	5	1	6	2	4

Table 6.1.1: Taxonomically significant characteristic of various genera of thraustochytrids (Dick 2001).

only one gene or protein and sometimes could be misleading. Therefore taxonomy today is based on both morphology and molecular sequences.

As an example, the ambiguity of the phylogenetic position of thraustochytrids was cleared only after mid 1980s when molecular tools were employed to classify thraustochytrids (Details are in Chapter 1).

Molecular taxonomy is based on the sequence of either a gene or an amino acid. The information based on sequences provides information also on the relationship between species. Such relationships based on sequences are simple, mathematically defined and are easy to quantitate (Long, 1998). While many genes, such as DNA gyrase, HSPs and Cytochrome B are frequently employed for molecular taxonomy, the rRNA gene is one of the most commonly used, for the following reasons (Woese, 1987; Lim, 1996).

- Universal Distribution and high copy number – occur in all organisms and therefore the relationships between all lineages can be studied. The copy no. is very high and even reach up to 5000 in some eukaryotes.
- Large size – their sizes are very big and consist for many domains.
- Can be sequenced directly – are easy to handle and cloning if not normally required for sequencing.
- Highly Conserved – the gene has various regions that are highly conserved and therefore making it possible to study a wide range or organisms. It also has highly variable regions to study relationships between very close organisms also.

- No lateral gene transfer – The gene does neither undergo gene duplication nor foreign DNA gets incorporated in it.
- Large database – a large number of organisms have been sequenced and therefore minute comparisons can be drawn between various organisms.

6.1.1. The rRNA gene and taxonomy

The rRNA gene is a tandemly repeated multigene family and the gene clusters are found on more than one locus of a chromosome (Schlőtterer, 1998; Hwang and Kim, 1999). In eukaryotic systems, they are also found on different chromosomes. The structure of the rRNA gene is shown in Fig. 6.1. 1.

The nuclear rRNA spacer regions have evolved much faster than the nuclear coding regions and therefore the rRNA coding regions are more conserved than the spacer regions. Due to these differences, the various regions of the rRNA gene are used to infer a broad spectrum of phylogenetic relationships (Schlőtterer, 1998; Hwang and Kim, 1999).

The LSU (28S) and SSU (18S) regions consist of some highly conserved and some highly variable regions. The rapidly evolving segments of high variability are called expansion segments or variable regions. The SSU can be used to construct deep phylogenetic branches in the whole gamut of taxonomy, from Kingdoms to species. The 5.8S region is as conserved as SSU but is too small (~ 150 bp) to contain enough phylogenetic information. The LSU region is much larger than SSU and shows more variations in its different domains.

Therefore it is used to study the phylogenetic relationships at levels below that of Orders.

Molecular taxonomy depends on comparative sequence analysis (Whelan et al., 2001). This uses accumulated mutations to estimate the relative divergence of organisms from their recent ancestor. The archived sequences provide a reference database, to compare the sequence in consideration. The data is first analyzed by aligning the sequences and the conserved regions of the sequences serve as landmarks in initial alignment. The sequences are further analyzed by a variety of available methods using computers. These analyses can be computationally intense as the data is very large. A number of computational algorithms for inferring phylogenies from molecular data are currently in use (Long, 1998). Statistical tests are used to access the robustness and reliability of the phylogenetic inferences such as bootstrap method.

The approaches usually used for estimating the phylogeny are distance matrix method, parsimony analysis and maximum likelihood methods (Long, 1998). Distance matrix method calculates the similarity value (S) between each pair of homologous sequences in a given data set. The best tree is the one that minimizes the difference between the distance values of the tree branch length and the actual distance values from the original data set. The most common distance based method is the Neighbor-joining method. Parsimony methods compare sequences on nucleotide per nucleotide basis. It seeks a phylogenetic tree topology, which minimizes the number of postulated evolutionary changes required to construct the evolutionary history of a given set of characters. The

best parsimony analysis is that which requires the fewest no. of nucleotide substitutions. Maximum likelihood method works backwards, providing estimates of the likely hood of a given data set, giving a specific phylogenetic tree topology. The best maximum likelihood tree is that which maximizes the likelihood of observing the actual data set, given that specific tree topology.

These methods are coming into vogue in the taxonomy of thraustochytrids. Honda et al. (1999) studied the taxonomy and phylogeny of several species based on rRNA gene sequences and concluded that thraustochytrids, can be classified into two major groups namely, labyrinthulid phylogenetic group and thraustochytrid phylogenetic group. Molecular taxonomy has also been used to define and describe new taxa, such as the aplanochytrid (Leander and Porter, 2000) and the clam parasite QPX (Stokes et al., 2002). Leander *et al.* (2004) have made extensive use of 18S rRNA gene sequences to clarify the taxonomic position of several species of aplanochytrids. They authors have noticed three phylogenetic groups among the Labyrinthulomycetes, namely the labyrinthulid, the aplanochytrid and the thraustochytrid phylognetic groups.

This chapter describes the taxonomy of 3 isolates of thraustochytrids used in this study, based both on morphological, as well as molecular taxonomy.

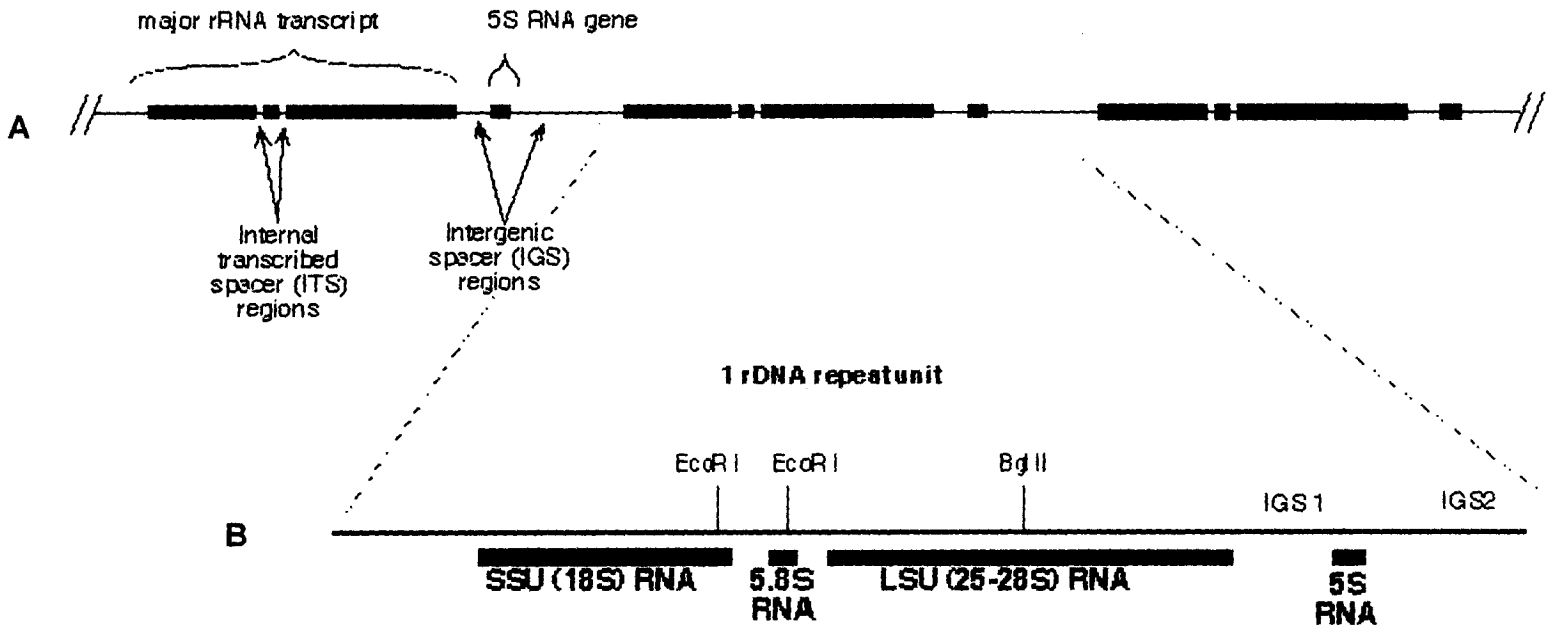


Fig. 6.1.1. Schematic representation of the rRNA gene. A. tandem repeats of the rRNA operon; B. Subunits of the rRNA gene.

6.2. Materials and Methods

6.2.1. Culture and culture conditions

Isolates NIOS-1, NIOS-4 and NIOS-6 were studied with regard to their morphology and molecular taxonomy. The source of isolation and culture methods are given in Chapter 2.

6.2.2. Observations on morphology and development

Two day old cultures grown either a liquid MV medium or seawater / pinepollen culture (Appendix 1) were examined for their morphology and various developmental stages. Colony morphology was examined by growing the isolates on MV agar for 5 days. All observations were made using bright field microscopy with an Olympus BX 60 or Zeiss Axioskop 2 plus microscopes. Digital images were captured using Olympus, Camedia P-200.

6.2.3. Extraction of DNA

Total genomic DNA was isolated according to the procedure of Sherriff *et al.* (1994). The isolates NIOS-1, NIOS-4 and NIOS-6 were grown in M4 medium as described in Chapter 2 for 5 days. Cells were harvested by centrifugation at 7500 rpm (5600 g) for 15 min and washed thrice with 0.22 µm filtered seawater (FSW). The cell pellets were transferred to Eppendorf tubes (~ 0.3 ml) and the cells were disrupted by repeated freezing in liquid nitrogen and thawing. The cells were suspended in 0.5 ml lysis buffer (50 mM Tris pH 7.2, 50 mM EDTA and 3 % SDS, to which 10 µl proteinase K (10 mg ml^{-1}) was added. The

suspension was mixed thoroughly and incubated at 52 °C for 30 min and a further 15 min at 70 °C. Tris-saturated phenol (400 µl) and chloroform : isoamyl alcohol(24:1) (400 µl) were added to this suspension and mixed for 10 min by inverting the tubes. The tubes were then centrifuged at 10000 rpm for 15 min. The upper phase was transferred to a fresh Eppendorf tube to which 800 µl of chloroform : isoamyl alcohol (24:1) was added and mixed as above. The tubes were once again centrifuged at 10000 rpm for 15 min. The upper phase was transferred to a fresh tube and 10 µl of RNAse (10 mg ml $^{-1}$) was added. The tubes were incubated at 37 °C for 30 min. This was again followed by phenol, chloroform, isoamyl alcohol treatment. DNA was precipitated using 3 volumes of absolute alcohol and one-tenth volume of 3 M sodium acetate (pH 5.4) and pelleted by centrifugation at 10000 rpm for 15 min. The pellet was washed with 70 % ethanol. The pellet was dried and suspended in minimum amount of TE buffer (pH 7.4) composition of which is given in appendix 2. The pellet was stored at –4 °C till further use. The concentration and purity of the DNA was checked on 0.8% agarose gel containing ~ 0.5 µg ml $^{-1}$ ethidium bromide in 1% TAE buffer (Appendix 2), run at 80 V.

