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Published in:
Bioresource Technology

Link to article, DOI:
10.1016/j.biortech.2020.123707

Publication date:
2020

Document Version
Peer reviewed version

Journal Pre-proofs

Review

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PII: S0960-8524(20)30979-2
DOI: https://doi.org/10.1016/j.biortech.2020.123707
Reference: BITE 123707

To appear in: *Bioresource Technology*

Received Date: 14 May 2020
Revised Date: 15 June 2020
Accepted Date: 16 June 2020

Please cite this article as: Wang, J., Ledesma-Amaro, R., Wei, Y., Ji, B., Ji, X-J., Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica*, *Bioresource Technology* (2020), doi: https://doi.org/10.1016/j.biortech.2020.123707

Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica*

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Highlights

- *Y. lipolytica* is a superior industrial host for the production of lipids.
- Biosynthesis and degradation pathways of lipid in *Y. lipolytica* and the potential metabolic engineering targets are introduced.
- Metabolic engineering strategies for increasing lipid accumulation in *Y. lipolytica* are summarized.
- Multi-omics analysis, *in silico* metabolic models, and synthetic biology tools will further push the lipid yields to theoretical limits.
- Perspectives for novel engineering approaches for increasing lipid accumulation in *Y. lipolytica* are discussed.

Abstract

Current energy security and climate change policies encourage the development and utilization of bioenergy. Oleaginous yeasts provide a particularly attractive platform for the sustainable production of biofuels and industrial chemicals due to their ability to accumulate high amounts of lipids. In particular, microbial lipids in the form of triacylglycerides (TAGs) produced from renewable feedstocks have attracted considerable attention because they can be directly used in the production of biodiesel and oleochemicals analogous to petrochemicals. As an oleaginous yeast that is generally regarded as safe, *Yarrowia lipolytica* has been extensively studied, with large amounts of data on its lipid metabolism, genetic tools, and genome sequencing and annotation. In this review, we highlight the newest strategies for increasing lipid accumulation using metabolic engineering and summarize the research advances on the overaccumulation of lipids in *Y. lipolytica*. Finally, perspectives for future engineering approaches are proposed.

Keywords

Yarrowia lipolytica, Metabolic engineering, Lipid accumulation, Triacylglycerides, Biodiesel

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1. Introduction

Concerns about energy supply and the environment have driven the development and utilization of biofuels (Kim et al., 2019a). Recently, lipid-derived biodiesel has attracted widespread attention as a renewable fuel due to its high energy density, clean-burning properties, non-toxicity and biodegradablility (Ma et al., 2018). *Yarrowia lipolytica* is a non-conventional oleaginous yeast with "generally regarded as safe" (GRAS) status (Groenewald et al., 2014). Its genome has been sequenced and annotated (Liu et al., 2015a; Liu and Alper, 2014), metabolic pathways have been extensively studied (Dourou et al., 2018; Beopoulos et al., 2009a), and many genetic engineering tools have been developed (Larroude et al., 2018b; Shi et al., 2018; Gao et al., 2016). Moreover, *Y. lipolytica* can utilize a variety of inexpensive renewable substrates as carbon sources and can accommodate high flux of acetyl-CoA (Magdouli et al., 2017; Ledesma-Amaro and Nicaud, 2016a; Nambou et al., 2014; Poli et al., 2014). These characteristics make *Y. lipolytica* a remarkable industrial host for the production of many products, including lipid-derived biodiesel (Ledesma-Amaro and Nicaud, 2016b; Ledesma-Amaro, 2015); alkanes (Bruder et al., 2019); odd-chain fatty acids (Park et al., 2020; Park et al., 2018); plant-derived terpenoids, such as α-farnesene (Liu et al., 2019c; Yang et al., 2016), carotenoids (Jacobsen et al., 2020; Larroude et al., 2018a), linalool (Cao et al., 2017), and others (Ma et al., 2019); organic acids, such as citric acid (Kamzolova and Morgunov, 2017), isocitric acid (Rzechonek et al., 2019), succinic acid (Li et al., 2018; Li et al., 2017), α-ketoglutarate (Lei et al., 2019; Zeng et al., 2017), pyruvic acid (Kamzolova and Morgunov, 2018) and crotonic acid (Wang et al., 2019); as well as sugar alcohols, such as erythritol (Liu et al., 2019b; Liu et al., 2017c), erythrulose (Carly et al., 2018), isomaltulose (Zhang et al., 2018). Even more value-added functional polyunsaturated fatty acids (PUFAs), such as γ-linolenic acid (Sun et al., 2017), arachidonic acid (Liu et al., 2019a; Liu et al., 2017a; Liu et al., 2017b), eicosapentaenoic acid (Xue et al., 2013), and docosahexaenoic acid (Gemperlein et al., 2019) were produced in engineered *Y. lipolytica* strains.

Metabolic engineering strategies for increasing lipid accumulation in *Y. lipolytica* have expanded considerably. The early efforts focused on: (i) Maximizing the flux towards lipid biosynthesis, such as deleting genes related to β-oxidation involved in the lipid degradation pathway (Dulermo and Nicaud, 2011), tuning the activity of transcription factors involved in lipid metabolism (Leplat et al., 2018), overexpressing fatty acid (FA) and triacylglyceride (TAG) synthesis genes (Tai and Stephanopoulos, 2013), overexpressing functional heterologous lipid synthetic related genes in *Y. lipolytica* (Friedlander et al., 2016; Zhang et al., 2014). (ii) Minimizing the flux towards competing metabolic pathways, such as the inhibition of glycogen and citric acid biosynthesis pathways (Bhutada et al., 2017; Sagnak et al., 2018). The current novel strategies focus on: (i) Expanding the range of available substrates (Johnravindar et al., 2018; Ledesma-Amaro and Nicaud, 2016a; Dobrowolski et al., 2016; Sestric et al., 2014); (ii) Designing new approaches to secrete intracellular fatty acids into the culture medium (Ledesma-Amaro et al., 2016a); (iii) Engineering alternative acetyl-CoA pathways to decouple nitrogen limitation, enabling *Y. lipolytica* to produce large amounts of acetyl-

CoA and accumulate lipids even in the presence of nitrogen (Xu et al., 2016); (iv) Engineering cytosolic NADPH pathways to provide more NADPH (Qiao et al., 2017); (v) Engineering oxidative stress defense pathways to mitigate lipotoxicity and improve cell morphology (Xu et al., 2017b); (vi) Evolutionary metabolic engineering combining adaptive evolution with metabolic engineering, resulting in strains that have both high biomass yields and high lipid production capacity (Liu et al., 2015c); (vii) Screening different metabolic modification targets based on predictions made using genome-scale metabolic models (GEMs) (Kim et al., 2019b). In order to assess the extent to which different engineering approaches increase lipid production, the theoretical limits of process yield must first be determined. Papanikolaou et al. (2011) used stoichiometric ratios to calculate the theoretical limits of lipid production from different substrates. They indicated that in terms of glucose (and similar sugars such as lactose, fructose, etc), about 1.1 moles of acetyl-CoA are generated from 100 grams of glucose (~0.56 moles) catabolized. If all the acetyl-CoA produced is channeled towards lipid synthesis, the maximum theoretical yield of lipid produced per gram of glucose consumed is around 0.32 g/g. Similarly, xylose can be either catabolized by the phosphoketolase reaction, which is the most efficient pathway producing around 1.2 moles of acetyl-CoA per 100 grams of xylose (~0.66 moles) utilized, or through the pentose phosphate pathway, where around 1.0 mole of acetyl-CoA is formed per 100 grams of xylose utilized. Assuming that oleaginous microorganisms utilize exclusively the phosphoketolase pathway to assimilate xylose, the maximum theoretical yield of lipid produced per gram of xylose consumed is around 0.34 g/g. As far as glycerol is concerned, the maximum theoretical yield of lipid is around 0.30 g/g.

In this review, we summarize the recent research advances on lipid accumulation in *Y. lipolytica*. We first introduced the lipid biosynthesis and degradation pathways of *Y. lipolytica* and the potential metabolic engineering targets. Then, we focus on strategies for increasing lipid accumulation in *Y. lipolytica* using metabolic engineering. Finally, we propose future perspectives for new engineering approaches.

