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Research review paper

Using oils and fats to replace sugars as feedstocks for biomanufacturing: Challenges and opportunities for the yeast *Yarrowia lipolytica*

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ABSTRACT

More than 200 million tons of plant oils and animal fats are produced annually worldwide from oil, crops, and the rendered animal fat industry. Triacylglycerol, an abundant energy-dense compound, is the major form of lipid in oils and fats. While oils or fats are very important raw materials and functional ingredients for food or related products, a significant portion is currently diverted to or recovered as waste. To significantly increase the value of waste oils or fats and expand their applications with a minimal environmental footprint, microbial biomanufacturing is presented as an effective strategy for adding value. Though both bacteria and yeast can be engineered to use oils or fats as the biomanufacturing feedstocks, the yeast *Yarrowia lipolytica* is presented as one of the most attractive platforms. *Y. lipolytica* is oleaginous, generally regarded as safe, demonstrated as a promising industrial producer, and has unique capabilities for efficient catabolism and bioconversion of lipid substrates. This review summarizes the major challenges and opportunities for *Y. lipolytica* as a new biomanufacturing platform for the production of value-added products from oils and fats. This review also discusses relevant cellular and metabolic engineering strategies such as fatty acid transport, fatty acid catabolism and bioconversion, redox balances and energy yield, cell morphology and stress response, and bioreaction engineering. Finally, this review highlights specific product classes including long-chain diacids, wax esters, terpenes, and carotenoids with unique synthesis opportunities from oils and fats in *Y. lipolytica*.

1. Introduction

Most biomanufacturing processes use sugar-based feedstocks as the main carbon and energy source to produce value-added compounds. The most predominate feedstocks are C6 sugars such as glucose or C5 sugars such as xylose, derived from agriculture-based starch or cellulosic biomass. However, some alternative and more sustainable feedstocks are beginning to be considered for biomanufacturing due to reduced costs and the potential for improved bioconversion or yield. Among possible choices, hydrophobic substrates such as plant oils or animal fats are often overlooked as a biomanufacturing substrate even though they are relatively prevalent. For example, the global production of vegetable oils was estimated at 209 million tons in 2020/2021, greater than that of sugars, about 186 million tons (Cabrera et al., 2022; Tridge, 2021). From a *de novo* or *ex novo* metabolic engineering pathway perspective, there are additional advantages. When producing lipid-derived products such as diacids, wax esters, or terpenes, fewer enzymatic steps are necessary, and the synthesis may also require less NADPH (nicotinamide adenine dinucleotide phosphate) and ATP (adenosine triphosphate), relative to glucose.

Currently, most vegetable oils are used in the production of food, while the remainder is diverted to biofuel production (Balat, 2011). Both in the manufacturing process and after use, many of these oils become waste, estimated as high as 15%-20% (Cabrera et al., 2022). Recent advances in synthetic biology and metabolic engineering have accelerated the development of microbial cell factories for biomanufacturing with various feedstocks (Dourou et al., 2018; Sitepu et al., 2014). Due to a fast growth rate and ability to efficiently metabolize and assimilate hydrophobic substrates (*e.g.* alkanes, fatty acids, and glycerides) as a sole carbon source (Fickers et al., 2005a), the oleaginous yeast *Yarrowia lipolytica* is considered as one of the most promising platform organisms for bioproduction from oils or fats (Soong et al., 2021; Soong et al.,

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2019). Previously considered a source for single cell oil (SCO) with applications in supplements or animal feeds, *Y. lipolytica* now shows promise to manufacture higher-value lipid derived products (Beopoulos et al., 2009). Recent progress in developing efficient genetic engineering tools and fermentation technology for the yeast make it an ideal host for large-scale biomanufacturing process (Abdel-Mawgoud et al., 2018; Ledesma-Amaro and Nicaud, 2016).

With the concept of sustainability occupying an important position on the global agenda, the use of abundant waste plant oils or animal fats as feedstocks for biomanufacturing may support a circular bioeconomy (Lad et al., 2021; Liepins et al., 2021). Many oils and fats are wasted during their intended use in food preparation and other industries, which further causes concerns of pollution and environmental release (Lopes et al., 2020). While the production cost of raw sugar and cooking oils are approximately equal per pound ($0.30 and $0.45 USD, respectively), waste feedstocks are potentially much cheaper, as many restaurants currently pay companies to handle their removal and disposal (Ates and Bukowski, 2022; Abadam, 2023). Waste cooking oil (WCO), produced after cooking, is the most common form of waste lipids. WCO is mainly composed of triglycerides (TAGs); as a result of repeated high heating during the frying process, TAGs are partially hydrolyzed into free fatty acids (FFAs) and glycerol (Win and Trabold, 2018). The pristine sources of cooking oil which then become waste are often made up of plant oils, such as palm, soybean, canola, olive, sunflower, corn, coconut, and grapeseed. Popular animal based oils include animal fat, bacon grease, butter/ghee, and fish oil. The EPA has estimated that approximately 14 pounds of WCO are generated per person every year in the United States (Lad et al., 2021) and the global annual creation of WCO is estimated at up to 29 million tons (Lisboa et al., 2014; Maddikeri et al., 2015).

In this review, we first address why the oleaginous yeast *Y. lipolytica* should be considered as one of the most promising organisms for biomanufacturing from oil or fat feedstocks. Next, we summarize the overall biochemistry, molecular biology, and bioprocess engineering considerations for utilizing hydrophobic carbon sources. In particular, we highlight major challenges and opportunities for *Y. lipolytica* as a new platform host to produce value-added products from oils and fats, which includes fatty acid transport, catabolism and bioconversion pathways, redox balances and energy yield, example lipid-based products, considerations regarding cell morphology, stress, substrate uptake and product formation, and bioreactor and bioprocess engineering with hydrophobic substrates. Finally, we cover several biomanufacturing product examples that are made entirely or partially from oil substrates and compare production capabilities with more traditional, hydrophilic substrates.

2. *Yarrowia lipolytica* as the platform organism for biomanufacturing with feedstocks of oils and fats

Recently, oleaginous yeasts have been demonstrated in literature to produce oleochemicals, biofuels and acetyl-CoA- derived metabolites. To use oils or fats as alternative carbon source for biomanufacturing, the microbial host must have a capability to metabolize hydrophobic substrates and tolerate potential inhibitors (*e.g.*, salts, alkanes, and toxic fatty acids) (Lad et al., 2021). Many species of oleaginous yeasts within the genera *Candida*, *Cryptococcus*, *Cutaneotrichosporon*, *Lipomyces*, *Rhizopus*, *Rhodotorula*, and *Yarrowia* meet these criteria, and are currently being engineered to increase utilization (Yaguchi et al., 2018). Among all oleaginous species, *Y. lipolytica* is one of the most attractive hosts since it is a "generally regarded as safe" (GRAS) organism and has a high flux of essential metabolites such as cytosolic acetyl-CoA, which can direct metabolic flux toward to energy generation and many biosynthesis pathways (Liu et al., 2021a; Soong et al., 2019).

Additionally, *Y. lipolytica* possesses biomanufacturing potential. *Y. lipolytica* has already been demonstrated as a chemical producer at industrial scale, although primarily from sugar substrates and other non-oil substrates such as glycerol. As example, the chemical company DuPont has constructed an eicosapentaenoic acid (EPA) producing strain (Zhu and Jackson, 2015). Additionally, many large chemical companies possess patents for production of various terpenes (also from sugar substrates) in *Y. lipolytica* (Miller and Alper, 2019). Other companies have demonstrated industrial recombinant protein production in this host (Madzak, 2021). As feedstocks contribute to a substantial portion of the total cost in biomanufacturing, cheaper alternative feedstock sources should be considered (Green, 2011). Alternative substrates such as oils (especially waste oils) can have lower cost and higher theoretical yield for certain products. Compared to traditional industrial biomanufacturing yeast *Saccharomyces cerevisiae*, *Y. lipolytica* offers improved metabolism of oils and fats as well as higher or comparable titers of a wide variety of substrates (Kim et al., 2020). Fatty acyl-CoA, especially acetyl-CoA, is the crucial precursor for numerous value-added lipid-derived products. As lipid catabolism is different from glucose, the amount of intracellular fatty acyl-CoA may be increased via either a *de novo* lipid synthesis or an *ex novo* lipid degradation pathway (Fig. 1). *Y. lipolytica* is a noteworthy bioproduction yeast for the conversion of hydrophobic substrates into acetyl-CoA derived products, as it possesses a high fatty acyl-CoA flux and an exceptional ability to accumulate lipids and metabolize hydrophobic substrates.

Another benefit of biomanufacturing in this host is that the complete genome sequence of *Y. lipolytica* is readily available and relatively well annotated, allowing for diverse molecular biology tools and various metabolic engineering strategies for optimal production (Gálvez-López et al., 2019; Larroude et al., 2019; Markham and Alper, 2018). While not as well understood as *S. cerevisiae,* complex enzymatic and metabolic pathways in *Y. lipolytica* related to oil and fat metabolism are becoming more elucidated with experimental evidence as well as computational modeling and predictions. Various genome scale models are also being developed, which can be combined with flux balance analysis and data-driven approaches to predict strategies to obtain optimal production levels (Czajka et al., 2021; Xu et al., 2020). This knowledge supports the consideration of *Y. lipolytica* as one of the most powerful workhorses for biomanufacturing value-added products from economical hydrophobic feedstocks such as oils and fats.

3. Uptake and transport of oils and fatty acids in *Y. lipolytica*

The first step of hydrophobic substrate metabolism is its import into the cell (Fig. 2). While hydrophobic molecule uptake in yeasts was originally thought to occur passively via diffusion across the cellular membrane, it is now known to be active and mediated by proteins (Claus et al., 2019). Extracellular alkane import into the cell is known to occur through size-dependent ATP transporters, and once inside, alkanes are oxidized into fatty alcohols before their catabolism (Thevenieau et al., 2007; Watanabe et al., 2022). Although the mechanism and related proteins involved in extracellular fatty acid import are not fully understood, it is likely that multiple proteins are involved in this process and they exhibit substrate and length preferences, as is the case in *S. cerevisiae* (Dulermo et al., 2014). Recent literature suggests that YlUP1p-UP4p (proposed new name by the authors: YlEfbp1-4) may be involved in short and medium chain fatty acid import in *Y. lipolytica* (Onésime et al., 2022). Once inside the cell, subcellular localization/transport is not completely understood, however; the many known enzymes described herein may serve multiple functions in activation, transport, storage, and/or metabolism.

3.1. Secreted lipases and biosurfactants to help with uptake and utilization of oil substrates

Extracellular oils are usually stored in the form of triglycerides, which cannot passively cross cell membranes and must be degraded into FFAs and glycerol before cellular uptake (Fig. 2). This process is assisted by natively secreted extracellular lipases and surfactants, which are

natively produced with both hydrophobic and hydrophilic carbon sources (Bankar et al., 2009). The surface of the cell membrane of *Y. lipolytica* is hydrophobic, and secreted surfactants and emulsifiers such as liposan facilitate the attachment of lipid droplets to the cell surface (Thevenieau et al., 2010). With lipase catalyzing the degradation of TAGs into FFAs and the presence of biosurfactants to promote oil droplet availability, the mixing and transport process of extracellular oil substrates in the aqueous medium can be significantly enhanced (Gonçalves et al., 2014). The addition of exogenous surfactants has also been shown to increase hydrophobic substrate uptake. For example, Arabic gum has been used to increase waste cooking oil uptake in *Y. lipolytica* (Lopes et al., 2019). Another group has shown that a biosurfactant produced from a *Bacillus ceres* culture increased *Y. lipolytica* growth and substrate assimilation on palm oil mill effluent (POME) (Louhasakul et al., 2020). The authors hypothesized that surfactant addition may increase membrane fluidity, in turn releasing intracellular lipases that may assist with lipid metabolism. However, it should be noted that not all surfactants are shown to be helpful in oil uptake, and the effects are often specific to the culture conditions (Lopes et al., 2020).

Y. lipolytica has twenty known genes in the *LIP* family, *LIP1-LIP20* (Table 1; extracellular, membrane-bound, and intracellular). *Y. lipolytica* also possesses five known genes in the *TGL* family, *TGL1-4* and *TGL32*. The lipases encoded by the *LIP* and *TGL* gene families are most known for their ability to hydrolyze TAG into free fatty acids and glycerol (EC 3.1.1.3). However, the *LIP1, LIP3, LIP6* and *LIP20* genes instead encode intercellular carboxylesterases (EC 3.1.1.1). The majority of the other *LIP* genes are likely extracellular, with the exception of *LIP7* and *LIP8*, which have been shown to encode membrane-bound lipases, although they are secreted during the stationary phase (Table 1) (Fickers et al., 2003). Conversely, the five genes in the *TGL* family are intercellular, and their protein product is involved in the metabolism of lipid bodies within the cell. The specific amino acid residues which compose a catalytic triad of Ser, His, and Asp have been determined for many lipases in the *LIP* family (Syal and Gupta, 2017). Lipase activity varies widely from author to author and is clearly dependent on media composition and environmental conditions (Fickers et al., 2003; Guerzoni et al., 2001). RNA-seq supported transcriptome analysis has shown that many, but not all, *Y. lipolytica* lipases are induced in the presence of hydrophobic

