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Bioprocessing for Value-Added Products from Renewable Resources
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Chapter 11. Production of High-Value Products by Marine Microalgae Thraustochytrids

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1. INTRODUCTION

Man has made use of natural products since ancient times. One example is the use of microalgae as food, dating back to 2000 years ago when *Nostoc flagelliforme*, a cyanobacterium, was utilized as a food source in China [1]. Indeed, microalgae constitute an extremely diverse group of organisms encompassing prokaryotic cyanobacteria and eukaryotic protists. By definition, microalgae are a group of microscopic organisms; the majority possess photosynthetic and accessory pigments for undergoing photosynthesis [2]. Modern applications of microalgal biotechnology have only become the subject of intensive studies in recent decades after the potential of certain microalgae to produce numerous highly useful or high-value products, such as biohydrogen, pigments (e.g., carotenoids, phycobiliproteins, etc.), polyunsaturated fatty acids (PUFAs), sterols, and vitamins was recognized [3–7].

Reviews concerning the benefits and uses of microalgae in biotechnology are numerous [e.g., 8–12]. Unanimously, these reviews concern the potential of using microalgae as producers of high-value products. Specifically, high-value products include those that are extracted for direct human consumption, mainly in the form of functional foods, which are dietary supplements for immune enhancement and disease prevention [13]. In addition, microalgal high-value products also include those that are used as feed additives in the aquaculture industry [14], which are mainly applied as supplements of rare but essential nutrients, such as PUFAs for conditioning zooplankton, which are subsequently employed as food for cultivated aquatic animals [15].

Thraustochytrids have been recently classified as microalgae, especially in a commercial sense [8, 16]. For decades, scientific investigations on thraustochytrids by taxonomists and ecologists mainly focused on their distribution and abundance in natural environments. The biotechnological potential of thraustochytrids was not recognized until the discovery that thraustochytrids contain substantial amounts of docosahexaenoic acid (22:6 DHA ω-3) [17]. Since then, numerous studies have been conducted to investigate the DHA production

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potential of certain strains of thraustochytrids for marketing as single cell oils [16, 18]. More recently, the potential for using thraustochytrids for the co-production of astaxanthin, squalene, and sterol has also been suggested [7, 19–20] and has led to the concept of using thraustochytrids as cell factories for the production of novel high-value natural products [21].

Although thraustochytrids have drawn more and more attention and have been suggested as cell factories for novel products, up to now, there have been no comprehensive reviews concerning the production of high-value products by thraustochytrids. Singh and Ward [22] provided the only relevant review about the effects of environmental and nutritional factors on DHA production in thraustochytrids. The present review, therefore, intends to focus on some recent advances in the biotechnological applications of thraustochytrids with an emphasis on the production of various high-value products. Moreover, the use of thraustochytrids as feed additives in aquaculture and the poultry industry as well as safety issues that concern their biotechnological applications are also addressed.

2. MODES OF MICROALGAL CULTIVATION

The development of efficient large-scale microalgal cultivation systems is essential for the production of commercially important algal products [8]. In general, microalgal cultivation systems have been broadly categorized into photoautotrophic and heterotrophic systems. The former is light-driven and is suitable for most microalgal species, while the latter requires organic substrates to be used as the sole carbon and energy source and is applicable only to a small number of microalgae [10–11]. Nevertheless, heterotrophic cultivation is considered superior to conventional photoautotrophic cultivation due to the fact that light, the growth-limiting factor, is eliminated [10]. Since there are numerous cultivation modes that can be operated under heterotrophic cultivation, for example, batch, fed-batch, and continuous cultures, the following sections focus on relevant cultivation examples associated with DHA production by thraustochytrids.

Commercially, there are two main types of photoautotrophic systems: open pond and closed photobioreactors; both use natural sunlight or artificial illumination for microalgal cultivation [8]. Open pond is considered as the oldest and simplest system employed for the mass cultivation of microalgae. This system offers culture conditions identical to the external environment and takes advantage of “free” sunlight as its energy source, but the high risk of microbial contamination has restricted the use of such systems to a limited number of microalgae, such as *Dunaliella*, which can tolerate high salinity [8], and *Spirulina*, which can grow well at high pHs [23]. To the best of our knowledge, there is no report on DHA production by microalgae using an open pond system.

Closed photobioreactors, such as tubular, fiber-optical, and helical reactors, use either natural sunlight or artificial illumination. This type of photoautotrophic system has been suggested as the alternative to the disadvantageous open-pond cultivation [24]. Common advantages of using closed photobioreactors include the large illumination surface to volume ratio, the minimization of contamination from the environment, and the efficient control of culture conditions [10]. Close photobioreactors have been employed for DHA production using *Isochrysis galbana* [25–26]. Poisson and Ergan [26] reported a DHA yield of 0.96 mg

L^{-1} by *I. galbana* grown in a conventional bioreactor with cool white fluorescent tubes for continuous illumination. By using optical fiber photobioreactors, the DHA yield in *I. galbana* was further enhanced to 5.4 mg L^{-1}. Vazhappilly and Chen [27] obtained a DHA yield of 1 mg L^{-1} for *Thraustochytrium aureum* ATCC 28211 grown under photoautotrophic conditions in flask cultures (0.1 g L^{-1} biomass after 23 days). The relatively low production of DHA by microalgae using photoautotrophic cultures, coupled with the costly construction and maintenance of closed photobioreactors, has diverted the focus to the heterotrophic mode of production [4].

Heterotrophic systems are a cost-effective alternative for culturing microalgae since light is eliminated for cultivation, and, at the same time, organic substrates are utilized as the sole source of carbon and energy, leading to a high biomass concentration and productivity [10]. Heterotrophic cultivation of microalgae, such as *Crypthecodinium cohnii* and thraustochytrids, for DHA production has been reported using conventional bioreactors or fermenters [28]. Vazhappilly and Chen [29] reported that when *T. aureum* ATCC 28211 was used as the producing organism on 5% glucose, the DHA yield was increased to 4.0 mg L^{-1}, compared to 1.0 mg L^{-1} under photoautotrophic conditions. This illustrates that the heterotrophic mode of growth is superior to the photoautotrophic mode in terms of biomass and DHA production. Bowles *et al.* [30] reported that the high DHA yield of 1.6 g L^{-1} and productivity of 0.49 g L^{-1} day^{-1} were achieved by *Thraustochytrium* strain G13 in bioreactors. More efficiently, a DHA yield of 3 g L^{-1} and productivity of 3.1 g L^{-1} day^{-1} were reported for *Schizochytrium* sp. SR21 when grown in conventional batch bioreactors with 12% glucose [31]. Modifying the batch culture system to become a fed-batch, chemostat or perfusion culture may further enhance the biomass yield and DHA productivity. Thus, heterotrophic systems may provide a cost-effective way for continuously supplying high quality microalgae-produced DHA on a large scale [28].

3. THRAUSTOCHYTRIDS

3.1. The taxonomy of thraustochytrids

Conventionally, thraustochytrids are taxonomically defined as a group of obligate, eukaryotic marine microorganisms characterized by monocentric thalli that can attach to their substrata by means of ectoplasmic net elements that arise from an organelle termed the sagenogenetosome [32]. Reproduction is usually by the asexual formation of biflagellate, heterokont, and zoospores from zoosporangia. Amoeboid stages are also frequently observed. The anteriorly directed flagellum is of the tinsel type, which has a bilateral row of mastigonemes and is the longer of the two, while the posterior flagellum is of the whiplash type.

Thraustochytrids were first described by Sparrow [33] and were placed in the family Thraustochytriaceae and order Saprolegniales (Öomycetes) to include chytrid-like organisms with biflagellate zoospores. However, the presence of L-galactose as the primary monomer of the cell walls [34], the possession of a sagenogenetosome and associated ectoplasmic net elements [35], and the absence of sexual reproduction do not suggest a close relationship with the Oomycota [36]. The inability of *T. aureum* to synthesize lysine via the aminoadipic acid

(AAA) or diaminopimelic (DAP) pathways indicates that they are not related to fungi [37]. Rather, a closer affinity with *Labyrinthula* and *Aplanochytrium* (Labyrinthulids) has been demonstrated [36, 38–39]. Thus, the Thraustochytriaceae and Labyrinthulaceae form a natural grouping [40]. Genera are separated primarily on the morphology of the thallic stage and differences in sporogenesis and spore release, such as sporangium size, and cleavage patterns. However, morphological characters used for species delimitation are sometimes variable and difficult to use for species characterization [41–42].

Advances in molecular techniques have assisted scientists in resolving the problematic phylogenetic position of thraustochytrids, which was impossible to determine with morphological characteristics alone. 5S ribosomal RNA data analysis from *Ulkenia visurgensis* and *Schizochytrium aggregatum* showed a high dissimilarity between thraustochytrids and other protists [43]. Mo and Rinkevich [44] developed a simple, reliable, and fast protocol for DHA extraction in thraustochytrids. The application of such a protocol has expedited data generation for phylogenetic studies of thraustochytrids.

At present, thraustochytrids are members of the phylum Heterokonta of the stramenopiles of the kingdom Straminipila [45]. Stramenopiles organisms are characterized by having tripartite, tubular, flagellar hairs and mitochondria with tubular cristae [45–46]. Sequence analysis of the 18S ribosomal RNA data confirms the unique position of thraustochytrids in the stramenopiles and separates the thraustochytrids into two major groupings: the labyrinthulid phylogenetic group and the thraustochytrid phylogenetic group [47]. Recently, Leander and Porter [48] also indicated that the labyrinthulids and thraustochytrids are closely related when the partial small-subunit ribosomal DNA region was sequenced. The phylum consists of three distinct groupings, namely, the labyrinthulids, the thraustochytrids, and the labyrinthuloids [48]. Alternatively, Huang *et al*. [49] proposed the use of fatty acid profiles of individual species to classify thraustochytrids and have confirmed the groupings derived from PUFAs profile corroborated with the 18S data in resolving the phylogenetic position of the thraustochytrids. Currently, the thraustochytrids contains seven genera: *Althornia, Diplophyhrys, Elina, Japonochytrium, Schizochytrium, Thraustochytrium,* and *Ulkenia* [45, 47, 50].

