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Biotechnology in Future Food Lipids: Opportunities and Challenges

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Keywords

microbial lipids, metabolic engineering, enzymatic modification, structural lipids, phospholipids

Abstract

Lipids are a large group of essential nutrients in daily diets that provide energy and maintain various physiological functions. As the global population is rapidly expanding, there is an urgent need to enhance the production and quality of food lipids. The development of modern biotechnology allows the manipulation of oil production in plants and microorganisms and the improvement of the nutritional value of food lipids. Various metabolic engineering strategies have been exploited to increase oil production and produce value-added oils in traditional oil crops and other novel lipid sources (e.g., plant vegetative tissues, microalgae, and oleaginous microorganisms). Furthermore, natural lipid structures can be modified by lipases to prepare functional lipids, e.g., diacylglycerols, medium–long–medium-type structured triacylglycerols, human milk-fat substitutes, and structural

phospholipids, for specific nutritional demands. In this review, we focus on the recent advances in metabolic engineering of lipid production in plants and microorganisms, and the preparation of functional lipids via biocatalysis.

1. INTRODUCTION

In light of an ever-growing population but increasingly limited natural resources, a major challenge we are facing today is how to feed the world (Hamrick & Chen 2021, Lv et al. 2021). Nearly a billion people all around the world still suffer from hunger or malnourishment (Ghazani & Marangoni 2022), and the demand for food is expected to double over the coming decades because of the ballooning population (Vanhercke et al. 2019b). Lipids are a large group of macronutrients in our diets that provide energy and maintain various physiological functions in our bodies, such as energy storage, cellular signaling, and regulation of the cellular membrane (Ghazani & Marangoni 2022). Insufficient lipid intake can lead to a deficiency in essential fatty acids and fat-soluble vitamins, which is associated with a variety of human pathologies and disorders, including immune dysfunction, cardiovascular disease, vision problems, retinopathy, and inflammation (Shahidi et al. 2021, Wu et al. 2022). Plants and animals are the traditional sources of food lipids, but the current production level barely meets the growing demand of the world. Considering the increasing competition for land, water, and other resources (Godfray et al. 2010), there is a need to develop alternative dietary lipid sources to keep the human food system sustainable while maintaining safety and nutrition (Lv et al. 2021).

Novel lipid sources such as plant vegetative tissues and oleaginous microorganisms (mainly yeast, fungi, microalgae, and bacteria) hold great promise in supporting and complementing the traditional food lipid supply from plants and animals. In plants, more than 60% of the oils are derived from seeds, whereas other tissues, such as plant vegetative tissues, do not normally accumulate oils. Owing to the high biomass in plant vegetative tissues, there is a strong interest in improving oil production from them. Moreover, oleaginous microbes are excellent hosts for lipids and are able to accumulate large quantities of lipids (20–90% of dry cell weight) (Uprety et al. 2022) and assemble the desirable fatty acids into lipid molecules (Kamineni & Shaw 2020). However, the commercially desirable lipids in microbial oils, i.e., those rich in polyunsaturated fatty acids (PUFAs), are often present at low levels and generally mix with lipids of low commercial interest (Uprety et al. 2022). Therefore, different metabolic engineering strategies have been exploited in plants, microalgae, and other microorganisms to improve oil production and produce value-added oils for food purposes (**Figure 1**).

At the other extreme, the world is facing a growing epidemic of obesity and related disorders. High consumption of triacylglycerols (TAGs) with low nutritional value has been a huge trigger for these problems (Ando et al. 2017). Recent findings show that functional lipids such as diacylglycerols (DAGs), structured TAGs (STs), and structured phospholipids (SPLs) can offer better nutritional or health value. Nevertheless, these desirable components are present at low levels in natural lipids and the development of preparation methods is needed. Chemical and enzymatic methods are commonly used for their preparation, but chemical methods are less accepted by consumers when compared with enzymatic methods, along with excessive by-products. Therefore, enzymatic modification of the composition and/or position of fatty acids in lipids represents an effective approach to meeting special nutritional demands (**Figure 1**).

This manuscript reviews advances in the metabolic engineering of plants, microalgae, and microorganisms for improving food lipid production and value and highlights recent progress made in developing functional lipids using enzymatic modification approaches.

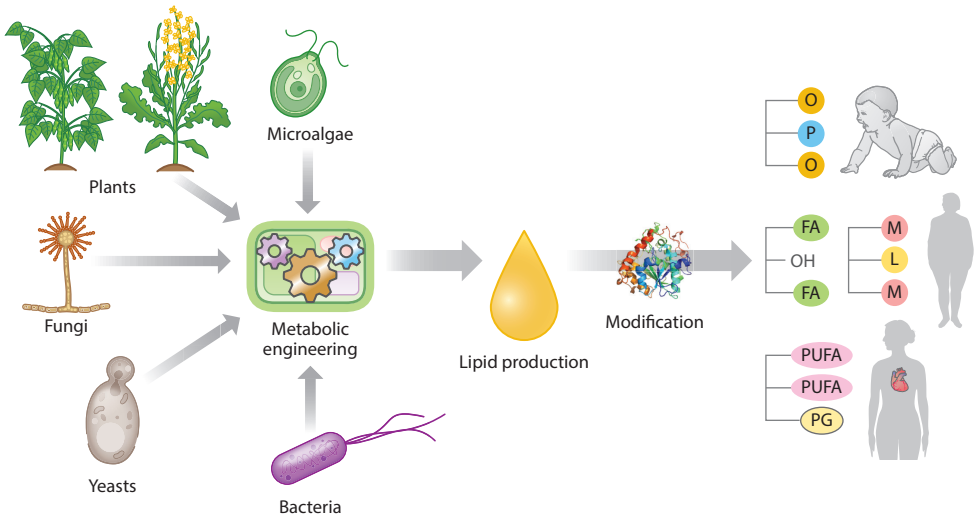

Figure 1

Biotechnology in the development of future food lipids. Metabolic engineering of oleaginous plants, microalgae, fungi, yeast, and bacteria can increase lipid production and/or value. Enzymatic modification of lipids can further produce structured lipids such as the human milk-fat substitute for infant formulas, diacylglycerols and medium–long–medium-type structured lipids to control obesity, and PUFA-rich phospholipids for the prevention of cardiovascular diseases. Abbreviations: FA, fatty acids; L, long-chain fatty acids; M, medium-chain fatty acids; O, oleic acid; P, palmitic acid; PG, phosphate group; PUFA, polyunsaturated fatty acids. Figure adapted from images created using BioRender.com and stock.adobe.com.

2. METABOLIC ENGINEERING TO INCREASE LIPID PRODUCTION AND VALUE IN PLANTS

In plant cells, TAG formation is highly compartmentalized and involves the coordinated action of multiple pathways in different subcellular compartments. In brief, TAG biosynthesis starts with de novo fatty acid biosynthesis in the plastids, which are then exported to the cytosol and incorporated into TAGs in the endoplasmic reticulum (ER). The assembled TAGs accumulate between the leaflets of the ER membrane and eventually pinch off the ER to deposit lipid droplets in the cytosol. The lipid droplets contain a TAG core surrounded by a monolayer of phospholipids (PLs) with various proteins embedded (Pyc et al. 2017). Those lipid droplet membrane-associated proteins are important in the formation and turnover of lipid droplets (Pyc et al. 2017). Given the fact that TAG formation requires multiple metabolic modules, various metabolic engineering strategies have been explored to enhance TAG production at different metabolic levels, including increasing fatty acid biosynthesis (Push), enhancing TAG assembly (Pull), packaging TAGs into lipid droplets (Package), and preventing TAG turnover (Protect). These metabolic engineering strategies have been exploited individually or in combination to manipulate oil production in oil crops, plant vegetative tissues, and microalgae (**Figure 2**).