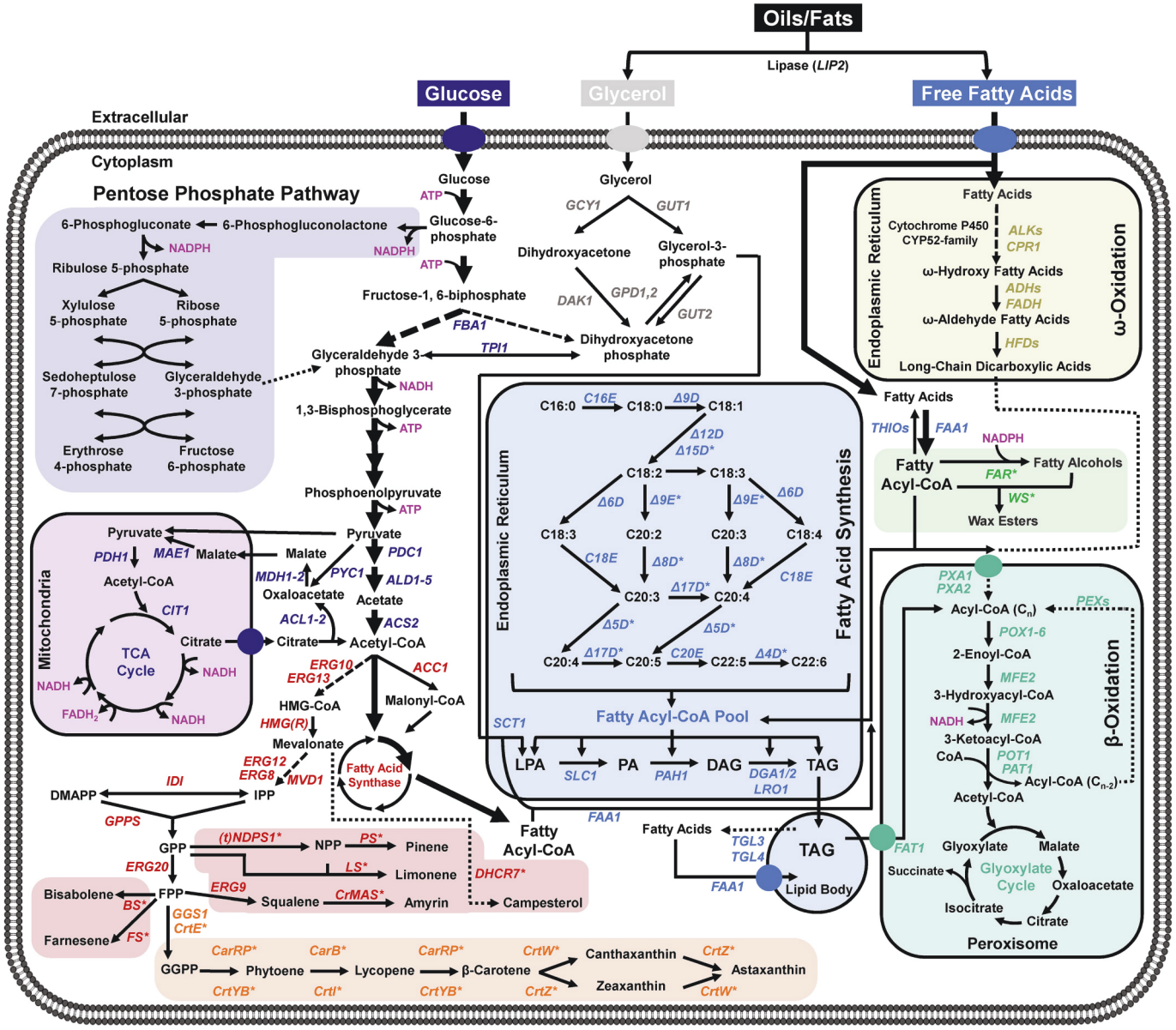

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Fig. 1. Overview of metabolic engineering of *Yarrowia lipolytica* to produce high-value products from oils and fats. Bolded arrows represent contrasting length metabolic pathways to synthesize fatty acyl-CoA from glucose and lipids. Shaded circles/ovals represent transport across a membrane or lipid body, which is represented by a black outline. The outer cell membrane is represented by a lipid bilayer. Genes non-native to *Y. lipolytica* are denoted by an asterisk (*). Pink box: relevant mitochondrial metabolism; Purple shaded area: Native pentose phosphate pathway; Blue box: unsaturated fatty acid synthesis in the endoplasmic reticulum (ER); Blue circle: fatty acid storage and mobilization in lipid bodies; Teal box: relevant metabolic pathways in the peroxisome; Green shaded area: Metabolic engineering for wax ester and fatty alcohol synthesis; Yellow box: Metabolic engineering for ω-fatty acid and long chain dicarboxylic acid biosynthesis in the ER; Orange box: metabolic engineering for the production of carotenoids, Red box: metabolic engineering for the production of terpenes; Grey area, glycerol metabolism. Pink abbreviations: ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; $FADH_2$, flavin adenine dinucleotide. Purple abbreviations: *PDH1*, pyruvate dehydrogenase; *CIT1*, citrate synthase; *MAE1*, malic enzyme; *PDC1*, pyruvate decarboxylase; *ALD1-5*, aldehyde dehydrogenase 1 through 5; *ACS2*, acetyl-CoA synthetase; *ACL1-2*, ATP citrate lyase 1 and 2; *MDH1-2*, cytosolic malate dehydrogenase 1 and 2; *PYC1*, pyruvate carboxylase; *FBA1*, fructose bisphosphate aldolase; *TPI1*, triosephosphate isomerase. Blue abbreviations: *C16E, C18E, C20E* and *Δ9E* are fatty acid elongases increasing the chain length by 2 carbons; *Δ4D, Δ5D, Δ8D, Δ9D, Δ12D, Δ15D* and *Δ17D* are fatty acid desaturases at carbon n; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; *SCT1*, glycerol-3-phosphate o-acyltransferase; *SLC1*, LPA acyltransferase; *PAH1*; phosphatidate phosphatase; *DAG1/2*, DAG acyltransferase; *LRO1*, phospholipid: DAG acyltransferase; *TGL4*, intracellular lipase; *TGL3*, a positive regulator of *TGL4*; *FAA1*, cytoplasmic fatty acyl-CoA synthetase; *THIOs*, Acyl-CoA thioesterases. Teal abbreviations: *PXA1* and *PXA2*, peroxisomal acyl CoA transporter 1 and 2, respectively; *POX1* to *POX6*, acyl-CoA oxidases 1–6, respectively; *MFE2*, peroxisomal multifunctional enzyme 2; *POT1*, 3-ketoacyl-CoA thiolase; *PAT1*, acetoacetyl-CoA thiolase; *PEXs*, peroxisome biogenesis factors; *FAT1*, fatty acid transport protein. Green abbreviations: *FAR*, fatty acyl CoA reductase; *WS*, wax ester synthase. Yellow abbreviations: *ALKs*, Cytochrome P450 enzymes; *CPR1,* NADPH cytochrome P450 reductase; *ADHs,* alcohol dehydrogenases; *FADH,* fatty alcohol dehydrogenase, *HFDs*, fatty aldehyde dehydrogenases. Orange abbreviations: *GGS1* and *CrtE*, endogenous and exogenous geranylgeranyl pyrophosphate synthases, respectively; GGPP, geranylgeranyl pyrophosphate; *CarRP* and *CrtYB*, bifunctional phytoene synthases/lycopene cyclases; *CarB* and *CrtI*, phytoene desaturases; *CrtW*, β-carotene ketolase; *CrtZ*, β-carotene hydroxylase. Red abbreviations: *ERG10*, acetoacetyl-CoA thiolase; *ERG13*, hydroxymethylglutaryl-CoA synthase; *ACC1*, acetyl-CoA carboxylase; *HMG(R)*, 3-hydroxy-3-methylglutaryl-CoA reductase (often truncated, also called *HMG1*); *ERG12*, mevalonate kinase; *ERG8*, phosphomevalonate kinase; *MVD1* (also called *ERG19*), mevalonate pyrophosphate decarboxylase; *IDI*, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; *ERG20*, geranyl/farnesyl diphosphate synthase; *BS*, bisabolene synthase; *FS*, farnesene synthase; NPP, neryl disphosphate; *(t)NDPS1*, (truncated) neryl disphosphate synthase 1; *PS*, pinene synthase; *LS*, limonene synthase; *ERG9*, squalene synthase (also called *SQS1*); *CrMAS*, *Catharanthus roseus* amyrin synthase; *DHCR7*, 7-dehydrocholesterol reductase. Grey abbreviations: *GCY1*, glycerol dehydrogenase; *GUT1* and *GUT2*, glycerol kinase 1 and 2; *DAK1*, dihydroxyacetone kinase; *GPD1* and *GPD2*, glycerol-3-phosphate dehydrogenase 1 and 2. Extracellular abbreviation: *LIP2*, extracellular lipase.

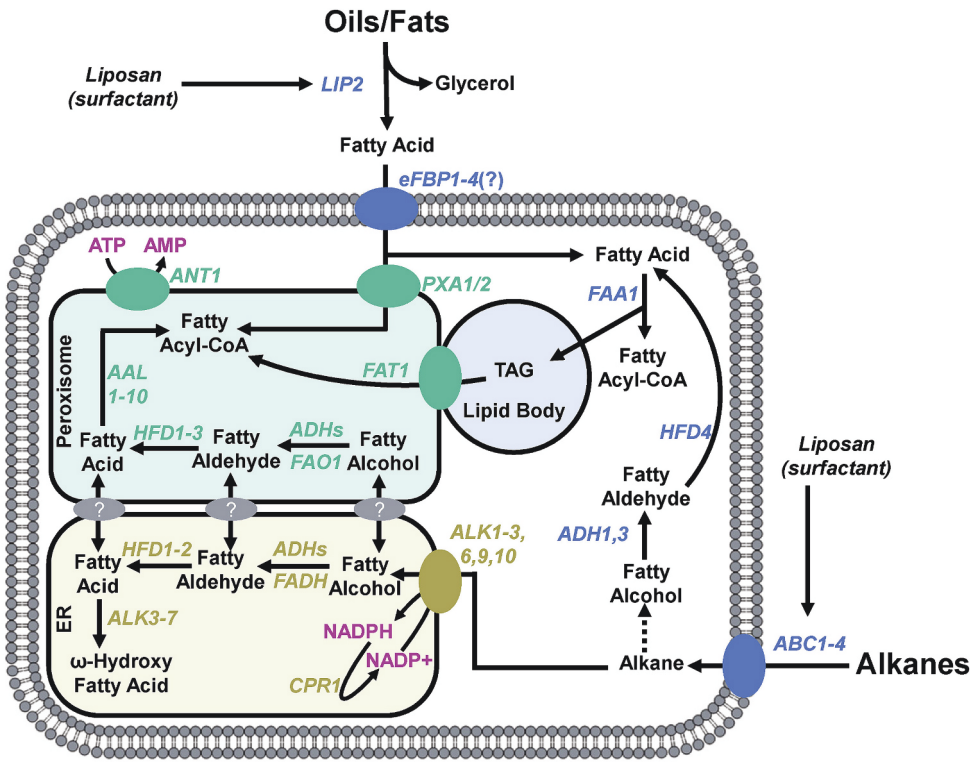

Fig. 2. Oils, fats and alkanes uptake and assimilation by *Yarrowia lipolytica*. The size of oil droplets is reduced by secreted biosurfactants such as liposan and extracellular lipases (mainly Lip2p) that hydrolyze triacylglycerol. Then, free fatty acids and alkanes are imported into *Y. lipolytica*, which is thought to be size dependent, and assisted by *eFBP1-4* and *ABC1-4*, respectively. Once inside, these hydrophobic carbon sources are activated to fatty acyl-CoA in the peroxisome (green) or ω-hydroxy fatty acids in the ER (yellow) which can then form other lipid-derived products. Black abbreviation: TAG, triglyceride. Purple abbreviations: ATP, adenosine triphosphate; AMP, adenosine monophosphate; NADPH, nicotinamide adenine dinucleotide phosphate; NADP+, oxidized NADPH. Blue abbreviations: *LIP2*, extracellular lipase 2; *eFBP1-4*, extracellular fatty acid binding protein 1-4, respectively; *ABC1-4*, ATP-binding cassette transporter 1-4, respectively; *FAA1*, fatty acyl-CoA synthetase; *HFD4*, fatty aldehyde dehydrogenase 4; *ADH1,3*, alcohol dehydrogenase 1 and 3. Teal abbreviations: *PXA1/2*, peroxisomal acyl-CoA transporter complex; *FAT1*, fatty acyl-CoA synthetase and transporter; *ADHs*, aldehyde dehydrogenases; *FAO1,* fatty alcohol oxidase 1*; HFD1-3*, fatty aldehyde dehydrogenase 1-3, respectively; *AAL1-10*, acyl/aryl-CoA ligase 1-10, respectively; *ANT1*, peroxisomal ATP/AMP transporter. Yellow abbreviations: *ALK1-3,6,9,10,* Cytochrome P450 enzymes, 1-3, 6, 9, and 10 respectively, with alkane hydroxylase activity; *CPR1*, cytochrome P450 reductase 1, *ADHs*, aldehyde dehydrogenases; *FADH*, fatty alcohol dehydrogenase; *HFD1-2*, fatty aldehyde dehydrogenase 1 and 2; *ALK3-7*, Cytochrome P450 enzymes 4-7 respectively, with fatty acid ω-hydroxylase activity.

substrates such as oleic acid or tributyrin (Meunchan et al., 2015). It has also been reported that the addition of biosurfactants improve Lip2p activity, with a possible mechanism being improved contact of TAG with the active site (Janek et al., 2020).

3.2. Import of extracellular alkanes and free fatty acids, and intracellular activation

Although the mechanisms governing oil and fatty acid import and

Table 1
Native *Yarrowia lipolytica* lipases, gene annotation, localization, regulatory information, and specificity. All Gene IDs were sourced from GYRC (https://gryc.inra.fr/). If localization is unknown, it was predicted by SignalP 6.0 and/or SecretomeP 1.0 and is denoted as "predicted" (Bendtsen et al., 2004; Teufel et al., 2022).

Y. lipolytica lipase gene	Gene ID (E150/CLIB122)	Localization	Protein regulatory information, specificity and/or notes	Reference
LIP1	YALI0E10659g	Intracellular	Carboxylesterase. When codon optimized for heterologous expression in *Pichia pastoris*, had a substrate preference for C4 ester of pNP.	(Gottardi et al., 2021; Zhang et al., 2010)
LIP2	YALI0A20350g	Extracellular	Main extracellular lipase, most widely conserved lipase across the *Yarrowia* clade. C8:0 and C18:1 pNP ester preference, but can act on C12,14,16 as well. Transcriptionally repressed by glucose, and hexokinase Hxk1p is involved in this repression. Deletion of *SOA1-2* genes reduce expression of Lip2p. Meunchan et al. reported that in medium containing oleic acid, *LIP2* has the most abundant RNA transcripts (95%) of the *LIP* family. The presence of Ca^{+} and bile salts is thought to increase activity.	(Aloulou et al., 2007; Desfougères et al., 2009; Fickers et al., 2005c, 2011; Meunchan et al., 2015)
LIP3	YALI0B08030g	Intracellular	Carboxylesterase.	(Gottardi et al., 2021)
LIP4	YALI0E08492g	Predicted extracellular	Not expressed in media containing glucose, oleic acid, or tributyrin. Heterologous expression in *E. coli* showed substrate preference for C6 pNP ester.	(Meunchan et al., 2015; Syal and Gupta, 2017)
LIP5	YALI0E02640g	Predicted extracellular	Heterologous expression in *E. coli* showed substrate preference for C8 pNP ester.	(Syal and Gupta, 2017)
LIP6	YALI0C00231g	Intracellular	Carboxylesterase.	(Gottardi et al., 2021)
LIP7	YALI0D19184g	Membrane bound or periplasm; secreted in stationary phase	One of the main lipases contributing to extracellular lipase activity along with LIP2p and LIP8p. Substrate preference for C6 pNP ester.	(Fickers et al., 2005b; Meunchan et al., 2015; Syal and Gupta, 2017)
LIP8	YALI0B09361g	Membrane bound or periplasm; secreted in stationary phase	Secondary to LIP2p as the most important extracellular lipase. Overexpressing hexokinase *HXK1* decreases gene expression. Meunchan et al. reported that in medium containing glucose, *LIP8* has the most abundant transcripts of the *LIP* family (approximately 90%). Fickers et al. reported substrate preference for C8 and C10 pNP ester while Kumari and Gupta reported substrate preference for C16 pNP ester and triolein.	(Fickers et al., 2005b; Hapeta et al., 2021; Kumari and Gupta, 2012; Meunchan et al., 2015)
LIP9	YALI0E34507g	Predicted extracellular	Heterologous expression in *E. coli* showed substrate preference for C10 pNP ester. Overexpressing hexokinase *HXK1* decreases gene expression. Transcriptionally induced by media containing tributyrin.	(Hapeta et al., 2021; Syal and Gupta, 2015)
LIP10	YALI0F11429g	Predicted extracellular	Transcriptionally induced by media containing tributyrin.	(Meunchan et al., 2015)
LIP11	YALI0D09064g	Predicted extracellular	Longest full-length protein in the *Yarrowia LIP* clade (429 AA). Heterologous expression in *E. coli* showed preference for long chain fatty acid glycerides. Transcriptionally expressed with glucose as the carbon source.	(Kumari et al., 2012; Meunchan et al., 2015)
LIP12	YALI0D15906g	Predicted extracellular	Heterologous expression in *E. coli* showed preference for medium chain fatty acid glycerides.	(Kumari et al., 2012)
LIP13	YALI0E00286g	Predicted extracellular	Induced by glucose. Overexpressing hexokinase *HXK1* increases gene expression. Heterologous expression in *E. coli* showed preference for C16 pNP ester.	(Hapeta et al., 2021; Syal and Gupta, 2017)
LIP14	YALI0B11858g	Predicted extracellular	Heterologous expression in *E. coli* showed preference for C16 pNP ester and triolein.	(Kumari and Gupta, 2012)
LIP15	YALI0E11561g	Predicted extracellular	Heterologous expression in *E. coli* showed preference for C4 pNP ester.	(Syal and Gupta, 2017)
LIP16	YALI0D18480g	Predicted extracellular	Heterologous expression in *P. pastoris* showed preference for C10 pNP ester. Likely no signal peptide although the N-terminal residues are important for function.	(Zhao et al., 2011)*
LIP17	YALI0F32131g	Predicted extracellular	Overexpressing hexokinase *HXK1* increases gene expression. Transcriptionally induced by media containing tributyrin.	(Hapeta et al., 2021; Meunchan et al., 2015)
LIP18	YALI0B20350g	Predicted extracellular	Heterologous expression in *E. coli* showed preference for C12 pNP ester and triolein.	(Kumari and Gupta, 2012)
LIP19	YALI0A10439g	Predicted extracellular	Not expressed in media containing glucose, oleic acid, or tributyrin.	(Meunchan et al., 2015)
LIP20	YALI0E05995g	Predicted intracellular	Carboxylesterase.	(Gottardi et al., 2021)
TGL1	YALI0E32035g	Intracellular	Overexpression increased lipid production and relative amount of C16:1 lipid. Upregulated in media containing ammonia.	(Pomraning et al., 2017; Silverman et al., 2016)
TGL2	YALI0E31515g	Intracellular	Upregulated in media containing ammonia.	(Pomraning et al., 2017)
TGL3	YALI0D17534g	Intracellular	Localized within the lipid body. Positively regulates *TGL4*.	(Dulermo et al., 2013)
TGL4	YALI0F10010g	Intracellular	Localized at the interface between lipid bodies, main lipase that degrades intercellular TAGs. Overexpression increased lipid production, potentially by increasing phospholipid biosynthesis. Upregulated in media containing ammonia.	(Dulermo et al., 2013; Pomraning et al., 2017; Silverman et al., 2016)
TGL32	YALI0D16379g	Intracellular	Increasing activity reduces the size of the lipid body which may promote the production of lipid-derived organic acids.	(Gatter et al., 2016)

