



## Review

# Yarrowia lipolytica as a biotechnological chassis to produce usual and unusual fatty acids

Rodrigo Ledesma-Amaro <sup>a,\*</sup>, Jean-Marc Nicaud <sup>a</sup><sup>a</sup> INRA, UMR1319 Micalis, F-78350 Jouy-en-Josas, France<sup>b</sup> AgroParisTech, UMR Micalis, Jouy-en-Josas, France

## ARTICLE INFO

## Article history:

Received 24 September 2015

Received in revised form 2 December 2015

Accepted 8 December 2015

Available online 15 December 2015

## Keywords:

Yarrowia lipolytica

Lipid metabolism

Oleaginous yeasts

Unusual fatty acids

Systems metabolic engineering

## ABSTRACT

One of the most promising alternatives to petroleum for the production of fuels and chemicals is bio-oil based chemistry. Microbial oils are gaining importance because they can be engineered to accumulate lipids enriched in desired fatty acids. These specific lipids are closer to the commercialized product, therefore reducing pollutants and costly chemical steps. *Yarrowia lipolytica* is the most widely studied and engineered oleaginous yeast. Different molecular and bioinformatics tools permit systems metabolic engineering strategies in this yeast, which can produce usual and unusual fatty acids. Usual fatty acids, those usually found in triacylglycerol, accumulate through the action of several pathways, such as fatty acid/triacylglycerol synthesis, transport and degradation. Unusual fatty acids are enzymatic modifications of usual fatty acids to produce compounds that are not naturally synthesized in the host. Recently, the metabolic engineering of microorganisms has produced different unusual fatty acids, such as building block ricinoleic acid and nutraceuticals such as conjugated linoleic acid or polyunsaturated fatty acids. Additionally, microbial sources are preferred hosts for the production of fatty acid-derived compounds such as  $\gamma$ -decalactone, hexanal and dicarboxylic acids. The variety of lipids produced by oleaginous microorganisms is expected to rise in the coming years to cope with the increasing demand.

© 2015 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Contents

1.	Introduction	41
2.	<i>Yarrowia lipolytica</i> : an industrial yeast	41
3.	Systems metabolic engineering tools	41
4.	Lipid metabolism in <i>Yarrowia lipolytica</i> : from basic knowledge to usual lipid production	42
4.1.	Fatty acid synthesis	42
4.2.	Triacylglycerol synthesis	43
4.3.	Lipid remobilization	43
4.4.	FA transport and activation	43
4.5.	Fatty acid degradation	43
5.	Unusual fatty acid production in <i>Yarrowia lipolytica</i>	43
5.1.	Conjugated linoleic acid (CLA)	43
5.2.	Ricinoleic acid (RA)	44
5.3.	Polyunsaturated fatty acids: EPA and DHA	45
5.4.	Unusual compounds derived from fatty acids	46
5.4.1.	$\gamma$ -decalactone production	46
5.4.2.	Hexanal production	46
5.4.3.	Dicarboxylic acids production	47
6.	Conclusions and perspective	47

**Abbreviations:** GEMs, Genome scale metabolic models; MFA, metabolic flux analysis; TAG, triacylglycerol; DAG, diacylglycerol; CLA, conjugated linoleic acid; EPA, eicosapentanoic acid; DHA, docosahexanoic acid; LA, linoleic acid; ALA, alpha-linoleic acid; RA, ricinoleic acid; DCW, dry cell weight; DCA, dicarboxylic acid.

\* Corresponding authors at: Institut Micalis, INRA-AgroParisTech, UMR1319, Team BIMLip: Biologie Intégrative du Métabolisme Lipidique, CBAI, F-78850 Thiverval-Grignon, France.

E-mail addresses: [Rodrigo.Ledesma@grignon.inra.fr](mailto:Rodrigo.Ledesma@grignon.inra.fr) (R. Ledesma-Amaro), [jean-marc.nicaud@grignon.inra.fr](mailto:jean-marc.nicaud@grignon.inra.fr) (J.-M. Nicaud).

Acknowledgments	47
References	47

## 1. Introduction

General concerns about climate change and the increasing search for renewable sources of energy are boosting bio-lipids as promising alternatives to fossil fuels. Over the last few decades, the chemical industry has been developing technology that uses oils and fats as renewable sources of precursors in the synthesis of polymers, plasticizers, lubricants, coatings, surfactants, drugs, and fuels, among others [1]. Normally, lipids are produced in animals, plants and microbes in the form of triacylglycerols (TAGs), which are thereafter modified by chemical processes such as oxidation, C–C bond-forming additions, metathesis or C–H activation [2] to constitute the final products.

Notably, in contrast to animals and plants, microorganisms can be easily engineered and accepted by industry and society. The recent developments in systems metabolic engineering—an emergent discipline that combines synthetic and systems biology and metabolic modeling with traditional metabolic, genetic and protein engineering techniques—facilitates the modification of microorganisms to directly produce unusual fatty acids. These unusual fatty acids are one step closer to the final product, thus reducing or completely avoiding chemical processes during the manufacture [1].

In the recent years, the bio-based production of unusual fatty acids has attracted significant attention in both plants [3] and microbial sources (see below).

Some microorganisms, called ‘oleaginous microorganisms’, are able to accumulate high amounts of usual lipids (more than 20% of their cell dry weight), and they are considered the most suitable chassis for bio-oil production. Nonetheless, several approaches have been employed to convert model organisms into oleaginous organisms by genetic engineering. For example, *E. coli* has been extensively used to produce free fatty acids [4], *S. cerevisiae* has been engineered to be able to accumulate up to 50% of its DCW as lipids [5], and *A. gossypii* modified to harbor genes from oleaginous organisms was able to accumulate up to 70% of lipids [6].

Among oleaginous microorganisms, *Yarrowia lipolytica* is the most studied due to both its interesting biotechnological characteristics and its suitability to be manipulated in the laboratory. Here, we review the lipid metabolism of *Y. lipolytica*, the latest advances and techniques in its manipulation and the different approaches employed in recent years to produce unusual fatty acids.

## 2. *Yarrowia lipolytica*: an industrial yeast

*Yarrowia lipolytica* is a dimorphic, non-pathogenic ascomycetous yeast. It is often found in environments with the presence of hydrophobic substrates such as dairy products and oily waste. Therefore, several strains have been isolated in soils contaminated with oils, in marine environments, in sediment samples and in waste waters [7]. Importantly, the safety of this yeast has recently been assessed [8] because food supplements have recently been approved for commercialization [9]. This safety approval is particularly important for broadening the range of possible applications of the products derived from the fermentation of *Yarrowia lipolytica*.

The safety status of *Yarrowia lipolytica* and its unique physiological features, such as the growth in hydrophobic substrates, has made it an important biotechnological yeast. *Yarrowia lipolytica* has been used for many food-related applications [10], such as the production of meat and dairy products, the production of aromas such as gamma decalactone, the synthesis of organic acids (citric, isocitric, alpha-ketoglutaric, pyruvic and succinic), spoilage yeasts for discoloration, the production of polyalcohol for sucrose replacement, the production of emulsifiers and surfactants and the production of single cell oils and proteins for food. It has

also been proposed for the treatment and degradation of pollutants [7] such as hydrocarbons, oils, nitro, halogenated and organophosphate compounds, for the reduction of metals and for the treatment of wastewater.

Additionally, it is an advantageous host for the production of proteins, and more than 130 different proteins with academic or commercial applications have been produced in this yeast [11].

Finally, as we introduce above and describe below, *Yarrowia lipolytica* has been widely used in the production of lipids and lipid-derived compounds [12] such as biodiesel, edible oils or dicarboxylic acids as building blocks for polymers.

## 3. Systems metabolic engineering tools

The huge number of industrial applications has boosted both basic research to understand the physiological features of *Y. lipolytica* and the development of metabolic engineering tools. The genome of this yeast (strain W9, CLIB122) has been sequenced [13] and manually annotated by a network of expert curators in the program Génolevures [14]. Additionally, the genome of the strain Po1f, commonly used in metabolic engineering approaches, has also been recently sequenced [15].

For decades, molecular biology techniques have been developed in *Y. lipolytica*. It has been engineered to increase the efficiency of homologous recombination to improve the frequency of the transformation, which has facilitated gene overexpression and deletion [16]. Examples of molecular biology tools developed in this yeast are replicative plasmids, constructions for genomic insertions, constitutive and inducible promoters, fluorescence tags, protein expression and secretion vectors. A recent review covering the topic was published by Madzak [17]. These molecular biology tools, along with systems biology, synthetic biology and mathematical models, permit systems metabolic engineering, which is currently boosting industrial biotechnology [18].

Genome scale metabolic models (GEMs) have been shown to be useful tools in both basic and applied research [19]. Accordingly, they help in the re-annotation of genomes and metabolic network analysis, and they also permit the identification of non-obvious targets for metabolic engineering. In recent years, three genome-scale metabolic models of *Y. lipolytica* have been developed [20–22], although none of them has yet been used in metabolic engineering approaches.

Synthetic biology has been defined as ‘the design and construction of new biological systems (e.g., genetic control systems, metabolic pathways, chromosomes, cells) that do not exist in nature through the assembly of well-characterized, standardized, reusable components’ [23]. Therefore, novel tools must be developed to facilitate and to increase the complexity of synthetic systems. In *Yarrowia lipolytica*, a method for the one-step integration of multiple genes has been established [24]. Additionally, synthetic terminators [25] and plasmids have been developed for increased expression levels and copy numbers [26] and hybrid synthetic promoters [27,28]. In recent years, the CRISPR-Cas9 system, a tool for genome engineering, has been developed and shown to be useful for manipulating a wide range of organisms, including yeasts [29,30]. This technology has not yet been fully implemented in *Yarrowia*, but this is expected to change in the near future [31].

High-throughput technologies have enabled the analysis of large amount of omics data for investigating cellular metabolism and physiology at the systems level [32]. These systems biology data have recently been obtained and analyzed for *Y. lipolytica*. In this regard, transcriptomic analysis revealed four different transcription profiles over 32 h of fermentation and identified genes potentially involved in the metabolism of oleaginous species [33]. Additionally, transcriptomic and proteomic analyses of *Y. lipolytica* have been conducted in relation

to amino acid catabolism, which is important in cheese production [34]. Proteomic analysis was performed to study the proteins involved in the transition yeast hyphae [35] and in the osmotic response to erythritol [36]. Finally, fluxomics in this yeast has been developed through metabolic flux analysis (MFA) with C13 [37,38]. Notably, metabolic flux analysis has recently revealed the pentose phosphate pathway as the major source of the cofactor required for lipid production [39]. Very recently, metabolomics and lipidomics studies have revealed cell-wall biogenesis proteins as possible targets to increase lipid accumulation [40].

