THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

Multidimensional engineering for the production of fatty acid derivatives in *Saccharomyces cerevisiae*

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CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2019

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ISBN 978-91-7905-174-7

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 4641
ISSN 0346-718X

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Cover: Multidimensional engineering strategies enable yeast as the cell factory for the production of diverse products.

Printed by Chalmers Reproservice
Gothenburg, Sweden 2019

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Abstract

Saccharomyces cerevisiae, also known as budding yeast, has been important for human society since ancient time due to its use during bread making and beer brewing, but it has also made important contribution to scientific studies as model eukaryote. The ease of genetic modification and the robustness and tolerance towards harsh conditions have established yeast as one of the most popular chassis in industrial-scale production of various compounds. The synthesis of oleochemicals derived from fatty acids (FAs), such as fatty alcohols and alka(e)nes, has been extensively studied in *S. cerevisiae*, which is due to their key roles as substitutes for fossil fuels as well as their wide applications in other manufacturing processes. Aiming to meet the commercial requirements, efforts in different engineering approaches were made to optimize the TRY (titer, rate and yield) metrics in yeast.

The major aim of this thesis was to enable a versatile yeast platform for the production of FA derivatives through diverse engineering strategies. We tested several membrane transporters for the potential to mediate fatty alcohol export in *S. cerevisiae*. A novel function of the mammalian transporter FATP1 was identified as it was able to benefit fatty alcohol efflux in a high fatty alcohol production strain. According to the results, human FATP1 led to an improvement of extracellular fatty alcohols (2.6-fold increase) and cell fitness compared with the control strain. FATP1 was then introduced into an engineered *S. cerevisiae* strain carrying a heterologous 1-alkene biosynthetic pathway for improved 1-alkene secretion and production. Combined with an optimization of fatty acid metabolism and the electron transport system, a final titer of 35.3 mg/L of 1-alkenes was achieved with more than 80% being secreted.

Medium-chain fatty acids (MCFAs) are non-inherent fatty acids in yeast whose microbial synthesis is considered to be challenging. Through expressing either an engineered native fatty acid synthase (FAS) or an engineered bacterial type I FAS, the synthesis of MCFAs has been successfully implemented in yeast. In our work, directed evolution of the native transporter Tpo1 and adaptive laboratory evolution were performed to increase the tolerance against MCFAs. Together with further augmentation of the metabolic flux towards MCFAs and optimization of the cultivation process this resulted in >1 g/L MCFA production. Based on the MCFA production platform, we attempted to synthesize medium-chain fatty alcohols (MCFOHs, C6-C12) in yeast. Different protein engineering strategies were designed to engineer the carboxylic acid reductase from *Mycobacterium marinum* (MmCAR), a key enzyme involved in fatty acid conversion. We successfully changed the substrate specificity towards MCFAs and improved the enzyme catalytic activity via directed evolution, using both rational and semi-rational approaches. With further deleting the *TPO1* transporter gene and combining different MmCAR mutations, a final production of 250 mg/L MCFOHs was achieved, a 3-fold increase compared with the control strain.

In conclusion, we provided new insight into the establishment of yeast platforms for the production of FA derivatives through multidimensional engineering strategies.

Keywords: *Saccharomyces cerevisiae*, fatty acid derivatives, metabolic engineering, protein engineering, transporter, tolerance

List of Publications

This thesis is based on the work contained in the following papers:

Paper I: Heterologous transporter expression for improved fatty alcohol secretion in yeast

Yating Hu, Zhiwei Zhu, Jens Nielsen and Verena Siewers (2018), *Metabolic Engineering*, 45:51-58.

Paper II: Engineering 1-alkene biosynthesis and secretion by dynamic regulation in yeast

Yongjin Zhou, Yating Hu, Zhiwei Zhu, Verena Siewers and Jens Nielsen (2018), *ACS Synthetic Biology*, 7:584-590.

Paper III: Multidimensional engineering of *Saccharomyces cerevisiae* for efficient synthesis of medium-chain fatty acids

Zhiwei Zhu*, Yating Hu*, Paulo Teixeira, Rui Pereira, Yun Chen, Verena Siewers and Jens Nielsen (2019), Manuscript submitted

Paper IV: Engineering a carboxylic acid reductase for selective synthesis of medium-chain fatty alcohols

Yating Hu*, Zhiwei Zhu*, Margit Winkler, Verena Siewers and Jens Nielsen (2019), Manuscript

Additional publications not included in the thesis:

Paper V: Metabolic engineering of *Saccharomyces cerevisiae* for production of germacrene A, a precursor of beta-elemene

Yating Hu, Yongjin Zhou, Jichen Bao, Luqi Huang, Jens Nielsen and Anastasia Krivoruchko (2017), *Journal of Industrial Microbiology & Biotechnology*, 44(7): 1065-1072.

Paper VI: Engineering *Saccharomyces cerevisiae* cells for production of fatty acid derived biofuels and chemicals

Yating Hu, Zhiwei Zhu, Jens Nielsen and Verena Siewers (2019), *Open Biology*, 9(5):190049.

Paper VII: Integrative metabolic modeling and machine learning reveal thermal determinants of yeast cell growth

Gang Li, Yating Hu, Ibrahim EI-Semman, Verena Siewers, Boyang Ji and Jens Nielsen (2019), Manuscript

*Authors contributed equally to this work

Contribution summary

Paper I

Designed the study, performed all the experimental work, including strain construction and data analysis, and wrote the manuscript.

Paper II

Performed part of the experimental work (investigation of FATP1 transporter) and assisted in writing the manuscript.

Paper III

Designed the study of Tpo1 transporter engineering, assisted in metabolite analysis and fermentation, assisted in writing the manuscript.

Paper IV

Assisted in designing the study, performed all the experimental work, and wrote the manuscript.

Paper V

Designed the study, performed all the experimental work and wrote the manuscript.

Paper VI

Designed the structure and wrote the manuscript.

Paper VII

Designed and performed the experimental work, assisted in writing the manuscript.

Preface

This dissertation is submitted as partial fulfillment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden. The PhD project was carried out between July 2015 and August 2019 at the Division of Systems and Synthetic Biology (SysBio) under the supervision of Prof. Jens Nielsen and Dr. Verena Siewers. This project was mainly funded by the Swedish Foundation for Strategic Research.

Yating Hu

August 2019

Acknowledgements

First and foremost, I am very grateful to my co-supervisor Prof. Jens Nielsen for giving the opportunity to work in Sysbio since 2014 the last year of my master studies and continue to develop myself by following PhD studies. I have felt supported by you all the time during these five years. Thank you for believing in me and giving me the encouragement and freedom to explore and grow. I also have learned a lot from your efficiency and intensity, and it was a great experience to work with you. And I feel so lucky that could continuously work as postdoc in Sysbio.

I would like to thank my supervisor Verena for all the helps and suggestions during my PhD studies. Thanks for always showing your patience with my incessant mistakes (especially during my thesis writing). Thanks to Dina for all your supports during my teaching, it was definitely a great experience.

I received many helps from lots of people throughout my studies. Thanks to Zhiwei for all of fantastic scientific collaborations, and I would not take the work on protein engineering so far without your help. Every discussion we've had are meaningful to me. Thanks to Yongjin for all your great advices and helps on how to improve writing and every detail about how to do experiment in the beginning of my PhD studies. I have learned a lot from you. Anastasia for your assistance and supervision when I began my work in the lab. Gang for your many suggestions and assistance in the dry lab and allowing me to join your amazing project.

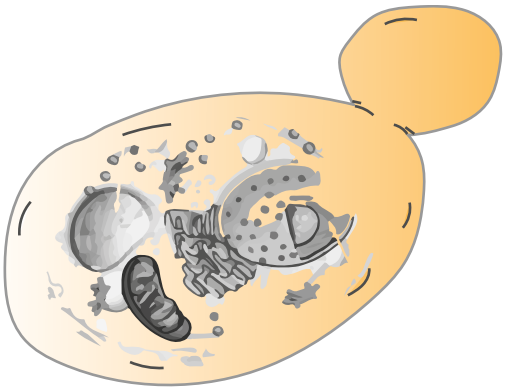
I have been so appreciated to meet and work with all the great people in Sysbio. Thanks to Quanli, Yi, Xin and Yu for you accompany during these years as great colleagues and friends, and I feel fortunate to meet you here. I will remember our exciting trip in Holland, Portugal and Russia forever. Thanks to Yanyan, Lucy, Xiaowei, Tao, Qi for your helps and many unforgettable discussions. I am so glad that I could share the office with you Rosemary, and thank for giving suggestions on my paper writing. Yassi and Leonie for many fun talks and the wonderful time in the gym. I would like to thank the research engineers and the administrators Erica, Martina, Angelica, Emelie, Elin, Erik, Fredrik, Shaq, Anne-Lise, Gunilla and Jacob for your professional assistance in the office and lab. In addition, I'd like to thank all the people who have made my stay comfortable and colorful. Thanks to Ivan, Joakim, Christer, Martin, Sakda, Yun, Jiufu, Guodong, Zongjie, Zheng W, Yiming, Mingji, Lei, Boyang, Jun, Feiran, Peishun, Hao L, Hao W, Michi, Ximena, Kate, Tyler, Ed, Klass, Promi, Stefan, Avlant, Ben, Rui, Johan B, Olena, Alex B, Paulo, Raghu, Carl, Jens C, Demi, Oliver, Veronica, David, Florian, Abder, Shadi, Ivan D, Filip, Christoph, Dany, Max, Ela, Han L, Hongzhong, Jing F, Santosh and Julia.

A very special thanks to my dear friends, Ying, Ge, Hui, Yehao, Xiaoli and Ping. Thanks for always being with me.

Last but not least, my biggest thanks go to my family and my parents. Thank you for your unconditional love and support. Thanks to Jichen for all your support on my life, work and making our life better. Thanks to my lovely cats (Daidai, Yongyong and Nono) for the accompany and cheering me up. I love you.

Contents

1 Introduction



1.1 *Saccharomyces cerevisiae* as cell factory for production of diverse molecules

The yeast *Saccharomyces cerevisiae* has been one of most frequently used microorganism since ancient times. In past decades, the traditional role of *S. cerevisiae* for bread making and beer brewing has been extended to the production of diverse chemicals[1]. *S. cerevisiae*, an intensively studied model eukaryote, has the advantage of a well-characterized genetic background. The ease of manipulation, the robustness and the resistance to phage contamination allow it to act as one of the most popular industrially applied microorganisms[1]. Additionally, the functional expression of eukaryotic cytochrome P450 enzymes enables it to be a suitable host for the production of natural products[2]. Along with these advantages, the great interest of reconstructing biosynthesis pathways in *S. cerevisiae* has given access to the industrial production of various chemicals (Fig. 1). Owing to the dramatic advances in biotechnology in recent years, the biosynthesis of several natural products with commercial purpose as well as alternative biofuels was comprehensively demonstrated in yeast, which is expected to benefit the sustainable development of society. A number of prominent examples are presented in the following chapters.

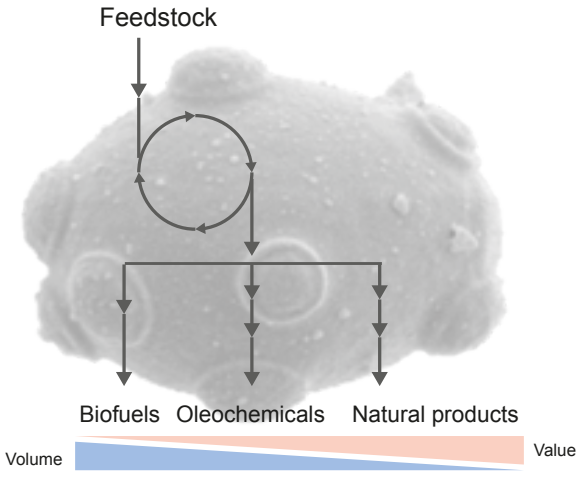


Figure 1. Reconstructing heterologous biosynthesis pathway enables the production of ethanol, oleochemicals and natural products.

1.1.1 Biosynthesis of natural products in S. cerevisiae

Natural products are the metabolites produced by living organisms, of which a branch of secondary metabolites derived from primary metabolites is considered to be high-value compounds with great commercial interests as cosmetics, medicines and food additives. The traditional way to obtain these metabolites through directly extracting them from plants is often considered an inefficient and uneconomical manner that too much relies on climate conditions and plant life cycles[3]. In addition, a low yield production can be aggravated by a complex extraction process. Therefore, the biosynthesis of natural products in microorganisms as alternative method has received increasing attention and can be optimized to meet commercial requirements[4]. In many cases, the biosynthetic pathways of plant secondary metabolites contain multiple distinct P450 enzymes, which are anchored in the endoplasmic reticulum membrane by amino-terminal hydrophobic anchors[5]. Compared with the prokaryotic model *E. coli*, the yeast *S. cerevisiae* therefore represents the perfect host for natural product synthesis and enables further large-scale industrial production.

Isoprenoids

Isoprenoids, also known as terpenoids, are one of the largest classes of natural products that widely exist in plants, animals and microbes with currently over 40,000 molecules identified[6]. Isoprenoids can be classified as primary metabolites and secondary metabolites that serve a range of important physiological and societal functions[7]. According to the isoprene rule, terpenoids are formed by the repetitive joining of isoprene units linked head to tail[8]. They are typically classified, based on the number of isoprene units, as hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40) and polyterpenoids (C > 40)[8,9]. Isoprenoids are derived from the universal precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) that contain the C_5 isoprene units (Fig. 2). Both of the precursors can be synthesized through either the mevalonate (MVA) pathway in eukaryotes and cytoplasm of plants or the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway in prokaryotes and plant plastids. The MVA pathway starts from the condensation of three acetyl-CoA molecules, while the MEP pathway is initiated by the condensation of pyruvate and glyceraldehyde-3-phosphate (G-3-P). In *S. cerevisiae*, the endogenous MVA pathway is the only route to synthesize isoprenoids (Fig. 2). The head-to-tail coupling of two isoprene units results in the formation of geranyl diphosphate (GPP), which is the precursor to monoterpenes. Additional isoprene units delivered through the MVA pathway are added to generate farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), the precursors of diverse terpenoids[10].

Various isoprenoids have been successfully synthesized in *S. cerevisiae*, such as limonene[11], taxadine[12], miltiradiene[13], ferruginol[14] and β-carotene[15]. The most successful story of terpenoid synthesis in yeast is the production of artemisinic acid, the precursor of semi-

synthetic artemisinin that reached production at industrial scale[16]. In the initial strain, an engineered MVA pathway was used to increase FPP production in yeast; several genes involved in the FPP synthesis route were upregulated and one gene responsible for FPP degradation to sterol was downregulated[17]. An amorphadiene synthase (ADS) responsible for the first committed reaction of FPP conversion, together with a novel cytochrome P450 monooxygenase (CYP71AV1) that performs a three-step oxidation of amorphadiene to artemisinic acid was successfully implemented to obtain the substrate for further artemisinin production[17]. The biosynthesis of artemisinic acid represents a significant progress in the development of pharmaceuticals by using synthetic biology in *S. cerevisiae*.

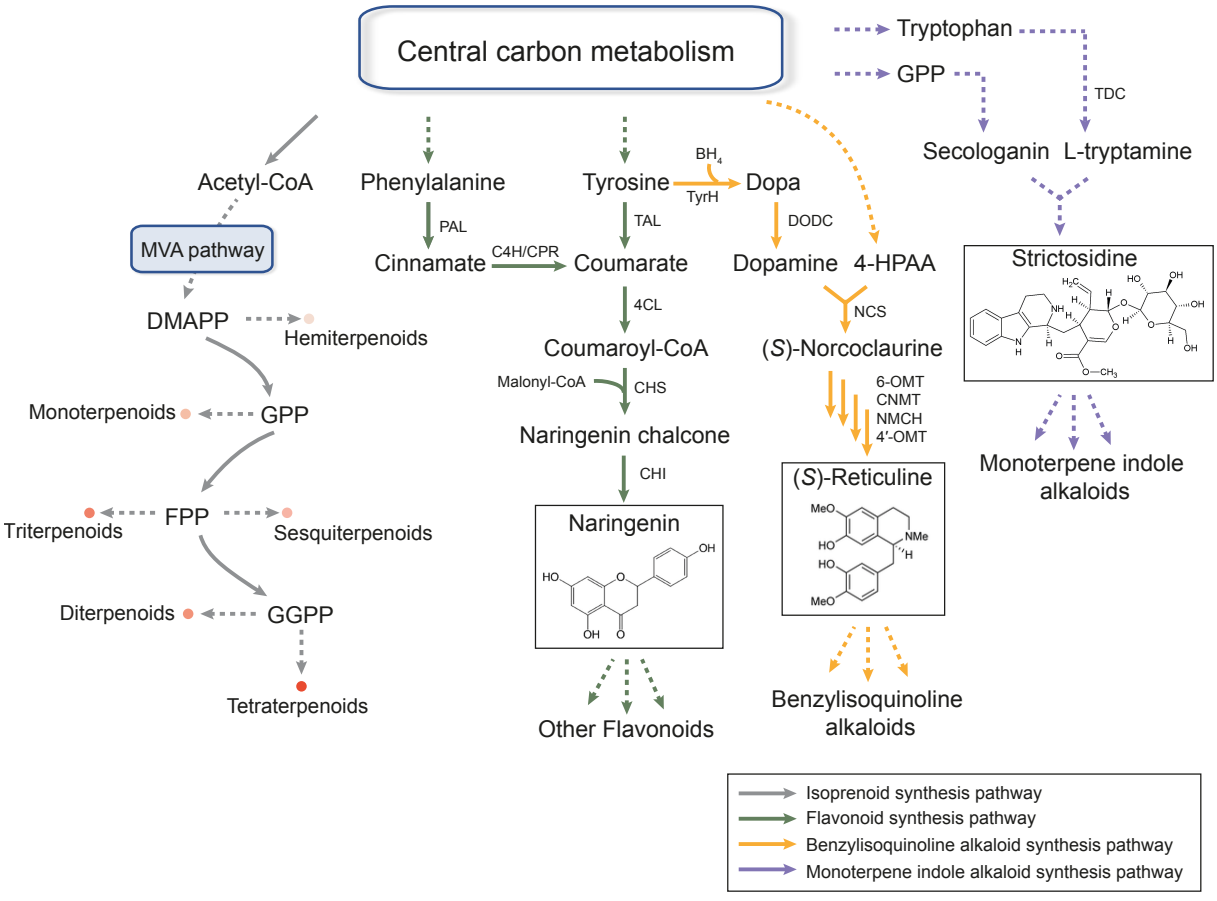


Figure 2. Reconstructing heterologous biosynthesis pathways in yeast to produce various natural products, including isoprenoids, flavonoids and alkaloids.

Flavonoids

Flavonoids, including flavanones, isoflavones, flavones, catechins and anthocyanins, are also an important class of natural products containing a 15-carbon skeleton phenylpropanoid core, which are widely found in plants and have multiple functions. Flavonoids have been associated with a broad spectrum of health-promoting effects, which has stimulated large efforts towards the identification of their biosynthetic networks as well as the establishment of production platforms in microbes[18–20]. Flavonoids are derived from the phenylpropanoid

pathway in plants, which is initiated by the non-oxidative deamination of phenylalanine to *trans*-cinnamic acid via a key enzyme phenylalanine ammonia lyase (PAL)[21]. *Trans*-cinnamic acid is subsequently hydroxylated at the para position to generate *p*-coumaric acid by *p*-coumaric acid cinnamate-4-hydroxylase (C4H), in conjunction with cytochrome P450 reductase (CPR). Then, *p*-coumaric acid can be converted to naringenin via three enzymes, 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) (Fig. 2).

PAL and C4H as the entry point of the route were co-expressed in *S. cerevisiae* together with the reductase CPR to successfully drive the carbon flux into the phenylpropanoid pathway; this work was first performed by Ro and Douglas[22]. Naringenin serves as a key intermediate, from which various flavonoids can be synthesized (Fig. 2). The production of naringenin was significantly increased by expressing a set of genes from *Arabidopsis thaliana*, together with the deregulation of aromatic amino acid biosynthesis by alleviating feedback inhibition and elimination of byproduct formation resulting in a 40-fold increase of naringenin formation in *S. cerevisiae*[23]. The naringenin biosynthesis pathway in yeast can also be derived from tyrosine by introducing a tyrosine ammonia-lyase (TAL)[24]. With the efforts of increasing the malonyl-CoA supply, eliminating the feedback inhibition mediated by tyrosine and downregulating the competing pathways, around 90 mg/L of naringenin were obtained. Based on efficient synthesis of naringenin, a series of downstream flavonoid products were synthesized in *S. cerevisiae*, such as genistein[25], kaempferol and quercetin[26]. *p*-Coumaric acid has been proven to play a crucial role in product scope determination, and also serves as the precursor from which the flavonoid products resokaempferol and fisetin were firstly synthesized in yeast[26].

Alkaloids

As one of the large groups of natural products, alkaloids possess extremely variable chemical structures. Normally, an alkaloid contains at least one basic nitrogen atom in an amine-type structure. Alkaloids were found to exist in various organisms, such as fungi, plants and animals, but the functions of alkaloids in each species are diverse and yet to be fully understood[27]. Owing to the molecular complexity, the production of alkaloids through microbial biosynthesis is a more feasible and efficient approach compared to synthetic chemistry strategies. The most well-known alkaloids produced by yeast are benzylisoquinoline alkaloids (BIAs) and monoterpene indole alkaloids (MIAs) (Fig. 2).

