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Compatible solutes and docosahexaenoic acid accumulation of thraustochytrids of the *Aurantiochytrium* group

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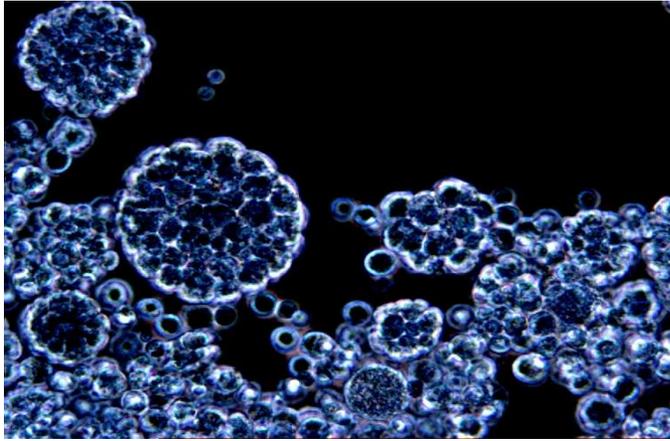
Aurantiochytrium sp. strain T66 in liquid medium

ACKNOWLEDGEMENT

SUMMARY

Docosahexaenoic acid (DHA; 22:6 n-3), a polyunsaturated fatty acid (PUFA), is linked to various health benefits in humans including cognitive and visual development of infants and reduced risk of cancer, cardiovascular diseases and mental illnesses of adults. Fish oil, with an annual production of about 600,000 tons is at present the major source of DHA. However, the production of fish oils is expected to become inadequate for supplying an expanding market within few years. Thraustochytrids are marine heterotrophic producers of PUFA-rich triacylglycerols which represent an alternative source of DHA.

The focus of this thesis has been split between a basic study of the osmolyte system of traustochytrids and work towards an understanding of their growth kinetics, effects of nutrient depletion, and lipid and DHA accumulation. Three new osmotolerant thraustochytrid isolates (T65, T66, and T67) and the previously known *Schizochytrium* sp. strain S8 (ATCC 20889) were assigned to the genus *Aurantiochytrium* based on 18S ribosomal DNA phylogeny, morphology and PUFA profiles (approximately 80% DHA). Strains T66 and S8 displayed a nearly linear increase in cellular content of endogenously synthesized (-)-*proto*-quercitol and glycine betaine with increasing osmotic strength. This represented the first demonstration of accumulation of principal compatible solutes in thraustochytrids. A less osmotolerant isolate (Thraustochytriidae sp. strain T29), which was closely phylogenetically related to *Thraustochytrium aureum* (ATCC 34304) did not accumulate glycine betaine or (-)-*proto*-quercitol, illustrating a variation in osmolyte systems and osmotolerance levels among thraustochytrids.

To study the effects of nutrient limitations, *Aurantiochytrium* sp. strain T66 was grown in batch bioreactor cultures in a defined glutamate and glycerol containing medium with various medium limitations. N and P starvation and O_2 limitation initiated lipid accumulation. N starvation alone or in combination with O_2 limitation yielded the highest lipid contents obtained in this study, i.e., 54 to 63% of cell dry weight with a corresponding cell density of 90 to 100 g l^{-1} dry biomass. The DHA-content of N starved cells was 29% of total fatty acids, while O_2 limitation increased the DHA-content up to 52%. Simultaneously, O_2 limitation abolished accumulation of monounsaturated fatty acids. We inferred that the biological explanation is that O_2 limitation hindered activity of

the O_2-dependent desaturase(s) responsible for production of monounsaturated fatty acids, and favored the O_2-independent PUFA synthase. The highest DHA-productivity observed was 93 mg l^{-1} h^{-1}, obtained during sequential N starvation and O_2 limitation. This productivity approaches the highest values previously reported for thraustochytrids, and indicates that T66 may become a candidate organism for a future large-scale microbial PUFA production process.

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Paper II

Anita N. Jakobsen, Inga M. Aasen, Kjell D. Josefsen and Arne R. Strøm
Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid *Aurantiochytrium* sp. strain T66: effects of N and P starvation and O_2 limitation. Accepted for publication in Appl. Microbiol. Biotechnol.

1 INTRODUCTION

1.1 n-3 oils and their market

There is increasing evidence that the long-chain polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA; 22:6 n-3) and eicosapentaenoic acid (EPA; 20:5 n-3) display beneficial effects for human health, ranging from prevention of cancer and cardiovascular diseases to treatment of mental illnesses (12). Furthermore, DHA is regarded essential for cognitive and visual development of fetuses and infants (63). Plant oils do not contain long-chain n-3 PUFA (see section 1.2) (54). The major source of EPA and DHA is therefore oily fish such as salmon, herring and sardines, as well as their processed oils.

Due to the beneficial effects for human health, it is an increasing trend to include n-3 oils in infant formula and for enrichment of food products such as bread, eggs, spreadable fats, milk and dairy products. It is also an increasing demand for high-quality food-grade PUFA-oils for use as pharmaceuticals and nutraceuticals. The use as pharmaceutical products includes both fish oils and individual fatty acids (12, 60).

The world's increasing demand for n-3 PUFA is mainly due to the growth of the aquaculture industry (86). As n-3 fatty acids of marine fish originate from the fish's diet, fish oil (up to 40%) has traditionally been added to farmed Atlantic salmon feed (78). In 2005, the aquaculture feed industry consumed more than half of the world's fish oil supply and by 2010 this number is expected to increase to 80 to 100% of total supply (12, 86), indicating a future inadequate supply and a possible need for alternative sources. In 2006, the global production of fish oil was 594,000 tons. In the beginning of 2007 the price reached USD 890 per ton and the price is expected to increase further (36).

1.1.1 Single cell oils as an alternative to fish oils

Oils from the fish industry are often processed from fish by-products, resulting in varying oil quality (e.g., due to rancidity). Furthermore, application of fish oil to human consumption can cause problems due to a typical fishy smell and unpleasant taste,

concerns by vegetarians, and possible contamination by heavy metals, polychlorinated biphenyls or dioxins (86, 104, 117).

Microbial oils or single cell oils, is a relatively new concept, but they are now a commercial reality and constitute alternative sources of n-3 and n-6 PUFA (97). However, only a relatively small number of eukaryotic microorganisms are capable of accumulating triacylglycerols rich in C20-C22 PUFA. Yeasts were the first such microorganisms described (100), but PUFA-producing species have since been identified among marine microalgae (26, 109), thraustochytrids (17, 22, 126) and fungi (24, 116 and references therein). The PUFA profile and the productivity of these microorganisms depend on the selected strain and the growth conditions. The microorganisms are considered to be oleaginous if they accumulate lipid to a level of above 20-25% of their cell dry weight (97, 99).

Certain marine bacteria have PUFA-containing phospholipids in their cell membrane, but the PUFA account for only a small part of the cell dry weight (12, 87). Bacteria that accumulate triacylglycerols are rare, but examples are found among the actinomycetes group, particularly species of *Mycobacterium, Nocardia, Rhodococcus and Streptomyces* (5, 125). Triacylglycerols from these bacteria are composed of even- and odd-numbered saturated and monounsaturated fatty acids and branched fatty acids (4, 5). For these lipids to become suitable for nutritional purposes metabolic engineering will be required to alter their fatty acid profile.

The main focus in the single cell oil research has so far been γ-linolenic acid (GLA; 18:3 n-6), arachidonic acid (AA; 20:4 n-6), DHA and EPA (for reviews confer references 23, 26, 43, 100, 116). Several genera of the fungi *Mortierella, Mucor* and *Cunninghamella* have been identified to produce GLA at concentrations of 15-25% of total fatty acids (116). In fermentations with a mutant of *Mortierella ramannia*, a biomass concentration above 50 g l^{-1} and a GLA concentration above 5 g l^{-1} have been achieved (49). Several species of *Mortierella* are producers of AA. Hwang et al. (53) developed a fed-batch process for high-level production of AA by *M. alpina*, achieving 45.8 g lipid l^{-1} and 18.8 g AA l^{-1} after 12.5 days of cultivation. *M. alpina* strains are used in industrial AA production by Martek Biosciences Corporation, USA (7). Several heterotrophic algae

produce EPA and a productivity of 0.175 g l^{-1} h^{-1} has been obtained by using the diatom *Nitzschia laevis* (121 and references therein). EPA has been found in a wide variety of autophototrophic marine microalgae; however the productivities are low due to low specific growth rates and low cell densities in the cultures (116, 121).

A variety of microorganisms have been evaluated as DHA producers (105). The dinoflagellate *Crypthecodinium cohnii* and the thraustochytrid *Schizochytrium* sp. are heterotrophic marine microorganisms used for commercial production of DHA by Martek Biosciences Corporation, USA (7). Both types of organisms accumulate large amounts of triacylglycerols. DHA is the only PUFA represented in oil from *C. cohnii* (104), whereas the PUFA profile of thraustochytrids varies between different genera/strains. DHA is however, the dominant PUFA in most cases (22, 52). Commercially, oil from *C. cohnii* is used mainly in infant formula and food, and oil from thraustochytrids is used in the adult nutritional market (97, 116). Dried products of thraustochytrids have also been commercialized as feed for rotifers used in marine aquaculture and as feed supplement to egg-laying hens (12). It is believed that thraustochytrids have the potential to become important production organisms for the commercial DHA market (12, 68). The exploitation of *C. cohnii* and thraustochytrids for production of DHA is further considered in section 1.7.