All the cutting-edge technologies described above set the basis for systems metabolic engineering approaches in *Y. lipolytica*, which is expected to boost the current limitation in lipid production, to enhance yields and productivity and to improve the synthesis of unusual fatty acids.

#### 4. Lipid metabolism in *Yarrowia lipolytica*: from basic knowledge to usual lipid production

The importance of *Y. lipolytica* as a biolipid producer has made it a unicellular model organism to study fatty acid metabolism and lipid accumulation. Here, we summarize the most important process and genes involved in usual lipid synthesis and degradation (summarized in Fig. 1). We here define usual lipids as those that are naturally and abundantly synthesized by yeast and can be accumulated and stored (such as triacylglycerols or steryl esters).

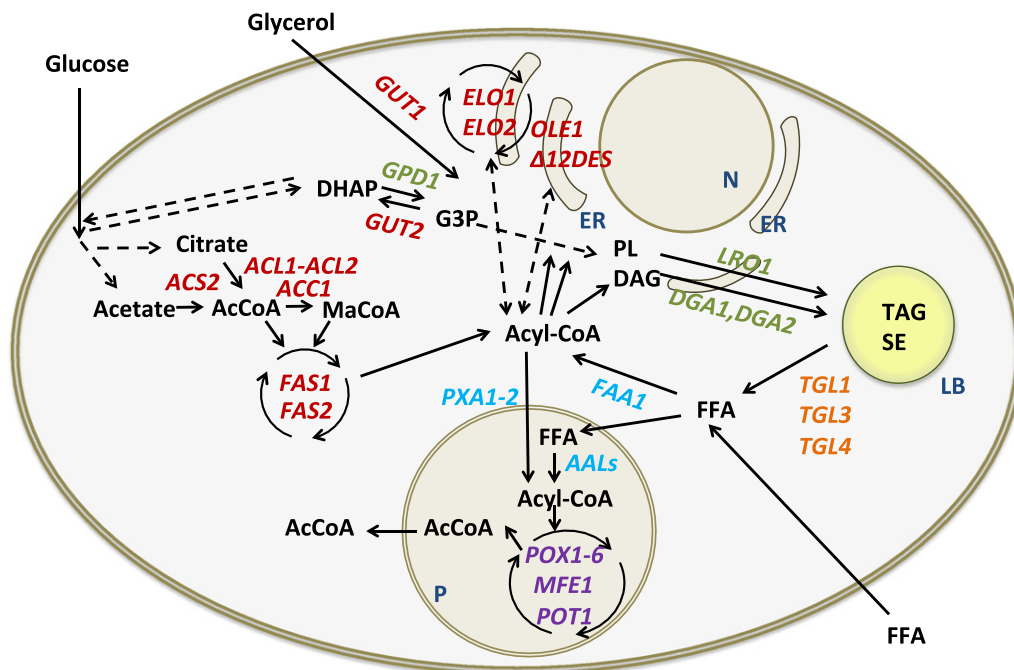
##### 4.1. Fatty acid synthesis

Fatty acids are produced in the cytosol of *Yarrowia* from the building block acetyl-CoA, which can come from ACS genes (acetyl-CoA synthetase, YALIOF05962g), a pyruvate dehydrogenase complex, an amino acid degradation pathway or ACL genes (ATP citrate lyase YALIOE34793g and YALIOD24431g) [41]. ACL genes are present only in the genome of

oleaginous yeast. Despite their overexpression [42] or heterologous expression [43], they produce no increase or a slight increase in lipid content, and they have been considered a hallmark of oleaginous microorganisms [44]. *Y. lipolytica* is able to use different carbon sources to produce biomass and lipids, the most studied of which are glucose and glycerol. Glycerol is a relevant and inexpensive industrial byproduct, and it is therefore a desirable substrate for the industrial applications of this yeast [45]. Nonetheless, glucose is still the most widely used in lab-scale research. Recently, *Y. lipolytica* has been engineered to be able to degrade other carbon sources, such as sucrose [46], fructose [47], galactose [48], cellulose [49], cellobiose [50] or raw starch [51].

Acetyl-CoA carboxylase YALIOC11407g (*ACC1*) can transform acetyl-CoA into malonyl-CoA. The overexpression of *ACC1* has been associated with lipid overproduction [52]. The fatty acid synthase (*FAS*) enzymatic complex (YALIOB15059g and YALIOB19382g) produces acyl-CoA using acetyl-CoA as an initiation molecule and malonyl-CoA as elongation unit, adding two carbons to the fatty acid backbone. Most of the released acyl-CoA corresponds to a chain length of 16 or 18 carbons. Thereafter, these 16:0 and 18:0 activated molecules can be substrates of elongases and desaturases. Elongases are responsible for chain elongation and will produce long chain fatty acids (YALIOB20196g) or very long chain fatty acids (YALIOF06754g). Desaturases, located in the ER, produce either a first desaturation (*OLE1*;  $\Delta 9$  desaturase, YALIOC05951g) to produce palmitoleic (16:1) or oleic acid (18:1) or a second double bond ( $\Delta 12$  desaturase *FAD2*, YALIOB10153g) to produce primarily linoleic acid (18:2). Unexpectedly, the overexpression of *OLE1* contributed to an increased lipid yield in a previously engineered strain, producing higher biomass titers and subsequently more fatty acids [53].

Alternatively, when *Y. lipolytica* grows in an oily environment, a large battery of extracellular lipases produce fatty acids out of the oils, which will be rapidly incorporated into the cell. In the cytosol, these free fatty acids can be activated by *FAA1* (fatty acyl-CoA synthetase, YALIOD17864g) to produce acyl-CoA [54].



**Fig. 1.** Lipid metabolism in *Y. lipolytica*. Schematic representation of the metabolic pathways leading to the production of neutral lipids (TAG, triacylglycerol and SE, steryl esters) from either glucose or free fatty acids (FFA). Dashed lines indicate multiple steps. DHAP (dihydroxyacetone phosphate), G3P (glycerol-3-phosphate), AcCoA (acetyl-CoA), MaCoA (malonyl-CoA), PL (phospholipid), DAG (diacylglycerol). Gene names in italics are detailed in the text, including their functionality and *Yarrowia* name. The colors of the genes indicate different metabolic pathways, as divided in the text: In red fatty acid synthesis and elongation and desaturation system, in green triacylglycerol synthesis, in orange lipid remobilization, in light blue fatty acid activation and transport, and in purple fatty acid degradation. The different organelles are indicated by the dark blue letters where N is the nucleus, ER the endoplasmic reticulum, LB the lipid body and P the peroxisome.

## 4.2. Triacylglycerol synthesis

*Y. lipolytica* accumulates lipids in an intracellular particle called a lipid body. It is primarily formed of neutral lipids, particularly TAGs (85%) and some SE (8%) [55]. TAGs are formed through the Kennedy pathway, where preformed DAG is converted into TAG either from phospholipid by *LRO1* (phospholipid:diacylglycerol acyltransferase, YALIOE16797g) or from acyl-CoA by *DGA1* and *DGA2* (YALIOE32769g/YALIOD07986g, respectively). SEs are formed from acyl-CoA, and sterol is formed by *ARE1* (YALIOF06578g) [56,57]. These reactions may take place between the ER and the lipid body surface, where the responsible enzymes have been found [55]. Metabolic engineering approaches over-expressing *DGA1* and *DGA2* have successfully improved lipid production [52,56,58,59]. This effect can be associated with the faster depletion of cytosolic acyl-CoA, which is a well-known negative regulator of fatty acid synthesis [60,61].

## 4.3. Lipid remobilization

Lipid bodies are dynamic structures that represent an additional carbon source once the nutrients of the media have been depleted. Therefore, TAGs are substrates of intracellular lipases, which will release free fatty acids at the surface of the lipid bodies. In *Y. lipolytica*, in contrast to *S. cerevisiae*, there are two genes: *TGL4* (YALIOF10010g), an active lipase localized in the interface of the lipid body, and *TGL3* (YALIOD17534g), a positive regulator of *TGL4*. Notably, the spatial organization of these two enzymes varies depending on the media composition and the physiological state of the cell, suggesting a complex regulation. Importantly, from a biotechnological point of view, the single or double deletion of these genes results in a two-fold increase in the lipid-accumulation capacity [62].

## 4.4. FA transport and activation

Free fatty acids, from either the extracellular media or TGL genes, must subsequently be activated and transported to the peroxisome to carry out beta-oxidation. Recently, a model for fatty acid transport and activation has been proposed [54]. According to the model, free fatty acids can 1) enter the peroxisome directly by unknown transporters or be activated by *FAA1* to acyl-coA, which can be 2) stored in lipid bodies, as mentioned in the *triacylglycerol synthesis* section, or 3) enter the peroxisome in an activated form through the specific transporters *Pxa1/Pxa2* (YALIOA06655g/YALIOD04246g). Free fatty acids in the

peroxisome must be activated to be usable by the beta-oxidation enzymes. The *AAL* genes control this activation process (Dulermo R, manuscript under submission). However, *AAL* activation requires ATP, which enters the peroxisome through the *ANT1* protein (encoded by YALIOE03058g). The model also suggests an alternative remobilization pathway mediated by *FAT1* [63] (YALIOE16016g) that can directly transport FA from the lipid body to the peroxisome. Notably, alternative pathways show different chain-length preferences.

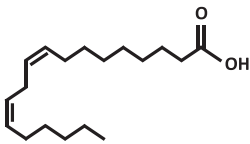
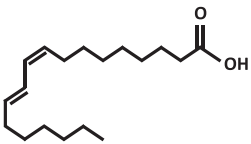
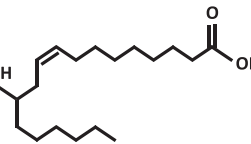
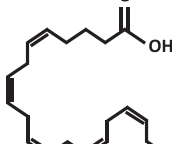
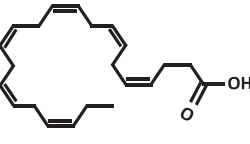
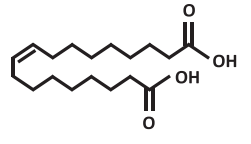
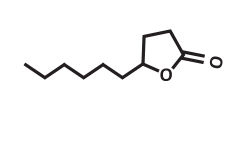
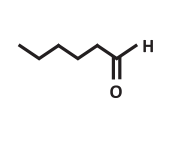
## 4.5. Fatty acid degradation

Fatty acid degradation takes place in the peroxisome following the beta-oxidation pathway, which is a four-reaction cycle resulting in two carbon shortenings of the fatty acid backbone, accompanied by the release of one molecule of acetyl-CoA. In *Y. lipolytica*, the first step is carried out by six acyl-CoA oxidase *POX* genes (YALIOE32835g, YALIOF10857g, YALIOD24750g, YALIOE27654g, YALIOC23859g and YALIOE06567g). The *POX* genes show different chain-length preferences, and the sextuple knockout strain is unable to degrade fatty acids, which leads to a lipid over-accumulation phenotype [64–66]. The second and third steps in beta-oxidation are catalyzed by the multifunctional enzyme *MFE* (YALIOE15378g), which, in contrast to *POX*, is encoded by a single gene. The deletion of *MFE* has been extensively studied in engineered strains for lipid production due to its technical simplicity compared to *POX1-6* deletions [58,67]. The fourth and last step in beta-oxidation is carried out by the thiolase *POT1* (YALIOE18568g). Because beta-oxidation takes place in the peroxisome, several strategies to block this pathway address the abolition of peroxisome biogenesis through the deletion of *PEX3*, *PEX10* [68] and *PEX11* [69].

