Microbial Oils as Nutraceuticals and Animal Feeds

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Abstract

Lipids and oils are produced by all single-cell organisms for essential structural and functional roles; however, the term single cell oils (SCOs) is mainly restricted to describe the lipids produced by a limited number of oleaginous microorganisms (archaea, bacteria, yeast, fungi, and microalgae) with oil contents higher than 20% of biomass weigh. SCOs have different fatty acid compositions from those of plant seed or fish oils and are nowadays considered as new sources of nutraceuticals and animal feeds. In spite of the current commercial success of some SCOs, the development of more efficient microbial fermentation processes and the possibility of manipulating by systems metabolic engineering the lipid composition of cells require new biotechnological strategies to obtain high yields of the desired SCOs. Understanding the synthesis and regulatory mechanisms involved in the production of SCOs is fundamental to eliminate the metabolic bottlenecks that impair achieving high oil yields.

1 Introduction

The term, single cell oils (SCOs), also named microbial lipids or microbial oils, was created by Ratledge and Wynn (1974) to identify those lipids of single-cell organisms – microorganisms – that would be suitable for human and animal consumption as an alternative to plant and animal oils and fats. SCO was initially created to designate the triacylglycerol (TAG) fraction of the total cell lipids to be equivalent to the commercial plant and animal oils. However, SCO is now used to include all types of fatty acid (FA) containing lipids, and therefore, SCO includes not only free fatty acids and TAGs but also other complex lipids such as glycosylated and sulfur-containing lipids.

Lipids and oils are produced by all single-cell organisms for essential structural and functional roles; however, the term SCO is nowadays mainly restricted to

describe the lipids produced by a limited number of organisms, named oleaginous microorganisms (see below), which are those species able to accumulate 20–80% of lipids per dry biomass as a reserve storage material.

Although the prospects of obtaining useful and cheap SCO have been considered more than 60 years ago (Ratledge 2013), it was only in the past three decades that they have begun to be produced commercially at large scale for food and feed markets (Béligon et al. 2016). More recently, SCO has been considered as possible source of biofuels opening new perspectives for the energy and transport industrial sectors (Hu et al. 2008).

One of the main advantages of SCOs is that their production processes are independent from season, climate, and location, and moreover, they can be synthesized using a wide range of carbon sources including organic waste and renewable carbon sources (see below).

On the other hand, because SCOs have different FA compositions from those of plant seed or fish oils, they are considered as new sources of nutraceuticals highly valuable for human life. Nutraceuticals can be defined as foodstuffs, such as a fortified food or dietary supplement that provides health benefits in addition to its basic nutritional value. Nevertheless, this term and its applications have not attained a common regulatory definition in all countries so far.

In spite of the current commercial success of some SCOs, the development of more efficient microbial fermentation processes and the possibility of manipulating the lipid composition of cells require new biotechnological strategies to obtain high yields of the desired SCOs. Although recombinant oleaginous microorganisms can be engineered and accepted by industry and society, nonetheless, several approaches have been also employed to convert other model GRAS (generally regarded as safe) microorganisms into oleaginous cells by genetic engineering.

Therefore, taking into account all these considerations, there is a growing interest to know how the microbial oils can be produced in a cost-effective manner and how they can be tailor-made to meet the demands of the two most important branches of the oil industries: oils for human and animal consumption and oils for biofuels. However, only the food and feed uses of microbial oils will be reviewed in this chapter. In this sense, other recent journal reviews can provide complementary information on this matter (Béligon et al. 2016; Bellou et al. 2016; Lee et al. 2016; Ochsenreither et al. 2016; Yoshida et al. 2016; Bharathiraja et al. 2017).

2 Oleaginous Microorganisms

As mentioned above oleaginous microorganisms are defined as cells with oil contents higher than 20% of biomass weigh. Single cell oils are produced by different microorganism groups such as archaea, bacteria, algae, yeasts, and fungi. When growing on limiting concentrations of a key nutrient (typically nitrogen) and enough or excess of carbon source, these microorganisms will utilize the available carbon to synthetize and store it as reduced lipids like TAG or sterol esters (SE). Table 1 summarized the most important oleaginous microorganisms described so far. While

the eukaryotic microorganisms synthesize TAGs with similar compositions to vegetable oils, prokaryotic cells also synthesize other neutral lipids (e.g., polyhydroxyalkanoates (PHAs) and wax esters (WEs)), and thus the peculiarities of these organisms are described below case by case.

2.1 Archaea

Archaea do not synthesize fatty acyl esters, instead, their lipids are based on isoprenoid chains. Archaeal membrane lipids are very different from those of bacteria and eukaryotes as they are made up of saturated chains containing methyl branches, attached to glycerol by ether linkages with a stereochemistry in the two positions of the glycerol opposite that of conventional mesophilic lipids. So far, no accumulation of TAGs has been reported yet in archaea. However, as in bacteria, archaea produce PHA under conditions of nutrient limitation when carbon is available in excess.

2.2 Bacteria

Generally, bacteria produce SCO with a composition quite different from other microorganisms. Bacteria can produce a large variety of complex neutral and polar lipids, but only few of them can accumulate large amounts of TAGs. Oleaginous bacteria have the advantage of showing high cell growth rates under simple cultivation methods. In bacteria, the most abundant class of neutral lipids used as intracellular carbon and energy storage compounds are PHAs (bioplastics), such as polyhydroxybutyrate (PHB) or polyhydroxyvalerate (PHV), TAG, WE, and, to a lesser extent, SE.

Unlike eukaryotes only a minority of prokaryotes can accumulate TAG or WE. The highest level of TAG accumulations has been reported in the actinomycetes group, such as the genera *Mycobacterium*, *Streptomyces*, *Rhodococcus, Micromonospora, Dietzia, Gordonia*, and *Nocardia* (Kosa and Ragauskas 2011). TAGs in cells of *Rhodococcus opacus* cells accounted up to 87% of the cellular dry weight. Some bacteria such as *Rhodococcus ruber* are capable of accumulating both PHAs and TAGs (Garay et al. 2014). Accumulation of WE of about 200 nm diameter has been reported in some *Acinetobacter* spp. (Wältermann and Steinbüchel 2005). Some marine-related *λ-Proteobacteria* like *Alcanivorax borkumensis* accumulate while growing in petroleum hydrocarbons' lipid droplet (LD) reserves that consist of mixtures of TAGs and WE (Kalscheuer et al. 2007). Numerous bacterial species of marine origin have now been shown to produce very long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Such isolates have been found to be particularly prevalent in high-pressure, low-temperature deep-sea habitats and permanently cold marine environments (DeLong and Yayanos 1986; Yano et al. 1997).

Table 1 Most relevant oleaginous microorganisms and marketed products

Organism	Product	Company
Bacteria	Lipids	
Rhodococcus opacus	Lipids	
Rhodococcus jostii	Lipids	
Yeast		
Yarrowia lipolytica	EPA. EPA-SCO	E.I. du Pont (USA)
Rhodosporidium toruloides	Lipids	
Rhodotorula glutinis	Lipids	
Lipomyces starkeyi	Lipids	
Cryptococcus albidus	Lipids	
Candida curvata	Lipids	
Fungi		
Mucor (javanicus) circinelloides	Oil of Javanicus (GLA-rich oil)	J & E Sturge (UK)
Cunninghamella echinulata	GLA. DHA-SCO	Martek/DSM (The Netherlands)
Mortierella isabellina	ALA. GLA	Sigma (USA)
Mortierella alpina	ARA. EPA. ALA. ARA-SCO. CABIO-oil	DSM (The Netherlands). Cargill Alking Bioengineering Co. Ltd. (Hubei, China). Suntory Co (Japan)
Pythium ultimum	Lipids	
Rhizopus arrhizus	Lipids	
Microalgae		
Nannochloropsis sp.	EPA	Aurora. Cellana. Qualitas Health. Necton. Reed Mariculture. Fitoplanton Marino. Yantai. Astaxa. Proviron. Archimede
Schizochytrium sp.	DHA. DHA-SCO-S. DHA for plus. DHAgold. NeoGreen. AlgaPrime DHA. EPA	Martek/DSM (The Netherlands). Alltech (USA). DSM Nutritional Products (USA). Coppens International BV (The Netherlands). TerraVia Holdings Inc. (formerly Solazyme, USA) and Bunge Ltd. (USA). Veramaris (DSM-Evonick)
Phaeodactylum tricornutum	DHA. EPA	Micoperi Blue Growth (Italy)
Porphyridium cruentum	EPA	Asta Technologies Ltd. (The Netherlands)

(*continued*)

Table 1 (continued)

Organism	Product	Company
Chaetoceros sp.	DHA	
Isochrysis galbana	DHA	Symrise AG (Germany)
Crypthecodinium cohnii	EPA	Martek/DSM (The Netherlands)
Chlorella sp.	DHA	
Thraustochytriidae sp.	DHA	
Aurantiochytrium sp.	DHA DHA-natur	Lubrizol Corp (USA) ADM Animal Nutrition (USA)
Ulkenia sp.	DHA. DHA-CL. DHA-*Aid*	Lonza (Switzerland)
Algae	Onavita DHA	ADM (USA)
Algae	Onavita ALA	ADM (USA)

2.3 Yeasts

Most oleaginous yeast can accumulate lipids at levels of more than 40% of their dry weight and as much as 70% under nutrient-limiting conditions. However, the lipid content differs, and fatty acid profiles differ between species. The oily yeast genera include *Candida, Cryptococcus, Lipomyces, Rhodosporidium, Rhodotorula, Rhizpus, Trichosporon*, and *Yarrowia*. Yeasts exhibit advantages for lipid production due mainly to their low duplication times and metabolic versatility. They can utilize many different carbon sources (e.g., glucose, xylose, glycerol, starch, cellulose hydrolysates, and industrial and municipal organic wastes) for the production of different lipids like TAG, surfactants, or PUFAs. The yeast *Lipomyces starkeyi* is unique because it does not reutilize their own lipids accumulating high amounts of lipids (Holdsworth et al. 1988).

2.4 Fungi

Oleaginous fungi are reported to accumulate intracellular lipid up to 50–70% in the form of cytosolic lipid bodies mainly composed of TAGs (Sancholle and Lösel 1995). The distribution and diameters of their lipid bodies vary with the organism, growth phase, and environmental conditions. Glucose, lactose, starches, oils, corn steep liquor, and agricultural waste have been used as carbon sources for lipid production in fungi (Thevenieau and Nicaud 2013).

The diversity of fungal species has facilitated the selection of oleaginous strains as they can compete at commercial scale with the traditional lipid production from plant and animal sources on the basis of several reasons: (i) high growth rates, (ii) the

ability to grow on cheap waste materials as substrate for oil production, (iii) the controlled environment that is not affected by seasonal and climatic conditions, and (iv) the possibility to engineer the key steps of lipid synthesizing enzymes to end up with the formation of lipid and other valuable products such as PUFAs. Fungi species, such as *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, or *Mortierella isabellina*, are reported to accumulate lipids (Bellou et al. 2012). These fungi are explored mainly for the production of special lipids such as PUFAs. The oleaginous fungus, *Mucor rouxii*, is known to accumulate high amounts of intracellular lipids and γ-linolenic acid (GLA). *Mucor alpine* and *Mortierella alliacea* have a high productivity of arachidonic acid (ARA) (Eroshin et al. 2000; Aki et al. 2001). The production of PUFAs is related to the age of the mycelia, since the production decreases as the cells grew older (Fakas et al. 2009).