2. Biosynthesis and degradation of lipids in *Y. lipolytica*

Intracellular lipids include neutral lipids (TAGs and steryl esters) and non-neutral lipids (free fatty acids (FFAs), glycolipids, and phospholipids). In general, the former serve as an energy reserve for the cell, while the latter perform special physiological functions (Darvishi et al., 2017). Approximately 95% of the intracellular lipids in *Y. lipolytica* are stored in lipid bodies (LBs) in the form of TAGs (Zeng et al., 2018). TAGs can be synthesized via two pathways (Papanikolaou and Aggelis, 2011): (i) *de novo* synthesis, which requires hydrophilic substrates such as sugars, organic acids, or alcohols to produce FA precursors, primarily acetyl-CoA, and integrates them into the *Kennedy* pathway to synthesize TAGs; (ii) *ex novo* biosynthesis, which involves the hydrolysis of hydrophobic substrates (such as alkanes, FFAs, and TAGs) to produce FAs and glycerol, and transports them inside the cell to reassemble them into TAGs.

2.1 *De novo* TAG synthesis

The *de novo* TAG synthesis pathway is stimulated when nitrogen is depleted and carbon is abundant in the medium (Mathiazhakan et al., 2016). The nitrogen depletion process involves a series of regulatory events related to the central metabolism. Initially, the nitrogen depletion leads to increased activity of adenosine monophosphate (AMP) deaminase (*AMPD*), which converts AMP into inosine monophosphate (IMP) and ammonia (Evans et al., 1983). Isocitrate dehydrogenase (*IDH*) is an AMP-dependent dehydrogenase that is inhibited by low AMP concentrations (Botham and Ratledge, 1979). As a result, the tricarboxylic acid (TCA) cycle is downregulated, and the carbon flux from isocitric acid no longer flows toward α-ketoglutarate. The citric acid accumulated in the mitochondria is transported into the cytosol by malate/citrate transferase in the mitochondrial membrane (Palmieri et al., 1996). Subsequently, citrate is cleaved by ATP-citrate lyase (*ACL1* and *ACL2*) to produce acetyl-CoA and oxaloacetate. In this way, *Y. lipolytica* can produce abundant cytosolic acetyl-CoA, which is the direct precursor of FA biosynthesis (Figure 1).

FA biosynthesis in the cytosol involves a series of reactions that convert the precursor acetyl-CoA to long-chain fatty acids. Firstly, malonyl-CoA is generated by carboxylation of acetyl-CoA with acetyl-CoA carboxylase (*ACC1*) (Al-Feel et al., 1992). Then, acetyl-CoA and malonyl-CoA are condensed to acyl-CoA under the action of the FA synthase complex (*FAS1* and *FAS2*). The FA synthase use NADPH as the reducing cofactor, and each step in the elongation of the acyl-CoA chain requires two NADPH molecules. The chain length of naturally synthesized acyl-CoAs is typically 16 or 18 carbon atoms. In the next step, these C16:0 and C18:0 molecules are delivered to the endoplasmic reticulum (ER) for further elongation and desaturation (Ledesma-Amaro and Nicaud, 2016b) (Figure 1).

TAG synthesis generally follows the *Kennedy* pathway, which uses acyl-CoA and glycerol-3-phosphate (G3P) as the direct substrates (Papanikolaou and Aggelis, 2003). TAG assembly begins with the action of G3P acyltransferase (*SCT1*), which catalyzes the conversion of G3P to lysophosphatidic acid (LPA) (Beopoulos et al., 2008). LPA is then further acylated by LPA acyltransferase (*SLC1*) to generate phosphatidic acid (PA). PA is further dephosphorylated to diacylglycerol (DAG) by phosphatidic acid phosphatase (*PAP*) (Silverman et al., 2016). In the final step of TAG assembly, DAG is acylated at the sn-3 position, either by an acyl-CoA-dependent or acyl-CoA-independent reaction, to form TAG (Tai and Stephanopoulos, 2013; Beopoulos et al., 2009b). The acyl-CoA-dependent reaction is catalyzed by DAG acyltransferases (*DGA1* or *DGA2*), with acyl-CoA as the final acyl group donor. The acyl-CoA-independent reaction is catalyzed by phospholipid DAG acyltransferase (*LRO1*), which uses glycerophospholipid as the acyl group donor (Figure 1). Moreover, steryl ester synthetase encoded by *ARE1* has been demonstrated to promote DAG acylation by acting as acyltransferase in an acyl-CoA-dependent reaction. The primary enzymes involved in these pathways are summarized in Table 1.

2.2 *Ex novo* TAG synthesis

Y. lipolytica can also utilize hydrophobic substrates such as TAGs, alkanes, and FFAs to synthesize lipids, and this pathway requires the hydrolysis of hydrophobic substrates. TAGs are hydrolyzed by a large battery of extracellular lipases, which release FAs and glycerol for cellular uptake (Dulermo et al., 2015b). Alkanes entering the cell are hydroxylated in the endoplasmic reticulum (ER) using the cytochrome P450-dependent alkane monooxygenases. The thus formed corresponding fatty alcohols are oxidized to aldehydes by dehydrogenases and further to FAs by long-chain-aldehyde oxidases (Fukuda, 2013). The released FAs can be activated by binding to coenzyme A into acyl-CoAs through the acyl-CoA synthetase (*FAA1*), which are then reassembled into TAGs in the ER. Both the *de novo* and *ex novo* TAG synthesis pathways are summarized in Figure 1.

2.3 TAG degradation

The accumulated intracellular TAGs stored in LBs serve as an energy reserve for the cell. Once carbon is insufficient, the TAG will be degraded to release acetyl-CoA for maintaining cellular metabolism. Initially, FFAs can be released from TAGs under the action of the intracellular lipases *TGL3* and *TGL4* (Dulermo et al., 2013). The released FFAs can be activated to acyl-CoAs by the enzyme *FAA1* and then transported into the peroxisome by the specific transporters Pxa1 and Pxa2 (Dulermo et al., 2015b). Alternatively, the released FFAs can also be transported into the peroxisome and then activated there to acyl-CoAs by acyl/aryl-CoA ligase (*AAL*) (Dulermo et al., 2016). Subsequently, acyl-CoAs are degraded through the β-oxidation pathway in the peroxisomes to release acetyl-CoA. Cyclical β-oxidation encompasses the four steps of oxidation, hydration, dehydrogenation, and thiolysis, which are repeated for each C2 unit. Six acyl-CoA oxidases (*POX1-6*) catalyze the first step, while the multifunctional enzyme *MFE1* catalyzes the second and third steps (Mlickova et al., 2004). The last step is catalyzed by 3-ketoacyl-CoA thiolase (*POT1*) (Berninger et al., 1993). Because β-oxidation occurs in peroxisomes, peroxisome biogenesis is usually downregulated by deleting *PEX3*, *PEX10*, and *PEX11* to prevent the degradation of TAGs in industrial strains (Dulermo et al., 2015a; Xue et al., 2013). Additionally, genes related to the β-oxidation pathway are also often targets for deletion to increase lipid accumulation (Lazar et al., 2018).

3. Enhancing lipid production through classical metabolic engineering

The ability of *Y. lipolytica* to accumulate lipids can be improved by increasing the biosynthetic precursors (such as acetyl-CoA, malonyl-CoA, NADPH, ATP), shuting down the degradation pathways, and adjusting the fermentation conditions (such as culture medium, C/N ratio, pH, inoculation volume, fermentation mode). With the development of metabolic engineering and synthetic biology, the biosynthesis of lipids has been further improved by optimizing the complex metabolic network of *Y. lipolytica*. In the past few years, researchers used a variety of approaches to increase lipid production

in *Y. lipolytica*. These metabolic engineering strategies are summarized in Table 2.