3.2. Uniqueness of tropical/subtropical thraustochytrids for fermentation

Thraustochytrids are cosmopolitan and have been isolated from a wide range of habitats throughout the world, including some extreme environments, such as hyper-saline lakes and the deep sea [51]. Their roles in the mineralization of substrata from plant and animal origins are well documented [50]. Recently, thraustochytrids from subtropical mangroves have been investigated and suggested as one of the major colonizers of mangrove detritus [52–53]. In mangrove environments, thraustochytrids are found to be associated with decaying mangrove leaves and can also colonize, penetrate, and decompose mangrove leaves with their hydrolytic enzymes [54–55]. Apart from fulfilling their ecological roles, mangrove thraustochytrids are superior to their counterparts in the temperate regions in their biotechnological applications. This is largely because of their abilities to tolerate fluctuating environmental conditions (e.g., growing in various saline environments) and their potential to produce substantial biomass yield [52, 56]. The unique ability of mangrove thraustochytrids to grow at low salinities and

to achieve high biomass concentrations offers advantages for the biotechnological production of valuable metabolites through fermentation. Among the mangrove isolates, *Schizochytrium mangrovei* containing numerous oil globules is an ideal candidate for the exploitation of oil production, especially DHA (Fig. 1).

Thraustochytrids can tolerate a range of salinity ranging from as low as 1 ‰ (parts per thousand or g L^{-1}) to full-strength seawater (37 ‰). However, they generally exhibit optimal growth in salinity proximal to the latter [57]. However, from a biotechnological perspective, the high concentration of sodium chloride in full-strength seawater is not suitable for fermentation because of the corrosive effect of chloride ions on conventional bioreactors [58]. Therefore, strains that require minimal sodium chloride for fermentation are ideal candidates because fermentation under low sodium chloride concentrations minimizes the production cost. Tropical and subtropical thraustochytrids, including *Schizochytrium limacinum* and *S. mangrovei*, that only require half-strength seawater for optimal growth are desirable and advantageous from a production point of view [52, 59].

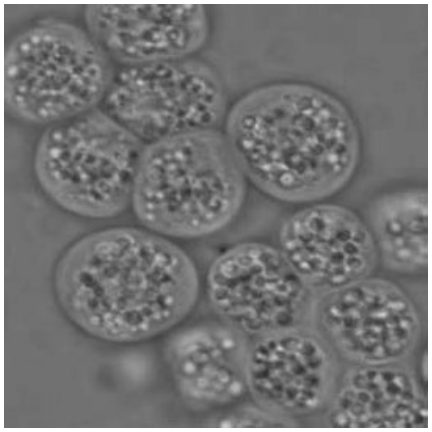


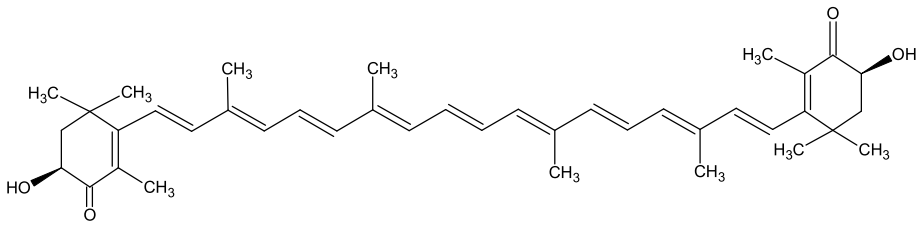
Fig. 1. *Schizochytrium mangrovei* containing numerous oil globules.

Thraustochytrids with the potential to produce high biomass concentrations are beneficial for the production of high-value products. Thraustochytrids isolated from the subtropical regions are high biomass producers. For example, *S. limacinum* SR21 isolated from Yap Island, Japan, can produce a biomass concentration of as high as 48.1 g L^{-1} when grown under optimal conditions [60]. However, selecting the appropriate species is important because biomass production potential does vary from species to species, although they may be isolated from the same locality. Bowles *et al*. [30] conducted an extensive study to investigate the influence of geographical distribution on the biomass concentration of 52 strains on glucose media. Their findings indicated that strains isolated from subtropical locations (25–27ºN) tended to produce higher biomass than their counterparts in the cool (59–61ºN) and cold (50–51ºN) temperate regions. A closer observation of the isolated strains indicated the variability of biomass concentrations among strains within each geographical zone [30]. Fan *et al.* [56] demonstrated that of the 9 strains of thraustochytrids isolated from decaying *Kandelia candel* leaves, only two *S. mangrovei* strains produced biomass concentrations high enough to be considered for potential biotechnological applications. These observations concluded that thraustochytrids isolated from tropical and subtropical regions are robust in terms of biomass concentration but it is also important to select the appropriate species for exploitation (e.g., thraustochytrids with high biomass production as well as DHA content).

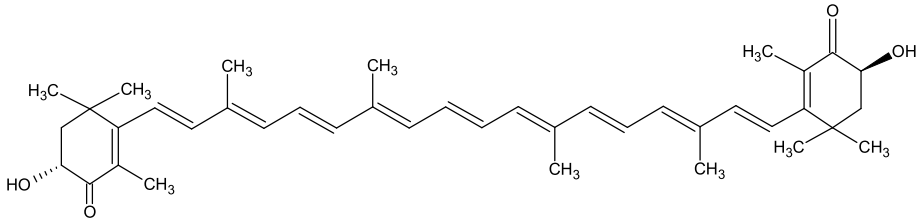
4. HIGH-VALUE PRODUCTS FROM THRAUSTOCHYTRIDS

4.1. Astaxanthin

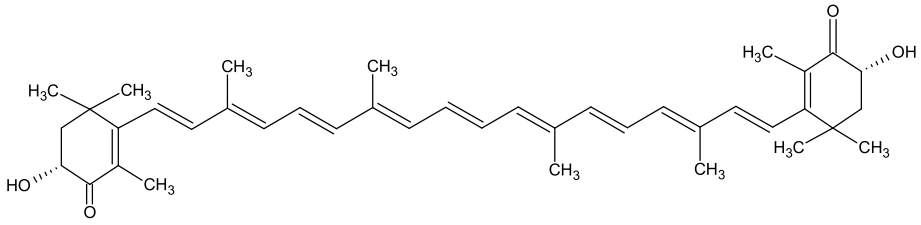
Astaxanthin (3,3'-dihydroxy-ß,-ß,carotene 4,4'-dione), a ketocarotenoid, is widely distributed in nature and is responsible for imparting the distinctive orange-red coloration in animals, particularly in shrimp, crabs, lobster, and salmonids of marine origin [6]. Structurally, astaxanthin exists in three stereoisomers (i.e., 3S, 3´S; 3R, 3´S; and 3R, 3´R) depending on the projection of the hydroxyl group from the two asymmetric carbon atoms located at the C3 and C3´ positions (Fig. 2.) [61]. In red yeast, *Xanthophyllomyces dendrorhous* (previously known as *Phaffia rhodozyma*), the isomer 3R, 3´R is present as the predominant form while synthetic astaxanthin contains a mixture of the three isomers [62]. The provision of 3R, 3´R isomer from *X. dendrorhous*, however, is not a preferred choice for incorporation into feed additives for aquaculture because fish are predominantly pigmented with the 3S, 3´S isomer. The green microalga *Haematococcus pluvialis* is by far the most intensively studied species, owing to the high content of astaxanthin (0.7–3.4% of biomass) within its cell [63]. In *H. pluvialis*, although astaxanthin is produced in the form of 3S, 3´S, the preferred isomer for incorporation in aquaculture feed for salmonids [61], the slow growth rate in heterotrophic cultures and the susceptibility of photoautotrophic cultures to contamination are the major drawbacks for the use of *Haematococcus* for astaxanthin production [64]. Thraustochytrids are capable of heterotrophic growth on an organic substrate in the dark and synthesizing astaxanthin. The potential of astaxanthin production by thraustochytrids is thus of high interest [19, 65].



Astaxanthin 3S, 3'S



Astaxanthin 3R, 3'S



Astaxanthin 3R, 3'R

Fig. 2. Stereoisomers of astaxanthin.

4.1.1. Health benefits and industrial applications

Astaxanthin is a superior antioxidant and is known as "super vitamin E' because its antioxidant activity surpasses that of other carotenoids, such as β-carotene, canthaxanthin, lutein, zeaxanthin, as well as vitamins C and E, especially in quenching singlet oxygens and scavenging free radicals [66]. Increasing evidence has demonstrated the role of astaxanthin in protecting against chemically-induced cancers [67], enhancing the immune system [68], preventing the damaging effects of ultraviolet radiation [69], and treating a number of human diseases, such as atherosclerosis and age-related macular degeneration [70]. Astaxanthin is being marketed as a functional food ingredient in many places of the world [63].

In addition to its role as functional food ingredients or as dietary supplements, the major application of astaxanthin is as feed additive in aquaculture and the poultry industry [61]. In aquaculture, astaxanthin plays an important role in enhancing flesh coloration (i.e., the pink color) of farmed salmonids which is desired by consumers. Since salmonids lack the ability to synthesize astaxanthin *de novo*, the pigment must be incorporated into their diets [61]. In the poultry industry, astaxanthin is effective for increasing yolk coloration in eggs [71].

4.1.2. Production potential

Although Goldstein [72] recognized the formation of bright orange-red pigmentation in *Thraustochytrium roseum*, he did not attempt to characterize or identify the pigments involved in the coloration. It was more than ten years later when the presence of carotenoids, such as β-carotene and canthaxanthin, were confirmed in the thraustochytrid *S. aggregatum* [73]. With the development of modern instrumentation, such as HPLC-MS, the presence of other important carotenoids, astaxanthin and phoenicoxanthin, were recently confirmed to exist in thraustochytrids [66].

