Trends in Biotechnology

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Review

# Holistic Approaches in Lipid Production by *Yarrowia lipolytica*

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**Concerns about climate change have driven research on the production of lipid-derived biofuels as an alternative and renewable liquid fuel source. Using oleaginous yeasts for lipid synthesis creates the potential for cost-effective industrial-scale operations due to their ability to reach high lipid titer, yield, and productivity resulting from their unique metabolism. *Yarrowia lipolytica* is the model oleaginous yeast, with the best-studied lipid metabolism, the greatest number of genetic tools, and a fully sequenced genome. In this review we highlight multiomics studies that elucidate the mechanisms allowing this yeast to achieve lipid overaccumulation and then present several major metabolic engineering efforts that enhanced the production metrics in *Y. lipolytica*. Recent achievements that applied novel engineering strategies are emphasized.**

## Highlights

*Yarrowia lipolytica* is a model oleaginous yeast for the production of lipids and lipid-derived biofuels, studies of lipid metabolism, and the biosynthesis of various industrially important metabolites.

Multiomics measurements and *in silico* metabolic modeling for *Y. lipolytica* deepens our understanding of the organism's metabolism and aids in identifying the limiting steps in lipid biosynthesis.

A variety of metabolic engineering attempts to enhance lipid production have been conducted, exploring the overexpression of fatty acid and triacylglyceride synthesis pathways and the deletion of antagonistic degradation pathways.

More recent strategies involve a holistic understanding of limiting factors in lipid biosynthesis. These studies target the bottlenecks identified in previous omics studies and formulate metabolic engineering strategies accordingly.

## Lipid Production and Its Metabolism in *Yarrowia lipolytica*

Contemporary society relies heavily on fossil fuels (petroleum, coal, and natural gas) as the source of energy [1]. However, concerns about climate change have prompted research into the development of renewable liquid fuels [2]. These new technologies need to supply fuels in a cost-effective and sustainable manner while contributing to the reduction of greenhouse gases [3]. Biofuels produced from microbes, primarily bioethanol and biodiesel, are such promising alternatives (Box 1). In particular, lipid-derived biodiesels are garnering much attention due to their high energy density, which makes them superior substitutes for diesel fuels and jet fuels compared with other forms of renewable energy. The cost-effective production of biodiesel relies on several important criteria, including **lipid content** (see Glossary), **lipid titer**, **lipid productivity**, and **lipid yield**. Therefore, oleaginous organisms, which excel at accumulating intracellular lipids, are often chosen as the industrial workhorse. *Y. lipolytica*, an oleaginous yeast belonging to the *Yarrowia* clade [4,5], is widely regarded as the model organism for this purpose [6]. Its lipid metabolism and supporting pathways has been studied extensively, it has a plethora of genetic engineering tools, and its genome has been fully sequenced [7–14]. More recently, the ubiquitous genome editing technique CRISPR–Cas9 has also been demonstrated in *Y. lipolytica*, allowing high-frequency homologous recombination as well as targeted gene insertion and deletion [15,16]. These characteristics enhance the potential of *Y. lipolytica* in achieving economic biodiesel production at an industrial scale.

To engineer *Y. lipolytica* efficiently for enhanced lipid accumulation, a thorough understanding of its metabolism must be developed first. *De novo* lipid synthesis requires cells to coordinate various biochemical pathways that can produce triacylglycerides (TAGs) from glucose or other small-molecule carbon substrates such as sugars, organic acids, and alcohols. This process is activated when nitrogen in the medium becomes scarce, causing a series of regulatory events to cascade. Initially, nitrogen limitation causes a rapid decrease in intracellular AMP levels due

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## Box 1. The Importance of Microbial Biofuels

Due to the concerns regarding climate change and environmental pollution caused by extensive use of fossil fuels, there has been a growing demand for alternative forms of renewable energy. Biofuels produced from engineered microbes (Figure I) represent a promising example.

### First-Generation Biofuels

These are primarily bioethanol produced by microbial fermentation of sugars obtained from food crops such as corn and sugarcane. Commonly used organisms include *Saccharomyces cerevisiae* and *Zymomonas mobilis* [27,28]. In addition, biodiesel produced from the transesterification of FAs fall into this category. The FAs are often extracted from vegetable oils and animal fats [1]. Nearly all-first generation biofuels utilize substrates that are derived from food crops, causing a major competition with food supply.

### Second-Generation Biofuels and Biodiesel

To avoid such an issue, the industry has now shifted its focus towards second-generation biofuel production from nonedible feedstocks such as plant biomass, forestry residues, energy crops (e.g., lignocellulosics), waste oils, crude glycerol, municipal wastes, and gaseous substrates [77]. The end fuel product remains the same compared with the previous generation. One major avenue of research in this area involves the synthesis of microbial biolipids as a substrate for biodiesel production. The lipids are accumulated either *de novo* or *ex novo* by oleaginous microorganisms and can be converted downstream into FA methyl esters (FAMEs) (i.e., biodiesel) using either acid- or base-catalyzed transesterification. These FAME molecules exhibit chemical and physical properties similar to those of conventional diesel and do not require extensive modification before use [68]. Furthermore, biodiesel has a high energy density, comparable with other forms of renewable energy, and is biodegradable, nontoxic, and essentially free of sulfur and aromatic components [69,70].

### Oleaginous Organisms

These organisms are classified as bacteria, yeast, or microalgae capable of accumulating more than 20% of their cell dry mass as lipids. Oleaginous yeasts are the best studied due to their fast growth rate and ability to reach high biomass densities, which is crucial for fast and abundant lipid production. Examples of oleaginous yeast species include *Rhodosporidium*, *Lipomyces*, *Candida*, and *Yarrowia* [11,71,72]. Typically in oleaginous yeasts, lipids are mostly stored in the form of triacylglycerols in special organelles called LBs.