## 5. Unusual fatty acid production in *Yarrowia lipolytica*

### 5.1. Conjugated linoleic acid (CLA)

Conjugated linoleic acid is a group of octadecadienoic acids isomers of linoleic acid (18:2, n9, n12) (Fig. 2). They are functional food, and therefore, they are used as dietary supplement ingredients. The potential benefits of CLA are the prevention of metabolic diseases and cancer, anti-atherogenic and anti-obesity effects, and immune-system modulation [70]. Most of these effects have been associated with two isomers: cis-9, trans-11 CLA and trans-10, cis-12 CLA [71,72].

			
Linoleic Acid	Conjugated Linoleic Acid	Ricinoleic Acid	Eicosapentanoic Acid
			
Docosahexaenoic Acid	18:1 Dicarboxylic Acid	Gamma-Decalactone	Hexanal

**Fig. 2.** Chemical structure of the unusual fatty acids produced in *Y. lipolytica*. Scheme of the chemical structure of the most relevant fatty acids and fatty acid-derived compounds produced in *Y. lipolytica* and described in the text.



CLAs are naturally found in foods originating in ruminant animals and dairy products. They are produced by probiotic microorganisms, which use the linoleic acid present in milk [73].

CLAs have been chemically produced by the alkaline isomerization of LA present in plant oils [74]. However, this process generates a mixture of different CLAs, which is undesirable for medical and nutritional requirements. Therefore, microbiological synthesis represents a feasible alternative to produce specific isomers. In this regard, the bioconversions of ricinoleic acid into a mixture of CLAs (cis-9, trans-11 and trans-9, trans-11 octadecadienoic acids) were studied using *Lactobacillus planetarium*, which produced up to 2.7 mg/ml of CLAs from 30 mg/ml of castor oil [75,76]. More recently, the specific production of cis-9, trans-11 octadecadienoic acid (10.5 mg/mL) was achieved using *Delacroixia coronata* and trans-vaccenic acid as substrates (33.3 mg/ml) [77]. No microorganism was found in nature with a sufficient bioconversion capacity to produce high amounts of trans-10, cis-12-CLA.

Different isomerases have been characterized: one trans-10, cis-12 CLA-producing isomerase from *Propionibacterium acnes* and two from cis-9, trans-11 CLA-producing isomerases from *Clostridium sporogenes* and *Lactobacillus reuteri* [78,79]. These findings have boosted metabolic engineering approaches to producing trans-10, cis-12-CLA in industrial friendly strains by the heterologous expression of these enzymes. Therefore, codon-optimized isomerase from *Propionibacterium acnes* has been expressed in *S. cerevisiae* and tobacco plants, where CLA production rose up to 5.7% and 15% of total free fatty acids, respectively [80]. A similar approach has been developed in rice to produce up to 1.3% of total fatty acids in seeds [81]. Additionally, bioconversions of LA into CLA were conducted when the same isomerase was expressed in *E. coli* and *Lactococcus lactis*, with transformation rates of 50% and 30%, respectively [82].

Recently, metabolic engineering efforts have focused in the oleaginous yeast *Yarrowia lipolytica*, which is able to cope with high amounts of free fatty acids, the substrates of isomerases. In 2012, Zhang et al. [83] expressed in high copy number a codon-optimized version of *Propionibacterium acnes* in *Y. lipolytica* Polh, which produced trans-10, cis-12-CLA with up to 5.6% of total fatty acids (0.23% of DCW) in the neosynthesis condition from glucose. Additionally, the best strain was able to show, in a biotransformation condition from linoleic acid, an 80% conversion rate of LA into CLA. Nonetheless, this strain was unstable, and the yield of CLA gradually decreased to 0.08% of DCW. One year later, the same authors generated one improved strain of *Y. lipolytica* able to produce CLA with up to 10% of total fatty acids (0.4% of DCW) [84]. The same strain was able to produce 4 g/L of CLA

from soybean oil-based media (44% of total fatty acids, which represented 30% of DCW). In this approach, the codon-optimized isomerase was coexpressed with a  $\Delta 12$  desaturase from *Mortierella alpina* under the control of a modified promoter, hp4d, fused to 12 additional copies of UAS1B. A multi-copy integration plasmid was used to further enhance the expression of both genes. Therefore, the resulting *Y. lipolytica* strain is a promising candidate platform to produce trans-10, cis-12-CLA. Schematic Fig. 3 represents the metabolic engineering approach carried out in *Y. lipolytica* to produce CLA.

## 5.2. Ricinoleic acid (RA)

Ricinoleic acid (RA; 12-hydroxy-octadeca-cis-9-enoic acid: C18:1-OH) is a hydroxylated unusual fatty acid that can be exploited in several industrial applications (Fig. 2). RA is considered a renewable material for the chemical industry, which can use it as a substrate for double-bond reactions (oxidation, polymerization, hydrogenation, epoxidation, halogenation, addition reactions, sulfonation and metathesis) and for hydroxyl-group reactions (dehydration, hydrolysis, caustic fusion, pyrolysis, alkoxylation, esterification, halogenation, urethane formation and sulfonation) [85]. Its derivatives can be used as plasticizers, lubricants, dyes, inks, soaps, adhesives, pharmaceuticals, paints, food additives, textiles, paper, electronic components, perfumes, cosmetics and biofuels [86,87].

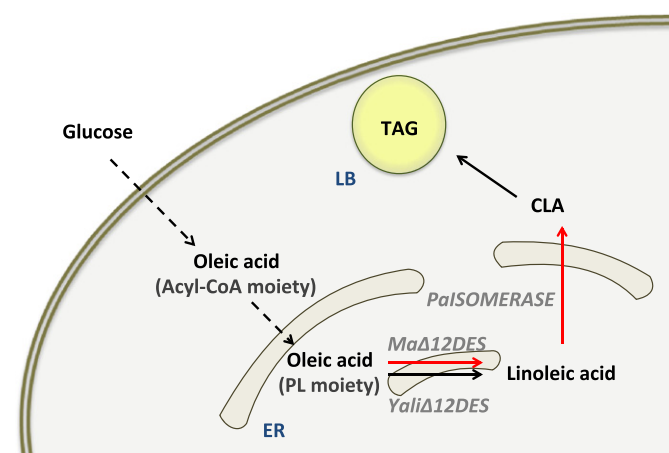
The major source of RA is castor oil, extracted from the seeds of the castor plant (*Ricinus communis*). RA represents 90% of the total fatty acids of seed oil. However, the castor plant is not a suitable crop for a variety of reasons: 1) Its cultivation is limited to tropical and subtropical regions. 2) It is primarily produced in areas with economic and political instability, which produces fluctuations in price and supply. 3) The seeds must be harvested by hand, which is not convenient for large-scale production. 4) The seeds contain ricin, a toxic protein that causes health problems to workers. Finally, 5) the refining process is long and tedious and includes many steps, such as removing undesired colors, odors, flavors and ricin.

Therefore, the increasing number of potential applications of RA and the drawbacks associated with its traditional production have pushed the scientific community to find alternative sources of this unusual fatty acid.

The molecular mechanisms involved in RA biosynthesis are known, which has permitted the development of metabolic engineering approaches to producing fatty acids in other plants and microorganisms. RA is formed in a reaction catalyzed by a  $\Delta 12$ -hydroxylase (FAH12), which adds a hydroxyl group ( $-OH$ ) to the 12th carbon of oleic acid moieties [88] esterified to the sn-2 position of primarily phosphatidylcholine in the endoplasmic reticulum membrane [89]. This FAH12 has been heterologously expressed in tobacco [90], where low levels of RA were produced (<1%), and in the model plant *Arabidopsis thaliana*, which produced RA up to 20% of total fatty acids when the endogenous  $\Delta 12$ -desaturase (which competes for the substrate) was deleted [91–94]. These production titers were increased to 30% of total fatty acids when RcdGAT2 (*Ricinus communis* diacylglycerol acyltransferase) was coexpressed with *R. communis* FAH12 in *A. thaliana* [95,96]. Nonetheless, these levels remain far lower than those in the native castor seeds.

Alternatively, the production of RA in microorganisms has been studied both in model organisms such as *S. cerevisiae* and *S. pombe* and in the oleaginous yeast *Y. lipolytica*. Baker's yeast has been used to express RcdFAH12 under the control of GAL10, but only 0.8% of total fatty acids were RA [92].

More recently, Meesapyodsuk and Qiu [97] isolated the first non-plant  $\Delta 12$ -oleate hydroxylase from the sclerotium tissue of the fungus *C. purpurea* (CpFAH12). Its expression in *S. cerevisiae* under a GAL1 promoter produced up to 19% of RA when the cells were grown in oleate media. The co-expression of CpFAH12 with the fungal diacylglycerol



**Fig. 3.** Metabolic engineering strategy to produce CLA in *Y. lipolytica*. PL, phospholipid; Ma, *Mortierella alpina*; Yali, *Yarrowia lipolytica*; Pa, *Propionibacterium acnes*. Dashed lines indicate multiple enzymatic reactions. The purple line indicates the heterologous reaction. The different organelles are indicated by the dark blue letters where ER is the endoplasmic reticulum and LB the lipid body.

acyltransferase *CpDGAT2* also increased by approximately 30% the total production of RA content [98].

