Metabolic engineering of *Saccharomyces cerevisiae* for production of medium-chain fatty acids and their derivatives

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Content

1 Summary

The oleochemical and petrochemical industries provide diverse chemicals used in personal care products, food and pharmaceutical industries or as fuels, oils, polymers and others. However, fossil resources are dwindling and concerns about these conventional production methods have risen due to their strong negative impact on the environment and contribution to climate change. Therefore, alternative, sustainable and environmentally friendly production methods for oleochemical compounds such as fatty acids, fatty alcohols, hydroxy fatty acids and dicarboxylic acids are desired. The biotechnological production by engineered microorganism could fulfill these requirements. The concept of metabolic engineering, which is the modification of metabolic pathways of a host organism for increased production of a target compound, is a widely used strategy in biotechnology to generate cell factories or chassis strains for robust, efficient and high production. In this work, the versatile model and industrial yeast *Saccharomyces cerevisiae* was manipulated by metabolic engineering strategies for increased production of the medium-chain fatty acid octanoic acid and *de novo* production the derived 8-hydroxyoctanoic acid.

Octanoic acid production was enabled by the fatty acid biosynthesis pathway by use of a mutated fatty acid synthase (FAS^{RK}) in a wild type FAS deficient strain. The yeast fatty acid synthase (FAS) consists of two polypeptides, α and β, which assemble to a α6β6 complex in a co-translational manner by interaction of the subunits. Because this step might be subject to cellular regulation, the α- and β- subunits of fatty acid synthase were fused to form a single-chain construct ($fusFAS^{RK}$), which displayed superior octanoic acid production compared with split FAS^{RK}. Thus, FAS^{RK} expression was identified as a limiting step of octanoic acid production. But the strains that produce octanoic acid have a severe growth defect that is undesirable for biotechnological applications and could lead to lower production titers. One reason is the strong inhibitory effect of octanoic acid. Another possibility is that the mutant FAS no longer produces enough essential long-chain fatty acids. To compensate for this, the mutated split and fused FAS variants were co-expressed individually in a strain harboring genomic wild type FAS alleles. In addition, mutant and wild type variants of fused and split FAS were co-expressed together in a FAS deficient strain. However, both cases resulted in decreased octanoic acid titers potentially by physical and/or metabolic crosstalk of the FAS variants.

1 Summary

The fatty acid biosynthesis relies on cytosolic acetyl-CoA for initiation and derived malonyl-CoA for elongation and requires NADPH for reductive power. To increase production of octanoic acid, engineering strategies for increased acetyl-CoA and NADHP supply were investigated. First, the flux through the native cytosolic acetyl-CoA and NADPH providing pyruvate dehydrogenase bypass was enhanced by overexpression of the target genes *ADH2*, *ALD6* and *ACS*^{L461P} from *Salmonella enterica* in combination or individually. Next, the acety-CoA forming heterologous phosphoketolase/phosphotransacetylase pathway was expressed and NADPH formation was increased by redirecting the flux of glucose-6-phosphate into the NADPH producing oxidative branch of the pentose phosphate pathway. In particular, the flux through glycolysis and pyruvate dehydrogenase bypass was reduced by downregulating the expression of the phosphoglucose isomerase *PGI1* and deleting the acetaldehyde dehydrogenase *ALD6*. Glucose-6-phosphate was guided into the pentose phosphate pathway by overexpressing the glucose-6-phosphate dehydrogenase *ZWF1*. The first approach did not influence octanoic acid production but the latter increased yields in the glucose consumption phase by 65 %. However, combining the superior fusFAS^{RK} with acetyl-CoA and NADPH supply engineering strategies did not result in additive production effects, indicating that other limitations hinder high octanoic acid accumulation. Limitations could be caused in particular by the strong inhibitory effects of octanoic acid or by intrinsic limitations of the FAS^{RK} mutant.

To enlarge the octanoic acid production platform towards other derived valuable oleochemical compounds the *de novo* production of 8-hydroxyoctanoic acid was targeted. Since short- and medium-chain fatty acids have a strong inhibitory effect on *Saccharomyces cerevisiae*, the inhibitory effect of hydroxy fatty acid and dicarboxylic with eight or ten carbon atoms were compared and revealed only little or no growth impairment. Subsequently, the formation of 8-hydroxyoctanoic acid was targeted by a terminal hydroxylation of externally supplied octanoic acid in a bioconversion. For that, three heterologous genes, encoding for cytochromes P450 enzymes and their cognate cytochrome P450 reductases were expressed and 8-hydroxyoctanoic acid production was compared. In addition, the use of different carbon sources was compared. A cytochrome P450 from *Fusarium oxysporum f. sp. Lycopersici* displayed highest production when ethanol/glycerol was used as the carbon source. Hence, it was chosen for a *de novo* biosynthesis by combinatorial expression with a FAS^{RK} for octanoic acid supply. This resulted in

production of 3 mgL^{-1} of 8-hydroxyoctanoic acid. However, at the end of fermentation a large amount of octanoic acid was still present and was not converted, revealing that the activity of the cytochrome P450 and its cognate P450 reductase are limiting 8-hydroxyoctanoic acid production.

In summary, in this work octanoic acid biosynthesis in *Saccharomyces cerevisiae* was successfully increased with the fatty acid biosynthesis pathway through a novel fusion strategy of the FAS genes and engineering of precursor supply pathways. Current challenges and potential limitations in octanoic acid production were discovered and are discussed. In addition, the platform for medium-chain fatty acid production was extended to include a derivatization pathway for the first *de novo* production of 8-hydroxyoctanoic acid in *Saccharomyces cerevisiae*.

2 Introduction

2.1 The oleochemical industry and market

The industry that processes renewable vegetable oils and animal fats into suitable products is known as the oleochemical industry. Their products are used in food, as biofuels or oleochemicals, which are utilized in industrial products such as soaps, detergents, personal care products, solvents, surfactants, pharmaceuticals and polymers among many others. The biggest share of the annual production of vegetable oils of more than 80 % was used in human food in 1998, but more than 14 % was also used for production of chemicals (Hill, 2000) equaling to 14-17 million tons (MT) per year. The market of oleochemical products has grown steadily, due to increased prices for petrochemicals and the rising demand for production from sustainable sources (Oleochemicals Market Size, Share & Trends Analysis Report By Product, 2020). Thus, the annual consumption of vegetable oils, which reached 100-105 MT in the year 2000 (Hill, 2000; Metzger and Bornscheuer, 2006) more than doubled to 206 MT in 2019/20 (Oilseeds: World Markets and Trade, 2020), representing a market size for oleochemicals of more than 20 billion USD in 2019 (Oleochemicals Market Size, Share & Trends Analysis Report By Product, 2020). For comparison, world consumption of fossil oil processed by the petrochemical industry reached an estimated volume of 400 MT in the year in 2002 (Metzger and Bornscheuer, 2006). The oil crops used by oleochemical industry are palm, soybean and rapeseed which together accounted for about 60 % of the vegetable oil market in 2006 (Rupilius and Ahmad, 2007). Especially palm oil has increased its market share and production tremendously surpassing soybean production for the first time in 2006 (Rupilius and Ahmad, 2007 and reference therein). A logical consequence considering its superior productivity of about 4 MT/Ha*year compared to 0.4 MT/Ha*year for soybeans (Rupilius and Ahmad, 2007).

Two oils are extracted from the fruit of the oil palm, palm oil and palm kernel oil, which are mainly composed of fatty acids (FA) bound in triacylglycerides (TAG) but differ in their exact composition (Norhaizan et al., 2013). Being the major constituents of vegetable oil, FA are the biggest share in the oleochmicals market (Oleochemicals Market Size, Share & Trends Analysis Report By Product, 2020). The dominant FA components of palm oil are long-chain fatty acids

(C16-C18, LCFA), which are equally divided between saturated and monounsaturated FA. In contrast, the largest FA fraction of 66-80 % of palm kernel oil are mainly saturated medium-chain fatty acids (C8-C14, MCFA) (Norhaizan et al., 2013). Palm kernel oil, with the exception of coconut oil (Rupilius and Ahmad, 2007), is the only vegetable oil or fat rich in MCFA and also short-chain fatty acids (C4-C6, SCFA) such as hexanoic acid (Norhaizan et al., 2013).

The increasing demands for palm oil have in consequences led to an expansion of oil palm plantations in tropical regions. Although it is a renewable raw resource, there is a growing awareness of the negative environmental impacts that oil plantations cause (Schmidt, 2015). They cause deforestation and fragmentation of tropical forests (Vijay et al., 2016), loss of biodiversity (Fitzherbert et al., 2008; Vijay et al., 2016), air and water pollution (Comte et al., 2015), increased greenhouse gas emission (Reijnders and Huijbregts, 2008) and flood risks (Khatun et al., 2017) as well as multiple social problems (Khatun et al., 2017 and references therein). But, oleochemicals are important products of modern industry and daily life. However, their steadily growing demand has a strong negative impact on the environment. Therefore, the search for a more environmentally friendly and sustainable production method is a current key challenge.

2.2 Applications and uses of fatty acids and their derivatives

While several vegetable oils or fats are used directly for industrial purposes, for instance shea or cocoa butter in cosmetic products, isolated FA and derived compounds have multiple applications depending on their physiochemical properties defined by chain length, functional groups and degree of saturation. The carboxyl head group and double C-C bonds of unsaturated fatty acids (UFA) allow chemical modification into an almost unlimited amount of chemicals and compounds, which include bulk and fine chemicals, precursor compounds and more. Chemical synthesis reactions for the modification of FA and derived compounds have consequently been intensively researched and reviewed (Biermann et al., 2000; Hou, 2000; Behr and Gomes, 2010). The main FA derivatives, namely fatty acid methyl esters (FAME), fatty acid ethyl esters (FAEE), fatty alcohols, hydroxy fatty acids (HyFA), dicarboxylic acids (DCA), alkanes, and many more (Fig. 1), are introduced in this section in regard to their uses.

Biofuels

Fuels produced from renewable sources instead of fossil resources are termed biofuels and are considered a more environmentally friendly alternative. Such biofuels must have the same characteristics as current fuels and ideally be compatible with current infrastructure and combustion engines in order to be used as a 'drop in' variant (Peralta-Yahya et al., 2012). Fuels are a complex mixture of hydrocarbons consisting of branched, linear and cyclic alkanes and aromatics that determine their characteristics of energy content, octane or cetane number, volatility, viscosity, corrosiveness and freezing point (Lee et al., 2008), which must be achieved by 'drop in' biofuels. FA themselves are not suitable biofuels due to their ionic nature caused by the carboxyl group, but FA derived alcohols, alkanes (Peralta-Yahya et al., 2012) and FAME in the medium- to long-chain range share characteristics with diesel and jet fuels (Lee et al., 2008). Especially short and medium chain fatty alcohols from C5-C12 have been explored for use as biofuels in diesel blends and have improved properties in comparison to others (reviewed in Rajesh Kumar and Saravanan, 2016).

Surfactants, lubricants, detergents and emulsifiers

The importance of FA and their derivatives for use in consumer and industrial products has increased considerably from the 1940s to the 1950s in the USA (Ruston, 1952). Since then, they have been used mainly in the form of surfactants in household, cleaning and cosmetic products as shampoos, liquid soaps, lotions, suspensions, foam stabilizers, and further in fabric softeners and textile auxiliaries, oil and paint additives and surface coatings (Ruston, 1952; Farris, 1979; Maag, 1984; Johansson and Svensson, 2001). Surfactants are surface-active agents with the dual characteristics of a hydrophilic head group and a hydrophilic tail and with the characteristic of forming micelles when a critical concentration is reached (Dave and Joshi, 2017). As such, they act as detergents with cleansing properties or emulsifiers for fat or oil and water mixtures. To reach these needed characteristics M/LCFA and their derivatives are esterified with *e.g*. glycerol and glucosides or are aminated, ethoxylated, condensed and sulfated to a large quantity of different compounds with the desired properties (Maag, 1984; Johansson and Svensson, 2001; Xue et al., 2018).

Biopolymers and plastics

Frequently used polymers such as polyethylene, polypropylene and polystyrene are derived from fossil resources, but the development of polymers from renewable resources is considered an important advance in the market of commodity plastics and medical products (Williams and Hillmyer, 2008). Additionally, these traditional plastics are chemically stable and not biodegradable or compostable, resulting in a large amount of plastic waste ending up in the environment (Zink et al., 2018), causing to pollution of the oceans (Eriksen et al., 2014) and soil (Liu et al., 2014) in the form of macro- or mircoplastics. Therefore, in addition to being produced from renewable sources, biodegradability or compostability is an important property of new plastics for packaging and other purposes (Ciriminna and Pagliaro, 2020). For production of biodegradable polymers used as drug delivery particles, FA and their derivatives are increasingly incorporated into the structures due to their hydrophobic nature, which can provide improved material properties of flexibility, melting point, degradation and release properties (Jain et al., 2008; Sokolsky-Papkov et al., 2009). However, for polymer reactions at least two functional groups are necessary, so that only modified bifunctional FA are suitable. Therefore, FA derivatives applied in polymerization reactions *i.e.* for polyesters carry either an additional hydroxyl- or carboxyl group. For instance, modified monomers based on DCA like adipic acid (hexanedioic acid) or sebacic acid (decanedioic acid) have been used (Jain et al., 2008; Sokolsky-Papkov et al., 2009). Additionally, UFA such as oleic acid and others have been modified at their double bond to meet the requirements for polymer reactions (Galià et al., 2010; Hinzmann et al., 2020).

Pharmaceuticals and uses of fatty acids as antimicrobials

FA in the range of short- to long-chain have long been known to exhibit antimicrobial properties and have received more attention in recent years for their potential applications in pharmaceutical and other industries (reviewed in Desbois and Smith, 2010; Desbois, 2012; Churchward et al., 2018)**.** Antimicrobial properties against bacteria, fungi, viruses and algae were demonstrated for FA from chain length of 8-22 carbon atoms for different degrees of saturation (Desbois and Smith, 2010)**.** Although the mode of action is not fully understood yet, their inhibitory or cidal effects are associated with membranes interaction at high concentrations that solubilize fractions

of the membrane and thereby interfere with membrane processes (Desbois and Smith, 2010). But recent evidence supports the view that their mode of action is more complex than incorporation into membranes and further research is regarded as necessary (Churchward et al., 2018).

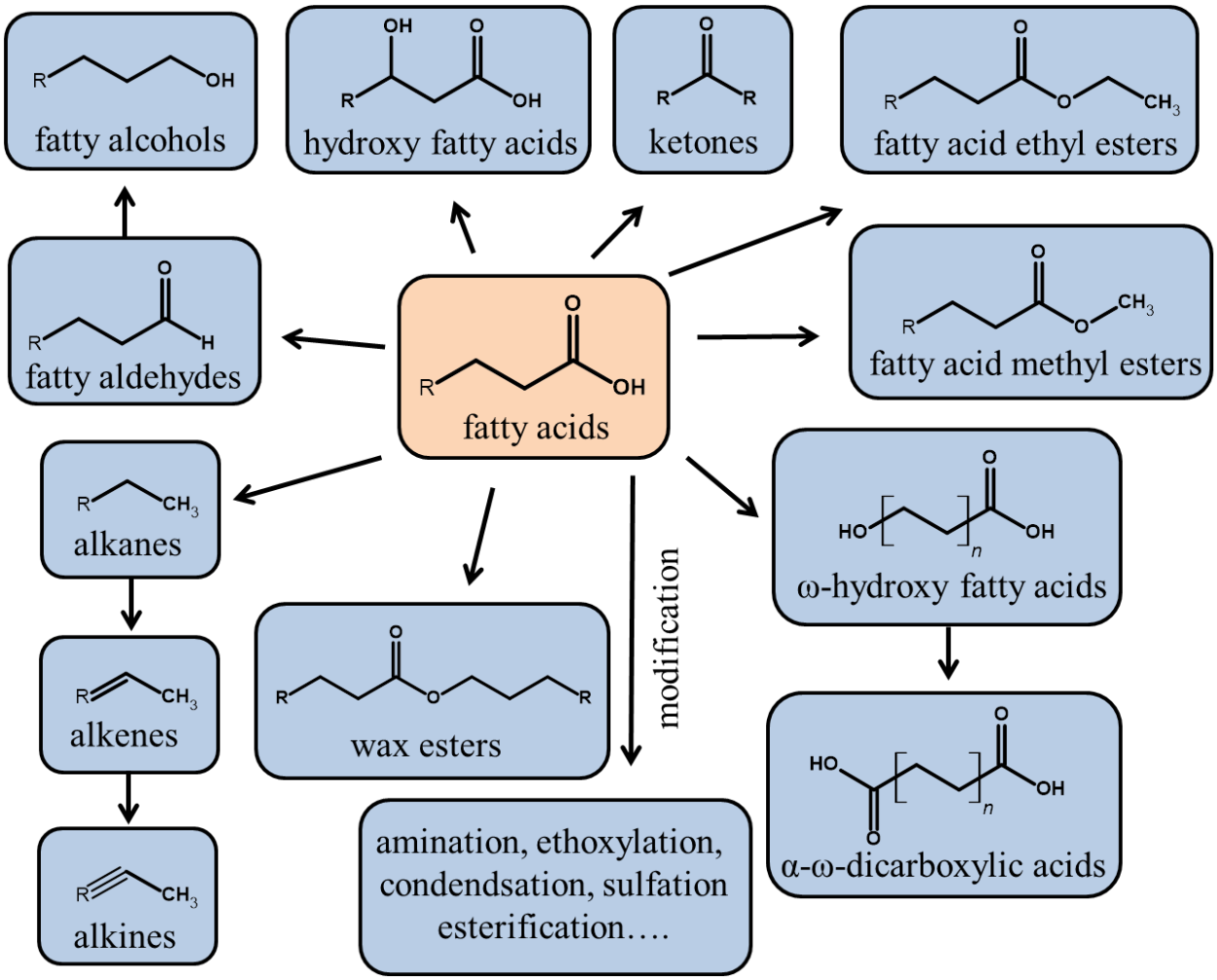

Fig. 1: Oleochemicals derived from fatty acids. Structure and features of different fatty acid derived oleochemicals are shown. Modification of fatty acids and derivatives include esterification, amination, sulfation, ethoxylation, oxidation and polymerization and others to an almost unlimited amount of different compounds. R denotes hydrocarbon chains of variable lengths.

The efficacy of FA as an antimicrobial agent depends on chain length and degree of saturation, with highest activity for saturated FA in the medium-chain range of 10-12 carbon atoms, gradually decreasing with longer or shorter-chain length (Desbois and Smith, 2010). Having such potent antimicrobial properties, FA have multiple applications in medicine or in agriculture and

food production (Desbois, 2012). For example, the rapidly emerging resistance to conventional antibiotics and drugs by human pathogens is a growing threat (Ventola, 2015). In addition, health concerns of consumers have risen for the use of conventional antibiotics in animal food industry and there restrictions have been placed on their use to avoid the potential emergence of resistant bacterial populations that could be transmitted to humans (Dibner and Richards, 2005). Therefore, the use of FA against drug resistant pathogens and human infections, as well as to control infections in live stocks and against plant pathogens, is being discussed in various studies as an alternative to conventional methods (Desbois and Smith, 2010; Desbois, 2012).

2.3 Biotechnology and metabolic engineering

Current methods of FA production through the processing of vegetable oils have raised concerns about a negative impact on the environment. Hence, suitable, environmentally friendly, sustainable and cost-effective production methods are required. One such alternative could be the production by microbial systems through biotechnological applications. Microorganisms can be cultivated in bioreactors in fermentation processes, using not only renewable resources but also waste materials as a carbon source (Weber et al., 2010). This could reduce excessive land use for plantations and competition with food production. Fermentation processes are often carried out under milder conditions in aqueous solutions, in contrast to the use of solvents and harsh conditions in chemical synthesis. In addition, the extraction of secondary metabolites from plants is dependent on the availability of the raw material and is often a time-consuming, low yield but high effort process. Biotechnological production with microorganisms could offer a more economical process independent of weather or season.

Biotechnology is a broad field, which uses living organisms or derived systems for industrial applications for commercial purposes. It is classified according to the field of application in “white” or “industrial biotechnology” for technical applications, “red biotechnology” for production of pharmaceuticals and “green biotechnology” for use of plants and plant systems for agriculture (Schüler, 2016). Advances in biology and related fields especially related to genetic manipulation of organisms (genetically modified organism; GMO) have initiated modern biotechnology. While agricultural biotechnology of genetically modified plants is viewed

critically and there is controversy in regards to food safety, environmental risks, labeling and consumer rights (McCullum et al., 2003) as well as patentability, social and ethical risks (Nezhmetdinova et al., 2020), which is more pronounced in Europe and coupled to a stricter regulation (Falk et al., 2002; McCullum et al., 2003), there is less controversy expected for industrial biotechnology by use of GMO (discussed by Paula and Birrer, 2006).

Within modern pharmaceutical or industrial biotechnology, the field of metabolic engineering, which can be defined as the rational manipulation of metabolic pathways of an organisms to enable or optimize biosynthesis of a desired substance, has become an impactful strategy. Different milestones in the development of molecular biology, biochemistry and genetics as well as methodological achievements, have contributed to modern metabolic engineering. Following the understanding of basic genetic principles, molecular structure and functionality, heterologous gene expression in *E. coli* paved the way into the field of biotechnology by recombinant DNA technology (Cohen et al., 1973). Further milestones in genetic engineering techniques (reviewd in Nielsen, 2001) and understanding of metabolic fluxes and their regulation (Woolston et al., 2013) have led to rational approaches of pathway design and analysis of the performance of the modified strains for production of the desired product and improved yields (Nielsen, 2001).

Recent developments have reduced the costs and time for genetic modification of strains and their analysis. These include cloning methods like Gibson Assembly (Gibson et al., 2009) or Golden Gate Cloning (Lee et al., 2015), artificial gene synthesis (Hughes and Ellington, 2017), next generation sequencing (Goodwin et al., 2016) and genetic engineering techniques by CRISPR/Cas9 (Jinek et al., 2012). Lately, computational modelling of metabolic networks (Kerkhoven et al., 2015) and high throughput engineering and screening methods (Liu and Jiang, 2015) have emerged. In addition, the availability of protein structures and databases is increasing steadily, making a large number of alternative enzymes and information rapidly accessible.

Although a lower environmental impact of microbial biotechnology is evident in contrast to petro- or oleochemistry, the success of a biotechnological production depends on its ability to compete on the market with conventional production methods in terms of market price and availability. Many different compounds can be produced by engineered microorganisms including biofuels like ethanol, 1-butanol and fatty alcohols (Jang et al., 2012, 2012; Nielsen et al., 2013; Cho et al., 2015; Kim et al., 2016), bulk or fine chemicals (Cho et al., 2015; Suástegui

and Shao, 2016), pharmaceuticals and drug precursors (Lee et al., 2009), organic compounds (Park and Lee, 2008), food additives like flavors and antioxidants (Lin et al., 2014; Schempp et al., 2018) and plant secondary metabolites (Marienhagen and Bott, 2013). But industrial production is often ineffective due to low yields and high production costs for extraction and downstream processing, which prevents a market introduction in competition to petro- or oleochemical industry. Nevertheless, large-scale commercial production of compounds with various applications has been launched or industrial production is being pursued (reviewed in Jullesson et al., 2015). These include production of pharmaceuticals like hydrocortisone (Szczebara et al., 2003) or the antimalarial drug artemisinin (Paddon et al., 2013), the flavoring compound vanillin (Gallage and Møller, 2015), chemical building blocks like 1,4-butandiol or advanced biofuels like farnesene (Meadows et al., 2016) and many others (Jullesson et al., 2015). Taken together, metabolic engineering and biotechnology are important technologies at present and are likely to increase their industrial importance in the future.

Metabolic engineers have worked on a variety of prokaryotic and eukaryotic organisms for utilization in biotechnological applications. Among those, the budding yeast *S. cerevisiae* is one of the most intensively studied model organisms (Karathia et al., 2011). Therefore, there is a deep understanding of its physiology and genetics, which is merged in freely available data bases such as the Saccharomyces Genome Database (SGD) (Cherry et al., 1998). Due to the interest and its research history, many tools for molecular biology are available and have been improved over time, ensuring easy manageability of *S. cereviasiae* for metabolic engineering and making it one of the most potent candidates for biotechnological applications. In addition, the yeast has been used in the production of bread, wine and beer for hundreds of years, which makes it a ‘generally recognized as save’ (GRAS) organisms. Throughout this thesis, the yeast *S. cerevisiae* is used as the biotechnological host organism, so the following sections focus mainly on the metabolism and engineering aspects of this yeast.

2.4 *De novo* biosynthesis of fatty acids in *Saccharomyces cerevisiae*

In all organisms, FA are fundamental compounds of life. They are essential for membrane formation in form of phospholipids (Carman and Han, 2011), function as signaling molecules (Georgiadi and Kersten, 2012) or as energy storage in form of TAG or sterol esters (SE) (Welte

and Gould, 2017). The biosynthesis of FA is mediated by fatty acids synthases (FAS). Although the underlying chemistry is conserved, there are two distinct architectures of FAS machinery: either dissociated enzymes (type II) or a multifunctional complex (type I), which carries all reaction centers. The type I FAS is present in the cytosol of eukaryotes and some prokaryotes such as *Actinomycetales*, *Mycobacteria*, *Corynebacteria*, *Rhodococcus* and *Nocardia* (Schweizer and Hofmann, 2004), whereas type II FAS is mainly present in bacteria (White et al., 2005), but can also be found in mitochondria of eukaryotes (Hiltunen et al., 2009). Type I FAS can additionally differ in their gene organization and are encoded in a single gene in mammals (Schweizer and Hofmann, 2004), but in fungi in either one or two genes (i.e. *FAS1* and *FAS2* in *S. cerevisiae*) (Fischer et al., 2020). As the focus of this thesis is the fungal type I FAS machinery of *S. cerevisiae* it is introduced in more detail below.

The structure of yeast FAS was first solved in 2006 (Jenni et al., 2006) followed by publications of structures with higher resolution (Lomakin et al., 2007; Johansson et al., 2008; Johansson et al., 2009), which describe the organization and reaction process of yeast FAS in detail. FAS subunits are encoded by the genes *FAS2* (α-chain) and *FAS1* (β-chain) and assemble as a α6β6 hetro-dodecamer of 2.6 MDa, forming a rigid, barrel-shaped structure which encapsulates all active centers in two dome-like structures (Jenni et al., 2006).

FA biosynthesis is a circular mechanism (Fig. 2) of activation, priming, (multiple) elongation step(s), chain modification and termination (Lomakin et al., 2007). Biosynthesis begins with a priming reaction mediated by the acetyl-transferase domain (AT), which transfers acetyl from Acetyl-CoA (AcCoA), to the acyl carrier protein (ACP). ACP serves as a substrate shuttle for the active center within the reactive chamber. The acetyl moiety is handed over to the ketoacyl synthase domain (KS) and the elongation unit malonyl-CoA (MalCoA) is loaded to the empty ACP by the malonyl/palmitoyl transferase (MPT). ACP delivers the malonate to the KS domain, where the malonate and acetate are condensed to a 3-ketoacyl intermediate. Following a reduction by the ketoacyl reductase (KR) with NADPH, the maturing FA is dehydrated by the dehydratase (DH), until reduced a second time by the enoyl reductase (ER), which uses NADPH for reduction. The reduced acyl chain is then guided to several rounds of elongation with malonate, until reaching its final length. The acyl chain is released from FAS and bound to CoA by the MPT domain. The FA can be released from CoA by activity of thioesterases (TE), which

form free CoA and free FA. The FA released from yeast type I FAS are exclusively saturated acyl chains in the range of 14- 18 carbon atoms. The formation of the saturated C16 FA palmitate requires net 8 AcCoA, 7 ATP and 14 NADPH (van Rossum et al., 2016b).

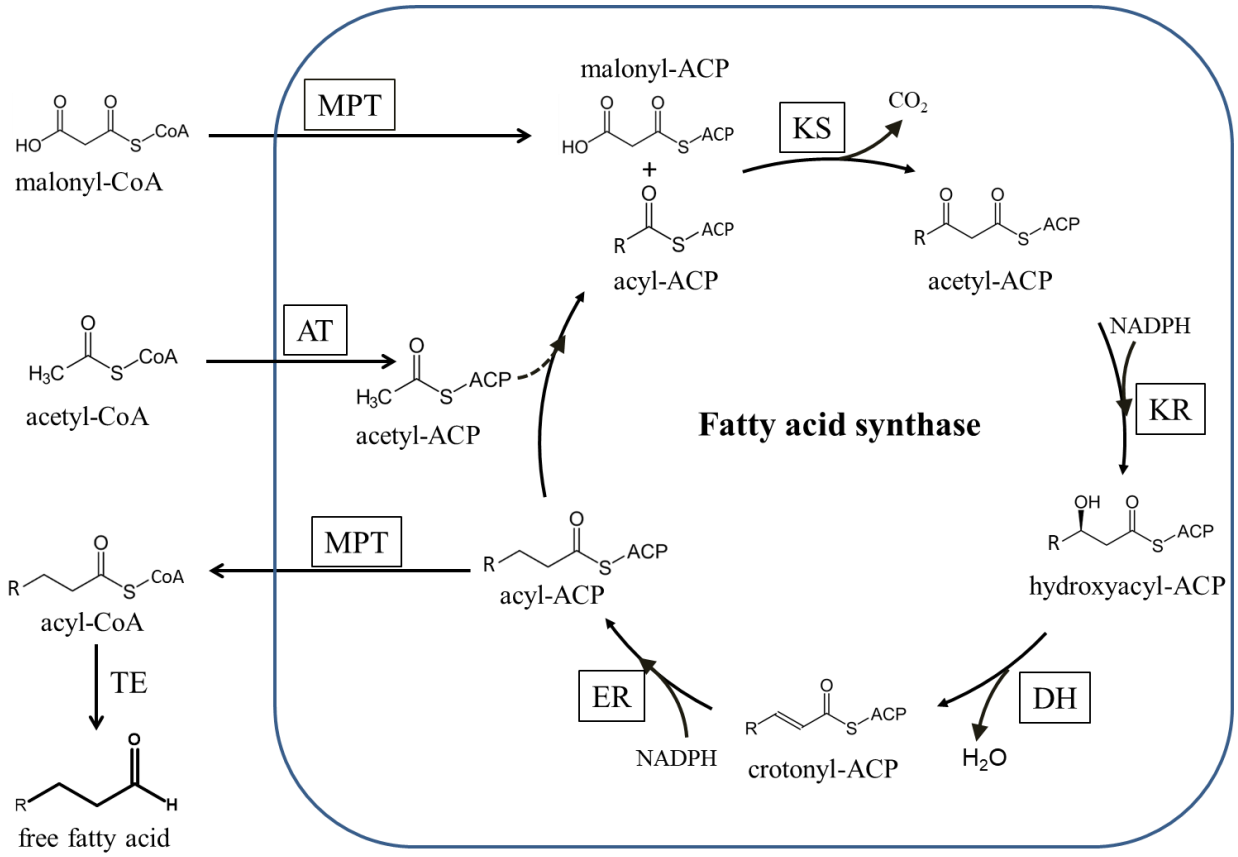

Fig. 2: Fatty acid biosynthesis cycle of *Saccharomyces cerevisiae*. The circular mechanism of fatty acid biosynthesis is displayed schematically. Acetyl-CoA and malonyl-CoA are transferred to the FAS by the acetyl transferase (AT) and malonyl/palmitoyl transferase (MPT) domains and are covalently bound to the acyl carrier protein (ACP). They are condensed by the ketosynthase (KS) and processed by ketoreductase (KR), dehydratase (DH) and enoyl reducatse (ER). The acyl chain is either released by the MPT domain or condensed with another malonyl-ACP for a new cycle of elongation and processing.

Besides saturated FA, UFA are also essential components of membranes and energy storage and an estimated amount of 70-80 % of the acyl chains contain a double bond at the Δ^9 position (Martin et al., 2007). The responsible Δ^9 fatty acid desaturase (encoded in *OLE1*), is essential and its gene expression is tightly regulated by the OLE pathway, which senses the FA composition of membranes (Ballweg and Ernst, 2017). This regulation allows balancing between UFA and

saturated FA, which determine membrane properties of thickness, rigidity, phase behavior and fluidity (Holthuis and Menon, 2014) upon environmental or nutritional changes.

