Genetic modification of enzymes and metabolic pathways for the improvement of fatty acid synthesis in the yeast *Saccharomyces cerevisiae*

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The mystery of human existence lies not in just staying alive,
but in finding something to live for.

Fyodor Dostoyevsky

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Summary

The baker's yeast *Saccharomyces cerevisiae* is a valuable and increasingly important microorganism for industrial applications (Hong and Nielsen, 2012). Its robustness concerning process conditions like low pH, osmotic and mechanical stress as well as toxic compounds is an advantage. Moreover, *S. cerevisiae* is 'generally regarded as safe' (GRAS). The model organism has been studied intensively. The collected data, including genomic, proteomic and metabolic information, can be used to genetically modify and improve its metabolism. Fatty acids and fatty acid derivatives have wide applications as biofuels, biomaterials, and other biochemicals. Several studies have been dealing with the overproduction of fatty acids and derivatives thereof in *S. cerevisiae*. The fatty acid biosynthesis starting with acetyl-CoA requires two enzymes, the acetyl-CoA carboxylase (Acc1p) and the fatty acid synthase complex (FAS), to produce acyl-CoA esters with predominantly 16 to 18 carbon atoms chain length (Lynen et al., 1980). For the synthesis of monounsaturated fatty acids in *S. cerevisiae* the ER bound acyl-CoA desaturase, Ole1p is essential (Tamura et al., 1976; Certik and Shimizu, 1999). Using *S. cerevisiae*, the first section of this work dealt with the heterologous characterization of potential ω1-desaturases. Due to the fact that unsaturated fatty compounds can be modified further by hydrosilylations, hydrovinylations, oxidations to epoxides, acids, aldehydes, ketones or metathesis reactions, the interest in ω1-fatty acids is tremendous (Behr and Gomes, 2010). With the intention to find enzymes in fungi, that have a terminal desaturase activity a search in different genome databases was performed. The sequences of Pex-Desat3 and Obr-TerDes were used as reference sequences. The analysed proteins from *Schizophyllum commune* (EFI94599.1), *Schizosaccharomyces octosporus* (EPX72095.1), *Wallemia mellicola* (EIM20316.1), *Wallemia ichthyophaga* (EOR00207.1) and *Agaricus bisporus var. bisporus* (EKV44635.1), however, finally turned out to be Δ9 desaturases. A fungal desaturase with ω1-activity could not be found. The Δ9 desaturase SCD1 from *Mus musculus* was crystallized by Bai et al. (2015) and the information for specific amino acids responsible for the substrate specificity or enzyme activity were allocated. In combination with sequence and enzyme activity data form ChDes1 from *Calanus hyperboreus*, Desat2 from *Drosophila melanogaster*, Pex-Desat3 from *Planotortrix excessana* and Obr-TerDes from *Operophtera brumata* single amino acid exchanges were performed in the Δ9 desaturase Ole1p from *S. cerevisiae*. For all mutants, only fatty acids (C_{16} - C_{18}) with a double bond between carbon C9 and C10 could be found. This indicates, that all inserted amino acid exchanges do not affect the substrate specificity or the position of the introduced double bond.

In the second section the focus was in the development of a production system for fatty acids in *S. cerevisiae* with regard to the previously established procedures by metabolic engineering. The combination of cytosolic malate dehydrogenase (*MDH3*), cytosolic malate enzyme (*MAE1*) and a citrate- α-ketoglutarate- carrier (*YHM2*) should improve the availability of acetyl-CoA in the cytosol, which is an important precursor for the fatty acid biosynthesis. If the major pathway (acetyl-CoA carboxylase and fatty acid synthase) was already optimized by high expression levels than no positive effect on increased fatty acid synthesis was detectable. Only non-optimized strains, with the additional overexpression of ATP-citrate lyase and cytosolic malate dehydrogenase, lead to a 41 % (20 mg/g dcw) improvement of fatty acid synthesis. In order to increase the fatty acid content further, the additional overexpression of *DGA1* and *TGL3* was performed. Hence, the highest amount of fatty acids could be observed with the strain *S. cerevisiae* WRY1Δ*FAA1*Δ*FAA4* (2.5 g/L ± 0.8 g/L). The additional elimination of acyl-CoA synthetase Fat1p did not improve the yield.

It was recently reported, that chain length control of the fatty acid synthesis of bacterial FAS can be changed by rational engineering (Gajewski et al., 2017a). The knowledge about bacterial FAS was transferred in this work to *S. cerevisiae* FAS. Mutating up to five amino acids in the FAS complex enabled *S. cerevisiae* to produce medium chain fatty acids (C_6 - C_{12}). Further improvement was done by metabolic pathway engineering (promoter of alcohol dehydrogenase II from *S. cerevisiae* (p*ADH2*), deletion of acyl-CoA synthetase *FAA2*) and optimization of fermentation conditions (YEPD-bacto medium buffered with potassium phosphate). The production of medium chain fatty acids resulted in the highest yield of 464 mg/L (C_6 to C_{12} fatty acids). Furthermore, strains were created specifically overproducing hexanoic acid (158 mg/L) and octanoic acid (301 mg/L). The characterization of transferases, which could be responsible for the de-esterification of CoA-bound fatty acids, was analysed in an additional approach. It could be shown, that the genes *EHT1*, *EEB1* and *MGL2* have an influence on the MCFA yield in the supernatant. Generally speaking, the data from the single and double deletion strains suggest that Eeb1p has a selective hydrolytic activity for hexanoic acid-CoA ester, while Eht1p shows selective hydrolytic activity for octanoic acid-CoA ester, which is in line with Saerens et al. (2006).

1.Introduction

Fatty acids are generally composed of a hydrophobic hydrocarbon chain of any length and a hydrophilic carboxylic acid functional group (McNaught and Wilkinson, 1997). As an essential part of all living organisms, they are used by nature due to their hydrophobic characteristic as structural components of membrane lipids, major storage form of metabolic energy (β-oxidation to build acetyl-CoA), components of post translationally modified proteins, as well as building blocks for secondary metabolites and signalling molecules (Tehlivets et al., 2007). The characterisation of physiochemical properties of membrane lipids and identification of different strains can be obtained due to their specific fatty acid profile and pattern (McElhaney and Souza, 1976). Manipulation of specific external conditions, like temperature or pH can influence the fatty acids profile of phospholipids in cellular membranes (Cronan, 1978).

Fatty acids can be categorized into three groups. Short chain fatty acids (SCFAs) are represented by carboxylic acids that contain aliphatic tails with less than six carbon atoms. Medium chain fatty acids (MCFAs) possess between six and twelve carbons in the hydrocarbon chain. Long chain fatty acids (LCFAs) are fatty acids with aliphatic tails longer than twelve carbons (Talukdar et al., 2011). Fatty acids can be found in saturated and unsaturated states. Saturated fatty acids have straight hydrocarbon chains with single bonds, whereas unsaturated fatty acids contain one or more double bonds in the hydrocarbon chain (Sado-Kamdem et al., 2009). The typical intracellular forms of fatty acids are as fatty acyl-acyl carrier protein (acyl-ACP), fatty acyl-coenzyme A ester (acyl-CoA), storage lipids, eicosanoids, and free fatty acids (Tehlivets et al., 2007; Peralta-Yahya et al., 2012). Free fatty acids have potentially harmful properties as membrane and protein perturbing detergents (Suutari et al., 1990; Peralta-Yahya et al., 2012). Free fatty acids can be activated by esterification with coenzyme A. Fatty acids are an important component of lipids, which constitute of a large and diverse group of compounds like fats, waxes, sterols, monoglycerides, diglycerides, triglycerides, phospholipids, and others (Fahy et al., 2009). A common feature of all lipids is their hydrophobic nature and solubility in non-polar organic solvents.

The main sources for the industrial use of lipids are animal fats and plant oils, and can thus serve as raw materials for the production of functional polymers such as polyurethanes, polyamides and polyesters (Behr and Rothstock, 2008; Song and Narine, 2008; Behr and Gomes, 2010).

1.1. Fatty acid biosynthesis and degradation

Fatty acids can derive from three different sources in case of *S. cerevisiae*. Firstly, the cells can take up fatty acids from the culture medium and activate them by adding a coenzyme A, secondly by endogenous lipid (protein) turnover and thirdly by *de novo* synthesis and elongation (Tehlivets et al., 2007).

De novo fatty acids synthesis is highly conserved within the kingdoms of life due to its importance. Despite high similarities in the biosynthetic reaction sequence, the structural organization of the enzyme activities is entirely different. In the cytoplasm of yeast, fungi, and animals, but also in some bacteria like corynebacteria and mycobacteria, the type I fatty acid synthases (FAS I) caries out all steps of fatty acid biosynthesis as one multifunctional protein complex (Kolter, 2007; Janßen and Steinbüchel, 2014). The fungal FAS I is encoded by two genes and is assembled to a α_6-β_6-heterododecamer of 2.6 MDa. The animal FAS I is encoded by a single gene, and the translated peptide chains form an α_2-homodimer of about 540 kDa (Schweizer and Hofmann, 2004; Leibundgut et al., 2008; Maier et al., 2008). In plants, bacteria and archaea, but also in mitochondria of animal and yeast cells, the fatty acid biosynthesis is catalysed by independent enzymes that belong to the type II fatty acid synthase (FAS II) (White et al., 2005; Kolter, 2007). In the following section the focus will be on fatty acid synthesis in *S. cerevisiae*.

1.1.2 Fatty acid biosynthesis in *S. cerevisiae*

In *S. cerevisiae* fatty acid biosynthesis requires two enzymes: acetyl-CoA carboxylase (Acc1p) and the fatty acid synthase complex (FAS), which comprises eight different enzyme activities. Acc1p is responsible for the carboxylation of acetyl-CoA to form malonyl-CoA. It is the first known and rate-limiting step in fatty acid biosynthesis. The protein is assumed to be a promising target for optimization of fatty acid synthesis. The introduction of site mutation at Ser1157 and/or Ser659 of Acc1p resulted in an increase in Acc1p activity (Shi et al., 2014). The fatty acid synthase complex (FAS) in *S. cerevisiae* is encoded by two genes. *FAS1* codes for Fas1p (β-chain) with the domains acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH) and major part of the malonyl/palmitoyl transferase (MPT). The gene *FAS2* codes for Fas2p (α-chain) with the domains acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KS), minor part of the malonyl/palmitoyl transferase (MPT) and the phosphopantetheine transferase (PPT) (Figure 1).

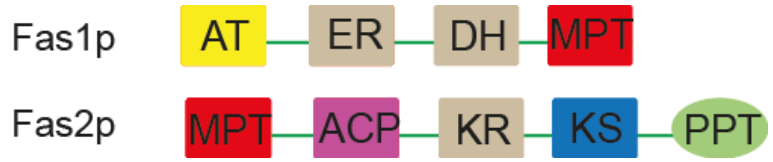

Figure 1: The fatty acid synthase complex (FAS) in *S. cerevisiae* is encoded by two genes, *FAS1* and *FAS2*. Fas1p (β-chain) comprises the domains acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH) and major part of the malonyl/palmitoyl transferase (MPT). The Fas2p (α-chain) comprises the acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KS), phosphopantetheine transferase (PPT) and the minor part of the malonyl/palmitoyl transferase (MPT).

Multiple copies of the two polypeptides are assembled to a α_6-β_6-heterododecamer, which catalyses all reactions required for synthesis of fatty acids: activation, priming, multiple cycles of elongation, and termination (Lynen et al., 1980). The activation is achieved by the PPT domain, attaching the 4'-phosphopantetheine prosthetic group of CoA to the acyl carrier protein (ACP) domain (Fichtlscherer et al., 2000). The 4'-phosphopantetheine prosthetic group acts as a long "arm" that fixes the acyl chain, but provides it with the necessary flexibility in the enzyme to reach the different catalytic sites. PPT and ACP are translated on the same polypeptide chain of the yeast FAS (Schweizer and Hofmann, 2004). The priming reaction is catalysed by the acetyltransferase (AT) domain. Acetyl-CoA is incorporated in the FAS complex as acetyl-ACP (Schuster et al., 1995). The elongation reaction begins with the delivery of the acetyl-ACP to the KS domain. The malonyl-CoA, synthesised by Acc1p, is acquired from the MPT domain by ACP and delivered to the KS domain for chain elongation. Malonyl-ACP is then decarboxylated, and the keto-synthase catalyses its condensation with acetyl-ACP to a 3-ketoacyl intermediate (Landriscina et al., 1972; von Wettstein-Knowles et al., 2006). The 3-ketoacyl intermediate is processed by the KS domain, DH domain, and ER domain in series to form a saturated acyl-ACP (Landriscina et al., 1972; von Wettstein-Knowles et al., 2006). ACP shuttles the intermediates and brings the saturated acyl-ACP back to the KS domain for the next cycle of elongation with malonyl-CoA. The termination reaction is mainly initiated in case of a saturated acyl-ACP with a carbon chain length of 16 or 18 carbon atoms. The ACP domain delivers the saturated acyl-chain to the active centre of the MPT domain, where it is transferred to the SH-group of CoA and then released (Lynen et al., 1980).

In addition to the cytoplasmic fatty acid biosynthesis pathway in *S. cerevisiae*, a FAS II-dependent pathway exists in the mitochondria. Mitochondrial FAS generates octanoyl-ACP used for the synthesis of lipoyl moieties (Zhang et al., 2013). The inactivation of this pathway leads to small mitochondria and a respiratory-deficient phenotype, loss of spectrally detectable cytochromes, low lipoate content and defects in mitochondrial RNA processing (Beopoulos et al., 2011).

1.1.3 Elongation and synthesis of unsaturated fatty acids

The released fatty acyl-CoAs from the FAS complex are nearly completely composed of even-length and straight carbon chains. Desaturases are hydrophobic, membrane bound proteins (Beopoulos et al., 2011), that introduce double bonds into these carbon chains. The enzymes act specifically regarding location, number and stereometry of already present double bonds (Uemura, 2012). If speaking of Δ desaturases the numeration of the position of the double bond starts from the carboxyl end, while in case of ω-desaturases the numbering begins from the methyl end (Los and Murata, 1998).

Desaturases can be divided into three groups: the acyl-CoA, acyl-ACP and acyl-lipid desaturases. Acyl-CoA desaturases use a fatty acid CoA ester as a substrate (Los and Murata, 1998). To generate monounsaturated fatty acids in *S. cerevisiae* the ER bound acyl-CoA desaturase, Ole1p is essential (Tamura et al., 1976; Certik and Shimizu, 1999). The 57 kDa large protein is encoded by the gene *OLE1* (Stukey et al., 1990). It consists of four transmembrane domains with several hydrophobic regions (Stukey et al., 1990; Bai et al., 2015). The N-terminus consists of 397 residues, while the remaining 100 amino acids represent the cytochrome b5-like domain, which is an electron donor for fatty acid desaturases during the reaction (Tamura et al., 1976; Certik and Shimizu, 1999). Bai et al. (2015) crystallized and solved the structure of mouse SCD1 bound to stearoyl-CoA at 2.6Å resolution. This structural information helps to understand the design of Ole1p, due to its high similarity to SCD1. Figure 2 (A) shows a topology diagram of SCD1, with four predicted transmembrane domains, like in Ole1p. The structural data indicate nine conserved histidine motifs, which are necessary for the coordination of the dimetal centre. The long and narrow substrate tunnel, in the cytosolic domain, surrounds the acyl-CoA (Figure 2 B). At the binding point of C9 and C10 of the acyl chain, the tunnel has a kink (Figure 2 D). Amino acids responsible for the formation of the kink are Trp258, Thr257, Gln143 and Trp149. The end section of the tunnel is completed by Tyr104 and Ala108 (Figure 2 C). Ole1p desaturates the carbon atoms at the same position as SCD1 from *M. musculus* to form cis-monounsaturated C16:1$^{\Delta 9}$ (palmitoleic acid or (9Z)-Hexadec-9-enoic acid) and C18:1$^{\Delta 9}$ (oleic acid or (9E)-Octadec-9-enoic acid) fatty acids. Ole1p is not able to synthesise polyunsaturated fatty acids (Stukey et al., 1990; Uemura, 2012). Deletion of *OLE1* leads to a growth deficiency of *S. cerevisiae* and therefore oleic acid has to be supplemented to the culture medium (Stukey et al., 1990). The expression of *OLE1* is highly regulated on different levels (gene expression, mRNA stability, protein degradation and inhibition) and dependent on various exposures, like carbon source, fatty acid availability, oxygen and metal ion concentrations (Martin et al., 2007).

As an example the ER membrane protein Mga1p, a key player in the transcriptional and post-transcriptional regulation, can be mentioned. The enzyme destabilizes the mRNA of *OLE1* upon exposure to unsaturated fatty acids (Chellappa et al., 2001; Kandasamy et al., 2004). Typically, 70-80 % of yeast fatty acids are monounsaturated (Martin et al., 2007). While growing under normal growth conditions palmitoleic acid is the most prominent unsaturated fatty acids. The profile changes in favour to stearic acid and oleic acid, if ethanol concentration in the medium is increased (Yazawa et al., 2011).

To generate fatty acids with more than 18 carbon atoms in *S. cerevisiae* elongases are essential. The maximal chain length is limited to 26 carbon atoms. The elongation process takes place in the ER (Tehlivets et al., 2007). *S. cerevisiae* has three different elongases. Elo1p is required for the elongation of saturated and unsaturated MCFA (in particular C_{14} and $C_{14:1}^{\Delta 9}$) to produce LCFA (in particular C_{16}, $C_{16:1}^{\Delta 11}$ and C_{18}) (Toke and Martin, 1996; Dittrich et al., 1998). The remaining two elongases, Elo2p and Elo3p form very long chain fatty acids (C_{24} - C_{26}) required for the sphingolipid synthesis (Uemura, 2012).

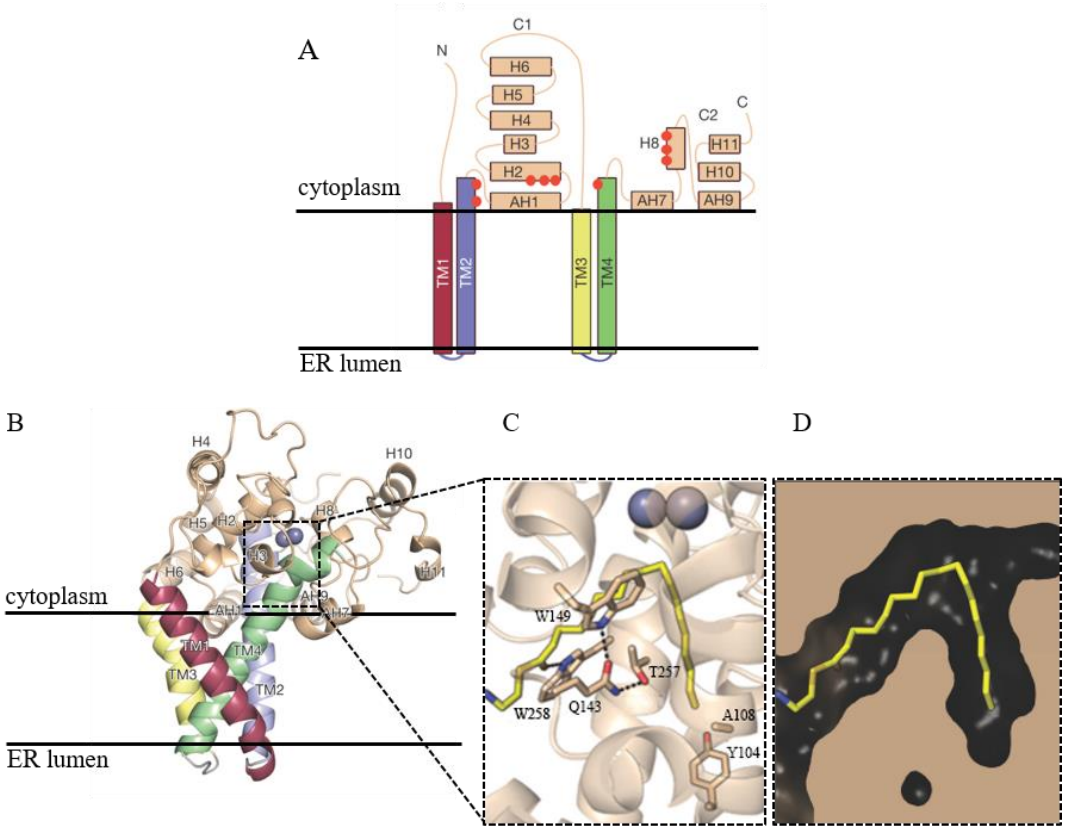

Figure 2: Overview of structure and topology of SCD1 from *M. musculus* A) General view of topology of SCD1 with four transmembrane domains and the nine conserved histidine residues (orange circles) with regard to the localization inside the ER membrane. B) The crystal structure of SCD1 with a bound stearoyl-CoA (yellow, red and blue sticks) and the two zinc ions (blue-grey circles). C) Close-up view of the stearoyl-CoA binding sites with residues forming the kink (Trp258, Thr257, Gln143 and Trp149), and capping the end of the substrate tunnel (Tyr104 and Ala108). D) The substrate tunnel housing the acyl chain shown a surface cross-section (from Bai et al. (2015), modified).

1.1.4 Fatty acids as energy storage

Fatty acids can be used as an efficient energy storage system in various eukaryotic cells. Therefore, lipid depot formation in the cytosol is used to build intracellular lipid droplets (LD) (Zweytick et al., 2000; Brown, 2001). LPs consist of a membrane monolayer of phospholipids spanning the hydrophobic core of neutral lipids (Clausen et al., 1974; Christiansen, 1978). A small amount of proteins, mainly involved in lipid metabolism, are incorporated in the membrane monolayer (Athenstaedt et al., 1999). LPs accumulate during stationary growth phase, reach an approximate diameter of 400 nm and can make 20 % - 70 % of the total lipid content of the cell (Clausen et al., 1974; Bailey and Parks, 1975; Rattray, 1984; Leber et al., 1994). Triacylglycerol (TAG) and sterol ester (SE) are the major neutral lipids in LPs, with up to 95 % share and are not soluble in the cytosol (Leber et al., 1994; Czabany et al., 2008). TAGs have a low biological toxicity compared to free fatty acids, provide as a fatty acid donor for membrane biogenesis (Daum and Paltauf, 1980) and serve as a sink for toxic fatty acids (Hundová and Fencl, 1977, 1978) as well as for diacylglycerol (DAG).

The diacylglycerol acyltransferase (Dga1p) and acyl-CoA-independent acyltransferase (Lro1p) are the main enzymes involved in the TAG synthesis in *S. cerevsiaie* (Dahlqvist et al., 2000; Oelkers et al., 2002). Regulation of Dga1p is crucial for increasing lipid content, which has been shown by disruption studies (Lardizabal et al., 2001; Oelkers et al., 2002; Sandager et al., 2002; Sorger and Daum, 2002). Lro1p seems to be exclusively localized in the endoplasmic reticulum (ER), while Dga1p is localized in the ER and LPs (Sorger and Daum, 2002). Sterol esters (SE), the esterified form of sterols linked to a long chain fatty acid, are synthesized by two sterol acyltransferases named Are1p and Are2p in *S. cerevisiae* (Yang et al., 1996; Yu et al., 1996). Both enzymes are localized in the ER (Zinser et al., 1993).

In *S. cerevisiae* TAG synthesis requires formation of its precursors phosphatidic acid and DAG. The *de novo* biosynthetic pathways (Kennedy, 1962a, 1962b), which yield phosphatidic acid utilize either glycerol 3-phosphate (G3P) or dihydroxyacetone phosphate (DHAP) as precursors (Athenstaedt and Daum, 1999). Briefly, glycerol 3-phosphate (G3P) is acylated successively by G3P acyltransferase and LPA acyltransferase to phosphatidic acid. This is followed by dephosphorylation by phosphatidic acid phosphohydrolase to release DAG. The final acylation step to form TAG can be synthesized either by Dga1p or Lro1p (Czabany et al., 2007; Daum et al., 2007; Beopoulos et al., 2008). Manipulation of specific external conditions, like temperature, pH, nitrogen and carbon source concentration, metal ion supplementation and mineral composition can influence the neutral lipid accumulation behaviour (Beopoulos et al., 2011).

1.1.5 Degradation and export of fatty acids

Peroxisomes are dynamic organelles, present in yeast, fungi, plants and animals, whose number and function are continuously adapted to cellular needs (Veenhuis et al., 1987; McCammon et al., 1990; Marshall et al., 1995). Peroxisome biogenesis is controlled by peroxins encoded by various *PEX* genes and is induced during growth on oleic acid (Nuttall et al., 2011).

Medium and long chain fatty acids degradation occurs mainly via the β-oxidation pathway in the peroxisomes of yeast, which is like the *de novo* synthesis pathway running in a reverse direction (Hiltunen et al., 1992; Kurihara et al., 1992; Kunau et al., 1995). The pathway breaks down two carbons, in the form of acetyl-CoA from the fatty acid chain, in a repeating spiral of four steps (Trotter, 2001; Wanders et al., 2003). The degradation begins with the addition of coenzyme A to a free fatty acid and rate-limiting oxidation of the acyl-CoA substrate into trans-2-enoyl-CoA by an acyl-CoA oxidase Pox1p (also known as Fox1p). *S. cerevisiae* with Δ*pox1* is unable to grow on LCFAs as a sole carbon source (Dmochowska et al., 1990). LCFAs are activated into long chain acyl-CoAs by acyl-CoA synthetases (Faa1p, Faa4p and Fat1p) in the cytosol and are afterwards transported into peroxisomes (Johnson et al., 1994). Faa1p and Faa4p can activate fatty acids of a wide range of sizes, but have preferences for LCFAs. *S. cerevisiae* with Δ*faa1*Δ*faa4* has an impaired fatty acid uptake (Færgeman et al., 2001). Fat1p is required for LCFA import and activation of very LCFAs (C_{20} - C_{26}) (Schaffer and Lodish, 1994; Færgeman and Knudsen, 1997; DiRusso et al., 2000). *S. cerevisiae* with Δ*fat1* has a decreased fatty acid uptake and accumulates very LCFAs, with decreased activation of those (Choi and Martin, 1999; Watkins et al., 2000). MCFAs can freely enter peroxisomes by passive diffusion and are activated inside the peroxisomes by acyl-CoA synthetase Faa2p (Johnson et al., 1994). Faa2p is localized on the peroxisomal periphery (Hettema et al., 1996). *S. cerevisiae* with Δ*faa2* is unable of degradation of MCFAs and synthetase activity is completely abolished (Hettema et al., 1996). The second and third reactions of the β-oxidation are catalysed by Fox2p (also known as Mfe2p or Pox2p), which comprises the 3-hydroxyacyl-CoA and 2-enoyl-CoA hydratase activities (Hiltunen et al., 2003). The reaction product is 3-ketoacyl-CoA. The 3-ketoacyl-CoA thiolase Pot1p (also known as Fox3p or Pox3p) produces acetyl-CoA and a two carbon-shortened acyl-CoA (Mathieu et al., 1997). The shortened acyl-CoA acts as substrate for Pox1 p/Fox1 p for a following cycle of β-oxidation until only two or three carbons remain. Both saturated and unsaturated fatty acids are degraded by β-oxidation. For processing unsaturated fatty acids with the double bond at even positions *S. cerevisiae* possesses the NADPH- dependent 2,4-dienoyl-CoA reductase (Sps19p) (Gurvitz et al., 1997; van Roermund et al., 1998).

In vitro activity experiments with rat liver peroxisomes indicate that palmitoyl-CoA undergoes 2-5 cycles of β-oxidation (Osmundsen et al., 1980). *S. cerevisiae* Δ*pex* mutants are unable to grow on oleic acid (Lefevre et al., 2013). The reactions of the β-oxidation produce a high amount of NADH and acetyl-CoA, which makes the utilization of fatty acids as a carbon source for numerous species interesting (Lefevre et al., 2013).

1.2. Metabolic engineering

Metabolic engineering, meaning optimizing genetic and regulatory processes within microorganisms, is the topic of interest when it comes to production of fatty acids and their derivatives. Extensive research has been carried out in the past years to find the suitable microorganisms, medium and conditions that would allow an economic production of lipids.

1.2.1 Fermentation medium

In order to lower production costs of lipids various fermentation media have been studied. So far examined carbon and nitrogen sources include glycerol (Fakas et al., 2009), oils and fats (Papanikolaou and Aggelis, 2002), wastewater (Xue et al., 2008), wastes from food industry (Zhu et al., 2008), rice straw (Huang et al., 2009), wheat straw (Yu et al., 2011), and bagasse (Tsigie et al., 2011). In *S. cerevisiae* cultivation medium with glucose as carbon source, the increase of histidine concentration (or *HIS3* overexpression) significantly increased stearidonic acid production and cell growth (Kimura et al., 2014). This positive effect could be obtained also with the amino acid leucine or the overexpression of *LEU2* (Kamisaka et al., 2007).

1.2.2 Oleaginous microorganisms

Diverse oleaginous microorganisms, that accumulate large quantities of lipids (>25 % of the cell dry weight) when grown under nitrogen-limited conditions, such as bacteria, fungi and yeasts are an attractive alternative to higher plants and algae for lipid production (Sijtsma and de Swaaf, 2004; Xue et al., 2008, 2010; Subramaniam et al., 2010; Beopoulos et al., 2011). The accumulated lipids have a high content of TAGs and the fatty acid composition is similar to plant seed oils (Verwoert et al., 1995). Some oleaginous yeasts are able to accumulate up to 70-80 % lipids of their cell dry weight in oil-containing medium (Ageitos et al., 2011; Beopoulos et al., 2011) and grow to high cell densities with biomass yields of 10 - 100 g/L reported over 3-7 days (Li et al., 2008). Biofuel production with *Rhodotorula glutinis, Yarrowia lipolytica* and *Lipomyces starkeyi* is already well researched (Angerbauer et al., 2008; Tsigie et al., 2011; Yen et al., 2012).

R. glutinis accumulates up to 72 % lipids with a biomass production of 180 g/L, when growing on glucose or rich fermentation medium (Pan and Rhee, 1986; Pan et al., 1986; Beopoulos et al., 2011). The most frequent fatty acids are C_{16} (18%), $C_{18:1}^{\Delta 9}$ (60%) and $C_{18:2}^{\Delta 9,12}$ (12 %) (Li et al., 2008). *L. starkeyi* accumulates up to 73 % lipids, when growing on glucose, xylose, ethanol or L-arabinose. The most frequent fatty acids are C_{16} (33%) and $C_{18:1}^{\Delta 9}$ (55%) (Li et al., 2008). *Y. lipolytica* can grow on a variety of hydrophobic substrates and accumulates up to 40% lipids of its cell dry weight (Beopoulos et al., 2011). The most frequent fatty acids are C_{16} (11 %), C_{18} (28 %) and $C_{18:2}^{\Delta 9,12}$ (51 %) (Beopoulos et al., 2009a; Beopoulos et al., 2009b).
The baker's yeast *S. cerevisiae*, a non-oleaginous yeast, producing 15 % of TAG and SE of cell dry weight (Daum et al., 2007), has a well-studied genetic and physiological background, and is one of the most widespread and industrially relevant cell factories. The availability of a large collection of genetic tools, the resistance to phage infection, and the high tolerance against toxic inhibitors and products (Hong and Nielsen, 2012), made it a successful platform microorganism for the production of a wide variety of products including ethanol, organic acids, amino acids, enzymes and proteins (Du et al., 2011). *S. cerevisiae* is a more direct host for the production of fatty acids derived fuels and chemicals (Kocharin et al., 2012, 2013), such as free fatty acids (Tang et al., 2013), fatty alcohols (FAL) (Lian et al., 2014), and fatty acid ethyl esters (FAEE), due to the FAS I in the cytosol (Lian and Zhao, 2015), for structure see Figure 3). The released fatty acyl-CoA can be converted to the desired products (FFAs, FALs, FAEEs) by the corresponding enzymes (thioesterases, fatty acyl-CoA reductases, and wax-ester synthases) without prior activation to a CoA ester like in the case of type II FAS.

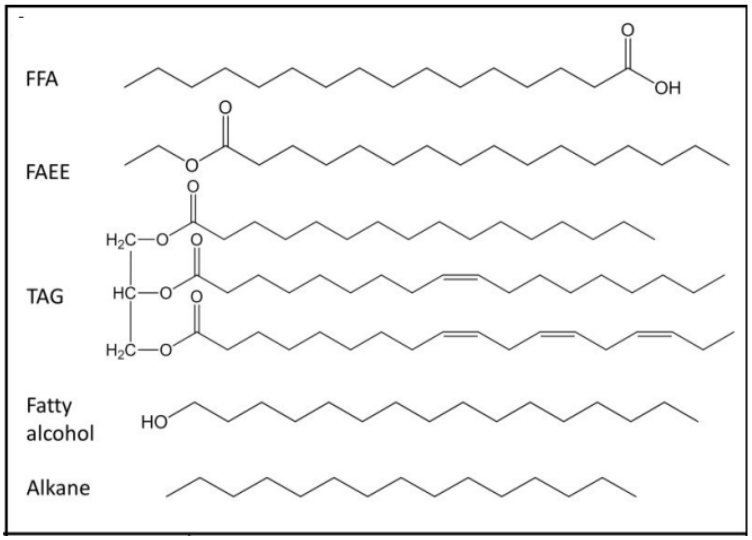

Figure 3: Chemical structure of fuels derived from fatty acid biosynthesis (from Ruffing (2013). FFA free fatty acids, FAEE fatty acid ethyl ester, TAG triacylglycerol.

1.2.3 Pathway optimization

The construction of genetically engineered strains for fatty acid production in *S. cerevisiae* mainly focuses on the overexpression of rate-limiting enzymes and removing feedback inhibitions in the pathway. In order to accumulate high amounts of lipids the precursors acetyl-CoA, malonyl-CoA, glycerol 3-phosphate and the cofactor NADPH have to be provided in excess (Rossi et al., 2011).

Acetyl-CoA is a key precursor in the biosynthesis of fatty acids, lipids, sterols, amino acids, and other (Tai and Stephanopoulos, 2012; Vorapreeda et al., 2012; Lian et al., 2014). In *S. cerevisiae* the acetyl-CoA synthesis occurs in the mitochondria, the peroxisomes and the cytosol, but is not able to cross the corresponding membranes (Roermund et al., 1995; Pronk et al., 1996; Strijbis and Distel, 2010). Thus, *S. cerevisiae* lacks the machinery to export acetyl-CoA from mitochondria to the cytosol (Strijbis and Distel, 2010), where acetyl-CoA is generated through the direct activation of cytosolic acetate via the pyruvate dehydrogenase (PDH)-bypass pathway (Guest et al., 1989; Pronk et al., 1996). The decarboxylation of pyruvate to acetaldehyde (by PDCs), followed by synthesis of acetate and subsequent activation by the acetyl-CoA synthetase (ACS) leads to acetyl-CoA in the cytosol (Shiba et al., 2007). In addition, the acetaldehyde can be converted to ethanol by alcohol dehydrogenases (ADHs), which occurs generally during growth on glucose containing medium (Crabtree effect) (van Hoek et al., 1998; Vemuri et al., 2007). Figure 4 shows the weak points in the metabolic flux towards acetyl-CoA and the heterologous biosynthetic pathways with higher efficiency and lower energy input requirements for the improved of fatty acid synthesis and thus lipid synthesis. Acetaldehyde is the branch point to control the flux distribution between ethanol and acetyl-CoA. The deletion of ADHs should direct the flux to acetyl-CoA and thereby reduce the amount of ethanol. Smidt et al. (2012) show that decreased ADH activity led to increased production of glycerol. *S. cerevisiae* is in general only able to produce small amounts of n-butanol on rich medium via endogenous pathways (Branduardi et al., 2013; Si et al., 2014). By additional inactivation of glycerol formation (deletion of *GPD1* and *GPD2*) the metabolic flux was redirected to acetyl-CoA, which was measured in a 4-fold product increase of n-butanol (Krivoruchko et al., 2013). Further deletions of a peroxisome citrate synthase (*CIT2*) or cytosolic malate synthase (*MLS1*) decreased n-butanol production (Krivoruchko et al., 2013). By removing the mitochondrial targeting sequences from the PDH genes (*PDA1*, *PDB1*, *PDX1*, *LAT1*, *LPD1*) the enzymes localized in the cytosol (cytoPDH) increased n-butanol production by additional 3-fold (Guest et al., 1989; Shiba et al., 2007; Lian et al., 2014).

A different approach was published by Schadeweg and Boles (2016), where an overexpression of the pantothenate kinase *coaA* from *E. coli* and ATP independent acetylating acetaldehyde dehydrogenase, adhE$^{A267T/E568K}$ (stably integrated into the genome), deletion of *ADH1-6* and *GPD2*, and supplementation with pantothenate lead to a titer of 130 mg/L n-butanol. The PDH-bypass pathway, where acetaldehyde is produced by pyruvate decarboxylase isozymes (*PDC1*, *PDC5*, *PDC6*) from pyruvate, represents an additional point for enhancement of acetyl-CoA supply (Hohmann, 1991). Deletion of all three genes leads to a complete loss of alcoholic fermentation capability, accumulation of pyruvate and impaired growth (Flikweert et al., 1997). Expression of cytoPDH and ATP-dependent citrate lyase (ACL) in a Δ*pdc* strain can again enabled the cells to grow in the presence of low concentration of glucose. ACL uses citrate and ATP to produce acetyl-CoA and oxaloacetate (Zaidi et al., 2012). ACL is only present in oleaginous yeast (Lian et al., 2014). This difference to non-oleaginous yeasts shows the potential significance of ACL as an key enzyme for the synthesis of acetyl-CoA. Expression of various ACLs from oleaginous yeasts (Lian et al., 2014), plants (Fatland et al., 2002), and mammalian cells lead in *S. cerevisiae* to an increase of fatty acids production up to 1.17-fold (Tang and Chen, 2014).

The additional deletion of mitochondrial NAD^+-dependent isocitrate dehydrogenases (*IDH1*, *IDH2*), could increase the citrate level up to 5-fold in the cytosol and thus fatty acids production (Anoop et al., 2003; Tang et al., 2013). In the patent from Gunawardena et al. (2008) butanol production was described by overexpressing *PDC1* and *ALD6* from *S. cerevisiae* and a heterologous ACS. The optimized reaction pathway shifts pyruvate via PDC to acetaldehyde. The aldehyde dehydrogenase (ALD) produces acetate, which is converted to acetyl-CoA by the acetyl-CoA synthetase (ACS) at the cost of two ATP molecules (Pronk et al., 1996; Starai and Escalante-Semerena, 2004). A feedback inhibition resistant ACS mutant from *Salmonella enterica*, with a point mutation at position L641P has been described to be more efficient than overexpression of the endogenous *ACS1* or *ACS2* genes (Chen et al., 2013), which lead only to a 2-5-fold increased acetyl-CoA level (Chen et al., 2012). The production of up to 10 g/L 3-hydroxypropionic acid (3-HP) was optimized by a similar approach (Kildegaard et al., 2016). *S. cerevisiae* was engineered for improved translation of PDC, ALD, ACS, a mutated ACC and MCR (malonyl-CoA reductase from *Chloroflexus aurantiacus*). Acc1p contained two mutations at dephosphorylation sites to prevent inactivation by Snf1p, to increase malonyl-CoA. The MCR reduces malonyl-CoA to 3-HP using NADPH as a cofactor.

Due to redox imbalance within the metabolic process the NAD dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was additionally replaced with an NADP dependent GAPDH variant (Kildegaard et al., 2016).

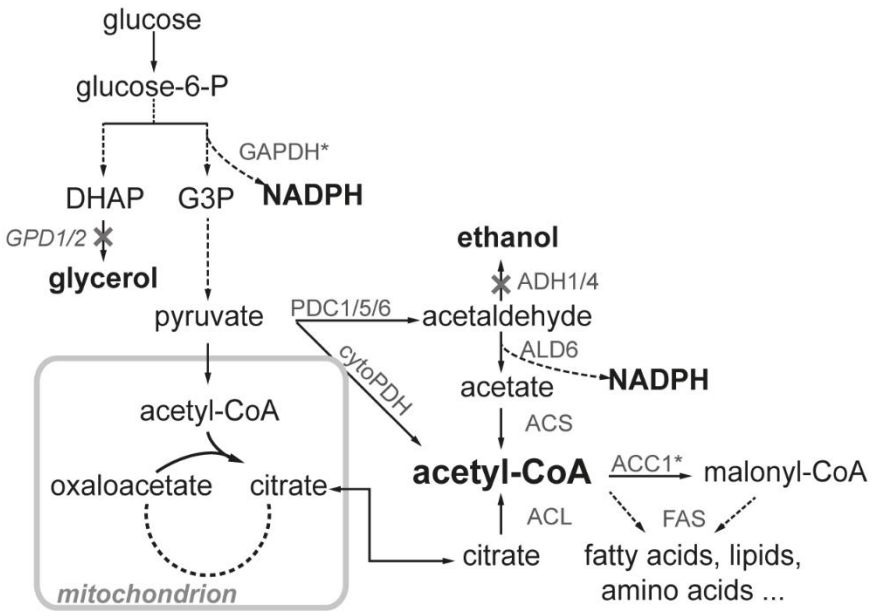

Figure 4: Overview of main strategies to increase acetyl-CoA pool in *S. cerevisiae*. Deletion of competing pathways, like glycerol synthesis (*GPD1*, *GPD2*), ethanol metabolism (*ADH1*, *ADH4*) and expression of heterologous pathways to enhance the acetyl-CoA level in the cytosol. The cytosolic pyruvate dehydrogenase (cytoPDH) and the pyruvate decarboxylases (*PDC1, PDC5, PDC6*) should increase the flux from pyruvate to acetyl-CoA and acetaldehyde, respectively. The ATP-dependent citrate lyase (ACL) can use citrate derived from the TCA, while the acetyl-CoA synthetase (ACS) can use acetate derived from acetaldehyde for acetyl-CoA formation. The *ALD6* and $NADP^+$-dependent $GAPDH^*$ increase the NADPH pool. Overexpression of $ACC1^*$ and *FAS1*/*2* genes by *TEF* promoter increases the fatty acid synthesis (from Lian and Zhao (2015), modified).

In order to accumulate high amounts of lipids, malonyl-CoA is also of substantial importance. The group of J. Keasling could show, that by replacing the native promoter of *ACC1* and the FAS complex genes *FAS1* and *FAS2* with a strong *TEF1* promoter, the expression level was increased by 7 to 16-fold (Runguphan and Keasling, 2014). Furthermore, could the additional overexpression of cytosolic *tesA* from *E. coli* and the deletion of *FAA1* and *FAA4* facilitate the production of free fatty acids (400 mg/L). Another approach is the deletion of storage lipid synthesis and accumulation relevant genes (*ARE1*, *ARE2*, *DGA1*, *LRO1*), which leads to a 3-fold increase in FFA production. A 5-fold increase (17.2 mg/L FAEEs) could be obtained by additional deletion of the initial step of the β-oxidation (acyl-CoA oxidase Pox1p) (Valle-Rodriguez et al., 2014). Leber et al. (2015) obtained 2.2 g/L long chain FFA due to expression of *DGA1* and *TGL3*, with simultaneous deletion of *FAA1, FAA2, FAA4*, *FAT1*, *PXA1* and *POX1*.

For the synthesis of medium chain fatty acids (C_6 - C_{10}) the expression of *hFAS* from *H. sapiens* with replaced thioesterase domain by *TEII* from *R. norvegicus*, and 2µ plasmid based expression of *Sfp* from *B. subtilis* was used. The obtained yield of 111 mg/L is currently the highest published for the yeast *S. cerevisiae* (Leber and DaSilva, 2014). In case of FAL and FAEE production Runguphan and Keasling (2014) could not see a positive effect in yield by disruption of the β-oxidation. Furthermore, Kamisaka et al. (2007) suggested a transcriptional control over TAG and fatty acid biosynthesis of *SNF2*, whose disruption increased TAG accumulation. Table 1 lists a summary of different approaches in *S. cerevisiae* for the synthesis of fatty acids and fatty acid derivatives.

Table 1: Current strategies of metabolic engineering in *S. cerevisiae* for production of fatty acids related fuels and chemicals.

strain	**product**	**strategy**	**result**	**reference**
BY4742	FAL (C_6)	Expression of *FAR* from *Tyto alba*, *ACC1* and *ACL1* (from *Y. lipolytica*); deletion of *RPD3*	1.1 g/L*	Feng et al. (2015)
BY4742	LC-FFA	Expression of truncated '*tesA* from *E. coli*, *ACC1*, *FAS1*, and *FAS2*; deletion of *FAA1* and *FAA4*	400 mg/L	Runguphan and Keasling (2014)
BY4741	FFA	Expression *ACOT5* from *M. musculus*; deletion of *FAA1* and *FAA4*	493 mg/L	Chen et al. (2014)
CEN.PK2-1C	FAEE (C_8 -C_{10})	Expression of the reversed β-oxidation and *EEB1* or *EHT1*	0.75 mg/L	Lian et al. (2014)
BJ5464	FFA (C_6 -C_{10})	Expression of *hFAS* from *H. sapiens* with *TEII* from *R. norvegicus*, and *Sfp* from *B. subtilis*	111 mg/L	Leber and DaSilva (2014)
BY4741	FFA (C_{16}/C_{18})	Expression of *DGA1* and *TGL3*; deletion of *FAA1*, *FAA4* and *FAT1*, deletion of *FAA2*, *PXA1* and *POX1*	2.2 g/L	Leber et al. (2015)
CEN.PK113-11C	FFA	Expression of truncated '*tesA* from *E. coli*, *CTP1* and cytMDH3, FAS, ME and ACL; promoter exchange of native *ACC1* to *TEF1*; deletion of *FAA1*, *FAA4*, *HFD1* and *POX1*	1 g/L 10.4 g/L* *fed-batch fermentations	Zhou et al. (2016)

1.3. Aim of this work

The demand for renewable, bio-based products as an alternative to those of petroleum origin is rapidly increasing. Using microorganisms to produce novel compounds from fatty acids and derivatives is an essential component to this approach, to avoid competition with food production, price increase, questionable land-use practices and environmental concerns associated with oil production from fatty acid of plants and animals (Hill et al., 2006; Steen et al., 2010). Fatty acid derivatives have an immense number of applications as biofuels, biomaterials, lubricants, stabilisers, nutritional additives, surfactants, polymers and pharmaceuticals and other biochemicals (Lennen and Pfleger, 2013; Runguphan and Keasling, 2014). Yeasts are good candidate microorganisms for such production because of uncomplicated culture conditions, simple genetically manipulation and high fatty acid accumulation potential. The baker's yeast *S. cerevisiae* was one of the first yeasts in which lipid metabolism was studied and elucidated (Oelkers et al., 2002; Sandager et al., 2002; Sorger and Daum, 2002, 2003).