2.5 Microalgae (Cyanobacteria and Algae)

The term microalgae, in applied phycology, usually includes the microscopic algae sensu stricto and the photosynthetic bacteria (i.e., cyanobacteria), formerly known as *Cyanophyceae*. The cell structure is eukaryotic in microalgae and prokaryotic in cyanobacteria. Several microalgae species are able to produce large amounts of neutral lipids, typically in the form of TAGs as storage products for carbon and energy under specific environmental stress conditions, such as nitrogen or phosphate limitations. The lipid content can reach up to 80% in dry biomass, and therefore these microorganisms are referred to as oleaginous microalgae (Chisti 2007; Hu et al. 2008). The interest in algae lipids arises mainly from the fact that these organisms are able to synthesize considerable quantities of PUFAs that reach humans or animals via the food chain or are used as food supplements (Bellou et al. 2014).

In some species belonging to *Porphyridium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Tetraselmis*, *Phaeodactylum*, *Chlorella*, and *Schizochytrium*, lipid content varies between 30 and 50%. Higher productivities can be reached by varying the culture conditions, like, for example, temperature, irradiance, or nutrient availability. The indisputable advantage of microalgae is that they can use carbon dioxide and sunlight as carbon and energy sources, respectively, for photoautotrophic growth. In addition, they can use organic carbon under heterotrophic or mixotrophic culture conditions.

Microalgae applications range from human and animal nutrition to cosmetics and the production of high-value molecules. The majority of applications concern biomass production destined for animal or human consumption. There is also an increasing interest in the use of microalgae lipids in numerous commercial applications, such as food, chemical, and pharmaceutical industries (Bellou et al. 2014). However, there are still some limitations in using microalgae to produce PUFAs in large scale due to the low biomass density in the reactors under industrial conditions.

There are few strains with special fatty acids biosynthetic capacity and therefore of industrial interest. The most prominent DHA producer among microalgae is the heterotrophic dinoflagellate *Crypthecodinium cohnii* containing more than 50%

(w/w) DHA of total fatty acids (Jiang and Chen 2000; Ratledge et al. 2001; de Swaaf et al. 2003a,b). Other significant DHA producers are microalgae of the genus *Schizochytrium*, e.g., *Schizochytrium* sp. S31 (Wu et al. 2005), *Schizochytrium* G13/2S (Ganuza and Izquierdo 2007), and *Schizochytrium limacinum* (Chi et al. 2007). *Amphidinium* sp. and *Prorocentrum triestinum* are also known as efficient DHA producers (Makri et al. 2011). *Porphyridium cruentum* and *Nannochloropsis salina* are able to synthetize EPA with content of 25% of total lipids (Bellou and Aggelis 2013).

3 Microbial Oil Biosynthesis

3.1 Pathways

There are two sequential steps in the synthesis of microbial oils: FA and TAG syntheses (Ratledge 2004; Garay et al. 2014). TAGs are also known as "neutral lipids."

The pathway for saturated FA synthesis is conserved among microbial species. It starts from acetyl-CoA. In yeast, fungi, and bacteria, acetyl-CoA is produced from organic carbon sources (glucose, acetate, etc.). Microalgae, on the other hand, may use either inorganic carbon (CO_2) or organic carbon sources (Liang and Jiang. 2013). Carboxylation of acetyl-CoA forms malonyl-CoA. This molecule serves as a building block for several steps of condensation and reduction, resulting in FAs of different chain length.

The synthesis of saturated FAs is catalyzed by the enzyme fatty acid synthase (FAS). There are two groups of FAS, based on the organization of their catalytic units. Type I FAS, present in fungi and yeast genomes, carries out all steps of fatty acid biosynthesis as a multimeric protein complex (Schweizer and Hofmann 2004). Type II FAS is composed of independent polypeptides and is found in bacteria and microalgae (White et al. 2005). Regardless of their classification, both types of FAS include the same seven enzymatic components: a malonyl/acetyltransferase, an acyl carrier protein, a ketoacyl synthase, a ketoacyl reductase, a dehydrase, an enoyl reductase, and a thioesterase.

The first committed step of FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC) and consists of an ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA (Fig. 1a). This reaction takes place in the cytosol in heterotrophs or both, cytosol and plastids, in autotrophs (Bellou et al. 2016). As in the case of FAS, there are two different forms of ACC. The first one, present in prokaryotes (AccABCD) and archaea, consists of three functional enzymes: biotin carboxylase (BC, AccC), biotin carboxyl carrier protein (BCCP, AccB), and carboxyltransferase (CT, AccA, and AccD) (Guchhait et al. 1974; Lombard and Moreira 2011). The eukaryotic form (Acc1) consists of three functional domains, homologous to the bacterial subunits, on a single polypeptide (Konishi and Sasaki 1994). The reaction proceeds in two steps and is biotin-dependent, as shown in Fig. 1b (Tran et al. 2015).

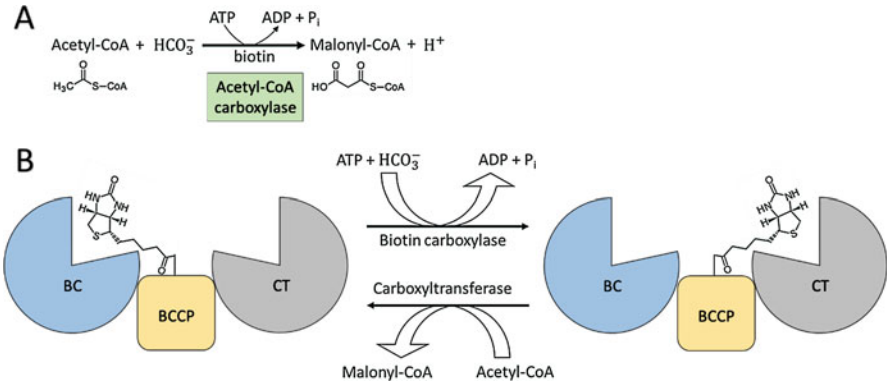

Fig. 1 Reaction catalyzed by the ACC. (**a**) Overall reaction catalyzed by ACC. (**b**) The two steps of the reaction catalyzed by ACC. In the first step, BC catalyzes the carboxylation of biotin, a cofactor covalently linked to BCCP. In the second step, the carboxyl group is transferred from carboxyl-BCCP to acetyl-CoA by CT

Malonyl-CoA is subsequently transformed into malonyl-ACP by the action of malonyl-CoA: ACP transacylase (MAT). In type I FAS, this reaction is catalyzed by FabD. The next step is carried out by the β-ketoacyl-ACP synthases (KS). These enzymes are involved in FA chain extension via Claisen condensation of fatty acyl-thioesters and malonyl-ACP to form a β-ketoacyl-ACP intermediate elongated by two carbon atoms.

Typically, there are two or three KSs involved in type II FAS: FabH (KS III), FabF (KS II), and FabB (KS I). In many cases, the *fabB* gene is absent (Kuo and Khosla 2014; Wang and Cronan 2004). The initial cycle of elongation is catalyzed by FabH, involving condensation of malonyl-ACP and acetyl-CoA, while subsequent cycles of elongation are performed by FabB or FabF. On the other hand, type I FAS contains only one KS. Following the condensation reaction, the β-ketoacyl-ACP is reduced to β-hydroxyacyl-ACP using NADPH and H^+. This reaction is catalyzed by a β-ketoacyl-ACP reductase (KR). The same enzyme is denominated FabG in type II FAS. After that, β-hydroxyacyl-ACP is dehydrated to trans-2-enoyl-ACP by water elimination. The enzyme involved in this reaction is a β-hydroxyacyl-ACP dehydrase (DH). In type II FAS, this reaction may be catalyzed by FabA or FabZ. However, in some organisms in which desaturation step is not dependent of type II FAS, such as cyanobacteria or gram-positive bacteria, FabA is absent. The last step in each elongation cycle is carried out by an enoyl-ACP reductase (ER). This enzyme catalyzes the reduction of 2-enoyl-ACP to fatty acyl-ACP at the expense of H^+ and NADPH/NADH. In type II FAS, this enzyme is denominated FabI. Finally, acyl-ACP-thioesterase (FAT) cleaves the acyl chain and liberates the FA. The pathway is represented in Fig. 2.

There is considerable diversity in the mechanisms used by bacteria to generate unsaturated FAs (uFAs). In most bacteria, the bifunctional FabA is involved in the synthesis of uFAs (Heath and Rock 1996) (Fig. 3a). In *Streptococcus pneumoniae*, FabM is the enzyme responsible for introducing the double bond, which is unrelated

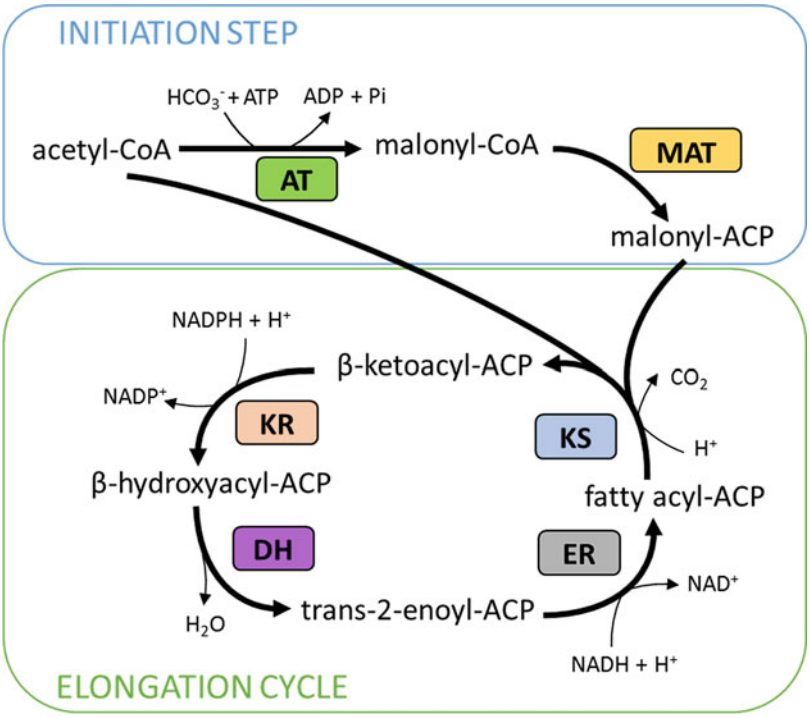

Fig. 2 Fatty acid synthesis pathway

to FabA, but it is a member of the hydratase/isomerase superfamily (Marrakchi et al. 2002). In some bacteria, there is an alternative route for the generation of uFAs after the elongation cycle. *Bacillus subtilis* expresses a desaturase, Des, which insert a double bond in FA chains (Altabe et al. 2003). *Pseudomonas aeruginosa* produces uFAs using FabA/B pathway. However, it possesses two aerobic desaturases: DesA, introduces the double bond into acyl chains attached to phospholipids and has a similar structure to the *B. subtilis* Des enzyme, and DesB, an inducible acyl-CoA Δ9-desaturase (Zhu et al. 2006).

