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Review Article

Engineering *Yarrowia lipolytica* to produce nutritional fatty acids: Current status and future perspectives

Lizhen Cao [a], Mingxue Yin [a], Tian-Qiong Shi [b], Lu Lin [a], Rodrigo Ledesma-Amaro [c], Xiao-Jun Ji [a,*]

[a] State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No. 30 South Puzhu Road, Nanjing, 211816, People's Republic of China
[b] School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, No. 1 Wenyuan Road, Nanjing, 210046, People's Republic of China
[c] Department of Bioengineering and Imperial College Centre for Synthetic Biology, Imperial College London, London, SW7 2AZ, United Kingdom

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ABSTRACT

Due to their vital physiological functions, nutritional fatty acids have great potential as nutraceutical food supplements for preventing an array of diseases such as inflammation, depression, arthritis, osteoporosis, diabetes and cancer. Microbial biosynthesis of fatty acids follows the trend of sustainable development, as it enables green, environmentally friendly and efficient production. As a natural oleaginous yeast, *Yarrowia lipolytica* is especially well-suited for the production of fatty acids. Moreover, it has a variety of genetic engineering tools and novel metabolic engineering strategies that make it a robust workhorse for the production of an array of value-added products. In this review, we summarize recent advances in metabolic engineering strategies for accumulating nutritional fatty acids in *Y. lipolytica*, including conjugated fatty acids and polyunsaturated fatty acids. In addition, the future prospects of nutritional fatty acid production using the *Y. lipolytica* platform are discussed in light of the current progress, challenges, and trends in this field. Finally, guidelines for future studies are also emphasized.

1. Introduction

Nutritional chemicals, an important class of food supplements, can supply the nutrients that the human body lacks or loses. They are not only building blocks of cells in the human body, but also indispensable for good health and the prevention of various diseases such as inflammation, depression, arthritis, osteoporosis, diabetes and cancer [1–3]. Nutritional fatty acids, which are traditionally isolated and sourced from animals and plants, are vital dietary nutrients that offer an array of benefits, from strong brain development to lower risk of heart disease and cancer [4–9]. In recent years, conjugated fatty acids (CFAs) and polyunsaturated fatty acids (PUFAs) are the two most widely studied nutritional fatty acids, especially in the area of dietary supplements and functional foods (Fig. 1) [10,11]. CFAs, mainly including conjugated linoleic acids (CLAs) and conjugated linolenic acid (CLNAs), possess various health promoting effects such as inhibiting cancer cell growth and suppressing inflammatory responses [12,13]. The two main families of PUFAs, ω-3 and ω-6, are both of high physiological significance for human health [2]. Growth in the area of nutritional fatty acid applications is promoted by increasing interest in human health. The global market for ω-3 PUFAs alone reached US$ 13 billion in 2021 [14]. However, traditional sources of these nutritional fatty acids such as plant seeds and marine fish are facing a series of problems. The increase of fish consumption and the expansion of marine pollution, as well as limitations of plant growth due to season, climate, and environmental variations both threaten the traditional supply of these products [15,16]. Hence, it is significant to spur a sustainable, environmentally friendly, and efficient method for high-quality nutritional fatty acid production. Metabolic engineering of oleaginous microorganisms to increase the production of nutritional fatty acids has become a promising eco-friendly approach [17].

As a broadly studied oleaginous yeast, *Yarrowia lipolytica* has a mature genetic toolbox and a strong industrial track-record. It is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) due to its non-pathogenic nature [18]. It also can accumulate over 90% of lipids in its dry cell weight (DCW) and utilize an array of renewable substrates as carbon sources [19,20]. Furthermore, the whole genome sequence of *Y. lipolytica* has been clearly annotated,

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* Corresponding author.
E-mail address: xiaojunji@njtech.edu.cn (X.-J. Ji).

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greatly expanding the power of mature genetic engineering tools such as DNA assembly techniques, Gateway cloning, Golden gate, CRISPR/Cas9 and so on [21–23]. For these reasons, *Y. lipolytica* is emerging as an attractive host for the biomanufacturing of various native and non-native biochemicals. Notable examples include fatty acids, fatty acid-derived products, terpenoids, flavonoids, and polyketides, among others [24–28].

In this review, we first describe the metabolism of fatty acids in microorganisms. Then, we highlight the various engineering strategies and efforts to increase the accumulation of nutritional fatty acids in *Y. lipolytica*, including CFAs and PUFAs. In general, this review aims to provide detailed and attractive development strategies for the production of nutritional fatty acids in *Y. lipolytica*.

2. Cellular pathways for the biosynthesis and accumulation of nutritional fatty acids

CFAs and PUFAs, two main well-studied nutritional fatty acid classes, are produced through different biosynthetic pathways in different parts of the cell. Two different pathways for the synthesis of PUFAs are known in nature - the aerobic desaturase/elongase pathway and the anaerobic polyketide synthases (PKS) pathway. Many genes encoding CFA and PUFA biosynthesis pathways have been identified to date, and various novel genes have been explored, providing a solid basis for metabolic engineering of microorganisms to increase the production of nutritional fatty acids (Fig. 2).

2.1. Conjugated fatty acid biosynthesis pathway

The most well-known CFAs are CLAs and CLNAs with two and three conjugated double bonds, respectively. In CFAs, the double bonds at different positions can be either *cis* (*c*) or *trans* (*t*). CLA biosynthesis occurs in the cytosol and starts with free linoleic acid (LA), which can be produced by the degradation of triacylglycerol (TAG). Therefore, other forms of LA, such as CoA- and triacylglycerol-, are not suitable as substrates. The conversion of free LAs into CLAs is catalyzed by LA isomerase. For example, the isomerases from *Lactobacillus reuteri* and *Clostridium sporogenes* can produce *c*9, *t*11-CLA, and the isomerase from *Propionibacterium acnes* (Pai) can produce *t*10, *c*12-CLA [29].

In contrast to CLAs, the conjugation of CLNAs is introduced in the endoplasmic reticulum (ER), and starts with LA from phosphatidylcholine (PC). Oleic acid, firstly synthesized in the cytosol and inserted into the sn-2 position of PC, is then desaturated to LA by fatty acid desaturase (Fad2). Subsequently, fatty acid conjugases (Fadxs), divergent forms of Fad2, modify LA at the sn-2 position of PC to synthesize CLNAs. Various Fadxs from different organisms such as *Punica granatum*, *Momordica charantia*, and *Calendula officinalis* were investigated, and found to respectively produce punicic acid, α-eleostearic acid, and calendic acid [30].