BIAs are a large and structurally diverse family that exhibits a broad range of pharmacological activities, which contribute to the increasing interest in their synthesis in microbes. BIAs share the common intermediate *(S)*-norcolaurine, which is generated by condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA). Then a series of reactions catalyzed by norcoclaurine 6-O-methyltransferase (6-OMT), coclaurine-N-methyltransferase (CNMT), coclaurine *N*-methyltransferase (NMCH) and 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'-OMT), is performed to produce the major branch point *(S)*-reticuline (Fig. 2). However, the two BIA backbones derived from tyrosine

were substantial engineering challenges, since the enzymes from plants involved in the conversion reactions were still not identified[28]. As yeast could endogenously produce 4-HPAA, the synthesis of dopamine was the major problem for BIA production. Subsequently, a mammalian tyrosine hydroxylase (TyrH), bacterial DOPA decarboxylase (DODC), and four enzymes associated with biosynthesis of the electron carrier cofactor tetrahydrobiopterin (BH_4) were co-expressed in *S. cerevisiae* to enable the *de novo* production of the key intermediate *(S)*-reticuline[29], which represented a viable microbial production platform for biosynthesis of diverse BIA molecules. For example, the opioids thebaine and hydrocodone were recently successfully synthesized in yeast based on the optimized reticuline producing platform[30].

MIAs with complex structures possess diverse ranges of function as anticancer, antimalarial and antiaddiction drugs. The biosynthesis of strictosidine, the common precursor of MIAs, was recently reconstituted in yeast[31]. Strictosidine was formed through the condensation of tryptamine and monoterpene secologanin, where the tryptamine can be derived from tryptophan via tryptophan decarboxylase (TDC) and GPP can be converted to secologanin by a series of enzymes (Fig. 2). Together with some additional modifications, such as elimination of the byproduct pathways, the enhanced flux enabled production of 0.5 mg/L strictosidine. The further optimization of nepetalactol production in *S. cerevisiae* was performed, resulting in a positive effect on metabolic flux redirection towards the strictosidine synthesis pathway[32]. All these works played crucial roles in the development of *S. cerevisiae* for the sustainable production of MIAs and MIA derivatives.

These studies demonstrated the superiority of *S. cerevisiae* as the chassis for the production of high-value natural products. The additional examples regarding the production of biofuels will highlight that *S. cerevisiae* possesses the potential as a versatile engineering and production platform.

1.1.2 Biosynthesis of advanced biofuels in S. cerevisiae

The increasing demand of fossil fuels along with environmental concerns have stimulated the scientific research for the development of alternative sustainable energy resources, such as biofuels. Bioethanol, widely applied as gasoline substitute, is mainly derived from sugar cane, corn or wheat. However, the use of feedstock that competes with food production prevents this process from further application[33]. Similar, the current biodiesel from oilseed crops faces the fact of limiting production scale due to the high demand of cultivation areas[34]. Taken these concerns, advanced biofuels produced by microbes that can be used as drop-in fuels were put forward to replace the first generation of biofuels. The yeast *S. cerevisiae* is the most popular workhorse for current advanced biofuel production, such as farnesene, butanol and hydrocarbons.

Butanol

Owing to the longer chain length, butanol is in principal a more attractive biofuel than ethanol as it has a higher energy content, lower vapor pressure and lower hygroscopicity. Moreover, it has high blending percentage with gasoline, which can reach 85% compared with only 10% of ethanol-gasoline blending ratio[33,34]. The relatively high tolerance against butanol (up to 2%) makes *S. cerevisiae* a suitable chassis for microbial butanol synthesis[35]. *S. cerevisiae* can natively produce small amounts of butanol on rich medium through a pathway dependent on threonine or glycine catabolism, and deletion of Adh1 was found as a switch for the endogenous butanol synthesis pathway[36,37]. Overexpression of the pathway enzymes together with elimination of the competing pathways resulted in up to 242.8 mg/L butanol in yeast. In addition, a heterologous butanol biosynthesis pathway was reconstructed in yeast, which is initiated by thiolase Erg10 from acetyl-CoA, and then a set of heterologous enzymes, including 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), trans-enoyl-CoA reductase (*ter*) and butyraldehyde dehydrogenase/butanol dehydrogenase (*adhE2*) enable butanol formation in yeast[39]. Isobutanol is one of the isomers of butanol whose biosynthesis pathway was fully demonstrated in yeast. Isobutanol synthesis was driven through valine biosynthesis pathway in *S. cerevisiae*, by which pyruvate can be converted to 2-acetolactate via acetolactate synthase (Ilv2 and Ilv6) in the mitochondria. Then, 2-acetolactate is converted to the key intermediate 2-ketoisovalerate through acetohydroxyacid reductoisomerase (Ilv5) and dihydroxyacid dehydratase (Ilv3). Improving the valine biosynthesis pathway to enhance the supply of 2-ketoisovalerate was a feasible strategy to increase the production of isobutanol in yeast, by which the isobutanol yield was increased from 0.28 mg/g glucose to 3.86 mg/g glucose in minimal medium[39]. By the Ehrlich pathway - similar to butanol synthesis - the ketoacid is converted into isobutanol in the cytosol through ketoisovalerate decarboxylases (KIVDs) and alcohol dehydrogenases (ADHs). The pathway was optimized by expressing the *Lactococcus lactis* KivD and endogenous dehydrogenase Adh6 that have higher catalytic activities than other endogenous enzymes[40]. Isobutanol production using *S. cerevisiae* has attracted much commercial interest that was demonstrated by a range of patents by Butalco, Butamax and Gevo[41]. With the efforts of pathway optimization, the isobutanol yield has reached 0.33 g/g glucose (80.3% of the maximal theoretical yield) and a final titer of 18.6 g/L of isobutanol was produced in complex medium[42].

Farnesene

Farnesene is suggested to play an essential role in plants and animals and has two isomers α-farnesene and β-farnesene. Besides, it is also a fuel alternative that serves as a potential substitution for jet fuel. Farnesene, an isoprenoid hydrocarbon with a 15-carbon skeleton, can be synthesized based on the MVA pathway in yeast and assembled from IPP and DMAPP as precursors. FPP as a branch point of diverse terpenoids is natively generated in *S. cerevisiae*, while a heterologous enzyme responsible for FPP conversion needs to be introduced into yeast for farnesene formation[43]. In a previous study, both the farnesene

synthase and the endogenous MVA pathway were overexpressed, resulting in farnesene production from glucose at >50% of the theoretical mass yield[44]. Moreover, optimization of the central carbon metabolism was recently conducted by the company Amyris to enable a high-level farnesene producing platform in *S. cerevisiae*. Four non-native metabolic reactions performed by xylulose-5-phosphate specific phosphoketolase (xPK), phosphotransacetylase (PTA), acetaldehyde dehydrogenase (acylating) (ADA) and an NADH-specific HMG-CoA reductase (NADH-HMGr) were introduced to rewrite the central carbon metabolism, enabling a reduced ATP requirement for acetyl-CoA synthesis, reducing the loss of carbon and balancing the cofactor consumption[45]. The strains with optimized central metabolism produced 25% more farnesene than the control strain with comparable sugar consumption and 75% less oxygen consuming. A final titer of 130 g/L of farnesene was achieved by cultivating the engineered strain using industrial bioreactors after two weeks, which envisions the potential future large-scale industrial farnesene production in *S. cerevisiae*.

Fatty acids

Fatty acids (FAs) as versatile compounds can be utilized for industrial manufacturing of detergents, cosmetics and pharmaceutical ingredients. Besides, FAs also serve as the common precursors for a variety of biofuels, such as alka(e)nes, fatty alcohols and fatty acid ethyl esters. In *S. cerevisiae*, FAs are the basic elements of complex lipids; they can be incorporated into phospholipids and sphingolipids or serve as an energy reservoir in steryl esters (SE) and triacylglycerols (TG) stored in lipid droplets[46,47]. The spectrum of FAs in yeast is rather simple. The major components are palmitoleic acid (C16:1), oleic acid (C18:1), palmitic acid (C16) and stearic acid (C18); 80% of yeast fatty acids are monounsaturated (C18:1 and C16:1) through a reaction catalyzed by the ER-resident Δ9 desaturase Ole1[48,49]. Palmitic acid is the predominant fraction among the about 20% of saturated FAs. The minor species including myristic acid (C14) and cerotic acid (C26) are suggested to play essential functions in protein modification or as components of sphingolipids and GPI-anchors, respectively[50]. Cellular fatty acids can be obtained from different sources either by *de novo* synthesis, hydrolysis of complex lipids and delipidation of proteins, and external supply[46,50]. The synthesis pathway of fatty acids has been comprehensively demonstrated, which is initiated by the conversion of acetyl-CoA to malonyl-CoA via acetyl-coenzyme A carboxylase (ACCase) encoded by *ACC1* in yeast. There are two systems involved in the *de novo* synthesis of fatty acids, which are carried out by type I fatty acid synthase (type I FAS) and type II FAS that take place in the cytoplasm and the mitochondria, respectively. In yeast, type I FAS contains two multifunctional polypeptides, an α- and a β-chain encoded respectively by *FAS2* and *FAS1* that form a $\alpha_6\beta_6$ heterododecamer, whereas the type II FAS consists of monofunctional polypeptides. The mitochondrial FAS II pathway is suggested to support octanoic acid supply, which is the precursor for the generation of lipoic acid and plays an essential role in maintaining the function of several mitochondrial enzyme complexes where it serves as cofactors[51]. Since the synthesis of important storage lipids and fatty acids with distinct chain length all occur in the cytoplasm, the attempts for engineering fatty acid

metabolism are mainly focused on the type I FAS. The type I FAS catalyzes all the reactions to synthesize long acyl chains (mainly C14 to C18) as shown in **Figure 3**, and it must be activated by an intrinsic phosphopantetheine transferase (PPT), by which the acyl carrier protein (ACP) can be activated via post-translational modification to install a CoA-derived 4'-phosphopantetheine arm (PPant) that provides thiol group to link the growing fatty acyl chain[52,53].

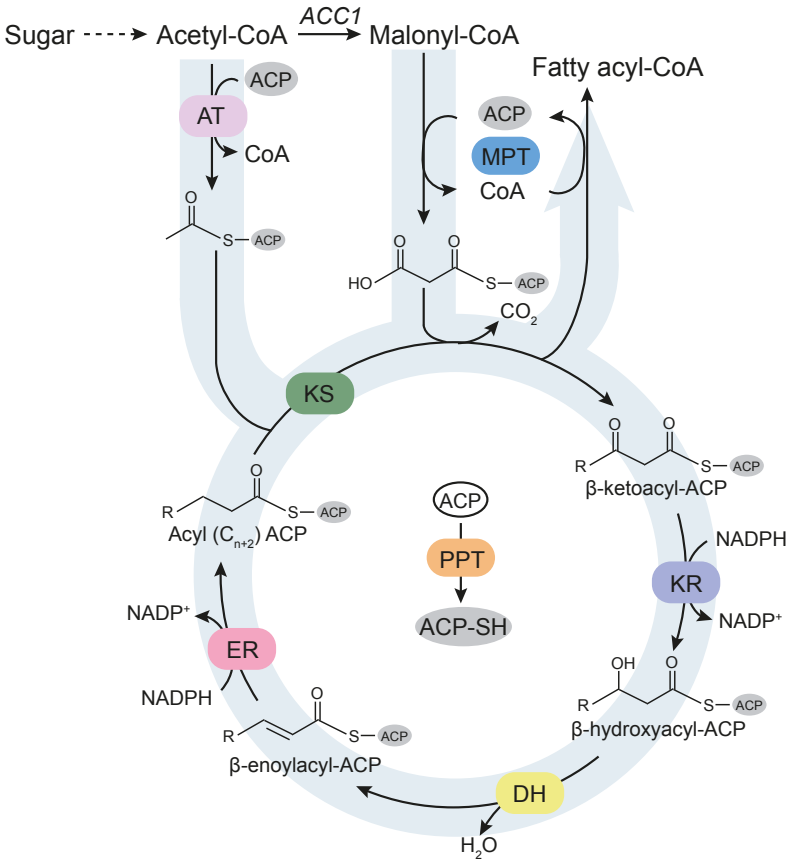


Figure 3. The catalytic reaction cycle of fatty acid biosynthesis and the domains in the yeast type I FAS system.

The reactions catalyzed by the yeast FAS are initiated by transferring the acetyl primer from acetyl-CoA and the malonyl elongation substrate from malonyl-CoA to the ACP pantetheine arm with the help of the acetyl transferase (AT) and malonyl/palmitoyl transferase (MPT), respectively (Fig. 3). Then they are condensed by the ketoacyl synthase (KS) to form β-ketoacyl-ACP through a malonyl decarboxylation reaction, after which the acetoacetyl-ACP will be further modified at its β-carbon position by ketoacyl reductase (KR), dehydratase (DH) and enoyl reductase (ER) yielding acyl-ACP[54]. The central ACP domain is located between the different active sites within the complex, and is responsible for shuttling the processed acyl-chain back to the KS domain initiating the next round of the elongation cycle[55]. In each cycle, the growing acyl-chain is elongated by C2 units provided by malonyl-CoA until the chain length of the fatty acid reaches 16 or 18 carbon atoms. Then the end product will be shuttled by ACP from ER to MPT, where it is transferred to CoA and released[56]. Recently, the metabolism of *S. cerevisiae* was successfully changed to a pure lipogenesis metabolism through blocking ethanol fermentation, together with additional

metabolic engineering strategies for increasing acetyl-CoA and cofactor supply, enabling production of 33.4 g/L free fatty acids in a fed-batch cultivation[57]. Moreover, the fatty acid metabolism has been tailored to generate diverse fatty acids with distinct chain lengths. For example, rewiring the native fatty acid elongation system and introducing a heterologous *Mycobacteria* FAS I system, enabled a very-long- chain fatty acids (VLCFAs, C_{22}-C_{26}) producing platform in yeast[58]. On the other hand, the formation of short to medium-chain fatty acids was achieved in yeast as well by controlling the chain-length through modification of the yeast type I FAS[59,60].

1.2 Objectives and structure of the thesis

The yeast *S. cerevisiae* as a versatile microbial cell factory, and recent studies about the production of natural products such as isoprenoids, flavonoids and alkaloids were introduced in the first part of this thesis. As mentioned, *S. cerevisiae* is considered as an attractive chassis with many advantages for future industrial biofuel production. Although the microbial synthesis of fatty acid-derived chemicals using *S. cerevisiae* has drawn much attention, still, many challenges and obstacles are yet to be overcome. Studies focusing on advanced biofuel production were also introduced in the first chapter of the thesis. The aim of our project was to explore the capability of yeast to enable a production platform for fatty acid derivatives. Details about the production of these chemicals will be discussed in the second chapter, including fatty alcohols, alka(e)nes and fatty acid ethyl esters. In this work, we applied novel multidimensional engineering approaches, such as protein engineering and adaptive laboratory evolution (ALE) to enable the improved production of fatty acid derivatives. The following parts will give a brief introduction into the work.

The first part of the project addressed the secretion of long-chain fatty alcohols (LCFOHs, C12-C18) in *S. cerevisiae*. The accumulation of intracellular LCFOHs leads to impaired growth of yeast cells, which is a major obstacle of high-level production of fatty alcohols. We found that the heterologous fatty acid transporter FATP1 from human could also mediate fatty alcohol export in a high fatty alcohol producing yeast strain. With the expression of *FATP1*, improvements in fatty alcohol secretion and cell fitness were observed. Furthermore, we also identified the protein domain of FATP1 involved in fatty alcohol secretion through domain swapping with FATP4, a transporter from same family as FATP1. Engineering product secretion is attractive for improving the process economy and environmental friendliness[61]. In paper II, improved secretion and production of 1-alkenes in yeast were obtained through expression of the same transporter and other metabolic pathway engineering strategies.

Medium-chain fatty acids (MCFAs, C6-C12) are valuable molecules that can be the substrates for the production of jet-fuels, gasoline, plastics and cosmetics. However, due to the toxicity of these non-inherent fatty acids, it is quite challenging to synthesize them by microbes[62]. We therefore attempted to improve the resistance of yeast cells against MCFAs by

performing directed evolution of a native membrane transporter Tpo1 and adaptive laboratory evolution (ALE). The work about Tpo1 transporter engineering will be introduced in the protein engineering section in the third chapter of this thesis. The resistance improvement against MCFAs using ALE will be covered in the last section of third chapter.

Based on the high MCFA producing strain, we also attempted to synthesize medium-chain fatty alcohols (MCFOHs) in yeast. Although the MCFA producing strain represents a great platform for the synthesis of MCFOHs in *S. cerevisiae*, the lack of enzymes that specifically convert MCFAs limits the high-level production of MCFOHs. Instead of traditional metabolic pathway engineering, different protein engineering approaches were designed to develop an improved carboxylic acid reductase from *Mycobacterium marinum* (MmCAR), which is the key enzyme catalyzing the reduction of fatty acids to fatty aldehydes, which can be further converted to alcohols. Both the results from *in vitro* and *in vivo* tests demonstrated that we successfully altered the substrate specificity of MmCAR towards MCFAs and significantly improved the catalytic activity of the enzyme. This part of work mainly focusses on the engineering of the key enzymes that will be discussed in the protein engineering section. Accordingly, different strategies depending on random approaches and rational or semi-rational design used to engineer MmCAR will be introduced separately.

This thesis will give in the following a detailed introduction about how we performed the multidimensional engineering approaches to obtain the versatile yeast platforms for production of fatty acid derivatives.

2 Microbial synthesis of fatty acid-derived chemicals

2.1 Fatty alcohols

Fatty alcohols (FOHs) are considered as important chemicals widely used for the production of cosmetics, lubricants, plastics and potential biofuels. Microbial synthesis of fatty alcohols offers a solution that prevents the production from strongly relying on plant oils, which could enable a sustainable supply rather than competing with food oil production. Different routes of fatty alcohol synthesis have been established in *S. cerevisiae* as shown in **Figure 4**, which start from fatty acyl-CoA, fatty acyl-ACPs and fatty acids, respectively. The enzymes fatty acyl-CoA/ACP reductase (ACR/AAR), fatty acid reductase (FAR) and carboxylic acid reductase (CAR), respectively, are involved in the corresponding steps[63–65]. Normally, these enzymes catalyze a two-electron reduction to form fatty aldehydes as the intermediates, then followed by a second reduction via aldehyde reductases (ALRs) or alcohol dehydrogenases (ADHs) towards fatty alcohol generation (Fig. 4). Nevertheless, several fatty acyl-CoA reductases catalyzing the four-electron reduction of fatty acyl-CoA directly to the corresponding fatty alcohol (FaCoAR) have been identified in the jojoba plant, *Arabidopsis thaliana* and *Marinobacter aquaeolei* VT8, eliminating the requirement of a separate fatty aldehyde reductase[66,67].

The expression of NADPH-dependent FaCoAR from *Mus musculus* (*mFAR1*) in *S. cerevisiae* led to the production of 47.4 mg/L fatty alcohols[68]. In addition, improving the fatty acid synthesis pathway through replacing the native promoter of *ACC1* and overexpression of *FAS1/2* and malic enzyme, resulted in an increase in the final fatty alcohol titer to 98 mg/L. Being the precursors of fatty alcohols, many efforts towards optimization of fatty acid production have been proven to benefit fatty alcohol formation. The fatty acid degradation pathway was blocked through deleting fatty acyl-CoA oxidase (encoded by *POX1* in yeast) together with deletion of fatty acyl-CoA synthetase (encoded by *FAA1* and *FAA4*) and abolishing the reversal of aldehyde formation by deleting aldehyde dehydrogenase (encoded by *HFD1*), which has been reported leading to an improvement of fatty acid production in

yeast[69]. Consequently, through co-expressing the FaCoAR from *Marinobacter aquaeolei* VT8, the CAR enzyme from *Mycobacterium marinum* and the native alcohol dehydrogenase encoded by *ADH5*, this yielded up to 120 mg/L fatty alcohols in shake flasks. A significant increase in fatty alcohol production was achieved in another study, in which the authors deleted the competing reactions encoded by *DGA1*, *HFD1* and *ADH6*, controlled cofactor usage by deleting *GDH1* to slow down the glutamate synthesis and overexpressed the Δ9-desaturase encoded by *OLE1*. The final strain produced 1.2 g/L fatty alcohols in shake flasks[70].

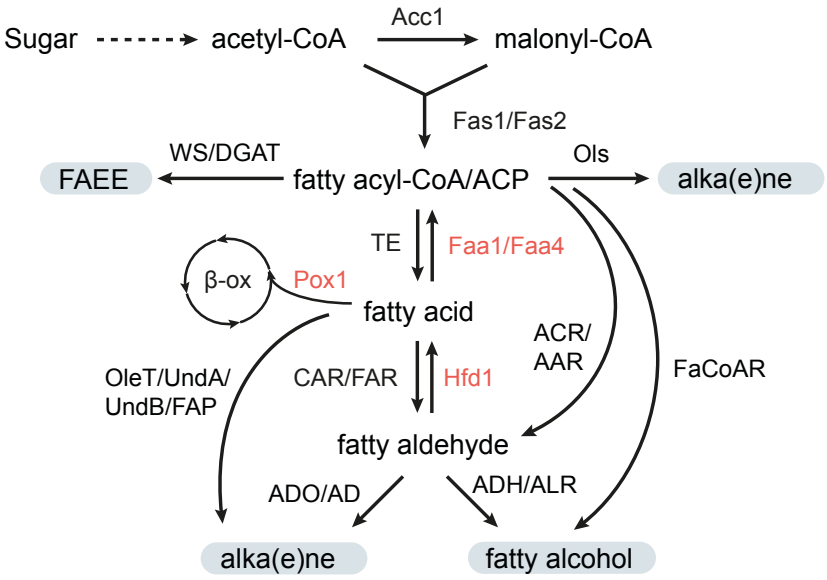


Figure 4. Diverse heterologous pathways for synthesis of fatty acid-derived chemicals. Acc1, acetyl-coenzyme A carboxylase; Fas1/2, fatty acid synthetase; Faa1/4, fatty acyl-CoA synthetase; Pox1, fatty acyl-CoA oxidase; TE, thioesterase; Hfd1, aldehyde dehydrogenase; ADO, aldehyde deformylating oxygenase; AD, aldehyde decarbonylase; ADH, alcohol dehydrogenase; ALR, aldehyde reductase; ACR/AAR, fatty acyl-CoA/ACP reductase; FAR, fatty acid reductase; CAR, carboxylic acid reductase; FAP, fatty acid photodecarboxylase; Ols, olefin synthetase; WS/DGAT, wax ester synthase/acyl-CoA: diacylglycerol acyltransferase.The enzymes in black are involved in the synthesis process, and the knockout of the enzymes in red has been shown to have a positive effect on the production of FA derivatives.

