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MINI-REVIEW

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Recent advances in biosynthesis of fatty acids derived products in *Saccharomyces cerevisiae* via enhanced supply of precursor metabolites

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Abstract Fatty acids or their activated forms, fatty acyl-CoAs and fatty acyl-ACPs, are important precursors to synthesize a wide variety of fuels and chemicals, including but not limited to free fatty acids (FFAs), fatty alcohols (FALs), fatty acid ethyl esters (FAEEs), and alkanes. However, *Saccharomyces cerevisiae*, an important cell factory, does not naturally accumulate fatty acids in large quantities. Therefore, metabolic engineering strategies were carried out to increase the glycolytic fluxes to fatty acid biosynthesis in yeast, specifically to enhance the supply of precursors, eliminate competing pathways, and bypass the host regulatory network. This review will focus on the genetic manipulation of both structural and regulatory genes in each step for fatty acids overproduction in *S. cerevisiae*, including from sugar to acetyl-CoA, from acetyl-CoA to malonyl-CoA, and from malonyl-CoA to fatty acyl-CoAs. The downstream pathways for the conversion of fatty acyl-CoAs to the desired products will also be discussed.

Keywords Fatty acids · Acetyl-CoA · Malonyl-CoA · Fatty acyl-CoAs · *Saccharomyces cerevisiae*

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Introduction

Microbial conversion of renewable feedstock into fuels and chemicals has been intensively investigated due to increasing concerns on sustainability and global climate change [16]. Besides *Escherichia coli* and *Saccharomyces cerevisiae*, cyanobacteria [46] and oleaginous yeasts [1] have also been explored as cell factories. Compared with its counterparts, *S. cerevisiae* is more industrially relevant thanks to the well-studied genetic and physiological background, the availability of a large collection of genetic tools, the compatibility of high-density and large-scale fermentation, the resistance to phage infection, and the high tolerance against toxic inhibitors and products [27]. Therefore, *S. cerevisiae* is one of the most popular cell factories and has been successfully used in modern fermentation industry to produce a wide variety of products including but not limited to ethanol, organic acids, amino acids, enzymes, and therapeutic proteins [16, 27].

Increasing efforts have been devoted to the production of advanced biofuels, such as *n*-butanol, long-chain alcohols, fatty acid ethyl esters (FAEEs or biodiesels), and alkanes, which have similar properties to current transportation fuels [24, 25, 82]. Notably, most of these "drop-in" fuels are derived from fatty acids (fatty acyl-CoAs or fatty acyl-ACPs), which can be produced by the endogenous fatty acid biosynthetic pathways. Fatty acid biosynthesis (FAB) is catalyzed by fatty acid synthases (FASs), which are rather conserved in nature. Based on the architecture, FASs can be divided into two classes, type I FASs and type II FASs, which are mainly present in eukaryotes and prokaryotes, respectively [3]. The type I FASs consist of large multifunctional polypeptides that carry all the proteins necessary for FAB on one or two large polypeptide chains. In the case of type II FASs, mono-functional proteins are

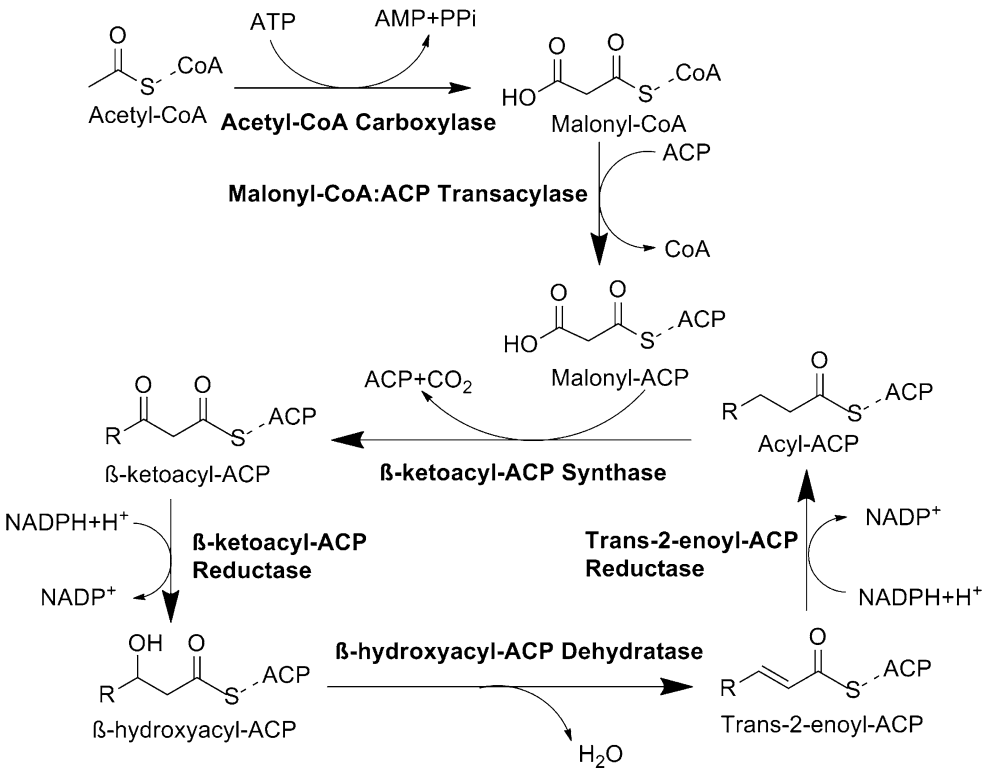

Fig. 1 Overview of the general schemes for fatty acid biosynthesis. Malonyl-ACP is used as the extender unit, and fatty acyl chain is extended by two carbon units after each elongation cycle including condensation, reduction, dehydration, and reduction

discretely expressed from a series of separate genes. The type II FASs are found mostly in bacteria, but also in eukaryotic organelles such as mitochondria and plastids [69]. In some actinomycetes, such as *Mycobacterium* species, both type I and type II FASs are present [23]. The canonical FAB involves in the elongation of acyl-ACP precursors using malonyl-ACP as the extender unit [69]. Although the organization of the FAS system varies between different organisms, the individual enzymatic reactions of FAB are essentially the same [3, 69]. FAB begins with the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase (ACC) at the cost of ATP. Malonyl-CoA is then transferred to ACP by malonyl-CoA:ACP transacylase. The generated malonyl-ACP is used as an extender unit, condensing with acyl-ACP to form β-ketoacyl-ACP with two more carbon units. The extended β-ketoacyl-ACP is subject to an NADPH-dependent reduction to form β-hydroxyacyl-ACP, whose hydroxyl group is removed by β-hydroxyacyl-ACP dehydratase, leading to the formation of *trans*-2-enoyl-ACP. The double bond is then reduced in another NADPH-dependent reaction by the *trans*-2-enoyl-ACP reductase. Each elongation cycle results in the synthesis of acyl-ACP with the fatty acyl chain extended by two carbon units (Fig. 1).

In the cytosol of *S. cerevisiae*, FAB is catalyzed by a type I FAS system, with all the functional domains organized into two subunits, encoded by *FAS1* (β-subunit) and *FAS2* (α-subunit) (Fig. 2). Since all functional domains including the ACP are organized in the FAS complex, the whole FAB process is performed within the fatty acid elongation chamber after malonyl-CoA is loaded. Then fatty acyl-CoAs, mainly palmitoyl-CoA, are released from the FAS complex by the malonyl:palmitoyl transferase (MPT) domain. The released fatty acyl-CoAs can be converted to the desired products, such as free fatty acids (FFAs), fatty alcohols (FALs), and fatty acid ethyl esters (FAEEs) by the corresponding terminal enzymes, namely the thioesterase (TE), fatty acyl-CoA reductase (FAR), and wax-ester synthase (WS), respectively (Fig. 2). In type II FAS, the direct product is in the form of fatty acyl-ACPs, which need to be hydrolyzed by a TE to release FFAs and subsequently activated to fatty acyl-CoAs by a fatty acyl-CoA ligase. Therefore, compared with *E. coli* and other prokaryotes, *S. cerevisiae* is a more direct host for the production of fatty acids derived fuels and chemicals, such as FFAs, FALs, and FAEEs (Fig. 2). On the other hand, neither the endogenous nor heterologous proteins have access to fatty acyl-ACPs, which are constrained in the fatty acid elongation center.