A preliminary study of astaxanthin in *Thraustochytrium* sp. CHN-1 indicated that the dominant form of astaxanthin was present as 3S, 3´S, the all trans-isomer, and was synthesized from β-carotene with echinenone and canthaxanthin as intermediates [65, 74]. This study also indicated that content of carotenoids was positively associated with biomass concentration, reaching the highest content of 0.45 mg g^{-1} after 8 days of incubation, and astaxanthin can comprise as much as 50% of the total carotenoids [66]. In addition, Yamaoka *et al.* [74] tested the effect of different light emitting diodes (LEDS) on astaxanthin production in *Thraustochytrium* sp. CHN-1 and indicated that blue LEDS at 470 nm stimulated astaxanthin production in *Thraustochytrium* sp. CHN-1 to a higher cellular content of 0.5 mg g^{-1} as compared to 0.1 mg g^{-1} obtained for the same strain under darkness after 15 days of cultivation. During the same period, a more in-depth study on the carotenoid profile in *Schizochytrium* sp. KH105 was documented [19]. In the study, the effects of carbon and nitrogen concentrations on astaxanthin production were investigated. The result indicated that a high C/N ratio (10% glucose and 0.27% yeast extract and polypeptone) led to the highest astaxanthin yield of 6.1 mg l^{-1} [19]. Long [75] has recently filed for a patent on a formula consisting of corn sugar, processed corn steep liquor, sea salts and thiamine for increasing astaxanthin production in *Thraustochytrium* sp. After 7 days of incubation, an astaxanthin content of 1.5 mg g^{-1} and yield of 8.35 mg L^{-1} were achieved in the *Thraustochytruium* sp.

Due to the ease of heterotrophic cultivation and downstream processing, including the extraction of astaxanthin with acetone, Aki *et al.* [19] suggested the potential of thraustochytrids for the commercial production of astaxanthin. Nevertheless, astaxanthin content in thraustochytrids remains very low as compared to the green microalga *Haematococcus* and thus must be further increased in order to justify commercial production. It is known that a number of environmental and cultural stimulants, such as temperature and metal ions, increase the production of astaxanthin in microorganisms. Further investigations of possible enhancements of astaxanthin production should focus on the effects of these stimulants on astaxanthin synthesis in thraustochytrids [76].

4.2. Docosahexaenoic acid (DHA)

There are two major groups of polyunsaturated fatty acids (PUFAs), namely, ω-3 and ω-6, which are distinguished primarily by the location of the terminal double bond. In ω-3 PUFAs, the terminal double bond is located 3 carbon atoms from the methyl terminus, whereas the ω-6 PUFAs contain a terminal double bond that is 6 carbon atoms from the methyl terminus [22]. Among the PUFAs, DHA, composed of 22 carbon atoms and 6 double bonds in the ω-3 family, has received the most attention because of its importance in the functional development and possible treatment of various diseases in humans [77]. The chemical structure of DHA is shown in Fig. 3.

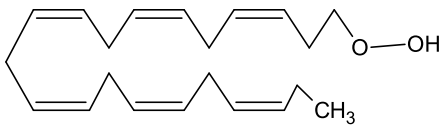


Fig. 3. Chemical structure of docosahexaenoic acid.

Currently, fatty marine fish are the main commercial source of DHA in human diets [78]. With an increasing demand for fish for human consumption and a threatening decrease in fish stocks due to over-fishing, fish and their oils may not be able to sustain the world's future requirements for DHA [79]. Moreover, fish can only synthesize a minute amount of DHA, and most of their PUFAs are acquired from the food chain and originated in marine phytoplankton, including microalgae [79]. Therefore, it is desirable to explore other potential sources for DHA production. Up to now, most research efforts have explored the possibility of producing DHA using marine microalgae [28–29]. The use of microorganisms as a source of DHA is advantageous because there is no seasonal limitation to production, which can be easily achieved using industrial fermentation processes [80]. Moreover, microorganisms usually contain relatively simple fatty acid profiles with a high level of the desired fatty acid, for example, DHA in *Schizochytrium* sp. SR21 [18]. This simplifies purification and reduces unpleasant flavors, which are often associated with impurities.

Among the microalgal groups, thraustochytrids are one of the most promising sources of DHA [21]. Spray-dried *Schizochytrium* sp. biomass has been marketed as Algamac® 2000 and Algamac®3000, and is currently available as a source of DHA to enrich aquaculture species (Aquafauna Bio-Marine, USA). Depending on the strain and growth conditions, the total lipid

content of thraustochytrids can be as high as 77% of cell dry weight, as in the case of *S. limacinum* [60]. DHA may represent as much as 50% of the total fatty acids in thraustochytrids [81–82]. Because of the exceptionally high DHA content, the production potential of DHA in thraustochytrids is tremendous.

4.2.1. Health benefits

Because of its various health benefits, DHA has attracted much research and commercial interest [78]. DHA and other ω-3 PUFAs are essential for human development *in utero* and in infancy as well as throughout the lifespan. DHA in biological tissues is mostly present in an esterified form of phospholipids, triglycerides or cholesterol, and is mainly localized in the sn-2 position of the glycerol backbone [83]. DHA is also a major component in many human tissues, including the grey matter of the brain, the membrane of the retina, and spermatozoa [84–86]. Maintaining a sufficient amount of DHA in the brain is essential for normal growth in infants [87]; a deficiency of DHA is shown to be strongly associated with learning disabilities [88]. Therefore, an adequate supply of DHA from mother's milk and/or infant formulae is required to meet the high demand of DHA for normal brain development in pre-terms and infants [86].

Furthermore, evidence from clinical and epidemiological studies has also strongly suggested the beneficial and protective roles of DHA in alleviating atherosclerosis, rheumatoid arthritis, myocardial infarction, and malignant diseases [78, 89]. Thus, a balanced dietary intake of PUFAs that contain sufficient DHA is recommended in order to prevent pathological symptoms related to DHA deficiency. Although the optimal daily intake of PUFAs and the optimal ratio of n-6 to n-3 PUFAs remain unknown, a dietary intake of 1.2–1.6 g day^{-1} of ω-3 PUFAs, as suggested by a panel of nutritional scientists in the US, with a ratio of ω-3 to ω-6 fatty acids of 2.3:1 is recommended for health protection [90].

4.2.2. Distribution of DHA in various lipid classes

Knowing the distribution of DHA in the different lipid classes in thraustochytrids would provide important information for commercial consideration because DHA in the triacylgycerol layer is the only economically feasible form for commercialization and down-stream processing [91]. There are, however, only a few reports investigated in detail the lipid class distribution of thraustochytrids, which are discussed below [18, 92].

Fig. 4. summarizes the distribution of the major lipid classes in thraustochytrids and indicates that the major lipid class in thraustochytrids is neutral lipids, making up from 44.7–95.0% of total lipids. Glycolipids and phospholipids are the minor constituents in thraustochytrids, comprising approximately 0–27.1% and 8.9–28.2% of total lipids, respectively. Nakahara *et al*. [18] found that *Schizochytrium* sp. SR21 contained about 50.0% of biomass as lipids, which was composed of 95.0% neutral and 5.0% polar lipids. Similarly, *Thraustochytrium* sp. ATCC 26185 was found to contain 32.0% of lipids in its biomass with phospholipids accounting for approximately 8.0% of the total lipids [92]. The proportions of different lipid classes in *T. aureum* ATCC 34304 varied [93, 95] when grown in different media, indicating the profound influence of media composition on lipid composition (Fig. 4.).

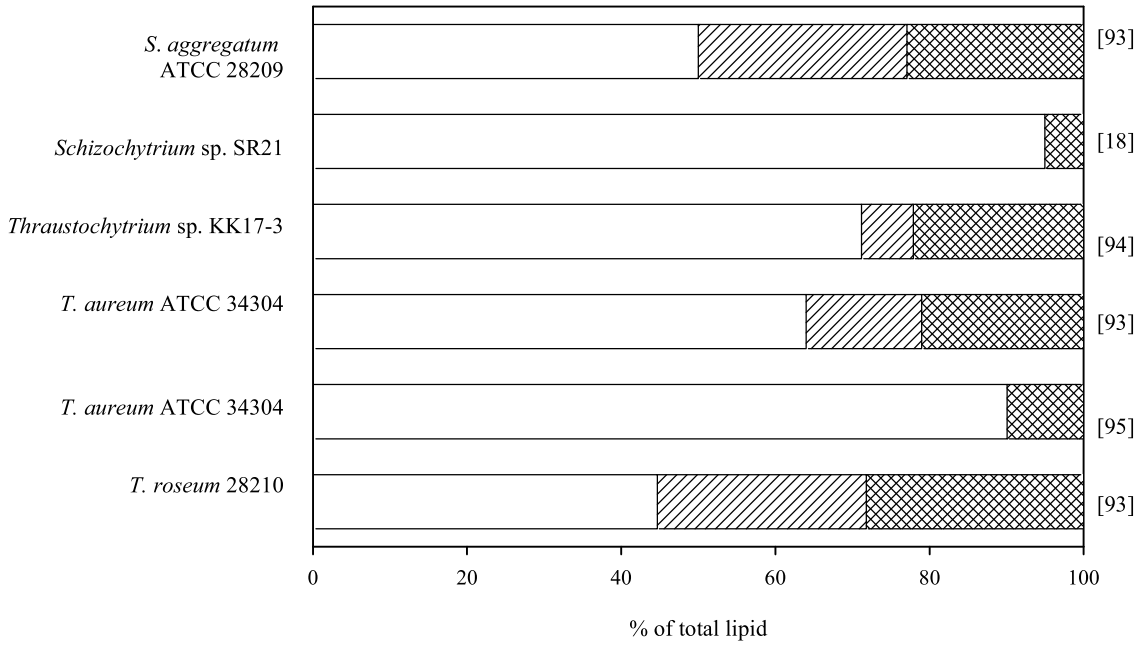


Fig. 4. Distribution of lipid classes in thraustochytrids. (Neutral lipids ▭, Glycolipids ▨, Phospholipids ▩)

In the neutral lipid fractions, triacylglycerols were the major component followed by diacylglycerols and monoacylglycerols (Table 1). Triacylglycerols account for 86.2% of the neutral lipids in *T. aureum* ATCC 34304, but in *Schizochytrium* sp. SR21, it can constitute 98.0% of the neutral lipids. Diacylglycerols and monoacylglycerols, however, were reported to be minor components (< 10.0%) of the neutral lipids of thraustochytrids [93].