6.2.4. Polymerase Chain Reaction (PCR) of genomic DNA

The small subunit rRNA gene was amplified by polymerase chain reaction (PCR) on the DNA thermocycler PTC 200 (MJ Research), using the 18S rDNA specific terminal primers 18S001(5'-AACCTGGTTGATCCTGC CAGTA-3') and 18S13 (5'-CCTTGTTACGACTTCACCTTCCTCT-3') (Honda *et al.*, 1999). The

primers corresponded to the nucleotides 1-22 and 1,733-1,757 of the *Ochromonas danica* 18S rRNA gene (accession number: M32704, J02950). Approximately 25 ng of genomic DNA was taken in 25 µl reaction volume containing 1 X PCR buffer, 200µM each of dATP, dCTP, dGTP and dTTP, 5 pmoles of each primer, 1.5 mM $MgCl_2$, and 1.0 U Ampli *Taq* Gold DNA polymerase (Applied Biosystems). Amplification conditions comprised an initial denaturation step of 10 min at 94 °C followed by 35 cycles of I min denaturation at 94 °C, 1 min of annealing at 55 °C and 2 min of extension step at 72 °C and in the end a final extension step of 10 min at 72 °C. The PCR products were checked on 1.5 % agarose gel containing ~ 0.5 µg ml^{-1} ethidium bromide in 1 % TAE buffer at 120 V.

6.2.5. Cloning of PCR product in TA vector

The PCR products were ligated into pCR®2.1 vector using the TA cloning kit (Invitrogen) according to the instructions of the manufacturer. Briefly, 3 µl of fresh PCR product (`90 ng) was added to a mixture of 1 µl, 10X ligation buffer, 1 µl of pCR®2.1 vector and 4 µl of sterile distilled water. To this solution 1 µl of T4 DNA ligase (4.0 Weiss units) was added. The ligation reaction was incubated at 14 °C for overnight. The constructs were transformed into *E. coli* DH5α competent cells by heat shock method. The transformation was carried out following the protocol of Sambrook *et al.* (1989). The competent cells (50 – 100 µl) were thawed, to which 3 µl of ligation mix was added and gently mixed and kept on ice for 30 min. Further the cells were given a heat shock for 2 min in a

water bath maintained at 42 °C. The tubes were placed on ice for 2 min, to which 1 ml LB broth was added. The cells were incubated at 37 °C for 1 h under shaken conditions (200 –250 rpm). The cells were then plated on LB agar (containing 1 µg ml^{-1}) on which previously 40 µl X-gal (20 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and 10 µl IPTG (200 mg ml^{-1} isopropylthio-β-D galactoside) was spread. The plates were incubated at 37 °C for overnight or till the blue / white selection occurs.

6.2.6. Plasmid Isolation

Plasmid extraction was carried out by following the protocol of Sambrook *et al.* (1989). A single white colony was transfered into 1 ml LB broth containing 1 µg ml^{-1} ampicillin in an Eppendorf tube and incubate at 37 °C for 16 – 20 h with vigorous shaking. The tubes were then centrifuged at 10000 g at 4 °C for 15 min. The medium was decanted and the pellet was dried at room temperature. The pellet was resuspended in 100 µl of ice-cold solution I (see Appendix 2) by vigorous shaking. Two hundred microliters of freshly prepared Solution II was added. This suspension was mixed by inverting the tube rapidly and 150 µl of ice-cold solution III (see Appendix 2) was added. This solution was mixed and the tubes are centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was transferred to a fresh tube and 2 volumes of ethanol was added and incubated at –4 °C for 30 min. The precipitated DNA was pelleted by centrifugation at 10000 rpm for 15 min at 4 °C and the pellet is washed with 70 % ethanol. The pellet was dried and resuspended in 0.1 % TE (pH 8).

6.2.7. Polymerase Chain Reaction (PCR) of inserts

The inserts were amplified using M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-AACAGCTATGACCATG-3') universal primers. Approximately 25 ng of plasmid DNA was taken in 20 µl reaction volume containing 1 X PCR buffer, 200µM each of dATP, dCTP, dGTP and dTTP, 5 pmoles of each primer, 1.5 mM $MgCl_2$, and 1.0 U Ampli *Taq* Gold DNA polymerase (Applied Biosystems). Amplification conditions comprised an initial denaturation step of 10 min at 94 °C followed by 35 cycles of 30 s denaturation at 94 °C, 30 s of annealing at 55 °C and 2.5 min of extension step at 72 °C and in the end a final extension step of 5 min at 72 °C. The PCR products were checked on 1.5 % agarose gel containing ~ 0.5 µg ml^{-1} ethidium bromide in 1 % TAE buffer at 120 V.

6.2.8. Sequencing of Inserts

Amplified inserts were then sequenced for both the strands on an automated DNA sequencer ABI-Prism 3700 using the fluorescence-based detection approach and dideoxy-termination sequencing chemistry as per the manufacturere's details (Applied Biosystems). For sequencing, two additional internal primers, NS3 (5'-GCAAGTCTGGTGCCAGCAGCC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White *et al.*, 1990) were used in conjunction with the M13 universal primers that were originally used to amplify the cloned inserts.

6.2.9. BLAST and Phylogenetic analysis

The 18S rRNA gene sequences were edited and assembled using Autoassembler program (AppliedBiosystems) and deposited in the NCBI nucleotide sequence databank as given for each isolate. The final sequences were used to identify and retrieve 36 related homologous sequences of reference organisms (Table 1) available in the GenBank database (National Center for Biotechnology Information, USA: NCBI Home page http://www.ncbi.nlm.nih.gov), using the BLASTn program (Altschul *et al.* 1990). The 18S rDNA sequences obtained for the organism were aligned with corresponding sequences belonging to the 34 reference Labyrinthulomycetes (Table-2) using the programs Clustal X (http://www-igbmc.u-strasbg.fr/BioInfo/) (Higgins *et al.*, 1989) and GenDoc (http://www.psc.edu/biomed/genedoc) (Nicholas *et al.*, 1997) and also checked manually for large gaps. The aligned sequences were flushed at the ends to avoid missing information for any compared reference entries. This resulted in a final alignment of 1600 bp, which was used for further comparisons. The aligned sequences were then used to derive corrected Kimura two-parameter distance (Kimura, 1980) estimates and infer phylogenetic relationships using both distance based as well as direct character-state based methods, namely, neighbor joining (Saitou *et al.*, 1987) and maximum likelihood, with analytical routines available in the software packages PHYLIP 3.5c (Felsenstein, 1993) (http://evolution.genetics.washington.edu/phylip.html) and Phylo_win (Galtier *et al.*, 1996). For phylogenetic analysis, the two alveolates, *Prorocentrum micans* (Dinoflagellata) and *Oxytricha granulifera* (Ciliophora) were selected as

outgroups to root the phenetic trees as they often form sister groups with the straminopiles in the 18S rDNA eukaryotic tree. The robustness of the phenetic clustering was ascertained by 500 bootstrap resamplings (Felsenstein, 1985).

Organism	GenBank AccessionNumber
Oxytricha granulifera	X53486
Prorocentrum micans	M14649
Aplanochytrium kergulense	AB022103
Japanochytrium Sp.	AB022104
Labyrinthuloides haliotidis	U21338
Labyrinthula Sp.	AB022105
Schizochytrium aggregatum	AB022106
Schizochytrium limacinium	AB022107
Schizochytrium minutum	AB022108
Thraustochytrium aggregatum	AB022109
Thraustochytrium aureum	AB022110
Thraustochytrium kinnei	L34668
Thraustochytrium multirudimentale	AB022111
Thraustochytrium striatum	AB022112
Thraustochytrium pachydermum	AB022113
Ulkenia profunda	L34054
Ulkenia profunda (#29)	AB022114
Ulkenia radiata	AB022115
Ulkenia visurgensis	AB022116
Thraustochytriidae sp. N4-103	AB073309
Thraustochytriidae sp. N1-27	AB073308
Thraustochytriidae sp. H6-16	AB073306
Thraustochytriidae sp. H1-14	AB073305
Thraustochytriidae sp. F3-1	AB073304
Thraustochytrium sp. CHN-1	AB126669
Aplanochytrium stocchinoi	AJ519935
Schizochytrium sp. KH105	AB052555
Thraustochytrium sp. KK17-3	AB052556
Labyrinthulid quahog parasite QPX 1	AY052664
Labyrinthulid quahog parasite QPX	AF155209
Aplanochytrium sp. SC1-1	AF348520
Aplanochytrium sp. PR1-1	AF348516
Aplanochytrium sp. PR12-3	AF348517
Aplanochytrium sp. PR15-1	AF348518
Labyrinthula sp 1	AF348522
Thraustochytriidae sp. M4-103	AB073307

Table 6.2.1: List of 18S rRNA gene sequences of related Labyrinthulomycota species used as reference taxa in this study for ascertaining the generic affiliation of the new isolate.

6.3. Results

6.3.1. Taxonomy of isolate NIOS-1

6.3.1.1. Developmental stages

Colonies on MV agar medium were round, white and comprised of globose to irregular cells (Fig. 6.3.1a). Prominent ectoplasmic nets were present (Fig 6.3.1 b). Vegetative cells ranged in size from 4 –20 µm in size (Fig. 6.3.1 c). Developmental stages consisted of diads (Fig. 6.3.1 d,) tetrads (Fig. 6.3.1 e) and zoosporangia with several zoospores (Figs. 6.3.1 f –i). Amoeboid cells were also frequently observed (Figs. 6.3.1 j and k). Typical heterokont biflagellate zoospores with one long anterior flagellum and one short posterior flagellum were seen (Figs. 6.3.1 l). Zoospores were elliptical in shape and the flagella were sub-terminally attached.

Repeated binary division of the cells of NIOS-1 is a characteristic feature of the genus *Schizochytrium* Goldstein and Belsky (Goldstein and Belsky, 1964). The present isolate morphologically resembles this genus.

A culture of this isolate has been deposited in Microbial Type culture collection (MTCC), India, under the accession number MTCC 5121.

6.3.1.2. Molecular Characteristics

Amplification of the 18S rRNA gene of NIOS-1 gave a single PCR product of 1747 bp. The sequence has been deposited at the NCBI gene bank under the accession number AY 705757. A BLAST, search (Altschul et al, 1990) showed that the isolate NIOS-1 was homologous to *Schizochytrium limacinium*. All the

sequences that showed the highest homology to NIOS-1 in the BLAST search belonged to the thraustochytrid phylogenetic group. The isolate belongs to the Labyrinthulomycetes, as clearly shown by the phylogenetic analyses. Phenetic trees obtained by neighbor joining (NJ) (Fig 6.3.4 a) and maximum likelihood (ML) method (Fig 6.3.4 b) showed an overall similar topology except for minor differences in the branch lengths. In both the phenetic trees NIOS-1 appeared to be a distinctive new member and also formed a new clade with *Schizochytrium limacinum.* This was supported with a very high bootstrap of 100 indicating its strong relationship.