1.2   Nomenclature of fatty acids

Fatty acids are composed of a hydrocarbon chain and a terminal carboxyl group. Most naturally occurring fatty acids (Table 1) consist of an even number of carbon atoms (4 to 24) and they may have various degrees of unsaturation (0 to 6 double bonds). The configuration of most double bonds in naturally occurring fatty acids is *cis*. Fatty acids are described in short by x:y, where x represent the number of carbon atoms and y the number of double bonds. Unsaturated fatty acids are in addition classified according to the position of the first double bond: n or ω as counted from the methyl terminus or Δ if counted from the carboxyl terminus. For example, Δ4, Δ7, Δ10, Δ13, Δ16, Δ19 22:6 denotes a fatty acid composed of 22 carbon atoms with 6 double bonds at position 4, 7, 10, 13, 16 and 19 as counted from the carboxyl terminus. As counted from the methyl

terminus this fatty acid has the first double bond at position 3 and it is therefore an n-3 or ω-3 fatty acid (Figure 1).
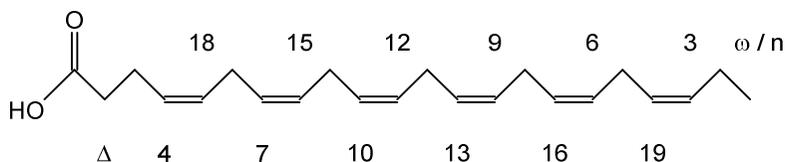
Figure 1. The structure of docosahexaenoic acid (DHA; 22:6 n-3). The fatty acid has the first double bond at carbon 3 as counted from the methyl terminus and is therefore an n-3 or ω-3 fatty acid. Alternatively, if counted from the carboxyl terminus, the fatty acid is denoted Δ4, Δ7, Δ10, Δ13, Δ16, Δ19 22:6.

Table 1. Common, systematic and short names of selected fatty acids (Based on 25, 85).

Common name	Systematic name [a]	Short name
Saturated fatty acids		
Palmitic acid	Hexadecanoic acid	16:0
Stearic acid	Octadecanoic acid	18:0
Monounsaturated fatty acids		
Palmitoleic acid	9-Hexadecenoic acid	16:1 n-7
Oleic acid	9-Octadecenoic acid	18:1 n-9
n-6 Polyunsaturated fatty acids		
Linoleic acid (LA)	9,12-Octadecadienoic acid	18:2 n-6
γ-linolenic acid (GLA)	6,9,12-Octadecatrienoic acid	18:3 n-6
Arachidonic acid (AA)	5,8,11,14-Eicosatetraenoic acid	20:4 n-6
Docosapentaenoic acid (DPA)	4,7,10,13,16-Docosapentaenoic acid	22:5 n-6
n-3 Polyunsaturated fatty acid		
α-linolenic acid (ALA)	9,12,15-Octadecatrienoic acid	18:3 n-3
Eicosapentaenoic acid (EPA)	5,8,11,14,17-Eicosapentaenoic acid	20:5 n-3
Docosahexaenoic acid (DHA)	4,7,10,13,16,19-Docosahexaenoic acid	22:6 n-3

a) All double bonds are in *cis*-configuration

Polyunsaturated fatty acids are composed of 18 or more carbon atoms and have two or more double bonds in the carbon chain. PUFA can be classified into two major groups;

the n-6 and the n-3 family. Several PUFA are listed in Table 1 and the syntheses of the two major groups are illustrated in Figure 4.

1.3   PUFA in human nutrition

PUFA are essential components in higher eukaryotes (12). PUFA of both the n-6 and n-3 families are important structural components of phospholipids in cell membranes of animal tissues, where they affect important functions such as membrane fluidity, flexibility and permeability (82).

The position of the double bond in the PUFA strongly affects the properties of its derivates. Linoleic acid (LA; 18:2 n-6, precursor of the n-6 family) and α-linolenic acid (ALA; 18:3 n-3, precursor of the n-3 family) are the parent essential fatty acids for humans. The term essential implies that humans are not able to synthesize these fatty acids in sufficient amounts; and they are therefore indispensable components of our diet (83). LA and ALA compete for the rate-limiting Δ6-desaturase (Figure 4). The resulting PUFA AA, dihomo-gamma-linolenic acid (20:3 n-6) and EPA are precursors of eicosanoids (12, 43, 82). Eicosanoids are signal molecules containing 20 carbon atoms. The eicosanoids derived from n-6 PUFA have strong inflammatory properties; whereas those produced from n-3 PUFA have anti-inflammatory properties (43, 82). An imbalance in intake of n-6 and n-3 PUFA are linked to many diseases including cancer, cardiovascular diseases and mental illnesses (12 and references therein). It appears to be important to sustain a balanced dietary intake between n-6 and n-3 PUFA to ensure homeostasis and normal development. In the literature and in recommendations by several scientific authorities, the optimal ratio of n-6/n-3 PUFA varies from 1/1 to 4/1 (12 and references therein).

DHA and AA are major structural components of the central nervous system, and are important for foetal growth and development of infants (63). DHA comprises up to 60% of the fatty acids of the retina and 15-20% of the cerebral cortex. Infants are capable of converting LA into AA and ALA into DHA, but the rates of these endogenous syntheses are considered to be too low to keep up to the demand (63, 82, 83). Evidence for potential

benefits of dietary supplement of PUFA during pregnancy, lactation and early childhood are promising, but not yet conclusive (32, 63, 73, 83).

1.4 General characteristics and classification of Thraustochytrids

Thraustochytrids are unicellular eukaryotic protists commonly found in coastal and oceanic waters and sediments (95, 96). Depending on the nutrient conditions, the number of thraustochytrids in the water column varies from below detection level to 10^6 cells l^{-1} (95 and references therein). Dense populations of thraustochytrids have been detected even at depth of 2000 meter (96) and at their maxima their biomass concentration in the water column can be similar to that of bacteria (95). Due to their high content of triacylglycerols rich in PUFA, particularly DHA, they are believed to be important primary producers of PUFA for the marine food chains (95).

1.4.1 Morphological features of thraustochytrids are the basis of classic taxonomy

In classic taxonomy based on morphology, the thraustochytrids have been characterized by the presence of an ectoplasmic net (Figure 2) (except for the genus *Althornia*) produced by a unique organelle termed the sagenogenetosome (sagenogen) or bothrosome. The elements of the ectoplasmic net form a branched network of plasma membrane extensions. Thus, it contributes to increase the cell surface area and help to absorb nutrients and to attach vegetative cells to solid substrates. When grown in rich liquid media the ectoplasmatic net is mostly absent (95). Thraustochytrids produce extracellular enzymes (19), often secreted from the ectoplasmic net (95), that are capable of breaking down several complex organic compounds (19). They are believed to play an important role in mineralization of organic material such as plant, algae and phytoplankton detritus (95, 96). In their amoeboid cell form, they can feed on bacteria (94).
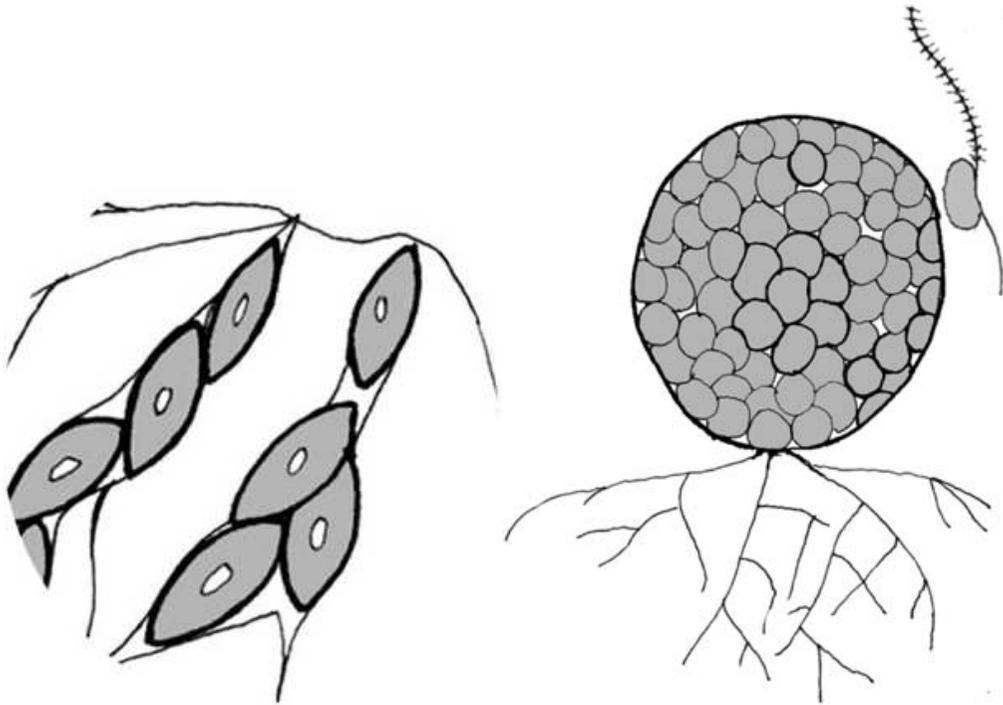
Figure 2. Illustrations of the two groups of Labyrinthulomycetes[1]. Left: Labyrinthulids, represented by *Labyrinthula* sp. with a spindle-shaped vegetative cells enrobed by the ectoplasmic net. Right: Thraustochytrids, represented by a non-proliferous *Thraustochytrium* sp. with unilateral non-motile ectoplasmic net.

In the vegetative stages, thraustochytrids are single cells with a diameter of 4 to 20 μm (95). The cell wall is composed of sulfated polysaccharides and proteins. Most thraustochytrids reproduce by means of biflagellate, heterokont zoospores. Historically, the mode of zoospore production forms the major taxonomic criterion to distinguish the different genera of thraustochytrids. Species of the genus *Thraustochytrium* have been characterized by their globose sporangia, with or without proliferous bodies (a part of cytoplasm that remains behind after release of zoospores). They divide directly into zoospores caused by partial dissolution of the cell wall. Strains assigned to the genus *Schizochytrium* undergo repeated binary division of vegetative cells, resulting in a cluster of cells, each of which develops into a zoosporangium or zoospores. The genus *Ulkenia*

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[1] This illustration was published in (65). Copyright Elsevier (2004). Reproduced with permission from Elsevier GmbH.

has been described by its release of amoeboid cells before forming sporangia (95, 131 and references therein). Several authors have listed numerous morphological features useful to characterize and distinguish thraustochytrids; such as the presence of amoeboid cells or proliferous bodies, repeated binary cell division, the number and size of zoospores and the condition of the cell wall after zoospore release (35, 51, 131).