The encapsulation of FA synthesis in the barrel-shaped interior reaction chamber of FAS and shuttling of intermediates to the active centers by covalent binding to the ACP domain (Jenni et al., 2006) contributes to an rapid and efficient process. Taken together FA biosynthesis by FAS complex can be seen a substrate channeling system (Huang et al., 2001; Schmitt and An, 2017). Substrate channeling is defined as the active or passive passing of metabolites from one active center to the next without diffusion into the cellular fluid (Srere, 1987). Thus, the transport and path of reactants alongside cascade reactions is efficiently controlled (Wheeldon et al., 2016). This withholds many advantages over free diffusion of reactants (Huang et al., 2001) *e.g.* the transit time from one reaction to the next is reduced, intermediates are shielded from side reactions and unfavorable chemical equilibria are circumvented (Huang et al., 2001 and references therein).

2.5 Fatty acid degradation by the β-oxidation pathway

Besides the ability of *de novo* FA biosynthesis, microorganisms can take up FA from the environment and use them as a carbon source, which is advantageous over an energy demanding biosynthesis (Hiltunen et al., 2003). In addition, FA which are stored as an energy source in the form of TAG or SE must be effectively broken down into utilizable units. This catabolic pathway is known as the β-oxidation (reviewed in Hiltunen et al., 2003; van Roermund et al., 2003), which is compartmentalized in the peroxisomes in *S. cerevisiae*. Before the actual β-oxidation takes place, FA must be converted into an activated form by esterification with CoA, which is mediated by fatty acyl-CoA synthases (Hiltunen et al., 2003). Several genes encode for fatty acyl-CoA synthases, which differ in their substrate preference and localization. Associated with lipid particles are the LCFA specialized Faa1p, Faa4p and Fat1p. The latter has a substrate preference for very LCFA (C20-C26) and is a bifunctional enzyme involved in activation and transport into the peroxisomes (Black and DiRusso, 2007). In contrast, LCFA are imported into peroxisomes in their activated from by activity of the ABC transporters Pxa1p and Pxa2p (Hettema et al., 1996). MCFA are thought to enter peroxisome by passive diffusion (Hettema and Tabak, 2000) or a flip-

flop mechanism (Hettema et al., 1996), where they are then activated by the MCFA specific peroxisomal Faa2p (Hiltunen et al., 2003). The first step of β-oxidation of saturated activated FA is the introduction of a double bond to form a trans-2-enoyl-CoA intermediate and hydrogen peroxide. In *S. cerevisiae* this reaction is catalyzed by the fatty acyl-CoA oxidase Pox1p, which is the only oxidase for this purpose. The trans-2-enoyl-CoA intermediate is hydrated and oxidized, under formation of NADH, by the bifunctional enoyl-CoA hydratase and 3-hydroxyacy-CoA dehydrogenase Pox2p. In the last step, AcCoA is cleaved of the intermediate by activity of the 3-ketoacyl-CoA thiolase Pot1p. As a result, the FA is shortened by two carbon atoms and can undergo another round of β-oxidation. For degradation of UFA additional enzymes including $\Delta^{3,5}$- $\Delta^{2,4}$ dienoyl-CoA isomerase (Dci1p) 2,4-dieonyl-CoA reductase (Sps19p) and Δ^{2}-Δ^{3}-enoyl-CoA isomerase (Eci1p) are required (van Roermund et al., 2003).

2.6 Engineering strategies for short- and medium-chain fatty acid biosynthesis

In recent years different studies have developed engineering strategies for *S. cerevisiae* and other organisms to increase production of FA and enable FA derivative formation. Although having compelling properties for industrial applications, FA in the short- and medium-chain range are only produced in trace amounts in *S. cerevisiae*, in contrast to LCFA (Cotrell et al., 1986). In order to realize production of S/MCFA two alternative engineering strategies were developed. The manipulation of the chain length control mechanisms of the FA biosynthesis and reversal of the β-oxidation. Both strategies are reviewed in details as part of this thesis in (Baumann et al., 2020) and are introduced briefly in this section.

The β-oxidation (reviewed by Hiltunen et al., 2003) is the degradation pathways of FA (see 2.5). Its reactions, however, are equilibrium balanced and are therefore reversible, which would lead to production of FA instead of degradation. Functional reversal of the β-oxidation cycle was first demonstrated by expression of the individual genes in *E. coli* (Dellomonaco et al., 2011) and later in *S. cerevisiae* (Lian et al., 2014) and initially resulted in increased production of n-butanol and enabled S/MCFAEE production. In the first round of the reversed cycle two AcCoA moieties are converted to acetoacetyl-CoA by a thiolase and further processing and the activity of an acyl-CoA TE results in formation of the C4 product n-butanol. Additional rounds of the cycle increase

the chain length by two carbon atoms each. The choice of the termination enzyme and the thiolase defines the length of the acyl-CoA and can result in the formation of C4-C10 compounds in *E. coli* (Kim et al., 2015) or improve C9-C12 FA production in *S. cerevisiae* (Teixeira et al., 2017).

The FAS machinery of *S. cerevisiae* naturally releases acyl-CoAs in the long-chain range by regulating additional elongation rounds for the maturing chains as well as release after reaching the appropriate length. Then FA can be released from CoA by activity of soluble TE. Manipulating the chain length control mechanism for a premature release of FA in *S. cerevisiae* has been targeted to enable synthesis of S/MCFA. Expression of soluble heterologous TE, which exhibit activity towards short- and medium-chain acyl variants, was established and combined with different heterologous FAS variants like the human type I FAS or bacterial type II FAS (Leber and Da Silva, 2014; Fernandez-Moya et al., 2015), which increased accumulation of S/MCFA. However, yeast FAS encapsulates the FA biosynthesis in its interior reaction chambers, thereby reducing accessibility of external TE to the acyl-ACP intermediate for premature cleavage. Nevertheless, some type I FAS, for instance from *Rhodosporidium toruloides*, harbor two redundant ACP domains located inside of the reaction chamber for substrate shuttling (Zhu et al., 2017). Replacing one of the ACP domains by a medium-chain specific TE successfully placed the TE inside of reaction chamber and increased MCFA biosynthesis by premature release of the FA from ACP (Zhu et al., 2017). Later it was demonstrated in *E. coli* that the specificity of the TE TesA can be modulated by protein engineering from long-chain to medium-chain (Deng et al., 2020), so that more improvements in S/MCFA production by TE expression can be expected in future.

Besides the introduction specific TE, a premature release of FA was achieved by manipulating FAS directly. Different mutation in key active sites of domains, which are involved in chain length regulation of released acyl chains including the KS, AT and MPT domains, have been evaluated for their FA profile (Gajewski et al., 2017). The rationale of the mutations is to minder binding of longer-chain acyl intermediates in the KS binding channel (G1250S, M1251W and F1279Y in KS domain) by building a steric hindrance and thereby promoting release instead of condensation for a new round of elongation. Additionally, the MPT domain has been engineered for a lowered affinity for the elongation unit MalCoA by weakening the stabilization of the

carboxyl group (R1834K) and thereby favoring release of shorter acyl chains. Lastly, the loading of the priming substrate AcCoA by the AT domains was elevated by introduction of a previously evaluated I3016A mutation, because an increased priming by AcCoA in combination with reduced elongation by MalCoA promotes production of S/MCFA. Single or combined aforementioned point mutations enabled production of S/MCFA (Gajewski et al., 2017). Later, the rational FAS mutations were combined with an embedded medium-chain specific TE in a *Mycobacterium vaccae* FAS which successfully increased MCFA biosynthesis in *S. cerevisiae* (Zhu et al., 2020) although titers remained below the initial study with *S. cerevisiae* FAS (Gajewski et al., 2017).

2.7 Engineering of fatty acid derivatives production by expression of a cytochrome P450 system

The *de novo* biosynthesis of FA derivatives, which are valuable products with numerous applications (see 2.2) is desired and has been targeted before in *S. cerevisiae* and other organism by design of artificial pathways and expression of heterologous enzymes. For instance, production of the biofuel compound 1-octanol was achieved by expression of a heterologous carboxylic acid reductase and a phosphopantetheinyl transferase required for activation of the reductase in a strain previously engineered for octanoic acid (OA) production (Henritzi et al., 2018).

Recently, for modification of FA the ω-oxidation pathway, a pathway which is involved in FA degradation in higher eukaryotes like humans, animals, plants and insects when β-oxidation is blocked (Miura, 2013), has been implemented. In this two-step pathway FA are terminally hydroxylated (terminal carbon is referred to as ω-position) resulting in ω-hydroxy fatty acids (ω-HyFA), followed by further oxidation of the hydroxyl group to α,ω-dicarboxylic acids (α,ω-DCA) which are then routed to a bilateral β-oxidation (Verkade, 1938). The initial terminal hydroxylation reaction in the ω-oxidation pathway is carried out by a versatile superfamily of enzymes referred to as cytochrome P450s (CYP).

CYP are heme carrying moonogygenases, which can be found in mammals, plants and some yeast and bacteria and new insights in the past decades have contributed to the understanding of

their structure, mode of action and biological role, which have been reviewed in detail (Munro et al., 2007b; Munro et al., 2013). The name P450 results from a characteristic spectrum at 450 nm, which originates from the binding of carbon monoxide to the heme iron (Omura and Sato, 1964). Their mode of action is the transfer of one oxygen atom from molecular oxygen (O_2) to a substrate, which usually leads to hydroxylation, while the other is used to form H_2O (Katagiri et al., 1968). This reaction requires the transfer of two electrons, which are delivered by one or more redox partners primordially from NADH or NADPH (Munro et al., 2007b). Usually P450 systems consists of 3 components redox systems (class I) mostly found in prokaryotes or two component redox systems (class II), which are mainly found in eukaryotes (Munro et al., 2007b). However more recently some exceptions to this rule have been discovered (Munro et al., 2013). In the class I system the electrons are transferred from iron-sulphur proteins to the CYP, while the iron-sulphur proteins receive their electrons from NADH/NADPH binding FAD-reductases (Munro et al., 2013). In contrast, in the class II system, electrons are transferred from NADPH to CYP by membrane bound FAD- and FMN- biding cytochrome P450 reductase (CPR) (Munro et al., 2013).

Over the years of research history more and more CYP were discovered with versatile substrate preference and redox partners and deployed to metabolic engineering of desired products (Renault et al., 2014). For instance, CYP52 of the yeast *Candida tropicalis* naturally ω-hydroxylates FA in the range of C12-C18 (Eschenfeldt et al., 2003). With the objective to selectively bioconvert externally supplied MCFA into ω-HyFA in *S. cerevisiae* Durairaj et al., 2015 expressed and characterized two different heterologous CYP and CRP from *Fusarium oxysporum* and succeed in production of 8-hydroxyoctanoic acid, 10-hydroxydecanoic acid and 12-hydroxydodecanoic acid. A combined reversal of the β-oxidation cycle and expression of the ω-oxidation pathway in *E. coli* resulted in *de novo* synthesis of α,ω-DCA in the range of C6-C10 (Clomburg et al., 2015). C10-C12 α,ω-DCA were later produced in *S. cerevisiae* by bioconversion of supplied FA by expression of the heterologous ω-oxidation pathway genes CYP94C1 and the CPR *ATR1* from *Arabidopsis thaliana* (Han et al., 2017). In this study the majority of the ω-HyFA were converted to DCA by endogenous mechanisms, which however, were not further elucidated. *De novo* biosynthesis of long-chain C16-C18 HyFA (at the ω or ω-1 position) was achieved in yeast by blocking β-oxidation for increased FA accumulation followed

by expression of a CYP from *Starmerella bombicola* and a CPR from *Arabidopsis thaliana* (Liu et al., 2019).

2.8 Provision of precursors and cofactors for fatty acid biosynthesis

The biosynthesis of FA requires AcCoA for priming, AcCoA derived MalCoA for elongation and NADPH for reductive equivalents. Providing the precursors and cofactors in sufficient amounts is a function of central metabolisms (Fig. 3). AcCoA is not only part of FA metabolisms but also a key substrate of the tricarboxylic acid (TCA) and glyoxylate cycle (Krivoruchko et al., 2015) and is involved in acetylation of proteins and histones (Galdieri et al., 2014; Krivoruchko et al., 2015). Due to its central role AcCoA metabolism is compartmentalized in the cytosol, peroxisomes (as part of β-oxidation see 2.5), nucleus and mitochondria, where the majority of AcCoA is formed (Krivoruchko et al., 2015). In the nucleus it is majorly used for acetylation and is formed from acetate by nuclear acetyl-CoA synthases (ACS) (Krivoruchko et al., 2015). In the mitochondria AcCoA is synthesized by the pyruvate dehydrogenase (PDH) complex from pyruvate which is translocated into mitochondria prior to conversion (Pronk et al., 1996). But AcCoA cannot freely cross the mitochondrial membrane and there is no effective transport system of AcCoA from mitochondria to the cytosol. In the carnitine shuttle the acetyl group of AcCoA is bound to carnitine, transported over the membrane into the other compartment where it is regenerated by transfer of the acetyl group to CoA. However, the transcription of genes involved in the carnitine shuttle is repressed by glucose (Schmalix and Bandlow 1993; Elgersma 1995). Furthermore, *S. cerevisiae* does not have a carnitine biosynthesis pathway and is dependent on exogenous carnitine supply (van Roermund et al., 1995; Swieger et al., 2001) and if active *in vivo*, the carnitine shuttle is directional and functions as a transport system of AcCoA from the cytolsol into the mitochondria only (van Rossum et al., 2016a). Therefore, FA biosynthesis depends on the successive and cytosolic reactions from pyruvate to AcCoA, which are referred to as the pyruvate dehydrogenase bypass (PDH-bypass). First, pyruvate is decarboxylated to acetaldehyde by the pyruvate decarboxylases (PDC). Then, acetaldehyde is converted to acetate by aldehyde dehydrogenases, which generate either NADH (Ald2p) or NADPH (Ald6p). Finally acetate is esterified with CoA by acetyl-CoA synthethases (Acs1p and Acs2p), which requires the hydrolysis of ATP. Due to the Crabtree effect of *S. cerevisiae*,

alcoholic fermentation of acetaldehyde towards ethanol by alcohol dehydrogenases (ADH) is the dominant pathway on fermentable carbon sources. Upon the diauxic shift from glucose to ethanol consumption, ethanol is converted back to acetate and into AcCoA.

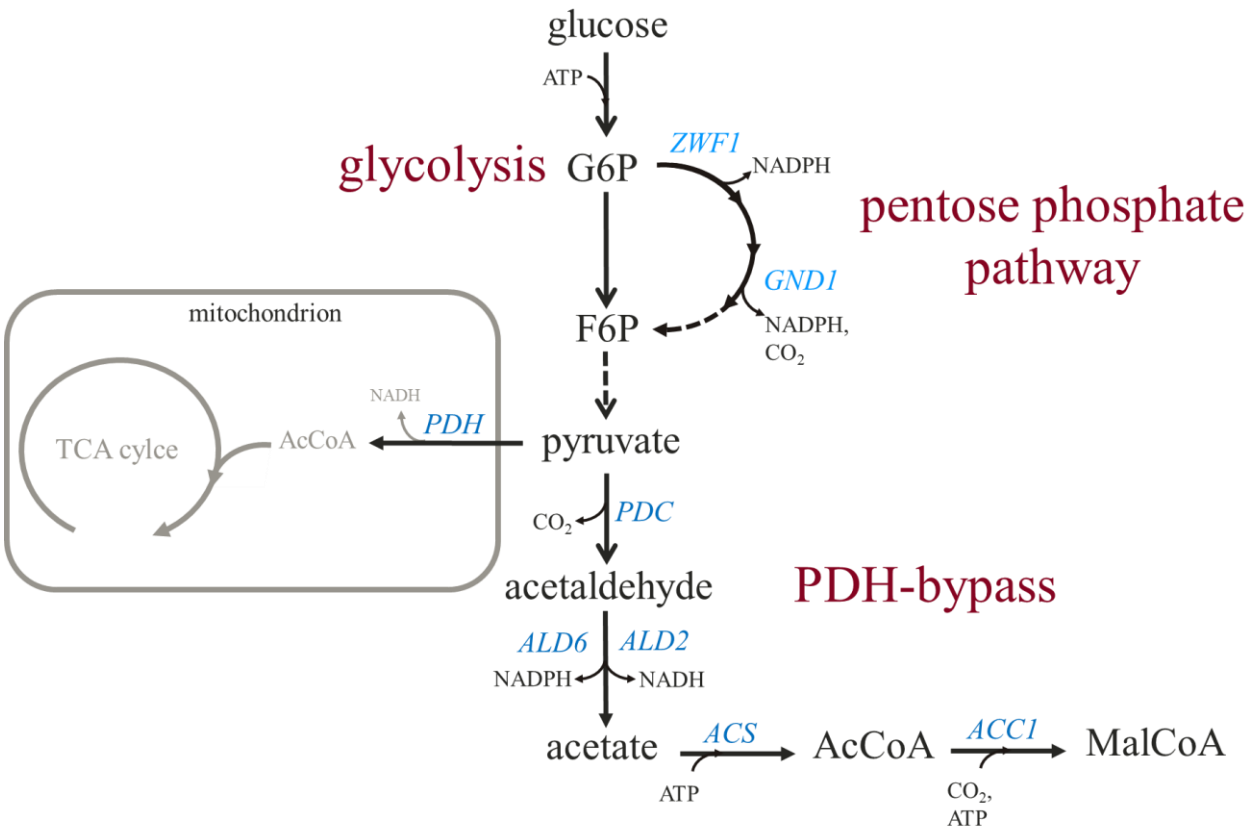

Fig. 3: Pathways for acetyl-CoA and NADPH formation for fatty acid biosynthesis from glucose in *S. cerevisiae*. Genes or gene classes are displayed in blue, pathway names in red or grey, one-step reactions as solid arrows and multiple-step reactions as dashed arrows. Only relevant cofactors and genes are shown for clarity. Abbreviations are used as follows: TCA cycle, tricarboxylic acid cycle; PDH-bypass, pyruvate dehydrogenase bypass; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; AcCoA, acetyl-CoA; MalCoA; malonyl-CoA, PDC, pyruvate decarboxylases; ACS, acetyl-CoA synthethsases.

The universal electron donor NADPH serves different functions in reductive biosynthesis, detoxification, oxidative defense and regulation of protein activity (Agledal et al., 2010). Its function in metabolisms is, with exceptions, dedicated to anabolic pathways like the FA biosynthesis. In contrast, NADH is more involved in catabolic pathways (Bakker et al., 2001). Since NADPH must be available for FA biosynthesis and other functions in the cytosol, the ratio of NADPH/$NADP^+$ in this compartment, which was determined by a sensor reaction under glucose limited and chemostatic conditions, is strongly in favor of NADPH (ratio

NADPH/$NADP^+$ 15.60 ± 0.60), which is more than 10 times higher than for whole cell measurements (ratio NADPH/$NADP^+$ of 1.05 ± 0.08) (Zhang et al., 2015).

The NADPH provision has been attributed to two main dehydrogenases in yeast when grown on glucose. The major cytosolic NADPH source is the glucose-6-phosphate dehydrogenase (Grabowska and Chelstowska, 2003), encoded by *ZWF1*, which catalyzes the first and rate limiting step of the pentose phosphate pathway (PPP) (reviewed in Stincone et al., 2015) and is expressed constitutively (Nogae and Johnston, 1990; Thomas et al., 1991). A metabolic flux analysis with C13 labeled glucose revealed that 24 % of the glucose that enters the cell is fluxing towards the PPP in glucose-limited chemostat cultures (van Winden et al., 2005). An important role for NADPH formation has the PDH-bypass in the reaction catalyzed by aldehyde dehydrogenase Ald6p. Deletion of either *ZWF1* or *ALD6* generates viable mutants, while a double deletion mutant is not viable or at least strongly growth-impaired on glucose (Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2005), suggesting that *ALD6* is essential in the absence of *ZWF1* (Grabowska and Chelstowska, 2003). A third source for cytosolic NADPH was discovered in the isocitrate dehydrogenase Idp2p, which catalyzes the oxidation of isocitrate to alpha-ketogluterate. *IDP2* expression is reduced on glucose but elevated in presence of non-fermentable carbon sources (Loftus et al., 1994). Thus, its role in NADPH formation lies in growth of non-fermentable carbon sources and after the diauxic shift when the cells are grown with glucose (Minard and McAlister-Henn, 2005). However, when treated with 3 mM of the external oxidizing agent hydrogen peroxide and grown on ethanol as the sole carbon source, a *Δzwf1* mutant fails to grow, while in contrast a *Δidp2* and *Δald6* mutant strains shows growth (Minard and McAlister-Henn, 2005). This indicates that *ZWF1* is also the major and essential source of NADPH when ethanol is the carbon source.

2.9 Engineering strategies for enhanced cytosolic acetyl-CoA and NADHP supply in *Saccharomyces cerevisiae*

The requirement of cytolsolic AcCoA and NADPH for FA biosynthesis and other metabolites has motivated metabolic engineers to modulate the metabolism towards an increased supply of these target compounds. There are several ways to achieve increased AcCoA and NADPH supply for

FA in *S. cerevisiae*, which are outlined in details in a publication as part of this thesis (Baumann et al., 2020) and are briefly introduced in this section.

One extensively studied and straight forward strategy to increase the cytosolic AcCoA formation is to increase flux through the native PDH-bypass (Fig. 3). This is achieved by overexpression of the relevant genes (*ALD6*, *ACS1* or a superior mutated and heterologous variant from *Salmonella enterica*, $^{Se}ACS^{L641P}$; Shiba et al., 2007) and additionally by minimizing ethanol formation as a competing pathway. Different studies confirmed the beneficial impact of *ALD6* and $^{Se}ACS^{L641P}$ on production titers of various different FA and derived compounds (Krivoruchko et al., 2013; Jong et al., 2014; Lian et al., 2014; Feng et al., 2015). A positive effect by the reduction of ethanol formation was demonstrated by deleting either the main (*ADH1*) (Li et al., 2014) or multiple ADH (*ADH1*, *ADH4*) (Lian et al., 2014).

The most promising alternative strategy is the expression of a phosphoketolase (PK)/phosphotransacteylase (PTA) pathway. First acetyl-phosphate and either erythrose-4-phosphate or glyceraldehyde-3-phosphate is formed by the PK from the substrates fructose-6-phosphate or xylulose-5-phosphate. Then acetyl-phosphate is converted to AcCoA by the PTA. If the pathway is implemented with a xylulose-5-phosphate specific PK (xPK) there are two advantages over AcCoA formation by the PDH-bypass. Xylulose-5-phosphate is formed in the PPP downstream of the oxidative branch (Stincone et al., 2015), which is the main NADPH providing pathway in *S. cerevisiae* (see 2.8). Additionally the PTA reaction does not require ATP in contrast to AcCoA formation by Acs1p and Acs2p, which implies an energetic advantage. Therefore, the implementation of an xPK/PTA pathway for FA biosynthesis with redirection the metabolic flux *via* the PPP leads to the highest theoretical yield of all known precursor-supply pathways (van Rossum et al., 2016b). Indeed, expression of the PK/PTA pathway for production of FAEE led to increased production titers (Jong et al., 2014). Later it was discovered, that the acetyl-phosphate intermediate formed by a PK can be dephosphorylated by an endogenous glyceraldehyde-3-phosphate phosphatase (*GPP1*), whose deletion should be implemented to increase production titers (Meadows et al., 2016).

AcCoA formation in *S. cerevisiae* is highly compartmentalized and partially formed in the mitochondria by the PDH complex, but cannot freely cross the membrane. To access this AcCoA

pool for cytosolic FA biosynthesis a heterologous citrate-oxaloacetate shuttle was implemented, which can transport AcCoA indirectly over the membranes. Such strategies naturally occur in the oleaginous yeast *Yarrowia lipolytica* (*Y. lipolytica*) (Vorapreeda et al., 2012), which accumulates lipids of up to 40 % of its cellular dry weight (Ageitos et al., 2011). However, this strategy compromises with the lowest theoretical yields of all supply pathways (van Rossum et al., 2016b). In the citrate-oxaloacetate shuttle, mitochondrial AcCoA is loaded to oxaloacetate by the citrate synthase (CS) to form citrate. Citrate is then exported from the mitochondria and AcCoA and oxaloacete are released in the cytosol by a heterologous ATP-citrate lyase, which requires ATP for the reaction. This strategy was first implemented alone (Tang et al., 2013) and further optimized by overexpressing the mitochondrial pyruvate carrier (Yu et al., 2018) and combined with strategies to increase NADPH formation by increased PPP flux (Yu et al., 2018) and expression of a transhydrogenase cycle consisting of a malate enzyme and malate dehydrogenase transforming NADH into NADPH (Zhou et al., 2016; Yu et al., 2018). In fed batch fermentations these combined strategies have shown the highest reported titers for LCFA production in *S. cerevisiae* so far (Yu et al., 2018). In a different strategy, a bacterial PDH complex from *Enterococcus faecalis* was expressed directly in the cytosol of *S. cerevisiae* for AcCoA supply and increased free FA production of mainly unsaturated C16:1 and C18:1 FA by 83 % when combined with other engineering strategies (Zhang et al., 2020).

2.10 Aim of this thesis

In this thesis, the aim was to engineer and optimize S. *cerevisiae* for biotechnological production of MCFA and FA derivatives. The focus should be on the production of the C8 FA OA and its derivative 8-hydroxyoctanoic acid. To enable synthesis of specifically OA, the native FAS was exchanged for a previously developed FAS variant with an arginine to lysine exchange in the MPT domain (R1834K; FAS^{RK}) (Gajewski et al., 2017).

To improve OA biosynthesis, FAS should be investigated as an engineering target. *S. cerevisiae* FAS is split in two polypeptides α and β, which are co-translationally assembled into the FAS complex. Because this step may be subject to cellular regulation and may be rate-limiting, the two polypeptides were fused into a single-chain construct. This fused FAS variant was investigated for OA production and showed improved performance to the split version (Wernig et al., 2020b). Furthermore, it should be investigated whether co-expression of the mutated (fused and split) FAS^{RK}, which causes growth impairments in contrast to the wild type (WT) version, and a WT-FAS is beneficial for cell viability and OA production.

Fatty acid biosynthesis is dependent on AcCoA and NADPH. An aim of this work was to evaluate different approaches to engineering the supply pathways of acety-CoA and NADPH for OA production. The native PDH-bypass should be evaluated as an engineering target by overexpression of the genes involved in ethanol degradation to AcCoA. In addition, AcCoA and NADPH supply should be enhanced by expression of a heterologous PK/PTA pathway and by increasing the metabolic flux into the PPP.

Furthermore, an aim of this thesis was the construction of a *de novo* biosynthesis of 8-hydroxyoctanoic acid by hydroxylation of the terminal carbon of OA through expression of a heterologous CYP and a CPR. Potential limitations of the pathway, such as inhibitory effects of 8-hydroxyoctanoic on cell growth, supply of the CYP cofactor heme and the use of different carbon sources in the fermentation should be evaluated for the production.

3 General Discussion

3.1 Production of octanoic acid and 8-hydroxyoctanoic acid in *Saccharomyces cerevisiae*

In this thesis the optimization of OA production and engineering of *de novo* biosynthesis of 8-hydroxyoctanoic acid in *S. cerevisiae* is addressed. Throughout this work OA was produced by a mutated FAS (FAS^{RK}) by the FA biosynthesis pathway. The production of OA from priming cytosolic AcCoA requires furthermore three elongation rounds with MalCoA. MalCoA is generated from AcCoA by Acc1p and requires hydrolysis of ATP and in each elongation round two NADPH are used for reductions. The net reaction of OA from AcCoA is:

$$4\text{acetyl-CoA} + 6\text{NADPH} + H^+ + 3\text{ATP} + H_2O \rightarrow \text{OA} + 4\text{CoA} + 6NADP^+ + 3\text{ADP} + P_i \quad (1)$$

When OA is further converted to 8-hydroxyoctanoic acid by a CYP pathway as described in (Wernig et al., 2020a) the net reaction changes to:

$$4\text{acetyl-CoA} + 7\text{NADPH} + H^+ + 3\text{ATP} + O_2 + 2H^+ \rightarrow \text{8-hydroxyoctanoic acid} + 4\text{CoA} + 7NADP^+ + 3\text{ADP} + P_i \quad (2)$$

In consequence, OA and 8-hydroxyoctanoic acid production have a high demand for AcCoA, MalCoA and NADPH. Therefore, in one publication the engineering for AcCoA and NADPH supply for OA production were evaluated for the first time by different strategies (Wernig et al., 2021). For further improvement of OA production one publication deals with optimization of the mutated FAS by fusing the distinct β-chain (*FAS1*) and the α-chain (*FAS2*) ($fusFAS^{RK}$), which increased production compared to the separate expression of the FAS genes ($FAS1^{RK}$/*FAS2*) (Wernig et al., 2020b). Many FA derived compounds such as ω-HyFA are valuable industrial products (see 2.2). By expression of a heterologous CYP and a CPR in combination with the OA producing FAS, the first *de novo* biosynthesis of 8-hydroxyoctanoic acid was demonstrated here (Wernig et al., 2020a).

In the following chapters, the different aspects of OA production and 8-hydroxyoctanoic acid biosynthesis are discussed and evaluated. The different possibilities of S/MCFA production by reverse β-oxidation or FA biosynthesis are compared (3.2) and different engineering strategies in other organisms than *S. cerevisiae* are discussed (3.3). Current potential limitations in the production of OA are shown (3.4), potential strategies to improve 8-hydroxyoctanoic acid production are discussed (3.5) and a future outlook of S/MCFA production in microorganisms is given (3.6)

3.2 Comparison of short- and medium-chain fatty acid production strategies in *Saccharomyces cerevisiae*: Modified fatty acid biosynthesis and reverse β-oxidation

In this work and in the literature different strategies for production of S/MCFA have been examined. Either the native FA biosynthesis is modified for an early termination of FA production or the β-oxidation cycle is reversed to operate in an anabolic way (see 2.6). These two different strategies are compared and discussed in this section.

For performing one cycle of the reversed β-oxidation, at least four different enzymes are necessary (Dellomonaco et al., 2011). Initiated by condensation of two AcCoA, the execution of one round of the cycle leads to production of butyryl-CoA, which can be either dedicated for further elongation or termination and conversion to 1-butanol, for instance. This however requires additional enzymes (Dellomonaco et al., 2011). Specifically, for an additional round of the cycle, a condensation of the acyl-intermediate with another AcCoA and specific termination for a product with desired chain-length *e.g.* by TE, is necessary. The reversed β-oxidation cycle is executed with only AcCoA as a starter and elongation unit. The intermediates are bound to CoA and NADH or NADPH can be used for reductive steps. Many challenges in the development of the reversed β-oxidation exist in yeast (Teixeira et al., 2017), but are not only restricted to *S. cerevisiae*.

The fact that only AcCoA is used for priming and also elongation of acyl-CoA products in the reverse β-oxidation cycle bears an energetic advantage and a higher theoretical yield compared to production *via* FA biosynthesis, where the elongation unit MalCoA is generated by carboxylation

of AcCoA at the expense of ATP (Dellomonaco et al., 2011). Additionally the use of NADH instead of NADPH for reductive power could be advantageous as yeast naturally has a high turnover of NADH (Bakker et al., 2001). However the AcCoA and NADH requirement of the pathway seems to be higher than the natural occurrence in *E. coli* and yeast (Teixeira et al., 2017), as deletion of NADH consuming pathways in *E. coli* (Dellomonaco et al., 2011) and increased NADH supply in *S. cervisiae* (Lian and Zhao, 2015) improved the performance of the pathway. Additionally, since all intermediates are bound to CoA an increased CoA demand exists and an improvement of the CoA supply is necessary as demonstrated for 1-butanol production in *S. cerevisiae* (Schadeweg and Boles, 2016a), which adds to the engineering challenges.