In the polar lipid fractions, phospholipids were the major lipid class present in thraustochytrids [93]. Of the different types of phospholipids, phosphatidylcholine was the major component, comprising 49.0–76.2% of the total phospholipids, followed by phosphatidylethanolamine (2.3–11.0%). In *Schizochytrium* SR21, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PL) were the major phospholipids, accounting for 71.0%, 11.0% and 5.0%, respectively, of the total phospholipids [18].

PUFAs make up a large proportion of polar lipids, ranging from 36.5% to 72.0%. In general, the proportion of PUFAs in polar lipid is higher than that in neutral lipid (Fig. 5), owning to the regulatory role of PUFAs on membrane fluidity [94]. The major molecules present in the PCs of *Schizochytrium* sp. SR21 are 1-palmitic acid-2-DHA-PC and 1,2-DHA-PC, comprising 70% of the PCs, indicating the abundance of DHA molecules in thraustochytrids [60]. Ashford *et al*. [96] also indicated that 71.8% of the PUFAs in phospholipids was DHA.

Table 1
Composition of neutral lipids and phospholipids from thraustochytrids

	Schizochytrium sp. SR21	*S. aggregatum* ATCC 28209	*Thraustochytrium* sp. ATCC 26185	*T. aureum* ATCC 34304	*T. aureum* ATCC 34304	*T. roseum* ATCC 28210
Composition of neutral lipids (%)						
Triacylglycerols	98.0%	>90.0%	NP	>90.0%	86.2%	>90.0%
Diacylglycerols and monoacylglycerols	NP*	<10.0%	NP	<10.0%	NP	<10.0%
Composition of phospholipids (%)						
Diphosphatidylglycerol	NP	NP	3.7%	NP	NP	NP
Phosphatidylcholine	71.0%	NP	76.2%	NP	49.0%	NP
Phosphatidylethanolamine	11.0%	NP	2.3%	NP	9.0%	NP
Phosphatidylglycerol	NP	NP	1.3	NP	NP	NP
Phosphatidylinositol	5.0%	NP	16.9%+	NP	NP	NP
References	[18]	[93]	[92]	[93]	[95]	[93]

*NP means data not provided
+Data is collectively represented of lysophosphatidylcholine and phosphatidylinositol

PUFAs comprise 25.9–61.5% of neutral lipids (Fig. 5.) and DHA is one of the dominant PUFAs in neutral lipids of thraustochytrids (e.g., 51.2% in *Thraustochytrium* sp. KK17-3 [94] and 69.3% in *T. aureum* [95]). Nakahara *et al*. [18] and Ashford et al. [96] provided evidence to support that DHA was also abundant in triacylglycerols and comprised 27.6% and 56.7%, of total fatty acids in *Schizochytrium* sp. ATCC 20888 and *Schizochytrium* sp. SR1, respectively. Nakahara *et al*. [18] indicated that seven triacylglycerol species were identified in *Schizochytrium* sp. SR21 with PA-PA-DHA as the major species (27.4%) followed by PA-DHA-DHA (16.9%). Ashford *et al*. [96] observed that DHA and DPA were preferentially esterified in the sn-2 position (71.0–75.0%) of the glycerol backbone of triacylglycerols and found the existence of alternating light (11.7 Å) and dark (29 Å) staining bands in triacylglycerols using high pressure freeze substitution. The dark band corresponded to PUFAs while the light band corresponded to saturated and mono-unsaturated fatty acids. Consequently, they proposed a structural model of triacylglycerols and suggested that the light staining band containing saturated and mono-unsaturated fatty acids might be segregated end to end to form a layer, while DHA arranged in sn-2 position was segregated into the dark staining layer [96]. This structural observation explained the abundance of DHA molecules in triacylglycerols of oleaginous thraustochytrids.

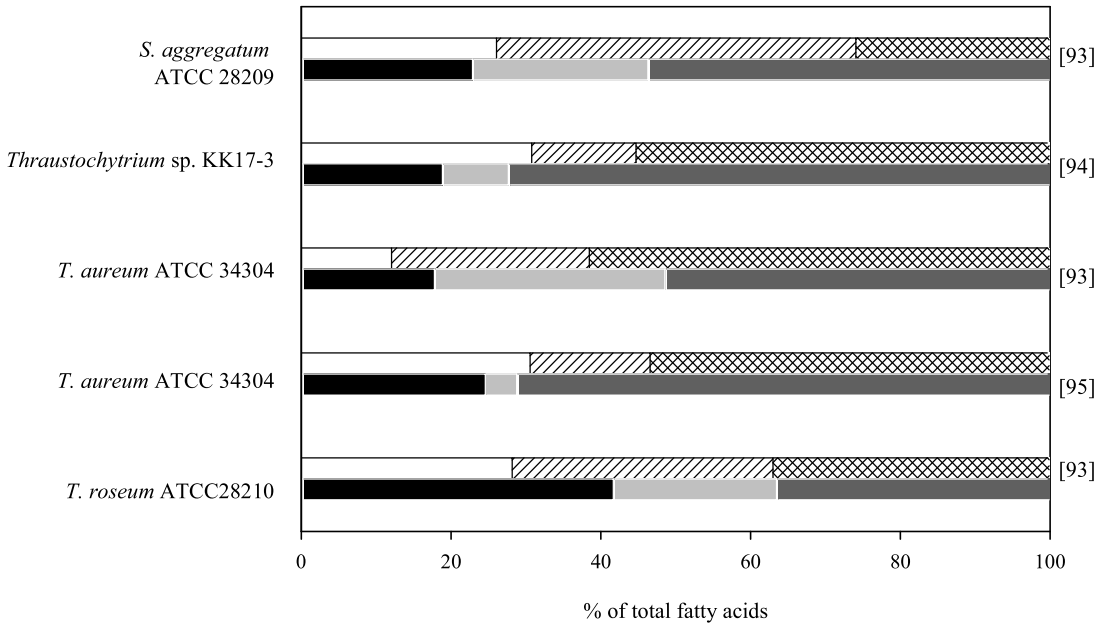


Fig. 5. Percentage of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in neutral (NL) and polar (PL) lipid classes in thraustochytrids. NL-SFA, NL-MUFA, NL-PUFA, PL-SFA, PL-MUFA, PL-PUFA

4.2.3. Production potential

The most important criterion for selecting a microbial source for the commercial production of DHA is the potential of the microorganisms to produce substantial biomass and total lipids [28]. A simple fatty acid profile with a large proportion of DHA in the lipids is another important criterion to be considered when selecting DHA producing microorganisms [97]. Of the various microbial sources, thraustochytrids are the most successful microalgal group in fulfilling these criteria. Table 2 summarizes some of the selected DHA-producing

thraustochytrids reported in papers and patents published from 1991 to 2003. Overall, seven species of thraustochytrids from three genera, *Schizochytrium*, *Thraustochytrium* and *Ulkenia*, have been investigated and considered to have DHA production potential. *Thraustochytrium* spp., in general, contained a larger proportion of DHA, ranging from 38.5%–59.5% of the total fatty acids, than *Schizochytrium* spp. and *Ulkenia* sp. SAM 2179, which contained only 30.5%–46.2% of DHA in their total fatty acids. In contrast, however, *Schizochytrium* spp. and *Ulkenia* sp. SAM 2179 contained generally much higher amounts of total lipids than *Thraustochytrium* spp. The total lipid contents of the former might be as high as 77.5% of the biomass while the total lipid content of the latter accounted for in many cases only approximately 20.0% of the biomass. There was, however, an exception: *Thraustochytrium* sp. G13 was reported to contain 78.0% of its biomass as lipids although further investigation might be necessary to confirm the findings [30]. *Schizochytrium* spp. and *Ulkenia* sp. SAM 2179 grew much faster than *Thraustochytrium* spp. Therefore, *Schizochytrium* spp. and *Ulkenia* sp. SAM 2179 have been considered as more promising organisms for the production of DHA. *S. limacinum* is so far the most successful species in terms of biomass, lipid production, and DHA production, followed by *Ulkenia* sp. SAM 2179 and *Thraustochytrium* sp. G13 [30, 60, 101]. The DHA content and productivity of *S. limacinum* were 276.5 mg g^{-1} and 3325.0 mg l^{-1} day^{-1}, respectively. This level of DHA productivity is the highest of any microalgal sources ever reported in the literature.

4.2.4. Factors affecting DHA production

Culture conditions are always important for the optimal production of PUFAs in microalgae, however, they may be species specific [4]. As illustrated in Table 2, the culture conditions for DHA production were different for different species of thraustochytrids, and the production might influenced by the culture mode, growth phase, environment (e.g. medium pH, temperature, etc.), and nutrition (e.g. carbon, nitrogen, phosphorus, sodium, trace elements, growth factors, etc.) [22].

There are many different culture modes available for the cultivation of microalgae in heterotrophic cultures, including batch, fed-batch, continuous, and perfusion cultures [10], but the most widely adopted strategy for DHA production in thraustochytrids is still batch culturing (see Table 2). However, there are obvious limitations associated with this culture mode because of the problem of substrate inhibition with high initial substrate concentrations. A fed-batch strategy developed to overcome the substrate inhibition problem resulted in a two-fold increase in DHA yield in *T. roseum* ATCC 28210 over the batch culture using the same algal strain [100]. Growth phase can also profoundly influence the lipid production of oleaginous microorganisms. Lipid accumulation usually follows a sigmoid pattern, with maximum PUFA production in the culture during the late exponential or early stationary phase [80]. DHA production in thraustochytrids follows the same pattern, with maximum DHA production in most species from day 2 to 4, which corresponds to either their late exponential or early stationary phase.

Table 2
Selected DHA producing thraustochytrids extracted from literatures and patents from 1991-2003.

