Development and implementation of strategies to improve octanoic acid production in *Saccharomyces cerevisiae*

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Contents

1 Summary

Octanoic acid (C8 FA) is a medium-chain fatty acid which, in nature, mainly occurs in palm kernel oil and coconuts. It is used in various products including cleaning agents, cosmetics, pesticides and herbicides as well as in foods for preservation or flavoring. Furthermore, it is investigated for medical treatments, for instance, of high cholesterol levels. The cultivation of palm oil plants has surged in the last years to satisfy an increasing market demand. However, concerns about extensive monocultures, which often come along with deforestation of rainforest, have driven the search for more environmentally friendly production methods. A biotechnological production with microbial organisms presents an attractive, more sustainable alternative.

Traditionally, the yeast *Saccharomyces cerevisiae* has been utilized by mankind in bread, wine, and beer making. Based on comprehensive knowledge about its metabolism and genetics, it can nowadays be metabolically engineered to produce a plethora of compounds of industrial interest. To produce octanoic acid, the cytosolic fatty acid synthase (FAS) of *S. cerevisiae* was utilized and engineered. Naturally, the yeast produces mostly long-chain fatty acids with chain lengths of C16 and C18, and only trace amounts of medium-chain fatty acids, i.e. C8-C14 fatty acids. To generate an *S. cerevisiae* strain that produces primarily octanoic acid, a mutated version of the FAS was generated (Gajewski et al., 2017) and the resulting *S. cerevisiae* FAS^{R1834K} strain was utilized in this work as a starting strain.

The goal of this thesis was to develop and implement strategies to improve the production level of this strain. The current mode of quantification of octanoic acid includes labor-intensive, low-throughput sample preparation and measurement – a main obstacle in generating and screening for improved strain variants. To this end, a main objective of this thesis was the development of a biosensor. The biosensor was based on the *pPDR12* promotor, which is regulated by the transcription factor War1. Coupling *pPDR12* to GFP as the reporter gene on a multicopy plasmid allowed *in vivo* detection via fluorescence intensity. The developed biosensor enabled rapid and facile quantification of the short- and medium-chain fatty acids C6, C7 and C8 fatty acids (Baumann et al., 2018). This is the first biosensor that can quantify externally supplied octanoic acid as well as

octanoic acid present in the culture supernatant of producer strains with a high linear and dynamic range. Its reliability was validated by correlation of the biosensor signal to the octanoic acid concentrations extracted from culture supernatants as determined by gas chromatography. The biosensor's ability to detect octanoic acid in a linear range of 0.01-0.75 mM ($\approx$1-110 mg/L), which is within the production range of the starting strain, and a response of up to 10-fold increase in fluorescence after activation was demonstrated.

A high-throughput FACS (fluorescence-activated cell sorting) screening of an octanoic acid producer strain library was performed with the biosensor to detect improved strain variants (Baumann et al., 2020a). For this purpose, the biosensor was genomically integrated into an octanoic acid producer strain, resulting in drastically reduced single cell noise. The additional knockout of *FAA2* successfully prevented medium-chain fatty acid degradation. A high-throughput screening protocol was designed to include iterative enrichment rounds which decreased false positives. The functionality of the biosensor on single cell level was validated by adding octanoic acid in the range of 0-80 mg/L and subsequent flow cytometric analysis. The biosensor-assisted FACS screening of a plasmid overexpression library of the yeast genome led to the detection of two genetic targets, *FSH2* and *KCS1*, that in combined overexpression enhanced octanoic acid titers by 55 % compared to the parental strain. This was the first report of an effect of *FSH2* and *KCS1* on fatty acid titers. The presented method can also be utilized to screen other genetic libraries and is a means to facilitate future engineering efforts.

In growth tests, the previously reported toxicity of octanoic acid on *S. cerevisiae* was confirmed. Different strategies were harnessed to create more robust strains. An adaptive laboratory evolution (ALE) experiment was conducted and several rational targets including transporter- (*PDR12*, *TPO1*) and transcription factor-encoding genes (*PDR1*, *PDR3*, *WAR1*) as well as the mutated acetyl-CoA carboxylase encoding gene $ACC1^{S1157A}$ were overexpressed or knocked out in producer or non-producer strains, respectively. Despite contrary previous reports for other strain backgrounds, an enhanced robustness was not observable. Suspecting that the utilized laboratory strains have a natively low tolerance level, four industrial *S. cerevisiae* strains were evaluated in growth assays with octanoic

acid and inherently more robust strains were detected, which are suitable future production hosts.

The first transcriptomics analysis via RNA-Seq of an *S. cerevisiae* octanoic acid producer strain gave a profound insight into the producer strain's transcriptional response to octanoic acid production in different phases of fermentation (Baumann et al., 2021). The analysis revealed that the transcriptomic response to internally produced octanoic acid is markedly disparate from the responses of wild type strains to externally supplied octanoic acid. In addition, a negative effect of octanoic acid production on strain growth was confirmed. The data suggests that the reduced growth of the octanoic acid producer strain could partly be a result of diminished long-chain fatty acid production. Differential expression analysis of the RNA-Seq data of three sampling times led to the identification of an unreported target, *RPL40B*, that when overexpressed increased octanoic acid titers by 40%. This was the first report of Rpl40b to play a role in fatty acid biosynthesis, the exact mechanism of which remains to be investigated.

In summary, in this thesis new strategies were successfully developed and established tools harnessed to improve the yeast *S. cerevisiae* as cell factory for production of octanoic acid. An octanoic acid biosensor was designed, verified, and optimized for implementation in a high-throughput FACS screening. The developed method is highly versatile and can be implemented for various library screenings of short- and medium-chain fatty acid producer strains. A transcriptomics analysis gave a comprehensive picture of strain physiology during octanoic acid production. Overall, three unreported targets were identified and will improve production, ideally in an industrial strain that showed high inherent robustness.

2 Introduction

2.1 Fatty acid metabolism in *Saccharomyces cerevisiae*

Fatty acids play an inevitable role in cells, contributing to many cellular processes such as cell signaling and energy supply. In addition, they serve as building blocks for membrane lipids (phospholipids and sphingolipids) and storage lipids (steryl esters and triacylglycerols). Storage lipids are kept in specialized cellular compartments, so-called lipid droplets, and can be mobilized when necessary as a source of energy for the cells. Cellular fatty acids originate from three sources: uptake from the environment, intracellular lipid turnover and *de novo* lipid biosynthesis (Tehlivets et al., 2007; Klug & Daum, 2014).

2.1.1 Fatty acid biosynthesis

The *de novo* lipid biosynthesis pathway is a well-conserved mechanism among eukaryotes and has been studied extensively for several decades. In yeasts such as *Saccharomyces cerevisiae*, fatty acid biosynthesis takes place in mitochondria and the cytosol. Subsequent modification steps are carried out in the endoplasmic reticulum. Here, we will focus on the cytosolic biosynthesis pathway, which is the main source of cellular fatty acids in the absence of external supply (Tehlivets et al., 2007; Klug & Daum, 2014). Cytosolic fatty acids are synthesized from the precursor molecules acetyl-coenzyme A (acetyl-CoA) and its derivative malonyl-CoA by the fatty acid synthase (FAS) as illustrated in Figure 1. Cytosolic acetyl-CoA is generated in the pyruvate dehydrogenase (PDH) bypass pathway from pyruvate produced from glucose (Pronk et al., 1996). The second precursor, malonyl-CoA, is generated via carboxylation of acetyl-CoA, a reaction that is catalyzed by the acetyl-CoA carboxylase Acc1 (Roggenkamp et al., 1980).
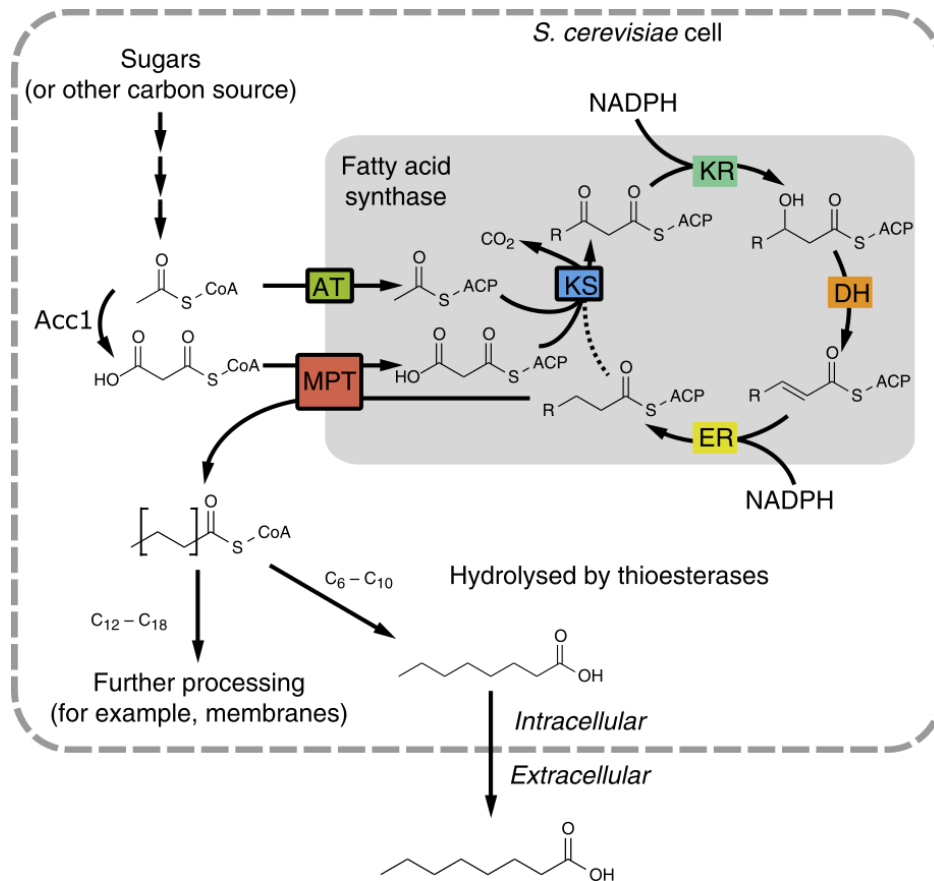
Figure 1: Fatty acid biosynthesis in *Saccharomyces cerevisiae*. The precursor acetyl-CoA is derived from sugars like glucose and converted to malonyl-CoA by an Acc1-catalyzed carboxylation. The acetyl transferase (AT) domain loads an acyl substrate (starter molecule acetyl or ACP-bound, elongated acyl-chain). A malonyl/palmitoyl transferase (MPT) loads a malonyl molecule which is subsequently bound to the acyl carrier protein (ACP). The two substrates are condensed by the ketoacyl synthase (KS). The resulting β-ketoacyl intermediate is modified in a series of reaction steps performed by the ketoacyl reductase (KR), dehydratase (DH) and enoyl reductase (ER). The resulting acyl-chain serves as a starter for the next cycle and the process is repeated until the final product is released as an acyl-CoA ester. Acyl-CoAs can be cleaved by thioesterases, releasing free fatty acids. The fatty acid synthesis enzyme can be engineered to produce short- and medium-chain fatty acids (here: C6-C10). Figure modified from Gajewski et al. (2017).

Fatty acids are synthesized by iterative reactions in the so-called "FAS cycle" starting from an acetyl moiety which is successively elongated by the addition of malonyl-CoA molecules. This process is catalyzed by the FAS enzyme, a large complex that consists of six Fas1 and six Fas2 subunits encoded in *S. cerevisiae* by the genes *FAS1* and *FAS2*, respectively. The FAS complex exhibits different catalytic activities which are embedded in its various domains. Briefly, the single steps of the FAS cycle occur as following: An acetyl transferase (AT) loads an acyl substrate which can be the priming molecule acetyl-CoA or an ACP (acyl carrier protein)-bound, elongated acyl-chain. A malonyl/palmitoyl transferase (MPT) loads

a malonyl molecule which is subsequently bound to the ACP. These two substrates are condensed by the ketoacyl synthase (KS). The resulting molecule, a β-ketoacyl intermediate bound to the ACP, is further processed in a series of reaction steps performed by the domains ketoacyl reductase (KR), dehydratase (DH) and enoyl reductase (ER) to a fully reduced acyl-chain. The two reduction steps catalyzed by KR and ER require the cofactor NADPH. The reduced acyl-chain either serves as a starter for the next cycle or is released as the final product. The last step, the unloading of the final product, is performed by the MPT (Gajewski et al., 2017; Heil et al., 2019).

The main products of the yeast cytosolic FAS cycle are saturated C16 and C18 fatty acids with a minor fraction constituted of saturated C14 fatty acids. A part of the synthesized fatty acids are modified by desaturation, hydroxylation and elongation in other cellular organelles (Klug & Daum, 2014). The majority of saturated C16 and C18 fatty acids undergo desaturation in the endoplasmic reticulum catalyzed by Ole1, resulting in monounsaturated C16:1 (palmitoleic acid) and C18:1 (oleic acid) fatty acids (Viljoen et al., 1986; Stukey et al., 1989, 1990; Tuller et al., 1999). Furthermore, a fraction of the synthesized fatty acids is elongated to very long-chain fatty acids up to C26 by elongases in the endoplasmic reticulum (Toke & Martin, 1996; Oh et al., 1997).

Fatty acid metabolism is regulated on several levels. The amount of FAS enzyme in the cell is regulated, for instance, on both the gene expression and protein level. Even though Fas1 and Fas2 are present in an equimolar ratio in the FAS enzymatic complex, the overall amount of FAS enzyme is solely determined by the level of Fas1 (Tehlivets et al., 2007). Enhanced Fas1 levels lead to an increase in *FAS2* expression, thereby ensuring equimolar amounts of the two parts constituting the complex (Wenz et al., 2001). Apart from the FAS complex, regulation of Acc1 is also critical because malonyl-CoA formation is a rate-limiting step in fatty acid biosynthesis. To adjust cellular malonyl-CoA abundance, regulation on both the transcriptional and protein level is essential (Tehlivets et al., 2007). The expression of *ACC1* is controlled by several transcription factors (TFs), e.g. Ino2/Ino4 and Opi1 (Hasslacher et al., 1993; Chirala et al., 1994). On the protein level, Acc1 is inhibited by phosphorylation mediated by the protein kinase Snf1 (Woods et al. 1994). Snf1-

mediated inhibition was shown to be abolished through mutation of two serine residues of Acc1 (S659A, S1157A), leading to enhanced activity of Acc1 and increased fatty acid production (Choi & Da Silva, 2014; Shi et al., 2014).

2.1.2 Fatty acid uptake and degradation

Besides *ne novo* biosynthesis, *S. cerevisiae* can also take up externally supplied fatty acids (Klug & Daum, 2014). The biochemical mechanisms behind fatty acid uptake are poorly understood. There are two main theories about how fatty acids cross biological membranes. The first model states that long-chain fatty acids primarily cross membranes by diffusion which is driven by concentration gradients. According to this theory, fatty acids bind to the membrane and flip through the lipid bilayer without any transporters involved. Many research efforts, however, have also brought evidence for the second scenario (Claus et al., 2019). According to this model, fatty acids mainly cross the plasma membrane by protein-facilitated transport (Higgins, 1994; Zou et al., 2002; Tarling et al., 2013). This mechanism allows for a selective exchange of compounds with the external environment. Furthermore, selective transport can be tightly controlled, thereby avoiding toxic effects, e.g. caused by the accumulation of toxic molecules. Possibly, the mechanism of uptake is dual, depending on the cell's requirements and fatty acid availability (Claus et al., 2019).

Internalized or synthesized free fatty acids are readily activated to their acyl-CoA form, which is mediated in *S. cerevisiae* by the fatty acyl-CoA synthetases Faa1, Faa2, Faa3, Faa4 or Fat1. Activated fatty acids are either used for the synthesis of triacylglycerols and steryl esters for storage in lipid droplets or degraded by β-oxidation in the peroxisomes. Long-chain fatty acids are activated primarily by Faa1 and Faa4 prior to transport into the peroxisomes by the transporter Pxa1-Pxa2 (Hettema et al., 1996; Scharnewski et al., 2008). In contrast, medium-chain fatty acids enter peroxisomes presumably by passive diffusion or spontaneous flipping with the aid of the auxiliary proteins Ant1 and Pex11 (van Roermund et al., 2000, 2001) and are activated thereafter by peroxisomal Faa2 (Knoll et al., 1994; Hettema et al., 1996). The peroxisomal fatty acid degradation process in *S. cerevisiae* is a cyclic mechanism catalyzed by three enzymes, Pox1, Fox2 and Pot1. In each degradation cycle one acetyl-CoA is released and this process is

repeated until complete degradation of the fatty acid is achieved (Hiltunen et al., 2003).

2.2 Metabolic engineering for octanoic acid production in *Saccharomyces cerevisiae*

The FAS biosynthesis in *S. cerevisiae* can be modified via metabolic engineering to shift the product spectrum from primarily long-chain fatty acids, i.e. >C16, to short- and medium chain fatty acids, i.e. C6-C14. The medium-chain fatty acid octanoic acid (C8 fatty acid) is a compound of high industrial interest.

2.2.1 The concept of metabolic engineering

The aim of metabolic engineering is to modify the metabolic network of a microorganism to use it for the biotechnological production of pharmaceuticals, chemicals, foods, or biofuels (Woolston et al., 2013). To generate such a microbial cell factory, endogenous metabolic pathways can be improved and rewired, or entirely new pathways are established. Both approaches require a modulation of e.g. gene expression. Considering that the entire cellular network of a microorganism plays a role in the formation of products from substrates, this task can be rather challenging. Targeted genes not only include metabolic genes from the desired pathway but also genes encoding enzymes involved in interconnected reactions, pathway regulation or product degradation. Once the desired pathway is established in laboratory-scale in the host organism, the product titer, yield and productivity need to be maximized which is achieved through iterative rounds of the so-called "design-build-test" cycle. Eventually, the goal of every metabolic engineering effort is a cost-effective production of the target compound in industrial scale (Woolston et al., 2013; Nielsen & Keasling, 2016).

The biotechnological production of chemicals and biofuels can be a sustainable alternative to current production methods, i.e. petrochemistry or extraction from plants (Saling, 2005). One of the great advantages of a biotechnological production is that renewable resources, for instance sugars like glucose, xylose or arabinose, can be used as starting material. Nowadays, most commercial biotechnological processes utilize sugars derived from sugar cane or starch. However, to prevent

competition with food production, the use of non-edible waste materials like lignocellulose, which accumulates in other industrial production processes as side product, is increasingly investigated (Gustavsson & Lee, 2016).

The metabolic engineering field has developed at an incredible speed in the last three decades. This is the result of a plethora of new emerging technologies which at the same time have become more easily available, faster and cheaper. The most important developments include chemical DNA synthesis, which allows for the expression of heterologous genes in any host organism, the advances in whole genome sequencing, and the rapid increase of omics-based tools as well as high-throughput screening methods (Woolston et al., 2013). In addition to that, genetic engineering tools like the CRISPR/Cas system (Jinek et al., 2012) have simplified and accelerated genome editing of microorganism. Furthermore, through synthetic biology it was demonstrated that it is possible to create entire synthetic chromosomes (Gibson et al., 2010; Postma et al., 2021). Intertwining metabolic engineering with synthetic biology and a systems biology approach, i.e. considering the cell in its entirety, will likely allow for production of a bigger variety of products and the use of a wider spectrum of microbial hosts in the near future (Nielsen & Keasling, 2016).

To date, a number of microbial production processes are already commercialized. Some prominent examples are the production of the feed supplement lysine by *Corynebacterium glutamicum* (Brautaset & Ellingsen, 2011), the chemical building block 1,4-butanediol via *Escherichia coli* (Burgard et al., 2016), production of antibiotics with the filamentous fungus *Penicillium chrysogenum* (van den Berg, 2010) and the production of bioethanol with *S. cerevisiae* (Mohd Azhar et al., 2017).

2.2.2 Yeast as a microbial production host

S. cerevisiae – often simply referred to by "budding yeast" or "baker's yeast" – is a unicellular fungus of the phylum Ascomycota. *S. cerevisiae* has been employed by mankind for thousands of years for bread baking and production of alcoholic beverages like beer, wine and sake. The molecular basis of these processes is well understood by now and *S. cerevisiae* is the predominant yeast species used in these and other biotechnological processes (Schmelzer et al., 2020). One of the

most important products in industrial biotechnology is bioethanol, the production of which takes advantage of the baker's yeast intrinsic ability to produce high amounts of ethanol (Mohd Azhar et al., 2017).

However, *S. cerevisiae* cannot only be used to produce primary metabolites like ethanol, but also for non-inherent products like secondary metabolites and recombinant enzymes by applying metabolic engineering. Roughly 13 % of biopharmaceuticals approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) were produced in *S. cerevisiae* in the years 2003-2014. In 1982, insulin was the first recombinant protein (produced in *E. coli*) that was licensed as biopharmaceutical for use in humans (Baeshen et al., 2014). Only four years later, in 1986, insulin was successfully produced in *S. cerevisiae* (Markussen et al., 1986; Thim et al., 1986) and nowadays, the producer Novo Nordisk has a worldwide market share of about 50 % with its yeast-produced insulin products (Schmelzer et al., 2020). With globally rising incidence of diabetes, the annual sales of insulin are rapidly increasing (Kaplan et al., 2016; Saeedi et al., 2019) – demonstrating the importance of biotechnological processes for today's society.

With the desire for a bigger variety of biotechnological products, the need for host organisms with very specific characteristics is growing. Therefore, the interest in other, less characterized yeast species has increased in the past years. Some of these so-called non-conventional yeasts have inherent properties that are desirable in industrial production processes (Steensels et al., 2014; Thorwall et al., 2020). The yeast *Yarrowia lipolytica*, for example, can accumulate more than 50 % of its dry cell weight in the form of lipids, making it a suitable host for bio-oil production (Beopoulos et al., 2009). *Kluyveromyces marxianus* is a fast-growing, highly thermotolerant yeast, able to use a broad range of carbon substrates – traits that are desirable for industrial fermentations (Cernak et al., 2018). Another example is *Kluyveromyces lactis*, which produces the enzyme lactase enabling it to degrade the "milk sugar" lactose. *K. lactis* has been widely used in dairy industry since the 1950s to generate lactose-free products but has also been used as a host for the expression of over 40 heterologous proteins to date (van Ooyen et al., 2006).

Despite advances in the use of a variety of yeast species for biotechnological purposes, *S. cerevisiae* is still the host-of-choice for most industrial and lab-scale applications (Hong & Nielsen, 2012). *S. cerevisiae* was the first eukaryote with sequenced genome in the year 1996, which strongly facilitated metabolic engineering efforts (Goffeau et al., 1996). Today, it is one of the best studied microorganisms with well-understood physiology, genetics and metabolism. *S. cerevisiae* has an inherent ability for homologous recombination which can be combined with an array of molecular and genetic engineering tools that are available today, such as adapted CRISPR/Cas systems (Da Silva & Srikrishnan, 2012; Generoso et al., 2016). Another advantage is its ease in handling, as well as its FDA-approved GRAS (generally regarded as safe) status (Da Silva & Srikrishnan, 2012).

2.2.3 Applications of octanoic acid and global market

Octanoic acid, also known as caprylic acid, is a saturated medium-chain fatty acid consisting of an eight-carbon backbone and a terminal carboxyl group. It is a colorless oily liquid with a mild, slightly unpleasant odor (PubChem, 2020). Octanoic acid naturally occurs in coconut and palm kernel oil and in some dairy products (Rupilius & Ahmad, 2007; Deen et al., 2020). The industrial range of applications of octanoic acid is expansive: It is used as antimicrobial in food preservation as well as in household cleaning products, as flavoring agent in baked goods to confer a cheesy and buttery taste, as ingredient in infant formulas and as emulsifier in cosmetics. It is utilized in the synthesis of dyes and drugs and as component of lubricants, pesticides, herbicides and insecticides (PubChem, 2020). Furthermore, a variety of studies have demonstrated possible applications of octanoic acid in medicine. For instance, its antimicrobial properties were shown to be beneficial for treatment of candidiasis infections and it was investigated for the treatment of high cholesterol levels and essential voice tremor (Wilson et al., 2006; Xu et al., 2013; Bae & Rhee, 2019; Lowell et al., 2019).

The global demand for octanoic acid and thus production have steadily increased over the last years (Yan & Pfleger, 2020). Octanoic acid is mainly sourced from palm kernel oil and coconut oil, in which it is contained in the form of triglycerides (Berger, 2003; Deen et al., 2020; Yan & Pfleger, 2020). Approximately 4 % of the

fatty acids contained in palm kernel oil and 7.5 % in coconut oil constitutes octanoic acid. For the extraction of octanoic acid from these oils, the triglycerides are split into the glycerin backbone and the fatty acids, followed by separation of the different fatty acids of the mixture (Rupilius & Ahmad, 2007; Deen et al., 2020). The global production volume of palm kernel oil is projected to reach 8.9 million tons and of coconut oil 3.6 million tons in the years 2019/20 (IndexMundi, 2020).

The growing demand for these oils, especially for use as biodiesel, has led to an enhanced production of plant oil crops alongside with rising concerns about the environmental impact (Fargione et al., 2008). In countries like Malaysia and Indonesia, large areas of rainforest are cleared to establish new plantations for palm oil plants, thereby contributing to increasing greenhouse gas emissions. Another problem is the ethical dilemma of cultivating edible crops for use as chemical compounds or biofuels instead of human nutrition (Fargione et al., 2008). In addition to these concerns, the extraction and purification process from plants is complex and associated with excessive water consumption (Schmidt, 2015).

A possible solution to these problems is the biotechnological production of fatty acids through microorganisms like *S. cerevisiae* as schematically depicted in Figure 2.
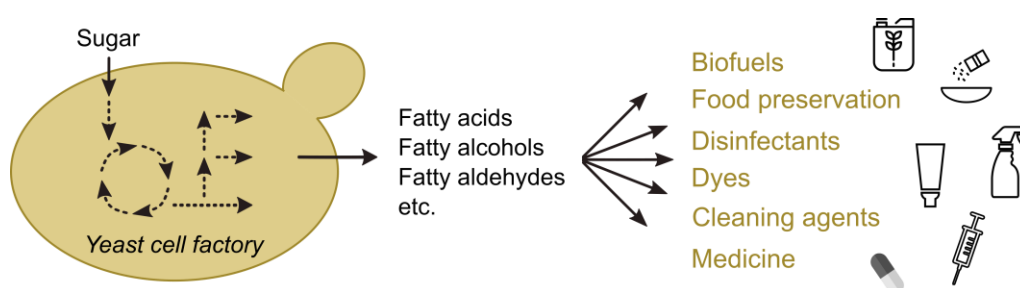
Figure 2: Biotechnological production of fatty acids and derivatives via yeast. Abundant, non-edible materials like lignocellulose can be used as sugar source for a microbial conversion to valuable compounds. Yeast is used as a "cell factory" that produces fatty acids, alcohols, aldehydes and other derivatives which find application in various consumer products.

A biotechnological approach allows for the production of highly specific compounds, thereby avoiding complex purification processes. Furthermore, fatty acids can be microbially converted to derivatives like fatty alcohols (biofuels) or other chemical compounds widely used in industry, e.g. fatty acid esters, aldehydes and alkanes/alkenes (Pfleger et al., 2015; Baumann et al., 2020b). Most

importantly, the microbial production approach can reduce the motivation for converting tropical rainforest into farmland, as non-edible waste materials can be used as starting material to feed the microbe (D'Espaux et al., 2017).

2.2.4 Octanoic acid production in *Saccharomyces cerevisiae*

Naturally, *S. cerevisiae* mainly produces C16 and C18 fatty acids with minor amounts of C14 and C26; but production of medium-chain fatty acids like octanoic acid is rare and therefore requires metabolic engineering. There are different strategies to establish short- and medium-chain fatty acid production in *S. cerevisiae*. Several studies have shown that expression of bacterial or human FAS systems combined with short- and medium-chain specific heterologous thioesterases, which cleave ACP-bound fatty acyl chains, enables increased release of short- and medium-chain fatty acids (Leber & Da Silva, 2014; Fernandez-Moya et al., 2015; Zhu et al., 2017). Another strategy is to manipulate chain-length control of the native yeast FAS, and relies on site-directed mutagenesis of the FAS-encoding genes. Gajewski et al. (2017) applied this approach to reduce the malonyl affinity of the MPT domain of Fas1. Reduced malonyl uptake supposedly favors the release of incompletely elongated acyl chains. This rational was successfully demonstrated by the exchange of an arginine by a lysine residue (R1834K) in the MPT domain, leading to production of short- and medium-chain fatty acids, in particular octanoic acid (Gajewski et al., 2017). Such a manipulated FAS enzyme is a good basis for an *S. cerevisiae* chassis strain for octanoic acid production.

To advance octanoic acid titers, several bottlenecks need to be addressed. These include the supply of adequate amounts of precursors, i.e. acetyl-CoA and malonyl-CoA, co-factors (NADPH), and energy (ATP), minimizing degradation of the product, and maximizing excretion of the product. Another issue that needs to be considered is the toxicity of octanoic acid for *S. cerevisiae*.

2.3 Tools for improving octanoic acid production in *Saccharomyces cerevisiae*

Key requirements for increasing octanoic acid production in *S. cerevisiae* is a detailed knowledge about the cell's metabolism as well as good tools for manipulation. Even though some knowledge has been gathered, there are still many unknows concerning the cell's metabolism during fatty acid production. Rational engineering to establish an octanoic acid production pathway in *S. cerevisiae* was already successful and some improvements in the biosynthesis could be achieved; however, classical tools have reached their limits and need to be complemented with new strategies to reach industry-scale titers and yields (Nielsen & Keasling, 2016; Baumann et al., 2020b).

2.3.1 Rational engineering

Rewiring of the cellular metabolism is accomplished traditionally through rational engineering, thereby shifting the flux towards a desired product, in this case medium-chain fatty acids. Rational engineering relies on a broad knowledge of the cellular network, enzyme kinetics, pathway regulation as well as possible bottlenecks. Commonly applied methods include knockout, overexpression and targeted mutation of metabolic genes (Woolston et al., 2013).

Significant efforts have been devoted to improving the precursor supply routes to provide carbon, energy and redox power and an extensive outline of strategies can be found in the attached publication in chapter 6.2 (Baumann et al., 2020b). Synthesis of the precursor acetyl-CoA in the yeast cytosol via the PDH bypass route is uneconomical for overproduction of fatty acids (van Rossum et al., 2016). Therefore, research has focused on establishing other, more energy-efficient routes for acetyl-CoA supply, e.g. by introducing heterologous pathways into *S. cerevisiae* – with mixed success rates (Baumann et al., 2020b). Acc1 has been another prominent target for rational engineering since it provides malonyl-CoA (chapter 2.1.1 and below). Apart from precursor supply, the FAS enzyme complex has also been rationally engineered. The beforementioned mutations in relevant domains (e.g. R1834K in the MPT domain) led to a drastically altered fatty acid profile. Furthermore, it was reported that a fused version of the α and β subunits of

FAS, namely fusFAS, showed superior performance (Wernig et al., 2020). Another common method is the overexpression of rate-limiting enzymes. For instance, the exchange of the native *FAS* promoters by the strong constitutive promoters *pTDH3*, *pTEF1* and truncated *pHXT7* increased final octanoic acid titers (Wernig et al., 2020).

To prevent octanoic acid degradation, i.e. chain length specific β-oxidation, gene knockouts were used. In particular knockout of *FAA2*, encoding a short- and medium-chain specific fatty acyl-CoA synthetase, led to strongly increased octanoic acid titers (Leber et al., 2016; Henritzi et al., 2018).

Two rational strategies have been employed to counteract octanoic acid toxicity on yeast. Octanoic acid leads to a disruption of the plasma membrane composition, causing membrane leakage and in certain concentrations cell death (Legras et al., 2010; Liu et al., 2013). These effects were to a certain extent reduced by altering the plasma membrane composition via enhanced oleic acid supply. The oleic acid content in the membrane was increased by external supply or via engineered Acc1^{S1157A}. The higher tolerance towards octanoic acid was attributed to an increase in the average chain length of membrane fatty acids and higher *cis*-monounsaturated fatty acid levels (Liu et al., 2013; Besada-Lombana et al., 2017).

Taken together, these rational approaches allowed to produce octanoic acid at titers ranging in the order of mg/L – amounts that are insufficient for an industrial-scale production. The applied methods are centered around an in-depth understanding of the fatty acid biosynthesis pathway as well as its associated fluxes and regulatory system. However, many genes and enzymes that are connected to fatty acid biosynthesis, degradation or robustness in the framework of global regulatory networks are unknown. Oftentimes, multiple enzymes are involved in a poorly understood manner in generating a certain phenotype. This impedes the use and impact of rational approaches (Woolston et al., 2013). To unravel the role of such enzymes and connections, combinatorial approaches and new strategies are necessary.

2.3.2 Transcriptomics

In a transcriptomics analysis the entirety of a cell's transcriptome is identified and quantified. This not only allows for a global analysis of gene expression at a snapshot in time but also of changes in the expression profile under different conditions. The method has become highly popular over the last years as a result of improved technology in combination with decreased costs. The prevalent technique used today is RNA-Seq, i.e. next-generation sequencing of the transcriptome. RNA-Seq analysis can elucidate the producer strain's physiology during production of the compound-of-interest and factors can be detected which were previously unknown to play a role in the biosynthesis (Wang et al., 2009; McGettigan, 2013). For instance, RNA-Seq analysis has been used to study *S. cerevisiae's* response to the production of fatty alcohols (Dahlin et al., 2019) and cyanobacterial response to free fatty acid production (Ruffing, 2013), revealing condition-specific responses of the host organisms.

Two transcriptome-wide studies analyzed a wild type strain's response to externally supplied octanoic acid. The strains were supplemented with 0.05 mM octanoic acid (7 mg/L) for 20 minutes and 0.3 mM octanoic acid (43 mg/L) until mid-log growth, respectively (Legras et al., 2010; Liu et al., 2013). The first study by Legras et al. (2010) revealed that the transporters Pdr12 and Tpo1 are involved in octanoic acid expulsion and that the yeast showed an oxidative stress response. The second study by Liu et al. (2013) suggested that octanoic acid supply leads to membrane leakiness and thereby cell disruption. The group demonstrated that oleic acid supplementation alleviated this toxic effect. Both studies revealed important response mechanisms to supplied octanoic acid, however, they did not analyze the transcriptomics of an *S. cerevisiae* octanoic acid producer, which might strongly differ.

2.3.3 Adaptive laboratory evolution

For microbial adaptive laboratory evolution (ALE), microorganisms are cultivated and growth is monitored in a controlled laboratory setting for long periods of time, usually in the range of several weeks to months. The cells are put under pressure by environmental stress factors like high temperature, low pH, or the presence of

toxic compounds, which reduce growth rates. Having to cope with this pressure, some cells develop improved phenotypes, which accumulate in the cell population during long term selection. The underlying basis of such phenotypic changes are genetic mutations and selection for improved traits (Dragosits & Mattanovich, 2013). The steps of an ALE experiment are schematically shown in Figure 3.
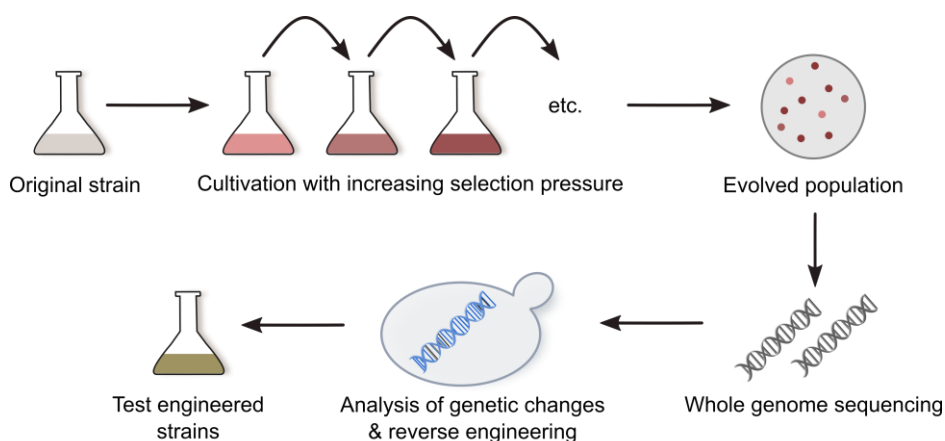
Figure 3: Adaptive laboratory evolution (ALE). A microbial strain is cultivated over several generations in serial shake flask cultures with a selection pressure such as increasing toxic compound concentration or decreasing pH. Evolved populations include individual cells with improved phenotypes. The genome of selected cells is sequenced, genetic changes identified and reversely engineered into the original strain, resulting in improved strain performance.

The common laboratory procedure for ALE is a serial batch cultivation in multiple shake flasks. An aliquot of the cell culture is transferred to a new flask with fresh medium at regular intervals and the serial transfers are repeated until a desired phenotype – usually improved growth – is observable. Eventually, single cells are extracted from the culture and genetic changes can be analyzed by whole genome re-sequencing. This allows for easy and rapid phenotype-genotype correlations. Common changes that can be observed after an ALE experiment are single-nucleotide polymorphisms (SNPs), small-scale insertions and deletions (indels) as well as deletions and duplications of larger genomic regions up to entire chromosomes. By reversely engineering these changes into the starting strain through classical genetic methods, identified targets can be validated, and a superior, more robust production strain can be generated (Dragosits & Mattanovich, 2013; Mans et al., 2018).

ALE has been used to select cells with an enhanced tolerance to an array of industrially relevant compounds. For instance, ALE of *E. coli* with high concentrations of octanoic acid resulted in evolved strains with changes in membrane composition and fluidity. These changes conferred enhanced tolerance to octanoic acid as well as increased production titers (Royce et al., 2015). ALE has also been performed with *S. cerevisiae*, for example for increased resistance to acetic acid (González-Ramos et al., 2016) and medium-chain alcohols (González-Ramos et al., 2013; Davis López et al., 2018). The artificial evolution of microorganisms in combination with trait analysis and subsequent reverse engineering of mutations into production hosts, is a vital tool for future cell factory development. It can enable yeast to catabolize substrates which are found in renewable resources like lignocellulose or to tackle toxicity issues which are encountered in the production of fatty acids and biofuels (Abatemarco et al., 2013; Mans et al., 2018). An inherent limit of ALE is that it relies on easily detectable phenotypes like increased growth. Although some phenotypes, like stress tolerance, are tied to growth, it is difficult to couple the production, and hence titers of molecules like fatty acids, to growth. Therefore, it is also necessary to develop additional screening and selection strategies to further improve fatty acid production (Nielsen & Keasling, 2016).

2.3.4 Biosensors and high-throughput screenings

To quantify fatty acids produced by a microbial strain, they must be extracted, undergo methylation to fatty acid methyl esters (FAMEs) and subsequently be analyzed by chromatography. Sample preparation for FAME quantification from each individual culture is a laborious, time-consuming procedure and analysis relies on low-throughput chromatography-based methods (Michener et al., 2012; Schallmey et al., 2014; Nielsen & Keasling, 2016).

An elegant solution to circumvent this issue is a biosensor. Biosensors can detect small molecules in the cell and respond by regulating host processes. Hence, biosensors can be utilized as devices for sensitive and specific small-molecule detection, thereby substantially speeding up detection and quantification. Genetic elements that are commonly used as biosensors are transcription factors (TFs). TFs respond to one or more inducing molecules by targeting promoters with

specific recognition sites, thereby dynamically regulating gene expression. When coupling TF-responsive promoters to a gene encoding for a fluorescent protein, the presence of a molecule is transformed into an easily detectable, quantifiable output. Such TF-based biosensors can be applied in metabolic engineering to live-monitor titers or even dynamically regulate production pathways (Schallmey et al., 2014; D'Ambrosio & Jensen, 2017). Concerning fatty acid production in *S. cerevisiae*, TF-based biosensors have been applied to monitor and regulate supply of the precursor malonyl-CoA. For this purpose, several research groups have adapted the bacterial FapR-*fapO* system to yeast, which enabled sensing of intracellular malonyl-CoA levels (Li et al., 2015; David et al., 2016). Another approach is the use of the bacterial transcriptional repressor FadR. FadR is a ligand-responsive TF that controls the expression of several genes involved in fatty acid biosynthesis, degradation and transport (Zhang et al., 2012). FadR represses transcription in the absence of long-chain fatty acids/acyl-CoAs. By coupling FadR-responsive operator sites to synthetic yeast promoters and a GFP-encoding gene, an *S. cerevisiae* biosensor mainly responsive to C14 fatty acid was generated (Teo et al. 2013).

Biosensors for intracellular molecules are valuable tools, especially to monitor pathway intermediates and to determine rate-limiting enzymes. These biosensors, however, do not serve for determining the overall produced amount of a compound when it is secreted from the cell. This is the case for many small molecules of industrial interest, including octanoic acid, which is readily secreted to the environment. To sense extracellular octanoic and decanoic acid, Mukherjee et al. (2015) expressed heterologous G-protein coupled receptors (GPCRs) in yeast. These receptors were linked to the yeast mating pathway for signaling and GFP expression as an output. Unfortunately, the linear and dynamic ranges of the biosensors were low and they were not proven to be functional in culture broth (Mukherjee et al., 2015), which would be indispensable for a useful biosensor.

Functional biosensors facilitate rapid and multiplexed phenotypic evaluation of cells. Therefore, biosensors can be used in high-throughput screenings of genetically diverse populations consisting of up to 10^9 clone variants to detect best-performing strains (Schallmey et al., 2014). The common experimental setup is

schematically shown in Figure 4. Strain libraries can be generated by a plethora of ways, for instance, by random mutagenesis with UV-radiation, error prone PCR, promoter shuffling of pathway genes, or plasmid cloning of genome-wide libraries (D'Ambrosio & Jensen, 2017).
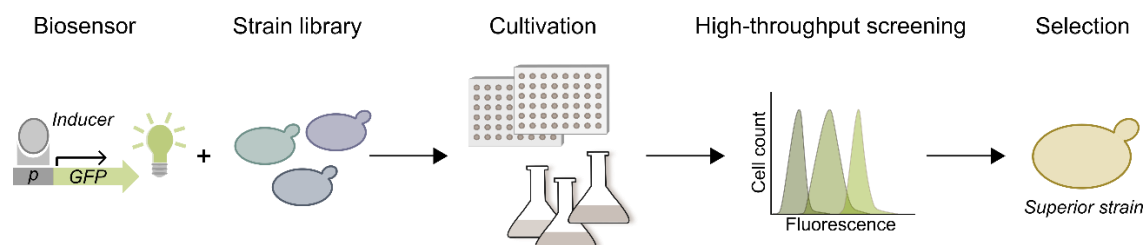

Figure 4: Application of biosensors for high-throughput screenings of strain libraries. High-throughput screenings in combination with biosensors allow for the identification of yeast strains with superior phenotypes among millions of strain variants. A biosensor consisting of an inducible promoter (p) coupled to *GFP* enables the quantification of small molecules by generating an easily detectable output (here: fluorescence). To identify strains with the desired property, strain libraries are cultivated in shake flasks or multiwell plates and tested in biosensor-aided flow cytometry screens.

For an efficient screening, cultivation and detection of strains must be high throughput. Massive parallel cultivation is achieved in multiwell plate formats in small volumes, or for bigger volumes in common shake flasks. For screening, the most ubiquitous and facile method is microfluidic cell sorting, in particular fluorescence-activated cell sorting (FACS). Biosensors that couple increased production of an intracellular metabolite with expression of fluorescent proteins like GFP, enable high-throughput FACS of strain libraries (Schallmey et al., 2014; Nielsen & Keasling, 2016). Following the sorting of highest fluorescent individual cells in a screening, chosen strains are further evaluated. The nature of the genetic element that is responsible for the desired phenotypic change, can be identified with classical molecular genetic methods. By reintroducing it into different producer strain backgrounds, positive effects can be evaluated and strain performance further optimized (Woolston et al., 2013). FACS screenings have been successfully employed recently for the identification of improved *S. cerevisiae* strains with higher xylose transportation capacity (Wang et al., 2014) and increased fatty acyl-CoA pools (Dabirian et al., 2019).

2.4　Aim of this thesis

The aim of this thesis was to optimize octanoic acid production in *S. cerevisiae* through development of new strategies as well as exploitation of classical approaches, and to investigate its physiological impact on the producing strain. Previous to this work, an *S. cerevisiae* strain was engineered, which has a fatty acid production spectrum that is shifted from long-chain fatty acids to primarily octanoic acid. This was achieved by rationally engineering the native FAS enzyme, generating the modified variant *FAS^{R1834K}* (Gajewski et al., 2017). This strain was to be used as a starting strain for further optimization.

To quantify octanoic acid produced by yeast, at the beginning of this thesis, it had to be extracted, methylated and analyzed via GC, which is a laborious and time-consuming work. For this purpose, a biosensor was to be developed that comes with a linear detection range within currently produced levels and an easily quantifiable output, ideally fluorescence. This biosensor should subsequently be used in a high-throughput screening of a whole genome multicopy plasmid library to detect improved strain variants. For such a screening, the biosensor would have to be modified and verified on single cell level in a FACS setup. The high-throughput FACS screening of the strain library would allow for detection of genes that when overexpressed increase octanoic acid titers of the producer strain.

The first transcriptomics analysis during fermentation of an *S. cerevisiae* octanoic acid producer strain was to be conducted. Such an analysis will give profound insights into the yeast's transcriptional response to octanoic acid production. Subsequent analysis of differential gene expression in comparison to a reference strain aims for identification of promising genetic targets for engineering.

Rational approaches, i.e. knockout, overexpression or mutation of genes encoding for enzymes involved in fatty acid biosynthesis, transport or transcriptional regulation were to be evaluated. With the aim to increase strain robustness, an ALE experiment should be conducted in parallel shake flasks in the presence of increasing octanoic acid concentrations and cell growth was to be monitored over prolonged fermentation time. Toxicity of octanoic acid on *S. cerevisiae* strains used in industrial fermentations was to be evaluated to identify future production hosts.

3 General discussion

The first goal of this thesis was to develop and apply new strategies to improve octanoic acid production in yeast. Secondly, the aim was to generate further understanding of the physiology of the yeast during octanoic acid production. In the following subchapters, the results of the attached publications and additional results section will be discussed in a wider context, i.e. how they can be expanded, and by which tools they can be complemented to guide future strain engineering efforts. Furthermore, it will be discussed which factors restrict octanoic acid production in yeast to date and how to overcome them.

3.1 Octanoic acid biosensor and high-throughput screenings – achievements and opportunities

This thesis contains one publication (chapter 6.1; Baumann et al., 2018) that describes the development of the first TF-based biosensor for screening of short- and medium-chain fatty acid production. Our first attempt had been to use a previously published GPCR-based octanoic acid biosensor (Mukherjee et al., 2015), improve its low linear and dynamic range and make it functional in culture broth. However, it was not possible to reproduce the published results (data not shown), which led us to develop an entirely new biosensor system for octanoic acid detection.

As depicted in chapter 2.3.4, TF-based biosensors enable facile and rapid detection of small molecules through combination with an output such as fluorescence. A biosensor based on the *pPDR12* promoter was developed, which is regulated solely by the TF War1. A quantifiable output was achieved by coupling *pPDR12* to GFP expression. The biosensor responded to C6, C7 and C8 fatty acids, respectively, in a concentration-dependent manner over high linear and dynamic ranges. It was able to reliably detect 0.01 mM up to 2 mM octanoic acid and generated an up to 10-fold increase in fluorescence. Furthermore, this is the first biosensor that reliably quantified octanoic acid in culture supernatants of a producer strain – and is even functional in complex medium. This was confirmed by transforming a non-producer strain with a multicopy plasmid containing the biosensor and incubating this biosensor strain with culture supernatants containing

different amounts of octanoic acid (chapter 6.1, Baumann et al., 2018).

The application of the biosensor is not restricted to screen octanoic acid producer strains. For instance, it can be applied to screen producer strains of fatty acids of shorter chain lengths (C6, C7) to which it is also responsive (Holyoak et al., 1999; Hatzixanthis et al., 2003; Baumann et al., 2018). C6 and C7 fatty acids are technologically relevant, for example as precursors for biofuels (Peralta-Yahya et al., 2012). Furthermore, the development, optimization and verification process of the biosensor can serve as a model for similar approaches for other industrially relevant compounds.

As a complimentary future strategy, it could also be of interest to screen for clones with an increased fatty acid precursor supply. Sufficient precursor supply is a common bottleneck in microbial pathways. To detect *S. cerevisiae* strains with increased malonyl-CoA levels, another established biosensor system could be utilized. It was developed based on the bacterial TF FapR-*fapO* system which allows for sensing of intracellular malonyl-CoA levels (Li et al., 2015; David et al., 2016). David et al. (2016) established a system in which the biosensor detects malonyl-CoA accumulation and thereupon induces expression of a heterologous pathway which uses malonyl-CoA for 3-hydroxypropionate production. Li et al. (2015) successfully adapted the FapR-*fapO* biosensor system for use in a FACS screening of a genetic library and detected two new targets that, when overexpressed, increased 3-hydroxypropionte levels. The application of this biosensor to screen an octanoic acid producer strain library for increased malonyl-CoA levels would have to be considered carefully though. It has been shown that elevated malonyl-CoA levels do not necessarily lead to higher octanoic acid titers. On the contrary, enhanced malonyl-CoA levels (generated by expression of mutated *ACC1* variants) have favored chain elongation of medium-chain fatty acids (Choi & Da Silva, 2014; Zhou et al., 2016; Besada-Lombana et al., 2017; Zhu et al., 2020), leading to an increase in long-chain fatty acids.

