THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Synthesis of jojoba-like wax esters in metabolically engineered strains of *Saccharomyces cerevisiae*

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Cover:
An engineered yeast cell producing the three most common jojoba-like wax esters, docosenoyl eicosenoate (C22:1-C20:1), eicosenoyl eicosenoate (C20:1-C20:1) and eicosenoyl docosenoate (C20:1-C22:1).

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Abstract

Yeast has a long-standing tradition in human history as a production organism of choice. Besides being used for the production of fermented products like bread or beer, it has also been extensively explored for the production of proteins and chemicals. In the past, yeast research in this respect was often focused on the production of biofuels as an alternative to fossil fuels to enable the independence of crude oil. However, crude oil also functions as a source for a wide range of chemicals.

Some of these chemicals can be substituted by alternatives derived from plant oils, like jojoba oil. Jojoba seeds contains approximately 50% (w/w) oil, which consists mostly (up to 97%) of wax esters (WEs). Minor parts include phytosterols, triacylglycerols (TAGs) and fatty alcohols (FOHs). This makes jojoba exceptional, since plants usually accumulate TAGs as storage compounds. Jojoba oil can among other applications be used in cosmetic and personal care products as well as lubricants. Currently, around 4,000 tons/year of jojoba oil is produced, with an estimated demand of up to 200,000 tons/year. Because of this, oil produced from the jojoba plant will not be enough to meet the demand in the future, even if huge land areas in various parts of the world are planted.

Therefore, jojoba oil production in modified microorganisms represents a very promising approach. In this thesis, the yeast *Saccharomyces cerevisiae* was explored as a production organism for jojoba-like WEs. Jojoba-like WEs are naturally derived from the fatty acid (FA) metabolism of the plant, more specifically from very long-chain monounsaturated fatty acids (VLCMUFAs) with a carbon chain length of 20/22 (C20/C22). These VLCMUFAs can be converted to FOHs by the action of fatty acyl reductases (FARs). WEs are synthesized by wax synthases (WSs), which esterify an activated FA (fatty acyl-CoA) with a FOH molecule.

In this thesis, the synthesis of jojoba-like WEs in *S. cerevisiae* was established by making use of various enzymes derived from bacterial and plant sources as well as tuning *S. cerevisiae* FA metabolism towards the increased synthesis of VLCMUFAs. In this way a *S. cerevisiae* strain was created that produces 14.38 ± 1.76 mg WEs/g CDW. Of these WEs, 39.2 mol% are jojoba-like diunsaturated C38:2-WEs to C42:2-WEs, with the most abundant ones being C42:2-WEs (18.3 mol% of all WE species). These are also the most abundant WEs in natural jojoba oil (46.8 mol% of all WE species).

Keywords: *Saccharomyces cerevisiae*, very long-chain monounsaturated fatty acids, very long-chain monounsaturated fatty alcohols, jojoba-like wax esters

List of Publications

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

(I) **Wenning L, Yu T, David F, Nielsen J, Siewers V.** 2017. Establishing very long-chain fatty alcohol and wax ester biosynthesis in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **114**:1025–1035.

(II) **Yu T, Zhou YJ, Wenning L, Liu Q, Krivoruchko A, Siewers V, Nielsen J, David F.** 2017. Metabolic engineering of *Saccharomyces cerevisiae* for production of very long chain fatty acid-derived chemicals. *Nat. Commun.* **8**:15587.

(III) **Wenning L, Ejsing CS, David F, Sprenger RR, Nielsen J, Siewers V. 2018.** Increasing jojoba-like wax ester production in *Saccharomyces cerevisiae* by enhancing very long-chain, monounsaturated fatty acid synthesis. *Submitted manuscript*.

(IV) **Bergman A, Wenning L, Siewers V, Nielsen J.** 2018. Investigation of putative regulatory acetylation sites in Fas2p of *Saccharomyces cerevisiae*. *Manuscript, available at bioRxiv* (https://doi.org/10.1101/430918).

Contribution to manuscripts

Paper I

Designed the study, performed the experimental work, analyzed the data and wrote the manuscript.

Paper II

Performed part of the cloning and assisted in writing the manuscript.

Paper III

Designed the study, performed the experimental work (except for the analysis of the molecular composition of the wax esters), analyzed the data (except for the molecular composition of the wax esters) and wrote the manuscript.

Paper IV

Took part in performing experimental work, helped analyzing the data and writing the manuscript.

Preface

This dissertation serves as partial fulfillment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden. The PhD project was carried out between April 2014 and March 2018 in the Division of Systems and Synthetic Biology under the supervision of Dr. Verena Siewers and co-supervision of Prof. Jens Nielsen. In this project, the yeast *Saccharomyces cerevisiae* has been metabolically engineered to produce jojoba-like wax esters. The research was mainly funded by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 606795, project acronym: YEASTCELL. Other funding sources include the Swedish Foundation for Strategic Research (SSF), the Novo Nordisk Foundation, the Knut and Alice Wallenberg Foundation and the European Union's Horizon 2020 Framework Programme for Research and Innovation – Grant Agreement No. 720824.

Leonie Wenning

October 2018

In memory of a good friend and colleague –
Shanna Sichwart (*30.01.1984 ✞04.01.2018)

Everything will be okay in the end. If it's not okay, it's not the end.

- ***John Lennon***

Acknowledgements

I am very grateful to my co-supervisor Prof. Jens Nielsen and my main supervisor Dr. Verena Siewers for giving me the opportunity to prove myself by following the PhD studies at the division of Systems and Synthetic Biology (Sysbio) at Chalmers University of Technology. I still remember the phone interview very well that I had with Verena and Jens in autumn 2013 for the position as a "Early Stage Researcher" in the YEASTCELL ITN and Jens asking me if I am aware of that it is an additional (at least) 4 years of research lying ahead of me, before (hopefully) reaching the finish line. Also, the personal interview with Verena in January 2014 in Delft was a great and new experience for me, since I directly met some of my new colleagues of the YEASTCELL ITN.

In connection to that, I want to thank my fellow PhD students of the YEASTCELL ITN, Anja, Anna (Meier), Ângela, Manu, David, Frederico, Javier, Matthias, Nuri, and Raúl, for being such a great crowd to be with. Somehow it felt like we were a natural group right from the start and it was very helpful to have some "fellow sufferers" along the way. At the end of YEASTCELL (or probably already half way through) it was not only the common goal that kept us together, but also and in particular friendship. This thanks also goes to all the PIs in YEASTCELL who did a very great job at educating us as well as guiding us along the way.

I would also like to thank all the great people I met at Sysbio, especially Alexandra, Anna (Wronska), Ievgeniia, Kathleen, Min, Stefan, Yassi and Yating for being great office mates, colleagues and friends to discuss with about science and life in general. Also, a special thanks to Kathleen and Yassi for our short, but intensive Tysk/Svensk learning lunch group. I would also like to thank Florian and Tao for a great collaboration and input on various projects and the whole "metabolic engineering" formerly "biofuels" subgroup for all the meetings and valuable discussions about science. Moreover, I would like to thank all the current and previous research engineers at Sysbio and the people from Chalmers Mass Spectrometry Infrastructure (CMSI) for supporting the lab and our research efforts. I would also like to thank Rahul for supplying Alexandra and me with important data on acetylation for the study on acetylation sites of Fas2p. In addition to that I would like to thank Associate Prof. Christer S. Ejsing for his help with the identification of the molecular composition of the wax ester species.

A very special thanks goes to my dear friends from the two places that I consider home, Bocholt and Münster: Angela, Kati, Lena, Stephi, Dominique, Elena, Franzi, Gabriel, Gundi, Renata, Shanna and Steff. Thanks for always being there and supporting me in different ways.

My biggest thanks goes to my family, my husband Klaas, my parents and Timo. Thank you for your continuous love and support.

Leonie

List of abbreviations

acetyl-CoA synthetase	-	ACS
Acinetobacter baylyi ADP1	-	*Ab*
adenosine diphosphate	-	ADP
adenosine monophosphate	-	AMP
adenosine triphosphate	-	ATP
alcohol dehydrogenase	-	ADH
aldehyde dehydrogenase	-	ALD
amino acid	-	AA
AMP activated protein kinase	-	AMPK
Apis mellifera	-	*Am*
Arabidopsis thaliana	-	*At*
atmospheric pressure chemical ionization	-	APCI
Brassica napus	-	*Bn*
Calanus hyperboreus	-	*Ch*
cell dry weight	-	CDW
Crambe abyssinica	-	*Ca*
deoxyribonucleic acid	-	DNA
diunsaturated wax ester	-	DUWE
electron ionization	-	EI
electrospray ionization	-	ESI
endoplasmic reticulum	-	ER
enoyl-CoA reductase (ECR)	-	ECR
Euglena gracilis	-	*Eg*
fatty acid desaturase	-	FAD

fatty acid elongase (β-ketoacyl-CoA synthase)	-	FAE/KCS
fatty acid methyl ester	-	FAME
fatty acid synthase	-	FAS
fatty acid	-	FA
fatty acyl reductase	-	FAR
fatty acyl-ACP	-	FAACP
fatty acyl-CoA	-	FACoA
fatty alcohol	-	FOH
fatty aldehyde reductase	-	FAldhR
fatty aldehyde	-	FAldh
flame ionization detector	-	FID
Fourier transform	-	FT
free fatty acid	-	FFA
cell fresh weight	-	CFW
gas chromatography	-	GC
glucose-6-phosphate dehydrogenase	-	G6PH
glycosylphosphatidylinositol	-	GPI
high erucic acid	-	HEA
high pressure liquid chromatography	-	HPLC
inositol/choline-responsive element	-	ICRE
inositolphosphoryl ceramide	-	IPC
inositol-responsive upstream activating sequence	-	UAS_{INO}
low erucic acid	-	LEA
Lunaria annua	-	*La*
lysine acetyltransferase	-	KAT

lysine deacetylase	-	KDAC
mannose-diinositolphosphoryl ceramide	-	$M(IP)_2C$
mannose-inositol-phosphoryl ceramide	-	MIPC
Marinobacter aquaeolei VT8	-	*Ma*
mass spectrometry	-	MS
monounsaturated fatty acid	-	MUFA
monounsaturated fatty alcohol	-	MUFOH
nicotinamide adenine dinucleotide	-	NAD^+/NADH
nicotinamide adenine dinucleotide phosphate	-	$NADP^+$/NADPH
pentose phosphate pathway	-	PPP
phosphatidic acid	-	PA
phospholipid	-	PL
phytyl ester synthase	-	PES
polyhydroxyalkanoates	-	PHAs
pyruvate decarboxylase	-	PDC
pyruvate dehydrogenase	-	PDH
ribonucleic acid	-	RNA
Saccharomyces cerevisiae	-	*Sc*
Salmonella enterica	-	*Se*
Simmondsia chinensis	-	*Sci*
steryl ester	-	SE
total fatty acids	-	TFAs
transmembrane helix	-	TMH
triacylglycerol	-	TAG
tricarboxylic acid cycle	-	TCA

Triticum aestivum	-	*Ta*
Tropaeolum majus	-	*Tm*
unsaturated fatty acid	-	UFA
(very) long-chain fatty acid	-	(V)LCFA
(very) long-chain fatty acyl-CoA	-	(V)LCFACoA
(very) long-chain fatty alcohol	-	(V)LCFOH
(very) long-chain monounsaturated fatty acid	-	(V)LCMUFA
(very) long-chain wax ester	-	(V)LCWE
wax ester	-	WE
wax synthase	-	WS
wax synthase/diacylglycerol acetyltransferase	-	DAcT
wax synthase/diacylglycerol acyltransferase	-	WS/DGAT(D)
β-hydroxyacyl-CoA dehydratase	-	HCD
β-ketoacyl-CoA reductase	-	KCR
β-ketoacyl-CoA synthase (fatty acid elongase)	-	KCS (FAE)

Nomenclature of fatty acids, fatty alcohols and wax esters

fatty acid

CX:Y-FA, X indicating the number of carbon atoms and Y indicating the number of double bonds (if the position of the double bond is indicated, it is counted from the carboxyl group (Δ) and unless otherwise noted, the Z (cis) species (e.g. C16:1Δ9-FA, cis-Δ9 hexadecenoic acid)

hydroxylated fatty acid

CX:Y-OH, X indicating the number of carbon atoms and Y indicating the number of double bonds

fatty alcohol

CX:Y-FOH, X indicating the number of carbon atoms and Y indicating the number of double bonds

wax ester

CX:Y-WE, X indicating the total number of carbon atoms and Y indicating the total number of double bonds

molecular wax ester species

$CX_1:Y_1$-$CX_2:Y_2$, the first part of the abbreviation referring to the fatty alcohol moiety and the second part referring to the fatty acyl-CoA moiety; X indicating the number of carbon atoms in the alcohol and acyl moiety, respectively, and Y indicating the number of double bonds in each moiety

Gene and protein nomenclature

In this thesis, the *Saccharomyces cerevisiae* "Genetic Nomenclature Guide" (1998) for genes and proteins has been followed.

This means that gene symbols comprise three italic letters, and an Arabic number, with lowercase italic used for recessive alleles (e.g. *gen1*) and uppercase italic used for dominant alleles (e.g. *GEN1*).

Allele designations consist of the gene symbol, a hyphen and an italic Arabic number (e.g. *gen1-1*). Alleles created by recombinant DNA technology are named by use of the symbol for the gene that is altered, followed by a symbol to indicate the nature of the alteration: disruption (::) (e.g. *GEN1*::*GEN2*, a disruption of the gene *GEN1* by integration of the functional gene *GEN2*); deletion (Δ) (e.g. *GEN1*Δ, deletion of the *GEN1* gene); replacement (Δ::) (e.g. *GEN1*Δ::*GEN2*, replacement of *GEN1* by the *GEN2* gene).

For mating-type loci, special rules apply: the two wildtype alleles of the mating-type (*MAT*) locus are designated as *MAT*a and *MAT*α.

Proteins are referred to by the relevant gene symbol, non-italic, initial letter uppercase and with the suffix "p" (e.g. Gen1p). If unambiguous, the suffix can be omitted e.g. "the Gen1 protein".

If an amino acid in a protein is exchanged against another amino acid, this is indicated by the gene symbol followed by the abbreviation of the wildtype amino acid, its position and the mutant amino acid written in superscript (e.g. *GEN1*^{a1b}, replacement of amino acid "a" at position 1 by amino acid "b").

Contents

1 Introduction

1.1 *Saccharomyces cerevisiae* as a production organism

S. cerevisiae as a production organism has a long-standing history. It has been used to naturally produce fermented products like beer, bread and wine for centuries (Legras et al., 2007), but is also an important organism for the heterologous production of proteins (Bonander and Bill, 2012; Martínez et al., 2012; Porro et al., 2005) and chemicals (Krivoruchko and Nielsen, 2015; Marella et al., 2018).

To produce proteins and chemicals in *S. cerevisiae*, its metabolism needs to be modified and additional genes might need to be introduced or intrinsic genes overexpressed or deleted. The advantages of *S. cerevisiae* as a production host are that it is very efficient in fermenting sugars to ethanol, which makes it a favorable organism in beer and wine production, but also to produce bioethanol. Moreover, it shows a fast growth rate and is robust towards low pH, high sugar and ethanol concentrations, high osmotic pressure as well as general fermentation inhibitors (Hahn-Hägerdal et al., 2007). Another point making it an attractive choice is that it has a "generally recognized as safe" (GRAS) status (Borodina and Nielsen, 2014). Moreover, since it is extensively used in industry, a broad knowledge about its metabolism has been acquired and a range of useful evolutionary and metabolic engineering tools have been developed (Çakar et al., 2012; Krivoruchko and Nielsen, 2015). In terms of protein production, another advantage of *S. cerevisiae* compared to bacterial hosts is that it possesses the ability of post-translational modifications and secretion of proteins (Schmidt 2004).

One of the most prominent examples for protein production in *S. cerevisiae* concerns insulin, which is used in the treatment of diabetes (Kjeldsen et al., 1999; Kjeldsen et al., 2002; Liu et al., 2012). In terms of production of chemicals in yeast, interesting targets include alkanes/alkenes (usable as advanced biofuels), 1-butanol (usable as biofuel and chemical building block), fatty acids (FAs) (used in the production of biodiesel, dietary supplements and pharmaceuticals), fatty alcohols (FOHs) (usable as biofuels and chemical building blocks), isoprenoids (a very broad class of chemicals used as biofuels, dietary supplements, fine fragrances, flavors, pharmaceuticals and vitamins), polyhydroxyalkanoates (PHAs) (biopolymers), polyketides (used as pharmaceuticals), polyphenols (another broad class of chemicals used as antioxidants or neutraceutical ingredients), sterols (used as dietary supplements) and wax esters (WEs) (usable in detergents, as lubricants and in cosmetics) (Krivoruchko et al., 2015; Nielsen, 2014). All aforementioned biochemicals are derived from the central carbon metabolism in yeast, more precise from the precursor molecule acetyl-CoA which will be described in more detail in the following section.

1.1.1 Acetyl-CoA metabolism in *S. cerevisiae*

When *S. cerevisiae* is grown on glucose, glucose is transported via different hexose transporters (HXTs) inside the yeast cell, where it enters glycolysis and is transformed through several steps into pyruvate (**Figure 1**) (Boles and Hollenberg, 1997). Pyruvate can then further be converted into acetyl-CoA in the cytosol in three consecutive steps. In the first step, pyruvate is decarboxylated to acetaldehyde, catalyzed by a pyruvate decarboxylase (PDC). *S. cerevisiae* possesses three PDCs (Pdc1p, Pdc5p and Pdc6p), whereof Pdc1p is most important in glucose rich medium (Pronk et al., 1996). Acetaldehyde can then either be converted into ethanol, catalyzed by an alcohol dehydrogenase (ADH) or it can be converted to acetate by an aldehyde dehydrogenase (ALD). The former is the preferred reaction in high glucose concentrations, the latter occurs to a lower extent and is the second step in cytosolic acetyl-CoA formation (Gombert et al., 2001; Heyland et al., 2009). Of the five different ALDs present in *S. cerevisiae*, Ald6p is the one responsible for most of the acetate formation in the cytosol in glucose rich medium (Dickinson, 1996; Meaden et al., 1997). Ald2p and Ald3p are cytosolic enzymes that are involved in the synthesis of β-alanine which is an intermediate in pantothenic acid (vitamin B5) and coenzyme A (CoA) synthesis (White et al., 2003). Ald4p and Ald5p are mitochondrial enzymes with Ald4p being the major mitochondrial form (Boubekeur et al., 1999; Boubekeur et al., 2001; Remize et al., 2000). The expression of *ALD2*, *ALD3* and *ALD4* is repressed by glucose, whereas *ALD5* and *ALD6* are constitutively expressed (Tessier et al., 1998; Wang et al., 1998). The last step in cytosolic acetyl-CoA formation is catalyzed by one of two acetyl-CoA synthetases (ACSs) in *S. cerevisiae* (Acs1p and Acs2p). In glucose containing medium, *ACS1* is repressed and Acs2p is solely responsible for acetyl-CoA formation in the cytosol (van den Berg et al., 1996; Kratzer and Schüller, 1995; Starai and Escalante-Semerena, 2004; Takahashi et al., 2006). Besides being localized in the cytosol, Acs1p was also detected in peroxisomes and the nucleus, whereas Acs2p localizes to the cytosol, the nucleus and perhaps the endoplasmic reticulum (ER) (Chen et al., 2012; Huh et al., 2003; Kals et al., 2005; Takahashi et al., 2006).

Some of the cytosolic pyruvate enters the mitochondria, via the mitochondrial pyruvate carrier (MPC) (Bricker et al., 2012; Herzig et al., 2012). Another source of mitochondrial pyruvate is malic enzyme (Mae1p) which catalyzes the oxidative decarboxylation of malate to pyruvate (**Figure 2**) (Boles et al., 1998). Pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) which consists of several subunits (Behal et al., 1989; Dickinson et al., 1986; Lawson et al., 1991; Maeng et al., 1994; Mortimer et al., 1992; Niu et al., 1988; Steensma et al., 1990). Mitochondrial acetyl-CoA can either enter the tricarboxylic acid cycle (TCA cycle) via a citrate synthase (Cit1p/Cit3p), be used as an acetyl donor for protein acetylation or be converted to acetate. Cit1p and Cit3p catalyze the condensation of acetyl-CoA and oxaloacetate to form citrate which is considered the rate limiting step of the TCA cycle. Cit1p is the major functional citrate synthase in the mitochondria, whereas Cit3p is only a minor functional isoform (Jia et al., 1997; Suissa et al., 1984). Generally, glucose represses the TCA cycle and thereby respiration (Gombert et al., 2001; Heyland et al., 2009; Weinert et al., 2014).

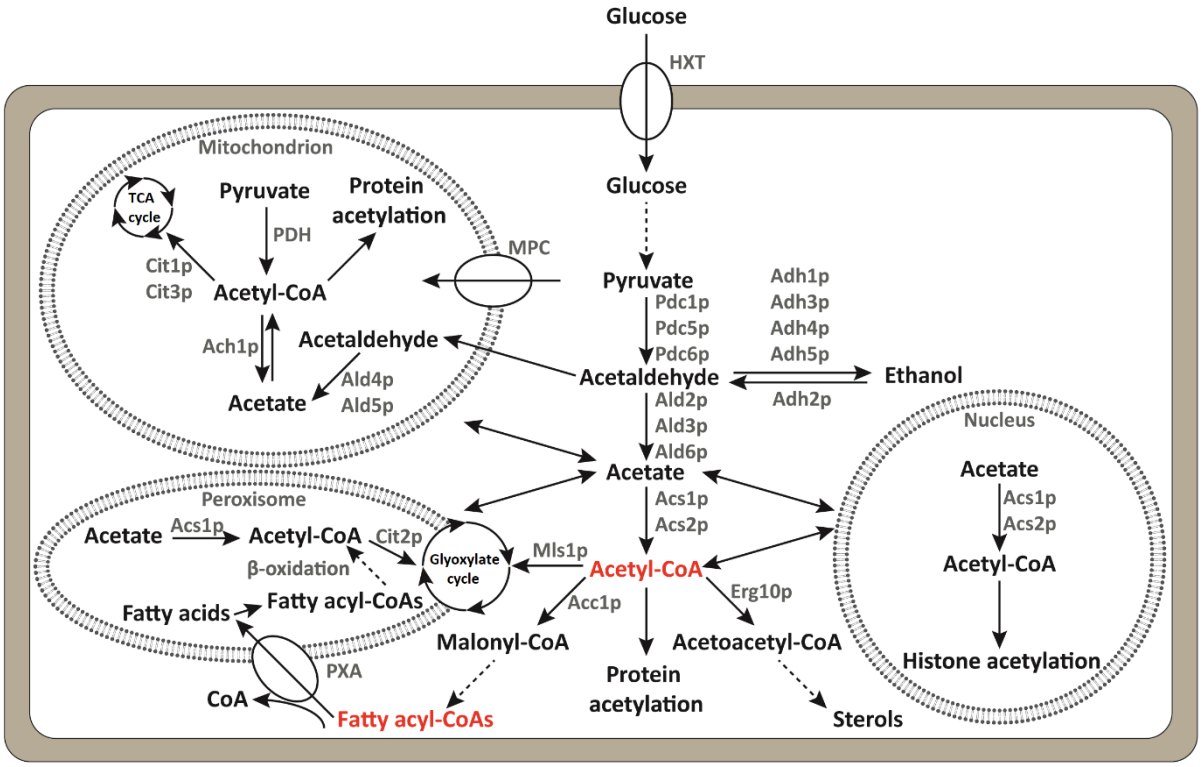

Figure 1 General acetyl-CoA metabolism in *S. cerevisiae*. Acc1p, acetyl-CoA carboxylase; Ach1p, acetyl-CoA hydrolase; Acs1p/Acs2p, acetyl-CoA synthetase; Adh1p/Adh2p/Adh3p/Adh4p/Adh5p, alcohol dehydrogenase; Ald2p/Ald3p/Ald4p/Ald5p/Ald6p, aldehyde dehydrogenase; Cit1p/Cit2p/Cit3p, citrate synthase; Erg10p, acetyl-CoA C-acetyl transferase (acetoacetyl-CoA thiolase); HXT, hexose transporter; Mls1p, malate synthase; MPC, mitochondrial pyruvate carrier; Pdc1p/Pdc5p/Pdc6p, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex; PXA, peroxisomal ABC transporter; TCA cycle, tricarboxylic acid cycle. Adapted from Galdieri et al. (2014). ⇢ indicates multiple enzymatic steps and → indicates a single enzymatic step.

Peroxisomal acetyl-CoA is the product of FA degradation via β-oxidation but is also synthesized from acetate, catalyzed by Acs1p. The transport of FAs from the cytosol into peroxisomes occurs via a peroxisomal ABC transporter (PXA), consisting of Pxa1p and Pxa2p (Hettema et al., 1996; Shani et al., 1995; Shani et al., 1996; Shani and Valle, 1996). New evidence suggests that cytosolic fatty acyl-CoAs (FACoAs) are hydrolyzed by the PXA complex before their FA moieties are transported into peroxisomes where they are re-esterified by the acyl-CoA synthetases Faa2p and Fat1p prior to their oxidation (van Roermund et al., 2012). Acetyl-CoA in the nucleus is synthesized from acetate catalyzed by Acs1p and Acs2p and functions as a substrate for histone acetylation.

A study in which the *Salmonella enterica* Acs (*Se*Acs) was targeted either to the nucleus, the cytosol or the mitochondria in a yeast strain harboring a temperature sensitive *ACS2* allele (*ACS2-TS1*) showed that only the variants targeted to the nucleus and the cytosol could complement the strain, but not the variant which was targeted to the mitochondria. This suggests that the acetyl-CoA in the cytosol and the nucleus forms a single pool and that acetyl-CoA can pass freely through the nuclear pore complex, but that mitochondrial and nucleocytosolic acetyl-CoA pools are biochemically separated (Takahashi et al., 2006). It has also been shown that the membrane of peroxisomes is impermeable to acetyl-CoA and NAD(H). This demonstrates that acetyl-CoA is compartmentalized in the mitochondria, the nucleocytosol and the peroxisomes and cannot directly be transported between these compartments. Therefore, the cell developed different transport systems for acetyl-CoA (van Roermund et al., 1995).

One way for the cell to shuttle acetyl-CoA between different compartments is via acetate which can freely diffuse between the cytosol, the mitochondria, the nucleus and peroxisomes. In the mitochondria, acetyl-CoA can be hydrolyzed to acetate and CoA catalyzed by the acetyl-CoA hydrolase Ach1p, whose expression is repressed in glucose (Buu et al., 2003; Lee et al., 1989; Lee et al., 1990). Ach1p was shown to also catalyze the CoA transfer from succinyl-CoA to acetate to form acetyl-CoA and succinate, which is the preferred reaction of the enzyme (Fleck and Brock, 2009). In a recent study with a PDC-negative *S. cerevisiae* strain also containing a deletion in *MTH1*, it was shown that Ach1p can potentially also catalyze the transfer of CoA from acetyl-CoA to succinate to form acetate and succinyl-CoA under glucose derepressed conditions (Chen et al., 2015).

A second way for acetyl-CoA transport is via acetyl-carnitine which can be formed in the cytosol, the mitochondria and the peroxisomes from carnitine and acetyl-CoA by carnitine acetyl-CoA transferases (CATs). Three CATs are known in yeast with Cat2p being localized in peroxisomes and mitochondria, Yat2p in the cytosol and Yat1p in the outer mitochondrial membrane. Cat2p has been identified as the main CAT in cells grown on FAs, whereas Yat2p becomes important in ethanol-grown cells (Elgersma et al., 1995; Swiegers et al., 2001). Yat1p is induced by ethanol, but only has a minor contribution to CAT activity (Schmalix and Bandlow, 1993). Acetyl-carnitine can be transported from the peroxisomes to the cytosol and from the cytosol into mitochondria via the transporter Crc1p (Palmieri et al., 1999; van Roermund et al., 1999). However, the carnitine/acetyl-carnitine shuttle is only active when carnitine is externally supplied, since it has been shown that *S. cerevisiae* is not able to *de novo* synthesize carnitine (van Roermund et al., 1999).

The third option for shuttling of acetyl-CoA is the glyoxylate cycle, which is located in the cytosol and the peroxisomes and is essential for growth on C_2 compounds like acetate and ethanol, but also for degradation of FAs via β-oxidation (**Figure 2**) (van Roermund et al., 1995). Since it requires the TCA cycle as well as gluconeogenesis to function, all three pathways are coordinately regulated. The glyoxylate cycle has an anaplerotic role by providing the TCA cycle with succinate. The acetyl-CoA pool in the peroxisomes is connected to the glyoxylate cycle via the citrate synthase Cit2p which catalyzes the conversion of acetyl-CoA and oxaloacetate (Oac) to citrate (Kim et al., 1986; Lewin et al., 1990). The net reaction of the glyoxylate cycle is the formation of 1 mol succinate, 2 mol CoA and 1 mol NADH + H^+ from 2 mol of acetyl-CoA and 1 mol NAD^+. Succinate is transported into the mitochondria where it is converted to malate in the TCA cycle. Malate can then be transported into the cytosol where it is converted to oxaloacetate by malate dehydrogenase (Mdh2p) (Minard and McAlister-Henn, 1991), further to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Pck1p) and finally to glucose (Valdes-Hevia et al., 1989). The reverse reaction, the formation of pyruvate from phosphoenolpyruvate, is catalyzed by a pyruvate kinase (Pyk1p = Cdc19p, Pyk2p) (Boles et al., 1997; Mortimer et al., 1989). The malate synthase Mls1p connects the cytosolic acetyl-CoA pool with the glyoxylate cycle by catalyzing the formation of malate from glyoxylate and acetyl-CoA. This enzyme is localized in the cytosol when cells are grown

on two carbon compounds but localizes to the peroxisomes under growth on oleic acid (Hartig et al., 1992; Kunze et al., 2002). Although many reactions of the glyoxylate cycle in the peroxisomes are identical with the ones of the TCA cycle in the mitochondria, they are still catalyzed by different isoenzymes. The key steps of the glyoxylate cycle are the ones catalyzed by isocitrate lyase (Icl1p) (Fernández et al., 1992), malate synthase (Mls1p) and malate dehydrogenase (Mdh2p/Mdh3p)(Minard and McAlister-Henn, 1991; Steffan and McAlister-Henn, 1992). These enzymes are repressed in medium containing glucose and derepressed in non-glucose containing medium (Duntze et al., 1969).

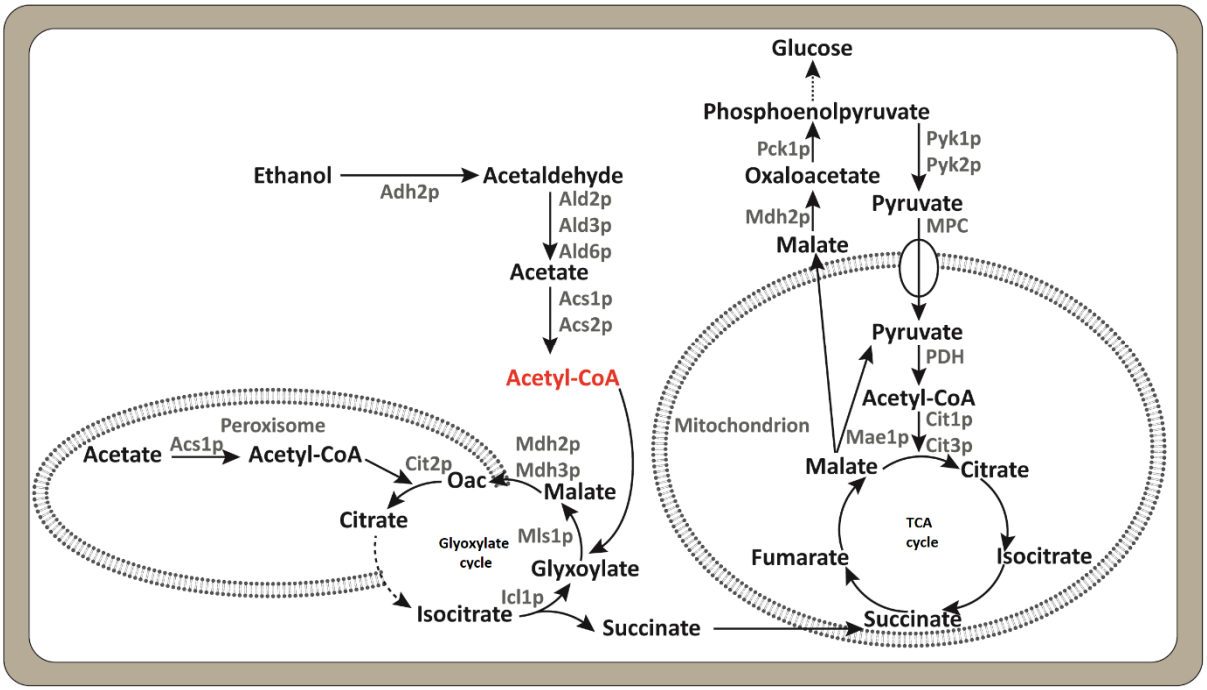

Figure 2 Acetyl-CoA metabolism in *S. cerevisiae* grown on C_2 compounds. Acs1p, acetyl-CoA synthetase; Adh2p, alcohol dehydrogenase; Ald2p/Ald3p/Ald6p, aldehyde dehydrogenase; Cit1p/Cit2p/Cit3p, citrate synthase; Icl1p, isocitrate lyase; Mae1p, malic enzyme; Mdh2p/Mdh3p, malate dehydrogenase; Mls1p, malate synthase; MPC, mitochondrial pyruvate carrier; Pck1p, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase complex; Pyk1p = Cdc19p/Pyk2p, pyruvate kinase; TCA cycle, tricarboxylic acid cycle. ⇢ indicates multiple enzymatic steps and → indicates a single enzymatic step.

