



*Review*

## **Recent advances in bioengineering of the oleaginous yeast *Yarrowia lipolytica***

**Murtaza Shabbir Hussain, Gabriel M Rodriguez, Difeng Gao, Michael Spagnuolo, Lauren Gambill and Mark Blenner\***

Department of Chemical and Biomolecular Engineering, Clemson University, Clemson, USA

\* **Correspondence:** Email: [blenner@clemson.edu](mailto:blenner@clemson.edu); Tel: +864-656-0290.

**Abstract:** The oleaginous yeast, *Yarrowia lipolytica*, is becoming increasingly popular for metabolic engineering applications. Advances in synthetic biology and metabolic engineering have allowed microorganisms such as *Y. lipolytica* to be tailored for specific chemical production. Significant progress has been made to understand the genetics of *Y. lipolytica* and towards developing novel genetic engineering tools, leading to accelerated metabolic engineering efforts for a variety of different products. In this review, we discuss recent advances in genetic engineering tools and metabolic engineering achievements specific to *Y. lipolytica*. Topics covered in this review include genetic manipulation and expression systems, lipid-based products, peroxisome-based products and alternative sugar utilization.

**Keywords:** *Yarrowia lipolytica*; lipids;  $\beta$ -oxidation; xylose; CRISPR-Cas9; promoter; hybrid promoter; integration; metabolic engineering

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### **1. Introduction**

The non-conventional oleaginous yeast, *Yarrowia lipolytica*, has been used in industry for the over 60 years. Its applications include single cell protein production, citric acid production and animal feed [1,2]. Advances in genetic engineering tools have enabled synthetic biology and metabolic engineering of microorganisms allowing for the manipulation of cellular metabolism to

produce valuable chemicals economically. The oleaginous property of *Y. lipolytica* allows this yeast to accumulate lipids greater than 20% of dry cell weight [1]. This trait along with continued improvements in genetic engineering tools has led to increased interest in engineering this host for the production of lipid-based products.

Over the years, significant effort has been made to understand the genetics of *Y. lipolytica* and to develop novel genetic engineering tools. Transformation protocols, basic expression cassettes, and gene deletion tools have been established for several years [3,4]. More recently, episomal plasmid vectors, high expression synthetic promoters, and CRISPR-Cas9 genome editing has been developed for use in *Y. lipolytica* [5–9]. Such advances in genetic engineering tools have accelerated metabolic engineering efforts in *Y. lipolytica*. Engineering efforts improved lipid production to as high as 90% of dry cell weight, with titers up to 85 g/L and lipid productivity of ~1 g/L-hr [10–14]. *Y. lipolytica* has been engineered to produce specific products such as omega-3 fatty acids [15], dicarboxylic acids [16], polyhydroxyalkanoates (PHAs) [17], itaconic acid [18], free fatty acids [13,19], alpha ketoglutarate [20,21], alkanes [13], esters [13], and alcohols [13,22,23].

In parallel, there have been significant efforts to engineer *Y. lipolytica* for the utilization of alternative sugars such as xylose, cellobiose, and galactose [25–32]. Alternative sugar substrates have become increasingly abundant due to advances in lignocellulose degradation [33]. The native xylose pathway of *Y. lipolytica* has been recently elucidated which has led to several studies achieving robust xylose utilization [25,26,28,29]. In this review, we discuss the recent advances in developing genetic engineering tools and in metabolic engineering efforts specific to *Y. lipolytica*.

## 2. Genetic Engineering Tool Advances in *Y. lipolytica*

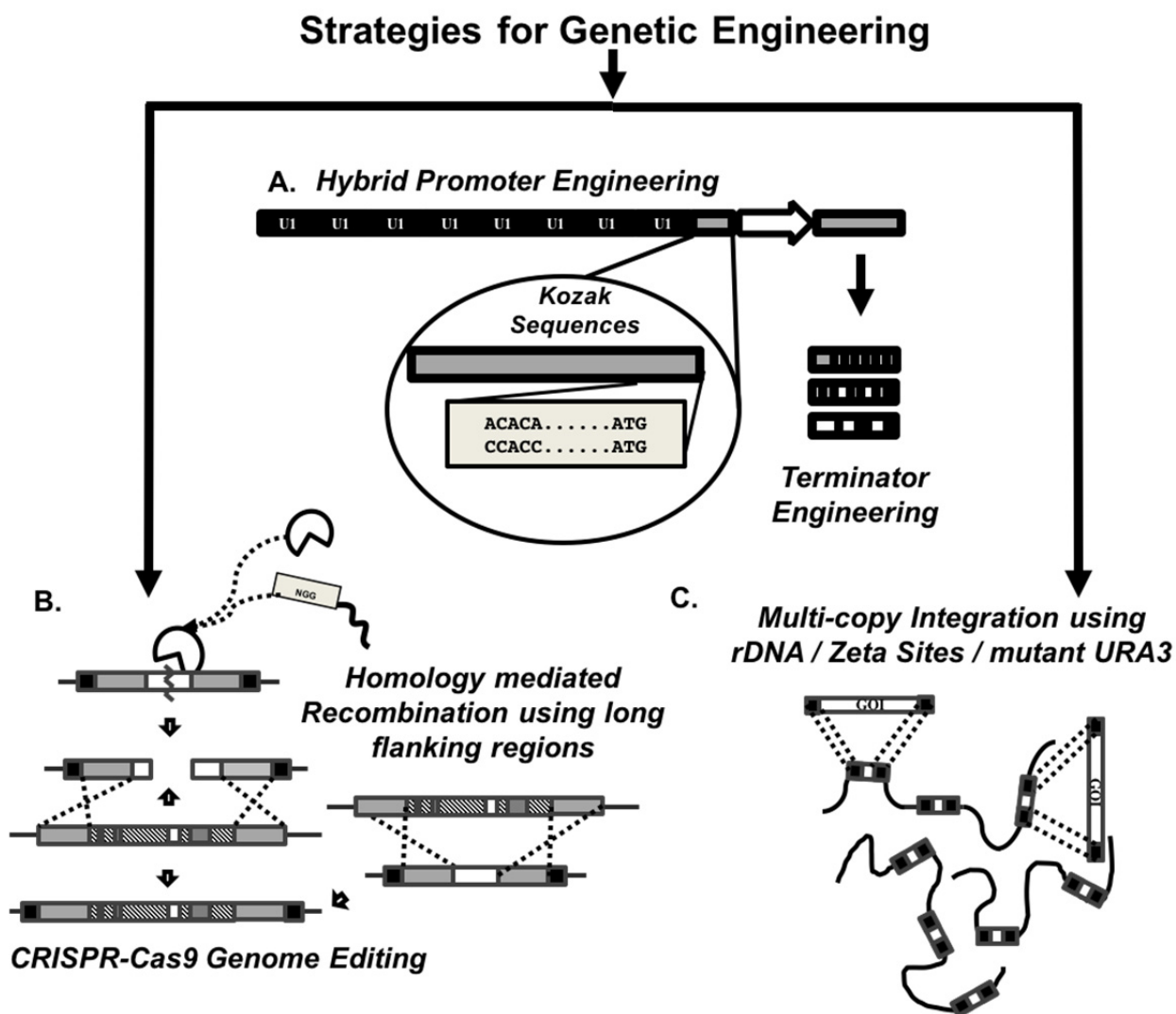
Efficient transformation protocols in tandem with access to fully annotated and sequenced genomes of *Y. lipolytica* strains has greatly facilitated the development of genetic engineering tools over the past three decades [34,35,36]. These genetic engineering tools include but are not limited to the creation of hybrid, carbon responsive, and inducible promoter systems alongside quick and efficient genome editing efforts (Figure 1).

### 2.1. Transformation methods

Original transformation methods developed for *Y. lipolytica* utilized a PEG-protoplast transformation [37]. Advances to this protocol soon followed with a lithium acetate (LiAc) protocol adapted from *Saccharomyces cerevisiae* to obtain site-directed integrative transformation efficiencies of up to  $1 \times 10^4$  transformants/g of linearized DNA [38]. Modifications to the LiAc transformation and development of an electroporation protocol superseded the above technique allowing for highly efficient replicative transformation [39]. To date, the LiAc protocol is the more commonly practiced method for transformation of plasmids and electroporation is more efficient for transforming linearized integrative vectors [3,39]. Recently, improvements to the overall transformation efficiency of linearized integrative DNA has been accomplished by using a combinatorial approach of LiAc and electroporation, with yields reaching  $2 \times 10^4$  transformants/g of linearized DNA [40].

Although considerable efforts have been made to improve transformation efficiencies in this

oleaginous yeast, transformation efficiencies remain about 2 orders of magnitude lower than the conventional *S. cerevisiae*. Despite this, it has been demonstrated that given the current efficiencies, *Y. lipolytica* could still be used as a suitable host for molecular evolution of proteins using both rational and directed evolution strategies [41,42].



**Figure 1.** General Strategies applied towards the development of genetic tools. A: At the promoter level, genetic tools can be engineered by constructing strong hybrid responsive or inducible systems. Kozak sequence modifications enable improved post-transcriptional expression. Furthermore, engineering synthetic promoters could help to improve mRNA stability and half-life, thereby improving expression levels. B: CRISPR Cas9 for knockouts and homologous recombination of DNA at high efficiencies. Homology mediated recombination requiring the use of large flanking DNA homologies are most efficient in  $\Delta ku70$  strain. C: Multi-copy integration performed using zeta docking sites, rDNA sites or restoring URA3 function by multi-copy integration DNA fragments containing URA3 alleles with gene of interest.