Many of the metabolic engineering approaches have been applied to enable an efficient yeast cell factory with high levels of fatty alcohols being produced. However, the impaired growth attributed to the accumulation of fatty alcohols and the high extraction costs for intracellular products are major obstacles for large-scale industrial production[69]. The aim of the first part of this project (**Paper I**) was therefore to identify fatty alcohol exporters that could benefit the cell growth and fatty alcohol secretion.

The metabolism for fatty alcohol production had been optimized in a previous study from our group[69], and around 1.5 g/L of fatty alcohols were obtained in a fed-batch culture from the final strain, FOH33, in which the enzymes involved in fatty alcohol synthesis including heterologous reductases MmCAR and FaCoAR and endogenous alcohol dehydrogenase Adh5 were overexpressed. In addition, the genes *POX1*, *FAA1*, *FAA4*, *HFD1,* and *ADH6* responsible

for competing pathways were deleted in the same strain. However, the large amount of intracellular fatty alcohols accumulated in this strain led to impaired cell growth. In order to relieve the cellular toxicity, we attempted to identify transporters that facilitate fatty alcohol export in this strain. As no specific fatty alcohol transporters had been reported and substrate specificity of known transporters was the theoretical basis for choosing transporter candidates, we assumed that transporters for fatty acids could also recognize fatty alcohol as the substrate due to the similar structures. Thus, several fatty acid and wax transporters from different species were selected and expressed in *S. cerevisiae*. The candidate transporters are listed in Table 1.

Table 1. List of fatty alcohol transporter candidates.

Name	Species	Description	Reference
ABCB1	*H. sapiens*	Regulation of the efflux of phospholipid, long-chain fatty acids, cholesterol, etc.	71,72
ABCG11	*A. thaliana*	Epidermal plasma membrane cuticular lipid exporter. Functions as homodimer or heterodimer with ABCG9, ABCG12, and ABCG14.	73
ABCG12	*A. thaliana*	Plant cuticular wax exporter. Functions as a heterodimer with ABCG11.	74,75
ABCG9	*A. thaliana*	Half transporter, which works with ABCG11 as heterodimer or homodimer. It is involved in lipid/sterol homeostasis regulation.	75
ABCG14	*A. thaliana*	Half transporter, which works with ABCG11 as heterodimer. It is involved in lipid/sterol homeostasis regulation.	75–77
FATP1	*H. sapiens*	Long-chain fatty acid uptake transporter of the FATP family, which has acyl-CoA synthetase activity.	78,79
FATP4	*H. sapiens*	Transporter of the FATP family that facilitates long-chain and very long-chain fatty acid uptake.	80,81
CD36	*H. sapiens*	A transporter protein that enhances cellular fatty acid uptake.	82,83

All of the transporter genes were codon-optimized and evaluated in strain FOH33 through assessing the extracellular fatty alcohols, among which FATP1 was the only one found to dramatically increase the final titer of extracellular fatty alcohols, resulting in 70 mg/L compared to 16 mg/L produced by the control strain (Fig. 5). In addition to elevated secretion of fatty alcohols, FATP1 expression in yeast also resulted in a higher biomass yield, i.e. an 83% increase of final cell mass (Fig. 5), suggesting that the expression of FATP1 was beneficial for relieving the cellular toxicity by exporting the intracellular fatty alcohols out of the cells. For the other transporters, their expression negatively affected the final biomass yield (OD600) and the final titer of extracellular fatty alcohols, which were even lower than found for the control strain.

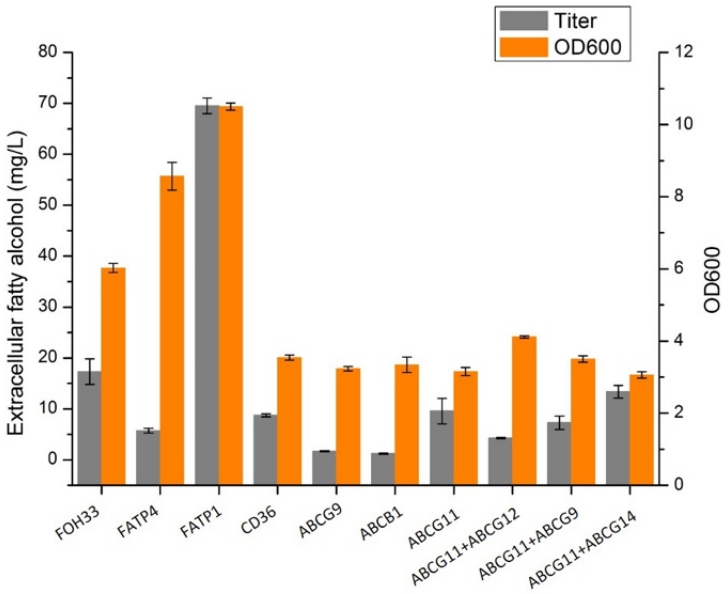


Figure 5. Extracellular fatty alcohol titers and final OD600 of *S. cerevisiae* strain FOH33 expressing different heterologous transporters. Adapted from Hu et al. [84]

FATP1 belongs to the FATP family in human containing six FATP isoforms (FATP1-6). All of the members have been identified as dual-functional proteins that mediate fatty acid uptake and have fatty acyl-CoA synthetase activity in human cells[78,85–88]. Although all the members share a well-conserved 311-amino acid sequence, each of them showed different capacities in fatty acid uptake and activation[79]. Based on previous studies, FATP1 was known as fatty acid transporter that facilitates the uptake of long-chain fatty acids (LCFAs)[89], and the similar property of LCFAs uptake was observed in a yeast strain expressing FATP1 using LCFAs as the sole carbon source[79]. As the modifications in the host strain FOH33 including deletion of *FAA1/4* and overexpression of FaCoAR might cause the scarcity of acyl-CoA that is essential for cell growth, we therefore speculated that the increase in biomass formation might partially be due to the long-chain fatty acyl-CoA synthetase activity of FATP1, since the activation of long-chain fatty acids to form acyl-CoA could potentially complement the deficiency of Faa1/4 in this strain.

In order to investigate if other FATPs have similar effects on fatty alcohol secretion, we thus tested all six isoforms (FATP1-6) in the FOH33 strain. However, even less extracellular fatty alcohols were detected by GC-MS compared with control strain, and none of the these was able to facilitate fatty alcohol export except FATP1 (Fig. 6a). In addition, according to the final OD600, the expression of FATP2, FATP3, FATP5 and FATP6 had no positive effect on the cell growth except FATP4, as the strain containing FATP4 resulted in a relatively high final OD600 that was around 9. (Fig. 6b). However, although the expression of FATP4 did benefit the cell growth of FOH33, an increase in extracellular fatty alcohols was still not observed, suggesting that the fatty alcohol export activity was specific to FATP1. The result was also consistent with our hypothesis that the fatty acyl-CoA synthetase activity could potentially also contribute to the improvement in cell growth.

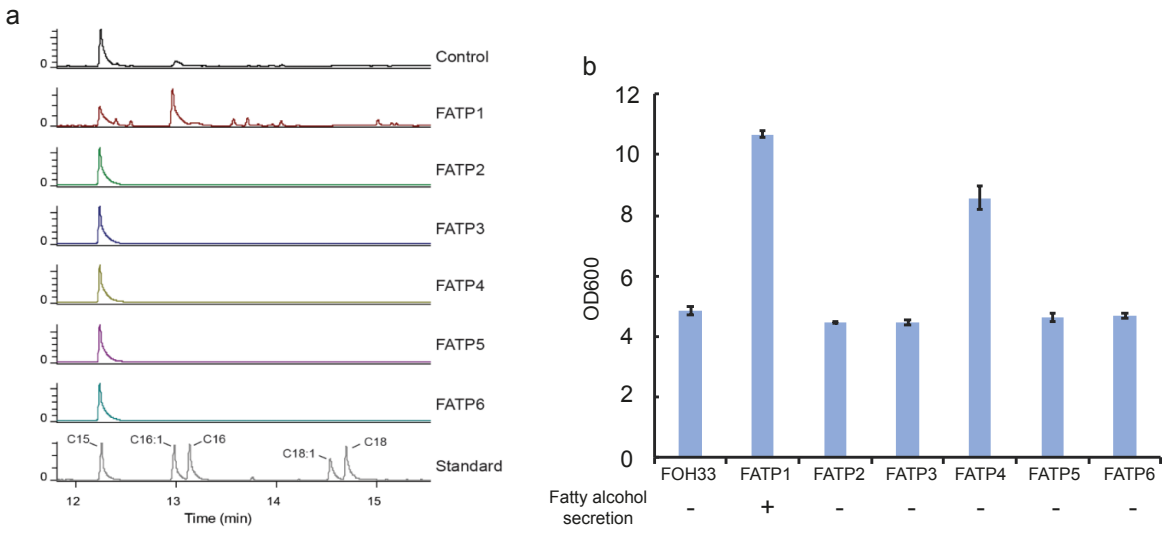


Figure 6. The effect of expression of FATP family transporters in a high fatty alcohol production yeast strain, FOH33. (a) GC spectra of strains based on FOH33 containing FATP transporter isoforms. FOH33 carrying the empty vector pIYH01 was used as control. (b) The final biomass (represented as OD600) of FOH33-derived strains expressing FATP proteins or carrying an empty vector. Increased production of extracellular fatty alcohols (+) or no effect (-) compared to control strain is indicated according to (a). Adapted from Hu et al.[84]

We analyzed the profile of fatty alcohols produced by the FATP1 expressing strain. Around 70 mg/L extracellular fatty alcohols were obtained from shake-flask cultivations, which was 4.5-fold higher than the final titer of the control strain (ca. 15 mg/L) (Fig. 7a). In addition, an increase in the titer of intracellular fatty alcohols was also obtained in the FATP1 expressing strain, resulting the production of 170 mg/L intracellular fatty alcohols compared to 120 mg/L in the control strain (Fig. 7b). Moreover, extracellular fatty acids were also detected, and the final titer was around 190 mg/L, showing that the level of extracellular fatty acids in the FATP1 expression strain was also enhanced compared with the control strain (60 mg/L) (Fig. 7c). The growth curves of the control strain and FATP1 expressing strain are shown in Figure 7d. FATP1 enabled both a faster growth rate and higher final cell mass than the control strain (Fig. 7d). The specific yield co-efficient of fatty alcohols was investigated to determine whether the increased extracellular fatty alcohols resulted from the higher biomass concentration. Although the specific yield of total fatty alcohols in the FATP1 expressing strain was even slightly lower than for the control strain, according to the results, the FATP1 expression strain produced 6.4 mg/L/OD of extracellular fatty alcohols, which accounted for 29% of the total fatty alcohol products, while the control strain only produced 2.4 mg/L/OD of extracellular fatty alcohols (Fig. 7e). In conclusion, with the FATP1 transporter expression, the final titer of extracellular and total fatty alcohols increased around 4.5- and 2-fold, respectively, and the cell growth also benefitted from FATP1 expression as a 2-fold increase in the final biomass was obtained.

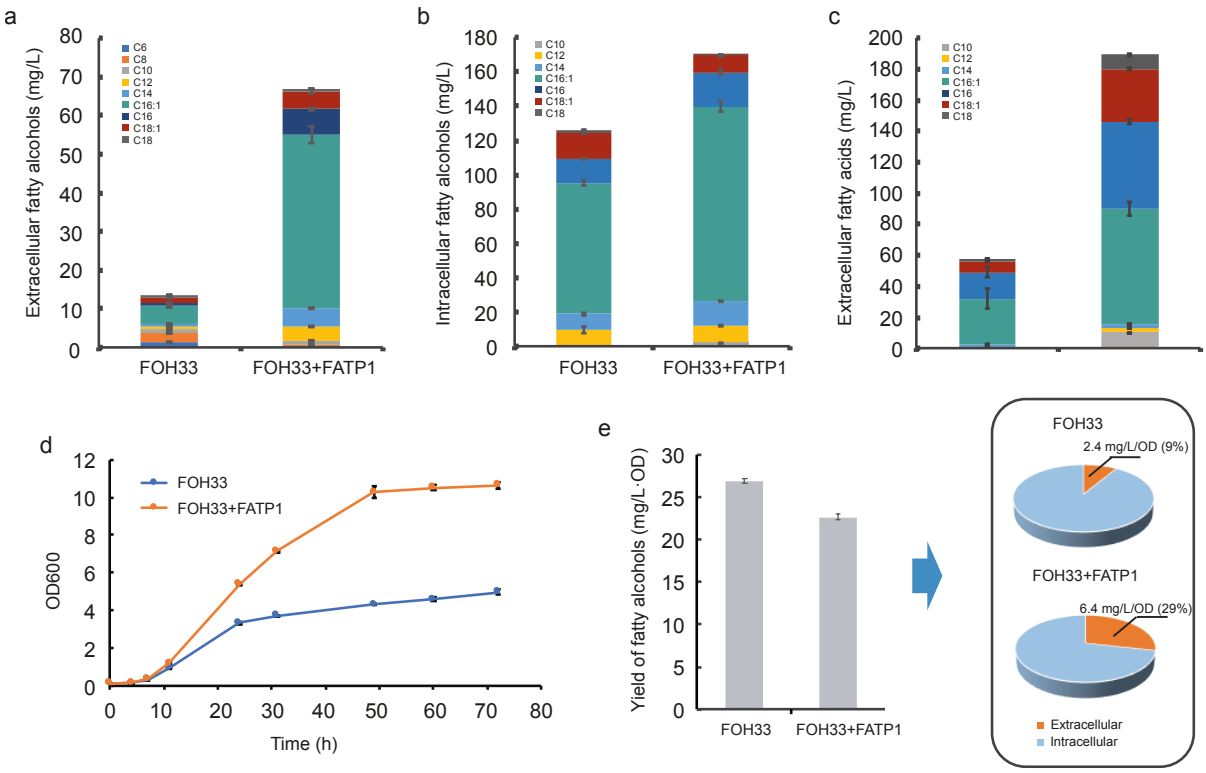


Figure 7. The effect of FATP1 expression on extracellular fatty alcohols, intracellular fatty alcohols and cell growth. (a) Final titers of extracellular fatty alcohols produced by FOH33 and FATP1 expressing strain. (b) Intracellular fatty alcohol titers produced by control strain FOH33 and FATP1 expressing strain. (c) Final titers of extracellular fatty acid produced by FOH33 and FATP1 expressing strain. (d) Growth curves of FOH33 and FATP1 expressing strain. (e) The yield of fatty alcohols (including both extracellular and intracellular fatty alcohols) from FOH33 and FATP1 expressing strain, and the distribution of extracellular and intracellular fatty alcohols for each strain. Adapted from Hu et al.[84]

FATP4 has been proven to benefit the growth of FOH33 with no fatty alcohols being secreted, which potentially resulted from its function as acyl-CoA synthetase. Therefore, there might be no direct connection between fatty alcohol export and fatty acid activation activities. As FATP1 was found to be the only functional transporter for fatty alcohols, we wanted to identify which protein domain of FATP1 can confer the ability to transport fatty alcohols to FATP4. Thus, a domain exchange strategy was applied between FATP1 and FATP4. All members of FATP family contain an intrinsic well-conserved FATP/VLCS motif and an ATP/AMP motif, which have been reported to contribute to the fatty acyl-CoA synthetase activity a and are commonly found in all adenylate-forming enzymes[90,91]. Each gene was divided into three domains based on the corresponding amino acid sequence. The first part (F1 and F4) at the N-terminus was the region upstream of the respective ATP/AMP motifs of FATP1/4, the middle part (F2 and F5) contained the ATP/AMP motifs, and the last part (F3 and F6) covered 143 amino acids containing the FATP/VLCS motif (Fig. 8a). The three domains from FATP1 and FATP4, respectively, were fused with each other to generate six new FATP chimeras, FATP423, FATP153, FATP126, FATP156, FATP426 and FATP453 (Fig. 8a).

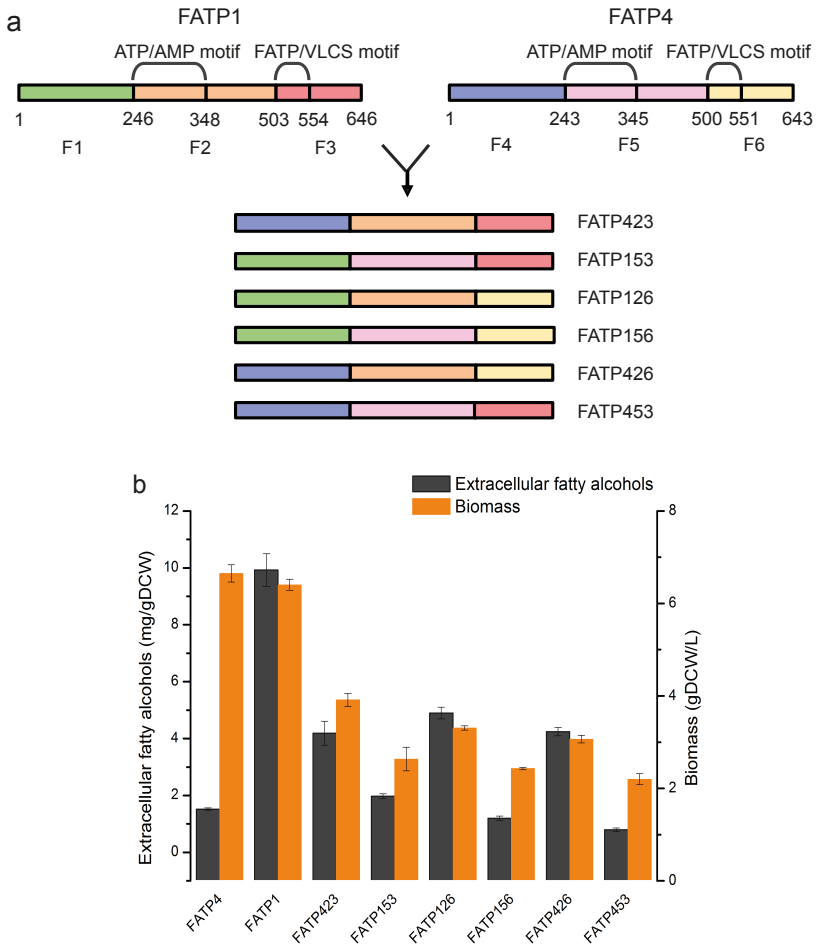


Figure 8. Effect of six FATP chimeras resulting from domain swapping. (a) Division of FATP1 and FATP4 into three domains to construct six new FATP chimeras. (b) Effect of expressing six new chimeras on yield of extracellular fatty alcohols and the final biomass of each strain. Adapted from Hu et al.[84]

After transforming FOH33 with the chimeric constructs, the final biomass of all transformants was much lower compared to the FATP1 and FATP4 expressing strains, suggesting that the fatty acyl-CoA activity in the new isoforms may have been affected after the domain exchange (Fig. 8b). However, these chimeras possess distinct efficiencies in fatty alcohol transport, as an increased yield of extracellular fatty alcohols was only observed in the strains with FATP423, FATP126 and FATP426 (Fig. 8b). According to the results, 3.9, 4.9 and 4.1 mg/g DCW was the yield of extracellular fatty alcohols obtained from strains containing FATP423, FATP126 and FATP426, respectively a more than 2-fold increase compared to the strain with FATP4. Interestingly, all the three efficient FATP chimeras shared a common F2 domain that contains the ATP/AMP motif from FATP1, which indicated that the F2 domain contributed the most to the fatty alcohol export activity. This suggested a conclusion distinct to a former report, which suggested that the FATP/VLACS motif of yeast FATP orthologue Fat1 showed specific functions for fatty acid uptake[92]. However, after the further introduction of two mutations into Asp^{508} to abolish ATP binding in FATP1 (see Figure S4 in Paper I), the mutants lost their function as fatty alcohol transporter, which reveals a consistent conclusion that the ATP/AMP motif played a critical role in fatty alcohol export in FATP1.

In this study, we identified a novel function of transporter FATP1, and significantly improved the fitness of a biofuel production strain as well as the secretion and production of fatty alcohols. This work highlights the possibility that transporter engineering could be a promising strategy to enable the successful commercialization of biofuel production.

2.2 Alka(e)nes

Alkanes and alkenes synthesized by microbes are attractive drop-in biofuels that could be utilized as gasoline, diesel and jet fuel. They are produced naturally by many organisms where they have distinct functions, for example, as plant cuticular waxes and insect pheromones[93,94]. The first success of heterologous *de novo* synthesis of alkanes was obtained in *E. coli*[95]. The authors identified an alkane biosynthesis pathway from cyanobacteria and introducing the pathway into *E. coli* by expressing a cyanobacterial AAR and a fatty aldehyde deformylating oxygenase (ADO), the fatty acid metabolism was successfully directed towards alka(e)ne production (Fig. 4). Later, the *de novo* synthesis pathway was elucidated in yeast as well.