2.1. Engineering Oil Production in Oil Crops

Many genes have been considered key targets for manipulating seed oil production by pushing carbon flux for de novo fatty acid biosynthesis, such as *Wrinkled 1* (*WRI1*) and *acetyl-CoA*

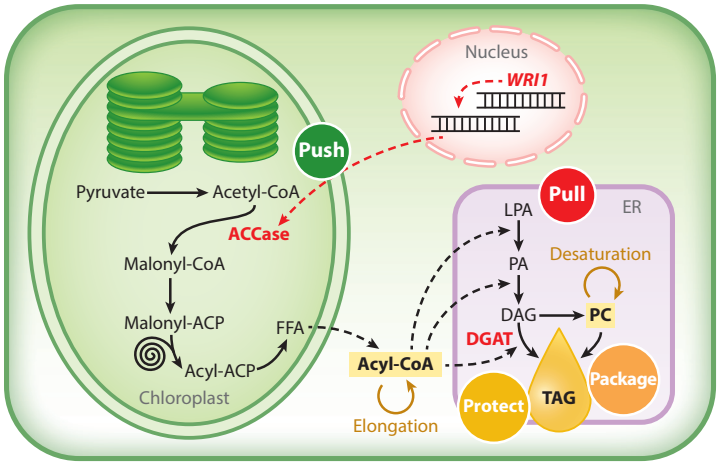

Figure 2

Metabolic engineering strategies to modify triacylglycerol (TAG) production in plants and microalgae, including increasing fatty acid biosynthesis (Push), enhancing TAG assembly (Pull), packaging TAGs into lipid droplets (Package), and preventing TAG turnover (Protect). Enzymatic reactions are shown by solid black arrows, substrate diffusion/transport is shown by dashed black arrows, transcriptional regulation is shown by dashed red arrows, and acyl modification is shown by solid brown arrows. Abbreviations: ACP, acyl carrier protein; ACCase, acetyl-CoA carboxylase; CoA, coenzyme A; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FFA, free fatty acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; *WRI1*, *Wrinkled 1*.

carboxylase (*ACCase*), and pulling fatty acids into TAGs, such as *DAG acyltransferase* (*DGAT*). WRI1 is a master transcription factor that regulates the expression of genes involved in fatty acid biosynthesis and glycolysis (Baud et al. 2009, Ruuska et al. 2002). WRI1 was first identified in the model plant *Arabidopsis thaliana*, in which disruption of *WRI1* led to an 80% reduction in seed oil content (Focks & Benning 1998). Later, WRI1 orthologs have also been identified in many crop species, which show a similar regulatory role in oil biosynthesis, including in soybean (*Glycine max*) (Chen et al. 2020), canola (*Brassica napus*) (Liu et al. 2010), and maize (*Zea mays*) (Shen et al. 2010). Overexpression of *WRI1* led to increased TAG content in seeds and vegetative tissues in various plant species (Cernac & Benning 2004, Chen et al. 2020, Q. Li et al. 2015, Liu et al. 2010, Sanjaya et al. 2011, Shen et al. 2010). For example, overexpression of maize *WRI1* increased seed oil content by up to 46% in maize without affecting seed germination, grain yield, or seedling growth (Shen et al. 2010). In soybean, soybean *WRI1* overexpressing lines showed up to ~15% increases in seed oil content and improved agronomic traits such as increased seed number per plant, leading to a 33–53% increase in total seed oil production per plant (Guo et al. 2020). These findings highlight WRI1 as a promising target for improving oil production in crops.

ACCase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is the first committed step of plastid fatty acid synthesis (Salie & Thelen 2016). In most plant plastids, ACCase is a heteromeric form consisting of four subunits: biotin carboxylase, biotin carboxyl carrier protein (BCCP), and α- and β-carboxyltransferases (CTs). A homomeric form of ACCase, whereby all four enzymatic components are in a single polypeptide, is present in the cytosol of plants for fatty acid elongation. ACCase is considered the rate-limiting step in de novo fatty acid synthesis and has been subjected to modifications with the aim of improving fatty acid biosynthesis. Enhancing ACCase activity by targeting an *Arabidopsis* homomeric ACCase to the plastids boosted the oil

content by 5% in canola (Roesler et al. 1997). In *Arabidopsis*, α-CT and β-CT subunits are physically associated, possibly forming heterotetramers (α2β2), but α-CT is threefold to tenfold less abundant than β-CT and appears to be the limiting subunit in the ACCase complex (Ke et al. 2000, M.M. Wang et al. 2022). Overexpression of pea *α-CT* resulted in the successful formation of active α/β-CT subunits and increased ACCase activity, eventually leading to an 8–15% increase in oil production in *Arabidopsis* and camelina seeds (M.M. Wang et al. 2022). Recently, three small plastid proteins of the envelope membrane, CT interactors, were found to interact with α-CT to mediate the docking of ACCase to the plastid envelope membrane, thereby attenuating fatty acid biosynthesis. CT interactor knockout lines showed a 22% increase in oil content in *Arabidopsis* leaves (Ye et al. 2020). Biotin/lipoyl attachment domain-containing (BADC) proteins are negative regulators to ACCase, which are BCCP analogs that interact with BCCP but are not biotinylated and thus compete with BCCP to bind with other ACCase subunits (Salie et al. 2016). Downregulation of *BADC*s increased the seed oil content by up to 32% (Keereetaweep et al. 2018).

DGAT catalyzes the last and committed step of acyl-CoA-dependent TAG assembly by transferring acyl-CoA to the *sn*-3 position of 1,2-DAG to yield TAG, which appears to play a critical role in determining the flux of carbon into oils (Xu et al. 2018). Overexpression of canola *DGAT1* in canola increased the seed oil content by up to ∼14% and ∼8% (a 3.5% increase in absolute oil content) in greenhouse and field conditions, respectively (Taylor et al. 2009, Weselake et al. 2008). In addition to native DGAT, high-performance DGAT variants represent promising candidates for manipulating oil production. A high-oil DGAT allele was identified in maize, which contains a phenylalanine insertion at position 469 and is responsible for the increased enzyme activity and oil content (Zheng et al. 2008). Overexpression of the high-oil *DGAT* allele increased maize seed oil content by up to 41% (Zheng et al. 2008). Directed evolution has been used to engineer the enzyme performance of canola and soybean DGAT1 (Roesler et al. 2016, Siloto et al. 2009), and overexpression of performance-enhanced soybean *DGAT1* variants increased soybean seed oil content by up 16% in field trials (Roesler et al. 2016).

