THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Metabolic engineering of the yeast Saccharomyces cerevisiae to enable biosynthesis of long-chain alkanes and olefins

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Cover:
Elimination of fatty aldehyde dehydrogenase Hfd1 and expression of a ferredoxin reducing system is required to enable alkane biosynthesis by the yeast Saccharomyces cerevisiae.

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Preface

This dissertation serves as a partial fulfilment of the requirement to obtain the degree of doctor of philosophy at the Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden. The research project described in this thesis was carried out in the Systems Biology group under the supervision of Professor Jens Nielsen and cosupervisor Dr. Verena Siewers. In this project, the yeast *Sacharomyces cerevisiae* has been metabolically engineered to enable the heterologous biosynthesis of alkanes and olefins. The project has been funded by FORMAS, Vetenskapsrådet and the European Research Council (grant no. 247013).

Nicolaas A.A. Buijs, May 2016

VLADIMIR: A - . What are you insinuating? That we've come to the wrong place?

From: *Waiting for Godot* by Samuel Beckett

Metabolic engineering of the yeast *Saccharomyces cerevisiae* to enable biosynthesis of long-chain alkanes and olefins

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Abstract

Industrial-scale production of renewable transportation biofuels has been developed as an alternative to fossil fuels. During the last decade, ethanol has become the most prominent biofuel and yeast has been the production organism of choice. However, ethanol has a lower energy content then fossil fuel derived diesel and kerosine. Therefore it is less applicable to heavy-duty and maritime transportation as well as aviation. Therefore, new types of more advanced biofuels are being developed. These advanced biofuels can ideally be used as drop-in fuels and substitute gasoline, diesel, and kerosine. A specific type of advanced biofuels are alkanes. Alkanes can potentially be manufactured in a renewable fashion through microbial biosynthesis. Thus far, examples of microbial synthesis of alkanes have been mostly limited to (cyano)bacteria. This is remarkable because yeast has been the production organism of choice for industrial biofuel production.

This thesis addresses this knowledge gap and describes introduction of a heterologous metabolic pathway for the biosynthesis of long-chain alkanes by the yeast *Saccharomyces cerevisiae*. We show that efficient synthesis is obstructed by parts of endogenous lipid and aldehyde metabolism. Elimination of the fatty aldehyde consuming hexadecenal dehydrogenase Hfd1 proved essential. Furthermore, expression of a redox system is shown to be required for alkane biosynthesis in yeast. Deletion of *HFD1* together with expression of an alkane biosynthesis pathway resulted in the production of the alkanes pentadecane, and heptadecane. Furthermore, we evaluated a compartmentalization strategy to improve alkane biosynthesis, and extended this strategy towards an alternative pathway leading to the synthesis of olefins.

In conclusion, this thesis provides a proof of principle for biosynthesis of long-chain alkanes in the industrial workhorse *S. cerevisiae*. We anticipate that these findings will be important for further engineering of the alkane biosynthesis pathway, and important for yeast engineering to enable industrial production of alkane based drop-in biofuels. Such a development could allow the biofuel industry to diversify beyond bioethanol.

Key words: biofuels, advanced biofuels, yeast, metabolic engineering, Saccharomyces cerevisiae, fatty acids, fatty acid derived biofuels, alkanes, alkenes, olefins.

Acknowledgements

The first paper in this thesis describes how I managed to make a yeast produce a very small amount of alkanes. For some, it might be important for moving to a sustainable future, or not. For me, the detection of that first alkane peak will remain connected to the loss of my father. At Landvetter airport I noticed the first alkane peaks and without them that day the project would have been over. It was July 7, 2013 and that Sunday I was going home to be there during my father's last two weeks. To stand next to him during his final moments.

My father is the person who I owe my gratitude most to. He nearly passed away when I was twelve, and in the years after that he dedicated himself to my brother, sister, and me. Despite his suffering he was always there for me and he had an understanding of me that is hard to replace. Without him I would never have moved up from mavo to vwo, and thus be able to pursue a PhD years later. I am grateful that I still got to spent so many more years, for the lessons he taught me, and I owed it to him to finish this thesis.

That Sunday, upon arrival home I could at least tell my family that the problem I was struggling with had been solved. The problem that had something to do with a fungus, sugar cubes, and diesel. I couldn't care less though. From then on things could move forward and the future would be amazing, I remember myself saying that. However, perhaps not surprisingly, the dark ages of my PhD were yet to come. It was bad.

The fact that the center did hold I owe that to some very great people. First of all, my girlfriend Leonie, my sister Laury, my brother Hein and his girlfriend Yvonne, and my close friends Maarten, and Ramon. Leonie had to endure what might have seemed a never ending story, and also had to tolerate me writing when I was actually coming to visit her in Göteborg. The person that served as my guide and mentor was Rahul. Rahul and I have had many long (scientific) discussions, lost illusions, and enjoyed many concerts and operas. Over the years we became close friends and I am very thankful for that. Ximena was like a mother to me, and I am grateful to have met such a dedicated and kind research engineer and woman. My gratitude is also to Peter, who has been a good friend to me. I also would like to thank Marieke and her parents Marja and Ton. It did not end as I hoped for and I bear part of the blame, but nevertheless they were there for me. Special thanks should go to Margreet and Trudy, who went out of their way to help my family during the last year of my father's life. It does not happen very often that one meets people like them and I am not sure if I have told them that often enough.

I would like to thank Prof. Jens Nielsen for giving me the opportunity to come to Sweden. He has created an environment in his lab in which there are no limits. That really helped me to develop as a person and a scientist. I am thankful for his patience with my sometimes harsh comments, optimism, and that he was always willing to find time in his schedule to discuss things. Verena, my cosupervisor, I could always walk into her office to ask for help and advice. Florian and I, we worked on a research project and founded a company together. I enjoyed our discussions and it was a nice experience. My gratitude is also to Kuk-ki and Luis who helped me find my way as a new PhD student.

The FORMAS collaboration with Prof. Mathias Uhlén and Paul Hudson's group at KTH came at a pivotal time in my project. It is no understatement that the discussions with Paul and Heribert helped to push the project past its nadir. I enjoyed the discussions with Danuta, Ivana, Josefine, and Håkan. Furthermore, I am grateful for the fruitful discussions with visiting Prof. Brian Pfleger, and the discussions with my opponent Prof. Jack Pronk. I would like to thank

Prof. Jerker Mårtensson, who has been very patient with me as a student and helped me understand a little bit of organic synthesis. My gratitude is also to Prof. Ivan Mijakovic, Prof. Christer Larsson, and Associate Professor Dina Petranovic for their helpful suggestions during discussions of my project. I am also indebted to André Kelkkanen and Bo Norrman from CIPRA/Chalmers Innovation, who taught me about the business side of science and spent a lot of time helping us to get funding for the startup.

The SysBio group is a place were people respect each other, strive to publish quality research, and share. Yongjin is the person from that group from whom I learned several things. We worked together over several years. During these years you taught me various yeast genetic engineering skills. I also valued our many (heated) scientific discussions. I was also lucky to have supervised three talented students: Anne-Sofie, Diego, and Elsa. Thank you for the hard work, valuable lessons, and new insights that you provided with. Furthermore, I am happy to have shared an office with Jichen, who was always willing to listen to the latest news about an eagle owl in Holland. Also my former office mates Ana Joyce, Lifang, Marta, Bouke, Christoph, Natapol, and Pramote made going to Chalmers a pleasure. My gratitude is also to the people from the small lab in the big lab who made it a nice place to work there: Nina, Ana Joyce, Tatiana, Xin, Min, Lei, Yating, Aida, Hülya, Eugenio, Magnus, Abder, Payam, António, Zongjie, Yongjin, Wendel, Martin E, Ed, and Andreas. After work it was nice to focus on something else, and I would like to thank everyone who joined the many afternoons of playing basketball at Olofshöjd.

I owe my gratitude to the many people who taught me new skills along the way. There have been more people helping me than I can remember, but to give it a try: Sakda, Suwanee, Pramote, Kanokarn, Mikael (Kovalent), Michi, and Julia educated me on the analysis part of the project; Lilli and Nils-Gunnar saved the days when our equipment was down and we also had nice discussions; Xin helped me with nile red staining and fluorescence microscopy; José Luis, Mark, Andreas, and Stefan helped with setting up fed-batch fermentations (not those in the paper); José Luis, Lifang, and Kuk-ki supported me with protein purification and analysis; Eugenio and Ana Joyce with mitochondrial staining and fluorescence microscopy; Eugenio also taught me how to isolate RNA and do RT-PCR; Maurizio was very helpful in teaching how to deal with the Bioscreen; Tobias, Saeed and Rasmus were of great help getting started with modelling; Adil on how to apply for funding and how to get papers published; Yiming, Lei, Clara, Ming-Tao, Siavash, Zongjie, and Guodong for helping finding the shortest path to yeast transformants and making it an enjoyable experience; Jichen showed me how to make – by far– the best *E. coli* competent cells; Juan shared his deletion strains with me; Zongjie probably switched to *HIS* marker because I used up all his *URA* template; and I had many nice discussions with Alexandra, Min, Zongjie, Frederik, Joakim, Yun, Ming-Tao, Andreas, Zhiwei, Yongjun, Amir, Payam, Petri, Ed, Zheng, Ibrahim, Antonio M, Jorge, Paulo, Rui, Martin (E), Florian and Jiufu. I also enjoyed working with Ausra, Valeria, Jenny V, Peter, Ruifei, George, Charis, Niklas, Zhongpeng, David M, Kalle, Hampus, and Christian and the others from the IndBio group. Last but not least, I owe my gratitude to my savior in the lab Il-Kwon, who always had a stock of everything and was always willing to help and offer good advice.

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Before moving to Sweden from Holland, Anna, Robert, and Alma were very kind and helpful in helping me find accommodation in Göteborg. I also would like to thank Professor Sten Ljungström and his wife for their hospitality.

In conclusion, this thesis might suggest it was all me who did it but I owe it to the help and support of many others. Some I probably have forgotten to mention.

List of publications

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

Paper I. Long-chain alkane production by the yeast *Saccharomyces cerevisiae*.
Buijs NA[*], Zhou YJ[*], Siewers V, Nielsen J..
Biotechnol. Bioeng. 2015, **112**, 1275–9.

Paper II. Harnessing peroxisomes for production of fatty acid derived chemicals and fuels by yeast.
Zhou YJ[*], Buijs N[*], Zhu Z, Orol Gómez D, Boonsombuti A, Siewers V, Nielsen J

Paper III. The role of biofuels in the future energy supply.
Caspeta L, Buijs NA, Nielsen J
Energy Environ. Sci., 2013, **6**, 1077-1082.

Paper IV. Advanced biofuel production by the yeast *Saccharomyces cerevisiae*.
Buijs NA, Siewers V, Nielsen J.
Curr. Opin. Chem. Biol. 2013, **17**, 480–8. (review)

[*]= equal contribution

Contribution to manuscripts:

Paper I. Designed experiments; performed experiments; analyzed the data; wrote the manuscript.

Paper II. Assisted in designing of research; performed olefin related experiments; analyzed olefin related data; assisted in manuscript preparation.

Paper III. Performed part of literature study; assisted in manuscript preparation.

Paper IV. Performed literature study; wrote the manuscript.

List of abbreviations

Abbreviation:	Definition:
AAR	(Fatty) Acyl-CoA/ACP reductase (aldehyde forming)
ACC	Acetyl-CoA carboxylase
αDOX	α-dioxygenase
FA	Fatty acid
FaCoAR	Fatty acyl-CoA reductase (alcohol forming)
FACS	Fatty acyl-CoA synthase
FADO	Fatty aldehyde deformylating oxygenase
FAld	Fatty aldehyde
FaOH	Fatty alcohol
FAR	Fatty acyl-CoA/ACP reductase (aldehyde forming)
FAS	Fatty acid synthase
FD	Ferredoxin
FFA	Free fatty acid
FNR	Ferredoxin NAD(P)H reductase
F/FNR	Ferredoxin and ferredoxin NAD(P)H reductase
Fld	Flavodoxin
Fld/FNR	Flavodoxin and ferredoxin/flavodoxin NAD(P)H reductase
MmCAR	*Mycobacterium marinum* carboxylic acid reductase

Gene and protein nomenclature

The *Saccharomyces cerevisiae* Genetic Nomenclature Guide (1998) for genes and proteins has been followed in this thesis. In short, wild-type yeast gene names are denoted in all-caps and italic as *GEN1*, and consist of three letters followed by up to three numbers. The corresponding protein derived from, for example, *GEN1* are written as Gene1. Non-italic, and starting with a capital letter followed by small caps letters and ending with the numbers.

A deviation from the nomenclature guide is that in this thesis deletions of wild-type genes are denoted in italic by Δ*GENE1* or as *GENE1*-.

Heterologous proteins that are expressed in yeast are denoted as *Xx*Prot, where "*Xx*" is an abbreviation of the species of origin, and "Prot" the protein name abbreviation similar to the yeast protein notation.

Contents

Part I: Prologue

Introduction

Biofuels

Renewable biofuels have emerged as an alternative energy source over the past decade in an effort to move away from fossil based transportation fuels. The most prominent biofuel at the moment is ethanol and it is not a good substitute for conventional gasoline due to intrinsic disadvantages. Ethanol usage as a fuel requires engine modifications and it has a lower energy density than fossil oil derived gasoline. This lower energy density prohibits application in heavy duty and maritime transportation, as well as aviation. Furthermore, restrictions on blending with gasoline (i.e. the blend wall) currently limit the commercial success and further development of ethanol into a carbon neutral biofuel (Peplow, 2014). Therefore, new types of biofuels are being developed to overcome such limitations. Biofuels that can be used as drop-in fuels and replace the whole plethora of fossil transportation fuels. This new category of biofuels is often referred to as advanced biofuels (Fairley, 2011).

Advanced biofuels such as alkanes are similar to the high energy components of fossil transportation fuels (Sheppard et al., 2016). Alkanes can be used as gasoline, kerosine, or diesel. Their applicability as different fuel types would depend on the alkane chain length distribution, with increasing combustion energy per molecule with increasing chain length. Besides alkanes, there are many other types of molecules that can be synthesized by microorganism and are being pursued as such. These moleculs have been excellently reviewed (Liao et al., 2016) and are partially covered in *Paper IV*, but will not be further discussed here.

Microbial alkane synthesis

Microorganisms, plants, and several insects can synthesize non-isoprenoid alkanes from fatty acids, which in turn can be constructed *de novo* by the organism from a carbon source such as glucose. Microorganisms such as cyanobacteria synthesize long-chain alkanes (Ladygina et al., 2006; Valderrama, 2004), possibly to maintain redox balance and partitioning during adaptation to low temperatures (Berla et al., 2015). Plants and insects have been shown to produce very-long chain alkanes (waxes) to avoid dehydration or to act as pheromones (Valderrama, 2004).

Fatty acids are ultimately decarboxylated to yield a hydrocarbon. The fatty acid saturation state will determine whether the reaction(s) yield an alkane or alkene. In this thesis, to both products will be referred when mentioning alkanes, unless stated otherwise.

From the fatty acid precursor there are three known biosynthetic routes to alkanes (Beller et al., 2015). Of these only the first two are of interest for heterologous biofuel production since the third route leads only to very long-chain, wax-like, alkanes. The three metabolic routes to non-isoprenoid alkane biosynthesis in microorganisms are listed below and the first two will be discussed in more depth in the following sections.

1. Fatty acid reduction followed by deformylating oxygenation (releasing formate) or decarboxylation (releasing CO or CO_2) by cyanobacterial and plant enzymes (Bernard et al., 2012; Choi and Lee, 2013; Schirmer et al., 2010). The fatty acids can be free fatty acids or the activated fatty acyl-CoA or –ACP form.

2. Fatty acid decarboxylation, or elongation followed by decarboxylation by a cyanobacterial P450 decarboxylase or polyketide synthase (PKS), respectively (Mendez-Perez et al., 2011; Rude et al., 2011).
3. Fatty acid head to head condensation by a (cyano)bacterial OleABCD complex which results in very-long chain alkenes (Beller et al., 2010; Beller et al., 2011; Frias et al., 2011a; Frias et al., 2011b; Sukovich et al., 2010a; Sukovich et al., 2010b).

The existence of a non-decarboxylative pathway through fatty aldehyde and fatty alcohol intermediates has been claimed in the bacterium *Vibrio furnissii* M1 (Park, 2005), but this study has been refuted (Wackett et al., 2007).

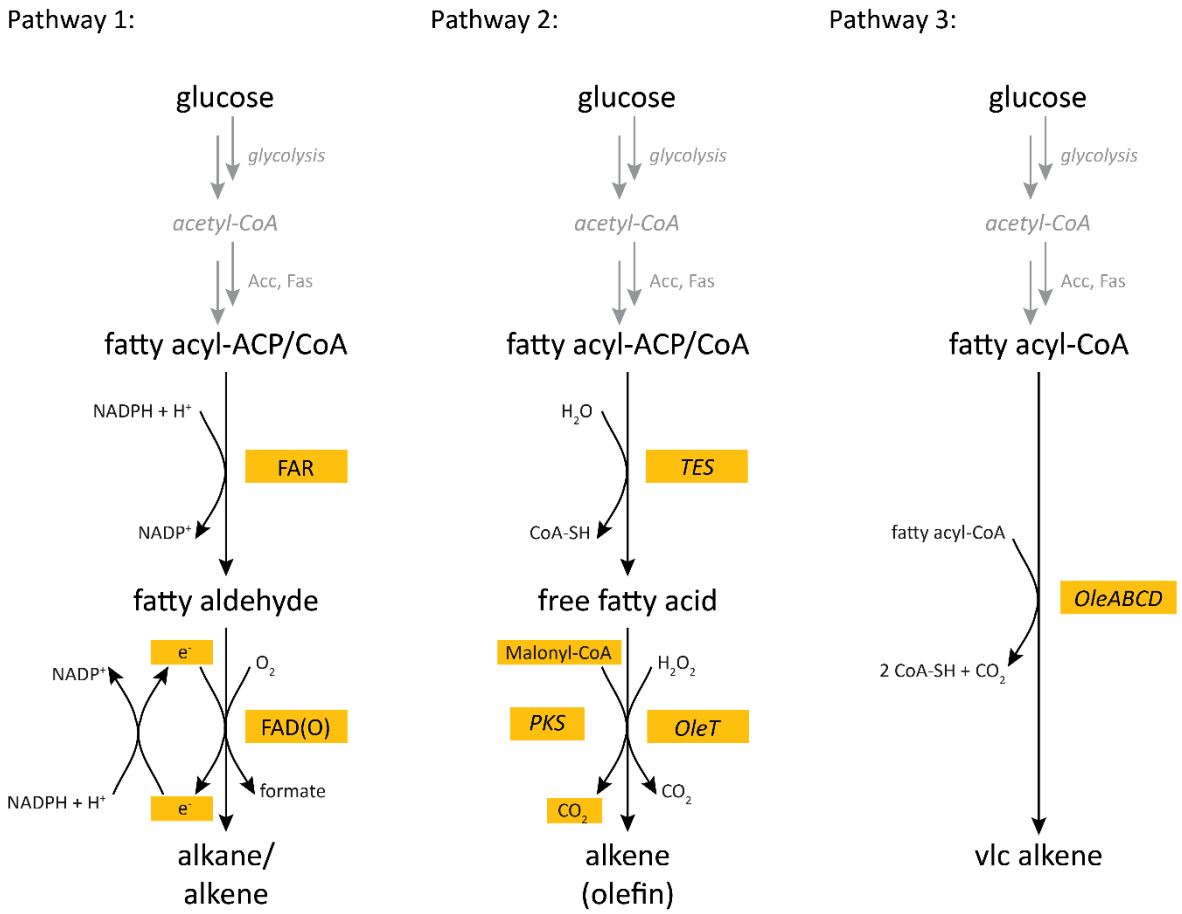


Figure 1: Straight-chain alkanes and alkenes can be synthesized by microorganisms through various routes. Three routes utilizing (activated) fatty acids have been described in literature. The first route involve a fatty acid reduction, catalysed by a fatty acyl-ACP/CoA reductase (FAR). In addition, fatty acid reductases have been described that can utilize free fatty acids instead of fatty acyl-ACP/CoA (not shown). The second step involves either fatty acid deformylation, decarboxylation or decarbonylation (FAD(O)) reaction of the fatty aldehyde and this yields an alkane or alkene, which depends on the saturation state of the fatty aldehyde. The fatty aldehyde deformylating oxygenase is shown as an example. The second route involves a hydrogen peroxide dependent P450 decarboxylase or a polyketide synthase (PKS). The latter extends the fatty acid followed by decarboxylation. The third route involves head-to-head condensation of two fatty acids, which leads to the synthesis of very long chain alkenes. A PKS that has recently been described by Liu *et al* (2015) has not been included in the figure. This PKS utilizes malonyl-CoA as substrate and produces an alkene as final product through several iterations of chain elongation.

Pathway 1: alkane and alkene biosynthesis

In 2010, the landmark study on heterologous alkane biosynthesis by Schirmer *et al* was published(Schirmer et al., 2010). This study was very important for the progression of the field because it identified soluble non-membrane bound proteins in cyanobacteria from the first pathway (as listed above and shown in Figure 1). The soluble character of these proteins meant that they could easily be expressed in other microbial hosts. The described biosynthetic process proceeds via a two-step reduction/deformylation metabolic pathway. This pathway consisted of the *Synechococcus elongatus* PCC7942 fatty acyl-ACP reductase (FAR) and fatty aldehyde deformylating oxygenase (FADO). Expression of these two enzymes in *Escherichia coli* enabled *de novo* biosynthesis of long-chain alkanes from glucose. The introduction of *Bacillus subtilis* FabH2 and the *S. elongatus* PCC7942 FAR and FAD in *E. coli* by Harger *et al* (2012) enabled the synthesis of even-chain alkanes(Harger et al., 2013). Subsequently, others have expressed alternatives enzymes in *E. coli* to synthesize long-chain alkanes: Akhtar et al (2013) utilized a *Mycobacterium marinum* fatty acid reductase and a *Synechocystis* species PCC 6803 fatty aldehyde decarbonylase (*in vitro*), and Howard *et al* (2013) utilized the *Photorhabdus luminescens* LuxCDE fatty acid reductase complex and a *Nostoc punctiforme* fatty aldehyde deformylating oxygenase (Akhtar et al., 2013; Howard et al., 2013). Choi and Lee (2013) modified the fatty acid pool composition by means of expressing a mutated thioesterase, and utilized the *Arabidopsis thaliana* fatty aldehyde decarbonylase Cer1 (Choi and Lee, 2013). The expression of this thioesterase has enabled the controlled biosynthesis of short and medium chain alkanes. Substitution of the FAR by a *Mycobacterium marinum* carboxylic acid reductase (CAR; as has been described by Akthar *et al*), expression of the *S. elongatus* FADO reducing system, and aeration optimization by Kallio *et al* (2014) enabled the biosynthesis of propane (Kallio et al., 2014). Following that study, modification of a *Clostridial* butanol pathway by expression of a CAR and ADO by Menon *et al* (2015) led to the synthesis of propane (Menon et al., 2015). The studies by Kallio *et al* and Menon *et al* have illustrated the broad substrate specificity of the fatty aldehyde deformylating oxygenase. Furthermore, based on the crystal structure of a *Procholorococcus marinus* FADO, Khara *et al* (2013) mutated the substrate binding site and obtained a mutant that preferred shorter chain aldehydes (Khara et al., 2013). Next, Sheppard *et al* (2016) have expressed this mutant FADO together with a *Nocardia iowensis* CAR in a background strain in which six endogenous aldehyde reductases had been deleted (Sheppard et al., 2016). Similar to Kallio *et al,* this enabled the synthesis of gasoline-range alkanes. In addition, Sheppard *et al* also introduced the substrate specificity conferring point mutation in a *Nostoc punctiforme* PCC73102 FADO, and extended the product profile to branched chain alkane isopentane. Finally, Rahman *et al* (2014) have been able to boost titers from 5 to 44 mg/L using the FAR+FADO pathway using a scaffolding strategy (Rahman et al., 2014).

Biosynthesis of alkanes via the FAR+FADO pathway has also been engineered in the cyanobacteria *Synechocystis*, *Nostoc punctiforme, Synechococcus*, and *Anabaena*. Overexpression of the endogenous FAR and FADO in *Synechocystic* sp. PCC 6803 increased titers to 26 mg l^{-1} (1.1% of cell dry weight) in photo-bioreactors (Wang et al., 2013). A similar FAR and FADO overexpression approach in *N. punctiforme* together with optimization of light conditions and overexpression of a putative lipase increased the heptadecane content to 12.9% of cell dryweight (Peramuna et al., 2015). Interestingly, Yoshino *et al* (2015) have introduced the *S. elongatus* FAR+FADO pathway in a *Synechococcus* sp. NKBG15041c, which contains a polyketide synthase like protein of the second pathway (see below). The resulting low observed heptadecane yield has been attributed to the lack of a (overexpressed) reducing

system, low levels of precursor, and competition with aldehyde dehydrogenases (Yoshino et al., 2015).

In the past, there have been several reports on alkane biosynthesis involving a fatty aldehyde intermediate by using crude cell extract of plants. For example, *Pisum sativum* cell extracts have been shown to catalyze the conversion of fatty acyl-CoA and the decarbonylation of octadecanal, thereby releasing carbonmonoxide while consuming oxygen and NADPH (Cheesbrough and Kolattukudy, 1984; Schneider-Belhaddad et al., 2000). The responsible enzyme was inhibited by metal chelators and utilizes a copper containing proto-porphyrin IX to catalyze the carbonyl bond scission (Schneider-Belhaddad et al., 2000). A cobalt proto-porphyrin prosthetic IX group has also been shown to be involved in very-long chain alkane biosynthesis by *Botyrococcus braunii* (Dennis and Kolattukudy, 1991). Unfortunately, the utilization of proto-porphyrin enzymes for heterologous alkane biosynthesis is challenging because there is a high-risk of mismetalation of the active site. This mismetalation is due to the use of scarce metals. Furthermore, these enzymes are typically membrane-bound or -associated, which makes their expression, isolation and characterization difficult. Interestingly, the pine tree *Pinus jeffrey* also possess a pathway for synthesizing heptane from fatty acid intermediates. This tree uses heptane as a biological weapon against bark beetle. The proteins involved have not yet been identified (Savage et al., 1996). Lastly, a putative decarbonylase from *Arabidopsis thaliana* (*SCD2*/At4g37470) with homology to thioesterases/hydrolases has been described in a patent application and has to our knowledge not been further characterized. Plants with an Scd2 mutant displayed increased aldehyde and reduced alkane levels, and alkane levels were increased upon SCD2 overexpression (Zeng and He, 2009).

Pathway 2: olefin biosynthesis

The second pathway has not been explored to the same extent as the FADO pathway. The decarboxylation step results in formation of a terminal double bond and this pathway therefore yields only 1-alkenes instead of the mixture of alkanes and alkenes that result from the first (FAR+FADO) pathway. 1-Alkenes, or terminal alkenes, are also called olefins. The second pathway has been shown to involve a P450 decarboxylase or a PKS like protein. An addition to these two enzyme has been the nonheme iron oxidase. Such a fatty acid decarboxylating nonheme iron oxidase from *Pseudomonas aeruginosa* PA14 yielding medium-chain alkenes has been described recently (Rui et al., 2014; Rui et al., 2015).

Rude *et al* (2011) were the first to have described a P450 fatty acid decarboxylase (OleT) from *Jeotgalicoccus* sp, and have showed that it is involved in nonadecene formation in that organism (Rude et al., 2011). OleT is a hydrogenperoxide dependent P450, which makes it difficult to function efficiently in micro-organism. To circumvent the necessity of hydrogenperoxide supply, Li *et al* (2014) have fused OleT to the reductase domain from *Rhodococcus* sp. which renders the enzyme NADPH-dependent (Liu et al., 2014b). Unfortunately this also reduced the enzyme acitivity and did not result in higher titers *in vivo*. OleT has also been heterologously expressed in *E. coli* together with a *Thermomyces lanuginosus* lipase to convert fed triacylglycerides into olefins (Yan et al., 2015). Alternatively, Dennig *et al* (2015) have shown that *in vitro* activity of OleT can also be supported by putidaredoxin CamAB (Dennig et al., 2015). Recently, OleT homologues from different cyanobacteria have been extensivly characterized and the pathway optimized in *S. cerevisiae* (Chen et al., 2015).

Mendez-Perez *et al* (2011) have described a polyketide synthase Ols from *Synechococcus sp.* Strain PCC 7002, which through elongation and decarboxylation produces nonadecene and nonadecadiene (Mendez-Perez et al., 2011). Expression optimization of this enzyme did unfortunately not yield any product in *E. coli* (Mendez-Perez et al., 2012). There have been no studies so far describing expression of Ols in yeast. Another iterative PKS has been described by Liu *et al* (2015). Expression in *E. coli* of the *Streptomyces* iterative PKS SgcE and its thioesterase components SgcE10 led to the biosynthesis of pentadecane as sole product, after hydrogenation. (Liu et al., 2014a).

Heterologous alkane biosynthesis in yeast

All the aforementioned studies used (cyano)bacteria while yeast has been the organism of choice for industrial ethanol production (Hong and Nielsen, 2012). In 2013, industrial interest as inferred from patent (applications) on microbial alkane synthesis was limited to examples of alkane biosynthesis in *E. coli* or cyanobacteria (Baynes et al., 2009; Behrouzian et al., 2009; Burk and Osterhout, 2011; Friedman and da Costa, 2007; Howard et al., 2012; Hu et al., 2010; Lee et al., 2010; Reppas and Ridley, 2009a; Reppas and Ridley, 2009b; Rude and Schirmer, 2010; Schirmer et al., 2008; Schirmer et al., 2009; Schirmer and Trinh, 2010). The exception being expression of OleT in *S. cerevisiae* for olefin biosynthesis (Alibhai et al., 2007a; Alibhai et al., 2007b). Before publishing Paper I and Paper II, the only study on alkane biosynthesis in yeast was carried out by Bernard *et al* (2012), who described very-long chain alkane biosynthesis. They expressed the fatty acyl-CoA reductase/decarbonylase complex consisting of *Arabidopsis thaliana* CER1 and CER3 in the yeast *Saccharomyces cerevisiae* (Bernard et al., 2012). This resulted in very long chain alkane synthesis, which cannot be used directly as a fuel. Using a lipoxygenase in *Yarrowia lipolytica*, Blazeck *et al* (2013) have synthesized 4.98 mg/L of pentane by cleaving linoleic acid (Blazeck et al., 2013). However, this would be an inefficient way of synthesizing short-chain alkanes since it requires the *de novo* synthesis of long-chain fatty acids and leads to a dicarboxylic fatty acid byproduct. Therefore there was still a need for an advanced biofuel producing yeast strain that can be implemented in existing (cellulosic) ethanol plants and in addition could allow the biofuel industry to diversify beyond ethanol.

The aim of this thesis was to explore heterologous long-chain alkane and olefin biosynthesis by the yeast *S. cerevisiae*.

Table 1: studies on (heterologous) biosynthesis of alkanes

Alkane type[1]:	Titer (mg l^{-1}):		Reference:
Studies in bacteria (*Escherichia coli)*			
Long-chain	300		(Schirmer et al., 2010)
Long-chain	440	[2]	(Lennen et al., 2010)
Long-chain	81.3		(Harger et al., 2013)
Long-chain	5		(Howard et al., 2013)
Short- and medium-chain	581		(Choi and Lee, 2013)
Short-chain	0.46	[3]	(Khara et al., 2013)
Short-chain	32		(Kallio et al., 2014)
Long-chain	44		(Rahman et al., 2014)
Short-chain	3.4		(Menon et al., 2015)
Long-chain	57		(Coursolle et al., 2015)
Long-chain	256		(Song et al., 2016)
Long-chain	56	[4]	(Yan et al., 2016)
Short-chain	0.3	[5]	(Zhang et al., 2016)
Short-chain	4.3		(Sheppard et al., 2016)
Studies in cyanobacteria			
Long-chain	2.3	[6]	(Tan et al., 2011)
Long-chain	4.7	[7]	(Hu et al., 2013)
Long-chain	26		(Wang et al., 2013)
Long-chain	0.01	[7]	(Yoshino et al., 2015)
Long-chain	3.8	[7]	(Kageyama et al., 2015)
Long-chain	305	[7]	(Peramuna et al., 2015)
Studies in yeast (*Saccharomyces cerevisiae)*:			
Very-long chain	0.43-0.10	[8]	(Bernard et al., 2012)
Long-chain	0.074		(Foo et al., 2015)

[1]= short-chain: C4-C8, medium-chain: C10-C14, long-chain: C16-C18, very-long chain: ≥C20.
[2]= catalytic conversion of fatty acids produced by *Escherichia coli*
[3]= with supplementation of 72 mg L^{-1} butanal in medium.
[4]= assuming 7 gDW L^{-1} biomass. Reported value 8.05 mg gDW^{-1} (Yan *et al*).
[5]=valine derived pathway.
[6]= assuming an OD_{730} of 14, reported value: 161.562 μg L^{-1} OD_{730}^{-1} (Tan *et al*).
[7]= assuming 2.36 gDW L^{-1} biomass. Reported values: 0.2% of dryweight (Hu *et al*), 4.2 μg/gDW (Yoshino *et al*), 1600 μg gDW^{-1} (Kageyama et al), and 12.9% of dryweight (Peramuna *et al*).
[8]= assuming 5 gDW L^{-1} biomass. Reported value 86-19 μg gDW^{-1} (Bernard *et al*).

Scope of the thesis

The aim of this study was to enable and improve long-chain alkane biosynthesis by the yeast *S. cerevisiae*.

Paper I

This paper is foundational and describes a proof of principle of heterologous biosynthesis of long chain alkanes in the industrial workhorse *S. cerevisiae*.

The main contribution of this paper is that it describes the necessity of expression of a reducing system and elimination of a main competing fatty aldehyde dehydrogenase. Introduction of a fatty acyl-CoA derived alkane biosynthesis pathway from *Synechococcus elongatus* initially did not yield any product. This pathway consisted of a fatty acyl-CoA/ACP reductase (FAR) and a fatty aldehyde deformylating oxygenase (FADO), and was discovered and expressed in *Escherichia coli* by Schirmer *et al* (2010). Trouble-shooting of the pathway uncovered two obstacles to alkane biosynthesis. First, we demonstrated that expression of a reducing sytem from *E. coli,* consisting of ferredoxin and ferredoxin reductase, is required for functionality of the last, FADO catalyzed step in the alkane pathway. Second, using fatty alcohols as indicator for fatty aldehyde accumulation we identified that the fatty aldehyde-consuming enzyme Hfd1 prevented production of alkanes as it efficiently converts fatty aldehydes to fatty acids.

Paper II

This paper studies the pathway from Paper I further and explores a few strategies to increase alkane biosynthesis.

The results described in the first paper indicated that the first step in the pathway catalyzed by *Se*FAR did not contribute much to the overall alkane titer and showed that the fatty alcohol bypdroduct formation seemed to consume the majority of the fatty aldehyde intermediates. Furthermore, in the meantime studies in *E. coli* had shown that an alternative class of enzymes can synthesize fatty aldehydes from unactivated free fatty acids (Akhtar et al., 2013; Howard et al., 2013). In addition, expression of the cyanobacterial pathway in the first paper did not shed light on whether the alkane titer was low because the fatty acyl-CoA and fatty aldehyde were insufficiently available to the FAR and FADO, or because it was much slower than lipid metabolic enzymes, promiscuous aldehyde reductases or alcohol dehydrogenases. Therefore, to separate these factors we considered compartmentalization. By placing the enzymes in an enclosed environment, they would be shielded off from competing enzymes as well as be in close proximity to the other enzymes in the pathway. This would allow use to test different strategies to increase pathway efficiency, and to increase producitivity by increasing the number of compartments. Other studies have also uncovered the natural redox partner of the FADO enzyme (Zhang et al., 2013), and expression of a fusion of the native reducing system to the FADO enzyme in *E. coli* could improve activity (Wang et al., 2014), and that hydrogen peroxide inhibits the activity of the FADO (Andre et al., 2013) providing us with several strategies to test and possibly increase the alkane titer in *S. cerevisiae*.

The cellular compartment we decided to use was the peroxisome organelle. In yeast, this organelle is where fatty acids are degraded to acetyl-CoA through β-oxidation. Due to this function of the peroxisome, we assumed that the transport of fatty acids into this compartment would not be a limiting factor.

Paper III

This paper is a commentary in which we describe why biofuels could play a role in a sustainable solution that replaces fossil fuels.

Paper IV

This paper is a review of the field, in 2012, of advanced biofuel production by the yeast *S. cerevisiae*.

Materials and Methods

The sections below are a description of the experiment carried out for the additional figures presented in this thesis.

Yeast strains, plasmids and reagents

The background yeast strain *S. cerevisiae* CEN.PK113-11C (*MAT**a** MAL2-8c SUC2 his3Δ1 ura3-52*) was kindly provided by P. Kötter, University of Frankfurt, Germany. The background yeast strain *S. cerevisiae* BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) carrying *HFD1::KanMX* was ordered from ThermoFischer Scientific (Stockholm, Sweden). The Δ*FAA1* Δ*FAA4* Δ*POX1* strain (YJZ06) and the Δ*HFD1* Δ*POX1* strain (YJZ54) carrying genomic integration of *Mycobacterium marinum* CAR, Aspergillus nidulans npgA, *Synechococcus elongatus* ferredoxin and ferredoxin reductase in the *GAL80* and *ADH5* loci was kindly constructed by Y. Zhou (as described in paper II and unpublished data). The *Synechococcus elongatus* orf1594 FAR and orf1593 FADO, ferredoxin, and ferredoxin NADPH reductase, FADO homologues from *Nostoc punctiforme* PCC73102, *Thermosynechococcus elongatus*, *Hapalosiphon welwitschii* IC-52-3, *Planktothrix agardhii* NIVA-CYA 126/8, *Lyngbya aestuarii* (strain CCY9616), *Crocosphaera watsonii* WH 8501, and *Prochlorococcus marinus* (strain MIT 9313), *Escherichia coli* thioesterase TesA', *Photorhabdus luminescens* LuxC, LuxD, and LuxE, *Mycobacterium marinum* carboxylic acid reductase, and *Aspergillus nidulans* npgA were synthesized and codon-optimized for expression in yeast (Genscript, Piscataway, NJ, USA). All synthetic genes sequences, except for the FADO homologues, are listed in Buijs *et al* (Buijs et al., 2013). The cofactor system encoded by *E. coli* fldA (NCBI Gene ID: 945293) and fpr (NCBI Gene ID: 12932121) was amplified from *E. coli* DH5α. The genes *PEX34*, *TES1*, and *CTT1* were amplified from yeast genomic DNA. PrimeStar DNA polymerase was purchased from TaKaRa Bio (Segeltorp, Sweden). Oligonucleotides were custom synthesized by Eurofins (Ebersberg, Germany). Restriction enzymes, DNA gel purification and plasmid extraction kits were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Genetic engineering

All the pathways for alkane and olefin production were constructed on the plasmid backbone pYX212 (FADO homologues) or p423GPD (olefin plasmids) by using a modular pathway engineering strategy as described before (Buijs et al., 2013). Plasmids were extracted from single yeast colonies using the Zymoprep Yeast Plasmid Miniprep II kit (Nordic Biolabs, Täby, Sweden) and transformed into *E. coli* DH5α competent cells. After purification of the plasmid, verification by restriction analysis, and sequencing, the plasmids were transformed into yeast. Yeast competent cells were prepared and transformed with 1 μg of plasmid DNA according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods, 2002).

Strain cultivation

Yeast strains for preparation of competent cells were cultivated in YPD consisting of 10 g/L yeast extract (Merck Millipore, Darmstadt, Germany), 20 g/L peptone (Difco, VWR, Stockholm, Sweden), and 20 g/L glucose (Merck Millipore). During genetic manipulation, yeast strains were selected on Synthetic Dextrose (SD) medium, which contained 6.9 g/L yeast nitrogen base without amino acids (Formedium, Hunstanton, UK), 0.77 g/L complete supplement mixture without uracil (Formedium), 20 g/L glucose (Merck Millipore) and 20 g/L agar (Merck Millipore). *E. coli* strains were grown at 37 °C on Luria−Bertani medium (10 g/L tryptone (BD Biosciences, Stockholm, Sweden), 5 g/L yeast extract (Merck Millipore), 10 g/L NaCl (Merck Millipore)) supplemented with ampicillin (80 μg/mL) if required. Agar plates

were prepared with the corresponding liquid medium supplemented with 15 g/L agar powder (Merck Millipore).

Shake flask batch fermentations were carried out in minimal medium containing 30 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, and vitamins and 2x trace metal solutions as has been described by Verduyn *et al* (1992) and pH 5.6 (Verduyn et al., 1992). For the FADO homologue comparison experiment the KH_2PO_4 was raised to 14.4 g/L. Cultures were inoculated from overnight precultures at 0.1 OD_{600} in 20 ml minimal medium supplemented with histidine (40 mg/L; Sigma Aldrich) or uracil (60 mg/L; Sigma Aldrich) in 250 ml shake flasks. The shake flasks were incubated at 30 °C and 200 rpm orbital shaking. After 72 hours, the cells were harvested by centrifugation (5 minutes; 1000 g) and washed once with 5 ml phosphate buffer (10 mM KH_2PO_4, pH 7.5). The supernatant was removed, and the pellet frozen in liquid nitrogen and freeze-dried (Christ Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 48 hours.

Hydrocarbon analysis by GC-MS

Alkanes and olefins were extracted from the freeze dried cell pellets using the lipid extraction method that has been described before (Khoomrung et al., 2013). The only deviation from that method was the use of 6 ml $CHCl_3$:MeOH extraction solvent and in the final step extractants were redissolved in hexane. Hexadecane (0.5 μg/L) was used as an internal standard. For the extraction method comparison, hydrocarbons were also extracted from liquid cell cultures, cell pellets, and freeze dried cell pellets using the methods described by Schirmer *et al* (Schirmer et al., 2010), Rodriguez *et al* (Rodriguez et al., 2014), and Khoomrung *et al* (Khoomrung et al., 2012), respectively. Samples were analyzed by gas chromatography (FocusGC, ThermoFisher Scientific) coupled to mass spectrometry (DSQII, ThermoFisher Scientific) using a Zebron ZB-5MS Guardian capillary GC column (30 m × 0.25 mm × 0.25 μm, Phenomenex, Værløse, Denmark). For alkane/alkene analysis the following method was run: initial temperature 50 °C hold for 5 minutes, ramp by 10 °C per minute to 140 and hold for 10 minutes, ramp by 15 °C per minute to 310 °C and hold for 7 minutes, inlet temperature 250 °C, mass transfer line 300 °C, splitless flow of 1.0 mL/min, ion source temperature 230 °C, full scan mode 50-650 m/z. Samples were analyzed by single 2 μL injections. For olefin analysis the following method was run: initial temperature 50 °C hold for 5 minutes, ramp by 10 °C per minute to 310 °C and hold for 6 minutes, inlet temperature 250 °C, mass transfer line 300 °C, splitless flow of 1.0 mL/min, ion source temperature 230 °C, full scan mode 50-650 m/z. Samples were analyzed by single 2 μL injections. Analytical standards for alkanes (Sigma Aldrich) and olefins (TCI Europe, Zwijndrecht, Belgium) were analyzed during the same run for peak identification and quantification.

Lipid staining with nile red

Duplicate samples of 0.2 ml cell culture were taken aseptically and prior to staining an additional 5 μl of culture was checked for contamination by microscopy and plating on cyclohexamide plates. Next, one set of samples was collected by centrifugation for 3 minutes at 1200 rcf, supernatant was removed, cells were washed twice with 0.4 ml PBS buffer, and resuspended in 0.2 ml PBS buffer. Both sets were then stained with 0.5 μl nile red (1 mg ml^{-1} in DMSO) and cells were left in the dark for 30 minutes.

Results

In the following paragraph a brief overview of the main results from Paper I and Paper II will be presented and some additional unpublished data are also presented.

Results at a glance: Paper I

Succesful introduction of an cyanobacterial pathway in *Saccharomyces cerevisiae* proved more complicated than we anticipated. As Paper I demonstrates, the newly introduced pathway intersects with endogenous sphingolipid metabolism. Furthermore, intracellular accumulation of minor quantifities of heptadecane in our background strain required the application of a lipid extraction method to get the product out. Conventional methods that had been used to detect alkanes in Escherichia coli (Howard et al., 2013; Schirmer et al., 2010), fatty acids (Khoomrung et al., 2012) or the branched-chain alkane squalene in yeast (Rodriguez et al., 2014) proved not applicable. Below the results from Paper I will be briefly highlighted. Subsequently, unpublished results will be presented on the alkane extraction method we used to get the results described in Paper I. My contributions to this paper are that I developed the extraction method, identified Hfd1 as a target after troubleshooting the alkane pathway extensively, performed all alkane related experiments in the paper, analyzed the data, prepared the figures, and wrote the manuscript.

The *Synechococcus elongatus* alkane biosynthesis pathway constists of a fatty acyl-CoA/ACP reductase (FAR) and a fatty aldehyde deformylating oxygenase (FADO) (see Figure 1, pathway 1). Upon expression of this pathway in a wild-type yeast strain, no alkanes or byproducts could be detected (Figure 2, and Paper I, Figure 2). Schirmer *et al* (2010) have shown that the FADO enzyme *in vitro* requires a reducing system consisting of ferredoxin and ferredoxin NAD(P)H reductase (F/FNR). At the time of our study, the native redox partners of *Se*FADO were not known. Therefore we chose to express the *E. coli* ferredoxin (Fdx) and ferredoxin NADPH reductase, which had been shown to work *in vivo* by Schirmer *et al*. Subsequent co-expression of this reducing system with the FAR and FADO resulted in the detection of small amounts of heptadecane. We suspected that part of the intermediates were consumed by a competing enzyme due to the absence of C15 products. C15 products were expected because C16 and C18 fatty acids make up the bulk of lipids in a yeast cell (Khoomrung et al., 2012; Khoomrung et al., 2013). Kaiser *et al* (2013) have described the importance of deleting the fatty aldehyde dehydrogenase AldE in *S. elongatus* in order to accumulate fatty aldehydes in *S. elongatus* (Kaiser et al., 2013). Based on the strong homology of AldE to Hfd1 (34% identity, E-value 1e-69) we therefore decided to eliminate the Hfd1 encoding gene. To circumvent any other potential problems with the FAR or FADO, we used fatty alcohols as an indicator to test our hypothesis (Paper I, Figure 2b). Interestingly, deletion of *HFD1* was enough to supply endogenous alcohol dehydrogenases and FADO with substrate to synthesize fatty alcohols and alkanes, respectively. Additional expression of *Se*FAR in a *HFD1Δ* background slightly increased the fatty alcohol and alkane titers (Paper I, Figure 2a-b).