Several advantages and disadvantages arise from the fact that the reversed β-oxidation is carried out by individual enzymes. Because intermediates are bound to CoA it is difficult to measure them effectively thereby investigating performance of the individual enzymes. Nevertheless, in enzyme assays, the performance of different enzymes on C4-CoA intermediates was analyzed in cell extracts (Lian and Zhao, 2015). However, not for compounds with other chain lengths leaving the optimal substrate of the tested enzymes unexplored (Teixeira et al., 2017). For improvement of the β-oxidation pathway in *E. coli* or *S. cerevisiae,* most studies therefore rely on systematic evaluation of different enzymes and fine tuning the gene expression (Kim et al., 2015; Lian and Zhao, 2015; Wu et al., 2017), which is a rather time consuming and inefficient process considering the countless possibilities when four to six different enzymes are expressed.
Furthermore, the intermediates of the pathway are freely accessible to side reaction or early termination. This withholds the possibility of production and accumulation of FA intermediates but potentially reduces the titers of the desired product by unwanted side reactions and termination. Therefore, the choice of the right termination enzyme *i.e.* the TE is crucial. However, most TE, as tested in *E. coli,* display a broad chain length substrate specificity and are not very specific (McMahon and Prather, 2014; Grisewood et al., 2017). Due to the challenging fine tuning of further elongation and specific termination, reversed β-oxidation in *S. cerevisiae* is so far either restricted to high production of C4 compounds (Schadeweg and Boles, 2016a, 2016b) or S/MCFA production (C6-C12) at total titers below 12 mgL^{-1} (Lian and Zhao, 2015) or 13 mgL^{-1} (Teixeira et al., 2017).

In contrast to reverse β-oxidation enzymes, the FAS is an evolved complex machinery, which harbors all the catalytic sites inside of a rigid structure (Jenni et al., 2006). The reaction chamber of FAS therefore can be seen as its own compartment for substrate channeling (see 2.4) executed by the ACP domain, which is advantageous over freely accessible intermediates. The intermediates are rapidly transferred to the active centers while side reactions are avoided. This makes the FAS complex a highly efficient production machine. However, the accessibility of soluble enzymes like heterologous TE for early termination and production of S/MCFA is decreased and needs a more challenging engineering strategy including heterologous FAS and enzyme engineering. Thus, only low production titers where achieved with this strategy (Zhu et al., 2017; Zhu et al., 2020). But as demonstrated before, the FAS chain length control machinery can be modulated by single amino acid exchanges, so that the exchange of a single arginine to lysine in the MPT domain is enough for specific and high production titers of OA (Gajewski et al., 2017). Overall these findings result in a more easy manipulable system than expression and fine tuning of multiple enzymes in the reverse β-oxidation.

Reversal of the β-oxidation for S/MCAF production is a pathway with a higher theoretical yield than using FA biosynthesis. However engineering the specific production of FA in the chain length of C6-C12 is more challenging for reverse β-oxidation than by a mutated FAS, which is reflected in lower reported titers to date. Both strategies for S/MCFA and derivative production harbor different advantageous and disadvantageous over one another and many challenges need to be addressed for increased production (see above and 3.4).

3.3 Engineering strategies for the production of fatty acids and their derivatives in different organisms

In this thesis S. *cerevisiae* has been used for engineering of OA and 8-hydroxyoctanoic acid production. Other microorganisms like the bacterium *E. coli* and other yeasts, especially *Y. lipolytica* have also been exploited and engineered for FA and derived compounds like TAG, S/MCFA, fatty acohols, ω-HyFA and α,ω-DCA (Steen et al., 2010; Tai and Stephanopoulos, 2013; Kim et al., 2015; Rutter et al., 2015; Rigouin et al., 2017; Shin and Lee, 2017; Xu et al., 2018; Kim et al., 2019; Kim and Park, 2019; Deng et al., 2020; Hernández Lozada et al., 2020).

The use of different microorganisms for FA biosynthesis has different challenges, risks and advantages over the others. In this section the engineering strategies and achievements (also reviewed in Marella et al., 2018) in different organisms are compared and advantages and disadvantages in use of the different microorganisms are discussed.

Besides *S. cerevisiae* the model organism *E. coli* is the most intensively studied one and has the longest history of research. Unsurprisingly, many studies focused on engineering of *E. coli* for FA and derived compound production. The strategies for enhanced FA applied in *E. coli* are similar to the approaches in *S. cerevisiae*. This includes the engineering of precursor supply of AcCoA and MalCoA (Lee et al., 2011; Shin and Lee, 2017; Hernández Lozada et al., 2020), blocking of the β-oxidation (Deng et al., 2020) and expression of specific TE for release of free FA or termination for production for S/MCFA (Lu et al., 2008; Deng et al., 2020). AcCoA formation is highly compartmentalized in yeast, which needs to be considered in the engineering strategy for improved AcCoA supply. In contrast *E. coli* has the advantage of no compartmentalization of AcCoA metabolisms and therefore requires a more simple engineering. For production of S/MCFA in *E. coli* two different strategies were mainly applied. The implementation of a reversed β-oxidation (Dellomonaco et al., 2011; Kim et al., 2015; Wu et al., 2017) and/or expression of (modified) short/medium-chain specific TE for termination of fatty acid elongation by reversed β-oxidation or the type II FAS system (Steen et al., 2010; Kim et al., 2015; Deng et al., 2020). When combined with the type II FAS of *E. coli* TE have a more easy access to acyl- intermediates due to the dissociated nature of the single FAS enzymes. In a recent study the TE TesA was modified to increased substrate specificity for MCFA and together with blockage of the FA degradation led to production of 2.7 gL^{-1} of OA in a fed batch fermentation process from 104 g of glucose (Deng et al., 2020). Production of FA derivatives like 1-octanol was achieved by expanding previous strategies for OA production with expression of a specific acyl-CoA reductase (Hernández Lozada et al., 2020). *E. coli* was also engineered for biotransformation of multiple different supplied FA precursors to ω-HyFA and α,ω-DCA (reviewed in Kim and Park, 2019).

Due to short generation time and availability of genetic tools *E. coli* is a suitable candidate for metabolic engineering. But yeasts are favored in industrial production settings due to several advantages. They can be advantageous in expression of eukaryotic enzymes, can tolerate higher

cell density and lower pH leading to an overall higher fermentation performance (Aronsson and Rönner, 2001; Ageitos et al., 2011), and are more stable and robust than *E. coli*. Even though engineering of increased production of FA and derivative is more challenging in *S. cerevisiae,* it is compensated by advantages in industrial production.

Another logical progression in biotechnological production of FA and derivatives is the use and engineering of organisms, which naturally accumulate high amounts of lipids. Such organisms are highly interesting for an industrial production as they potentially reach higher titers by an already adapted metabolism. Microorganisms, which synthesize more than 20 % of their dry cellular weight in lipids, are known as oleaginous organisms (Papanikolaou, 2012). Some bacteria even reach lipid accumulation of more than 80 % of their dry cell weight (Alvarez and Steinbüchel, 2002). Belonging to the most researched oleaginous organisms in recent years are the yeasts *Yarrowia lipolytica* (*Y. lipolytica*) and *Rhodopsoridium turoliodes* (*R. turoloides*), which accumulate lipids in the form of TAG to 40 % and 76 % of their dry cell weigh (Ageitos et al., 2011). Especially *Y. lipolitica* has been in the focus (Adrio, 2017), because compared to other oleaginous yeasts it has a comparable longer research history with deeper understanding of its physiology and genetics and has more microbiological tools available than others.

An increased lipid production, especially of TAG in *Y. lipolytica* has consequently been targeted in multiple studies. The strategies include overexpression of genes encoding of the TAG and MalCoA forming Dga1p and Acc1p (Tai and Stephanopoulos, 2013), combining this with overexpression of a fatty acyl desaturase, which was shown to be a rate liming step of FA biosynthesis (Qiao et al., 2015) or evolutionary engineering of the strains (Liu and Jiang, 2015). Also S/MCFA biosynthesis has been targeted in *Y. lipolytica* by expression of medium-chain specific TE and *e.g.* reached production of mainly decanoic acid and some OA comprising to 40 % of total lipids produced (Rutter et al., 2015). Additionally, the type I FAS of *Y. lipolytica* has also been engineered for MCFA biosynthesis by narrowing the binding channel of the KS domain by exchanging I1220 to the bulky tryptophan, which enabled production of C14 FA of up to 11 % of total FA (Rigouin et al., 2017).

In recent years the use of non-model organisms has been increased. Therefore, the development of the necessary toolboxes for genetic manipulation has been enhanced (Fatma et al., 2020). But

although being the most researched oleaginous yeast, there still is a lack of efficient toolboxes for metabolic engineering in *Y. lipolytica* compared to *S. cerevisae*. For instance the availability of selection markers is limited resulting in a more challenging and time consuming engineering process (Larroude et al., 2020). Some recent advances like a more efficient CRISPR/Cas9 systems for easier genetic manipulation have been achieved (Larroude et al., 2020) but development of more tools for fast and easy genetic manipulation in the future is necessary and will only then show the full potential of oleaginous yeast for FA production (Ganesan et al., 2019).

3.4 Current challenges and limitations in octanoic acid production by mutated fatty acid synthases

In this work two publications (Wernig et al., 2020b; Wernig et al., 2021) deal with increasing production of OA in *S. cerevisiae*. Within this thesis OA was produced by a mutated FAS (FAS^{RK}) by the FA biosynthesis pathway and was increased by fusing the two distinct genes of *S. cerevisiae* FAS (*FAS1* and *FAS2*) into a single-chain construct (fusFAS) (Wernig et al., 2020b). Furthermore OA production was increased by engineering of the AcCoA and NADPH supply (Wernig et al., 2021). The latter study revealed, that the combination of both strategies does not lead to an additive effect (Fig. 5D in Wernig et al., 2021) and does not further increase OA production. Two possibilities could prevent additive effects of the two strategies and are discussed in this section in details: (i) intrinsic limitation of the FAS^{RK} mutant, (ii) strong inhibitory effects of OA. Additionally, strategies which could increase production of OA are discussed.

(i) Within the scope of this work, the FAS mutant of choice for OA production harbors an arginine to lysine (R1834K) exchange in the MPT domain (Gajewski et al., 2017). The rationale of this mutation was developed by Gajewski, 2017 due to the fact that the ratio between AcCoA and MalCoA concentration can influence the chain length control of a FAS *in vitro, i.e.* higher concentrations of the FA elongation unit MalCoA favor longer-chain FA production and higher concentrations of the starter unit AcCoA favors shorter FA (Kawaguchi et al., 1980). This effect was mimicked by reducing the affinity of the MPT domain for MalCoA, which acts similarly to

the reduced MalCoA concentrations. Additionally, the MPT domain releases acyl chains from FAS by using the same active site as for malonate binding. In competition for the binding site, weakening of the malonate binding was reasoned to favor the release of the acyl chain (Gajewski, 2017). It was previously established in a crystalized MPT domain of human FAS that MalCoA binding is stabilized by a highly conserved arginine residue (Bunkoczi et al., 2009). The MalCoA binding then was weakened for S/MCFA production *in vitro* in a *Corynebacterium ammoniagenes* FAS by an exchange of the equivalent arginine to lysine (Gajewski, 2017). Later, this mutation was transferred to the FAS of *S. cerevisiae* (R1834K; FAS^{RK}) which displayed production of mainly OA *in vivo* (Gajewski et al., 2017). The in the scope of this study developed $fusFAS^{RK}$ and its enhanced expression by strong promoters increases OA production in an otherwise unmodified strain (Wernig et al., 2020b), demonstrating that FAS^{RK} itself is a limiting factor for OA biosynthesis. The reason might be the intrinsic functionality of the FAS^{RK} mutant, which could be operating at its maximum capacity due to reduced MalCoA loading. Therefore, increasing AcCoA and NADPH supply might not further increase production and the effects are not additive (Wernig et al., 2021). Additionally, by increasing the supply of AcCoA by strain engineering (Wernig et al., 2021) the natural AcCoA/MalCoA ratio is influenced. MalCoA is formed from AcCoA by activity of an acetyl-CoA carboxylase (Acc1p, Fig. 3). Although *ACC1* expression was not modified in the engineered OA producing strain (Wernig et al., 2021), to minimize LCFA production, MalCoA concentration might have been increased as well thereby counteracting the functionality of the RK mutant in an unfavorable way for OA production. It remains unknown, which AcCoA/MalCoA ratio is optimal for OA production by $fusFAS^{RK}$ and how the distribution of AcCoA/MalCoA is effected in the developed strains (Wernig et al., 2021), but fine tuning of AcCoA and MalCoA concentrations is certainly important and should be investigated in the future.

(ii) S/MCFA like OA are long known as strong inhibitors of *S. cerevisiae* growth (Viegas et al., 1989; Alexandre et al., 1996; Legras et al., 2010; Borrull et al., 2015; Henritzi et al., 2018; Wernig et al., 2020a). As the cause, two different mechanisms have been discussed (also discussed in Wernig et al., 2020a). On the one hand, OA is a weak acid (pK_a 4.89), which is thought to enter the cell in a protonated form by passive diffusion (Soumalainen and Nurmien, 1976) or by an yet unknown carrier (Borrull et al., 2015) and subsequently dissociates in the

neutral cytosol of yeast, causing an accumulation of protons and pH decrease (Viegas and Sá-Correia, 1997). Indeed, a pH drop in the yeast cytosol and ER was measured upon exposure to 150 mgL^{-1} external OA by expression of a pH sensitive GFP variant in these compartments (Reifenrath and Boles, 2018). This acidification activates H^+-ATPases (Cabral et al., 2001), resulting in elevated use of ATP with increasing OA concentrations and reduced growth as a consequence. Additionally, biosynthesized OA is exported from the cells and found in majority in the fermentation supernatant (Pavlovic, 2016), where it is protonated again in the acidic medium and can re-enter cells. To counteract this inhibitory effect, the production medium was buffered at pH 6.5 with a phosphate buffer (Wernig et al., 2020a; Wernig et al., 2020b; Wernig et al., 2021), which was demonstrated to increase OA production before (Pavlovic, 2016).

One the other hand, S/MCFA can interfere with membranes causing growth inhibition. For instance in one study the majority of cell internal OA was found in the cell wall fraction of the yeast (Borrull et al., 2015). Recently, it was demonstrated that a S/MCFA production strain also exhibited increased incorporation of these FA into phospholipids in membranes (Xue et al., 2020). Nevertheless, for functionality and activity of membrane proteins, membranes need to maintain their properties *i.e.* of thickness and fluidity, which directly correlates with packing, chain length and degree of saturation of incorporated FA (van der Rest et al., 1995). By interaction or incorporation of high concentrations of S/MCFA however, natural membrane integrity is disturbed and in consequence membrane leakiness is induced (Liu et al., 2013; Borrull et al., 2015). Unsurprisingly, the inhibitory effect of S/MCFA can therefore be counteracted by supplementation of the LCFA oleic acid (C18:1) (Liu et al., 2013), potentially by balancing membrane perturbation by S/MCFA with longer FA. However, supplementation of LCFA is not suitable for OA production for two reasons: (i) it is an undesirable scenario in terms of industrial production, as production costs would increase enormously and become uneconomical, and (ii) if an S/MCFA producing strain is supplemented with oleic acid, the production of SCFA is greatly reduced and shifted to longer-chain FA (*i.e.* C12) (Pavlovic, 2016), most likely due to down-regulation of the native FA biosynthesis. Although buffering the medium counteracts the inhibitory effect of acidification by OA, its incorporation into the membrane could not be avoided in this way, causing a growth defect of OA producing strains (Wernig et al., 2020b; Wernig et al., 2020a; Wernig et al., 2021).

An increased production of OA, by combining different strategies, might be prevented by the strong inhibitory effect as discussed above. To overcome this issue, either yeast strains need to be engineered for an increased robustness, the toxic compounds could be converted intracellularly to a non-toxic substance, of which they can be recovered after extraction or the product could be removed by an *in situ* extraction. However, the only reported engineering strategy to date to increase robustness towards externally supplied OA was an increased production of oleic acid (C18:1 FA), achieved by overexpression of a mutated superior $Acc1^{A1157}$ for increased MalCoA formation (Besada-Lombana et al., 2017). As discussed above this is not an applicable strategy for OA production.

Derivatization of FA to FAEE by esterification with ethanol is considered a mechanism of FA detoxification (Peddie, 1990; Borrull et al., 2015). Intracellular conversion of FA to FAEE, extraction of these compounds and recovery of the FA by chemical ester hydrolysis could be performed. But FAEE are highly volatile likely resulting in loss of product via outgassing, when formed in high concentration. In addition, we have shown that ω-HyFA and α,ω-DCA are less inhibitory to yeast than their corresponding FA (Wernig et al., 2020a). However, due to their chemical structure they are not suitable for an easy recovery of the FA by chemical reactions after extraction. Otherwise a process based solution like *in situ* extraction of the product by an adsorbing agent *i.e*. the anion exchange resin amberlite or others should be considered. Initial experiments indicated a feasibility of this strategy (Pavlovic, 2016).

Among the different FAS mutants, which were developed for S/MCFA production many, including FAS^{RK}, displayed a “leaky” FA production, meaning that besides S/MCFA also LCFA are produced (Gajewski et al., 2017). This fact harbors some advantages and disadvantages, which are discussed in the following section.

The main advantage is that S/MCFA production is enabled rather conveniently by implementation of one mutation in the FAS gene or expression of the mutated version in a FAS deficient strain and this mutant also provided the essential LCFA for proliferation. However this also results in reduced production titers as the capacity of FAS is split between S/MCFA and LCFA production. In addition, this may contribute to the growth defect of production strains (Wernig et al., 2020a; Wernig et al., 2020b; Wernig et al., 2021) as reduced amounts of essential LCFA are formed. Recently, a FAS variant with the mutations I306A/

G1250S/M1251W/F1279W that cannot complement an FAS-deficient strain for LCFA (Pavlovic, 2016; Schrodt, 2019) was characterized as a FAS variant, that only produces S/MCFA in the range of C6, C8, C10 (Schrodt, 2019). Thereby, the full capacity of the FAS could be used for S/MCFA production. For such a FAS variant LCFA need to be provided either externally, which is not a feasible option as described above, or by intrinsic production. In Schrodt, 2019 the FAS variant was characterized in a strain, which additionally harbors a genomic WT-FAS copy for LCFA provision. However, as demonstrated, co-expression of a mutated and WT-FAS reduces OA production (Wernig et al., 2020b) possibly by (i) competition for AcCoA, MalCoA and NADPH, (ii) elongation of octanoyl-CoA released from a mutated FAS by WT-FAS or (iii) formation of heterogeneous complexes of mutated peptides and WT peptides in the same α6β6 complex, individually or by combinations (Wernig et al., 2020b). To avoid formation of heterogeneous complexes of mutated and non-mutated FAS variants, co-expression of other non-fungal FA biosynthesis systems like the human FAS (Leber and Da Silva, 2014), bacterial type II FAS from *E.coli* (Fernandez-Moya et al., 2015), bacterial type I from *Mycobacerium* (Yu et al., 2017) or fungal FAS from *Rhodospuridium toruloides* (Zhou et al., 2016), which have been successfully expressed in *S. cerevisiae* before, could be tested.

An alternative strategy for avoiding these pitfalls could be the separation of a growth- from a production phase. In an optimal scenario, the production of LCFA would be restricted to the growth phase and S/MCFA could be produced with full capacity by mutated FAS in a production phase. Indeed, it was demonstrated for the C22 fatty alcohol 1-docosanol, that separation of growth and production phase can increased production (Yu et al., 2017). The separation was achieved by a dynamic control of the 1-docosanol forming enzymes by expression from the *GAL1* promoter, which is repressed by high glucose levels and activated by exposure to the inductor galactose. After enough biomass was gained 1-docosanol production was activated and showed increased titers compared to immediate expression (Yu et al., 2017). However, implementation of such a system is more challenging for OA production due to multiple reasons. Simultaneous expression of a WT-FAS for LCFA production and a mutated FAS for OA production should be avoided as this reduces production titers (Wernig et al., 2020b). Therefore, activation of expression of the OA producing FAS^{RK} variant in a production phase is likely not enough for increasing the production titers. The expression of the WT-FAS additionally needs to be deactivated after the growth phase. Activation or repression could be achieved by use of the

galactose activated *GAL* promoters (Johnston and Davis, 1984) or the methionine repressible *MET3* (Mao et al., 2002) and *MET25* promoters. Other systems for a dynamic expression control in *S. cerevisiae* were derived from the bacterial *tet* operator (*tetO*), in which gene expression is regulated for activation or repression in response to an inductor. The *tet* repressor system was shown to be feasible in *S. cerevisiae* (Cuperus et al., 2015) and a distinct homolog was engineered for specific off-switch by the inexpensive molecule camphor (Ikushima et al., 2015). However, the FAS complex is highly stable and long-lived with a half-live of more than 20 h (Enger et al., 1993), showing that switching off the expression in the production phase is not sufficient but additionally, efficient degradation of the complex must be induced, to avoid interference of the WT-FAS with mutated FAS. Being a large complex of 2.4 MDa (Jenni et al., 2006), the FAS is not degraded *via* the proteasome but is delivered to the vacuole by autophagy (Shpilka et al., 2015) although many details of this mechanism yet remain elusive. Nevertheless, degradation by the proteasome can be induced by an auxin-inducible degron system, in which a degradation signal (degron) is fused to the desired protein and degradation is induced by supplementation of auxin (Morawska and Ulrich, 2013). However, to the best of my knowledge there is no tool available yet to initiate rapid vacuolar degradation of a protein in *S. cerevisiae*.

3.5 Strategies to increase production of 8-hydroxyoctanoic acid in *Saccharomyces cerevisiae*

The *de novo* production of 8-hydroxyoctanoic was demonstrated in *S. cerevisiae* by production of OA from the FAS^{RK} and sequential conversion to 8-hydroxyoctanoic acid by a CYP/CPR and improved further by changing the carbon source from glucose to ethanol/glycerol (Wernig et al., 2020a). The production titer however remained low (3 mgL^{-1}) and need to be improved for a profitable industrial application. In this section potential strategies to improve the production are presented and discussed.

In the publication (Wernig et al., 2020a), we demonstrated that CYP/CPR activity is limiting the production of 8-hydroxyoctanoic acid, because high amounts of produced OA remained in the media and were not converted further. Therefore, to increase the production, improving the CYP/CPR activity should be considered first. CYP belong to the most studied enzyme class and

many new CYP have been discovered in recent years (Renault et al., 2014). We have selected and compared three different CYP/CPR pairs for 8-hydroxyoctanoic acid formation based on their substrate specificity. Due to the growing number of newly characterized CYP, many new heterologous candidates, which possibly have improved substrate preference and high activity in *S. cerevisiae* could be selected and compared to the ones used in Wernig et al., 2020a.

Otherwise, a recent review (Jiang et al., 2020) summarized and discussed frequently applied strategies to improve the expression and performances of heterologous CYP in yeast. These include codon optimization, N-terminal modification, protein engineering, co-expression of CYP and CPR, fusion proteins of CYP-CPR and strain modifications to improve the microenvironment for CYP expression (Jiang et al., 2020). Codon optimization of heterologous genes is a well-established strategy that can improve gene expression (Elena et al., 2014). Several examples demonstrated that codon optimization of heterologous CYP can improve protein expression in *S. cerevisiae* (Semiz and Sen, 2015), improve production titers (Wang et al., 2019) or enable high production (Liu et al., 2018), although in this last study codon-optimized CYP expression was not compared to non-codon optimized expression. Hence, codon optimization, which was not applied in Wernig et al., 2020a, should be considered as a future strategy to increase 8-hydroxyoctanoic acid production.

Most eukaryotic CYP contain an N-terminal transmembrane helix and are anchored at the ER membrane. Although *S. cerevisiae* is a preferred host due to presence of an ER membrane, modifications of the N-terminus was investigated for improved expression in one exemplary study. Thus, the expression of a CYP from wheat was improved in *S. cerevisiae* by substituting the 5’ segment by the N-terminus of the endogenous CYP51 (Cabello-Hurtado et al., 2001).

Natural substrate specificity of heterologous CYP not always meets the exact demands for a desired production pathway. Therefore, multiple studies have focused on rational enzyme engineering to improve the substrate specificity of expressed CYP. For instance, forskolin production was enhanced by engineering of the substrate recognition site of a CYP, which was shown to catalyze the rate limiting reaction in the pathway (Forman et al., 2018). Another example is the biotransformation of acetylated cortexolone to hydrocortisone by a heterologous CYP, which was enhanced by 3-fold by engineering of the CYP (R126D/Y398F) (Chen et al.,

2020). As an alternative to rational engineering, random mutagenesis was applied. For instance, a mutant library of CYP102A1 was generated by error-prone PCR and tested by a newly developed synthetic RNA biosensor to link the CYP activity to GFP fluorescence and resulted in a CYP with a 30-fold increased activity (Michener and Smolke, 2014). Another example for a performed error-prone PCR to introduce mutations into a CYP that performs the hydroxylation of genistein was given by (Hatakeyama et al., 2017). The authors isolated a triple mutant variant from more than 2000 mutants, which displayed improved activity. Rational engineering and random mutagenesis were demonstrated to improve production by enhanced product specificity. Rational engineering however requires the availability of the protein structure and detailed knowledge of the active site, which is not always elucidated. Random mutagenesis requires a powerful high throughput screening system. While OA binding for the best performing CYP (CYP539A7 in Wernig et al., 2020a) was simulated computationally and allowed identification of the key residues of OA binding (Durairaj et al., 2015), there is no high throughput analysis method for 8-hydroxyoctanoic acid. Thus, rational engineering for improved substrate specificity should be considered to improve 8-hydorxyoctanoic acid production.

The activity of eukaryotic CYP requires efficient electron transfer from a CPR redox partner (Munro et al., 2013). A suitable CPR should be co-expressed because endogenous CPRs may not be compatible with the heterologous CYP (Jiang et al., 2020). For the reaction of OA to 8-hydroxyoctanoic acid in Wernig et al., 2020a, efficient electron transfer to the CYP was ensured by expression of a cognate CPR. Although high expression of the CPR from strong promoters did not increase production titers (Wernig et al., 2020a) simple co-expression might not be enough for high CYP activity. To improve CYP-CPR pairing an artificial channeling system could be implemented. To perform channeling, enzymes must consist of multifunctional domains, be associated in complexes or clustered under certain conditions (Oreb et al., 2020). The first two are known as direct channeling, in which the intermediates of the reactions are directly channeled to the next active site avoiding diffusion and accessibility of reactants to side reactions. Clustering into protein agglomerates does not fully prevent diffusion of substrates but accelerates active site and substrate or protein-protein encounters and thereby increases metabolic rates (Castellana et al., 2014). To differentiate these, the latter is referred to as proximity channeling (Bauler et al., 2010; Castellana et al., 2014). The understanding of this concept has evoked

construction of artificial substrate- or proximity channeling systems to improved efficiency in desired pathways (Whitaker and Dueber, 2011; Chen and Silver, 2012; Thomik et al., 2017; Besada-Lombana et al., 2018; Oreb, 2020). For the construction, close proximity of the enzymes is mediated directly by fusion into one polypeptide chain, by fusion with protein-protein interaction domains to their termini, or by embedding them in artificial scaffold proteins (Oreb, 2020). To bring the CYP and CPR partners into close proximity and create an artificial proximity channeling system for electron transfer, fusion of the CYP and CPR has been examined in multiple studies (reviwed in Munro et al., 2007a). For instance, the production of long-chain ω-HyFA was improved by fusing the responsible CYP with its cognate CRP with a short linker, which outperformed their individual co-expression (Liu et al., 2019). As alternative strategies for proximity channeling, fusion with protein-protein interaction sites or usage of a scaffold protein could be investigated to increase CYP and CPR encounters. Additionally CYP can interact with other redox partners like cytochrome b5, which are considered to be involved in and increase the electron transfer (Schenkman and Jansson, 2003; Zhang et al., 2007). Indeed, co-expression of a cognate cytochrome b5 with CYP and CPR was shown to enhance production of artemisinin (Paddon et al., 2013) or glycyrrhetinic acid (Wang et al., 2019), for instance. Thus, fusion of CYP and CPR and co-expression of cytochrome b5 should be considered furthermore to improve CYP activity. In addition, CYP are heme dependent enzymes and the endogenous provision of heme has been considered to limit CYP activity and 8-hydroxyoctanoic acid production (Wernig et al., 2020a). We demonstrated, that supplementation of hemin improves production of 8-hydroxyoctanoic acid from glucose (Wernig et al., 2020a). Therefore, enhancement of heme supply by metabolic engineering strategies (Michener et al., 2012; Savitskaya et al., 2019) should be considered to increase the production.

In conclusion, many different studies have focused on increasing the activity of heterologous CYP in *S. cerevisiae* by different strategies. Due to the low conversion of OA to 8-hydroxyoctanoic acid, the CYP reaction was identified as the limiting step (Wernig et al., 2020a) and the strategies presented here should be elucidated in future to increase CYP activity and 8-hydroxyoctanoic acid production.

3.6 A future perspective on microbial production of oleochemiclas

Various successes and challenges in the production of OA and 8-hydroxyoctanoic acid were reached and revealed in this work. The production of OA was successfully increased by different engineering strategies and *de novo* production of 8-hydroxyoctanoic acid was achieved. The final goal however, is to achieve high production titers and yields to implement an industrial production of S/MCFA and their derivatives. The here presented production titers remained too low for an economical industrial production. But further development in the field of S/MCFA production can be expected in the future. Much depends on the development of the crude oil and vegetable oil market prices, with which biotechnological production must compete and how much biotechnological production can be improved further.

Multiple possibilities were outlined to overcome the mentioned challenges and to further increase production. The utilization of oleaginous organisms like *Y. lipolytica* and others will increase with more genetic tools in the future, providing thrilling host organism for oleochemicals production. Additionally, high throughput methods were developed recently to analyze production of S/MCFA (Baumann et al., 2018; Xue et al., 2020) of many samples in parallel. These include a *S. cerevisiae* biosensor that transforms S/MCFA concentrations in the media in a correlated GFP readout signal (Baumann et al., 2018) and a colony based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of membrane lipids (Xue et al., 2020). The authors of the latter study reasoned and demonstrated, by a previously established S/MCFA producing FAS variant, that an increased free S/MCFAFA production in *S. cerevisiae* increases the rate of S/MCFA in membrane lipids which were analyzed with a performance rate of 2s per sample (Xue et al., 2020). The establishment of rapid and efficient high throughput analytic methods enables the utilization of high throughput engineering approaches such as screening of strains and gene libraries, directed evolution and random mutagenesis, which could speed up engineering of producer strains and reveal engineering strategies, which so far have not been on the agenda of researchers.

Additionally, biotechnological production of hexanoic acid and OA was established by anaerobic open culture microbiome consortia by chain elongation of short carboxylates from organic waste

materials such as acetate with ethanol as a source for carbon, energy and reducing equivalents (reviewed in Angenent et al., 2016). The reverse β-oxidation was identified as the responsible pathway for elongation. For instance, production of mainly hexanoic acid was achieved with a production rate of more than 57 $gL^{-1}d^{-1}$ by using ethanol and acetate as substrates (Grootscholten et al., 2013). However, undefined microbiome consortia, strict anaerobic conditions and the fact that only process conditions can be manipulated to achieve production goals are challenges that need to be addressed for a market introduction of this process.

Even if a biotechnological production of OA and other S/MCFA is not able to compete with conventional production in the near future, S/MCFA are valuable platform chemicals that can be converted into or are the precursor to many different compounds of high market value. New *de novo* production pathways, based on FA or derived compounds can be established in future. For instance, the heterologous biosynthesis pathway of the pharmaceutical compounds of cannabinoids depend on hexanoyl-CoA as precursor (Luo et al., 2019). In conclusion, the here presented achievements and the outlined challenges are a valuable contribution to the biotechnological production of not only S/MCFA but of many other products derived from or involving FA.

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5 Publications

5.1 Fusing α and β subunits of the fungal fatty acid synthase leads to improved production of fatty acids

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(3) Compilation of data-sets and figures

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Fusing α and β subunits of the fungal fatty acid synthase leads to improved production of fatty acids

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Most fungal fatty acid synthases assemble from two multidomain subunits, α and β, into a heterododecameric FAS complex. It has been recently shown that the complex assembly occurs in a cotranslational manner and is initiated by an interaction between the termini of α and β subunits. This initial engagement of subunits may be the rate-limiting phase of the assembly and subject to cellular regulation. Therefore, we hypothesized that bypassing this step by genetically fusing the subunits could be beneficial for biotechnological production of fatty acids. To test the concept, we expressed fused FAS subunits engineered for production of octanoic acid in *Saccharomyces cerevisiae*. Collectively, our data indicate that FAS activity is a limiting factor of fatty acid production and that FAS fusion proteins show a superior performance compared to their split counterparts. This strategy is likely a generalizable approach to optimize the production of fatty acids and derived compounds in microbial chassis organisms.

Fungal fatty acid synthases (FAS) are prototypical multi-domain molecular machines that form a barrel-like structure. Acyl carrier protein (ACP) domains, spanning their reaction chambers and shuttling substrates and intermediates between the individual catalytic domains, facilitate compartmentalized FA synthesis[1,2].