6.3.2. Taxonomy of isolate NIOS-4

6.3.2.1. Developmental stages

The development cycle of the isolate NIOS-4 is given in Fig. 6.3.2. The organism produced minute globose colonies on MV agar with a glistening texture (Fig. 6.3.2 a). They also produced a light orange pigment after 7 days. Feeble ectoplasmic nets were produced (Fig. 6.3.2 b). Vegetative cells ranged from 4-10 µm in size (Fig. 6.3.2 c). These cells on further growth became irregular and amoeboid in shape (Fig. 6.3.2 d). The amoeboid cells divided into four cells (Figs. 6.3.2 e – g). These became the zoosporangia, the contents of whic divided to form zoospores in multiples of 4 (Figs 6.3.2 h and i). Typic heterokont biflagellate zoospores with one long anterior flagellum and one sh posterior flagellum were seen (Figs. 6.3.2. j), which were elliptical in shape the flagella were sub-terminally attached.

The developmental cycle comprised an amoeboid stage, which is typical of the genus *Ulkenia* Gaertner (Gaertner, 1977) and a tetrad stage, as in the genus *Schizochytrium*. This isolate is deposited in Microbial Type culture collection (MTCC), India, and American Type Culture Collection under the accession numbers MTCC 5123 and ATCC PRA-147 respectively.

6.3.2.2. Molecular Characteristics

Amplification of the 18S rRNA gene of the isolate NIOS-4 gave a single PCR product of 1744 bp and the sequence is submitted to the NCBI gene bank under the accession number AY 705741. A BLAST (Altschul et al, 1990), search showed that the isolate NIOS-4 was homologous to *Schizochytrium* sp. KH105. All the sequences that showed the highest homology to NIOS-4 in the BLAST search belonged to the thraustochytrid phylogenetic group. Phylogenetic analyses placed the organism distinctly within the group of Labyrinthulomycetes. Phenetic trees obtained by NJ (Fig. 6.3.5 a) and ML phenetic trees (Fig 6.3.5 b) showed an overall similar topology except for the minor differences in the branch lengths. In both the phenetic trees NIOS-4 appeared to be a distinctive new member and also formed a new clade with *Schizochytrium* sp. KH105. This was supported with a very high bootstrap of 100 indicating its strong relationship.

6.3.3. Taxonomy of isolate NIOS-6

6.3.3.1. Developmental stages

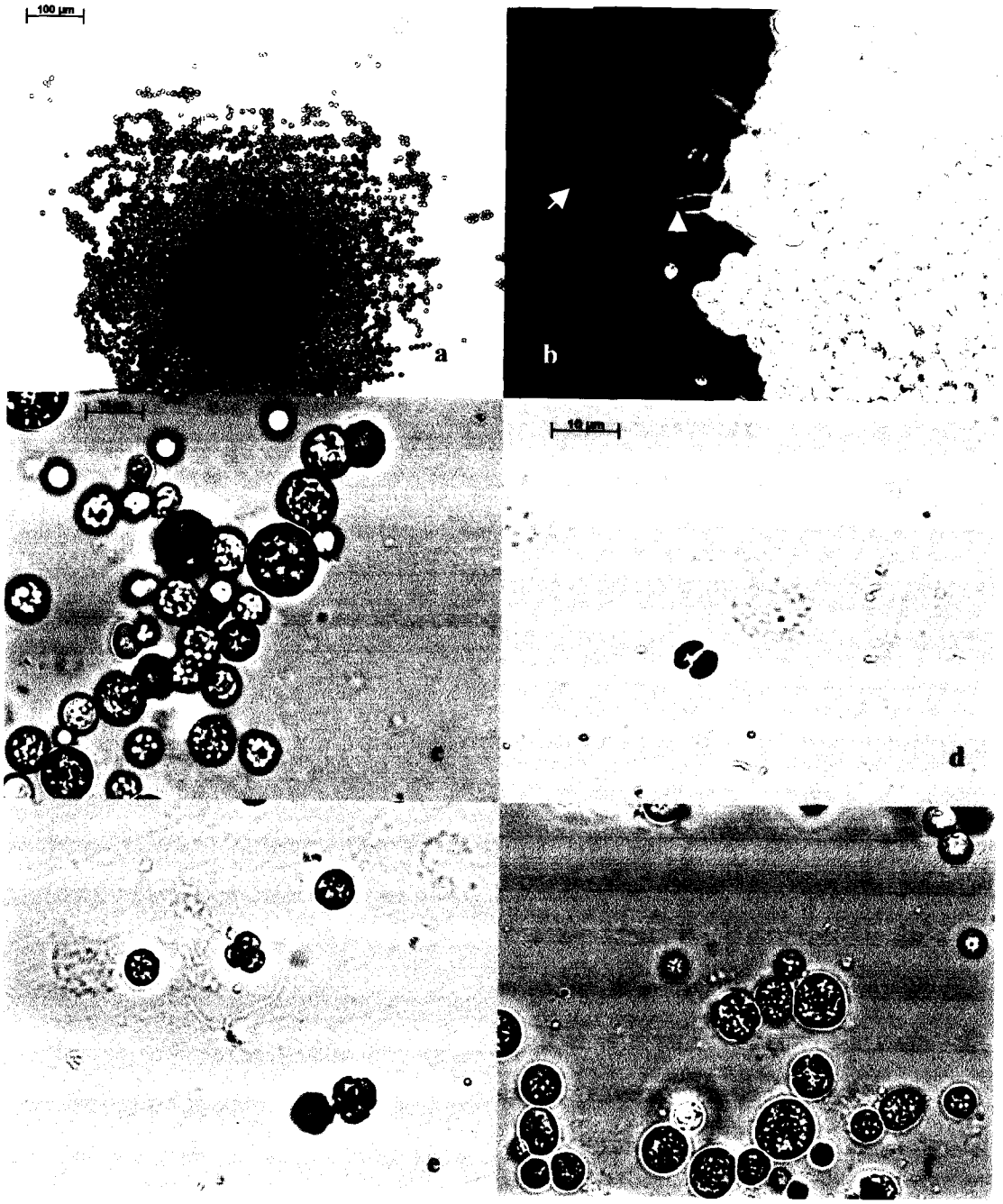
The development cycle of the isolate NIOS-6 is given in Fig. 6.3.3. The colonies were round and produced globose to irregular cells in shape (Fig. 6.3.3. a). Extensive ectoplasmic nets were formed (Fig. 6.3.3 b). Vegetative cells ranged from 5 – 15 µm in size and were filled with oil droplets (Figs. 6.3.3 c and d). The contents of these cells became granular (Fig. 6.3.3 e) and divided to form zoospores. The zoosporangium contained more than 30 zoospores (Fig. 6.3.3 f). The zoospores were released through a tear in the cell wall (Fig. 6.3.3 g). Typical heterokont biflagellate zoospores with one long anterior flagellum and one short posterior flagellum were seen (Figs. 6.3.3 g). Amoeboid cells were also frequently seen (Fig. 6.3.3 h).

In the isolate NIOS-6, the entire contents of the zoosporangia divided to form zoospores. This is a characteristic feature of the genus *Thraustochytrium* Sparrow (see Dick, 2001). This isolate is deposited in Microbial Type culture collection (MTCC), India, and American Type Culture Collection under the accession number s MTCC 5122 and ATCC PRA-148 respectively.

6.3.3.2. Molecular Characteristics

Amplification of the 18S rRNA gene of the isolate NIOS-6 gave a single PCR product of 1784 bp and the sequence is submitted to the NCBI gene bank under the accession number AY705752. A BLAST (Altschul et al, 1990), search showed that the isolate NIOS-6 was homologous to *Thraustochytrium kinnei.* All

the sequences that showed the highest homology to NIOS-6 in a BLAST search belonged to the thraustochytrid phylogenetic group. Phylogenetic analyses based on the 18S rRNA gene sequence robustly established the protistan identity of the isolate NIOS-6, belonging to Labyrinthulomycetes. Apart from some minor differences in branch lengths, the phenetic trees showed an overall similar topology (Fig 6.3.6 a and b). In both the phenetic trees NIOS-4 appeared to be a distinctive new member with *Thraustochytrium kinnei* as the closest relative. A high bootstrap value of 95 indicated its strong relationship with this species. Isolate NIOS-6 could also be evolutionary basal to the three undescribed *Thraustochytrium* species whose accession numbers are given in Table 6.2.1.