* The authors of Zhao et al., 2011 refer to *LIP16* as *LIP9*

activation have been comprehensively studied in the model yeast *S. cerevisiae*, they are not completely clear in *Y. lipolytica*. However, a comprehensive table of relevant *Y. lipolytica* genes is detailed in Table 2. Generally speaking, medium-chain fatty acids ($\leq$12 carbons) are toxic to *S. cerevisiae* resulting in stress to the cellular membrane, production of ROS (reactive oxygen species), cytoplasmic acidification and disruption of the electron transport system (Alexandre et al., 1996). This toxicity was shown to be reduced with the deletion of *ScHFD1*, which is

hypothesized to be due to an increase in cell fitness (Zhu et al., 2017). Medium-chain fatty acids are less toxic to *Y. lipolytica*, and a strain engineered for their production (C8:0 and C10:0) showed no difference in lipid titer relative to the wild type (Rutter et al., 2015). The engineered strain did have a slower growth rate, but this could be due to the stress of metabolic modifications, unrelated to the presence of medium chain fatty acids. *Y. lipolytica* appears to have a preference for fatty acid assimilation; it assimilates long-chain unsaturated fatty acids (C18:1, C18:2 and C18:3) at a much higher rate than saturated fatty acids (C16:0 and C18:0) (Aggelis et al., 1997; Papanikolaou et al., 2001).

Various strains of *Y. lipolytica* have shown the ability to metabolize medium and long chain alkanes (C9-C19) (Zinjarde et al., 2014). These alkanes are mildly toxic, and longer chain (C14, C16) are imported by YlABC1p (reducing toxicity) while shorter chain (C10, C12) are hypothesized to be imported by YlABC2-4p (Thevenieau et al., 2007). Once imported, alkanes are oxidized to fatty alcohols, aldehydes/ketones, and fatty acids in the endoplasmic reticulum and peroxisome (Fig. 2) (Fukuda and Ohta, 2017). While extracellular fatty acid transport and activation mechanisms are similar to those of *S. cerevisiae*, peroxisomal transport and activation mechanisms are largely different (Dulermo et al., 2015). For example, the membrane-bound ScPxa1p/ScPxa2p heterodimer is essential for growth on long-chain fatty acids in *S. cerevisiae*, while deletion of both *pxa1* and *pxa2* in *Y. lipolytica* (which are thought to also form a heterodimer) does not impair growth on fatty acids. Extracellular fatty acids are transported into *Y. lipolytica* via an unknown transporter (potentially Efbp1p-4p for medium and short chains), while cytosolic fatty acids are activated to fatty acyl-CoA by fatty acyl-CoA synthetase, YlFaa1p (Table 2) (Dulermo et al., 2015). Once activated, fatty acids are either stored as TAGs in the lipid body or imported into the peroxisome via YlPxa1p/YlPxa2p. Nonactivated fatty acids may also enter the peroxisome, although the protein involved in this process is currently unknown (Dulermo et al., 2015). The fatty acids derived from lipid remobilization of intercellular lipid bodies can also directly enter the peroxisome via the YlFat1p transporter (Liu et al., 2021b). Typically, intracellular short or medium chain fatty acids are converted into fatty acyl-CoA via Aal1p-10p in the peroxisome for further degradation via β-oxidation, while long-chain fatty acids are converted into fatty acyl-CoA via Faa1p in the cytosol (Fig. 2).

3.3. Catabolism of intracellular fatty acids

Once transported and activated, intracellular fatty acids are typically degraded through β-oxidation in the peroxisome or ω-oxidation in the endoplasmic reticulum, as shown in Fig 1. While both β-oxidation and ω-oxidation can degrade fatty acids, typically, β-oxidation is the more active and energetically efficient degradation route. The rate of fatty acid metabolism depends on carbon chain length and is regulated by fatty acyl-CoA synthetases Fat1p, and Aal1p-10p (Fig. 2, Table 2). Shorter, volatile fatty acids (such as acetate) are converted into acetyl-CoA and metabolized via the glyoxylate shunt pathway. In these conditions, NADPH is generated through the oxidative pentose phosphate pathway (Liu et al., 2016). For medium and longer chain fatty acids, each cycle of β-oxidation consists of a four-step enzymatic reaction, shortening the fatty acid by two carbons, which is released into acetyl-CoA. The first step, generating 2-enoyl-CoA, is catalyzed by six acyl-CoA oxidases (*POX1-6* encoding Aox1p-Aox6p, respectively) with different chain length preferences (Table 2, Fig. 1). The second and third steps, generating 3-hydroxylacyl-CoA and 3-ketoacyl-CoA, respectively, are catalyzed by multifunctional enzyme Mfe2p with hydratase and dehydrogenase activities. The fourth step, producing acetyl-CoA and fatty acyl CoA two less carbons, is carried out by Pot1p and/or Pat1p (Mlíčková et al., 2004; Yamagami et al., 2001).

Many groups desiring to produce lipids or related products have reduced β-oxidation activity by the deletion of *POX* and related genes. For example, deletion of *POX1-POX6* and mitochondrial glycerol-3-phosphate dehydrogenase *GUT2* led to a 3-fold increase in lipid content (from 13% to 42%, based on the g lipids/g DCW) and exhibited a vigorous lipid accumulation phenotype with large lipid bodies (Beopoulos et al., 2008). Mutations or deletions in *MFE2* alone have been extensively studied for lipid production as it is simpler than deleting six *POX* genes (Blazeck et al., 2014; Dulermo and Nicaud, 2011). Another strategy involves the reduction of peroxisomal formation (where β-oxidation occurs) via deletion of *PEX* genes such as *PEX3* and *PEX11* (Table 2) (Hong et al., 2009). However, this metabolic engineering strategy is unsuccessful when oils or fats are the sole carbon source, because β-oxidation is essential for biogenesis in these situations (as well as a supply of acetyl-CoA), and dual carbon feeding strategies must be applied. It should be noted that for cytosolic bioproduction of acetyl-CoA derived products, the peroxisomal acetyl-CoA generated during β-oxidation must often be imported to the cytosol. However, this mechanism of import is not entirely understood in *Y. lipolytica* (Worland et al., 2020a). It has been suggested this may occur through the shuttling of glyoxylate cycle intermediates from the peroxisome through the mitochondria and eventually into the cytoplasm, or the carnitine shuttle, which transports acetyl-CoA between the cytosol and mitochondria (Fig. 1) (Worland et al., 2020a). Use of this pathway as a metabolic engineering strategy is challenged by the limited knowledge of the specific enzymes and general information available relative to better studied organisms such as *S. cerevisiae*.

Y. lipolytica is also known to metabolize various alkanes, alkanols, and alcohols through β-oxidation (Fickers et al., 2005a). The assimilation of alkanes in *Y. lipolytica* has been reviewed recently (Fukuda, 2023). A reduction of peroxisomal formation and therefore β-oxidation should eliminate alcohol and potentially alkane catabolism, but may have other effects on metabolism (Titorenko et al., 2002). Intracellular fatty acid degradation may also occur through ω-oxidation in the endoplasmic reticulum (Fig. 2). Once imported, fatty acids are hydroxylated by the cytochrome P450 *ALK* genes, each with different substrate and hydroxylation preferences (Table 2) (Fickers et al., 2005a; Iwama et al., 2016). Then, the ω-hydroxy fatty acids are converted into ω-aldehyde acids by *ADH* family proteins or Fao1p, and the ω-aldehyde acids are converted into long-chain diacids (LCDAs) by *FALDH* family proteins (Fig. 1, Table 2). Following their microbial synthesis, LCDAs may be further metabolized via β-oxidation (Scheller et al., 1998). Other lipid species, such as fatty alcohols, aldehydes, or alkanes, will be oxidized into fatty acids by proteins detailed in Table 2 and may also participate in these biosynthetic pathways. Just as the ω-oxidation metabolic pathway can be engineered to synthesize ω-hydroxy fatty acids and α, ω-dicarboxylic acids, the β-oxidation metabolic pathway can be engineered to produce β-hydroxy fatty acids and lactones (Liu et al., 2021a).

3.4. Ex novo fatty acid biosynthesis from oil substrates

Fatty acids are *de novo* synthesized in *Y. lipolytica* when a hydrophilic substrate (*e.g.*, glucose and glycerol) is the carbon source. The *de novo* pathway of fatty acid biosynthesis requires ATP, reducing agent NADPH, and precursors such as acetyl- and malonyl-CoA (Kohlwein, 2010). This is followed by fatty acid through cytosolic fatty acid synthase (*FAS*) (Fig. 1). Naturally, *Y. lipolytica* mainly produces C16 and C18 fatty acids (Beopoulos et al., 2009). Long-chain polyunsaturated fatty acids, such as oleic acid (C18:1) or linoleic acid (C18:2) are synthesized by desaturases located in the endoplasmic reticulum (Fig. 1) (Liu et al., 2021b). Fatty acids can be stored in lipid bodies, phospholipids, as part of a structural lipid, or degraded. YlFaa1p is involved in the storage in lipid bodies in *Y. lipolytica*, whereas fatty acid remobilization is largely associated with YlFat1p and YlPxa1p/YlPxa2p (Dulermo et al., 2015). Generally, shorter-chain saturated fatty acids (*i.e.* C12:0, C14:0, and C16:0) are more likely to be degraded, while longer-chain saturated or unsaturated fatty acids are more likely to participate in lipid accumulation (Papanikolaou et al., 2002).

In *ex novo* fatty acid biosynthesis, a crucial variable is the availability

Table 2
Genes involved in oil and fat transport, activation, and catabolism (β- and ω- oxidation) in Yarrowia lipolytica. All Gene IDs and functions were sourced from the cited literature, UniProt (https://www.uniprot.org/) or GRYC (https://gryc.inra.fr/).

Y. lipolytica Gene abbreviation	Gene ID (E150/CLIB122)	Protein localization	Protein function	Notes	Reference
AAL1	YALI0E11979g	Peroxisome	Peroxisomal fatty acid activation, acyl-CoA ligase	Upregulated in oleate medium. When expressed without peroxisomal localization in strain *faa1*Δ*ant1*Δ (lacking cytoplasmic fatty acid activation), sufficient for growth on fatty acids. Likely no substrate specificity (compliments growth on C6:0, C10:0, and C18:1 fatty acids).	(Dulermo et al., 2016)
AAL2,3,5,6,9,10	YALI0A14234g, YALI0E05951g, YALI0F06556g, YALI0C05885g, YALI0A15103g, YALI0D17314g	Peroxisome	Peroxisomal fatty acid activation, acyl-CoA ligases	Upregulated in oleate medium. When any of these genes are expressed without peroxisomal localization in strain *faa1*Δ*ant1*Δ (lacking cytoplasmic fatty acid activation), growth is partially complimented on C6:0 fatty acids only.	(Dulermo et al., 2016)
AAL4,7	YALI0E12419g, YALI0E20405g	Peroxisome	Peroxisomal fatty acid activation, acyl-CoA ligases	Dulermo et al. reported these genes are constitutively expressed and Tenagy et al. reported that their transcripts are upregulated in the presence of n-alkanes and oleic acid. When either gene is expressed without peroxisomal localization in strain *faa1*Δ*ant1*Δ (lacking cytoplasmic fatty acid activation), growth is partially complimented on C6:0 fatty acids only. Aal4p has specificity for C18:0 fatty acids and both Aal4p and Aal7p may have weak specificity for C16:1 and C18:1. Aal7p may be more involved in short chain fatty acid assimilation.	(Dulermo et al., 2016; Tenagy et al., 2020)
AAL8	YALI0B07755g	Peroxisome	Peroxisomal fatty acid activation, acyl-CoA ligase	Constitutively expressed. When overexpressed without peroxisomal localization in strain *faa1*Δ*ant1*Δ (lacking cytoplasmic fatty acid activation), growth is partially complimented on C6:0 fatty acids only.	(Dulermo et al., 2016)
ABC1	YALI0E14729g	Outer cell membrane	Import and/or export of C14 and C16 alkanes	ATP-binding. Transcript level did not change in the presence of C8-C12 alkanes.	(Chen et al., 2013; Thevenieau et al., 2007)
ABC2	YALI0C20265g	Outer cell membrane	Import and/or export of C10 and C12 alkanes	ATP-binding. Transcript level increased greatly in the presence of C8-C10 alkanes and slightly in the presence of C11-C12 alkanes.	(Chen et al., 2013; Thevenieau et al., 2007)
ABC3	YALI0B02544g	Outer cell membrane	Import and/or export of C10 and C12 alkanes	ATP-binding. Transcript level slightly increased in the presence of C8-C10 alkanes but not C11 or C12.	(Chen et al., 2013; Thevenieau et al., 2007)
ABC4	YALI0B12980g	Outer cell membrane	Import and/or export of C10 and C12 alkanes	ATP-binding. Transcript level did not change in the presence of C8-C12 alkanes.	(Chen et al., 2013; Thevenieau et al., 2007)
ACS2	YALI0F05962g	Cytosol	Acetyl-CoA synthetase	Converts acetate to acetyl-CoA. In the short chain clade of acetyl-CoA synthetases. However, Acl1p and Acl2p, which convert citrate from the mitochondria into acetyl-CoA, are thought to be the main producers of cytoplasmic acetyl-CoA.	(Dulermo et al., 2016; Fakas, 2017)
ADH1,3	YALI0D25630g, YALI0A16379g	Cytosol, potentially also endoplasmic reticulum or peroxisome	Alcohol dehydrogenase	Supplementation of either of these genes is sufficient to restore growth on fatty alcohols in a strain unable to utilize fatty alcohols for growth. These enzymes are likely more involved in exogenous fatty alcohol assimilation than metabolism of fatty alcohols derived from alkanes.	(Iwama et al., 2015)
ADH2,4-7	YALI0E17787g, YALI0E15818g, YALI0D02167g, YALI0A15147g, YALI0E07766g,	Not mentioned	Alcohol dehydrogenase	Supplementation of these genes is not sufficient to restore growth on fatty alcohols in a strain unable to utilize fatty alcohols for growth.	(Iwama et al., 2015)
ALK1	YALI0E02684g	Endoplasmic reticulum (membrane)	Hydroxylates n-alkanes	An Δ*alk1-12* mutant with *ALK1* added back under a strong promoter grows like wild type on C10-C18 alkanes. Of the *ALK* family, *ALK1* is the gene most upregulated in the presence of n-alkanes. Regulated by transcription factors Yas1p, Yas2p, and Yas3p.	(Fukuda and Ohta, 2013; Hirakawa et al., 2009; Iwama et al., 2016)
ALK2	YALI0F01320g		Hydroxylates n-alkanes		(Iwama et al., 2016)

