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INVITED REVIEW

Thraustochytrid Marine Protists: Production of PUFAs and Other Emerging Technologies

Seshagiri Raghukumar

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Abstract Thraustochytrids, the heterotrophic, marine, straminipilan protists, are now established candidates for commercial production of the omega-3 polyunsaturated fatty acid (ω-3 PUFA), docosahexaenoic acid (DHA), that is important in human health and aquaculture. Extensive screening of cultures from a variety of habitats has yielded strains that produce at least 50% of their biomass as lipids, and DHA comprising at least 25% of the total fatty acids, with a yield of at least 5 g L^{-1}. Most of the lipids occur as triacylglycerols and a lesser amount as phospholipids. Numerous studies have been carried out on salinity, pH, temperature, and media optimization for DHA production. Commercial production is based on a fed batch method, using high C/N ratio that favors lipid accumulation. *Schizochytrium* DHA is now commercially available as nutritional supplements for adults and as feeds to enhance DHA levels in larvae of aquaculture animals. Thraustochytrids are emerging as a potential source of other PUFAs such as arachidonic acid and oils with a suite of PUFA profiles that can have specific uses. They are potential sources of asataxanthin and carotenoid pigments, as well as other lipids. Genes of the conventional fatty acid synthesis and the polyketide-like PUFA synthesis pathways of thraustochytrids are attracting attention for production of recombinant PUFA-containing plant oils. Future studies on the basic biology of these organisms, including biodiversity, environmental adaptations, and genome research are likely to point out directions for biotechnology explorations. Potential areas include enzymes, polysaccharides, and secondary metabolites.

S. Raghukumar (✉)
Myko Tech Pvt. Ltd., 313 Vainguinnim Valley,
Dona Paula, Goa 403 004, India
e-mail: s_raghukumar@mykotech.com

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Introduction

Thraustochytrids, once an obscure group of marine protists, have now come into the limelight by virtue of their biotechnological importance in the production of docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (ω-3 PUFA) that is important for human health and in aquaculture (Lewis et al. 1999; Ward and Singh 2005; Barclay et al. 2005). They are also potential candidates for many other applications (Fan and Chen 2007a). This review briefly summarizes present applications and emerging areas in biotechnology of thraustochytrids, besides suggesting future directions for research.

Thraustochytrids, aplanochytrids, and labyrinthulids are related groups belonging to the Labyrinthulomycetes of the kingdom Straminipila (Leander and Porter 2001; Raghukumar 2002). Labyrinthulomycetes, also called the straminipilan fungi, are osmoheterotrophs, producing a network of plasma membrane extensions, the ectoplasmic net elements (EN; see Porter 1990). Thraustochytrids and aplanochyrids are unicellular. Labyrinthulids are colonial, the cells being enrobed by the EN. Reproduction is mostly through heterokont, biflagellate, straminipilan zoospores that possess a long, anterior, tinsel and a shorter posterior whiplash flagellum (see Porter 1990).

Several interesting physiological and biochemical properties of thraustochytrids were known even by the early 1970s (Goldstein 1973). The obligate requirement of one or more of the B vitamins by several thraustochytrids was demonstrated even in the 1950s and 1960s (see Goldstein 1973), a fact confirmed later by Bahnweg (1979a). Helen

Vishniac even suggested using a thraustochytrid as an assay organism for thiamine (Goldstein 1973). Thraustochytrids have an obligate requirement for Na^+ ions that cannot be replaced by K^+. They are ubiquitous in both coastal and oceanic habitats where they often attain substantial biomass and possibly play an important role in the marine ecosystem (Raghukumar 2002). Ellenbogen et al. in 1969 discovered DHA to be a signature fatty acid of thraustochytrids (see Goldstein 1973). Subsequently, Findlay et al. in 1986 used the presence of DHA and docosapentaenoic acid (DPA) to assess the biomass of these organisms in decomposing mangrove leaf litter (see Raghukumar 2002). These formed the basis of the technology for DHA production from thraustochytrids by Omega Tech, now part of Martek Biosciences Corporation, USA (Barclay 1994a, b).

A study in the early 1970s revealed that despite heavy fat ingestion, the Eskimo populations of Greenland had a low incidence of coronary diseases. This was attributed to their predominantly fish diet rich in the ω-3 PUFAs DHA and eicosapentaenoic acid (EPA; see Connor 2000). DHA is an important constituent of the phospholipids of cell membranes (Stillwell and Wassall 2003) and is crucial to the development of the brain and retinal vision in infants. They also prevent several diseases in adults, particularly coronary ailments. Phospholipids containing the ω-3 PUFAs DHA and EPA and the ω-6 PUFA, arachidonic acid (ARA), are precursors to eicosanoids which regulate inflammatory responses, blood clotting, and blood pressure. Fish oil was almost the only source of DHA in the 1980s. The odor, the possibility of heavy metal contamination, and the presence of unfavorable PUFAs encouraged DHA production from alternative sources. DHA is now commercially produced from the marine dinoflagellate *Crypthecodinium coehnii* and the thraustochyrid, *Schizochytrium* sp. (Sijtsma and de Swaaf 2004). A major advantage with both *Crypthecodinium* and thraustochytrids is that these organisms are heterotrophic and can be grown to produce high biomass in fermentors. Cohen and Ratledge (2005) cover the latest aspects in microbial production of single cell oil. Owing to the increasing demand of this fatty acid, numerous researchers are actively engaged in developing their own technology or in improving the present technology on DHA production by thraustochytrids.