Acetyl-CoA is not only used for the generation of energy in the form of adenosine triphosphate (ATP) via the TCA cycle and respiration, and as an acetyl donor for protein acetylation but is also an important building block for cellular constituents. The acetyl-CoA carboxylase Acc1p converts acetyl-CoA to malonyl-CoA which is the building block for FAs (Al-Feel et al., 1992; Roggenkamp et al., 1980). Malonyl-CoA is also the precursor for industrially relevant polyketides and polyphenols (Nielsen, 2014). Acetoacetyl-CoA is the product of the acetyl-CoA C-acetyl transferase (acetoacetyl-CoA thiolase) Erg10p in the cytosol and is the precursor for sterols like ergosterol which is an important membrane constituent in *S. cerevisiae* (Hiser et al., 1994; Karst and Lacroute, 1977). Acetoacetyl-CoA is also the precursor for industrially interesting compounds like PHAs, 1-butanol and isoprenoids. FAs in turn are the precursors for the industrially interesting compounds alkanes/alkenes, FOHs as well as WEs (Nielsen, 2014). The next section will therefore describe the FA metabolism in *S. cerevisiae.*

1.1.2 Fatty acid metabolism in *S. cerevisiae*

FA biosynthesis is dependent on acetyl-CoA as a precursor. *S. cerevisiae* possesses a mitochondrial and a cytosolic FA machinery. The main function of mitochondrial FA biosynthesis is probably the formation of octanoyl-ACP, a precursor of lipoic acid, an essential cofactor in oxidative decarboxylation reactions. Mutants lacking mitochondrial FA biosynthesis are respiratory incompetent, but viable, which indicates that this pathway is not needed for bulk FA biosynthesis (Tehlivets et al., 2007). Therefore, only the cytosolic pathway will be discussed further. Cytosolic FA biosynthesis in *S. cerevisiae* releases FACoAs, which can be used for the synthesis of membrane lipids, including phospholipids (PLs), sphingolipids and cardiolipins (Klug and Daum, 2014), or for the synthesis of storage lipids, including steryl esters (SEs) and triacylglycerols (TAGs) (Czabany et al., 2007; Kohlwein, 2010; Korber et al., 2017). Lipid turnover reactions can release free fatty acids (FFAs) (de Kroon, 2007; Kurat et al., 2006), which can be reactivated to FACoAs by the action of fatty acyl-CoA synthetases (Faa1p-Faa4p/Fat1p) (Black and DiRusso, 2007). FACoAs can also be used for the posttranslational modification of proteins in the form of acylation (not shown here) (Deschenes et al., 1990) or be degraded back to acetyl-CoA via β-oxidation in the peroxisomes (Hiltunen et al., 2003; Poirier et al., 2006)(**Figure 3**).

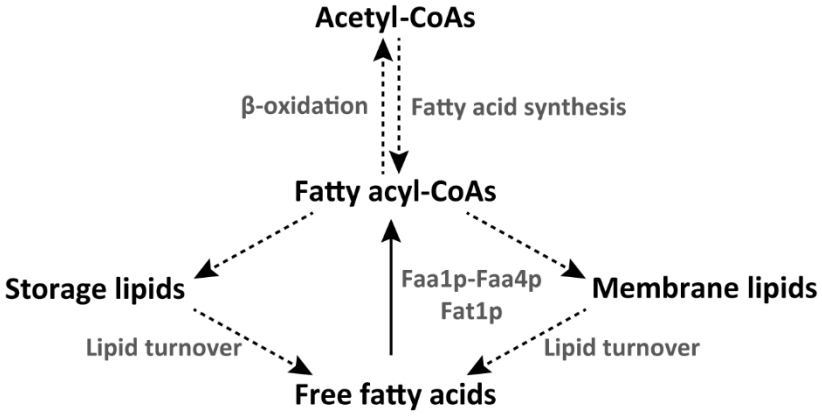

Figure 3 Fates of fatty acyl-CoAs in *S. cerevisiae*.
Faa1p/Faa2p/Faa3p/Faa4p/Fat1p, fatty acyl-CoA synthetases. ⇢ indicates multiple enzymatic steps and → indicates a single enzymatic step.

The initial and rate-limiting step in cytosolic FA biosynthesis is the carboxylation of acetyl-CoA to malonyl-CoA catalyzed by Acc1p, which requires the cofactors biotin and ATP to be functional. Biotin is covalently attached to the Acc1p apoprotein by the enzyme biotin:apoprotein ligase (Bpl1p). Acc1p harbors three functional domains, which are all located on one polypeptide chain (Cronan and Wallace, 1995; Mishina et al., 1980). Its product malonyl-CoA serves as a C_2 donor in a cyclic series of reactions catalyzed by fatty acid synthase (FAS) (**Figure 4**) (Tehlivets et al., 2007).

S. cerevisiae cytosolic FAS and FAS from other yeasts (as well as mammals) belong to the type I FAS systems. This means that individual functions involved in cytosolic FA biosynthesis are represented as discrete domains on a single or two different polypeptide chains. In contrast to that, type II FAS systems in most bacteria, but also in eukaryotic mitochondria or chloroplasts, consist of individual proteins (Schweizer and Hofmann, 2004). The *S. cerevisiae* cytosolic FAS is composed of two subunits, Fas1p (β-subunit) and

Fas2p (α-subunit) which are organized as a hexameric α6β6 complex. Fas1p is composed of the four domains acetyl transferase (AT), enoyl reductase (ER), enoyl dehydratase (DH) and malonyl-palmitoyl transferase (MPT) (Schweizer et al., 1986), whereas Fas2p harbors the domains acyl carrier protein (ACP), β-ketoacyl reductase (KR), β-ketoacyl synthase (KS) and phosphopantetheine transferase (PPT) (Mohamed et al., 1988). The initial FA biosynthesis cycle is composed of six reactions (**Figure 4**). In the first step, the AT domain catalyzes the transfer of the acetyl-CoA primer to the KS domain in Fas2p. The second step is the transacylation of malonate from malonyl-CoA to the pantetheine SH-residue, which is covalently attached to the ACP domain in Fas2p, catalyzed by the MPT domain. The third step is acetoacetyl-[ACP] formation by acyl-transfer and condensation (decarboxylation) with ACP-bound malonate catalyzed by the KS domain. The fourth step is the NADPH-dependent reduction of acetoacetyl-[ACP] to β-hydroxybutanoyl-[ACP] catalyzed by the KR domain. The fifth step is the dehydratisation of β-hydroxybutanoyl-[ACP] to crotonyl-[ACP] catalyzed by the DH domain. The sixth and final step is the NADPH-dependent reduction of crotonyl-[ACP] to butyryl-[ACP] catalyzed by the ER domain. This saturated acyl-[ACP] enters the next FA synthesis cycle together with a malonyl-[ACP] molecule (**Figure 5**). FA biosynthesis in the cytosol of *S. cerevisiae* yields saturated acyl-residues that are extended by two carbon atoms (acyl$_{(n+2)}$-[ACP]) in each cycle (Tehlivets et al., 2007).

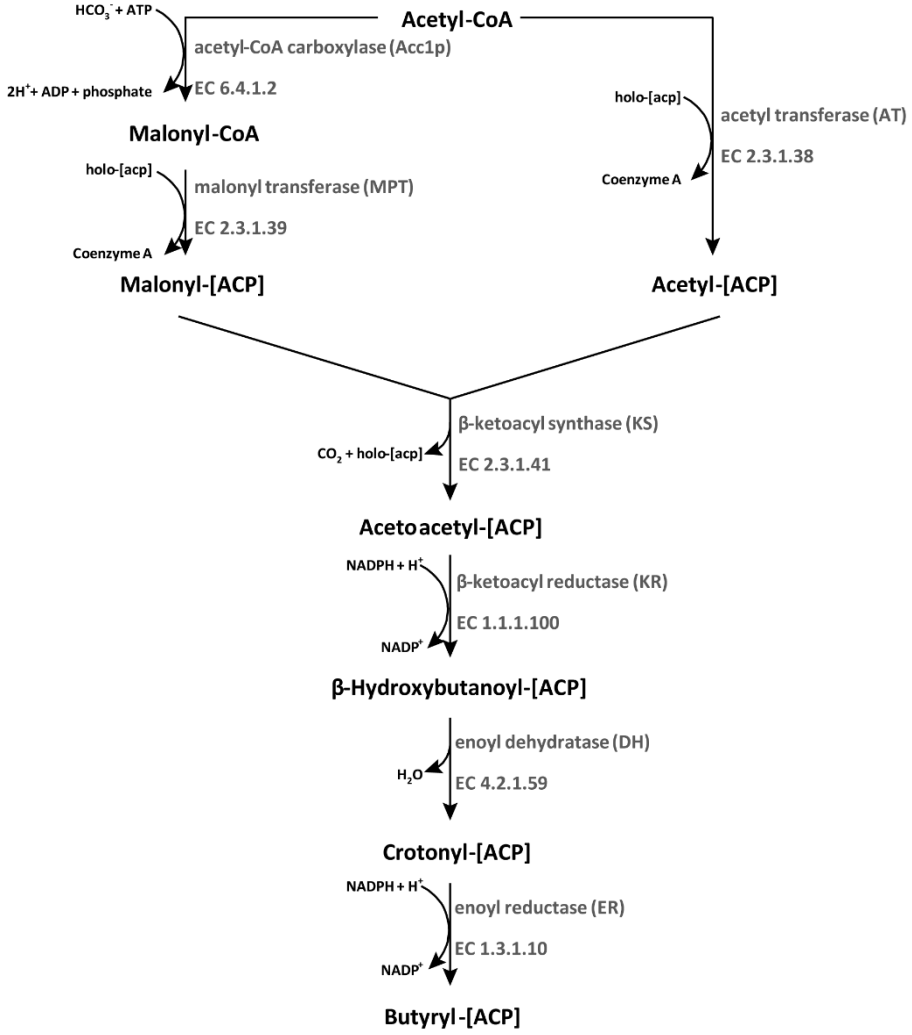

Figure 4 Initial cytosolic fatty acid biosynthesis cycle in *S. cerevisiae*.

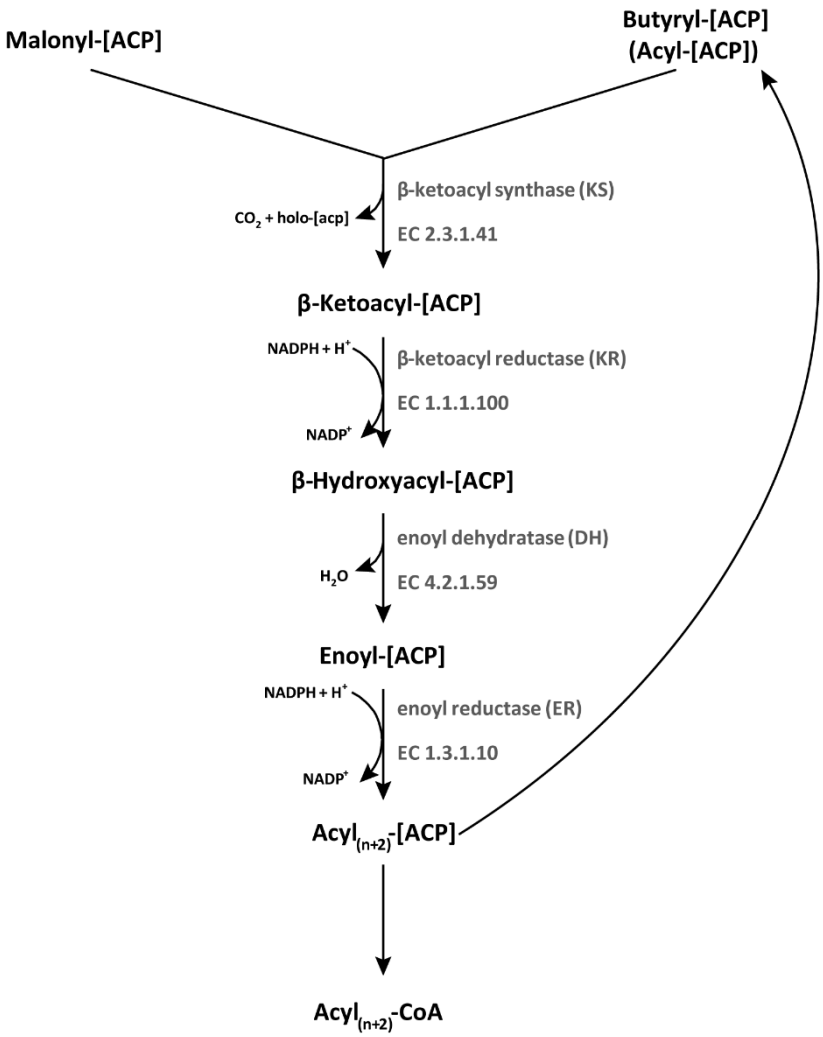

Figure 5 Cytosolic fatty acid biosynthesis pathway in *S. cerevisiae*.
n, number of carbon atoms.

FAs from cytosolic *de novo* FA synthesis in *S. cerevisiae* are released as acyl-CoAs. The transfer of acyl-residues from ACP to CoA is catalyzed by an intrinsic acyltransferase activity of yeast cytosolic FAS, yielding long-chain fatty acyl-CoAs (LCFACoAs), mostly palmitoyl-CoA (C16:0-CoA) and stearoyl-CoA (C18:0-CoA).

In contrast to that, most bacteria catalyze the direct formation of lipids from acyl-ACP substrates (Tehlivets et al., 2007). Also in plants, acyl-ACPs such as palmitoyl-ACP or stearoyl-ACP, can be used directly by acyltransferases to produce phosphatidic acid (PA), a precursor for lipid synthesis (Harwood, 1996). Moreover, archaea, bacteria, mammals and plants harbor thioesterases which function as acyl-[ACP] hydrolases to cleave off FFAs from acyl-[ACP]s (Cantu et al., 2010).

Regulation of fatty acid biosynthesis

The FA biosynthesis machinery is regulated on various levels, including the transcriptional and (post-)translational ones. *ACC1* as well as *FAS1*/*FAS2* are transcriptionally regulated by the membrane lipid precursors inositol and choline in coordination with PL biosynthesis (Carman and Henry, 1999; Chen et al., 2007). This means that *ACC1* as well as *FAS1/FAS2* transcription is controlled by the transcription factors Ino2p/Ino4p and Opi1p. The Ino2p/Ino4p activator complex binds to inositol/choline-responsive elements (ICREs), also called inositol-responsive upstream activating sequence (UAS_{INO}) which are located in the promoter regions of *ACC1* (Chirala et al., 1994; Hasslacher et al., 1993), *FAS1*/*FAS2* (Chirala, 1992; Schüller et al., 1992) and genes involved in PL biosynthesis (Schüller et al., 1995). Binding of the Ino2p/Ino4p complex activates expression of genes downstream of the UAS_{INO} element (Ambroziak and Henry, 1994; Loewy and Henry, 1984; Schwank et al., 1995). In contrast to that, Opi1p is a negative regulator of the expression of *ACC1*, *FAS1*/*FAS2* and genes involved in PL biosynthesis. In the absence of inositol, Opi1p binds to PA, a precursor of PLs, and is thereby trapped at the ER membrane. Therefore, the signal for Opi1p- mediated repression is not inositol itself, but rather PA levels. When inositol is present in high concentrations, PA levels decrease, Opi1p is released from the ER and translocates to the nucleus, where it interacts with Ino2p and thereby inhibits transcription (Chen et al., 2007; Henry et al., 2014; Loewen et al., 2004). Interestingly, it has been shown that Opi1p prefers binding to C16 over C18-PA-species. Therefore, C16-PA-species bind Opi1p more effectively to the ER, which in turn reduces repression of Opi1p controlled genes (**Figure 6**) (Hofbauer et al., 2014).

Regulation by Opi1p is connected to the Snf1p kinase, the yeast ortholog of mammalian AMP activated protein kinase (AMPK) (**Figure 6**) (Carling et al., 1994; Woods et al., 1994; Zhang et al., 2013). Snf1p is essential for releasing genes from glucose repression and undergoes an increase in activity during growth in glucose-limited conditions (Carling et al., 1994; Woods et al., 1994). In case of Acc1p, Snf1p catalyzed phosphorylation leads to inactivation of the enzyme (Witters and Watts, 1990; Woods et al., 1994). Snf1p is actually part of a complex (SNF1 complex), which is composed of the catalytic α-subunit Snf1p, one of three different regulatory β-subunits (Sip1p, Sip2p or Gal83p) and the stimulatory γ-subunit Snf4p (Jiang and Carlson, 1997). One of the regulatory β-subunits of the SNF1 complex (Sip2p) is subject to acetylation (Lin et al., 2009). The level of acetylation is dependent on the acetyl-CoA concentration in the cell and is decreased in *snf1*Δ mutants (Galdieri and Vancura, 2012; Zhang et al., 2013).

In a state of high nucleocytosolic acetyl-CoA levels, the acetylation of Sip2p leads to an increased interaction with Snf1p and thereby inhibition of Snf1p, which in turn leads to raised activity of Acc1p by decreased phosphorylation. This effect of a raised Acc1p activity is accompanied by elevated histone acetylation at the promoter region of *ACC1*, which results in increased *ACC1* expression levels (**Figure 6**) (Galdieri et al., 2014). Similarly, the deletion of *SNF1* results in raised Acc1p activity and an increased

conversion of acetyl-CoA to malonyl-CoA which reduces the pool of acetyl-CoA in the nucleocytosol and thereby leads to a decreased global histone acetylation. Moreover, *snf1*Δ mutants show a reduced fitness and reduced stress resistance (Zhang et al., 2013).

On the other hand, lower acetyl-CoA levels lead to hypoacetylation of Sip2p, and thereby increased activity of Snf1p, which in turn leads to increased phosphorylation and inhibition of Acc1p as well as decreased conversion of acetyl-CoA to malonyl-CoA (Lu et al., 2011). In addition, lower acetyl-CoA levels lead to a decreased expression of *ACC1*, because of a reduced acetylation of histones in the promoter region of *ACC1* (Galdieri et al., 2014). The reduced expression of *ACC1* in turn leads to an increased global histone acetylation by an elevated level of acetyl-CoA in the nucleocytosol, which results in an altered transcriptional regulation (**Figure 6**) (Galdieri and Vancura, 2012; Zhang et al., 2013).

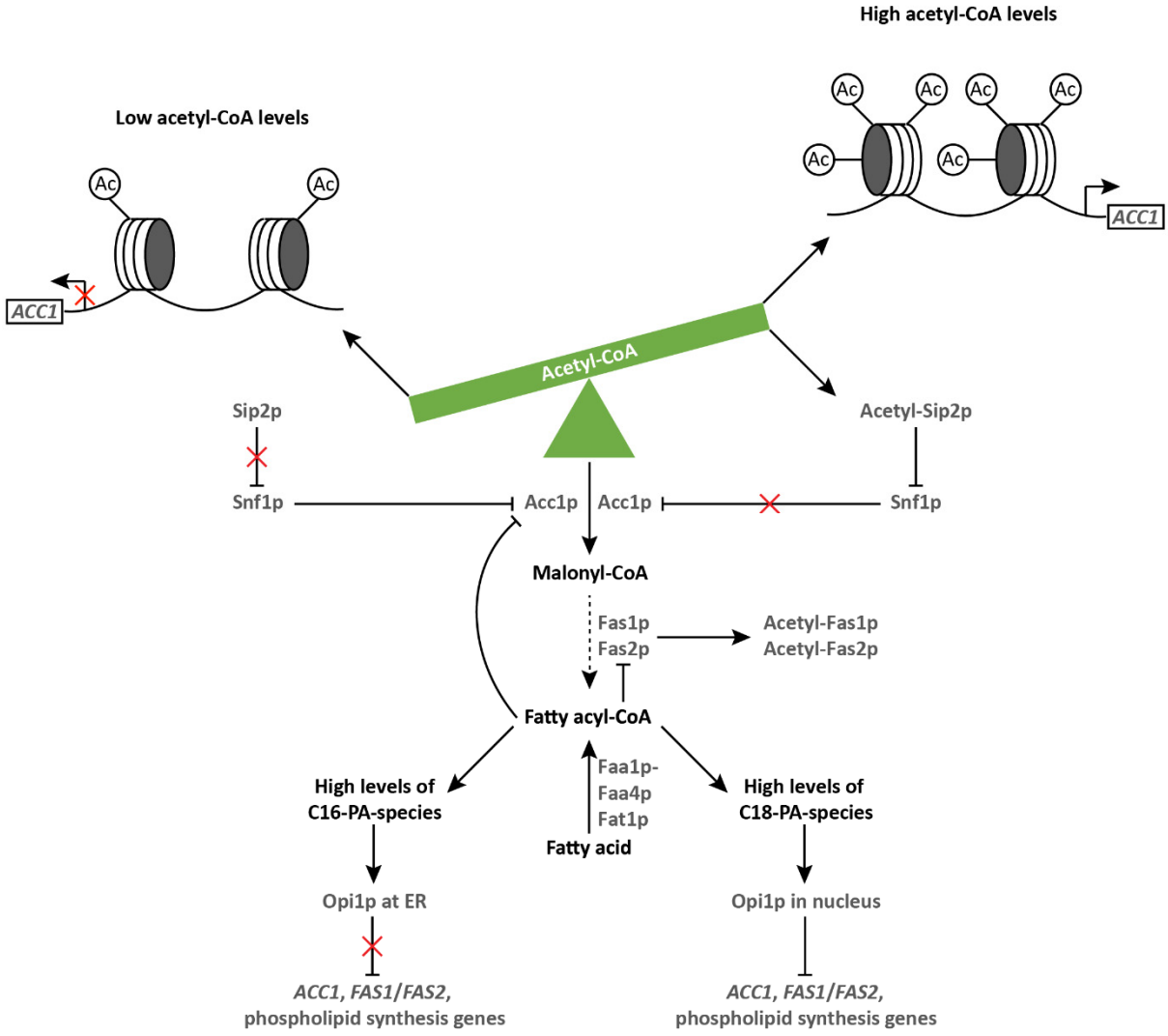

Figure 6 Regulation of genes/enzymes involved in fatty acid biosynthesis in *S. cerevisiae*. Ac, acetyl-CoA; *ACC1*, gene coding for acetyl-CoA carboxylase; Acc1p, acetyl-CoA carboxylase; ER, endoplasmic reticulum; Faa1p/Faa2p/Faa3p/Faa4p/Fat1p, fatty acyl-CoA synthetases; *FAS1/FAS2*, genes coding for fatty acid synthase 1/2; Fas1p/Fas2p, fatty acid synthase; Opi1p, "over producer of inositol" protein (transcriptional regulator); PA, phosphatidic acid; Sip2p, "Snf1p interacting protein" (one of three β-subunits of the SNF1 complex); Snf1p, "sucrose non-fermenting" protein (AMP-activated S/T protein kinase). Adapted from Galdieri et al. (2014). In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps and → indicates a single step; ⊣ indicates inhibition; ✕ indicates prevention of a process/step.

The phosphorylation of Acc1p occurs at a serine (Ser, S) residue in the protein, S1157, and putatively also at S659 (Ficarro et al., 2002; Shi et al., 2014a). Studies have shown that the modification of S1157 from serine to alanine (Ala, A) can abolish the phosphorylation of Acc1p by Snf1p which in turn leads to a constitutively active version of Acc1p (Hofbauer et al., 2014; Shi et al., 2014a). Cells harboring the mutant enzyme show elevated levels of malonyl-CoA and an increased C18-FAs/C16-FAs ratio, with wildtype cells having a ratio of around 0.5, while cells harboring the mutant enzyme (*ACC1*^{S1157A}) show a ratio of around 2 (Hofbauer et al., 2014). A yeast strain harboring the double mutated enzyme (*ACC1*$^{S659A, S1157A}$), in addition to the wildtype Acc1p, shows an increase in the total FA content as well as in Acc1p activity, compared to a strain harboring two copies of the wildtype *ACC1* or a strain harboring one copy of the wildtype *ACC1* and one copy of the single mutated enzyme (*ACC1*^{S1157A}) (Shi et al., 2014a).

Another way of regulation of Acc1p as well as Fas1p/Fas2p is their repression by exogenous FAs (Chirala, 1992; Kamiryo et al., 1976; Kamiryo and Numa, 1973; Numa and Tanabe, 1984), which is probably due to the formation of FACoAs catalyzed by fatty acyl-CoA synthetases inside the yeast cell. This theory is supported by the fact that mutant strains of *S. cerevisiae* defective in fatty acyl-CoA synthetase activity were hardly affected by addition of exogenous FAs (Kamiryo et al., 1976). Since FACoAs are a central precursor for storage- and membrane lipids and play an important role in cellular differentiation, gene expression, growth regulation, protein modifications, secretory processes as well as signal transduction, it is of utmost importance for the cell to balance FACoA levels (Faergeman and Knudsen, 1997; Neess et al., 2015; Rose et al., 1992). In this context it has been shown that yeast FAS is strongly inhibited by its own products, palmitoyl-CoA and stearoyl-CoA, *in vitro* (Lust and Lynen, 1968; Sumper and Träuble, 1973). This inhibitory effect is probably due to a reduced cellular content of the enzymes involved in *de novo* FA synthesis (Acc1p, Fas1p, Fas2p). This conclusion is based on the finding that the catalytic efficiency per Acc1p molecule was the same for an enzyme preparation from yeast cells grown with or without exogenous FAs (Kamiryo and Numa, 1973). In contrast to that, it has been hypothesized for rat-liver acetyl-CoA carboxylase that LCFACoAs can also act as an allosteric inhibitor of the enzyme (Nikawa et al., 1979; Ogiwara et al., 1978).

Another enzyme playing a role in regulation of acyl-CoA concentrations is the acyl-CoA binding protein (Acb1p). This enzyme transports newly synthesized LCFACoAs from FAS to FACoA-consuming processes (Rose et al., 1992; Schjerling et al., 1996). Depletion of Acb1p causes a dramatic reduction of C26:0 very long-chain fatty acids (VLCFAs) and sphingolipids, which in turn leads to a changed sphingolipid composition in the plasma membrane and thereby a perturbed plasma membrane structure. It also leads to a slight increase in monounsaturated fatty acids (MUFAs), an aberrant ER to Golgi transport and an accumulation of vesicles as well as autophagocytotic-like bodies. Supplementation of exogenous palmitic acid (C16:0-FA) cannot complement the reduced level of VLCFAs in Acb1p-depleted cells, indicating that Acb1p is also required for elongation of exogenous FAs (Gaigg et al., 2001). Moreover, it has been shown that disruption of *ACB1* results in an increase of stearoyl-CoA (C18:0-CoA), whereas the levels of other FACoAs were not

changed significantly, except for myristoyl-CoA (C14:0-CoA), which could not be detected in the *ACB1*-disrupted strain (Schjerling et al., 1996). The accumulation of the end product of FA biosynthesis (C18:0-CoA) and the simultaneous reduction of C26:0-FAs indicates that Acb1p is required for termination of FA biosynthesis and transport of FACoAs to the elongation machinery (Gaigg et al., 2001). Moreover, overexpression of bovine or yeast ACBP leads to increased FACoA levels (Knudsen et al., 1994; Mandrup et al., 1993). This is also supported by the observation that overexpression of Acb1p can boost fatty acid ethyl ester production in yeast (de Jong et al., 2014; Shi et al., 2014b). On a transcriptional level it could be shown that depletion of Acb1p results in an upregulation of genes encoding proteins involved in FA biosynthesis (*ACC1*, *FAS1*, *FAS2*, *OLE1*) and PL biosynthesis (e.g. *INO1*). The level of expression could be normalized by addition of exogenous FAs or increased *de novo* FA synthesis, whereas the expression of an Acb1p mutant with an inability of binding FACoAs could not revert the transcriptional changes caused by Acb1p depletion. This suggests that gene expression is either regulated by the Acb1p–FACoA complex directly or by its ability to transport FACoA to sites of FACoA consumption. This observation also shows that transcription of genes controlled by the UAS_{INO} cannot fully be repressed by inositol and choline, but instead full repression requires a functional Acb1 protein (Feddersen et al., 2007).

Besides regulation of Acc1p and the FAS by FAs/FACoAs, it has been shown that Fas1p and Fas2p contain potential lysine (Lys, K)-acetylation sites. The acetylation of proteins can have different effects, depending on the protein. In case of histones, which are the most studied acetylated proteins, acetylation enables the control of aging, cell cycle progression, DNA repair, replication and transcription. In general, increased acetylation of histones located at promoter regions results in increased transcription (Galdieri et al., 2014). Besides histones, also several other proteins involved in cell cycle progression, cytokinesis, metabolism, RNA processing, stress response and transcription have been identified as targets for acetylation (Duffy et al., 2012; Lin et al., 2009; Weinert et al., 2014). In terms of metabolism, a large proportion of enzymes involved in glycolysis, gluconeogenesis and amino acid (AA) metabolism were found to be acetylated in yeast (Henriksen et al.,2012). Since numerous of these enzymes (as well as their acetylation sites) are highly conserved in other organisms, regulation by acetylation might have a conserved role in cellular metabolism. Fas1p/Fas2p carry the highest number of detected unique acetylation sites in *S. cerevisiae* (29 and 50 sites, respectively). This equals an acetylation frequency of 1.41 and 2.65 detected acetylations/100 AAs, respectively. Therefore, Fas1p and Fas2p likely belong to the top 20% and top 10% of yeast proteins with the highest frequency of acetylation, respectively (Henriksen et al., 2012). Three of these K-acetylation sites in Fas2p (K83, K173 and K1551) have been confirmed in a study by Kumar et al. (unpublished). Two of the sites are located either near or within the ACP domain of Fas2p (K83 and K173, respectively) and one site is located close to the catalytic site of the KS domain (K1551) (Lomakin et al., 2007). So far, it has not been investigated if the detected acetylation sites in Fas1p and/or Fas2p have a regulatory function.

Cofactors involved in fatty acid biosynthesis

De novo FA synthesis in *S. cerevisiae* consumes high amounts of ATP, NADPH and acetyl-CoA. ATP is needed for activation of CO_2 during the carboxylation of acetyl-CoA as well as for the formation of CoA from pantothenate. NADPH is oxidized during the two reduction steps of FA biosynthesis, catalyzed by the ER and KR domains of Fas1p and Fas2p, respectively. Therefore, FA biosynthesis is dependent on high ATP/AMP levels, reduction equivalents as well as acetyl-CoA (Tehlivets et al., 2007).

The source of most ATP and some of the NADPH are mitochondria, where ATP is generated during respiration and NADPH is synthesized during the oxidative decarboxylation of malate to pyruvate, catalyzed by malic enzyme. Nevertheless, the main source of NADPH in *S. cerevisiae* is the pentose phosphate pathway (PPP) in the cytosol. In contrast to that the main part of NADPH in oleaginous microorganisms (one exception being *Yarrowia lipolytica*) comes from the reaction catalyzed by cytosolic malic enzyme, which is not present in *S. cerevisiae* (Boles et al., 1998; Dulermo et al., 2015; Ratledge, 2014).

Like acetyl-CoA, NAD^+ and ATP are compartmentalized and cannot move between different compartments of the cell, but instead need to be actively transported. NAD^+ is either *de novo* synthesized from tryptophan or regenerated from nicotinamide via a salvage pathway. Both pathways are situated in the cytosol and are conserved from yeast to humans (Bedalov et al., 2003). Cytosolic NAD^+ can be transported into mitochondria via the mitochondrial NAD^+ carrier protein Ndt1p (Yia6p) (El Moualij et al., 1997; Todisco et al., 2006). NAD^+ can further be converted into NADH or $NADP^+$/NADPH via different pathways in the cytosol as well as in the mitochondria. In the mitochondria, two main pathways for the generation of NADPH have been described (Miyagi et al., 2009). Both pathways involve the mitochondrial NADH kinase Pos5p, which is able to phosphorylate NADH to NADPH and NAD^+ to $NADP^+$(Outten and Culotta, 2003). During the conversion of acetaldehyde to acetate, $NADP^+$ is reduced to NADPH, catalyzed by Ald4p/Ald5p (mostly Ald4p) (Miyagi et al., 2009; Tessier et al., 1998). The cytosolic conversion of NAD^+ is catalyzed by the two kinases Utr1p and Yef1p, with Utr1p being the major cytosolic form. Whereas Yef1p is a NADH kinase and thereby also able to phosphorylate NADH, Utr1p has been shown to only act as a NAD^+ kinase (Kawai et al., 2001; Shi et al., 2005). The deletion of *POS5* and *UTR1* is lethal in yeast. Yef1p can only compensate when Pos5p is still functional. This demonstrates that Pos5p and Utr1p can partially compensate for the loss of each other (Bieganowski et al., 2006). It also shows that NAD^+ and its derivatives are essential metabolites in yeast (and all other organisms). Various enzymes in the cytosol can convert $NADP^+$ to NADPH. Ald6p and the glucose-6-phosphate dehydrogenase Zwf1p, which catalyzes the first step in the PPP, are considered as the main suppliers of NADPH in the cytosol (Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2001; Nogae and Johnston, 1990). A third enzyme that catalyzes the reduction of $NADP^+$ to NADPH is the cytosolic isocitrate dehydrogenase (Idp2p) (Haselbeck and McAlister-Henn, 1993; Loftus et al., 1994). It has been shown that the

source of cytosolic NADPH is dependent on the carbon source. While Ald6p and Zwf1p are important for NADPH production in glucose-containing medium, Idp2p becomes essential for growth on the non-fermentable carbon sources lactate, acetate and oleate (Minard and McAlister-Henn, 2005). Although the redox potentials of NAD^+/NADH and $NADP^+$/NADPH are almost the same, most cellular NAD is found in the oxidized form NAD^+ and NADP in the reduced form NADPH (Tehlivets et al., 2007). Besides H^+, O_2 and H_2O, NAD^+ derivates are integrated with more biochemical reactions than any other biochemical agent (Gossmann et al., 2012).