Although manipulating single gene expression has significantly enhanced the seed oil content, combinational metabolic engineering by stacking genes involved in different lipid biosynthetic pathways may hold more promise. Gene stacking has proved to be more effective for improving oil production in plant vegetative tissues (see Section 2.2), but only a few reports have focused on seed oils. For example, the combined expression of *WRI1* and *DGAT1* in *Arabidopsis* led to a 16% increase in the seed oil content, which further increased by 20% when combined with the suppression of *Sugar Dependent Lipase 1* (*SDP1*), encoding a lipase for TAG turnover (van Erp et al. 2014). In some cases, however, lipid gene manipulation had only limited or no success. Co-expression of *Arabidopsis WRI1* and *DGAT1* in soybean did not increase the seed oil content; further transcriptomic and metabolomic analyses revealed lipid droplet packaging and fatty acid biosynthesis may be inhibited, whereas TAG turnover and starch/polysaccharide biosynthesis may be enhanced (Arias et al. 2022). These results demonstrate the complexity of lipid metabolism and its interplay with other competing pathways. Indeed, mounting evidence shows that WRI1, DGAT1, and ACCase are regulated at the post-translational level, and various regulators, interactors, and metabolites can affect their stability, activity, and performance in oil biosynthesis (Caldo et al. 2017, Kong et al. 2020, Ma et al. 2015, Salie et al. 2016).

2.2. Engineering Oil Production in Plant Vegetative Tissues and Microalgae

In addition to engineering oil production of existing oil crops, substantial research has been carried out to develop novel sources of oils such as plant vegetative tissues and microalgae, with the aim to fulfill the potential of oil production and secure a growing supply of vegetable oils.

Vegetative tissues such as leaves and stems make up the majority of the plant biomass and represent a novel platform for oil production (Vanhercke et al. 2019a). Tobacco plants (*Nicotiana tabacum* and *Nicotiana benthamiana*) can produce a high amount of biomass and have been commonly used in engineering oil production. Leaves typically contain no significant levels of oil (<0.5% on a dry weight basis) but metabolic engineering efforts have achieved up to 15–33% (dry weight) of oil accumulation in engineered tobacco leaves so far, highlighting the potential to outperform current oil crops (Vanhercke et al. 2014, 2017). The integrated Push–Pull–Package–Protect metabolic engineering strategy has been found to be more effective than an individual Push, Pull, Package, or Protect strategy at producing high concentrations of oils in vegetative tissues by mimicking metabolic fluxes in oilseeds, where carbon flux is efficiently channeled into TAGs for storage (Vanhercke et al. 2019a). For example, the combined overexpression of *WRI1* (Push), *DGAT1* (Pull), and the lipid droplet protein *OLEOSIN* (Package) yielded up to 15% (dry weight) of oil in engineered tobacco leaf tissues (Vanhercke et al. 2014). Furthermore, silencing *SDP1* (Protect) in this high-oil tobacco background elevated the oil accumulation by up to 33% (Vanhercke et al. 2017). These Push–Pull–Package–Protect strategies have also been used to increase oil content in the vegetative tissues of crops such as sugarcane and potato, where up to 1.9% and 3.3% (dry weight) of oil were achieved in sugarcane leaves and potato tubers, respectively (Hofvander et al. 2016, Liu et al. 2017, Zale et al. 2016). Microalgae hold promise as a sustainable resource for lipid production because of their high lipid accumulation ability and phototrophic growth rate and the fact that they do not need to compete with agricultural crops for arable land (Hu et al. 2008). But microalgal oil production faces many challenges and is still not economically viable (Sun et al. 2019). Thus, similar metabolic engineering approaches have been exploited to manipulate oil production in various microalgal species, such as *Nannochloropsis* spp. and *Pheaodactylum tricornutum* (Hamilton et al. 2014, Liu et al. 2022, Radakovits et al. 2011, Wang et al. 2021, Xu 2022, Xue et al. 2015).

2.3. Producing Value-Added Oils in Plants and Microalgae

The property and value of oils in food, nutrition, or the oleochemical industry are largely determined by the composition of fatty acids, which can be classified based on carbon chain length (e.g., long chain, >C16; short chain, <C8) and saturation degree (saturated, no double bonds; monounsaturated, 1 double bond; polyunsaturated, >2 double bonds). For example, PUFAs such as eicosapentaenoic acid (EPA; $20{:}5\Delta^{5,8,11,14,17cis}$) and docosahexaenoic acid (DHA; $22{:}6\Delta^{4,7,10,13,16,19cis}$) are well recognized for their health-promoting benefits and have been widely used in nutritional supplements (Boyd et al. 2021, Keaney & Rosen 2019); conjugated linolenic acids (18 carbon fatty acids bearing three conjugated double bonds) such as punicic acid ($18{:}3\Delta^{9cis,11trans,13cis}$) have been shown to display various beneficial bioactivities (Holic et al. 2018). Owing to growing demand and scarce supply, metabolic engineering to produce these unusual functional fatty acids in plants and microalgae has attracted substantial attention.

To date, considerable achievements have been made to produce PUFAs such as EPA and DHA in crops by introducing alternate fatty acid desaturases and elongates from the aerobic PUFA metabolic pathways (Napier et al. 2019). By assembling multiple *desaturase* and *elongase* genes, up to 24% EPA and 12% DHA have been achieved in seed oils of transgenic *Camelina sativa*, an emerging oilseed crop (Petrie et al. 2014, Ruiz-Lopez et al. 2014). In field-grown transgenic *C. sativa*, up to 16% EPA and 9% EPA plus 5–10% DHA were accumulated in seed oils, respectively (Han et al. 2020, Usher et al. 2017). The oilseed crop canola has also been engineered to produce considerable levels of EPA and DHA (Petrie et al. 2020). Recently, two different transgenic canola lines, which were developed by BASF and Cargill and Nuseed, CSIRO, and GRDC, that accumulate up to 12% EPA and/or DHA have been approved for commercial growth in

the United States (Napier et al. 2019). Similarly, EPA and DHA production in microalgae *Nannochloropsis oceanica* and *P. tricornutum* have been optimized by modulating the PUFA metabolic pathway and the TAG assembly enzyme DGAT (Hamilton et al. 2014; Haslam et al. 2020; Liu et al. 2022; Xin et al. 2017, 2019; Xu 2022).

As for conjugated fatty acids, modest success has been achieved in crops and plant vegetative tissues in terms of their production using metabolic engineering. The creation of conjugated double bonds stems from the catalytic action of fatty acid conjugases (FADXs), a divergent form of fatty acid desaturase 2 (FAD2) (Holic et al. 2018). By overexpressing a pomegranate *FADX* in an *Arabidopsis fad3fae1* mutant and canola-type *B. napus* (with *elongase* knocked out, equivalent to the *fae1* mutation in *Arabidopsis*), punicic acid accumulated up to 11.5% and 11% in the seed oils (Mietkiewska et al. 2014, Xu et al. 2020). The punicic acid content in transgenic *Arabidopsis* seeds increased up to 21% and 24.8% when *FAD2* and *FAD2* plus *DGAT2*, respectively, from pomegranate were coexpressed with *FADX* (Mietkiewska et al. 2014, Weselake & Mietkiewska 2014). Similarly, coexpression of tung tree *FADX* with *DGAT2* showed a synergistic effect and resulted in up to 12% of α-eleostearic acid (18:3 $\Delta^{9cis,11trans,13trans}$) production in *Arabidopsis* leaves (Yurchenko et al. 2017). The level of conjugated fatty acids in transgenic plants, however, has been much lower than that of plants that naturally produce them, which is likely due to the inefficient trafficking of conjugated fatty acids from membrane lipids to storage TAG (Holic et al. 2018). Therefore, further identifying unusual fatty acid selective acyltransferases and introducing them together with other important enzymes may be necessary for alleviating the metabolic bottleneck of unusual fatty acid production. Indeed, significant levels of hydroxy fatty acid were achieved in transgenic *Arabidopsis* seed oils by the coexpression of specialized acyltransferases that selectively incorporate hydroxy fatty acid at each stereochemical position of TAG (Lunn et al. 2019).