Extraction method to get to Paper I

In order to measure hydrocarbons, a modified extraction method had to be used. This extraction method seemed to extract alkanes from the internal membranes of the yeast cells. In Figure 3, the results of a comparison of four extraction methods are shown. The first three methods have been applied to extract alkanes from bacteria (Schirmer et al., 2010), squalene and ergosterol from yeast (Rodriguez et al., 2014), and (esterified) fatty acids from yeast cells (Khoomrung et al., 2012). The squalene extraction method involves boiling of cells in an alkaline solution

followed by extraction with dodecane. This method was included because it might have been a feasible alternative to the laborious lipid extraction method. Squalene accumulates mainly in lipid droplets (Spanova et al., 2010), and the same has been suggested for alkanes in cyanobacteria (Peramuna et al., 2015), thus a method that works for squalene could perhaps be applied to alkanes too. The esterified fatty acid extraction method involves boiling of the dried biomass samples and simultaneous esterification by methylation, which results in fatty acid methyl esters (FAMEs). This method was included because the boiling step could break up the cells and release the alkanes into the hexane solvent; we suspected that this might have been the problem when applying the first method to yeast. The fourth method is a slightly modified lipid extraction method that has been used in Paper I and II. The modification is that the final solvent was changed from chloroform:methanol to hexane to avoid loading lipid into the gas chromatograph. The unmodified lipid extraction has been described by Khoomrung *et al* (2013) and was originally developed for HPLC-CAD analysis.

As can be seen in Figure 3, the extraction of hydrocarbon from cell cultures with ethyl acetate did not yield any detectable product. Extraction from a 20 ml liquid cell culture using an equal volume of ethyl acetate followed by concentration using a rotarory evaporator did not yield any detectable hydrocarbon (data not shown). However, the strain used in that extraction experiment was a wild-type background strain expressing the complete FAR+FADO pathway, meaning that it cannot be excluded that Hfd1 pushed titers below the detection limit. With the lipid extraction method it was possible to detect heptadecane in that strain (Figure 2, and Paper I Figure 2). The modified lipid extraction method resulted in detection of both C15 and C17 hydrocarbons.

In Figure 3B and Figure 3C, the detected pentadecane and heptadecane titers are shown for different starting volumes of cell cultures. Different culture volumes were used to test the linearity of the extraction method and the lower limit of our method. The numbers are not corrected for background signal observed in a control strain carrying an empty plasmid. Using an equal volume of starting cell culture, lipid extraction of samples presented in Figure 3A resulted in a pentadecane titer of 17.2 ± 1.1 $\mu g\ L^{-1}$, versus 16.5 ± 0.2 $\mu g\ L^{-1}$ for the samples in Figure 3C (n=3), which is not a significant difference. The average percentage of heptadecane of the combined heptadecane and pentadecane titer was on 15.8 ± 0.82%, excluding the 2 mL samples for which 7.5% was heptadecane.

The data does not show a downward trend at higher culture volumes, which would be expected if the extraction solvent becomes saturated with product. The strain used for this experiment is the same strain as shown in Figure 7 carrying the *Se*FADO and empty pYX212. However, the strains shown in Figure 7 were cultivated in minimal medium with increased buffer capacity which resulted in higher final OD_{600}. Lastly, it should be noted that for Paper I, biomass was collected by weighing approximately 10-15 mgDW after freeze drying. For the data shown in Paper II and the additional data presented in this thesis, the amount of biomass was inferred from the OD_{600} and a corresponding cell culture volume was freeze dried after removal of the supernatant.

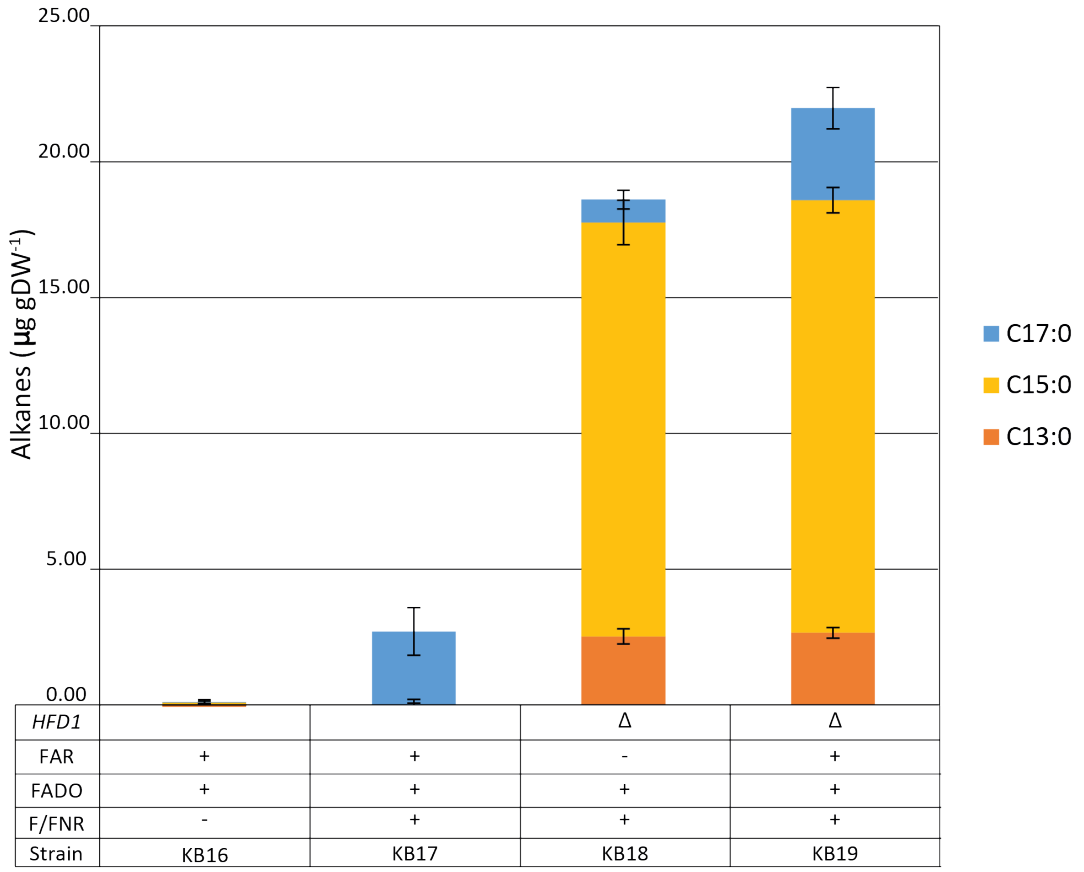


Figure 2: Alkane titer data as presented in Paper I, Figure 2. A *S. elongatus* alkane biosynthetic pathway was introduced in the yeast *S. cerevisiae*. This pathway consists of a *S. elongatus* fatty acyl-CoA reductase (*Se*FAR) and a *S. elongatus* fatty aldehyde deformylating oxygenase (*Se*FADO). Furthermore, the pathway intersects with endogenous metabolism of fatty aldehydes by promiscuous aldehyde reductases (ALR) and fatty alcohol dehydrogenases (ADH) and the hexadecenal dehydrogenase Hfd1 (encoded by *HFD1*/*YMR110C*), which catalyzes the last step in the sphingolipid breakdown pathway. *E. coli* ferredoxin (*Ec*Fdx) and ferredoxin NADPH reductase (*Ec*Fpr), denoted by F/FNR, was introduced to provide the cofactor required for the FADO enzyme.

Results at a glance: Paper II

From Paper I we had learned that metabolism in yeast is intrinsically more complex than that of bacterial hosts. Before discovering the role of Hfd1 in the alkane pathway, we suspected that ferredoxin could be inactive due to its iron-sulphur cluster. The expression of a bacterial mevalonate pathway in yeast was hampered by difficulties with expression of iron-sulfur cluster proteins (Carlsen et al., 2013; Partow et al., 2012). Iron-sulfur cluster biosynthesis takes mainly place in the mitochondria. As such, we had explored localization of the *Se*FAR+*Se*FADO pathway in the mitochondria in an earlier stage of the project. But given the low amounts and short chain length of fatty acids synthesized by the mitochondrial FAS system and the resulting complications with detection of heptane, this seemed a dead end. However, the idea of compartmentalization was interesting and could provide us with a model system to study our pathway. Therefore moving the pathway into the peroxisomes was a natural extention of our previous work. My main contribution in this paper is that I performed the olefin experiments described in the paper. The olefin project was originally initiated by Dr. A. Boonsombuti, and D. Orol Gómez continued it under my supervison after which I subsequently completed it.

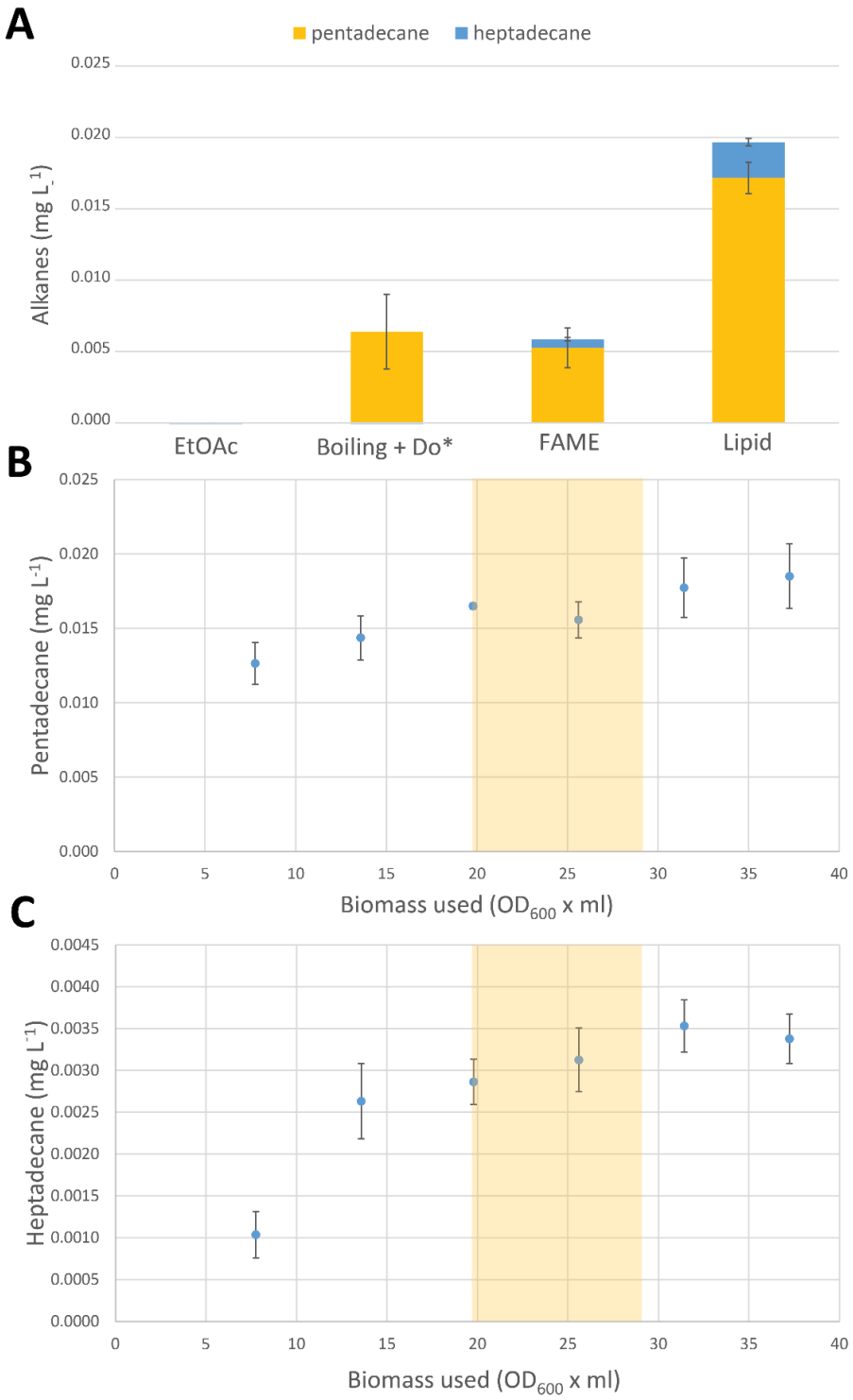


Figure 3: (A) comparison of different extraction methods. Heptadecane titers, and Boiling + Do and FAME pentadecane titers were outside calibration range. (B) extraction of pentadecane from different starting volumes of yeast cell broth. First data point outside calibration range. (C) extraction of heptadecane using different culture volumes. First two data points outside calibration range. The yellow square represents the range of biomass amounts that has been used typically for alkane extraction in our studies. Bars represent average ± standard deviation of triplicate measurements. *= without correction for losses by a cholesterol internal standard, heptadecane could not be detected.

Potential problems with moving a pathway into peroxisomes could arise from mislocalization or inactivity of the enzyme upon addition of a peroxisomal targeting signal (PTS) or insufficient precursor or NADPH and ATP cofactor supply. Therefore, as in Paper I, we used fatty alcohol biosynthesis as a model system to verify the feasibility of using peroxisomes for biosynthesis of fatty acid derived compounds. In Paper I we relied on unidentified endogenous enzymes for conversion of the fatty aldehyde intermediates into fatty alcohols. However, for relocalization of the pathway, peroxisomal targeting signals have to be added to the proteins and therefore we opted for a different approach. We chose to express a fatty alcohol forming fatty acyl-CoA reductase, which is a type of enzyme that has been shown to be efficient in converting fatty acyl-CoAs into fatty alcohols (Runguphan and Keasling, 2014; Steen et al., 2010). As such, a fatty acyl-CoA reductase (FaCoAR) from *Marinobacter aquaeolei* VT8 (Willis et al., 2011) was targeted into the peroxisome to obtain a proof-of-principle for the compartmenalization strategy.

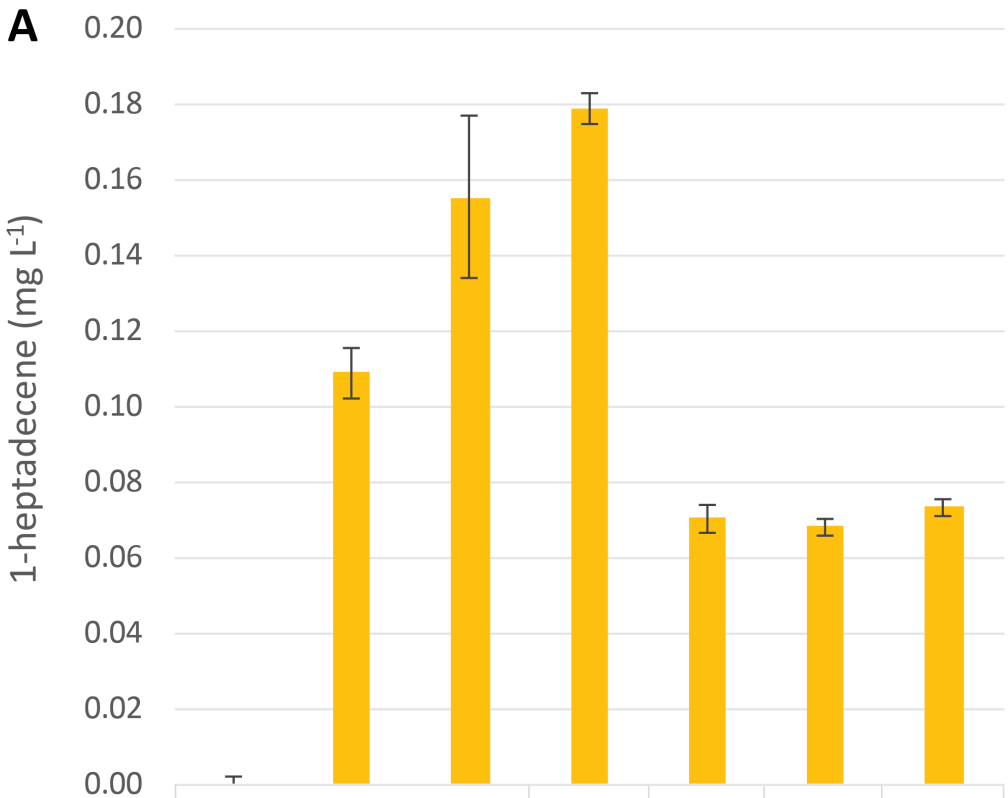
The FaCoAR was expected to be directed to the peroxisomes after addition of a C-terminal PTS. The actual localization of the reductase was studied by expressing an N-terminal fusion of the enzyme with a green fluorescent protein (GFP). Fluorescence microscopy showed that the FaCoAR-GFP fusion protein was successfully targeted to the peroxisome (Paper II, Figure 1b); proper localization could be inferred from the colocalization of the fusion protein with a Pex3-RFP fusion. Pex3 is a peroxisomal membrane protein, and together with Pex19 required for the import of other peroxisomal membrane proteins (Fang et al., 2004; Hettema et al., 2000). Peroxisomal compartmentalization of FaCoAR increased the titer, reduced the average chain length and saturation degree of the fatty alcohols in comparison with the cytosolic pathway. Elimination of *POX1*, which encodes the first step of the beta-oxidation (i.e. the main pathway competing for fatty acyl-CoA in the peroxisome), did not increase the titer further but moved the average chain length and saturation degree towards that of the cytosolic pathway (Paper II, Figure 1c-e).

With the positive result of the fatty alcohol biosynthesis pathway in hand, the next step was to move an alkane biosynthesis pathway into the peroxisome (Paper II, Figure 2a). First, the pathway described in Paper I was moved by adding PTSs to the *Se*FAR, *Se*FADO, *Ec*F/FNR. Relocalization of this fatty acyl-CoA dependent pathway improved the heptadecane titer (Paper II, Figure 2b). Without interference from other pathways due to shielding of the intermediate by the compartmentalization, several strategies were evaluated to improve the efficiency of the last step in the peroxisomal pathway. For example, comparison of the *E. coli* reducing system with the *S. elongatus* reducing system showed slightly less variability with the latter system (Paper II, Suppl. Figure 2). Given this result, and assuming that the FADO would work more efficient with its natural reducing system, we chose to continue with the *Se*F/FNR system. Next, to test whether the *Se*FAR was limiting, a fatty aldehyde forming *Mycobacterium marinum* carboxylic acid reductase (*Mm*CAR) was expressed and localized to the peroxisomes. This increased the alkane titer in comparison with the cytosolic pathway and the *Se*FAR+*Se*FADO pathway (Paper II, Figure 2c). The *Mm*CAR+*Se*FADO pathway was able to utilize unsaturated fatty acids and led to alkene synthesis in the presence of Hfd1. Subsequently, deletion of *HFD1* increased the alkane titers of both the cytosolic and the peroxisomal pathway. Interestingly, this deletion did affect the fatty alcohol byproduct titers differently, with a much lower increase for the peroxisomal pathway (Paper II, Figure 2d). Elimination of two suspected fatty aldehyde consuming alcohol dehydrogenases encoded by *ADH5* and *SFA1* led to a drop in fatty alcohols formed by the strain expressing a cytosolic pathway, but did not affect the titer of the peroxisomal pathway (Paper II, Figure 2e).

The next step was to try to increase the number of peroxisomes in the yeast cells by modulating peroxisome biogenesis. Peroxisome biogenesis is controlled by a class of proteins called peroxins. These proteins regulate the peroxisome size and numbers. For example, the peroxins Pex30, Pex31, and Pex32 have been described as negative regulators (Vizeacoumar et al., 2004), and Pex11 and Pex34 as positive regulators (Huber et al., 2012; Tam et al., 2003; Tower et al., 2011). Deletion of these three negative regulators and overexpression of the two positive regulators could shed light on the potential of the pathways and the peroxisome as compartment. Using FaCoAR again as a proof of principle, deletion of the peroxins Pex31 and Pex32 increased the fatty alcohol titer produced by the peroxisomal pathway (Paper II, Figure 3b and Suppl. Figure 4). Extending this strategy to the *Mm*CAR+*Se*FADO peroxisomal alkane pathway showed an improvement in alkane titer. Overexpression of *PEX34* had a positive effect on the alkane titer, and combined deletion of *PEX31*, *PEX32*, and overexpression of *PEX34* seemed to increase the final titer more than threefold (Paper II, Figure 3c and Suppl. Figure 5-7).

Building up on the positive result of the free fatty acid derived alkane pathway, the compartmentalization strategy was applied to olefin biosynthesis. Olefins are alkenes with the double bond at the terminal carbon-carbon bond. Olefin biosynthesis can be achieved by introduction of a *Jeotgalicoccus sp*. ATCC8456 fatty acid decarboxylase (OleT), which uses hydrogen peroxide as cofactor (Rude et al., 2011). A study by Chen *et al* (2015) has shown that the productivity of this enzyme benefits significantly from a substantial precursor boost by *FAA1* and *FAA4* deletion (Chen et al., 2015). Therefore these two deletions were introduced in the *ΔPOX1* strain. Peroxisomomal localization of OleT and *POX1Δ* would mean that the hydrogen peroxide levels could be limiting. Liu *et al* (2014) have shown *in vitro* that OleT can be supported by flavodoxin (Fld) and flavodoxin (/ferredoxin) NADPH reductase (Fpr) from *E. coli* (Liu et al., 2014b). Flavodoxin can be reduced by ferredoxin NADPH reductase (Wan and Jarrett, 2002), which is the same enzyme that was expressed for the alkane pathway. In the same study by Liu *et al* (2014), a fusion between OleT and a *Rhodococcus sp.* P450 reductase domain has been constructed that renders the enzyme NADPH dependent, instead of H_2O_2. In our study, we also expressed this OleT-RhFRED fusion enzyme and targeted it to the peroxisome. Proper localization verification of this fusion enzyme was not pursued.

Thus we tested the effect of the reducing system on a cytosolically localized OleT (Figure 4, and Paper II, Figure 4b). The slightly higher titer seems to suggest that OleT can at least partially be supported by Fld and Fpr *in vivo*. Next, the pathway was directed to the peroxisome by adding PTS sequences to each gene. This led to a slight increase in the olefin titer, similar to what was observed for the alkanes and fatty alcohols. Plasmid based overexpression of *PEX34* reduced the volumetric olefin titers (Figure 4), including the biomass specific titers (data not shown). Please note that overexpression of PEX34 and PEX11 was achieved by genomic integration in case of the fatty alcohol and alkane producing strains.



Location	-	C	C	P	C	P	P
OleT	-	+	+	+	-	-	+
Fld/FNR	-	-	+	+	-	-	+
OleT-FRED	-	-	-	-	+	+	-
PEX34	-	-	-	-	-	-	+
Strain	KB111	KB113	KB112	KB114	KB116	KB117	KB122

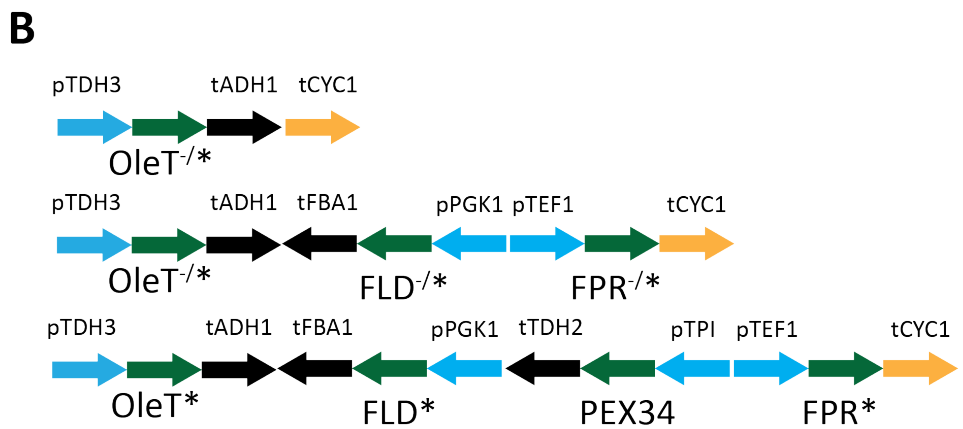


Figure 4: (A) Olefin data as shown in Paper II, extended with data from supplementary information of Paper II and unpublished data of plasmid based overexpression of *PEX34*. The localization of the enzymes is indicated with C for cytosol and P for peroxisome. Bars represent the mean of triplicate biological clones and error bars represent the standard deviation. (B) modular pathway constructs that were introduced on a pYX212 2m multicopy plasmid backbone. OleT, FLD, and FPR denoted with -/* contained an additional peroxisomal targeting signal (PTS) in case of peroxisomal localization. An OleT fusion with *Rh*FRED, with and without PTS was expressed using the same promoter and terminator as OleT. The alkenes 1-pentadecene, 1,7-pentadecadiene, and 1,8-heptadecadiene could be detected but not reliably quantified.

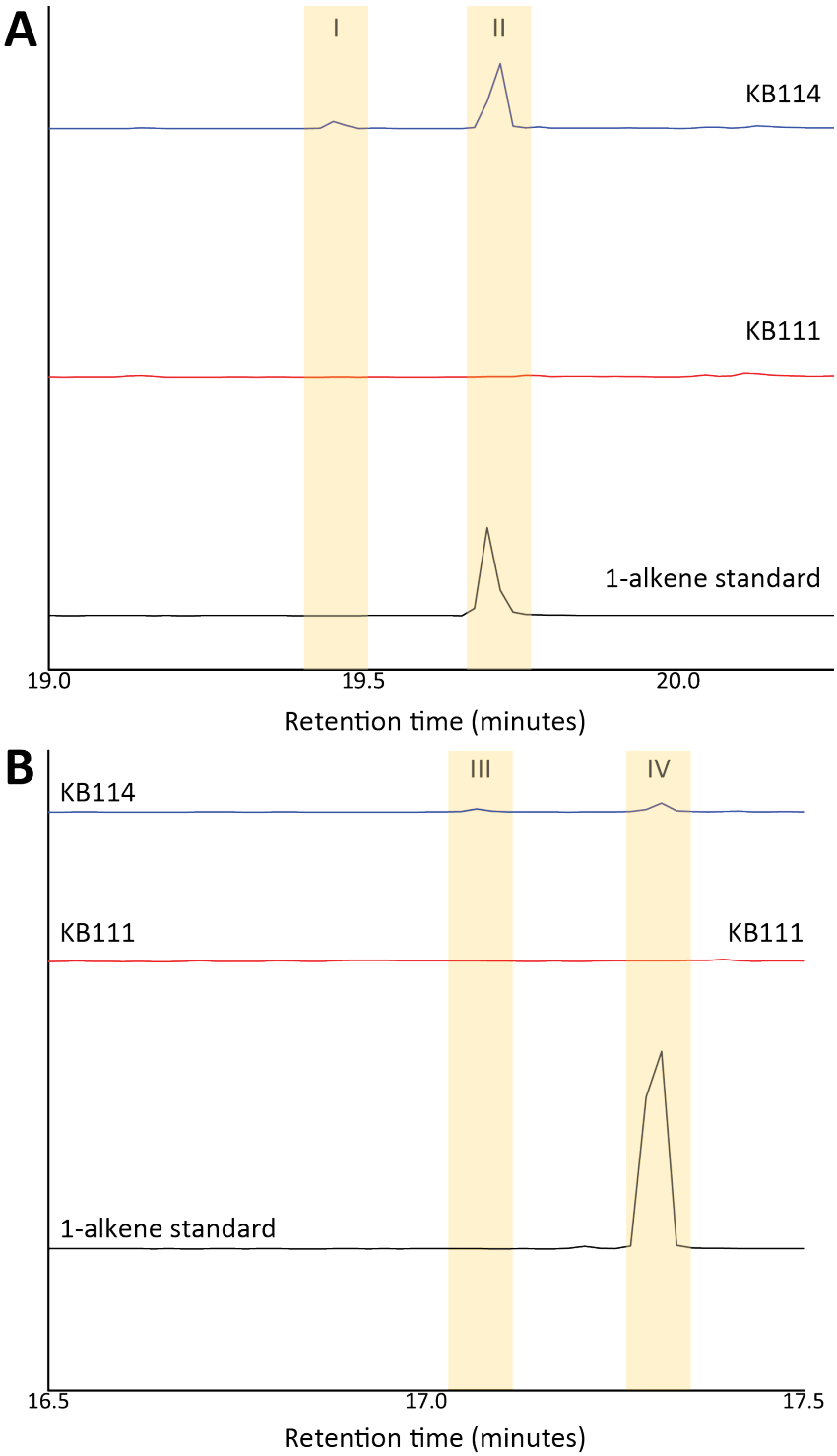


Figure 5: GC spectra of olefin producing strain (blue/top trace), a control strain (red/middle trace), and an olefin standard (black/bottom trace). Subplot A shows the area in a gas chromatography (GC) spectrum where C17 alkenes are eluting. Subplot B shows the area of the GC spectrum were C15 alkenes are eluting. The peaks labelled peaks represent 1,8-heptadecadiene (I) and 1-heptadecene (II), 1,7-pentadecadiene (III), and 1-pentadecene (IV). The C15 and C17 dialkene were assumed based on its expected retention time, as inferred from elution times of alkane and alkene standards, as well as their mass spectrum. The 1-heptadecene (II) matched the standard retention time and mass spectrum. The peak areas of the alkenes I, III, and IV could not be quantified because they were outside the range of the calibration standards. The GC spectra show the spectra for m/z values in full scan mode in the range of 50-650 m/z.

Expression of the LuxCDE fatty acid reductase complex

The luminescent bacterium *Photorhabdus luminescens* applies a fatty acid reductase complex to provide the fatty aldehyde substrate for its luciferase. This complex is composed of acyl-protein reductase LuxC, fatty acyl-transferase LuxD, and fatty acyl-protein synthetase LuxE (Meighen, 1991).

In Figure 6, the results are shown of expression of the *Pl*LuxCDE complex in a BY4741 Δ*HFD1* background (which does not require expression of F/FNR). The LuxCDE complex was expressed together with a *Se*FADO or *Np*FADO, and *E. coli* thioesterase TesA' to increase the free fatty acid levels. Quantification of the alkane titers were not pursued after it became apparent that expression in a CEN.PK background would require coexpression of a F/FNR reducing system. Therefore, LuxCDE became incompatible with single plasmid based pathway expression, which in our study has space for five genes.

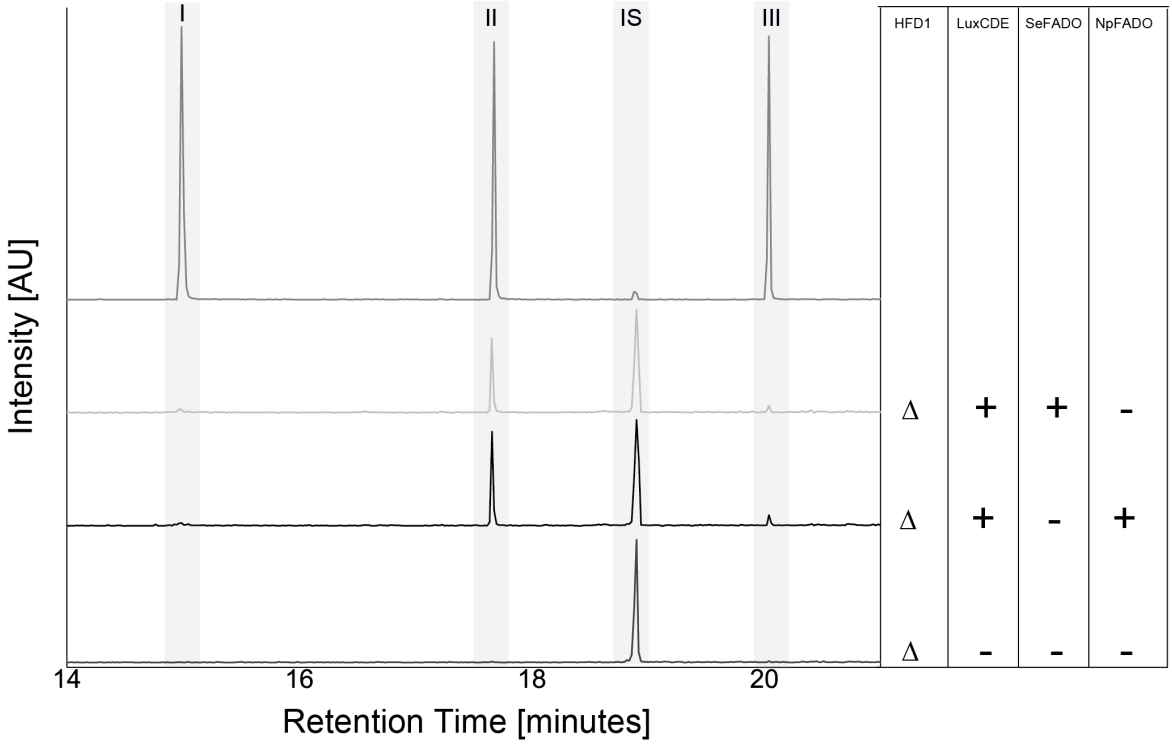


Figure 6: GC spectra of strain expressing the *Photorhabdus luminescens* LuxCDE fatty acid reductase complex, *E. coli* TesA' and a *S. elongatus* or *N. punctiforme* FADO in a BY4741 ΔHFD1 background. Shake flask cultures were incubated for 48 hours in glucose minimal medium, and alkanes extracted. The control strain was carrying an empty vector pYX212 (bottom trace). A C7-C30 alkane analytical standard (top trace) was used as a reference. The highlighted peaks labeled with I, II, IS, and III represent tridecane (I), pentadecane (II), hexadecane (IS; internal standard), and heptadecane (III), respectively. The shown spectra are for the m/z values 184, 212, and 240.

FADO homologue comparison

A recent study by Coates *et al* (2014) has characterized the hydrocarbon profile of a wide variety of cyanobacterial species possessing a FAR+FADO pathway or a Ols polyketide synthase (Coates et al., 2014). Interestingly, their results have shown a range of hydrocarbon titers and product spectra. In Paper I we demonstrated that the FADO enzyme is not able to

convert the bulk of the fatty aldehydes, and this study made us curious how these FADO homologues of *Se* and *Np*FADO would affect the product profile and titer in *S. cerevisiae*. Therefore we chose to express FADO homologues from *Hapalosiphon welwitschii* IC-52-3, *Planktothrix agardhii* NIVA-CYA 126/8, *Lyngbya aestuarii*, and *Crocosphaera watsonii* WH 8501 described in the Coates *et al* study, which represent the different profiles observed in that study. In addition, we chose to express the *Procholorococcus marinus* FADO used in a previous study in *E. coli* (Sheppard et al., 2016) as well as a *Thermosynechococcus elongatus* homologue. The latter homologue would be an interesting candidate for enzyme engineering due to its ability to operate at high temperatures and associated refolding capabilities/structural stability (Dr. M. Engqvist, personal communication).

In Figure 7 the results of the FADO comparison are shown. The FADO homologues were expressed from 2μ pYX212 multicopy plasmid from a glucose repressed pGAL1 promoter in a ΔGAL80 strain carrying single chromosomal integrations of *Mm*CAR, *Aspergillus nidulans* NpgA, and *Se*F/FNR (constructed by Dr. Y.J. Zhou, unpublished data). The low expression of *Mm*CAR proved important for qualification of pentadecene and heptadecene due to overlap of these peaks with fatty alcohols and fatty aldehydes. This has also been extensively studied and described before (Akhtar et al., 2013).

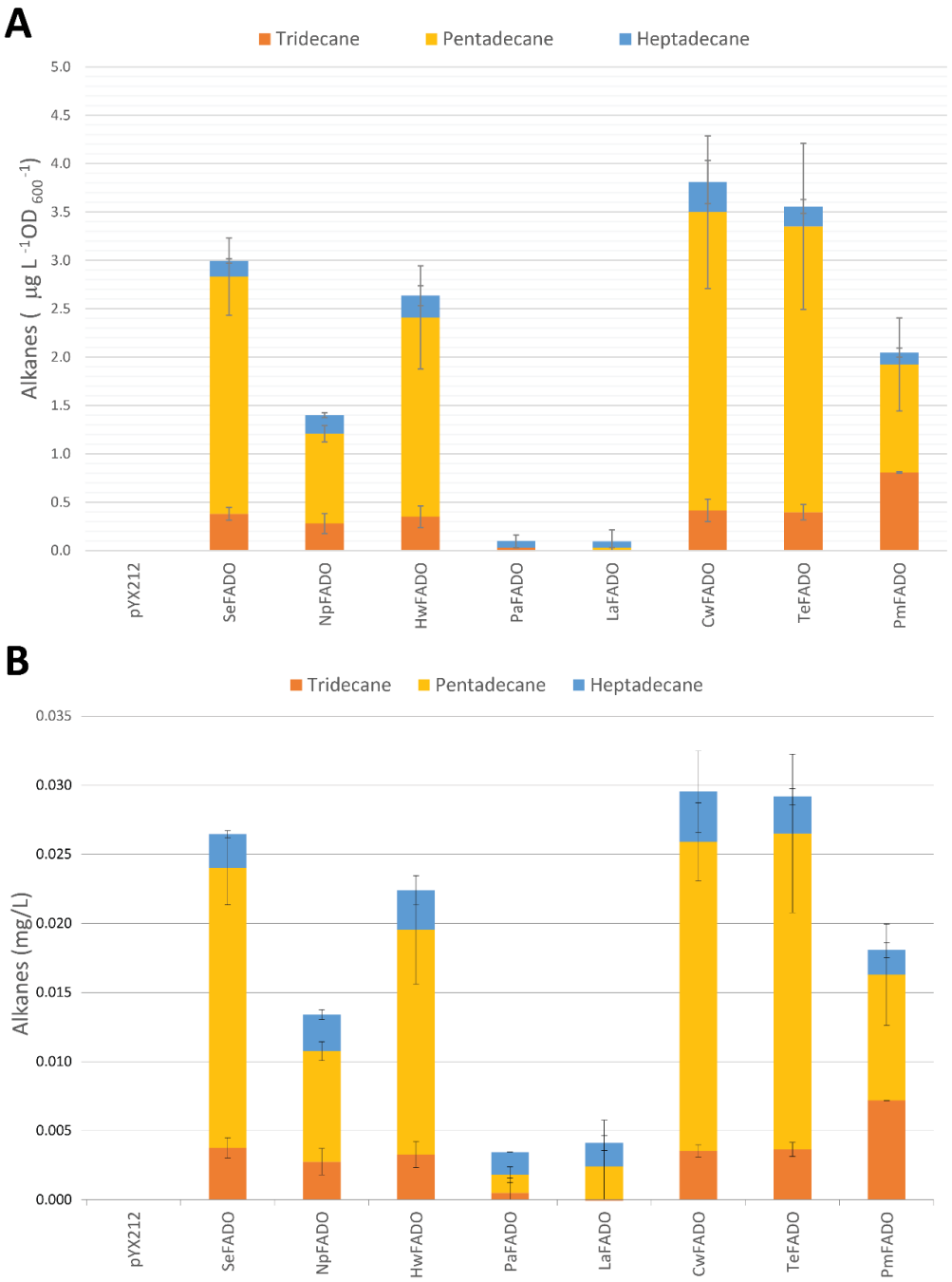


Figure 7: (A) biomass specific and (B) volumetric hydrocarbon titers of strains YJZ54 (Δ*HFD1* Δ*POX1* Δ*GAL80*; YJ Zhou, unpublished data) expressing a single genomic integration of *Mm*CAR, *An*NpgA, *Se*F/FNR. Different homologues of the fatty aldehyde deformylating oxygenase were expressed from a *GAL1* promoter on multicopy plasmid. Titers were corrected for any background signal observed in the control strain. Expressed FADO homologues were codon-optimized for yeast and derived from *S. elongatus* (SeFADO), *Nostoc punctiforme* PCC73102 (NpFADO), *Hapalosiphon welwitschii* IC-52-3 (HwFADO), *Planktothrix agardhii* NIVA-CYA 126/8 (PaFADO), *Lyngbya aestuarii* (strain CCY9616) (LaFADO), *Crocosphaera watsonii* WH 8501 (CwFADO), *Thermosynechococcus elongatus* (TeFADO), and *Prochlorococcus marinus* (strain MIT 9313) (PmFADO). Error bars represent the standard deviation of three individual biological clones, except for *Cw*FADO for which the average and standard deviatons of two clones are shown.

Discussion

In Paper I we have demonstrated that to enable heterologous alkane biosynthesis, the main obstacle proved to be endogenous aldehyde metabolism by Hfd1. The main byproducts were found to be fatty alcohols, most likely formed through reduction of fatty aldehydes by endogenous aldehyde reductases (ALR) and alcohol dehydrogenases (ADH). Subsequently, in Paper II we used peroxisomal compartmentalization to evaluate the alkane biosynthesis pathway and improve its titers. In addition, we expressed an olefin forming fatty acid decarboxylase and showed that its activity can be partially supported by a flavodoxin reducing system.

In the following sections the results presented and summarized in the previous chapter will be discussed and placed in a broader context.

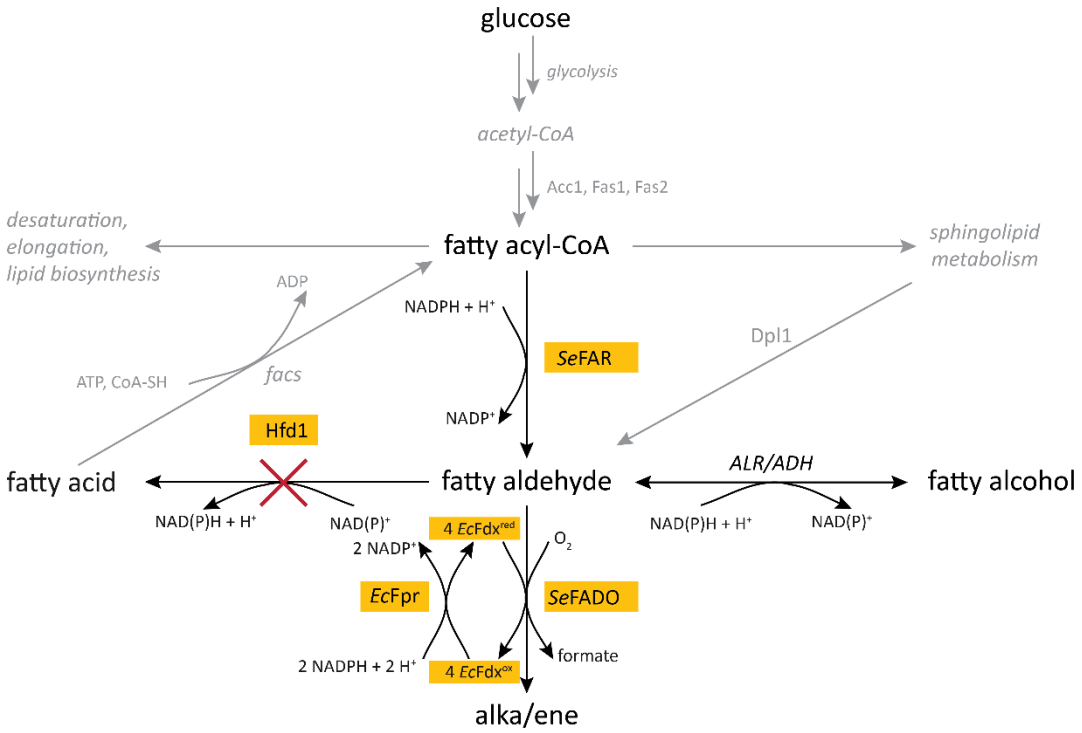


Figure 8: the main conclusions from Paper I were that Hfd1 has to be eliminated to enable biosynthesis of tridecane and pentadecane. Hfd1 is involved in sphingolipid metabolism, and converts the product of the Dpl1 catalyzed reaction into a fatt acid. Furthermore, expression of an *E. coli* reducing system to support the fatty aldehyde deformylating oxygenase (FADO) proved essential in a CEN.PK background strain. This reducing system consisted of ferredoxin (Fdx) and ferredoxin NADPH reductase (Fpr).

Competition for fatty aldehydes by Hfd1 and perhaps other aldehyde dehydrogenases

In Paper I (Figure 2) we have shown that the main obstacle for alkane biosynthesis by the cyanobacterial *Se*FAR+*Se*FADO pathway was the fatty acid forming fatty aldehyde dehydrogenase Hfd1 (see Figure 8). Hfd1 has been detected in the mitochondrial and lipid droplet proteome (Burri et al., 2006; Currie et al., 2014). For its *Homo sapiens* (human) orthologue encoded by *ALDH3A2* Keller *et al* (2014) have proposed that it forms dimers and that it is membrane bound with its active site facing the membrane (Keller et al., 2014). Keller *et al* have also shown that a helix controls the enzyme activity by acting like a lid on the active

site or substrate selection mechanism. This helix starts with a glutamine residue 445 and its removal diminished the enzyme activity towards hexadecanal without affecting the activity on dodecanal and octanal. Removing this helix could be a metabolic engineering target to avoid accumulation of unwanted short chain fatty aldehydes while still being able to produce long-chain alkanes. Lastly, Kelson *et al* (1997) have shown that human FALDH encoded by *ALDH3A2* is active on saturated and unsaturated short- to long-chain aldehyde substates (Kelson et al., 1997).

In retrospect, it should have been obvious that hexadecenal dehydrogenase Hfd1 would be an obstacle to alkane biosynthesis. However, it is also an example of a blind spot in some genome scale metabolic models that lack the dept and connectivity to expose such reactions. Furthermore, Hfd1 orthologs involved in fatty aldehyde metabolism in *Yarrowia lipolytica*, a model yeast that can consume alkanes, have been described only recently (Iwama et al., 2014) thereby complicating troubleshooting of the pathway. Also, industrial interest in Hfd1 at the time of writing of Paper I was limited to five patent applications describing overexpression or deletion of *HFD1*. Overexpression of *HFD1* has been was claimed to increase saffron production (Raghavan et al., 2011) or fatty acid production (Beardslee et al., 2011), or as a putative enzyme that might act on aldehyde intermediates in a 1,3-butanediol biosynthesis pathway (Burgard et al., 2011). Deletion of *HFD1* has been claimed as a possibility to improve isobutanol formation (Buelter et al., 2010; Lies et al., 2010).