The transfer of ADP/ATP between the cytosol and the mitochondria occurs via mitochondrial inner membrane carriers. They import ADP from the cytosol into the mitochondrial matrix, where it can be phosphorylated by ATP synthase, and export newly synthesized ATP into the cytosol, replenishing the cell with metabolic energy (Ruprecht et al., 2014). *S. cerevisiae* possesses three adenine nucleotide translocators, namely Aac1p, Aac2p (=Pet9p) and Aac3p, of which Pet9p is the major ADP/ATP carrier of the mitochondrial inner membrane (Kolarov et al., 1990; Lawson and Douglas, 1988; Smith and Thorsness, 2008). *AAC1* is expressed at a very low level compared to *PET9*, and *AAC3* is primarily expressed under anaerobic conditions (Kolarov et al., 1990). Under certain conditions (e.g. aerobic exponential growth on glucose) these transporters can also catalyze transport in the opposite direction, importing ATP into mitochondria (Traba et al., 2008).

Other cofactors required for FA biosynthesis are biotin and pantothenate. Biotin, which is an essential cofactor for Acc1p and other carboxylases, is usually supplemented to the medium and transported into the cell by the Vht1p transporter localized in the plasma membrane (Stolz et al., 1999). Pantothenate is a precursor of CoA, which acts as an acyl-carrier in *S. cerevisiae*, and is synthesized from pyruvate and the two AAs valine and aspartate. CoA is synthesized by condensation of pantothenate and cysteine, which yields 4'P-pantetheine, and subsequent adenylation by ATP. Pantetheine represents the flexible arm attached to the ACP of FAS. The PPT domain of the Fas2p subunit of FAS is required for self-pantetheinylation of the ACP domain and thereby autoactivation of the FAS enzyme (Fichtlscherer et al., 2000; Tehlivets et al., 2007). FAS self-pantetheinylation was the first identified example of an apo-enzyme being capable of post-translational autoactivation.

1.1.3 Fatty acid elongation and desaturation in *S. cerevisiae*

The elongation of FAs to VLCFAs of 20-26 carbons (C20-C26) is performed at the ER of *S. cerevisiae.* The enzymes are localized to the ER membrane, facing the cytosol, and the reactions are reminiscent of cytosolic *de novo* FA biosynthesis, with the exception that distinct enzymes are catalyzing the elongation reactions in contrast to the cytosolic FAS which contains two subunits with seven functional domains. Moreover, the elongation enzymes at the ER use FACoAs as substrates, in contrast to the cytosolic FAS which is dependent on acyl-ACPs (Denic and Weissman, 2007; Tehlivets et al., 2007).

The first step in FA elongation at the ER is the coupled condensation and decarboxylation of Acc1p-derived malonyl-CoA with a LCFACoA derived from *de novo* FA biosynthesis, generating a β-ketoacyl-CoA (**Figure 7**). This step is catalyzed by a β-ketoacyl-CoA synthase (KCS), also known as fatty acid elongase (FAE). In *S. cerevisiae*, three different KCS enzymes exist: Elo1p, Elo2p and Elo3p. Elo1p elongates C12-C16 to C16/C18 FACoAs and is also able to elongate monounsaturated FACoAs, C14:1Δ9-FA to C16:1Δ11-FA and C16:1Δ9-FA to C18:1Δ11-FA. C16:1Δ9-FA is elongated with a ~12-fold lower efficiency *in vivo* than C14:1Δ9-FA for cells externally supplemented with 0.5 mM C16:1Δ9-FA and C14:1Δ9-FA, respectively (Dittrich et al., 1998; Rössler et al., 2003; Schneiter et al., 2000; Toke and Martin, 1996). Elo2p elongates C16/C18 up to C24 FACoAs and Elo3p can elongate C18 to C20-26 FACoAs, being essential for the elongation of C24 to C26 (Oh et al., 1997; Rössler et al., 2003). The second step is the reduction of the β-ketoacyl-CoA to β-hydroxyacyl-CoA, catalyzed by a β-ketoacyl-CoA reductase (KCR), encoded by *IFA38* (*YBR159w*) in *S. cerevisiae* (Beaudoin et al., 2002; Han et al., 2002). The third step is the dehydration of β-hydroxyacyl-CoA to an enoyl-CoA catalyzed by a β-hydroxyacyl-CoA dehydratase (HCD), encoded by *PHS1* (*YJL097w*) (Denic and Weissman, 2007). The last step is the reduction of enoyl-CoA to a saturated FACoA that is extended by two carbon atoms, catalyzed by an enoyl-CoA reductase (ECR), encoded by *TSC13* (Kohlwein et al., 2001). The elongated FACoA can re-enter the elongation cycle. Since the microsomal Elo proteins of yeast are lacking significant homology to the mitochondrial β-ketoacyl synthase (Cem1p) as well as the KS domain of cytosolic FAS, definite proof for the function of Elo1p, Elo2p and Elo3p as condensing enzymes had long been lacking. However, using purified Elo1p, Elo2p, Elo3p, Ifa38p, Phs1p as well as Tsc13p inserted into proteoliposomes, Denic and Weissman (2007) were able to show that the Elo proteins of *S. cerevisiae* indeed form a novel family of condensing enzymes that specify VLCFA length (Denic and Weissman, 2007).

In *S. cerevisiae*, the major VLCFA species is C26-FA, either occurring as saturated FA (C26:0-FA) or as hydroxylated FA (C26:0-OH), which are mostly present in sphingolipids (Dickson, 2008; Oh et al., 1997). The simplest sphingolipid is a ceramide, which consists of a long-chain base (sphingoid backbone) connected to a FA via an amide bond. Yeast sphingolipids either contain phytosphingosine (18-20 carbons) or dihydrosphingosine (16-20 carbons) as long-chain base. The attached FA is predominantly C26:0-FA or C26:0-OH. Ceramides can become more complex by attaching a head group. In yeast, the formation of three different complex sphingolipids, namely inositolphosphoryl ceramide (IPC), mannose-inositol-phosphoryl ceramide (MIPC) and mannose-diinositolphosphoryl ceramide ($M(IP)_2C$) is possible. IPC is formed by the addition of an inositol phosphate group to a ceramide. MIPC is the product of IPC mannosylation and $M(IP)_2C$ can be synthesized when a second inositol phosphate group is attached to MIPC. Sphingolipids are mostly found in the plasma membrane, but also in organelle membranes. Sphingolipids fulfill a range of different roles in *S. cerevisiae*, e.g. they are required for the formation of glycosylphosphatidylinositol (GPI) lipid anchors for proteins in the plasma membrane and the trafficking of proteins in the secretory pathway (Dickson, 2008; Toulmay and Schneiter, 2007).

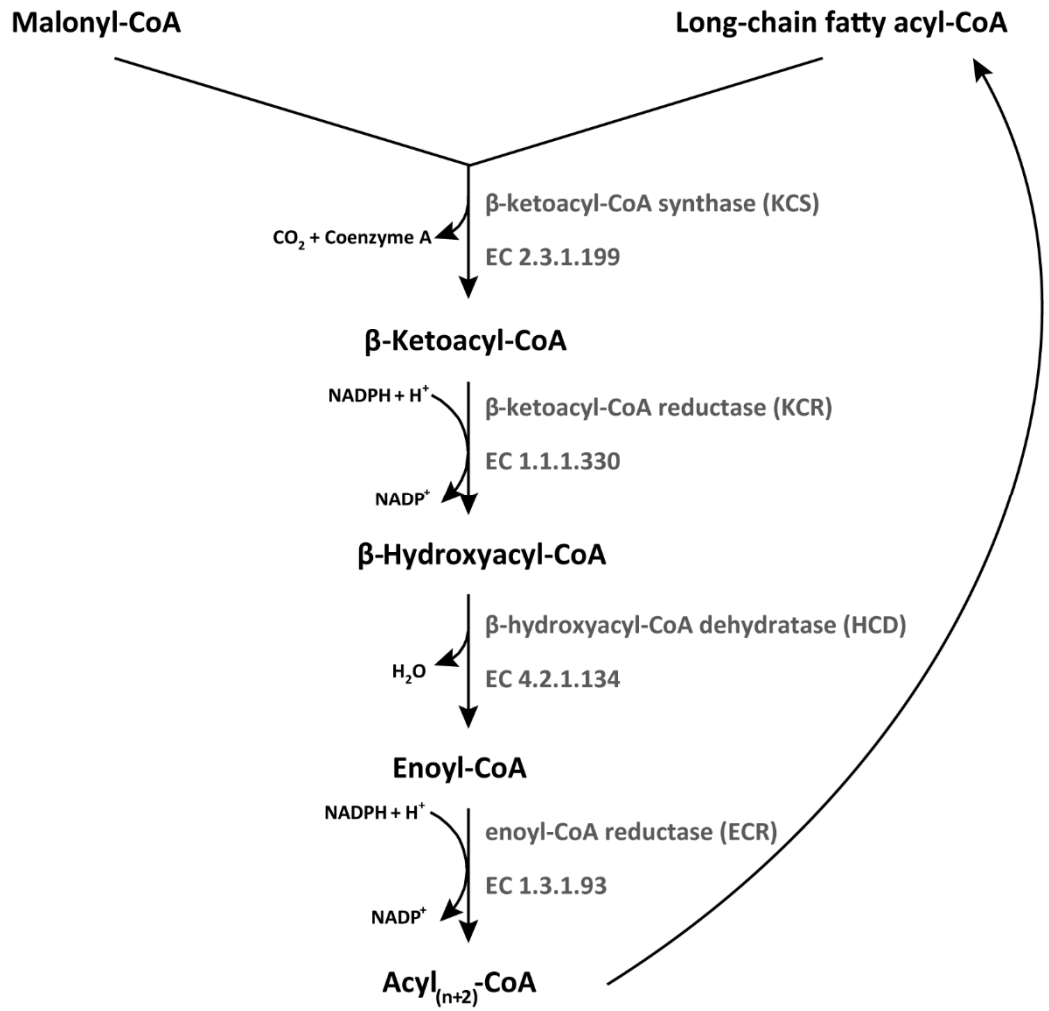

Figure 7 Fatty acid elongation at the endoplasmic reticulum in *S. cerevisiae*.
n, number of carbon atoms.

It has been shown that disruption of *ELO2* or *ELO3* mainly leads to a change in the VLCFA composition, with the *elo3*Δ strain being unable to synthesize C26:0-FA and C26:0-OH, but containing increased levels of C20-FAs and C22-FAs, with C22:0-FA being the most abundant VLCFA (~ 3% of total FAs, corresponding to a 10-fold increase compared to the wildtype). Moreover, the formation of hydroxylated FAs with a chain length of C16-C24 could be observed in this strain (Oh et al., 1997). In terms of the sphingolipid composition, disruption of *ELO3* leads to accumulation of long-chain bases, especially phytosphingosine, and an increase in IPC species with a simultaneous reduction of the mannosylated complex sphingolipids MIPC and $M(IP)_2C$ (Ejsing et al., 2009; Oh et al., 1997). The decreased sphingolipid concentrations might also explain why deletion of *ELO3* was reported to affect vacuolar organization (Kohlwein et al., 2001), protein trafficking to the cell surface (Proszynski et al., 2005), (H^+)-ATPase in the plasma membrane (García-Arranz et al., 1994), resistance to sterol biosynthesis inhibitors as well as bud localization defects, resulting in a prolonged doubling time (Revardel et al., 1995).

Typically, ~ 80% of *S. cerevisiae* FAs are monounsaturated via a reaction catalyzed by the ER-localized Δ9 fatty acid desaturase (FAD) Ole1p (Martin et al., 2002; Martin et al., 2007; Stukey et al., 1989). Ole1p can introduce a double bond in cis-configuration at the Δ9-position of FACoAs with a chain length of C12-C19 (**Figure 8**) (Martin et al., 2002). It is the only FAD present in *S. cerevisiae*. This is in contrast to other fungi, which usually also contain Δ12 and Δ15 FADs. Therefore, *S. cerevisiae* is naturally only able to synthesize saturated and monounsaturated, but no polyunsaturated FAs (Martin et al., 2007). The most abundant FAs in *S. cerevisiae* under standard growth conditions are palmitoleic acid (C16:1Δ9-FA), followed by oleic acid (C18:1Δ9-FA), palmitic acid (C16:0-FA) and stearic acid (C18:0-FA) (Oh et al., 1997; Welch and Burlingame, 1973). Minor species include C14:0-FA, C14:1Δ9-FA and C26:0-FA (Martin et al., 2007). Since MUFAs are important constituents of cell membranes, the *OLE1* gene is essential, unless the medium is supplemented with MUFAs (Stukey et al., 1989). In order for the FAD to be functional, it requires oxygen, NADH, cytochrome b5 as well as cytochrome b5 reductase. In contrast to mammalian Δ9 desaturases, the yeast enzyme is a chimeric protein, consisting of an N-terminal desaturase domain linked to a C-terminal cytochrome b5 domain, which acts as an electron donor in FA desaturation. Additionally, *S. cerevisiae* expresses an independent membrane bound cytochrome b5 (*CYB5*) which can potentially contribute electrons to heterologously expressed ER membrane-bound desaturases that lack the cytochrome b5 moiety (Mitchell and Martin, 1995). The formation of the double bond by Ole1p is catalyzed through transfer of reducing equivalents from NADH, via cytochrome b5 reductase, to the Ole1p cytochrome b5 domain and then to the diiron-oxo catalytic center of the enzyme to finally form the MUFA as well as H_2O (Martin et al., 2007).

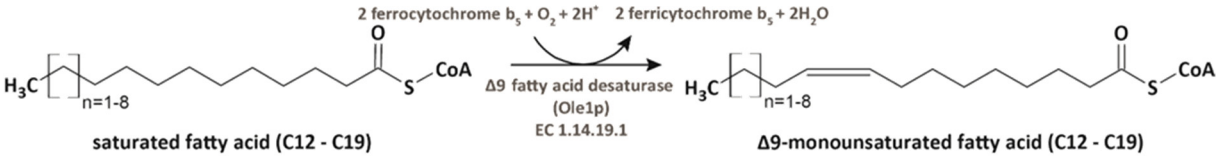

Figure 8 Desaturation of fatty acids at the endoplasmic reticulum in *S. cerevisiae*.

Regulation of fatty acid elongation and desaturation

One way for the cell to regulate VLCFA biosynthesis is via transcriptional regulation of certain genes encoding FA elongation enzymes, e.g. the expression of the *ELO1* gene is upregulated by C14:0-FA and repressed by C16:0-FA (Toke and Martin, 1996). Moreover, it has been shown that the expression of *ELO1*, *ELO2* and *ELO3* is downregulated in stationary phase and under nitrogen limitation (Gasch et al., 2000). Besides transcriptional regulation it has also been shown that certain K-residues, which are located in the sixth transmembrane helix (TMH6) region proximal to the luminal face of the membrane in the condensing enzymes Elo2p and Elo3p, determine VLCFA length (Denic and Weissman, 2007). The subfamily of Elo2 proteins in the yeast species *A. gossypii, C. glabrata, K. lactis* and *S. cerevisiae* contains a conserved K-residue at position -4 in the TMH6 (corresponding to AA 255 in *S. cerevisiae* Elo2p) and a conserved aspartic acid (Asp, D) or leucine (Leu, L) at position 0 in TMH6 (corresponding to AA 259 in *S.*

cerevisiae Elo2p), which is situated near the luminal end of TMH6. In contrast to that, the subfamily of Elo3 proteins in those species contains a conserved K-residue at position 0 in the TMH6 (corresponding to AA 266 in *S. cerevisiae* Elo3p) and a conserved phenylalanine (Phe, F) at position -4 in the TMH6 (corresponding to AA 262 in *S. cerevisiae* Elo3p) (**Figure 9**). When those sites in Elo3p were mutated to the conserved AAs present in Elo2p (*ELO3*$^{F262K, K266L}$), the mutated Elo3p lost its ability to form C26:0-FA and instead synthesized C22:0-FA and C24:0-FA. This indicates that the placement of K-residues in the TMH6 close to the cytosol results in shortening of the final VLCFA product, which in turn means that by exchanging certain AA residues in TMH6 with K, the final chain length of the VLCFAs can be controlled. This hypothesis is confirmed by the fact that exchanging the alanine (Ala, A)-residue at position TMH6(-8) in Elo2p (*ELO2*^{A251K}) leads to a mutant enzyme that is only able to elongate C14/C16 to C18 FACoAs *in vitro*, whereas exchanging the isoleucine (Ile, I)-residue a position THM6(-11) in Elo2p (*ELO2*^{I248K}) leads to a mutant enzyme only able to elongate C14 to C16 FACoAs (Denic and Weissman, 2007).

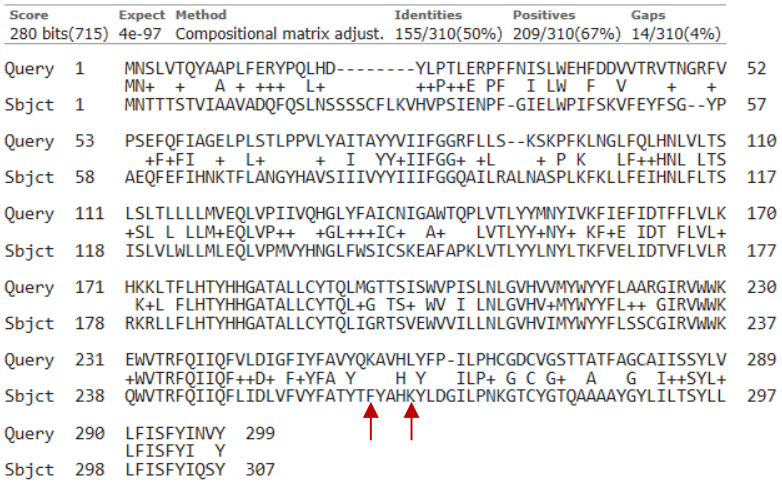

Figure 9 Alignment of Elo2p (query) and Elo3p (subject) of *S. cerevisiae*. The conserved lysine (Lys, K) residues at amino acid positions 255 in Elo2p and 266 in Elo3p, respectively, are indicated by red arrows.

Regulation of *OLE1* occurs on the level of transcription as well as mRNA stability. *OLE1* transcription is repressed by Δ9 unsaturated fatty acids (UFAs)(Bossie and Martin, 1989; Choi et al., 1996; McDonough et al., 1992), and also the half-life of the *OLE1* mRNA is dramatically reduced upon exposure to UFAs (Gonzalez and Martin, 1996). In contrast to that, *OLE1* transcription is increased in response to low oxygen and low temperature (Kwast et al., 1999; Nakagawa et al., 2002). This response is mediated by two homologous ER membrane-bound transcription factors, Spt23p and Mga2p, which activate *OLE1* expression through N-terminal polypeptides that are released from the membrane through an ubiquitin/proteasome-mediated mechanism and afterwards targeted to the nucleus (Chellappa et al., 2001; Hoppe et al., 2000; Zhang et al., 1999). In addition, Mga2p also contributes to the regulation of *OLE1* mRNA stability (Kandasamy et al., 2004).

1.2 Enzymes involved in wax ester synthesis

WEs are oxoester of (very) long-chain fatty acyl-CoAs ((V)LCFACoAs) and primary, (very) long-chain fatty alcohols ((V)LCFOHs. Like TAGs, WEs belong to the class of neutral lipids (Röttig and Steinbüchel, 2013). Their formation is catalyzed by two different enzyme classes based on FACoA substrates and NADPH as cofactor (**Figure 10**). The FACoA molecule is first reduced to a fatty aldehyde (FAldh) intermediate, catalyzed by a fatty acyl reductase (FAR). Dependent on the source of the enzyme (meaning pro- or eukaryotic), the FAldh is either released or not released. All so far described eukaryotic enzymes do not release the FAldh, but directly reduce it further to a FOH. In contrast to that, it had long been assumed that all prokaryotic enzymes release the FAldh intermediate and a second enzyme converts it further to a FOH (Hofvander et al., 2011). Recently, however, it was demonstrated that two prokaryotic enzymes, namely Maqu_2220 (*Ma*FAldhR) (NCBI accession no. YP_959486) and Maqu_2507 (NCBI accession number YP_959769), derived from the Gram-negative bacterium *Marinobacter aquaeolei* VT8 (*Ma*), are also able to catalyze both reduction steps (Hofvander et al., 2011; Lenneman et al., 2013; Wahlen et al., 2009; Willis et al., 2011). In bacteria, FOHs can also be synthesized from FAACP substrates, since those are the products of bacterial *de novo* FA synthesis. The second step in WE formation is the esterification of a FOH with a FACoA molecule, catalyzed by a wax synthase (WS), leading to a WE molecule (Hofvander et al., 2011).

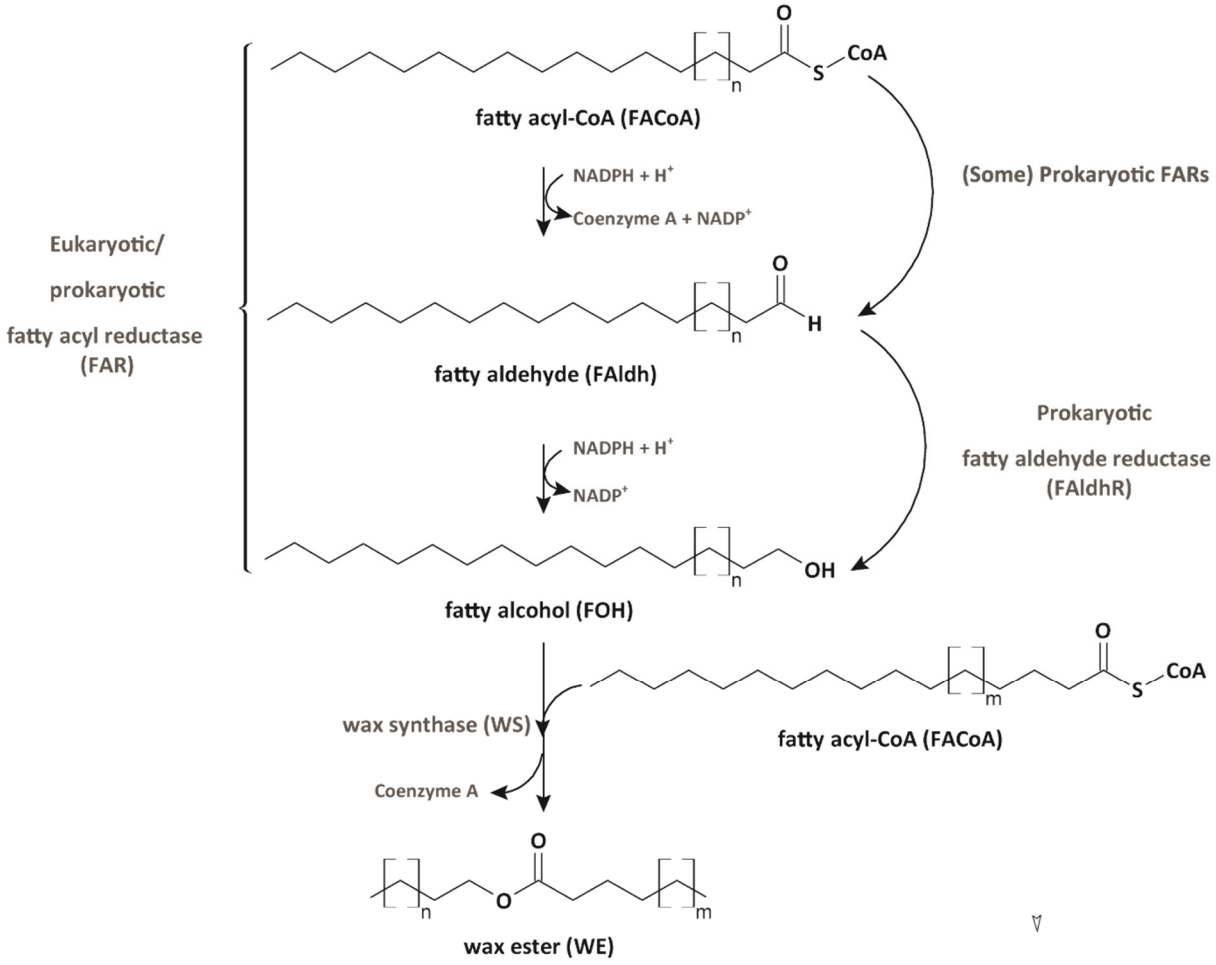

Figure 10 Enzymes involved in wax ester synthesis.

1.3 Natural occurrence and function of fatty alcohols and wax esters

Naturally, FOHs and WEs occur in pro- as well as eukaryotes, fulfilling different purposes depending on the species.

1.3.1 In prokaryotes

WEs occur as a form of carbon storage in prokaryotes, but they are much less common compared to TAGs or PHAs, with the latter one being the most common storage compound in the majority of all bacteria (Röttig and Steinbüchel, 2013). The ability of synthesizing huge amounts of TAGs, with up to more than 80% of the cell dry weight (CDW), is widespread among species of the Gram-positive actinomycetes, e.g., *Rhodococcus opacus* (Alvarez et al., 1996). Besides *R. opacus*, also other genera of the *Actinomycetales* store TAGs as their main storage lipid (Röttig and Steinbüchel, 2013). In contrast to that, species belonging to the Gram-negative genus *Acinetobacter* accumulate mainly WEs as storage lipids. They are also able to synthesize TAGs, but only accumulate those in minor amounts (Fixter et al., 1986; Makula et al., 1975). Also, the first discovery of WEs in bacteria, over 40 years ago, was in *Acinetobacter* species. In addition to that, some genera of marine hydrocarbonoclastic bacteria, e.g., *Alcanivorax*, *Marinobacter* or *Thalassolituus*, as well as *Psychrobacter*, *Micrococcus* or *Moraxella* have been shown to be able to accumulate WEs (Barney et al., 2012; Bredemeier et al., 2003; Bryn et al., 1977; Gallagher, 1971; Holtzapple and Schmidt-Dannert, 2007; Kalscheuer et al., 2007; Russell and Volkman, 1980). Moreover, also some Gram-positive actinomycetes, e.g. species of *Corynebacterium* or *Nocardia*, have been identified as WE producers (Bacchin et al., 1974; Raymond and Davis, 1960).

1.3.2 In eukaryotes

FOHs and WEs are synthesized in a range of eukaryotes, including birds (Biester et al., 2012; Hellenbrand et al., 2011; Wang and Kolattukudy, 1995), copepods (Teerawanichpan and Qiu, 2012), insects (Löfstedt, 1993; Teerawanichpan et al., 2010; Tillman et al., 1999), mammals (Cheng and Russell, 2004a; Cheng and Russell, 2004b), plants (Doan et al., 2009; Domergue et al., 2010; Lardizabal et al., 2000; Li et al., 2008; Metz et al., 2000; Rowland et al., 2006; Wang et al., 2002) and protozoa (Meesapyodsuk and Qiu, 2014; Teerawanichpan and Qiu, 2010). In these organisms, FOHs as well as WEs have very specialized purposes depending on the species. Birds preen their feathers with waxes that are produced in the uropygial gland, whereupon the type and the composition of the secreted WEs are dependent on the bird species, but also on the age and sex of the bird as well as on the season (Biester et al., 2012; Hellenbrand et al., 2011). Copepods use WEs as a carbon storage, similar to prokaryotes (Teerawanichpan and Qiu, 2012). In insects, specific FOHs (or their derivatives) can act as sex pheromones (Löfstedt, 1993; Tillman et al., 1999), whereas WEs can have a structural function as a component of beeswax (Blomquist et al., 1980; Tulloch, 1971). In mammals, sebaceous glands produce a lipid-rich secretion termed sebum that is exuded onto the surface of the skin. These glands are found in the dermis of a wide range of animals, but the chemical composition

of sebum is distinct in each species, e.g. human sebum is composed mainly of wax monoesters (25% of total lipids), TAGs (41%), FFAs (16%) and squalene (12%), whereas mouse sebum is composed of wax monoesters (5%), wax diesters (65%), TAGs (6%) as well as free and esterified sterols (23%) (Nikkari, 1974). WEs also form a major part of mammalian meibum, produced by meibomian glands in the eyelids, responsible for preventing evaporation of the eye's tear film (Driver and Lemp, 1996). In protozoa, like *Euglena gracilis* (*Eg*), WEs function as an energy storage under dark or anaerobic conditions (Meesapyodsuk and Qiu, 2014; Teerawanichpan and Qiu, 2010). In plants, WEs are part of the cuticular wax and thereby protect the cells from desiccation, UV light or pathogens (Post-Beittenmiller, 1996). The amount of WEs in cuticular waxes is usually rather low, e.g. in *Arabidopsis thaliana* (*At*), only around 0.1 to 2.9% of the cuticular wax is made up of WEs. In contrast to that, leaves of the carnauba palm (*Copernicia cerifera*) have a thick layer which consists of up to 85% of WEs (Li et al., 2008). A special plant in terms of lipid storage is *Simmondsia chinensis* (*Sci*), also known as jojoba plant, since it stores high amounts of WEs inside its seeds, contrary to other plants, which store TAGs (Murphy, 2001). Also in the spermaceti organ in the heads of sperm whales high amounts of WEs can be found, which are needed to regulate buoyancy (Clarke, 1970). In the past, sperm whales were haunted extensively, because of their high storage of WEs. Since whale hunting has been banned, jojoba and carnauba are nowadays the major natural sources for WEs (Lardizabal et al., 2000; Li et al., 2008).

1.4 Composition of jojoba oil

Jojoba (*S. chinensis*) is a perennial, woody shrub which is native to the semiarid regions of Southern Arizona, Southern California and Northwestern Mexico. It is the sole species of the family of *Simmondsiaceae*. Seeds of the jojoba plant contain approximately 50% (w/w) oil, which consists mostly (up to 97%) of WEs and only to a minor extent of phytosterols, TAGs and FOHs (van Boven et al., 1997; El-Mallah and El-Shami, 2009; Greene and Osborn Foster, 1933; McKinney and Jamieson, 1936).

The composition of the WE part of jojoba seed oil has been analyzed in various studies, using different methods, either involving hydrolysis of WEs and analysis of their constituents or the direct analysis of intact WE species. So far, the most prominent method for quantification of WEs has been hydrolysis of the WEs and subsequent quantification of the derivatized FA and FOH moieties by gas chromatography coupled with flame ionization detection (GC-FID) or electron ionization mass spectrometry (GC-EI-MS) (El-Mallah and El-Shami, 2009; Miwa, 1971; Tada et al., 2005). The disadvantage of this method is the loss of information about the molecular species composition of the individual WEs. Nowadays, it is also possible to analyze intact WEs via GC-MS, due to the development of GC capillaries that are stable at high temperatures. This method has already been used for the analysis of the WEs from various sources, including human meibum (Butovich et al., 2012), skin surface lipids (Michael-Jubeli et al., 2011) and human hair (Fitzgerald and Murphy, 2007). Moreover, more than 150 WE standards have been analyzed by GC-MS, and their EI mass spectra represent a reference source, facilitating species identification based on mass spectra interpretation (Urbanová et al., 2012).

Nevertheless, definite identification of unsaturated WEs via GC-MS remains challenging, because these species show very low abundant molecular ions and low abundant diagnostic ions (Butovich et al., 2012; Urbanová et al., 2012). The analysis of WEs in jojoba seed oil, beeswax as well as human hair has also been performed by non-aqueous reversed-phase (RP) high pressure liquid chromatography (HPLC) and the subsequent detection applying atmospheric pressure chemical ionization (APCI)-MS (Butovich et al., 2009; Medvedovici et al., 2002; Tada et al., 2005; Vrkoslav et al., 2010). Another method used for analysis of jojoba seed oil as well as human hair involves silver-ion (Ag) HPLC-MS. In contrast to RP HPLC/APCI-MS, Ag-HPLC/APCI-MS is able to separate WEs based on the number, position and geometry of double bonds. The analysis of jojoba seed oil via Ag-HPLC/APCI-MS led to an identification of 39 different WE species, with a chain length of C38 to C48 and zero to three double bonds. Of those 39 different WE species, only seven accounted for 90% of the total WE species. They were composed of C18-C24 monounsaturated fatty alcohol (MUFOH) and MUFA residues. The most prominent ones, comprising over 50%, were the C42:2-WEs (Vrkoslav et al., 2013). For a quantitative output, calibration of many internal standards is necessary when using chromatography-based methods. This can be circumvented by using a direct infusion into the mass spectrometer (shotgun approach). To enable high-throughput quantitative profiling of molecular WE species, nano-electrospray ionization tandem mass spectrometry (nanoESI-MS/MS) has been used (Iven et al., 2013). Using this method, the three WE species C22:1-C20:1 (32 mol%), C20:1-C20:1 (18 mol%) and C20:1-C22:1 (7 mol%) were identified as main WE species of jojoba seed oil (**Figure 11**). The diunsaturated wax ester (DUWE) species C38:2-WE (4 mol%), C40:2-WE (21 mol%), C42:2-WE (47 mol%) and C44:2-WE (6 mol%) together made up 77.8 mol% of the total WE species. The data by Iven et al. (2013) are in good agreement with Vrkoslav et al. (2010/2013) as well as earlier published work involving hydrolysis of jojoba WEs and analysis of their constituents by GC-FID or GC-MS (El-Mallah and El-Shami, 2009; Miwa, 1971; Tada et al., 2005).