Using *S. cerevisiae*, with its simple fatty acids synthesis pathway, the first section of this work deals with the heterologous characterization of potential ω1-desaturases. Due to the fact that unsaturated fatty compounds can be modified further by hydrosilylations, hydrovinylations, oxidations to epoxides, acids, aldehydes, ketones or metathesis reactions, the interest in ω1-fatty acids is tremendous (Behr and Gomes, 2010). In the second section the focus is in the development of a production system for fatty acids in *S. cerevisiae* with regard to the previously established procedures by metabolic engineering. Extensive X-ray structural studies have recently led to a deep understanding of the FAS structural properties (Johansson et al., 2008; Enderle et al., 2015). Using this information in the third section of this work, FAS was manipulated and the medium chain fatty acid synthesis in *S. cerevisiae* was studied and subsequently enhanced.

2. Materials and Methods

In the following section the used materials will be listed and the applied methods will be described.

2.1. Medium, microorganisms and cultivation

For this work used medium and microorganisms will be listed in the following section. The cultivation methods and condition will be described in chapter 2.1.2.

2.1.1 Medium

Medium for cultivation of *E. coli* and *S. cerevisiae* is listed in Table 2. Stock solutions for medium preparation are listed in Table 3. For solid medium (plates) 20 g/l agar was added before autoclaving. Antibiotics for selection for dominant markers were added after autoclaving and cooling medium to at least 55 °C (Table 4). To prepare yeast synthetic medium selective for plasmids with auxotrophic markers, the respective amino acid or nucleobase was omitted. For yeast medium carbon sources were added after autoclaving. Sugar solutions were autoclaved separately, as 10 x stock solutions. Ethanol was added from a 100 % (v/v) stock after cooling medium to at least 55 °C. Carbon sources used in this work are: D-glucose [D], D-galactose [G], raffinose [R] and ethanol [E].

Table 2: List of cultivation medium for bacteria and yeast used in this work. Solid medium contained 2 % (w/v) agar. [1]Added from stock solutions.

medium	**composition**	**reference**
LB	10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5	Sambrook and Russell (2001)
YEP-oxoid	10 g/l Bacto Yeast Extract, Technical (288620), 20 g/l Oxoid bacteriological peptone (LP0037)	Zimmermann et al. (1975)
YEP-bacto	10 g/l Bacto Yeast Extract (212750), 20 g/l BD Bacto Peptone (211677)	Zimmermann et al. (1975)
YEP^+ oleic acid	10 g/l Bacto Yeast Extract (212750), 20 g/l BD Bacto Peptone (211677), 0.5 mM oleic acid	Zimmermann et al. (1975)
SC	1.7 g/l yeast nitrogen base (w/o amino acids and ammonium sulfate), 5 g/l (NH4)2SO4, 1x AA-solution[1] uracil, L-leucine, L-tryptophan and L-histidine as needed for selection, pH 6.3 (with KOH)	Zimmermann et al. (1975)

Table 3:List of stock solutions used in this work.

name	substance	concentration	
		stock solution [g/l]	final [mM]
AA-solution	Adenine	0,224	0.083
	L-tyrosine	0.288	0.079
	L-arginine	0.768	0.220
	L-methionine	0.768	0.257
	L-phenylalanine	0.96	0.291
	L-isoleucine	1.152	0.439
	L-lysine monohydrate	1.152	0.351
	L-valine	1.152	0.492
	L-threonine	1.152	0.484
Uracil	Uracil	1,92	0.171
L-leucine	L-leucine	2,4	0.094
L-tryptophan	L-tryptophan	2,4	0.124
L-histidine	L-histidine	3,6	0.439

Table 4: Antibiotics used in this work

substance	concentration	
	stock solution [mg/ml]	final [µg/ml]
ampicilin	100 in H_2O_{bidest}	100
carbinicillin	100 in 50 % (v/v) ethanol	100
kanamycin	50 in H_2O_{bidest}	50
G418	200 in H_2O_{bidest}	200
Hygromycin B	200 in H_2O_{bidest}	200
clonNAT	100 in H_2O_{bidest}	100

2.1.2 Microorganisms and their cultivation

S. cerevisiae and *E. coli* strains were cultivated at 30 °C, respectively at 37 °C. Liquid cultures in shake flasks or tubes were shaken at 180 rpm on rotary shakers.

Table 5: Bacterial and yeast strains used in this work.

name	genotype	reference
E. coli		
DH10B	F', *mcrA* Δ(*mrr*$^-$*hsdRMS*$^-$*mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ139 Δ(*ara, leu*)7697 *galU galK* λ$^-$*rpsL endA1 nupG*	Thermo Fisher Scientific
S. cerevisiae		
CEN.PK2-1C	MATa; *leu2-3,112*; *ura3-52*; *trp1-289*; *his3-Δ1*; *MAL2-8c*; *SUC2*	EUROSCARF, Frankfurt

W303	MATa; *his3-11*,15; *leu2-3,112*; *ade2-1*; *ura3-1, trp1Δ2; can1-100*	EUROSCARF, Frankfurt
BY4741	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	EUROSCARF, Frankfurt
WRY1	*his3Δ1*; *leu2*Δ*0*; *lys2*Δ*0*; *ura3*Δ*0*; *acc1*::P_{TEF1}-*ACC1*; *fas1*::P_{TEF1}-*FAS1*; *fas2*::P_{TEF1}-*FAS2*	University of California, Berkeley, Department of Chemical & Biomolecular Engineering, Prof. J. Keasling, USA
WRY1Δ*FAA1*Δ*FAA4*	*his3*Δ*1*; *leu2*Δ*0*; *lys2*Δ*0*; *ura3*Δ*0*; *acc1*::P_{TEF1}-*ACC1*; *fas1*::P_{TEF1}-*FAS1*; *fas2*::P_{TEF1}-*FAS2*; Δ*faa1*; Δ*faa4*	University of California, Berkeley, Department of Chemical & Biomolecular Engineering, Prof. J. Keasling, USA
BY4743Δ*fas1*Δ*fas2*	MATa; *his3*Δ*0*; *leu2*Δ*0*; *TRP1*; *lys2*Δ*0*; *MET15; ura3*Δ*0;* Δ*fas1*::*kanMX4;* Δ*fas2*::*kanMX4*	Goethe Universität Frankfurt, Prof. Dr. Grininger, Frankfurt

Table 6: *S. cerevisiae* strains constructed in this work.

name	**genotype**
RPY10	WRY1 Δ*ole1*
RPY18	WRY1 Δ*faa1*Δ*faa4*Δ*fat1*
RPY21	BY4743 Δ*fas1*Δ*fas2*Δ*faa2*
RPY23	BY4743 Δ*fas1*Δ*fas2*Δ*mgl2*
RPY15	BY4743 Δ*fas1*Δ*fas2*Δ*eeb1*
RPY25	BY4743 Δ*fas1*Δ*fas2*Δ*eht1*
RPY29	BY4743 Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1*Δ*mgl2*

2.2. Plasmids

During this work different plasmids were used. In Table 7 provided plasmids are listed, whereas Table 8 summarizes plasmids constructed in this work.

Table 7: List of plasmids used in this work.

name	**description**	**reference**
p425	2µ-plasmid, *LEU2*-marker, expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Becker and Boles (2003)

pUG6-K	plasmid as a template for PCR-amplification of a loxP-flanked deletion cassette with *kanMX4*-marker (G418) under control of the *A. gossypii TEF1*-promoter and -terminator, *E. coli* Ampicillin-marker and pBR322-origin	Güldener et al. (1996)
p426	2µ-plasmid, *URA3*-marker, expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Becker and Boles (2003)
pUG6-H	plasmid as a template for PCR-amplification of a loxP-flanked deletion cassette with *hphNT1*-marker (Hygromycin B) under control of the *A. gossypii TEF1*-promoter and -terminator, *E. coli* Ampicillin-marker and pBR322-origin	Güldener et al. (1996)
p423	2µ-plasmid, *HIS3*-marker, expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Becker and Boles (2003)
pRS72-H	2µ-plasmid, *hphNT1*-marker (Hygromycin B), expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Prof. Boles group, Frankfurt
pRS72-N	2µ-plasmid, *natNT2* -marker (clonNAT), expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Prof. Boles group, Frankfurt
pRS72-K	2µ-plasmid, kanMX4-marker (G418), expression of genes under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator with optional 6His-affinitytag, *E. coli* Ampicillin-marker and pBR322-origin	Prof. Boles group, Frankfurt
pUG6-N	plasmid as a template for PCR-amplification of a loxP-flanked deletion cassette with *natNT2* -marker (clonNAT) under control of the *A. gossypii TEF1*-promoter and -terminator, *E. coli* Ampicillin-marker and pBR322-origin	Güldener et al. (1996)

pRS315-*FAS1*^{wt}	CEN6/ARSH4-plasmid with *LEU2* marker, expression of *FAS1* wildtype under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS315-*FAS1*^{I306A}	CEN6/ARSH4-plasmid with *LEU2* marker, expression of *FAS1* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS315-*FAS1*^{R1834K}	CEN6/ARSH4-plasmid with *LEU2* marker, expression of *FAS1* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS315-*FAS1*$^{I306A\ R1834K}$	CEN6/ARSH4-plasmid with *LEU2* marker, expression of *FAS1* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*^{wt}	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* wildtype under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*^{G1250S}	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*$^{G1250S\ M1251W}$	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*^{F1279Y}	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*^{F1279W}	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*$^{G1250S\ F1279Y}$	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*$^{G1250S\ M1251W\ F1279Y}$	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt

pRS313-*FAS2*$^{G1250S\ F1279W}$	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
pRS313-*FAS2*$^{G1250S\ M1251W\ F1279W}$	CEN6/ARSH4-plasmid with *HIS3* marker, expression of *FAS2* mutant under control of natural promoter and natural terminator, *E. coli* Ampicillin-marker	Goethe Universität Frankfurt, Jun. Prof. Grininger, Frankfurt
p6MDHSpMae	2µ-plasmid, *URA3*-marker, expression of cytosolic *MDH3* under control of the truncated constitutive *HXT7*-promoter and the *CYC1*-terminator, *E. coli* Ampicillin-marker	Prof. Boles group, Frankfurt

Table 8. List of plasmids constructed in this work.

name	**description**	**template**
desaturase		
pRS72-K_Pex-Desat3	like pRS72-K, for expression of Pex-*Desat3* from *Planotortrix excessana*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K_Pex-Desat3
pRS72-K_Obr-TerDes	like pRS72-K, for expression of *Obr-TerDes* from *Operophtera brumata*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K_Obr-Desat
pRS72-K_CAP70780.1	like pRS72-K, for expression of CAP70780.1 from *Podospora anserina*, codon-optimized for *S. cerevisiae* glycolytic code	pMK-RQ_ CAP70780.1
pRS72-K_EFI94388.1	like pRS72-K, for expression of EFI94388.1 from *Schizophyllum commune*, codon-optimized for *S. cerevisiae* glycolytic code	pMK-RQ_ EFI94388.1
pRS72-K_EFI94599.1	like pRS72-K, for expression of EFI94599.1 from *Schizophyllum commune*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K_ EFI94599.1
pRS72-K_EIM20316.1	like pRS72-K, for expression of EIM20316.1 from *Wallemia mellicola*, codon-optimized for *S. cerevisiae* glycolytic code	pMK-RQ_ EIM20316.1
pRS72-K_EKV44635.1	like pRS72-K, for expression of EKV44635.1 from *Agaricus bisporus* var. *bisporus*, codon-optimized for *S. cerevisiae* glycolytic code	pOK-RQ_ EKV44635.1

pRS72-K_EOR00207.1	like pRS72-K, for expression of EOR00207.1 from *Wallemia ichthyophaga*, codon-optimized for *S. cerevisiae* glycolytic code	pMK-RQ_ EOR00207.1
pRS72-K_EPX72095.1	like pRS72-K, for expression of EPX72095.1 from *Schizosaccharomyces octosporus*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K_ EPX72095.1
pRS72-K_*OLE1*	like pRS72-K, for overexpression of *OLE1* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
pRS72-K_*OLE1*^{C120G}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{Y149L}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{Y149T}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{G153V}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{G153M}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{T318L}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-K_*OLE1*^{T318M}	like pRS72-K, for overexpression of mutated *OLE1* from *S. cerevisiae*	pRS72-K_*OLE1*
pRS72-N_p*PDC1-Cyb5*-t*PGK1*	like pRS72-N, for overexpression of *CYB5* with *PDC1* promoter and *PGK1* terminator from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
fatty acid synthase		
pRS315-p*ADH2-FAS1*^{wt}	like pRS315-*FAS1*^{wt}, for overexpression of *FAS1* with *ADH2* promoter from *S. cerevisiae*	pRS315-*FAS1*^{wt}
pRS315-p*ADH2-FAS1*^{IA}	like pRS315-*FAS1*^{I306A}, for overexpression of mutated *FAS1* with *ADH2* promoter from *S. cerevisiae*	pRS315-*FAS1*^{I306A}
pRS315-p*ADH2-FAS1*^{RK}	like pRS315-*FAS1*^{R1834K}, for overexpression of mutated *FAS1* with *ADH2* promoter from *S. cerevisiae*	pRS315-*FAS1*^{R1834K}
pRS315-p*ADH2-FAS1*^{IARK}	like pRS315-*FAS1*-^{I306AR1834K}, for overexpression of mutated *FAS1* with *ADH2* promoter from *S. cerevisiae*	pRS315-*FAS1*^{I306AR1834K}
pRS313-p*ADH2-FAS2*^{wt}	like pRS315-*FAS2*^{wt}, for overexpression of *FAS2* with *ADH2* promoter from *S. cerevisiae*	pRS313-*FAS2*^{wt}

pRS313-p*ADH2-FAS2*^{GS}	like pRS315-*FAS2*^{G1250S}, for overexpression of mutated *FAS2* with *ADH2* promoter from *S. cerevisiae*	pRS313-*FAS2*^{G1250S}
pRS313_p*ADH2-FAS2*^{GSMW}	like pRS315-*FAS2*^{G1250SM1251W}, for overexpression of mutated *FAS2* with *ADH2* promoter from *S. cerevisiae*	pRS313-*FAS2*^{G1250SM1251W}
thioesterase		
pRS72-N_*EHT1*	like pRS72-N, for overexpression of *EHT1* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
pRS72-N_*EEB1*	like pRS72-N, for overexpression of *EEB1* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
pRS72-N_*tesA*	like pRS72-N, for expression of *tesA* from *E. coli*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K_*tesA*
pRS72-N_*MGL2*	like pRS72-N, for overexpression of *MGL2* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
other		
pRS72-N_*DGA1*	like pRS72-N, for overexpression of *DGA1* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
pRS72-H_*TGL3*	like pRS72-H, for overexpression of *TGL3* from *S. cerevisiae*	*S. cerevisiae* BY4741 gDNA
p425_*ACL1*	like p425, for expression of *ACL1* from *Mus musculus*, codon-optimized for *S. cerevisiae* glycolytic code	pUC57-K-*ACL1*
p423_*YHM2*	like p423, for expression of *YHM* from *S. cerevisiae*	*S. cerevisiae* W303 gDNA
p426_*MDH3*	like p426, for expression of *MDH3* from *S. cerevisiae* in the cytosol	p6MDHSpMae
pRS72-K_*MAE1*$^{-90N}$	like pRS72-K, for expression of *MAE1* from *S. cerevisiae* in the cytosol, starting at the start codon ATG 90 nucleotides were removed	*S. cerevisiae* W303 gDNA

2.3. Oligonucleotides

The DNA oligonucleotides and primers used in this work were synthesized by biomers.net GmbH in Ulm, Germany and are listed in the appendix (section II).

2.4. Materials, chemicals and equipment

A detailed listing of used material and chemicals is provided in Table 9. Table 10 summarizes the used equipment.

Table 9: List of enzymes and chemicals used in this work.

manufacturer	chemicals and enzymes
Alfa Aesar (Karlsruhe, Germany)	Stearic acid
BD Difco™ (Becton, Dickinson and Company, USA)	Bacto yeast extract, Bacto yeast extract technical Bacto tryptone, Difco yeast nitrogen base (w/o amino acids and ammonium sulfate), Bacto peptone
Biomers.net (Ulm, Germany)	Primer and oligonucleotides
Cayman Chemical Company (Ann Arbor, Michigan, USA)	Palmitic acid
Fermentas (St. Leon-Rot, Germany)	GeneJet™ Plasmid Miniprep Kit
Fluka (St. Gallen, Germany)	DMDS
Gibco BRL/ Invitrogen (Carlsbad, California, USA)	UltraPure Agarose
ICN Biomedicals (Eschwege, Germany)	Dithiothreitol (DTT)
Macherey- Nagel (Düren, Germany)	NucleoSpin Extract II
Merck (Darmstadt, Germany)	Silica 60 TLC plate
New England Biolabs (Ipswich, Massachusetts, USA)	Restriction endonucleases, T4 DNA ligase, Q5 High-Fidelity PCR Kit, Crimson Taq DNA Polymerase
Oxoid (Basingstoke, GB)	bacteriological peptone
Roth (Karlsruhe, Germany)	All chemicals except those listed elsewhere
Santa Cruz Biotechnology (Dallas, Texas, USA)	1-Nonadecene
Sigma- Aldrich (St. Louis, Missouri, USA)	G418, salmon sperm DNA (D1626), PEG MW3350, Kanamycin, Tergitol solution NP-40, Amberlite IRA-410 chloride form, fatty acid standard except those listed elsewhere
Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)	GeneJET Plasmid Miniprep Kit, GeneRuler 1 kb DNA Ladder, dNTP mix, Phusion High Fidelity Polymerase
WERNER BioAgents (Jena, Germany)	clonNAT (Nourseothricin)

Table 10: Equipment and other materials used in this work

manufacturer	equipment
Agilent Technologies	Agilent 7890A with a Series 5975C mass selective detector and DB5ht column (30 m x 0.25 mm, 0.1 µm film thickness)
Aquilabiolabs GmbH cell growth quantifier (CGQ)	Cell Growth Quantifier (CGQ)
Bio Rad	Pulse Controller II + Gene Pulser II
Martin Christ Gefriertrocknungsanlagen	Alpha 1-4 LD Freeze Dryer with Lyo Chamber guard (121517 PMMA)

ELGA LabWater	ELGA PureLab Classic Mk1, ELGA Maxima purification cartridge RO FEED
Eppendorf	Centrifuges (5415D, 5415R, 5702, 5810R), heating blocks (Thermo Stat plus, Thermomixer comfort),
IKA	Rocker 2D basic, VXR Vibrax basic with VX 2E "Eppendorf" attachment, Combimag RCT, MAG Red, KM02 electronic
Infors HT	Orbitron shaker Type N
Knick	pH Meter 765 calimatic
Memmert	Water bath with shaking device, Minitron
Motic	B3 Professional Series microscope
Perkin Elmer	Perkin Elmer Clarus 400 system equipped with an Elite FFAP capillary column (30m x 0,25mm, film thickness 0.25 µm)
SensoQuest	Labcycler Basic with triple block
Thermo Fisher Scientific Inc.	GE Healthcare Ultrospec™ 2100 pro UV/Visible Spectrophotometers, NanoDrop 1000 Spectrophotometer, Dionex UltiMate 3000 (Semipreparative Autosamplers, ISO-3100SD Isocratic Analytical Pump, Solvent Rack, TCC-3000SD Thermostatted Column Compartment), refractive index detector Shodex RI-101, HyperREZ XP Carbohydrate H^+8 µm column; HERAfreeze HFU T Series Freezer
Vilber Lourmat	FUSION-SL with Fusion SL3 Xpress; Gel Documentation System
Welch	Vacuum Pump Type 300883

2.5. Molecular biological methods

In the following chapters all biomolecular methods will be described.

2.5.1 Isolation of plasmid DNA from *E. coli*

Small-scale preparations of plasmid DNA from *E. coli* cultures (5 - 10 ml) were isolated by means of a commercial preparation kit (GeneJET™ Plasmid Miniprep Kit) as specified by the manufacturer. Plasmid preparations were stored at -20 °C.

2.5.2 Isolation of plasmid and genomic DNA from *S. cerevisiae*

Cells of *S. cerevisiae* were grown overnight in 5 ml SCD or YEPD medium at 30 °C in presence of an appropriate antibiotic if necessary. Harvested cells (2.500 rcf, 1 min) were washed with H_2O_{bidest} and resuspended in 400 µl buffer P1 (Table 11).

200 µl buffer P2 was added, and the cell suspension was incubated with 500 µl glass beads in a VXR basic Vibrax cell disrupter for 10 min at 4 °C. After 30 s of centrifugation (16.000 rcf), 600 µl of the supernatant was transferred to a fresh Eppendorf tube. For protein precipitation 300 µl buffer P3 was added. The sample was centrifuged for 15 min at 16,000 rcf at 4 °C. For precipitation of DNA, 700 µl of the supernatant were transferred to a fresh Eppendorf tube and 700 µl isopropanol was added. After mixing vigorously, the sample was centrifuged for ≥ 45 min at 16,000 rcf. The DNA-pellet was washed twice with 500 µl 70 % (v/v) ethanol (-20 °C), centrifuged for 5 min at 16,000 rcf, dried at RT for 15 min and resuspended in 20 - 30 µl ddH_2O. The isolated DNA was stored at -20 °C.

Table 11: Composition of buffers needed for the isolation of plasmid and genomic DNA from *S. cerevisiae*

P1	
2 M Glucose	2.5 ml
500 mM EDTA	2 ml
1 M Tris-HCl pH 8.0	2.5 ml
RNase A (1000 x)	100 mg/ml in ddH_2O
ddH_2O	add to 100 ml
store at 4 °C	
P2	
1 M NaOH	20 ml
10 % (w/v) SDS	10 ml
ddH_2O	add to 100 ml
store at room temperature	
P3	
3M Potassium acetate	29.45 g
store at 4 °C	

2.5.3 Determination of DNA concentration

DNA concentrations and quality were measured spectrophotometrically using the NanoDrop 1000 Spectrophotometer.

2.5.4 Digestion of DNA with restriction endonucleases (restriction digestion)

The site-specific cleavage of plasmid DNA was performed with restriction endonucleases according to the instructions of the manufacturer.

2.5.5 Polymerase chain reaction (PCR)

For different PCR experiments various polymerases were used. In the case of confirmation of genomic deletions and insertions the Crimson Taq polymerase was used according to the manufacturer's protocol. For amplification of genes and integrative cassettes for further cloning the Phusion® High-Fidelity DNA Polymeras was used according to the manufacturer's protocol. Q5® High-Fidelity DNA Polymerase was used according to the manufacturer's protocol for the amplification of plasmids needed for CrispRCas9 modifications in *S. cerevisiae*. Annealing temperatures of primer pairs were calculated with the Tm calculator tool on the NEB homepage (NEB Tm Calculator v1.8.1). All PCRs were performed in a thermocycler and were stored at -20 °C if necessary.

2.5.6 Agarose gel-electrophoresis for DNA separation

Fragments of DNA samples of different lengths were separated using 0.8 - 1.5 % (w/v) agarose gels. TAE-buffer (40 mM Tris, 2.5 mM EDTA, 20 mM acetic acid) was used as the electrophoresis buffer. Gels were run in horizontal electrophoresis chamber. The GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc.) or 1 kb DNA Ladder (NEB) were used for sizing of the DNA fragments. After addition of 1:6 volume of 6x DNA loading buffer, DNA samples were pipetted into the gel pockets. Separation took place at 100 - 150 V for 30 - 60 min, depending on gel percentage and expected fragment size. Subsequently, the agarose gel was stained for 10 - 20 min in an aqueous 0.5 µg/ml ethidium bromide solution. DNA fragments were visualized with UV light using a Vilber Lourmat gel documentation system.

2.5.7 DNA-purification and DNA-extraction form agarose gels

DNA fragments of interest were cut from stained agarose gels and the slices were transferred to fresh Eppendorf tubes. In the case of PCR reactions or restriction digestions the whole reaction mix was used. The DNA was purified using the NucleoSpin Extract II Kit according to the manufacturer's protocol. Purified DNA was stored at -20 °C.

2.5.8 DNA sequencing

Sequencing samples were prepared by mixing 30 - 100 ng/µl (plasmid) or 10 - 50 ng/µl (PCR product) of DNA with suitable primers (10 µM) and send to GATC Biotech AG (Konstanz, Germany). The obtained sequence data were analysed with the help of Clone Manager V9 (Scientific & Educational Software, USA).

2.6. Genetic methods

The chapter summarizes the genetic methods and all affiliated cloning experiments.

2.6.1 Transformation of *E. coli*

E. coli cells were transformed by electroporation according to the protocol of Dower (Dower et al., 1988) and Wirth (Wirth, 1989) using a Bio-Rad Gene Pulser. Briefly, frozen aliquots of competent *E. coli* cells were mixed with DNA (from *E. coli* or yeast DNA preparations) and thawed on ice before being transferred to electroporation cuvettes. The Bio-Rad Gene Pulser was set to a voltage of 1.5 - 2.5 kV/cm, a resistance of 200 Ω and a capacity of 25 µF. Immediately after the pulse 700 µl of LB medium was added. For regeneration of the cells an incubation at 37 °C for 30 - 60 min was required. 200 - 400 µl of the transformation preparation were spread on a selective LB agar plate with ampicillin or kanamycin, which subsequently were incubated over night at 37 °C.

2.6.2 Transformation of *S. cerevisiae*

S. cerevisiae was transformed according to two protocols of the LiAc/SS carrier DNA/PEG method from Gietz et al. (Gietz and Schiestl, 2007a, 2007b). Briefly, early exponentially grown *S. cerevisiae* cells (OD_{600} 0.6- 1.5) were washed twice with sterile H_2O_{bidest} (3 min, 3.000rcf) resuspended in FCC solution (5 % (v/v) glycerin, 10 % (v/v) DMSO), aliquoted and frozen at -80 °C. To transform DNA (approx. 1 µg for single plasmids, ≥ 1.5 µg each for co-transformations with multiple plasmids and ≥ 2.5 µg and more for integrative DNA-cassettes) 0.1 M lithium acetate (LiAc), 270 µg/ml single stranded DNA (ssDNA) and 36 % (w/v) polyethylene glycol (PEG) 3350 were added and the pelleted cells were resuspended. Subsequently, plasmid DNA or PCR samples were added. The duration of the heat-shock at 42 °C was 35 - 50 min (depending on *S. cerevisiae* strain). After cell harvest, the pelleted cells were directly plated on selection medium or, in case of transformations with a dominant selection marker, transferred to 5 ml of appropriate liquid medium for a 4 h - regeneration. Once the cells were resuspended in 100 - 250 µl medium and plate out on selective medium, an incubation at 30 °C for 2 - 4 days was necessary.

2.6.3 Codon optimization of genes

The codon usage of some target genes, that do not originate from *S. cerevisiae* were adapted to the codon usage of *S. cerevisiae*. The used algorithm preferred codons of the glycolytic genes and exchanged them for the native codons. A detailed description is available in (Wiedemann and Boles, 2008). Genes were synthesized by Life Technologies Corporation or GENEWIZ, Inc.

2.6.4 Plasmid construction by recombinational cloning

Most plasmids used in this work were constructed *in vivo* by homologous recombination described by (Oldenburg et al., 1997). Target ORFs were amplified by PCR (2.5.5) using primers with DNA sequences (> 40 bp) at the 5'-end homologous to the integration position in the target plasmid. The vector was linearized at the site of insertion by restriction digestion (2.5.4). *S. cerevisiae* was transformed with the linearized vector and PCR product (2.6.2) and transformants were plated out on selective medium. All colonies were used to inoculate selective liquid medium (5 ml). Subsequently, DNA was isolated (2.5.2) from these cultures and used for transformation of *E. coli* (2.6.1). Plasmids were isolated from *E. coli* single-colony cultures (2.5.1) and verified by restriction digestion (2.5.4) and DNA sequencing (2.5.8).

2.6.5 Plasmid construction by Gibson assembly

For plasmid construction, where less than three fragments were assembled, the Gibson assembly was used. Regardless of fragment length, one or more overlapping DNA fragments could be joined in a single isothermal reaction by the concerted action of a T5 exonuclease, a DNA Phusion polymerase and a DNA Taq ligase. The T5 exonuclease chews back the PCR products from 5´ to 3´but closed circular DNA molecules are not degraded. Phusion polymerase fills in the gaps and the DNA Taq ligase seals the single stranded nicks (Gibson et al., 2009). The product of a Gibson assembly is a ligated double-stranded DNA molecule (fragment or plasmid). PCR amplified fragments (2-3 µl) (2.5.5) and plasmids (50 ng) were mixed and incubated at 50 °C for 60 min in 5 µl 2x Gibson mix (Table 12).

Table 12: Composition of 5x Isothermal reaction buffer and 2x Gibson mix.

5x Isothermal reaction buffer	
PEG-8000	1.5 g
1 M Tris HCl pH 7,5	3 ml
2 M $MgCl_2$	150 µl
1 M DTT	300 µl
Each dNTP 100 mM	µl
100 mM NAD	300 µl
add H_2O_{bidest}	6 ml
aliquoted 500 µl and store at -20 °C	

2x Gibson mix	
5x Isothermal reaction buffer	64 µl
10 U/µl T5 exonuclease	0,13 µl
40 U/µl Taq DNA ligase	32 µl
2 U/µl Phusion polymerase	4 µl
add H_2O_{bidest}	60 µl
aliquoted 10 µl and store at -20 °C	

2.6.6 Genomic gene deletion by Delitto Perfetto

To inactivate encoded enzymes in *S. cerevisiae*, antibiotic resistance cassettes were integrated into the respective gene by double-strand break mediated homologous recombination with *Delitto Perfetto* method (Sauer, 1987; Güldener et al., 1996; Carter and Delneri, 2010). The marker cassettes were amplified by PCR (2.5.5) using primers with 5' ends homologous to the target gene to enable site-specific insertion (45 bp). The cassettes are composed of a dominant marker gene (hygromycin, geneticin or nourseothricin resistance), flanked by a promoter (*TEF1*), a terminator (*TEF1*), a I-*Sce*I recombinase (mitochondrial endonuclease) with a galactose inducible promoter (*GAL*) and a SEAM1/SEAM2 sequence (repetitive sequence for second homologous recombination step). For the first homologous recombination the marker cassette is transformed into *S. cerevisiae* and can be incorporated into the genome by the 45 bp long homologous region for the target gene. After cultivation in galactose the second homologous recombination step is initiated, by which the *Sce*I recombinase cuts at the recognition sites and induced a double strand break. To repair the break the cell can use the SEAM1 and SEAM2 sequences, which are identical and close the gap. Since the sequences are identical at the 3' end and at the 5' end, the gap can be closed without a "scar". After replica plating of transformants, the correct replacement was confirmed by PCR with isolated genomic DNA.

2.6.7 Genomic gene deletion by CRISPR/Cas9

In the case of not successful deletion of target genes with *Delitto Perfetto* a second approach using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) was followed up (Cong et al., 2013; DiCarlo et al., 2013; Heigwer et al., 2014). The Cas9 endonuclease is part of a typ 2 bacterial CRISPR-Cas system from *Streptococcus pyrogenes*, which is able to form a complex with a guide RNA (gRNA) to cut at a specific sequence.

Cas9 is led by a 20 bp long genomic target sequence at the 5'of the gRNA to the genomic target. At the 3' of the target sequence is a protospacer-associated-motif (PAM), which makes sure that the Cas9 induces a double strand break at this point. The next step is homologous recombination with a PCR fragment, plasmid or 120 bp (60 bp downstream and 60 bp upstream) double strand oligonucleotide as donor DNA (DiCarlo et al., 2013). Through a plasmid with nourseothricin resistance marker (pRCCN) a guide sequence for the Cas9 protein was provided to determine the gene which should be deleted. The target sequence was analysed by DNA2.0 gRNA Design Tool (https://www.dna20.com/eCommerce/ cas9/input) to find an appropriate guide sequence. The plasmid containing the gRNA is transformed into *S. cerevisiae* with a repair template as donor DNA.

2.7. Methods for cell cultivation and fermentation experiments

The chapter summarizes the cultivation procedure and all affiliated growth experiments.

2.7.1 Spectrophotometrical determination of cell density

The turbidity of a liquid culture was determined photometrically by measuring the optical density at 600 nm (OD_{600}) using an Ultrospec 2100 pro spectrophotometer in polystyrene (PS) cuvettes with a path length of 1 cm. If the measured OD_{600} exceeded 0.4, the sample was diluted with water in order to ensure the accuracy of the measurement (linear absorption). For precise growth curves the Aquilabiolabs GmbH cell growth quantifier was used (Bruder et al., 2016).

2.7.2 Glycerol stock cultures

For long-time cryo conservation, all strains were frozen at -80 °C as glycerol stocks. For this, the specific *S. cerevisiae* strains and plasmid-containing *E. coli* strains were cultivated up to stationary growth phase in the presence of suitable antifungal or antibiotics and appropriate medium. A 600 µl aliquot of the culture was mixed with 400 µl 60 % (w/v) glycerol and stored in sterile screw cap tubes. If necessary strains could be reactivated by steaking on appropriate agar medium with suitable antifungal or antibiotic.

2.7.3 Serial dilution spot assay

For comparison of growth of different *S. cerevisiae* strains with various growth medium a serial dilution spot assay was performed. Yeast cells were grown in liquid medium to exponential phase, harvested by centrifugation (3.000 rcf, 30 s), washed once with sterile water and then resuspended to an OD_{600} of 1.0 in selective medium. Subsequent tenfold dilutions were made and 5 µl were spotted on plates of the medium to be examined. Once the spots dried, the plates were incubated at 30 °C.

2.7.4 Aerobic batch fermentations

Cells of *E. coli* were grown in shaking flasks without baffles (50 ml) or sterile test tubes (15 ml). The culture volumes did not exceed 30 % of the flask or test tube volume. Aerobic batch fermentations of *S. cerevisiae* strains was done in shake flasks of varying sizes (50 ml, 100 ml, 300 ml) and did not exceed 20 % of the flask volume. For every cultivation a pre-culture in 10 ml -50 ml was inoculated and grown for 24 h to 48 h, if not otherwise stated. The main culture was inoculated with a starting optical density of 0.1 and incubated for 24 h, 48 h or 72 h on rotary shakers (180 rpm) at 30 °C.

2.7.5 Aerobic batch fermentations with ion exchanger

Aerobic batch fermentations with an ion exchange resin of *S. cerevisiae* strains were performed in shake flasks of 300 ml size and did not exceed 20 % of the flask volume. The ion exchange resin (Amberlite IRA410 Cl) was washed with 70 % ethanol, harvested by centrifugation (3.000 rcf, 30 s) and added to the medium. The cultivation was performed on rotary shakers (180 rpm) at 30 °C.

2.7.6 Lyophilisation of cell pellets and determination of cell dry weight

Analysis of intracellular lipid composition and fatty acid distribution was done from lyophilized yeast cells. Therefore, cells from 50 ml samples were harvested in 50 ml falcon tubes and lyophilized with open falcon lid using an Alpha 1-4 LD Freeze Dryer with Lyo Chamber guard (Martin Christ, Osterode, Germany) for 18 h to 24 h. The falcons were weighted prior to filtering and after drying to measure the cell dry weight.

2.8. Metabolite analysis by HPLC

For analysis of metabolites in culture medium a UHPLC+ system by Thermo Scientific (Dionex UltiMate 3000) equipped with a HyperREZ XP Carbohydrate H+ 8 µm column and a refractive index detector (Thermo Shodex RI-101) was used. The supernatant of 1 ml culture medium (16.000 rcf, 5min) was mixed with 1/9 volumes of 50 % (w/v) 5-sulfosalicylic acid and centrifuged (13.000 rcf, 5 min, 4 °C). Samples of 1 µl were injected, and substances were separated at column temperature of 65 °C with 5 mM sulfuric acid as mobile phase with a flow rate of 0.6 ml/min. Compounds were identified by comparing their retention with those of standard substances. Four standards (mixtures of D-glucose, acetate, glycerol and ethanol with concentrations of 0.05 - 2 % (w/v)) were analysed for quantification. System control and data evaluation was performed with the Chromeleon 6.80 Chromatography Data Systems software (Dionex GmbH, Idstein).

2.9. Fatty acid analysis

The chapter summarizes the fatty acid analysis procedure and all affiliated preparation experiments.

2.9.1 Extraction of lipids from culture medium

For the extraction of fatty acids present in the culture medium the cells were separated from the medium (3.500 rcf, 10 min). An internal standard (0.2 mg heptanoic acid) was added to 10 ml supernatant and mixed with 1 ml 1M HCl and 2.5 ml methanol-chloroform solution (1:1). The solution was vigorously vortexed (5 min) and then centrifuged for 10 min at 3.000 rcf. The lower chloroform layer was recovered and evaporated with nitrogen gas. The samples were dissolved in 200 µl of toluene for methylation (2.9.3).

2.9.2 Extraction of lipids from dry cell pellet

For the extraction of hydrophobic substances like LCFAs and wax esters from whole freeze-dried cells, 30 mg of cell dry mass were mixed with 1.5 ml chloroform/ methanol (2:1 v/v). The mixtures were shaken (800 rpm) for 1.5 h at room temperature and then centrifuged for 1 min at 16.000 rcf to sediment the cell debris. The supernatants were transferred to clean reaction tubes for FAME preparation (2.9.3) or TLC analysis (2.9.7).

2.9.3 Preparation of fatty acid methyl esters

Fatty acids from dried cells or supernatant were determined by GC (2.9.4). For this, 30 mg of cell dry weight (2.7.6) or extracted free fatty acids (2.9.1; dissolved in 200 µl of toluene) were mixed with 1.5 ml of methanol and 300 µl of 8 % (w/v) HCl solution (conc. HCl (35 % w/w; 9.7 ml) was diluted with 41.5 ml of methanol), vortexed vigorously and incubated at 100 °C in an oven for 3 h to form fatty acid methyl ester (FAME). Once the samples were cooled on ice (10 min) 1 ml H_2O and 1 ml hexane could be added. The mixtures were vortexed thoroughly for 30 s and the organic phase was withdrawn and transferred to gas chromatography vials.

2.9.4 Determination of fatty acid methyl esters by GC

The gas chromatographic measurements were carried out with an Perkin Elmer Clarus 400 system (Perkin Elmer, Germany) equipped with an Elite FFAP capillary column (30 m x 0,25 mm, film thickness: 0.25 µm; PerkinElmer, Germany) and a flame ionization detector (Perkin Elmer, Germany). A 1 µl portion of sample was analysed after split injection (1:10); helium was used as carrier gas (90 kPa). The temperatures of the injector and detector were 200 °C and 250 °C, respectively. The following temperature program was applied: 50 °C for 5 min, increase of 10 °C/min to 120 °C and hold for 5 min, increase of 15 °C/min to 180 °C and hold for 10 min, increase of 20 °C/min to 220 °C which were uphold for 30 min. FAMEs were identified and quantified by comparison of their retention times with the retention times of authentic standard substances.

2.9.5 Preparation of fatty acids with DMDS

Fatty acids with unknown desaturation pattern were analysed by GC-MS. For this, 30 mg of cell dry weight were treated like described in sections 2.9.2 and 2.9.3. The withdrawn organic phase (500 µl n-hexane) was blended with 500 µL of DMDS (Fluka AG, Buchs, Switzerland) and 50 µL of iodine solution (60 mg in 1 mL of diethyl ether). The reaction was carried out in vials that can be closed with Teflon-lined caps and incubated at 42 °C for 16 h. Samples were diluted by addition of 1 ml of n-hexane, and the iodine was removed by treatment with 2 ml of $Na_2S_2O_3$ solution (5 % w/v in H_2O). For GS/MS analysis (2.9.6) the organic phase was removed and concentrated to 200 µl.

2.9.6 Fatty acid analysis by GC-MS

The gas chromatographic/mass spectrometric measurements were carried out with an Agilent 7890A (Agilent, Waldbronn, Germany) equipped with a Series 5975C (Agilent, Waldbronn, Germany) mass selective detector. Samples were separated on a DB5ht column (lenght of 30 m, 0.25 mm of an inner diameter, 0.1 µm film thickness; Agilent, Waldbronn, Germany). Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The temperature of the ion source was held at 230 °C and its quadrupole temperature at 150 °C. The following temperature program was applied: 70 °C for 2.5 min, increase of 10 °C/min to 240 °C, increase of 30 °C/min to 325 °C and hold for 5 min, decrease of 120 °C/min to 70 °C which were uphold for 1 min. For data evaluation the Data Analysis tool from MSD ChemStation E.02.00.493 (Agilent, Waldbronn, Germany) was used.

2.9.7 Thin layer chromatography (TLC)

For the qualitative analysis of lipids in extracts of *S. cerevisiae*, the concentrated extract (2.9.2) from 30 mg (dissolved in 20 µl hexane) was use and applied. Together with 10 µg of suitable standard substances to a Silica 60 TLC plate (Merck, Darmstadt, Germany). The mobile phase was hexane/diethyl ether/ formic acid (80:20:2, v/v/v). Spots were visualized by sublimated iodine. Substances were identified by comparing with standard substances carried along. Standard substances used were oleic acid, triolein and oleyloleate.

2.10. *In Silico* Methods & Software

DNA sequences from moth, fungi and bacteria were obtained from GenBank®, a genetic sequence database (http://www.ncbi.nlm.nih.gov/nuccore). In case of *S. cerevisiae* the *Saccharomyces* Genome Database (Cherry et al., 2012) was used. Sequence alignments for desaturases were conducted using the PRALINE multiple alignment server (http://www.ibi.vu.nl/programs/pralinewww/; (Simossis and Heringa, 2005) with standard settings plus PHOBIUS transmembrane structure prediction (Käll et al., 2004) and YASPIN secondary structure prediction. Similarities and identities between protein sequences were calculated from PRALINE alignments using SIAS (http://imed.med.ucm.es/Tools/sias.html) with BLOSUM62 matrix. Jalview was used for the evaluation of the multiple sequence alignments (Waterhouse et al., 2009). The Pfam 29.0 (December 2015, 16295 entries) was used to predict domains inside a protein sequence (Finn et al., 2016).

3. Results

The following chapter presents the performed work on desaturase characterization, fatty acid synthesis optimization and establishment of medium chain fatty acid synthesis in *S. cerevisiae*.

3.1. Desaturases

Using *S. cerevisiae*, with its simple fatty acids synthesis pathway, the following section deals with the characterization of potential ω1-desaturases.

3.1.1 Screening for desaturases with terminal desaturase activity

Fatty acid desaturases can be divided into soluble and integral membrane classes. The soluble enzymes, acyl-ACP desaturases are found in the plastids of higher plants, whereas the integral membrane acyl-CoA desaturases are found in endomembrane systems in prokaryotes and eukaryotes (Chen et al., 2013). The only acyl-CoA Δ9 desaturase in *S. cerevisiae* is Ole1p (Stukey et al., 1989, 1990). The enzyme converts palmitic acid-CoA ($C_{16:0}$) and stearic acid-CoA ($C_{18:0}$) esters to palmitoleic acid-CoA ($C_{16:1}^{\Delta 9}$) and oleic acid-CoA ($C_{18:1}^{\Delta 9}$) esters, respectively (Viljoen et al., 1987; Tuller et al., 1999). *S. cerevisiae* cannot produce polyunsaturated fatty acids such as $C_{18:2}$ and $C_{18:3}$ (Kajiwara, 2002). Desaturases from other organisms, like moth, have not only the function to produce unsaturated fatty acid but also to provide precursors for pheromone synthesis. The moth *Operophtera brumata* has a Δ11-like desaturase, Obr-TerDes. This desaturase acquired a novel methyl-terminus activity, but has lost the usual Δ11-function (Ding et al., 2011). The enzyme is thus able to convert $C_{20:3}^{\Delta 11,14,17}$-CoA into $C_{20:4}^{\Delta 11,14,17,19}$-CoA, and thereby introduce a terminal double bond to the acyl chain. Albre et al. (2012) described a desaturase with terminal desaturase activity from *Planotortrix excessana*. Pex-Desat3 is able to produce $C_{14:1}^{\Delta 13}$-CoA, $C_{16:1}^{\Delta 15}$-CoA and $C_{18:1}^{\Delta 17}$-CoA, which were identified as methyl ester by GCMS analysis.

With the intention to find enzymes in fungi, that have a terminal desaturase activity, a search in different genome databases was performed. The sequences of Pex-Desat3 and Obr-TerDes (Table 28) were used to search for similar annotated proteins inside the fungi taxa.

The search was performed in cooperation with Prof Dr Marco Thines and PhD student Deepak Kumar Gupta from the Biodiversity and Climate Research Centre, Senckenberg Research Institute in Frankfurt am Main, Germany. The best accordance regarding the protein sequence similarity is shown in Figure 5.