Unfortunately, due to the limited supply of precursors (acetyl-CoA and malonyl-CoA) and tight regulation of the FAS systems, *S. cerevisiae* does not naturally produce fatty acids to high levels [69, 71, 73]. Therefore, extensive metabolic engineering efforts have been made to convert *S. cerevisiae* into oleaginous yeast. This review will focus on the engineering of the precursor supply (acetyl-CoA and malonyl-CoA) and the FAS system for producing fatty acids related

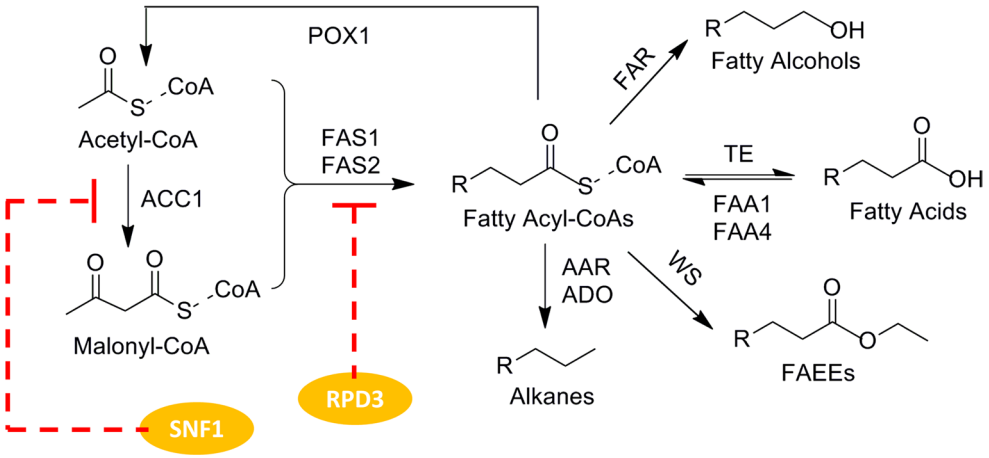

Fig. 2 Development of yeast cell factories to produce fatty acids related fuels and chemicals. After malonyl-CoA is loaded, all the fatty acid biosynthesis reactions occur in the type I fatty acid synthase (FAS) elongation chamber. Then fatty acyl-CoAs (mainly palmitoyl-CoA) are released from the FAS complex, which can be converted to free fatty acids (FFAs), fatty acid ethyl esters (FAEEs), and fatty alcohols (FALs) by a thioesterase (TE), a wax-ester synthase (WS), and a fatty acyl-CoA reductase (FAR), respectively. Fatty acid biosynthesis is highly regulated at multiple levels, such as SNF1 mediated ACC1 activity inactivation and inositol mediated *FAS1* and *FAS2* repression (RPD3)

fuels and chemicals in *S. cerevisiae*. Since the generation of precursor metabolites and the FAS complex are highly regulated at multiple levels, current engineering efforts targeted at both structural and regulatory genes will be discussed.

From sugars to acetyl-CoA

As a central metabolite, acetyl-CoA plays important roles in a series of cellular functions. It is also a key precursor in the biosynthesis of sterols, amino acids, fatty acids, and polyketides. In *S. cerevisiae*, acetyl-CoA metabolism occurs in various compartments, including mitochondria, peroxisomes, nucleus, and cytosol [65]. In the cytosol where biosynthesis generally occurs, acetyl-CoA is generated via the pyruvate dehydrogenase (PDH)-bypass pathway, from pyruvate to acetaldehyde and then to acetate, which is activated to acetyl-CoA by the acetyl-CoA synthetase (ACS) at the cost of two ATP equivalents (Fig. 3). Due to the feedback inhibition of ACS and high energy input requirement, the activation of acetate is a rate-limiting step. In addition, the glycolytic fluxes mainly go through ethanol formation and the supply of acetyl-CoA is very limited (known as Crabtree effect). Several metabolic engineering strategies have been attempted to boost the availability of acetyl-CoA in the cytosol of yeast, such as to redirect the metabolic fluxes to acetyl-CoA biosynthesis, to inactivate the competing pathways, and to introduce heterologous biosynthetic pathways with higher efficiency and lower energy input requirement (Fig. 3).

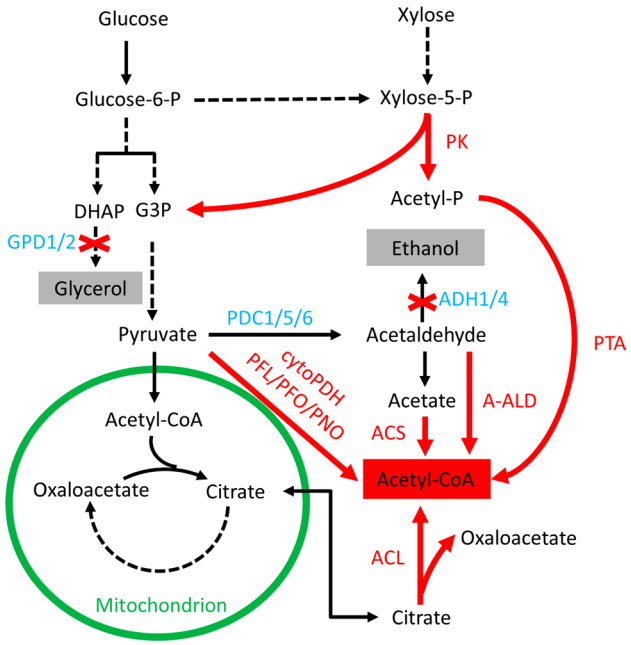

Fig. 3 Design and construction of acetyl-CoA overproducing yeast strains. Competing pathways, *GPD1* and *GPD2* for glycerol production and *ADH1* and *ADH4* for ethanol formation, were inactivated to redirect the glycolytic fluxes to acetyl-CoA biosynthesis. Heterologous pathways, including pyruvate dehydrogenase (PDH), pyruvate: formate lyase (PFL), pyruvate:ferrodoxin oxidoreductase (PFO), pyruvate: $NADP^+$ oxidoreductase (PNO), engineered PDH-bypass pathway, ATP-dependent citrate lyase (ACL), acetylating aldehyde dehydrogenase (A-ALD), and phosphoketolase pathway (PK), which demonstrated higher efficiency and/or lower energy input requirement, were introduced to enhance the acetyl-CoA level in the cytosol of yeast. *ACS* acetyl-CoA synthetase, *PTA* phosphotransacetylase

Host engineering

Redirect the metabolic flux to acetyl-CoA biosynthesis

Due to the Crabtree effect, most of the metabolic fluxes go through ethanol formation during glucose fermentation in *S. cerevisiae*. Therefore, the central metabolism of yeast should be engineered to redirect the metabolic flux to acetyl-CoA biosynthesis. As shown in Fig. 3, acetaldehyde is the branch point to control the flux distribution between ethanol and acetyl-CoA, and alcohol dehydrogenases (*ADH*s) were chosen as the metabolic engineering targets. By the deletion of major *ADH*s in the cytosol (Δ*adh1*–Δ*adh4*), acetyl-CoA level was increased around 2-fold and the production of *n*-butanol, an important biofuel molecule derived from acetyl-CoA, was increased more than 4-fold [43]. In another study, 1.9-fold improvement in fatty acids production was achieved by knocking out *ADH1* in a fatty acids producing host [42].