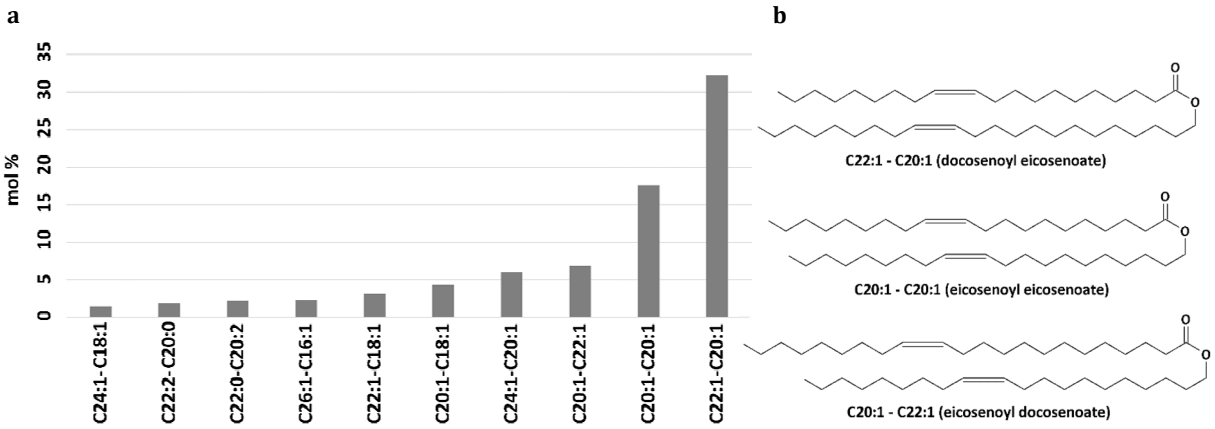

Figure 11 Wax ester profile of jojoba seed oil (mol% of total wax esters). (a) Relative accumulation of the ten most abundant wax ester species in mol % of total wax ester accumulation (means of four replicate measurements) (adapted from Iven et al. (2013)). **(b)** Molecular structure of the three most abundant wax ester species.

1.5 Commercial exploitation of jojoba oil

Jojoba oil can be used in cosmetic and personal care products, and it even has an anti-inflammatory effect, making it able to treat skin infections and accelerate wound healing. Therefore, it can also be used as an oil phase in skin ointments (topical drugs) to treat burns, wounds and sores (Pazyar et al., 2013; Ranzato et al., 2011). In addition, jojoba oil can be used for the synthesis of lubricants, candles, varnishes, printing inks, detergents, resins, and plastics (Miwa, 1984) or as a coating material for fruits and pills (El-Anany et al., 2009; Jannin and Cuppok, 2013).

The current production of jojoba seed oil is around 4,000 tons/year, with an estimated demand up to 200,000 tons/year (Sánchez et al., 2016). This increased demand primarily comes from the cosmetic and personal care industry, because of the ability of jojoba oil to protect the skin and prevent aging. It mimics the sebum, produced by glands in the human skin. Therefore, it has a lasting moisturizing effect and can be used in shampoos, conditioners, other hair products, cosmetic formulations, massage products, skin creams, sunscreens, lotions, exfoliates, moisturizers, lipsticks and balms.

Moreover, jojoba oil also has the potential to be used in the pharmaceutical industry as a carrier for medicine preparation, stabilizer for antibiotics (e.g. penicillin) as well as appetite depressant. In addition, there is a growing trend towards herbal medicines which is supposed to push the demand for jojoba oil.

Because of its high viscosity index, compatibility with different additives, and miscibility with mineral oil, jojoba oil can also be used in adhesives, electric insulators, factices, foam control agents and plasticizers. Its lubricant properties can be exploited in various high-temperature and pressure applications, e.g. hydraulics. The advantage of jojoba oil as a lubricant are its high oxidative as well as thermal stability.

Since the demand for naturally derived products is rising, the market for jojoba oil is increasing steadily. Because of the increased demand and improved production capabilities, the production of jojoba oil has increased over the past few years especially in Argentina, Australia, Mexico and Peru (https://www.grandviewresearch.com/industry-analysis/jojoba-oil-market).

Since the need for jojoba oil for the lubricant and cosmetic/medical industry rises, the oil produced from the jojoba plant will not be enough to meet the demand, even if huge land areas in various parts of the world are planted. Therefore, jojoba oil production in modified microorganisms represents a very promising approach.

1.6 Heterologous production of wax esters

WE production has been implemented in a range of prokaryotes (not included in this thesis) as well as eukaryotes. This section focuses on the heterologous production of WEs in yeasts (**Section 1.6.1, Table 1**) and plants (**Section 1.6.2, Table 2**).

1.6.1 In yeasts

The heterologous expression of FARs and WSs in *S. cerevisiae* has so far mostly been performed to investigate the functionality of enzymes, rather than to specifically produce high amounts of WEs. Most studies were conducted with substrate feeding of FAs, FOHs or both precursors (**Table 1**). FARs and WSs of bacterial, bird, insect, mammalian (*Mus musculus*), plant or protozoan origin were examined. Most studies, including the ones by Kalscheuer et al. (2004), King et al. (2007), Li et al. (2008), Miklaszewska et al. (2013), Miklaszewska and Banaś (2016), Teerawanichpan et al. (2010), Teerawanichpan and Qiu (2010), Yu et al. (2018) and Zhang et al. (2017) do not specify the amounts of WEs produced by modified *S. cerevisiae* strains. The study by Biester et al. (2012) showed the production of up to 2 µmol WEs/g cell fresh weight (CFW) for *S. cerevisiae* cells heterologously expressing WS1 from *Gallus gallus* (*Gg*WS1) under supplementation of C10:0-FOH to C18:0-FOH, whereas cells expressing *Gg*FAR1 and *Gg*WS4 under supplementation of C14:0-FA produced 550 nmol WEs/g CFW. The combined expression of a *M. musculus* FAR, lacking its putative peroxisomal signal and fused to *Arabidopsis* oleosin (Oleo3:mCherry:*Mm*FAR1Δc), together with the WS from *M. musculus*, also fused to *Arabidopsis* oleosin (Oleo3:EYFP::*Mm*WS), led to the synthesis of ~ 80 ng WEs/mg CFW (Heilmann et al., 2012). Bansal and Durrett (2016) reported the production of ~ 30 ng/mg CDW for C18:0 – C2:0 (stearyl acetate), which is actually not considered as a WE, since it is derived from acetic acid. The expression of various *E. gracilis* wax synthase/diacylglycerol acetyltransferases (WSDs) led to the synthesis of up to 2500 µg WEs/g CFW under supplementation of C14-FA and C14-FOH substrates (Tomiyama et al., 2017). The highest WE production in *S. cerevisiae* reported in the literature was achieved by expression of a WS derived from *M. hydrocarbonoclasticus* (*Mh*WS2)[1], which led to the synthesis of ~ 20.57 ± 1.67 mg WEs/g CDW under supplementation of 0.5 mg/mL hexadecanol (C16:0-FOH) (Miklaszewska et al., 2018). Most of the studies show a synthesis of WEs in the chain length range of C26-C36 (**Table 1**). Only when *S. cerevisiae* cells were supplied with very long-chain substrates (C18:0-FOH, C24:0-FOH or C28:0-FOH), the synthesis of C40:0-WEs and C44:0-WEs was reported.

[1] Márquez and Ventosa (2005) reviewed literature data concerning the two species *M. hydrocarbonoclasticus* and *M. aquaeolei*. They also conducted studies concerning the FA composition, G+C content and DNA–DNA hybridization for clarification of the taxonomic positions of these two species. Their results as well as the information about phenotypic and phylogenetic traits found in literature, led them to the proposal to unite the two species under the same name, *M. hydrocarbonoclasticus*.

Table 1 Heterologous production of wax esters in *S. cerevisiae.*

Organism	Fatty acyl reductase (FAR)	Wax synthase (WS)/ diacylglycerol acetyltransferase (DAcT)/ acyl-CoA: diacylglycerol acyltransferase (DGAT/D)	Predominant wax ester species (alcohol-acyl moiety)	Substrate feeding (fatty acid (FA)/ fatty alcohol (FOH))	Reference
***Saccharomyces cerevisiae* H1246**	-	*Acinetobacter baylyi* ADP1 AtfA (WS/DGAT) (*Ab*WS)	ethyl (C2) esters, isoamyl (C5) esters	-	Kalscheuer et al., 2004
***S. cerevisiae* JCY500**	-	*Petunia hybrida* (*Ph*WS1)	C5:0 – C16:0 C5:0 – C18:0 C5:0 – C20:0 C5:0 – C22:0 C5:0 – C24:0	-	King et al., 2007
***S. cerevisiae* H1246**	-	*Arabidopsis thaliana* WSD1 (*At*WS)	C18:0 – C16:0 C24:0 – C16:0 C28:0 – C16:0	C16:0-FA C18:0/ C24:0/ C28:0-FOH	Li et al., 2008
***S. cerevisiae* H1246**	*Euglena gracilis* (*Eg*FAR)	*Eg*WS	C14:0 – C14:0 C14:0 – C16:1 C14:0 – C16:0 C16:0 – C14:0	C14:0-FA	Teerawanichpan and Qiu, 2010
***S. cerevisiae* H1246**	*Apis mellifera* (*Am*FAR1)	*Eg*WS	C14:0 – C14:0 C16:0 – C14:0 C16:0 – C16:0 C18:0 – C14:0 C18:0 – C16:0 C18:0 – C16:1	C14:0-FA	Teerawanichpan et al., 2010
S. cerevisiae* BY4741 *lro1Δ* *dga1Δ	-	*Gallus gallus* (*Gg*WS1/2/4/5; *Gg*DGAT1)	C12:0/C14:0-FOH C16:1/C18:1-FA	C10:0 to C18:0-FOH	Biester et al., 2012
	-	*Anser domesticus* (*Ad*WS4)	C12:0/C14:0-FOH C16:1/C18:1-FA	C10:0 to C18:0-FOH	
	-	*Tyto alba* (*Ta*WS4)	C12:0/C14:0-FOH C16:1/C18:1-FA	C10:0 to C18:0-FOH	
	*Gg*FAR1	*Gg*WS4	Not specified	C14:0-FA	
***S. cerevisiae* H1246**	*Mus musculus* (mCherry:*Mm*FAR1)	*M. musculus* (EYFP::*Mm*WS)	Not specified	-	Heilmann et al., 2012
	Oleo3:mCherry:*Mm*FAR1Δc (lacking putative peroxisomal signal + fusion with *Arabidopsis* oleosin)	EYFP::*Mm*WS	Not specified	-	
	Oleo3:mCherry:*Mm*FAR1Δc	Oleo3:EYFP::*Mm*WS (protein fusion with *Arabidopsis* oleosin)	Not specified	-	
***S. cerevisiae* H1246**	-	*M. musculus*	C16:0 – C16:1 C16:0 – C16:0 C16:0 – C18:1 C16:0 – C14:0	C16:0-FOH	Miklaszewska et al., 2013

***S. cerevisiae* H1246**	*Am*FAR1	*Euonymus alatus* (*Ea*DAcT)	C18:0 – C2:0 C16:0 – C2:0	-	Bansal and Durrett, 2016
***S. cerevisiae* H1246**	-	*S. chinensis* (*Sci*WS)	C18:0 – C16:0 C18:0 – C14:0 C18:0 – C16:1	C18:0-FOH	Miklaszewska and Banaś, 2016
***S. cerevisiae* H1246**	-	*Eg*WSD2	C14:0 – C14:0	C14:0-FA C14:0-FOH	Tomiyama et al., 2017
	-	*Eg*WSD5	C14:0 – C14:0	C14:0-FA C14:0-FOH	
	-	*Eg*WSD2	C14:0 – C14:0 C16:0 – C16:0 C18:0 – C18:0	C14:0 to C18:0-FA C14:0 to C18:0-FOH	
	-	*Eg*WSD5	C14:0 – C14:0 C16:0 – C16:0 C18:0 – C18:0	C14:0 to C18:0-FA C14:0 to C18:0-FOH	
***S. cerevisiae* H1246**	-	*Thraustochytrium roseum* (*Tr*WSD4)	C16:0 – C14:0 C16:0 – C16:0	C16:0-FA C16:0-FOH	Zhang et al., 2017
	-	*Tr*WSD5	C16:0 – C12:0 C16:0 – C14:0 C16:0 – C16:0 C2:0 – C16:0	C16:0-FA C16:0-FOH	
S. cerevisiae* BY4741 *lro1Δ dga1Δ	-	*Marinobacter hydrocarbonoclastic us* DSM 8798 (*Mh*WS2)	C16:0>C14:0> C18:0-FOH C16:1>C18:1> C16:0>C14:0> C18:0>C12:0-FA	C14:0 to C18:0-FOH	Miklaszewska et al., 2018
	A. thaliana (*At*FAR5)	*Mh*WS2	C18:0 – C16:0 C18:0 – C16:1 C18:0 – C18:0 C18:0 – C18:1	-	
	Tetrahymena thermophila (*Tt*FAR)	*Mh*WS2	C16:0 – C16:0 C16:0 – C16:1 C18:0 – C16:0	-	
***S. cerevisiae* H1246**	-	*Sci*WS-*M. aquaeolei* VT8 (*Ma*FAldhR) as fusion protein	Not specified	C18:1-FOH	Yu et al., 2018
	-	*Sci*WS-*Ma*FAldhR as fusion protein	Not specified	-	
	*Ma*FAldhR	*Sci*WS	Not specified	C18:1-FOH	
	*Ma*FAldhR	*Sci*WS	Not specified	-	
	-	*SciWS*	Not specified	C18:1-FOH	
	-	*Mm*WS	Not specified	C18:1-FOH	
	-	PCO*Ab*WS (optimized for plant codon usage)	Not specified	C18:1-FOH	
	-	TM*Mm*WS- *Ab*WS (fusion protein of first 60 AAs of *Mm*WS and N-terminal end of *Ab*WS)	Not specified	C18:1-FOH	

1.6.2 In plants

Besides the heterologous production of WEs in yeasts, also the production in transgenic plants has been investigated intensively (**Table 2**). It is an interesting aspect for this study, since a lot of enzymes tested in transgenic plants have also been used, or could potentially be used, in *S. cerevisiae*. In contrast to yeast, plants show a more favorable composition of FAs for synthesis of jojoba-like WEs, since they naturally contain high amounts of VLCMUFAs. Transgenic plants studied for their ability to synthesize jojoba-like WEs include the model organism *A. thaliana,* the oil crop species *Brassica carinata, Camelina sativa* and *Crambe abyssinica,* the wild oil species *Lepidium campestre* as well as *Nicotiana benthamiana*, a close relative of the commercially used tobacco plant. *B. carinata*, *C. sativa* and *C. abyssinica* are oilseed crops, with *C. sativa* showing an oil content of 30-40% of seed weight (Yu et al., 2018). In contrast to other oilseed crops, like rapeseed or soybean, *B. carinata*, *C. sativa* and *C. abyssinica* oilseed species have the advantage of not being used on a commodity-scale for edible oil production. Even though niche food markets exist for carinata and camelina oil, crambe oil is entirely used as an industrial oilseed. The use of non-food oilseed crops excludes unintended mixing of seeds for edible and industrial markets (Zhu et al., 2016). The highest production of WEs in transgenic plants so far (~ 100 mg WEs/g seeds) was achieved in *A. thaliana* by heterologous expression of *Ma*FAldhR together with *Sci*WS. The most prominent WE species in this case was C18:1-C20:1 (17.7 mol%) (Iven et al., 2016). The highest yield of WEs in transgenic *C. abyssinica* (~ 85 mg WEs/g seeds), *B. carinata* and *C. sativa* was achieved by heterologous expression of *Sci*FAR together with *Sci*WS. The most prominent WE species in this case was C22:1-C20:1 (~ 30 mol%), which is also the most abundant WE species in jojoba seed oil (Iven et al., 2013; Zhu et al., 2016).

Table 2 Heterologous production of wax esters in different plant species.

Organism	Fatty acyl reductase (FAR)	Wax synthase (WS)/ phytyl ester synthase (PES)	Predominant wax ester species (alcohol-acyl moiety)	Substrate feeding (fatty acid (FA)/ fatty alcohol (FOH))	Reference
Arabidopsis thaliana **(seeds)**	*Simmondsia chinensis* (*Sci*FAR) + elongase of *Lunaria annua* (*La*FAE1)	*S. chinensis* (*Sci*WS)	C20 – C24 FOH C16 – C24 FA	-	Lardizabal et al., 2000
A. thaliana **(seeds)**	*Mus musculus* (Oleo3:*Mm*FAR1Δc)	Oleo3:*MmWS*	C20:1 – C18:2	-	Heilmann et al., 2012
A. thaliana fae1Δ fad2Δ **(seeds)**	Oleo3:*Mm*FAR1Δc	Oleo3:*MmWS*	C18:1 – C18:1	-	
A. thaliana **(seeds)**	*Marinobacter aquaeolei* VT8 (*Ma*FAldhR)	*Sci*WS	C18:1 – C20:1 C20:1 – C20:1	-	Iven et al., 2013
Nicotiana benthamiana **(leaves - chloroplasts)**	*A. thaliana* (*At*FAR6)	*At*PES2	C16:0 > C18:0 FOH C12:0, C14:0 FA	-	Aslan et al., 2014

	*At*FAR6 + *A. thaliana* WRINKELD1 (*At*WRI1)	*At*PES2	C16:0 > C18:0 FOH C12:0, C14:0 FA	-	
	*At*FAR6	*M. hydrocarbonoclasticus* (tp*Mh*WS2) (tp = transit peptide sequence; 76 AA N-terminal of *At*FAR6)	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
	*At*FAR6 + *At*WRI1	tp*Mh*WS2	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
	tp*Ma*FAldhR	*At*PES2	C16:0 > C18:0 FOH C12:0, C14:0 FA	-	
	tp*Ma*FAldhR + *At*WRI1	*At*PES2	C16:0 > C18:0 FOH C12:0, C14:0 FA	-	
	tp*Ma*FAldhR	tp*Mh*WS2	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
	tp*Ma*FAldhR + *At*WRI1	tp*Mh*WS2	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
	tp*Ma*FAldhR	*Mh*WS2	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
	tp*Ma*FAldhR + *At*WRI1	*Mh*WS2	C16:0 > C18:0 FOH C16:0 > C18:0 FA	-	
***N. benthamiana* (leaves & stems)**	tp*Ma*FAldhR	tp*Mh*WS2	C16:0 > C18:0 FOH C16:0, C18:0, C18:2, C20:0, C22:0 FA	-	Aslan et al., 2015
***A. thaliana* (seeds)**	Oleo3:*Mm*FAR 1Δc	Oleo3:*Mm*WS	C16:0 – C18:2	-	Iven et al., 2016
***Camelina sativa* (seeds)**	Oleo3:*Mm*FAR 1Δc	*Sci*WS	C18:1 – C20:1	-	
***C. sativa* (seeds)**	*Ma*FAldhR	*Sci*WS	C18:1 – C20:1	-	
***Crambe abyssinica* (seeds)**	*Sci*FAR + elongase of *S. chinensis* (*Sci*FAE1)	*Sci*WS	C22:1 – C20:1	-	Zhu et al., 2016
***Brassica carinata* (seeds)**	*Sci*FAR + elongase of *S. chinensis* (*Sci*FAE1)	*Sci*WS	C24:1 – C24:1	-	

C. sativa **(seeds)**	*Sci*FAR + *La*FAE1	*Sci*WS	C24:1 – C24:1	-	
Lepidium campestre **(seeds)**	*Sci*FAR	*Sci*WS	C22:1 – C20:1 C22:1 – C22:1	-	Ivarson et al., 2017
	*Sci*FAR + *Sci*FAE	*Sci*WS	C22:1 – C20:1 C24:1 – C20:1	-	
A. thaliana **(seeds)**	*Ma*FAldhR	*Sci*WS	C18:1-FOH C20:1-FA	-	Yu et al. 2018
	-	*Sci*WS-*Ma*FAldhR as fusion protein	C20:1-FOH C20:1-FA	-	
	-	2x *Sci*WS-*Ma*FAldhR as fusion protein	C20:1-FOH C20:1-FA	-	
	*Ma*FAldhR	*Sci*WS-*Ma*FAldhR as fusion protein	C20:1-FOH C20:1-FA	-	
	*Ma*FAldhR	*Ab*WS	C18:1-FOH C18:0-FA C20:1 – C18:1 (16 mol%) C18:1 – C18:1 (11 mol%)	-	
	*Ma*FAldhR	PCO*Ab*WS	C18:1-FOH C18:0-FA	-	
	*Ma*FAldhR	TM*Mm*WS- *Ab*WS	C18:1-FOH C18:0-FA	-	
	*Ma*FAldhR	*Ma*WS2	C18:1-FOH C18:0-FA	-	
A. thaliana fae1Δ fad2Δ **(seeds)**	*Ma*FAldhR	*Ab*WS	C18:1 – C18:1 (62 mol%) C18:0 – C18:1 (9 mol%)	-	
C. sativa **(crossings between *Ma*FAldhR/*Sci*WS and high oleate (HO) line) (seeds)**	*Ma*FAldhR	*Sci*WS	C18:1 – C18:1 (27-34 mol%) C18:1 – C16:0 (11-14 mol%)	-	

1.7 Overview of the thesis

The goal of this study was to establish and increase the synthesis of very long-chain jojoba-like WEs in *S. cerevisiae*.

Paper I

As shown in **Table 1**, the synthesis of WEs in *S. cerevisiae* was so far limited to a chain length up to C36, which does not represent the predominant WE species in jojoba oil, namely C42:2-WEs (46.8 mol%) and C40:2-WEs (20.7 mol%) (Iven et al., 2013). The synthesis of very long-chain WEs (VLCWEs) up to C44 in *S. cerevisiae* had so far only been achieved after substrate feeding. Therefore, the goal of this study was to enable the synthesis of jojoba-like WEs in *S. cerevisiae* without the additional feeding of FOH or FA precursors. For this purpose, various combinations of heterologous FARs and WSs were screened. The FARs from *A. mellifera* (*Am*FAR1) and *M. aquaeolei* VT8 (Maqu_2220/*Ma*FAldhR) enabled the synthesis of FOHs, with a chain length of C16-C22, when expressed in a *S. cerevisiae* strain carrying a deletion in *ELO3* and overexpressing *ELO2*. In addition, the combined expression of *MaFAldhR* together with the WS from *S. chinensis* (*SciWS*) in a *S. cerevisiae* strain carrying a deletion in *ELO3* and overexpressing *ELO2* led to the synthesis of jojoba-like WEs up to chain length of C42. However, DUWE formation was only observed up to a chain length of C36.

Paper II

In this study, we investigated a different route for production of VLCFAs and VLCFOHs in *S. cerevisiae* than presented in **Paper I**. We expressed a heterologous FAS I system from *Mycobacterium vaccae* (*Mv*) which is able to produce C16-FAs/C18-FAs as well as C22/C24/C26 VLCFAs (Kaneda et al., 1995). As a model compound, we followed the production of docosanol (C22:0-FOH) in *S. cerevisiae* under expression of different FARs. A FAR from *A. thaliana* (*At*FAR1) (Rowland and Domergue, 2012) was found to be able to catalyze the synthesis of docosanol when expressed in a *S. cerevisiae* strain defective in storage lipid synthesis and *ELO3*, while overexpressing *ELO2*. Since this strain suffered a severe growth defect, probably due to a lack of VLCFACoAs, we developed a dynamic control strategy for separating cell growth from docosanol production, involving galactose inducible promoters. The combined expression of *At*FAR1 and *Mv*FAS, fused to its PPT domain, in a background strain carrying a deletion in *ELO3,* while overexpressing *ELO1, ELO2* and a constitutively active version of *ACC1* (*ACC1***), led to the highest docosanol production reported so far.

Paper III

Paper I demonstrated that it is possible to synthesize VLCWEs in *S. cerevisiae*. Since DUWE formation was only observed up to a chain length of C36, we hypothesized that the natural pool of very long-chain monounsaturated fatty acids (VLCMUFAs) (> C18) in *S. cerevisiae* is too low to enable DUWE synthesis > C36. Therefore, the goal of this study was to increase the amount of VLCMUFAs of a chain length of C20-C24. For this purpose, we screened a range of heterologous FAEs/KCSs as well as FADs. A plant-derived FAE1/KCS from *Crambe abyssinica* (*Ca*FAE1) together with the yeast intrinsic Ole1p was shown to enable a steep increase in C20:1-FA and C22:1-FA. In contrast to that, a FAE1/KCS derived from *Lunaria annua* (*La*FAE1) led to a high increase in C24:1-FA. The combined expression of *CaFAE1*, *OLE1*, *MaFAldhR* and *SciWS* in a *S. cerevisiae* strain, carrying several other modifications, led to a yeast strain capable of producing high amounts of MUFOHs (up to C22:1-FOH) as well as DUWEs (up to C46:2-WE). Moreover, the analysis of the molecular composition of the WE species revealed that the final *S. cerevisiae* WE production strain showed a clear trend towards jojoba-like C42:2-WEs at the expense of C34:0-WEs.

Paper IV

The goal of this study was to investigate the potential regulatory role of lysine acetylation sites in Fas2p of *S. cerevisiae*, with the idea of identifying a way to increase the FA pool. The FAS enzyme, consisting of the subunits Fas1p and Fas2p, belongs to the most highly acetylated proteins in yeast. Since it is located at a branchpoint of acetyl-CoA metabolism, a regulatory function of the lysine acetylation sites is not unlikely. We used a residue replacement system, in which the AAs glutamine (Q) or arginine (R) were introduced at three lysine sites (K) (K83, K173 and K1551) in Fas2p, either separately or simultaneously. The goal of this AA replacement was to either mimic a constitutively acetylated (Q) or non-acetylatable state (R). Our results indicate that the three lysine residues studied do probably not fulfill a regulatory role, since both triple mutations (designated as *FAS2*^{QQQ} and *FAS2*^{RRR}, respectively) reduced the amount of secreted FFAs in a *S. cerevisiae* strain harboring deletions in *FAA1* and *FAA4*.

2 A route for jojoba-like wax ester synthesis in *S. cerevisiae*

As described in **section 1.6.1** and shown in **Table 1**, the heterologous expression of FARs and WSs in *S. cerevisiae* had so far mostly been performed to study the functionality of enzymes, rather than to specifically produce high amounts of WEs. Most studies were conducted under substrate feeding of FAs, FOHs or both precursors and only a few studies reported titers, yields or productivity.

Previous studies had shown that, among others, the FARs derived from *Apis mellifera* (*Am*FAR1) (Teerawanichpan et al., 2010), *A. thaliana* (*At*FAR1, *At*FAR4, *At*FAR5) (Domergue et al., 2010) and *E. gracilis* (*Eg*FAR) (Teerawanichpan and Qiu, 2010) are able to catalyze the formation of FOHs in *S. cerevisiae*. In the meantime, the formation of FOHs in *S. cerevisiae* was also successfully achieved by expression of a FAR derived from *M. aquaeolei* VT8 (Maqu_2507) (Zhou et al., 2016), four FAR homologues of *Triticum aestivum* (*Ta*FAR1/2/3/4) and the FAR from *S. chinensis* (*Sci*FAR) (Miklaszewska and Banaś, 2016).

WSs tested to enable WE synthesis in *S. cerevisiae* include enzymes from *A. baylyi* ADP1 (*Ab*WS) (Kalscheuer et al., 2004), *A. domesticus* (*Ad*WS4) (Biester et al., 2012), *E. gracilis* (*Eg*WS, *Eg*WSD2, *Eg*WSD5) (Teerawanichpan et al., 2010; Teerawanichpan and Qiu, 2010; Tomiyama et al., 2017), *G. gallus* (*Gg*WS1/2/4/5, *Gg*DGAT1) (Biester et al., 2012), *M. hydrocarbonoclasticus* DSM 8798 (*Mh*WS2) (Miklaszewska et al., 2018), *M. musculus* (*Mm*WS)(Heilmann et al., 2012; Miklaszewska et al., 2013; Yu et al., 2018), *Petunia hybrida* (*Ph*WS1) (King et al., 2007), *S. chinensis* (*Sci*WS) (Miklaszewska and Banaś, 2016; Yu et al., 2018) *Thraustochytrium roseum* (*Tr*WSD4) (Zhang et al., 2017) and *T. alba* (*Ta*WS4) (Biester et al., 2012) (**Table 1**). The highest WE yield in transgenic yeasts and plants reported in literature were achieved by expression of enzymes derived from *Marinobacter* species. *S. cerevisiae*, expressing a WS derived from *M. hydrocarbonoclasticus* (*Mh*WS2), showed the synthesis of ~ 20.57 ± 1.67 mg WEs/g CDW under supplementation of 0.5 mg/mL hexadecanol (C16:0-FOH) (Miklaszewska et al., 2018). However, this study did not describe the composition of the WEs produced. Transgenic *A. thaliana* expressing a FAR derived from *M. aquaeolei* VT8 (*Ma*FAldhR) together with a WS derived from *S. chinensis* (*Sci*WS) achieved a WE production of ~ 100 mg WEs/g seeds, with C18:1-C20:1 being the most abundant WE species (17.7 mol%) (Iven et al., 2016). The combined expression of a FAR and a WS both derived from *S. chinensis* (*Sci*FAR + *Sci*WS) led to the highest WE yield in transgenic *C. abyssinica* (~ 85 mg WEs/g seeds), with C22:1-C20:1 being the most abundant WE species (~ 30 mol%). This WE species is also the most abundant WE species in jojoba oil (32.2 mol%) (Iven et al., 2013; Zhu et al., 2016).

Based on the literature available when we started our work on jojoba-like WE production in *S. cerevisiae* CEN.PK 113-5D (*MAT*a *MAL2-8 SUC2 ura3-52*), we decided to test a range of FARs, including the ones from *A. mellifera* (*Am*FAR1) (NCBI accession no. ADJ56408),

M. aquaeolei VT8 (Maqu_2220/*Ma*FAldhR) (NCBI accession no. YP_959486), *S. chinensis* (*Sci*FAR) (NCBI accession no. AF149917) and *T. aestivum* (*Ta*FAR) (TAA1a; NCBI accession no. CAD30692), for their ability to synthesize very long-chain fatty alcohols (VLCFOHs) in *S. cerevisiae*. The WSs we tested for their ability to catalyze jojoba-like WE formation in *S. cerevisiae* included the ones from *A. baylyi* ADP1 (*Ab*WS) (NCBI accession no. AF529086), *A. thaliana* (*At*WS) (NCBI accession no. NP_568547), *E. gracilis* (*Eg*WS) (NCBI accession no. ADI60058), and *S. chinensis* (*Sci*WS) (NCBI accession no. AF14991).

Since *S. cerevisiae* naturally mainly produces C16:1Δ9-FA, C18:1Δ9-FA, C16:0-FA and C18:0-FA (Oh et al., 1997; Welch and Burlingame, 1973), we tested the expression of FARs and WSs in a background strain in which the synthesis of VLCFAs was boosted by deletion of *ELO3* and overexpression of *ELO2* as well as a constitutive active version of *ACC1* (*ACC1***)(**Figure 12**) (Hofbauer et al., 2014; Shi et al., 2014a). This led to a strain incapable of synthesizing C26:0-FA, showing decreased amounts of C18:1-FA, but increased amounts of the VLCFAs C20:0-FA, C20:1-FA, C22:0-FA, C22:1-FA, C24:0-FA and C24:1-FA, as previously described by Oh et al. (1997) (**Figure 13**).