Despite the necessity for high-throughput screening techniques, to date, biosensors are one of the very rare devices for efficient strain analysis. Recently, an alternative method has been reported for high-throughput quantification of the fatty acid composition in yeast cells (Xue et al., 2020). It is based on the

observation that produced fatty acids of different chain lengths are proportionally present in phosphatidylcholines of yeast membrane lipids. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) measurement of yeast colonies allowed for analysis of the fatty acid spectrum produced by individual strains within about 2 seconds per sample. Verification of the method was achieved by screening 288 colonies of a *FAS2* (KS domain) mutant library combined with automated peak analysis via a Python script. The setup allowed for identification of strain variants producing medium-chain fatty acids in varying ratios (Xue et al., 2020). This method could be an interesting alternative to the use of the *pPDR12-GFP* biosensor to measure octanoic acid producer strains as it is comparable regarding speed, throughput and accuracy, and comes along with easy sample preparation. However, a major drawback is the need for very costly equipment. The choice of method therefore also depends on the resources and equipment at hand.

The successful development of the *pPDR12-GFP* octanoic acid biosensor enabled us to conduct a high-throughput screening to identify new targets that increased octanoic acid production, as described in the attached manuscript in chapter 6.4 (Baumann et al., 2020a). For use of the *pPDR12-GFP* biosensor in the envisioned screening, some aspects were modified. A combined biosensor and octanoic acid producer strain was constructed by genomically integrating *pPDR12-GFP* in a producer strain. The genomic integration of the biosensor drastically decreased the single-cell noise in comparison to expression from a multicopy plasmid. Additional knockout of *FAA2* (chapter 2.3.1) and integration of a positive feedback loop ($\Delta pWAR1::pPDR12$) led to a strain amenable to high-throughput screening via FACS.

The FACS screening was designed to include consecutive enrichment rounds to reduce the rate of false positive strains. Eventually, the screening of a gene overexpression library resulted in the detection of two new targets, *Kcs1* and *Fsh2*, that had never been described to be involved in fatty acid metabolism and that, in combined overexpression, led to a 55 % increase in octanoic acid titers (chapter 6.4, Baumann et al., 2020a). To date, the role of the enzyme encoded by *FSH2* remains obscure. However, Fsh1, which is about 25 % homologous, plays a role

in lipid metabolism, and *FSH1* overexpression was shown to favor the production of free fatty acids (Ramachandran et al., 2020). Therefore, it can be speculated that *FSH2* also plays a – yet unknown – role in lipid metabolism. *KCS1* encodes an inositol pyrophosphate synthase that phosphorylates different inositol phosphates, which are important signaling molecules in the cell (Saiardi et al., 1999, 2000; Dubois et al., 2002). A recent publication showed that *KCS1* knockout affects S-adenosyl-L-methionine, glycolytic intermediates' and ATP levels (Chen et al., 2021). Therefore, it is likely that *KCS1* overexpression affects glycolysis, leading to increased octanoic acid titers. Further analyses, e.g. transcriptomics or proteomics analyses, with *FSH2* and *KCS1* overexpression strains could be conducted to unravel the exact molecular mechanisms.

The methodology of the performed high-throughput FACS screening has been developed with diverse applications in mind. Firstly, the developed method can be used to screen various genetic libraries. The library that was chosen consisted of the entire yeast genome cloned on multicopy plasmids with native promoter and terminator regions comprising of 1588 plasmids in total (Jones et al., 2008). This library enables the overexpression of genes under control of their native regulatory sequences; however, it must be considered that several genes are contained on each plasmid. To identify single genes responsible for an observed phenotype laborious cloning and testing must be conducted. To circumvent this, a library consisting of only one gene per plasmid would be advantageous. An example for this is the Molecular Barcoded Yeast Open Reading Frame (MoBY-ORF) collection which comprises 4956 genes (Ho et al., 2009). The MoBY-ORF library is composed of centromeric plasmids, each carrying a single yeast ORF flanked by endogenous promoter and terminator sequences. These sequences are flanked by unique oligonucleotide barcodes which facilitate the correlation of an interesting phenotype to the responsible gene. The usability of the MoBY-ORF library was demonstrated recently in a FadR biosensor-assisted screening for genes enhancing fatty acyl-CoA pools, eventually resulting in increased fatty alcohol levels (Dabirian et al., 2019). To identify genes, library plasmids are purified, and barcodes sequenced, which makes the use of this library very straightforward. Despite the advantages of the MoBY-ORF library, both described libraries have the same scope as they comprise the native yeast genome.

Another interesting approach could be the screening of a library of mutated variants of pathway genes. For instance, analogous to the abovementioned MALDI/ToF MS screening of *FAS2* KS domain variants by Xue et al. (2020), a library of mutated variants of *FAS1* could be screened. The MPT domain of *FAS1* would state a promising target for mutation as it plays an eminent role in the loading of malonyl-CoA elongation units as well as the release of elongated fatty acyl-CoA chains (chapter 2.2.4). Rational engineering or epPCR could be employed to generate a library of mutated *FAS1*-MPT domain variants. In a *pPDR12-GFP* biosensor-assisted mutant library screening, new versions could be detected that lead to enhanced release of medium-chain acyl-CoAs. Thereby, new enzyme variants with superior properties to the already known variant *FAS1^{R1834K}* (Gajewski et al., 2017) could be detected.

For screening strain libraries, single cell flow cytometry in combination with cell sorting, i.e. FACS, is the predominant method of choice. This is mainly due to widespread equipment availability and rapid and ultra-high throughput measurements (Dietrich et al., 2010). However, single cell FACS screens are based on the assumption that the production capacity of a cell is proportional to the intracellular content. For compounds that are mostly released from the cell via passive diffusion, a FACS screening is likely effective. For compounds that are mostly excreted by active transport mechanisms, however, FACS may favor the selection of transport-deficient cells that retain the product intracellularly (Wagner et al., 2018). The mode of release of octanoic acid is not entirely clear yet but it is likely secreted from the cell by a mixture of passive diffusion and active transport (Legras et al., 2010; Leber & Da Silva, 2014; Borrull et al., 2015). Preliminary FACS experiments revealed a correlation between intra- and extracellular octanoic acid amounts. It was shown that strains with higher octanoic acid titers in the supernatant (determined by GC) also showed a higher biosensor activation as measured via single cell fluorescence (Baumann et al., 2020a, chapter 6.4). Based on these observations it was concluded that a FACS screening is suitable to detect strains with increased intra- *and* extracellular octanoic acid titers.

Nevertheless, due to the toxicity of octanoic acid on the yeast cells (Viegas et al., 1989; Borrull et al., 2015), it is highly desirable to have cells with a high secretion

rate. Therefore, the development of screenings that detect strains with increased octanoic acid secretion is worthwhile considering. For this aim, the extracellular amount of the compound needs to be quantified. This could be achieved, for instance, in a two-cell system with separate cultivation of the producer and biosensor strains in microtiter plates. Supernatants of the producers are added to the biosensor strain and fluorescence evaluated in e.g. multiwell plate readers. Despite a lower throughput in comparison to single cell FACS, an advantage of this method is that the limits of the linear detection range can be circumvented by diluting the samples. For a microtiter plate screening, strain cultivation, handling, and metabolite analyses need to be performed on automated robotic platforms to reach the aspired throughput. However, the lack of such facilities is often an obstacle for realizing this type of screening (Dietrich et al., 2010).

An interesting alternative that circumvents the need for these facilities, is a droplet-based flow cytometry screening. In such a two-cell system, producer and biosensor cells are encapsulated and cultivated in microdroplets, e.g. alginate capsules. The compound-of-interest is synthesized by the producer cell, secreted into the capsule, and consequently taken up by the biosensor cell (Meyer et al., 2015; Wagner et al., 2018). Meyer et al. (2015) applied this approach to identify *Bacillus subtilis* cells with improved vitamin B production. For the screening of the *B. subtilis* strain library, it proved to be essential to use a biosensor strain with an orthogonal nutrient requirement. This prevented competition between producer and biosensor cells for growth substrates. Furthermore, the microcapsules that contained the biosensor strain and the *B. subtilis* library, were embedded in a hydrophobic solvent to minimize crosstalk between library strains. The resulting readout, biosensor fluorescence, was evaluated via droplet FACS analysis. The advantage of this method is that it can quantify secreted molecules in very high throughput. In the framework of this project, this method was evaluated for a biosensor strain in combination with octanoic acid producer cells (data not shown). Preliminary tests revealed that the hydrophobic solvent used to embed the microcapsules did not prevent crosstalk between library strains. Due to the molecular characteristics of octanoic acid, it was impossible to generate an appropriate (hydrophobic) solvent and therefore this screening approach was not pursued further.

Despite various challenges regarding biosensor and screening development, many successful examples – including this work – have shown the potential of the technology, which is predicted to drastically facilitate metabolic engineering in the upcoming years (Nielsen & Keasling, 2016).

3.2 *Saccharomyces cerevisiae* physiology during octanoic acid production

One of the aims of this project was to address the lack of knowledge of the producer strain's physiology during octanoic acid production. In the attached publication Baumann et al. (2021) in chapter 6.3 this issue was addressed by conducting the first transcriptomics analysis of an octanoic acid producer strain, SHY34/*fusFAS*^{RK}, in comparison to a reference strain, SHY34/*fusFAS*^{WT}. The transcriptomic landscape was analyzed at three sampling times during octanoic acid production and compared to literature data of the transcriptomic response of wild type strains to supplemented octanoic acid. Thereby, a new target, *RPL40B*, plasmid-based overexpression of which led to a 40 % increase in octanoic acid titers, was identified. This was the first report of an involvement of *RPL40B*, which encodes for a subunit of the ribosome, in fatty acid biosynthesis (chapter 6.3, Baumann et al., 2021). It would be of great interest to investigate this further, e.g. by analyzing knockout or knockdown mutants of *RPL40B* or of its paralog *RPL40A*. To gain insight into the mechanism behind this effect, proteome analyses or ribosome profiling, so-called RiboSeq (Weinberg et al., 2016), could be performed.

Another interesting finding of the RNA-Seq experiment was that the (plasmid-based) expression of *fusFAS*^{RK} was much higher than of the wild type analogue *fusFAS*^{WT}. Chain length control of the modified *fusFAS*^{RK} is leaky enabling synthesis of long-chain fatty acids (Gajewski et al., 2017), which are essential for instance as components of membranes and energy supply (Klug & Daum, 2014). Whilst the majority of fatty acyls is released from the FAS^{RK} complex when reaching a chain length of C8, a fraction of the fatty acyls is extended with malonyl-CoA units to generate long-chain fatty acids. The RNA-Seq results suggest that the gene expression of SHY34/*fusFAS*^{RK} is probably not optimized for maximum octanoic acid production but rather the synthesis of enough long-chain fatty acids for

survival. Another factor that limits titers is the cell's utilization of fatty acid precursors for other metabolic processes (van Rossum et al., 2016). This competition for cellular resources might explain the limit in octanoic acid production that was observed in various experiments, stressing the importance of also improving precursor supply – as reviewed in chapter 6.2 (Baumann et al., 2020b).

A crucial conclusion of the transcriptomic analysis was that increasing octanoic acid titers correlated with increased differential gene expression and therefore seemed to influence overall strain physiology. The effect of overexpression and deletion, respectively, of all genes that were up- or downregulated at more than one sampling time was evaluated. Interestingly, the majority thereof did not have any effect on octanoic acid titers or growth. The differential expression of these genes during octanoic acid production therefore seems to serve other purposes, e.g. to cope with octanoic acid toxicity. This would be in accordance with another finding of the RNA-Seq experiment, i.e. negative effects of octanoic acid on the producer cell's growth. The final OD of the producer strain remained lower than that of the reference strain. The toxicity of octanoic acid on different *S. cerevisiae* strains was also observed in separate experiments (chapter 5.1.1). Strategies that could mitigate the toxic effects will be discussed in the following subchapter.

3.3 Strategies to increase strain robustness and reduce octanoic acid toxicity

Increasing the robustness of *S. cerevisiae* to octanoic acid is vital for generating a high performing producer strain. The mechanisms of octanoic acid toxicity and transport are not entirely elucidated yet. Octanoic acid that is produced by the yeast cell is readily secreted, likely by a mixture of passive diffusion and active transport (Legras et al., 2010; Leber & Da Silva, 2014; Borrull et al., 2015). At low media pH, which is the common condition in industrial processes, octanoic acid is highly toxic because it is mostly present in its undissociated form which presumably can easily enter the cell by passive diffusion. In the cytosol of the cell, it dissociates, leading to a drop in intracellular pH and an accumulation of toxic anions. To counteract, the cell activates plasma membrane H^+-ATPases which pump out excessive protons (Viegas et al., 1998). In addition to this mechanism, octanoic acid, due to

its hydrophobic character, incorporates into the membrane. This disturbs membrane integrity and composition and its essential function as transport system and selective barrier. Eventually these impairments lead to membrane leakage and cell death (Viegas et al., 1998; Cabral et al., 2001; Legras et al., 2010; Liu et al., 2013).

Legras et al. (2010) analyzed a wild type strain's response to octanoic acid supplementation by transcriptomics analysis and subsequent study of knockout mutants. They identified oxidative stress as well as the transporter Tpo1 and the transporter-regulating TFs War1 and Pdr3 to be minorly involved in adaptation to the acids. The main role in yeast resistance to octanoic acid was ascribed to the transporter Pdr12. In the framework of this project, different strategies were used aiming to increase yeast robustness, including rational engineering of such reported targets (chapter 5.1.3). Despite the reported decrease in octanoic acid resistance for the knockout mutants Δ*pdr12*, Δ*tpo1*, Δ*war1* and Δ*pdr3* (Legras et al., 2010), overexpression of neither of these genes increased growth of a wild type strain in the presence of octanoic acid (Figure 9). Possible reasons for this are discussed in the additional results chapter 5.1.3 in detail.

As this rational engineering strategy was unsuccessful in generating a robust strain, an ALE experiment was conducted. However, the improved phenotype of an evolved population was not reproducible from isolated single cells, as shown in the additional results section (chapter 5.1.2). The engineering of targets reported from an ALE experiment from another group (Zhu et al., 2020) did also not result in an improved phenotype in our producer strain (Figure 8). Despite ALE being a successful instrument in many cases to improve yeast strain robustness e.g. to aromatic acids at low pH (Pereira et al., 2020), acetic acids (González-Ramos et al., 2016), or high temperatures (Caspeta et al., 2014), it doesn't seem to be the ideal method to elevate yeast tolerance to high levels of octanoic acid. When performing ALE with *E. coli* strains with high octanoic acid concentrations, a strain with strongly increased tolerance that was able to grow in the presence of up to 30 mM (=4.3 g/L) octanoic acid was generated (Royce et al., 2015). It is important to mention, however, that these experiments were conducted at neutral pH, when octanoic acid toxicity is much lower than at acidic pH (Viegas & Sá-Correia, 1997).

The increased tolerance of the *E. coli* strain was mainly attributed to changes in the bacterial membrane integrity and rigidity and an increase in the average lipid tail length (Royce et al., 2015; Chen et al., 2020). Such changes cannot be transferred one-to-one to yeast due to differences in membrane architecture and composition. There have been several attempts to engineer the yeast membrane for increasing its stress tolerance, which have recently been summarized (Qi et al., 2019). A beforementioned example (chapter 2.3.1) for this strategy is the modification of *ACC1*. Expression of a modified version, *ACC1^{S1157A}*, was reported to result in an enhanced oleic acid content in the membrane, conferring the strain with higher robustness to octanoic acid and even leading to higher titers in a production strain (Liu et al., 2013; Besada-Lombana et al., 2017). In our experiments, however, a strain overexpressing *ACC1^{S1157A}* did not show a change of growth in the presence of octanoic acid, possibly due to masking effects of the native *ACC1* gene expressed from the strain background. Despite these contradictory results, it cannot be excluded that membrane engineering could be a beneficial complimentary strategy for generating a more robust production strain.

Despite some advances, current robustness levels would be insufficient for an industrial-scale production of octanoic acid via yeast. An octanoic acid market report estimates that titers need to reach at least 60 g/L for an economically feasible production at competitive prices (personal communication, H. Marckmann, Clariant). Considering the limited success of the above stated attempts for increasing robustness, it is unlikely that laboratory yeast strains can be generated for the required tolerance level. The primary optimization of the production strain for increasing titers should certainly be conducted in laboratory strains due to their ease in handling and genetic modification. The following steps should then be conducted in an inherently more tolerant strain. For determining the tolerance levels of different strains, toxicity tests with five *S. cerevisiae* strains in different media and growth conditions at an initial pH around 6.5 were conducted (Figure 5, Figure 6 in chapter 5.1.1). Hereby, the total inhibitory concentration was shown to differ widely between 400 and 800 mg/L octanoic acid. All four tested industrial strains were more robust than the laboratory strain CEN.PK2-1C, and even more so, when preadapted to octanoic acid.

As the tolerance levels of the evaluated industrial strain is still not high enough, in the next step, the biosynthesis could be coupled to a product detoxification step. One possibility is an *in situ* product removal in a two-phase fermentation with an appropriate solvent (Henritzi et al., 2018; Zheng et al., 2020). Henritzi et al. (2018) added dodecane to an octanoic acid production strain and achieved an increase in overall titers, i.e. in the aqueous and dodecane phase, by about 40 %. Nevertheless, only a fraction of the produced octanoic acid was sequestered by dodecane. Hence, for an efficient *in situ* extraction of octanoic acid, other solvents would need to be evaluated.

An alternative strategy to detoxify octanoic acid is the transformation of octanoic acid into a non-toxic derivative. For instance, octanoic acid could be captured in the form of nonpolar storage lipids like triacylglycerols (TAGs). TAGs, which are stored in lipid droplets in the cell, can serve as a sink for free fatty acids, thereby avoiding toxic and membrane-disturbing effects (Friedlander et al., 2016). The activated form of octanoic acid, octanoyl-CoA, could be incorporated into TAGs by heterologous, medium-chain specific enzymes, which have been discovered in plants and fungi (Lardizabal et al., 2002; Aymé et al., 2015; Reynolds et al., 2019; Yuan et al., 2019). The captured fatty acids could be retrieved from TAGs by activation of lipases in a late phase of fermentation when toxic effects of free fatty acids on growth are negligible. A specific lipase or an endogenous lipase with a broad product spectrum including medium-chain acyl moieties, such as Tgl3 (Athenstaedt & Daum, 2003; Schmidt et al., 2013), could be used for this purpose. Cloning Tgl3 under control of an inducible promotor, would enable controlled activation at a late fermentation phase, thereby releasing octanoic acid.

3.4 Future perspectives for octanoic acid production in *Saccharomyces cerevisiae*

Octanoic acid is applied in a wide range of products and its market demand has constantly increased over the last years (Yan & Pfleger, 2020; IndexMundi, 2020). The biotechnological production in *S. cerevisiae* offers a promising alternative to current production methods. The basis for this approach has already been created but the strains must be further improved.

An *S. cerevisiae* production strain based on the FAS^{RK} variant in combination with a knockout of medium-chain specific β-oxidation (Δ*faa2*) was used as a basis for initial experiments. The overexpression of the genes *RPL40B*, *FSH2* and *KCS1* that were identified in the framework of this thesis, improved production titers, however, their exact role in fatty acid metabolism remains to be unraveled. The developed biosensor and screening strategy present a means to screen various genetic libraries for identification of additional targets which can enhance octanoic acid production. For an efficient production, an improved precursor supply and balanced cofactors are vital and can be engineered based on knowledge from previous research as summarized in the publication in chapter 6.2 (Baumann et al., 2020b). Once a well-performing laboratory strain is established, the strain development could be transferred to an inherently more tolerant strain like EthanolRed, including overexpression of the three here identified targets. In addition, it would be of great interest to evaluate yeasts like *Y. lipolytica* as host organisms (Beopoulos et al., 2009; Friedlander et al., 2016). *Y. lipolytica* is an oleaginous yeast that naturally accumulates large amounts of lipids and fatty acids and could therefore be an interesting host for octanoic acid production (Beopoulos et al., 2009). Irrespective of the host organism, due to the toxicity of octanoic acid, production will have to be coupled to a detoxification strategy, such as sequestration in non-toxic TAGs.

Ideally, the sugar substrate for yeast to produce octanoic acid could derive from inexpensive, renewable resources, e.g. non-food bioenergy crops or agricultural waste (Gustavsson & Lee, 2016; D'Espaux et al., 2017). Such lignocellulosic biomass commonly contains, in addition to glucose, the pentose sugars xylose and arabinose, which cannot naturally be fermented by yeast. For an efficient utilization of all sugars, *S. cerevisiae* needs to be genetically engineered, a challenge that has been addressed extensively and with some success (Laluce et al., 2012). Despite considerable progress in this field as well as in the engineering of yeast for fatty acid production, major improvements in octanoic acid titer and yield are necessary to advance to a sustainable and competitive large-scale production.

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5 Additional Results

5.1 Additional results and discussion – Strategies to increase *Saccharomyces cerevisiae* robustness

Octanoic acid has previously been shown to exert toxic effects on yeast. The toxicity of octanoic acid is attributed to different mechanisms. Octanoic acid is a lipophilic weak acid that crosses the yeast plasma membrane by passive diffusion in the protonated state or active transport and can dissociate in the cytosol. This leads to a decrease of pH in the cytosol and an accumulation of toxic anions. Furthermore, octanoic acid disturbs the membrane's organization and integrity. This perturbs the transport system of the membrane and its function as barrier (Viegas et al., 1998; Cabral et al., 2001; Legras et al., 2010; Liu et al., 2013).

Several strategies have been tested to counteract toxicity and increase robustness and will be described in the following. The toxicity of octanoic acid on a variety of *S. cerevisiae* strains was evaluated and ALE with the aim of increasing strain robustness was performed. In addition, rational engineering of targets via overexpression and knockout was evaluated regarding effects on tolerance.

5.1.1 Evaluation of octanoic acid toxicity on *Saccharomyces cerevisiae* strains

To assess growth inhibition, five *S. cerevisiae* strains were supplied with a range of octanoic acid concentrations under different conditions. The common haploid laboratory strain CEN.PK2-1C and four diploid industrial strains, i.e. IndSc-1, IndSc-2, IndSc-3, and EthanolRed, were tested. Firstly, pre-cultures of all strains were inoculated in triplicate shake flask cultures in YPD. In addition, cultures of all strains were preadapted to 200 mg/L octanoic acid in two consecutive rounds of overnight cultures in YPD. For evaluating growth inhibition, all shake flask cultures were cultivated until exponential growth phase was reached, diluted in fresh media and incubated with 0-800 mg/L octanoic acid in a 96-well plate without shaking. After 20 h, the OD_{600} was measured in a platereader (Figure 5).
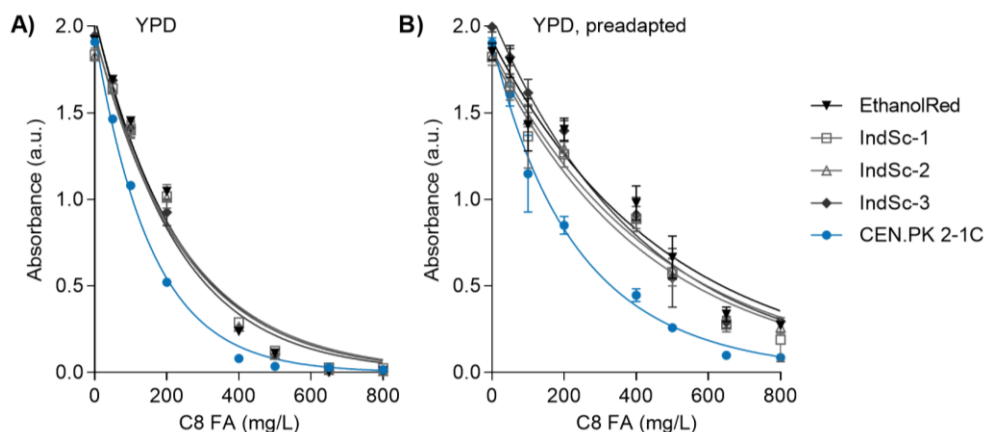
Figure 5. Assessing octanoic acid toxicity on industrial *S. cerevisiae* strains. Strains were inoculated to an OD_{600} of 0.05 and supplied with octanoic acid in (A) YPD or (B) YPD with two rounds of preadaptation to 200 mg/L octanoic acid before the test. Growth was measured by absorbance after 20 h (no agitation) in a platereader. $n = 2$, error bars = ± standard deviation. Growth curves estimated with nonlinear regression, $R^2 > 0.95$.

From this experiment several important conclusions could be drawn. Firstly, a preadaptation to octanoic acid led to better growth of all strains. Preadaptation to octanoic acid has previously been shown to be beneficial. It was suspected that protective changes in the membrane had occurred during adaptation and were responsible for decreased toxicity (Liu et al., 2013). Secondly, all industrial strains grew better than the laboratory strain CEN.PK2-1C under both conditions. Thirdly, octanoic acid inhibition is dose dependent. Increasing octanoic acid concentrations led to decreased growth. For non-adapted strains, 400-600 mg/L octanoic acid led to total growth arrest in YPD and for preadapted strains in YPD, 800 mg/L octanoic acid led to almost entire growth arrest.

To confirm that differences in growth not only occur in non-shaking conditions, CEN.PK2-1C and EthanolRed were cultivated in shake flask cultures in YPD medium supplemented with 0, 100, 200 and 400 mg/L octanoic acid, respectively, for 40 h with shaking on a cell growth quantifier (Aquila Biolabs). EthanolRed was chosen to represent the industrial strains as in the first experiment no difference between them had been observable. Again, better growth of EthanolRed than CEN.PK2-1C was observable under all octanoic acid concentrations and even without octanoic acid challenge (Figure 6).
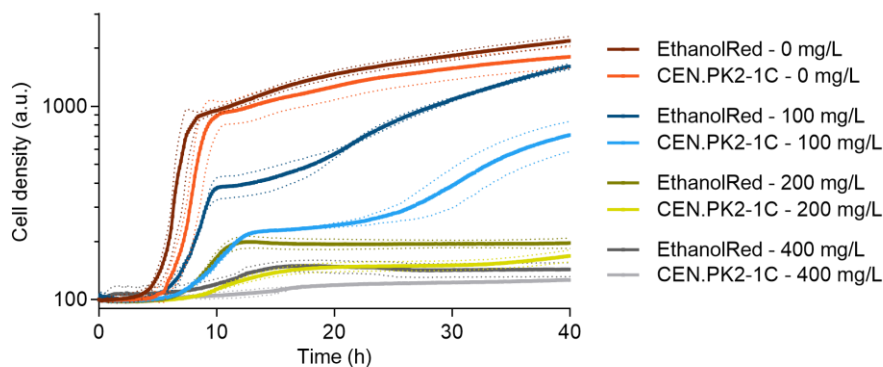
Figure 6. Assessing growth of CEN.PK2-1C and EthanolRed under octanoic acid challenge. Strains were inoculated to an OD$_{600}$ of 0.1 in YPD medium in shake flasks and supplied with octanoic acid. Growth was measured continuously by a cell growth quantifier over 40 h. $n = 3$, error bars = ± standard deviation.

In summary, *S. cerevisiae* industrial strains were more robust to octanoic acid than the haploid CEN.PK2-1C laboratory strain. However, creating and analyzing genetic modifications as well as general handling are much easier with haploid strains. For this reason, CEN.PK2-1C is commonly used in primary experiments to establish a production pathway and evaluate molecular modifications. Once a good laboratory strain has been established for producing high titers, the genetic modifications can be transferred to an industrial strain such as EthanolRed.

5.1.2 Adaptive laboratory evolution and engineering of identified target genes

ALE is a useful method to increase a strain's robustness against a toxic compound. This method is introduced in chapter 2.3.3. ALE is usually achieved by exposing the cells to increasing concentrations of the compound-of-interest in serial batch cultures. A slight variation of this procedure is to alternate the cultivation cycles in the presence and absence of the compound, which was shown to confer constitutive tolerance (González-Ramos et al., 2016). Both approaches were tested with the aim to generate a yeast strain that is more robust to octanoic acid. As a starting strain, a prototrophic *S. cerevisiae* strain was used in which β-oxidation was knocked out by deletion of *POX1*. Without preventing the degradation of fatty acids, it is likely that one would evolve and select for strains that grow better in the presence of octanoic acid due to enhanced degradation.

The resulting strain LBY3 (CEN.PK113-7D Δ*pox1*) was cultivated in YPD medium spiked with 525 mg/L octanoic acid in triplicates for each of the two approaches (steadily increasing/ alternating concentrations) over three months (approximately 60 generations). Cultures were transferred to fresh media every 2-4 days and octanoic acid concentrations were increased three times over the course of the experiment (Figure 7A). An aliquot of each of the six final cultures as well as the original strain LBY3 were inoculated in fresh YPD medium and cultivated in the presence of 650 mg/L and 750 mg/L octanoic acid, respectively (Figure 7B).

The increased growth rate of all cultivated strains seemed to suggest that they had evolved for faster growth in the presence of high octanoic acid concentrations. For the next verification step, single colonies were isolated from the evolved cultures. This step is essential for whole genome sequencing, to be able to pinpoint genetic changes responsible for the improved phenotype. Several colonies were isolated from each culture and cultivated again in the presence of 650 mg/L octanoic acid. The growth curves are shown for exemplary colonies in Figure 7C. Only about half of all colonies showed higher growth rates than the parental strain and the difference between evolved colonies and the parental strain was much smaller as for the mixed cultures and mostly not significant.

Possibly, the mixed cultures contained cells with different genotypes that complemented each other and thereby enabled increased growth rates. However, it would be uneconomical to screen and sequence dozens to hundreds of single colonies to get a picture of the genotypic changes present in all six mixed cultures. Therefore, the focus was set on an alternative strategy.
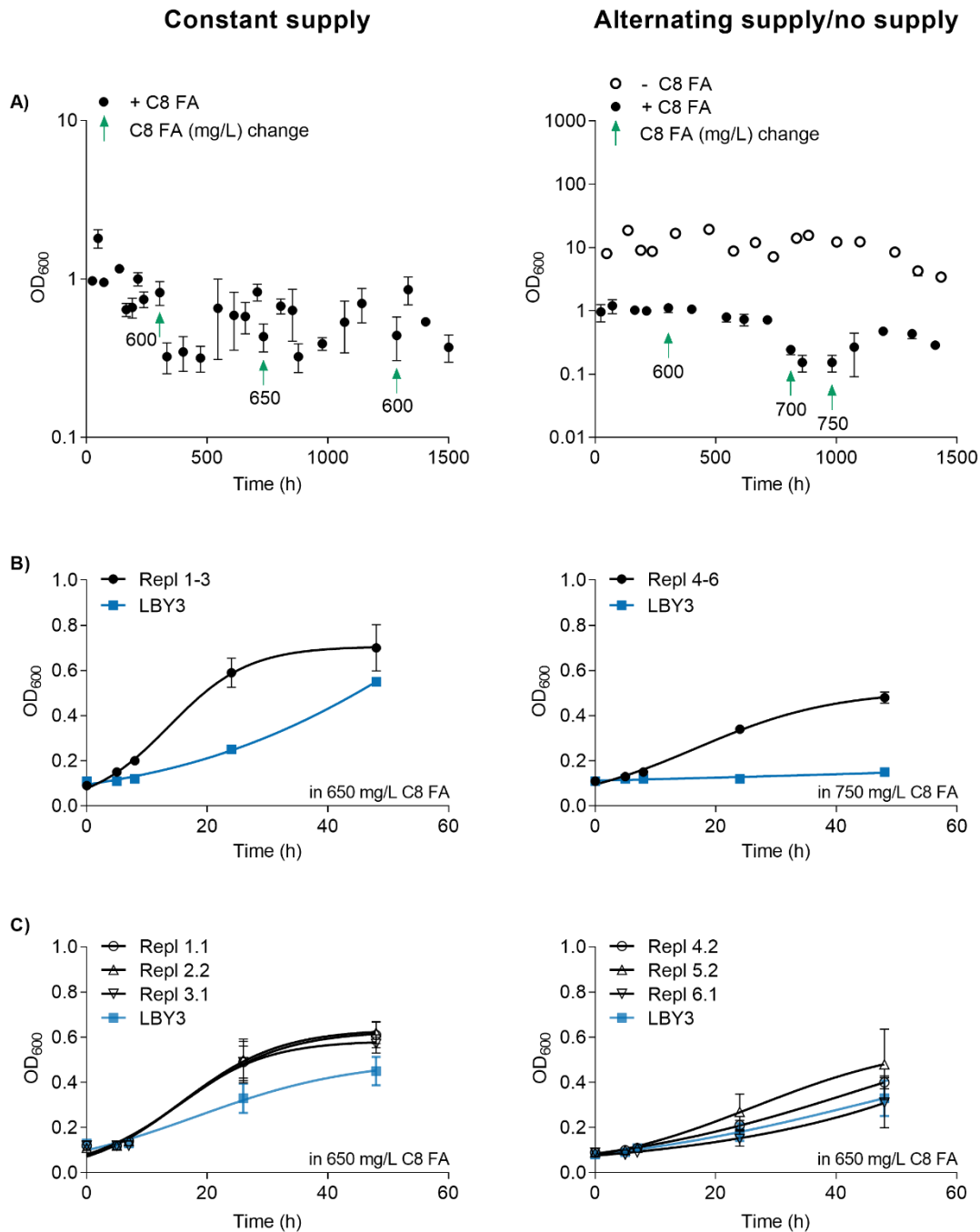
Figure 7. Adaptive laboratory evolution procedure and assessment of evolved strains. ALE was performed with strain LBY3 (CEN.PK113-7D Δ*pox1*) under two conditions with three replicates (Repl) each: Constant octanoic acid supply (left panel) and an alternating approach (right panel). (A) Both experiments were performed in YPD medium with a starting concentration of 525 mg/L octanoic acid which was adjusted over the course of ALE as indicated with arrows. (B) Evolved mixed cultures from the six parallel flask cultivations (Replicates 1-3 and 4-6, respectively) were grown with indicated octanoic acid amounts to verify improved evolved phenotypes. (C) Exemplary growth of cultures originating from single colonies of the mixed evolved population from each flask.

In parallel to this thesis another group conducted an ALE experiment with octanoic acid (Zhu et al., 2020) similar to the one presented here. As parental strain they used an *S. cerevisiae* CEN.PK113-11C $\Delta pox1$ strain which was grown with octanoic acid over approximately 100 generations in parallel shake flasks in Delft medium. At the final stages of the ALE experiment, the cultures were supplied with 500 mg/L octanoic acid. The growth rates and final biomasses were increased in several evolved strains compared to the parental strain. Whole genome sequencing revealed mutations in seven genes, two of which were described to be causal for the observed phenotype. Combined knockout of the affected genes *PDR1* and *OSH2* resulted in increased resistance close to that of the final evolved strain and even separate knockout of each gene individually increased resistance markedly. *PDR1* encodes a TF that regulates expression of multidrug resistance genes and *OSH2* encodes a member of an oxysterol-binding protein family that is involved in sterol metabolism. The same group also reported a new variant of the transporter Tpo1, namely Tpo1-M49, which contains three amino acid exchanges and was obtained via directed evolution for decanoic acid tolerance. Tpo1-M49 was shown to also increase tolerance of cells to octanoic acid and lead to increased medium-chain fatty acid titers (Zhu et al., 2020).

Intrigued by this report, the aim was set to test the effect of $\Delta pdr1$, $\Delta osh2$ and Tpo1-M49 on robustness as well as octanoic acid production in the octanoic acid producer strain LBY38 (*FAS1^{RK}*, $\Delta faa2$). Knockout mutants were generated in the LBY38 strain background, namely LBY42 (=LBY38 $\Delta pdr1$) and LBY45 (=LBY38 $\Delta osh2$) and genomically introduced *TPO1-M49* into the *pyk2* locus of LBY38 (=LBY44). To test the robustness of the strains, they were supplied with 0-325 mg/L octanoic acid and growth was measured after 42 h (Figure 8A). In addition, octanoic acid production of the strains was tested in YPD and SCD^{HTLU} medium, respectively, (Figure 8B, C) because increased robustness to octanoic acid eventually would only be a benefit if it also leads to elevated titers.
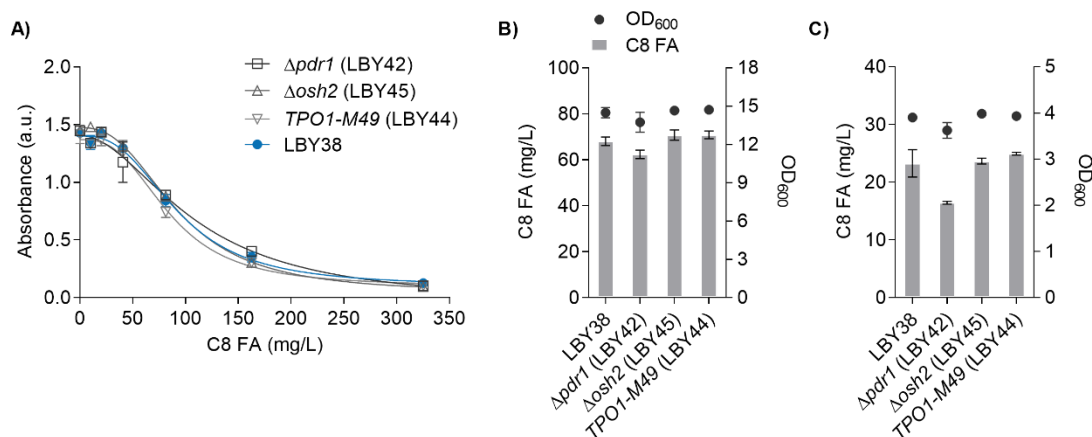
Figure 8. Influence of engineered targets on octanoic acid titers and toxicity. (A) Strains were inoculated to an OD_{600} of 0.05 and supplied with octanoic acid in buffered SCD^{HTLU} medium. Growth was measured by absorbance after 42 h (no agitation) in a platereader. $n = 3$, error bars = ± standard deviation. (B, C) OD_{600} (dots) and octanoic acid titers (bars) of engineered strains compared to parental strain LBY38. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered YPD (B) and buffered SCD^{HTLU} medium (C), respectively. $n = 2$, error bars = ± standard deviation

Surprisingly, none of the strains showed improved growth or production over the parental strain LBY38. The *pdr1* knockout even led to decreased growth and octanoic acid titers in both SCD^{HTLU} and YPD medium. The reason for the differing phenotypes reported by Zhu and colleagues could possibly be attributed to differing experimental conditions such as media (Delft vs. SC/YP medium) or strain background. The knockouts of *PDR1* and *OSH2*, respectively, were all generated in CEN.PK background strains, however, our knockouts were introduced into a producer strain whereas Zhu *et al.* introduced them in non-producer strains for toxicity tests. Possibly, this led to varying responses to supplied octanoic acid.

Here, one copy of Tpo1-M49 was integrated genomically into a strain that still contains the wild type *TPO1* version and production and tolerance were tested in buffered SCD^{HTLU} medium. Zhu et al. (2020), on the other hand, showed improved tolerance for a Δ*tpo1-TPO1-M49* strain in CSM medium. The increase in octanoic acid production was shown to depend on the copy number of *TPO1-M49*. Therefore, testing the effect of several copies of *TPO1-M49* in a LBY38 Δ*tpo1* strain could be worthwhile. Furthermore, the *TPO1-M49* variant was detected in a screening for improved decanoic acid transport, hence, a similar screening could be conducted to detect improved variants specifically for octanoic acid transport.

In summary, the outcome of an ALE experiment is dependent on the individual experimental setup, evolved phenotypes of mixed cultures are not always detectable after single colony isolation, and if detectable, not necessarily confer the same phenotype in another strain background and experimental setup.

5.1.3 Overexpression of transporters, transcriptional regulators and mutated acetyl-CoA carboxylase

Previous to this thesis, several enzymes had been described to be involved in the response to, or transport of octanoic acid. Therefore, the encoding genes were presumed to be promising targets for rational engineering. The transporters Pdr12 and Tpo1 have been shown to be involved in octanoic acid expulsion from yeast cells (Legras et al., 2010; Borrull et al., 2015). The expression of *PDR12* was found upregulated 3.5-fold in wild type *S. cerevisiae* cells under octanoic acid challenge and a Δ*pdr12* strain was more sensitive to octanoic acid than the wild type strain. Knockout of another transporter, Δ*tpo1*, also resulted in increased sensitivity to octanoic acid (Legras et al., 2010). It was hypothesized that increased amounts of transporters could lead to enhanced octanoic acid expulsion and therefore better resistance and consequently growth.

Legras et al. (2010) also found that knockout of the transcriptional regulator-encoding genes *WAR1* and *PDR3* resulted in reduced growth when octanoic acid was supplied. War1 and Pdr3 are TFs that regulate the expression of transporters that could be involved in octanoic acid export. War1 is a positive regulator of *PDR12* expression in response to weak acids including octanoic acid (Holyoak et al., 1999; Hatzixanthis et al., 2003; Kren et al., 2003). Pdr3 was shown to regulate the expression of ABC transporters that are involved in multidrug resistance (Schüller et al., 2007; Thakur et al., 2008). The fifth chosen target for overexpression was a mutated version of the acetyl-CoA carboxylase encoding *ACC1* gene. It was reported that a mutated version, namely Acc1[S1157A], conferred increased resistance to octanoic acid due to an increase in oleic acid in the membrane (Besada-Lombana et al., 2017), similar to the effect of externally supplied oleic acid (Liu et al., 2013).

The overexpression of *PDR12* did not result in increased growth or octanoic acid titers in our hands (Baumann et al., 2021). To test the effect of overexpression of the four other targets, they were expressed under the control of medium strong promotors on multicopy plasmids in the non-producer strain CEN.PK113-7D and resistance to supplied octanoic acid was evaluated (Figure 9).
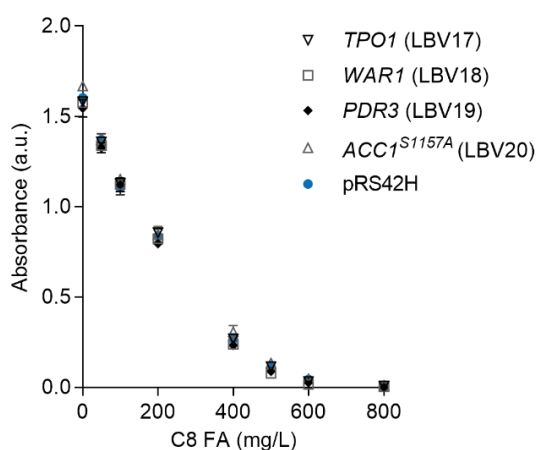

Figure 9: Influence of overexpressed genes on growth of CEN.PK113-7D with octanoic acid supply. CEN.PK113-7D was transformed with plasmids containing the indicated genes or the control plasmid (pRS42H), respectively. All strains were inoculated to an OD_{600} of 0.05 and supplied with octanoic acid in buffered SCD^{hygro} medium. Growth was measured by absorbance after 20 h (no agitation) in a platereader. $n = 3$, error bars = ± standard deviation.

The experiment showed that neither of the overexpressions led to an increase in resistance to octanoic acid in the non-producer background CEN.PK113-7D. For the overexpression of the transporter-encoding genes *PDR12* and *TPO1* and the TF-encoding *WAR1* (regulating *PDR12* expression) this could be a hint that export of octanoic acid is not a bottleneck for detoxification – at least not at near neutral pH (~6.5). It is possible that the active export of octanoic acid plays a minor role in comparison to passive diffusion. Furthermore, the toxicity test showed no effect of *PDR3* overexpression on resistance to octanoic acid. This suggests that the transporters under control of Pdr3 do not mediate octanoic acid transport. However, considering that Δ*war1* and Δ*pdr3* strains exerted reduced growth under octanoic acid supplementation (Legras et al., 2010), it is more likely that native expression levels of involved transporters are sufficient. Another possibility is that active export becomes important only at low pH, when the protonated form can easily enter the cell and rapid active export becomes a bottleneck for detoxification.

In contrast to previous reports, a positive effect from *ACC1*^{S1157A} expression on octanoic acid resistance was not observable. The "*S1157A*" amino acid exchange was described to abolish Snf1-mediated repression of Acc1 (Choi & Da Silva, 2014; Shi et al., 2014), leading to increased resistance to octanoic acid (Besada-

Lombana et al., 2017). The contradictory results could be due to the expression of the native *ACC1* from the strain background, which might mask positive effects expected from expression of the mutated version.

5.1.4 Conclusion

The results presented above show that toxicity is a complex trait to tackle. The aim of the presented approaches was to assess the inherent robustness of different *S. cerevisiae* strains to determine the most suitable production host. Furthermore, ALE and engineering of rational targets were to confer an *S. cerevisiae* strain with increased resistance to octanoic acid.

A robust yeast strain is essential for industrial octanoic acid production in which high titers would need to be produced. The here presented approaches were not effective in increasing robustness. However, it was shown that industrial yeast strains, like EthanolRed, are suitable candidates as future production hosts due to increased tolerance to octanoic acid, especially after preadaptation. Nevertheless, even for industrial strains, octanoic acid toxicity is a vital issue that needs to be minimized. To achieve this goal, future work should focus on evaluating other approaches, for instance, engineering membrane composition, *in situ* extraction of octanoic acid, or transformation of octanoic acid into non-toxic derivatives as discussed in detail in chapter 3.3.

5.2 Material and methods

5.2.1 Strains and plasmid construction

Yeast strains and plasmid used in the additional results are listed in Table 1 and Table 2, respectively. Plasmids LBV18 and LBV19 and were constructed as follows: Promoters *pTPI1* and *pPYK1* as well as genomic regions *WAR1-tWAR1* and *PDR3-tPDR3* were amplified from CEN.PK113-11C genomic DNA with primers containing respective overhangs (Table 3) for homologous recombination in yeast. Plasmid pRS42H was digested with *Eco*RV and transformed with the respective insertion fragments into CEN.PK2-1C for homologous recombination. Yeast transformations were performed according to Gietz & Schiestl (2007). Cells were streaked out on YPD^{hygro} (200 µg/mL) medium to select for hygromycin-resistant colonies. All colonies on the selective plates were collectively transformed into electrocompetent *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) and transformants were selected on lysogeny broth (LB) agar plates supplemented with 100 µg/mL ampicillin (Sambrook et al., 1989). Selected plasmids were extracted according to standard procedures and verified via Sanger sequencing.

Knockout of *PDR1* and *OSH2* and replacement of *PYK2* open reading frame by *TPO1-M49*, respectively, were performed via CRISPR/Cas9. CRISPR/Cas9 plasmids were amplified each in two PCR fragments, assembled *in vitro* in an isothermal reaction using T5 exonuclease, polymerase and ligase (Gibson et al., 2009) and transformed into *E. coli* DH10β. Plasmids were verified via Sanger sequencing. The insertion fragment *TPO1-M49* was amplified from plasmid p416GPD-*TPO1-M49* with overhangs for up- and downstream regions, respectively, of *PYK2*. CRISPR/Cas9 plasmids were transformed into LBY38 with the respective donor/insertion fragments. Clones were analyzed via genomic DNA extraction and PCR verification according to standard procedures.

Table 1. Yeast strains used in the additional results.

Strain	Characteristics	Reference
CEN.PK2-1C	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1; leu2-3_112; trp1-289*	Euroscarf, Frankfurt am Main, Germany
CEN.PK113-7D	*MATa; MAL2-8c; SUC2*	Euroscarf, Frankfurt am Main, Germany
CEN.PK113-7D Δ*pox1*	*MATa; MAL2-8c; SUC2; Δpox1*	(Wernig et al., 2019)
CEN.PK113-11C	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1*	Euroscarf, Frankfurt am Main, Germany
Ind-Sc1	Industrial *S. cerevisiae* ethanol production strain	This group
Ind-Sc2	Industrial *S. cerevisiae* ethanol production strain	This group
Ind-Sc3	Industrial *S. cerevisiae* ethanol production strain	This group
EthanolRed	Industrial *S. cerevisiae* ethanol production strain	Lesaffre/Fermentis
LBY38	CEN.PK2-1C Δ*pFAS1-1-300::pHXT7-1-392* Δ*pFAS2-1-200::pHXT7-1-392, FAS1^{R1834K}, Δfaa2*	(Wernig et al., 2021)
LBY42	LBY38 Δ*pdr1*	This thesis
LBY44	LBY38 Δ*pyk2::pTDH3-TPO1-M49 (F322L, T54S, I432N)-tCYC1*	This thesis
LBY45	LBY38 Δ*osh2*	This thesis

Table 2. Plasmids used in the additional results.