## 2.2. Promoter level regulation

Studies of metabolic pathways in *Y. lipolytica* have revealed several highly expressed native genes that are induced and/or repressed by different carbon sources and physiological conditions [43]. The best characterized native promoters are Glycerol-3-phosphate dehydrogenase (G3P), isocitrate lyase (ICL1), 3-oxo-acyl-CoA thiolase (POT1), aceto-acetyl-CoA thiolase (PAT1), the acyl-CoA oxidases (POX 1 through 5), extracellular lipase 2 (Lip2), and alkaline extracellular protease (XPR2). A list of the native promoters commonly used and their associated responsiveness to different conditions are summarized in Table 1.

While the inducible nature of these native promoters can serve as a regulatory switch to control metabolic pathways, the regulation associated with these promoters can be complex and exhibit unpredictable behavior. Furthermore, the need to promoters of various strength and inducibility for metabolic and pathway engineering is a desired outcome that cannot be met exclusively by native promoters. Over the past decade, there have been several studies aimed at developing and characterizing hybrid promoters in *Y. lipolytica* that confer very high and tunable expression [7,44,45]; however, hybrid promoters that have programmed regulatory behavior have not been reported yet.

**Table 1.** Commonly used native promoters for metabolic engineering & substrate inducibility.

Substrate(s)	Induced Promoter Systems	Repressed Promoter Systems
Glycerol	Glycerol-3-phosphate dehydrogenase (G3P)	Acyl-CoA-oxidase 2 (POX2)
	Translation Elongation Factor-1 $\alpha$ (TEF 1- $\alpha$ )	3-oxo-acyl-CoA thiolase (POT1)
Glucose	Glycerol-3-phosphate dehydrogenase (G3P)	Acyl-CoA-oxidase 2 (POX2)
		3-oxo-acyl-CoA thiolase (POT1)
Oleic acid	Acyl-CoA-oxidase 2 (POX2)	
Ricinoleic acid	Isocitrate lyase (ICL1)	Glycerol-3-phosphate
methyl ester	3-oxo-acyl-CoA thiolase (POT1)	dehydrogenase (G3P)
n-Decane	Translation Elongation Factor-1 $\alpha$ (TEF 1- $\alpha$ )	
Ethanol	Cytomchrome P450 (ICL1)	Acyl-CoA-oxidase 2 (POX2)
		Isocitrate lyase (ICL1)
Acetone		Glycerol-3-phosphate
		dehydrogenase (G3P)

*Y. lipolytica* hybrid promoters are made by deconstructing the native promoters to identify upstream activating sequences (UAS) that confer transcriptional activation, and creating tandem repeats of these sequences to accomplish high levels of transcription. A commonly used UAS in *Y. lipolytica* is the UAS1B that was first isolated from the complexly regulated XPR2 promoter [46].

Functional dissection of the XPR2 promoter was used to identify the UAS1B sequence that was devoid of regulation by pH, nitrogen and peptone levels [47]. Placing 4 UAS1B elements in tandem in front of a minimal LEU2 core promoter containing a TATA box demonstrated the first efforts at creating a constitutive synthetic hybrid hp4d promoter that conferred strong transcriptional activation [47,48]. Even stronger transcriptional activity resulted when additional tandem repeats of the UAS1B elements were used, exhibiting more than a 400-fold increase in transcriptional levels effectively bypassing enhancer limitations associated with natural eukaryotic promoter systems [8]. We recently showed that the UAS1B elements, while constitutive with respect to nitrogen, elicit carbon source dependent regulation of expression with oleic acid eliciting the strongest transcriptional activation. Expression from the UAS1B hybrid promoters with glucose is strong, albeit weaker than oleic acid, while glycerol elicits a weak transcriptional activation [49].

An interesting feature of the UAS1B hybrid promoters is that they are growth phase dependent promoters. These promoters produce limited gene expression during exponential phase growth but increase significantly during late exponential/early stationary phase [48,49]. This characteristic is particularly important in heterologous protein production where it is often important to segregate the growth and expression phases. This segregation contributes to improved cell productivity and alleviates toxicity issues related to heterologous protein expression.

The UAS1B hybrid promoter would be less efficient in metabolic engineering efforts to rewire pathways for substrate utilization, as the desired outcome in this particular example would be early phase enzyme expression. In this instance, strong constitutive promoters such as the transcriptional elongation factor-1 $\alpha$  (TEF1- $\alpha$ ) and the RPS7 genes in *Y. lipolytica* are better suited [50]. Accordingly, the UAS (TEF) was systematically identified from truncations of the native TEF promoter and used to create strong constitutive hybrid promoters [7]. These elements also demonstrated that tandem usage leads to a 4-fold increase in expression relative to the TEF (404) promoter. Other interesting features of the UAS (TEF) elements were the earlier growth phase transcriptional activation and a more consistent expression level independent of the carbon substrate (sucrose, glucose, glycerol and oleic acid) used compared to the UAS1B elements [7].

The discovery of modular UAS elements has significantly improved gene expression in *Y. lipolytica*, and aided metabolic engineering and heterologous protein production efforts. However, there is little known to date about the regulatory sequences embedded within these sequences. A conceptual understanding of the enhancers and/or repressors within these elements could enable the development of smaller and more tightly regulated hybrid promoter systems. The only example of a hybrid promoter designed from tandem enhancer sequences in *Y. lipolytica* is the use of Alkane Responsive Element 1 (ARE1) [45]. An n-decane inducible hybrid promoter could be designed by using tandem ARE1 sequences upstream of a minimal core promoter.

Kozak sequences present in the core promoter proximal to the ATG initiator codon can serve as another modular genetic component to control gene expression. These small sequences play a major role in the initiation of translation in eukaryotic systems [51,52]. The CCACC Kozak sequence is commonly used in *Y. lipolytica* for efficient ribosome initiation and high gene expression. [53,54]. Other modifications of Kozak sequences used for enhanced translational efficiency include ACA/CAAA [55,56] and a CACA sequence [57]. In most examples of Kozak sequence use in *Y. lipolytica*, A in the +3 position is advantageous. A separate study examined 47 eukaryotic species

and showed that there was a strong bias towards having a A/G at the  $-3$  position, an A/C in the  $-2$  position and a C at the  $+5$  position [58]. However, more experimental data are required to determine whether this downstream consensus sequence improves translational initiation in *Y. lipolytica*.

### 2.3. Terminators to regulate gene expression

Terminators are another important component of promoters because these sequences dictate the efficiency of transcription termination and play a role in determining the half-life of synthesized mRNA. The terminator from the gene encoding cytochrome c oxidase (CYC1t) from *S. cerevisiae* is a commonly used terminator sequence in *Y. lipolytica* [7,8,26,49,59,60,61]. Native terminators are also used in *Y. lipolytica* without additional engineering efforts. The use of short synthetic terminators to improve gene expression has been explored recently [61]. The synthetic designs were constructed in *S. cerevisiae* and translated into *Y. lipolytica* to attain a 2-fold improvement relative to the native TEF terminator. Furthermore, terminators and promoters can interact to form loops that regulate gene expression [62] however, this phenomena has not been studied in *Y. lipolytica*.

### 2.4. Genome repair and editing mechanism in *Y. lipolytica*

Double stranded break (DSB) repair in yeast can occur via homologous recombination (HR), single strand annealing (SSA) and non-homologous end-joining (NHEJ) mechanisms such as micro-homology mediated end-joining (MMEJ) and illegitimate recombination (IR) [63,64,65]. Based off a comparative genetic analysis with DNA repair proteins in *S. cerevisiae*, it has been hypothesized that hemi-ascomycetous species such as *Y. lipolytica* would predominantly utilize the NHEJ pathway for DSB repair [66]. Previous studies aimed at using HR for genome editing and repair required the use of up to 1 kb of homologous flanking fragments for site-directed gene insertion [67]. Long homologous flanking regions are required to yield  $\sim 50\%$  frequency for site-specific insertion in *Y. lipolytica* [63]. Otherwise, exogenous DNA randomly integrates into the genome to repair DSB. This suggests that NHEJ is dominant over HR, although the two repair mechanisms are known to work independently in yeasts [68].

The core component of the NHEJ pathway, the ku70/ku80 heterodimer was knocked out to improve the frequency of HR in *Y. lipolytica* [69,70]. Knockout of ku70 alone led to decreased transformation efficiencies but improved HR frequencies with 1 kb flanking homologies on both ends. Meanwhile, reducing the length of the flanking homology from 1000 bp to 50 bp did not have a dramatic effect on HR frequencies, reducing it from 56% to 43%, respectively [69]. The use of short homology lengths for homologous recombination was recently improved by using hydroxyurea-mediated cell cycle arrest leading to enriched S-phase cells and increased homologous recombination [71]. The CRISPR-Cas9 system from *Streptococcus pyogenes* further improved the HR efficiency for genome integration in *Y. lipolytica* [59]. HR in the wild type strain was reported to occur at a 64% frequency while HR frequency was 100% in the  $\Delta KU70$  strain. It needs to be noted, however, the HR frequency varies as a function of genomic loci, chromatin modification and DNA packaging [39,70,72,73].