3. METABOLIC ENGINEERING TO INCREASE LIPID PRODUCTION IN MICROORGANISMS

An overview of anabolism and catabolism in eukaryotic microorganisms is shown in **Figure 3**. Maximizing flux through lipid biosynthesis can be achieved by combining general metabolic engineering strategies: (*a*) blocking pathways that compete with synthesis of key precursors or consume oleochemical products, (*b*) pushing flux into lipid biosynthesis by overexpressing rate-limiting enzymes or enzymes that produce intermediates dedicated to lipid biosynthesis, (*c*) pulling flux through lipid biosynthesis by overexpressing enzymes or regulatory proteins that bypass native regulation, and (*d*) expressing auxiliary proteins that alleviate stresses and other problems caused by producing specific oleochemicals.

3.1. Increasing the Acetyl-CoA Pool

Acetyl-CoA is the key building block of all oleochemicals and a central metabolite involved in many biochemical systems. The synthesis of acetyl-CoA is highly regulated and competes with the production of several fermentation products such as acetate, ethanol, and lactate. In *Escherichia coli*, deleting such competing pathways is crucial for directing flux via the reversed β-oxidation pathway (Cintolesi et al. 2014; Clomburg et al. 2012, 2015; Dellomonaco et al. 2011; Wu et al. 2020). However, such procedures lead to diminished cell growth rates or substrate uptake rates. For instance, disrupting three pyruvate decarboxylase isozymes eliminates ethanol production in *Saccharomyces cerevisiae* (Dai et al. 2018). Furthermore, these pyruvate decarboxylase mutants exhibit slow growth rates on glucose and accumulate mutations that lead to reduced glucose uptake. To overcome these challenges, a chimeric ATP:citrate lyase–malic enzyme–malate dehydrogenase–citrate transporter Ctp1 was overexpressed and a 20% increase in fatty acid production was observed. Deleting

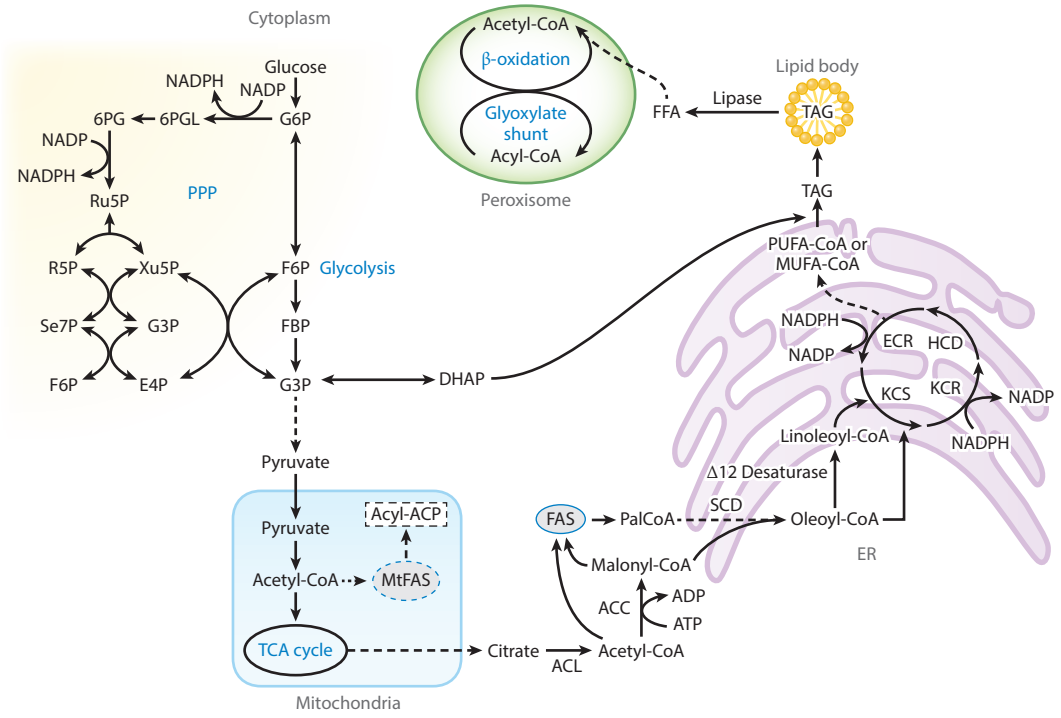

Figure 3

The native and designed synthetic pathways involved in lipid biosynthesis in eukaryotic oleaginous microorganisms. The solid arrows denote a single step reaction and the dashed arrows denote the process containing more than one biochemical reaction. Abbreviations: 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; ACP, acyl carrier protein; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; ECR, *trans*-2,3-enoyl-CoA reductase; ER, endoplasmic reticulum; F6P, fructose 6-phosphate; FAS, fatty acid synthase; FBP, fructose-bisphosphatase 1; FFA, free fatty acids; G6P, glucose 6-phosphate; G3P, glyceraldehyde 3-phosphate; HCD, 3-hydroxacyl-CoA dehydratase; KCR, 3-ketoacyl-CoA reductase; KCS, 3-ketoacyl-CoA synthase; MtFAS, mitochondria fatty acid synthase; MUFA, monounsaturated fatty acid; PalCoA, palmitoyl coenzyme A; PPP, pentose phosphate pathway; PUFA, polyunsaturated fatty acid; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; SCD, stearoyl-CoA desaturase; Se7P, sedoheptulose 7-phosphate; TAG, triacylglycerol; TCA, tricarboxylic acid cycle; Xu5P, xylulose 5-phosphate. Figure adapted with permission from Xu et al. (2016).

acyl-CoA synthases *FAA1* and *FAA4* and acyl-CoA oxidase *HFD1* and the overexpression of *Rhodosporidium toruloides FAS*, *TesA′*, and *ACC1* produced 10.4 g/L free fatty acids (FFAs) in fed-batch fermentation (Zhou et al. 2016). Another study demonstrated that combining adaptive laboratory evolution and metabolic engineering transformed a host strain into a Crabtree-negative yeast (Dai et al. 2018). Further experiments resulted in a strain capable of producing 33.4 g/L FFAs, exhibiting a fourfold improvement over the initial strain in glucose-limited and nitrogen-restricted fed-batch cultivation (Yu et al. 2018). Increasing the acetyl-CoA pool has been shown to be beneficial for increasing lipid production in both nonoleaginous and oleaginous organisms.