Plasmid	Characteristics	Reference
pRS42H	2μ, *HPH^R*, *AmpR*, multiple cloning site including *Eco*RV	(Taxis & Knop, 2006)
pRCC-K	2μ, *kanMX*, *AmpR*, *pROX3-Cas9^{opt}-tCYC1*, *pSNR52-gRNA*	(Generoso et al., 2016)
p416GPD-TPO1-M49	*CEN6/ARS4, AmpR, URA3, pTDH3-TPO1(F322L, T54S, I432N)-tCYC1*	(Zhu et al., 2020)
LBV17	pRS42H with *pPGK1-TPO1-tTPO1* integrated in *Eco*RV site	(Baumann et al., 2018)
LBV18	pRS42H with *pFBA1-WAR1-tWAR1* integrated in *Eco*RV site	This thesis
LBV19	pRS42H with *pTPI1-PDR3-tPDR3* integrated in *Eco*RV site	This thesis
LBV20	pRS42H with *pPYK1-ACC1^{S1157A}-tACC1* integrated in *Eco*RV site	(Baumann et al., 2018)
LBV42	pRCC-K with *gRNA* for *PDR1* locus	This thesis
LBV43	pRCC-K with *gRNA* for *OSH2* locus	This thesis
LBV103	pRCC-K with *gRNA* for *PYK2* locus	(Baumann et al., 2021)

Table 3. Oligonucleotides used in the additional results.

Primer	Sequence 5' → 3'	Application
LBP92	GGTCGACGGTATCGATAAGCTTGATGCGGCCGCTGGGTCATTACGTAAATAATGATAGG	Amplification and sequencing of *pFBA1* and *WAR1-tWAR1* with overhangs to pRS42H
LBP93	CCAGTTATTGCAATCTGCGTGTCCATTTTGAATATGTATTACTTGGTTATGG	
LBP94	CCATAACCAAGTAATACATATTCAAAATGGACACGCAGATTGCAATAACTGG	
LBP95	GCTGGAGCTCCACCGCGGTGGCGGCCGCCAACAACTTATTATCACCGTACGATTTCGC	
LBP96	GTTGAAGGGATACAACTCTG	
LBP97	GCTTCATAATAGTTCCTCTGG	
LBP86	GAGGTCGACGGTATCGATAAGCTTGATGAGCTCGAATAGCTCATCCAATCGATCCAAG	Amplification and sequencing of *pTPI1* and *PDR3-tPDR3* with overhangs to pRS42H
LBP87	CTACAAAAAACACATACATAAACTAAAAATGAAAGTGAAGAAATCAACTAGATCAAAAG	
LBP88	CTTTTGATCTAGTTGATTTCTTCACTTTCATTTTTAGTTTATGTATGTGTTTTTTGTAG	
LBP89	CCCTCACTAAAGGGAACAAAAGCTGGAGCTCGATTTAAACTGTGAGGACCTTAATAC	
LBP90	CCTGATACTTCCAATAATCC	
LBP91	GTGTCATTGCTAGAAATCC	
WGP234	CTTGGTGGTGTTCGTCGTATCTCTTAATCATAGAAGCAGACAATGGAG	Amplification of CRISPR/Cas9 plasmid backbone
WGP235	TGTTGTCTGACATTTTGAGAGTTAACACCGAAATTACCAAGGCTC	
MGP126	GGGAAACGCCTGGTATC	Sequencing of gRNAs
LBP259	CTGGATAAACGTCGCTCCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Amplification of pRCC-K with gRNA for *PDR1*
LBP260	GTGGAGCGACGTTTATCCAGGATCATTTATCTTTCACTGCGGAG	
LBP261	CAGCCAAGAATATACAGAAAAGAATCCAAGAAACTGGAAGACGTATACGTTTGTATAGATAAAAGTTCTCAAAAACTTCC	Donor DNA for *PDR1* knockout
LBP262	GGAAGTTTTTGAGAACTTTTATCTATACAAACGTATACGTCTTCCAGTTTCTTGGATTCTTTTCTGTATATTCTTGGCTG	
LBP263	TCAAAACAACCTTATTCGCC	Sequencing of *PDR1* locus
LBP264	ACAGACAGCATTAGACATTTC	
LBP271	AAATCACATCTAACCCCTCC	
LBP272	AGTAAGCATGAAGTGCCC	
LBP265	ACTAGAACCGCTGCATCAGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	Amplification of pRCC-K with gRNA for *OSH2*
LBP266	GCTGATGCAGCGGTTCTAGTGATCATTTATCTTTCACTGCGGAG	
LBP267	TAATCATAAGCTTAAGCTCGCCAACTGAAAACTTCACAATCACGCCTTTTTTATGCGAGCTTTTTTCCTGGTACTTGTAT	Donor DNA for *OSH2* knockout
LBP268	ATACAAGTACCAGGAAAAAAGCTCGCATAAAAAAGGCGTGATTGTGAAGTTTTCAGTTGGCGAGCTTAAGCTTATGATTA	
LBP276	GTTATAACAAGGGCTCCTCC	Sequencing of *OSH2* locus
LBP277	GCCAAAAGAAACAAATGTGC	
LBP273	CCTCATCAACACATCAACC	
LBP274	TCATAACGCCCATCTTCC	

LBP282	CAACTATATTTTACTTTCATCCTCTACGTCCATTGTAAGATT ACAACAAAAGCACTATCGCGTCCCAAAACCTTCTCAAG	Amplification and sequencing of Δ*pyk2::pTDH3-TPO1-M49-tCYC1*
LBP283	CTGACACAATGGACAATTAAATAAAATTAAGTAAAAAAATA AGGACTTTAATTTTTAACAGTTTATTCCTGGCATCC	
LBP118	CAGAGCGGTGAAACGCAAC	
LBP119	CGCAGTTTGCGAACATTACCTG	

5.2.2 Cultivation of yeast strains

For *S. cerevisiae* cultures, pre-cultures were inoculated from several colonies and combined in 20 mL SCD (with amino acids histidine, tryptophan, lysin and uracil, if applicable) or YPD with or without 100 mM potassium phosphate buffer (pH 6.5) as indicated for each experiment. After shaking at 180 r.p.m. at 30 °C overnight, the main cultures were inoculated to an OD_{600} of 0.1 in 20-50 mL of the respective medium - containing octanoic acid if applicable - and cultured in 100 mL or 300 mL shake flasks under the same conditions. OD_{600} was measured in a spectrophotometer or a cell growth quantifier from Aquila biolabs as indicated for each Figure. For sampling, cultures were harvested after 48 h of fermentation by centrifugation and 10 mL of the supernatant was used for fatty acid extraction.

For the preadaptation to octanoic acid, precultures were inoculated from single colonies in YPD without buffer and grown overnight (180 r.p.m., 30 °C). Strains were inoculated to an OD of 0.1 to fresh YPD containing 200 mg/L octanoic acid and cultivated overnight. This procedure was performed twice before strains were used for the toxicity test (chapter 5.2.4).

5.2.3 Fatty acid extraction, derivatization and GC-FID analysis

Fatty acid extraction, derivatization and GC analysis were performed as described previously (Henritzi et al., 2018). Fatty acids were extracted from 10 mL culture supernatant after 48 h of cultivation, derivatized to FAMEs and measured on a Perkin Elmer Clarus 400 instrument (Perkin Elmer, Germany) equipped with an Elite FFAP capillary column (30 m × 0.25 mm, film thickness: 0.25 μm; PerkinElmer, Germany) and a flame ionization detector (Perkin Elmer, Germany).

5.2.4   Toxicity tests

Pre-cultures were inoculated in YPD or SCD medium (with supplements if applicable) as indicated for each experiment and grown overnight (30 °C, 180 r.p.m.). For main cultures, strains were inoculated to an OD_{600} of 0.2 and cultivated (30 °C, 180 r.p.m.) for 4-6 hours. Cultures were then diluted in fresh media to an OD_{600} of 0.05 and aliquots of 50 µl transferred into wells of a 96-well plate (clear with flat bottom, greiner bio-one). An octanoic acid (Sigma-Aldrich, GC grade) dilution series was made in fresh media. 200 µl aliquots of these octanoic acid dilutions were transferred into the wells containing the 50 µl strain solutions to reach final volumes of 250 µl per well. The starting absorbance was measured in a platereader (CLARIOstar®, BMG Labtech, Ortenberg, Germany) and plates were incubated for the indicated time period at 30 °C without shaking before absorbance was measured again. From final absorbance values, a blank value (media without strain) was subtracted.

5.2.5   Adaptive laboratory evolution

ALE was performed with strain LBY3 (CEN.PK113-7D Δ*pox1*) in two parallel approaches. For both approaches, three shake flasks each were used (180 r.p.m., 30 °C). At the beginning, LBY3 was inoculated to an OD_{600} of 0.01 in all six flasks in YPD medium supplied with 525 mg/L octanoic acid. Cultures were serially transferred to fresh medium every 2-4 days and growth was measured periodically with a spectrophotometer. In the first approach, octanoic acid was always present in the medium. For the second approach, every second transfer was performed into fresh medium without octanoic acid, thereby releasing toxicity pressure. The octanoic acid amounts were varied over the course of the experiments as indicated in Figure 7A. Evolved cultures were assessed as described in chapter 5.2.2.

5.2.6   Software

Data tables were stored in Microsoft Excel 2016. Graphs were made using the software Prism 9.0.0 (GraphPad, USA). Design of plasmids was accomplished with Geneious Prime 2020.2.

5.3 References for the additional results

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6 Publications

6.1 A yeast-based biosensor for screening of short- and medium-chain fatty acid production

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Contributions of doctoral candidate and co-authors

(1) Concept and design

Doctoral candidate LB: 65%

Co-authors AR, EB, MO: 10%, 10%, 15%

(2) Conducting tests and experiments

Doctoral candidate LB: 100%, yeast molecular biology, biosensor and growth measurements, fermentations, metabolite extraction and GC analysis

(3) Compilation of data sets and figures

Doctoral candidate LB: 100%, biosensor assays, metabolite titers

(4) Analysis and interpretation of data

Doctoral candidate LB: 70%, biosensor assays, metabolite titers

Co-authors EB, MO: 10 %, 20%, supervision and advice

(5) Drafting of manuscript

Doctoral candidate LB: 80%

Co-authors JM, EB, MO: 5%, 5%, 10%

ACS SyntheticBiology

A Yeast-Based Biosensor for Screening of Short- and Medium-Chain Fatty Acid Production

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S *Supporting Information*

ABSTRACT: Short- and medium-chain fatty acids (SMCFA) are important platform chemicals currently produced from non-sustainable resources. The engineering of microbial cells to produce SMCFA, however, lacks high-throughput methods to screen for best performing cells. Here, we present the development of a whole-cell biosensor for easy and rapid detection of SMCFA. The biosensor is based on a multicopy yeast plasmid containing the SMCFA-responsive *PDR12* promoter coupled to GFP as the reporter gene. The sensor detected hexanoic, heptanoic and octanoic acid over a linear range up to 2, 1.5, and 0.75 mM, respectively, but did not show a linear response to decanoic and dodecanoic acid. We validated the functionality of the biosensor with culture supernatants of a previously engineered *Saccharomyces cerevisiae* octanoic acid producer strain and derivatives thereof. The biosensor signal correlated strongly with the octanoic acid concentrations as determined by gas chromatography. Thus, this biosensor enables the high-throughput screening of SMCFA producers and has the potential to drastically speed up the engineering of diverse SMCFA producing cell factories.

KEYWORDS: *biosensor, high-throughput screening, short-chain fatty acids, medium-chain fatty acids, octanoic acid, PDR12*
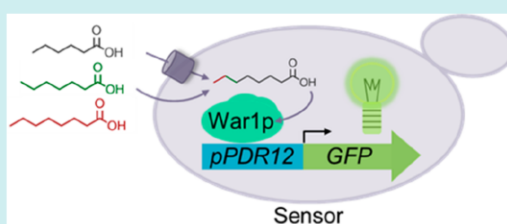
Short- and medium-chain fatty acids (SMCFA) and their derivatives have a wide range of industrial applications such as pharmaceuticals, antimicrobials or biofuels.[1] Rising concerns about nonsustainable production methods, such as deforestation and competition with the food industry, makes the fermentative production from renewable resources by microbial cell factories increasingly attractive. In recent years, several reports about the successful engineering of strains for the production of SMCFA have been published, but yields, titers and productivities remain low.[2] One of the reasons for this is the lack of high-throughput screening methods for randomly mutated or rationally engineered strains to identify highly producing cells, as the common screening methods currently applied are mostly based on low-throughput gas (GC) or liquid chromatography (LC).[3]

The use of biosensors for screening has become a popular alternative to chromatography and can substantially accelerate the optimization of production strains.[4] Most developed biosensor systems rely on the expression of sensing elements, *e.g.*, genes encoding extracellular receptors, under the control of an inducible promoter, and are coupled with an easily detectable output such as fluorescence, luminescence, colorimetry or growth rate.[4]

To our knowledge only one whole-cell SMCFA biosensor was developed so far, which was also based on this principle.[5] It made use of heterologous G-protein coupled receptors

(GPCRs) in combination with the yeast mating pathway for signaling and a reporter construct inducing GFP expression upon exposure to octanoic acid (C_8 FA) and decanoic acid (C_{10} FA). Although this GPCR-based sensor system has the advantage of being amenable to the sensing of other compounds by exchanging the receptor unit,[6] it has some drawbacks including complicated strain construction (several gene deletions and insertions), the need for functional expression of heterologous plasma membrane localized receptors as well as a low linear range for C_8 FA detection. Most importantly, the functionality of this sensor could not be verified in a mixture with other secreted acids, *i.e.*, myristic and palmitic acid (C_{14} and C_{16} FA), which was partially attributed to the toxicity of FA on cell growth.[5] However, functionality of a sensor in a mixture of different acids, as found in culture supernatants, would be essential for practical application.

Due to these limitations, we assumed that a promoter-based biosensor for SMCFA detection could be a more promising approach. There are several examples of transcription factor/promoter-based biosensors developed for the detection of environmental pollutants but not so many for the screening of mutant libraries to identify best performing cells.[7−11] A prerequisite for the use of such systems as biosensors is the

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identification of a substrate-specific transcription factor and its respective promoter.[12] The *PDR12* promoter appeared as a suitable candidate for the sensor system that we envisioned, as it is rapidly activated by only one transcription factor[13,14] and responds to a narrow spectrum of substrates.[13,15−17] *PDR12* encodes an ATP-binding cassette transporter and is under control of the Zn_2Cys_6 transcription factor War1p.[13,14] War1p constitutively binds to a weak acid response element (WARE) in the *PDR12* promoter. Upon weak acid stress, War1p is phosphorylated and undergoes conformational changes leading to its activation and thereby *PDR12* expression.[14,18,19] *PDR12* has been shown so far to be induced by multiple substrates, *i.e.*, propanoic, butanoic, pentanoic, hexanoic and heptanoic acids (C_3-C_7 FA),[15,17,20] C_8 FA, sorbate and benzoate[13] but not by acetate,[16] succinate, citrate[17] or organic alcohols.[15] The role of Pdr12p in response to C_3-C_8 FA has been demonstrated with a Δ*pdr12* mutant that was hypersensitive and the activation of a *PDR12*-lacZ reporter construct in response to these FA.[15,17] Furthermore, a microarray analysis revealed the activation of *PDR12* expression upon exposure of yeast cells to C_8 FA.[20] In addition to these characteristics, the *PDR12* promoter has a low constitutive activity in the absence of stress,[14] but upon the appropriate signal exposure, Pdr12p becomes one of the most abundant plasma membrane proteins.[21]

These previous findings led us to the assumption that GFP expression driven by the *PDR12* promoter could be suitable as a biosensor for SMCFA. We further sought to develop it as a two-cell sensor system in which SMCFA sensing is decoupled from SMCFA production for several reasons. First, with increasing titers, the producer microbe supernatant can simply be diluted to stay in the linear range of the sensor, which also helps to evade detrimental effects of SMCFA on yeast growth at higher concentrations.[22] A one-cell sensor system could lead to false-negatives due to a saturation of the sensor signal at concentrations exceeding the linear range. Second, the producer microbe can be further engineered or modified without affecting the sensor function. Finally, we want to select for cells producing high extracellular titers. In a one-cell system, an intracellular sensor will only measure the cytosolic amount of produced SMCFA, which can lead, for example, to the identification of transport-deficient mutants that accumulate SMCFA intracellularly instead of producing higher titers.

Previously, we engineered an *S. cerevisiae* strain producing mainly C_8 FA with titers of up to 245 mg/L (1.7 mM) and minor amounts of C_6, C_{10} and C_{12} FA.[23] Interested in improving this strain for higher titers, we aimed at developing a biosensor assay for rapid detection of SMCFA in culture supernatants of this strain and mutants thereof.

RESULTS AND DISCUSSION

Design of a *PDR12* Promoter-Based Biosensor for SMCFA. We sought to develop a two-cell sensor system in which SMCFA sensing is decoupled from SMCFA production as schematically illustrated in Figure 1. The biosensor was designed by fusing the *PDR12* promoter (corresponding to 1168 bp upstream of the *PDR12* coding sequence)[15] to the open reading frame of enhanced GFP, namely Envy,[24] and inserted into a multicopy plasmid that was transformed into *S. cerevisiae* CEN.PK113−11C. The sensor's functionality relies on the uptake of exogenous SMCFA, such as C_8 FA, by the yeast cells.[25] At physiological pH, C_8 FA are taken up *via* active transport, whereas at acidic pH the undissociated acid form can enter the cell by passive diffusion.[22,26] Once taken up by
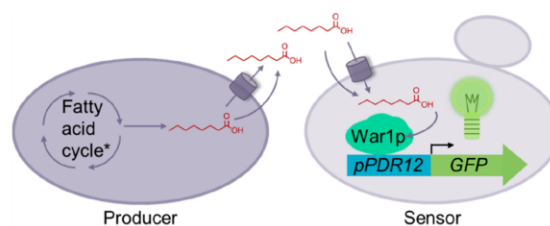

Figure 1. Short- and medium-chain fatty acid detection by a whole-cell biosensor. A producer microbe secretes short- and/or medium-chain fatty acids (SMCFA), such as octanoic acid shown here, which are produced through a modified fatty acid cycle (*). The culture supernatant is added to the yeast biosensor strain, which takes up the SMCFA *via* diffusion or active transport. The biosensor consists of the *PDR12* promoter (*pPDR12*) coupled to an enhanced version of green fluorescent protein (*GFP*). The transcription factor War1p, which is constitutively bound to *pPDR12*, undergoes phosphorylation and conformational changes upon signal exposure and initiates the expression of GFP.

the cell, the C_8 FA activates War1p, leading to transcription from the *PDR12* promoter. We found that GFP expression is activated only in the presence of the inducing signal (here: C_8 FA); in the absence of signal, we observed only low constitutive fluorescence (Figure S1). To prove the functionality of the biosensor in culture supernatants, we used a previously constructed *S. cerevisiae* strain (RPY21/FAS^R1834K) that produces mainly C_8 FA, which is secreted from or diffuses out of the cells.[25]

Characterization and Verification of Functionality of the Biosensor. We analyzed the biosensor response to C_8 FA concentrations between 0 and 1 mM for an initial validation in defined medium (SCD). We observed a correlation of the RFI (relative fluorescence intensity) of the biosensor to C_8 FA over the entire concentration range and a linear response between 0.01 and 0.75 mM (Figure 2A), which is a strong improvement over a previously published GPCR-based C_8 FA sensor (0.019−0.25 mM linear range).[5] The highest intensity of the signal after activation showed a nearly 10-fold increase after 2 h of incubation with C_8 FA, displaying a high dynamic range (Figure 2A, Figure S2). To make sure that the activation of the biosensor is specific, we tested a control strain with a plasmid expressing GFP under control of the *MET25* promoter, which is methionine repressible. In this control strain we observed no C_8 FA concentration-dependent induction of GFP expression in SCD medium without methionine (Figure 2A).

Industrial microbial fermentations commonly use complex medium rather than defined medium. Therefore, we next tested the biosensor in a complex medium, *i.e.*, YPD. Again, we observed a correlation of the RFI to the tested C_8 FA concentrations (0−3 mM) and an identical linear range between 0.01 and 0.75 mM (Figure 2B, Figure S3). While the dynamic range of the sensor was lower than in SCD medium even after twice the incubation time (Figure S4), this can be attributed to the higher background fluorescence of YPD. Nevertheless, the maximum increase in the intensity of the signal after activation was more than tripled after 4 h of incubation. Even though there is a discrepancy in previous reports about the *PDR12* induction by C_8 FA,[15,17,20] we could clearly show that the *PDR12* promoter's C_8 FA-dependent response allows it to be used as a biosensor. To our knowledge, this is the first SMCFA biosensor shown to function reliably in
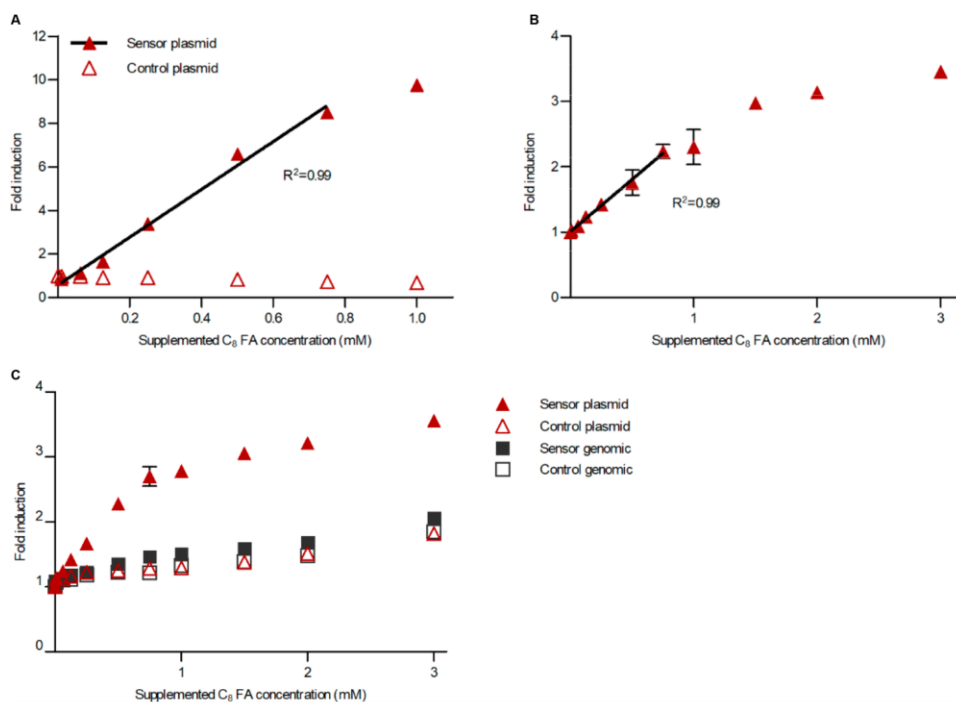
Figure 2. C_8 fatty acid-dependent response of plasmid-based and genomically integrated biosensors. Response and linear range of the plasmid-based biosensor after 2 h incubation with C_8 fatty acids (FA) in SCD (A) and after 4 h in YPD medium (B). Error bars represent two technical replicates. Experiments were conducted in three biological replicates with comparable results (Figure S2, Figure S3). (C) Response of the genomically integrated biosensor after 4 h incubation with C_8 FA in YPD. Sensor plasmid: CEN.PK113−11C + p426pPDR12-GFP. Control plasmid: CEN.PK113−11C + p426pMET25-GFP. Sensor genomic: LBY27. Control genomic: CEN.PK113−11C. For fold induction, fluorescence intensities (FI) were divided by optical densities (OD_{600}) and normalized to FI/OD_{600} values of samples without C_8 FA.

complex medium. This is of particular importance since the highest SMCFA titers produced in yeast so far were reached in complex medium.[23,27,28]

To avoid possible stability problems by using a multicopy plasmid-based biosensor, we integrated the sensor construct into the *S. cerevisiae* CEN.PK113−11C genome resulting in strain LBY27. This sensor strain, however, only showed a barely detectable increase in the intensity of the signal upon C_8 FA addition in comparison to the control CEN.PK113−11C strain (Figure 2C). Therefore, a multicopy plasmid seems to be necessary for a good dynamic range, and we continued to work with the plasmid-based biosensor.

The Biosensor Also Responds to C_6 and C_7 but Not C_{10} or C_{12} FA. It was shown in previous work that *PDR12* expression is influenced by several weak acids.[13,15−17] As none of these compounds—except other SMCFA—are usually produced by *S. cerevisiae*, we reasoned that they should not influence the biosensor's ability to detect SMCFA in *S. cerevisiae* culture supernatants.

The previously engineered *S. cerevisiae* strain RPY21/ FAS^{R1834K} mainly produces C_8 FA and in minor amounts C_6, C_{10} and C_{12} FA. To test whether the biosensor also detects these byproducts, we analyzed its response upon addition of C_6, C_{10} and C_{12} FA to YPD. As depicted in Figure 3, the RFI of the biosensor correlates to C_6 FA levels between 0 and 3 mM, and the biosensor showed a linear response between 0.01 and 2 mM in all replicates (Figure S3). C_{10} FA seems to be very toxic to the cells, as growth is already strongly decreased at the
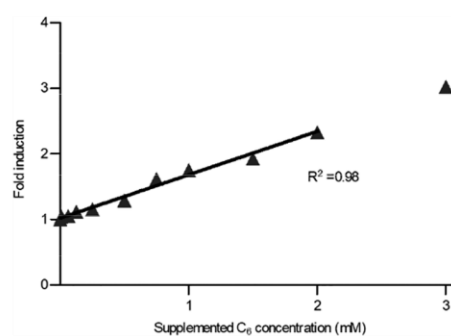

Figure 3. C_6 fatty acid-dependent response of the biosensor. Response and linear range of the biosensor after 4 h incubation with C_6 fatty acids (FA) in YPD medium. Error bars represent two technical replicates. Experiments were conducted in three biological replicates with comparable results (Figure S3). For fold induction, fluorescence intensities (FI) were divided by optical densities (OD_{600}) and normalized to FI/OD_{600} values of samples without FA.

lower concentrations, which leads to false high inductions when dividing FI by OD_{600} values (Figure S3). This is in accordance with previous findings about high toxicity of C_{10} FA.[22] Overall, we cannot exclude a very slight response of the biosensor to C_{10} FA at low concentrations (0.01−0.25 mM), which would be in accordance to a previous report suggesting a discrete role of Pdr12p in C_{10} FA response.[17] Nevertheless, we
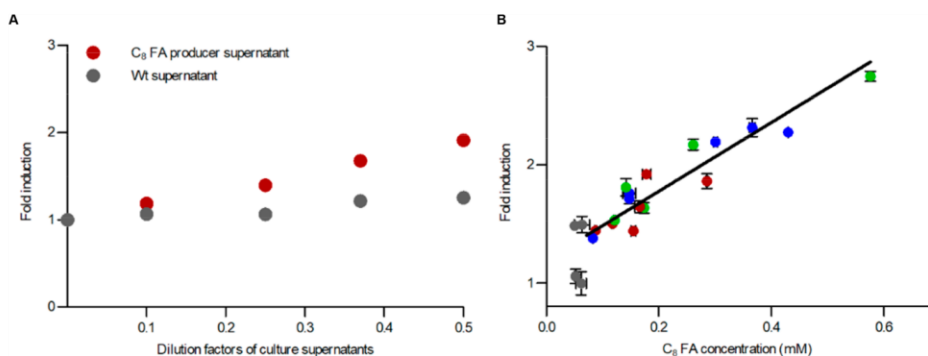
Figure 4. Biosensor response to C_8 fatty acids in *S. cerevisiae* culture supernatants and correlation to GC measurement. (A) Response of the biosensor to differently diluted culture supernatants of a C_8 fatty acid (FA) producer (RPY21/FAS^{R1834K}/pRS42H) and a wildtype strain (Wt, CEN.PK113−7D). (B) Linear correlation of the fold induction of biosensor signal in 0.5 dilutions of culture supernatants with GC measurements of the same supernatants. Strains: CEN.PK113−7D (gray), RPY21/FAS^{R1834K}/pRS42H (red), RPY21/FAS^{R1834K}/LBV17 (blue), RPY21/FAS^{R1834K}/LBV20 (green).

could not observe a linear response range of the biosensor to C_{10} FA in all three biological replicates.

For C_{12} FA, we found a concentration-dependent decrease in fluorescence over the entire concentration range tested and an increase in the OD_{600} at C_{12} FA concentrations between 0.25 and 1 mM (Figure S5). We also observed this when adding C_{12} FA to plain medium not containing the biosensor strain (data not shown). Therefore, this is probably due to incomplete solubility and light scattering by C_{12} FA micelles. In addition, we also tested the biosensor response to the odd chain C_7 FA and observed a linear response between 0.01 and 1.5 mM (Figure S6). This is in accordance to previous studies reporting that *PDR12* is induced by C_7 FA.[15,17]

In summary, we show that the biosensor responds to C_6, C_7 and C_8 FA in YPD medium over linear ranges from 0.01 up to 2, 1.5, and 0.75 mM, respectively, whereas it does not show a linear response to C_{10} FA and no concentration-dependent response at all to C_{12} FA.

Detection of C_8 FA by the Biosensor in *S. cerevisiae* Culture Supernatants. To validate that the biosensor can discriminate between different SMCFA concentrations in culture supernatants, we first conducted a pretest. We cultivated a C_8 FA producer strain (RPY21/FAS^{R1834K}/pRS42H) and a wildtype strain (CEN.PK113−7D) in buffered YPD (with hygromycin if applicable) for 72 h and added the culture supernatants in different dilutions to the biosensor, and in parallel we measured the C_8 FA content by GC. As shown in Figure 4A, the lowest dilution (0.5) of the C_8 FA producer supernatant led to a 1.9-fold increase in the intensity of the signal after 4 h of incubation. Furthermore, we observed a constant increase in signal intensity with decreasing dilution factors confirming the C_8 FA concentration-dependent activation of the biosensor in *S. cerevisiae* culture supernatants. The supernatant of the wildtype culture also led to a slight increase in signal intensity of the biosensor, which can be attributed to minor amounts of C_8 FA that it produced naturally. As determined by GC, the wildtype strain produced 0.02 mM C_8 FA, whereas RPY21/FAS^{R1834K}/pRS42H produced 0.28 mM C_8 FA.

To obtain supernatants with a wide range of C_8 FA concentrations we not only tested the original RPY21/FAS^{R1834K} strain but also two derivatives thereof. We transformed the strain with one of two plasmids containing

overexpressed genes or the control plasmid (pRS42H), respectively. One plasmid harbored an overexpression construct of *TPO1* (LBV17). The transporter Tpo1p seems to be involved in the resistance to C_8 FA[20] and therefore has the potential to decrease C_8 FA toxicity. The second construct encoded the acetyl-CoA carboxylase *ACC1* and harbored mutations leading to amino acid exchanges of two serine residues to alanine (*ACC1^{S659AS1157A}*; LBV20). These two mutations impair the deactivation of Acc1p *via* phosphorylation.[29−31] A similarly modified Acc1p (*ACC1^{S1157A}*) was shown to have a positive effect on yeast resistance to C_8 FA,[32] and therefore we reasoned that it could also influence C_8 FA product titers of RPY21/FAS^{R1834K}. We cultivated RPY21/FAS^{R1834K} with overexpression or control plasmids as well as a wildtype strain (CEN.PK113−7D) in two independent experiments in two to three replicates and obtained a wide range of C_8 FA titers between 0.05 and 0.58 mM as measured by GC. We added the culture supernatants to the biosensor (corresponding to a 0.5 dilution) and measured the RFI. As shown in Figure 4B, the RFI of the biosensor clearly correlates with the C_8 FA concentrations measured by GC. A linear correlation could be observed for concentrations of produced C_8 FA from 0.08 mM up to the highest concentration tested, 0.58 mM. We additionally tested the supernatants in a dilution of 0.25 with the biosensor and received—despite lower fold inductions—an identical linear range (Figure S7A). In all samples, the C_6 FA content was below 6% of the total SMCFA titers and did not alter the linear correlation of the biosensor response to C_8 FA concentrations (Figure S7B).

This is the first report of a whole-cell promoter-based biosensor for the detection of SMCFA in culture supernatants. In contrast to previously generated sensor systems,[5,6,8−10] the here presented biosensor solely consists of an *S. cerevisiae* strain transformed with a multicopy plasmid containing the *PDR12* promoter coupled to GFP without further strain engineering, fine-tuning or expression of additional components needed. We demonstrate that it linearly responds to C_8 FA in defined and complex medium as well as to C_6 and C_7 FA in complex medium. Furthermore, the biosensor responds to C_8 FA concentrations from 0.08 up to at least 0.58 mM in *S. cerevisiae* culture supernatants and therefore in a mixture of other secreted FA. The biosensor response showed a clear correlation of RFI to C_8 FA values determined by GC and

therefore can be used to compare SMCFA titers of different culture supernatants. On the basis of these results, we envision two possible applications. It can be used as a screening system of microbial producer strain libraries of one specific SMCFA, as shown for the C_8 FA producing strain here, or to screen for increased overall SMCFA titers. For the latter, GC measurements can be used to determine the exact SMCFA composition of the culture supernatants once a biosensor-aided screening resulted in several good performing candidate strains.

The currently produced amounts of C_6 FA by yeast vary from 0.17 to 0.62 mM[23,28,33] and fall within the linear range of the biosensor. On the other hand, C_8 FA titers produced by yeast vary from 0.45 to 1.7 mM[23,28,33] and therefore partly exceed the linear range of the biosensor. However, we can simply dilute culture supernatants before adding them to the biosensor to retain a linear response. The biosensor also has a wide linear range for C_7 FA detection and could be a valuable tool to screen a C_7 FA-producer library; however, yeast has not been engineered to produce C_7 FA so far.

For future use, a more stable, plasmid-independent sensor strain might be beneficial. This could be achieved by a multicopy integration of the sensor into the genome. Thereby, the low dynamic range of the sensor response as seen with the single genomic integration might be increased. The dynamic range of the sensor could be further optimized by the genomic replacement of the native WAR1 promoter with the PDR12 promoter.[11] Assuming low "leaky" expression levels of WAR1, such a positive-feedback loop would accelerate WAR1 expression in the presence of inducing molecules. These higher War1p levels would then result in a higher expression of PDR12 promoter-coupled GFP, possibly leading to higher dynamic ranges of the sensor. To improve the linear range of the biosensor, the saturation of the sensor signal would have to be shifted toward higher FA concentrations. One possibility to achieve this could be to decrease the binding affinity of War1p to the WARE site in the PDR12 promoter. This might be accomplished either by mutating WAR1 or the WARE site. Alternatively, War1p could be mutated to exhibit a lower binding affinity for its inducers; however, it is known neither if the inducing molecules directly bind War1p nor at which molecular site.[14,18,19] In order to try any of these approaches, detailed War1p structural data would be needed.

Nevertheless, the here presented biosensor has the appropriate linear and dynamic ranges to use it for high-throughput screening of yeast SMCFA production. Needing only 4 h of incubation of sample supernatants with the biosensor in multiwell format, hundreds of strains can be rapidly screened to select the best performing strains. For future use, we envision a broader application spectrum of the biosensor as its use is not restricted per se to S. cerevisiae or even yeast SMCFA producers. The biosensor's SMCFA response certainly will have to be confirmed in the supernatants of other microbes, i.e., other yeasts or bacteria, as different species also produce different metabolites that could influence the biosensor's response to SMCFA in other culture supernatants. Overall, this biosensor represents a valuable and ready-to-use system, which fills a need to rapidly improve microbial SMCFA production.

■ METHODS

Strains and Plasmid Construction. Yeast strains and plasmids used throughout this study are listed in Table S1. The PDR12 promoter, TPO1 and $ACC1^{S659AS1157A}$ as well as promoters (pPGK1, pPYK1) and endogenous terminators (tTPO1, tACC1) were amplified from CEN.PK113−11C genomic DNA with primers containing the respective over-hangs for cloning via homologous recombination (oligonucleotides are listed in Table S2). The PDR12 promoter was integrated into the SacI site of p426pMET25-GFP, thereby replacing pMET25. The genomic insertion of pPDR12-GFP was performed via CRISPR/Cas9 as described previously.[34] The CRISPR/Cas9 plasmid was amplified in two PCR fragments and transformed for in vivo assembly. The insertion fragment was amplified from plasmid p426pPDR12-GFP. Yeast transformations were performed according to Gietz and Schiestl[35] or for RPY21 via an adapted procedure.[23] To generate RPY21/FAS^{R1834K}, RPY21 was transformed with a plasmid carrying the wildtype version of FAS2 and another plasmid carrying FAS1 with mutations leading to an amino acid replacement (R1834K), thereby redirecting the FA production from long chain FA to SMCFA.[23] Cells were streaked out on selective YPD (1% yeast extract, 2% peptone, both produced by BD, Difco Laboratories, Sparks, USA; 2% D-glucose, purchased from Roth, Karlsruhe, Germany) containing hygromycin or G418 (200 μg/mL) to select for hphNT1 or kanMX or on selective SCD medium[36] lacking leucine, tryptophan, uracil and/or histidine (± LWUH) to select for LEU2, URA3 or HIS3 markers, respectively. Electrocompetent E. coli DH10β (Gibco BRL, Gaithersburg, MD) was used for subcloning according to standard procedures, and trans-formants were selected on lysogeny broth (LB) agar plates[37] supplemented with 100 μg/mL ampicillin.

Cultures for Fatty Acid Production. C_8 FA producer and control (wildtype) strains were grown as previously described[23] with minor adjustments. For precultures, several colonies of the strains were picked and combined in 20 mL YPD with 100 mM potassium phosphate buffer (pH 6.5). After shaking at 180 r.p.m. at 30 °C overnight, the main culture was inoculated to an OD_{600} of 0.1 in 30 mL buffered YPD medium and cultured in 300 mL shake flasks under the same conditions. After 72 h, the cultures were harvested by centrifugation and 20 mL of the supernatant was used for FA extraction (see below) whereas remaining supernatant was used for the biosensor assay.

Fatty Acid Extraction and Quantification via GC. FA extraction and GC analysis were performed as described previously.[25] For each culture, two 10 mL aliquots from the same culture supernatant were separately processed and measured by GC. The standard deviation (SD) between the two measurements from the same culture was for all samples below 2 mg/L.

Cultures for the Biosensor Assay. For the biosensor assay, strains (CEN.PK113−11C/p426pMET25-GFP or p426pPDR12-GFP) were grown overnight with 180 r.p.m. shaking at 30 °C in SCD-L-W-U and under addition of repressive concentrations of methionine (2 mM) for the strain harboring plasmid p426pMET25-GFP. Precultured cells were used to inoculate 20 mL of fresh SCD-L-W-U (+2 mM methionine) to an OD_{600} of 0.1 and grown for 4−6 h until an OD_{600} of about 0.4. Cells were centrifuged and resuspended in fresh YPD or SCD-L-W-U without methionine and transferred into black 96-well plates with clear flat bottom (ref. 655097, Greiner Bio-one, Frickenhausen, Germany) with 100 μL per well. To keep conditions sterile, well plates were sealed with a gas permeable sealing membrane (Breathe-Easy, Diversified

Biotech, Dedham, MA, USA). For the primary verification of the sensor, 90 μL of fresh media was added together with 10 μL of 70% v/v ethanol (control) or 10 μL of differently diluted fatty acids (in 70% v/v ethanol) to always reach a final volume of 200 μL/well and have the same ethanol concentrations in all wells. C_6, C_7, C_8, C_{10} and C_{12} FA (Sigma-Aldrich) were diluted in 70% v/v ethanol in concentrations between 0.2 and 60 mM to reach final dilutions of 0.01 to 3 mM in the wells. For the biosensor test with culture supernatants, 100 μL of culture supernatant (for cultivation conditions see above) was added to 100 μL of the biosensor strain. Experiments were performed in biological triplicates with technical duplicates.

Biosensor Measurements. Directly after pipetting of all components into the 96-well plate, it was incubated at 30 °C and 600 r.p.m. shaking in a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). Measurements of OD_{600} and FI with excitation at 470 ± 15 nm and emission at 515 ± 20 nm were taken every 30 min over several hours. To normalize for different cell numbers in wells, the FI value of each well was divided by the respective OD_{600} value. For calculating the fold induction, these FI/OD_{600} values were divided for the different FA concentrations by the FI/OD_{600} values of the "0 mM" controls (without FA). For calculation of the fold induction response to the culture supernatants, FI/OD_{600} values of the wells with supernatant and biosensor were divided by FI/OD_{600} values only containing biosensor. Values are shown as mean ± the standard deviation (SD).

Data analyses including the calculation of linear regression and R^2 values were performed using the software Prism 5 (GraphPad, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00309.

Supporting figures and tables (PDF)

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Author Contributions
LB, EB and MO conceived the study. ASR and JPM provided ideas and constructive input. LB performed all experiments. LB, EB and MO analyzed the data and wrote the paper. All authors read and approved the manuscript.

Notes
The authors declare the following competing financial interest(s): EB is inventor of EP patent application No. 15 162 192.7 filed on April 1, 2015, and of EP patent application No. 15 174 342.4 filed on June 26, 2015, by Goethe-University Frankfurt, concerning short-chain acyl-CoA producing FAS variants. There are no other competing interests.

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

SMCFA, short- and medium-chain fatty acid(s); FA, fatty acid(s); C_6, C_7, C_8, C_{10}, C_{12} FA, hexanoic, heptanoic, octanoic, decanoic, dodecanoic acid(s); FAS, fatty acid synthase; PDR12, plasma membrane ATP-binding cassette transporter; TPO1, polyamine transporter of the major facilitator superfamily; ACC1, acetyl-CoA carboxylase; GFP, green fluorescent protein; GC, gas chromatography; OD_{600}, optical density at λ = 600 nm; FI, fluorescence intensity; RFI, relative fluorescence intensity.

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SUPPORTING INFORMATION

Table S1. Yeast strains and plasmids used in this study.

Strain	Characteristics	Reference
CEN.PK113-7D	*MATa; MAL2-8c; SUC2*	Euroscarf, Frankfurt am Main, Germany
CEN.PK113-11C	*MATa; MAL2-8c; SUC2; ura3-52; his3Δ1*	Euroscarf, Frankfurt am Main, Germany
LBY27	CEN.PK113-11C *Δpyk2::pPDR12*-GFP	This study
RPY21/FAS^R1834K	*MATα; ura3Δ0; his3Δ0; leu2Δ0; TRP1; lys2Δ0; MET15; ΔFAS1::kanMX4; ΔFAS2::kanMX4; Δfaa2*; transformed with plasmids pRS315-FAS1^R1834K and pRS313-FAS2	[1]
Plasmid	**Characteristics**	**Reference**
Plasmids used for fermentations		
p426pMET25-GFP	2μ, *URA3, Amp^r, pMET25*-GFP-*tCYC1*	This study
p426pPDR12-GFP	2μ, *URA3, Amp^r, pPDR12*-GFP-*tCYC1*	This study
pRS42H	2μ, *hphNT1, Amp^r*, multiple cloning site including *Eco*RV	[2]
LBV17	pRS42H with *pPGK1-TPO1-tTPO1* integrated in *Eco*RV site	This study
LBV20	pRS42H with *pPYK1-ACC1^S659AS1157A-tACC1* integrated in *Eco*RV site	This study
pRS315-FAS1^R1834K	*CEN6/ARS4, LEU2, Amp^r, pADH2-FAS1^R1834K-tFAS1*	[3]
pRS313-FAS2	*CEN6/ARS4, HIS3, Amp^r, pADH2-FAS2-tFAS2*	[3]
Plasmids used for CRISPR/Cas9		
pRCC-K	2μ, *kanMX, Amp^r, pROX3-Cas9^opt-tCYC1, pSNR52-gRNA*	[4]
pRCC-K-PYK2	pRCC-K with *gRNA* for *PYK2* locus	This study

Table S2. Oligonucleotides used in this study.

