Studies in Natural Products Chemistry

Atta-ur-Rahman, FRS

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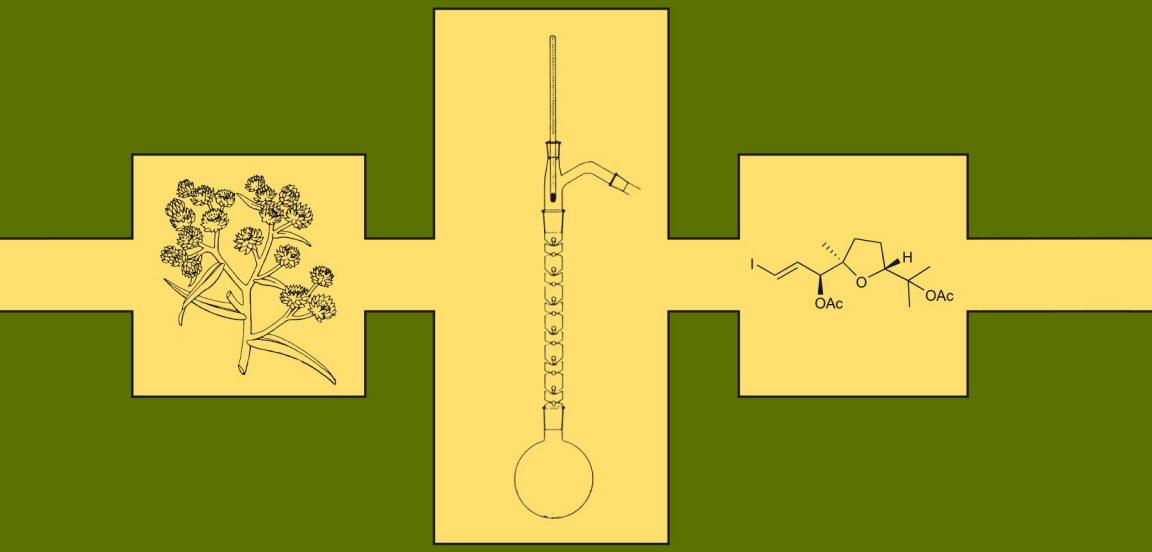

Volume 74

Bioactive Natural Products

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Studies in Natural Products Chemistry

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Preface

Volume 74 of the book *Studies in Natural Products Chemistry* contains a number of remarkable reviews covering various important aspects of bioactive natural products. Chapter 1 by Ishiwata et al. presents a broad review on various glucans and their synthesis by employing the recent advances in stereoselective bimodal glycosylation. Many diterpenes exhibit a wide range of biological activities including antitumor effects. In Chapter 2, Panis et al. present the new perspectives and current advances in plant-derived diterpenes for the treatment of breast cancer. In Chapter 3, Süntar et al. provide a detailed review on anticancer activity of selected flavonoids and their chemical structures.

Dihydrophenanthrenes have been frequently isolated from plants. Li et al. have summarized the recent literature on natural dihydrophenanthrenes and their activities in Chapter 4. In Chapter 5, Maraschin et al. review the applications of metabolomics in the discovery of novel bioactive compounds having pharmaceutical potential. Metabolic engineering involves the intentional modification of cellular metabolism for the production of desired compounds. A review on the current metabolic engineering strategies used in the manufacturing and biosynthesis of some nutraceuticals is presented by Řezanka et al. in Chapter 6. In Chapter 7, Cao et al. present a comprehensive review on the isolation, characterization, and biological activities of *Aspergillus versicolor*, which was isolated from a marine sponge and served as a source of several interesting bioactive metabolites.

Hyperuricemia and gout are clinical conditions in which there is excess production of uric acid in the body, which leads to inflammation and intense pain in the joints. Fu et al. provide a detailed review on the understanding of the mechanism of antihyperuricemic peptides in Chapter 8. In Chapter 9, Kilic has discussed some novel cold unit operations for the preservation of bioactive components of natural functional foods.

Essential oils are secondary metabolites extracted from potentially beneficial plants having antimicrobial and some other biological activities. Mutlu-Ingok et al. present a review on the recent advances in biological activities of essential oils in Chapter 10. Some natural polymers are significantly attractive wound dressing materials owing to their bioactivity and biocompatibility. Chapter 11 by Yaşayan et al. emphasizes the use of natural polymers and their functions in the wound healing process. Finally, Chapter 12 by Sharma et al. focuses on the hot topic of the recent pandemic year with

insights into coronaviruses and natural product-based therapeutic targets and strategies.

This volume contains many excellent reviews by eminent experts on bioactive natural products. The volume proves to be of great interest to researchers working in the field of medicinal and natural products chemistry.

I express my sincere thanks to Mr. Mahmood Alam for his support in the preparation of this volume. I also thank Dr. Taqdees Malik for her secretarial assistance.

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Chapter 6

Biosynthesis of polyunsaturated fatty acids by metabolic engineering of yeast *Yarrowia lipolytica*

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Chapter Outline

Introduction

The rapid development of society, especially Western civilization after World War II, has led to a change in diets. Mainly due to the high consumption of vegetable oils, a change in the type of fatty acids consumed has occurred, especially in the amounts of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs)

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TABLE 6.1 Names of PUFAs

ω-x Nomenclature	Abbreviation	Systematic Abbreviation
16:0	Palmitic	16:0
18:0	Stearic	18:0
18:1ω9	Oleic	9-18:1
18:2ω6	Linoleic or LA	9,12-18:2
18:3ω3	ALA	9,12,15-18:3
18:3ω6	GLA	6,9,12-18:3
18:4ω3	STA	6,9,12,15-18:4
20:2ω6	EDA	11,14-20:2
20:3ω6	DGLA	8,11,14-20:3
20:3ω3	ETrA	11,14,17-20:3
20:4ω6	ARA	5,8,11,14-20:4
20:4ω3	ETA	8,11,14,17-20:4
20:5ω3	EPA	5,8,11,14,17-20:5
22:4ω6	DTA	7,10,13,16-22:4
22:5ω3	DPA	7,10,13,16,19-22:5
22:6ω3	DHA	4,7,10,13,16,19-22:6

All double bond(s) have *cis* configuration(s), e.g., all-*cis* 6,9,12,15-18:4 or better (6*Z*,9*Z*,12*Z*,15*Z*-18:4)[a].