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Table 2 (*continued*)

Y. lipolytica Gene abbreviation	Gene ID (E150/CLIB122)	Protein localization	Protein function	Notes	Reference
		Endoplasmic reticulum (membrane)		An Δ*alk1-12* mutant with *ALK2* added back under a strong promoter grows like wild type on C16-C18 alkanes. Upregulated transcription in the presence of n-alkanes.	
ALK3	YALI0E23474g	Endoplasmic reticulum (membrane)	Hydroxylates both n-alkanes and the ω-end of dodecanoic acid	An Δ*alk1-12* mutant with *ALK3* added back under a strong promoter grows like wild type on C10-C11, and C14-C18 alkanes. Under its native promoter, an overexpression of *ALK3* cannot support growth.	(Iwama et al., 2016)
ALK4	YALI0B13816g	Endoplasmic reticulum (membrane)	Hydroxylates the ω-end of dodecanoic acid	An Δ*alk1-12* mutant with *ALK4* added back under a strong promoter does not grow on any C10-C18 alkanes. The ω-oxidation activity of Alk4p is lower than Alk3p, Alk5p, and Alk7p.	(Iwama et al., 2016)
ALK5,7	YALI0B13838g, YALI0A15488g	Endoplasmic reticulum (membrane)	Hydroxylates the ω-end of dodecanoic acid	An Δ*alk1-12* mutant with either *ALK5 or ALK7* added back under a strong promoter does not grow on any C10-C18 alkanes.	(Iwama et al., 2016)
ALK6	YALI0B01848g	Endoplasmic reticulum (membrane)	Hydroxylates both n-alkanes and the ω-end of dodecanoic acid	An Δ*alk1-12* mutant with *ALK6* added back under a strong promoter grows like wild type on C14-C18 alkanes. Under its native promoter, *ALK6* transcription is upregulated in the presence of n-alkanes. The ω-oxidation activity of Alk6p is lower than Alk3p, Alk5p, and Alk7p.	(Iwama et al., 2016)
ALK9	YALI0B06248g	Endoplasmic reticulum (membrane)	Hydroxylates n-alkanes	An Δ*alk1-12* mutant with *ALK9* added back under a strong promoter does not grow well on C10-C18 alkanes.	(Iwama et al., 2016)
ALK10	YALI0B20702g	Endoplasmic reticulum (membrane)	Hydroxylates n-alkanes	An Δ*alk1-12* mutant with *ALK10* added back under a strong promoter grows like wild type on C10-C15 alkanes. Under its native promoter, an overexpression of *ALK10* cannot support growth.	(Iwama et al., 2016)
ALK8,11,12	YALI0C12122g, YALI0C10054g, YALI0A20130g	Endoplasmic reticulum (membrane)	Little to no hydroxylase activity	An Δ*alk1-12* mutant with either *ALK8, ALK11 or ALK12* added back under a strong promoter does not grow on any C10-C18 alkanes.	(Iwama et al., 2016)
ANT1	YALI0E03058g	Peroxisome	Peroxisomal ATP transporter, involved in fatty acid activation	Essential for growth on C10 fatty acid. An Δ*ant1* mutant strain also resulted in reduced growth on short and long chain fatty acids.	(Dulermo et al., 2015)
CPR1	YALI0D04422g	Not mentioned	NADPH-cytochrome P450 reductase	Overexpression suggested as a method to increase production of ω-hydroxy fatty acids.	(Gatter et al., 2014)
FAA1	YALI0D17864g	Cytoplasm and membranes	Sole cytoplasmic fatty acyl-CoA synthetase	Deletion of *FAA1* results in reduced lipid bodies and growth on C10-C18 alkanes. May have more activity towards long chain fatty acids. It is thought that Faa1p is required for the storage of external fatty acids in lipid bodies.	(Dulermo et al., 2016, Dulermo et al., 2015; Tenagy et al., 2015)
FADH	YALI0F09603g	Endoplasmic reticulum	Alcohol dehydrogenase	Not sufficient to restore growth on fatty alcohols, expression in the presence of alkanes in was low.	(Gatter et al., 2014; Iwama et al., 2015)
FAO1	YALI0B14014g	Peroxisome	Fatty alcohol oxidase	Sufficient to restore growth on fatty alcohols. Preference for C12 to C18 fatty alcohols. Involved in the conversion of ω-hydroxy fatty acid to ω-aldehyde fatty acid.	(Gatter et al., 2014; Iwama et al., 2015)
FAT1	YALI0E16016g	Peroxisome (membrane)	Fatty acyl CoA synthetase, exports fatty acids from lipid bodies into the peroxisome	YlFat1p is not essential for growth on fatty acids, unlike the *S. cerevisiae* homolog. YlFat1p only has one transmembrane domain while ScFat1p has two. Deletion resulted in increased fatty acid accumulation.	(Dulermo et al., 2014; Tenagy et al., 2015)
FAT2-4	YALI0E12859g, YALI0B05456g, YALI0C09284g	Not mentioned	Fatty acyl CoA synthetase	A single deletion of *FAT2*, *FAT3*, or *FAT4* did not show growth defects on C10-C18 alkanes.	(Tenagy et al., 2015)
HFD1	YALI0F23793g	Endoplasmic reticulum and peroxisome	Fatty aldehyde dehydrogenase	Transcript upregulated in media containing alkanes and oleic acid relative to glucose. More upregulated in the presence of C10 alkanes relative to C12, and C14, C16.	(Iwama et al., 2014)
HFD2	YALI0E15400g	Endoplasmic reticulum and peroxisome	Fatty aldehyde dehydrogenase	Transcript upregulated in media containing alkanes relative to glucose or oleic acid. Two transcriptional variants exist, one that likely contains a peroxisomal targeting	(Iwama et al., 2014)

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Table 2 (*continued*)

Y. lipolytica Gene abbreviation	Gene ID (E150/CLIB122)	Protein localization	Protein function	Notes	Reference
				sequence and one that does not. More upregulated in the presence of C10 alkanes relative to C12, C14, and C16.	
HFD3	YALI0B01298g	Peroxisome	Fatty aldehyde dehydrogenase	Transcript upregulated in media containing alkanes relative to glucose or oleic acid. More upregulated in the presence of C16 alkanes relative to C10, C12, and C14.	(Iwama et al., 2014)
HFD4	YALI0A17875g	Intracellular (not endoplasmic reticulum or peroxisome)	Fatty aldehyde dehydrogenase	Transcript not upregulated in media containing alkanes relative to glucose or oleic acid.	(Iwama et al., 2014)
*MFE2**	YALI0E15378g	Peroxisome	Multifunctional β-oxidation enzyme	Involved in β-oxidation; deletion of this enzyme greatly reduces β-oxidation.	(Blazeck et al., 2013)
PAT1	YALI0E11099g	Peroxisome	Acetyl-CoA acyltransferase	Overexpression of *PAT1* is hypothesized to reduce metabolic inhibition of acetyl-CoA on the mevalonate pathway.	(Marsafari and Xu, 2020)
PEX1	YALI0C15356g	Peroxisome (membrane)	AAA family ATPase	Required for peroxisomal membrane fusion.	(Titorenko and Rachubinski, 2000)
PEX2	YALI0F22539g	Peroxisome (membrane)	Peroxisomal biogenesis	Pex2p travels to the peroxisome via the endoplasmic reticulum.	(Lambkin and Rachubinski, 2001; Titorenko and Rachubinski, 1998)
PEX3	YALI0F01012g	Peroxisome	Peroxisomal biogenesis	Deletion of *PEX3* reduces formation of peroxisomes.	(Bascom et al., 2003)
PEX5	YALI0F28457g	Cytosol and Peroxisome	Peroxisomal protein import	Pex5p is required for PTS1-tagged protein import but not sufficient.	(Szilard and Rachubinski, 2000)
PEX6	YALI0C18689g	Cytosolic	AAA family ATPase	Pex6p is required for peroxisomal membrane fusion.	(Titorenko and Rachubinski, 2000)
PEX7	YALI0F18480g	Cytosol and Peroxisome	Peroxisomal protein import	Pex7p is involved in importing proteins tagged with PTS2 into the peroxisome.	(Chang and Rachubinski, 2019)
PEX9	YALI0F00748g	Not mentioned	Peroxisome biogenesis	Pex9p is involved in peroxisomal protein secretion.	(Titorenko et al., 1997)
PEX10	YALI0C01023g	Peroxisome	Peroxisome biogenesis transcription factor	Deletion of *PEX10* reduces formation of peroxisomes.	(Blazeck et al., 2013)
PEX11	YALI0C04092g	Peroxisome (membrane)	Peroxisomal biogenesis	Deletion of *PEX11* reduces formation of peroxisomes.	(Chang et al., 2015)
PEX11C, PEX11/25	YALI0C04565g, YALI0D25498g	Peroxisome	Peroxisomal replication	A strain with a deletion of either of these genes grown on glucose does not have a change in peroxisome count, but grown on oleic acid, there is a reduction in peroxisomes.	(Chang et al., 2015)
PEX13,14,17	YALI0C05775g, YALI0E09405g, YALI0D00891g	Peroxisome (membrane)	Peroxisomal protein import	Pex13p, 14p, and Pex17p form a peroxisomal docking complex that is involved in protein import.	(Chang and Rachubinski, 2019)
PEX16	YALI0E16599g	Peroxisome (membrane)	Peroxisomal replication	Typically represses peroxisomal division. In mature peroxisomes, Pex16p interacts with an Aoxp protein complex which inhibits Pex16p and allows for peroxisomal division. Pex16p travels to the peroxisome via the endoplasmic reticulum.	(Guo et al., 2003)
PEX19	YALI0B22660g	Peroxisome	Peroxisomal membrane stability	Deletion of *PEX19* does not change formation of peroxisomes, but these mutants cannot use oleic acid as a carbon source. Interacts with Pex2p. Pex19p is thought to be involved in peroxisomal membrane protein stability.	(Lambkin and Rachubinski, 2001)
PEX20	YALI0E06831g	Cytosol and Peroxisome	Peroxisomal protein import	Imports acyl-CoA oxidases into the peroxisome that are not tagged by PTS1 or PTS2. Assists with the import of PTS2-tagged proteins into the peroxisome.	(Chang and Rachubinski, 2019)
PEX23	YALI0D27302g	Peroxisome (membrane)	Peroxisomal biogenesis	Deletion of *PEX23* reduces formation of peroxisomes.	(Brown et al., 2000)
PEX24	YALI0D11858g	Peroxisome (membrane)	Peroxisomal biogenesis	Deletion of *PEX24* reduces formation of peroxisomes.	(Tam and Rachubinski, 2002)
POT1	YALI0E18568g	Peroxisome	3-ketoacyl-CoA thiolase	Pot1p is PTS2 tagged, and Pex5p, Pex7p, and Pex20p are involved in its processing into the mature protein form. Overexpression of *POT1* can increase the amount of cytoplasmic acetyl-CoA, which may inhibit the mevalonate pathway.	(Chang and Rachubinski, 2019; Marsafari and Xu, 2020)
POX1,6	YALI0E32835g, YALI0E06567g	Peroxisome	Fatty acyl CoA oxidase	Encodes Aox1p and Aox6p, respectively. These proteins are specific for dicarboxylic acid degradation.	(Onésime et al., 2022)
POX2	YALI0F10857g	Peroxisome	Fatty acyl CoA oxidase	Encodes Aox2p. This protein prefers long chain fatty acyl-CoAs.	(Onésime et al., 2022)

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Table 2 (*continued*)

Y. lipolytica Gene abbreviation	Gene ID (E150/CLIB122)	Protein localization	Protein function	Notes	Reference
POX3	YALI0D24750g	Peroxisome	Fatty acyl CoA oxidase	Encodes Aox3p. This protein is specific to short chain fatty acyl-CoAs.	(Onésime et al., 2022)
POX4,5	YALI0E27654g, YALI0C23859g,	Peroxisome	Fatty acyl CoA oxidase	Encodes Aox4p and Aox5p, respectively. These proteins do not appear to have a chain length preference.	(Onésime et al., 2022)
PXA1,2	YALI0A06655g, YALI0D04246g	Peroxisome (membrane)	Fatty acid transport	The YlPxa1p/YlPxa2p heterodimer is not essential for growth on long chain fatty acids, unlike the *S. cerevisiae* homolog heterodimer. The heterodimer is involved in short, medium and long chain fatty acid transport from the cytosol into the peroxisome.	(Dulermo et al., 2015)
*UP1***	YALI0D03245g	Extracellular	Medium and short chain fatty acid import	Involved in the import of C10, C12 and C14 fatty acids, but little growth is observed when *UP1* is overexpressed in a strain lacking *UP1-4*.	(Onésime et al., 2022)
*UP2,4***	YALI0F04598g, YALI0F04620g	Extracellular	Medium and short chain fatty acid import	Involved in import of C12 and C14 fatty acids.	(Onésime et al., 2022)
*UP3***	YALI0C05687g	Extracellular	Medium and short chain fatty acid import	Involved in the import of C10, C12 and C14 fatty acids. Overexpression of this gene in a strain with *UP1-4* knocked out restored growth to wild-type level.	(Onésime et al., 2022)

* Some literature refers to this gene as *MFE1*
** New name for this protein family, proposed by the authors of Onésime et al., 2022: *eFBP*

of reducing agent NADPH, a cofactor of fatty acid synthase. When glucose is the carbon source, there are two known pathways that produce a strong NADPH pool in yeasts. One metabolic pathway involves decarboxylation via the cytosolic NADP+-dependent malic enzyme MEp (EC 1.1.1.40) (Zhang et al., 2016); the other is from the oxidative pentose phosphate pathway (oxPPP) (Wasylenko et al., 2015). While malic enzyme is linked to lipid overproduction in other oleaginous organisms (Wynn et al., 1997; Zhang et al., 2007), that is not the case in *Y. lipolytica* (Zhang et al., 2013). This may be because *Y. lipolytica* lacks a NADP+ dependent cytosolic malic enzyme, and the mitochondrial malic enzyme present is NAD+-dependent instead (Worland et al., 2020a). Furthermore, the heterologous introduction of a cytosolic NADP+ dependent malic enzyme did not increase lipid productivity (Worland et al., 2020a). With glucose as the carbon source, NADPH was synthesized via the oxPPP at the same rate as it was consumed via TAG biosynthesis in an engineered strain (Wasylenko et al., 2015). This result has received additional support, as many studies have shown that the overexpression of pathway enzymes enhanced the oxPPP flux and increased lipid production (Dobrowolski and Mirończuk, 2020; Silverman et al., 2016; Yuzbasheva et al., 2019). However, when other carbon sources besides glucose are used, the mechanism of NADPH production is less clear. It is likely that *Y. lipolytica* generates NADPH from both isocitrate dehydrogenase ICDHp or the mannitol cycle, with transcriptional evidence showing these pathways are upregulated during lipid biosynthesis (Worland et al., 2020a). Taken together, *de novo* fatty acid synthesis requires the cofactor NADPH, which is mainly produced by the oxPPP with glucose as the carbon source, less likely through other pathways with other carbon sources. It should also be noted that *Y. lipolytica* has a high flux of NADPH relative to other organisms (Zhang et al., 2022).