Primer	Sequence 5'-3'	Application
Plasmid construction or sequencing		
LBP63	CAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTGATATCTTTGTTTTGCATTTTAC	Amplification of *pPDR12* from CEN.PK113-11C with overhangs to the p426pMET25-GFP backbone
LBP64	CTACACCTGTAAACAATTCCTCGCCTTTAGACATTTTTTTATTAATAAGAACAATAAC	
LBP103	CTAATGTAGGCCATGGAAC	Sequencing of GFP
LBP76	GGTCGACGGTATCGATAAGCTTGATCCCGGGATAGTAGAAAAAAAAGGGGATATCACTAC	Amplification of *TPO1-tTPO1* from CEN.PK113-11C with overhangs to pRS42H and *pPGK1*, respectively
LBP77	GTAATTATCTACTTTTTACAACAAATATAAAACAATGTCGGATCATTCTCCCATTTCTAA	
LBP78	TTAGAAATGGGAGAATGATCCGACATTGTTTTATATTTGTTGTAAAAAGTAGATAATTAC	Amplification of *pPGK1* from CEN.PK113-11C with overhangs to *TPO1* and pRS42H, respectively
LBP79	GGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGAATTACCGTCGCTCGTGATTTG	
LBP80	GCTACTGCTGAGAACCTG	Sequencing of LBV17
VSP159	CGTGTGACAACAACAGCC	
LBP81	GACTCACTATAGGGCGAATTGGGTACCGGGCCCCGACAGATTGGGAGATTTTCATAGTAG	Amplification of *pPYK1* from CEN.PK113-11C with overhangs to pRS42H and *ACC1*, respectively
LBP82	GAAGACTCGAATAAGCTTTCTTCGCTCATTGTGATGATGTTTTATTTGTTTTGATTGGTG	
LBP83	CACCAATCAAAACAAATAAAACATCATCACAATGAGCGAAGAAAGCTTATTCGAGTCTTC	Amplification of *ACC1* with *S659A* and *S1157A* and *tACC1* with overhangs to *pPYK1* and pRS42H, respectively
RPP108	CTATGGCAATCAAAAGACCACCATCAGCTAGTTGAC	
RPP107	GATATCATACTGCGTCAACTAGCTGATGG	
RPP088	CATATGACAAATCTGAAACAGCAACAGCCCTGTTCATAC	
RPP087	ATGGGTATGAACAGGGCTGTTGCTGTTTCAGATTTGTCATATGTTG	
LBP84	GTACTCTGAAGGATCTGTTTGAGCGCTTCCATCGGGCCCATCGAATTCCTGCAGCCCGGG	
LBP98	CTTGTCATCCAATCTGTTC	Sequencing of LBV20
LBP99	CCAAATAAGCACCGATACC	
LBP100	GCAACCATTCCTTAACAGG	
LBP101	GACATACAGAACTTCCAGG	
LBP102	GGAACATAGTTTGCAGTAGG	
RPP89	TTCGAAACCTTCTGTAGAAGCAACACAAAC	
RPP90	CGGTCAAGGAAAGAACTGAACAAATTGAAC	
RPP109	TCCAACTCTTGCCGTCATTTGC	
RPP056	CACACAGGAAACAGCTATGAC	Sequencing of p426pPDR12-GFP, LBV17 and LBV20
LBP85	CGTTACCCAACTTAATCGCC	
Insertion of *pPDR12*-GFP in *PYK2*		
LBP108	GTCCATTGTAAGATTACAACAAAGCACTATCGGGCGAATTGGGTACCGG	Amplification of *pPDR12*-GFP-*tCYC1* from p426pPDR12-GFP with overhangs to *PYK2*
LBP109	ATTAAGTAAAAAAAATAAGGACTTTAATTTTTAGATATCTTTGTTTTGCATTTTACATTC	
LBP118	CAGAGCGGTGAAACGCAAC	Amplification and sequencing of Δ*pyk2*::*pPDR12*-GFP-*tCYC1*
LBP119	CGCAGTTTGCGAACATTACCTG	
Amplification of CRISPR/Cas9 plasmid pRCC-K		
WGP234	CTTGGTGGTGTTCGTCGTATCTCTTAATCATAGAAGCAGACAATGGAG	Amplification of pRCC-K with gRNA for *PYK2*
WGP235	TGTTGTCTGACATTTTGAGAGTTAACACCGAAATTACCAAGGCTC	
MGP193	TTGCAATTCGGAGTCCGCAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	

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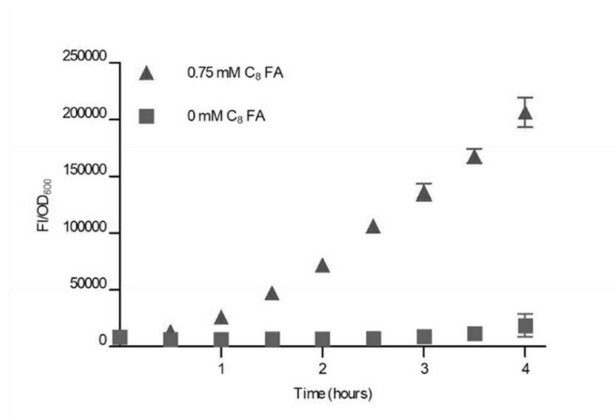| MGP194	CTTGCGGACTCCGAATTGCAAGATCATTTATCTTTCACTGCGGAG	
gRNA sequences used for deletion of *PYK2*		
PYK2 gRNA	TTGCAATTCGGAGTCCGCAA	

Figure S1. Time-dependent response of the biosensor in SCD medium with and without C_8 fatty acids. Relative fluorescence intensity (fluorescence intensity (FI) divided by OD_{600}) of the plasmid-based sensor in response to 0 (grey squares) and 0.75 mM (red triangles) C_8 fatty acids (FA), respectively. The background fluorescence of the biosensor strain not exposed to C_8 FA is very low over the entire time course. Error bars represent two technical replicates.
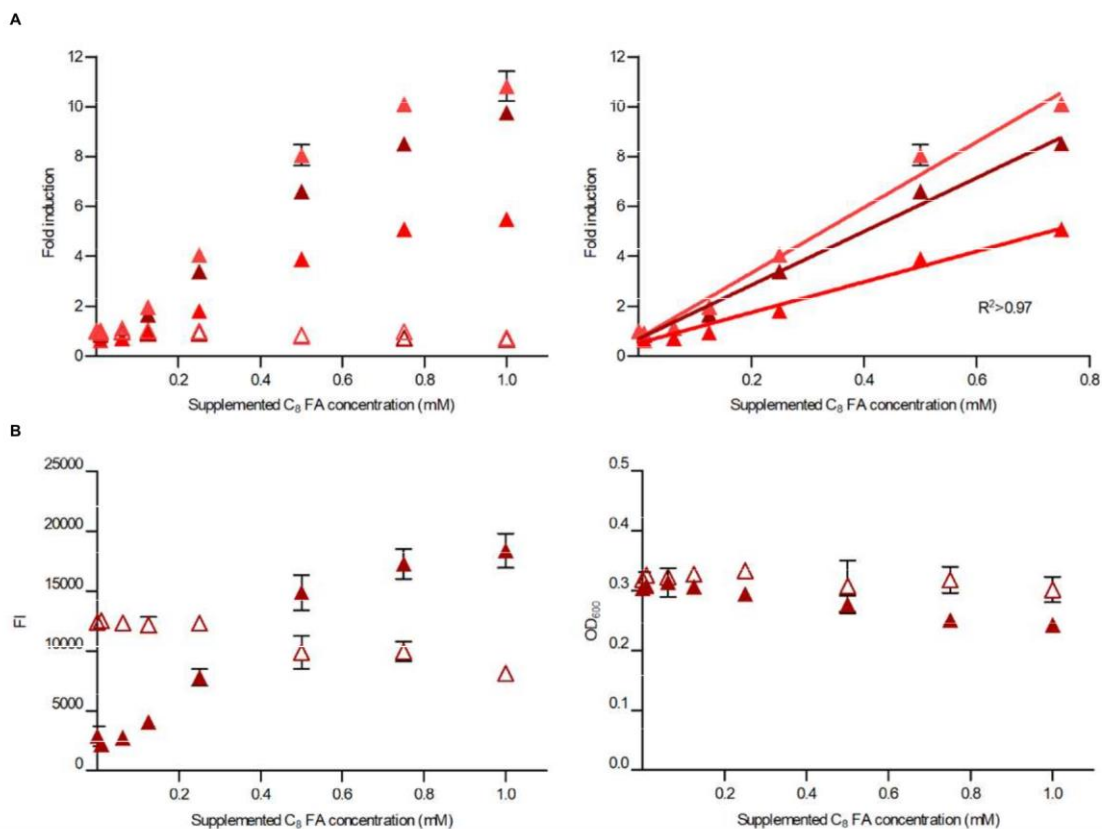
Figure S2. C_8 fatty acid-dependent response of the biosensor in SCD medium. (A) Response (left) and linear range (right) of the biosensor after 2 hours incubation with supplemented C_8 fatty acids (FA) in SCD medium. Shown are three biological replicates with two technical replicates each. For fold induction values, fluorescence intensities (FI) were divided by optical densities (OD$_{600}$) and normalized to FI/OD$_{600}$ values of samples without C_8 FA. (B) FI (left) and OD$_{600}$ (right) of all three biological replicates. Filled triangles: CEN.PK113-11C + p426pPDR12-GFP. Clear triangles: CEN.PK113-11C + p426pMET25-GFP.
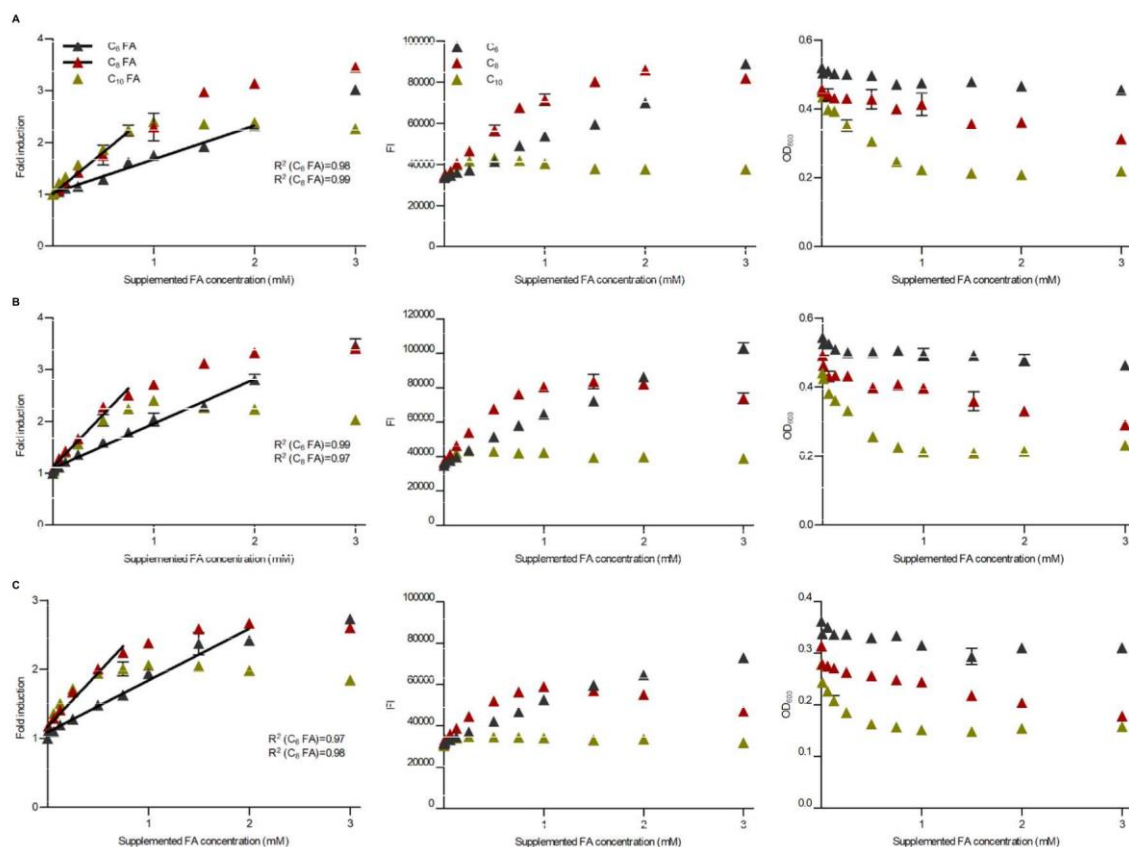
Figure S3. C_6, C_8 and C_{10} fatty acid-dependent response of the biosensor in YPD medium of three biological replicates (A, B, C). Response and linear range (left), fluorescence intensities (FI; middle) and optical densities (OD_{600}; right) after 4 hours incubation with supplemented C_6, C_8 or C_{10} fatty acids (FA) of all three biological replicates. Linear ranges were only observed in response to C_6 and C_8 FAs (left). Error bars represent two technical replicates. For fold induction values, FIs were divided by OD_{600} values and normalized to FI/OD_{600} values of samples without FA.
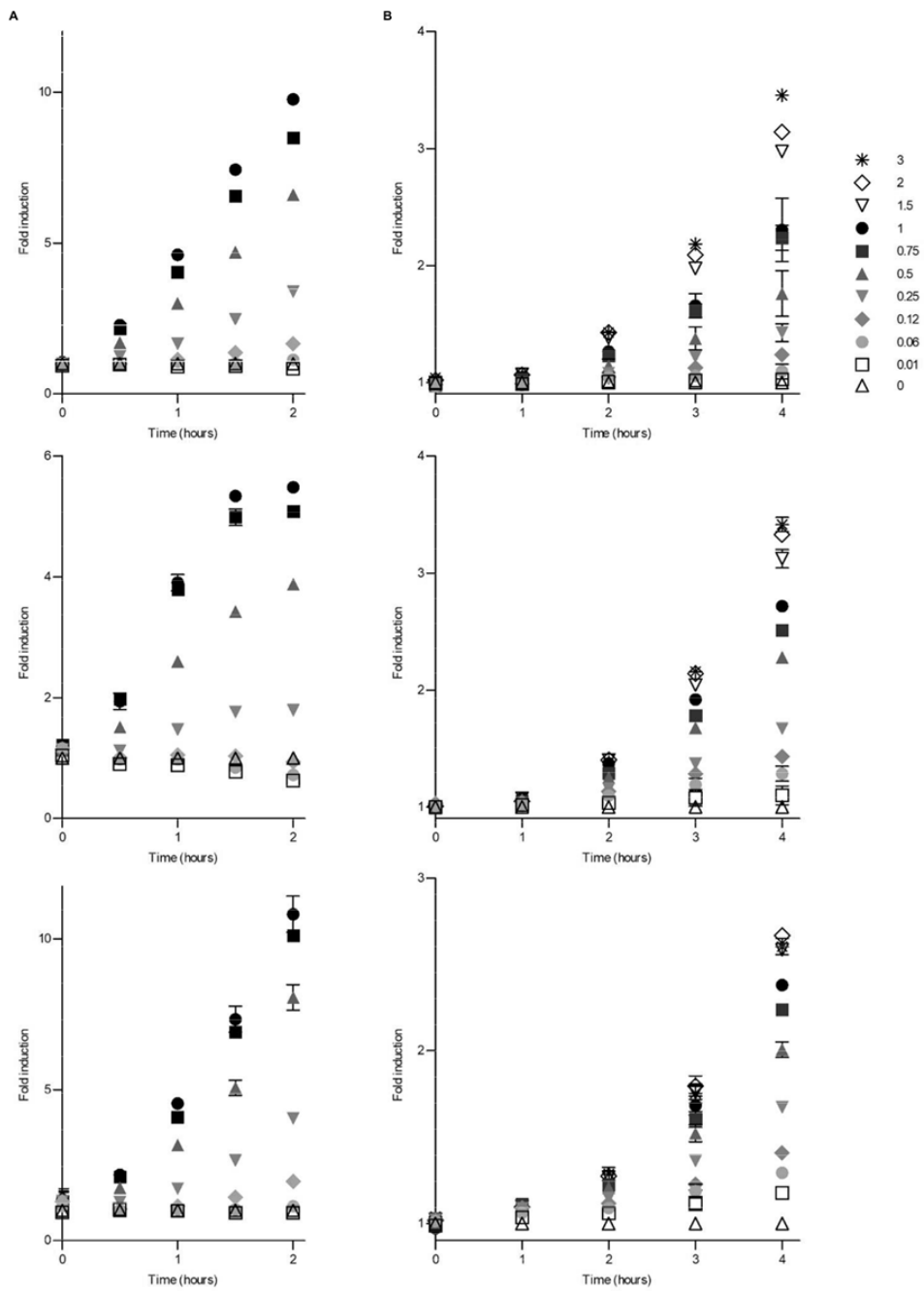
Figure S4. Time-dependent response of the biosensor to different C_8 fatty acid concentrations. Response over a 2 hour incubation period to supplemented 0-1 mM C_8 fatty acids (FA) in SCD (A) and over a 4 hour incubation period to supplemented 0-3 mM C_8 FA in YPD medium (B) of three biological replicates. Error bars represent two technical replicates. For fold induction values, fluorescence intensities (FI) were divided by optical densities (OD_{600}) and normalized to FI/OD_{600} values of samples without C_8 FA (0 mM).

Figure S5. C_{12} fatty acid-dependent growth and fluorescence of the biosensor. Fluorescence intensities (FI; left) and optical densities (OD_{600}; right) in response to supplementation with C_{12} fatty acids (FA) after 4 hours incubation in YPD medium. Error bars represent two technical replicates.
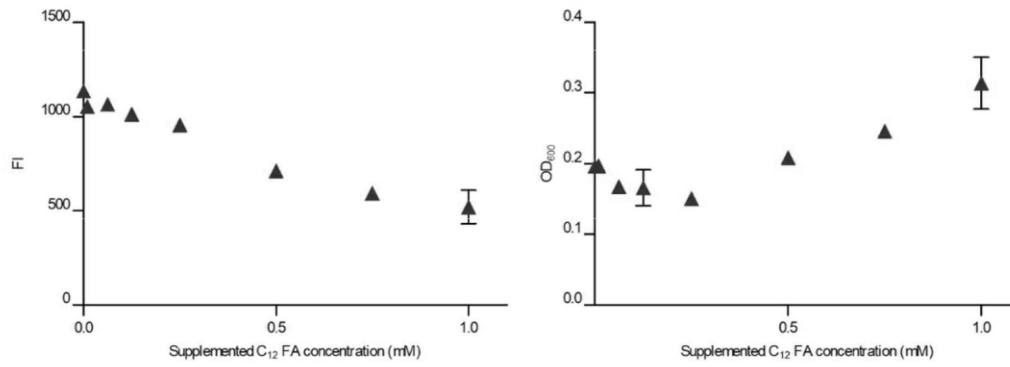
A

B
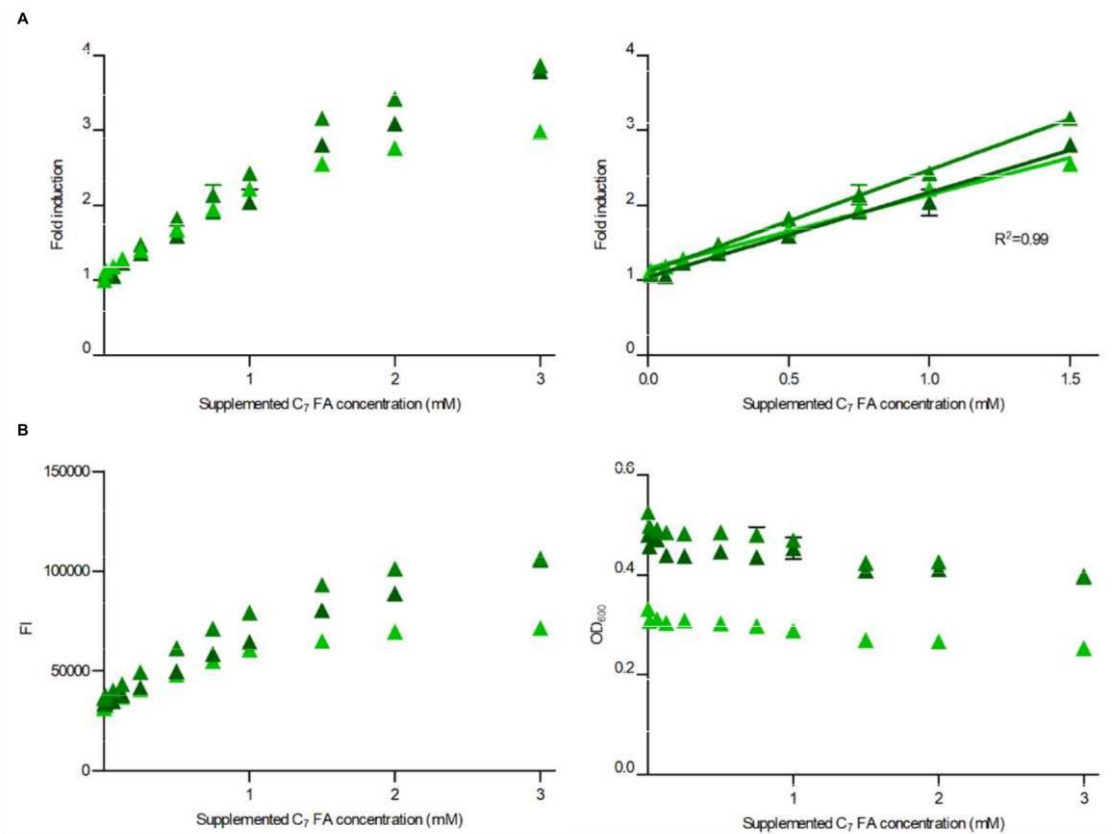
Figure S6. C_7 fatty acid-dependent response of the biosensor. (A) Response (left) and linear range (right) of the biosensor after 4 hours incubation with supplemented C_7 fatty acids (FA) in YPD medium. For fold induction values, fluorescence intensities (FI) were divided by optical densities (OD_{600}) and normalized to FI/OD_{600} values of samples without C_7 FA. (B) FI (left) and OD_{600} (right) values of all three biological replicates. Shown are three biological replicates with error bars representing two technical replicates.

Figure S7. Biosensor response to fatty acids in *S. cerevisiae* culture supernatants and correlation to GC measurement. (A) Linear correlation of the fold induction of biosensor signal in 0.25 dilutions of culture supernatants with GC measurements of C_8 fatty acids (FA) of the same supernatants. (B) Linear correlation of the fold induction of biosensor signal in 0.5 dilutions of culture supernatants with GC measurements of C_6 and C_8 FA 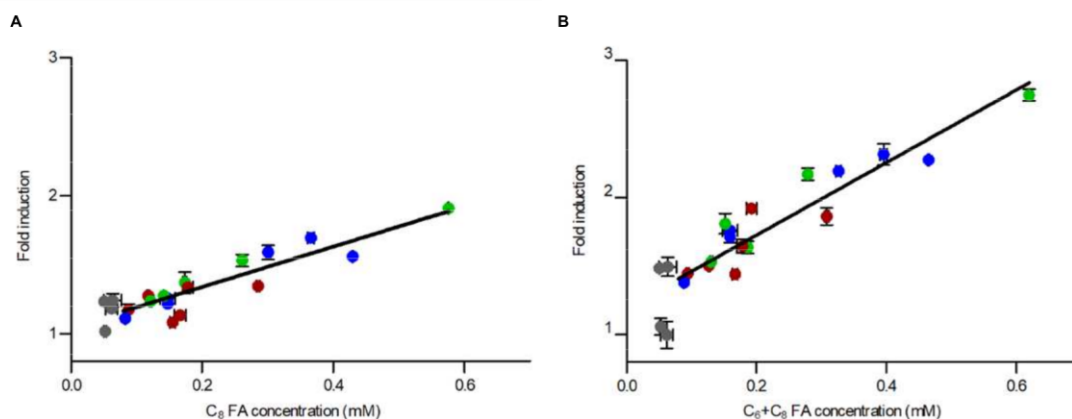of the same supernatants. Strains: CEN.PK113-7D (grey), RPY21/FAS^{R1834K}/ pRS42H (red), RPY21/FAS^{R1834K}/ LBV17 (blue), RPY21/FAS^{R1834K}/ LBV20 (green).

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6.2 Engineering *Saccharomyces cerevisiae* for production of fatty acids and their derivatives

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(2) Conducting tests and experiments

n/a

(3) Literature collection

Doctoral candidate LB: 35%

Co-authors FW, SB, MO: 15%, 15%, 35%

(4) Analysis and interpretation of data

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(5) Drafting of manuscript

Doctoral candidate LB: 35%

Co-authors FW, SB, MO: 15%, 15%, 35%

14   Engineering *Saccharomyces cerevisiae* for Production of Fatty Acids and Their Derivatives

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CONTENTS

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Abbreviations

AcCoA	Acetyl-CoA
AcP	Acetyl-phosphate
ALE	Adaptive laboratory evolution
CoA	Coenzyme A
FA	Fatty acid(s)
FACS	Fluorescence-activated cell sorting
FAEE	Fatty acid ethyl ester(s)
GAP	Glyceraldehyde-3-phosphate
gTME	Global transcription machinery engineering
MalCoA	Malonyl-CoA
PPP	Pentose phosphate pathway
SE	Steryl ester
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TE	Thioesterase
X5P	Xylulose 5-phosphate

I. Introduction

Fatty acids (FAs) and their derivatives, such as fatty alcohols, dicarboxylic acids, FA esters, alkanes, and alkenes, are frequently summarized under the generic term "**oleochemicals.**" The diversity of physicochemical properties of oleochemicals is determined by their functional groups and by the length of the aliphatic chains. This is reflected by a variety of products containing these classes of compounds, including fuels, lubricants, surfactants, detergents, cosmetics, food additives, and pharmaceuticals. Consequently, the production volume of different oleochemicals is measured in millions of

tons per year (Rupilius and Ahma 2006), and the demand is steadily increasing. They are currently produced from petroleum (in which case the term "petrochemicals" is also used) or biomass-derived (mainly plant) fats. While the concerns associated with the exploitation of fossil resources have been known for a long time, there is an increasing awareness of problems caused by mass cultivation of oily plants (e.g., palms), for instance, excessive land use and water consumption (Schmidt 2015). The biorefinery concept, in which **engineered microbes** are used to convert various biomass feedstocks into desired products (Nielsen et al. 2013), offers a potentially more effective exploitation of resources and thereby reduces the environmental impact compared to extraction from plants. Indeed, significant advances in production of oleochemicals with different chain lengths and functionalities have been achieved in diverse microbial hosts (for review, see Yu et al. 2014; d'Espaux et al. 2015; Pfleger et al. 2015; Sarria et al. 2017; Marella et al. 2018; Xue et al. 2018; Zhang et al. 2018). Since rapid progress is made in the field, we feel that a critical re-evaluation of the literature is justified. Here, we will focus on strategies developed in *S. cerevisiae*, one of the best characterized and most popular biotechnological host organisms (see also Chap. 13 by Schmelzer et al.), in which highest yields of microbially produced FA have been recently reported (Yu et al. 2018). Different approaches for engineering precursor and cofactor supply, chain length control, elimination of by-product formation, and overcoming product toxicity as well as the development of biosensors for accelerating the screening for best performing strains are summarized and evaluated. Throughout the manuscript, we consciously do not collect all data on achieved titers and yields for different FA-derived products, as another recent review has provided a very detailed summary (Fernandez-Moya and Da Silva 2017). Strategies that have been developed in other host organisms (e.g., *Escherichia coli*) are occasionally discussed, if they appear suitable to expand the pathways already established in *S. cerevisiae*. Lastly, we give an overview of the production of FA-derived products in *S. cerevisiae* as well as methods for optimizing fermentation conditions, enabling the scale-up of current lab-scale fermentation processes.

II. Providing Carbon, Redox Power, and Energy for Fatty Acid Synthesis: The Options for Precursor Supply Routes

S. cerevisiae, an ethanologenic yeast, only requires relatively small amounts of FA as building blocks of membrane lipids. The FA synthesis naturally occurs in the cytosol by the FA synthase (FAS) that uses **acetyl-CoA (AcCoA)** and its derivative **malonyl-CoA (MalCoA)** as precursor molecules. AcCoA metabolism in yeast is compartmentalized, whereby the major part is synthesized inside mitochondria via the pyruvate dehydrogenase (PDH) complex (Krivoruchko et al. 2015) and the mitochondrial AcCoA cannot be exported to the cytosol (van Rossum et al. 2016a, c). The native pathway for the synthesis of cytosolic AcCoA in *S. cerevisiae* is referred to as **pyruvate dehydrogenase (PDH) bypass,** and it diverts only a minor part of the pyruvate produced from glucose (Pronk et al. 1996). Following decarboxylation of pyruvate by pyruvate decarboxylases, acetaldehyde is oxidized to acetate by **acetaldehyde dehydrogenases (ALDs),** and acetate is subsequently ligated to CoA by the **acetyl-CoA synthetases (ACS)**. This reaction is thermodynamically driven by hydrolysis of ATP to AMP and inorganic pyrophosphate, which is subsequently hydrolyzed by the inorganic pyrophosphatase. Thus, each AcCoA molecule is synthesized at the expense of two ATP equivalents by the PDH bypass. This reaction scheme implies that the glycolytic ATP supply (net two ATP moles per mol glucose) is insufficient, because energy equivalents are also needed for cell proliferation and maintenance. Thus, a significant proportion of the available (sugar) substrate must be diverted to the tricarboxylic acid (TCA) cycle and respiration for the supply of energy equivalents. This reduces the attainable product yield (van Rossum et al. 2016b) and, considering that most FA and derived products have a relatively low commercial value, makes their production through the PDH bypass uneconomical.

AcCoA is not only a building block of FA but also of many compound classes that are of interest in biotechnology, such as isoprenoids (e.g., artemisinic acid; Paddon et al. 2013), polyesters (e.g., polyhydroxybutyric acid; Kocharin et al. 2012), polyketides (e.g., 6-methylsalicylic acid; Wattanachaisaereekul et al. 2008), or flavonoids (e.g., naringenin; Koopman et al. 2012). To avoid constraints such as limited metabolic capacity of organelles, availability of cofactors, and transport of products across organellar membranes, heterologous pathways for production of these compounds are preferably expressed in the cytosol. Therefore, significant effort has been devoted to improving the cytosolic AcCoA supply in *S. cerevisiae*. An excellent review article (van Rossum et al. 2016b) provided a systematic analysis of possible pathways to optimize the supply of cytosolic AcCoA under consideration of reaction stoichiometry, energy conservation, and maximally attainable yields for four model AcCoA-derived compounds including FA. Here, we will revisit the current literature that exploited some of those different possibilities (summarized in Fig. 1) to produce FA and other AcCoA-derived products. Since the provision of **reducing equivalents**—either NADH or NADPH—is equally important for FA production and intrinsically dependent on the choice of the AcCoA synthesis route, the redox cofactor supply will be discussed throughout this chapter. The synthesis of FA can be engineered to occur either via FAS that strictly requires NADPH or by reversing β-oxidation, a FA degradation pathway, which allows for more flexibility regarding the **cofactor dependence** (see Sect. III.B). Therefore, the choice of the appropriate AcCoA pathway also depends on the synthesis mode of FA.

A. Engineering the Pyruvate Dehydrogenase Bypass

In most proof-of-concept studies, the production of AcCoA-derived products in *S. cerevisiae* relied on the native PDH bypass or engineered variants of it, without introducing heterologous AcCoA routes. Even when various alternative AcCoA pathways were introduced, the native

PDH bypass was present in the background of all engineered strains, with one exception (Meadows et al. 2016) known to us (see Sect. II.C).

An increased flux through the PDH bypass can be achieved by redirecting acetaldehyde away from reduction to ethanol by alcohol dehydrogenases (ADHs) toward oxidation to acetate by ALDs. In a first study (aiming to increase the production of the isoprenoid amorphadiene), it could be shown that overexpressing the acetaldehyde dehydrogenase **ALD6** alone substantially increased the accumulation of acetate (Shiba et al. 2007). Since Ald6 is NADP-specific, this step is also important to provide NADPH required by FAS. Next, the authors tested different ACS variants, whereby only the expression of a heterologous enzyme from *Salmonella enterica*, mutated to prevent inactivation by acetylation (**SeACS**^{L641P}), led to a substantial increase in amorphadiene production (Shiba et al. 2007). In the same study, the overexpression of endogenous enzymes had no (for Acs2) or little (for Acs1) effect on amorphadiene titers, which was attributed to negative regulatory mechanisms at the post-translational level, possibly acetylation. In subsequent studies, a positive effect of combined Ald6/SeACS^{L641P} overexpression on production of several AcCoA-derived products, including FA derivatives such as FA ethyl esters (FAEEs) and hexadecanol, was confirmed by different groups (Chen et al. 2013b; Krivoruchko et al. 2013; Jong et al. 2014; Lian et al. 2014; Feng et al. 2015).

As a strategy to utilize the major fermentation product ethanol for AcCoA synthesis, the ADH isoform II (Adh2), which is the main enzyme responsible for oxidation of ethanol to acetaldehyde, was co-expressed with Ald6/SeACS^{L641P} in some variants (Chen et al. 2013b; Krivoruchko et al. 2013; Jong et al. 2014; Feng et al. 2015). Unfortunately, the effect of Adh2 overexpression alone remained unclear, since it was overexpressed only in combination with other enzymes (e.g., acetoacetyl-CoA thiolase Erg10) that affect the product yield (Kocharin et al. 2012; Chen et al. 2013b; Krivoruchko et al. 2013) or together with Ald6 and SeACS^{L641P}, without a direct comparison to a control not overexpressing Adh2 (Jong et al. 2014; Feng et al. 2015). Considering that the interconversion of acetaldehyde and ethanol is rather driven by the chemical equilibrium, it remains yet to be demonstrated that Adh2 overexpression indeed diverts the flux toward AcCoA in engineered strains in the presence of glucose.
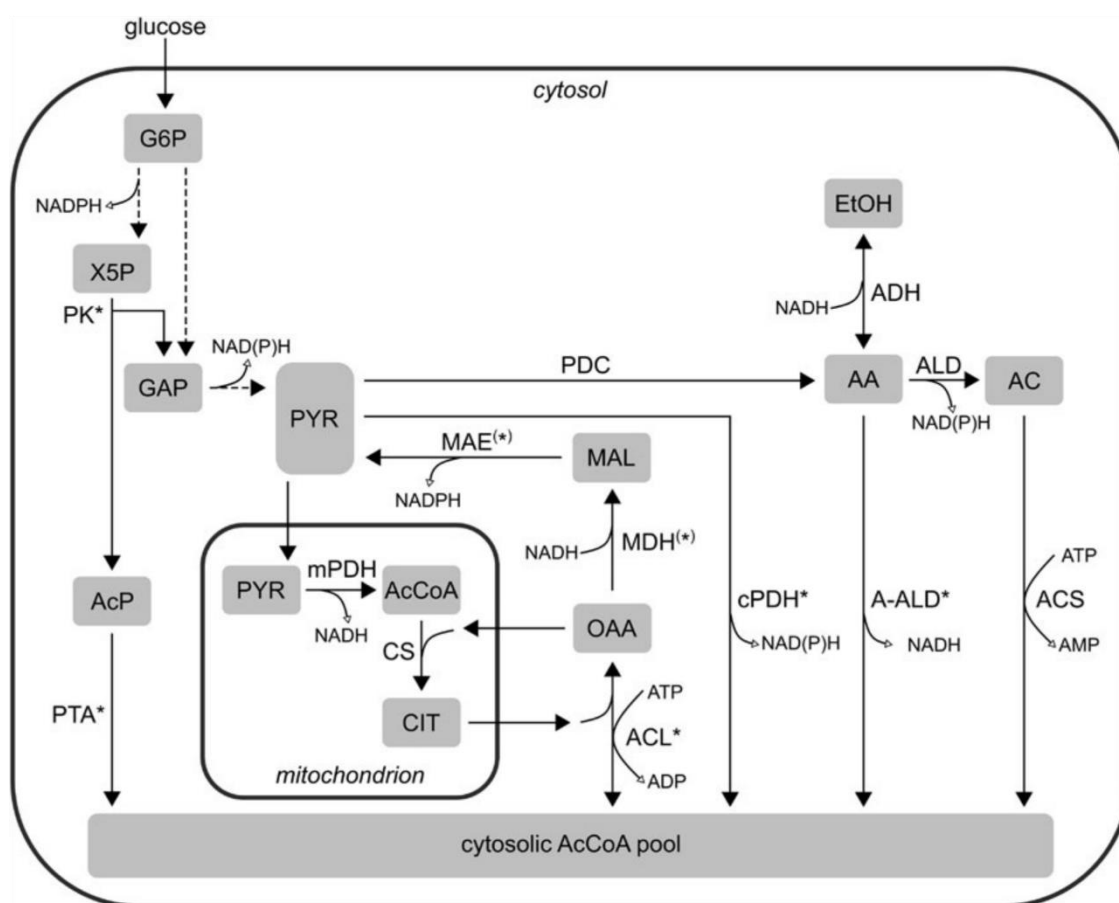
Fig. 1 A simplified scheme of precursor supply pathways for production of acetyl-CoA (AcCoA) derivatives in the cytosol of *S. cerevisiae*. Only the relevant pathway intermediates are shown in gray boxes: glucose-6-phosphate (G6P), xylulose 5-phosphate (X5P), glyceraldehyde-3-phosphate (GAP), pyruvate (PYR), acetaldehyde (AA), ethanol (EtOH), acetate (AC), acetyl-phosphate (AcP), citrate (CIT), oxaloacetate (OAA), and malate (MAL). Solid lines depict single-step reactions, whereas dashed lines stand for multiple enzymatic steps. Reversibility of reactions is indicated by double-headed arrows only if relevant in the context of AcCoA supply. For reactions that can be catalyzed by enzymes with different specificities, the generic notation "NAD(P)H" is used; otherwise, the specific cofactor is denoted. For clarity, only the reduced form of the redox cofactors is shown. The reaction stoichiometries are not considered, and energy equivalents are shown only for the AcCoA supply reactions. Enzyme names are abbreviated as follows: *PK* phosphoketolase, *PTA* phosphotransacetylase, *PDC* pyruvate decarboxylase, *ALD* aldehyde dehydrogenase, *mPDH* mitochondrial pyruvate dehydrogenase, *cPDH* cytosolic pyruvate dehydrogenase, *CS* citrate synthase, *ACL* ATP citrate lyase, *MDH* malate dehydrogenase, *MAE* malic enzyme, *A-ALD* acetylating aldehyde dehydrogenase, *ACS* acetyl-CoA synthetase; heterologous enzymes are marked with an asterisk * and endogenous enzymes that are expressed in a different compartment with an asterisk in brackets $^{(*)}$. Transporters are not shown. Influx/efflux of (pyro)phosphate, carbon dioxide, and coenzyme A (CoA) moieties is omitted for clarity

A logical approach to boost the production of cytosolic AcCoA via the PDH bypass is the **elimination of ethanol production** by deleting the ADH genes. Li et al. (2014) first demonstrated that the deletion of *ADH1* is beneficial for FA production. Lian et al. (2014) went a step further and deleted the isoforms *ADH1* and *ADH4* with a concomitant deletion of glycerol-3-phosphate dehydrogenases *GPD1* and *GPD2* to prevent accumulation of glycerol, a trait pre-

viously observed in ADH-deficient strains (Smidt et al. 2012). This elimination of competing products already increased the flux toward AcCoA, measured as an increase in n-butanol production through a simultaneously expressed heterologous pathway (Lian et al. 2014). Schadeweg and Boles (2016b) could later show that a deletion of ADH isoforms 1–5, even without a GPD deletion, substantially increases the production of n-butanol through a similarly designed pathway. When Ald6/ SeACS^{L641P} were overexpressed in an ADH-/ GPD-deficient strain, the production of n-butanol unexpectedly decreased due to a strong accumulation of acetate (Lian et al. 2014). Conversely, overexpression of SeACS^{L641P} alone improved the productivity in the same strain background (Lian et al. 2014; Lian and Zhao 2015). Taken together, the analyses performed in ADH positive (Shiba et al. 2007) and negative (Lian et al. 2014; Lian and Zhao 2015) strains suggest that the **balance of ALD and ACS activity** is a critical and context-dependent variable in optimizing the flux through the cytosolic PDH bypass due to the rate-limiting role of ACS and the accumulation of toxic acetate levels.

B. ATP-Independent Pyruvate-to-Acetyl-CoA Routes

The high energy requirement makes the PDH bypass a rather inefficient precursor supply pathway for manufacturing AcCoA-derived products. Therefore, several alternative ATP-independent AcCoA yielding pathways were tested in *S. cerevisiae* for different products. One possibility to convert acetaldehyde to AcCoA is via **acetylating aldehyde dehydrogenases (A-ALD)**. Their functionality in yeast was demonstrated by the ability to complement the growth defect of an ACS-deficient strain (Kozak et al. 2014b) and to replace endogenous acetaldehyde dehydrogenases (Kozak et al. 2016). Schadeweg and Boles (2016b) demonstrated increased n-butanol production via reverse β-oxidation when they overexpressed A-ALD from *E. coli*, mutated to favor the (nonphysiological) reaction direction from acetalde-

hyde to AcCoA (adhE$^{A267T/E568K}$; Membrillo-Hernandez et al. 2000) in an ADH-deficient strain. However, a positive effect of A-ALD overexpression could only be seen when the supply of **coenzyme A (CoA)** was concomitantly increased by overexpression of a heterologous pantothenate kinase (coaA from *E. coli*) and pantothenate feeding, demonstrating that the availability of not only acetyl moieties but also of the coenzyme is a factor limiting the synthesis of AcCoA (Schadeweg and Boles 2016b).

This notion likely applies to all AcCoA pathways, as the overexpression of the pantothenate kinase and pantothenate supplementation was also beneficial in combination with an engineered PDH bypass (Ald6/ SeACS^{L641P} overexpression) in a strain constructed for production of the flavonoid naringenin (Liu et al. 2017). The endogenous pantothenate supply can be improved by overexpressing the polyamine oxidase Fms1 (Schadeweg and Boles 2016a), which catalyzes the limiting step of its biosynthesis.

A-ALD pathway is a very promising alternative to the PDH bypass to produce AcCoA derivatives, owing to its lower energy requirement. However, the theoretically attainable yield for FA produced by FAS is lower when A-ALD is used compared to PDH bypass (van Rossum et al. 2016b) due to cofactor incompatibility (the A-ALD pathway yields NADH, while FAS requires NADPH). Thus, only if the FAs are produced via reverse β-oxidation, which can be engineered to utilize exclusively NADH, the implementation of the A-ALD pathway appears feasible. The same is true for other alternative pathways that convert pyruvate to AcCoA in an ATP-independent manner and thereby yield NADH, namely, (1) pyruvate formate lyase (PFL) combined with formate dehydrogenase, and (2) **cytosolic pyruvate dehydrogenase complex (cPDH)** (van Rossum et al. 2016b). Although the functionality of PFL in yeast cytosol has been demonstrated (Waks and Silver 2009; Kozak et al. 2014b), it has not been used for high-level production of FA or other AcCoA derivatives so far, due to its complex biochemical properties, including strong oxygen sensitivity (Knappe et al. 1969). In contrast, a significant improvement of n-butanol produc-

tion was reported when a bacterial PDH or the native PDH components lacking the mitochondrial targeting sequence were overexpressed in yeast cytosol (Lian et al. 2014).

It has to be noted, however, that the cytosolic PDH activity was not directly demonstrated in this study. In another study (Kozak et al. 2014a), it has been shown that the activity of PDH in the yeast cytosol is strictly dependent on supplementation of lipoic acid, a cofactor that is normally synthesized inside mitochondria and must be covalently linked to the E2 subunit of PDH. Moreover, overexpression of enzymes involved in lipoylation of E2 in the cytosol is crucial (Kozak et al. 2014a). Since Lian et al. (2014) did neither supplement lipoic acid nor express the components of the lipoylation machinery, the mechanism by which cPDH led to an improvement of n-butanol production in their work remains enigmatic.

Of particular interest for FA synthesis via the (NADPH-dependent) FAS would be an implementation of NADPH-yielding cPDH variants. Indeed, a mutated (NADP$^+$-accepting) PDH was developed (Bocanegra et al. 1993) and implemented to produce the polyketide triacetic acid lactone in yeast cytosol. Although the cytosolic PDH activity was not directly measured, this strategy led to a significantly increased NADPH/NADP ratio and AcCoA levels in whole cell extracts as well as to improved product titers in fermentations (Cardenas and Da Silva 2016). Since this approach has the same cofactor yield (one NADH and one NADPH per molecule AcCoA) but a better energetic balance compared to the PDH bypass, it could potentially improve the production of FA. However, the study did not address the abovementioned requirement for lipoylation, and this aspect will therefore require further investigation before this approach can be employed for FA production.

C. Phosphoketolase Pathway

Among all individually considered alternative AcCoA pathways, the phosphoketolase/transacetylase variant was predicted to enable the highest yield of FAS-derived FA (87% of the theoretical maximum), when the carbon flux is rerouted from glycolysis to the oxidative **pentose phosphate pathway (PPP)** to increase the NADPH pool (van Rossum et al. 2016b). In this scheme, glucose-6-phosphate is converted to xylulose 5-phosphate (X5P) through a consecutive action of the endogenous enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase, and D-ribulose-5-phosphate 3-epimerase, whereby the first and the third reaction yield one NADPH molecule each (for a review of the PPP, see Stincone et al. 2015). X5P is then converted to **acetyl-phosphate (AcP)** and **glyceraldehyde-3-phosphate (GAP)** by a heterologous **phosphoketolase (PK)**. Subsequently, AcP is directly converted to AcCoA by a heterologous **phosphotransacetylase (PTA)**. The PK/PTA pathway was expressed in a strain engineered to produce FAEE and indeed led to a significant increase of product titers compared to the reference strain containing only the native PDH bypass (Jong et al. 2014).

Another variant to convert PKA-derived AcP to AcCoA involves the acetate kinase (ACK), which yields acetate and ATP by transferring the phosphate residue from AcP to ADP. Although ATP is produced in this reaction, the production of AcCoA via this route is energetically less favorable than via PTA since two ATP have to be invested into the subsequent activation of acetate by ACS (see above). The implementation of this strategy led to a significant improvement of polyhydroxybutyrate (Kocharin et al. 2013) and, to a lower extent than with the PK/PTA variant, FAEE production (Jong et al. 2014).

In one recent study (Meadows et al. 2016), the PK/PTA pathway was combined with the A-ALD to produce the isoprenoid farnesene, as the **combinatorial configuration** was calculated to lead to the highest possible product yields (van Rossum et al. 2016b). Interestingly, the authors found that AcP produced by PK is partly dephosphorylated by endogenous (promiscuous) glycerol-3-phosphate phosphatases and their activity needs to be reduced to favor the PTA reaction. This study stands out as the only one in which the endogenous PDH bypass was fully substituted (by *ald6 acs1 acs2* deletion) with heterologous AcCoA synthesis routes

(A-ALD and PK/PTA). Indeed, this approach led to the highest yields ever reported of an isoprenoid produced in *S. cerevisiae* and impressively demonstrated how an "ideal" combination of precursor supply pathways based on stoichiometric analyses can be designed to produce AcCoA derivatives at an industrially competitive level.

D. Citrate-Oxaloacetate Shuttle

The major breakthroughs regarding production of oleochemicals in *S. cerevisiae* have been recently achieved by implementing the citrate-oxaloacetate shuttle. If NADPH supply is not concomitantly engineered, this pathway, like the ATP-independent pyruvate to AcCoA routes, has the lowest expected yield for FA produced via FAS (74% of the theoretical maximum; van Rossum et al. 2016b) due to the imbalance of NADH production and NADPH requirement of FAS. Nevertheless, many oleaginous yeasts, such as *Yarrowia lipolytica*, which produce large amounts of lipids in the cytosol, rely on this system to provide cytosolic AcCoA (Vorapreeda et al. 2012). The term "shuttle" refers to the fact that AcCoA is formed from pyruvate inside mitochondria via the native PDH complex. Subsequently, the acetate moiety is transferred to oxaloacetate by **citrate synthase (CS)**. The resulting citrate either can enter the TCA cycle inside the mitochondria or be exported to the cytosol to serve as a shuttle for acetyl moieties. A cytosolic **ATP citrate lyase (ACL)**, an enzyme that is not present in *S. cerevisiae*, is required to form AcCoA and oxaloacetate under the expense of one ATP molecule. The cycle is closed by transporting oxaloacetate back into the mitochondria through a citrate-oxaloacetate antiporter. In a first attempt, the overexpression of a murine ACL in *S. cerevisiae* led to a slightly increased titer of total FA (Tang et al. 2013). The effect was mostly pronounced during the stationary phase, probably reflecting the redirection of the metabolism toward respiration of the accumulated ethanol.

In the same study, isocitrate dehydrogenase genes *IDH1* and *IDH2* were deleted to promote the accumulation of citrate; somewhat surprisingly, this intervention did not affect the total content but only the saturation profile of FA, suggesting that ACL activity and/or other factors were rate limiting. Later studies, performed in strains engineered for n-butanol (Lian et al. 2014), hexadecanol (Feng et al. 2015), or mevalonate (Rodriguez et al. 2016) production, showed that the choice of the heterologous enzyme has a certain impact on the pathway efficiency.

In most recent studies that reported the highest titers of FA or their derivatives (Zhou et al. 2016b; Yu et al. 2018), the citrate-oxaloacetate shuttle was further optimized and combined with strategies that increase the **NADPH supply**. The overexpression of ACL alone in a strain that was already engineered to block FA degradation led to a moderate improvement (up to 50%) of free FA titers, which is consistent with previous studies (Tang et al. 2013; Lian et al. 2014). A further improvement was achieved by introducing a **transhydrogenase**-like reaction sequence to increase NADPH supply, exemplifying the importance of redox cofactors as a driving force for FA production. In this scheme, a cytosolic **malate dehydrogenase (MDH)** reduces oxaloacetate to malate (oxidizing NADH), and a cytosolic **malic enzyme (MAE)** subsequently transfers hydrogen to NADP, whereby malate is converted to pyruvate, which then re-enters mitochondria and undergoes a new cycle (Fig. 1). Additional overexpression of the endogenous citrate transporter Ctp1 appeared to have no beneficial effect on the FA production, suggesting that the export of citrate into the cytosol may not have been a limiting factor in this approach. In a follow-up study (Yu et al. 2018), several strategies to enhance the citrate-oxaloacetate shuttle were tested. Overexpression of the endogenous (mitochondrial) PDH components to enhance the mitochondrial synthesis of AcCoA did not lead to an increase in FA titers in this study. However, the overexpression of the **mitochondrial pyruvate carrier** subunits Mpc1 and Mpc3 and introduction of a heterologous CS in addition to the native one appeared to increase the flux through the

citrate-oxaloacetate shuttle, measured as an increase in FA titers by about 20%.

An incremental improvement was achieved by introducing an additional ACL gene from *Aspergillus nidulans* (a murine ACL was already present in the engineered strain). Simultaneously the isocitrate dehydrogenase Idp2 and the citrate-α-ketoglutarate antiporter Yhm2, which also accepts oxaloacetate instead of α-ketoglutarate, were overexpressed. Idp2 was supposed to oxidize citrate to α-ketoglutarate and thereby provide additional NADPH for FA synthesis. This, however, can only occur at the expense of the cytosolic citrate and therefore AcCoA pool. Yhm2 overexpression was intended to increase the antiport of citrate with oxaloacetate. Unfortunately, since three genes were introduced at once as compared to the reference strain, their individual contribution to the improvement cannot be disentangled.

A further substantial improvement of FA synthesis in this strain background was achieved by **diverting the glucose flux from glycolysis to the oxidative PPP** to increase the NADPH pool. To this end, the expression of the phosphoglucose isomerase (*PGI1*) was downregulated, whereas the endogenous genes encoding PPP enzymes glucose-6-phosphate dehydrogenase (*ZWF1*), 6-phosphogluconate dehydrogenase (*GND1*), transketolase (*TKL1*), and transaldolase (*TAL1*) were concomitantly overexpressed. A downregulation of the mitochondrial isocitrate dehydrogenase *IDH2* to prevent the degradation of citrate in the TCA led to a further improvement but only in a strain with the upregulated PPP. In its precursor strain, lowering Idh2 activity had no beneficial effect, and this notion is consistent with previous results, obtained in a strain that contained the citrate-oxaloacetate shuttle, but was not engineered for an increased supply of NADPH (Tang et al. 2013). Collectively, these studies show that sufficient reducing power is essential for efficient FA production.

E. Strategies to Manipulate NADPH Level Independently of Precursor Supply Routes

Besides abovementioned possibilities of overexpressing enzymes that yield NADPH (e.g., Ald6, Zwf1) or creating transhydrogenase cycles (e.g., oxaloacetate-malate-pyruvate), the level of this cofactor can be manipulated through interventions into glycolysis or amino acid metabolism. One possibility to increase the pool of NADPH is bypassing the main source of cellular NADH—the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction—by a NADP-dependent enzyme. The expression of a heterologous non-phosphorylating **NADP-dependent GAPDH** in strains engineered for polyhydroxybutyrate (Kocharin et al. 2013) or FAEE (Shi et al. 2014a) production led to considerable product yield improvements, but not in a strain designed to produce fatty alcohols (d'Espaux et al. 2017). It has to be noted that—besides different product pathways—the genetic interventions into the carbon metabolism of these strains largely differed, suggesting that the applicability of this strategy is context-dependent. One important consideration is that the activity of a non-phosphorylating GAPDH will reduce the ATP yield of glycolysis, which, as outlined above, ultimately will lead to a reduction of the maximally attainable yield. The implementation of a phosphorylating NADP-dependent GAPDH does not suffer from this downfall, but its utility for production of oleochemicals in *S. cerevisiae* could not yet be demonstrated (d'Espaux et al. 2017). A complementary approach is **reducing the NADPH consumption** through competing pathways. Based on the observation that a large proportion of NADPH is oxidized by NADP-dependent glutamate dehydrogenase Gdh1, d'Espaux et al. (2017) demonstrated that a deletion of the *GDH1* gene is beneficial for fatty alcohol production.