[a]*According to the current nomenclature, the geometric isomers should be used instead of the* cis–trans *nomenclature, the so-called* E–Z *nomenclature (see below), but by convention is still an outdated designation, i.e.,* cis. *Configuration* E–Z *is the IUPAC preferred method for describing the absolute stereochemistry of double bonds in organic chemistry according to the Cahn–Ingold–Prelog priority rules [15], i.e., the configuration* E *(from entgegen, the German word for "opposite") and the configuration* Z *(from zusammen, the German word for "together").*

(Table 6.1 and Fig. 6.1) [1]. According to WHO reports, excessive use of vegetable oils has shifted the ratio of ω-6/ω-3 from the ideal value of 1:1–1:0.25 to an almost unbelievable 1:0.1–1:0.03, i.e., values typical of European and US residents. This change in ratio brings with it a number of complications including an increased incidence of cardiovascular disease, increased risk of cancer and autoimmune diseases, mental disorders, worsening memory in the form of Alzheimer's disease, developmental retardation, visual disturbances, muscle weakness and tremor, dyslipidemia, insulin resistance, high blood pressure,

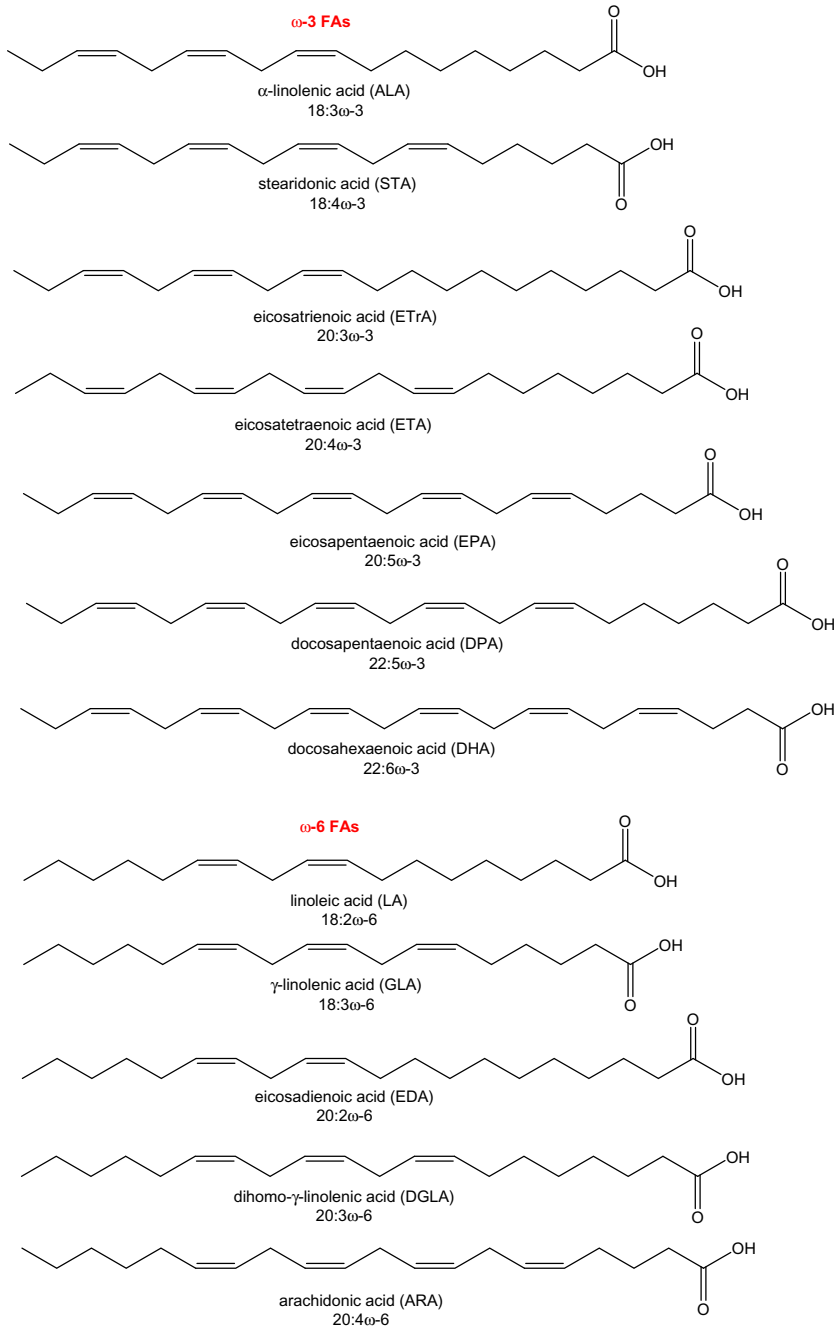

FIG. 6.1 Structures of the most common polyunsaturated fatty acids.

thrombosis, diabetes, asthma, and obesity [2–10]. Humans have the natural ability to biosynthesize eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) from linolenic acid, but production is insufficient to meet daily needs [11]. In humans, DHA is therefore either obtained from food or is biosynthesized in small amounts from eicosapentaenoic acid (EPA, 20:5, ω-3) by "Sprecher's shunt" [12] via the metabolic intermediate docosapentaenoic acid (DPA, 22:5 ω-3). The ability of human enzymes to desaturate and elongate essential linoleic acid (LA) and α-linolenic acid (ALA) to EPA and especially to DHA is low and insufficient to provide adequate levels of LC-PUFA to meet needs for mental and cardiovascular health. In addition, biosynthesis decreases with increasing age and in some diseases [13,14]. Therefore, DHA is now considered an essential PUFA.

The current production of PUFA from natural sources is not sufficient for commercial use. The main source of PUFAs is currently sea fish, but fish stocks are declining and may not represent a truly sustainable resource (Table 6.2) [16].

Furthermore, heavy metal pollution, toxic organic molecules, and recently, microplastics represent serious contamination problems for sea fish. Feeding fish on fish farms or freshwater ponds with wheat or corn leads to only minor levels of EPA and DHA in these fish; in addition, the ω-6/ω-3 ratio has shifted toward the ω-3 PUFA [1]. At present, as an alternative to fish oils, some ω-3 PUFA-rich oils, mostly from microorganisms [17] or agriculture crops, are added to foods sold on the market. Vegetable oils obtained from agricultural crops such as sunflower, soy, canola, palm and olive oils are relatively inexpensive and are not contaminated with pollutants. However, PUFAs found in commercially available vegetable oils are typically limited to LA (18:2ω-6) and ALA (18:3ω-3) [18].

In conventional PUFA biosynthesis, saturated long-chain fatty acids produced by fatty acid synthase (FAS) are modified by a series of elongation and desaturation reactions. A number of separate desaturase and elongase enzymes (approximately 30 different enzymes and 70 reactions; [19]) are required to produce unsaturated and longer chain PUFAs, typically EPA and/or DHA. See also a simplified scheme of biosynthesis (Fig. 6.2).