The examples above describe improvements on strategies to perform scarless, single copy

integrations in *Y. lipolytica*. The use of an auxotrophic marker for selection of genome integration makes screening easy; however, the number of marker genes are limited in *Y. lipolytica*. In some instances, conserving the selectable marker is of interest for future applications and therefore one must rescue the marker post integration. For this purpose, the Cre-Lox system has been used [67]. The selectable marker in the disruption cassette is flanked by the LoxP/LoxR sites which are 34 bp sequences containing 13 bp identical, inverted repeats separated by an 8 bp spacer [74]. Activation of the heterologous bacteriophage Cre-recombinase allows for the excision of the selectable marker after screening for site-directed integration, enabling the marker to be used again. This editing mechanism, however, does leave a genomic scar as a result, whilst the CRISPR-Cas9 genome editing mechanism is a scar-free process. An alternative means of marker recovery is to replace the selectable marker with an inactive gene by HR [15].

### 2.5. Genome integration: cassettes and tools for integration

Stable cell lines are preferred for industrial hosts. Stability can be accomplished by integration of desired genes into the genome. Multi-copy gene integration is often used to improve heterologous gene expression. One of the earlier multi-copy integration methods in *Y. lipolytica* was homology based linear DNA integration into the ribosomal DNA (rDNA) loci [75]. Here, it was reported that up to 60 copies of the transformed gene were integrated. The integrated genes, however, were mostly tandemly repeated copies of the gene. A linear relationship between copy number and reporter gene expression was reported for up to 10 copies per cell line. Additional copies resulted in a toxic phenotype, leading to de-selection. Over 200 such rDNA clusters have been identified in the *Y. lipolytica* genome [76] allowing for not only multi-copy integration but also the integration of multiple genes in these loci.

Another commonly used loci for multi-copy integration in *Y. lipolytica* is the retrovirus-like mobile genetic element, Ylt1 retrotransposon, which was first identified upstream of the glyoxylate pathway regulator gene, *GPR* in this yeast species [77]. Ylt1 is flanked by long terminal repeats (LTRs) called zeta sites. Unlike the rDNA cluster, zeta elements are not present in all strains. The repetitive zeta elements were not found in the W29 strain or its derivative strains such as PO1d [78]. In the series of strains derived from B204-12C, around 35 copies of the Ylt1 retrotransposon with about 55 copies of the zeta sites were detected [77]. Therefore, expression cassettes containing flanking zeta regions have been used for successful multi-copy gene integration [79]. Interestingly, flanking zeta regions on an expression cassette aided in random integration in *Y. lipolytica* Ylt1-free strains although the copy number of integrated genes was not reported [78].

To date, characterization of docking sites for multi-copy integration in *Y. lipolytica* has been challenging because controlled integration is not possible. More controlled approaches of multi-copy gene integration have been accomplished by using a two-copy cassette at a single integration site [22]. Another method used to control copy number insertions into the genome is the use of a mutant *URA3* allele which is targeted either to the rDNA site, for 12–60 copies per cell or to the *XPR2* site, for about 30 gene copies per cell [80,81]. Interestingly, these integrations were found to occur in tandem at one or two sites in the genome. Site preferences for random gene integration and the relationship between loci and gene expression strength are yet to be understood in *Y. lipolytica*. A

better understanding of these components would prove beneficial for future strain engineering efforts. Challenges remain in precisely controlling copy numbers for integration, while determining the exact copy number post integration can be accomplished using real-time PCR on extracted genomic DNA (gDNA).

### 3. Lipid Production in *Y. lipolytica*

Developing lipid accumulating strains is desirable for the production of biofuels and fatty acid derived bioproducts. This host has already been engineered to produce a number of products using its lipid producing capabilities (Table 2). This list is not intended to be an exhaustive list; rather it is meant to show a number of directly relevant products and the degree to which titer has been improved through metabolic engineering.

**Table 2.** Summary of maximum titers achieved in *Y. lipolytica*.

Products	Maximum Titer	Reference
Triacylglycerides (TAGs)	85 g/L	[14]
Free Fatty Acids (FFA)	10.4 g/L	[19]
Fatty Alcohols	2.15 g/L	[13]
Alkanes	23.3 mg/L	[13]
Fatty Acid Ethyl Esters (FAEE)	142.5 mg/L	[13]
Polyhydroxy Alkanoates (PHAs)	1.11 g/L	[82]
Itaconic Acid	4.6 g/L	[18]
Omega-3 Fatty Acids (EPA)	56.6% of Total Lipids	[15]
Alpha ketoglutarate	186 g/L	[21]

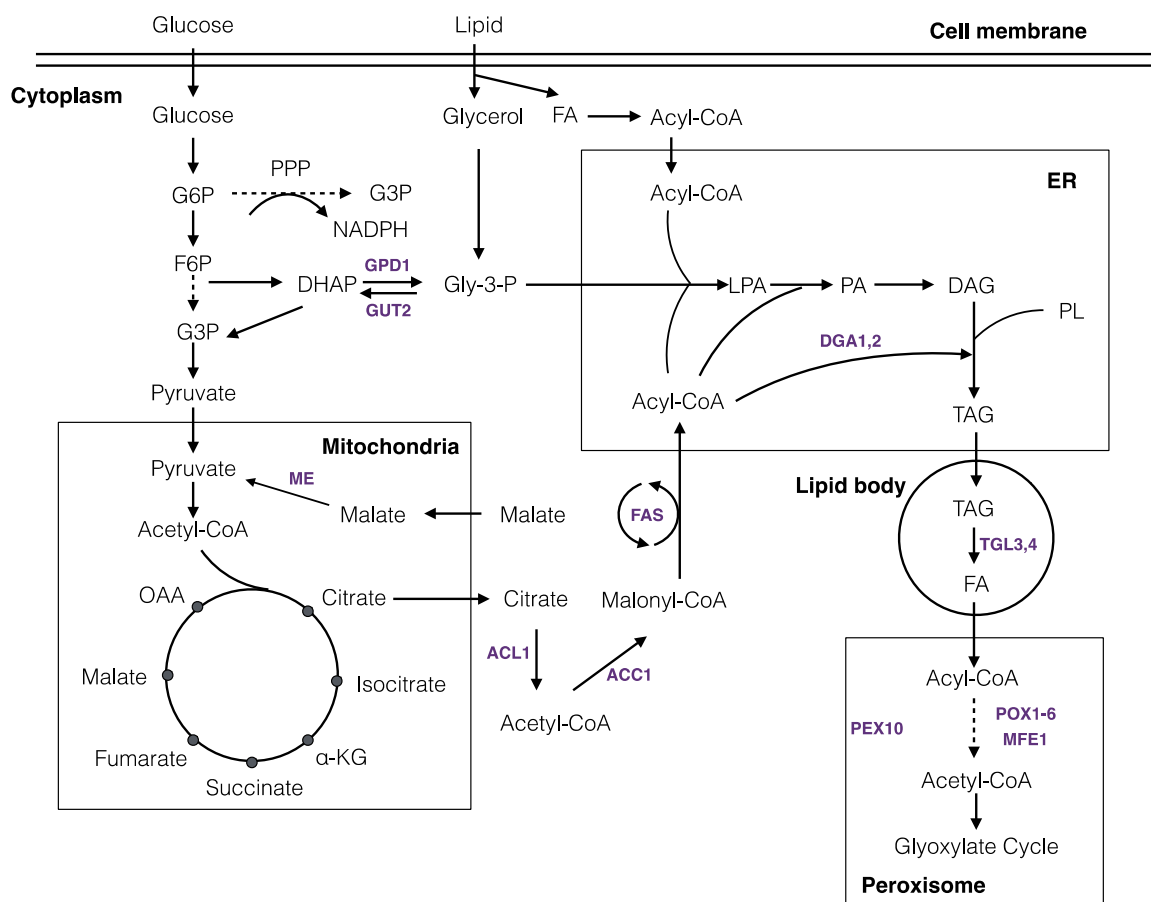
Lipogenesis (Figure 2) can be induced by nitrogen limited conditions or by high carbon to nitrogen (C/N) ratios. Nitrogen exhaustion leads to increased activity of AMP deaminase (AMPD), which decreases the concentration of cytosolic AMP. The activity of AMP-dependent isocitrate dehydrogenase (IDH) is therefore inhibited, resulting in the accumulation of isocitrate. Citrate generated from accumulated isocitrate by aconitase then exits the mitochondria and is cleaved by cytosolic ATP-citrate lyase (ACL) to generate acetyl-CoA [83,84]. The first step of fatty acid synthesis is the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). NADPH generated by malic enzyme (ME) provides the reducing power for fatty acid synthesis in other oleaginous yeast [85,86]. However, recent research has demonstrated that for *Y. lipolytica*, the pentose phosphate pathway is the major source for NADPH generation [87,88]. When acetate is used as a substrate, NADPH for fatty acid synthesis is produced through gluconeogenesis and the oxidative pentose phosphate pathway [89]. In fact, a recent study showed that no significant change in the IDH expression level was detected during lipid accumulation. However, the gene encoding isocitrate lyase (ICL), which is involved in converting isocitrate to glyoxylate, was observed to be strongly up-regulated in *Y. lipolytica* [90]. TAG synthesis in *Y. lipolytica* involves three acyltransferases. The first step is the incorporation of acyl-CoA into glycerol-3-phosphate (G3P) by



glycerol-3-phosphate acyltransferase (*GPAT*), leading to the formation of lysophosphatidic acid (LPA). LPA and acyl-CoA can be converted to phosphatidic acid (PA) by lysophosphatidic acid acyltransferase (*LPAT*). The phosphate group is then removed from PA to form diacylglycerol (DAG) by phosphatidic acid phosphatase (PAP). The last step of TAG synthesis is conducted by one of two types of diacylglycerol acyltransferases that incorporate an acyl group into a DAG. Acyl-CoA: diacylglycerol acyltransferase (*DGAT* or *DGA*) transfers an acyl group from acyl-CoA to a DAG, while phospholipid: diacylglycerol acyltransferase (*PDAT*) transfers an acyl group from a phospholipid to a DAG.

