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MINI-REVIEW

Thraustochytrids as production organisms for docosahexaenoic acid (DHA), squalene, and carotenoids

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Abstract Thraustochytrids have been applied for industrial production of the omega-3 fatty acid docosahexaenoic (DHA) since the 1990s. During more than 20 years of research on this group of marine, heterotrophic microorganisms, considerable increases in DHA productivities have been obtained by process and medium optimization. Strains of thraustochytrids also produce high levels of squalene and carotenoids, two other commercially interesting compounds with a rapidly growing market potential, but where yet few studies on process optimization have been reported. Thraustochytrids use two pathways for fatty acid synthesis. The saturated fatty acids are produced by the standard fatty acid synthesis, while DHA is synthesized by a polyketide synthase. However, fundamental knowledge about the relationship between the two pathways is still lacking. In the present review, we extract main findings from the high number of reports on process optimization for DHA production and interpret these in the light of the current knowledge of DHA synthesis in thraustochytrids and lipid accumulation in oleaginous microorganisms in general. We also summarize published reports on squalene and carotenoid production and review the current status on strain improvement, which has been hampered by the yet very few published genome sequences and the lack of tools for gene transfer to the organisms. As more sequences now are becoming available, targets for strain improvement can be identified and open for a system-level metabolic engineering for improved productivities.

Keywords DHA · Squalene · Astaxanthin · Biosynthesis · Process development · Genetic tools

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Introduction

Thraustochytrids are unicellular, eukaryote, heterotrophic, and obligate marine microorganisms, commonly found in seawater and sediments, with the highest abundance in nutrient-rich areas, such as mangrove forests (Raghukumar 2002; Singh et al. 2014). Thraustochytrid strains are able to accumulate high levels of lipids as triacylglycerols, with a high content of the long-chain omega-3 (ω3) fatty acid docosahexaenoic acid (DHA). High-productivity strains can be cultivated to cell densities above 100 g/l dry weight in 4 days and accumulate lipid levels in the range of 50–70 % of cell dry weight (CDW) with DHA constituting 30–70 % of the total fatty acids (Chang et al. 2013a; Li et al. 2015; Raghukumar 2008). The first reports on use of thraustochytrids for production of DHA appeared early in the 1990s and were initiated by the rapidly increasing understanding of the benefits of long-chain polyunsaturated (LC-PUFA) ω3 fatty acids for human health. Production of DHA-rich oils based on thraustochytrids was commercialized a few years later by the US company OmegaTech, which later was acquired by Martek, and is now a part of DSM. The history of the development of the industrial production of DHA-rich oils from thraustochytrids by OmegaTech has been reviewed by Barclay et al. (2010), describing their strategies for strain isolation and further developments to the 150-m^3 production scale.

The taxonomy and phylogeny of thraustochytrids have been extensively described by others, and DHA-producing species of thraustochytrids have been identified within all the thraustochytrid genera (Honda et al. 1999; Yokoyama and Honda 2007; Yokoyama et al. 2007). Taxonomic reclassifications within the thraustochytrids have resulted in the establishment of several new genera. For instance, *Aurantiochytrium* was established as a separate genus in 2007, and some species previously classified as *Schizochytrium* were moved to the new genus (Yokoyama and Honda 2007). The highest cell densities and DHA productivities have been reported for species of *Schizochytrium*, *Aurantiochytrium*, and *Ulkenia*. The ability of thraustochytrids to produce high levels of DHA is attributed to the presence of an alternative pathway for DHA synthesis, catalyzed by a polyketide synthase (PKS) enzyme complex. The PKS also generates another LC-PUFA, docosapentaenoic acid (DPA; C22:5 ω6), which is a characteristic fatty acid for thraustochytrids (Hauvermale et al. 2006; Lippmeier et al. 2009; Metz et al. 2001).

The extensive research on thraustochytrids has mainly been motivated by their properties as DHA producers. However, during the strain screening programs, also other commercially interesting products have been identified. For instance, some strains produce high levels of carotenoids, others squalene. These products have established, and growing, markets in food, feed, and pharma, currently produced from other raw materials. As saprophytic organisms, thraustochytrids also produce extracellular enzymes, and some strains have been reported to produce extracellular polysaccharides, see, e.g., Gupta et al. (2012) and Singh et al. (2014). However, based on the reported production levels, the intracellular, lipid-related compounds squalene and carotenoids seem to have the highest potential for a future industrial production by thraustochytrids. Figure 1 shows the biosynthetic pathways for DHA, squalene, and carotenoids.

The most recent reviews on thraustochytrids thoroughly cover isolation methods and analytical methods (Gupta et al. 2012), ecological impacts, distribution and role in marine habitats (Singh et al. 2014), and metabolic pathways and enzymes involved in DHA synthesis (Xie and Wang 2015). Results from the high number of process optimization studies are also referred, but not as a main topic. In the present review, we extract the main findings from the reports on process optimization for DHA production and interpret these in light of the current knowledge of DHA synthesis in thraustochytrids and lipid accumulation in oleaginous microorganisms in general. We also summarize the published reports on squalene and carotenoid production and review the current status on strain improvement and available genetic tools for thraustochytrids.