3.1 Tuning endogenous genes involved in lipid metabolism

Lipid-overaccumulation phenotypes can be produced by combining the overexpression of genes related to lipid biosynthesis with the deletion of genes related to lipid degradation (Dulermo and Nicaud, 2011). For instance, Lazar et al. (2014) combined the deletion of *POX1-6* and *TGL4* with the overexpression of *GDP1* and *DGA2*, and the resulting strain JMY3501showed reduced FA degradation and increased intracellular G3P concentration, which improved TAG accumulation. Blazeck et al. (2014) used a combinatorial strategy to synergistically regulate multiple key genes involved in lipid degradation and biosynthesis in *Y. lipolytica*, including *PEX10*, *MFE1*, *AMPD*, *ACL1*, *ACL2*, *MAE*, *DGA1*, and *DGA2*, which generated 57 distinct genotypes. The most effective modifications were the double deletion of *PEX10* and *MFE1* and the overexpression of *DGA1*. After optimizing the bioreactor conditions, the engineered strain produced a lipid titer of 25 grams of lipid per liter of culture, with a lipid content of 71% in cell dry weight, representing a 60-fold improvement over the starting strain. Tai and Stephanopoulos (2013) adopted a push-and-pull strategy by overexpressing *ACC1* and *DGA1* simultaneously. Compared with the starting strain Po1g, the engineered strain exhibited an increase of the lipid content from 8.7 to 41%. After 120 h of fermentation in a 2-L bioreactor, the lipid content of the engineered strain reached 61.7%, with a lipid yield of 0.195 grams of lipid per gram of carbon substrate consumed, and lipid productivity of 0.143 g/(L·h) (grams of lipid per liter of culture per hour). Qiao et al. (2015) adopted a promoter engineering strategy by replacing hp4d with the TEFin promoter to co-overexpress *ACC1* and *DGA1* and overexpress the stearoyl-CoA desaturase (*SCD*) in *Y. lipolytica*. In a 1.5-L bioreactor with glucose as the substrate, the engineered strain produced a lipid content and titer of 67% and 55 g/L, respectively. It is possible that the overexpression of *SCD* can release the potential allosteric inhibition of *ACC1* by saturated fatty acids and increase fatty acid biosynthetic flux by continuously converting inhibitory saturated fatty acids into monounsaturated fatty acids (Flowers and Ntambi, 2008). Moreover, the engineered strain also exhibited a variety of desirable phenotypes, such as fast growth, high sugar tolerance, and high unsaturated fatty acid content (Qiao et al., 2015). Finally, channeling the metabolic flux towards lipid synthesis can also improve lipid accumulation to a certain extent. Bhutada et al. (2017) identified and deleted the *GSY1* gene encoding a glycogen synthetase, and TAG accumulation increased by 60% compared with the wild-type strains, proving that glycogen synthesis is a competing pathway whose elimination promotes the production of neutral lipids.

3.2 Tuning metabolic regulators involved in lipid metabolism

The tuning of cellular metabolic regulators can effectively enhance the carbon flux and facilitate the accumulation of lipids (Leplat et al., 2018; Wang et al., 2018). For instance, the glucose repression regulator *SNF1* is involved in the transition from the growth phase to the oleaginous phase, and can allow the strain to accumulate high levels of lipids even in the presence of nitrogen. Seip et al. (2013) deleted the *SNF1*, resulting

in 2.6-fold higher lipid content than in the wild-type strain. Similarly, the deletion of the glucose repression regulator *MIG1* enhanced the lipid content from 36 to 48.7%. The authors observed that the deletion of *MIG1* resulted in the downregulation of *MFE1* transcription and the upregulation of many genes related to lipid biosynthesis. These changes of the gene expression profile might be responsible for the increased LB formation and lipid content in the gene deletion strain (Wang et al., 2013). In a separate study, Liu et al. (2015b) sequenced the previous evolved strains E13 and E26 and identified the lipogenesis factor *YALI0F26191g* (*UGA2*), which encodes succinate semialdehyde dehydrogenase that takes part in the gamma-aminobutyric acid (GABA) assimilation pathway. The deletion of *UGA2* enhanced the lipid content by 50% compared to the starting strain (Po1f Δ*pex10* Δ*mfe*). The reduction in GABA assimilation could lead to an imbalance in the intermediates of the TCA cycle, thereby reducing the flux through the TCA cycle and channeling more carbon towards the lipogenic pathway. In another similar study, Liu et al. (2015c) discovered that *Mga2p* is a regulator of desaturase gene expression and an effective lipogenesis factor that is involved in regulating the metabolism of unsaturated fatty acids in *Y. lipolytica*. The authors introduced a single amino acid mutation into *Mga2p* and combined it with previous metabolic modifications to obtain a producer strain with a lipid titer of 25 g/L, which showed an imbalance between glycolysis and the TCA cycle can be used as a driving force for lipogenesis.

3.3 Heterologous gene expression in *Y. lipolytica*

Heterologous gene expression in *Y. lipolytica* was successfully used to increase lipid accumulation. For instance, Friedlander et al. (2016) combined the overexpression of *DGA1* from *Rhodosporidium toruloides* and *DGA2* from *Claviceps purpurea* with the deletion of the endogenous *TGL3*, and the resulting strain showed a lipid content of 77% and a lipid yield of 0.21 g/g in batch fermentation. Similar modifications, combining the overexpression of heterologous Δ-15 desaturase (Δ-*15D*) from flax and endogenous genes (*ACC1*, *DGA1*, *SCD*, Δ-*12D*) with the deletion of the endogenous *PEX10* and *MFE1*, led to a superior lipid producer with a lipid content and titer of 77.8% and 50 g/L, respectively, using glucose as substrate in a 5-L stirred-tank bioreactor (Yan et al., 2020). Although *ACL* is considered to be the key enzyme for generating acetyl-CoA in oleaginous yeasts, the overexpression of the native *ACL* gene did not significantly increase the lipid content of *Y. lipolytica* (Blazeck et al., 2014). Notably, the native *ACL* has a high K_M value of 3.6 mM, which means that it has a low affinity for the substrate. Consequently, a heterologous *ACL* from *Mus musculus*, with a much lower K_M value for citrate of 0.05 mM, was overexpressed in *Y. lipolytica*, which improved the lipid content from 7.3 to 23.1% (Zhang et al., 2014). Similarly, the heterologous expression of hemoglobin from *Vitreoscilla* in *Y. lipolytica* increased the utilization of oxygen by the strain during fermentation, thereby improving the growth and increasing the final lipid yield. This modification resulted in an increase in the lipid content by 40% compared with the parental strain when dissolved oxygen (DO) was controlled at 30% of the atmospheric value (Zhang et al., 2019). In a separate study, Walker et al. (2020) investigated the effect of thiamine on the physiology of *Y. lipolytica* and found that thiamine deficiency decreased the protein abundance related to the lipid biosynthesis pathways and energy metabolism. The authors further

used the thiamine-regulated promoter P3 to express 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase (*THI13*) from *S. cerevisiae* and proved that restoring thiamine synthesis can boost lipid accumulation in *Y. lipolytica*.

4. Novel strategies for enhancing lipid production in *Y. lipolytica*

In addition to the regulation of the target gene listed above, several novel strategies have emerged in the past few years. These studies emphasize a deeper understanding of the limiting factors and bottlenecks in lipogenesis and rely on the design of reasonable approaches to eliminate these effects. Different approaches for increasing lipid accumulation in *Y. lipolytica* are summarized in Figure 2.

4.1 Engineering alternative acetyl-CoA pathways

In *Y. lipolytica*, lipogenic acetyl-CoA is only generated by the splitting of citrate by ATP-citrate lyase when the TCA cycle is repressed under nitrogen-limited conditions (Beopoulos et al., 2009b). Therefore, the availability of acetyl-CoA limits lipid biosynthesis. To decouple acetyl-CoA formation from nitrogen starvation and enhance acetyl-CoA supply, Xu et al. (2016) proposed five effective approaches based on establishing an alternative cytosolic acetyl-CoA formation pathway to decouple lipid synthesis from nitrogen starvation. One of the most efficient approaches was the overexpression of heterologous peroxisomal carnitine acetyltransferase (*perCAT2*) from *S. cerevisiae* and endogenous genes (*ACC1*, *DGA1*). The results showed that *perCAT2* improved both the lipid titer and content compared with the starting strain with *ACC1* and *DGA1* overexpression. They also overexpressed the phosphoketolase–phosphotransacetylase (PK-Pta) pathway to couple NADPH regeneration with cytosolic acetyl-CoA formation, which increased the lipid yield to 0.225 g/g in shake-flask cultures. When cultured in a 3-L bioreactor, the *perCAT2* overexpression strain produced 66.4 g/L of lipids with a yield of 0.229 g/g of glucose and productivity of 0.565 g/(L·h), representing a 3.1-fold increase of lipid production compared with the starting strain. Additionally, the engineered strains accumulated lipids at the initial stages of growth and continued throughout the entire fermentation process. In another study, Niehus et al. (2018) overexpressed the endogenous xylitol dehydrogenase (*XDH*), xylose reductase (*XR*), and xylulose kinase (*XK*) in the Pold strain, which allowed it to grow with xylose as the sole carbon source. The authors further designed two additional pathways to generate acetyl-CoA from xylulose-5-P, and both pathways increased lipid production from xylose. One of the most efficient approaches based on overexpressing heterologous phosphoketolase (*XPKA*) and acetate kinase (*ACK*), which results in the conversion of xylose to acetate, which is further converted to lipogenic acetyl-CoA by acetyl-CoA synthetase (*ACS*). The resulting strain produced a lipid content of 67%, lipid titer of 16.5 g/L, lipid yield of 0.344 g/g, and lipid productivity of 1.85 g/(L·h) in a fed-batch bioreactor using lignocellulosic hydrolysate as the sole carbon source.