In contrast to *S. cerevisiae*, *S. pombe* produces oleic acid approximately 75% of total fatty acids, which make it a more appropriate organism to produce RA *de novo*. Holic and colleagues [99] expressed *CpFAH12* in *S. pombe* and produced 52.6% of RA, a total of 137.4 µg/ml. Notably, RA showed a toxic effect with associated growth defects at 30 °C, which were completely solved at 37 °C. Thus, their strategy included a first growth phase at 37 °C, followed by a production phase at 20 °C. The RA content was further improved 1.98-fold after the deletion of the three triglyceride lipases (TG lipases Tgl3, Tgl4 and Tgl5) involved in storage-lipid remobilization [100]. Later, Yazawa et al. [101] found in a cDNA library from *S. Pombe* that *PGL7*, which encodes for a phospholipase A2, restored growth defects after the expression of *CpFAH12*. It is speculated that this lipase activity may remove RA from phospholipids, suppressing its toxicity. This same strain was able to secrete up to approximately ~185 µg/ml, and the intracellular amount remained the same (~200 µg/ml), thus improving the total production titer [102]. Finally, the overexpression of *PTL2*, one of the intracellular TG lipases that also showed phospholipase activity, increased the secreted and intracellular RA levels by 1.2- and 1.3-fold, respectively [103].

Despite the above-mentioned enhancement of the production of RA in *S. pombe*, the lipid-accumulation capacity of this yeast, which is typically approximately 5% of the DCW, limits its use as a substitute of castor oil. Therefore, microorganisms with the ability to accumulate high amounts of lipids have to be considered. In this regard, *Y. lipolytica*, which is a well-known oleaginous yeast that accumulates high amounts of oleic acid, has been regarded as the most suitable candidate for RA production.

In 2013, Beopoulos et al. [104] constructed a multiple engineered strain that could accumulate RA up to 43% of its total lipids and more than 60 mg/g of DCW. This strain was unable to perform beta-oxidation due to the deletion of the six *POX* genes blocking the  $\beta$ -oxidation pathway, which permits a higher lipid accumulation. Additionally, the endogenous  $\Delta 12$ -desaturase (*FAD2* gene) was deleted to avoid competition by the substrate oleic acid. Moreover, the strain was deleted for three DAG acyltransferases (*DGA1*, *DGA2* and *LRO1*) and overexpressed for *Lro1p*, rerouting TAG synthesis only through phospholipids. In this case, the best production of RA was found with the expression of two copies of *CpFAH12*, whereas slight RA production was shown with *RcFAH12* instead, and RA reduction took place after the co-expression of *RcDGAT2* or *CpDGAT2*. This best producer strain was further improved by the expression of three copies of *CpFAH12* and

two copies of *LRO1* [105]. When this strain was cultured in a 10-L bioreactor using a defined medium with 160 g/L of glucose and 24 g/L of oleic acid, it produced 12 g/L of RA, which represented 60% of total lipids. Schematic Fig. 4 represents the metabolic engineering approach employed in *Y. lipolytica* to produce RA.

### 5.3. Polyunsaturated fatty acids: EPA and DHA

Over the last few decades, polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA; 20:5 $\Delta^{5,8,11,14,17}$ ) and docosahexaenoic acid (DHA; 22:6 $\Delta^{4,7,10,13,16,19}$ ), have attracted scientific attention because they have been associated with several health benefits (Fig. 2). They are essential for proper visual and neurological development, they reduce the incidence of cardiovascular diseases, and they prevent myocardial infarction, bronchial asthma, inflammatory bowel disease, major depression and several types of cancer. A recent review has been published on the positive effects of EPA and DHA on human health [106]. Humans and mammals are not able to synthesize these omega-3 fatty acids *de novo*, so we need to uptake them from our diets. Although humans are able to produce EPA and DHA from exogenous LA and ALA, the efficiency of the conversion is very low. Therefore, the supplementary intake of these omega-3 fatty acids has been recommended by several health organizations [107].

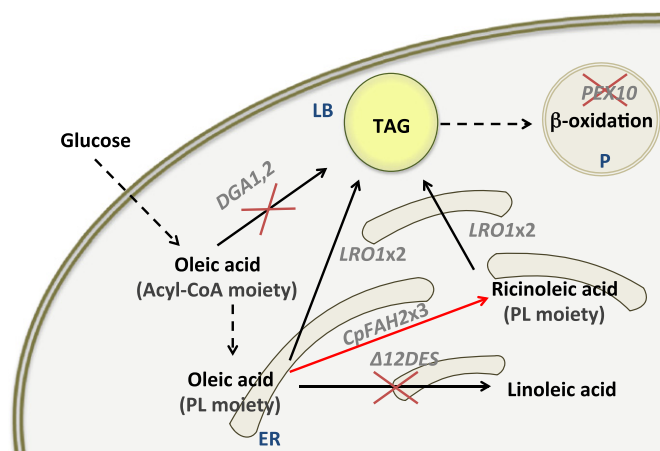
Currently, the most important source of DHA and EPA is fish oils. Fish acquire omega-3 fatty acids from marine microorganisms and phytoplankton. Unfortunately, fish oils are not able to cope with the ever-increasing global demand because of the decline of worldwide fish stocks and the environmental pollution of marine ecosystems [108].

The search for alternatives has revealed two major candidates: plants and microbial oils. Both plants and microbes must be engineered to produce enough of the desired fatty acids. Several approaches have succeeded in the generation of transgenic plants with oilseed enriched in omega-3 fatty acids, although the desired yield and oil composition have not yet been achieved [109]. Remarkably, as a proof of concept, Ruiz-Lopez et al. generated a transgenic oilseed crop (*Camelina sativa*) with fish oil-like levels of EPA and DHA [110].

In parallel, a vast number of studies have focused on the production of EPA and DHA in microorganisms. Some microalgae (*Cryptocodinium cohnii* and *Schizochytrium sp.*) are good producers of DHA, and a few market products are already available. Nonetheless, difficulties in large-scale cultivation often limit their applicability. For a comprehensive review of microalgae omega-3, see [111].

Synthetic biology advances enable the heterologous expression of omega-3 pathways in industrial-friendly microorganisms. Therefore, different metabolic engineering approaches have been employed in a wide range of microorganisms, which has been recently reviewed [112]. The omega-3 pathway comprises a variety of desaturases and elongases with different substrate specificities in chain length, the number of double bonds and lipid moiety.

Although significant efforts have been made to express elongases and desaturases in different microorganisms, such as *S. cerevisiae* [113], *Mortierella alpina* [114] and *Lipomyces starkeyi* [115], the best EPA producer to date is a modified *Y. lipolytica* strain [116]. This strain was constructed by DuPont following a strategy hereafter summarized (for more detailed information, the authors recommend the recent reviews by Zhu et al. [116,117]). First, a screening over 40 *Y. lipolytica* strains was revealed as the most suitable for lipid production of strain (ATCC) #20362 with DCW greater than 100 g/L, a lipid content greater than 30% DCW, and lipid productivity greater than 1 g/L/h. Second, this strain was transformed with  $\Delta 6$  desaturase, C18/20 elongase,  $\Delta 5$  desaturase and  $\Delta 17$  desaturase under the control of a TEF promoter, which enables it to produce 3% of EPA through the  $\Delta 6$  pathway. This first approach served as a proof of concept and as a way to identify the bottlenecks of the pathway: 1) low flux into the engineered pathway, 2) low efficiency of the elongase and 3) an insufficiently high expression of the genes of the pathway. Accordingly, the approach improved the



**Fig. 4.** Metabolic engineering strategy to produce RA in *Y. lipolytica*.  $\times 2$  or  $\times 3$ , two or three copies of the gene were overexpressed. PL, phospholipid. Dashed lines indicate multiple enzymatic reactions. Red crosses indicate deleted reactions. The purple line indicates the heterologous reaction. The different organelles are indicated by the dark blue letters where ER is the endoplasmic reticulum, LB the lipid body and P the peroxisome.

expression of the genes by identifying a battery of strong promoters, by setting up a system of codon optimization and by developing an URA/FOA method for multiple copy integration. To increase the flux through the  $\Delta 6$  pathway, an elongase to produce stearic acid (18:0) from palmitic acid (16:0) of *M. alpina* [118] and a  $\Delta 12$  desaturase from *Fusarium moniliforme* [119] were overexpressed. This strain (Y9027) harbors a total of 19 copies of different heterologous genes and is able to produce up to 40% of EPA [120]. However, this strain still accumulates up to 21% of GLA, an undesired omega-6 fatty acid, suggesting a rate-limiting acyl exchange between the phospholipid and CoA pools in the ER membrane.

GLA is an omega-6 fatty acid that has been considered undesired in omega-3 supplements. In fact, a low omega-6/omega-3 ratio has been associated with several health benefits [121]. To avoid the concomitant production of GLA during EPA production, the  $\Delta 9$  pathway was used instead of the  $\Delta 6$  pathway described above. The enzymes of the  $\Delta 9$  pathway are  $\Delta 9$ -elongases [122] and  $\Delta 8$ -desaturases [123]. Bifunctional fusion genes of  $\Delta 9$ -elongase and  $\Delta 8$ -desaturase with increased activity were developed [124]. Again, multiple copies of the selected codon-optimized genes under strong promoters were integrated into the genome ( $\Delta 9$ -elongase,  $\Delta 8$ -desaturase, C16/18-elongase,  $\Delta 12$ -desaturase gene,  $\Delta 17$ -desaturase,  $\Delta 5$ -desaturase and cholinephosphotransferase). The casual integration of one of the constructions at the *PEX10* locus was shown to be highly advantageous for EPA production. The first generated strain Y4305 [9] contained 30 copies of 9 different genes and produced EPA at 56.6% FAMES and approximately 15% DCW, without GLA accumulation. This strain was further improved to generate Z1978 with a total of 35 copies of 17 different genes. Z1978 produced EPA at >58% FAME and approximately 20% DCW [125]. On this, the best strain was constructed and called Z5567, which contained 41 copies of 19 different genes, producing EPA at approximately 50% FAME and approximately 25% DCW [125].

Finally, the successful development and optimization of the fermentation processes resulted in two commercial products, New Harvest™ EPA oil and Verlasso® salmon, using *Y. lipolytica* as source of EPA. Schematic Fig. 5 represents the metabolic engineering approach employed in *Y. lipolytica* to produce EPA.

#### 5.4. Unusual compounds derived from fatty acids

Both usual and unusual fatty acids can serve as substrates for enzymatic activities that convert them into industrial products. Here, we summarize three of these industrial fatty acid-derived compounds

that have been produced in *Y. lipolytica*: dicarboxylic acids (DCA; extensively reviewed in [126]), which derive from usual fatty acids and  $\gamma$ -decalactone (reviewed in [127]), and hexanal, which derive from unusual fatty acids. In contrast to the production of fatty acids, up to now in these processes, lipids are added to the media to serve as biotransformation sources. Nonetheless, advances in biolipid production from other common carbon sources, such as glucose, or inexpensive ones, such as lignocelluloses, are expected to lower the production costs in the coming years.