Early attempts to improve lipid accumulation focused on redirecting the carbon flux towards the glycerol pathway by deleting *GUT2*, preventing the reaction of glycerol-3-P to DHAP, thereby generating more precursor glycerol-3-P for TAG synthesis. In this study, lipid degradation through  $\beta$ -oxidation was also hindered by deletion of *POXI-6* genes which encodes six acyl-coenzyme A oxidases [91]. In *Y. lipolytica*, *DGA2*, which encodes the DGAT1 enzyme and *DGAI*, which encodes the DGAT2 enzyme, are the only genes contributing to the acylation of DAG. The latter gene is suggested to be the major contributor of TAG synthesis. However, *DGA2* showed great potential in acyltransferase activity when expressed in the quadruple mutant strain under a strong constitutive promoter [92]. A push-and-pull strategy was developed by overexpression of both *ACC* and *DGAI* to enable high levels of lipid accumulation. Double expression of *ACC* and *DGAI* under the control of a strong TEF<sub>intron</sub> promoter carries out the first and last step of TAG synthesis, providing enhanced driving force to redirect the carbon flux toward lipid synthesis and resulting in an increased lipid content of 41.4% [11]. Further enhancement of lipid accumulation was achieved by simultaneous expression of the *SCD* (delta-9 stearoyl-CoA desaturase) gene, *ACC* and *DGAI*. *SCD* was identified as a rate limiting step and a target for the metabolic engineering of lipid synthesis pathway by reverse engineering the mammalian cellular obese phenotypes. The high flux created by overexpression of *ACC* and *DGAI* is encouraged and sustained by preventing allosteric pathway inhibition. Overexpression of *SCD* enables the conversion of saturated to monounsaturated fatty acids, providing increased sequestration of the pathway products in a lipid sink. Moreover, the engineered strain obtained other favorable phenotypes including fast growth, high sugar tolerance and lipid productivity up to 22 g/l/d [12].

Several efforts have focused on redirecting carbon flux to fatty acid synthesis by modifying glucose repression regulators. Disruption of the *MIG1* gene, encoding a transcriptional regulator that binds to several glucose repression genes, enhanced lipogenesis through depression of several genes relevant to lipid synthesis including *GPD1*, *ICL*, *ME1* and *ACL1* and through repression of  $\beta$ -oxidation genes including *MFE1* [93]. Another glucose repression regulator Snf1 from the Snf1/AMP-activated protein kinase (AMPK) pathway was identified as a lipid accumulation regulator. Deletion of *SNF1* led to constitutive accumulation of lipid up to 2.6-fold higher than those of the wild type [94]. Disruption of  $\beta$ -oxidation has been explored to prevent TAG degradation. Pex10p, encoded by the *PEX10* gene, is involved in peroxisome biogenesis. Deletion of *PEX10* in an eicosapentaenoic acid (EPA) producing strain resulted in inactivation of  $\beta$ -oxidation and increased total lipid accumulation as well as EPA production [15].



**Figure 2.** Lipid biosynthesis in *Y. lipolytica*. Triacylglyceride (TAG) biosynthesis in *Y. lipolytica*. GPD1: glycerol-3-phosphate dehydrogenase, GUT2: glycerol-3-phosphate dehydrogenase, ACL: ATP-citrate lyase, ACC1: acetyl-CoA carboxylase, ME: malic enzyme, DGA1&2: diacylglycerol acyltransferase, TGL3&4: triacyl glyceride lyase, PEX10: peroxisomal biogenesis factor, POX1-6: Peroxisomal Acyl-CoA Oxidase, MFE:  $\beta$ -oxidation multi-function enzyme.

The *MFE1* gene is another target for disrupting of  $\beta$ -oxidation. Coupling the deletion of *MFE1* and improving G3P synthesis increased both *de novo* and *ex novo* TAG synthesis [95]. Combinatorial multiplexing of several lipogenesis targets, including deletion of both *MFE1* and *PEX10* genes, overexpression of DGA1 and restoration of a complete leucine biosynthetic pathway, generated a significantly lipogenic strain with a lipid content of 74% [10]. This study also demonstrated that lipid accumulation could be uncoupled from nitrogen starvation and established links between leucine-mediated signaling and lipogenesis. In *Y. lipolytica*, the only source of cytosolic acetyl-CoA is from splitting citrate by ACL when TCA cycle is repressed under nitrogen-limited conditions. Therefore, uncoupling lipid accumulation and nitrogen starvation can also be achieved by rewiring the acetyl-CoA pathway. Five alternative cytosolic acetyl-CoA pathways were engineered separately, including the pyruvate-acetate route, pyruvate-aldehyde route, pyruvate formate lyase, acetyl-CoA shuttling pathway and nonoxidative pentose-phosphate pathway [13]. The

engineered strains not only show improved lipid production but are also less sensitive to C/N ratio regulation. TGL3 and TGL4 are intracellular lipases that are responsible for the degradation of TAG in lipid body. Deletion of TGL3 has a positive effect on preventing degradation of TAG in the late phase of lipid accumulation and thus increases the overall lipid titer [14]. By combining a TGL3 knockout with overexpression of a heterologous DGA1 (*Rhodospiridium toruloides*) and DGA2 (*Claviceps purpurea*), 77% lipid content and 0.21 lipid yield was achieved in a batch fermentation.

Aside from rational metabolic engineering efforts, a rapid evolutionary metabolic engineering approach linked with a floating cell enrichment process was used to develop highly lipogenic strains. This screen led to a strain with a mutation of the succinate semialdehyde dehydrogenase, *UGA2*, achieving a high lipid content of 78% [96] and suggesting an important role of gamma-aminobutyric acid assimilation in lipogenesis. Another evolved strain had a mutant MGA2 protein that served as a regulator of desaturase gene expression and exhibited high lipid content with elevated unsaturated fatty acid levels. The mutant MGA2 regulator resulted in a drastically altered transcriptome, with glycolysis upregulated and the TCA cycle downregulated. This suggested that imbalance between glycolysis and the TCA cycle could serve as a driving force for lipogenesis [97].

## 4. $\beta$ -oxidation Products

### 4.1. $\beta$ -oxidation summary

In yeast, the process of  $\beta$ -oxidation primarily occurs within specialized organelles known as peroxisomes (Figure 3). The number, size and content of peroxisomes varies with environmental and genetic stimuli. Some  $\beta$ -oxidation has also been reported to occur within the mitochondria [98]. The peroxisomal  $\beta$ -oxidation cycle consists of five major steps. First, the substrate of interest, often a fatty acid (FA), is transported into the peroxisome with the aid of Acyl-CoA Binding Proteins (ACBP) [98]. During transport, the FA is acetylated by the two peroxisomal acyl-CoA Synthases (PXA1 & PXA2) in an ATP-dependent reaction [99]. The newly acylated fatty acid is then desaturated by acyl-CoA oxidases (POX) at the vinyl position, consuming  $FAD^+$  and producing  $H_2O_2$  as a byproduct. *Y. lipolytica* has six *POX* genes (*POX1-POX6*) which have been shown to have different chain length and substrate specificities [100]. The newly formed desaturated FA-CoA ester is then hydrated across the double bond by Multi-Function Enzyme 2 (MFE2-C domain), encoded by the *MFE1* gene, such that the addition of a hydroxyl occurs at the  $\beta$ -carbon position, forming a 3-hydroxyacyl-CoA intermediate. From here, the MFE2 enzyme (A/B domains) acts again to oxidize the 3-hydroxy intermediate to 3-ketoacyl-CoA and forms NADH in the process. Finally, the 3-ketoacyl-CoA is cleaved at the alpha carbon by peroxisomal 3-oxyacyl-thiolase (POT1), releasing a molecule of acetyl-CoA and producing a fatty acyl-CoA, which is two carbons shorter than the substrate that entered the cycle. From this point, the product can continue the cycle beginning with the POX reaction.

### 4.2. Plastic production in *Y. lipolytica*

The highly active peroxisomes of *Y. lipolytica* have been exploited to produce polymers known

as polyhydroxyalkanoates (PHAs). PHAs are naturally occurring polyesters produced in a variety of microorganisms as a means of carbon and energy storage. Structurally, PHAs are composed of three (or less commonly four) carbon monomers often containing an alkyl side-chain at the beta carbon position (Figure 3). This side-chain can vary in length, depending on the monomer composition and has a major impact on the thermal and physical properties of the polymer. Shorter side-chains (one to three carbons) tend to produce thermoplastics, while longer side-chains (four to eleven carbons) tend to impart elastomeric properties to the polymer [101]. This side-chain-dependent variability is of great practical interest as it allows for the tuning of the mechanical properties of the final polymer.