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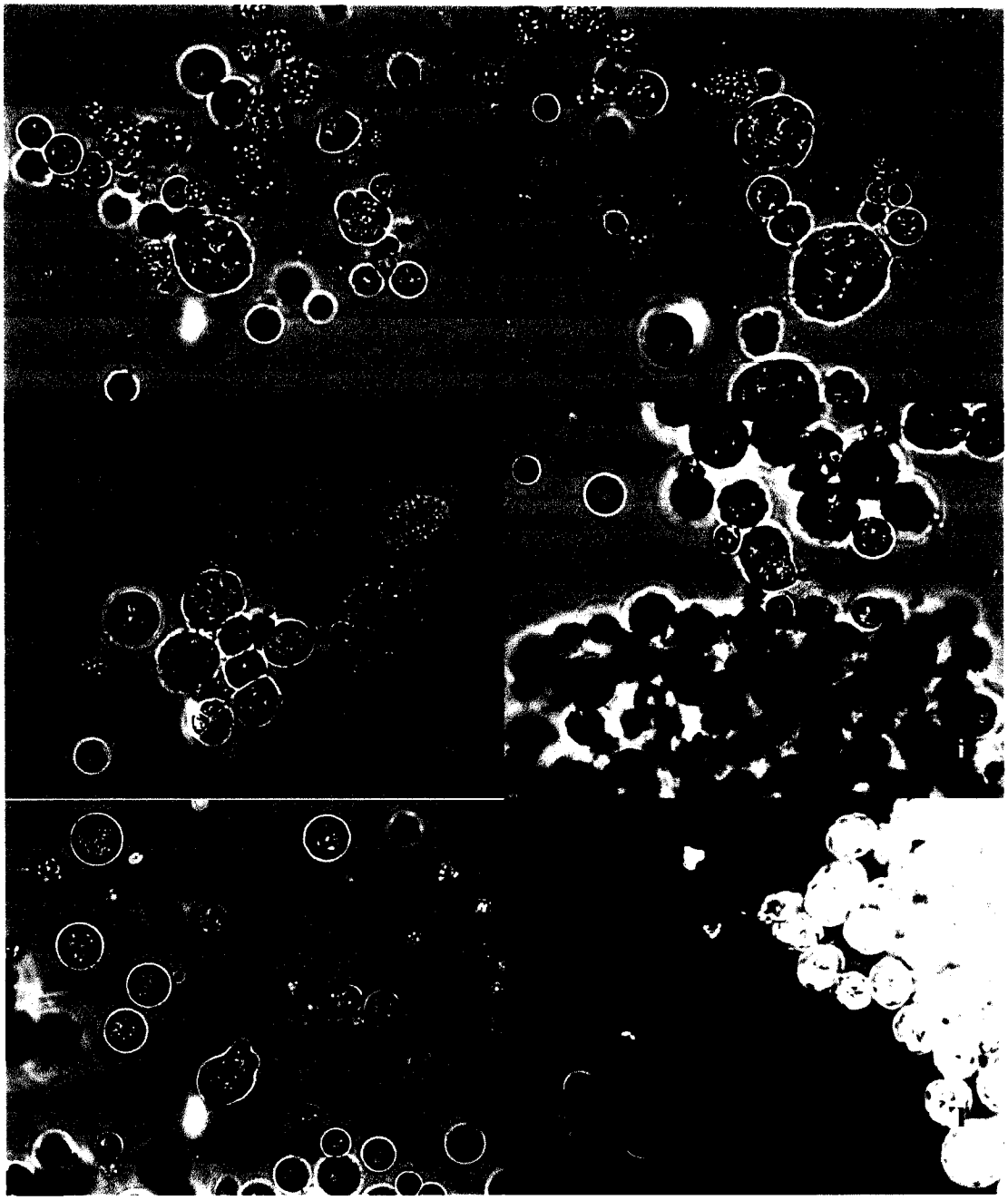
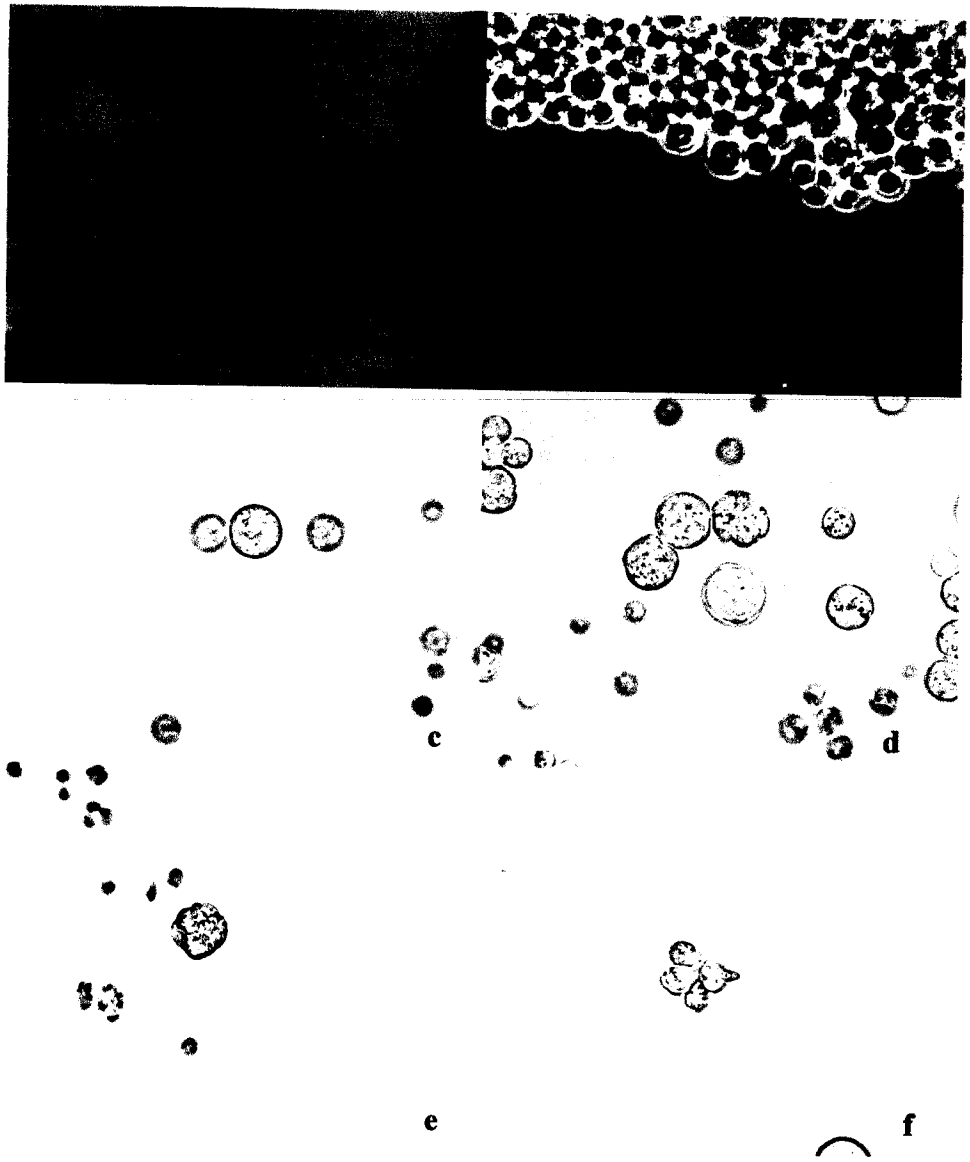


Fig. 6.3.1: Photomicrographs of various developmental stages of isolate NIOS-1. a. Colony on MV agar medium. Bar represents 100 µm; b. Ectoplasmic net elements (arrow) on MV agar. Bar represents 10 µm; c. Vegetative cells of various sizes; d. diad; e. tetrad; f-i. Zoosporangia in various developmental stages and formation of zoospores; j and k. amoeboid cells. l. heterokont zoospores. Bars in fig c - l represents 10 µm.



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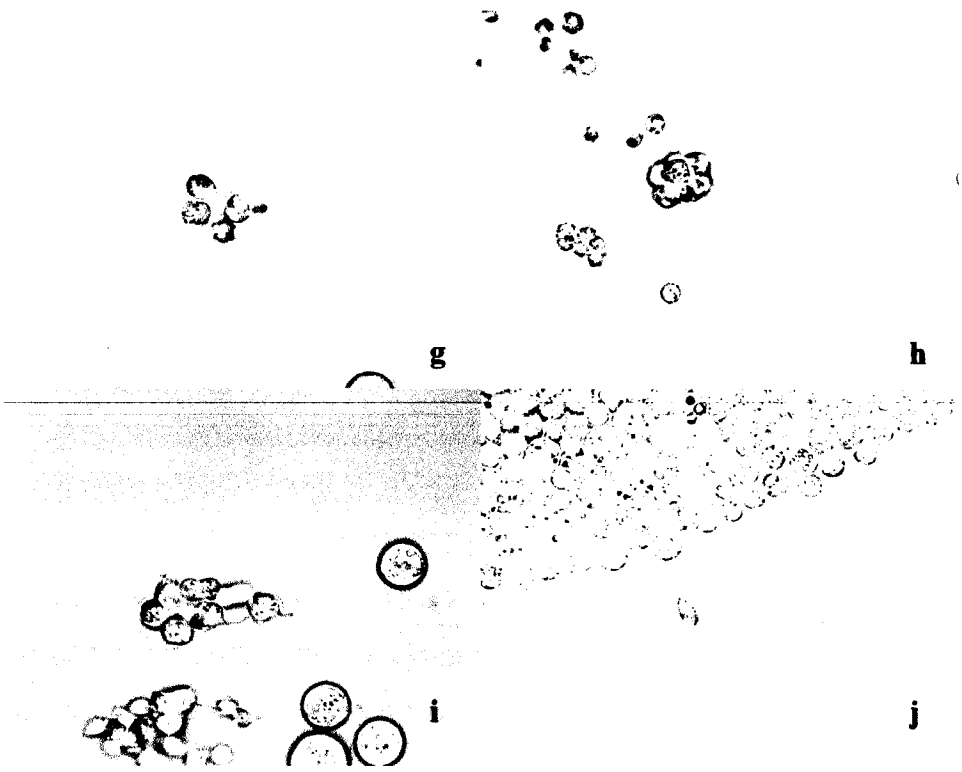

Fig. 6.3.2. Photomicrographs for various development stages of isolate NIOS-4. a. Colony on MV agar medium. Bar represents 100 µm; b. Ectoplasmic net elements on MV agar. c. Mature thallus with granular contents; d. Cells becoming irregular and amoeboid; e. Amoeboid cells showing initiation of zoospore cleavage; f and g. Division to form a tetrad; h. Initiation of further cleavage of zoospores; i. Zoospore formation; j. Heterokont zoospore on MV agar. Scale in Fig. b applies to Figs. b to j. Bar represents 10 µm.