DHA—yet the only commercial product from thraustochytrids

Applications of DHA and market prospects

DHA-rich oils from thraustochytrids are currently on the market as dietary supplements. The main source of the marine ω3 fatty acids eicosapentaenoic acid (EPA) and DHA are fish oils. Approximately 200,000 t fish oils are used in products for the human markets, while the production of microbial ω3-rich oils constituted only 5000 t in 2011 with thraustochytrids and the heterotrophic microalgae *Chrypthecodinium cohnii* as production organisms. Dietary supplements constitute the largest market share of 55 % for ω3 products, followed by functional food and beverages, and pharmaceuticals. The ω3-PUFA market is projected to show an annual growth rate of 12.8 % between 2014 and 2019 and is expected to be worth USD 4300 millions by 2019 (www.marketsandmarkets.com).

EPA and DHA are also important constituents in the feed for marine aquaculture. The annual production of fish oil is in the order of 1×10^6 t, of which 71 % was used for aquafeed in 2010 (FAO 2014). The global catches of the fish stocks used for oil production have reached its maximum limit. A future growth of the marine aquaculture will therefore require new sources for these fatty acids in substantial volumes, and microbial oils have been proposed as a solution (Olsen 2011). This will represent a large-volume market but with far lower prices than for the current human products.

DHA synthesis in thraustochytrids

In oleaginous yeast, phototrophic microalgae, and bacteria (e.g., *Rhodococcus* spp.), saturated fatty acids dominate in the storage lipids, mainly C16:0 but also C14:0 and C18:0 (Alvarez and Steinbüchel 2002; Goold et al. 2015; Wang 2015). The only known oleaginous microorganisms that produce LC-ω3-PUFA as a major part of their storage lipids are thraustochytrids, the closely related labyrinthulids, and the heterotrophic dinoflagellate *C. cohnii*. However, EPA and DHA frequently occur in the membrane lipids of marine microorganisms, both bacteria and microalgae (Mühlroth et al. 2013; Valentine and Valentine 2004).

The thraustochytrids use a standard fatty acid synthase (FAS) enzyme complex for synthesis of the shorter, saturated fatty acids, mainly C14:0 and C16:0 (Hauvermale et al. 2006). The thraustochytrids also express some desaturases and elongases, since low levels of C16:1, C18:1, C18:2, arachidonic acid (ARA), EPA, and other unsaturated fatty acids can be found (Yokoyama and Honda 2007; Yokoyama et al. 2007). Although proven in only a few strains, it is likely that all high-level DHA-producing strains use the PKS

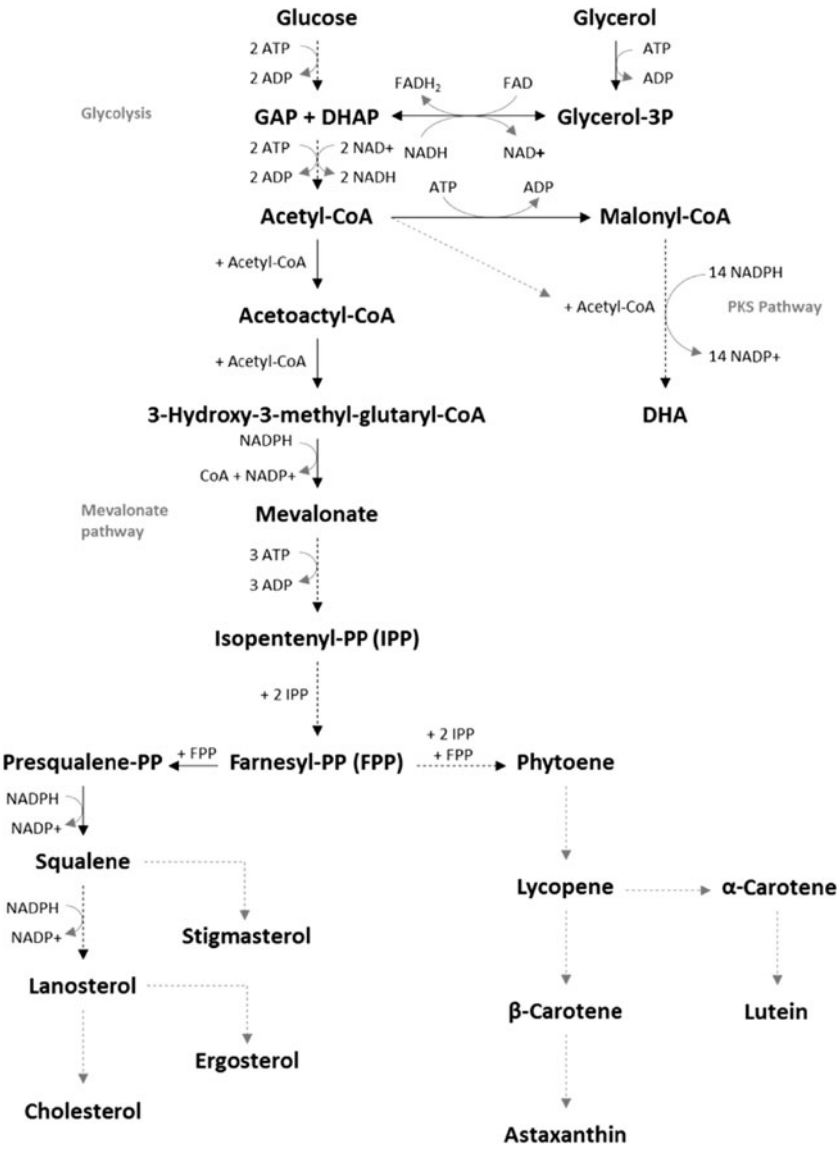

Fig. 1 Metabolic network for the biosynthesis of DHA, squalene, steroids, and carotenoids from glucose and glycerol. DHA is produced by the PKS pathway (see Fig. 2), while squalene, steroids, and carotenoids are produced via the mevalonate pathway (Sun et al. 2014). *Dashed arrows* indicate multiple enzymatic steps, and *dashed*, *grey arrows* in addition indicate reactions where energy carriers have been omitted