Given the importance of Hfd1 elimination we were curious about which other enzymes could play a similar role. To get an idea of which other endogenous enzymes could take over Hfd1 aldehyde dehydrogenase activity, Hfd1 was blasted (Altschul et al., 1990) against the yeast proteome. It was found that Hfd1 shares homology (25-32% identity, E-value < $2e^{-11}$) with the aldehyde reductases Ald2-Ald6, cytoplasmic succinate-semialdehyde dehydrogenase Uga2, mitochondrial 1-pyrroline-5-carboxylate dehydrogenase Put2, and Msc7. Multiple sequence alignment of these yeast proteins using Omega Clustal (Sievers et al., 2011) is shown in Figure 9.

The aldehyde dehydrogenases Ald2 and Ald3 are cytoplasmic, NAD^+ dependent, glucose repressed, stress induced, and are involved in β-alanine biosynthesis (a precursor to CoA) (Christie et al., 2004). Ald3 has also been shown to be involved in phenylacetaldehyde oxidation (Kim et al., 2014). Ald4 is a mitochondrial, $NAD(P)^+$ dependent, glucose repressed, aldehyde dehydrogenase which is required for growth on ethanol. Deletion of *ALD4* results in decreased respiratory growth and sensitivity to oleic acid. *ALD4* expression can be induced by decanoic acid (Legras et al., 2010) and oleic acid (Koerkamp et al., 2002). Ald5 is a mitochondrial, $NADP^+$ dependent, constitutively expressed aldehyde dehydrogenase that is involved in regulation or biosynthesis of electron transport chain components (Kurita and Nishida, 1999) and involved in acetate metabolism. Ald6 is a cytosolic and mitochondrial, $NADP^+$ dependent aldehyde dehydrogenase that is involved in acetate biosynthesis. *ALD6* deletion results in inability of the yeast to utilize ethanol as carbon source and a reduced growth rate on complex medium containing glucose (Meaden et al., 1997). Uga2 is involved in glutamate degradation (via Gdh2, glucose repressed) and utilization of γ-aminobutyric acid (Bach et al., 2009; Christie et al., 2004). Uga2 orthologues from *E. coli* and *Salmonella typhimurium* have been shown to be able to convert short- to medium chain aldehydes into their acid derivatives. Given their low expression during growth (Bach et al., 2009), Uga2 activity is not very likely to play a big role in fatty aldehyde reduction. Put2 is involved in utilization of proline as nitrogen source and it has been shown that its substrate binding site has

some conformational flexibility (Pemberton et al., 2014). Msc7 is a protein with unknown function though partial disruption seems to indicate a role in meiosis (Thompson and Stahl, 1999).

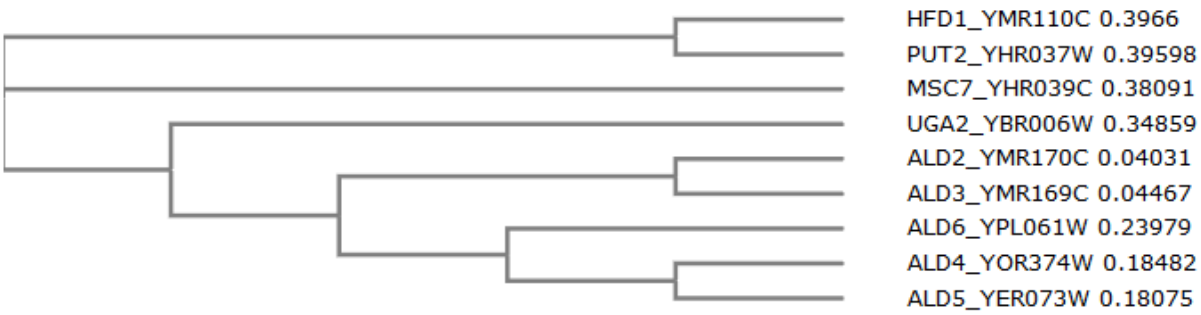


Figure 9: multiple sequence alignment using Omega ClustalW of Hfd1 and its homologues in yeast.

The involvement of several of these aldehyde dehydrogenases in important metabolic processes, such as coenzyme A biosynthesis and NADPH formation, makes them less attractive deletion targets. Furthermore, as has been shown in Paper I, deletion of *HFD1* resulted in over 400 µg/gDW of fatty alcohols, while less than 20 µg/gDW alkanes were formed upon introduction of *Se*FADO and F/FNR. Therefore, focusing on avoiding competition with fatty alcohol forming aldehyde reductases/alcohol dehydrogenases seemed a more promising approach.

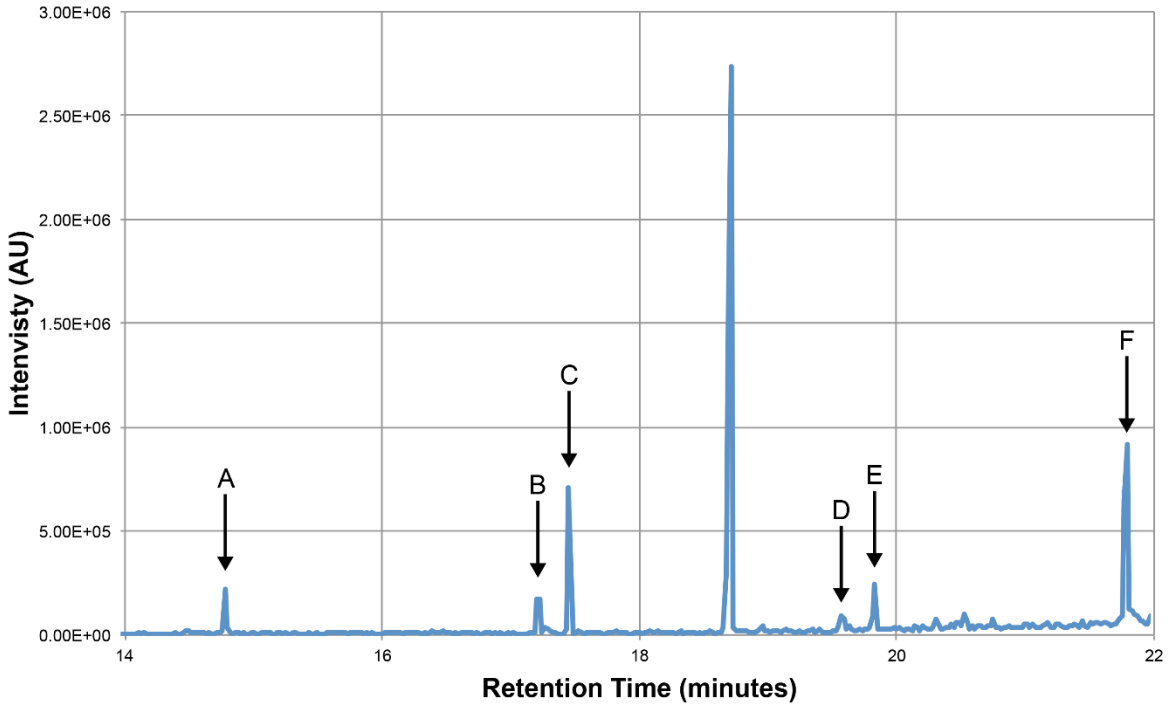


Figure 10: Typical GC spectrum of Δ*HFD1* strain expressing FAR, FADO, and F/FNR. The labelled peaks represent tridecane (A), a fatty alcohol and pentadecene (B), pentadecane (C), a fatty alcohol and heptadecene (D), heptadecane (E), and a fatty alcohol (F).

Peroxisomal compartmentalization to improve alkane and olefin biosynthesis

Since different factors seemed to be at play in limiting the alkane biosynthesis, compartmentalization of the pathway was considered. Compartmentalization could possibly enable better substrate channeling, concentration of the enzymes, and help to avoid competition for the substrate and intermediate by other enzymes. In the past, compartmentalization has been shown to be a useful strategy to increase productivity and has for example been used to improve isoprenoid (Farhi et al., 2011), isobutanol (Avalos JL et al., 2013), and penicillin biosynthesis (Herr and Fischer, 2014). Besides improving productivity, shielding the pathway from the cytosol could provide us with a model system to evaluate different versions of the alkane pathway.

The compartment of choice was the peroxisome, as it is the organelle in which fatty acids are degraded and therefore it should have ample supply of (activated) fatty acids (Hiltunen et al., 2003). One of the first studies employing the peroxisome for fatty acid-derived product formation has been described by Poirier *et al* (2001). In that study, compartmentalization was used as a means to improve medium-chain length polyhydroxyalkanoates (mcl-PHA) (Poirier et al., 2001). Mcl-PHAs are synthesized by PHA synthases from 3-hydroxyacyl-CoAs (Huijberts et al., 1994), which are intermediates of fatty acid biosynthesis and an intermediate of the Pox2/Fox2 catalyzed β-oxidation reaction (Hiltunen et al., 2003). In order to tap into the β-oxidation intermediates in the peroxisome, a PHA synthase from *Pseudomonas aeruginosa* was fused to the C-terminal 35 amino acids of a *Brassica napus* isocitrate lyase (which encodes a peroxisomal targeting signal). By expressing a mutant of *POX2*, the mcl-PHA titers could be increased 7-fold (De Oliveira et al., 2004).

In Paper II we applied compartmentalization to create an enclosed environment in which different strategies could be evaluated. Given the overlap with Paper I, in the following sections the results will be discussed topic wise.

Lesson from compartmentalization of the FADO enzyme

The rationale for our compartmentalization strategy was that concentration and shielding off the enzymes would improve titers. The idea of the FADO enzyme being the bottleneck has been described before, but in our study we might have shown a way how its activity can be improved. The higher increase in alkane titer of the peroxisomal versus the cytosolic pathway seems to suggest that proximity to a membrane and accumulation of fatty aldehydes in them stimulates the turnover by the FADO.

The idea of a membrane controlling the formation rate of a hydrophobic product has been observed for the yeast fatty acid synthase (Sumper, 1974; Sumper and Träuble, 1973). Sumper *et al* have shown that the yeast fatty acid synthase can only work efficiently (*in vitro*) if the hydrophobic substrate can be released into a membrane. This can be explained by the product being hydrophobic while the enzyme is surrounded by a hydrophilic environment. A sharp drop in activity, as observed without membrane presence, shows similarity to the *in vitro* activity of the FADO. For the latter enzyme, Li *et al* (2012) have observed an initial burst in product formation followed by a slowing down of the reaction to a steady state rate (Li et al., 2012). This slowing down seems to occur at the moment the product titer reaches its maximum solubility. For example, in case of heptanal the slow-down sets in after formation of 0.1-0.2 mM (as measured by the formation of formate). Deformylating oxygenation of heptanal leads

to formation of hexane, which has a solubility of 0.11 mM in water. In addition, Peramuna *et al* (2015) have suggested that high alkane titers upon FAR+FADO overexpression in cyanobacteria are supported by lipid droplet mediated alkane trafficking. According to that study, this was supported by the observation that the FAR (Npun-F1710) is most abundant when the *N. punctiforme* cells accumulate small lipid droplets in which the alkanes are presumably deposed (Peramuna et al., 2015). It is interesting that the *Pisum sativum* decarbonylase has been shown to require the presence of phospholipid phosphatidylcholine (PC) for reaching its potentially maximum activity (Schneider-Belhaddad et al., 2000). Furthermore, it has been suggested before that substrate availability is also limiting turnover (Das et al., 2013; Warui et al., 2011). This is probably because of the low solubility of long-chain fatty aldehydes and their tendency to form micelles, something which has been observed by Warui *et al*. Observations by Buer *et al* seem to support this hypothesis further since part of the long-chain substrate extends into the solvent (Buer et al., 2014), thereby creating an unfavorable environment for the fatty aldehyde compared to being inside a membrane. Furthermore, it has been shown that substrate delivery can be accommodated by addition of catalytic amounts of BSA (Eser et al., 2011; Eser et al., 2012). All this points in the direction of the transfer of the intermediate and product to and from the FADO enzyme as being a rate-limiting step. Part of this problem is solved by the FAR+FADO complex itself. Warui *et al* (2014) have shown that the FAR and FADO strongly interact and that this leads to efficient channeling of the (fatty) aldehyde intermediate (Warui et al., 2015). A solution could be to target the enzyme complex to an organelle membrane, or to place it on scaffold in the vicinity of it. Scaffolding has already been applied to increase the turnover of the FAR+FADO pathway (Rahman et al., 2014). Yeast cells control the ability of phosphatidate phosphatase Pah1 to access its phospholipid substrate by phosphorylation of its membrane anchor (Karanasios et al., 2010). When the anchor, which is an N-terminal helix, is phosphorylated, then Pah1 cannot dock onto the ER membrane. Without ER docking Pah1 cannot access its hydrophobic substrate and thus its activity is diminished. By adding for example such a helix (without the phosphorylation site) to *Se*FAR and *Se*FADO, the activity of the alkane pathway might be increased.

Interestingly, *E. coli* reaches much higher alkane titers compared to yeast. The *E. coli* plasma membrane contains mainly (75%) zwitterionic phosphatidylethanolamine and anionic (20%) phosphatidylglycerol (Cronan and Rock, 2008; Raetz and Dowhan, 1990). To the contrary, the yeast plasma (inner) membrane has been described to contain around 55% negatively charged phospholipids (Zinser et al., 1991), while the ER and mitochondria contain relatively more neutral/zwitterionic phosphatidylcholine (see (van Meer et al., 2008) for a review), similar to bacteria. This higher level of neutral/zwitterionic lipids might explain the accumulation of aldehyde and alkanes intracellularly and the effectiveness of Hfd1.

The low impact of peroxisomal targeting of the olefin pathway to the peroxisome pathway could contradict this explanation. However, a plausible explanation for the observation that the olefins showed a much smaller benefit is that the hydrogen peroxide substrate is the main limiting factor. This has also been observed by Chen *et al* (2015), who reported a big increase in titer upon hydrogen peroxide supplementation to the medium. Furthermore, in our study overexpression of cytosolic catalase *CTT1* diminished titers for the cytosolic OleT (data not shown).

At the moment this is merely a hypothesis and more research is required to support it, but given its potential it could be a very interesting follow-up study. A feature setting the alkane pathway

apart from other biofuels such as isobutanol and ethanol is that those products are fairly water soluble, even hygroscopic in case of ethanol. Furthermore, hydrophobic isoprenoid pathways typically make use of endogenous enzymes for precursor supply and eukaryotic terpene synthase (Tippmann et al., 2013), which could make their compatability with yeast less of an issue. In conclusion, the idea of a soluble cytosolic alkane plugin pathway might be misguided and transfer and deposition of the substrates and products should be considered in a subcellular contained environment, such as an organelle.

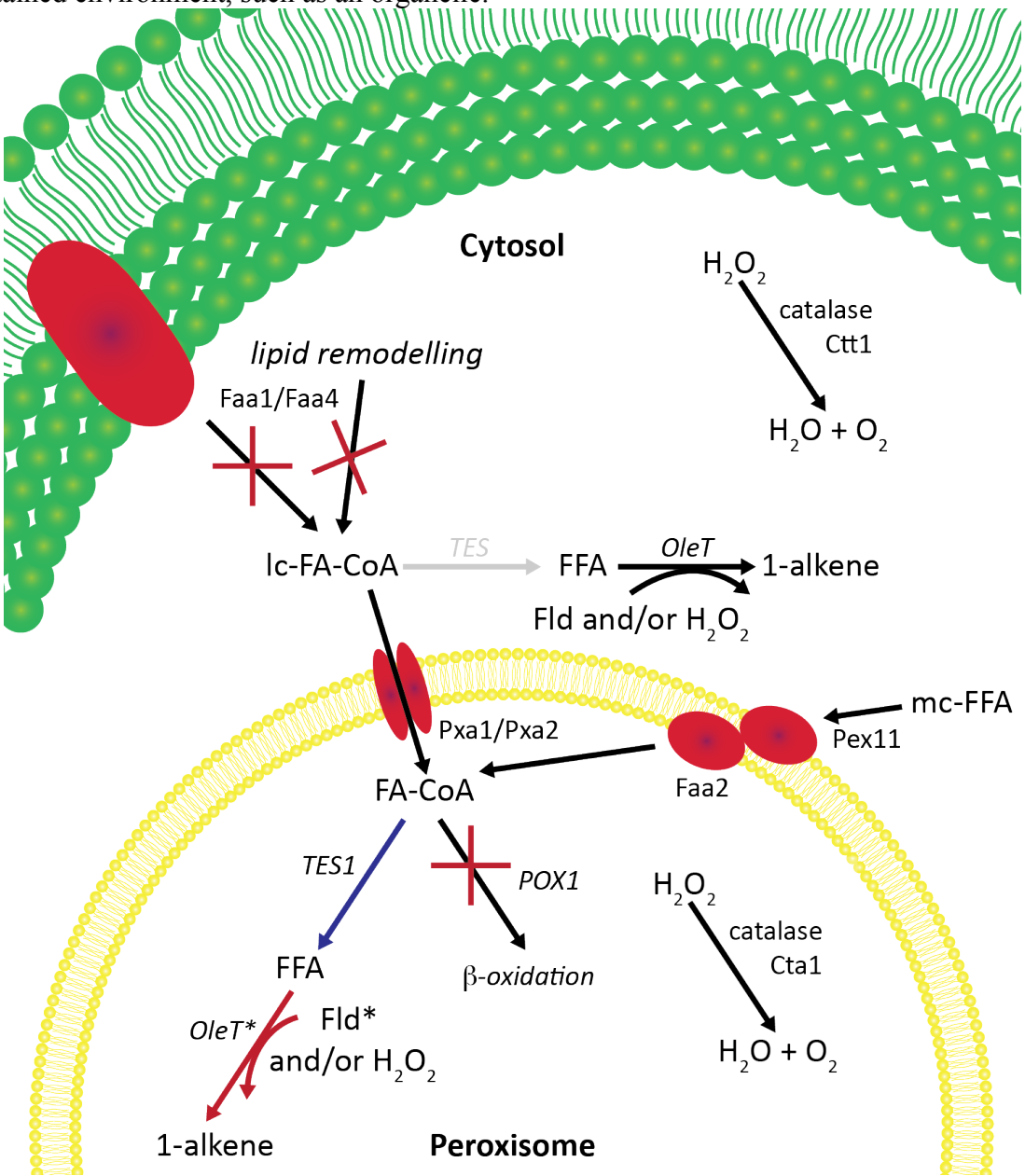


Figure 11: Peroxisomal compartmentalization as it has been applied to the terminal alkene pathway in Paper II. OleT is a decarboxylase that utilizes hydrogen peroxide (H_2O_2) as a cofactor. Flavodoxin (Fld) and flavodoxin NADPH reductase (not shown in figure) were tested as an alternative reducing system for OleT. H_2O_2 is degraded by cytosolic catalase Ctt1 and the peroxisomal catalase Cta1. Import of long-chain fatty acyl-CoAs (lc-FA-CoA) into the peroxisome is facilitated by the transporters Pxa1 and Pxa2. Import of medium-chain (mc) free fatty acids occurs through Pex11 and activation by Faa2. Fatty acyl-CoA (FA-CoA) can be hydrolysed by peroxisomal thioesterase Tes1. Free fatty acid titers were boosted by deletion of the genes encoding fatty acyl-CoA synthetases Faa1 and Faa4, and the oxidase Pox1. Targetting to the peroxisomes of OleT and flavodoxin was achieved by addition of a PTS, as denoted with a *.

The F/FNR reducing system of FADO

Paper I showed the importance of a reducing system for the FADO reaction in yeast. In paper II we tested *S. elongatus* and *E. coli* ferredoxin homologues in parallel and did not observe a significant difference (Paper II, SI Figure 2). This seems to indicate that the redox partner is not a limiting factor. However, this comparison was done in a Δ*POX1* strain, meaning that presence of Hfd1 could mask a beneficial effect of the reducing system from *E. coli* or *S. elongatus*. To our knowledge other studies have not compared both reducing systems *in vivo* by overexpression. Thus far, it has only been demonstrated that overexpression of the *S. elongatus* F/FNR is beneficial in *E. coli* (Kallio et al., 2014; Menon et al., 2015; Pásztor, 2015). Given its importance it might be interesting to discuss a publication by Schultz *et al* (2000) and the types of ferredoxin cofactors.

As has been described by Schultz *et al*, ferredoxin homologues can be divided into photosynthetic and heterotrophic classes for phototrophic organisms (Schultz et al., 2000). This classification was originally based on the tissue where the enzyme is expressed and whether it is regulated by light. Heterotrophic ferredoxin variants are more efficient at accepting electrons from NADPH than photosynthetic variants, which are more efficient at the reverse reaction (Schultz *et al*., 2000, and references therein). Schultz *et al* showed that for various plant acyl-ACP desaturases, which are structural homologues of the FADO (Krebs et al., 2011; Waugh, 2015), the activity was increased up to 20-fold *in vitro* by utilizing heterotrophic ferredoxin homologues. In that study, the heterotrophic *Impatients balsamina* ferredoxin supported roughly a two-fold higher cytochrome C reduction rate than heterotrophic *Anabaena sp.* ferredoxin. However, the *Anabaena sp.* ferredoxin supported a two-fold higher *in vitro* desaturase activity than the *I. balsamina* ferredoxin in some cases.

Zhang *et al* (2013) have identified petF and observed a 26.6% higher pentadecane yield *in vitro* by FADO supported by PetF in comparison with *Spinacia olera* ferredoxin, a photosynthetic ferredoxin (Zhang et al., 2013). The latter has been used *in vitro* by Schirmer *et al* to show that the FADO requires a cofactor (Schirmer et al., 2010). PetF was identified by screening for essential ferredoxin genes in *S. elongatus*. Screening for essential genes most likely results in detection of the phototropic ferredoxin homologue since that cofactor will be involved in essential electron transfer during photosynthesis. Multiple sequence alignment using Omega Clustal (Sievers et al., 2011) of the ferredoxin homologues described by Schulz *et al*, and the ferredoxin homologues used in paper 2 (*E. coli* Fdx and *S. elongatus* PetF1) is shown in Figure 12. The sequences of *E. coli* ferredoxin clusters together with the uncharacterized *S. elongatus* petF2 and the other heterotrophic ferredoxins. The *S. elongatus* petF clusters in its own branch, similar to the *Anabaena sp.* ferredoxin.

In light of the observations by Schultz *et al* and since the *E. coli* F/FNR has been ascribed a heterotrophic role (Wan and Jarrett, 2002), it might be beneficial to express other ferredoxin candidate genes from *S. elongatus*, or for example the *Impatiens balsamina* ferredoxin.

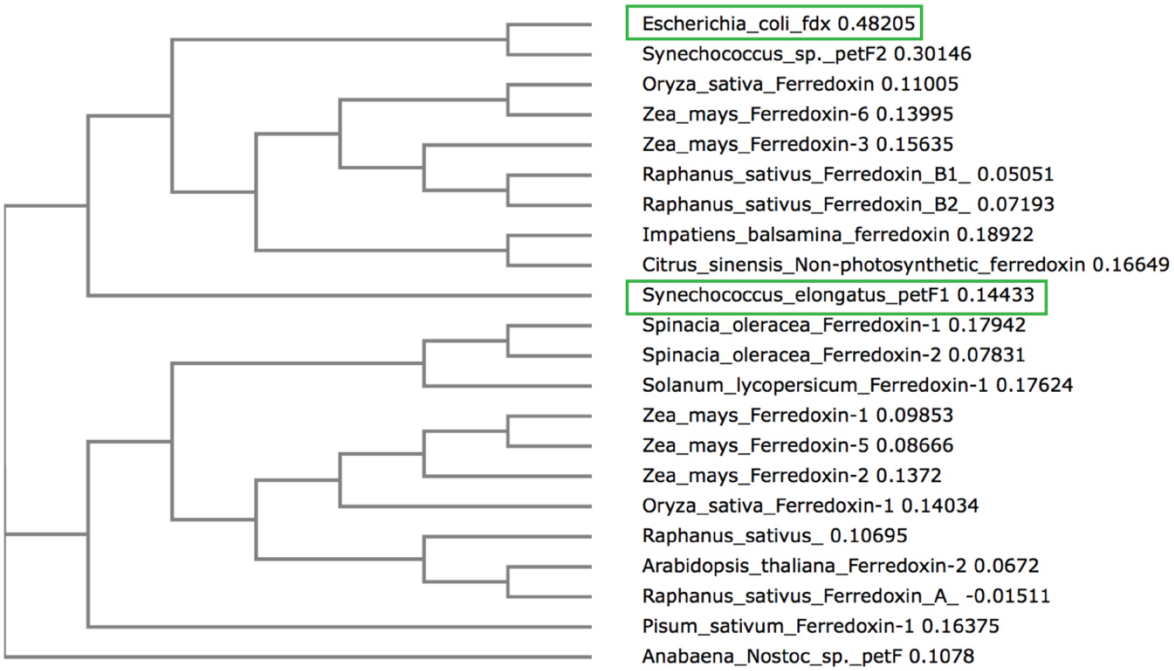


Figure 12: Multiple sequence alignment with Omega Clustal of ferredoxin homologues. *E. coli* ferredoxin fdx and *S. elongatus* petF1 have been expressed in our studies and are marked with green boxes.

Increasing peroxisome size and number

After compartmentalization of the alkane pathway had resulted in improved titers, we sought to increase the number and size of peroxisomes. A study by Vizeacoumar *et al* (2004) has investigated the effect of *PEX30*, *PEX31*, and *PEX32* deletion on these parameters for cells grown on oleate (Vizeacoumar et al., 2004). In that study they have found that these three peroxins are negative regulators of peroxisome size and numbers. Pex11 (alias Pmp27) is considered to be a positive regulator of peroxisome number. This has been concluded from the observation that Δ*PEX11* show few and enlarged peroxisomes (Erdmann and Blobel, 1995), and strains overexpressing *PEX11* contain more but smaller peroxisomes (Marshall et al., 1995). Furthermore, overexpression of the *PEX11* homologues pexK and PEX11 in *Aspergillus nidulans* and *Penicillium chrysogenum*, respectively, increased the number of peroxisomes and increased penicillin production on lactose over twofold (Herr and Fischer, 2014; Kiel et al., 2005). *PEX34* overexpression has been shown to increase the number of peroxisomes when cells are grown on glucose, while it does not lead to obvious peroxisome size differences (Tower et al., 2011). In our study, overexpression of *PEX34* had a negative effect on the biomass yield (Paper II, SI Figure 5), possibly due to increased energy expenditure on peroxisome generation.

Pex11 has also been shown to be essential for short and medium chain fatty acid degradation (Van Roermund et al., 2000), which suggests that it acts as a transporter for the substrate of acyl-CoA synthethase Faa2. This dual role as a transport protein has been confirmed by Mindthoff *et al* (2016), who have shown that Pex11 forms a membrane channel (Mindthoff et al., 2016). Transport through this channel has been proposed to be controlled by phosphorylation since it controlled the rate of β-oxidation. Phosphorylation of Pex11 is also required for its activity as promoter of peroxisome proliferation and phosphorylation has been shown to be induced by glucose presence (Knoblach and Rachubinski, 2010). Induction of phosphorylation by glucose has been explained from the higher growth rate of cells on this substrate and the need for peroxisome proliferation to keep up with cell division. Pex11

phosphorylation is mediated by the kinase Pho85 and its overexpression has been shown to enhance peroxisome proliferation and elongation in glucose and oleate containing medium (Knoblach and Rachubinski, 2010). Another mechanism of Pex11 activity regulation is oligomerization with other peroxins and inhibition of dimer formation has been described to stimulate peroxisome proliferation (Marshall et al., 1996). Marshall *et al* also have demonstrated that a point-mutation in Pex11 could disrupt homo-oligomerization of Pex11 and this subsequently leads to hyper-proliferation of peroxisomes. Pex11 transcription is repressed by glucose (Knoblach and Rachubinski, 2010), which could explain the positive effect of *PEX11* overexpression observed in our study.

In our experiments, *PEX11* overexpression in combination with *PEX34* reversed the increase observed in an alkane producing strain overexpressing *PEX34* alone. Pex34 is known to interact with other peroxins, including Pex11 (Tower et al., 2011). Given the role of oligomerization in the control of Pex11 activity, the similar titers in the *PEX11* and *PEX34* overexpressing strain compared to the reference could possibly be explained by formation of heterodimers, which would deactive Pex34. This supports the observation that biomass and product titers recover to the levels of the control strains without *PEX11* or *PEX34* overexpression. The results presented in Paper II Figure 3 represent an attempt to increase titers, and above all to study the pathway dynamics by increasing the number of peroxisomes. Based on titer data and limited fluorescence images alone not much can be concluded about the exact influence that the deletions and overexpressions have on the peroxisomes.

Extending the story towards olefins

At the start of this project, two pathways had been described in detail by Schirmer *et al* (2010) and Rude *et al* (2011) that could be expressed in yeast to enable hydrocarbon biosynthesis. The first one being the FAR+FADO pathway, yielding alkanes and alkenes, and the second the OleT catalyzed pathway, yielding olefins. We initially started with both projects but failed to detect products for the olefin pathway. After we had solved the problems with the FAR+FADO pathway we decided to pick up the work on the OleT pathway again. Given the promising results with the alkane pathway in the peroxisome we chose to apply the same strategy to the OleT (Figure 11). Furthermore, a study by Liu *et al* (2014) had shown that OleT could potentially be supported by flavodoxin, which could be an important improvement of this reaction (Liu et al., 2014b).

In another recent study using *E. coli*, the olefin forming fatty acid decarboxylase OleT has been shown to convert free fatty acids (FFA) released by an extracellular lipase into the medium (Yan et al., 2015). FFA levels have been increased in *S. cerevisiae* by deletion of *FAA1* and *FAA4* (Leber et al., 2014; Scharnewski et al., 2008). Furthermore, Chen *et al* (2015) have applied the same strategy in *S. cerevisiae* to OleT and were able to boost olefin titers by 8-fold to 383 $\mu g\ L^{-1}$ (Chen et al., 2015). In addition, these deletions have been shown to result in fatty acid accumulation and excretion, followed by reimport during the stationary phase (Scharnewski et al., 2008). Therefore, with the study by Chen *et al* in mind, we decided to use a Δ*FAA1* Δ*FAA4* Δ*POX1* as a background strain for the evaluation of the OleT enzyme in the cytosol and peroxisomes.

In Figure 4 and Paper II Figure 4 the results are shown for expression of OleT with or without a supporting flavodoxin reducing system consisting of *E. coli* flavodoxin (Fld) and flavodoxin NADPH reductase (Fpr). The main olefin produced by the yeast was 1-heptadecene, suggesting a preference for C18:0 fatty acids. This is in agreement with what has been observed by Rude

et al (2011), who have shown that OleT prefers C18:0 fatty acids over C18:1 and C16:0. In addition, it is in agreement with the results obtained by Chen et al, who have found that C17:0 is the main olefin in a *ΔFAA1 ΔFAA4* background. Chen *et al* have also described detection of C19:0 olefins, we have not quantified C19 alkenes. The beneficial effect of flavodoxin seems to indicate it can support activity of OleT. However, based on our data it can not be excluded that this could be through generation of hydrogen peroxide by a reversed electron transfer reaction of its flavin mononucleotide group with oxygen. Furthermore, in our study the observed titers did not reach similar levels as in *E. coli* or as observed by Chen *et al*. The cytosolic and the peroxisomal OleT yielded less than half the titer described in the latter study. The difference between the titer observed by Chen *et al* can perhaps be partially explained by the different type of medium used (synthetic dropout medium, 1% raffinose, 2% galactose). Elimination of the main fatty acyl-CoA synthetases had the expected effect of releasing the fatty acid substrate into the medium, but the uptake did not seem take place. As shown in Figure 13, extracellular FFAs clouded the medium and formed vesicles that could be stained with nile red. A difference between yeast and bacteria might be that components excreted by the yeast increase the solubility of the hydrophobic compounds in the medium by acting as surfactants (Heeres et al., 2015), thereby making it easier for fatty acids to diffuse into the medium from the cell membrane. Interestingly, it has been shown that a large fraction of fatty acids adsorp to glass (shake flasks) or move to the water air interface (Vorum and Brodersen, 1994), thereby complicating reuptake by cells. In summary, deletion of fatty acyl-CoA synthetases is not an efficient solution for increasing free fatty acid levels for OleT in yeast.

Relocalization of OleT to the peroxisome led to a small improvement in olefin titer (Figure 4 and Paper II Figure 4), and additional expression of a peroxisomal targeted flavodoxin reducing system could increase the titer similar to what has been observed in the cytosol (data not shown). Overexpression of *PEX34* in case of the peroxisomal pathway lowered volumetric titers almost threefold. Furthermore, overexpression of the peroxisomal fatty acyl-CoA thioesterase *TES1* had no significant effect on the olefin titer either (data not shown). This suggests that OleT is not limited by its substrate, nor benefits from compartmentalization as discussed earlier for the FADO, and that the bottleneck may be elsewhere. Perhaps increasing the number of peroxisomes dilutes the substrate and enzyme, thereby reducing the overall efficiency of the enzyme. An alternative strategy could be to improve OleT activity by increasing levels of its cofactor and cosubstrate prior to overexpressing *PEX34*. This is again supported by the study of Chen *et al* (2015), who have demonstrated that olefin titers are boosted by heme and hydrogenperoxide supplementation.

Interestingly, Dennig *et al* (2015) have suggested that OleT could also act as a monooxygenase, utilizing O_2 instead of H_2O_2 as a cosubstrate. Oxygen as possible cosubstrate could fit with our observation that expression of cytosolic OleT still results in olefin formation in a strain overexpressing the cytosolic catalase *CTT1* (data not shown), and the observation that peroxisomal OleT yields only slightly lower titers in the presence of peroxisomal catalase Cta1 and absence of the flavodoxin reducing system (data not shown). The latter would still require confirmation by overexpression of *CTA1*, since its expression has been found to be repressed by glucose (Hörtner et al., 1982). Another reasonable explanation could be the presence of an unknown cofactor. Lastly, in our study we expressed OleT, flavodoxin, and flavodoxin/ferredoxin NADPH reductase from constitutive promoters (pTDH3, pPGK1, and pTEF1, respectively). Expression levels could affect the activity of OleT as has been shown by Dennig *et al* who observed that the ratio of a putidaredoxin reducing system CamAB versus OleT affects the amount of 1-heptadecene formed *in vitro* (Dennig et al., 2015). In their study,

in which OleT was extensively characterized, Dennig *et al* observed a drop of over two-fold in olefin formation after increasing the CamAB level ten-fold. In addition, Chen *et al* (2015) have demonstrated that expression of OleT from a high-copy plasmid is detrimental to the olefin titer in comparison with a low-copy plasmid.

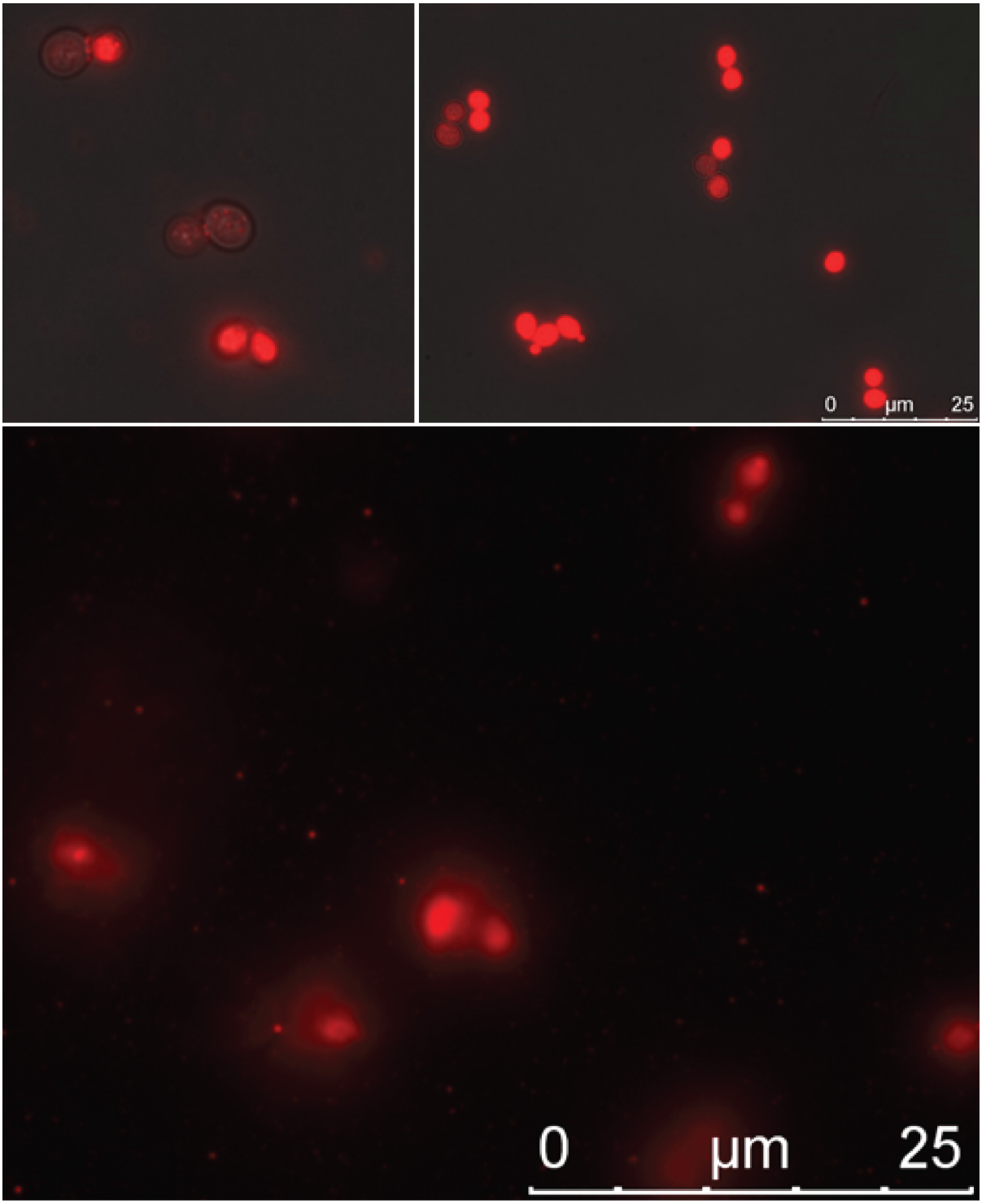


Figure 13: Nile red staining of KB111 (YJZ06 Δ*FAA1* Δ*FAA4* Δ*POX1* + empty pYX212 vector) cell cultures. The top left photo shows stained cells in focus, the bottom photo shows stained cells and droplets with the droplets in focus, the top right figure shows cells that were washed with PBS prior to nile red staining. The extracellular droplets that were observed could be stained by nile red and droplets dissappaered after washing cells with PBS buffer before staining. Cultures were checked for contamination by plating and microscopy.

The LuxCDE fatty acid reductase complex and FADO comparison

The *Photorhabdus luminescens* LuxCDE fatty acid reductase complex is an alternative to the *Mm*CAR utilized in Paper II. The complex consists of a fatty acyl reductase LuxC, fatty acyl transferase LuxD, and acyl-protein synthetase LuxE. LuxE activates free fatty acids using ATP to form an fatty acyl-AMP intermediate. This intermediate subsequently acylates LuxE, a reaction that releases AMP. This bound acyl group is then transferred from LuxE to the reductase LuxC. However, LuxC acylation can also by fatty acyl-CoA and LuxC can also reduce acyl groups bound to LuxE. The reduction is NADPH-dependent and the acylation step controls the reaction rate (Wall et al., 1986). There is still some ambiguity regarding the function of LuxD. LuxD has been described as a myristoyl-ACP thioesterase that acts as an acyl transferase and a study has suggested its involvement in the stability of the LuxC-LuxE complex (Howard et al., 2013; Li et al., 2000). The free fatty acids titers that have been detected by Howard *et al* seem to suggest that LuxD is not a very efficient thioesterase, in comparison with for example *E. coli* thioesterase TesA' overexpression (Xu et al., 2013). To avoid that the substrate could become limiting we chose to express *E. coli* thioesterase TesA', together with the *P. luminescens* LuxCDE complex, and either *Se*FADO or *Np*FADO.

In Figure 6 the results are shown of expression of the Lux pathway. These results demonstrate the functionality of this pathway in a BY4741 *ΔHFD1* background. Its application for alkane biosynthesis in *E. coli* has been described by Howard *et al* (2013). In that study, the observed titers of 5 mg L^{-1} were lower than what has been found by Schirmer *et al* (2010) with a FAR+FADO pathway. However, Howard *et al* have observed that the LuxCDE+NpFADO pathway did lead to an approximately threefold higher titer than a *Np*FAR+*Np*FADO pathway. Interestingly, addition of tetradecanoic acid to the medium resulted to a sharp decrease in alkane formation and increased fatty alcohol titers. We abandoned the idea of utilizing *Pl*LuxCDE because it requires expression of at least three genes, while for *Mm*CAR only *An*NpgA needs to be coexpressed. Therefore titers were not quantified in a CEN.PK background strain.

In Paper II we used *Mm*CAR instead of *Se*FAR as it seemingly resulted in higher titers. Part of the low activity of *Se*FAR (beside it not being the preferred substrate) could come from competition for fatty acyl-CoA, as has been shown for FaCoAR in Paper II. Unfortunately we could not obtain a fatty acyl-CoA overproducing strain (Valle-Rodríguez et al., 2014) that also contained an Δ*HFD1*, complicating further engineering of strains with a *Se*FAR+*Se*FADO pathway.

In Figure 7, alkane titers are shown as observed for strains expressing various FADO homologues. None of the FADOs displayed a significantly higher titer than the *Se*FADO that has been used thus far in our studies. The *Np*FADO, *Pa*FADO, and *La*FADO performed worse than the reference strain. It should be noted that expression was induced during the ethanol phase, which had a severe negative effect on the overall titer. The observation that the PaFADO produces much less pentadecane seems to conflict with the hydrocarbon profile that has been obtained for the strain *Planktothrix agardhii* NIVA-CYA 126/8 (Coates et al., 2014). In that study this species was the only one showing pentadecane accumulation (approximately 12% of total hydrocarbon content). Possibly, efficient functioning of the FADO requires coexpression of its FAR counterpart, as it has been shown to form a complex (Warui et al., 2015).

Results in context of other studies on alkane biosynthesis

The only other point of reference regarding heterologous long-chain alkane synthesis by yeast is the recent study by Foo *et al* (2015). The biosynthesis of very-long chain alkanes has been described by Bernard *et al* (2012). The study by Foo *et al* was interesting since it took an alternative route to alkane biosynthesis. To avoid competition for NADPH by a FAR, and to have the flexibility to add fatty acids as a feedstock, they chose to express an α-dioxygenase to catalyze the conversion of free fatty acids into fatty aldehydes (Foo et al., 2015). Dioxygenases catalyse the first reaction in the α-oxidation cycle of fatty acids (presumably absent in *S. cerevisiae*). This reaction requires only oxygen and leads to the formation of a 2-hydroperoxy fatty acid intermediate, which spontaneously degrades into a fatty aldehyde with the loss of one carbon in the form of CO_2 (Kaehne et al., 2011). In addition to a *Oryza sativa* α-dioxygenase, a *S. elongatus* FADO was expressed to subsequently convert the fatty aldehydes into alkanes. Foo *et al* did not express a reducing system. Since they used a BY4741 background strain this is consistent with our observation that alkanes could be detected in this background without expression of the *Ec*F/FNR (Paper I, SI Figure 3), in contrast with a CEN.PK background.

Initially, the yeast constructed by Foo *et al* produced less than 3 μg/L alkanes during growth in medium supplemented with 200 mg/l fatty acids. By increasing the medium pH to 7.0, titers were shown to increase to approximately 350 μg/L. This increase in titer has been explained by the authors by the increased in pH, which should raise the solubility of fatty acids. To synthesize alkanes without addition of fatty acids, Foo *et al* have subsequently expressed the alkane pathway in a Δ*FAA1* Δ*FAA4* knock-out strain. This did not yield any alkanes, even though a similar strain expressing only the dioxygenase was reported to accumulate 1.2 mg/L fatty aldehydes. The authors have speculated that this could be because of accumulation of reactive aldehydes intermediates. Expression of the FADO from a constitutive promoter and the αDOX from a galactose inducible promoter resulted in 73.5 μg/L alkanes, though the control strain with a nonfunctional FADO produced around 42 μg/L. No extracellular alkanes were detected (Foo et al., 2015).

A recent study on odd-chain fatty alcohol biosynthesis in *S. cerevisiae* could give an idea of how the αDOX pathway compares to the FAR pathway in an *HFD1* background strain. In a recent study, Jin *et al* (2016) have expressed *Oryza sativa* α-dioxygenase (DOX) in a CEN.PK *ΔFAA1 ΔFAA4 ΔHFD1* background to synthesize odd-chain fatty alcohols (Jin et al., 2015). Remarkably, deletion of *HFD1* in a CEN.PK *ΔFAA1 ΔFAA4* strain was shown to lead to an overall decrease in fatty alcohol titer in comparison with its ancestor (from 20.9 to 12.9 mg/L). Only the titer of C15:0 fatty alcohols increased in the triple deletion strain and the apparent increase is in the same range as observed in Paper I. The decrease in titer is an interesting observation, because fatty acyl-CoA synthetases are downstream of Hfd1 in the spingholipid metabolic pathway. Jin *et al* have speculated that Hfd1 may play a key role in the metabolism of unsaturated fatty acids. The *ΔFAA1 ΔFAA4* strain expressing αDOX showed a 75% growth inhibition compared with a strain carrying an empty plasmid. Therefore, another explanation could be that the increased fatty aldehyde levels decrease cell growth further and thereby decrease the overall fatty aldehyde and alcohol titers. This hypothesis is supported by the observation by Nakahara *et al* (2012) that C16 fatty aldehyde accumulation in a 2.5-5 μM range and above is toxic to *HFD1* knock-out strains. The *ΔFAA1 ΔFAA4* αDOX expressing strain was shown to accumulate approximately 25 μM and 50 μM of pentadecanal and pentadecenal

(please note that the dioxygenase reaction leads to a carbon loss). Furthermore, Foo *et al* have also observed a detrimental effect on product titer with high expression levels of αDOX.