Thraustochytrids	*S. limacinum* SR21	*S. limacinum* SR21	*S. mangrovei* KF2	*Thraustochytrium* sp. ATCC 20892	*Thraustochytrium* sp. G13	*T. aureum* ATCC 34304	*T. aureum* ATCC 34304	*T. roseum* ATCC 28210	*T. roseum* ATCC 28210	*Ulkenia* sp. SAM 2179
Growth parameters										
Biomass (g l^{-1})	48.1	38.0	13.3	7.1	14.0	3.8	4.9	9.8	17.1	19.5
Total lipid (% of dry weight)	77.5	37.0	59.1	16.9	78.0	16.5	20.3	17.5	24.0	61.0
DHA (% TFA)	35.6	30.5	35.3	59.5	38.5	48.5	51.0	49.8	48.8	46.2
DHA in biomass (mg g^{-1})	276.5	116.6	204.3	100.3	140.4	70.4	103.8	85.9	117.0	282.1
DHA yield (mg l^{-1})	13300.0	4200.0	2762.0	707.4	1965.6	269.6	510.5	841.8	2000.0	5500.0
DHA productivity (mg l^{-1} day^{-1})	3325.0	840.0	1280.6	176.9	1150.6	44.9	85.1	168.4	166.7	1833.3
Environmental and nutritional parameters										
Culture mode	Bioreactor Batch	Shake flask Batch	Shake flask Batch	Shake flask Batch	Bioreactor Batch	Shake flask Batch	Shake flask Batch	Shake flask Batch	Shake flask Fed batch	Bioreactor Batch
Culture period (day)	4	5	2 days 4 h	4	1 day 17 h	6	6	5	12	3
Medium pH	4	NP	6	7	NP	NP	NP	6	6	4
Temp.(ºC)	28	25	25	25 followed by 15	24	25	25	25	25	28
Carbon source										
Glucose (g l^{-1})	120.0	90.0	60.0	40.0	40.0	20.0	_	_	_	60
Starch (g l^{-1})	_	_	_	_	_	_	25.0	25.0	25.0 (10.0)	_
Nitrogen source										
Corn steep liquor (g l^{-1})	1.4	20.0	_	_	_	_	_	_	_	0.7
$(NH4)_2SO_4$ (g l^{-1})	4.0	_	_	0.2	_	0.2	0.2	0.2	0.2	2.0
Sodium glutamate (g l^{-1})	_	_	_	2.0	_	2.0	2.0	2.0	2.0 (0.8)	_
Yeast extract (g l^{-1})	_	_	10.0	2.0	5.0	_	_	2.0	2.0	_
C/N ratio	51.9	32.7	24.0	39.2	32.0	33.0	_	_	_	51.9

Other medium components										
Artificial sea salts ($g\ l^{-1}$)	15.0	15.0	15.0	_	_	_	_	_	_	15.0
$CaCO_3$ ($g\ l^{-1}$)	_	_	_	0.2	_	0.2	0.2	0.2	0.2	_
KCl ($g\ l^{-1}$)	_	_	_	1.0	_	1.0	1.0	1.0	1.0	_
KH_2PO_4 ($g\ l^{-1}$)	3.0	_	_	0.2	_	0.1	0.1	0.1	0.2 (0.08)	3.0
MgSO4 ($g\ l^{-1}$)	_	_	_	5.0	_	5.0	5.0	5.0	5.0 (2.0)	_
NaCl ($g\ l^{-1}$)	_	_	_	25.0	_	25.0	25.0	25.0	10.0	_
$NaHCO_3$ ($g\ l^{-1}$)	_	_	_	_	_	0.1	0.1	0.1	0.1	_
Na_2SO_4($g\ l^{-1}$)	_	_	_	_	20.0	_	_	_	_	_
Trace elements										_
Citric acid ($mg\ l^{-1}$)	_	_	_	5.0	_	_	_	_	_	_
$CoCl_2.6H_2O$ ($mg\ l^{-1}$)	_	_	_	0.13	_	_	_	_	0.26	_
$CuSO_4.5H_2O$ ($mg\ l^{-1}$)	_	_	_	0.01	_	_	_	_	0.02	_
$FeCl_3.6H_2O$ ($mg\ l^{-1}$)	_	_	_	1.45	_	_	_	_	2.9	_
$MnCl_2.4H_2O$ ($mg\ l^{-1}$)	_	_	_	4.3	_	_	_	_	8.6	_
$ZnCl_2$ ($mg\ l^{-1}$)	_	_	_	0.3	_	_	_	_	0.6	_
Thiamine ($\mu g\ l^{-1}$)	_	_	_	10.0	_	10.0	10.0	_	10.0	_
Vitamin B12 ($\mu g\ l^{-1}$)	_	_	_	1.0	_	1.0	1.0	_	1.0	_
References	[60]	[31]	[56]	[98]	[30]	[99]	[82]	[83]	[100]	[101]

Thraustochytrids grow over a wide range of temperatures from 5 to 37ºC with an optimum between 20 and 25°C [57]. The optimum temperature for DHA production in thraustochytrids is from 25 to 28ºC [60, 83]. Yongmanitchai and Ward [80] found that a lower temperature promotes the biosynthesis of PUFAs in microorganisms because more PUFAs are required in order to regulate the membrane fluidity under a cold environment. For this reason, Nichols *et al*. [102] suggested the biotechnological potential of Antarctic thraustochytrids which require a lower temperature optimum for growth and thus may produce more PUFAs at their optimal temperature, at which both biomass and PUFA yields could be at their maxima. Although lower temperatures might increase DHA biosynthesis, for most other species, it also decreases overall growth. Consequently, the best strategy for DHA production should consider both biomass and DHA production. Singh and Ward [22] suggested that temperature shift might be an effective way of obtaining sufficient biomass with high DHA content in a relatively short time. For example, DHA production was found to increase in shifting temperature conditions (i.e., 3 days at 25ºC followed by one day at 15ºC) [98].

Thraustochytrids grow at pH ranging from 5.0 to 11.0, with an optimum between 6.0 and 8.0 [103]. Adjusting the initial pH to 6.0–7.0 was the best for DHA production in a number of *Thraustochytrium* spp., such as *T. roseum* ATCC 2810, and *Thraustochytrium* sp. ATCC 20892 [83, 98]. On the contrary, an acidic pH (pH 4.0) was preferred for optimal growth and DHA production in *S. limacinum* SR21 and *Ulkenia* sp. SAM 2179 [31, 101].

In addition to environmental conditions, nutritional conditions are also important. Carbon and nitrogen sources are essential nutrients for cell growth and protein biosynthesis in all organisms including thraustochytrids. Thraustochytrids can utilize a wide variety of carbon source for DHA production, with glucose and starch being the two most efficient and preferred sources [60, 83]. *Schizochytrium* spp. and *Ulkenia* sp. SAM 2179 tend to grow and produce DHA best on glucose, whereas *Thraustochytrium* spp. grow best on starch granules [83]. For instance, a medium with 2.5% starch was found to be the best for biomass production (9.8 g L^{-1}) and DHA production (841.8 mg L^{-1}) in *T. roseum* ATCC 28210 [83]. *Schizochytrium* spp. exhibited the best DHA production in a medium containing 6%–12% glucose, without suffering from substrate inhibition, whereas the optimal glucose concentration in *Thraustochytrium* spp, is less than 4% (Table 2). Although the type and amount of carbon sources used are important for achieving high lipid and DHA production, the amount of carbon in relation to the nitrogen source is also important. A high C/N ratio is generally preferred for lipid accumulation, which can be triggered by nitrogen exhaustion [22]. In oleaginous thraustochytrids, a C/N ratio of 24 or above was reported to be optimal for lipid accumulation (Table 2). For example, *S. limacinum* SR21 accumulated 77.5% of lipids in biomass when grew in a medium with a C/N ratio of 51.9. Likewise, *Thraustochytrium* sp. G13 also attained a high lipid content of 78.0% in biomass when a medium with a C/N ratio of 39.1 was used.

Thraustochytrids utilize undefined organic nitrogen substrates (e.g. corn steep liquor) more efficiently than inorganic nitrogen (e.g., ammonium). However, a combination of corn steep liquor and ammonium sulfate was found to be the best nitrogen source for DHA production in *S. limacinum* SR21 [60]. *Thraustochytrium* spp. prefers sodium glutamate but cannot grow in a medium containing ammonium sulfate [22].

Phosphorous is equally vital to cell growth as well as nucleic acid formation. Its importance also extends to the formation of the phospholipids layer, which, in thraustochytrids, contains a substantial amount of PUFAs [94]. Singh and Ward [100] observed that DHA production significantly increased in *T. roseum* ATCC 28210 when the amount of phosphorous, in the form of KH_2PO_4, increased from 0.1 to 0.2 g L^{-1} in the medium. The optimal concentration of KH_2PO_4 for DHA production in *S. limacinum* SR21 and *Ulkenia* sp. SAM 2179 was even higher (3 g L^{-1}), compared to 0.1–0.2 g L^{-1} in most *Thraustochytrium* spp. [60, 101].

Sodium is another important element for growth in thraustochytrids because sodium ions are essential for phosphate uptake [104]. It can be supplied either as sodium chloride or artificial sea salt. Half-strength seawater was as effective as full-strength seawater; a comparable biomass and DHA yield were obtained for *T. roseum* in the medium containing 25 g L^{-1} and 10 g L^{-1} of NaCl [100]. For DHA production, a medium with half-strength seawater in the form of artificial sea salts is an effective replacement for a defined medium, particularly in *Schizochytrium* spp. (Table 2).

In addition, thraustochytrids require certain trace elements and growth factors, such as vitamins B_{12} (cobalamine) and B_1 (thiamine), for growth [103]. The effect of vitamins on *T. aureum* was tested using a defined medium containing glucose and glutamate [95]. A combination of biotin, cobalamine, nicotinic acid, pantothenic acid, riboflavin, and thiamine produced the highest biomass. *Thraustochytrium globsum, T. roseum* and *S. aggregatum* were all found to require cobalamine for growth [103].

4.2.5. Statistics-based approach for medium optimization

Traditionally, the one-at-a-time strategy is the most popular method employed for optimizing medium components and environmental factors for DHA production in thraustochytrids [31, 98]. The one-at-a-time method is to keep the level of all components constant, with the exception of the one investigated [105]. A major drawback with this method is its inability to account for interactions among factors. Recently, statistics-based experimental design has been employed for the production of PUFAs in microalgae, such as diatoms [106] and thraustochytrids [107]. This method involves three steps, including experimental design (e.g., central composite, factorial, Plackett and Burman designs, etc.), optimization (e.g., response surface methodology, steepest ascent, canonical analysis, etc.), and verification [105].