3.2. Increasing the Malonyl-CoA Pool

Malonyl-CoA, the elongation unit used in fatty acid biosynthesis, is produced from ACCase, a rate-limiting reaction in fatty acid biosynthesis in several organisms. The production of FFAs was increased via a balanced overexpression of ACCase subunits associated with the coexpression of a thioesterase in *E. coli* (Lennen et al. 2010). In yeast, several strategies were involved to

improve malonyl-CoA production, including (*a*) replacing the native promoter of ACC1 with a strong constitutive promoter (Qiao et al. 2015), (*b*) overexpressing a mutated ACC1 (Ser659Ala, Ser1157Ala) that abolishes post-translational phosphorylation inhibition (d'Espaux et al. 2017), (*c*) bypassing the ACC pathway through the expression of phosphoenolpyruvate carboxylase and methyl malonyl-CoA CT (Shin & Lee 2017), and (*d*) resolving an allosteric feedback inhibition due to C16–C20 saturated acyl-CoAs via the overexpression of Δ9 stearoyl-CoA desaturase in *Yarrowia lipolytica* (Qiao et al. 2015) or the overexpression of a Δ9-desaturase in *S. cerevisiae* to improve membrane fluidity and the fatty acyl-CoA pool (d'Espaux et al. 2017). Furthermore, it was found that a mutant Mga2p regulator in *Y. lipolytica* resulted in elevated unsaturated fatty acid biosynthesis and lipid accumulation, which might be due to the reduced feedback inhibition of ACC (Liu et al. 2015). Indirect malonyl-CoA biosensors have also been created in *E. coli*, *Pseudomonas putida*, and *Corynebacterium glutamicum* by employing RppA, a type III polyketide synthase that may generate flaviolin, a red-colored pigment, from malonyl-CoA. A genome-wide sRNA knockdown library was also used together with this sensor to identify high malonyl-CoA producers (Yan et al. 2018).

3.3. Leveraging β-Oxidation as a High-Yielding Synthetic Pathway

β-oxidation enzymes may be used in a synthetic direction (r-BOX) to produce medium- and long-chain oleochemicals via acyl-CoAs. The advantage of this pathway is the larger theoretical yields that can be achieved by avoiding ATP consumption required for malonyl-CoA synthesis. The r-BOX is also ideally suited for producing fatty alcohols as this biosynthetic pathway is redox balanced to glucose catabolism. This β-oxidation pathway was first demonstrated by Dellomonaco et al. (2011), and several groups have employed the r-BOX pathway to increase oleochemical biosynthesis in bacteria or yeast. One relevant study found that *E. coli* type II FAS could be employed to make up the pathway (Clomburg et al. 2018). Another study found that the relative expression of essential component enzymes was beneficial for generating medium-chain TAGs (MCTs) (Wu et al. 2017b). The same group further showed that improving NADH availability is important to increasing medium-chain fatty acid production derived from r-BOX in *E. coli* (Wu et al. 2017a). The r-BOX pathway has also been assembled in the cytosol of *S. cerevisiae* to produce butanol, MCTs, and ethyl esters (Mehrer et al. 2018). One main hurdle of assembling r-BOX in yeast is the compartmentalization of r-BOX in the peroxisome away from pyruvate dehydrogenase in the cytosol. The key enzyme in the r-BOX pathway is the reversible thiolase, which elongates the acyl-chains. Specific thiolase variants have different substrate preferences and help determine the ultimate product chain-length. The original description of the r-BOX pathway employs a short-chain acyl-CoA transferase, YqeF, resulting in the accumulation of C4 acyl-CoA and butanol (Clomburg et al. 2012), whereas *Ralstonia eutropha* BktB has been widely employed in r-BOX supporting synthesis of acyl-CoA of up to 10 carbons (Clomburg et al. 2015, Wu et al. 2020).

3.4. Safety of Oleaginous Microbes

The safety of food microorganisms has been strongly highlighted by several global organizations. The United States Pharmacopeia convention develops and publishes standards for food ingredients in the Food Chemicals Codex (Reynolds 1964). The European Food Feed Cultures Association defined food cultures as "safe live bacteria, yeasts or molds used in food production which are in themselves a characteristic food ingredient" (Bourdichon et al. 2018). The Qualified Presumption of Safety (QPS) was developed to provide a generic safety evaluation for biological agents in Europe (Koutsoumanis et al. 2021). Generally recognized as safe (GRAS) rules are used for US Food and Drug Administration regulatory approval (Singh & Gaur 2021). The Chinese

government publishes a microbe list for food usage and allows the usage of those microorganisms in traditional foods (Yao et al. 2022).

Oleaginous microbes are preferred for use in the production of food lipids, especially those in the safe lists published by governments. A couple of oleaginous microorganisms have been characterized as GRAS and QPS status, such as the oleaginous yeasts *Y. lipolytica* and *Xanthophyllomyces dendrorhous* (Koutsoumanis et al. 2021, Nicaud 2012) and the oleaginous fungi *Schizochytrium limacinum* and *Aspergillus oryzae* (Borowitzka 2013, Rousta et al. 2021). The qualification of these microbes is described in the official documents that provide guidance to not only producers but also researchers.

4. BIOCATALYTIC APPROACHES FOR LIPID MODIFICATION

Functional lipids are important food constituents for meeting desired nutritional demands. For example, overconsumption of TAGs, which are the major source of energy within the human diet, has been related to obesity and various health problems (Ando et al. 2017). Replacing TAGs with DAGs can efficiently suppress the accumulation of body fat and help reduce body weight (Prabhavathi Devi et al. 2018). Medium- and long-chain fatty acid TAGs (MLCTs) can reduce calories in lipids and reduce fat accumulation (Nagao & Yanagita 2010). Other STs, for example, *sn*-2 palmitate TAGs, are the main components in human milk-fat substitutes for infant formulas and can meet specific nutritional or functional demands (Wei et al. 2019). Incorporation of PUFAs into STs or SPLs increases their availability and metabolic absorption (Ang et al. 2019).

Lipid modification can be achieved chemically or enzymatically. Enzymatic methods provide superior chemo- and regioselectivity and are more environmentally friendly than chemical methods. Lipases (TAG hydrolases E.C. 3.1.1.3) are the most commonly used enzymes for lipid modification and can catalyze hydrolysis, esterification, and transesterification reactions of lipids. Many of them are commercially available and display regioselectivity to either the *sn*-1,3 or *sn*-2 position of TAGs and have different chain-length preferences. With new developments in protein engineering and bioinformatics, enzymatic functions can also be adjusted to meet different modification purposes.

4.1. Production of Diacylglycerols

DAGs are natural components in edible oils, with levels lower than 10% in natural lipids, and consist mainly of 1,2-DAGs and 1,3-DAGs (3:7). In an era when obesity has become an intractable problem worldwide, DAGs have attracted widespread attention due to their specific physiological functions. The different metabolic pathways of TAGs and 1,3-DAGs (the main component in DAGs) contribute to health-beneficial properties (Prabhavathi Devi et al. 2018). DAGs have, for example, been found to have obviously positive effects in regulating postprandial lipids, relieving type 2 diabetes, and suppressing the accumulation of visceral fat (Li et al. 2019). However, DAGs cannot exert their beneficial effects unless their content is above 27.3% in the edible lipids (Saito et al. 2010). Generally, higher levels of DAGs showed better beneficial effects (Saito et al. 2010); thus, it is necessary to maximize the DAGs content in edible oils by enzymatic methods.