pathway as the main generator of DHA. In this pathway, DHA is synthesized by successive elongation steps with malonyl-CoA, similar to those carried out by FAS, but omitting the removal of the double bond introduced from malonyl-CoA in most of the cycles, thereby saving reducing power (NADPH) and eliminating the need for molecular oxygen and NADPH required for the desaturases. The pathway is not fully unraveled, but hypothetical models have been proposed (Fig. 2) (Metz et al. 2001; Ratledge 2004). The PKS pathway also generates DPA (C22:5, ω6), in the order of 10 % of the fatty acids (Chaisawang et al. 2012; Hauvermale et al. 2006; Matsuda et al. 2012). This indicates some plasticity early in the DHA biosynthesis pathway allowing for the synthesis of C6:0 instead of the usual C6:1 intermediate. When new two-carbon units are added to C6:0 following the same set of reactions as shown in Fig. 2, the result will be DPA.

FAS and PKS use the same precursors, acetyl-CoA and NADPH (Fig. 1). Reducing power in the form of NADPH is assumed to be limiting for lipid accumulation (Ratledge 2014). Malic enzyme (ME) is a main generator of NADPH for fatty acid synthesis in most, but not all, studied oleaginous microorganisms (Dulermo et al. 2015; Garay et al. 2014; Ratledge 2014). In order for ME to act as an NADPH generator for FA synthesis, it has been proposed that the enzyme has to form an integrated complex with the ATP:citrate lyase enzyme (ACL) and the FAS complex to ensure a direct channeling of acetyl-CoA into fatty acids (Ratledge 2002). It is not known whether the PKS in thraustochytrids would require a specific ME. ME activity has been demonstrated in cell-free extracts of thraustochytrids (Chaisawang et al. 2012; Chang et al., 2013b; Ren et al. 2013; Ren et al. 2009; Song et al. 2013). In time course studies, the activity of ME increased rapidly at the initiation of

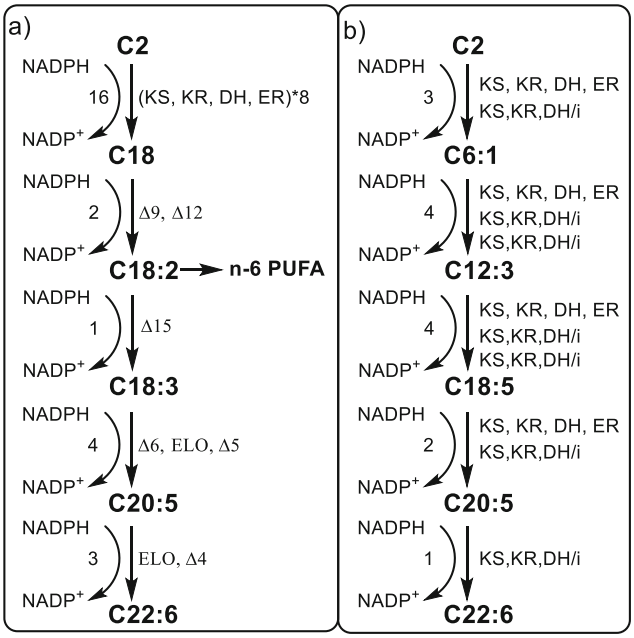

Fig. 2 Alternative pathways for DHA biosynthesis. **a** The FAS pathway followed by elongation and desaturation. Only the ω3 pathway to DHA is depicted, while the branching point to the ω6 pathway is indicated. **b** The PKS pathway (Metz et al. 2001; Ratledge 2004). The number of NADPH needed at each step, or sequence of steps, is displayed to the *left of the arrows*, while the enzyme activities involved are indicated to the *right*. *KS* ketoacyl synthase; *KR* ketoreductase; *DH* dehydratase; *DH/i* bifunctional dehydratase and trans-cis isomerase, the isomerase may also move the double bond; *ER* enoyl reductase; *Δ* desaturase, the *number* indicates which bond (counted from the carboxyl end) is desaturated; and *ELO* elongase, a multifunctional enzyme with KS, KR, DH, and ER activities

triacylglycerol (TAG) accumulation and continued to increase in parallel with the lipid accumulation (Ren et al. 2009; Song et al. 2013). The first NADPH-generating enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD), had its maximum activity during the active cell growth. However, more than 50 % of the maximum activity was still maintained at the end of the lipid accumulation phase (Ren et al., 2009, 2013). In a study by Song et al. (2013) using *Aurantiochytrium* sp. SD116, the FAS was inhibited by supplementing the growth medium with valeric acid (pentanoic acid) added continuously as pH control. Despite far less total lipids when valeric acid was added, the DHA concentration was unaffected (20 g/l at the end in both cases). The ME activity was significantly lower than in the control, but when the supply of valeric acid was terminated, the ME activity and the synthesis of C16:0 and other FAS products were restored. This study indicated that the measured ME activity was linked to the FAS, and the authors suggested that PKS does not depend on ME for NADPH generation. However, a study by Ren et al. (2009) supports a role of ME in DHA production in *Schizochytrium* sp. HX-308. Addition of malic acid in the rapid lipid accumulation phase increased the fraction of DHA of total fatty acids (TFAs) from 35 to 60 % and from ~8 to ~15 % of the biomass. The TFA content of the biomass increased from ~23 to ~26 %, meaning that the synthesis of other fatty acids was reduced.