3.5. Intracellular lipid biosynthesis and remobilization

Y. lipolytica and other oleaginous yeasts store most of their lipids in lipid bodies. Lipid bodies are dynamic organelles at the center of lipid and energy homeostasis that store intracellular neutral lipids, particularly triglycerides (85% of lipid bodies) and sterol esters (8% of lipid bodies) (Athenstaedt et al., 2006). Under nutrient limitation, these stored lipids can be remobilized and degraded for energy. Mechanisms of lipid biosynthesis and metabolic engineering strategies in *Y. lipolytica* (although mainly from sugar substrates) have been reviewed recently (Wang et al., 2020). In brief, intercellular lipid formation starts with a condensation of fatty acyl-CoA and glycerol-3-phosphate by Sct1p into lysophosphatidic acid (LPA). Next, Slc1p converts LPA into phosphatidic acid (PA), which is then converted into diacylglycerol (DAG) by Pah1p. DAGs receive an additional fatty acyl group to become TAGs; this fatty acyl group is either donated from a phospholipid and mediated by Lro1p, or obtained from free fatty acyl-CoA by DAG acyltransferases by Dga1p and Dga2p (Fig. 1). Steryl esters (SEs) are also generated from acyl-CoA at the interface between the endoplasmic reticulum and an intracellular lipid body. Overexpression of *DGA1* and *DGA2* have successfully enhanced lipid production (Beopoulos et al., 2012; Gajdoš et al., 2015; Tai and Stephanopoulos, 2013). The deletion of *MGA2*, a gene encoding a regulatory lipid biosynthetic protein, shows a shift towards saturated fatty acids, specifically a reduction of C16:1 and C18:1 coupled with an increase in C16:0 and C18:0 fatty acids (Liu et al., 2015).

TAGs are substrates of intracellular lipases in the *TGL* family, which will release FFAs at the surface of the lipid bodies. Two *Y. lipolytica* proteins, TGL4p, a lipase that is localized at the edge of lipid bodies, and TGL3p, a positive regulator of *TGL4*, are thought to be involved in lipid remobilization, although others also exist (Table 1). The inactivation of either *TGL3* or *TGL4*, or both, greatly increases the ability to accumulate lipids and specifically TAGs (Dulermo et al., 2013). It should also be noted that reactive oxygen species (ROS) may accumulate during lipid metabolism and are inhibitory to growth. To reduce this effect, one group created a strain ALDH, with expressions of *ALDH* (*E. coli* aldehyde dehydrogenase), *ZWF1* (NADPH regeneration, *S. cerevisiae* glucose-6-phosphate dehydrogenase), and *GSR-GPO* (oxidative stress defense, *Y. lipolytica* glutathione disulfide reductase and glutathione peroxidase) (Xu et al., 2017). This strain showed increased growth and glucose consumption, in addition to higher lipid production.

4. Redox balance in the metabolism of fatty acids by *Y. lipolytica*

A balanced cellular redox system is essential for homeostasis and proliferation (Vemuri et al., 2007). The pyridine cofactors NAD+/NADH (nicotinamide adenine dinucleotide) and NADP+/NADPH (NAD+/NADH with an additional phosphate group) have critical, but distinct, roles in maintaining intracellular redox balance (Bloem et al., 2015). NADH is primarily used by the cell as a reducing substance in catabolism, and the regeneration of ATP from ADP (adenosine diphosphate)

through the aerobic respiratory chain (Liu et al., 2018a). Conversely, NADPH is mainly involved in anabolism, including the formation of amino and fatty acids (Liu et al., 2018b).

In the metabolism of glucose by yeasts, NADH is primarily generated in the cytosol during glycolysis and in the mitochondria by the TCA cycle. During fatty acid metabolism, NADH is primarily generated in the peroxisome from fatty acid oxidation by the β-oxidation pathway and the TCA cycle. The generation of NADH and ATP in the metabolism of glucose and stearic acid (C18:0) are shown in Fig. 3. The oxidation of one molecule stearic acid yields 35 molecules of NADH (1.94 molecules of NADH per carbon), while the oxidation of one molecule of glucose yields 10 molecules of NADH (1.67 molecules of NADH per carbon). This difference in yield is due to fatty acids being in a more reduced form with more stored bond energy. Most reductive anabolic reactions require NADPH rather than NADH, such as the biosynthesis of fatty acids and TAGs, which are highly reduced species, from glucose in an oleaginous yeast (Ratledge, 2014). In the biosynthesis of fatty acids, two NADPH are required for each elongation step and one NADPH is consumed for each desaturation reaction (Wasylenko et al., 2015). Therefore, 16, 17 and 18 moles of NADPH required for the biosynthesis of stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) from acetyl-CoA in *Y. lipolytica*, respectively.

Since NAD and NADP are involved in many cellular processes and countless redox reactions, a balanced redox system is a prerequisite for both catabolism and anabolism (de Graef et al., 1999). Redox balance can be achieved by three strategies: (i) regulating the redox potential of the medium by providing reductive environmental conditions; (ii) improving the balance of NAD+/NADH through overflow metabolism, the respiratory chain, and mitochondrial redox shuttles (as NADH is mainly formed in glycolysis, fatty acid oxidation, and the TCA cycle); (iii) engineering a redox balance of NAD+/NADH and NADP+/NADPH (Chen et al., 2014; Qiao et al., 2017). Regarding (iii), the strategies and applications of redox cofactor engineering in industrial microorganisms has been reviewed recently (Liu et al., 2018b). In summary, intentional manipulation of redox potential may shift the flux of different metabolic pathways in ways that support growth and bioproduction. Besides pathway modification, the requirements of NADH and NADPH in a synthetic bioproduction pathway should be considered and potentially optimized. To this end, techniques which can elucidate cofactor flux, such as 13-C metabolic flux analysis, may help to better understand pathway requirements (Liu et al., 2016). However, understanding of the regulation mechanisms of redox balance in microorganisms is far from enough. Further studies are required to elucidate this mechanism, which can provide guidance for future redox cofactor engineering and metabolic engineering research.

5. Considerations regarding cell morphology, biomanufacturing scheme, and stress response

Morphological engineering strategies have been used to increase the growth rate of bacteria, expand cell volume, and simplify downstream separation (Huo et al., 2020; Jiang and Chen, 2016; Wang et al., 2019; Zhao et al., 2019; Zheng et al., 2020). This allows for maintaining the shape of dimorphic yeast in the spherical, yeast-like state (Jiang and Chen, 2016; Liu et al., 2022a; Zakhartsev and Reuss, 2018). Genetic manipulation of morphology-related pathways allows for a controlled, or eliminated, transition from spherical to hyphae, or vice versa (Hurtado et al., 2000; Hurtado and Rachubinski, 2002; Hurtado and Rachubinski, 1999; Jiménez-Bremont et al., 2012; Ruiz-Herrera and Sentandreu, 2002). Hyphal transition in *Y. lipolytica* and the related genes have been reviewed recently (Soong et al., 2019). Mhy1p is the most commonly discussed gene required for hyphal formation, but the transcription factor Msn2p is also required for this transition, and is regulated by histidine kinases Chk1p and Nik1p as well as the MAP kinases Ssk2p, Pbs2p, and Hog1p (Pomraning et al., 2018). It was recently shown that deletions of *RAS2*, *RHO5*, and *SFL1* genes also reduce hyphal formation, similar to *MHY1* (Lupish et al., 2022). Oftentimes, hyphal formation is dependent on media and culture conditions, and may be undesirable for bioprocessing as it can cause issues with mass transfer and mixing in bioreactors (Worland et al., 2020b). Some literature has

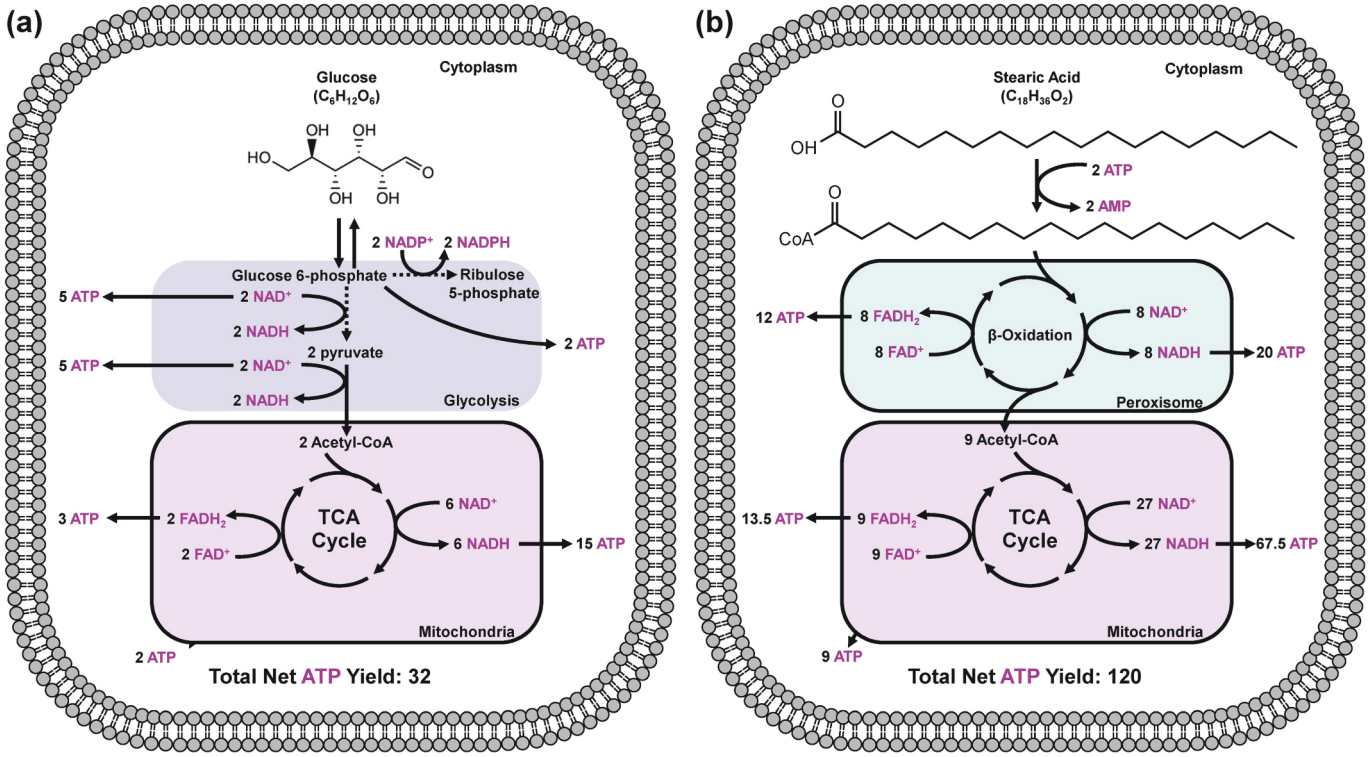

Fig. 3. The generation of NADH and ATP in the (a) glucose and (b) stearic acid metabolism in *Y. lipolytica*. Stearic acid metabolism yields net 120 ATP (6.7 ATP/carbon) while glucose yields net 32 ATP (5.3 ATP/carbon).

reported that the nitrogen source is important in this transition, however, other literature disagrees (Bellou et al., 2014). Additionally, some authors have noted that hyphal formation is more likely to occur with hydrophilic carbon sources as opposed to hydrophobic (Braga et al., 2016). Overexpression of *MHY1* forms hyphal filaments, but lowers lipid accumulation and citric acid titer, a precursor to lipid production (Liu et al., 2021a). Conversely, the absence of the *MHY1* gene in *Y. lipolytica* is associated with increased lipid bodies and production of citric acid, as well as dry cell weight. The deletion of *MHY1* increased metabolic flux through the lipid biosynthetic pathway and consequently, intracellular oil accumulation (Wang et al., 2018a).

In *Y. lipolytica*, hyphal morphology is often associated with stress response and decreased lipid production either due to reduced dissolved oxygen (DO), nutrient limitations or the presence of reactive oxygen species (ROS) (Bellou et al., 2014; Worland et al., 2020b; Xu et al., 2017). Bioreactor fermentations themselves also induce stress due to the heterogeneity of the environment, including pressure, oxygen, and nutrient differences (Worland et al., 2020a). It should be noted that while *MHY1* deletion may remove the formation of hyphal filaments, it does not change the stress tolerance of *Y. lipolytica* (Konzock and Norbeck, 2020). In contrast to the examples previously described, *MHY1* deletion may negatively impact bioproduction; a *MHY1* knockout significantly decreased the titer of lycopene in an engineered strain (Lupish et al., 2022). If the yeast-to-hyphae transition is left intact, hyphal formation may be a good indicator of cell stress and non-optimal culture conditions. In fact, efforts to decrease oxidative stress in *Yarrowia* saw reduced hyphal formation as well as increased growth and lipid production (P. Xu et al., 2017). In this scenario, quantitative image analysis (QIA) may be useful for at-line monitoring of the morphological transition during process development (Braga et al., 2016). It should also be noted that when cell morphology is heterogenous in a culture, OD_{600} is no longer strongly correlated to cell count (CFU, colony forming units) and is more closely related to total cell mass (Worland et al., 2020b). While not all literature is in consensus, it appears that improving the stress tolerance of *Y. lipolytica* (or reducing stress) without deletion of *MHY1* or other genes should promote the biomanufacturing of products from oils and fats in a sustainable and economically profitable manner.

Metabolite overflow, or when the microbial production of intermediates (such as citric acid or acetic acid) exceeds their consumption, may divert metabolic flux away from a desired product, increase cell stress, or otherwise hinder culture performance. As many of these metabolites are often acidic, this can greatly lower pH and challenge pH control. However, *Y. lipolytica* is often tolerant to low pH conditions, and this tolerance has been exploited for metabolic engineering (Zinjarde et al., 2014). When glucose is the carbon source, acetic acid is a common overflow metabolite, and the deletion of *ACH1* can reduce the formation of acetate to increase the production of succinic acid (Yu et al., 2018). However, low pH may be beneficial for some fermentations. Yu et al., 2018 were able to produce 53.6 g/L succinic acid in *Y. lipolytica* in their *ACH1* deletion strain (with additional overexpression of *ScPGK* and native *SCS2*) and without controlling pH, which remained around 2 for the course of the fermentation. The same trends may also hold for oil-based fermentations. It was found that approximately 5-fold more erythritol was produced from waste cooking oil at initial pH 3.0 and high osmotic pressure relative to initial pH 6.0 and lower osmotic pressure (Liu et al., 2018b). However, the authors note that erythritol biosynthesis is reduced at pH greater than 5.0. Another group showed the production of astaxanthin at 167 mg/L without pH control from vegetable oil (Li et al., 2020). While reaching pH 3.0 around day 2, astaxanthin production continued through day 8, and oxidative stress was much reduced compared to a control culture treated with 0.15 mM menadione (known to cause ROS). The authors also measured the effect of autoclaving vegetable oil on oxidative stress and found no difference between autoclaved and un-autoclaved vegetable oil, indicating that autoclaving is a suitable method of oil sterilization. While metabolite overflow may be an issue in both hydrophobic and hydrophilic based fermentations, careful consideration of cellular metabolism in conjunction with biomanufacturing scheme may instead benefit the fermentation. Additionally, techniques such as adaptive laboratory evolution (ALE) may increase acid tolerance of bioproduction strains. Recently, a bioproduction strain of *Y. lipolytica* genetically engineered to produce the polyketide triacetic acid lactone (TAL) was evolved to grow in waste containing acetic, butyric and propionic acid (Coleman et al., 2023). Evolved isolates consumed more acid than the control strain and produced a greater or equal amount of TAL.