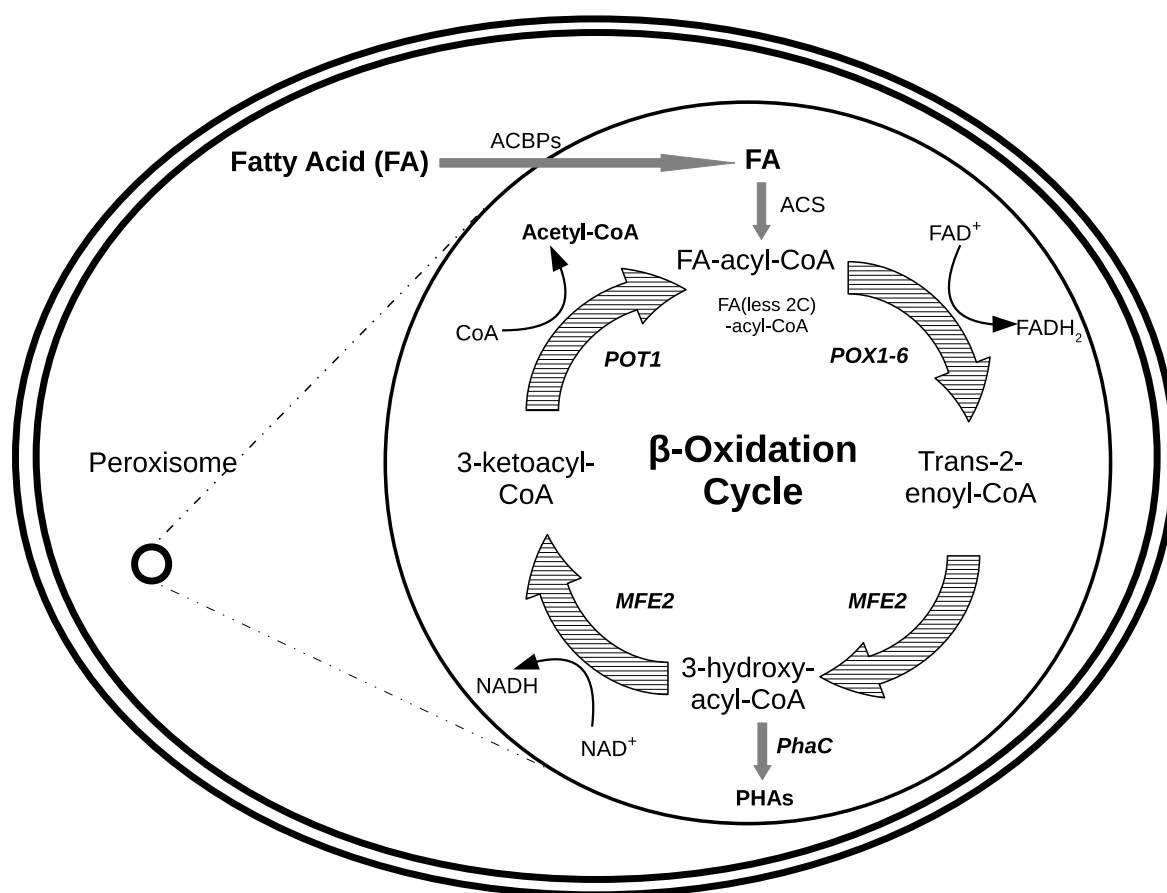
There are multiple routes to produce PHAs in *Y. lipolytica*, however they all end with one enzyme -PHA synthase. As the name suggests, this type of enzyme is solely responsible for polymerizing the PHA monomer subunits. The exact mechanism by which polymerization occurs is yet to be verified, however the current theory is as follows. The enzyme is most active in its dimerized form and thus two independent PHA Synthases must assemble to being the process [102]. To polymerize the monomer, a 3-hydroxyacyl-CoA terminated precursor is captured by nucleophilic attack on the terminal carbonyl by the sulfur atom of the active-site cysteine in one of the PHA synthases. This produces a CoA leaving group and results in monomer capture. The process is repeated for the other PHA synthase in the dimer. A second attack on the carbonyl is initiated by active-site aspartic acid-mediated attack from the hydroxyl oxygen of one captured monomer. This results in chain-transfer and elongation as the newly-freed site is filled with another monomer. This continues until either dissociation of the synthase or until exhaustion of precursor occurs, thus producing the final polymer (Figure 3).

Previous work has demonstrated that relatively minor manipulation of the metabolic pathway upstream of the PHA synthase can greatly affect the final product. Early work by Haddouche et al. characterized the role and sometimes complex interactions, of the acyl-CoA oxidases in the production of PHAs [17,100]. Flux through  $\beta$ -oxidation was able to be manipulated by eliminating the contribution of one or more *POX* genes. This work also provided additional evidence to support the individual role of each of the six *POX* genes.

More recent work focused on the next enzyme involved in  $\beta$ -oxidation: MFE2. This study demonstrated that peroxisomal  $\beta$ -oxidation is the primary mechanism by which fatty acid chains are shortened, however minor fatty acid acyl-CoA degradation capability existed outside of the peroxisome. MFE2 consists of three domains, two of which act as dehydrogenases (A/B) and one which acts as a hydratase (C). By removing the domains responsible for MFE2's dehydrogenase activity and recovering the hydratase domain, the chain-shortening activity of  $\beta$ -oxidation was almost entirely eliminated while production of the desired 3-hydroxyacyl-CoA intermediate was achieved. Through this manipulation, it was shown that feedstock chain length characteristics could be retained and incorporated into the final PHA product. Additionally, this work demonstrated that by expressing MFE2-C in a strain lacking the acyl-transferases *MFE2*, *DGA1*, *DGA2*, *ARE1* and *LROI*, PHA was able to be accumulated up to 7.3% DCW, the highest reported yield at the time of this writing [17,103].

Gao et al. explored the applicability of multi-copy integration to the production of PHAs in *Y. lipolytica* [60]. Their work concluded that multiple copies of a codon-optimized PHA synthase with a proper Kozak element was able to produce PHAs in significant quantities relative to literature.

When grown on triolein, the multi-copy strain was able to accumulate up to 1.11 g/L of PHAs (5% of DCW) within a 72 hour growth period [82]. At the time of this writing, this is the highest reported titer of PHAs by *Y. lipolytica*. Finally, there has been substantial work demonstrating that choice of and modifications to, the PHA synthase itself can also yield substantial changes in the terminal polymer. Chuah et al. demonstrated that by manipulating as few as one amino acid in the appropriate position, monomer preference can be affected [104]. Specifically, the mutation of amino acid 479 could have a drastic effect on the actions of the PHA synthase from *Chromobacterium sp.* strain USM2 [104]. Most notably, when alanine in this position was changed to glycine, a 160% increase in PHA accumulation and a change in preference from short-chain-length to medium-chain length PHAs was observed. By changing the composition of this site, not only can preference be manipulated, but so too can the monomer composition. Therefore, it is possible to produce PHAs as co-polymers between monomers with differing side-chain lengths. By affecting the blending, monomer type and length of polymer, the mechanical properties of PHAs can be modified and, theoretically, controlled.



**Figure 3.**  $\beta$ -oxidation in *Y. lipolytica*. The enzymes involved in  $\beta$ -oxidation in *Y. lipolytica*. ACBP: Acyl-CoA Binding Proteins, ACS: acyl-CoA synthase, POX1-POX6: peroxisomal acyl-CoA oxidases, MFE2: multi-function enzyme 2, POT1: peroxisomal 3-oxoacyl-CoA thiolase, PhaC: polyhydroxyalkanoate synthase.

## 5. Alcohols and other Oleochemicals

Recently several groups have begun producing other oleochemicals including alcohols, alkanes and esters [13,22,23]. Medium and long chain alcohols are used as moisturizers in cosmetics as well as lubricants and surfactants. Alcohol production in *Y. lipolytica* is typically achieved using a fatty acyl-CoA reductase (FAR) enzyme also using a carboxylic acid reductase. The FAR1 from *Tyto alba* (Tafar1) was used to enable production of hexadecanol [22]. The deletion of the fatty alcohol oxidase (FAO1) gene from *Y. lipolytica* and increasing the copy number of the *Tafar1* gene lead to a ~5 fold increase in titers. Deletion of the *DGAI* gene responsible for TAG synthesis and the introduction of 5 copies of the *Tafar1* gene led to a titer of ~690 mg/L from 160 g/L glucose after 6 days.

The production of 1-decanol was demonstrated using the FAR from *Arabidopsis thaliana* using a previously engineered *Y. lipolytica* for C8–C10 medium chain fatty acids [23]. The deletion of the *PEX10* gene while expressing FAR greatly increased 1-decanol titers by preventing peroxisome formation and thus alcohol degradation. A number of fatty acyl-ACP thioesterase (FAT) enzymes were also tested to release fatty acids from biosynthesis. The FAT enzyme from *Cuphea palustris* yielded the best decanol titers (550 mg/L). The majority of the decanol (~90%) was found to be secreted outside the cell and into the media.