Inactivate acetyl-CoA consuming pathways

Since most of the acetyl-CoA involved reactions are essential to maintain cellular functions, there were not many successful examples by eliminating the acetyl-CoA consuming pathways. Most current efforts targeted toward the glyoxylate shunt, which contributes to the transport and consumption of cytosolic acetyl-CoA in yeast [7, 65]. The glyoxylate shunt could be disrupted by knocking out *CIT2* or *MLS1*, which encodes peroxisomal citrate synthase and cytosolic malate synthase, respectively [7]. Compared with the reference strain with an intact glyoxylate shunt, the inactivation of *CIT2* or *MLS1* increased the production of α-santalene by 1.36- and 2.27-fold [6], 3-hydroxypropionic acid (3-HP) by 1.19- and 1.20-fold [75], and *n*-butanol by 1.58- and 1.36-fold [36], respectively. Interestingly, it was found that the production of polyhydroxybutyrate (PHB) was impaired in the Δ*cit2* or Δ*mls1* yeast strain [33].

Although the individual push and pull engineering efforts improved the acetyl-CoA availability, the combined host engineering strategy failed to further increase the acetyl-CoA level. Additional deletion of *CIT2* or *MLS1* in the Δ*adh1*-Δ*adh4* strain impaired acetyl-CoA biosynthesis and accordingly decreased *n*-butanol production [43]. The unexpected results might be caused by the impaired C_2 metabolism, since the decreased production of acetyl-CoA derived products was accompanied with the accumulation of acetate. Since excessive acetate was already accumulated, further disruption of the C_2 metabolism (glyoxylate shunt) would increase the acetate concentration to a cytotoxicity level.

Heterologous acetyl-CoA biosynthetic pathways

Due to feedback inhibition and high energy input requirement, the endogenous PDH-bypass pathway suffered from low efficiency for acetyl-CoA biosynthesis. To overcome such limitations, a feedback inhibition insensitive ACS mutant from *Salmonella enterica* ($SeAcs^{L641P}$) [6, 33, 36, 59] and/or alternative acetyl-CoA biosynthetic pathways with less energy input requirement (Fig. 3) were introduced to boost the availability of acetyl-CoA in yeast [34, 67]. Notably, improved production of amorphadiene [59], α-santalene [6], PHB [33, 34], *n*-butanol [36, 43], 3-HP [75], and fatty acids [67] were achieved by engineering the acetyl-CoA pool in *S. cerevisiae*. Considering the significance of acetyl-CoA in cellular metabolism, nature has evolved various routes to synthesize acetyl-CoA under different conditions. Here we only discuss the biosynthetic routes of acetyl-CoA from central metabolites, such as pyruvate, acetate, and citrate, and their (potential) applications in improved production of fuels and chemicals in yeast.

Engineered PDH-bypass pathway

Although ACS was determined to be rate-limiting, the overexpression of the endogenous *ACS1* or *ACS2* led to no or limited improvement in acetyl-CoA levels in yeast [43, 59]. It was found that ACS was post-translationally deactivated via acetylation [64]. In other words, ACS was subject to feedback inhibition by acetyl-CoA. A point mutation at the acetylation position (L641P) could maintain ACS at the active form and increase the acetyl-CoA supply. By introducing the above-mentioned $SeAcs^{L641P}$ into the Δ*adh1*–Δ*adh4* yeast strain, the production of *n*-butanol was increased from 10 mg/L to about 15 mg/L. If the DNA sequence was codon-optimized for yeast expression, the production of *n*-butanol could be further increased to 20 mg/L [43].

In wild-type yeast strains, the supply of acetate was found to limit acetyl-CoA biosynthesis as well. Thus, upstream genes including ethanol dehydrogenase (*ADH2*) and/or aldehyde dehydrogenase (*ALD6*) were co-expressed with $Se\mathit{Acs}^{L641P}$ to direct the metabolic fluxes to acetate and then acetyl-CoA biosynthesis. By introducing the engineered PDH-bypass pathway into wild-type yeast strains, the production of α-santalene was increased by 1.75-fold [6], amorphadiene by 1.22-fold [59], 3-HP by about 1.50-fold [75], PHB by 18-fold [33], and *n*-butanol by 3.10-fold [36], respectively. In another study, the production of FALs could be increased from 140 mg/L to 236 mg/L by introducing such an acetyl-CoA boosting pathway into the engineered yeast strain with increased fluxes to FAB.

Acetylating aldehyde dehydrogenase (A-ALD)

The activation of acetate is so ATP intensive that it constrains the maximal yield of the acetyl-CoA derived products. In addition, the supply of acetyl-CoA is even more limited under anaerobic conditions, a preferred process for industrial applications. Many prokaryotes contain an acetylating acetaldehyde dehydrogenase (A-ALD), which catalyzes the reversible conversion of acetaldehyde and acetyl-CoA in an ATP-independent manner (Fig. 3). As a proof of concept, A-ALD was tested in Δ*ald* (Δ*ald2*-Δ*ald3*-Δ*ald4*-Δ*ald5*-Δ*ald6*) and/or Δ*acs* (Δ*acs1*-Δ*acs2*) yeast strains as an alternative route for acetyl-CoA biosynthesis [35]. Either Δ*ald* or Δ*acs* yeast strain could not grow on glucose as the sole carbon source due to the lack of a route for cytosolic acetyl-CoA generation. Among different bacterial A-ALDs, EutE from *E. coli* (EcEutE) was determined to possess the highest activity and enable the highest specific growth rate on glucose. Although A-ALD could fully replace the endogenous acetyl-CoA biosynthetic route, the engineered strains still grew slower than the wild-type yeast strain. In addition, the use of A-ALD was only demonstrated by growth complementation and its energetic benefits for the synthesis of acetyl-CoA derived products still needs further evaluation. Recently, EcEutE was introduced into a fatty acids producing host but only marginal improvement was observed in fatty acids production [42].

ATP-dependent citrate lyase (ACL)

ATP-dependent citrate lyase (ACL) uses a tricarboxylic acid (TCA) cycle intermediate citrate as the substrate (Fig. 3), which is enzymatically converted to acetyl-CoA and oxaloacetate at the cost of one ATP molecule [79]. Notably, comparative genomic studies revealed the presence of *ACL* in oleaginous yeasts such as *Yarrowia lipolytica*, while not in non-oleaginous yeasts such as *S. cerevisiae* [74]. Such difference may imply the significance of ACL in providing precursor metabolites for the biosynthesis of fatty acids and lipids. *ACL*s from oleaginous yeasts [43], plants [20], and mammalian cells [66, 67] have been functionally expressed in *S. cerevisiae* and were able to increase the production of fatty acids by 1.17-fold [66, 67] and *n*-butanol by about 2-fold [43]. In another study, *ACL* from *Arabidopsis thaliana* and *Y. lipolytica* were introduced into a yeast strain engineered for overproducing fatty alcohols, whose titer was increased from 140 mg/L to 217 mg/L and 330 mg/L, respectively [21].

Due to the Crabtree effect and compartmentalization of cellular metabolism, citrate concentration in the cytosol might be low and rate-limiting for ACL activity (Fig. 3). To increase the intracellular citrate level, both metabolic engineering strategies and process optimization were attempted. To decrease the consumption of citrate through TCA cycle, *IDH1* and/or *IDH2* were deleted and citrate level was increased about 5-fold [67]. Under nitrogen-limited conditions, a 3-fold increase in intracellular citrate concentration was achieved [66]. Accordingly, the introduction of an ACL to the citrate accumulating strain led to increased production of fatty acids [66, 67].

Pyruvate dehydrogenase (PDH), pyruvate:ferrodoxin oxidoredutase (PFO), and pyruvate:NADP$^+$ oxidoredutase (PNO)

PDH catalyzes the oxidative degradation of pyruvate to generate acetyl-CoA in an ATP-independent manner (Fig. 3), which is the most widely existed and probably the most efficient route for acetyl-CoA generation. Unfortunately, PDHs are only found in prokaryotes and eukaryotic organelles. By removing the mitochondrial targeting sequences, the PDH structural genes (cyto*PDA1*-cyto*PDB1*-cyto*PDX1*-cyto*LAT1*-cyto*LPD1*) could be relocated and functionally reconstituted in the cytosol of yeast (cytoPDH). Similarly, PDH from *E. coli* (Ec*LpdA*-Ec*AceE*-Ec*AceF*) was also successfully assembled in this desired compartment of yeast. By introducing functional cytoPDHs into the Δ*adh1*–Δ*adh4* strain, acetyl-CoA concentration and *n*-butanol production was further increased by 1.38- and 3.01-fold, respectively [43].