Many plants, including algae and some fungi, have the ability to synthesize LA and ALA. They express genes encoding Δ12-desaturase (*fad*2) and ω3-desaturase (*fad*3). The direct identification of the respective enzymes, i.e., Δ12-desaturase and ω3-desaturase, has been performed only a few times, for example, for Δ12-desaturase [20] and ω3-desaturase [21] from *Saccharomyces kluyveri*. Coexpression of two genes from *Kluyveromyces lactis,* structurally similar to Δ12-desaturase and ω3-desaturase of *Saccharomyces cerevisiae,* led to the endogenous production of both PUFAs, i.e., LA and ALA [22]. Furthermore, from the fungus *M. alpina*, Δ6-desaturase and Δ12-desaturase cDNA

TABLE 6.2 Sources of Polyunsaturated Fatty Acids [16]

PUFA	Conventional Sources	Microbial Sources
GLA	Plants (Evening primrose, Borage, Blackcurrant)	Fungi (*Mucor circinelloides, M. mucedo, Mortierella isabellina, M. ramanniana, Cunninghamella echinulata, C. elegans, C. japonica, Rhizopus arrhizus, Thamnidium elegans*) Algae (*Spirulina platensis, Chlorella vulgaris*)
DGLA	Human milk, animal tissues, fish (*Scomber scrombrus*), mosses (*Pogonatum urnigerum*)	Fungi (*Mortierella* spp., *Mortierella alpina, Conidiobolus nanodes, Saprolegnia ferax*)
ARA	Animal tissues, fish (*Brevoortia, Clupea*), mosses (*Ctenidium molluscum*)	Fungi (*Mortierella* spp., *M. alpina, Cladonia nanodes, Entomophthora exitalis, Blastocladiella emersonit*) Algae (*Porphyridium cruentum, Sargassum salicifolium, Euglena gracilis*)
EPA	Fish (herring, menhaden), shellfish (blue crab, oyster, lobster, mussel)	Fungi (*M. alpina, Mortierella elongata, Pythium irregulare, Pythium ultimum*), algae (*Chlorella minutissima, C. minitissima, Monodus subterraneus, Polysiphonia latifolium, P. cruentum, Phaeodactylum tricornutum, Nannochloropsis oculata, Amphidinium carteri, Thalassiosira pseudonana*)
		Bacteria (*Shewanella putrefaciens*)
ETA	Animal tissues	Fungi (*M. alpina*)
DPA	Fish	Fungi (*Schyzochytrium* sp.)
DHA	Fish (tuna, herring, cod, sardine, salmon, menhaden), shellfish (blue crab, mussel, lobster, oyster)	Fungi (*Thraustochytrium aureum, T. roseum, Schyzochytrium* SR21, *Schyzochytrium aggregatum*), algae (*Crypthecodinium cohnii, Gyrodinium nelsoni, A. carteri, Gonyaulax lyedra*), bacteria (*Vibrio* spp., *Rhodopseudomonas* spp.)

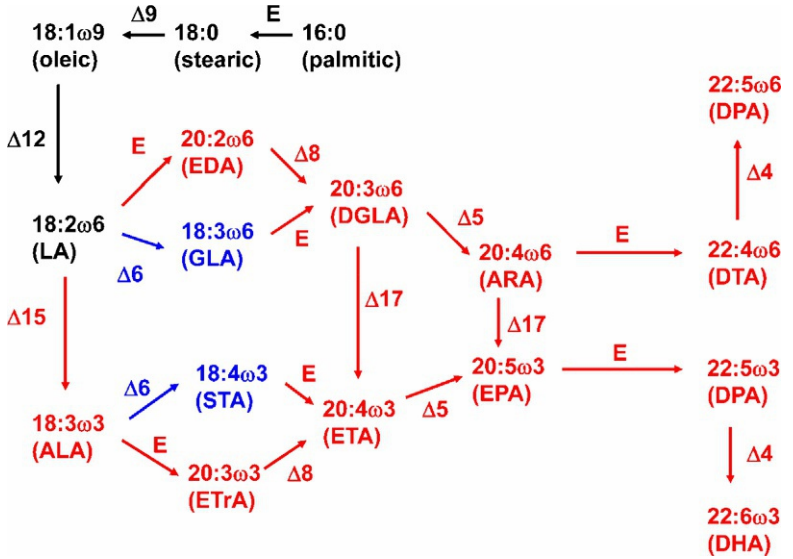

FIG. 6.2 Metabolic engineering in *Yarrowia lipolytica* for PUFA production. The native fatty acid pathway is indicated in black and the genetically modified pathway for PUFA production is indicated in red. Undesirable metabolic pathways leading to ω-6 PUFAs, i.e., mainly GLA, are indicated in blue.

were isolated and their coexpression in *S. cerevisiae* showed that the yield of γ-linolenic acid reached up to 8% of total fatty acids. The yeast was cultured at 15°C for 2 days in a synthetic medium containing 2% galactose [23].

Genetic engineering strategy inducing PUFA production

The metabolic engineering of nutraceutical products provides an attractive alternative to chemical synthesis or extraction from natural material, allowing the production of pure compounds under environmentally acceptable conditions. Model microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, or *Corynebacterium glutamicum* are used for this purpose [24–26]. These traditional hosts were usually selected based on their well-described metabolism, safe use status in the pharmaceutical and food industries and high genetic traceability [27]. Despite the prospects provided by metabolic engineering, regulation is required for the use of genetically modified organisms (GMOs) in commercial production processes. In this respect, GMO regulation includes approval processes, risk assessment, and other requirements that vary considerably from country to country [28–30].

From the very beginning, GMO foods [31–34], or food supplements, including PUFAs produced by genetically modified *Yarrowia lipolytica*, have

been widely discussed both as a result of their industrial applications and the possible consequences of their use. Ethical concerns include adverse effects on human health, the decline of overall biodiversity and, most importantly, the risk of release into the wild, as can be observed in some conspiratorial rumors about the escape of Covid-19 virus from the laboratory. These concerns can affect a person's psychological state when consuming GM products. The issue of labeling of GMO foods, for example, in the United States, in contrast to 64 other world nations, including the EU, is different and is also accepted differently by the public. In contrast, GMOs benefit humanity when used for purposes such as increasing the availability and quality of food, medical care and contributing to a cleaner environment. Used wisely, they could result in improved economic activity and alleviate food deficiencies. However, the full potential of GMOs cannot be realized without close attention to the risks associated with each new GMO case.

In recombinant microorganisms, the microbial profile of fatty acids can be altered both by creating new biosynthetic pathways in the host and/or by suppressing undesirable biosynthetic pathways. This can result in increased production of desirable, and reduced production of undesirable PUFA(s). In the case of PUFA production, this primarily means to influence the ratio of ω-3 and ω-6 fatty acids synthesized. This possibility leads to specific biosynthesis of, e.g., EPA without significant production of, e.g., γ-linolenic acid (GLA; 18:3ω-6). Microbial production of EPA using recombinant strains of microorganisms has advantages over production of EPA from natural microbial sources, e.g., diatoms *Cyclotella* sp. or *Nitzschia* sp., the bacterium *Shewanella*, filamentous fungi of the genera *Pythium* sp., *Mortierella elongata* or isolation from fish oil and/or marine plankton.

For the development and optimization of *Y. lipolytica* in order to improve production mainly of fatty acids, the whole bioprocess, including the preparation of the starting strain, must be performed on a larger scale, i.e., at least in a pilot plant in fermenters of tens of liters. Production must also be optimized. A study has already been published [35] that convincingly demonstrates that metabolic engineering, systems biology integration, synthetic biology, and evolutionary engineering can enable microbial strains to produce chemicals efficiently. One way to optimize cellular metabolism is to reduce the production of undesirable metabolites (i.e., biosynthesis of certain fatty acids, e.g., GLA) [36,37]. Other low-cost carbon sources can be used to reduce fermentation costs, especially single-carbon substrates, which can be metabolized to fatty acids by metabolic engineering of *Y. lipolytica*. We believe that genetically modified strains of *Y. lipolytica* can be used to produce fatty acid-based chemicals, holding great promise for researchers working in this field.