2.2. Polyunsaturated fatty acid biosynthesis pathway

According to the requirement for molecular oxygen, there are two PUFA biosynthesis pathways, including the aerobic desaturase/elongase pathway and the anaerobic PKS pathway [31]. The former pathway begins with oleic acid and is mainly found in fungi, microalgae and plants. The latter pathway, which operates in eukaryotic microalgae and marine bacteria, starts with acetyl units and mainly produces very long chain PUFAs such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) [32].

2.2.1. Aerobic desaturase/elongase pathway

The aerobic desaturase/elongase pathway is the main PUFAs synthesis pathway in *Y. lipolytica* (Fig. 2) [24]. De novo fatty acid synthesis starts in the cytosol. The precursor acetyl-CoA, from which the fatty acid synthetic (Fas) enzymatic complex starts its biosynthesis process, is formed by pyruvate dehydrogenase (Pdh) in the glycolysis pathway. Acetyl-CoA carboxylase (Acc1) converts acetyl-CoA into the elongation unit of the FAS system, malonyl-CoA. The initiation molecule acetyl-CoA can then be elongated by two carbon atoms from malonyl-CoA by the FAS enzymatic complex to produce acyl-CoA, in a process that mostly ends at a chain length of 16 or 18 carbon atoms. Acyl-CoA is then transported into the ER for further modification, such as elongation and desaturation, which gives the desaturase/elongase pathway name. Acyl-CoA can not only be elongated by elongases to form long-chain fatty acids, but can also be desaturated by a Δ-9 desaturase (Ole1) to produce the first desaturation and by Fad2 to obtain the second double bond. It is worth noting that the elongation and the first double bond are both introduced in the form of acyl-CoA, but the substrate of Fad2 and other desaturases is the acyl moiety of phospholipid (PL) [33].

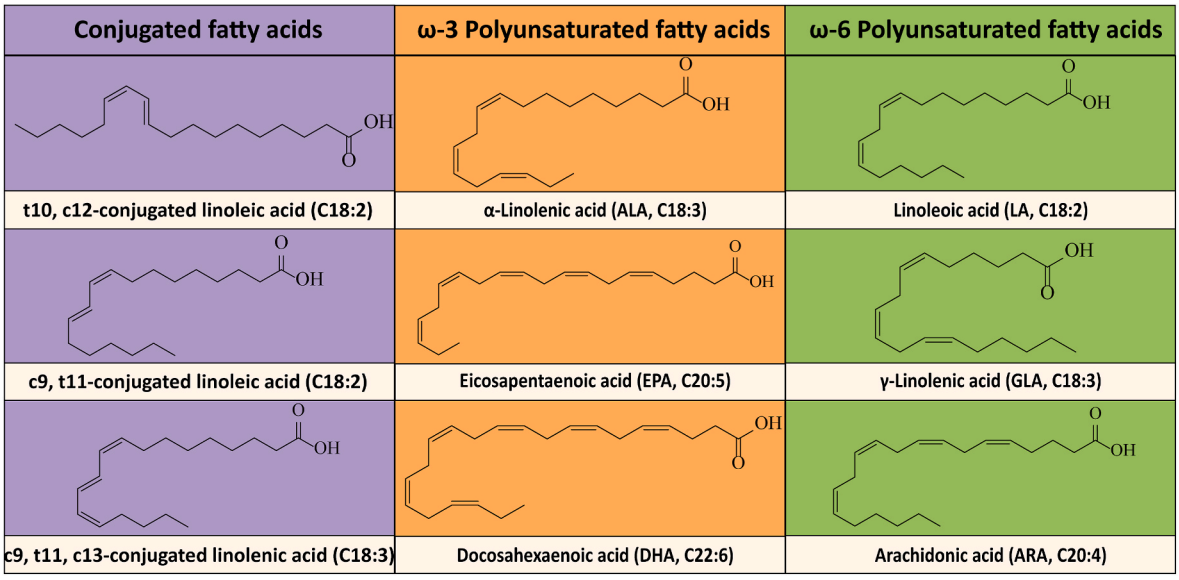

Fig. 1. Chemical structures of common nutritional fatty acids. Purple color represents conjugated fatty acids, orange color represents ω-3 polyunsaturated fatty acids, green color represents ω-6 polyunsaturated fatty acids; *t* means *trans* and *c* means *cis*.