4.2 Engineering cytosolic NADPH pathways

To better understand the mechanisms and bottlenecks of lipid metabolism, the gene encoding malic enzyme of *Y. lipolytica* was expressed in *Escherichia coli*, indicating that malic enzyme used NAD^+ as the major cofactor. Furthermore, when the $NADP^+$-dependent malic enzyme from *Mortierella alpina* (*MCE2*) was expressed in *Y. lipolytica*, the resulting strain showed no significant changes in lipid content or fatty acid composition, which indicated that the malic enzyme is not the primary source of NADPH for lipogenesis in *Y. lipolytica* (Zhang et al., 2013). Wasylenko et al. (2015) conducted ^{13}C metabolic flux analysis (MFA) of *Y. lipolytica* grown with glucose as the carbon source. By comparing the flux distribution of wild-type and engineered strains, the authors concluded that the oxidative pentose phosphate pathway (oxPPP) is the major NADPH source for lipogenesis. Qiao et al. (2017) established a quantitative model to analyze the global metabolic network during lipid accumulation. After verification and sensitivity analysis, it was found that the supply of NADPH limited lipogenesis, suggesting lipid accumulation can be improved by increasing the supply of NADPH (Ji and Huang, 2019). Qiao et al. (2017) then designed four engineered biosynthetic pathways to convert glycolytic NADH into cytosolic NADPH. The first strategy was overexpression of two $NADP^+$-dependent G3P dehydrogenases, *GapC* from *Clostridium acetobutylicum* and *GPD1* from *Kluyveromyces lactis*, which respectively increased the lipid yield by 20.0 and 17.8%. The second strategy was activating the pyruvate/oxaloacetate/malate (POM) cycle by cloning and expressing *MCE2* from *Mucor circinelloides* in AD strains (overexpression of *ACC1* and *DGA1*), which increased the lipid yield from 0.17 to 0.21 g/g. The third approach was co-expression of a phosphoketolase from *Leuconostoc mesenteroides* and a phosphate acetyltransferase from *Clostridium kluyveri* in AD strain to activate the NOG pathway, which can generate 3 mol acetyl-CoA from 1 mol glucose to increase the supply of acetyl-CoA. The engineered strain showed a 16% increase in lipid content. Finally, co-expression of a heterologous *GapC* and a heterologous *MCE2* further increased the lipid yield to 0.231 g/g. In an optimized bioreactor, the highest lipid titer, productivity, and yield reached 99.3 g/L, 1.2 g/(L·h), and 0.279 g/g, respectively.

4.3 Engineering oxidative stress defense pathways

Lipid accumulation in oleaginous yeasts is stimulated by nitrogen starvation, which triggers global physiological rearrangements and a variety of cellular stress responses (Rosenwasser et al., 2014; Morin et al., 2011). One of the main stress factors during lipid accumulation is the increased concentration of reactive oxygen species (ROS) due to lipid oxidation and peroxidation (Zhang et al., 2020; Li et al., 2011). ROS can attack the nucleophilic centers of many bioactive molecules (such as DNA, proteins, and lipids) and cause oxidative damage (Shi et al., 2017), triggering a series of cellular stress events, including cellular aging (Zimniak, 2011), replicative DNA damage (Mano et al., 2002), and protein adducts (Grimsrud et al., 2008). As natural molecules with fatty acids as the main components, neutral lipids are vulnerable to be attacked by oxygen free radicals through a chain reaction mechanism, and the resulting lipid peroxides can undergo further

autolysis to produce reactive aldehydes (Xu et al., 2017b). The carbonylation caused by reactive aldehydes attacks lysine, histidine, and cysteine residues, which inactivates critical enzymes of lipid metabolism, resulting in reduced cell viability and inefficiency of biosynthetic pathways (Grimsrud et al., 2008). Moreover, increased oxidative stress may result in the consumption of stored lipids as an energy source to maintain cellular homeostasis (Shi et al., 2017). Therefore, Xu et al. (2017b) adopted a metabolic engineering strategy by overexpressing glutathione peroxidase (*ylGPO*), glutathione disulfide reductase (*ylGSR*), and glucose-6-phosphate dehydrogenase (*ScZwf*) to upregulate the oxidative stress defense pathways, while simultaneously overexpressing aldehyde dehydrogenase (*EcAldH*) to detoxify reactive aldehydes, which could effectively increase the cellular resistance to oxidative stress and improve cell morphology. The resulting strain showed a lipid content of 81.4%, a lipid titer of 72.7 g/L, and lipid productivity of 0.97 g/(L·h) in a controlled bench-top bioreactor. These results indicate that engineering the oxidative defense pathway is vital for increasing lipid accumulation.

4.4 Engineered fatty acid secretion pathways

The high cost of recovery and separation processes often limits the economics of microbial lipid production (Ji and Ledesma-Amaro, 2020; Meullemiestre et al., 2016). To overcome these limitations, Ledesma-Amaro et al. (2016a) focused on designing unique approaches to secrete FAs into the culture medium, followed by an *in situ* separation method using organic layers. They engineered two synthetic pathways to achieve these aims. The first strategy enhanced FFA flux by combining the overexpression of genes involved in TAG synthesis and degradation (*DGA2*, *TGL4*, and *KlTGL3*) with the deletion of genes involved in FFA activation and degradation (*FAA1* and *MFE1*), which resulting in FFA accumulation and subsequent secretion. The second strategy aimed to abolish LB formation altogether and redirect FA synthesis to the cytosol by mimicking bacterial pathways with re-localized acyl-CoA thioesterases. They engineered a strain by deleting four genes (*ARE1*, *DGA1*, *DGA2*, and *LRO1*) to completely abolish the formation of neutral lipids (TAG and SE), combined with the deletion of *FAA1* and *MFE1*, as well as the overexpression of *RnTEII*, which increased the secretion of FAs. After using an optimized bioreactor to cultivate the best engineered strains, a titer of secreted FAs 10.4 g/L, a yield of 0.20 g/g, and total equivalent lipid content of 120.4% of the DCW was achieved, which significantly exceeded the storage limit of individual cells. These synthetic approaches decoupled production from biomass formation and facilitated product extraction, which may be a significant breakthrough for overcoming current limitations.

4.5 Adaptative evolution strategies

Rational metabolic engineering often results in a decrease of biomass while increasing product yield, and therefore compromises overall productivity (Trentacoste et al., 2013). Evolutionary metabolic engineering may provide a balance between these two competing factors (Abatemarco et al., 2013). For instance, Liu et al. (2015b) established

a rapid evolutionary metabolic engineering method based on floating cell enrichment screening. The authors observed that when the culture was stabilized, cells with high lipid content could float on top of the culture medium, and normal cells settled to the bottom. Here, the authors used ethyl methanesulfonate (EMS) to mutate floating cells randomly, screened mutants with higher lipid content by mild centrifugation (e.g., 100 × g), and finally measured the lipid content and cell growth ability. This iterative process increased the lipid titer, productivity, and yield to 39.1 g/L, 0.56 g/(L·h), and 0.244 g/g, respectively. Importantly, the floating cell enrichment scheme resulted in increased lipid production without reducing biomass. Moreover, the evolved strain exhibited lipid accumulation during the cell growth phase without the need for nitrogen starvation. In another study, Daskalaki et al. (2019) adopted an adaptive laboratory evolution strategy to improve lipogenesis by alternately changing the culture medium and continuously screening high-lipid cells, which ultimately coupled growth with lipid accumulation. Strains developed after 77 generations had a lipid content of 44%, which was 30% higher than that of the starting strain. Therefore, evolutionary strategies can boost lipid accumulation without affecting biomass, and combined with metabolic engineering and multi-omics technologies can maximize the robustness and productivity of oleaginous yeast.