Generally, oleaginous microorganisms accumulate lipids as TAGs at conditions where an essential nutrient, often nitrogen, is limiting cell division and organic carbon is in excess. The underlying mechanisms have been extensively reviewed by Ratledge (2004) and Ratledge and Wynn (2002), mainly based on studies on yeasts and filamentous fungi. Briefly, nitrogen limitation leads to a low level of AMP, reduced activity of the AMP-dependent isocitrate dehydrogenase of the tri-carboxylic acid (TCA) cycle, and accumulation of citrate, which is transported to the cytosol. ATP:citrate lyase splits citrate to oxaloacetic acid and acetyl-CoA, thereby providing a continuous supply of the fatty acid precursor acetyl-CoA in the cytosol. This enzyme has been found in all oil-accumulating microorganisms investigated, including the thraustochytrids (Chaisawang et al. 2012; Chang et al., 2013b; Janthanomsuk et al. 2015; Ren et al. 2009). Few thorough studies on the initiation of lipid accumulation in thraustochytrids have been reported. However, from studies where a defined nitrogen source has been used, it is evident that lipid accumulation is initiated when N is depleted (Jakobsen et al. 2008; Janthanomsuk et al. 2015; Qu et al., 2013b; Ren et al. 2010). When grown on media with a high N content supplied as yeast extract or peptones, TAGs accumulate also when N is still available (Chang et al., 2013a; Chang et al. 2014; Huang et al. 2012). Any explanations were not discussed by the authors but could be that the depletion of the most easily utilized amino acids and concomitant reduction in growth rate onsets the lipid accumulation, despite still high concentrations of nitrogen in the medium. Also, in continuous cultures with N as the growth-limiting compound, lipid accumulation occurred (Ethier et al. 2011; Ganuza and Izquierdo 2007). Phosphorus (P) limitation also initiated TAG accumulation in thraustochytrids (Jakobsen et al. 2008; Ren et al. 2013), but the effect of P limitation on DHA synthesis and lipid accumulation is far less studied than the effects of N limitation.

In fermentation studies where N was completely depleted, the lipid production rates decreased 1–2 days (20–50 h) after onset of the TAG accumulation. The production rates of the FAS products decreased at an earlier stage than the DHA production rates, thereby increasing the fraction of DHA of the total fatty acids towards the end of the fermentation (Chaisawang et al. 2012; Jakobsen et al. 2008; Li et al. 2015; Ren et al. 2010). A similar reduction in lipid accumulation rates in oleaginous fungi was shown to be due to inactivation of ME, and varying stability of ME was assumed to determine the maximum lipid levels obtained in different species and strains (Ratledge and Wynn 2002). In a study where high concentrations of yeast extract and peptone were fed continuously, no reduction in production rates occurred (Huang et al. 2012). In other studies where nitrogen was

supplied during the lipid accumulation phase, also oxygen was limiting (see below), complicating the data interpretation. Lower DHA fraction of TFA during the lipid accumulation phase than during exponential growth has often been observed (Chaung et al. 2012; Ren et al. 2014a, b). This can, at least partly, be explained by phospholipids (PLs) as the dominating lipid class during exponential growth with nitrogen in excess, since the PLs have a higher DHA fraction than the storage lipids (Fan et al. 2007; Qu et al. 2013a; Ren et al. 2014a). During exponential growth with N in excess, PLs are the dominating or only lipid class, constituting in the order of 10 % of CDW of thraustochytrids. The PLs constitute a decreasing fraction of the total lipids (TLs) during the lipid accumulation phase. At TL contents above 60 % of CDW, neutral lipids, dominated by TAGs, constituted 90–95 % of the TL (Fan et al. 2007; Ren et al. 2014a; Yaguchi et al. 1997).

The fatty acid yields on the carbon source are generally not reported but can be calculated to 0.29 g/g glucose in the lipid accumulation phase after N depletion in the study by Chaisawang et al. (2012). This is close to the theoretical yield, which is approximately 0.3 g/g glucose (Ratledge, 2014).

Other parameters influencing the DHA levels and production rates

Reduced oxygen supply, resulting in zero dissolved oxygen (DO) in the medium, has been shown to increase the relative fraction of DHA of TFA but also to a certain extent of the fraction of the biomass. A direct effect of oxygen limitation is that the content of unsaturated fatty acids produced by the oxygen-dependent desaturases is decreased, thereby increasing the relative fraction of the other fatty acids (Jakobsen et al. 2008). More importantly, low oxygen transfer rate (OTR) also reduced the fraction of the FAS products of TFA, thereby increasing the fraction of DHA. This was more evident the lower the OTR (Chang et al. 2014; Jakobsen et al. 2008; Qu et al. 2011; Ren et al. 2010) and indicates that the FAS activity is more affected by the oxygen supply than the PKS activity.

Results from experiments where the temperature was reduced within a range where the cell yields and lipid contents were not significantly affected (between 15 - 20 and 30 °C) indicate increasing DHA fractions with decreasing temperatures. For instance, the DHA fraction of TFA in *Aurantiochytrium mangrovei* Sk-02 increased from 29 to 42 % in the late lipid accumulation phase when the temperature was decreased from 30 to 12 °C; however, the DHA content of the cell mass was the same (~12 %) since the TFA content (mainly C16:0) of CDW decreased at lower temperatures (Chodchoey and Verduyn 2012). Several other studies also show a higher DHA fraction at lower temperatures but with smaller effects and not always clear trends. The temperature effect was even more evident for DPA than for DHA but with an opposite response. The DPA fraction of TFA, as well as the cell mass, decreased with decreasing temperature (Chodchoey and Verduyn 2012; Taoka et al. 2009; Unagul et al. 2005; Zeng et al. 2011). Hence, the synthesis of the two PKS products seems to react differently to temperature.