Rather than PDH, some anaerobic fungi use pyruvate:ferrodoxin oxidoredutase (PFO) [52] or pyruvate:NADP$^+$ oxidoredutase (PNO) [30] for oxidative degradation of pyruvate to generate acetyl-CoA. Instead of NADH, reduced ferrodoxin and NADPH are released as reducing agents by PFO and PNO, respectively (Fig. 3). Compared with other routes for acetyl-CoA generation, PNO possesses several advantages to construct an acetyl-CoA overproducing strain for FAB, since it supplies both acetyl-CoA and NADPH and functions under anaerobic conditions in an ATP-independent manner [30]. Interestingly, PNO is a fusion protein with an N-terminal PFO domain fused to a C-terminal flavoprotein domain, which transports electrons from the FeS clusters of the PFO domain to NADPH [47].

Pyruvate:formate lyase (PFL)

Similar to A-ALD, pyruvate:formate lyase (PFL) could also fully replace the endogenous pathway for cytosolic acetyl-CoA generation [35]. PFL is generally found in prokaryotes for acetyl-CoA biosynthesis under anaerobic conditions, but also in some anaerobic eukaryotes. Different from PDH, PFO, or PNO, which generate acetyl-CoA via oxidative decarboxylation of pyruvate, PFL follows a radical chemistry to convert pyruvate into acetyl-CoA and

formate (Fig. 3). The introduction of PFL into the Δ*ald* and/or Δ*acs* yeast strains rescued the growth on glucose as the sole carbon source under strictly anaerobic conditions, accompanied with the production of formate [35]. The growth rate of the Δ*ald* or Δ*acs* strain with PFL was lower than that of the wild-type strain, and the application for the production of acetyl-CoA derived molecules has not been evaluated yet. Considering the accumulation of formate in the PFL containing yeast strain, NADH can be generated by co-expression of a formate dehydrogenase (*FDH*) [48] and used as reducing force for biosynthesis.

Phosphoketolase pathway (PK)

Phosphoketolase, which catalyzes the cleavage of D-xylulose-5-phosphate into acetyl phosphate and glyceraldehyde-3-phosphate, was generally found in some filamentous fungi for cytosolic acetyl-CoA biosynthesis, such as *Aspergillus nidulans* [50]. The phosphoketolase pathway was originally introduced into *S. cerevisiae* for improved xylose utilization [63], and later was found to enhance the synthesis of acetyl-CoA as well [13, 34, 51] (Fig. 3). By heterologous expression of the phosphoketolase pathway in a yeast strain producing PHB, the production could be improved from 4 mg/g dry cell weight in the reference strain to 28 mg/g dry cell weight, while only 16 mg/g dry cell weight was produced in the same background strain with the engineered PDH-bypass pathway [34]. The phosphoketolase pathway was also introduced to the FAEEs producing yeast strain, and the production of FAEEs was increased by 5.7-fold compared with the reference strain [13]. Since the metabolic flux to pentose phosphate pathway is limited during glucose fermentation, the intracellular concentration of D-xylulose-5-phosphate can be rate-limiting for acetyl-CoA biosynthesis via the phosphoketolase pathway. On the contrary, xylose is consumed via D-xylulose-5-phosphate. Therefore, it will be interesting to test the production of acetyl-CoA derived fuels and chemicals from xylose using the *S. cerevisiae* strains containing the phosphoketolase pathway (Fig. 3).

From acetyl-CoA to malonyl-CoA

The activation of acetyl-CoA to malonyl-CoA is catalyzed by acetyl-CoA carboxylase (ACC), encoded by *ACC1* in *S. cerevisiae*, which was determined to be critical and rate-limiting for FAB [42]. In addition, the ACC activity in *S. cerevisiae* is highly regulated, at both transcriptional level and post-translational level. Therefore, both the structural gene (*ACC1*) and the regulatory network were engineered to increase the ACC activity in yeast.

Overexpression of ACC1 *led to improved malonyl-CoA level*

Since ACC is a rate-limiting enzyme for FAB, the most straightforward strategy is to overexpress the *ACC1* gene. Indeed, the overexpression of *ACC1* could increase the malonyl-CoA level and the corresponding production of a wide range of fuels and chemicals, including FFAs [53], FALs [21, 53], FAEEs [56], 3-HP [56, 75], resveratrol [61], and polyketides [76].

Engineering of the ACC1 *regulatory machinery*

Although *ACC1* overexpression is a simple and straightforward strategy to increase malonyl-CoA level, the production of malonyl-CoA derived molecules can only be improved to a very limited extent. Specifically, less than 2-fold improvement was achieved in most cases simply by *ACC1* overexpression [21, 53, 56, 61, 75, 76]. In some studies, *ACC1* overexpression did not improve the production at all [42]. Such results indicated the complicated regulation of ACC1 enzyme, making it necessary to engineer the regulatory machinery. The activity of ACC1 was post-translationally regulated by SNF1 [42] (Fig. 2). SNF1 is a protein kinase and the phosphorylated ACC1 lost its catalytic activity. Therefore, SNF1 was chosen as the major target for malonyl-CoA level engineering in *S. cerevisiae*.

Based on the phosphorylation recognition motif of SNF1, two putative phosphorylation sites were identified at Ser659 and Ser1157 of ACC1 [56]. By mutating the putative phosphorylation sites from serine to alanine ($ACC1^{S659A,S1157A}$), the ACC activity was increased more than 3-fold and the production of two malonyl-CoA derived metabolites, FAEEs and 3-HP, was increased by more than 3-fold accordingly. On the contrary, the overexpression of the wild-type *ACC1* only resulted in less than 1.5-fold improvement in the ACC activity, FAEEs titer, and 3-HP production. Interestingly, another study trying to eliminate phosphorylation-mediated deactivation identifies the same critical phosphorylation site (Ser1157). Introduction of this mutant ($ACC1^{S1157A}$) resulted in 9-fold higher ACC activity and 3-fold higher production of a polyketide and fatty acids, both of which were derived from malonyl-CoA [9]. Since ACC1 was negatively regulated by SNF1, another straightforward strategy was to delete the *SNF1* coding sequence in the genome. Similar ACC activity was detected in the Δ*snf*1 strain overexpressing either the wild-type *ACC1* or the *ACC1* mutant ($ACC1^{S659A,S1157A}$), which was more than 2-fold higher than the wild-type strain overexpressing native *ACC1*. Unfortunately, the inactivation of *SNF1* led to lower production of FALs, a malonyl-CoA-derived molecule, than the wild-type strain either with or without *ACC1* overexpression [21]. This is probably

due to the multiple roles of SNF1 in maintaining cellular functions [83]. Previously, it was found that the deletion of *SNF1* results in a reduced pool of cellular acetyl-CoA, globally decreased histone acetylation, and reduced fitness and stress resistance. In addition, the histone acetylation and the overall fitness of the Δ*snf*1 mutant were improved by increasing the cellular acetyl-CoA concentration [84]. Therefore, it will be interesting to test the production of FALs and other malonyl-CoA derived fuels and chemicals in the Δ*sfn*1 strain with acetyl-CoA overproducing pathways.

Besides its regulation at the post-translational level, ACC1 is also regulated at the transcriptional level. For example, *ACC1* and *HMG1* were found to be transcriptionally co-regulated [60], since the overexpression of *ACC1* led to increased production of both fatty acids and squalene/sterols. Interestingly, the overexpression of *ACC1* in a squalene overproducing strain led to decreased fatty acids production and increased level of squalene/sterols [60]. Since malonyl-CoA biosynthesis and squalene/sterols biosynthesis compete for the same precursor acetyl-CoA, squalene/sterols biosynthetic pathway should be down-regulated in the *ACC1* overexpression strain to achieve the maximal metabolic fluxes to malonyl-CoA and FAB.