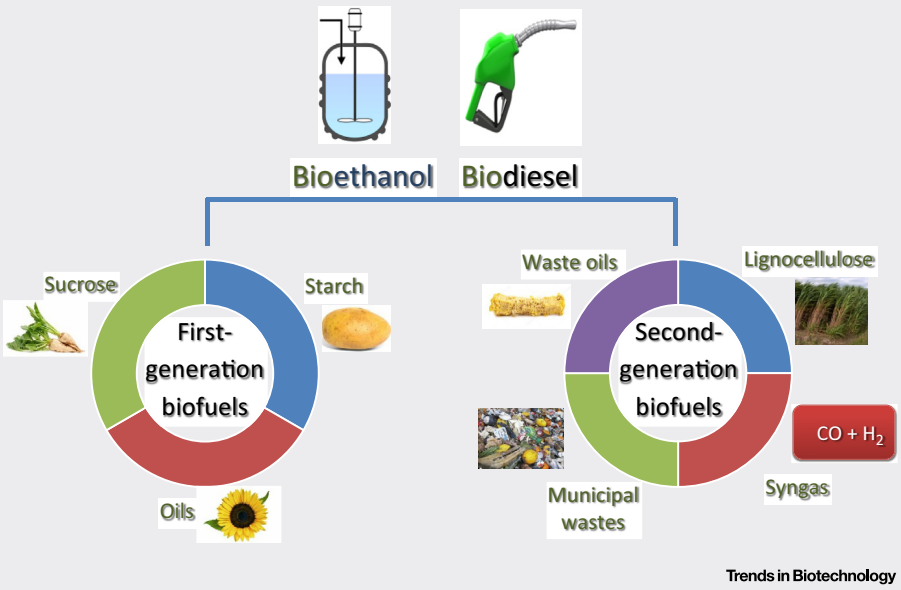


Figure I. A Comparison between First- and Second-Generation Biofuels.

## Glossary

**Acetate kinase (*ACK*):** catalyzes the formation of acetyl phosphate from acetate.
**Acetyl-CoA carboxylase (*ACC*):** carboxylates acetyl-CoA to malonyl-CoA.
**Acetyl-CoA synthetase (*ACS*):** catalyzes the production of acetyl-CoA from acetate.
**AMP deaminase 1 (*AMPD1*):** converts AMP into inosine monophosphate.
**ATP-citrate lyase (*ACL*):** catalyzes the formation of acetyl-CoA and oxaloacetate from citrate.
***DGA1*, *DGA2*:** DAG acyltransferases; catalyze the terminal step of TAG formation.
***FAA1*:** long-chain FA-CoA ligase 1; esterifies long-chain FAs into active CoA thioesters.
***FAS1*, *FAS2*:** FA synthases; synthesize palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH.
**Fatty acid desaturase 2 (*FAD2*):** required for linoleic acid synthesis.
***GapC*:** glyceraldehyde 3-phosphate dehydrogenase; catalyzes oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using the cofactor $NADP^+$.
***GPD1*:** NAD-dependent G3P dehydrogenase; key enzyme in glycerol synthesis.
***GSY1*:** glycogen synthase; transfers the glycosyl residue from UDP-Glc to alpha-1,4-glucan.
**Lipid content:** grams of lipid per gram CDW.
**Lipid productivity:** grams of lipid per liter of culture volume per hour.
**Lipid titer:** grams of lipid per liter of culture volume.
**Lipid yield:** grams of lipid per gram of carbon substrate consumed.
***LRO1*:** triacylglycerol formation enzyme; transfers acyl groups from the *sn*-2 position of a phospholipid to DAG.
***MAE*:** mitochondrial malic enzyme; catalyzes oxidative decarboxylation of malate to pyruvate while providing NADPH.
***MCE2*:** cytosolic $NADP^+$-dependent malic enzyme.
**Metabolic flux analysis (MFA):** an experimental fluxomics technique used to determine the production

to **AMP deaminase 1 (*AMPD1*)** recycling the amine group. Low AMP concentration causes the inhibition of isocitrate dehydrogenase (*IDH*). This leads to downregulation of the tricarboxylic acid (TCA) cycle, accumulation of citrate in the mitochondria, and export of excess citrate from the mitochondria to the cytosol. In the next step, citric acid is cleaved by **ATP-citrate lyase (*ACL*)** into cytosolic acetyl-CoA and oxaloacetate. Overall, this pathway, distinct from the pyruvate–acetaldehyde–acetate pathway used by conventional yeasts, is characteristic of oleaginous organisms, and it allows the formation of cytosolic acetyl-CoA, which is the starting material for lipid synthesis.

Once cytosolic acetyl-CoA is formed, it can be used as a two-carbon building block for fatty acid (FA) synthesis. The elongation of FAs begins with the action of **acetyl-CoA carboxylase (*ACC*)**, catalyzing the transformation of acetyl-CoA into malonyl-CoA. The FA synthase complex (*FAS1* and *FAS2*) then acts on malonyl-CoA to build C16 acyl-ACPs, which are then transported into the endoplasmic reticulum (ER) for further elongation and desaturation [17–19]. Desaturases in the ER produce palmitoleic (16:1) and oleic (C18:1) acids due to ***OLE1*** activity or linoleic acid (18:2) due to **FA desaturase 2 (*FAD2*)** activity. The overall stoichiometry of FA synthesis is

$$\frac{n}{2}\mathrm{AcCoA} + \frac{n}{2}\mathrm{ATP} + (n - 2 + m)\mathrm{NADPH} \rightarrow \mathrm{C}(n:m)\mathrm{FA}, \quad [1]$$

where $n$ and $m$ are the chain length and degree of unsaturation, respectively. This process relies heavily on the availability of ATP and NADPH.

To form TAGs, three FAs condense with one glycerol 3-phosphate (G3P) through the Kennedy pathway [9]. Initially, G3P is acylated by G3P acyltransferase (***SCT1***) to form lysophosphatidic acid (LPA). LPA is subsequently acylated by LPA acyltransferase (***SLC1***), which produces phosphatidic acid (PA). PA is then dephosphorylated by PA phosphatase (*PAP*) and diacylglycerol (DAG) is released. Finally, TAGs are synthesized either by DAG acyltransferase (***DGA1*** or ***DGA2***), which uses acyl-CoA as the final acyl group donor, or by phospholipid DAG acyltransferase (***LRO1***), which utilizes glycerophospholipids as the acyl group donor. These reactions occur between the ER and the lipid body (LB) surface, where the relevant enzymes have been located [20]. The pathways involved in lipid synthesis are summarized in Figure 1.

In addition to synthesis pathways, lipid degradation pathways are also relevant in engineering *Y. lipolytica* for lipid overproduction as they often become targets for deletion. Free FAs (FFAs) can be released through the action of the intracellular lipase ***TGL4*** [11,21]. The released FFAs must be activated to acyl-CoAs through the enzyme acyl-CoA synthetase (***FAA1***) to be further processed biologically [22]. The degradation of these long-chain acyl-CoAs by beta-oxidation (Figure 2) occurs in the peroxisome. There are four reactions occurring in a cyclic manner in beta-oxidation where the combined effect is to release an acetyl-CoA molecule, thereby shortening the acyl chain by two carbon units. In *Y. lipolytica* six acyl-CoA oxidases (***POX1–6***), which catalyze the first reaction, were identified, each having different chain-length preferences [11,23,24]. The second and third reactions are catalyzed by a multifunctional enzyme (***MFE2***) and the last reaction is catalyzed by 3-ketoacyl-CoA thiolase (***POT1***) [11,25,26]. These enzymes are often the targets for deletion.