##### 5.4.1. $\gamma$ -decalactone production

$\gamma$ -decalactone is a natural peach aroma widely used in food and beverages. Its biotechnological production has been studied with interest (Fig. 2). The biosynthetic pathway of  $\gamma$ -decalactone begins with the hydroxylation of oleic acid to produce RA. This step is followed by four rounds of beta-oxidation, which leads to the production of 4-hydroxy-decanoic acid, the precursor of  $\gamma$ -decalactone. This precursor is thereafter isomerized and lactonized to form the final product. *Y. lipolytica* has been widely studied in the biotransformation of exogenous RA or castor oil into  $\gamma$ -decalactone [127]. This process was optimized by metabolic engineering approaches comprising combinatorial deletions in the *POX* genes involved in the first step of  $\beta$ -oxidation. Because each of the six *POX* genes is known to have different chain-length specificities, the deletion of short chain oxidase (*POX3*) increased the availability of the precursor 4-hydroxy-decanoic acid and therefore total  $\gamma$ -decalactone [128,129]. Additionally, the decrease in the activity in the long-chain acyl-CoA oxidases resulted in higher  $\gamma$ -decalactone versus 3-hydroxy- $\gamma$ -decalactone amounts [130]. 3-hydroxy- $\gamma$ -decalactone accumulates when NAD supply is not sufficient, and thus, the flux reduction through  $\beta$ -oxidation permits a better supply of the required cofactor to specifically produce  $\gamma$ -decalactone. The production of  $\gamma$ -decalactone was further optimized by Belo et al. and other groups who studied and optimized different types of fermentation [131,132], parameters such as aeration and PH [133,134] and cell-immobilization materials [135]. More recently, different rounds of UV irradiation and genome shuffling modified *Yarrowia* strains, which were able to produce a 6.5-fold higher  $\gamma$ -decalactone than the wild type and a total 3.75 g/L [136]. This titer was further improved to 8 g/L through cell immobilization in attapulgit along with the use of ionic liquid as a cosolvent [137]. Efforts to decipher the complete synthetic pathway along with novel metabolic engineering approaches may improve lactone production by yeast in the next few years.

##### 5.4.2. Hexanal production

Hexanal is a C-6 aldehyde, a green-note molecule with characteristic flavors of fruits, vegetables and green leaves (Fig. 2) [138]. It is a high-value product widely used by the aroma industry.

These fatty acid aldehydes are naturally produced by plants, which synthesize them either from linoleic or linolenic acid in a two-step pathway. First, fatty acids are converted by a lipoxygenase (LOX) into 13-hydroperoxides, which are thereafter transformed into hexanal or trans-2-hexenal by a hydroperoxide lyase (HPL) [139]. The current industrial production of these aldehydes relies on plant homogenates as enzyme sources characterized by low enzyme concentration and low specificity [140]. As an alternative, these enzymes have been expressed in microbes, *E. coli* and yeasts. On the one hand, *E. coli*-expressing HPL was able to produce hexanal up to 140  $\mu$ g per 10  $\mu$ l of cell lysate [141]. On the other hand, *S. cerevisiae* was first engineered to express an HPL from banana and to produce cis-3-hexenal [142]. More recently, the co-expression of both HPL from watermelon and LOX from soybean resulted in strains producing approximately 60 mg/L of 3-hexenal by the bioconversion of linoleic acid using resting cells [140].

In *Y. lipolytica*, Bourel et al. [139] expressed an HPL from green bell pepper. In this approach, a maximum production of C-6 aldehydes of 350 mg/L hexanal and 50 mg/L of trans-2-hexenal was reached when

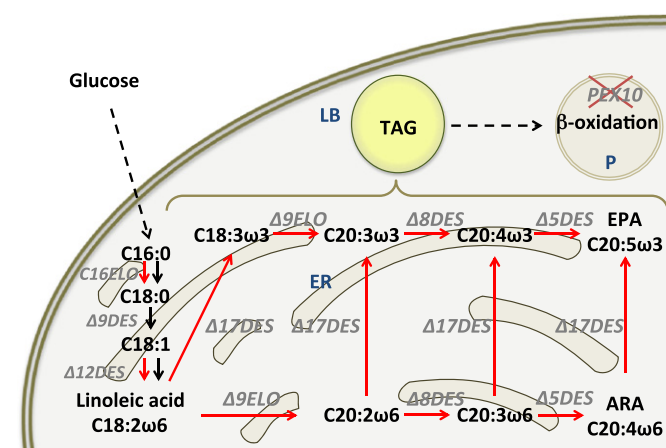


Fig. 5. Metabolic engineering strategy to produce EPA in *Y. lipolytica*. Dashed lines indicate multiple enzymatic reactions. The red cross indicates the deleted gene. The purple line indicates the heterologous reaction. ARA, arachidonic acid. EPA, eicosapentaenoic acid. ELO, elongase. DES, desaturase. The different organelles are indicated by the dark blue letters where ER is the endoplasmic reticulum, LB the lipid body and P the peroxisome.



hydroperoxides were added to the media. In spite of the rapid bioconversion from the first hours to the maximum production peak, there was a strong decrease in hexanal, likely due to the action of fatty aldehyde dehydrogenases. Although this strategy so far represents the highest bioconversion rate using microbial systems, it is still far from plan-based systems, and therefore, further improvements must be made, such as the deletion of degrading reactions or the enhancement of enzymatic activity. Additionally, the use of hydroperoxides as substrates has been shown to be toxic for the cells due to its interactions with the phospholipids of the membranes, which significantly reduce the efficiency of the conversion [143]. The co-expression of a LOX enzyme in the *Yarrowia* strain expressing the HPL would allow for the direct biotransformation from linoleic acid and not from its hydroperoxide form, reducing its toxicity. In addition, this approach could boost endogenous linoleic acid conversion into hexanal, which could therefore be produced from cheaper carbon sources such as glycerol.

#### 5.4.3. Dicarboxylic acids production

Dicarboxylic acids (DCA) are important building blocks for bioplastics such as polyamide and polyester (Fig. 2). Currently, long-chain DCA are primarily produced by a chemical process, although eco-friendly biotechnological fermentation has been developed. Long-chain DCA are produced by the functionalization of alkanes or fatty acids via biooxidation. Briefly, the omega terminal part of the fatty acid is oxidized by the hydroxylase complex, formed by a Cytochrome P450 monooxygenase and a NADPH-dependent Cytochrome P450 oxidoreductase. Then, the produced omega-hydroxy fatty acid is further oxidized by a fatty alcohol oxidase into a fatty acid aldehyde. Finally, DCA is synthesized by the action of a NAD-dependent fatty aldehyde dehydrogenase. These DCA can thereafter be degraded via  $\beta$ -oxidation in the peroxisome.

The biotransformation of alkanes or fatty acids into DCA was first reported in *C. tropicalis*, where the deletion of two of the three POX genes coding for the acyl-CoA oxidases was essential for a high accumulation [144,145]. An increase of DCA was also found after impairing the degradation of fatty acids by the deletion of carnitine acetyl-transferase (CAT gene) [146].

Another way to increase DCA yield is the overexpression of the enzymes of the pathway. Following this rationale, the co-expression of CYP monooxygenase and reductase genes in *C. tropicalis* increased by 30% the total production of DCA [145].

*Y. lipolytica* is also capable of degrading alkanes and therefore of producing DCA. Nicaud et al. [147] showed a higher production of DCA when a higher number of POX genes was deleted. Further experiments with tagged mutants identified interesting genes related to alkane utilization, which could be potentially used in biotechnological approaches, such as thioredoxin reductase, peroxinase, succinate–fumarate carrier and isocitrate lyase [148]. In the same manner as in *C. tropicalis*, the overexpression of some of the CYP monooxygenases and the reductase increased DCA production [149]. Recently, the enzyme responsible for fatty alcohol oxidase (FAO) in *Y. lipolytica* was identified, and its overexpression led to an increase in DCA production [150]. In spite of the efforts to produce DCA in *Y. lipolytica*, the bioconversion titers are not high enough to compete with *C. tropicalis*, and thus, further strain development by metabolic engineering and bioreactor-optimization approaches must be employed [126].

## 6. Conclusions and perspective

In the exhaustive search for alternatives to fossil fuels, oil-based chemistry is gaining importance due to its similar properties and possibilities. In particular, microbial oils are attracting considerable attention because they are industrially and socially accepted and can be easily adapted to market needs.

Among the lipid-producer microorganisms, *Yarrowia lipolytica* is the most studied, with an extensive toolbox for metabolic engineering. It

has been shown to be suitable for large-scale fermentations. The unusual fatty acids are one step closer to the commercial product than the usual lipids, which make them highly valuable products and more economically viable. As reviewed in this article, metabolically engineered *Y. lipolytica* is able to produce CLA, RA and EPA and has thus already led to several commercialized products. The recent advances in systems metabolic engineering along with the novel discovery of different enzymes involved in fatty acid modification will broaden the range of unusual fatty acids produced by this yeast. Some of these potential products are medium-chain fatty acids, double bond-end fatty acids, other conjugated and hydroxyl fatty acids, and epoxy and acetylenic fatty acids. Moreover, the development of novel synthetic biology techniques along with the growing interest in an integrated view of the system due to omics data will boost oleochemical production in oleaginous yeasts. Synthetic biology tools may facilitate gene modification and speed up metabolic engineering approaches, saving both time and resources. Systems biology may also help identify novel, non-obvious targets of manipulation to enhance lipogenesis. Additionally, fermentation technologies, downstream processes and upscaling from lab to industrial scales require more rigorous studies not only to control and to maximize yield but also to reduce competing undesired reactions, such as the usual co-production of citric acid during lipid production.

## Acknowledgments

This work has benefited of aid managed by the Agence Nationale de la Recherche under the “Investissements d’avenir” program with the reference ANR-11-BTBR-0003. R. Ledesma-Amaro received financial support from the European Union in the framework of the Marie-Curie FP7 COFUND People Program in the form of an AgreenSkills’Fellowship (grant agreement no. FP7-267196).