One pathway for PUFA synthesis involves the concerted action of desaturases and elongases (Uttaro 2006) (Fig. 3b). It is found in lower eukaryotes and is denominated the aerobic pathway, because desaturation is an aerobic process that uses molecular oxygen. Some yeast species, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, synthesize FAs up to oleic acid (C18:1n-9). These species only possess a Δ9-desaturase (OLE1). Other yeasts, such as *Saccharomyces kluyveri* and *Kluyveromyces lactis*, are able to produce up to ALA (alpha-linolenic acid; C18:3n-3). Both possess a Δ9-desaturase, as well as Δ12-desaturase and Δ15-desaturase (also known as ω3-desaturase) (Uemura 2012).

A number of lower eukaryotes such as fungi and microalgae produce large amounts of PUFAs with chain lengths of C20 and greater (Uttaro 2006), following the aerobic pathway. It is predicted that these organisms contain the complete set of

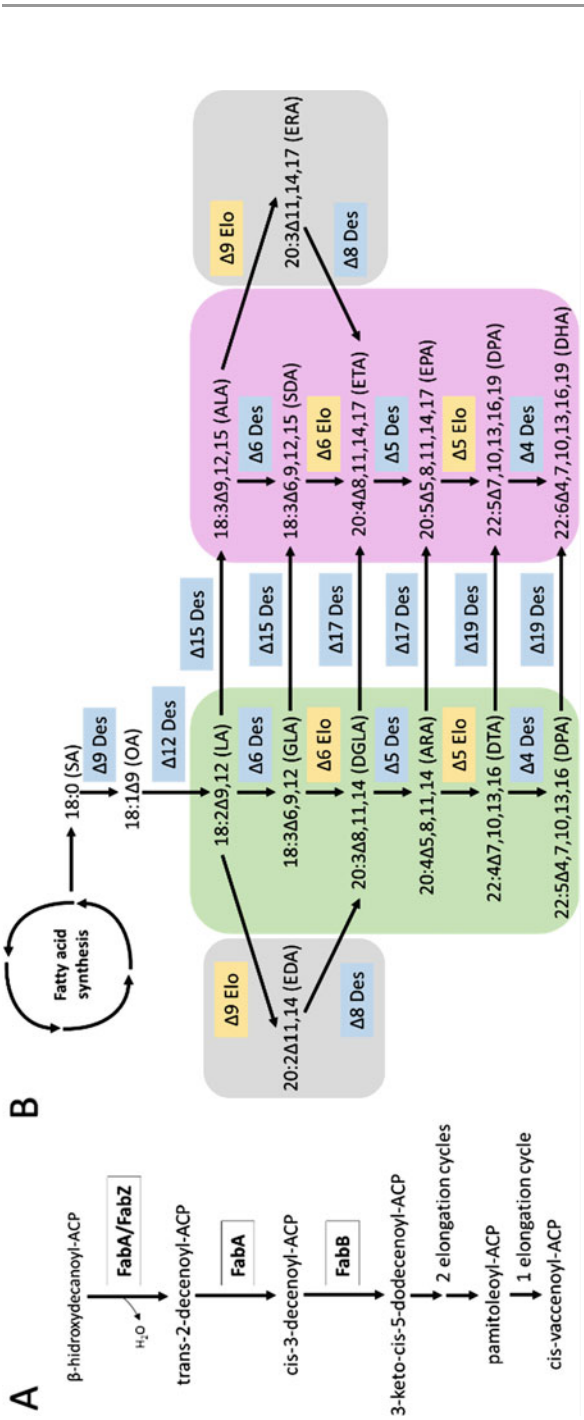

Fig. 3 Unsaturated fatty acid synthesis. (**a**) Synthesis of unsaturated fatty acids via FAS II in most bacteria. FabA carries out the dehydration of β-hydrodecanoyl-ACP to trans-2-decenoyl-ACP. After that, it performs the isomerization of trans-2-decenoyl-ACP to cis-3-decenoyl-ACP. The product of FabA isomerization reaction is used by FabB, skipping the reductase step and initiating the elongation of a monounsaturated FA. The product of the first two elongation cycles is palmitoleoyl-ACP, which may be the substrate for the next elongation cycle to produce cis-vaccenoyl-ACP. (**b**) Synthesis of PUFAs via aerobic pathway. The green-shaded and the pink-shaded areas represent omega-6 and omega-3 biosynthesis pathway (conventional Δ6-pathway), respectively. The gray-shaded area represents the alternative Δ8-pathway. Desaturases and elongases are represented by blue and yellow rectangles, respectively. Fatty acids are OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, di-homo-γ-linolenic acid; ARA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid

enzymes involved in the transformation of stearic acid to EPA (20:5n-3) and DHA (C22:6n-3). Other lower eukaryotes also hold a Δ8-desaturase involved in an alternate aerobic pathway to produce C20 PUFAs (Arao and Yamada 1994; Qi et al. 2002; Wallis and Browse 1999). Most of the higher producers of EPA and DHA are microalgae and fungi, inhabitants of cold marine environments.

The anaerobic pathway occurs in eukaryotic microalgae and some bacteria (marine bacteria and terrestrial myxobacteria). In these organisms, PUFA synthases are huge enzyme complexes with multiple catalytic domains, denominated polyketide synthases (PKSs). PKSs carry out the same reactions as FAS and use the same small protein, an acyl carrier protein (ACP), as a covalent attachment site for the growing carbon chain (Metz et al. 2001; Hayashi et al. 2016; Cronan and Thomas 2009).

The most important anaerobic pathway to produce TAGs is the *sn*-glycerol-3-phosphate (G3P) or Kennedy pathway (Fig. 4). In the first step, G3P is acylated with an acyl-CoA to form lysophosphatidate (LPA), which is catalyzed by G3P O-acyltransferase (GPAT). LPA is further condensed by LPA acyltransferase (LPAT), with another acyl-CoA to produce phosphatidate (PA). Afterward, PA can be dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG). Finally, synthesis of TAG is catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT), which incorporates the third acyl-CoA into DAG (Amara et al. 2016). In yeast, in addition to DGAT-catalyzed TAG formation, there is an acyl-CoA-independent biosynthesis of TAG. The enzyme involved in this process, phospholipid:diacylglycerol acyltransferase (PDAT), transfers an acyl group from the *sn*-2 position of phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine) to *sn*-3 position of diacylglycerol, yielding TAG and sn-1-lysophospholipid (Banas et al. 2013). It was also found that *Acinetobacter*

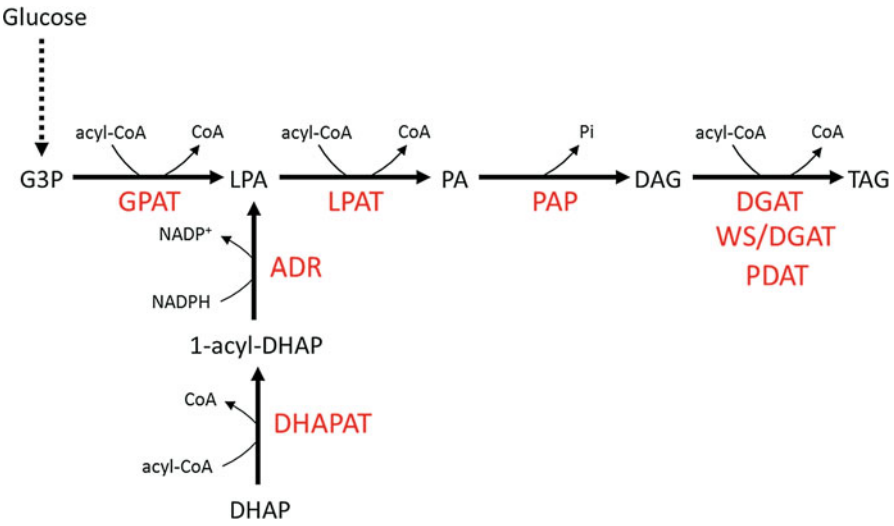

Fig. 4 Schematic diagram of the main lipid classes and biochemical pathways involved in the production of TAG

calcoaceticus ADP1, a bacteria able to accumulate both WE and TAG, has a bifunctional WE synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) that exhibits both WE synthase and DGAT activities (Stöveken et al. 2005).

In yeast, LPA is synthetized from two precursors: G3P and dihydroxyacetone phosphate (DHAP) (Athenstaedt et al. 1999). Synthesis of LPA from DHAP occurs in two acylation steps. The first is catalyzed by a DHAP acyltransferase (DHAPAT), which transform DHAP into 1-acyldihydroxyacetone phosphate (1-acyl-DHAP). In the second step, 1-acyl-DHAP is reduced in an NADPH-dependent reaction catalyzed by 1-acyldihydroxyacetone phosphate reductase (ADR), yielding LPA (Carman and Han 2009). The reactions sequence is also represented in Fig. 4.

3.2 Regulation

There is little information about the regulation of TAG synthesis in oleaginous microorganisms. In the most widely studied and engineered oleaginous yeast, *Yarrowia lipolytica*, the regulatory network of TAG biosynthesis is starting to be elucidated (Zhu and Jackson 2015). *Y. lipolytica* is a promising microbial cell factory for the production of TAGs, because it can accumulate a large amount of fatty acids in the form of the storage lipid TAG in the cell (Seip et al. 2013; Kerkhoven et al. 2016) reconstructed a genome-scale metabolic model of this yeast and used this for integrative analysis of multilevel omics data. Metabolite profiling and lipidomics were used to quantify the cellular physiology, while regulatory changes were measured using RNAseq. Analysis of the data showed that lipid accumulation in *Y. lipolytica* does not involve transcriptional regulation of lipid metabolism but is associated with regulation of amino acid biosynthesis, resulting in redirection of carbon flux during nitrogen limitation from amino acids to lipids.