## Understanding the Biology of Lipid Synthesis through Multiomics Approaches

To further expand on the knowledge of *Y. lipolytica* lipid metabolism and to identify bottlenecking locations in the metabolic network, quantitative biological measurements have become increasingly popular in the investigation of this organism. These studies often guide subsequent

and consumption rates of metabolites.
***MFE2*:** peroxisomal multifunctional enzyme type 2; acts on the beta-oxidation pathway.
***MIG1*:** transcription factor involved in glucose repression; regulated by the SNF1 kinase.
**Multiomics:** a new approach in biological systems combining data from genomics, transcriptomics, proteomics, lipidomics, and metabolomics; reveals the holistic picture of the investigated system.
***OLE1*:** Δ9 FA desaturase; required for monounsaturated FA synthesis.
**Peroxisomal carnitine acetyl-CoA transferase (*perCAT2*):** transfers acetyl groups to carnitine, which can then be shuttled across membranes.
***PEX3*, *PEX10*, *PEX11*:** peroxisomal membrane proteins required for peroxisome biogenesis.
***POT1*:** 3-ketoacyl-CoA thiolase; cleaves acetyl-CoA from 3-ketoacyl-CoA during beta-oxidation.
***POX1–6*:** fatty acyl-CoA oxidases; involved in the beta-oxidation pathway.
***SCT1*:** G3P/dihydroxyacetone phosphate *sn*-1 acyltransferase.
***SLC1*:** 1-acyl-sn- G3P acyltransferase; catalyzes the acylation of LPA to form PA.
***SNF1*:** AMP-activated S/T protein kinase; acts as a regulatory protein for glucose-repressed transcription, heat shock, sporulation, and peroxisome biogenesis.
**Stearyl-CoA desaturase (*SCD*):** desaturates fatty acyl-CoAs at the Δ9 position.
***SUC2*:** *Saccharomyces cerevisiae* invertase responsible for sucrose hydrolysis into glucose and fructose.
***TGL3*:** triacylglycerol lipase; in *Yarrowia lipolytica* serves as a regulatory protein for TGL4.
***TGL4*:** lipase 4; releases FAs from TAGs.
***XPKA*:** xylulose 5-phosphate phosphoketolase; forms acetyl phosphate from xylulose 5-phosphate.
***yIYEF*:** ATP-NADH kinase with phosphorylation activity of both NADH and $NAD^+$ to produce NADPH and $NADP^+$.

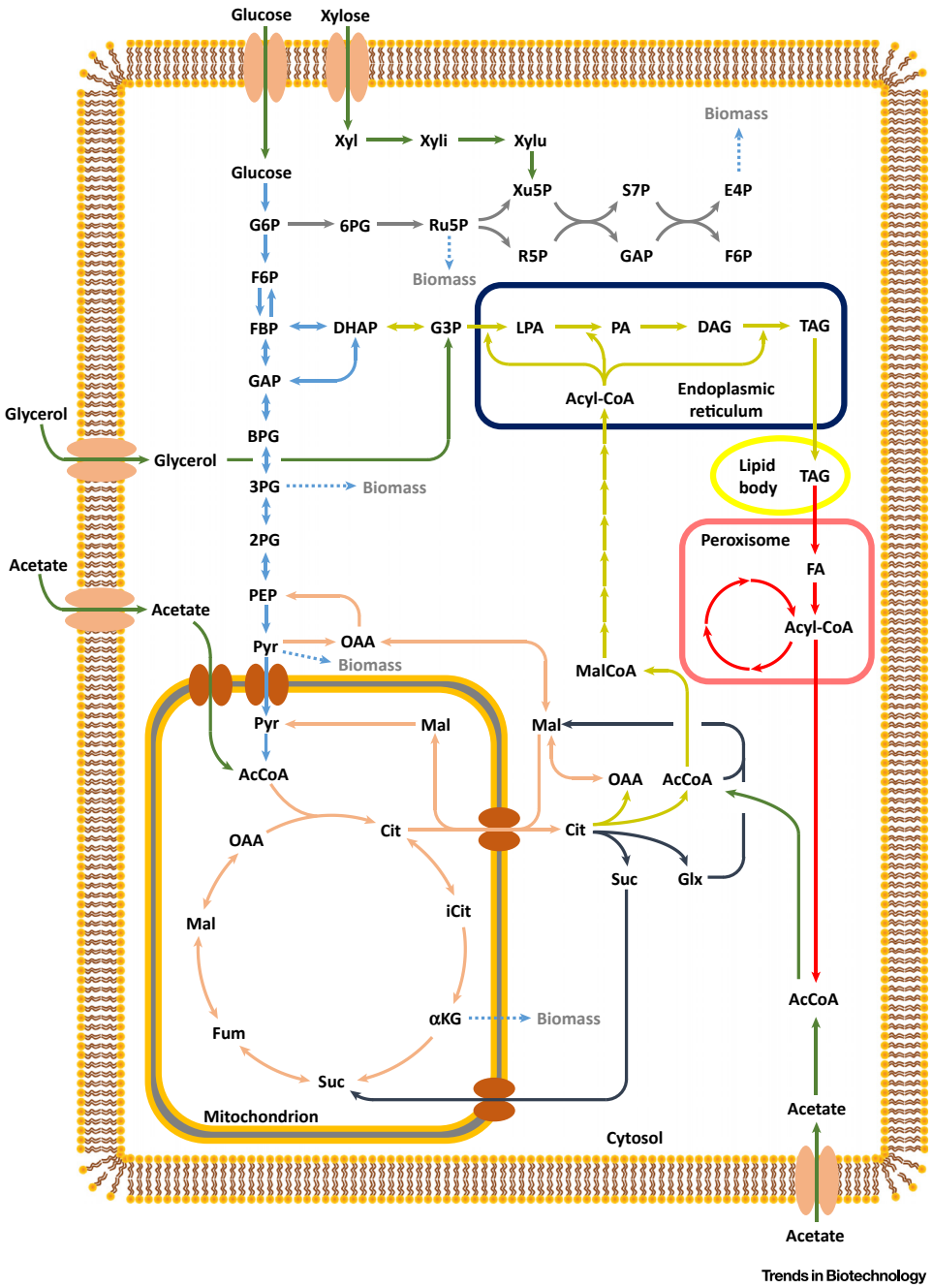


Figure 1. Overview of *Yarrowia lipolytica* Lipid Synthesis Metabolism. Differently colored arrows are used to represent different metabolic pathways: green, substrate incorporation reactions; blue, glycolysis/gluconeogenesis; orange, tricarboxylic acid (TCA) cycle and related anaplerotic reactions; gray, pentose phosphate pathway; dark blue, glyoxylate shunt; yellow, triacylglyceride (TAG) synthesis reactions; red, beta-oxidation pathway. Various intracellular organelles (mitochondrion, peroxisome, endoplasmic reticulum, and lipid body) and their relations to the metabolic pathways are also depicted.