Genome sequence analyses has characterized fungal FAS as heterogeneous family comprising currently six gene-topological variations. Most fungal species encode FAS subunits as two multi-domain polypeptides, α and β, that assemble to a hetero-dodecamer ($\alpha_6\beta_6$). In an elegant study, Bukhari *et al.*[3] have shown that fungal multifunctional FAS have evolved from monofunctional enzymes that were fused to form a single-gene encoded enzyme as an evolutionary intermediate. Intriguingly, at a later point, gene splitting at various (species specific) positions led to a set of two-genes encoded fungal FAS[3–6]. As an evolutionary late event, gene splitting was non-invasive to the overall structure, and also did not affect the assembly pathway, which was already established before on the single-gene variant[3,6]. This view is in line with recent findings proposing that protein complexes are under strong evolutionary selection for ordered assembly pathways[7].

Recently, Shiber *et al.*[8] revealed that yeast FAS assembly is initiated via the cotranslational interaction of the subunits α and β. It was shown that the N-terminus of the α-subunit (encoded by *FAS2*) is engaged by the β-subunit (*FAS1*) for cotranslational substructure folding. As substantiated in a further study on the molecular basis of cotranslational assembly, the C-terminus of β and the N-terminus of α undergo specific interactions while forming the MPT domain. Cotranslational assembly of yeast FAS is not restricted to the naturally occurring splitting site, but the protein can also assemble when subunit borders are shifted. Further, yeast FAS assembles when subunits are fused to a single polypeptide[9].

Here, we investigated whether these recent findings can be translated into biotechnological application. Given that many industrially relevant compounds are based on fatty acids (FA) and their derivatives (which are currently extracted from plants or synthesized from petrochemicals), engineering microbial cells for production of FA has recently become one of the major targets in biotechnology. Whereas a plethora of strategies to improve the supply of precursor molecules (acetyl-CoA and malonyl-CoA) or redox-cofactors (NADPH) for FA biosynthesis was developed and led to considerable successes as previously reviewed[10,11], only few studies focused on engineering of FAS enzymes in *S. cerevisiae*, mainly with the aim to control the chain length of produced FA[12–14]. Since upstream pathway engineering can unfold its full potential only if FAS has sufficient capacity to process the precursor molecules, FAS genes are usually overexpressed, e.g. by using strong promoters and/or plasmids[12,15,16].

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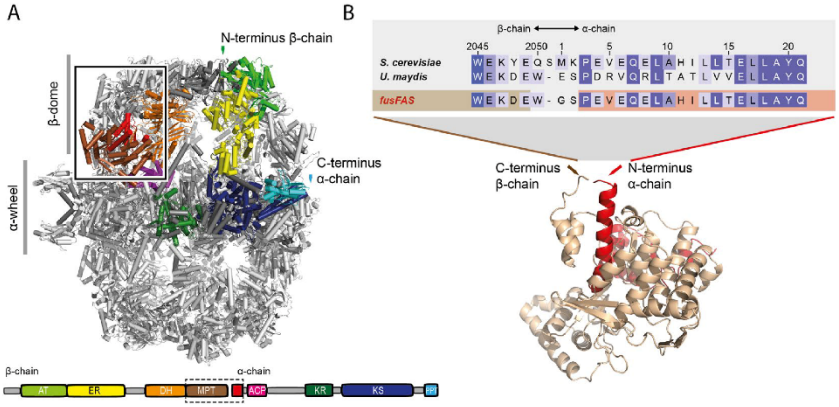

Figure 1. Structure of yeast FAS and the α/β interface. (**A**) Cartoon representation of the X-ray crystallographic structure of *S. cerevisiae* FAS (PDB-code: 3hmj)[28] with one β subunit and one α subunit shown in color code of the functional domains as schematically depicted below. The MPT fold is comprised of both subunits (β part in brown and α part in red). The region shown in more detail in (**B**) is framed in the structure. Nomenclature: acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH), malonyl-palmitoyl-transferase (MPT), acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KS) and phosphopantetheine transferase domain (PPT). (B) Structure of the MPT domain of *S. cerevisiae* FAS in cartoon representation and color coded as in (A). In fusFAS, chains are linked by a short sequence derived from the single-chain *Ustilago maydis* FAS. An alignment of the relevant sequence regions is shown.

This can lead to a disbalanced synthesis of FAS α and β subunits in engineered cells and, as a consequence, to degradation of superfluous subunits in the proteasome to maintain the stoichiometry of the complex[17]. We reasoned that fusing α and β subunits in one polypeptide chain[9] could be beneficial to avoid such undesired side-effects of a deregulated expression and promote cotranslational assembly of the FAS complex. To test the concept with a reliable readout, we used a FAS variant previously developed[12,18] for production of octanoic acid (OA), a C8 FA that is secreted out of the cells and readily detected in culture supernatants. We show that the single-polypetide FAS is superior to the split-subunit version. The underlying principle likely represents a generically aplicable strategy to increase type I FAS-based production of FA and derived chemicals.

Results and Discussion

Construction and functionality of fused FAS subunits. To synthesize both *S. cerevisiae* FAS subunits as a single polypeptide ("fusFAS"), *FAS1* (encoding the β subunit) and *FAS2* (α subunit) open reading frames (ORFs) were connected by a sequence encoding a linker derived from the single-chain *Ustilago maydis* FAS[9] (Fig. 1).

The fused ORFs were placed under the control of the *FAS1* promoter and *FAS2* terminator and inserted into centromeric plasmids. As a control, we used a plasmid containing *FAS1* and *FAS2* as separate ORFs flanked by their native promoters and terminators (split FAS). First, we compared the ability of these constructs to complement the growth defect of the FAS deficient strain SHY34 in FA-free media. Both plasmids conferred the same growth rate (Supplementary Fig. S3), demonstrating that the fusion strategy does not negatively affect the FAS function. For production of OA, we introduced the R1834K substitution within the Fas1 chain, which was previously shown to promote the production of short and medium chain FA[12,18] into the fusion construct (fusFAS^{RK}) and into the split FAS plasmid (FAS^{RK}). In accordance with previous observations[12], the mutated constructs conferred slower growth rates compared to the wildtype fusFAS (Fig. 2A), due to their reduced ability to synthesize the essential long chain (C16 and C18) FA[12] and cytotoxicity of the produced OA[19].

Next, we compared OA titers produced by SHY34 expressing different FAS variants in shake flask fermentations (Fig. 2B). In addition to the deletion of genomic *FAS1* and *FAS2* copies, *FAA2* gene encoding the medium chain fatty acyl-CoA synthetase was deleted in this strain to minimize degradation of octanoic acid via β-oxidation as described previously[15,20]. fusFAS^{RK} expression resulted in a higher accumulation of extracellular OA compared to the two-gene-encoded FAS^{RK} at both time points. Strikingly, with fusFAS^{RK} the difference was more pronounced at the earlier time point (corresponding to an increase of 58% compared to the split enzyme) indicating that a higher productivity (defined as product formation per time) can be achieved by the fusion of subunits. Moreover, the production of two byproducts with different chain lengths, hexanoic acid and decanoic acid, was also increased with the fusion construct (Supplementary Fig. S4), suggesting that the approach is generalizable and not restricted to the production of OA.

Based on these data, it may be hypothesized that the assembly of the FAS complex occurs faster, as anticipated. Moreover, the equimolar stoichiometry of both subunits in the fusion protein can indirectly have a positive effect on cellular physiology by obviating the energetically wasteful cycles of synthesis and degradation of superfluous

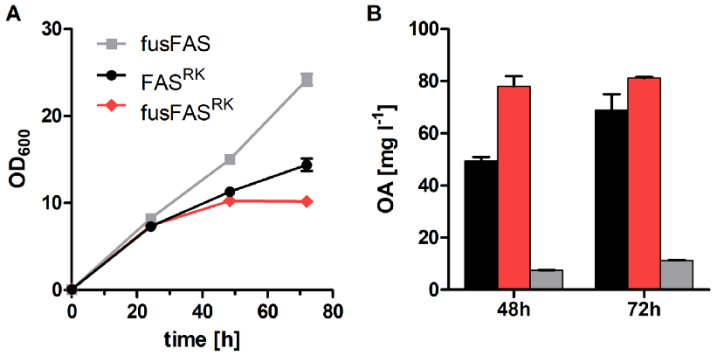

Figure 2. Functionality of FAS fusion constructs. The FAS variants FAS^{RK}, fusFAS^{RK} and fusFAS were expressed in the strain SHY34 (*Δfas1 Δfas2 Δfaa2*) cultivated in buffered YPD medium. The growth was assessed by measuring OD_{600} over time (**A**). Octanoic acid titers in culture supernatants were determined by gas chromatography after 48 and 72 h of cultivation (**B**). The same color code is used in both panels. Mean values and standard deviations of biological duplicates are shown. Error bars may be smaller than the symbols.

subunits[17], which is likely to occur if two genes are separately overexpressed with strong constitutive promoters. Although we cannot rule out that the FAS complex assembled from fused subunits is more resistant to proteolytic degradation, this hypothesis contradicts our observations, since autophagy of FAS is initiated during starvation[21] (i.e. at later stages of cultivation), where the benefit of fusFAS^{RK} expression was less pronounced (see Fig. 2B at 72 h).

Improving the expression of engineered FAS fusion constructs. The results presented above indicate that FAS activity is at least one of the limiting factors for OA production. Increased transcription and translation efficiency could therefore lead to further improvements of the production rate. We first performed a codon-optimization of the FAS sequences (for details see SI), but this had only a marginal, if any, effect on OA titers (see Supplementary Fig. S5). We next sought to improve the transcriptional control of FAS constructs. For this, well-known and extensively characterized strong constitutive promoters *pHXT7* $^{1-392}$, *pTDH3* and *pTEF1*[22,23] were selected. We expressed the fusFAS^{RK} under the control of these three promotors or *pFAS1* as a reference in SHY34 and compared the growth (Fig. 3A) and OA production (Fig. 3B) of the transformants.

Again, with all fusFAS^{RK} constructs the maximum titer was reached after 48 h hours independently of the promoter. The plasmid with the truncated *pHXT7* led to the highest titer after 48 h ($133.00 \pm 0.6\ mg\ l^{-1}$) and 72 h ($131.9 \pm 3.6\ mg\ l^{-1}$) of fermentation, an increase of 50% compared to the native *pFAS1* ($87.1 \pm 1.4\ mg\ l^{-1}$) after 72 h. Interestingly, the highest titers at 24 h were reached with the *pTEF1* construct, which correlated with decreased cell growth of the corresponding strain, likely due to OA toxicity. To take into account the trade-off between the biomass and OA yields, we calculated the specific OA titers ($mg\ l^{-1}\ OD_{600}^{-1}$, Fig. 3C). This analysis shows that, if cell proliferation is not desired (e.g. in high cell density fermentations), *pTEF1* is the promoter of choice, whereas *pHXT7* (or *pTDH3*) can be preferably used for a low inoculum culture.

Co-expression of WT and engineered FAS variants. In our previous work, the mutated FAS^{RK} variant was expressed in FAS deficient (*Δfas1 Δfas2*) strains to unambiguously characterize the properties of the engineered enzyme. As observed before[12] with FAS^{RK} and confirmed in Fig. 2A for fusFAS^{RK}, the mutated enzyme does partially complement the requirement of the strain for C16 and C18 FA due to its leaky chain length control, but there is a significant growth defect correlating with the production of OA (see Fig. 3). Since slow growth is an undesired trait from a biotechnological viewpoint, we wondered whether the mutated enzymes could be expressed in a FAS WT background to produce OA in a normally proliferating strain. An obvious pitfall of simultaneously expressing different variants of the same FAS subunits is the possible formation of heterogeneous complexes (i.e. assembling Fas1^{R1834K} and Fas1^{WT} β chains in the same $\alpha_6\beta_6$ dodecamer). We hypothesized that the concomitant expression of fusFAS^{RK} and (split) WT FAS would favor two homogenous FAS entities, as the topology of the fusion construct (aminoterminus-β-α-carboxyterminus) would not allow for the interaction with the termini of the split subunits (which engage via an interaction of the C-terminus of β with the N-terminus of the nascent α-chain)[8,9]. Hence, we expressed fusFAS^{RK} or FAS^{RK} in SHY24, which has WT *FAS1* and *FAS2* alleles in the genome and measured OA titers in culture supernatants (Fig. 4A).

In line with results shown in Fig. 2B, the fused enzyme exhibited superior performance, but the titers were overall lower compared to the FAS deficient strain (compare Figs. 2B and 4A). To rule out any unspecific effects of the strain background, we transformed plasmids with fused and split FAS variants with or without the R1834K mutation in different combinations into the FAS deficient strain SHY34. In accordance with other results presented here, fusFAS^{RK} showed higher productivity than FAS^{RK} and the presence of WT FAS reduced the OA titers in all combinations tested (Fig. 4B). The latter observation could be hypothetically explained by

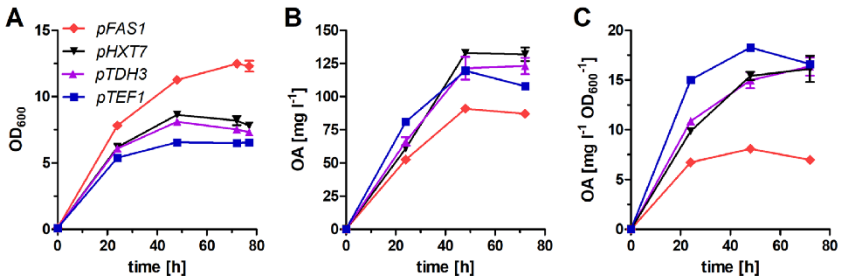

Figure 3. Expression of fusFAS with different promotors. fusFAS^{RK} was expressed from strong promotors (*pTEF1*, *pTDH3*, *pHXT7*$^{1-329}$) or *pFAS1* in strain SHY34 (*Δfas1 Δfas2 Δfaa2*). Comparison of growth (**A**) and OA production (**B**) in buffered YPD medium over a period of 72 h is shown. In (**C**), the titers were normalized to the OD_{600} of the respective culture. Mean values and standard deviations of biological duplicates are shown. Error bars may be smaller than the symbols.

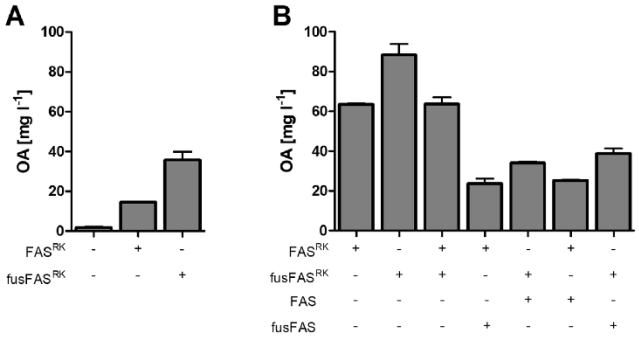

Figure 4. Co-expression of engineered and WT-FAS variants. (**A**) FAS^{RK} and fusFAS^{RK} were expressed from plasmids where indicated ("+") in the strain SHY24 (*Δfaa2*) that additionally has native *FAS* alleles in the genome. As a control, the cells transformed with the empty vector were used (left column). In (**B**), the indicated combinations of FAS plasmids were expressed in the FAS-deficient strain SHY34 (*Δfas1 Δfas2 Δfaa2*). The cells containing only one FAS plasmid (in the first two columns) were additionally transformed with appropriate empty vectors. Strains were cultivated in YPD with potassium phosphate buffer. For plasmid maintenance hygromycin (100 mg l^{-1}) (A) or hygromycin (100 mg l^{-1}) plus G418 (200 mg l^{-1}) (B) were used. Mean values and standard deviation of two biological replicates at 72 h of fermentation are shown.

different mechanisms (including combinations thereof): (i) competition for substrates and cofactors (acetyl-CoA, malonyl-CoA, NADPH) between mutated and WT FAS; (ii) elongation of octanoyl-CoA released from mutated FAS by WT FAS as observed *in vitro* (Pirson *et al.*, 1973) and (iii), as outlined above, formation of heterogeneous complexes, in which the WT subunits could elongate octanoyl-CoA released by mutated ones within the same FAS reaction chamber. However, the surprising finding that the co-expression of FAS^{RK} and fusFAS^{RK} yields less OA than fusFAS^{RK} alone cannot be explained by hypotheses (i) and (ii) and suggests that an interaction, including the formation of a heterogeneous complex, may occur between fused and singular subunits at some stage of complex assembly. In such a scenario, the physical interaction between fused and split subunits could have a negative kinetic effect on the assembly of the FAS complex and consequently lead to lower OA titers. However, other hypotheses to explain the observed effect cannot be ruled out at present. Regardless of the underlying mechanism, our data indicate that the interferences between engineered and WT FAS activities cannot be circumvented by expressing the fusion proteins.

Conclusion

Taken together, our data demonstrate that fusing α and β subunits of FAS in one polypeptide chain leads to a substantially higher FAS activity, measured as increased production of OA. Although the elucidation of the underlying mechanism is not in the scope of this study, it is - based on previously published research - reasonable to assume that an increased assembly rate and balanced stoichiometry of the subunits may be responsible for the observed effect. To optimize the expression of the fused FAS constructs, we identified a set of suitable promoters.

Strain name	Relevant genotype	Reference/Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(Brachmann *et al.*, 1998)[29]
SHY24	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δfaa2*	This work
SHY34	*MATα ura3Δ0 his3Δ0 leu2Δ0 TRP1 lys2Δ0 MET15 Δfas1 Δfas2 Δfaa2*	This work

Table 1. Yeast strains used in this study.

Importantly, we further show that the simultaneous presence of WT and engineered FAS variants decreases the OA titers by physical and/or metabolic crosstalk of different enzyme populations, which cannot be circumvented by expressing a single-chain version of the engineered FAS. The principles described here very likely apply to the biotechnological production of any FAS-derived molecules. Moreover, fusing the subunits of cotranslationally assembled protein complexes may be a generically applicable strategy, reaching beyond the production of FA.

Materials and Methods

Strain construction and transformation. Yeast strains used in this study are listed in Table 1. The strain SHY24 was constructed by deleting the *FAA2* locus in BY4741 using the plasmid pRCC-N-faa2 (see Supplementary Table S1) by CRISPR-Cas9 meditated gene deletion as described previously[24]. For this, a donor DNA together with the CRISPR-Cas9 plasmid encoding for the Cas9 and the guide RNA with a protospacer sequence targeting specifically *FAA2* (GAAGATTTTGAAACCTTACG) was transformed into yeast cells. The strain SHY34 resulted from the previously described strain RPY21[15] by deletion of two *kanMX4* markers which were present in the RPY21 genome as remnants of *FAS1* and *FAS2* deletion by the same CRISPR-procedure (pRCC-N-kanMX4; protospacer sequence: TTACTCACCACTGCGATCCC). RPY21 has a BY background and is based on strain BY.PK1238_1A_KO[12], in which *FAA2* was previously deleted[15] as described above for SHY24.

Transformations were performed following the frozen competent cell protocol[25], whereas SHY34 was transformed by a slightly modified method previously described[12]. Specifically, because strain SHY34 is FAS deficient, the cells were cultivated in YPD medium supplemented with oleic acid (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 1.42% (v/v) Tergitol^{TM} solution NP-40, 0.016% (v/v) oleic acid) before transformation with the appropriate plasmid coding for FAS. Transformed yeasts were plated on solid YPD (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose) containing appropriate antibiotics hygromycin (100 mgl^{-1}) or G418 (200 mgl^{-1}) for plasmid selection and grown at 30 °C for two to four days.

Plasmid construction. Nucleotide sequences of FAS variants used in this study are shown in Supplementary Information and plasmids are listed in Supplementary Table S1. Plasmids were constructed via homologous recombination in yeast[26]. Plasmid fragments were amplified by PCR using oligonucleotides listed in Supplementary Table S2. The assembled plasmids were propagated in and extracted from *E. coli* DH10B by standard procedures.

For replacement of auxotrophy markers by dominant markers, *hphNT1* or *kanMX4* cassettes were amplified from pRS62-H or pRS62-K, respectively, and inserted into the EcoRV cut site of *LEU2* in pRS315 or the MscI cut site of *HIS3* in pRS313 based plasmids.

Media and cultivation. *Saccharomyces cerevisiae* liquid cultures were grown in shake flasks at 30 °C and 180 rpm in YPD medium as described previously[12] without supplementation of free FA or with supplementation of oleic acid (0.5 mM and 1% (v/v) Tergitol NP-40 solution Sigma Aldrich, Germany) for the FAS deficient strain. For maintaining plasmids with *hphNT1* or *kanMX4* marker appropriate antibiotics hygromycin (100 mgl^{-1}) or G418 (200 mgl^{-1}) were used. The medium was additionally buffered with 100 mM potassium phosphate and adjusted to a pH of 6.5. Main cultures of 50 mL were inoculated from pre cultures to an OD_{600} of 0.1 and grown for 72 h at 30 °C with shaking (200 rpm). Samples for compound extraction were taken at given time points.

Compound extraction and derivatization. Extraction of free fatty acids in the culture medium was performed as described before[15]. Cells were separated from the medium by centrifugation (3,500 rcf, 10 min) and 10 ml of culture supernatant was mixed with an internal standard (0.2 mg heptanoic acid), 1 mL of 1 M HCl and 2.5 ml of methanol:chloroform (1:1) solution. After phase separation (3,000 rcf, 5 min) the organic phase layer was taken and evaporated in a vacuum concentrator (Concentrator 5301, Eppendorf, Germany). Fatty acids were methylated for GC analysis as described[27]. The extract was dissolved in 200 µL toluene, mixed with 1.5 mL of methanol and 300 µL of 8.0% (w/v) HCl solution and incubated at 100 °C for 3 h to form fatty acid methyl esters (FAME). FAMEs were extracted from the mixture by addition of 1 ml H_2O and 1 ml hexane. The organic phase was taken for gas chromatography analysis.

Gas chromatography. The gas chromatography analysis was performed on a Perkin Elmer Clarus 400 system (Perkin Elmer, Germany) equipped with an Elite-5MS capillary column (Ø 0.25 mm; length 30 m; film thickness 1.00 µm) and a flame ionization detector (Perkin Elmer, Germany). 1 µL of sample was analyzed after split injection (1:10) and helium was used as carrier gas (90 kPa). For FAME quantification, the temperatures of the injector and detector were set to 200 and 250 °C, respectively. The following temperature program was applied: run time 42.67 min, start at 50 °C and hold for 5 min; ramp at 10 °C min to 120 °C and hold for 5 min, ramp at

15 °C to 220 °C and hold for 10 min, ramp at 20 °C to 300 °C and hold for 5 min. FAMEs were identified and quantified by comparison with authentic standard substances.

Data availability

The authors will make available all data (underlying the described findings) without restriction.

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Author contributions

F.W. and S.B. performed the experiments, analyzed the data and drafted the manuscript. M.G. designed the fusion strategy. E.B. and M.O. were involved in the experimental design. M.O. guided the project and finalized the manuscript, which was approved by all authors.

Competing interests

E.B. and M.G. are co-inventors of EP patent application 15 162 192.7 filed on 1 April 2015, and of EP patent application 15 174 342.4 filed on 26 June 2015, by Goethe-University Frankfurt, describing short-chain acyl-CoA producing FAS variants. There are no other competing interests.

Additional information

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Supplementary Information

Fusing α and β subunits of the fungal fatty acid synthase leads to improved production of fatty acids

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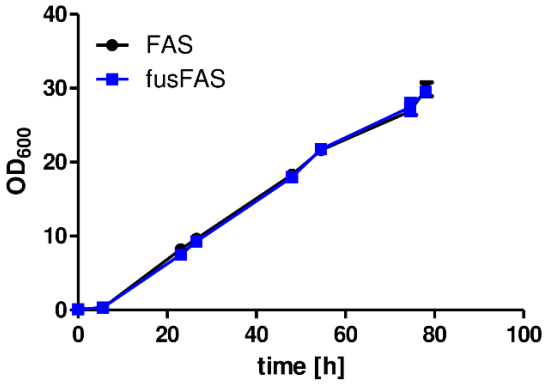
Supplementary Table S1 | Plasmids used in this study. Under "description" the relevant genetic elements are listed. AmpR, Ampicillin resistance; prefixes "p" and "t" denote promoters and terminators, respectively; MCS, multiple cloning site. Other elements listed follow conventional nomenclature or (for FAS variants) the nomenclature used in the manuscript.

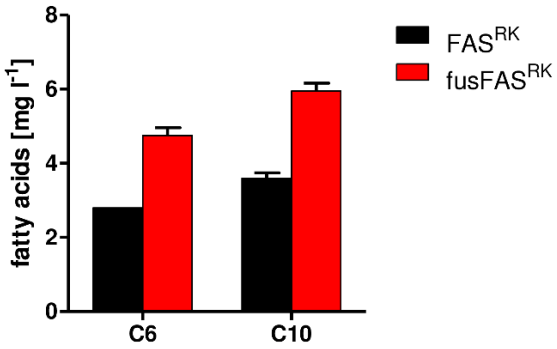
Laboratory stock code	Plasmid name	Description	Used in Figure	Reference
ASB22	pRS62-H	*2µ, AmpR, HphNT1, pHXT7-MCS-tCYC1*	-	Farwick et al., 2014
ASB23	pRS62-K	*2µ, AmpR, kanMX4, pHXT7-MCS-tCYC1*	-	Farwick et al., 2014
SHV61	pRS315-FAS1^{RK}-FAS2	*CEN6/ARS4, AmpR, LEU2, pFAS1-FAS1^{R1834K}-tFAS1, pFAS2-FAS2-tFAS2*	Fig. 2, S4	This study
FWV113	pRS315K-FAS1^{RK}-FAS2	*CEN6/ARS4, AmpR, kanMX4, pFAS1-FAS1^{R1834K}-tFAS1, pFAS2-FAS2-tFAS2*	Fig. 4a,b; S5	This study
FWV124	pRS315K- FAS1$^{opt;RK}$-FAS2^{opt}	*CEN6/ARS4, AmpR, kanMX4, pFAS1-FAS1$^{opt;R1834K}$-tFAS1, pFAS2-FAS2^{opt}-tFAS2*	S5	This study
FWV125	pRS315K- fusFAS$^{opt;RK}$	*CEN6/ARS4, AmpR, kanMX4, pFAS1-FAS1$^{opt;R1834K}$-FAS2-tFAS2*	S5	This study
FWV128	pRS313K-fusFAS^{RK}	*CEN6/ARS4, AmpR, kanMX4, pFAS1-FAS1 ^{R1834K} -FAS2-tFAS2*	Fig. 4a,b; S5	This study
FWV129	pRS315K-FAS1-FAS2	*CEN6/ARS4, AmpR, kanMX4, pFAS1-FAS1-tFAS1, pFAS2-FAS2-tFAS2*	Fig. 4b; S3	This study
FWV132	pRS313H-fusFAS	*CEN6/ARS4, AmpR, HphNT1, pFAS1-FAS1-FAS2-tFAS2*	Fig. 4b; S3	This study
FWV133	pRS313H-fusFAS^{RK}	*CEN6/ARS4, AmpR, HphNT1, pFAS1-FAS1^{R1834K}-FAS2-tFAS2*	Fig. 4b	This study
FWV155	pRS315H-FAS1^{RK} -FAS2	*CEN6/ARS4, AmpR, hphNT1, pFAS1-FAS1 ^{R1834K} -tFAS1, pFAS2-FAS2-tFAS2*	Fig. 4b	This study
SHV29	pRCC-N-kanMX4	*2µ, AmpR, natNT2, pROX3-opt.CAS9^{op}-tCYC1, pSNR52-gRNA[kanMX4]-tSUB4*	-	This study
SHV42	pRCC-N-faa2	*2µ, AmpR, natNT2, pROX3-opt.CAS9^{op}-tCYC1, pSNR52-gRNA[faa2]-tSUB4*	-	Henritzi et al., 2018
SHV69	pRS313-fusFAS	*CEN6/ARS4, AmpR, HIS3, pFAS1-FAS1-FAS2-tFAS2*	Fig. 2	This study
SHV70	pRS313-fusFAS^{RK}	*CEN6/ARS4, AmpR, HIS3, pFAS1-FAS1^{R1834K}-FAS2-tFAS2*	Fig. 2; Fig. 3; S4	This study
SHV72	pRS313-pHXT7-fusFAS^{RK}	*CEN6/ARS4, AmpR, HIS3, pHXT7^{-1-392}-FAS1^{R1834K}-FAS2-tFAS2*	Fig. 3	This study
SHV73	pRS313-pTEF1-fusFAS^{RK}	*CEN6/ARS4, AmpR, HIS3, pTEF1-FAS1^{R1834K}-FAS2-tFAS2*	Fig. 3	This study
SHV74	pRS313-pTDH3-fusFAS^{RK}	*CEN6/ARS4, AmpR, HIS3, pTDH3-FAS1^{R1834K}-FAS2-tFAS2*	Fig. 3	This study

Supplementary Table S2 | Oligonucleotides used in this study

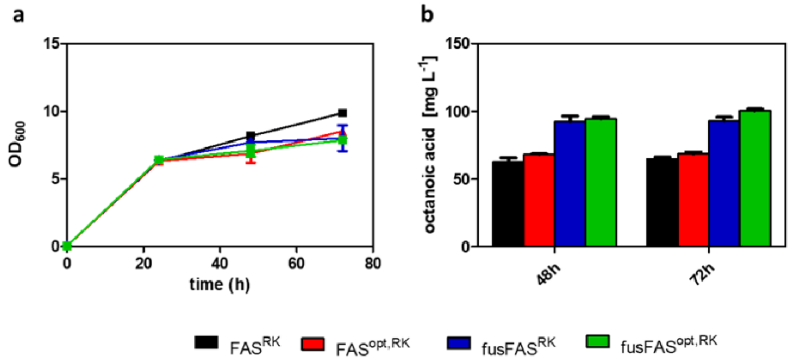
Primer Name	Sequence 5′-3′	Application
FWP220	AGTTTATGTACAAATATCATAAAAAAAGAGAATC TTTCAGCGACATGGAGGCC	Amplification of *kanMX4* with overhangs for pRS315 backbone to replace *LEU2*
FWP221	TTCTCAACAAGTAATTGGTTGTTTGGCCGAGCGG TCTGACACTGGATGGCGGCG	
FWP263	CGGCATCAGAGCAGATTGTACTGAGAGTGCACCA TCGTCCCAAAACCTTCTCAAG	Amplification of *hphNT1* with overhangs for pRS313 backbone to replace *HIS3*
FWP264	GCATCTGTGCGGTATTTCACACCGCATATGATCC GCAGCGACATGGAGGC	
FWP275	GGCATCAGAGCAGATTGTACTGAGAGTGCACCAT AATTCCAGCGACATGGAGGCC	Amplification of *kanMX4* with overhangs for pRS313 backbone to replace *HIS3*
FWP276	TACGCATCTGTGCGGTATTTCACACCGCATATGA TCCGTGACACTGGATGGCGGC	
FWP312	ATAAAGTTTATGTACAAATATCATAAAAAAAGAG AATCTTTCGTCCCAAAACCTTCTC	Amplification of *hphNT1* with overhangs for prS315 backbone to replace *LEU2*
FWP313	CTCTATTTCTCAACAAGTAATTGGTTGTTTGGCC GAGCGGTCTCAGCGACATGGAGG	
SHP100	ATGACCTTGATGATTAGCGAAGTAAGTCGACCTA GGTTCTATCCGAACTCCCCTTTTG	Amplification of pFAS2-FAS2-tFAS2 with overlaps to pRS315-FAS1-RK backbone
SHP101	CCCTCACTAAAGGGAACAAAAGCTGGGTACCGGG CATGCATAGAGCTGCTACGCGGAC	
SHP145	TGAATTGTAATACGACTCACTATAGGGCGAATTG GAGCTCCGCTCGTAGGAACAATTTCG	Amplification of *pHXT7*$^{-1--392}$ with overhangs for pRS313-fusFAS^{RK} backbone to replace *pFAS1*
SHP26	GTGAGATAGGGTTAATGGTCTTGTGGAGTAAGCG TCCATTTTTTGATTAAAATTAAAAAAACTTTTTG TTT	
SHP150	GAATTGTAATACGACTCACTATAGGGCGAATTGG AGCTCCACAGTTTATTCCTGGCATCC	Amplification of *pTDH3* with overhangs for pRS313-fusFAS^{RK} backbone to replace *pFAS1*
SHP151	ATAGGGTTAATGGTCTTGTGGAGTAAGCGTCCAT TTTGTTTGTTTATGTGTGTTTATTCG	
SHP152	TGAATTGTAATACGACTCACTATAGGGCGAATTG GAGCTCCCCTTGCCAACAGGGAGTTC	Amplification of *pTEF1* with overhangs for pRS313-fusFAS^{RK} backbone to replace *pFAS1*
SHP153	GGGTTAATGGTCTTGTGGAGTAAGCGTCCATTTT GTAATTAAAACTTAGATTAGATTGCTATGC	
SHP78	CTCATGACGAGCTCCGTATGCTAAATGAATGTAC TTATGACGATTTGGAACACATTCAAA	Donor-DNA fragment for faa2 deletion
SHP62	CCAAGGGAAAAATATAAAAAAGTACATTGGGCCT TTTCATGGCGGCCGACTGGTTTCTTAAGTGATTA TATAACCGTAAGAAATATTTAACTT	Donor-DNA fragment for *kanMX4* cassette deletion in *Δfas1* locus
SHP63	TTTTCACATGCTACCTCATTCGCCTCGTAACGTT ACGACCGGGCCCCCTGCGGCTCCGGTAAGAGAGC ACTACGTAGTCCCTCTTTTAATATGTAACGTGT	Donor-DNA fragment for *kanMX4* cassette deletion in *Δfas2* locus



Supplementary Figure S3 | Complementation of FAS deficiency by FAS constructs. The wildtype split FAS (FAS) and fused FAS (fusFAS) were expressed in the strain SHY34 (*Δfas1 Δfas2 Δfaa2*) cultivated in buffered YPD medium. The growth was assessed by measuring OD_{600} over time. Mean values and standard deviations of biological duplicates are shown. Error bars may be smaller than the symbols.