A statistics-based approach was employed to optimize medium composition and environmental factors for DHA production by *Schizochytrium* sp. ATCC 20888. A fractional factorial design was initially used for screening a range of variables [107]. Concentrations of glucose, yeast extract, and sodium chloride as well as the pH value were the significant variables identified. These variables were further optimized using central composite design and determined using canonical analysis. After optimization, an experimental DHA yield of 498 mg L^{-1} confirmed the validity of the predicated value and was 41% higher than the result from the one-at-a-time experiment (352 mg L^{-1}) [107].

4.2.6. Molecular manipulation of DHA production

Modern biotechnology has witnessed the development of many molecular techniques and their applications for gene manipulation. These molecular techniques have recently been used to discover the pathways leading to DHA production in thraustochytrids [108, 109]. Elucidating the DHA biosynthetic pathway in thraustochytrids is the initial step towards using genetic engineering approaches for DHA enhancement and for genetic manipulation. *Schizochytrium* and *Thraustochytrium* are the two well-recognized genera of thraustochytrids for DHA production [31, 99], but their biosynthetic pathways have only recently been established [108, 109]. Though *Schizochytrium* and *Thraustochytrium* are taxonomically related and are grouped in the same family, it is surprising that two distinct pathways exist for their DHA biosyntheses, namely, the anaerobic pathway, which is also known as a novel polyketide synthase system (PKS) in *Schizochytrium* sp. ATCC 20888 and the aerobic pathway, which involves a series of desaturases and elongases in *Thraustochytrium* sp. ATCC 26185 [110]. Both pathways utilize acetyl-CoA and malonyl-CoA as the principal building blocks for DHA biosynthesis [22, 110].

Schizochytrium sp. ATCC 20888 has been demonstrated to utilize the PKS for DHA biosynthesis [108] (Fig. 6). The exact route for its biosynthesis remains to be determined, but it likely involves condensation and isomerization for introducing double bonds and chain elongation [111]. This pathway differs from the aerobic pathway in that it involves polyketide synthase instead of fatty acid synthase for the repeated full cycle of condensation, ketoreduction, and dehydration reactions. The pathway often omits steps from the full cycle, such as dehydration and reduction, and does not require molecular oxygen and membrane-bound desaturases for introducing double bonds in the developing fatty acid chain [110]. In *Schizochytrium* sp. ATCC 20888, three open reading frames with 11 domains were identified as genes for PUFAs biosynthesis and eight of them had a high homology with the PKS genes of *Shewanella*. The findings suggested the possibility of laterally transferring PKS genes from prokaryotes to eukaryotes [108].

Alternatively, the biosynthesis of DHA in *Thraustochytrium* sp. ATCC 26185 involves the aerobic pathway, which requires two major steps (Fig. 6). Biosynthesis starts with the *de novo* biosynthesis of stearic acid (18:0) from repeated additions of two carbon atoms from acetyl-CoA or malonyl-CoA through a series of condensation, reduction and dehydration reactions [22]. The biosynthetic process proceeds subsequently from 18:0 through a series of desaturation and elongation processes (Fig. 6).

Of particular interest in the acyl chain desaturation and elongation is the controversial debate over the existence of a Δ4 desaturase that introduces a final double bond to docosapentaenoic acid (22:5 DPA ω-3) in order to biosynthesize DHA [112]. Experimental evidence has recently been made available that confirms the presence of Δ4 desaturase in *Thraustochytrium* sp. ATCC 26185 [109, 113]. The Δ4 desaturase is a front-end desaturase, similar to other front-end desaturases, and is capable of desaturating substrata of both ω-3 e.g., DPA and ω-6 e.g., docosatetraenoic acid (22:4 DTA ω-6) [109].

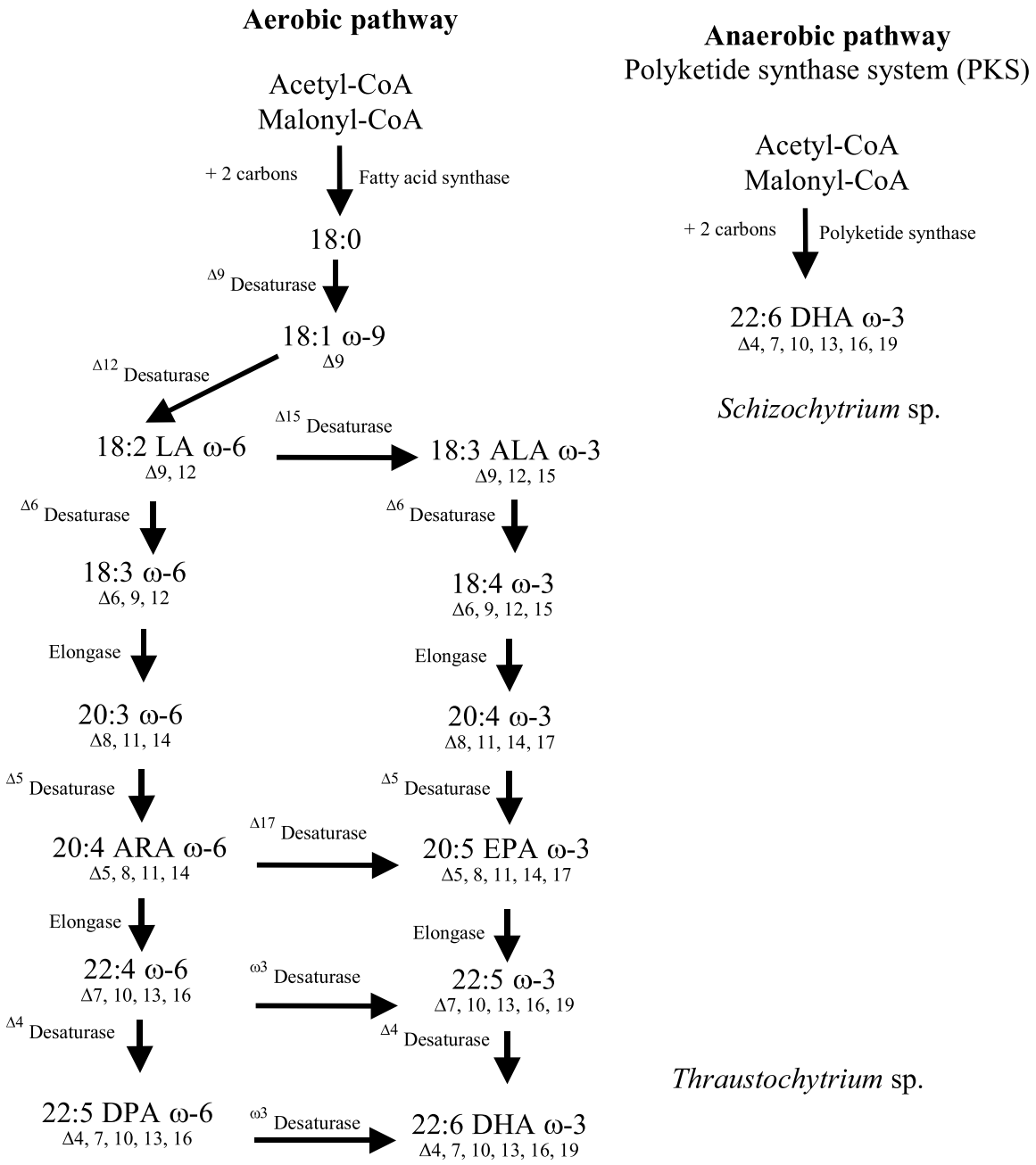


Fig. 6. The aerobic and anaerobic pathways for DHA biosynthesis in thraustochytrids.

The recent successful elucidation of DHA biosynthesis in thraustochytrids has created the possibility of transgenically producing DHA in other systems, especially in oilseed plants [110]. The concept of producing PUFAs by using plant as a 'chemical factories' has been proposed and considered as a potential way to meet future oil demand, owing to the availability of oilseed plant-based production systems [114]. Qiu *et al*. [109] heterologously expressed the Δ4 desaturase gene (FAD4) in the oilseed plant, *Brassica juncea*, which resulted in DHA accumulation (about 6% of total fatty acids). Similarly, Meyer *et al*. [113]

have discovered the existence of ω-3 desaturase gene that channels ω-6 substrata to ω-3 substrata at different stages of the DHA biosynthetic pathway from *Thraustochytrium sp*. ATCC 26185. This gene allows the conversion of linoleic acid (18:2 LA ω-6), which is especially abundant in oilseed plants, and may increase the production of ω-3 PUFAs in transgenic plants [113]. In addition, the discovery of the PKS pathway allows further choices for genetically manipulating DHA production [111].

4.2.7. Downstream processing

Downstream processing involves multi-step procedures for the recovery and purification of products of interest [115]. From a commercial point of view, the most important objective in downstream processing is to maximize product recovery and, at the same time, minimize the cost of production [115]. As for the recovery of biomass and PUFAs from microalgae, the recovery process may well exceed 60% of the production costs [116]. Thus, knowledge on the procedures for purifying PUFAs is required in order to identify the steps that may be omitted or improved in order to reduce the production cost. Robles Medina *et al*. [117] provided an extensive summary of the various useful methods for recovering PUFAs and indicated that the downstream processing of PUFAs includes three major steps: recovering biomass, extracting lipids, and concentrating and purifying the PUFAs [117]. The biomass of microalgae can be harvested and separated from the culture broth by either centrifugation or filtration. The addition of flocculants prior to separation might enhance cell aggregation, which would facilitate the recovery of biomass [116]. Harvested cells are usually subject to lyophilization, as lipids are more readily extracted from freeze-dried biomass than wet biomass. In addition, reports also indicate that spray drying and freeze drying are the two preferred drying methods for thraustochytrids prior to lipid extraction [97, 118]. However, Molina Grima *et al*. [119] showed that lipid is equally and efficiently extracted from both dry and wet biomass of *Phaeodactylum tricornutum*. Thus, the omission of the drying step could significantly reduce the production cost [119].