1.4.2 Classification of thraustochytrids based on 18S ribosomal DNA taxonomy and PUFA profiles

Adl et al. (2) rank thraustochytrids under Labyrinthulomycetes which again are ranked under Stramenopiles (Chromista). Labyrinthulomycetes are divided into two groups: Thraustochytriaceae (thraustochytrids) comprising seven genera and Labyrinthulaceae (labyrinthulids) with only one genus, *Labyrinthula*. The two groups are separated based on their 18S ribosomal RNA gene sequences. The 18S ribosomal RNA genes of thraustochytrids carry a variable insert of approximately 14 to 28 bp which is reported to be absent in all other organisms, including the Labyrinthulaceae (51). Labyrinthulids are morphologically distinguished from thraustochytrids by their spindle-shaped cell bodies which are enrobed by and glide through the ectoplasmic net (95) (Figure 2). The seven genera of Thraustochytriaceae listed by Adl et al. (2) are: *Thraustochytrium*, *Schizochytrium*, *Ulkenia*, *Althornia*, *Elnia*, *Japonochytrium*, and *Aplanochytrium* (2). *Aplanochytrium* has by others been ranked directly under Labyrinthulomycetes (64).

However, the genus-level classification of thraustochytrids is problematic due to a poor correlation between the classic taxonomy based on morphological features and the taxonomy based on molecular phylogenetic analyses (51, 52, 131). Recently, thraustochytrids have been divided into two subgroups based on molecular 18S ribosomal DNA taxonomy (61). Huang et al. (52) found five defined PUFA profiles among thraustochytrids isolated from the coast of Japan and Fiji (Profile type A to E of Table 2). Because strains with the same PUFA profile clustered on the same 18S ribosomal DNA–based phylogenetic branch they proposed to use PUFA profiling as criterion for grouping of thraustochytrids.

Table 2. PUFA profiles of thraustochytrids isolated from coastal water of Japan and Fiji (52).

Profile type	PUFA [a]
A	DHA, DPA
B	DHA, DPA, EPA
C	DHA, EPA
D	DHA, DPA, EPA, AA
E	DHA, DPA, EPA, AA, DTA

a) Abbreviations: DHA, docosahexaenoic acid (22:6 n-3); DPA, docosapentaenoic acid (22:5 n-6); EPA, eicosapentaenoic acid (20:5 n-3); AA, arachidonic acid (20:4 n-6) and DTA, docosatetraenoic acid (22:4 n-6).

Burja et al. (22) screened thraustochytrid isolates from 19 different collection sites throughout Atlantic Canada and determined four C20-C22 PUFA profiles. Three were identical to the profiles described by Huang et al. (52), namely type B, C and D. PUFA profile of type D was the dominant profile, comprising 74% of the isolates. The fourth profile consisting of DHA, EPA and AA, was observed for 5.6% of the isolates. It is noteworthy that most thraustochytrids accumulate docosapentaenoic acid (DPA; 22:5 n-6) (constitutes up to 10% of the total fatty acids) which otherwise is a minor PUFA in nature (61)**.**

Yokoyama and Honda (131) have recently evaluated the classification of the *Schizochytrium*–like thraustochytrids and proposed a taxonomic rearrangement of the genus *Schizochytrium*. The rearrangement appears appropriate since species assigned to *Schizochytrium* occur in three separate lineages based on 18S ribosomal DNA phylogeny (51, 52, 131). Apparently, the characteristic successive binary division of vegetative cells has been gained in several lineages (131). Based on a combination of morphology, 18S ribosomal DNA taxonomy and PUFA and carotenoid profiles, the genus *Schizochytrium* has been proposed divided into three genera: *Schizochytrium* sensu stricto, *Aurantiochytrium* gen. nov., and *Oblongichytrium* gen. nov. Strains belonging to the monophyletic group of *Aurantiochytrium* are characterized by a PUFA profile composed of approximately 80% DHA and less than 5% AA, accumulation of astaxanthin and growth phase cells that do not form large colonies or develop ectoplasmic net elements. Characteristics of the genera *Schizochytrium* sensu stricto and *Oblongichytrium*, have

been discussed by Yokayama and Honda (131). These authors pointed out that further taxonomic rearrangements of thraustochytrid genera may be necessary.

In this thesis, strains reported to be members of the genus *Aurantiochytrium* by Yokoyama and Honda (131) are referred to as *Aurantiochytrium* regardless of their original genus assignments.

1.5   The function of lipids in eukaryotic microorganisms

Lipids have two major physiological roles in eukaryotic microorganisms; as components of cell membranes and as storage material. The depot fat serves as a source of building blocks of lipophilic components and as a reserve of carbon and energy (29, 133).

1.5.1 Structural lipids

Phospholipids, sterols and glycolipids are essential structural and functional components of eukaryotic cell membranes (11, 12)**.** These lipids are amphipathic, which make them ideal as building blocks of the lipid bilayers that form the boundaries of all living cells. Eukaryotic cells, as distinct from prokaryotic cells, typically possess intracellular organelles and nucleus enclosed by lipid membranes. In addition to the lipid bilayer, biological membranes contain proteins and carbohydrates (11)**.**

Phospholipids are the major class of membrane lipids and are abundant in all biological membranes. A phospholipid is composed of fatty acid(s) attached to a glycerol or a sphingosine molecule which further binds a phosphate group attached to an alcohol such as ethanolamine, serine choline, inositol or glycerol. The fatty acids represent the hydrophobic interior of the membranes, while phosphate and groups linked to it are hydrophilic and are oriented towards the water phases at both sides of the membrane. Phospholipids derived from glycerol are called phosphoglycerids (11).

Steroids are lipids built from four linked hydrocarbon rings. A hydrocarbon tail is attached at one end of the molecule and a hydroxyl group is linked at the other end. Sterols are rigid, planar molecules and their presence stabilizes the cell membrane (71).

Eukaryotic microorganisms produce a variety of sterols as reviewed by Volkman (115). Ergosterol is typically found in cell membranes of yeasts and fungi (132). Studies have demonstrated that cholesterol is the dominant sterol in two *Thraustochytrium* species (69, 118).

1.5.2 Depot fat

Several yeasts (29), marine microalgae (109) and protists (126) accumulate triacylglycerols and/or steryl esters as storage materials (80, 133). Triacylglycerols are composed of three fatty acids linked to a glycerol molecule. Triacylglycerols are highly reduced and are therefore concentrated storages of metabolic energy (11). Steryl esters are particularly found in non-oleaginous yeasts with ergosterol as the main sterol (29). The depot lipids are stored in cytosolic lipid bodies (8, 79, 80, 118, 133). Lipid bodies contain a hydrophobic matrix of neutral lipids which is surrounded by a monolayer of phospholipids, glycolipids and/or sterols and a small amount of proteins (29, 80, 133). The proteins are proposed to have a structural role, maintaining the integrity of the lipid body and avoiding coalescence with neighboring lipid bodies (100). In eukaryotes, lipid bodies are assumed to arise from the endoplasmic reticulum (81). In for instance yeasts and microalgae, accumulation of lipid bodies seems to be induced by environmental stress such as N limitation or osmotic stress (80). Cytosolic lipid bodies vary in size, composition and numbers. In yeasts, the diameter of the majority of lipid bodies is 0.3-0.4 µm (80). In the thraustochytrid strain *A. limacinum* SR21, lipid bodies with diameter less than 1 µm are reported for zoospores. The lipid bodies increase in both size (1 to 3 µm) and numbers in vegetative cells as they grow (79). Studies imply that lipid bodies are not only an inert storage of lipid, but they also play a role in biosynthesis, mobilization and trafficking of intracellular neutral lipids (80, 81).

1.6   Fatty acid biosynthesis

1.6.1 Fatty acid synthase

Fatty acid biosynthesis is usually carried out by the ubiquitous fatty acid synthase (FAS) system with palmitic acid (16:0) or stearic acid (18:0) as the major end product, but the chain length may vary, depending on the particular organism and its FAS system (103).

The principal reaction mechanism of FAS is essentially the same in all organisms (Table 3); although the FAS variants may be divided into two classes. In the type II system of most bacteria, protozoa and eukaryotic organelles of prokaryotic descent (chloroplasts and mitochondria), the FAS enzymes, including the acyl carrier protein (ACP), are localized on separate proteins which are encoded by a series of separate genes. The system is therefore also termed "dissociated FAS" (90, 103). In contrast, the FAS enzymes of the type I system are highly integrated multienzymes which consist of a single (animal FAS) or two (fungal FAS) large polypeptide(s) composed of several distinct functional domains. The type I FAS are particularly found in the eukaryotic cytoplasm, but a prokaryotic exception is found among a subgroup of the *Actionomycetales* (103 and references therein).

The first step in fatty acid synthesis (Table 3) is the carboxylation of acetyl-CoA to malonyl-CoA, an ATP-dependent irreversible reaction that is catalysed by acetyl-CoA carboxylase. Acetyl transacylase catalyses the transfer of the acetyl-group of acetyl-CoA to an acyl carrier protein (ACP), creating acetyl-ACP (Step 2). Similarly, malonyl transacylase catalyses the transfer of the malonyl–group of malonyl-CoA to ACP, creating malonyl-ACP (Step 3). Fatty acids with an odd number of carbons are synthesized starting with propionyl-ACP (instead of acetyl-ACP), which is formed from propionyl-CoA by acetyl transacylase. Acetyl-malonyl-ACP condensing enzyme catalyses the condensation reaction of acetyl-ACP and malonyl-ACP into acetoacetyl-ACP (step 4). The CO_2 involved in the first step of the fatty acid synthesis is released in this condensation reaction, thus all carbon atoms of fatty acids with an even number of carbons are derived from acetyl-CoA. In the three subsequent reactions, acetoacetyl-ACP is reduced to butyryl-ACP by a reduction (step 5), a dehydration (step 6) and second reduction (step 7). The reductant in fatty acid synthesis is NADPH.

Table 3. Principal reactions in fatty acid synthesis (11).