Supplementary Figure S4 | Production of other SMCFA by FAS constructs. The production of hexanoic acid (C6) and decanoic acid (C10) by two gene encoded FAS (FAS^{RK}) and fused FAS ($fusFAS^{RK}$) was quantified in the same samples (at 48 h) as shown in Fig. 2b in the main manuscript. Mean values and standard deviations of biological duplicates are shown.



Supplementary Figure S5 | Codon-optimization of FAS-constructs. Mutated two gene encoded FAS (FAS^{RK}) and fused FAS ($fusFAS^{RK}$) were expressed in strain SHY34 (*Δfas1 Δfas2 Δfaa2*) as codon optimized (opt.) or non-optimized variants. Comparison of (a) growth) and octanoic acid production (b) in buffered YPD medium over a time of 72h. For plasmid maintenance hygromycin (100 mg l^{-1}) was used. Mean values and standard deviations of biological duplicates are shown.

Sequences of promoters and FAS constructs. Sequences of promoters used in Figure 3 are listed. The online tool JCat (http://www.jcat.de) was used to codon-optimize the nucleotide sequence and calculate the codon adaptation index (CAI) of FAS ORFs. The change in CAI resulting from optimization is indicated, where appropriate. To introduce the R1834K mutation, the respective native codon "AGA" in *FAS1* was replaced by "AAA".

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>pFAS2
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>pTDH3
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>pTEF1
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>fusFAS based on native FAS1 and FAS2 ORFs
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>codon-optimized fusFAS, CAI change: 0.29 > 0.97
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5.2 Production of octanoic acid in *Saccharomyces cerevisiae*: Investigation of new precursor supply engineering strategies and intrinsic limitations

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Co-author EB: 10 %; Co-author MO: 20 %

(2) Conducting test and experiments:

Doctoral candidate FW: 90 % yeast molecular biology, fermentations, growth and metabolite analysis, strain robustness analysis

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(3) Compilation of data-sets and figures

Doctoral candidate FW: 90 % metabolite titers, strain growth, strain robustness assay

Co-author LB: 10 %; growth assay

(4) Analysis and interpretation of data

Doctoral candidate FW: 70 %; analysis and interpretation of all data

Co-author LB: 5 %; analysis and interpretation of Fig. 1

Co-author EB: 10 %; analysis and interpretation of all data

Co-author MO: 15 %, analysis and interpretation of all data

(5) Drafting of manuscript

Doctoral candidate FW: 80 %

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Production of octanoic acid in *Saccharomyces cerevisiae*: Investigation of new precursor supply engineering strategies and intrinsic limitations

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Highlights

- Construction of an easily manipulable octanoic acid producing strain
- First implementation of the phosphoketolase pathway for production of a fatty acid
- Increased yields of octanoic acid during the glucose consumption phase

Keywords

octanoic acid, acetyl-CoA, phosphoketolase, phosphotransacetylase

Abstract

The eight-carbon fatty acid octanoic acid (OA) is an important platform chemical and precursor of many industrially relevant products. Its microbial biosynthesis is regarded as a promising alternative to current unsustainable production methods. In *Saccharomyces cerevisiae*, the production of OA had been previously achieved by rational engineering of the fatty acid synthase. For the supply of the precursor molecule acetyl-CoA and of the redox cofactor NADPH, the native pyruvate dehydrogenase bypass had been harnessed, or the cells had been additionally provided with a pathway involving a heterologous ATP-citrate lyase. Here, we redirected the flux of glucose towards the oxidative branch of the pentose phosphate pathway and overexpressed a heterologous phosphoketolase/phosphotransacetylase shunt to improve the supply of NADPH and acetyl-CoA in a strain background with abolished OA degradation. We show that these modifications lead to an increased yield of OA during the consumption of glucose by more than 60% compared to the parental strain. Furthermore, we investigated different genetic engineering targets to identify potential factors that limit the OA production in yeast. Toxicity assays performed with the engineered strains suggest that the inhibitory effects of OA on cell growth most likely impose an upper limit to attainable OA yields.

1. Introduction

Fatty acids (FA) with various chain lengths and their derivatives with different functional groups are important compounds in modern industry, which have numerous applications like fuels, cosmetics, pharmaceuticals and food additives. Recently, engineering microbial FA production has attracted attention as an alternative to established methods such as petrochemistry or oil palm cultivation, which have lately been criticized for their environmental impact.

In *Saccharomyces cerevisiae*, biosynthesis of FA in the cytosol is catalyzed by the large multidomain fatty acid synthase (FAS) complex, which naturally generates long-chain FA (LCFA, C14-C18) as building blocks of membranes or for storage lipids. The biosynthesis is initiated by cytosolic acetyl-CoA (AcCoA) and maturing FAs are elongated by AcCoA-derived malonyl-CoA, until reaching their final length. NADPH is required during the process for reductive power (for review, see (Baumann et al., 2020). In *S. cerevisiae*, AcCoA is compartmentalized in the cytosol, mitochondria, and peroxisomes and there is no free exchange of the intermediate across organelle membranes(Chen et al., 2012; Krivoruchko et al., 2015). The majority of cytosolic AcCoA for FA biosynthesis is generated by the cytosolic pyruvate dehydrogenase bypass (PDH-bypass), in which pyruvate is decarboxylated to acetaldehyde by the pyruvate decarboxylases followed by conversion of acetaldehyde to acetate by aldehyde dehydrogenases. Finally, acetate is ligated to CoA by acetyl-CoA synthetase at the expense of two ATP equivalents (van Rossum et al., 2016).

For improved production of various AcCoA-derived products, engineering the precursor supply was the approach of choice and many studies targeted an increased flux through the PDH-bypass (de Jong et al., 2014; Krivoruchko et al., 2013; Li et al., 2014; Lian et al., 2014; Shiba et al., 2007). Due to the Crabtree-effect in yeast, the largest fraction of acetaldehyde is converted into ethanol by dominant alcohol dehydrogenases even under aerobic conditions, which competes with the aldehyde dehydrogenase reaction (Nielsen, 2014) and thereby decreases cytosolic AcCoA formation from pyruvate. Indeed, it was demonstrated that deletion of the dominant alcohol dehydrogenase gene *ADH1* improves LCFA production (Li et al., 2014). Other strategies relied on the overexpression of PDH-pathway genes, which resulted in increased carbon flux through the PDH-bypass bypass (de Jong et al., 2014; Krivoruchko et al., 2013; Li et al., 2014; Lian et al., 2014; Shiba et al., 2007). Increased acetate and NADPH accumulation was achieved by overexpressing the aldehyde dehydrogenase *ALD6*. Additionally, expression of a heterologous acetyl-CoA synthetase with an L641P substitution ($^{Se}ACS^{L641P}$) to prevent its inactivation by acetylation (Starai et al., 2005) proved beneficial compared to the overexpression of endogenous variants (Shiba et al., 2007). In a subsequent study targeting FA ethyl ester (FAEE) production, concomitant overexpression of the alcohol dehydrogenase *ADH2* to increase acetate formation from ethanol, *ALD6* and $^{Se}ACS^{L641P}$ led to increased product formation (de Jong et al., 2014). Very recently, the AcCoA and NADPH supply were engineered by introducing a heterologous ATP-citrate lyase, a mitochondrial citrate transporter, a cytosolic malate dehydrogenase and a cytosolic malic enzyme to optimize the production of LCFA (Yu et al., 2018), short and medium chain FA (SMCFA) (Zhu et al., 2020) and FA derivatives (Zhou et al., 2016). These modifications were introduced in addition to the endogenous PDH bypass.

Van Rossum et al (van Rossum et al., 2016) calculated the theoretical maximum yields for palmitic acid (C16) production via different AcCoA providing pathways. The highest FA yield was predicted for a heterologous phosphoketolase (PK)/phosphotransacetylase (PTA) pathway, when NADPH formation occurs via the pentose phosphate pathway (PPP). In this scheme, glucose-6-P is converted to xylulose-5-P by four sequential reactions catalyzed by glucose-6-P-dehydrogenase (Zwf1), 6-phospogluconolactonase (Sol3), 6-phosphogluconate dehydrogenase (Gnd1) and ribulose-5-P-epimerase (Rpe1). In the two dehydrogenase reactions NADPH is formed, resulting in two moles NADPH for one mole of xylulose-5-P. Cytosolic AcCoA is then provided by the consecutive reactions of a xylulose-5-P specific PK (xPK) which converts xylulose-5-P to acetyl-P (AcP) and glyceraldehyde-3-P and conversion of acetyl-P to AcCoA by a PTA. Expression of the xPK/PTA pathway proved to increase formation of FAEE (de Jong et al., 2014) and, more recently, of farnesene (Meadows et al. 2016). In the latter study, it was shown that AcP formed by PK can be hydrolyzed by endogenous glycerol-3-P phosphatases (*GPP1* alias *RHR2*) and deletion of *GPP1* is therefore beneficial.

To the best of our knowledge, engineering precursor and cofactor supply via the PPP/xPK/PTA pathway has not yet been employed for FA production. For the proof of concept, we targeted the

production of octanoic acid (OA), an eight-carbon (C8) FA. Production of OA (and other SMCFA) can be achieved by a minimal invasive strategy through mutations in the FAS enzyme, which favor premature termination of the FA elongation cycle (Gajewski et al., 2017). For instance, a single R1834K substitution in the malonyl-palmitoyl transferase domain of Fas1 (herein referred to as FAS^{RK}) allows for a favored biosynthesis of OA.

Here, we investigated if PDH-bypass engineering and PK/PTA pathway expression are applicable to increase OA production by FAS^{RK} in *S. cerevisae*. The targets for employed genetic engineering are summarized in Fig. 1. We show that known PDH-bypass engineering targets have no positive effect on OA production, whereas the expression of xPK/PTA in combination with interventions that increase the flux through the oxidative PPP improve the production of OA.

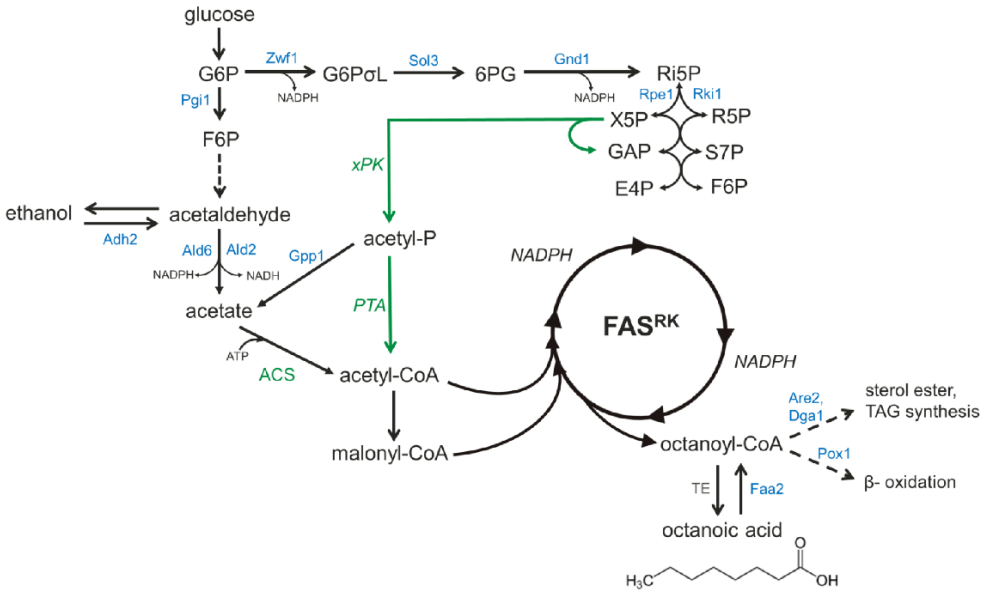

Figure 1: Overview of genetic engineering targets. The metabolic intermediates and enzymes relevant for this work are shown. Dashed lines depict multiple metabolic steps. In blue the endogenous enzymes, whose genes were overexpressed, knocked-down or knocked out are shown as described in the Result section. Heterologously expressed enzymes are shown in green (xPK, xylulose-5-P specific phosphoketolase; PTA, phosphotransacetylase; ACS, acetyl-CoA synthetase). The multiple endogenous enzymes with thioesterase activity are depicted with a generic abbreviation (TE).

2. Results and Discussion

2.1 Base strain construction and elimination of SMCFA degradation. In our previous work on OA production by a mutated FAS in *S. cerevisae* we have been using the FAS deficient strain SHY34 (*Δfas1Δfas2Δfaa2)*, derived from the BY-series (Wernig et al., 2020). However, SHY34 depends on supplementation of FA like oleic acid, since *FAS1* and *FAS2* genes are essential (Giaever et al., 2002) and their knockout leads to auxotrophy for LCFA, which are necessary for cell survival. Although growth can be restored by LCFA (e.g. oleic acid) supplementation, *FAS* gene knockout resulted in strongly reduced growth rate and a lower final cell density compared to a BY4741 WT strain (Fig. 2A). Due to these characteristics, handling of the strain is difficult, resulting in low transformation efficiencies and extended experimental times, which is an undesired trait for a biotechnological application. When the FAS deficient strain is transformed with a plasmid for production of OA (SHV61 carrying *FAS1*^{RK}*/FAS2),* oleic acid supplementation becomes redundant due to leaky production of LCFA (Gajewski et al., 2017), but growth is still strongly impaired (Fig. 2B). Therefore, we sought to generate a superior OA producer strain by eliminating these drawbacks. We chose the strain CEN.PK2-1C, belonging to the popular CEN.PK-series that is used for both academic (e.g. (Van Dijken et al., 2000) and industrial applications (e.g. (Meadows et al., 2016) as a parental strain. Using CRISPR/Cas9, we introduced a mutation encoding the amino acid exchange R1834K into the genomic *FAS1*. To increase expression of the FAS genes, we exchanged the endogenous promoters of both *FAS1* and *FAS2* by the strong *pHXT7*$^{-1--392}$ promotor (Hamacher et al., 2002). The resulting strain VGY2 is simpler in handling (such as for plasmid transformations), has twice the maximum growth rate of SHY34 + *FAS1*^{RK}*/FAS2* (0.65 ± 0.08 vs. 0.33 ± 0.05) and grows to higher final cell densities (Fig. 2B). To eliminate degradation of SMCFA, which was observable after longer incubation periods (48 h - 72 h) of VGY2 (Supplementary Fig. S1), we knocked out the medium chain fatty acyl-CoA synthetase *FAA2* (Henritzi et al., 2018; Leber et al., 2016), resulting in strain LBY38. LBY38 maintained OA amounts constant even 72 h after inoculation (Supplementary Fig. S1) and accumulated increased OA amount compared to its precursor strain VGY2 (Fig. 2C).

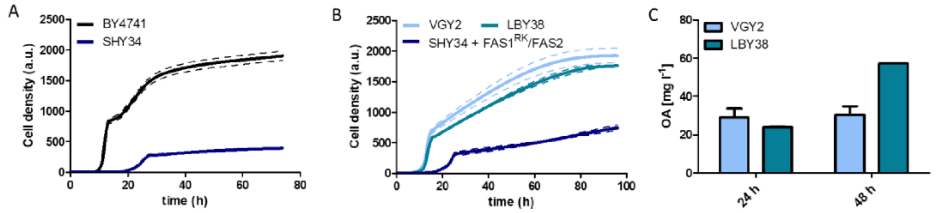

Figure 2: Growth behavior and octanoic acid production of strains expressing a mutated fatty acid synthase.
In (A), growth of the BY-derived SHY34 strain (Δ*fas1*Δ*fas2*Δ*faa2*) is compared to the BY4741 wildtype in YPD media supplemented with oleic acid. In (B) growth of strains VGY2 (*ΔpFAS1::pHXT7*$^{1-392}$*::FAS1*^{R1834K}, *ΔpFAS2::pHXT7*$^{1-392}$*::FAS2*), LBY38 (VGY2 Δ*faa2*) and SHY34 transformed with plasmid SHV61 (FAS1^{RK}/FAS2) in YPD medium without oleic acid supplementation is shown. Growth analyses were performed on a cell growth quantifier (Aquila Biolabs). The curves represent the mean (solid lines) and standard deviation (dashed lines) of three biological replicates. Octanoic acid production of strains VGY2 and LBY38 grown in phosphate buffered YPD medium is shown in (C). The data represent mean and standard deviation of two biological replicates.

FA biosynthesis depends on cytosolic AcCoA and derived malonyl-CoA as precursor and elongation units, respectively, and utilizes NADPH as reduction equivalent. To increase production of OA by a mutated FAS, we aimed to enhance the precursor and cofactor supply and investigated the PDH-bypass as a first engineering target in the LBY38 background. Although decreased alcoholic fermentation, achieved by deletion of the main alcohol dehydrogenase *ADH1,* proved to be beneficial for LCFA biosynthesis (Li et al., 2014) we rejected this approach for OA production, considering its strong negative effect on cell growth rates (Paquin and Williamson, 1986), which we also observed in preliminary experiments that resulted in very low OA titers. Instead, we decided to enhance the utilization of ethanol (by overexpressing *ADH2*) and the PDH bypass (by overexpressing *ALD6* and $^{Se}ACS^{L641P}$) since this strategy was reported to improve the biosynthesis of LCFA-derived (de Jong et al., 2014). However, neither the overexpression of *ADH2* alone nor in combination with *ALD6* and $^{Se}ACS^{L641P}$ had a positive effect on OA production (Supplementary Fig. S2). On the contrary, when the cells were transformed with the $^{Se}ACS^{L641P}$/*ALD6* plasmid, growth and OA titers decreased (Supplementary Fig. S2A,B), possibly due to accumulation of toxic acetate as a consequence of increased Ald6 activity (Shiba et al., 2007). The specific OA titers (defined as OA production over OD_{600}), which were calculated to take the tradeoff between growth and production into account (Supplementary Fig. S2C), were comparable to the empty vector control, indicating that the decrease in titers was mainly due to the growth defect. It is noteworthy that LBY38 transformed with two empty high copy plasmids (2µ origin) accumulated about 50 % less OA in comparison to plasmid-free LBY38 (compare Fig. 2C at 48 h and Fig. 3B), possibly due to plasmid burden effects (Karim et al., 2013).

Improvements in FA production have been previously reported for deletion of the acyl-CoA oxidase (*POX1*) gene, catalyzing the first step of β-oxidation (Leber et al., 2016; Runguphan and Keasling, 2014) and for the knockouts of the non-essential storage lipid formation of steryl esters (AcCOA sterol acyltransferase, *ARE2*) or triacylglycerols (diacylglycerol acyltransferase, *DGA1*) (Valle-Rodríguez et al., 2014). Deletion of *POX1* in LBY38 increased OA titers in the resulting strain FWY32

(Supplementary Fig. S3). In contrast, in FWY32-derived FWY40 (Δ*are2*) or FWY41 (Δ*dga1*) no further positive effect was observed (Supplementary Fig. S3), suggesting that Are2 and Dga1 are specific for LCFA and do not utilize OA as substrate. Thus, FWY32 was used to further investigate the influence of precursor and cofactor supply engineering strategies on OA production.

2.2 Re-routing the flux of glucose through the oxidative pentose phosphate pathway and expression of a heterologous phosphoketolase/phosphotransacetylase shunt

In our previous work and initial experiments described above, we observed that OA production mainly occurs after exhaustion of glucose, i.e. during the utilization of ethanol. This can be readily explained by the fact that ethanol catabolism must occur via AcCoA formation, whereas only a minor fraction of pyruvate produced from glucose is entering the PDH bypass (Pronk et al., 1996). The abovementioned observation that overexpressing *ADH2*, $^{Se}ACS^{L641P}$, and *ALD6* does not lead to increased OA titers (Supplementary Fig. S2), suggests that the capacity of the endogenous PDH bypass enzymes is not limiting and low productivity on glucose is rather due to inherent physiological constraints. We therefore reasoned that a higher yield of OA on glucose – which is highly desirable from a biotechnological prospective – could be achieved by modifying the sugar metabolism. Among different alternative pathways for AcCoA (and NADPH) supply, the highest yields of FA were predicted for concomitantly rerouting glucose flux to the oxidative PPP (for NADPH production) and expressing heterologous xPK and PTA (van Rossum et al. 2016; see introduction). We therefore decided to test this scheme for OA production.

The Zwf1/Gnd1 (oxidative PPP) and Ald6 catalyzed reactions are the main source for NADPH supply so that a *zwf1 ald6* double deletion mutant is not viable (Grabowska and Chelstowska, 2003). To enhance the metabolic flux over the PPP and increase NADPH formation, overexpression of *ZWF1* alone is not sufficient (Kwak et al., 2019; Yu et al., 2018). Therefore, we forced the metabolism into the direction of the PPP by deleting the *ALD6* gene in strain FWY32, leaving the Zwf1 reaction as the only source of NADPH. Next, we overexpressed *ZWF1* by exchanging its promoter by the strong $pHXT7^{-1—392}$ (Hamacher et al., 2002). To increase the availability of the Zwf1 substrate glucose-6-P, we exchanged the promotor of the phosphoglucoseisomerase gene (*PGI1*) by the weak *pCOX9*, as described previously (Yu et al., 2018). This approach is advantageous over *PGI1* deletion, which causes a severe growth phenotype (Aguilera, 1986). The resulting stain FWY38 harboring the combined strategy of *ALD6* deletion, *PGI1* knock-down and *ZWF1* overexpression (denoted as PPP flux in the figures) showed slightly reduced growth (Fig. 3A), comparable OA titers to parental strain LBY38 (Fig. 3B) but increased specific titers of OA (Fig. 3C).

Several different PKs have been functionally expressed in *S. cerevisae* before (Bergman et al., 2016; de Jong et al., 2014; Meadows et al., 2016). We genomically integrated the PK gene of *Clostriudium acetobutylicum*, which showed highest specific activity for xylulose-5-P (xPK) in a previous report

(Bergman et al., 2016), and a PTA gene from *Bacillus subtilis* (^{Bs}PTA) (de Jong et al., 2014) into the *GPP1* locus of strains FWY32 and FWY38. Thereby, *GPP1*, which encodes for a glycerol-3-P-phosphatase and is able to dephosphorylate acetyl-P produced by a PK (Meadows et al., 2016), was deleted. The resulting strain FWY43 showed 18 % increased OA titers (Fig. 3B) and about 33 % increased specific titers compared to strain LBY38 (Fig. 3C). Combined strategies of PPP flux increase and xPK/PTA expression (strain FWY45) led to an increase in OA titers by 29 % (Fig. 3B) and in specific titers by about 45 % (Fig. 3C) compared to parental strain LBY38. Besides ^{Bs}PTA expression, we chose to test and compare an additional promising PTA *eutD* from *Salmonella enterica* (^{Se}*EutD*) (Brinsmade and Escalante-Semerena, 2004), but the titers of OA did not differ between the variants (Supplementary Fig. S4).

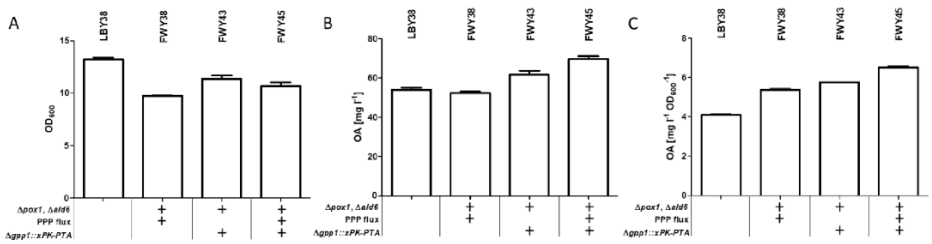

Figure 3: Increasing OA production by PPP and xPK/PTA pathway engineering. Growth (as final OD_{600}, A), OA titers (B) and specific OA titers (normalized to OD_{600}, C) of engineered strains are shown. Strains were grown in potassium phosphate buffered YPD media and samples were taken after 48 h of fermentation. Values and error bars represent mean and standard deviation of two biological replicates.

2.3 Growth phase and carbon source dependent production of OA.

As explained above, one of the premises of redirecting carbon flux via the PPP and xPK/PTA pathway was to increase OA yield during the glucose consumption phase. To investigate this hypothesis, we analyzed the growth phase-dependent production of OA (Fig. 4) and compared the parental strain LBY38 (Fig. 4A) with strains expressing the xPK/PTA pathway FWY43 (Fig. 4B) and FWY45 (Fig. 4C). Interestingly, about two-thirds of total OA are produced, regardless of the strain background, in the late phase of fermentation during ethanol consumption. This behavior is in accordance with production of LCFA in yeast, which can be increased in late growth phases due to loss of competition between production and biomass formation (Yu et al., 2018). To get a clearer insight into the flux distribution changes by the introduced genetic modifications, we calculated the yields of OA (mol OA per mol AcCoA equivalents) at late stages of glucose (16.5 h) and ethanol consumption (48.0 h) (Fig. 4D). Yields achieved by the engineered strains FWY43 and FWY45 increased by about 25% on ethanol but even more considerably by approximately 65% during the glucose consumption phase (Fig. 4D) in comparison to LBY38, which is consistent with the expectation. The increased yields of OA on ethanol compared to LBY38 despite the deletion of *ALD6* in FWY43 and FWY45 could be explained by overcompensation of NADPH supply via the enhanced Zwf1 reaction in these strains, whereas acetate for AcCoA can still be produced by the (NAD^+-dependent) Ald2.

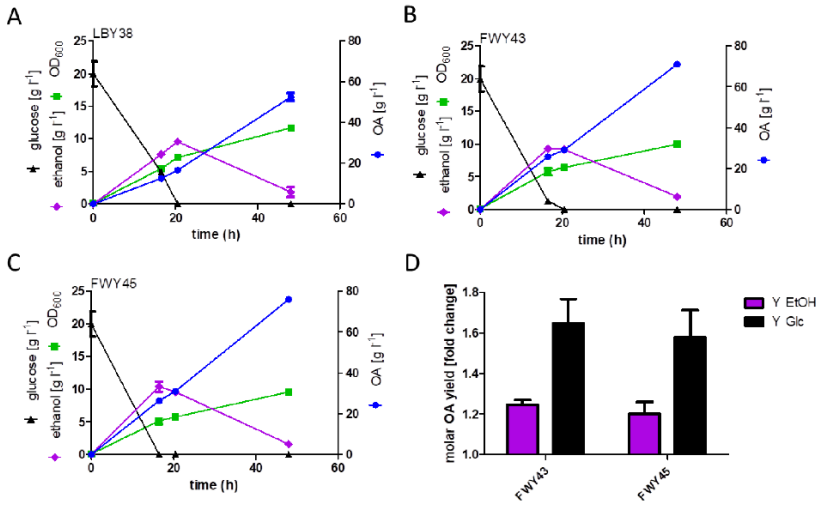

Figure 4: Growth phase-dependent production of octanoic acid in strains LBY38, FWY43 and FWY45. Strains LBY38 (A), FWY43 (B) and FWY45 (C) were grown in potassium phosphate buffered YPD media. Samples for OA, glucose and ethanol measurements were taken at indicated time points. (D) Yields of OA per theoretical AcCoA equivalents from consumed glucose or ethanol were calculated as fold change over the parental strain LBY38. Values and error bars represent mean and standard deviation of two biological replicates.

Despite the higher relative yield on glucose, the strains FWY43 and FWY45 still produce two-thirds of OA during the ethanol consumption. We therefore investigated whether cultivating the cells on ethanol as sole carbon source could increase the OA production. In the course of glycolysis and PDH-bypass, one mole of consumed glucose theoretically results in formation of two moles AcCoA (two AcCoA equivalents) while catabolism of one mole ethanol via PDH-bypass results in one mole AcCoA equivalents. To account for this, strains LBY38 and FWY45 were cultivated in double molar amounts of ethanol (corresponding to 9.3 g l^{-1}) compared to glucose (corresponding to 20 g l^{-1}). Both strains showed barely any growth and OA production with our standard cell inoculum (starting OD_{600} 0.1) (Supplementary Fig. S5), although the used ethanol concentration is known to be sufficient for growth of a wildtype strain. As a known enhancer of OA toxicity (Legras et al., 2010), ethanol in combination with intrinsic OA production apparently has a strong negative effect on cell proliferation. OA production became detectable by increasing the cell inoculum of fermentations (high OD fermentation, starting OD_{600} of 8.0), but titers remained below those with glucose as carbon source (Supplementary Fig. S5).

2.4 Systematic analysis of reactions limiting OA production.

Expression of the xPK/PTA pathway and enhanced PPP-flux successfully increased OA biosynthesis, but various reactions in the pathway could be limiting OA titers. Hence, we systematically analyzed potential targets in the pathway. Higher accumulation of xylulose-5-P and following reaction of xPK/PTA towards AcCoA were targeted by overexpression of ribulose-5-P-epimerase *RPE1*, which was achieved by exchanging its native promoter by the strong *pPFK1*, and integration of a second genomic copy of the heterologous xPK / PTA cassette in strain FWY45. Resulting strains (FWY47 and FWY48, respectively) did not show changed growth or production compared to parental strain FWY45 (Fig. 5A,B). In strain FWY45 the gene encoding the NADPH dependent aldehyde dehydrogenase Ald6 is deleted, leaving the NAD^+-dependent Ald2 as the main aldehyde dehydrogenase of the PDH-bypass. To create a stronger driving force for rerouting carbon flux to the PPP/xPK/PTA pathway, *ALD2* gene was additionally deleted in the strain FWY45, thereby decreasing AcCoA production via the PDH bypass. However, the new strain FWY50 showed slightly reduced OA titers compared to the parental strain FWY45 (Fig. 5B), demonstrating that some flux through PDH-bypass is important even when xPK/PTA is expressed. Furthermore, we reasoned that, even if OA formation increases in the strain FWY45 (mainly) during the glucose growth phase, this advantage should be diminished after diauxic shift from glucose to ethanol consumption. For increased metabolic flux and NADPH formation during ethanol degradation, we re-integrated *ALD6* under the control of the glucose repressed *pADH2* into FWY45. However, the resulting strain FWY58 did not shown any increase in OA compared to the other strains (Fig. 5B).

We next targeted further up and downstream reactions of OA biosynthesis. This includes the oxidative part of PPP consisting of three reactions catalyzed by Zwf1, Gnd1 and Sol3. To evaluate whether a limitation originates from one of these reactions, we concomitantly overexpressed *ZWF1*, *GND1* and *SOL3* from a high copy plasmid with strong promoters (module named oxPPP (plasmid FWV171)) in FWY45. To test a possibly limiting role of FAS, we overexpressed a superior mutated FAS variant ($fusFAS^{RK}$), consisting of a fusion construct of $FAS1^{RK}$ and *FAS2* (Wernig et al., 2020) in addition to the oxPPP module or separately. Surprisingly, additional expression of $fusFAS^{RK}$ in strain FWY45 did not influence OA production nor did the combined expression of oxPPP and $fusFAS^{RK}$ (Fig. 5D). Hence, although the individual expression of $fusFAS^{RK}$ (Wernig et al., 2020) and PPP/xPK/PTA (Fig. 3) increase OA production, these effects appear not to be additive.