In order to maintain the maximum lipid accumulation rate, the concentration of the carbon source should be above 15–20 g/l. When the concentration drops below this level, for instance, towards the end of fermentations, the rates decrease. This effect was also evident when the carbon source was added by pulsing, allowing the concentration to decrease to ~5 g/l (Qu et al. 2013a). Limiting the carbon source by slow feeding gave similar, or slightly reduced, lipid contents of CDW as obtained with carbon in excess, however with reduced lipid production rates (Janthanomsuk et al. 2015; Qu et al. 2013a). At decreasing feeding rates, the production rates of the FAS products decreased more than the DHA production rates, resulting in a higher DHA fraction of the fatty acids.

Summary of the main factors affecting the DHA production rates

Total lipid contents of the cell mass above 80 % (Li et al. 2015) and DHA contents above 80 % of TFA (Huang et al. 2012) have been reported. However, such extremes have never been obtained simultaneously. The variations in DHA fraction of TFA observed when oxygen transfer rates and feeding strategies (N, P, and C) are changed seem to a larger degree to be caused by variations in the production rates for the fatty acids generated by FAS than the DHA production rates. In order to improve the DHA productivity, factors specifically increasing the rates of the PKS need to be identified. For a comparison of reported effects, the specific productivity (q_P) of DHA related to the "fat-free" cell mass have been calculated (Table 1). The highest q_P values were obtained when very high concentrations of complex nitrogen sources were applied. When nitrogen is available during the lipid accumulation phase, new synthesis of the rate-limiting enzymes is possible, and high enzyme activities can be maintained throughout the fermentation. However, more direct comparisons of different strategies for N supply, using the same strain, are needed. The stability of ME is assumed to be important to maintain a high lipid production rate in yeast and fungi. However, for thraustochytrids, it is yet unclear whether FAS and PKS use the same ME for NADPH generation, if they have their specific MEs, or even if the NADPH for PKS is generated by other enzymes. More knowledge about the number of MEs and possible associations with the two enzyme complexes will be provided by genome analyses. The distribution of the carbon flow between

Table 1 Specific productivities of DHA and C14-C18 fatty acids based on "fat-free" cell dry weight, approximate values calculated from data presented in the cited references

Strain	C source (in excess)	N source and concentration (g/l)[a]	N supply during lipid accumulation	Dissolved oxygen	q_p [mg/(g h)]		Reference
					DHA	C14–C18	
Thraustochytrium sp. ONC-T18	Glucose	MSG + YE (8 + 2)	No	NA	6.3	–	Burja et al. (2006)
Schizochytrium sp. S31[b]	Glycerol	YE (14)	No	DO = 0, 0.6 vvm, 450 rpm	7.3	5.1	Chang et al. (2013b)
Schizochytrium sp. S31[b]	Glycerol	YE (50)	NH_3 as pH control	DO = 0, 0.6 vvm, 700 rpm	9.1	12.7	Chang et al. (2013a)
A. limacinum SR21	Glycerol	YE + Pep (4 + 4)	Feeding of YE + Pep[c]	DO = 50 %	21	–	Huang et al. (2012)
A. limacinum SR21	Glucose	MSG + YE (45 + 30)	No	DO = 0	17	–	Li et al. (2015)
A. limacinum SR21	Glycerol	CSL (5)	Continuous culture	DO = 50 %	3.8	7.7	Ethier et al. (2011)
Schizochytrium G13/2S	Glucose	MSG (4)	Continuous culture	–	7	7.5	Ganuza and Izquierdo (2007)
Aurantiochytrium sp. T66	Glycerol	MSG (22)	No	DO = 30 %	3.3	8.7	Jakobsen et al. (2008)
Schizochytrium sp. HX-308	Glucose	MSG (22)	No	NA	6	–	Qu et al. (2013b)
Schizochytrium sp. HX-308	Glucose	MSG (22)	No	NA	6	7	Ren et al. (2014a)
Aurantiochytrium sp. B-072	Glucose	MSG (8)	No	NA	23[d]	85[d]	Chaisawang et al. (2012)
Aurantiochytrium sp. B-072	Glucose	$(NH_4)_2SO_4$ (5)	NH_3 as pH control	DO = 30 %	50–55[d]	85[d]	Janthanomsuk et al. (2015)

The values refer to the period with highest production rates during the lipid accumulation. Fat-free dry weight is calculated as CDW minus TFA if not otherwise stated

NA data not available

[a] *MSG* mono-sodium glutamate, *YE* yeast extract, *Pep* peptone

[b] DHA fraction and calculated fat-free CDW based on TL, not TFA

[c] A solution of 20 g/l YE + 20 g/l Pep was fed continuously

[d] Calculated by the cited authors as mmol/(g h). Recalculated to mg/(g h) by using the molecular masses of DHA and C16:0, respectively

the two pathways will also be affected by the K_m values for the enzymes belonging to the respective systems. No such studies have been reported.

The overall volumetric productivity of the processes has not been emphasized in the sections above. However, the highest reported DHA productivities in the scientific literature are 7–8 g/l day. These were obtained with *Schizochytrium* sp. S31 at 150 g/l CDW (Chang et al., 2013a) and with *Aurantiochytrium limacinum* SR21 at 88 g/l CDW (Li et al. 2015). In a patent, 13 g/l day at 190 g/l CDW has been reported (Bailey et al. 2003).