Step	Reaction	Enzyme
1	Acetyl-CoA + HCO_3^- + ATP → Malonyl-CoA + ADP + P_i +H^+	Acetyl-CoA carboxylase
2	Acetyl-CoA + ACP ↔ Acetyl-ACP + CoA	Acetyl transacylase
3	Malonyl-CoA + ACP ↔ Malonyl-ACP + CoA	Malonyl transacylase
4	Acetyl-ACP + Malonyl-ACP → Acetoacetyl-ACP + ACP + CO_2	Acyl-malonyl-ACP condensing enzyme
5	Acetoacetyl-ACP + NADPH + H^+ ↔ D-3-Hydroxybutyryl-ACP + $NADP^+$	β-Ketoacyl-ACP reductase
6	D-3-hydroxybutyryl-ACP ↔ Crotonyl-ACP + H_2O	3-Hydroxyacyl-ACP dehydratase
7	Crotonyl-ACP + NADPH + H^+ → Butyryl-ACP+ $NADP^+$	Enoyl-ACP reductase

The formation of butyryl-ACP completes the first elongation cycle. The growing fatty acid chain is elongated by the sequential addition of two-carbon units derived from acetyl-CoA. The activated donor of the two-carbon units is malonyl-ACP. The elongation process continues until the acyl chain reaches 14 to 18 C-atoms. The enzyme acyl-ACP thioesterase then removes ACP from the fatty acids, and thus determines the chain length of the fatty acid. Overall stoichiometry for the synthesis of palmitic acid is:

$$8 \text{ Acetyl-CoA} + 7 \text{ ATP} + 14 \text{ NADPH} + 6 \text{ H}^+$$

$$\rightarrow \text{palmitic acid} + 14 \text{ NADP}^+ + 8 \text{ CoA} + 6 \text{ H}_2\text{O} + 7 \text{ ADP} + 7 \text{ P}_i$$
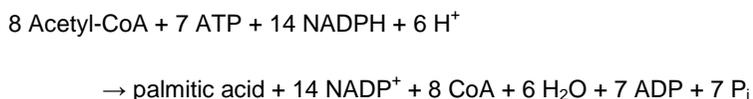
The synthesis of fatty acids in eukaryotic cells takes place in the cytoplasm with acetyl-CoA as the basic building block. However, acetyl-CoA is primarily found in the mitochondrion, formed from pyruvate, the end product of the glycolysis. Hence, acetyl-CoA must be transferred to the cytoplasm, but the mitochondrial membrane is relatively impermeable to acetyl-CoA (11, 97). How oil-accumulating microorganisms supply the cytoplasm with sufficient acetyl-CoA and NADPH is discussed in section 1.6.4.

1.6.2 The conventional aerobe desaturase/elongase route for biosynthesis of PUFA

In most eukaryotes, synthesis of PUFA occurs by the conventional aerobic route catalyzed by sequential desaturation and elongations reactions of fatty acids at the cytoplasmic face of the endoplasmic reticulum. Double bonds are introduced to the fatty acid chain by an O_2-dependent desaturase system composed of at least three types of enzymes: NAD(P)H-cytochrome b_5 reductase, cytochrome b_5 and the terminal fatty acid desaturase as illustrated in Figure 3 (11, 23, 116). The various desaturases are named according to the position at which they introduce the double bond in the fatty acyl chain, counted from the carboxyl terminus. Most of these enzymes are membrane-bound, but desaturases can be soluble (114).
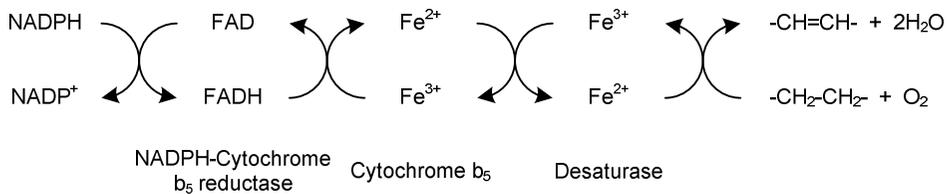
Figure 3. Formation of unsaturated fatty acids by the aerobic desaturation complex. Modified from Ratledge (98).

In the aerobic pathway, the first double bond is introduced by the $\Delta9$-desaturase in the $\Delta9$ position of palmitic acid (16:0) forming palmitoleic acid (16:1, n-7) or in the $\Delta9$ position of stearic acid (18:0), forming oleic acid (18:1 n-9). Those are the most common monounsaturated fatty acids in microorganisms (23). Oleic acid is then further desaturated by $\Delta12$ desaturase to yield linoleic acid (LA; 18:2 n-6) which is the precursor for the PUFA n-6 family. LA can be converted to α-linolenic acid (ALA; 18:3 n-3), the precursor of the PUFA n-3 family. The latter conversion is catalysed by $\Delta15$ desaturase. Mammals lack the $\Delta12$ desaturase and $\Delta15$ desaturase enzymes, thus LA and ALA are considered as dietary essential fatty acids for humans (Figure 4) (67).

Elongation of fatty acids involves the same four steps as seen for fatty acid synthesis by the FAS system (Table 3): condensation of a long-chain acetyl-primer to malonyl-CoA, reduction of the γ-keto group, dehydration and a second reduction, resulting in elongation of the fatty acid with a two carbon unit. The elongases are essential multiunit membrane-associated enzymes for synthesis of PUFA (116, 117). The formation of DHA from EPA occurs via two different mechanisms in the aerobic pathway of eukaryotes. Identification of Δ4 desaturase (93) in lower eukaryotes like *Thraustochytrium* sp. indicates that n-3 DPA can be directly converted to DHA (Figure 4). This enzyme has not been detected in higher eukaryotes like mammals. The synthesis of DHA from EPA in mammals has been reported to follow the "Sprecher" pathway: elongation of n-3 DPA to tetracosapentaenoic acid (24:5 n-3) followed by a Δ6 desaturation and an oxidation of tetracosahexaenoic acid (24:6 n-3) to form DHA (see Figure 4) (67, 117).

1.6.3 Anaerobe route for biosynthesis of PUFA

The existence of an anaerobic pathway for PUFA synthesis in bacteria was previously known, but poorly characterized until Metz and colleagues (76) described a novel, polyketid synthase-like (PKS) system in 2001. A cluster of five genes (designated *pfa*A, *pfa*B, *pfa*C, *pfa*D, and *pfa*E) is reported to be responsible for EPA production in *Shewanella* sp. strain SCRC-2738. Four of these genes represent subunits of an enzyme complex capable of EPA synthesis (88 and references therein). This PUFA synthase enzyme possesses multiple domains, eight of which are strongly related to PKS proteins, whereas three are homologs of bacterial FAS (76). The fifth gene encodes a phosphopantetheinyl transferase that activates the acyl carrier protein (ACP) domain (88 and references therein). Expression of the gene cluster in *E. coli* under anaerobic conditions resulted in EPA production, demonstrating that its synthesis is independent of the "conventional" aerobic desaturase/elongase pathway (76). The PUFA synthase complex is termed anaerobic because double bonds are introduced to the fatty acid chain by an O_2-independent mechanism. However, the term does not imply that this pathway only occurs in anaerobic organisms, the organisms which display anaerobic PUFA synthesis are in fact obligate aerobes (97).
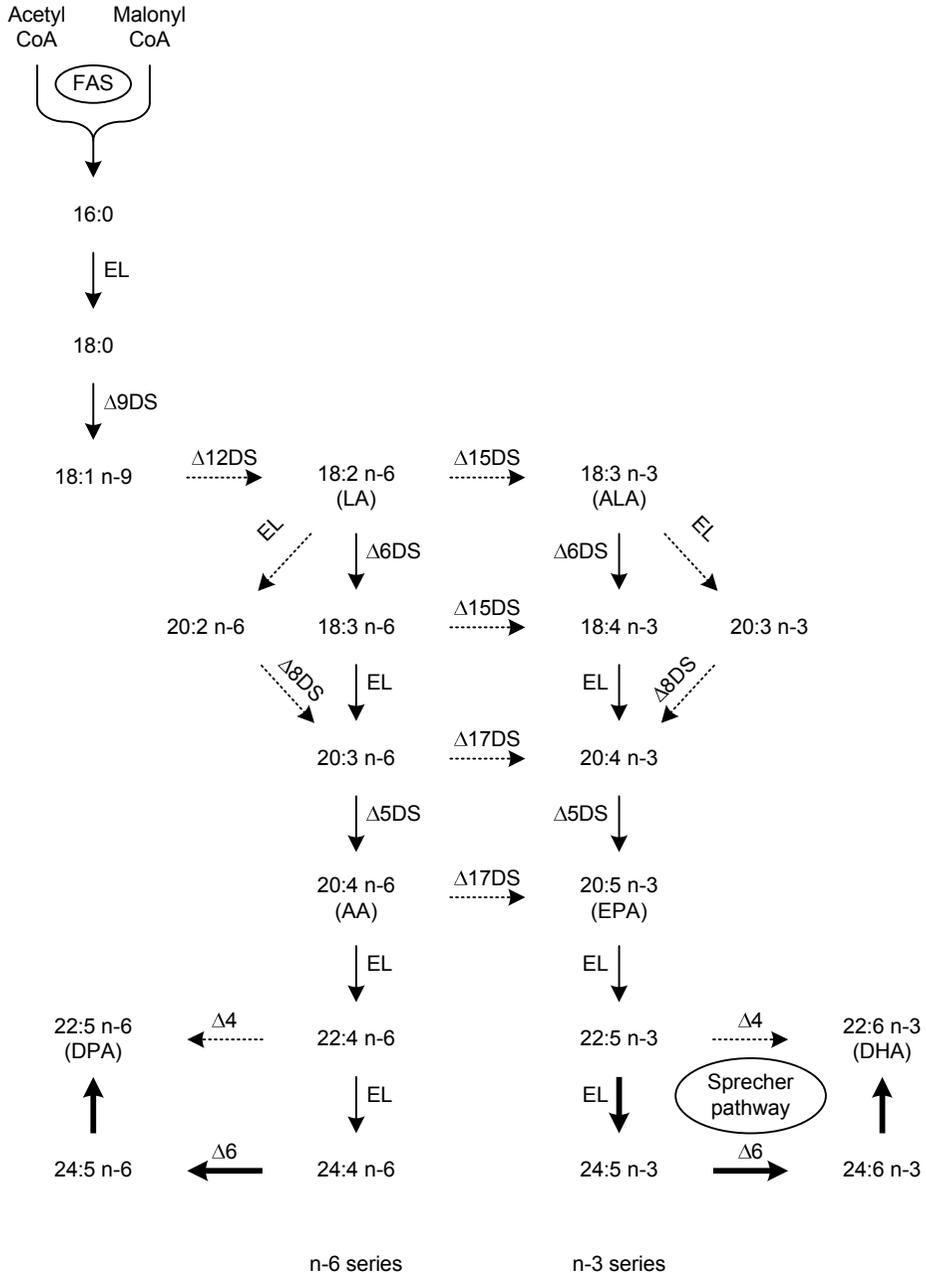
Figure 4. Biosynthesis of polyunsaturated fatty acids by the conventional aerobic route. Enzymatic reactions represented by solid line arrows ($\longrightarrow$) are found in mammals and lower eukaryotes, those represented by dotted line arrows ($\cdots\cdots\blacktriangleright$) are exclusively reported in lower eukaryotes and those represented by heavy weight arrows ($\blacktriangleright$) are exclusive for mammals (based on 12, 67, 97, 117). The abbreviations DS and EL indicate reactions catalyzed by desaturases and elongases, respectively. The common names of several fatty acids are indicated (see Table 1).