As a basis for the successful production of lipids in yeast, knowledge of the complete genome is necessary. In addition, genome-scale models

(GSMs) of *Y. lipolytica* [38,39] should cover multiple reactions, and its reconstruction should involve annotations of unknown protein sequences predicted from the entire genome. Furthermore, much more attention must be paid to the physiological conditions under which the yeast is grown. One possibility is to influence the carbon-to-nitrogen ratio, the amount of phosphorus, and other elements such as magnesium. High transformation efficiency is also important to avoid time losses due to the need for repetitive experiments. It is necessary to integrate genes into specific loci and to insert the entire pathway, which would allow integration of more than one DNA fragment and thus significantly speed up strain engineering. In the field of metabolite analysis, in this case PUFAs, it is first necessary to simplify the preparation of methyl esters or even to analyze free fatty acids and to reduce the time required for analysis, see, e.g., Rezanka et al. [40]. It is also necessary to suppress the activity of enzymes causing, for example, the hydrolysis of triacylglycerols as end products of lipid biosynthesis and the degradation of fatty acids (β-oxidation). There are many possible solutions for implementation of more efficient engineering of *Y. lipolytica*. Tools and methods developed in the past could serve as starting points for the development of more productive strains. Furthermore, it would be possible to include other yeasts such as *S. cerevisiae* in screenings, which could lead to an expansion of possible strains to use and may be of great practical importance.

Biosynthesis of microbial PUFAs

For a long time, species of yeasts *S. cerevisiae* or *Schizosaccharomyces pombe,* used in the production of beer or bread, have served as model organisms in the development of metabolic engineering strategies for the synthesis of certain metabolites [41]. Wild strains of *S. cerevisiae* produce saturated and monounsaturated fatty acids, mainly with 16- and 18-carbons, because they contain only one fatty acid desaturase, i.e., Δ9-desaturase (OLE1), which is capable of producing monounsaturated palmitoleic (16:1ω-7) and oleic (18:1ω-9) acids [42]. Both *S. cerevisiae* and *Sch. pombe* are unable to synthesize linoleic acid (18:2ω-6) [43,44]. However, in some yeasts, oleic acid can be desaturated by introducing a second double bond at the Δ12 position with Δ12-fatty acid desaturase, followed by desaturation of ω3-fatty acids (Fig. 6.3) to form ALA. Examples include *S. kluyveri* or *K. lactis* that can produce both PUFAs (18:2ω-6 and 18:3ω-3) [22,44]. In addition to *S. kluyveri* and *K. lactis*, *Candida albicans* and *C. tropicalis* are able to produce ALA to a greater or lesser extent, but other yeasts such as *Candida boidinii* and *Zygosaccharomyces rouxii* are only able to produce LA [43]. Oleaginous yeasts include *Y. lipolytica* (max lipid content 36%) and *Trichosporon pullulans* (65%) producing about 1% ALA, but *Lipomyces starkeyi* (63%), *Cryptococcus albidus* (65%), and *Rhodotorula glutinis* (72%) can only produce LA [45].

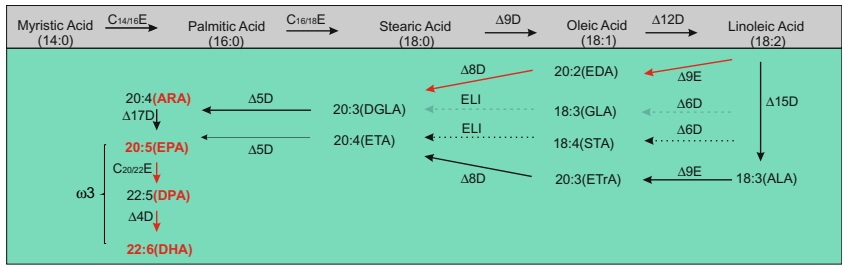

FIG. 6.3 Metabolic engineering in *Yarrowia lipolytica* for omega-3 EPA and DHA production (indicated in *red*). The native fatty acid pathway is indicated in the gray box and the engineered pathway for ω-3 EPA and DHA production is indicated in green.

PUFA synthesis occurs in mammals, including humans, predominantly by the Δ6-desaturation route, with dietary LA and/or linolenic acids being desaturated by Δ6 desaturase to γ-linolenic (18:3ω-6) and stearidonic (18:4ω-3) acids, see Fig. 6.3. Hydrocarbon chain elongation and further desaturation leads to arachidonic (20:4ω-6), possibly dihomo-γ-linolenic acid (20:3ω-6) in the ω-6 pathway, and eicosapentaenoic (20:5ω-3) and docosahexaenoic (22:6ω-3) acids via eicosatetraenoic acid (20:4ω-3) in the ω-3 pathway (Fig. 6.3). Therefore, suitable desaturase and elongase genes must be introduced into yeast for the production of PUFAs; see several examples in Table 6.3.

In order for *S. cerevisiae* to produce PUFAs with 20 or 22 carbon atoms, it is necessary to insert genes encoding Δ4-, Δ5-, and Δ6-fatty acid desaturases. As mentioned above, (see Table 6.3), various organisms have been used, including higher plants, algae, mosses, fungi, nematodes, and also mammals. In addition, elongase genes of *S. cerevisiae* are necessary. These are the three elongations of fatty acids genes that unfortunately are not functional for the biosynthesis of the PUFAs described above. The first of these, i.e., Elo1p mainly elongates myristic and palmitic acids, and Elo2p and Elo3p are involved in the formation of very long-chain saturated fatty acids, which are a part of sphingolipids. Therefore, elongase genes from other organisms were used. In essence, these organisms produce C_{20} and C_{22} PUFAs. Because Δ5 and Δ6 desaturases are active in both series (ω-6 and ω-3), the resulting products can be both arachidonic and eicosapentaenoic fatty acids. For examples see paper [46], which used elongase (*Caenorhabditis elegans*), Δ5-desaturase (*M. alpine*), and Δ6-desaturase (borage). *S. cerevisiae* was cultured for 4 days at 25°C in a synthetic medium containing 2% galactose, and two fatty acid starters (LA and/or ALA) were added at a concentration of 0.5 mM. After cultivation, the fatty acid composition was as follows: γ-linolenic 6.8%, dihomo-γ-linolenic 1.4%, arachidonic 0.2%, and eicosapentaenoic 0.2%.