Screening, media and culture optimization, and development of large-scale fermentation protocols are the three crucial steps in the development of microbial technologies in general. Extensive screening of cultures from a wide variety of habitats and environmental conditions has yielded useful thraustochytrid strains and important ecological information on DHA production by thraustochytrids (Table 1). Strains with a low salinity and mesophilic temperature requirements are particularly advantageous.

Culture Parameters and DHA Production

Thraustochytrids vary largely in biomass production, total lipids, and DHA content (Ward and Singh 2005 and Fan and Chen 2007a; see Table 2). The DHA content of total lipids was more or less constant in two *Thraustochytrium* strains, although the total lipid content varied depending on culture conditions (Singh and Ward 1997a). However, this may not always be the case (Bajpai et al. 1991). Maximum lipids are produced at the end of the exponential or the early stationary phase. Obtaining the maximum possible biomass with a high amount of total lipids in the shortest possible time is the strategy in media development for DHA production. The best time to harvest for DHA varies generally from 4 to 7 days (Bajpai et al. 1991; Singh et al. 1996; Yaguchi et al. 1997).

Table 1 Conclusions based on large-scale screening of thraustochytrids for DHA production

No. of isolates	Habitats	Conclusions	Reference
151	Saline warm springs, playas, tidal pools, and estuaries using a rapid sandwich filtration and plating technique, USA	Isolation of strains with enhanced fatty acid and DHA yields and wider salinity tolerance	Barclay (1994a)
–	Australian waters	Strains with different PUFA profiles; one containing only arachidonic acid	Lewis (1998)
57	Cold temperate littoral, cool temperate littoral, and subtropical mangroves	Thraustochytrids from cold temperate environments were generally richer in DHA	Bowles et al. (1999)
68	Seagrass, salt marsh grass, and coastal sediments at 19 different Atlantic Canadian locations	than those from tropical environments. The growth rate of tropical isolates, however, was much better	Burja et al. (2006)
6	Subtropical mangroves	*Labyrinthula* species (labyrinthulids) are potential sources of PUFAs	Sakata et al. (2000)
>300	Coastal seawater, Japan	Thraustochytrids have distinct PUFA profiles	Huang et al. (2003)
6	Subtropical mangroves	Selection of a strain belonging to *Schizochytrium mangrovei*	Fan et al. (2002)

Table 2 Representative examples of biomass, lipid, and DHA yields in different thraustochytrids and labyrinthulids

Organism	Biomass (g L^{-1})	Percent lipids in biomass	Percent DHA in lipids	g DHA L^{-1}	Reference
Thraustochytrium aureum ATCC 34304 (4 days growth)	4.9	20.3	51.0	0.5	Bajpai et al. (1991)
T. aureum ATCC 34304; 69 h growth	5.7	8.0	40.0	–	Iida et al. (1996)
T. roseum ATCC 28210 (5 days) fed batch culture	17.1	25	50.0	2.1	Singh and Ward (1997a)
Thraustochytrium sp. 20892 (4 days)	6.1	15.2	53.1	0.7 (growth at 25°C for 3 days, followed by 15°C for 1 day	Singh et al. (1996)
Thraustochytrium sp. (*striatum*?) ONC-T18 (4 days)	28.0	81.7	31.5	4.6	Burja et al. (2006)
Thraustochytrid strain 12B (3 days)	31.0	57.8	43.1	6.8	Perveen et al. (2006)
Labyrinthula sp. S3-2 (14 days)	4.9	–	–	–	Kumon et al. (2002)
Labyrinthula L25; 21 days	–	–	–	2.1	Kumon et al. (2005b)
Labyrinthula L72; 14 days	–	27.4	45.9	0.7	Kumon et al. (2005a)
Labyrinthula L95-2; 7 days (30°C or 3 days, then 16°C for 1 day)	–	–	80.0	–	Sakata et al. (2000)
Thraustochytrium sp. KK17-3 (3 days)	7.1	19.9	52.1	0.3	Huang et al. (2001)
Schizochytrium KH105; 4 days in a fermentor	30.0	43.0	25.8	3.4	Yamasaki et al. (2006)
Schizochytrium limacinum SR21; 5 days	38.0	50.0	43.1	4.2	Yokochi et al. (1998)
Schizochytrium limacinum SR21; 4 days in a fermentor	48.0	77.5	35.6	13.3	Yaguchi et al. (1997)
Schizochytrium limacinum SR21; 7 days	22.1	–	–	4.9	Chi et al. (2007)