Limitation of nitrogen during continued growth of *Y. lipolytica* provokes a dramatic response in the biomass composition and an increase in virtually all lipids. *Y. lipolytica* under nitrogen limitation attempts to minimize the usage of nitrogen. In these conditions, transcripts related to amino acid metabolism are downregulated. Nitrogen restriction triggers nitrogen catabolite repression, which is regulated by interaction of four GATA transcription factors: Gln3, Gat1, Gzf3, and Dal80 (Kerkhoven et al. 2016). These transcription factors are characterized by their ability to bind to the DNA sequence "GATA." As an alternative route to recycle nitrogen, *Y. lipolytica* has an ortholog of the *Aspergillus nidulans* xanthine dehydrogenase (Cultrone et al. 2005), which is absent in *S. cerevisiae*. By means of this pathway, purines can be degraded via allantoin to release ammonia. Several genes in this pathway are under control by Gat1 and Gln3, whereas this whole pathway is upregulated during nitrogen restriction. A more central role in nitrogen sensing and signalling is played by the TOR complex (Zhang et al. 2011). High-quality nitrogen sources such as ammonium or glutamine stimulated the activation of TOR complex (Stracka et al. 2014), whereas nitrogen starvation inhibits this complex (Zaman et al. 2008). Based on a study of differential gene expression profile, Kerkhoven et al. determined the upregulation of autophagy, proteasome, peptidases,

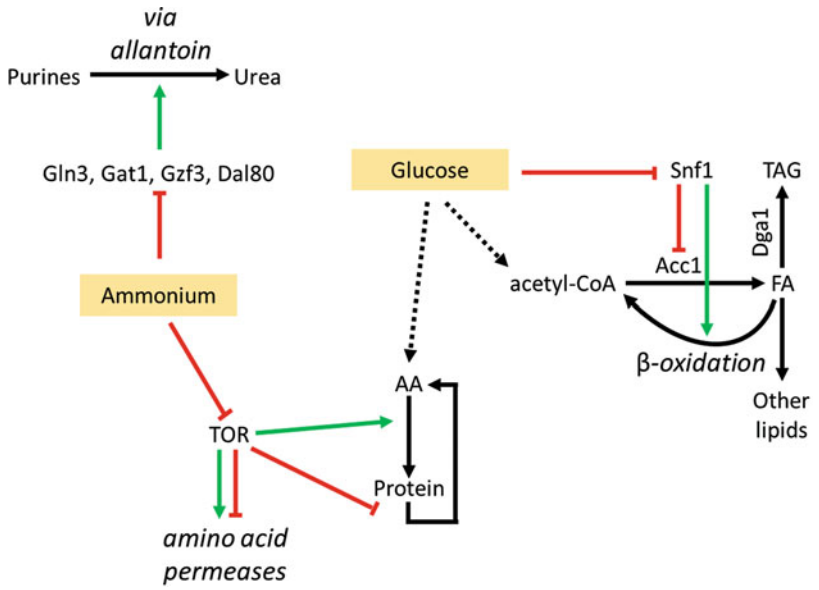

Fig. 5 Schematic overview of regulation that occurs during nitrogen limitation in a high lipid-producing strain. (Figure adapted from Fig. 5 in Kerkhoven et al. 2016)

and ubiquitylation mediated by TOR complex during nitrogen starvation (Kerkhoven et al. 2016). Another key regulator of metabolism is Snf1, a protein kinase that is involved in many signalling pathways. In *S. cerevisiae* Snf1 inhibits the activity of the acetyl-CoA carboxylase Acc1 by phosphorylation (Shi et al. 2014), and these phosphorylation sites on Acc1 are conserved in *Y. lipolytica* Acc1. Moreover, Snf1 increases expression of β-oxidation. Nonetheless, it is likely that additional regulation takes place such as posttranslational modifications, as it is known that Acc1 activity is repressed by phosphorylation by Snf1 (Kerkhoven et al. 2016). This regulatory pathway is represented in Fig. 5.

In microalgae, the mechanism of nitrogen starvation induced neutral lipid accumulation. When the nitrogen levels required for the protein synthesis of growing cells are insufficient, the excess of carbon from photosynthesis is diverted into storage molecules, such as TAGs (Scott et al. 2010). The phenomenon of nitrogen starvation in microalgae has been analyzed using diverse omics approaches (Blaby et al. 2013; Dong et al. 2013; Schmollinger et al. 2014; Park et al. 2015). These studies have notably contributed to the elucidation of TAG biosynthetic pathway in microalgae; however, limited putative regulators of this metabolic response have been pointed out. Nitrogen response regulator 1 was identified in *Chlamydomonas* as a putative transcription factor with a regulatory role in nitrogen assimilation and TAG accumulation in nitrogen-depleted conditions (Boyle et al. 2012). An insertional mutation of nitrogen response regulator 1 led to a 50% reduction in TAG accumulation; it is possible that there are other key regulators controlling lipid synthesis under nitrogen stress. Another study identified TAG accumulation

regulator 1, a tyrosine kinase involved in the control of TAG accumulation upon both nitrogen and sulfur deficiencies (Kajikawa et al. 2015). The changes in TAGs cannot only be explained by modulation in intracellular nitrogen or carbon/nitrogen availability. These changes appear to be controlled by sensing mechanisms involving transcription factors (Park et al. 2015). An early study showed that silicon starvation not only induces TAG synthesis but also modulates FA profile, with increasing proportions of saturated and monounsaturated fatty acids (MUFAs) being observed under silicon-starvation conditions (Roessler 1988). An increase up to 50% in lipid content was observed under silicon-starvation conditions in several microalgae (Griffiths et al. 2012).

In bacteria, a few studies suggest that TAG is synthesized during times of stress and resource depletion. Moreover, TAGs are used to generate precursors that will be converted to phospholipids or other products when food supplies improve and growth resumes (Alvarez et al. 2000, 2001, 2013; Olukoshi and Packter 1994). For example, in *Rhodococcus* members and other actinomycetes, the biosynthesis and accumulation of TAGs seems to be a process linked to the stationary growth phase or as a response to stress. Detailed research of TAG metabolism in these microorganisms started only a few years ago. Thus, the fundamental understanding of this process and its regulation remain to be clarified. Recently, a regulatory protein (NlpR: Nitrogen lipid Regulator), which contributes to the modulation of nitrogen metabolism and TGA accumulation in oleaginous rhodococci was identified (Hernandez et al. 2017). NlpR acts as a pleiotropic transcriptional regulator by activating of nitrate/nitrite assimilation genes and others genes involved in fatty acid and TAG biosynthesis, in response to nitrogen deprivation. Moreover, this regulator contributes to the distribution of carbon into the different lipid fractions in response to nitrogen levels, increasing the rate of carbon flux into lipid metabolism (Hernandez et al. 2017).

3.3 Oil Accumulation (Subcellular Structures)

TAG needs to be stored within the cell in a way that allows FA mobilization when needed. This function is fulfilled by cytosolic organelles called lipid bodies. Lipid bodies consist primarily of TAGs and cholesterol esters surrounded by a phospholipid monolayer rich in characteristic proteins and are present in the cytoplasm as a form of energy storage (Ryckebosch et al. 2014a). These lipid-rich compartments are formed in all eukaryotic organisms (Zweytick et al. 2000), including fungi and yeasts, as well as in a few prokaryote genera such as *Rhodococcus* and *Streptomyces* (Wältermann et al. 2005). All oleaginous microorganisms contain lipid bodies in the cells, where most lipids are concentrated as neutral lipids (Garay et al. 2014).

Most yeasts produce small numbers of cytosolic lipid bodies, but oleaginous yeasts accumulate up to 25% (w/w) storage lipid in response to a high carbon: nitrogen ratio (Holdsworth and Ratledge 1991). In *S. cerevisiae*, lipid bodies contain equal amounts of TAGs and sterol esters (Leber et al. 1994). LD biogenesis takes place between two membrane leaflets of the endoplasmic reticulum (Choudhary

et al. 2011). DGATs synthesize TAGs in the inner and outer leaflet of the endoplasmic reticulum, which begin to accumulate, generating lenslike protrusions and promoting the recruitment of structural proteins. When there is enough accumulation of TAG between the leaflets, the outer buds off and the LD formed (Adeyo et al. 2011; Bozaquel-Morais et al. 2010).

In microalgae, lipid bodies emerge from plastidial membranes (Fan et al. 2011; Goodson et al. 2011). They grow facing the cytosol or toward the inside of the plastid, facing the stroma, which is the major aqueous fluid surrounding the thylakoids inside the chloroplast (Liu and Benning 2013). The mechanisms underlying the orientation of LD growth in plastid membranes are not well understood.

Many prokaryotes accumulate lipophilic compounds as lipid bodies in the cytoplasm. Members of most genera synthesize polymeric lipids such as PHB or other PHAs (Steinbüchel 2001), whereas accumulation of TAGs and WEs in lipid bodies is a property of only a few prokaryotes. Like the formation of PHA, TAG and WE biosynthesis is also promoted in response to stress imposed on the cells during imbalanced growth, for example by nitrogen limitation, if an abundant carbon source is present. Lipids act as storage compounds for energy and carbon, needed for maintenance of metabolism and synthesis of cellular metabolites during starvation and in particular when growth resumes.

Large amounts of TAGs have been reported mainly in nocardioforms such as *Mycobacterium* sp., *Nocardia* sp., *Rhodococcus* sp., *Micromonospora* sp., *Dietzia* sp., *Gordonia* sp., and *Streptomyces* sp., which accumulate LDs in cells and mycelia (Akao and Kusaka 1976; Alvarez et al. 1996; Alvarez and Steinbuchel 2002; Barksdale and Kim 1977; Hoskisson et al. 2001; Olukoshi et al. 1994), as well as in *Alcanivorax* sp. and other hydrocarbonoclastic marine bacteria (Kalscheuer et al. 2007). Moreover, TAGs frequently accumulate in members of the gram-negative genus *Acinetobacter*, though the amounts are small in comparison to accumulated WEs (Stöveken et al. 2005). In general, TAGs are stored in spherical lipid bodies, with quantities and diameters depending on the respective species, growth stage, and cultivation conditions. One interesting example is *R. opacus* PD630, where lipids can exceed 70% of the dry weight (Alvarez et al. 1996).

The first reports on WE biosynthesis in gram-negative bacteria were published more than 30 years ago, mainly involving the genus *Acinetobacter* (Fixter and Fewson 1974; Fixter and McCormack 1976; Gallagher 1971; Scott and Finnerty 1976). Meanwhile, accumulation of WEs was also described for *Moraxella*, *Micrococcus*, and *Fundibacter* (Bredemeier et al. 2003; Bryn et al. 1977; Russell and Volkman 1980). WE biosynthesis has also been reported in actinomycetes: for example, in *Corynebacterium*, *Mycobacterium tuberculosis*, and *Nocardia* (Bacchin et al. 1974; Raymond and Davis 1960; Wang et al. 1972). In *Acinetobacter calcoaceticus*, WEs can reach 25% of the dry weight, indicating that WEs act as main storage compound (Fixter and Fewson 1974; Wältermann et al. 2005), but only one or a few WE bodies are observed per cell (Scott and Finnerty 1976; Wältermann et al. 2005). WEs are not exclusively produced as intracellular bodies. Some strains of *Acinetobacter* sp. and the marine bacterium *Fundibacterium jadensis* also produce extracellular WEs from alkanes (Bredemeier et al. 2003; Dewitt et al. 1982;

Makula et al. 1975; Singer et al. 1985). The function of extracellular WEs and the mechanisms of export are not known yet.