Enzymatic production of DAGs is generally performed in three ways: (*a*) partial hydrolysis of TAGs with water to produce DAGs, monoacylglycerols (MAGs), and FFAs; (*b*) glycerolysis reaction between TAGs and glycerol yielding DAGs; and (*c*) esterification of FFAs with glycerol to form DAGs (Li et al. 2021). Moderate hydrolysis and glycerolysis are commonly used methods for DAG production. However, the obtained DAG level is less than 60% even after purification, and high levels of by-products (MAGs and FFAs) are observed (Lee et al. 2020, Li et al. 2021). Enzymatic esterification has been widely applied in the synthesis of DAGs because it is the most

direct method for DAG production. Furthermore, the usage of lipase with *sn*-1,3-regioselectivity (mainly Lipozyme RM IM and Novozym 435) at suitable ratios between the FFAs and glycerol limits the synthesis of TAGs, which is very hard to separate from DAGs (Lee et al. 2020). However, the DAG content obtained from the reactions catalyzed by these lipases is still limited to the range of 60% to 90% (Li et al. 2016). Certain levels of TAGs are nevertheless generated because of acyl migration during the esterification process. MAG and DAG lipases such as lipase SMG1 from *Malassezia globosa* showed great potential to solve this problem because of their strict specificity for MAGs and DAGs (Li et al. 2016). Wang et al. (2014) adopted SMG1 to produce high-purity DAGs (97% purity after purification). However, preparation of FFAs is a prerequisite step for esterification, which adds to the complexity and cost (Li et al. 2021). Li et al. (2021) used a combination of partial hydrolysis and esterification to solve this problem, and high-purity DAGs (99.3% after purification) were successfully obtained.

It is concluded that high-purity DAGs (generally with DAG levels higher than 80%) could be successfully prepared and commercialized. Moreover, DAGs derived from various sources have been produced to meet individual preferences and needs (Cheong et al. 2007, D.M. Li et al. 2015, Liu et al. 2018, Wang et al. 2019). The challenge encountered is whether higher-purity DAGs (>99%) can be synthesized to facilitate the exploration of their applications in the pharmaceutical field. Also, owing to the high commercial value of DAGs, fast detection methods need to be developed as a means to avoid counterfeit products in the market.

4.2. Production of Structured Triacylglycerols

STs with medium-chain and long-chain fatty acids on the glycerol backbone can be divided into, for example, MLM, MML, LML, and LLM types (M, medium-chain fatty acids; L, long-chain fatty acids). Among them, MLM is gaining more attention (Utama et al. 2019). The medium-chain fatty acids at *sn*-1,3 positions could be hydrolyzed easier in the body than the long-chain fatty acids and thus could be utilized as a quick energy source (Ding et al. 2009). Free medium-chain fatty acids have lower tendencies to be deposited in human adipose tissue (Nagao & Yanagita 2010, Vistisen et al. 2006). Meanwhile, the remaining 2-MAGs with long-chain fatty acids still provide essential fatty acids and can be absorbed well through the intestinal wall. It has been reported that MLM could be used to control obesity, fat malabsorption, and other related disorders (Utama et al. 2019). For example, CyOCy (Cy, caprylic acid; O, oleic acid) was used to treat patients with pancreatic insufficiency as well as to provide people with a rapid energy supply. However, levels of MLM are low in natural oils and fats (Utama et al. 2019); therefore, it is necessary to develop methods for preparation.

There are three commonly used methods for ST production (Utama et al. 2019) (**Figure 4*a***): (*a*) transesterification/exchange of acyl groups between two TAG molecules; (*b*) direct esterification of FFAs to the glycerol molecules; and (*c*) acidolysis between TAG molecules and FFAs. The low yield of expected STs (Y.D. Wang et al. 2022) and high cost of the substrate (FFAs) (Yang et al. 2014) limit the application of the esterification method. Transesterification between two natural plant oils could create a complex product with various TAG species, which is not quite suitable for production of STs with certain molecular structures (Y.D. Wang et al. 2022). However, the usage of two single TAG molecules and an *sn*-1,3-specific lipase might be a good choice to solve this problem. Bai et al. (2013) adopted tricaprylin and trilinolenin as substrates and Lipozyme RM IM or Novozym 435 as a catalyst. CyLnCy and CyLnLn (Ln, linolenic acid) were found to be the most dominant TAGs. Acidolysis of long-chain TAGs with medium-chain fatty acids using *sn*-1,3-specific lipases (*Rhizomucor miehei* lipase and *Thermomyces lanuginosus* lipase) is also commonly used for producing MLM (Caballero et al. 2014). Caballero et al. (2014) studied the