Squalene production in thraustochytrids

Applications of squalene and market prospects

Some thraustochytrids, in particular some species belonging to the genus *Aurantiochytrium*, produce squalene in quantities of more than 30 % of the CDW. Squalene is an intermediate in the biosynthesis of sterols like cholesterol and ergosterol (Fig. 1) and is widespread in nature. In the livers of deep-sea sharks, it may constitute more than 80 % of the oil (Bakes and Nichols 1995). Squalene is extensively used as an excipient in pharmaceutical emulsions for the delivery of vaccines, drugs, and other medicinal substances. It improves the immune system and is therefore used as a protective agent in cancer treatment, and it is also used as a hydrating and antioxidant agent in cosmetics (Huang et al. 2009; Reddy and Couvreur 2009). The squalene market is currently growing and is expected to reach 4000 t and a value of USD 177 million by 2019 (www.marketsandmarkets.com). The shark liver oil has been the traditional source for squalene, but the uncontrolled killing of these animals has caused growing environmental concerns. Combined with governmental regulations, it has restricted the growth of this segment. Alternative squalene sources include vegetable oils, where the highest quantities are found in amaranth and olive oils, on average 7–8 % (*w*/*w*) and 1 %, respectively (Popa et al. 2015). Due to the higher demand of processing plants when extracting squalene from

vegetable oils compared to shark livers, the potential of biotechnological production in microbial cell factories has attracted increasing attention (Ghimire et al. 2016).

Squalene-producing strains and reported production data

Squalene is produced from farnesyl diphosphate (FPP) via the mevalonate pathway (Fig. 1). The enzymes preceding FPP have not been mapped in thraustochytrids. However, several enzymes related to squalene and sterol synthesis have been shown to be expressed in *Aurantiochytrium* sp. SD116, including sterol 24-C-methyltransferase, cycloartenol synthase, cholesterol transport protein, and squalene synthetase (Ma et al. 2015). The squalene synthase of *Aurantiochytrium* sp. KRS101 has been produced recombinantly and shown to catalyze the conversion of two molecules of FPP into squalene in the presence of NADPH and Mg^{2+} (Hong et al. 2013b).

The highest reported squalene levels so far are 32 and 20 % of CDW and were obtained with the strains *Aurantiochytrium* spp., Yonez5–1 and 18 W-13a (Table 2). In strain 18 W-13a, squalene constituted 69 % of TL and in Yonez5–1 ~94 % (Nakazawa et al., 2012, 2014). The kinetics of the accumulation is not known, as the first sampling was made when the maximum level of biomass, total lipids, and squalene already was reached (Kaya et al. 2011). Also, strains primarily selected for their TAG and/or DHA contents have been shown to produce high levels of squalene, up to 6.6 % of TL or 3.3 % of the cell mass (Hoang et al. 2014; Qu et al. 2013a). In *A. mangrovei* PQ6 (formerly classified as *Schizochytrium mangrovei* PQ6), squalene was accumulated during the lipid accumulation phase, but faster than the increase in lipids, and reached a maximum of 33 mg/g of CDW (6 % of TL) and 1.0 g/l after 4 days (Hoang et al. 2014). In contrast, *Schizochytrium* sp. CCTCC M209059 produced relatively high amounts of squalene in the growth and early lipid accumulation phase, comprising 38 % of TL (30–40 % TL of CDW) and 11 % of CDW (~3 g/l). The TL continued to increase to 70 %, but the squalene content decreased in actual concentrations (g/l), and had disappeared at the end of the TAG accumulation phase (Ren et al. 2014a). The reported volumetric productivities are in the order of 0.2–1.4 g/l day (Table 2). The cell dry weights for the two strains accumulating 20–30 % squalene of CDW were only 3.4 and 6 g/l, respectively, indicating that a considerable potential for improved productivities if the cell density can be increased.

In conclusion, thraustochytrids are promising candidate production organisms for squalene. However, more work is needed on strain selection and studies of the production kinetics. For instance, to which degree the squalene production is growth associated or related to the lipid accumulation seems to vary between strains. A strain accumulating high levels with high volumetric productivities would be the selection for a process targeting squalene as the main product, while squalene associated with the biomass residues after oil extraction could be a valuable co-product to DHA production.

Thraustochytrids as carotenoid producers

Application of carotenoids and market prospects

Thraustochytrids are often pigmented and have been found to synthesize carotenoids (Aki et al. 2003; Burja et al. 2006; Raghukumar 2008; Singh et al. 2014). Carotenoids are a diverse group of natural pigments, mainly produced by plants and also by some microorganisms. The carotenoids can be divided into xanthophylls and carotenes. The two groups have similar molecular structures, but xanthophylls contain oxygen, while carotenes are purely hydrocarbons. The commercially

Table 2 Squalene levels and productivities in high producing thraustochytrids

Strain	CDW (g/l)	Squalene (mg/g CDW)	Squalene (g/l)	Squalene (g/l day)	Lipid (g/l)	V^a (l)	Time (day)	Reference
Aurantiochytrium sp. 18 W-13a	6.5	198	1.3	0.32	3.9	0.2	4	Kaya et al. (2011)
Aurantiochytrium sp. Yonez 5–1	3.4	318	1.1	0.27	1.1	0.2	4	Nakazawa et al. (2014)
A. mangrovei PQ6	30	33	1.0	0.25	15	15	4	Hoang et al. (2014)
A. mangrovei PQ6	31	33	1.0	0.25	16	100	4	Hoang et al. (2014)
Schizochytrium sp. CCTCC M209059[b]	25	11	0.3	0.18	8	1000	1.5	Ren et al. (2010)
Schizochytrium sp. CCTCC M209059[b]	50	84	4.3	1.4	21	7	3	Ren et al. (2014a)