Finding an appropriate solvent system is important for extracting lipids from algal cells. Lewis *et al*. [120] indicated that the extraction of lipids from thraustochytrids was more efficient when solvents were added in the order of increasing polarity (chloroform > methanol > water). After lipid extraction, PUFAs can be concentrated by chromatographic methods, supercritical fluid extraction, or urea fractionation [117]. Of the various PUFA concentration methods, the HPLC-based method is the most appropriate for the recovery of high purity products. Yamamura and Shimomura [97] described a chromatographic method based on preparative HPLC with reverse phase octadecyl silane and showed that the method was effective for separating DHA with 99% purity from *Schizochytrium sp*. SR21.

4.3. Squalene

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22 hexaene) is a dehydro-triterpenic hydrocarbon ($C_{30}H_{50}$) with six double bonds (Fig. 7) and is an intermediate for the biosynthesis of phytosterol and cholesterol in plants and animals [121]. Liver oils of deep-sea sharks, currently, represent the richest natural source of squalene [122–123]. The future supply and availability of shark liver oils, however, are questionable because of concerns

about the over-exploitation of fishery stock [124]. For this reason, alternative sources, such as those from plant and microorganisms, are being explored for squalene production [123, 125]. Among these potential new sources, microalgae represent one of the best candidates for future exploration because of the well-established fermentation technology for their mass cultivation [20]. Squalene has recently been reported in thraustochytrids when the lipid profiles in the selected thraustochytrids were studied [92, 126]. In addition, a squalene recovery process is recently available and can be handled easily in a simple downstream process based on counter-current chromatography, which has led to the successful purification of squalene with 96% purity [127]. Thus, the production of squalene using thraustochytrids has great potential.

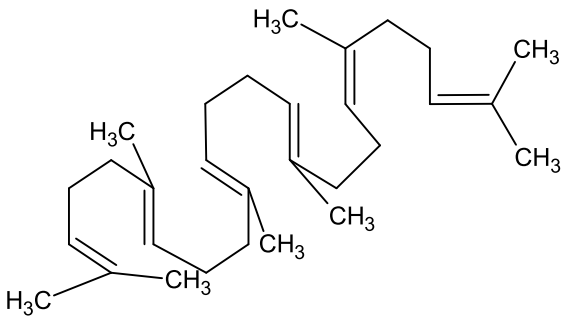


Fig. 7. Chemical structure of squalene.

4.3.1. Health benefits

Squalene has recently been recognized to possess health beneficial properties, owing largely to its strong oxygen scavenging abilities and anti-tumor activities [128–129]. Dessì *et al*. [128] showed that squalene is a strong antioxidant and is capable of protecting PUFAs against temperature and UVA-induced oxidation. Moreover, the antioxidative and protective roles of squalene in alleviating against skin irritation and UVB-induced skin cancer are notable [130]. The high content (10%) of squalene in human skin lipid, therefore, may play an important role in protecting against skin damage [131].

The anti-carcinogenic role of squalene against chemically-induced colon and lung cancers has also been demonstrated in animal models [129]. The mechanism by which squalene may inhibit tumor formation is based on its inhibitory role on ß-hydroxy-ß-methylglutaryl-CoA reductase catalytic activity and subsequent inhibition on farnesylation of Ras oncoproteins [132].

4.3.2. Production potential

Squalene was first reported in *Thraustochytrium* sp. and was noticed to represent 63% of the nonsaponifiable lipid when its lipid profile was analyzed [92]. Weete *et al*. [92], however, did not investigate the effects of nutritional and culture conditions on squalene content [92]. Lewis *et al*. [126] later investigated the influences of culture age, temperature and dissolved oxygen level on the squalene content of thraustochytrid ACEM 6063, a possible member of the genus, *Schizochytrium*. Thraustochytrid ACEM 6063 grown under a low dissolved oxygen level (0–5% saturation) contained a higher squalene content (exceeding 1.0 mg g^{-1}) than that

grown at higher dissolved oxygen levels (40%–60%) (0.01 mg g^{-1}) [126]. It was suggested that the high squalene content observed at low oxygen levels might be related to the reduced efficiency of the enzyme, squalene monooxygenase, which requires oxygen molecules to catalyze the oxidation of squalene to 2,3-oxidosqualene for sterol biosynthesis [126].

Squalene content in thraustochytrid ACEM 6063 was found to be inversely associated with culture temperature; for example, a squalene content of 1.2 mg g^{-1} was obtained at 15°C, which decreased to 0.7 mg g^{-1} at 20°C [126]. Lewis *et al*. [126] also showed that squalene content decreased in aged cultures of thraustochytrid ACEM 6063. A similar reduction in squalene content (e.g., 0.16 mg g^{-1} to 0.03 mg g^{-1} in *S. mangrovei* FB1 from day 3 to day 5) was observed in three strains of *S. mangrovei* as the culture aged [20]. A recent study found that a further enhancement of squalene content can be achieved through the application of enzyme inhibitor (K.W. Fan, F. Chen and Y. Jiang, unpublished data). A squalene content of 0.53 mg g^{-1} was observed in *S. mangrovei* FB3 when terbinafine (100 mg L^{-1}), a squalene monooxygenase inhibitor, was applied as compared to the untreated control in which the squalene content was only 0.37 mg g^{-1}. Nonetheless, squalene content in thraustochytrids is still relatively low (0.01–1.8 mg g^{-1}) as compared to conventional sources (e.g., 4.24 mg g^{-1} in olive oil) [20, 126, 123]. Thus, Jiang *et al*. [20] suggested that further optimization of media and other conditions is necessary for enhancing the squalene content in thraustochytrids.

4.4. Other novel bioactive compounds

Over the past decades, thraustochytrids have also been known to produce a number of other bioactive compounds that may be targets for future biotechnological applications [18–19, 133]. The applications of sterols as precursors for steroid production and as natural surfactants are widely recognized in the biotechnology industry [7]. Thraustochytrids are known to produce significant amounts of sterols and thus may be considered as potential candidates for sterols production [92, 126]. Weete et *al*. [92] analyzed the sterol content of *Thraustochytrium* sp. ATCC 26185 and found that cholesterol and 24-ethylcholesta-5,7,22-trien-3β-ol are the major sterols, representing 69% of total sterols. Recently, a thraustochytrid (strain ACEM 6063) isolated from Australian coast was shown to produce 20 sterols, of which 13 were identified. Brassicasterol, cholesterol, stigmasterol, and 24-ethylcholesta-5,7,22-trien-3β-ol were the dominant sterols, representing 50% to 90% of the total sterols [126]. In addition, marine lycosphingolipids are well known to possess excellent bioactivities and are also found in thraustochytrids. Jenkins *et al*. [133] identified glycosphingolipids and thraustochytrosides A-C in *T. globosum* CNK-018, but their bioactivities remained to be determined.

In addition to DHA production, there is evidence to support the possible production of other PUFAs from certain species of thraustochytrids [21]. For example, a substantial amount of DPA was produced with a productivity of 0.44 g $L^{-1}day^{-1}$ in a DHA producing *Schizochytrium* sp. that was isolated from Japanese coasts [18]. In addition, canthaxanthin, a potent antioxidant similar to astaxanthin, was also produced in *Schizochytrium* sp. KH105 at a yield of 10 mg L^{-1} [19]. Thraustochytrids have also been shown to have the ability to penetrate and colonize recalcitrant materials such as cellulose and sporopollenin in the walls of pollen grains [134], suggesting that thraustochytrids may also be a source of degrading enzymes that could be of great industrial importance.

5. OTHER APPLICATIONS OF THRAUSTOCHYTRIDS

5.1. Thraustochytrids in aquaculture

Due to the increasing demand for seafood and the rapid depletion of natural fisheries, 25% of the worldwide seafood production is now from the aquaculture industry [135]. The rapid expansion of aquaculture has led to an increased demand for aquaculture feeds, including microalgae. Indeed, microalgae have played a vital role in modern aquaculture; they are used not only as feeds for adult aquatic animals, but are also essential nutrients for these animals during their larval stages [136]. Of particular interest is the necessity of DHA derived from microalgae for the normal development of juvenile crustaceans, larval fish, and their bloodstock, since most marine animals have a limited ability to synthesize DHA themselves [137–138]. At present, however, fish oil is still the major source of PUFAs for farmed fish, but thraustochytrids have become more and more popular. It has been reported that thraustochytrids are used as feed additives in the aquaculturing of marine mussels, salmonids, and tropical sea cucumbers [139–141].

The first documented application of thraustochytrids in the aquaculture industry was to enrich the DHA content of brine shrimp and rotifers with spray-dried biomass of *Schizochytrium* sp. [118]. Brine shrimp and rotifers are used extensively as live prey for feeding to marine fish larvae, but the PUFA content in these two organisms are insufficient for larval development [142]. Thus, an enrichment procedure with microalgae is commonly required to improve their PUFA content prior to feeding [143]. It was reported that the PUFA content, especially that of DHA in brine shrimp nauplii (100 brine shrimp nauplii per mL) enriched with 400 mg L^{-1} of spray-dried *Schizochytrium* sp., increased from an undetectable level to 0.8% of the biomass [118]. Similarly, DHA content in enriched rotifers (400 rotifers per mL) increased from an undetectable level to 1.38% of the dry biomass after feeding 70 mg L^{-1} of spray-dried cells. The success of *Schizochytrium* sp. used for feeding live prey is mainly due to their high content of DHA (>30% of total fatty acids), suitable size for ingestion, and excellent suspension characteristics in seawater [118].

Spray-dried *Schizochytrium* sp. has also been successfully applied as partial replacement in feeding juvenile mussels, *Mytilus galloprovincialis* [141]. The application of live prey and microalgae as feeds for the larval stages of marine animals is desirable in the aquaculture industry, but the cost is relatively high [144]. Attempts to provide a cost-effective alternative to conventional feed by using non-living algae have been made [144]. When juvenile mussels are fed an inert diet containing 70% spray-dried biomass of *Schizochytrium* sp. and 30% ground biomass of *Spirulina* twice a day at a ration of 20 mg diet per day the juvenile mussels showed a 60% increase in organic dry weight over the control group [141]. This has led to a 50% reduction in feed production cost.