## References

- [1] Ledesma-Amaro R. Microbial oils: a customizable feedstock through metabolic engineering. *Eur J Lipid Sci Technol* 2015;117:141–4.
- [2] Biermann U, Bornscheuer U, Meier MAR, Metzger JO, Schäfer HJ. Oils and fats as renewable raw materials in chemistry. *Angew Chem Int Ed* 2011;50:3854–71.
- [3] Napier JA. The production of unusual fatty acids in transgenic plants. *Annu Rev Plant Biol* 2007;58:295–319.
- [4] Liu H, Yu C, Feng D, Cheng T, Meng X, Liu W, et al. Production of extracellular fatty acid using engineered *Escherichia coli*. *Microb Cell Factories* 2012;11:41.
- [5] Kamisaka Y, Kimura K, Uemura H, Yamaoka M. Overexpression of the active diacylglycerol acyltransferase variant transforms *Saccharomyces cerevisiae* into an oleaginous yeast. *Appl Microbiol Biotechnol* 2013;97:7345–55.
- [6] Ledesma-Amaro R, Santos MA, Jimenez A, Revuelta JL. Strain design of *Ashbya gossypii* for single-cell oil production. *Appl Environ Microbiol* 2014;80:1237–44.
- [7] Zinjarde S, Apte M, Mohite P, Kumar AR. *Yarrowia lipolytica* and pollutants: interactions and applications. *Biotechnol Adv* 2014;32:920–33.
- [8] Groenewald M, Boekhout T, Neugeglise C, Gaillardin C, van Dijck PW, Wyss M. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. *Crit Rev Microbiol* 2014;40:187–206.
- [9] Xue Z, Sharpe PL, Hong SP, Yadav NS, Xie D, Short DR, et al. Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. *Nat Biotechnol* 2013;31:734–40.
- [10] Zinjarde SS. Food-related applications of *Yarrowia lipolytica*. *Food Chem* 2014;152:1–10.
- [11] Madzak C, Gaillardin C, Beckerich JM. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *J Biotechnol* 2004;109:63–81.
- [12] Beopoulos A, Cescut J, Haddouche R, Uribelarrea JL, Molina-Jouve C, Nicaud JM. *Yarrowia lipolytica* as a model for bio-oil production. *Prog Lipid Res* 2009;48:375–87.
- [13] Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, et al. Genome evolution in yeasts. *Nature* 2004;430:35–44.
- [14] Sherman DJ, Martin T, Nikolski M, Cayla C, Souciet JL, Durrens P. Genolevures: protein families and synteny among complete hemiascomycetous yeast proteomes and genomes. *Nucleic Acids Res* 2009;37:D550–4.
- [15] Liu L, Alper HS. Draft genome sequence of the oleaginous yeast *Yarrowia lipolytica* PO1f, a commonly used metabolic engineering host. *Genome Announc* 2014;2:4.
- [16] Verbeke J, Beopoulos A, Nicaud JM. Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains. *Biotechnol Lett* 2013;35:571–6.
- [17] Madzak C. *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering. *Appl Microbiol Biotechnol* 2015;99:4559–77.



- [18] Cho C, Choi SY, Luo ZW, Lee SY. Recent advances in microbial production of fuels and chemicals using tools and strategies of systems metabolic engineering. *Biotechnol Adv* 2014;33:1455–66.
- [19] Blazeck J, Alper H. Systems metabolic engineering: genome-scale models and beyond. *Biotechnol J* 2010;5:647–59.
- [20] Loira N, Dulermo T, Nicaud JM, Sherman DJ. A genome-scale metabolic model of the lipid-accumulating yeast *Yarrowia lipolytica*. *BMC Syst Biol* 2012;6:35.
- [21] Pan P, Hua Q. Reconstruction and in silico analysis of metabolic network for an oleaginous yeast, *Yarrowia lipolytica*. *PLoS ONE* 2012;7, e51535.
- [22] Kavsek M, Bhutada G, Madl T, Natter K. Optimization of lipid production with a genome-scale model of *Yarrowia lipolytica*. *BMC Syst Biol* 2015;9:72.
- [23] Nielsen J, Fussenegger M, Keasling J, Lee SY, Liao JC, Prather K, et al. Engineering synergy in biotechnology. *Nat Chem Biol* 2014;10:319–22.
- [24] Gao S, Han L, Zhu L, Ge M, Yang S, Jiang Y, et al. One-step integration of multiple genes into the oleaginous yeast *Yarrowia lipolytica*. *Biotechnol Lett* 2014;36:2523–8.
- [25] Curran KA, Morse NJ, Markham KA, Wagman AM, Gupta A, Alper HS. Short synthetic terminators for improved heterologous gene expression in yeast. *ACS Synth Biol* 2015;4:824–32.
- [26] Liu L, Otupul P, Pan A, Alper HS. Increasing expression level and copy number of a *Yarrowia lipolytica* plasmid through regulated centromere function. *FEMS Yeast Res* 2014;14:1124–7.
- [27] Blazeck J, Reed B, Garg R, Gerstner R, Pan A, Agarwala V, et al. Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 2013;97:3037–52.
- [28] Blazeck J, Liu L, Redden H, Alper H. Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Appl Environ Microbiol* 2011;77:7905–14.
- [29] Jakociunas T, Bonde I, Herrgard M, Harrison SJ, Kristensen M, Pedersen LE, et al. Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*. *Metab Eng* 2015;28:213–22.
- [30] DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res* 2013;41:4336–43.
- [31] Frisch R, Bendezu F, Fan X, Hong R, Jackson E. A non-conventional gene editing method in a non conventional yeast: using Cas9 for genome editing in *Yarrowia lipolytica*. Abstract in the Cold Spring Harbor meeting: Genome Engineering: The Crispr/Cas revolution; 2015.
- [32] Jang YS, Park JM, Choi S, Choi YJ, Seung do Y, Cho JH, et al. Engineering of microorganisms for the production of biofuels and perspectives based on systems metabolic engineering approaches. *Biotechnol Adv* 2012;30:989–1000.
- [33] Morin N, Cescut J, Beopoulos A, Lelandais G, Le Berre V, Uribealarea JL, et al. Transcriptomic analyses during the transition from biomass production to lipid accumulation in the oleaginous yeast *Yarrowia lipolytica*. *PLoS ONE* 2011;6, e27966.
- [34] Mansour S, Bailly J, Delettre J, Bonnamme P. A proteomic and transcriptomic view of amino acids catabolism in the yeast *Yarrowia lipolytica*. *Proteomics* 2009;9:4714–25.
- [35] Morin M, Monteoliva L, Insenser M, Gil C, Dominguez A. Proteomic analysis reveals metabolic changes during yeast to hypha transition in *Yarrowia lipolytica*. *J Mass Spectrom* 2007;42:1453–62.
- [36] Yang LB, Dai XM, Zheng ZY, Zhu L, Zhan XB, Lin CC. Proteomic analysis of erythritol-producing *Yarrowia lipolytica* from glycerol in response to osmotic pressure. *J Microbiol Biotechnol* 2015;25:1056–69.
- [37] Christen S, Sauer U. Intracellular characterization of aerobic glucose metabolism in seven yeast species by <sup>13</sup>C flux analysis and metabolomics. *FEMS Yeast Res* 2011;11:263–72.
- [38] Blank LM, Lehmbeck F, Sauer U. Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS Yeast Res* 2005;5:545–58.
- [39] Wasylenko TM, Ahn WS, Stephanopoulos G. The oxidative pentose phosphate pathway is the primary source of NADPH for lipid overproduction from glucose in *Yarrowia lipolytica*. *Metab Eng* 2015;30:27–39.
- [40] Pomraning KR, Wei S, Karagiosis SA, Kim YM, Dohnalkova AC, Arey BW, et al. Comprehensive metabolomic, lipidomic and microscopic profiling of *Yarrowia lipolytica* during lipid accumulation identifies targets for increased lipogenesis. *PLoS ONE* 2015;10, e0123188.
- [41] Vorapreeda T, Thammarongtham C, Cheevadhanarak S, Laoteng K. Alternative routes of acetyl-CoA synthesis identified by comparative genomic analysis: involvement in the lipid production of oleaginous yeast and fungi. *Microbiology* 2012;158:217–28.
- [42] Dulermo T, Lazar Z, Dulermo R, Rakicka M, Haddouche R, Nicaud JM. Analysis of ATP-citrate lyase and malic enzyme mutants of *Yarrowia lipolytica* points out the importance of mannitol metabolism in fatty acid synthesis. *Biochim Biophys Acta* 1851;2015:1107–17.
- [43] Ledesma-Amaro R, Lozano-Martinez P, Jimenez A, Revuelta JL. Engineering *Ashbya gossypii* for efficient biolipid production. *Bioengineered* 2015;6:119–23.
- [44] Ratledge C. Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans* 2002;30:1047–50.
- [45] Rakicka M, Lazar Z, Dulermo T, Fickers P, Nicaud JM. Lipid production by the oleaginous yeast *Yarrowia lipolytica* using industrial by-products under different culture conditions. *Biotechnol Biofuels* 2015;8:104.
- [46] Lazar Z, Rossignol T, Verbeke J, Crutz-Le Coq AM, Nicaud JM, Robak M. Optimized invertase expression and secretion cassette for improving *Yarrowia lipolytica* growth on sucrose for industrial applications. *J Ind Microbiol Biotechnol* 2013;40:1273–83.
- [47] Lazar Z, Dulermo T, Neuveglise C, Crutz-Le Coq AM, Nicaud JM. Hexokinase—a limiting factor in lipid production from fructose in *Yarrowia lipolytica*. *Metab Eng* 2014;26C:89–99.
- [48] Lazar Z, Gamboa-Melendez H, Le Coq AC, Neuveglise C, Nicaud JM. Awakening the endogenous Leloir pathway for efficient galactose utilization by *Yarrowia lipolytica*. *Biotechnol Biofuels* 2015;8:185.
- [49] Wei H, Wang W, Alahuhta M, Vander Wall T, Baker JO, Taylor 2nd LE, et al. Engineering towards a complete heterologous cellulase secretome in *Yarrowia lipolytica* reveals its potential for consolidated bioprocessing. *Biotechnol Biofuels* 2014;7:148.
- [50] Guo Z, Duquesne S, Bozonnet S, Ciochi G, Nicaud JM, Marty A, et al. Development of cellobiose-degrading ability in *Yarrowia lipolytica* strain by overexpression of endogenous genes. *Biotechnol Biofuels* 2015;8:109.
- [51] Ledesma-Amaro R, Dulermo T, Nicaud JM. Engineering *Yarrowia lipolytica* to produce biodiesel from raw starch. *Biotechnol Biofuels* 2015;8:148.
- [52] Tai M, Stephanopoulos G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metab Eng* 2013;15:1–9.
- [53] Qiao K, Imam Abidi SH, Liu H, Zhang H, Chakraborty S, Watson N, et al. Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metab Eng* 2015;29:56–65.
- [54] Dulermo R, Gamboa-Melendez H, Ledesma-Amaro R, Thevenieau F, Nicaud JM. Unraveling fatty acid transport and activation mechanisms in *Yarrowia lipolytica*. *Biochim Biophys Acta* 2015.
- [55] Athenstaedt K, Jolivet P, Boulard C, Zivy M, Negroni L, Nicaud JM, et al. Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. *Proteomics* 2006;6:1450–9.
- [56] Beopoulos A, Haddouche R, Kabran P, Dulermo T, Chardot T, Nicaud JM. Identification and characterization of DGA2, an acyltransferase of the DGAT1 acyl-CoA:diacylglycerol acyltransferase family in the oleaginous yeast *Yarrowia lipolytica*. New insights into the storage lipid metabolism of oleaginous yeasts. *Appl Microbiol Biotechnol* 2012;93:1523–37.
- [57] Athenstaedt K, YALIOE32769g (DGA1) and YALIOE16797g (LRO1) encode major triacylglycerol synthases of the oleaginous yeast *Yarrowia lipolytica*. *Biochim Biophys Acta* 1811;2011:587–96.
- [58] Blazeck J, Hill A, Liu L, Knight R, Miller J, Pan A, et al. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun* 2014;5:3131.
- [59] Gajdos P, Nicaud JM, Rossignol T, Certik M. Single cell oil production on molasses by *Yarrowia lipolytica* strains overexpressing DGA2 in multicopy. *Appl Microbiol Biotechnol* 2015.
- [60] Black PN, Faergeman NJ, DiRusso CC. Long-chain acyl-CoA-dependent regulation of gene expression in bacteria, yeast and mammals. *J Nutr* 2000;130:305S–9S.
- [61] Feddersen S, Neergaard TB, Knudsen J, Faergeman NJ. Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA-binding-protein-dependent mechanism in *Saccharomyces cerevisiae*. *Biochem J* 2007;407:219–30.
- [62] Dulermo T, Treton B, Beopoulos A, Kabran Gnankon AP, Haddouche R, Nicaud JM. Characterization of the two intracellular lipases of *Y. lipolytica* encoded by TGL3 and TGL4 genes: new insights into the role of intracellular lipases and lipid body organisation. *Biochim Biophys Acta* 2013;1831:1486–95.
- [63] Dulermo R, Gamboa-Melendez H, Dulermo T, Thevenieau F, Nicaud JM. The fatty acid transport protein Fat1p is involved in the export of fatty acids from lipid bodies in *Yarrowia lipolytica*. *FEMS Yeast Res* 2014;14:883–96.
- [64] Wang HJ, Le Dall MT, Wach Y, Laroche C, Belin JM, Gaillardin C, et al. Evaluation of acyl coenzyme A oxidase (Aox) isozyme function in the n-alkane-assimilating yeast *Yarrowia lipolytica*. *J Bacteriol* 1999;181:5140–8.
- [65] Mickova K, Roux E, Athenstaedt K, d'Andrea S, Daum G, Chardot T, et al. Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol* 2004;70:3918–24.
- [66] Beopoulos A, Mrozova Z, Thevenieau F, Le Dall MT, Hapala I, Papanikolaou S, et al. Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol* 2008;74:7779–89.
- [67] Dulermo T, Nicaud JM. Involvement of the G3P shuttle and beta-oxidation pathway in the control of TAG synthesis and lipid accumulation in *Yarrowia lipolytica*. *Metab Eng* 2011;13:482–91.
- [68] Hong SP, Sharpe PL, Xue Z, Yadav NS, Zhu Q. Peroxisome biogenesis factor protein (pex) disruptions for altering polyunsaturated fatty acids and total lipid content in oleaginous eukaryotic organisms. US Patent Application 20090117253. 2009.
- [69] Remi D, Thierry D, Gamboa-Melendez H, Thevenieau F, Nicaud JM. Role of Pex11p in lipid homeostasis in *Yarrowia lipolytica*. *Eukaryot Cell* 2015.
- [70] Crumb DJ, Vattam DA. Conjugated linoleic acid (CLA)—an overview. *Int J Appl Res Nat Prod* 2011;4:12–8.
- [71] Lee Y, Thompson JT, de Lera AR, Vanden Heuvel JP. Isomer-specific effects of conjugated linoleic acid on gene expression in RAW 264.7. *J Nutr Biochem* 2009;20:848–59. e1–5.
- [72] Churrua I, Fernandez-Quintela A, Portillo MP. Conjugated linoleic acid isomers: differences in metabolism and biological effects. *Biofactors* 2009;35:105–11.
- [73] Gholami Z, Khosravi-Darani K. An overview of conjugated linoleic acid: microbial production and application. *Mini-Rev Med Chem* 2014;14:734–46.
- [74] Haas MJ, Kramer JK, McNeill G, Scott K, Foglia TA, Sehat N, et al. Lipase-catalyzed fractionation of conjugated linoleic acid isomers. *Lipids* 1999;34:979–87.
- [75] Kishino S, Ogawa J, Ando A, Omura Y, Shimizu S. Ricinoleic acid and castor oil as substrates for conjugated linoleic acid production by washed cells of *Lactobacillus plantarum*. *Biosci Biotechnol Biochem* 2002;66:2283–6.
- [76] Ando A, Ogawa J, Kishino S, Shimizu S. Conjugated linoleic acid production from castor oil by *Lactobacillus plantarum* JCM 1551. *Enzym Microb Technol* 2004;35:40–5.
- [77] Ando A, Ogawa J, Sugimoto S, Kishino S, Sakuradani E, Yokozeki K, et al. Selective production of cis-9,trans-11 isomer of conjugated linoleic acid from trans-vaccenic acid methyl ester by *Delacroixia coronata*. *J Appl Microbiol* 2009;106:1697–704.