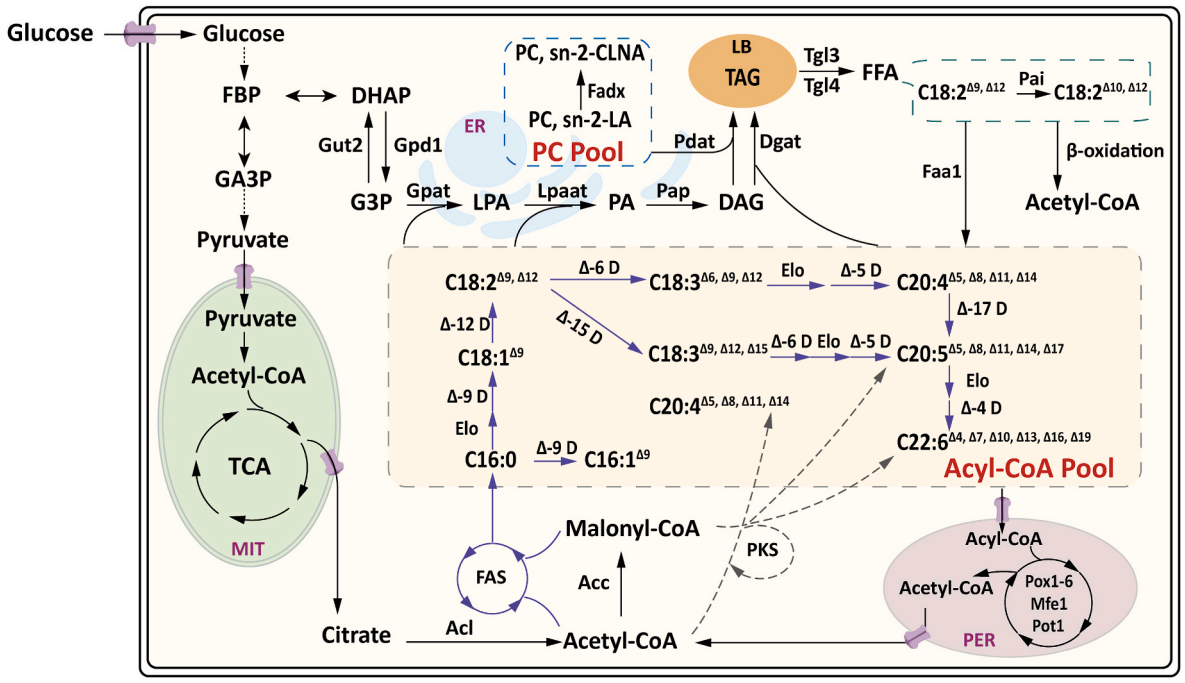

Fig. 2. Overview of metabolic pathways for the production of native fatty acids from glucose. Solid lines represent one-step reactions and dashed lines represent multistep reactions. Purple lines represent the desaturase/elongase pathway and gray lines represent the PKS pathway. Abbreviations: ER, endoplasmic reticulum; LB, lipid body; MIT, mitochondria; PER, peroxisome; FBP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; FFA, free fatty acid; LA, linoleic acid; CLNA, conjugated linolenic acid; Gpd1, NAD^+-dependent G3P dehydrogenase; Gut2, FAD^+-dependent G3P dehydrogenase; Gpat, G3P acyltransferase; Lpaat, lysophosphatidic acid acyltransferase; Pap, PA phosphohydrolase; Pdat, phospholipid-dependent phospholipid DAG acyltransferase; Dgat, acyl-CoA dependent DAG acyltransferase; Tgl3&4, TAG lipases 3 and 4; Acl, ATP-citrate lyase; Acc, acetyl-CoA carboxylase; FAS, fatty acid synthase; Faa1, fatty acyl-CoA synthase; Pai, *Propionibacterium acnes* isomerase; Pox1-6, fatty-acyl-CoA oxidases 1–6; Mfe1, peroxisomal multifunctional enzyme type 1; Pot1, 3-ketoacyl-CoA thiolase; Elo, elongase; D, desaturase; Fadx, fatty acid conjugase; TCA, Tricarboxylic acid; PKS, polyketide synthase.

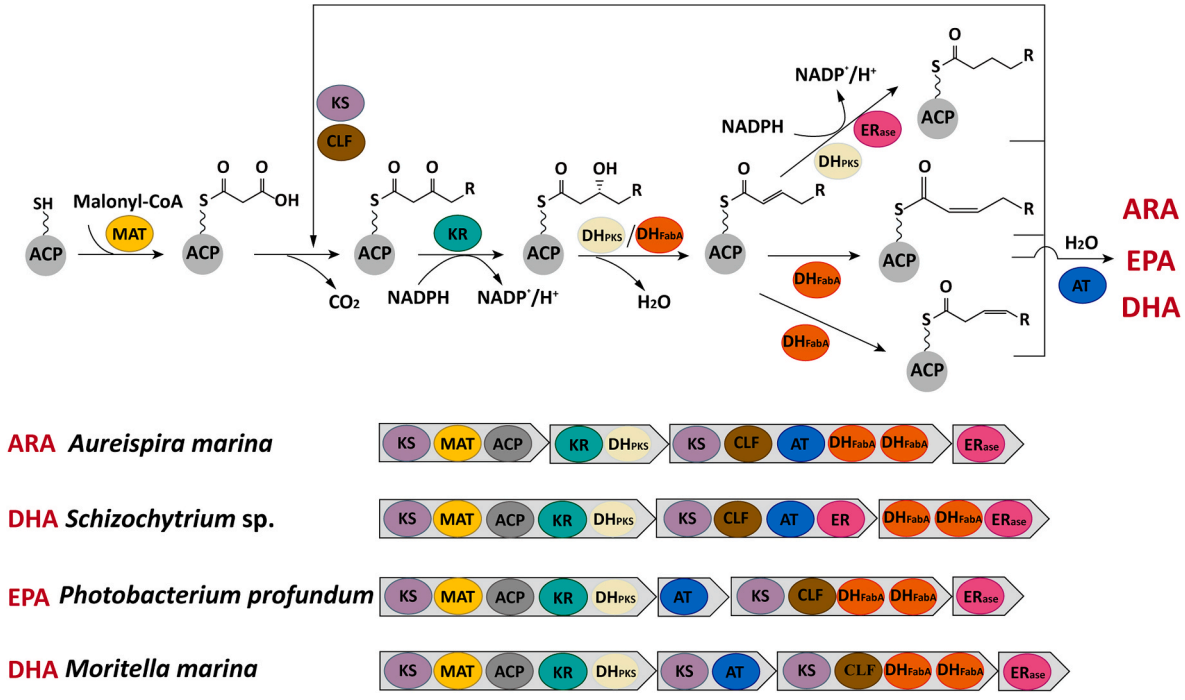

Fig. 3. Polyketide synthases pathway of polyunsaturated fatty acids biosynthesis and domain organizations of polyunsaturated fatty acid synthases from different microorganisms. Abbreviations: ACP, acyl carrier protein; MAT, malonyl-CoA acyltransferase; KS, 3-ketoacyl-ACP synthase; CLF, chain length factor; KR, 3-ketoacyl-ACP reductase; DH_{PKS}, polyketide synthase-type dehydratase; ERase, enoyl reductase; DH_{FabA}, tandem FabA-type dehydratase; AT, acyltransferase.

2.2.2. Anaerobic PKS pathway

The anaerobic PKS pathway is another important native microbial pathway for the biosynthesis of PUFAs [20]. Although the PKS pathway is mostly found in eukaryotic microalgae and marine bacteria, it exhibited great potential for the production of PUFAs in *Y. lipolytica*, especially DHA and ARA. So far, all identified anaerobic PUFA synthases are very large multienzyme complexes with 3–5 subunits that share a similar domain structure, composed of chain length factor (CLF), malonyl-CoA: ACP acyltransferase (MAT), acyltransferase (AT), enoyl reductase (ERase), acyl carrier protein (ACP), 3-ketoacyl-ACP reductase (KR), 3-ketoacyl-ACP synthase (KS) and two types of 3-hydroxyacyl-ACP dehydratase (DH_{PKS} and DH_{FabA}) (Fig. 3). In recent years, the functions of these diverse domains have been dissected by many researchers. The different domains not only have different functions such as controlling the carbon length, regulating unsaturated bond formation and dominating the release of fatty acids, but the number, location and order of the domains also govern the ultimate metabolites produced by the multienzyme complex [32,34].