6. Bioprocess and bioreaction engineering

Y. lipolytica is able to grow and thrive in a wide range of environmental conditions. Additionally, its efficient lipid metabolism and ability to produce a variety of valuable chemicals make it well-suited for use in a variety of industrial bioprocesses (Park and Ledesma-Amaro, 2023). While fundamental research in cellular and metabolic engineering of *Y. lipolytica* is critical to achieve high titer, rate, and yield (TRY) of a desired product from a substrate of oil(s) and/or fat(s), bioreaction and/or bioprocess engineering with hydrophobic substrates should also be considered. Specifically, this plays an important role in the final success of a biomanufacturing process with economically viable production at large scale. Compared with conventional biomanufacturing with hydrophilic substrates such as glucose, the following bioprocess and bioreaction engineering strategies for fermentation with oil(s) and fat(s) should be considered:

1) Pre-hydrolysis of oil/fat feedstocks prior to the use in the fermentation: Oils are typically present in the form of triglycerides, which must be hydrolyzed into free fatty acids by extracellular lipases so that they can be imported into the cell (Fig. 2). As many authors have observed reduced lipase activity during a fermentation, especially in stationary phase, it may become necessary to pre-hydrolyze triglycerides into free fatty acids with an appropriate commercially available lipase prior to the use in fermentation. An overexpression of native *LIP* genes may avoid or minimize the need of the pre-hydrolysis step, as summarized in Table 1, but that may also lead intracellular toxicity or intracellular TAG degradation. If intracellular TAG is a product or a storage pool for other desired intracellular products such as carotenoids, overexpression of a *LIP* gene may not be a preferred option.
2) Foaming control in fermentation: Foaming during a fermentation is a common challenge, which is thought to be related to the accumulation of extracellular proteins, carbohydrates, and lipids (Moeller et al., 2010). However, the exact foaming mechanism for each specific fermentation may be distinct. Additionally, oil/fat fermentations may generate more foaming due to the formation of soap (reaction of TAG/fatty acids with base) caused by the need of base addition for pH control. Careful considerations should be made in the bioreaction engineering design phase for a specific process to account for this increased foam, such as foam removal, using additional antifoam agents, or new antifoams that can be fed in higher concentrations without increasing cellular toxicity and/or hindering mass transfer.
3) Feeding strategy optimization: The feeding schedule and flowrate of oils/fats should be optimized based on the observed rates of cell growth and product formation during the entire fermentation process. Liquid oils/fats can be directly fed into the bioreactor without dissolving them in an aqueous solution like other substrates such as glucose, meaning a higher concentration of feed can be used with a lower dilution effect. This may lead to higher cell densities and product titers in fermentation. However, as oils and fats are typically more viscous than aqueous media, care should be taken to ensure that the pumping system can handle these fluids. Additionally, if an oil containing saturated fats that are solid or semi-solid at room

temperature (such as palm oil) is used, the feed and tank should be kept at a temperature higher than its melting point to maintain a liquid state. In addition, some fermentation processes may require the cofeeding of oils and fats with another substrate such as glucose. This is especially necessary for strains with a disrupted β-oxidation pathway.

4) Fermentation process optimization: Fermentation media and process parameters including airflow rate, agitation speed, temperature, pH, and dissolved oxygen (DO) setpoints should be optimized to maximize the final production, especially when oils/fats are used as the substrate in the medium. For example, feeding oils to fermentation medium may lead to lower mass transfer coefficient (k_La) and require higher agitation speeds to maintain DO levels (Xie et al., 2018). However, in later stages of the fermentation, the presence of foam may increase the gas holdup and reduce the need of agitation. Therefore, a time- or growth phase- dependent adjustment of agitation speed may be beneficial to implement. It should also be noted that the hydrolysis of triglycerides to produce free fatty acids may lower the media pH and require extra base addition.
5) Advanced and intelligent fermentation technologies: While most current fermentation facilities are designed for batch or fed-batch operations, continuous fermentation process, especially a two-stage continuous fermentation process that decouples cell growth and product formation in two sequential bioreactors, is promising for achieving significantly higher production titer and productivity (Xie, 2022; Xie et al., 2017). This is also applicable to fermentations containing with oils and fats, which may require different medium and process conditions for optimal cell growth and product formation. In addition, the increase in process complexity presented by cofeeding both sugars and oils/fats may warrant the use of artificial intelligence (AI)-based process control. This may help cope with potential risks involving contamination, strain mutations, feed quality variations, lack or failure of biosensors, and changes in process robustness (Xie et al., 2021; Zheng et al., 2022). A conceptual continuous fermentation process for biomanufacturing of high-value products from oils and/or fats utilizing this strategy is illustrated in Fig. 4a.

In addition to the engineering strategies above, a special attention should be paid to the mixing of the mass transfer in the bioreactor due to the use of the hydrophobic substrate (Liu et al., 2021a). A uniform distribution of cells, nutrients/metabolites, O_2, and CO_2 throughout a bioreactor is desirable for optimal microbial growth (Rathore et al., 2016). Achieving uniform mixing in large-scale bioreactors is nontrivial, especially with water-immiscible substrates. Additionally, in large-scale biomanufacturing with hydrophobic substrates, mass transfer and optimal surface area between extracellular lipids and the aqueous phase must be considered (Liu et al., 2021b). As most lipids are insoluble in aqueous medium, the major challenge for the growth of microorganisms on the lipid substrates in a bioreactor system is the poor mixing and mass transfer between phases (Lucatero et al., 2003) (Fig. 4b i, ii). *Y. lipolytica* is known to secrete surfactants that can emulsify hydrophobic molecules in the aqueous phase, reducing droplet size and increasing specific surface areas of lipid droplets, which improves substrate availability. However, mass transfer from the organic phase (hydrophobic substrate) within an aqueous mixture (medium) to the extracellular membrane of the cell is still the limiting step in hydrophobic bioprocesses (Liu et al., 2022a). To solve the overall mixing and mass transfer issues involved in bioprocesses, optimization of process parameters such as impeller speed, position, and type, media viscosity and density, inlet gas flow rate, oxygen mass transfer coefficient k_La, vessel geometry and number/location of baffles are considered as promising solutions (Gelves et al., 2013; Menisher et al., 2000; Xie et al., 2018).

As mass transfer occurs at the aqueous/organic interface, increasing surface area through optimization of impeller design and agitation speed to create dispersed droplets will increase bioconversion potential (Fig. 4b i, ii). Spatial distribution and the size of oil droplets play a critical role in overall oil substrate mixing behavior and bioreactions, which has been previously investigated experimentally and described by empirical equations (Borzani and Podlech, 1976; Stokes and Harvey, 1973). Theoretically, increasing agitation speed and using pitched-blade impellers reduce the size of oil droplets, and distributes them into the lower part of the bioreactor, thus improving the overall mixing and homogeneity. This, especially combined with metabolic pathway engineering, may lead to faster cell growth and/or higher product formation (Liu et al., 2022b; Xie et al., 2018) (Fig. 4b ii, iii). Increased oxygen availability and transfer is also a motivation for increased agitation rate. Specifically, higher oxygen transfer rate (OTR), manipulated by agitation speed, increased growth in *Y. lipolytica* bioreactor fermentation with waste fish oil as the carbon source (Snopek et al., 2021). OTR was also shown to be the most influential variable for growth in a bioreactor fermentation with waste pork lard (Lopes et al., 2018). Interestingly, the solubility of oxygen in vegetable oils may be higher than water, so hydrophobic based bioproduction may potentially increase cellular oxygen availability (Cuvelier et al., 2017).

Computational fluid dynamic (CFD) simulations have also emerged recently to predict multiphase flow hydrodynamics in biochemical reactors. Recently, CFD was shown to predict extracellular oil mixing and mass transfer rates in a stirred-tank bioreactor (Liu et al., 2021a), which suggests a combination of two pitched-blade impellers on top and one Rushton-type impeller in bottom may improve the mixing of oil substrate in aqueous medium. The CFD results informed the optimization of impeller types and agitation speed, and these adjustments enhanced oil uptake and bioconversion in a fed-batch fermentation (Fig. 4b iv). Other modeling techniques have also been used to understand the multi-phase bioprocesses in various bioreactor systems. A fermentation in an internal loop airlift reactor in a gas-liquid system has been described with a Tanks-in-Series model (TIS) (Sikula et al., 2007; Znad et al., 2004). This TIS model could be applied to a bioreactor to simulate an aqueous/oil fermentation system. The effective bioreactor domain in a TIS model is divided into several inter-connected smaller volumes, each with homogeneous properties such as velocity and nutrient concentration. Unfortunately, the TIS configuration often fails to accurately model mixing, since these segments are too coarse to represent all local conditions in a bioreactor (Bellandi et al., 2019). However, the use of a CFD compartmental model based on hydrodynamic studies can account for local conditions as well as recirculation, unlike TIS. This CFD-based compartment modeling can accurately predict mixing in stirred bioreactors (Delafosse et al., 2014; Laakkonen et al., 2006). Additionally, it can accurately model the complex relationship between fluid flow and biological reactions in stirred-tank bioreactors (Delvigne et al., 2005; Guha et al., 2006; Le Moullec et al., 2010). Therefore, future work may use CFD-based compartmental modeling is to simulate and optimize oil mixing, uptake, and bioconversion.

7. Selected examples for biosynthesis of value-added products from oil substrates

As evidenced from the prior sections, the microbial production of oil-derived products is still an emerging field, and there are still limited examples of microbial production from oil substrates. Many compounds are still only able to be produced at the mg/L scale. However, this section provides a comprehensive understanding of the field through examples of value-added products sourced from both hydrophobic and hydrophilic substrates. Specifically, the production of long-chain dicarboxylic acids, fatty alcohols, wax esters, terpenes, terpenoids, carotenes, and carotenoids by *Y. lipolytica* are detailed below. Specific examples of note are outlined in Table 3. In many cases, metabolic pathway engineering, involving co-optimizing for increased product flux and decreased lipid body accumulation, is the most viable strategy. Many groups have also found that peroxisomal or other subcellular localization is beneficial to production in *Y. lipolytica* (Ma et al., 2021;

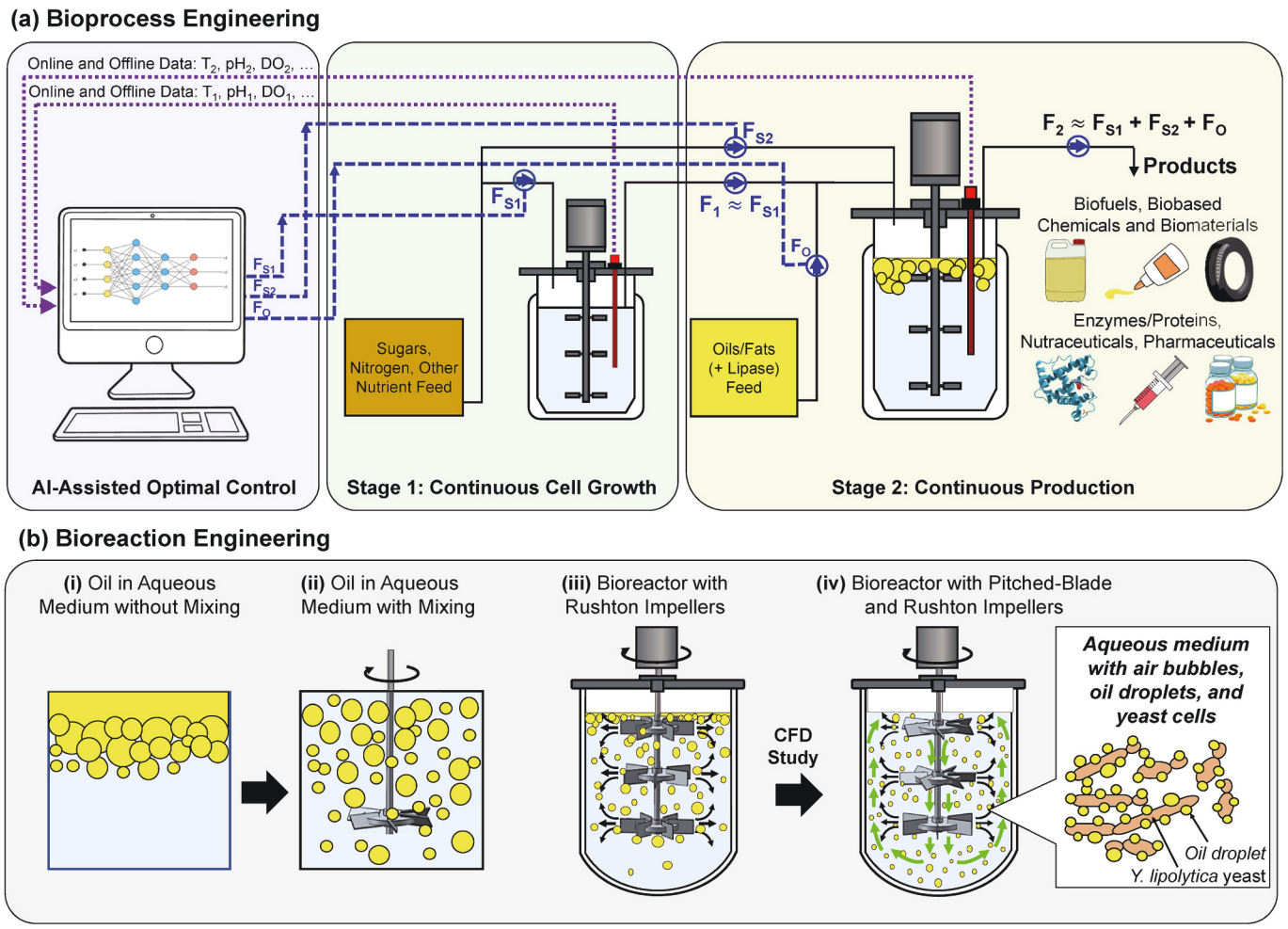

Fig. 4. Potential bioprocess and bioreaction engineering strategies for biomanufacturing of high-value products from oils or fats. (a) A conceptual AI-assisted continuous oil fermentation process that decouples cell growth and product formation with advanced process controls. (b) A schematic diagram showing how oil is dispersed into droplets in a bioreactor containing aqueous medium: (i) Oil in aqueous medium without agitation. (ii) Oil is dispersed into droplets by powerful agitation. (iii) and (iv) Computational fluid dynamics (CFD) can be used to simulate mixing, oil uptake, and bioconversion, which can help improve bioreactor design (*e.g.*, using pitched-blade impellers) and operating conditions (*e.g.* using higher agitation speed) (Liu et al., 2021a).

Yang et al., 2019; Yocum et al., 2021). It should be noted that in some cases, hydrophobic carbon sources are shown to increase titers relative to hydrophilic, and in others, the opposite is true.

7.1. *Biosynthesis of long-chain dicarboxylic acids (LCDAs) from oils*

The sustainable biotransformation of LCDAs from renewable feedstocks has recently received much attention. LCDA monomers typically have a higher market value than traditional fatty acids. The LCDA market size was valued at $148 million USD in 2015 with a compound annual growth rate (CAGR) of 7.0% projected until 2025 (Grand View Research, 2016). These monomer building blocks are precursors for products such as polyamides, polyesters, and polyurethanes, which have applications in the bio-plastic and coating industries (Chung et al., 2015). In plastics, they are involved in the formation of novel polymers with attractive characteristics such as translucence, increased stability, or flexibility (Díaz et al., 2014). LCDAs also have applications in the transportation, chemical, food and healthcare industries as adhesives, surfactants, lubricants and corrosion inhibitors (Werner and Zibek, 2017).

