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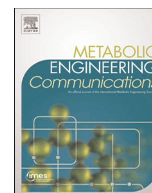

Engineering the fatty acid metabolic pathway in *Saccharomyces cerevisiae* for advanced biofuel production

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ABSTRACT

Fatty acid-derived fuels and chemicals have attracted a great deal of attention in recent decades, due to their following properties of high compatibility to gasoline-based fuels and existing infrastructure for their direct utilization, storage and distribution. The yeast *Saccharomyces cerevisiae* is the ideal biofuel producing candidate, based on the wealth of available genetic information and versatile tools designed to manipulate its metabolic pathways. Engineering the fatty acid metabolic pathways in *S. cerevisiae* is an effective strategy to increase its fatty acid biosynthesis and provide more pathway precursors for production of targeted products. This review summarizes the recent progress in metabolic engineering of yeast cells for fatty acids and fatty acid derivatives production, including the regulation of acetyl-CoA biosynthesis, NADPH production, fatty acid elongation, and the accumulation of activated precursors of fatty acids for converting enzymes. By introducing specific enzymes in the engineered strains, a powerful platform with a scalable, controllable and economic route for advanced biofuel production has been established.

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1. Introduction

The rapid urbanization and industrialization in modern society has resulted in an increasing need for fossil fuels. However, traditional fossil fuel has a variety of problems: there are serious environmental concerns, its supply is limited and its price fluctuates erratically in the market (Tee et al., 2014; Hill, 2007). Hence there is a need to look for an alternative renewable fuel source. Recent research has focused on the production of biofuels by microorganisms, since they are renewable and rich sources of natural products (Wackett, 2011; Peralta-Yahya et al., 2012). The application of microbial produced biofuels is expected to be the solution to the problems of large landscale needed and potential environmental pollution associated with fossil fuels. The cost of microbial

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produced-biofuels could be significantly lowered, as they could be grown on a wide range of substrates, such as ligocellulosic biomass, waste water, waste food and other byproducts (Enweremadu and Mbarawa, 2009; Heeres et al., 2014). All these advantages make microbial production of biofuels an ideal alternative source to fossil fuels.

With the development of genetic and metabolic engineering, the biosynthetic pathways of microorganisms such as bacteria, yeast and micro-algae could be easily modified (Hollinshead et al., 2014; Xu et al., 2014; Pfleger et al., 2015). Based on the wealth of genetic information available and versatile tools to manipulate the metabolic pathways, the yeast *Saccharomyces cerevisiae* is considered as the model organism in improving our understanding of the metabolic mechanisms in fatty acid biosynthesis, and serving as a host strain for the enhanced production of biofuels (Nielsen, 2009; Tang et al., 2013; Zhou et al., 2014).

In the last few decades, the production of biofuels with short carbon chain such as bio-ethanol and butanol by engineered yeast cells has led to significant success. However, the application of bioethanol or butanol suffers considerable challenges: their energy density is low and the vapor pressure is relative high given the existing infrastructure. Besides, their corrosiveness nature prevents their widespread utilization (Yan and Liao, 2009). Thus, research focus is gradually moving towards other advanced fuels such as fatty acid-derived biofuels, which are thought to be more valuable and suitable for industrial use. Fatty acids are the starting materials for advanced biofuel production and they can be converted and used in the biological production of a wide range of applications such as fatty acid derived biodiesels, bioalcohols and alkanes/alkenes with different length of carbon chains (Steen et al., 2010; Lennen and Pfleger, 2013). Hence through the strategy of combining the natural fatty acids metabolic pathways with engineered synthetic pathways in yeast cells, the production of desired chemicals and fuels are expected to be realized.

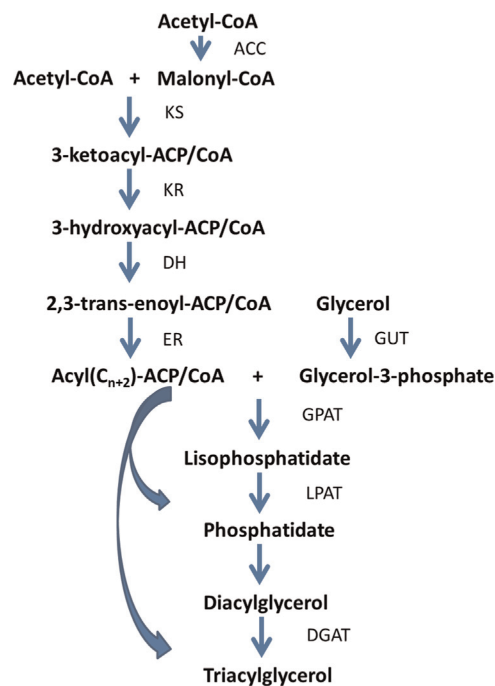

Fig. 1. Schematic diagram of fatty acid biosynthesis, elongation and storage as TAGs in yeast cells. ACC: acetyl-CoA carboxylase; KS: 3-ketoacyl synthase; KR: 3-ketoacyl reductase; DH: enoyl dehydratase; ER: enoyl reductase; GUT: glycerol kinase; GPAT: glycerol-3-phospate acyltransferase; LPAT: lysophosphatidic acid acyltransferase; DGAT: diacylglycerol acyltransferase.

2. Fatty acid metabolism in *Saccharomyces cerevisiae*

2.1. Fatty acid de-novo biosynthesis

The *de novo* fatty acid biosynthesis in yeast cells is an anabolic biochemical process (Fig. 1). It starts from acetyl-CoA, which is carboxylated to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC). Acetyl-CoA, together with malonyl-CoA, then feeds into the fatty acid synthesis pathway. The end products of fatty acids vary between yeast species. The further elongation and desaturation are carried out in the endoplasmic reticulum (ER) by the specific elongase and desaturase of yeast species. After cyclic series of elongation reactions, different carbon length containing fatty acyl-CoAs are formed, mainly of the C14, C16 and C18 saturated acyl-CoAs (Tehlivets et al., 2007). The various unsaturated acyl-CoAs are formed by the respective desaturases, which introduce the double bond into the saturated fatty acyl-CoA derivatives. The most common unsaturated fatty acyl-CoAs in yeasts are C16:1, C18:1, C18:2 and C18:3. They are regulated by different functional desaturases (Martin et al., 2007): The Δ-9 fatty acid desaturase works on the formation of monounsaturated C16:1 and C18:1, whereas the Δ12 and Δ15 desaturases are responsible for introducing the additional double bonds into the location of Z12 and Z15 of the mono-unsaturated acyl chains, to form C18:2 and C18:3 fatty acyl-CoAs.