More recently, production of a range of oleochemicals was demonstrated by targeting various pathways to the different organelles involving fatty acid biosynthesis and degradation [13]. Fatty acid ethyl ester (FAEEs) production was achieved by expression of AtfA from *Acinetobacter Abaylyi*. When targeting this enzyme to the ER or Peroxisome, 136 mg/L and 111 mg/L of FAEE was produced, respectively, whereas only 7 mg/L was produced when targeting the cytosol. Alkanes were produced using a similar organelle targeting approach by expressing the aldehyde deformylating oxygenase (ADO) and carboxylic acid reductase (CAR). Up to 23 mg/L of fatty alkanes were made by expressing CAR from *Mycobacterium marinum* and ADO from *Prochlorococcus marinus*. Alcohols were also produced by expression of *E. coli* FadD and *Marinobacter aquaeolei* FAR. In a scale-up 3L bioreactor, 2.15 g/l of alcohols were made from *Y. lipolytica*.

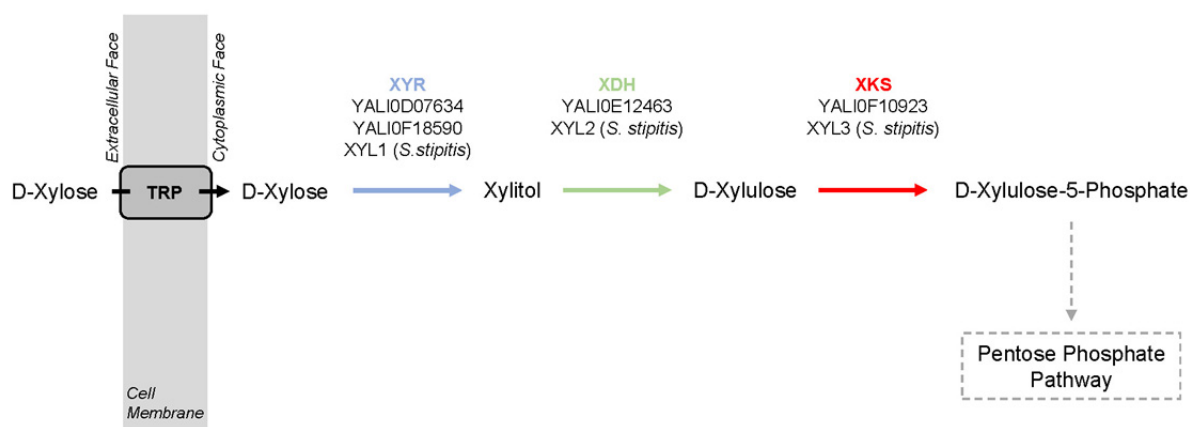
## 6. Xylose Utilization

Advances in lignocellulosic biomass degradation have made alternative sugars more abundant. The xylan polysaccharide accounts for up to 35% of plant biomass and is primarily made of xylose. Unfortunately, many industrial microorganisms do not utilize alternative sugars such as xylose efficiently. As a result, a great deal of effort has been directed towards engineering microorganisms for xylose utilization.

Efforts to characterize the inherent xylose metabolizing abilities of *Y. lipolytica* have resulted in conflicting reports. Blazeck et al. observed growth of a modified strain of PO1f on xylose as the sole carbon source [10]. Similarly, Tsigie et al. demonstrated growth of *Y. lipolytica* on xylose using the PO1g strain [105]. While these results are promising, a majority of the literature reports that *Y. lipolytica* cannot grow on xylose without modification [11,106,107,108].

In the last year, several manuscripts have been published focused on engineering *Y. lipolytica* for growth on xylose (Figure 4), with each work using different strategies to accomplish this goal.

Approaches taken include adaptation of the native xylose pathway [25], using a heterologous pathway [28,29] and overexpression of the native xylose pathway without adaptation [26].



**Figure 4.** Xylose utilization pathway in *Y. lipolytica*. *Y. lipolytica* and *S. stipitis* enzymes have been used to enable xylose utilization in *Y. lipolytica*. TRP: transporter, XYR: xylose reductase, XDH: xylitol dehydrogenase, XKS: xylulose kinase.

Ryu et al. recently uncovered the native xylose enzymes in *Y. lipolytica* grown on mixed sugars [25]. By using transcriptional analysis, the native xylose reductase (XYR), xylitol dehydrogenase (XDH) and xylulose kinase (XKS) were identified along with several putative transporters for glucose, xylose and cellobiose. The xylose enzymes were further characterized *in vitro*. By overexpressing the *XDH* gene followed by adaptation of the strain on xylose, a xylose utilizing strain of *Y. lipolytica* was obtained. Based on this observation, XDH was found to be the rate limiting step in xylose utilization.

Rodriguez et al. also recently systematically elucidated the native xylose pathway of *Y. lipolytica* [26]. BLAST analysis was used to identify candidate xylose enzymes. To assess their true functionality, a metabolic engineering strategy using *E. coli* mutants lacking the xylose genes (*xylA* and *xylB*) was developed. The putative xylose enzymes from *Y. lipolytica* were expressed in the mutants to see if the *Y. lipolytica* enzymes were able to recover growth of the *E. coli* mutants on xylose. It was found that the XKS enabled growth of *E. coli*  $\Delta xylB$  on xylose and the XDH enabled growth of *E. coli*  $\Delta xylA$  on xylitol. The XYR2 from *Y. lipolytica* was also found to enable *E. coli*  $\Delta xylA$  to grow on xylose. A complete xylose pathway was proven using *E. coli* mutants as a test bed for efficient enzyme testing. It was later found that XYR1 was also a functional xylose reductase. By overexpressing XDH and XKS in *Y. lipolytica*, growth was dramatically improved on xylitol and growth was enabled in xylose without the need for adaptation. Disruption of the XDH or XKS genes using a CRISPR-Cas9 system [9] showed that these genes were essential for growth on xylitol. Overexpression of XKS was found to improve xylose and xylitol metabolism, leading to the conclusion that both XDH and XKS are rate limiting.

Ledesma-Amaro et al. heterologously expressed the XYR and XDH from *S. stipitis* along with the native XKS to achieve efficient growth on xylose [29]. It was found that the XYR and XDH were

necessary but not sufficient to achieve efficient growth and that XKS was a significant bottleneck in the pathway. Integration of xylose gene from *S. stipitis* followed by adaptive evolution has also led to efficient xylose utilization [28]. In sum, these papers demonstrate *Y. lipolytica* is a robust host capable of high product titers on alternative substrates.

## 7. Conclusion

*Y. lipolytica* continues to increase in popularity for metabolic engineering efforts as a result of further advances in genetic tool development. The long-standing and growing industrial applications using this oleaginous yeast is a testimony for continued studies. Genetic engineering tools have advanced from early promoters and integration transformations to novel highly expressing engineered hybrid promoters, episomal vectors and CRISPR-Cas9 genome editing. The number of chemicals and fuels that have been demonstrated by engineering *Y. lipolytica* have also grown dramatically. However, genetic engineering tools and therefore research speeds, continue to lag compared to other model organisms such as *Saccharomyces cerevisiae* and *Escherichia coli*. A better understanding of the genetic and regulatory systems of *Y. lipolytica* is required, as well as more powerful genome editing tools to facilitate rapid strain engineering. Despite these current limitations, it is clear that *Y. lipolytica* will continue to play a role in industrial microbial applications to improve society.

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## Conflict of Interests

All authors declare no conflicts of interest in this paper

## References

1. Goncalves FA, Colen G, Takahashi JA (2014) *Yarrowia lipolytica* and its multiple applications in the biotechnological industry. *Sci World J* 2014: 476207.
2. Zhu Q, Jackson EN (2015) Metabolic engineering of *Yarrowia lipolytica* for industrial applications. *Curr Opin Biotechnol* 36: 65–72.
3. Cordero Otero R, Gaillardin C (1996) Efficient selection of hygromycin-B-resistant *Yarrowia lipolytica* transformants. *Appl Microbiol Biotechnol* 46: 143–148.
4. Chen DC, Beckerich JM, Gaillardin C (1997) One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 48: 232–235.