As mentioned in the introduction, Bernard *et al* (2012) have described the biosynthesis of very-long chain alkanes. In their study they have expressed the *Arabidopsis thaliana* fatty aldehyde reductase/decarbonylase *CER1* and *CER3* and cytochrome b5 in a very-long chain fatty acid overproducing strain. This led to the biosynthesis of 19 μg/gDW very-long chain alkanes, which is similar to the 22 μg/gDW long-chain alkanes that were detected in our study (Paper I, Figure 2). In the study by Foo *et al* (2015), the even-chain alkanes tetradecane and hexadecane have been observed to be synthesized *de novo* by the yeast expressing a αDOX and *Se*FADO. This is consistent with detection of pentadecane and heptadecane in our strains expressing the FAR+FADO, LuxCDE+FADO, and *Mm*CAR+*Se*FADO pathway. The long-chain alkane titer in the ΔHFD1 is a significant improvement over the titer observed for the background strain, but still low in comparison with (cyano) bacteria. To put this in perspective, a recent study has described a naturally occurring *Aureobasidium pullulans var. melanogenum* yeast excreting 32.5 g L^{-1} of alkanes (Liu et al., 2014c). Further work should focus on improving the FADO reaction.

Future Directions

From the results discussed in the previous chapter and current literature it can be concluded that the alkane titers are mostly limited by the FADO enzymes.

Avenues for increasing FADO activity

Activity of the FADO depends on iron being present in its active site. Schirmer *et al* have shown that titers could be boosted from approximately 25 mg L^{-1} to over 300 mg L^{-1} by utilizing a modified medium. In this medium, among other things, the Fe(III)Cl concentration has been increased over threefold to 0.1 mM and surfactant Triton X 100 added. Jia *et al* (2014) have shown that increasing the iron content in the growth medium (complex medium +2 mmol l^{-1} $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) affects the ratio of enzyme with bound iron. However, they have also observed that it was very hard to obtain proteins with two iron atoms bound, most likely because of flexibility of the FADO's iron binding pocket. Interestingly, an abandoned patent application has claimed overexpression of a component of a siderophore (iron scavenger) synthesis to improve fatty aldehyde biosynthesis (Hu and Greenfield, Derek L. Arlagadda, 2011).

Long-chain fatty aldehyde do not completely fit in the active site of the FADO, which leaves them exposed to the hydrophilic environment (Andre et al., 2013; Buer et al., 2014). In addition, Buer *et al* have shown that the substrate can enter the active site in different orientations, including an orientation in which the aldehyde extends into the solvent, and through an alternative channel which would inhibit the enzyme. Waugh (2015) has suggested to create a hybrid enzyme in between the methane monooyxgenase and FADO (Waugh, 2015). Such a hybrid could accommodate the difficulties with substrate loading and product offloading. As discussed in the previous section, substrate and product transfer could possibly be accommodated by bringing the FAR+FADO complex in the vicinity of organel membranes.

Improving extraction and detection method

As described in the Results section, a lipid extraction method was used to extract intracellular alkanes from yeast. The main problems with the current extraction and measurement method is that it co-purifies fatty acids and fatty alcohols together with the hydrocarbons, and that the recovery of the internal standard is poor. The presence of of fatty acids pollutes the GCMS analytical system. The reason that we chose to accept this is that initially the titers were so low that a derivatization method would push the titers below the detection limit. Furthermore, reliable quantification of pentadecadiene and heptadecadiene would require verification with analytical standards to remove any possible doubt of their levels and presence. As a way forward lipid extraction could be followed by an additional FAME derivatization.

Screening methods for improved fatty aldehyde and alkane biosynthesis

The current extraction method does not allow for high-throughput screening due to the requirement of relatively high culture volumes (4-8 mL). Therefore, a screening method based on intracellular product levels would be a big improvement. A screening method for alkane synthesis could be based on the alkane sensing mechanism of the yeast *Yarrowia lipolytica*, which has been studied in some detail. In this organism, the expression of alkane metabolic genes are controlled by the transcription activators Yas1 and Yas2 (Endoh-Yamagami S et al., 2007; Yamagami et al., 2004), and the transcriptional repressor Yas3 (Hirakawa et al., 2009;

Kobayashi et al., 2013). The transcriptional activators bind to alkane response elements (AREs) in the promoter of several *Y. lipolytica* alkane catabolic genes (Iida et al., 2000). Others have applied prokaryotic alkane responsive transcription factors to construct heterologous alkane biosensors in *E. coli.* For example, the transcription factors *Pseudomonas putida* AlkS (Reed et al., 2012) and *Acinetobacter baylyi* ADP1 AlkR (Wu et al., 2015) have been used to detect short- to medium-chain, and medium- to long-chain alkanes, respectively.

Besides alkanes, a biosensor for the fatty aldehyde intermediate could help to improve the FAR reaction in absence of an efficient FADO. The fatty aldehyde intermediate is also part of the bacterial bioluminescence pathway (Meighen, 1991) of which the first half was used by Howard *et al* (2013). For example, fluorescence by the fatty aldehyde consuming *Vibrio fischeri* lucifarase has been applied to show functionality of the α-dioxygenase in *E. coli* (Kaehne et al., 2011).

Conclusion

The aim of this project was to engineer a *Saccharomyces cerevisiae* yeast strain to enable *de novo* alkane biosynthesis from glucose. To this end, a cyanobacterial alkane biosynthesis pathway was introduced in yeast. This cyanobacterial pathway from *Synechococcus elongatus* consisted of a fatty acyl-CoA/ACP reductase (FAR) and a fatty aldehyde deformylating oxygenase (FADO).

As described in Paper I, we discovered upon expression in yeast that the FADO requires the additional expression of a reducing system consisting of ferredoxin and ferredoxin NADPH reductase (F/FNR). Interestingly, this F/FNR reducing system was not required in a BY4741 background. Besides the reducing system, we also found that the heterologous alkane biosynthesis pathway intersect with sphingolipid metabolism at the fatty aldehyde intermediate. This had the consequence that a hexadecenal dehydrogenase Hfd1 had to be deleted to enable conversion of C16 and shorter fatty aldehydes into alkanes by the FADO enzyme. The main detected byproducts were fatty alcohols, which were possibly formed by promiscuous endogenous ALRs and ADHs.

In order to avoid such byproduct formation and to create a model system in which we could evaluate alternative strategies for the alkane pathway, we explored peroxisomal compartmentalization in Paper II. The results showed that alkane biosynthesis benefited from the concentration of the pathway enzymes and presence of an enclosing bacterial-like membrane. Conversion of fatty aldehyde by Hfd1 could not be prevented by localization of the pathway in the peroxisome, but the fatty alcohol byproduct titer could be reduced. However, these byproducts were still present at much higher levels than the hydrocarbons and further strategies need to be developed to overcome these challenges.

The compartmentalization strategy was also extended to olefin biosynthesis. Olefin biosynthesis proceeds through a one-step reaction from fatty acids, catalyzed by *Jeotgalicoccus* sp fatty acid decarboxylase OleT. This enzyme did not benefit to the same extent as the alkane pathway from compartmentalization, but we could show that OleT could utilize the cofactor flavodoxin *in vivo* in addition to its natural cosubstrate. However, more work is required to establish its role as a redox partner.

In conclusion, this thesis describes the first example of heterologous alkane biosynthesis in the yeast *Saccharomyces cerevisiae*. Future work should focus on improving the FADO catalyzed reaction as it was shown to be the main rate-limiting enzyme. This can possibly be achieved by facilitating the transfer of the hydrophobic intermediates and products of the pathway into lipid membranes.

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Long-chain Alkane Production by the Yeast *Saccharomyces cerevisiae*

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ABSTRACT: In the past decade industrial-scale production of renewable transportation biofuels has been developed as an alternative to fossil fuels, with ethanol as the most prominent biofuel and yeast as the production organism of choice. However, ethanol is a less efficient substitute fuel for heavy-duty and maritime transportation as well as aviation due to its low energy density. Therefore, new types of biofuels, such as alkanes, are being developed that can be used as drop-in fuels and can substitute gasoline, diesel, and kerosene. Here, we describe for the first time the heterologous biosynthesis of long-chain alkanes by the yeast *Saccharomyces cerevisiae*. We show that elimination of the hexadecenal dehydrogenase Hfd1 and expression of a redox system are essential for alkane biosynthesis in yeast. Deletion of *HFD1* together with expression of an alkane biosynthesis pathway resulted in the production of the alkanes tridecane, pentadecane, and heptadecane. Our study provides a proof of principle for producing long-chain alkanes in the industrial workhorse *S. cerevisiae*, which was so far limited to bacteria. We anticipate that these findings will be a key factor for further yeast engineering to enable industrial production of alkane based drop-in biofuels, which can allow the biofuel industry to diversify beyond bioethanol.

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KEYWORDS: alkanes; fatty alcohols; fatty aldehydes; *Saccharomyces cerevisiae*; aldehyde dehydrogenase; advanced biofuels

Communication to the Editor

Renewable biofuels have been developed over the past decade in an effort to move away from fossil based transportation fuels. Of these biofuels, ethanol is the most prominent fuel type, but it is not an optimal fuel due to its low energy density. Furthermore, restrictions on blending of ethanol with gasoline (i.e. the blend wall) also limit the further expansion on the use of this biofuel (Peplow, 2014). As a consequence, new types of biofuels are being developed that can be used as drop-in fuels; these fuels are often referred to as advanced biofuels (Fairley, 2011). Medium- and long-chain alkanes, a specific type of advanced biofuels, are of interest as kerosene and diesel substitutes because of their high similarity to fossil oil derived transportation fuels (Lennen and Pfleger, 2013). The first example of *de novo* heterologous production of alkanes was achieved in *Escherichia coli* by the introduction of a cyanobacterial fatty acyl-ACP/CoA reductase (FAR) and a fatty aldehyde deformylating oxygenase (FADO) (Schirmer et al., 2010), which resulted in an alkane titer of approximately 25 mg/L. Subsequently, alternative FAR and FADO enzymes have been expressed in *E. coli* (Akhtar et al., 2013; Howard et al., 2013; Kallio et al., 2014), with a highest reported titer of 580 mg/L (Choi and Lee, 2013). So far, these studies have been limited only to bacteria and the feasibility of long-chain alkane production in yeast has not been explored, despite yeast being the organism of choice for industrial ethanol and isobutanol production (Buijs et al., 2013). For the purpose of alkane biosynthesis, yeast has only been used to elucidate the very long chain alkane biosynthesis pathway found in plants (Bernard et al., 2012). Such alkanes are of little interest from a biofuel perspective because of their long chain length. Furthermore, several studies have described the successful production of other alternative fatty acid-derived biofuels and chemicals, such as fatty alcohols and fatty acid ethyl esters by yeast (Zhou et al., 2014). This makes the absence of a proof of principle of long-chain alkane biosynthesis by yeast even more peculiar. Therefore, there is an urgent need for an alkane producing yeast strain that will allow the biofuel industry to diversify beyond ethanol by producing a direct substitute for fossil oil based transportation fuels.

This study delivers a proof of principle of heretofore unexplored alkane biosynthesis in the yeast *Saccharomyces cerevisiae*. We demonstrate this metabolic capability by expression of an alkane biosynthetic pathway consisting of a FAR encoded by *Synechoccocus elongatus* orf1594, and a FADO encoded by *S. elongatus* orf1593 (Fig. 1). However, upon the first instance of expression of the *Se*FAR and *Se*FADO in a *S. cerevisiae* CEN.PK background, no alkanes could be detected (Figure 2A, KB16). We suspected that an explanation

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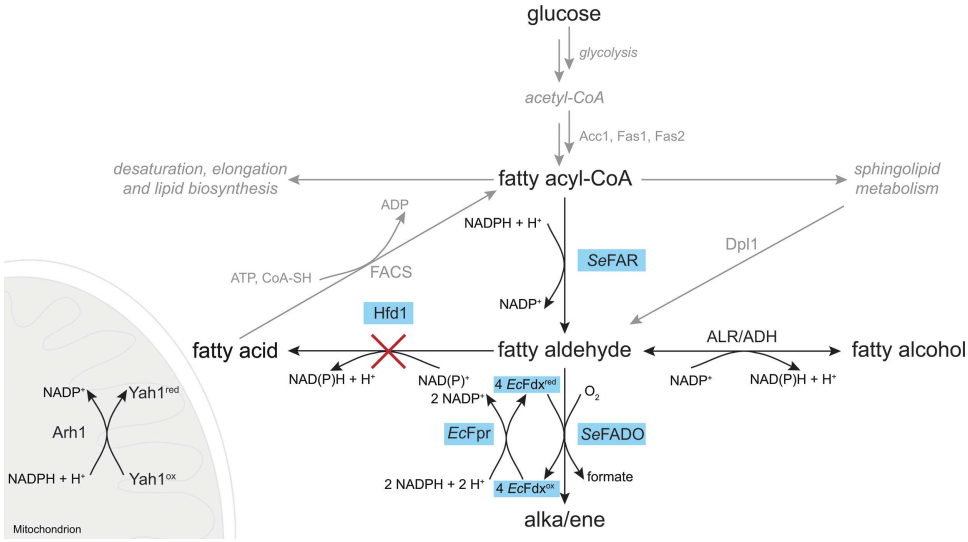

Figure 1. Alkane biosynthetic pathway and fatty aldehyde metabolism in *Saccharomyces cerevisiae*. A heterologous alkane biosynthetic pathway, consisting of a *S. elongatus* fatty acyl-CoA/ACP reductase (*Se*FAR; encoded by orf1594) and a *S. elongatus* fatty aldehyde deformylating oxygenase (*Se*FADO; encoded by orf1593), was introduced in the yeast *S. cerevisiae*. This pathway intersects with endogenous metabolism of fatty aldehydes by promiscuous aldehyde reductases (ALR) and fatty alcohol dehydrogenases (ADH) and the hexadecenal dehydrogenase Hfd1 (encoded by *HFD1/YMR110C*), which catalyzes the last step in the sphingolipid breakdown pathway. The *E. coli* ferredoxin (*Ec*Fdx)/ferredoxin reductase (*Ec*Fpr) system was introduced to provide the cofactor required for the FADO enzyme. The endogenous ferredoxin and ferredoxin reductase homologues Yah1 and Arh1, respectively, are localized to the mitochondria.

for the absence of alkanes could be the lack of a compatible redox partner that is required by the FADO enzyme in the CEN.PK background strain.

For the FADO enzyme, it has been shown *in vitro* that it requires ferredoxin (F) and ferredoxin NADP+ reductase (FNR) to supply electrons (Schirmer et al., 2010). *S. cerevisiae* possesses the ferredoxin and the ferredoxin reductase homologs Yah1 and Arh1, respectively. But these proteins reside in the mitochondria (Lill et al., 2006), which makes them inaccessible as redox partners for the cytosolic alkane pathway. Thus to provide a cytosolic reducing system, we chose to co-express the *E. coli* ferredoxin (F) Fdx and ferredoxin NADP+ reductase (FNR) Fpr since *E. coli* was able to support *in vivo* alkane production.

The co-expression of the *Ec*F/FNR reducing system resulted in the biosynthesis of 2.7 ± 0.9 μg/gDW heptadecane (Fig. 2A, KB17) and no detection of pentadecane. This result is in contrast with the alkane profile that was found in *E. coli* as well as the fatty acid profile of *S. cerevisiae*, in which C16 and C18 are the predominant fatty acid species (Khoomrung et al., 2013). We speculated that there might be a problem with the supply of C16 fatty aldehydes for the deformylation reaction.

Limited fatty aldehyde availability for the FADO reaction could be a result of low efficiency of the FAR reaction and/or competing reactions. Therefore, we chose to verify the fatty aldehyde synthesis by the FAR to ensure efficient functionality of the pathway. To confirm the supply of fatty aldehydes, fatty alcohol synthesis was used as an indicator. The detection of fatty alcohols as byproducts of alkane biosynthesis has been observed in *E. coli* (Schirmer et al., 2010) and is suspected to be a result of the activity of endogenous promiscuous aldehyde reductases and alcohol dehydrogenases (Rodriguez and Atsumi, 2014). *S. cerevisiae* contains around 40 homologues of such reductases and dehydrogenases, and fatty alcohol synthesis was consequently expected to occur after the introduction of the FAR. Nevertheless, when we overexpressed *Se*FAR in the background yeast strain, it yielded only trace amounts of fatty alcohols (Fig. 2B).

These results indicated that there could be an additional (irreversible) reaction, not present in *E. coli*, which competes for the fatty aldehyde substrate. In the case of *S. elongatus*, it has recently been shown that such an enzyme is present and that overexpression of FAR results in fatty acid secretion due to the presence of the fatty aldehyde dehydrogenase AldE (Kaiser et al., 2013). This enzyme converts fatty aldehydes very efficiently into fatty acids. Alignment of AldE against the *S. cerevisiae* proteome yielded the hexadecenal dehydrogenase Hfd1 as the primary candidate.

To test the hypothesis that Hfd1 prevents the biosynthesis of fatty alcohols by converting fatty aldehydes into fatty acids, *HFD1* was knocked-out followed by *Se*FAR overexpression. Remarkably, the deletion of *HFD1* alone sufficed to enable fatty alcohol production (0.43 ± 0.04 mg/gDW, Figure 2B). The fatty aldehydes observed in this *hfd1*Δ strain most likely resulted from the sphingolipid breakdown pathway in which Hfd1 catalyzes the final step (Nakahara et al., 2012). The additional overexpression of *Se*FAR increased the fatty alcohol titer to 0.52 ± 0.03 mg/gDW. The main fatty alcohol was hexadecanol (C16:0; 79%), followed by tetradecanol (C14:0; 11%), hexadecenol (C16:1; 7.3%), and dodecanol (C12:0; 2.8%). The drastic increase of C16 fatty alcohols illustrates that Hfd1 presumably catalyzes the oxidation of C16 fatty aldehydes toward the corresponding fatty acids.

The detection of heptadecane in strain KB17 carrying *Se*FAR, SeFADO, and *Ec*F/FNR, and the absence of the fatty alcohol octadecanol in the *hfd1*Δ *SeFAR* strain suggests that Hfd1 and the endogenous aldehyde reductases/alcohol dehydrogenases cannot use octadecanal as a substrate. This is in agreement with the detection of

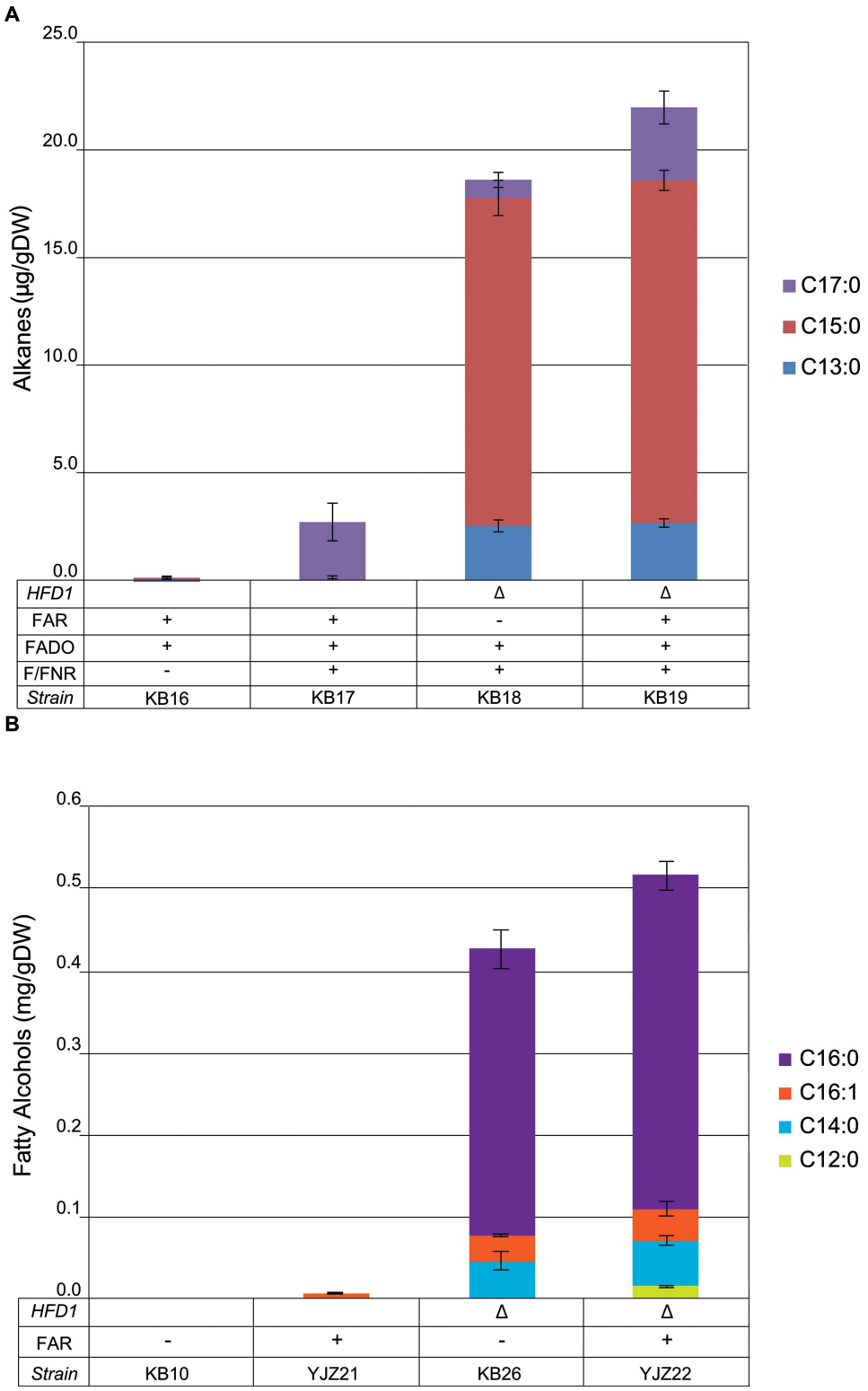

Figure 2. Analysis of alkane (**A**) and fatty alcohol production (**B**) in engineered *S. cerevisiae* strains. Strains carry either the WT allele or a deletion of the *HFD1* gene encoding hexadecenal dehydrogenase and express *S. elongatus* fatty acyl-CoA/ACP reductase (FAR), *S. elongatus* fatty aldehyde deformylating oxygenase (FADO), and/or *E. coli* ferredoxin/ferredoxin reductase (F/FNR). The error bars represent the standard deviation of three biological replicates.

very long chain alkanes upon expression of an alkane biosynthesis pathway from plants without deletion of *HFD1* (Bernard et al., 2012). The modest increase in fatty alcohol titer after *Se*FAR expression in an *hfd1*Δ strain is most likely due to the low affinity of *Se*FAR for fatty acyl-CoA. *Se*FAR prefers fatty acyl-ACP (Schirmer et al., 2010), which in *S. cerevisiae* is only available in the mitochondria (Hiltunen et al., 2010). In light of this, expression of alternative (activated) fatty acid reductases (Akhtar et al., 2013; Howard et al., 2013) may be worth pursuing. These results clearly illustrate the importance of *HFD1* deletion to enable fatty aldehyde supply.

Subsequently, the *Se*FADO and the *Ec*F/FNR reducing system were introduced in the *hfd1*Δ strain, as deletion of *HFD1* alone is sufficient to provide fatty aldehydes for the upstream part of the alkane pathway (which had been shown by the increased production of fatty alcohols). As expected, the alkane production increased drastically to 18.6 ± 1.4 μg/gDW in this *hfd1*Δ *Se*FADO *Ec*F/FNR strain (Figure 2A, KB18). Accumulation of tridecane and pentadecane was observed together with heptadecane, which had been the sole product in the wild-type genetic background strain KB17. The chain length profile of these alkanes is in agreement with those of the observed fatty alcohols. Additional expression of *Se*FAR in the *hfd1*Δ strain resulted in a titer of 22.0 ± 1.4 μg/gDW. The slight increase in titer suggests again that the *Se*FAR has low catalytic efficiency on fatty acyl-CoAs. No alkanes were detected extracellularly indicating that the alkanes are not excreted, which is in contrast with the detection of 80% of the produced alkanes in the extracellular medium in *E. coli* (Schirmer et al., 2010). An explanation for this could be the low titer and the hydrophobic nature of alkanes, which probably stimulates alkane accumulation in the yeast cell membranes. The observed hydrocarbon titer of 22.0 ± 1.4 μg/gDW is in the same range as measured by Bernard et al. 2012, who observed around 19 μg/gDW of very long chain alkanes after overexpression of *A. thaliana* CER1 and CER3. The relatively low hydrocarbon titer compared with the fatty alcohol titer indicates that the FADO enzyme may be a bottleneck in the alkane pathway. Elimination of the remaining competing enzymes is thus a promising engineering target, as has been shown in *E. coli* (Rodriguez and Atsumi, 2014). In addition, expression of the cyanobacterial reducing system (Zhang et al., 2013) instead of the *E. coli* F/FNR reducing system might improve the catalytic efficiency of the FADO enzyme.

Similarly, we also realized long-chain alkane production in a BY4741 background in which *HFD1* was disrupted. This commercially available knockout strain was initially used to test the requirement of Hfd1 elimination for alkane biosynthesis. Interestingly, expression of only *Se*FAR and *Se*FADO in this strain resulted in pentadecane and heptadecane biosynthesis (Supplementary Figure 3), possibly indicating the presence of a reducing system that is absent in the CEN.PK background strain.

In conclusion, this study provides a proof of principle of producing long-chain alkanes in the industrial workhorse *S. cerevisiae*. Our findings could provide an important first step towards industrial production of alkane drop-in biofuels in current production facilities. Future work among other challenges should focus on circumventing the competition with the remaining (promiscuous) reductases and dehydrogenases, utilization of alternative fatty acid reductases, and improving the efficiency of the deformylation reaction for the purpose of higher titers of alkanes in yeast.

Materials and Methods

Yeast strains, Plasmids and Reagents

The strains and primers used in this study are listed in the Supplementary Tables I and II, respectively. The background yeast strain *S. cerevisiae* CEN.PK113–11C (*MAT*a *MAL2–8*^{c} *SUC2 his3Δ1 ura3–52*) was kindly provided by P. Kötter, University of Frankfurt, Germany. The *S. elongatus* orf1594 (NCBI Gene ID: 3775018) and orf1593 (NCBI Gene ID: 3775017) were synthesized and codon-optimized for expression in yeast (Genscript, Piscataway, NJ, USA). The cofactor system encoded by *E. coli fdx* (NCBI Gene ID: 12931590) and *fpr* (NCBI Gene ID: 12932121) was amplified from *E. coli* DH5α. PrimeStar DNA polymerase was purchased from TaKaRa Bio (Segeltorp, Sweden). Oligonucleotides were custom synthesized by Sigma Aldrich (Stockholm, Sweden). Restriction enzymes, DNA gel purification and plasmid extraction kits were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Genetic Engineering

The *HFD1* disruption was performed as described previously (Akada et al., 2006) by using *Kluyveromyces lactis URA3* as a selection marker. The pathways for alkane and alcohol production were constructed on the plasmid backbone pYX212 by using a modular pathway engineering strategy as described before (Zhou et al., 2012). The modular pathways are shown in Supplementary Figure 1. Plasmids were extracted from single yeast colonies using the Zymoprep Yeast Plasmid Miniprep II kit (Nordic Biolabs, Täby, Sweden) and transformed into *E. coli* DH5α competent cells. After purification of the plasmid, verification by restriction analysis, and sequencing, the plasmids were transformed into yeast. Yeast competent cells were prepared and transformed with 1 μg of plasmid DNA according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods, 2002).

Strain Cultivation

Yeast strains for preparation of competent cells were cultivated in YPD consisting of 10 g/l yeast extract (Merck Millipore, Darmstadt, Germany), 20 g/L peptone (Difco, VWR, Stockholm, Sweden), and 20 g/L glucose (Merck Millipore). During genetic manipulation, yeast strains were selected on Synthetic Dextrose (SD) media, which contained 6.9 g/L yeast nitrogen base without amino acids (Formedium, Hunstanton, UK), 0.77 g/L complete supplement mixture without uracil (Formedium), 20 g/L glucose (Merck Millipore) and 20 g/L agar (Merck Millipore). *E. coli* strains were grown at 37 °C on Luria – Bertani medium (10 g/L tryptone (BD Biosciences, Stockholm, Sweden), 5 g/L yeast extract (Merck Millipore), 10 g/L NaCl (Merck Millipore)) supplemented with ampicillin (80 μg/mL) if required. Agar plates were prepared with the corresponding liquid medium supplemented with 15 g/L agar powder (Merck Millipore).

Shake flask batch fermentations were carried out in minimal medium containing 30 g/L glucose (Verduyn et al., 1992). Cultures were inoculated, from overnight precultures, at 0.1 OD_{600} in 25 ml minimal medium supplemented with histidine (40 mg/L; Sigma

Aldrich) in 250 ml shake flasks. The shake flasks were incubated at 30 °C and 200 rpm orbital shaking. After 48 hours, the cells were harvested by centrifugation (5 minutes; 1000 g) and washed once with 5 ml phosphate buffer (10 mM KH_2PO_4, pH 7.5). The supernatant was removed and the pellet frozen in liquid nitrogen and freeze-dried (Christ Alpha 2–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 48 hours.

Hydrocarbon Aanalysis by GC-MS

Alkanes and fatty alcohols were extracted from the freeze dried cell pellets as described before (Khoomrung et al., 2013), with the exceptions that the extracted fraction was dissolved in hexane (alkanes) or ethyl acetate (fatty alcohols) and that hexadecane (alkanes) or pentadecanol (fatty alcohols) was used as an internal standard. Samples were analyzed by gas chromatography (FocusGC, ThermoFisher Scientific) coupled to mass spectrometry (DSQII, ThermoFisher Scientific) using a Zebron ZB-5MS Guardian capillary GC column (30 m × 0.25 mm × 0.25 μm, Phenomenex, Værløse, Denmark). The GC-MS conditions are described in the Supplementary Information. Analytical standards for alkanes (Sigma Aldrich) and fatty alcohols (Sigma Aldrich) were analyzed during the same run for peak identification and quantification.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supporting Information

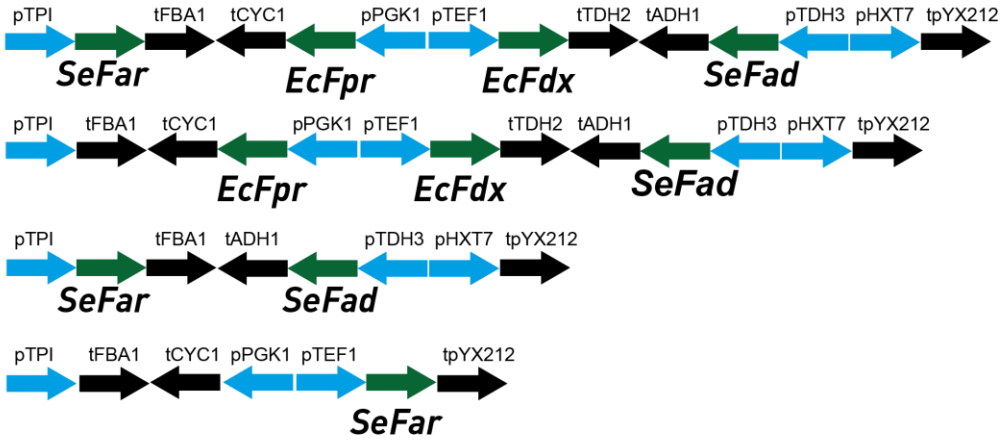
Yeast strains and primer sequences

Supplementary Table I: Yeast strains used in this study.

Name	Description	Resource
CEN.PK113-11C	*MAT*a *MAL2-8*^{c} *SUC2 his3Δ1 ura3-52*	Kötter, University of Frankfurt, Germany
KB10	CEN.PK113-11C; pYX212(-)	This study
YJZ01	CEN.PK113-11C *hfd1*Δ	This study
KB26	YJZ01; pYX212(-)	This study
YJZ21	CEN.PK113-11C; pYX212+FAR	This study
YJZ22	YJZ01; pYX212+FAR	This study
KB16	CEN.PK113-11C; pYX212 +FAR +FADO	This study
KB17	CEN.PK113-11C; pYX212 +FAR +FADO +F/FNR	This study
KB18	YJZ01; pYX212 +FADO +F/FNR	This study
KB19	YJZ01; pYX212 +FAR +FADO +F/FNR	This study
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ThermoScientific, Germany
KB20	BY4741; pSPGM1(-), pIYC04(-)	This study
KB21	BY4741; pSPGM1 +FAR +FADO, pIYC04 +F/FNR	This study
BY4741 6550	BY4741 *hfd1*Δ	ThermoScientific, Germany
YJZ24	BY4741 6550; pYX212(-)	This study
YJZ25	BY4741 6550; pYX212 +FAR +FADO	This study
YJZ26	BY4741 6550; pYX212 +FAR +FADO +F/FNR	This study
YJZ27	BY4741 6550; pYX212 +FADO +F/FNR	This study

Supplementary Table II: Primers used for part cloning, module construction, and colony PCR.

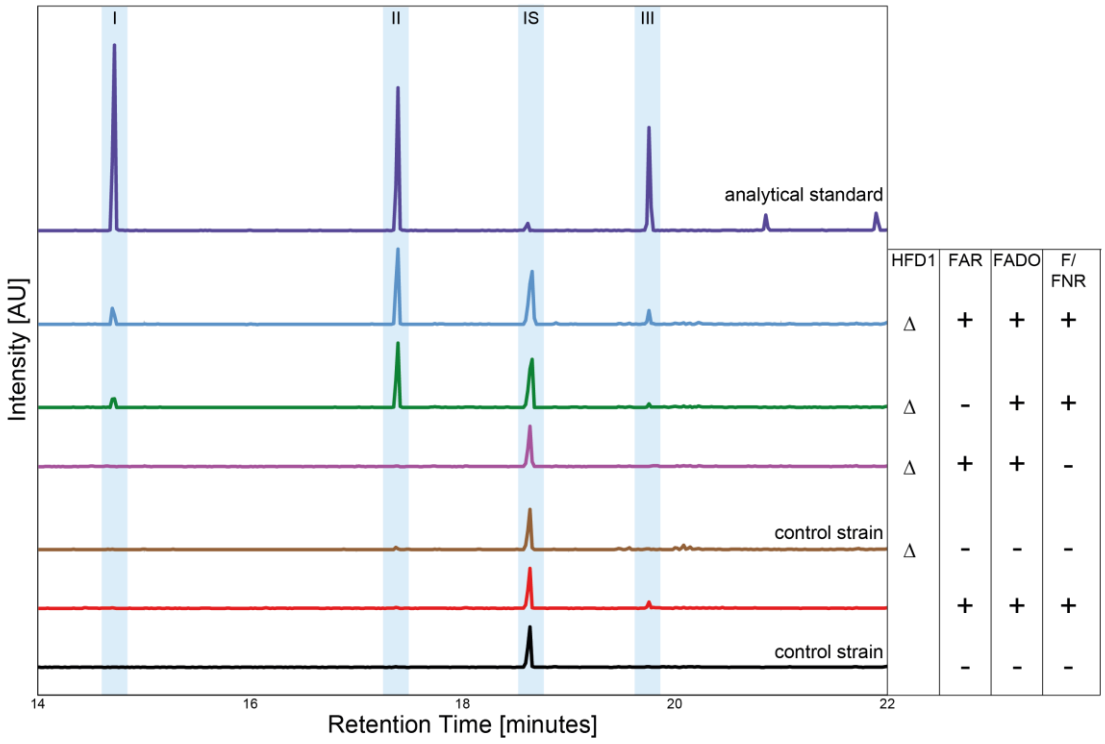
Seq No.	Name	Sequence (5'-3')
1	hfd1(up)-F	GATTATCAATGTCCCAGTTATACG
2	hfd1(up)-R	CGAAAGGTTACTTATACATCAAATAATTAATTAACCTTAAACATTACGTTCACATGTTGGTGATAAATTACTATG
3	hfd(dn)-F	GAGTACGAGGATCTTGATGAGAC
4	hfd(dn)R	CACTTGTTATTGCCATTTCTGTC
5	hfd1(up)-URA3-R	CGAAAGGTTACTTATACATCAAATAATTAATTAACCTTAAACATTACGTTCACATGTTGGTGATAAATTACTATG
6	URA3(hfd1)-F	GGTTAATTAATTATTTGATGTATAAGTAACCTTTCGTTTAAAAATTTCATATGGGCGATAATATATCGTGATTCTGGGTAGAAGATCG
7	URA3(hfd1)-R	CTATTATCTTGTTAATGGTCTCATCAAGATCCTCGTACTCCATCGATAAGCTTGATATCG
8	SeFAR-F2	CTATAACTACAAAAAACACATACATAAACTAAAAATGTTCGGTTTAATAGGTCAC
9	SeFAR-R2	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCAGATTGCTAAGGCTAAAG
10	SeADO-F1	CAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGCCACAATTAGAAGCCTC
11	SeADO-R1	CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTTAGACTGCTGCCAAACCGTATG
12	EcFd-F1	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGCCAAAGATTGTTATTTTG
13	EcFd-R1	CTAAATCATTAAAGTAACTTAAGGAGTTAAATTTAATGCTCACGCGCATGGTTG
14	EcFNR-F1	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTACCAGTAATGCTCCGCTG
15	EcFNR-R1	GGAAGTAATTATCTACTTTTTACAACAAATATAACAAAATGGCTGATTGGGTAACAGG
16	FAR COL FW	GGATCCAAAACAATGTTCGG
17	FAR COL RV	GATTGCTAAGGCTAAAGGTTGG
18	FAD COL FW	GCCACAATTAGAAGCCTCCTTAG
19	FAD COL RV	CTGCTGCCAAACCGTATGC
20	F FW	ATCGAAGCGGCCGCAAAACAATGCCAAAGATTGTTATTTTGC
21	F RV	ATCGTCGAGCTCTTAATGCTCACGCGCATG
22	FNR COL FW	ATGGCTGATTGGGTAACAGG
23	FNR COL RV	ACAGCGGAGCATTACTGGTAA



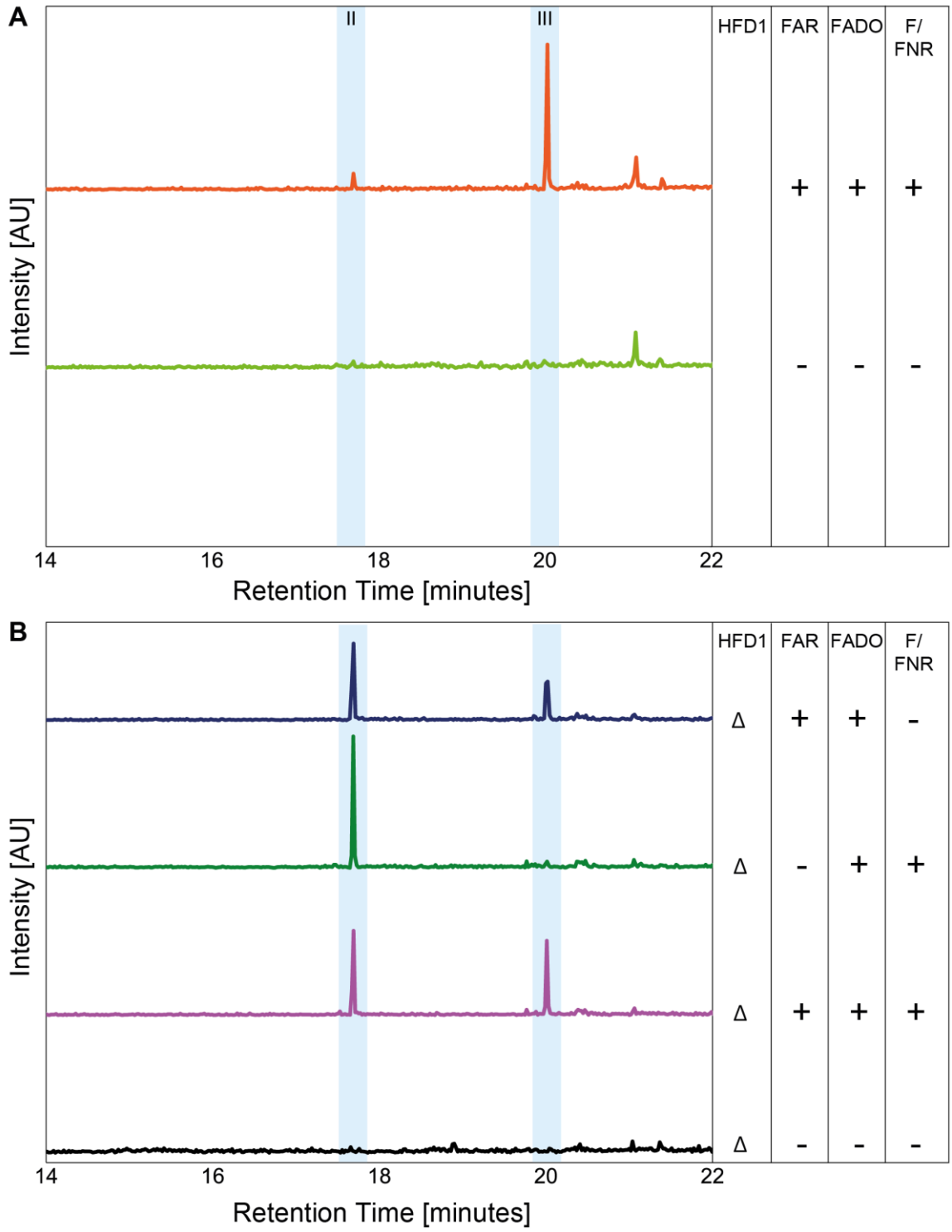
Supplementary Figure 1: DNA pathway assembly constructs. *Synechoccocus elongatus* fatty acyl-ACP/CoA reductase (SeFar) and *S. elongatus* fatty aldehyde deformylating oxygenase (SeFad) genes were synthesized and codon-optimized. *Escherichia coli* ferredoxin (EcFdx) and *E. coli* ferredoxin NADP+ reductase (SeFpr) genes were amplified from *E. coli* DH5α. The promoter pTPI and the terminator tpYX212 are homologous to the respective promoter and terminator on the pYX212 plasmid. All four plasmids were constructed using using the modular pathway engineering strategy (Zhou et al., 2012).

GC-MS analysis

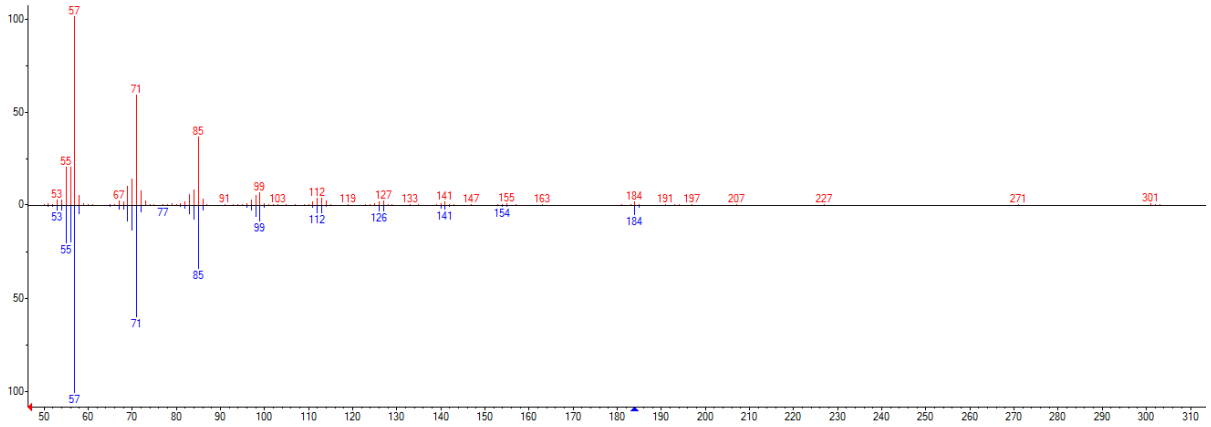
The following program was run for alkane analysis: initial temperature 50°C hold for 5 minutes, ramp by 10°C per minute to 310°C and hold for 6 minutes, inlet temperature 250°C, mass transfer line 300°C, splitless flow of 1.0 mL/min, ion source temperature 230°C, full scan mode 50-650 m/z. Samples were analyzed by single 2 µL injections. The following program was run for fatty alcohol analysis: initial temperature 45°C hold for 2.5 minutes, ramp by 20°C per minute to 220 and hold for 2 minutes, ramp by 20°C per minute to 300°C and hold for 5 minutes, inlet temperature 300°C, mass transfer line 300°C, splitless flow of 1.0 mL/min, ion source temperature 200°C, full scan mode 50-650 m/z. Samples were analyzed by single 2 µL injections.



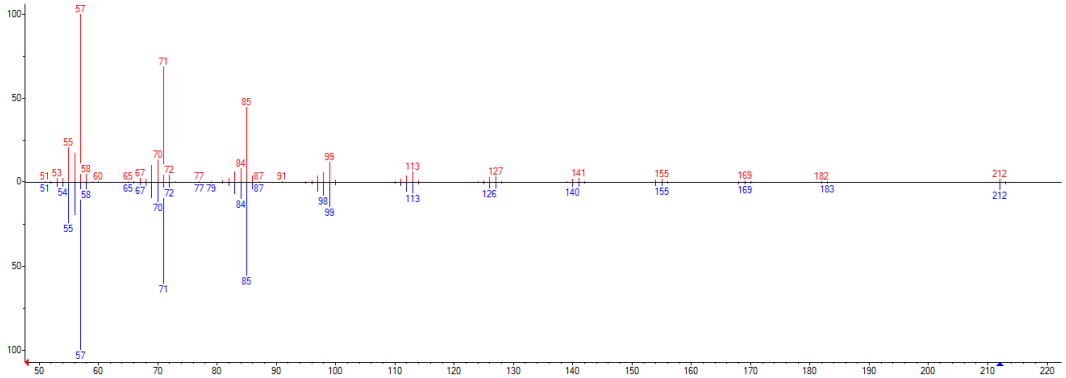
Supplementary Figure 2: Alkane biosynthesis. Gas chromatograms of shake flask cultures incubated for 48 hours in glucose minimal medium. The lines represent *S. cerevisiae* CEN.PK113-11C strains that express *S. elongatus* FAR and FADO as well as the *E. coli* reduction system consisting of Fdx (F) and Fpr (FNR). The *S. cerevisiae* strains carrying an empty vector pYX212 (black and brown traces) are shown as a control. A C7-C30 alkane analytical standard (top trace) was used as a reference. The peaks highlighted by the blue bars labeled with I, II, IS, and III represent tridecane (C13), pentadecane (C15), hexadecane (C16; internal standard), and heptadecane (C17), respectively. The shown spectra are for the *m/z* values 184, 212, and 240. The mass spectra for the labeled peaks in comparison with a NIST library standard are shown in the Supplementary Figures 4-6.