The fatty acyl-CoA precursors are then destined to the formation of lipids, such as triglycerides (TAGs) and steryl esters (SEs) (Ratledge and Wynn, 2002) (Fig. 1). The lipid storage pathway starts from the generation of glycerol-3-phosphate (G-3-P), which is synthesized by glycerol-3-phosphate dehydrogenase (GPD1) from dihydroxyacetone phosphate (DHAP), or by acylation of glycerol *via* glycerol kinase (GUT1). The lysophosphatidic acid (LPA) is subsequently formed from the condensation of G-3-P and fatty acyl-CoA *via* the enzyme G-3-P acyltransferase (GPAT). The acylation of LPA results in the formation of phosphatidic acid (PA) *via* the enzyme LPA acyltransferase (LPAT). Finally, after dephosphorylation of PA, diacylglycerol (DAG) is formed and it is catalyzed by DGA acyltransferase (DGAT) to generate triacylglycerols (TAGs) (Courchesne et al., 2009). The lipids are stored in the yeast cells as lipid particles or lipid bodies (Sandager et al., 2002), which contribute more than 70% of the total lipid content. The stored lipids serve as energy reserves.

The fatty acid *de novo* synthesis requires substrates and cofactors. Acetyl-CoA is the most important precursor for fatty acid synthesis. The fatty acids synthesis is initiated by loading acetyl-CoA onto the FAS complex and during this process, acetyl-CoA and malonyl-CoA are required in constant supply. Non-oleaginous and oleaginous yeasts have different metabolic pathways for the generation of cytosol acetyl-CoA. In non-oleaginous yeasts, the pyruvate–acetaldehyde–acetate pathway produces the actyl-CoA from glycolysis pathway (Fatland et al., 2002; Hynes and Murray, 2010). The sugars first undergo fermentation process in yeasts cells to generate pyruvate. By the enzyme of pyruvate decarboxylase, the pyruvate is converted to acetaldehyde, and later by the enzymes of aldehyde dehydrogenase and acetyl-CoA synthetase, the acetaldehyde is converted to acetate, which is finally catalyzed to generate cytosol acetyl-CoA.

In oleaginous yeasts, an additional cytosol acetyl-CoA generation pathway exists, in addition to the pruvate–acetaldehyde–acetate pathway. The citrate molecule generated from the citric acid cycle (TCA cycle) could be transported out of the mitochondria to cytosol, where it is then cleaved by the enzyme ATP-citrate lyase (ACL), which exists widely in the oleaginous yeasts, but is absent in the non-oleaginous yeasts (Tang and Chen, 2014; Zhu et al., 2012). The existence of this ACL enzyme contributes to the oleaginous character of the oleaginous yeasts (Liu et al., 2009;

Tang et al., 2013).

NADPH is required as a cofactor for fatty acids synthesis. In each step of the chain elongation, two molecules of NADPH are required by the FAS complex. In oleaginous yeasts, intracellular NADPH is generated mainly by malic enzyme (ME) (Beopoulos et al., 2009a). This enzyme is responsible for the conversion of malate into pyruvate, and the generation of NADPH from $NADP^+$, to provide the vital cofactor for fatty acid synthesis. In non-oleaginous yeasts such as *S. cerevisiae*, NADPH is mainly generated by the pentose phosphate pathway in the cytosol, which includes the enzyme activity of glucose-6-dehydrogenase, 6-phosphogluconate dehydrogenase, and NADPH-dependent isocitrate dehydrogenase (Minard and McAlister-Henn, 2005).

In addition, fatty acid bio-synthesis is a process which is dependent on the condition of high energy load. ATP is the main energy source and during fatty acid synthesis, the ATP/AMP ratio has been found to be increased in the yeast cells.

2.2. Fatty acids catabolism

Fatty acids synthesized by yeast cells either enter the TAGs biosynthesis pathway, to be stored in the form of TAGs as an important source of energy, or are directed to the β-oxidation pathway which occurs in the peroxisomes (Fig. 2). The catabolism of fatty acids generates energy and primary metabolites for the cell. In yeasts, long chain fatty acid catabolism starts from its activation by fatty acyl-CoA synthetase (FAA1 and FAA4) in cytoplasm and are then transported to peroxisomes by the ATP-binding cassette (ABC) transporters (PXA1 and PXA2), while medium chain fatty acids are directly transported to and then activated by the fatty acyl-CoA synthetase (FAA2) in peroxisomes (Hiltunen et al., 2003).

Once inside the peroxisomes, fatty acyl-CoAs undergo the degradation cycle, resulting in a two-carbon shortened acyl-CoAs from each reduction step, and the latter then enters the degradation cycle again. The acyl-CoA oxidase (POX1/FOX1) in peroxisomes is responsible for the first step of β-oxidation. In *S. cerevisiae*, POX1 is the only acyl-CoA oxidase which accepts both the long-chain and short-chain substrates, allowing the completion of β-oxidation and catalyzes the acyl-CoAs to *trans*-2-enoyl-CoA. Followed by the reaction of POX1, the MFE/FOX2 gene with the functional domains for 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities catalyzes the second/third step of the β-oxidation pathway, to form 3-ketoacyl-CoA. The 3-ketoacyl-CoA is then cleaved to form n-2 acyl-CoA and one molecule of acetyl-CoA by the POT1/FOX3 gene, which exhibits ketoacyl-CoA thiolase activity. Through the above repeated reaction cycles, the breakdown of fatty acyl-CoAs is finally completed and totally degraded to acetyl-CoA (Hiltunen et al., 2003; Beopoulos et al., 2011). The generated acetyl-CoA is then transported by carnitine acetyltransferases (CATs) into the mitochondria where it enters the TCA cycle (Strijbis et al., 2010), providing necessary energy required by the cells.