TABLE 6.3 Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs

Biological Material	Enzymes	GenBank Accession No.
Pavlova lutheri	Δ4 fatty acid desaturase	AY332747
Caenorhabditis elegans	Δ5 fatty acid desaturase	AAC95143.1
Dictyostelium discoideum	Δ5 fatty acid desaturase	AB022097
Homo sapiens	Δ5 desaturase	AF199596
Mortierella alpina	Δ5 fatty acid desaturase	AF067654
Mus musculus	Δ5 desaturase D5D	AB072976
Phytophthora megasperma	Δ5 fatty acid desaturase	AJ510244
Pythium irregulare	Δ5 fatty acid desaturase	AF419297
Rattus norvegicus liver	Δ5 desaturase	AF320509
Thraustochytrium sp.	Δ5 fatty acid desaturase	AF489588
Argania spinosa	Δ6 desaturase	AY131238
Borago officinalis	Δ6 desaturase	AF007561
Ceratodon purpureus	Δ6 fatty acid desaturase	AJ250735
Echium gentianoides	Δ6 desaturase	AY055117
Echium pitardii var. *pitardii*	Δ6 desaturase	AAL23581.1
H. sapiens	Δ6 fatty acid desaturase	AF126799
Mortierella alpina	Δ6 fatty acid desaturase	AF465283
Mortierella isabellina	Δ6 fatty acid desaturase	AF465281, AF110510, AF465282
Mucor circinelloides	Δ6 fatty acid desaturase	AB052086
Mucor rouxii	Δ6 desaturase	AF296076
M. musculus	Δ6 fatty acid desaturase	AF126798
Pythium irregulare	Δ6 fatty acid desaturase	AF419296
Synechocystis sp.	Δ6 desaturase	L11421
Acheta domesticus	Δ9 desaturase 3	AF338466

TABLE 6.3 Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs—Cont'd

Biological Material	Enzymes	GenBank Accession No.
Anabaena sp.	Δ9 desaturase	E11368
Emericella nidulans	Δ9 stearic acid desaturase	AY504633
Mortierella alpina	Δ9 desaturase	AF085500
Ogataea (*Pichia*) *angusta*	Δ9 fatty acid desaturase	D83185
Picea glauca	Δ9 desaturase	AF438199
Synechococcus vulcanus	Δ9 acyl-lipid fatty acid desaturase	U90417
Synechococcus vulcanus	Δ9 acyl-lipid fatty acid desaturase	U90417
Arabidopsis thaliana	Δ12 desaturase microsomal	BAB01960.1
C. elegans	Δ12 fatty acid desaturase FAT-2	AF240777
Emericella (*Aspergillus*) *nidulans*	Δ12 desaturase oleate	AAG36933
Chlorella vulgaris	Δ12 desaturase CvFad2	AB075526
Mortierella alpina	Δ12 fatty acid desaturase	AAL13300
Mortierella alpina	Δ12 fatty acid desaturase	AB020033
Mortierella alpina	Δ12 fatty acid desaturase	AF110509
Mortierella alpina	Δ12 fatty acid desaturase	AF417244
Mucor rouxii	Δ12 desaturase	AF161219
Spirulina platensis	Δ12 desaturase	X86736
Perilla frutescens	Δ15 desaturase	AAL36934
Synechocystis sp.	Δ15 desaturase	BAA18302.1
Synechocystis sp.	Δ15 desaturase	BAA02924
Chlamydomonas reinhardtii	ω6 desaturase for chloroplast	AB007640
C. elegans	Elongation of very long chain fatty acids protein 6	NM_068396
Mortierella alpina	Elongase gene	AX464731

Continued

TABLE 6.3 Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs—Cont'd

Biological Material	Enzymes	GenBank Accession No.
M. musculus	ELOVL family member 5, elongation of long chain fatty acids	NM_134255
R. norvegicus	ELOVL fatty acid elongase 5	NM_134382
R. norvegicus	ELOVL fatty acid elongase 6	NM_134383
Brassica oleracea	Stearoyl-ACP desaturase	AAK14969.1
A. thaliana	3-Ketoacyl-CoA synthase 18	NM_119617

Another long-chain fatty acid, docosahexaenoic acid, was also produced by introducing genes into *S. cerevisiae*. This involved coexpression of Δ6-elongase (alga *Thalassiosira pseudonana*) and Δ5-elongase (alga *Ostreococcus tauri*) with algal Δ5-desaturase (*Phaeodactylum tricornutum*) and Δ4-desaturase (*Euglena gracilis)* or three bifunctional elongases (fish *Oncorhynchus mykiss*), algal Δ5-desaturase (*P. tricornutum*) and Δ4-desaturase (*E. gracilis*) [47]. The yeasts were cultured at 20°C for 4 days in a synthetic medium containing 2% galactose and 0.25–0.5 mM stearidonic acid (18:4ω-3) as a precursor. The yield was about 0.5% DHA, based on total fatty acids.

Although not all examples of successful gene transfer responsible for the production of PUFAs in *S. cerevisiae* are given above, it can be seen (see also paper of [48]) that the yields are not completely comparable with sources used for production of PUFAs. Therefore, attention was focused on another yeast, e.g., *Y. lipolytica*. There is, of course, the possibility of increasing the production of lipids in *S. cerevisiae*, either by the classical procedure, i.e., by changing the culture conditions, or by means of genetically modified yeast. As an example, we should mention article [49] where the authors successfully reprogramed yeast metabolism from alcoholic fermentation to lipogenesis. In essence, the key step was to prevent the formation of acetyl-CoA in the cytosol and the transfer of pyruvate formed from glucose via glucose β-phosphate and phosphoenolpyruvate into the mitochondrial matrix. Here, pyruvate is involved in the tri-carboxylic acid (Krebs) cycle and the resulting acetyl-CoA is further metabolized to malonyl-CoA and fatty acids. Although the goal was to create a strain producing free fatty acids (predominantly C10 to C18, palmitoleic and oleic acids, with palmitic, palmitoleic and oleic acids being identified as the majority), it is certainly possible to create a strain producing PUFAs.

Biosynthesis of lipids in modified *Yarrowia lipolytica*

Next to standard organisms, many other microorganisms have been investigated for the production of nutraceuticals, e.g., *Y. lipolytica* [50,51] has been extensively studied for its innate ability to produce large quantities of lipids [52]. Oleaginous yeasts are defined as yeasts that are naturally capable of biosynthesis and oil storage, with an oil content of at least 25% dry cell weight (DCW). Thanks to metabolic engineering efforts, PUFAs have also been produced (e.g., Table 6.4).

Y. lipolytica belongs to the Ascomycota family. The ascigerous teleomorph of *Candida lipolytica*, previously classified as *Endomycopsis lipolytica* and *Saccharomycopsis lipolytica*, has been assigned to the new genus *Yarrowia*. *Yarrowia lipolytica* is a typical species of this genus [53]. The complete genome of the strain *Y. lipolytica* CLIB122 has been published (Gen Bank: GCA000002525). It is approximately 20.5 Mb in size and is organized into 6 chromosomes that encode ~6472 genes. *Y. lipolytica* is a nonpathogenic yeast that can utilize various fats or hydrocarbons as carbon sources. Therefore, it is often isolated from foods such as cheese and sausage, or from natural sources such as oil fields. *Y. lipolytica* is only very distantly associated with other yeasts. It shares several properties with filamentous fungi, including resolution to true mycelial form under certain growth conditions. *Y. lipolytica* does not undergo mating type switching and is used as a model organism in basic research as well as for industrial applications such as the production of nutraceuticals [54].