4.6 Computer-aided metabolic engineering

By using *in silico* modeling to guide metabolic engineering, new strains with high lipid accumulation have been engineered (Xu et al., 2020; Kerkhoven et al., 2016; Kavšček et al., 2015; Pan and Hua, 2012). For instance, Kim et al. (2019b) used a genome-scale model of metabolism (GEM) to identify potential genetic engineering targets for increasing lipid production in *Y. lipolytica*. Several overexpression and knockout targets that might increase lipid accumulation were identified, including diglyceride acyltransferase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase et al. One of the knockout targets *YALI0F30745g*, encoding an enzyme involved in one-carbon/methionine metabolism, was experimentally validated. The resulting mutant showed increased the lipid content by 45% compared to the wild-type. Similarly, Wei et al. (2017) applied another GEM to predict six gene amplification targets that could enhance the precursor supply of acetyl-CoA and malonyl-CoA, and lead to 34.1% increasement of TAG biosynthesis. Moreover, the simulations showed that supplementation of six amino acids in growth medium, including L-glycine, L-alanine, L-cysteine, L-serine, L-threonine, and L-aspartic acid, could increase TAG accumulation. Among them, the addition of L-threonine and L-aspartic acid might increase the yield of TAG by 55.5%. In conclusion, such mathematical modeling approach may provide an attractive platform for better understanding of *Y. lipolytica* metabolic network and guiding the rational metabolic engineering.

5. Challenges and future perspectives

Although substantial improvements of lipid production have been achieved with *Y. lipolytica*, high costs still limited the economic benefits of biofuels and oleochemicals derived from microbial lipids. Consequently, inexpensive substrates, such as

lignocellulose and industrial by-products, were often used to minimize production costs in industrial-scale plant. However, toxic compounds present in inexpensive substrates would be potential inhibitors that can lead to unacceptably low growth rates and limit the industrialization of current processes. To this end, it is possible to improve the tolerance of engineered strains to toxic compounds by designing detoxification pathways or adding antidotes. Therefore, the use of inexpensive industrial composite substrates should be a considerable direction in future applied research. Furthermore, the third-generation biofuels aim to utilize microbial cell factories to convert CO_2 into fuels and chemicals. Future research directions should consider using *Y. lipolytica* to produce lipid directly from CO_2, including artificial photosynthesis, carbon fixation and utilization, feedstock tolerance, and strain process engineering.

Recent developments in systems and synthetic biology have made it possible to transform living organisms to serve humanity. Constructing an efficient cell factory based on *Y. lipolytica* requires the combination of multi-omics technologies to understand the regulatory mechanisms and bottlenecks in lipid metabolism, the guidance of *in silico* metabolic models to design and optimize more efficient synthetic pathways, and the development of synthetic biology tools to assemble and fine-tune *Y. lipolytica* metabolism, so that the precursors in the metabolic pathway are adequately supplied, the metabolic fluxes and cofactors are balanced, and feedback inhibition is alleviated, which would further push the lipid production towards the theoretical maximum limits. We believe that with the advancement of synthetic biology, more novel tools and regulatory methods will be developed, which would accelerate the construction of *Y. lipolytica* cell factories with desired lipogenesis phenotype.

6. Conclusions

Synthesis of microbial lipids from renewable feedstocks is one of the most promising strategies for biodiesel and oleochemicals production. Metabolic engineering of *Y. lipolytica* for increasing lipid accumulation has achieved considerable success. Future approaches to further increase lipid production using glucose or other inexpensive substrates in *Y. lipolytica* are expected to require the combination of systems metabolic engineering, multi-omics integration analysis, computer-aided designs, and synthetic biology approaches, which would further push the lipid production towards theoretical maximum limits.

Acknowledgments

This work was financially supported by the National Science Fund for Excellent Young Scholars of China (No. 21922806), the National Key Research and Development Program of China (No. 2018YFA0903700), the National Natural Science Foundation of China (Nos. 21776131, 31800079), the Six Talent Peaks Project in Jiangsu Province of China (No. 2018-SWYY-047), the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (No. XTD1814), and the BBSRC grant BB/R01602X/1.

Declaration of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "**Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica***".

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Tables

Table 1 The primary enzymes involved in TAG metabolism in *Y. lipolytica*.

Table 2 Summary of metabolic strategies for increasing lipid accumulation in *Y. lipolytica*. Δ followed by lowercase letters represents gene knockouts. Gene overexpression is represented by all uppercase letters. Desaturase genes are expressed through the following notation: Δ#D. "× #" refers to gene copy number. Some genes are preceded by an uppercase letter and a lowercase letter, which refer to the source of the gene. For example, *ScSUC2* refers to the *SUC2* gene from *Saccharomyces cerevisiae*. Specially, "hp4d" and "TEFin" refer to promoters, *f-Δ15D* means *Δ15D* from flax.

Table 1 The primary enzymes involved in TAG metabolism in *Y. lipolytica*.

Gene	EC number	YL name	Encoded enzyme
ACL1	EC 2.3.3.8	YALI0E34793g	ATP-citrate lyase, subunit 1
ACL2	EC 2.3.3.8	YALI0D24431g	ATP-citrate lyase, subunit 2
ACC1	EC 6.4.1.2	YALI0C11407g	Acetyl-CoA carboxylase
GUT1	EC 2.7.1.30	YALI0F00484g	Glycerol kinase
GPD1	EC 1.1.1.18	YALI0B02948g	NAD^+-dependent G3P dehydrogenase
GUT2	EC 1.1.5.3	YALI0B13970g	FAD^+-dependent G3P dehydrogenase
SCT1	EC 2.3.1.15	YALI0C00209g	Glycerol-3-phosphate acyltransferase
SLC1	EC 2.3.1.51	YALI0E18964g	1-acyl-sn-G3P acyltransferase
PAP	EC 3.1.3.4	YALI0D27016g	Phosphatidic acid phosphatase
DGA1	EC 2.3.1.20	YALI0E32769g	Acyl-CoA diacylglycerol acyltransferase 1
DGA2	EC 2.3.1.20	YALI0D07986g	Acyl-CoA diacylglycerol acyltransferase 2
LRO1	EC 2.3.1.158	YALI0E16797g	Phospholipid diacylglycerol acyltransferase
ARE1	EC 2.3.1.26	YALI0F06578g	Acyl-CoA sterol acyltransferase
TGL3	EC 3.1.1.3	YALI0D17534g	Triacylglycerol lipase 3
TGL4	EC 3.1.1.3	YALI0F10010g	Triacylglycerol lipase 4
FAA1	EC 6.2.1.3	YALI0D17864g	Long chain fatty acyl-CoA synthetase
PXA1	-	YALI0A06655g	Peroxisomal acyl-CoA transporter, subunit 1
PXA2	-	YALI0D04246g	Peroxisomal acyl-CoA transporter, subunit 2
AAL1	EC 6.2.1.12	YALI0E11979g	Acyl/aryl-CoA ligase
AAL2	EC 6.2.1.12	YALI0A14234g	Acyl/aryl-CoA ligase
AAL3	EC 6.2.1.12	YALI0E05951g	Acyl/aryl-CoA ligase
AAL4	EC 6.2.1.12	YALI0E12419g	Acyl/aryl-CoA ligase
AAL5	EC 6.2.1.12	YALI0F06556g	Acyl/aryl-CoA ligase
AAL6	EC 6.2.1.12	YALI0C05885g	Acyl/aryl-CoA ligase
AAL7	EC 6.2.1.12	YALI0E20405g	Acyl/aryl-CoA ligase
AAL8	EC 6.2.1.12	YALI0B07755g	Acyl/aryl-CoA ligase
AAL9	EC 6.2.1.12	YALI0A15103g	Acyl/aryl-CoA ligase
AAL10	EC 6.2.1.12	YALI0D17314g	Acyl/aryl-CoA ligase
POX1	EC 1.3.3.6	YALI0E32835g	Fatty-acyl-CoA oxidase
POX2	EC 1.3.3.6	YALI0F10857g	Fatty-acyl-CoA oxidase
POX3	EC 1.3.3.6	YALI0D24750g	Fatty-acyl-CoA oxidase
POX4	EC 1.3.3.6	YALI0E27654g	Fatty-acyl-CoA oxidase
POX5	EC 1.3.3.6	YALI0C23859g	Fatty-acyl-CoA oxidase
POX6	EC 1.3.3.6	YALI0E06567g	Fatty-acyl-CoA oxidase
MFE1	EC 4.2.1.119	YALI0E15378g	Peroxisomal multifunctional enzyme type 1
POT1	EC 2.3.1.16	YALI0E18568g	3-ketoacyl-CoA thiolase
PEX3	-	YALI0F22539g	Peroxisomal biogenesis factor 3
PEX10	-	YALI0C01023g	Peroxisomal biogenesis factor 10
PEX11	-	YALI0C04092g	Peroxisomal membrane protein
MAE	EC 1.1.1.38	YALI0E18634g	Mitochondrial malic enzyme

Note: All information listed above comes from GRYC (http://gryc.inra.fr) and NCBI.