Schizochytrium limacinum, a species originally described from the mangroves of Yap Island, Japan (Honda et al. 1998), is a model species for commercial production of DHA. The original strain, SR21, has been studied by Nakahara et al. (1996), Yaguchi et al. (1997), Yokochi et al. (1998), and Chi et al (2007). Detailed studies by Yokochi et al. (1998) on this strain yielded maximal biomass values of 48.1 g biomass L^{-1} medium in 4 days, up to 77% of lipids in biomass and 43.1% of DHA in lipids, amounting to 13.3 g L^{-1} medium. It showed a salinity tolerance of 0–200% that of seawater, the optimum being 50–100%. Glucose, fructose, and glycerol were the best C sources, di- and polysaccharides being poorly utilized. Surprisingly, oils did not serve as precursors for lipid and DHA, although oleic acid and linseed oil supported good biomass. Cornsteep liquor and ammonium were most suitable for growth and DHA production. Application of a Plackett–Burman experimental design by Chi et al. (2007) showed that trace metals, ammonium chloride, ammonium acetate, and temperature significantly affected growth and DHA concentration.

Thraustochytrids vary widely in their ability to use carbon and nitrogen sources (Bahnweg 1979a, b). Glucose is generally the preferred carbon source (Singh et al. 1996; Burja et al. 2006), although several strains utilize maltose and starch well (Bajpai et al. 1991; Li and Ward 1994). Sijtsma and de Swaaf (2004) have suggested that it would be interesting to examine if C2 compounds such as acetic acid and ethanol could be utilized as carbon sources by these organisms, as is the case with the marine dinoflagellate, *Crypthecodinium cohnii.*

Various peptones are good nitrogen sources for biomass production. While a few thraustochytrids utilize inorganic nitrogen like ammonium and nitrate efficiently (Barclay 1994a; Huang et al. 2003), most appear to be inefficient in this (Bahnweg 1979b; Iida et al. 1996; Singh and Ward 1997b). Inorganic nitrogen sources are much cheaper than organic ones and the capability of a strain to grow on them is very useful in large-scale cultivation. Sodium glutamate is an excellent nitrogen source for many thraustochytrids (Iida et al. 1996; Singh et al. 1996; Singh and Ward 1997a) and can also be utilized as a carbon source.

Several, but not all thraustochytrids, have an obligate requirement for one or more B vitamins, but this may be taken care of by growing them in complex organic media. Phosphate, in the form of KH_2PO_4, and vitamins enhance fatty acid and DHA yields in several strains. Temperatures of 25–30°C generally favor optimal growth in thraustochytrids and labyrinthulids (Perveen et al. 2006; Yokochi et al. 1998; Burja et al. 2006; Kumon et al. 2002). Low temperatures stimulate DHA production, but negatively affect growth, thus resulting in low overall DHA yields. Thraustochytrids may initially be grown at a higher temperature (e.g., 25°C) to stimulate growth and later under cold conditions (15°C) to enhance DHA yield (Singh et al. 1996; Sakata et al. 2000). Jain et al. (2004) stored harvested biomass at 10°C for 24–48h to increase absolute levels of DHA, thus circumventing the expense involved in large-scale growth at below-ambient temperatures. They also enhanced DHA yields by slightly increasing the viscosity of the medium using polyvinyl pyrrolidone. DHA yields in these cases were either the result of higher amounts of lipids with constant DHA percentage, or an increase in both lipids and percentage DHA, or only the increase in DHA with lipid levels remaining constant. Higher PUFA contents under low temperatures may be an adaptation to favorably alter the fluidity of the membrane that is negatively affected at low temperatures.

Thraustochytrids require seawater for growth. Salinity optima and tolerance levels for growth vary among strains, some growing even at salinities as low as 2ppt (Burja et al. 2006). Strains from mangrove environments, where salinity fluctuates highly, may show lower salinity optima or wider salinity tolerance. Optimal salinity in thraustochytrids, as well as *Labyrinthula* sp., a labyrinthulid common in mangroves, generally corresponds to 50–100% seawater (Iida et al. 1996; Kumon et al. 2002; Perveen et al. 2006). Thraustochytrids have a broad pH tolerance of 5 to 8 for growth and DHA production (Iida et al. 1996; Singh and Ward 1997b; Kumon et al. 2002; Perveen et al. 2006). They do not require light for growth and DHA production, although a strain of *Thraustochytrium aureum* produced higher levels of DHA when grown in light (see Singh and Ward 1997a).