Genes with homology to the *pfa* genes have been cloned from various EPA- and DHA-producing marine bacteria, indicating that most, or all, PUFA producing bacteria utilize this PUFA synthase complex (48, 88). Among eukaryotes, such PUFA synthase appears to be unique to thraustochytrids. It has therefore been suggested that *Schizochytrium* has obtained the PUFA synthase genes from bacteria by lateral gene transfer (48, 76). Recently, three genes encoding the PUFA synthase of *Schizochytrium* sp. was expressed in *E. coli*, together with a gene for phosphopantetheinyl transferase. Accumulation of both DPA and DHA confirmed that production of both fatty acids occur by the PUFA synthase complex (48). Synthesis of short-chain saturated fatty acids (14:0 and 16:0) in *Schizochytrium* sp. has recently been proposed to occur by a separate FAS system of type I which resembles a fusion of the two fungal FAS subunits (48). Thus, the combined action of the PUFA and fatty acid synthases can account for the simple fatty acid profile of *Schizochytrium* sp. (48) which lacks the 18-, 20-, and 22-carbon intermediates seen in eukaryotes that synthesize PUFA by an desaturase/elongase pathway.

The PUFA synthase complex carries out the same basic reactions as FAS. It uses the same acyl carrier protein (ACP) as a covalent attachment site for the growing carbon chain and acetyl-CoA and malonyl-CoA are the essential building blocks (97, 116). There is, however, one significant difference; whereas all enzyme activities in FAS are utilized repeatedly until the desired chain length is reached, the PUFA synthase complex can omit steps resulting in intermediates with many keto, hydroxyl and carbon double bonds (97, 116). An isomeriase activity can determine the location and configuration of the double bonds of the PUFA. The exact mechanism of DHA synthesis by the PKS route remains to be defined.

There is a disagreement in the literature whether all thraustochytrids utilize the PUFA synthase complex for synthesis of PUFA (92, 97). As previously mentioned, many thraustochytrids have a rather complex PUFA profile (section 1.4.2) and an aerobic desaturase/elongase pathway for synthesis of DHA has been proposed for *Thraustochytrium* sp. (92). A key enzyme in this pathway is a Δ4 desaturase that catalyzes the synthesis of DHA from 22:5 (n-3) fatty acid and also n-6 DPA from 22:4 (n-6) fatty acid (93). Additionally, a Δ6 elongase from *Thraustochytrium* sp. has been

described that shows activity against both C18 and C20 fatty acids, but prefers the shorter ones (124). Thus, different pathways for synthesis of PUFA may exist in thraustochytrids.

1.6.4 Regulation of lipid accumulation in oleaginous yeasts and fungi

Accumulation of storage lipid in oleaginous filamentous fungi and yeasts is generally induced by a nutrient imbalance in the culture medium (97). Thus, batch fermentations for lipid production can be considered as a biphasic process, where the first phase involves biomass production and the second involves lipid accumulation with excess carbon and limited amount of N (or another key nutrient) (66, 100). Non-oleaginous yeasts do not accumulate lipid even when they are placed in the same N-limited growth medium. However, the biosynthesis of lipids in both oleaginous and non-oleaginous species occurs by the same biosynthetic pathway. Ratledge (97) has reviewed the possible reasons for oleaginicity to be twofold, i.e., the ability to supply sufficient amounts of the precursor molecules of fatty acid biosynthesis acetyl-CoA and NADPH to the cell cytoplasm.

Ratledge (97) suggested that N-limited growth of oleaginous yeasts and fungi induces a cascade of reactions (Figure 5) which lead to a continuous supply of cytosolic acetyl-CoA:

- N exhaustion induces increased AMP deaminase activity in order to release extra ammonium ions (reaction catalyzed by AMP deaminase: AMP→inosine 5'-monophosphate + NH_3).
- Increased AMP deaminase activity decreases the cellular content of AMP.
- The activity of isocitrate dehydrogenase (enzyme of the tricarboxylic acid cycle) is inhibited because its activity is strictly dependent on AMP in oleaginous species (no such dependency occurs with the enzyme from non-oleaginous species).
- Thus, isocitrate accumulates in the mitochondrion and is readily equilibrated with citrate (via aconitase).
- Citrate is transported across the mitochondrial membrane to the cytoplasm (in exchange for malate) where it is cleaved by ATP:citrate lyase to form acetyl-CoA

and oxaloacetate at the expense of one ATP. This reaction does not occur in the majority of non-oleaginous species.

- Oxaloacetate is converted to malate, which re-enters the mitochondrion.
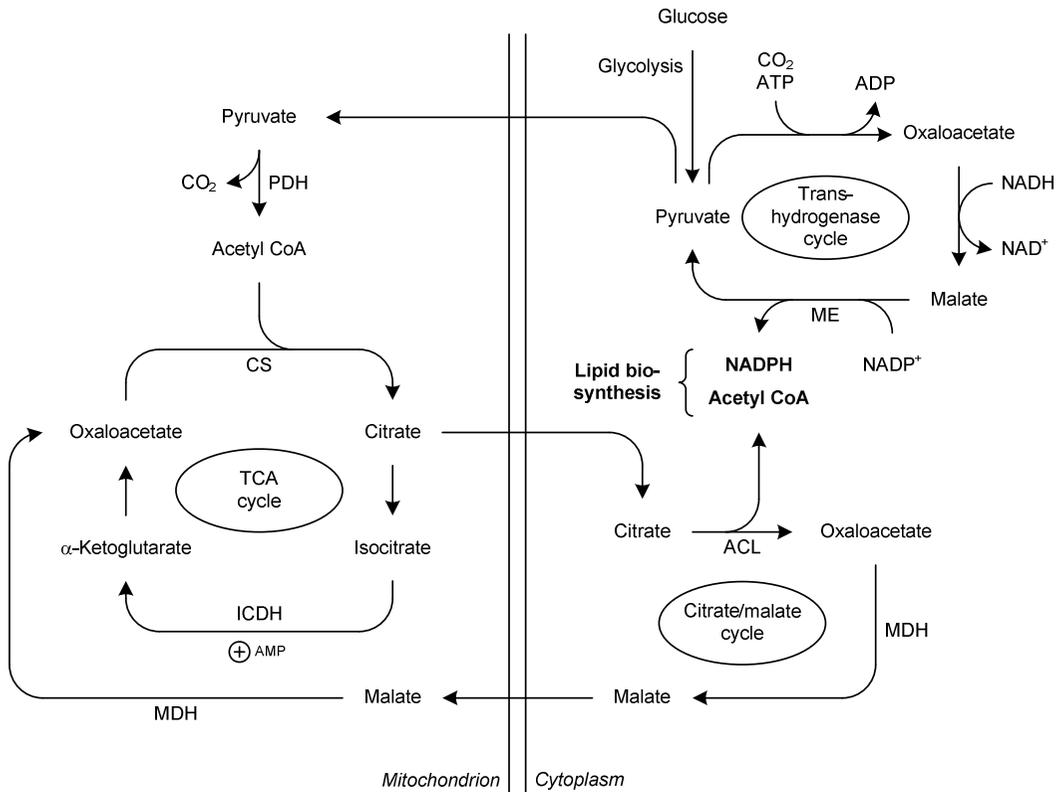
Figure 5. A suggested model for how oil accumulating filamentous fungi supply their cytoplasm with sufficient precursors of acetyl-CoA and NADPH for lipid biosynthesis (modified from 97). Key enzymes are: ME, malic enzyme; ACL, ATP:citrate lyase; MDH, malate dehydrogenase; ICDH, isocitrate dehydrogenase; PDH, pyruvate dehydrogenase; CS, citrate synthase. Abbreviation: TCA cycle, tricarboxylic acid cycle.

In most oleaginous species the majority of NADPH is considered to be provided be the following reaction catalyzed by the malic enzyme (Figure 5) (97):

$$\text{Malate} + NADP^+ \rightarrow \text{pyruvate} + CO_2 + NADPH.$$
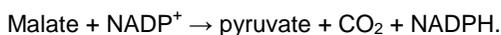
Ratledge and Wynn (100) hypothesized that the malic enzyme, the ATP:citate lyase and FAS combine in a complex to ensure a direct channeling of acetyl-CoA into fatty acids

which are further esterified to triacylglycerols and incorporated via the endoplasmic reticulum into lipid droplets.

1.7   Microbial heterotrophic production of DHA

The dinoflagellate *C. cohnii* and species of thraustochytrids are heterotrophic microorganisms used for commercial production of DHA (97). *C. cohnii* accumulates up to 60% (of dry biomass) triacylglycerols with DHA (25 to 60% of total fatty acids) as the only PUFA present (104). The highest volumetric productivity of DHA reported for this alga is 53 mg DHA $l^{-1}h^{-1}$, obtained in fed-batch cultivation with pure ethanol as feed. The cultivation resulted in a production of 83 g dry biomass l^{-1}, 35 g lipid l^{-1} and 11.7 g DHA l^{-1} in 220 h (Table 4).