Malonyl-CoA synthetase

Although the activation of acetyl-CoA by ACC is the major route, malonyl-CoA can also be directly synthesized from malonate by the malonyl-CoA synthetase (MCS). *MCS*s from plants [75] and bacteria [5] have been cloned, characterized and functionally expressed in *S. cerevisiae*. Unfortunately, *S. cerevisiae* does not naturally produce malonate. Therefore, malonate supplementation is required and malonate uptake system should be introduced. By overexpression of *MAE1*, a dicarboxylic transporter from *Schizosaccharomyces pombe*, the engineered *S. cerevisiae* strain was able to uptake the exogenously added malonate [5]. Since malonyl-CoA is the precursor for the synthesis of a wide variety of value-added compounds, such as polyketides, malonate supplementation and MCS overexpression may still be a viable route to generate cellular malonyl-CoA in yeast.

From malonyl-CoA to fatty acyl-CoAs

In yeast, fatty acid biosynthesis is carried out by the type I FAS system with all functional domains organized in two polypeptides, FAS1 and FAS2. Since the endogenous FAS system suffer from low efficiency and high regulation [41], such as coordinated induction of FAB genes, feedback inhibition, and inositol-mediated repression [8, 69], fatty acids do not naturally accumulate to high levels (Fig. 2). Therefore, the FAB regulatory network was hijacked by engineering of the transcriptional factors and heterologous FASs were overexpressed to overcome the limitations of the endogenous system. Besides the canonical FAB mechanisms shared by most of organisms, nature also evolved several special mechanisms to synthesize fatty acids *de novo*, such as the elongase system [38, 39] (Fig. 4a) and the fermentative pathway [26, 29] (Fig. 4b). Although not widely distributed in nature, these special pathways possess significant advantages over the canonical ones, in terms of pathway efficiency and controllability of the fatty acids chain lengths.

Endogenous FAS engineering

Engineering of structural genes

To direct the malonyl-CoA pool to FAB, the most straightforward strategy was to overexpress *FAS*1 and *FAS*2. By replacing the native promoters of *ACC1*, *FAS1*, and *FAS2* with strong and constitutive promoters, their expression levels were increased about 7–16-fold. Accordingly, the production of FFAs, FALs, and FAEEs were increased by about 11-, 2-, and 4-fold, respectively. Compared with the strain overexpressing *ACC1*, additional overexpression of *FAS1* and *FAS2* increased the production of FALs and FAEEs by about 1.7- and 3.6-fold, respectively [53].

To improve the fatty acyl-CoA levels, competing pathways were also engineered in *S. cerevisiae*. For example, the β-oxidation cycle, contributing to the degradation of fatty-CoAs, can be disrupted by the deletion of *POX1*. Unfortunately, the disruption of β-oxidation cycle led to lower production of FALs and FAEEs [21, 53]. Besides β-oxidation, a large fraction of fatty acids are utilized to synthesize steryl esters (SEs) and triacylglycerols (TAGs) in *S. cerevisiae*. Compared with the wild-type strain, FFAs content was increased about 3-fold in a strain (Δ*are1*-Δ*dga1*-Δ*are2*-Δ*lro1*) devoid of TAG and SE formation, 4-fold in a strain (Δ*pox1*) incapable of β-oxidation, and 5-fold in a strain (Δ*are1*-Δ*dga1*-Δ*are2*-Δ*lro1*-Δ*pox1*) lacking both TAG and SE biosynthesis and β-oxidation pathways [72].

Engineering of regulatory genes

Due to the limited success in improving the efficiency of FAB by engineering structural genes, another study targeted the transcriptional regulators involved in FAB (Fig. 2) to increase the production of FALs [21]. By manipulating the structural genes in fatty acids synthesis and degradation pathways, the production of FALs was increased less than 1.6-fold. On the contrary, the disruption of the regulatory

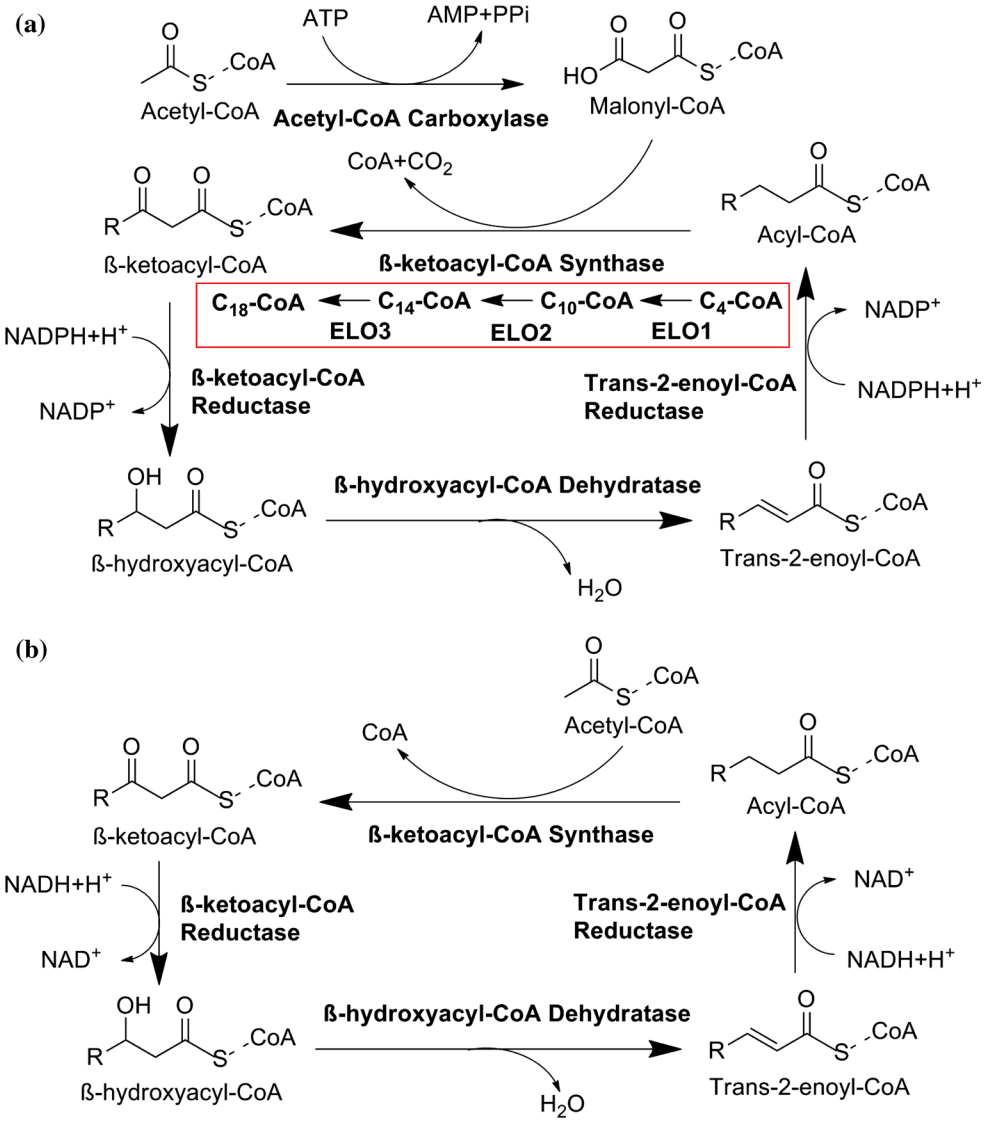

Fig. 4 Non-canonical fatty acid biosynthesis schemes, namely the elongase system **a** and the fermentative pathway **b**. Different with the canonical scheme, which shows ATP/malonyl-CoA, ACP, and NADPH dependence, the elongase system is ATP, CoA, and NADPH dependent (ACP independent), while the fermentative pathway is CoA and NADH dependent (ATP, ACP, and NADPH independent)

network by knocking out transcriptional factors improved the production of FALs by 1.6- to 2.7-fold. Specifically, the deletion of *RPD*3 (Fig. 2), a negative regulator of phospholipids metabolism, enabled the highest production of FALs at a titer around 122 mg/L. Since the phospholipids synthesis requires fatty acids as the precursors, the deletion of negative regulators of phospholipids metabolism would enhance the phospholipids production and create a driving force for FAB.