F. Engineering Malonyl-CoA Supply

MalCoA, the extender unit for FA synthesis via FAS, is synthesized by carboxylation of AcCoA in an ATP-dependent manner by the endogenous enzyme **AcCoA carboxylase (Acc1)**. In some studies, the native form of Acc1 was overexpressed to boost the production of FA derivatives, which indeed led to higher product titers (Shin et al. 2012; Runguphan and Keasling 2014). As an alternative approach, Wang

et al. (2014b) overexpressed a plant MalCoA synthetase, which ligates malonic acid and CoA to generate MalCoA directly. This improved the productivity of strains engineered for polyketide production and increased the total FA amount. Since the native Acc1 is known to be inactivated by phosphorylation by the kinase Snf1 upon glucose depletion, different strategies were developed to circumvent this regulatory mechanism, including relocalization of the mitochondrial ACC from *S. cerevisiae* (Hfa1) into the cytosol (d'Espaux et al. 2017) and site-directed **mutagenesis to prevent phosphorylation** (Choi and Da Silva 2014; Shi et al. 2014b). All of them led to remarkable improvements in yields of MalCoA-derived products.

The specific activity of the phosphorylation-resistant mutant Acc1^{S1157A} was higher compared to wild-type Acc1 even when measured in extract from glucose-grown cells (Choi and Da Silva 2014), suggesting that the protein might be partly phosphorylated also on this carbon source (not only on ethanol). In another study, a second (in silico predicted) phosphorylation site (S659) was mutated to alanine in addition to S1157, which led to an increase in specific activity and total FA yields compared to a control harboring the S1157A mutation alone (Shi et al. 2014b). Considering that Snf1 is activated by AMP (Sanz 2003), we hypothesize that phosphorylation resistance may become even more relevant on glucose-grown cells when the PDH bypass, which produces AMP in the ACS reaction (Fig. 1), is overexpressed.

Another important consideration for strain design is the **sensitivity of FA chain lengths to the AcCoA/MalCoA ratio**. Elevated concentration of MalCoA is known to favor the elongation of the FA carbon chains by FAS (Sumper et al. 1969). Indeed, the expression of Acc1^{S1157A} (Choi and Da Silva 2014; Hofbauer et al. 2014; Besada-Lombana et al. 2017) and Acc1$^{S1157A, S659A}$ (Zhou et al. 2016b) led to a higher proportion of longer FA chains. This demonstrates that, especially if tight chain length control is desired, the relative abundance of the precursor molecules must be subtly balanced. By affecting the lipid composition of the membranes, the ACC hyperactivity not only changes the product profiles but also

appears to **negatively influence cell growth**, which was attributed to an imbalanced synthesis of long-chain FA or depletion of intermediates (Shi et al. 2014b). On the other hand, the expression of Acc1^{S1157A} was shown to improve the resistance of yeast to medium-chain FA (see Sect. IV.B). All these observations demonstrate that balancing the ACC activity is a non-trivial task and several factors such as the AcCoA abundance, product chain length, and toxicity must be taken into account. The development of product-specific biosensors (see Sect. IV.C) will accelerate the screening of strains producing the desired chain length in a high-throughput manner, which will greatly facilitate the balancing of AcCoA and MalCoA supply.

To summarize, the endogenous supply of cytosolic precursors (AcCoA and MalCoA), redox cofactors (NADH or NADPH), and energy equivalents (ATP) via the PDH bypass is not sufficient for production of FA and derivatives in *S. cerevisiae* beyond the proof-of-concept level. Although there are no systematic studies comparing the FA yields in dependence on different precursor supply routes, stoichiometric analyses (van Rossum et al. 2016b) and experimental data outlined above show that there is no "one best" precursor supply pathway valid for all AcCoA-derived products. Since the choice of the FA elongation system (FAS vs. reverse β-oxidation; see Sect. III) dictates the redox cofactor requirement, the appropriate precursor supply pathway must be chosen accordingly. Moreover, to minimize the amount of carbon that needs to be diverted for energy supply, the native PDH bypass must be fully replaced by a combinatorial precursor supply configuration, as predicted by stoichiometric analyses for different model compounds (van Rossum et al. 2016b) and experimentally shown for the isoprenoid farnesene (Meadows et al. 2016). Considering that very promising improvements in FA productivity, reaching total yields up to 30% of the theoretical maximum, have been recently achieved (Yu et al. 2018), it is conceivable that similar combinatorial approaches can lead to further advances in the production of oleochemicals.

III. Strategies for Chain Length Control

The majority of microbially synthesized FAs are 14–20 carbon atoms in length (referred to as long-chain FA). Mainly saturated and mono-unsaturated FA with 16 or 18 carbon atoms are synthesized in *S. cerevisiae* (Cottrell et al. 1986). Engineering microbes like *E. coli*, *S. cerevisiae*, or oleaginous yeasts (e.g., *Yarrowia lipolytica*) toward FA and FA derivatives with alternating chain length is desired due to their compelling properties as biofuels; for the cosmetic, healthcare, and pharmaceutical industries; or as platform compounds (Lennen and Pfleger 2013).

In microorganisms however, biosynthesis of **short- (≤C4), medium- (C6–C12), or very long- (≥C22) chain FA** is rare and low in quantity. For this reason strategies for chain length control through a focus on the main anabolic (FA biosynthesis) and catabolic (β-oxidation) pathways have been developed.

A. Manipulation of the Natural Fatty Acid Biosynthesis and Elongation Machinery

Fatty acid biosynthesis follows a conserved chemistry among different types of FAS enzymes which differ in their general organization. **Type I FAS** systems (mainly eukaryotic) carry all catalytic domains in one multifunctional complex (Schweizer and Hofmann 2004). However, fungal type I FAS greatly differ from animal type I FAS in their architecture (Leibundgut et al. 2008).

In type I systems, FA biosynthesis is initiated by the transfer of the starter unit AcCoA to the acyl-carrier protein (ACP) by the acetyltransferase (AT) domain of FAS. Subsequently, the ACP domain delivers the acetyl residue to the ketosynthase (KS) domain. Similarly, the extension unit MalCoA is loaded onto the free ACP by the malonyl-palmitoyl transferase (MPT) domain. Acetyl and malonyl moieties are condensed by the KS domain to a β-ketoacyl intermediate, which is then further processed. The β-ketoacyl intermediate is reduced by the ketoacyl reductase (KR) domain

to form 3-hydroxyacyl-ACP, which is dehydrated by the dehydratase (DH) domain and eventually reduced by the enoyl reductase (ER) domain to a saturated acyl chain containing two additional carbon atoms. The reductive steps at the KR and ER domains are strictly NADPH-dependent. After completion of one cycle, the acyl chain is either condensed with another malonyl moiety for further elongation or released by the MPT domain as a CoA-bound thioester in fungal FAS (Lomakin et al. 2007). After release of acyl-CoA esters from the FAS complex, **thioesterases (TE)** cleave the thioester bond between the acyl chain and CoA to release free FA.

As a strategy for enrichment of short- and medium-chain FA, expression of short- and medium-chain-specific TE for early termination of FA biosynthesis has been established in multiple studies (Leber and Da Silva 2014; Fernandez-Moya et al. 2015; Xu et al. 2016; Zhu et al. 2017b). Combined with fungal FAS enzymes, soluble TE only have limited or no access to FAS-bound acyl-CoA or acyl-ACP esters, and protein engineers have therefore developed strategies to locate TE within the FAS complex. For instance, some type I FAS (e.g., from *Rhodosporidium toruloides* and *Aplanochytrium kerguelense*) harbor two redundant ACP domains, which are located inside of the FAS scaffold. It is therefore possible to replace one of them by a short-chain TE to enable a direct access to acyl intermediates inside the reaction chamber. The implementation of this strategy proved to be more efficient than the expression of free TE and increased short- and medium-chain FA production by 3- to 15-fold (Zhu et al. 2017b). In *Y. lipolytica* FAS, replacement of the MPT domain by a short- and medium-chain-specific TE resulted in an increase of up to 29% of C12–C14 FA of the total FA content (Xu et al. 2016).

As an alternative approach, the yeast FAS has been replaced by non-fungal enzymes. Human FAS (hFAS) carries its own TE domain, which releases the acyl moieties directly from the ACP (Leibundgut et al. 2008) and has a more flexible structure than yeast FAS (Brignole et al. 2009). Expression of hFAS, in which its own TE domain was deleted, in com-

bination with the short-chain-specific TE CpFatB1 (*Cuphea palustris*) or TEII (*Rattus norvegicus*) as free proteins enabled the production of C6–C10 FA (Leber and Da Silva 2014). Substituting the TE domain of hFAS by TEII in the same polypeptide chain substantially increased the production of short- and medium-chain FA.

In contrast to type I FAS, the catalytic domains of the **bacterial type II FAS** system are expressed as single enzymes. This allows a simpler manipulation of the subunits, and acyl intermediates are freely accessible for TE. Expression of the *E. coli* system (acpS, acpP, fabB, fabD, fabG, fabH, fabI, fabZ) in combination with the TE fatB from *Ricinus communis* in a FAS-deficient *S. cerevisiae* strain significantly increased the total FA titer and shifted the FA profile toward C14 FA production (Fernandez-Moya et al. 2015).

A minimally invasive strategy to rewrite the chain length control relies on **rational engineering of yeast FAS** by site-directed mutagenesis. For instance, an early release of shorter acyl chains (C6–C10 or C14) was achieved by introducing bulky residues like tryptophan into the KS domain to shorten the acyl-ACP binding channel in *S. cerevisiae* (Gajewski et al. 2017; Zhu et al. 2017b) or *Y. lipolytica* (Rigouin et al. 2017) FAS. Furthermore, it was shown that decreasing the affinity of the MPT domain for malonyl moieties by introducing one point mutation disfavors the chain elongation and promotes the release of shorter-chain (C6–C10) FA (Gajewski et al. 2017). One additional mutation was introduced into the AT domain for a more frequent priming with AcCoA, as this was expected to shift the product profile toward shorter chains; however, this modification proved only effective in combination with mutations in the MPT and/or KS domain. Single or multiple mutations in the three domains caused different ratios of C6–C10 FA, with some of the mutants exhibiting a considerable specificity, e.g., for C8 FA (Gajewski et al. 2017). Thus, rational engineering of FAS holds a great promise for narrowing down the product profile to the desired chain length.

Very long-chain FAs (C22–C26) are present in *S. cerevisiae* at low abundance (Welch and Burlingame 1973) but are essential compounds, e.g., for sphingolipids (Oh et al. 1997). After their release from the FAS complex, FAs are elongated at the ER membrane from (long-chain) acyl-CoA precursors. **Elongases** with different product specificities (Elo1, Elo2, and Elo3) carry out the elongation of acyl-CoA intermediates with MalCoA. Elo1 elongates medium- and long-chain compounds (C14–C16) to C18 FA, Elo2 elongates compounds up to 22 C atoms, and Elo3 elongates compounds up to 26 C atoms (Toke and Martin 1996; Oh et al. 1997). Reminiscent of cytosolic FA biosynthesis, two reductions and one dehydration step are necessary to complete very long-chain FA synthesis. To selectively increase the pool of C22, overexpression of *ELO2* and deletion of *ELO3* are necessary (Yu et al. 2017), whereas overexpression of only *ELO3* is sufficient for enrichment of C26 FA (Wenning et al. 2017). Alternatively, FAS I from mycobacteria has been shown to naturally generate FA with 22–26 carbon atoms (Kaneda et al. 1995). Expression of FAS I from *Mycobacterium vaccae* in an Elo2-/Elo3-deficient strain increased the C22 FA pool by fourfold (Yu et al. 2017).

B. Reversal of β-Oxidation as an Orthogonal Pathway for Fatty Acid Biosynthesis

The **β-oxidation** cycle naturally is an FA degradation pathway (for review see Hiltunen et al. 2003). In each turn of the cycle, FAs are truncated by removing two carbon atoms from the FA chain, thereby generating AcCoA.

Reversing all reactions of the β-oxidation can consequently be used as an alternative synthetic pathway for FA production. The single reactions of the β-oxidation are **equilibrium-balanced**, and the functionality of the enzymes in the reverse direction has been demonstrated (Dellomonaco et al. 2011; Clomburg et al. 2012). The reverse β-oxidation pathway starts with the condensation of two AcCoA to acetoacetyl-CoA by a **thiolase**, followed by the reduction of the β-ketogroup to 3-hydroxyacyl-CoA by a **reductase/dehydrogenase**, dehydration to trans-enoyl-CoA by a **hydratase/dehydratase**, and a final reduction by another reductase/dehydro-

genase, yielding butyryl-CoA. In this cycle, NADH or NADPH can serve as **electron donors** for the reductive steps (depending on the choice of heterologous enzymes). By iterating this reaction sequence, the acyl chain can be elongated by two carbon atoms per cycle. Depending on the desired chain length and functional groups of the products, the reverse β-oxidation can be **terminated through different enzymes**. For instance, TE can release free FA from the CoA-bound form, and aldehyde/ alcohol dehydrogenases can reduce the acyl-CoA esters to n-alcohols (see Sect. V).

Elongation beyond a length of four carbon atoms requires multiple turns of reverse β-oxidation, which is hampered by a **competition between the thiolase and the termination enzymes**. Thus, although the synthesis of minor amounts of longer-chain FA via reverse β-oxidation could be demonstrated at the proof-of-concept level in *E. coli*, the product yields gradually decreased with each iteration of the cycle (Dellomonaco et al. 2011; Clomburg et al. 2012). Follow-up studies therefore focused on improving the selectivity of reverse β-oxidation for longer-chain products. To this end, the core pathway was expanded by thiolases that accept acyl-CoA intermediates with various chain lengths, such as the β-ketothiolase BktB, which proved suitable to produce C6–C10 compounds in multiple studies (Dekishima et al. 2011; Clomburg et al. 2015; Kim et al. 2015). The chain length specificity of termination enzymes (i.e., TE for free FA) was shown to be the second key determinant for selective production of longer acyl chains (Clomburg et al. 2015; Kim and Gonzalez 2018). To prevent a premature termination of the cycle, the deletion of endogenous TE genes was critical in *E. coli* strains. These observations are likely transferrable to *S. cerevisiae*. To provide sufficient carbon and reducing equivalents, heterologous reverse β-oxidation pathways were expressed in the yeast cytosol. As a proof of concept, n-butanol production, for the synthesis of which only one functional turn of the cycle is needed, has been targeted. Using a variety of enzymes from different organisms and in combination with different precursor supply pathways (see

above), titers of up to 1 g L^{-1} could be reached (Lian and Zhao 2015; Schadeweg and Boles 2016a, b). Less effort has been dedicated to the production of **medium-chain products** in *S. cerevisiae* so far, but the feasibility could be demonstrated by combining reverse β-oxidation with a medium-chain-specific TE, CpFatB1, thereby enabling the production of C6–C10 FA, albeit at a low yield (Lian and Zhao 2015). It is certain that by transferring the expanded enzyme toolbox developed in *E. coli* (Kim and Gonzalez 2018) and fine-tuning the expression of endogenous TE, many of which were recently characterized (e.g., Kruis et al. 2018), the production of short- and medium-chain FA via reverse β-oxidation will be further optimized in *S. cerevisiae* in the future.

Directly compared, canonical FA biosynthesis and reverse β-oxidation both bear certain advantages and drawbacks. The most obvious advantage of FA biosynthesis is a rather strict chain length control that can be easily manipulated by protein engineering of FAS, as outlined above, whereas specific production of longer carbon chains via reverse β-oxidation is still very challenging. While the FAS functional modules operate as a perfectly synchronized machine within one macromolecular complex, it is difficult to fine-tune the activity of individual reverse β-oxidation enzymes. At the current stage of technology, reverse β-oxidation can compete with FA biosynthesis in the product range of up to ten carbon atoms. On the other hand, FA biosynthesis is energetically more expensive than reverse β-oxidation, since each elongation cycle by FAS requires one MalCoA that is synthesized at the expense of one ATP molecule. In contrast, reverse β-oxidation has the advantage of using only AcCoA for elongation steps, which also makes it insensitive to the AcCoA/MalCoA ratio, a parameter that has a significant influence on the chain length control by FAS and overall cellular fitness. Whereas the reverse β-oxidation can be designed to use the easily available NADH, FAS is strictly dependent on the less abundant NADPH. Thus, the redox cofactor supply of reverse β-oxidation requires far less interventions into the central

carbon metabolism compared to the FA biosynthesis route and offers a larger choice of potential precursor supply routes (see Sect. II, Fig. 1).

IV. Chassis Engineering

To achieve high FA production levels in *S. cerevisiae*, several obstacles have to be addressed within the chosen production pathway. Blocking the degradation of FA has been achieved through the disruption of different enzymes involved in β-oxidation. An issue that remains to be tackled is the toxicity of some FA. We will discuss different methods that have or could be employed to engineer more robust chassis. Furthermore, several biosensor systems have been developed, which can be used in high-throughput screenings to further speed up the search for best performing strains.

A. Elimination of By-product Formation and Fatty Acid Degradation

To increase FA pools, it is important not only to increase precursor pools (see Sect. II, Fig. 1) but also to downregulate competing pathways as well as to prevent degradation through specific gene deletions or overexpressions.

1. Prevention of β-Oxidation

The degradation, i.e., β-oxidation, of the already synthesized free FA or their activated forms, the acyl-CoAs, takes place in the peroxisomes (Hiltunen et al. 2003; van Roermund et al. 2003). In *S. cerevisiae*, β-oxidation of saturated FA is a cyclic mechanism mainly catalyzed by the three enzymes Pox1, Fox2, and Pot1 (Hiltunen et al. 2003). Pox1 is an acyl-CoA oxidase catalyzing the first step of the degradation of an acyl-CoA molecule. The dehydrogenated intermediate is then modified by the multifunctional enzyme Fox2, which acts as an enoyl-CoA hydratase as well as a 3-hydroxyacyl-CoA dehydrogenase, followed by thiolytic cleavage through Pot1, a 3-ketoacyl-CoA thiolase. These cyclic steps are repeated until the FA

molecule is completely degraded (Hiltunen et al. 2003).

Prior to peroxisomal β-oxidation, free FAs are activated to their **acyl-CoA form**. In *S. cerevisiae*, this reaction can be catalyzed by five enzymes, Faa1, Faa2, Faa3, Faa4, and Fat1 (Black and DiRusso 2007). Long-chain FAs are primarily activated by Faa1 and Faa4 in the cytosol (Scharnewski et al. 2008) and transported into the peroxisomes by the heterodimeric ATP-binding cassette transporter Pxa1/Pxa2 (Hettema et al. 1996). Fat1 is a multifunctional enzyme, which imports long-chain FA into the peroxisomes and also has an acyl-CoA synthetase activity for very long-chain FA (Zou et al. 2002). Medium-chain FAs, on the other hand, are assumed to enter peroxisomes by passive diffusion or spontaneous flipping and are then activated by the peroxisomal Faa2 (Knoll et al. 1994; Hettema et al. 1996; Hettema and Tabak 2000). The role of Faa3 is not entirely clear yet. It showed some activity on very long-chain FA; however, its in vitro activity on long-chain FA was much lower than that of Faa1 and Faa2 (Johnson et al. 1994; Knoll et al. 1994).

The effects of disrupted FA activation, β-oxidation, or both, on FA titers have been studied extensively. In *S. cerevisiae* strains engineered for increased FA production, a positive effect on free FA titers was detected when deleting *FAA1* and *FAA4* separately, as well as together; however, only minimal increases were observed when deleting *POX1* (Li et al. 2014; Runguphan and Keasling 2014). Another group found that the two triple deletion mutants Δ*faa2*Δ*pxa1*Δ*pox1* and Δ*faa1*Δ*faa4*Δ*fat1* produced more free FA than the original strain (Leber et al. 2015). The combination of all six knockouts in one strain showed the highest titers with 1.3 g L^{-1} free FA, corresponding to 18% of the maximum theoretical yield (Leber et al. 2015).

Apart from these rather general β-oxidation disruptions, there have also been approaches for a **chain length-specific disruption of β-oxidation**, involving *FAA2*, *PEX11*, and *ANT1* (Leber et al. 2016). Pex11 is thought to transport short- and medium-chain FA into the peroxisomes, where they are activated by

Faa2 (Knoll et al. 1994; van Roermund et al. 2000). Ant1 is an adenine nucleotide transporter, which exchanges AMP by ATP across the peroxisomal membrane, thereby providing the energy for the acyl-CoA activation of the free FA (van Roermund et al. 2001). To decrease β-oxidation of specifically short-chain FA, a strain with three knockouts (Δfaa2Δant1Δpex11) was generated, which displayed a much stronger increase in hexanoic and octanoic acid levels compared to the "full" β-oxidation-deficient Δfaa2Δpxa1Δpox1 mutant (Leber et al. 2016). This observation was attributed to the disruption of the AcCoA recycle mechanism in the latter strain (Leber et al. 2016). Overall, the ideal combination of fatty acyl-synthetase and β-oxidation gene knockouts must be evaluated carefully, depending on the desired chain length of the final FA. Nevertheless, most reported gene knockouts of this pathway have turned out to be beneficial for increasing FA titers (Buijs et al. 2015; Zhou et al. 2016b).

2. Disruption of Triacylglycerol and Steryl Ester Synthesis

A competing pathway that can decrease FA levels is the incorporation into storage lipids, primarily triacylglycerols (TAGs) or steryl esters (SEs). In *S. cerevisiae*, TAGs are synthesized from diacylglycerols and fatty acyl-CoAs by the acyltransferases Dga1 and Lro1 (Oelkers et al. 2000, 2002). Steryl ester synthesis of sterols and fatty acyl-CoAs is catalyzed by the acyl-CoA:sterol acyltransferases Are1 and Are2 (Yang et al. 1996). In engineered yeast strains, the knockout of one or several of these four TAG/SE genes has led to increased production of FA derivatives such as 1-hexadecanol (Tang and Chen 2015), fatty alcohols (d'Espaux et al. 2017; Tang et al. 2017), and FAEEs (Valle-Rodríguez et al. 2014). In another strain optimized for FA production (by disruption of β-oxidation and acyl-CoA-activating enzymes), TAG synthesis was increased by overexpressing Dga1. This was combined with an overexpression of the lipid recycle via the triacylglycerol

lipase Tgl3, leading to 2.2 g L^{-1} extracellular free FA (Leber et al. 2015).

B. Engineering Yeast Product Tolerance and Excretion

Some FA as well as some of their derivatives are **toxic** to *S. cerevisiae*. In general, yeast is a robust cell factory that can sense stress and adapt its metabolism accordingly. However, to avoid loss of cell viability and ensure economically competitive yields, yeast strains are needed, which are tolerant to high product concentrations even at low pH (Deparis et al. 2017). Over the last years, much progress has been made to understand the underlying principles of the toxicity of these compounds, and random as well as rational approaches have been applied to improve yeast robustness and thereby product yields.

1. Toxicity Mechanisms and Transport of Fatty Acids and Derivatives

FAs that are toxic to *S. cerevisiae* include **hexanoic, octanoic, and decanoic acids** (Lafon-Lafourcade et al. 1984; Viegas et al. 1989; Liu et al. 2013). At acidic pH, which is the common fermentation condition, the undissociated FA can enter the cells by passive diffusion (Viegas 1997). In the neutral cytosol, they dissociate, thereby causing a decrease of the intracellular pH and an accumulation of the toxic anions (Viegas et al. 1989, 1998; Viegas 1997). Furthermore, they disturb the plasma membrane integrity, causing changes in membrane composition, permeability, and fluidity (Alexandre et al. 1996; Legras et al. 2010; Liu et al. 2013). To generate strains with higher tolerance, it is important to ensure a **rapid transport/secretion** of FA out of the cell, as well as to avoid their re-entrance into the cells. A transcriptome analysis of octanoic- and decanoic acid-stressed cells, respectively, revealed a partly overlapping response mechanism, which was similar to an oxidative stress response, but it also showed a compound-specific activation of

genes encoding transcription factors and transporters (Legras et al. 2010). The role of the identified transporters Tpo1 and Pdr12 in octanoic and decanoic acid efflux was further analyzed in growth tests with knockout mutants (Legras et al. 2010). An overexpression of such efflux-involved transporters is one approach that could increase product yields—in the case, that secretion is a bottleneck. For example, overexpression of Pdr12 was shown to increase the secretion of short branched-chain FA at early time points of production (Yu et al. 2016). However, transporter overexpression can also have unwanted side effects, such as slower growth, depleting ATP from other cellular processes or altering the plasma membrane composition, as was hypothesized based on other Pdr12 overexpression studies (Nygård et al. 2014). Due to a lack of yeast efflux pumps known to specifically transport FA and their derivatives, another viable approach is the screening of heterologous transporters. In a recent study, 12 human or *Arabidopsis thaliana* (putative) transporters were screened, and one of them, human FATP1, improved overall cell fitness and fatty alcohol (Hu et al. 2018) as well as 1-alkene (Zhou et al. 2018) production and secretion in *S. cerevisiae*. For alkane resistance of *S. cerevisiae*, endogenous efflux pumps, namely, Snq2 and Pdr5, as well as heterologous transporters, namely, *Y. lipolytica* ABC2 and ABC3, were shown to have a positive effect (Chen et al. 2013a; Ling et al. 2013).

2. Methods for Increasing Yeast Robustness

To avoid the re-entrance of the products, the robustness of the **plasma membrane** needs to be improved. Octanoic acid, for instance, disrupts the plasma membrane composition, leading to membrane leakage and cell death (Legras et al. 2010; Liu et al. 2013). This effect was reduced by rationally engineering plasma membrane composition through increasing the oleic acid content, either by external supply (Liu et al. 2013) or by expression of a mutated AcCoA carboxylase, Acc1^{S1157A} (Besada-

Lombana et al. 2017). The increase in the average chain length of membrane FA, as well as higher *cis*-monounsaturated FA levels, was shown to provide higher tolerance to toxic FA (Liu et al. 2013; Besada-Lombana et al. 2017).

As tolerance is usually a complex phenotype, which cannot solely be improved by rational engineering of single genes, **adaptive laboratory evolution (ALE)** is an interesting alternative. In an ALE experiment, a strain is grown over many generations, with increasing concentrations of the toxic compound, for selection of cells with an enhanced tolerance phenotype. Causal mutations in the final strain are identified by whole genome sequencing (Dragosits and Mattanovich 2013; Mans et al. 2018). When performed with *E. coli*, an evolved strain not only showed higher tolerance to octanoic acid but also produced higher titers, which was attributed to changes in membrane composition and fluidity (Royce et al. 2015). A similar approach would be possible for *S. cerevisiae*, for which ALE has been performed successfully, for example, for increased resistance to high temperatures (Caspeta et al. 2014) and acetic acid (González-Ramos et al. 2016) or alcohol tolerance (González-Ramos et al. 2013; Davis López et al. 2018), but not for short- or medium-chain FA tolerance.

A method that can lead to similar results as ALE was termed **global transcription machinery engineering, gTME** (Alper et al. 2006). It can induce a remodeling of the transcriptome and therefore target polygenic traits, such as tolerance. gTME relies on the random mutagenesis of a transcription factor that regulates the transcription of several genes and was successfully applied to enhance *S. cerevisiae* tolerance to ethanol (Alper et al. 2006), as well as to adapt to growth on lignocellulosic hydrolysates through improved xylose utilization (Liu et al. 2011). When using this method in combination with an appropriate selection for mutants with increased growth in the presence of the toxic FA, it could be a valuable tool for future tolerance engineering but has so far not been applied to *S. cerevisiae* for FA tolerance.

C. Dynamic Pathway Control and Biosensors

The metabolic engineering of yeast to produce a desired compound can result in an imbalanced metabolism due to the disruption of a tightly regulated, complex biological system, which has evolved for growth and survival. However, inherent control components of this system, such as promoters, transcription factors, or gene copy number, can be utilized for a dynamic pathway control, thereby minimizing metabolic imbalances and increasing product titers (Michener et al. 2012; Schallmey et al. 2014; Shi et al. 2018).

1. Altering Gene Expression Level and Timing

The level and timing of pathway gene expression is crucial in metabolic engineering and can be adjusted, for instance, by using constitutive **promoters** of different strengths or promoters that are inducible/repressible (Da Silva and Srikrishnan 2012). This strategy was applied successfully, for example, to increase 1-alkene production and secretion in *S. cerevisiae*. By replacing the strong e*TDH3* promoter for expression of the membrane-bound enzyme PfUndB with the *GAL7* promoter, which is activated upon glucose depletion, 1-alkene **production was decoupled from growth** (Zhou et al. 2018). The same strategy, i.e., to separate growth from production by expressing pathway genes under the control of carbon source-dependent promoters, was also effective in increasing docosanol production (Yu et al. 2017). A similar approach was applied for *S. cerevisiae* short- and medium-chain FA production, again with a glucose-repressed promoter. Here, *FAS1* and *FAS2* were expressed on a low copy plasmid under control of the alcohol dehydrogenase II promoter of *S. cerevisiae*, p*ADH2*, leading to increased short- and medium-chain FA titers (Gajewski et al. 2017). In another approach, several promoters of different strengths were tested for downregulating the expression of *IDH2* (carbon flux redistribution into free FA) and *PGI1* (increasing NADPH supply), respectively, as the deletion of either

gene led to growth defects. The expression of the genes was reduced by using weaker promoters, leading to increased long-chain FA production in *S. cerevisiae* (Yu et al. 2018). These examples underpin the importance of an appropriate control of gene expression and have much potential to be further exploited for increasing short- and medium-chain FA production—especially as promoters and terminators of different strengths have been described in great detail in recent years (Alper et al. 2005; Da Silva and Srikrishnan 2012; Curran et al. 2013; Lee et al. 2015).

2. Biosensors

Currently, the analysis of short- and medium-chain FA titers is time-consuming and laborious and is usually achieved through chromatography-based methods. A more rapid and convenient alternative are biosensors. They detect the concentration of a molecule—ideally over a wide range of concentrations—and transform it into an easily detectable, quantifiable output, such as growth rate or fluorescence, eventually enabling **high-throughput screenings**, as depicted in Fig. 2 (Michener et al. 2012; Schallmey et al. 2014; Shi et al. 2018). One example of in vivo biosensors is transcription factors, which, in nature, are inevitable for the dynamic control of gene expression. Once their inducing molecules and target promoters are known, transcription factors can be used in metabolic engineering to regulate production pathway expression or even live-monitor titers. Several bacterial **transcription factor-based systems** have been adapted to yeast (Teo et al. 2013; Li et al. 2015; Skjoedt et al. 2016; Wang et al. 2016), especially dynamic sensor-regulator systems of the FA intermediate MalCoA (Johnson et al. 2017). The prokaryotic transcription factor-based **FapR-*fapO*** system has been engineered in *S. cerevisiae* and enabled the sensing of intracellular MalCoA levels (Li et al. 2015; David et al. 2016). This system has been expanded to dynamically control production by coupling the expression of 3-hydroxypropionic acid path-
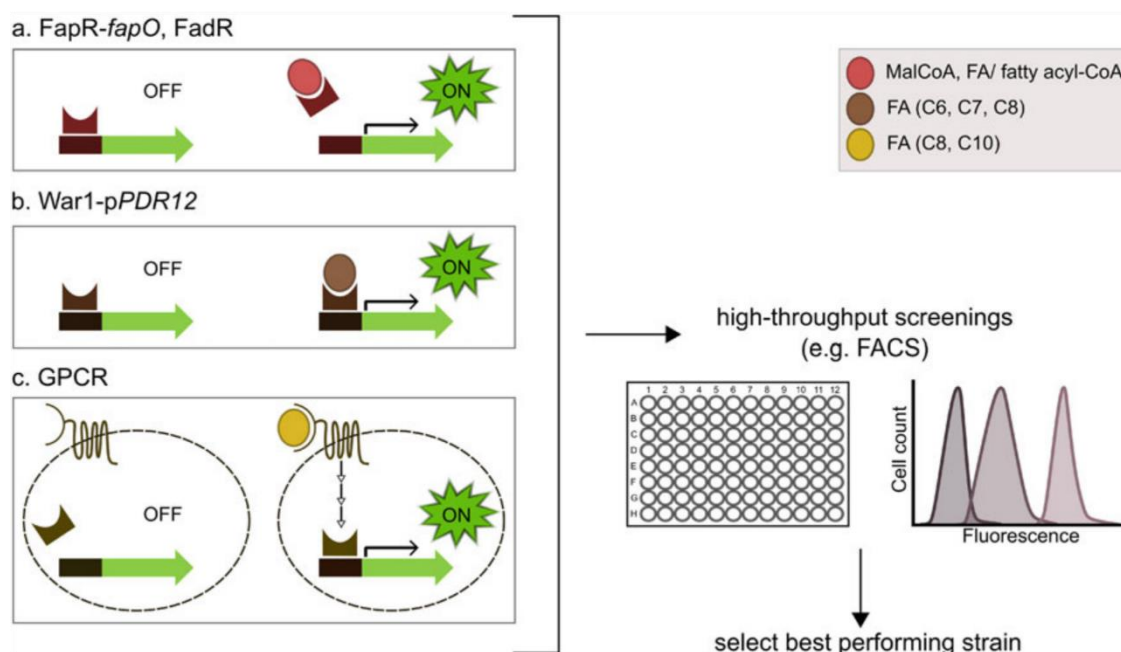
Fig. 2 Yeast biosensors developed for high-throughput screenings of fatty acids or pathway intermediates. (**a**) FapR and FadR are bacterial repressors (OFF state) which have been adapted to yeast for biosensing. Once they are bound by MalCoA (FapR) or FA/fatty acyl-CoA (FadR), expression is enabled, e.g., of a downstream-located fluorescent protein (ON state). (**b**) The yeast endogenous *PDR12* promoter (p*PDR12*) is regulated by the transcription factor War1 that is thought to constitutively bind to the promoter. War1p changes to an active form upon C6, C7, or C8 FA presence. (**c**) A G-protein-coupled receptor responsive to C8 and C10 FA was coupled to the yeast mating pathway for signaling and GFP expression as a quantifiable output. For high-throughput screenings with biosensors, high-throughput cultivation platforms and/or screening methods, such as fluorescence-activated cell sorting (FACS), are needed to select best performing strains

way genes to the intracellular MalCoA level (David et al. 2016). Another bacterial regulation system is based on the transcriptional repressor **FadR**, which is repressed in the presence of FA/fatty acyl-CoAs and cannot bind to specific operator sites (Zhang et al. 2012). This property was exploited to monitor FA levels in *S. cerevisiae* by coupling it to synthetic yeast promoters and GFP expression (Teo et al. 2013). While these MalCoA sensor-regulator systems represent a promising method to fine-tune intracellular precursor supply, systems that directly sense the final, excreted FA, constitute another valuable tool for metabolic engineering. However, reports about such biosensors are scarce to date. The first short- and medium-chain FA yeast biosensor was shown to respond to octanoic and decanoic acid via heterologous G-protein-coupled receptors that were linked to the yeast mating pathway for signaling and GFP expression as a concentration-dependent output (Mukherjee et al. 2015). Besides the rather low linear and dynamic ranges of this biosensor, it was not proven to be functional in culture broth—an indispensable feature for an applicable biosensor. Another biosensor, which was developed for para-hydroxybenzoic acid, was based on the weak acid-inducible ***PDR12* promoter** which is regulated by the transcription factor War1 (Williams et al. 2017). Our group recently adapted this system for the sensing of hexanoic, heptanoic, and octanoic acid reaching high linear and dynamic ranges (Baumann et al. 2018). This biosensor can sense short- and medium-chain FA in *S. cerevisiae* culture broth, which facilitates the monitoring of end-product concentrations and opens the path to high-throughput screenings

of producer strain libraries. High-throughput screening, with the help of appropriate cultivation facilities, **fluorescence-activated cell sorting (FACS)**, or microfluidics, permits the rapid screen of hundreds and thousands of strains to identify best performing cells (Becker et al. 2004; Dietrich et al. 2010; Wang et al. 2014a). High-throughput screening technologies have the potential to substantially speed up metabolic engineering efforts and strain selection in the next years (Schallmey et al. 2014).

V. *S. cerevisiae* as a Production Platform for Fatty Acid Derivatives

Several biosynthetic pathways have recently been engineered to produce a broad spectrum of FA derivatives in various microbes. In this part, we will give a brief overview of some recently reported biosynthetic pathways in *S. cerevisiae* to produce FA esters, fatty alcohols, fatty aldehydes, and alkanes/alkenes as well as dicarboxylic acids. FA-derived compounds can be synthesized on the one hand from fatty acyl-CoA, the end product of the yeast FA biosynthesis, and on the other hand from free FA, which arise after the cleavage of CoA by endogenous yeast TE. In contrast to acyl-CoA—the biosynthesis of which is tightly regulated by feedback inhibition—free FA can be accumulated to much higher levels in yeast (Foo et al. 2017; Teixeira et al. 2017). Both precursors can be processed by several heterologous enzymes as illustrated in Fig. 3. All downstream pathways have in common that the length of the acyl
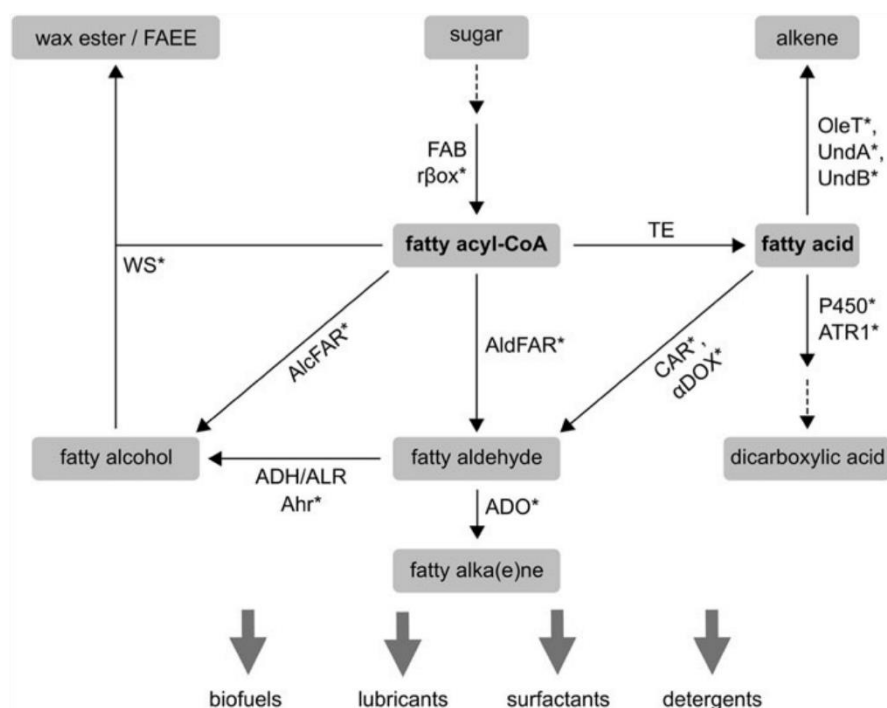
Fig. 3 Enzymatic routes for the production of FA-derived compounds in *S. cerevisiae*. FA-derived compounds can be synthesized from the end product of FA biosynthesis (FAB) or reverse β-oxidation (rβox), the fatty acyl-CoA, and from free FA by several heterologous enzymes in yeast. FAs result from the cleavage of CoA by endogenous thioesterases (TE). Heterologous enzymes are marked with an asterisk. *OleT* H_2O_2-dependent P450 FA decarboxylase, *UndA* medium-chain FA-preferring nonheme iron oxidase, *UndB* membrane-bound desaturase-like enzyme, *P450* cytochrome P450 CYP94C1, *ATR1* cytochrome reductase from *A. thaliana*, *CAR* carboxylic acid reductase from *M. marinum*, αDOX α-dioxygenase from *O. sativa* (rice), *FAR* fatty acyl-CoA reductase, *ADO* aldehyde deformylating oxygenase, *ADH* alcohol dehydrogenase, *ALR* aldehyde reductase, *Ahr* aldehyde reductase from *E. coli*, *WS* wax ester synthase

chain in the end product is determined by the acyl chain of the FA precursor and by the specificity of the downstream enzymes.

A. Alkenes

Free FA can be converted to terminal alkenes by a one-step decarboxylation pathway, catalyzed by an H_2O_2-dependent cytochrome P450 enzyme, OleT (Chen et al. 2015; Zhou et al. 2018); a medium-chain FA-preferring nonheme iron oxidase, UndA (Zhu et al. 2017a; Zhou et al. 2018); or a membrane-bound desaturase-like enzyme, UndB (Zhou et al. 2018), together with a reduction system. The major challenge when applying these iron-dependent enzymes is the inefficient electron transfer, resulting in low titers in yeast due to inefficient cofactor or reducing systems (Zhou et al. 2018).

B. Dicarboxylic Acids

Utilizing the **ω-oxidation pathway** in *S. cerevisiae* enables the production of ω-hydroxy- and α, ω-dicarboxylic acids (ω meaning "last" C atom when counted from the carboxyl group) from free FA. Such α, ω-dicarboxylic acids can serve as raw material for commodities and polymers (Han et al. 2017). Free FA can be terminally hydroxylated by a cytochrome P450 enzyme, followed by an oxidation to a carboxyl group by alcohol dehydrogenases and aldehyde dehydrogenases. Feeding of medium-chain FA to an *S. cerevisiae* strain expressing the cytochrome P450 enzyme CYP94C1, together with a cytochrome reductase, ATR1 from *A. thaliana*, allowed the production of ω-hydroxy- and α, ω-dicarboxylic acids with chain lengths ranging from C10 to C16 (Han et al. 2017).

C. Fatty Aldehydes, Alkanes, and Alcohols

For the production of alkanes and alcohols, a fatty aldehyde intermediate is used as a precursor. There are several possible routes for the synthesis of **fatty aldehydes**. For instance, FA can be reduced through action of a carboxylic

acid reductase (CAR) from *Mycobacterium marinum* (Zhou et al. 2016b; Tang et al. 2017; Henritzi et al. 2018), or it can be oxidatively decarboxylated by an α-dioxygenase (α-DOX) from *Oryza sativa* (rice) (Jin et al. 2016; Foo et al. 2017). To be active, CAR requires a phosphopantetheinylation by a phosphopantetheinyl transferase (Akhtar et al. 2013). A third possibility is the reduction of CoA-bound FA to the corresponding aldehyde by fatty acyl-CoA reductases (AldFARs). Compared to CAR, it was shown that AldFAR-type enzymes are rather inefficient in yeast (Buijs et al. 2015; Zhou et al. 2016b). Aldehydes can be converted to **odd-chain fatty alkanes** (C_{n-1}) by cyanobacterial aldehyde deformylating oxygenases (ADO) or to **fatty alcohols** by endogenous alcohol dehydrogenases (ADHs) and aldehyde reductases (ALR) (Buijs et al. 2015; Zhou et al. 2016b; Kang et al. 2017; Zhu et al. 2017a). Zhou et al. (2016b) showed that the expression of a CAR together with its activating enzyme, phosphopantetheine transferase NpgA from *A. nidulans*, and an ADO, led to the synthesis of long-chain alkanes (Zhou et al. 2016b). To supply sufficient electrons, a reducing system was expressed additionally (Buijs et al. 2015). As previous studies showed that CAR also shows high activity toward medium-chain FA, Zhou et al. (2016a) produced medium-chain alkanes by screening different ADO orthologs and engineering their substrate binding sites (Zhu et al. 2017a). However, it is reported that ADO was not able to compete with the alcohol-forming enzymes (ADHs/ALRs) due to its low catalytic efficiency (Buijs et al. 2015; Zhou et al. 2016b; Foo et al. 2017; Kang et al. 2017; Zhu et al. 2017a). Another **two-step pathway to produce alkanes** was demonstrated by Foo et al. (2017). Expression of an α-DOX from rice led to the production of long odd-chain fatty aldehyde intermediates, which can subsequently be deformylated to even-chain alkanes (C_{n-2}) by ADO (Foo et al. 2017). The advantage of a dioxygenase is that it uses dioxygen instead of NADPH for the production of aldehydes (Foo et al. 2017). Odd-chain fatty aldehydes produced by α-DOX can also be oxidized to odd-chain fatty alcohols through endogenous yeast ADHs/ALRs (Jin et al. 2016). Zhou et al. (2016b)

explored ADHs/ALRs of yeasts and could show that Adh5 plays an important role in the production of long-chain alcohols, as overexpressing *ADH5* together with CAR increased the amount of long-chain alcohols (Zhou et al. 2016b). So far, the expression of fatty alcohol pathways resulted in the production of a pool of long- or medium-chain fatty alcohols with different chain lengths. Recently, our group was able to selectively produce the diesel-like alcohol 1-octanol in *S. cerevisiae* by combining a C8 FA-producing FAS with a two-step reduction pathway composed of CAR together with the phosphopantetheinyl transferase Sfp from *Bacillus subtilis* and the aldehyde reductase Ahr from *E. coli* (Henritzi et al. 2018). It has been shown that Ahr accepts a broad range of aliphatic aldehydes with chain lengths from C4 to C16 (Akhtar et al. 2013).

Another way to produce fatty alcohols is a **one-step reduction** of the fatty acyl-CoA, catalyzed by fatty acyl-CoA reductases (AlcFARs) (Runguphan and Keasling 2014). Zhou et al. (2016b) expressed the CAR/Adh5 pathway together with a fatty acyl-CoA reductase to produce fatty alcohols simultaneously from free FA and acyl-CoAs (Zhou et al. 2016b). Other labs have also reported the functional expression of several heterologous AlcFARs (Feng et al. 2015; d'Espaux et al. 2017). The advantage of one-step reactions, like the direct conversion from fatty acyl-CoAs to alcohols by AlcFARs or the FA decarboxylation pathway for alkene production, is the reduced intermediate metabolite loss and the circumvention of toxic intermediates (Chen et al. 2015). Besides medium-chain and long-chain FA-derived chemicals, **very long-chain FA-derived products** also play an important role as ingredients for lubricants, detergents, polymers, photographic film-processing agents, coatings, cosmetics, and pharmaceuticals (Yu et al. 2017). Yu et al. (2017) were able to selectively produce docosanol (C22) from very long-chain FA by rewiring the native FA elongation system and overexpressing a heterologous mycobacteria FAS I system, which provides high levels of C22 FA as direct precursor, together with a specific AlcFAR (Yu et al. 2017).

D. Wax Esters and Fatty Acid Ethyl Esters

Further compounds of interest include wax esters, which are typically esters of long-chain FA and long-chain alcohols and are used in personal care products, lubricants, or coatings (Wenning et al. 2017). They can be synthesized by **wax ester synthases (WS)** from alcohols and fatty acyl-CoA thioesters (Shi et al. 2012; Runguphan and Keasling 2014). Esters of ethanol and FA with chain lengths ranging from C14 to C20 (**FAEE**) represent suitable diesel fuels (Shi et al. 2012). Shi et al. (2012) could functionally express different WSs from different species and characterize their substrate preference. Unfortunately, some of the WSs from bacteria are bifunctional enzymes, which function as WS and acyl-CoA:diacylglycerol acyltransferase (DGAT), resulting in TAG formation, leading to a depletion of the acyl-CoA precursor pool (Shi et al. 2012). Eriksen et al. (2015) investigated the heterologous expression of a FAS I from *Brevibacterium ammoniagenes* coupled with a WS/DGAT to produce FAEEs. This strategy has the advantage of providing additional FA for growth supplementation and to supply the FA needed for FAEE synthesis (Eriksen et al. 2015). The microbial synthesis of wax esters by the esterification of a (very) long-chain fatty acyl-CoA with a primary, very long-chain fatty alcohol was reported by Wenning et al. (2017). The group combined the expression of a heterologous FAR with the expression of a plant WS to synthesize different jojoba-like wax esters. The used FAR reduced long-chain fatty acyl-CoAs, which resulted from de novo FA biosynthesis and elongation, to a long-chain alcohol. The plant-derived WS enabled the esterification of this alcohol with a long-chain fatty acyl-CoA in *S. cerevisiae* (Wenning et al. 2017).

Overall, there are still many **obstacles** that have to be overcome when using these heterologous enzymes, such as the challenging expression in the heterologous yeast host, low catalytic efficiency, inefficient electron transfer, or loss of intermediates by competing pathways. These issues have to be tackled in order to increase yields and titers of FA-derived compounds. One promising strategy could be a

compartmentalization into peroxisomes by a peroxisomal targeting of pathway enzymes (Sheng et al. 2016; Zhou et al. 2016a). Similarly, blocking competing pathways through deletion of specific genes is a common strategy. For instance, the deletion of *HFD1* was shown to be a crucial step for alkane and alcohol biosynthesis (Buijs et al. 2015; Zhou et al. 2016b). *HFD1* encodes an aldehyde dehydrogenase involved in sphingolipid degradation and coenzyme Q biosynthesis and catalyzes the oxidation of fatty aldehydes to FA (Zhu et al. 2017a). Additionally, many engineering strategies for increasing the supply of the precursor AcCoA and free FA also led to an increase in the production of FA-derived products (Zhou et al. 2016b; Teixeira et al. 2017).