[a] Culture volume

[b] Data derived from graphs in the cited reference

most important carotenoids are the xanthophylls astaxanthin, cantaxanthin, lutein, and zeaxanthin and the carotenes β-carotene and lycopene. The main application of carotenoids is as colorant in food and feed but with an increasing market in nutrition, pharmaceuticals, and cosmetics (Berman et al. 2015). The dominating carotenoid products in both quantities and value are chemically synthesized astaxanthin and cantaxanthin for aquaculture feed. Astaxanthin is the economically most important carotenoid globally, with a market size of above 350 million USD. In 2010, the global market for carotenoids was reported to be 1200 million USD and with an estimated annual growth of 2.3 % (Cutzu et al. 2013). In 2014, the Global Information (http://www.giiresearch.com/report/bc199439-global-market-carotenoids.html) reported the market value to 1500 million USD, with an estimate to reach 1800 million USD in 2019, indicating an annual growth rate of 3.9 %.

Today, most carotenoids are either chemically synthesized (e.g., astaxanthin, β-carotene, and cantaxanthin) or obtained from plant extracts. Up to now, microbially produced carotenoids have not been cost competitive with the chemically synthesized products, thus only serving higher-price, niche applications (Schmidt et al. 2011).

Carotenoid-producing strains and reported production data

Carotenoids, both xanthophylls and carotenes, are synthesized by the mevalonate pathway via the common intermediate lycopene (Fig. 1). No studies on the involved enzymes and genes in thraustochytrids have been reported.

A *Thraustochytrium* strain-denoted ONC-T18 was found to produce β-carotene and the xanthophylls astaxanthin, zeaxanthin, cantaxanthin, phoenicoxanthin, and echinenone, with total levels in the order of 30 μg/g CDW (Armenta et al. 2006; Burja et al. 2006). Higher carotenoid levels were reported for *Thraustochytrium* CHN-1, which produced 160 μg/g astaxanthin and 450 μg/g total carotenoids (Carmona et al. 2003), while *Aurantiochytrium* sp. KH105 produced 1.5–3.4 mg/g total carotenoids depending on media and cultivation conditions, with astaxanthin levels up to ~1.4 mg/g (Aki et al. 2003; Yamasaki et al. 2006). Highest carotenoid levels so far are reported for the strain Thraustochytriidae sp. AS4-A1 (*Ulkenia* sp.), producing up to 40 mg/g astaxanthin or 4 % of CDW. The cell densities were 8–12 g/l and the astaxanthin concentrations 0.30–0.45 g/l after 6 days. The astaxanthin production followed the DHA accumulation profile with the maximum concentration obtained somewhat later than the maximum DHA concentration (Quilodran et al. 2010). Interestingly, recombinant expression of the *Vitreoscilla* hemoglobin protein resulted in ninefold increased astaxanthin content in *Aurantiochytrium* sp. SK4 under microaerobic conditions, and this engineering approach also positively affected cell growth and PUFA production (Suen et al. 2014).

The maximum reported astaxanthin level of 4 % of CDW by thraustochytrids is far higher than the 6–7 mg/g obtained by the yeast *Phaffia rhodozyma* (Schmidt et al. 2011), and in the same order as for the phototropic microalgae *Haematococcus pluvialis*, both used for commercial production of astaxanthin. Thraustochytrids are therefore highly interesting production organisms for this carotenoid and for a simultaneous production of DHA and astaxanthin for salmon feed. Due to the low cell densities, the reported volumetric productivities are yet low for thraustochytrids. If high-cell-density fermentations can be developed, it is not unlikely that also the volumetric productivities will exceed the productivities reported for other potential production organisms. Further, current progress in development of genetic tools and genome sequencing of thraustochytrids should open for engineering of thraustochytrids as future production hosts for a range of carotenoids, utilizing the knowledge generated from the ongoing engineering efforts on other microorganisms, such as the yeasts *P. rhodozyma* and *Xanthophyllomyces dendrorhous* (Schmidt et al. 2011; Gassel et al. 2013).

Available genome sequences and tools for engineering of thraustochytrids

The scarcity of thraustochytrids genome data has so far limited the identification of targets for strain development. A partial genome of a thraustochytrid (QPX) identified as a parasite on the hard clam quahog has been published (Garcia-Vedrenne et al. 2013). More recently, the genome sequence of a good DHA producer *Schizochytrium* sp. CCTCC M209059 was published (Ji et al. 2015). Draft genome data of two thraustochytrids, *A. limacinum* SR21 and *S. aggregatum* ATCC 28209, are available from the Joint Genome Institute (JGI; http://genome.jgi.doe.gov/), and a genome scale metabolic model of *A. limacinum* SR21 was recently built and analyzed for DHA production (Ye et al. 2015). A few transcriptome studies of thraustochytrids have also been published (Ma et al. 2015; Rubin et al. 2014).