Few prokaryotes accumulate TAGs as energy stores. They tend instead to sequester glycogen, polyphosphates, and PHAs. Biosynthesis of PHAs is characteristic among prokaryotes (Koutinas et al. 2014), and, as in the case of TAGs, PHAs are stored as insoluble cytosolic inclusions (Poirier et al. 1995). The species that are able to produce PHAs accumulate these compounds in lipid bodies, which are also called carbonosomes (Bartz et al. 2007; Jendrossek 2009). The PHA bodies are surrounded by amphipathic, small surface proteins called phasins (Grage et al. 2009; Jendrossek 2009). They contain PHB, polyhydroxyvalerate, or copolymers such as poly (3-hydroxybutyrate-co-3-hydroxyvalerate). PHAs have thermoplastic and elastomeric properties and are recyclable materials that can be easily degraded into carbon dioxide and water (Philip et al. 2007).

Accumulation of PHA was first reported in *Bacillus megaterium* (Lemoigne 1926). Since then, many reports on the occurrence of PHAs have been published, including reports on members of the halobacteria, whereas PHA was not detected in lactobacilli, *Enterobacteriaceae*, and methanogens (Garay et al. 2014). *Ralstonia eutropha* is the best characterized bacterium related to PHA metabolism. These bacteria accumulate 10 to 20 intracytoplasmic inclusions of PHB per cell and amounting up to 90% of the cell dry weight (Eggers and Steinbuchel 2014). A few actinomycetes, for example, *R. ruber*, simultaneously synthesize and accumulate similar amounts of TAGs and the copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from unrelated substrates such as gluconate, glucose, and acetate (Alvarez et al. 2000).

4 Commercial Oils, Applications, and Market

4.1 The Diversity of Fatty Acids

FAs differ by the length of the aliphatic chain, the degree of unsaturation, the location, and the *cis* or *trans* conformation of double bonds. In general, the FAs are classified as saturated fatty acids (e.g., palmitic, stearic), MUFAS (e.g., palmitoleic, oleic), and PUFAs. Moreover, PUFAs can be classified in several families (groups or classes) such as omega-3 (ω-3, v-3, or n-3), omega-6 (ω-6, v-6, n-6), and other groups. The ω-3 family includes ALA (C18:3n-3), EPA (C20:5n-3), and DHA (C22:6n-3), while the ω-6 family gathers linoleic acid (LA (C18:2n-6)), GLA (C18:3n-6), ARA (C20:4n-6), and conjugated linoleic acid (CLA).

PUFAs of the ω-3 and ω-6 families are essential for maintaining many functions in mammalians including humans. ALA and LA are the precursors for the synthesis of more highly unsaturated and longer-chained fatty acids of ω-3 and ω-6 families, respectively. Because mammals lack the ability to synthesize LA and ALA, they must be supplied by the diet from different foods sources.

LA is practically found in all foods and is the predominant PUFA in land-based meats, dairy, vegetables, vegetable oils, cereals, fruits, nuts, legumes, seeds, and

breads. GLA can be found in some plant oils such as evening primrose and borage oils. DHA and ARA are found in mother's milk which provides their requirements for neural development and visual acuity to newborns. However, since DHA and ARA are absent from cow's milk, when it is used in place of mother's milk, these PUFAs should be added to the diet of babies to ensure a normal development.

Although fish oil is the best source of PUFAs, the inclusion of fish oil into the infant milk formulas is not recommended due to the presence of environmental pollutants in fishes. Moreover, the production of fish oil is now reaching their limits (see below). Therefore, oleaginous microorganisms can provide an alternative and economically feasible source of PUFAs, provided that most of the PUFAs occur in TGAs which is the preferred form to take lipids within the diet. For instance, it has been shown that microalgae oils contain sufficient ω-3 PUFA to serve as an alternative for fish oil (Ryckebosch et al. 2014b). Thus, this topic will be focused in the applications and market of PUFAs and in particular PUFA-containing microbial oils.

4.2 PUFAs for Human Nutrition and their Nutraceutical Properties

As mentioned above, strictly, EPA and DHA are nonessential ω-3 FAs as the human body can convert essential ALA into EPA and DHA. However, in humans, this conversion is not efficient enough to meet the EPA and DHA demand to impart beneficial health effects; thus, it is expected to obtain these fatty acids from dietary sources (Lee et al. 2016; Béligon et al. 2016).

DHA is an essential component of cell membranes in some human tissues. For instance, it accounts for over 60% of the total fatty acids in the rod outer segment in the retina. Moreover, DHA is an essential nutrient during early human development (Sijtsma and de Swaaf 2004). DHA also can act as breast and colon cancer chemopreventive agent (Hou et al. 2016).

DHA and EPA are involved in early neural and retinal development being essential for the proper visual and neurological development of infants. As preterm and young infants are unable to synthesize DHA at a rate fast enough to keep up with the demand from the rapidly growing brain, they should obtain these compounds from their diet. As mentioned, breastfeeding serves as a good source of PUFAs. In general, while total fat levels in the typical Western diet are too high, the intake of long-chain ω-3 PUFA is too low. DHA supplementation at either 50 mg/day or 100 mg/day for 6 weeks was effective in increasing plasma phospholipid DHA contents of children.

The nutritional benefits of EPA are less clear than those for DHA. Nevertheless, EPA has been advocated as a highly desirable PUFA that can exert beneficial effects on hypertension, thrombosis, arteriosclerosis, arthritis, and various inflammatory responses (Lenihan-Geels et al. 2013). The baseline blood levels of EPA are inversely related to the risk of sudden death due to cardiovascular disease. It has been used to prevent arteriosclerosis and coronary heart disease, and a low dose of 20 mg/kg/day appears to protect against cardiac arrhythmia. It has also been used for the alleviation of some neuropsychiatric disorders, including manic depression

(bipolar disorder), depression, schizophrenia, and also attention deficit hyperactivity disorder in children (Ratledge 2013).

Interestingly, DHA, when administered as a single PUFA, either to young children or to adults, can be retro-converted into EPA by simple loss of a C2 unit. EPA was, however, contraindicated in babies, and thus, DHA was not as effective as originally hoped. However, when DHA was given to infants along with ARA, ARA prevents the retro-conversion of DHA to EPA by blocking the degradative pathway. A combination of two volumes of ARA and one volume of DHA is the most effective ratio for providing these PUFAs to newly born infants, including premature babies (Sinclair and Jayasooriya 2010).

GLA has been also incorporated into infant formula and used for treatments of atopic eczema, rheumatoid arthritis, multiple sclerosis, and premenstrual tension.

Combinations of EPA and DHA can be satisfied by consumption of fish oils particularly those from the so-called oily fish. But not every person and particularly some people that do not eat animals are willing to have fish products in their diet.

ARA is the most abundant PUFA in humans and has a major role as a structural lipid associated predominantly with phospholipids and is a direct precursor of a number of eicosanoids regulating lipoprotein metabolism, blood rheology, leukocyte function, and platelet activation.

The supplementation with ARA and DHA appears to be beneficial in reducing the risk of HIV-1 transmission, particularly during the period of breastfeeding.

Antibacterial activity of PUFAs on *Propionibacterium acnes* and *Staphylococcus aureus* to treat acne and superficial infections has been also investigated (Desbois and Lawlor 2013).

The combination of multifunctional factors with synergic effect including PUFAs is now considered as a possibility to develop more effective multifunctional foods (Yamada 2017).

4.3 Animal Feed

Microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to farm animals. The main applications of microalgae in aquaculture are associated with nutrition as a source of proteins, vitamins, and PUFAs. Fish meal and fish oil were used to complement pig and poultry diets in the last century, but with the growth of aquaculture from the 1960s onward, these products have been diverted toward feeding fish. Today diets complemented with fish oil are used for companion animals.

Fisheries are currently providing fish not only for human consumption but also for fish feed, and they are supplying fish at a so large mass scale that it appears no longer sustainable. In fact, the Food and Agriculture Organization (FAO) of the United Nations suggests that fish oil demand will be soon over the production capacity.

Currently, aqua feed relies on the inclusion of marine ingredients, fish meal, and fish oil since, as with humans, farmed carnivorous marine fish species are inefficient at converting ALA into significant levels of EPA and DHA. Therefore, these PUFAs

must be supplied in the fish diet usually from marine ingredients and fish oil derived from pelagic fisheries (Sprague et al. 2016).

Microalgae are required for larval nutrition during a brief period, either for direct consumption in the case of mollusks or directly as food for the live prey fed to small fish larvae. The most frequently used species in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema,* and *Thalassiosira*. Microalgae such as *Dunaliella salina*, *Haematococcus pluvialis,* and *Spirulina* are also used as a source of natural pigments for the culture of prawns, salmonid fish, and ornamental fish. Mainly the microalgae *Arthrospira* and *Chlorella* are used also to feed many types of animals: cats, dogs, aquarium fish, ornamental birds, horses, poultry, cows, and breeding bulls (Spolaore et al. 2006).

However, the aquaculture industry is experiencing so rapid increases in fish oil price due to flat supply and increased global demand for this commodity that these finite marine ingredients have gradually been replaced with alternatives of terrestrial agricultural origin. Rapeseed oil is the most commonly utilized fish oil alternative in Europe. Nevertheless, terrestrial plants are not so efficient since they only contain shorter-chain PUFAs such as ALA and are completely devoid of any EPA and DHA. Because of that, PUFA-containing microalgae biomass has been used directly as an alternative feed additive to plant oils (Chauton et al. 2015; Sprague et al. 2016 2017).

4.4 Market

As mentioned, fish oil is the main source of PUFAs, but the world supply of fish oil is currently stabilized at around one million t per year (Finco et al. 2017). Because approximately 70% of the available fish oil is used for fish feed production for salmonids, only a small portion of the captured fish is now used to produce functional foods for humans.

The global PUFA market has been estimated to be valued at US$ 4212 M by 2016 end and is expected to witness a compound annual growth rate (CAGR) of 10.7% over the period 2016–2026 (FMI 2016). Growth of the global PUFA market is mainly driven by increasing prevalence of chronic diseases such as cardiovascular diseases, stroke, cancer, and diabetes. On the basis of product type, the market is categorized into ω-3 and ω-6 PUFAs.

The ω-3 market segment is further segmented into ALA, DHA, and EPA. According to FAO (2014) and Grand View Research (2014), the global demand for ω-3 PUFAs was 21,900 t in 2012, and it is expected to increase to more than 135,500 t in 2025. Other studies estimate a market of $9.94 billion for ω-3 PUFAs in 2015 with a projected grow at a CAGR of 13.8% from 2015 to 2020 (Marketsandmarkets 2016). The particular DHA segment is expected to register a high CAGR of 11.7% during the 2016–2026 periods (FMI 2016).

The ω-6 market segment is further segmented into LA and ARA. The ARA segment is expected to dominate over LA segment and is estimated to account for the highest market share of 73.2% by 2016 (FMI 2016). The global market for ARA was

$176.48 million in 2016 and is expected to grow at a CAGR of 6.8% during 2017–2022 (Mordor Intelligence 2017).