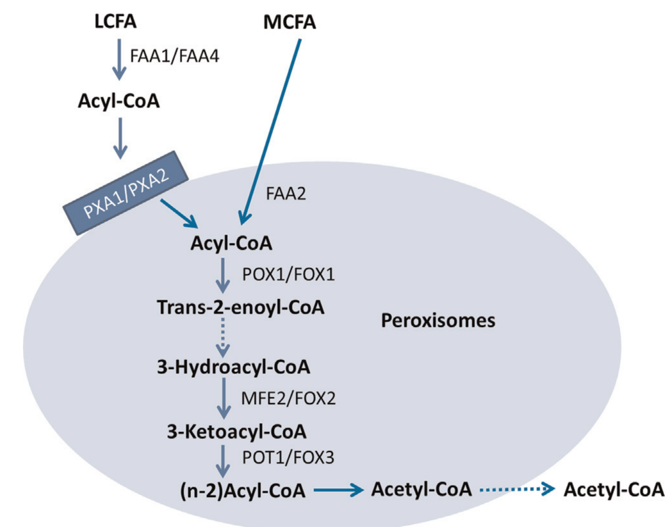

Fig. 2. Schematic diagram of β-oxidation in yeast peroxisomes. LCFA: long-chain fatty acid; MCFA: medium-chain fatty acid.

3. Engineering pathway for enhanced fatty acid production

3.1. Regulation of acetyl-CoA biosynthesis pathway

Regulating the fatty acid metabolic pathways in yeasts could dramatically increase fatty acid production levels. Based on the mechanism of its *de-novo* biosynthesis process, modification of yeast cells for sufficient supply of precursors and cofactors could be an effective strategy for enhanced production of fatty acid. Acetyl-CoA is a central molecule for yeast metabolism and also the building block for fatty acid synthesis. Thus, increasing the acetyl-CoA level becomes an efficient strategy to enhance the fatty acid biosynthesis in yeast (Krivoruchko et al., 2015).

The interesting phenomenon of fatty acid accumulation under a nitrogen limited culture condition was first discovered in oleaginous yeasts. When oleaginous yeasts were cultured in a medium with an excess carbon source but limited nitrogen supply, the lipid contents accumulated to a much higher level (Morita et al., 2013; Beopoulos et al., 2009b; Liu et al., 2013). This phenomenon was found to be linked with the increase of intracellular citrate level, which could be cleaved to acetyl-CoA for fatty acid biosynthesis. In oleaginous yeasts, the activity of mitochondrial NAD^+-dependent isocitrate dehydrogenase (IDH) is dependent on the cellular adenosine monophosphate (AMP) concentration. Once the nitrogen source is exhausted, the activity of AMP deaminase in yeast cells will increase to break down the AMP as a source of ammonium supply. This reaction decreases the intracellular AMP concentration, which is the co-substrate for mitochondrial $NADP^+$-isocitrate dehydrogenase (IDH) (Wynn et al., 2001; Ratledge, 2002). The inhibition of IDH activity results in the accumulation of citrate, which is then transported to the cytosol *via* the citrate/malate shuttle (Ma et al., 2005). The citrate can be further cleaved by the enzyme ATP-citrate lyase (ACL) to acetyl-CoA, for fatty acid synthesis. In this mechanism, the sufficient supply of acetyl-CoA from excess citrate is responsible for the increase in fatty acid yield. Since acetyl-CoA cannot directly be transported out of the mitochondrial matrix into cytosol, engineering a supply of the enzyme ACL could be a promising pathway to increase fatty acid synthesis in *S. cerevisiae*.

However in non-oleaginous yeasts, there is no correlation between fatty acid accumulation and nitrogen limited condition. It has been predicted that IDH activity in non-oleaginous yeast has no definite dependency on intracellular AMP concentration. Due to its lack of ACL, the non-oleaginous yeasts produce acetyl-CoA only from the pyruvate–acetaldehyde–acetate pathway (Yang et al., 2012). To overcome this, mitochondrial IDH in *S. cerevisiae* was engineered to be replaced by one from the oleaginous yeast *Rhodosporidium toruloides* (RtIDH). The original IDH in *S. cerevisiae* was deleted, and RtIDH gene was expressed in the ΔIDH mutant strain. It was found that when the engineered strain was cultured under nitrogen limited conditions, the citrate concentration was increased. Together with the use of increased carbon/nitrogen molar ratio in the culture media, the citrate level was enhanced more obviously (Yang et al., 2012).

To increase the intracellular citrate in *S. cerevisiae*, both of the genes *idh1* and *idh2* which encode for the two subunits of

mitochondrial IDH were knocked out. The citrate level in the $\Delta idh1/2$ gene disrupted strain was found to be significantly increased to about 4- to 5-times to that in the wild type strain. The accumulated citrate could serve as a precursor for acetyl-CoA production. The expression of the heterologous ACL in the $\Delta idh1/2$ gene disrupted strain resulted in a successful construction of a second acetyl-CoA pathway similar to that in the oleaginous yeasts. This metabolic engineered pathway resulted in significant increase in fatty acids production levels, especially in the mono-unsaturated fatty acids: In the $\Delta idh1/2$ strain expressing heterologous ACL, there was a 92% increase in 9-hexadecanoic acid (C16:1) and 77% increase in 9-octadecenoic acid (C18:1). The increased production of mono-unsaturated fatty acids is desirable for biodiesel fuels for their cold flow property (Tang et al., 2013).