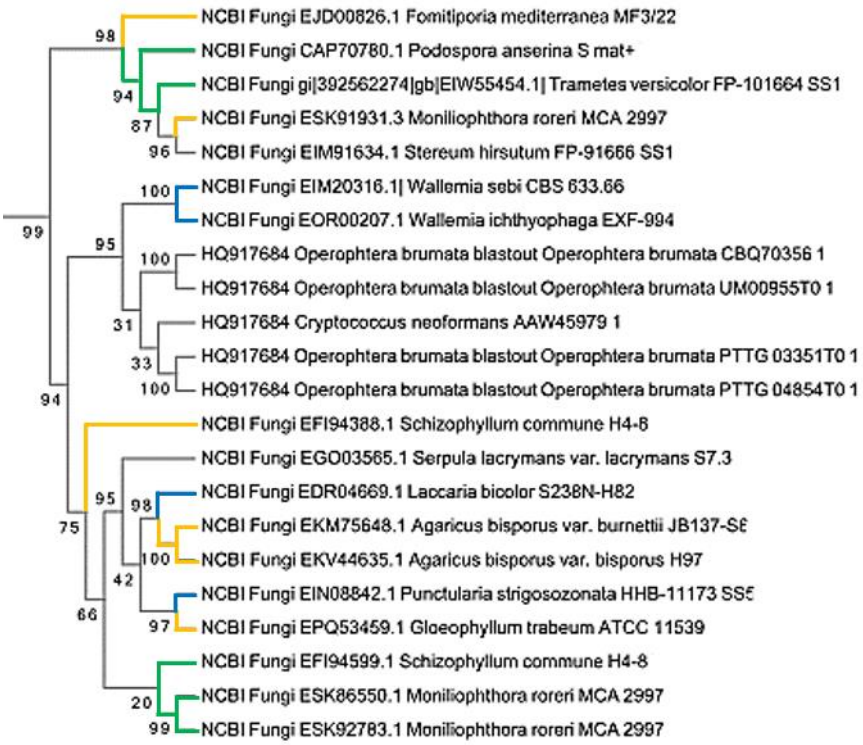

Figure 5: The sequences of Pex-Desat3 and Obr-TerDes were used to generate a neighbor-joining phylogenetic tree with all annotated proteins in the NCBI database for the kingdom fungi (339 complete genomes, February 2014). The section represents the best hits for desaturases with similar protein sequence, whereas green lines present hits for Pex-Desat3, yellow lines for Obr-TerDes and blue for both protein sequences. All other sequences represent proteins that align with other desaturases from moth published by Albre et al. (2012).

The obtained potential candidate desaturases are derived from different fungi. Since the desaturases from *Fomitiporia mediterranea*, *Gloeophyllum trabeum*, *Laccaria bicolor*, *Punctularia strigosozonata* and *Trametes versicolor* are already characterized as Δ9 desaturases, there will be no further analysis of those (Table 13). Among the remaining fungi, *Moniliophthora roreri* has three desaturases, of which two (ESK86550.1 and ESK91931.1) are Δ9 desaturases. The fungus itself is a basidiomycete causing frosty pod rot disease, one of the most severe problems for cacao production (Pod, 1989). *Agaricus bisporus*, known as white button mushroom, is a basidiomycete and one of the most commonly consumed mushrooms in the world (Raper et al., 1972). The two protein sequences (EKM75648.1 and EKV44635.1) align with Obr-TerDes. For *Podospora anserina*, an unnamed protein product was assigned to CAP70780.1 as a match for Pex-Desat3.

The filamentous ascomycete, is a model organism to study genetics, aging, ascomycete development as well as mitochondrial and peroxisomal physiology. *P. anserina* is able to produce pentaketides with antifungal, antibacterial, and cytotoxic activity (Wang et al., 1997). *Schizophyllum commune*, *Wallemia ichthyophaga* and *Wallemia mellicola* (*sebi*), match with at least one moth ω1-desaturase and have the synthesis of hydrophobins, group of small (~100 amino acids) cysteine-rich proteins, in common. Hydrophobins were first discovered and separated in *Schizophyllum commune* (Wessels et al., 1991), whereas the other two species contain predicted hydrophobins with unusually high proportion of acidic amino acids (Zalar et al., 2005). Furthermore, the fungi *Stereum hirsutum* and *Serpula lacrymans* are represented by hypothetical proteins with no match for Obr-TerDes or Pex-Desat3, respectively.

Table 13: List of candidate desaturases with potential terminal desaturase activity. The proteins from the neighbor-joining phylogenetic tree are listed with information concerning the organism, gene name and short description of the enzyme activity.

organism	**gene**	**description**
Agaricus bisporus var. *bisporus* H97	EKV44635.1	hypothetical protein
Agaricus bisporus var. *burnettii* JB137-S8	EKM75648.1	hypothetical protein
Fomitiporia mediterranea MF3/22	EJD00826.1	Δ9-CoA desaturase
Gloeophyllum trabeum ATCC 11539	EPQ53459.1	Δ9-CoA desaturase
Laccaria bicolor S238N-H82	EDR04669.1	Δ9-CoA desaturase
Moniliophthora roreri MCA 2997	ESK86550.1	Δ9-CoA desaturase
Moniliophthora roreri MCA 2997	ESK92783.1	acyl-CoA desaturase
Moniliophthora roreri MCA 2997	ESK91931.1	Δ9-CoA desaturase
Podospora anserina S mat+	CAP70780.1	unnamed protein product
Punctularia strigosozonata HHB-11173 SS5	EIN08842.1	Δ9-CoA desaturase
Schizocaccharomyces octosporus yFS286	EPX72095.1	acyl-CoA desaturase
Schizophyllum commune H4-8	EFI94599.1	hypothetical protein
Schizophyllum commune H4-8	EFI94388.1	hypothetical protein
Serpula lacrymans var. *lacrymans* S7.3	EGO03565.1	hypothetical protein
Stereum hirsutum FP-91666 SS1	EIM91634.1	hypothetical protein
Trametes versicolor FP-101664 SS1	EIW55454.1	Δ9-CoA desaturase
Wallemia ichthyophaga EXF-994	EOR00207.1	putative acyl-CoA desaturase
Wallemia mellicola CBS 633.66	EIM20316.1	hypothetical protein

The already characterized Δ9 desaturases were aligned with Ole1p. The alignment shows the presence of highly conserved sections (Figure 6). Characteristic conserved histidine motifs were present in all protein sequences. Additionally by using the Pfam database the presence of a cytochrome b5-like Heme/Steroid binding domain could be predicted (Finn et al., 2016).

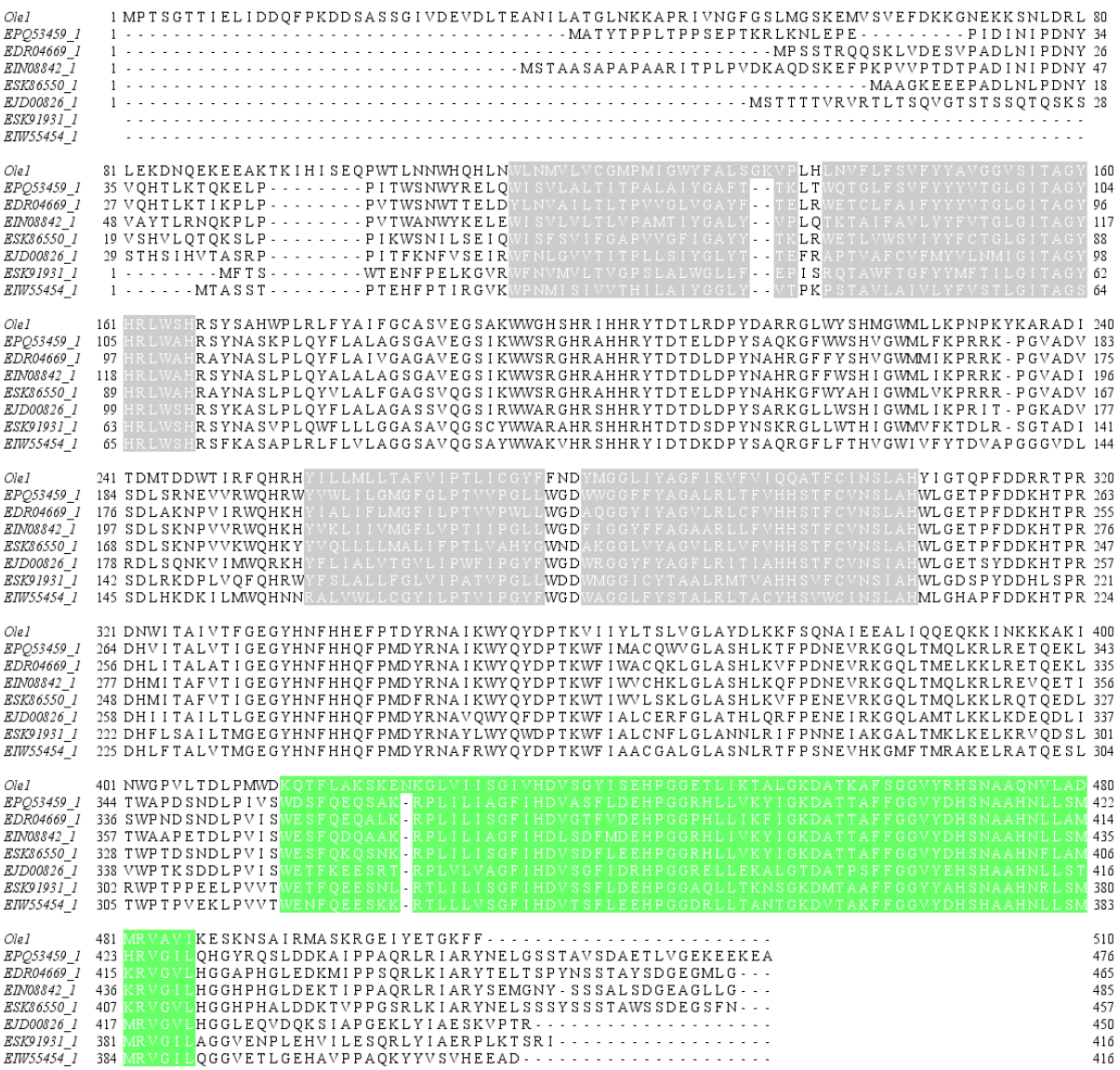

Figure 6: Multiple sequence alignment of Δ9 desaturases with Ole1p. Protein sequences from *F. mediterranea* (EJD00826.1), *G. trabeum* (EPQ53459.1), *L. bicolor* (EDR04669.1), *M. roreri* (ESK86550.1 and ESK91931.1), *P. strigosozonata* (EIN08842.1) and *T. versicolor* (EIW55454.1) were used for comparison with Ole1p Δ9 desaturase from *S. cerevisiae*. Sequence alignment was conducted using the PRALINE multiple alignment server (Simossis and Heringa, 2005). Green areas represent the by Pfam database predicted cytochrome b5 domain (Finn et al., 2016), while grey areas show the predicted Ole1p transmembrane domains.

For purposeful selection of enzymes a sequence alignment was performed with the remaining protein sequences (Table 30) and compared to Pex-Desat3 and Obr-TerDes (Figure 7, for complete alignment see Figure 40). Using the PRALINE multiple sequence alignment tool similarities concerning conserved histidine motifs for desaturases can be observed in all protein sequences (Simossis and Heringa, 2005). Recent publication of Bai et al. (2015) allows the comparison of single, for the activity potentially important, amino acids. The green framed amino acid may determine the length of bound acyl chains.

Desaturases Pex-Desat3 and EIM91634.1 have at the first position (139) the amino acid leucine, Obr-TerDes asparagine, while all other proteins have the hydrophobic tyrosine. The second position (143) has for Obr-Ter and CAP70780.1 the amino acid isoleucine, Pex-Desat3 valine, while all other proteins have the amphoteric glycine. For EIM91634.1, within this alignment, no amino acid is assigned. The blue framed amino acids are presumably responsible for the binding of the acyl chain. The first position (178) shows a glutamine for Pex-Desat3, ESK92783.1,EFI94388.1,CAP70780.1 and EIM91634.1. Obr-TerDes has a threonine, while the majority of the protein sequences has the amino acid glutamic acid. For the second position (Tryptophan 184) no difference in the amino acid can be found. The last position shows an isoleucine and phenylalanine for the terminal desaturases, whereas the other unknown desaturases have a threonine. The purple framed amino acid, arginine represents a highly conserved amino acid for all proteins. The presence of a cytochrome b5-like Heme/Steroid binding domain could be predicted for all desaturases with exception of Pex-Desat3 and Obr-TerDes (Figure 40).

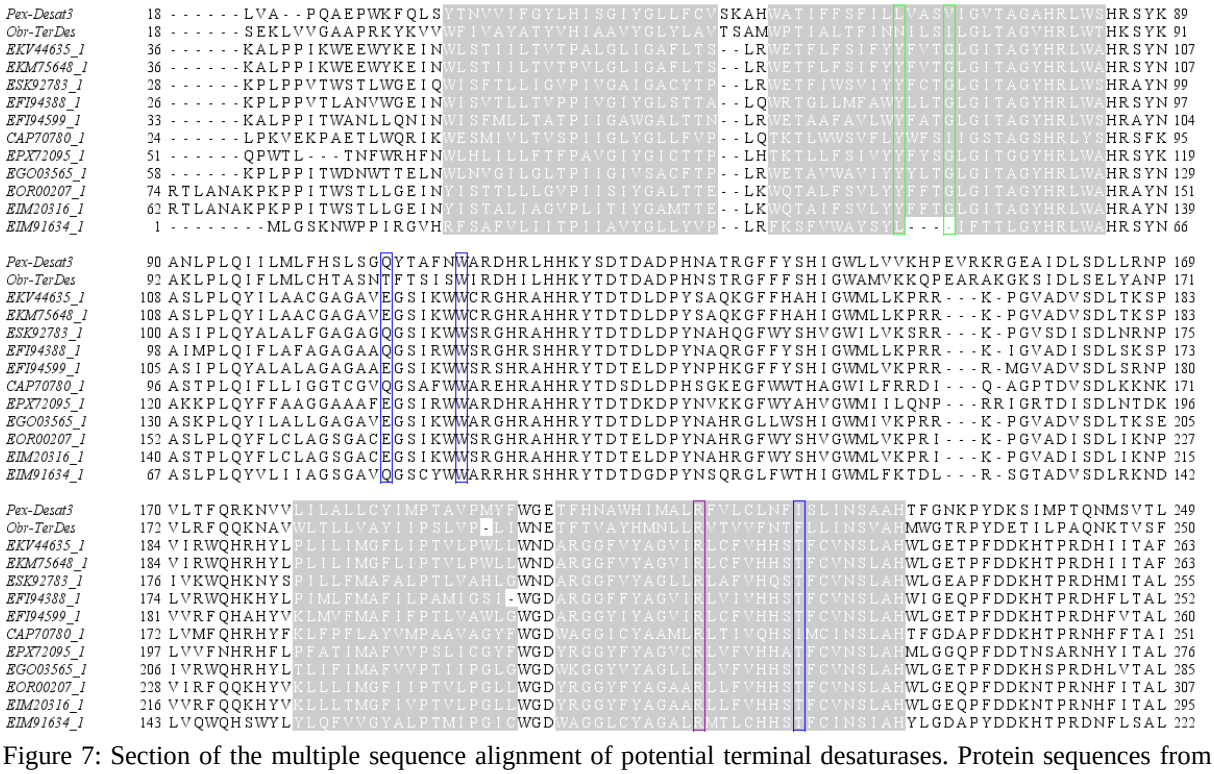

Figure 7: Section of the multiple sequence alignment of potential terminal desaturases. Protein sequences from *A. bisporus* var. *bisporus* (EKV44635.1), *A. bisporus* var. *burnettii* (EKM75648.1), *M. roreri* (ESK92783.1), *P. anserina* (CAP70780.1), *S. octosporus* (EPX72095.1), *S. commune* (EFI94599.1), *S. lacrymans* var. *lacrymans* (EGO03565.1), *S. hirsutum* (EIM91634.1), *W. ichthyophaga* (EOR00207.1) and *W. mellicola* (EIM20316.1) were used for comparison with Pex-Desat3 from *P .exessana* and Obr-TerDes from *O. brumata*. Sequence alignment was conducted using the PRALINE multiple alignment server (Simossis and Heringa, 2005). The green framed amino acid, may determine the length of bound acyl chains. Framed with a blue box are amino acid that could bind to the acyl-chain. The purple framed amino acid represents a highly conserved amino acid, which interaction may help stabilize the kink for double bond introduction. Grey boxes represent predicted transmembrane domains in Ole1p. The complete sequence alignment is shown in Figure 40.

A summary of further analysed proteins is listed in Table 14. The protein from *P. anserina* (CAP70780.1) is interesting, due to the amino acid profile regarding the length of bound acyl chains. From *S. commune*, the hypothetical protein EFI94388.1 has an interesting amino acid pattern regarding the binding of the acyl chain. The proteins from *Serpula lacrymans* var. *lacrymans* (EGO03565.1), *Moniliophthora roreri* (ESK92783.1) and *Agaricus bisporus* var. *burnettii* (EKM75648.1) will not be analysed due to the high similarity to EFI94388.1 from *S. commune* (Table 31). Since the protein sequence EKV44635.1 from *Agaricus bisporus var. bisporus* is highly similar to the sequences of EKM75648.1, ESK92783.1, EFI94599.1, EFI94388.1 and EGO03565.1, it will be included representatively for further investigations.

Table 14: Selection of proteins for further analysis.

gene	**description**	**reason for analysis**
CAP70780.1	unnamed protein product	length of bound acyl chain
EFI94388.1	hypothetical protein	binding of the acyl chain
EFI94599.1	hypothetical protein	lack of similarity
EIM20316.1	hypothetical protein	lack of similarity
EKV44635.1	hypothetical protein	similarity to other proteins
EOR00207.1	putative acyl-CoA desaturase	lack of similarity
EPX72095.1	acyl-CoA desaturase	lack of similarity

For the analysis of potential desaturases, the existing vector pRS72K was used (Table 7). pRS72K is a multicopy vector, that drives expression of the insert through the *HXT7* promoter (Figure 8). It contains a multiple cloning site to introduce DNA fragments and a *kanMX4* marker (with *TDH3* promoter and *TEF1* terminator) for G418 resistance for selection in yeast. For selection in *E. coli* the β-lactamase gene (*bla*) was used. After transformation of *S. cerevisiae* WRY1Δ*ole1* (Table 6) with the constructed plasmids, the strains were plated on YEPD medium containing 0.5 mM oleic acid.

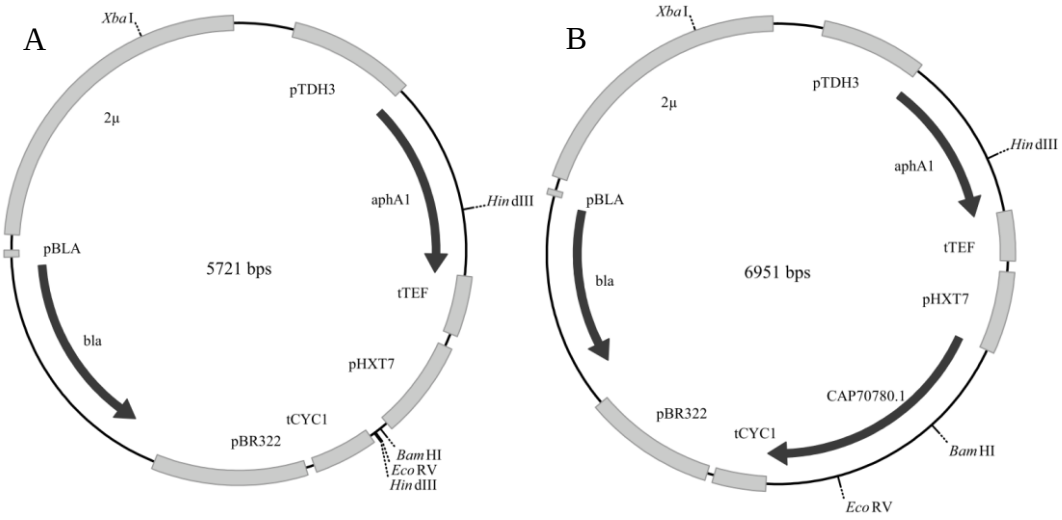

Figure 8: Plasmid map of A) pRS72K and B) pRS72K-CAP70780.1. Transcription in *S. cerevisiae* is mediated by *HXT7* promoter and *CYC1* terminator. Enzymes *Xba*I, *Hind*III, *BamH*I and *Eco*RV indicate sites for verification of plasmid size after restriction. Further elements: 2µ origin, *kanMX4* (*aphA1* gene) marker, ampicillin resistance gene (*bla*) and *E. coli* multicopy origin of pBR322.

The results of a serial dilution spot assay with YEPD medium containing oleic acid and without oleic acid are presented in Figure 9. After 48 h of incubation at 30 °C in YEPD^{+}oleic acid medium all strains were able to grow comparable to the *OLE1* expressing strain. For strains expressing EFI94599.1 from *S. commune* and EOR00207.1 from *W. ichthyophaga*, growing on medium without oleic acid, a wildtype-like growth can be observed. Minor growth deficiency can be observed for strains expressing EPX72095.1, EIM20316.1 and EKV44635.1. For the genes from *S. commune* (EFI94388.1) and *P. anserina* (CAP70780.1) no growth can be observed, which is comparable to the growth of *S. cerevisiae* WRY1Δ*ole1* expressing Pex-Desat3 and Obr-TerDes. To identify the specific fatty acids produced after expression of the desaturases a GC-MS analysis was performed. For localization of the double bond inside the methyl esters of the fatty acids further reactions with DMDS were necessary. Dimethyl disulphide adducts are specific derivatives of unsaturated fatty acids, which enable the locating of double bonds. The mass spectrometric analysis shows, in case of one double bond two important fragment ions, because the cleavage occurs between the carbons with the double bond (Dunkelblum et al., 1985). In case of a terminal double bond introduced by a ω1-desaturase the specific fragment size would be 61 m/Q.

The plasmid containing strains were grown for 48 h in YEPD medium without supplementation of oleic acid. For all strains, which could grow like in the spotting assay, only fatty acids with a double bond between carbon C9 and C10 could be found. The strains with plasmids containing the genes EFI94388.1 and CAP70780.1 could not grow.

Hence, addition of oleic acid should bypass this growth incapability. Subsequent analysis of the cell pellet showed no fatty acids with terminal double bonds neither polyunsaturated fatty acids like $C_{18:2}$. The product spectrum did not differ from the empty plasmid control.

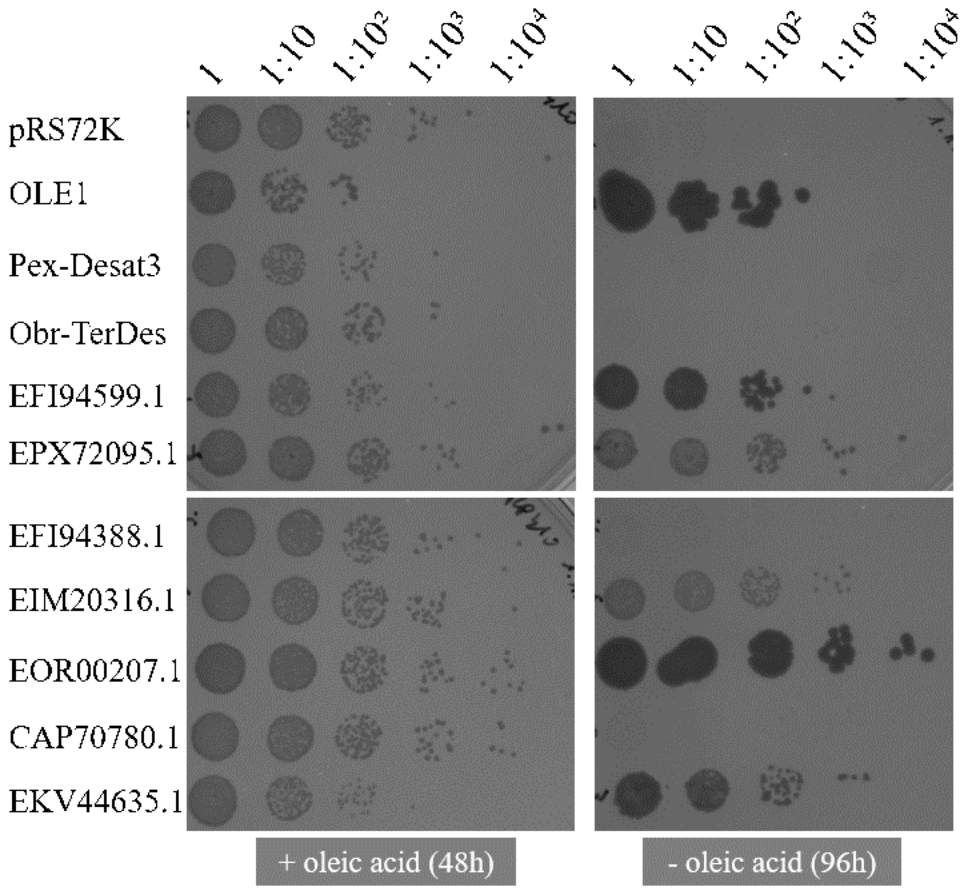

Figure 9: Growth analysis of the *S. cerevisiae* Δ*ole1* strain with different genes being expressed using *HXT7* promoter with the pRS72K plasmid backbone. Serial dilutions of washed cells were dropped on solid YEPD medium with and without 0.5 mM oleic acid, respectively. Cells were grown at 30°C for 2 (with oleic acid) or 4 days (without oleic acid).

Based on the stated facts could the proteins from *S. commune* (EFI94599.1), *S. octosporus* (EPX72095.1), *W. mellicola* (EIM20316.1), *W. ichthyophaga* (EOR00207.1) and *A. bisporus* var. *bisporus* (EKV44635.1) be classified as Δ9 desaturases.

3.1.2 Site directed mutagenesis of Δ9 desaturase Ole1p

The protein SCD1, an integral membrane protein located in the ER catalysing the formation of a *cis*-double bond between the ninth and tenth carbons of stearoyl- or palmitoyl-CoA from *M. musculus*, was crystallized and the structure with bound stearoyl-CoA (2.6 Å) described by Bai et al. (2015). The acyl chain is surrounded in a tunnel, hidden in the cytosolic domain.

By analysis of the geometry conclusions can be drawn for the regioselectivity and stereospecificity. With this information specific mutation of single or multiple amino acids can be performed in order to change the substrate specificity or enzyme activity regarding double bond integration. For the analysis of amino acids, that could have an influence on the activity of Ole1p a sequence alignment was performed with the protein sequences from SCD1 from *M. musculus*, Pex-Desat3 from *P. excessana*, Obr-TerDes from *O. brumata* and Ole1p from *S. cerevisiae* (Figure 10). Due to the localisation at the end of the substrate channel, the amino acid Tyr104 and Ala108 (in SCD1), corresponding to amino acids Tyr149 and Gly153 in Ole1p, could have an influence on the length of the bound acyl chain. For Ole1p, at the position Tyr149, the same amino acid is found in SCD1, while Pex-Desat3 and Obr-TerDes have a leucine and asparagine, respectively. The amino acid Gly153 in Ole1p corresponds to alanine in SCD1, valine in Pex-Desat3 and isoleucine in Obr-TerDes. Six amino acids have been highlighted to have an influence on the substrate specificity, by comparison of mouse desaturases SCD1 and SCD3. The first position, Cys120 in Ole1p, is very divers throughout the aligned protein sequences. SCD1 has a valine, while Pex-Desat3 and Obr-TerDes have a glycine and threonine, respectively. The non-polar amino acid Val109 in Ole1p correlates with the non-polar amino acids leucine in SCD1 and Obr-TerDes and the non-polar amino acid isoleucine in Pex-Desat3. For the amino acids Pro319 and Thr329 from Ole1p the corresponding amino acids for the terminal desaturases do not change. The amino acid Thr318 in Ole1p corresponds to leucine in Obr-TerDes, to methionine in Pex-Desat3 and to glutamine in SCD1. Notably the amino acids of the terminal desaturases are non-polar, while the Δ9 desaturases have a polar amino acid at this position. The last position is Phe330 in Ole1p, which corresponds to valine in SCD1 and leucine in both terminal desaturase sequences. The purple marked amino acid, located within the fourth transmembrane region of the proteins, represents a highly conserved arginine, which interaction may help stabilize the kink for double bond introduction.

Together with the information obtained from the sequence alignment, the following point mutations in Ole1p were introduced (Table 15). For substrate specificity change the amino acid Cys120 was substituted with glycine, which is present in Pex-Desat3 at the same position. Threonine (318) was replaced by methionine and leucine, which represent the amino acids in Pex-Desat3 and Obr-TerDes, respectively.

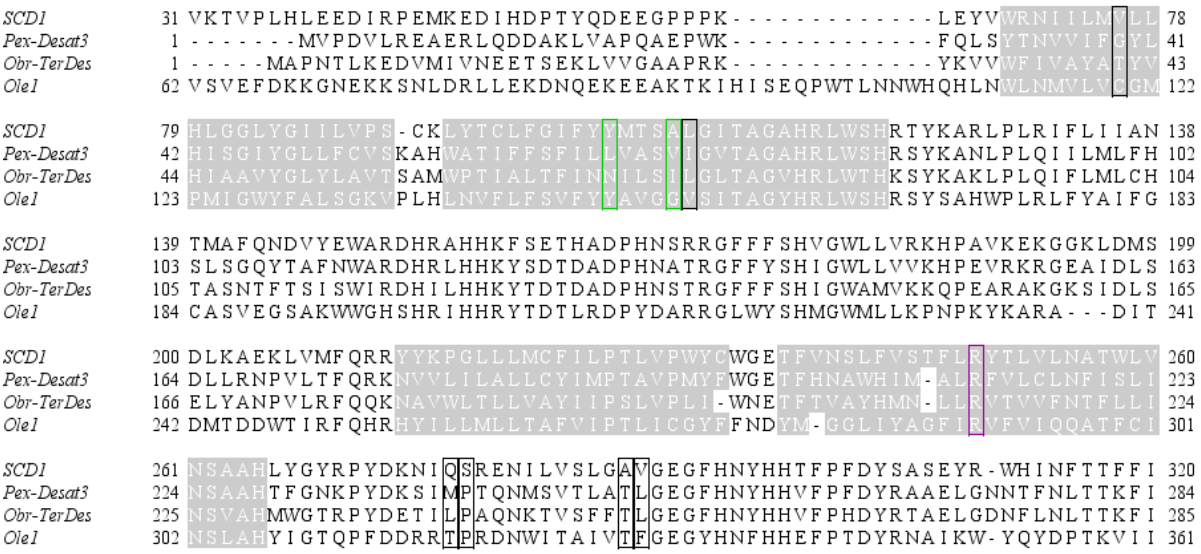

Figure 10: Sequence alignment of desaturases from *M. musculus* (SCD1), *P. excessana* (Pex-Desat3), *O. brumata* (Obr-TerDes) and *S. cerevisiae* (Ole1p). The alignment was conducted using the PRALINE multiple alignment server (Simossis and Heringa, 2005). The amino acids framed with the green box, may determine the length of bound acyl chains. The purple marked amino acid represents a highly conserved amino acid, which interaction may help stabilize the kink for double bond introduction. Amin acids indicated with a black box, change the substrate specificity in mouse SCD3 (Bai et al., 2015). Grey boxes represent transmembrane domains as predicted in SCD1. The complete sequence alignment is shown in Figure 41.

To influence the length of the bound acyl chain amino acid Tyr149 in Ole1p was substituted by leucine and threonine, while amino acid Gly153 was exchanged with valine and methionine, respectively. Threonine is present in ChDes1 from *C. hyperboreus*, which is a desaturase that preferentially acts on very long-chain fatty acyl-CoA (C_{22} - C_{26}). The activity is lost, when threonine was mutated to tyrosine, while desaturation of C_{18} was retained (Meesapyodsuk and Qiu, 2014). Methionine is present in Desat2 from *D. melanogaster*, which can only accept acyl substrates up to 14 carbons long (Dallerac et al., 2000). All other amino acid substitutions are based on the sequences of Pex-Desat3 and Obr-TerDes, which could lead to a novel terminal desaturase activity in Ole1p by chaining the length of the bound acyl chain and substrate specificity.

Table 15: Amino acid positions in Ole1p, which can have an influence on the enzyme activity and substrate specificity. For mutations of amino acid, sequence comparison to Pex-Desat3 and Obr-TerDes, as well as recent publication form Bai et al. (2015) was used.

position in Ole1p	mutation to amino acid	selected on basis of
Cys120	Gly	Pex-Desat3
Tyr149	Leu	Pex-Desat3
	Thr	ChDes1 from *C. hyperboreus*
Gly153	Met	Desat2 from *D. melanogaster*
	Val	Pex-Desat3
Thr318	Leu	Obr-TerDes
	Met	Pex-Desat3

For the analysis of amino acid substitutions in Ole1p, the vector pRS72K-*OLE1* (Table 8) was used as a template. The gene was amplified with two PCRs (two fragments of the gene), which have overlapping regions towards each other and the plasmid pRS72K (linearized with *Bam*HI-HF and *Eco*RV-HF). The primers used for the amplification contained the critical nucleotide substitutions (Table 23). Transformation of *S. cerevisiae* BY4741 with the linearized plasmid and the PCR fragments resulted in a plasmid containing the amino acid exchanged in Ole1p. After transformation of *S. cerevisiae* WRY1Δ*ole1* with the constructed plasmids, the strains were plated on YEPD medium containing 0.5 mM oleic acid. Due to the addition of oleic acid, all strains should be able to grow, even though no wildtype desaturase was expressed. Growth analysis via serial dilution spotting assay was performed subsequently (Figure 11). The strain expressing *OLE1*^{C120G} shows very weak growth compared to the strain expressing the wildtype *OLE1*. For the growth on medium without oleic acid supplementation, nearly all strains have a weak growth compared to the strain expressing the wildtype *OLE1*. Only mutants *OLE1*^{C120G}, *OLE1*^{Y149L}, *OLE1*^{T318L} and *OLE1*^{T318M} show comparable growth behaviour. The empty vector control harbouring strain is not able to grow without addition of oleic acid.

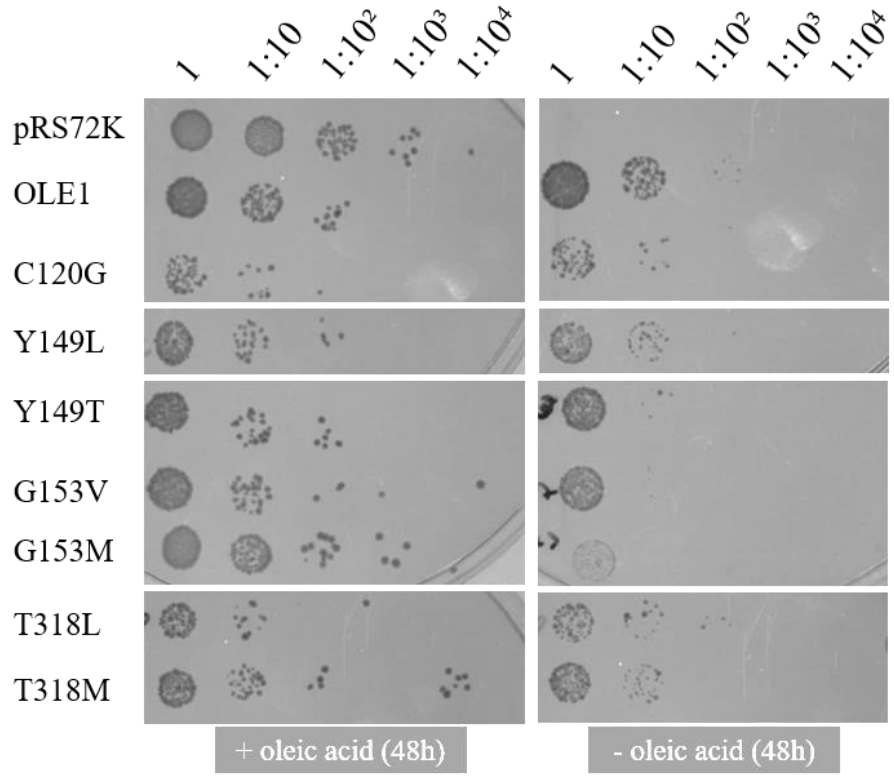

Figure 11: Growth analysis via serial dilution spotting assay of the *S. cerevisiae* WRY1Δ*ole1* strain with different mutated *OLE1* being expressed using *HXT7* promoter with the pRS72K plasmid backbone. Serial dilutions of washed cells were dropped on solid YEPD medium with and without 0.5 mM oleic acid, respectively. Cells were grown at 30°C for two days.

At the same time a GC-MS analysis of 10 - 30 mg cell pellet was performed. All strains were grown in liquid YEPD medium without oleic acid for 48 h. For all strains, only fatty acids (C_{16} - C_{18}) with a double bond between carbon C9 and C10 could be found. This indicates, that all inserted amino acid exchanges do not affect the enzyme activity.

3.2. Improving fatty acid synthesis

In this section the focus is on the development of a production system for fatty acids in *S. cerevisiae* with regard to the previously established procedures by metabolic engineering.

3.2.1 Optimization of acetyl-CoA synthesis

Acetyl-CoA is a very important intermediate in the fatty acid biosynthesis pathway and most relevant substrate for an optimal synthesis rate. Different organisms have various ways of providing sufficient amount of acetyl-CoA inside the cytosol.

In *S. cerevisiae* the pyruvate dehydrogenase bypass is responsible for an indirect synthesis via the pyruvate decarboxylase (PDC) (Flikweert et al., 1997; van Maris et al., 2003). In case of PDC deletion, the only way for the yeast cells to grown during glucose limiting and aerobe conditions is the supplementation of ethanol or acetate (Flikweert et al., 1997, 1999; van Maris et al., 2003). Subsequently the acetate can be transferred into acetyl-CoA with the acetyl-CoA synthetase (ACS). *S. cerevisiae* does not have the ability to transport acetyl-CoA from the mitochondria to the cytosol, like oleaginous yeasts (Pronk et al., 1996; Flikweert et al., 1997; van Maris et al., 2003).

In the past years numerous studies in yeast and bacteria have shown, that the fatty acid pathway can be optimized at different points inside the pathway by genetic modification (Lu et al., 2008; Nawabi et al., 2011; Blazeck et al., 2014; Runguphan and Keasling, 2014). Li et al. (2014) could show a yield of 140 mg/L free fatty acids, when deleting the β-oxidation, overexpressing *ACC1* and different thioesterases, as well as disabling the acyl-CoA synthetases. A different approach was the deletion of the isocitrate dehydrogenase genes *IDH1* and *IDH2* combined with the expression of a heterologous ATP-Citate lyase (Tang et al., 2013) to increase the synthesis of LCFA synthesis in *S. cerevisiae*. In Figure 12 the fatty acid synthesis and tricarboxylic acid (TCA) cycle in *S. cerevisiae* with highlighted steps of preformed manipulation is shown.

In order to test the effect on the fatty acid synthesis of the ATP-citrate lyase (*ACL1*) in *S. cerevisiae* W303, the gene was overexpressed with a *HXT7* promoter in a multi-copy vector (Shanehsaz, 2014). The activity of the enzyme could be verified with expression in a Δ*acs2* strain background, when growing on glucose. The strain was able to grow without notable growth deficiency. Hence, the ATP-citrate lyase is able to provide sufficient acetyl-CoA for a normal growth comparable to the wildtype strain. The combination of cytosolic malate dehydrogenase (*MDH3*), cytosolic malate enzyme (*MAE1*) and a citrate- α-ketoglutarate-carrier (*YHM2*), should improve the availability of acetyl-CoA in the cytosol (Figure 12). The expression of the genes did not negatively impair the growth with glucose or rafffinose as a carbon source of the strain. The combination of ATP-citrate lyase and cytosolic malate dehydrogenase did lead to an overall increase of LCFAs (C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$) of 41,3 % (20 mg/g dcw) compared to the empty vector control (Shanehsaz, 2014).

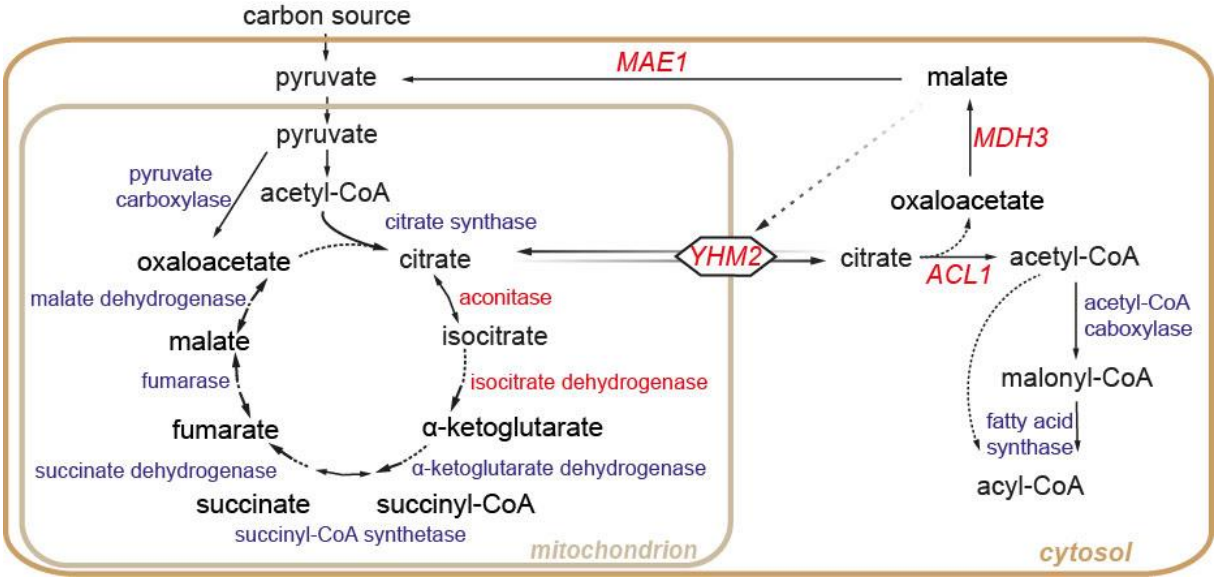

Figure 12: Schematic illustration of fatty acid synthesis and TCA cycle in *S. cerevisiae* with highlighted steps for manipulation. During glycolysis glucose is converted to pyruvate, which is transported to the mitochondria. Pyruvate can be further processed to acetyl-CoA (pyruvate dehydrogenase) and oxaloacetate (pyruvate carboxylase). Both products are converted to citrate via citrate synthase. The first step of manipulation starts with the transport of citrate into the cytosol (*YHM2*), where the ATP-Citrate Lyase (*ACL1*) can synthesise acetyl-CoA required for fatty acid synthesis. The deletion of aconitase and isocitrate dehydrogenase with expression of a transporter could increase the amount of exported citrate. The by-product of Acl1p is oxaloacetate. By the use of cytosolic malate dehydrogenase (*MDH3*) and malic enzyme (*MAE1*), oxaloacetate can be reintegrated into the cycle via pyruvate formation.

The combination of *ACL1*, *MDH3* and *MAE1* was not suitable for improvement due to the fact, that the fatty acid content decreased by 44 % (~10 mg/ g dcw), when compared to the empty vector harbouring strain (~18 mg/ g dcw).

The following deletion of the isocitrate dehydrogenase genes *IDH1* and *IDH2* was performed in *S. cerevisiae* WRY1 und WRY1ΔFAA1ΔFAA4, where the fatty acid synthesis is optimized by exchange of the native promoters of *ACC1*, *FAS1* and *FAS2* with a *TEF1* promoter. The expression of *ACL1* was tested in both strains in medium containing 2 % glucose as a carbon source for 48 h and 96 h. In all tested approaches no significant increase in fatty acid content could be observed (Abakumov, 2015).

In summary, no positive effect in increased fatty acid synthesis is detectable, when the major pathway (acetyl-CoA carboxylase and fatty acid synthase) are already optimized by high expression level due to promoter exchange. Only non-optimized strains, with the additional overexpression of *ACL1* and cytosolic *MDH3*, lead to improvement of fatty acid synthesis.

3.2.2 Increasing fatty acid content by *DGA1* and *TGL3* expression

S. cerevisiae forms non-essential lipid droplets (LD), which serve as a storage compartment (Czabany et al., 2008). LDs consist of 95 % non-polar lipids and are composed of roughly 50 % triacylglycerols (TAG) and 50 % sterol esters (SE) (Sandager et al., 2002; Koch et al., 2014). The main pathway for TAG formation is the acyl-CoA-dependent esterification of DAG by Dga1p and Are1p (Wagner and Paltauf, 1994). Dga1p is localized to LDs and acylates the major part of diacylglycerol using acyl-CoA as an acyl donor. Dga1p has been identified and overexpressed in yeast hosts contributing to an increase in intracellular and extracellular fatty acid levels (Runguphan and Keasling, 2014). Lipases, like Tgl3p, which are required for TAG and SE conversion to FFAs, are abundant in yeast (Athenstaedt and Daum, 2005). Tgl3p is a bifunctional triacylglycerol lipase and LPE acyltransferase. In the absence of lipid droplets the protein stability and level is reduced. Deletion of *TGL3* can lead to an increase in the cellular level of TAGs. Leber et al. (2015) could enhance carbon flux into neutral lipid droplet formation and degradation by overexpressing *DGA1* and *TGL3*, to increase extracellular FFAs.

Figure 13 shows the metabolic pathway of TAG and SE in *S. cerevisiae* with indicated manipulations introduced in this work. The formation of TAG and SE takes place in the ER. Lipid droplets are formed in case of higher amount of storage lipids. The utilisation of TAG and SE occurs via β-oxidation. To analyse the effect of *DGA1* and *TGL3* overexpression and thereby enhance carbon flux into neutral lipid droplet formation and degradation, different *S. cerevisiae* strains were used. The control strain without optimization of fatty acid synthesis (BY4741) was compared to *S. cerevisiae* WRY1 (Figure 14A). The WRY1 strain has an optimized fatty acid synthesis due to the exchange of the native promoters of *ACC1*, *FAS1* and *FAS2* with the *TEF1* promoter.