5. Liu L, Otoupal P, Pan A, et al. (2014) Increasing expression level and copy number of a *Yarrowia lipolytica* plasmid through regulated centromere function. *FEMS Yeast Res* 14: 1124–1127.
6. Hussain MS, Gambill L, Smith S, et al. (2016) Engineering promoter architecture in oleaginous yeast *Yarrowia lipolytica*. *ACS Synth Biol* 5: 213–223.
7. Blazeck J, Reed B, Garg R, et al. (2013) Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 97: 3037–3052.
8. Blazeck J, Liu L, Redden H, et al. (2011) Tuning gene expression in *Yarrowia lipolytica* by a hybrid promoter approach. *Appl Environ Microbiol* 77: 7905–7914.
9. Schwartz CM, Hussain MS, Blenner M, et al. (2016) Synthetic RNA polymerase III promoters facilitate high-efficiency CRISPR-Cas9-mediated genome editing in *Yarrowia lipolytica*. *ACS Synth Biol*.
10. Blazeck J, Hill A, Liu L, et al. (2014) Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun* 5: 3131.
11. Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metab Eng* 15: 1–9.
12. Qiao K, Imam Abidi SH, Liu H, et al. (2015) Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metab Eng* 29: 56–65.
13. Xu P, Qiao KJ, Ahn WS, et al. (2016) Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals. *P Natl Acad Sci USA* 113: 10848–10853.
14. Friedlander J, Tsakraklides V, Kamineni A, et al. (2016) Engineering of a high lipid producing *Yarrowia lipolytica* strain. *Biotechnol Biofuels* 9: 77.
15. Xue Z, Sharpe PL, Hong SP, et al. (2013) Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. *Nat Biotechnol* 31: 734–740.
16. Smit MS, Mokgoro MM, Setati E, et al. (2005) alpha,omega-Dicarboxylic acid accumulation by acyl-CoA oxidase deficient mutants of *Yarrowia lipolytica*. *Biotechnol Lett* 27: 859–864.
17. Haddouche R, Poirier Y, Delessert S, et al. (2011) Engineering polyhydroxyalkanoate content and monomer composition in the oleaginous yeast *Yarrowia lipolytica* by modifying the ss-oxidation multifunctional protein. *Appl Microbiol Biotechnol* 91: 1327–1340.
18. Blazeck J, Hill A, Jamoussi M, et al. (2015) Metabolic engineering of *Yarrowia lipolytica* for itaconic acid production. *Metab Eng* 32: 66–73.
19. Ledesma-Amaro R, Dulermo R, Niehus X, et al. (2016) Combining metabolic engineering and process optimization to improve production and secretion of fatty acids. *Metab Eng* 38: 38–46.
20. Zhou J, Yin X, Madzak C, et al. (2012) Enhanced alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06 by alteration of the acetyl-CoA metabolism. *J Biotechnol* 161: 257–264.
21. Yovkova V, Otto C, Aurich A, et al. (2014) Engineering the alpha-ketoglutarate overproduction from raw glycerol by overexpression of the genes encoding NADP<sup>+</sup>-dependent isocitrate dehydrogenase and pyruvate carboxylase in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 98: 2003–2013.
22. Wang G, Xiong X, Ghogare R, et al. (2016) Exploring fatty alcohol-producing capability of *Yarrowia lipolytica*. *Biotechnol Biofuels* 9: 107.

23. Rutter CD, Rao CV (2016) Production of 1-decanol by metabolically engineered *Yarrowia lipolytica*. *Metab Eng* 38: 139–147.
24. Wang W, Wei H, Knoshaug E, et al. (2016) Fatty alcohol production in *Lipomyces starkeyi* and *Yarrowia lipolytica*. *Biotechnol Biofuels* 9: 227.
25. Ryu S, Hipp J, Trinh CT (2015) Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica*. *Appl Environ Microbiol* 82: 1334–1345.
26. Rodriguez GM, Hussain MS, Gambill L, et al. (2016) Engineering xylose utilization in *Yarrowia lipolytica* by understanding its cryptic xylose pathway. *Biotechnol Biofuels* 9: 149.
27. Ledesma-Amaro R, Nicaud JM (2016) Metabolic engineering for expanding the substrate range of *Yarrowia lipolytica*. *Trends Biotechnol* 34: 798–809.
28. Li H, Alper HS (2016) Enabling xylose utilization in *Yarrowia lipolytica* for lipid production. *Biotechnol J* 11: 1230–1240.
29. Ledesma-Amaro R, Lazar Z, Rakicka M, et al. (2016) Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. *Metab Eng* 38: 115–124.
30. Ledesma-Amaro R, Dulermo T, Nicaud JM (2015) Engineering *Yarrowia lipolytica* to produce biodiesel from raw starch. *Biotechnol Biofuels* 8: 148.
31. Celinska E, Bialas W, Borkowska M, et al. (2015) Cloning, expression, and purification of insect (*Sitophilus oryzae*) alpha-amylase, able to digest granular starch, in *Yarrowia lipolytica* host. *Appl Microbiol Biotechnol* 99: 2727–2739.
32. Yang CH, Huang YC, Chen CY, et al. (2010) Heterologous expression of *Thermobifida fusca* thermostable alpha-amylase in *Yarrowia lipolytica* and its application in boiling stable resistant sago starch preparation. *J Ind Microbiol Biotechnol* 37: 953–960.
33. Xu J, Xiong P, He B (2016) Advances in improving the performance of cellulase in ionic liquids for lignocellulose biorefinery. *Bioresour Technol* 200: 961–970.
34. Dujon B, Sherman D, Fischer G, et al. (2004) Genome evolution in yeasts. *Nature* 430: 35–44.
35. Liu L, Alper HS (2014) Draft genome sequence of the oleaginous yeast *Yarrowia lipolytica* PO1f, a commonly used metabolic engineering host. *Genome Announc* 2.
36. Magnan C, Yu J, Chang I, et al. (2016) Sequence assembly of *Yarrowia lipolytica* strain W29/CLIB89 shows transposable element diversity. *PLoS One* 11: e0162363.
37. van Heerikhuizen H, Ykema A, Klootwijk J, et al. (1985) Heterogeneity in the ribosomal RNA genes of the yeast *Yarrowia lipolytica*; cloning and analysis of two size classes of repeats. *Gene* 39: 213–222.
38. Davidow LS, Apostolakos D, O'Donnell MM, et al. (1985) Integrative transformation of the yeast *Yarrowia lipolytica*. *Curr Genet* 10: 39–48.
39. Barth G, Gaillardin C (1996) *Yarrowia lipolytica*, In: Wolf K, *Nonconventional Yeasts in Biotechnology*, NewYork: Springer.
40. Wang JH, Hung W, Tsai SH (2011) High efficiency transformation by electroporation of *Yarrowia lipolytica*. *J Microbiol* 49: 469–472.
41. Duquesne S, Bordes F, Fudalej F, et al. (2012) The yeast *Yarrowia lipolytica* as a generic tool for molecular evolution of enzymes. *Methods Mol Biol* 861: 301–312.
42. Theerachat M, Emond S, Cambon E, et al. (2012) Engineering and production of laccase from *Trametes versicolor* in the yeast *Yarrowia lipolytica*. *Bioresour Technol* 125: 267–274.

43. Juretzek T, Wang HJ, Nicaud JM, et al. (2000) Comparison of promoters suitable for regulated overexpression of  $\beta$ -galactosidase in the alkane-utilizing yeast *Yarrowia lipolytica*. *Biotechnol Bioproc Eng* 5: 320–326.
44. Wang HJ, Le Dall MT, Wach Y, et al. (1999) Evaluation of acyl coenzyme A oxidase (Aox) isozyme function in the n-alkane-assimilating yeast *Yarrowia lipolytica*. *J Bacteriol* 181: 5140–5148.
45. Yamagami S, Morioka D, Fukuda R, et al. (2004) A basic helix-loop-helix transcription factor essential for cytochrome p450 induction in response to alkanes in yeast *Yarrowia lipolytica*. *J Biol Chem* 279: 22183–22189.
46. Blanchin-Roland S, Cordero Otero RR, Gaillardin C (1994) Two upstream activation sequences control the expression of the XPR2 gene in the yeast *Yarrowia lipolytica*. *Mol Cell Biol* 14: 327–338.
47. Madzak C, Blanchin-Roland S, Cordero Otero RR, et al. (1999) Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter. *Microbiology* 145 (Pt 1): 75–87.
48. Madzak C, Treton B, Blanchin-Roland S (2000) Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J Mol Microbiol Biotechnol* 2: 207–216.
49. Shabbir Hussain M, Gambill L, Smith S, et al. (2016) Engineering promoter architecture in oleaginous yeast *Yarrowia lipolytica*. *ACS Synth Biol* 5: 213–223.
50. Muller S, Sandal T, Kamp-Hansen P, et al. (1998) Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* 14: 1267–1283.
51. Kozak M (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res* 12: 857–872.
52. Kozak M (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44: 283–292.
53. Merkulov S, van Assema F, Springer J, et al. (2000) Cloning and characterization of the *Yarrowia lipolytica* squalene synthase (SQS1) gene and functional complementation of the *Saccharomyces cerevisiae* erg9 mutation. *Yeast* 16: 197–206.
54. Zhang B, Rong C, Chen H, et al. (2012) De novo synthesis of trans-10, cis-12 conjugated linoleic acid in oleaginous yeast *Yarrowia lipolytica*. *Microb Cell Fact* 11: 1–8.
55. Kramara J, Willcox S, Gunisova S, et al. (2010) Tay1 protein, a novel telomere binding factor from *Yarrowia lipolytica*. *J Biol Chem* 285: 38078–38092.
56. Farrel C, Mayorga M, Chevreux B (2013) Carotene hydroxylase and its use for producing carotenoids. In: Ltd DNP.
57. Gasmi N, Fudalej F, Kallel H, et al. (2011) A molecular approach to optimize hIFN alpha2b expression and secretion in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 89: 109–119.
58. Nakagawa S, Niimura Y, Gojobori T, et al. (2008) Diversity of preferred nucleotide sequences around the translation initiation codon in eukaryote genomes. *Nucleic Acids Res* 36: 861–871.