An appropriate C/N ratio is necessary for optimal biomass formation, low nitrogen levels leading to decrease in biomass (Yokochi et al. 1998). Thraustochytrids appear to have a C/N ratio of about 10.4 (Kimura et al. 1999). On the contrary, oil accumulation in oleaginous organisms requires a high C/N ratio. Under nitrogen depletion, the microbes cannot multiply but continue to assimilate C as oil, thus becoming 'obese' (Ratledge 2004) (Figs. 1 and 2). Carbon increase up to 100 g L^{-1} increased lipid and DHA yield in *Thraustochytrium* sp. ONC-T18 (Burja et al. 2006). However, some strains may be inhibited by such high glucose levels (Bowles et al. 1999; Iida et al. 1996).

A summary of the essential culture medium criteria for DHA production by thraustochytrids is presented in Table 3.

Large-scale Production of DHA

Medium composition, incubation temperature, pH, culture age, seawater concentration, and impeller speed and shape in fermentors affect DHA production (Yaguchi et al. 1997;

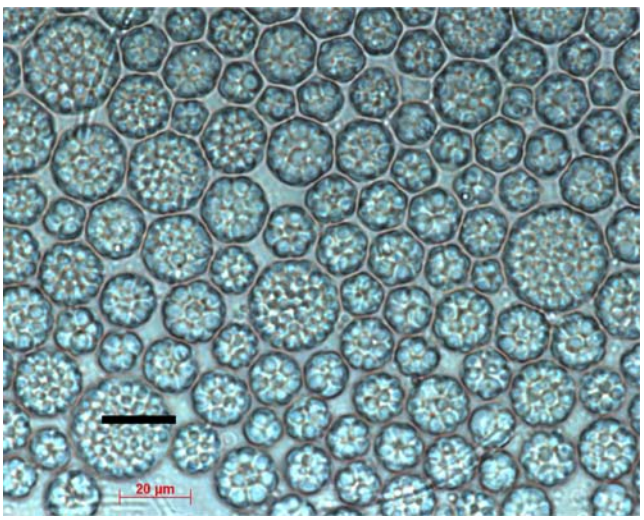

Fig. 1 Cells of a *Schizochytrium* isolate showing numerous lipid bodies. *Bar* represents 20 µm

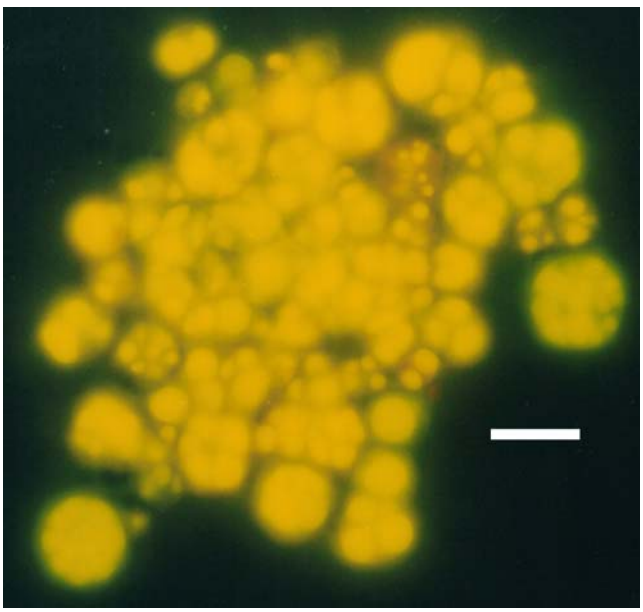

Fig. 2 Epifluorescence microscopy of cells of *Schizochytrium* sp. stained with Nile blue and showing fluorescent lipids. *Bar* represents 20 µm

Table 3 Some essential growth criteria for production of DHA by thraustochytrids

Parameter	Requirement
Carbon source	Glucose, fructose, or glycerol
Nitrogen source	Ammonium salt (for *Schizochytrium*) and organic nitrogen (peptone or glutamate) for others
Macroelements	Seawater or a Na^+ salt and other major cations and anions in seawater; KH_2PO_4
Microelements	Various trace elements of seawater
Vitamins	Thiamine, biotin, riboflavin, and cobalamin depending on the organism
C/N ratio	Based on cell ratio for biomass production; excess C/N for lipid accumulation
Temperature	25–30°C for biomass production; ~15°C for lipid accumulation
Salinity	50–100% of that of seawater
pH	5–8
Oxygen levels	>4% for growth and <3% for DHA production (*Schizochytrium*)
Preferred culture mode	Fed batch
Age of culture	Stationary phase