Fatty acids, fatty acyl-ACP/CoA and fatty aldehydes are the major precursors that can be used to synthesize alka(e)nes in *S. cerevisiae* via corresponding enzymes. $OleT_{JE}$ was reported as a cytochrome P450 enzyme from the CYP152 family that is responsible for the conversion of C12-C20 fatty acids to corresponding terminal olefins (1-alkenes) using H_2O_2 as the sole electron and oxygen donor[96,97]. UndA and UndB were identified as fatty acid decarboxylases that also use fatty acids as the precursor, but with a substrate specificity towards medium-chain fatty acids (C10-C16) (Fig. 4), yielding medium-chain 1-alkenes. Furthermore, an algal fatty acid photodecarboxylase (FAP) driven by light was recently found that can facilitate the conversion of fatty acids to their corresponding alka(e)nes, and this enzyme was applied successfully in *E. coli* to generate hydrocarbons in the presence of visible light[98]. The synthesis of alka(e)nes can also be initiated from fatty aldehydes by aldehyde deformylating oxygenase (ADO) or aldehyde decarbonylase (AD). ADO is considered as a non-haem di-iron oxygenase requiring molecular oxygen and an external reducing system to provide four electrons, yielding hydrogen peroxide (H_2O_2) and formate as by-products[99]. AD catalyzes the decarbonylation of fatty aldehydes to alka(e)nes. *Drosophila* CYP4G1 and *Arabidopsis* CER1 with the associated protein CER3, the most well-known ADs, are naturally involved in long-chain alkane biosynthesis and have been successfully expressed in yeast to generate alka(e)nes[100,101]. Finally, a multi-domain polyketide synthase (PKS) from cyanobacteria, referred to as Ols (Fig. 4), can catalyze the reaction from fatty acyl-CoA/ACP to alka(e)nes through an elongation/sulfonation/decarboxylation mechanism[102].

Many studies focusing on the biosynthesis of alka(e)nes in *S. cerevisiae* achieved significant progress during recent years. However, one reason for the suboptimal titers of alka(e)ne

production in yeast is the low efficiency of the enzymes involved and the strong competition of fatty alcohol accumulation[103]. Compartmentalization by targeting the enzymes involved in alka(e)ne production to the peroxisomes in this case, has been proven to be a feasible strategy with the advantage of isolating the synthesis pathway from the competing routes in the cytosol. In a previous study, *Synechococcus elongatus* ADO (*Se*ADO) together with *Mycobaterium marium* CAR (*Mm*CAR) was targeted to the peroxisomes in yeast resulting in around 0.12 mg/L alkanes, 90% more alkanes than produced by the cytosolic pathway[104]. A similar approach was applied successfully to improve medium chain alka(e)ne (C7-C13) production in yeast[105]. Besides the inefficiency of the synthesis pathway, also the toxicity caused by accumulation of alka(e)nes limits production in the cells, which has stimulated some studies focusing on the tolerance increase against alka(e)nes. Both endogenous and heterologous transporters were identified in *S. cerevisiae* to benefit the cellular tolerance against alka(e)nes, for example, the native efflux pumps Snq2 and Pdr5 and the exporters Abc2 and Abc3 from *Yarrowia lipolytica*[106,107]. As part of the project, we aimed to engineer an ideal yeast cell factory for the production and secretion of 1-alkenes by pathway engineering, overall dynamic metabolic balancing and transporter expression (**Paper II**).

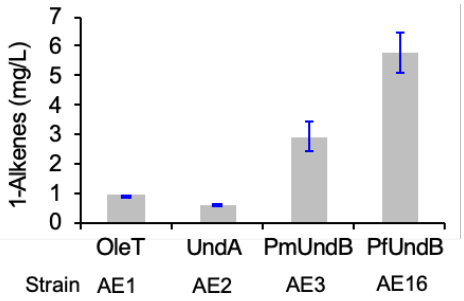


Figure 9. The titer of 1-alkenes produced by *S. cerevisiae* strains expressing different heterologous enzymes. The host strain used for evaluation was the fatty acid over-producing strain YJZ06 carrying deletions of *POX1*, *FAA1* and *FAA4*. Adapted from Zhou et al.[108]

Concerning alka(e)ne production, there is a performance variation observed in yeast and *E. coli* with the potential reasons for these differences being protein expression level and/or enzymatic activity in the cellular environment. We therefore initiated this work with a screening for more efficient pathways for terminal alkene production in the fatty acid over-producing yeast strain YJZ06[69], which carries deletions of *FAA1/4* and *POX1*. Besides H_2O_2-dependent P450 enzyme OleT, we also tested the recently identified nonhaem iron oxidase UndA and desaturase-like enzyme UndB. All these fatty acid decarboxylase genes were expressed under control of an enhanced strong promoter *eTDH3p* from the pYX212 plasmid backbone. Expression of OleT from *Jeotgalicoccus* enabled production of 0.92 mg/L 1-alkenes, while PpUndA from *Pseudomonas putida* F1 led to a lower 1-alkene titer of 0.57 mg/L (Fig. 9). The distinct efficiency of PpUndA for medium-chain 1-alkene production observed previously was reflected in the fact that this enzyme seemed to have poor activity towards long-chain fatty acids in our study[109]. Interestingly, UndB, the desaturase-like enzyme, catalysed the biosynthesis of 1-alkenes in *S. cerevisiae* with the highest efficiency. (Fig. 9). Two UndB enzymes, PmUndB from *Pseudomonas mendocina* and pfUndB from

Pseudomonas fluorescens Pf-5, were evaluated in the yeast strain, of which the latter showed higher activity in 1-alkene production, resulting in 5.76 mg/L of 1-alkenes.

The correlation between free fatty acid accumulation and 1-alkene production is of great interests to be investigated (Fig. 10). From the results, marginal effects on 1-alkene production were observed in strains deficient of Pox1 and/or Faa4, while a 36% increase in the final titer was obtained after the deletion of *FAA1*. The triple deletion led to 6.20 mg/L of 1-alkenes, which was a 2.8-fold increase compared to the strain with wild-type background. Furthermore, we found that the production of 1-alkenes was well correlated with the free fatty acid levels, which was consistent with previous studies [110](Fig. 10). These results suggested fatty acid decarboxylase needs sufficient levels of precursor free fatty acids.

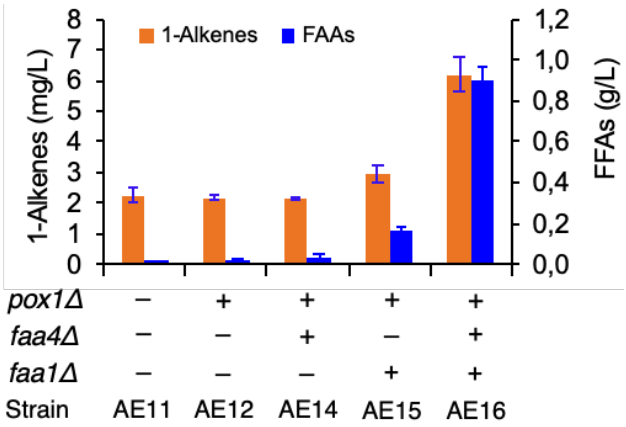


Figure 10. The effect of manipulating the levels of free fatty acids on biosynthesis of 1-alkene. Adapted from Zhou et al.[108]

Electron transfer is involved in terminal alkene biosynthesis, as it is an oxidation process. From several previous studies, it was shown that an efficient reduction system can positively affect fatty acid decarboxylases both *in vitro* and *in vivo*[111]. We thus tried to introduce and compare three electron transfer systems, which were ferredoxin-ferredoxin reductase (Fd/Fpr) and flavodoxin-ferredoxin reductase (Fld/Fpr) from *E. coli* using NADPH as cofactor, putidaredoxin-putidaredoxin reductase from *P. putida* (Pdx/Pdr, encoded by *camB* and *camA*, respectively) using NADH as cofactor (Fig. 11a). According to the results, the ferredoxin and flavodoxin based electron transfer system enabled a 42% and 58% improvement in 1-alkene production, respectively, while a 108% increase in 1-alkene production was obtained with the putidaredoxin reductase system (Fig. 11b). However, efforts towards electron channelling optimization through fusing the Pdx/Pdr system to PfUndB with different linkers did not yield a higher 1-alkene production (see Figure S1 in Paper II). It is still worth to mention that the utilization of NADH as electron donor provides a great advantage for 1-alkene synthesis in yeast, which has much higher (>7-fold) levels of NADH compared NADPH[112].

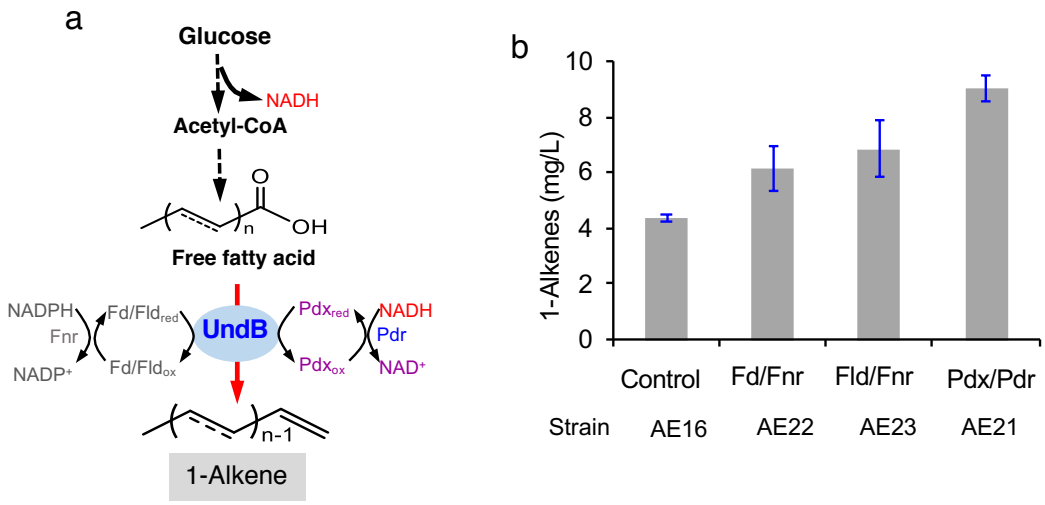


Figure 11. The effects of different electron transfer systems on total 1-alkene production. (a) Overview of the 1-alkene synthesis pathway including Fd/Fnr, Fld/Fnr and Pdx/Pdr electron transfer systems. (b) The titer of 1-alkenes in different engineered strains. Adapted from Zhou et al.[108]

We found that there was high ratio of terminal alkene secreted, as UndB was reported as a membrane-bound desaturase-like enzyme [113](see Figure S2 in Paper II). Fluorescence microscopy analysis of a UndB-GFP carrying strain AE17 showed that the GFP signal mainly localized to the membrane (Fig. 12a and 12b). Here, the strong promoter *eTDH3p* was used to enable high-level expression of PfUndB, but this resulted in impaired growth in strain AE24 (Fig. 12c), which might be attributed to the *eTDH3p*-based transcription being strongly upregulated in the glucose phase and the high-level expression of a membrane protein might cause stress resulting in poor cell growth. We therefore fine-tuned the expression of *undB* by utilizing a dynamic control system, in which the cell growth and 1-alkene production could be separated by controlling the enzyme expression via carbon source dependent promoter *GAL7*. Thus, the expression of PfUndB was repressed at high glucose levels and activated at glucose depletion under the control of *GAL7p* combined with the deletion of the transcription factor Gal80 in the strain AE38. The final titer of 1-alkenes reached 25 mg/L, leading to a 100% higher final titer of 1-alkenes compared to strain AE24 constitutively expressing *PfUndB* under control of *eTDH3p* (Fig. 12c). Both strains had high proportion of extracellular 1-alkenes (>60%) and the dynamic control of *PfUndB* improved the 1-alkene production, both intracellular and extracellular (see Figure S2 in Paper II). In addition, a better cell growth was observed in strain AE38, which had a higher final biomass and higher glucose consumption rate compared to AE34 (Fig. 12c and see Figure S3 in Paper II). Besides, compared with AE34, a higher 1-alkene production (Fig. 12c) and lower accumulation of FFA were obtained from strain AE38 (see Figure S4 in Paper II). As consequences of performing the dynamic control, the toxicity of 1-alkenes was relieved by expressing the membrane protein PfundB and the dynamic control of the expression of the membrane protein enabled an increase in the production, which indicated a feasible approach to benefit a cell factory producing toxic chemicals.

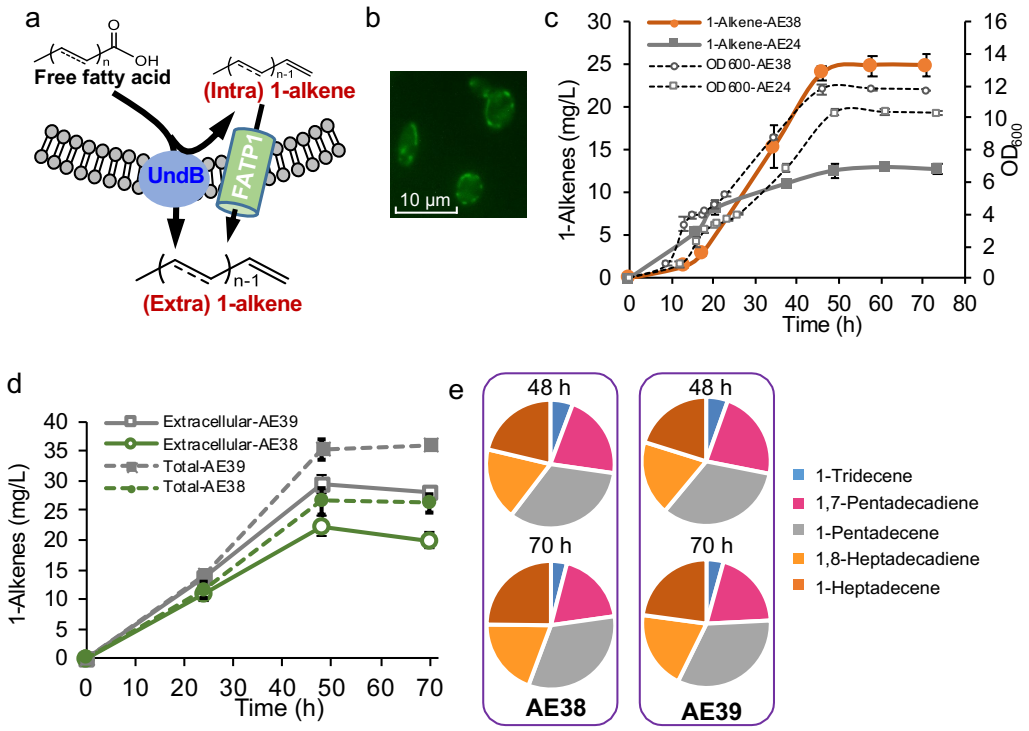


Figure 12. The effects of dynamic control of the expression of membrane enzyme UndB and transporter expression. (a) Overview of 1-alkene production and secretion (b) Fluorescence microscopy of strain AE17 containing *undB* fused with GFP. (c) The comparison of 1-alkene production and OD600 of strain AE38 and AE34. The UndB gene was controlled by *GAL7p* in AE38 and controlled by *eTDH3p* in AE34. (d) The effect on total 1-alkene production with LCFA transporter FATP1 expression. Strain AE39 contains FATP1 and strain AE38 does not. (e) The profiles of total 1-alkenes in strain AE38 and AE39 at 48 h and 70 h. Adapted from Zhou et al.[108]

The improvement of secretion has been proven to be a cost-efficient manner that could reduce the cost for downstream extraction and purification. Many transporters, such as resistance-nodulation-cell division (RND) or ATP-binding cassette (ABC) transporters[114,115], have been successfully utilized to enhance the production and secretion in previous studies. As introduced previously, we identified the novel function of FATP1 as LCFOH exporter in *S. cerevisiae* in **Paper I**. Here, we wanted to explore the potential capability of FATP1 to facilitate 1-alkene secretion based on the similar hydrophobic property of both LCFAs and 1-alkenes (Fig. 12a). As FATP1 is a membrane protein, the inducible promoter *HXT6p* was used to separate the cell growth and production process. Coupled to *HXT6*p *FATP1* was expected to be highly expressed after glucose depletion and thus coupled to 1-alkene production. A marginal effect on cell growth and fatty acid production was obtained after integration of the *FATP1* expression cassette in strain AE39 (see Figure S5 in Paper II), but the extracellular and total 1-alkene production both showed a significant improvement after 70 h cultivation, which was 40% and 37% higher than for the control strain AE38, respectively (Fig. 12d). The FATP1 expressing strain had more than 81% 1-alkenes secreted out of the cells. Although there was little difference in the 1-alkene profiles between AE38 and AE39, slightly more C17 1-alkenes were accumulated at 70 h compared with at 48 h (Fig. 12e).

In this part of work, we engineered a high 1-alkene production and secretion yeast platform by cofactor engineering, transporter engineering and dynamic enzyme control. This study also indicated that the product secretion would provide great benefits for industrial production as it will decrease the separation costs.

2.3 Fatty acid ethyl esters

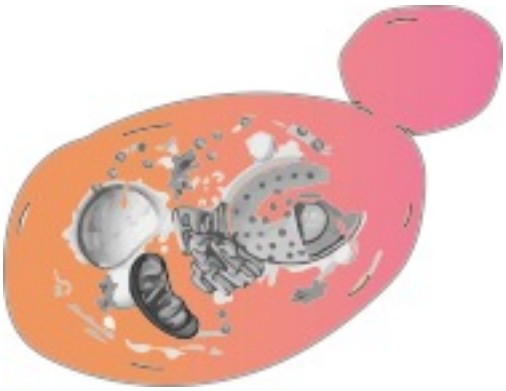
Fatty acid ethyl esters (FAEEs) are secondary metabolites naturally produced by *S. cerevisiae* that usually play key roles as flavor compounds. Biodiesel consists of monoalkyl esters of long-chain fatty acids with short-chain alcohols, primarily methanol and ethanol, i.e. fatty acid methyl esters (FAMEs) and FAEEs. Due to the high toxicity of methanol to the cells[116], the precursor of FAMEs, an increasing interest has been put on the microbial synthesis of FAEEs as a substitute of diesel and bunker oil. Ethanol and acyl-CoAs are the essential precursors that are involved in the biosynthesis of FAEEs, a process with esterification as the mechanism. Esterification is defined as the formation of an ester from alcohols and carboxylic acids, here catalyzed by the wax ester synthases/acyl-CoA: diacylglycerol acyltransferases (WS/DGAT) (Fig. 4), which naturally accept acyl groups with a chain length of C16 or C18 and linear alcohols with a chain ranging from C12 to C20[117]. WSs can have different preferences for substrates with distinct chain length, which enables diverse applications for ester products, such as jojoba-like wax esters and fatty acid ethyl esters[118].

A highly unspecific WS/DGAT from *Acinetobacter calcoaceticus ADP1* was firstly tested in *S. cerevisiae*, resulting a poor performance in FAEE production[119]. Then, five heterologous WSs from bacteria and mammals were evaluated in yeast to investigate their substrate preferences. The results showed that the WS from *Marinobacter hydrocarbonoclasticus* had the best performance towards ethanol as the substrate *in vitro* and led to the highest FAEE titer of 6.3 mg/L in *S. cerevisiae*[118]. Then, chromosomal integration of multiple gene copies yielded a six-fold improvement, up to 34 mg/L, of FAEEs compared to the plasmid-based producer. A further 40% increase in FAEE production was obtained when the precursor supply was improved through overexpression of acyl-CoA binding protein (encoded by *ACB1*) and when the NADPH level was elevated by expression of a bacterial $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (*GAPN*)[120]. In order to achieve efficient synthesis of FAEEs, the concentration of free fatty acids as the precursor would affect the final titer. In *S. cerevisiae*, FAs are used to synthesize phospholipids and storage neutral lipids. The major FA reserves, triacylglycerols (TAGs) and steryl esters (SEs), can constitute about 97% of the storage lipid content of the cell and are not essential to the cell[121]. Blocking the pathways competing for FAs by deleting the genes involved in TAG and SE synthesis (*DGA1*, *LRO1*, *ARE1* and *ARE2*) was beneficial for FA accumulation and in turn increased the FAEE production 2.5-fold[122]. Meanwhile, also acetyl-CoA as the common precursor for fatty acid derivatives was considered to play a key role in FAEE production. The flux towards

acetyl-CoA was improved by overexpressing alcohol dehydrogenase (encoded by *ADH2*), acetaldehyde dehydrogenase (encoded by *ALD6*) and ACS encoded by heterologous gene ace_{SE}^{L641P}, together with the integrated ws2, which led to a three-fold increase of FAEE production[123]. Then, acetyl-CoA was further increased by the phosphoketolase (PHK) pathway through the expression of *xpkA* and either *ack* from *Aspergillus nidulans* or *pta* from *Bacillus subtilis* (a more detailed description of the enzymes will be provided in the following chapter). The introduction of the heterologous PHK pathways helped to produce around 5 mg/g cell dry weight FAEEs, an up to a 1.7-fold improvement. Instead of traditional carbon sources, such as glucose and sucrose, the microbial fermentation using glycerol has been extensively studied due to several advantages including a highly reduced substrate and the cost advantage of anerobic processes with less energy requirements. By increasing the ethanol formation from glycerol, together with blocking the glycerol export route and adding exogenous fatty acids, the titer of FAEEs reached 0.52 g/L which is the highest reported FAEE production to date in yeast[124].

Although it seems unlikely that the concentration of FAEEs reported so far is toxic to the cells, the engineering of transporters specific towards FAEEs that potentially benefit the secretion would still be a feasible strategy to further improve the production of FAEEs in the long term.

3 Strategies for improving the production of fatty acid derivatives



3.1 Modifications of central carbon metabolism

Glucose is the most widely studied carbon source of wild-type *S. cerevisiae* that can be transported into yeast cells by the membrane hexose transporters via facilitated diffusion. The members of the hexose transporter (HXT) family differ with each other in their transcriptional and posttranscriptional regulation, substrate specificity and affinity for glucose[125]. After the uptake, glucose is then cleaved into three-carbon units via the glycolysis pathway.