59. Schwartz CM, Hussain MS, Blenner M, et al. (2016) Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR-Cas9-Mediated Genome Editing in *Yarrowia lipolytica*. *ACS Synth Biol* 5: 356–359.
60. Gao S, Han L, Zhu L, et al. (2014) One-step integration of multiple genes into the oleaginous yeast *Yarrowia lipolytica*. *Biotechnol Lett* 36: 2523–2528.
61. Curran KA, Morse NJ, Markham KA, et al. (2015) Short synthetic terminators for improved heterologous gene expression in yeast. *ACS Synth Biol* 4: 824–832.
62. Laine JP, Singh BN, Krishnamurthy S, et al. (2009) A physiological role for gene loops in yeast. *Genes Dev* 23: 2604–2609.
63. Kanaar R, Hoeijmakers JH, van Gent DC (1998) Molecular mechanisms of DNA double strand break repair. *Trends Cell Biol* 8: 483–489.
64. Pastink A, Lohman PH (1999) Repair and consequences of double-strand breaks in DNA. *Mutat Res* 428: 141–156.
65. Pastink A, Eeken JC, Lohman PH (2001) Genomic integrity and the repair of double-strand DNA breaks. *Mutat Res* 480: 37–50.
66. Loeillet S, Palancade B, Cartron M, et al. (2005) Genetic network interactions among replication, repair and nuclear pore deficiencies in yeast. *DNA Repair (Amst)* 4: 459–468.
67. Fickers P, Le Dall MT, Gaillardin C, et al. (2003) New disruption cassettes for rapid gene disruption and marker rescue in the yeast *Yarrowia lipolytica*. *J Microbiol Methods* 55: 727–737.
68. Van Dyck E, Stasiak AZ, Stasiak A, et al. (1999) Binding of double-strand breaks in DNA by human Rad52 protein. *Nature* 398: 728–731.
69. Verbeke J, Beopoulos A, Nicaud JM (2013) Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains. *Biotechnol Lett* 35: 571–576.
70. Kretzschmar A, Otto C, Holz M, et al. (2013) Increased homologous integration frequency in *Yarrowia lipolytica* strains defective in non-homologous end-joining. *Curr Genet* 59: 63–72.
71. Vasiliki T, Elena B, Gregory S, et al. (2015) Improved gene targeting through cell cycle synchronization. *PLoS One* 10.
72. Isaac RS, Jiang F, Doudna JA, et al. (2016) Nucleosome breathing and remodeling constrain CRISPR-Cas9 function. *Elife* 5.
73. Daer R, Cutts JP, Brafman DA, et al. (2016) The impact of chromatin dynamics on Cas9-mediated genome editing in human cells. *ACS Synth Biol*.
74. Hamilton DL, Abremski K (1984) Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. *J Mol Biol* 178: 481–486.
75. Le Dall MT, Nicaud JM, Gaillardin C (1994) Multiple-copy integration in the yeast *Yarrowia lipolytica*. *Curr Genet* 26: 38–44.
76. Casaregola S, Feynerol C, Diez M, et al. (1997) Genomic organization of the yeast *Yarrowia lipolytica*. *Chromosoma* 106: 380–390.
77. Schmid-Berger N, Schmid B, Barth G (1994) Ylt1, a highly repetitive retrotransposon in the genome of the dimorphic fungus *Yarrowia lipolytica*. *J Bacteriol* 176: 2477–2482.

78. Mauersberger S, Wang HJ, Gaillardin C, et al. (2001) Insertional mutagenesis in the n-alkane-assimilating yeast *Yarrowia lipolytica*: generation of tagged mutations in genes involved in hydrophobic substrate utilization. *J Bacteriol* 183: 5102–5109.
79. Bordes F, Fudalej F, Dossat V, et al. (2007) A new recombinant protein expression system for high-throughput screening in the yeast *Yarrowia lipolytica*. *J Microbiol Meth* 70: 493–502.
80. Pignede G, Wang HJ, Fudalej F, et al. (2000) Autocloning and amplification of LIP2 in *Yarrowia lipolytica*. *Appl Environ Microbiol* 66: 3283–3289.
81. Nicaud JM, Madzak C, van den Broek P, et al. (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. *FEMS Yeast Res* 2: 371–379.
82. Gao C, Qi Q, Madzak C, et al. (2015) Exploring medium-chain-length polyhydroxyalkanoates production in the engineered yeast *Yarrowia lipolytica*. *J Ind Microbiol Biotechnol* 42: 1255–1262.
83. Ratledge C (2002) Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans* 30: 1047–1050.
84. Beopoulos A, Cescut J, Haddouche R, et al. (2009) *Yarrowia lipolytica* as a model for bio-oil production. *Prog Lipid Res* 48: 375–387.
85. Ratledge C (2014) The role of malic enzyme as the provider of NADPH in oleaginous microorganisms: a reappraisal and unsolved problems. *Biotechnol Lett* 36: 1557–1568.
86. Wynn JP, Kendrick A, Hamid AA, et al. (1997) Malic enzyme: a lipogenic enzyme in fungi. *Biochem Soc Trans* 25: S669.
87. Zhang H, Zhang L, Chen H, et al. (2013) Regulatory properties of malic enzyme in the oleaginous yeast, *Yarrowia lipolytica*, and its non-involvement in lipid accumulation. *Biotechnol Lett* 35: 2091–2098.
88. Wasylenko TM, Ahn WS, Stephanopoulos G (2015) The oxidative pentose phosphate pathway is the primary source of NADPH for lipid overproduction from glucose in *Yarrowia lipolytica*. *Metab Eng* 30: 27–39.
89. Liu N, Qiao K, Stephanopoulos G (2016) <sup>13</sup>C Metabolic Flux Analysis of acetate conversion to lipids by *Yarrowia lipolytica*. *Metab Eng* 38: 86–97.
90. Morin N, Cescut J, Beopoulos A, et al. (2011) Transcriptomic analyses during the transition from biomass production to lipid accumulation in the oleaginous yeast *Yarrowia lipolytica*. *PLoS One* 6: e27966.
91. Beopoulos A, Mrozova Z, Thevenieau F, et al. (2008) Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol* 74: 7779–7789.
92. Beopoulos A, Haddouche R, Kabran P, et al. (2012) Identification and characterization of DGA2, an acyltransferase of the DGAT1 acyl-CoA:diacylglycerol acyltransferase family in the oleaginous yeast *Yarrowia lipolytica*. New insights into the storage lipid metabolism of oleaginous yeasts. *Appl Microbiol Biotechnol* 93: 1523–1537.
93. Wang ZP, Xu HM, Wang GY, et al. (2013) Disruption of the MIG1 gene enhances lipid biosynthesis in the oleaginous yeast *Yarrowia lipolytica* ACA-DC 50109. *Biochim Biophys Acta* 1831: 675–682.
94. Seip J, Jackson R, He H, et al. (2013) Snf1 is a regulator of lipid accumulation in *Yarrowia lipolytica*. *Appl Environ Microbiol* 79: 7360–7370.

95. Dulermo T, Nicaud JM (2011) Involvement of the G3P shuttle and beta-oxidation pathway in the control of TAG synthesis and lipid accumulation in *Yarrowia lipolytica*. *Metab Eng* 13: 482–491.
96. Liu L, Pan A, Spofford C, et al. (2015) An evolutionary metabolic engineering approach for enhancing lipogenesis in *Yarrowia lipolytica*. *Metab Eng* 29: 36–45.
97. Liu L, Markham K, Blazeck J, et al. (2015) Surveying the lipogenesis landscape in *Yarrowia lipolytica* through understanding the function of a Mga2p regulatory protein mutant. *Metab Eng* 31: 102–111.
98. Poirier Y, Antonenkov VD, Glumoff T, et al. (2006) Peroxisomal beta-oxidation—a metabolic pathway with multiple functions. *Biochim Biophys Acta* 1763: 1413–1426.
99. Dulermo R, Gamboa-Melendez H, Ledesma-Amaro R, et al. (2015) Unraveling fatty acid transport and activation mechanisms in *Yarrowia lipolytica*. *Biochim Biophys Acta* 1851: 1202–1217.
100. Haddouche R, Delessert S, Sabirova J, et al. (2010) Roles of multiple acyl-CoA oxidases in the routing of carbon flow towards beta-oxidation and polyhydroxyalkanoate biosynthesis in *Yarrowia lipolytica*. *FEMS Yeast Res* 10: 917–927.
101. Hazer B, Steinbuchel A (2007) Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. *Appl Microbiol Biotechnol* 74: 1–12.
102. Rehm BH (2003) Polyester synthases: natural catalysts for plastics. *Biochem J* 376: 15–33.
103. Sabirova JS, Haddouche R, Van Bogaert I, et al. (2011) The “LipoYeasts” project: using the oleaginous yeast *Yarrowia lipolytica* in combination with specific bacterial genes for the bioconversion of lipids, fats and oils into high-value products. *Microb Biotechnol* 4: 47–54.
104. Chuah JA, Tomizawa S, Yamada M, et al. (2013) Characterization of site-specific mutations in a short-chain-length/medium-chain-length polyhydroxyalkanoate synthase: in vivo and in vitro studies of enzymatic activity and substrate specificity. *Appl Environ Microbiol* 79: 3813–3821.
105. Tsigie YA, Wang CY, Truong CT, et al. (2011) Lipid production from *Yarrowia lipolytica* Po1g grown in sugarcane bagasse hydrolysate. *Bioresour Technol* 102: 9216–9222.
106. Ruiz-Herrera J, Sentandreu R (2002) Different effectors of dimorphism in *Yarrowia lipolytica*. *Arch Microbiol* 178: 477–483.
107. Fontes GC, Amaral PF, Nele M, et al. (2010) Factorial design to optimize biosurfactant production by *Yarrowia lipolytica*. *J Biomed Biotechnol* 2010: 821306.
108. Pan LX, Yang DF, Shao L, et al. (2009) Isolation of the oleaginous yeasts from the soil and studies of their lipid-producing capacities. *Food Technol Biotechnol* 47: 215–220.



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