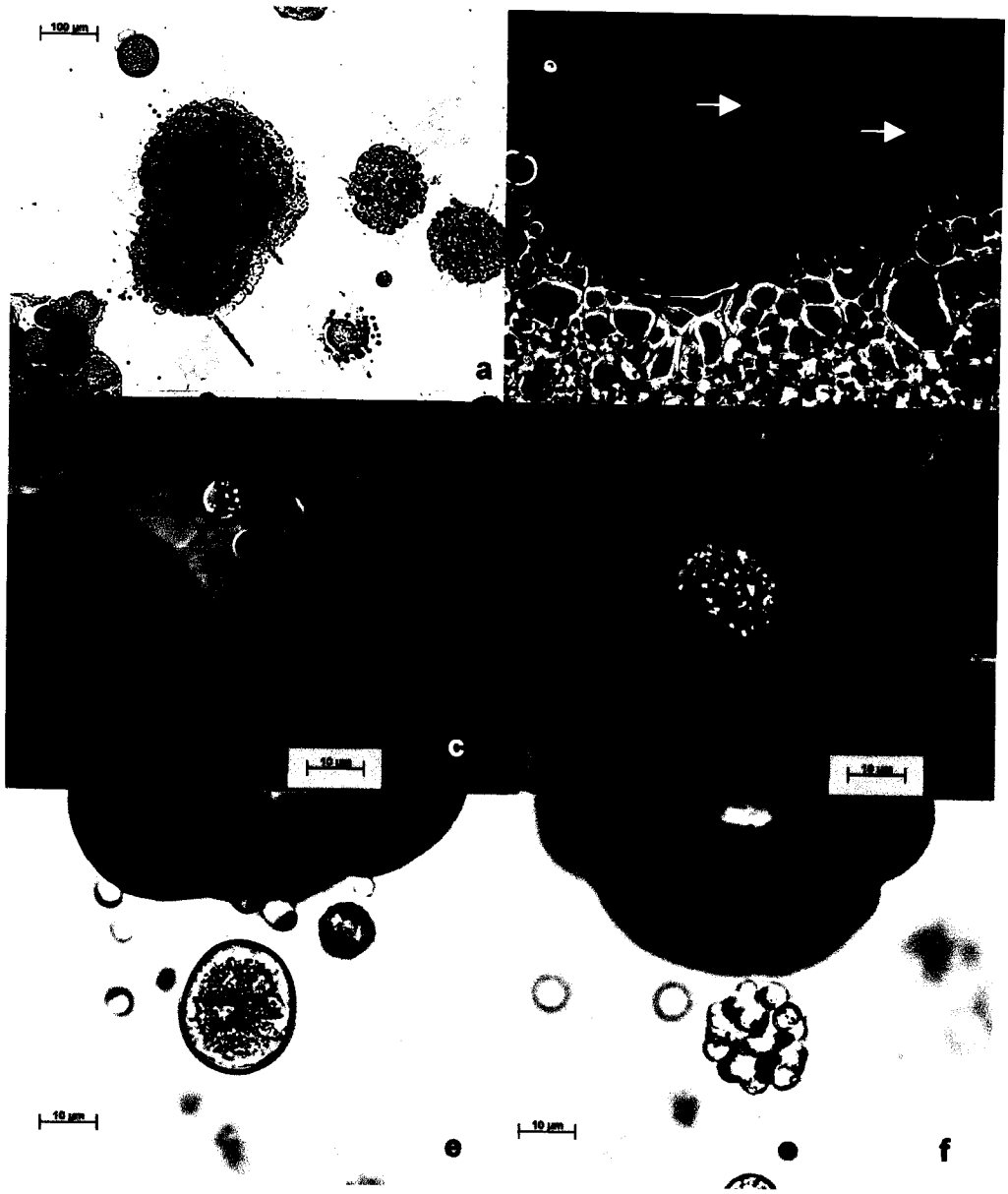

Fig. 6.3.3: Contd. overleaf

Fig. 6.3.3: Photomicrographs of various developmental stages of isolate NIOS-6. a. Colony on MV agar medium. Bar represents 100 µm; b. Ectoplasmic net elements (arrow) on MV agar. Bar represents 50 µm; c. Freshly encysted zoospore on pine pollen; d. Young thallus with prominent globules; e. Mature thallus with granular contents; f. Young zoosporangium showing initiation of zoospore cleavage; g. Mature zoosporangium with zoospores; h. Liberation of heterokont zoospores; i. An irregularly shaped cell. Bar in Figs c - i represents 10 µm.

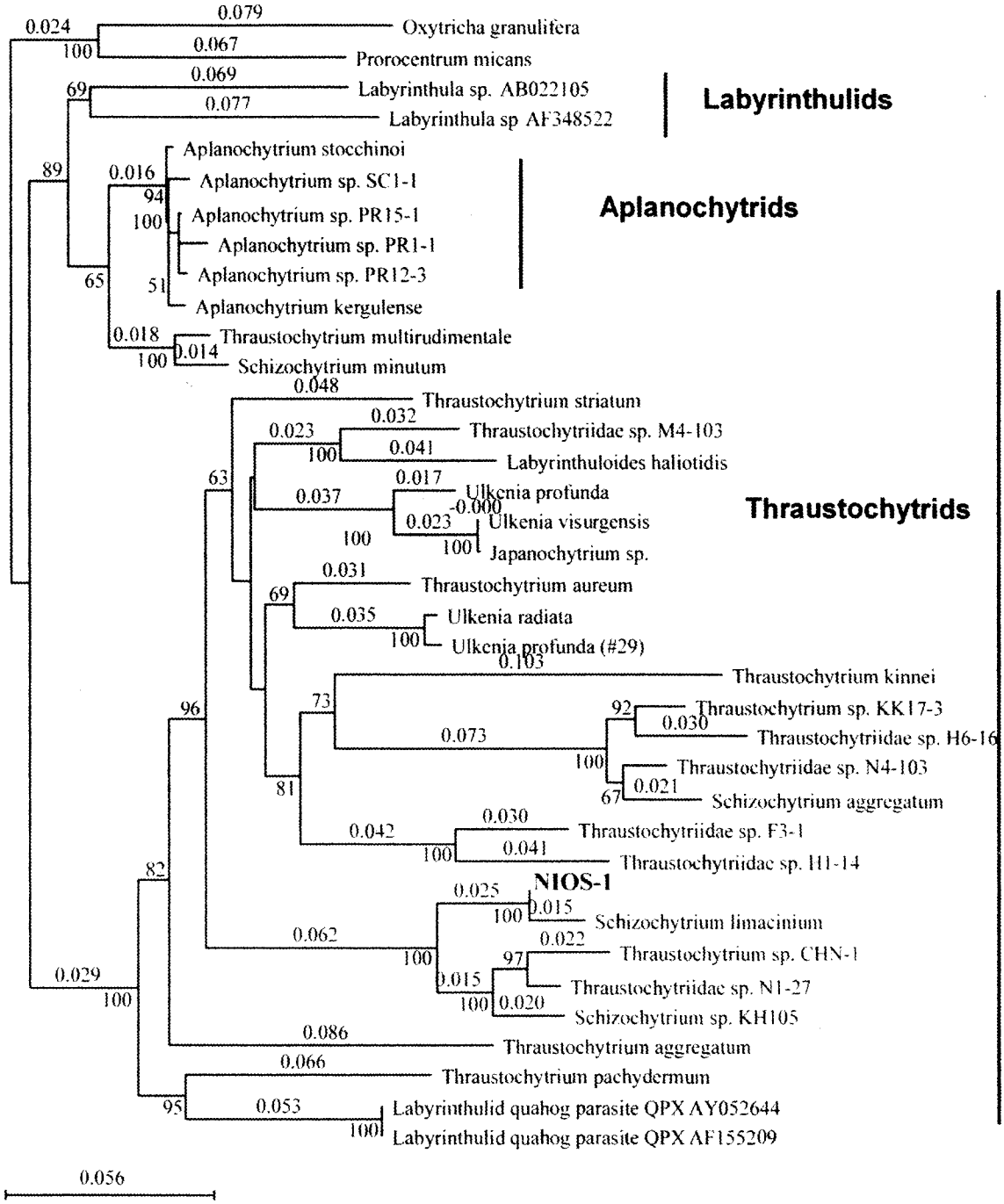

Fig. 6.3.4 a: Phylogenetic analysis of the NIOS-1 and other thraustochytrids aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The Neighbor-joining tree is constructed using Kimura 2 parameter. The numbers at each internal branching shows bootstrap values or for the nodes supported by more than 50 % (500 replicates). The numbers on t branches are the branch lengths. Bar represents 0.056 mutations per site.

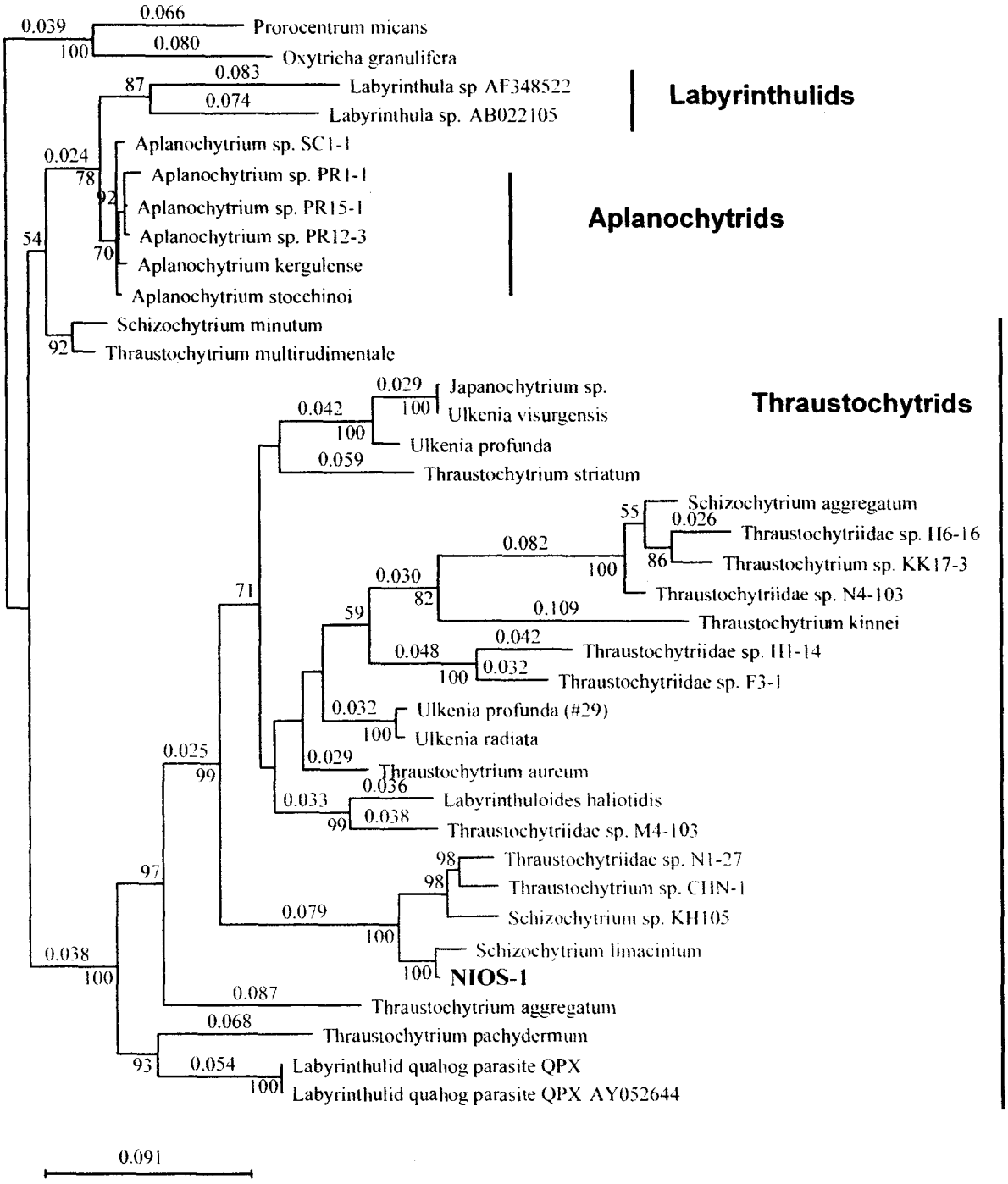

Fig. 6.3.4 b: Phylogenetic analysis of NIOS-1 and other thraustochytrids, aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The phylogram is the best maximum likelihood tree (ln(L)=-9724.886) and distance is calculated using gamma corrected Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50 % (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.091 mutations per site.