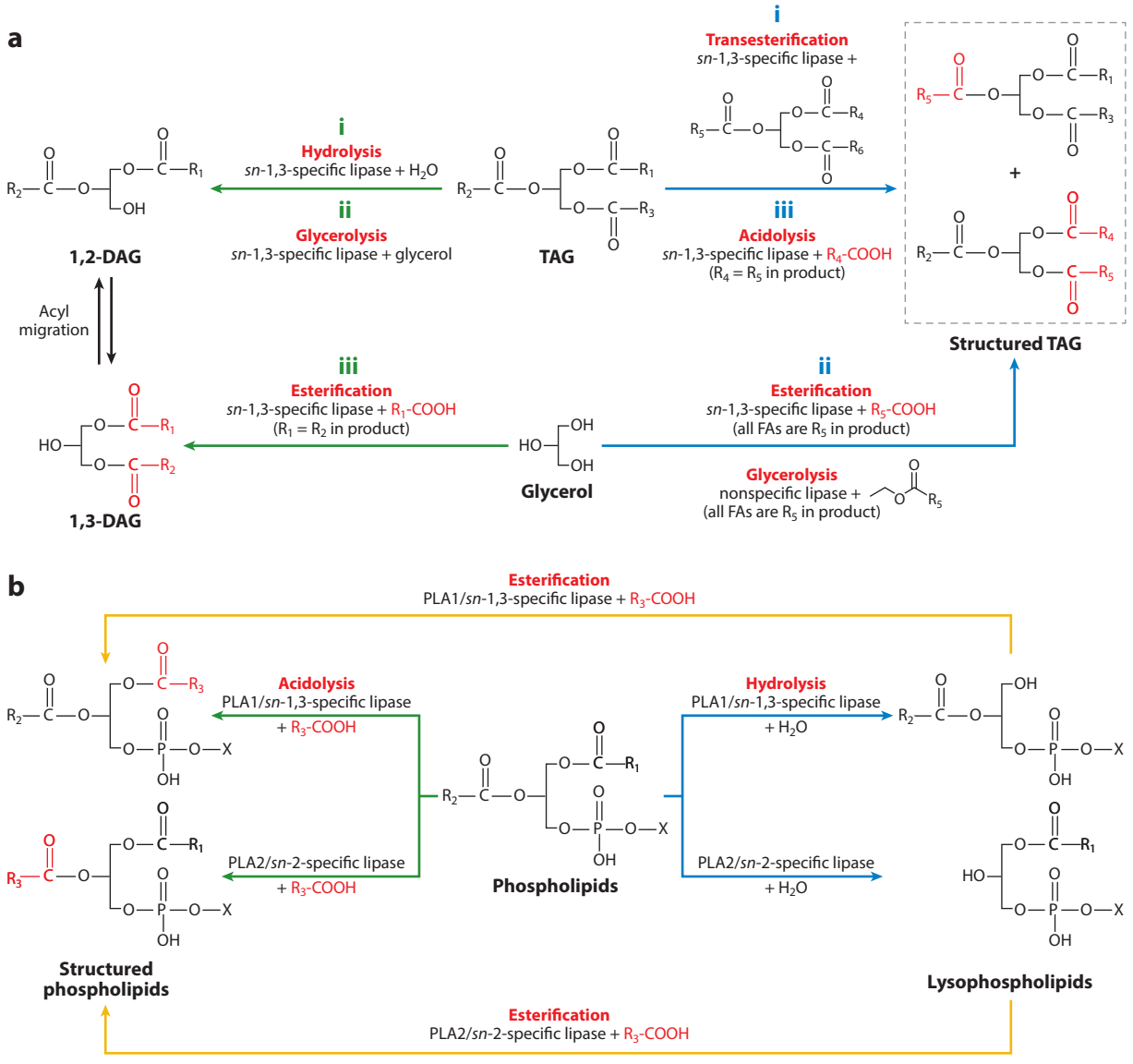

Figure 4

Strategies for lipase-catalyzed lipid modification. (*a*) Production of diacylglycerols (*green arrows*) through (i) partial hydrolysis of triacylglycerol (TAG), (ii) glycerolysis reaction between TAG and glycerol, or (iii) esterification of free fatty acids (FFAs) with glycerol, catalyzed by *sn*-1,3-specific lipases. Production of structured TAGs (*blue arrows*) through (i) transesterification between two TAG molecules, (ii) direct esterification of FFA with glycerol, or (iii) acidolysis between TAG and FFAs, catalyzed by *sn*-1,3-specific lipases. PUFA-rich TAGs can also be produced by glycerolysis between glycerol and ethyl ester, catalyzed by nonspecific lipases. Substituted or newly added fatty acids (FAs) are highlighted in red. Abbreviations: DAG, diacylglycerol; PUFA, polyunsaturated fatty acid. (*b*) Production of structured phospholipids through acidolysis (*green arrows*), esterification of lysophospholipids through esterification (*orange arrows*), and hydrolysis of structured phospholipids to produce lysophospholipids (*blue arrows*), catalyzed by phospholipase A1 (PLA1), phospholipase A2 (PLA2), *sn*-1,3-, or *sn*-2-specific lipases. Substituted FAs are highlighted in red. X = −H, phosphatidic acid (PA); X = $-CH_2CH_2NH_2$, phosphatidylethanolamine; X = $-CH_2CH_2N{+}(CH_3)_3$, phosphatidylcholine; X = $-CH_2CH(OH)CH_2OH$, phosphatidylglycerol; X = $-CH_2CH(NH_2)COOH$, phosphatidylserine.

acidolysis between caprylic acid and avocado oil and found that 29.2% of caprylic acid could be incorporated at the *sn*-1,3 positions after the optimization. To date, it is still challenging to obtain high-purity MLM because of the difficulty of separating the different TAG species. More importantly, the current detection methods are not yet able to easily differentiate the location of fatty acids, which means it is difficult to distinguish between isomers.

Human milk is the best source of energy and nutrition for all healthy infants (Wei et al. 2019). However, breastfeeding is not always possible, and infant formulas are widely considered a good alternative to human milk. Fats are crucial components in human milk and provide energy, essential fatty acids, fat-soluble vitamins, and hormones to improve the growth of infants (Wei et al. 2019). Vegetable oils and milk fat are normally added to infant formula to stimulate the fatty acid composition of human milk fat. However, TAG profiles of human milk fat, plant oils, and bovine milk fat differ substantially, which might influence the health status of infants. Thus, it is necessary to develop a human milk-fat substitute that has similar fatty acid composition and TAG profiles to human milk fat.

The distinguishing feature of human milk fat is the high level of palmitic acid at the *sn*-2 position in TAG molecules (more than 70%) (Wei et al. 2019). Moreover, OPO and OPL (O, oleic acid; P, palmitic acid; L, linoleic acid) are the major TAGs in human milk fat, accounting for 20–40% of total TAGs (Wei et al. 2019). It is thus clear that methods for *sn*-2 palmitate TAGs production must be developed. There are two schemes to prepare the *sn*-2 palmitate TAGs (**Figure 4*a***): (*a*) one-step reactions between TAGs and another TAG/fatty acid ester/FFA and (*b*) two-step reactions that combine hydrolysis and esterification. Lipases with *sn*-1,3 regiospecificity (from, e.g., *R. miehei*, *Candida lipolytica*, *Candida antarctica*, *Tidestromia lanuginosa*) are important catalysts for modifying the fatty acids at the *sn*-1,3 position in both schemes (Wei et al. 2019). *sn*-2 Palmitate TAGs (65% of palmitic acid at *sn*-2 position) are produced from tripalmitin with oleic acid catalyzed by lipase (*R. miehei*) as the main lipid ingredient for the infant milk formula Betapol®. High-purity (96% of palmitic acid at the *sn*-2 position) *sn*-2 palmitate TAGs can be successfully produced using the two-step route (Ganske & Bornscheuer 2005). For now, it is not a problem to imitate the large number of TAGs in human milk-fat substitutes. There is also a certain level of MLCTs (8–10%, mainly in the form of MLL) and branched-chain fatty acids (Dingess et al. 2017, Wei et al. 2019); however, their importance has yet to be elucidated or examined. This makes it impossible to add these ingredients to current human milk-fat substitutes and simulate breast milk well; thus, it is important to further investigate the importance of the various components in breast milk for the better development of human milk-fat substitutes.

PUFA-rich TAGs are another vital ST for providing important fatty acids to humans. The human body has a limited ability to synthesize PUFAs, but these fatty acids show great benefits with respect to chronic inflammatory diseases, cancer, and sudden death. Thus, PUFAs must be obtained from the diet, for example, via consumption of fish oils. In natural fish oils, PUFAs show low purity, and further processing is necessary. At present, PUFA-rich ethyl esters are the main PUFA source on the market. However, the metabolism of ethyl esters could lead to low bioavailability and the production of ethanol, which tends to cause intoxication in humans with poor alcohol tolerance (Wang et al. 2017). In recent decades, EPA and DHA in the TAG form attracted interest because of their higher stability than that found in FFA forms and higher bioavailability than that found in ethyl ester forms. As a result, a great deal of attention has been given to the synthesis of PUFA-rich TAGs. Current breakthroughs in the preparation of PUFA-rich TAGs are focused on two methods (**Figure 4*a***): (*a*) esterification of free PUFAs to glycerol and (*b*) glycerolysis between glycerol and ethyl esters. Wang et al. (2017) applied MAS1 from marine *Streptomyces* sp. strain W007 in the esterification of PUFAs and glycerol to produce PUFA-rich TAGs, and a good catalytic reaction effect was found: 92% of PUFAs could be incorporated into TAGs. However, the

free PUFAs are easily oxidized, which limits the application of the esterification method (Wang et al. 2016). Besides, cheap and easily available PUFA-rich ethyl esters are more suitable for the industrial production of PUFA-rich TAGs. Wang et al. (2016) obtained high-purity PUFA-rich TAGs (96.2%) after purification when using PUFA-rich ethyl esters and glycerol as substrates and immobilized MAS1 as a catalyst. It is noted that lipases with no regiospecificity are more suitable catalysts for the glycerolysis of PUFA-rich ethyl esters (Wang et al. 2016).