The emerging problem is to provide enough food resources containing PUFAs to an increasing population. Thus, the potential demand for ω-3 PUFAs is 1.274 million t based on a supply of 500 mg/day. However, the supply by oil fish is only 0.84 million t, so the gap is 0.434 million t.

Some of the major company players in this market are Koninklijke DSM N.V., Enzymotec Ltd., Aker BioMarine AS, Croda International PLC, GlaxoSmithKline plc, FMC Corporation, Omega Protein Corporation, BASF SE, Cargill Incorporated, Polaris Nutritional Lipids, Zymes LLC, Denomega Nutritional Oils, Barlean's Organic Oils, Vega Nutritionals Ltd., Arista Industries, Copeinca ASA, Horizon Organic, Pharma Marine USA LLC, Cabio Bioengineering, Cayman Chemicals, Guangdong Runke, Kingdomway, Suntory, Zhejiang Weiss (Wecan), Lonza Group AG, GC Rieber Oils AS, Kerry Group, Cellana Inc., Clover Corporation, Archer Daniels Midland Company, Nordic Naturals Inc., Smit & Zoon, Solutex GC, Stepan Company, Neptune Wellness Solutions, FrieslandCampina, and others. The manufacturers, marketers, and supporters of EPA and DHA omega-3 fatty acids have created the GEOED association (http://www.goedomega3.com/).

Several sources have been evaluated as alternative market sources of PUFAs, but due to their wide availability and ability to accumulate large amounts of lipids in a short time, oleaginous microorganisms have been used in the production of oils rich in PUFAs, mainly ω-3 acids, from the mid-1990s. In this sense, while oleaginous plants can reach 500–5000 kg/ha/yr. of lipids, microorganisms can achieve up to 2000 kg/m^3/yr. of lipids (Finco et al. 2017).

Commercial production of microbial oils is mainly restricted to yeasts, filamentous fungi, and algae. The first microbial oil that was produced at commercial scale was the GLA-rich oil, named Oil of Javanicus, from *Mucor circinelloides*. This oil was produced by J&E Sturge (UK) from 1985 to 1990 competing with the GLA from the seed oil of *Oenothera biennis*. This oil is mainly sold in the UK as a dietary supplement for the alleviation of premenstrual tension in women and due to price competitions only low volumes of the microbial oil have been produced (i.e., 5–10 t) (Ratledge 2006).

After the initial approach of microbial oil to the market in the 1990s, David Kyle at Martek Inc. (USA) company began to explore the possibility of producing long-chain microbial PUFAs. The key targets were initially EPA and DHA (Boswell et al. 1992). The company exploited *Crypthecodinium cohnii* as DHA producer.

Currently DHA is also produced commercially by other microorganisms such as *Schizochytrium*, *Aurantiochytrium*, and *Ulkenia* (also known as *Labyranathula*). The company that has originally developed this process was OmegaTech (USA) led and pioneered by Bill Barclay that was acquired by Martek in 2002. This oil is now also produced by other companies including Lonza Group (Switzerland), Jiangsu Tiankai Biotechnology Co. Ltd. (China), and several smaller companies in the USA and the UK. The FDA gave approval and GRAS status to DHA as DHASCO oil in 2002. About 20 countries with 150 companies were producing DHA in 2015 (Finco 2017).

In addition, in the 1980s, a process for the microbial ARA production was identified in Japan using *Mortierella alpina* (Totani et al. 1987). However, the production of ARA by fermentation was developed by the company, Gist-brocades (Netherlands). An agreement was subsequently reached between Martek Inc. and Gist-brocades for the latter company to produce the ARA-rich oil for exclusive sale to Martek. The ARA-rich oil is now also produced by Suntory in Japan, as SUNTGA40S, and by Cargill, as CABIO oil, together with Wuhan Alking Bioengineering Co. Ltd. in China, where it is used for infant nutrition. In 2012, the EU gave approval to allow for sales of the ARA-rich oil for infant nutrition in Europe.

As commented above, the combination of ARA and DHA oils was found to be most appropriate for providing these key PUFAs to newly born infants. Safety trials have shown the oil to have an unimpeachable safety record (Ryan et al. 2010). Sales of DHA and ARA oils have steadily increased since they were launched to the market and are now commercialized into over 70 countries for more than 20 companies.

DSM (Dutch State Mines) (Netherlands) has the global leadership on the production of ω-3. The company is the main supplier of DHA to the US market (with 80% of the market share) and to European and Asian markets (excluding China). The position of DSM is because the company Martek one of the largest suppliers of these oils was taken over by DSM, who had previously acquired Gist-brocades in 2011. For its last year of trading as an individual company, Martek Biosciences recorded a revenue of US $ 317 million for sales of the oil for infant nutrition. Although the selling price of the oil is commercially sensitive, it is considered that at least 2000 t of microbial DHA are sold annually.

Alltech, a global animal nutrition company, produces DHA from heterotrophic algae in closed stainless steel fermenters in one of the largest algae production sites in the world in Winchester (Kentucky, USA) aimed at the animal feed market.

Microbial EPA is mainly produced using microalgae. The EPA producer companies include Qualitas Health Ltd. (Israel), Photonz (New Zealand), and Algisys LLC (USA). The oil produced by Qualitas Health is sold as EicoOil, being a mixture of EPA with other PUFAs. DuPont (USA) has developed an alternative to microalgae producing EPA using a genetically modified *Y. lipolytica* (Xue et al. 2013). However, by using naturally occurring oleaginous yeast, DuPont has been also able to produce EPA (Damude et al. 2011), and now this EPA-rich oil has received the GRAS status from the FDA.

Concerning the patents on microbial oils, it is possible to classify them according to different general subjects: (i) downstream processes for the isolation of microbial oils, (ii) microbial oil production and uses, and (iii) engineering microorganisms for oil production (Table 2).

Table 2 Examples of patents concerning to microbial oils

Subject	Organism	Patent	Proprietary
Downstream processes	Algae, fungi	WO2015095688A1	Dsm Ip Assets B.V.
	All	EP2419520A4	Solazyme, Inc.
	All	US6166231	Martek Biosciences Corp
	All	US4905761	IIT Research Institute
	Algae, fungi	US6255505	DSM Gist BV
	All	WO2008151149	Solazyme, Inc.
	All	WO2006046943A2	Martek Biosciences Corp
	All	WO2003049832A1	Martek Biosciences Corp
	C. cohnii	WO1991011918A1	Martek Biosciences Corp
	All	WO2011153246A2	Kanegafuchi Kagaku Kogyo KK
	Algae, fungi	EP0207475A2	DSM Gist BV
	Algae, fungi	US6255505	
Microbial oil production and uses	*C. cohnii*	US20040072330	University of Hull
	Algae, fungi	US6428832	Koninklijke DSM NV
	M. schmucker	US5882703	OmegaTech Inc.
	Fungi	EP0269351A3	Lion Corp
	Schizochytrium	US5130242	Phycotech Inc.
	All	US5374657	Martek Biosciences Corp
	M. schmucker	US7666657	Martek Biosciences Corp
	Mortierella	US4783408	Agency Ind Sci & Technol
	Mortierella	EP0276541A2	Suntory Ltd.
	Ulkenia	US6509178	Nagase and Co Ltd.,
	Dinoflagellates	US5397591	Suntory Ltd.
	Marine	WO1989000606A1	Martek Biosciences Corp
	S. limacinum	US8232090	Maricultura, Incorporated
	S. limacinum	WO2013010090	ABL Biotechnologies Ltd.
			Allthech Inc

(*continued*)

Table 2 (continued)

Subject	Organism	Patent	Proprietary
Engineering microorganisms	Yeasts	US8951776	MIT USA
	All	US8765404	MIT USA
	Y. lipolytica	US20130344548	MIT USA
	S. cerevisiae	US7736884	Fluxome Sciences AS
	Yeasts	US7198937	E I du Pont de Nemours and Co
	Yeasts	US20060160193	E I du Pont de Nemours and Co
	Cyanobacteria	US20100081178	Targeted Growth Inc.
	Y. lipolytica	US8435758	E I du Pont de Nemours and Co
	All	US20110223641	MIT USA
	S. cerevisiae	WO2005118814	Fluxome Sciences As & Authors
	Cyanobacteria	WO2012087963	Targeted Growth, Inc.
	Cyanobacteria	WO2012087982	Matrix Genetics, Llc
	Pseudomonas YS-180	US6207441	Seong Gu Ryu
	Y. lipolytica	US7550286	E I du Pont de Nemours and Co

5 Industrial Production Processes

A crucial point for the establishment of microbial lipids utilization is the cost-effective production and purification of fuels or products of higher value. This topic will analyze the state of the art on the production and extraction of microbial oils.

5.1 Culture Conditions

The development of a microbial PUFA production process requires the selection of the proper microorganism and optimized cultivation techniques. Bacteria, algae, fungi, and yeasts are able to accumulate lipids, but in fact, there are only few studies on PUFAs produced by bacteria. Moreover, the potential for lipid production is a species-specific characteristic, as well as the ability to produce PUFAs.

In order to select appropriate lipid producer strains, the most important parameters to be considered include the specific growth rate, the biomass production under optimal culture conditions, the total lipid content, and their PUFA proportion. Therefore, to control, to validate, and to optimize the processes, one of the most important parameters for lipid production is the lipid content of the cell mass. The yield and the composition of the microbial lipids depend on the type of fermentation and the particular operational conditions (e.g., culture medium, nitrogen source, pH, temperature, aeration, etc.).

In general, the production of microbial oils can be realized by submerged (SmF) or solid-state fermentations (SSF). In SmF conditions oleaginous microorganisms

can be cultivated as batch, fed-batch, or continuous cultures, using baffled and unbaffled flasks as well as stirred tank reactors (Ochsenreither et al. 2016). On the other hand, SSF reproduces the natural microbiological processes such as food production, composting, and ensiling. In general, the advantages of SSF are a higher productivity, the possibility to use low-cost media and to reduce energy and waste water costs. The disadvantages of SSF are, for example, the difficulties in scale-up, in the control of process parameters, and an increasing cost for product recovery (Ochsenreither et al. 2016).

One advantage of using microorganisms to produce PUFAs is that they can be cultivated on various types of carbon substrates, even on different organic industrial waste. Since carbon sources constitute over 60% of the total production cost in typical fermentation processes, the use of low-cost carbon sources, such as organic waste, should therefore be considered as an important factor to make the process economically feasible. As well organic and inorganic nitrogen sources are used individually or in combination including yeast extract, urea, peptone, glycine, KNO_3, NH_4NO_3, and $(NH_4)_2SO_4$ (Ochsenreither et al. 2016).

In addition to oleaginous yeast and fungi, microalgae are currently produced at large scale as a microbial oil source. In fact, microalgae are currently cultivated at large scale to produce lipids for biofuels as well as food and feed products. The cost of microalgae production is a challenge for the commercial utilization of this biomass as biofuels. However, as the target price level of microalgae as food and feed ingredients is higher, the economic feasibility appears to be closer for nutrition purposes than that of biofuel uses.