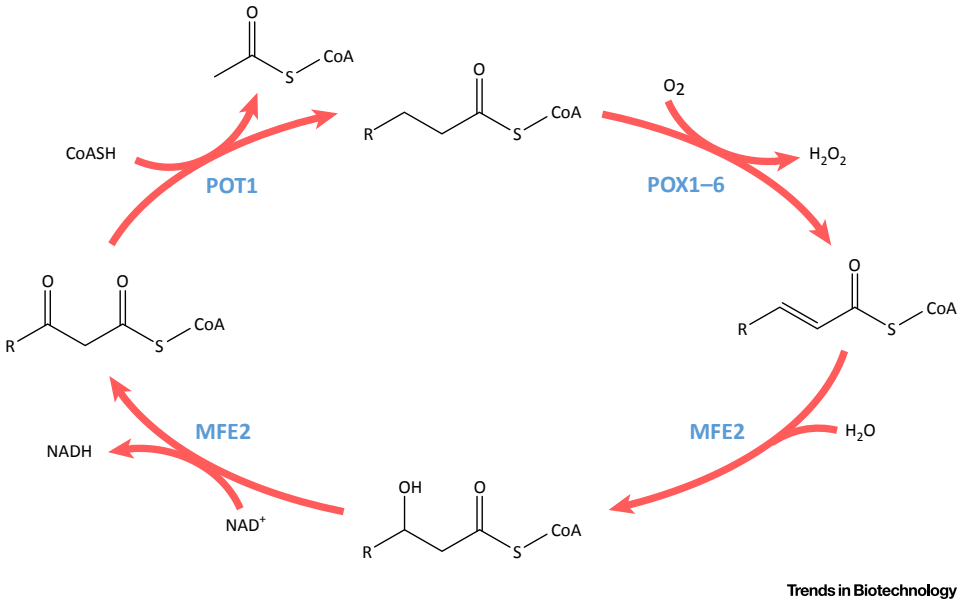


Figure 2. Beta-Oxidation Pathway, Which Degrades Fatty Acids into Acetyl-CoA in a Cyclic Manner.

engineering strategies in achieving the best results. Wasylenko and colleagues performed $^{13}C$ **metabolic flux analysis (MFA)** on *Y. lipolytica* cultured on glucose and compared the flux distribution between a wild-type strain and an engineered strain [12]. The authors concluded that the availability of NADPH is limiting lipid synthesis. By comparing how fluxes change between the two strains, they were also able to deduce that the lipogenic NADPH is synthesized almost exclusively from the oxidative pentose phosphate pathway (PPP). This corroborated previous genomic studies showing that *Y. lipolytica* did not have a cytosolic copy of malic enzyme [29], which makes this yeast unique compared with other oleaginous organisms. A similar study analyzed the flux distribution of *Y. lipolytica* cultured on acetate [13]. It demonstrated the importance of gluconeogenesis in supporting both biomass precursor synthesis and lipogenic NADPH synthesis. A regulatory node that controls the flux through gluconeogenesis was also identified.

Many other analyses have also elucidated important mechanisms of lipid metabolism in *Y. lipolytica*. The approaches applied include genome-scale model reconstruction and analysis as well as transcriptomic, metabolomic, lipidomic, and proteomic measurements [29–34]. A transcriptomic analysis performed by Morin and colleagues demonstrated that the expression levels of many genes differ significantly depending on whether *Y. lipolytica* was actively dividing or synthesizing lipids [29]. Furthermore, they concluded that lipid accumulation could be a consequence of passive carbon flux rerouting to cytosolic acetyl-CoA as opposed to being controlled at the transcriptomic level. Liu and colleagues [31] observed a similar phenomenon where excess carbon directed to lipid biosynthesis could be a consequence of glycolysis and TCA cycle activity imbalance. However, genes related to protein synthesis, including ones encoding ribosomal subunits and translation initiation and elongation factors, were actively downregulated [31]. This observation was confirmed by **multiomics** analysis of *Y. lipolytica* strains exhibiting a lipid-overproducing phenotype under carbon- and nitrogen-limiting conditions [30]. In particular, lipid accumulation did not involve transcriptional regulation of lipid

metabolism but was clearly associated with the regulation of amino acid biosynthesis. Genes related to amino acid metabolism were downregulated, whereas those involved in protein turnover and autophagy, which provides alternative supplies of nitrogen, were overexpressed. An especially important factor that affects lipid synthesis appeared to be leucine metabolism [34]. Interestingly, the authors provided evidence pointing towards several specific regulations that are associated only with nitrogen limitation in combination with *DGA1* overexpression, demonstrating how metabolic engineering of the cells could alter gene regulation. Finally using proteomic methods, Pomraning and colleagues [33] showed that *ACL*, *ACC*, and lecithin cholesterol acyl transferase are phosphorylated during nitrogen limitation [33]. This observation suggested the importance of post-translational modification in the regulation of lipid accumulation.

## Enhancing Lipid Production Metrics through Metabolic Engineering

Wild-type strains of *Y. lipolytica* are not the most efficient producers of lipids. For instance, *Rhodosporidium toruloides* can naturally accommodate 70% lipid content in the cell, whereas *Y. lipolytica* typically achieves 20–40% natively [35–37]. Nevertheless, the advantage of using *Y. lipolytica* for lipid production lies in the ability to alter its metabolism [14,16,38]. Studies focused on improving lipid production in *Y. lipolytica* have expanded considerably in the past several years and various research groups have applied different engineering strategies (Box 2).