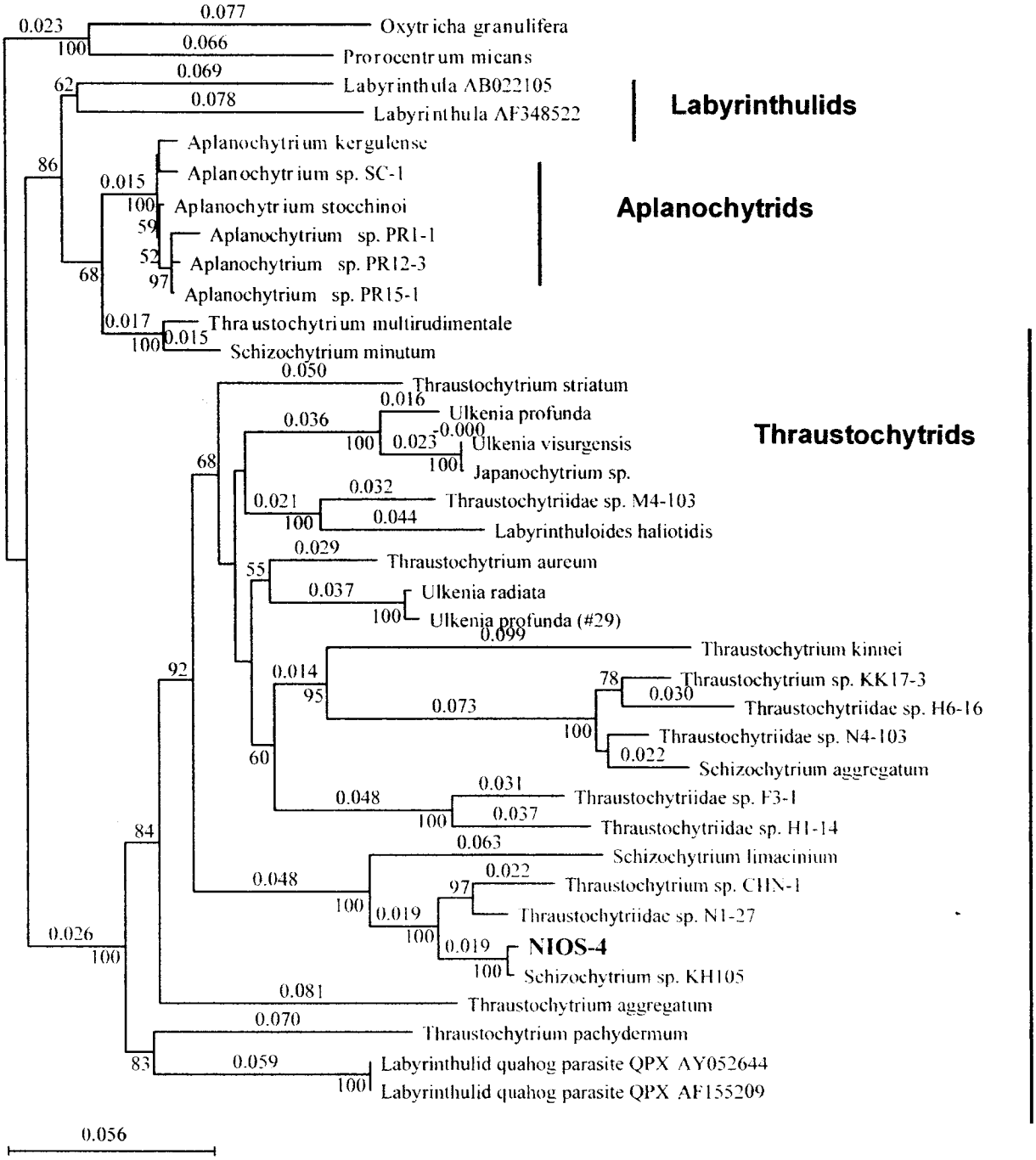

Fig. 6.3.5 a: Phylogenetic analysis of the NIOS-4 and other thraustochytrids, aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The Neighbor-joining tree is constructed using Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50 % (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.056 mutations per site.

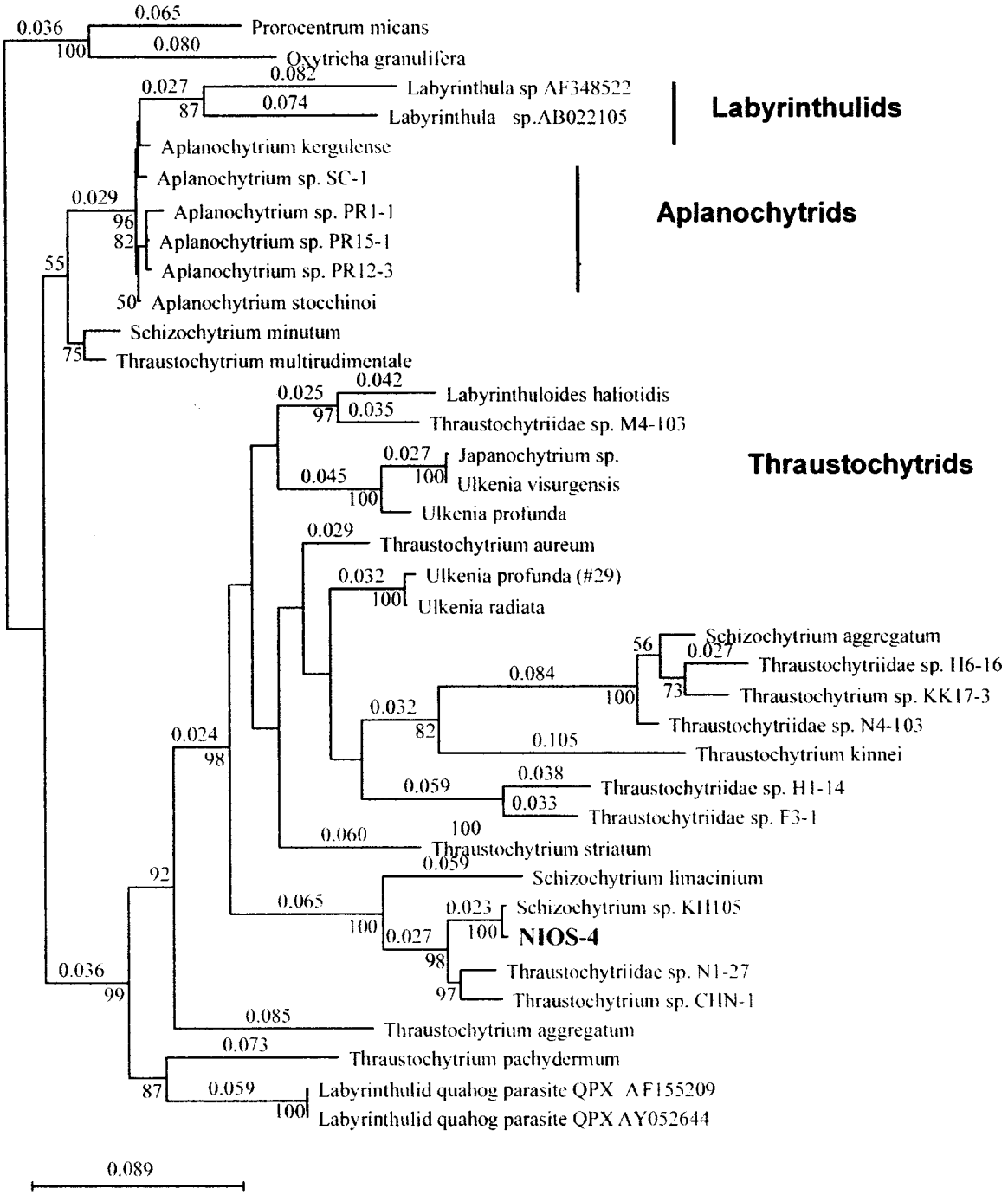

Fig. 6.3.5 b: Phylogenetic analysis of NIOS-4 and other thraustochytrids, aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The phylogram is the best maximum likelihood tree (ln(L)=-9814.179) and distance is calculated using gamma corrected Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50 % (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.091 mutations per site.

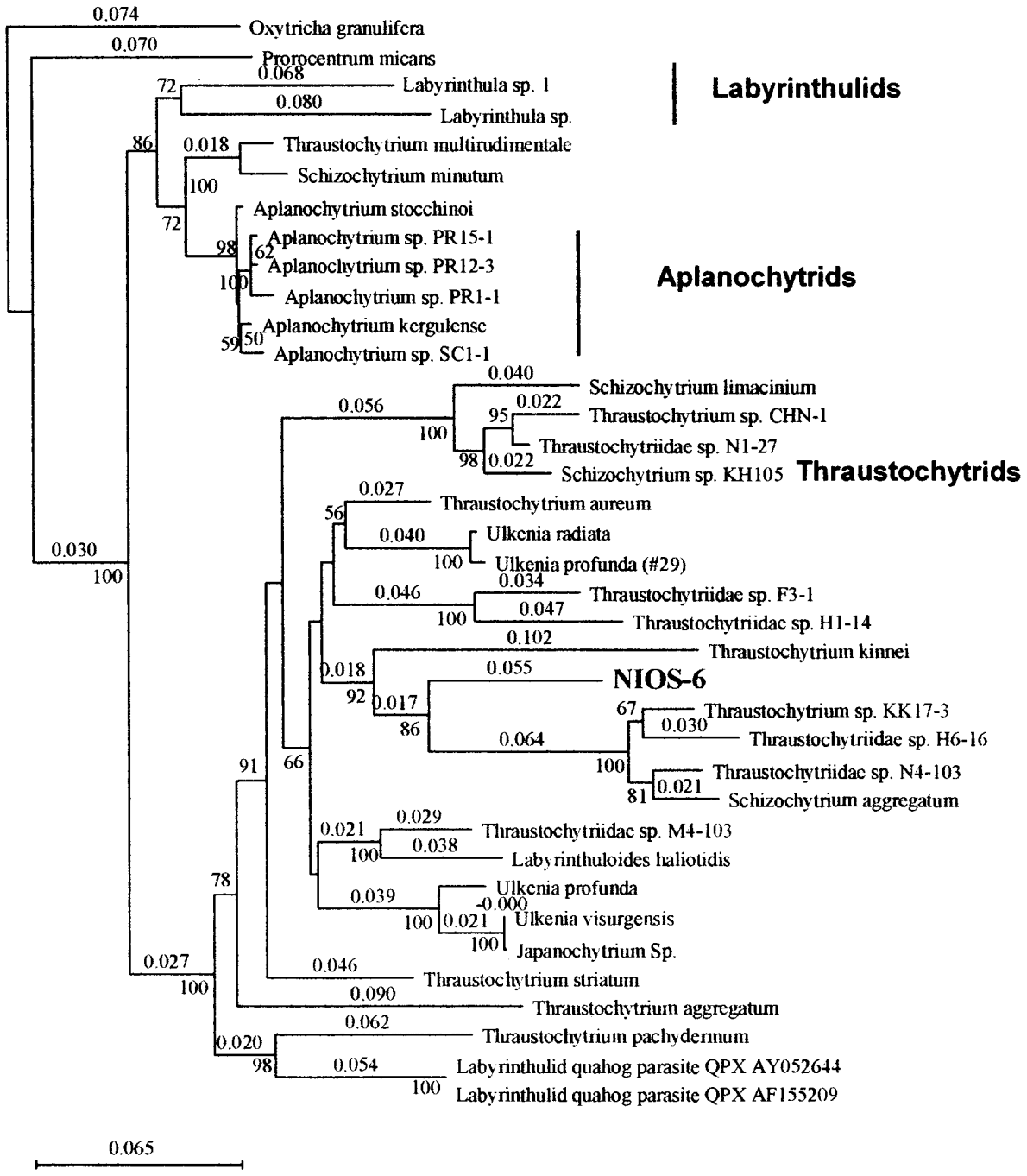

Fig. 6.3.5 a: Phylogenetic analysis of the NIOS-6 and other thraustochytrids, aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The Neighbor-joining tree is constructed using Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50 % (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.065 mutations per site.