Non-oleaginous yeasts use the pyruvate–acetaldehyde–acetate pathway (pyruvate dehydrogenase bypass: PDH bypass) for acetyl-CoA biosynthesis. Several strategies have been efficiently applied to increase acetyl-CoA production levels. Generally, yeast cells intake sugars from the culture medium. The sugars then undergo glycolysis, generating pyruvate which is the starting material for acetyl-CoA formation (Hynes and Murray, 2010). Pyruvate could enter into the mitochondria to be converted into acetyl-CoA for the TCA cycle. Regarding this route, the above described engineered pathway has successfully solved the problem of acetyl-CoA accumulation and its transportation to cytosol for fatty acid biosynthesis. On the other hand, pyruvate could also be converted to acetaldehyde by the enzyme pyruvate decarboxylase (PDC), and later into acetate by the enzyme aldehyde dehydrogenase (ADH). Acetate is then activated and converted to acetyl-CoA by the enzyme acetyl-CoA synthetase (ACS). The activation of acetate is reported as the rate-limiting step, which results from the high energy input requirement and low activity of acetyl-CoA synthetase (Shiba et al., 2007). The intermediate acetaldehyde, from the PDH bypass pathway, is a branch point which could finally be converted to acetyl-CoA, or could also enter the pathway towards ethanol production. This phenomenon is known as the “Crabtree effect” and exists widely in yeast cells during alcoholic fermentation (Vemuri et al., 2007). Besides, after sugars are taken in the yeast cells, it can also be partially directed towards glycerol biosynthesis (de Smidt et al., 2012). All of these effects could decrease the glycolytic flux towards the acetyl-CoA biosynthesis.

Taking above factors into consideration, the aim is to re-engineer the PDH bypass pathway in *S. cerevisiae* cells and direct the carbon flux as much as possible towards acetyl-CoA production. Through this strategy, the activity of ACS responsible for the rate-limiting step in the pyruvate–acetaldehyde–acetate pathway for acetyl-CoA biosynthesis could also be strengthened. In recent years, several metabolic engineering strategies have focused on the following: The overexpression of both ACS1 and ACS2 in *S. cerevisiae* resulted in significant increase in intracellular acetyl-CoA levels, which is about two to five fold higher as compared to the wild type strain (Chen et al., 2013); In order to prevent the original ACS in *S. cerevisiae* from being inactivated by acetylation, a heterologous *Salmonella enterica* ACS (SeACS) gene was overexpressed, and the result showed increased acetyl-CoA levels in the mutant strain (Shiba et al., 2007); Besides, together with the heterologous expression of SeACS, the ADH2 and ALD6 gene, which are responsible for the catalysis of ethanol to acetaldehyde and the conversion of acetaldehyde to acetate were also co-expressed in the yeast strain. This engineered pathway of ethanol degradation pathway successfully directed the carbon flux towards the biosynthesis of acetyl-CoA, resulting in a yield of $408 \pm 270\ \mu g\ g\ CDW^{-1}$ of fatty acid ethyl esters (FAEE) (Chen et al., 2013; de Jong et al., 2014).

In another approach, the competing pathways to the carbon flux to acetyl-CoA biosynthesis could be blocked, to both decrease

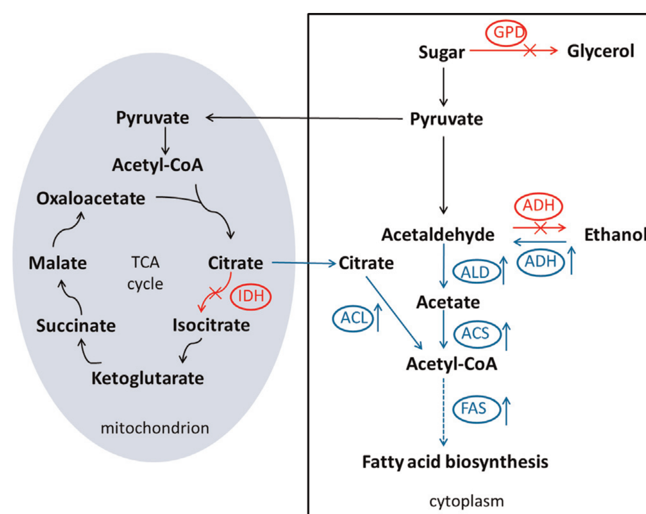

Fig. 3. Regulation of acetyl-CoA biosynthesis pathways for enhanced production of fatty acids. The strengthened steps involved in acetyl-CoA generation were indicated in blue line and the blocked steps were indicated in red line. IDH: NAD+-dependent isocitrate dehydrogenase; ACL: ATP-citrate lyase; GPD: glycerol-3-phosphate dehydrogenases; ADH: alcohol dehydrogenase; ALD: aldehyde dehydrogenase; ACS: acetyl-CoA synthetase; FAS: fatty acid synthase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the formation of ethanol and eliminate the generation of glycerol. To inactivate the glycerol pathway, both GPD1 and GPD2 genes which encoded for the glycerol-3-phosphate dehydrogenases, that are involved in the rate-limiting step of glycerol biosynthesis were deleted. Hence the glycerol production from carbon source was eliminated (Lian et al., 2014). ADH1 and ADH4 genes which encoded for two isoenzymes of alcohol dehydrogenases were knocked out to block the reduction of acetaldehyde to ethanol. By doing so, acetaldehyde and acetate were found to be accumulated to a much higher level compared with that in the wild type strain, and they were then converted to the formation of acetyl-CoA. In this study, the n-butanol biosynthesis pathway was taken as a model to detect increased acetyl-CoA levels, and the production of n-butanol was found to be four times higher than that in the wild type strain (Lian et al., 2014). The accumulated acetyl-CoA can also promisingly serve as precursor for fatty acid biosynthesis.

Overall, the strategies to increase the starting material acetyl-CoA for fatty acids synthesis, in *S. cerevisiae* were described in Fig. 3. Both the engineered PDH bypass pathway and heterologous expressed acetyl-CoA biosynthesis pathway demonstrated their feasibility in increasing acetyl-CoA production levels and accumulating fatty acid in the yeast cells.

3.2. Regulation of fatty acid elongation pathway

In addition to engineering pathways for the increase of acetyl-CoA pool in *S. cerevisiae*, strategies to strengthen the subsequent fatty acids biosynthesis pathway for its enhanced production can be achieved by regulating the activity of involved enzymes.