The expression of *DGA1* and *TGL3* causes a 35 % increase in overall fatty acid content (in the medium and inside the cells) in strain BY4741, when compared to the empty vector control. The genetically manipulated strain WRY1 shows a 40 % higher amount of fatty acids in total, when compared to strain BY4741. The expression of *DGA1* and *TGL3* induces additional fatty acid accumulation up to 2.6 g/L (± 1.2 g/L) in *S. cerevisiae* WRY1. The distribution of TAGs and SEs was additionally analysed by TLC (Figure 14B). The expression of *DGA1* and *TGL3* causes an increase of TAG, when comparing to the corresponding control strain.

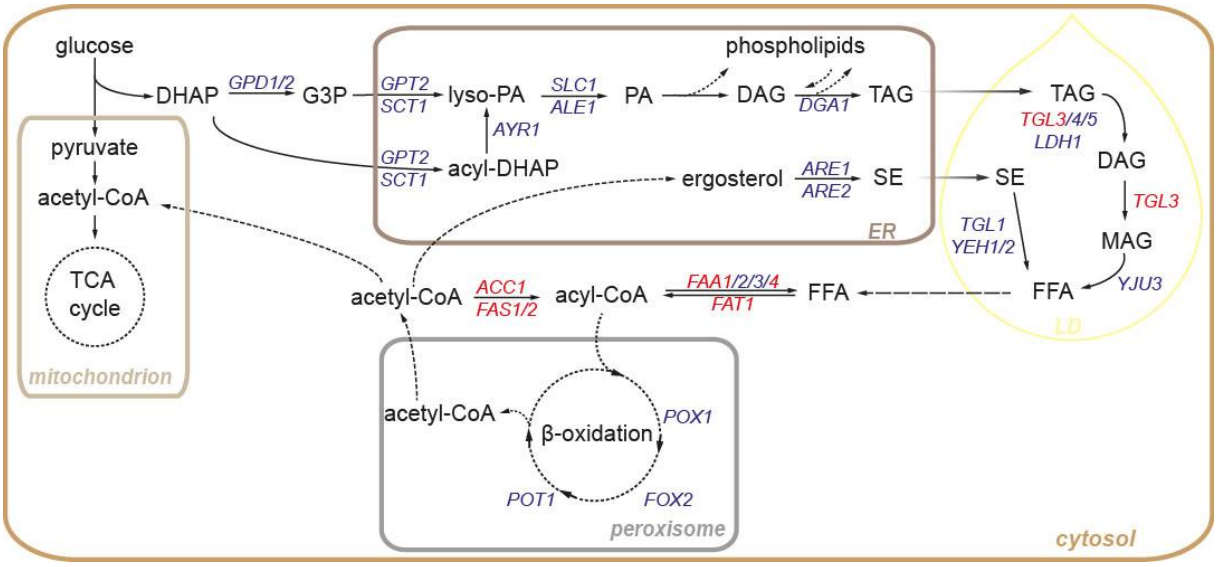

Figure 13: The metabolic pathway of TAG and SE in *S. cerevisiae* (adopted from Kohlwein (2010) and Wang (2015)). Dashed lines indicate multiple enzymatic reactions or unknown/unverified reactions. Enzymes, which were modified for this work are indicated in red. The formation of TAG and SE takes place in the ER. As soon as a higher amount of the storage lipids are synthesised, lipid droplets are formed. For the utilisation of TAG and SE, in late stationary growth phase, when no carbon source is available, the degradation via β-oxidation can occur. The free fatty acids, produced during the degradation reaction inside the LDs, can be esterified by fatty acid synthetases in acyl-CoAs. Compounds: acyl-DHAP acyl dihydroxyacetone phosphate, DAG diacylglycerol, DHAP dihydroxyacetone phosphate, FFA free fatty acid, G3P glycerol 3-phosphate, lyso-PA lyso phosphatidic acid, PA phosphatidic acid, SE sterol esters, TAG triacylglycerol. Genes: *ACC1* acetyl-CoA carboxylase, *ALE1* lysophospholipid acyltransferase, *ARE1*/*ARE2* acyl-CoA:sterol acyltransferase, *AYR1* bifunctional triacylglycerol lipase and 1-acyl DHAP reductase, *DGA1* diacylglycerol acyltransferase, *FAA1*/*FAA3*/*FAA4* long chain fatty acyl-CoA synthetase, *FAA2* medium chain fatty acyl-CoA synthetase, *FAS1*/*FAS2* fatty acid synthase, *FAT1* very long chain fatty acyl-CoA synthetase and fatty acid transporter, *FOX2* 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, *GPD1*/*GPD2* glycerol-phosphate dehydrogenase, *GPT2* glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase, *LDH1* serine hydrolase, *POT1* 3-ketoacyl-CoA thiolase, *POX1* fatty-acyl coenzyme A oxidase, *SCT1* glycerol 3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase, *SLC1* 1-acyl-sn-glycerol 3-phosphate acyltransferase, *TGL1* sterol ester hydrolase, *TGL3* bifunctional triacylglycerol lipase and LPE acyltransferase, *TGL4* multifunctional lipase/hydrolase/phospholipase, *TGL5* bifunctional triacylglycerol lipase and LPA acyltransferase, *YEH1*/*YEH2* sterol ester hydrolase, *YJU3* monoglyceride lipase (Cherry et al., 2011).

The free fatty acids, produced during the degradation reaction of SEs and TAGs, can be esterified by fatty acid synthetases. The proteins Faa1p, Faa2p, Faa3p, Faa4p and Fat1p are responsible for the import and activation of those fatty acids (Watkins et al., 1998). To maximize the effect of acyl-CoA accumulation in the medium, the strain WRY1ΔFAA1ΔFAA4 was compared to WRY1ΔFAA1ΔFAA4Δ*fat1* (Figure 14A). The highest amount of fatty acids can be observed with the strain *S. cerevisiae* WRY1ΔFAA1ΔFAA4 p*HXT7-DGA1* p*HXT7-TGL3* (2.5 g/L ± 0.8 g/L), while the deletion of *FAT1* does not increase the maximum overall yield.

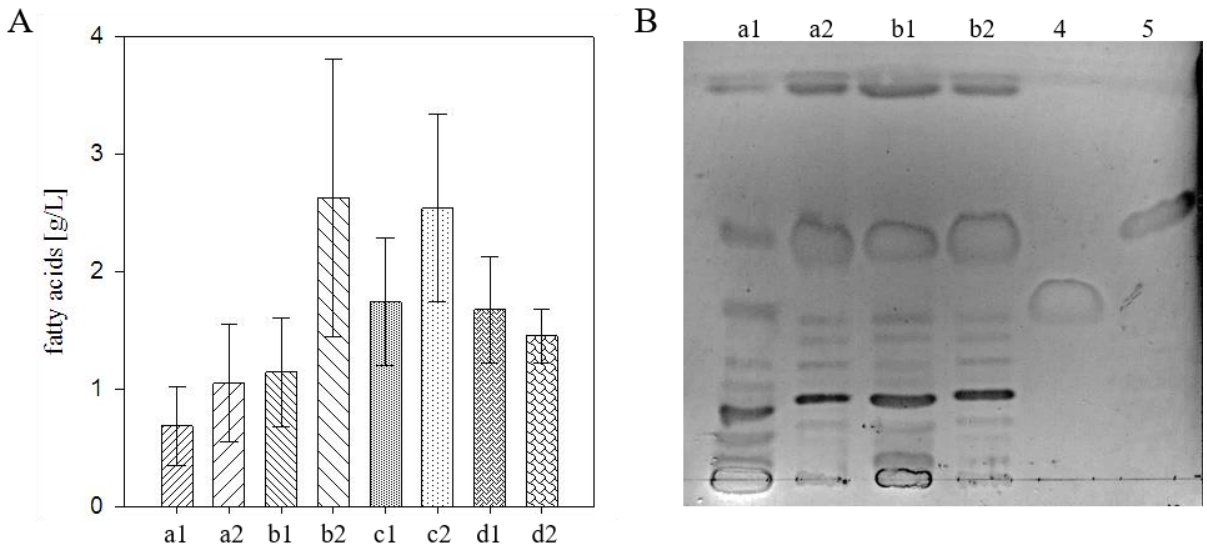

Figure 14: Fatty acid content and distribution during expression of a triacylglycerol lipase and an acyltransferase in *S. cerevisiae* BY4741 (a), *S. cerevisiae* WRY1 (b), *S. cerevisiae* WRY1ΔFAA1ΔFAA4 (c) and *S. cerevisiae* WRY1ΔFAA1ΔFAA4Δ*fat1* (d). The control strains contained the empty plasmids pRS72H and pRS72N (1). The genes *TGL3* and *DGA1* were expressed using the *HXT7* promoter (2). The fatty acid content of the supernatant and dried cell pellet after 48 h cultivation in YEPD-bacto medium was determined by gas chromatography (A). Results are mean of two (WRY1, WRY1ΔFAA1ΔFAA4, WRY1ΔFAA1ΔFAA4Δ*fat1*) and three (BY4741) independent experiment (with three technical replicates), respectively. The dried pellet was further processed for TLC to determine the lipid composition and wax ester accumulation behaviour (B). Standard substances used were oleic acid (4) and triolein (5).

In conclusion, the additional expression of *DGA1* and *TGL3* is contributing to the overall yield of fatty acids. The genetic background of the used yeast strain is critical, as it can be seen for *S. cerevisiae* WRY1ΔFAA1ΔFAA4Δ*fat1*, in which case the additional deletion of *FAT1* does not improve the yield.

3.3. Medium chain fatty acid synthesis

Fatty acid synthase (FAS) is a large enzymatic complex responsible for the entire synthesis of fatty acids in yeast. In *S. cerevisiae* FAS is composed of two subunits distributed on two genes, *FAS1* (β-chain) and *FAS2* (α-chain) (Maier et al., 2008). The two polypeptides have been object of extensive X-ray structural studies (Maier et al., 2008; Grininger, 2014), which have led to a deep understanding of the reaction mechanisms of the enzyme family (Schweizer and Bolling, 1970; Kapur et al., 2012). It was recently reported, that rational engineering of the fatty acid synthesis of bacterial FAS can change the catalytic properties and therewith chain length control (Gajewski et al., 2017a). By combining enzyme kinetics with quantitative computational modelling, molecular understanding of parameters regulating iterative synthesis in FAS was achieved (Johansson et al., 2008; Anselmi et al., 2010; Gipson et al., 2010; Gajewski et al., 2017a). The knowledge about FAS in bacteria was transferred in this work to *S. cerevisiae* FAS, in order to synthesize a broad spectrum of fatty acids by changing the catalytic properties of the enzyme. Further improvement of the outcome was done by metabolic engineering of precursors and degradation pathways. The characterization of transferases, which could be responsible for the de-esterification of CoA bound fatty acids was analysed in an additional approach.

3.3.1 Effect of FAS mutations on fatty acid synthesis

Fatty acid chain length regulation of *de novo* synthesis is typically influenced by cellular concentrations of malonyl-CoA and acetyl-CoA (Runguphan and Keasling, 2014). Increased acetyl-CoA concentrations would shift the product spectrum towards shorter acyl-CoA esters, while increased malonyl-CoA concentrations towards longer products. To bypass this regulation, FAS was manipulated in all enzymatic centres that play key roles in chain length control; the condensation domain (KS) and the transferases (MPT and AT) (Figure 15). The modifying domains (KR, DH and ER), which presumably have no direct influence in chain length regulation, were not included in engineering.

The MPT domain is responsible for malonyl-CoA loading as well as product release. For FAS loading, a CoA-bound substrate is covalently bound to the active serine of the transferase, releasing free CoA, and then transferred onto the ACP domain. The opposite direction is used for product unloading (Maier et al., 2008). It has been reported, that the MPT domain tolerates binding of acyl chains with various chain length. Due to the dual function there is a continuous competition in the MPT domain.

The amino acid arginine at position 1834 was mutated into lysine to reduce the malonyl-CoA affinity, without eliminating the whole protein activity (Table 16). The effect should be disfavouring malonyl-CoA uptake as well as favouring the competing acyl release. The AT domain is responsible for loading the FAS with acetyl-CoA. Previous studies on the hMAT-transferase reported a mutation responsible for an increased acetyl binding (Rangan and Smith, 1997; Bunkoczi et al., 2009). For this the amino acid isoleucine at position 306 was promising (Enderle et al., 2015). The ratio acetyl/malonyl would be beneficial to increase synthesis of shorter chained fatty acids. *In vitro* data support the significance of this mutation in opening a novel binding channel to host acyl chains with a length of up to C_{10} (Gajewski et al., 2017a). The KS domain catalyses the acyl chain elongation. The condensation reaction, in which the acyl intermediate is loaded into the KS, and then the malonyl decarboxylation, have to be inhibited to limit the elongation of the acyl chain. Previous structural characterization indicated a gatekeeper function of methionine at position 1251 (Johansson et al., 2008). By direct mutation the effect should be enhanced due to replacement with a bigger residue tryptophan (Table 16). The second position is glycine (at 1250), which should influence the conformational flexibility of the neighbouring gatekeeper M1251 and thereby increasing the energy barrier of its movement (Christensen et al., 2007).

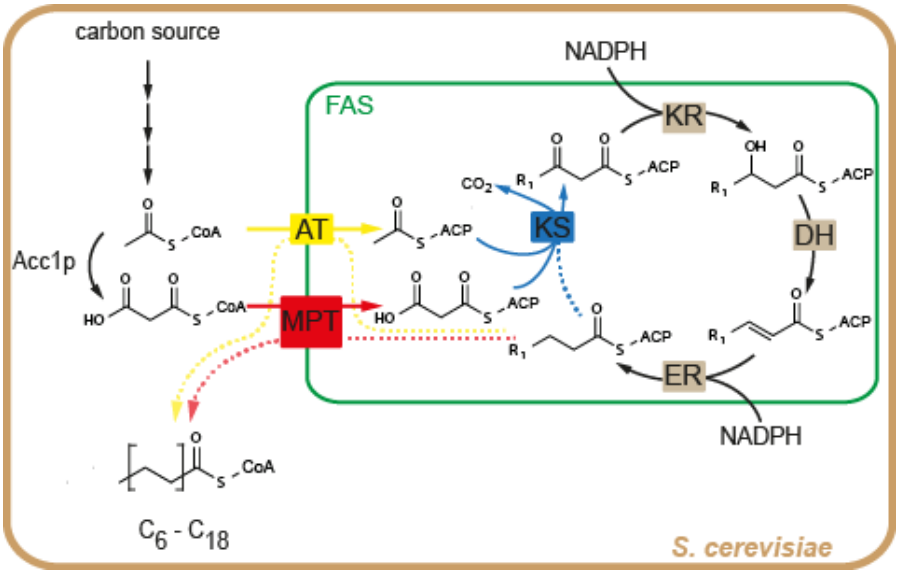

Figure 15: Fatty acid synthesis and FAS domains important for manipulation of product spectrum. Fatty acid synthesis starts with the uptake of a carbon source and its conversion to acetyl-CoA. The acetyl-CoA carboxylase (Acc1p) catalyses the carboxylation of acetyl-CoA to produce malonyl-CoA. The FAS carries modifications in the domains KS (blue), AT (yellow) and MPT (red) to produce shorter fatty acids from acetyl-CoA, malonyl-CoA and NADPH. Mutations change substrate specificities of active sites and substrate binding channels. The FAS of *S. cerevisiae* naturally produces fatty acid CoA esters (changed according to Gajewski et al. (2017b)).

A third promising position was selected based on sequence alignments with organisms known to produce hexanoic acid, such as *Aspergillus parasiticus* and *Aspergillus flavus* (Hitchman et al., 2001). The amino acid F1279 is located on the opposite side of the amino acids G1250-M1251 in the KS binding channel. It is assumed to inhibit binding of acyl chains beyond the length of six carbon atoms.

Table 16: Positions of potentially interesting amino acid exchanges to increase medium chain fatty acid synthesis. Directed mutagenesis of specific FAS domains in *S. cerevisiae*.

gene	**domain**	**position**	**substitution**	**short**
FAS1	AT	I306	Ala	IA
	MPT	R1834	Lys	RK
	AT/ MPT	I306 / R1834	Ala / Lys	IA RK
FAS2	KS	G1250	Ser	GS
		G1250 / M1251	Ser / Try	GS MW
		F1279	Tyr	FY
		F1279	Try	FW
		G1250 / F1279	Ser / Tyr	GS FY
		G1250 / M1251 / F1279	Ser / Try / Tyr	GS MW FY
		G1250 / F1279	Ser / Try	GS FW
		G1250 / M1251 / F1279	Ser / Try / Try	GS MW FW

By the use of a fatty acid synthase deletion strain, *S. cerevisiae* Δ*fas1*Δ*fas2* (Table 5) the fatty acid profile of the mutant FAS could be determined. Some of in Table 16 listed mutants were analysed for the production capacity of medium chain fatty acids in YEPD-bacto medium. For strains, which were unable to grow in YEPD-bacto medium, oleic acid was supplemented (Table 2). The combinations of *FAS1* and *FAS2* mutants were selected according to *in vitro* data (Gajewski et al., 2017a). The expression of *FAS1* and *FAS2* was performed with low copy vectors pRS315 (for *FAS1*) and pRS313 (for *FAS2*) with wildtype promoters and terminators (Figure 16).

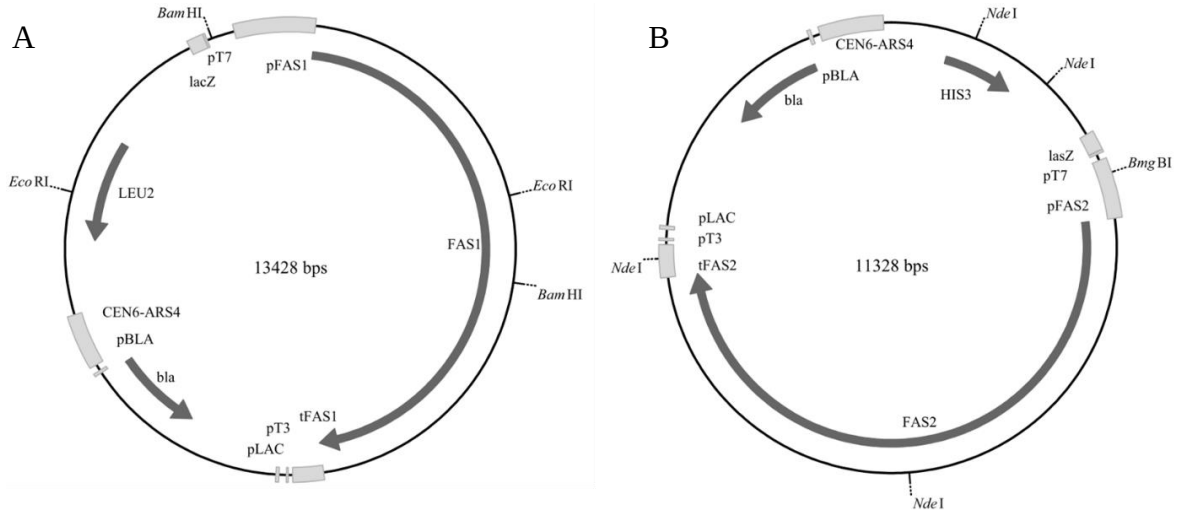

Figure 16: Plasmid map of A) pRS315-*FAS1* and B) pRS313-*FAS2*. Transcription in *S. cerevisiae* is mediated by natural gene promoters and natural terminators. Enzymes *BamH*I-*Eco*RI and *Nde*I-*BmgB*I indicate sites for verification of plasmid size after restriction. Further relevant elements: CEN6-ARS4 origin, leucine or histidine marker and ampicillin resistance gene (*bla*).

Since *S. cerevisiae* Δ*fas1*Δ*fas2* can only grow in medium with supplementation of oleic acid, no empty vector control could be measured. The control used in this study was the wildtype FAS from *S. cerevisiae* expressed under the same conditions like the mutant FAS regarding plasmids, medium, temperature etc. If not otherwise specified fatty acids were extracted from the culture medium at given time points. All strains could be cultivated in YEPD-bacto medium, except for the most strains with mutations within the KS domain at position F1279 (Figure 17). The combinations IA FY and IARK FY were able to grow in YEPD-bacto medium. The highest amount of medium chain fatty acids (C_6 - C_{12}) could be gained by using the mutant IARK GSMW (114 mg/L), which is also the best mutant for octanoic acid (78 mg/L) and decanoic acid production (22 mg/L). The mutant IARK FY has a narrower product spectrum and the synthesised octanoic acid has a maximal purity of 96 % (48 mg/L). With regard to the hexanoic acid synthesis the mutant IARK GS has the highest yield (49 mg/L), while mutant IA GS is the most specific hexanoic acid synthesising strain (88 % hexanoic acid, 17 mg/L).

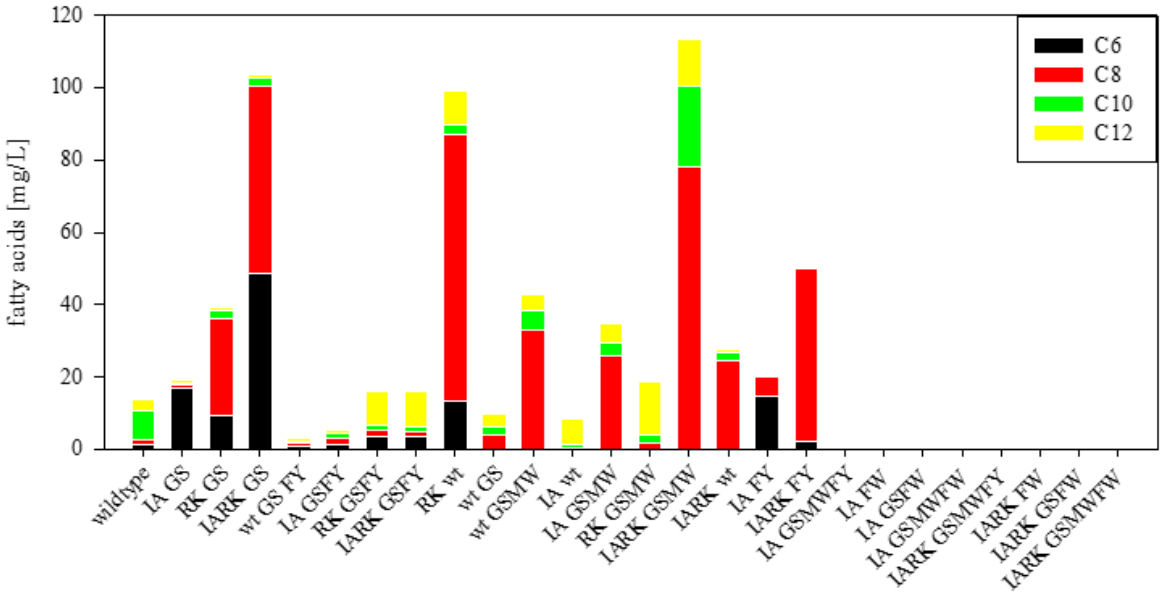

Figure 17: Medium chain fatty acid spectrum of mutated FAS in *S. cerevisiae* Δ*fas1*Δ*fas2*. The fatty acids were isolated from the YEPD-bacto medium after 48 h cultivation at 30 °C. Values are the mean of two to four biological replicates with three technical replicates for each experiment. Data are in publication from Gajewski et al. (2017b).

Since not all strains were able to grow, the supplementation of oleic acid at the beginning of the cultivation was necessary to define the product spectrum of these FAS mutants. The direct result was that all strains grew and an isolation of fatty acidy from the culture medium could be performed (Figure 18). The strains with mutations in the KS domain at position F1279 show no exceptionally high amounts of medium chain fatty acids. It is worth mentioning the drastically increased amount of dodecanoic acid in the culture medium (up to 160 mg/L) of the strains wildtype, RK wt, wt GS, wt GSMW, IA wt, IA GSMW, RK GSMW, IARK GSMW and IARK wt. The best strains for medium chain fatty acid synthesis in YEPD-bacto medium show no or very low synthesis rate in YEPD-bacto^{+}oleic acid medium.

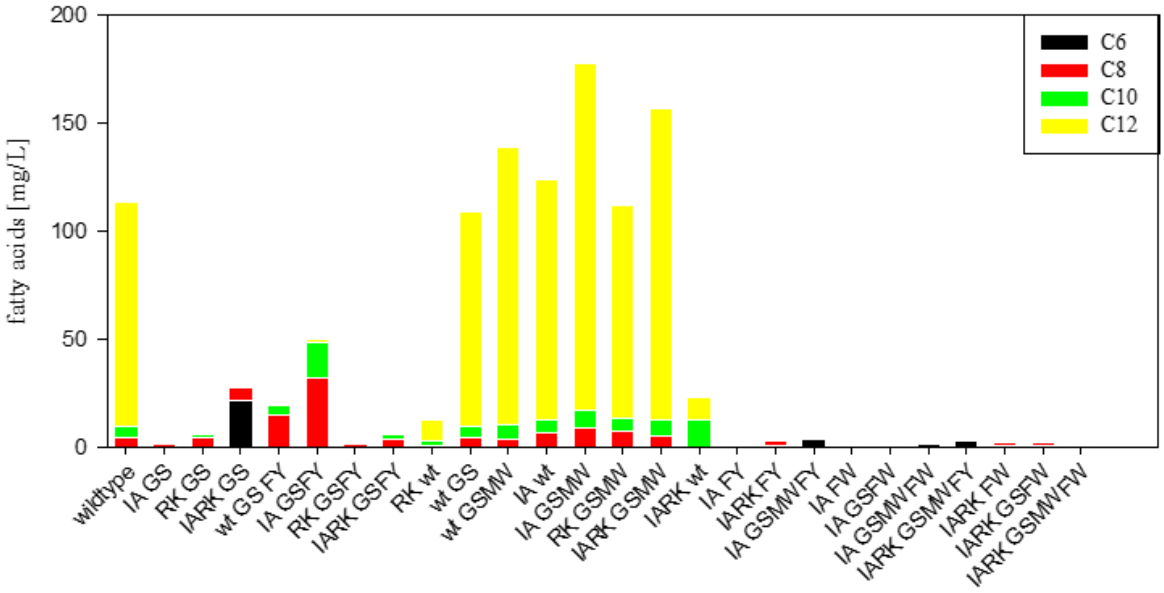

Figure 18: Fatty acid product spectrum of mutated FAS in *S. cerevisiae* Δ*fas1*Δ*fas2*. The fatty acids were isolated from the YPD-bacto medium with 0,5 mM oleic acid after 48 h cultivation at 30 °C. Values are the mean of two biological replicates.

The analysis of the growth behaviour was extended for four strains to determine the growth rate, time period in exponential phase and entering time for stationary phase (Figure 19). The testes strains RK wt, IA wt and IA GSMW show no difference compared to the FAS wildtype strain. Only mutant strain IARK GSMW has a decreased growth rate and end optical density.

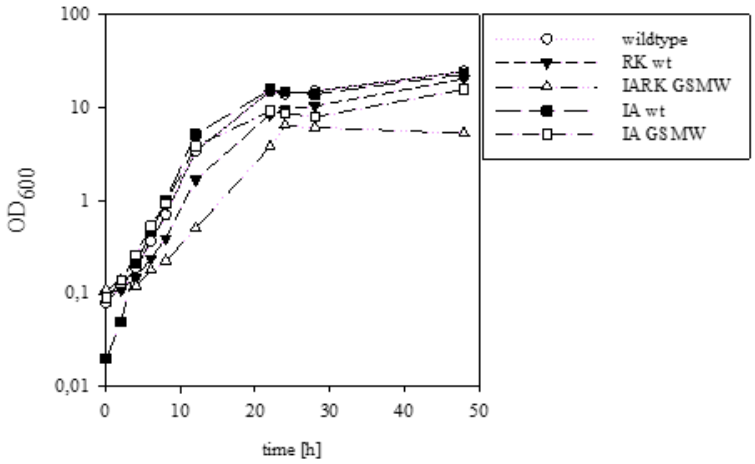

Figure 19: Growth curve of selected *S. cerevisiae* Δ*fas1*Δ*fas2* FAS mutant strains in YEPD-bacto medium. Indicated is the optical cell density at 600 nm for the wildtype FAS and the mutants RK wt, IARK GSMW, IA wt, IA GSMW. Cultivation was performed in 300 ml shaking flasks without baffles for 48 h at 30 °C with 180 rpm.

Changing the product spectrum of the *S. cerevisiae* FAS could lead to an impaired behaviour in triacylglycerol (TAG) accumulation due to changed fatty acids spectrum. TAGs are the most relevant storage form for energy and fatty acids and can be utilized for membrane biogenesis, especially during depletion of fatty acids (Sorger and Daum, 2002). The *S. cerevisiae* strains were grown in YEPD-bacto medium for 48 h. After separation of the culture medium from the cells, the cell pellet was dried and subsequently analysed by TLC. The comparison of the neutral lipid composition of wildtype FAS, RK wt, IA GS and IARK GS mutants shows no difference regarding the distribution of TAG or SE as well as various mono- and diacylglycerols (Figure 20).

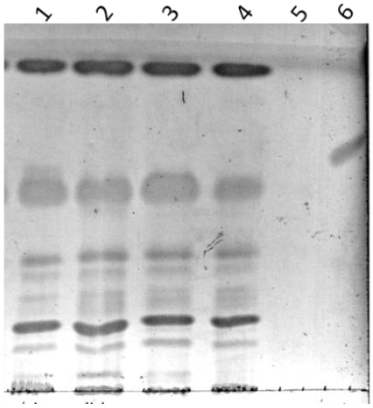

Figure 20: Lipid composition and wax ester accumulation behaviour of *S. cerevisiae* Δ*fas1*Δ*fas2* with FAS mutants. Cells were grown in YEPD-bacto medium for 48 h and 30 mg of dried cell material was analysed by TLC. 1) *S. cerevisiae* Δ*fas1*Δ*fas2* wildtype, 2) *S. cerevisiae* Δ*fas1*Δ*fas2* RK wt, 3) *S. cerevisiae* Δ*fas1*Δ*fas2* IA GS, 4) *S. cerevisiae* Δ*fas1*Δ*fas2* IARK GS, 5) heptanoic acid 7 µg 6) triolein 10 µg.

Prior studies have highlighted the toxicity of medium chain fatty acids, like octanoic and decanoic acid (Legras et al., 2010; Liu, Chernyshov, et al., 2013). Nevertheless, the sensitivity differs in various yeast strain and genomic background. Therefore a growth test with *S. cerevisiae* BY4741 was performed with supplementation of hexanoic acid and octanoic acid (Figure 21). The strain *S. cerevisiae* BY4741, parental strain of *S. cerevisiae* Δ*fas1*Δ*fas2*, was grown as a pre-culture in YEPD-bacto medium (10 ml) without octanoic and hexanoic acid supplementation. After 24 h the cells were centrifuged, washed with water and a new culture was inoculated in YEPD-bacto medium, which contained various amounts of octanoic acid and hexanoic acid. *S. cerevisiae* BY4741 shows a normal growth behaviour, if hexanoic acid is present up to a concentration of 0.5 g/L (4.3 mM). In case of octanoic acid supplementation the growth is inhibited above a concentration of 0.3 g/L (2.1 mM). Hence, for the optimization of medium chain fatty acids synthesis the aspect of toxicity should be taken into account.

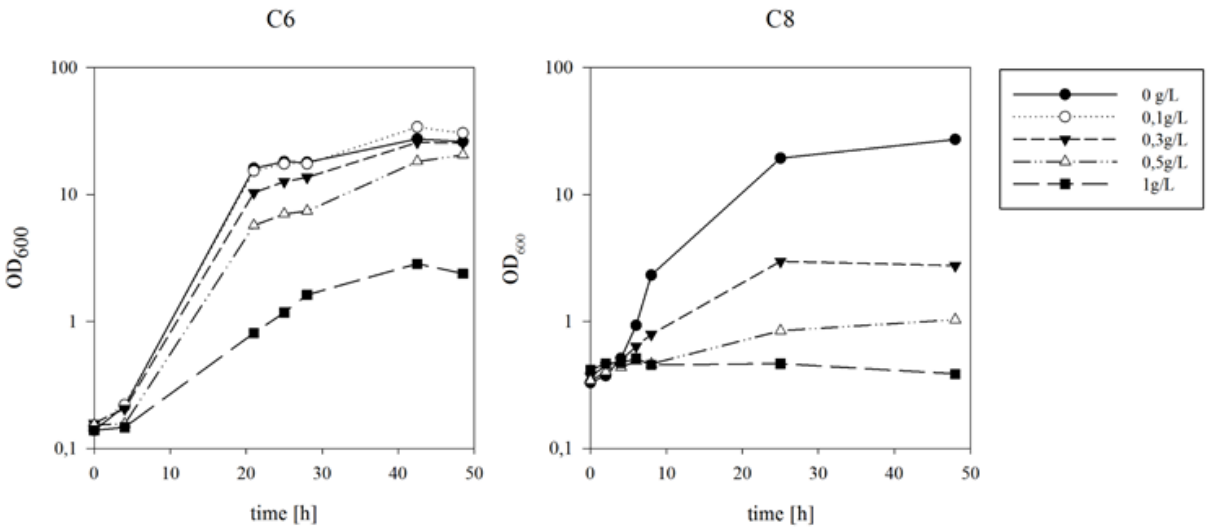

Figure 21: Growth curve of *S. cerevisiae* BY4741 in YEPD-bacto medium containing 0 g/l - 1 g/L hexanoic acid (C6) or 0 g/L - 1 g/L octanoic acid (C8). Cultivation was performed in 300 ml shaking flasks without baffles for 48 h at 30 °C with 180 rpm.

3.3.2 Effect of culture medium on fatty acid synthesis

It could be shown that the supplementation of oleic acid to the culture medium had an influence on fatty acid profile and growth behaviour of the FAS mutant harbouring strains (Figure 17 and Figure 18). Therefore, the effect of a wildtype FAS, which can synthesise oleic acid in necessary amounts, in combination with a mutant FAS in the same strain under similar gene expression conditions was tested. The expression strain was *S. cerevisiae* BY4741, with the genomic wildtype FAS. The expression of both FAS, genomic wildtype FAS and low-copy plasmid-based mutant FAS, was controlled by the wildtype promoter and terminator. Using SCD$^-$L$^-$H growth medium no difference in medium chain fatty acid synthesis between wildtype FAS and mutant FAS in *S. cerevisiae* BY4741 could be observed (Table 17). When the expression was performed in *S. cerevisiae* Δ*fas1*Δ*fas2* in SCD$^-$L$^-$H medium a significant increase in hexanoic acid (69 %) and octanoic acid (99 %) synthesis could be observed compared to the yield in *S. cerevisiae* BY4741. The highest amount of medium chain fatty acids could be obtained by using the mutant IARK GS (48.4 mg/L), which is furthermore the best mutant for hexanoic acid (4.7 mg/L) synthesis. From the overall extracted medium chain fatty acids of mutant RK wt more than 80 % is octanoic acid. With regard to the hexanoic acid synthesis 33 % of the fatty acid spectrum of the mutant IA GS is hexanoic acid.

Table 17: Medium chain fatty acid profile of *S. cerevisiae* BY4741 and *S. cerevisiae* Δ*fas1*Δ*fas2* with plasmids expressing mutated FAS determined in SCD$^-$L$^-$H medium. Cultivation was performed in 300 ml shaking flasks without baffles for 48 h at 30 °C with 180 rpm. Values are the mean of three biological replicates ± standard deviation.

SCD$^-$L$^-$H (mg/L)

***S. cerevisiae* BY4741**	**C6**	**C8**	**C10**	**C12**
wildtype	0.93 ± 0.38	0.27 ± 0.06	1.58 ± 0.43	1.22 ± 0.54
RK wt	0.77 ± 0.13	0.46 ± 0.16	0.65 ± 0.29	0.70 ± 0.61
IA GS	1.24 ± 0.20	0.63 ± 0.33	0.65 ± 0.45	1.09 ± 0.31
IARK GS	1.47 ± 0.21	0.30 ± 0.09	0.86 ± 0.58	0.85 ± 0.33
S. cerevisiae* Δ*fas1*Δ*fas2	**C6**	**C8**	**C10**	**C12**
wildtype	1.01 ± 0.28	5.04 ± 1.10	2.66 ± 1.59	4.18 ± 1.78
RK wt	1.04 ± 0.53	29.18 ± 3.16	3.52 ± 1.66	2.47 ± 0.12
IA GS	4.25 ± 1.48	2.38 ± 0.23	3.72 ± 1.15	2.16 ± 0.99
IARK GS	4.77 ± 0.80	33.13 ± 3.29	6.35 ± 1.41	4.11 ± 1.33

Another option is complex medium, where the amino acid source (yeast extract) contains a variety of different compounds and the use of peptone, which includes fats, salts, vitamins and other. The analysis using different compositions and manufacturer for YEPD medium, indicates a variation on fatty acid distribution and yield (Table 18).

Table 18: Medium chain fatty acid profile in *S. cerevisiae* Δ*fas1*Δ*fas2* in different YEPD medium. YEPD-oxoid medium contains peptone obtained from Oxoid and YEPD-bacto medium contains peptone obtained from BD. Cultivation was performed in 300 ml shaking flasks without baffles for 48 h at 30 °C with 180 rpm. Values are the mean of three biological replicates ± standard deviation.

YEPD-oxoid (mg/L)

S. cerevisiae* Δ*fas1*Δ*fas2	**C6**	**C8**	**C10**	**C12**	**OD_{600}**
wildtype	0.13 ± 0.08	0.23 ± 0.06	0.35± 0.16	0.95 ± 0.14	6.85 ± 0.59
RK	2.19 ± 0.33	19.61 ± 2.25	1.12 ± 0.58	0.11 ± 0.02	3.26 ± 0.11
IA GS	7.96 ± 0.48	0.33 ± 0.18	0.51 ± 0.10	0.51 ± 0.22	3.96 ± 0.43
IARK GS	7.55 ± 0.46	22.61 ± 0.71	3.10 ± 0.58	1.40 ± 0.59	3.09 ± 0.93

YEPD-bacto (mg/L)

S. cerevisiae* Δ*fas1*Δ*fas2	**C6**	**C8**	**C10**	**C12**	**OD_{600}**
wildtype	1.17 ± 0.09	1.72 ± 0.12	7.82 ± 1.75	3.26 ± 0.28	21.90 ± 0.14
RK	14.24 ± 1.49	78.61 ± 6.14	2.67 ± 1.33	9.46 ± 1.24	26.05 ± 0.63
IA GS	19.78 ± 0.98	1.35 ± 0.53	0.49 ± 0.13	0.86 ± 0.16	26.84 ± 0.75
IARK GS	48.65 ± 4.54	51.96 ± 11.66	2.07 ± 0.85	0.86 ± 0.42	22.18 ± 0.92

The YEPD-oxoid medium contained peptone from Oxoid and Bacto Yeast Extract- technical. The highest amount of octanoic acid was 22.6 mg/L obtained with mutant IARK GS. For YEPD-bacto medium Bacto Peptone and Bacto Yeast Extract were used. In this case, the amount of octanoic acid with the RK wt strain was 78.6 mg/L. The amount of C_6 increased by 84 % compared to YEPD-oxoid medium. Since the YEPD-bacto medium showed the best result regarding fatty acid content and benefits the growth, it will be used for all further experiments.

In summary, it can be stated that, the use of synthetic medium or YEPD-oxoid medium compared to previous used YEPD-bacto medium is not beneficial to medium chain fatty acid synthesis. For further improvement of medium chain fatty acid synthesis YEPD-bacto medium should be used.

3.3.3 Effect of promoter exchange on fatty acid synthesis

The expression of heterologous or homologous genes is a multi-stage process. Insertion of genes into an expression vector does not guarantee a high level of protein. For this reason one of the first steps in optimisation of gene expression is the promoter exchange. Most powerful promoters, like glycolytic promoters are poorly regulated. In the case of expressing toxic proteins or proteins with toxic products (like shown for the growth of *S. cerevisiae* in presence of C_6 or C_8 in Figure 21), it is the better choice to use regulated promoters. Hence, a separation of growth and expression phase can ensure optimal production conditions. Examples of promoters regulated primarily by glucose-repression are those of the *ADH2*, *SUC2* and *CYC1* genes (Romanos et al., 1992). The promoter used in this study is from the alcohol dehydrogenase II from *S. cerevisiae*. The *ADH2* promoter (p*ADH2*) is strongly repressed (over 100-fold) in the presence of glucose (Gancedo, 1998). Therefore, no inducer for expression is needed and activation is in stationary phase, when cell biomass and ethanol concentration is high and glucose not available (Lee and DaSilva, 2005).

To determine, if the p*ADH2* is suitable for the expression of mutated FAS, the glucose consumption and ethanol synthesis were observed upfront. Therewith, initially the strains with the wildtype promoter were used, to see if the ethanol synthesis is impaired or glucose consumption slow (Figure 22). Based on this results the glucose is consumed after approx. 10 - 15 h in both strains (wildtype and RK wt). The ethanol concentration in the medium increases continuously after 8 h. For the strain with the wildtype FAS decrease in ethanol concentration is initiated after 28 h and no ethanol is detectable after 48 h.

For RK wt mutant 0.5 % (wt/vol) ethanol remains in the medium even after 48 h. This leads to the conclusion, that using the p*ADH2*, the gene expression would be initiated after 8 - 15 h due to the glucose depletion. An optimal expression level would be reached, when ethanol concentration in the cells is high (approx. 20-25 h). Consequently, a longer cultivation time would be necessary to obtain the maximum amount of fatty acids, because of later induced gene expression.

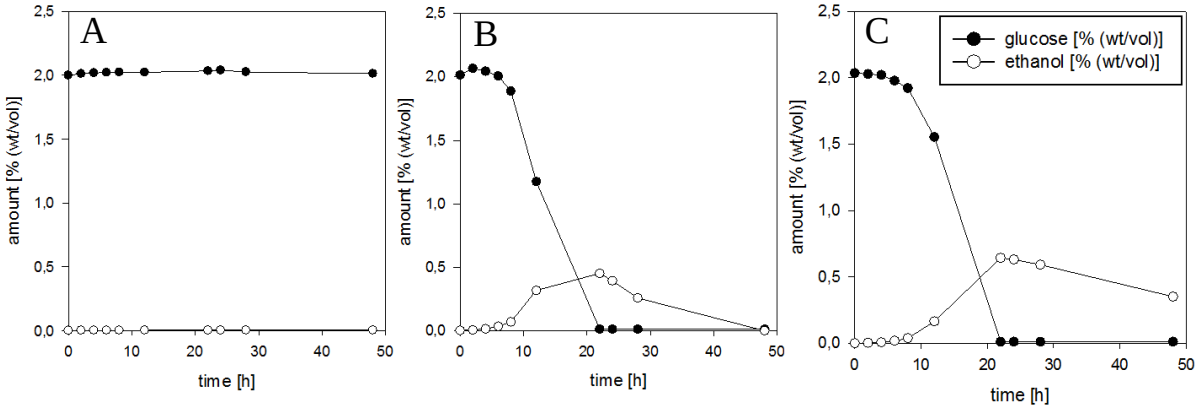

Figure 22: Glucose and ethanol concentration of *S. cerevisiae* Δ*fas1*Δ*fas2* during 48 h cultivation in YEPD-bacto medium. For the A) pure medium, B) the wildtype FAS and the C) RK wt mutant the medium was examined at several time points. The amount of remaining glucose and ethanol in the culture medium was measured by HPLC. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. The shown data are results from one experiment with three technical replicates.

In order to obtain the best point in time to extract the fatty acids from the medium, growth experiments with subsequent fatty acids extractions were performed for 48 h, 72 h and 96 h (Figure 23). In case of p*ADH2* derived expression of the mutated FAS, the yield of medium chain fatty acids in the medium was lower after 48 h compared to use of the wildtype promoter. The amount of synthesised octanoic acid by mutant RK wt was 9-fold less after 48 h of cultivation. In consequence of using the p*ADH2*, the highest amount of octanoic acid (176 mg/L) was reached after 72 h of cultivation. The overall maximal yield of 285 mg/L medium chain fatty acids was obtained with RK wt mutant after 72 h cultivation. In case of synthesis of hexanoic acid using mutant IA GS, the highest amount could be measured after 96 h, while the other medium chain fatty acids decrease in the medium.

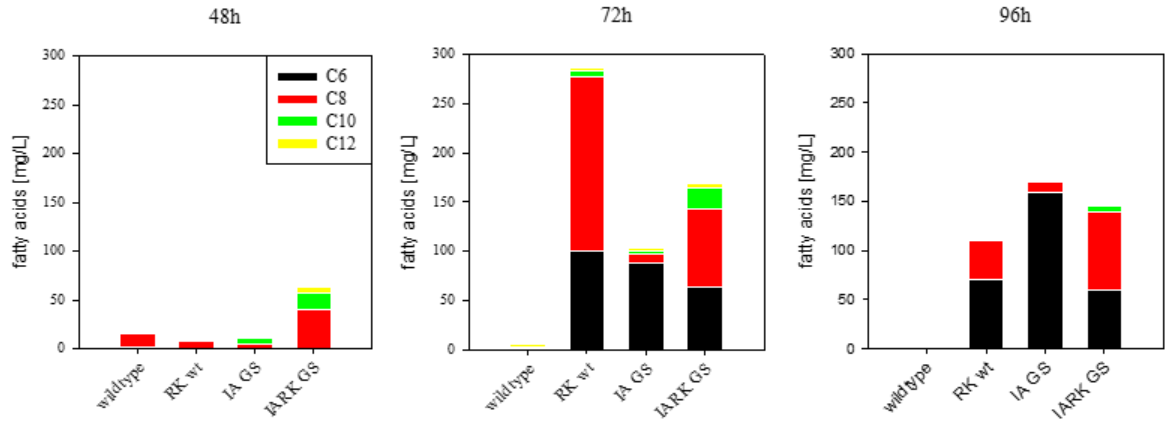

Figure 23: Medium chain fatty acid profile after 48 h, 72 h and 96 h of *S. cerevisiae* Δ*fas1*Δ*fas2* with *ADH2* promoter mediated expression of mutated FAS (RK wt, IA GS, IARK GS) in YEPD-bacto medium. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates. The relative standard deviation for all measurements after 48 h is below 10 %, for 72 h below 8 % and for 96 h below 3 %.