4.3. Production of Structured Phospholipids

PLs are a class of lipid compounds containing phosphate groups, which are important components of lipids and exhibit unique biological activities (Sun et al. 2018, Zhang et al. 2020). The amphiphilic property of PLs facilitates the bioavailability of fatty acids (Ulven & Holven 2015). It has been confirmed that the ingestion of lipids in the PL form exerts stronger biological effects compared with the TAG form (Ang et al. 2019). Furthermore, PLs with specific fatty acids may change the fatty acid composition of membrane PL in a certain cell type, which might be useful in targeting specific diseases and metabolic conditions (Ang et al. 2019, Kim & Akoh 2015). However, specialized PLs rarely exist in natural lipids and are difficult to obtain via fractionation and extraction. Thus, the synthesis of SPLs was triggered and researched.

SPLs refer to the modification of the structure and position of natural PLs through specific modification technologies (Castejón & Señoráns 2020). There are two general methods used in the modification of PLs (**Figure 4*b***): (*a*) one-step acidolysis (direct transesterification) between PLs and FFAs and (*b*) a two-step method in combination of hydrolysis and esterification. One-step acidolysis is the direct method for SPL production, but the purity cannot be guaranteed (Adlercreutz et al. 2002). Vikbjerg et al. (2007) adopted an acidolysis reaction for preparing SPLs with caprylic acid and soybean phosphatidylcholine, and the highest incorporation rate of caprylic acid was 36%. The two-step method has the advantage of synthesizing the highly purified SPLs but is more laborious (Vikbjerg et al. 2007) because of the need to separate FFAs in the first step. Currently, the most widely used enzymes [phospholipase A1 (PLA1), phospholipase A2 (PLA2), and lipase] in PL modification vary in their regiospecificity. Both *sn*-1,3-specific lipases (Novozym 435 and Lipozyme TL IM) and PLA1 cleave the *sn*-1 position, whereas *sn*-2-specific lipases (immobilized *C. antarctica* lipase A) and PLA2 act on fatty acids at the *sn*-2 position of PLs (He et al. 2017). The position distribution of fatty acids exerts a great influence on the metabolism and biological effects of SPLs (Hu et al. 2017). For example, higher adsorption and oxidation stability could be obtained when PUFAs are esterified at the *sn-2* position (Ang et al. 2019). In the future, the design of new SPLs, the analysis of SPL metabolic pathways, and nutritional mechanisms combined with lipidomics technology are expected to advance this field further.

4.4. Enzyme Engineering and Bioinformatics for Developing Catalysts in Lipid Modification

Although many lipases display outstanding catalytic abilities in lipid modification, there are still high demands to obtain lipases with better performance regarding their regioselectivity, fatty acid chain-length selectivity, fatty acid specificity, and higher stability to heat, extreme pH, organic solvents, and processing in industrial applications. Development of modern biotechnology enables engineering of existing lipases and the discovery of novel lipases to obtain desired enzyme functions. Rational design and directed evolution are two main strategies for protein engineering (Kourist et al. 2010). Until now, thousands of 3D lipase structures have been resolved, and their reaction mechanisms, catalytic residues, and enzyme-substrate interactions have been

widely studied (Casas-Godoy et al. 2018, Kazlauskas 1994, Schmid & Verger 1998). With this information, key residues can be predicted and mutated to the desired amino acids, depending on different purposes (Soni 2022, Zorn et al. 2016). Directed evolution is an efficient method to create mutant libraries (i.e., with more than 10^4–10^7 variants) that provide a much bigger space for mutagenesis and exploration of enzyme functions (Arnold 2018). It can be a completely random mutagenesis at any residue of the enzyme or limited to certain key residues that are mutated to all 19 other amino acids. Owing to the large quantity of mutants, high-throughput screening methods or selection systems are required, such as colorimetric assays, to detect the hydrolysis products of the product p-nitrophenyl (pNP) from hydrolysis of pNP esters on microtiter plates and the selection of colonies containing active lipases in TAG-agar plates. With the assistance of computational predictive algorithms and machine learning, prediction of protein mutagenesis for desired characteristics has become more precise (Mazurenko et al. 2020, Yang et al. 2019). On the other hand, unlike traditional extraction of lipases from plant/animal tissues or microorganisms, metagenomic approaches enable the discovery and exploration of novel lipases from different resources (Almeida et al. 2020, Shahraki et al. 2022, Verma et al. 2021).

5. CONCLUSIONS AND PERSPECTIVES

Food lipids are essential components in our daily diets, and some lipids (such as EPA and DHA-enriched lipids) have important health-promoting roles and have long been used as nutritional supplements. There is an urgent need to improve oil production to meet the social demand for food and energy. In the past decades, substantial efforts have been devoted to engineering oil production (enhancing oil content and producing value-added oils such as PUFAs) in traditional oil crops and newly developed lipid sources (e.g., plant vegetative tissues, microalgae, and other oleaginous microorganisms), highlighting the possibility of customizing value-added lipids in the future. Although various metabolic engineering strategies have been exploited, only moderate levels of success have been achieved so far, which is largely because of the complexity of lipid metabolism in plants and microorganisms. Therefore, further exploration of the underlying mechanisms of lipid biosynthesis, transport, and storage and their regulation is necessary.

The emergence of functional lipids may help address a substantial problem in the world, namely obesity and related disorders caused by excessive consumption of lipids with low nutritional value. Also, some diseases are caused by underconsumption of certain beneficial lipids, such as how a PUFA deficiency may affect the development of the brain and is detrimental to the prevention of cardiovascular diseases. In recent years, a lot of research has been done on the synthesis of functional lipids, mainly focusing on structural lipids (DAGs, human milk-fat substitutes, MLM, PUFA-rich TAGs, and SPLs). In turn, many functional lipids have been commercialized. However, there is still a problem of insufficient purity, and further research is needed.

Precise nutrition for different groups of the population is considered to be a promising future direction for functional lipid development, requiring customization of the right lipids according to the health status of individuals. Based on the above prospects, there are two main challenges that need to be addressed. First, it is still difficult to figure out the type of fats and oils needed by specific individuals, as current functional lipids can only meet a single need, and it is difficult to meet all the needs of individuals simultaneously. Second, the purity of the functional lipids is still difficult to guarantee. To address the above challenges, efforts need to be made to precisely describe what kind of lipids individuals need. Furthermore, upgrading metabolic engineering strategies and enzyme engineering and separation technology is needed to rationally design structured lipid production in plants and microorganisms and to achieve the preparation and acquisition of high-purity single-structured lipids at an industrial scale.

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Errata

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