It is well-known that FAB is also subject to feedback inhibition by fatty acyl-CoAs or fatty acyl-ACPs. In eukaryotes, acyl-CoA binding protein (encoded by *ACB1*) plays an important role in transporting fatty acyl-CoAs in different compartments and attenuating the feedback inhibitory effect of acyl-CoAs on FAB enzymes, such as ACC1, FAS1, and FAS2 [32]. A recent report found that overexpression of *ACB1* could enhance the supply of fatty acyl-CoAs and increase the production of FAEEs by around 1.2-fold [58]. On the contrary, another study found that the deletion of *ACB1* could enhance the cytosolic acyl-CoAs and increase the production of FAEEs by 1.48-fold [70].

Heterologous FAS engineering

Bacterial type I FAS (bFAS)

Due to their coordinated biosynthesis, type I FASs are generally considered to be kinetically more efficient than the dissociated type II FASs. However, FAS may still be rate-limiting for FAB in yeast, because of its highly regulated network. Type I FASs are mainly present in eukaryotes, but also found in some actinomycetes, such as *Mycobacterium* species [23]. Considering the significant differences between prokaryotes and eukaryotes,

heterologous expression of a bacterial type I FAS (bFAS) was proposed for efficient FAB in yeast, by taking the advantage of the type I FAS while bypassing the endogenous regulation. It was found that a bFAS (FAS-B) from *Brevibacterium ammoniagenes* (also known as *Corynebacterium ammoniagenes*) together with its activation protein (phosphopantetheinyl transferase, PptA) could complement the growth of a Δ*fas*1 mutant on fatty acids free medium, indicating that *FAS-B* could be functionally expressed in yeast and synthesize all necessary fatty acids or fatty acyl-CoAs to maintain cellular functions [18]. Subsequent introduction of a wax synthase gene could redirect the additional fatty acids fluxes to the synthesis of FAEEs, whose titer was 6-fold higher than that in the wild-type strain. It was also shown that a FAS-B mutant (Y2226F), whose ketoacyl-ACP reductase activity was eliminated by point mutation at the catalytic center, enabled the production of triacetic acid lactone (TAL) at a titer higher than 50 mg/L in *S. cerevisiae* [80].

Homo sapiens type I FAS (hFAS)

Considering the kinetics advantage of the type I FASs, another strategy is to use the *Homo sapiens* type I FAS (hFAS). Different with the yeast counterpart, whose structure is well organized and highly complex, hFAS is not constrained by a scaffolding structure and displays a remarkable degree of flexibility [40]. Especially for the TE domain, this fatty acids cleaving unit is on the C-terminus and isolated from the core scaffold. It was found that the removal of the TE domain (hFASΔTE) eliminated the native TE activity, but had no negative effect on the activity of other domains [37]. Coexpression of a short-medium chain specific *TE* (*FatB1* from *Cuphea palustris* or *TEII* from *Rattus norvegicus*) together with the engineered hFAS resulted in the shift of fatty acids profiles. C_6, C_8, and C_{10} fatty acids were detected at high levels in the engineered strain, and the production of the major fatty acid (C_8) was increased more than 17-fold compared with the wild-type strain. By replacing the native TE domain, the short-medium chain specific TEs were linked to hFAS and the closer proximity of TE and FAS domains resulted in a 4- to 9-fold increase in C_8 fatty acid levels over the unlinked counterparts and a 64-fold increase over the wild type strain [37]. The flexibility, high activity, and probably feedback inhibition insensitivity make hFAS a promising platform to produce fatty acids related molecules with different chain lengths.

Elongase

As mentioned above, the traditional FAS system utilizes malonyl-ACP as the extender unit and NADPH as the reducing force to elongate the acyl chains, highlighting the ATP, NADPH, and ACP dependence [69]. A different *de novo* FAS system, the microsomal elongase, was described in *Trypanosoma brucei*, a eukaryotic human parasite that causes sleeping sickness [38, 39]. Genome sequence indicates the presence of a putative type II FAS system, but experimental results suggest that the type II FAS is not responsible for *de novo* FAB. For example, 1) the components are membrane-associated proteins, while soluble proteins are generally found for a type II FAS system. 2) Several type II FAS inhibitors, such as triclosan and cerulenin, which inhibit *trans*-2-enoyl-ACP reductase and β-ketoacyl-ACP synthase [45], respectively, will not affect FAB. 3) RNA interference (RNAi) silencing of ACP, a key component of type II systems, has no effect on *de novo* FAB either. Later, the system was characterized to use three elongases instead of a type II synthase (Fig. 4a).

In this microsomal elongase system, malonyl-CoA serves as the extender unit and CoA is the acyl chain carrier (Fig. 4a). In other words, this system is ATP, NADPH, and CoA-dependent. In addition, trypanosomes have different fatty acids requirements during their life cycle, as they encounter various growth environments [39]. It was found that the fatty acids requirement was achieved via the chain specificity of elongases, with ELO1 converting C_4- to C_{10}-CoA, ELO2 extending C_{10}- to C_{14}-CoA, and ELO3 elongating C_{14}- to C_{18}-CoA (Fig. 4a). The modular feature of the elongase system will allow the accessibility for chain length engineering. For example, if only ELO1 is included into the cyanobacterial alkane biosynthetic pathway, C_7 and C_9 alkanes will be the major products, which are the same as gasoline. Compared with *E. coli*, *S. cerevisiae* possess the intracellular membrane network and may be a better host for functional reconstitution of the elongase system, whose components are membrane-associated proteins.

Fermentative pathway via the reversed β-oxidation cycle

Another special mechanism for *de novo* FAB was described in the mitochondria of *Euglena gracilis* [26, 29]. So far, five different FAS systems have been reported for *E. gracilis*, two type II FAS localized in the chloroplasts, one type I FAS in the cytosol, one microsomal FAS, and one mitochondrial FAS. The mitochondrial system is involved in anaerobic wax ester fermentation. This fermentative pathway is malonyl-CoA (or ATP) independent, and has the ability to synthesize fatty acids directly from acetyl-CoA as both starter and extender units [29]. In addition, NADH, instead of NADPH, serves as the reducing power (Fig. 4b). Due to the ATP and ACP independence and CoA and NADH dependence, the fermentative FAB proceeds by reversal of the β-oxidation cycle. Compared with the canonical FAB, the fermentative pathway is advantageous for its energetic benefits of ATP independence and

availability of CoA and NADH versus ACP and NADPH inside the cell (Fig. 4b). Although genes coding the fermentative fatty acid synthesis pathway are not well-characterized yet, the availability of the genome sequence of *E. gracilis* will definitely help to decipher the genetic code and benefit in designing efficient fatty acids derived biofuel synthetic pathways.

The advantages of such system were demonstrated in recent reports to reverse the β-oxidation cycles for efficient production of a series of fatty acids derived fuels and chemicals with different chain lengths [11, 14]. By manipulating several global transcriptional regulators involved in fatty acids metabolism and carbon catabolite repression, all β-oxidation enzymes were constitutively expressed under anaerobic conditions even with the absence of fatty acids and presence of glucose [14]. The resultant strain could reverse the β-oxidation cycle to produce *n*-butanol, long chain alcohols and fatty acids at much higher titers and yields than previous studies. Similarly, functional reversal of the β-oxidation cycle was achieved via the synthetic biology approach by overexpressing all the structural genes of the β-oxidation cycle [11]. Although the efficiency of the latter system was much lower than the former one, it represented a well-defined platform that could be readily transferred to other hosts, such as *S. cerevisiae*. Due to its ubiquitous nature, β-oxidation cycle was also functionally reversed in yeast, demonstrated by the synthesis of *n*-butanol, medium-chain fatty acids, and medium-chain fatty acid ethyl esters [44].