The distribution of LCFA (C_{16} - C_{18}) in the culture medium was measured at the same time points (Figure 24). For the strain with the wildtype FAS a maximal overall yield of 165 mg/L after 48 h of cultivation is detectable. The amount of LCFA decreases over time to 26 mg/L in the culture medium after 96 h. The same decrease can be observed for all three tested mutant FAS. However, the concentration of LCFA after 48 h is drastically lower (44 %) compared to the wildtype and decreases to a minimum of 23 mg/L after 96 h in case of mutant RK wt.

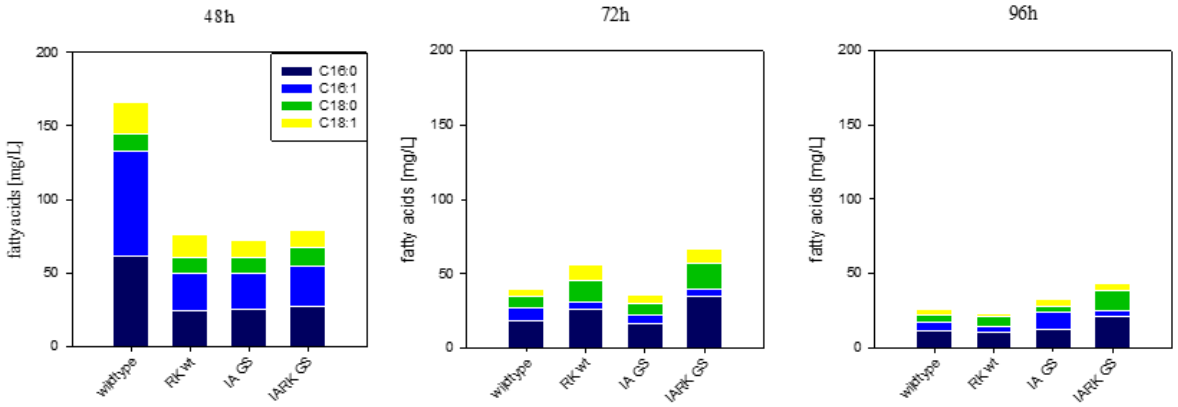

Figure 24: LCFA profile after 48 h, 72 h and 96 h of *S. cerevisiae* Δ*fas1*Δ*fas2* with *ADH2* promoter mediated expression of mutated FAS (RK wt, IA GS, IARK GS) in YEPD-bacto medium. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates. The relative standard deviation for all measurements after 48 h is below 12 %, for 72 h below 15 % and for 96 h below 9 %.

Possibly, due to medium chain fatty acid synthesis a drop in the pH of the culture medium could be observed for all mutant FAS harbouring strains (Table 19). The growth of the mutant strains was negatively affected compared to the wildtype strain. To avoid pH reduction potassium phosphate buffered medium was used and the pH was adjusted to 6.5 at the beginning of the cultivation process.

Table 19: PH values of fermentations with *S. cerevisiae* Δ*fas1*Δ*fas2* and *ADH2* promoted expression of FAS mutants in YEPD-bacto medium after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates.

S. cerevisiae* Δ*fas1*Δ*fas2	**OD_{600}**	**pH**
wild type	21.55 ± 3.25	6.30 ± 0.10
RKwt	11.10 ± 0.21	5.68 ± 0.13
IA GS	11.35 ± 0.71	6.28 ± 0.04
IARK GS	6.35 ± 0.07	5.97 ± 0.04

For cultivations with 100 mM buffered medium an increase in fatty acid content in all tested mutant strains could be observed (Figure 25, 100 mM). Especially, mutant IARK GS has a 2.6-fold and 3-fold increase in octanoic acid and hexanoic acid, respectively. The pH reduction remains the same as in not buffered YEPD-bacto medium. The highest amount of octanoic acid could be obtained with mutant RK wt (223 mg/L), where the pH difference compared to not buffered medium is the highest. The cultivation of *S. cerevisiae* in 250 mM potassium phosphate buffered medium shows a slight increase in fatty acids in the medium (Figure 25, 250 mM). The maximal yield of medium chain fatty acids increases from 430 mg/L to 464 mg/L for the IARK GS mutant. The amount of octanoic acid could be increased by 13 %.

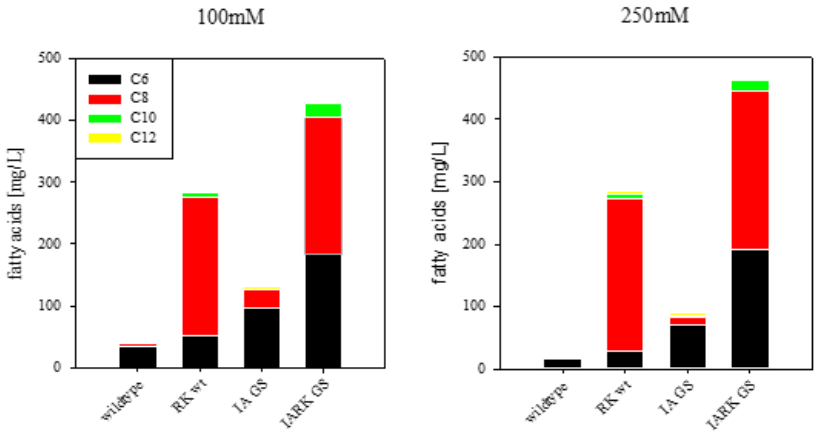

Figure 25: Medium chain fatty acid profile after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2* and *ADH2* promoter for the expression of mutated FAS in 100 mM and 250 mM potassium phosphate buffered YEPD-bacto medium. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates. The relative standard deviation for all measurements for 100 mM is below 4 % and for 250 mM below 7 %.

When regarding the questions of the pH, it partly becomes clear that the pH of the culture medium did not substantially decrease from the initial pH adjusted at the beginning of the cultivation (Table 20). Moreover, the use of 250 mM potassium phosphate buffered medium lead to a cultivation pH comparable with the wildtype. The growth was not positively affected by constant pH.

Table 20: PH values and optical density of fermentations with *S. cerevisiae* Δ*fas1*Δ*fas2* and *ADH2* promoted expression of FAS mutants in buffered YEPD-bacto medium (pH 6,5) after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates with standard deviation.

S. cerevisiae	**100 mM**		**250 mM**	
Δ*fas1*Δ*fas2*	**OD_{600}**	**pH**	**OD_{600}**	**pH**
wild type	21.05 ± 2.83	6.39 ± 0.01	16.77 ± 0.25	6.34 ± 0.02
RKwt	10.93 ± 0.18	6.29 ± 0.01	11.15 ± 0.13	6.36 ± 0.01
IA GS	10.20 ± 0.07	6.41 ± 0.01	7.82 ± 0.13	6.35 ± 0.01
IARK GS	9.38 ± 0.60	6.03 ± 0.04	6.33 ± 0.41	6.27 ± 0.02

Changing the carbon source from glucose to ethanol, the p*ADH2* should lead to an immediate expression of the FAS. No positive effect could be observed, as the culture grew poorly and to no high optical density, as observed with glucose (Figure 26). The amount of medium chain fatty acids could not be radically improved. Moreover, for the IARK GS mutant a significant decrease could be observed. Only mutant IA GS indicates a 22 % improvement of hexanoic acid synthesis after 72 h compared to buffered YEPD-bacto (100 mM). The overall amount of LCFA does not differ between wildtype, RK wt and IA GS mutant. The mutant IARK GS has a higher amount of stearic acid and oleic acid compared to the wildtype. An increase in oleic acid content cannot be observed for the other mutants.

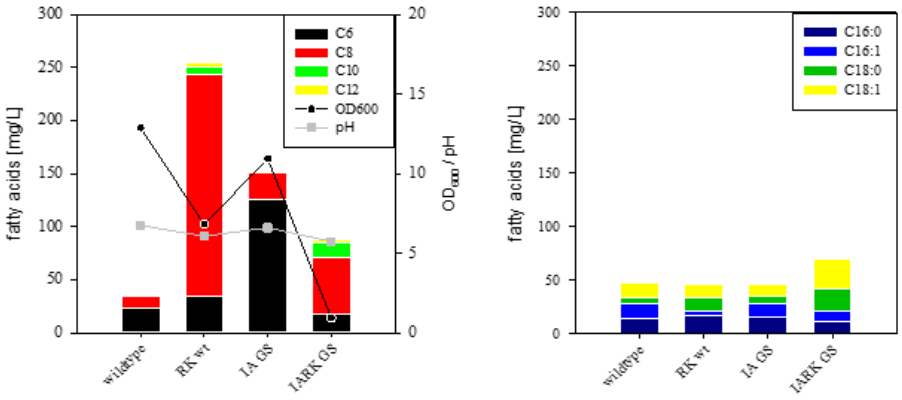

Figure 26:Fatty acid profile in supernatant with optical density and pH value of MCFA and LCFA in YEPE-bacto medium after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2* and *ADH2* promoter. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates. The relative standard deviation for all measurements is below 12 %.

In summary, it can be stated that, p*ADH2* mediates improved medium chain fatty acid synthesis after 72 h incubation. Shortening or prolonging incubation time (48 h or 96 h) is not generally beneficial for product yield. Due to pH reduction in YEPD-bacto medium addition of potassium phosphate could positively influence the fatty acid synthesis by stabilizing the pH of the culture medium. An overall yield of 254 mg/L medium chain fatty acids was obtained with RK wt mutant after 72 h of cultivation in YEPE-bacto medium.

3.3.4 Optimization of MCFA synthesis by metabolic engineering

In this section the focus is on the development of an improved MCFA production system in *S. cerevisiae* by metabolic engineering.

3.3.4.1 Deletion of acyl-CoA synthetase Faa2p

For the utilisation of free fatty acids *S. cerevisiae* needs acyl-CoA synthetases. Acyl-CoA synthetases esterify free fatty acids to acyl-CoA esters (Black and DiRusso, 2007). There are six described acyl-CoA synthetases in *S. cerevisiae* encoded by *FAA1*, *FAA2*, *FAA3*, *FAA4*, *FAT1* and *FAT2* (Black and DiRusso, 2007). The medium chain fatty acyl-CoA synthetase Faa2p is localized in peroxisomes, is required for β-oxidation and activates imported fatty acids within a range of C_7 - C_{17} (Black and DiRusso, 2007).

To increase the amount of hexanoic acid and octanoic acid, the *FAA2* gene was deleted. The resulting strain *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* (Table 6) was tested in 100 mM potassium phosphate buffered YEPD-bacto medium (Figure 27). The mutant RK wt is able to produce 301 mg/L octanoic acid, 25 % more than with unchanged *FAA2*. The amount of decanoic acid and dodecanoic acid have significantly increased for all mutants. Mutant IA GS has synthesised 94 % more decanoic acid, while mutant IARK GS has a 61 % increase compared with yield in *S. cerevisiae* Δ*fas1*Δ*fas2*. While there is no substantial difference between the pH of the tested strains, the optical density is significantly different. The wildtype strain grows, as shown before, to the highest optical density, while the IA GS mutant has a 74 % lower growth. Nonetheless, the IA GS mutant has a higher yield in medium chain fatty acids.

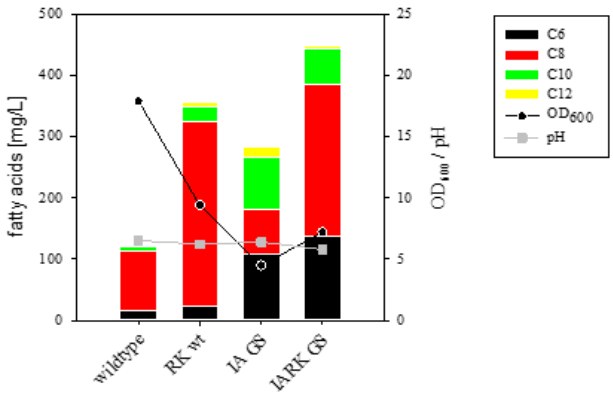

Figure 27: Fatty acid profile after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* and *ADH2* promoter for the expression of mutated FAS in 100 mM potassium phosphate buffered YEPD-bacto medium. The optical density (OD_{600}) and the pH are measured at the end of the cultivation. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates. The relative standard deviation for all measurements is below 9 %.

The presence of medium chain fatty acids in the culture medium can have a negative influence on the growth of yeast cells, like shown for the *S. cerevisiae* BY4741 in Figure 21. To evaluate the impact for the *FAS1*, *FAS2* and *FAA2* deletions a growth test with different concentrations of octanoic acid in the culture medium was performed (Figure 28). The growth difference between the tested strains *S. cerevisiae* Δ*fas1*Δ*fas2* and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* is visible even without addition of octanoic acid.

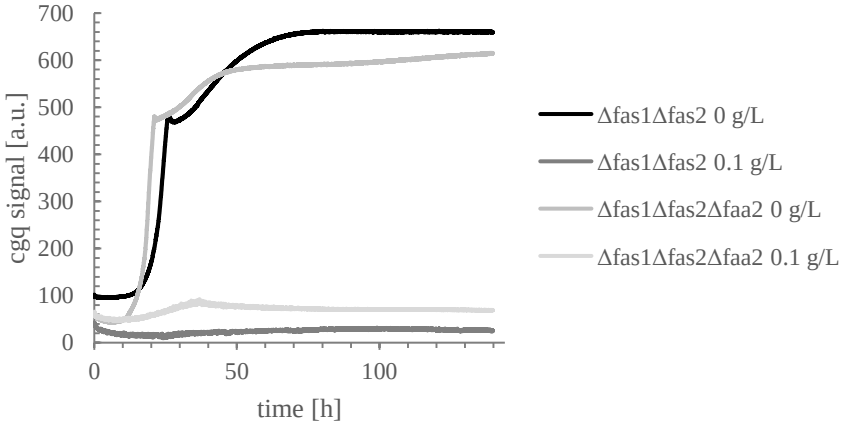

Figure 28: Growth curve of *S. cerevisiae* Δ*fas1*Δ*fas2* and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* in YEPD-bacto medium containing 0,5 mM oleic acid with addition of 0 g/L and 0,1 g/L octanoic acid, respectively. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm and monitored with a cell growth quantifier (Bruder et al., 2016).

S. cerevisiae Δ*fas1*Δ*fas2*Δ*faa2* has a higher growth rate and enters the stationary phase after 48 h, while the corresponding control strain enters the stationary phase after 60 h. The end optical density after 140 h (< 6 days) differs in 3.6 %. The addition of 0.1 g/L octanoic acid leads to a low growth rate for both strains, while *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* shows a better ability to handle the added amount of octanoic acid. This is reflected in a difference of 62 % in the end optical density. The sensitivity towards octanoic acid decreased, since the cells are able to grow to a higher optical cell density compared to the wildtype.
In summary, the deletion of *FAA2* improves the yield of MCFA, with a maximal outcome of 301 mg/L octanoic acid with RK wt mutant. The *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* strain shows in general a better ability to handle the presence of octanoic acid.

3.3.4.2. Degradation of triacylglycerol

S. cerevisiae forms non-essential lipid droplets (LD), which serve as a storage compartment (Czabany et al., 2008). LDs consist of 95 % non-polar lipids and are composed of roughly 50 % triacylglycerols (TAG) and 50 % sterol esters (SE) (Sandager et al., 2002; Koch et al., 2014). The main pathway for the formation of TAG in *S. cerevisiae* is the acyl-CoA-dependent esterification of DAG by Dga1p and Are1p (Wagner and Paltauf, 1994). Due to its low biological toxicity compared to free fatty acids (FFA), TAG provides an appropriate form of fatty acid storage (Daum and Paltauf, 1980; Leber et al., 1994). Unusual fatty acids that might have a toxic effect on the cell when incorporated into membranes, can be stored in TAG and are excluded from membrane formation (Lehner and Kuksis, 1996; Sorger and Daum, 2003). Dga1p has been identified as one of the primary proteins involved in TAG formation and has been overexpressed in yeast hosts contributing to an increase in intracellular and extracellular fatty acid levels (Runguphan and Keasling, 2014). Lipases, which are required for TAG and SE conversion to FFAs, are abundant in yeast. Among over fourteen enzymes currently identified, Tgl3 is the primary TAG lipase (Athenstaedt and Daum, 2005). Leber et al. (2015) could enhance carbon flux into neutral lipid droplet formation and degradation by overexpressing *DGA1* and *TGL3*, to increase extracellular FFAs. To imitate the effect of *DGA1* and *TGL3* expression shown by Leber et al. (2015) and increase extracellular FFAs, the strain *S. cerevisiae* BY4741 was used for expression and the neutral lipid distribution was analysed by TLC (Figure 29).

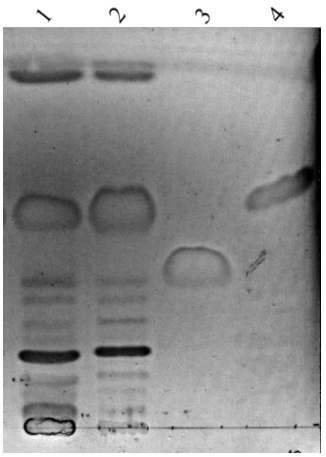

Figure 29: Wax ester accumulation behaviour of *S. cerevisiae* BY4741 expressing *DGA1* and *TGL3* grown in YEPD-bacto medium for 72 h analysed by TLC. 1) *S. cerevisiae* BY4741 pRS72N pRS72H, 2) *S. cerevisiae* BY4741 pRS72N-*DGA1* pRS72H-*TGL3*, 3) oleic acid (18µg), 4) triolein 18 µg.

The results from the TLC point out, that the amount of TAG is higher than in the empty vector harbouring strain. In case of using *S. cerevisiae* Δ*fas1*Δ*fas2* with FAS mutant expression via p*ADH2*, the accumulation and subsequent degradation effect of TAGs via *DGA1* and *TGL3* expression was directly analysed by GC (Figure 30). The enzymes *TGL3* and *DGA1* were expressed using the *HXT7* promoter with a 2 µ plasmid. For all strains, including the wildtype, a decreased growth can be observed after 72 h in YEPD-bacto medium. The presence of empty plasmids pRS72H and pRS72N cause a decrease of octanoic acid with RK wt mutant by 68 % compared to the approach using *S. cerevisiae* Δ*fas1*Δ*fas2* with FAS mutant expression via p*ADH2* (Figure 23). Mutant IARK GS has an overall decrease of 93 % of medium chain fatty acids. The pH in the empty plasmid approach of the wildtype strain is at 6.4, while all mutants have a pH of approx. 5.5 after 72 h of cultivation. Through *DGA1* and *TGL3* expression no major difference in pH can be observed, when compared to the empty plasmid approach. For the cell density the negative effect of *DGA1* and *TGL3* expression is evident for the wildtype (-21 %) and the RK wt (-26 %) mutant. Mutants IA GS and IARK GS have a slight increase of 25 % and 16 % respectively in cell density while expressing *DGA1* and *TGL3*.

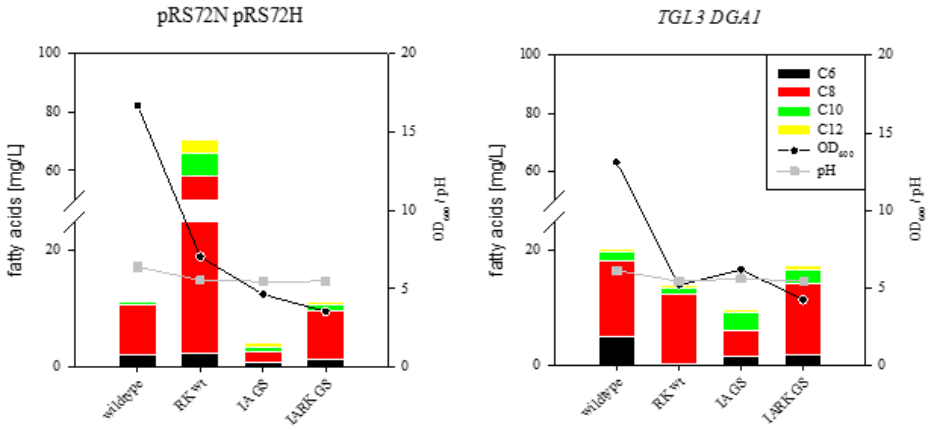

Figure 30: Expression of triacylglycerol lipase (*TGL3*) and diacylglycerol acyltransferase (*DGA1*) in combination with mutated FAS in *S. cerevisiae* Δ*fas1*Δ*fas2*. Fatty acid synthases were expressed by *ADH2* promoter, while *DGA1* and *TGL3* were expressed with *HXT7* promoter. The fatty acid pattern of the supernatant after 72 h cultivation in YEPD-bacto medium was determined by gas chromatography. The control strain contained the empty plasmids pRS72H and pRS72N. The optical cell density and pH were measured after 72 h cultivation. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Results are mean of two independent experiment with three technical replicates for each experiment. The break line starts at 25 mg/L and ends at 50 mg/L.

The effect on LCFAs, ranging from C_{16} to C_{18}, for the wildtype does not change with *DGA1* and *TGL3* expression (Figure 31).

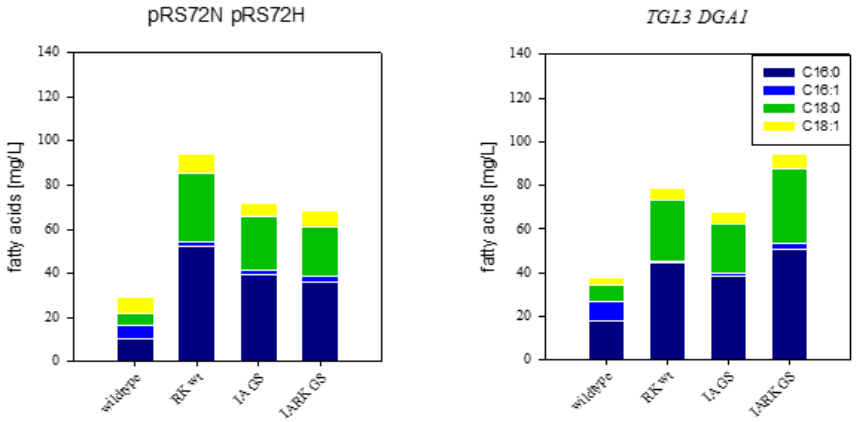

Figure 31: LCFA profile in supernatant of *S. cerevisiae* Δ*fas1*Δ*fas2* during expression of triacylglycerol lipase (*TGL3*) and diacylglycerol acyltransferase (*DGA1*) in combination with mutated FAS. Fatty acid synthases were expressed by *ADH2* promoter, while *DGA1* and *TGL3* were expressed with *HXT7* promoter. The fatty acid pattern of the supernatant after 72 h cultivation in YEPD-bacto medium was determined by gas chromatography. The control strain contained the empty plasmids pRS72H and pRS72N. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Results are mean of two independent experiment with three technical replicates for each experiment.

S. cerevisiae Δ*fas1*Δ*fas2* IARK GS shows a slight increase in total LCFA yield in the supernatant compared to the empty plasmid approach. The amount of saturated fatty acids, palmitic acid and stearic acid, is in all mutant strains significantly higher than in the wildtype strain. To verify, if MCFAs are incorporated into TAGs the pellet fraction of the cells has to be analysed (Figure 32).

In case of the wildtype strain expressing the genes for the triacylglycerol lipase and diacylglycerol acyltransferase, a positive effect can be observed for decanoic acid and dodecanoic acid. No increased hexanoic acid or octanoic acid can be found in the pellet fraction of the FAS mutant strains.

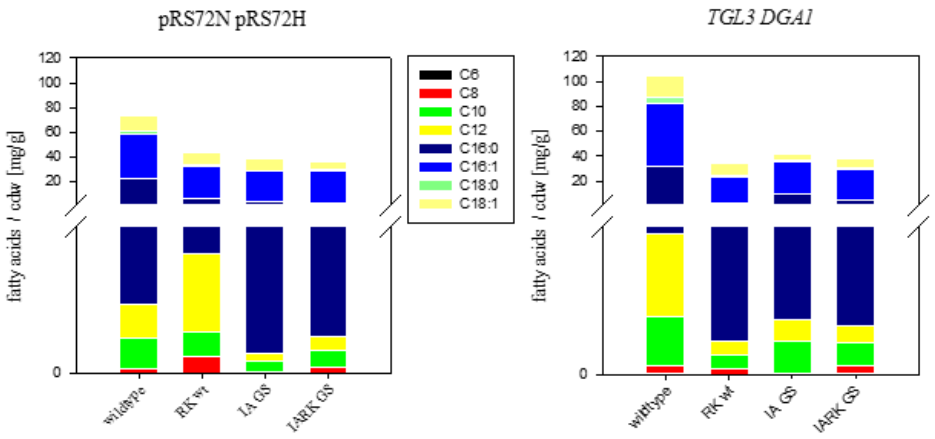

Figure 32: The fatty acid pattern of the freeze dried pellet fraction after 72 h cultivation in YEPD-bacto medium during expression of triacylglycerol lipase Tgl3p and diacylglycerol acyltransferase Dga1p in combination with mutated FAS in *S. cerevisiae* Δ*fas1*Δ*fas2*. Fatty acid synthases were expressed by *ADH2* promoter, while *DGA1* and *TGL3* were expressed with *HXT7* promoter. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. The fatty acid pattern was determined by gas chromatography. The control strain contained the empty plasmids pRS72H and pRS72N. Results are mean of two independent experiment with three technical replicates for each experiment. The break lines start and end at 1 mg/g.

Given these points, no beneficial effect for all strains of Dga1p and Tgl3p for the production of MCFA can be observed. With regard to the saturated LCFA outcome in the supernatant a positive effect can be noticed.

3.3.5 Extraction of MCFA from medium with ion exchanger

Biotechnological formation of industrially interesting compounds, like different fatty acids often has limited productivity. Reasons for this can be various, among them toxicity of the product for the producing microorganism. Hence, one way to bypass this issue, is to keep the concentration of the particular fatty acids in the culture medium low. Thereby the cells would not be exposed to toxic concentrations, process membrane rearrangement and increase activity of the β-oxidation. A similar compound regarding the toxicity is perillic acid. Perillic acid, present in *Perilla frutescens*, has anticancer and antimicrobial activity. The chemical synthesis is an environmentally challenging process (Wang et al., 1993), while the biocatalytic synthesis using a solvent tolerant *Pseudomonas putida* DSM 12264 is an efficient and ecological method (Berger, 2007). Mars et al. (2001) could gain 11 g/L perillic acid by biotransformation of limonene after about six days of fed-batch cultivation.

For further improvement of productivity, a continuous removal of perillic acid, selectively adsorbed on an anion exchange resin, could reduce product inhibition effects. Various anion exchange resins were tested, with Amberlite IRA 410 Cl (1,39 mmol perillic acid/g resin) being best suitable for selective absorption of perillic acid. Amberlite IRA 410 Cl is a strongly basic anion exchange resin (Type II anion exchanger), with a pale yellow translucent bead-gel structure (Zagorodni, 2006). It is a styrene divinylbenzene copolymer (with a $N^{+}(CH_3)_2C_2H_4OH$ functional group) and has a good regeneration efficiency and excellent rinse performance. The best elution system for perillic acid was composed of ethanol and 1 M HCl (60:40 v/v). Since octanoic acid and perillic acid have a similar pK_S of 4.89 and 4.94, respectively, at pH 7 both are completely dissociated. Therefore, both can be counted into weak up to medium-strong acids. To demonstrate the octanoic acid binding capacity of amberlite, an experiment including different pH and amount of amberlite was performed (Figure 33). The pH values (3, 5 and 6.5) were selected according to previous results with the medium chain fatty acids producing strains. Due to the fact that the pH decreases over time, it is important to comprise the whole range of possible pH during cultivation, to obtain optimal binding conditions. Since the experiment design differs to Mars et al. (2001), where an ISPR (*in situ* product removal) method for binding of perillic acid in bypass to the fed-batch fermenter was used, the amount of amberlite was added directly to the culture medium. When using amberlite to determine the octanoic acid binding capacity, the concentration was set to 200 mg/L. Due to the fact, that the best elution system for perillic acid was a solution of ethanol and 1 M HCl (60:40 v/v), the identical was applied for octanoic acid. Figure 33 shows the best binding conditions are at pH 6.5 using 3 g amberlite, when using the given elution system. The difference between 1 g amberlite and 3 g amberlite is at +15 % for pH 6.5 and at +30 % for pH 3, respectively.

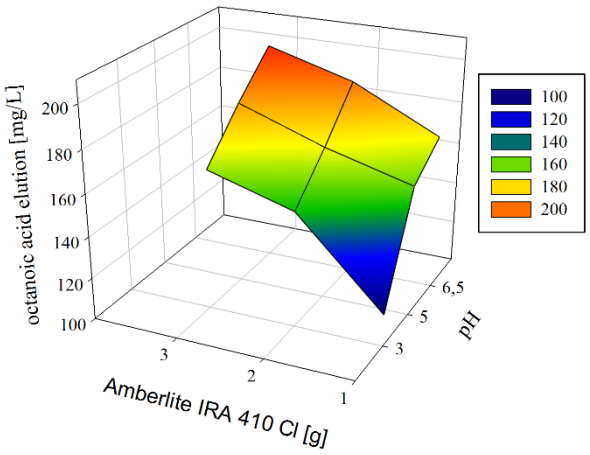

Figure 33: Amberlite IRA 410 Cl binding capacity for octanoic acid. 30 ml of YEPD-bacto medium, adjusted to pH 3, 5 and 6.5 were incubated for 16 h with 1 g, 2 g and 3 g Amberlite IRA 410 Cl with 200 mg/L octanoic acid. The incubation was performed at room temperature and 80 rpm. Elution was performed for 16 h with ethanol and 1 M HCl (60:40 v/v). Results show values of one experiment, with two technical replicates.

With this in mind, Amberlite was used in a cultivation of *S. cerevisiae* Δ*fas1*Δ*fas2* with p*ADH2* regulated expression of mutated FAS (Figure 34). The used YEPD-bacto medium was adjusted to pH 6.5. Due to the low difference of binding capacity between 1 g and 3 g Amberlite IRA 410 Cl the lower amount was added at the beginning of the cultivation. After 72 h the amberlite was separated from the cells and the supernatant, for elution and subsequent gas chromatographic analysis. The yield of MCFAs in the supernatant was significantly lower compared to the cultivation without amberlite (Figure 23). For mutant RK wt the amount of octanoic acid decreased by 31 % and for hexanoic acid by 66 %. The most pronounced difference for decanoic acid could be observed for mutant IARK GS, with 67 % less outcome. At the same time mutant IARK GS has a 50 % and 31 % increased hexanoic acid and octanoic acid yield, respectively. The analysis of the amberlite elution fraction indicates the preferred binding of longer MCFA (decanoic acid and dodecanoic acid). With regard to the overall yield mutant RK wt has a 16 % increased outcome, with 75 % more decanoic acid and 95 % dodecanoic acid compared to the cultivation without amberlite (Figure 23). The least difference can be observed for mutant IA GS, while mutant IARK GS has a 2.7-fold overall increase. The pH of all cultivations was not unusually differing (< 8 %), although the cell density was slightly higher compared to growth without amberlite. Only for the strain with the wildtype FAS a decrease by 13 % in cell density can be observed. Mutant IARK GS has an 1.8-fold increase in cell density at the end of the cultivation.

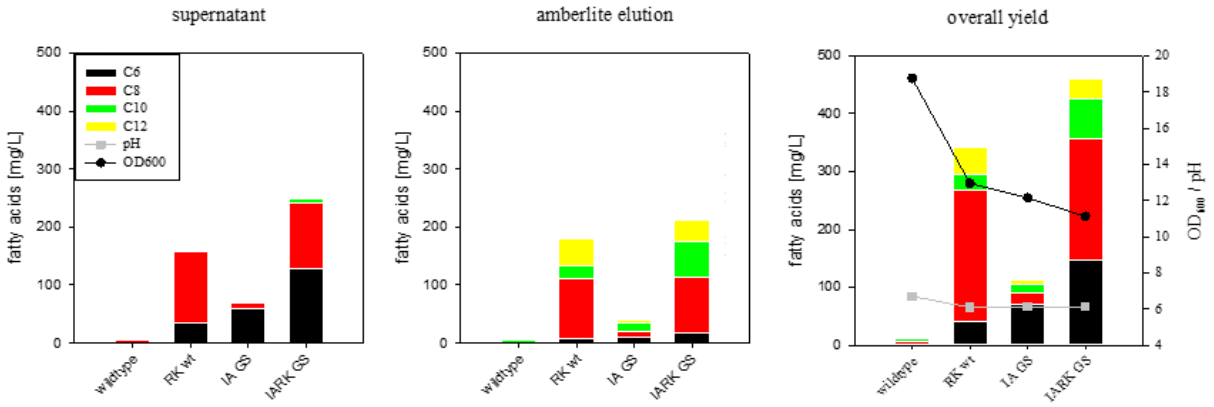

Figure 34: Medium chain fatty acid distribution of *S. cerevisiae* Δ*fas1*Δ*fas2* and p*ADH2* regulated expression of mutated FAS in YEPD-bacto medium (pH 6.5) containing 1 g Amberlite IRA 410 Cl. Analysis of supernatant and elution fraction of Amberlite was performed after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of two biological replicates.

For the purpose of evaluating Amberlite IRA 410 Cl as a potential ion exchange resin to be used in an industrial scale, the effect of other available fatty acids, like palmitic acid should be considered. The amount of LCFAs bound to Amberlite is compared to the amount present in the supernatant very high (Figure 35). For all tested strains more than 80 % of the overall yield of LCFAs is bound to Amberlite.

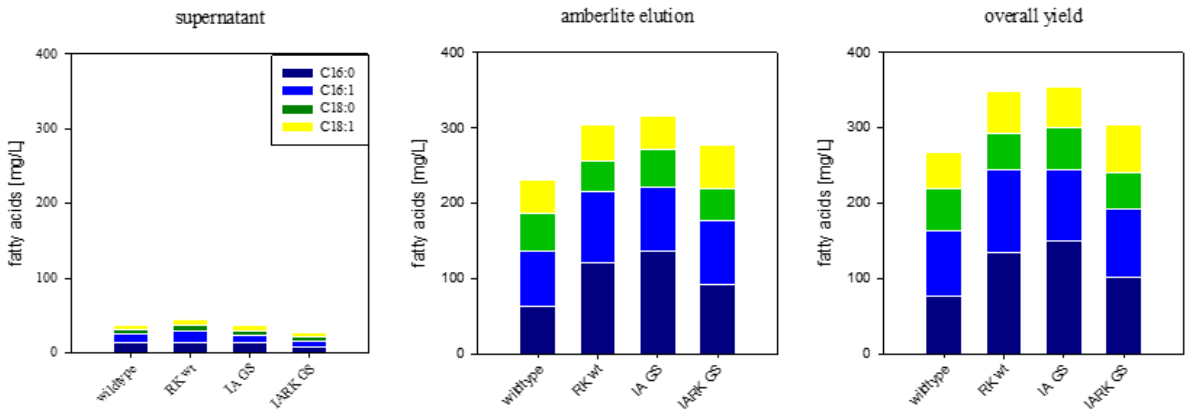

Figure 35: LCFA distribution of *S. cerevisiae* Δ*fas1*Δ*fas2* and *ADH2* regulated expression of mutated FAS in YEPD-bacto medium (pH 6.5) containing 1 g Amberlite IRA 410 Cl. Analysis of supernatant and elution fraction of amberlite was performed after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of two biological experiments with three technical replicates for each experiment.

Altogether the application of Amberlite IRA 410 Cl for the extraction of MCFA is a viable approach. The presence of LCFA decreases the efficiency, due to using up binding positions on the anion exchange resin. In addition, a negative effect of the resin, that can act as glass beads for the cells, cannot be excluded.

3.3.6. Analysis of putative hydrolases for MCFA production

Hydrolases catalyse the hydrolysis of a chemical bond (Shah et al., 2012). Different members of this enzyme family can act on ester bonds. The acyl-CoA hydrolase (EC 3.1.2.20) is an enzyme that catalyses the chemical reaction from acyl-CoA with water to CoA and a carboxylate, thereby specifically acting on thioester bonds (Alexson et al., 1989). A more common name is acyl-CoA thioesterase. The enzyme can be localized in different cellular compartments such as ER, peroxisomes and cytosol. Maintaining correct levels of acyl-CoA, CoA-SH, and free fatty acids is source of speculation for regulation of lipid metabolism (Hunt and Alexson, 2002). The availability of various acyl-CoA thioesterases having activities toward a narrow or broad range of substrates could provide important checkpoints in the oxidation of medium chain fatty acids. For *S. cerevisiae*, several thioesterases were suggested to hydrolyse the medium CoA esters produced by the fatty acids synthase to free fatty acid. This is relevant in strain engineering, as thioesterases might interfere in chain length regulation by selective hydrolysation of short and medium chain acyl-CoAs, and thereby preventing their reloading for further elongation by FAS.

3.3.6.1 Identification of putative hydrolases

Using the *Saccharomyces* Genome Database (SGD), where broad information about the *S. cerevisiae* genome and its genes, proteins, and other encoded features is provided, annotated hydrolases were investigated (Cherry et al., 2011). The six listed enzymes stand for putative hydrolases acting on ester bonds, proteins with CoA transferase activity or already characterized proteins (Table 21). The proteins Mgl2p, Eeb1p and Eht1p are the most promising for further analysis regarding the activity towards medium chain fatty acids (Saerens et al., 2006; Knight et al., 2014), while Ach1p is involved in mitochondrial acetate detoxification, and not of greater importance for hydrolysis of C_6 - C_{10} acyl-CoAs (Fleck and Brock, 2009). The gene YDR444W encodes for a protein whose biological role is unknown, and therefor will not be considered for further analysis. Tes1p, a acyl-CoA thioesterase, is only involved in fatty acid oxidation within the peroxisomes (Jones et al., 1999).

Table 21: Results for hydrolases in *S. cerevisiae* with unknown function or with activity on ester bonds. Information are obtained by search for hydrolases from SGD website (Cherry et al., 2011).

systematic name	gene	alias	description
YBL015W	*ACH1*	acetyl-CoA hydrolase	protein with CoA transferase activity
YDR444W		putative hydrolase	putative hydrolase acting on ester bonds
YMR210W	*MGL2*	putative carboxylic ester hydrolase	putative acyltransferase with similarity to Eeb1p and Eht1p
YJR019C	*TES1*	acyl-CoA thioesterase PTE1	peroxisomal acyl-CoA thioesterase
YPL095C	*EEB1*	medium-chain fatty acid ethyl ester synthase/esterase	acyl-coenzymeA:ethanol O-acyltransferase
YBR177C	*EHT1*	medium-chain fatty acid ethyl ester synthase/esterase	acyl-coenzymeA:ethanol O-acyltransferase

3.3.6.2 Effect of *EEB1*, *EHT1* and *MGL2* deletion

Knight et al. (2014) described Eht1p as an octanoyl-CoA:ethanol acyltransferase (AEATase) and as a thioesterase. This dual activity is assumed to be valid for the AEATase Eeb1p and Mgl2p, too (Saerens et al., 2006). The genes of three AEATases (Mgl2p, Eeb1p and Eht1p) were deleted in different combinations to identify the origin of acyl-CoA hydrolyzing activity in combination with FAS mutants. The separate deletion of thioesterases in the strain *S. cerevisiae* Δ*fas1*Δ*fas2* was performed using the CrispR/Cas method. The obtained combinations were single deletion strains Δ*eht1*, Δ*eeb1* and Δ*mgl2*, double deletion strains Δ*eeb1*Δ*eht1*, Δ*eht1*Δ*mgl2* and triple deletion strain Δ*eeb1*Δ*eht1*Δ*mgl2*. With this set of strains the above observed effect of free medium chain fatty acids in the medium can maybe be explained due to specific AEATase activity.

The strains were transformed with plasmid harbouring the modified fatty acid synthase genes with the p*ADH2* responsible for the expression. The fatty acid profile of the triple deletion strains in YEPD-bacto medium buffered with 100 mM potassium phosphate (initial pH 6.5) with optical density and pH after 72 h was compared to the initial strain *S. cerevisiae* Δ*fas1*Δ*fas2* (Figure 36). The triple gene deletion led to basically eliminated production of medium chain fatty acids. The total fatty acid yield in the supernatant was between 3.1 mg/L for the wildtype FAS and 7.3 mg/L for the IARK GS mutant (Table 22).

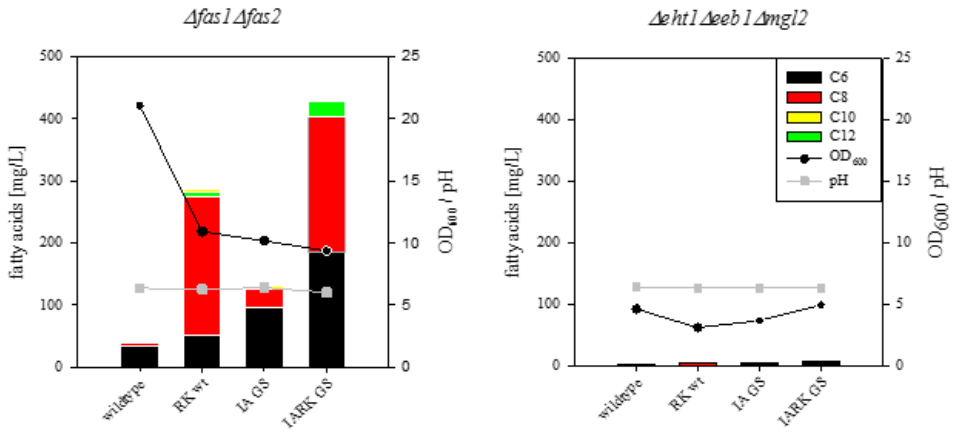

Figure 36: Fatty acid profile of supernatant after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2* and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1*Δ*mgl2* in 100 mM buffered YEPD-bacto medium. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. The optical density (OD_{600}) and pH are measured after 72 h. Values are the mean of three biological replicates with three technical replicates for each experiment. For details standard deviation see Table 22.

Table 22: Medium chain fatty acid profile in supernatant of *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1*Δ*mgl2* in 100 mM buffered YEPD-bacto medium after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates with three technical replicates for each experiment ± standard deviation.

MCFA in supernatant (mg/L)					
S. cerevisiae* Δ*eht1*Δ*eeb1*Δ*mgl2	**C6**	**C8**	**C10**	**C12**	**total**
wildtype	0.77 ± 0.09	1.76 ± 0.07	0.52 ± 0.04	0.06 ± 0.01	3.10 ± 0.08
RK wt	0.86 ± 0.08	4.04 ± 0.10	0.49 ± 0.03	0.05 ± 0.01	5.45 ± 0.13
IA GS	1.61 ± 0.05	1.81 ± 0.10	1.20 ± 0.06	0.09 ± 0.02	4.72 ± 0.13
IARK GS	2.08 ± 0.09	3.62 ± 0.08	1.48 ± 0.04	0.15 ± 0.01	7.33 ± 0.07

The two double knockout strains generally caused reduced extracellular MCFA levels. In order to analyse the expanded effect on fatty acid yield the strains *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1* and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*mgl2* were compared to the initial strain *S. cerevisiae* Δ*fas1*Δ*fas2* (Figure 37). The strain *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*mgl2* has the least observable effect. The end optical density is lower compared to the initial strain, but the fatty acid distribution does not change. The maximum fatty acid yield of 260 mg/L is obtained with mutant IARK GS. This represents a 39 % decrease compared to the initial strain. The mutant RK wt produces 39 % less octanoic acid compared to *S. cerevisiae* Δ*fas1*Δ*fas2*.

For *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1* it can be observed that the maximum yield of octanoic acid decreases down to 90 mg/L, which represents a 65 % decrease compared to *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*mgl2* and a approx. 5-fold decrease compared to the initial strain *S. cerevisiae* Δ*fas1*Δ*fas2*. The mutants IA GS and IARK GS show an tremendously reduced fatty acid yield.

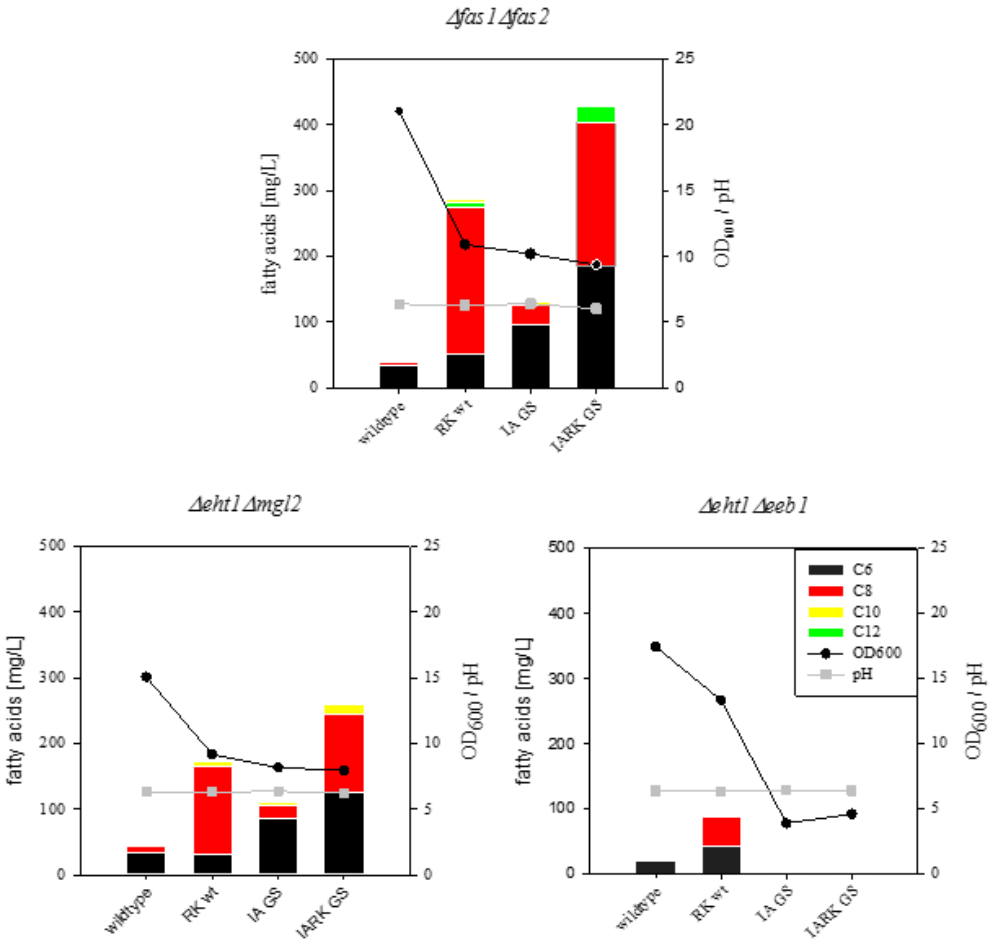

Figure 37: Fatty acid profile of supernatant after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2*, *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*mgl2* and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1* in 100 mM buffered YEPD-bacto medium. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. The optical density (OD_{600}) and pH are measured after 72 h. Values are the mean of three biological replicates with three technical replicates for each experiment. The relative standard deviation for the initial strain is below 12 % and for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*mgl2* below 14 % and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*Δ*eeb1* below 21 %.