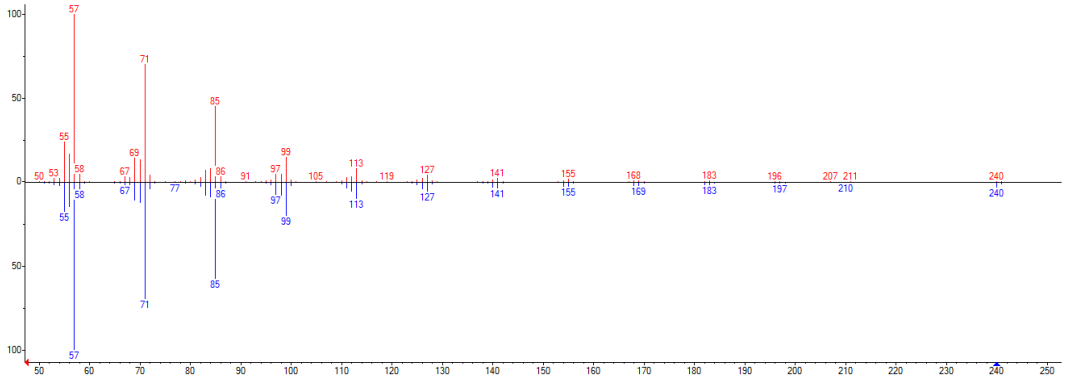
Supplementary Figure 3: A. Gas chromatograms of intracellular hydrocarbons produced by *Saccharomyces cerevisiae* BY4741 strain expressing *S. elongatus* orf1594 (FAR) and *S. elongatus* orf1593 (FADO) with *E. coli* Fdx (F) and *E. coli* Fpr (FNR) reducing system (top); and a BY4741 control strain carrying an empty plasmid (bottom). **B.** Gas chromatograms of intracellular hydrocarbons produced by *Saccharomyces cerevisiae* BY4741 *hfd1*Δ (6550) strains expressing *S. elongatus* orf1594 (FAR) and *S. elongatus* orf1593 (FADO) without reducing system (top); *S. elongatus* orf1593 with *E. coli* Fdx (F) and *E. coli* Fpr (FNR) reducing system (2nd from top); *S. elongatus* orf1594 and *S. elongatus* orf1593 with the F/FNR reducing system (2nd from bottom); and a control strain carrying an empty plasmid (bottom). The peak in the left blue rectangle represents pentadecane (II), the peak in the right rectangle heptadecane (III). The shown traces are for the *m/z* values 212 and 240.



Supplementary Figure 4: Mass spectra of the observed tridecane (I) GC peaks compared to the mass spectra of the NIST library standards for this compound (at the bottom).



Supplementary Figure 5: Mass spectra of the observed pentadecane (II) GC peaks compared to the mass spectra of the NIST library standards for this compound (at the bottom).



Supplementary Figure 6: Mass spectra of the observed heptadecane (III) GC peaks compared to the mass spectra of the NIST library standards for this compound (at the bottom).

Harnessing peroxisomes for production of fatty acid derived chemicals and biofuels in yeast

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Abstract

Synthetic pathways for microbial production of biochemicals often suffer from bypass pathway competition and insufficient precursor supply. Compartmentalization in cellular organelles can shield such pathways from competing pathways, and provide more compact and suitable environments for biosynthesis. Peroxisomes are cellular organelles where fatty acids are degraded. Fatty acid degradation is inhibited under typical glucose batch growth conditions, which makes peroxisomes an interesting candidate. Here, we show that moving heterologous biosynthesis pathways into peroxisomes can increase the production of fatty acid-derived fatty alcohol, alkane and olefin. In addition, we demonstrate that peroxisomal compartmentalization significantly decreased by-product accumulation by secluding the heterologous pathways from more efficient competing enzymes. Furthermore, we evaluated productivity by increasing the peroxisome population by engineering peroxisome biogenesis controlling peroxins. The strategies described here could also be used for increasing peroxisome based production of other biochemicals.

Introduction

The yeast *Saccharomyces cerevisiae* is an attractive cell factory for production of biochemicals as it has been the organism of choice for industry[1]. Yeast fatty acid biosynthesis has attracted significant attention for production of high energy densities biofuels and oleo-chemicals[2]. However, the productivity and yield of oleo-chemicals produced by the well characterized model yeast *S. cerevisiae* is still relatively low[3–5], which may be attributed to the more complicated cellular metabolism, more complex regulatory mechanisms, and cellular compartmentalization. Thus, novel approaches are urgently required to increase the productivity of these biochemicals and biofuels.

In *S. cerevisiae*, fatty acid biosynthesis and degradation takes place in the cytosol and peroxisome, respectively. To our knowledge, all heterologous fatty acid-derived biosynthetic pathways have thus far been localized in the cytoplasm[3–7]. An example of a fatty acid-derived biofuels are alkanes. Alkanes are an ideal gasoline and diesel substitute because of their high similarity to fossil oil derived liquid fuels[2]. We recently established long-chain alkane production in *S. cerevisiae* by introducing a fatty acyl-ACP/CoA reductase (*Se*AAR) and an fatty aldehyde-deformylating oxygenase (*Se*ADO) from *S. elongatus*, however, the productivity was extremely low of 22.0 µg $gDCW^{-1}$ (0.037 mg l^{-1}) and there was much higher accumulation of by-product fatty alcohols[4]. Limited conversion of fatty aldehydes has also been observed in another study on alkane biosynthesis in yeast[8]. The high level accumulation of fatty alcohols may attributed to cytosolic promiscuous aldehyde reductases/alcohol dehydrogenases (ALRs/ADHs)[9]. These enzymes compete for the intermediate fatty aldehyde with the less efficient ADO[10]. Compartmentalization of the alkane metabolic pathway could prevent the loss of the intermediate fatty aldehydes toward fatty alcohol biosynthesis by secluding the alkane biosynthetic pathways. Peroxisome is such an organelle that has at least three potential advantages for alkane production (Fig. 3a): 1) provide spatial proximity for substrate channelling[11]; 2) has the peroxisomal catalase CTA1 that may relieve the H_2O_2 inhibition of the ADO; 3) seclude competing enzymes such as aldehyde reductases, which would decrease fatty alcohol accumulation. Furthermore, the peroxisome has the advantages of high fatty acyl-CoA[12].

In this study, the yeast peroxisomes were harnessed to produce the fatty acid derived fatty alcohols, alkanes and olefins. To prove this concept, we first constructed a one-step pathway into peroxisomes for production of fatty acyl-CoA derived fatty alcohols. We then constructed a multi-step fatty acid-derived pathway for alkane biosynthesis, which suffers from strong competition from by-pass fatty alcohol pathways catalysed by efficient endogenous ALRs/ADHs. By using this model system, we show that peroxisomal compartmentalization not only increases the production of target molecules, but also decreases the by-product formation. We also show that enhancing the peroxisome population could further improve the biosynthesis. The peroxisome

population was engineered by deletion and overexpression of several peroxins. Finally, we extended peroxisome compartmentalization to olefin biosyntheis. Overal, these results demonstrate that peroxisome targeting is a feasible strategy for improving production of fatty acid derived chemicals. We anticipate that future research could extend this to acetyl-CoA derived chemicals too.

Results

Peroxisomal compartmentalization to produce fatty acyl-CoA derived fatty alcohol

In order to show that peroxisomes can be used as an compartment, we wanted to verify that the peroxisomes have a high enough level of precursors and NADPH to initially support a heterologous pathway. As a proof concept, we used a simple one-step fatty alcohol biosynthesis pathway by expressing a fatty acyl-CoA reductase (FaCoAR) from *Marinobacter aquaeolei*[13] (Fig. 1a). FaCoAR catalyses the conversion of fatty acyl-CoAs into fatty alcohols with a broad substrate specificity. Peroxisomal compartmentalization of FaCoAR (Fig. 1b) increased fatty alcohol production by 3.7-fold to 12.4 mg l^{-1} in a wild-type background (Fig. 1c). The observed titer after peroxisomal targeting deletion is approximately half of what has been observed in *Escherichia coli* for which expression of FaCoAR in a ΔFadE, fatty acyl-CoA synthetase (FadD) overexpressing strain led to a titer of 21.7 mg/L fatty alcohols[14]. It is worth mentioning that utilization of a GGGS linker and a 12 residue C-terminal peroxisome targeting signal from the yeast peroxisomal enzyme Fox2 improved production (Supplementary Fig. 1). Successful translocation of the pathway into the peroxisome was shown by fusion of FaCoAR with a green fluorescent protein (GFP), followed by fluorescence microscopy (Fig. 1b). Besides increasing the titer, peroxisomal targeting of FaCoAR resulted in shorter and unsaturated fatty alcohols in comparison with the cytosolic pathway (Fig. 1d-e). The increase upon peroxisomal localization indicates that cytosolic FaCoAR was limited in substrate and struggled to compete. Peroxisomal FaCoAR only has to compete with fatty acid degradation through β-oxidation. To eliminate this competition, fatty acid degradation was blocked by deletion of fatty oxidase encoding gene *POX1.* Deletion of *POX1* was expected to redirect fatty acids from lipid degradation towards FaCoAR. In agreement with this, the deletion increased cytosolic fatty alcohol production by 68%. This was consistent with our previous observation that FaCoAR struggled to compete with cytosolic fatty acyl-CoA consuming enzymes. However, it did not yield an increased peroxisomal titer, which showed that substrate was not the bottleneck in the peroxsome. Without competition of Pox1, the peroxisomal product profile shifted towards longer and saturated fatty acids (Fig. 1d and e). The average fatty alcohol chain length after *POX1* deletion is interesting. In the *POX1* knockout strain the average increased from 16.55 to 16.75 carbons, it moved to the average that was observed for the cytosolic pathway. In a previous study FaCoAR has been characterized *in vitro* and observed a Km of 4 μM for palmitoyl-CoA and a specific activity of 0.057 μM min^{-1} mg^{-1}. In that

study, it has been demonstrated that compared to its preferred substrate palmitoyl-CoA, FaCoAR displays 69% and 78% activity towards steroyl-CoA and oleyl-CoA, respectively[13]. This substrate preference and the observation that palmitate and palmitoleic acid make up 70% of the lipids in a yeast cell seems to suggest that *POX1* elimination leads to a relative increase of unsaturated C18 fatty acids that are transported to the peroxisome[15]. This could possibly be because this type of fatty acids are less preferred by Dga1 for incorporation into TAGs[16–18]. Regardless of the limitations of FaCoAR, the significant improvement in fatty alcohol titer after peroxisomal compartmentalization indicated that peroxisomes contain a high enough level of precursors and NADPH. This result gave us confidence to engineer peroxisome for production of alkanes.

Peroxisomal production of fatty acid-derived alkanes

Next, we set to engineer peroxisomes for production of alkanes. We compartmentalized the *S. elongatus* fatty acyl-CoA derived pathway (*Se*AAR, *Se*ADO with the electron transfer system of *E. coli* ferredoxin *Ec*Fd and ferredoxin NADPH reductase *Ec*FNR) into peroxisomes, which enabled an increase of alkane production in a wild-type background (Fig. 2b). This increase was modest compared to the fatty alcohol pathway. A possible explanation for this is the low efficiency of *Se*AAR with fatty acyl-CoA substrates, something which we also observed as well in our previous study[4]. The low *in vivo* activity on fatty acyl-CoA has been corroborated by a study[19]. In that study, overexpression of *Se*AAR in a ΔFadE, FadD overexpressing *E. coli* strain in the presence of exogenous fatty acids led to the synthesis of 20.7 mg/L fatty alcohols, while FaCoAR overexpression in the same background resulted in 319.7 mg/L. A very likely explanation for the low *Se*AAR in vivo activity is its substrate preference. A previous study has reported A Km of 130 μM for oleoyl-CoA, versus 8 μM for oleoyl-ACP and a V_{max} of 7.6 and 19.8 μM hr^{-1} (0.13 and 0.33 μM min^{-1}), respectively[20]. More recently, a more detailed study has shown that activity of FAR on fatty acyl-ACP is superior to fatty acyl-CoA[21]. Therefore, fair evaluation of SeFAR would require activation of FFAs to fatty acyl-ACP (FA-ACP). Activation of FFAs to FA-ACP would require expression of the *S. elongatus* ACP and fatty acyl-ACP synthethase[22]. Alternatively, short-chain fatty acyl-CoAs could also be interconverted into fatty acyl-ACP by a *Pseudomonas aeruginosa* FabY [23]. Furthermore, less efficient peroxisomal import of *Se*FAR and *Se*FADO than FaCoAR cannot be ruled out. FAR and FADO have been shown to form a tight complex[21] and it has been shown that complex formation can hamper import of proteins into the peroxisome[24].

In response to this result, we chose to evaluate a *Mycobacterium marinum* carboxylic acid reductase (*Mm*CAR)[25] together with SeFADO and S. elongatus F/FNR reducing system (Fig. 2a). This reductase requires expression of a 4'-phosphopantetheinyl transferase and to that end we coexpressed NpgA from *Aspergillus nidulans. Mm*CAR has the advantage that it taps into the free fatty acid pools, but the disadvantage that it consumes an additional ATP (to AMP) and could require hydrolysis of fatty acyl-CoAs (and thus 1 ATP invested). Free fatty acids can be

accumulated too very high levels in *S. cerevisiae* due to the absence of feed-back inhibition that has been observed for fatty acyl-CoAs[3,26,27]. Furthermore, the feasibility of such a route in yeast has been shown for biosynthesis of alkanes[8]. This free fatty acid-derived pathway resulted in a 1-fold higher alkane production (0.06 mg l^{-1}) than the fatty acyl-CoA-based pathway in a wild-type background (Fig. 2b). Peroxisomal compartmentalization of this FFA-derived pathway (strain A10) resulted a 90% higher alkane titer compared to cytosolic pathway. Analogous to the fatty alcohol pathway, we then tried to increase the precursor supply by blocking the β-oxidation. However, *POX1* deletion led to a marginal improvement for alkane production (Fig. 2c).

In our previous study we had found that elimination of the fatty aldehyde dehydrogenase Hfd1 is essential to enable synthesis of tridecane and pentadecane. Deletion of *HFD1* encoding increased alkane production by 10 fold, which was similar to the effect of *hfd1Δ* to cytosolic alkane biosynthesis (Fig. 2c). Peroxisomal compartmentalization showed similar improvements (about 1-fold) for alkane production in different strain backgrounds as we observed for fatty alcohols. The byproduct fatty alcohol titer decreased much more in *hfd1Δ pox1Δ* background (50% decrease, strain A14 vs A12) than *pox1Δ* background (32 % decrease, strain A13 vs A11) (Fig. 2c).

Accumulation of (fatty) alcohols has been the motivation for elimination of alcohol dehydrogenases and aldehyde reductases in *E. coli*[9,28,29]. The advantage of this approach is that contribution of each enzyme to aldehyde consumption can be inferred from a reduction in alcohol titer. Deletion of twelve aldehyde reductases did yield *E. coli* the ability to accumulate fed medium-chain aldehydes instead of converting them into alcohols. In our study, deletion of *ADH5* and *SFA1* led to a slight increase of the cytosolic alkane titer (Fig. 2d), but the biomass specific titer did not improve significantly (Supplementary Fig. 5). The deletion of *ADH5* and *SFA1* (Fig. 2d) showed that peroxisomal localization could shield the fatty aldehyde intermediate from cytosolic ALRs, but not from Hfd1. This can possibly be explained by accumulation of the fatty aldehydes in the peroxisomal membrane, followed by migration through interorganelle contact sites to the ER and/or mitochondrial membrane, after which it would come into contact with Hfd1. The observation that deletion of promiscuous aldehyde dehydrogenase activity comes from improved growth characteristics, and thereby titers, is in agreement with a previous study[30]. Their study is to our knowledge the only study in which such an ALR deletion was combined with long-chain alkane biosynthesis. In that study an increased long-chain alkane titer was observed after YqhD deletion in *E. coli*. The increase in alkane titer seemed to be mainly due to an increase in biomass yield of the YqhD strain. Our results show that compartmentalization can significantly increase alkane production by sequestering the alkane biosynthetic enzymes. The peroxisome provided us with a model system in which we could evaluate several strategies to improve the ADO catalysed step. For example, we evaluated an alternative ADO from *Nostoc punctiforme* PCC73102, which has been shown more efficient than the *Se*ADO[20]. Upon expression in the peroxisome, the *Np*ADO proved less efficient (Supplementary Fig. 2). Furthermore, we combined the *Se*ADO with its native

reducing system (*Se*FNR and *Se*Fd)[31] and this improved alkane production a little and thus was used for further study. Fusion of this reducing system to the ADO, which has been shown to improve *in vitro* ADO activity[32], lowered the alkane titer compared with expression of the separate enzymes (Supplementary Fig. 2). The fusion of the reducing system reduced fatty aldehyde channelling efficiency, possibly by obstructing the interaction between the AAR and FADO.

Engineering the peroxisome number for improving biosynthesis

Eventhough peroxisomal targeting improved the production of alkanes, the titer needs to be further enhanced to make it an industrial viable process. As there is only a very small number of peroxisomes in cells grown on glucose (Fig. 1b and 3d), we explored the possibility to increase the peroxisome population in order to improve alkane production (Fig. 3a). Peroxisomes are highly controlled organelles and their growth and division is regulated by different mechanisms such as metabolite induction and peroxins[33]. Several reports have showed different peroxisomal integral membranes are involved in regulating peroxisome population. For example, Pex28-31 have been shown to be involved in peroxisome proliferation and their deletion resulted in changes in peroxisome number and size[34,35].

As a proof of concept, we investigated the combinatorial deletion of *PEX30-32* on the production of fatty acyl-CoA derived fatty alcohols by using the available knockout strains in a S288C background[34]. Among the deletion combinations, *pex31,32Δ* increased 20% fatty alcohol production for the peroxisomal pathway, but had no effect on the cytosolic fatty alcohol production (Supplementary Fig. 3). These results indicated *pex31,32Δ* had increased peroxisomes for fatty alcohol biosynthesis without interrupting the cytosolic fatty acyl-CoA metabolism. We further introduced *pex31,32Δ* into CEN.PK derived strain YJZ03. With peroxisomal FacoAR expression, *pex31Δ* increased fatty alcohol production by 28% and *pex31Δ pex32Δ* resulted in a more significant increase of fatty alcohol production (77%, Fig. 3b). Studying the cell by fluorescence microscopy showed that *pex31,32Δ* strain had more and bigger peroxisomes (Fig. 3d). Previous studies described that *pex31,32Δ* can increase peroxisome number as a result of oleic acid supplementation[34], here we show that the double deletion also increased the peroxisome size and condition in glucose media. Of the single peroxin deletion strains (Paper II, SI Figure 4), strain ΔPEX30 showed the highest fatty alcohol titer. This strain would also have the highest peroxisome density when grown on oleate[34].

We then implemented this strategy for improving alkane production. Consistently, the *pex31*Δ increased alkane production by 22% and *pex31*,32Δ had a 25% higher alkane production (Supplementary Fig.4 and Figure 3c). However, the accumulation of by-product fatty alcohols also increased much more by 50%, which might be attributed to higher permeability of peroxisome membrane for fatty aldehydes (which are then converted by cytosolic ADHs/ALRs resulting in fatty alcohol biosynthesis). Fluorescence microscopy showed that the peroxisomal

membrane protein PEX3-GFP and matrix protein SeADO-GFP were properly localized to the peroxisome (Fig. 3d). These results indicated that the *pex31,32Δ* had a looser membrane structure that increased the diffusion of fatty aldehydes.

Beside the induction by peroxins, some peroxisome integral membrane proteins are responsible for constitutive peroxisome division, which may serve as further engineering targets. Pex34 is such a peroxin that works with Pex11 to control the peroxisome population of cells under conditions of both peroxisome proliferation and constitutive peroxisome division[36,37]. Overexpression of *PEX11* increased alkane production slightly (Supplementary Fig. 4c), while *PEX34* overexpression significantly improved alkane production by 54% (Fig. 3c), though the strain has a lower biomass yield (Supplementary Fig. 4b). More importantly, the fatty alcohol accumulation in a *PEX34* overexpression strain A20 was 26% lower compared to the control strain (Fig. 3c), which indicted *PEX34* overexpression resulted in a compact peroxisome membrane structure with less diffusion of fatty aldehydes. Consistently, fluorescence microscopy showed that PEX34 overexpression cells had much more peroxisomes compared to the control strain, and both matrix protein SeADO-GFP and membrane protein Pex3-GFP were targeted to the peroxisome (Fig. 3d). Combined PEX34 overexpression to *pex31*,*32*Δ (strain A22) further increased alkane to 3.55 mg l^{-1}, which was a 3-fold increase compared to the wild-type ground (strain A14) and 7-fold higher compared to the cytosolic pathway. It should be emphasized that the biomass specific alkane titer in strain A22 increased even much more (3.5-fold) than control strain A14 (Supplementary Fig. 5), which is because engineering peroxins reduced biomass yields slightly. However, *pex31*,32Δ and PEX34 overexpression had no effect on cytosolic alkane production (Supplementary Fig. 6), which verified that engineering these peroxins actually increased the peroxisome number without interruption of cytosolic fatty acid metabolism.

Expanding peroxisomal compartmentalization for olefin production

We also explored peroxisomal compartmentalization for production of olefins, which are used for example as surfactants and lubricants. Olefins can be synthesized from free fatty acids by polyketide synthases[38–40], and H_2O_2 dependent P450 or iron dependent fatty acid decarboxylases[41–43]. Cytosolic expression of a codon-optimized *oleT*, encoding a P450 fatty acid decarboxylase from *Jeotgalicoccus sp*. ATCC8456[25], enabled production of olefins at 0.15 mg l^{-1} in a fatty acid overproducing background YJZ06 (Fig. 4). The main product was 1-heptadecene (C17:1), similar to a previous report[42,44]. This titer is lower than what previously has been observed for expression in a ΔFAA1 ΔFAA4 deletion strain[44], which could be due to differences in strain background and cultivation conditions. Since *OleT* is hydrogen peroxide dependent, which could limit the reaction, a previous study sought to find alternative redox partners for this enzyme[45]. In that study OleT was fused to a P450 reductase domain from *Rhodococcus* sp to render the enzyme NADPH dependent. Furthermore, that study showed that *Escherichia coli* flavodoxin and

flavodoxin reductase can support OleT activity *in vitro,* but overexpression *in vivo* was not reported. Therefore, we tested both strategies. The OleT-RhFRED fusion protein decreased the olefin production (Supplementary Fig. 7), similar to what was observed in the fatty acid overproducing *E. coli* strain[45]. OleT together with the potential electron transfer system from *E coli* (consisting of flavodoxin FldA and flavodoxin/ferredoxin NADPH reductase Fnr; Fld/FNR) increased olefin production by 43% (Fig. 4b). Alternatively, overexpression of the molecular chaperones GroEL/GroES for improving P450 folding did not further increase olefin production. Next, we expressed OleT together with FldA/FNR in the peroxisomes. Peroxisomal compartmentalization improved olefin production by 15% compared to the cytosolic pathway, but within measurement error. These results demonstrated that peroxisomal compartmentalization can be a general strategy for producing oleo-chemicals derived from fatty acyl-CoAs and free fatty acids in the peroxisome.

Discussion

In this study, we explored the yeast peroxisome for production of fatty acid-derived chemicals. Though peroxisomes play a prominent role in oxidative metabolism of waste molecules such as fatty acids, we show peroxisome can be harnessed for reductive biosynthesis of fatty acid derived chemicals. This comparmentalization concept combined several advantages of the peroxisome such as a relatively high level of precursors, more compact space for substrate channelling and an insulated environment from competing pathways.

Peroxisomal targeting the fatty acyl-CoA reductase FaCoAR enabled a 3-fold higher production of fatty alcohols than the cytosolic pathway, which verified the concept that the peroxisome have sufficient fatty acyl-CoA levels and indicated that there are sufficient cofactors (eg. NADPH) available for the biosynthesis of highly reduced molecules. The peroxisomal compartmentalization strategy proved to be even more advantageous for alkane production. In this case, the alkane biosynthesis suffered from the strong competition by cytosolic promiscuous ALRs/ADH[4]. Bypass pathway competition is always a big problem in metabolic engineering, which will limit the yield of targeted products. Although blocking the by-pass pathway by deleting corresponding genes is a common strategy, it is challenging to delete all ALRs/ADHs, because some of them are involved in reductive biosynthesis of some essential metabolites. More importantly, many of these ALRs/ADHs play an important role in relieving toxicity of inhibitors from biomass hydrolysates such as furfural and 5-hydroxymethylfurfural[46], which is very essential for future efficient biomass utilization. Peroxisomal compartmentalization improved the alkane production by 1-fold and significantly decreased accumulation of the by-product fatty alcohols (Fig. 2), which indicated that peroxisomal targeting could shield the intermediate from cytosolic aldehyde reductases.

Protein fusion and scaffold strategies have been successfully used for improving cellular substrate channelling[47]. However, these strategies sometimes suffer from the loss of enzyme activity and can be challenging for multistep (>3 step) pathways due to the difficulty in constructing the functional complex protein fusion or scaffolds. Alternatively, sub-organelle compartmentalization of synthetic pathways has been shown helpful for biosynthesis. For example, mitochondrial compartmentalization of the metabolic pathways improved the production of isoprenoids[48,49] and isobutanol[50]. All these studies have shown the organelle compartmentalization can provide a more suitable environment (high level of precursor[50,51] and sub-organelles specific localized cofactors[48]) for biosynthesis of specific products. Peroxisomes have also been harnessed for production of penicillin in *Aspergillus nidulans* by targeting a cytosolic step of penicillin biosynthesis into peroxisomes for enhanced substrate channelling[51], we show that re-construction of the heterologous pathways in peroxisome can also improve production of a new group of chemicals.

As *S. cerevisiae* contains only a very small number of peroxisomes are present under glucose rich conditions (Fig. 1b). Here we showed that deletion of *PEX31* and *PEX32* resulted in more and larger peroxisomes and further increased the biosynthesis of fatty acyl-CoA derived fatty alcohols (Fig. 3b and d). However, there was much less of a benefit for alkane production, where the by-product fatty alcohols increased instead by 50% (Fig. 3c). These results could indicate that *pex31,32Δ* could increase leakage of fatty aldehydes to the cytoplasm were for ADH/ALRs catalysed fatty alcohol biosynthesis. In addition, an increased peroxisome area could reduce the efficiency of substrate channeling between the *Mm*CAR and FADO. This was in agreement with that the small amount of fatty alcohol accumulation observed for the peroxisomal pathways, and with the observation that *hfd1Δ* is still essential for peroxisomal alkane biosynthesis (Fig. 2c). In contrast, PEX34 overexpression improved alkane production without elevated fatty alcohol accumulation, which indicated this constitutively expressed peroxin promoted the peroxisome proliferation without changing membrane permeability. These observations bring to the attention that engineering peroxisome proliferation may affect the peroxisome structure and function, which could affect primary metabolism beyond fatty acid degradation, because peroxisomes plays an essential role in those processes too. On the other hand, our observation here may give some insights for understanding the biogenesis and metabolism of peroxisome.

A disadvantage of peroxisomal compartmentalization would be the increased the ATP cost of the pathway. The ATP cost increases due to the import of long chain fatty acyl-CoAs, which requires transport via Pxa1/Pxa2 at the cost of an ATP to ADP; short to medium chain FAs require activation by Faa2 at the cost of ATP to AMP. Furthermore, utilization of *Mm*CAR in the free fatty acid-dependent alkane pathway also require their subsequent hydrolysis, followed by ATP to AMP dependent acylation of *Mm*CAR. Furthermore, upscaling production in the peroxisomes will most likely also require an increase in NADPH supply. NADPH is used for reduction of double bonds in

fatty acids in the peroxisome and the isocitrate dehydrogenase Idp3 has been shown to be the main NADPH source[52,53]. The transcription of *IDP3* has been demonstrated to be negatively regulated by glucose[53]. This repression makes it an obvious engineering target. Furthermore, compartmentalization can be used for *in vivo* evaluating different enzymes or cofactors (Supplementary Fig. 2), which should be helpful for constructing more efficient pathways by screening of better enzymes or cofactors.

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Author contributions

Y.J.Z and J.N. conceived the study, Y.J.Z. designed and performed all the experiments, analysed the data, and wrote the manuscript; Y.J.Z., Z.Z. N.A.B, V.S, J.N., revised the manuscript.

Competing financial interests

Most of the authors have filed for patent protection of part of the work described herein.

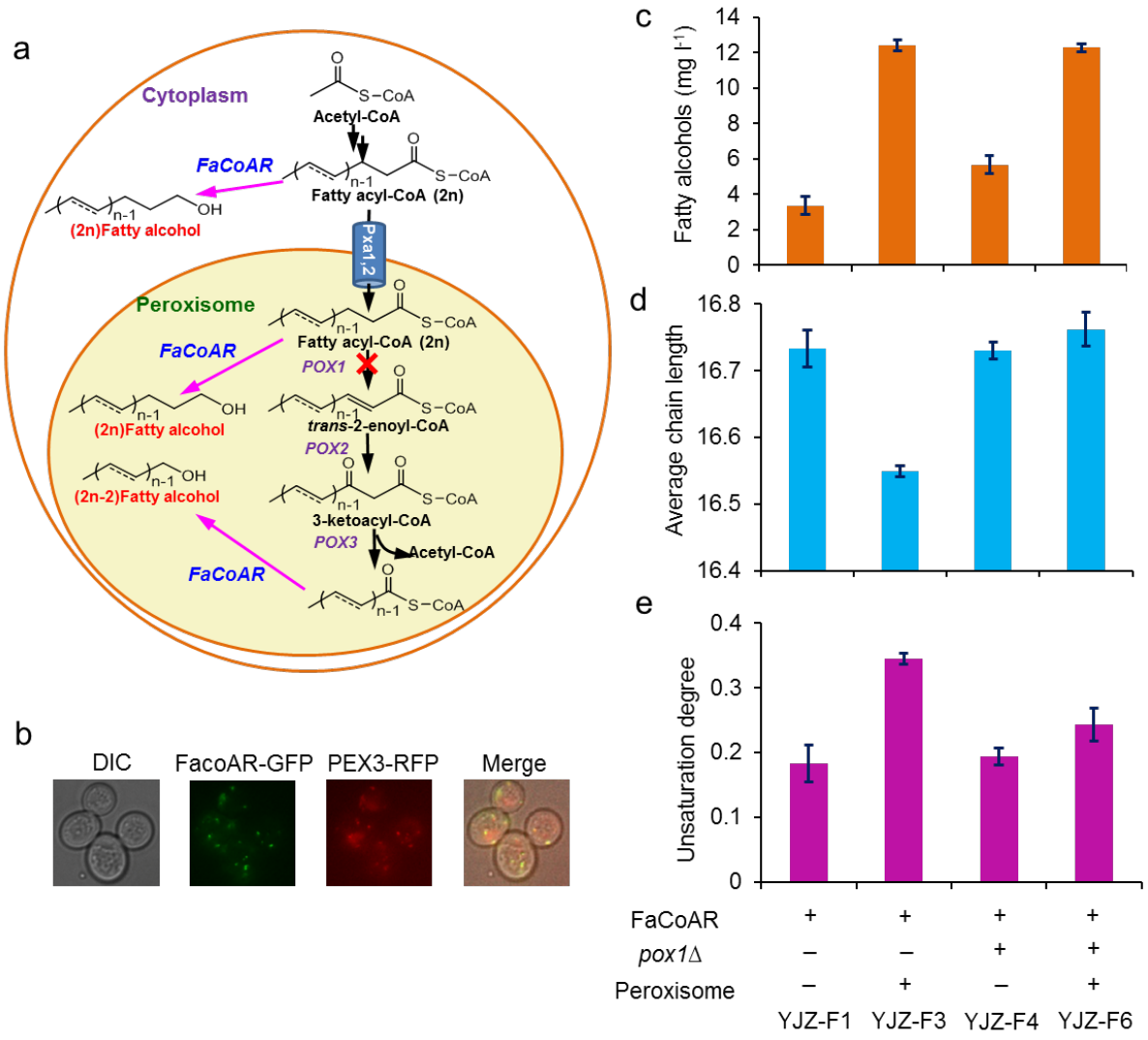

Figure 1 Peroxisomal compartmentalization improved fatty alcohol production from fatty acyl-CoA. (**a**) Schematic view of the peroxisomal or cytosolic pathway for fatty alcohol production. Peroxisomal β-oxidation cycle provides shorter and unsaturated fatty acyl-CoAs, which then can be converted to shorter and unsaturated fatty alcohols catalyzed by FaCoAR. (**b**) Confirmation of the peroxisomal targeting of FacoAR by its co-localization with peroxisomal membrane protein Pex3. FacoAR was fused to a GFP protein and then transformed into yeast strain EY1673 carrying a RFP targeting PEX3. The strains were cultivated 48 h in SC-URA media (**c**)-(**e**) the titers, average chain lengths and unsaturation degrees of fatty alcohols. Unsaturation degrees represents the average double bond number in the fatty alcohols. All data represent the mean±s.d. of three yeast clones, which were cultivated in shake flasks containing Delft media for 72h at 200 rpm, 30 °C.

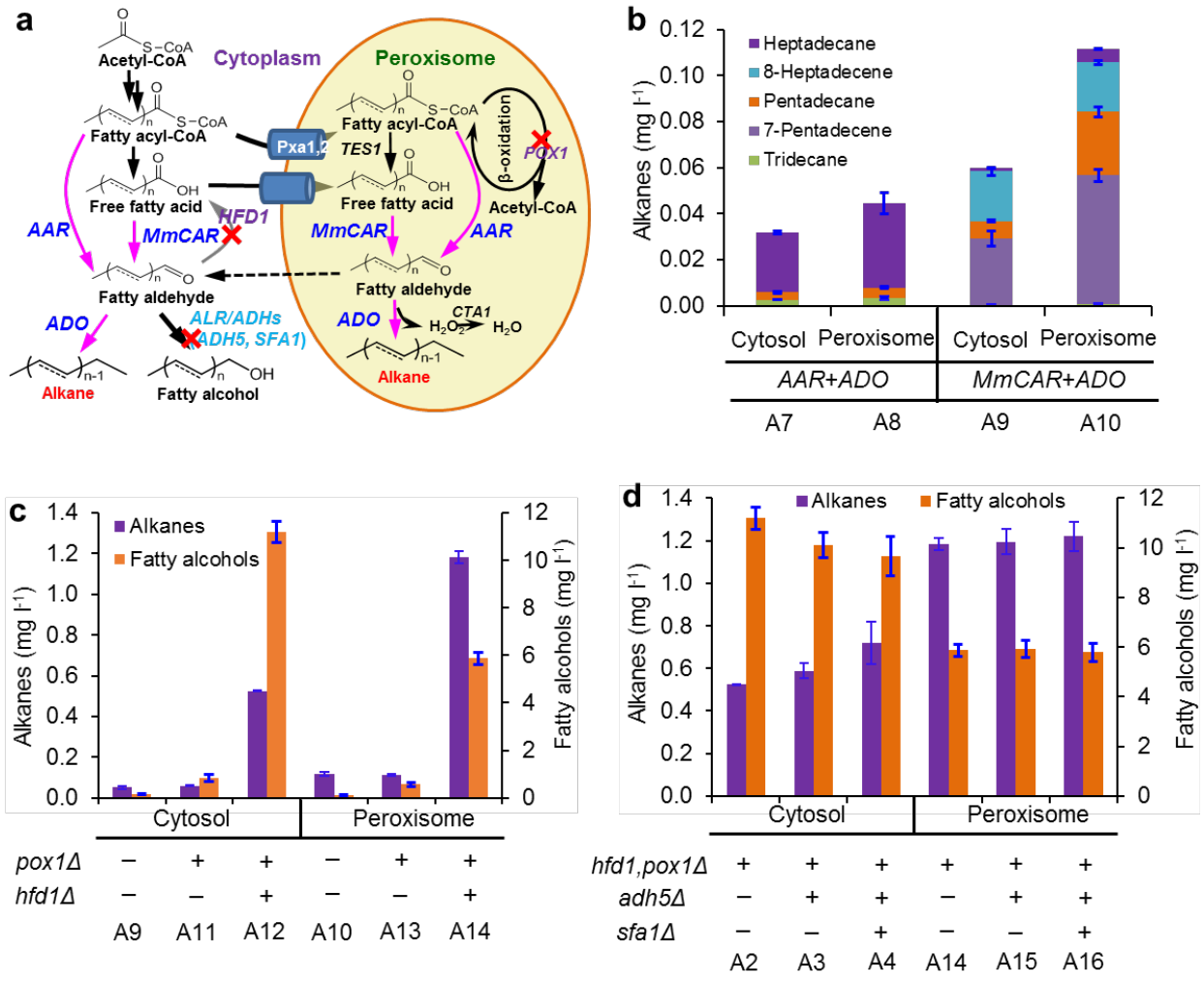

Figure 2 Peroxisomal compartmentalization improved alkane production. (**a**) Schematic view of the peroxisomal and cytosolic pathway for alkane production. (**b**) Peroxisomal compartmentalization improved the alkane production for the fatty acyl-CoA- derived pathway in a wild-type background. (**c**) Peroxisomal compartmentalization improved the alkane production for the fatty acid-derived pathway in a wild-type background. (**d**) Comparing the effect of peroxisome targeting on alkane production in different genetic background. (**e**) Deletion of alcohol dehydrogenase ADH5 and aldehyde reductase SFA1 for alkane production. All data represent the mean±s.d. of three yeast clones, which were cultivated in shake flasks for 72h at 200 rpm, 30 °C.

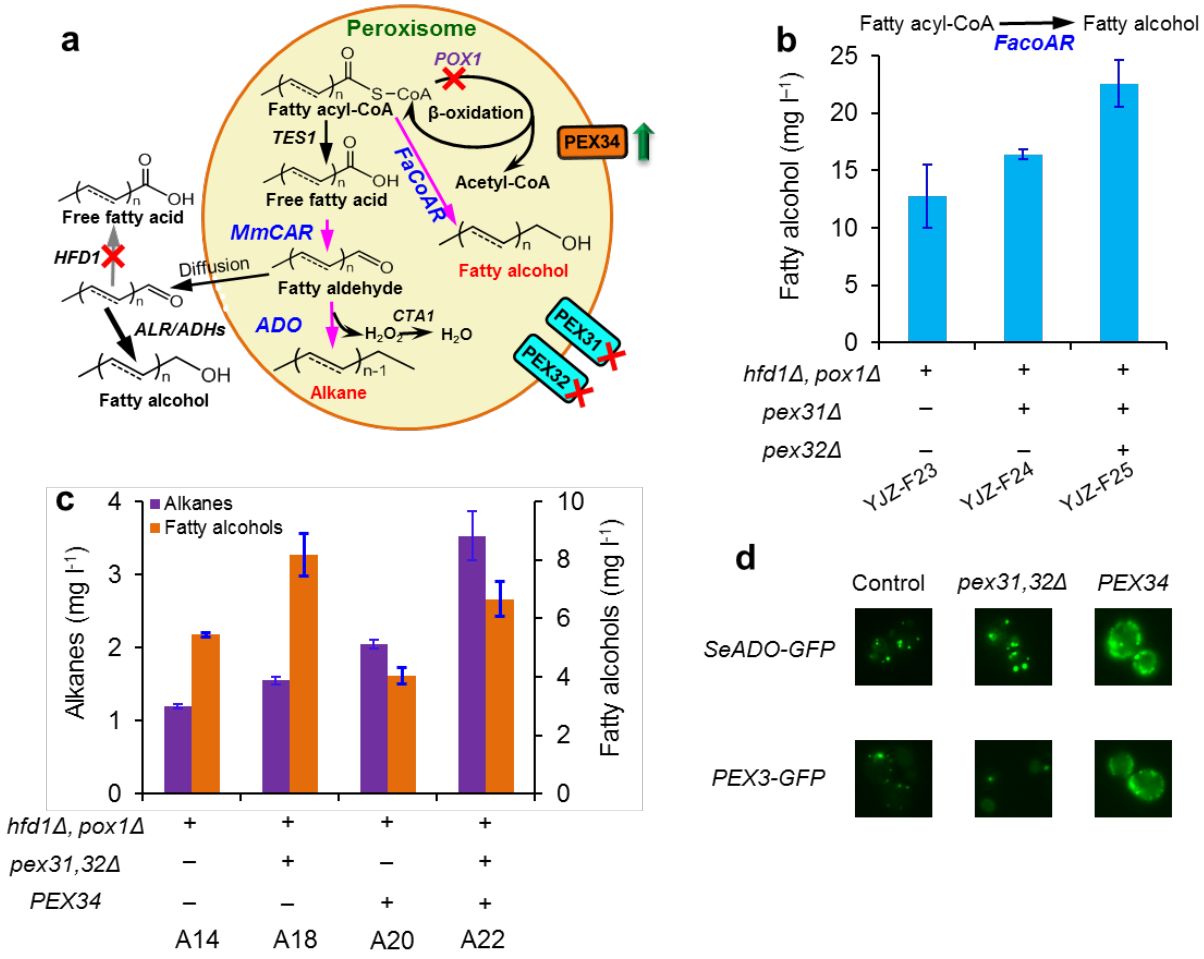

Figure 3 Engineering peroxisome population for improving biosynthesis. (**a**) Schematic view of the peroxisomal pathways for production of fatty acyl-CoA-derived fatty alcohols and FFA-derived alkanes. (**b**) Deletion of *PEX31* and *PEX32* improved production of fatty acyl-CoA-derived fatty alcohols. (**c**) Engineering peroxins for improving alkane production from free fatty acids. All data represent the mean±s.d. of three yeast clones, which were cultivated in shake flasks for 72h at 200 rpm, 30 °C. (**d**) Microscopy visualization of cells carrying the GFP-tagged matrix protein SeADO-GFP or membrane protein PEX3-GFP.

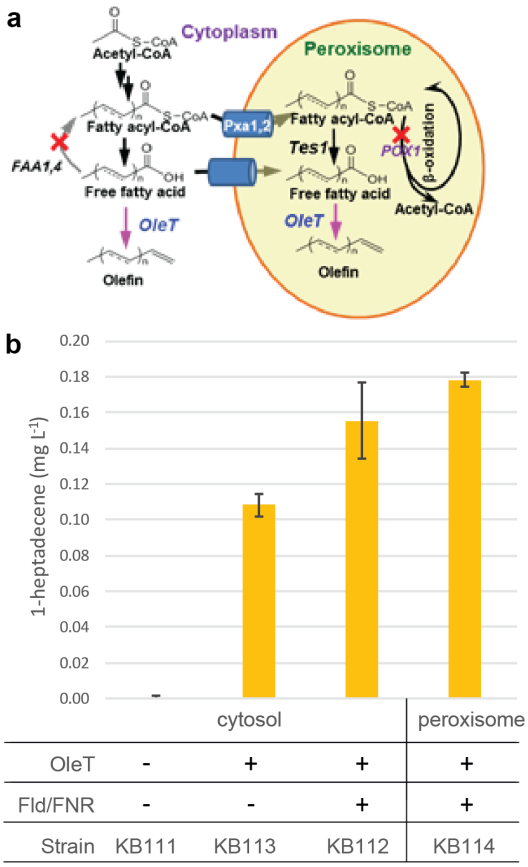

Figure 4 Peroxisomal compartmentalization improved olefin production in yeast. (**a**) Schematic view of the metabolic pathway for olefin production. (**b**) The olefin titers from the strains carrying the cytosolic or peroxisomal pathways. 1,7-pentadecadiene, 1-pentadecene, and 1,8- heptadecadiene could be detected but not reliably quantified. All data represent the mean±s.d. of three clones, which were cultivated in shake flasks for 72h at 200 rpm, 30 °C.

Online methods

Yeast strains, Plasmids and Reagents

The plasmids and strains used in this study are listed in the Supplementary Tables 1 and 2, respectively. The primers (supplementary table 3) were custom synthesized ordered from Sigma-Aldrich. The *SeAAR* and *SeADO* were codon-optimized and synthesized as described before[12]. *EcFNR* and *EcFd* was cloned from the genome of *E. coli* DH5α as described before[12]. *FacCoAR, MmCAR, SeFNR*, SeFd, *NpADO* and OleT (Supplementary Table. 4) were codon-optimized for yeast expression and synthesized by Genscript. PrimeStar DNA polymerase was purchased from TaKaRa. Zymoprep™ Yeast Plasmid Miniprep II was supplied by Zymo Research Corp. Restriction enzymes, DNA gel purification and plasmid extraction kits were purchased from ThermoFisher Scientific. Analytical standards for quantification of alkanes, fatty alcohols and terminal alkenes were supplied by Sigma-Aldrich.

Strain Cultivation

Yeast strains were normally cultivated in YPD media consisting of 10 g l^{-1} yeast extract (Merck Millipore), 20 g l^{-1} peptone (Difco), and 20 g l^{-1} glucose (Merck Millipore). Strains containing *URA3* and/or *HIS3* based plasmids/cassettes were selected on synthetic complete media without uracil or L-histidine (SC-URA, SC-HIS or SC-URA-HIS), which consisted of 6.7 g l^{-1} yeast nitrogen base (YNB) without amino acids (Formedium), 20 g l^{-1} glucose (MerckMillipore) and 0.77 g l^{-1} complete supplement mixture without corresponding nutrition (CSM-URA, CSM-HIS or CSM-HIS-URA, Formedium). The *URA3* maker was removed and selected against on SC+5-FOA plates, which contained 6.7 g l^{-1} YNB, 0.77 g l^{-1} complete supplement mixture and 0.8 g l^{-1} 5-fluoroorotic acid. Strain containing the *amdSYM*[37] cassettes were selected on SM media (5 g l^{-1} $(NH_4)_2SO_4$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \bullet 7H_2O$, 3.3 g l^{-1} K_2SO_4, 0.6 g l^{-1} acetamide, 30 g l^{-1} glucose) trace metal and vitamin solutions[38] supplemented with 40 mg l^{-1} histidine and/or 60 mg l^{-1} uracil if needed). Strains containing the *kanMX* cassettes were selected on YPD plates containing 200 mg l^{-1} G418 (Formedium).