Collectively, the results presented here suggest that some intrinsic factor(s) limit(s) higher OA accumulation. One possibility is the strong inhibitory effect of OA on yeast growth (Alexandre et al., 1996; Borrull et al., 2015; Henritzi et al., 2018; Legras et al., 2010; Viegas et al., 1989; Wernig et al., 2020). SMCFA inhibitory effects are attributed to different mechanisms. They act as weak acids, which enter the cell in a protonated form by passive diffusion and dissociate intracellularly, thereby acidifying the cytosol (Cabral et al., 2001; Viegas and Sá-Correia, 1997). This triggers the activity of

H^+-ATPase (Cabral et al. 2001), causing a strong energetic effort. Additionally, OA is known to disturb the integrity of the plasma membrane and cause its leakiness (Borrull et al., 2015; Liu et al., 2013). The membrane related stress can be counteracted by supplementation of LCFA like oleic acid

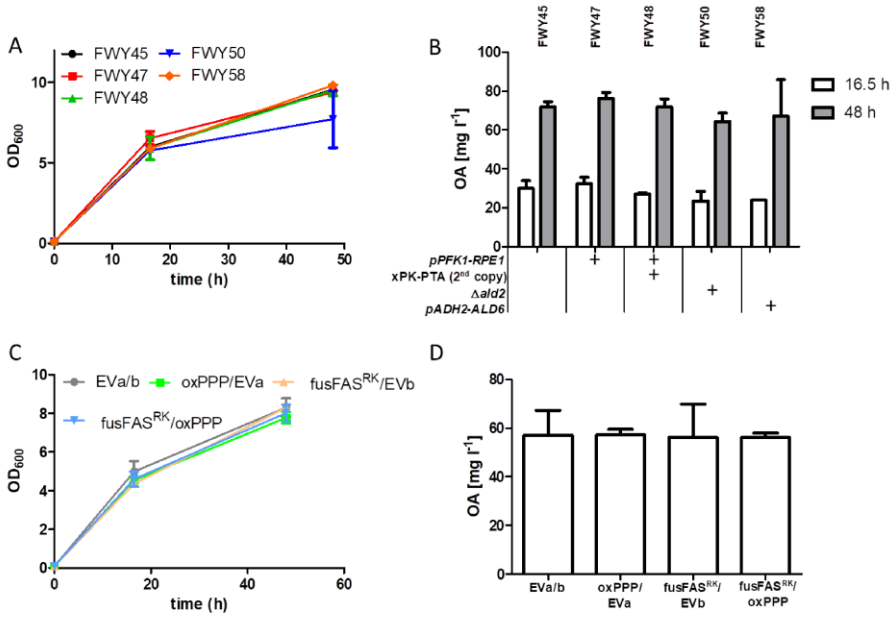

Figure 5: Systematic investigation of reactions that potentially limit AcCoA supply. Growth (A and C) and OA production (B and D) of engineered strains (A and B) or strain FWY45, which was transformed with plasmids FWY169 (oxPPP) for overexpression of three enzymes of the oxidative PPP (P_{TEF2}-*ZWF1*, P_{CCW12}-*SOL3*, P_{TEF1}-*GND*) and/or with the plasmid FWY133 (*pFAS1*-fusFAS^{RK}) for overexpression of an additional mutated FAS copy (C and D) are shown. Appropriate empty vectors (Ev a and b) were used for controls. Strains were grown in in potassium phosphate buffered YPD media and samples were taken after 16.5 (late glucose consumption phase) or 48 h (late ethanol consumption phase) (B) or after 48 h (D). Values and error bars represent the mean and standard deviation of two biological replicates.

(Besada-Lombana et al., 2017; Liu et al., 2013), which increase membrane stability. To test the sensitivity of engineered strains to external OA, we exposed LBY38 and FWY45 as well as the wildtype CEN.PK2-1C to increasing OA concentrations in microtiter plates and measured the growth of the cells as a function of OA concentration. As shown in Fig. 6 the producer strains are almost completely growth-inhibited already at 200 mg l^{-1} of supplemented OA in contrast to the wildtype cells. This observation can be readily explained by (i) the additive toxic effect of internally produced and external OA, (ii) compromised synthesis of LCFA and (iii) the inability of the producer strains to degrade OA (due to the *faa2/pox1* deletions).

This suggests that the inhibitory effect of OA might be the main limitation for higher OA production and hinder an additive effect of different engineering strategies. However, other limiting factors cannot be ruled out at present.

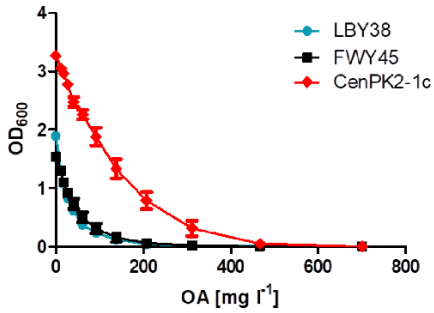

Figure 6: Strain robustness of strains LBY38, FWY45 and CEN.PK2-1c towards ocanoic acid. Strain were grown in 96-well plates in different concentration of supplemented octanoic acid and cell growth was analyzed by OD_{600} measurement in a plate reader after 18 h. Values show mean and standard deviation of three biological replicates.

Conclusion

In this work, we engineered a robust, CEN.PK-based strain for OA production that has clear advantages in respect to handling and genetic manipulation in comparison to the previously developed BY-derived strains. We demonstrated that engineering of precursor supply routes for cytosolic AcCoA and NADPH are important targets to increase the production of OA by mutant fatty acid synthases. To the best of our knowledge, the heterologous xPK/PTA in combination with increased flux over PPP was tested for the first time in the context of fatty acid production in yeast. This strategy is favorable over PDH-bypass engineering, especially for obtaining higher yields of OA from glucose, which is consistent with theoretical considerations and a desirable trait from an industrial point of view. However, our experiments show that further improvements of OA yields are likely constrained by product toxicity. Therefore, improving strain robustness or process development for *in situ* product removal will be important challenges to reach industrial-scale OA production in the future.

Materials and Methods

Strain construction and transformation

Yeast strains used in this study are listed in Table 1. CRISPR/Cas9 was used to modify strains with deletions, integrations and exchange of promoters as described previously (Generoso et al., 2016). For this, yeast strains were transformed with the CRISPR/Cas9 plasmid encoding Cas9 and guide RNA together with an appropriate donor DNA (synthetic double-stranded DNA for deletions, PCR-amplified expression cassettes or promotors for integrations). Donor DNA carried at least 30 bp overhangs to the desired locus. Specific gRNA sequences were selected with the freely availably tool (https://www.atum.bio/eCommerce/cas9/) to cut in the desired gene or promoter region. Gene deletions of *POX1*, *ALD6*, *ALD2*, *ARE2* and *DGA1* and integrations into the gene loci of *GPP1* and *URA3* were carried out by removing the entire gene ORF. The native promoters of *ZWF1* (from -500 bp to 0 bp), and *PGI1* (from –405 bp to 0 bp) were replaced by the truncated $pHXT7^{1-392}$ (amplified from plasmid pRS62-K) and *pCOX9* amplified from genomic DNA of CEN.PK2-1C, respectively. For construction of strain FWY47 a *pPFK1-RPE1-tRPE1* cassette was amplified from the plasmid pHD8 (Demeke et al., 2013) and integrated into the *URA3* locus. ^{Ca}xPK (Bergman et al., 2016) and either ^{Bs}PTA (de Jong et al. 2014) or ^{Se}EutD (Brinsmade and Escalante-Semerena, 2004) where amplified from plasmids FWV163, FWV164 or FWV165 and integrated into the *GPP1* locus, which was thereby deleted. Oligonucleotides used for amplification or deletions (synthetic double-stranded DNA) are listed in Table S3. Relevant genes sequences are listed in the Supplementary Information. Transformations were performed following the frozen competent cell protocol (Gietz and Schiestl, 2007), whereas SHY34 was transformed by a slightly modified method previously described (Gajewski et al., 2017). Transformed yeasts were plated on solid YPD (2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose) containing appropriate antibiotics hygromycin (100 mg l^{-1}) or G418 (200 mg l^{-1}) for plasmid selection and grown at 30 °C for two to four days.

Table 1: Yeast strains used in this study

Strain name	Relevant genotype	Reference/ Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al., 1998
SHY34	*MATa ura3Δ0 his3Δ0 leu2Δ0 TRP1 lys2Δ0 MET15 Δfas1 Δfas2 Δfaa2*	Wernig et al., 2020
CEN.PK2-1C	*MATa; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2*	Euroscarf, Germany
VGY2	CEN.PK2-1C *pFAS1:: $pHXT7^{1-392}$-$FAS1^{R1834K}$*, *pFAS2::$pHXT7^{1-392}$-FAS2*	This study
LBY38	VGY2 Δ*faa2*	This study
FWY32	LBY38 Δ*pox1*	This study
FWY36	FWY32 Δ*ald6*	This study
FWY37	FWY36 *pZWF1::$pHXT7^{1-392}$*	This study

FWY38	FWY37 *pPGI1::pCOX9*	This study
FWY40	FWY32 Δ*are2*	This study
FWY41	FWY32 Δ*dga1*	This study
FWY45	FWY38 Δ*gpp1::*^{Ca}*xPK-*^{Bs}*PTA*	This study
FWY46	FWY38 Λ*gpp1::*^{Ca}*xPK-EutD*	This study
FWY47	FWY45 Λ*ura3::pPFK1-RPE1-tRPE1*	This study
FWY48	FWY47 Λ*faa2::* ^{Ca}*xPK-*^{Bs}*PTA*	This study
FWY50	FWY45 Δ*ald2*	This study
FWY58	FWY45 Δ*ura3::pADH2-ALD6*	This study

Plasmid construction

Plasmids were constructed via homologous recombination in yeast (Oldenburg et al., 1997) or Golden Gate cloning (Lee et al., 2015) (PTA genes) by standard procedures. Plasmid fragments were amplified by PCR using oligonucleotides listed in Supplementary Table S2. The assembled plasmids were propagated in and extracted from *E. coli* DH10B by standard procedures. For construction of FWV171 the dominant marker *hphNT1* was completely replaced by *kanMX4* in plasmid TWRV1 by amplification of *kanMX4* from plasmid pRS52-K and insertion into the NdeI cut site of *hphNT*. ^{Ca}xPK, ^{Bs}PTA and ^{Se}EutD were provided by Dr. Arun Rajkumar, cloned into the PYTK001 backbone of the Golden Gate system. ^{Ca}xPK was sub-cloned into the plasmid pRS62-H by homologous recombination. ^{Bs}PTA and ^{Se}EutD under the control of *pHHF1* and *tSSA1* by Golden Gate cloning (Lee et al., 2015).

Media and cultivation

If not stated otherwise, *Saccharomyces cerevisiae* liquid cultures were inoculated to an OD_{600} of 0.1 and grown in shake flasks at 30 °C and 180 rpm in YPD medium without supplementation of free FA or with supplementation of oleic acid (0.5 mM and 1 % (v/v) Tergitol NP-40 solution Sigma Aldrich, Germany) for the FAS-deficient strain SHY34. For maintaining plasmids with *hphNT1* or *kanMX4* marker appropriate antibiotics hygromycin (100 mg l^{-1}) or G418 (200 mg l^{-1}) were used. The medium was additionally buffered with 100 mM potassium phosphate and adjusted to a pH of 6.5. If not stated otherwise, glucose was used as a carbon source at concentrations of 20 g l^{-1}. Samples for compound extraction were taken at given time points. Growth was either monitored by measurement of optical density at 600 nm (OD_{600}) or online by use of a Cell Growth Quantifier, Aquila Biolabs (Bruder et al., 2016).

Compound extraction and derivatization

Extraction of free fatty acids in the culture medium was performed as described before (Henritzi et al., 2018). Cells were separated from the medium by centrifugation (3,500 rcf, 10 min) and 10 ml of culture supernatant was mixed with an internal standard (0.2 mg heptanoic acid), 1 ml of 1M HCl and 2.5 ml of methanol:chloroform (1:1) solution. After phase separation (3,000 rcf, 5min) the organic phase layer was taken and evaporated in a vacuum concentrator (Concentrator 5301, Eppendorf, Germany). Fatty acids were methylated for GC analysis as described before (Ichihara and Fukubayashi, 2010). The extract was dissolved in 200 µl toluene, mixed with 1.5 ml of methanol and 300 µl of 8.0% (w/v) HCl solution and incubated at 100 °C for 3 h to form fatty acid methyl esters (FAME). FAMEs were extracted from the mixture by addition of 1 ml H_2O and 1 ml hexane. The organic phase was taken for gas chromatography analysis.

3.5 Gas chromatography

The gas chromatography analysis was performed on a Perkin Elmer Clarus 400 system (Perkin Elmer, Germany) equipped with an Elite-5MS capillary column (Ø 0.25 mm; length 30 m; film thickness 1.00 µm) and a flame ionization detector (Perkin Elmer, Germany). 1 µL of sample was analyzed after split injection (1:10) and helium was used as carrier gas (90 kPa). For FAME quantification, the temperatures of the injector and detector were set to 200 and 250 °C, respectively. The following temperature program was applied: run time 42.67 min, start at 50 °C and hold for 5 min; ramp at 10 °C min to 120 °C and hold for 5 min, ramp at 15 °C to 220 °C and hold for 10 min, ramp at 20 °C to 300 °C and hold for 5 min. FAMEs were identified and quantified by comparison with authentic standard substances.

Quantificaion of glucose and ethanol by HPLC

Samples for analysis were centrifuged (16,000 rcf, 5 min) and 450 µl of the supernatant was mixed with 50 µl of 50 % (w/v) 5-sulphosalicylic acid for protein precipitation. The supernatant was analyzed by a HPLC (Dionex, Thermo Fisher Scientific, G) equipped with a HyperREZ XP Carbohydrate H^+ column (300 × 700 mm, 8 micron; Thermo Fisher Scientific, Germany) at 30 °C. 0.5 mM H_2SO_4 was used as mobile phase with constant flow of 0.6 ml min^{-1}. Metabolites were identified and quantified by use of authentic standards.

2.4 Octanoic acid toxicity assay

For the toxicity assay a pre-culture of the strains was inoculated into fresh YPD medium to an OD_{600} of 0.2 and cultivated for 5-6 h until an OD_{600} of 0.8 -1.0 was reached. The culture was re-diluted in YPD medium to an OD_{600} of 0.05 and 50 µl was used to inoculate 200 µL of YPD medium in 96-well plates with a dilution series of OA. OA was dissolved in YPD medium at the highest concentration, filtered for sterility (0.2 µm) and subsequently diluted to desired concentrations. Cultivations were

performed for 18 h at 30 °C without agitation and cells were mixed thoroughly before OD_{600} measurement in a plate reader (ClarioStar, BMG Labtech, Germany).

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Author contributions and acknowledgements

F.W. and L.B. performed the experiments, analyzed the data and drafted the manuscript. E.B. and M.O. were involved in the experimental design. M.O. guided the project and finalized the manuscript, which was approved by all authors. We thank Arun Stephen Rajkumar for providing plasmids pB14, pB15 and pB20 and Sandra Born for providing strain VGY02.

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Supplementary information

Octanoic production in *Saccharomyces cerevisiae*: Investigation of new precursor supply engineering strategies and intrinsic limitations

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Table S1: Plasmids used in this study

Laboratory stock code	Plasmid name	Relevant elements	Reference
ASB13	pRS41-H	*CEN6/ARS4,, AmpR, hphNT1*	Taxis and Knop., 2006
ASB16	pRS42-H	*2micron, AmpR, hphNT1*	Taxis and Knop., 2006
ASB20	pRS52-K	*2micron, AmpR, kanMX4,*	Boles lab stock
ASB22	pRS62-H	*2micron, AmpR, hphNT1*	Boles lab stock
ASB23	pRS62-K	*2micron, AmpR, kanMX4,*	Farwick et al., 2014
AB02	pRCC-N-POX1	*2µ, AmpR, natNT2, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[POX1]-tSUB4*	This study
MR02	pRCC-K-URA3	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[URA3]-tSUB4*	Boles lab stock
HDV10	pHD8	*2µ, AmpR, kanMX4, pPFK1-RPE1-tRPE1*	Demeke et al., 2013
FWV169	prRS52-K-oxPPP	*2µ, AmpR, kanMX4, pTEF2-ZWF1-tZWF1, pCCW12-SOL3-tSOl3, pTEF1-GND1-tGND1*	This study
FWV133	pRS313H-fusFAS^{RK}	*CEN6/ARS4, AmpR, hphNT1, pFAS1-FAS1^{R1834K}-FAS2-tFAS2*	Wernig et al., 2020
SHV61	pRS315-fusFAS^{RK}	*CEN6/ARS4, AmpR, LEU2, pFAS1-FAS1^{R1834K}-tFSA1, pFAS2-FAS2-tFAS2*	Wernig et al., 2020
TWRV1	pRS42-H-^{Sc}ACS-ALD6	*2µ, AmpR, hphNT1, pPGI1-ALD6-tRPL15A, pPFK1-^{Se}ACS^{L641P}- tTDIT1*	This study
FWV171	pRS42-K-^{Se}ACS-ALD6	*2µ, AmpR, kanMX, pPGI1-ALD6-tRPL15A, pPFK1-^{Se}ACS^{L64}-tTDIT1*	This study
VSV11	pRCC-K-ALD6	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[ALD6]-tSUB4*	Schadeweg & Boles, 2016
ISOV115	pRS42-H-ADH2	*2µ, AmpR, hphNT1, pHXT7^{1--392}-ADH2-tFBA1*	Brat & Boles, 2012
FWV156	pRCC-K-P$_{PGI1}$	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[pPGI1]-tSUB4*	This study
FWV157	pRCC-K-P$_{ZWF1}$	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[pZWF1]-tSUB4*	This study
FWV158	pRCC-K-ARE2	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[ARE2]-tSUB4*	This study
FWV159	pRCC-K-DGA1	*2µ, AmpR, kanMX4, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[DGA1]-tSUB4*	This study
pB14	PYTK001-^{Ca}xPK-B14	*CamR,* ^{Ca}xPK	Arun S. Rajkumar
pB15	PYTK001-^{Bs}xPTA-B15	*CamR,* ^{Bs}xPTA	Arun S. Rajkumar
pB20	PYTK001-^{Sc}EutD-B20	*CamR,* ^{Sc}EutD	Arun S. Rajkumar
FWV163	pRS62-H-^{Ca}xPK	*2µ, AmpR, hphNT1, pHXT7^{1--392}-^{Ca}xPK-tFBA1*	This study
FWV164	cGG-^{Bs}PTA	*pHHF2-^{Bs}PTA-tSSA1*	This study
FWV165	cGG-^{Se}EutD	*pHHF2-^{Se}EutD- tSSA1*	This study
FWV168	pRCC-N-GPP1	*2µ, AmpR, natNT2, pROX3-opt.CAS9^{Sp}-tCYC1, pSNR52-gRNA[GPP1]-tSUB4*	This study

Table S2: Oligonucleotides used in this study

Primer Name	Sequence 5'-3'	Application
FWP322	CTGTCACCGTCAGAAAAATATGTCAATGAG GCAAGAACCGGGCTGGGCGATCTTCCTTG	Amplification of *pCOX9* with overhangs to *PGI1* locus to replace *pPGI1*
FWP323	GCCAGTTTGAAGTTAGTGAATGAGTTATTG GACATGTCTGTGTAAGTCGCTTGTAGTTAG	
SBP176	CTCTATTCCACGAGGCATTC	Amplification of $P_{HXT7}^{-1—392}$ with overhangs to *ZWF1* locus to replace P_{ZWF1}
FWP327	ACCCGTGTACATAAGCGTGAAATCACCACA AACTGTGTGTAGCTCGTAGGAACAATTTCG	
FWP331	ACACATTACGTTAGCAAAAGCAACAATAAC AAACACAACCGGCATCCTGCAACTGTTCTG TGGAGCTATTAAATCTTTAT	Donor DNA for deletion of *ARE2*
FWP332	ATAAAGATTTAATAGCTCCACAGAACAGTT GCAGGATGCCGGTTGTGTTTGTTATTGTTGC TTTTGCTAACGTAATGTGT	
FWP337	TAAGGAAACGCAGAGGCATACAGTTTGAAC AGTCACATAATAATGAATTCATTGGAAAAC ACAAAATATGTTAGAATAAA	Donor DNA for deletion of *DGA1*
FWP338	TTTATTCTAACATATTTTGTGTTTTCCAATG AATTCATTATTATGTGACTGTTCAAACTGTA TGCCTCTGCGTTTCCTTA	
FWP342	TTGATTGCCATTTTTTTCTTTCCAAGTTTCCT TGTTATAAAATTAAAGTAGCAGTACTTC	Amplification of xPK-PTA cassette with overhangs for *GPP1* locus
FWP343	TTTATTTTTAGCGTAGTAGTTTTATCAAAAA AATAAAAGAAAACACCCATGAACCACAC	
FWP362	TTGAAGAAACATGAAATTGCCCAGTATTCT TAACCCAAAATGGATATTGATCTAGATGG	Amplification of *pPFK1-RPE1-tRPE1* with overhangs to *URA3* locus
FWP363	AATCATTACGACCGAGATTCCCGGGTAATA ACTGGAAAAATATAAGGATGAGAAAGTG	
FWP366	GTCGACGGTATCGATAAGCTTGATATCGAA TTCCTGCAGTTGATAGGTCAAGATCAATG	Amplification of *pTEF2* with overhangs to prS52-K and *ZWF1* for cloning of FWV169
FWP367	ATTTTTTTCGAATTTGACGGGGCCTTCACTC ATGTTTAGTTAATTATAGTTCGTTGAC	
FWP368	CTTGTTTTTAGAATATACGGTCAACGAACT ATAATTAACTAAACATGAGTGAAGGCCC	Amplification of *ZWF1-tZWF1* with overhangs to *pTEF2* and pRS52-K for cloning of FWV169
FWP369	AAATTTGTATTTGTAGAGTGCATCCTATATA TTCAATTCATATTTTATCTCTTTTTTTTTTTT TTTTTTC	
FWP170	AAAAAAAAAAGAGATAAAATATGAATTGA ATATATAGGATGCACTCTACAAATAC	Amplification of *SOL3-tSOL3* with overhangs to *pCCW12* and pRS52-K for cloning of FWV169
FWP171	AGAAATTAATCTTCTGTCATTCGCTTAAACA CTATATCAATAAATGGTGACAGTCGGTG	
FWP172	AGCCCTCTCAGAAAACACACCGACTGTCAC CATTTATTGATATAGTGTTTAAGCGAATG	Amplification of *pCCW12* with overhangs to prS52-K and *SOL3* for cloning of FWV169
FWP173	TTTTGAGCCTCCATGTCTCTGAAGAACTCCC TGTTGGCAAGGCACCCATGAACCACAC	
FWP174	ATCTGAACTGCCCCTTTTGGACTAACCGTGT GGTTCATGGGTGCCTTGCCAACAGGG	Amplification of *pTEF1* with overhangs to prS52-K and *GND1* for cloning of FWV169
FWP175	AAACCAATCAAACCGAAATCAGCAGACATT TTGTAATTAAAACTTAGATTAGATTGC	
FWP176	GAAAGAAAGCATAGCAATCTAATCTAAGTT TTAATTACAAAATGTCTGCTGATTTCGG	Amplification of *GND1-tGND1* with

FWP177	TTAACCCTCACTAAAGGGAACAAAAGCTGG AGCTCCACCGCGGTCTACTCTACTTCTATCA TGATAATAG	overhangs to *pTEF1* and pRS52-K for cloning of FWV169
FWP418	TCATTATAGAAATCATTACGACCGAGATTC CCGGGTAATAACTGGGCAAAACGTAGGG	Amplification of *pADH2* with overhangs to *ALD6* and *URA3* for integration
FWP402	GGTTCAGCAGTGTCAAAGTGTAGCTTAGTC ATTGTGTATTACGATATAGTTAATAG	
FWP410	AATCAACTATCAACTATTAACTATATCGTA ATACACAATGACTAAGCTACACTTTGAC	Amplification of *ALD6- tALD6* with overhangs to *pADH2* and *URA3* integration
FWP419	TTGAAGAAACATGAAATTGCCCAGTATTCT TAACCCAATCCACGTTAGTTTTCTTTGG	
FWP413	TTTTGCATTGCCTTATCTTTTGCCGCCAGAA GAAACAAGGTGACGACGGATGAATATGTTG ACAGTCTAGCAAACAGTAG	Donor DNA for deletion of *ALD2*
FWP414	CTACTGTTTGCTAGACTGTCAACATATTCAT CCGTCGTCACCTTGTTTCTTCTGGCGGCAAA AGATAAGGCAATGCAAAA	
FWP415	AACCCTTAATATAACTTCGTATAATGTATGC TATACGAAGTTATCAGCGACATGGAGGC	Amplification of *kanMX4* with overhangs to PRS42-H for replacement of *hphNT*
FWP416	ATATCACCTAATAACTTCGTATAGCATACA TTATACGAAGTTATGACACTGGATGGCGG	

Sequences of relevant genes.

>^{Ca}xPK

ATGCAGAGTATAATTGGAAAACATAAGGATGAAGGCAAGATAACACCTGAGTATTTAAAAAAGATTGACGCGTATTGGCGTGCGGCCAATTTTATAA
GCGTCGGGGCAGTTGTACCTACTTGATAACCCGCTGCTACGTGAGCCTCTTAAACCCGAGCATTTGAAGAGAAAGGTGGTTGGCCACTGGGGCACCAT
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CAGTACATTAGGGAGGTTGGCGAAGATCTTCCCGAAATTACGAACTGGCAGTGGCACGTGTAG

>^{Bs}PTA

ATGGCAGATTTATTCAGCACCGTGCAAGAAAAAGTGGCCGGTAAAGACGTAAAAATAGTATTCCCTGAAGGCCTGGACGAACGTATATTGGAAGCGG
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AA

>^{Se}EutD
ATGATCATAGAACGTGCCAGAGAATTAGCCGTACGTGCCCCCGCGCGTGTCGTGTTCCCGGATGCTCTGGATGAGCGTGTCTTGAAGGCCGCTCATT
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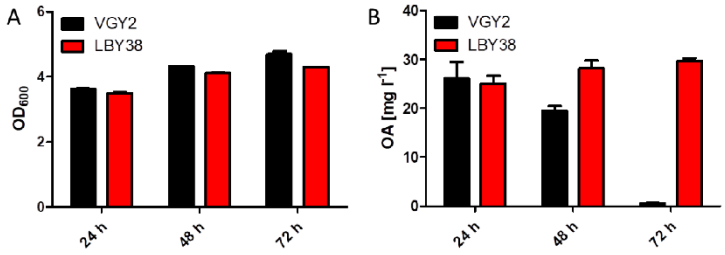
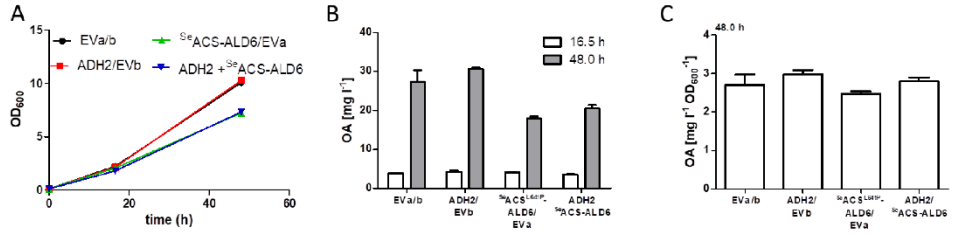
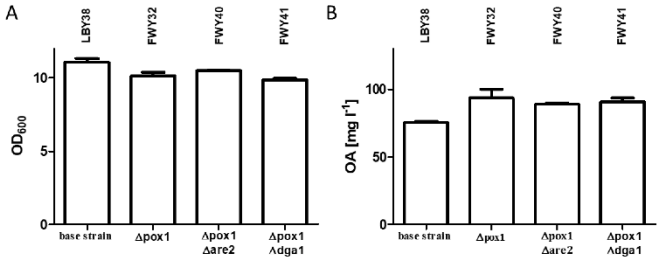


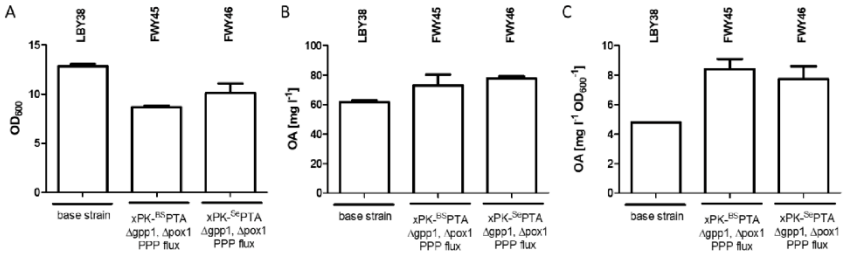
Supplementary Figure S1: Octanoic acid production of strains VGY2 and LBY38. Strains were grown in phosphate buffered SCD medium and growth (A) and octanoic acid production (B) was monitored over 72 h. Samples represent mean and standard deviation of two biological replicates. Error bars may be smaller than symbols.



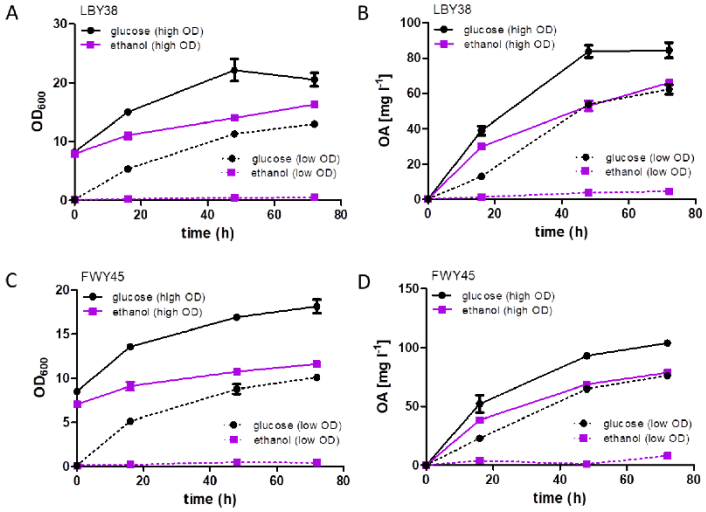
Supplementary Figure S2: Overexpression of PDH-bypass genes for octanoic acid biosynthesis. Strain LBY38 expressing high copy plasmids TWRV1 (*pHXT7$^{1-392}$-ADH2*) or FWV171 (*pPGI1-ALD6, pPFK1-^{Se}ACS^{L641P}*) was grown in potassium phosphate buffered YPD media for 48 h with appropriate antibiotics for plasmid selection. Two appropriate high copy empty vectors (EVa and b) were used as control. (A) cell growth, (B) OA concentrations and (C) specific titers were analyzed. Values and error bars represent mean and standard deviation of two biological replicates.



Supplementary Figure S3: Deletion of β-oxidation and storage lipid formation in octanoic acid producing strians. (A) Growth and (B) octanoic acid production of strain with deletion in genes relevant for β-oxidation (*POX1*) and storage lipid formation (*ARE2*, *DGA1*). Samples were taken after 48 h of fermentation. Values represent mean and standard deviation of two biological replicates.



Supplementary Figure S4: Comparison of two different PTAs. (A) Growth, (B) OA production and (C) specific titers of a control strain (LBY38) or strains either expressing a PTA from *Bacillus subtilis* (^{BS}PTA) or *Salmonella enterica* (^{Sc}PTA). Samples were taken after 48 h. Values show mean and standard deviation of two biological replicates.



Supplementary Figure S5: Octanoic acid production from glucose or ethanol in high OD fermentations compared to low OD fermentations. Strains LBY38 (A and B) or FWY45 (C and D) were inoculated to an OD of 0.1 (low OD, dashed lines) or 8.0 (high OD, solid lines) in potassium buffered YPD media containing either 0.1 mol/L (20.0 g l^{-1}glucose (black) or 0.2 mol l^{-1} (9.3 g l^{-1}) ethanol (violet). Samples show mean and standard deviation of two biological replicates.