- [78] Rosson RA, Grund AD, Deng MD, Sanchez-Riera F. Linoleate isomerase. United States: Patent 6743609. 2004.
- [79] Peng SS, Deng M-D, Grund AD, Rosson RA. Purification and characterization of a membrane-bound linoleic acid isomerase from *Clostridium sporogenes*. *Enzym Microb Technol* 2007;40:831–9.
- [80] Hornung E, Krueger C, Pernstich C, Gipmans M, Porzel A, Feussner I. Production of (10E,12Z)-conjugated linoleic acid in yeast and tobacco seeds. *Biochim Biophys Acta* 1738;2005:105–14.
- [81] Kohno-Murase J, Iwabuchi M, Endo-Kasahara S, Sugita K, Ebinuma H, Imamura J. Production of trans-10, cis-12 conjugated linoleic acid in rice. *Transgenic Res* 2006;15:95–100.
- [82] Rosberg-Cody E, Johnson MC, Fitzgerald GF, Ross PR, Stanton C. Heterologous expression of linoleic acid isomerase from *Propionibacterium acnes* and anti-proliferative activity of recombinant trans-10, cis-12 conjugated linoleic acid. *Microbiology* 2007;153:2483–90.
- [83] Zhang B, Rong C, Chen H, Song Y, Zhang H, Chen W. De novo synthesis of trans-10, cis-12 conjugated linoleic acid in oleaginous yeast *Yarrowia lipolytica*. *Microb Cell Factories* 2012;11:51.
- [84] Zhang B, Chen H, Li M, Gu Z, Song Y, Ratledge C, et al. Genetic engineering of *Yarrowia lipolytica* for enhanced production of trans-10, cis-12 conjugated linoleic acid. *Microb Cell Factories* 2013;12:70.
- [85] Mutlu H, Meier MAR. Castor oil as a renewable resource for the chemical industry. *Eur J Lipid Sci Technol* 2010;112:10–30.
- [86] da Silva NL, Maciel MR, Batistella CB, Maciel FR. Optimization of biodiesel production from castor oil. *Appl Biochem Biotechnol* 2006;129–132:405–14.
- [87] Ogunniyi DS. Castor oil: a vital industrial raw material. *Bioresour Technol* 2006;97:1086–91.
- [88] Galliard T, Stumpf PK. Fat metabolism in higher plants. 30. Enzymatic synthesis of ricinoleic acid by a microsomal preparation from developing *Ricinus communis* seeds. *J Biol Chem* 1966;241:5806–12.
- [89] Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S. Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. *Biochem J* 1991;280(Pt 2):507–14.
- [90] van de Loo FJ, Broun P, Turner S, Somerville C. An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. *Proc Natl Acad Sci U S A* 1995;92:6743–7.
- [91] Broun P, Somerville C. Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic *Arabidopsis* plants that express a fatty acyl hydroxylase cDNA from castor bean. *Plant Physiol* 1997;113:933–42.
- [92] Smith MA, Moon H, Chowrira G, Kunst L. Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. *Planta* 2003;217:507–16.
- [93] Lu C, Fulda M, Wallis JG, Browne J. A high-throughput screen for genes from castor that boost hydroxy fatty acid accumulation in seed oils of transgenic *Arabidopsis*. *Plant J* 2006;45:847–56.
- [94] Kumar R, Wallis JG, Skidmore C, Browne J. A mutation in *Arabidopsis* cytochrome b5 reductase identified by high-throughput screening differentially affects hydroxylation and desaturation. *Plant J* 2006;48:920–32.
- [95] Burgal J, Shockey J, Lu C, Dyer J, Larson T, Graham I, et al. Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. *Plant Biotechnol J* 2008;6:819–31.
- [96] Lee KR, Chen GQ, Kim HU. Current progress towards the metabolic engineering of plant seed oil for hydroxy fatty acids production. *Plant Cell Rep* 2015;34:603–15.
- [97] Meesapyodsuk D, Qiu X. An oleate hydroxylase from the fungus *Claviceps purpurea*: cloning, functional analysis, and expression in *Arabidopsis*. *Plant Physiol* 2008;147:1325–33.
- [98] Mavraganis I, Meesapyodsuk D, Vrinten P, Smith M, Qiu X. Type II diacylglycerol acyltransferase from *claviceps purpurea* with ricinoleic acid, a hydroxyl fatty acid of industrial importance, as preferred substrate. *Appl Environ Microbiol* 2010;76:1135–42.
- [99] Holic R, Yazawa H, Kumagai H, Uemura H. Engineered high content of ricinoleic acid in fission yeast *Schizosaccharomyces pombe*. *Appl Microbiol Biotechnol* 2012;95:179–87.
- [100] Yazawa H, Kumagai H, Uemura H. Characterization of triglyceride lipase genes of fission yeast *Schizosaccharomyces pombe*. *Appl Microbiol Biotechnol* 2012;96:981–91.
- [101] Yazawa H, Holic R, Kumagai H, Uemura H. Toxicity of ricinoleic acid production in fission yeast *Schizosaccharomyces pombe* is suppressed by the overexpression of plg7, a phospholipase A2 of a platelet-activating factor (PAF) family homolog. *Appl Microbiol Biotechnol* 2013;97:8193–203.
- [102] Yazawa H, Kumagai H, Uemura H. Secretory production of ricinoleic acid in fission yeast *Schizosaccharomyces pombe*. *Appl Microbiol Biotechnol* 2013;97:8663–71.
- [103] Yazawa H, Ogiso M, Kumagai H, Uemura H. Suppression of ricinoleic acid toxicity by pt2 overexpression in fission yeast *Schizosaccharomyces pombe*. *Appl Microbiol Biotechnol* 2014;98:9325–37.
- [104] Beopoulos A, Verbeke J, Bordes F, Guichard M, Bressy M, Marty A, et al. Metabolic engineering for ricinoleic acid production in the oleaginous yeast *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 2014;98:251–62.
- [105] Nicaud J, Marty A, Beopoulos A, Verbeke J, Bordes F, Guichard M. Levures mutantes capables de produire un acide gras inhabituel. World Patent Application WO 2014/178014 A1. 2014.
- [106] Swanson D, Block R, Mousa SA. Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Adv Nutr* 2012;3:1–7.
- [107] Meyer BJ, Mann NJ, Lewis JL, Milligan GC, Sinclair AJ, Howe PR. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids* 2003;38:391–8.
- [108] Domingo JL, Bocio A, Falco G, Llobet JM. Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. *Toxicology* 2007;230:219–26.
- [109] Chen Y, Meesapyodsuk D, Qiu X. Transgenic production of omega-3 very long chain polyunsaturated fatty acids in plants: accomplishment and challenge. *Biocatalysis Agric Biotechnol* 2014;3:38–43.
- [110] Ruiz-Lopez N, Haslam RP, Napier JA, Sayanova O. Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oil-seed crop. *Plant J* 2014;77:198–208.
- [111] Martins DA, Custodio L, Barreira L, Pereira H, Ben-Hamadou R, Varela J, et al. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. *Mar Drugs* 2013;11:2259–81.
- [112] Gong Y, Wan X, Jiang M, Hu C, Hu H, Huang F. Metabolic engineering of microorganisms to produce omega-3 very long-chain polyunsaturated fatty acids. *Prog Lipid Res* 2014;56:19–35.
- [113] Tavares S, Grotkjaer T, Obsen T, Haslam RP, Napier JA, Gunnarsson N. Metabolic engineering of *Saccharomyces cerevisiae* for production of Eicosapentaenoic Acid, using a novel Delta5-Desaturase from *Paramecium tetraurelia*. *Appl Environ Microbiol* 2011;77:1854–61.
- [114] Sakuradani E, Ando A, Shimizu S, Ogawa J. Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 15-4. *J Biosci Bioeng* 2013;116:417–22.
- [115] Salunke D, Manglekar R, Gadre R, Nene S, Harsulkar AM. Production of polyunsaturated fatty acids in recombinant *Lipomyces starkeyi* through submerged fermentation. *Bioprocess Biosyst Eng* 2015.
- [116] Xie D, Jackson EN, Zhu Q. Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: from fundamental research to commercial production. *Appl Microbiol Biotechnol* 2015;99:1599–610.
- [117] Zhu Q, Jackson EN. Metabolic engineering of *Yarrowia lipolytica* for industrial applications. *Curr Opin Biotechnol* 2015;36:65–72.
- [118] Macool DJ, Xue Z, Zhu Q, A *Mortierella alpina* C16/18 fatty acid elongase. US Patent 7470532. 2008.
- [119] Yadav NS, Zhu Q, Zhang H.  $\Delta 12$  desaturases suitable for altering levels of polyunsaturated fatty acids in oleaginous yeast. US Patent 7504259. 2009.
- [120] Zhu Q, Xue Z, Yadav N, Damude H, Pollak D, Rupert R, et al. Metabolic engineering of an oleaginous yeast for the production of omega-3 fatty acids. In: Cohen Z, Ratledge C, editors. *Single Cell Oils*. second ed. Urbana Illinois: AOCS Press; 2010. p. 51–73.
- [121] Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomol Pharmacother* 2002;56:365–79.
- [122] Damude HG, McGonigle B, Zhu Q, Zhixiong X. Delta-9 elongases and their use in making polyunsaturated fatty acids. US Patent Application US20070118929. 2007.
- [123] Damude HG, Zhu Q. Delta-8 desaturase and its use in making polyunsaturated fatty acids. US Patent US 7256033. 2007.
- [124] Damude HG, Kinney AJ, Ripp KG, Zhu Q. Multizymes and their use in making polyunsaturated fatty acids. US Patent Application 20080254191. 2008.
- [125] Hong SP, Seip J, Sharpe PL, Xue Z, Yadav NS, Zhang H, et al. Recombinant microbial host cells for high eicosapentaenoic acid production. US Patent Application 20120052537. 2011.
- [126] Huf S, Krügener S, Hirth T, Rupp S, Zibek S. Biotechnological synthesis of long-chain dicarboxylic acids as building blocks for polymers. *Eur J Lipid Sci Technol* 2011;113:548–61.
- [127] Romero-Guido C, Belo I, Ta TM, Cao-Hoang L, Alchihib M, Gomes N, et al. Biochemistry of lactone formation in yeast and fungi and its utilisation for the production of flavour and fragrance compounds. *Appl Microbiol Biotechnol* 2011;89:535–47.
- [128] Wache Y, Laroche C, Bergmark K, Moller-Andersen C, Aguedo M, Le Dall MT, et al. Involvement of acyl coenzyme A oxidase isozymes in biotransformation of methyl ricinoleate into gamma-decalactone by *Yarrowia lipolytica*. *Appl Environ Microbiol* 2000;66:1233–6.
- [129] Guo Y, Song H, Wang Z, Ding Y. Expression of POX2 gene and disruption of POX3 genes in the industrial *Yarrowia lipolytica* on the gamma-decalactone production. *Microbiol Res* 2012;167:246–52.
- [130] Wache Y, Aguedo M, Choquet A, Gatfield IL, Nicaud JM, Belin JM. Role of beta-oxidation enzymes in gamma-decalactone production by the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol* 2001;67:5700–4.
- [131] Gomes N, Teixeira JA, Belo I. Fed-batch versus batch cultures of *Yarrowia lipolytica* for gamma-decalactone production from methyl ricinoleate. *Biotechnol Lett* 2012;34:649–54.
- [132] Reis N, Goncalves CN, Aguedo M, Gomes N, Teixeira JA, Vicente AA. Application of a novel oscillatory flow micro-bioreactor to the production of gamma-decalactone in a two immiscible liquid phase medium. *Biotechnol Lett* 2006;28:485–90.
- [133] Garcia EE, Belin JM, Wache Y. Use of a Doehlert factorial design to investigate the effects of pH and aeration on the accumulation of lactones by *Yarrowia lipolytica*. *J Appl Microbiol* 2007;103:1508–15.
- [134] Aguedo M, Gomes N, Garcia EE, Wache Y, Mota M, Teixeira JA, et al. Decalactone production by *Yarrowia lipolytica* under increased O<sub>2</sub> transfer rates. *Biotechnol Lett* 2005;27:1617–21.
- [135] Braga A, Belo I. Immobilization of *Yarrowia lipolytica* for aroma production from castor oil. *Appl Biochem Biotechnol* 2013;169:2202–11.
- [136] Zhao Y-P, Mu X-Q, Xu Y. Improvement in gamma-decalactone production by *Yarrowia* sp. after genome shuffling. *Chem Pap* 2014;68:1030–40.
- [137] Zhao Y, Xu Y, Jiang C. Efficient biosynthesis of gamma-decalactone in ionic liquids by immobilized whole cells of *Yarrowia lipolytica* G3-3.21 on attapulgit. *Bioprocess Biosyst Eng* 2015.