Shake flask batch fermentations for production of alkanes, fatty alcohols and olefins were carried out in minimal medium containing 5 g l^{-1} $(NH_4)_2SO_4$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \bullet 7H_2O$, 30 g l^{-1} glucose, trace metal and vitamin solutions[38] supplemented with 40 mg l^{-1} histidine and/or 60 mg l^{-1} uracil if needed. Cultures were inoculated, from 24 h precultures, at an initial OD_{600} of 0.1 in 15 ml minimal medium and cultivated at 200 rpm, 30 °C for 72 h.

Genetic engineering

All the episomal vectors or genome-integrated pathways (Supplementary Fig. 8) were constructed by the modular pathway engineering (MOPE) strategy as previously described[30].

Scarless gene deletion was performed as described previously[39] by using a *Kluyveromyces lactis URA3* as a selection marker. An *amdSYM* cassette[37] with 80-bp homologous arms was used for *PEX31* deletion.

During construction of YJZ74, it proved difficult to delete *PEX32* using the same strategy as for *pex31*Δ*::amdSYM* in strain YJZ63. Because the same promoter and terminator in *amdSYM* cassette, are used in the *kanMX* cassette, this cassette was not suitable for subsequent *PEX32* deletion in strain YJZ63 harbouring *pex31*Δ*::amdSY*. We first tried a *URA3* cassette with 80-bp homologous arms, however, the loop-out *URA3* failed three times. Then we constructed three alternative *kanMX* cassettes: *kanMX*-PX1 with promoter *TEF1p* and terminator *PEX32t*, *kanMX*-PX2 only containing *kanMX* open reading frame without promoter and terminator, *kanMX*-PX3 with promoter *tHXT7p* and terminator *PEX32t*. Interesting, *kanMX*-PX3 succeed in replacing *PEX32* in YJZ63, which indicated the expression level of *kanMX* should be carefully tuned for deletion of some specific genes.

Products extraction and quantification

Extraction and Quantification of fatty alcohols, alkanes, and olefins were performed as a previously reported[3] with slight modifications. Briefly, fatty alcohols were analysed by gas chromatography (Focus GC, ThermoFisher Scientific) equipped with a Zebron ZB-5MS GUARDIAN capillary column (30 m x 0.25 mm x 0.25 μm, Phenomenex) and a flame ionization detector (FID, ThermoFisher Scientific). The GC program for fatty alcohol quantification was as follows: initial temperature of 45 °C hold for 2.5 min; then ramp to 220 °C at a rate of 20 °C per min and hold for 2 min; ramp to 300 °C at a rate of 20 °C per min and hold for 5 min. The temperature of the inlet and detector were kept at 280 °C and 300 °C respectively.

Alkanes and olefins were analysed by a GC-MS (Focus GC with a DSQII mass spectrometer ThermoFisher Scientific) equipped with a Zebron ZB-5MS GUARDIAN capillary column (30 m x 0.25 mm x 0.25 μm, Phenomenex). The GC program for alkanes was as follows: initial temperature of 50 °C, hold for 5 min; then ramp to 140 °C at a rate of 10 °C per min and hold for 10 min; ramp to 310 °C at a rate of 15 °C per min and hold for 7 min. The olefin GC program was: initial temperature of 50 °C, hold for 5 min; then ramp to 310 °C at a rate of 10 °C per min and hold for 6 min. The temperature of inlet, mass transfer line and ion source were kept at 250 °C, 300 °C and 230 °C, respectively. The flow rate of the carrier gas (helium) was set to 1.0 ml per minute, and data were acquired at full scan mode (50-650 m/z) and then analyzed using the Xcalibur software.

Fluorescence microscopy analysis

For confirmation of the protein localization, the proteins were fused to a green fluorescent protein (GFP) with a flexible linker GGTGGTGGTTC, and then the encoding genes were transformed to the yeast strain EY1673 carrying a peroxisome marker protein Pex3 with a C-

terminal tagged RFP [40]. The cells were cultivated in SC-URA or Delft media for 48 h at 30 °C, 200 rpm. 3 µL cell cultures were dropped onto microscope slides and then viewed with a LEICA DM2000 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

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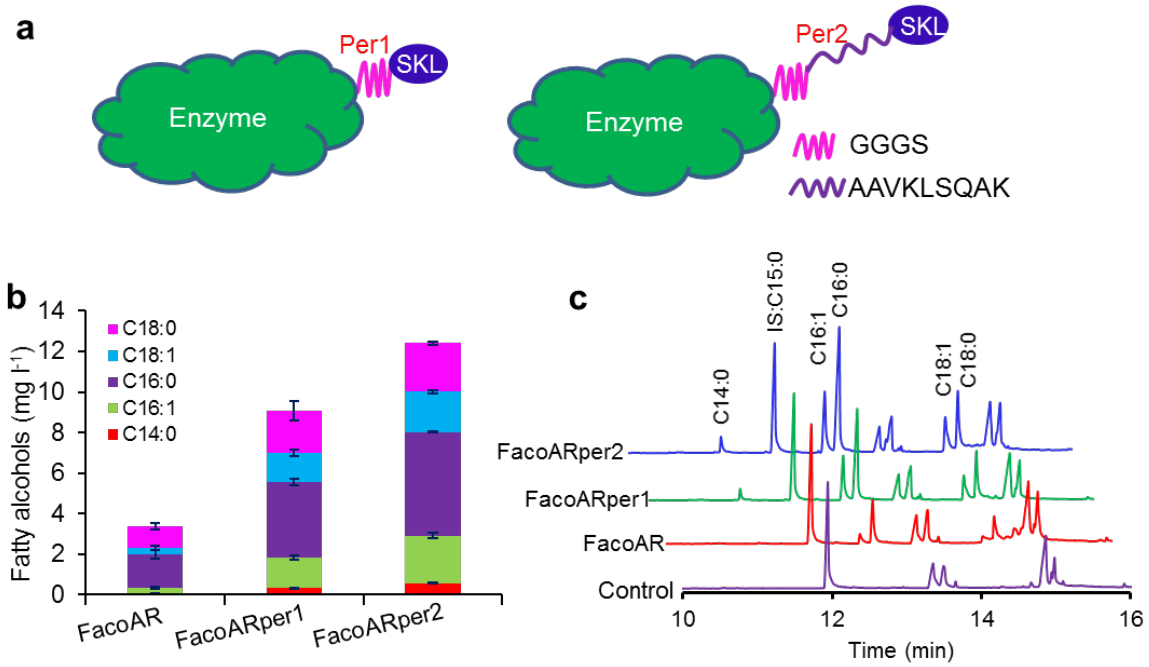
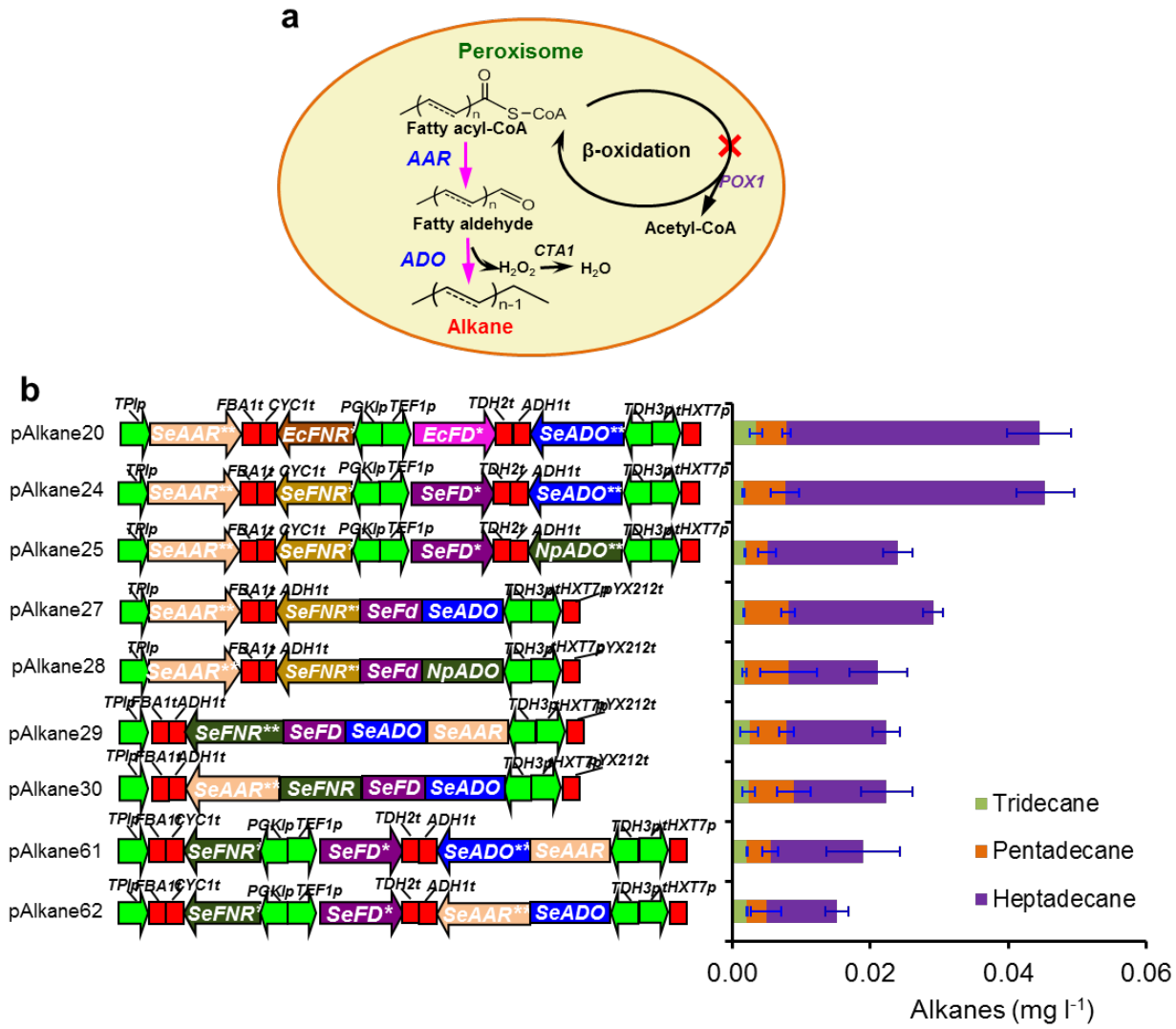
Supplementary Information for

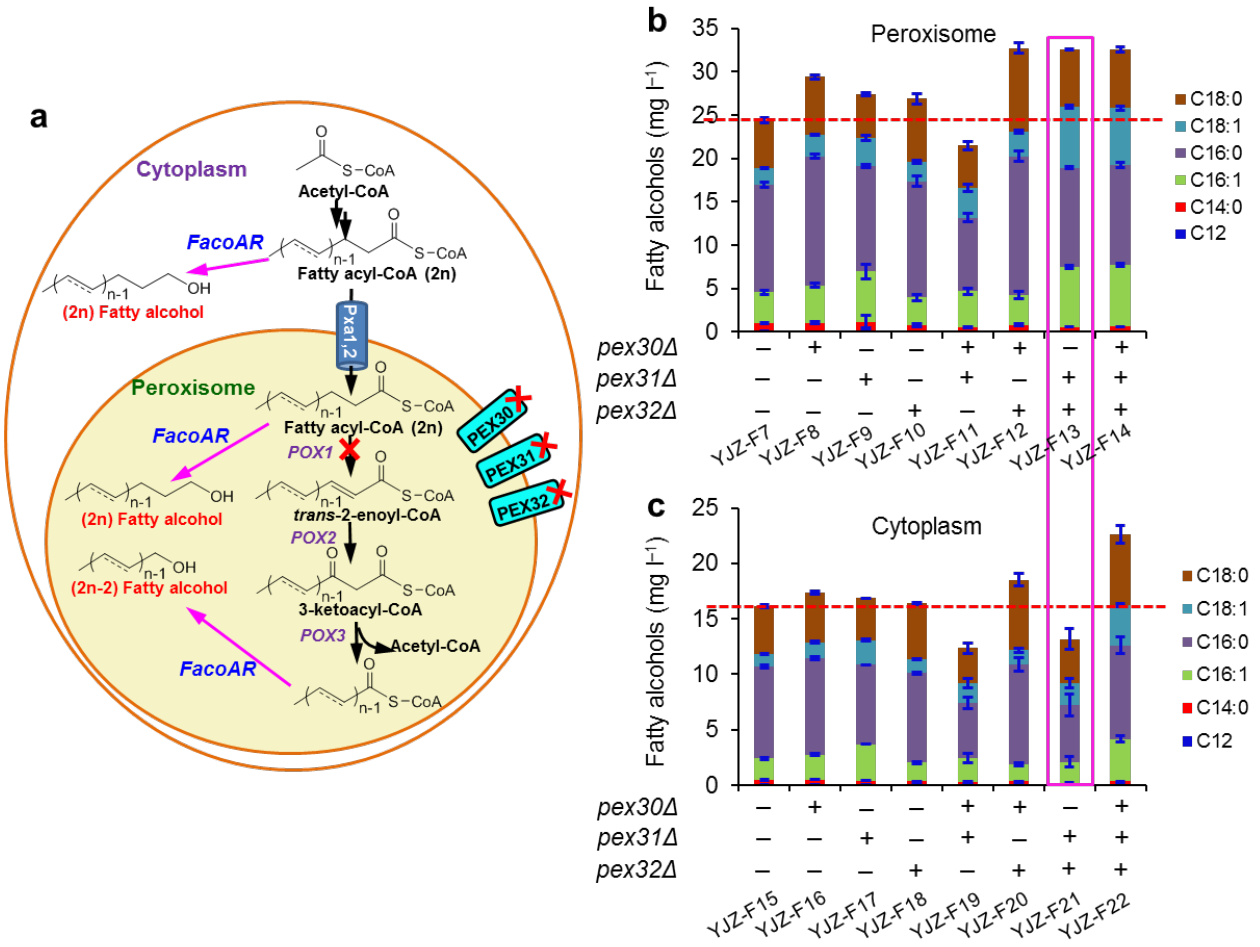
Harnessing peroxisomes for production of fatty acid derived chemicals and biofuels in yeast



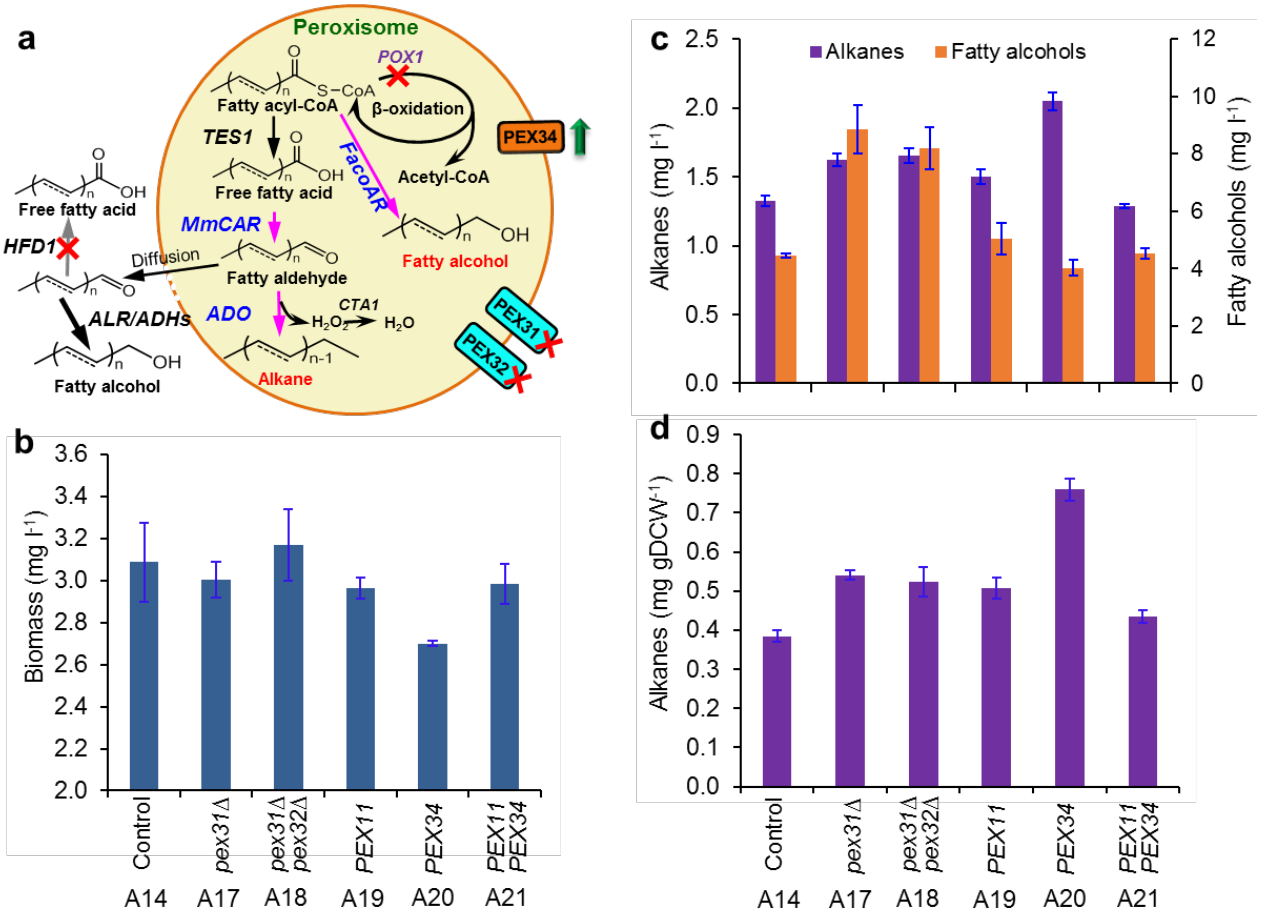
Supplementary Fig. 1 Optimization of the signal peptides for peroxisomal enzyme targeting. (**a**) Schematic representation of designed signal sequence for peroxisomal targeting. The peroxisomal targeting signal per1 is composed by a flexible linker sequence GGGS[1] and a PTS1 signal tri-peptide SKL; the peroxisomal targeting signal per2 had a another flexible loop AAVKLSQAK[2] to increase the accessibility of the PTS1 signal. (**b**) The fatty alcohol titers in the cells harboring the FacoAR, FacoARper1 or FacoARper1. Peroxisomal targeting fatty acyl-CoA reductase FacoAR with signal per1 (FacoARper1) enabled a 2.7-fold improvement in fatty alcohol production in wild-type background. And alternative peroxisomal targeting signal per2 (FacoARper2) improved fatty alcohol production by 3.7 fold. These results shows that per2 signal is better than per1 for peroxisomal enzyme targeting. (**c**) The GC chromatograph of products extraction from corresponding cells that carrying cytosolic FacoAR, peroxisomal FaCoARper1 or FaCoARper1. **Note:** per1: GGGSSKL. Per2: GGGSAAVKLSQAKSKL. The strain variants were cultivated in shake flasks for 72 h, at 30°C, 200 rpm. The data represent the mean ± s. d. of three independent clones.



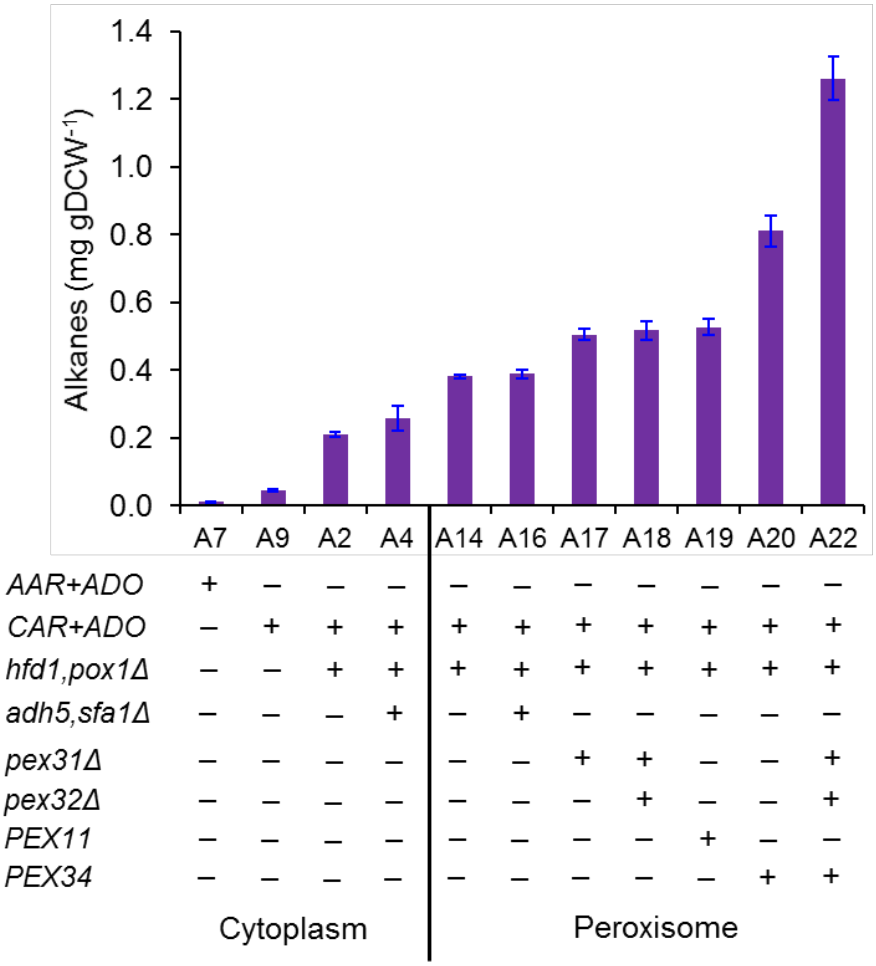
Supplementary Fig. 2 Alkane production by different pathways harboring different aldehyde-deformylating oxygenases and electron transfer systems. All the pathways was constructed on the pYX212 backbone by modular pathway engineering strategy[1] and then all the plasmids were transformed to strain CENPK 113-11C. *Se*AAR, fatty acyl-ACP/CoA reductase encoding gene from *Synechococcus elongatus*; *Se*ADO, aldehyde-deformylating oxygenase encoding gene from *S. elongatus*; *NpADO*, *ADO* from *Nostoc punctiforme* PCC73102; *EcFNR*, ferredoxin reductase encoding gene from *E. coli*; *EcFd*, ferredoxin encoding gene from *E. coli*; *SeFNR*; *FNR* ferredoxin reductase encoding gene from *S. elongatus*; *SeFd,* ferredoxin encoding gene from *S. elongatus*; "**" means peroxisome targeting signal per2: GGGSAAVKLSQAKSKL and "*" represents signal per1: GGGSSKL.



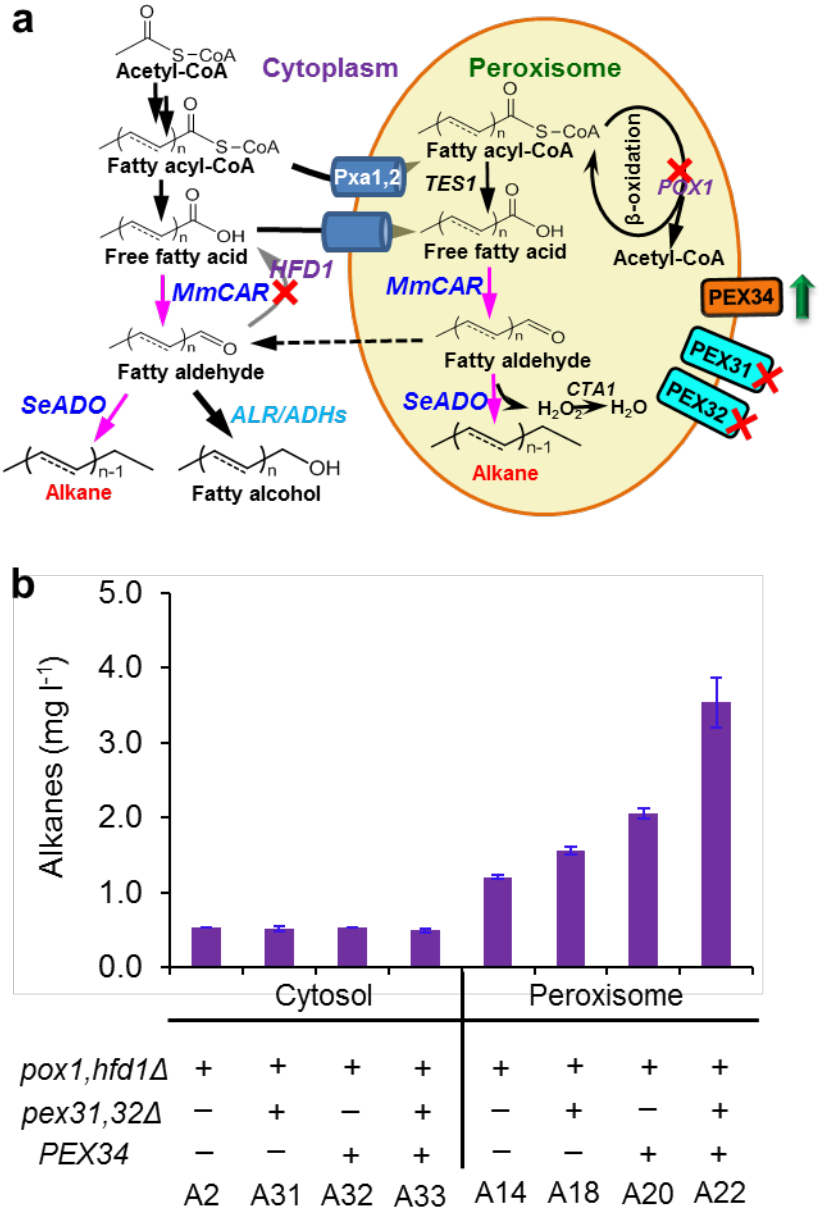
Supplementary Fig. 3 Production of peroxisomal or cytosolic fatty alcohol in S288c background strain carrying of PEX30, 31 and/or 32 deletion[3]. Peroxisomal (pFcoARper2) or cytosolic (pFcoAR) expression plasmids were transformed to S288c background strains carrying of PEX30, 31 and/or 32 deletion (Supplementary Table 2). The *PEX31, 32*Δ had improved production of peroxismal fatty alcohols but not cytosolic fatty alcohols, which indicated *PEX31, 32*Δ increased the peroxisome number not perturbed the fatty acyl-CoA metabolism. The data represent the averages ± standard deviations of three independent clones.



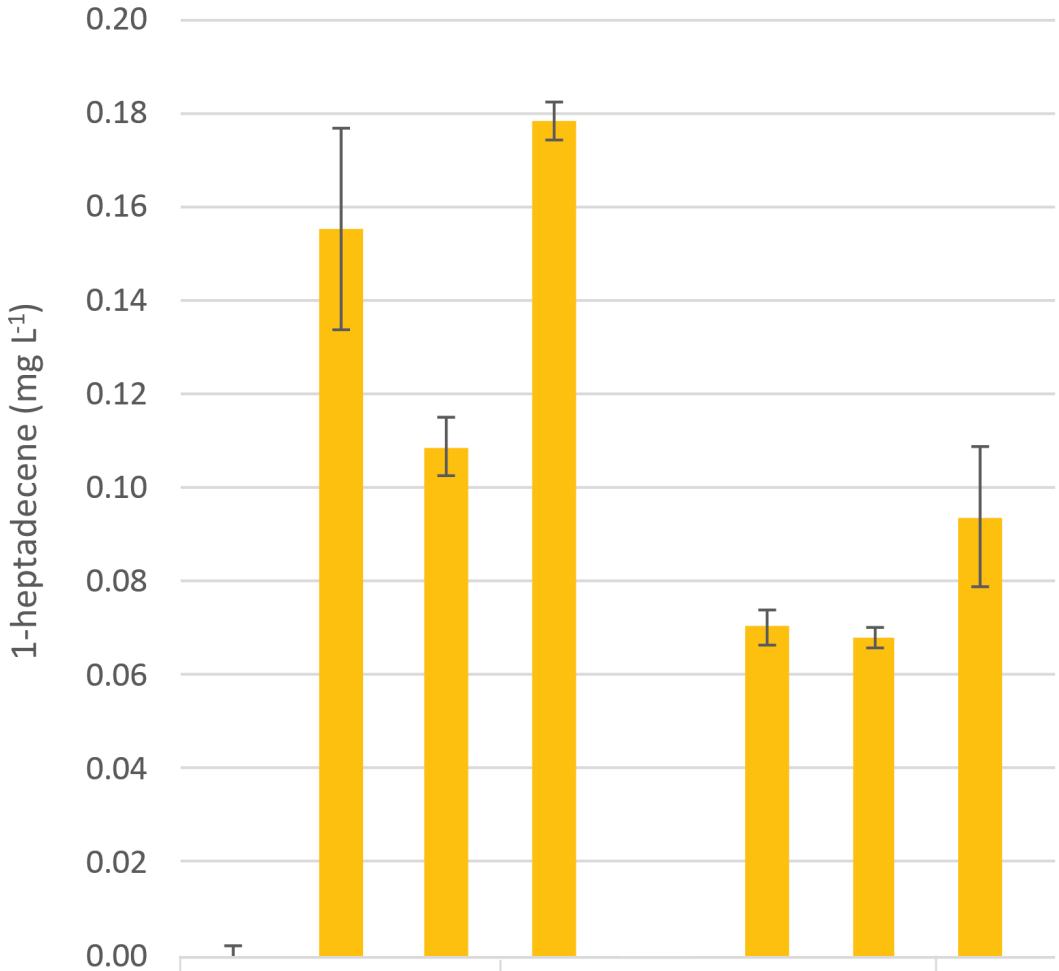
Supplementary Fig. 4 Engineering peroxisome population for alkane production. (**a**) The scheme of rewired peroxisome biosynthetic pathways for alkane production. (**b**) The final biomasses of strain variants. (**c**) The titers of alkane and by-product fatty alcohols from the strains with engineered peroxins. (**d**) The specific titers of alkane from the strains with engineered peroxins. The strain variants were cultivated in shake flasks for 72 h, at 30°C, 200 rpm. The data represent the mean ± s. d. of three independent clones.



Supplementary Fig. 5 The biomass specific alkane titers of the yeast cells with cytosolic and peroxisomal alkane pathways.

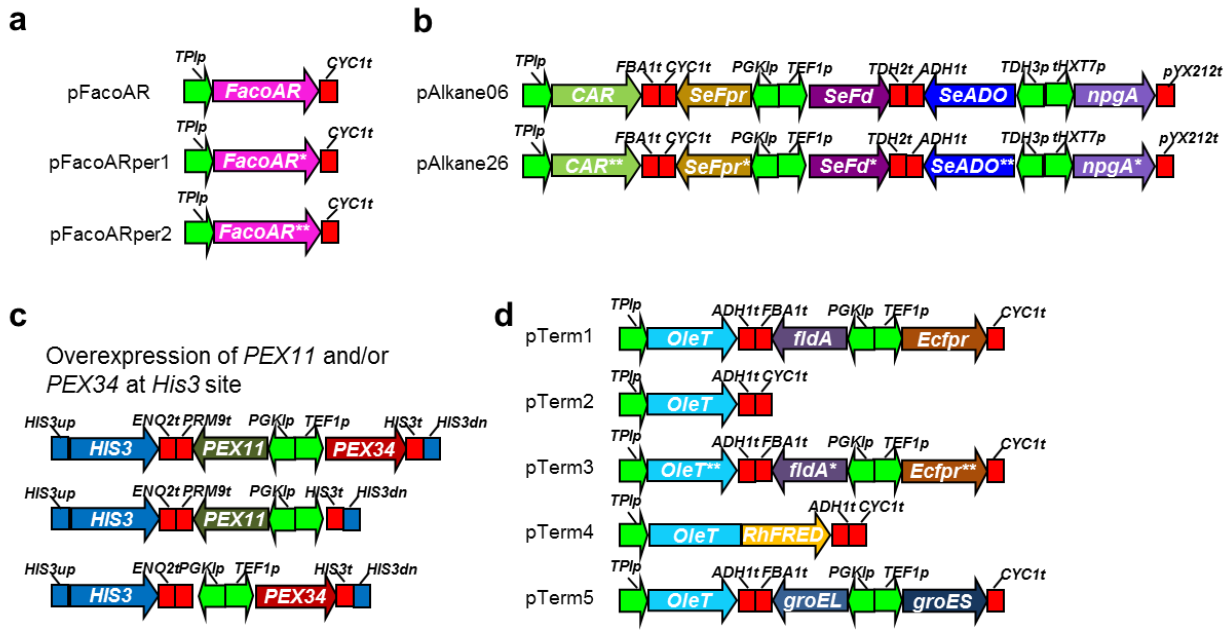


Supplementary Fig. 6 Engineering peroxins improved alkane production from peroxisomal pathways but not the cytosolic ones. Deletion of PEX31, 32 and overexpression had little effect on cytosolic pathway-based alkane production, while engineering these peroxins significantly improved peroxisomal-based alkane production. The strain variants were cultivated in shake flasks for 72 h, at 30°C, 200 rpm. The data represent the mean ± s. d. of three independent clones.



Localization	-	C	C	P	C	P	P
OleT	-	+	+	+	-	-	+
Fld/FNR	-	+	-	+	-	-	+
OleT-FRED	-	-	-	-	+	+	-
GroEL-GroES	-	-	-	-	-	-	+
Strain	KB111	KB113	KB112	KB114	KB116	KB117	KB122

Supplementary Fig. 7 Comparing the cytosolic and peroxisomal pathways for olefin production. The strain variants were cultivated in shake flasks for 72 h, at 30°C, 200 rpm. The data represent the mean ± s. d. of three independent clones.



Supplementary Fig 8 The genetic arrangement of pathways on episomal plasmids or chromosome. (**a**) Episomal plasmids for production of fatty acyl-CoA derived fatty alcohols. (**b**) Episomal plasmids for production of alkanes. (**c**) Genome-overexpression of *PEX11* and/or *PEX34* at *HIS3* site for regulating the peroxisome population. See strain YJZ66, YJZ67, YJZ68 and YJZ75. (**d**) Episomal plasmids for production of olefins. "**" means peroxisome targeting signal per2: GGGSAAVKLSQAKSKL and "*" represents signal per1: GGGSSKL.

Supplementary Table 1 Plasmids used in this study

Plasmids	Genotype or characteristic	Resource
pYX212	2 µm, AmpR, *URA3*, *TPI*p, *pYX212*t	R&D systems
P423GPD	2 µm, AmpR, *HIS3*, *TDH3*p, *CYC1*t	
pFacoAR	pYX212-(*TPIp*-***FacoAR***-*pYX212t*)	This study
pFacoARper1	pYX212-(*TPIp*-***FacoARper1***-*pYX212t*)	This study
pFacoARper2	pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)	This study
pAlkane7	pYX212-(*TPIp*-***SeAAR***-*FBA1t*)+(PGK1p-***EcFNR***-*CYC1t*)+(*TEF1p*-***EcFD***-*TDH2t*)+ (*TDH3p*-***SeADO***-*ADH1t*)	[4]
pAlkane20	pYX212-(*TPIp*-***SeAARper2***-*FBA1t*)+(PGK1p-***EcFNRper1***-*CYC1t*)+(*TEF1p*-***EcFDper1***-*TDH2t*)+ (*TDH3p*-***SeADOper2***-*ADH1t*)	This study
pAlkane24	pYX212-(*TPIp*-***SeAARper2***-*FBA1t*)+(PGK1p-***SeFNRper1***-*CYC1t*)+(*TEF1p*-***SeFDper1***-*TDH2t*)+ (*TDH3p*-***SeADOper2***-*ADH1t*)	
pAlkane06	pYX212-(*TPIp*-***MmCAR***-*FBA1t*)+(PGK1p-***SeFNR***-*CYC1t*)+(*TEF1p*-***SeFD***-*TDH2t*)+(*TDH3p*-***SeADO***-*ADH1t*)+(tHXT7p-***npgA***-*pYX212t*)	This study
pAlkane26	pYX212-(*TPIp*-***MmCARper2***-*FBA1t*)+(PGK1p-***SeFNRper1***-*CYC1t*)+(*TEF1p*-***EcFDper1***-*TDH2t*)+(*TDH3p*-***SeADOper2***-*ADH1t*)+(tHXT7p-***npgAper1***-	This study
pTerm1	p423-(*TDH3p*-***oleT***-*ADH1t*)+(*PGK1p*-***EcfldA***-*FBA1t*)+(*TEF1p*-***EcFNR***-*CYC1t*)	This study
pTerm2	p423-(*TDH3p*-***oleT***-*CYC1t*)	This study
pTerm3	p423-(*TDH3p*-***oleTper2***-*ADH1t*)+(*PGK1p*-***EcfldAper2***-*FBA1t*)+(*TEF1p*-***EcFNRper2***-*CYC1t*)	This study
pTerm5	p423-(*TDH3p*-***oleT-RhFRED***-*CYC1t*)	This study
pTerm9	p423-(*TDH3p*-***oleT***-*ADH1t*)+(*PGK1p*-***groEL***-*FBA1t*)+(*TEF1p*-***groES***-*CYC1t*)	This study

Supplementary Table 2 *S. cerevisiae* strains used in this study.

Strains	Genotype or characteristic	Resource
Background strains		
CEN.PK113-11C	*MATa MAL2-8c SUC2 his3Δ1 ura3-52*	ATCC
YJZ01	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ*	[4]
YJZ02	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ*	This study
YJZ03	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ*	This study
YJZ06	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ*	This study
ZW31	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ*	This study
ZW32	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ sfa1Δ*	This study
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[5]
BY4742*pex30Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex30::KanMX4*	[5]
BY4742*pex31Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex31::KanMX4*	[5]
BY4742*pex32Δ*	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex32::KanMX4*	[5]
DK1	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4*	3
DK2	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex32::KanMX4*	3
DK3	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex31::KanMX4 pex32::KanMX4*	3
TKO	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4 pex32::NAT*	3
YJZ63	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31::amdSYM*	This study
YJZ74	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31::amdSYM pex32::KanMX4*	This study
YJZ66	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ::HIS3*+(*PGK1p-**PEX11**-PRM9t*)+(*TEF1p-**PEX34**-HIS3t*)	This study
YJZ67	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ::HIS3*+(*PGK1p-**PEX11**-PRM9t*	This study
YJZ68	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ::HIS3*+(*TEF1p-**PEX34**-HIS3t*)	This study
YJZ75	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31::amdSYM pex32::KanMX4 his3Δ::HIS3*+(*TEF1p-**PEX34**-HIS3t*)	This study
Fatty alcohol producing strain		
YJZ-F1	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pYX212-(*TPIp-**FacoAR**-pYX212t*) =[CEN.PK113-11C, pFacoAR]	This study
YJZ-F2	*MATa; MAL2-8c SUC2 his3Δ1 ura3-52* pYX212-(*TPIp-**FacoARper1**-pYX212t*) =[CEN.PK113-11C, pFacoARper1]	This study
YJZ-F3	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pYX212-(*TPIp-**FacoARper2**-pYX212t*) =[CEN.PK113-11C, pFacoARper2]	This study

YJZ-F4	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ* pYX212-(*TPIp*-***FacoAR***-*pYX212t*) =[YJZ02, pFacoAR]	This study
YJZ-F5	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ* pYX212-(*TPIp*-***FacoARper1***-*pYX212t*)=[YJZ02, pFacoARper1]	This study
YJZ-F6	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[YJZ02, pFacoARper2]	This study
YJZ-F7	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[BY4742 pFacoARper2]	This study
YJZ-F8	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex30::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[BY4742*pex30Δ* pFacoARper2]	This study
YJZ-F9	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex31::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[BY4742*pex31Δ* pFacoARper2]	This study
YJZ-F10	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex32::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[BY4742*pex32Δ* pFacoARper2]	This study
YJZ-F11	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[DK1 pFacoARper2]	This study
YJZ-F12	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex32::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[DK2 pFacoARper2]	This study
YJZ-F13	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex31::KanMX4 pex32::KanMX4* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[DK3 pFacoARper2]	This study
YJZ-F14	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4 pex32::NAT* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[TKO pFacoARper2]	This study
YJZ-F15	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[BY4742 pFacoAR]	This study
YJZ-F16	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex30::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[BY4742*pex30Δ* pFacoAR]	This study
YJZ-F17	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex31::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[BY4742*pex31Δ* pFacoAR]	This study
YJZ-F18	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pex32::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[BY4742*pex32Δ* pFacoAR]	This study
YJZ-F19	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[DK1 pFacoAR]	This study
YJZ-F20	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex32::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[DK2 pFacoAR]	This study
YJZ-F21	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex31::KanMX4 pex32::KanMX4* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[DK3 pFacoAR]	This study
YJZ-F22	*MATα his3Δ1 leu2Δ0 ura3Δ0 pex30::KanMX4 pex31::KanMX4 pex32::NAT* pYX212-(*TPIp*-***FacoAR***-*pYX212t*)=[TKO pFacoAR]	This study
YJZ-F23	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[YJZ03 pFacoARper2]	This study
YJZ-F24	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[YJZ63 pFacoARper2]	This study
YJZ-F25	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ pex32Δ* pYX212-(*TPIp*-***FacoARper2***-*pYX212t*)=[YJZ74 pFacoARper2]	This study

Alkane producing strain		
A1	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ* pAlkane06	This study
A2	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ* pAlkane06	This study
A3	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ* pAlkane06	This study
A4	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ sfa1Δ* pAlkane06	This study
A7	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pAlkane7	This study
A8	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pAlkane20	This study
A9	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pAlkane06	This study
A10	*MATa MAL2-8c SUC2 his3Δ1 ura3-52* pAlkane26	This study
A11	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ* pAlkane26	This study
A13	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ* pAlkane26	This study
A14	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ* pAlkane26	This study
A15	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ* pAlkane26	This study
A16	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ adh5Δ sfa1Δ* pAlkane26	This study
A17	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ::amdSYM* pAlkane26	This study
A18	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ::amdSYM pex32Δ::kanMX* pAlkane26	This study
A19	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ:: HIS3+*(*PGK1p-**PEX11**-PRM9t*) pAlkane26	This study
A20	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ:: HIS3+*(*TEF1p-**PEX34**-His3t*) pAlkane26	This study
A21	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ:: HIS3++*(*PGK1p-**PEX11**-PRM9t*)+(*TEF1p-**PEX34**-His3t*) pAlkane26	This study
A22	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ::amdSYM pex32Δ::kanMX his3Δ:: HIS3+*(*TEF1p-**PEX34**-His3t*) pAlkane26	This study
A31	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ::amdSYM pex32Δ::kanMX* pAlkane06	This study
A32	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ his3Δ:: HIS3+*(*TEF1p-**PEX34**-His3t*) pAlkane06	This study
A33	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 hfd1Δ pox1Δ pex31Δ::amdSYM pex32Δ::kanMX his3Δ:: HIS3+*(*TEF1p-**PEX34**-His3t*) pAlkane06	This study
Terminal alkene producing strains		
KB111	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* p423GPD	This study
KB112	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* pTerm1	This study

KB113	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* pTerm2	This study
KB114	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* pTerm3	This study
KB116	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* pTerm5	This study
KB120	*MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ faa1Δ faa4Δ* pTerm9	This study

Supplementary Table 3 Primers used in this study

Primer No.	Name	Sequence (5'-3')
Primers for episomal pathways construction		
1	TPIp-F	GTTTAAAGATTACGGATATTTAACTTACTTAGAATAATG
2	TPIp-R	CATTTTTAGTTTATGTATGTGTTTTTTGTAG
3	PGK1p-R	CATTTTGTTATATTTGTTGTAAAAAGTAGATAATTAC
4	TEF1p-F	ATAGCTTCAAAATGTTTCTACTCCTTTTTTACTC
5	TEF1p-R	CATTTTGTAATTAAAACTTAGATTAGATTGCTATGC
6	TDH3p-F	CTCGAGTTTATCATTATCAATACTGCCATTTC
7	TDH3p-R	GTTTGTTTATGTGTGTTTATTCGAAACTAAGTTCTTGGTG
8	tHXT7p-F	GTATTCTTTGAAATGGCAGTATTGATAATGATAAACTCGAGCTCGTAGGAACAATTTCG
9	tHXT7p-R	CATTTTTTGATTAAAATTAAAAAAACTTTTTGTTTTTGTG
10	FBA1t-F	GTTAATTCAAATTAATTGATATAGTTTTTTAATGAG
11	CYC1t-F	GATACCGTCGACCTCGAGTCATGTAATTAGTTATGTC
12	CYC1t-R	GGGTACCGGCCGCAAATTAAAGCCTTCGAGCGTCC
13	TDH2t-F	
14	ADH1t-F	GCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAG
15	pYX212t-F	TAGGGCCCACAAGCTTACGCGTCGACCCGGGTATCC
16	pYX212t-R	GCCGTAAACCACTAAATCGGAACCCTAAAGG
17	FaCoAR-F	GCTTAAATCTATAACTACAAAAAACACATACATAAACTAAAAATGAATTATTTCTTGACAGGTGG
18	FaCoAR-R	GATACCCGGGTCGACGCGTAAGCTTGTGGGCCCTATTACCAATAGATACCTCTCATAATGG
19	FacoARper1-R	GGATACCCGGGTCGACGCGTAAGCTTGTGGGCCCTATTATAGTTTAGAAGAACCACCACCCCAATAGATACCTCTCATAATGG
20	FacoARper2-R	GATACCCGGGTCGACGCGTAAGCTTGTGGGCCCTATTATAGTTTAGATTTTGCCTGCGATAGTTTTACAGCGGCAGAACCACCACCCCAATAGATACCTCTCATAATG
21	SeAAR-F	CTATAACTACAAAAAACACATACATAAACTAAAAATGTTCGGTTTAATAGGTCAC
22	SeAAR-R	CATTAAAAAACTATATCAATTAATTTGAATTAACTCAGATTGCTAAGGCTAAAG
23	SeAARper2-R	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCATAGTTTAGATTTTGCCTGCGATAGTTTTACAGCGGCAGAACCACCACCGATTGCTAAGGCTAAAGGTTG
24	EcFNR-F	GGAAGTAATTATCTACTTTTTACAACAAATATAACAAAATGGCTGATTGGGTAACAGG
25	EcFNR-R	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTACCAGTAATGCTCCGCTG
26	EcFNRper-R1	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTATAGTTTAGAAGAACCACCACCCCAGTAATGCTCCGCTGTCATATGG
27	SeFNR-F	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGCCAAAGATTGTTATTTTG
28	SeFNR-R	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTAATAAGTTTCTACGTGCCATC

Supplementary Table 3 (continued)

29	SeFNRper1-R	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTATAGTTTAGAAGAACCAC CACCATAAGTTTCTACGTGCCATC
30	EcFd-F	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGCCAAAGATTGTTATTTTG
31	EcFd-R	CTAAATCATTAAAGTAACTTAAGGAGTTAAATTTAATGCTCACGCGCATGGTTG
32	EcFDper1-R	CTAAATCATTAAAGTAACTTAAGGAGTTAAATTTATAGTTTAGAAGAACCACCACCA TGCTCACGCGCATGGTTGATAG
33	SeFd-F	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCTACTTACAAGGTCAC TTTG
34	SeFd-R	CTAAATCATTAAAGTAACTTAAGGAGTTAAATTTAGTACAAGTCTTCTTCCTTATGTG
35	SeFdper1-R	CTAAATCATTAAAGTAACTTAAGGAGTTAAATTTATAGTTTAGAAGAACCACCACCG TACAAGTCTTCTTCCTTATGTG
36	SeADO-F	CAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGCCACAATTAGAAGC CTC
37	SeADO-R	CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTTAGACTGCTGCCAAACC GTATG
38	SeADOper2-R	CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTTATAGTTTAGATTTTGC CTGCGATAGTTTTACAGCGGCAGAACCACCACCGACTGCTGCCAAACCGTATGC
39	MmCAR-F	CTATAACTACAAAAAACACATACATAAACTAAAAATGTCACCTATCACCAGAGAAG
40	MmCAR-R	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCACAACAAACCCAACAATCTC
41	MmCARper2-R	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCATAGTTTAGATTTTGCCTGC GATAGTTTTACAGCGGCAGAACCACCACCCAACAAACCCAACAATCTC
42	OleT-F	GAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGGCTACATTGAAGAGAG ACAAG
43	OleT-R1	CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTCAAGTTCTATCTACTAC TTCTCTG
44	OleTper2-R	CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTCATAGTTTAGATTTTGC CTGCGATAGTTTTACAGCGGCAGAACCACCACCAGTTCTATCTACTACTTCTCTG
45	OleT-R2	CTGCTGGTTCACCTATGGTAACTGGTTGATGTCTATGCAATACAGTTCTATCTACTAC TTCTCTG
46	RhFRED-F	CAGAGAAGTAGTAGATAGAACTGTATTGCATAGACATCAACCAGTTACCATAGGTG AACCAGCAG
47	RhFRED-R	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTACAATCTCAAGGCTAATC TGTC
48	fldA-F	GGAAGTAATTATCTACTTTTTACAACAAATATAACAAAATGGCTATCACTGGCATCTT TTTCG
49	fldA-R	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCAGGCATTGAGAATTTCGTCG AGATG
50	fldA-R2	CTCATTAAAAAACTATATCAATTAATTTGAATTAACTCATAGTTTAGAAGAACCACCA CCGGCATTGAGAATTTCGTCGAGATG
51	EcFNR-F2	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCTGATTGGGTAACAGG CAAAGTC
52	EcFNR-R2	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTACCAGTAATGCTCCGCTG TCATATG
53	EcFNRper-R2	GACATAACTAATTACATGACTCGAGGTCGACGGTATCTTATAGTTTAGATTTTGCCT GCGATAGTTTTACAGCGGCAGAACCACCACCCCAGTAATGCTCCGCTGTCATATG

Supplementary Table 3 (continued)

54	GroES-R	TAAAAAACTATATCAATTAATTTGAATTAACTCAGGCATTGAGAATTTCGTCG
55	GroES-F	TTATCTACTTTTTACAACAAATATAACAAAATGGCTATCACTGGCATCTTTTTC
56	GroEL-F	CATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCTGCTAAAGAAGTTAAATTCG
57	GroEL-R	CTAATTACATGACTCGAGGTCGACGGTATCTTACATCATA CCACCCATACCAC
58	PEX32:KanMX-F	CTACTTCATACTTTCTCTTTTCTTTCATTGATCTTAATTTAGTTCAGTGGACATCATTTTGCTAATTTCAAGGAAAGAAATGGGTAAGGAAAAGACTCACG
59	PEX32:kanMX-R	GTATGAGAAAATTCTTCATATCATGATAATGTTAGGCAACCAACTATATATGCAGTTTAGAGGCTTAAAGCAATACTATTAGAAAAACTCATCGAGCATC
60	PEX31:amdSYM-F	GCTCACCAAAATTATTATCAATAAAGCGGCACTTTTAAATTTGGTATTCTTCCCTGGTTGTCAAGCCTTGGTTTCCCTTTATTTGATAGTATGGTACGCTGCAGGTCGACAAC
61	PEX31:amdSYM-R	CCATGCAGCCAATGAAAGAAACTCTGAGATTGCTGTGTGCCCTTGCACCGACCAGTGTGAACGTTGTTGTCCATATGGGGCATGCACTCACTAGTGGATCTGATATCAC
62	TEF1p(PEX32)-F	CACTCGAAACTACTTCATACTTTCTCTTTTCTTTCATTGATCTTAATTTAGTTCAGTGGACATCATTTTGCTAATTTCAAGGAAAGAAATAGCTTCAAAATGTTTCTACTC
63	TEF1p(Kan)-R	GGCCTCGAAACGTGAGTCTTTTCCTTACCCATTTTGTAATTAAAACTTAGATTAG
64	KanMX(ORF)-F	ATGGGTAAGGAAAAGACTCACG
65	tHXT7p(PEX32)-F	CACTCGAAACTACTTCATACTTTCTCTTTTCTTTCATTGATCTTAATTTAGTTCAGTGGACATCATTTTGCTAATTTCAAGGAAAGAACTCGTAGGAACAATTTCGGGCCCCTG
66	tHXT7p(Kan)-R	GGCCTCGAAACGTGAGTCTTTTCCTTACCCATTTTTTGATTAAAATTAAAAAAACTTTTTG
67	HIS3-F	ACTCTTGGCCTCCTCTAGTACACTC
68	HIS3-R3	GCAGAAAAGACTAATAATTCTTAGTTAAAAGCACTCTACATAAGAACACCTTTGGTGG
69	PRM9t-R3	CAGTGATATGCATATGGGAGATGGAGATGATACCTATTTTCAACATCGTATTTTCCGAAG
70	PRM9t-F3	CCTTGGTATGCAAGACATGTGGAAAGCTACATGAACAGAAGACGGGAGACACTAGCAC
71	PEX11-R1	TCATGTAGCTTTCCACATGTCTTGCATACC
72	PEX11-F1	GGAAGTAATTATCTACTTTTTACAACAAATATAACAAAATGGTCTGTGATACACTGGTATATC
73	PEX34-F	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGTTTCGAAGAAAAATACGGCTG
74	PEX34-R	CGTATGCTGCAGCTTTAAATAATCGGTGTCATTATACAATTATTCTACAAAGTG
75	PRM9t-F4	GGAAGTAATTATCTACTTTTTACAACAAATATAACAAAACAGAAGACGGGAGACACTAGCAC

Supplementary Table 4 Codon optimized genes used in this study.

Synthesized genes	Sequence (5'-3')
FaCoAR	ATGAATTATTTCTTGACAGGTGGTACAGGTTTTATCGGTAGATTCTTGGTTGAAAAG TTGTTAGCCAGAGGTGGTACAGTTTATGTTTTAGTTAGAGAACAATCTCAGGATAAG TTGGAAAGATTGAGAGAAAGATGGGGTGCCGATGACAAACAAGTCAAGGCTGTAA TAGGTGACTTGACATCTAAAAATTTGGGTATCGATGCTAAGACCTTGAAGTCTTTAA AGGGTAACATCGATCATGTATTCCACTTAGCTGCTGTTTATGATATGGGTGCCGACG AAGAAGCTCAAGCCGCTACTAATATTGAAGGTACAAGAGCAGCCGTCCAAGCTGCT GAAGCTATGGGTGCTAAACATTTCCATCACGTTTCTTCAATCGCTGCTGCTGGTTTGT TCAAGGGTATTTTTAGAGAAGACATGTTTGAAGAAGCTGAAAAATTGGATCATCCA TATTTGAGAACTAAGCACGAAAGTGAAAAAGTTGTCAGAGAAGAATGTAAAGTTCC TTTTAGAATCTACAGACCTGGTATGGTTATTGGTCATTCTGAAACCGGTGAAATGGA TAAAGTTGACGGTCCATACTACTTTTTCAAGATGATCCAAAAGATTAGACACGCTTT GCCACAATGGGTTCCTACTATCGGTATTGAAGGTGGTAGATTAAACATCGTACCTGT TGATTTTGTAGTTGATGCATTGGACCATATTGCCCACTTAGAAGGTGAAGATGGTAA TTGTTTCCATTTGGTCGATTCTGACCCATACAAAGTAGGTGAAATTTTAAACATATTT TGCGAAGCAGGTCACGCCCCTAGAATGGGTATGAGAATCGATTCAAGAATGTTCGG TTTCATTCCACCTTTTATAAGACAATCTATTAAAAATTTGCCACCTGTTAAGAGAATT ACTGGTGCTTTGTTAGATGACATGGGTATTCCACCTTCTGTTATGTCATTCATAAACT ACCCAACCAGATTTGACACTAGAGAATTGGAAAGAGTTTTGAAGGGTACAGATATA GAAGTCCCAAGATTACCTTCTTATGCTCCAGTTATATGGGATTACTGGGAAAGAAAC TTAGATCCAGATTTGTTTAAAGATAGAACATTGAAGGGTACTGTAGAGGGTAAAGT TTGTGTCGTAACAGGTGCTACCTCCGGTATTGGTTTGGCTACAGCAGAAAAATTGGC CGAAGCTGGTGCAATCTTGGTTATTGGTGCAAGAACTAAGGAAACATTGGATGAAG TTGCCGCTAGTTTAGAAGCAAAAGGTGGTAATGTCCATGCCTATCAATGTGATTTCT CTGACATGGATGACTGCGATAGATTCGTTAAGACTGTCTTGGATAATCATGGTCACG TTGATGTATTAGTTAATAACGCTGGTAGATCCATAAGAAGAAGTTTGGCATTATCTT TTGATAGATTCCATGACTTCGAAAGAACAATGCAATTGAACTACTTCGGTTCAGTTA GATTGATTATGGGTTTTGCCCCAGCTATGTTGGAAAGAAGAAGAGGTCATGTTGTC AATATATCCAGTATCGGTGTATTAACAAACGCTCCTAGATTCTCAGCATACGTTTCTT CAAAATCAGCTTTGGACGCATTTTCCAGATGCGCAGCCGCTGAATGGTCCGATAGA AACGTCACCTTTACTACAATTAACATGCCATTGGTAAAGACCCCAATGATTGCTCCTA CTAAAATCTATGATTCTGTTCCAACCTTGACTCCTGACGAAGCAGCCCAAATGGTTG CAGATGCCATAGTCTACAGACCAAAGAGAATCGCTACTAGATTGGGTGTCTTCGCA CAAGTATTGCATGCTTTGGCACCTAAGATGGGTGAAATCATCATGAACACAGGTTAC AGAATGTTTCCAGATTCACCAGCTGCTGCTGGTTCTAAGAGTGGTGAAAAACCTAA GGTTTCCACAGAACAAGTAGCATTTGCCGCCATTATGAGAGGTATCTATTGGTAA
SeAAR	ATGTTCGGTTTAATAGGTCACTTAACAAGTTTAGAACAAGCCAGAGATGTCAGTAG AAGAATGGGTTACGATGAATACGCAGACCAAGGTTTAGAATTTTGGTCTTCAGCCC CACCTCAAATCGTAGATGAAATTACAGTTACCTCTGCTACTGGTAAAGTCATTCATG GTAGATACATCGAATCATGTTTCTTGCCAGAAATGTTGGCTGCAAGAAGATTCAAAA CTGCAACAAGAAAGGTTTTGAATGCAATGTCCCATGCCCAAAAGCACGGTATCGAT

	ATTTCCGCATTGGGTGGTTTTACAAGTATAATCTTCGAAAACTTCGATTTGGCTAGTT TGAGACAAGTTAGAGACACTACATTGGAATTCGAAAGATTCACCACTGGTAACACC CACACTGCTTACGTCATTTGTAGACAAGTAGAAGCCGCTGCAAAAACCTTGGGTATA GATATCACACAAGCCACCGTTGCTGTTGTCGGTGCTACTGGTGACATCGGTTCCGCA GTATGCAGATGGTTGGATTTGAAATTGGGTGTTGGTGACTTAATCTTGACAGCTAG AAACCAAGAAAGATTGGATAACTTGCAAGCAGAATTAGGTAGAGGTAAAATCTTGC CATTGGAAGCCGCTTTGCCTGAAGCCGATTTTATCGTTTGGGTCGCTTCTATGCCAC AAGGTGTAGTTATTGATCCAGCTACCTTAAAACAACCTTGCGTTTTGATAGACGGTG GTTATCCTAAAAATTTGGGTTCTAAGGTTCAAGGTGAAGGTATCTATGTCTTGAACG GTGGTGTCGTAGAACATTGTTTCGATATAGACTGGCAAATCATGTCAGCAGCCGAA ATGGCAAGACCTGAAAGACAAATGTTTGCCTGCTTCGCTGAAGCAATGTTGTTAGA ATTTGAAGGTTGGCACACTAATTTCTCTTGGGGTAGAAACCAAATTACAATAGAAAA GATGGAAGCCATCGGTGAAGCCTCTGTTAGACACGGTTTCCAACCTTTAGCCTTAGC AATCTGA
MmCAR	ATGTCACCTATCACCAGAGAAGAAAGATTAGAAAGAAGAATACAAGACTTATACGC CAACGATCCTCAATTCGCCGCTGCCAAGCCAGCAACAGCCATCACCGCTGCAATTGA AAGACCAGGTTTGCCATTGCCTCAAATCATCGAAACTGTTATGACAGGTTATGCTGA TAGACCTGCTTTGGCACAAAGATCAGTAGAATTTGTTACAGATGCAGGTACTGGTC ATACTACATTGAGATTGTTACCACACTTCGAAACTATCTCTTACGGTGAATTATGGG ACAGAATTTCTGCCTTGGCTGATGTTTTATCAACCGAACAAACTGTTAAACCTGGTG ACAGAGTCTGTTTGTTGGGTTTTAATTCTGTTGACTACGCAACTATAGATATGACATT GGCCAGATTAGGTGCAGTAGCCGTTCCATTGCAAACCTCTGCCGCTATTACTCAATT ACAACCAATAGTCGCTGAAACACAACCTACCATGATAGCAGCCTCTGTAGATGCTTT GGCAGACGCCACTGAATTGGCTTTATCAGGTCAAACTGCAACAAGAGTCTTAGTATT CGACCATCACAGACAAGTTGATGCCCATAGAGCTGCTGTTGAATCCGCTAGAGAAA GATTGGCAGGTAGTGCCGTTGTCGAAACTTTAGCTGAAGCAATAGCTAGAGGTGAC GTTCCAAGAGGTGCTTCTGCTGGTTCTGCTCCTGGTACAGACGTCTCCGATGACAGT TTGGCATTGTTAATCTATACCTCTGGTTCAACTGGTGCCCCAAAAGGTGCTATGTAC CCTAGAAGAAATGTTGCTACATTTTGGAGAAAGAGAACCTGGTTCGAAGGTGGTTA CGAACCATCTATCACTTTGAACTTCATGCCTATGTCACATGTTATGGGTAGACAAATC TTGTATGGTACTTTATGCAACGGTGGTACAGCATACTTTGTTGCCAAGTCTGACTTG TCAACATTATTCGAAGATTTGGCTTTAGTCAGACCAACTGAATTAACATTCGTCCCTA GAGTATGGGATATGGTTTTTGACGAATTTCAATCAGAAGTCGATAGAAGATTGGTA GATGGTGCTGACAGAGTAGCTTTAGAAGCACAAGTTAAGGCAGAAATAAGAAACG ATGTTTTGGGTGGTAGATATACATCTGCCTTAACCGGTTCTGCTCCAATATCAGACG AAATGAAGGCTTGGGTAGAAGAATTGTTAGATATGCATTTGGTTGAAGGTTACGGT TCAACTGAAGCTGGTATGATATTAATCGACGGTGCAATTAGAAGACCAGCCGTTTT GGATTATAAATTGGTTGATGTCCCTGACTTGGGTTACTTTTTAACTGATAGACCACA CCCTAGAGGTGAATTGTTGGTTAAGACAGATTCTTTGTTCCCAGGTTATTACCAAAG AGCTGAAGTTACAGCAGATGTCTTTGATGCTGACGGTTTCTATAGAACCGGTGACAT TATGGCAGAAGTCGGTCCTGAACAATTCGTATACTTAGATAGAAGAAACAACGTTTT GAAATTGTCTCAGGGTGAATTTGTAACTGTTTCAAAGTTGGAAGCTGTATTCGGTGA CTCTCCATTAGTTAGACAAATATATATATACGGTAATTCAGCCAGAGCTTATTTGTTA GCAGTCATAGTACCAACACAAGAAGCCTTGGATGCTGTTCCTGTCGAAGAATTGAA

	AGCCAGATTGGGTGACTCCTTGCAAGAAGTTGCAAAGGCCGCTGGTTTGCAAAGTT ACGAAATCCCAAGAGATTTCATCATCGAAACCACTCCTTGGACCTTAGAAAACGGTT TGTTAACTGGTATCAGAAAATTGGCTAGACCACAATTGAAAAAGCATTACGGTGAA TTGTTAGAACAAATATATACTGACTTGGCCCACGGTCAAGCTGATGAATTGAGATCC TTAAGACAAAGTGGTGCAGATGCCCCAGTATTAGTTACAGTCTGTAGAGCAGCCGC TGCATTGTTAGGTGGTTCCGCTAGTGATGTTCAACCTGACGCACATTTTACCGATTT GGGTGGTGACTCTTTGTCAGCTTTATCTTTTACAAATTTGTTGCACGAAATCTTCGAT ATAGAAGTACCAGTTGGTGTCATTGTATCACCTGCTAACGATTTGCAAGCATTGGCA GATTATGTTGAAGCCGCTAGAAAACCAGGTTCTTCAAGACCTACTTTTGCTTCTGTTC ATGGTGCATCAAATGGTCAAGTTACAGAAGTCCACGCTGGTGACTTGTCTTTGGATA AGTTCATTGATGCAGCCACTTTGGCCGAAGCTCCAAGATTACCTGCTGCAAACACTC AAGTAAGAACAGTTTTGTTAACCGGTGCTACTGGTTTCTTGGGTAGATATTTGGCAT TAGAATGGTTAGAAAGAATGGATTTGGTTGACGGTAAATTGATTTGCTTAGTCAGA GCAAAGTCCGACACTGAAGCAAGAGCCAGATTGGATAAAACATTCGATAGTGGTG ACCCAGAATTGTTAGCACATTACAGAGCTTTAGCAGGTGACCACTTGGAAGTTTTAG CCGGTGACAAGGGTGAAGCTGACTTGGGTTTAGATAGACAAACATGGCAAAGATT GGCTGATACCGTAGACTTAATCGTTGATCCAGCCGCTTTAGTCAACCATGTATTGCC ATACTCCCAATTGTTCGGTCCTAACGCATTGGGTACTGCTGAATTGTTGAGATTGGC TTTGACTTCTAAAATTAAGCCTTACTCCTACACCAGTACTATCGGTGTTGCAGATCAA ATTCCACCTTCAGCCTTCACTGAAGATGCTGACATAAGAGTCATCTCCGCAACAAGA GCCGTAGATGACAGTTATGCTAATGGTTACTCCAACAGTAAATGGGCAGGTGAAGT TTTGTTAAGAGAAGCCCATGATTTGTGTGGTTTACCAGTTGCTGTCTTTAGATGCGA CATGATTTTGGCAGATACAACCTGGGCCGGTCAATTGAACGTTCCAGATATGTTCAC AAGAATGATCTTGTCCTTAGCAGCCACCGGTATAGCTCCTGGTAGTTTCTATGAATT GGCTGCTGATGGTGCTAGACAAAGAGCACATTACGATGGTTTGCCAGTTGAGTTTA TTGCCGAAGCTATCTCCACCTTAGGTGCTCAAAGTCAAGATGGTTTCCATACTTATCA CGTAATGAATCCATACGATGACGGTATTGGTTTGGACGAATTTGTTGATTGGTTAAA CGAATCTGGTTGTCCTATTCAAAGAATAGCTGATTATGGTGACTGGTTACAAAGATT CGAAACTGCTTTGAGAGCATTACCAGATAGACAAAGACATTCCAGTTTGTTACCTTT GTTACACAATTACAGACAACCAGAAAGACCTGTCAGAGGTTCTATTGCTCCTACAGA TAGATTCAGAGCCGCTGTACAAGAAGCAAAAATAGGTCCAGATAAGGACATCCCTC ATGTTGGTGCTCCTATTATCGTAAAGTATGTATCAGATTTGAGATTGTTGGGTTTGTT GTAA
npgA	ATGGTGCAAGACACATCAAGCGCAAGCACTTCGCCAATTTTAACAAGATGGTACAT CGACACCCGCCCTCTAACCGCCTCAACAGCAGCCCTTCCTCTCCTTGAAACCCTCCAG CCCGCTGATCAAATCTCCGTCCAAAAATACTACCATCTGAAGGATAAACACATGTCT CTCGCCTCTAATCTGCTCAAATACCTCTTCGTCCACCGAAACTGTCGCATCCCCTGGT CTTCAATCGTGATCTCTCGAACCCCAGATCCGCACAGACGACCATGCTATATTCCACC CTCAGGCTCACAGGAAGACAGCTTCAAAGACGGATATACCGGCATCAACGTTGAGT TCAACGTCAGCCACCAAGCCTCAATGGTCGCGATCGCGGGAACAGCTTTTACTCCCA ATAGTGGTGGGGACAGCAAACTCAAACCCGAAGTCGGAATTGATATTACGTGCGTA AACGAGCGGCAGGGACGGAACGGGGAAGAGCGGAGCCTGGAATCGCTACGTCAA TATATTGATATATTCTCGGAAGTGTTTTCCACTGCAGAGATGGCCAATATAAGGAGG TTAGATGGAGTCTCATCATCCTCACTGTCTGCTGATCGTCTTGTGGACTACGGGTAC

	AGACTCTTCTACACTTACTGGGCGCTCAAAGAGGCGTATATAAAAATGACTGGGGA GGCCCTCTTAGCACCGTGGTTACGGGAACTGGAATTCAGTAATGTCGTCGCCCCGG CCGCTGTTGCGGAGAGTGGGGATTCGGCTGGGGATTTCGGGGAGCCGTATACGGG TGTCAGGACGACTTTATATAAAAATCTCGTTGAGGATGTGAGGATTGAAGTTGCTG CTCTGGGCGGTGATTACCTATTTGCAACGGCTGCGAGGGGTGGTGGGATTGGAGCT AGTTCTAGACCAGGAGGTGGTCCAGACGGAAGTGGCATCCGAAGCCAGGATCCCT GGAGGCCTTTCAAGAAGTTAGATATAGAGCGAGATATCCAGCCCTGTGCGACTGGG GTGTGTAATTGCCTATCCTAA
SeADO	ATGCCACAATTAGAAGCCTCCTTAGAATTAGACTTTCAATCAGAATCATATAAAGAT GCTTACAGTAGAATCAACGCAATCGTCATTGAAGGTGAACAAGAAGCATTTGATAA CTACAACAGATTGGCAGAAATGTTACCAGATCAAAGAGACGAATTGCATAAATTGG CCAAGATGGAACAAAGACACATGAAAGGTTTCATGGCTTGTGGTAAAAATTTGTCC GTTACTCCTGATATGGGTTTCGCACAAAAGTTTTTCGAAAGATTGCATGAAAACTTC AAAGCTGCAGCCGCTGAGGGTAAAGTTGTCACATGTTTGTTGATCCAATCTTTGATA ATCGAATGCTTTGCTATCGCAGCCTATAATATCTACATTCCAGTCGCTGATGCATTCG CCAGAAAGATTACCGAAGGTGTAGTTAGAGACGAATATTTGCACAGAAACTTCGGT GAAGAATGGTTGAAGGCAAACTTCGATGCTTCTAAGGCAGAATTGGAAGAAGCTA ATAGACAAAACTTGCCTTTAGTCTGGTTGATGTTAAATGAAGTAGCCGATGACGCTA GAGAATTGGGTATGGAAAGAGAATCATTAGTTGAAGACTTCATGATCGCATACGGT GAAGCCTTAGAAAACATCGGTTTTACTACCAGAGAAATAATGAGAATGTCCGCATA CGGTTTGGCAGCAGTCTAA
NpADO	ATGCAACAATTAACAGACCAATCAAAGGAATTAGACTTCAAATCAGAAACTTACAAA GATGCCTACTCCAGAATCAACGCAATCGTCATTGAAGGTGAACAAGAAGCACATGA AAACTACATCACCTTGGCCCAATTATTACCAGAATCCCATGATGAATTGATCAGATT GTCTAAGATGGAATCAAGACACAAAAAGGGTTTTGAAGCCTGTGGTAGAAATTTGG CTGTTACTCCTGACTTACAATTTGCCAAAGAATTTTTCTCTGGTTTGCACCAAAACTT CCAAACTGCTGCAGCCGAGGGTAAAGTTGTCACATGTTTGTTGATCCAATCATTAAT AATCGAATGCTTTGCTATCGCTGCATATAATATCTACATTCCAGTTGCCGATGACTTC GCTAGAAAAATTACAGAAGGTGTAGTTAAGGAAGAATATTCCCATTTGAACTTTGG TGAAGTCTGGTTAAAAGAACACTTCGCAGAGAGTAAGGCCGAATTGGAATTAGCAA ATAGACAAAACTTGCCTATCGTCTGGAAAATGTTAAATCAAGTAGAAGGTGACGCT CATACCATGGCAATGGAAAAGGATGCTTTGGTTGAAGACTTCATGATTCAATACGG TGAAGCATTATCAAACATAGGTTTTTCTACCAGAGACATTATGAGATTGAGTGCTTA CGGTTTGATAGGTGCTTGA
SeFNR	ATGTTAAATGCCTCAGTCGCTGGTGGTGCTGCAACCACTACTTATGGTAACAGATTG TTTATATATGAAGTAATAGGTTTGAGACAAGCTGAAGGTGAACCTTCCGATTCCAGT ATAAGAAGATCAGGTTCAACATTTTTCAAAGTACCATACAGTAGAATGAACCAAGA AATGCAAAGAATATTGAGATTGGGTGGTAAAATCGTTTCAATTAGACCAGCTGAAG AAGCCGCTGCAAATAACGGTGCCGCTCCTTTACAAGCAGCCGCTGAAGAACCAGCA GCCGCTCCAACTCCTGCTCCAGCAGCCAAAAAGCATTCTGCTGAAGATGTCCCTGTA AACATCTATAGACCTAACAAACCATTTGTTGGTAAAGTCTTGTCAAACGAACCATTA GTTCAAGAAGGTGGTATCGGTGTAGTTCAACATTTGACATTCGACATTTCTGAGGGT GACTTGAGATACATCGAAGGTCAATCAATAGGTATAATACCTGACGGTACTGACGA TAAGGGTAAACCACACAAATTGAGATTGTACTCTATCGCTTCAACAAGACATGGTGA

	CCACGTTGATGATAAGACCGTCTCCTTGTGTGTAAGACAATTACAATACCAAAACGA AGCAGGTGAAACTATAAACGGTGTTTGTAGTACATTTTTGTGCGGTTTAAAACCTGG TGACGATGTTAAGATCACTGGTCCTGTCGGTAAAGAAATGTTGTTACCAGCCGACA CAGATGCTAATGTTATTATGATGGGTACAGGTACTGGTATAGCACCTTTTAGAGCCT ATTTGTGGAGAATGTTCAAGGATAACGAAAGAGCTATTAACTCCGAATACCAATTCA ATGGTAAAGCATGGTTGATATTCGGTATCCCTACCACTGCCAACATCTTGTACAAGG AAGAATTGGAAGCATTACAAGCCCAATATCCAGATAATTTCAGATTGACTTACGCCA TTTCAAGAGAACAAAAGAACGAAGCTGGTGGTAGAATGTATATTCAAGACAGAGTT GCTGAACATGCAGATGAAATATGGAATTTGTTGAAGGATGAAAAGACCCACGTCTA TATTTGTGGTTTACGTGGTATGGAAGACGGTATCGATCAAGCTATGACTGTAGCTGC TGCTAAAGAAGATGTTGTATGGTCCGATTATCAAAGAACATTAAAGAAAGCAGGTA GATGGCACGTAGAAACTTATTAA
SeFd	ATGGCTACTTACAAGGTCACTTTGGTAAATGCTGCTGAAGGTTTGAACACTACAATA GATGTTGCTGATGACACATATATCTTGGACGCCGCTGAAGAACAAGGTATTGATTT GCCTTACTCCTGTAGAGCTGGTGCATGTAGTACCTGCGCTGGTAAAGTTGTCTCTGG TACTGTTGACCAATCCGATCAATCATTTTTGGATGACGATCAAATCGCAGCCGGTTT CGTATTAACATGCGTTGCTTATCCAACCTCTGATGTTACTATTGAAACACATAAGGA AGAAGACTTGTACTAA
OleT	ATGGCTACATTGAAGAGAGACAAGGGTTTAGACAACACATTGAAAGTATTGAAGCA AGGTTACTTATACACCACCAACCAAAGAAATAGATTGAACACTTCTGTTTTCCAAAC AAAGGCATTAGGTGGTAAACCTTTCGTTGTCGTAACTGGTAAAGAAGGTGCCGAAA TGTTCTACAACAACGATGTTGTCCAAAGAGAAGGCATGTTGCCAAAGAGAATCGTT AACACTTTGTTCGGTAAAGGTGCCATCCATACAGTCGATGGTAAAAAGCACGTAGA CAGAAAAGCTTTGTTCATGTCATTGATGACTGAGGGTAATTTGAACTACGTCAGAG AATTGACCAGAACTTTATGGCATGCCAATACACAAAGAATGGAATCTATGGATGAA GTCAACATATACAGAGAATCAATCGTATTGTTGACAAAGGTTGGTACCAGATGGGC TGGTGTACAAGCACCACCTGAAGACATCGAAAGAATTGCAACAGATATGGACATAA TGATCGATTCCTTTAGAGCCTTGGGTGGTGCTTTCAAAGGTTACAAAGCAAGTAAA GAAGCTAGAAGAAGAGTTGAAGATTGGTTGGAAGAACAAATCATCGAAACCAGAA AGGGTAACATTCATCCACCTGAAGGTACTGCCTTGTATGAATTTGCTCACTGGGAAG ATTACTTAGGTAACCCTATGGACTCCAGAACATGTGCTATTGATTTGATGAATACCTT CAGACCATTGATCGCTATAAACAGATTCGTTTCTTTCGGTTTGCATGCAATGAATGA AAACCCTATAACCAGAGAAAAGATTAAATCAGAACCAGATTACGCTTACAAGTTCG CACAAGAAGTTAGAAGATATTACCCATTTGTCCCTTTCTTACCTGGTAAAGCTAAGG TTGATATCGACTTCCAAGGTGTTACAATTCCAGCAGGTGTCGGTTTGGCCTTAGACG TATATGGTACTACACATGATGAATCCTTGTGGGATGACCCTAATGAATTCAGACCAG AAAGATTCGAAACATGGGATGGTAGTCCTTTTGACTTAATTCCACAAGGTGGTGGT GACTACTGGACCAACCACAGATGCGCTGGTGAATGGATTACCGTTATCATCATGGA AGAAACTATGAAGTACTTCGCAGAAAAGATTACTTACGATGTACCTGAACAAGATTT GGAAGTTGACTTAAACTCTATTCCAGGTTATGTAAAGAGTGGTTTCGTTATTAAAAA TGTCAGAGAAGTAGTAGATAGAACTTGA
RhFRED[a]	ATG**GTATTGCATAGACATCAACCAGTTACCATAGGTGAACCAGCAGCCAGA**GCCG TCAGTAGAACAGTTACCGTAGAAAGATTGGACAGAATTGCCGATGACGTTTTGAGA TTAGTCTTGAGAGATGCAGGTGGTAAAACTTTACCAACTTGGACACCTGGTGCTCAT

	ATTGATTTGGATTTGGGTGCATTATCCAGACAATATAGTTTGTGTGGTGCTCCAGAT GCACCTTCCTACGAAATCGCTGTCCATTTGGACCCAGAATCAAGAGGTGGTTCAAG ATACATACACGAACAATTAGAAGTTGGTAGTCCATTGAGAATGAGAGGTCCTAGAA ATCATTTTGCTTTAGATCCAGGTGCAGAACACTACGTTTTCGTCGCTGGTGGTATTG GTATAACCCCTGTTTTGGCTATGGCTGATCATGCAAGAGCCAGAGGTTGGTCTTATG AATTACACTACTGTGGTAGAAACAGATCAGGCATGGCATATTTGGAAAGAGTCGCC GGTCATGGTGACAGAGCTGCATTACACGTATCCGAAGAAGGTACAAGAATAGACTT GGCCGCTTTGTTAGCCGAACCAGCTCCTGGTGTTCAAATCTACGCTTGCGGTCCAGG TAGATTGTTAGCCGGTTTAGAAGATGCTTCAAGAAATTGGCCTGACGGTGCTTTGCA TGTAGAACACTTTACTTCTTCATTAGCAGCTTTAGATCCAGACGTTGAACATGCATTC GATTTGGAATTGAGAGACTCAGGTTTAACAGTAAGAGTTGAACCAACCCAAACTGT TTTAGATGCATTGAGAGCCAATAACATTGATGTCCCTTCCGACTGCGAAGAAGGTTT ATGTGGTAGTTGCGAAGTCGCTGTATTGGATGGTGAAGTAGATCATAGAGACACAG TTTTGACCAAGGCAGAAAGAGCTGCAAATAGACAAATGATGACTTGCTGTAGTAGA GCTTGCGGTGACAGATTAGCCTTGAGATTGTAA

a: the bolded sequence encoding a linker peptide VLHRHQPVTIGEPAAR[6] for fusion with OleT

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The role of biofuels in the future energy supply

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In recent years several different arguments have been raised against the use of biofuels and their role in our future energy supply. These arguments can be divided into issues related to costs, food *versus* fuel, and lack of sustainability. Here we address these three points and argue that biofuels represent an essential contribution to our future energy supply and more importantly will contribute to a reduction in carbon dioxide emissions.

Broader context

Here we address three issues that, in recent years, have been raised as arguments against the use of biofuels and their role in our future energy supply. These issues are related to their costs of production, land use (or food *versus* fuel debate), and lack of sustainability. We argue that biofuels represent an essential contribution to our future energy supply and will contribute to a reduction in carbon dioxide emissions, and that most of the disapproval is related to the first generation of biofuels produced from edible feed stocks. We argue that giving up on the development and production of advanced biofuels, produced from biomass, is to neglect the opportunity of applying the advances in biotechnology, synthetic biology, systems biology, and metabolic engineering for the development of production processes for bio-based transportation fuels. By capitalizing on the advances in metabolic engineering it will be possible to develop efficient cell factories that can ensure a stable supply of transportation fuels for the future in addition to the substantial reduction of CO_2 emissions at proper costs and hereby contribute to a reduction in global warming.

There is presently a scientific consensus about the environmental damage caused by emissions of carbon dioxide (CO_2) and other greenhouse gases (GHGs). This has resulted in an increasing pressure to decrease and eventually retire fossil fuels, which today not only represent our main source of energy but also represent the main source of CO_2 emissions. For example, in 2009 fossil fuels provided 80% of the about 16 TeraWatts (TW) of energy used worldwide and the combustion of these released about 7.8 billion metric tons of carbon (GtC), *i.e.* 28.6 Gt of CO_2.[1,2] Due to the growth of population and average income, these numbers will increase, despite the introduction of technologies for more efficient energy usage, and the estimated energy demand by 2050 is at least 27.0 TW.[3] Today the atmospheric CO_2 concentration is 394.5 parts per million by volume (ppmv), and the net accumulation has been progressively increased at a yearly rate of 1.03–2.13 ppmv during the last four decades (The Maua Loa Observatory). Thus, it will be expected to reach about 500 ppmv in 2050 if emissions remain unchecked. The biggest concern associated with global warming is the melting of ice sheets in the West Antarctic and in Greenland, which will result in a dramatic rise of the sea level, and changes in the thermohaline circulation (THC), which will result in dramatic climate changes.[4] It has been suggested that stabilization of CO_2 levels below 450 ppmv may prevent the disintegration of the West Antarctic Ice Sheet and the shutdown of the THC.[4] Hoffert and Covey (1992) suggested that concentrations of 550 ppmv, if sustained, could cause global warming comparable in magnitude but opposite in sign to the cooling of the world in the last Ice Age.[5] Various scenarios to stabilize atmospheric CO_2 concentrations at 450–650 ppmv over the next few hundred years have been calculated,[6,7] and these show that total annual CO_2 emissions from 2050 and onwards should not exceed 6.0 GtC in order to stabilize the atmospheric CO_2 concentration at 450 ppmv by 2100. In order to reach this level, it will be necessary to reduce actual emissions at an approximate rate of 44.2 million metric tons of carbon (MtC) per year, and assuming that the current proportions of oil, gas and coal will be maintained, this would require yearly replacement of approximately 8.0, 5.5, and 7.3 Gigawatts (GW) of these fossil fuels, respectively by carbon-constrained fuels. Additionally, it will also be necessary to produce 0.29 TW of carbon emission free energy in order to support the growth in energy consumption. Overall, we calculated that the amount of carbon-neutral fuel that has to be produced in 2050 has to be approximately 12 TW in order to reach the CO_2 emission target of 44.2 MtC. The Intergovernmental Panel on Climate Change (IPCC) Working Group III reported similar values.[8]

To overcome this grand challenge it will be necessary to implement a range of new CO_2-constrained technologies for energy supply.[3,9,10] The IPCC Working Group III has recently recommended the use of all available technologies to better reach environmental goals at lower economic costs.[8] Although

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there are many alternatives for providing electricity and heat, *i.e.* nuclear, hydro, wind and solar, there are a few alternative options for transportation fuels (Fig. 1). There is currently much focus on electric cars, and their use is compelling in terms of energy efficiency, which is about 80% compared with about 20% for cars with internal combustion engines. However, the energy density in batteries is much lower than for liquid fuels[11,12] and battery panels of 272–408 kg have to be used to cover distances of 145–400 km, which should compare with a standard gasoline tank of 45 liters weighing 50 kg. Therefore reduction in cost and especially improvement in energy density are mandatory requirements for electric cars to become a major substitute for cars with internal combustion engines.[8,11,12] There are also other issues with the implementation of electric cars such as mismatch with the current infrastructure and requirement of a stable supply of rare elements used in the batteries.[9] For these reasons the National Petroleum Council (NPC) in the United States of America (USA) and the International Energy Agency (IEA) projected that electricity will be a moderate contributor to the transportation energy in 2050 (Fig. 2).[13,14] Most likely, electricity will therefore only be used for short distance transportation using personal cars (city driving) as well as for locomotives, trains, trams and busses where electricity can be provided through existing overhead power lines or an electrified third rail (power lines required high investment), whereas for ships, aircrafts and trucks the use of internal combustion engines using diesels and jet fuels with an energy density of 12–13 kW h kg^{-1} is likely to be maintained for many years to come (Fig. 1).

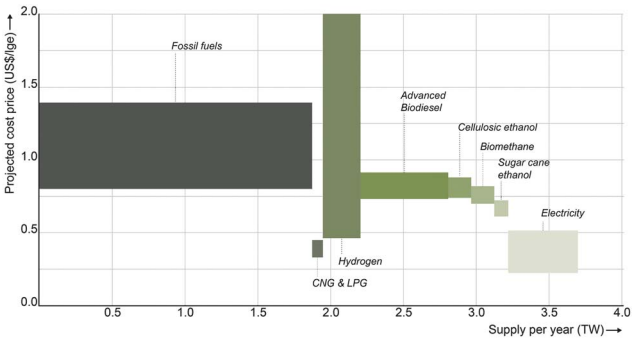

Fig. 2 Transportation energy supply for 2050 and projected retail prices. Prices are expressed in 2010 US$ per liter gasoline equivalent (lge). Sources: oil prices,[14,19] compressed natural gas (CNG) and liquefied petroleum gas (LPG),[20] hydrogen,[21] electricity,[19] advanced biodiesel, cellulosic ethanol, biomethane, and sugar cane ethanol.[14]

Nowadays global consumption of liquid transportation fuels is about 2.9 TW and these fuels are mainly derived from oil.[1]

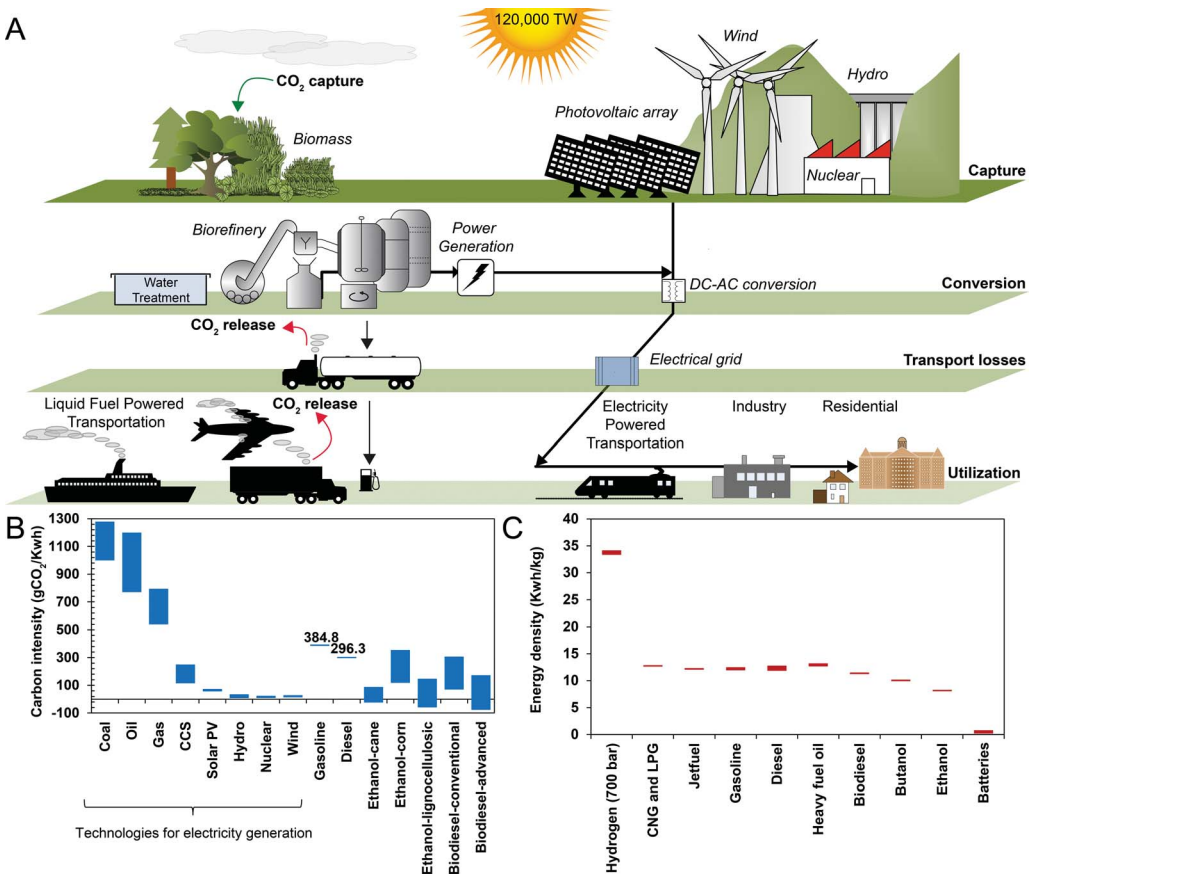

Fig. 1 Renewable energy alternatives for transportation. (A) Current renewable alternatives to provide energy for transportation include biofuels from biomass and electricity from different sources including photovoltaics, wind, hydro and nuclear. (B) Comparison of the carbon intensity among fossil and renewable energy options – data obtained from ref. 15–18. (C) Energy density in key fossil fuels and biofuels as well as in batteries.[11,12]

Between 1980 and 2008 there was a 31% increase in oil consumption, but interestingly the known reserves increased comparably due to improved exploration and extraction techniques.[9] Despite this fact oil prices have increased drastically in this period, and it has been argued that even though the oil reserves are increasing, access to cheap oil is limited at a supply of about 75 million barrels per day, and this causes dramatic increases in the oil price when the demand approaches this upper bound.[22,23] This should be seen in the context of the estimated requirement for a transportation fuel supply of around 110 million barrels per day in 2020.[23] Liquid transportation fuels can be derived from coal through the Fischer–Tropsch process, but this is very energy intensive and has a very large CO_2 footprint. Carbon capture and storage (CCS) can be used for decreasing CO_2 emissions, but such a technology is still neither well developed nor economically competitive.[9,24] The discovery of large natural gas resources that can be extracted at low costs in the USA is expected to result in a wider use of liquefied natural gas (LNG) as a transportation fuel, and even though the investment is higher than for standard trucks, the payback time is, with the current price difference between diesel and LNG, only 3–4 years.[9] The technology for use of LNG in internal combustion engines has been developed and implemented for busses, but a wider implementation will require investment in the necessary infrastructure. Furthermore, the combustion of natural gas will result in net CO_2 emissions of about 443 g CO_2 per kW h (Fig. 1). Natural gas can also be used, along with CCS, to produce electricity in a CO_2 neutral way, but as mentioned above this technology is still not available cost competitively.[24]

Today biofuels represents about 2.7% of the global transportation energy (~0.12 TW),[8] and it is dominated by ethanol (84.6 billion liters in 2011) and biodiesel derived from rapeseed or soybean oils (19.0 billion liters in 2011). Ethanol is primarily produced in the USA from corn (52.7 billion liters) and in Brazil from sugar cane (21.1 billion liters); both processes are often referred to as first generation bioethanol. In their forecast for transportation fuel usage in the USA by 2050, the National Petroleum Council (NPC) in the USA predicts that oil, natural gas and biofuels will dominate, but corn ethanol will be absent and substituted by lignocellulosic ethanol, generally referred to as second generation bioethanol (Fig. 2).[13]

Despite the already extensive use of biofuels and their predicted wider use, criticism is often raised against the production and use of biofuels. This was particularly strong in a recent editorial in *Angew. Chem., Int. Ed.* by Dr Hartmut Michel with the title "The Nonsense of Biofuels".[25] In the editorial Dr Michel argues that using plants for energy production is a poor use of the land as the upper efficiency of photosynthesis is 4.5% with a likely efficiency of about 1%. He compares this with the 15% of the current solar panels and argues for the use of solar photovoltaic (PV) combined with the use of electric cars, as the use of land will be about 600 times more efficient than for the biomass–biofuels–combustion engine combination. Although these arguments are not debatable for ethanol and biodiesel produced from corn and soybean oil respectively, which have a net energy gain of 5.2 mW h ha^{-1} and 4.0 mW h ha^{-1}, the case of ethanol from sugar cane or lignocellulose obtained from low-input high-diversity grass is less dramatic, *i.e.* ~120 times less efficient with net energy gains of 26.0 and 28.0 mW h ha^{-1} respectively.[15,16] Furthermore, biofuels produced from biomass waste, namely bagasse and other solid residues from the agro-industry, do not have a land-use issue as these materials are accumulated concomitant with the processing of crops, in addition to represent a potential solution to the disposal problems. Dr Michel also argues, in lines with others, that the bioethanol is not a CO_2 neutral fuel. Besides the arguments against biofuels raised by Dr Michel, there are also often raised issues of costs and issues with use of agricultural land for production of fuels rather than food (food *versus* fuel issue). In the following we will address each of these issues and hereby hopefully demonstrate that much of the criticism is really linked to the current technologies, whereas emerging technologies will address all these issues and hereby represent attractive potential solutions to our future problems by ensuring sustainable supply of liquid transportation fuels.