Table 2 Summary of metabolic strategies for increasing lipid accumulation in *Y. lipolytica*. Δ followed by lowercase letters represents gene knockouts. Gene overexpression is represented by all uppercase letters. Desaturase genes are expressed through the following notation: Δ#D. "× #" refers to gene copy number. Some genes are preceded by an uppercase letter and a lowercase letter, which refer to the source of the gene. For example, *ScSUC2* refers to the *SUC2* gene from *Saccharomyces cerevisiae*. Specially, "hp4d" and "TEFin" refer to promoters, *f-Δ15D* means *Δ15D* from flax.

Product	Substrate	Scale	Parental strain	Genetic modifications	Content (%)	Titer (g/L)	Yield (g/g)	Productivity (g/(L·h))	References
TAG	Glucose	Flask	Po1d	*Δgut2, Δpox1-6, GPD1*	70.0	-	-	-	Dulermo and Nicaud (2011)
TAG	Glucose	Flask	Po1d	*Δpox1-6, GPD1*	65.0	-	-	-	Dulermo and Nicaud (2011)
TAG	Glucose	Flask	Po1d	*Δmfe1, GPD1*	54.0	-	-	-	Dulermo and Nicaud (2011)
FA	Glycerol	Flask	W29	*Δdga1, Δdga2, Δlro1,Δare1, DGA2×4*	53.7	9.90	-	-	Gajdoš et al. (2017)
TAG	Glycerol	Flask	Po1d	*Δgsy1, Δtgl4, DGA2, GPD1*	52.4	2.62	-	-	Bhutada et al. (2017)
TAG	Sucrose	Flask	Po1d	*Δdga1, Δdga2, Δlro1, Δare1, Δmfe1, DGA2×3, ScSUC2*	49.0	6.70	-	-	Gajdos et al. (2015)
TAG	Glucose	Flask	ACA-DC 50109	*Δmig1*	48.7	2.44	-	-	Wang et al. (2013)
TAG	Glucose	Flask	ACA-DC 50109	*Δmhy1*	43.1	-	-	-	Wang et al. (2018)
FA	Glucose	Flask	ATCC 20362	*Δsnf1*	30.0	1.10	-	-	Seip et al. (2013)
TAG	Glucose	Flask	W29	*Δpex10, ZWF1, ACBP*	30.0	-	-	-	Yuzbasheva et al. (2017)
TAG	Glycerol	Flask	Po1h	*MmACL*	23.1	1.70	-	-	Zhang et al. (2014)
TAG	Seawater/ glycerol	Flask	AJD	*DGA1*	21.0	-	0.070	-	Dobrowolski et al. (2019)
TAG	Starch	Flask	Po1d	*Δpox1–6, Δtgl4, DGA2, GPD1, (riceAlphaAmylase, Glucoamylase)×2*	-	2.84	0.13	-	Ledesma-Amaro et al. (2015)
FFA	Glycerol	Flask	W29	*Δgpd1, Δgut2, Δpex10, ACC1*	-	2.03	-	-	Yuzbasheva et al. (2018)
TAG	Galactose	Bioreactor	Po1d	*GAL1, GAL7, GAL10E, GAL10M*	16.6	3.22	0.056	-	Lazar et al. (2015)

TAG	Sucrose	Bioreactor	Po1d	Δ*tgl4*, Δ*pox1–6*, *DGA2*, *GPD1*, *HXK1*, *ScSUC2*	26.0	9.15	0.063	-	Lazar et al. (2014)
FFA	Glucose	Bioreactor	W29	Δ*faa1*, Δ*mfe1*, *DGA2*, *TLG4*, *KlTGL3*	120.4	10.40	0.200	-	Ledesma-Amaro et al. (2016a)
TAG	Xylose	Bioreactor	E26	(Δ*pex10*, Δ*mfe1*, *DGA1*, *LEU2*, *URA3*, evolved) *SsXYL1*, *SsXYL2*, starved	-	15.00	-	0.190	Li and Alper (2016)
TAG	Glucose/ xylose/ acetate	Bioreactor	Po1d	Δ*pox1–6*, Δ*tgl4*, *GDP1*, *DGA2*, *XR*,*XDH*, *XK*, *AnXPKA*, *AnACK*	67.0	16.50	0.344	1.850	Niehus et al. (2018)
TAG	Glucose	Bioreactor	Po1g	*hp4d-ACC1*, *TEFin-DGA1*	61.7	17.60	0.195	0.143	Tai and Stephanopoulos (2013)
FAMEs	Glucose/ Phosphite	Bioreactor	NS18	*DGA1*, *DGA2*, *PsPTXD*	-	23.0	-	-	Shaw et al. (2016)
TAG	Inulin	Bioreactor	Po1d	Δ*pox1–6*, Δ*tgl4*, *DGA2*, *GPD1*, *HXK1*, *GAL1*, *GAL7*, *GAL10E*, *GAL10M*, *ScSUC2*, *kmINU1*	43.0	23.82	0.160	0.158	Hapeta et al. (2017)
TAG	Molasses/ glycerol	Bioreactor	Po1d	Δ*pox1–6*, Δ*tgl4*, *DGA2*, *GPD1*, *HXK1*, *ScSUC2*	31.0	24.20	0.100	0.430	Rakicka et al. (2015)
TAG	Glucose	Bioreactor	L36	*DGA1*, *LEU2*, *URA3*	-	25.00	0.213	0.145	Liu et al. (2015b)
TAG	Glucose	Bioreactor	Po1f	Δ*pex10*, Δ*mfe1*, *DGA1*, *LEU2*, *URA3*	71.0	25.30	-	0.210	Blazeck et al. (2014)
TAG	Glucose	Bioreactor	Po1f	Δ*pex10*, Δ*mfe1*, *DGA1*, *LEU2*, *URA3*, evolved	78.0	39.10	0.244	0.560	Liu et al. (2015c)
TAG	Glucose	Bioreactor	Po1f	*ACC1*, *DGA1*, *SCD*, Δ*12D*, *f-Δ15D*, Δ*pex10*, Δ*mfe1*	77.8	50.00	-	-	Yan et al. (2020)
TAG	Xylose/ glycerol	Bioreactor	Po1d	Δ*pox1–6*, Δ*tgl4*, *GDP1*, *DGA2*, *XK*, *SsXR*, *SsXDH*	42.0	50.50	0.120	-	Ledesma-Amaro et al. (2016b)

TAG	Glucose	Bioreactor	Po1g	*TEFin-ACC1*, *TEFin-DGA1*, *TEFin-SCD*	67.0	55.00	0.234	0.707	Qiao et al. (2015)
TAG	Glucose	Bioreactor	Po1g	*ACC1*, *DGA1*, *ScCAT2*	70.0	66.40	0.229	0.565	Xu et al. (2016)
TAG	Glucose	Bioreactor	Po1g	*ACC1*, *DGA1*, *URA3*, *EcALDH*, *ScZWF*, *GSR*, *GPO*	81.4	72.70	0.252	0.970	Xu et al. (2017b)
TAG	Glucose	Bioreactor	NS18	*RtDGA1*, *CpDGA2*, Δ*tgl3*	77.0	85.00	0.210	0.730	Friedlander et al. (2016)
FAMEs	Glucose	Bioreactor	Po1g	*ACC1*, *DGA1*, *McMCE2*, *CaGAPC*	66.7	99.30	0.279	1.200	Qiao et al. (2017)
TAG	Acetic acid	Bioreactor	Po1g	*ACC1*, *DGA1*	59.0	115.00	0.160	0.800	Xu et al. (2017a)

Notes: Lipid content (%), grams of lipid per gram CDW; Lipid titer (g/L), grams of lipid per liter of culture; Lipid yield (g/g), grams of lipid per gram of carbon substrate consumed; Lipid productivity (g/(L·h)), grams per lipid per liter of culture per hour.

Figures

Figure 1 Synthesis and degradation of TAGs in *Y. lipolytica*. Differently colored arrows indicate different metabolic pathways: black, glycolysis; green, oxidative pentose phosphate pathway; light blue, tricarboxylic acid (TCA) cycle and related anaplerotic reactions; yellow, *de novo* TAG synthesis pathway; purple, *ex novo* TAG synthesis pathway; navy blue, *Kennedy* pathway; red, β-oxidation pathway. Abbreviations: MIT, mitochondria; N, nucleus; ER, endoplasmic reticulum; LB, lipid body; PER, peroxisome; OAA, oxaloacetate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglyceride; FFA, free fatty acid.