Lewis et al. 1999). A typical commercial production of DHA has been described by Bailey et al. (2003) and Barclay et al. (2005). Production comprises a biomass producing and a lipid enhancing stages. During the first, the biomass is increased by supplying carbon and nitrogen in a fed batch mode (Bailey et al. 2003). The glucose level is kept constant at 7 g/L, using corn syrup. Nitrogen is supplied in the form of ammonium sulfate and ammonium hydroxide, the latter also functioning to buffer the medium in such a way that the pH does not become acidic in the presence of ammonium sulfate. In the second phase, carbon alone is supplied to enhance lipid levels. Contrary to earlier belief that PUFA synthesis requires oxygen, Bailey et al. (2003) found that low oxygen levels were actually conducive to DHA production in *Schizochytrium*. Thus, oxygen levels are maintained at 4% to 8% saturation levels during the biomass production stage and 1% or less during the DHA production stage. This brings down numerous fermentation problems associated with high aeration. Using this protocol, Martek has been able to produce 170–210 g dry weight biomass per liter, of which about 50% (80 g L^{-1}) is lipid, containing at least 50% of this as DHA. Thus, the final yield of DHA was up to 40 g L^{-1} medium. A remarkable invention has been the use of non-chloride sodium salts, preferably sodium sulfate, for growing thraustochytrids, thus avoiding corrosion problems caused by chlorides (Barclay 1994b). Sodium sulfate also reduced clumping of cells.

DHA Products

Martek Biosciences now dominate the market for production of DHA from *Schizochytrium*, as well as *C. cohnii* (Ratledge 2004; Sijtsma and de Swaaf 2004; Barclay et al. 2005). Several international bodies and government agencies have recommended the use of DHA in infant formulae, such that they mimic human breast milk (Ward and Singh 2005). It has also been recommended for pregnant and nursing women. Infant formulae constitute the major market for DHA, the fatty acid coming from *C. cohnii*, rather than *Schizochytrium*. On the other hand, *Schizochytrium*-derived DHA is finding use in adult food supplements, including various additives such as yoghurt, cheese, spreads, dressings, breakfast cereals, and softdrinks (Lewis et al. 1999; Ward and Singh 2005). Dried *Schizochytrium* has GRAS status for use as feed to broiler chickens and laying hen feed in order to enhance DHA in the meat and eggs (Barclay 1994a; Ward and Singh 2005). *Schizochytrium* oil, known as DHA Gold, is marketed by Omega Tech Inc to supplement animal feeds (Ratledge 2004).

Marine finfish and crustacean larvae require DHA and other PUFAs (Lewis et al. 2000). *Schizochytrium* has been used in aquaculture for more than a decade to significantly enrich DHA levels of rotifers and *Artemia* larvae before they are fed to larval fish and shrimp (Barclay and Zeller 1996). Examples are 'Algamac' products of Aquafauna Bio-Marine Inc., USA and 'Docosa Gold' of Sanders Brine Shrimp Company, USA. Such enriched feeds increased survival rate and reduced pseudoalbinism in juvenile turbot fish (Song et al. 2007). Thraustochytrids enriched in B_{12} vitamins prior to feeding rotifers significantly improved their growth rate (Hayashi et al. 2007). Different aquaculture species may have specific PUFA requirements, and thraustochytrids with different PUFA profiles could be used to meet specific demands in aquaculture (Lewis et al. 2000). Further research in this area is in progress (Song et al. 2007).

Thraustochytrid Genes for Recombinant Technology

The DHA biosynthetic pathway in thraustochytrids has presently attracted attention because of efforts by many researchers to produce recombinant plants or yeasts capable

of producing PUFAs (see Wallis et al. 2002; Damude and Kinney 2007). There are two distinct PUFA synthesis pathways in thraustochytrids. The one in *Thraustochytrium* sp. ATCC 26185 follows the typical fatty acid synthesis (FAS) pathway found in many organisms, comprising a series of alternating desaturation and elongation steps of short-chain saturated fatty acids. DPA (22:5ω3) produced from EPA (20:5ω3) is further converted to DHA (22:6ω3) by Δ4 desaturase enzyme. This pathway requires molecular oxygen for the desaturation steps. The Δ4 desaturase gene from *Thraustochytrium* has been cloned in the yeast *Saccharomyces cerevisiae* and the mustard plant, *Brassica juncea* (Qiu et al. 2001). In *Schizochytrium* ATCC 20888, the short-chain fatty acids (14:00 and 16:00) appear to be synthesized by the FAS pathway, while the PUFAs appear to be synthesized by a distinct PUFA synthesis pathway. Three open reading frames with 11 domains were identified as genes of PUFA synthase in *Schizochytrium*, eight of which were highly homologous to those in the marine bacterium *Shewanella* (Hauvermale et al. 2006; Fan and Chen 2007a). The bacterial PUFA synthase, in turn, had been found to be homologous to the polyketide synthase system (PKS), responsible for producing a broad group of secondary metabolites that are catalyzed by reactions analogous to the fatty acid synthesis. PUFA synthesis by this pathway is much shorter compared to the FAS pathway, being produced by dehydration and isomerization of keto groups in cycles of polyketide-forming chain elongation reaction. This pathway does not require aerobic conditions. Thraustochytrids with this pathway, therefore, may not require oxygen for the buildup of high levels of PUFAs. It is unwise to call these pathways the aerobic *Thraustochytrium* and the anaerobic *Schizochytrium* pathways, since these genera do not seem to have a phylogenetic basis (Yokoyama and Honda 2007). Besides, if the PKS-like PUFA synthesis pathway is indeed the result of lateral gene transfer from bacteria as suggested by others, it is likely that it occurred across genera in thraustochytrids. The distribution of the two pathways in other thraustochytrids warrants further studies.