The applications of *Schizochytrium* sp. also extend to the rearing of salmonids [140]. PUFAs are essential for normal development in juvenile fish [138]. Carter *et al*. [140] have recently studied the possibility of incorporating freeze-dried biomass of *Schizochytrium* sp. into feeds for Atlantic salmonids (*Salmo salar*). The results indicated that a *Schizochytrium* sp. based feed can replace fish-oil based feed or canola-oil based feed because of the

satisfactory performance in terms of weight gain, whole body chemistry, and immune function [140].

Schizochytrium sp. has been successfully applied as an inducing agent for artificial spawning in *Holothuria scabra*, a commercially important tropical sea cucumber [139] with a high demand worldwide [145]. However, one of the major obstacles to the commercial production of sea cucumbers is the difficulty in artificially achieving reliable spawning and a regular supply of eggs [139]. It is thus possible to use a combination of thermal stimulation and *Schizochytrium* sp. to induce artificial spawning in a number of tropical sea cucumbers [139].

5.2. Enriching foods with thraustochytrids

An insufficient intake of dietary ω-3 PUFAs in western diets has led to increased incidents of cardiovascular diseases and other illnesses [146]. One of the potential methods for increasing the dietary intake of ω-3 PUFAs is to enrich commonly consumed food products with DHA [147]. Some of the ω-3 PUFAs enriched foods on the market include bakery products, dressings, eggs, infant formula, milk, and poultry products [147]. Thraustochytrids have been successfully employed as a source of DHA to enrich food products, including eggs, chicken and lamb meats, as well as milk products from ewes and cows [148–150].

Eggs are one of the first food sources enriched with thraustochytrids [151, 152]. Laying hens that were fed on a diet of *Schizochytrium* sp. equivalent to 165 mg of DHA per hen per day showed a 5-fold increase in egg DHA content (135 mg per egg) compared with eggs produced from hens fed on a control diet with only 28 mg of DHA per egg. Moreover, a further increase in DHA content to 220 mg per egg was achieved when a ration of *Schizochytrium* sp. equivalent to 825 mg of DHA per hen per day was provided [153].

There are a number of benefits claimed for hens fed on an algal-based diet containing *Schizochytrium* sp., including an increased DHA content in eggs and enhanced egg production, feed conversion, and mean body weight [153]. Moreover, eggs enriched with DHA from *Schizochytrium* sp. do not differ in terms of sensory characteristics from eggs produced from hens fed on a control diet and do not negatively impact consumer acceptance. Thus, it is possible to utilize enriched eggs as a food ingredient in other foods that contain eggs, for instance, egg noodles [148, 152].

In addition, research has revealed the promising results of enriching milk fat with PUFAs [154]. The milk fat of dairy cows and ewes can be modified by dietary enrichment with *Schizochytrium* sp., which results in an improved fatty acid profile while retaining acceptable flavor [150, 155]. Franklin *et al*. [155] indicated that cows fed with a diet containing 910 g day^{-1} of *Schizochytrium* sp. produced milk with PUFA and DHA contents of 6.53% and 0.46%, respectively, in milk fat as compared to only 4.03% PUFA and no DHA in milk fat in cows fed with a control diet. Similarly, ewes fed with a diet containing 52 g day^{-1} of *Schizochytrium* sp. had a content of 1.2% DHA in milk fat, but the milk fat of ewes fed with a control diet had an undetectable level of DHA [150].

Manipulating the fatty acid composition of animal meats has also been recently considered as a way to introduce ω-3 PUFAs into diets [156]. The applications of *Schizochytrium* sp. to enrich chicken and lamb meats have been documented [149, 157]. Broiler chicken fed with a

diet supplemented with 2.2% of *Schizochytrium* sp. had 2.8-fold and 4.0-fold increases in ω-3 PUFA and DHA content, respectively, and had acceptable sensory characteristics [157]. Similarly, lambs fed with a diet supplemented with 15.5% *Schizochytrium* sp. contained the highest percentage of DHA in the phospholipids and neutral lipid classes of the longissimus muscle, as compared with lambs fed with other diets without algal supplements [149].

6. UTILIZATION OF RENEWABLE RESOURCES

The development of products from the byproducts of agricultural and food industries is one of the major foci for the utilization of renewable resources throughout the globe [158–159]. Vegetable oils contain nutritious components (e.g., fatty acids) that may be used by single-cell microorganisms as organic substrates for the production of high-value products. The three most prominent fatty acids in vegetable oils are oleic acid (OA, 18:1ω-9), LA, and α-linolenic acid (ALA; 18:3 ω-3), comprising well over 70% of the total fatty acids [160]. Lali *et al*. [161] demonstrated that certain strains of thraustochytrids are able to utilize LA and ALA as their sole carbon sources for growth and DHA production. Results indicate that all of these fatty acids are able to sustain growth in the tested thraustochytrids, but DHA accumulation was observed in only three of the tested strains [161]. Of the three fatty acids, ALA was the best source for DHA accumulation, particularly with *Thraustochytrium* F3-1. The ability of thraustochytrids to utilize canola, corn and linseed oils for growth and DHA production further indicates the potential for converting vegetable oils to biomass of oleaginous thraustochytrids [82, 83].

In addition to using vegetable oils as a renewable resource for the production of thraustochytrid biomass, there is an increasing interest in the transformation and conversion of low-value raw materials into higher value products [112, 159]. One such application is the use of thraustochytrids for the transformation of food-processing wastes into valuable oils. Fan *et al*. [162] showed that *S. mangrovei* KF6 is able to utilize industrial food wastes, including bread crusts, soymilk residue (okara powder), and grain husks, for DHA production. Bread crusts fermented with *S. mangrovei* KF6 contained the highest DHA content (12.6 mg g^{-1}), followed by okara powder (7.3 mg g^{-1}) and brewing grain (6.2 mg g^{-1}). Evidence indicated that the 18-carbon fatty acids, particularly LA in the industrial food wastes, are preferentially utilized by thraustochytrids for DHA production [56, 162].

7. SAFETY ISSUES

The increasing number of commercial food products containing DHA derived from thraustochytrids (e.g., DHA enriched eggs) on the market has led to concern over the safe use of thraustochytrids [148]. It seems obvious that DHA has no apparent detrimental effects and is safe for human consumption as observed in the long history of seafood consumption throughout the world. Nevertheless, scientific studies are required to establish beyond doubt that there are no adverse effects associated with the consumption of thraustochytrids-derived oil.

Objective approaches are available to assess the safety of algal-based oils that contains DHA [163–167]. Investigators from Monsanto, Omega Tech (presently known as Martek Biosciences), and Pharmacia have jointly conducted a series of studies specifically to examine the safety of *Schizochytrium* sp. [163–167]. The common algal toxins, such as domoic acid and *Prymnesium* toxin, were not detected in *Schizochytrium* sp. [164, 165]. Furthermore, dietary administration of *Schizochytrium* sp. to Sprague-Dawley rats in subchronic (3 g kg^{-1} body weight day^{-1}), developmental toxicity evaluation (30% of diet), and single generation reproduction (30% of diet) studies indicated that there was no apparent adverse effect on the test rats [164–166]. Similarly, no adverse effects were observed in New Zealand white rabbits and growing swine fed with the biomass of *Schizochytrium* sp. at 1.8 g kg^{-1} body weight day^{-1} and 2.68 kg per pig over 120 days, respectively [163, 165]. *Schizochytrium* sp. was further confirmed not to be mutagenic in the Ames reverse mutation assay [167]. More recently, *Schizochytrium* sp. has been affirmed as Generally Recognized as Safe (GRAS) by the FDA for use in food application [168].

In addition, Kroes *et al*. [169] provided an independent and comprehensive study to test and evaluate the safe use of DHA45-oil, a food-grade oil derived from the marine microalga *Ulkenia* sp. produced in a multi-step fermentation process and containing >95% triacylglycerols and having a DHA content of 45% in the oil. The study affirmed that DHA45-oil is not genotoxic, as demonstrated in the negative results from the Ames and chromosomal aberration assays [169]. Moreover, acute (2 g oil kg^{-1} body weight), subchronic (2 g kg^{-1} body weight day^{-1}), and one-generation reproductive (7.5% of diet day^{-1}) studies also indicated that feeding Sprague-Dawley and Wistar rats with DHA45-oil did not produce significant differences in the evaluated parameters, such as mortality, neurological response, ophthalmology, gross pathology, and histopathology, between the control and DHA45-oil-fed group. This study, along with the studies on *Schizochytrium* sp., conclusively supports the safe use of thraustochytrid-derived oil as a safe dietary source of DHA.

8. CONCLUSIONS

Microalgae represent a largely under-exploited group of natural resources with a tremendous potential to produce high-value natural products. Thraustochytrids not only play an important role in the ecosystem as nutrient recyclers, but also serve as a supreme source of single cell oils with great commercial prospects. We have witnessed the application and commercialization of thraustochytrids for the production of DHA from its initial screening and medium optimization stage to the maturation stage in which the safe consumption of thraustochytrids is evaluated. Through years of dedicated scientific efforts, the commercial production of DHA using thraustochytrids as single-cell factories has now become a reality, and thraustochytrid-derived products have entered the marketplace as aquaculture feed and PUFA-fortified food for the fishery and healthcare sectors.

Thraustochytrids offer great potential for the production of high-value products. Future approaches are to explore means for maximizing DHA production and to further explore the potential of thraustochytrids to produce new products. To maximize DHA production, the use of economical substrates and genetic engineering approach are suggested. Pilot testing using

renewable resources, such as plant-based oils and food industry wastes, as cheap substrates for thraustochytrids fermentation has been preliminarily investigated. The use of renewable resources opens up new possibilities for DHA production in thraustochytrids. Nevertheless, the feasibility of these substrates for large-scale production of DHA remains to be assessed. The elucidation of DHA biosynthetic pathways in thraustochytrids using molecular techniques represents an exciting and potentially rewarding subject for future research. The knowledge acquired on DHA biosynthesis in thraustochytrids can be used in the future for genetic manipulation; for example, the construction of thraustochytrids with ultra-high DHA yields.

Thraustochytrids can be further manipulated and manifested as cell factories for the production of other high-value products, such as astaxanthin, squalene, and other valuable metabolites. The isolation and screening of new strains from rare and extreme environments represents a practically sound method for discovering new high-value products. Moreover, the cultivation conditions for the production of each new high-value product require individual optimization.

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