The single deletions had a minor impact on extracellular fatty acid levels. During expression of the wildtype fatty acid synthase the difference in fatty acid yield between the thioesterase deletion strains is below 20 %. Only, *S. cerevisiae* Δ*mgl2* shows significantly lower yield of fatty acids in the supernatant (2 mg/L). For the synthesis of octanoic acid with the RK wt mutant, the deletion of *EHT1* shows the highest impact on synthesis.

By comparison with the initial strain a 2-fold decrease was observed. The deletion of *MGL2* shows a minimal negative impact of 15 % on octanoic acid yield in the supernatant. For the synthesis of hexanoic acid using the IA GS mutant, no negative effect in all tested deletion strains could be observed. In case of the mutant IARK GS, the difference of the overall yield in all single deletion strains compared to the initial strain does not exceed 24 %. In terms of hexanoic acid synthesis a 10 % decrease with the Δ*mgl2* strain can be stated, while the Δ*eeb1* strain has only a 3 % decrease compared to the initial strain. A different distribution profile can be observed for octanoic acid. The strain Δ*eht1* has the lowest amount (132 mg/L), while the other two deletion strain show maximum 22 % decrease in outcome compared to *S. cerevisiae* Δ*fas1*Δ*fas2* with 220 mg/L.

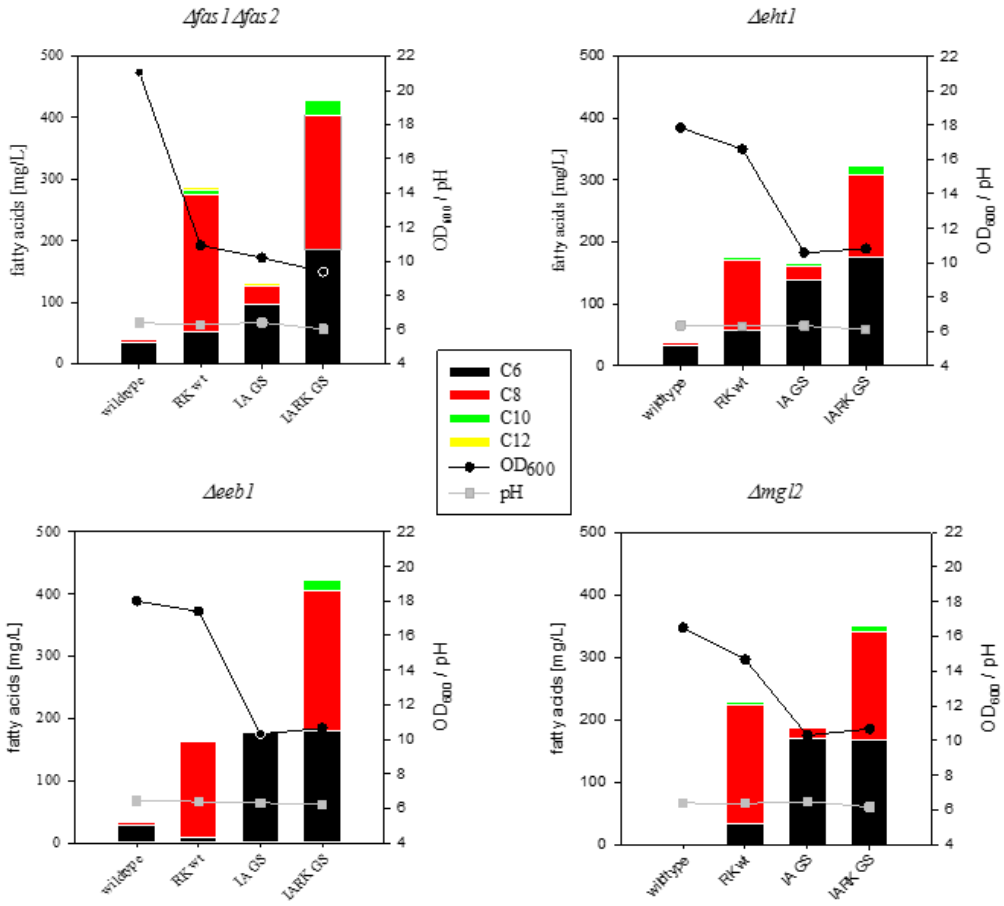

Figure 38: Fatty acid profile of supernatant after 72 h with *S. cerevisiae* Δ*fas1*Δ*fas2*, *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1*, *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eeb1*and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*mgl2* in 100 mM buffered YEPD-bacto medium. The optical density (OD_{600}) and pH are measured after 72 h. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Values are the mean of three biological replicates with three technical replicates for each experiment. The relative standard deviation for the initial strain is below 12 %, for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1* 10 % for the mutant FAS and 52 % for the wildtype FAS, for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eeb1* below 17 % for the mutant FAS and 76 % for the wildtype FAS, and for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*mgl2* below 14 %.

In *S. cerevisiae* the degradation of TAG is mainly catalysed by Tgl3p. A deletion of all lipases (Tgl3p, Tgl4p, Tgl5p and Ldh1p) would lead to an accumulation of TAG. It was stated by Selvaraju et al. (2016), that Mgl2p (*YMR210w*) does not play a significant role in ethyl ester synthesis, but encodes for a monoacylglycerol lipase. Therefore, the effect on TAG availability was tested with TLC in all single thioesterase deletion strains (Figure 39). The comparison between the individual strains with different fatty acid synthases for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eeb1* show no difference in neutral lipid composition and arrangement. The same effect can be observed for *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*mgl2*. For *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1* a difference in TAG accumulation can be observed for the mutants RK wt and IARK GS. The distribution of SE, DAG, MAG and free fatty acid does not differ significantly.

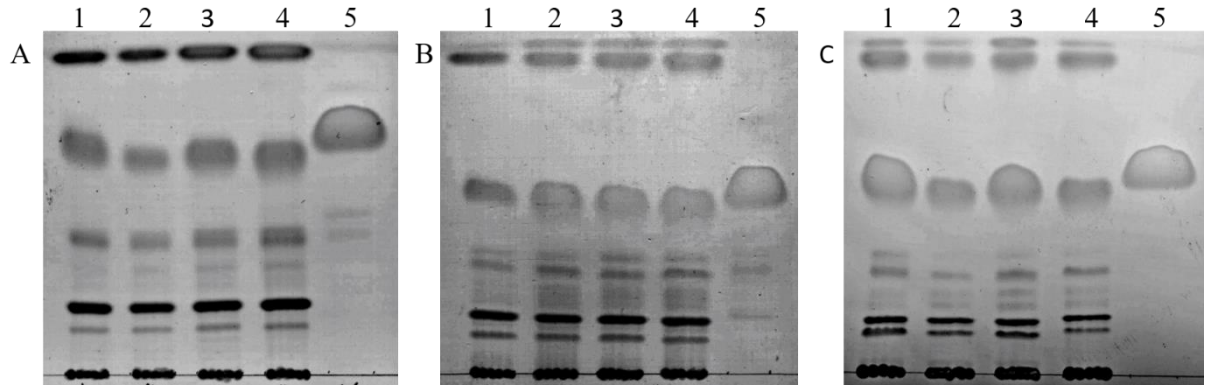

Figure 39: Lipid composition and wax ester accumulation behaviour of *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eeb1* (A), *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*mgl2* (B) and *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*eht1* (C) with FAS mutants. Cultivation was performed in 300 ml shaking flasks without baffles at 30 °C with 180 rpm. Cells were grown in YEPD-bacto medium for 72 h and 30 mg of dried cell material was extracted and subsequently analysed by TLC. 1) FAS wildtype, 2) FAS mutant RK wt, 3) FAS mutant IA GS, 4) FAS mutant IARK GS, 5) triolein 10 µg.

It could be shown, that the genes *EHT1*, *EEB1* and *MGL2* have an influence on the MCFA yield in the supernatant. For the Δ*eht1* strain a significant drop in octanoic acid concentration could be observed, which is in line with Saerens et al. (2006) reported specificity of Eht1p for octanoic acid CoA ester. Generally speaking the data from the single and double deletion strains suggest that Eeb1p has a selective activity for hexanoic acid CoA ester.

4. Discussion

The call for renewable and bio based products as an alternative to those of petroleum origin is rapidly increasing. Using microorganisms to produce novel compounds from fatty acids is an essential component to this approach (Hill et al., 2006; Steen et al., 2010). Yeasts are good candidate microorganisms for fatty acid derivatives production because of uncomplicated culture conditions, simple genetically manipulation and high fatty acid accumulation potential (Oelkers et al., 2002; Sandager et al., 2002; Sorger and Daum, 2002, 2003). Three aspects of fatty acid synthesis in *S. cerevisiae* were addressed in this work. The first aspect deals with the characterization of potential ω1-desaturases. The second with the development of a production system for fatty acids in *S. cerevisiae,* while the third aspect specifies on medium chain fatty acid synthesis.

4.1. Desaturases

Unsaturated fatty acids are essential components of membranes and storage lipids. They are generated through desaturation and elongation reactions on a carbon chain backbone of fatty acid. Various types of fatty acid desaturases with different substrate specificities and region specificities are found in prokaryotes and eukaryotes. To generate monounsaturated fatty acids in *S. cerevisiae* the ER bound acyl-CoA desaturase, Ole1p is essential (Tamura et al., 1976; Certik and Shimizu, 1999). Bai et al. (2015) crystallized and solved the structure of mouse SCD1 bound to stearoyl-CoA. This structural information helps to understand the design of Ole1p, due to its high similarity to SCD1 and furthermore to identify amino acids having an influence on the enzyme activity and substrate selectivity. The data indicate nine conserved histidine motifs necessary for the coordination of the dimetal centre. The substrate tunnel surrounds the acyl-CoA (Figure 2B). At the binding point of C9 and C10 of the acyl chain, the tunnel has a kink (Figure 2D). Amino acids likely responsible for the formation of the kink are Trp258, Thr257, Gln143 and Trp149. Based on previous kinetic isotope data of plant desaturases, Broadwater et al. (1998) and Shanklin et al. (1997) hypothesised that the C9 and C10 bond for Δ9 desaturases is precisely positioned with respect to the diiron center (Shanklin et al., 2009). The end section of the tunnel is completed by Tyr104 and Ala108 (Figure 2C).

4.1.1 Characterization of fungi desaturases

The Δ11-like desaturase, Obr-TerDes is able to convert $C_{20:3}^{\Delta 11,14,17}$-CoA into $C_{20:4}^{\Delta 11,14,17,19}$-CoA, and thereby introduce a terminal double bond to the acyl chain (Ding et al., 2011). Albre et al. (2012) described a second desaturase with terminal desaturase activity from *Planotortrix excessana*. Pex-Desat3 is able to produce $C_{14:1}^{\Delta 13}$-CoA, $C_{16:1}^{\Delta 15}$-CoA and $C_{18:1}^{\Delta 17}$-CoA. The results from this study show, that the comparison of sequences, in order to identify enzymes with similar or same reaction activity is not possible. Although the sequence alignment and subsequent neighbour-joining phylogenetic tree indicates a few potential protein sequences, the activity of the tested is in most cases similar to Ole1p. The proteins from *S. commune* (EFI94599.1), *S. octosporus* (EPX72095.1), *W. mellicola* (EIM20316.1), *W. ichthyophaga* (EOR00207.1) and *A. bisporus* var. *bisporus* (EKV44635.1) could be classified as Δ9 desaturases. To verify the Δ9 enzyme activity the *S. cerevisiae* strains were spotted on medium with and without addition of oleic acid. The strains should only be able to grow in the presence of oleic acid, like shown by Schweizer and Bolling (1970). This means, the spotting assay is reliable regarding the fatty acids needed for growth. These proteins also have in common the lack of similarity to Pex-Dast3 and Obr-TerDes. Which supports the importance of the mentioned amino acids that may determine the length of bound acyl chains (position in Ole1p 139 and 143). Pex-Desat3 has a leucine and valine, while Obr-TerDes has an asparagine and isoleucine at those positions. The classified Δ9 desaturases have all tyrosine and glycine, respectively. Leucine is a non-polar amino acid with an isobutyl side chain, while asparagine, a aliphatic amino acid, has a carboxamide group. Compared to tyrosine, an aromatic, partially hydrophobic, amino acid the tunnel should be longer and allow longer fatty acids to be bound. This can be confirmed by the product spectrum of Ob-TerDes ($C_{20:3}^{\Delta 11,14,17}$). The second amino acid that may determine the length of bound acyl chains is valine and isoleucine for the terminal desaturases, which are both aliphatic, hydrophobic, amino acids. Valine is preferably substituted by isoleucine, methionine or leucine (Betts and Russell, 2003). This means that the terminal desaturases do not differ drastically at this position. The Δ9 desaturases have a glycine, a small amino acid, with a hydrogen as a side chain. Glycine can play a distinct functional role, such as bind to phosphates due to the small side chain (example ATP binding site in a protein kinase) (Betts and Russell, 2003). This residue is by implication highly important for the substrate specificity as it is conserved in most of the tested (Figure 7) and already described Δ9 desaturases (Figure 6). The protein sequences with the similar amino acids, responsible for the length of the bound acyl chain (CAP70780.1) and binding of the acyl chain (EFI94388.1), could not be clearly identified with the applied methods.

CAP70780.1 shares the amino acid isoleucine at position 143 (in Ole1p) with the terminal desaturase Obr-TerDes, while EFI94388.1 shares the amino acid glutamine with Pex-Desat3 and CAP70780.1 at position 178 (in Ole1p). Obr-TerDes has a threonine (178), while the majority of the other proteins have the amino acid glutamic acid. Glutamine is a polar amino acid with a amide functional group. It is preferably substituted by other polar residues, in particular glutamic acid, which contains an oxygen in place of the amino group. Threonine is considered a slightly polar amino acid, though it is fairly neutral with regard to mutations (Betts and Russell, 2003). From the point of view of insertion of mutations these amino acids are very closely related to each other, and should not have a big influence on the protein conformation or activity. For the strains expressing the genes from *S. commune* (EFI94388.1) and *P. anserina* (CAP70780.1) no growth could be observed, which is comparable to the growth of *S. cerevisiae* WRY1Δ*ole1* expressing Pex-Desat3 and Obr-TerDes. In the *P. anserina* genome two other membrane fatty acid desaturase-like proteins are predicted, the hypothetical protein CAP70336.1 (PODANS_3_4190) with assumed Δ9 desaturase activity and the hypothetical protein CAP64892.1 (PODANS_5_4330) described as an ω6-fatty acid desaturase (Δ12 desaturase) (Espagne et al., 2008). In this case the fungus should be able to synthesise oleoyl-CoA ($C_{18:1}^{\Delta 9}$) and linoleoyl-CoA ($C_{18:1}^{\Delta 9,12}$). It can be assumed, that CAP70780.1 is responsible for the introduction of the third bond in an already unsaturated fatty acids. Oleic acid is no substrate for the enzyme, due to the fact, that addition of oleic acid to the medium did not lead to synthesis of $C_{18:2}^{\Delta 9,12}$. To verify the assumption the yeast growth medium would have to be supplemented with different types of unsaturated fatty acids to identified the substrate spectrum of the enzyme. Linoleoyl-CoA could be a potential candidate substrate for CAP70780.1, which in that case could have a Δ6 desaturase activity and the product would be γ-linolenoyl-CoA ($C_{18:1}^{\Delta 6,9,12}$) or Δ15 desaturase activity and with α-linolenoyl-CoA ($C_{18:1}^{\Delta 9,12,15}$) as product. The *S. commune* fatty acid metabolism is not resolved, but has the same predictions for the synthesis of oleoyl-CoA ($C_{18:1}^{\Delta 9}$) and linoleoyl-CoA ($C_{18:1}^{\Delta 9,12}$) (Ohm et al., 2010). Again linoleoyl-CoA could be a potential candidate substrate for EFI94388.1. If supplementation of growth medium wants to be avoided the expression host could be changed. Other yeasts are naturally able to synthesise unsaturated fatty acids like linoleoyl-CoA, which could be used as substrates. Another option would be an enzyme assay, to determine the specific activity with purified proteins.

4.1.2 Site directed mutagenesis in Ole1p

With the structural information of Δ9 desaturases from mouse and human (Bai et al., 2015; Wang et al., 2015) specific mutation of single amino acids can be performed in order to change the substrate specificity and enzyme activity of Ole1p. The selected amino acid exchanges were founded on these findings and the comparison with the sequences of other known desaturases with terminal desaturase activity or unusual substrate specificity. Six amino acids have been highlighted to have an influence on the substrate specificity. Two of them were tested (C120, T318) with no effect on the substrate specificity, due to no new product formation. For the amino acid localised at the end of the substrate channel, Tyr149 and Gly153, an influence on the length of the bound acyl chain was described (Bai et al., 2015). Pex-Desat3 has a leucine and valine, while Obr-TerDes has an asparagine and isoleucine, respectively. In case of position Tyr149 a mutation to a smaller or more polar amino acids could have an influence on the substrate length due to prolongation of the tunnel length. The desaturase from *C. hyperboreus* has a threonine at position 149 and preferentially acts on C_{22} - C_{26} acyl-CoAs. Threonine, a slightly polar amino acid, can often be replaced by serine, but is unlikely to be replaced by tyrosine (Betts and Russell, 2003). The tyrosine is present in Δ9 desaturases, which accept up to C_{18} acyl chains (like Ole1p). The change of Ole1p to a ChDes1-like desaturase could not be achieved by this single mutation, although the unsaturated substrates can be synthesised by *S. cerevisiae* elongases Elo2p and Elo3p (C_{24} - C_{26}) (Uemura, 2012). The mutation to leucine did not result in Ole1p desaturase with Pex-Desat3 like activity, because the cells were able to grow in the absence of oleic acid. This is a clear indicator of Δ9 desaturase activity and is supported by GC-MS data. For Gly153 the exchange to valine did not result in the expected Pex-Desat3 like activity. For the desaturase Desat2 from *D. melanogaster* (with methionine at position 153) a substrate specificity towards MCFA could be observed, although the enzyme remains a Δ9 desaturase (Dallerac et al., 2000). Desat2 preferentially acts on myristic acid and leads to the synthesis of $C_{14:1}^{\Delta 9}$. The yeast desaturase deficiency should be complemented by production of this unsaturated fatty acid because trace amounts of $C_{16:1}^{\Delta 9}$ and $C_{18:1}^{\Delta 9}$ fatty acids are formed (Dallerac et al., 2000). Desat1 from *D. melanogaster* has a 65 % identity to Desat2 and interestingly the equivalent amino acid to Tyr149 is in Desat1 and Desat2 a tyrosine. It can be stated that the equivalent amino acid to Tyr149 is not responsible for the substrate specificity towards C_{14} fatty acids. The analysis of the *S. cerevisiae* strain with Ole1p^{G153M} did not show any formation of unsaturated myristic acid, although the strain was growing worse without addition of oleic acid. Again a single mutation was not sufficient to change the activity of Ole1p.

Broun et al. (1998) substituted five amino acids located to a cluster of active-site histidine residues from an oleate 12-hydroxylase (Van De Loo et al., 1995) and convert the enzyme into an oleate Δ12 desaturase. Broadwater et al. (2002) confirmed that only two of the five locations were predominantly influencing the functional outcome.

All things considered, it is not sufficient to change one amino acid in order to change the substrate specificity or enzyme activity. Fatty acid desaturases are closely related homologs based on their amino acid sequences, but perform highly regio- and stereo selective reactions on fatty acid. The crystallization and elucidation of the 3D structure of terminal desaturases with different substrates would support the intentions for minimal invasive metabolic engineering on proteins in order to produce rare fatty acids in *S. cerevisiae* or other yeast.

4.2. Improving fatty acid synthesis in general

Fatty acids can be easily converted into different chemicals such as alkanes, alcohols, and biodiesel by microorganisms (Steen et al., 2010; Choi and Lee, 2013; Liu et al., 2013). In the yeast *S. cerevisiae* the fatty acid synthesis is regulated on two levels, the protein level and gene expression level (Smedsgaard and Nielsen, 2005). Additionally, co-substrates and metabolites such as acetyl-CoA, ATP, and NADPH are responsible for the actual synthesis rate (Tehlivets et al., 2007).

4.2.1 Implementation of co-substrate and metabolite synthesis

S. cerevisiae is able to produce acetyl-CoA from the mitochondrial pyruvate dehydrogenase pathway and the cytosolic pyruvate-acetaldehyde-acetate pathway (Pronk et al., 1996; Shiba et al., 2007; Chen et al., 2012). A major part of pyruvate is metabolized to ethanol by alcohol dehydrogenases and only a small part to acetyl-CoA (van Hoek et al., 1998; Vemuri et al., 2007). The oleaginous yeast *Candida tropicalis* directs nearly all pyruvate into the fatty acid synthesis. Pyruvate enters the TCA cycle, is withdrawn as citrate, and subsequently transformed to acetyl-CoA by ATP-Citrate Lyase (ACL). The by-product oxaloacetate can be degraded to malate by malate dehydrogenase (MDH). Malate can be re- incorporated in the TCA cycle or malic enzyme (ME) can use malate for pyruvate formation. Thus, the yeast is able to generate required electron carriers via MDH and ME without wasting any carbon flux by degradation of glutamine into glutamate, aspartate, CO_2, pyruvate, lactate, alanine and citrate (Liu et al., 2013).

The implementation of the established metabolic pathway of *C. tropicalis* in *S. cerevisiae* by combination of cytosolic malate dehydrogenase (*MDH3*), cytosolic malate enzyme (*MAE1*), citrate- α-ketoglutarate- carrier (*YHM2*) and ATP-Citrate Lyase (*ACL1*) to improve the availability of acetyl-CoA in the cytosol (Figure 12) was not sufficient to increase the fatty acid content. These results are comparable to the findings from Beopoulos, Cescut, et al. (2009), where the expression of a native *MAE1* in *Y. lipolytica*, which has the other necessary enzymes encoded in the genome, did not yield in a higher lipid content. In contrast to this Zhang et al. (2007) overexpressed the gene in *M. circinelloides*, under the control of the strong constitutive promoter of the glyceraldehyde 3-phosphate dehydrogenase gene and could increase lipid accumulation by a factor of 2.5.

For the synthesis of NADPH, an electron donor used by the FAS complex, the pentose phosphate pathway (PPP) is the main source. Furthermore, can the NADPH isocitrate dedydrogenase, NAD kinase and the aldehyde dehydrogenases provide the cells with NADPH (Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2005; Tehlivets et al., 2007). The main enzymes in the PPP are glucose-6-phosphate dehydrogenase (G6PDH, gene *ZWF1*) and phosphogluconate dehydrogenase (GND, gene *GND1*) (Maaheimo et al., 2001). In most oleaginous yeasts, the NADPH supply is hypothesized to origin from the malic enzyme (ME) (Ratledge, 2014). Dos Santos et al. (2004) removed the mitochondrial target sequence of yeast ME, demonstrated its functionality in the cytosol and implemented an additional source of cytosolic NADPH. Subsequently, this led to a decreased pentose phosphate pathway (PPP) flux. Mishra et al. (2016) indicated via metabolic modelling experiments and *in silico* analysis, that the G6PDH and GND have a higher capability to regenerate the NADPH when compared to ME and the isocitrate dehydrogenase (IDH). Ultimately, the PPP enzymes must be targeted rather than ME to improve fatty acid synthesis. Tang et al. (2013) proved that the disruption of the isocitrate dehydrogenase genes lead to no significant decrease of NADPH level but rather a decrease in ATP level. This is plausible, due to the accumulation of citrate in the TCA and its impairment. The deletion of *IDH1* and *IDH2* in *S. cerevisiae* WRY1Δ*FAA1*Δ*FAA4* did not lead to an increase in fatty acid content, although the accumulated citrate from the TCA could be processed into acetyl-CoA by the co-expressed ATP-Citrate Lyase. This results could correlate with the reduced ATP level in the cells, as ACL consumes ATP for acetyl-CoA synthesis. Chen et al. (2016) identified for a yeast strains with *FAA1* and *FAA4* deletion an upregulation of enzymes in the TCA cycle (Cit1, Idh1, Idh2, Kgd1, Fum1, and Mdh1). Moreover, four enzymes involved in acetate metabolism (Ald6, Ald4, Acs2, and Ach1) (Saint-Prix et al., 2004) and two enzymes (Acc1 and Acb1) from the fatty acid metabolism were upregulated.

Due to the deletion of *IDH1* and *IDH2* and a constitutive active promoter in front of *ACC1* these regulation mechanisms might have been overridden. This may have led to an imbalance in general metabolic processes in the cells and subsequent constant fatty acid yield. The acetyl-CoA synthetase from *S. enterica* could be an alternative to ACL in order to improvement the supply of acetyl-CoA and NADPH (de Jong et al., 2014).

4.2.2 Deletion of fatty acyl-CoA synthetases

In the past years numerous studies in yeast and bacteria have shown, that the fatty acid pathway can be optimized at different points inside the pathway by genetic modification (Lu et al., 2008; Nawabi et al., 2011; Blazeck et al., 2014; Runguphan and Keasling, 2014). In a latest study, Leber et al. (2015) found that disruption of *FAA1*, *FAA4*, and *FAT1* yielded 490 mg/L FFA. When combined with disruption of the β-oxidation pathway and co-expression of *DGA1* and *TGL3*, 2.2 g/L FFA were produced. The results are compatible with findings in this study. The *S. cerevisiae* BY4741 strain was also able to produce approx. 0.69 g/L (± 0.34 g/L) fatty acids (intracellular and extracellular) after 48 h, and the deletion of fatty acyl-CoA synthetases results in a further increase. In contrast to Leber et al. (2015) the additional deletion of *FAT1* did not influence the maximum overall yield, when compared to the double deletion strain Δ*faa1*Δ*faa4*. Furthermore, did the overexpression of *ACC1*, *FAS1* and *FAS2* lead to a higher synthesis with approx. 1.7 g/L overall fatty acid compared to BY4741Δ*FAA1*Δ*FAA4*Δ*FAT1*. The highest amount of overall fatty acids tested in this study was with *S. cerevisiae* WRY1Δ*FAA1*Δ*FAA4* p*HXT7-DGA1* p*HXT7-TGL3* (2.5 g/L ± 0.8 g/L), which is comparable to the sextuple mutant BY4741Δ*FAA1*Δ*FAA4*Δ*FAT1*Δ*FAA2*Δ*PXA1*Δ*POX1* overexpressing p*TEF-DGA1* and p*TEF-TGL3* (2.2 g/L FAA). Leber et al. (2015) claim that 80-90 % of total detected fatty acids (intracellular and extracellular) are secreted from the cell as FFAs. In this study the amount of secreted fatty acids is 64 % of total detected fatty acids (1.6 g/L FFA) for the best strain. This leaves scope for improvement. Remains to mention that it is unusual to detect only saturated and unsaturated C16 and C18 fatty acids, although the *FAA2* gene was deleted in the sextuple mutant, which is a medium chain fatty acyl-CoA synthetase. Faa2p acts on C7:0 - C17:0 fatty acids (Johnson et al., 1994). In experiments during this study with the *S. cerevisiae* Δ*fas1*Δ*fas2* strain with wildtype FAS expressed via plasmids the deletion of *FAA2* leads to secretion of dodecanoic acid, decanoic acid, octanoic acid and hexanoic acid (Figure 27).

4.3. Medium chain fatty acids

Fatty acids are considered strategically important platform compounds for the sustainable production of fine chemicals. The control of the fatty acid chain-length to increase the variety of available chemicals has not been achieved in an acceptable extent to date. Here, the approach on engineering the *de novo* fatty acid production in *S. cerevisiae* for the production of medium chain fatty acids is described. The aim was to create a *S. cerevisiae* production strain for medium chain fatty acids by rationally engineering substrate specificities of the FAS complex, in particular the enzymatic domains responsible for loading and release of the acyl chain as well the elongation. Short and medium chain fatty acids are rarely synthesized by microorganisms in contrast to LCFA. As an exception the lipoic acid production pathway can be mentioned (Hiltunen et al., 2010). In *S. cerevisiae*, the mitochondrial FAS II pathway produces octanoyl-ACP, the precursor for lipoic acid synthesis (Schonauer et al., 2009).

4.3.1 Selective production of MCFA by reprograming of the FAS

The successful reprogram of the FAS into a MCFA producing system could be achieved. The FAS was manipulated in all enzymatic centers that play key roles in chain length control; the condensation domain (KS) and the transferases (MPT and AT). The three mutations G1250S, M1251W and F1279Y/F1279W in the KS domain are interfering in the elongation reaction of the intermediates. The single mutation R1834K in the MPT domain limits the loading of the malonyl-CoA, needed for elongation and could in addition facilitated product release. The I306A mutation in the AT domain enlarged the acetyl binding channel and thereby integrated a novel way for product release. A selective production of MCFA was demonstrated with the expression of mutated FAS with wildtype *FAS1/FAS2* promoter. The overall titer of 114 mg/L MCFA could be gained by using the mutant IARK GSMW, which is also the best mutant for octanoic acid (78 mg/L) and decanoic acid production (22 mg/L). The mutant IARK FY has a narrower product spectrum and the synthesised octanoic acid has a maximal purity of 96 % (48 mg/L). With regard to the hexanoic acid synthesis the mutant IARK GS has the highest yield (49 mg/L), while mutant IA GS is the most specific hexanoic acid synthesising strain (88 % hexanoic acid, 17 mg/L). The strains with mutations in the KS domain at position F1279 cannot grow without supplementation of oleic acid. Exceptions represent the mutants wt GSFY, IA GSFY RK GSFY, IARK GSFY, IA FY and IARK FY. The amino acid phenylalanine is located on the opposite side of the amino acids G1250 and M1251 in the KS binding channel.

By combining the three mutations in the binding channel, the FAS complex seams to lose activity or significantly increase the selectivity for substrates. It is assumed to inhibit binding of acyl chains beyond the length of six carbon atoms, which could lead to complete loss of LCFA synthesis. This is supported by the data from the growth experiment with oleic acid, where the G1250-M1251-F1279 mutants are able to grow but not to produce significant amounts of MCFAs. Schweizer and Bolling (1970) showed the dependence of *S. cerevisiae,* when FAS complex was malfunction on the presence of myristic acid, palmitic acid, stearic acid, or oleic acid, added to the medium. These fatty acids are, in different amounts, the natural end products of saturated fatty acid synthesis in yeast (Lynen et al., 1964, 1980). If supplied with any of the LCFA, a certain other fatty acid may be replaced without loss of viability. The fatty acids with less carbon atoms than myristic acid do not support growth, but even increasingly inhibit the growth (Schweizer and Bolling, 1970). The supplemented oleic acid can not only be used as source of energy, if glucose is consumed, but also as a substrate for β-oxidation in peroxisomes and as a building block for complex lipids (TAG, SE) (Hiltunen et al., 2003). The exchange of phenylalanine to tyrosine (F1279Y) gives the impression not to drastically change the binding channel, due to the fact that all exceptional mutants (see above) can grow and produce MCFA. This can be explained by the smaller side chain of the amino acid, which differs only in that it contains a hydroxyl group in place of the hydrogen on the benzene ring. The exchange of phenylalanine to tryptophan (F1279W), with a side chain indole with a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring, results in all tested strains to no growth. If oleic acid is added to the culture medium the strains can again grow but the MCFA synthesis is below wildtype FAS production rate. The examination of the combined mutations G1250-F1279 leads to the conclusion that the FAS complex has not completely changed the substrate specificity and allows a sufficient synthesis of LCFA, while the single mutation F1279Y has a positive effect on octanoic acid and hexanoic acid synthesis regarding the specificity of the product spectrum. The amino acid M1251 is placed in the middle of the acyl-binding channel of the KS domain. It is assumed to rotate and unlock the inner part of the binding cavity (Johansson et al., 2008). The second position is G1250, which should influence the conformational flexibility of the neighbouring M1251 and thereby change the conformation of the G1250-M1251-G1252 turn and increasing the energy barrier of M1251 movement. The importance of the movement is represented by the cerulenin resistance of *S. cerevisiae* strains mutated at the glycine position (G1250S).

The resistance for cerulenin, an inhibitor of the fatty acid synthesis by blocking the interaction of malonyl-CoA with the ketoacyl synthase, increases (Inokoshi et al., 1994) and *S. cerevisiae* FAS is able to produce high amounts of decanoic acid and ethyl caproate (Aritomi et al., 2004). The amount of ethyl caproate corresponded to the size of side chain of the G1250. While the glycine with a hydrogen atom and alanine with the methyl group have low concentrations the exchange to serine (hydroxyl group) and cysteine (sulfhydryl group) resulted in a greater inhibition of FAS (Aritomi et al., 2004). This effect could be confirmed with two tested FAS mutants. The combinations wt GS and wt GSMW show increased octanoic acid and decanoic acid synthesis compared to the wildtype FAS. The double mutation has an 4-fold higher MCFA concentration after 48 h compared to the single FAS mutant. It must be taken into account that the KS domain does not reflect the entire enzymatic characteristics of the FAS complex. The MPT domain is also involved in chain length determination due to the release of fatty acid with a defined chain length (Chakravarty et al., 2004; Lomakin et al., 2007).

The MPT domain of the *S. cerevisiae* FAS is responsible for loading malonyl-CoA to the active center of the enzyme and for unloading of CoA ester products. It was previously shown in structural studies by Bunkoczi et al. (2009) for human mitochondrial and cytosolic transferases, that the active site has three conserved residues (cytosolic/mitochondrial: Ser581/ Ser117, His683/ His234 and Arg606/ Arg142). By replacing Arg142 with glutamine, glycine, or alanine a significantly reduced catalytic activity and lowered affinity for malonyl-CoA was noticed. Kawaguchi et al. (1980) showed for the *Brevibacterium ammoniagen* FAS that the relative proportion of palmitic acid to stearic acid depends on the concentration of malonyl-CoA and on the ratio of acetyl-CoA to malonyl-CoA. Rangan and Smith (1997) replaced the arginine with the smaller lysine and the resulting enzyme exhibited increased activity toward acetyl and medium chain acyl substrates. In the present study, the R1834K mutation was introduced in the MPT domain. Destabilizing malonyl binding, which would have a comparable effect to a decrease in malonyl-CoA concentration, and shifting the equilibrium to product unloading resulted in an increase of MCFA in *S. cerevisiae*. The effect could be observed in the FAS mutant strain RK wt with 74 mg/L octanoic acid synthesis (43-fold increase over the wildtype) and 99 mg/L overall yield of MCFA in the medium.

The acetyl transferase (AT) of the *S. cerevisiae* FAS is responsible for the loading of acetyl-CoA onto the FAS. Since the studies on the hMAT-transferase from different laboratories (Rangan and Smith, 1997; Aritomi et al., 2004) lead to an increased acetyl binding, similar engineering was performed (Gajewski et al., 2017a).

The I306A mutation was suggested to increase acetyl-CoA loading and to theoretically introduce a novel way for product release of medium chain length acyl chains (Figure 15). The combination IA wt did not show high amounts of MCFAs in the supernatant (8 mg/L), while the combination with other mutations changed the fatty acids pattern severely. The MPT mutation R1834K and AT mutation I306A in combination with a wildtype KS domain resulted in a 3-fold increase in MCFA concentration compared to the single mutant IA wt. Merge the mutations in the KS domain, MPT domain and AT domain results in the mutant IARK GSMW, the best for overall MCFA production (grew only to approx. $1/3$ of end optical cell density in YEPD-bacto medium compared to the other mutants). Whereas the mutant IARK GS, shows a crucial increase in hexanoic acid synthesis (49 mg/L). The previously observed effect of a 4-fold higher MCFA concentration, if the methionine was additionally mutated, could not be repeated. The effect of the mutations is not additive regarding the substrate yield. Generally speaking, the mutations G1250S and I306A lead in existing mutants, such as IARK GS, to a higher yield in hexanoic acid. Mutations M1251W and R1834K lead in existing mutants, such as RK GS or IARK wt, to an increase in octanoic acid. It was observed, that most of the tested strains, when grown in presence of oleic acid produce less MCFA (Figure 18). The combination of genomic FAS and a plasmid based expression of mutant FAS (with wildtype promoter and terminator, low-copy vector) could have been an alternative choice to supplementation of LCFA. The wildtype FAS could synthesise the necessary amount of LCFA for growth and other cellular processes, and the mutant FAS could produce MCFA. Using synthetic medium no difference in MCFA synthesis between wildtype and mutants in *S. cerevisiae* BY4741 could be observed (Table 17). The strains produce no MCFA. The synthesis of fatty acids utilizes substantial amounts of metabolites, like acetyl-CoA, ATP and NADPH, and other cellular processes dependent on these compounds as well, which leads to the assumption that the amount of metabolites is not sufficient for both FAS complexes to synthesise fatty acids. On the other hand, in *S. cerevisiae* the homologous recombination is very efficient (Houston et al., 2004) and could have occurred during the cultivation and lead to a recombination event by which the mutated FAS genes were exchanged with the wildtype and lead to a wildtype strain with wildtype-like fatty acid synthesis.

4.3.2 Culture medium composition

Furthermore, is the use of different medium composition crucial for optimal fatty acid synthesis in yeast. Growth of yeast cells is faster and the biomass produced is greater in medium containing complex ingredients (Narendranath et al., 2001). An assumption has been, that the greater amount of nutrients leads to an improved growth. Now it is recognized, that the ingredients of complex medium play non-nutritional roles in promoting the growth and survival of yeast and stimulate growth in the presence of organic acids (Thomas et al., 1994). It could also be discovered that the same amount of different peptone and yeast extract can influence the yield of fatty acids produced. Miller et al. (1986) showed that the used Bacto peptone contains estrone, which leads to estrogenic activity in *S. cerevisiae* and conversion to estradiol. Estradiol might be a responsible for rapid cell growth, which could be observed in comparison to medium with Peptone from Oxoid. Kamekura et al. (1988) analysed different peptones and found that the amount of present bile acids is different. For Bacto-Peptone and Peptone Bacteriological Technical 9.5 mg/g and 0.05 mg/g of total bile acids could be verified, respectively. In case of Oxoid Bacteriological Peptone L37 0.28 mg/g of bile acids were detectable. In Bacto-Peptone the major bile acid was taurocholic acid with a total of 40% followed by glycocholic acid, taurodeoxycholic acid, and glycodeoxycholic acid(Kamekura et al., 1988). The anti-aging effectiveness of these bile acids correlates with their hydrophobicity. Lithocholic acid (LCA), which has the most hydrophobic characteristic, showed in experiments from Goldberg et al. (2010) the highest ability to delay chronological aging of *S. cerevisiae* BY4742 Δ*pex5* and wild type strain under caloric restricted conditions. Chronological lifespan is defined as the time that a yeast cell can maintain viability and vitality in a non-dividing state (Wasko et al., 2013). Three positions in the six-member rings of the steroid nucleus are important for the ability of LCA to extend the mean and maximum chronological life span of yeast. Goldberg et al. (2010) provides evidence that bile acids suppress the aging process of lipid induced necrotic cell death. Thereby the ability to reduce the intracellular level of free fatty acids and DGA could be observed, which are known to trigger such death. Since the amount of bile acids is very high in Bacto-Peptone (~ 9 mg/g), and the difference in growth and synthesis of MCFAs is significantly higher, the Oxoid-Peptone should not be used. Aris et al. (2013) could show that the leucine status influences the chronological life span during calorie restriction. Adding leucine to growth medium will subsequently extended chronological life span.

On the other hand Kamisaka et al. (2007) indicated that the expression of the *LEU2* gene or the addition of leucine into the culture medium leads to an increase in the total fatty acid content. The effect of plasmid based expression of the *LEU2* gene, which is the selection marker for the plasmid harbouring *FAS1*, seems not to have a great influence regarding the prolongation of life span or fatty acid content. The effect of estradiol supplementation on growth of *S. cerevisiae* should be tested in further experiments, to determine the exact reason for the significant difference in the YEPD-bacto and YEPD-oxoid medium.

Another effect of the synthesis of MCFA is the pH value of the medium. It could be shown that in the absence of good buffering, the pH of a growth medium is lower when MCFA are produced (Table 19, Table 20). Predominantly this it observed when octanoic acid and decanoic acid are produced in higher amounts (mutant IARK GS). In general it can be stated that *S. cerevisiae* is an acidophilic organism and prefers acidic growth conditions. However, the optimal pH range is dependent on temperature, oxygen availability, and the yeast strain itself. In the case of a extracellular pH of 3, the intracellular pH of *S. cerevisiae* is maintained between 5.5 and 5.75 (Imai and Ohno, 1995a). An extracellular pH between 6 and 10, results in a intracellular level between 5.9 and 6.75 (Imai and Ohno, 1995b). When the extracellular pH is lower than the optimal, the cells need to pump out hydrogen ions at the expense of ATP level in order to maintain the intracellular pH within the range that enables growth and survival (Narendranath et al., 2001; Thomas et al., 2002). The usage of ATP results in decreased growth (Warth, 1988; Pampulha and Loureiro-Dias, 2000). The reduction of cytosolic pH by acids challenges cells and the electrochemical potential maintained across the membrane is impaired (Buzas et al., 1989; Nielsen and Arneborg, 2007; Yalcin and Ozbas, 2008; Arroyo-López et al., 2009). An efficient transport of MCFA to the medium could maintain the cytosolic pH at optimal level. Yu et al. (2016) engineered *S. cerevisiae* for the production of short branched-chain fatty acids and increased the secretion of those by overexpression of Prd12p. Pdr12p, an ATP-dependent plasma membrane protein, gives *S. cerevisiae* resistance to monocarboxylic acids with chain lengths of from C1 to C7 by removal of the anions from the cytosol at pH 5.8 (Piper et al., 1998; Holyoak et al., 1999). Borrull et al. (2015) examined the Pdr12 and Tpo1 transporters regarding their involvement during the detoxification process of C8 and C10 fatty acids. Octanoic acid was mostly expelled by the Pdr12p.The resistance to decanoic acid requires Tpo1p, several genes in the β-oxidation pathway and ethyl ester synthesis (Legras et al., 2010). Narendranath and Power (2005) could show, that a lower initial pH results in a reduction of ethanol concentration and increase of remaining sugar in the medium.

Buffering the culture medium to pH 6 with citrate phosphate buffer or low salt MES buffer was shown by Wasko et al. (2013) to result in doubling chronological lifespan under standard conditions in synthetic medium. Due to this, the buffering of culture medium, during MCFA synthesis is crucial to increase lifespan of producing cells. In this study the culture medium was buffered with potassium phosphate buffer to a pH of 6.5. The extracellular pH was chosen to be closer to the intracellular pH, to place less stress on the yeast cells and reduce energy loss on maintaining intercellular pH (Casal et al., 1996). In general, the pH was set higher than the pK_a of octanoic acid (4.89) and decanoic acid (4.9), to reduces the concentration of undissociated acid, and to decrease the inhibitory effect on yeast growth and metabolism (Weast et al., 1990). The use of 250 mM potassium phosphate buffered medium lead to a cultivation pH comparable with the wildtype and higher yield in MCFA. In further experiments the medium provides an opportunity for optimization. The potassium phosphate buffer was used to maintain the pH of the culture medium during the growth of *S. cerevisiae* at a constant level. The exchange to urea, could benefit in two ways. Urea can be used as a nitrogen source and can decreases acidification of the culture. The second mode of action would be by using urea also to decrease the amount of available nitrogen, which is shown to be beneficial for the synthesis of fatty acids (Leber and DaSilva, 2014; Li et al., 2014). Another method to modify the medium would be the use of a bi-phase medium, where volatile esters, which are also products of the yeast thioesterases (Saerens et al., 2006; Zhuang et al., 2015) could be prevented from evaporation. It was analysed in *E. coli* that dodecane could improve the yield by capturing produced FAEEs (674 mg/l compared to 427 mg/L) (Steen et al., 2010). Hence, a decrease of the toxic effect of MCFA and the equivalent esters on membrane fluidity/stability could be prevented.

4.3.3 Change expression pattern of mutated FAS

To optimize the protein amount of mutated FAS and thereby increase the MCFA synthesis the native *FAS1* and *FAS2* promoter in the plasmids was exchanged by the alcohol dehydrogenase 2 promoter (Ciriacy, 1975) from *S. cerevisiae*. The p*ADH2* (573 bp) is normally in an inactive conformation in the yeast chromosome. For the maximal expression the transcription factor Adr1p is necessary (Ciriacy, 1975; Denis et al., 1981). The de-repression of the promoter occurs by binding of two Adr1p monomers to the 22-bp palindromic sequence of UAS1 (Thukral et al., 1991). The transcription of *ADR1* is inhibited in the presence of glucose (Price et al., 1990).