An often raised argument against biofuels is the *high cost* and hence the requirement of governmental subsidy for ensuring cost-competitive production of biofuels. It is clear that biofuels cannot compete with fuels produced from conventional oil (*e.g.* oil supplied by the Organization of Petroleum Exporting Countries (OPEC)), whereas, depending on feedstock, they can be competitive with non-conventional oil derived from deep waters or the Arctic as well as oil extracted with enhanced oil recovery techniques (EOR oil).[23] The transportation fuel supply curve for 2020 will therefore incorporate sugar cane ethanol as a biofuel that will not require subsidies, *i.e.* cheaper or in the same range of total-production cost as non-conventional oil derived fuels. Ethanol from corn, which is a less efficient process than sugar cane ethanol, will not be able to compete with any form of oil, and it will only survive on the market through subsidies and mandates.[23] It is therefore expected that corn-ethanol will not be sustained after 2050 when cellulosic ethanol and advanced biodiesel will have an important participation in the supply curve (Fig. 2). Lignocellulosic ethanol, so-called second generation bioethanol, will be cost-competitive and is likely to replace existing corn based ethanol production in the next 10–15 years.[14] Production of conventional biodiesel, *i.e.* biodiesel from vegetable oils, is also not cost competitive, and this is mainly due to the low energy yield per hectare (mW h ha^{-1}) of rapeseed and soybean cultivations (5.7 and 9.1 mW h ha^{-1} respectively compared with 18.8 mW h ha^{-1} for corn and 36.12 mW h ha^{-1} for sugar cane).[26] Production of advanced biodiesel through microbial fermentation is with current technology quite expensive, but with a change to lignocellulosic feedstock use (or biomass feedstock) the cost of production will decrease dramatically. Thus, the argument of high cost for biofuels is an issue with two out of the three current technologies, *i.e.* bioethanol production from corn and biodiesel from rapeseed oil, whereas bioethanol from sugar cane is competitive in terms of production costs. But, with emerging technologies, such as biomass based production of ethanol and advanced biofuels, production of biofuels is likely to be cost competitive.

Another criticism often raised is that biofuels do not contribute to a reduction in CO_2 emissions compared with oil-based fuels, *i.e.* current biofuels are *not CO_2 neutral*. As shown in Fig. 1B this is partly true when it comes to corn based ethanol as this only has an average of 12% lower carbon intensity than gasoline.[17] This is mainly due to the large amounts of energy that have to be invested in the farming and for distillation of the ethanol.[17] The two other types of biofuels currently being produced, however, result in significant reduction of CO_2 emission compared with gasoline or diesel (Fig. 1B). Their use is not CO_2 neutral but biodiesel from soybean has a 41% lower carbon intensity on average than diesel from oil and ethanol from sugar cane has a reduction of 60% compared with gasoline.[16,17,27] The second generation of biofuels using biomass as a feedstock is expected to give a similar 60% reduction in carbon intensity. However, this can significantly increase and become CO_2 negative if there is a utilization of lignocellulosic residues, *e.g.* utilization of forest, agricultural and livestock residues; short-rotation forest plantations; energy crops; the organic component of municipal solid waste; and other organic waste streams. Some calculations have shown that low-input high-diversity grassland biomass can provide greater reductions of greenhouse gas emissions, or even be carbon-negative because of net carbon dioxide sequestration (Fig. 1B).[15] However, it is important to perform proper evaluations of land-use change as the conversion of forest and grassland into a new cropland, like corn or switchgrass, can contribute to increased emissions, emphasizing the importance of the use of waste products,[28] or restructuring current farming, *e.g.* change from tobacco to energy crops. Residues from the current most important crops accumulate approximately 2 TW,[29] and low-input high-diversity grassland biomass can easily provide an additional 2 TW.[15,30,31] Transformation of this amount of energy into ethanol or advanced biofuels can yield between 0.7 and 1.6 TW, which is about 25–55% of the current energy use for transportation. In addition, this biomass may generate 0.8–1.8 kW h L^{-1} biofuel of electricity by burning the lignin[32] (Fig. 1A). The use of 1 TW of biofuels produced from these resources can reduce CO_2 emission by about 1.45 GtC or even higher when considering the sequestration of carbon related to the use of abandoned and degraded land, which will almost alone ensure the required reduction of CO_2 emissions.[15]

With a growing population and shift in dietary habits (towards eating more meat) there is an increased demand for land to be used for food production. It has therefore been argued that we should not be using land for biofuel production, *i.e.* a *food versus fuel* issue. In order to address this issue we evaluated the total biomass production in the World. The sun provides the earth with around 120 000 TW per year and global photosynthesis uses part of this energy along with water and CO_2 to produce around 100 billion tons of dry biomass annually.[33] Approximately 6.5% of this biomass is diverged for human consumption, but one third is lost or wasted annually mostly in the industrialized world where this loss accounts for 95–115 kg per year per person due to mechanical damage, spillage during harvesting, degradation, loss of edible parts, and wastes during household consumption and managing in supermarkets.[34] In low-income countries the loss accounts for 6–11 kg per year per person, and is mainly associated with technical, storage, packing and marketing limitations.[34] Low-income countries are also limited in agronomic resources including technical assistance, low income per unit of land, migration of human labor, and earth erosion which limit the potential utilization of land for the production of food.[35,36] There are at present 35 countries requiring external assistance for food; most of them in the Sub-Saharan Africa and zones of Asia with severe civil conflicts.[37] In recent years several commitments and programs have been facing agricultural, food supply, and rapid population growth problems in sub-Saharan Africa with the aim to decrease hunger and poverty as well as to improve livelihoods, social equity and sustainable development with Africa's own renewable means.[35,38] This continent can be a potential exporter of cereals if political, agricultural and trade barriers get solved.[39] Latin America is also a potential region for increasing the production of food and lignocellulosic biomass. Here there is a better, but still insufficient, infrastructure for financing and improving agriculture to better exploit the potential of this region.[36] The former considerations are only related to the efficient use of arable land in low-income countries, but there is a vast region of exhausted and low quality land extended of about 385–472 million hectares around the world that can be used for the cultivation of low-input, perennial grasses.[29] Cultivation of plants of the Agavaceae family, which can grow in arid and degraded lands, has for example been considered an important option for food supply and the agave bagasse can be used for biofuel production.[40] Resolving problems of food waste, losses, production and distribution can be sufficient to ensure future food supply, while residues from the related agro-industrial activities can be used for biofuel production. Thus, there is basically no food *versus* fuel issue if the right infrastructure is implemented for handling of agricultural products.

From our arguments above it is quite clear that much of the criticism raised against biofuels is related to the current biofuel production, in particular corn ethanol and vegetable biodiesel. However, these processes are not likely to be part of the future biofuel industry (Fig. 2), which is moving towards the production of second generation bioethanol and advanced biofuels. The production of second generation bioethanol using biomass is much driven from a cost perspective and the terms for fitting technical improvements and feedstock-sustainability milestones are between 5 and 25–35 years respectively.[14] However, if biofuel use will be expanded, there is a need for production of advanced biofuels. Ethanol is in fact not a good fuel as it has a low energy density (8.0 kW h kg^{-1}) and is highly hygroscopic, and it is only produced due to the very high efficiency of the conversion of sugars to ethanol by yeast fermentation. However, there is much interest in advanced biofuels that can fit even better into the current infrastructure, and ensure blending to high levels or even completely replace fossil derived transportation fuels (Fig. 1C). Butanol (10.0 kW h kg^{-1}) is one example of advanced biofuel with improved characteristics, and currently two companies, Butamax (a Dupont-BP joint venture) and Gevo, are aiming at developing commercial butanol production. Other high density biofuels, called advanced

biodiesels, are farnesane (12.8 kW h kg^{-1}), fatty-acyl ethyl esters (11.25 kW h kg^{-1}), and olefins (13.08 kW h kg^{-1}). These biodiesels are being developed, tested and produced at the pilot scale by companies like Amyris, LS9 and Solazyme, respectively. Farnesane meets the ASTM D975 diesel standard and has received EPA certification to be blended at up to 35% with petroleum diesel – up to 15–20% is normally recommended for conventional biodiesel. It is also claimed that the other advanced biodiesels can be used in existing engines without modification as have been evaluated in naval ships, for example. Therefore, with the current developments of technologies for the production of advanced biofuels, demonstration of their economic feasibility is going to take between 8 and 20 years, and we are therefore likely to first see implementation sometime between 2020 and 2030.[14,41] These developments away from corn based ethanol and vegetable biodiesel and towards biomass derived ethanol and advanced biofuels are clearly supported by a number of reports.[8,13,14,23,41,42]

Advanced biofuels produced from lignocellulose can positively impact current concerns about the use of biofuels, breaking the barriers for their full integration in the current end-used technology – internal combustion engines. These technologies, called conventional transportation, will not suddenly change to other technologies based on electricity or hydrogen, which will require a gradual introduction since it requires the development of many sectors, *i.e.* production of advanced transportation engines and related industries of materials, components, control systems, installers, and business services.[24,41] This kind of growth and diffusion of technology requires a considerable amount of time (between 2 and 7 decades[24]). Thus solar PV technologies, which nowadays account for about 0.04 TW (Renewables 2011), have been predicted to reach between 0.2 and 6.9 TW by 2050 based on historical growth.[24] This is to compare with the predicted increased use of biofuels from 0.12 TW in 2011 to between 1.2 and 7.4 TW by 2050.[13,41]

Advances in biofuel development and production not only stay at the development and optimization of technologies for the conversion of lignocellulose to the desired fuel, some research groups are currently working on the development of less-recalcitrant lignocellulosic materials to decrease pretreatment efforts and costs for the conversion of biomass into fermentable sugars by hydrolysis.[43–45] Increase of photosynthetic capacity by replacing photosystem I by a new reaction center, with farther-red-absorbing pigments, can double the length of wave absorption,[33,46] whilst engineering of the ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase can increase CO_2 assimilation.[47,48] Furthermore, displaying hydrolytic activities in cells could lead to the generation of an integrated process that can convert lignocellulose to biofuels with one unit operation, with the concomitant decrease in production costs.[49,50] Recently, an electro-microbial process for the conversion of sunlight and CO_2 into alcohols has been reported.[51] Much more advances have been generated in the last decade for redirecting the economy towards a bio-based economy. Ignoring the development and production of biofuels is to give up on the opportunity of applying the whole range of biotechnological applications (*i.e.* synthetic biology, systems biology, and metabolic engineering) to tackle the big problem of ensuring stable supply of transportation fuels for the future and at the same time reduce CO_2 emissions at proper costs and hereby contribute to a reduction in global warming.

Conflict of interest

The authors declare no conflict of interest.

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Current Opinion in Chemical Biology

Advanced biofuel production by the yeast *Saccharomyces cerevisiae*

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Replacement of conventional transportation fuels with biofuels will require production of compounds that can cover the complete fuel spectrum, ranging from gasoline to kerosene. Advanced biofuels are expected to play an important role in replacing fossil fuels because they have improved properties compared with ethanol and some of these may have the energy density required for use in heavy duty vehicles, ships, and aviation. Moreover, advanced biofuels can be used as drop-in fuels in existing internal combustion engines. The yeast cell factory *Saccharomyces cerevisiae* can be turned into a producer of higher alcohols (1-butanol and isobutanol), sesquiterpenes (farnesene and bisabolene), and fatty acid ethyl esters (biodiesel), and here we discusses progress in metabolic engineering of *S. cerevisiae* for production of these advanced biofuels.

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Introduction

After 2004, the oil prices went from being elastic to inelastic; nowadays the oil supply cannot meet the demand at prices to which the global economy is accustomed [1•]. In 2008, 55% of crude oil was used for production of transportation fuels [2]. Other exhaustible sources that can be converted into transportation fuels, such as tar sands, shale oil, shale gas, natural gas (gas-to-liquid) and coal (coal-to-liquid), cannot replace conventional oil because of their limited reserves [1•,3] or problems with large green-house gas emissions associated with the conversion process [4]. Also alternative technologies (e.g. fuel cells, hydrogen, and electricity) will probably not play a substantial role in heavy duty vehicles, shipping, and aviation because of their limited capacity or energy density [5]. Biofuels can be used in current internal combustion engines and hence as drop-in fuels, and this compatibility with the current infrastructure makes them attractive for replacing fossil transport fuels in the future.

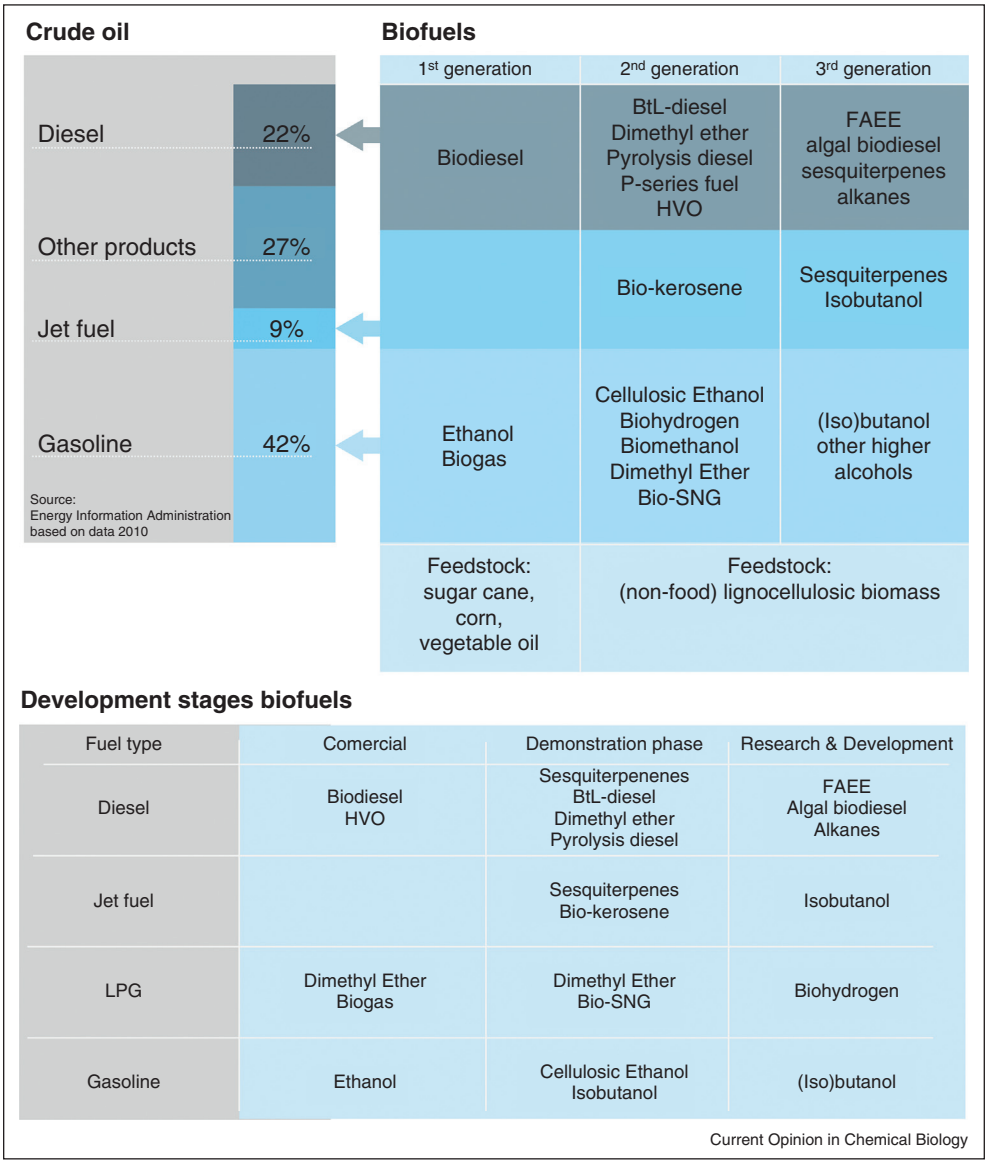
Presently, the most widely used biofuel is ethanol produced from sugar cane, corn or wheat, but the use of feedstock that can be used for food production prevents this process from expanding further [6–8]. Another dominant biofuel is biodiesel, a vegetable oil derived diesel substitute that because of its feedstock cannot be produced at a very large scale and the energy yield per hectare is quite low [9,10]. These disadvantages will most likely lead to a replacement of 1st generation biofuels by 2nd generation biofuels that are derived from biomass [8,11,12] (see Figure 1). However, development of 2nd generation bioethanol processes will not enable replacement of diesel (for heavy duty vehicles and ships) or jet fuels [5,12]. For these applications new processes, or a 3rd generation biofuels have to be developed, to which we will here refer to as advanced biofuels. Advanced biofuels are seen as disruptive technologies that can be used as drop-in fuels, though most of them are still in the research and development stage (see Figure 1). The feasibility of biofuels was studied by the US Department of Energy, and it has been estimated that sufficient biomass can be generated to replace at least 30% of the present petroleum consumption by 2030 [6].

The yeast *Saccharomyces cerevisiae* is the workhorse in the current biofuel industry as it is used for production of ethanol [13]. *S. cerevisiae* has been metabolically engineered to produce several advanced biofuels: 1-butanol [14] and isobutanol [15•,16••,17•,18], the sesquiterpenoids farnesene [19,20], bisabolene [21•], and amorphadiene [22,23], and fatty acid ethyl esters (FAEEs) (biodiesel) [24•]. 1-Butanol and isobutanol are gasoline substitutes (far better than ethanol), farnesene, bisabolene and FAEEs are diesel substitutes, and amorphadiene is a jet fuel substitute [25].

Butanol

Since butanol is less hygroscopic and has a longer chain length than ethanol, it can be blended up to 85% with gasoline [12]. Compared to ethanol, which can only be blended up to 10% due to limits set by regulation and requirements of engine modification [13], the high percentage of butanol-blending renders it an attractive biofuel. Butanol also has a higher energy density than ethanol, albeit still lower than gasoline [26]. There are four isomers of butanol of which 1-butanol [13] and

Figure 1



Biochemical and chemical alternatives to fossil transportation fuels. All transportation fuel alternatives are synthesized chemically except (cellulosic) ethanol, biohydrogen, FAEE, algal biodiesel, sesquiterpenes, alkanes, (iso)butanol, and higher alcohols, which are synthesized biochemically.
BtL = biomass to liquid; HVO = hydrotreated vegetable oil; FAEE = fatty acid ethyl esters (biodiesel).
This figure was adapted from the IEA World Energy Outlook 2010, the Technology Roadmap for Biofuels, and other reports [8,9,11,12,82,83].

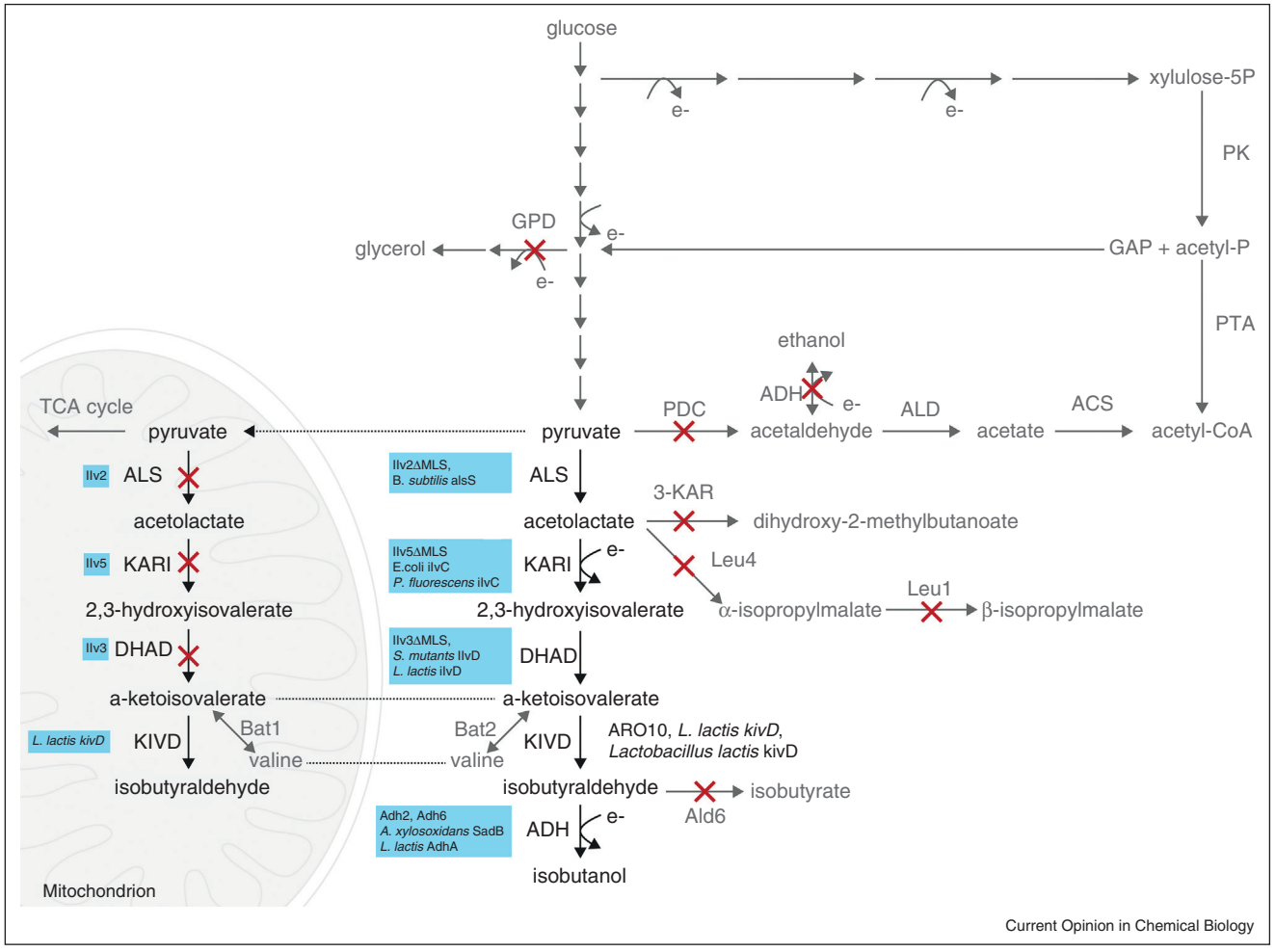
isobutanol have been produced in yeast. Since most recent studies focused on isobutanol we will only discuss this isomer. Besides being a gasoline substitute, isobutanol can also be dehydrated into isobutylene [27] which can be processed into a jet fuel (paraffinic kerosene) [28].

The isobutanol biosynthesis pathway consists of the anabolic synthesis of ketoisovalerate (an intermediate of valine biosynthesis) in the mitochondria and catabolism of this ketoacid into isobutanol in the cytosol (the Ehrlich pathway) (see Figure 2) [29].

Isobutanol production by *S. cerevisiae* was first reported by Chen *et al.* [15•] who overexpressed the endogenous genes of the mitochondrial valine biosynthesis pathway (*ILV2*, *ILV5*, and *ILV3*). With this approach, they increased the yield from 0.28 mg g^{-1} to 3.86 mg g^{-1} on glucose.

To optimize the last steps of the pathway, Kondo *et al.* [17•] measured the *in vivo* conversion of externally added α-ketoisovalerate into isobutanol by different ketoisovalerate decarboxylases (KIVDs) and alcohol

Figure 2



Isobutanol biosynthesis in *S. cerevisiae*. Upper cases: general enzyme types; lower cases: specific proteins. GPD = glycerol-3-phosphate dehydrogenase; TCA = tricarboxylic acid; ALS = acetolactate synthase; KARI = ketoacid reductoisomerase; DHAD = dihydroxyacid dehydrogenase; KIVD = ketoisovalerate dehydrogenase; ADH = alcohol dehydrogenase; PDC = pyruvate decarboxylase; 3-KAR = 3-ketoacid reductase; ALD = aldehyde dehydrogenase; ACS = acetyl-CoA synthase; PK = phosphoketolase; PTA = phosphotransacetylase. Red crosses: enzymatic steps eliminated by gene deletion.

dehydrogenases (ADHs) in a *pdc1*Δ strain. *Lactococcus lactis* KivD and endogenous dehydrogenase Adh6 were found to be the best candidates. Subsequently, they overexpressed *ILV2*, *L. lactis KIVD*, and *ADH6*, combined with disruption of *PDC1* (one of the three pyruvate decarboxylase encoding genes), which should increase the availability of pyruvate for isobutanol production. This resulted in a yield of 6.6 mg g^{-1} on glucose.

It was possible to further improve the product yield by moving the valine biosynthetic pathway to the cytosol [15•] so that α-ketoisovalerate could directly be converted by KIVD in the cytosol. This is was done by Lee *et al.* [18]. They tested various KIVD candidates and found that *L. lactis* KivD had the highest *in vitro* specific activity. Subsequently, they expressed this KIVD together with overexpression of the endogenous *ILV2*, *ILV5*, and *ILV3* genes stripped of their mitochondrial targeting signals. With this strategy Lee *et al.* were able to increase the isobutanol titer from 20 mg L^{-1} to 151 mg L^{-1}.

Recently, Brat *et al.* combined the overexpression of all steps of isobutanol biosynthesis [16••]. Since they observed that the expression of a cytosolic pathway did not increase production in the presence of an active mitochondrial pathway, they suspected competition between both routes. Therefore, the mitochondrial pathway was entirely replaced with a cytosolic pathway, contrary to Lee *et al.* who kept the mitochondrial pathway intact [18]. Then they tested *in vitro* activities of KIVD candidates Aro10 (previously studied in [30–32]) and *L. lactis* KivD in

a *pdc1*Δ*pdc5*Δ*pdc6*Δ (pdc−) strain, and all endogenous ADH candidates expressed in an *adh1*Δ*adh3*Δ*adh5*Δ strain. They found the highest specific activity with Aro10 and Adh2, which is different from the *in vivo* results of Kondo *et al.* [17•]. Finally, expression of a cytosolic pathway consisting of *ILV2*, *ILV5*, *ILV3*, *ARO10*, and *ADH2*, and deletion of the first gene of the mitochondrial pathway *ILV2* resulted in a titer and yield of 630 mg L^{-1} and 15 mg g^{-1}, respectively. Interestingly, additional deletion of the remaining two genes of the mitochondrial pathway resulted in a decreased production.

Isobutanol production is being commercialized by three companies: Butalco, Butamax, and Gevo. Efforts are made to increase the pyruvate pool, eliminate by-product formation, increase activity of the iron–sulfur cluster protein dihydroxyacid dehydratase (DHAD), and balance the cofactors in the pathway. A survey of the most recent patent (applications) provided insight into their research approaches, which will be discussed briefly in the following sections.

Butamax retains the mitochondrial pathway [33], while Butalco [34] and Gevo [35] opted for the introduction of a cytosolic pathway. Butalco uses only endogenous genes [34], while the other two companies use heterologous enzymes, too. Among the potential choices are *Bacillus subtilis* acetolactase synthase AlsS [35,36] for the first step in the pathway. This enzyme also has KIVD activity [37] and is preferred because it has increased affinity for pyruvate over ketobutyrate [38]. For the third step, catalyzed by an iron–sulfur cluster protein, Butamax uses *Streptococcus mutans* IlvD, which is less oxygen sensitive than the endogenous DHAD [39]. Gevo uses *L. lactis* IlvD or endogenous cytosolic targeted Ilv3 [35]. Both companies make use of *L. lactis* KivD for step 4 [35,40]. For step 5, Butamax isolated *Achromobacter xylosoxidans* sadB [36] and Gevo uses a protein engineered version of *L. lactis* AdhA [41].

All three companies tried to increase the pyruvate pool by deleting all three pyruvate decarboxylase encoding genes [34,42,48]. Such a strain cannot grow on glucose as the sole carbon source [43–45] since it lacks a pathway to synthesize cytosolic acetyl-CoA, which is essential for growth. To overcome this problem metabolic engineering strategies have been developed. Reduced growth, even with C_2 supplementation, is probably due to pyruvate accumulation and NAD^+ depletion, and can partially be circumvented by increasing NADH oxidation [46]. The strategy proposed by Butalco is the introduction of pyruvate formate lyase, an acetyl-CoA carnitine shuttle, or reducing instead of deleting pyruvate decarboxylase activity [34]. Butamax on the other hand pursued the introduction of a phosphoketolase pathway [47], which led to equal growth rates in the engineered strain as in the pdc− strain on media with C_2 supplementation (μ_{max} = 0.23 $hour^{-1}$). Gevo chose a directed evolution strategy and evolved their pdc− strain for growth without addition of a C_2 carbon source, glucose tolerance and faster growth. They reached a μ_{max} of approximately 0.10 $hour^{-1}$ in complex medium [48], which is lower than the pdc− strain evolved in the group of Pronk [49,50]). Knocking out all six ADH genes would also cease ethanol production and still maintain the pathway to acetyl-CoA [51], but conversion of acetaldehyde into ethanol by isobutyraldehyde dehydrogenase may still result in ethanol production in such a strain.

An increase of the pyruvate pool by elimination of other pyruvate and α-ketoisovalerate consuming reactions profoundly affected isobutanol production [41]. Overexpression of the valine pathway, introduction of *L. lactis* KivD into the mitochondria, elimination of threonine deaminase (Ilv1) and isopropylmalate synthase (Leu4), reduction of pyruvate dehydrogenase (Pda1) and branched chain amino acid transaminase activities (Bat1), resulted in a 15-fold increase [40]. Gevo reduced by-product formation by eliminating *YMR226C*/*TAM29* and *ALD6* [41].

Deletion of the endogenous iron–sulfur cluster containing proteins isopropylmalate dehydratase (Leu1) and mitochondrial DHAD (Ilv3) by Butamax enhanced the activity of DHAD by 1.5-fold and 7-fold, respectively [42]. Manipulation of the cytosolic iron–sulfur cluster biosynthesis machinery further enhanced this activity [39]. It was found that the fraction of active DHAD increased when *FRA2* (7–31%) or *GRX3* (19–31%) was deleted. Deletion of *FRA2* and overexpression of Aft1-L99A (constitutively active mutant) in a pdc− and *gpd2*Δ strain with integrated DHAD (*S. mutans* IlvD) and secondary ADH (*A. xylosoxidans* SadB) decreased the dihydroxyisovalerate level by 50%. Gevo tried to improve DHAD activity by overexpressing parts of the mitochondrial (*NFS1*, *ISD11*) and cytosolic (*CFD1*, *NAR1*, *NBP35*) iron–sulfur cluster machinery, as well as the transcription factor genes *AFT1* and *AFT2* [41,48]. Iron–sulfur cluster biogenesis in yeast has been reviewed by Lill *et al.* [52,53].

Altering cofactor specificity of either acetohydroxy acid reductoisomerase or glyceraldehyde-3-phosphate dehydrogenase can address the inherent cofactor imbalance of the isobutanol pathway [34]. Butamax made *Pseudomonas fluorescens* Pf-5 IlvC (KARI) specific for NADH instead of NADPH [38] and thereby effectively replaced the ethanol fermentation with isobutanol fermentation.

Combining some of the strategies discussed above, the introduction of *B. subtilis alsS*, *Escherichia coli ilvC*, and *L. lactis ilvD*, *L. lactis kivD*, *L. lactis adhA* in an *ald6*Δ *tma29*Δ

*gpd1*Δ *gpd2*Δ pdc− strain overexpressing *AFT1* led to an isobutanol yield of 0.33 g g^{-1} (80.3% of the maximal theoretical yield) and a titer of 18.6 g L^{-1} in complex medium [41]. This is an order of magnitude higher than the yield reported by Brat *et al.* [16••].

Sesquiterpenoids

Sesquiterpenoids are a class of isoprenoids that contain three isoprene units. The isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate are products of the mevalonate (MVA) or the 2-methylerythritol 4-phosphate (MEP) pathway (see Figure 3, for an extensive description see [54]). Amyris is a producer of the sesquiterpene biofuel farnesene that after saturation by hydrogenation can be used as diesel [55,56] and jet fuel [25]. Another sesquiterpene product is bisabolene that upon hydrogenation yields bisabolane, a diesel substitute [21•].

Levels of acetyl-coA, the precursor of the MVA pathway, were increased successfully by overexpressing the enzymes Ald6 and *Salmonella enterica* Acs1 (engineered to relieve glucose repression) from the pyruvate to acetyl-CoA route [57]. Recently it was shown that overexpression of Adh2 can further improve this strategy [58•]. Further optimization studies showed that repression of squalene synthase encoding *ERG9* leads to higher production of farnesyl pyrophosphate (FPP) derived sesquiterpenoids [22,59–61]. Also, overexpression of *HMG1*, encoding HMG1-CoA reductase, increased farnesol, nerolidol and geranylgeraniol production. Truncating *HMG1*, resulting in a fed-back insensitive enzyme, increased productivities slightly more, especially that of farnesol [62]. Repression of *ERG9* was combined with overexpression of a truncated *HMG1* [63]. On the basis of an *in silico* genome scale model of *S. cerevisiae*, modifications in the ammonium assimilation pathway were introduced that led to an increased sesquiterpene production (87% compared to the reference strain) though this was also accompanied by a 35% reduction in growth rate [64].

The first study in which a sesquiterpene diesel substitute was produced by yeast was performed by Peralta-Yahya *et al.* [21•]. The authors screened six bisabolene synthases in *E. coli*, followed by optimization of the production by metabolic engineering. Subsequently they introduced these synthases in a FPP overproducing *S. cerevisiae* strain. In this strain, the truncated Hmg1, the FPP synthase Erg20, and the global transcription regulator Upc2-1 were overexpressed, and combined with repression of *ERG9*. The synthase derived from *Abies grandis* resulted in the highest bisabolene titer of 994 ± 241 mg L^{-1} on complex galactose/glucose medium.

Researchers at Amyris engineered *S. cerevisiae* to increase production of amorphadiene and artemisinic acid [23]. Every gene of the MVA pathway was overexpressed, including three copies of the truncated HMG-CoA reductase gene. With this, an amorphadiene titer of 37 g L^{-1} and yield of 18.67 Cmol% was achieved during a fed-batch restricted in ethanol, as this alcohol leads to a higher supply of acetyl-CoA [65]. Higher titers, when glucose is used as carbon source, have been observed before [59]. Amyris' patents describe how yeast can be modified further to overproduce sesquiterpenoids. For example, *E. coli* acetyl-CoA thiolase AtoB, *Staphylococcus aureus* or *Enteroccous faecalis* HMG-CoA synthase MvaS, and *S. aureus* (MvaA) or *E. faecalis* (MvaE) HMG-CoA reductase have all been suggested as alternatives to endogenous enzymes [19]. *S. aureus* MvaA has been shown to have higher activity than the endogenous equivalent [66].

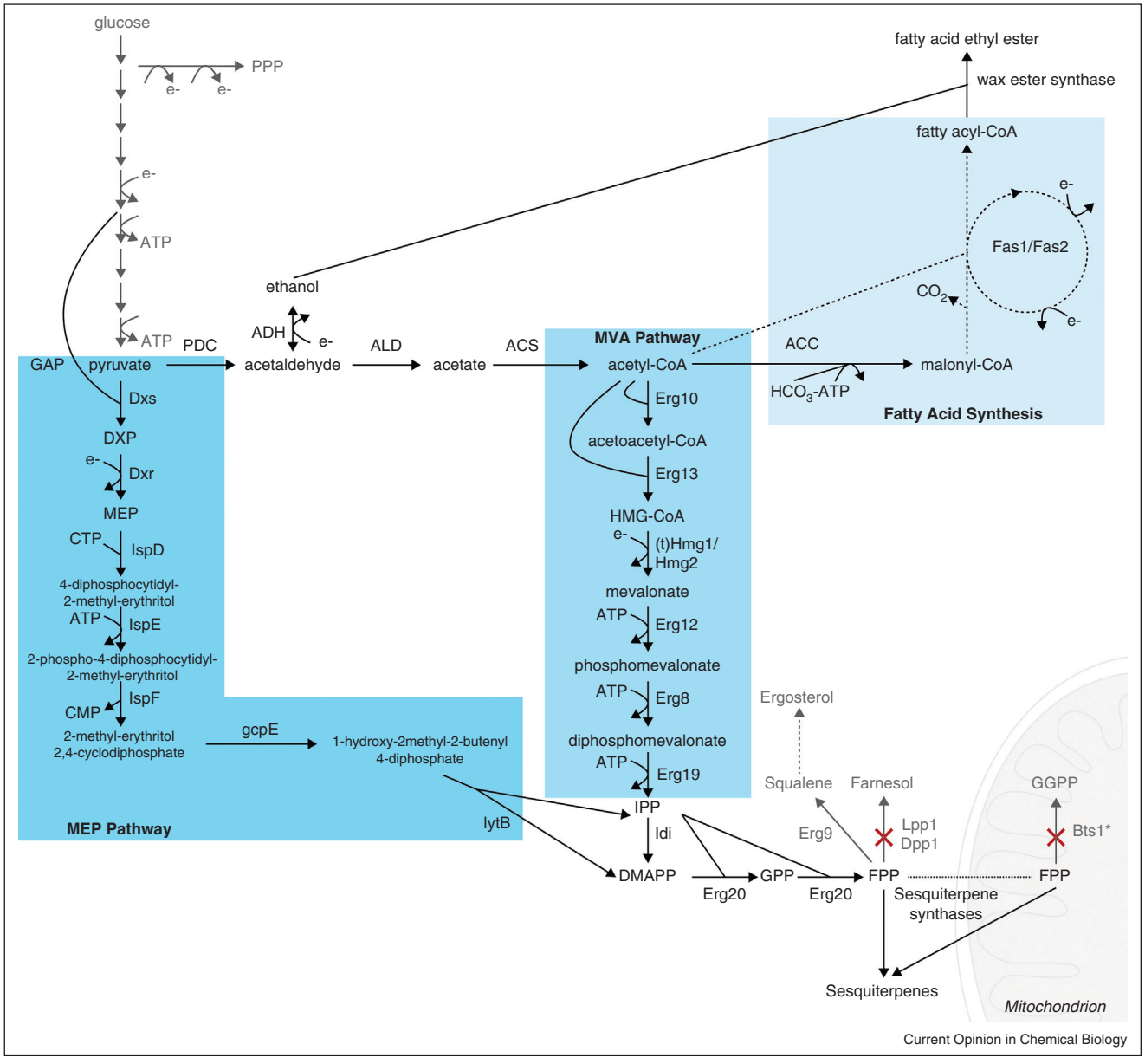
In most cases of heterologous sesquiterpene production of the last reaction of the pathway, that is, the reaction catalyzed by the sesquiterpene synthase, is rate limiting [67]. Amyris patented a screening method for these synthases [67] based on the observation that intermediates of the pathway are toxic at elevated levels [68]. This method is effective because only cells that have a functioning synthase can survive as they prevent the accumulation of the toxic intermediates. Hereby, the inventors drastically reduced the number of clones that have to be characterized. This method can be used to optimize a specific protein by enzyme engineering or to screen genomic libraries created from environmental samples for presence of synthases [68]. To minimize the loss of pathway intermediates terpene synthases have been fused to enzymes catalyzing either the upstream or downstream reaction of the respective pathway. Creation of such fusions led to almost 3-fold and 2-fold improvement of the production of geranylgeraniol [69] and patchoulol [70], respectively. However, there are also reports on the contrary [71], and in general it seems more important to coordinate the expression of enzymes in the pathway.

Inspired by the observation that the sesquiterpenoid precursors can be found in different cellular compartments, Farhi *et al.* localized FPP and valencene or amorphadiene synthases to the mitochondria [72••]. First, they tried to improve product yields by elimination of by-product formation. However, elimination of both Lpp1 and Dpp1 (FPP consuming phosphatases) and Bts1 (geranylgeranyl diphosphate synthase) did not improve sesquiterpene synthesis but led, respectively, to a 10% and 50% decrease compared to the reference strain. Expression of a truncated *HMG1*, *Arabidopsis thaliana* FPP synthase, and *Artemisia annua* amorphadiene synthase (mitochondrial targeting signals were added to both synthases) led to a production improvement of approximately 20-fold.

Fatty acid ethyl esters

Fatty acid (m)ethyl esters can be synthesized microbiologically, chemically, enzymatically, or with whole cell

Figure 3



Sesquiterpenoid and fatty acid ethyl ester biosynthesis in *S. cerevisiae*. Upper cases: general enzyme types; lower cases: specific proteins. PPP = pentose phosphate pathway; GAP = glyceraldehyde 3-phosphate; ATP = adenosine triphosphate; DXP = 1-deoxy-D-xylulose 5-phosphate; MEP = 2-methylerythritol 4-phosphate; CTP = cytidine triphosphate; CMP = cytidine monophosphate; IPP = isopentenyl diphosphate; DMAPP = dimethylallyl diphosphate; GPP = geranyl diphosphate; FPP = farnesyl diphosphate; GGPP = geranylgeranyl diphosphate; PDC = pyruvate decarboxylase; ADH = alcohol dehydrogenase; ALD = aldehyde dehydrogenase; ACS = acetyl-CoA synthase; MVA = mevalonate; ACC = acetyl-CoA carboxylase. * = localization based on Yeast GFP Fusion Localization Database [84]. Red crosses: enzymatic steps eliminated by gene deletion.

catalysts expressing lipases and using triacylglycerides and (m)ethanol [73]. FAEEs can also be formed by esterification of fatty acids and ethanol, catalyzed by FAEE synthases/carboxylesterases, or by alcoholysis, through the action of acyl-CoA:ethanol O-acyltransferases [73].

The first study in which FAEEs were produced in *S. cerevisiae* was carried out by Kalscheuer *et al.* [74]. More recently, Shi *et al.* expressed five different wax ester synthases in *S. cerevisiae* to study their activity *in vitro* (crude cell extracts) and *in vivo* [24•]. The activity of these enzymes was tested with alcohols ranging from ethanol to octadecanol with palmitoyl-CoA as the fatty acid substrate. Shi *et al.* found that the wax ester synthase from *Marinobacter hydrocarbonoclasticus* was the best candidate because it performed well, even with short chain

alcohols. This enzyme also produced the highest titer of FAEEs, 6.3 mg L^{-1}. Overexpression of Acc1 (acetyl-coA carboxylase), the presumed rate limiting step in fatty acid synthesis, led to an FAEE titer increase to 8.2 ± 1.1 mg L^{-1}. One additional benefit of this wax ester synthase is that it does not exhibit acyl-CoA:diacylglycerol acyltransferase (DGAT) activity, thereby avoiding the formation of the by-product triacylglycerol (TAG) [75].

Conclusion

In order to completely replace fossil fuels with biofuels, the whole range of products derived from crude oil needs to be replaced by bio-based production routes, that is, not only transportation fuels. Bio-based production of petrochemicals is still in its infancy [76–78], but *S. cerevisiae* is also likely to play a role here [79••].

At the moment, isobutanol production is the most developed advanced biofuel. Current research directs toward improvement of the product yield by eliminating by-product formation, such as ethanol. Reduction of ethanol formation is also appealing for production of sesquiterpenes and FAEE because more ATP is produced via oxidative respiration [49]. In order to further improve the yield of these advanced biofuels it may be necessary to further elucidate the regulation in the endogenous MVA and fatty acid biosynthetic pathways.

At bulk scale production, the environment is typically not sterile and biomass is often reused in consecutive batches. Therefore, low growth rates will be a problem for industrial applications since the described strains will not be able to compete against wild-type species in a non-sterile environment. Wild-type species have evolved over centuries to become the best adapted strains and that is what strains engineered for industry should become in an industrial setting. Another remaining challenge to successfully implement 2nd generation and advanced biofuels is the use of enzymes to hydrolyze lignocellulosic biomass and the subsequent use of both hexose and pentose sugars [8]. This is important to reduce production costs and to address the food-versus-fuel problem [80].

However, even after addressing all technological hurdles, the main concern remains the cost competitiveness of biofuels [81], but it may be time to begin considering the social and environmental costs associated with the use of conventional fossil fuels [4].

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