Strains of thraustochytrids have been reported to accumulate large amounts of triacylglycerols (e.g., 50 to 80% of dry biomass) with a high fraction of PUFA. DHA commonly account for 20-40% of total fatty acids (12). Depending on the strain, thraustochytrids accumulate one or several other PUFA (section 1.4.2). Lipid and DHA production by thraustochytrids is further considered in section 1.7.1. Examples of fatty acid profiles of some selected thraustochytrids strains, other PUFA-producing microorganisms and fish oil are given in Table 5. It should be pointed out that, depending on growth conditions, the percentage of each fatty acid in the profiles presented in Table 5 may vary considerably.

1.7.1 Lipid and DHA production by thraustochytrids

Accumulation of storage lipid in oleaginous yeasts and filamentous fungi is generally induced by a nutrient imbalance in the culture medium (section 1.6.4). However, it has not been clear whether a nutrient imbalance is required to induce lipid accumulation in thraustochytrids. Previous reports have claimed that lipid accumulation in thraustochytrids occurs at non-limiting conditions in growth phase (41, 100). On the other hand, several studies have demonstrated that high C-to-N ratio is favorable for high-level lipid accumulation in thraustochytrids grown in complex media, presumably caused by N starvation (17, 22, 113, 126). However, the kinetics of cell growth, nutrient limitation and

high-level lipid accumulation have not been studied in sufficient detail to decide the modes of lipid accumulation in thraustochytrids.

The highest volumetric productivity of DHA reported for thraustochytrids is 138 mg l^{-1} h^{-1}, obtained with *A. limacinum* SR21 (126). When grown in a bioreactor in a glucose (12%) containing media and corn steep liquor and ammonium sulfate as sources of N, this strain was able to produce 48.1 g dry biomass l^{-1} of which the total fatty acids comprised 77.5% and DHA accounted for 35.6% of the total fatty acids. This resulted in a volumetric DHA concentration of 13.3 g l^{-1}. In early growth phase, the lipid content of the cells was approximately 20% of cell dry weight and equally divided by neutral (triacylglycerol) and polar (phospholipids) lipids. The share of triacylglycerols increased to about 95% as the total lipid content increased to its maximum level in late growth phase. Reduced dissolved O_2 level in the fermentation broth (e.g., below 3%) during late growth phase was found to promote DHA production in *Schizochytrium* sp. (9). Production data of selected thraustochytrid strains are given in Table 4.

The most dominant triacylglycerol in SR21 is reported to be 1,3-dipalmitoyl-2-DHA-triacylglycerol and it constituted 27% of the triacylglycerols (84). The polar lipids are mainly composed of phosphatidylcholine (74%), phosphatidylethanolamine (11%) and phosphatidylinositol (5%). 70% of the phosphatidylcholine were 1-palmiotyl-2-DHA-phosphatidylcholine and 1,2-di-DHA- phosphatidylcholine (126). Phosphatidylcholine is reported as the major phospholipid also in other thraustochytrids (34, 55, 118). Besides triacylglycerols and phospholipids, small amounts of sterols, squalene, mono-and diacylglycerols, free fatty acids and glycolipids have been detected in thraustochytids (34, 56, 69, 118).

Table 4. Biomass, lipid and DHA production of selected heterotrophic marine microorganisms [a].

Strain	Device	Time (h)	Biomass (g l⁻¹)	Lipid (% of d.w)	DHA (% of TFA)	DHA cons. (g l⁻¹)	Ref.
A. limacinum SR21	B	96	48	78 [b]	36	13.3	(126)
A. limacinum SR21	S	120	36	39 [b]	35	4.2	(130)
ONC-T18	B	120	25	70 [b]	21	3.7	(22)
S. mangrovei Sk-02	S	48	24	55 [b]	42	5.5	(113)
S. mangrovei KF2	S	52	13	-	35	2.8	(33)
Thraustochytrium G13	B	41	14	78	18 [c]	2.2	(17)
T. aureum (ATCC 34304)	S	144	5	20	51 [c]	0.5	(10)
C.cohnii	B	220	83	42	33 [c]	11.7	(109)
C.cohnii	B	400	109	56	32 [c]	19.0	(108)

a) Abbreviations: B, bioreactor; S, shake flasks; d.w., dry weight; TFA, total fatty acids.
b) Total fatty acids in % of d.w.
c) DHA value given in % of total lipid.

Table 5. Fatty acid profiles (%, (w w^{-1}) of total fatty acids) of some selected microorganisms used for heterotrophic production of PUFA. The fatty acid profile of oil from herring is included as an example of marine fish oil [a].

Strain	14:0	15:0	16:0	16:1 n-7	18:0	18:1 n-9	18:2 n-6	20:4 n-6	20:5 n-3	22:5 n-6	22:6 n-3	Ref.
DHA producers												
A. limacinum SR21	3	6	49	t	1	0	0	t	1	6	33	(130)
ONC-T18	10	0	40	15	1	7 [b]	0	0	1	5	20	(22)
S. mangrovei Sk-02	4	3	43	0	1	0	0	0	0	8	41	(113)
S. mangrovei KF2	4	3	48	1	0	0	0	t	t	7	39	(33)
KH105 [c]	7	7	32	0	t	t	t	t	2	10	35	(3)
T. aureum	0	0	21	1	9	15 [b]	3	1	1	6	43	(10)
C. cohnii [d]	17	0	17	1	2	10 [b]	0	0	0	0	44	(110)
AA producer												
M. alpina [d]	0	0	18	0	6	7	9	42	2	0	12	(53)
EPA producer												
N. laevis	11	0	23	27	0	4 [b]	3.2	2	24	0	0	(122)
Fish oil												
Herring [e]	5-8	0	10-19	6-12	1-2	9-25	0	0	4-15	0	2-8	(12)

a) Abbreviation: t, trace.
b) The report does not state whether the fatty acid is 18:1 n-9 or n-7.
c) The fatty acid profile contains in addition 6% of the fatty acid 17:0.
d) The fatty acid content is given in percent of total lipid (w w^{-1}).
e) The fatty acid profile contains in addition 7-20% of the fatty acid 20:1 n-9 and 7-30% of 22:1 n-11.

1.8   Microbial osmoadaptation

1.8.1 Positive turgor pressure is essential for growth

The ranges of osmolality of natural microbial habitats differ greatly: from freshwater environment to hypersaline lakes. Drought, freezing and rainfall can rapidly change the osmolality of the microorganism's niches. The salinity of the open ocean salinity varies between 33 and 37‰ (ca. 097-1.06 osmol kg^{-1}), gradually decreasing from the tropics towards the polar sea. In near-shore waters and in semi-enclosed seas, the seawater may be diluted by river water, and the salinity is influenced by seasons and by tidal actions. In these environments, the salinity may range from 0 to 37‰ [58]. In many industrial fermentation processes, cells are exposed to both high and fluctuating osmolality, primarily due to medium components and product accumulation, particularly in production processes of low-molecular-weight products. The ability to adapt to fluctuations in environmental osmolality is of fundamental importance for the growth and survival of microorganisms, as a positive turgor pressure (outward-directed pressure) is regarded as the driving force for cell division [57, 77, 120].

1.8.2 Strategies of osmoadaptation

Changes in the external osmolality direct water fluxes along the osmotic gradient. Swelling (and subsequent lysis) may occur if microorganisms are exposed to a sudden decrease (osmotic downshock) in environmental salinity. However, the result is most often only a minor increase in cell volume [28], as the organism adapts to the osmotic change. In bacteria, mechanosensitive ion channels in the cytoplasmic membrane respond to membrane tension due to excessive turgor, and rapidly release solutes from the cytoplasm to reduce its osmolality [72]. Mechanosensitive ion channels are also found in eukaryotic membranes, for instance in plastids and in fission yeasts [46], but little is known about their physiological function [72]. An increase in external osmolality (osmotic upshock) causes efflux of water from the cytoplasm, and a resulting decrease in turgor pressure and cell shrinkage is observed.

The extreme halophilic Archaea, some anaerobic fermentative or sulfate reducing Bacteria (40 and references therein) and the obligatory aerobic *Salinibacter ruber* [89]

balance the osmotic pressure by active uptake of high concentrations of inorganic ions, mainly K^+ and Cl^- (40, 57). The cytoplasm of these organisms is exposed to high ionic strength. They have enzymes that not only tolerate, but also require high ionic conditions compared to their non-halophilic counterparts (62), and they can only grow in environments of high salt concentrations (106).

In current literature, all other organisms tested respond to osmotic stress by adjusting their cytoplasmic osmolality by synthesis or uptake of certain organic osmolytes commonly called compatible solutes. The term indicates that accumulation of these solutes occurs without perturbing cellular macromolecules performing vital cellular processes, even when the solutes exist at high concentrations in the cytoplasm (20, 39, 129).

1.8.3 Compatible solutes and their function

Osmoadaptation by accumulation of compatible solutes is used in all three domains of life (101, 102, 119, 127, 129). Accumulation of compatible solutes after an osmotic upshock increases the osmolality of the cytoplasm to the level or above the level of the surroundings. This prevents further dehydration and in turn makes free water to re-enter the cell and restore cell volume within the limits that allow growth. Compatible solutes are either synthesized endogenously or taken up from the environment (57).

In general, effective compatible solutes are uncharged molecules (such as polyols) or zwitterions that are neutral at physiological pH (such as glycine betaine). They have high solubility in water, and they exert a stabilizing effect on macromolecules. Thus, high intracellular concentrations can be reached without disturbing cellular functions (39, 128, 129). Osmotolerant organisms have been reported to through evolution select compatible solutes that stabilize intracellular proteins (129). The protein-stabilizing effect of compatible solutes is often explained in terms of the preferential exclusion model; i.e. the solutes are preferentially excluded from the hydration layer of proteins and other cytoplasmic macromolecules (6) (Figure 6). According to this theory, the preferential exclusion pushes the proteins towards their compact native state which have the smallest amount of bound water. In contrast to the stabilizing solutes, denaturing solutes such as

inorganic ions and urea tend to enter the hydration layer, bind to proteins and thereby causing them to unfold (Figure 6).

An alternative explanation that does not involve the properties of bound water is that compatible solutes or denaturing solutes are preferentially excluded or accumulated, respectively, around the protein backbone (107). Thus, for stabilizing compatible solutes, the repulsion from the protein backbone is greater than the combined attraction by the amino acid side groups. The thermodynamic effect is the same as describe above, the proteins will tend to fold up more compactly and the equilibrium between the unfolded and the native protein is pushed towards the native state (16, 107).