Two main approaches are taken to improve the production economics of phototrophic microalgae, that is, increasing the productivity of the cultivation systems and reducing both capital and operational production costs. In this sense, large-scale industrial cultivation of phototrophic microalgae can be conducted in open pond systems or in closed photobioreactor systems (CPS). Open ponds are often cheap raceway constructions which provide large volumes, but these systems have a low productivity and high energy costs required to harvest the cells at low densities of 0.1–0.2 g dry cell weight/l. CPS are more expensive to build, but have much higher productivity, since they are designed to maximize the utilization of light energy and to achieve efficient uptake of nutrients and CO_2 and have higher cell densities at 2–4 g dry weight/l, which lower the harvesting costs.

The economics of microalgae production depend on the photosynthetic productivity, and there are ongoing efforts to increase the microalgae productivity following different strategies (Chauton et al. 2015). The first strategy is to exploit the cultivation conditions to direct the metabolism toward lipid production. The second is to improve biomass productivity or lipid yield by mutagenesis and selective breeding, and the third strategy is to improve strains by genetic modifications to optimize light absorption and increase the biosynthesis of EPA and DHA (see below).

Lipids serve as a carbon and energy storage in microalgae under limiting growth conditions when photosynthesis exceeds the limitations for growth. To control the balance between biomass production and lipid accumulation, physiological variables can be adjusted to induce lipid accumulation and also shift the lipid composition toward the desired lipids: growth restriction due to limited illumination and

deprivation of nitrogen, sulfur, or phosphate is used to induce lipid accumulation and composition changes.

On the other hand, many efforts are focused on how to achieve maximum biomass productivity by increasing the photosynthetic efficiency (PE), which is the percentage of the solar irradiation that is converted to biomass. The overall PE of a cultivation system will depend both on the technological efficiency of the cultivation system and the biological efficiency of the production strain to convert the irradiation into chemical energy. Then photosynthetic productivity is directly depending on the intensity of the solar irradiation. Only the light within the wavelength range of 400 to 700 nm, called photosynthetically active radiation (PAR), can be utilized by plants and algae, which in practice means that only 40–45% of total solar energy can be utilized for photosynthesis.

5.2 Downstream Processes

Downstream processing cost is one of the major obstacles to be solved for full economic efficiency of microbial lipids (Béligon et al. 2016; Ochsenreither et al. 2016). Because microbial oils are intracellular for storage purposes, they have to be extracted upon further applications. Natural oleaginous strains have not been engineered so far to excrete TAGs or free fatty acids in order to simplify downstream processing. However, metabolic engineering efforts have been conducted for secretion of fatty acids in *Escherichia coli* (Liu et al. 2012; Meng et al. 2013) and *S. cerevisiae* (Michinaka et al. 2003; Leber et al. 2015).

Furthermore, for downstream processing, it is important to know if PUFAs are present as part of the membrane structure, e.g., in phospholipids, or as part of TAGs in the cytosol, as well as to know the type of PUFAs present in the cells.

The extraction method and the lipid quantification must be fast, efficient, and applicable to an industrial scale-up. Therefore, the extraction methods, that may be highly suitable for analytical purposes, might not be applicable in industrial large-scale operations due to high costs or simply a non-scalable extraction setup. The optimal extraction method should also enable a reproducible, quantitative, cost-effective, and nontoxic removal of lipids under mild conditions to prevent oxidative damage to PUFAs. The final processing of oils involves purification or refining and modification in order to stabilize the crude oil. Extraction methods, using solvents, developed to extract lipids from fishes or vegetables have been modified and adapted for microbial lipids extraction. Microbial lipids applied in food industry cannot be extracted with toxic solvents or should in the best case avoid any solvents to prevent solvent residues in food or contaminations with heavy metals (Uematsu et al. 2002). Additionally, the optimal method in terms of oil recovery has to be elucidated for each strain.

Extraction methods include Soxhlet extraction, Folch extraction, pressurized liquid extraction, and extraction with supercritical fluids (Ochsenreither et al. 2016). Several automated Soxhlet extraction systems have been developed and are commercially available. The Folch method, similar to the method developed by

Bligh and Dyer, is the most reliable extraction method for total lipids and is often used as a standard technique in extracting microbial lipids. The pressurized liquid extraction is similar to Soxhlet extraction but uses liquid solvents at elevated temperatures and pressures. For extracting lipids supercritical CO_2 is a good solvent (Sahena et al. 2009).

For the extraction of lipids from microbial biomass, cell disruption is very important, because efficiency of cell disruption directly influences subsequent downstream operation and overall extraction efficiency (Senanayake and Fichtali 2006). A multitude of cell disruption and lipid extraction methods are available which can be roughly divided in mechanical and nonmechanical methods. Nevertheless, depending on microorganism, scale, economics, and lipid application, the method spectrum is narrowed to a few.

Cell disruption by mechanical methods is achieved by bead milling, high-pressure homogenization, and ultrasound (Ochsenreither et al. 2016). Cell disruption by nonmechanical methods is achieved by physical or chemical disruption methods.

Physical methods for disruption include decompression, osmotic shock, microwave treatment, pulsed electrical fields, and (freeze)-drying, but, their scale-up is limited for most of them.

Disruption by chemical methods includes the permeabilization by a variety of chemicals such as antibiotics, chelating agents, chaotropes, detergents, solvents, alkalis, and acids. Acid-catalyzed in situ transesterification which combines cell disruption, lipid extraction, and transesterification to fatty acid methyl (FAME) or ethyl (FAEE) esters has been used for biodiesel production. Cell disruption with lytic enzymes, a process that can be carried out at mild reaction conditions, has been used especially for oleaginous yeasts. The enzymatic treatment in combination with solvent extraction, pressing, or ultrasound has been demonstrated for the lipid extraction of microalgae. However, some chemical treatments are excluded in food applications or require intensive downstream processing to eliminate them.

A direct application of solvents offers the possibility of combining cell disruption and lipid extraction without further pretreatment, but the use of large amounts of organic hazardous solvents, like chloroform and methanol or even less toxic one, like hexane/isopropanol, is not always efficient since the cell walls of most microorganisms are usually impermeable to most solvents (Ochsenreither et al. 2016).

6 Metabolic Engineering Strategies for Producing Microbial Oils

6.1 Genetic Engineering (Mutagenesis)

Improvement in the production of microbial oils involved the use of classical genetic techniques, such as mutagenesis, together with genetic engineering (Liang and Jiang 2013; Gong et al. 2014; Ledesma-Amaro 2015; Shi and Zhao 2017). Despite the low cost of genome sequencing, only few oleaginous yeasts genomes have been sequenced. Therefore, genetic tools remain scarce or under development for this

kind of yeast. Among them, only *Y. lipolytica* was used as a model organism (Fickers et al. 2005), so its genome was made public a few years ago, and many genetic tools for its modification are available (Bredeweg et al. 2017; Dujon et al. 2004).

The first attempts at enhancing lipid accumulation were performed by modification of the expression of key enzymes situated in the crossroads of metabolic routes. In *Y. lipolytica* the increase of G3P pools by modifying gene expression of the enzymes leading to its production and/or its degradation resulted in threefold increase in lipid accumulation compared to the wild-type strain. The simultaneous abolishment of the ß-oxidation gave rise to an obese yeast capable of accumulating more than 80% of its cell dry weight in lipids (Beopoulos et al. 2008; Dulermo and Nicaud 2011). In this organism, overexpression of the DGAT resulted in a great increase of TAG content of the cells (Beopoulos et al. 2012; Silverman et al. 2016).

PUFAs are highly susceptible to oxygen radical attack, and the resulting oxidative species are detrimental to cell metabolism and limit lipid productivity. Xu et al. (2017) investigated cellular oxidative stress defense pathways in *Y. lipolytica* to improve the lipid titer, yield, and productivity.

The oleaginous fungus *M. alpina* has been engineering for the production of PUFAs (Sakuradani et al. 2013; Okuda et al. 2015; Shi et al. 2016; Kikukawa et al. 2016).

In microalgae, most of the studies of genetic engineering have targeted lipid biosynthesis in order to enhance TAG accumulation. In several cases endogenous or heterologous (from *S. cerevisiae* or *C. reinhardtii*) DGATs have been overexpressed in different *Nannochloropsis* species (Beacham and Ali 2016; Iwai et al. 2015; Li et al. 2016; Xin et al. 2017; Zienkiewicz et al. 2017; Wei et al. 2017a). Overexpression of the endogenous DGAT1a-encoding gene in *Nannochloropsis oceanica* resulted in a 39% increase in TAG content per cell, and RNAi repression resulted in a 20% decrease in TAG content per cell following N deprivation (Wei et al. 2017a). Overexpression of the endogenous DGAT number 5 in *N. oceanica* resulted in a 3.5-fold increase in TAG (Zienkiewicz et al. 2017). Furthermore, the DGAT number 7 has also been overexpressed in *N. oceanica* IMET1, resulting in 69 and 129% increase in dry weight (DW) of TAG content under N-replete and N-deprived conditions (Li et al. 2016). The overexpression of malonyl-CoA ACP transacylase (MCAT) of *N. oceanica* IMET1 has been resulted in a 36% DW increase in lipids (Chen et al. 2017).

Overexpression of malic enzyme in transgenic *P. tricornutum* markedly increased the total lipid content (Xue et al. 2015). A similar approach has been followed with the green microalga *Chlorella pyrenoidosa* (Xue et al. 2016). *P. tricornutum* has been engineered to accumulate DHA using the Δ5-elongase from the picoalga *Ostreococcus tauri* (Hamilton et al. 2014, 2016). EPA was also produced by using a similar method (Peng et al. 2014; Wang et al. 2017) that used a sequential metabolic engineering strategy to overcome the metabolic bottlenecks in *P. tricornutum* in order to overproduce ARA and DHA. For this purpose, the malonyl-CoA acyl carrier protein transacylase and desaturase 5b were cloned and coordinately expressed, increasing the production of ARA and DHA. Niu et al.

(2013) have improved the neutral lipid and PUFAs biosynthesis by overexpressing a type II diacylglycerol acyltransferase in *P. tricornutum*.

Escherichia coli could be an alternative fatty acid producer, since it has been successfully used to generate many valuable platform chemicals and biofuels; however, it is not an oleaginous microorganism, so its low yield of intracellular lipids restricts its utility for lipid production. Nevertheless, *E. coli* has been genetically engineered to increase its fatty acid biosynthesis (Lennen and Pfleger 2012; Meng et al. 2011; Lee et al. 2014; Wu and San 2014; Cao et al. 2016; Xiao et al. 2018).

Some experiments have been performed in yeast to improve TAGs accumulation by random mutagenesis using ultraviolet irradiation. One of these experiments was carried out in *Lipomyces starkeyi*-selecting mutants by cerulenin, a compound displaying inhibitory effects on lipid biosynthesis. This strategy resulted in an increase in lipid productivity up to 31% (Tapia et al. 2012). The same procedure was done in the oleaginous yeast *Rhodosporidium toruloides,* and the lipid productivity in this yeast was successfully improved (Yamada et al. 2017). UV mutagenesis was also used to improve TAGs accumulation in the green microalgae *Scenedesmus obliquus*. For this aim, starchless mutants were obtained in which TAG content reached up to 49.4% DW (de Jaeger et al. 2014; Breuer et al. 2014).