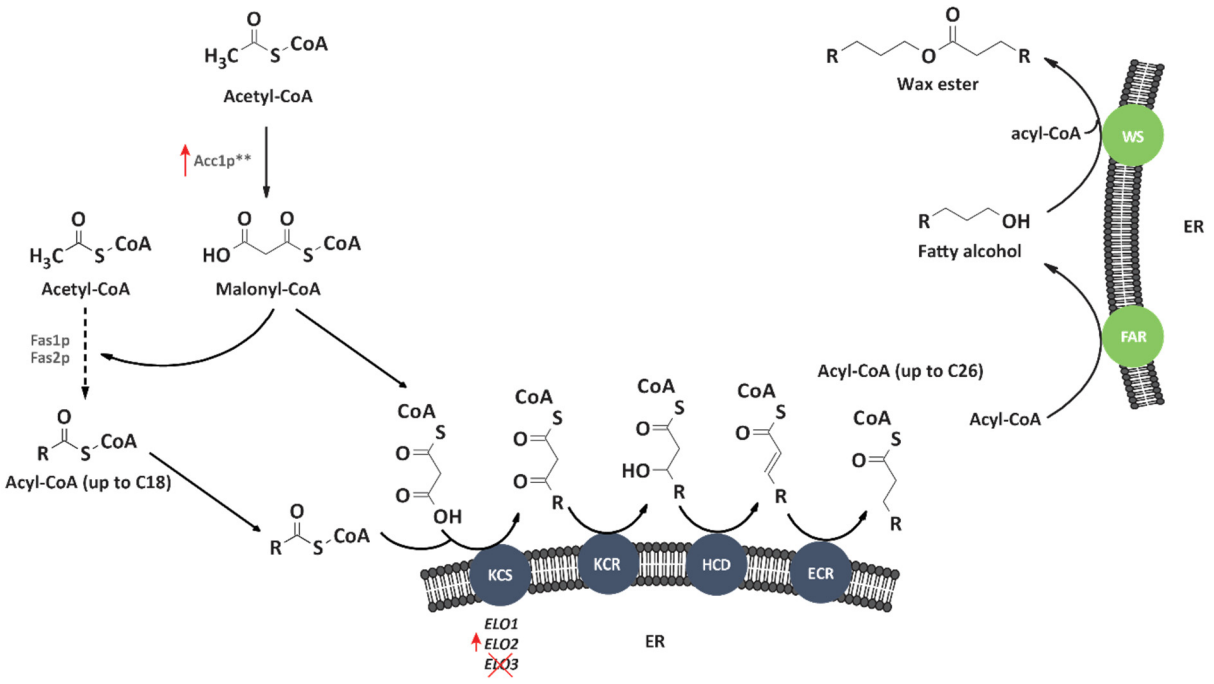

Figure 12 Wax ester synthesis pathway in engineered *S. cerevisiae* strains. Synthesis of fatty acids (FAs), fatty alcohols (FOHs), and wax esters (WEs) in modified *S. cerevisiae* strains, catalyzed by intrinsic and heterologous enzymes (indicated in grey/blue and green, respectively). *De novo* FA synthesis in yeast starts with carboxylation of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (Acc1p). The next steps in cytosolic FA biosynthesis are catalyzed by the fatty acid synthases 1 (Fas1p; β-subunit) and 2 (Fas2p; α-subunit), finally leading to C12–C18 fatty acyl-CoAs (FACoAs). The elongation of the C12–C18 FACoAs to C16-C26 FACoAs occurs at the endoplasmic reticulum (ER), catalyzed by four different enzymes in yeast, including a β-ketoacyl-CoA synthase (KCS) (fatty acid elongases Elo1p, Elo2p and Elo3p), the β-ketoacyl-CoA reductase (KCR), the β-hydroxyacyl-CoA dehydratase (HCD), and the enoyl-CoA reductase (ECR). FACoAs from *de novo* FA biosynthesis and elongation can be reduced by a heterologous fatty acyl-CoA reductase (FAR) to a FOH. Another enzyme, the wax synthase (WS), catalyzes the esterification of a FOH with another FACoA molecule, leading to a WE. In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps, → indicates a single step; ↑ indicates overexpression of a gene and ✕ indicates deletion of a gene. Adapted from Wenning et al. (2017).

Figure 13 Fatty acid composition. Concentration of fatty acids ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* CEN.PK 113-5D, *S. cerevisiae* CEN. PK 113-5D *elo3*Δ, *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1***, *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212) and *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*ELO2*). All strains were grown in minimal medium containing 2% glucose for 48 h. Adapted from Wenning et al. (2017).

When we tested the combined overexpression of the codon optimized sequences of either *Sci*FAR or *Ta*FAR (TAA1a) with *ELO2* in *S. cerevisiae*, we were not able to detect the formation of FOHs (data not shown). This is in contrast to a study by Miklaszewska and Banaś (2016) which showed that the expression of *Sci*FAR in *S. cerevisiae* led to the formation of mostly C18:0-FOH. In case of *Ta*FAR (TAA1a), four homologous enzymes with 75%, 55%, 49%, and 95%-AA identity, respectively, to *Ta*FAR (TAA1a) (*Ta*FAR1, *Ta*FAR2, *Ta*FAR3, and *Ta*FAR4) were successfully expressed in *S. cerevisiae* and the production of C22:0-FOH, C18:0-FOH, C24:0-FOH, and C28:0-FOH, respectively, could be detected (Wang et al., 2015, 2016). Reasons for the observed differences between previous studies and our study could be that we used codon optimized genes for expression in *S. cerevisiae* which might not be favorable for the investigated enzymes. Moreover, in the study by Miklaszewska and Banaś (2016), Wang et al. (2015) and Wang et al. (2016) different background strains were used compared to our study.

In contrast to that, we observed that the combined overexpression of *AmFAR1* and *ELO2* in the *S. cerevisiae* background strain CEN.PK 113-5D *elo3*Δ *ACC1*** led to the formation of C16:0-FOH, C16:1-FOH, C18:0-FOH, C18:1-FOH, C20:0-FOH, C20:1-FOH, C22:0-FOH and C22:1-FOH, with the highest concentration of C22:0-FOH/C22:1-FOH followed by C18:0-FOH/C18:1-FOH (**Figure 14**). The total FOH production was 3.22 ± 0.36 mg FOHs/g CDW after 48 h.

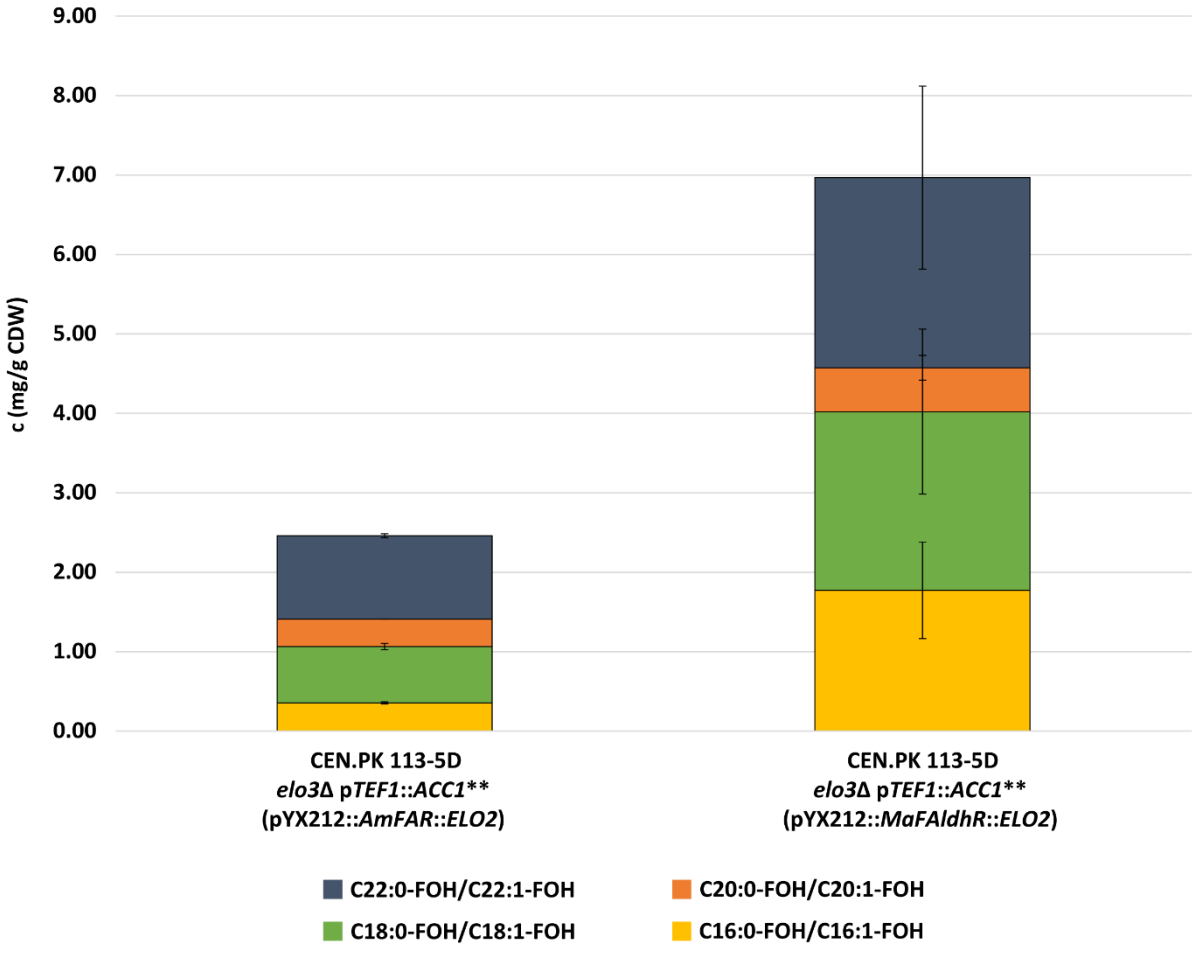

Figure 14 Fatty alcohol composition. Concentration of fatty alcohols (FOHs) ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*ELO2*::*AmFAR*) and *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*ELO2*::*MaFAldhR*). Both strains were grown in minimal medium containing 2% glucose for 48 h. Adapted from Wenning et al. (2017).

In comparison to that, Teerawanichpan et al. (2010) identified C18:0-FA as the most preferred substrate of *Am*FAR1 when the enzyme activity was tested in *S. cerevisiae* under feeding of 250 µM of FAs. In general, the enzyme was capable of converting FAs of a chain length between C16 and C22.

Interestingly, most of the FAs in the background strain CEN.PK 113-5D *elo3*Δ *ACC1*** have a chain length of C16 (70.73% of the total fatty acids (TFAs)). Nevertheless, only 11.07% of the produced FOHs in strain CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*AmFAR1*::*ELO2*) have the same carbon chain length. In contrast to that, only 0.99% of the TFAs have a chain length of C22, while at the same time 32.60% of the produced FOHs in strain CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*AmFAR1*::*ELO2*) have the same carbon chain length. This strongly indicates that *Am*FAR1 has a preference towards C22-FAs as substrate, but that it can convert a range of other substrates as well. This observation stands in contrast to the one by Teerawanichpan et al. (2010), which identified C18:0-FA as preferred substrate of this enzyme. One reason for this difference could be that the FAs that were fed to the yeast cells in the study by Teerawanichpan et al. (2010) have a different uptake rate and/or efficiency, which leads to a varied substrate availability inside the yeast cells. Moreover, the activation rate and/or efficiency of FAs to FACoAs inside the yeast cells could be different for different FA chain lengths. In conclusion, the higher C18:0-FOH titers observed by Teerawanichpan et al. (2010) might be the result of a more efficient C18:0-FA uptake and/or activation compared to the same process for C20:0-FA and C22:0-FA.

In case of the combined overexpression of *MaFAldhR* (Maqu_2220) and *ELO2* in the *S. cerevisiae* background strain CEN.PK 113-5D *elo3*Δ *ACC1***, the formation of C16:0-FOH, C16:1-FOH, C18:0-FOH, C18:1-FOH, C20:0-FOH, C20:1-FOH, C22:0-FOH and C22:1-FOH, with the highest concentration of C22:0-FOH/C22:1-FOH followed by C18:0-FOH/C18:1-FOH could be observed (**Figure 14**). The total FOH production was 7.84 ± 3.09 mg FOHs/g CDW after 48 h.

Maqu_2220 has also been used for the synthesis of FOHs in a range of other organisms, including bacteria, cyanobacteria, oleaginous yeasts as well as plants. The best production strain of *Escherichia coli* (derived from MG1655), harboring the Maqu_2220 enzyme, produced 3.82 ± 0.05 g/L FOHs after 71 h in shake flaks. After 71 h, the most abundant FOH was C16:1-FOH, followed by C16:0-FOH, C14:1-FOH and C18:1-FOH (Haushalter et al., 2015). To achieve an increased synthesis of medium chain FOHs in *E. coli*, expression of Maqu_2220 or Maqu_2507 was combined with the expression of various thioesterases (Liu et al., 2013; Youngquist et al., 2013). An *E. coli* strain (Δ*fadE*) overexpressing its intrinsic fatty acyl-CoA synthetase (*Ec*fadD) in combination with Maqu_2220 and the thioesterases from *Cinnamomum camphorum* (CCTE) as well as *Umbellularia californica* (BTE), produced 258.3 mg/L FOHs, with the most prominent one being C12:0-FOH. In case of the cyanobacterium *Synechocystis* sp. PCC 6803, the initial production strain, containing the Maqu_2220 enzyme, reached an FOH level of 2.87 ± 0.29 mg/g CDW after 140 h. FOHs detected included C16:0-FOH and C18:0-FOH (Yao et al., 2014). This

productivity could be increased to 10 mg FOHs/g CDW by partial repression of six genes, including *slr1510*, encoding the essential phosphate acyltransferase enzyme PlsX (Kaczmarzyk et al., 2018). When Maqu_2220 was expressed in the oleaginous yeast *Y. lipolytica* together with *Ec*fadD, a FOH tier of 205.4 mg/L could be observed (Xu et al., 2016). The highest overall alcohol titer of 8 g/L was reached in the oleaginous yeast *Rhodosporidium toruloides* through fed-batch fermentation after 75 h. In this case, the most abundant FOH was C18:1-FOH, followed by C18:0-FOH and C16:0-FOH (Fillet et al., 2015). The reported alcohol titer is the highest so far reached in microbial production.

Transgenic plants expressing Maqu_2220 also show the synthesis of C20:1-FOH, in addition to C16:0-FOH, C18:0-FOH and C18:1-FOH (Aslan et al., 2014; Aslan et al., 2015; Iven et al., 2013; Iven et al., 2016; Yu et al., 2018). Except for the studies conducted in plants, all previous studies involving expression of Maqu_2220 in heterologous organisms only report the synthesis of FOHs up to a chain length of C18. In contrast to that, we could show in our study, that Maqu_2220 can also catalyze the formation of C20-FOHs and C22-FOHs in heterologous microorganisms. However, our study could not detect the formation of FOHs shorter than C16 or longer than C22. Similar to what has been observed for *Am*FAR1 expression, the FOH product range of *Ma*FAldhR seems to be determined by a combination of substrate availability as well as enzyme specificity.

Because of the observed results for FOH production in *S. cerevisiae*, we tested *Am*FAR1 and *Ma*FAldhR as candidate enzymes for the production of WEs in *S. cerevisiae* under combined expression with a WS. We analyzed the combined overexpression of a FAR (*Am*FAR1, *Ma*FAldhR), a WS (*Ab*WS, *At*WS, *Eg*WS or *Sci*WS) and *ELO2* in the background strain CEN.PK 113-5D *elo3*Δ *ACC1***. Expression of *Am*FAR1 in combination with *Ab*WS, *At*WS, or *Eg*WS, did not lead to any WE formation (data not shown). In contrast to that, the strain expressing *Am*FAR1 in combination with *Sci*WS and *ELO2* showed the formation of saturated, mono- and DUWEs with a chain length ranging from C30 to C42, with C34:0-WE being the most prominent one, followed by C40:0-WE (**Fig. 5B in paper I**). Peak identification was performed based on appropriate WE standards and on comparison with the spectra in Urbanová et al. (2012).

The highest WE production and the broadest spectrum of WEs in *S. cerevisiae* was observed by the combined expression of *Ma*FAldhR, *Sci*WS and *ELO2*. WEs synthesized included saturated, mono- and diunsaturated ones with a chain length ranging from C30 to C42 (**Fig. 5C in paper I; Figure 15**). The 31 different WE species identified were composed of a FOH residue with a chain length of C16–C22 and a FACoA residue with a chain length of C14–C22 (**Table 3**). The most common WEs in this strain were C34-WEs. The total WE production was 12.24 ± 3.35 mg WEs/g CDW, which corresponds to a concentration of 14.98 mg/L in culture and a yield of 0.749 mg WEs/g glucose. The modified strain produced 3.31 ± 1.13 mg WEs/g CDW with a chain length >C38. This corresponds to a percentage of 27.1% of all WEs produced in this particular strain (**Figure 15**). DUWEs were only synthesized up to a chain length of C36. The most likely reason for this is that the concentration of VLCMUFAs in the modified yeast strains is too low to

promote the formation of DUWEs above a chain length of C36. Therefore, the results presented in **paper I** clearly showed that it is important to promote the increased formation of VLCMUFAs (C22-C24) in *S. cerevisiae* to enable the synthesis of jojoba-like WEs in this yeast species.

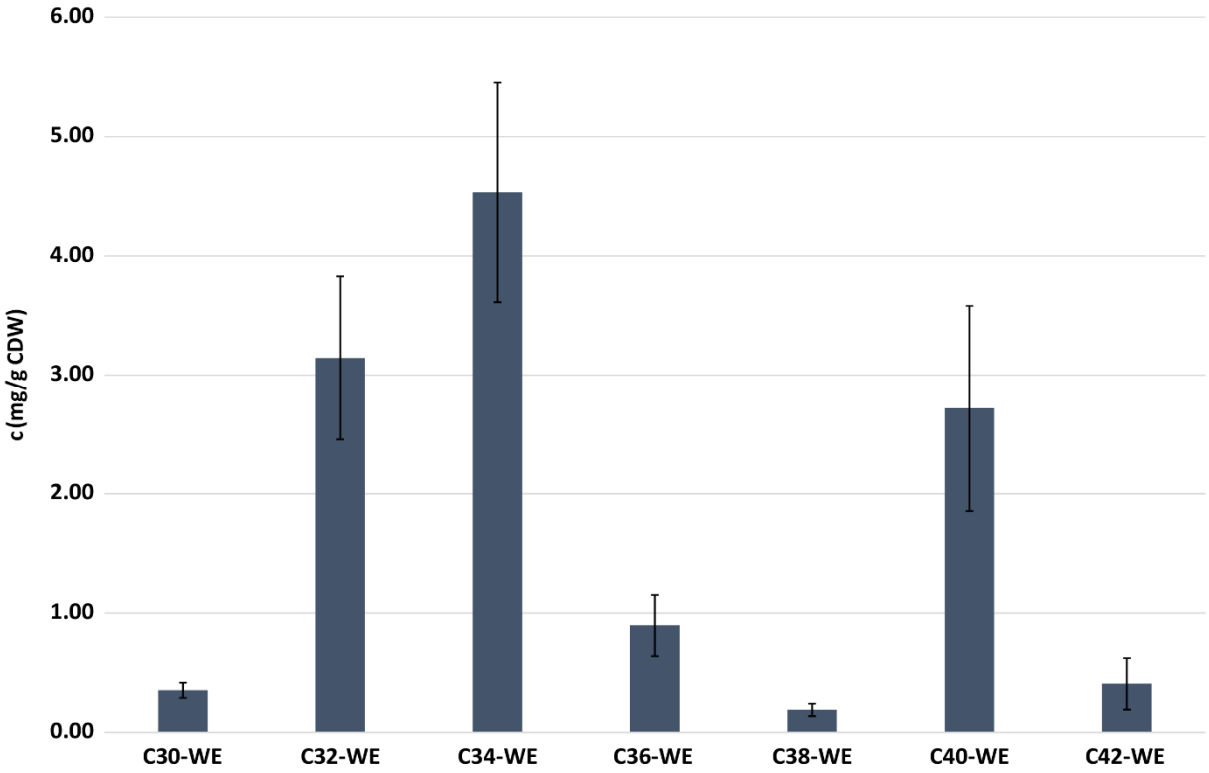

Figure 15 Wax ester composition. Concentration of wax esters (WEs) ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*MaFAldhR*::*SciWS*::*ELO2*). The strain was grown in minimal medium containing 2% glucose for 48 h. Adapted from Wenning et al. (2017).

Since the FAR from *M. aquaeolei* VT8 (*Ma*FAldhR/Maqu_2220) has been proven to be a very efficient enzyme in different hosts (including cyanobacteria, oleaginous yeasts, *S. cerevisiae* as well as plants) it might be of interest to further investigate *Marinobacter* FARs from different strains, e.g. *M. algicola* DG893 (NCBI accession no. ZP_01892457) or *M. adhaerens* HP15 (NCBI accession no. ADP96574) (Hofvander et al., 2011), to reveal their mode of action. Moreover, it might be interesting to express a combination of *Ma*FAldhR together with the WS from *M. hydrocarbonoclasticus* DSM 8798 (*Mh*WS2) in *S. cerevisiae* or another yeast, since those enzymes are both derived from the same organism and therefore the substrate channeling between the two enzymes might be favorable. In addition to that it has been shown previously, that expression of *Mh*WS2 in *S. cerevisiae* can lead to a high WE titer (Miklaszewska et al., 2018).

Table 3 Composition of wax ester (WE) species detected in strain *S. cerevisiae* CEN.PK 113-5D *elo3*Δ *ACC1*** (pYX212::*MaFAldhR*:: *SciWS*::*ELO2*). The strain was grown in minimal medium containing 2% glucose for 48 h. Adapted from Wenning et al. (2017).

WE chain length	Alcohol moiety	Acyl moiety	Name
C30:0	C16:0	C14:0	Palmityl myristate
C30:1	C16:1	C14:0	Palmitoleyl myristate
C32:0	C16:0	C16:0	Palmityl palmitate
C32:0	C18:0	C14:0	Stearyl myristate
C32:1	C16:0	C16:1	Palmityl palmitoleate
C32:1	C18:0	C14:1	Stearyl myristoleate
C32:2	C16:1	C16:1	Palmitoleyl palmitoleate
C34:0	C20:0	C14:0	Arachidyl myristate
C34:0	C16:0	C18:0	Palmityl stearate
C34:0	C18:0	C16:0	Stearyl palmitate
C34:1	C18:0	C16:1	Stearyl palmitoleate
C34:1	C16:0	C18:1	Palmityl oleate
C34:2	C18:1	C16:1	Oleyl palmitoleate
C34:2	C16:1	C18:1	Palmitoleyl oleate
C36:0	C20:0	C16:0	Arachidyl palmitate
C36:0	C22:0	C14:0	Behenyl myristate
C36:0	C16:0	C20:0	Palmityl arachidate
C36:0	C18:0	C18:0	Stearyl stearate
C36:1	C20:0	C16:1	Arachidyl palmitoleate
C36:2	ND[1]	ND[1]	ND[1]
C38:0	C22:0	C16:0	Behenyl palmitate
C38:0	C16:0	C22:0	Palmityl behenate
C38:0	C20:0	C18:0	Arachidyl stearate
C38:0	C18:0	C20:0	Stearyl arachidate
C38:1	C22:0	C16:1	Behenyl palmitoleate
C38:1	C20:0	C18:1	Arachidyl oleate
C40:0	C22:0	C18:0	Behenyl stearate
C40:0	C20:0	C20:0	Arachidyl arachidate
C40:0	C18:0	C22:0	Stearyl behenate
C40:1	C22:0	C18:1	Behenyl oleate
C42:0	C20:0	C22:0	Arachidyl behenate
C42:0	C22:0	C20:0	Behenyl arachidate

[1] Could not be determined by comparison to the mass spectra of analyzed wax ester standards or to mass spectra in Urbanová et al. (2012).

3 Alternative route for very long-chain fatty acid and alcohol synthesis in *S. cerevisiae*

To investigate an alternative route for VLCFA synthesis in yeast, we tested the expression of a bacterial type I FAS system derived from *M. vaccae* in *S. cerevisiae*. Usually, bacteria harbor a type II FAS system which consists of several separate proteins catalyzing the synthesis of FAs. In contrast to that, Mycobacteria harbor a special type I FAS system, which is responsible for producing C16-FAs/C18-FAs for the intrinsic cellular demand, as well as C22/C24/C26 VLCFAs. The FAS I system from *M. vaccae* is reported to specifically synthesize C16-FAs/C18-FAs as well as C22/C24 VLCFAs. Those VLCFAs are used as building blocks for the synthesis of mycolic acids, major constituents of the mycobacterial cell envelope with a chain length up to 90 carbons (Kaneda et al., 1995). The mycobacterial FAS I is a large multifunctional enzyme which shares homology with fungal FASs (Boehringer et al., 2013). Using a heterologous enzyme for the synthesis of VLCFAs in yeast has the advantage of not underlying natural regulation mechanisms, unlike the yeast intrinsic elongases Elo1p, Elo2p and Elo3p.

As an example output molecule of VLCFA metabolism in yeast, this study focused on the production of docosanol, a saturated VLCFOH with a chain length of 22 carbons. It is used as an emollient, emulsifier, thickener in cosmetics and nutritional supplements with a market volume of 40,000 ton per year. In addition to that, docosanol has been approved as a pharmaceutical antiviral agent for reducing the duration of cold sores caused by the herpes simplex virus (Katz et al., 1991). As described earlier, a FAR is needed for conversion of FAs to FOHs. In a previous study, a FAR from *A. thaliana* (*At*FAR1) had been identified as being specific for the conversion of docosanoic acid (C22:0-FA) to docosonaol (C22-FOH) in planta and mainly converting C18:0-FA and C22:0-FA into their respective FOHs in yeast (Domergue et al., 2010). Therefore, *At*FAR1 was included in this study to enable the production of docosanol in *S. cerevisiae* (**Figure 16**).

The synthesis of docosanoic acid (C22:0-FA) was first investigated in a *S. cerevisiae* strain (JV03) accumulating LCFAs. This strain carries defects in storage lipid synthesis and β-oxidation (CEN.PK 113-5D *MAT*a *MAL2-8c SUC2 ura3-52 HIS3 are1*Δ *dga1*Δ *are2*Δ *lro1*Δ *pox1*Δ) (**Figure 17a**). Naturally, strain JV03 does not produce C22:0-FA. Only by deletion of *ELO3* and overexpression of *ELO2*, C22:0-FA production is triggered. Despite using an engineered background strain, the overall production of C22:0-FA was below 1 mg/g CDW (**Figure 17b**).

To become independent from the yeast native elongation system, we focused on establishing the FAS I from *M. vaccae* (*Mv*FAS) (NCBI reference sequence: WP_003928293.1) in yeast. The *Mv*FAS was selected as it would provide high levels of C22:0-FA as direct precursors for docosanol production. Even if there are other mycobacterial species described that solely produce C22:0-FA as VLCFA, in contrast to *M. vaccae* which also produces C24:0-FA, only in case of *M. vaccae* the sequence encoding the FAS was known when this study was conducted.

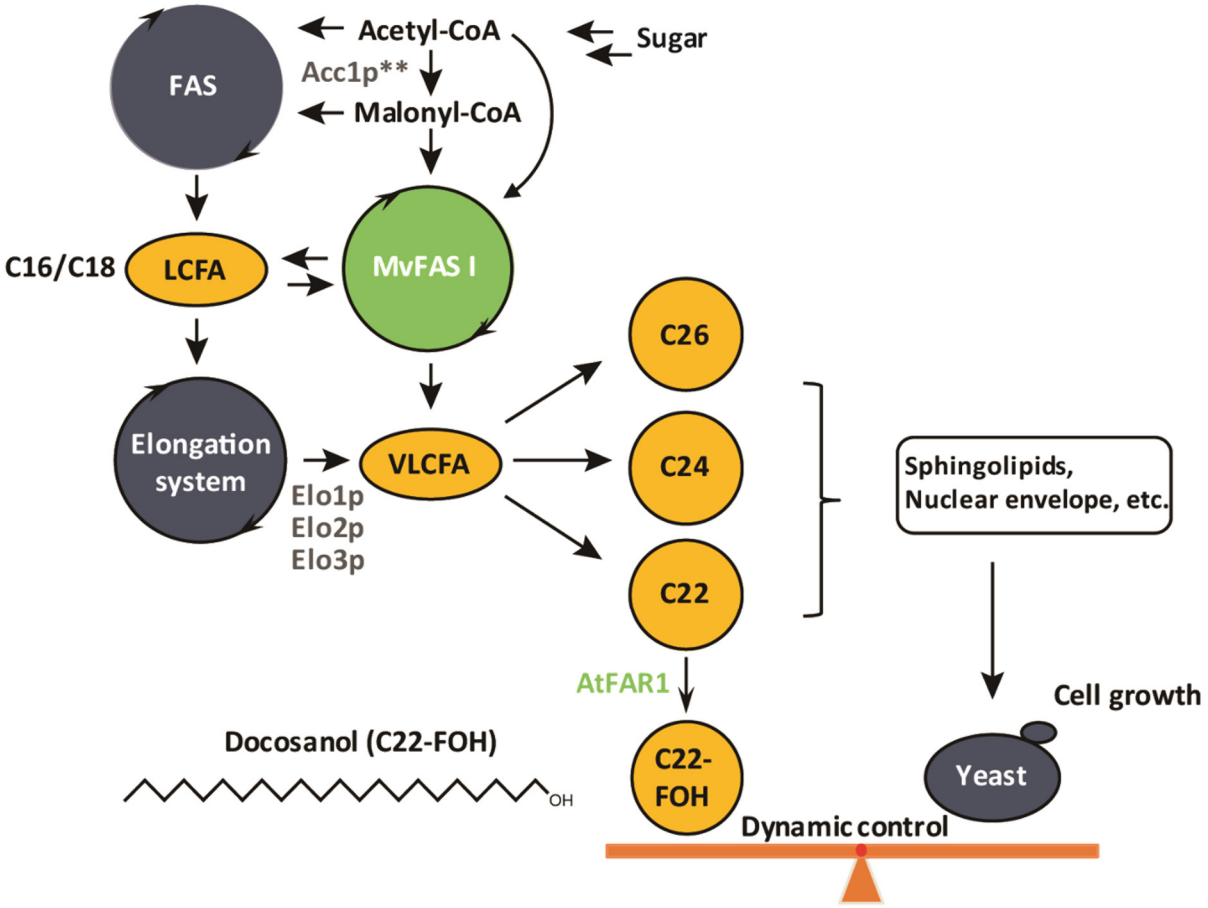

Figure 16 General strategy for docosanol production in modified *S. cerevisiae*. Carbon sources, like glucose, are converted into acetyl-CoA in the cytosol of *S. cerevisiae.* The acetyl-CoA carboxylase (Acc1p) catalyzes the conversion of acetyl-CoA to malonyl-CoA. Malonyl-CoA can then either be used by the intrinsic yeast fatty acid synthase (FAS) or by the heterologous FAS of *M. vaccae* (*Mv*FAS I) to generate C16/C18 long-chain fatty acids (LCFAs). LCFAs are elongated to very long-chain fatty acids (VLCFAs) via the intrinsic elongation system (represented by the yeast fatty acid elongases Elo1p, Elo2p and Elo3p) or *Mv*FAS I and further modified to the target product of interest. VLCFAs can be converted into fatty alcohols (FOHs) by action of a fatty acyl reductase (FAR), e.g. one from *A. thaliana* (*At*FAR1). The product selectivity is determined by the choice of enzymes and the background strain. Adapted from Yu et al. (2017).

In contrast to the FAS system from *S. cerevisiae* which is able to perform self-pantetheinylation of the ACP domain and thereby autoactivation of the FAS enzyme, catalyzed by the PPT domain, the mycobacterial FAS lacks the PPT domain and does therefore require an additional protein, an acyl carrier protein synthase (AcpS), for activation of ACP by pantetheinylation (Boehringer et al., 2013). Therefore, the *Mv*FAS used in this study was fused at its carboxy-terminus via a 3*GGGGS linker to an AcpS derived from *M. vaccae*, generating *Mv*FAS-AcpS. This chimeric *Mv*FAS-AcpS was able to restore the growth of the FA auxotrophic yeast strain PWY12 (*MAT*a *ura3*Δ *leu2*Δ *his3*Δ *trp1*D *can1*D *fas1*Δ::*HIS3* *fas2*Δ::*LEU2*) (Wenz et al., 2001) on yeast extract peptone dextrose medium (YPD) or complete supplement mixture (CSM) synthetic medium without FA addition (**Supplementary Fig. 2 in paper II**). This observation shows that *Mv*FAS-AcpS is functional in *S. cerevisiae* and able to replace the natural yeast FAS system, consisting of Fas1p/Fas2p, in a *fas1*Δ *fas2*Δ background strain.