From fatty acyl-CoAs to advanced biofuels and chemicals

The generated long chain fatty acyl-CoAs can be converted to a wide variety of fuels and chemicals by introducing the corresponding terminal enzymes. For example, the synthesis of FFAs, biodiesels (FAEEs), and FALs can be achieved by heterologous expression of a thioesterase (*TE*), a wax-ester synthases (*WS*), and a fatty acyl-CoA reductase (*FAR*), respectively (Fig. 2). Fatty acids related fuels and chemicals produced by engineered *S. cerevisiae* stains are summarized in Table 1.

Free fatty acids (FFAs)

TEs, catalyzing the release of free fatty acids from fatty acyl-CoAs or fatty acyl-ACPs, were well-characterized and showed broad diversity, ranging from long-chain (C_{14}–C_{18}) and medium-chain (C_8–C_{12}) to short-chain (C_4–C_6) specificity [31]. Overexpression of the cytosolic form of the *E. coli* thioesterase I (‘*TesA*) enabled the production of long chain FFAs in yeast. Combined with the overexpression of genes (*ACC1*, *FAS1*, and *FAS2*) for enhanced precursor supply and deletion of degrading pathways (*FAA1* and *FAA4*), more than 400 mg/L of FAA was synthesized from 20 g/L glucose in shake flask fermentation [53]. Similarly, another study also attempted to increase FFAs production by engineering of the fatty acyl-CoA metabolism [4]. Total FFAs production could be increased from 77 mg/L to 327 mg/L by deletion of *FAA*1 and *FAA*4, the major fatty acyl-CoA synthetases contributing to the degradation of long chain fatty acids. Additional overexpression of a cytosolic thioesterase from *Mus musculus* (*ACOT5* s) further increased the titer of total FFAs to 493 mg/L [4]. On the contrary, another study found that the deletion of *FAA1* and *FAA4* only marginally increased the production of FFAs and the overexpression of TEs from different species led to decreased FFAs production, except for the endogenous one (*PTE1*), which slightly increased the production of FFAs [42]. Such contradictory results may result from the use of different yeast strains in these studies (Table 1). By introducing a medium chain specific TE (FatB1 from *Cuphea palustris*, CpFatB1) into the yeast strains with engineered hFAS [37] or reversed β-oxidation pathway [44] mentioned above to supply medium chain fatty acyl-ACPs or fatty acyl-CoAs, medium chain fatty acids ranging from C_6 to C_{12} could be synthesized (Table 1).

Fatty alcohols (FALs)

The production of fatty alcohol could be simply achieved by overexpression of a *FAR* gene, such as that from mouse (m*FAR*) [53] and *Tyto alba* (Ta*FAR*) [21]. Around 50 mg/L of fatty alcohols could be produced in the wild-type yeast strain expressing m*FAR* or Ta*FAR*. Additional overexpression of *ACC1*, *FAS1*, and *FAS2* resulted in the production of FALs to about 95 mg/L. FALs production was also increased in another study by manipulating the fatty acids metabolism related transcriptional regulators, followed by acetyl-CoA supply engineering and process optimization, which led to the highest production of FALs in yeast with a titer at 1100 mg/L and a yield around 30 mg/g glucose (Table 1).

Fatty acid ethyl esters (FAEEs)

The synthesis of FAEEs could be achieved by heterologous expression of a promiscuous acyl-CoA:alcohol acyltransferase or wax-ester synthase (*WS*) [53, 56–58, 72, 78]. Five WSs from various bacteria, including *Acinetobacter baylyi* ADP1 (also known as *Acinetobacter calcoaceticus* ADP1), *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6 and *Psychrobacter arcticus* 273-4, were cloned and characterized to have different substrate specificities. Among various

Table 1 Production of fatty acids related fuels and chemicals in *S. cerevisiae*

	Final titer	Host	Genetic manipulations	Reference
Free fatty acids	400 mg/L	BY4742	Overexpression of '*TesA*, *ACC1*, *FAS1*, and *FAS2* Deletion of *FAA1* and *FAA4*	[53]
	493 mg/L	BY4741	Overexpression of *Mus musculus ACOT5* in cytosol Deletion of *FAA1* and *FAA4*	[4]
	145 μg/10^8 cells	BY4741	Overexpression of *Mus musculus ACL* Deletion of *IDH1* and *IDH2*	[67]
	140 mg/L	YPH499	Deletion of *FAA1* and *ADH1* Evolved for better growth on glucose	[42]
	111 mg/L (short chain)	BJ5464	Overexpression of a *Homo sapiens FAS* mutant whose TE domain was replaced by *Rattus norvegicus* TEII	[37]
	330 mg/L	BJ5464	Overexpression of *ACC1*^{S1157A}	[9]
	11 mg/L (medium chain)	CEN.PK2	Overexpression of the reversed β-oxidation pathway and Se*Acs*^{L641P} Deletion of *ADH1*, *ADH4*, *GPD1*, and *GPD2*	[44]
	1.6 % DCW	CEN.PK113	Deletion of *ARE1, DGA1, ARE2, LRO1,* and *POX1*	[72]
Fatty alcohols	98 mg/L	BY4742	Overexpression of mouse *FAR*, *ACC1*, *FAS1*, *FAS2*, and *Mortierella alpina ME*	[53]
	1100 mg/L	BY4741	Overexpression of *Tyto alba FAR*, *ACC1*, and *Yarrowia lipolytica ACL* Deletion of *RPD3* Fed-batch and high OD fermentation	[21]
Fatty acid ethyl esters	5.44 mg/L	BY4742	Overexpression of Ab*WS*, *ACC1*, *FAS1*, and *FAS2* Deletion of *POX1*	[53]
	25 mg/L	BY4741	Overexpression of *WS2* Deletion of *FAA2, ACB1, PXA2* Fed-batch fermentation with nitrogen limitation	[70]
	10498 μg/gDCW	BY4741	Overexpression of *WS2*, *Fas-B*, *PptA*, and *FAA1*	[18]
	6.3 mg/L	CEN.PK113	Overexpression of *WS2*	[57]
	17.2 mg/L	CEN.PK113	Overexpression of *WS2* Deletion of *ARE1, DGA1, ARE2, LRO1,* and *POX1*	[72]
	408 μg/gDCW	CEN.PK113	Overexpression of *WS2*, *ADH2*, *ALD6*, and Se*Acs*^{L641P}	[13]
	48 mg/L	CEN.PK113	Overexpression of *WS2* (integration, 6 copies), *ACB1*, and *GAPN*	[58]
	5100 μg/gDCW	CEN.PK113	Overexpression of *WS2* (integration, 6 copies) and phosphoketolase pathway	[13]
	15.8 mg/L	CEN.PK113	Overexpression of *WS2* and *ACC1*^{S659A,S1157A}	[56]
	0.75 mg/L (medium chain)	CEN.PK2	Overexpression of the reversed β-oxidation pathway and *EEB1* or *EHT1*	[44]
	520 mg/L	YPH499	Overexpression of Ab*WS*, *GUP1*, *GCY1*, and *DAK1* Deletion of *FPS1*, and *GPD2* Glycerol fermentation with oleic acid supplementation	[78]
Alkanes	N.A. (very long chain)	INVSc1	Overexpression of *SUR4*^{F262A,K266L} and *Arabidopsis thaliana CER1* and *CER3*	[2]

candidates, the WS from *M. hydrocarbonoclasticus* DSM 8798 (WS2) was found to be the most efficient for biodiesel production (6.3 mg/L) in *S. cerevisiae* [57] (Table 1), which was consistent with other studies to obtain lower FAEEs production using WS from *A. baylyi* ADP1(AbWS) [53, 78]. In the following studies, acetyl-CoA boosting pathways, such as the engineered PDH-bypass pathway and phosphoketolase pathway, were introduced into the *WS2* containing yeast strain, and the production of FAEEs was increased for additional 3-fold [13]. Unfortunately, these FAEEs producing strains suffered from plasmid instability and huge clone variations in terms of the titer of the desired product [13, 58]. Therefore, *WS2* expression cassette was integrated into the chromosome rather than cloned into the 2μ based plasmid. Besides the consistency of FAEEs production, the titer was also significantly increased, especially with high copy number integration of the *WS2* expression cassette. By several rounds of transformation with gradually increased selection pressure, recombinant yeast strains with the *WS2* expression cassette integrated