A minimal expression level is achieved with little binding of Adr1p. When yeast cells are starved for glucose the Adr1p level increases and the transcription of the *ADH2* gene is initiated (Di Mauro et al., 2000; Young et al., 2000). Cook and Denis (1993) showed that the de-repression of Adr1p occurs within 40 - 60 min of glucose depletion. Since the p*ADH2* is repressed in the presence of glucose, the activation of gene expression occurs in stationary growth phase of the yeast cells, when cell biomass and ethanol concentration is high. Lee and DaSilva (2005) could show by measuring specific β-galactosidase activities in glucose containing growth medium that after 12 h no *ADH2* activation was detectable. The p*ADH2* promoter was fully induced by late stationary phase. The comparison of MCFA yield at different time points (Figure 23) indicates that the early stationary phase starts after 48 h and the maximal p*ADH2* activity is reached after 72 h. The glucose is depleted and the level of ethanol is high, which leads to a high protein concentration of FAS and subsequently a high level of free fatty acids in the culture medium. A comparable effect was observed by Kealey et al. (1998), who used the p*ADH2* for the expression of a fungal polyketide synthase 6-methylsalicylic acid synthase. The end product, 6-methylsalicylic acid, could be increased by 2-fold. The increase for the mutant RK wt was approx. 3-fold, when p*ADH2* derived expression was compared to the wildtype promoter. The replacement of glucose as carbon source with ethanol was additionally tested in order to increase the expression level of mutated FAS in *S. cerevisiae*. No positive effect regarding the MCFA synthesis could be observed, as the culture grew poorly and to no high optical density, as observed with glucose. It has to be considered, that the cells first have to grow and build a stable membrane, before a resistance to MCFA is present. The amount of LCFA in the medium is comparable to the approach in glucose containing medium, where the mutants showed approx. the same amount after 72 h of growth. The distribution of saturated and unsaturated fatty acids did not change. Whereby oleic acid, the most abundant unsaturated fatty acid in *S. cerevisiae* strains, is the most effective in overcoming toxic effects of ethanol during the growth phase (You et al., 2003). Only for mutant IARK GS an increase in oleic acid content in the medium could be observed. This increase does not counteract the negative effect of ethanol regarding the membrane fluidity, which is observable in the growth behaviour of the mutant strain. The amount of MCFA produced by the mutant IA GS increases which may be due to the low toxicity of hexanoic acid compared to octanoic acid and decanoic acid, which are the most prominent MCFA synthesised by the other mutants.

The application of glucose repressed promoters in industrial fermentations has a few disadvantages, due to the fact that high glucose concentrations are required for high cell densities but limited glucose for optimal expression of desired proteins. The optimal inducible promoter would have to be tightly regulated, economical for induction and highly active after induction. None of the presently available promoters for gene expression in *S. cerevisiae* meet all specifications (St John and Davis, 1981; Walker, 1998).

4.3.4 Best approach for optimal yield of MCFA

The promoter exchange is not the only method to increase the MCFA yield via metabolic engineering. The used CEN/ARS vectors are present in low copy numbers (1 to 2 copies per cell) and are mitotically highly unstable. This would lead to plasmid-free cells (up to 20 % per generation), due to inefficient transmission to daughter cells during cell division (Clarke and Carbon, 1980; Murray and Szostak, 1983). The implementation of 2 µ vectors for the expression would lead to a higher copy number (10 to 40 copies per cell) and the lost during cell division would be minimal (up to 3% per generation). Irani et al. (1987) showed that Adr1p, can become limiting for *ADH2* transcription from multi-copy plasmids, which makes the usage of p*ADH2* on 2 µ vectors not suitable for MCFA production. Plasmid stability is a very important requirement for highly efficient plasmid-based production processes in biotechnology. In order to use those in further approaches, the establishment of a plasmid addiction system, similar to the one published by Kroll et al. (2010) for *E. coli* could be developed. The chromosomal integration offers the most stable alternative, due to the fact, that the point mutations in the *FAS1* and *FAS2* can be inserted via CrispR/Cas9 very easily (Hinnen et al., 1978; DiCarlo et al., 2013; Generoso et al., 2016) and the usage of an already optimized yeast strain for fatty acid synthesis (*TEF1* promoter for *ACC1*, *FAS1* and *FAS2*) would shorten the general optimization process (Runguphan and Keasling, 2014). Gorgens et al. (2001) confirmed, that strong overexpression of a gene is not always the best approach for optimal yield of a specific product. It can cause a burden on the cell, due to increased energy demand, dilution of required cofactors and precursors for transcription and translation and limited space (Görgens et al., 2001). It could be confirmed by different research groups, that a moderate rather than strong multi copy gene expression leads to an optimized process in *S. cerevisiae* (Tao et al., 2005; Lee and DaSilva, 2006). Alternatively, the copy number of mutated *FAS1* and *FAS2* could be increased by stepwise integration of several copies into the yeast chromosome. If using a heterologous FAS complex in *S. cerevisiae* it should be considered to adapt the codon usage to ensure maximal translation efficiency (Wiedemann and Boles, 2008).

Thereby the level of mRNA containing rare codons could be removed and the available tRNA (such as from glycolytic genes) would be sufficient for optimal expression (Purvis et al., 1987). If the supplementation of LCFA has to be taken into account, due to toxicity of the MCFA, the integration of a foreign FAS with the respective mutations in the MPT, AT and KS domain should be considered. By using the sequence of alternative yeasts, homologous recombination with *S. cerevisiae* FAS could be avoided.

4.3.5 Improved metabolic fluxes towards MCFA synthesis

A different target for metabolic engineering is the elimination of by-products and product degradation. Therefore it is often necessary to modify enzymes of a pathway for improved metabolic fluxes towards the desired pathway. MCFAs are taken up in the yeast peroxisomes by diffusion. The ABC transporters, Pxa1p and Pxa2p, hydrolyze fatty acids -CoA esters prior to their entry into peroxisomes, releasing CoA into the cytoplasm. Fatty acids are activated by the peroxisomal acyl-CoA synthetase Faa2p, which is localized on the peroxisomal periphery, prior to β-oxidation (van Roermund et al., 2012). Faa2p acts on C_7 - C_{17} fatty acids (Johnson et al., 1994). Hence, the deletion of *FAA2* is crucial to obtain higher amounts of C_8 - C_{12} during growth. It appears that by deletion of *FAA2* the wildtype strain is able to secrete hexanoic acid and octanoic acid in higher amount than before. One explanation could be the mitochondrial lipoic acid synthesis. In *S. cerevisiae*, the mitochondrial FAS II synthesizes octanoic acid, which in form of a octanoyl-ACP acts as a substrate for *de novo* lipoic acid synthesis (Schonauer et al., 2009). The produced octanoic acid could be diffusing through the mitochondrial membrane and subsequently be detected in the medium. On the other hand, the cytosolic FAS is in the wildtype form able to synthesise octanoic acid, which is in general degraded via β-oxidation, due to the toxic effect it has on the cells. Since the Faa2p is not present in the strain, a degradation is not possible and leads to an increase of octanoic acid in the medium. The strain *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* has a higher growth rate and in general a decreased sensitivity towards octanoic acid compared to the *S. cerevisiae* Δ*fas1*Δ*fas2* strain. The secretion of decanoic acid, which is toxic in already low concentrations, is noteworthy higher when Faa2p is not present. This shift in fatty acid composition is the result of the impaired ability of the strains to detoxify the fatty acids C_6 - C_{10} by β-oxidation. For the mutant IA GS in *S. cerevisiae* Δ*fas1*Δ*fas2*Δ*faa2* the poor growth can be explained by the high concentration of decanoic acid. Decanoic acid acts as a weak acid and can thereby affect the cell wall structure and influence the lipid organization and function of cellular membranes (Liu et al., 2015).

The deletion of other acyl-CoA synthetases, like Faa1p, Faa4p and Fat1p would not be beneficial for the MCFA production. *S. cerevisiae* with Δ*faa1*Δ*faa4* or Δ*fat1* has only an impaired LCFA uptake (Schaffer and Lodish, 1994; Færgeman and Knudsen, 1997; DiRusso et al., 2000; Færgeman et al., 2001).

In general, free fatty acids are oxidized to provide energy very quickly or are incorporated in storage lipids because they are harmful to cells and can perturb membrane properties. Free fatty acids can be preserved as triacylglycerols (TAG) or sterol esters (SE) in specific lipid droplets. TAG and SE accumulate mainly during the stationary growth phase and can be mobilized to provide building blocks for membrane biosynthesis (Zanghellini et al., 2008). TAG are mobilized by TAG lipases like Tgl3p, Tgl4p, Tgl5p and Ayr1p (Athenstaedt and Daum, 2005; Ploier et al., 2013). Leber et al. (2015) could show that with the diacylglycerol acyltransferase, Dga1p, and the triacylglycerol lipase, Tgl3p, the *S. cerevisiae* strain BY4741 Δ*FAA1* Δ*FAA4* Δ*FAT1* Δ*FAA2* Δ*PXA1* Δ*POX1* yielded 2.2 g/L extracellular free fatty acids. The main composition of these fatty acids was C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$. In our case, no great effect could be observed for the release of theoretically bound MCFAs. Dga1p seems to prefer incorporation of oleoyl-CoA and palmitoyl-CoA), while myristoyl-CoA and stearoyl-CoA are not frequently used substrates (Oelkers et al., 2002). MCFA, according to our result, appear not to be in the substrate spectrum of Dga1p and do not appear to be preserved as TAGs (Figure 32). It could also be shown by TLC, that the degradation efficiency of Tgl3p is in general not sufficient to prevent the accumulation of TAG. When comparing the results of the empty vector approach with the results from Leber et al. (2015) it becomes clear, that *S. cerevisiae* has a problem regarding the expression of two genes simultaneously, when using a strong constitutive promoter. The difference between the approaches was the usage of the *TEF1* promoter in contrast to the *HXT7* promoter in this study. The observation were very similar, the extracellular free fatty acid yield and cell density decreased. It is assumed, that the reduction of the fatty acid yield may be due to the decreased cell density and higher metabolic burden. No significant difference in LCFA synthesis can be observed in case of *DGA1* and *TGL3* expression. The pattern of LCFA stays almost the same for all mutant strains. The wildtype strain though has a significantly lower amount of unsaturated fatty acids, which indicates again that the addition of unsaturated fatty acids helps yeast cells to maintain a normal membrane morphology in presence of MCFA.

For further use of the established yeast production strains several point of improvement could be implemented. The supplementation of precursors required for the synthesis of fatty acids is essential in the approach to improve the synthesis. The initial substrate for fatty acid synthesis is acetyl-CoA. Since the production of cytosolic acetyl-CoA needs two substrates, acetate and CoA, the level of CoA might limit acetyl-CoA production. Schadeweg and Boles (2016) improve the synthesis of CoA in *S. cerevisiae* by overexpression of the pantothenate kinase *coaA* gene from *E. coli*. NADPH is also described as the bottle neck of fatty acid synthesis. Mishra et al. (2016) indicated via metabolic modelling experiments, that the G6PDH and GND have a higher capability to regenerate the NADPH. An overexpression could lead to more NADPH, which could subsequently be used as an electron donor by FAS.

Yarrowia lipolytica is a promising microbial cell factory for the production of fatty acids. Therefore, the establishment of the FAS mutations in the *Y. lipolytica* genome could reach higher yield, without great influence on the metabolic processes of the cell (Bankar et al., 2009; Beopoulos, Chardot, et al., 2009; Blazeck et al., 2014; Ledesma-Amaro and Nicaud, 2016). An alternative choice could be *Schwanniomyces occidentalis*, a oleaginous yeast from the class *Saccharomycetes*, which can grow on different carbon sources (glucose, fructose, galactose, arabinose, xylose glycerol, cellobiose, starch, maltose, sucrose) and has the ability to grow at a broad temperature (25 - 33 °C, with 80 % of the max. growth rate) and pH range (3.5 - 6.5) (Lamers et al., 2016). It is Crabtree negative, thus the oxidative metabolism is not repressed by high sugar concentrations and high-efficiency electro-transformation protocols are already established (Costaglioli et al., 1994).

4.3.6 Three acyl-CoA thioesterase with different substrate spectrum

It could be demonstrated, that the FAS complex is able to synthesizes MCFA. The fatty acids of interest have to be further hydrolysed by specific enzymes, because the end product of the FAS complex are CoA ester. A transport across the membrane via active transporters to the medium is subsequently possible. The impact of transporter proteins on the fatty acid spectrum was not analysed in this study. A special focus was placed on the acyl-CoA hydrolysing enzymes. Three acyl-CoA thioesterases (Mgl2p, Eeb1p and Eht1p) were examined regarding their influence on the MCFA production (Saerens et al., 2006; Knight et al., 2014). The genes were deleted in different combinations to identify the origin of acyl-CoA hydrolysing activity in combination with FAS mutants. Saerens et al. (2006) reported specificity of Eht1p for octanoic acid CoA ester, which could be confirmed. The strain Δ*eht1* has the lowest amount of octanoic acid, when compared to the parental strain.

The data suggest also a substrate specificity of Eeb1p towards hexanoic acid CoA ester. This knowledge can be used in further improvement tasks to increase the product purity by deletion and expression of relevant thioesterases. For example, could the Δ*eeb1* strain be used for the RK wt FAS with an overexpression of *EHT1* and lead in doing so to higher product yield and purity. Leber and DaSilva (2014) engineered *S. cerevisiae* for the synthesis of fatty acids (C_6 - C_{10}) by expression of various thioesterases with different substrate spectrum. Alexson and Nedergaard (1988) could isolate and characterize two acyl-CoA hydrolases, with preferred substrate spectrum from propionyl-CoA to nonanoyl-CoA. And Tes1, a peroxisomal acyl-CoA thioesterase in *S. cerevisiae*, could be used if the peroxisome targeting signal is removed. The enzyme is required for the degradation of short chain fatty acids and branched fatty acids, and showed high activity toward butyryl-CoA, decanoyl-CoA and 8-methyl-nonanoyl-CoA (Maeda et al., 2006). Hence, using recombinant thioesterases with a narrow product spectrum might lead to better productivity.

The deletion of *EHT1, MGL2* and *EEB1* has a negative influence on the growth of the cells. The strains, wildtype and FAS mutants, show decreased end optical densities. The toxic effect of the MCFA on the cell membrane and metabolism could be a possible reason, because the cells cannot export the MCFA as fee fatty acids out of the cytoplasm. On the other hand, the wildtype strain is also affected by the deletion, which leads to the conclusion that the thioesterases have also other functions inside the metabolic pathways of the yeast (Knight et al., 2014). For the Eht1p enzyme a localization in LD was predicted with a interaction with Osh4p, a member of the oxysterol binding protein family in yeast (Grillitsch et al., 2011; Currie et al., 2014). Osh4p is involved in the regulation of the Golgi complex secretory function (Fang et al., 1996) and in the regulation of lipid metabolism (Raychaudhuri and Prinz, 2010). Eht1p may in this case be important for Osh4p function and lead to disturbance in the lipid metabolism or error in the Golgi secretory system. It has to be mentioned that Zhuang et al. (2015) overexpressed *EHT1* and *EEB1* in *Pichia pastoris*, which lead to the formation of volatile C_6 - C_{14} methyl and ethyl esters. No difference between the two enzymes regarding the substrates could be determined, which is in contradiction with previous findings in *S. cerevisiae*. The verification of volatile FAME or FAEE during or after fermentation were not performed in this study. It cannot be excluded, that a portion of the synthesized medium chain acyl-CoA esters are processed into volatile esters. The fruity aromas from ethyl hexanoate (sour apple) or ethyl octanoate (sour apple) could not be clearly verified by olfactory examination during this study (Van Laere et al., 2008; Lorenzoni et al., 2012).

4.3.7 Continuous product removal with a ion exchange resins

To optimize the process of MCFA synthesis without metabolic engineering the cells were incubated with an ion exchange resin, that can absorb the synthesised MCFA from the culture medium and bind it. Amberlite IRA 410 Cl is a strongly basic anion exchange resin, with a styrene divinylbenzene copolymer (with a $-N^{+}(CH_3)_2C_2H_4OH$ functional group). With the binding of the fatty acids to Amberlite IRA 410 Cl a negative influence on the cell membrane, cell metabolism and cell growth could be avoided, like presented by Srivastava et al. (1992) for the production of lactic acid.

It was confirmed, that the cells grow to a higher end optical density and the overall yield for example for mutant RK wt has increased by 16 % compared to the cultivation without amberlite. The evaluation of Amberlite IRA 410 Cl as a potential ion exchange resin shows that not only MCFA are bound, but the largest part of overall fatty acids eluted from the resin after the fermentation were LCFA (230 - 320 mg/L). Therewith, the available "space" is occupied with LCFA and the MCFA cannot be bound. This means that, additional ion exchange resins should be tested to find optimal binding conditions and optimize elution protocols. In the development of a large-scale industrial fermentation process, the first step is transfer to small fermenters. Thereby, the possibility of controlling a number of culture parameters, like oxygen availability, carbon source, nitrogen and others, can result in higher cell densities and product yield (King et al., 1989). And the downstream costs in the process could drop, with the a high selectivity for the end product (Woodley, 2008). With the transfer of the process to a small fermenter, a continuous product removal with a ion exchange resin can be performed. Mirata et al. (2010) could show by production of perillic acid, that the utilisation of an ion exchange resin during a fed-batch cultivation leads to a 2.8-fold increase in product yield compared to fed-batch fermentation without resin. With this approach the MCFA concentration in the culture medium could be kept to a minimal and cells would not have to adapt to the toxic environment.

4.4. Outlook

MCFA have been shown to have a toxic effect on *S. cerevisiae*, as a result the implementation of a sink towards a different compound resulting from MCFA would be advantageous.

MCFA could be converted to FAEEs through an esterification reaction with ethanol, performed by a bacterial wax ester synthase (WS) (Kalscheuer et al., 2006) or the WS2 from *Marinobacter hydrocarbonoclasticus*, which has been described as the most active enzyme in *S. cerevisiae* (Shi et al., 2012). Fatty alcohols and petroleum-derived alkanes have numerous applications as fuels, fragrances and detergents.

The conversion of MCFA by carboxylic acid reductase (CAR) from *Mycobacterium marinum* into corresponding aldehydes could be a different implementation of a sink. In *E. coli* CAR needs free fatty acids, NADPH and ATP for the production of fatty aldehydes (Akhtar et al., 2012). The produced aldehyde could serve as a substrate for a cyanobacterial aldehyde decarbonylase and an aldehyde reductase to produce fatty alkanes and fatty alcohols, respectively. A short form to produce fatty aldehyde in *S. cerevisiae* would be the use of the fatty acyl-CoA reductases from *Acinetobacter* (*ACR1*, NADPH dependent), which needs acyl-CoA esters as substrate, the products of the FAS (Akhtar et al., 2012). A more general approach would be the expression of a aldehyde/ alcohol dehydrogenase from *Clostridium acetobutylicum* (Yoo et al., 2016). The enzyme has a wide substrate spectrum and could use MCFA CoA ester to produce alcohols (Atsumi et al., 2008; Schadeweg and Boles, 2016). Another way for the synthesis of alkanes is the use of the aldehyde deformylating oxygenase from cyanobacteria (Schirmer et al., 2010), which catalyses the oxygen-dependent conversion of aldehyde to an alkane and formate (Warui et al., 2011; Li et al., 2012). The native hydrocarbon chain-length is between C_{15} - C_{17}. The production of medium chain-length alkanes (C_9 - C_{14}) was established in *E. coli* by the expression of *CER1*, a fatty aldehyde decarbonylase from *Arabidopsis thaliana* (Aarts et al., 1995; Choi and Lee, 2013). A greater challenge would be the implementation of medium chain length polyhydroxyalkanoate (PHA) synthesis in *S. cerevisiae*. The synthesis of PHA in the peroxisome of *S. cerevisiae* by using intermediates of β-oxidation is already established (Poirier et al., 2001). *S. cerevisiae* expressing a cytosolic medium chain length PHA polymerase from *Pseudomonas oleovorans* can also use the 3-hydroxyacyl CoA intermediates from fatty acid metabolism to synthesize PHAs (Zhang et al., 2006). The next step would be to manipulate FAS domain KR and DH to release the 3-hydroxy-acyl-ACP, which could serve as a substrate for the 3- hydroxyacyl-ACP thioesterase from *Pseudomonas putida* (PhaG). The CoA addition to the free 3-hydroxy fatty acids could be performed by a fatty acid CoA-ligase. The resulting 3-hydroxyacyl-CoA, the substrate of the PHA-Synthase (PhaC1), could be polymerized to poly-3-hydroxyalkanoate (PHA) (Heinrich et al., 2016).

5. Zusammenfassung

Der Biotechnologie wird eine große Rolle bei der zukünftigen Bereitstellung von alternativen Energieträgern sowie Grund- und Feinchemikalien aus nachwachsenden Rohstoffen zugesprochen. Dabei bietet die Biotechnologie eine Chance, völlig neuartige chemische Substanzen zu entwickeln, die sonst nur über aufwendige chemische Synthesen herzustellen wären. Heutzutage können Mikroorganismen in Betracht gezogen werden, um diese Substanzen mit verbesserten oder äquivalenten Eigenschaften zu produzieren.

Verschiedene Studien behandeln das Thema der Fettsäureproduktion und dessen Folgeprodukte in der Bäckerhefe *Saccharomyces cerevisiae*. Bei *S. cerevisiae* handelt es sich um einen weit verbreiteten Organismus, welcher in der Nahrungsmittelindustrie sowie bei der Produktion von Bioethanol, therapeutischen Proteinen und Impfstoffen zum Einsatz kommt (Hong and Nielsen, 2012). Durch die langjährige Erfahrung in der Bioethanolproduktion sind Fermentationsprozesse sehr gut realisierbar (Chen et al., 2013). *S. cerevisiae* hat für die industrielle Produktion von Basis- sowie Spezialchemikalien wie z.B. Fettsäuren oder Fettsäurederivate viele Vorteile, da es etablierte molekularbiologische Arbeitsmethoden gibt, eine einfache Zusammensetzung von Kultivierungsmedien ausreicht, eine kurze Verdopplungszeit vorherrscht sowie eine Robustheit gegenüber rauen äußeren Einflüssen ausgeprägt ist.

Fettsäuren sind essentielle Moleküle der Zelle, die durch die hydrophobe Art der Acyl-Ketten für die Bildung von Membran-Bilayer-Strukturen genutzt werden. Außerdem dienen Fettsäuren der Speicherung von Energie, welche während der β-Oxidation in Form von Acetyl-CoA freigesetzt werden kann (Tehlivets et al. 2007). Bei der Fettsäuresynthese werden zwei Hauptenzyme benötigt, die Acetyl-CoA Carboxylase (Acc1p) und der Fettsäuresynthase Komplex (FAS). Es werden überwiegend langkettige Acyl-CoAs synthetisiert (C_{16} - C_{18}) (Lynen et al., 1980). Die Interaktion mit Faktoren wie z.B. der Verfügbarkeit von Malonyl-CoA oder Acetyl-CoA, bestimmen die Acyl-Kettenlänge *in vivo* (Tehlivets et al., 2007). Die gebildeten Acyl-CoAs können in *S. cerevisiae* durch die im ER membrangebundene Δ9 Desaturase Ole1p mit Doppelbindungen versehen werden (Tamura et al., 1976; Certik and Shimizu, 1999). Ole1p ist nicht in der Lage mehrfachungesättigte Fettsäure zu synthetisieren (Uemura, 2012). Der Ablauf der Reaktion benötigt neben Sauerstoff noch zwei Elektronen. Bei Pilzen wird das Cytochrom b5 als Elektronendonor verwendet (Los and Murata, 1998).

In der vorliegenden Arbeit wurden im ersten Abschnitt unterschiedliche Desaturasen aus Pilzen charakterisiert. Ungesättigte Fettsäuren können in unterschiedlichen enzymatischen und chemischen Reaktionen, wie der Hydrosilylierung, Hydrovinylierung, Oxidation in Epoxide, Säuren, Aldehyde oder Ketone umgewandelt werden, wodurch das Interesse an ω1-Fettsäuren deutlich steigt (Behr and Gomes, 2010). Mit dem Ziel, Desaturasen aus Pilzen in *S. cerevisiae* zu exprimieren und auf ω1-Desaturase Aktivität zu testen wurden Sequenzen aus unterschiedlichen Genomdatenbanken von Pilzen mittels Sequenzalignments untersucht. Die Nummerierung bei ω-Desaturasen beginnt vom Methyl-Ende (Los and Murata, 1998), womit bei ω1-Desaturasen eine endständige Doppelbindung in der Acyl-Kette entstehen würde. Die Desaturasen aus den Motten *Operophtera brumata* (Obr-TerDes) und *Planotortrix excessana* (Pex-Desat3), mit Fähigkeit zu terminaler Desaturierung (Ding et al., 2011; Albre et al., 2012), wurden als Referenzsequenzen genutzt. Die untersuchten Enzyme der Pilze *Schizophyllum commune* (EFI94599.1), *Schizosaccharomyces octosporus* (EPX72095.1), *Wallemia mellicola* (EIM20316.1), *Wallemia ichthyophaga* (EOR00207.1) und *Agaricus bisporus var. bisporus* (EKV44635.1) konnten als Δ9 Desaturasen charakterisiert werden. Bei den Enzymen von *S. commune* (EFI94388.1) und *Podospora anserina* (CAP70780.1) kann angenommen werden, dass es sich um Δ6 Desaturasen, die γ- Linolensäure ($C18:1^{\Delta6,9,12}$) oder Δ15 Desaturasen, die α-Linolensäure ($C_{18:1}{}^{\Delta9,12,15}$) synthetisieren, handelt. Für beide Pilze wird die Synthese der Vorläuferfettsäure, Linolsäure ($C_{18:2}{}^{\Delta9,12}$) vorhergesagt (Espagne et al., 2008; Ohm et al., 2010). Eine Desaturase mit terminaler Desaturierung konnte nicht mittels der angewandten Methoden identifiziert werden.

Ein weiterer Ansatz zur Synthese von endständig ungesättigten Fettsäuren war die Modifikation der Hefe-eigenen Desaturase Ole1p mittels gezielter Punktmutationen. Die durch Bai et al. (2015) aufgelöste Struktur der Δ9 Desaturase aus der Maus (*M. musculus*) konnte dabei entscheidende Anhaltpunkte für Aminosäuren, die wichtig für die Substratspezifität und Enzymaktivität sind, liefern. Zusätzlich wurden zuvor publizierte Daten zu den Desaturasen von *Calanus hyperboreus* (ChDes1), *Drosophila melanogaster* (Desat2), *Planotortrix excessana* (Pex-Desat3) und *Operophtera brumata* (Obr-TerDes) aufgenommen, um gezielte Aminosäureaustausche in Ole1p in *S. cerevisiae* durchzuführen (Dallerac et al., 2000; Ding et al., 2011; Albre et al., 2012; Meesapyodsuk and Qiu, 2014). Alle untersuchten Ole1p Mutanten konnten ausschließlich Δ9 Desaturaseaktivität ausführen, da das Produktspektrum nicht vom Wildtypspezifischen zu unterscheiden war. Dies deutet drauf hin, dass alle Aminosäureaustausche die Substratspezifität und die Position der einzuführenden Doppelbindung nicht verändern konnten.

Im zweiten Abschnitt der vorliegenden Arbeit wurde der Fokus auf die Optimierung der Fettsäuresynthese gelegt. *S. cerevisiae* hat naturgemäß einen geringen Fettsäureanteil, der nicht für eine industrielle Etablierung von Produktionsprozessen ausreicht. Der Lipidanteil in oleogenen Hefen liegt zwischen 30 - 50 % und bei der Bäckerhefe zwischen 5 - 7 % (Uemura 2012). Beim Vergleich des Metabolismus von oleogenen und nicht- oleogenen Hefen wurde festgestellt, dass z.B. *Candida tropicalis* einen guten Ausgangspunkt für die Erhöhung des Acetyl-CoA Pools und damit der Fettsäuresynthese in *S. cerevisiae* darstellt (Liu et al., 2013). Die Etablierung eines Hauptteils des Fettsäuremetabolismus von *C. tropicalis* in *S. cerevisiae* durch Expression der zytosolischen Malat Dehydrogenase (*MDH3*), des zytosolischen Malat Enzyms (*MAE1*), des Citrat - α-Ketoglutarat Transporters (*YHM2*) sowie der ATP-Citrat Lyase (*ACL1*) konnte die Acetyl-CoA Verfügbarkeit im Zytosol nicht hinreichend steigern um einen positiven Effekt in der Fettsäureausbeute festzustellen. Für alle getesteten *S. cerevisiae* Stämme mit optimierter Expression der Acetyl-CoA Carboxylase und Fettsäuresynthase, die schon eine hohe Fettsäureausbeute aufweisen, ist keine Verbesserung nachzuweisen. Nur beim Einsatz von nicht-optimierten *S. cerevisiae* Stämmen kann eine zusätzliche Expression der ATP-Citrat Lyase in Kombination mit der zytosolischen Malat Dehydrogenase zu einem Anstieg des Fettsäuregehalts von 41 % (20mg/g Ztm) führen.

Ein weiterer Ansatz zur Steigerung der Fettsäuresynthese wurde mit der Expression von *DGA1* und *TGL3* verfolgt. Die Diacylglycerol Acyltransferase Dga1p ist eines der Hauptenzyme in der Triacylglycerol (TAG) Synthese in *S. cerevisiae* (Dahlqvist et al., 2000; Oelkers et al., 2002; Sandager et al., 2002), wohingegen Tgl3p die Hydrolyse der gebildeten TAG in Diacylglycerol und eine freie Fettsäure katalysiert (Athenstaedt and Daum, 2003). Leber et al. (2015) konnte eine Verbesserung zu Gunsten der Neutrallipidsynthese und dem anschließende Abbau durch die Überexpression von *DGA1* und *TGL3* erzielen und dabei 2.2 g/L langkettige freie Fettsäuren produzieren, die im Medium nachweisbar waren. Der beste Produktionsstamm der vorliegenden Arbeit, *S. cerevisiae* WRY1Δ*FAA1*Δ*FAA4* p*HXT7-DGA1* p*HXT7-TGL3*, konnte 2.5 g/L ± 0.8 g/L intra- und extrazelluläre Fettsäuren herstellen. Davon waren 64 % (~1.6 g/L) freie Fettsäuren, die im Wachstumsmedium nachweisbar waren. Eine zusätzliche Deletion der Acyl-CoA Synthetase Fat1p führte zu keiner Verbesserung der Fettsäureausbeute.

In der vorliegenden Arbeit wurde im dritten Abschnitt die Charakterisierung und Optimierung der Fettsäuresynthase und damit verbunden die Synthese von Fettsäuren mit 6 - 12 Kohlenstoffatomen mittels der Bäckerhefe *S. cerevisiae* untersucht. Mittellange Fettsäuren können unter anderem als Fettalkohole oder Alkane eine Bandbreit von Applikationen als Biokraftstoffe, Fungizide, Duftstoffe oder Detergenzien haben.

Der Fettsäuresynthase Komplex wird aus zwei Untereinheiten zusammengesetzt, Fas1(β) und Fas2 (α). Die β-Untereinheit besteht aus der Acetyltransferase (AT), Enoyl Reduktase (ER), Dehydratase (DH) und dem Großteil der Malonyl-Palmitoyl Transferase (MPT). Die α-Untereinheit fasst die Acyl Transportsproteine (ACP), 3-Ketoreduktase (KR), 3-Ketosynthase (KS), Phosphopanthein Transferase (PPT) und einen Teil der Malonyl-Palmitoyl Transferase (MPT) zusammen (Tehlivets et al., 2007; Gipson et al., 2010). Vor kurzem wurde durch Gajewski et al. (2017a) gezeigt, dass der bakterielle Fettsäuresynthase Komplex hinsichtlich der Selektion der Kettenlänge von synthetisierten Acyl-CoA modifiziert werden kann. Indem die korrespondierenden Aminosäuren in den Domänen AT, MPT und KS (Vorbereitungs-, Beladungs- und Verlängerungsschritt) ausgetauscht wurden, konnte dieses Wissen auf die FAS in *S. cerevisiae* übertragen werden. Durch die AT Domäne wird Acetyl-CoA in die Kettenverlängerung einbracht, wohingegen die MPT Domäne, das durch Acc1p gebildete Malonyl-CoA aufnimmt und mittels ACP der KS Domäne zur Verfügung stellt. Für die MPT Domäne (Aminosäure R1834) sollte die Affinität gegenüber Malonyl-CoA verringert und ein erleichterter Weg der Freigabe von Acyl-CoAs hergestellt werden. Die AT Domäne (Aminosäure I306) stellt eine Möglichkeit dar, das Verhältnis von Acetyl zu Malonyl zu verbessern und einen zusätzlichen Bindekanal für Acylketten bis zu einer Länge von zehn Kohlenstoffatomen bereitzustellen. Innerhalb der KS Domäne stellen drei Aminosäuren eine Option dar, die Kettenverlängerung vorzeitig zu unterbrechen (M1251, G1250, F1279) (Christensen et al., 2007b; Johansson et al., 2008). Bei der Mutante IARK GSMW, die in allen drei Domänen manipuliert wurde, konnte bei Plasmid-basierter Expression und Nutzung der *FAS1* und *FAS2* Wildtyppromotoren eine Gesamtausbeute von 114 mg/L C_6-C_{12} Fettsäuren erzielt werden. Im selben Schritt konnte die größte Menge an Oktansäure (78 mg/L) und Decansäure (22 mg/L) synthetisiert werden. Hinsichtlich der Synthese von Hexansäure stellte sich die Mutante IARK GS mit 49 mg/L als bester Stamm heraus, während die Mutante IA GS vorherrschend C_6 synthetisiert (88 % Hexansäure).

Ein weiterer Ansatz war die Steigerung der Ausbeute durch *metabolic engineering* und Anpassung der Fermentationsbedingungen. Die Wildtyppromotoren von *FAS1* und *FAS2* wurden durch den Promotor der Alkohol Dehydrogenase II aus *S. cerevisiae* (p*ADH2*) ersetzt. In Anwesenheit von Glukose im Medium ist p*ADH2* reprimiert, und initiiert die Genexpression in der stationären Wachstumsphase der Bäckerhefe, wenn die Ethanolkonzentration steigt.

Außerdem wurde die Acyl-CoA Synthetase Faa2p mittels der CRISPR/Cas9 Methode ausgeschaltet. Faa2p ist ein membranständiges peroxisomales Protein (Hettema et al., 1996), welches C_7-C_{17} Fettsäuren für die β-Oxidation aktivieren kann (Johnson et al., 1994). Der Hefestamm *S. cerevisiae* Δ*faa2* kann dementsprechend keine mittellangen Fettsäuren abbauen. *S. cerevisiae* kann neben der β-Oxidation freie Fettsäuren in Triacylglycerol (TAG) oder Sterolester (SE) einbinden. Um die Ausbeute zu erhöhen wurden die Diglyceride Acyltransferase (*DGA1*) und Triacylglycerol Lipase (*TGL3*) überexprimiert. Ein nutzbringender Effekt konnte in Bezug auf die Ausbeute nicht festgestellt werden. Die Anpassung der Fermentationsbedingungen wurde durch Auswahl eines besseren Wachstumsmediums (YEPD-bacto) eingeleitet, das sich durch eine besondere Zusammensetzung des eingesetzten Peptons im Vergleich zum YEDP-oxoid Medium hervorhebt. Bei der Untersuchung des pH Wertes des Wachstumsmediums konnte eine Verringerung dessen bei steigender Syntheserate von mittellangen Fettsäuren festgestellt werden. Aus diesem Grund wurde 100 - 250 mM mit Kaliumphosphat gepuffertes YEPD-bacto Medium eingesetzt. Dies führte zu einem pH Wert, der zu vergleichen war mit dem des Wildtyp *S. cerevisiae* Stammes und zeitgleich zu einer Steigerung der Ausbeute. Die Synthese von mittellangen Fettsäuren konnte mit den oben genannten Verbesserungen auf ein Maximum von 464 mg/L (C_6-C_{12}) gesteigert werden. Für die spezifischen Ausbeuten konnten 158 mg/L Hexansäure und 301 mg/L Oktansäure erzielt werden. Die Charakterisierung von Transferasen, die für die Hydrolyse der CoA-gebundenen Fettsäuren verantwortlich sein könnten, wurden in einem weiteren Ansatz untersucht. Für *S. cerevisiae* wurden mehrere Thioesterasen aufgelistet, welche die Hydrolyse von mittellangen Acyl-CoA Estern in freie Fettsäuren katalysieren. Im Rahmen dieser Arbeit konnte gezeigt werden, dass die Gene *EHT1*, *EEB1* und *MGL2* einen großen Einfluss auf die Ausbeute der mittellangen Fettsäuren haben. Die Daten deuten darauf hin, dass Eeb1p eine selektive Aktivität gegenüber C_6-CoA Estern und Eht1 gegenüber C_8-CoA Estern aufweist.

6. References

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7. Appendices

I. Abbreviations

%	percent
Δ	delta/ deletion of a gene
°C	degrees Celsius
µF	microfarad
A	ampere
mA	milliampere
g	gram
g (in x g)	multiple of normal gravitational acceleration
g dcw	gram dry cell weight
h	hour
kDa	kilodalton
l	litre
µ	micro (10^{-6})
M	molar
m	meter, milli (10^{-3})
mg	milligram
µg	microgram
min	minute
ml	milliliter
µl	microliter
M	molar concentration
mM	millimolar
mmol	millimole
mol	mole
m/Q	mass-to-charge ratio
ng	nanogram
nm	nanometer
s	second
rpm	revolutions per minute
V	volt
v/v	volume per volume
W	watt
w/o	without
w/v	weight per volume
AA-solution	amino acid solution
ACP	acyl carrier protein
acyl-DHAP	acyl dihydroxyacetone phosphate
AEATase	octanoyl-CoA:ethanol acyltransferase
AT	acetyl transferase
AMP	adenosine-5‘-monophosphate
ADP	adenosine-5‘-diphosphate
ATP	adenosine-5‘-triphosphate
approx.	approximately
bp	basepairs
BLAST	basic local alignment search tool
CoA	coenzyme A
C-terminus	carboxyl-terminus
D	D-glucose (medium)
DAG	diacylglycerol
DH	dehydratase
DHAP	dihydroxyacetone phosphate
DMDS	dimethyl disulphide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside
DTT	dithiothreitol
E	ethanol (medium)
ed./eds.	editor/editors
e.g.	*exempli gratia*
EDTA	ethylene diamine tetraacetic acid
ER	endoplasmatic reticulum
ER	enoyl reductase
et al.	*et alii*
EtBr	ethidium bromide
FAEE	fatty acid ethyl ester
FAL	fatty alcohol
FAME	fatty acid methyl ester
FAS	fatty acid synthase complex
FAS I	type I fatty acid synthase
FAS II	type II fatty acid synthase

FFA	free fatty acid
G	D-galactose (medium)
G3P	glycerol 3-phosphate
G418	geneticin
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GRAS	generally regarded as safe
gRNA	guide RNA
3-HP	3-hydroxypropionic acid
H^+	proton
H_2O_{bidest}	double-distilled water
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
ISPR	*in situ* product removal
kb	kilo base pairs
KR	ketoacyl reductase
KS	ketoacyl synthase
LB	lysogeny broth (medium)
LC	long chain
LCA	lithocholic acid
LCFA	long chain fatty acid
LD	lipid droplet
LiAc	lithium acetate
LPA	lysophosphatidic acid
lyso-PA	lyso-phosphatidic acid
MC	medium chain
MCFA	medium chain fatty acid
MCFAL	medium chain fatty alcohol
MPT	malonyl/palmitoyl transferase
MS	mass spectrometry
mRNA	messenger RNA
NAD^+/ NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/ NADPH	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NaOH	sodium hydroxide
OD/ OD_{600}	optical density, usually at 600 nm
ORF	open reading frame
P	phosphate
p	promoter
PA	phosphatidic acid
p*ADH2*	*ADH2* promoter
PAM	protospacer-associated-motif
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	decimal logarithm of the reciprocal of the hydrogen ion activity
PHA	polyhydroxyalkanoate
P_i	inorganic phosphate
PPP	pentose phosphate pathway
PPT	phosphopantetheine transferase
R	raffinose (medium)
RNA	ribonucleic acid
RT	room temperature
SC	synthetic complete (medium)
SCFA	short chain fatty acid
SDS	sodiumdodecylsulfate
SE	sterol ester
SGD	*Saccharomyces* Genome Database
SH-group	sulphydryl group
sp.	species
ss	single stranded
t	terminator
TAE-buffer	TRIS-Acetat-EDTA buffer
TAG	triacylglycerol
Taq	*Thermus aquaticus*
TCA cycle	tricarboxylic acid cycle
TLC	thin layer chromatography
TM	transmembrane domain
UV	ultraviolet
WE	wax ester

wt	wildtype
YEP	yeast extract peptone (medium)
YEP-bacto	peptone from BD Difco™ in yeast extract peptone (medium)
YEP-oxoid	peptone from Oxoid in yeast extract peptone (medium)
Ztm	Zelltrockenmasse

II. DNA oligonucleotides

The primers are divided into different sections. In Table 23 DNA oligonucleotides are listed, which were used for cloning. Table 24 summarized the primers for amplification of deletion cassettes or CrispRCas9 plasmids (including donor DNA). Primers for PCR-based verification of the correct integration of a deletion cassette in the respective ORF or scarless deletion of respective ORF are listed in Table 25. Primers used for the targeted replacement of codons in the respective ORF are listed in Table 26. Table 27 specifies primers used for sequencing DNA fragments and plasmids.