6.2 Systems and Synthetic Biology

Increasingly, there are a number of studies that use various systems biology tools to understand the metabolic switches in oleaginous microorganisms. However, their applications to metabolic engineering of oleaginous microorganisms are still in their infancy. Actually, studies on metabolic engineering of oleaginous microorganisms often involve target screening processes which require extensive experimental works (Silverman et al. 2016; Friedlander et al. 2016). Systems biology provides easier and more efficient ways to guide metabolic engineering of oleaginous microorganisms.

In systems biology, both bottom-up and top-down approaches are central to assemble information from all levels of biological pathways that must coordinate physiological processes (de Lorenzo and Galperin 2009). The top-down approaches, which are based on "omics" tools for high-throughput measurement of cellular components, enable data-driven discovery of key players controlling the systems and their interactions. Meanwhile, the bottom-up approaches employ predictive mechanistic models developed based on existing knowledge to perform systematic analysis of the cellular processes (Lee et al. 2005; Park and Lee 2008).

Studies using top-down approaches reveal that lipid accumulation in most oleaginous fungi is a consequence of reduced fluxes through central carbon metabolism caused by nitrogen limitation. In contrast, for oleaginous bacteria, central carbon metabolism is upregulated during nitrogen limitation together with activation of the Kennedy pathway. The difference between oleaginous fungi and bacteria implies that engineering of central carbon metabolism in oleaginous fungi to simulate the

regulatory patterns in oleaginous bacteria would be an effective strategy for lipid overproduction (Park et al. 2017).

Related to bottom-up approaches, genome-scale models of metabolism (GEMs) and constraint-based modeling (CBM) methods are used to understand and engineer microbial oil synthesis (Kavscek et al. 2015; Kerkhoven et al. 2016). A GEM is a structured collection of all metabolic reactions that exist in the cell, which can be built systematically using genome annotations and biochemical knowledge (O'Brien et al. 2015).

Recently, reflecting the growing interests on oleaginous microorganisms, several GEMs for oleaginous microorganisms have been published (Table 3). In general, special attention to lipid-related pathways was made during the reconstruction. Modeling results highlight that the pentose phosphate pathway is preferred for the generation of NADPH in *Y. lipolytica* (Kavscek et al. 2015) and *Candida tropicalis* (Mishra et al. 2016), while the malic enzyme is identified as a key node in the regulation of NADPH regeneration in *Mortierella alpina* (Ye et al. 2015).

Although oleaGEMs have been used for understanding mechanisms of lipid production and optimizing fermentation conditions, currently there is no report on the use of oleaGEMs for metabolic engineering. During the past decade, various computational strain design algorithms (CSOMs) have been developed, and majority of them are looking for a design, which enables growth-coupled production of target compounds (Maia et al. 2016). However, as oleaginous microbes produce lipids in the nutrient-limited nongrowing phases, it is hard to apply existing CSOMs to oleaGEMs for designing improved lipid producers. Furthermore, until a recent date, there was no objective function suitable for predicting metabolic fluxes in the nutrient-limited conditions (Park et al. 2017).

Synthetic biology emerged around the year 2000 as a new biological discipline, and many different definitions have been applied to this field. However, one commonly used way to describe synthetic biology is as the design and construction of new biological functions that are not found in nature (Serrano 2007).

Y. lipolytica was modified to secrete FAs by considering two synthetic approaches, firstly where FAs are produced by enhancing the flux through neutral lipid formation, as typically occurs in eukaryotic systems, and secondly by mimicking the bacterial system to produce free FAs (Ledesma-Amaro et al. 2016).

TALEN (transcription activator-like effector nucleases)-based genome-editing technology was applied to *Y. lipolytica* inducing targeted genome modifications. This is an illustration of how a combination of molecular modeling and genome-editing technology can offer novel opportunities to rationally engineer complex systems for synthetic biology in order to obtain a significant increase of myristic acid (C14) production (Rigouin et al. 2017).

Hayashi et al. (2016) have investigated in *E. coli* the biological function of the tandem ACP domains in PUFA synthases to construct PUFA synthase derivatives with less and more active ACP domains than the native enzyme and examined the effects on PUFA productivity. Tee et al. (2014) developed in *E. coli* a rational strain design process in systems biology, an integrated computational and experimental approach for carboxylic acid production, as an alternative method.

Table 3 Metabolic maps at genomic scale of some oleaginous fungi, bacteria, and microalgae

Organism	GSM model	Reference
Fungi		
Yarrowia lipolytica	*i*NL895 2002 reactions 1847 metabolites 895 genes	Loira et al. 2012
Yarrowia lipolytica	*i*YL619_PCP 1142 reactions 843 metabolites 619 genes	Pan and Hua 2012
Yarrowia lipolytica	*i*MK735 1336 reactions 1111 metabolites 735 genes	Kavscek et al. 2015
Yarrowia lipolytica W29	*i*YALI4 1942 reactions 1691 metabolites 847 genes	Kerkhoven et al. 2016
Yarrowia lipolytica	*i*YLI647 1347 reactions 1119 metabolites 647 genes	Mishra et al. 2018
Yarrowia lipolytica	iYL_2.0 1471 reactions 1083 metabolites 645 genes	Wei et al. 2017b
Candida tropicalis	iCT646 945 reactions 712 metabolites 646 genes	Mishra et al. 2016
Mucor circinelloides	*i*WV1213 1326 reactions 1413 metabolites 1213 genes	Vongsangnak et al. 2016
Mortierella alpina	iCY1106 1854 reactions 1732 metabolites 1106 genes	Ye et al. 2015
Gram-positive bacteria		
Rhodococcus jostii RHA1	*i*MT1174 1935 reactions 1243 metabolites 1174 genes	Tajparast and Frigon 2015
Cyanobacteria		
Synechocystis sp. *PCC*	*iJN678* *863 reactions* *795 metabolites* *678 genes*	Nogales et al. 2012
Synechocystis sp. PCC 6803	*i*Syn731 1156 reactions	Saha et al. 2012

(*continued*)

Table 3 (continued)

Organism	GSM model	Reference
	996 metabolites 731 genes	
Cyanothece sp. ATCC 51142	*i*Cce806 667 reactions 587 metabolites 806 genes	Vu et al. 2012
Cyanothece sp.	*i*Cyt773 946 reactions 811 metabolites 773 genes	Saha et al. 2012
Chlorella vulgaris UTEX 395	*i*CZ843 2294 reactions 1770 metabolites 843 genes	Zuñiga et al. 2016
Synechococcus sp. PCC 7002	*i*Syp728 742 reactions 754 metabolites 728 genes	Hendry et al. 2016
Synechococcus elongatus PCC7942	*i*Syf715 851 reactions 838 metabolites 715 genes	Triana et al. 2014
Synechococcus 2973	*i*Syu683 1178 reactions 1028 metabolites 683 genes	Mueller et al. 2017
Synechococcus sp. PCC 7002	*i*Syp611 552 reactions 542 metabolites 611 genes	Hamilton and Reed 2012
Synechococcus sp. PCC 7002	*i*Syp708 746 reactions 581 metabolites 702 genes	Vu et al. 2013
Microalgae		
Chlamydomonas reinhardtii	*i*RC1080 2190 reactions 1068 metabolites 1080 genes	Chang et al. 2011
AlgaGEM *Chlamydomonas reinhardtii*	AlgaGEM 1725 reactions 1862 metabolites 866 genes	Dal'Molin et al. 2011
Chlamydomonas reinhardtii	*i*Cre1355 2394 reactions 1133 metabolites 1355 genes	Imam et al. 2015

(*continued*)

Table 3 (continued)

Organism	GSM model	Reference
Chlorella variabilis	*i*AJ526 1455 reactions 1236 metabolites 526 genes	Juneja et al. 2016
Phaeodactylum tricornutum	iLB1027 4456 reactions 2172 metabolites 1027 genes	Levering et al. 2016
Nannochloropsis salina	*i*NS934 2345 reactions 1985 metabolites 934 genes	Loira et al. 2016

7 Research Needs

PUFAs have many health benefits and are essential for supporting the development of children. Although several microorganisms produce PUFAs naturally, native microorganisms frequently have low growth rates and produce low yields of these FAs, which are usually far below the desirable level to be commercially industrialized. Therefore, it is required to increase the research efforts to modify these microorganisms through metabolic engineering to accumulate higher amounts of lipids enriched in the desired FAs demanded by the industry. Additionally, these microorganisms should be further engineered to release the synthetized FAs to the culture medium, to facilitate the downstream processing. Metabolic engineering has the potential not only to improve yields but also to generate novel sources of PUFAs from food-grade microorganisms.

The SCO production cost depends mainly upon the species chosen for cultivation, lipid concentration within cells, and the concentration of cells produced. On the first sense, more screening efforts should be done to increase the number of available oleaginous microorganisms. Concerning lipid concentration, the overall yield and productivity of SCOs is normally constrained by different metabolic and regulatory bottlenecks. Thus, systems biology should contribute to proposing metabolic alternatives that can be further implemented by using novel synthetic biological tools. Different strategies such as limiting the acyl exchange of intermediates, increasing the metabolic flux toward the products, enhancing precursors supply, and reducing the use of precursors and end products by competing pathways should be the targets to alleviate some of the bottlenecks. The use of heterologous enzymes such as acyltransferases, desaturases, elongases, and others will allow to better control the fluxes. Synthetic biology will contribute to redesigning gene clusters and pathways by chemically synthesizing them with optimized codons, promoters, or intergenic sequences, to facilitate their heterologous expression in the most productive microbial hosts. In fact, the integration of classical genetic, metabolic and protein

engineering, system biology, synthetic biology, and evolutionary engineering, recognized as the new field of systems metabolic engineering, has been suggested some years ago as a global approach to increase the FA production in the oleaginous microorganism (Tee et al. 2014).

From commercial and industrial standpoints, it is important to reduce operating costs. In this sense, developing fermentation procedures using low-cost media and efficient product separation processes can lower operating costs. Even with a high level of productivity, SCOs are too expensive to compete with chemical (lubricants) or commodity (biofuels) products. This explains why industrial developments are focused on the high-value products as PUFAs for dietary supplements and for infant nutrition (Thevenieau and Nicaud 2013). Nevertheless, the use of renewable substrates must also be considered as an ecological added value. Organic waste can be used to grow oleaginous microorganisms to be converted into PUFAs decreasing the final price of the product, in a biorefinery concept (Huang et al. 2013; Béligon et al. 2016). The endogenous production of FAs can reduce cell viability due to the loss of inner membrane integrity (Lennen et al. 2011), and thus the secretion of endogenous FAs could moderate the toxicity effect while reducing product extraction costs (Ledesma-Amaro et al. 2016).

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