from 1 to 6 copies per genome were obtained. Accordingly, FAEEs production was also gradually increased, and the highest titer of 34 mg/L was obtained with 6 copies of the *WS2* expression cassette integrated into yeast chromosome [58]. Further engineering of the fatty acyl-CoAs supply by overexpression of *ACB1* and NADPH supply by overexpression of *GAPN* (an $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase) resulted in a final FAEEs titer of 48 mg/L [58], which represented the highest titer for FAEEs produced in *S. cerevisiae* from glucose (Table 1). Other efforts to increase the production of FAEEs include the overexpression of *ACC1-FAS1-FAS2* [53], engineering of the post-translational regulation of ACC1 [56], the deletion of competing pathways [72], and the optimization of fermentation medium [70], but with only limited success (Table 1).

Besides glucose fermentation, *S. cerevisiae* was also engineered to produce FAEEs from other carbon sources such as glycerol. By overexpression of glycerol utilization genes and deletion of glycerol formation routes, ethanol could be produced at higher titer and yield from glycerol and used for the biosynthesis of FAEEs [78]. Unfortunately, the production of FAEEs was not improved at all. If exogenous fatty acids (oleic acid) were supplemented into the fermentation medium, FAEEs could be produced at a titer as high as 0.52 g/L [78]. These observations indicated that the production of FAEEs from glycerol was limited by the supply of fatty acyl-CoAs. Therefore, it will be interesting to transfer the FAS engineering strategies to this efficient glycerol utilization yeast strain. To produce biodiesel from biodiesel by-product will make the whole process more economically competitive.

Different with the long chain FAEEs, yeast can naturally produce small amount of medium chain FAEEs. The formation of medium-chain FAEEs is catalyzed by acyl-CoA:ethanol *O*-acyltransferases, encoded by *EEB1* and *EHT1* [54]. The supply of medium chain fatty acyl-CoAs could be increased by the yeast strain containing the reversed β-oxidation pathway, and the production of ethyl octanoate and ethyl decanoate was increased by 2.8- and 2.1-fold, respectively [44] (Table 1).

Alkanes

Hydrocarbons (alkanes) can be synthesized by introducing a heterologous pathway from cyanobacteria containing an acyl-ACP reductase (AAR) and an aldehyde deformylating oxygenase (ADO) [55]. Although the success in engineering *E. coli* to produce alkanes with different chain lengths [10, 25, 28, 55], there has been no report on the reconstitution of the cyanobacterial alkane pathways in yeast so far. The difficulty in the construction of an alkane producing yeast lies on the lack of free ACP and fatty acyl-ACPs in the cytosol, which are constrained in the type I FAS complex, since AAR showed much higher affinity and activity toward acyl-ACPs than acyl-CoAs [55]. Functional expression of bacterial genes in yeast remains another challenge. On the contrary, the alkane biosynthetic pathway from plant (*A. thaliana*) has been functionally reconstituted in yeast to produce very long chain alkanes, mainly C_{27}, C_{29}, and C_{31} alkanes [2] (Table 1). Notably, a fatty acid elongase mutant ($SUR4^{F262A,K266L}$) was co-expressed to enable the synthesis of very long chain acyl-CoAs such as C_{28}-CoA and C_{30}-CoA in *S. cerevisiae*. Alkane biosynthesis in plant is carried out by the CER1/CER3 heterodimer, with fatty acyl-CoA reductase and fatty aldehyde decarbonylase activities. Besides very long chain alkanes, CER1 was found to enable the production of C7 and C9 alkanes [10] and was proposed to demonstrate broad substrate specificities. Thus, the application of the plant pathway is limited by the substrate specificity of CER3. To construct a yeast strain that can produce medium- or long- chain alkanes, protein engineering should be carried out to switch the substrate specificity of CER3 [19].

Besides fuels and commodity chemicals mentioned above, some value-added compounds, such as poly-unsaturated fatty acids (PUFA) can also be generated from fatty acid biosynthetic pathways. For example, the production of omega-3 fatty acids has been demonstrated in an engineered *S. cerevisiae* strain [68] and a closely related yeast strain, *Y. lipolytica* [77].

Perspectives

Considering the significance of acetyl-CoA in FAB, the supply of this precursor metabolite must be further engineered in *S. cerevisiae*. Besides the introduction of heterologous acetyl-CoA biosynthetic pathways, including engineered PDH-bypass pathway, A-ALD, PFL, PFO, PNO, cytoPDH, ACL, and phosphoketolase pathway, host engineering is at least equally important to redirect the glycolytic fluxes to acetyl-CoA and inactivate the competing pathways (Fig. 3). Although acetyl-CoA level was significantly increased by knocking out major ADHs, acetaldehyde and acetate were accumulated to high levels and ethanol was still the major product in the Δ*adh* strain. Therefore, the inactivation of the upstream enzymes, the pyruvate decarboxylases (*PDC*s), was performed to construct an acetyl-CoA overproducing yeast strain. It was found that the Δ*pdc* strain could not grow on glucose as the sole carbon source, due to the lack of a route to generate cytosolic acetyl-CoA [22]. Then an acetyl-CoA biosynthetic pathway such as cyto*PDH* and

ACL was introduced into the Δ*pdc* strain, which enabled the cell growth in the presence of low concentration glucose. An acetyl-CoA overproducing yeast strain based on the Δ*pdc* strain can be constructed with the aid of protein engineering, pathway engineering [17, 19], and genome-wide engineering tools. Since growth rate was the direct readout of acetyl-CoA levels in the cytosol, a high throughput selection/screening platform could be readily developed.

Another route to enhance acetyl-CoA supply and redirect the fluxes to FAB is to disrupt other acetyl-CoA consuming pathways, such as the biosynthesis of sterols and amino acids. Nevertheless, these pathways are all essential for cell survival. Rather than completely eliminating the enzyme activity by gene knockout, down regulating the expression of genes encoding the initial or rate-limiting enzymes of these biosynthetic pathways, such as *ERG10* and *HMG1* in the sterols pathway, will be more desirable. Basically, the expression of essential genes can be knocked down by replacing the endogenous promoter with a very weak or inducible/repressible one [49]. A more powerful and flexible strategy is to reconstruct the RNA interference (RNAi) machinery in *S. cerevisiae* and knock down the targeted genes with the customized small interfering RNA [12, 15, 62].

The synthesis of fatty acids is rather energy intensive, and *S. cerevisiae* does not need fatty acids in large quantities naturally. Therefore, FAB is tightly regulated at many levels, from transcription (such as *ACC1*, *FAS1*, and *FAS2*) and post-translational modification (such as the inactivation of ACC1 activity by SNF1 phosphorylation) to the supply of precursors (acetyl-CoA and malonyl-CoA) (Fig. 2). Although extensive metabolic engineering efforts have been made to engineer a fatty acids overproducing yeast strain, the achievement is still limited and far from commercialization. With the development of genome editing tools, genome-wide engineering can be performed to enhance the supply of acetyl-CoA and malonyl-CoA and bypass the FAB regulatory network. For example, RNA interference (RNAi) machinery was reconstituted in *S. cerevisiae* [12, 15] and RNAi-assisted genome evolution (RAGE) was developed by targeting any gene in the yeast genome [62]. Using RAGE, an acetate-tolerant yeast strain was constructed after three rounds of screening. To facilitate genome-wide engineering, high throughput screening tools, such as *in vivo* biosensors, should be developed simultaneously [81]. In terms of the production of fatty acids related molecules, the development of biosensors for acetyl-CoA, malonyl-CoA, fatty acyl-CoA, and even the specific product is highly desirable.

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