Because of the protein-stabilizing effect of compatible solutes, they can protect proteins and organisms against many adverse conditions (120, 127). Some examples of protective properties are polyols, taurine and hypotaurine serving as antioxidants, trehalose contributing to increased thermostability, sugars and polyols increasing freeze tolerance and carbohydrates protecting against desiccation. Accumulated compatible solutes might also function as an intracellular storage material of carbon, energy and nitrogen (120).
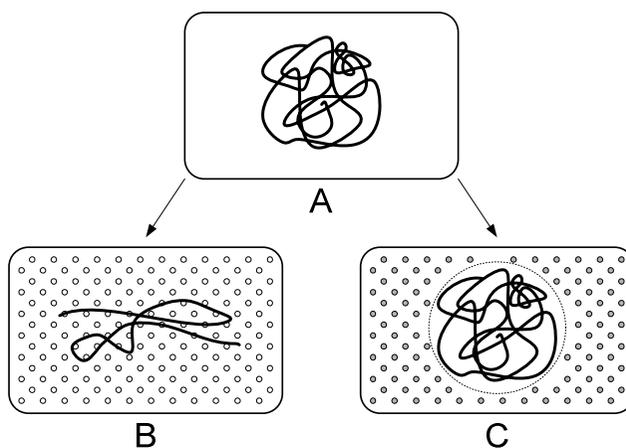
**Figure 6.** Illustration of the effect of stabilizing compatible solutes or denaturing solutes on protein conformation and stability. A: Protein in its native conformation; B: denatured protein in an environment of denaturing (binding) solutes such as urea; C: stabilized form of protein in the presence of compatible solutes which are preferentially excluded from the hydration layer of proteins (Modified from 39).

1.8.4 Osmoadaptation in eukaryotic microorganisms

All eukaryotic microorganisms analyzed to date accumulate compatible solutes to provide osmotic balance and turgor in response to increased salinity in the environment. The compatible solutes of eukaryotic microorganisms comprise a few major chemical categories, as listed in Table 6.

Table 6. Examples of compatible solutes accumulated in eukaryotic microorganisms by de novo synthesis or active uptake from the surroundings (Based on 15, 59, 119, 120).

Chemical category Example of solute	Examples of eukaryotic microorganisms that accumulate the solute
Polyols	
Glycerol	Microalgae, fungi
Sorbitol	Microalgae, fungi
Mannitol	Microalgae, fungi
Arabitol	Fungi
Sugars	
Sucrose	Microalgae
Trehalose	Fungi
Heteroside	
Floridoside	Microalgae
Cyclitols	
D-(+)-1,4/2,5-Cyclohexanetetrol	Microalgae
1,3,5/1,4-Cyclohexanepentol	Microalgae
Amino acids and derivates	
Proline	Microalgae
Methylated tertiary N compounds	
Glycine betaine	Microalgae
Methylated S-compound	
Dimethylsulphoniopropionate (DMSP)	Microalgae

In marine microalgae exposed to an osmotic upshock, a rapid uptake of ions, mainly K^+, often precedes accumulation of compatible solutes (13, 30, 31, 38, 58). Accumulation of compatible solutes then partially replaces the unfavorable high concentration of ions (13). Many microalgae species are able to accumulate more than one compatible solute (13, 37, 58, 119). The known compatible solutes of microalgae belong to several chemical categories; sugars (such as sucrose and trehalose), polyols (such as glycerol, mannitol, sorbitol), heterosides (such as floridoside and isofloridoside), methylated tertiary N-compounds (such as glycine betaine and homarine), methylated S-compound (dimethylsulphoniopropionate) and amino acids (such as proline and glutamate) (13, 119,

120). In addition, some microalgae accumulate species of cyclitols, such as 1D-1,4/2,5-cyclohexanetetrol (27, 37, 38) and 1,3,5/2,4-cyclohexanepentol (59). Halotolerant fungi often accumulate glycerol or other polyols such as mannitol, arabitol and sorbitol (15, 50)

1.8.5 Osmoadaptation in thraustochytrids

Thraustochytrids have a wide distribution in coastal and oceanic waters and sediments and can be subjected to rapid changes in environmental osmolality (95). Several strains that are capable of growth in a wide range of salinity have been described (22, 130), while other strains, such as *T. aureum* (ATCC 34304) have been reported to be rather salt sensitive (55). The proline content of *T. aureum* and *T. roseum* has been reported to rise linearly with the external salt concentration in cells grown in 50 to 100% seawater, but proline and other free amino acids provided only a small contribution to the osmotic pressure of the cytoplasm (123). It was proposed that K^+ and Na^+ ions are major cytoplasm osmolytes in these organisms (123), but this proposal was later retracted (42). No further reports on the osmolyte system of thraustochytrids have previously been published.

2   AIMS OF THE STUDY

The aim of this thesis was to achieve a better understanding of the basic physiology of thraustochytrids. In addition to an increased understanding of their biology, these investigations were motivated by the potential of these organisms to represent a larger share of the future commercial production of n-3 PUFA. Within few years, marine fish oils may become a limiting factor for marine fish farming (86). Additionally, the demand for n-3 PUFA for human consumption is increasing (12). At present, microbial oil is consumed mainly as high-quality food-grade PUFA-oils for use in infant formula and in pharmaceutical and neutraceutical products due to their high price compared to fish oils. To become or remain competitive and to enlarge the application area, the volumetric productivity of microbial DHA has to increase in order to lower the production costs (104).

This thesis includes studies of the fundamental properties of growth kinetics and accumulation of lipid and DHA in thraustochytrids, in addition to an investigation of their osmolyte system. Findings within these areas should contribute to the basic understanding of the biology of thraustochytrids. Additionally, from an applied point-of-view, this knowledge may contribute towards high volumetric productivities of n-3 PUFA from microbial systems. Future industrial fermentation processes are dependent on rapid growth and lipid accumulation in addition to strains with high osmotolerance that can withstand the high osmotic pressures of the cultivation broth.

3   SUMMARY OF RESULTS AND DISCUSSION

3.1   Isolation and preliminary screening of new thraustochytrids

More than 70 new strains of thraustochytrids were isolated by using the so-called pine pollen baiting method (18, 59). The isolates were collected from near-shore marine sediments and seawater, mainly from the coast of Norway but also from more distant areas such as Portugal and France. A preliminary screening in order to select strains with high growth rate and high lipid- and DHA- content was performed on solid medium, in shake flasks and in bioreactors in various complex media. Based on the preliminary screening, further work was concentrated on four isolates designated T29, T65, T66 and T67. Strains T65, T66 and T67 were isolated from a mixture of marine sediment and seawater sampled from the coast of Madeira, Portugal, while T29 was isolated from seawater sampled from the south coast of Norway near Kristiansand.

3.2   Endogenously synthesized (-)-*proto*-quercitol and glycine betaine are principal compatible solutes of *Schizochytrium* sp. strain S8 (ATCC20889) and three new isolates of phylogenetically related thraustochytrids (Paper I)

3.2.1   Classification of thraustochytrids

We investigated the taxonomy of the previously known *Schizochytrium* sp. strain S8 (ATCC 20889), and our four new isolates (T29, T65, T66 and T67) based on 18S ribosomal DNA phylogeny. Pair-wise comparison of the 18S ribosomal RNA gene sequences of the strains S8, T65, T66 and T67 showed that they are closely related (99% identity), and that they belong to the previously described thraustochytrid group 1 (61) (see Figure 1 of Paper I). The strains formed a cluster with several previously known strains such as *Schizochytrium* sp. strain N1-27 (52, 112), Thraustochytriidae sp. strain FJN-10 (accession number AY773276) and *Schizochytrium* sp. strain KH105 (52) and they displayed 89% identity with *S. limacinum* SR21 (84). These strains were later reassigned to *Aurantiochytrium* (see below).

An earlier report (52) suggested the use of PUFA profiles for taxonomic classification of thraustochytrids. We therefore characterized the PUFA profile of S8, T65, T66 and T67 and demonstrated that the strains displayed a simple profile consisting of DHA (approx. 80%) and DPA, previously described as PUFA profile type A (52), indicating a close taxonomic relationship to strains KH105 and SR21 (52). We furthermore observed that strains with PUFA profile type A (DHA and DPA) and type B (DHA, DPA and EPA) were scattered in the same phylogenetic branch and therefore proposed that for taxonomic purposes, strains with PUFA profiles type A and type B should be joined.

Due to the uncertainty of the genus-level classification of *Schizochytrium* spp. and other thraustochytrids, S8, T65, T66 and T67 were not assigned to any genus in Paper I. However, a rearrangement of the genus *Schizochytrium* has recently been proposed, erecting two new genera *Aurantiochytrium* and *Oblongichytrium* (131). The 18S ribosomal DNA-based monophyletic group of *Aurantiochytrium* now includes strains KH105, N1-27, FJN-10 and SR21 which all are close relatives of S8, T65, T66 and T67. Furthermore, the genus *Aurantiochytrium* is suggested to be characterized by a PUFA profile consisting of approximately 80% DHA (131). Based on the results above, we concluded that strain T66 (see Paper II) and also T65, T67, and S8 should be classified in the genus *Aurantiochytrium*.

The 18S ribosomal RNA gene sequence of T29 and subsequent pair-wise alignments with those of previously known thraustochytrids demonstrated 85% identity with the other four strains characterized by us and an almost 100% identity compared to the 18S ribosomal RNA gene sequence of *Thraustochytrium aureum* (ATCC 34304) (10, 44). The close relationship between these two strains was also reflected by their similar PUFA profile consisting of DHA, DPA, AA and EPA. The PUFA profile of T29 contained in addition a small amount of eicosatrienoic acid (20:3 n-6).

3.2.2 Identification of (–)-proto-quercitol and glycine betaine acting as compatible solutes

Even though several salt tolerant strains of thraustochytrids have been described previously (22, 130), their main osmolyte systems have remained unknown (see section 1.8.5). We showed that *Aurantiochytrium* sp. strain S8 (ATCC 20889) and the three new isolates T65, T66 and T67 adapted well to defined media of elevated osmotic strength. Cell extracts of the osmotically stressed cells were analyzed qualitatively by NMR (^{1}H and ^{13}C). Two-dimensional ^{1}H-^{1}H correlated spectroscopy showed two dominating compounds. These were identified as glycine betaine and *proto*-quercitol based on the obtained NMR spectra combined with literature search, and accurate molecular mass determination. Measurement of specific optical rotation showed that the *proto*-quercitol species of strain T66 was levorotatory; that is (-)-*proto*-quercitol (1D-1,3,4/2,5-cyclohexanepentol).