Figure 2. Overview of the different approaches for increasing lipid accumulation in *Y. lipolytica*. Abbreviations: TAG, triacylglyceride; Δ, gene knockout; TEF, gene overexpression; ROS, reactive oxygen species; DAG, diacylglycerol; FFA, free fatty acid; β, β-oxidation pathway. (I) β-oxidation pathway is deleted to hinder lipid degradation; Regulating endogenous genes involved in lipid metabolism to push and pull lipid synthesis; Overexpressing heterologous genes to enhance enzyme activity or change cell physiology; Blocking other competing metabolic pathways to channel the metabolic flux towards lipid synthesis. (II) Engineering cytosolic NADPH pathways to provide more cofactors for lipogenesis; Engineering alternative acetyl-CoA pathways to decouple nitrogen limitation and provide more precursors for lipogenesis; Engineering oxidative stress defense pathways to mitigate lipotoxicity caused by ROS. (III) Engineering fatty acid secretion pathways to exceed the lipid storage limit of individual cells and facilitate product extraction. (IV) Growth-related adaptive laboratory evolution combined with metabolic engineering can maximize the robustness and productivity of engineered strain. (V) Expanding the range of available substrates to minimize production costs. (VI) Multi-omics analysis to understand the regulatory mechanisms and bottlenecks in lipid metabolism, *in silico* metabolic models to design and optimize more efficient synthetic pathways, and synthetic biology tools to assemble and fine-tune *Y. lipolytica* metabolism.

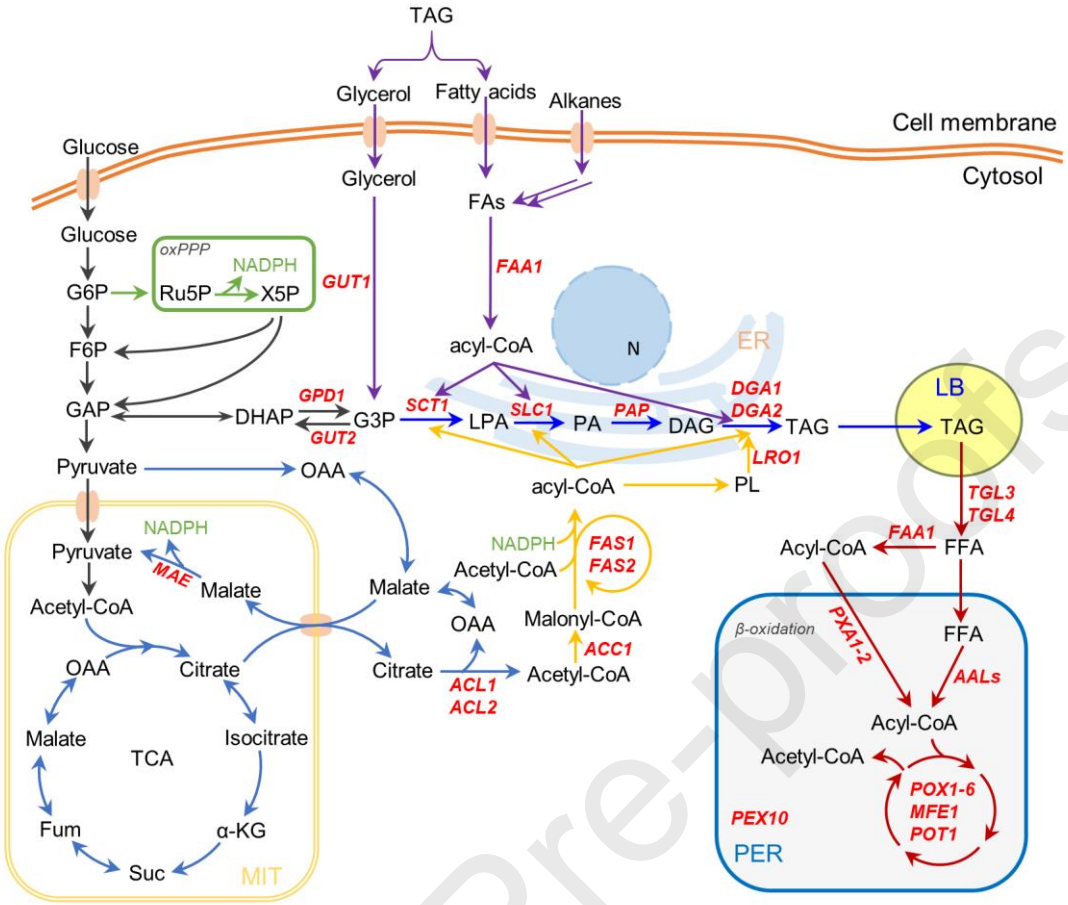


Figure 1 Synthesis and degradation of TAGs in *Y. lipolytica*. Differently colored arrows indicate different metabolic pathways: black, glycolysis; green, oxidative pentose phosphate pathway; light blue, tricarboxylic acid (TCA) cycle and related anaplerotic reactions; yellow, de novo TAG synthesis pathway; purple, ex novo TAG synthesis pathway; navy blue, *Kennedy* pathway; red, β-oxidation pathway. Abbreviations: MIT, mitochondria; N, nucleus; ER, endoplasmic reticulum; LB, lipid body; PER, peroxisome; OAA, oxaloacetate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglyceride; FFA, free fatty acid.