Other PUFAs and Lipids

Kumon et al. (2005a) reported the presence of DHA as the sole PUFA in a labyrinthulid, a rarity that would be useful in downstream processing for DHA extraction. Most thraustochytrids produce one or more of other PUFAs, namely arachidonic acid (ARA, 20:4 ω-6), docosapentaenoic acid (DPA, 22:5 ω-6), eicosapentaenoic acid (EPA, 20:5 ω-3), and docosatetraenoic acid (DTA, 22:4, ω-6) (Barclay 1994a). *S. limacinum* SR21 contained up to 7.2% of ω-6 DPA in total lipids (Morita et al. 2006). This PUFA is universally rare, except in labyrinthulids and thraustochytrids and has also been found in the brain and retina under certain conditions. *Labyrinthula* isolate L59 contained only ω-6 DPA as the PUFA (Kumon et al. 2003). Six different PUFA profiles have been reported in thraustochytrids (Huang et al. 2003; Burja et al. 2006). These are: (1) DHA and DPA; (2) DHA, DPA, and EPA; (3) DHA and EPA; (4) DHA, DPA, EPA, and ARA; (5) DHA, DPA, EPA, ARA, and DTA; and (6) DHA/EPA/ARA. These profiles do not seem to change with different culture media, although Burja et al. (2006) found that PUFA profiles may be more diverse on agar plates than in submerged cultures. This suggests an adaptive mechanism for the different PUFA. These findings have three implications: (1) Thraustochytrids may be considered potential candidates for other PUFAs useful in human health. (2) It may be possible to produce designer oils with different combinations of fatty acids for different applications. (3) The different profiles may provide us an insight into the various biosynthetic pathways that will be further useful in recombinant technologies (Burja et al. 2006). Metabolic engineering may lead us to alter the fatty acid profiles of thraustochytrids. For example, cultivation of thraustochytrids in media enriched with the B_{12} vitamin resulted in reduction of odd-numbered saturated fatty acids, a biochemical explanation for which has been offered (see Hayashi et al. 2007).

DHA is found in neutral or storage lipids, as well as the cell membrane phospholipids (Morita et al. 2006). DHA-containing thraustochytrid oil is derived from neutral lipids (predominantly triacylglycerols), since these contribute more than 90% of the total lipids in mature cells of thraustochytrids (Iida et al. 1996; Yaguchi et al. 1997). Phospholipids in mature cells of *S. limacinum* SR21 amounted to only 10% of the total lipids, although young cells contained nearly 50% of their lipids in phospholipids (Morita et al. 2006). Percentage DHA in total fatty acids was also only 8.7% in phospholipids in mature cells of this organism compared to 62% in young ones, suggesting the transfer of the fatty acids of polar lipids to neutral lipids. DHA contributed 39.1% of the polar lipids compared to only 28.2% of the neutral lipids in a *Thraustochytrium* sp. (Huang et al. 2003). The dynamics between the various lipid components in thraustochytrids might shed interesting light. Phospholipid DHA has its own biotechnological value (see Okuyama et al. 2007), if their levels could be enhanced. This was achieved in a thraustochytrid by transferring cells rich in triacylglycerols to a glucose-deficient medium, thus stimulating an in vivo conversion of triacylglycerols to phospholipids (Okuyama et al. 2007). Phosphatidylcholine is the major component of the phospholipids, the others being phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol, lysophosphatidylcholine, diphosphatidylacylglycerols, phosphatidylglycerols, phos-

phatidylserine, and various unidentified phospholipids (Morita et al. 2006; Fan et al. 2007b). Abe et al. (2006) have recently reported a novel phosphatidylcholine in *Schizochytrium* sp. F26-b.

Squalene, a precursor of phytosterol, has recently been studied in thraustochytrids (Jiang et al. 2004; see Fan and Chen 2007a). This compound is presently obtained from livers of deep-sea sharks and has several bioactive properties, including antioxidant and anticancer ones. Thraustochytrids are also a rich source of sterols that have the potentials for production of various steroids and surfactants (Lewis et al. 2001; Fen and Chen 2007a).