Currently, LCDAs are almost exclusively produced by chemical synthesis from non-renewable petrochemical substrates. As a result, LCDAs have a limited product range and require multiple synthesis steps, yielding unnecessary, often hazardous byproducts. Biologically based manufacturing may overcome the shortcomings of the chemically based process (Huf et al., 2011; Mobley, 1999). In biological production of LCDAs, cells can use many types of substrates (*i.e.*, glucose, alkanes, and fatty acids) to enzymatically produce currently unavailable diacid products in an environmentally friendly manner. In fact, many yeasts can natively convert long-chain fatty acids (a renewable resource) directly into LCDAs. Alkane-assimilating yeasts such as *Candida tropicalis* or *Candida viswanathii* are historically documented as hosts for dicarboxylic acid (DCA) biotransformation (Lee et al., 2018). Currently, LCDAs are mainly produced in China through the fermentation of *C. tropicalis* with alkanes as the feedstock (Picataggio et al., 1992; Xie et al., 2018). Picataggio et al. reported that the engineered *C. tropicalis* produced 140 g/L DCAs. However, due to the pathogenicity of *C. tropicalis* (BSL 2), the BSL-1 and GRAS yeast *Y. lipolytica* is a promising alternative (Werner and Zibek, 2017). To engineer *Y. lipolytica* for production of DCAs/LCDAs from oils or fats, flux through the ω-oxidation pathway must be amplified, which starts by oxidization of fatty acids to the corresponding ω-hydroxyl-fatty acids (Fig. 1). This step is catalyzed by an enzyme complex consisting of a cytochrome P450 monooxygenase (*ALKs*) and an NADPH-dependent cytochrome P450 reductase (*CPR1*) (Fig. 2) (Gatter et al., 2014; Nthangeni et al., 2004; Sakaki and Inouye, 2000; Werner and Zibek, 2017). Then the ω-hydroxyl-fatty acids are further oxidized to ω-aldehyde fatty acids with the catalysis by fatty-alcohol dehydrogenases or fatty acid oxidases (Werner and Zibek,

Table 3
Metabolic engineering strategies with oils or fats as the feed source of note in *Yarrowia lipolytica*.

	Substrate and media conditions	Titer and yield	Metabolic engineering strategy	Production scale	Notes	Reference
α-farnesene	YP with 50 g/L oleic acid, more added throughout as the feed.	10.2 g/L, 0.1g/g oleic acid	Strain F10 is *Y. lipolytica* PO1f with the following genetic integrations under the ut8, hp4d, or TEF promoter, all randomly integrated unless specified: *AtoB* and *HMG(R)* (*AtoB* and *HMG(R)* are codon optimized from *Bordetella petrii,* one copy), *ERG12* (native *Y. lipolytica* gene, three copies), *FS-ERG20* (α-Farnesene synthase from apple seeds, codon optimized for *Y. lipolytica,* fused with native gene *ERG20*, four copies), *ERG13*, *IDI, GPPS*, *ERG8*, and *ERG19* (native *Y. lipolytica* genes, one copy) and *Vitreoscilla stercoraria* hemoglobin (VHb, one copy, codon optimized for *Y. lipolytica*, integrated into the CAN1 locus).	5L fed-batch bioreactor	Swapping oleic acid for waste cooking oil (also at 50 g/L) reduced the titer, and similar lower titers were also seen when using other plant oils (olive, soybean, palm, rapeseed). OD_{600} reached 167 at the end of the bioreactor fermentation.	(Liu et al., 2021b)
α-humulene	2 x YP with 7.5% (v/v) WCO. The feed started at 36h and consisted of pure WCO at 8mL/hour.	5.9 g/L, yield not reported	Strain GQ2012-C3 is *Y. lipolytica* PO1f with overexpression of many genes, most tagged with a peroxisomal targeting sequence (PTS): one copy of native *ERG10* and *ERG13*, two copies of native *ANT1*, *IDI*, *ERG8*, *ERG12*, *ERG19, ERG20*, *POT1*, and *POX1*, three copies of codon-optimized α-humulene synthase from *Aquilaria crassna ACHS*, five copies of codon-optimized NADH-HMG(R) from *Ruegeria pomeroyi*, and downregulation of native genes *GCY16* and YALI0B21142g.	5L fed-batch bioreactor	The authors noted that *Y. lipolytica* preferred WCO over glucose at the same amount of carbon source; *i.e.* 60 g/L WCO growth rates were higher than 60 g/L glucose growth rates.	(Guo et al., 2022)
β-farnesene	2 x YP with 50 g/L oleic acid, more added throughout as the feed.	35.2 g/L, 0.17 g/g oleic acid	Strain Q26 is *Y. lipolytica* PO1f with the following random genetic integrations under the TEF, hp4d, EXP, GPD, or ut8 promoter: *AtoB*, *HMG(R)*, and *ERG13* (*AtoB* and *HMG(R)* are codon optimized from *Bordetella petrii, ERG13* is native *Y. lipolytica gene,* two copies of each gene, one with ePTS1 peroxisomal localization sequence), *ERG20* and *AanFS* fusion gene (four copies, codon optimized, K197T/F180H mutation in β-farnesene synthase *AanFS* from *Artemisia annua*, *ERG20* is native *Y. lipolytica* gene), *ERG12* and *IDI* (native *Y. lipolytica* genes, three copies), *GPPS*, *ERG8*, *ERG19* and *POT1* (native *Y. lipolytica* genes, one copy). Additionally, deletion of *DGA1/DGA2*.	5L fed-batch bioreactor	Titer of 31.9 g/L was achieved with waste cooking oil at 50 g/L. OD_{600} reached 310 at the end of the bioreactor fermentation.	(Liu et al., 2022a)
Lycopene	The medium contained 3.4 g/L YNB, 2.5 g/L YE (yeast extract), 8.8 g/L ammonium sulfate, 30mM palmitic acid, 100 g/L glucose, and 10% dodecane. 37.5 mM isoprenol was added after two days.	4.2 g/L, yield not reported	Strain YLIyc-9 is *Y. lipolytica* PO1f with random integrations of the following genes under the TEF promoter: *IDI* (two copies; codon optimized from *Pseudescherichia vulneris*), *CrtE*, *CrtB*, and *CrtI* (codon optimized from *Lamprocystis purpurea*), *IPK* (codon optimized from *Arabidopsis thaliana*), *CHK* (codon optimized from *S. cerevisiae*), and *ERG20* (native *Y. lipolytica* gene).	3-L fed-batch bioreactor	Increasing lipid content by either feeding palmitic acid or overexpressing lipid biosynthesis genes *ACC1* and *DGA1* lead to higher lycopene titers.	(Luo et al., 2020)
Long-chain diacids (LCDAs)	Fermentation medium contained 50 g/L glucose, 40 g/L yeast extract, 20 g/L peptone, 15 g/L ammonium sulfate. The continuous feed consisted of both glucose (700 g/L) and pure ethyl palmitate.	119 g/L, yield not reported	*Y. lipolytica* strain D3928, derived from ATCC 20362 with deletions of *DGAT1*, *DGAT2*, *PEX3*, *POX2-4*, and *PEX*20, and overexpressions of *VsCPR* and *VsCYP* (codon optimized from *Vicia sativa*), *CtCYP* and *CtALDH*, (codon optimized from *Candida tropicalis*), *CcFAO* (codon optimized from *Candida cloacae*), and *AAL4* (native *Y. lipolytica* gene).	5-L fed-batch bioreactor	Starting at 28 hours, ethyl palmitate was fed so that its concentration within the bioreactor was maintained between 1- 20 g/L. At the end of the fermentation, the majority of LCDAs were C16:0.	(Zhu et al., 2019)
Wax ester	2.48% (w/v) hydrolyzed waste cooking oil as well as 2.5 g/L yeast extract, 1 g/L $(NH4)_2SO_4$, 6 g/L KH_2PO_4, 2 g/L K_2HPO_4, 1 mM $MgSO_4$, 0.05 X trace metal. More hydrolyzed waste cooking oil, phosphate, and carbonate was supplemented throughout.	7.58 g/L, 0.31 g/g waste cooking oil	Strain VSWE1 is *Y. lipolytica* ATCC 20362 with random integration of the following genes under the TEF promoter: *MhFAR* (from *Marinobacter hydrocarbonoclasticus*) and *AbWS* (codon optimized from *Acinetobactor baylyi*)	Shake flask	The titer of wax esters was higher with waste cooking oil as the carbon source compared to glucose, oleic acid, or soybean oil.	(Soong et al., 2021)

2017). DCAs are then synthesized by converting the aldehyde group of the ω-aldehyde fatty acids to the carboxyl group, with the catalysis of a fatty-aldehyde dehydrogenase (*HFD*) (Werner and Zibek, 2017).

To further improve DCA/LCDA production, metabolic flux towards ω-oxidation should be increased while flux away should be reduced. Current strategies includes blocking β-oxidation by the deletion of *MFE2* or *POX* genes (Table 2) and reducing FFA accumulation in TAGs by deleting genes such *DGA1/2* (Fig. 1) (Abghari et al., 2017; Zhu et al., 2019). For example, one group showed that deletion of the sole cytoplasmic fatty acyl-CoA synthetase *FAA1* (increasing ω-oxidation flux) as well as overexpressing of ω-oxidating *YlALK5* and *CPR1* produced 330 mg/L LCDA in a shake flask. The titer was increased to 3.49 g/L in a 1L fermentation with glycerol as the sole carbon source under nitrogen limitation (Abghari et al., 2017; Soong et al., 2019). Since β-oxidation is usually removed or decreased in DCA production, a hydrophilic carbon source such as glucose or glycerol must be co-fed to support cellular biogenesis. While it could be argued that only hydrophilic carbon sources are necessary, this leads to poor DCA production due to the many enzymatic steps required to synthesize FFA from glucose, and later convert this FFA to DCA (Abghari et al., 2017). Therefore, approaches involving co-feeding of hydrophobic and hydrophilic substrates may allow for high-yield and low-cost production of LCDAs as well as decoupling growth from production. Applying this strategy, one group achieved titers up to 23 g/L LCDAs by co-feeding of glucose and sunflower oil (Nicaud et al., 2010). DuPont has also reported a titer of 119 g/L LCDAs by co-feeding ethyl palmate and glucose in fed-batch bioreactors (Zhu et al., 2019).

7.2. Biosynthesis of fatty alcohols and wax esters from oils

Wax esters, or an ester condensation of a fatty acid and a fatty alcohol, naturally exist in plants, microbes, insects such as the honeybee, and mammals such as whales and humans. These neutral lipids from a structurally diverse class of molecules that are generally very hydrophobic. As is typical of lipids, the physical and chemical properties of wax esters are a result of chain length, degree of unsaturation, and the presence of any other functional groups. Their applications in nature range from protective hydrophobic coatings to buoyancy and insulation (Jetter and Kunst, 2008; Liu et al., 2022a; Wältermann et al., 2004). Wax esters have many commercial applications, in food, cosmetics, lubricants, and the pharmaceutical industry (Doan et al., 2017; Fiume et al., 2015; Petersson et al., 2005). The global market size of wax esters is projected to hit $11.3 billion in 2027 with a projected CAGR of 2.7% (IndustryARC, 2019). Despite their widespread prevalence in nature, only a few organisms, such as sperm whales and the jojoba plant, accumulate high amounts of wax intercellularly. The hunting ban for sperm whales and the agricultural challenges of cultivating jojoba mean that there is little supply (Al-Obaidi et al., 2017). There is great potential for microbial production of wax esters, as many microorganisms already possess an abundant fatty acid and alcohol precursor pool. In fact, bacteria genera such as *Acinetobactor*, *Streptomyces*, and *Rhodococcus* have been shown to produce wax esters (Ishige et al., 2002; Liu et al., 2022a, 2022b; Röttig et al., 2016; Round et al., 2019; Santala et al., 2014). Prokaryotic wax ester synthesis is a three-step process. Initially, fatty acyl-CoA reductase (*FAR)* reduces long-chain fatty acyl-CoA into a fatty aldehyde. This aldehyde is then reduced to a fatty alcohol by fatty aldehyde reductase. Lastly, the fatty alcohol is esterified with fatty acyl-CoA to form a wax ester through wax ester synthase/diacylglycerol acyltransferase (*WS/DGAT*) (Liu et al., 2022a).

In eukaryotic organisms such as *Y. lipolytica*, wax ester formation proceeds similarly (Fig. 1). Fatty alcohol intermediates also have industrial applications, and thus their production has been explored as both a metabolic node for wax ester synthesis and as a final product. Recently, two groups have shown that fatty alcohols can be produced from glucose in *Y. lipolytica* through heterologous expression of codon optimized *FAR* from *Marinobacter hydrocarbonoclasticus* (*MhFAR*) strain VT8. One group produced fatty alcohols at 5.75 g/L in YPD medium with a high amount of glucose (91 g/L glucose in shake flasks) (Zhang et al., 2019a) and another showed 5.8 g/L in a fed-batch fermentation with minimal media (Cordova et al., 2019). Further work has shown that the introduction of *WS* can produce fatty acid methyl and ethyl esters from glucose at the gram per liter scale (Gao et al., 2018; Liu et al., 2022a; Xu et al., 2016). *FAR* genes from other organisms have also been explored, although they are largely inferior. For example, a combination of *MhFAR* and *AbWS* from *Acinetobactor baylyi* had a higher titer of fatty alcohols and wax esters relative *ScFAR* from *Salvia chinensis* or *MmFAR* from *Mus musculus* and *AbWS* with glucose as the carbon source (Zhao, 2017).

The production of wax esters from hydrophobic carbon sources in *Y. lipolytica* is a much more emergent field. It was shown recently that *Y. lipolytica* containing copies of genes *MhFAR* and *AbWS* produced enhanced lipid bodies when grown on oleic acid or oil-containing media (Soong et al., 2021). Additionally, by switching from glucose to oils as main carbon source, the wax ester biosynthetic pathway becomes much shorter, which led to a 70-fold increase in wax ester production. In shake flask experiments, the wax ester titer reached 7.58 g/L and the intracellular wax esters contributed up to 56% of the dry cell wright (DCW) (Soong et al., 2021), which is so far the highest reported wax ester production level in *Y. lipolytica* (Table 3). Because the difference between wax esters produced *de novo* from glucose and *ex novo* from fatty acids is so pronounced, it is likely that *ex novo* production is favored for higher product titers and should be the focus of future research.

7.3. Biosynthesis of terpenes and terpenoids from oils

Terpenes consist of five main classes of molecules: monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes (carotenes, detailed in the below section). While the word terpene is often used interchangeably with terpenoid, terpenes refer to hydrocarbons while terpenoids refer to terpenes modified with new functional groups such as hydroxyl, aldehyde or ketone (Perveen, 2018). Terpenes range in a wide variety of color, flavor, and fragrance and have applications in the pharmaceutical, food and medicinal cannabis industries (Baron, 2018; Moser and Pichler, 2019). The global terpene market was valued at $610 million USD in 2020 with a projected CAGR of 6.4% up until 2027 (Absolute Reports, 2022). Some of the most common terpenes include limonene, a lemony flavoring found in citrus fruits, or pinene, a piney scent found in pine trees, as well as herbs such as parsley and rosemary (Baron, 2018). While terpenes are prevalent in nature, they are only present at low concentrations which limits their industrial production (Wang et al., 2018b). However, there has been growing industrial interest in this compound, with Amyris notably announcing in 2015 that they could produce farnesene from yeast at the low cost of $1.75 per liter (Wang et al., 2018a).