Eicosapentaenoic acid and docosahexaenoic acid

Considerable efforts have been made at the laboratory level to express elongases and desaturases for PUFA biosynthesis in various microorganisms such as *S. cerevisiae* [59], *Mortierella alpina* [60], and *Lipomyces starkeyi* [61]. In the field of commercialization, only modified *Y. lipolytica* strain Y4305, which was developed by the DuPont company, has been used in a genetic engineering strategy [56,62–64]. The following genes encoding proteins were used for PUFA biosynthesis: synthetic Δ9 elongase derived from *E. gracilis*, C16/18 elongase from *Mortierella alpina*, Δ5 desaturase from *Peridinium* sp., Δ8 desaturase from *E. gracilis*, Δ12 and Δ15 desaturases from *Fusarium moniliforme*, Δ17 desaturase from *Phytophthora ramorum*; codons were always optimized for expression in *Y. lipolytica* [64]. Various strains of *Y. lipolytica* from the American Type Culture Collection were first screened and strain ATCC 20362 was selected because its DCW production was greater than 100 g/L, with lipid production up to 30% DCW. This yield meant that lipid productivity was more than 1 g/L/h. In the next step, this strain was transformed with three desaturases (Δ5, Δ6, and Δ17) and C18/20 elongases under the control of the translation elongation factor (TEF) promoter. As a result of this transformation, a yeast producing 3% EPA was obtained [65].

TABLE 6.4 Production of PUFAs in Engineered *Y. lipolytica*

Product	Production	Strain	Medium, Fermentation Type and Parameters	References
α-Linolenic acid	1400 mg/L	L36DGA1	YSC medium contained 80 g/L glucose; a pulse of 80 g glucose was added at 72 h/fed-batch 2 L bioreactor, 20°C	[55]
EPA	56.6% of total lipids with 15% DCW	ATCC 20362	Nitrogen-rich medium contained 20 g/L glucose for the first stage fermentation, nitrogen-limited medium contained 80 g/L glucose for the second stage fermentation/two-stage flask, 30°C	[56]
EPA	50% of total lipids with 25% DCW	Y4305	Two-stage 2 L bioreactor, 30°C	[57]
DHA	5.6% of total lipids	Y4305	Cells were grown in MM medium containing 20 g/L glucose for 48 h, then transferred to HGM medium containing 80 g/L glucose for additional 72 h fermentation/two-stage fermentation, 30°C	[58]

Unfortunately, although this procedure proved to be viable, three basic deficiencies were identified and gradually eliminated in the next steps: low flow of metabolites into the engineered pathway, low efficiency of elongases and low levels of gene expression. Improvements have been achieved by eliminating these shortcomings. To increase flow to the Δ6 pathway, overexpression of elongase from *M. alpina* was performed, which increased the conversion of palmitic acid to stearic acid and Δ12 desaturase from *F. moniliforme*. This strain already contained 19 copies of various heterologous genes and was capable of producing up to 40% EPA relative to DCW. However, expression also had an adverse effect. The expressed Δ6 desaturase, and lack of CoA pools in the membrane of the endoplasmic reticulum, produced undesirable PUFA ω-6 fatty acids, and especially 21% of GLA.

Therefore, a completely different procedure for EPA biosynthesis was developed, based on replacing the Δ6 pathway with the Δ9 pathway, see Fig. 6.1. Multiple copies of selected genes were integrated into the genome, and an optimized codon with a strong promoter was obtained, including genes encoding C16/18-elongase, Δ5, Δ8, Δ12, Δ17-desaturases, and choline phosphotransferase. The strain prepared in this way already contained 30 copies of nine different genes and production achieved 56.6% EPA as a proportion of total fatty acids and DCW yield was approximately 15%. GLA was not produced. This strain was further improved to produce a mutant strain that contained a total of 35 copies of 17 different genes. EPA production reached 58% of total FAs, and the DCW yield was approximately 20%. To fully optimize the culture conditions, an industrially usable strain was constructed containing 41 copies of 19 different genes and EPA production was about 50% based on total FAs; DCW yield was approximately 25%. This yield was achieved by optimizing the fermentation processes.

Interest in the production of PUFAs using genetically modified yeasts has been reflected in the number of patents that have been granted. Just a few of the most significant examples are US patents. Two patents relate mainly to the preparation of modified strains of the oleaginous yeast *Y. lipolytica* capable of producing more than 25% of eicosapentaenoic acid (EPA) based on total lipids [66], or more than 5.6% docosahexaenoic acid (DHA), again based on total lipids [67]. The other two patents [68,69] granted to the same company relate to processes for the production of ω-3 and/or ω-6 fatty acids in oleaginous yeasts of the genus *Yarrowia*. They have been genetically modified with desaturases and elongases, for example, from *Mortierella alpina* and *Saprolegnia diclina* capable of catalyzing the elongation and desaturation of acids (LA-GLA-DGLA-ARA-EPA, ALA-STA-ETA-EPA-DPA, and/or DGLA-ETA). The patent of Knutzon et al. [70] relates to fatty acid desaturases capable of desaturating C18 acids. This is again a biosynthetic pathway in the O-LA-GLA or ALA-STA sequences. One can also mention some other patents; we chose two of them as examples. The first [71] describes genetically engineered strains of the oil yeast *Y. lipolytica*.

Production of strain Y8672 reached up to 61.8% EPA, based on total fatty acids. Another patent [72], again used the genetically modified yeast *Y. lipolytica* strain Y2214, and describes the production of arachidonic acid to a level of 14% of total fatty acids.

Triacylglycerols

Major metabolic engineering efforts in *Y. lipolytica* have focused on increasing the production of lipids, especially triacylglycerols (TAGs). Because TAGs are nonpolar, they are not part of the cell membrane phospholipid bilayer, and as lipid bodies, are located within the cell [73]. Many approaches have been developed to increase targeted lipogenesis, in particular the regulation of lipase activity (reverse hydrolysis of TAGs to FFAs), and the reduction of enzymes that catalyze β-oxidation or peroxisome formation. Overexpression of natural diacylglycerols was used for their extensive potential in the biosynthesis of triacylglycerols. These overexpression targets were catalyzed by acyltransferase (DGA1) with concomitant deletions of genes (pex10 and mfe1) that promote β-oxidation and peroxisome formation [74] followed by lipid yields of 25.3 g/L (90% dry matter) in such a modified *Y. lipolytica*. Similarly, expression of DGA1 and acetyl-CoA carboxylase led to the formation of a mutant producing 17.6 g/L lipids (61.7% based on dry matter) [75]. In addition, transcriptomic analysis of the high-production mutant strain of *Y. lipolytica* showed Δ9 desaturase activity that reduced the tricarboxylic acid cycle flux and increased the glycolytic flux. It was further reported that overexpression of DGA1 in this mutant strain resulted in lipid production of 25 g/L, which represented 86% of dry matter [76]. Floating cells were also selected after random mutagenesis in *Y. lipolytica* and a strain was found that produced almost 40 g/L lipids (87% of dry matter) [76].

Enhanced PUFA production using different carbon sources

Further attention has been paid to modifications that allowed cells to utilize less traditional and especially cheaper substrates. Most of the cultures mentioned above were performed on glucose, which is an economically unsuitable source of carbon. On the contrary, lignocellulosic biomass (hemicellulose), which is a polysaccharide composed of xylose, appears to be a suitable (cheap) source. Following genetic engineering of enzymes capable of degrading xylose (xylose reductase, xylitol dehydrogenase, and xylulokinase), *Y. lipolytica* was able to utilize this source, with lipids accounting for 21% of the dry matter [77]. Similarly, Li and Alper [78] used heterologous enzymes from *Scheffersomyces stipitis,* the resulting lipid production was 15 g/L. Many other polymers (oligomers) of carbohydrates have also been used as carbon sources. These include, for example, sugarcane bagasse,

molasses, inulin, etc. From the above overview, it is clear that *Y. lipolytica*, after appropriate genetic modification, is able to utilize essentially any oligo- and/or polysaccharide.