Glycolysis is the critical metabolic pathway for the growth of all organisms and is responsible for the assimilation of carbon for either respiration or fermentation[126,127]. In the beginning of glycolysis, glucose is phosphorylated by a hexokinase (encoded by *HXK1*, *HXK2* and *GLK1*) yielding glucose-6-phosphate (G6P) through transferring the phosphoryl group from ATP to the hydroxyl group of glucose. Followed by an isomerization and another phosphorylation that are catalyzed by phosphoglucose isomerase (encoded by *PGI1*) and phosphofructokinase (encoded by *PFK1* and *PFK2*), respectively, glucose is converted into fructose 1,6-bisphosphate (F1,6P). Next, the six-carbon F1,6P is cleaved into two three-carbon metabolites glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) under a reversible condensation catalyzed by fructose-biphosphate aldolase (encoded by *FBA1*). There has been no energy generated so far, to the contrary, two molecules of ATP have already been invested. In the remaining steps of glycolysis, the flux will continue from G3P to form pyruvate under a series of reactions with two molecules of ATP being generated. The first ATP in glycolysis is produced during the conversion from 1,3-bisphosphoglycerate (BPG) to 3-phosphoglycerate (3PG) via phosphoglycerate kinase (encoded by *PGK1*). And another ATP is generated along with the pyruvate formation from phosphenolpyruvate (PEP) catalyzed by pyruvate kinase (encoded by *PYK1* and *PYK2*). In yeast, pyruvate is located at a major junction of assimilatory and dissimilatory reactions, which is also considered as the branch-point between respiratory dissimilation of sugars and

alcoholic fermentation[128]. As an essential precursor for acetyl-CoA synthesis, pyruvate has two major metabolic fates. First, pyruvate can be transported into mitochondria facilitated by the mitochondrial pyruvate carrier (MPC) protein (encoded by *MPC1*, *MPC2* and *MPC3*), and then oxidized by the pyruvate dehydrogenase complex (PDH) to generate acetyl-CoA to enter the tricarboxylic acid (TCA) cycle[128,129]. Second, pyruvate can be converted to acetyl-CoA in the cytosol via three reactions catalyzed by pyruvate decarboxylase (encoded by *PDC1*, *PDC5* and *PDC6*), acetaldehyde dehydrogenase (encoded by *ALD2/3/4/5/6*) and acetyl-CoA synthetase (encoded by *ACS1* and *ACS2*). In addition, pyruvate can be the substrate for alcoholic fermentation reducing it to ethanol primarily catalyzed by alcohol dehydrogenase 1 (encoded by *ADH1*) with acetaldehyde as the intermediate.

Eliminating alcoholic fermentation

Whenever yeast exhibits a fermentative sugar metabolism, ethanol and carbon dioxide are the predominant fermentation products[130]. The wide application of microbial synthesis has stimulated the further improvement of the yield of the desired products. In the case of yeast as the host organism, attempts of redirecting the carbon flux away from alcoholic fermentation towards the desired products have been made in recent years. Acetaldehyde, as the intermediate generated from pyruvate, is also the precursor for ethanol production. Thus, the deletion of the ADH genes, together with overexpression of the ALD and the heterologous ACS were conducted to prevent the conversion from acetaldehyde to ethanol and augment the flux towards acetyl-CoA[131]. However, the formation of ethanol is not entirely eliminated in yeast by simply deleting ADH genes, as there is a large number of promiscuous ADHs that could catalyze the reaction to generate ethanol and many of those ADHs are also involved in the production of other essential metabolites within the cells[132].

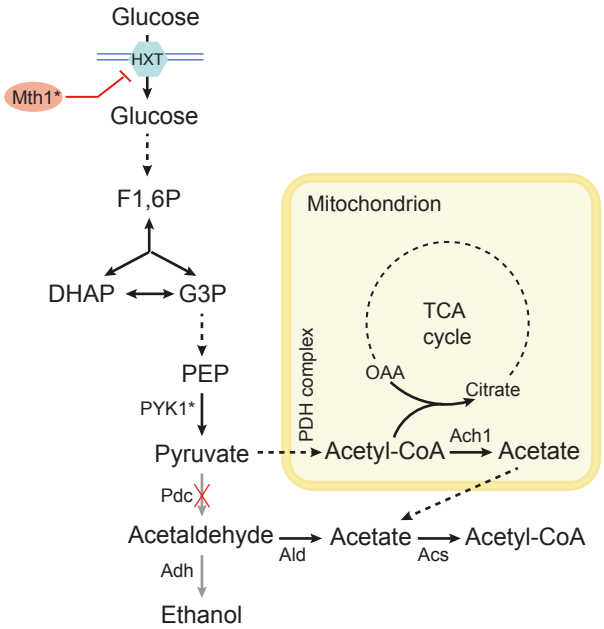


Figure 13. Engineering strategies for eliminating alcoholic fermentation in yeast cells. Pyk, pyruvate kinase; Pdc, pyruvate decarboxylase; Adh, alcohol dehydrogenase; Ald, acetaldehyde dehydrogenase; Acs, acetyl-CoA synthetase; Ach, acetyl-CoA hydrolase.

Instead, ethanol formation was inhibited by the knockout of all three Pdc enzymes (*PDC1*, *PDC5* and *PDC6*)[133] (Fig. 13). However, the glucose sensitivity, as the general characteristic of the Pdc-negative strain, makes it unable to grow in excess glucose, which is due to the repression of respiratory metabolism and the deficiency of C2 supply in the cytosol. The extended cultivation under conditions in which the Pdc-negative strain would not grow offered a solution through generating spontaneous mutants. The problem was solved by evolving the Pdc-deficient strain, which succeeded in obtaining a C2-independent Pdc-negative strain that could also grow in excess glucose[133]. From the evolved strain, an internal deletion in a transcriptional regulator, Mth1, which is involved in glucose sensing in yeast, was found to be associated with the restored growth of the Pdc-negative strain[134]. With this mutated Mth1, the glucose flux was reduced thus resulting in decreased repression of respiration in the evolved strain (Fig. 13). As mentioned above, the block of ethanol synthesis by deletion of all three *PDC* genes will also inhibit the formation of cytosolic acetyl units that serve as precursors for various downstream products, such as fatty acids. Later, the mitochondrial Ach1 was found to facilitate the formation of acetate from acetyl-CoA (Fig. 13), which can then enter the cytosol to provide C2 units for cytoplasmic acetyl-CoA synthesis and thus compensates for the lack of cytosolic acetyl-CoA supply via the PDH bypass[135]. However, this route cannot function under glucose-repressed conditions and is restricted by the limited mitochondrial acetyl-CoA supply due to the stringent regulation of the PDH complex. Alternatively, the expression of a heterologous PDH complex from *Enterococcus faecalis* was shown to fully complement the cytosolic acetyl-CoA supply in an ACS-deficient yeast strain[136]. Most recently, *S. cerevisiae* metabolism was successfully altered from alcoholic fermentation to lipogenesis metabolism with a high-level production of free fatty acids[137]. All three *PDC* genes were deleted from an engineered yeast strain with improved acetyl-CoA supply. After adaptive laboratory evolution (ALE) in glucose, the evolved strain showed that the lipogenesis metabolism could replace the traditional ethanol fermentation. A mutated *PYK1* was proven to be able to restore the growth of this Pdc-negative strain in glucose (Fig. 13). Through this mutation, the glycolytic flux was downregulated to reduce the high NADH level in the cell.

Engineering acetyl-CoA metabolism

The costs of the feedstock can contribute up to 75% of the total costs of the processes for producing chemicals of commercial interest from carbohydrates[138]. This requires maximizing the product yields and avoiding carbon loss in order to achieve an economical production process. Acetyl-CoA, the essential molecule carrying C2 units in yeast, is a key precursor for many metabolites, the acetyl donor for protein acetylation and the fuel for the TCA cycle and glyoxylate cycle[139,140]. In *S. cerevisiae*, acetyl-CoA is involved in the metabolic network in the cytosol, nucleus, peroxisome and mitochondrion, and cannot be transported freely between all compartments. As the major substrate for *de novo* fatty acid synthesis in yeast, the native synthesis of cytosolic acetyl-CoA from pyruvate is referred to as the pyruvate-

dehydrogenase bypass (PDH bypass), in which the AMP-forming ACS catalyzes the last step from acetate with the concomitant hydrolysis of ATP to AMP and PP_i:

$$acetate+ATP+CoA \rightarrow acetyl\text{-}CoA+AMP+PP_i$$

The formation of acetyl-CoA through the native cytosolic PDH bypass from glucose can be described as:

$$glucose+4NAD(P)^{+}+2ATP+2CoA+2H_2O \rightarrow 2acetyl\text{-}CoA+2(NAD(P)H+H^{+})+2CO_2+2ADP+2P_i$$

Since the native cytosolic PDH bypass pathway is suboptimal in terms of acetyl-CoA supply as it results in loss of CO_2 and energy, diverse strategies were implemented to benefit the process economy of acetyl-CoA synthesis.

ATP citrate lyase (ACL) is involved in the production of cytosolic acetyl-CoA from citrate and is present in oleaginous yeasts such as *Yarrowia lipolytica*, but not in non-oleaginous yeasts such as *S. cerevisiae*[138]. ATP is required in the reaction with the formation of ADP instead of AMP, resulting in lower energy consumption compared with the native route. Five ACL genes from different sources were integrated into the yeast genome to compare their activities, of which the ACL from *A. nidulans* exhibited the highest activity[141]. Together with other modifications, the production of acetyl-CoA derived mevalonate was significantly increased. The endogenous mitochondrial citrate transporter Ctp1 was overexpressed to ensure a sufficient precursor supply, together with the overexpression of the ACL from *Mus musculus*, malic enzyme from *R. toruloides* (RtME) and malate dehydrogenase ´Mdh3 (Fig.14), resulting an optimized acetyl-CoA pathway with a remarkable improvement in fatty acid production[69]. Another reaction that yields acetyl-CoA from pyruvate is catalyzed by pyruvate-formate lyase (PFL) with formate being generated. PFL has to be activated by a PFL-activating enzyme (PFL-AE) (Fig. 14). The catalytic activity of PFL depends on a radical residue that makes it sensitive to oxygen. Thus, the PFL and PFL-AE from *E. coli* were first expressed in *S. cerevisiae* to enable a functional replacement of the native PDH bypass as the sole pathway for acetyl-CoA synthesis during anaerobic culture[142] (Fig. 14). In a later study, PFL and PFL-AE from *E. coli* were co-expressed with two different electron donors, reduced ferredoxin or reduced flavodoxin, respectively, elucidating that the PFL-driven bypass with these electron donors can improve growth of a Pdc-negative strain even under aerobic conditions[143]. A reversible reaction directly from acetaldehyde to acetyl-CoA that yields NADH without consuming ATP catalyzed by acetylating acetaldehyde dehydrogenase (A-ALD) was also introduced into *S. cerevisiae* (Fig. 14). Different heterologous A-ALDs were evaluated in a yeast strain with all five native *ALD* genes deleted (*ALD2/3/4/5/6*Δ), showing that all of the candidates enabled a faster growth but low final biomass formation[142]. This phenomenon might be due to the acetaldehyde toxicity in the cells. The route towards cytosolic acetyl-CoA synthesis catalyzed by phosphoketolase and phosphotransacetylase (XFPK/PTA) has drawn much attention due to the complete carbon conversion in the acetyl-CoA formation (Fig. 14). The XFPK enzymes can utilize either fructose-6-P (F6P), xylulose-5-P (X5P) or ribulose-5-P (R5P) as substrates. The sugar phosphates and inorganic phosphate are converted to acetyl-P and either

erythrose-4-P or glyceraldehyde-3-P[144]. The PTA is responsible for the reaction that converts AcP and CoA into acetyl-CoA and P_i. In *S. cerevisiae*, the co-expression of XFPK from *Aspergillus nidulans* and PTA from *Bacillus subtilis* was implemented to enhance the production of FAEEs significantly[145]. However, the flux through the XFPK/PTA pathway in yeast using glucose as substrate appeared to be low[146]. As the XFPK/PTA bypass results in a negative ATP yield, some carbon has to pass through native glycolysis to promote ATP generation, thus preventing complete carbon conversion.

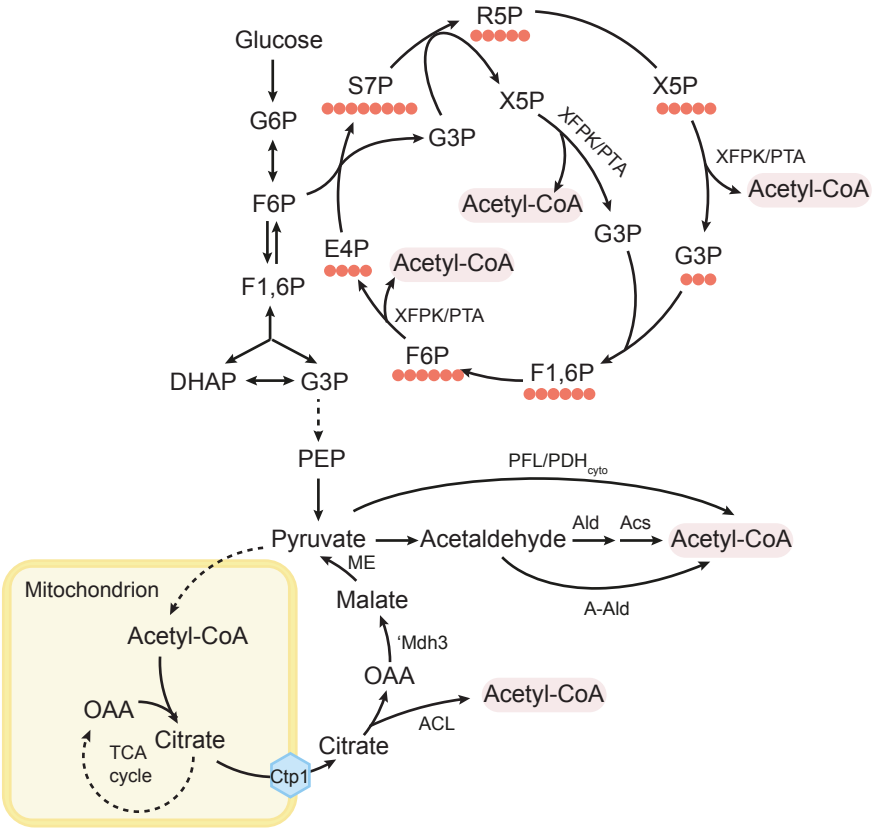


Figure 14. Engineering strategies for improving cytosolic acetyl-CoA supply in yeast cells. ME, malic enzyme; Mdh, malate dehydrogenase; ACL, ATP citrate lyase; Ald, acetaldehyde dehydrogenase; Acs, acetyl-CoA synthetase; Pfl, pyruvate-formate lyase; A-Ald, acetylating acetaldehyde dehydrogenase; Pdh, pyruvate dehydrogenase; XFPK, phosphoketolase; PTA, phosphotransacetylase.

3.2 Redox and cofactor engineering

NADH and NADPH are two essential reducing equivalents involved in the conversion of many metabolites in yeast, where NADH predominantly participates in catabolic reactions and NADPH is mainly required for anabolic reactions. For example, every elongation cycle of fatty acid synthesis requires two molecules of NADPH, thus 16 NADPH molecules are needed for the formation of one C18 fatty acid. Since yeast cells naturally produce excess NADH as the electron carrier, the NADPH supply is usually limiting for the synthesis of fatty acids and its

derivatives. NADPH is predominantly generated from the pentose phosphate pathway (PPP) in yeast (Fig. 15). The pathway is initiated by oxidizing G6P via glucose-6-phosphate dehydrogenase (G6PDH, encoded by *ZWF1*), which is the first rate-limiting step in the PPP. Then, ribulose-5-phosphate is generated through 6-phosphogluconate dehydrogenase (6PGDH, encoded by *GND1* and *GND2*), thereby yielding two molecules of NADPH. Many attempts in metabolic engineering have been made to increase the NADPH supply in the cytosol. In the recent study for improved fatty acid production, extra NADPH was produced by overexpression of the otherwise mitochondrial malic enzyme (Fig. 14), which converts malate into pyruvate, in the cytosol[69].

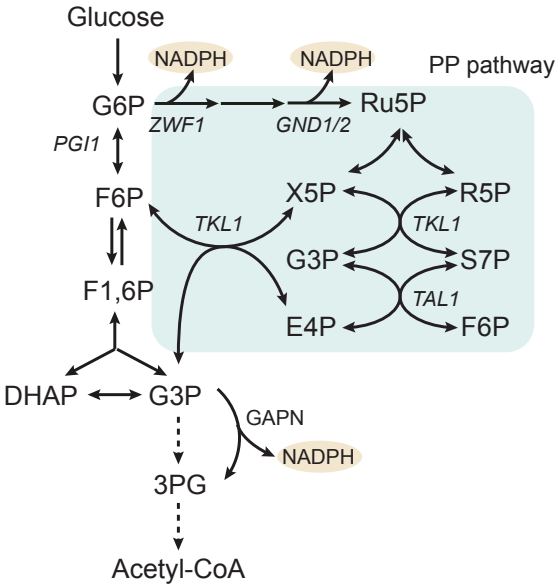


Figure 15. An overview of NADPH synthesis in yeast and engineering strategy for improved NADPH production. GAPN, glyceraldehyde-3-phosphate dehydrogenase; *TKL1*, transketolase; *TAL1*, transaldolase; *ZWF1*, glucose-6-phosphate dehydrogenase; *GND1/2*, 6-phosphogluconate dehydrogenase.

In order to facilitate NADPH regeneration and reduce the loss of carbon, a non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phospate dehydrogenase (GAPN) from *Bacillus cereus* was expressed in a yeast strain with deletion of *GPD1* encoding NAD^+-dependent glycerol-3-phosphate dehydrogenase[147] (Fig. 15). In previous work from our group, fine tuning the flux distribution between the PPP and glycolysis by overexpression of the *GND1* gene, transketolase (encoded by *TKL1*) and transaldolase (encoded by *TAL1*), together with downregulation of phosphoglucose isomerase (encoded by *PGI1*) in yeast to provide additional NADPH led to a 28% increase in free fatty acid production[137].

3.3 Protein engineering

Enzymes are considered as versatile biocatalysts catalyzing diverse chemical reactions in nature, and evolution has enabled them to possess high activities and high specificities towards challenging substrates. Protein engineering is a process with the goal of obtaining modified enzymes that are more suitable for a particular application or purpose than the unmodified enzymes, which generally follows two main strategies, the random approach or the rational design. Both can also be combined in a semi-rational design strategy[148].

Random approach (Paper III & IV)

The random approach referred to as directed evolution is mainly conducted by introducing random mutations into either a single protein sequence or the sequence encoding the active site coupled to high-throughput screening and selection strategies. Screening and selection are two of the most important procedures involved in protein engineering, of which the selection process could automatically eliminate less-functional variants, while the screening facilitates the evaluation of each protein for the desired property[149]. Directed evolution can be conceptualized as series of mutational steps in a fitness landscape that represents the phenotype of all the variants in the library. The goal of directed evolution studies is to take these steps within the fitness landscape that "climb" towards peak activity levels, during which the beneficial mutations will result in a successively improved pheotype[150]. Various information including 3D structure information of the target protein is unnecessary, which is one of the advantages of the random approach (Fig. 16). Theoretically, this means that the approach could be applied towards any protein.

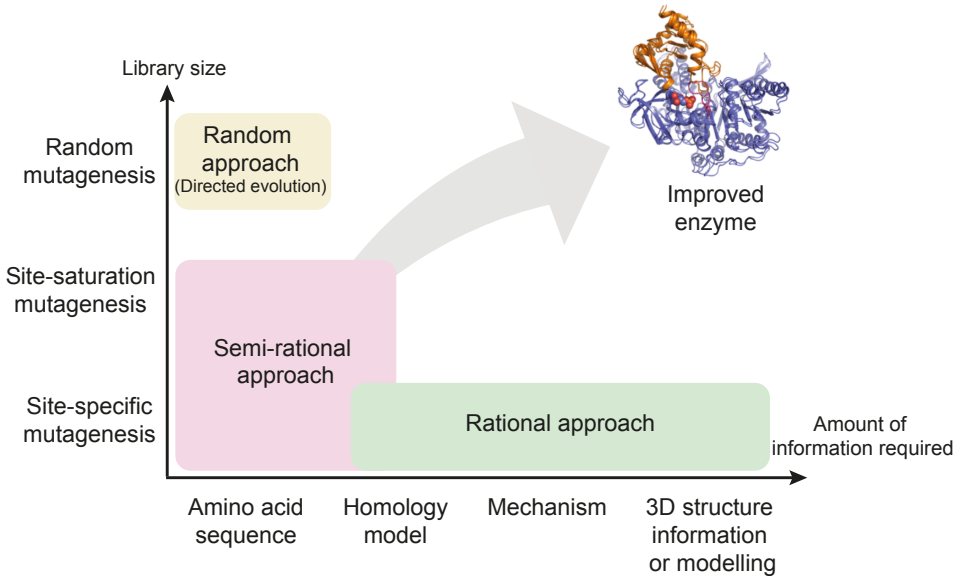


Figure 16. The features of different protein engineering methods in terms of the sizes of the library and the amount of information required. Adapted from Romas et al. and Liu et al. [151,152].

Before performing directed evolution, the library creation is the first crucial step, since the diversity of library affects the exploration of a protein's potential properties. Methods for

the library construction have been developed in recent years, such as error-prone PCR and DNA shuffling. Error-prone PCR is the most commonly used way to create genetic variation nowadays, during which the gene is amplified in the presence of manganese ions or nucleotide analogs to promote mutagenesis through the DNA polymerase or by using a low fidelity DNA polymerase[153]. In the laboratory, the level of mutagenesis can be controlled by the concentration of template and the number of reaction cycles[154]. DNA-shuffling is widely applied to effectively recombine homologous genes from different species, which requires high similarity of the DNA sequences of multiple parent genes[155,156]. However, it is still impossible to cover the entire mutational space of a typical protein with all known library creation methods, as approximately 10^{13} unique mutants are required for a complete randomization of a mere decapeptide[150].