2.3. Nutritional fatty acid accumulation pathways

Lipids produced by *Y. lipolytica* are stored in lipid bodies, which are primarily composed of TAGs (85%) and sterol esters (8%) [24,35]. The de novo synthesized and modified fatty acids bound to CoA are generally ligated with glycerol-3-phosphate (G3P) to form TAGs in the Kennedy pathway. First, Gpat (G3P acyltransferase) binds acyl-CoA to the sn-1 position of glycerol-3-phosphate (G3P) to produce lysophosphatidic acid (LPA). Subsequently, an acyl group is transferred from CoA to the sn-2 position of LPA under the action of LPA acyltransferase (Lpaat), which produces phosphatidic acid (PA). Then, diacylglycerol (DAG) is formed through dephosphorylation of PA by phosphatidic acid phosphatase (Pap). TAGs can be formed via DAG through either the acyl-CoA-dependent pathway or the acyl-CoA-independent pathway. The first pathway utilizes DAG acyltransferases (Dga1 and Dga2) to transfer an acyl group from CoA to DAG to form TAG, which occurs in the stationary phase of cell growth. The second pathway is catalyzed by phospholipid DAG acyltransferase (Pdat), which can transfer an acyl group from PC to DAG to form TAG, which occurs in the exponential phase.

3. Engineering *Yarrowia lipolytica* to produce nutritional fatty acids

Due to the beneficial effects of nutritional fatty acids, microbial production is increasingly being developed to meet the growing demand. In recent years, different metabolic engineering strategies have been used to synthesize a diverse range of fatty acids in *Y. lipolytica* [15,19,24,36,37]. The engineering strategies mainly include the overexpression of heterologous synthetic pathways for de novo fatty acid synthesis, improving the precursor supply of acetyl-CoA, inhibition of competing pathways to push the metabolic process, regulation the lipid metabolism-related genes to pull the fatty acids accumulation, inhibition of the β-oxidation pathway to block fatty acids degradation, and some other approaches such as engineering the relevant transcription

Table 1
Summary of the production of nutritional fatty acids using the *Y. lipolytica* platform.

Product	Parental strain	Genetic modifications	Scale	Production level	References
Trans10, cis12-CLA	*Y. lipolytica* Po1h	Multicopy expression of *PAI*	Flask	5.9% of TFAs	[29]
Trans10, cis12-CLA	*Y. lipolytica* Po1h	Co-expression of *PAI* and *FADS12* from *Mortierella alpina*	Bioreactor	4 g/L	[41]
CLA	*Y. lipolytica* Po1d	Integration of two copies of *PAI* and deletion of *POX1-6* and *DGA1*, *DGA2*, *LRO1*	Bioreactor	302 mg/L	[42]
Trans10, cis12-CLA	*Y. lipolytica* Polh-pINA1292-spopaid12-16	Whole-cell biocatalyst based on *Y. lipolytica*	Flask	15.6 g/L	[43]
Trans10, cis12-CLA	*Y. lipolytica* Po1g	Expression of *PAI*, native *DGA1* and *MaFADS12* from *Mortierella alpina*	Flask	132.6 mg/L	[44]
ALA	*Y. lipolytica* QU22	/	Flask	9.52% of TFAs	[45]
ALA	*Y. lipolytica* ATCC 76982	Expression of *Fm1* from *Fusarium moniliforme*	Flask	28.1% of TFAs	[46]
ALA	*Y. lipolytica* L36DGA1	Expression of three copies of *Rk12-15* from *R. kratochvilovae*	Bioreactor	1.40 g/L	[47]
ALA	XUS-B and ALA-A	Strain mating	Bioreactor	1.42 g/L	[48]
EPA	*Y. lipolytica* ATCC 20362	Expression of 30 copies of 9 different genes. Deletion of *PEX10*, *LIP1* and *SCP2*	Flask	56.6% of TFAs	[49]
EPA	*Y. lipolytica* ATCC 20362	Expression of 35 copies of 17 different genes	Flask	58% of TFAs	[50]
EPA	*Y. lipolytica* ATCC 20362	Expression of 41 copies of 19 different genes	Flask	25% of DCW	[50]
EPA	*Y. lipolytica* ATCC 20362	Expression of 41 copies of 23 different genes	Bioreactor (two-stage fermentation)	48% of TFAs	[51,52]
EPA	*Y. lipolytica* Y4184	Deletion of *YlSNF1*	Flask	7.6% of DCW	[53]
DHA	*Y. lipolytica*	Expression of Δ-4, Δ-5, Δ-6, Δ-7 desaturase, C18-20 elongase, C20-22 elongase and deletion of native acyltransferase and desaturase	Flask	5.6% of TFAs	[20,54]
DHA	*Y. lipolytica* Po1h	Expression of artificial *pfa* BGC from *Aetherobacter fasciculatus*	Bioreactor	350 mg/L	[55]
GLA	*Y. lipolytica* Po1g	Coexpression of Δ-12 desaturase and Δ-6 desaturase from *Mortierella alpina*	Flask	20% of TFAs	[56]
GLA	*Y. lipolytica* Po1f	Expression of Δ-6 desaturase from *Mortierella alpina*	Flask	71.6 mg/L	[57]
ARA	*Y. lipolytica* Po1f	Expression of Δ-6 desaturase, Δ-6 elongase and Δ-5 desaturase from *Mortierella alpina*.	Flask	0.4% of TFAs	[58]
ARA	*Y. lipolytica* Po1f	Expression of a fusion enzyme of Δ-9 elongase and Δ-8 desaturase with a GGGGS linker.	Flask	118.1 mg/L	[59]
ARA	*Y. lipolytica* Po1h	Expression of artificial *pfa* BGC from *Minicystis rosea*	Flask	10.9 mg/L	[55]

Abbreviations: CLA, conjugated linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; GLA, γ-linolenic acid; ARA, arachidonic acid; *PAI*, *Propionibacterium acnes* isomerase; *FADS12*, Δ-12 desaturase; *POX1-6*, fatty-acyl-CoA oxidase 1–6; *DGA1&2*, acyl-CoA diacylglycerol acyltransferase 1&2; *LRO1*, phospholipid diacylglycerol acyltransferase; *PEX10*, peroxisomal biogenesis factor 10; *LIP1*, lipase 1; *SCP2*, sterol carrier protein; *YlSNF1*, AMP-activated protein kinase; *pfa* BGC, PUFA biosynthesis gene clusters; YNB, yeast nitrogen base; TFAs, total fatty acids; DCW, dry cell weight.

factors [20,38]. At the same time, optimization of fermentation processes is also considered as an effective measure to increase fatty acid accumulation [39,40]. Examples of nutritional fatty acids produced in *Y. lipolytica* are described below and summarized in Table 1. CFAs and PUFAs are especially emphasized due to their important nutritional functions.