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5.3 *De novo* biosynthesis of 8-hydroxyoctanoic acid via a medium-chain length specific fatty acid synthase and cytochrome P450 in *Saccharomyces cerevisiae*

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De novo biosynthesis of 8-hydroxyoctanoic acid via a medium-chain length specific fatty acid synthase and cytochrome P450 in *Saccharomyces cerevisiae*

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ABSTRACT

Terminally hydroxylated fatty acids or dicarboxylic acids are industrially relevant compounds with broad applications. Here, we present the proof of principle for the *de novo* biosynthesis of 8-hydroxyoctanoic acid from glucose and ethanol in the yeast *Saccharomyces cerevisiae*. Toxicity tests with medium-chain length ω-hydroxy fatty acids and dicarboxylic acids revealed little or no growth impairments on yeast cultures even at higher concentrations. The ability of various heterologous cytochrome P450 enzymes in combination with their cognate reductases for ω-hydroxylation of externally fed octanoic acid were compared. Finally, the most efficient P450 enzyme system was expressed in a yeast strain, whose fatty acid synthase was engineered for octanoic acid production, resulting in *de novo* biosynthesis of 8-hydroxyoctanoic acid up to 3 mg/l. Accumulation of octanoic acid revealed that cytochromes P450 activities were limiting 8-hydroxyoctanoic acid synthesis. The hydroxylation of both externally added and intracellularly produced octanoic acid was strongly dependent on the carbon source used, with ethanol being preferred. We further identified the availability of heme, a cofactor needed for P450 activity, as a limiting factor of 8-hydroxyoctanoic acid biosynthesis.

1. Introduction

The industrial production of fatty acids (FA) and derived compounds is mainly based on extraction from plant oils or animal fats, or on petrochemical syntheses routes (Haupt et al., 1984; Akaike, 1985; Rupilius and Ahmad, 2007). However, environmental concerns regarding the use of fossil resources, deforestation and excessive land and water use for oil plant cultivation (Schmidt, 2015) as well as challenges in chemical synthesis (*i.e.* ω-hydroxy fatty acids) (Scheps et al., 2013) have increased interest in production through microbial biosynthesis. The engineering of microbial cell factories therefore holds the potential for a "greener" industry through environmentally friendly production conditions and increased sustainability. Hydroxy fatty acids and dicarboxylic acids are fatty acid derived compounds which harbor additional hydroxyl or carboxyl groups. They have compelling chemical properties and are relevant for chemical, pharmaceutical, food and cosmetic industries (Kim and Oh, 2013), are used as additives for lubricants, emulsifiers and can have antibiotic, anti-inflammatory and anticancer properties (Kim and Oh, 2013). Additionally, they can serve as building blocks for polymers and bioplastics (Liu et al., 2011; Scheps et al., 2013).

There is great interest in microbial biosynthesis of ω-hydroxy fatty acids (HyFA) and α,ω-dicarboxylic acids (DCA) (Lu et al., 2010; Clomburg et al., 2015; Durairaj et al., 2015; Han et al., 2017; Haushalter et al., 2017; Liu et al., 2019). In *Saccharomyces cerevisiae*, a host organism with a long tradition in the biotech industry, the production of medium-chain (C8–C14) HyFA variants by bioconversion (Durairaj et al., 2015; Han et al., 2017) or long-chain (C16–C18) variants by *de novo* biosynthesis (Liu et al., 2019) has been achieved by terminal hydroxylation of FAs. Subsequent oxidation of the terminal hydroxyl group by endogenous alcohol and aldehyde dehydrogenases has led to formation of DCAs (Han et al., 2017).

Formation of HyFAs from FA precursors requires efficient hydroxylation of the terminal carbon. Such reactions can be catalyzed by enzymes belonging to the superfamily of cytochromes P450 (CYPs), which are active on a large variety of different substrates (Renault et al., 2014). CYPs are hemeproteins which act as monooxygenases by activating molecular oxygen and transferring one atom to its substrate and reducing the other to water (Munro et al., 2013). Two electrons are required for the CYP reaction which are transferred by donor proteins. The membrane anchored eukaryotic CYPs mainly act in a two-component system, in which the electrons are transferred from NADPH to CYP by a cytochrome P450 reductase (CPR) (Munro et al., 2013). Several eukaryotic CYPs have

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been identified to specifically ω-hydroxylate medium-chain FAs (Fisher et al., 1998; Durairaj et al., 2015; Han et al., 2017).

The aforementioned bioconversions of medium-chain FAs to HyFAs were achieved by supplementation of FAs. In contrast, in this work we aimed to construct a complete pathway from glucose or ethanol to the medium-chain length 8-hydroxyoctanoic acid (C8-HyFA) in *S. cerevisiae*. This, however, requires direct synthesis of octanoic acid (C8-FA) from those carbon sources. In the yeast cytosol, FAs are synthesized *de novo* by the type I fatty acids synthase (FAS), a large enzymatic complex (Lomakin et al., 2007), which is encoded by the two genes *FAS1* and *FAS2*. Intermediates of FA biosynthesis are covalently linked to FAS via the ACP domain, and are released in a coenzyme A (CoA) bound form with a length of mainly 16 or 18 carbon atoms (Lomakin et al., 2007). Subsequent cleavage of the CoA-FA-thioester bound by endogenous thioesterases yields free FA. Multiple studies have addressed the enrichment of medium-chain FAs by heterologous expression of medium-chain specific thioesterases (Leber and Da Silva, 2014; Fernandez-Moya et al., 2015; Zhu et al., 2017). An alternative approach for medium-chain FA biosynthesis has been carried out by rational engineering of the FAS (Gajewski et al., 2017). Mutations in relevant domains increased medium-chain FAs production. A single mutation (*FAS1*^{R1834K}) allowed highly selective production of C8-FA (about 90% of secreted fatty acids) (Gajewski et al., 2017). Thus, we considered the FAS1^{R1834K}/FAS2 system combined with expression of medium-chain FA specific CYPs to be optimal for selective *de novo* biosynthesis of C8-HyFA.

Here, we present for the first time the proof of principle for the *de novo* biosynthesis of C8-HyFA in *S. cerevisiae*. We constructed a complete pathway from glucose or ethanol to C8-HyFA (Fig. 1) by combining C8-FA production via a mutated FAS (*FAS1*^{R1834K}*/FAS2*) with direct ω-hydroxylation of the C8-FA by various CYPs.

2. Materials and methods

2.1. Strain construction and transformation

Yeast strains used in this study are listed in Table 1. Yeast strain SHY24 was constructed by deletion of the *FAA2* locus in BY4741. Strain SHY34 resulted from the previously described strain RPY21 (Gajewski et al., 2017) by deletion of two *kanMX* markers which were present in the RPY21 genome as remnants of *fas1* and *fas2* deletion. RPY21 has a BY background and is based on strain BY.PK1238_1A_KO.

RPY21 and SHY34 carry additional deletions in *FAA2* (medium chain fatty acyl-CoA synthetase).

Strain LBY3 was constructed from strain CEN.PK113-7D by deletion

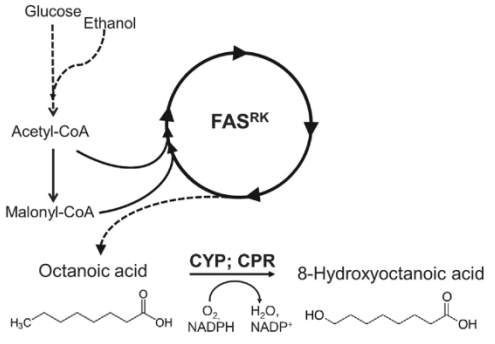

Fig. 1. Schematic presentation of *de novo* biosynthesis of 8-hydroxyoctanoic acid. A mutated fatty acid synthase FAS1^{R1834K}/FAS2 (FAS^{RK}) produces octanoic acid which is ω-hydroxylated by a cytochrome P450 (CYP) resulting in 8-hydroxyoctanoic acid formation. The electrons required for the CYP reaction are transferred from NADPH to CYP by a cytochrome P450 reductase (CPR).

Table 1
Yeast strains used in this study.

Strain Name	Relevant features	Reference/Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al. (1998)
CEN.PK113-7D	*MATa MAL2-8c SUC2*	Euroscarf, Frankfurt am Main, Germany
SHY24	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δfaa2*	This work
SHY34	*MATα ura3Δ0 his3Δ0 leu2Δ0 TRP1 lys2Δ0 MET15 Δfas1 Δfas2 Δfaa2*	This work
LBY3	CEN.PK113-7D *Δpox1*	This work

of *POX1*. The deletions of *kanMX*, *FAA2* and *POX1* were carried out by CRISPR-Cas9 meditated gene deletion as described previously (Generoso et al., 2016). Transformations were performed following the frozen competent cell protocol (Gietz and Schiestl, 2007), whereas SHY34 was transformed by a slightly modified method as previously described (Gajewski et al., 2017). FAS deficient strains are auxotrophic for fatty acids and, to allow growth, their medium was supplemented with 0,5 mM oleic acid and Terigitol-NP-40 (70% Sigma Aldrich Germany) to solubilize it. Transformed yeasts were plated on solid YPD (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose) containing appropriate antibiotics hygromycin (100 mg/L) or G418 (200 mg/L) for plasmid selection and grown at 30 °C for two to four days.

2.2. Plasmid and strain construction

All plasmids used in this study are listed in Table 2. Plasmids carrying cytochrome P450s (CYP) and cytochrome reductases (CPR) were constructed using the Golden Gate cloning system (Lee et al., 2015) by following the published protocol. Genes of cytochrome P450 and cytochrome reductases were purchased as DNA Strings from Thermo Fischer Scientific, Germany with flanking BsmBI and BasI restriction site overhangs for Golden Gate cloning. The *MET25* promoter was included in the Golden Gate toolkit by amplification from BY4741 genomic DNA and

Table 2
Plasmids used in this study.

Plasmid	Description	Reference
pYTK-CYP4B1-POR	2micron, *KanR*, *HygR*, *pTDH3*-CYP4B1-*tENO1*, *pMET25*-POR-*tSSA1*	This study
pYTK-CYP94C1-ATR1	2micron, *KanR*, *HygR*, *pTDH3*-CYP94C1-*tENO1*, *pMET25*-ATR1-*tSSA1*	This study
pYTK-CYP53A7-cprA	2micron, *KanR*, *HygR*, *pTDH3*_CYP539A7-*tENO1 pMET25*-cprA-*tSSA1*	This study
pYTK- CYP539A7	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1*	This study
pYTK- CYP539A7-*pPGK1*-cprA	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1 pPGK1*-cprA-*tSSA1*	This study
pYTK- CYP539A7-*pTEF2*-cprA	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1 pTEF2*-cprA-*tSSA1*	This study
pYTK- CYP539A7-*pALD6*-cprA	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1 pALD6*-cprA-*tSSA1*	This study
pYTK- CYP539A7-*pRNR1* -cprA	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1 pRNR1*-cprA-*tSSA1*	This study
pYTK- CYP539A7-*pRAD27* -cprA	2micron, *KanR*, *HygR*, *pTDH3*-CYP539A7-*tENO1 pRAD27*-cprA-*tSSA1*	This study
pRS315-FAS1-RK	CEN6/ARS4, *AmpR*, *LEU2*, *pFAS1-FAS1*^{R1834K}*-tFAS1*	Gajewski et al. (2017)
pRS313-FAS2	CEN6/ARS4, *AmpR*, *HIS3*, *pFAS2-FAS2-tFAS2*	Gajewski et al. (2017)
pRS315-FAS1^{RK}-FAS2	CEN6/ARS4, *AmpR*, *kanMX*, *pFAS1-FAS1*^{R1834K}, *pFAS2-FAS2-tFAS2*	This study
pRS315-FAS1^{RK}-FAS2-2μ	2micron, *AmpR*, *kanMX*, *pFAS1-FAS1*^{R1834K}, *pFAS2-FAS2-tFAS2*	This study
pRS62-K	2micron, *AmpR*, *kanMX*,	Farwick et al. (2014)

KanR and *AmpR* stand for genes which are responsible for kanamycin or ampicillin resistance for *E. coli*. *HygR* and *kanMX* stand for genes which are responsible for hygromycin or G418 resistance for *S. cerevisiae*.

cloning into pYTK backbone. CYPs used were: CYP4B1 from *Oryctolagus cuniculus*, CYP94C1 from *Arabidopsis thaliana*, CYP539A7 from *Fusarium oxysporum f. sp. lycopersici* (UniProtKB accession numbers: P15128; Q9ZUX1; A0A0D2X892) and their respective CPRs POR from *Oryctolagus cuniculus*, ATR1 from *Arabidopsis thaliana*, cprA from *Fusarium oxysporum f. sp. lycopersici* (UniProtKB accession numbers: P00389, Q9SB48, A0A0D2XWA9). CYPs and CPRs were cloned into a 2micron backbone with hygromycin resistance marker.

The plasmid pRS315-*FAS1*^{RK}-*FAS2* was constructed by homologous recombination in strain BY4741.

pFAS2-FAS2-tFAS2 and *kanMX* fragments were amplified with oligonucleotides (Supplementary Table 1) that contained at least 30 bp overhangs to the plasmid backbone for homologous recombination. *pFAS2-FAS2-tFAS2* was integrated into XhoI cut site of pRS315-*FAS1* backbone and *kanMX* was integrated into EcoRV cut site (*LEU2*) with overhangs to replace *LEU2* gene. Fragments of *pFAS2-FAS2-tFAS2* and *kanMX* were amplified from pRS313-FAS2 and pRS26-K, respectively. Plasmids containing auxotrophic markers (LEU2 or HIS3) were selected in synthetic media lacking the corresponding compound (leucine or histidine), whereas the plasmids carrying hygromycin (*HygR*) and G418 (*kanMX*) resistance genes were selected by adding antibiotics (200 μg/ml) to the media.

2.3. Media and cultivation

Several colonies of yeast strains or transformed yeasts were used to inoculate liquid medium pre-cultures. Liquid cultures were grown in shake flasks at 30 °C and 180 rpm using either SC (0.17% (w/v) yeast nitrogen base without amino acids, 0.5% (w/v) ammonium sulfate, addition of amino acids as previously described (Bruder et al., 2016)) or YP (2% (w/v) peptone, 1% (w/v) yeast extract) medium. 2% (w/v) glucose was used as carbon source for pre-cultures. Main cultures were inoculated from pre-cultures to high cell density (OD_{600} of 8–10) with either 2% (w/v) glucose, 2% (w/v) galactose, or 2% (v/v) ethanol plus 2% (v/v) glycerol as carbon sources as indicated. Glycerol was added to ethanol containing media with the rationale to provide the cells with C3 carbon skeletons.

High cell density bioconversion experiments were performed by supplementation with 50 mg/L octanoic acid. In bioconversion experiments with SC medium supplementation of 2 mM methionine was done for pre-cultures to repress CPR expression from *pMET25*. For the bioconversions and *de novo* biosynthesis with YP medium 100 mM potassium phosphate buffer (pH 6.5) was added. 5-Aminolevulinic acid (ALA) and hemin were purchased from Sigma Aldrich, Germany and dissolved as 100x stock solutions in H_20 and DMSO, respectively. They were added to the main cultures with final concentrations of 300 μg/L and 50 μg/L, respectively. Samples for compound extraction were taken at given time points.

2.4. Toxicity assay and degradation analysis

For the toxicity assay a BY4741 pre-culture was inoculated into fresh YPD medium to an OD_{600} of 0.2 and cultivated for 5–6 h until an OD_{600} of 0.8–1.0 was reached. The culture was re-diluted in YPD medium to an OD_{600} of 0.05 and 50 μl was used to inoculate 200 μL of YPD medium in 96-well plates with a dilution series of C8- or C10 fatty acids, ω-hydroxy fatty acids or dicarboxylic acids. The pH values were approximately 5.0 for the media with dicarboxylic acids and approximately 5.9 for the media with ω-hydroxy fatty acids. The compounds were dissolved in YPD medium at their highest concentrations, filtered for sterility (0.2 μm) and subsequently diluted to desired concentrations. Cultivations were performed for 18 h at 30 °C without agitation and cells were mixed thoroughly before OD_{600} measurement in a plate reader (ClarioStar, BMG Labtech, Germany).

For 8-hydroxyoctanoic acid or octanedioic acid degradation analysis, strains were inoculated from pre-cultures in fresh YPD medium supplemented with 50 mg/L 8-hydroxyoctanoic acid or octanedioic acid to high cell density (OD_{600} = 10). The pH values were approximately 6.2 for medium with 8-hydroxyoctanoic acid and 5.9 for medium with octanedioic acid. Samples for extraction of compounds were taken at given time points.

2.5. Compound extraction and derivatization

For extraction of fatty acids or hydroxy fatty acids and dicarboxylic acids, yeast cultures were separated from the medium (16 000 rpm, 5 min). Hydroxy fatty acids or dicarboxylic acids were extracted from 1 ml culture supernatant. 0.02 mg heptanoic acid was added as an internal standard followed by acidification with 200 μL 1 M HCl and extraction with equal volumes of ethyl acetate. After phase separation the organic phase was taken and evaporated in a vacuum concentrator (Concentrator 5301, Eppendorf, Germany). The resulting extract was dissolved in 200 μL ethyl acetate and mixed with equal volumes of N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Alfa Aesar, Germany) for derivatization. Derivatization was performed for 45 min at 75 °C and samples were analyzed by gas chromatography (GC).

Fatty acids were derivatized to methyl esters for GC analysis and extracted from 10 ml culture supernatant. 0.2 mg heptanoic acid was added as an internal standard followed by acidification with 1 ml 1 M HCl and extraction by 2.5 ml of 1:1 mixture of methanol and chloroform. After centrifugation for phase separation (4000 rpm, 10 min) the organic phase was taken and evaporated in a vacuum concentrator (Concentrator 5301, Eppendorf, Germany). The resulting extract was dissolved in 200 μL toluol and mixed with 10 ml methanol and 300 μL 8% HCl for methylation. Derivatization was performed at 100 °C for 3 h. Fatty acid methyl esters were extracted by addition of 1 ml water and 1 ml hexane. The hexane phase was collected and analyzed by GC.

2.6. Gas chromatography

Analysis was performed on a Clarus 400 gas chromatograph (PerkinElmer, Germany) equipped with a FID detector and an Elite-5MS column (Ø 0.25 mm; length 30 m; film thickness 1.00 μm). 1 μL of sample was injected with 1:10 split and helium was used as carrier gas (90 kPa). 8-Hydroxy fatty acids and dicarboxylic acids– BSTFA derivates were separated with by the following method: run time 25.5 min, start at 50 °C and hold for 5 min; ramp at 20 °C min to 240 °C and hold for 2 min, ramp at 20 °C–300 °C and hold for 5 min. Octanoic acid methyl esters were separated by the following method: run time 42.67 min, start at 50 °C and hold for 5 min; ramp at 10 °C min to 120 °C and hold for 5 min, ramp at 15 °C–220 °C and hold for 10 min, ramp at 20 °C–300 °C and hold for 5 min.

3. Results & discussion

3.1. Evaluating possible toxic effects of medium-chain ω-hydroxy fatty acids and dicarboxylic acids on yeast

We first evaluated possible toxic or inhibitory effects of HyFAs and DCAs on yeast cells. Medium-chain FAs like C8-FA and decanoic (C10-FA) acid and derivatives like 1-octanol are well known to affect the growth of yeast cells (Viegas et al., 1989; Alexandre et al., 1996; Legras et al., 2010; Borrull et al., 2015; Henritzi et al., 2018). 1-Octanol for instance even causes a stronger growth impairment than C8-FA (Henritzi et al., 2018). As the biosynthesis of products with toxic properties can reduce product yields e.g. by inhibition of cellular processes or even can lead to cell death, we investigated possible toxic effects of HyFAs and DCAs with eight or ten straight carbon atoms. Measurements of cell density (recorded as changes in OD_{600}) were used as proxy to assess the toxicity of extracellularly supplemented compounds to a wild type (BY4741) yeast culture. The toxicity was compared to the well-known toxic compounds C8-FA and C10-FA. As expected, C8 and C10–FAs

strongly influenced growth of the yeast strain at rising concentrations (Fig. 2a). At about 500 mg/L and 300 mg/L, respectively, no growth was detectable anymore. In contrast, the C8 or C10-HyFAs and DCAs caused little or no growth impairment even at high concentrations (Fig. 2a).

The toxicity of C8–FAs or C10–FAs has been attributed to various mechanisms. *S. cerevisiae* rapidly acidifies its growth medium and under such conditions weak acids, like medium-chain FAs, are present in their protonated form. In this state, they are more liposoluble and can pass the membrane by passive diffusion (Viegas and Sá-Correia, 1997). In the neutral cytosol, dissociation leads to a decrease of cellular pH and accumulation of acyl-anions, which lead to an inhibition of cellular processes (Cabral et al., 2001). Indeed, the influence of a decreasing pH on increasing toxicity of C8–FAs has been demonstrated (Liu et al., 2013). C8–FAs rapidly enter yeast cells (Henritzi et al., 2018) although the majority is found in cell wall fractions and not inside the cell, which is attributed to energy expensive expulsion by the Pdr12 carrier (Borrull et al., 2015). Additionally, exposure of yeast to C8–FAs is causing membrane stress and disturbs integrity leading to membrane leakage (Liu et al., 2013). For medium-chain FAs, toxicity is increasing with increasing chain length (C10 > C8 > C6) (Liu et al., 2013) possibly because of stronger membrane perturbation due to higher liposolubility. Interestingly, dependency of chain-length on toxicity was also demonstrated here for HyFAs and DCAs, whereby the longer C10 variants cause a growth defect while the C8 variants do not (Fig. 2a). However, due to the additional hydroxyl or carboxyl group HyFAs and DCAs, respectively, are less liposoluble than their corresponding FAs, which likely results in a reduced ability to integrate into or diffuse across the plasma membrane. However, other reasons for lower toxicity cannot be ruled out.

3.2. Degradation of medium-chain ω-hydroxy fatty acids and dicarboxylic acids in yeast

It has been suggested that ω-hydroxy lauric acid can be rapidly degraded via β-oxidation in *S. cerevisiae* (Durairaj et al., 2015). In order to test the stability of C8-HyFA and C8-DCA in a yeast culture, a wild type (BY4741) strain was inoculated to a high cell density in YPD medium supplemented with either 50 mg/L C8-HyFA or C8-DCA. The amounts of C8-HyFA were reduced by 37% after 72 h (Fig. 2b). To assess if this decrease can be attributed to degradation via the same route as that observed for C8-FA (Leber et al., 2016; Henritzi et al., 2018), *FAA2* (encoding the medium-chain fatty acyl CoA synthetase) or *POX1* (encoding the acyl-CoA oxidase, the initial enzyme of β-oxidation) were deleted. In these two strains, the degradation of C8-HyFA was reduced by more than 50% compared to the WT (Fig. 2b). This suggests that activation by Faa2 and (subsequent) β-oxidation are the main but not the only reason for reduction of C8-HyFA levels. The remaining decrease of C8-HyFA in *faa2* or *pox1* deletion strains could be due to competing reactions, e.g. esterification (Borrull et al., 2015). Moreover, the deletions of *POX1* and *FAA2* could also indirectly (e.g. by altering the lipid composition of the membrane) affect the uptake and intracellular fate of C8-HyFA.

Interestingly, the supplemented C8-DCA showed only small decreases with similar results in all three strains (Fig. 2c). This suggests that C8-DCA either is not degraded via β-oxidation or it does not cross the membrane due to the presence of the additional carboxyl group. The initial pH of the medium supplemented with 50 mg/L C8-HyFA or C8-DCA was around 6.2 and 5.9, respectively. However, cultures of *S. cerevisiae* acidify the medium rapidly and therefore C8-DCA (p*K*a: 4.53 and 5.50 (Bretti et al., 2006)) is likely to become increasingly protonated in the course of cultivation, which should facilitate its diffusion across the plasma membrane. This is reasonable to expect, as C8-FA, which exhibits an even lower p*K*a value (4.89 (Dean, 1987)), is readily taken up by the cells in phosphate-buffered media at starting pH of 6.5 (Henritzi et al., 2018). The results presented here show that medium-chain HyFAs and DCAs have only little or no toxic effects on yeast cells, and degradation of C8 variants either does not take place or can be reduced by blocking of the β-oxidation.

3.3. Bioconversion of octanoic acid results in C8-HyFA production but not of C8-DCA

CYPs from various organisms have been shown to ω-hydroxylate medium-chain FAs of eight to twelve carbon atoms by *in vitro* assays (Fisher et al., 1998) or by heterologous expression in *S. cerevisiae* and whole-cell bioconversion of externally added FAs (Durairaj et al., 2015; Han et al., 2017). We decided to systematically compare effective ω-hydroxylation of C8–FAs for three CYPs CYP4B1 (Fisher et al., 1998) CYP94C1 (Durairaj et al., 2015) and CYP539A7 (Han et al., 2017) based on their activity towards medium-chain FAs or C8-FA specifically. The hydroxylation reaction consists of a two component system with CYP and CPR. The CYPs were expressed under control of the strong and constitutive *TDH3* promoter while CPRs were under control of the methionine-repressible *MET25* promoter. The latter was chosen because an excess of CPR over CYP can induce the formation of reactive oxygen species and affect cell viability (Zangar et al., 2004). Therefore, balancing expression between CYP and its reductase can be crucial for cell health and high productivity (Paddon et al., 2013). By controlling expression of the CPRs by the *MET25* promoter we prevented its expression in the pre-culture by methionine supplementation, but reached higher expression in the main culture by omitting methionine for high productivity. The cytochromes and their reductases were expressed in strain SHY24 (Δ*faa2*) so that degradation of C8-HyFA was minimized (see Fig. 2b). Effective ω-hydroxylation was tested in high cell density bioconversions in SC-medium with supplementation of 50 mg/L C8-FA. Initial experiments were performed using 2% glucose as a carbon source. All three tested CYP-CPR variants showed production of C8-HyFA but titers remained below 1 mg/L (Fig. 3a). We additionally tested use of ethanol/glycerol and galactose as carbon sources. With galactose as carbon

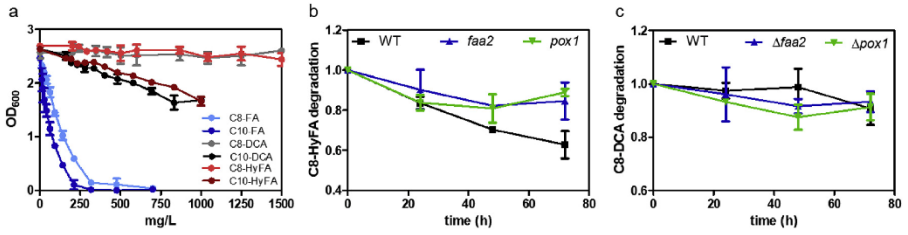

Fig. 2. Toxicity assays and degradation analyses of fatty acids, ω-hydroxy fatty acids and dicarboxylic acids. a) BY4741 wild type yeast strain was grown in 96-well plates in YPD medium with different concentrations of C8 or C10 fatty acids (FA), ω-hydroxy fatty acids (HyFA) or dicarboxylic acids (DCA). b) Supplementation of 50 mg/L of 8-hydroxyoctanoic acid (C8-HyFA) or c) supplementation of 50 mg/L octanedioic acid (C8-DCA) to yeast cultures of BY4741 (WT), SHY24 (Δ*faa2*) or LBY3 (Δ*pox1*). The values in (b) and (c) were normalized to a control sample without yeast cells. Values and error bars represent mean and standard deviation of two biological replicates.

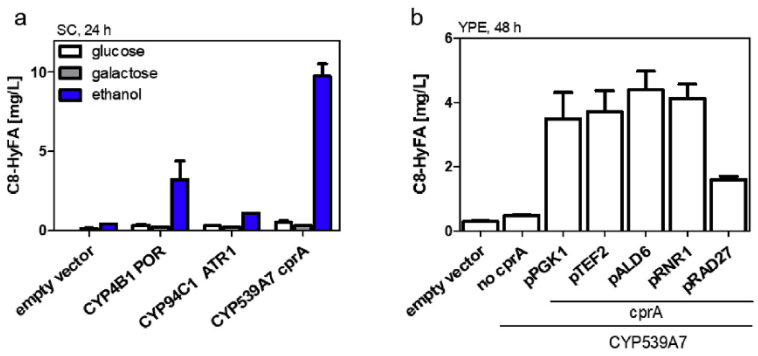

Fig. 3. Bioconversion of octanoic acid to 8-hydroxyoctanoic acid. Bioconversions were performed with strain SHY24 (*Δfaa2*) in SC (a) or YP medium (b) supplemented with 50 mg/L C8-FA. Note that ethanol media additionally contained glycerol (see Materials & Methods). a) Expression of different CYPs and CPRs in SHY24 with different carbon sources as indicated. b) WT strain (BY4741) was transformed with the empty vector, CYP539A7 alone (no cprA), or CYP539A7 in combination with cprA under the control of different promoters indicated at the bottom. Samples were taken after 24 h (a) or 48 h (b). Values and error bars represent mean and standard deviation of two biological replicates.

source C8-HyFA remained mainly below production levels with glucose. Interestingly, in ethanol/glycerol media, all three tested cytochromes displayed production of C8-HyFA. Activity of CYP94C1-ATR1 remained the lowest of the tested CYPs. This is consistent with the finding that its activity is mainly displayed on medium-chain FAs with ten or twelve carbon atoms (Kandel et al., 2007; Han et al., 2017). The best performing enzymes were CYP539A7-cprA which converted 20% of C8-FA to C8-HyFA. Thus, CYP539A7-cprA was chosen for further experiments.

With the aim of a *de novo* biosynthesis of C8-HyFAs, we wanted to adapt the fermentation conditions to the best conditions previously developed for C8-FA production. Selective C8-FA production was previously achieved by the expression of a mutated fatty acid synthase ($FAS1^{R1834K}$/FAS2) (Gajewski et al., 2017). However, the C8-FA titers were strongly dependent on the medium. In YPD medium with a potassium phosphate buffer, C8-FA titers were strongly increased (Gajewski et al., 2017). In contrast, production of C8–FAs remained low in SC medium compared to YP medium (Pavlovic, 2016). For a change from SC medium to YP medium the *MET25* promoter for controlling CPR expression is not feasible as it would be continuously repressed by methionine in the medium. To balance the expression of cprA we tested expression from five promoters with different strengths (*pPGK1* > *pTEF2* > *pALD6* > *pRNR1* > *pRAD27* (Lee et al., 2015)) and analyzed production of C8-HyFAs from C8–FAs in buffered YP medium with ethanol as carbon source (Fig. 3b). Because titers remained low after 24 h bioconversion, incubation times were increased to 48 h. Promoter strength of cprA expression had only limited effects on C8-HyFAs production. Four out of five promoters showed comparable C8-HyFA output and only the use of *pRAD27* reduced titers by 50% suggesting that expression was too low and cprA became limiting. Indeed, if no CPR was expressed only limited amounts of C8-HyFA were detectable. High expression of cprA *i.e.* from *pPGK1* did not reduce growth of cultures (final OD 26.8 compared to 23.3 for empty vector control) suggesting that there is no toxic effect of high cprA expression. These new bioconversion conditions (promoter, medium, buffering) reduced titers by about 60% compared to the initial experiment in synthetic medium. The best performing expression system with *pTDH3*-CYP539A7 and *pALD6*-cprA was chosen for further experiments.

DCAs acids can be formed from HyFAs by two consecutive oxidations catalyzed by alcohol and aldehyde dehydrogenases. It has been reported that expression of a CYP and blocking of the β-oxidation for production of HyFAs alone led to formation of DCAs by endogenous alcohol and aldehyde dehydrogenases (Han et al., 2017). We also considered formation of C8-DCAs from C8-HyFAs under our conditions, but C8-DCA titers always remained below the detection limit. Future strategies for C8-DCA formation should consider and test the overexpression of heterologous alcohol and aldehyde dehydrogenases with specific medium-chain activity.