Lipid degradation, as an antagonistic pathway to lipid synthesis, hinders lipid accumulation, and hence related genes have become the main targets for deletion [11,24]. For instance, *MFE2* deletion was performed in engineered strains and yielded a lipid-overaccumulation phenotype [26,39,40]. Similarly, genes encoding proteins responsible for peroxisome biogenesis, ***PEX3***, ***PEX10***, and ***PEX11***, have been deleted to abolish beta-oxidation activity entirely [41,42]. The deletion of cellular metabolism regulators can also benefit lipid production [43,44]. As an example, *Y. lipolytica* strains with an ***SNF1*** deletion accumulated FAs constitutively, reaching amounts that are 2.6-times higher than the wild type. Similar improvements were observed with the deletion of *SNF1* in a *Y. lipolytica* strain engineered to produce omega-3 eicosapentaenoic acid. These strains showed a 52% increase in EPA titers [7.6% of cell dry weight (CDW)] over the control [43]. Despite this success, the mechanism of how *SNF1* regulates the lipid synthesis pathway has not been resolved. A similar observation was found for strains with ***MIG1*** gene disruption [44]. The mutant cells exhibited more LBs than its parental strain as the lipid content increased to 48.7%. The *MIG1* disruption was hypothesized to have downregulated *MFE2* and upregulated other genes relevant to lipid biosynthesis. Finally, based on the assumption that glycogen and TAG synthesis compete with each other since they are the two major carbon storage units, Bhutada and colleagues performed null mutations on glycogen synthase (***GSY1***), achieving 60% higher amounts of TAGs compared with the wild-type strain [45].

Although the work listed above demonstrated the success of targeted gene deletion for lipid overaccumulation, performing knockouts in *Y. lipolytica* is challenging as this yeast preferentially uses the nonhomologous end-joining (NHEJ) mechanism for DNA repair over homologous recombination (HR) [46]. By contrast, gene overexpression can be conducted readily. A simple but efficient method for improving lipid production in *Y. lipolytica* involves the 'push-and-pull' strategy based on the overexpression of *ACC1* and *DGA1* [47]. The combined overexpression of the two genes increased lipid production significantly, reaching 61.7% lipid content, with the overall yield and productivity from glucose being 0.195 g/g and 0.143 g/l/h, respectively, in bioreactors.

## Box 2. Different Approaches to Improve Lipid Biosynthesis in *Yarrowia lipolytica*

Lipid biosynthesis can be improved in several different ways (Figure I). Each uses a unique approach but they end with the same ultimate goal of maximizing intracellular lipid accumulation.

(i) The traditional method of research involves wild-type strains and optimization of medium composition as well as process parameters such as temperature, pH, and aeration.

(ii) Shortly after the development of genetic tools, metabolic engineering has emerged as a means to alter an organism's function for various needs, including improved lipid synthesis. This takes into consideration the known genes and enzymes from the lipid biosynthesis and related pathways, changing their expression levels through overexpression or deletion and modulating the enzymatic properties.

(iii) Now, the availability of sequenced and annotated genomes (genomics) combined with methods for intra- and extracellular metabolite detection (metabolomics), analysis of entire sets of proteins (proteomics), and quantification of the transcriptome (transcriptomics) has allowed scientists to study in detail the lipid metabolism of *Y. lipolytica* as well as its regulation. The combination of different techniques that complement one another gave rise to a new discipline called systems biology.

(iv) *In silico* metabolic modeling involving the reconstruction of genome-scale models with certain software allowed the prediction of bottlenecking steps without performing large-scale wet-laboratory experiments. These methods, when properly implemented, can often lead to highly accurate predictions of how certain metabolic modifications can change an organism's phenotype. Hence, researchers often employ modeling to guide engineering.

(v) By applying *in silico* modeling and systems biology along with metabolic engineering, new strains with higher lipid synthesis potential can be constructed. Another round of process optimization will then need to be conducted to tailor the fermentation to the new strain. Several iterations may be required to achieve the full capacity of the system.

(vi) Currently the development of synthetic biology has greatly advanced the engineering of *Y. lipolytica* and other biological systems. Drawing principles from other engineering fields, researchers can now build artificial and tunable biological circuits, which significantly facilitate the channeling of carbon fluxes into the desired lipid and lipid-based molecule biosynthesis pathways.