VI. Optimization of Fermentation Conditions

Yeast FA titers keep rising through a plethora of metabolic engineering efforts; however, general production process optimization is just as important to unravel the full potential of producer strains. In oleaginous yeasts, like *Y. lipolytica*, lipid overproduction starts with the exhaustion of a primary nutrient, i.e., when entering stationary growth (Beopoulos et al. 2009). Consequently, **nitrogen and/or glucose limitation** has been utilized to increase lipid production in *S. cerevisiae* (Thompson and Trinh 2014; Yu et al. 2018). Such limiting conditions led to high lipid titers in a highly engineered *S. cerevisiae* strain, producing 33.4 g L^{-1} free FA in a fed-batch fermentation—the highest reported free FA titer by microbial fermentation to date (Yu et al. 2018). These results also emphasize the importance of the transition from flask cultivation to **fed-batch cultivation** in a fermenter. The latter permits a tight control of cultivation parameters, such as pH, aeration, and nutrient supply, thereby enabling higher titers of FA and derivatives (Thompson and Trinh 2014; Zhou et al. 2016b; Yu et al. 2018).

In a very recent study, the effect of "forced" FA synthesis on the metabolism and physiology of an engineered *S. cerevisiae* strain was analyzed (Gossing et al. 2018). Such a systematic characterization could help to determine the crucial parameters for improving yeast lipid production. Besides the expected increase in β-oxidation and storage lipids, the analyzed strain also showed higher levels of oxidative stress and decreased amino acid levels (Gossing et al. 2018). Therefore, a viable approach could be an adjustment of **media composition**, e.g., by providing higher amounts of **amino acids**, or overexpressing genes to improve the amino acid uptake or synthesis.

Several previous studies have demonstrated a positive effect of amino acid supplementation on lipid accumulation. The addition of methionine led to increased palmitoleic acid production (Kamisaka et al. 2015), whereas high leucine levels led to increased lipid accumulation in a Δ*snf2* strain (Kamisaka et al. 2007). The additional supplementation of several other amino acids has been demonstrated to contribute to ethanol tolerance in yeast (Hirasawa et al. 2007; Sekine et al. 2007; Pham and Wright 2008; Yoshikawa et al. 2009). Nevertheless, it is also important to consider the genetic background of the FA producing strain at hand, as working with auxotrophic strains can decrease growth (Baganz et al. 1997; Çakar et al. 1999; Basso et al. 2010). Additionally, for some strain series, such as the BY strains, the importance of sufficient amino acid supply for optimal growth has been emphasized (Hanscho et al. 2012).

Vitamins, such as biotin and pantothenate, also play pivotal roles for normal yeast growth and are essential in FA synthesis (Suomalainen and Keränen 1963; Tehlivets et al. 2007). The addition of pantothenate, for example, was used as a metabolic switch to regulate the synthesis of β-farnesene, an AcCoA-derived sesquiterpene (Sandoval et al. 2014). This method could possibly be transferred to FA production processes, as they, similarly to β-farnesene, depend on CoA intermediates.

The **fermentation temperature** has a major influence not only on yeast growth but also on FA tolerance and production (Piper 1995; Viegas and Sá-Correia 1995; Viegas 1997). An engineered *S. cerevisiae* produced more palmitoleic acid at low temperatures (20–25 °C) (Kamisaka et al. 2015), and the oleaginous yeast *Metschnikowia pulcherrima* was shown to produce high lipid levels at low temperatures and pH

(Santamauro et al. 2014). However, at lower temperatures, octanoic and decanoic acid are also more toxic (Viegas and Sá-Correia 1995; Viegas 1997). Furthermore, low temperatures might not be feasible in large-scale production, in which high gravity can lead to elevated temperatures, and cooling of big fermentation plants is cost-intensive (Gibson et al. 2007). Therefore, it might be more desirable to generate thermotolerant *S. cerevisiae* strains by introducing traits of thermotolerant yeasts, such as *Kluyveromyces marxianus* (Cernak et al. 2018), or even thermotolerant oleaginous yeasts, like *R. toruloides* (Wu et al. 2018). Another important cultivation parameter for FA production is the **pH** and media buffering. Short- and medium-chain FAs are more toxic to *S. cerevisiae* at lower pH values, causing decreased viability and yields (Viegas et al. 1989). Therefore, it was shown that the addition of potassium phosphate buffer to complex medium elevated short- and medium-chain FA production substantially (Gajewski et al. 2017).

A promising approach to avoid the re-uptake of the products by the cells and simultaneously decrease the effects of product toxicity is an **in situ extraction**. The addition of dodecane to the culture of a long-chain fatty alcohol-producing *S. cerevisiae* was successfully applied for the extraction of these compounds (Rungu-phan and Keasling 2014; d'Espaux et al. 2017). Upon addition of dodecane to a culture broth of a 1-octanol producing *S. cerevisiae* strain, however, production was reduced—likely due to the extraction of the precursor octanoic acid by dodecane (Henritzi et al. 2018). Therefore, when considering in situ extraction, an agent needs to be found, which is highly specific for each product, and does not remove important pathway intermediates from the culture.

Current efforts for yeast FA production were carried out in minimal (Zhou et al. 2016b; Yu et al. 2018) as well as complex medium (Leber et al. 2015; Gajewski et al. 2017). To be truly more sustainable than petroleum-based production, and to prevent a competition with food supply, starting materials, such as **lignocellulosic biomass** from agri-cultural waste, need to be utilized. To extract fermentable sugars from such biomass, it first must undergo pretreatment, in the process of which fermentation inhibitors arise. Therefore, the ideal FA producing yeast needs to be resistant to these inhibitors, as well as to be able to utilize glucose *and* five-carbon sugars, such as xylose, as carbon sources (Peralta-Yahya et al. 2012). D'Espaux et al. (2017) presented the first—and, so far, only—report about FA/alcohol production with *S. cerevisiae* solely from lignocellulosic feedstock. They fed the non-food crops sorghum and switchgrass, which were pretreated with ionic liquids, to an engineered *S. cerevisiae* strain and obtained up to 0.7 g L^{-1} fatty alcohols (d'Espaux et al. 2017). By combining the different engineering and cultivation strategies mentioned above, a further increase in FA titers produced from lignocellulosic biomass can be foreseen.

VII. Conclusions

Much progress has been made in recent years in the development of strategies for microbial production of FA and their derivatives from renewable feedstocks. The available literature shows that extensive interventions into the central carbon metabolism, relying on expression of heterologous pathways and manipulation of the activity of endogenous enzymes, are necessary to enable a high yield of oleochemicals in different chassis organisms. The highest yields of microbially produced oleochemicals reported to date were achieved in *S. cerevisiae*, owing to the great body of knowledge regarding the physiology of this yeast (0.1 g free FA/g glucose, i.e., approximately 30% of the theoretical yield). Moreover, FA production in *S. cerevisiae* has become more chain length-specific, and many FAs and a great variety of derivatives have already been produced successfully in lab-scale fermentations. The development of biosensors and high-throughput screening methods has become increasingly important and will considerably accelerate the development for well-performing strains. Once such challenges

as metabolic imbalances and product toxicity have been tackled, yields, titers, and productivity could reach economically viable levels before long.

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6.3 Transcriptomic response of *Saccharomyces cerevisiae* to octanoic acid production

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Contributions of doctoral candidate and co-authors

(1) Concept and design

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Co-authors TD, VS, JN, EB, MO: 10%, 5%, 5%, 5%, 10%

(2) Conducting tests and experiments

<u>Doctoral candidate LB</u>: 90%, yeast molecular biology, fermentations, metabolite extraction and GC analysis, toxicity test

Co-author TD: 10%, RNA extraction

(3) Compilation of data sets and figures

<u>Doctoral candidate LB</u>: 100%, RNA-Seq data, metabolite titers and growth, toxicity test

(4) Analysis and interpretation of data

<u>Doctoral candidate LB</u>: 70%, RNA-Seq data, differential expression analysis, metabolite titers and growth, toxicity test

Co-authors TD, EB, MO: 10%, 10%, 10%, RNA-Seq data, supervision and advice

(5) Drafting of manuscript

<u>Doctoral candidate LB</u>: 80%
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Transcriptomic response of *Saccharomyces cerevisiae* to octanoic acid production

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ABSTRACT: The medium-chain fatty acid octanoic acid is an important platform compound widely used in industry. The microbial production from sugars in *Saccharomyces cerevisiae* is a promising alternative to current non-sustainable production methods, however, titers need to be further increased. To achieve this, it is essential to have in-depth knowledge about the cell's physiology during octanoic acid production. To this end, we collected the first RNA-Seq data of an octanoic acid producer strain at three time points during fermentation. The strain produced higher levels of octanoic acid and increased levels of fatty acids of other chain lengths (C6-C18) but showed decreased growth compared to the reference. Furthermore, we show that the here analyzed transcriptomic response to internally produced octanoic acid is notably distinct from a wild type's response to externally supplied octanoic acid as reported in previous publications. By comparing the transcriptomic response of different sampling times, we identified several genes that we subsequently overexpressed and knocked out, respectively. Hereby we identified *RPL40B*, to date unknown to play a role in fatty acid biosynthesis or medium-chain fatty acid tolerance. Overexpression of *RPL40B* led to an increase in octanoic acid titers by 40 %.

KEYWORDS: *Octanoic acid, transcriptome response to medium-chain fatty acids, RNA-Seq, RPL40B*

INTRODUCTION

Medium-chain fatty acids like octanoic acid (C8 fatty acid) have a wide range of applications in antimicrobials, surfactants and cosmetics, and can also serve as precursors for biofuels.[1–4] At present, octanoic acid and its derivatives are produced from oil seed crops or crude oil.[5] In comparison to petrochemical industry, the extraction from oily plants, such as oil palm, might appear environmentally less harmful but there are rising concerns about its extensive cultivation. Oil palm cultivation is often associated with deforestation of rainforest and the ecological consequences thereof, and in addition competes with food production.[6] The engineering of microbes for octanoic acid production from renewable biomass is therefore a promising alternative.[5,7]

Yeast produces fatty acids in the fatty acid synthesis cycle through action of the cytosolic fatty acid synthase (FAS), a large enzymatic complex encoded by the two genes *FAS1* and *FAS2*. Naturally, *Saccharomyces cerevisiae* produces mostly saturated and monounsaturated long-chain fatty acids with chain lengths of C16 and C18.[8] There are different ways to redirect production from long-chain to short- and medium-chain fatty acids. Here, we use a yeast strain that carries a modified variant of *FAS1*, namely *FAS^RK*.[9] This enzyme contains an amino acid exchange from arginine to lysine ("R1834K") in the malonyl-palmitoyl transferase (MPT) domain of Fas1p. Thereby, the loading of the precursor malonyl-CoA is reduced, leading to premature release of short acyl chains, such as octanoyl-CoA. Octanoyl-CoA is then hydrolysed by thioesterases and free octanoic acid is released.[9] For an economically feasible production, further strain improvements and process optimizations are necessary.[10]

To increase titers, it is essential to advance the understanding of the producer strain's physiology in the course of octanoic acid production.[11] To our knowledge, such an analysis has not been undertaken yet. So far, two transcriptome-wide studies have been performed with extracellular addition of octanoic acid to wild type strains.[12,13] In the first one, a microarray analysis showed that incubation with 0.05 mM (7 mg/L) octanoic acid for 20 minutes caused oxidative stress and a similar response to decanoic acid was observed. Overall, 75 genes were found to be differentially regulated in comparison to the non-exposed control. Pdr12 was identified as the main involved transporter and the transporter Tpo1 was shown to play a minor role in octanoic acid expulsion.[12] In the second study, the exposure to 0.3 mM (43 mg/L) octanoic acid until mid-log growth phase revealed differential expression of at least 2-fold of 136 genes in comparison to the non-exposed control. Here, membrane leakiness was identified as a possible mechanism of cell disruption and increasing the oleic acid content was shown to enhance yeast tolerance to octanoic acid.[13]

To get a comprehensive insight into expression changes during production of octanoic acid, we performed RNA-Seq in three different growth phases of an *S. cerevisiae* octanoic acid producer strain and a reference strain. We used expression profiles from the different growth phases to guide strain engineering efforts and achieved a 40 % increase in octanoic acid production.

RESULTS

Fermentation profiles of an octanoic acid producer compared to reference. To get the first insight into transcriptional changes in different growth phases associated with octanoic acid production, we performed RNA-Seq transcriptome profiling. As a background strain we used SHY34,[14] a triple knockout strain (Δ*fas1*Δ*fas2*Δ*faa2*). In this strain the two *FAS* genes are knocked out as well as *FAA2*, encoding a fatty acyl-CoA synthetase, deletion of which prevents degradation of short- and medium-chain fatty acids.[3,15] We transformed this strain either with a reference plasmid containing *FAS1* and *FAS2* in their wild type form fused into one sequence, i. e. *fusFAS^wt*, or with a plasmid carrying *FAS1^RK* and *FAS2* fused into one sequence, namely *fusFAS^RK*. The *fusFAS^RK* sequence contains an amino acid exchange from arginine to lysine in the MPT domain (R1834K), generating an octanoic acid producer.[14]

The two strains grew with a similar growth rate in exponential phase and only after the diauxic shift, growth was slower in the octanoic acid producer strain compared to the reference strain leading to a lower final OD in the octanoic acid producer (Figure 1A). Such impairment of growth has similarly been observed for non-producers spiked with octanoic acid.[3] Samples were taken in early exponential growth phase (14 h, T1), shortly after the diauxic shift (22 h, T2) and in ethanol consumption phase (46 h, T3), respectively. To confirm octanoic acid production, samples were taken from the culture supernatants at each sampling time for fatty acid extraction and quantification via GC measurement (Figure 1B). In the reference strain, octanoic acid was produced in negligible amounts (< 7 mg/L) at all three sampling times whereas in the octanoic acid producer, the amount increased gradually from 7 mg/L (T1) to 43 mg/L (T2) to 87 mg/L (T3). We also observed an enhanced production of other short- and medium-chain fatty acids, namely C6, C10 and C12 fatty acids, in the octanoic acid producer strain, with the most pronounced difference to the reference strain at T3 (Figure S1). Interestingly, even secreted amounts of saturated long-chain fatty acids, i.e. C16:0 and C18:0 fatty acids, were higher in the octanoic acid producer compared to the reference strain at T2 and T3 (Figure 1C).
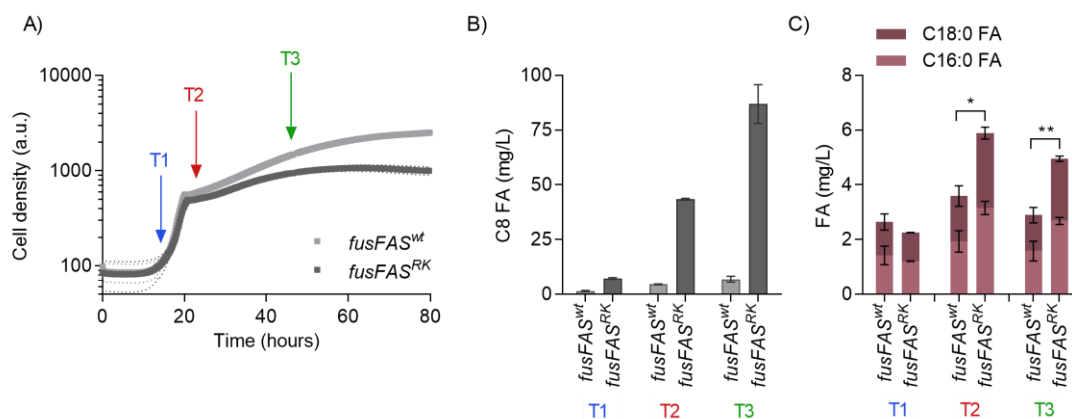
Figure 1. Characterization of growth and octanoic acid production. (A) Growth was monitored on a cell growth quantifier in triplicates for strains SHY34+*fusFAS^wt* (reference strain) and SHY34+*fusFAS^RK* (octanoic acid producer). Sampling times are indicated with arrows (T1 = 14 h; T2 = 22 h; T3 = 46 h). (B, C) C8, C16:0 and C18:0 fatty acids (FA) were extracted and quantified via GC. n = 3, error bars = ± standard deviation. Statistical analysis was performed using two-tailed t test (*$p < 0.05$, **$p < 0.01$).

Analysis of transcriptomic response in different phases of octanoic acid production. From the samples described above, we extracted RNA and performed RNA-Seq analysis. A principal component analysis (PCA) of the RNA-Seq data set revealed a close clustering of the three replicates at each sampling time in both strains (Figure S2).The data of the reference strain and the octanoic acid producer strain also clustered closely together, suggesting a limited number of differentially expressed genes (Figure S3). At T1, only 15 genes were upregulated ($\log_2$FC > 1, FDR < 0.01) in the octanoic acid producer in comparison to the reference strain – at T2 this was the case for 29 genes and at T3 for 85 genes. The number of downregulated genes was in a similar range, with 11 genes downregulated at T1, 44 genes at T2 and 144 genes at T3. The number of differentially expressed genes between the two strains therefore increased with fermentation time. A list of all differentially expressed genes can be found in a Supplementary File 1. GO term enrichment analyses for all three sampling times are attached as Supplementary File 2.

To get a comprehensive view of the transcriptomic landscape of the octanoic acid producer in different growth phases, we compared the RNA-Seq data of the three sampling times (Figure 2). We observed only eight genes upregulated and eleven genes downregulated at more than one sampling time compared to the reference. For upregulation, these included *FAS1^RK*, *FAS2* as well as *HIS3* – the latter being the selection marker of the *fusFAS^RK* plasmid used in the experiment. A higher copy number or expression of *fusFAS^RK* was recently shown to increase octanoic acid titers.[14] It is likely that the expression was upregulated in the octanoic acid producer to ensure that enough long-chain fatty acids are produced for cell growth since chain length control in

fusFAS[RK] is leaky.[9] *YOR203W* was found to be upregulated at all sampling times, however, according to the SGD database, it is unlikely to encode a functional protein, and its assigned sequence partially overlaps with *HIS3*, suggesting an incorrect assignment in the RNA-Seq read mapping. *HXT2*, a glucose transporter, was the only gene found to be downregulated at all three sampling times.
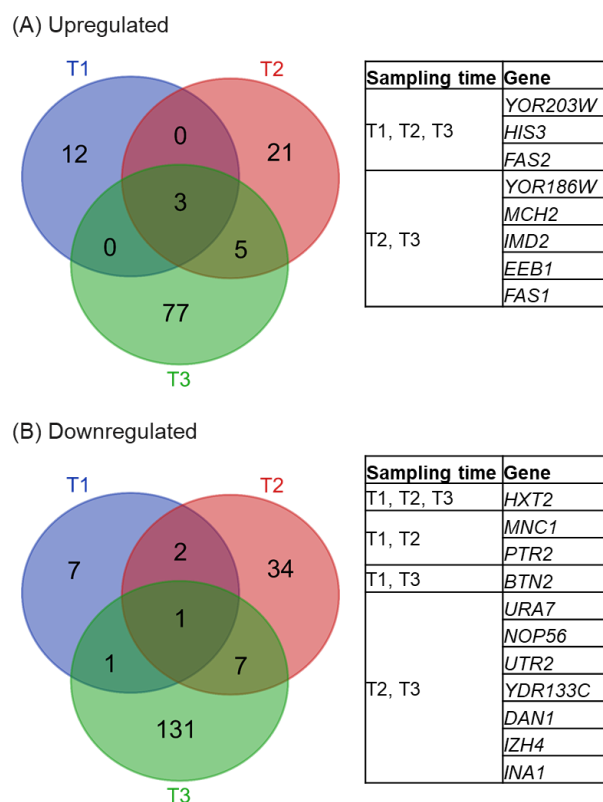
(A) Upregulated

Sampling time	Gene
T1, T2, T3	*YOR203W*
	HIS3
	FAS2
T2, T3	*YOR186W*
	MCH2
	IMD2
	EEB1
	FAS1

Venn diagram values: T1 = 12; T2 = 21; T1∩T2 = 0; T1∩T2∩T3 = 3; T1∩T3 = 0; T2∩T3 = 5; T3 = 77.

(B) Downregulated

Sampling time	Gene
T1, T2, T3	*HXT2*
T1, T2	*MNC1*
	PTR2
T1, T3	*BTN2*
T2, T3	*URA7*
	NOP56
	UTR2
	YDR133C
	DAN1
	IZH4
	INA1

Venn diagram values: T1 = 7; T2 = 34; T1∩T2 = 2; T1∩T2∩T3 = 1; T1∩T3 = 1; T2∩T3 = 7; T3 = 131.

Figure 2. Differential gene expression for the octanoic acid producer compared to reference. Number of genes that are upregulated (A) or downregulated (B) in the octanoic acid producer strain in comparison to the reference strain (log_2FC > 1, FDR < 0.01) between sampling times (T1 = 14 h; T2 = 22 h; T3 = 46 h). Gene lists include genes that are differentially regulated at more than one sampling time as indicated.

Comparison to published transcriptome data. Two other transcriptome-wide studies have been performed so far, however, both of them with extracellular addition of octanoic acid to wild type *S. cerevisiae* strains.[12,13] In the first study, a microarray analysis by Legras and colleagues,[12] 75 genes were found to be differentially regulated (log_2FC ≥ 0.3, FDR < 0.05) in comparison to the non-exposed control after incubation with only 0.05 mM (7 mg/L) octanoic acid for the short period of 20 minutes.[12] In the second study by Liu and colleagues,[13] the exposure to six times the amount, i. e. 0.3 mM (43 mg/L) octanoic acid, led to differential expression (log_2FC > 1, p < 0.01) in 136 genes in comparison to the non-exposed control.[13] Interestingly, the overlap between these two published studies and our analysis was limited to a very small number of genes (Figures S4, S5). In all three studies the transporter Pdr12 was found to be upregulated (RNA-Seq: T3), which

has previously been associated with octanoic acid expulsion.[12] However, additional plasmid-based expression of Pdr12 has not led to an increase in octanoic acid titers in our hands, suggesting that endogenous expression is sufficient (Figure S6).

To understand expression changes in the octanoic acid producer, we wanted to further analyse the effect of differentially expressed genes. To limit the number of genes for further analysis, we decided to focus on those genes that were differentially expressed at more than one sampling time in our data. We reasoned that such genes might have a more profound effect on octanoic acid production and constitute more promising targets for engineering than genes that are differentially regulated at only one sampling time.

Overexpression of identified genes with a multi-copy plasmid library. We decided to test the effect of plasmid-based overexpression of all genes that were found to be up- or downregulated, respectively, in at least two sampling times in our data (see gene tables in Figure 2). In addition, we also tested the effect of overexpression of some genes overlapping with data from Liu and colleagues.[13] For overexpressing the respective genes, we used a plasmid-library[16] that contains the majority of the yeast genome cloned as approximately 10 kb fragments into multi-copy plasmid backbones. Two of the genes (*DAN1*, *NOP56*) were not represented in the library and were therefore cloned separately on a multi-copy plasmid backbone. To avoid plasmid burden effects, we tested the plasmids in a strain that contains *FAS1^RK* and *FAS2* in their wild type loci (LBY38). Towards the end of the exponential growth phase (18 h) we evaluated the octanoic acid titers for some of the strains and observed a variation of titers in accordance with differing growth (Figure 3). LBY38 containing plasmids with *IMD2*, *HXT2* or *BTN2*, respectively, had OD_{600} values and titers of only about 50 % of the control, indicating delayed growth and therefore octanoic acid production.
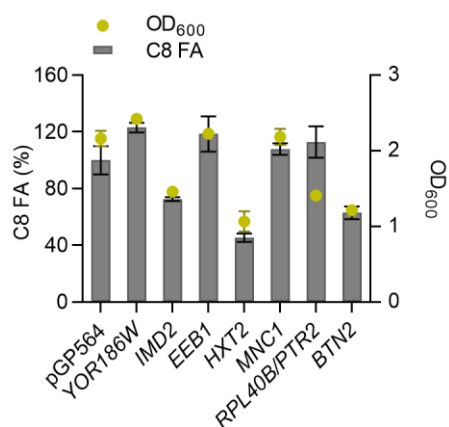

Figure 3. Fermentation of octanoic acid producer with overexpressed genes. The octanoic acid producer strain LBY38 was transformed with plasmids of a multi-copy library or a control plasmid (pGP564) and cultured for 18 h in buffered SCD^-leu medium. Tested library plasmids contained the genes indicated on the x-axis. Octanoic acid was extracted and quantified by GC measurement, and is shown in percent of the control (pGP564). *n* = 2, error bars = ± standard deviation.

In ethanol consumption phase (46 h), all strains had reached similar OD_{600} values, which facilitated comparison between octanoic acid titers (Figure 4A). After 46 h of fermentation, the expression of the library plasmid containing two neighbouring genes of interest, *RPL40B* and *PTR2*, yielded a 40 % higher octanoic acid titer; whereas *HXT2*, *BTN2* and *IMD2* expression led to a 25 %, 15 % and 13 % decrease, respectively. The expression of the library plasmid containing *ECI1* led to a decrease in titer to about 60 % of the reference. All other plasmids did not (< 10 % difference) influence octanoic acid titers. To further characterize the effects on octanoic acid production, we generated individual knockout strains for *HXT2*, *BTN2*, *IMD2* and *ECI1* in the LBY38 background. We analysed octanoic acid titers after 46 h of growth in buffered SCD medium. All four knockout strains, Δ*hxt2*, Δ*btn2*, Δ*imd2* and Δ*eci1*, showed similar growth as well as octanoic acid titers as the control LBY38 (Figure 4B).
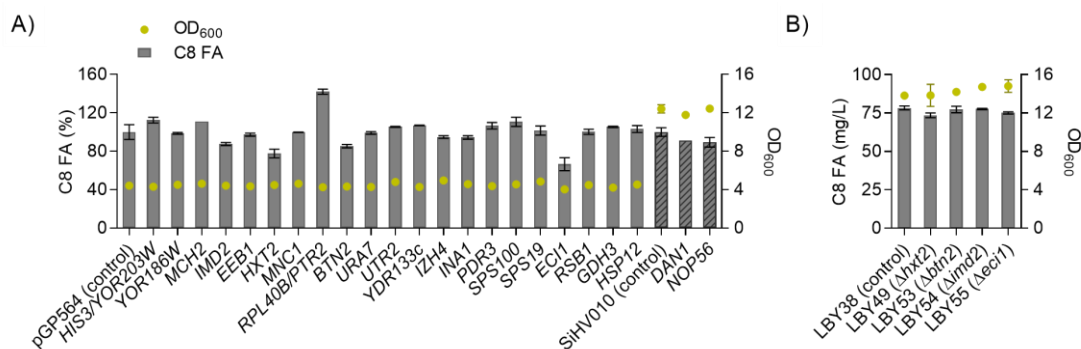

Figure 4. Test of overexpression and deletion of targets in octanoic acid producer. (A) The octanoic acid producer strain LBY38 was transformed with plasmids of a multi-copy library and cultured in buffered SCD^{-leu} medium. Tested library plasmids contained the genes indicated on the x-axis (dark grey bars). The genes *DAN1* and *NOP56* were not part of the library and therefore were cloned individually into SiHV010 multi-copy backbone, transformed into LBY38 and cultured in buffered YPD^{hyg} (striped bars). Titers are shown in percent of the respective control strains. (B) Four genes were knocked out individually in LBY38 and the strains were fermented in buffered YPD. For all samples, octanoic acid was extracted after 46 h of growth and quantified by GC measurement. n = 2, error bars = ± standard deviation.

Overexpression of *RPL40B* leads to increase in octanoic acid production. To determine which of the two genes - *RPL40B* or *PTR2* - is responsible for the observed positive effect, we cloned each with its native promotor and terminator on a separate plasmid backbone. The fermentation showed that the plasmid-based expression of *RPL40B* caused an increase in octanoic acid titers of 40 %, whereas *PTR2* expression did not influence octanoic acid titers (Figure 5A). The amount of secreted saturated C18 fatty acid was lower in the strain with plasmid-based *RPL40B* expression compared to the reference. When introducing a second copy of *RPL40B* into the genome under the control of the strong *pPGK1* promoter, we could not observe this increase in titers (Figure 5B), suggesting that the native *RPL40B* regulatory elements are required for the positive effect on

octanoic acid production. To investigate the effect of *RPL40B* on robustness towards octanoic acid, we transformed the non-producer CEN.PK2-1C with either a control plasmid or the multi-copy plasmid containing *RPL40B*. After incubation for 20 h in a 96-well plate (without shaking) with 0-300 mg/L octanoic acid, the strain with plasmid-based expression of *RPL40B* showed strongly decreased growth under all octanoic acid concentrations and even without addition of octanoic acid (Figure 5C).
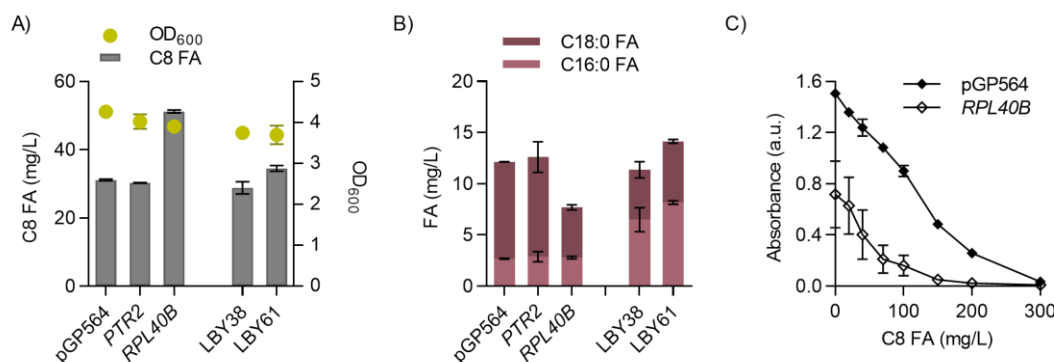

Figure 5. Effect of *PTR2* and *RPL40B* overexpression. (A, B) The octanoic acid producer strain LBY38 was transformed with plasmids containing *PTR2*, *RPL40B* or the control vector (pGP564). A second copy of *RPL40B* was integrated into the genome under control of *pPGK1* in the *PYK2* locus in LBY38 resulting in strain LBY61. Strains were cultured in buffered SCD medium and fatty acids (FA) were extracted after 46 h and quantified by GC measurement. n = 2, error bars = ± standard deviation. (C) Non-producer CEN.PK2-1C containing either the vector pGP564 (control) or *RPL40B* were inoculated in triplicates to OD_{600}=0.05 and supplied with 0-300 mg/L octanoic acid. Growth was measured by absorbance after 20 h. n = 3, error bars = ± standard deviation.

DISCUSSION

To date, no transcriptomics data of an *S. cerevisiae* octanoic acid producer strain was available. To fill this gap, we generated a high-quality RNA-Seq data set for three sampling times during fermentation. By comparing the growth, fatty acid and expression profile of the producer to a reference strain, we were able to get a profound insight into transcriptomic changes during octanoic acid production.

In the course of fermentation, octanoic acid biosynthesis increased in the producer strain from 7 mg/L after 14 h to 87 mg/L after 46 h. This increase correlated with the increase in differential expression in comparison to the reference strain, suggesting that increasing octanoic acid titers influence overall strain physiology. In terms of growth, the two strains behaved similarly until the diauxic shift when growth of the octanoic acid producer slowed down more quickly and reached lower final OD values. The main production of octanoic acid started shortly before or during the

diauxic shift, which is consistent with the production profile observed in our previous studies.[9,14] Although we did not measure the concentrations of glucose and ethanol in the course of fermentations shown in Figure 1, the sampling points can be clearly assigned to the glucose (T1) and ethanol consumption phases (T2, T3), respectively, based on the previously published correlation of precisely measured growth curves and carbon source profiles.[17] This implies that octanoic acid production mainly occurs during the consumption of ethanol, presumably due to an increased supply of the precursor molecule acetyl-CoA on this carbon source. The onset of octanoic acid production parallels the slowdown in growth; this can probably be attributed to toxic effects of octanoic acid on the cell physiology,[3,12,13,18] which is further potentiated in the presence of ethanol.[18,19] Octanoic acid disrupts the composition of the yeast plasma membrane leading to increased leakage and at high concentrations cell death.[12,13] This effect was shown to be mitigated by an increase in the average chain length of membrane fatty acids, i.e. more C18 than C16 fatty acids, as well as an increase in unsaturated fatty acid, i.e. oleic acid (C18:1).[13,20] In the culture supernatant, unsaturated long-chain fatty acids were not detectable. Interestingly, at T2 and T3, we observed that the octanoic acid producer secreted more saturated long-chain fatty acids of chain lengths C16 and C18 than the reference strain. Since the strain used in the experiment is a Δ*fas1*Δ*fas2*Δ*faa2* deletion strain, its only possibility to produce long-chain fatty acids is the leaky synthesis through plasmid-encoded *fusFAS*[RK]. Even though the "R1834K" amino acid exchange in the MPT domain of *FAS1* leads to premature release of short- and medium-chain fatty acids from the FAS cycle, this regulation is not completely tight.[9] As the pressure to synthesize enough long-chain fatty acids, which are essential for growth and survival, is probably high, the copy number of the plasmid containing *fusFAS*[RK] seems to have been increased. The evidence for this is twofold: Firstly, we saw higher expression of the *fusFAS*[RK] construct with RNA-Seq for all time points in the octanoic acid producer strain and secondly, the expression of the marker gene on the plasmid, *HIS3*, was increased accordingly. In summary, this suggests that gene expression is not optimized for a maximum production of octanoic acid but rather ideal for producing sufficient long-chain fatty acids to survive and mitigate toxic effects of octanoic acid. This could also explain the limit in octanoic acid production that we seem to encounter, as the precursor malonyl-CoA is increasingly used for C16 and C18 rather than for C8 fatty acid biosynthesis.

To get a deeper understanding of transcriptional changes, we analyzed differential expression at the three sampling times in the producer compared to the reference. For this comparison, we chose a $\log_2$FC of at least 1 as a threshold (FDR < 0.01) and chose a subset of the hits for further analysis. We evaluated the effect of additional, plasmid-based expression of all genes that were found to be up- or downregulated, respectively, at more than one RNA-Seq sampling time in the

octanoic acid producer. For this purpose, we chose to use a multi-copy library which contains the entire yeast genome cloned onto 2μ backbone plasmids and each plasmid contains roughly 2-10 genes.[16] Even though the other genes on the plasmid might also affect octanoic acid production, it is unlikely that they so much influence titers as to mask the possible effect of the gene-of-interest. The expression of *HXT2*, *BTN2*, *IMD2* and *ECI1* from library plasmids led to a decrease in octanoic acid titers after 46 h of fermentation whereas individual knockouts did not have any effect on growth or octanoic acid titers (Figure 4). Interestingly, growth and octanoic acid production were also delayed as a result of *HXT2*, *BTN2* and *IMD2* overexpressions (Figure 3). In our RNA-Seq data, *HXT2* was found significantly downregulated at all sampling times. In addition, *HXT2* expression has also been found downregulated with externally supplied octanoic acid.[13] As the downregulation was observable at all three sampling times, it seems to be rather independent of octanoic acid production. The expression of *BTN2*, similarly to *HXT2*, was downregulated at all sampling times in RNA-Seq ($\log_2FC > 1$, FDR < 0.01; except at T2: $\log_2FC = 0.7$) and its additional plasmid-based expression led to lower octanoic acid titers. *BTN2* encodes a v-SNARE protein involved in intracellular protein transport and regulation of pH.[21,22] In another study, Δ*btn2* strains showed an enhanced activity of vacuolar H^+-ATPase as well as diminished growth and decreased buffering capacity under sorbic acid supply, affecting intracellular pH homeostasis.[21] This contradicts the downregulation of *BTN2* expression at all sampling times in the octanoic acid producer, as the presence of octanoic acid might create more acidic conditions which the cell has to counteract. Also, our Δ*btn2* strain did not show any difference in octanoic acid titers. The reason for the decrease in octanoic acid titers following plasmid-based *BTN2* expression is also unclear. These partly contradictory results could also arise from differences in protein turnover or possible post-translational modifications. The expression of *IMD2* was significantly upregulated in our RNA-Seq data at all three sampling times ($\log_2FC > 1$, FDR < 0.01; except at T1 $\log_2FC = 0.65$) and additional plasmid-based expression led to a 13 % decrease in octanoic acid titers but did not influence growth. *IMD2* encodes an inosine monophosphate dehydrogenase, an enzyme involved in GTP biosynthesis. For *ECI1*, we observed a significant upregulation of expression at T3 ($\log_2FC = 1.4$) in our data as well as under octanoic acid supply in a non-producer strain in previously published data.[13] *ECI1* encodes a peroxisomal isomerase and is essential for β-oxidation of unsaturated fatty acids.[23] We observed that *ECI1* plasmid-based expression resulted in less octanoic acid whereas its knockout (LBY55) showed similar titers as the reference strain. We suspect that upregulation of *ECI1* leads to increased β-oxidation of unsaturated fatty acids, thereby possibly covering the cell's need for acetyl-CoA. Why this also leads to lower octanoic acid titers, remains obscure, as Eci1p has not been described to be involved in medium-chain fatty acid

degradation. The exact effect of all four enzymes on octanoic acid biosynthesis remains to be investigated.

We found that most genes identified to be differentially expressed at more than one sampling time, did not have a significant effect on octanoic acid titers when overexpressed. This suggests that the upregulation of these genes in the octanoic acid producer had effects not directly related to the octanoic acid titers. One possibility is that the observed gene expression changes help to mitigate effects on precursor availability or for instance toxic effects of the acid.

The library plasmid containing two genes of interest, namely *RPL40B* and *PTR2*, led to a strong increase in octanoic acid titer after 46 h of fermentation. Further analysis showed that plasmid-based expression of *RPL40B* was responsible for the improvement. Interestingly, secreted C18:0 fatty acid titers were halved in this strain. As *RPL40B* expression was downregulated at T3 in the transcriptomics analysis (log_2FC = 0.45), we suspect that this decrease reduces toxicity by lowering octanoic acid synthesis. In addition, a decrease in *RPL40B* expression may help maintain long-chain fatty acid synthesis at sufficient levels. Consequently, this would increase the strain's fitness. The genomic integration of a second copy of *RPL40B* under the control of the strong *pPGK1* promoter did not increase octanoic acid titers significantly and had no effect on C18 fatty acid titers. It seems that genomic expression of a second gene copy was not strong enough to exhibit the effects observed with expression from a multi-copy plasmid. Intriguingly, *RPL40B* expression from a multi-copy plasmid in non-producer CEN.PK2-1C, resulted in reduced growth. It is possible that this negative effect on growth is only observable in CEN.PK2-1C because LBY38 is already growth inhibited by inherent octanoic acid production. The decreased growth resulting from *RPL40B* overexpression in CEN.PK2-1C could also be attributed to lowered C18 fatty acid production by the native *FAS* complex and thereby impaired robustness. Rpl40b is a subunit of the ribosome[24] but to our knowledge has never been reported to play a direct role in fatty acid biosynthesis. Future work could investigate the global effect of increased Rpl40b expression via ribosome profiling ("RiboSeq"),[25] proteome analyses and ribosome assembly studies. Another interesting approach could be to study knockout or knockdown mutants of *RPL40B* or of other ribosomal subunits such as *RPL40A*.

In the aforementioned published transcriptomic data sets,[12,13] which were generated by adding octanoic acid externally to *S. cerevisiae* wild type strains, *RPL40B* had not been found to be differentially regulated. Overall, there was little overlap between our RNA-Seq data set and the two other data sets. On the one hand, the diverging results could be attributed to the difference in experimental conditions, such as strain background, media, and pH. More importantly,

differential expression seems to be highly growth phase-dependent and to differ for internally produced and externally supplied octanoic acid. This underlines the importance of collecting transcriptomic data not just for externally added compounds, but for actual producer strains. Our comprehensive transcriptomics data set facilitated the detection of an unreported genetic target by enabling an in-depth view into yeast physiology during octanoic acid production. We were able to identify the new target *RPL40B*, overexpression of which led to 40 % more octanoic acid.

MATERIALS AND METHODS

Strains and Plasmid Construction. Yeast strains and plasmids used throughout this study are listed in Table S1. The genes *DAN1* and *NOP56* for overexpression were amplified from SHY34[26] genomic DNA with primers containing the respective overhangs for cloning via Golden Gate[27] (oligonucleotides are listed in Table S2). The *PDR12-tPDR12* fragment as well as promoter *pTDH3* were amplified from CEN.PK113-11C genomic DNA with primers containing the respective overhangs for cloning via homologous recombination into pRS42H/*Eco*RV. The library plasmid containing *RPL40B* and *PTR2* (A10-C10 from [16]) was split into two plasmids as follows: Each gene was amplified with its endogenous up- and downstream regions from the original plasmid with primers containing overhangs for the pGP564 backbone for homologous recombination in yeast (CEN.PK2-1C). pGP564 was digested with *Bam*HI/*Pst*I prior to use. Genomic knockout of *HXT2* was performed via CRISPR/Cas9 as described previously.[28] The CRISPR/Cas9 plasmid for *hxt2* knockout was assembled via Golden Gate - SiHV138 was used and the GFP-dropout region was replaced by pre-assembled, double stranded gRNA oligonucleotides. For *BTN2*, *IMD2* and *ECI1* knockout via CRISPR/Cas9 was not possible and ORFs were knocked out with the Cre-*loxP* recombinase system with the *HPH*[R] marker as described in [29]. For this purpose, the resistance cassette was amplified from plasmid pUG6H with primers containing overhangs for the up- and downstream regions, respectively, of the targeted ORF. For genomic integration of a second copy of *RPL40B*, the promoter *pPGK1* was amplified via PCR from pYTK011 and *RPL40B* was amplified via PCR from the original plasmid including the downstream part. Used primers contained suitable overhangs for the parts or *PYK2* up- or downstream regions, respectively. Integration was achieved via CRISPR/Cas9 plasmid LBV103 (assembled via homologous recombination into pRCC-K backbone) into the *PYK2* locus, thereby removing the entire *PYK2* ORF. Yeast transformations were performed according to Gietz and Schiestl[30] or for SHY34 via an adapted procedure.[9] Cells were streaked out on selective YPD (1 % yeast extract, 2 % peptone, both by BD, Difco Laboratories, USA; 2 % D-glucose, Roth, Germany) containing hygromycin (200 µg mL^{-1}) to select for *hphNT1*, G-418 (200 µg mL^{-1}) to select for *KanMX* or on selective SCD medium[17] lacking leucine (SCD^{-leu}) to

select for *LEU2*. Electrocompetent *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) was used for subcloning according to standard procedures, and transformants were selected on lysogeny broth (LB) agar plates[31] supplemented with carbenicillin (100 μg mL^{-1}), chloramphenicol (20 μg mL^{-1}) or kanamycin (50 μg mL^{-1}).

Cultures for RNA-Seq analysis. For pre-cultures, several colonies of SHY34 with plasmid *fusFAS^{wt}* or *fusFAS^{RK}*, respectively, were inoculated in 20 mL YPD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and shaken (180 r.p.m.) at 30 °C overnight. The main cultures were inoculated in triplicates to an OD$_{600}$ of 0.1 in 50 mL buffered YPD medium in 300 ml flasks on a cell growth quantifier (aquila biolabs GmbH, Germany[17]). Samples were taken for fatty acid quantification as described below. For RNA extraction, ~10 OD of cells/sample were pelleted in a pre-cooled falcon at 4°C (3000 rcf) for 3 min. Falcons were then immediately dropped into liquid nitrogen for a few minutes and stored at -80 °C until further processing.

RNA extraction, processing and sequencing. RNA extractions were performed on samples that were mechanically lysed with 0.5 mm acid washed beads using an MP-Biomedicals™ FastPrep-24 for three one-minute cycles. Further extraction was performed using an RNeasy® Kit from Qiagen. Libraries were prepared using the TrueSeq mRNA Stranded HT kit. Sequencing was carried out using an Illumina NextSeq 500 High Output Kit v2 (75 cycles), with a minimum of 10 million single-end reads per replicate. The Novo Nordisk Foundation Centre for Biosustainability (Technical University of Denmark) performed the RNA sequencing and library preparation. RNA-Seq data sets can be found using SRA accession PRJNA575618. RNA-Seq read mapping was performed after analysis in FASTQC. RNA-Seq mapping for differential expression was mapped with STAR[32] and reads were assigned with featureCounts[33]. Differential expression results can be found in Supplementary File 1.

Cultures for Fatty Acid Production. *S. cerevisiae* strains were grown as previously described[9] with minor adjustments. For pre-cultures, several colonies of a strain were inoculated in 20 mL YPD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and antibiotic (200 μg mL^{-1} hygromycin), if applicable, or in similarly buffered SCD medium lacking leucine (SCD^{-leu}). After shaking (180 r.p.m.) at 30 °C overnight, the main culture was inoculated to an OD$_{600}$ of 0.1 in the respective medium and cultured in 300 mL shake flasks under the same conditions. For sampling, the cultures were harvested by centrifugation and 10 mL of the supernatant was used for fatty acid extraction.

Fatty Acid Extraction and Derivatization. Fatty acid extraction and derivatization were performed as described previously.[3] Cells were separated from the medium (3500 rcf, 10 min), an internal

standard (0.2 mg heptanoic acid) was added to 10 mL supernatant and mixed with 1 mL 1 M HCl and 2.5 mL methanol/chloroform solution (1:1). After vigorous shaking for 3 min, the mixture was centrifuged at 3000 rcf for 10 min and the chloroform layer was recovered and evaporated overnight. The methylation of the fatty acids was performed as previously described.[34] Samples were dissolved in 200 μL toluene, mixed with 1.5 mL methanol and 300 μL 8.0 % (w/v) HCl solution (diluted in methanol), vortexed, and incubated at 100 °C for 3 h to form fatty acid methyl esters (FAME). After cooling on ice for 10 min, 1 mL H_2O and 1 mL hexane were added to the sample, followed by thorough shaking, and the organic phase was transferred to a GC vial.

GC-FID analysis of FAMEs. GC analyses were carried out on a Perkin Elmer Clarus 400 instrument (Perkin Elmer, Germany) equipped with an Elite FFAP capillary column (30 m × 0.25 mm, film thickness: 0.25 μm; PerkinElmer, Germany) and a flame ionization detector (Perkin Elmer, Germany) as described previously.[3]

Toxicity test. CEN.PK2-1C was transformed with plasmid pGP564 or *RPL40B*, respectively, and plated on SCD[-leu]. Pre-cultures were inoculated in triplicates in buffered SCD[-leu] and grown over night at 30 °C (180 r.p.m.). For main cultures, strains were inoculated to an OD_{600} of 0.2 and incubated for 4 h under the same condition (30 °C, 180 r.p.m.). The cultures were then diluted in fresh media to an OD_{600} of 0.05 and transferred into a 96-well plate (clear with flat bottom) with 50 μl per well. A dilution series was made with octanoic acid diluted in the same media, to reach final concentrations in the wells of 0-300 mg/L. Of these dilutions, 200 μl were added per well. The three biological replicates per strain were inoculated in technical triplicates in the well plates. The starting absorbance was measured in a platereader (CLARIOstar®, BMG Labtech, Ortenberg, Germany) and plates were incubated for 20 h at 30 °C without shaking before absorbance was measured again. From final absorbance values, a blank value (media without strain) was subtracted.

Software. RNA-Seq read mapping and data analyses were performed with R packages limma, edgeR and piano. Data tables were stored in Microsoft Excel 2016. Fermentation graphs were made using the software Prism 9 (GraphPad, USA). Geneious Prime 2020.2 software was used for assembly planning.

Abbreviations. FAS: fatty acid synthase; FDR: false discovery rate; GC: gas chromatography; $\log_2$FC: $\log_2$ fold change; MPT: malonyl-palmitoyl transferase; OD_{600}: optical density at λ=600 nm.

Data availability. RNA-Seq data sets of data generated in this study can be found using SRA accession PRJNA575618.

ASSOCIATED CONTENT

Supporting Information

Supplementary Figures and Tables: Tables S1-S2 and Figures S1-S6

Supplementary File 1_differential expression

Supplementary File 2_GO term enrichment analysis

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Author Contributions

LB performed experiments including RNA-Seq fermentations, cloning, engineered strain fermentations and analysis. TD performed RNA extraction. LB and TD analysed RNA-Seq data and wrote the paper. MO and EB co-supervised the project. VS and JN provided advice and resources. All authors read and approved the manuscript.

Conflicts of interest

EB is inventor of EP patent application No. 15 162 192.7 filed on April 1, 2015, and of EP patent application No. 15 174 342.4 filed on June 26, 2015, by Goethe-University Frankfurt, concerning short-chain acyl-CoA producing FAS variants. There are no other competing interests.

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SUPPORTING INFORMATION

Table S1. Yeast strains and plasmids used in this study.