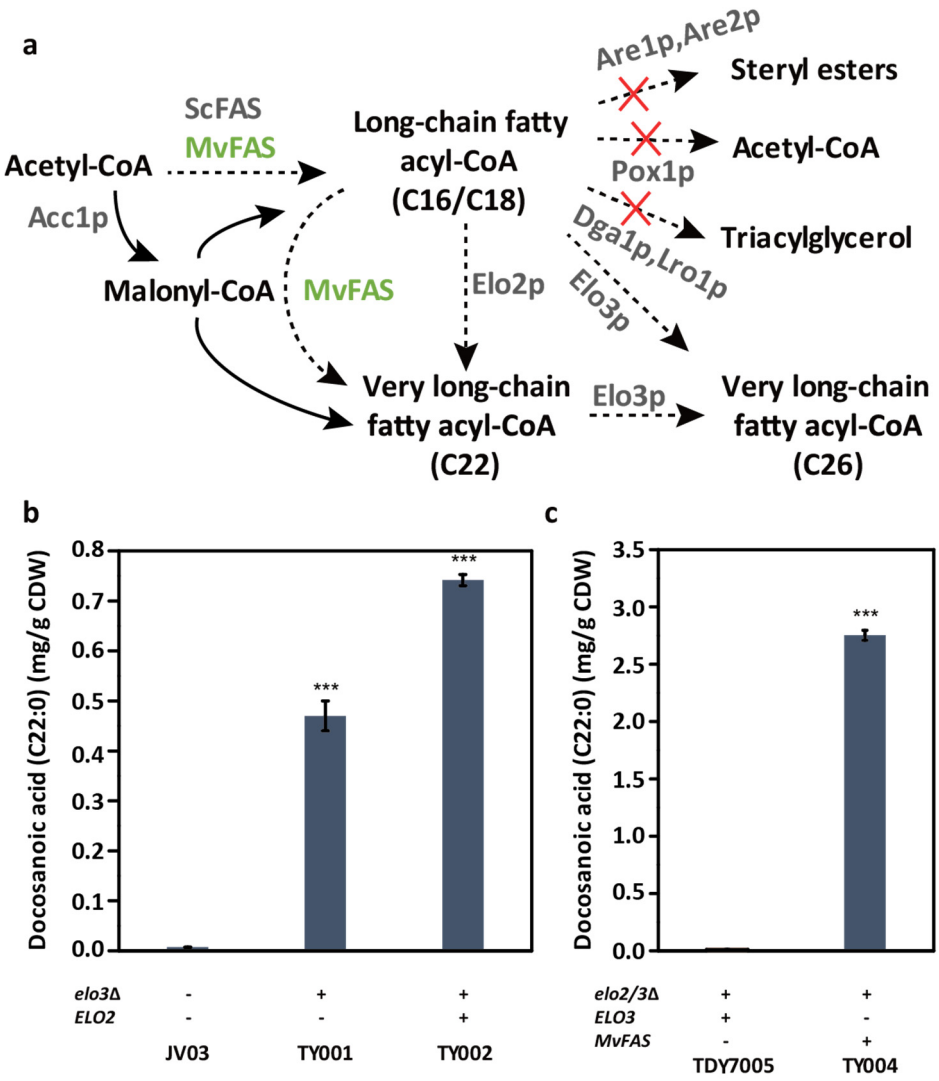

Figure 17 Expression of a mycobacterial fatty acid synthase (*Mv*FAS) in a storage lipid biosynthesis and β-oxidation defective strain of *S. cerevisiae* (CEN.PK 113-5D *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ *pox1*Δ) (JV03). (a) Schematic overview of strategies for chain length control towards very long-chain fatty acids. Overexpressed genes are shown in grey (endogenous) or green (heterologous). Storage lipid biosynthesis and β-oxidation were eliminated by deleting genes encoding Are1p/Are2p, steryl ester synthases 1/2; Dga1p/Lro1p, acyltransferases; Elo2p/Elo3p, fatty acid elongases 2/3 and Pox1p, acyl-CoA oxidase in *S. cerevisiae*. *Sc*FAS, fatty acid synthase of *S. cerevisiae*; *Mv*FAS, fatty acid synthase of *M. vaccae*. **(b)** Production of docosanoic acid (C22:0-FAME) ± SD (mg/g CDW) in strains TY001 (JV03 *elo3*Δ) and TY002 (JV03 *elo3*Δ p*ELO2*). **(c)** Production of docosanoic acid ± SD (mg/g CDW) in strain TDY7005 (*MAT*a *lys2 ura3–52 trp1*Δ *leu2*Δ *elo2*Δ::*kanMX elo3*Δ Δ::*TRP1*/p*ELO3*) and strain TY004 (*MAT*a *lys2 ura3–52 trp1*Δ *leu2*Δ *elo2*Δ*::kanMX elo3* Δ*::TRP1/*pGPD415-*MvFAS-AcpS*). Shown is the mean ± SD of three biological replicates of a representative measurement. *p<0.05; **p<0.01 and ***p<0.001 (Student's t-test, one-tailed, two-sample unequal variance). The strains were grown in minimal medium containing 3% glucose for 72 h. In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps, → indicates a single step; ✕ indicates deletion of a gene. Adapted from Yu et al. (2017).

Since *Mv*FAS-AcpS expression in the *S. cerevisiae* strain PWY12, harboring its natural elongation system, mainly showed the synthesis of C26:0-FA (**Supplementary Fig. 3 in paper II**), the next step was to test the expression of the *Mv*FAS-AcpS in an *ELO2/ELO3* deletion strain, TDY7005 (*MAT*a *lys2 ura3–52 trp1*Δ *leu2*Δ *elo2*Δ*::KAN elo3*Δ*::TRP1/*pRS316-*ELO3*) (Paul et al., 2006).

As described in previous studies, the double deletion mutant *elo2*Δ *elo3*Δ is not viable and shows growth deficiencies even when supplemented with VLCFAs (Oh et al., 1997; Revardel et al., 1995; Silve et al., 1996). Therefore, strain TDY7005 contains *ELO3* on a plasmid carrying a *URA3* marker (pRS316-*ELO3*) that can be used for counter-selection on 5-fluoroorotic acid (5-FOA) containing plates. To test the *Mv*FAS in an *elo2*Δ *elo3*Δ background strain, the plasmid p415GPD-*MvFAS-AcpS*, carrying a *LEU* marker, was introduced into strain TDY7005. The final strain TY004, which had lost pRS316-*ELO3*, was obtained by counter-selection on 5-FOA containing plates. Strain TY004 was able to produce C22:0-FA and C24:0-FA which clearly demonstrates the ability of *Mv*FAS to replace the natural yeast elongases Elo2p/Elo3p in an *elo2*Δ *elo3*Δ background strain (**Supplementary Fig. 4a in paper II; Figure 17c**). Expression of *Mv*FAS in *S. cerevisiae* led to an almost fourfold higher yield of C22:0-FA (mg/g CDW) compared to engineering of the yeast intrinsic elongation system (**Figure 17b/c**).

To implement docosanol production in yeast, several heterologous FARs, including the ones from *A. mellifera* (*Am*FAR1) (NCBI accession no. ADJ56408), *A. thaliana* (*At*FAR1) (NCBI accession no. NP_197642), *Calanus finmarchicus* (*Cf*FAR1) (NCBI accession no. JN243755), *S. chinensis* (*Sci*FAR) (NCBI accession no. AF149917) and *T. aestivum* (*TaFAR*) (TAA1a; NCBI accession no. CAD30692), were expressed in two JV03 (CEN.PK 113-5D *are1*Δ *dga1*Δ *are2*Δ *lro1*Δ *pox1*Δ) background strains, JV03 *elo3*Δ (TY001) and JV03 *elo3*Δ p*ELO2* (TY002). Of the tested FARs, only the one from *A. thaliana* was able to catalyze docosanol formation in the background strain JV03 *elo3*Δ p*ELO2* (resulting strain TY012) (**Supplementary Table 2 in paper II; Figure 18a/b**). Strain TY012 was able to produce 1.13 mg/L docosanol after 72 h of growth in minimal medium containing 3% glucose (**Figure 18b**). At the same time, expression of *At*FAR1 reduced the final OD_{600} of the strain from OD_{600} = 6.0 to OD_{600} = 0.3 (**Figure 18c**). This is probably due to the fact that the cell is lacking essential VLCFACoAs for growth under expression of *At*FAR1.

To restore cell growth and achieve higher docosanol production, the next step was to establish a dynamic control system. This was implemented by separating cell growth and docosanol production into two different phases through expression of genes under carbon source dependent promoters (**Figure 19**). For expression of *ELO3*, the *HXT1* promoter (*HXT1*p) was chosen which is activated by high levels of glucose and becomes repressed as the level of glucose decreases (Keren et al. 2013). In contrast to that, *AtFAR1* was expressed under various promoters (*HXT7*p, *ADH2*p or *ICL1*p) which have in common that they are all repressed by high glucose concentrations and activated at low levels of glucose. Another promoter tested for *AtFAR1* expression was the *GAL1* promoter (*GAL*1p) which is activated by galactose but repressed by glucose. The highest titer and biomass

specific yield of docosanol, 4.19 ± 0.2 mg/L and 12.85 ± 0.55 mg/OD_{600}, respectively, was achieved in a strain expressing *AtFAR1* under control of *GAL1*p in the background strain JV03 *elo3*Δ p*ELO2* (resulting strain TY018) (**Supplementary Table 3 in paper II, Figure 19c**). This titer is about fourfold higher compared to strain TY012 in which *AtFAR1* is expressed under the constitutive promoter *PGK1* (*PGK1*p). Strain TY018 showed a final OD_{600} of 6.59 ± 0.19, which was very similar to its parent strain (TY001), which showed a final OD_{600} of 8.71 ± 0.38 (**Figure 19c**). When *ELO3* was expressed in strain TY018, no docosanol production could be detected. This indicates that *ELO3* deletion is essential for docosanol production and that the separation of growth and docosanol formation can boost titers.

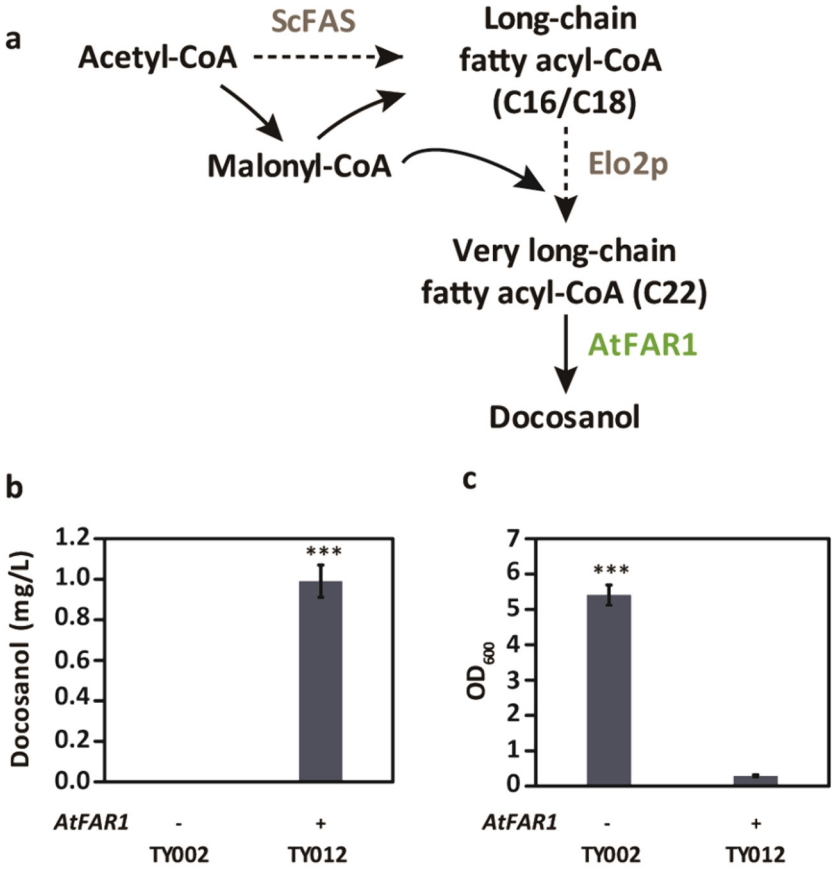

Figure 18 Expression of *At*FAR1 in a storage lipid synthesis and β-oxidation defective strain of *S. cerevisiae* (CEN.PK 113-5D *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ *pox1*Δ *elo3*Δ p*ELO2*) (TY002). (a) Schematic pathway for docosanol biosynthesis in *S. cerevisiae*. Overexpressed genes are shown in grey (endogenous) or green (heterologous). *At*FAR1, fatty acyl-CoA reductase 1 of *A. thaliana*; Elo2p, fatty acid elongase 2 of *S. cerevisiae*; *Sc*FAS, fatty acid synthase of *S. cerevisiae*. **(b)** Production of docosanoic acid ± SD (mg/g CDW) in strain TY002 (JV03 *elo3Δ* p*ELO2*) lacking or containing *AtFAR1*. **(c)** Final OD_{600} of strains TY002 and TY012, lacking or containing *AtFAR1*. Shown is the mean ± SD of three biological replicates of a representative measurement. *p<0.05; **p<0.01 and ***p<0.001 (Student's t-test, one-tailed, two-sample unequal variance). The strains were grown in minimal medium containing 3% glucose for 72 h. In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps and → indicates a single step. Adapted from Yu et al. (2017).

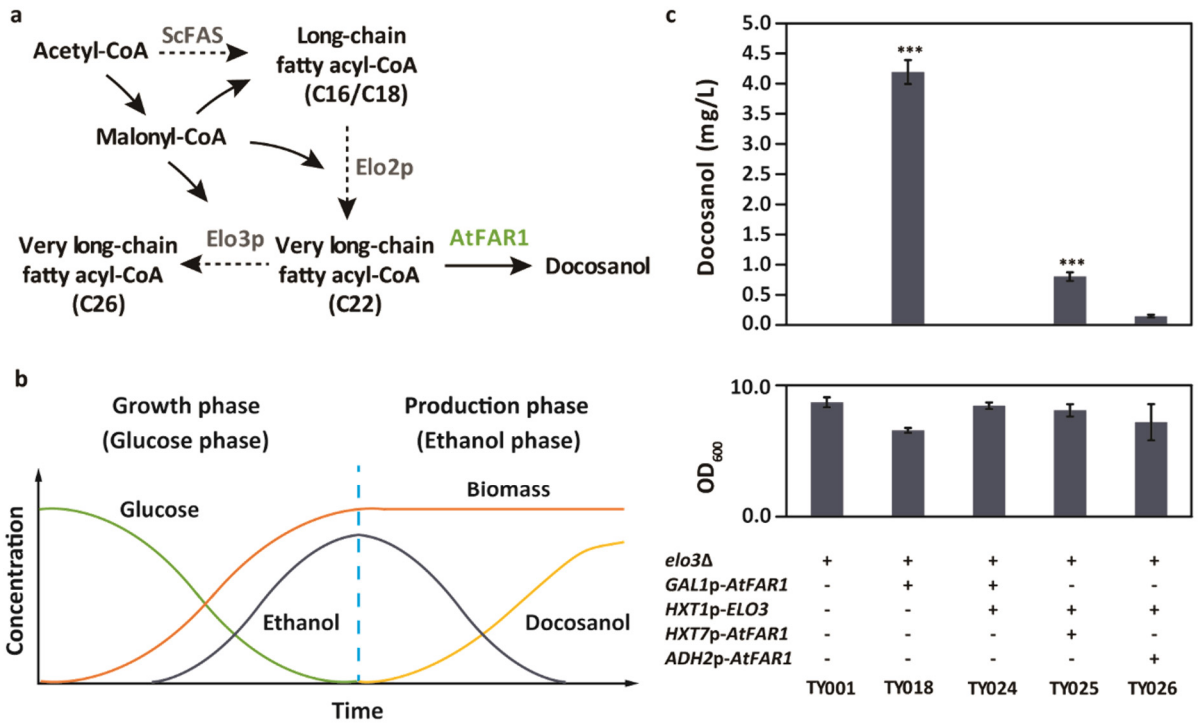

Figure 19 Dynamic control system separating cell growth from docosanol production in storage lipid biosynthesis and β-oxidation defective strains of *S. cerevisiae* (CEN.PK 113-5D *are1Δ are2Δ dga1Δ lro1Δ pox1Δ elo3Δ*) (TY001). (a) Schematic pathway for docosanol biosynthesis in *S. cerevisiae*, illustrating the effect on cell growth. Overexpressed genes are shown in grey (endogenous) or green (heterologous). *At*FAR1, fatty acyl-CoA reductase 1 of *A. thaliana*; Elo2p/Elo3p, fatty acid elongase 2/3 of *S. cerevisiae*; *Sc*FAS, fatty acid synthase of *S. cerevisiae*. **(b)** Schematic illustration showing the separation of cell growth and docosanol production by using carbon source dependent promoters. **(c)** Concentration of docosanol (mg/L) and final culture OD_{600} in strains expressing pathway genes under carbon source dependent promoters. Shown is the mean ± SD of three biological replicates of a representative measurement. *p<0.05; **p<0.01 and ***p<0.001 (Student's t-test, one-tailed, two-sample unequal variance). The strains were grown in minimal medium containing 3% glucose for 72 h and under supplementation of 0.5% galactose in case of strain TY018 expressing *AtFAR1* under the *GAL1* promoter (*GAL1*p). In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps and → indicates a single step. Adapted from Yu et al. (2017).

Since the growth rate and the final cell biomass of the storage lipid biosynthesis and β-oxidation defective *S. cerevisiae* strain JV03 are lower than those of the wildtype strain CEN.PK 113-5D (Valle-Rodríguez et al., 2014), the final docosanol production strain was constructed based on the background strain IMX581 (CEN.PK 113-5D *MAT*a *ura3-52 can1*Δ::*cas9-natNT2 TRP1 LEU2 HIS3*) (Mans et al., 2015) (**Figure 20**). This strain carries the *cas9* gene of *Streptococcus pyogenes* integrated at the *CAN1* locus and can therefore be used for integration of genes via CRISPR/Cas9 (Jakočiūnas et al., 2015; Mans et al., 2015).

The first implementation of the docosanol pathway in strain IMX581 by deletion of *ELO3* and simultaneous integration of *ELO2* under the *GAL10* promoter, as well as *AtFAR1* under the *GAL1* promoter at the *ELO3* site (strain TY029), led to a strain capable of producing 2.4 mg/L docosanol (**Figure 20b**). The further integration of *ELO1* under the *GAL7* promoter at the same site of the genome did not improve docosanol production significantly (strain TY030) (**Figure 20b**). To prevent putative consumption of the inductor molecule galactose and thereby enabling a stable expression of genes under control of *GAL* promoters, the *GAL1* gene was deleted by simultaneous integration of a constitutively active version of *ACC1* (*ACC1*$^{S659A, S1157A}$; *ACC1***), which should enable an increased precursor supply of malonyl-CoA (strain TY031) (Shi et al., 2014a). Deletion of

ELO3 and simultaneous integration of *AtFAR1* under the *GAL1* promoter at the *ELO3* site in strain TY031, resulted in a strain capable of producing 1.4 mg/L docosanol (strain TY033) (**Figure 20b**). When expressing *Mv*FAS in strain TY033, the docosanol production was significantly increased up to 40 mg/L (TY036) (**Figure 20b**). The additional integration of *ELO1* under the *GAL7* promoter and *ELO2* under the *GAL10* promoter at the *ELO3* site of the genome in strain TY036, led to the final docosanol production strain (TY037), capable of producing 83.5 mg/L of docosanol, which represents an approximately 80-fold increase compared to the proof-of-concept strain TY012 (**Figure 20b**). This clearly demonstrates the suitability of the heterologous, mycobacterial FAS (*Mv*FAS) for synthesis of VLCFAs in *S. cerevisiae* in concert with the yeast intrinsic elongases Elo1p and Elo2p and opens up the possibility to produce a broad range of VLCFA-derived products in *S. cerevisiae*.

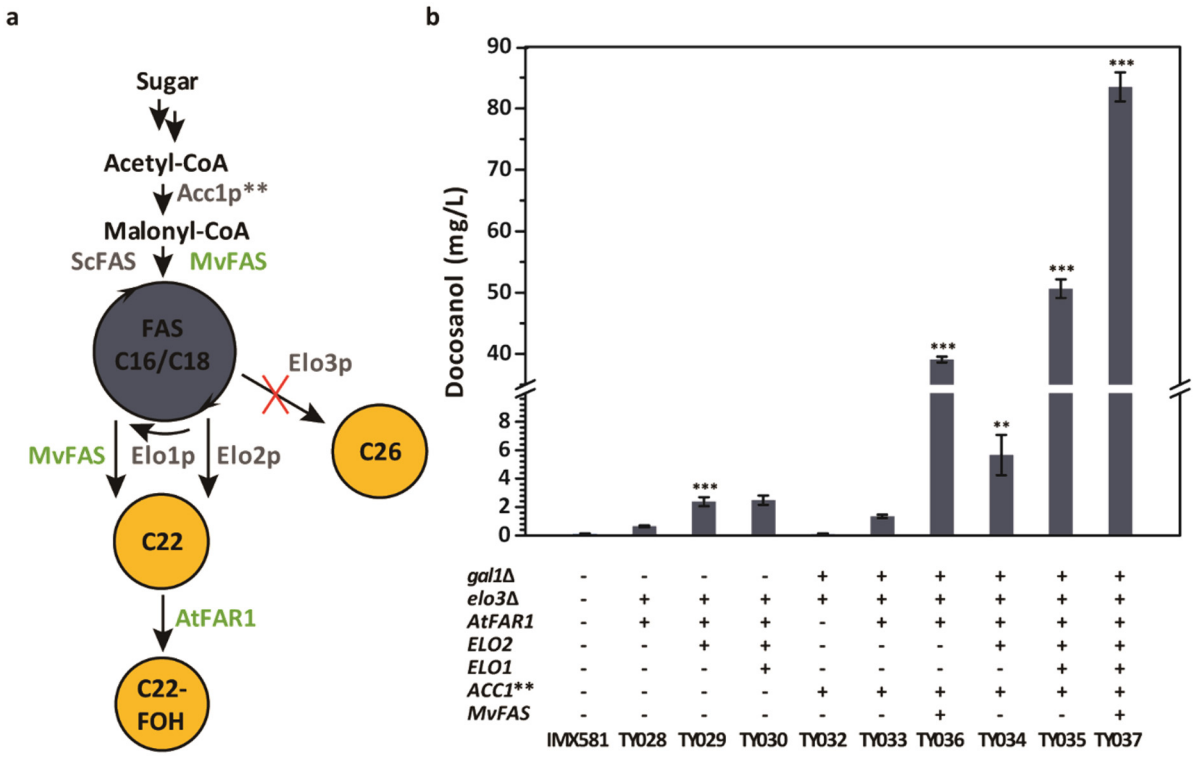

Figure 20 Docosanol production in various strains of *S. cerevisiae* IMX581 (CEN.PK 113-5D *MAT*a *ura3-52 can1*Δ::*cas9-natNT2 TRP1 LEU2 HIS3*). **(a)** Schematic overview showing the strategy used for docosanol production in modified *S. cerevisiae* strains. Overexpressed genes are shown in grey (endogenous) or green (heterologous). Acc1p, acetyl-CoA carboxylase from *S. cerevisiae*; Elo1p/Elo2p/Elo3p, fatty acid elongase 1/2/3 from *S. cerevisiae*; *Sc*FAS, fatty acid synthase from *S. cerevisiae; At*FAR1, fatty acyl-CoA reductase 1 from *A. thaliana; Mv*FAS, fatty acid synthase from *M. vaccae.* **(b)** Concentration of docosanol (mg/L) in various strains derived from *S. cerevisiae* IMX581. All strains were grown in minimal medium with 0.5% galactose. All genes were integrated into the genome of *S. cerevisiae* IMX58 except for *MvFAS*, for which a plasmid-based expression was used. Shown is the mean ± SD of three biological replicates of a representative measurement. *p<0.05; **p<0.01 and ***p<0.001 (Student's t-test, one-tailed, two-sample unequal variance). The strains were grown in minimal medium containing 3% glucose for 72 h and under supplementation of 0.5% galactose in case of strains expressing genes under *GAL* promoters. ╳ indicates deletion of a gene. Adapted from Yu et al. (2017).

4 Improving jojoba-like wax ester synthesis in *S. cerevisiae*

As described in chapters 2 and 3, it is possible to tune FA metabolism in *S. cerevisiae* towards the synthesis of VLCFAs and derived products. However, the synthesized VLCFAs and products thereof were mostly restricted to saturated compounds. In case of WE synthesis in *S. cerevisiae*, formation of DUWEs was only observed up to a chain length of C36. This indicated that the concentration of VLCMUFAs in the modified yeast strains was too low to promote the formation of DUWEs above a chain length of C36. Therefore, the goal of this study was to increase the formation of VLCMUFAs in *S. cerevisiae* to enable the synthesis of jojoba-like DUWEs of a chain length of C38-C44.

For this purpose, several endogenous as well as heterologous KCS and FAD enzymes were selected for expression in *S. cerevisiae*. FADs introduce double bonds into FAs and might therefore be able to increase the amount of UFAs in *S. cerevisiae* (Martin et al., 2007). KCS enzymes catalyze the first step in the elongation of FAs to VLCFAs at the ER and thereby determine the substrate- and product range. Studies have also indicated that this is the rate limiting step in VLCFA synthesis (Haslam and Kunst, 2013; Millar and Kunst, 1997).

It has been shown that the yeast intrinsic FA elongation system mainly leads to the formation of VLCFAs with a chain length of C26, either occurring as saturated VLCFAs (C26:0-FA) or as hydroxylated VLCFAs (C26:0-OH), which are mostly present in sphingolipids (Dickson, 2008; Oh et al., 1997). In contrast to that, plant KCS enzymes are able to elongate C18:1Δ9-FA further to VLCMUFAs, e.g. gondoic acid (C20:1Δ11-FA) and erucic acid (C22:1Δ13-FA). In *A. thaliana* seeds, around 28% (w/w) of the TFAs are VLCFAs, with C20:1Δ11-FA being the most abundant one (21% (w/w) of TFAs) (Millar and Kunst, 1997). The formation of VLCFAs in *A. thaliana* is initiated by one of its 21 KCS enzymes, with fatty acid elongation/elongase 1 (*At*FAE1) being the first KCS that has been identified in this species (and in the whole plant kingdom) (Blacklock and Jaworski, 2006; James and Dooner, 1990; Kunst et al., 1992; Lemieux et al., 1990; Trenkamp et al., 2004). The identification of *At*FAE1 occurred in mutant studies, which could show that mutation at the *At*FAE1 locus results in severely reduced levels of VLCFAs (<1% (w/w) of TFAs), whereupon only the VLCFA composition in developing seeds was affected. This demonstrates that *At*FAE1, and its homologous proteins in other species, are seed specific enzymes, and that the remaining 20 KCS enzymes in *A. thaliana* fulfill a different role in plant VLCFA synthesis. *B. napus* exists in two different cultivars, the low erucic acid (LEA) cultivar and the high erucic acid (HEA) cultivar. The HEA cultivar accumulates C22:1Δ13-FA as major VLCFA in its seed oil (45-60% (w/w) of TFAs), catalyzed by its intrinsic FAE1 KCS (*Bn*FAE1/*Bn*KCS) (Barret et al., 1998). Seeds of *C. abyssinica* plants contain up to 60% ((w/w) of TFAs) C22:1Δ13-FA, whose formation is initiated by the *C. abyssinica* intrinsic FAE1 KCS (*Ca*FAE1/*Ca*KCS) (Leonard, 1992; Mietkiewska et al., 2007; Zhu et al., 2016). Also seeds of *L. annua* contain mostly C22:1Δ13-FA (44% (w/w) of TFAs) but are in addition to that able to store nervonic acid (C24:1Δ15-FA). The formation of both of these VLCFAs is initiated by the *L. annua* intrinsic FAE1 KCS (*La*FAE1/*La*KCS) (Guo et al., 2009).

As described earlier, *S. chinensis* contains mostly C20:1Δ11-FA and C22:1Δ13-FA (58.3% and 31.2% (w/w) of TFAs, respectively), catalyzed by its intrinsic FAE1 KCS (*Sci*FAE1/*Sci*KCS) (Lassner et al., 1996). *Tropaeolum majus* is able to accumulate significant amounts of C22:1Δ13-FA (70-75% (w/w) of TFAs) in its seed oil, catalyzed by its intrinsic FAE1 KCS (*Tm*FAE1/*Tm*KCS) (Pollard and Stumpf, 1980).

In our study, we combined the expression of heterologous plant KCS enzymes together with the expression of heterologous FAD enzymes derived from plants or a marine copepod. The KCS enzymes tested are those derived from *A. thaliana* (*At*FAE1) (NCBI accession no. AT4G34520), *B. napus* (*Bn*FAE1) (NCBI accession no. AF490459), *C. abyssinica* (*Ca*FAE1) (NCBI accession no. AY793549), *L. annua* (*La*FAE1) (NCBI accession no. EU871787), *S. chinensis* (*Sci*FAE1) (NCBI accession no. AAC49186) as well as *T. majus* (*Tm*FAE1) (NCBI accession no. AAL99199). As a control, we also overexpressed the Elo2p from *S. cerevisiae*. The FADs tested are those derived from *C. hyperboreus* (*Ch*Des9-1) (NCBI accession no. AHL21604) and *S. chinensis* (*Sci*FAD-SP) (NCBI accession no. AAA33932), which was expressed without its N-terminal 31 AAs plastid localization signal. Moreover, two acyl-CoA desaturase-like (ADS) proteins from *A. thaliana* (*At*ADS1.2 and *At*ADS1.4) (NCBI accession no. AT1G06090 and AT1G06120, respectively) were tested in this study. As a control, we also overexpressed the Ole1p from *S. cerevisiae*.

Except for the KCS enzymes from *S. chinensis* (*Sci*FAE1) and *T. majus* (*Tm*FAE1) as well as the FAD from *S. chinensis* (*Sci*FAD-SP), all of these enzymes had been functionally expressed in *S. cerevisiae* before. *At*FAE1 produced C20:0-FA, C20:1-FA, C22:0-FA, C22:1-FA, C24:0-FA, C24:1-FA and C26:0-FA, with C20:1-FA as main product, when expressed in *S. cerevisiae* strain INVSc1 (Katavic et al., 2002; Trenkamp et al., 2004) and C20:1-FA, C22:1-FA as well as C20:0-FA when expressed in strain LRB520 (Millar and Kunst, 1997). It had also been shown before that *At*FAE1 is able to rescue the otherwise lethal phenotype *elo2*Δ *elo3*Δ in *S. cerevisiae* (Paul et al., 2006). Expression of *Bn*FAE1 from a HEA cultivar enabled *S. cerevisiae* strain INVSc1 to elongate C18:1Δ9-FA and C18:1Δ11-FA further to C20:1Δ11-FA, C20:1Δ13-FA, C22:1Δ13-FA and C22:1Δ15-FA, with C22:1-FAs being the main product (Han et al., 2001; Katavic et al., 2002). It could also be shown that *At*FAE1 as well as *Bn*FAE1 are both able to elongate C16:1Δ9-FA in *S. cerevisiae*, besides C18:1Δ9-FA (Katavic et al., 2002). *Ca*FAE1 expression in *S. cerevisiae* strain INVSc1 led to the formation of the non-native to yeast C20:1Δ11-FA, C20:1Δ13-FA, C22:1Δ13-FA, C22:1Δ15-FA, C26:1Δ17-FA and 26:1Δ19-FA (Mietkiewska et al., 2007). *In vitro* tests showed that the preferred substrate of the enzyme is C20:1-CoA with erucic acid (C22:1Δ13-FA) being synthesized as the main product. When *La*FAE1 was expressed in *S. cerevisiae* strain INVSc1, a similar product spectrum of C20:1Δ11-FA, C22:0-FA, C22:1Δ13-FA, C24:0-FA, C24:1Δ15-FA and C26:1Δ17-FA could be observed (Guo et al., 2009). Previous expression of *Tm*FAE1 in *S. cerevisiae* did not lead to a change in the observed FA spectrum (Mietkiewska et al., 2004).

*Ch*Des9-1, which is derived from *C. hyperboreus*, has been identified recently as a FAD specifically using VLCFAs (C20-C26) as substrates. Expression of *Ch*Des9-1 in *S. cerevisiae*

strain INVSc1 led to the production of two main products, C20:1Δ9-FA and C26:1Δ9-FA. When this yeast strain was supplemented with C18:0-FA, the synthesis of C22:1Δ9-FA, C22:1Δ11-FA, C24:1Δ9-FA, C24:1Δ11-FA and C24:1Δ13-FA was additionally enabled (Meesapyodsuk and Qiu, 2014). *At*ADS1.2 and *At*ADS1.4 both belong to the ADS gene family in *A. thaliana*, which in total comprises nine genes coding for FAD-like proteins. The expression of *At*ADS1.2 or *At*ADS1.4 in the yeast strain *fat1*Δ (BY4741 *MAT*a *his3D1 leu2D0 met15D0 ura3D0 YBR041w::kanMX4*) led to the synthesis of C22:1Δ9-FA, C24:1Δ9-FA and C26:1Δ9-FA (Smith et al., 2013).

In our study, the combined plasmid-based expression of KCS and FAD enzymes was performed in the *S. cerevisiae* background strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1*. This strain harbors a constitutively active version of Acc1p (encoded by *ACC1***) to increase the precursor supply for FA synthesis in the form of malonyl-CoA, a deletion of the *ELO3* gene to diminish C26-FAs formation as well as extra copies of the genes *IFA38*, *PHS1* and *TSC13* which encode the yeast intrinsic elongation enzymes KCR, HCD and ECR, respectively. In addition to that, it contains an extra copy of the *ACB1* gene encoding the acyl-CoA binding protein Acb1p to increase the FACoA pool, which has been demonstrated in previous studies to enable the increased production of fatty acid ethyl esters (de Jong et al., 2014; Shi et al., 2014b).