In order to obtain an improved phenotype through the random approach, several rounds of evolution usually have to be conducted, which is time- and labor-consuming. In addition, as the library size is relatively large, a convenient and efficient high-throughput screening method is required to benefit the accumulation of beneficial variants that would greatly reduce the chance of missing the desired mutant (Fig. 16). For example, fluorescent proteins that possess a screenable phenotype, such as GFP, have been widely used in directed evolution by fusion the GFP protein to the enzyme of interest; the enzymes with improved solubility or folding related to the florescence signal will thus be easily visualized by fluorescent microscopy[157,158]. Recently, the directed evolution strategy was also applied to benefit biofuel production. Variants of the AcrB efflux pump from *E. coli* that act on the non-native substrate n-butanol were generated and selected through this random approach. These variants conferred an improvement of the tolerance to n-butanol, resulting in enhanced growth rates of *E. coli* by up to 25%[159]. And a cellodextrin transporter CDT2 was engineered in *S. cerevisiae* under anaerobic conditions through directed evolution; a significant improvement of cellobiose uptake activity and ethanol productivity was observed after several rounds of evolution[160].

As the current production of MCFAs by yeast cells has already approached the inhibitory concentration that is around 1 mM for octanoic acid and decanoic acid[161,162], it is challenging to enable further accumulation of medium-chain fatty acids (MCFAs) in yeast, which makes it urgent to mitigate the toxicity for the cells. In **Paper III**, we evolved a native membrane efflux pump Tpo1 to increase its activity conferring cellular resistance against C10 fatty acid. Since the detailed mechanisms and structure information of Tpo1 are not known, we performed the random approach to engineer this protein.

Tpo1 was originally identified as a polyamide transporter that is located on the plasma membrane[163], and it was observed to benefit the resistance of yeast cells against diverse toxic compounds, such as decanoic acid[164]. To investigate the function of Tpo1 in yeast, we sought to cultivate the Tpo1 negative strain in medium with 50 mg/L of decanoic acid, which led to impaired growth in such toxic medium. Overexpression of Tpo1 from a single-copy plasmid under the control of the *TDH3* promoter restored the resistance against decanoic

acid (Fig. 17a). We then sought to use directed evolution to obtain a better Tpo1 variant which could enable the yeast cells to have improved tolerance against decanoic acid (Fig. 17b).

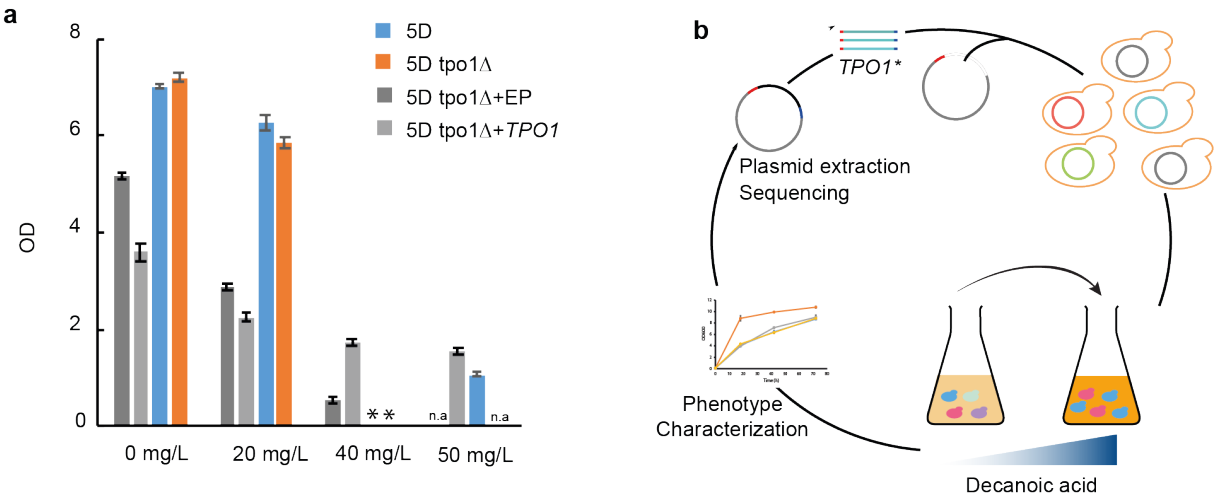


Figure 17. The design of Tpo1 transporter engineering. (a) The role of Tpo1 involved in cellular tolerance against decanoic acid. The CEN.PK 113-5D, 5D strain with Tpo1 deletion, Tpo1 deletion strains with empty plasmid (EP) or *TPO1* overexpression plasmid were tested in medium with different concentration of decanoic acid. The final OD600 was measured after 72 h in CSM media with decanoic acid as indicated. (b) The overview of the directed evolution process. Adapted from Paper III.

A random mutagenesis library was created through error-prone PCR with a library size of ~7.8 × 10^4 mutants, and three Tpo1 mutants (M11, M21 and M41) were selected after enrichment in medium with 60-90 mg/L of decanoic acid (Fig. 18 and see Supplementary Figure S7a in Paper III). The mutagenesis library for the second round of evolution was established based on the best variant M21 from the first round, then the best variant M49 with an improved activity was obtained from the screening with higher decanoic acid concentration (90-100 mg/L) (Fig. 18 and see Supplementary Figure S7b in Paper III).

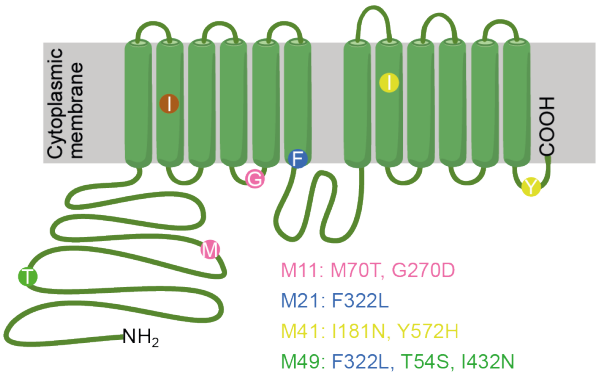


Figure 18. The positions of each mutation in Tpo1 variants M11, M21, M41 and M49. Adapted from Paper III.

The growth of yeast strains containing M49 (F322L, T54S, I432N) and M21 (F322L) was tested in medium with decanoic acid, showing that growth of both of the strains was significantly improved compared with the strain carrying the wild type Tpo1 (Fig. 19a). We then investigated the contribution of each mutation individually, the results also showed that mutation F322L conferred the major contribution to the observed phenotype (see Supplementary Figure S7c in Paper III). Normally, an improved tolerance could in turn benefit the production of the corresponding chemicals. Thus, we integrated the expression cassettes of wild type *TPO1* and the M49 mutant independently into chromosome of stain YHE02 with *POX1* and *TPO1* deleted to investigate if the evolved Tpo1 could improve the production of MCFAs, and the two strains were transformed with the MCFA production plasmid pScFAS28. The titer of extracellular fatty acids had significantly increased in the strain expressing M49, and each species of MCFAs, especially C10 to C14, was enhanced by 19% to 213% compared to the strain containing wild-type Tpo1 (Fig. 19b). We then introduced two copies of M49 into strain YHE02 to investigate if the production of MCFAs could be further improved. According to the results, the final titer of extracellular fatty acids reached 110 mg/L, and the titers of each MCFA species increased by 79-320% (Fig. 19b and Supplementary Figure S8a in Paper III). Owing to the poor understanding of these major facilitator superfamily (MFS) transporters, the functions of the altered amino acid residues were difficult to deduce. However, we successfully obtained a more efficient Tpo1 transporter to enhance the final titer of MCFAs in this work, which demonstrated that directed evolution-based transporter engineering could be a promising strategy to elevate the production of toxic chemicals resulting from improved cellular tolerance.

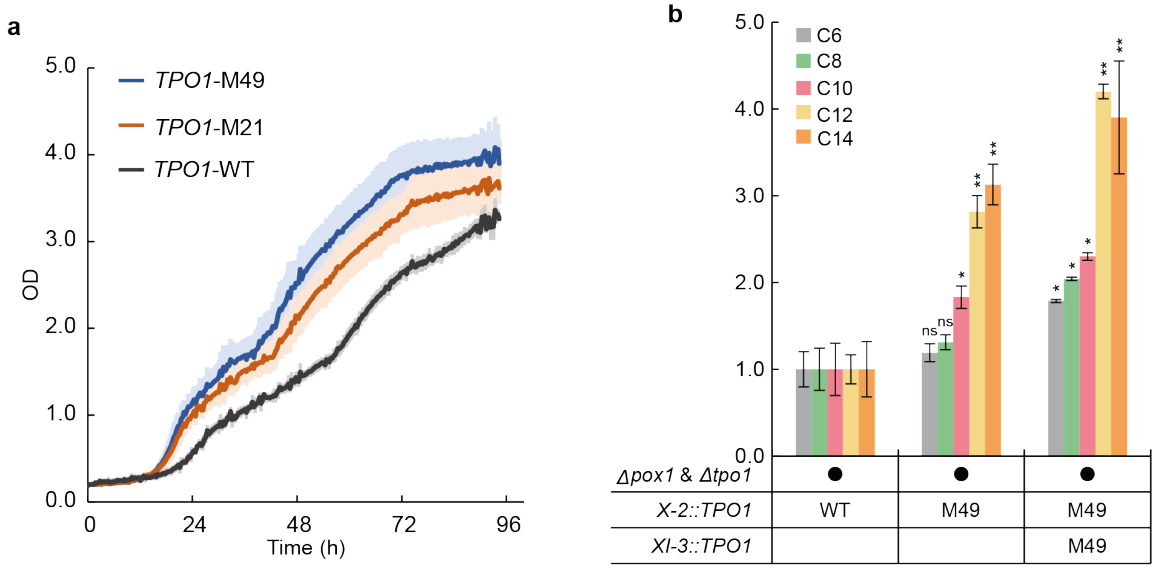


Figure 19. The effect of engineered Tpo1 on cellular tolerance and the production of MCFAs. (a)The growth curves of the strains YHE02 (*pox1Δ* & *tpo1Δ*) expressing Tpo1 variants M21, M49 and wild-type Tpo1 in CSM-URA medium with 100 mg/L decanoic acid. The cell densities were measured by Biolector. (b) The profile of MCFAs produced by strains containing wild-type Tpo1, one copy of M49 and two copies of M49 in the chromosomes. MCFA production plasmid pScFA28 was introduced as well. Adapted from Paper III.

In **Paper IV**, we focused on engineering a carboxylic acid reductase from *Mycobacterium marinum* (MmCAR), which is a key enzyme involved in fatty aldehyde production from fatty acids. Fatty aldehydes are the common precursors for different oleochemicals, such as fatty alcohols and alka(e)nes. The CAR enzyme consists of an adenylation domain (A domain) and a reductase domain (R domain) linked via a peptidyl carrier protein (PCP). The conformational dynamics of the CAR enzyme can be conferred with different nucleotide ligands, where the ATP-bound form represents the adenylation state, and AMP or AMP-acyl bound forms are always observed in the thiolation state[165]. In this work, in order to enable a selective medium-chain fatty alcohol (MCFOH) synthesis, we designed multiple strategies for multidimensional engineering of MmCAR, including a random approach via directed evolution, and structure-guided semi-rational design and rational approaches.

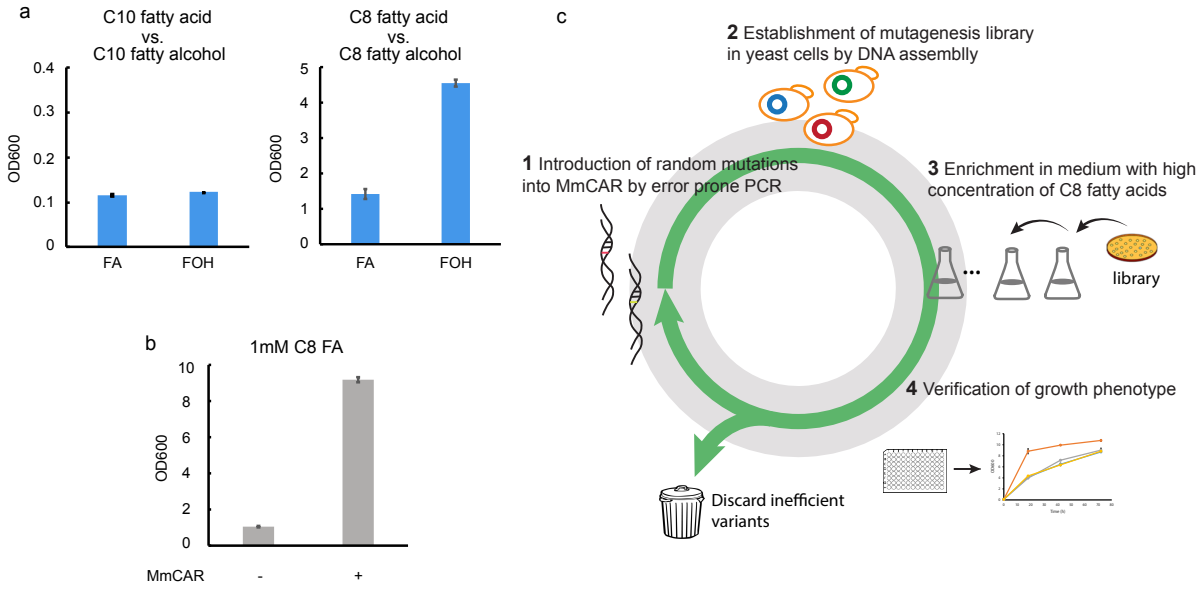


Figure 20. The design of a high-throughput screening approach for MmCAR variants. (a) Comparison of the cellular toxicity of MCFAs and the corresponding fatty alcohols. Strain YJZ03 (*Δpox1 Δhfd1*) was used to evaluate the toxicity by measuring the final OD600 of the strain cultivated in equivalent concentrations of MCFAs/MCFOHs for 72 h. (b) The final OD600 of strains with/without MmCAR after cultivation in 1mM C8 fatty acid (c) The workflow of the high-throughput growth-coupled enzyme engineering method. Adapted from Paper IV.

To improve the catalytic function of MmCAR for MCFA conversion, directed evolution was performed by first establishing a random mutagenesis library of the full-length *MmCAR*. Meanwhile, an efficient growth-coupled screening approach was created to assist the selection of MmCAR variants. As mentioned above, MCFAs are toxic to yeast cells, thus the toxicity could be utilized for the selection of enzyme variants related to their capacities of detoxification. We thus compared the toxicity of octanoic acid and decanoic acid and their corresponding fatty alcohols using strain YJZ03 with the deficiency of Pox1 and Hfd1. According to the results, equal toxicity to the cells was observed for the same concentration (70 mg/L) of C10 fatty acid and fatty alcohol, and distinct toxicity to the cells was observed

for the same concentration (150 mg/L) of C8 fatty acid and C8 fatty alcohol, where the C8 fatty acid showed much more toxicity to the cells than C8 fatty alcohol (Fig. 20a). Therefore, 1mM (150 mg/L) C8 fatty acid was used to investigate if the conversion from MCFAs to MCFOHs by MmCAR could be a potential detoxification pathway for MCFAs. The final OD600 of the strain containing MmCAR was much higher than the control strain in the presence of a high concentration of C8 fatty acid, suggesting that the expression of MmCAR benefits MCFA detoxification through formation of less toxic MCFOHs (Fig. 20b). Thus, a growth-coupled high-throughput screening scheme was established based on the detoxification of C8 fatty acid, where enzyme variants with a higher activity catalyzing the MCFA reduction can be selected (Fig. 20c).

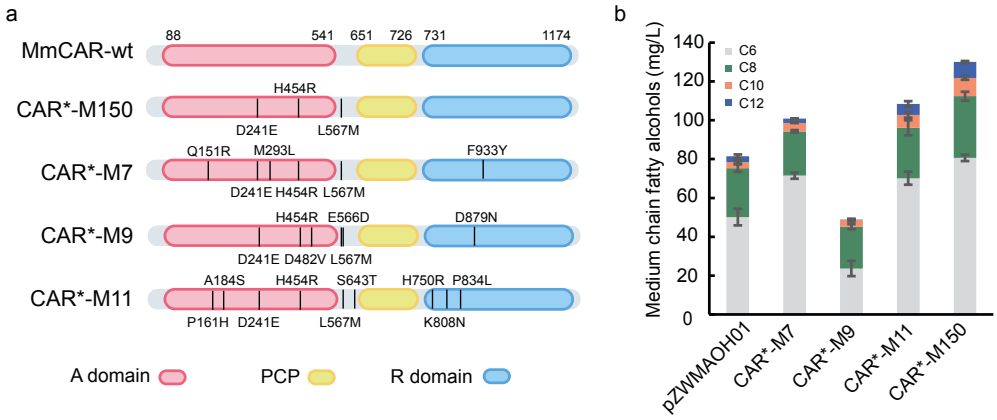


Figure 21. The synthesis of MCFOHs in *S. cerevisiae* expressing MmCAR variants. (a) Schematic illustration of the mutated MmCARs derived from two rounds of directed evolution based on the full-length MmCAR. (b) Production of MCFOHs *in S. cerevisiae* expressing mutated MmCARs derived from directed evolution based on full-length MmCAR. Adapted from Paper IV.

Using this efficient screening method, an increasing concentration of C8 FA from 290-330 mg/L was used to facilitate the variant selection for the first round of evolution based on random mutagenesis of the full-length *MmCAR*. The beneficial variant CAR*-M150 was isolated from the first round, whose expression enabled the best growth of yeast cells in medium with C8 fatty acids. In this mutant, two of the mutations D241E and H454R, were located in A domain of MmCAR, while the mutation L567M was located between A domain and PCP domain (Fig. 21a). Subsequently, the plasmid CAR*-M150 was extracted from YJZ03 and introduced into the high MCFA producing strain ZWE243 to test the production of MCFOHs. The results showed that CAR*-M150 enabled a titer of 120 mg/L MCFOHs, which was a more than 50% increase in MCFOH production compared with the wild type MmCAR (Fig. 21b). The CAR*-M150 was therefore used as the template to establish a mutagenesis library for the second round of evolution, during which a higher concentration of C8 FA (330–370 mg/L) was utilized for the library enrichment. From the second round of evolution, the variants CAR*-M7, CAR*-M9 and CAR*-M11 were selected as the most efficient mutants based on the cell growth. Their expression in strain ZWE243 also enabled the production of around 100 mg/L, 40 mg/L and 110 mg/L of MCFOHs, respectively (Fig. 21b). We identified

several beneficial mutants based on directed evolution of the full-length *MmCAR* that potentially enhanced the catalytic activity towards MCFAs. Although strains expressing the three variants could not produce as many MCFOHs as the strain expressing M150, two of the variants still showed higher catalytic activity towards MCFA conversion compared to the wild type as more MCFOHs were produced *in vivo* (Fig. 21b). Two of the mutated residues (D241E, H454R) from M150 appeared on the surface of the A domain rather than the active site, and the other one (L567M) located on the A_{sub} domain for which the conformation changes substantially between the adenylation and thiolation state, which indicated that these mutations affected the activity of MmCAR indirectly (see supplementary Figure S8).

Rational approach and semi-rational approach (Paper III & IV)

With the advances in acquiring protein structures or reliable models, biochemical data and computational methods, protein engineering is developing more and more from random approaches to data-driven rational or semi-rational design[166]. Rather than selecting from bigger libraries through repeated screening processes, many studies are moving beyond traditional directed evolution, instead advocating new strategies for designing smaller (Fig. 16), higher quality libraries[167]. In a rational or semi-rational approach, the focus on specific amino acid residues inferred from these reliable data significantly reduces the library size, which results in libraries with higher functional content (Fig. 16). Thus, less efforts and time have to be applied for screening of the library and this is especially advantageous if no high-throughput assay system is available. However, an in-depth understanding of the structural features of the active site and their functions is highly required for rational design (Fig. 16), which imposes restrictions on its general application[168,169]. On the other hand, semi-rational design, such as site saturation mutagenesis, could target multiple residues based on the *in silico* prediction and sequence information (Fig. 16), which can lead to the identification of the potentially crucial residues[170,171]. Sequence-guided enzyme redesign, performed by multiple sequence alignments (MSA), is the most popular strategy, which enables the exploration of amino acid conservation and evolutionary history among homologous protein sequences and structures[172]. Based on the sequence homology, it is beneficial for protein engineering in identifying functional hotspots, assessing local amino acid variability and guiding back-to-consensus design[167,172]. The development of protein structure databases and homology modeling offer valuable assistance for structure-based enzyme redesign. More specific residues located near the key active sites and domain interfaces could be identified to benefit an efficient superior library design. For example, Cahn and co-workers established a structure-guided, semi-rational strategy for reversing enzymatic nicotinamide cofactor specificity[173]. The efficacy of this strategy was demonstrated by inverting the cofactor specificity of four structurally diverse NADP-dependent enzymes towards NAD. Recently, computational enzyme redesign has become a promising strategy due to the development of *in silico* modeling. The advanced computational method can efficiently estimate the energetics of amino acid variation on the overall protein structure with the advantage of reducing experimental protein engineering to the evaluation of only a handful of rational

designs[167]. Chen and co-workers reported a computational, structure-based redesign to switch the substrate specificity of the phenylalanine adenylation domain in the nonribosomal peptide synthetase enzyme gramicidin S synthetase A[174]. Furthermore, a computational method was also applied to reconstruct a critical loop in human guanine deaminase to enable a 100-fold increase of enzyme activity towards ammelide[175]. Besides the random approaches mentioned above, also rational and semi-rational strategies were implemented in our work to enable an improved enzyme activity.

The fungal FASI (fFASI) was engineered by embedding thioesterases into the reaction chamber to release MCFAs in our previous study[60]. Due to the high similarity between bacterial FASI (bFASI) and fFASI, we assumed that the same engineering strategies could also be applied to bFASI for changing the chain length of fatty acid products. In **Paper III**, we performed rational design to both of a bFASI (from *Mycobacterium vaccae*, MvFAS) and fFASI (from *S. cerevisiae*, ScFAS), resulting high-level production of MCFAs through the co-expression of these FAS variants.