3.1. Conjugated fatty acids

CFAs have various isomers with diverse conjugated double bonds at different positions (Fig. 1). In recent years, CFAs have gained increasing attention due to their beneficial physiological effects, including anti-inflammatory, antioxidant, anti-obesity, anti-atherogenic and anti-cancer activities [10,13], which have been demonstrated by various animal experiments and clinical trials [60]. The most widely studied CFAs include CLAs, which mainly come from dairy and meat [10, 61–63], and CLNAs, which can be obtained from plant seeds [64,65]. Different studies have successfully constructed CLA-producing microbial strains, but only a few researchers focused on the microbial production of CLNAs, although they possess stronger benefits than CLAs [66,67].

In *Y. lipolytica*, *t*10, *c*12-CLA is the most widely studied CFA. The key enzyme in the synthesis of *t*10, *c*12-CLA is the LA isomerase Pai from *P. acnes*, which uses free LA as the only substrate. Zhang et al. [29] firstly reported the de novo biosynthesis of CLA by *Y. lipolytica* Po1h. They increased the expression level by codon optimizing *PAI* and utilizing the multicopy vector pINA1292, resulting in 5.9% *t*10, *c*12-CLA of total fatty acids (TFAs). However, the engineered strain was unstable and the yield of CLA decreased with prolonged cultivation. Therefore, they further improved the titer of *t*10, *c*12-CLA through the co-expression of *PAI* and the Δ-12 desaturase gene (*FADS12*) from *Mortierella alpina* under the control of the hp14d promoter rather than the previously used hp4d, which increased CLA production up to 10% of TFAs and 45 mg/L after 72h of shake-flask cultivation. Importantly, when the engineered strain was cultivated in medium supplemented with 2% (w/v) soybean oil, which contains a high proportion of LA, 3.1g CLA/L was produced in the biomass, corresponding to 0.9g CLA/L in the overall culture broth [41]. However, the substrate LA and the product CLA can both be degraded by the β-oxidation pathway, which limited the LA conversion rate. In another study, the elimination of β-oxidation (Δ*pox1-6*) and the inhibition of TAG synthesis (Δd*ga1,* Δ*dga2,* Δ*lro1*) in a strain of *Y .lipolytica* with two copies of *PAI* and one copy of endogenous *YlFAD2* resulted in a high concentration of CLA (52 mg/L) in shake-flasks without exogenous LA addition [42]. Zhang et al. [43] subsequently utilized recombinant *Y. lipolytica* as a whole-cell biocatalyst and optimized the reaction conditions to further increase CLA production. The cells were permeabilized through freeze/thawing, and produced 15.6 g/L CLA under the optimized conditions of 1.5 g/L sodium acetate, 100 g/L wet cells, and 25 g/L LA at 28 °C, pH 7, and 200 rpm, corresponding to an LA conversion rate of 62.2%. Furthermore, considering the sustainable development of biodiesel production, raw glycerol was chosen as the sole carbon source in recent work. The engineered strain WXYLO37 co-expressing *PAI*, *MaFADS12* and *DGA1* was cultured under optimized conditions including 0.2 g/L $CaCl_2$, glycerol and soy peptone as the carbon and organic nitrogen sources with a C/N ratio of 50:1, resulting in a CLA titer of 132.6 mg/L. This is the first report of the production of *t*10, *c*12-CLA by *Y. lipolytica* using glycerol as the sole carbon source [44]. Through efforts in the engineering of *Y. lipolytica*, metabolic engineering efforts are enabling the high level production of CFAs.

3.2. Polyunsaturated fatty acids

PUFAs contain two or more unsaturated carbon bonds, mostly at the ω-3 or -6 positions, both of which play important roles in human health. Studies have shown that ω-3 and -6 PUFAs can be converted into anti- and pro-inflammatory mediators, which gives them anti-inflammatory and immunomodulatory properties. They are also necessary for the proper functioning of the cardiac, musculoskeletal, gastrointestinal and immune systems in humans [68–70]. Traditionally, plant seed and fish oils are two predominant sources of PUFAs [71,49]. For example, EPA and DHA can be accumulated by some ocean fishes such as wild salmon, Pacific sardine, herring and mackerel [14,50]. By contrast, α-linolenic acid (ALA) is mostly found in vegetables and plant seeds such as flax, walnuts, and cress [72]. With the increase of health awareness in recent years, PUFAs have attracted increasing attention, while the traditional supplies of PUFAs from seed and ocean fish oils are becoming increasingly scarce. Consequently, novel sustainable strategies are needed to meet the growing market demand for PUFAs [14]. Among these approaches, microbial cell factories are powerful tools to guarantee the economically viable and sustainable production of PUFAs [38,40].

3.2.1. Omega-3 polyunsaturated fatty acids

In ω-3 PUFAs, the first carbon double bond is located at the third carbon from the methyl end. Omega-3 PUFAs have been shown to possess numerous physiological activities, which makes them important for good health throughout life, from infancy to senescence [73,74]. ALA, EPA and DHA are the most important ω-3 PUFAs and have been studied for many years [75,76].

ALA, which has the shortest chain length among natural ω-3 fatty acids, has been explored for many years not only due to its physiological activity, but also because it is a crucial precursor of EPA and DHA. Some *Y. lipolytica* strains have an innate ability of producing ALA, and after optimizing various fermentation parameters, such as cultivation time, nitrogen source, incubation temperature and shaking speed, the ALA content reached 9.52% in *Y. lipolytica* QU22 and 7.86% in QU137 (using peptone as nitrogen source and cultivation at 25 °C and 150 rpm for 3 days) [45]. In addition to controlling the culture conditions, metabolic engineering is another important strategy for increasing the production of ALA, which is biosynthesized by the desaturation of LA into ALA by Δ-15 desaturase. However, many Δ-15 desaturases are bifunctional and are also active on Δ-12 double bonds. Previously, bifunctional enzymes derived from *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* were studied in detail. The results demonstrated that *Y. lipolytica* could produce 28.1% ALA of TFAs by the expression of Fm1, which is originated from *F. moniliforme* and encodes a Δ-15 desaturase [46]. In addition, a bifunctional Δ12-15 desaturase from *Rhodosporidium kratochvilovae* is also considered as a potential enzyme for the synthesis of ALA. A study in 2018 integrated three copies of *Rk12-15* into the high lipid producing strain L36DGA1 (the isolated strain L36 with an overexpression of DGA1), and the ALA content reached 1.08 g/L at the optimal fermentation temperature of 20 °C. Furthermore, the engineered strain L36DGA1 produced 1.4 g/L ALA in a bioreactor with an operating volume of 2 L under the same culture conditions [47]. Additionally, Cordova et al. [77] also successfully explored the positive effect of trace metals, especially iron (II), on cell growth and production of PUFAs, such as ALA. They firstly examined strains Po1f and Po1f Δ*pex10* Δ*mfe1 DGA1* (abbreviated as Po1fpmD), and found that the addition of iron (II) not only strongly increased the LA content at the expense of stearic acid, but also significantly increased the final optical density and lipid accumulation. To investigate the mechanism through which iron shifts the fatty acid profile towards unsaturated fatty acids, they cultured strain L36DGA1 in YPD medium with the supplementation of iron (II), which resulted in ALA accumulation up to 50% of TFAs, confirming the importance of trace metals in lipid accumulation and fatty acid production. Considering economy and sustainability, lignocellulosic biomass is becoming an important renewable feedstock [78]. A strain mating approach was established to produce ALA from xylose. In this approach, an ALA-producing strain and a xylose-utilizing strain were mated to obtain a diploid *Y. lipolytica*, which produced an ALA titer of 0.52 g/L in shake flasks and 1.42 g/L in a 1.7 L bioreactor with xylose as the sole carbon source, representing a more than two-fold increase compared to the haploid ALA-producing strain which consumed glucose. Notably, diploid strains tended to be more robust in terms of