Analyzing the FA profiles of the resulting strains led to the conclusion that the combined overexpression of the yeast intrinsic Ole1p together with Elo2p, *Ca*FAE1 or *La*FAE1 can increase the pool of VLCMUFAs in *S. cerevisiae* (**Figure 21**). In contrast to that, no change in the FA composition could be observed upon expression of *At*FAE1, *Bn*FAE1 or *Tm*FAE1 together with Ole1p. The combined expression of *Ca*FAE1 together with Ole1p led to the highest concentration of C20:1-FA and C22:1-FA (2.19 ± 0.95 mg FAs/g CDW and 3.45 ± 1.09 mg FAs/g CDW, respectively), whereas the combined expression of *La*FAE1 together with Ole1p led to the highest concentration of C24:1-FA (0.48 ± 0.05 mg FAs/g CDW) in engineered *S. cerevisiae* strains. After identification of *Ca*FAE1 and *La*FAE1 as efficient enzymes for production of C20:1-FA, C22:1-FA and C24:1-FA in *S. cerevisiae*, the next goal was to study the influence of heterologous FADs on the synthesis of VLCMUFAs in *S. cerevisiae*. For this purpose, *Ca*FAE1 or *La*FAE1 were expressed together with a heterologous FAD in the background strain *S. cerevisiae* CEN.PK 113-5D *elo3*Δ X-2*::*p*MPC3::ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* (**Figure 22**)**.** The strain expressing *Ca*FAE1 together with *Ch*Des9-1 showed the highest formation of C20:1-FA and C22:1-FA (1.36 ± 0.20 mg FAs/g CDW and 2.91 ± 0.44 mg FAs/g CDW, respectively), which was however less compared with the amount of VLCMUFAs produced in the strain expressing *Ca*FAE1 together with Ole1p. The strain expressing *La*FAE1 together with *At*ADS1.2 showed the highest production of C24:1-FA (0.49 ± 0.05 mg FAs/g CDW), which was approximately the same amount produced by the strain expressing *La*FAE1 together with Ole1p. Since the combination of *Ca*KCS and Ole1p led to the highest concentration of C20:1-FA and C22:1-FA, of all enzyme combinations tested in this study, both enzymes were chosen to be expressed together with the WE synthesis enzymes *Ma*FAldhR and *Sci*WS to enable an increased synthesis of DUWEs in *S. cerevisiae*.

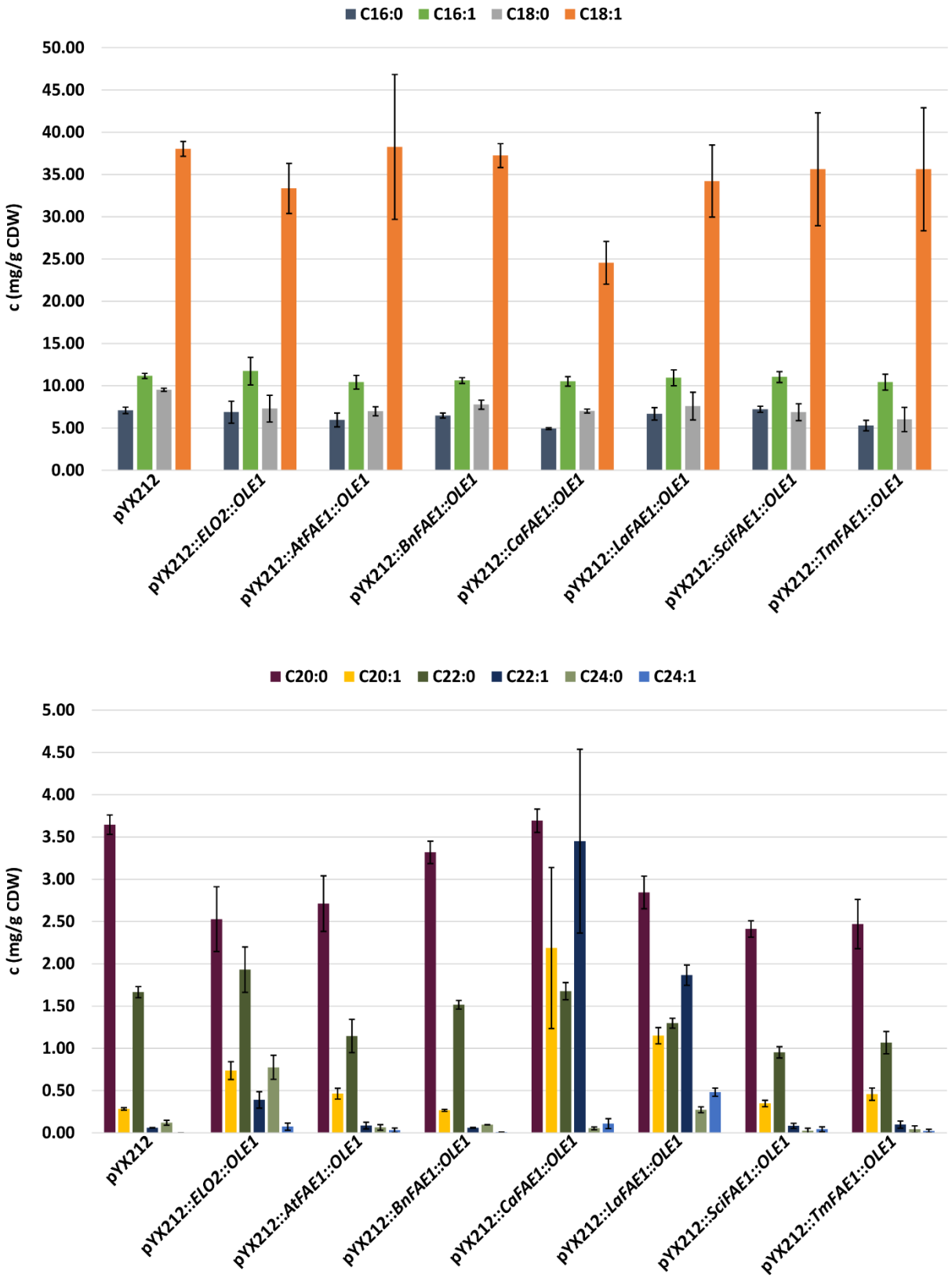

Figure 21 Fatty acid composition. Concentration of fatty acids ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* expressing different combinations of a fatty acid elongase (FAE)/β-ketoacyl-CoA synthase (KCS) and the intrinsic yeast desaturase Ole1p from the plasmid pYX212. All strains were grown in minimal medium containing 2% glucose for 48 h.

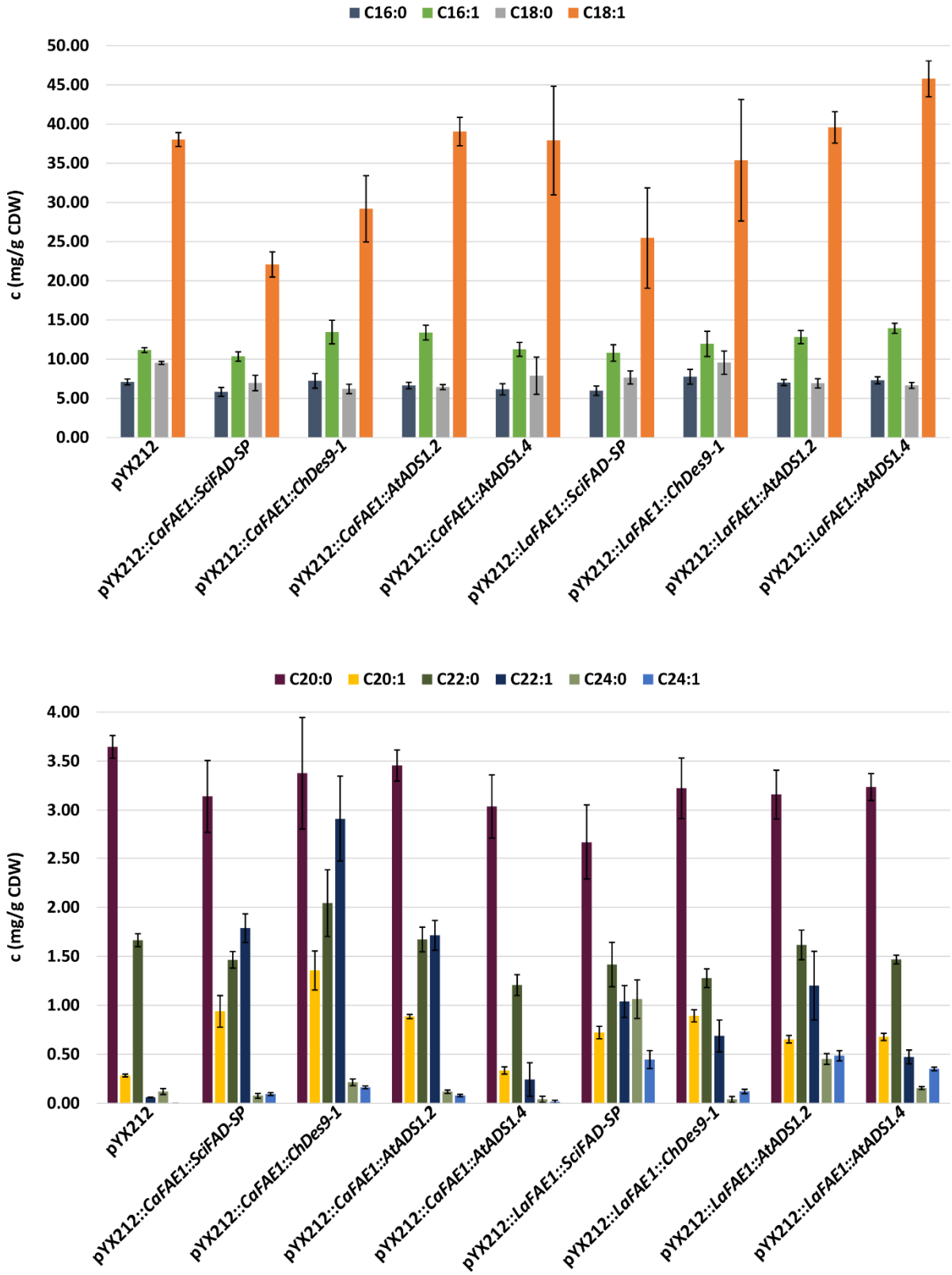

Figure 22 Fatty acid composition. Concentration of fatty acids ± SD (mg/g CDW) in in three biological replicates of *S. cerevisiae* strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* expressing different combinations of a fatty acid elongase (FAE)/β-ketoacyl-CoA synthase (KCS) and a fatty acid desaturase (FAD) from the plasmid pYX212. All strains were grown in minimal medium containing 2% glucose for 48 h.

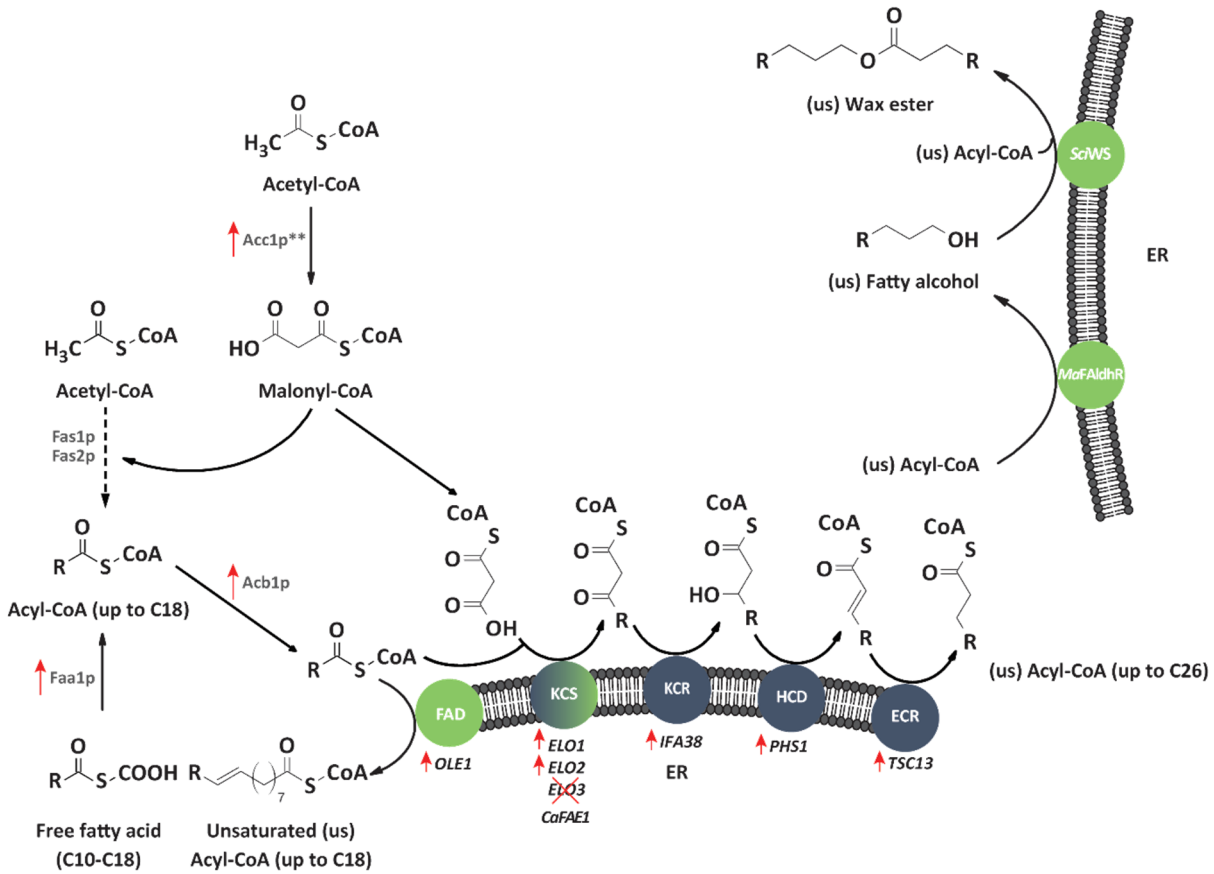

Figure 23 Wax ester synthesis pathway in engineered *S. cerevisiae* strain CEN.PK 113-5D *elo3Δ* X-2::p*MPC3*::*ACC1* X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* XII-5::*CaFAE1*::*OLE1* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW24).** Synthesis of fatty acids (FAs), fatty alcohols (FOHs), and wax esters (WEs) in modified *S. cerevisiae* strain LW24, catalyzed by intrinsic and heterologous enzymes (indicated in grey/blue and green, respectively). *De novo* FA synthesis in yeast starts with carboxylation of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (Acc1p). The next steps in cytosolic FA biosynthesis are catalyzed by the fatty acid synthases 1 (Fas1p; β-subunit) and 2 (Fas2p; α-subunit), finally leading to C12–C18 fatty acyl-CoAs (FACoAs). The acyl-CoA binding protein (Acb1p) transports newly synthesized long-chain acyl-CoAs from FAS to acyl-CoA-consuming processes, e.g. the elongation and/or desaturation of FAs at the endoplasmic reticulum (ER). The elongation of the C12–C18 FACoAs to C16-C26 FACoAs occurs at the ER, catalyzed by four different enzymes in yeast, including a β-ketoacyl-CoA synthase (KCS) (fatty acid elongases Elo1p, Elo2p and Elo3p), the β-ketoacyl-CoA reductase (KCR), the β-hydroxyacyl-CoA dehydratase (HCD), and the enoyl-CoA reductase (ECR). In strain LW24, the fatty acid elongase 1 (FAE1) KCS of *C. abyssinica* (*Ca*FAE1) is expressed additionally to the yeast intrinsic KCS enzymes. The desaturation of FACoAs at the ER is catalyzed by the yeast intrinsic fatty acid desaturase (FAD) Ole1p. Activation of FAs (C10-C18), resulting e.g. from lipid turnover, is catalyzed by a yeast intrinsic fatty acyl-CoA synthetase (Faa1p). The FACoAs which are released from *de novo* FA synthesis and elongation can be reduced by the heterologous fatty acyl-CoA reductase of *M. aquaeolei* VT8 (*Ma*FAldhR) to a FOH. A separate enzyme, the wax synthase of *S. chinensis* (*Sci*WS), catalyzes the esterification of a FOH with another FACoA molecule, leading to a WE. In case of enzyme-catalyzed reactions, ⇢ indicates multiple steps, → indicates a single step; ↑ indicates overexpression of a gene and ✕ indicates deletion of a gene.

To enable a stable expression of the genes *CaFAE1*, *ELO1*, *FAA1* and *OLE1*, they were integrated into the genome of *S. cerevisiae* strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* (**Figure 23**). It was also attempted to integrate *MaFAldhR* and *SciWS* into the genome of the same strain, but this proved unsuccessful, probably due to a lack of *ELO2* which was not part of the integration construct. For this reason, expression of *MaFAldhR*, *SciWS* and *ELO2* was kept plasmid based.

When the physiology of the resulting strains was investigated, it turned out that the combined overexpression of *Ca*FAE1, Elo1p, Faa1p and Ole1p in the background strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* (strain LW22) led to a growth defect of the strain with a final OD_{600} of 2.62 ± 0.05 after 48 h, compared to the same strain lacking the overexpression of *Ca*FAE1 and Ole1p (strain LW21) which showed a final OD_{600} of 10.9 ± 0.18 after 48 h. A similar growth defect was also observed in strain LW24 (expressing *Ca*FAE1, Ole1p, *Ma*FAldhR, *Sci*WS and Elo2p), but not in strain LW23 (expressing *Ma*FAldhR, *Sci*WS and Elo2p, but lacking *Ca*FAE1 and Ole1p) (**Supplementary Figure S3 in paper III**). The impaired growth of strains overexpressing *Ca*FAE1 and Ole1p (strains LW22 and LW24) indicates that the growth defect is due to an increased concentration of VLCMUFAs, which seem to be toxic to the cells as has been shown before for C18:1Δ9-FA and to a lesser extent also for C16:1Δ9-FA (Petschnigg et al., 2009).

The integration-based expression of *Ca*FAE1, Elo1p, Faa1p and Ole1p together with the plasmid-based expression of *Ma*FAldhR, Elo2p and *Sci*WS led to the formation of various FOH and WE species in strains LW23 and LW24. In contrast to that, the control strains LW21 and LW22, which do not contain the plasmid pYX212::*MaFAldhR*::*SciWS*::*ELO2*, did not show peaks corresponding to FOHs or WEs. In strain LW23, the synthesis of C16:0-FOH, C18:0-FOH, C18:1-FOH, C20:0-FOH and C22:0-FOH could be detected (**Supplementary Figure S6 in paper III**). The same FOH species were detected in strain LW24, which additionally synthesized C16:1-FOH, C20:1-FOH and C22:1-FOH (**Supplementary Figure S6 in paper III**). Moreover, strains LW23 and LW24 were both able to synthesize WEs with a chain length of C30-C44 (**Supplementary Figure S7 in paper III**).

The quantification of FOHs revealed that strain LW24 was able to produce 2.46 ± 0.71 mg FOHs/g CDW, of which 1.27 ± 0.46 mg/g CDW were MUFOHs. In comparison, strain LW23 produced 2.51 ± 0.29 mg FOHs/g CDW, of which only 0.014 mg ± 0.005/g CDW were MUFOHs (**Figure 24**). This demonstrates a significant increase ($p<0.05$) in the MUFOHs C18:1-FOH, C20:1-FOH and C22:1-FOH as well as a significant decrease ($p<0.05$) in the saturated FOHs C16:0-FOH, C18:0-FOH and C20:0-FOH in strain LW24 compared to strain LW23 (**Figure 24**). Strain LW23 and LW24 showed an overall titer of FOHs of 6.92 ± 0.85 mg/L and 2.10 ± 0.55 mg/L, respectively. These titers correspond to yields of 0.346 mg/g glucose and 0.105 mg/g glucose, respectively. The increased concentration of MUFOHs in strain LW24 triggered the formation of high amounts of DUWEs. The quantification of WEs revealed that strains LW23 and LW24 synthesized 13.13 ± 5.08 mg WEs/g CDW and 14.38 ± 1.76 mg WEs/g CDW, respectively (**Figure 25**). Compared to strain LW23, strain LW24 showed a significant decrease ($p<0.05$) in C34-WE species and a significant increase ($p<0.05$) in C42-WE species (**Figure 25**). Strain LW23 and LW24 showed an overall titer of WEs of 36.5 ± 16.71 mg/L and 11.92 ± 1.47 mg/L, respectively. These titers correspond to yields of 1.83 mg/g glucose and 0.60 mg/g glucose, respectively. Compared to jojoba seed oil which contains 77.8 mol% of C38:2 – C44:2 DUWEs, strain LW24 only contained 39.2 mol% of C38:2 – C44:2 DUWEs, but the overall composition of

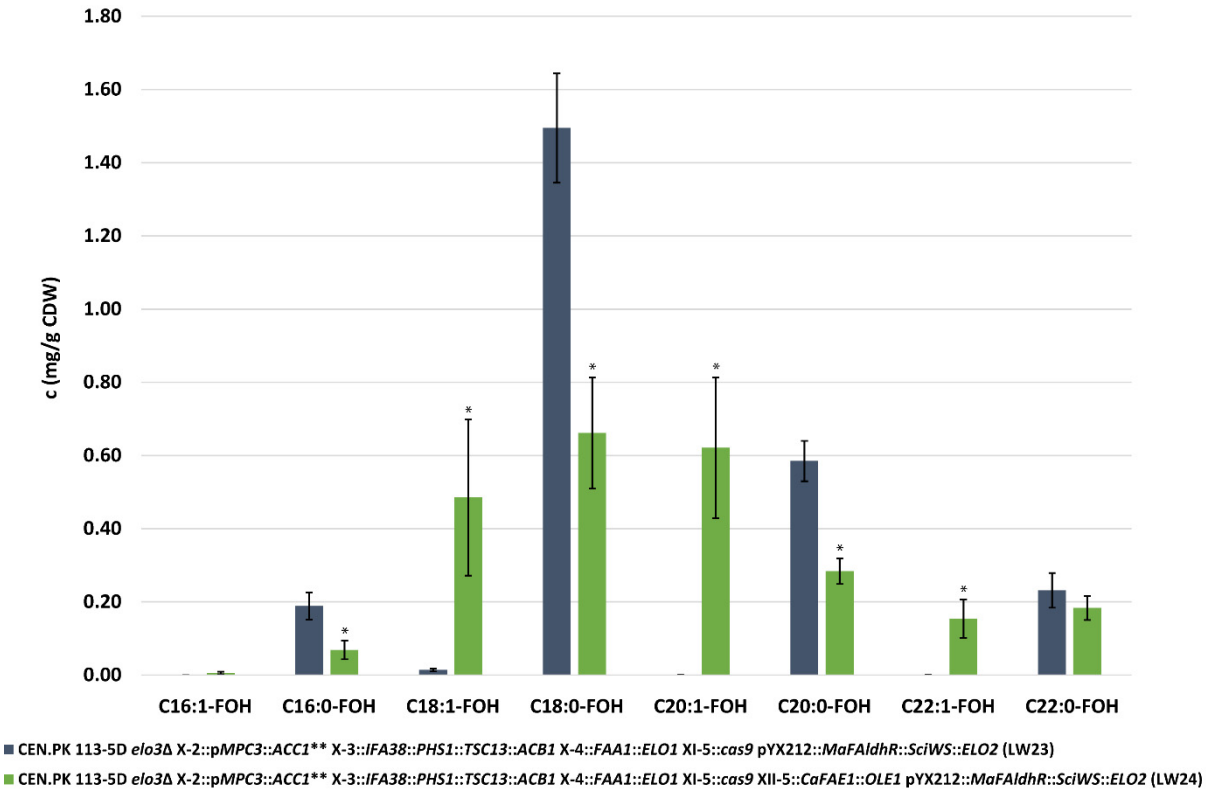

Figure 24 Fatty alcohol composition. Concentration of fatty alcohols (FOHs) ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* strains CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW23) and CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* XII-5::*CaFAE1*::*OLE1* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW24). Both strains were grown in minimal medium containing 2% glucose for 48 h. *p<0.05 (Students t-test, two-tailed, unequal variance assumed).

WE composition in the modified yeast strain was already very similar to the one in jojoba oil (**Figure 26**). Strains LW23 and LW24 were also analyzed for the molecular composition of their WEs, which in total identified 73 WE species (**Figure 27**). Strain LW23 mainly showed the synthesis of the WE species C18:0-C16:0 (25.30 ± 1.07 mol%), C22:0-C18:1 (17.25 ± 1.24 mol%) and C18:0-C16:1 (10.92 ± 0.82 mol%) (**Figure 27**). In comparison, strain LW24 had the tendency to produce WEs with an increased carbon chain length and desaturation degree, with the most abundant WE species being C22:0-C20:1 (12.52 ± 0.90 mol%), C22:1-C20:1 (10.67 ± 2.48 mol%) and C22:0-C18:1 (7.16 ± 1.38 mol%) (**Figure 27**). This study showed that it is possible to strongly increase the amount of VLCMUFAs in *S. cerevisiae* by making use of heterologous plant KCS enzymes and that high amounts of VLCMUFAs can trigger the synthesis of DUWEs of a chain length of C38-C46 under expression of a FAR and a WS in the yeast *S. cerevisiae*.

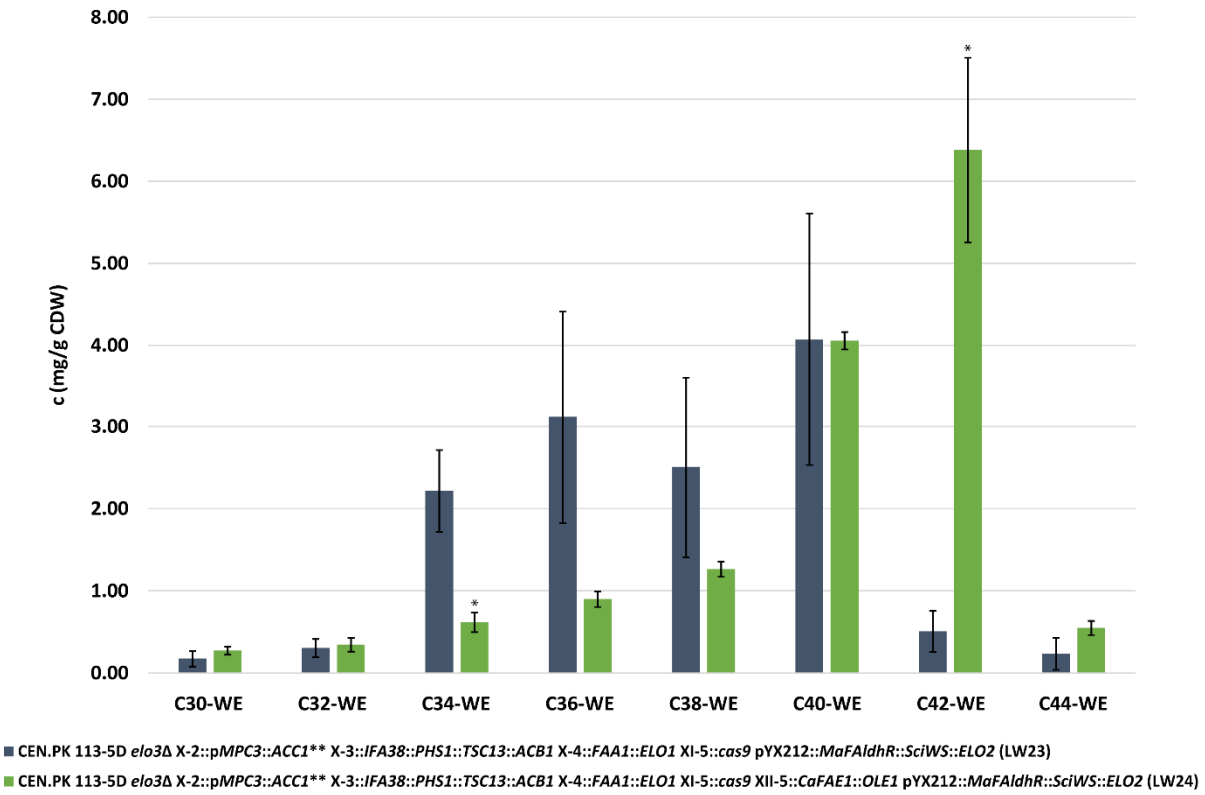

Figure 25 Wax ester composition. Concentration of wax esters (WEs) ± SD (mg/g CDW) in three biological replicates of *S. cerevisiae* strains CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW23) and CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* XII-5::*CaFAE1*::*OLE1* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW24). Both strains were grown in minimal medium containing 2% glucose for 48 h. *p<0.05 (Students t-test, two-tailed, unequal variance assumed).

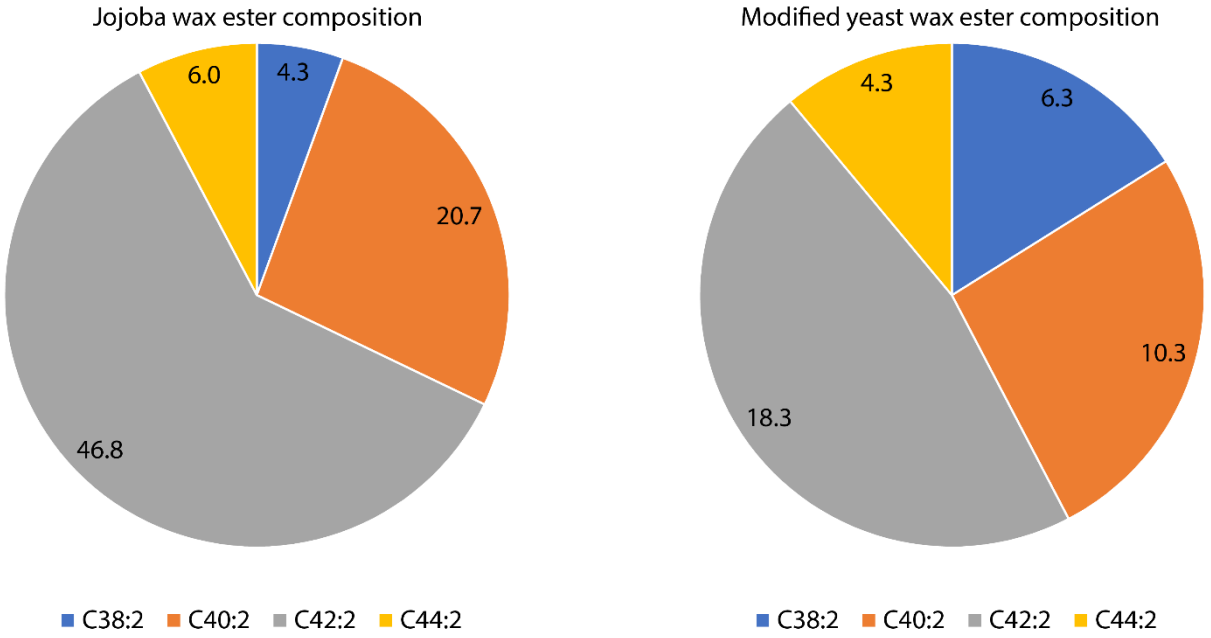

Figure 26 Wax ester composition (mol% of total wax esters) in jojoba seed oil (adapted from Iven et al. (2013)) vs. modified *S. cerevisiae* strain CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* XII-5::*CaFAE1*::*OLE1* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW24) (this study).

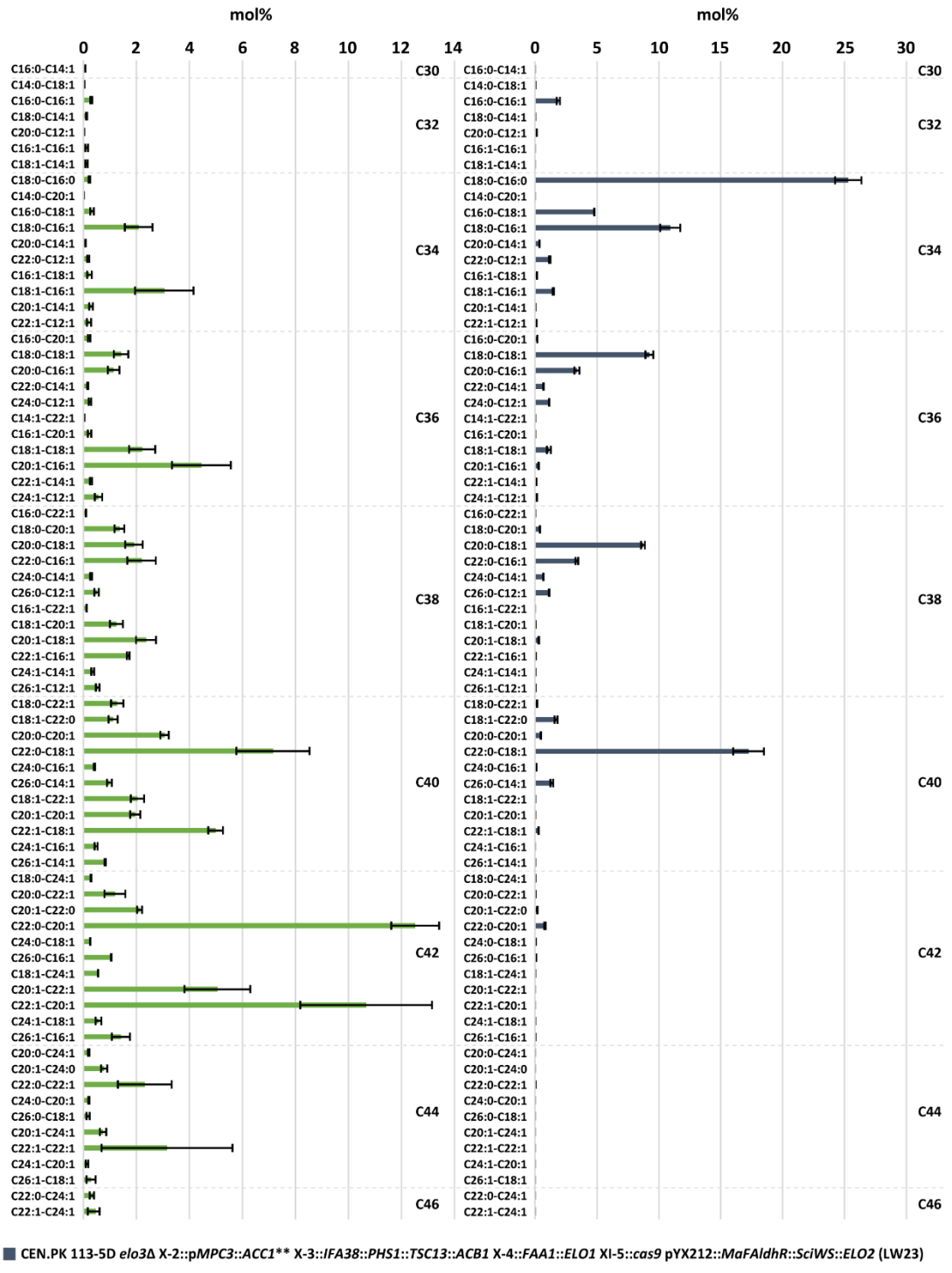

Figure 27 Molecular composition of wax ester species (mol% of total wax ester species) in three biological replicates of *S. cerevisiae* strains CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW23) and CEN.PK 113-5D *elo3*Δ X-2::p*MPC3*::*ACC1*** X-3::*IFA38*::*PHS1*::*TSC13*::*ACB1* X-4::*FAA1*::*ELO1* XI-5::*cas9* XII-5::*CaFAE1*::*OLE1* pYX212::*MaFAldhR*::*SciWS*::*ELO2* (LW24). Both strains were grown in minimal medium containing 2% glucose for 48 h.