The carboxylation of acetyl-CoA to malonyl-CoA by the acetyl-CoA carboxylase (ACC1) initiates the fatty acid biosynthesis, and it is considered as the rate-limiting step (Tehlivets et al., 2007). In *S. cerevisiae*, the activity of ACC1 is limited and it could be inhibited by a small yield of acyl-CoAs (Wang et al., 2014). Thus, the malonyl-CoA inside the cell is at a low concentration. Increasing the ACC1 activity could serve to increase fatty acid production. In a study, a heterologous ACC1 gene was overexpressed in *S. cerevisiae*, resulting in a significantly increased pool of both malonyl-CoA and fatty acyl-CoA. The final lipid content was also increased

by 58%, from 42.7 mg/L to 63.2 mg/L (Runguphan and Keasling, 2014). In addition, the ACC1 has been reported to be transcriptionally regulated in coordination with phospholipid biosynthesis by positive and negative regulatory factors and phosphorylation by *Snf1* protein kinase inactivates purified ACC1 (Shi et al., 2014). By abolishing posttranslational regulation of ACC1 *via* site-directed mutagenesis, which was suggested to function as phosphorylation sites recognized by *Snf1*, the ACC1 activity was significantly improved and the fatty acid production levels were increased (Shi et al., 2014).

In bacteria and plant cells, malonyl-CoA can be generated through condensing the malonic acid and CoA by malonyl-CoA synthetase (Chen and Tan, 2013; Wang et al., 2014). Overexpression of a plant malonyl-CoA synthetase (AAE13) in *S. cerevisiae* resulted in a 1.6 fold increase in lipid content, which suggested that supplying sufficient malonyl-CoA was a critical starting point for the engineering of fatty acid biosynthesis pathway (Wang et al., 2014).

When both the starting materials of acetyl-CoA and malonyl-CoA are present, the fatty acid synthase (FAS) complex is responsible for the subsequent elongation and final formation of acyl-CoAs. In *S. cerevisiae*, the FAS consists of two non-identical subunits, which are encoded by the genes FAS1 and FAS2, and they exhibit multiple functions. The overexpression of both FAS1 and FAS2 in *S. cerevisiae* successfully directed the acetyl-CoA and malonyl-CoA pool towards the fatty acid biosynthesis pathway. In comparison with the wild type strain, the strain with the plasmid-based FAS overexpression was shown to have a 30% increase in lipid content, from 42.7 mg/L to 70.6 mg/L (Runguphan and Keasling, 2014).

The multiple overexpression of ACC1 together with FAS1 and FAS2 in *S. cerevisiae* was shown to accumulate lipid contents to a much higher level. Since both ACC1 and FAS1/FAS2 have relative large gene sizes, simultaneous expression of these genes by plasmid-based method are difficult. Thus, to replace the native promoters of the genes with much stronger constitutive promoters could be a good option to regulate their activities (Nevoigt et al., 2006). The TEF1 promoter (P_{TEF1}) is a strong constitutive promoter, and it was used to replace the original promoters of ACC1, FAS1 and FAS2, respectively. By doing so, the engineered yeast strain increased fatty acid biosynthesis further, and this strain was successfully used as a host strain for the enhanced production of fatty acids and fatty acid derivatives.

An important consideration during the fatty acid biosynthesis pathway is the feedback effect of acetyl-CoA. In *S. cerevisiae*, the direct product generated by the FAS complex, acyl-CoA, resulted in a feedback inhibition. One efficient strategy to solve this problem is to direct fatty acyl-CoAs towards the production of free fatty acids, either by overexpressing endogenous acyl-acyl carrier protein (ACP) or by introducing heterologous acyl-CoA thioesterase. Another strategy is to strengthen the TAGs biosynthesis pathway, to enable acyl-CoAs to be stored in the form of TAGs, therefore, reducing the cellular concentration of fatty acyl-CoAs. The overexpression of acyl-CoA: diacylglycerol acyltransferase (DAGAT), responsible for the terminal and rate-limiting step of TAG synthesis by condensing acyl-CoAs and diacylglycerol, resulted in a significant increase in lipid content (Mora et al., 2012; Kamisaka et al., 2013). By combining DAGAT overexpression with the above described engineered pathway, in which the native promoter of ACC1, FAS1 and FAS2 were replaced by the TEF1 promoter respectively, the total fatty acid production increased from 42.7 mg/L to 171.5 mg/L, which is a 302% increase as compared to the wild type strain (Runguphan and Keasling, 2014).

3.3. Regulation of fatty acid catabolic pathway

Another strategy to increase fatty acid production levels in *S. cerevisiae* is *via* regulation of its catabolic pathway; in other words, to block the occurrence of β-oxidation. POX1 plays a critical role in fatty acid β-oxidation, and the deletion of it inhibited the growth of *S. cerevisiae* in the culture medium with oleic acid as sole carbon source (Chen et al., 2014a). However, the research showed that the deletion of POX1 did not change total lipid contents significantly (Valle-Rodríguez et al., 2014). The lipid content of the engineered strain with modified ACC1, FAS1 and FAS2 together with the deletion of POX1 or PXA2 was even lower than that in the original strain with a normal β-oxidation pathway (Runguphan and Keasling, 2014). PXA1 and PXA2 function as transporters for long chain fatty acid (LCFA) and the disruption of both resulted in the inability of LCFA to be degraded through β-oxidation (Li and Chen, 2014). Similarly, the single deletion of PXA1 or PXA2 did not increase fatty acid precursors for biofuel production, whereas the double deletion of PXA1 and PXA2 resulted in a slight increase in the final product level (Li and Chen, 2014).

Although β-oxidation is responsible for the breakdown of fatty acids to generate acetyl-CoA for the TCA cycle, it was suggested that the modification of this pathway may not be an ideal strategy to engineer the yeast cell for enhanced production of fatty acids. However, a few research papers found that the deletion of β-oxidation, together with the overexpression of a “converting enzyme” such as wax ester synthase, resulted in an increased production of fatty acid derivatives (FAEEs) (Valle-Rodríguez et al., 2014). The combination of this engineered pathway with blockage of other fatty acid storage pathways, led to fatty acid derivatives accumulating even more.