Strain	Characteristics	Reference
CEN.PK2-1C	*MATa*; *MAL2-8c*; *SUC2*; *ura3-52*; *his3Δ1*; *leu2-3_112*; *trp1-289*	Euroscarf, Frankfurt am Main, Germany
RPY21	*MATα; ura3Δ0; his3Δ0; leu2Δ0; TRP1; lys2Δ0; MET15; ΔFAS1::kanMX4; ΔFAS2::kanMX4; Δfaa2*; transformed with plasmids pRS315-*FAS1^{R1834K}* and pRS313-*FAS2*	[1]
SHY34	*MATα; ura3Δ0; his3Δ0; leu2Δ0; TRP1; lys2Δ0; MET15; Δfas1 Δfas2 Δfaa2*	[2]
LBY38	CEN.PK2-1C Δ*pFAS1*-1-300::*pHXT7*-1-392 Δ*pFAS2*-1-200::*pHXT7*-1-392, *FAS1^{R1834K}*, *Δfaa2*	This group
LBY49	LBY38 Δ*hxt2*	This study
LBY53	LBY38 Δ*btn2*	This study
LBY55	LBY38 Δ*eci1*	This study
LBY61	LBY38 Δ*pyk2*::*pPGK1-RPL40B-tRPL40B*	This study
Plasmid	**Characteristics**	**Reference**
Plasmids used for fermentations		
fusFAS^{wt}	pRS313-*pFAS-fusFAS12*-Wt	[3]
fusFAS^{RK}	pRS313-*pFAS-fusFAS12*-RK	[3]
pRS42H	2μ, *HPH^R*, *AmpR*, multiple cloning site including *Eco*RV	[4]
LBV16 (*PDR12*)	pRS42H with *pTDH3-PDR12-tPDR12* integrated in *Eco*RV site	This study
pGP564	2μ, *LEU2*, *KanR*	[5]
Library plasmids (containing genes): A10-F1 (*FAS1*), A16-E2 (*FAS2*), A15-A7 (*HIS3/YOR203W*), A15-D6 (*YOR186W*), A9-H11 (*MCH2*), A8-G4 (*IMD2*), A16-D6 (*EEB1*), A12-F8 (*HXT2*), A1-E10 (*MNC1*), A10-C10 (*PTR2*), A7-E2 (*BTN2*), A1-G6 (*URA7*), A5-A2 (*UTR2*), A3-B11 (*YDR133C*), A12-B2 (*INA1*), A14-D9 (*IZH4*) , A1-A8 (*PDR3*), A8-A2 (*SPS100*), A13-E10 (*SPS19*), A11-C10 (*ECI1*), A15-B2 (*RSB1*), A1-C1 (*GDH3*), A5-E11 (*HSP12*), A10-C10 (*RPL40B/PTR2*)	pGP564, yeast genomic DNA fragments of ~ 10 kb size	[5]
LBV100 (*PTR2*)	pGP564; 2μ, *LEU2*, *KanR*, *pPTR2-PTR2-tPTR2*	This study
LBV101 (*RPL40B*)	pGP564; 2μ, *LEU2*, *KanR*, *pRPL40B-RPL40B-tRPL40B*	This study
SiHV010	2μ, *HPH^R*, *KanR*	[6]
LBV71	SiHV010-*pYEF3-DAN1-tSSA1*	This study
LBV72	SiHV010-*pYEF3-NOP56-tSSA1*	This study

Plasmids used for CRISPR/Cas9		
pRCC-K	2μ, *kanMX, AmpR, pROX3-Cas9[opt]-tCYC1, pSNR52-gRNA*	7
SiHV138	2μ, *kanMX, AmpR, pROX3-Cas9[opt]-tCYC1, pSNR52-gRNA, GFP-dropout* (modified from pRCC-K)	7
LBV52	SiHV138 with *gRNA* (TGGGTTAACCTTAGGACGTC) for *HXT2* locus	This study
LBV103	pRCC-K with gRNA for *PYK2* (ATGTCTTTGGCGGACAAGGG) locus	This study
Plasmid used for Golden Gate assembly		
pYTK001	Golden Gate entry vector, *CamR*	6
Plasmids Cre-*loxP* recombinase system		
pUG6H	contains *loxP-HPH[R]-loxP* for knockout in yeast	8

Table S2. Oligonucleotides used in this study.

Primer	Sequence 5'-3'	Application
Cloning of LBV71, LBV72		
LBP321	CGTCTCGTCGGTCTCATATGTCTAGAATTAGTATATTAGCTGTCG	Amplification of *DAN1* from SHY34 genomic DNA with overhangs for Golden Gate cloning
LPB322	CGTCTCAGGTCGGTCTCAGGATCTATAACAATAGAGCGGCGGC	
LBP323	CGTCTCGTCGGTCTCATATGGCTCCTATTGAATACCTAC	Amplification of *NOP56* from SHY34 genomic DNA with overhangs for Golden Gate cloning
LBP324	CGTCTCAGGTCGGTCTCAGGATTTAATCCTTACTTTTCTTCTTTTTATCCTTC	
SiHSeq001	TCCTGGCCTTTTGCTGG	Sequencing of *DAN1* and NOP56 in pYTK001 backbone
SiHSeq002	GGACTCCTGTTGATAGATC	
Cloning of LBV100, LBV101		
LBP406	CTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGTGCTATGTATTCTTAGTGTACTGC	Amplification of *PTR2* including up- and downstream regions from A10-C10 of library[5]
LBP407	GTCGACGGTATCGATAAGCTTGATATCGAATTCTCGATTTATTACGATCTCCACAAATC	
LBP408	GCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGTGCGTTTAATTAACTTACTGTC	Amplification of *RPL40B* including up- and downstream regions from A10-C10 of library[5]
LBP409	CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCTATCCTAAAACGTGGCGTTA	
LBP237	GGAATTGTGAGCGGATAAC	Sequencing of LBV100, LBV101
LBP238	ACTATAGGGCGAATTGGG	
Cloning of LBV16		
LBP72	CCTCGAGGTCGACGGTATCGATAAGCTTGATATCACAGTTTATTCCTGGCATCCAC	Amplification of *pPGK1* from CEN.PK113-11C with overhangs to *PDR12* and pRS42H, respectively
LBP73	GTCTTTCTCAATATGTTCGTCAGTCGAAGACATTTTGTTTGTTTATGTGTGTTTATTCG	
LBP74	CGAATAAACACACATAAACAAACAAAATGTCTTCGACTGACGAACATATTGAGAAAGAC	Amplification of *PDR12-tPDR12* from CEN.PK113-

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LBP75	GATCCCCCGGGCTGCAGGAATTCGATGATATCTCCTTTTGAAGGT GATAGGGATATAATG	11C with overhangs to pRS42H and *pTDH3*, respectively
LBP85	GGCGATTAAGTTGGGTAACG	Sequencing of LBV16
RPP056	CACACAGGAAACAGCTATGAC	

Knockout of *BTN2*, *IMD2* and *ECI1* via Cre-*loxP* recombinase system

LBP325	CAACCAAAAGAAAATAACTAATAGACCCCATTACAATATAGAATT CGTACGCTGCAGGTCGAC	Amplification of *HPH^R* cassette with overhangs for *BTN2* up/downstream regions
LBP326	CGTAAAAATGAAAGATGGGGAGTATGTATTATCACCCAGCATAG GCCACTAGTGGATCTG	
LBP329	ATAATCAGTGCAATTAATAACTCCACAAGTAGCAAAAGCATTCGT ACGCTGCAGGTCGAC	Amplification of *HPH^R* cassette with overhangs for *IMD2* up/downstream regions
LBP330	CATCAGTAATACTGTATTGATGATGCCATTTTAACATGCATAGGC CACTAGTGGATCTG	
LBP333	GGCACAAATTGCTCGCACAGTAAAGGAAGGAAGAACAATTTCGT ACGCTGCAGGTCGAC	Amplification of *HPH^R* cassette with overhangs for *ECI1* up/downstream regions
LBP334	ATTGTGTGTGCGTTTTGTTTCACTGAGAAAGCGGACGGCATAGG CCACTAGTGGATCTG	
LBP309	GTCATGTAGCACTATTTCAGCC	Sequencing of *BTN2* locus
LBP310	GAATCACTTGCCATTCCACC	
LBP327	ACCATTACAGACCAACTTAC	
LBP328	AACTTCCTCATCCTCAAC	
LBP311	AGAGATGATGCAAGAGTAG	Sequencing of *IMD2* locus
LBP312	TCACCACCAAAATCACC	
LBP331	ACCAAGAGCCTACCAAGAC	
LBP332	CCAACATACCACCCATCATAAC	
LBP335	TCACTTAATGAACCCTGAC	Sequencing of *ECI1* locus
LBP336	CTCTCCATCTACCCAATAC	
LBP337	TCAAATAAACCTGCTGCC	
LBP338	ACGCAACTAAATGAGCAC	

Knockout of *HXT2*

LBP317	GATCTGGGTTAACCTTAGGACGTC	Amplification of gRNA for *HXT2* locus
LBP318	AAACGACGTCCTAAGGTTAACCCA	
LBP320	GATTATAAGAACAACAAATTAAATTACAAAAAGACTTATAAAGCA ACATAGAGATTATACTTAAACTAGCACTGATTTTTTTAAGGCTAAT GGCTACTAA	Donor for *hxt2* knockout
MGP126	GGGAAACGCCTGGTATC	Sequencing of gRNAs
LBP313	TCCCTCTCCACTCTTTCTC	Sequencing of *HXT2* locus
LBP314	ATCAGCCACAATACGCC	

Integration of *pPGK1-RPL40B* into *PYK2*

LBP106	ATGTCTTTGGCGGACAAGGGGTTTTAGAGCTAGAAATAGCAAGT TAAAATAAGG	Amplification of gRNA for *PYK2* locus
LBP107	CCCTTGTCCGCCAAAGACATGATCATTTATCTTTCACTGCGGAG	
LBP430	CATATTATTTCGAACATAGTATTCTGTATTCGCCGTAACATACATT TGCATGATCTTGTTTTATATTTGTTGTAAAAAG	Amplification of *pPGK1* from pYTK001 with overhangs to *pyk2*-downstream and *RPL40B*, respectively
LBP431	CTGACACAATGGACAATTAAATAAAATTAAGTAAAAAAAATAAG GACTTTAATTTTTAAACGGTGAGTAAGGAAAG	
LBP428	CTATATTTTACTTTCATCCTCTACGTCCATTGTAAGATTACAACAA AAGCACTATCGTGCGTTTAATTAACTTACTGTC	Amplification of *RPL40B* with downstream region from A10-C10 library[5]
LBP429	CAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATAA AACAAGATCATGCAAATGTATGTTACGG	

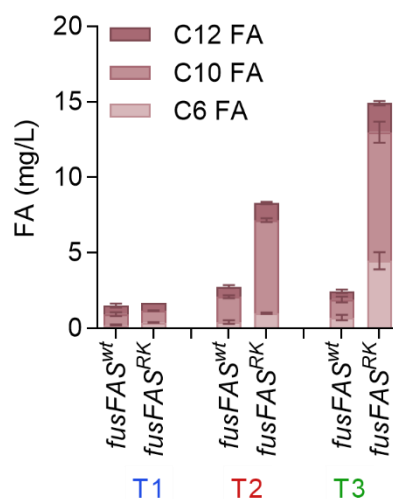| LBP118	CAGAGCGGTGAAACGCAAC	Sequencing of
LBP119	CGCAGTTTGCGAACATTACCTG	△*pyk2*::*pPGK1-RPL40B-*
VSP159	CGTGTGACAACAACAGCC	*tRPL40B*

Figure S1. Characterization of C6, C10 and C12 fatty acid production. Strains SHY34+*fusFAS^wt* (reference strain) and SHY34+*fusFAS^RK* (octanoic acid producer) were cultured for 46 h in buffered YPD medium. Sampling times are indicated with arrows (T1 = 14 h; T2 = 22 h; T3 = 46 h). Fatty acids (FA) were extracted and quantified via GC. *n* = 3, error bars = ± standard deviation.
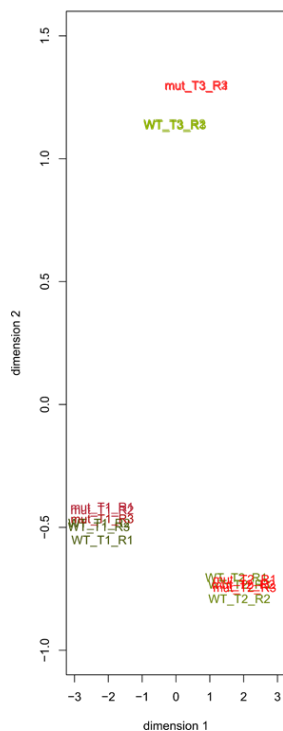
Figure S2. Principal component analysis (PCA) of RNASeq data sets. The RNASeq data of all three replicates (R1-R3) of the three sampling times (T1 = 14 h; T2 = 22 h; T3 = 46 h) of the reference strain (WT) and the octanoic acid producer strain (mut), respectively, were compared in PCA suggesting similar global expression.
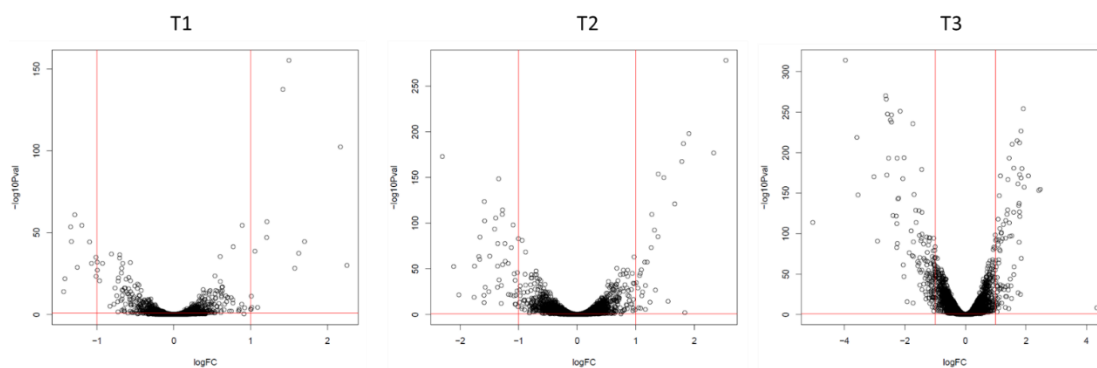

Figure S3. Differential gene expression analysis. Volcano plots show the $\log_2$FC of gene expression at the three sampling times (T1 = 14 h; T2 = 22 h; T3 = 46 h) for SHY34+$fusFAS^{RK}$ (octanoic acid producer) compared to SHY34+$fusFAS^{WT}$ (reference strain).

(A) Upregulated

Overlaps with…	Gene
…T1	*PDR12*

(B) Downregulated

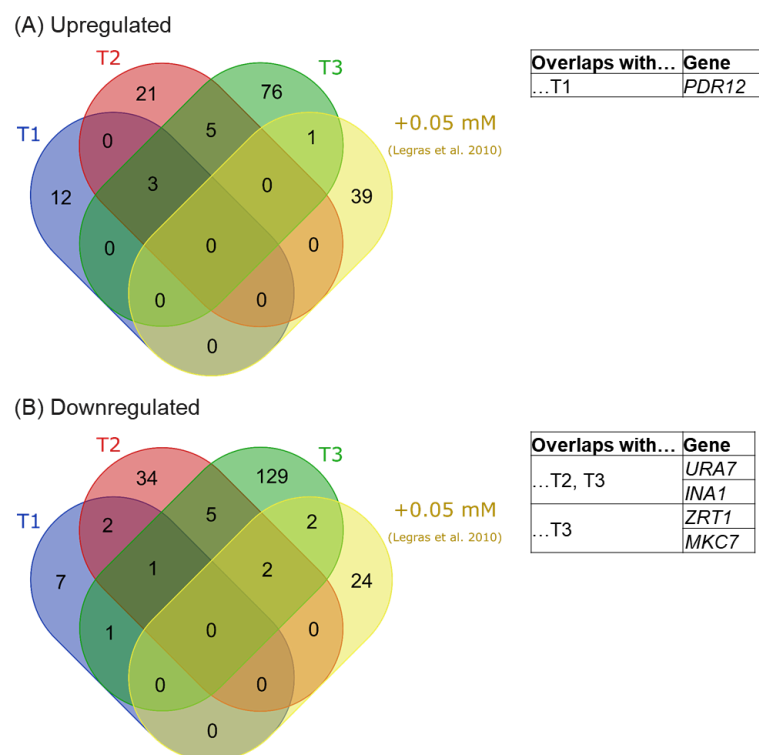| Overlaps with…	Gene
…T2, T3	*URA7*
	INA1
…T3	*ZRT1*
	MKC7

Figure S4. Comparison of transcriptome data of an octanoic acid producer with the response to externally supplied octanoic acid to a non-producer. Number of genes that are upregulated (A) or downregulated (B) in the two experiments. For RNA-Seq, an octanoic acid producer strain was analyzed in comparison to a non-producer strain ($log_2FC > 1$) at different sampling times (T1 = 14 h; T2 = 22 h; T3 = 46 h). Microarray differential expression data of a non-producer strain supplied with 0.05 mM octanoic acid for 20 minutes in comparison to non-supplied control ($log_2FC \geq 0.3$); data taken from Legras *et al*. 2010.[9] Gene lists include genes that overlap between data from Legras *et al*. 2010 and at least one sampling time of the RNA-Seq experiment.

(A) Upregulated

Overlaps with…	Gene
…T2, T3	IMD2
	EEB1
…T1	PHO5
…T2	PDC6
…T3	PDR3
	SPS100
	POX1
	SPS19
	ECI1
	PDR15
	RSB1
	PDR12
	GDH3

(B) Downregulated

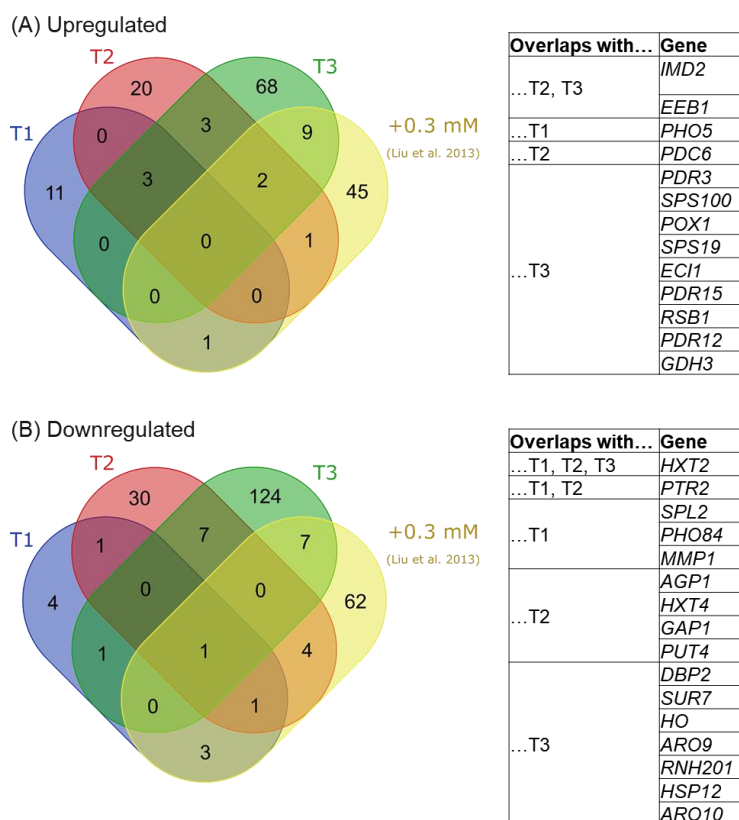| Overlaps with…	Gene
…T1, T2, T3	HXT2
…T1, T2	PTR2
…T1	SPL2
	PHO84
	MMP1
…T2	AGP1
	HXT4
	GAP1
	PUT4
…T3	DBP2
	SUR7
	HO
	ARO9
	RNH201
	HSP12
	ARO10

Figure S5. Comparison of transcriptome data of an octanoic acid producer with the response to externally supplied octanoic acid to a non-producer. Number of genes that are upregulated (A) or downregulated (B) in the two experiments. For RNA-Seq, an octanoic acid producer strain was analyzed in comparison to a non-producer strain ($log_2FC > 1$) at different sampling times (T1 = 14 h; T2 = 22 h; T3 = 46 h). Microarray differential expression data of a non-producer strain supplied with 0.3 mM octanoic acid in comparison to non-supplied control ($log_2FC > 1$) in mid-log growth; data taken from Liu *et al*. 2013.[10] Gene lists include genes that overlap between data from Liu *et al*. 2013 and at least one sampling time of the RNA-Seq experiment.
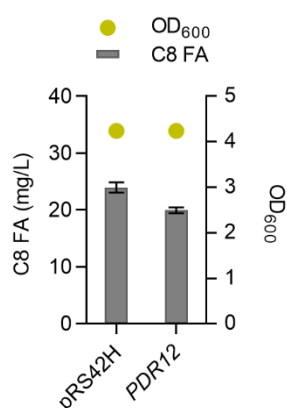

Figure S6. Effect of *PDR12* overexpression. The octanoic acid producer strain RPY21-*FAS1*[RK]-*FAS2* was transformed with a multi-copy plasmid containing *PDR12* or the control vector pRS42H. Strains were cultured in buffered YPD[hygro] medium and fatty acids were extracted after 72 h and quantified by GC measurement. *n* = 2, error bars = ± standard deviation.

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6.4   High-throughput screening of an octanoic acid producer strain library enables detection of new targets for increasing titers in *Saccharomyces cerevisiae*

Declaration of author contributions to the manuscript:
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Contributions of doctoral candidate and co-authors

(1) Concept and design

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Co-authors SB, JK, EB, MO: 5%, 5%, 5%, 5%

(2) Conducting tests and experiments

<u>Doctoral candidate LB</u>: 90%, yeast molecular biology, biosensor measurements, FACS screening, fermentations, metabolite extraction and GC analysis, toxicity tests

Co-author SB: 10%, FACS screening

(3) Compilation of data sets and figures

<u>Doctoral candidate LB</u>: 100%, biosensor assays, FACS screening, metabolite titers and growth, toxicity test

(4) Analysis and interpretation of data

<u>Doctoral candidate LB</u>: 70%, biosensor assays, FACS data, metabolite titers and growth, toxicity test

Co-authors SB, EB, MO: 10%, 10%, 10%, FACS data, supervision and advice

(5) Drafting of manuscript

<u>Doctoral candidate LB</u>: 85%

Co-authors SB, EB, MO: 5%, 5%, 5%

High-throughput screening of an octanoic acid producer strain library enables detection of new targets for increasing titers in *Saccharomyces cerevisiae*

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ABSTRACT: Octanoic acid is an industrially relevant compound with applications in antimicrobials or as a precursor for biofuels. Microbial biosynthesis through yeast is a promising alternative to current unsustainable production methods. To increase octanoic acid titers in *Saccharomyces cerevisiae*, we use a previously developed biosensor based on the octanoic acid responsive *pPDR12* promotor coupled to GFP. We establish a biosensor strain amenable for high-throughput screening of an octanoic acid producer strain library. Through development, optimization and execution of a high-throughput screening approach, we were able to detect two new genetic targets, *KCS1* and *FSH2*, which increased octanoic acid titers when overexpressed and have not been reported yet to be involved in fatty acid biosynthesis. The combined overexpression of both genes led to an increase in titers of 55 % compared to the parental strain. The presented methodology can be employed to screen any genetic library and thereby more genes involved in improving octanoic acid production can be detected in the future.

KEYWORDS: *Octanoic acid, C8 fatty acid, High-throughput Screening, overexpression library, FACS, yeast*
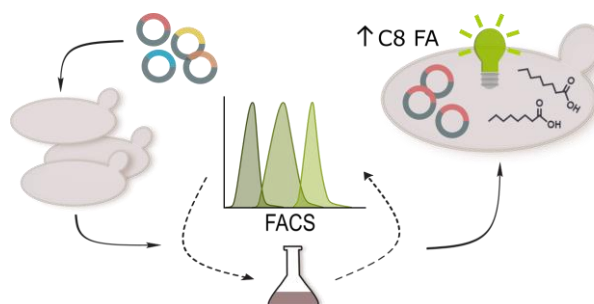
INTRODUCTION

Medium-chain fatty acids, such as octanoic acid (C8 fatty acid), are widely used in industry, for example as antimicrobials, or can be converted to fatty-acid derivatives like fatty alcohols, alkanes or alkenes, thereby widening the application range to biofuels, lubricants and surfactants.[1] The current supply is mostly based on the extraction from oily plants, such as coconut and palm oil plants. However, these vegetable oils mostly consist of long-chain fatty acids with only minor fractions of octanoic and decanoic acid.[2] Furthermore, the cultivation of oil plants oftentimes involves deforestation and extensive land use,[3] making other production methods from renewable substrates desirable. Engineering microbes like yeast to produce octanoic acid is a promising alternative with potentially less environmental impact.

In recent years, several reports have shown the successful engineering of *S. cerevisiae* to produce medium-chain (C6-C12) fatty acids.[4–7] Most recently more than 1g/L medium-chain fatty acids were reported to be produced by an engineered *S. cerevisiae* strain.[6] However, producing a mixture of chain lengths, reduces the yield of the desired fatty acid, and purification is difficult because different chain lengths have similar physicochemical properties.[1] One way to achieve strains only producing specific fatty acid chain lengths, is by rational engineering of the fatty acid synthesis (FAS) genes. Gajewski et al. (2017) introduced an amino acid exchange of arginine to lysine (R1834K) in the MPT domain of *FAS1*, thereby generating an *S. cerevisiae* strain producing mainly octanoic acid. Apart from strategies for chain-length control, engineering efforts to generate higher titers have mostly focussed on enzymes involved in precursor routes for increasing cytosolic acetyl-CoA and malonyl-CoA supply or on decreasing fatty acid degradation.[1,8,9] One example to achieve the latter, is knockout of *FAA2*, encoding a medium-chain fatty acyl-CoA synthetase, which activates imported fatty acids in the peroxisomes for degradation via the β-oxidation pathway.[10] Knockout of *FAA2* has previously been demonstrated to increase short- and medium-chain fatty acid titers considerably.[11,12]

Nevertheless, to construct efficient cell factories, targeted engineering strategies are insufficient. Due to the complex cellular network, there are many factors whose direct or indirect involvement in fatty acid biosynthesis, transport, degradation or detoxification mechanisms are unknown so far. An alternative strategy to rational engineering of specific target genes to generate strains with higher product titers is the generation of strain libraries. However, strain evaluation is currently the rate limiting step in such efforts because traditional analytical methods like gas chromatography (GC) are very laborious and time-consuming.[13,14] Biosensors in combination with a high-throughput detection technology, are a great tool to overcome this drawback. Biosensors

enable easy and rapid detection of the desired compound, e.g. via fluorescence quantification.[13,15–17] Previously, we have developed a whole-cell short-chain fatty acid biosensor amenable for high-throughput screenings.[18] The biosensor is based on the short-chain fatty acid responsive promotor *pPDR12* coupled to GFP. *PDR12* encodes an ATP-binding cassette transporter and its expression is regulated by the transcription factor War1p.[19,20] War1p activates GFP expression via *pPDR12* regulation in the presence of inducing molecules, i. e. hexanoic, heptanoic and octanoic acid, in a concentration-dependent manner. For octanoic acid, the linear detection range was between 0.01 - 0.75 mM (1.4 - 108 mg/L) and it showed a high dynamic range with up to 10-fold increase in signal after only 2 h of induction.[18]

Here, we modify the biosensor strain and verify its usability in flow cytometry. We aimed at detecting new targets that increase octanoic acid titers in *S. cerevisiae* through a high-throughput screening of a gene overexpression library via fluorescence-activated cell sorting (FACS). This approach enabled us to detect unexpected genes whose overexpression led to increased octanoic acid titers. To our knowledge, this is the first report of the application of an octanoic acid biosensor to identify genes enhancing octanoic acid levels in *S. cerevisiae*.

RESULTS AND DISCUSSION

Adaptation of an octanoic acid biosensor for use in a FACS-based screening. To adapt a previously developed octanoic acid biosensor[18] for screening via FACS, we carried out several modifications. The original biosensor relied on a multi-copy plasmid carrying *pPDR12*-GFP. For a single cell screening through flow cytometry, we assumed that GFP expression from a multi-copy plasmid would create high single cell variation as a result of differing plasmid copy numbers per cell. This assumption was confirmed in a flow cytometry experiment (Figure S1). To avoid this effect, we considered two options: (1) Normalization of copy numbers through constitutive expression of another fluorescent gene (e. g. mCherry) on the same centromeric plasmid with the biosensor or (2) genomic integration of the biosensor. The first option has been shown to be successful for PHBA detection,[21] however, it is more complex to execute because normalization of each measurement is inevitable. With the second option – a strain with genomically integrated biosensor – we had previously conducted measurements in a monochromator microplate reader. We had observed that the GFP signal was only barely distinguishable from a strain without sensor under octanoic acid supply.[18] To verify the functionality of a genomically integrated biosensor in flow cytometry, we cultured strain LBY27 – containing the *pPDR12*-GFP cassette genomically – for 2 h with octanoic acid concentrations ranging between 0-80 mg/L. After this induction period, the samples were screened employing flow cytometry and we observed an increase in fluorescence

signal with increasing octanoic acid concentrations (Figure 1). Even though the fluorescence values partly overlapped for different concentrations, the mean fluorescence rose in correlation with the octanoic acid concentration. This verified that supplied octanoic acid is taken up by the cells and induces GFP production in a concentration-dependent manner.[12,18] Furthermore, we asserted that the detection in the flow cytometer is much more sensitive than in the microplate reader confirming that the genomically integrated biosensor is generally suitable for the envisioned screening.
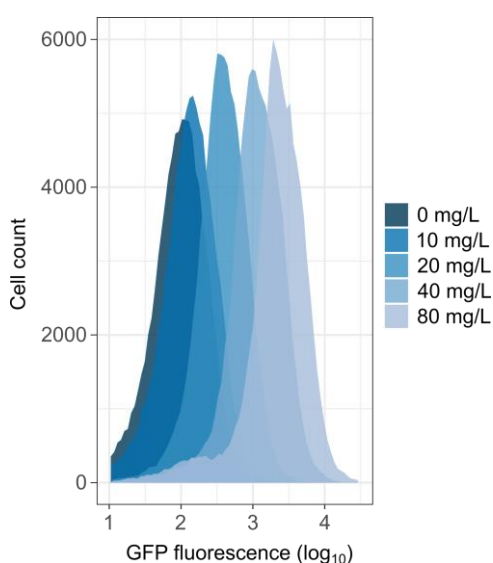

Figure 1: Evaluation of biosensor response to supplementation with octanoic acid. Strain LBY27, which contains a stable genomic integration of the biosensor *pPDR12-GFP*, was inoculated to an OD_{600} = 0.2 and grown in SCD medium for 3 h. The indicated octanoic acid amounts were added and the cultures were grown for another 2 h before measurement of GFP fluorescence in a flow cytometer (common logarithmic transformation of fluorescence area plotted). Results are shown for a single experiment; the experiment was repeated with comparable results (Figure S2).

In the next step, we genomically integrated the biosensor in an octanoic acid producer strain. To enable octanoic acid production, the strain contains a mutation in the *FAS1* gene at position 1834 bp, resulting in an amino acid exchange from arginine to lysine (*FAS1^RK*).[4] This strain, LBY31, furthermore contains a positive-feedback loop[21] to increase the dynamic range of the sensor. For the positive-feedback loop, the promotor of the transcriptional regulator-encoding gene *WAR1* was replaced with its target promotor *pPDR12*. This was shown to increase the dynamic range of GFP expression in response to para-hydroxybenzoic acid (PHBA), another activator of *pPDR12*.[21]

When growing LBY31 in buffered SCD medium, we observed that the 35 mg/L octanoic acid produced 24 h after inoculation were reduced to about 50% after 46 h. After 72 h of fermentation, almost no octanoic acid was detectable anymore (Figure 2). To prevent octanoic acid degradation, we deleted *FAA2*, encoding a fatty acyl-CoA synthetase involved in the activation of free medium-chain fatty acids prior to β-oxidation.[10] It has previously been shown that deletion of *FAA2* prevents the degradation of medium-chain fatty acids such as octanoic acid.[11,12] The resulting strain LBY39 indeed maintained the octanoic acid titer at the same level even 72 h after

inoculation. Therefore, the knockout of *FAA2* proved to be essential to maintain octanoic acid titers stable over several days of fermentation. Stable titers are important to prevent false-positive strains in the screening, which might have varying titers due to varying degrees of intracellular octanoic acid degradation. The resulting biosensor strain LBY39 has a high linear and dynamic range of detection and produced octanoic acid amounts within the detection range of the sensor (~35 mg/L).
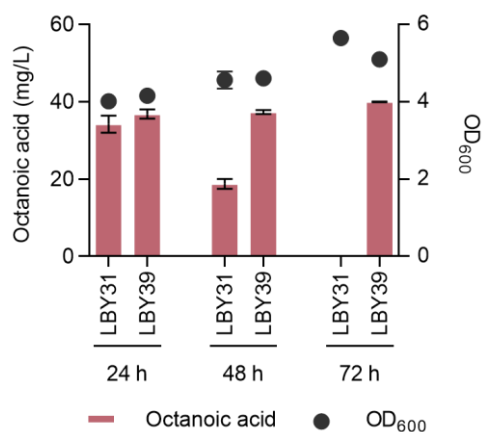

Figure 2: Effect of *FAA2* knockout on octanoic acid titers in biosensor strain. OD_{600} (dots) and octanoic acid titers (bars) of LBY31 and LBY39 fermented in buffered SCD medium over 72 h. LBY31 (producer): *pPDR12-GFP*, *FAS1RK*. LBY39 (producer): *pPDR12-GFP*, *FAS1RK*, Δ*faa2*. Fatty acids were extracted, methylated and quantified via GC. n = 2, error bars = ± SD.

We then sought to test whether the octanoic acid producer strain LBY39 can be distinguished from the non-producer LBY27 in flow cytometer analysis which we performed 4 h, 24 h and 48 h after inoculation (Figure 3). After only 4 h, LBY39 showed an increase in the mean fluorescence compared to the non-producer. Since octanoic acid production mainly occurs after glucose depletion (approx. 18 h), this difference was much more pronounced after 24 h. After 48 h, producer and non-producer populations become clearly distinct with median values differing by a factor of about 10^2. This suggests that the higher intracellular octanoic acid levels in LBY39 after 48 h also lead to higher GFP expression – despite loss of octanoic acid to the extracellular environment.
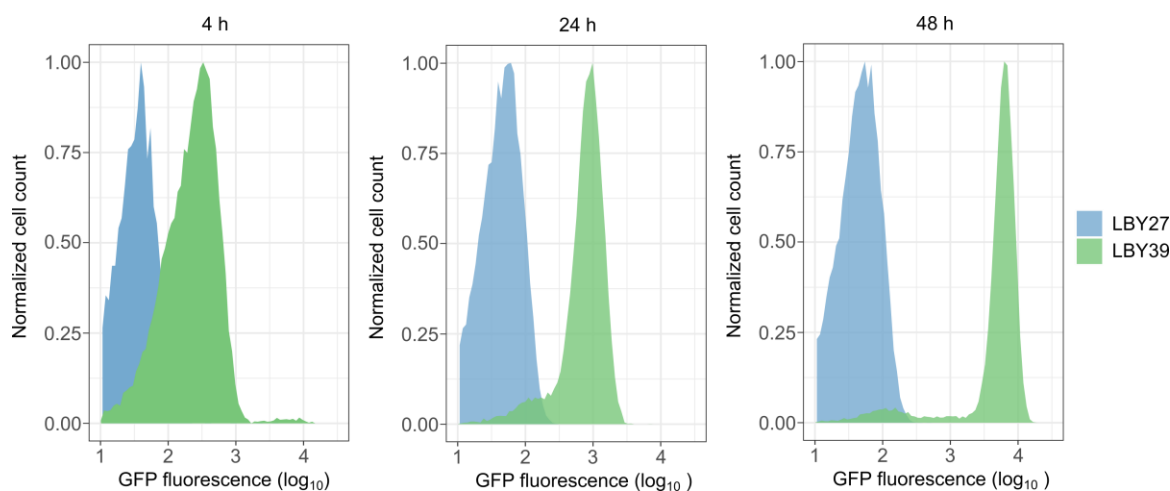
Figure 3: Fluorescence signal of the genomic octanoic acid sensor in producer and non-producer strain. Strains LBY27 and LBY39 contain a stable integration of the biosensor *pPDR12-GFP*. Strains were inoculated to OD_{600} = 0.1 and grown in buffered SCD medium. Aliquots were taken after 4 h, 24 h and 48 h for GFP fluorescence measurement in a flow cytometer (common logarithmic transformation of fluorescence area plotted). LBY27 (non-producer). LBY39 (producer): *FAS1^RK^*, Δ*faa2*.

High-throughput screening for genes enhancing octanoic acid levels. Our general procedure for a high-throughput screening is depicted in Figure 4. To find genes that when overexpressed lead to an increase in octanoic acid titers, we combined the sensor strain LBY39 with a gene overexpression library. Such libraries allow for detection of genes that when overexpressed (through the presence of high plasmid copy numbers) will have a positive effect on the titer of the produced compound.[22–24] We used a library[25] containing approximately 10 kb fragments of the entire yeast genome cloned on multi-copy (2μ) vectors with each vector comprising roughly 3-10 ORFs flanked by endogenous up- and downstream sequences. To prepare the library from *E. coli* glycerol stocks, we grew the 1588 strains individually in 96-deepwell plates ensuring thereby representation of all plasmids. Through transformation of LBY39 with a mixture of all library plasmids, about 22,000 colonies were generated – resulting in a theoretical coverage of the library of almost 14 times, assuming equal transformation probability of all plasmids. We inoculated eight shake flasks with colonies from 3-4 transformation plates each and grew them in buffered SCD medium (pH = 6.5). At pH 6.5, octanoic acid is mostly present in its dissociated form and hence unable to cross the plasma membrane of the cell for re-entry after secretion. By choosing this pH, we aimed at minimizing octanoic acid uptake from the medium, thereby avoiding cross-exchange of octanoic acid between cells.

The sampling was performed 48 h after inoculation to favour enrichment of cells with high octanoic acid titers and simultaneously good growth (Figure S3). Our high-throughput screening design was based on the attempt to decrease false positive results by integrating three

subsequent enrichment steps (E1-E3). Iterative screening rounds are essential to account for the variation of production on a single cell level. To avoid too strict cut-offs, we slowly decreased the percentage of highest fluorescent events sorted from E1 to E3. In the first round, we collected the highest 10 % of fluorescent events into fresh liquid medium from each of the 8 flasks separately (E1) and let them grow for 66 h. After this time period, we conducted another flow cytometry measurement. Here, the populations from flasks 1-6 showed a low fluorescence close to the negative control strain LBY27 (Figure S4). We therefore solely continued with flasks 7 and 8. To lower the rate of false positives, we conducted two subsequent cycles of enrichment after the first sorting, collecting the top 7.5 % fluorescent events (E2) and the top 5 % and top 1 % (E3), respectively. LBY39 containing the empty vector was grown and measured in the flow cytometer as a control. Fluorescence signals of the sorting steps are shown in Figure S3, S4 and S5. The populations from flasks 7 and 8 from E2 and E3 showed a lower mean fluorescence than the control strain LBY39 containing the empty vector (Figure S4, S5). The control strain, however, was always inoculated freshly to an OD=0.1 and not sorted like the populations from flasks 7 and 8. Therefore, the fluorescence signal might have varied due to growth-dependent octanoic acid amounts.
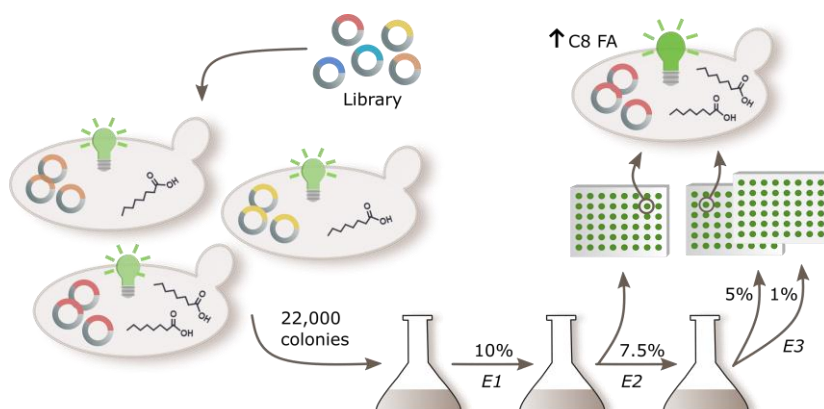

Figure 4: High-throughput screening of an octanoic acid producer strain library. The producer and biosensor strain LBY39 was transformed with a multi-copy yeast genomic library resulting in roughly 22,000 colonies. These were analysed in three consequent enrichment steps (E1-E3) and highest fluorescent events were sorted via FACS. Random colonies of enriched cultures were analysed further and strains that increase octanoic acid (C8 FA) titers can be detected.

Identification and verification of candidate plasmids. Next, we analysed the colonies of the enriched libraries. From the sorted events, about 80 % resulted in colony growth (approx. 200 per plate). To extract library plasmids from these colonies, we randomly picked about 10 colonies per plate, inoculated them, extracted plasmids and re-transformed them into *E. coli* for plasmid amplification. Not all transformations led to *E. coli* colony growth. Nevertheless, we were able to successfully extract six plasmids from flask 7 of E2 and one plasmid of E3. From flask 8, we

extracted eight plasmids of E2 and two plasmids of E3. To identify genes, we sequenced the ORFs on the plasmids and ran BLAST searches. Thereby, we successfully assigned all plasmids to the library (Table 1).

Table 1: Identification of library plasmids after high-throughput screening. Single colonies were analysed from flask 7 and 8 (F7, F8) after different enrichment rounds (E1-E3) from top fluorescent sorted events (top 7.5 %, 5 %, 1 %). Plasmids from these cells were extracted and the contained genes identified via BLAST search.

Flask	Enrichment	Name	Genes contained on library plasmid								
F7	E2 (top 7.5 %)	F7-E2.1	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F7-E2.2	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F7-E2.4	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F7-E2.5	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F7-E2.8	[YBL046W]&	COR1	YBL044W	ECM13	FUI1	[PRE7]*			
		F7-E2.11	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
	E3 (top 5 %)	F7-E3.1	[ESC1]&	ERG8	FMP42	FSH2	UBP8	MRE11	MRLP44	YMR226C TAF7	[MTF1]*
F8	E2 (top 7.5 %)	F8-E2.2	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F8-E2.3	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F8-E2.4	GDH3	[YAL061W]*							
		F8-E2.5	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F8-E2.6	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F8-E2.7	[PSF1]	RAD61	YDR015C	DAD1	[KCS1]				
		F8-E2.8	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
		F8-E2.10	[YML023C]*	APT1	UNG1	YML020W	OST6	YML018C [PSP2]*			
	E3 (top 5 %)	F8-E3.1	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					
	E3 (top 1 %)	F8-E3.2	[YKL183C-A]*	[LOT5]	FAS1	[PRS1]*					

[ ]	intact ORF, possibly missing up- or downstream regions
[ ]*	3' end of gene is missing
[ ]&	5' end of gene is missing

Of the 17 plasmids we identified, 12 corresponded to a library plasmid containing two complete ORFs (*LOT5*, *FAS1*) as well as two truncated ORFs (*YKL183C-A*, *PRS1*). It was striking that the library plasmid containing the wild type variant of *FAS1* was enriched in the screening after E2 and E3. We assumed that this might be the result of improved growth conferred through wild type *FAS1*. To evaluate this hypothesis, we transformed producer strain LBY38 with this library plasmid containing wild type *FAS1*, the library plasmid containing wild type *FAS2*, or the control vector pGP564, respectively. 48 h after inoculation, we extracted fatty acids and quantified via GC measurement. Indeed, we found that the *FAS1*-library plasmid led to slightly decreased octanoic acid titers but improved growth and, interestingly, higher C6, C10 and C12 fatty acid levels, whereas the library plasmid containing *FAS2* did not have an effect on either of these factors (Figure 5A, B). To confirm that this is the result of the *FAS* genes and not any of the other genes encoded on the library plasmids, we transformed LBY38 with plasmids solely containing *FAS1*, *FAS2* or *FAS1*[RK] or different combinations thereof. We were able to confirm that expression of wild type *FAS1* in LBY38 decreases octanoic acid titers (Figure S6).[26] Thereby, probably toxic effects on the cell are reduced. This suggests, that *FAS1* gives the cells an advantage over other cells and therefore appears enriched after iterative screening rounds. The observation that strains containing the *FAS1* library plasmid still have fluorescence in the range of the top fluorescing cells

of the population might result from increased production of C6 and C10 fatty acids. C6 fatty acid also strongly activates the biosensor but is less toxic than C8 fatty acid and for C10 fatty acid an activation of *pPDR12* could not be excluded in previous experiments.[18]
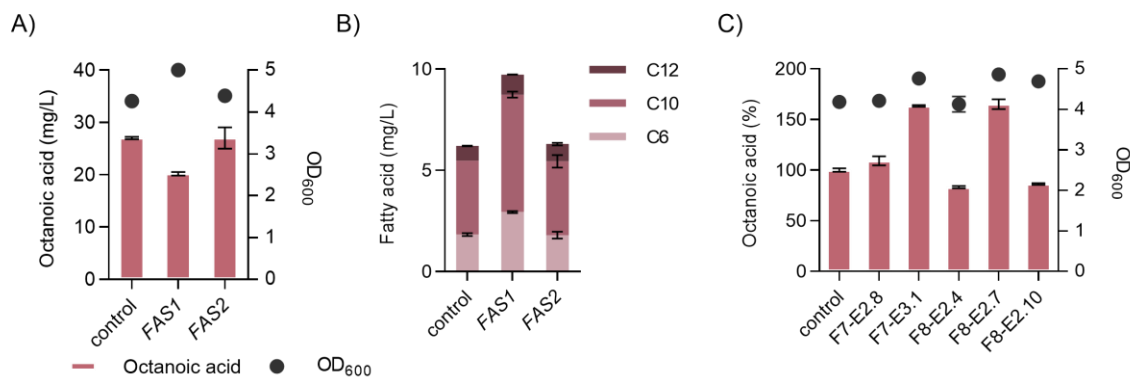

Figure 5: Effect of identified library plasmids on fatty acid titers and growth. OD_{600} (dots) and octanoic acid titers (bars) (A, C) and C6, C10 and C12 fatty acid titers (B) of octanoic acid producer strain LBY38 containing either the empty vector pGP564 (control) or library plasmids with wild type *FAS1* or *FAS2*, respectively, or other library plasmids as indicated. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered SCD medium. LBY38 (producer): *FAS1*[RK], Δ*faa2*. *n* = 2, error bars = ± SD.

Apart from the enriched *FAS1* library plasmid, we identified five more plasmids. Even though none of these plasmids was enriched, we assumed that they could improve growth and/or octanoic acid titers being present after at least one enrichment round. We transformed the producer LBY38 with each of the five plasmids and quantified produced octanoic acid 48 h after inoculation. Two of the plasmids, namely F7-E3.1 (F̲lask 7, E̲nrichment 3) and F8-E2.7 (F̲lask 8, E̲nrichment 2), led to an increase in octanoic acid titers as well as improved growth (Figure 5C).

Identification and verification of candidate genes. As both plasmids, F7-E3.1 and F8-E2.7, contain several genes (Table 1), we were seeking to find the one(s) responsible for the positive effect. For this purpose, we re-cloned the genes and repeated fermentations (Figure S7, Figure 6). Thereby, we identified *FSH2*, putatively encoding a serine hydrolase,[27] and *KCS1*, encoding an inositol hexa-/heptakisphosphate kinase,[28,29] as the genes responsible for the increase in growth and octanoic acid titer (Figure 6A, B). When overexpressing both *FSH2* and *KCS1* from one multi-copy plasmid, we observed an increase in octanoic acid titer of 55 % as well as higher OD (Figure 6C).