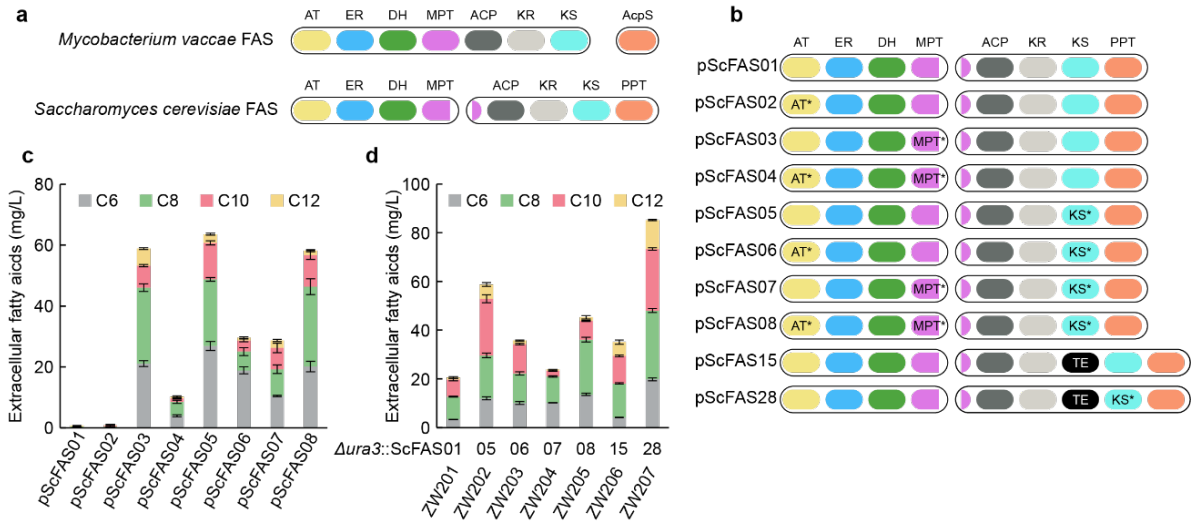


Figure 22. The synthesis of MCFAs in strains with engineered fFASI enzymes. (a) Overview of domain composition of the FASI enzymes from *S. cerevisiae* and *M. vaccae*. (b) Schematic overview of engineered fFASI. (c) The production of extracellular MCFAs by strains containing plasmids encoding engineered fFASI. (d) The production of extracellular MCFAs by strains with engineered fFASI genes integrated. Adapted from Paper III.

In the previous study, the incorporation of a heterologous short-chain thioesterase enabled the production of MCFAs[176]. We therefore evaluated different heterologous thioesterases in *S. cerevisiae* FAS (ScFAS15), among which the 'AcTesA from *Acinetobacter baylyi* was found to be the most efficient short-chain thioesterase (see Supplementary Figure S2 in Paper III). A mutated KS domain (equivalent to G1250S and M1251W in Fas2) was reported to confer steric restriction against long-chain acyl-ACP, and mutations in the AT and MPT domain (equivalent to I306A and R1834K in Fas1) could alter the substrate loading as well as acyl-CoA offloading efficiency[59,177]. Different combinations of mutated KS domain, AT domain and MPT domain were constructed based on wild type ScFAS (Fig. 22b). ScFAS05 only containing mutated KS was found to be the most efficient variant, in which the 'AcTesA thioesterase was introduced to generate ScFAS28 (Fig. 22c). The integration of ScFAS28 in ZW207

resulted in the production of 85 mg/L MCFAs, 45% higher than those produced by the strain with only the mutated KS domain, and 143% higher than those produced by ZW206 containing the FAS only with the ‘AcTesA thioesterase (Fig. 22d).

Different from fFASI, the type I FASs from the CMN (Corynebacteria, Mycobacteria and Nocardia) group of Actinobacteria are single-chain proteins with an auxiliary phosphopantetheinyl transferase (AcpS in the bacterial FAS system)[178]. As bFASI is highly similar to fFASI (Fig. 22a), and the expression of bFASI in yeast is not subjected to endogenous regulations[179], it would be interesting to introduce bFASI for MCFA production. In our work, a strategy equivalent to the one used for fFASI was applied to improve the activity of bFASI from *M. vaccae* towards production of MCFAs successfully. The co-expression of engineered bFASI and fFASI further increases in the production of MCFAs (see Figure 1h and i in Paper III).

In conclusion, the expression of two engineered FASI systems in yeast significantly facilitated the production of MCFAs, which also revealed that the engineering strategy for the FASI enzyme is applicable to variants from distant species.

For the downstream MCFOHs production, further rational and semi-rational redesign of MmCAR was conducted based on its distinct domains in **Paper IV**. As MmCAR possesses a broad substrate spectrum ranging from C4 to C18, which is suboptimal for MCFOH production, we sought to change the substrate specificity towards MCFAs through narrowing down the substrate binding pocket. The A domain of CAR exhibits a mechanism of substrate identification and activation similar to that of the ANL superfamily of adenylation enzymes, where the ATP is attacked by the oxygen from the carboxylic acid to generate an AMP-acyl phosphoester[180–182]. Enzymes from the superfamily of acyl-activating enzymes (AAEs), such as fatty acyl-AMP ligases (FAALs) and fatty acyl-CoA ligases (FACLs), are able to transfer the carboxylic groups of their substrates to other acceptor groups, suggesting that they share a function similar to the A domain of CAR[181,183]. A potentially crucial residue S306 (I303 in MmCAR) was identified after aligning the FAAL from *E. coli* (PDB id 3PBK) with the A domain of CAR from *Nocardia iowensis* (NiCAR PDB id 5MSD) (Fig. 23c). In a previous study, a bulk tryptophan was introduced to result in mutated proteins FAAL28 (I227W) and FACL13 (T214W) in the respective enzymes from *Mycobacterium tuberculosis* at the position equivalent to Q302 in MmCAR (I305 in NiCAR). The mutated enzymes had a significant increase in the activity towards C10 or C8 fatty acids, respectively, rather than longer acyl chains[183]. We thus replaced the amino acids of residues I303 and Q302 in MmCAR with a tryptophan. The variants containing I303W and Q302W enabled ∼130 mg/L and ∼110 mg/L of MCFOHs, respectively (Fig. 23a and b). According to the results, we speculated that the catalytic pocket of MmCAR was narrowed resulting from steric hindrance caused by the introduction of the bulkier tryptophan. Meanwhile, we also performed directed evolution towards the A domain of MmCAR coupled with the high-throughput screening method described above (Fig. 20c). After two rounds of evolution, CAR*-A69 from the first round and CAR*-A10 from the second round were isolated and expressed in high MCFA producing

strain ZWE243, both resulted in around 100 mg/L of MCFOHs (Fig. 23b). More C6 and C8 fatty alcohols were produced by the CAR*-A10 containing strain compared with CAR*-A69 strain. Four mutations (E176G, Q182R, Q371R, F501Y) were identified in the efficient variant CAR*-A10. However, all of them located on the surface of the A domain instead of the binding pocket. The distinct catalytic activities of the mutants derived from these approaches indicated that the rational design interfering with the binding pocket directly was potentially more efficient than the random approach in terms of altering substrate specificity.

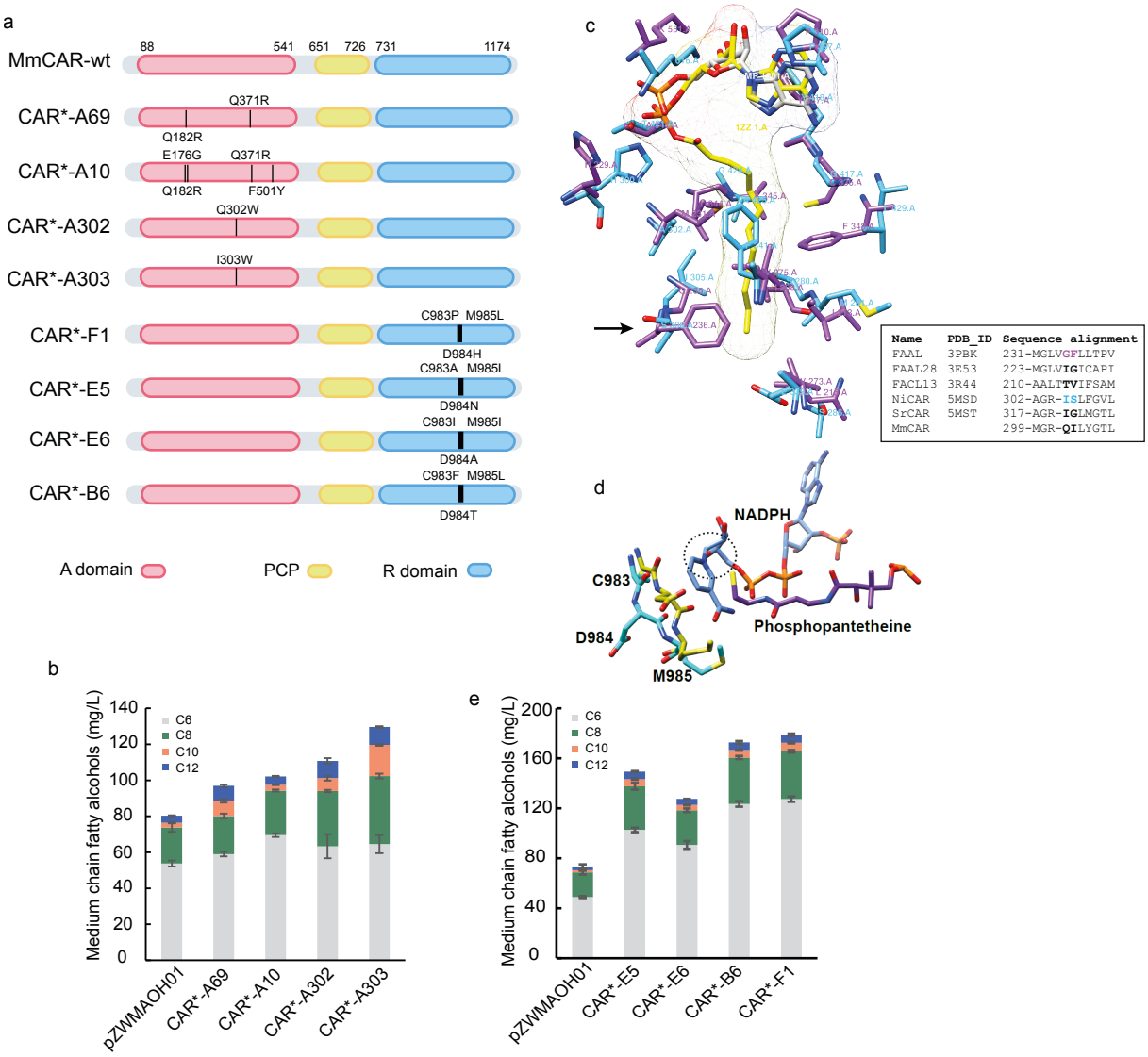


Figure 23. The synthesis of MCFOHs in *S. cerevisiae* by different MmCAR mutants. (a) Schematic overview of MmCAR mutants derived from different strategies. (b, e) Production of MCFOHs *in S. cerevisiae* by mutated MmCARs derived from adenylation domain modification (b) and reductase domain optimization (e). (c) Assignation of the amino acid residue related to the substrate chain length determination. And the alignment of sequences of FAALs and CARs are presented (d) The on- and off-states of the R domain determined by the conformational change of C983-M985. Adapted from Paper IV.

The R domain of MmCAR is where the thioester is reduced using NADPH; it enables a strict two-electron reduction of the acyl-PCP thioester to a fatty aldehyde. However, the backbone

reorientation of residues 983-985 was reported to affect the conformational equilibrium between on- and off-states of the MmCAR R domain towards a more efficient four-electron reduction[184]. The D984 will be positioned away from the nicotinamide in the active form, while it will locate with the nicotinamide binding pocket in the inactive form together with S983 (Fig. 23d). We introduced the mutations D984G and M985A into MmCAR individually with the aim to potentially change the final product to fatty alcohols as previously reported[184]. However, both mutants showed a negative effect on MCFOH production in yeast (see Supplementary Figure S3b and S3c in Paper IV). As our high-throughput screening approach was very effective for selection of enzyme variants from different mutagenesis libraries, in order to obtain an engineered R domain free of conformational regulation by C983-M985 or more efficient for reduction of medium-chain substrates, we applied this method for screening of a site-directed saturation mutagenesis library targeting residues 983-985 (Fig. 23a). Four variants (CAR*-F1/E5/E6/B6) that enabled a higher growth rate and final OD of yeast cells in the medium with 380 mg/L C8 FA were isolated and introduced into ZWE243 to investigate if they are also beneficial for MCFOH production (see Supplementary Figure S4 in Paper IV). Consistently, all these variants showed positive effects in MCFOH production, among which the CAR*-F1 (C983P, D984H, M985L) was the most efficient one that led to a final production of 170 mg/L MCFOHs (Fig. 22e). A common mutation (M985L or M985I) was found among these four MmCAR variants (Fig. 23a). However, after determining MCFOH production in a strain containing the individual mutation (CAR*-M985L), we found that the M985L mutant only led to a production of around 100 mg/L MCFOHs in yeast (see Supplementary Figure S5 in Paper IV). Meanwhile, the CAR*-F1w/o985 variant containing only the other mutations (C983P and D984H) from CAR*-F1 was also constructed and tested in strain ZWE243. The results showed an even slightly stronger effect on MCFOH production than for the CAR*-F1 mutant, indicating that these two mutations rather than M985L might play a more critical role in CAR*-F1 for MCFOHs production.

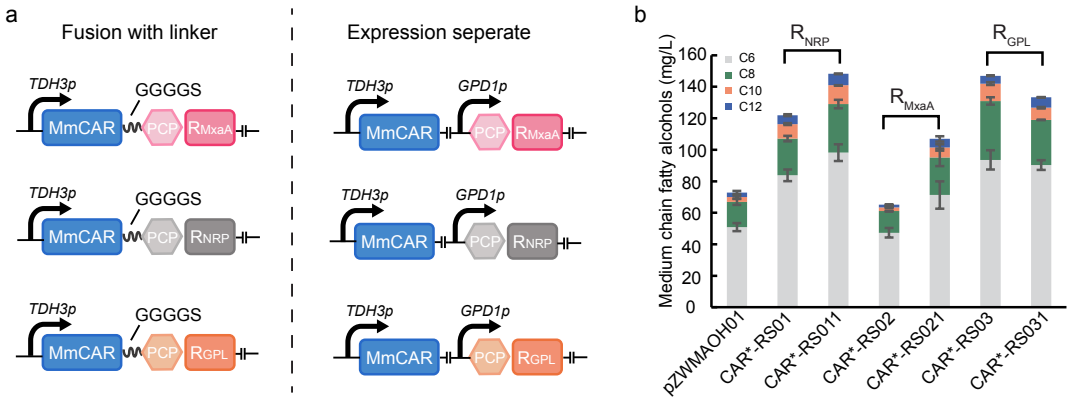


Figure 24. The synthesis of MCFOHs in *S. cerevisiae* by MmCAR chimeras with two PCP-R domains. (a) Schematic overview of MmCAR chimeras with two PCP-R domains are presented. CAR*-RS01, CAR*-RS02 and CAR*-RS03 are plasmids containing MmCAR separately co-expressed with R_{NRP}, R_{MxaA} and R_{GPL}, respectively. CAR*-RS011, CAR*-RS021 and CAR*-RS031 are plasmids containing MmCAR fused with R_{NRP}, R_{MxaA} and R_{GPL} using linker GGGGS, respectively (b) The production of MCFOHs in *S. cerevisiae* by MmCAR variants with di-domain. Adapted from Paper IV.

In a previous study, the comparison based on crystal structures between MmCAR and nonribosomal peptide synthetases (NRPSs) elucidated that they share a high similarity in the structures of the reductase domains[185,186]. We selected three reductase domains sharing high identity (50%) with the MmCAR R domain from a glycopeptidolipid (GPL) cluster of *Mycobacterium smegmatis* (R_{GPL}), an NRPS of *Mycobacterium tuberculosis* (R_{NRP}) and a terminal NRPS module (MxaA) of *Stimatella aurantiaca* Sga15 (R_{MxaA}) that had been reported to catalyze the 4-electron reduction to replace the native MmCAR R domain, respectively. All the chimeras, however, seem to have lost their function as no MCFOHs were detected in the yeast strains (data not shown). Instead, the heterologous PCP-R domains were introduced into yeast as shown in Figure 24a through either fusion with MmCAR using linker GGGGS or separate expression together with MmCAR in the cells to avoid misfolding of MmCAR. Surprisingly, most of the chimeras had positive effects on the MCFOHs production in yeast, of which the best variant CAR*-RS011 (MmCAR fused to the R_{NRP}) enabled around 150 mg/L of MCFOH production (Fig. 24b).

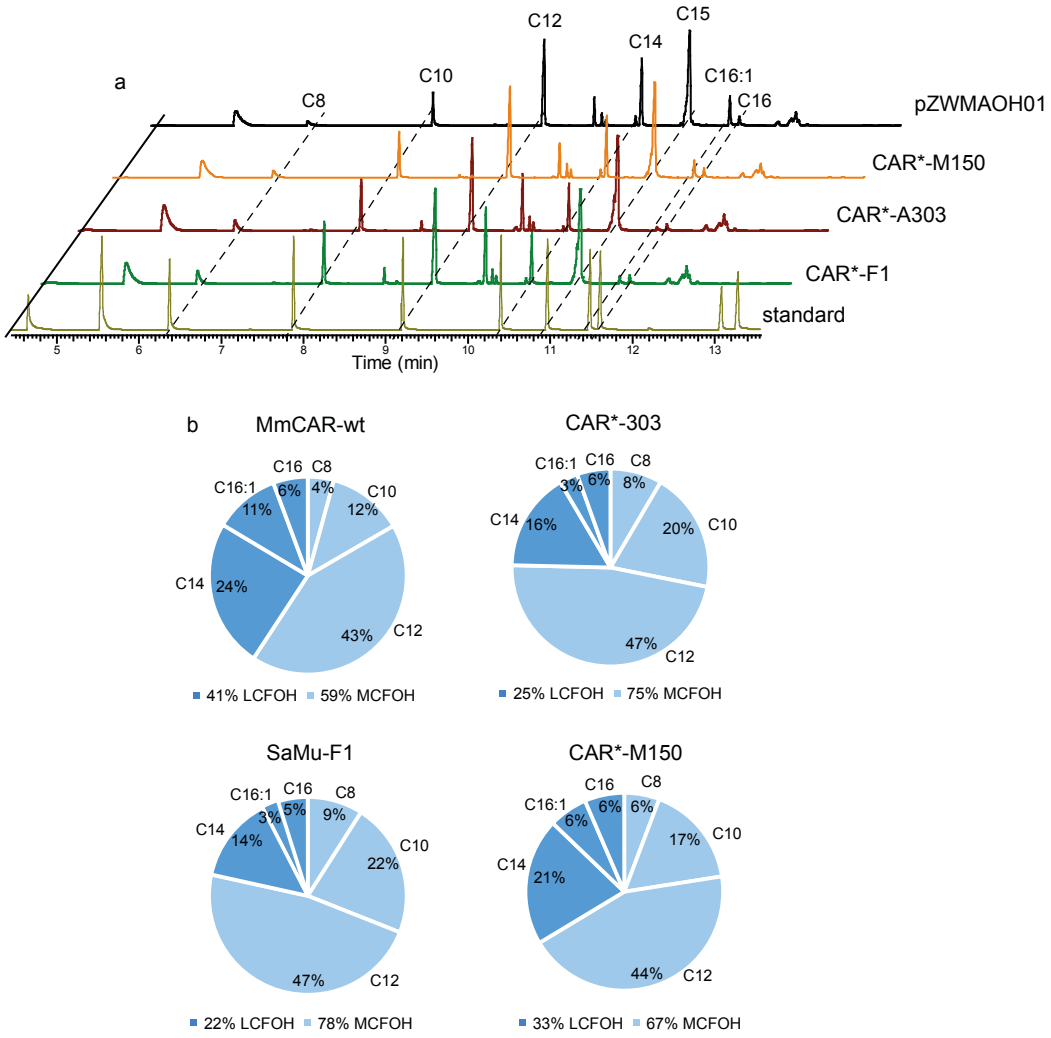


Figure 25. The substrate specificity of MmCAR mutants was characterized *in vivo*. (a) GC spectrum of intracellular fatty alcohols derived from strains ZWE243 with wild type CAR, CAR*-M150, CAR*-A303 and CAR*-F1, respectively. (b) Profiles of intracellular fatty alcohols in strain containing wild-type MmCAR, CAR*-M150, CAR*-A303 and CAR*-F1, respectively. Adapted from Paper IV.

Although the increase in production of MCFAs was achieved in those chimeras integrated with 4-electron R domains, it remained impossible to determine whether this enabled direct production of alcohols *in vivo*. In this part of the work, we have applied both rational and semi-rational design towards the R domain of MmCAR. The results elucidated that the smaller mutagenesis library from a semi-rational redesign led to a more efficient and direct effect on the desired function. Both of the approaches represent a less time-consuming and high efficiency manner compared with random approaches.

Besides assessing the production of total MCFOHs in yeast strains, we evaluated the substrate specificity of MmCAR mutants towards distinct chain-lengths of fatty acids *in vivo* and *in vitro* as well. We determined the intracellular fatty alcohols derived from strains containing the wild-type MmCAR, CAR*-A303, CAR*-M150 and CAR*-F1, respectively. Based on the GC spectra, we could clearly see that the major components of intracellular fatty alcohols were MCFOHs (C8-C12) and relatively less long chain fatty alcohols (LCFOHs, C14-C16) were detected (Fig. 25a). After comparing the ratio of each component, all the MmCAR variants enabled a higher proportion of MCFAs compared to the wild type. 75%, 78% and 67% of MCFOHs were produced by the strains containing CAR*-A303, CAR*-F1 and CAR*-M150, respectively, and the wild-type MmCAR had only 59% of MCFOHs (Fig. 25b). Furthermore, the activities for reducing fatty acids with different chain length were measured in crude protein extracts. According to the results, the activities towards C6-C10 fatty acids were improved in the presence of all the mutants compared to the wild type, except for the comparable activity of M150 on C10 fatty acids (Fig. 26). The I303W mutant seems the most efficient one that enabled about 2-fold increase in the activity towards C6 fatty acid compared to the wild type.