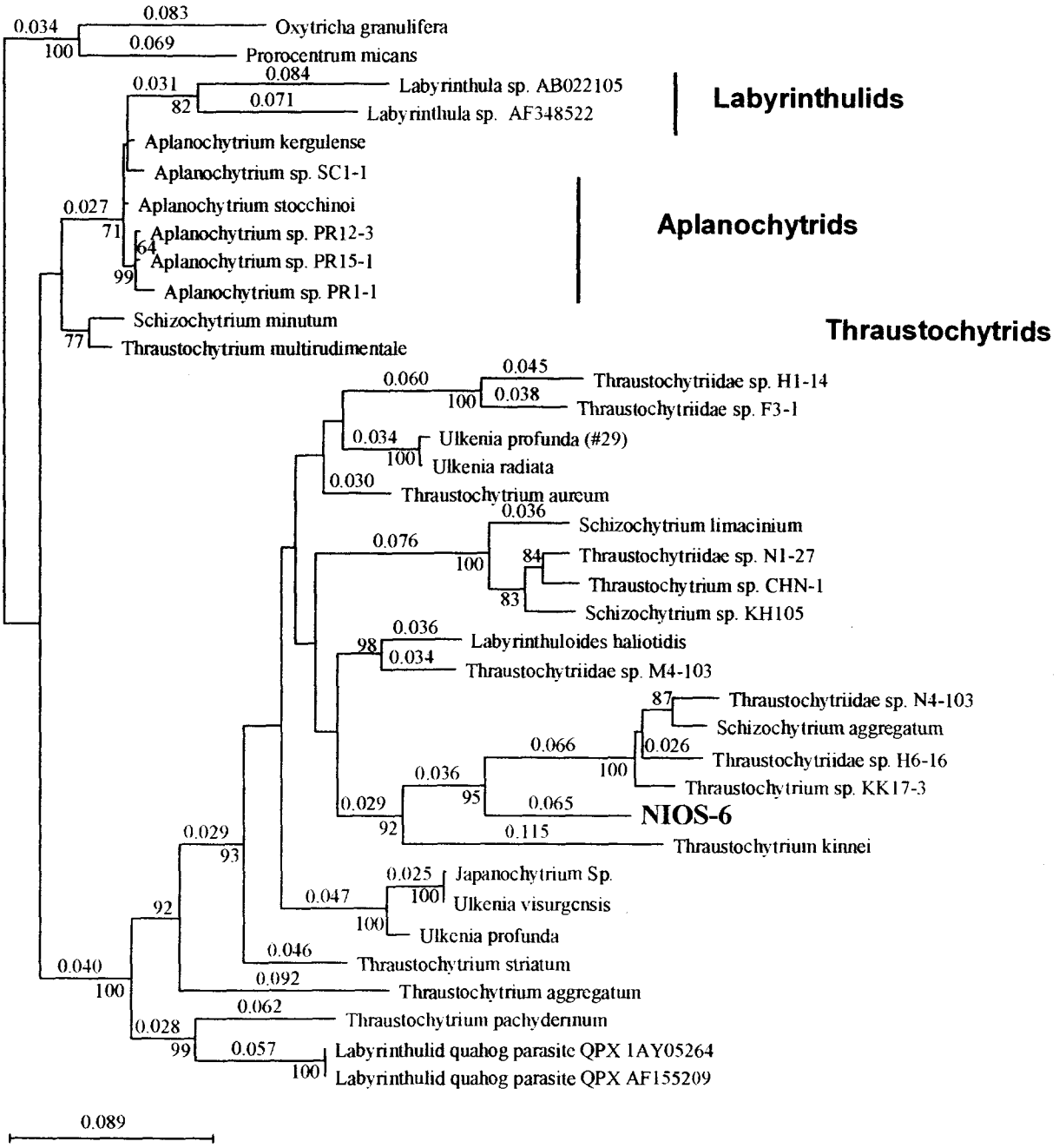

Fig. 6.3.6 b: Phylogenetic analysis of NIOS-6 and other thraustochytrids, aplanochytriums and labytrinthulids bases on the multiple alignments of 18S rDNA sequences. The phylogram is the best maximum likelihood tree (ln(L)=-10234.017) and distance is calculated using gamma corrected Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50 % (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.089 mutations per site.

6.4. Discussion

The phylogenetic trees presented in Figs. 6.3.4, 6.3.5 and 6.3.6 broadly agree with those generated earlier by Honda et al. (1999) and Leander and Porter (2001). Thus, three separate groups stand out, namely the labyrinthulids, comprising species of *Labyrinthula*, the aplanochytrids, consisting of *Aplanochytrium* spp. and the thraustochytrids.

The 18S SSU rRNA gene sequences of NIOS-1, NIOS-4 and NIOS-6 were distinctly different from the 34 sequences of Labyrinthulomycetes that are available in the NCBI gene bank (Fig. 6.3.4 - 6.6.6). Therefore, all three isolates appeared to belong to species not yet described.

Thraustochytrid taxonomy is generally based on morphology and life cycle based studies (Porter, 1990; Dick, 2001; Raghukumar, 2002). The general characters used are presented in Table 6.1.1. However there are only a few characters available for such a classification in thraustochytrids, as given in Table. 6.1.1. There has been a concern that many characters are either variable depending upon culture conditions or that characters may overlap between species (Booth and Miller, 1968). This has also been highlighted by the study of Honda et al., (1999), who examined the molecular phylogeny of thraustochytrids.

In the present study molecular sequences indicated that NIOS-1 and NIOS-4 belonged to the genus *Schizochytrium* (Figs. 6.3.4 and 6.3.5). This genus is characterized by repeated binary divisions of the mature vegetative cells, leading to the formation of zoospores (Goldstein and Belsky, 1964). NIOS-1 showed distinct diad and tetrad stages typical of this genus (Fig. 6.3.1 d and e).

Although NIOS-4 showed tetrad stages, linking it to the genus *Schizochytrium*, it also had an amoeboid stage of the mature vegetative cells similar to the genus *Ulkenia* Gaertner (Gaertner, 1977; see Table 6.1). In the absence of molecular sequences, it would have been difficult to assign this to the genus *Schizochytrium*.

Sequences of the 18S rRNA gene showed that although NIOS-1 and NIOS-4 had a close homology to species of *Schizochytrium*, they were set apart in the phylogenetic trees (Fig. 6.3.5 a and b). NIOS-1 formed a clade with *Schizochytrium limacinum*, while NIOS-4 formed a clade with an unidentified *Schizochytrium* sp. KH105. However, NIOS-4 was more closely related to two unidentified species of *Thraustochytrium*. Further, these trees show that in general species of *Schizochytrium* are spread in three distantly related clades, often closer to species of *Thraustochytrium*, than to each other. This yet again points to the problem of using morphological characters alone for the taxonomy of thraustochytrids.

Both morphological observations (Fig. 6.3.3), as well as the phenetic trees (Figs. 6.3.6) confirmed that NIOS-6 belonged to the genus *Thraustochytrium*, which is characterized by division of the zoosporangial contents into zoospores without the cell changing its shape (Dick, 2001). This isolate appeared to belong to a new species of *Thraustochytrium*. Among the known species of his genus it is most closely related to *T. kinnei* Gaertner.

In view of the above, it becomes necessary to combine morphological and developmental studies with molecular taxonomy. Molecular taxonomy of

thraustochytrids has shown that more studies have to be carried out to classify thraustochytrids more properly. Further critical observations on the morphology and life cycles of the isolates NIOS-1, NIOS-4 and NIOS-6 will help in a better understanding of their taxonomy.

So far, only a total of about 35 species are known, although thraustochytrids have been studied since they were first discovered nearly 70 years ago by Sparrow (Sparrow, 1936). This study indicates that several new species of thraustochytrids are yet to be discovered. Most of the earlier studies were carried out in temperate waters. Tropical waters are generally recognized as habitats of high marine biodiversity (Gray, 1997). It is likely that future studies on thraustochytrid diversity in these waters will reveal a high diversity of these organisms.

6.5. Conclusions

Three isolates of thraustochytrids, NIOS-1, NIOS-4 and NIOS-6 were examined for their morphology and development, as well as sequences of their 18S SSU rRNA gene. NIOS-1 morphologically corresponded to the genus *Schizochytrium*, while NIOS-4 showed characters resembling the genus *Ulkenia*, as well as *Schizochytrium*. NIOS-6 morphologically resembled the genus *Thraustochytrium*. Comparisons of their sequences with 34 other members of Labyrinthulomycetes and two outgroups revealed that all three were new to science. NIOS-1 and NIOS-4 corresponded to *Schizochytrium* and NIOS-6 to *Thraustochytrium*. The study highlights the importance of applying molecular sequence information together with morphological and developmental characters to classify thraustochytrids properly.

CHAPTER 7

Summary

Thraustochytrids, which comprise one of the groups of the marine, heterotrophic protists of the Kingdom Stramnipila, are ubiquitous and abundant in the sea. In addition to the reason that they play a potentially important role in the marine ecosystem, they have attracted much attention in recent years because they produce the polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA), docosapentanoic acid (DPA), eicosapentanoic acid (EPA) and arachidonic acid (ARA). DHA is particularly abundant in these organisms. This PUFA is known an essential nutrient for growth and maturation of crustaceans and some fishes. DHA is also extremely important in human nutrition and is a preventive of many diseases. Thraustochytrids might also be a potential source of other biotechnologically useful compounds. This thesis was aimed at: (1) Screening, optimizatation and enhancement of DHA production by thraustochytrids; (2) Screen them for the production of extracellular polymeric substances (EPS) and to chemically characterize them, and (3) to understand their importance to the cell.