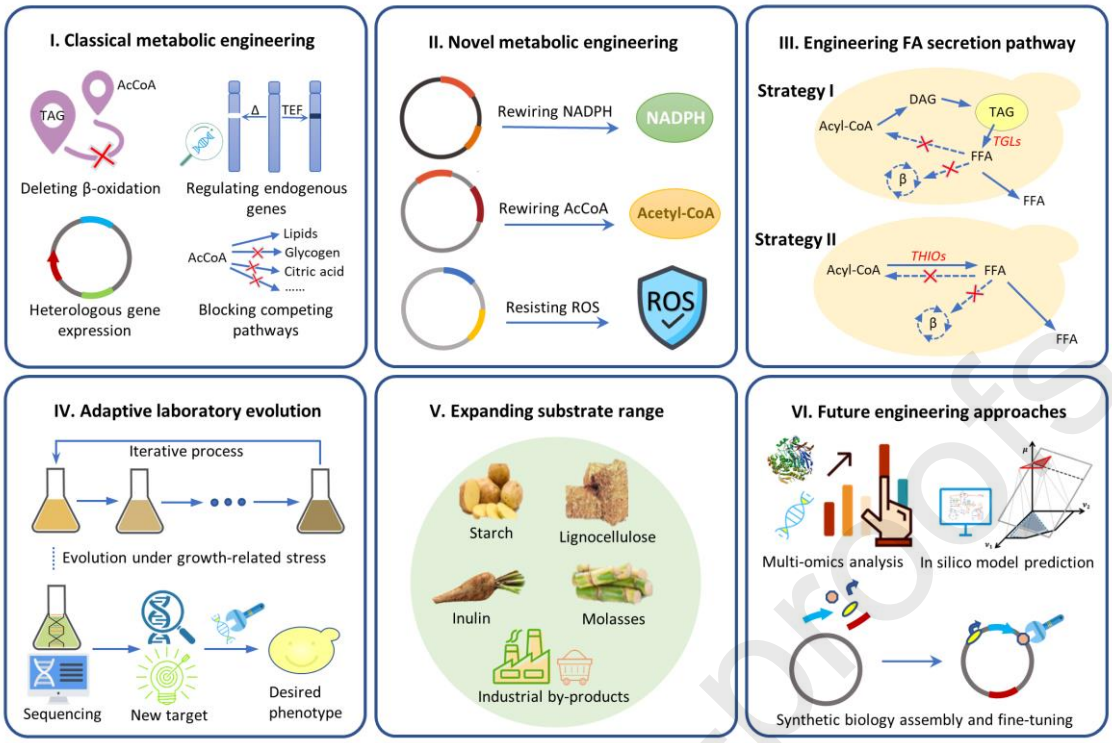
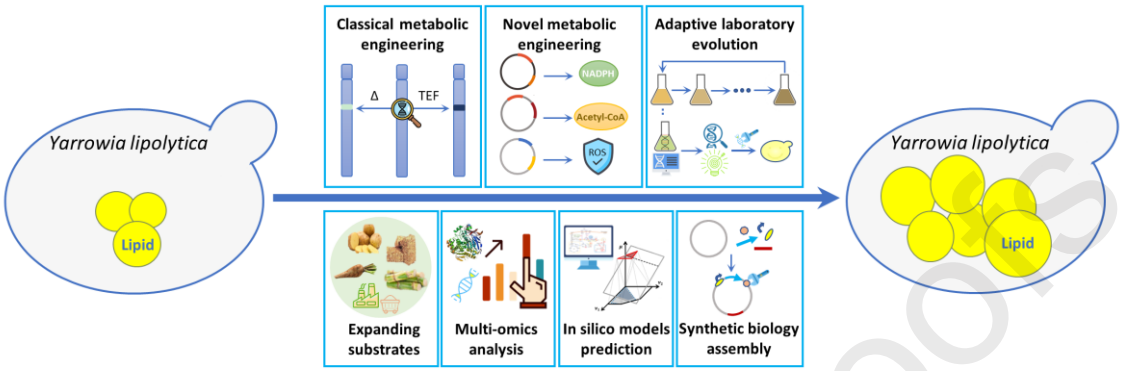
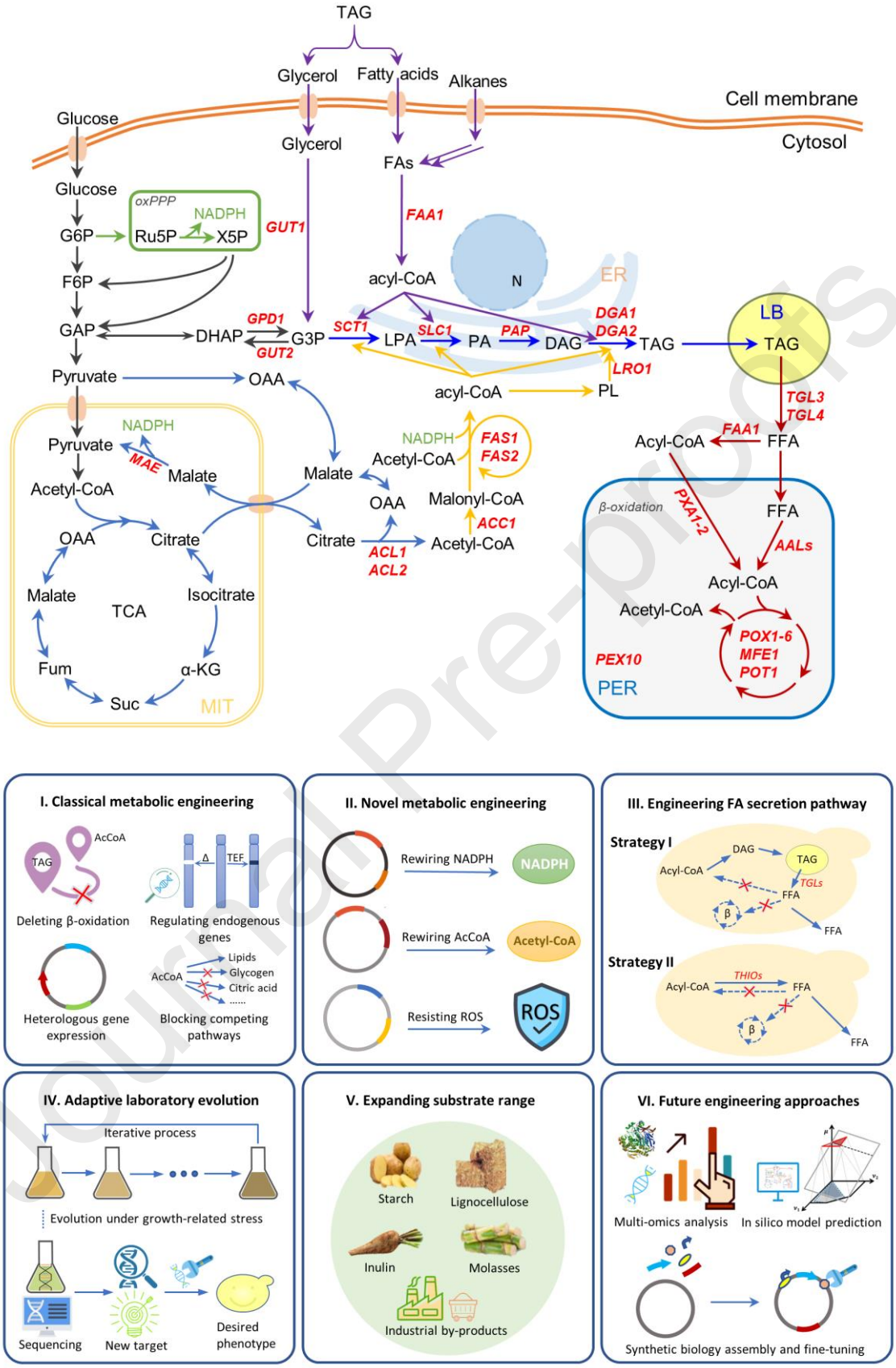


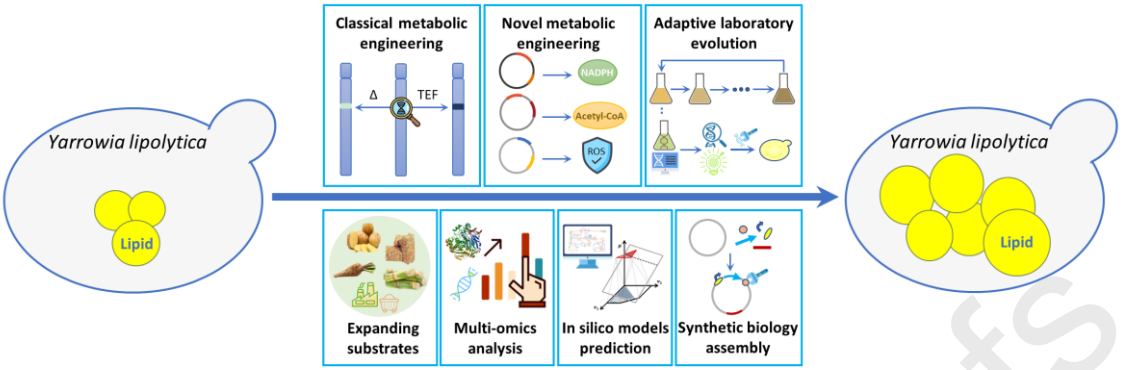
Figure 2. Overview of the different approaches for increasing lipid accumulation in *Y. lipolytica*. Abbreviations: TAG, triacylglyceride; Δ, gene knockout; TEF, gene overexpression; ROS, reactive oxygen species; DAG, diacylglycerol; FFA, free fatty acid; β, β-oxidation pathway. (I) β-oxidation pathway is deleted to hinder lipid degradation; Regulating endogenous genes involved in lipid metabolism to push and pull lipid synthesis; Overexpressing heterologous genes to enhance enzyme activity or change cell physiology; Blocking other competing metabolic pathways to channel the metabolic flux towards lipid synthesis. (II) Engineering cytosolic NADPH pathways to provide more cofactors for lipogenesis; Engineering alternative acetyl-CoA pathways to decouple nitrogen limitation and provide more precursors for lipogenesis; Engineering oxidative stress defense pathways to mitigate lipotoxicity caused by ROS. (III) Engineering fatty acid secretion pathways to exceed the lipid storage limit of individual cells and facilitate product extraction. (IV) Growth-related adaptive laboratory evolution combined with metabolic engineering can maximize the robustness and productivity of engineered strain. (V) Expanding the range of available substrates to minimize production costs. (VI) Multi-omics analysis to understand the regulatory mechanisms and bottlenecks in lipid metabolism, *in silico* metabolic models to design and optimize more efficient synthetic pathways, and synthetic biology tools to assemble and fine-tune *Y. lipolytica* metabolism.

Graphic Abstract



- *Y. lipolytica* is a superior industrial host for the production of lipids.
- Biosynthesis and degradation pathways of lipid in *Y. lipolytica* and the potential metabolic engineering targets are introduced.
- Metabolic engineering strategies for increasing lipid accumulation in *Y. lipolytica* are summarized.
- Multi-omics analysis, *in silico* metabolic models, and synthetic biology tools will further push the lipid yields to theoretical limits.
- Perspectives for novel engineering approaches for increasing lipid accumulation in *Y. lipolytica* are discussed.





Declaration of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "**Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica***".