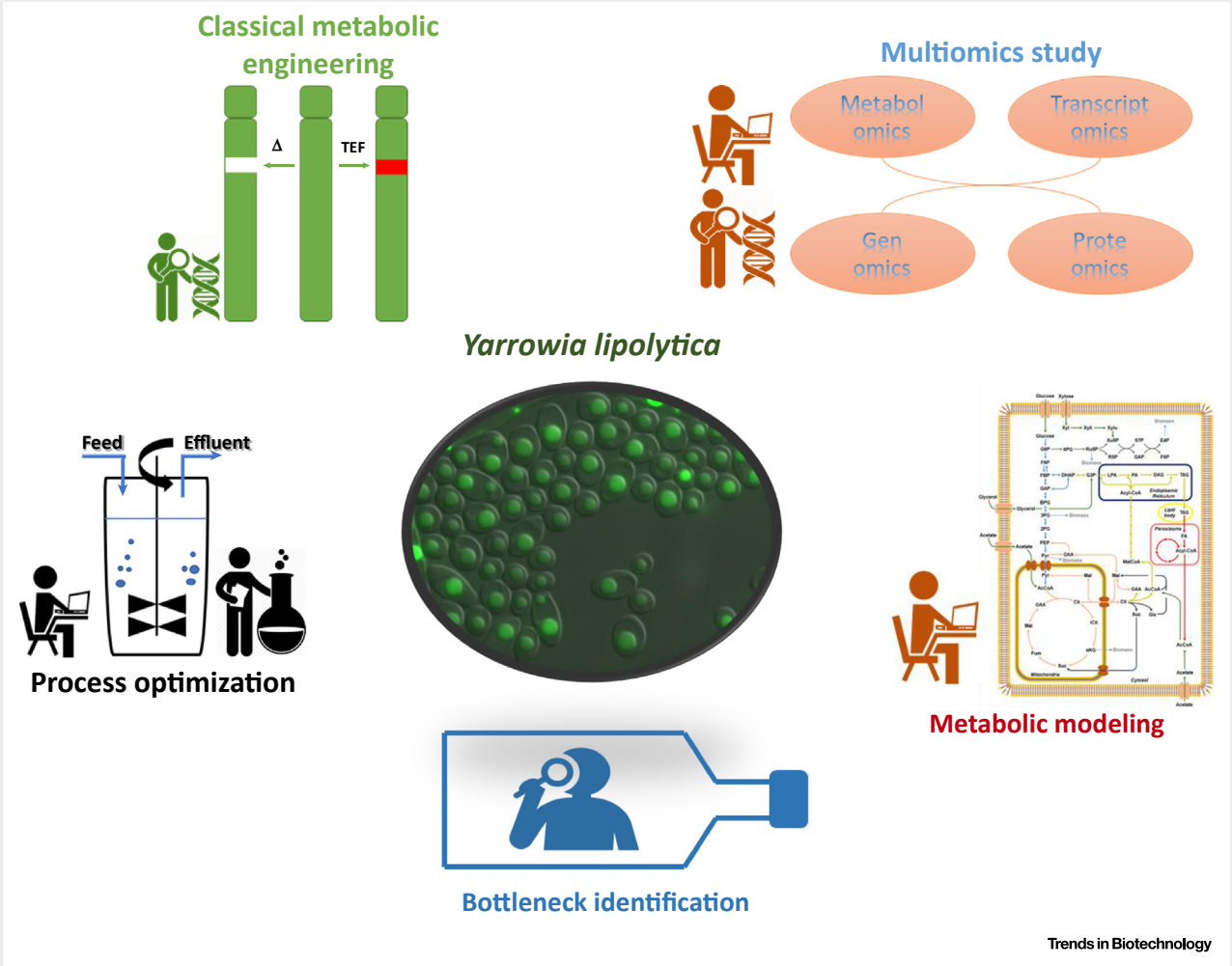


Figure I. Schematic Representation of the Different Ways to Boost Lipid Biosynthesis in *Yarrowia lipolytica*.

The combination of gene overexpression and deletion to heavily modify *Y. lipolytica* lipid-related metabolism prevailed in the earlier stages of strain development [48,49]. Lazar and colleagues combined the deletion of *POX1–6* and *TGL4* with the overexpression of *GDP1* and *DGA2* [39]. The resulting strain had diminishing FA degradation flux and increased intracellular G3P concentrations for enhanced TAG synthesis. These modifications were further expanded with the overexpression of hexokinase for improved fructose metabolism and the introduction of invertase for better sucrose utilization [48]. The resulting strain produced 9.15 g/l of lipids, with a lipid content of 26% from sucrose as the sole carbon source after 96 h culture time. The same strain was also demonstrated to successfully produce lipids from molasses and crude glycerol [49]. The highest lipid content, 40% of CDW, was obtained with a volumetric productivity of 0.43 g/l/h during continuous culture with glycerol. Gajdoš and colleagues engineered strains using a similar strategy [50]. When multiple copies of the native *Y. lipolytica DGA2* gene under the constitutive *TEF1* promoter were introduced into a strain where four acyltransferases (*Δdga1*, *Δdga2*, *Δlro1*, and *Δare1*) were deleted, lipid accumulation was improved. The modifications were then combined with *MFE2* deletion and ***SUC2*** overexpression for sucrose utilization. Both strains accumulated more than 40% of lipids content wise from sucrose, which was a twofold increase compared with the wild type. A more complex study performed by Blazeck and colleagues coupled combinatorial multiplexing of lipogenesis targets with phenotypic induction [26]. The authors analyzed overexpression of *AMPD*, *ACL*, and ***MAE*** for their potential to increase acetyl-CoA and NADPH supply. These modifications were multiplexed with overexpression of *DGA1* and *DGA2* and deletions of *MFE2* and *PEX10*. After characterization, the strain with *DGA1* overexpression and *MFE2* and *PEX10* deletion was determined to be most efficient. This engineered *Y. lipolytica* strain increased *de novo* lipid accumulation by >60-fold in lipid titer (25 g/l) compared with the parental strain, with lipid content approaching 90%.

Heterologous gene expression in *Y. lipolytica* has also been largely successful in engineering the lipid-overproducing phenotype. For instance, the *DGA1*-encoded enzyme from *R. toruloides* and *DGA2*-encoded enzyme from *Claviceps purpurea* were found to outperform the native ones of *Y. lipolytica* [51]. Overexpression of these heterologous genes was combined with the deletion of ***TGL3***, which encodes the regulatory protein for TAG lipase [21]. These combined modifications resulted in a lipid content of 77% and a yield of 0.21 g/g during batch culture [51]. Applying fed-batch mode with glucose as the only substrate allowed the strain to produce 85 g/l of lipids at a productivity of 0.73 g/l/h. Similarly, overexpression of heterologous *ACL* from *Mus musculus*, with a lower $K_M$ value for its substrate, was shown to improve TAG accumulation [26,52]. The authors reported an increase in lipid content from 7.3% to 23.1% resulting from this modification, indicating the importance of improving the supply of cytosolic acetyl-CoA for lipid synthesis [26,52].

Finally, there have been numerous studies focusing on expanding the substrate range of *Y. lipolytica*. Since a comprehensive review on this topic has been covered previously [53], we only touch on two new studies that demonstrated lipid synthesis on inexpensive and renewable feedstocks. The first study, by Niehus and colleagues [54], showed robust growth of highly engineered *Y. lipolytica* strains on lignocellulosic hydrolysates containing glucose and xylose. The authors expressed phosphoketolase (***XPKA***) and **acetate kinase (*ACK*)** along with the enzymes required for xylose assimilation (xylose reductase, xylitol dehydrogenase, and xylulose kinase) for the ultimate conversion of xylose into acetate, which can be further converted to lipogenic acetyl-CoA by **acetyl-CoA synthetase (*ACS*)**. Combining these genetic modifications with those mentioned above, they reached a lipid titer of 16.5 g/l with the maximum lipid content of 67% from lignocellulosic biomass hydrolysates alone. The second study, performed

by Xu and colleagues, demonstrated efficient lipid production from acetic acid using a semi-continuous, dynamically controlled bioreactor scheme with cell recycle [55]. They achieved simultaneous concentration and conversion of low-strength acetic acid (<30 g/l) into intracellular lipids. Exceptional results were reported where 115 g/l of lipids was produced after continuous feeding of 30 g/l acetic acid for 144 h. These results illustrate how *Y. lipolytica* can play an important role in upgrading the carbon sources from dilute waste streams to concentrated, value-added products biologically. Table 1 (Key Table) shows a summary of all of the studies discussed so far.

## Novel Strategies to Maximize the Lipid Production Potential of *Y. lipolytica*

In addition to the modulation of gene targets listed above, a number of novel strategies have emerged in the past several years [56–58]. These studies emphasize the importance of understanding the limiting factors of lipid production and have built on conclusions formulated in previous omics studies. The lipid production metrics from these studies are among the highest in the field, indicating the success of these approaches.