An isolate, designated 'NIOS-1' was selected for further studies on production of biomass, lipids and DHA contents, after screening a total of six isolates. NIOS-1 produced the best biomass of 19.12 g L^{-1} with the highest lipid concentration of 36 % and the maximum yield of DHA, amounting to 22.6 % of the total lipids.

A medium, designated 'M4' was selected for all further experimental work, based on preliminary media optimization studies. Several media from

literature were tested for production of biomass by NIOS-1 and a medium designated 'M3' was selected as the best. Further optimization of M3 helped in refining the medium for growth of NIOS-1 as follows: no requirements of vitamins and trace metals in addition to yeast extract; a pH optimum of 7.0 and a salinity optimum of 35 ppt. Among the 10 different carbon sources, glucose was the best for biomass production. This medium was designated 'M4'.

Levels of glucose, peptone and yeast extract in M4 medium were optimized for production of biomass, lipids and DHA using the 'One factor at a time' (OFAT) and Response Surface Methodology (RSM). According to the OFAT method, 3 % glucose, 0.15 % peptone and 1.5 % yeast extract gave the best biomass with optimum percent lipids. RSM trials, followed by multiple regression analyses indicated that glucose had a significant effect on biomass production and peptone on lipid concentration and DHA yield in biomass. High C:N ratios appeared to favour high DHA yields. The percentage of DHA in lipids, however, was not affected by glucose, peptone or yeast extract.

Total yields of lipids and the PUFAs, DHA and EPA in NIOS-1 could be enhanced by (a) refrigerating the harvested cell biomass or (b) addition of polyvinylpyrrolidone (PVP) to the medium. Experiments were carried out by harvesting cells and refrigerating them at different temperatures for 24 h and at 10°C for different durations of time. Refrigeration at 10 °C for 48 h was found to be optimal. Experiments were also carried out by addition of various concentrations of PVP to the medium and by testing the addition of 0.5 % PVP to the growth medium of 4 thrautochytrid isolates. While the various isolates

behaved differently, it was found that generally an addition of 0.5 % PVP resulted in increase of PUFA content. While both these treatments increased total yields of DHA, their percentage concentrations in total fatty acids remained constant. The combined effect of addition of 0.5 % PVP to growth medium and storage of the resulting biomass at 10 °C was found to be detrimental to total DHA yield.

Lipid bodies were used up during the transformation of vegetative cells to motile amoeboid cells and during the formation of ectoplasmic net elements. Experiments were carried out to understand the importance of high lipid and DHA accumulation to thraustochytrid cells, since this could have a direct implication in their ecology. Lipids were stained using nile blue and cells were examined under an epifluorescence microscope. Upon transformation of cells into amoeboid cells, the lipid bodies reduced in number and finally the cells became granular. Nile blue fluorescence, and therefore the lipids, diffused into ectoplasmic net elements when these were produced by cells that were originally devoid of the EN.

Total lipids were used up during starvation of cells. DHA appeared to be the primary energy reserve among lipids during starvation. When the cells of NIOS-1 were starved for a period of 28 d, the total lipids declined progressively. Cells, in which the lipid and DHA levels were stimulated, survived for a longer time. Fatty acid analysis of these cells revealed that DHA was the first fatty acid to decline in concentration, while palmitic acid concentration remained almost constant. Other fatty acids were present in minor quantities.

Increase in DHA levels was related to an increase in the specific gravity of cells. An increase in DHA content of the cells of isolates NIOS-1, NIOS-2, NIOS-4 and NIOS-10 resulted in a corresponding increase in their specific gravities, compared to controls.

The production of extracellular polymeric substances by thraustochytrids was confirmed both by microscopy, as well as quantitatively. This is the first report of EPS production by thraustochytrids. Production was confirmed in all 4 isolates examined. Phase contrast microscopy of cultures showed that EPS was produced as amorphous particles or fibrillar material. Cells were fully embedded in the EPS matrix as shown by scanning electron microscopy. EPS stained blue with alcian blue, revealed their anionic nature. All four isolates, NIOS-1, NIOS-4, NIOS-7 and NIOS-12 produced EPS when grown in high nutrient media. EPS was extacted and estimated gravimetrically. They were produced throughout the growth phase and continued in stationary phase. Studies with ^{14}C glucose indicated that about 7 % of carbon assimilated was converted to EPS in the late log phase.

EPS production was positively related to biomass, glucose concentration in the medium and total DHA yield. EPS production was estimated during the RSM trials carried out during experiments to optimize biomass and DHA yield. Correlation matrix analyses with biomass, total lipids, DHA concentration and total DHA yield were carried out to analyze their relation to EPS production.

EPS produced by isolate NIOS-1 and NIOS-4 consisted of both soluble as well as a matrix like insoluble forms, solubility being maximum in distilled water. Twelve solvents were tried, of which distilled water was the most efficient. The EPS produced by NIOS-1 was 99.4 % soluble whereas, that produced by NIOS-4 was 72.4 % soluble in distilled water.

EPS from two thraustochytrids were characterized in terms of molecular weight and gross biochemical composition. The EPS of the two isolates had a molecular weight in excess of 2000 kDa. Further, HPSEC confirmed that the polymers were more that 95 % pure. The EPS were acidic polysaccharides, containing uronic acids and sulfates apart form proteins. Sugars were the most abundant component of EPS in both the isolate – NIOS-1 and NIOS-4 comprising 53 and 39 % respectively in the total EPS. Sulfate content in both the polymers was above 10 %. Both the EPS samples produced viscous solutions in distilled water, that of NIOS –1 being more so. Infra red spectra of both the EPS samples confirmed the presence of uronic acids and sulfates.

EPS of the two thraustochytrids appeared to be predominantly a galactan, with α-1,4- linkages. Gas chromatograph analyses of the EPS produced by NIOS-1 and NIOS-4 revealed that they were comprised of galactose, mannose, arabinose and fucose or rhamnose. Galactose was the predominant sugar, contributing 65 – 89 % to the EPS. Based on periodate consumption, formic acid liberation and optical rotation, the EPS appeared to possess α-1,4- linkages. This was further validated by the enzyme hydrolysis studies.

EPS may help thraustochytrids to adhere to substrata and form a biofilm. The ecological significance of the extracellular polymeric substances produced by thraustochytrids was studied for the first time. The EPS produced by various isolates helped cells to adhere on glass slides. Therefore, they may help cells to adhere to various substrata in the ecosystem.

EPS may help thraustochytrids overcome desiccation. Cells from 7 day cultures with higher levels of EPS were more capable of surviving desiccation than those from younger cultures of 2 days. NIOS-1 and NIOS-4, which produced more EPS could withstand longer desiccation periods compared to NIOS –2 and NIOS-9, which produced less.

EPS in thraustochytrids contained various extracellular enzymes. The EPS produced by all the four isolates examined had the ability to bind extracellular enzymes in an active state. EPS showed proteolytic, lipolytic and glycosidase activities. Thus, they may prevent the loss of extracellular enzymes to the surrounding milieu by binding to them, retaining them close to cell and maintaining them in active form so that they are useful to the cell.

EPS may serve as a nutrient reserve to cells that produce it. Four isolates - NIOS-1, NIOS-2, NIOS-4 and NIOS-10 were grown using their own EPS as sole nutrient source. Only the isolate NIOS-1 and to some extent NIOS-

10 grew using their own EPS as nutrient source. The isolates, NIOS-2 and NIOS-9 did not. NIOS-1 utilized its EPS better, probably because it had a larger arsenal of enzymes tested.

The 18S SSU rRNA gene sequenc analyses and preliminary morphological studies suggest that all 3 isolates of thraustochytrids studied, NIOS-1, NIOS-4 and NIOS-6 were new to science. The three isolates were microscopically studied for their morphology and development. Their 18S SSU rRNA gene was sequenced and phylogenetic trees constructed, using the maximum likelihood and neighbor joining methods. Comparisons of their sequences were made with 34 other members of Labyrinthulomycetes and two outgroups. NIOS-1 and NIOS-4 corresponded to the genus *Schizochytrium*, while NIOS-6 corresponded to the genus *Thraustochytrium*.

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Appendix I

MV agar

Dextrose	4.0 g
Peptone	1.0 g
Gelatine hydrolysate	0.5 g
Yeast Extract	0.1 g
Liver extract	0.01 g
Agar – agar	8.0 g
Sea water	1000 ml

M3 Medium

Glucose	20 g
Peptone	15 g
Yeast extract	10 g
KH2PO4	0.25 g
Seawater	1000 ml
Vitamin solution	1 ml
Trace metal solution	1 ml

pH: 7.0 adjusted using 0.2 N NaOH.

M4 Medium

Glucose	20 g
Peptone	15 g
Yeast extract	10 g
KH2PO4	0.25 g
Seawater	1000 ml

pH: 7.0 adjusted using 0.2 N NaOH.

Vitamin Solution

Thiamine	0.2 g
Calcium Pantothenate	0.1 g
Pyridoxamine hydrochloride	0.02 g
Biotin	0.0005 g
Nicotinic acid	0.1 g
Pyridoxin Hydrochloride	0.04 g
p-Aminobenzoic acid	0.01 g
Cobalamin	0.00005 g
Distilled Water	100 ml

Trace Metal Solution

Na_2 EDTA	0.6 g
$FeCl_3.6H_2O$	0.029 g
H_3PO_4	0.684 g
$MnCl_2.4H_2O$	0.086 g
$ZnCl_2$	0.006 g
$CoCl_2.6H_2O$	0.0026 g
$NiSO_4.\ H_2O$	0.0052 g
$CuSO_4.5\ H_2O$	0.0002 g
$Na_2MoO_4.\ H_2O$	0.0005 g
Distilled Water	100 ml

Appendix II

Reagents for protein estimation by Petersons method (Peterson, 1977)

1. **Copper tartarate-carbonate (CTC)**
20 % Sodium carbonate in 50 ml distilled water
0.1 % Copper sulfate in 50 ml distilled water
0.2 % Sodium potassium tartarate

Reagent A: Mix equal parts of CTC solution, 0.8 N NaOH, 10 % SDS and distilled water.

Reagent B: One volume of Folin Ciocalteu solution + five volumes of distilled water.

Tris EDTA (TE) buffer (pH 7.4)
10 mM Tris chloride (pH 7.4)
1mM EDTA (pH 8.0)

0.1 TE (pH 8.0)
1 mM Tris chloride (pH 8.0)
1mM EDTA (pH 8.0)

50X Tris acetate EDTA (TAE) buffer (pH 8.0)
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Solutions for plasmid extraction by Alkali Lysis Method.

Solution I
50 mM glucose
25 mM Tris . Cl (pH 8.0)
10 mM EDTA (Ph 8.0)
This solution is autoclaved at 10 lb sq^{-1}. in for 15 min and stored at 4 °C

Solution II
0.2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

Solution III

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml