



Metabolic engineering of *Yarrowia lipolytica* for industrial applications

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Yarrowia lipolytica is a safe and robust yeast that has a history of industrial applications. Its physiological, metabolic and genomic characteristics have made it a superior host for metabolic engineering. The results of optimizing internal pathways and introducing new pathways have demonstrated that *Y. lipolytica* can be a platform cell factory for cost-effective production of chemicals and fuels derived from fatty acids, lipids and acetyl-CoA. Two products have been commercialized from metabolically engineered *Y. lipolytica* strains producing high amounts of omega-3 eicosapentaenoic acid, and more products are on the way to be produced at industrial scale. Here we review recent progress in metabolic engineering of *Y. lipolytica* for production of biodiesel fuel, functional fatty acids and carotenoids.

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Introduction

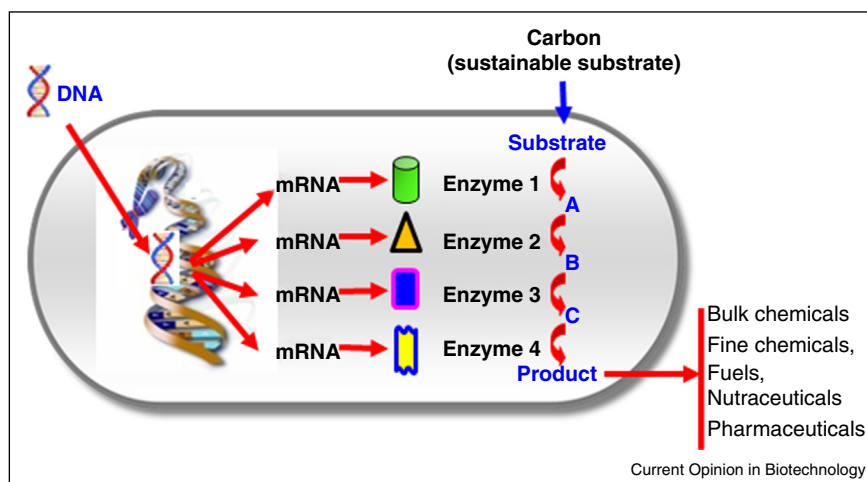
Metabolic engineering is a process of optimizing native metabolic pathways and regulatory networks or assembling heterologous metabolic pathways for production of targeted molecules using molecular, genetic and combinatorial approaches. The purpose of the metabolic engineering is to generate a cell factory that produces cost-effective molecules at industrial scale (Figure 1). In order to carry out a successful metabolic engineering project, the following factors should be considered: first, use of a safe and robust host organism with genetic and physiological advantages for the target product; second, understanding of the metabolic pathways, co-factor balances, and regulatory networks; third, selection of effective enzymes and genetic elements; fourth, analysis of stoichiometry and thermodynamics; fifth, availability of efficient transformation technology; and sixth, approaches to reduce potential toxic intermediates and products. Since

its inception [1,2], microbial metabolic engineering has mainly focused on traditional model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* [3–5].

Metabolic engineering of *E. coli* has achieved commercial production of 1,3-propanediol and 1,4-butanediol by DuPont [6] and Genomatica [7], respectively. *S. cerevisiae* has been metabolically engineered to produce artemisinic acid [8] and amorpho-4,11-diene [9], precursors of the antimalarial drug artemisinin commercially produced by Amyris. To reach cost-effective production of a product, host selection is very important. For example, it has been demonstrated that *S. cerevisiae* is a better host than *E. coli* for production of artemisinin precursors [10]. Both *E. coli* and *S. cerevisiae* have been selected as favored hosts for metabolic engineering largely due to the availability of classical genetic approaches, and accessibility of modern molecular biological tools for these two extensively studied model organisms.

After three decades of studies, *Yarrowia lipolytica* has emerged as a preferred non-conventional yeast for metabolic engineering, especially for the production of chemicals derived from acetyl-CoA, fatty acids (FA) and lipids [11–16]. *Y. lipolytica* is a hemiascomycetous yeast, with a history of industrial applications. It has been used as a model oleaginous organism for a wide range of studies including lipid accumulation and degradation, peroxisome biogenesis, dimorphism, and protein secretion pathways [12–14]. Extensive analyses show that *Y. lipolytica* is a safe organism for industrial applications [17]. Wild type (WT) *Y. lipolytica* strains can use glucose, fructose, glycerol and hydrophobic substrates as carbon sources; strain Po1g can also use xylose and sugarcane bagasse hydrolysate [18]. In addition, *Y. lipolytica* strains have been engineered to use sucrose [19,20]. When both glucose and fructose are available, *Y. lipolytica* uses glucose first; over-expression of a hexokinase can help *Y. lipolytica* to effectively use fructose [21]. As an aerobic organism; *Y. lipolytica* has high flux through the pentose phosphate pathway (PPP) that generates co-factor NADPH to support fatty acid (FA) biosynthesis [22,23]. Most *Y. lipolytica* strains are haploid, but can also exist in diploid form that is more tolerant to various conditions [13,16]. The whole genome sequence of strain CLIB122 [24] and a draft genome sequence of strain PO1f [25] have been published. Suitable genetic tools and transformation technologies have been developed [11,13,16,26]. In this review, we will focus on recent advances in metabolic engineering of *Y. lipolytica* for production of chemicals derived from acetyl-CoA, FA and lipids.

Figure 1



Metabolic engineering illustration. Metabolic engineering is a process to optimize an internal metabolic pathway and its regulatory networks or assemble and coordinate a heterologous metabolic pathway within a cell for production of a target molecule using molecular, genetic and combinatorial approaches.

Metabolic engineering of lipid biosynthesis and application for biodiesel production

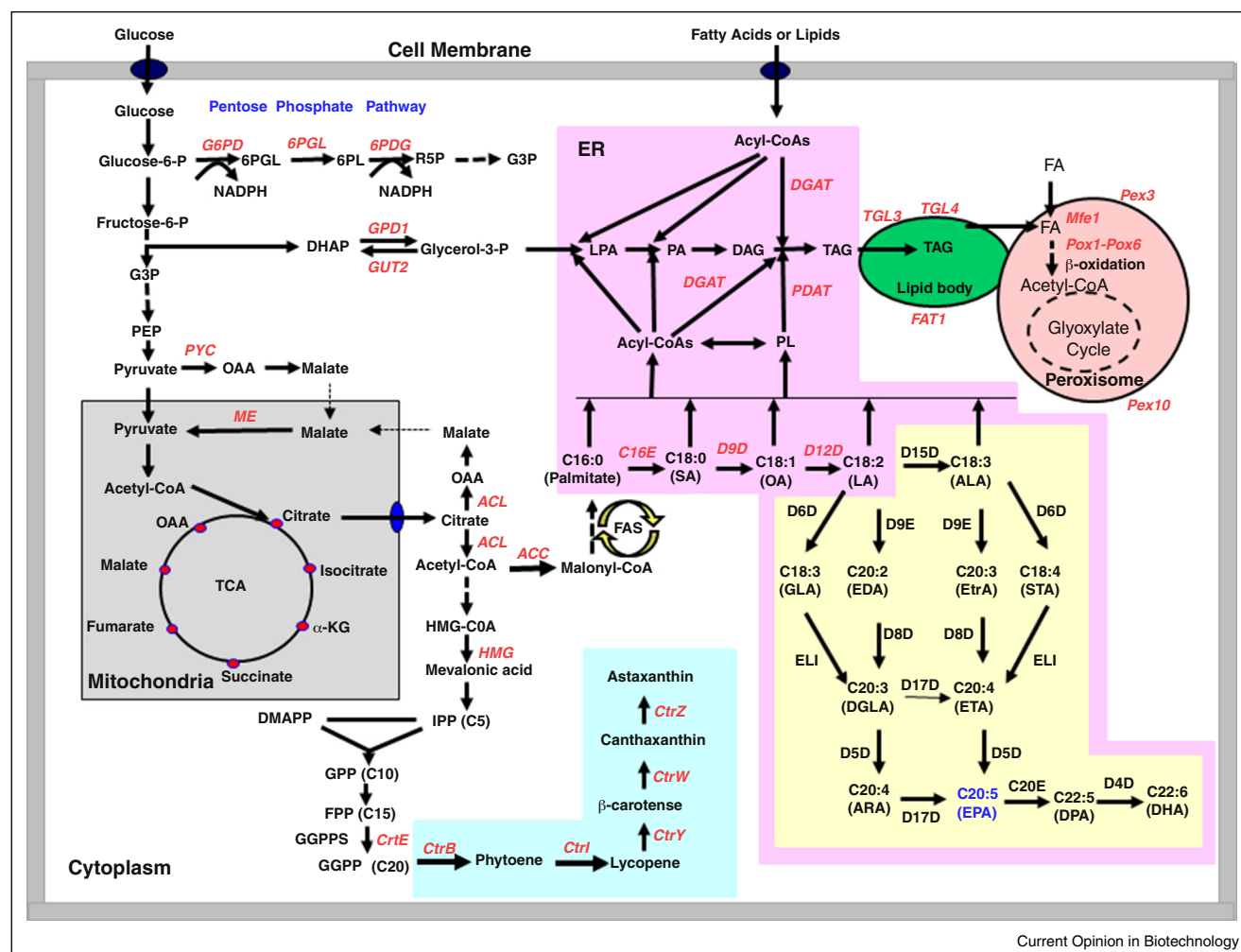
Biodiesel is a mixture of FA esters [27,28] that is currently produced from vegetable oil and animal fats. The global production reached 180 Mt/year in 2012 [27]. Ideally, biodiesel production should use renewable and sustainable substrates, and should not compete with the food and feed supply. Microbial oils with similar FA composition and energy value of plant oils are suitable for biodiesel production. The FA composition of *Y. lipolytica* lipid is similar to soybean oil, rich in C16 and C18 unsaturated FAs [12,15,27]. Biodiesel produced from these FAs will maintain liquid form under extreme cold conditions. Significant progress has been achieved toward production of cost-effective lipids from *Y. lipolytica*.

Some *Y. lipolytica* strains are oleaginous organisms that can synthesize FA from glucose or other sugars and accumulate lipids to more than 25% DCW [11,12]. More than 90% of *Yarrowia* lipid is accumulated in triacylglycerol (TAG) form from this *de novo* FA biosynthesis pathway [12,13,15]. *Y. lipolytica* can also use an *ex novo* process to accumulate intracellular lipids by incorporation of exogenous FAs and lipids. Both the *de novo* and *ex novo* lipid accumulation and the underlying mechanisms (Figure 2) have begun to be elucidated. The *de novo* FA synthesis starts from acetyl-CoA. When *Y. lipolytica* cells sense nutritional limitation in their environment, such as nitrogen starvation with excess of glucose in controlled fermentation processes, citric acid is over-produced and pumped out from the mitochondria. Cytosolic citric acid is converted to acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACL), an enzymatic activity which is crucial for FA biosynthesis. The conversion of acetyl-CoA

to malonyl-CoA by carboxylase (ACC1) is the committing step in FA biosynthesis. The synthesized C16 and C18 acyl-CoAs can be incorporated into lipids via the Kennedy pathway; and can be stored in lipid bodies (LB) or metabolized via the beta-oxidation pathway in peroxisomes. The required co-factor NADPH for FA biosynthesis is derived mainly from the PPP. Up-regulation of glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase of the PPP can increase NADPH supply [29]. Unlike some other oleaginous microorganisms, there is no cytosolic malic enzyme (ME) in *Y. lipolytica* [30]. Over-expression of the mitochondrial form of ME was not shown to affect FA biosynthesis [30]. Over-expression of a heterologous pyruvate carboxylase (PYC) increased lipid content more than 20%. When the engineered strain over-expressed both *PYC* and *ACL1*, the lipid content reached 45% DCW, which was about 50% increase over the parent strain [31]. These results suggest that over-expression of *PYC*, *ACL* and *ACC1* 'push' the carbon flux into FA biosynthesis pathway.

The regulatory network of lipid biosynthesis in *Yarrowia* is starting to be elucidated. The sucrose non-fermenting 1 (Snf1) protein kinase is a key regulator in glucose signal transduction in *S. cerevisiae* [32]; recent studies have demonstrated that SNF1 is also a negative regulator of *de novo* FA biosynthesis pathway, especially in the transition from growth to oleaginous phase in *Y. lipolytica* (Figure 3). The *snf1* null mutant constitutively synthesized FA at high levels, resulting in a 2.6-fold increase of lipid content over the control strain [33]. MIG1 is a repressor in the SNF1 network. The *mig1* defective mutant produced lipids at 48.7% DCW, while the WT strain produced lipids at 36% DCW [34]. The expression

Figure 2



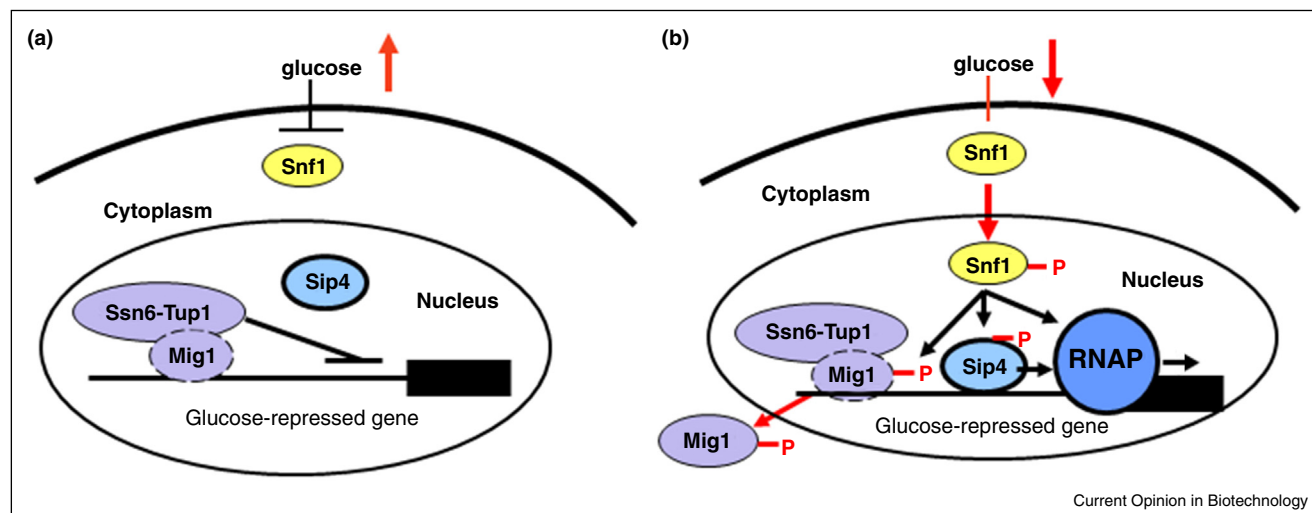
Overview of metabolic engineering of *Y. lipolytica* to produce molecules derived from fatty acid (FA), lipids or acetyl-coA. Cytosolic glucose enters glycolysis and pentose phosphate pathways. Pyruvate enters mitochondria where it is converted into acetyl-CoA then used in TCA cycle. Excess citrate is transported from the mitochondria into cytosol. ATP citrate lyase (ACL) converts the cytosolic citrate into acetyl-CoA that is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC), the first committing step of FA synthesis. After FA synthesis, triacylglycerol (TAG) is synthesized in the endoplasmic reticulum (ER) via Kennedy pathway and then accumulated in lipid bodies (LB). Acyl-CoA is used for acylation of glycerol-3-phosphate to form lysophosphatidic acid (LPA) that is further acylated to form phosphatidic acid (PA). PA is dephosphorylated to form diacylglycerol (DAG), which is then acylated to produce TAG catalyzed by DAG acyltransferase (DGAT). TAG can also be synthesized by phospholipid (PL):DAG acyltransferase (PDAT) using PL and DAG as substrates. *Ex novo* FA accumulation also uses Kennedy pathway. FA is metabolized by β-oxidation pathway in peroxisome. **Abbreviations:** α-KG, alpha-ketoglutarate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde3-phosphate; GPD1, glycerol-3-phosphate dehydrogenase; GUT2, glycerol-kinase; ME, malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pex3 and Pex10, peroxisome biogenesis factor 3 and 10, respectively; Pox1 to Pox6, acyl-CoA oxidases 1–6, respectively; TGL3 and TGL4, TAG lipase 3 and 4, respectively; PYC, pyruvatecarboxylase; TCA, tricarboxylic acid cycle. **Yellow box:** schematic diagram of aerobic pathways for ω-3 and ω-6 FA biosynthesis. **Abbreviations:** C16E, EL1, C20E and D9E are C16/C18, C18, C20/C22, Δ-9 elongases, respectively. D4D, D5D, D6D, D8D, D9D, D12D, D15D and D17D are Δ-4, Δ-5, Δ-6, Δ-8, Δ-9, Δ-12, Δ-15, and Δ-17 desaturases, respectively. **Light blue box:** schematic diagram of carotenoid biosynthesis pathway. **Abbreviations:** IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG, HMG reductase; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; CrtY, lycopene cyclase; CrtW, β-carotene ketolase; CrtZ, β-carotene hydroxylase. Solid ovals: transporters. Light dash lines: possible route, not confirmed. Thin line: weak activity.

of many lipid-related genes was changed in the *snf1* and *mig1* mutants [33,34].

The committing step for TAG biosynthesis is catalyzed by diacylglycerol (DAG) acyltransferase (DGAT). *Y.*

lipolytica contains three classes of DGAT: DGAT1, DGAT2, and a phospholipid:diacylglycerol acyltransferase (PDAT). Compared to the WT strain, the lipid content reduced 64% or 87% in strains with *dgat2* or *dagt1/dgat2/pdat* genotype [35], respectively. A similar

Figure 3



Schematic presentation of Snf1 signal transduction pathway in *Yarrowia*. **(a)** When glucose is unlimited (red arrow up), Ssn6-Tup1 and Mig1 repressors repress the transcription of responsive genes. **(b)** When glucose is limited (red arrow down), Snf1 is activated and removes Mig1 repressor from promoters to de-repress glucose repressible genes that include those for alternative C-source utilization like beta-oxidation, gluconeogenesis, and respiration (TCA). *Y. lipolytica* is an obligate aerobe, glucose repression is not as strict as in *S. cerevisiae*. Abbreviations: Mig1, a zinc finger repressor; P, phosphate; RNAP, RNA polymerase; Sip4, a Zn(2)Cys(6) transcriptional activator; Snf1, SNF1 protein kinase; Ssn6-Tup1, Ssn6-Tup1 repressor complex.

effect of *dgat2* was also observed in strain W29 [36]. When the *DGAT2* was over expressed in WT or *pex10* (peroxisomal biogenesis factor 10) strains, the lipid content reached 38% and 52% DCW [37**], respectively. *ACC1* over-expression in the WT strain resulted in about 2-fold lipid increase over control. When the *ACC1* and *DGAT2* were simultaneously over-expressed, the lipid content reached about 62% DCW. The overall lipid yield was 0.195 g/g glucose, which was about 61% of the theoretical maximum yield of 0.32 g/g [38**]. Over-expression of *DGAT2* in a strain with *pex10/mfe1* (a peroxisomal hydroxyacyl coenzyme A dehydrogenase) resulted in lipid production at 87% DCW [39**]. These results suggest that over-expression of *DGAT2* builds a sink to 'pull' the TAG biosynthesis.

Significant progress has been achieved in understanding and engineering the *de novo* lipid biosynthesis in *Y. lipolytica*. However it is still a challenge to produce cost-effective biodiesel from this process if glucose is used as the carbon source. Assuming a glucose price is of \$400/Mt, and an engineered strain produces lipids at maximum yield 0.32 g/g, the glucose cost will be \$1.25/kg lipid. Therefore, there is great potential if *Y. lipolytica* can use sugars from *in vivo* lignocellulose processes [40,41], which will significantly reduce the sugar cost.

Yarrowia can also utilize an *ex novo* process to accumulate lipids by incorporation of exogenous FAs and lipids (Figure 2). Elimination of beta-oxidation by knockout

of *POX* genes encoding the acyl-CoA oxidases increased lipid accumulation, and expression of *POX2* in the *Yarrowia* mutant (*pox2/pox3/pox5*) produced cells almost entirely occupied by LB when grown on oleic acid [42]. The *ex novo* lipid accumulation reached 80% DCW when the glycerol 3-phosphate (G3P) pool was increased by either *GUT2* deletion or *GPD1* over-expression in strains with eliminated β -oxidation [43,44]. The mutation of *FAT1*, determining FA export from LB, increased lipids 54% over control strain [45*]. Additionally, the capacity for lipid accumulation was doubled by inactivation of either one or both TAG lipases localized in LB [46*]. The *ex novo* lipid accumulation by *Y. lipolytica* has the potential to produce cost-effective biodiesel if appropriate agricultural and industrial oil wastes can be used as substrates.

Metabolic engineering of *Y. lipolytica* to produce functional fatty acids

Although the FA composition is similar to soybean oil [13,37**], the only polyunsaturated FA in *Yarrowia* lipid is linoleic acid (LA, C18:2n-6). The ω -3 FAs α -linolenic acid (ALA, C18:3n-3), stearidonic acid (STA, C18:4n-3), eicosapentaenoic acid (EPA, C20:4n-3), docosapentaenoic acid (DPA, C22:5n-3), docosahexaenoic acid (DHA, C22:6n-3); and the ω -6 FAs γ -linolenic acid (GLA, C18:3n-6), dihomo- γ -linolenic acid (DGLA, C20:3n-6) and arachidonic acid (ARA, C20:4n-6) are all commercially relevant products. *Y. lipolytica* has been developed as a platform cell factory to produce these ω -3 and ω -6 FAs [11,37**,47].

Co-expression of $\Delta 12$ -desaturase (D12D) and $\Delta 6$ desaturase (D6D) in *Y. lipolytica* resulted in GLA accumulation as over 25% total lipids [48,49]. Increasing copy number of D12D and D6D, and the over-expression of C16/C18 elongase (C16E) [50] in a *pex3* mutant significantly increased GLA production to reach 60% total lipids [Zhu *et al.*, unpublished data]. Similar strategies were employed to generate *Yarrowia* strains to produce DGLA and ARA to reach 42% and 35% total lipids, respectively [37**].

Yarrowia strains have been generated to produce EPA as the first commercial product. EPA is a key component in fish oil with known importance in human nutrition. A first generation strain Y4305 containing 30 copies of nine different heterologous genes was generated to produce EPA at 56.6% total lipids and about 15% DCW [37**]. Additional strains [51**] were later developed by combination of several strategies. First, an efficient EPA biosynthetic pathway was built using strong promoters, codon-optimized heterologous genes, and multiple gene copies for each step. Second, the carbon flux was pushed toward the EPA biosynthesis pathway by over-expression of *C16E* [50] and *D12D* [52], and was pulled for EPA biosynthesis by using multiple copies of $\Delta 17$ -desaturase genes [53]. Third, β -oxidation was eliminated by targeted deletion of peroxin genes [37**,54]. Fourth, EPA transportation was controlled by fine regulation of different acyltransferases [11,51**]. The new commercial strain Z5567 containing 41 copies of 19 different genes produced EPA at 50% total lipids and 25% DCW. Two commercial products have been developed based on EPA production strains. The purified EPA lipids have been used to develop NewharvestTM EPA oil, for a human nutritional supplement. The high-EPA biomass has been used to raise Verlasso[®], a sustainably farmed salmon.

The feasibility of engineered *Y. lipolytica* to produce DHA has also been demonstrated [55]. On the basis of strain Y4305, a DHA strain was constructed by over-expression of heterologous genes encoding for $\Delta 4$ -desaturase (D4D) and C20 elongase (C20E), or a multizyme having both D4D and C20E [56]. The DHA lipid contains about 5% DHA, 13.5% DPA and 26.5% EPA, which is similar to the seal oil [57] that contains high amounts of beneficial ω -3 DPA [58]. These studies have demonstrated that *Y. lipolytica* is a good host for metabolic engineering for production of ω -3 and ω -6 FAs. Strains can be developed to produce cost-effective products with desired FA compositions for specific applications.

Metabolic engineering of *Y. lipolytica* to produce carotenoids

Carotenoids such as lycopene, β -carotene, canthaxanthin, and astaxanthin are yellow to red-colored pigments; they are antioxidants with health benefits for humans and

animals. The global market is expected to reach \$1.4 billion in 2018 [59,60]. Currently, some carotenoids are chemically synthesized, while others are sourced from natural organisms. Metabolic engineering has the potential to produce these molecules more cost-effectively than current methods. Large efforts have been focused on using *E. coli* and *S. cerevisiae* as production hosts, and have been well reviewed [59,60]. In this section, we will review the progress toward production of carotenoids in metabolically engineered *Y. lipolytica*.

Carotenoids (Figure 2) are biosynthesized from two C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). As in other yeast, *Y. lipolytica* has the mevalonate pathway to produce IPP and DMAPP from acetyl-CoA. The geranyl pyrophosphate synthase converts IPP and DMAPP into geranyl pyrophosphate (GPP; C10), which is subsequently converted to farnesyl pyrophosphate (FPP; C15) and geranylgeranyl pyrophosphate (GGPP; C20). HMG reductase (HMG) is a rate-limiting step in the mevalonate pathway. If engineered correctly, the high acetyl-CoA flux in *Y. lipolytica* should produce more IPP and DMAPP, and then GGPP for carotenoid biosynthesis. Both DuPont and Microbia have separately selected *Y. lipolytica* as host for production of carotenoids [61,62].

Simultaneous over-expression of codon-optimized *CrtE*, *CrtB*, and *CrtI* in an EPA-producing strain accumulated lycopene to 2 mg/g DCW in flask assays [61,63]. When the codon-optimized *CrtE*, *CrtB*, *CrtI*, and *HMG* were over-expressed in a mutant (*pox1-pox6* and *gut2*), the engineered strain produced lycopene at 16 mg/g DCW in fed-batch cultures [64*]. The increased LBs of this mutant provided a sink for lycopene storage. As demonstrated in other systems, over-expression of a truncated *HMG* may further increase the lycopene production [65]. Co-expression of codon-optimized *CrtE*, *CrtB*, *CrtI* and *CrtY* in an EPA-producing strain accumulated β -carotene to 5.7 mg/g DCW, and the β -carotene was about 64% total carotenoids [61]. Microbia has also engineered *Yarrowia* to produce β -carotene [62]. Their safety profile studies showed that the β -carotene produced by *Y. lipolytica* is the same as other commercial products [66]. The feasibility of engineering *Y. lipolytica* to produce canthaxanthin and astaxanthin has also been demonstrated [61]. Additional research beyond what has been published would be necessary to increase the production rate, titer, and yield of these products to reach cost-effective commercial production.

Perspectives

Metabolic engineering of *Y. lipolytica* provides a unique platform for producing chemicals and fuels derived from acetyl-CoA, FA, and lipids. *Y. lipolytica* has now been metabolically engineered to produce omega-3 and omega-6 FAs, carotenoids and biodiesel. It is an attractive

platform for robust, sustainable fermentation manufacturing for a wide range of products. However, metabolic engineering of *Y. lipolytica* is still in its infancy, and there are a number of challenges to commercial success. Selection of the target molecule is very important for cost-effective production. The following criteria need to be considered. Does *Y. lipolytica* have physiological and genetic advantages for the selected molecule? What is the value of the product? Is there an existing chemical route, and if so, what are the economic targets to be met to make a biological route competitive? Products with high value and pathways that produce a battery of useful molecules will have obvious advantages. For lower value, higher volume products, three major challenges must be overcome.

First, the metabolic engineering research to build a production strain with optimal production rate, titer and yield must be accomplished much faster and cheaper than has been possible till date. More basic research is needed to understand the physiology, biochemistry and genetics of WT and engineered strains. Improved predictive modeling, combinatorial and automated gene cloning, and metabolic flux analysis will speed up the strain construction process. New technologies such as site-specific gene editing methods and sensor-based high throughput assays will be important to reduce time and cost of metabolic engineering.

Second, substrate cost must be substantially reduced. Since our knowledge is still limited, reaching theoretical yield is a big challenge for most products. Increased understanding of the physiology and biochemistry of WT and engineered strains will be valuable in optimizing yield. However, the cost of conventional substrates such as glucose, sucrose, or glycerol will remain a barrier to commercial success for lower value products. Alternative lower cost substrates, such as biomass-derived sugars, or low cost agricultural oils, must be utilized. Each of these will present different problems for *Yarrowia* physiology and for bioprocess development.

Third, capital investment for manufacturing assets must be reduced. Creative innovation in fermentation engineering and downstream processing will be required for industrial applications of biological manufacturing with using *Yarrowia* and (or other microorganisms) to become widely used. For example, a continuous fermentation process which performs with the same yield and titer as the fed batch fermentation will significantly increase the volumetric productivity of the process, and thereby decrease the capital investment in both fermentation and downstream process equipment.

Y. lipolytica shows considerable promise to meet the challenges in each of these three areas. With further advances in our knowledge and tools, metabolic engineering of

Y. lipolytica will play a more important role for industrial applications, and will help to provide more sustainable and renewable products to society in the future.

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