In the work by Qiao and colleagues, the potential allosteric inhibition of *ACC1* by saturated FAs was taken into consideration [56,59–61]. This issue was overcome through the conversion of saturated to monounsaturated FAs by the expression of stearoyl-**CoA desaturase (*SCD*)**, which is a central metabolic regulatory enzyme that catalyzes Δ9-desaturation of palmitoyl-CoA and stearoyl-CoA to palmitoleoyl-CoA and oleoyl-CoA, respectively [62]. The authors observed significant phenotypical changes after introducing this enzyme into the previously mentioned lipid-overproducing strain with *ACC1* and *DGA1* overexpression [56]. The level of oleic acid (C18:1) increased to 71% and palmitoleic acid reached 8% of the total FA pool. The concentration of neutral lipids at the end of a bioreactor culture reached 55 g/l with the overall yield of 0.234 g/g from glucose and productivity of 0.707 g/l/h. The yield achieved was 84.7% of the theoretical maximum. The authors hypothesized that the action of *SCD* released the inhibitory effect on *ACC1* caused by palmitoyl-CoA and stearoyl-CoA by pushing these acids to monounsaturated versions. The monounsaturated acyl-CoAs were then rapidly incorporated into TAG through the action of *DGA1* without further inhibition of the desaturase itself.

Ledesma-Amaro and colleagues focused on the secretion of intracellular FAs into the culture medium followed by *in situ* extraction using an organic layer [63]. They engineered two unique strategies to achieve this. The first involved the deletion of several genes related to free FA activation and degradation, which were *FAA1* and *MFE2*, respectively. As a result, the cells could no longer convert the immobilized FFAs into acyl-CoAs or utilize them in beta-oxidation, leading to FFA accumulation and subsequent secretion. The second approach reported in the study aimed at abolishing LB formation entirely and redirecting FA synthesis to the cytosol, thereby mimicking the bacterial pathway. The authors constructed a strain with deletions in four genes (*Δare1*, *Δdga1*, *Δdga2*, and *Δlro1*) that exhibited this phenotype and, combined with deletions in *FAA1* and *MFE2*, this resulted in significantly more FFA secretion. Using their best-performing strain and optimized reactor conditions, an extracellular FA concentration of 10.4 g/l was achieved, which amounted to a total of 1.2 g of lipid synthesized per gram CDW, well exceeding the storage limit of individual cells.

In a separate study, Xu and colleagues focused on lifting the bottleneck of lipogenic acetyl-CoA availability [57]. They noted that the action of *ACL* provides large amounts of cytosolic acetyl-CoA only when nitrogen becomes limited. However, a nitrogen source is required at the beginning of the culture to sustain high biomass production before lipid accumulation. As a

## Key Table

Table 1. Summary of the Lipid Production Metrics and Strain Genotypes in Various Studies

| Genotype | Lipid titer (g/l) | Lipid productivity (g/l/h) | Lipid yield (g/g) | Lipid content (%) | Substrate | Refs |
|---|---|---|---|---|---|---|
| Flask Cultures | | | | | | |
| Y4184 (ATCC 20362): Δsnf1 | 1.10 | – | – | 30.0 | Glucose | [43] |
| PO1h: mmACL | 1.70 | – | – | 23.1 | Glycerol | [52] |
| ACA-DC 50109: Δmig1 | 2.44 | – | – | 48.7 | Glucose | [44] |
| PO1d: Δtgl4, Δgsy1, DGA2, GPD1 | 2.62 | – | | 52.4 | Glycerol | [45] |
| PO1d: Δpox1–6, Δtgl4, DGA2, GPD1, (riceAlphaAmylase, glucoamylase)x2 | 2.84 | – | 0.130 | – | Starch | [73] |
| PO1d: Δdga1Δlro1Δare1Δdga2, Δmfe2, DGA2x3, scSUC2 | 6.70 | – | – | 49.0 | Sucrose | [50] |
| Bioreactor Cultures | | | | | | |
| PO1d: GAL1,7,10E,10M | 3.22 | – | 0.056 | 16.6 | Galactose | [74] |
| PO1d: Δpox1–6, Δtgl4, DGA2, GPD1, HXK1, scSUC2 | 9.15 | – | 0.063 | 26.0 | Sucrose | [48] |
| PO1d: Δfaa1 Δmfe1 DGA2 TLG4 klTGL3 | 10.40 | – | 0.200 | 120.4 | Glucose | [63] |
| E26 (Δpex10, Δ mfe1, DGA1, evolved): ssXYL1, ssXYL2, starved | 15.00 | 0.190 | – | – | Xylose | [75] |
| PO1d: Δpox1–6, Δtgl4, DGA2, GPD1, XPKA, ACK, XK, XDH, XR | 16.50 | 0.185 | 0.344 | 67.0 | Glucose/xylose | [54] |
| PO1g: ACC1, DGA1 | 17.60 | 0.143 | 0.195 | 61.7 | Glucose | [47] |
| PO1d: Δpox1–6, Δtgl4, DGA2, GPD1, HXK1, GAL1,7,10E,10M, scSUC2, kmINU1 | 23.82 | 0.158 | 0.160 | 43.0 | Inulin | [40] |
| PO1d: Δpox1–6, Δtgl4, DGA2, GPD1, HXK1, scSUC2 | 24.20 | 0.430 | 0.100 | 40.0 | Molasses/glycerol | [49] |
| PO1f: Δpex10, Δmfe2, DGA1 | 25.00 | 0.210 | – | 71.0 | Glucose | [26] |
| E26 (Δpex10, Δmfe1, DGA1, evolved): Δmga2 | 25.00 | 0.145 | 0.213 | – | Glucose | [31] |
| PO1d: Δpox1–6, Δtgl4, GDP1, DGA2, ssXR, ssXDH, ylXK | 50.50 | – | 0.120 | 42.0 | Xylose/glycerol | [76] |
| PO1g: ACC1, DGA1, SCD | 55.00 | 0.707 | 0.234 | 67.0 | Glucose | [56] |
| PO1g: ACC1, DGA1, scCAT2 | 66.40 | 0.565 | 0.229 | 70.0 | Glucose | [57] |
| NS18: rtDGA1, cpDGA2, Δtgl3 | 85.00 | 0.730 | 0.200 | 73.0 | Glucose | [51] |
| PO1g: ACC1, DGA1, caGapC, mcMCE2 | 99.00 | 1.200 | 0.270 | 66.8 | Glucose | [58] |
| PO1g: ACC1, DGA1 | 115.00 | 0.800 | 0.160 | 59.0 | Acetic acid | [55] |

result, *Y. lipolytica* cultivation is generally biphasic, requiring a growth phase (nitrogen present) and a lipid production phase (nitrogen depleted), which prolongs the fermentation. To decouple the onset of lipid synthesis from nitrogen availability, the engineering of other pathways that provide cytosolic acetyl-CoA even when nitrogen is present is essential [57]. The most efficient

method was the overexpression of **peroxisomal carnitine acetyltransferase (*perCAT2*)** from *Saccharomyces cerevisiae*. This *perCAT2* activity improved both lipid titer and content compared with the base strain with *ACC1* and *DGA1* overexpression. Coupling of the *perCAT2* reaction with the phosphoketolase (PK)–phosphotransacetylase (PTA) pathway for NAPDH regeneration increased the yield of lipids from glucose to 0.225 during shake-flask cultures. Furthermore, this effort resulted in lipid accumulation from the beginning of the culture and lasted throughout the entire fermentation, regardless of the C/N ratio. During bioreactor cultures the strain produced 66.4 g/l of lipids with a yield of 0.229 from glucose and a productivity 0.565 g/l/h.