3.4. Regulation of NADPH biosynthesis pathway

NADPH provides the reducing equivalents for the metabolic pathways in yeast cells, both for fatty acid biosynthesis and fatty acid derivatives production. In *S. cerevisiae*, NADPH is produced mainly from the pentose phosphate (PP) pathway in cytoplasm, together with the involvement of enzymes glucose-6-dehydrogenase, 6-phosphogluconate dehydrogenase and NADPH isocitrate dehydrogenase. The product ribulose-5-phosphate (R-5-P) is generated from the PP pathway, which could then be converted to xylulose-5-phosphate (X-5-P). X-5-P is reported as the precursor for the phosphoketolase (PHK) pathway, in which two molecules of NADPH is generated from one molecule of glucose. Thus, the PHK pathway was chosen to be introduced in *S. cerevisiae* to increase the intracellular NADPH yield, to enhance lipid production. The overexpression of the above mentioned enzymes in *S. cerevisiae* successfully drove carbon flow towards the PHK pathway. The combination of both the PP pathway and PHK pathway supplied additional NADPH required for acyl-CoA synthesis, as demonstrated by the increased production of fatty acid derivatives such as FAEEs (de Jong et al., 2014).

Another approach to create additional source of NADPH in *S. cerevisiae* is to introduce malic enzyme. Malic enzyme catalyzes the reaction of malate and $NADP^+$ to form NADPH, which is an important activity for regulation of lipid contents in yeast cells, especially in oleaginous yeasts (Zhang et al., 2007). However in non-oleaginous yeasts such as *S. cerevisiae*, the correlation between malic enzyme and lipid accumulation is weak, due to the low substrate affinity of the enzyme (Volschenk et al., 1997). Previous reports showed that overexpression of a malic enzyme gene from *Mucor circinelloides* resulted in a 2.5-fold increase in lipid content (Zhang et al., 2007) and overexpression of this gene in *Rhodotorula glutinis* led to more than 2-fold increase in its lipid contents (Li et al., 2013). However, except for a few reports about

the effect of malic enzyme on lipid accumulation, other research found that this enzyme did not function as a NADPH producer. When malic enzyme was overexpressed in *Y. lipolytica*, no apparent increase in lipid content was observed (Ratledge, 2014). Until now, there is no study showing the relationship between regulation of malic enzyme activity and lipid accumulation in *S. cerevisiae*. Hence the participation of malic enzyme in the fatty acid biosynthesis pathway in *S. cerevisiae* needs to be further investigated.

4. Converting enzymes for fatty acid derivatives production

Fatty acids are important precursors for the production of fatty acid derived fuels and chemicals. Production of specific fatty acids and their derivatives requires the participation of different converting enzymes in the biosynthesis pathways (Fig. 4).

Biodiesel is the most commonly fatty acid derived fuel composed of mono alkyl esters of long chain fatty acids. FAEE represents one of the important components of biodiesel, which is formed by a transesterification reaction between fatty acyl-CoAs and ethanol. Wax ester synthase (WS) (acyl-CoA: alcohol acyltransferase) is mainly responsible for this reaction. WS exists in microorganisms such as bacteria, plants and animals, and it accepts a wide range of alcohols as substrates (Shi et al., 2012). The expression of heterologous WS in *S. cerevisiae* can successfully enabled the strain to produce FAEEs directly from the sugars in the culture medium (de Jong et al., 2014; Valle-Rodríguez et al., 2014; Shi et al., 2012).

Fatty acyl-CoA reductase (FAR) is one of the most studied converting enzymes for fatty acid derivative production. It can be divided into two specific classes, depending on the formation of the end product (Hellenbrand et al., 2011). One class of FAR comprises aldehyde-forming enzymes, which catalyze a two-electron reduction of fatty acyl-CoAs and yields as the end product free fatty aldehydes. This class of enzyme has been studied in microorganisms such as bacteria, green algae and plant leaves (Metz et al., 2000; Willis et al., 2011). The generated fatty aldehyde can be further converted into fatty alcohols or alkanes/alkenes, by specific aldehyde reductases or aldehyde decarbonylases. Another class of FAR consists of the alcohol-forming enzymes, which catalyze the four-electron reduction of fatty acyl-CoAs, and yield the final product of fatty alcohols. This process includes two steps: the first step is similar to that of aldehyde-forming enzyme, in which the aldehyde is formed. In the second step, the aldehyde

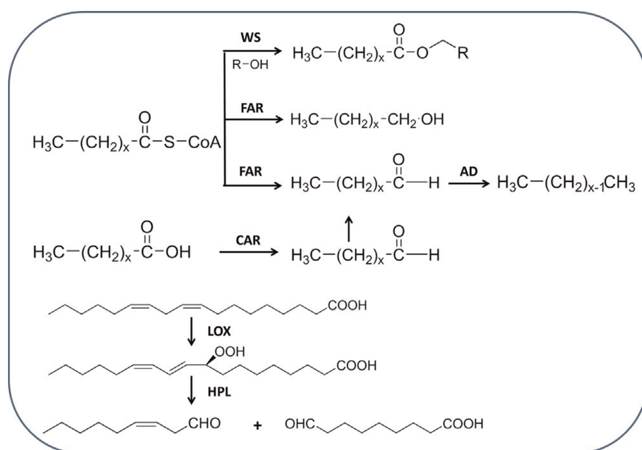

Fig. 4. Schematic diagram of fatty acid derivatives production by converting enzymes. WS: wax ester synthase; FAR: fatty acyl-CoA reductase; AD: aldehyde decarbonylase; CAR: carboxylic acid reductase; LOX: lipoxygenase; HPL: hydroperoxide lyase.

intermediate is subsequently reduced to fatty alcohol (Willis et al., 2011). This class of enzyme has been widely identified in plants, birds, insects and protozoa (Hellenbrand et al., 2011). It has been reported that the cloning and overexpression of the alcohol-forming FAR in *S. cerevisiae* resulted in the production of fatty alcohols with considerable yields (Tang and Chen, 2015; Runguphan and Keasling, 2014).