However, by far the most common alternative carbon source is glycerol. Glycerol is basically waste material in the production of biofuels from vegetable oils. *Y. lipolytica* has an innate ability to take advantage of this carbon source and the production of 3.5 g/L lipids (43% by dry cell weight) has been described [79]. Another strain of *Y. lipolytica* was able to produce 4.72 g/L lipid (21% dry matter) in fermenter batch culture using crude glycerol [80]. However, far better results have been achieved with the help of metabolic engineering. Heterologous glycerol dehydratase was expressed in *Y. lipolytica* and the lipid yield was 13 g/L (30% dry cell weight) [81]. Glycerol has also been used as an additive with another carbon source. Experiments were performed in which glycerol was added, for example, to xylose in a fed batch fermenter or was added to animal fats. These examples clearly show that high lipid production in *Y. lipolytica* can be achieved on various, especially waste sources, which considerably reduces the cost of industrial lipid production in genetically modified yeast.

Another way to increase the production of PUFAs is to change the carbon source. For example, after the addition of LA to the culture medium, the conversion of this acid to γ-linolenic acid was up to 69% by *Y. lipolytica* with heterologous overexpression of the Δ6-desaturase from *M. alpine* [82].

Pilot—Scale process for microbial PUFA production

To test the prepared recombinant strains, the authors used three types of equipment [62]. Firstly, 24-well blocks, test tubes, shake flasks, and microfermenters, secondly, several-liter laboratory fermenters and thirdly, a 5000 L pilot-scale fermenter. The cultivation of *Y. lypolitica* is a two-stage cultivation, i.e., growth and production (lipids and PUFAs). Therefore, the cells were first cultured in a simple bioreactor to a specific density, and after culture nitrogen had been depleted, glucose was added to produce lipids. The lipid composition of yeast, including the production of EPA and other fatty acids, was determined by gas chromatographic analysis. DCW and total lipid content was also determined in shake flasks and microfermenters. Most production strains were further cultivated in laboratory fermenters.

The inoculum prepared in the flasks was transferred to a 2 L laboratory fermenter with 950 mL of culture medium. The course of the culture, including production, is shown in Fig. 6.4, and for more details, see the Table of the US Patent [64]. To optimize the transfer of results from a laboratory fermenter to a pilot plant fermenter (5000 L) modeling was performed, where many parameters were optimized, particularly model equations of cell growth, substrate consumption, nitrogen utilization, oxygen uptake, lipid and EPA accumulation.

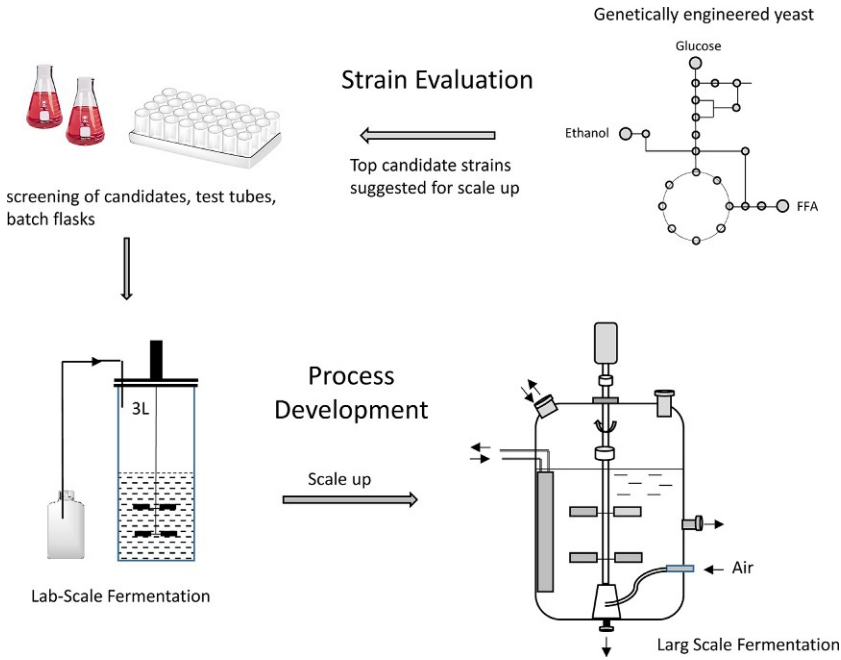

FIG. 6.4 Workflow of fermentation of PUFAs. Strains generated by metabolic engineering are first evaluated using test tubes and batch flasks, further tested in laboratory fermenters and finally, large-scale fermentation is performed.

The best strain, which was verified in pilot scale cultures of *Y. lipolytica* for the production of EPA, was finally grown in a 5000L fermenter, and ultimately resulted in patent filing and industrial production of two dietary supplements, i.e., New Harvest™ EPA oil and Verlasso® oil.

Engineering *Y. lipolytica* for production of lipid compounds other than PUFAs

The above examples show that *Y. lipolytica* can be used for biosynthesis of both "natural and atypical PUFAs," i.e., fatty acids that are not produced by wild strains, as well as other lipid compounds. For example, ricinoleic acid, a major component of castor oil. Ricinoleic acid (12-hydroxyoctadec-cis-9-enoic acid) has many specialized uses in bioproduction industries, and castor bean is currently the only commercial source of this fatty acid. The paper of Meesapyodsuk et al. [83] describes metabolic engineering of a microbial system (*Pichia pastoris*) to produce ricinoleic acid using a "push" (synthesis) and "pull" (assembly) strategy. CpFAH, a fatty acid hydroxylase from *Claviceps purpurea*, was used for synthesis of ricinoleic acid, and CpDGAT1, a diacylglycerol acyl transferase for triacylglycerol synthesis from the same

species, was used for assembly of the fatty acid. Coexpression of CpFAH and CpDGAT1 produced higher lipid contents and ricinoleic acid levels than expression of CpFAH alone. Coexpression in a mutant haploid strain defective in Δ12 desaturase activity resulted in a higher level of ricinoleic acid than in the diploid strain. Intriguingly, the ricinoleic acid produced was mainly distributed in the neutral lipid fractions, particularly the free fatty acid form, but with little in the polar lipids. This work demonstrates the effectiveness of the metabolic engineering strategy for industrial uses and excellent capacity of the microbial system for production of ricinoleic acid as an alternative to plant sources. The genetically modified *Y. lipolytica* strain produced more than 60 mg of ricinoleic acid per 1 g DCW, which was achieved by deletion of β-oxidation, TAG acyltransferases, and Δ12 desaturase. This production was achieved by overexpression of heterologous Δ12 hydroxylases from the plant *Ricinus communis* and fungus *Claviceps purpurea*, i.e., wild strains that normally produce ricinoleic acid [84].