However, even if more genome sequences now are becoming available, efficient methods for gene transfer are still scarce for thraustochytrids. To date, there are only 12 thraustochytrid strains, belonging to 4 different genera, which have been genetically manipulated, and only a few methods have been successfully employed for delivering transgenes into thraustochytrid cells. Except for one published study, where the authors established *Agrobacterium*-mediated transformation for *Schizochytrium* sp. (Cheng et al. 2012), electroporation or particle bombardment using linear DNA appear to be the choice (Table 3), but the reported methods

Table 3 DNA delivery methods, selection, and promoters used in thraustochytrids

Strain	Delivery methods	Selection	Promoters	Mutated/expressed genes	References
A. limacinum mh0186	Electroporation	G418	EF-1a/ubiquitin	NA	Sakaguchi et al. (2012)
A. limacinum mh0186	Electroporation	G418	EF-1a/ubiquitin	Expression of Δ12 desaturase	Matsuda et al. (2011)
A. limacinum OUC88	Electroporation	Chloramphenicol and zeocin	TEF1 and PGK	NA	Sun et al. (2015)
A. limacinum F26-b	Electroporation	Hygromycin	Ubiquitin	Mutation in lysophospholipid acyltransferase *PLAT1*	Abe et al. (2014)
Aurantiochytrium sp. MP4	Electroporation	Zeocin	Tubulin	Expression of hemoglobin	Suen et al. (2014)
Aurantiochytrium sp. SK4	Electroporation	Zeocin	Tubulin	Expression of hemoglobin	Suen et al. (2014)
Aurantiochytrium sp. KRS101	Electroporation	Cycloheximide	Constitutive GAP	Expression of Δ12 desaturase	Hong et al. (2013a)
Schizochytrium sp. 204-06 m	Particle bombardment	G418	EF-1a/ubiquitin	NA	Sakaguchi et al. (2012)
Schizochytrium sp. TIO01	Electroporation	Zeocin	TEF1	NA	Cheng et al. (2011)
Schizochytrium sp. TIO01	Electroporation	G418	TEF1	Expression of acetyl-CoA synthetase *ACS*	Yan et al. (2013)
Schizochytrium sp. TIO1101	*Agrobacterium tumefaciens*	G418	TEF1	NA	Cheng et al. (2012)
Schizochytrium sp. ATCC 20888	Particle bombardment	Zeocin	Tubulin	Mutation in PUFA synthase *PFA1*	Lippmeier et al. (2009)
Schizochytrium sp. ATCC 20888	Particle bombardment	Paromomycin	EF-1	Expression of hemagglutinin	Bayne et al. (2013)
T. aureum ATCC 34304	Particle bombardment	G418	EF-1a/ubiquitin	Mutation in Δ5 desaturase	Sakaguchi et al. (2012)
Parietichytrium sp. TA04Bb	Particle bombardment	G418	EF-1a/ubiquitin	NA	Sakaguchi et al. (2012)

NA not available

do not necessarily work for any thraustochytrid strain. However, other methods that have been successfully applied for photosynthetic microalgae may be applicable in thraustochytrids as well. Kim et al. (2014) reported that *Chlamydomonas reinhardtii* with an intact cell wall could be transformed by the use of positively charged aminoclay nanoparticles, and last year a bacterial conjugation-based method was established that directly transfers episomes from *Escherichia coli* to the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* with high transformation efficiency (Karas et al. 2015). These novel approaches might open new opportunities for thraustochytrid transformation. Recently, Sun et al. (2015) demonstrated that the *cre*/*loxP* system could be utilized to generate markerless mutations in *A. limacinum*, and this might enable the construction of strains with several mutations.

Besides the DNA delivery method, the selection method and the control of gene expression need to be taken into consideration when establishing genetic toolkits for thraustochytrids. Only a limited range of selection markers has been employed to select thraustochytrid transformants (Table 3). The concentrations needed for growth inhibition are strain dependent, indicating that the resistance to antibiotic selection of thraustochytrids need to be tested on a case-by-case basis (Cheng et al. 2011; Lippmeier et al. 2009; Suen et al. 2014). It has also been reported that mutating the gene encoding ribosomal protein L44 (P56E) renders *Aurantiochytrium* sp. KRS101 cycloheximide resistant (Hong et al. 2013a). In addition, it has been demonstrated that the enhanced fluorescence protein (EGFP) can be functionally expressed in *A. limacinum*, *T. aureum*, and *Schizochytrium* sp. (Cheng et al. 2012; Sakaguchi et al. 2012; Sun et al. 2015). In order to control transgene expression, promoters from highly expressed endogenous genes are often used. Table 3 also summarizes which promoters were used for controlling transgene expression in the cited studies.

The methods described above have mainly been used to elucidate the role of enzymes related to fatty acid synthesis, yet to a lesser degree for strain improvement. This will change when genome sequences now become available, enabling identification of targets for genetic engineering. In parallel with the efforts in sequencing, there is a need to further develop genetic toolkits.

Future prospects

Despite more than 20 years of research on DHA production by thraustochytrids, basic knowledge of biochemistry, genetics, and regulation of the synthesis is still lacking. Of particular importance for future strain and process improvements is the understanding of how the two main pathways used for fatty acid synthesis is regulated and how higher rates of the PKS pathway can be obtained. The few reported studies on squalene and carotenoids indicate a high production potential of these compounds. However, the information about the production kinetics is scarce, for instance, to which degree the production is growth associated or related to the lipid accumulation. The genome sequences that now are becoming available will help to generate hypotheses that can be verified experimentally by various omic analyses, biochemical studies of enzyme activities, and the use of labeled substrates. This will open for a system-level metabolic engineering for strain improvement.

DHA-rich oils produced by thraustochytrids are already commercialized for the human market. Production of DHA for other, lower-price markets, such as feed ingredients, as well as the production of squalene and carotenoids, will depend on the availability and price development for the current feedstocks for these products. Squalene and carotenoids could also be added-value co-products from a potential future large-volume production of DHA as a fish feed ingredient and thereby improve the process economy.

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