Carotenoids from Thraustochytrids

Thraustochytrids are a potential source of carotenoids such as β-carotene and oxygenated carotenoids such as the xanthophylls astaxanthin and canthaxanthin (Aki et al. 2003). These important antioxidants play a crucial role in human health by scavenging harmful free oxygen radicals (Fan and Chen 2007a). The green alga *Dunaliella* and the fungus *Blakeslea trispora* are present commercial sources of β-carotene, while astaxanthin is produced from the single-celled alga *Haematococcus* (Dufosse et al. 2005). Astaxanthin is used in aquaculture to enhance flesh coloration of salmonids and other animals. Thraustochytrids probably produce carotenoids to prevent storage fats from oxidation (Burja et al. 2006). Astaxanthin, zeaxanthin, canthaxanthin, beta-carotene, echinenone, and phoenicoxanthin have been found in thraustochytrids (Armenta et al. 2006; Burja et al. 2006). A strain of *Schizochytrium* accumulated 6.1 mg/L of astaxanthin in a 4-day cultivation in a medium with 10% glucose and less than 0.3% nitrogen sources in 50% strength seawater, while 10 mg L^{-1} canthaxanthin was produced with 6% nitrogen sources (Aki et al. 2003). Up to 8 mg of β-carotene L^{-1} was produced when grown in a glucose concentration of only 2%. Light was not necessary for the production of the carotenoids. The same strain produced up to 7.7 mg L^{-1} astaxanthin when grown in shochu alcoholic distillery waste (Yamasaki et al. 2006). Thraustochytrids could thus be a simultaneous source of both PUFAs, as well as xanthophylls. Improved extraction protocols are likely to provide higher yields of these pigments (Armenta et al. 2006). Yet another antioxidant of considerable interest in age-related problems is coenzyme Q, a carrier in the mitochondrial electron transport system. Armenta et al. (2006) have attempted to optimize extraction protocols of this compound from thraustochytrids. However, their results suggest that instead of 10 isoprene units that characterize coenzyme Q10 which is of biotechnological interest, thraustochytrids have a lesser number.

PUFA and carotenoid profiles may be characteristic of individual thraustochytrids and might be of phylogenetic and taxonomic relevance (Yokoyama and Honda 2007).

Future Directions and Other Potential Applications

Biotechnological applications are based on the unique properties of organisms which provide them the competence to survive competition and define the ecological niches that they occupy. Such adaptive characters of Labyrinthulomycetes are the result of their evolutionary history, as they are with all organisms. Labyrinthulomycetes, to which thraustochytrids belong, have had an evolutionary history of perhaps more than a billion years (Leipe et al. 1996) and are bound to be highly diverse in terms of taxonomy and ecological adaptations. Understanding these could foster their applications in biotechnology.

Unravelling the diversity of thraustochytrids from a wide variety of marine habitats should form the basis of ‘intelligent screening’ for various properties as outlined above. Such attempts should include coastal and oceanic waters, surface and deep-sea waters, sediments of various types including mangrove and oceanic ones, aerobic and anaerobic habitats, and tropical and temperate regions. Seawater appears to harbor an abundance of uncultured thraustochytrids and also a wealth of picoplanktonic ones, which need to be brought into culture by novel methods (Raghukumar 2006). Screening should also consider other members of the aplanochytrids and labyrinthulids. Aplanochytrids have rarely been examined for DHA production. Species of *Aplanochytrium* are common inhabitants of seagrasses and marine algae (Raghukumar 2002), and Labyrinthulids, comprising the single genus *Labyrinthula*, has attracted attention only recently (Kumon et al. 2002).

The Labyrinthulomycetes are probably the predominant group of obligate osmoheterotrophs among marine eukaryotes. They are abundant in oceanic waters and on a wide variety of decaying matter in coastal ecosystems, such as algal and seagrass beds, mangrove environments, and coastal sediments. They probably overcome competition with bacteria by producing unique degradative enzymes that could have industrial applications. Production of abundant proteases seems to be the common characteristic of the Labyrinthulomycetes (Bahnweg 1979a) and is worth detailed studies. Among proteases, leucine and valine arylamidases were noticed to be common among several thraustochytrids (Bongiorni et al. 2005). In addition, these authors also found esterase, esterase lipase, lipase, and acid and alkaline phosphatases to be prevalent in these organisms. Mangrove thraustochytrids are also capable of producing cellulases and xylanases (Bremer and Talbot 1995; Raghukumar et al. 1994). Even if thraustochytrids are

not directly put to industrial use for such enzymes, understanding the biochemistry and molecular biology of this aspect could be fruitful. Raikar et al. (2001) demonstrated hydrocarbon degradation by thraustochytrids. An isolate degraded up to 71% of tarballs added to sediments in a month and 30% added to a nutrient medium in a week. This shows their potential in bioremediation.

The apparent lack of resting cells in Labyrinthulomycetes suggests alternate modes for their long-term survival, such as the production of extracellular polysaccharides (EPS). Jain et al. (2005) reported sulfated extracellular polysaccharides from four strains of thraustochytrids. Up to 1.1 g L^{-1} of EPS was produced in the stationary growth phase under unoptimized culture conditions. The matrix-like EPS surrounding the cells could be visualized using scanning electron and phase contrast microscopy or by staining with alcian blue. Two strains produced EPS of 94 and 320 kDa molecular weights, respectively, with glucose being the major sugar, besides galactose, mannose, and arabinose. Extracellular polysaccharides have a wide variety of properties such as antitumor, antiviral, and anticoagulant ones and several applications in cosmetics and food industries (Sutherland 1998).