Another possibility is the production of γ-decalactone by β-oxidation of ricinoleic acid. Specifically, ricinoleyl-CoA can undergo four cycles of β-oxidation to produce 4-hydroxydecanoic acid, which can then be cyclized into γ-decalactone [85]. By optimizing the culture conditions, in particular oxygen transfer rate, cell density, and fed-batch cultivation in which an oil was added, a yield of up to 6.8 g/L was achieved [86].

Production of 4.98 mg/L pentane was achieved by overexpression of the soya lipoxygenase gene [87]. This production was made possible by *Y. lipolytica* having a high tolerance for ω-alkanes, aldehydes, and fatty alcohols. This fact was also used for the production (350 mg/L) of C6-aldehyde of [88], where the fatty acid hydroperoxide lyase from green bell pepper was over-expressed in *Y. lipolytica*. This yeast can also be used to produce fatty alcohols after insertion of the acyl-CoA reductase from the uropygial glands of the barn owl (*Tyto alba*). Production was more than 636 mg/L of intracellular and 53 mg/L of extracellular hexadecanol [89]. Acyl-CoA reductase from *Arabidopsis thaliana* inserted into the *Y. lipolytica* knockout strain, allowed the production of more than 500 mg/L decanol [90].

Engineering *Y. lipolytica* for biodiesel production

Many different processes have been used for the production of biofuels, and these have undergone further development of various technical and conceptual approaches over time, from first to third generation biodiesel production. For the production of first-generation biodiesel, edible vegetable oils such as soybean, canola, sunflower, palm, etc., were used, now encompassing almost 99% of all biodiesel production. However, the use of edible oils as an energy source has given rise to many objections and controversies regarding their use as fuel and also deforestation in order to obtain free land for the planting of oil palms. The second generation of biodiesel comes from lignocellulosic waste

in agriculture and inedible oils (e.g., *Jatropha curcas*), that also emerged as an attractive alternate raw material for the production of biodiesel on land unsuitable for agriculture. In the third generation, a procedure for more sustainable biodiesel production is being sought, and interest is focused on yeasts and fungi as a source of biodiesel (as well as microalgae).

Biodiesel is a mixture of alkyl esters (mostly methyl esters) of fatty acids obtained by transesterification of vegetable oil or animal fat [91]. However, biodiesel must meet existing standards, such as EN 14214 in EU, which limits the proportion of total unsaturated FAs (iodine value), methyl esters of linolenic acid, and PUFAs content with four or more double bonds [92,93]. PUFAs are responsible for low oxidative stability and a tendency to form gums [94]. The goal of high-quality biofuel production is a balanced mixture of 16:1, 18:1 and 14:0 fatty acids in a ratio of 5:4:1 [95]. This ideal mix has been identified as providing a good cetane number along with a low oxidation potential and a cold filter plugging point [96].

In most published articles, the authors focused on the genetic modification of yeast, which then produced PUFAs that wild-type strains do not normally produce [97]. However, the presence of PUFAs is undesirable for producing biofuels. Therefore, Tsakraklides et al. [98] used a completely different approach—to increase the production of oleic acid, see Fig. 6.5. A strain of *Y. lipolytica* that produced TAGs containing a high content of oleic acid was constructed. Deletions and over-expressions in the synthesis of fatty acids and TAGs that increased oleate levels were studied. The accumulation of oleic acid in TAGs was promoted by the exchange of native △9 fatty acid desaturase and glycerol-3-phosphate acyltransferase for heterologous enzymes, as well as the deletion of △12 fatty acid desaturase and fatty acid elongase. Deletion of the native Δ12 fatty acid desaturase led to the elimination of LA production.

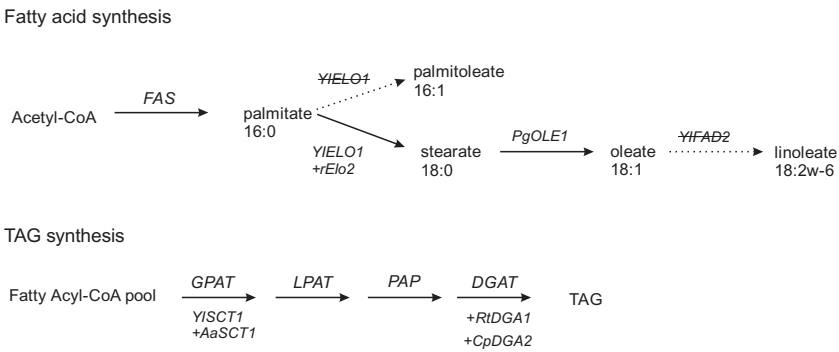

FIG. 6.5 A simplified pathway for fatty acid and TAG synthesis in *Y. lipolytica* was used with gene deletions (*Ylole1*, *Ylsct1*, *Ylfad2*) and heterologous gene expressions (*PgOLE1*, *AaSCT1*, *rELO2*, *RtDGA1*, *CpDGA2*) incorporated into the high-oleate strain. *DGAT*, diacylglycerolacyltransferase; *FAS*, fatty acid synthase, *GPAT*, glycerol-3-phosphate acyltransferase; *LPAT*, lysophosphatidic acid acyltransferase; *PAP*, phosphatidate phosphatase; *TAG*, triacylglycerol.

The wild-type and Δ*fad*2 strains were grown in nitrogen-free medium for 96 h, and analysis of fatty acid content by GC–MS after cultivation confirmed the absence of LA. Further, heterologous glycerol-3-phosphate acyltransferase expression in *Y. lipolytica* increased lipid production only minimally. By combining these genetic steps, polyunsaturated fatty acids were eliminated and a strain of *Y. lipolytica* was prepared that accumulated TAGs containing more than 90% oleate.

In addition, an oleaginous strain of *Rhodosporidium toruloides* (ATCC20409) for the production of microbial lipids such as biodiesel was investigated. The strain used a consolidated process to produce a high lipid content (76% of the dry weight of the biomass), which was then trans-esterified to produce biodiesel. This had the physicochemical properties in accordance with ASTM standards [99].

Lignocellulosic biomass represents raw material for producing biofuels. A study by Singh et al. [100] describes an integrated microbial fermentation process involving a thermoanaerobe and a marine thraustochytrid, aiming at the coproduction of docosahexaenoic acids and bioethanol using rice straw biomass as a substrate.

Conclusion

All of these examples, including the production of PUFAs, show that individual GMO strains of *Y. lipolytica* can be designed to produce almost any lipid compound. It is necessary to continue in this field, both with scientific experiments and with industrial uses of GMOs of *Y. lypolitica*, which have GRAS status. This phenomenon is reflected in the recognition of two industrial yeast products, named by DuPont as New Harvest™ EPA oil and oil Verlasso® for dietary supplements in sustainable salmon farming.

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Abbreviations

ALA α-linolenic acid
ARA arachidonic acid
DGLA dihomo-γ-linolenic acid
DHA docosahexaenoic acid
DPA docosapentaenoic acid
DTA docosatetraenoic acid
EDA eicosadienoic acid

EPA eicosapentaenoic acid
ETA eicosatetraenoic acid
ETrA eicosatrienoic acid
GLA γ-linolenic acid
GRAS generally regarded as safe
LA linoleic acid
STA stearidonic acid

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