S8 and T66 displayed a nearly linear increase in the cellular content of the glycine betaine and (-)-*proto*-quercitol with increasing osmotic pressure. This represents the first identification of the principal compatible solutes of thraustochytrids. Both organisms accumulated about 500 µmol (-)-*proto*-quercitol and 100 µmol glycine betaine per gram dry weight when stressed with 1.0 M NaCl (2.1 osmol kg $^{-1}$), and (-)-*proto*-quercitol was shown to be the dominating solute at all NaCl concentrations tested (0.25 to 1.0 M). Phosphatidylinositol and phosphatidylcholine have previously been detected in the related organism *A. limacinum* SR21 (126) and we speculated that (-)-*proto*-quercitol was synthesized de novo from *myo*-inositol (70, 111) and glycine betaine from cholin (47, 102).

When this investigation started, (-)-*proto*-quercitol had previously been detected only in eucalyptus (1, 74, 75) and no cyclohexanepentols had been shown to act as compatible solutes in any microorganism. Interestingly, at the time of our discovery, an optically inactive cyclohexanpentol species (1,3,5/2,4-cyclohexanepentol) was reported to be a compatible solute of the microalgae *Pavlova* (59), indicating that cyclohexanepentols in fact represent a group of compatible solutes that is used by several eukaryotic microorganisms. It is noteworthy that Kobayashi et al. (59) concluded that externally

supplied *myo*-inositol was taken up by the cells but not incorporated into 1,3,5/2,4-cyclohexanepentol.

T66 tolerated at least 2.0 M NaCl, but its doubling time increased considerably above 1 M NaCl (see Figure 5 of Paper I). The more salt sensitive isolate, T29 (a close relative to *T. aureum*), did not accumulate (-)-*proto*-quercitol or glycine betaine. This is in good agreement with earlier reports on the failure to identify any Dragendorff-positive quaternary ammonium compound such as glycine betaine in *T. aureum* (123).

The results summarized above show that the osmotolerance and the osmolyte systems vary among thraustochytrids and that endogenously synthesized (-)-*proto*-quercitol and glycine betaine constitute a potent osmolyte system among the members of the genus *Aurantiochytrium.*

3.3 Accumulation of docosahexaenoic acid-rich lipid in thraustochytrid *Aurantiochytrium* sp. strain T66: Effects of N and P starvation and O_2 limitation (Paper II)

Accumulation of storage lipid in oleaginous filamentous fungi and yeasts is generally induced by a nutrient imbalance in the culture medium (97). However, previous reports have claimed that lipid accumulation in thraustochytrids occurs at non-limiting conditions in growth phase (41, 100). In this study, we cultivated the strain *Aurantiochytrium* sp. T66 in high-cell density batch bioreactors in defined glutamate and glycerol containing medium with various medium limitations. While glycerol at all times was fed in excess, batch concentrations of PO_4^{3-} and glutamate were varied in order to obtain P or N starvation. At non-limiting O_2 conditions, dissolved O_2 was kept at 20% of saturation by automatic control of the stirring rate, while O_2 limitation was introduced by manually adjusting the stirring rate, thereby limiting the oxygen transfer rate.

3.3.1 Improved protocol for lipid extraction from thraustochytrids

To study the effects of nutrient limitations on lipid accumulation, an accurate protocol for lipid extraction from cells with a wide range of lipid content was required. We initially

used a modified Bligh and Dyer extraction protocol (14, 45), essentially as a previously described version reported to give the highest yield of lipids from freeze-dried thraustochytrids (21). However, we demonstrated that this protocol yielded incomplete lipid extraction from freeze-dried T66 cells. Since accumulated lipids of thraustochytrid strain *A. limacinum* SR21 are stored in lipid bodies (79), we suspected that proteins in the lipid bodies represented the major reason for the incomplete extraction. We demonstrated that heat and protease treatment prior to lipid extraction resulted in a complete extraction. This treatment increased the yield of extracted lipids up to 2.5-fold for cells harvested in exponential growth phase, while the yields from treated and untreated cells were essentially the same for stationary phase cells with high lipid content. Our new protocol for lipid extraction from thraustochytrids may be especially important for determination of lipid classes which requires complete lipid extraction.

3.3.2 N starvation induces lipid accumulation

We demonstrated that growth conditions can modulate the lipid content and the fatty acid composition of *Aurantiochytrium* sp. T66. The lipid content of the cells remained low (13%) during exponential growth and increased to 55% of dry biomass after glutamate exhaustion. Thus, T66 behaved as a typical oleaginous microorganism with respect to N starvation. This result is in agreement with observations that high C-to-N ratio of complex growth media is required to obtain high-level lipid accumulation in thraustochytrids (17, 22, 113, 126). The only PUFA detected in T66 were DHA and DPA. The maximum DHA content in the N starved culture was 9 g l^{-1}, achieved after 182 h of cultivation at a dry biomass concentration of 90 g l^{-1}, which to our knowledge is the highest biomass concentration reported for thraustochytrids in laboratory-scale experiments.

3.3.3 O$_2$ limitation induces lipid accumulation and increases relative PUFA-content

The lipid content of T66 increased from 13% to 33% of dry biomass after O_2 limitation (dissolved O_2 below 1% of saturation) was introduced. Thus, we demonstrated that O_2 limitation per se induced lipid accumulation. O_2 limitation strongly affected the fatty acid

composition of T66 and was shown to be advantageous for PUFA accumulation. The relative content of DHA and DPA increased from 25 and 7% in growth phase, to 52 and 13% at O_2 limitation, respectively. Simultaneously, O_2 limitation abolished accumulation of monounsaturated C16 and C18 fatty acids. This discriminating effect of O_2 limitation indicated that O_2 dependent desaturase(s) responsible for the production of monounsaturated C16 and C18 fatty acids did not function under these conditions while DHA and DPA both where synthesized by the PUFA synthase, which is known to function both in the presence and absence of O_2 (76).

To increase the total amount of DHA, we hypothesized that a combination of O_2 limitation and N starvation could bring together the high lipid level of N starvation and the high DHA level of O_2 limitaiton. By introducing O_2 limitation after glutamate was exhausted we demonstrated our highest volumetric concentration and productivity of DHA for strain T66, reaching 13.7 g DHA l^{-1} and 93 mg DHA l^{-1} h^{-1}, respectively. The biomass concentration and the maximum lipid content of the cells were about the same as for the N starved culture, combined with a high level of DHA, reaching 39% of the fatty acids. Although it was not the primary aim of this study to optimize DHA production, it is noteworthy that the achieved DHA productivity approaches some of the highest previously reported DHA productivities, i.e., 138 mg l^{-1} h^{-1} for *A. limacinum*, (126), 115 mg l^{-1} h^{-1} for *S. mangrovei* Sk-02, (113) and 117 mg l^{-1} h^{-1} for thraustochytrid strain 12B (91).

3.3.4 Sequential P and N exhaustion induces lipid accumulation and increases the relative content of PUFA

Sequential P and N starvation resulted in a maximum lipid content of 40% of dry biomass. Compared with N starvation, an elevated PUFA content was achieved (DHA, 40% and DPA, 16% of total fatty acids). As expected, the increase in the PUFA content was not accompanied by a reduction of the amount of monounsaturated C16 and C18 fatty acids as seen for O_2 limited cells (see above). We furthermore observed that lipid accumulation was induced from the time of phosphate depletion (in the presence of excess glutamate), demonstrating that P starvation per se induces lipid accumulation.

4 CONCLUDING REMARKS

Work described in this study constituted the first report on accumulation of compatible solutes in thraustochytrids. (-)-*proto*-Quercitol and glycine betaine comprise a potent osmolyte system of four members of the genus *Aurantiochytrium*. Several other strains within this genus have been reported to tolerate a wide range of salinities, and it may be tempting to speculate that the osmotolerance level and osmolyte system of species within the *Aurantiochytrium* genus may be phenotypical characteristics of taxonomic value. From an applied point-of-view, strains with high osmotolerance is advantageous for industrial fermentation processes, due to high osmotic pressure in the cultivation broth caused by e.g., high substrate concentrations.

Work described in this thesis expands the current knowledge of lipid accumulation in thraustochytrids. *Aurantiochytrium* sp. strain T66 displayed lipid accumulation primarily in the post-exponential growth phase, initiated by either N- or P-starvation or O_2 limitation. In oleaginous eukaryotic microorganisms, N starvation leads to activation of AMP deaminase that reduces the cellular AMP level. This leads to a cascade of reactions that supply the cytoplasm with acetyl-CoA (see section 1.6.4). The mechanism for AMP deaminase activation is unknown, but our findings indicate that the activation may be a general stress or starvation response rather than an activation to scavenge additional ammonia ions as previously proposed.

Additionally, we demonstrated that the fatty acid profile of T66 could be modulated by growth conditions resulting in a variation of DHA content from 25 to 52% of total fatty acids. The opportunity to strongly modulate the fatty acid profile is advantageous since different applications of the oil may need different amounts of DHA. O_2 limitation increased the relative level of PUFA in T66, while monounsaturated fatty acids were abolished, indicating that long-chain polyunsaturated fatty acids of T66 are synthesized by an O_2-independent PUFA synthase, further implying that O_2 limitation represents a simple experimental method for deciding the route of PUFA synthesis in thraustochytrids. Although it was not the primary aim of this study to optimize DHA and lipid productivity, the maximum DHA-productivity obtained approached the highest values

previously reported for thraustochytrids. Thus, with further optimization of fermentation conditions, T66 may be a good candidate for scale-up and commercialization.

An improved protocol for lipid extraction from dry thraustochytrid cell mass involving heat and protease treatment prior to extraction was developed. This method secures an accurate total lipid determination from cells within a wide range of lipid content, and may be especially important for determination of lipid classes which require complete lipid extraction.

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