overall growth than haploid stains, which indicated the feasibility of *Y. lipolytica* mating to expand the product profile from xylose [48].

EPA is a typical ω-3 PUFA traditionally derived from ocean fish oils, which has been prescribed to reduce triglycerides and inhibit arterial calcification [79]. Microbial production of EPA typically proceeds via the aerobic desaturase/elongase pathway. DuPont successfully created the best commercial EPA-producing *Y. lipolytica* strain based on the aerobic pathway. First, in order to acquire the optimal strain to produce EPA, scientists collected and screened more than 40 *Y. lipolytica* strains, and selected the strain ATCC 20362 based on its innate lipid content, lipid productivity, and DCW, which achieved the preliminary fermentation performance targets [50]. The selected strain was then genetically modified by introducing 30 copies of eight different heterologous genes, including 5 copies of Δ-12 desaturase from *F. moniliforme*, 7 copies of Δ-9 elongase from *Euglena gracilis* and *Eutreptiella* sp. CCMP389, 7 copies of Δ-8 desaturase from *E. gracilis*, one copy of C16/18 elongase from *M. alpina*, 5 copies of Δ-5 desaturase from *E. gracilis* and *Peridinium* sp. CCMP626, 3 copies of Δ-17 desaturase from *Pythium aphanidermatum* and one endogenous cholinephosphotransferase (*CPT*) gene under the control of promoters with diverse strength to elevate the desaturation and elongation rate. The modifications were further combined with the deletion of peroxisomal biogenesis factor 10 (*PEX10*), important for the normal peroxisome proliferation, as well as lipase 1 (*LIP1*) and sterol carrier protein (*SCP2*) associated with lipid metabolism. The ultimate engineering strain Gen I Y4305 was capable to accumulate EPA at the level of 56.6% of TFAs and 15% of the DCW [49]. Additionally, the strain Gen II = Z1987 which contained 35 copies of 17 different genes was subsequently constructed based on strain Y4305, and it could produce more than 58% EPA of TFAs, corresponding to approximately 20% of DCW. Furthermore, the strain Gen II = Z1987 was further modified to generate the strain Gen III HP Z5567 with an EPA content of 25% DCW [50]. The endogenous acyltransferases *DGAT2*, *PDAT* and *LPAAT* were also explored by DuPont, but only *LPAAT* contributes to the increase of the EPA titer [80]. Based on these research achievements, the commercial products New Harvest™ EPA oil and Verlasso® salmon have been developed and significantly reduced the use of fish-derived products [50]. DuPont also compared fed-batch, single-stage continuous and two-stage continuous fermentation through model simulation analysis and experimental validation to improve EPA production without compromising the EPA titer and substrate conversion ratio by the engineered *Y. lipolytica* strain Z7334, which produced at least 28% EPA of DCW. Compared with fed-batch fermentation, the two-stage continuous fermentation improved the EPA productivity by 80% and EPA concentration by 40%, while maintaining a similar EPA titer in biomass and EPA yield. This research successfully increased the volumetric productivity and concentration while largely maintaining the conversion yield [51]. In another study, Seip et al. [53] discovered that *Y. lipolytica* Snf1 is a key regulator of lipid synthesis and accumulation. Deletion of *YlSNF1* resulted in a 52% increase of the EPA titer compared to wild-type *Y. lipolytica*, reaching up to 7.6% of DCW.

Similar to EPA, DHA also offers health benefits to humans, including positive effects in fetal development as well as preventing osteoporosis, cardiovascular disease, diabetes, and Alzheimer's disease [81]. DHA is closely associated with nervous system development and has become an important component of infant milk powder [82]. A patent granted to DuPont disclosed the production of DHA by overexpressing 22 copies of eight different genes including heterologous Δ-4, Δ-5, Δ-6, and Δ-7 desaturases, as well as C18–20 and C20-22 elongases, combined with deletions of native acyltransferase and desaturase in *Y. lipolytica*, but the resulting strain only accumulated 5.6% DHA of TFAs [20].

The PKS pathway offers reduced NADPH consumption compared to the desaturase/elongase pathway. Multifunctional PKS complexes, encoded by PUFA (*pfa*) biosynthetic gene clusters (BGCs), utilize acyl-CoA as precursor to de novo synthesize DHA. The most well-studied *pfa* BGC for the production of DHA was cloned from the myxobacterium *Aetherobacter fasciculatus* (SBSr002), and was functionally reconstituted in *Pseudomonas putida* [83]. In 2019, the codon-optimized artificial *pfa* BGC encoding PUFA synthase and 4′-phosphopantetheinyl transferase (*PPT*) from *A. fasciculatus* were integrated into the site-specific locus YALI0_C05907 of *Y. lipolytica* Po1h. The resulting strain Po1h:Af4 accumulated DHA up to 10.5% of TFAs. By further limiting the level of phosphate buffer and removing the nitrogen limitation in the medium, the DHA titer reached 16.8% of TFAs, equivalent to 350 mg/L, which was the highest titer among all published studies [55]. However, further increasing DHA production in *Y. lipolytica* requires extensive studies of diverse fermentation strategies. For example, Guo et al. [84] designed a novel porous-membrane-impeller bioreactor and implemented a multistage control strategy, which resulted in the production of 44.3 g/L DHA by *Schizochytrium* sp.