3.4. Influence of heme on 8-hydroxyoctanoic acid titers in glucose conditions

The reactions of CYPs are dependent on heme in the active center (Munro et al., 2007b). Heme can also serve as a signaling molecule and efforts have been made to understand the regulation of heme biosynthesis in *S. cerevisiae* (Keng and Guarente, 1987; Zhang and Hach, 1999; Mense and Zhang, 2006). The cellular levels of heme have been found to be dependent on the carbon source and availability of oxygen (Zitromer and Lowrym, 1992). Under glucose/anaerobic conditions heme levels are low but are increased under ethanol/aerobic conditions (Zhang et al., 2017). We hypothesized that - when glucose is used as a carbon source - heme might be limiting the CYP activity, which could result in reduced titers of C8-HyFAs compared to ethanol/glycerol medium (Fig. 3a). Upregulation of known bottleneck reactions of heme biosynthesis *HEM2*, *HEM3* or *HEM12* (Michener et al., 2012) or deletion of heme oxygenase *HXM1* (Savitskaya et al., 2019), which is involved in heme degradation, have been shown to increase heme levels. It was reported that heme levels can also be increased by supplementation of hemin or by the heme precursor 5-aminolevulinic acid (ALA) (Zhang et al., 2017). Therefore, we tested if supplementation of ALA or hemin influences the production of C8-HyFAs in a bioconversion experiment using buffered YPD medium. ALA did not influence C8-HyFA titers compared to YPD medium without supplement (Fig. 4), which is consistent with the observation that the formation of ALA is not the rate limiting step of heme biosynthesis (Hoffman et al., 2003). However, titers were increased by about 2-fold when hemin was supplemented. Although hemin supplementation increased C8-HyFA titers in glucose medium, titers remained below those measured in ethanol/glycerol medium (about 50% reduced) (Figs. 3b and 4). Therefore, it is unlikely that heme availability is the only bottleneck for C8-FA hydroxylation under glucose conditions. Nevertheless, improvement of heme supply is certainly a target for future studies, and strategies to increase endogenous heme levels (Michener et al., 2012; Savitskaya et al., 2019) will have to be included in production strain engineering.

Furthermore, the limited capacity of the yeast cells to express functional membrane proteins such as CYPs must also be considered (Michener et al., 2012). A possible reason for increased hydroxylation activity in ethanol/glycerol media might be attributed to altered lipid composition on non-fermentative carbon sources (Alexandre et al., 1994; Huffer et al., 2011; Henderson and Block, 2014; Cray et al., 2015), which may be beneficial for functional expression of membrane bound CYP539A7-cprA. Additionally, ethanol increases the toxic effect of C8–FAs (Legras et al., 2010) which could trigger higher conversion rates to non-toxic HyFAs. An effect of the carbon source on the transcriptional level may also be considered, since the activity of the *TDH3* promoter on ethanol is reduced compared to its activity on glucose and galactose (Peng et al., 2015). A reduced transcript level may be beneficial to avoid

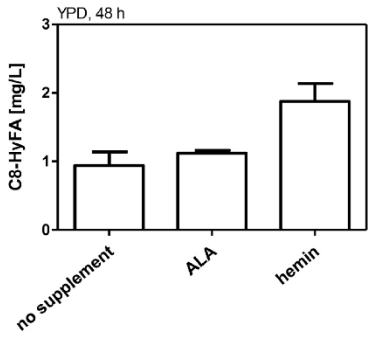

Fig. 4. Bioconversion of octanoic acid to 8-hydroxyoctanoic acid with hemin and ALA supplementation. Bioconversions were performed with strain SHY24 (*Δfaa2*) expressing CYP539A7 *pALD6-cprA* in buffered YPD medium supplemented with 50 mg/L C8-FA and additional supplementation of 5-aminolevulinic acid (ALA, 300 μg/mL) or hemin (50 μg/mL). Samples were taken after 48 h. Values and error bars represent mean and standard deviation of two biological replicates.

possible aggregation caused by the overexpression of a heterologous membrane protein. A very likely explanation for the beneficial effect of ethanol-containing media may also be found in the redox cofactor supply. Ethanol is converted to acetate via acetaldehyde by the consecutive action of the alcohol dehydrogenases and aldehyde dehydrogenases. The involvement of the NADP-dependent isoform Ald6 in ethanol catabolism may provide increased amount of NADPH, which is required for the hydroxylation by CYPs, compared to glucose-grown cells. Thus, redox-cofactor supply is also an important target for future studies.

3.5. *De novo* biosynthesis of 8-hydroxyoctanoic acid from glucose and ethanol

Recently, the biosynthesis of medium-chain FAs was established by rational engineering of the FAS (Gajewski et al., 2017). A mutation in the MPT domain of FAS1 (R1834K) enabled production of C8–FAs with high specificity. We wanted to combine C8-FA synthesis with ω-hydroxylation by CYP. For that, a plasmid expressing mutated fatty acids synthase (*FAS1*^{R1834K}/*FAS2*) was transformed together with the best performing CYP (*pTHD3*-CYP539A7 *pALD6*-crpA) in a *fas1/2* deficient strain with *faa2* deletion (SHY34) and fermented in YP medium with ethanol or glucose with or without hemin supplementation. The mutated FAS was expressed from a low copy (*CEN6/ARS4*) plasmid because a higher copy number (2micron) had no beneficial effect on octanoic acid production (Supplementary Fig. 1). Additionally, an empty vector control was used instead of *pTDH3*-CYP539A7 *pALD6*-cprA expression. All three fermentation conditions successfully resulted in *de novo* formation of C8-HyFA (Fig. 5a). Fermentations in glucose medium resulted in low titers, which did not exceed 1 mg/L. As already shown in the bioconversion experiments, hemin supplementation increased titers by 68%. The use of ethanol/glycerol media additionally increased titers by 2.3-fold. To quantitatively compare the utilization of different carbon sources for C8-HyFA production, we calculated the molar yields (μmol product per mol of carbon source; Supplementary Table 2) at the terminal time point (120 h). In ethanol/glycerol media, no detectable consumption of glycerol has occurred in the course of fermentation (Supplementary Fig. 2), so that the production of C8-HyFA occurred only from ethanol. Interestingly, the molar yields in glucose/hemin media were highest, but when the values were normalized to the cell density (OD_{600}), the values obtained in ethanol/glycerol were comparable to those obtained in glucose/hemin media, while the lowest performance was achieved in glucose media without hemin addition (Supplementary Table 2). This observation underlines the importance of heme for the functionality of CYPs. After 120 h of fermentation in all samples high amounts of C8-FA (52–100 mg/L) remained detectable. Whereas ethanol/glycerol media did not have a significant influence on C8-FA production, they yielded the highest C8-HyFA to C8-FA ratios (Fig. 5b). This again shows that activity of the CYP and not C8-FA production is the bottleneck for C8-HyFA production under all three conditions tested. Efficient electron transfer from CPR to CYP is important for high CYP activity. Artificial fusion proteins of CYP and CPR have been explored in many studies and can improve CYPs catalytic activity by forcing both partners into close proximity (Munro et al., 2007a). Therefore, in order to improve CYP activity, the fusion to CPR should be considered as a future engineering strategy.

4. Conclusions

Here, we present for the first time the proof of concept for *de novo* biosynthesis of 8-HyFA in *S. cerevisiae*. Production of 8-HyFA was achieved from glucose or ethanol by combining ω-hydroxylation of C8-FA by cytochrome P450 with C8-FA production via a mutated FAS. We show that the choice of fermentation conditions and of the carbon source are critical and that the ω-hydroxylation is the limiting step in the process. Possible targets for further strain improvement were identified. We show that medium-chain HyFAs and DCAs are little or non-toxic for *S. cerevisiae,* so that high production titers should be possible in the future.

Authors' contributions

FW, EB and MO conceived the study. FW conducted the experiments. FW, EB, and MO analyzed the data and wrote the paper. All authors read and approved the manuscript.

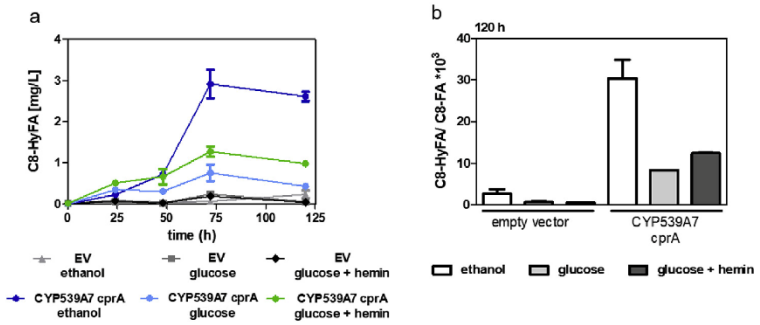

Fig. 5. *De novo* biosynthesis of 8-hydroxyoctanoic acid. Mutated fatty acid synthase (*FAS1*^{R1834K}/*FAS2*) and *pTDH3*-CYP539A7 with *pALD6*-cprA were expressed in the strain SHY34 which was grown in YP medium containing indicated carbon sources with or without hemin supplementation. Note that ethanol media additionally contained glycerol (see Materials & Methods). As a negative control, an empty vector was introduced instead of CYP539A7 and cprA. a) extracellular concentrations of C8-HyFAs and (b) the C8-HyFA/C8-FA ratio at 120 h is shown. Values and error bars represent mean and standard deviation of two biological replicates.

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Declaration of competing interest

E.B. is co-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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Abbreviations

OD600	optical density at 600 nm
CoA	coenzyme A
FAS	fatty acid synthase
CYP	cytochrome P450
CPR	cytochrome P450 reductase
ALA	5-Aminolevulinic acid
FA	fatty acid
HyFA	ω-hydroxy fatty acid
DCA	α,ω-dicarboxylic acid
SC	synthetic complete
YP	yeast extract and peptone

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2019.e00111.

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5.4 Engineering Saccharomyces cerevisiae for production of fatty acids and their derivatives [review]

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

Leonie Baumann[1], Florian Wernig[1], Sandra Born[1], Mislav Oreb[1]

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

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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 (Mal-CoA)** 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.

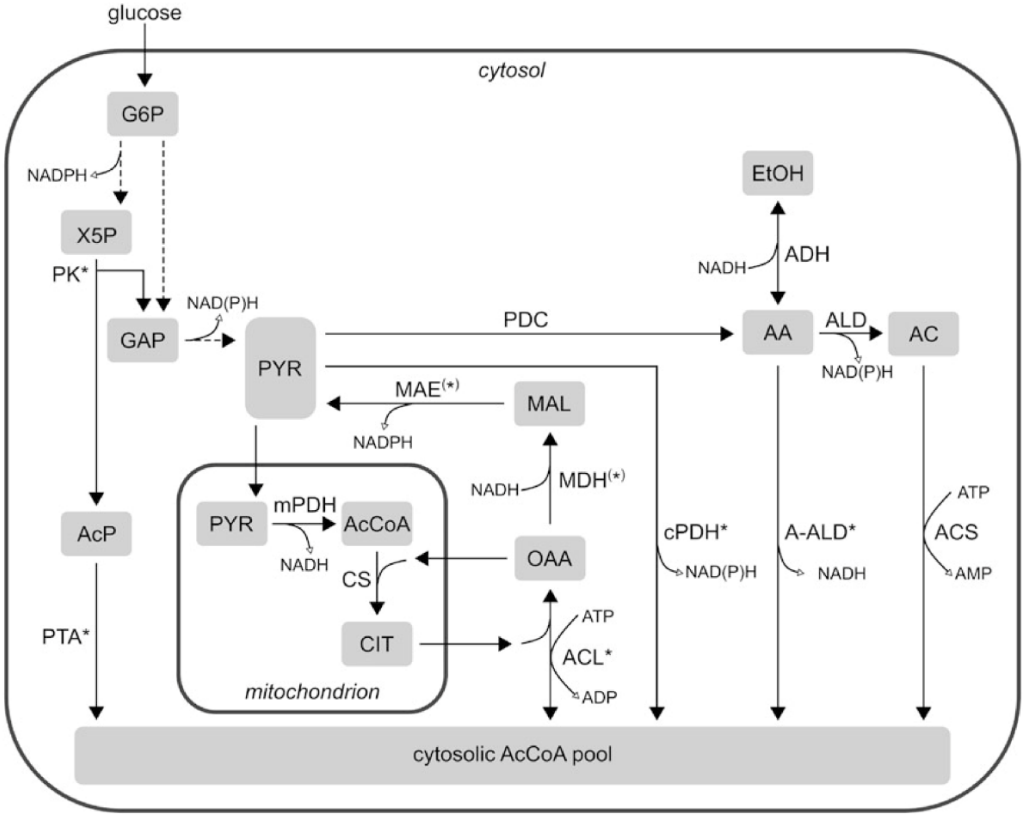

Fig. 1 A simplified scheme of precursor supply pathways for production of acetyl-CoA (AcCoA) derivatives in the cytosol of *S. cerevisiae*. Only the relevant pathway intermediates are shown in gray boxes: glucose-6-phosphate (G6P), xylulose 5-phosphate (X5P), glyceraldehyde-3-phosphate (GAP), pyruvate (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 (non-physiological) reaction direction from acetaldehyde 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 **acetylphosphate (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 (Δ*faa2*Δ*ant1*Δ*pex11*) was generated, which displayed a much stronger increase in hexanoic and octanoic acid levels compared to the "full" β-oxidation-deficient Δ*faa2*Δ*pxa1*Δ*pox1* 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-

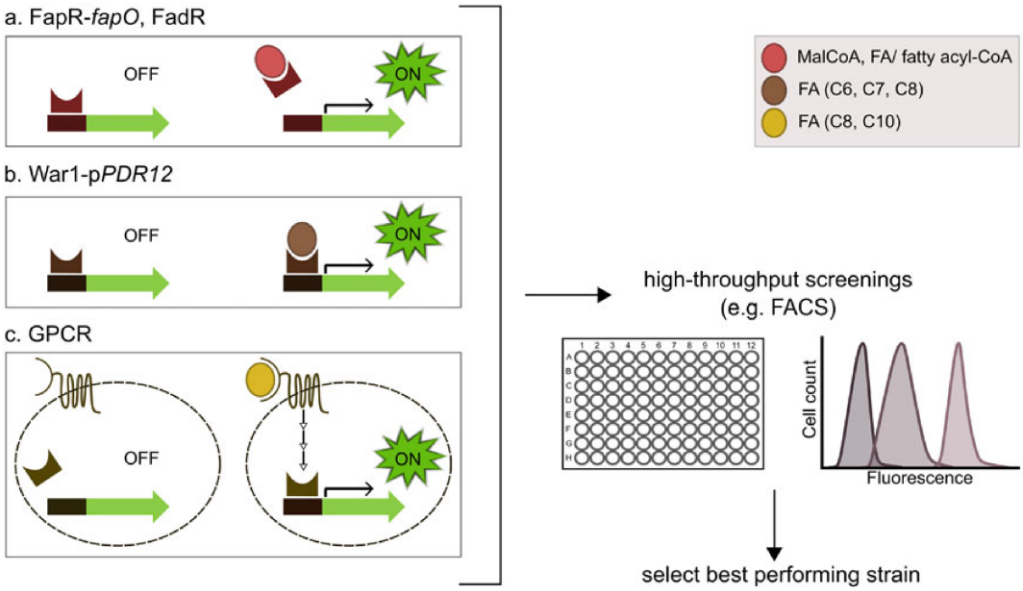

Fig. 2 Yeast biosensors developed for high-throughput screenings of fatty acids or pathway intermediates. (**a**) FapR and FadR are bacterial repressors (OFF state) which have been adapted to yeast for biosensing. Once they are bound by MalCoA (FapR) or FA/fatty acyl-CoA (FadR), expression is enabled, 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

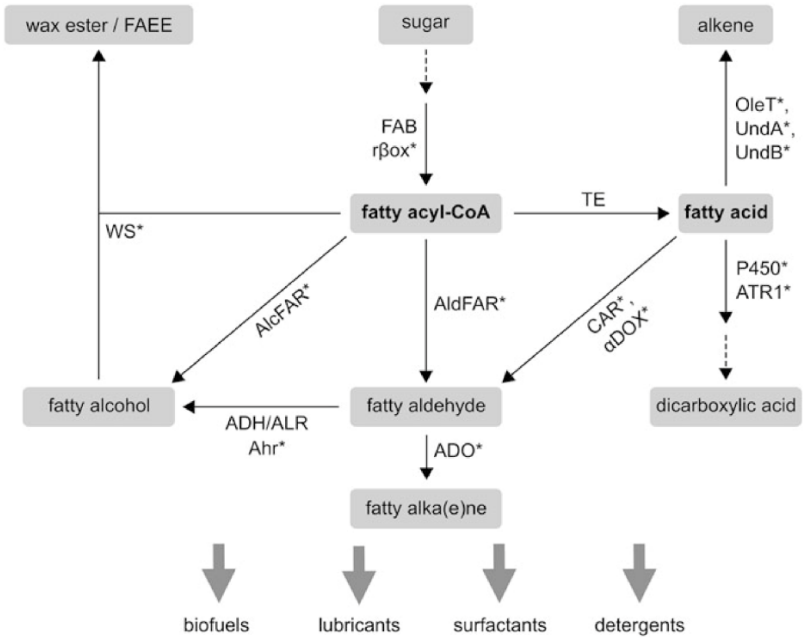

Fig. 3 Enzymatic routes for the production of FA-derived compounds in *S. cerevisiae*. FA-derived compounds can be synthesized from the end product of FA biosynthesis (FAB) or reverse β-oxidation (rβox), the fatty acyl-CoA, and from free FA by several heterologous enzymes in yeast. FAs result from the 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 reuptake 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 (Runguphan 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 agricultural 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 Deutsche Zusammenfassung

Viele wichtige Chemikalien und Stoffe werden von der petrochemischen Industrie aus Erdöl und der oleochemischen Industrie aus Pflanzenölen und Fetten gewonnen. Diese Produkte werden in den verschiedensten Bereichen der Industrie und des täglichen Lebens eingesetzt. So finden sie zum Beispiel Einsatz in Körperpflegeprodukten, in der Lebensmittel- und Pharmaindustrie oder als Kraftstoffe, Öle, Polymere und in vielen anderen Bereichen. Jedoch sind die fossilen Ressourcen endlich und geraten zunehmend durch ihren negativen Beitrag zum Klimawandel in die Kritik. Obwohl sie mit nachwachsenden Rohstoffen wie der Ölpalme oder der Kokosnuss arbeitet, ist auch die oleochemische Industrie aufgrund von negativen Auswirkungen auf die Umwelt zunehmend in die Kritik geraten. Ein steigender Bedarf an ihren Produkten hat zur Abholzung von Regenwäldern, den bevorzugten Anbaugebieten der Ölpalme, und an deren Stelle zu einem Ausbau von großen Monokulturen aus Ölpalmenplantagen geführt. Aufgrund dessen werden alternative, nachhaltige und umweltfreundliche Produktionsmethoden für eine Vielzahl von Oleochemikalien gesucht. Die Hauptprodukte der oleochemischen Industrie sind vor allem Fettsäuren und daraus abgeleitete Verbindungen wie Fettalkohole, Hydroxyfettsäuren und Dicarbonsäuren. Dabei sind Fettsäuren und deren Derivate mit kurzen oder mittellangen Kohlenstoffketten im Bereich von sechs bis zwölf Kohlenstoffatomen durch ihre vielseitigen Einsatzgebiete von besonderem Interesse.

Eine nachhaltige und umweltfreundliche Produktionsmethode für Oleochemikalien könnte die biotechnologische Produktion durch genetisch veränderte Organsimen sein. Dabei wird der Stoffwechsel eines Wirtsorganismus durch sogenanntes *metabolic engineering* gezielt so verändert, dass eine gewünschte Verbindung produziert wird. Grundlegende Methoden des *metabolic engineering* sind die Expression von heterologen Genen, die Überexpression oder Deletion von endogenen Genen oder Optimierung der beteiligten Enzyme. Das Ziel ist es, Zellfabriken oder Chassis-Stämme für eine robuste, effiziente und hohe Produktion zu erzeugen. Ein Organismus, der aufgrund seiner Eigenschaften wie Robustheit, einfacher Handhabung und wegen der Verfügbarkeit von vielen Methoden zur Genmanipulation oft verwendet wird, ist die vielseitige Modell- und Industriehefe *S. cerevisiae.*

In dieser Arbeit wurde die Hefe *S. cerevisiae* für eine Produktion von der C8 Fettsäure Oktansäure eingesetzt und durch *metabolic engineering* Strategien optimiert. Außerdem wurde eine *de novo* Produktion der von der Oktansäure abgeleiteten 8-Hydroxyoktansäure etabliert und optimiert. Die Oktansäure ist jedoch kein natürlicherweise in größeren Mengen vorkommendes Produkt der Hefe *S. cerevisiae.* Natürlicherweise liefert die Fettsäurebiosynthese Fettsäuren im Bereich mit einer Länge von 16-18 Kohlenstoffatomen. Jedoch wurde in einer vorherigen Arbeit die Oktansäure-Produktion über die Fettsäure-Biosynthese durch eine neu entwickelte mutierte Fettsäuresynthase (FAS^{RK}) ermöglicht. Durch den Einsatz der FAS^{RK} wird der natürliche Kettenlängenkontrollmechanismus hin zur Oktansäure manipuliert. Die FAS^{RK} Variante ist in der Lage, neben den essentiellen C16-C18 Fettsäuren auch Oktansäure zu produzieren. Um die Oktansäureproduktion in dieser Arbeit zu steigern wurden mögliche limitierende Faktoren und Reaktionen untersucht.

Die FAS der Hefe setzt sich aus zwei Untereinheiten (α und β) zusammen, die einen funktionalen Komplex aus α6β6 bilden. Die Bildung des FAS-Komplexes erfolgt cotranslational durch eine Interaktion der α- und β-Untereinheiten. Da dieser Schritt möglicherweise einer zellulären Regulation unterliegt, könnte er für eine Limitierung der Oktansäureproduktion sorgen. Daher wurden α- und β-Untereinheiten der Fettsäuresynthase zu einem einzigen Konstrukt ($fusFAS^{RK}$) genetisch fusioniert. Es zeigte sich, dass die Expression einer $fusFAS^{RK}$ im Vergleich zur FAS^{RK} zu einer höheren Produktion von Oktansäure führt. Dazu eingesetzt wurde ein zuvor etablierter *S. cerevisiae* Stamm, in dem beide FAS Gene *FAS1* und *FAS2* deletiert wurden. Zudem wurde die β-Oxidation, der Abbaumechanismus von Oktansäure, durch eine Deletion von *FAA2*, welches für eine Acyl-CoA Synthase codiert, blockiert. Die FAS^{RK}-Expression konnte dadurch als ein limitierender Schritt der Oktansäureproduktion identifiziert werden. Stämme, die Oktansäure oder andere kurz- und mittelange Fettsäuren produzieren, zeigen im Vergleich zu ihren parentalen Stämmen eine starkes Wachstumsdefizit, der für eine biotechnologische Anwendung unerwünscht ist und zu einer geringeren Produktion führen kann. Ein Grund dafür ist die stark inhibierende Eigenschaft der Oktansäure. Eine andere Möglichkeit besteht darin, dass die mutierte FAS nicht mehr genügend der essentiellen langkettigen Fettsäuren herstellt um ein normales Wachstum der Zellen zu ermöglichen. Um dies auszugleichen wurden die beiden mutierten FAS varianten (FAS^{RK} und $fusFAS^{RK}$) jeweils in einem Stamm mit genomischen WT-

FAS Allelen coexprimiert. Zudem wurden jeweils mutierte und WT-Varianten der fusionierten FAS und gespaltenen FAS in einem FAS-defizienten Stamm coexprimiert. In beiden Fällen zeigten sich jedoch verminderte Oktansäure-Titer im Vergleich zur Expression von ausschließlich einer (fus)FAS^{RK}, die möglicherweise durch physikalischen und/oder metabolischen Crosstalk der FAS-Varianten hervorgerufen werden.

Die Fettsäurebiosynthese, die im Zytosol der Hefe abläuft, wird eingeleitet durch die Bindung eines Acetyl-Restes an den FAS-Komplex, der von Acetyl-CoA übertragen wird. Zur Verlängerung der Fettsäurekette wird von Acetyl-CoA abgeleitetes Malonyl-CoA verwendet, welches mit dem gebundenen Acyl-Rest kondensiert wird. Zudem wird für jede Elongationsrunde NADPH zur Reduktion der Fettsäureketten benötigt. Da die Fettsäuresynthese strikt von diesen Vorläufern abhängt, wurden für eine Steigerung der Oktansäureproduktion verschiedene *engineering* Strategien für eine erhöhte Bereitstellung von Acetyl-CoA und NADHP untersucht. Der natürliche Bereitstellungsweg für zytosolisches Acety-CoA ist der sogenannte Pyruvatdehydrogenase-Bypass. Zur Steigerung der Acetyl-CoA Bereitstellung wurde zunächst der metabolische Fluss über diesen Stoffwechselweg durch Überexpression von Zielgenen erhöht. So wurden die Gene *AHD2*, welches für eine Alkoholdehydrogenase kodiert, *ALD6*, welches für eine $NADP^+$ abhängige Aldehyddehydrogenase kodiert und eine heterologe und mutierte Acetyl-CoA Synthase (*ACS1*^{L641P} aus *Salmonella enterica*) einzeln oder in Kombinationen überexprimiert. Es zeigte sich jedoch, dass dadurch keine gesteigerte Produktion von Oktansäure erreicht wird.

Als nächstes wurde die Expression eines alternativen heterologen Acetyl-CoA-Biosyntheseweges untersucht. In diesem Weg werden eine Xylulose-5-Phosphat spezifische Phosphoketolase eingesetzt, die Xylulose-5-Phosphat in Acetyl-Phosphat und Glyceraldehyd-3-Phosphat umwandelt. Das entstandene Acetyl-Phosphat wird dann durch eine Phosphotransacetylase in Acetyl-CoA konvertiert. Eine Xylulose-5-Phosphat spezifische Phosphoketolase wurde ausgewählt, da Xylulose-5-Phosphat ein Produkt aus dem Pentosephosphatweg ist, der für die hauptsächliche Bereitstellung von NADPH verantwortlich ist. Um die Xylulose-5-Phosphat- und die NADPH-Bildung zu erhöhen, wurde zudem der metabolische Fluss stärker in den Pentosephosphatweg geleitet. Dazu wurde der Fluss durch den Pyruvatdehydrogenase-Bypass und die Glykolyse durch eine Deletion des Acetaldehyddehydrogenase-Gens *ALD6* und

Herunterregulation des Phosphoglukose-Isomerase-Gens *PGI1* verringert. Durch Überexpression des *ZWF1*-Gens, das für das erste Enzym des oxidativen Pentosephosphatwegs (Glukose-6-Phosphat-Dehydrogense) kodiert, wurde der Fluss in den Pentosephosphatweg geleitet. Eine Expression des Phosphoketolase/Phosphotransacetylase-Wegs in Kombination mit der Umleitung des metabolischen Flusses in den Pentosephosphatweg erhöhte die Ausbeute der Oktansäureproduktion während der Wachstumsphase auf Glukose um 65 %. Weitere Stammmodifikationen zur Steigerung des Flusses über den Pentosephosphatweg durch eine zusätzliche Überexpression der Gene *ZWF1*, *SOL3* und *GND1*oder *RPE1* führten nicht zu einer weiteren Steigerung der Oktansäureproduktion. Auch die Expression des Phosphoketolase/Phosphotransacetylase-Wegs limitierte die Oktansäureproduktion nicht, da die Integration von jeweils einer weiteren genomischen Kopie keine Auswirkungen auf die erzielten Produkttiter hatte. Zudem konnte eine Kombination des Phosphoketolase/Phosphotransacetylase-Wegs mit *engineering* Strategien des Pyruvatdehydrogenase-Bypasses keine weitere Steigerung der Oktansäureproduktion herbeiführen. Im Einzelnen wurden die NAD^+ abhängige Aldehyde Dehydrogenase (*ALD2*) deletiert oder die $NADP^+$-abhängige Acetaldehyddehydrogenase (*ALD6*) durch einen Glukose-reprimierten/Ethanol-induzierten Promotor (*pADH2*) in der späteren Phase der Fermentation überexprimiert. Jedoch konnte dadurch keine Steigerung der Oktansäureproduktion erreicht werden.

Als Nächstes wurde getestet, ob eine Kombination der beiden erfolgreichen Strategien aus Expression der $fusFAS^{RK}$ und erhöhter Bereitstellung von Acetyl-CoA und NADPH durch Stamm-*Engineering* eine Steigerung der Oktansäureproduktion herbeiführt. Die Effekte waren aber nicht additiv und die Produktion veränderte sich nicht. Daraus lässt sich schließen, dass zusätzliche Faktoren eine Erhöhung der Produktion verhindern. Mögliche Gründe könnten die stark wachstumshemmenden Effekte der Oktansäure sein oder andere intrinsische Limitierungen, die durch die Mutation in der FAS entstehen könnten.

Neben kurzkettigen Fettsäuren sind viele davon abgeleitete Chemikalien wertvolle Stoffe für die Industrie. Eine Vielzahl von möglichen Anwendungen haben zum Beispiel Hydroxyfettsäuren, die über eine zusätzliche Hydroxylgruppe verfügen, oder Dicarbonsäuren, die über zwei Carboxylgruppen verfügen. Von besonderem Interesse sind dabei Hydroxyfettsäuren oder Dicarbonsäuren mit jeweils endständigen funktionellen Gruppen (ω-Hydroxyfettsäuren und α,ω-

Dicarbonsäuren). Zunächst wurden die wachstumsinhibierenden Eigenschaften von ω-Hydroxyfettsäuren und α,ω- Dicarbonsäuren mit einer Länge von acht bis zehn Kohlenstoffen untersucht und zeigten eine geringere Wachstumsbeeinträchtigung als die entsprechenden Fettsäuren. Dann wurde die Bildung von 8-Hydroxyoktansäure aus zugefütterter Oktansäure in einer Biokonversion durch eine terminale Hydroxylierung der Oktansäure angestrebt. Dazu wurde die Expression von drei heterologen Gene, die für verschiedene Cytochrom P450-Enzyme codieren und zusammen mit ihren zugehörigen Cytochrom P450-Reduktasen exprimiert wurden, getestet und verglichen. Zudem wurde die Verwendung verschiedener Kohlenstoffquellen, wie Glukose, Galaktose und Ethanol/Glycerin für die Biokonversion untersucht. Ein Cytochrom P450 aus *Fusarium oxysporum f. sp. Lycopersici* bewirkte die höchste Produktion, wenn Ethanol/Glycerin als Kohlenstoffquelle verwendet wurde. Anschließend wurde für die *de novo* Produktion von 8-Hydroxyoktansäure eine endogene Oktansäureproduktion durch eine mutierte FAS^{RK} mit der Hydroxylierungsreaktion von Cytochrom P450/Cytochrom P450 Reduktase in einem Stamm kombiniert. Dieser Ansatz ermöglichte eine Produktion von $3\ mgL^{-1}$ 8-Hydroxyoktansäure. Es zeigte sich jedoch, dass am Ende der Fermentation noch große Mengen der Oktansäure im Medium vorhanden waren, die nicht zur 8-Hydroxyoktansäure umgesetzt wurden. Dies deutet darauf hin, dass die Aktivität des Cytochrom P450 und seiner zugehörigen P450-Reduktase die 8-Hydroxyoctansäure-Produktion limitiert.

Zusammenfassend wurde in dieser Arbeit die Hefe *S. cerevisiae* erfolgreich durch *metabolic enigneering* Strategien für eine Produktion von Oktansäure optimiert. Es wurde gezeigt, dass die FAS selbst ein limitierender Faktor in der Oktansäure Produktion ist und dass eine Fusion der beiden FAS Gene *FAS1* und *FAS2* zu einer Steigerung der Oktansäureproduktion führt. Eine Co-Expression von einer WT-FAS mit der mutierten fusionierten FAS führte jedoch zu einer Verringerung des Produktionstiters. Zudem wurde der Stoffwechsel von *S. cerevisiae* zur Bereitstellung der Ausgangstoffe für die Fettsäurebiosynthese optimiert. Dazu wurden verschiedene erfolgsversprechende Strategien angewendet und verglichen. Aktuelle Herausforderungen und Limitierungen in der Oktansäureproduktion durch eine mutierte FAS wurden entdeckt und skizziert. Darüber hinaus, wurde die hier verwendete Plattform zur Produktion von Oktansäure durch ein Cytochrom P450 erfolgreich erweitert und die erste *de novo* Produktion von 8-Hydroxyoktansäure in *S. cerevisiae* erreicht.

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8 Curriculum vitae