The biosynthesis of alkanes/alkenes could be produced from decarbonylation of fatty aldehydes with "$n-1$" carbon chain length. Aldehyde decarbonylase (AD) mainly participates in the conversion of fatty aldehyde to alkane/alkene and they have been identified in plant such as *Arabidopsis thaliana* (Bernard et al., 2012) and microbes such as *Synechococcus elongatus* (Schirmer et al., 2010). In addition, other modified enzymes were also found to be similarly involved in alkane/alkene biosynthesis. A novel olefin-forming cytochrome P450 enzyme was identified to be able to decarboxylate fatty acids to terminal olefins (alkenes) (Rude et al., 2011), and a type I polyketide synthases alike enzyme was found to be involved in the production of medium-chain alpha olefins, in a *Synechococcus* strain with an elongation-decarboxylation mechanism (Mendez-Perez et al., 2011). Most of these enzymes have been studied for their characteristics and functions when introduced in *Escherichia coli* and the expression of them successfully led to the production of alkane/alkene (Choi and Lee, 2013; Wang and Lu, 2013; Liu et al., 2014). However, until now, very limited publications were found to report the engineering production of alkane/alkene in *S. cerevisiae*. The lack of investigation concerning the construction of the alkane biosynthesis pathway in *S. cerevisiae* could be attributed to the challenges from the complex yeast metabolism, alkane toxicity, and low efficiency of fatty aldehydes production and/or competing reactions (Zhou et al., 2014; Buijs et al., 2015). The deletion of hexadecenal dehydrogenase *Hfd1* together with expression of an alkane biosynthesis pathway resulted in the biosynthesis of long-chain alkanes, which was the first report to block the conversion of fatty aldehydes into fatty acids and increase the long-chain alkanes yields in *S. cerevisiae* (Buijs et al., 2015).

The conversion of fatty acids to the corresponding fatty acid derivatives by the above described converting enzymes always begins from the activated forms of fatty acids, such as fatty acyl-CoAs. Thus the activation of fatty acids is necessary for enhanced production of desired chemicals. The enzyme carboxylic acid reductase (CAR) was identified and characterized from *Mycobacterium marinum*, to be able to convert fatty acids directly into fatty aldehydes (Akhtar et al., 2013). CAR can accept a wide range of fatty acid substrates and together with the activities of fatty aldehyde reductase and fatty aldehyde decarbonylase, the generated fatty aldehydes can subsequently be converted to fatty alcohols and alkanes/alkenes, by undergoing the same route as above.

Starting from the fatty acid itself, another group of enzymes were identified in the aldehyde-producing hydroperoxide pathway, which catalyzes the hydroperoxidation of unsaturated fatty acids to produce medium chain or short chain fatty aldehydes (Santino et al., 2005; Mita et al., 2005). The enzymes involved in this pathway are classified as lipoxygenase (LOX) and hydroperoxide lyase (HPL), and they exist widely in plants. Generally, the substrates are polyunsaturated fatty acids (PUFA). LOX is responsible for inserting one peroxy group into the PUFA backbone, generating one unsaturated acid hydroperoxide. The unsaturated acid hydroperoxide is then cleaved by HPL, to form one molecule of aldehyde and one molecule of oxo-acid (Feussner and Wasternack, 2002). The enzymes can act on different carbon atom positions of the PUFA backbones, such as linoleic acid. The action on the carbon atom position 9 (9-LOX, 9-HPL) results in the formation of C9-aldehyde and C9-hydroperoxy fatty acid, and their action on carbon atom position 13 (13-LOX, 13-HPL) results in the

formation of C6-aldehyde and C13-hydroperoxy fatty acid. Both the 9-LOX and 9-HPL genes have been cloned and overexpressed in *S. cerevisiae* in order to construct the aldehyde-producing hydroperoxide pathway. The results showed that the engineered metabolic pathway resulted in the conversion of linoleic acid, with more than 1 mg/L 3(Z)-nonenal detected in culture medium (Li and Chen, 2014).

5. Enhanced production of activated forms of fatty acids for converting enzymes

Almost all of the converting routes from fatty acids to their derivatives start from either free fatty acids or activated forms of free fatty acids (fatty acyl-CoAs). However, in *S. cerevisiae*, the accumulated fatty acids from the engineered pathway are mainly stored in the form of TAGs. TAGs by themselves cannot be used directly as fatty acid derivative precursors. Thus, the metabolic modification of yeast cells for enhanced production of activated forms of precursors for converting enzymes is regarded as an efficient strategy to solve this bottleneck.

In *S. cerevisiae*, different pathways contribute to TAGs synthesis (Fig. 5). The various pathways are: (1) fatty acyl-CoAs released from the fatty acid biosynthesis pathways are destined for storage as TAGs by condensation of G-3-P and fatty acyl-CoAs, to form LPA. It is subsequently acylated to generate PA. PA is then dephosphorylated to form TAGs. During this process, acyl-CoAs needs to be continually supplied. DGAT, which is encoded by the *dga1* gene, is involved in the last step of TAGs biosynthesis and is identified as the rate-limiting enzyme (Oelkers et al., 2002). (2) TAGs can be formed in the acyl-CoA-independent pathway by condensation of phospholipid and DGAs by phospholipid: diacylglycerol acyltranferase, encoded by the *lro1 gene* (Kalscheuer et al., 2004). (3) TAGs can also be produced from free fatty acids. The reaction occurs by incorporating fatty acids on the DAGs in a phospholipase A_2-dependent deacylation–reacylation mechanism (Sorger and Daum, 2002). A small portion of fatty acyl-CoAs which are not used in the biosynthesis of TAGs are used for the biosynthesis of sterol esters (SEs). In *S. cerevisiae*, acyl-CoA: sterol acyltransferase encoded by *are1* and *are2* genes take part in this pathway (Sorger and Daum, 2002). It is assumed that the blockage of the lipid storage pathways could release more activated precursors from TAGs or SEs, and reduce competition with production of fatty acid derivatives (Fig. 6).