- [138] Hatanaka A. The biogenesis of green odour by green leaves. *Phytochemistry* 1993;34:1201–18.
- [139] Gr B, Nicaud J-M, Nthangeni B, Santiago-Gomez P, Belin J-M, Husson F. Fatty acid hydroperoxide lyase of green bell pepper: cloning in *Yarrowia lipolytica* and biogenesis of volatile aldehydes. *Enzym Microb Technol* 2004;35:293–9.
- [140] Buchhaupt M, Guder JC, Etschmann MM, Schrader J. Synthesis of green note aroma compounds by biotransformation of fatty acids using yeast cells coexpressing lipoxygenase and hydroperoxide lyase. *Appl Microbiol Biotechnol* 2012;93:159–68.
- [141] Whitehead I, Slusarenko A, Waspi U, Gaskin D, Brash A, Tijet N. Guava (*Psidium guajava*) 13-hydroperoxide lyase and uses thereof. WO Patent No 99/58648. 1999.
- [142] Hauml U, Silke N, Lerch K, Muheim A. Hydroperoxide lyase. US Patent No 6238898. 2001.
- [143] Tran Thanh H, Beney L, Simonin H, Nguyen TX, Gervais P, Belin JM, et al. Toxicity of fatty acid hydroperoxides towards *Yarrowia lipolytica*: implication of their membrane fluidizing action. *Biochim Biophys Acta* 1768;2007:2256–62.
- [144] Picataggio S, Deanda K, Mielenz J. Determination of *Candida tropicalis* acyl coenzyme A oxidase isozyme function by sequential gene disruption. *Mol Cell Biol* 1991;11:4333–9.
- [145] Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, et al. Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Biotechnology (N Y)* 1992;10:894–8.
- [146] Cao Z, Gao H, Liu M, Jiao P. Engineering the acetyl-CoA transportation system of *Candida tropicalis* enhances the production of dicarboxylic acid. *Biotechnol J* 2006;1:68–74.
- [147] Nicaud JM, Thevenieau F, Le Dall MT, Marchall R. World Patent WO/2006/064131. 2006.
- [148] Thevenieau F, Le Dall MT, Nthangeni B, Mauersberger S, Marchal R, Nicaud JM. Characterization of *Yarrowia lipolytica* mutants affected in hydrophobic substrate utilization. *Fungal Genet Biol* 2007;44:531–42.
- [149] Thevenieau F. Metabolic Engineering of the Yeast *Yarrowia lipolytica* for the Production of Long-Chain Dicarboxylic Acids from Renewable oil Feedstock. PhD thesis Paris: Institut National Agronomique Paris-Grignon; 2006.
- [150] Gatter M, Forster A, Bar K, Winter M, Otto C, Petzsch P, et al. A newly identified fatty alcohol oxidase gene is mainly responsible for the oxidation of long-chain omega-hydroxy fatty acids in *Yarrowia lipolytica*. *FEMS Yeast Res* 2014;14:858–72.