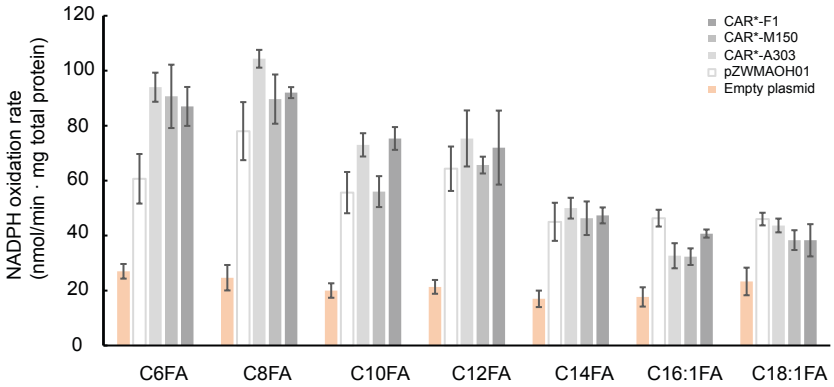


Figure 26. The substrate specificity of MmCAR mutants was characterized *in vitro*. The NADPH oxidation rate of the crude protein extracted from strain ZWE243 containing CAR*-F1, CAR*-M150, CAR*-A303, the wild-type MmCAR and an empty plasmid, respectively. Adapted from Paper IV.

The mutations evaluated above were combined based on I303W to achieve a further increase in MCFOH production. According to the results, the combination of CAR*-F1 and I303W (CAR*-F1+303) exhibited the strongest effect on MCFOH formation resulting in around 230 mg/L of MCFOH, 2.8-fold more MCFOHs than using the wild type MmCAR (Fig.

27a). The polyamine exporter Tpo1 had been reported to confer tolerance against MCFAs, and according to our previous work, an engineered Tpo1 was able to benefit the cellular tolerance against C10 and C8 fatty acid as well as the production of MCFAs. We therefore speculated that the deletion of it would potentially enable a further increase in MCFOH production through reducing the secretion of MCFAs. Thus, we deleted *TPO1* from yeast strain ZWE243, yielding YH28. Three plasmids pZWMAOH01, CAR*-A303, CAR*-F1+303 leading to distinct amounts of MCFOHs being produced were introduced into ZWE243 and YH28, respectively. The results showed that the improvement of MCFOH production was only observed in the Tpo1 deletion strain containing the most efficient variant CAR*-F1+303, for which 250 mg/L of MCFOHs were obtained with a 3-fold increase compared with the original strain (Fig. 27b). This suggested that the flux could only be redirected towards the MCFOH production by the variant with highest activity of MCFAs conversion in the *TPO1* deletion strain. Although the mechanistic details of how Tpo1 benefits the tolerance against MCFAs are still not fully clear, the possible mechanism inferred from our results in this work and previous work could be that Tpo1 might provide a channel for exporting the toxic substrates and the channel would be blocked in the absence of Tpo1.

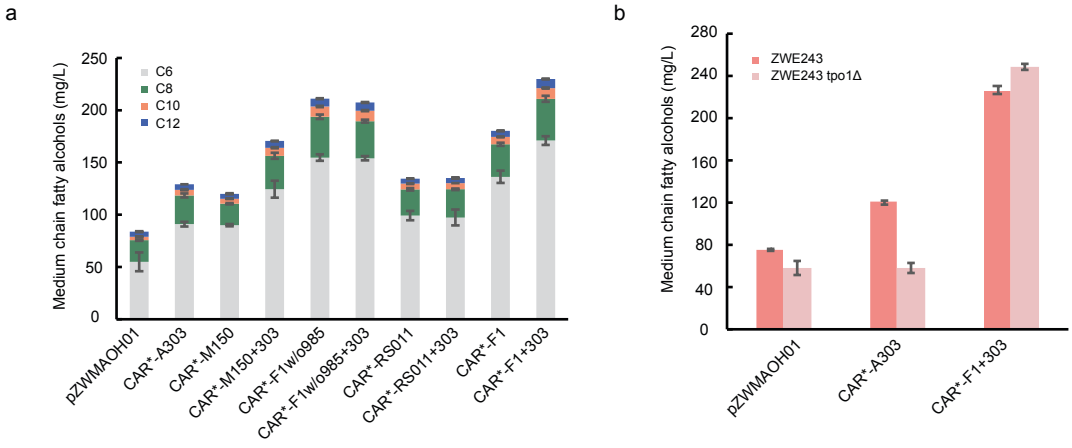


Figure 27. The synthesis of MCFOHs in strains with different combined mutants and *TPO1* deletion. (a) The production of MCFOHs in the ZWE243 strains containing plasmids pZWMAOH01, CAR*-M150, CAR*-A303, CAR*-M150+303, CAR*-F1w/o985, CAR*-F1w/o985+303, CAR*-RS011, CAR*-RS011+303, CAR*-F1 and CAR*-F1+303, respectively. (b) The effect of *TPO1* deletion on the MCFOH production. Adapted from Paper IV.

In conclusion, we implemented diverse protein engineering strategies to enable a significant improvement in the production of MCFAs and MCFOHs in *S. cerevisiae*. Here, protein engineering was applied to benefit the crucial catalytic reactions through optimizing the key enzymes as well as to improve product tolerance through modifying membrane transporters.

3.4 Adaptive laboratory evolution

Adaptive laboratory evolution (ALE) is a frequently used strategy for engineering microorganisms through accessing the basic mechanisms of molecular evolution and adaptive changes after long term selection under harsh growth conditions. Different from the protein engineering strategies mentioned above, ALE allows nonintuitive beneficial mutations to occur in diverse genes and regulatory regions in parallel[187,188]. Owing to the advances in next-generation sequencing technologies in recent years, ALE has been successfully applied to facilitate the establishment of production platforms for many organisms, such as *S. cerevisiae* and *E. coli*.

For instance, a high concentration of ethanol has been proven to result in cell wall instability and cell lysis[189,190]. Adaptation of a xylose-utilizing genetically engineered yeast strain to sugarcane bagasse hydrolysates improved multiple-inhibitor tolerance, while the ethanol yield in the adapted strain was improved 1.11-fold. Analysis of the evolved strain suggested a rearrangement in the composition of the cell wall[190,191]. A high concentration of organic acids, such as acetic acid, succinic acid and lactic acid, was observed to negatively affect the cell growth of yeast and *E. coli*[192–194]. A continuous culture was performed with a wild type *E. coli* strain in a gradually increasing concentration of succinate. The evolved strain was able to produce and sustain significantly higher titers of succinate (0.5 M) after the ALE. Reverse metabolic engineering was thus performed after identifying potentially beneficial mutations through DNA microarray analysis, and greater growth rates for the reverse engineered strains were observed under 0.592 M succinate stress conditions than for the wild-type[192]. In general, it would be beneficial for fermentations using yeast to conduct these at $\geq$ 40 °C in terms of increasing efficiency of the process and reducing costs. Therefore, ALE successfully facilitated the selection of yeast strains with improved growth and ethanol production at $\geq$ 40 °C[195]. Moreover, the underlying mechanism was revealed after genome sequencing, where a sterol composition change was found to contribute to this phenotype. In these successful examples, the potential of ALE in biotechnological engineering as a promising approach was highlighted. In our work, ALE was also implemented to improve the cell fitness against octanoic acid in order to benefit MCFA production in yeast.

In **Paper III**, the cellular resistance against decanoic acid was successfully improved through engineering the Tpo1 transporter, which was demonstrated to be beneficial for MCFA production. Therefore, we assumed that an improved cell fitness against these toxic chemicals could also be achieved by ALE. The ALE experiment was implemented to achieve improved MCFA tolerance through cultivation of a yeast strain in medium with octanoic acid. Thus, YJZ02 (*Δpox1*) as the parent strain was used for ALE, which has the advantage of preventing cells acquiring improved tolerance through an increased MCFA degradation activity. In order to perform ALE, cells were continuously transferred to fresh medium with octanoic acid for more than 100 generations (Fig. 28). We harvested the evolved strains with

significantly improved tolerance against octanoic acid from different stages during the evolution process, and seven protein-encoding genes were found having mutations after sequencing the genomes of three evolved strains (Fig. 28).

A M1I mutation at the start codon of *PDR1* that encodes a transcription factor regulating multidrug resistance genes was identified in ZWE01.29, and this mutation was passed down to the later isolated clones (Fig. 28, see Supplementary Figure S10 in Paper III). One nonsense mutation Y1142* was discovered in the pleiotropic ABC efflux transporter Pdr5 that is regulated by Pdr1 in ZWE03. A similar phenotype of conferring cellular tolerance against octanoic acid was enabled by the mutated *PDR1* allele (Pdr1, M1I) and other alleles (Pdr1, M1*; Pdr1, ΔN181; and *Δpdr1*), demonstrating that the mutated Pdr1 (Pdr1, M1I) was a null mutant (see Supplementary Figure S11 in Paper III). Osh2 has been reported to transport sterols between organelle membranes[196]. A frameshift mutation and a substitution mutation D1206Y in Osh2 were found in ZWE01.8 and ZWE01.29 of the ZWE01 population, respectively (Fig 28, see Supplementary Figure S10 in Paper III). According to the structure analysis, these two mutations in Osh2 might result in the loss-of-function of Osh2 (see Supplementary Figure S12 in Paper III).

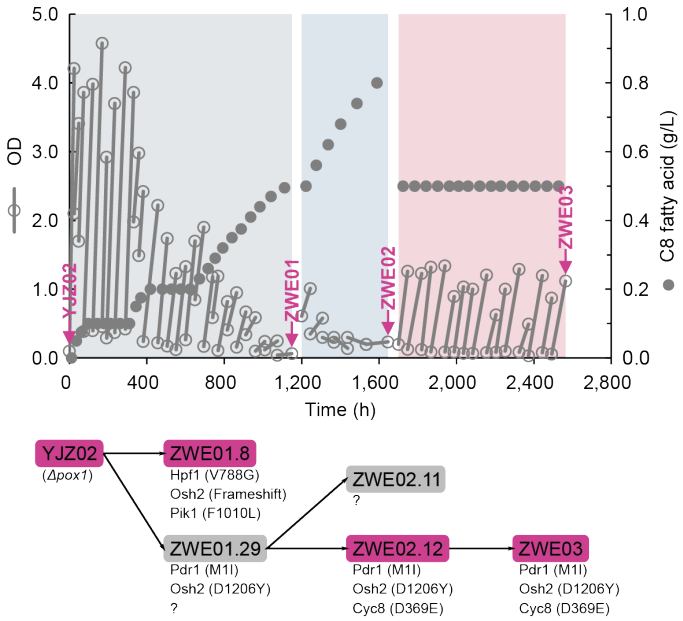


Figure 28. The ALE procedure for obtaining octanoic acid resistant strains.

To investigate if the deletion of both *OSH2* and *PDR1* in the original strain could lead to improved resistance to octanoic acid as well a comparison of cell growth in 400 mg/L octanoic acid was conducted for different strains. The results showed that the deletion of both *OSH2* and *PDR1* in strain YJZ02 had an effect similar to the final evolved strain ZWE03 on the level of resistance against octanoic acid (Fig. 29).

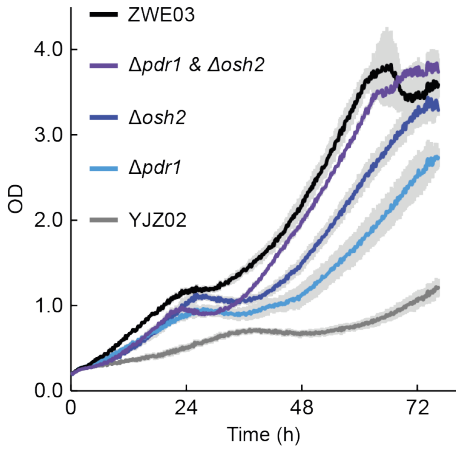


Figure 29. Growth comparison of evolved strain, parental strain and strains with *PDR1* and/or *OSH2* deletion.

Afterwards, the plasmid ScFAS28 was introduced into the evolved strains to evaluate the performance concerning MCFA production, and we found that they were able to produce more MCFAs than the parent strain, an improvement that ranged from 25-217% for each individual product (Fig. 30).

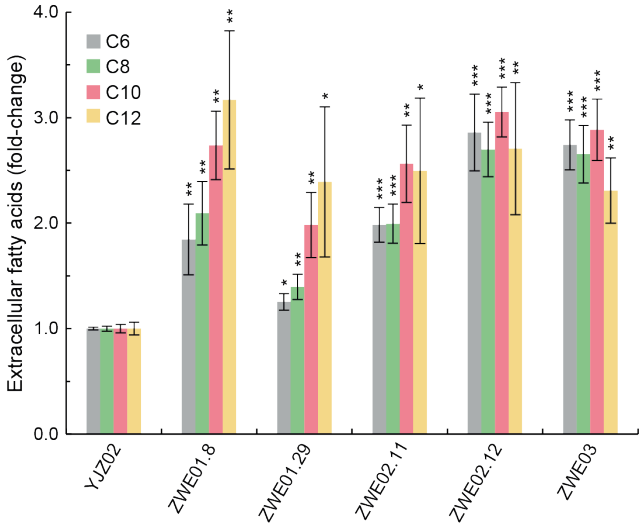


Figure 30. The synthesis of MCFAs from evolved strains compared to those from parental strain YJZ02.

Taken together, engineering efforts including protein engineering, pathway optimization and cellular fitness improvement were applied to dramatically improve the production of MCFAs. These versatile strategies could also be used to benefit the production of other toxic chemicals.

4 Summary, conclusion and perspectives

In past decades, the advances in biotechnology have offered great opportunities to expand the diversity of chemicals produced by microbes and enabled efficient microbial cell factories. The specific demands on titer, rate and yield are essential for the development from a proof-of-concept strain to a cell factory that could be utilized for industrial production. This is a time-consuming and most challenging road that requires numerous modifications and optimizations in metabolic pathways, enzymes of interest and the fermentation process. Combining diverse engineering strategies, free fatty acids have been produced at high titers of 33.4 g/L[57], providing a potential precursor pool for further formation of valuable chemicals. Compared with free fatty acids, fatty acid derivatives possess wider applications such as biofuels and biomaterials[197,198]. As described above, our work focused on the improved production of several fatty acid derivatives in *S. cerevisiae*, such as fatty alcohols, MCFAs and MCFOHs, through multidimensional engineering approaches.

The low viability of cells and the complicated extraction process are major obstacles for industrial fatty alcohol production. In order to solve these problems, we attempted to identify a transporter able to functionally export fatty alcohols from the yeast *S. cerevisiae*. Thus, in **Paper I**, mammalian transporter FATP1 was found in yeast to promote the export of fatty alcohols in yeast. The expression of FATP1 resulted an increased cell fitness and 4.5-fold higher extracellular fatty alcohols compared with the control strain. FATP1 was reported as a membrane-bound acyl-CoA synthetase specific to very long chain fatty acids, and involved in uptake of long chain fatty acids[199,200]. Further efforts towards functional domain identification were made through domain exchange between FATP1 and FATP4. In this part of the work, we highlight the possibility that transporter engineering could be a promising strategy to enable the successful commercialization of biofuel production. Transporter engineering was further applied in our next work.

In **Paper II**, the yeast *S. cerevisiae* was engineered to enable a relatively high production of 1-alkenes through dynamically regulating their synthesis and transporter engineering. PfUndB

from *P. fluorescens* Pf-5 showed high enzyme activity towards 1-alkene formation and was found to locate to the membrane. Dynamic control of PfUndB was performed to relieve the toxicity of expressing the membrane protein, together with cofactor engineering by expressing the NADH-dependent Pdx/Pdr system, yielding a 4-fold increase in 1-alkene production compared with the control strain. FATP1 was found to facilitate the export of 1-alkenes as well in this study; its expression contributed to a 40% increase in extracellular 1-alkene production. Different from the performance in the fatty alcohol producing strain, FATP1 only had a marginal effect on the cell growth. This suggested that the toxicity of 1-alkenes is not the bottleneck at the current stage in contract to fatty alcohols. The low catalytic activity of the key enzymes involved in 1-alkene formation is rather the major problem that demands prompt solution.

The optimization of a type I FAS in a previous study enabled the selective synthesis of MCFAs in yeast[59,60]. In **Paper III**, both the endogenous FAS and an orthogonal bacterial type I FAS were engineered for MCFA production in *S. cerevisiae*. Owing to the development of engineering strategies, the titers of MCFAs currently produced by engineered yeast cells have already approached the inhibitory concentration of MCFAs[105,201,202]. Thus, directed evolution of the transporter Tpo1 and adaptive laboratory evolution were implemented to increase cellular tolerance against toxic MCFAs with the consequence of 2.3- and 2.7- fold improvement in MCFA production, respectively. The mutants that conferred resistance to MCFAs during ALE were further characterized after performing genome sequencing of three evolved strains. However, due to the poor understanding of the Tpo1 transporter, it was difficult to deduce the possible mechanism of the altered amino acid residues. We further enlarged the precursor supply, which combined with these modifications, resulted in a final production of $>$1 g/L MCFAs and the highest yield (0.053 g/g glucose) reported in yeast so far. This high MCFA production platform strain could be considered as the provider of a precursor pool for the production of a range of MCFA-derived chemicals.

Based on our work described above, we further engineered MmCAR from *Mycobacterium marinum* for selective synthesis of MCFOHs in yeast through multiple protein engineering strategies in **Paper IV**. Based on high-throughput screening coupled with cell growth, a random mutagenesis approach was conducted based on the full-length MmCAR, and 50% more MCFOHs were produced by the best variant. Both a random mutagenesis approach and rational design based on its structure information were performed to the A domain of MmCAR, which successfully changed the substrate specificity to MCFAs, and the best mutation I303W resulted in a 1.6-fold increase in MCFOH production. The R domain modification through the semi-rational design toward residues 983-985 turned out to be the most efficient strategy that increased the final titer of MCFOHs 2.1-fold compared with the control strain. In combination with these efficient mutations, a final 3-fold increase in MCFOHs was achieved with the additional deletion of transporter Tpo1. In this part of work, the engineered MmCAR offered abundant production of MCFOHs and aldehydes as the versatile intermediates that enable broad applications for further formation of alka(e)nes

and FAEEs. However, the impaired growth caused by the toxicity of MCFOHs still is a major obstacle for future industrial production like MCFAs. Thus, the improvement of cellular tolerance against MCFOHs through fermentation process optimization and transporter engineering would be feasible strategies to obtain a further increase in MCFOH production.

We could clearly demonstrate that transporter engineering plays a very important role for the improved production of oleochemicals, especially of toxic chemicals that might cause impaired cell growth. In our work, the expression of a heterologous transporter has been proven to significantly benefit the export of fatty alcohols and alkenes, which would be of great commercial interest in reducing the cost of the final product extraction. However, the expression of heterologous transporters for improved production and secretion has not been widely studied due to many reasons. The poor understanding of transporters is one of the main reasons, as the substrates of many transporters have not been fully identified and the transporters for many products of commercial interests are still unknown. In addition, the difficulties of functionally expressing heterologous transporters could be caused by the discrepancy of membrane structures between different species and the negative effects on cell viability. We therefore brought up an alternative approach in our study, an improved activity of native transporter Tpo1 was achieved through protein engineering to benefit the cellular tolerance against toxic chemicals, resulting an improved production of MCFAs. This strategy could perfectly circumvent the necessity of functionally expressing a heterologous transporter, but it requires that the native one should ideally already possess some basic activity towards the substrates of interest. The protein engineering approach was also highlighted in our work where the key enzyme MmCAR involved in fatty alcohol formation was engineered to enable a changed specificity towards MCFAs. However, the application of this strategy is still limited by our knowledge of structural information and the screening method. The development in the accuracy of structural prediction based on computational structure modeling would play a very important role for further experimental investigation. Ultra-high-throughput screening method that could be achieved by the advances in instrumentation of reading and analysis would also confer critical contributions in future applications. Moreover, practical protein engineering methods towards non-typical proteins that have no structural information and screening method available would be a future direction to be developed along with the technical advances.

As mentioned, it is still challenging to meet the commercial requirements for the microbial production of fatty acid derivatives. The cellular toxicity caused by the accumulation of these chemicals is one of the major problems for large-scale industrial production. Transporter engineering, adaptive evolution and fermentation process optimization need to be further developed to improve the cellular tolerance and product secretion. Moreover, in many cases, the relatively low activity of the key enzymes is still the major obstacle, such as for alka(e)ne production in yeast. Besides achieving better performance by protein engineering towards the target enzymes, the development of high-throughput methods would also allow for rapid selection among large amounts of enzyme candidates. In addition, as the

oleochemicals possess relatively low value, increasing the utilization of carbon sources by changing to single carbon feedstocks, such as CO_2 and methanol, is also another feasible strategy to obtain high TRY metrics that needs to be extensively studied. The progress of metabolic engineering benefits from great developments in the field of biotechnology, for example, the CRISPR technology has significantly shortened the time of genetic manipulation in microbes. However, based on the "Design-Build-Test-Learn" (DBTL) cycle[203], learning is probably the most challenging part that requires more novel analysis tools to get new insight into optimization of microbes and proteins of interest. The development of next-generation sequencing in omics analysis and machine-learning techniques could provide a deeper understanding about the regulation and interaction of metabolites and proteins inside the cell, which would benefit the exploitation of new engineering targets.

In conclusion, this thesis clearly indicates that the engineered yeast cells provide great opportunities to produce diverse chemicals. Besides the traditional metabolic pathway engineering, multidimensional engineering at the protein level and the cellular level leading to an improved tolerance against toxic chemicals will also play crucial roles in enhancing the TRY metrics in yeast.

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