Fatty acyl-CoA-dependent pathways dominate in the lipid biosynthesis. Therefore the deletion of genes involved in the pathway results in fatty acyl-CoAs accumulation, to provide the key starting materials for specific converting enzymes, such as FARs (Tang and Chen, 2015). The *dga1* gene was disrupted in *S. cerevisiae* and the acyl-CoAs dependent pathway of TAGs synthesis was blocked. Next, a heterologous FAR gene was cloned and overexpressed in the gene disrupted strain, in order to convert the accumulated fatty acyl-CoAs into fatty acid derivatives. As a result, the engineered strain produced a large amount of fatty alcohols. Compared with the strain with overexpressed FAR gene, the yield of fatty alcohols in the *dga1* disrupted stain with overexpressed FAR gene was dramatically increased, from 26 mg/L to 45 mg/L, and even to 84 mg/L after culture medium optimization. The co-disruption of genes *dga1*, *lro1*, *are1* and *are2* blocked the non-essential fatty acids storage pathway to a great extent, leading to a 50% decrease in lipid content and 2.5-fold increase in the production of intracellular free fatty acids (Sandager et al., 2002). This multiple gene- disrupted strain was also transformed with a WS2 enzyme from *Marinobacter hydrocarbonoclasticus* strain, which led to an increase of FAEE production by 68% (Valle-Rodríguez et al., 2014).

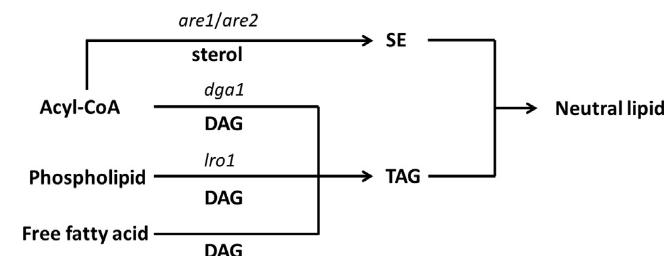

Fig. 5. Pathways of neutral lipid biosynthesis in *S. cerevisiae*. DAG: diacylglycerol; TAG: triacylglycerol; SE: sterol ester.

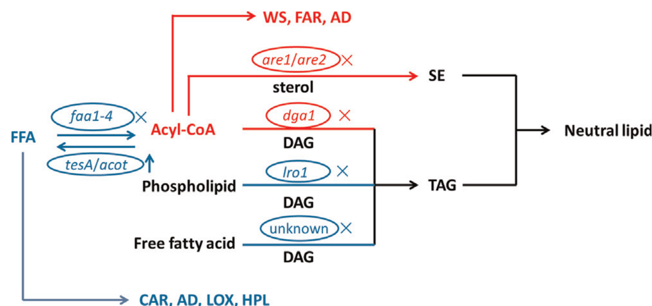

Fig. 6. Engineered pathway for enhanced production of activated forms of fatty acids. The red line indicated the engineered pathway for accumulation of fatty acyl-CoAs, which could be used directly by converting enzymes WS and FAR. The blue line indicated the engineered pathway for accumulation of free fatty acids, which could be converted directly by CAR and LOX. WS: wax ester synthase; FAR: fatty acyl-CoA reductase; AD: aldehyde decarbonylase; CAR: carboxylic acid reductase; LOX: lipoxygenase; HPL: hydroperoxide lyase; FFA: free fatty acid; DAG: diacylglycerol; TAG: triacylglycerol; SE: sterol ester. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In general, the blockage of TAGs or SEs biosynthesis is contrary side to action of fatty acid storage in yeast cells. The increased activated forms of fatty acyl-CoAs should result in a feedback inhibition for lipid accumulation. This was successfully prevented by the introduction of heterologous converting enzymes which converted the released fatty acyl-CoAs immediately. In addition, the above strategies could also be used to supply more activated precursors, for further scale up production of fatty acid derived chemicals.

Regarding the accumulation of free fatty acids, acyl-CoA thioesterase can be introduced into *S. cerevisiae*, to convert fatty acyl-CoAs to fatty acids. An *E. coli* acyl-ACP (CoA) thioesterase (tesA) lacking the membrane signal peptide expressed in *S. cerevisae* resulted in a significant enhancement of free fatty acid production levels (Runguphan and Keasling, 2014). The blockage of fatty acyl-CoAs from entering into the fatty acid degradation process could also be another efficient strategy. In *S. cerevisiae*, fatty acyl-CoA synthetases have been reported to be able to regulate fatty acid metabolism. The genes *faa1* and *faa4* which encode the acyl-CoA synthetase, also have the ability to activate LCFA in the cytoplasm (Beopoulos et al., 2011). The double deletion of *faa1* and *faa4* resulted in an increased free fatty acid level, consisting of 194 mg/L saturated fatty acids, which was 2.5-fold to that in the wild type strain, and 125 mg/L unsaturated fatty acids, which was not be detected in the wild type strain (Chen et al., 2014b). By expression of the acyl-CoA thioesterase (ACOT5) gene from *Mus musculus* in the gene disrupted strain, both the unsaturated free fatty acid and saturated free fatty acid further increased by 66% and 44%, respectively.

The feasibility of control between free fatty acids and fatty acyl-CoAs has been demonstrated by strategies described above. The engineered increment of fatty acyl-CoAs and free fatty acids as a

supply for specific converting enzymes could aid fatty acid derivatives production.

6. Prospects

The ongoing research of engineering the fatty acid metabolic pathway in *S. cerevisiae* for advanced biofuel production illustrated the importance of metabolic engineering in modern industrial biotechnology. Enhanced production of fatty aldehydes, fatty alcohols or alkanes/alkenes in *S. cerevisiae* makes this strain more attractive to meet the demand of biofuel products. By remodeling of its cellular pathways to provide more precursors and co-factors, and regulation of its enzymes involved in the elongation process, fatty acids biosynthesis was significantly improved. The strategies resulted in a release of more free fatty acids and fatty acyl-CoAs which would have been stored in the forms of TAGs or SEs. The introduction of heterologous converting enzymes in the modified strains also successfully converted the activated forms of precursors to fatty acid derivatives.

Hence, the metabolic engineered yeast strains herein provide a platform for the production of important fatty acid-derived chemicals and fuels with a scalable, controllable and economic route. The application of them for industrial use has the advantages of high productivity, considerable capacity and tolerance to environmental changes, and strong resistant to inhibitors which exists in biomass hydrolysates.

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