Thraustochytrids could serve as model organisms for a variety of molecular and biochemical studies. The remarkably rapid generation of membranes in the form of the ectoplasmic net elements (Fig. 3) in these organisms might help in understanding plasma membrane biosynthesis. If the inherent property of DHA production in Labyrinthulomycetes is related to their membrane structure under low temperatures and high pressure conditions, these properties are worth investigating in detail. There are clear indications that several thraustochytrids may be psychrophiles, with growth optima between 4 and 10°C. *Thraustochytrium antarcticum* described by Bahnweg and Sparrow and a strain of *Schizochytrium aggregatum* reported by Ulken (see Raghukumar 2002) are examples. Riemann and Schaumann (1993) have reported dense growth of thraustochytrids on the under surface of fast ice in the Weddell Sea of the Antarctic. A thraustochytrid isolated from salp fecal pellets in the Arabian Sea actively produced proteases under high hydrostatic conditions (see Raghukumar 2002).

The presence of hitherto unknown viruses in thraustochytrids is one area of unknown potential in biotechnology. While Kazama and Schornstein in 1972 and 1973 reported a herpes-type virus-like particles in *Thraustochytrium* sp., Takao et al. have recently reported a novel single-stranded RNA virus and a squashed-ball like ds-DNA virus in an isolate of *Schizochytrium* sp. (one strain being classified as the newly erected genus *Sicyoidochytrium* by the authors; see Takao et al. 2007).

Thraustochytrids obligately require Na^+ to enable phosphate uptake (Goldstein 1973). The osmotic stress thus caused in the cells is a threat to the intracellular, metabolic enzymes, necessitating the production of compatible solutes that stabilize intracellular proteins. Jakobsen et al. (2007) reported that endogenously synthesized (−)-*proto*-quercitol and glycine betaine were the principal compatible solutes of a *Schizochytrium* strain and three new osmotolerant isolates of thraustochytrids, the former found only in eucalyptus so far. Glycine betaine protects enzymes against thermal and salt inactivation in bacteria against high osmolality stress. In this context, it would be of interest to examine the tolerance of thraustochytrids to other metals, including heavy metals. An isolate from the shallow-water hydrothermal vents in the Azores grew well in the presence of iron and manganese at 15 and 70 μM and lead at 40 nm concentrations (Colaco et al. 2006). Further, the protease activities of this organism were not affected at these levels of these metals. The mechanisms of tolerance to such metals in thraustochytrids are worth pursuing. Yet another example of tolerance to extreme conditions might be that of aplanochytrids, which appear to overcome defense mechanisms of living algae consisting of phenolics and live within their tissues (see Raghukumar 2002).

Thraustochytrids are now well known to be associated with marine invertebrates. They are also common as parasites of mollusks (Raghukumar 2002). Primary cell cultures of sponges, cnidarians, crustaceans, mollusks, echinoderms, and tunicates are regularly contaminated with thraustochytrids, suggesting an unknown biological association between these protists and the invertebrates (see

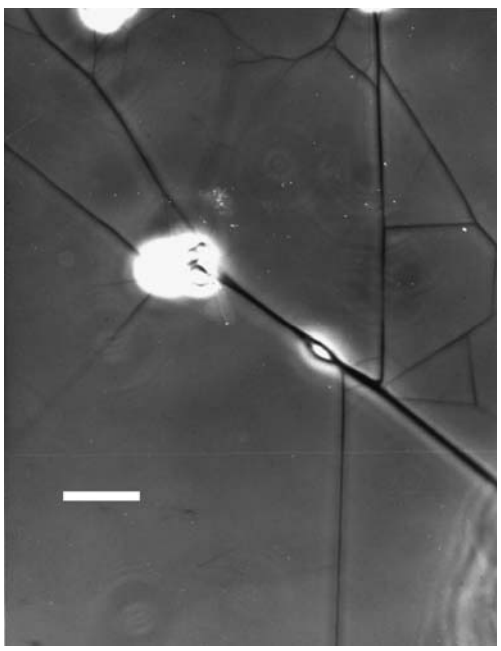

Fig. 3 Phase contrast microscopy of the dense network of the ectoplasmic net elements, which are plasma membrane extensions, in *Aplanochytrium minutum*. *Bar* represents 10 μm

Rabinowitz et al. 2006). Commensalistic and symbiotic microbes from marine invertebrates are an important target for novel secondary metabolites. Thraustochytrids associated with such animals are yet to form part of the campaign to discover novel drugs.

Given the increasing importance of thraustochytrids in biotechnology, it is surprising that no attempts have been made so far to sequence the complete genome of one or more thraustochytrids. As basic research on their biology progresses, the Labyrinthulomycetes may throw up a lot of surprises that are likely to have a significant impact on marine biotechnology.

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