A final approach that achieved remarkable lipid production metrics relied on the interconversion of two primary electron carriers in cells: NADH and NADPH [58]. The authors developed a stoichiometric model to analyze the overall metabolic network in lipid production and after validation and sensitivity analysis identified an imbalance in electron cofactors with NADH in excess and NADPH being limiting. This limitation in NADPH impaired lipid synthesis significantly and also confirmed previous studies that identified NADPH as the limiting resource for lipogenesis [12,13]. To convert the excess NADH to NADPH for lipid production, the authors constructed several pathway modules. Two $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenases, ***GapC*** from *Clostridium acetobutylicum* and ***GPD1*** from *Kluyveromyces lactis*, were expressed in an *ACC1–DGA1*-overexpressing background strain. A cytosolic $NADP^+$-dependent malic enzyme, ***MCE2*** from *Mucor circinelloides*, was also introduced to activate the pyruvate–oxaloacetate–malate cycle for further conversion from NADH to NADPH. The results showed that *GapC* and *GDP1* improved lipid yields by 20.0% and 17.8%, respectively. The introduction of malic enzyme also improved the yield by 23%. The *GapC* introduction was further coupled to endogenous $NAD^+$/NADH kinase (***yIYEF***) overexpression, which resulted in multiple functional synthetic pathways working simultaneously converting NADH to NADPH. The best lipid yield achieved by this study reached 0.28 g of lipid per gram of glucose in optimized bioreactors. The conversion of NADH to NADPH reduced the aeration needs of the culture, which contributed to higher cell density under the same aeration capacity. The achieved yield exceeded the theoretical maximum determined for wild-type *Y. lipolytica* strains, thus providing indirect evidence of the excess NADH conversion. The lipid titer, productivity, and yield achieved in this study reached 99 g/l, 1.2 g/l/h, and 0.27 g/g respectively, which are among the highest numbers reported in the literature.

## Concluding Remarks and Future Perspectives

*Y. lipolytica* has emerged as one of the most promising industrial hosts for biodiesel production. Metabolic engineering of this oleaginous yeast for enhanced lipid production has met with considerable success. Early efforts focused on enhancing lipid accumulation by knocking out competing pathways and overexpressing supporting pathways. These investigations were crucial in laying the groundwork for understanding the cellular metabolism of this organism and perfecting the genetic tools. More recently, with the advances in quantitative biology, multiomics measurements allowed a more in-depth understanding of the regulatory mechanisms that exist in lipid metabolism and specific bottlenecks were identified. Novel engineering schemes based on the analysis of the global metabolic network emerged as a consequence, pushing yields to the theoretical limit without sacrificing titer and productivity. These examples demonstrate the power of rational engineering based on a holistic understanding of all relevant cellular processes involved in the synthesis of lipids. We believe that this could be a promising methodology to accelerate the construction of strains with desired phenotypes.

### Outstanding Questions

Is *Yarrowia lipolytica*, the model oleaginous yeast, the best choice for lipid production? Should we focus on engineering this organism because it has established genomic tools or find a better organism with a higher natural capacity to accumulate lipids and develop the tools and techniques for modifications?

Would it be better to overexpress native enzymes where their expression is guaranteed in the native host or to find heterologous enzymes with the same function but potentially catalyze the reaction better? In the latter case, the expression of the enzyme may need additional tuning.

Where and how could we target specific heterologous enzymes into specific subcellular compartments in *Y. lipolytica*? Is it beneficial to create an artificial compartment in the cell to localize all relevant proteins and building blocks to accelerate the synthesis process?

Similarly, would it be feasible to engineer one particular strain to allow it to perform all steps of product biosynthesis? Would it be better to distribute the pathway among various strains to alleviate protein burden?

Haploid strains of *Y. lipolytica* have been the primary focus of research thus far due to ease of genetic manipulation. Is there any merit in using diploid or even polyploid strains for engineering or industrial fermentations? What about natural isolates versus established laboratory strains?

What is the ideal time of expression for various enzymes and pathways? Would it be better to constitutively express all proteins and potentially decouple the processes from fermentation phases or to develop new synthetic regulatory networks for timed expression even at the expense of longer culture time?

Economically, would it be better to develop a microorganism capable of full conversion from raw substrates to finalized products with potentially decreased titers and yields or to separate the substrate pretreatment,

fermentation, and end-product refining into discrete operation units for better control and optimization?

However, most of these successes have stemmed from laboratory environments where defined media are applied. In industrial biotechnology, inexpensive substrates, typically from variable sources, are utilized to minimize operation costs. Although several studies have attempted to address this issue by culturing *Y. lipolytica* on mixtures derived from industrial waste streams, it is unclear whether all engineered strains of *Y. lipolytica* can robustly grow and synthesize lipids under these conditions. Furthermore, the toxicity of compounds present in undefined media could still be a potential issue preventing the industrialization of current processes. To this end, the engineering of detoxification pathways or relevant cellular pumps could boost culture robustness and increase tolerance to inhibitors. Overall, shifting the focus away from using defined, single-carbon-substrate media to using industrially relevant complex substrates should be an important direction to take in future applied research. Accordingly, further engineering of established high-production strains for better robustness and reproducibility is also an important factor to consider (see Outstanding Questions).

Finally, fine-tuning of enzyme expression levels has thus far been limited in this organism. In most cases, overexpression has been conducted under constitutively active promoters such as the *Y. lipolytica TEF1* promoter [47,64,65], which may lead to unnecessarily high enzyme levels and protein burdens. An arsenal of various promoters with a wide spectrum of expression strength is needed at this time to better tailor fluxes through pathways and optimize product synthesis. The construction of synthetic inducible promoters is also an important area that needs exploring. Fortunately, several studies have begun to address these problems [66,67] which may lead to new metabolic engineering strategies in *Y. lipolytica* in the near future.

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