Terpenes have been produced in many hosts, including bacteria such as *E. coli*, *Synechoccus sp.*, *Synechocystis sp.*, *Rhodobacter sphaeroides*, *Streptomyces sp.*, and *Cupriavidus necator* as well as yeast such as *Y. lipolytica*, *S. cerevisiae*, and *Rhodosporidium toruloides* (Moser and Pichler, 2019; Wang et al., 2018a). The most studied organisms for terpene production are *E. coli* and *S. cerevisiae*, but the high amount of acetyl-CoA and flux through the mevalonate pathway motivates *Y. lipolytica* as a promising production host (Wang et al., 2018a). While not natively produced in *Y. lipolytica*, only a few genes must be added for their production (Fig. 1). The native mevalonate pathway converts acetyl-CoA into terpene precursors IPP (C5, isopentenyl diphosphate) and DMAPP (C5, dimethylallyl diphosphate). HMG-CoA reductase *HMG (R)* is often thought to the first rate-limiting step in this pathway, and its overexpression, along with pathway genes *ERG8,10,12,19* and IPP isomerase *IPI*, are often employed to increase terpene precursor flux (Ma et al., 2019). The production of terpenes in *Y. lipolytica* has been reviewed recently, from both hydrophilic and hydrophobic carbon sources (Arnesen et al., 2020; Li et al., 2021; Miller and Alper, 2019; Zhang et al., 2023). It should be noted that terpenes are often volatile,

and a dodecane overlay in *Y. lipolytica* cultures has been used to enhance recovery and yields as it is relatively inert (Miller and Alper, 2019; Pang et al., 2019). While most historical approaches for terpene production use glycose or glycerol, oil or fat-based carbon sources offer a more direct bioconversion route as acetyl-CoA is one of the main products of β-oxidation. An emergent metabolic engineering strategy for terpene production is compartmentalization to the peroxisome as it improved squalene production in *S. cerevisiae* (Liu et al., 2020) as well as in *Y. lipolytica* as detailed in some examples below.

Monoterpene titers are often reported at the low milligram scale relative to other terpenes as the majority of precursor GPP (geranyl pyrophosphate, C10) is converted immediately to FPP (farnesyl pyrophosphate, C15) by ERG20p (Dusséaux et al., 2020) (Dusséaux et al., 2020). As such, and given that *ERG20* is essential and cannot be knocked out, a common strategy to increase production of monoterpenes involves the conversion of GPP into isomer NPP (neryl diphosphate, C10) by neryl disphosphate synthase NDPS1p (Fig. 1) (Li et al., 2022). NPP is solely used for terpene synthesis unlike GPP and thus is a more reliable strategy. Metabolic pathway engineering to enhance flux has the potential to increase titers. For example, monoterpenes D-limonene and L-limonene were produced solely with the heterologous expression of their respective synthases from waste cooking oil at approximately 2 mg/L (Pang et al., 2019). However, this titer was reduced relative to production from glucose by approximately a factor of 5. This group found that by increasing metabolic flux through the mevalonate pathway, specifically introducing more copies of *HMG(R)*, *IDI* and truncated neryl disphosphate synthase *tNDPS1*, limonene titer was improved when waste cooking oil was the carbon source relative to glucose or glycerol (Li et al., 2022). Through fermentation optimization, they produced D-limonene and L-limonene at 91.24 and 83.06 mg/L respectively. A similar strategy was employed to produce monoterpene α-pinene from waste cooking oil as the sole carbon source with the overexpression of native pathway genes *ERG12*, *ERG8*, *ERG13*, *HMG(R)*, and heterologous *tNDPS1* for a titer of 33.8 mg/L (Wei et al., 2021).

Similar engineering efforts have been applied to sesquiterpenes, and their microbial production in yeasts has been reviewed recently (Mai et al., 2021). In *Y. lipolytica*, the titers of sesquiterpenes α-, β- and γ-bisabolene were increased by the overexpression of codon-optimized *HMG(R)* and ABC transporter *ABC-G1* from *Grosmania clavigera* (exports bisabolene out of the cell, hypothesized to reduce cellular toxicity) (Zhao et al., 2021). Bisabolene synthase genes were codon-optimized for *Y. lipolytica* from *Zingiber officinale* (α-, β- synthase) and *Helianthus annus* (γ- synthase). It should be noted that the titers of α-, β-, and γ- bisabolene with waste cooking oil as a carbon source (157.8 mg/L, 20.9 mg/L, 3.6 mg/L respectively) were reduced from glucose (973.1 mg/L, 68.2 mg/L, 20.2 mg/L respectively). The group hypothesized this was due to sub-optimal fermentation conditions. In a follow up study optimizing these conditions as well as magnesium supplementation, they reported a titer of 1058.1 mg/L α-bisabolene in a 5L bioreactor titer with waste cooking oil as the main carbon source, higher than reported glucose titers (Zhu et al., 2022). Another group produced sesquiterpene α-farnesene at a titer of 10.2 g/L with oleic acid as the substrate by improving upon a previous strain and increasing the copy number of previously determined rate-limiting mevalonate pathway enzymes *ERG12*, *FS-ERG20* fusion gene and hemogoblin *VHB* which enhances metabolism (Table 3) (Liu et al., 2021a). They also found that using waste cooking oil instead of glucose improved titer and yield of α-farnesene. However, this titer does not beat the highest reported α-farnesene titer, also reported by this group, 25.55 g/L obtained from glucose (Liu et al., 2019). It appears that similar metabolic engineering strategies were used, and it is possible that further metabolic flux and pathway optimization could help increase the titers of α-farnesene produced from oil. Specific to lipid metabolism, it was also shown that deletion of *DGA1/DGA2* to reduce the accumulation of intracellular lipids increased the titer of β-farnesene by approximately 45%, and with further optimization, produced 22.8 g/L of β-farnesene from glucose (Shi et al., 2021). Another group produced β-farnesene from 2x YP with oleic acid or WCO at a titer of 35.2 g/L or 31.9 g/L respectively, representing the highest reported titer to date (Liu et al., 2022a). This strategy involved the compartmentalization of the mevalonate pathway into the peroxisomes, enhancement of β-oxidation, metabolic pathway engineering, and protein engineering of β-farnesene synthase (Table 3). Peroxisomal localization was also used to enhance production of the sesquiterpene α-humulene, which was produced at titers of 5.9 g/L from waste cooking oil by expressing multiple copies of pathway genes tagged to the peroxisome as well as the downregulation of native gene expression by promoter swapping (Table 3) (Guo et al., 2022).

Recently, the triterpenoid amyrin was produced in *Y. lipolytica* from YP + WCO media at a maximum titer of 85 mg/L and 25 mg/L of α- and β- amyrin, respectively (Kong et al., 2022). The authors overexpressed pathway enzymes *HMG(R)*, *ERG20*, *ERG9*, and *ERG1* as well as codon optimized *Catharanthus roseus* amyrin synthase *CrMAS*. Semi-rational protein engineering also increased the activity of *CrMAS* to catalyze the final pathway step of 2,3-oxidosqualene into α- and β- amyrin (Kong et al., 2022). The effect of oxygen transfer parameter k_{La}, manipulated by changing filling volume in a flask, as well as different carbon sources (glucose and WCO) was investigated for its effect on amyrin titer. Interestingly, at k_{La} $84h^{-1}$ (corresponding to 25 mL media in a 250 mL flask), YPD media produced more amyrin than YP + WCO, but at k_{La} $39h^{-1}$ (50mL media in a 250mL flask), the opposite trend was seen. Metabolic engineering efforts to produce the triterpenoid derivative campesterol from codon optimized *DHCR7* (from *Danio rerio*) with sunflower seed oil as the carbon source showed that *HMG(R)* overexpression did not significantly increase the yield and overexpression of *MAE* (malic enzyme) significantly decreased the yield (Zhang et al., 2017). *MAE* overexpression may promote the synthesis of lipids and reduce flux towards campesterol (Blazeck et al., 2014; Zhang et al., 2017). Overexpression of *ACL* and *POX2* (ATP: citrate lyase and peroxisomal acyl-CoA oxidase 2, respectively) with the goal of increasing the amount of actetyl-CoA present in the cytoplasm did improve production. Specifically, Aox2p, which is unique relative to the rest of the *POX* family enzymes due to its high catalytic activity and long chain specificity, was hypothesized to be beneficial as long-chain fatty acids are the main component of sunflower seed oil (Zhang et al., 2017). With *POX2* overexpression and fermentation optimization, a maximum titer of 942 mg/L campesterol was obtained (Zhang et al., 2017).

7.4. Biosynthesis of carotenes and carotenoids from oils

Carotenoids are a class of mainly yellow, orange, or red lipid-soluble pigments naturally synthesized by plants, algae, or photosynthetic bacteria. Most carotenoids are tetraterpenoids containing forty carbons in their polyene backbones and two terminal rings, and their molecules are typically linear and symmetrical, with the order reversed in the center (Mezzomo and Ferreira, 2016). Tests in vitro and in vivo suggest that carotenoids act as excellent antioxidants in biological systems, scavenging and inactivating free radicals, especially quenching many reactions that lead to lipid peroxidation (Black et al., 2020; Erdman, 1999). The most prevalent carotenes and carotenoids in diets include lycopene, α-carotene, β-carotene, lutein, canthaxanthin, zeaxanthin, and β-cryptoxanthin (Krinsky et al., 2000). Dietary carotenoids provide health benefits in strengthening the immune system, decreasing the risk of degenerative diseases, particularly certain cancers and eye disease (Johnson, 2002). Currently, carotenoids are extensive used by the industry as nutraceuticals, pharmaceuticals, and poultry feed additives, as well as colorants in cosmetics and special foods (Meléndez-Martínez et al., 2019; Nabi et al., 2020), which are mainly recovered from plants by physicochemical extraction and produced through chemical synthesis. In carotenoid biosynthesis, acetyl-CoA is first converted to IPP and DMAPP through the mevalonate pathway, then GPP, then FPP, then geranylgeranyl pyrophosphate (GGPP) and finally carotenes/carotenoids such as lycopene, β-carotene, canthaxanthin, zeaxanthin and

astaxanthin (Fig. 1) (Liu, 2022). While *Y. lipolytica* does not natively produce carotenes, it does natively produce GGPP, which is only three enzymatic steps away from most carotenes (Fig. 1). Metabolic engineering strategies to produce carotenoids in *Y. lipolytica* should consider a high flux of precursor acetyl-CoA through the mevalonate pathway into IPP and DMAPP, often limited by HMG-CoA reductase *HMG(R)* and geranylgeranyl diphosphate synthase *GGS1* (Liu, 2022). Additionally, the downstream pathway conversion of IPP and DMAPP into carotenes should also be optimized.

Previous research has suggested that modifying the copy numbers of carotenoid biosynthesis (*CRT*) genes could increase the conversion of FPP into lycopene (Xie et al., 2015). This strategy, with multiple copies of *crtE*, *crtB* and *crtI*, and overexpression of the native AMP deaminase-encoding gene *AMPD*, produced 60 mg/g DCW lycopene in a 5L fed-batch fermentation with glucose as the carbon source (Zhang et al., 2019b). Lycopene production was further optimized in *Y. lipolytica* at 4.2 g/L with the supplementation of palmitic acid in addition to glucose, isoprenol (a precursor to lycopene), and dodecane to prevent isoprenol evaporation (Table 3) (Luo et al., 2020). When the engineered strains were fed [U-13C] glucose along with unlabeled isoprenol and palmitic acid, the majority (>90%) of fatty acids were unlabeled, suggesting they were derived from palmitic acid, while the majority of acetyl-CoA (75%) was labeled, suggesting they were derived from glucose. The authors suggest that the observed increase in lycopene titer with the addition of palmitic acid was likely due to increased intracellular hydrophobicity allowing for more lycopene storage, in addition to increased levels of lycopene precursors from palmitic acid metabolism such as acetyl-CoA (Luo et al., 2020). Compartmentalization may also be an interesting strategy for enhancing carotenoid production. *Y. lipolytica* grown on safflower oil produced 167 mg/L astaxanthin (Li et al., 2020) which was improved by localization to specific organelles, giving a final titer of 858 mg/L, although glucose was the substrate (Ma et al., 2021).

A similar strategy has been employed in β-carotene production. A screen of optimal promoter-gene pairs for β-carotene production (*GGS1*, *CarRP* and *CarB*) found that the strong TEF promoter led to increased production in *Y. lipolytica* relative to low or medium strength promoters. By introducing three copies of each of these genes, two under pTEF, as well as one copy of *tHMG(R),* the group produced β-carotene at a titer of 6.5 g/L in a 5L fed-batch bioreactor from glucose, the highest titer so far (Larroude et al., 2018). Another group added multiple copies of *CarB* (two) and *CarRP* (three) and a single copy of native genes *GGS1*, *ERG13*, and *HMG(R)* to produce 4.5 g/L β-carotene in a 5L bioreactor from glucose (Zhang et al., 2020). While this titer is lower, the authors noted that only eight genes were overexpressed and there may be potential to improve titer further. Additionally, the effect of carbon sources on the β-carotene production by the engineered strain was also examined. Relative to glucose, oil may increase biomass and cell sizes, but with the drawback of reduced protein content (Beopoulos et al., 2008; Vasiliadou et al., 2018; Worland et al., 2020b). Since carotenoids are hydrophobic and soluble in lipids, it was found that increasing the size of lipid bodies in *Y. lipolytica* significantly improved carotenoid production (Matthäus et al., 2014), which could be achieved by blocking the β-oxidation or by using oil substrates. For example, YP medium containing canola oil at 2 g/L produced a higher titer of β-carotene (121 mg/L) than YP medium with 2 g/L glucose, which yielded 53 mg/L β-carotene (Worland et al., 2020b). These examples demonstrate that *Y. lipolytica* has the potential to become a platform for carotene production from a hydrophobic substrate.

8. Conclusions

One of the major cost contributors to a biomanufacturing process is the raw material, or feedstock that is used for cell growth and product formation. While sugars, especially glucose, are used as the major carbon and energy source for biomanufacturing processes, other low-cost feedstocks should be explored to further reduce this cost. For example, the global production of plant oils and animal fats islarger than that of sugars, and a significant portion of oils and fats was wasted and released to the environment. This provides an opportunity to use oils, specifically waste oils or fats, as a plausible alternative carbon source for biomanufacturing. Due to the ability of oleaginous yeast *Y. lipolytica* to efficiently grow on oil substrates and its capability of further converting the derived fatty acids into many value-added products, it is a promising biomanufacturing platform for this purpose. With the availability of genomic information for the yeast and recent advances in the synthetic and systems biology tools for strain engineering, *Y. lipolytica* has been successfully engineered to produce many valuable products such as LCDAs, fatty alcohols, wax esters, terpenes, terpenoids and carotenoids from sugars and/or oil feedstocks. While most ocurrent studies on biomanufacturing with hydrophobic feedstocks involve only laboratory-scale production or proof-of-concept research, combining metabolic engineering strategies and systems biology tools will help determine the rate-limiting steps in the bioprocess. In addition, the hydrophobic nature of oil substrates may challenge efforts to achieve efficient mixing and mass transfer in large scale bioreactors. In many cases, it is necessary to further optimize the feeding strategy, fermentation conditions, and bioreactor design and operation to maximize product yield and minimize byproduct formation. Therefore, future directions should focus on integrating the research efforts from synthetic biology, cellular and metabolic engineering, and bioreaction engineering to establish a new biomanufacturing platform that can make a series of high-value products fromlow-cost oils and fats.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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