3.2.2. Omega-6 polyunsaturated fatty acids

Different from ω-3 PUFAs, which are mainly derived from marine fish, ω-6 PUFAs are more common in our daily life, such as LA, γ-linolenic acid (GLA) and ARA, obtained from vegetable oils and sunflower seeds [2]. Omega-6 PUFAs were also shown to possess various health-promoting effects. For instance, many preclinical and clinical studies have found a correlation between the intake of ω-6 PUFAs and the incidence of breast, prostate, and colon cancer [85].

The Δ-6 desaturase which converts LA into GLA catalyzes the rate-limiting step in the production of long-chain PUFAs. Therefore, it is necessary to identify a suitable Δ-6 desaturase. The function of Δ-12 and Δ-6 desaturases from *M. alpina* has been confirmed by coexpression in *Saccharomyces cerevisiae* [71]. Then, two expression cassettes encoding Δ-12 and Δ-6 desaturases from *M. alpina* under the control of the hp4d promoter were inserted into the pINA1269 vector and transformed into *Y. lipolytica*, allowing the synthesis of 20% GLA of TFAs [56]. In addition, optimization of culture conditions was also considered as a key strategy to achieve higher GLA titers. When cultivated using a temperature-shift fermentation process (from 28 to 20 °C after 1 day), the recombinant *Y. lipolytica* expressing codon-optimized Δ-6 desaturase from *M. alpina* successfully accumulated 71.6 mg/L GLA [57].

ARA is also an important ω-6 PUFA that has been produced in oleaginous microorganisms such as *M. alpina* and *Y. lipolytica* [86]. Liu et al. [58] firstly developed a one-step in vivo pathway assembly method to produce ARA in *Y. lipolytica.* The genes from *M. alpina* encoding Δ-6 desaturase, Δ-6 elongase and Δ-5 desaturase were codon-optimized and integrated into the 28S rDNA sites of *Y. lipolytica*, which leads to an ARA content of 0.4% of TFAs. They also conducted an in-depth analysis of the influence of the heterologous ARA synthesis pathway on *Y. lipolytica*, which showed that the introduced pathway can redirect carbon flux towards intracellular fatty acids instead of extracellular organic acids [87]. Subsequently, they cloned the novel Δ-9 pathway from *Isochrysis galbana*, which resulted in an ARA titer of 91.5 mg/L in *Y. lipolytica*. To further optimize the spatial domain organization, they attempted to utilize seven different linkers to develop fusion proteins of Δ-9 elongase and Δ-8 desaturase. The results showed that the novel fusion enzyme containing the rigid GGGGS linker was capable of increasing the ARA titer to 118.1 mg/L, representing a 29% increase compared to expression without linkers [59]. Additionally, Gemperlein et al. [55] attempted to produce ARA through the PKS pathway. However, overexpression of *pfa* BGCs from the myxobacterium *Minicystis rosea* (SBNa008) in *Y. lipolytica* Po1h only yielded 10.9 mg/L of ARA.

4. Challenges and future perspectives

Many researchers have developed efficient strategies to produce nutritional fatty acids in *Y. lipolytica* and made great progress in both CFAs and PUFAs (Fig. 4). However, only a few of them, such as Harvest™ EPA oil and Verlasso® salmon created by DuPont [50], were successfully applied on the industrial scale and commercialized. It seems that there are still some challenges that limit the further application of *Y. lipolytica.*