The biological role of *FSH2* is unknown. A large-scale study showed that Fsh1p localized to the cytosol[30] and in a study combining computational and experimental proteomics, it was assigned to a new group of serine hydrolases together with *FSH1* and *FSH3*.[27] Overexpression of *FSH1* was reported to decrease phospholipids and increase triacylglycerols, lipid droplets and free fatty

acids.[31] As *FSH1* and *FSH2* share 21-26 % homology,[31] it seems likely that *FSH2* also plays a role in lipid metabolism and thereby leads to increased octanoic acid titers when overexpressed - but due to a lack of knowledge about *FSH2*, this is speculative. The molecular and biological function of *KCS1*, is well characterized. It encodes an inositol pyrophosphate synthase which phosphorylates a variety of inositol phosphate substrates.[28,29,32] Inositol pyrophosphates serve as signalling molecules in many cellular processes. *KCS1* was shown to play a role in vesicular trafficking, cell wall integrity and stress response.[29,33] Deletion of *KCS1* resulted in strong phenotypes with increased cell size and impaired growth. The exact link of how *KCS1* overexpression leads to an increase in octanoic acid titers remains to be investigated. Suspecting that the detected genes might influence robustness towards octanoic acid, we transformed non-producer CEN.PK2-1C with plasmids containing *FSH2*, *KCS1* and both, respectively, and supplied octanoic acid in concentrations from 0-300 mg/L. However, both *FSH2* and *KCS1* did not increase robustness towards octanoic acid (Figure 6D).
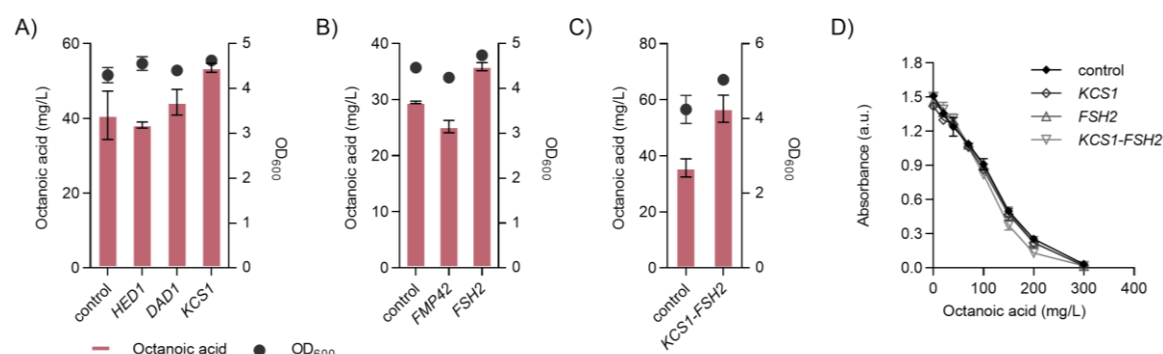

Figure 6: Identification of genes increasing octanoic acid titers and strain growth but not resistance. (A-C) OD_{600} (dots) and octanoic acid titers (bars) of octanoic acid producer strain LBY38 containing either the empty vector pGP564 (control) or plasmids with indicated genes. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered SCD medium. LBY38 (producer): $FAS1^{RK}$, $\Delta faa2$. n = 2, error bars = ±SD (D) Non-producer CEN.PK2-1C containing either the empty vector pGP564 (control) or plasmids with the indicated genes was inoculated to OD=0.05 and supplied with 0-300 mg/L octanoic acid. Growth was measured by absorbance after 20 h. n = 3, error bars = ±SD

We demonstrated the utility of employing a biosensor in a high-throughput screening via FACS to detect new targets that increase octanoic acid titers. This screening shows how useful the development of biosensors for industrially relevant compounds is. The developed method is very versatile and could also be employed with other gene libraries to detect even more new targets. This high-throughput screening approach enabled us to detect two entirely new genetic targets, *KCS1* and *FSH2*, which could not have been detected in a rational engineering approach. This is

the first report that *KCS1* and *FSH2* play a role in fatty acid biosynthesis and which, in combined overexpression, increased octanoic acid titers by 55 % compared to the parental strain.

METHODS

Strains and Plasmid Construction. Yeast strains used throughout this study are listed in Table 2, and plasmids and oligonucleotides in Supplementary Table S1 and S2, respectively.. For genomic integration of the biosensor (Δ*ura3*::*pPDR12-EnvyGFP-tCYC1*) into LBY31, CRISPR/Cas9 was used as described previously.[34] The CRISPR/Cas9 plasmid was amplified in two PCR fragments, assembled *in vitro* in an isothermal reaction using T5 exonuclease, polymerase and ligase[35] and transformed into *E. coli*. After verification via Sanger sequencing, it was transformed into the target strain together with the respective insertion fragment. The same approach was used for: integration of the positive feedback loop (Δ*pWAR1*::*pPDR12*) into LBY31 and Δ*faa2* knockout in LBY39. The insertion fragments were amplified with overhangs from the following template DNAs: biosensor from LBY27 genomic DNA; positive feedback loop from LBV14 plasmid DNA; Δ*faa2* donor DNA from SHY34[26] genomic DNA.

Table 2: Yeast strains.

Strain	Genotype	Reference
CEN.PK2-1C	*MATα ; MAL2-8c ; SUC2 ; ura3-52 ; his3Δ1; leu2-3_112 ; trp1-289*	Euroscarf, Frankfurt am Main, Germany
VGY2	CEN.PK2-1c Δ*pFAS1* -1-300::*pHXT7* -1-392 Δ*pFAS2* -1-200::*pHXT7* -1-392, *FAS1* [R1834K]	Wernig et al., 2021 (submitted)
LBY27	CEN.PK113-11C Δ*pyk2* ::*pPDR12-EnvyGFP-tCYC1*	Baumann et al., 2018
LBY31	VGY2 Δ*ura3* ::*pPDR12- EnvyGFP-tCYC1* Δ*pWAR1* ::*pPDR12*	This study
LBY38	VGY2 Δ*faa2*	Wernig et al., 2021 (submitted)
LBY39	LBY31 Δ*faa2*	This study

For cloning plasmids with one or more genes (LBV91-113), the desired fragments were amplified by PCR from the original library plasmids with overhangs to the sequences flanking the *Bam*HI/*Pst*I cut sites of pGP564. pGP564 was digested with *Bam*HI/*Pst*I and transformed together with the insertion fragments into CEN.PK2-1C and plasmids were assembled via homologous recombination in yeast. Yeast transformations were performed according to Gietz and Schiestl.[36] Cells were streaked out on selective SCD medium[37] lacking leucine (SCD[-leu]) to select for *LEU2*. The colonies from the yeast plates were collectively transformed into electrocompetent *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) and transformants were selected on lysogeny broth (LB) agar

plates[38] supplemented with kanamycin (50 µg mL^{-1}). Selected plasmids were extracted according to standard procedures and verified via Sanger sequencing.

Preparation of library plasmids for screening. The library was present in 17 96-well *E. coli* glycerol stocks. To ensure that all plasmids were represented, we inoculated the *E. coli* strains in 1 ml of LB^{Kan} in 17 96-deepwell plates (Flat-bottom blocks, Qiagen) and grew them overnight under shaking (250 r.p.m.). The cultures of each deepwell plate were mixed and plasmids extracted via 17 midi-preps (GeneJET™ Plasmid-Midiprep-Kit, Thermo Fisher Scientific). For later use, a mixture of all plasmid extracts was prepared.

Cultivation and flow cytometry analysis. For pre-cultures, several colonies of respective strains were inoculated in (selective) SCD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and shaken (180 r.p.m.) at 30 °C overnight. The main cultures were inoculated (in replicates as indicated for each figure) to an OD$_{600}$ of 0.1 in buffered SCD medium in shake flasks and grown for up to 72 h with shaking (180 r.p.m., 30 °C). Aliquots were taken for flow cytometry measurements and analyses were performed using a Sony SH800SA with a 488 nm argon laser and a 100 µm sorting chip. Instrument gains were set as follows: Forward scatter values were set to 6, thresholds for backscatter and FL2 channel ("GFP signal": 525 ± 50 nm) to 43 % and 30 %, respectively. Sample pressure was set to a maximum of 6, to keep the event per second rate below 10,000 events. For each analysis, 10^5 events were evaluated. For cell sorting, cell agglomerates (doublets) were excluded based on channels FSC-H/FSC-W, BSC-H/BSC-W and FSC-A/BSC-A.

High-throughput screening via FACS. For transformation of LBY39 with the library, a mixture of the 17 plasmid extracts was used. About 22,000 colonies resulted from the transformation and we inoculated eight shake flasks with colonies from 3-4 transformation plates each. These were grown in buffered SCD^{-leu} medium (pH = 6.5) alongside control strains (LBY27, LBY39+pGP564) in shake flasks (180 r.p.m., 30 °C). Aliquots were taken after 48 h (Enrichment 1) and measured via flow cytometry as described above. A gate was set to sort the 10 % events with highest fluorescence (~300,000 events, mode "purity") into 15 ml falcons containing fresh media. For all 8 flasks, sorting was performed, and samples were grown again for 66 h in shake flasks (approximated starting OD$_{600}$ ~ 0.0025). After 66 h, another round of sorting (Enrichment 2) was performed for flask 7 and 8. The 7.5 % highest fluorescent events were sorted into falcons as well as onto SCD^{-leu} solid medium (384 events). This procedure was repeated after another incubation period of 66 h in shake flasks, this time sorting only onto solid SCD^{-leu} of the top 5 % and 1 % fluorescent events, respectively (Enrichment 3).

Identification and verification of plasmids after screening. From the sorted plates resulting from the screening, we randomly picked about 10 colonies per sorted plate for growth in SCD[-leu]. Plasmids were extracted by standard procedures with an additional step of disrupting the yeast cells with glass beads (Ø0.25-0.5 mm, Roth, Karlsruhe, Germany) by vigorous shaking in lysis buffer for 8 min. For amplification, the plasmids were transformed in electrocompetent *E. coli* DH10β (Gibco BRL, Gaithersburg, MD). Plasmids were extracted from *E. coli* by standard procedures and sequenced with Sanger sequencing with primers LBP85, LBP237 or LBP238. Identification of library plasmids was performed via BLAST searching the *Saccharomyces cerevisiae* Genome Database (http:// www.yeastgenome.org/).

Cultures for Fatty Acid Production. *S. cerevisiae* strains were grown as previously described[4] with some adjustments. For pre-cultures, several colonies of a strain were inoculated in (selective) SCD with 100 mM potassium phosphate buffer adjusted to pH 6.5 and grown overnight (180 r.p.m., 30 °C). Main cultures were inoculated to an OD_{600} of 0.1 in 50 ml of the respective medium and incubated in 300 mL shake flasks under the same conditions. For sampling, cultures were harvested by centrifugation and 10 mL of the supernatant was used for fatty acid extraction.

Fatty Acid Extraction and Derivatization. Fatty acid extraction and derivatization were performed as described previously.[12] Cells were separated from the medium (3500 rcf, 10 min), an internal standard (0.2 mg heptanoic acid) was added to 10 mL supernatant and mixed with 1 mL 1 M HCl and 2.5 mL methanol/chloroform solution (1:1). After vigorous shaking for 3 min, the mixture was centrifuged at 3000 rcf for 10 min and the chloroform layer was recovered and evaporated overnight. The methylation of the fatty acids was performed as previously described.[39] Samples were dissolved in 200 µL toluene, mixed with 1.5 mL methanol and 300 µL 8.0% (w/v) HCl solution (diluted in methanol), vortexed, and incubated at 100 °C for 3 h to form fatty acid methyl esters (FAME). After cooling at 4 °C for 10 min, 1 mL H_2O and 1 mL hexane were added to the sample, followed by thorough shaking, and the organic phase was transferred to a GC vial.

GC-FID analysis of FAMEs. GC analyses were carried out on a Perkin Elmer Clarus 400 instrument (Perkin Elmer, Germany) equipped with an Elite FFAP capillary column (30 m × 0.25 mm, film thickness: 0.25 µm; PerkinElmer, Germany) and a flame ionization detector (Perkin Elmer, Germany) as described previously.[12]

Toxicity test. CEN.PK2-1C was transformed with plasmids pGP564, LBV106, LBV112 and LBV113, respectively, and plated on SCD[-leu]. Pre-cultures were inoculated in triplicates in buffered SCD[-leu] and grown over night with shaking (30 °C, 180 r.p.m.). For main cultures, strains were inoculated to an OD_{600} of 0.2 and incubated for about 5 hours. The cultures were then diluted in fresh media

to an OD_{600} of 0.05 and transferred into a 96-well plate (clear with flat bottom, greiner bio-one) with 50 µl/well. A dilution series was made with octanoic acid (Sigma-Aldrich, GC grade) diluted in the same media, to reach final concentrations in the wells of 0-300 mg/L when 200 µl of the respective dilutions was added to the strains per well. All three replicates of each strain were inoculated in technical triplicates in the well plates. The starting absorbance was measured in a platereader (CLARIOstar®, BMG Labtech, Ortenberg, Germany) and plates were incubated for about 20 h at 30 °C without shaking before absorbance was measured again. From final absorbance values, a blank value (media without strain) was subtracted.

Software. Flow cytometry data evaluations and graphical presentations were performed with R v3.6.3 using packages readr v1.3.1, plyr v1.8.6 and tidyverse v1.3.0. Data tables were stored in Microsoft Excel 2016. Other graphs were made using the software Prism 9 (GraphPad, USA).

Abbreviations. E1-E3: Enrichment 1-3; FACS: fluorescence-activated cell sorting; FAS: fatty acid synthase; GC: gas chromatography; MPT: malonyl-palmitoyl transferase; OD_{600}: optical density at λ=600 nm; ORF: open reading frame; SD: standard deviation

ASSOCIATED CONTENT

Supporting Information

Table S1. Table of strains and plasmids. **Table S2.** Table of oligonucleotides. **Figure S1.** Genomically integrated versus plasmid-based biosensor in flow cytometry. **Figure S2.** Biosensor response to supplemented octanoic acid. **Figure S3.** Fluorescence signal of library population, E1 sorting**. Figure S4**. Fluorescence signal of library population, E2 sorting. **Figure S5.** Fluorescence signal of library population, E3 sorting. **Figure S6.** Expression of *FAS* genes in LBY38. **Figure S7.** Identification of genes increasing octanoic acid titers and growth

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Author Contributions

LB and MO conceived the study. LB performed all experiments and wrote the paper. SB assisted with FACS experiments and data analysis. JK and EB provided advice and resources. All authors read, reviewed and approved the manuscript.

Notes

EB is inventor of EP patent application No. 15 162 192.7 filed on April 1, 2015, and of EP patent application No. 15 174 342.4 filed on June 26, 2015, by Goethe-University Frankfurt, concerning short-chain acyl-CoA producing FAS variants. There are no other competing interests.

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SUPPORTING INFORMATION

Table S1. Plasmids used in this study.

Plasmid	Characteristics	Reference
Plasmids from library		
pGP564 ("control")	2μ, *LEU2, KanR*	[1]
Library	pGP564, yeast genomic DNA fragments of ~ 10 kb size	[1]
Plate A10, well F1 ("*FAS1*")	pGP564, [*YKL183C-A*]*, [*LOT5*], *FAS1*, [*PRS1*]*	[1]
Plate A16, well E2 ("*FAS2*")	pGP564, [*SSO1*]&, *FAS2, YPL230W*, [*YPL229W*]*	[1]
Plate A1, well F6 ("F7-E2.8")	pGP564, [*YBL046W*]&, *COR1, YBL044W, ECM13, FUI1*, [*PRE7*]*	[1]
Plate A13, well A3 ("F7-E3.1")	pGP564, [*ESC1*]&, *ERG8, FMP42, FSH2, UBP8, MRE11, MRPL44, YMR226C, TAF7*, [*MTF1*]*	[1]
Plate A1, well C1 ("F8-E2.4")	pPGP564, *GDH3*, [*YAL061W*]*	[1]
Plate A3, well E7 ("F8-E2.7")	pPGP564, [*PSF1*], *RAD61, HED1, DAD1, KCS1*	[1]
Plate A12, well G7 ("F8-E2.10")	pPGP564, [*YML023C*]*, *APT1, UNG1, YML020W, OST6, YML018C*, [*PSP2*]*	[1]
Plate A13, well B3 ("*TAF7-MTF1*")	pPGP564, [*YMR226C*]*, *TAF7, MTF1, RRP5, RPS10B, YMR230W-A*, [*PEP5*]*	[1]
Plasmids for CRISPR		
pRCC-K	2μ, *kanMX, AmpR, pROX3-Cas9^opt-tCYC1, pSNR52-gRNA-tSUB4*	[2]
pRCC-N	2μ, *natNT2, AmpR, pROX3-Cas9^opt-tCYC1, pSNR52-gRNA-tSUB4*	[2]
pRCC-K-LBV32	pRCC-K with *gRNA* for *pWAR1* locus (TTGCTCCTACATTTATCGGA)	This study
pRCC-N-LBV47	pRCC-N with *gRNA* for *URA3* locus (AACGTTACAGAAAAGCAGGC)	This study
pRCC-K-LBV103	pRCC-K with *gRNA* for *PYK2* locus (ATGTCTTTGGCGGACAAGGG)	[3]
pRCC-N-SHV42	pRCC-N with *gRNA* for *FAA2* locus (GAAGATTTTGAAACCTTACG)	[4]
Other plasmids		
LBV14	2μ, *URA3, AmpR, pPDR12-EnvyGFP-tCYC1*	[3]
LBV91 ("*[PSF1]-RAD61*")	pGP564, [*PSF1*], *RAD61*	This study
LBV92 ("*HED1-DAD1-KCS1*")	pGP564, *HED1, DAD1, KCS1*	This study
LBV96 ("*[ESC1]&-ERG8*")	pGP564, [*ESC1*]&, *ERG8*	This study
LBV97 ("*FMP42-FSH2*")	pGP564, *FMP42, FSH2*	This study
LBV98 ("*UBP8-MRE11*")	pGP564, *UBP8, MRE11*	This study
LBV99 ("*MRPL44-YMR226C*")	pGP564, *MRPL44, YMR226C*	This study
LBV104 ("*HED1*")	pGP564, *HED1*	This study
LBV105 ("*DAD1*")	pGP564, *DAD1*	This study
LBV106 ("*KCS1*")	pGP564, *KCS1*	This study
LBV111 ("*FMP42*")	pGP564, *FMP42*	This study
LBV112 ("*FSH2*")	pGP564, *FSH2*	This study
LBV113 ("*KCS1-FSH2*")	pGP564, *KCS1, FSH2*	This study
LBGV023	ConLS'-gfp dropout-ConRE'-*LEU2-CEN6-ARS4-KanR*-ColE1	[5]
FWV26	pRS313, *CEN6-ARS4, AmpR, HIS3*	[6]

RPB34 ("*FAS1*")	pRS315, *CEN6-ARS4, AmpR, LEU2, pFAS1-FAS1wt*	6
RPB36 ("*FAS1RK*")	pRS315, *CEN6-ARS4, AmpR, LEU2, pFAS1-FAS1RK*	6
RPB38 ("*FAS2*")	pRS313, *CEN6-ARS4, AmpR, HIS3, pFAS2-FAS2wt*	6

Table S2. Oligonucleotides used in this study.

Primer	Sequence 5'-3'	Application
Insertion of biosensor in *ura3* locus		
MRP130	CGCCTGCTTTTCTGTAACGTTGATCATTTATCTTTCACTGCGGAG	Amplification of pRCC-N with gRNA sequence for cutting in *ura3*
MRP131	AACGTTACAGAAAAGCAGGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	
WGP234	CTTGGTGGTGTTCGTCGTATCTCTTAATCATAGAAGCAGACAATGGAG	
WGP235	TGTTGTCTGACATTTTGAGAGTTAACACCGAAATTACCAAGGCTC	
LBP195	GTATACATGCATTTACTTATAATACAGTTTTGATATCTTTGTTTTGCATTTTACATTC	Amplification of biosensor (*pPDR12-EnvyGFP-tCYC1*) from LBY27 genomic DNA
LBP196	CTTTAATTTGCGGCCGGTACCCAATTCGCC	
MRP141	AACGCATGAAATCCTTCATTTG	Amplification for proof of successful integration
MRP142	CGAAGGAAGGAGCACAGAC	
Replacement of *pWAR1* by *pPDR12*		
WGP234	CTTGGTGGTGTTCGTCGTATCTCTTAATCATAGAAGCAGACAATGGAG	Amplification of pRCC-K with gRNA sequence for cutting in *pWAR1*
WGP235	TGTTGTCTGACATTTTGAGAGTTAACACCGAAATTACCAAGGCTC	
LBP199	TTGCTCCTACATTTATCGGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG	
LBP200	TCCGATAAATGTAGGAGCAAGATCATTTATCTTTCACTGCGGAG	
LBP201	GAATTCTGTTGTTGTAATTGTCATAACTATTGAGCGATATCTTTGTTTTGCATTTTAC	Amplification of *pPDR12* with overhangs to *pWAR1* up- and downstream regions
LBP202	GACGGCAACGCCAGTTATTGCAATCTGCGTGTCCATTTTTTTATTAATAAGAACAATAAC	
LBP203	GACGCCACTGATATAAATCG	Amplification for proof of successful integration
LBP97	GCTTCATAATAGTTCCTCTGG	
Knockout of *FAA2*		
RPP266	GAAGTCCCGGTGTCCCTGACGTTATTGTAG	Amplification donor DNA for *faa2* knockout from SHY34 genomic DNA & proof of successful knockout
RPP267	GTGACCCATGTACTCCGCTAGATTGACCAG	
SHP80	TTAGCCGGTTACACCAAAGG	Amplification of an internal part of *FAA2*
LBP275	GCCACGAATTTGCAGTTC	
Cloning of LBV91-92, 96-99, 104-106, 111-113		
LBP375	GTGGCGGCCGCTCTAGAACTAGTGGATC	Amplification of fragment for LBV91 from E2-F8.7
LBP376	TATCGAATTCCTGCAGCCCGGGGGATCTCGTCTACCCGAAGTACTCTAGGCTTCCTATGC	
LBP377	CGGTGGCGGCCGCTCTAGAACTAGTGGATCTCCGCACCTTTTAAAAAAGGTTGAAAGGGC	Amplification of fragment for LBV92 from E2-F8.7
LBP393	CGGTATCGATAAGCTTGATATCGAATTCC	

|		
LBP383	GCTTGATATCGAATTCCTGCAGCCCGGGGGATCGTGCACACTTTCAAGCTAACACGCAC	Amplification of fragment for LBV96 from E3.F7.1
LBP390	GGCGGCCGCTCTAGAACTAGTG	
LBP384	CACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCTTAAGGTAGATAATAGTGGTCCAT	Amplification of fragment for LBV97 from E3.F7.1
LBP385	GTGAGCGCGCGTAATACGACTCACTATAGGAATTATTAATAACAAATAAAAAAAGCAGGG	
LBP386	GCGGTGGCGGCCGCTCTAGAACTAGTGGATCGGTTACAGCTATTAAATCTTATAGTCTTG	Amplification of fragment for LBV98 from E3.F7.1
LBP387	GCTTGATATCGAATTCCTGCAGCCCGGGGGATCGAAAATAAAGGCATCTACAAATCTCAT	
LBP388	GCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCAGTCGAGTTTTATCGGATCTG	Amplification of fragment for LBV99 from E3.F7.1
LBP394	GTATCGATAAGCTTGATATCGAATTCCGTTCTCTTTATCATCTATATATTACTCTTATAC	
LBP377	CGGTGGCGGCCGCTCTAGAACTAGTGGATCTCCGCACCTTTTAAAAAAGGTTGAAAGGGC	Amplification of fragment for LBV104 from E2-F8.7
LBP410	GTCGACGGTATCGATAAGCTTGATATCGAATTCAGTTGCGGTTCCCTCTGTCTCTC	
LBP411	CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGTTCAAAGAGGAGAACGTTTG	Amplification of fragment for LBV105 from E2-F8.7
LBP412	GTATCGATAAGCTTGATATCGAATTCAAAGAAAGGATAGAACTAATGAATATTCT	
LBP413	CCGCGGTGGCGGCCGCTCTAGAACTAGTGATGTACATATATCCTCACACGTCCG	Amplification of fragment for LBV106 from E2-F8.7
LBP393	CGGTATCGATAAGCTTGATATCGAATTCC	
LBP384	CACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCTTAAGGTAGATAATAGTGGTCCAT	Amplification of fragments for LBV111 from E3-F7.1
LBP431	CTGACACAATGGACAATTAAATAAAATTAAGTAAAAAAAATAAGGACTTTAATTTTTAAACGGTGAGTAAGGAAAG	
LBP385	GTGAGCGCGCGTAATACGACTCACTATAGGAATTATTAATAACAAATAAAAAAAGCAGGG	Amplification of fragment for LBV112 from E3-F7.1
LBP433	CACCGCGGTGGCGGCCGCTCTAGAACTAGTGTACAGTCTTTACTGCCCTAATA	
LBP413	CCGCGGTGGCGGCCGCTCTAGAACTAGTGATGTACATATATCCTCACACGTCCG	Amplification of fragments for LBV113 from E2-F8.7 and E3-F7.1
LBP436	GGCCAGTATTAGGGCAGTAAAGACTGTACGAAAAACACTTTTCTGTTCTTGTTTGTC	
LBP385	GTGAGCGCGCGTAATACGACTCACTATAGGAATTATTAATAACAAATAAAAAAAGCAGGG	
LBP437	GACAAACAAGAACAGAAAAGTGTTTTTCGTACAGTCTTTACTGCCCTAATA	
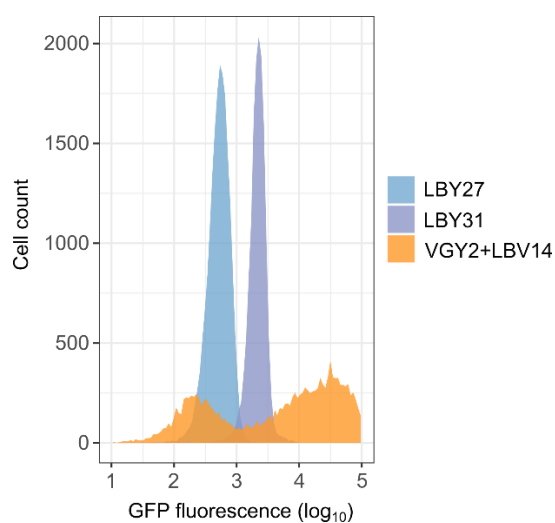
Figure S1. Fluorescence signal of genomically integrated vs. plasmid-based biosensor. The two strains LBY27 and LBY31 contain a stable genomic integration of the biosensor *pPDR12-GFP* whereas VGY2 was transformed with a multi-copy plasmid (LBV14) containing *pPDR12-GFP*. All strains were inoculated to an OD_{600} = 0.1 and grown in buffered SCD medium. Aliquots were taken after 4 h for GFP fluorescence measurement in a flow cytometer (common logarithmic transformation of fluorescence area plotted). LBY27 (non-producer). VGY2 (producer): $FAS1^{RK}$. LBY31 (producer): $FAS1^{RK}$, $\Delta pWAR1::pPDR12$.
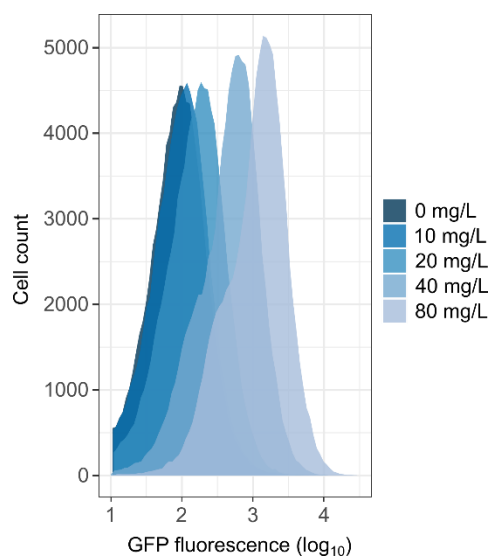

Figure S2. Evaluation of biosensor response to supplemented octanoic acid. Strain LBY27, which contains a stable integration of the biosensor *pPDR12-GFP*, was inoculated to an OD_{600} = 0.1 and cultivated until stationary phase. The indicated octanoic acid amounts were added and the cultures were cultivated for another 3 h before measurement of GFP fluorescence in a flow cytometer (common logarithmic transformation of fluorescence area plotted).
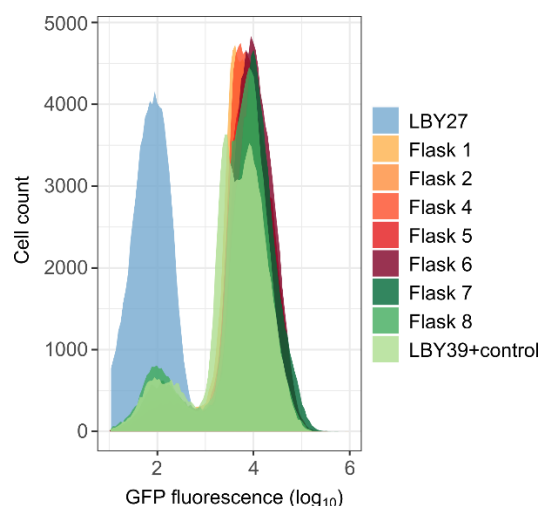
Figure S3. Fluorescence signals of library populations before the first enrichment (E1 sorting). Strain LBY27 (non-producer) and LBY39+control (producer + empty vector) serve as controls. All contain a stable integration of the biosensor *pPDR12-GFP*. Populations of Flask1-8 (LBY39 with library) were grown for 48 h in buffered SCD medium before measurement in a flow cytometer (common logarithmic transformation of fluorescence area plotted). Of the depicted populations, the top 10 % of fluorescent events were sorted. LBY39: *FAS1^{RK}*, Δ*faa2*.
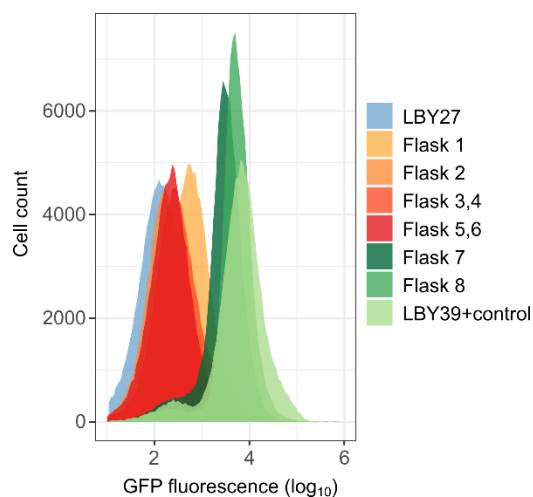

Figure S4. Fluorescence signals of library populations after the first enrichment (E2 sorting). Strain LBY27 (non-producer) and LBY39+control (producer + empty vector) serve as controls. All contain a stable integration of the biosensor *pPDR12-GFP*. Populations of Flask1-8 (LBY39 with library) were grown for 66 h in buffered SCD medium before measurement of GFP fluorescence in a flow cytometer (common logarithmic transformation of fluorescence area plotted). Of the depicted populations, the top 7.5 % of fluorescent events were sorted. LBY39: *FAS1^{RK}*, Δ*faa2*.
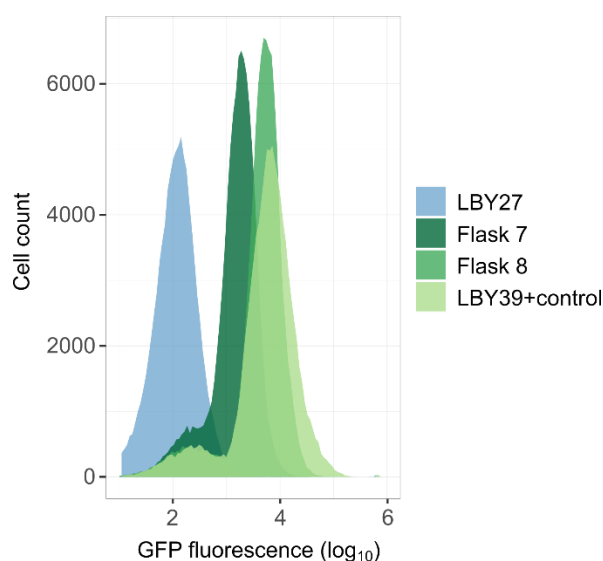
Figure S5. Fluorescence signals of library populations after the second enrichment (E3 sorting). Strain LBY27 (non-producer) and LBY39+control (producer + empty vector) serve as controls. All contain a stable integration of the biosensor *pPDR12-GFP*. Populations of Flask7-8 (LBY39 with library) were grown for 66 h in buffered SCD medium before measurement of GFP fluorescence in a flow cytometer (common logarithmic transformation of fluorescence area plotted). Of the depicted populations, the top 5 % and top 1%, respectively, of fluorescent events were sorted. LBY39: *FAS1RK*, *Δfaa2*.
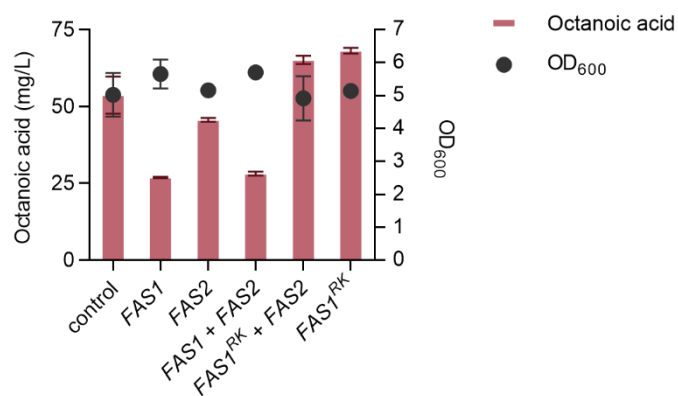

Figure S6. Effect of additional expression of *FAS* genes in LBY38 (*FAS1RK*) background strain. OD_{600} (dots) and octanoic acid titers (bars) of octanoic acid producer strain LBY38 with plasmids containing indicated genes (+ empty plasmid LBGV023 or FWV26, for those with only one gene indicated). As control served LBY38 carrying empty vectors FWV26 and LBGV023. Fatty acids were extracted, methylated and quantified via GC 72 h after inoculation in buffered SCD-leu-his medium. LBY38: *FAS1RK*, *Δfaa2*. *n* = 2, error bars = ± SD.
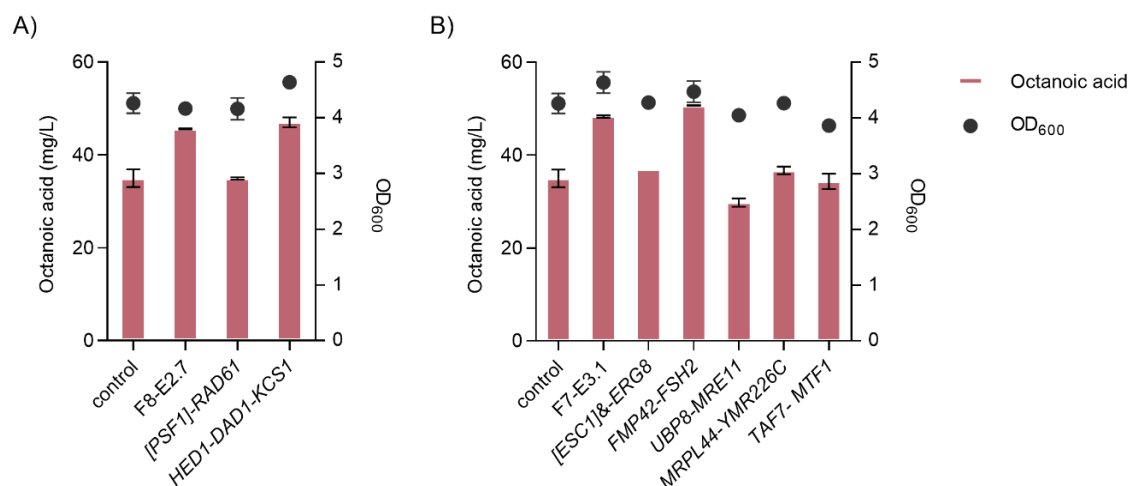
Figure S7. Identification of genes increasing octanoic acid titers and growth. OD_{600} (dots) and octanoic acid titers (bars) of octanoic acid producer strain LBY38 containing either the vector control or plasmids with the indicated genes. (A) Plasmid F8-E2.7 contains all five genes indicated on the x-axis (B) Plasmid F7-E3.1 contains all ten genes indicated on the x-axis. Fatty acids were extracted, methylated and quantified via GC 48 h after inoculation in buffered SCD medium. LBY38 (producer): *FAS1^{RK}*, *Δfaa2*. *[]*: intact ORF, possibly missing up- or downstream regions. *[]&:* 5' end of gene is missing. *n* = 2, error bars = ± SD

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7 Deutsche Zusammenfassung

Octansäure (C8 Fettsäure) ist eine mittelkettige Fettsäure, die in der Natur vor allem in Palmkernöl und Kokosnüssen vorkommt. Sie wird in verschiedenen Produkten wie Reinigungsmitteln, Kosmetika, Pestiziden und Herbiziden, sowie in Lebensmitteln zur Konservierung oder zum Aromatisieren eingesetzt. Darüber hinaus wird die Anwendung von Octansäure zur medizinischen Behandlung, z. B. von hohen Cholesterinwerten, untersucht. Um die seit Jahren steigende Nachfrage zu decken, hat der Anbau von Öl- und Kokospalmen in den letzten Jahren kontinuierlich zugenommen. Der Anbau insbesondere von Ölpalmen erfolgt oft in großflächigen Monokulturen, für welche Regenwald abgeholzt wird. Die damit einhergehende Umweltproblematik ist zunehmend im Fokus der Öffentlichkeit und befördert die Suche nach umweltfreundlicheren Produktionsmethoden. Eine biotechnologische Produktion mit Mikroorganismen wie Hefe, stellt dabei eine attraktive, nachhaltigere Alternative dar.

Die Bäckerhefe *Saccharomyces cerevisiae* wird seit Jahrtausenden für die Herstellung von Brot, Wein und Bier genutzt. Heutzutage kann basierend auf umfassenden Kenntnissen über Stoffwechsel und Genetik, der Metabolismus der Hefe so verändert werden, dass die Produktion einer Vielzahl von industriell relevanten Produkten möglich ist. Zur Herstellung von Octansäure wurde die zytosolische Fettsäuresynthase (FAS, *fatty acid synthase*) von *S. cerevisiae* genutzt. Natürlicherweise produziert Hefe im FAS-Zyklus hauptsächlich langkettige Fettsäuren mit Kettenlängen von C16 und C18, und nur geringe Mengen mittelkettiger Fettsäuren (C8-C14). Um einen *S. cerevisiae*-Stamm mit einem veränderten Fettsäurespektrum zu erzeugen, wurden die FAS-kodierenden Gene entsprechend verändert. Die hierbei erzeugte Variante FAS^{R1834K} bringt die Hefe zur vermehrten Produktion von Octansäure (Gajewski et al., 2017). Die Hefe produziert Octansäure im FAS-Zyklus und entlässt diese zunächst in ihrer aktivierten Form, dem Octanoyl-CoA. Dieses wird anschließend durch Thioesterasen gespalten, die Octansäure freigesetzt, und von der Zelle ins Medium abgegeben. Der FAS^{R1834K}-Hefestamm diente als Ausgangsstamm für die vorliegende Arbeit.

Das Ziel dieser Arbeit war es, die Grundlagen für eine Verbesserung der Octansäureproduktion in Hefe zu schaffen. Die Erhöhung der Octansäuretiter und -ausbeuten ist die Voraussetzung für eine wirtschaftlich rentable Herstellung im industriellen Maßstab. Eine der Herausforderungen hierbei ist die arbeitsintensive, durchsatzarme Probenvorbereitung und -messung von der Octansäure aus den produzierenden Hefen. Um diese zu vereinfachen und beschleunigen, wurde im Rahmen der vorliegenden Arbeit ein Biosensor für Octansäure entwickelt. Biosensoren ermöglichen die Erkennung kleiner Moleküle in Hefezellen, worauf diese mit einer spezifischen Regulierung der Genexpression reagieren. Im Fall eines GFP (*green fluorescent protein*) -gekoppelten Biosensors wird dies durch eine Veränderung der Fluoreszenz messbar.

Der entwickelte Biosensor basiert auf dem *pPDR12*-Promotor, welcher durch den Transkriptionsfaktor War1 reguliert wird. Die Kopplung von *pPDR12* mit GFP als Reportergen auf einem *multicopy*-Plasmid ermöglichte eine *in vivo*-Detektion durch Messung der Fluoreszenzintensität. Dadurch konnten mittels des Biosensors die kurz- und mittelkettigen Fettsäuren Hexan-, Heptan- und Octansäure schnell und einfach quantifiziert werden (Baumann et al., 2018). Dies ist der erste Biosensor, der sowohl zugefütterte Octansäure als auch im Kulturüberstand von Produzentenstämmen vorhandene Octansäure in einem hohen linearen und dynamischen Bereich messen kann. Die Funktionalität des Biosensors wurde durch die Korrelation des Biosensorsignals mit den gaschromatographisch bestimmten Octansäurekonzentrationen aus Kultur-überständen validiert. Es konnte gezeigt werden, dass der Biosensor Octansäure in einem linearen Bereich von 0,01-0,75 mM (1-110 mg/L) detektiert, was innerhalb des Produktionsspektrums des Ausgangsstammes liegt. Außerdem wurde die Fluoreszenzintensität durch Octansäureaktivierung um das bis zu 10-fache gesteigert, was ein hohes Reaktionsspektrum darstellt. Ein Biosensor mit den geschilderten Eigenschaften eignet sich, um große Stammbibliotheken zu screenen und dabei denjenigen Hefestamm zu identifizieren, der am meisten Octansäure produziert.

Mit dem Biosensor wurde ein Hochdurchsatzscreening mit Hilfe der Durchflusszytomerie (FACS, *fluorescence-activated cell sorting*) durchgeführt (Baumann et al., 2020a). Die Methode ermöglicht es in Kombination mit dem Biosensor innerhalb weniger Minuten Millionen von Hefezellen zu analysieren. Für das FACS-Screening wurde der Biosensor an die veränderten Anforderungen angepasst. Zunächst konnte die genomische Integration des Biosensors in einen Octansäure-Produzentenstamm das Einzelzellrauschen drastisch reduzieren. Die Funktionalität dieses Biosensors konnte durch Zugabe von Octansäure im Konzentrationsbereich von 0-80 mg/L und anschließender durchfluss-zytometrischer Analyse validiert werden. Des Weiteren wurde der Fettsäureabbau (β-Oxidation) von mittelkettigen Fettsäuren erfolgreich durch die Deletion von *FAA2* verhindert. *FAA2* kodiert für eine Acyl-CoA Synthetase, welche für die Aktivierung von mittelkettigen Fettsäuren zur peroxisomalen β-Oxidation verantwortlich ist.

Mit dem angepassten Biosensorstamm wurde ein Hochdurchsatzscreening-Protokoll entworfen, welches basierend auf Erkenntnissen aus verschiedenen Vorexperimenten optimiert wurde. So wurden im finalen Screening iterative Anreicherungen der Stämme mit der höchsten Fluoreszenz durchgeführt, um die Anzahl der falsch-positiven Stämme zu reduzieren. Für das Screening wurde der Biosensorstamm mit einer Bibliothek von *multicopy*-Plasmiden, die das gesamte Hefegenom abdeckte, transformiert. Ziel war es Gene zu finden, die wenn überexprimiert, die Octansäureproduktion steigern. Im FACS-Screening konnten zwei Gene, *FSH2* und *KCS1*, identifizieren werden, die in kombinierter Überexpression die Octansäuretiter um 55 % im Vergleich zum Ausgangsstamm erhöhten. Dies war der erste Bericht über einen Effekt von *FSH2* und *KCS1* auf die Octansäuretiter der Hefe. Die entwickelte Hochdurchsatzmethode kann auch für das Screening anderer genetischer Bibliotheken verwendet werden. Dies kann zukünftige Experimente zur Verbesserung von Hexansäure-, Heptansäure-, oder Octansäure-produzierenden Hefen deutlich beschleunigen.

Octansäure ist in hohen Konzentrationen für Hefezellen toxisch. Dies konnte in Wachstumstests mit *S. cerevisiae*-Stämmen bestätigen werden. Um robustere Produzentenstämme zu erzeugen wurden verschiedene Strategien evaluiert. Es

wurde eine adaptive Evolvierung (ALE, *adaptive laboratory evolution*) mit einem Wildtypstamm, den wir in Anwesenheit von hohen Octansäurekonzentrationen kultivierten, durchgeführt. Nach der adaptiven Evolvierung über etwa 60 Generationen zeigte die gemischte Hefepopulation eine erhöhte Wachstumsrate im Vergleich zum Ausgangshefestamm. Dieser Wachstumsvorteil konnte jedoch bei der Anzucht einzelner Hefestämme nicht reproduziert werden. Möglicherweise ist ALE keine geeignete Methode, um die Robustheit der Hefe gegenüber Octansäure wesentlich und konstitutiv zu erhöhen. Eine alternative Strategie bestand darin, den Effekt einer Überexpression bzw. Deletion einiger Gene auf die Octansäuretoleranz des Produktionsstammes zu evaluieren. Hierzu gehörten Transporter- (*PDR12*, *TPO1*) und Transkriptionsfaktor-kodierende Gene (*PDR1*, *PDR3*, *WAR1*) sowie das mutierte Acetyl-CoA-Carboxylase-kodierende Gen *ACC1^{S1157A}*. Frühere Untersuchungen durch ähnliche Experimente mit diesen Genen hatten von einer erhöhten Toleranz der Hefe gegenüber Octansäure berichtet. Entgegen der Erwartung war eine erhöhte Robustheit in unserem *S. cerevisiae*-Stamm jedoch nicht zu beobachten. Dies legte die Vermutung nahe, dass der verwendete *S. cerevisiae*-Stamm von Natur aus eine niedrige Toleranz aufweist. In Wachstumstests mit Octansäure wurde daraufhin die Toleranz von fünf *S. cerevisiae*-Stämmen untersucht und tatsächlich von Natur aus robustere Stämme entdeckt. Diese Stämme, z. B. *S. cerevisiae* EthanolRed, könnten in Zukunft als Ausgangsstamm für weitere Optimierungen dienen.

Im Rahmen eines weiteren Projekts wurde erstmalig eine Transkriptomanalyse (RNA-Seq) eines *S. cerevisiae*-Octansäureproduzenten realisiert. Sie wurde im Vergleich zu einem Wildtypstamm ausgeführt und es wurden in drei unterschiedlichen Fermentationsphasen Proben entnommen (Baumann et al., 2021) Solch eine Analyse ermöglicht einen umfangreichen Einblick in die globale Genexpression. Des Weiteren wurden die gesammelten Genexpressionsdaten mit bereits veröffentlichten Daten verglichen, welche von zwei Forschergruppen stammten, die die Genexpression in Wildtyp-Stämmen in Reaktion auf zugefütterte Octansäure untersucht hatten. Die vergleichende Analyse ergab, dass sich die Genexpression in Folge von intrazellulär produzierter Octansäure deutlich von der Genexpression in Reaktion auf zugefütterte Octansäure unterscheidet. Darüber hinaus konnte im RNA-Seq Experiment ein negativer Effekt der

Octansäureproduktion auf das Wachstum der Hefe bestätigt werden. Die Daten deuteten darauf hin, dass das reduzierte Wachstum des Octansäure-produzierenden Stammes – zumindest teilweise – auf eine verminderte Produktion von langkettigen Fettsäuren zurückzuführen sein könnte. Langkettige Fettsäuren erfüllen jedoch essentielle Funktionen in der Zelle, u. A. als Energiespeicher und Bestandteile von Zellmembranen. Basierend auf der vergleichenden Analyse der RNA-Seq-Daten der drei Fermentationsphasen wurden einzelne Gene zur Überexpression ausgewählt. Hierbei wurde das Gen *RPL40B*, kodierend für eine Untereinheit des Ribosoms, identifiziert, dessen Überexpression zu einer Erhöhung der Octansäuretiter um 40 % führte. Die Erkenntnis, dass Rpl40b eine Rolle in der Fettsäurebiosynthese spielt wurde hier erstmals berichtet – der genaue Mechanismus dieses Effekts ist allerdings noch unbekannt.

Zusammenfassend wurden in dieser Arbeit erfolgreich neue Strategien entwickelt und etablierte Methoden genutzt, um die Hefe *S. cerevisiae* als Zellfabrik für die Produktion von Octansäure zu verbessern. Ein Octansäure-Biosensor wurde konstruiert, verifiziert und für die Implementierung in einem Hochdurchsatz-FACS-Screening optimiert. Die entwickelte Methode ist sehr vielseitig und kann für Screenings verschiedenster Stammbibliotheken von Produzenten kurz- und mittelkettiger Fettsäuren eingesetzt werden. Eine Transkriptomanalyse ergab ein umfassendes Bild der Stammphysiologie während der Octansäureproduktion. Drei Gene wurden identifiziert, die durch Überexpression die Octansäuretiter erhöhen.

Die entwickelten Strategien und identifizierten Gene sind für die zukünftige mikrobielle Octansäureproduktion von wichtiger Bedeutung. Gefundene Gene können in einem natürlicherweise robusten Stamm, wie den im Rahmen dieser Arbeit evaluierten *S. cerevisiae* EthanolRed überexprimiert und mit weiteren Stammoptimierungen kombiniert werden. Im Gegensatz zu derzeitigen Produktionsverfahren müssen für die Octansäureproduktion mit Hefe keine neuen Landflächen erschlossen werden. Vielmehr kann Hefe andere Substrate wie landwirtschaftliche Abfallstoffe, z. B. Lignocellulose, als Energiequelle und Ausgangssubstrat nutzen.

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