5 Investigating the potential regulatory role of acetylation sites in Fas2p of *S. cerevisiae*

Besides being an important precursor for cellular constituents and essential for the generation of energy in the form of ATP, acetyl-CoA also functions as a substrate for protein acetylation, which represents a posttranslational modification. It is a widespread phenomenon observed in pro- and eukaryotic species and has been studied e.g. in bacteria, like *E. coli*, as well as in mammals, like *M. musculus* and *Homo sapiens* (Choudhary et al., 2014). Two main mechanisms have been proposed for the regulatory function of lysine acetylation. The first one is a change in protein structure and/or protein interactions with other cell components, triggered by neutralization of the positive charge of lysine by acetylation. The second mechanism suggests that lysine acetylation of proteins leads to the recruitment of "effectors", specifically recognizing acetylated proteins, e.g. chromatin-remodeling enzymes (Kamieniarz and Schneider, 2009). The acetylation of proteins enables the cell to respond fast to changes in its metabolic condition, which are reflected in the concentration of acetyl-CoA.

As described in **chapter 1, section 1.1.1**, in *S. cerevisiae* acetyl-CoA can only move freely between the cytosol and the nucleus, forming the nucleocytosolic acetyl-CoA pool, but it cannot move freely between the other compartments of the cell. Therefore, the degree of protein acetylation in the different cellular compartments is dependent on the acetyl-CoA concentration in the respective compartment and can vary considerably. In general, the level of acetyl-CoA in *S. cerevisiae* is highest during exponential growth on glucose and lower during the diauxic shift as well as the following ethanol phase and stationary phase (Cai et al., 2011; Seker et al., 2005).

The transfer of acetyl groups to the epsilon-amino groups of lysines (Lys, K) is catalyzed by lysine acetyltransferases. The removal of acetylations from proteins is catalyzed by lysine deacetylases (Galdieri et al., 2014). In the mitochondria, acetylation of proteins may also occur spontaneously, because of the higher concentration of acetyl-CoA as well as the higher pH compared to the cytosol or the nucleus (Wagner and Payne, 2013; Weinert et al., 2014).

The acetylation of proteins is best studied in case of histones. However, a broad range of other types of proteins are also targets for acetylation, including several ones involved in cell cycle progression, cytokinesis, metabolism, RNA processing, stress response and transcription (Duffy et al., 2012; Lin et al., 2009; Weinert et al., 2014). A study investigating a *S. cerevisiae* strain harboring a deletion in one of the lysine deacetylases (*rpd3*Δ) identified over 4,000 acetylation sites via high resolution mass spectrometry in batch grown cells harvested in the exponential phase ($OD_{600} \sim 0.5$) (Henriksen et al., 2012).

The study by Henriksen et al. (2012) also identified a range of metabolic enzymes as targets for acetylation, including some involved in glycolysis, gluconeogenesis and AA

metabolism. As several of these enzymes, and their acetylation sites, are highly conserved in other organisms, regulation by acetylation might have a conserved role in cellular metabolism. One enzyme in yeast for which the regulatory function of acetylation has already been demonstrated is Pck1p, catalyzing the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis (**Figure 2**). It has been shown that acetylation of K514 in Pck1p is essential for enzymatic activity and for the ability of yeast cells to grow on non-fermentable carbon sources (Lin et al., 2009). Another example is the regulation of bacterial and mammalian ACSs by acetylation. In case of *S. enterica* ACS, the acetylation of a conserved lysine residue leads to an almost complete inactivation of the enzyme, whereas deacetylation can reactivate the enzyme. In contrast to that, currently no direct evidence for the role of acetylation on the activity of *S. cerevisiae* ACS exists (Hallows et al., 2006; Schwer et al., 2006; Starai et al., 2002; Starai et al., 2003; Starai et al., 2004). Two examples for acetylated proteins in human are the glucose-6-phosphate dehydrogenase (G6PD) and human FAS. G6PD is a key enzyme in the PPP that plays an essential role in the oxidative stress response by producing NADPH. Acetylation of the G6PDH at K403 leads to inability of the enzyme to form active dimers and therefore to a complete loss of enzyme activity (Wang et al., 2014). In case of human FAS, acetylation destabilizes the enzyme by promoting its degradation via the ubiquitin-proteasome pathway. This in turn leads to a decreased *de novo* lipogenesis as well as tumor cell growth, making acetylation of human FAS a potential anticancer drug target (Lin et al., 2016).

Similar to human FAS, acetylation sites have also been identified in FAS of *S. cerevisiae*, with 29 unique acetylation sites detected in Fas1p and 50 unique acetylation sites detected in Fas2p. With an acetylation frequency of 1.41 and 2.65 (detected acetylations/100 AAs) in Fas1p and Fas2p, respectively, both proteins belong to the most highly acetylated proteins in *S. cerevisiae* (in terms of acetylation frequency) (Henriksen et al., 2012). Of the 50 lysine acetylation sites detected in Fas2p by Henriksen et al., 2012, three sites (K83, K173 and K1551) have been confirmed in a recent study, in which six different deletion strains (*sir2*Δ, *snf1*Δ, *snf1*Δ *sir2*Δ, *gcn5*Δ, *sir2*Δ *gcn5*Δ, *snf1*Δ *gcn5*Δ) were investigated (Kumar et al., unpublished). All three sites are located within different domains of FAS, with K83 being part of the MPT domain, which is located on both chains of FAS (Jenni et al., 2007), K173 being part of the ACP domain of Fas2p and K1551 being part of the KS domain of Fas2p (Lomakin et al., 2007).

As described in **chapter 1, section 1.1.2**, FA biosynthesis has been shown to be regulated on multiple levels in yeast. One factor influencing FA biosynthesis is the availability of acetyl-CoA, whose concentration in turn influences the acetylation status of proteins. Therefore, it is likely that there is a regulatory acetylation mechanism controlling FAS activity in yeast. To investigate this hypothesis, the objective of this study was to analyze the function of three lysine acetylation sites in Fas2p (K83, K173 and K1551), by exchanging these lysine residues against different AAs. In previous studies, a replacement system in which lysine is substituted by glutamine (Q), which abolishes the positive charge, or arginine (R), which retains the positive charge, to mimic a constitutively acetylated and non-acetylated state, respectively, has been applied (**Figure 28**)

(Kamieniarz and Schneider, 2009; Megee et al., 1990; Schwer et al., 2006; Wang et al., 2014). The use of this residue replacement system enables the investigation of the functional impact of protein acetylations.

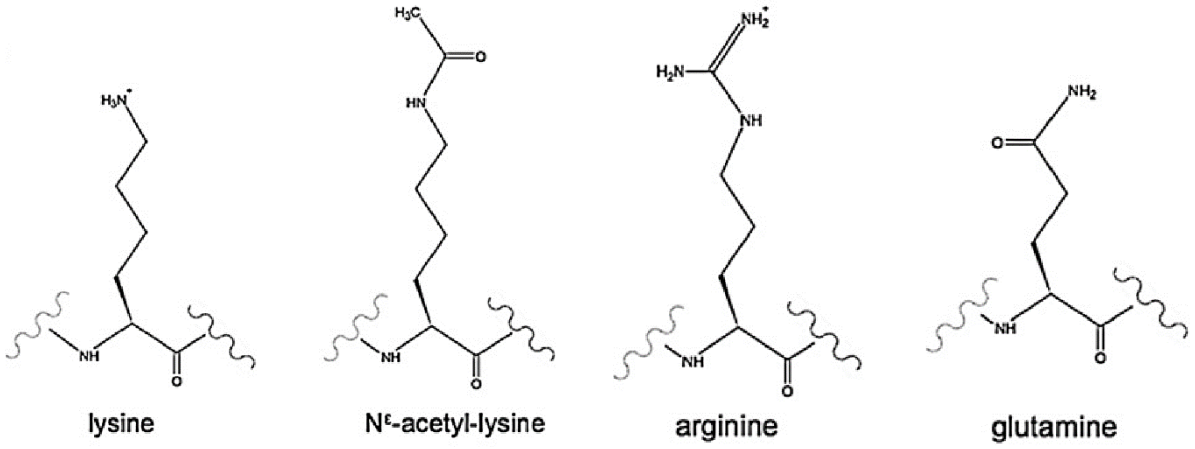

Figure 28 Structure of lysine, Nε-acetyl-lysine, arginine and glutamine. Adapted from Kamieniarz and Schneider (2009).

We applied the AA exchanges in Fas2p in two different background strains, CEN.PK113-7D and CEN.PK113-5D *faa1*Δ *faa4*Δ. In strain CEN.PK113-7D only the single mutations of *FAS2* (*FAS2*^{K83Q}, *FAS2*^{K83R}, *FAS2*^{K173Q}, *FAS2*^{K173R}, *FAS2*^{K1551Q} or *FAS2*^{K1551R}) were implemented, whereas in strain CEN.PK113-5D *faa1*Δ *faa4*Δ, the single mutations as well as the triple mutations of *FAS2* (designated as *FAS2*^{QQQ} and *FAS2*^{RRR}, respectively) were investigated. The latter strain carries deletions in two genes coding for the fatty acyl-CoA synthetases Faa1p and Faa4p, respectively, which are the two main enzymes responsible for the re-activation of LCFAs (C12-C18) from lipid turnover or from the external medium in *S. cerevisiae* (**chapter 1, section 1.1.2**) (Faergeman et al., 2001). This background strain has been chosen, since it has been shown previously that FACoAs can inhibit the activity of Fas1p/Fas2p (Chirala, 1992; Kamiryo et al., 1976). Therefore, in strain CEN.PK113-5D *faa1*Δ *faa4*Δ, FAS-activity would be partly de-regulated, triggering an increased overall flux through the FA biosynthesis pathway, FA excretion, and inhibition of FFA turnover, which in turn could enhance the effect of a regulatory alteration in Fas2p.

All resulting strains were investigated for their total FFA content and FA chain length composition. In case of the six modified strains derived from the background strain CEN.PK113-7D, no significant differences for the growth behavior, total FFA content and FA chain length composition could be observed for strains grown in minimal medium containing 2% glucose, harvested at three different time points (**Figure 1, Table 3 and Table 4 in paper IV**). In case of the eight modified strains derived from CEN.PK113-5D *faa1*Δ *faa4*Δ, no consistent changes in the total FFA content could be observed when strains were grown in minimal medium either containing 2% glucose, 2% (v/v) ethanol, 3% glucose, 3% (v/v) ethanol or 1% glucose + feed beads (corresponding to additional 2% glucose), harvested at two different time points in case of 2% and 3% glucose cultures and at one time point in case of the remaining cultures (**Figure 29 and Figure 30**).

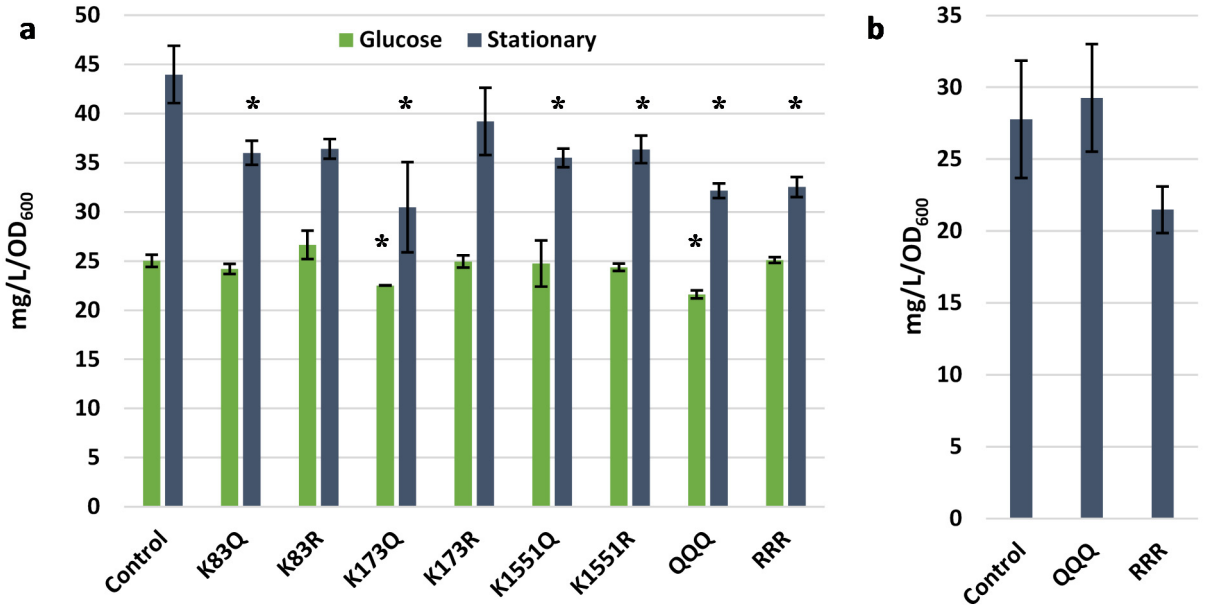

Figure 29 Total free fatty acid content (mg/L/OD_{600}) ± SD in three biological replicates of *S. cerevisiae* strains derived from CEN.PK113-5D *faa1*Δ *faa4*Δ (control) grown in minimal medium, **(a)** containing 2% glucose harvested around glucose depletion (Glucose) or after 72 h (Stationary) and **(b)** containing 2% (v/v) ethanol harvested at 72 h. The values for strain K173Q represent duplicate measurements). *p<0.05 (Students t-test, two-tailed, unequal variance assumed).

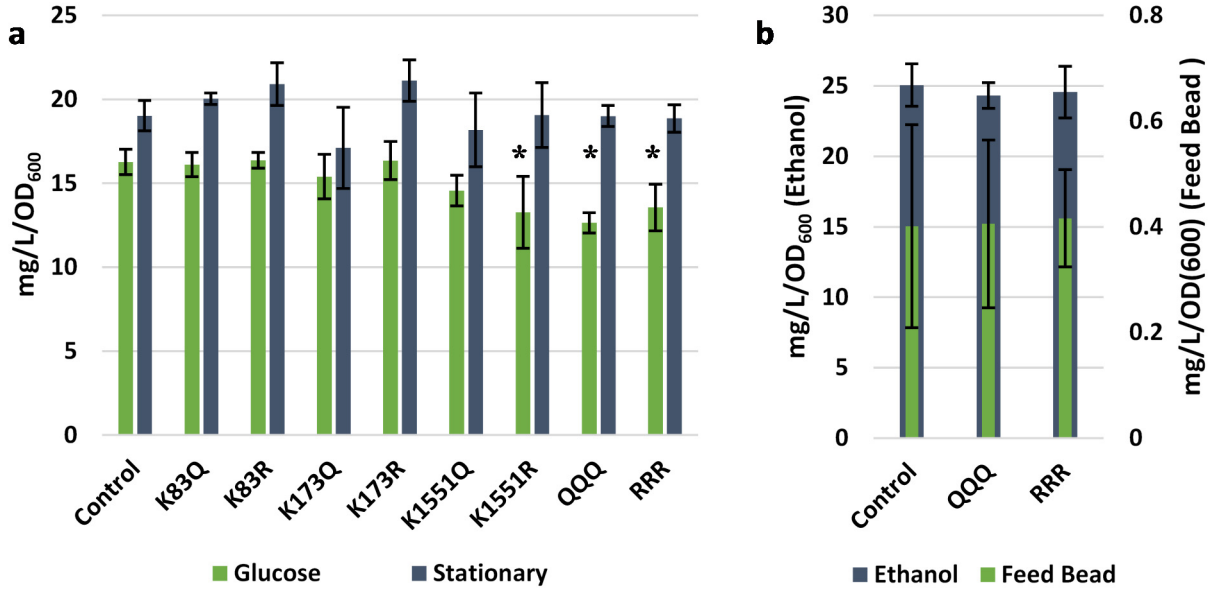

Figure 30 Total free fatty acid content (mg/L/OD_{600}) ± SD in four biological replicates of *S. cerevisiae* strains derived from CEN.PK113-5D *faa1*Δ *faa4*Δ (control) grown in minimal medium, **(a)** containing 3% glucose harvested around glucose depletion (Glucose) or after 72 h (Stationary) and **(b)** containing 3% (v/v) ethanol harvested at 72 h (Ethanol) or 1% dissolved glucose and 2% glucose supplied in feed bead format added after 24 h of culture, harvested after 96 h (Feed Bead). The values for strain K173R represent triplicate measurements. *p<0.05 (Students t-test, two-tailed, unequal variance assumed).

In case of the FA chain length composition of the strains, the only consistent change observed was an increased synthesis of C18:0-FA at the expense of C16:1-FA in strains CEN.PK113-5D *faa1*Δ *faa4*Δ *FAS2*^{K173Q} and CEN.PK113-5D *faa1Δ faa4Δ FAS2*^{QQQ}, grown in minimal medium containing either 2% or 3% of glucose and harvested around glucose depletion (**Table 5 and Table 6 in paper IV**). Other consistent changes seen were a general decrease of the total FFA content in higher concentrations of glucose (3% instead of 2%) and a drastic decrease in total FFA content under glucose limitation (feed beads compared to growth on glucose or ethanol), with a FFA concentration of 0.4 mg/L/OD_{600} compared to 15-25 mg/L/OD_{600} (**Figure 29 and Figure 30**).

Overall, this study could not confirm a regulatory function of the three lysine residues K83, K173 and K1551 in Fas2p, since both triple mutants, CEN.PK113-5D *faa1*Δ *faa4*Δ *FAS*^{QQQ} and CEN.PK113-5D *faa1*Δ *faa4*Δ *FAS2*^{RRR} showed a significant reduction in FFA content compared to the control strain (CEN.PK113-5D *faa1*Δ *faa4*Δ) when grown in minimal medium containing 3% glucose and harvested at glucose depletion and no change in FFA content compared to the control strain when grown in minimal medium containing 3% glucose and harvested at stationary phase (**Figure 30**). This indicates that the AA exchanges rather disturb the function of Fas2p and that the AA replacement system is not a very suitable method to study the function of lysine acetylation on metabolic proteins.

One problem with the substitution of N^{ϵ}-acetyl-lysine by glutamine or unmodified lysine by arginine is that the structure of these AAs is quite different (**Figure 28**) (Kamieniarz and Schneider, 2009). Compared to the unmodified lysine, arginine is bulkier, while glutamine is smaller than the N^{ϵ}-acetyl-lysine. Because of that, both substituted AAs could change the three-dimensional structure of the protein, and therefore negatively influence catalytic function in an acetylation-independent manner. Moreover, the replacement system assumes that the function of N^{ϵ}-acetyl-lysine merely depends on charge. Another point to consider is of course the vast number of acetylation sites detected in Fas1p and Fas2p (29 and 50, respectively) which makes it more challenging to identify N^{ϵ}-acetyl-lysines that fulfill a regulatory function in the protein. The three lysine sites in Fas2p (K83, K173, K1551) investigated in this study had been chosen based on two separate acetylation data sets (Henriksen et al., 2012; Kumar et al., unpublished). In addition to that, all three lysine sites are evolutionary conserved among fungal yeast species (Jenni et al., 2007). To identify lysine sites which have a regulatory function in *S. cerevisiae*, it might be interesting to look at the acetylation pattern of FAS from other yeast species, e.g. *Y. lipolytica.* A recently performed acetylome study in *Y. lipolytica* highlighted its FAS as an highly acetylated enzyme (Wang et al., 2017). A sequence alignment of the *S. cerevisiae* and *Y. lipolytica* FAS-sequences showed that 25 out of 50 of the detected acetylated lysines in *S. cerevisiae* Fas2p are conserved in *Y. lipolytica*, out of which four (K64 (MPT), K1092 (KS), K1446 (KS), K1821 (PPT)) are also acetylated in *Y. lipolytica*. In case of Fas1p, the sequence alignment showed that 18 out of 29 of the detected acetylated lysines in *S. cerevisiae* Fas1p are conserved in *Y. lipolytica*, out of which four were conserved in the acetylome data set of *Y. lipolytica* (K1426 (DH), K1639 (MPT), K1753 (MPT), K2017

(MPT)). In contrast to that, the lysine residues K83, K173 and K1551 found to be acetylated in Fas2p of *S. cerevisiae* were not identified to be acetylated in *Y. lipolytica* Fas2p. The eight lysine acetylation sites found in *S. cerevisiae* as well as in *Y. lipolytica* Fas1p and Fas2p represent an interesting set of enzyme residues to be investigated in potential future studies of FAS-acetylation.

The use of amber suppression technology to directly introduce unnatural AAs, like N^{ϵ}-acetyl-lysine, into a protein represents a superior method compared to the residue replacement system to study the impact of lysine acetylations. This is done by using a to the host orthogonal pair of aminoacyl-tRNA synthetase and tRNA (Neumann et al., 2018). This technique has been used previously to create a functional expression system for a pyrrolysyl-tRNA-synthetase/$tRNA^{Pyl}_{CUA}$ pair from *Methanosarcina barkeri* and *M. mazei* which was able to incorporate a range of unnatural AAs into proteins, including N^{ϵ}-acetyl-lysine (Hancock et al., 2010). Another study describes the addition of five unnatural AAs to the genetic code of *S. cerevisiae,* using the orthogonal pair tyrosyl–tRNA-synthetase/$tRNA_{CUA}$ from *E. coli* (Chin et al., 2003). Other orthogonal pairs used in *S. cerevisiae* include leucyl-tRNA-synthetase/$tRNA^{Leu}_{CUA}$ from *E. coli* (Wu et al., 2004) and glutaminyl-tRNA-synthetase from *E.coli*/$tRNA^{iMet}_{CUA}$ from human (Kowal et al., 2001). One of these systems could potentially also be used to study the regulatory function of the lysine acetylation sites in Fas1p and Fas2p.

Another possible strategy might be to focus on the general impact of lysine acetylation on the activity of FAS. This could be done by treating growing cells with the compound nicotinamide (NAM), acting as an inhibitor of the sirtuin family of deacetylases (Avalos et al., 2005), and/or trichostatin A (TSA), acting as an inhibitor of deacetylases of group I and II (Vigushin et al., 2001). In this way, proteins with an increased level of acetylations can be purified. Blotting of the purified enzyme from non-treated and NAM/TAS treated cells with a pan anti-acetyl lysine antibody would allow the identification of the group of deacetylase enzymes (I/II or III) responsible for removal of acetyl-groups. In addition to that, an *in vitro* assay for FAS-activity, including the FAS substrates acetyl-CoA and malonyl-CoA as well as the FAS cofactor NADPH, would allow to access the functional impact of general FAS-acetylation status by monitoring the consumption of NADPH.

In conclusion, even if our study was not able to confirm a regulatory function of the three studied lysine residues in Fas2p by the residue replacement system, there are still a range of interesting methods available which could provide information about the function of acetylation in the FAS enzyme of *S. cerevisiae*. As mentioned, the FAS enzyme as well as some of its lysine acetylation sites are highly conserved among different fungal species and therefore it might be possible to identify other lysine residues that could be studied for their functional role. Being able to control the activity of the intrinsic FAS in *S. cerevisiae* would allow the control of FA biosynthesis in this organism, which in turn could increase its potential as a production organism of FA-derived chemicals.

6 Conclusions and perspectives

The objective of this study was to implement the synthesis of very long-chain jojoba-like WEs in *S. cerevisiae*. Most previous studies on WE synthesis in *S. cerevisiae* had focused on investigating the functionality of heterologous FAR and WS enzymes in yeast (**Table I**), while their goal was not to specifically produce high amount of WEs. Moreover, the majority of the studies were performed under addition of external FAs and FOHs, which function as substrates for WE synthesis. The expression of heterologous FAR and WS enzymes in earlier studies led to the synthesis of WEs up to a chain length of C36 in *S. cerevisiae*. Only under substrate feeding, WEs up to a chain length of C44 were observed (**Table I**). However, natural jojoba oil mainly consists of C42:2-WEs (46.8 mol%) and C40:2-WEs (20.7 mol%) (Iven et al., 2013).

In **paper I** we were able to establish the synthesis of VLCWEs in *S. cerevisiae* without the additional feeding of FOH or FA precursors. This was achieved by expression of various enzymes, including the FARs from *A. mellifera* (*Am*FAR1) and *M. aquaeolei* VT8 (Maqu_2220/*Ma*FAldhR) as well as the WS from *S. chinensis* (*SciWS*). FOHs synthesized had a chain length of C16-C22, whereas the WEs synthesized had a chain length up to C42, with C34-WEs being the most abundant WE species. However, this yeast strain was not able to synthesize DUWEs with a chain length >C36. Therefore, the strain was lacking the most common jojoba WEs, C42:2-WEs and C40:2-WEs, which indicated a lack of VLCMUFAs.

This lack of VLC(MU)FAs in *S. cerevisiae* was addressed in **paper II** and **paper III**. In **paper II** we identified an alternative route for production of VLCFAs and VLCFOHs in *S. cerevisiae*. Increased VLCFA synthesis was achieved by expression of a heterologous bacterial FAS I system from *M. vaccae* which is able to produce C16-FAs/C18-FAs as well as C22/C24/C26 VLCFAs (Kaneda et al., 1995). The synthesis of docosanol (C22:0-FOH) was achieved by expression of a FAR from *A. thaliana* (*At*FAR1) (Rowland and Domergue, 2012). Since the initial strain suffered a severe growth defect, probably due to a lack of VLCFACoAs, we developed a dynamic control strategy for separating cell growth from docosanol production, involving galactose inducible promoters. The final *S. cerevisiae* strain showed the highest docosanol production reported so far.

In **paper III** we successfully increased the amount of VLCMUFAs of a chain length of C20-C24 in *S. cerevisiae*. The expression of the plant-derived FAE1/KCS from *C. abyssinica* (*Ca*FAE1) together with the yeast intrinsic Ole1p enabled the highest production of C20:1-FA and C22:1-FA, whereas expression of the FAE1/KCS derived from *L. annua* (*La*FAE1) led to the highest production C24:1-FA. The synthesis of jojoba-like MUFOHs (up to C22:1-FOH) as well as DUWEs (up to C46:2-WE) was achieved by combined expression of *CaFAE1*, *OLE1*, *MaFAldhR* and *SciWS* in *S. cerevisiae*. In addition to that, the most abundant WE species in the final *S. cerevisiae* strain were C42:2-WEs, whereas the amount of C34:0-WEs was strongly reduced.

The downside of the manipulation of the FA biosynthesis machinery towards the synthesis of VLCMUFAs is their negative effect on the growth of yeast, which is probably due to their influence on membrane structure. This has previously been described for oleic acid (C18:1Δ9-FA), but only in a *S. cerevisiae* strain defective in storage lipid biosynthesis, BY4742 *are1*Δ *are2*Δ *dga1*Δ *lro1*Δ (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 ycr048w*Δ::*KanMX4 ynr019w*Δ::*KanMX4 yor245c*Δ::*KanMX4 ynr008w*Δ::*KanMX4*), a descendant of strain S288C (Petschnigg et al., 2009). Growth of this strain was completely abolished at a concentration of 0.01% oleic acid, whereas wildtype cells were not affected. The quadruple mutant showed a PL to ergosterol ratio of 3.1 after 12 h incubation in medium containing 0.001% oleate, whereas wildtype cells show a ratio of 1.2. Moreover, the mutant showed a massively altered chain length distribution in membrane PLs, with accumulation of C18:1Δ9-FA. This indicates that TAG deficiency triggers the channeling of excess unsaturated FAs into PLs which in turn induces major membrane proliferations of the ER as confirmed by transmission electron microscopy (Petschnigg et al., 2009). Even if the strains constructed in **paper III** are not deficient in storage lipid biosynthesis, an elevated concentration of VLCMUFAs might still be able to change the composition of membrane lipids or the ratio of membrane to storage lipids. Therefore, in future studies it would probably be beneficial to separate cell growth from the WE production phase as has been done successfully for docosanol production in **paper II.** Furthermore, it might help to investigate the cause of the detrimental effect of VLCMUFAs on yeast cell growth, e.g. by analyzing the composition of membrane and storage lipids.

To further increase the flux through the FA biosynthesis machinery and thereby the concentration of WEs produced, the expression of *Mv*FAS could be implemented in the final WE production strain of **paper III.** Moreover, the copy number and integration sites of pathway genes should be studied to identify optimal expression conditions for genes involved in WE synthesis. Also, the position of the double bonds in the WEs synthesized by yeast should be investigated and compared to the position of the double bonds in jojoba WEs, since it might have an effect on the characteristics of the final product. The final product (the mix of WEs produced by the modified yeast strain) should be tested for its properties compared to natural jojoba oil. It would be especially important to investigate if other compounds in jojoba oil, e.g. the phytosterols, contribute to its characteristics.

Transgenic plants represent an interesting alternative to modified microorganisms for the production of jojoba-like oil. However, plants in general have some restrictions like the dependency on suitable agricultural area, longer growth time, fluctuation in harvest, and more difficulties in genetic modifications compared to microorganisms. In addition to that, controversies about transgenic plants are more likely compared to transgenic yeasts. No field trials are necessary to implement jojoba-like WE production in transgenic yeasts, which reduces the risk of unintended mixing of natural and genetically modified organisms compared to transgenic plants. Moreover, transgenic plants used for non-food purposes always compete with plants grown for food production. Since the jojoba-like

WEs in transgenic plants are solely produced in the plant seeds, the fate for the rest of the plant remains unclear, although it might potentially be suitable to use as an animal feed.

In contrast to that, it might be possible to engineer yeast cells in a way that they consist almost solely of WEs. It has already been shown in the case of TAGs, that *S. cerevisiae* cells can be engineered from an initial TAG content of only around 1% up to 25.4% TAGs/CDW, which could potentially be increased even further (Ferreira et al., 2018). The WE synthesis pathway could also be implemented in a natural oleaginous microorganism (oleaginous = more than 20% TAGs/CDW), like *Y. lipolytica*, which contains 30-40% TAGs/CDW (Beopoulos et al., 2009). The red oil yeast *R. toruloides* is capable of accumulating even up to 76% TAGs/CDW, with a biomass production of 100 g/L (Ageitos et al., 2011). This yeast has already been successfully engineered for high level production of FOHs by expression of Maqu_2220 (Fillet et al., 2015). The extension to WE production in this yeast might therefore be fairly easy. In addition to that, an increased formation of lipid bodies in *S. cerevisiae*, or in other yeasts, could potentially also trigger an increased WE synthesis (as has been shown for TAGs in *S. cerevisiae*) (Teixeira et al., 2018), since WEs are naturally stored in lipid bodies similar to TAGs (Wältermann et al., 2005; Wältermann and Steinbüchel, 2005).

Instead of using cellular resources for overexpression of a range of intrinsic *S. cerevisiae* enzymes involved in FA biosynthesis and elongation, it would be more attractive to improve the activity of these enzymes to increase the flux through the desired pathways. One enzyme playing an essential role in FA biosynthesis is the FAS complex, consisting of the subunits Fas1p and Fas2p. Both subunits were found to contain a substantial amount of lysine acetylation sites which might fulfill a regulatory function to control enzyme activity. In **paper IV** we investigated the potential regulatory role of lysine acetylation in Fas2p. Our results indicated that the three lysine sites studied (K83, K173 and K1551) do probably not fulfill a regulatory function in FAS activity, since both triple mutations (*FAS2*^{QQQ} and *FAS2*^{RRR}) reduced the amount of secreted FFAs in a *S. cerevisiae* strain harboring deletions in *FAA1* and *FAA4*. Since Fas2p of *S. cerevisiae* contains approximately 50 lysine residues that are acetylated under certain conditions, it might be very difficult to exactly pick the ones that fulfill a regulatory role. Nevertheless, it might be interesting to investigate the modification of lysine sites which have been found to be acetylated in other yeast species (e.g. common acetylation sites in Fas1p and Fas2p of *S. cerevisiae* and *Y. lipolytica*). This might allow the identification of regulatory lysine residues that could potentially be modified to achieve an increased activity of the yeast FAS and thereby increased FA levels.

In conclusion, this thesis clearly shows that jojoba oil production in *S. cerevisiae* can be achieved by structured rearrangement of yeast intrinsic FA biosynthesis and elongation as well as expression of a range of heterologous bacterial and plant derived enzymes. However, some challenges remain, e.g. the increase of the specific activity of enzymes involved in FA, FOH and WE synthesis.

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