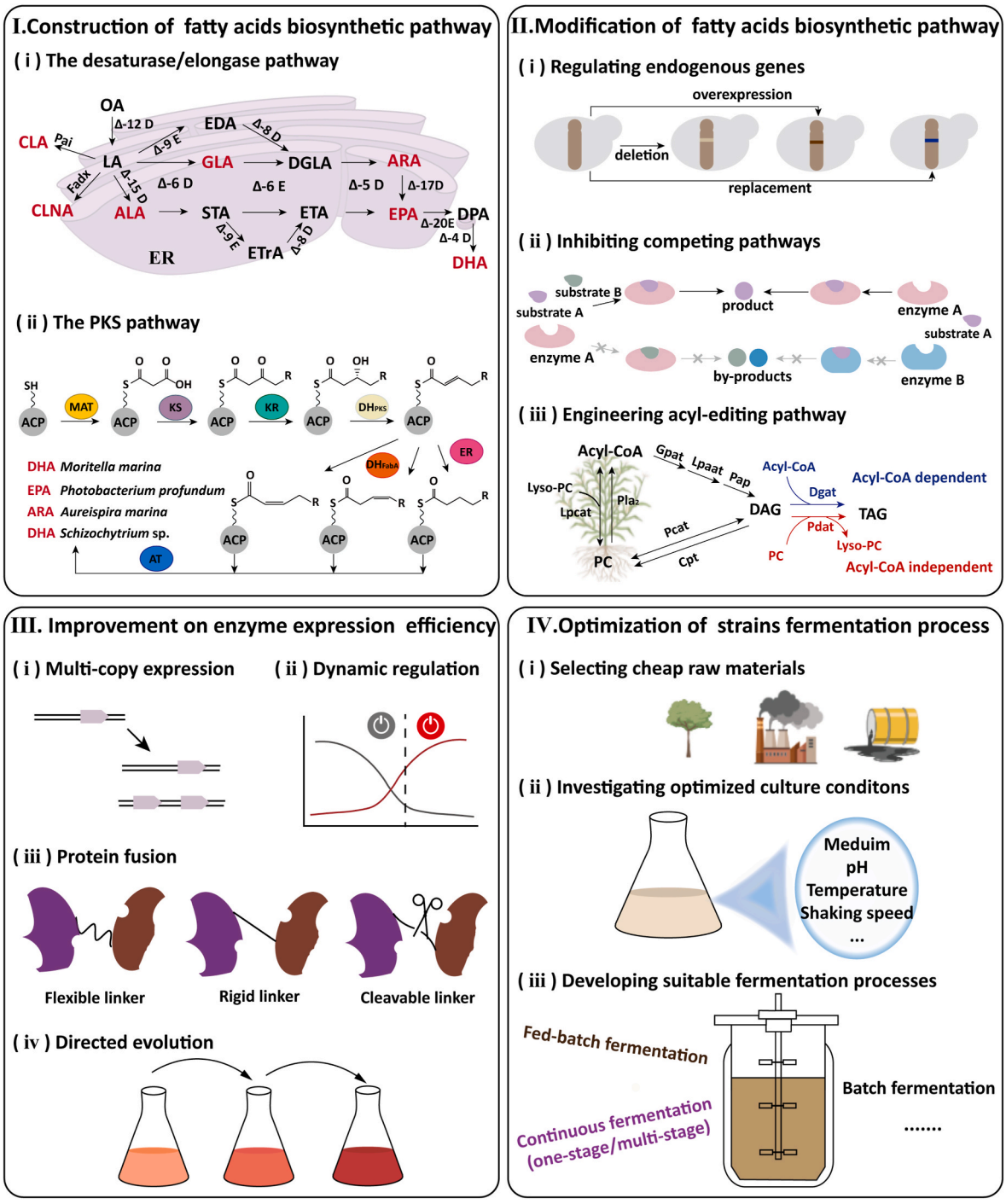

Fig. 4. Overview of different approaches for improving fatty acid accumulation in yeasts. I. Construction of heterologous desaturase/elongase and PKS pathways. II. Modifying fatty acid biosynthesis by combined regulation of endogenous genes, inhibition of competing pathways and engineering the acyl-editing pathway. III. Different strategies including multicopy expression, dynamic regulation, protein fusion and directed evolution. Ⅳ. Fermentation process optimization by utilization of cheap raw materials, suitable culture conditions and fermentation methods.

Firstly, the mechanism of anaerobic PKS pathway is unclear and only several studies focused on this pathway in *Y. lipolytica*. Aerobic desaturase/elongase pathway is the main PUFA production pathway in *Y. lipolytica* and has been investigated exhaustively. However, the anaerobic PKS pathway is more efficient due to the lower consumption of NADPH and simpler fatty acid profile [31]. The most successful example is the expression of *pfa* BGC from *A. fasciculatus* in *Y .lipolytica* for DHA production [55]. Nevertheless, the PKS pathway has been explored in other microorganisms and successfully increased the production of PUFAs. For example, the EPA BGC from *Shewanella japonica* was expressed in the thraustochytrid *Aurantiochytrium* leading to approximately 5-fold improvement of the EPA yield, which reached up to 2.7 g/L in fed-batch fermentation [88]. Hence, it is necessary to clarify the detailed PUFA synthase machinery and apply advanced strategies to optimize the production of these nutritional compounds.

Secondly, inefficient and unbalanced expression of pathway genes also limit minimal accumulation of intermediates and maximal product titers, as was achieved by DuPont scientists in the case of DHA. The desaturase conversion efficiency can reach up to 95% [49]. Recently, Wang et al. [89] established a two-layer “push-pull-block” strategy to

reprogram the fatty acid and lipid metabolism of *Y. lipolytica* to increase oleic oils production, which resulted in 56 g/L oleic microbial oil with 84% oleic acid in fed-batch fermentation. There are increasingly more novel strategies for balancing expression levels, such as utilization of codon-optimized genes and strong or regulated promoters, selection of heterologous enzymes with a narrow substrate preference, as well as improving enzyme activity. Tsakraklides et al. [90] achieved steric acid-preferred Δ-9 desaturase and oleic acid-preferred acyltransferase *GPAT* through the selection of 18 hetrologous genes, which signifiantly pulled and pushed the oleic acid production. Inefficient enzymes are another serious impediment preventing the balancing of metabolic fluxes, which can be solved by enzyme engineering. Directed evolution and semi-rational or rational design are two main strategies for obtaining higher catalytic activities [91].

Thirdly, the mechanism of the channeling from phospholipids to TAGs remains unclear, which has been considered as one of the main bottleneck of heterologous biosynthesis of nutritional fatty acids from plants. In plants, a number of enzymes related to PC acyl editing have been identified and characterized [92]. For example, Xu et al. [93] utilized a membrane-bound yeast two-hybrid system and bimolecular fluorescence complementation to explore the route of ALA from PC to TAG in flax seed oil, and uncovered intriguing physical interactions among transferases. In yeast, it is noteworthy that Δ-12 desaturation mainly occurs on the PC-bound 18:1, resulting in PC-bound 18:2, but the reaction and enzymes responsible for the transfer from PC to TAG remain to be explored [94]. Nevertheless, some progress has been made by selected plant-derived acyl-editing enzymes to improve the accumulation of PUFAs in microorganisms. The expression of *PgPDAT* and *PgLPCAT* from *Punica granatum* in *S. cerevisiae* successfully created an acyl channeling process to produce punicic acid, an important conjugated linolenic acid-derived nutritive fatty acid [95].

Finally, in order to achieve industrial scale manufacturing, the process costs must be reduced, which can be realized by using less expensive raw materials and optimizing fermentation conditions. Although there are still many barriers to settle for further improving the accumulation of nutritional fatty acids in *Y. lipolytica*, significant advances have been made. For example, many natural food substitutes, such as human milk fat substitutes and cocoa butter equivalents, have been produced by *Y. lipolytica* [96,97]. Nowadays, many emerging synthetic biology tools and gene editing technologies are on their way to become standard laboratory techniques for metabolic engineering of *Y. lipolytica*. *Y. lipolytica* therefore possesses enormous potential to commercially produce diverse nutritional products and natural nutrient substitutes.

5. Conclusions

With the continuous development of national economies and increasing average consumption levels, increasing attention is being paid to the physical and mental health of consumers. A large array of nutritional fatty acids are being explored and characterized due to their various beneficial activities, including especially CFAs and PUFAs. Engineering *Y. lipolytica* to produce nutritional fatty acids bypasses traditional limitations of chemical synthesis or extraction from animals and plants, offering a sustainable, environmentally-friendly, and efficient method for industrial production. In this review, we summarize the research on the production of nutritional fatty acids in *Y. lipolytica* through metabolic engineering and process optimization. Future studies aiming to further improve the accumulation of fatty acids and expand the nutrient spectrum of *Y. lipolytica* can take advantage of more detailed metabolic mechanisms and numerous innovative research tools, which will further promote the commercial production of nutritional supplements using *Y. lipolytica*.

Author contributions

Lizhen Cao: Writing - original draft, Writing - review & editing, Visualization; **Mingxue Yin:** partial writing; **Tian-Qiong Shi:** Resources, Visualization; **Lu Lin:** Investigation, Resources; **Rodrigo Ledesma-Amaro:** Investigation, Resources; **Xiao-Jun Ji:** Project administration, Conceptualization, Visualization, Supervision.

Declaration of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "**Engineering *Yarrowia lipolytica* to produce nutritional fatty acids: Current status and future perspectives**".

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