Table 23: DNA oligonucleotides used in this work for cloning.

name	**sequence (5'-3')**	**description**
desaturase		
Ole1-pRSK-fw	CACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGCCAACTTCTGGAACTACTATTGAATTGATTG	amplification of *OLE1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Ole1-pRSK-rev	CGTGAATGTAAGCGTGACATAACTAATTACATGACTCGAGTTAAAAGAACTTACCAGTTTCGTAG	amplification of *OLE1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Desat3 fw	CAAAAAGTTTTTTTAATTTTAATCAAAAAGTTAACATGGTTCCAGACGTCTTGAGAG	amplification of Pex-Desat3, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Desat3 rev	CGTGAATGTAAGCGTGACATAACTAATTACATGACTTAACCAGACTTCAAACCACCTG	amplification of Pex-Desat3, reverse, overhang homologous to the 5' end of the CYC1-promoter
Obr_Desat-S1	AAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGGCTCCAAACACTTTGAAGGAAGATG	amplification of Obr-TerDes, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Obr_Desat-S2	GCGTGAATGTAAGCGTGACATAACTAATTACATGACTCGAGTTAAGTGTTCTTTTCGTTG	amplification of Obr-TerDes, reverse, overhang homologous to the 5' end of the CYC1-promoter
Scom-S1	ACAAAGAATAAACACAAAAACAAAAAGTTTTTTTAATTTTAATCAAAAAATGTCCGACGTCGGTGTTG	amplification of *EFI94599.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Scom-S2	GTGAATGTAAGCGTGACATAACTAATTACATGACTCGAGTTAGAAACCAGCTTCAGAAGAG	amplification of *EFI94599.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter

Socto-S1	CAAAGAATAAACACAAAAACA AAAAGTTTTTTTAATTTTAATC AAAAAATGTCTTCTAACGGTAT CTCTC	amplification of *EPX72095.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Socto-S2	GTGAATGTAAGCGTGACATAA CTAATTACATGACTCGAGTTAG TTAGCAGCAGCGGCTTC	amplification of *EPX72095.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Cap1-hxt7-fw	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGTCCGTCACCGTCACCGAA GAACCG	amplification of *CAP70780.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Cap1-cyc1-rev	GGCGTGAATGTAAGCGTGACA TAACTAATTACATGACTCGAGT TAATGGCTTTCCTCCTCC	amplification of *CAP70780.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Ekv1-hxt7-fw	AAACACAAAAACAAAAAGTTT TTTTAATTTTAATCAAAAAATG CCAGCTTCTACTGCTACCAAGG	amplification of *EKV44635.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Ekv1-cyc1-rev	CGTGAATGTAAGCGTGACATA ACTAATTACATGACTCGAGTTA ACCCAACAAACCTTC	amplification of *EKV44635.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Eim1-hxt7-fw	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGGAATCTGCTAACGCTTAC ATTATTAC	amplification of *EIM20316.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Eim1-cyc1-rev	GAATGTAAGCGTGACATAACT AATTACATGACTCGAGTTACTT AGCCTTTCTAATGTG	amplification of *EIM20316.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Eor1-hxt7-fw	CACAAAAACAAAAAGTTTTTTT AATTTTAATCAAAAAATGGCTT TCGAACAAGTTGTTCC	amplification of *EOR00207.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Eor1-cyc1-rev	GTGAATGTAAGCGTGACATAA CTAATTACATGACTCGAGTTAA GAATCAGCTTGCTTC	amplification of *EOR00207.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter
Efi1/Scom2-hxt7-fw	TAAACACAAAAACAAAAAGTT TTTTTAATTTTAATCAAAAAAT GTCTGTTGAAAAGTACGTCC	amplification of *EFI94388.1*, forward, overhang homologous to the 3' end of the truncated HXT7-promoter
Efi1/Scom2-cyc1-rev	CGTGAATGTAAGCGTGACATA ACTAATTACATGACTCGAGTTA GTCAGATTGTTCACCAGA	amplification of *EFI94388.1*, reverse, overhang homologous to the 5' end of the CYC1-promoter

pPDC1	GCACAGATGCGTAAGGAGAAA ATACCGCATGAGTTTTTGGTGG TTCCGGCTTCCTTCCC	amplification of *PDC1* promoter, forward, overhang homologous to the plasmid pRS72N
pPDC1-Cyb5	CATTGTGTTCGGCAACTTCTTG GTAACTGTAAACTTTAGGCATT TTGATTGATTTGACTGTGTTAT TTTG	amplification of *PDC1* promoter reverse, overhang homologous to the *CYB5* gene
Cyb5-pPDC1	CATAACCTCACGCAAAATAAC ACAGTCAAATCAATCAAAATG CCTAAAGTTTACAGTTACCAAG	amplification of *CYB5* gene, forward, overhang homologous to the *PDC1* promoter
Cyb5-tPGK	GAAAAGAAAAAAATTGATCTA TCGATTTCAATTCAATTCAATT TATTCGTTCAACAAATAATAAG	amplification of *CYB5* gene, reverse, overhang homologous to the *PGK1* terminator
tPGK-Cyb5	GCTAGGTGTTGCTTATTATTTG TTGAACGAATAAATTGAATTG AATTGAAATCGATAGATC	amplification of *PGK1* terminator, forward, overhang homologous to the *CYB5* gene
tPGK	CGCGGCCTTTTTACGGTTCGGC GAATTGGGTACCGGCCAAATA ATATCCTTCTCGAAAGC	amplification of *PGK1* terminator, reverse, overhang homologous to the plasmid pRS72N
fatty acid synthase		
pADH2-Fas1	GAACCGTGAGATAGGGTTAAT GGTCTTGTGGAGTAAGCGTCCA TTGTGTATTACGATATAGTTAA TAGTTGATAG	amplification of *ADH2* promoter, forward, overhang homologous to the 3' end of *FAS1* gene in pRS315
Fas1-pADH2	GCATACAATCAACTATCAACTA TTAACTATATCGTAATACACAA TGGACGCTTACTCCACAAGACC ATTAAC	amplification of ADH2 promoter, reverse, overhang homologous to the 5' end of *FAS1* gene in pRS315
pADH2-Fas2	GCAAAATATGAGCTAATTCTTG CTCAACTTCCGGCTTCATTGTG TATTACGATATAGTTAATAG	amplification of *ADH2* promoter, forward, overhang homologous to the 3' end of *FAS2* gene in pRS313
Fas2-pADH2	GCATACAATCAACTATCAACTA TTAACTATATCGTAATACACAA TGAAGCCGGAAGTTGAGCAAG AATTAG	amplification of *ADH2* promoter, reverse, overhang homologous to the 5' end of *FAS2* gene in pRS313
thioesterase		
Eht1-pHXT7	AAACACAAAAACAAAAAGTTT TTTTAATTTTAATCAAAAAATG TCAGAAGTTTCCAAATG	amplification of *EHT1*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter
Eht1-tCYC1	GTGAATGTAAGCGTGACATAA CTAATTACATGACTCGAGTCAT ACGACTAATTCATCAAAC	amplification of *EHT1*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter
Eeb1-pHXT7	CACAAAAACAAAAAGTTTTTTT AATTTTAATCAAAAAATGTTTC GCTCGGGTTACTATCC	amplification of *EEB1*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter

Eeb1-tCYC1	GAATGTAAGCGTGACATAACT AATTACATGACTCGAGTTATAA AACTAACTCATCAAAG	amplification of *EEB1*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter
YMR210w-hxt7	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGCGTCTAAAAGAATTGTTA CC	amplification of *MGL2*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter
YMR210w-cyc1	GCGTGAATGTAAGCGTGACAT AACTAATTACATGACTCGAGCT AATTCGCGCGAAAGGTTGTGG	amplification of *MGL2*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter
TesA_opti-hxt7	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGGCTGACACCTTATTGATT TTGG	amplification of *TESA*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter
TesA_opti-woSignal	CGTGAATGTAAGCGTGACATA ACTAATTACATGACTCGAGTTA AGAGTCGTGGTTAACCAATG	amplification of *TESA*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter
other		
DGA1-hxt7	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGTCAGGAACATTCAATGA TATAAG	amplification of *DGA1*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter
DGA1-cyc1	GGAGGGCGTGAATGTAAGCGT GACATAACTAATTACATGACTC GAGTTACCCAACTATCTTCAAT TCTGC	amplification of *DGA1*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter
Tgl3-hxt7	GAATAAACACAAAAACAAAAA GTTTTTTTAATTTTAATCAAAA AATGAAGGAAACGGCGCAGGA ATACAAG	amplification of *TGL3*, forward, overhang homologous to the 3' end of the truncated *HXT7*-promoter
Tgl3-cyc1	GGCGTGAATGTAAGCGTGACA TAACTAATTACATGACTCGAGC TACCTACTCCGTCTTGCTCTTA TTATG	amplification of *TGL3*, reverse, overhang homologous to the 5' end of the *CYC1*-promoter

Table 24: List of DNA oligonucleotides for amplification of deletion cassettes or CrispRCas9 plasmids (including donor DNA).

name	**sequence (5'-3')**	**description**
desaturase		
dOle1-DelPe-for	CAACTTCTGGAACTACTATTGA ATTGATTGACGACCAATTTCCA AATAGGGATAACAGGGTAATC AGCGAC	amplification of a delitto perfetto deletion cassette for integration in *OLE1*, forward
dOle1-DelPe-rev	ATTTTTTTTTATGGTAGTTGCA GTTTTGTTATTGTAATGTGATA CCTTTGTTGTAATGTTTTAGTG CTGTTTATAATATGATCACCAC AATTTACGGTTCGGCGAATTGG GTACCG	amplification of a delitto perfetto deletion cassette for integration in *OLE1*, reverse

thioesterase		
CC_Eeb1_rv	TCCGGCAGGCCCATCGATGGG ATCATTTATCTTTCACTGCGGA G	amplification of a CrispRCas9 plasmid pRCCN for deletion of *EEB1*, reverse
CC_Eeb1_fw	CCATCGATGGGCCTGCCGGAG TTTTAGAGCTAGAAATAGCAA GTTAAAATAAGG	amplification of a CrispRCas9 plasmid pRCCN for deletion of *EEB1*, forward
DR_Eeb1	AGATTAGCAAAAAGATCAAGA TATCAAGTATTTTCATATTTGT CATTTTAATATGTTCTTAGATA AAATTCCTTACACATAATAATC TGTTCTTTTATTT	Donor-DNA fragment for *EEB1* deletion
CC_Eht1_rv	CTGCGAGACAGGTTTTCAGCG ATCATTTATCTTTCACTGCGGA GA	amplification of a CrispRCas9 plasmid pRCCN for deletion of *EHT1*, reverse
CC_Eht1_fw	GCTGAAAACCTGTCTCGCAGGT TTTAGAGCTAGAAATAGCAAG TTAAAATAAGG	amplification of a CrispRCas9 plasmid pRCCN for deletion of *EHT1*, forward
DR_Eht1	TTACATTGATAGTAGTTGCGTA AAAAACAAAGCTCATAAAAGT TTCCGATTGTCACACAATTTTC AAACCACTTTCCTATGTATTTA TACAACATTGGAT	Donor-DNA fragment for *EHT1* deletion
CC_YMR210w _rv	CTCGAACCCCCTGTTAATCCAG ATCATTTATCTTTCACTGCGGA G	amplification of a CrispRCas9 plasmid pRCCN for deletion of *YMR210w*, reverse
CC_YMR210w _fw	TGGATTAACAGGGGGTTCGAG TTTTAGAGCTAGAAATAGCAA GTTAAAATAAGG	amplification of a CrispRCas9 plasmid pRCCN for deletion of *YMR210w*, forward
DR_YMR210w	GTTATTTTAAAACTGTATTATA CAAACGCTGGTAAACTTCCAG AGACGATTTTGTAAATAAGTCA ACTTTTTAGATGTGTAACCTGA TAATTTTTTTTTT	Donor-DNA fragment for *YMR210w* deletion
fatty acyl-CoA synthetase		
CC_FAA2-rv	CGTAAGGTTTCAAAATCTTCGA TCATTTATCTTTCACTGCGGAG	amplification of a CrispRCas9 plasmid pRCCN for deletion of *FAA2*, reverse
CC_FAA2-fw	GAAGATTTTGAAACCTTACGGT TTTAGAGCTAGAAATAGCAAG TTAAAATAAGG	amplification of a CrispRCas9 plasmid pRCCN for deletion of *FAA2*, forward
DR_Faa2	GGAAGAATGCAGGTTACAAAA AACGGATAAGAACAAACTTGT TTCGAAATGTACTTATGACGAT TTGGAACACATTCAAACTAGA AAAAACTTTGATGTA	Donor-DNA fragment for *FAA2* deletion

Table 25: List of DNA oligonucleotides for PCR-based verification of the correct integration of a deletion cassette in the respective ORF or scarless deletion of respective ORF.

name	**sequence (5'-3')**	**description**
desaturases		
OLE1-A1	CATCGAGTCTTGCAAATCA	A1-primer for verification of the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome
OLE1-A2	TGGCATACCACAAACAAG	A2-primer for verification of the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome
OLE1 A3	GGTATTGTTCACGACGTATC	A3-primer for verification of the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome
OLE1 A4	ATGGCCGCCCATAGATTG	A4-primer for verification of the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome
Ole1-1kb-A1	GTCAAGGATTAGCGGATATGT AGTTCCAGTC	A1-primer for verification the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome (approx. 700 bp from ATG)
Ole1-1kb-A4	CCCTGAAGCCGCCTTGCATGGT GCTTTGTC	A4-primer for verification the correct integration of a deletion cassette in *OLE1* in the *S. cerevisiae* genome (approx. 700 bp from TAA)
thioesterase		
Eht1-A1	CGTCGTGGAGCTCACTCAAGA AGTTATCAG	A1-primer for verification of the correct deletion of *EHT1* in the *S. cerevisiae* genome
Eht1-A2	GTCAGCGGTGCAAACTCCACC ATCC	A2-primer for verification of the correct deletion of *EHT1* in the *S. cerevisiae* genome
Eht1-A3	GGATGAGCCAGGATTGGTGGT CAAG	A3-primer for verification of the correct deletion of *EHT1* in the *S. cerevisiae* genome
Eht1-A4	CTGTAAATATGACAGATATTGC CACTCAG	A4-primer for verification of the correct deletion of *EHT1* in the *S. cerevisiae* genome
Eeb1-A1	CCTTCAAGAGAACCGGCACCA AAGC	A1-primer for verification of the correct deletion of *EEB1* in the *S. cerevisiae* genome
Eeb1-A2	TGCGGTAAAGATTCTACGGGT GGTG	A2-primer for verification of the correct deletion of *EEB1* in the *S. cerevisiae* genome

Eeb1-A3	TGGAGCAGCCATGCTAACGAACTAC	A3-primer for verification of the correct deletion of *EEB1* in the *S. cerevisiae* genome
Eeb1-A4	CTGCCCAGAGATCAAATCAAGGAAG	A4-primer for verification of the correct deletion of *EEB1* in the *S. cerevisiae* genome
YMR210w-A1	CCAGTGAGCTAACCATTGGCGCTCCTCAAC	A1-primer for verification of the correct deletion of *YMR210w* in the *S. cerevisiae* genome
YMR210w-A4	ATTACCTGTACGGGCATCCCAAAGTAAGGC	A4-primer for verification of the correct deletion of *YMR210w* in the *S. cerevisiae* genome
fatty acyl-CoA synthetase		
Faa2-A1	GAAGTCCCGGTGTCCCTGACGTTATTGTAG	A1-primer for verification of the correct deletion of *FAA2* in the *S. cerevisiae* genome
Faa2-A4	GTGACCCATGTACTCCGCTAGATTGACCAG	A4-primer for verification of the correct deletion of *FAA2* in the *S. cerevisiae* genome
universal		
K2MX	TTGTCGCACCTGATTGCCCG	K2-primer for verification of the integration of a deletion cassette with kanamycin-resistance-marker (*kanMX4*) in the *S. cerevisiae* genome
K3MX	TATGGAACTGCCTCGGTGAG	K3-primer for verification of the integration of a deletion cassette with kanamycin-resistance-marker (*kanMX4*) in the *S. cerevisiae* genome
CloNAT-K2	GTACGCGACGAACGTCCGGGAGTCC	K2-primer for verification of the integration of a deletion cassette with nourseothricin -resistance-marker (*natNT2*) in the *S. cerevisiae* genome
CloNAT-K3	GAGGACATCGAGGTCGCCCCGGAGCAC	K3-primer for verification of the integration of a deletion cassette with nourseothricin -resistance-marker (*natNT2*) in the *S. cerevisiae* genome

Table 26: DNA oligonucleotides used for the targeted replacement of codons in the respective ORF. Changed DNA bases are written in small letters and underlined.

name	sequence (5'-3')	description
desaturase		
M1S2_C:G_fw	CCAACATTTGAACTGGTTGAAC ATGGTTCTTGTTggtGGTAT	amplification of mutated *OLE1-C120G*, forward
S1M1_C:G_rev	GAGCAAAGTACCAACCAATCA TTGGCATACCaccAACAAG	amplification of mutated *OLE1-C120G*, reverse
M2S2_Y:L_fw	CATTTAAACGTTTTCCTTTTCTC CGTTTTCTACctcGCTGTC	amplification of mutated *OLE1-Y149L*, forward
S1M2_Y:L_rev	CCGGCAGTAATAGAAACACCA CCGACAGCGAGGTAGAAAAC	amplification of mutated *OLE1-Y149L*, reverse
M2S2_Y:T_fw	CATTTAAACGTTTTCCTTTTCTC CGTTTTCTACacCGCTGTCG	amplification of mutated *OLE1-Y149T*, forward
S1M2_Y:T_rev	CGGCAGTAATAGAAACACCAC CGACAGCGgtGTAGAAAAC	amplification of mutated *OLE1-Y149T*, reverse
M3S2_G:V_fw	CTTTTCTCCGTTTTCTACTACGC TGTCGGTGTTGTTTCTAT	amplification of mutated *OLE1-G153V*, forward
S1M3_G:V_rev	CATAATCTATGGTAACCGGCA GTAATAGAAACAACACCGAC	amplification of mutated *OLE1-G153V*, reverse
M3S2_G:M_fw	CTTTTCTCCGTTTTCTACTACGC TGTCGGTatgGTTTCTAT	amplification of mutated *OLE1-G153M*, forward
S1M3_G:M_rev	CATAATCTATGGTAACCGGCA GTAATAGAAACcatACCGAC	amplification of mutated *OLE1-G153M*, reverse
M5S2_T:L_fw	CATCGGTACCCAACCATTCGAT GACAGAAGACTCCCTCGTG	amplification of mutated *OLE1-T318L*, forward
S1M5_T:L_rev	GTAACAATGGCAGTAATCCAG TTGTCACGAGGGAGTCTTCTG	amplification of mutated *OLE1-T318L*, reverse
M5S2_T:M_fw	CATCGGTACCCAACCATTCGAT GACAGAAGAatgCCTCGTG	amplification of mutated *OLE1-T318M*, forward
S1M5_T:M_rev	GTAACAATGGCAGTAATCCAG TTGTCACGAGGcatTCTTC	amplification of mutated *OLE1-T318M*, reverse

Table 27: List of DNA oligonucleotides used for sequencing DNA fragments and plasmids.

name	sequence (5'-3')	description
fatty acid synthase		
FAS1_rev	GTCTTCACTAAAGTTGTGTCGT TTTC	primer for sequencing, binding in *FAS1*, reverse
FAS1-I306A-fw	TCGGCGAGGGTAACGCACAAT TGGTAGC	primer for sequencing, verification of mutation in *FAS1*, forward
FAS1-I306A-rev	TAATGATTGTGGTGGGCCCGAT ACG	primer for sequencing, verification of mutation in *FAS1*, reverse
FAS1-R1834K-fw	CAACCTGTTACTACCTTCGTTT TCACTGG	primer for sequencing, verification of mutation in *FAS1*, forward

FAS1-R1834K-rev	GAACATTGGTAACGGTGTCTAAAGCTCTTAG	primer for sequencing, verification of mutation in *FAS1*, reverse
FAS2-G1250S-fw	TTCCACGGATGTGGCACAGGCACCAAC	primer for sequencing, verification of mutation in *FAS2*, forward
FAS2-G1250S-rev	TCGTCCATACACTGGTTGGGTTGATTCC	primer for sequencing, verification of mutation in *FAS2*, reverse
universal		
pBR322-rev	GAGCTTCCAGGGGGAAACG	primer for sequencing, binding in the pBR322 origin of replication, reverse
pPDC-for	GGTGGTTCCGGCTTCCTTCC	primer for sequencing, binding in the *PDC1*-promoter, forward
tPDC-rev	GTTCCTTAATCAAGGATCCTC	primer for sequencing, binding in the *PDC1*-terminator, reverse
tPGK1-for	GCTTTAACGAACGCAGAAT	primer for sequencing, binding in the *PGK1*-terminator, forward
pFBA1-rev	GGGTCATTACGTAAATAATGATAG	primer for sequencing, binding in the *FBA1*-promoter, reverse
SEQ-lasZ_a rep	CCATTCGCCATTCAGGCTGCGCAAC	primer for sequencing, binding in the *lacZ* reporter gene, forward
seq_HXT7	CAAGAACAAACAAGCTCAAC	primer for sequencing, binding in the *HXT7*-promoter, forward
pRS42_hxt7	CACACAGGAAACAGCTATGAC	primer for sequencing, binding in pRSx plasmid prior to *HXT7* promoter, forward
CYC1_rev	ACCTAGACTTCAGGTTGTC	primer for sequencing, binding in the *CYC1*-terminator, reverse
foreign		
afp148-Yep-rev	GCTGCAAGGCGATTAAG	primer for sequencing, binding in p42x and pRS31x plasmids, reverse (A. Farwick, Prof. Boles group, Frankfurt)
seq2_ColE1	AGGGAGCTTCCAGGGGG	primer for sequencing, binding in pBR322, forward (V. Schadeweg, Prof. Boles group, Frankfurt)
tCYC_Rw	CTCGAGTCATGTAATTAGTTATGTC	primer for sequencing, binding in *CYC1* terminator, reverse (W. Cardoso Generoso, Prof. Boles group, Frankfurt)

III. Supplemental figures and tables

Table 28: Protein sequence of desaturases Ole1p from *S. cerevisiae*, Pex-Desat3 from *P. excessana* and Obr-TerDes from *O. brumata*.

protein	protein sequence
Ole1p	MPTSGTTIELIDDQFPKDDSASSGIVDEVDLTEANILATGLNKKAP RIVNGFGSLMGSKEMVSVEFDKKGNEKKSNLDRLLEKDNQEKEE AKTKIHISEQPWTLNNWHQHLNWLNMVLVCGMPMIGWYFALSG KVPLHLNVFLFSVFYYAVGGVSITAGYHRLWSHRSYSAHWPLRLF YAIFGCASVEGSAKWWGHSHRIHHRYTDTLRDPYDARRGLWYSH MGWMLLKPNPKYKARADITDMTDDWTIRFQHRHYILLMLLTAFV IPTLICGYFFNDYMGGLIYAGFIRVFVIQQATFCINSLAHYIGTQPFD DRRTPRDNWITAIVTFGEGYHNFHHEFPTDYRNAIKWYQYDPTKV IIYLTSLVGLAYDLKKFSQNAIEEALIQQEQKKINKKKAKINWGPV LTDLPMWDKQTFLAKSKENKGLVIISGIVHDVSGYISEHPGGETLI KTALGKDATKAFSGGVYRHSNAAQNVLADMRVAVIKESKNSAIR MASKRGEIYETGKFF
Pex-Desat3	MVPDVLREAERLQDDAKLVAPQAEPWKFQLSYTNVVIFGYLHIS GIYGLLFCVSKAHWATIFFSFILLVASVIGVTAGAHRLWSHRSYKA NLPLQIILMLFHSLSGQYTAFNWARDHRLHHKYSDTDADPHNATR GFFYSHIGWLLVVKHPEVRKRGEAIDLSDLLRNPVLTFQRKNVVLI LALLCYIMPTAVPMYFWGETFHNAWHIMALRFVLCLNFISLINSA AHTFGNKPYDKSIMPTQNMSVTLATLGEGFHNYHHVFPFDYRAA ELGNNTFNLTTKFIDFFAMIGWATALKTVGHESIARRAQRTGDGS LTWKSDCEVVPGGLKSG
Obr-TerDes	MAPNTLKEDVMIVNEETSEKLVVGAAPRKYKVVWFIVAYATYV HIAAVYGLYLAVTSAMWPTIALTFINNILSILGLTAGVHRLWTHKS YKAKLPLQIFLMLCHTASNTFTSISWIRDHILHHKYTDTDADPHNS TRGFFFSHIGWAMVKKQPEARAKGKSIDLSELYANPVLRFQQKNA VWLTLLVAYIIPSLVPLIWNETFTVAYHMNLLRVTVVFNTFLLINS VAHMWGTRPYDETILPAQNKTVSFFTLGEGFHNYHHVFPHDYRT AELGDNFLNLTTKFIDFCAWMGQAYDRRYVPDDVIAARMKRTGE TNEKNT

Table 29: Protein sequence of Δ9 desaturases from *Fomitiporia mediterranea* MF3/22 (EJD00826.1), *Gloeophyllum trabeum* ATCC 11539 (EPQ53459.1), *Laccaria bicolor* S238N-H82 (EDR04669.1), *Moniliophthora roreri* MCA 2997 (ESK86550.1 and ESK91931.1), *Punctularia strigosozonata* HHB-11173 SS5 (EIN08842.1) and *Trametes versicolor* FP-101664 SS1 (EIW55454.1). All desaturases have a cytochrome b5 domain.

protein	**protein sequence**
EJD00826.1	MSTTTTVRVRTLTSQVGTSTSSQTQSKSSTHSIHVTASRPPITFKNF VSEIRWFNLGVVTITPLLSIYGLYTTEFRAPTVAFCVFMYVLNMIGI TAGYHRLWSHRSYKASLPLQYFLALAGASSVQGSIRWWARGHRS HHRYTDTDLDPYSARKGLLWSHIGWMLIKPRITPGKADVRDLSQ NKVIMWQRKHYFLIALVTGVLIPWFIPGYFWGDWRGGYFYAGFL RITIAHHSTFCVNSIAHWLGETSYDDKHTPRDHIITAILTLGEGYHN FHHQFPMDYRNAVQWYQFDPTKWFIALCERFGLATHLQRFPENEI RKGQLAMTLKKLKDEQDLIVWPTKSDDLPVISWETFKEESRTRPL VLVAGFIHDVSGFIDRHPGGRELLEKALGTDATPSFFGGVYEHSH AAHNLLSTMRVGVLHGGLEQVDQKSIAPGEKLYIAESKVPTR
EPQ53459.1	MATYTPPLTPPSEPTKRLKNLEPEPIDINIPDNYVQHTLKTQKELPPI TWSNWYRELQWISVLALTITPALAIYGAFTTKLTWQTGLFSVFYY YVTGLGITAGYHRLWAHRSYNASKPLQYFLALAGSGAVEGSIKW WSRGHRAHHRYTDTELDPYSAQKGFWWSHVGWMLFKPRRKPG VADVSDLSRNEVVRWQHRWYVWLILGMGFGLPTVVPGLLWGD WWGGFFYAGALRLTFVHHSTFCVNSLAHWLGETPFDDKHTPRDH VITALVTIGEGYHNFHHQFPMDYRNAIKWYQYDPTKWFIMACQW VGLASHLKTFPDNEVRKGQLTMQLKRLRETQEKLTWAPDSNDLPI VSWDSFQEQSAKRPLILIAGFIHDVASFLDEHPGGRHLLVKYIGKD ATTAFFGGVYDHSNAAHNLLSMHRVGILQHGYRQSLDDKAIPPA QRLRIARYNELGSSTAVSDAETLVGEKEEKEA
EDR04669.1	MPSSTRQQSKLVDESVPADLNIPDNYVQHTLKTIKPLPPVTWSNW TTELDYLNVAILTLTPVVGLVGAYFTELRWETCLFAIFYYYVTGL GITAGYHRLWAHRAYNASLPLQYFLAIVGAGAVEGSIKWWSRGH RAHHRYTDTDLDPYNAHRGFFYSHVGWMMIKPRRKPGVADVSD LAKNPVIRWQHKHYIALIFLMGFILPTVVPWLLWGDAQGGYIYAG VLRLCFVHHSTFCVNSLAHWLGETPFDDKHTPRDHLITALATIGE GYHNFHHQFPMDYRNAIKWYQYDPTKWFIWACQKLGLASHLKV FPDNEVRKGQLTMELKKLRETQEKLSWPNDSNDLPVISWESFQEQ ALKRPLILISGFIHDVGTFVDEHPGGPHLLIKFIGKDATTAFFGGVY DHSNAAHNLLAMKRVGVLHGGAPHGLEDKMIPPSQRLKIARYTE LTSPYNSSTAYSDGEGMLG
ESK86550.1	MAAGKEEEPADLNLPDNYVSHVLQTQKSLPPIKWSNILSEIQWISF SVIFGAPVVGFIGAYYTKLRWETLVWSVIYYFCTGLGITAGYHRL WAHRAYNASLPLQYVLALFGAGSVQGSIKWWSRGHRAHHRYTD TELDPYNAHKGFWYAHIGWMLVKPRRRPGVADVSDLSKNPVVK WQHKYYVQLLLLMALIFPTLVAHYGWNDAKGGLVYAGVLRLVF VHHSTFCVNSLAHWLGETPFDDKHTPRDHMITAFVTIGEGYHNFH HQFPMDFRNAIKWYQYDPTKWTIWVLSKLGLASHLKVFPENEVR KGQLTMQLKKLRQTQEDLTWPTDSNDLPVISWESFQKQSNKRPLI LISGFIHDVSDFLEEHPGGRHLLVKYIGKDATTAFFGGVYDHSNAA HNFLAMKRVGVLHGGHPHALDDKTVPPGSRLKIARYNELSSSYSS STAWSSDEGSFN

ESK91931.1	MFTSWTENFPELKGVRWFNVMVLTVGPSLALWGLLFEPISRQTA WFTGFYYMFTILGITAGYHRLWSHRSYNASVPLQWFLLLGGASA VQGSCYWWARAHRSHHRHTDTDSDPYNSKRGLLWTHIGWMVF KTDLRSGTADISDLRKDPLVQFQHRWYFSLALLFGLVIPATVPGLL WDDWMGGICYTAALRMTVAHHSVFCVNSIAHWLGDSPYDDHLS PRDHFLSAILTMGEGYHNFHHQFPMDYRNAYLWYQWDPTKWFI ALCNFLGLANNLRIFPNNEIAKGALTMKLKELKRVQDSLRWPTPP EELPVVTWETFQEESNLRTLILISGFIHDVSSFLDEHPGGAQLLTKN SGKDMTAAFFGGYYAHSNAAHNRLSMMRVGILAGGVENPLEHVI LESQRLYIAERPLKTSRI
EIN08842.1	MSTAASAPAPAARITPLPVDKAQDSKEFPKPVVPTDTPADINIPDN YVAYTLRNQKPLPPVTWANWYKELEWISVLVLTLVPAMTIYGAL YVPLQTKTAIFAVLYYFVTGLGITAGYHRLWAHRSYNASLPLQYA LALAGSGAVEGSIKWWSRGHRAHHRYTDTDLDPYNAHRGFFWS HIGWMLIKPRRKPGVADISDLSKNPVVRWQHKHYVKLIIVMGFLV PTIIPGLLWGDFIGGYFFAGAARLLFVHHSTFCVNSLAHWLGETPF DDKHTPRDHMITAFVTIGEGYHNFHHQFPMDYRNAIKWYQYDPT KWFIWVCHKLGLASHLKQFPDNEVRKGQLTMQLKRLREVQETIT WAAPETDLPVISWESFQDQAAKRPLILIAGFIHDLSDFMDEHPGGR HLIVKYIGKDATTAFFGGVYDHSNAAHNLLSMKRVGILHGGHPH GLDEKTIPPAQRLRIARYSEMGNYSSSALSDGEAGLLG
EIW55454.1	MTASSTPTEHFPTIRGVKWPNMISIVVTHILAIYGGLYVTPKPSTAV LAIVLYFVSTLGITAGSHRLWSHRSFKASAPLRLFLVLAGGSAVQG SAYWWAKVHRSHHRYIDTDKDPYSAQRGFLFTHVGWIVFYTDV APGGGVDLSDLHKDKILMWQHNNRALVWLLCGYILPTVIPGYFW GDWAGGLFYSTALRLTACYHSVWCINSLAHMLGHAPFDDKHTPR DHLFTALVTMGEGYHNFHHQFPMDYRNAFRWYQYDPTKWFIAA CGALGLASNLRTFPSNEVHKGMFTMRAKELRATQESLTWPTPVE KLPVVTWENFQEESKKRTLLLVSGFIHDVTSFLEEHPGGDRLLTA NTGKDVTAKFFGGVYDHSHAAHNLLSMMRVGILQGGVETLGEH AVPPAQKYYVSVHEEAD

Table 30: Protein sequence of various desaturases and hypothetical proteins from *Agaricus bisporus* var. *bisporus* H97 (EKV44635.1), *Agaricus bisporus* var. *burnettii* JB137-S8 (EKM75648.1), *Moniliophthora roreri* MCA 2997 (ESK92783.1), *Podospora anserina* S mat+ (CAP70780.1), *Schizocaccharomyces octosporus* yFS286 (EPX72095.1), *Schizophyllum commune* H4-8 (EFI94599.1 and EFI94388.1), *Serpula lacrymans* var. *lacrymans* S7.3 (EGO03565.1), *Stereum hirsutum* FP-91666 SS1 (EIM91634.1), *Wallemia ichthyophaga* EXF-994 (EOR00207.1) and *Wallemia mellicola* CBS 633.66 (EIM20316.1).

protein	**protein sequence**
EKV44635.1	MPASTATKVSTPTDDVVPADLNIPDNYVAHTLKTQKALPPIKWEE WYKEINWLSTIILTVTPALGLIGAFLTSLRWETFLFSIFYYFVTGLGI TAGYHRLWAHRSYNASLPLQYILAACGAGAVEGSIKWWCRGHR AHHRYTDTDLDPYSAQKGFFHAHIGWMLLKPRRKPGVADVSDLT KSPVIRWQHRHYLPLILIMGFLIPTVLPWLLWNDARGGFVYAGVI RLCFVHHSTFCVNSLAHWLGETPFDDKHTPRDHIITAFATIGEGYH NFHHQFPMDYRNAIKWYQYDPTKWFIWACQQMGLASHLKIFPD NEVKKGQLTMQLKKLRETQDKLVWPSDNSDLPVISWESYREQSL KRPLIVIAGFIHDVSDFIDEHPGGAHLLVKNIGKDATTAFFGGVYD HSNAAHNLLSMKRVGVLHGGSQHGVDEKAIPPGQRLRIARYNEL STSPYNSGTGYSDSEGLLG

EKM75648.1	MPASTATKVSTSTDDVVPADLNIPDNYVAHTLKTQKALPPIKWEE WYKEINWLSTIILTVTPVLGLIGAFLTSLRWETFLFSIFYYFVTGLGI TAGYHRLWAHRSYNASLPLQYILAACGAGAVEGSIKWWCRGHR AHHRYTDTDLDPYSAQKGFFHAHIGWMLLKPRRKPGVADVSDLT KSPVIRWQHRHYLPLILIMGFLIPTVLPWLLWNDARGGFVYAGVI RLCFVHHSTFCVNSLAHWLGETPFDDKHTPRDHIITAFATIGEGYH NFHHQFPMDYRNAIKWYQYDPTKWFIWVCQQMGLASHLKIFPD NEVKKGQLTMQLKKLRETQDKLVWPSDNSDLPVISWESYREQSL KRPLIVIAGFIHDVSDFIDEHPGGAHLLVKNIGKDATTAFFGGVYD HSNAAHNLLSMKRVGVLHGGSQHGVDEKAIPPGQRLRIARYNEL STSPYNSGTGYSDSEGLLG
ESK92783.1	MSDKPQLEEPADLHIPDNYVSHILKTTKPLPPVTWSTLWGEIQWIS FTLLIGVPIVGAIGACYTPLRWETFIWSVIYYFCTGLGITAGYHRL WSHRAYNASIPLQYALALFGAGAGQGSIKWWSRGHRAHHRYTD TDLDPYNAHQGFWYSHVGWILVKSRRKPGVSDISDLNRNPIVKW QHKNYSPILLFMAFALPTLVAHLGWNDARGGFVYAGLLRLAFVH QSTFCVNSLAHWLGEAPFDDKHTPRDHMITALVTIGEGYHNFHH QFPMDFRNAIKWYQYDPTKWAIWTMSKLGLASHLKVFPDNEVR KGELTMQLKKLRETQDNLTWPTDSNDLPVISWETFQNQSNKRPLI LISGFIHDVSDFLEEHPGGRHLLVKYIGKDATTAFFGGVYGHSNAA HNLLAMKRVGILYGGHPHALDDKTVPPGSRLKVARYTEIGLSSSS SAWSSDEA
CAP70780.1	MSVTVTEEPATMLAWEKNPLALPLPKVEKPAETLWQRIKWESMI VLTVSPIIGLYGLLFVPLQTKTLWWSVFLYWFSIIGSTAGSHRLYS HRSFKASTPLQIFLLIGGTCGVQGSAFWWAREHRAHHRYTDSDLD PHSGKEGFWWTHAGWILFRRDIQAGPTDVSDLKKNKLVMFQHR HYFKLFPFLAYVMPAAVAGYFWGDWAGGICYAAMLRLTIVQHSI MCINSLAHTFGDAPFDDKHTPRNHFFTAIVTAGEGYHNFHHQFPV DYRNGIKWYQYDPTKWFIYLSSRLGLSTQLQTFPQNEISKGELTM TLRRLKEKQEDIKWPARPDKLPLVSWETFQREAKKNSLILINGYIH DASEFENKHPGGKAIIRARVGKDATAAFGGGVYEHSNAAHNLLA MMRVAVLEGGVEHVKYVTPAERLRIIEHYKEEESH
EPX72095.1	MSSNGISQGSQASVHPSAGEDNTLRQRKIPVVPVVPERRFDPKAPK HIQEQPWTLTNFWRHFNWLHLILLFTFPAVGIYGICTTPLHTKTLL FSIVYYFYSGLGITGGYHRLWAHRSYKAKKPLQYFFAAGGAAAFE GSIRWWARDHRAHHRYTDTDKDPYNVKKGFWYAHVGWMIILQ NPRRIGRTDISDLNTDKLVVFNHRHFLPFATIMAFVVPSLICGYFW GDYRGGYFYAGVCRLVFVHHATFCVNSLAHMLGGQPFDDTNSA RNHYITALVTLGEGNHNYHHAFPNDYRNGLRWYEYDPTKAFIWA CSLVGLAYDLNTFPQNEIEKSLVQQRQKVLDRWRARLNWGTPLE VLPVMEFEEFLEESKTRPLVLINGVVHDVTGFQHPGGQGLLRSAF GKDATAAFNGGVYEHTNGAHNLLATYRIAVVRGGMEVEVWKSG AGEKMPMKDTFGQRIVRVGEQPTRLQPPIEAAAAN
EFI94599.1	MSDVGVEMRVTDEQSSDLNIPDNYVQHTLKTTKALPPITWANLL QNINWISFMLLTATPIIGAWGALTTNLRWETAAFAVLWYFATGLG ITAGYHRLWSHRAYNASIPLQYALALAGAGAAEGSIKWWSRSHR AHHRYTDTELDPYNPHKGFFYSHIGWMLVKPRRRMGVADVSDLS RNPVVRFQHAHYVKLMVFMAFIFPTLVAWLGWGDARGGYIYAG VIRLCFVHHSTFCVNSLAHWLGETPFDDKHTPRDHFVTALATIGE GYHNFHHQFPMDYRNAIKWYQYDPTKWFIATCRSIGLASHLKVF PDNEVRKGQLTMQLKRLRETQERLTWPSDSNDLPVITWESFQEQA

	ASRPLILIAGFIHDVSTFIDEHPGGPHLIVKFIGKDATTAFFGGVYD HSNAAHNLLGMKRVGVLHGGAPHILDLADEKKIPPSQRLKIARYS ELSSSGSSSEAGF
EFI94388.1	MSVEKYVPTDLNIPDNYVQHTLKTTKPLPPVTLANVWGEINWISV TLLTVPPIVGIYGLSTTALQWRTGLLMFAWYLLTGLGITAGYHRL WAHRSYNAIMPLQIFLAFAGAGAAQGSIRWWSRGHRSHHRYTDT DLDPYNAQRGFFYSHIGWMLLKPRRKIGVADISDLSKSPLVRWQH KHYLPIMLFMAFILPAMIGSIWGDARGGFFYAGVIRLVIVHHSTFC VNSLAHWIGEQPFDDKHTPRDHFLTALVTVGEGYHNFHHQFPMD YRNAIKWWQYDPTKWFIWANRRIGFASHLKVFPDNEVRKGQLA MQLKRLRRTQDNLQWPTTSNDLPVVTWETFQEQSKHRPLILIAGF IHDVSEFLEEHPGGEHLIRKFIGRDASTAFFGGVYDHSHAAHNLLA MKRVGILEGGVQIVNDIEKERRIPPSQRLRIARWEELSSGSSSGEQS D
EGO03565.1	MSSVYPSRKSPPPAMASITPPVTPKLANAHASKPKADEEPADMNIP DNYVTYTLKHQKPLPPITWDNWTTELNWLNVGILGLTPIIGIVSAC FTPLRWETAVWAVIYYYLTGLGITAGYHRLWAHRSYNASKPLQY ILALLGAGAVEGSIKWWARGHRAHHRYTDTDLDPYNAHRGLLW SHIGWMIVKPRRKPGVADVSDLTKSEIVRWQHRHYLTLIFIMAFV VPTIIPGLGWGDWKGGYVYAGLLRLVFVHHSTFCVNSLAHWLGE TPFDDKHSPRDHLVTALVTIGEGYHNFHHQFPMDYRNAIKWYQY DPTKWFIWVCKKAGLASHLKVFPDNEVRKGQLTMQLKRLRETQ DKLSWPEDNSHLPVINWESYLQQAQSRPLICVAGFIHDVGDFLDE HPGGRHLLTKNIGKDATTAFFGGVYDHSNAAHNLLAMKRVGVL HGGAPHGLEDKSIPPSQRLRIAQYNEMGGSGYSSAAWSDGEGLM G
EIM91634.1	MLGSKNWPPIRGVHRFSAFVLIITPIIAVYGLLYVPLRFKSFVWAYS YLIFTTLGYHRLWAHRSYNASLPLQYVLIIAGSGAVQGSCYWWA RRHRSHHRYTDTDGDPYNSQRGLFWTHIGWMLFKTDLRSGTADV SDLRKNDLVQWQHSWYLYLQFVVGYALPTMIPGICWGDWAGGL CYAGALRMTLCHHSTFCINSIAHYLGDAPYDDKHTPRDNFLSALL TMGEGYHNFHHQFPMDYRNAFRWYQYDPTKWFIALCGAVGLAS SLREFPSNEVEKGALTMKLKELKGVQDGLQWPVQADRLPLISWE SFQEQSKDRVLILVSGYIHDASGFLDEHPGGPTLLATSSGKDNTSA FFGGTYEHSNAAHNLLAMKRVGILAGGVECLGKSDHAVPPSQRL YIAQVTPGK
EOR00207.1	MAFEQVVPPPAATAATPATPASPVSSMSAPMSPPLTDQKGESDAY ISDNELPPPLNKDTDRPADADIEDNYVQRTLANAKPKPPITWSTLL GEINYISTTLLLGVPIISIYGALTTELKWQTALFSVLYYFFTGLGITA GYHRLWAHRAYNASLPLQYFLCLAGSGACEGSIKWWSRGHRAH HRYTDTELDPYNAHRGFWYSHVGWMLVKPRIKPGVADISDLIKN PVIRFQQKHYVKLLLIMGFIIPTVLPGLLWGDYRGGYFYAGAARL LFVHHSTFCVNSLAHWLGEQPFDDKNTPRNHFITALCTVGEGYHN FHHQFPMDFRNAIRWYQYDPTKWFISVCYALGLATHLKMFPSNEI KKGEYTMELKKLERVGEEINWPIDSNELPVISWTSFQQQAKERPL VLIHGFIHDVSSFMDEHPGGKHLLSKQVGKDGTTAFFGGVYDHSN AAHNLLAMMRVGILDGGMEVEILKIEEAQAKKKEQALSIVKDIEN EQDRKRAHDEAMKDVKRIEHEMQLALLRAPPQQVRSRKATDYFP PSQQLSIKKQADS

EIM20316.1	MESANAYIITPPSSVAELSPPLTPKQQAESEAYISDADLPPALNPDT DRPVDADIEDNYVQRTLANAKPKPPITWSTLLGEINYISTALIAGV PLITIYGAMTTELKWQTAIFSVLYYFFTGLGITAGYHRLWAHRAY NASTPLQYFLCLAGSGACEGSIKWWSRGHRAHHRYTDTELDPYN AHRGFWYSHVGWMLVKPRIKPGVADISDLIKNPVVRFQQKHYVK LLLTMGFIVPTVLPGLLWGDYRGGYFYAGAARLLFVHHSTFCVNS LAHWLGEQPFDDKNTPRNHFITALCTVGEGYHNFHHQFPMDFRN AIRWYQYDPTKWFISVCYALGLASHLKMFPSNEIKKGEYTMELKR LERVGEEIKWPIDSNELPVISWTSFQEQAKERPLILIHGFIHDVSSFM DEHPGGKHLLSKQIGKDATTAFFGGVYDHSNAAHNLLAMMRVG VLDGGMEVEVLKIEEAQAKKKEQALAIVKDIENEDERRVAHEKA MQDVKRIEEEMQSALLRSPPEHIRNKKAQDYFPPSQQLHIRKAK

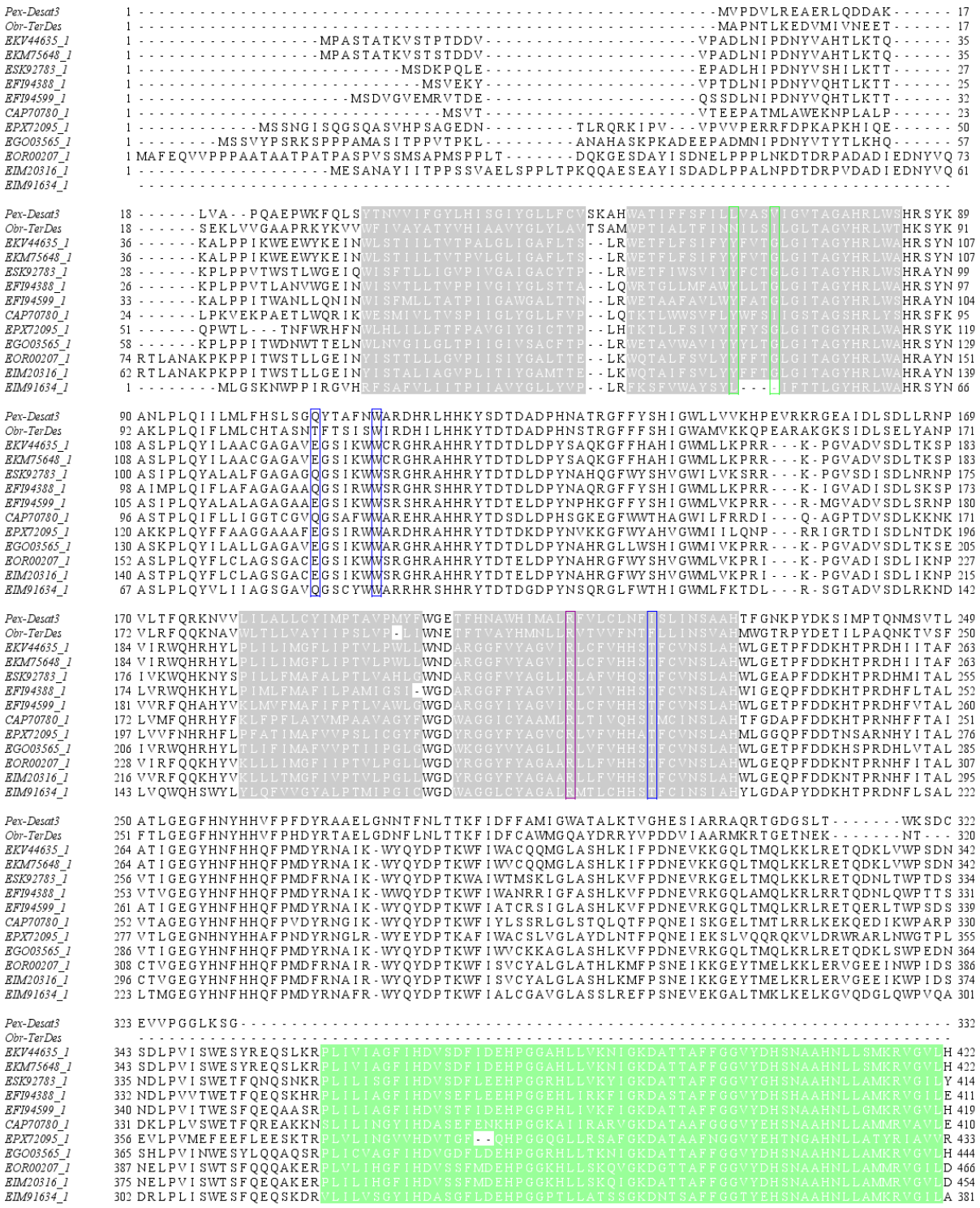

Figure 40 Multiple Sequence alignment of potential terminal desaturases. Protein sequences from *A. bisporus* var. *bisporus* (EKV44635.1), *A. bisporus* var. *burnettii* (EKM75648.1), *M. roreri* (ESK92783.1), *P. anserina* (CAP70780.1), *S. octosporus* (EPX72095.1), *S. commune* (EFI94599.1), *S. lacrymans* var. *lacrymans* (EGO03565.1), *S. hirsutum* (EIM91634.1), *W. ichthyophaga* (EOR00207.1) and *W. mellicola* (EIM20316.1) were used for comparison with Pex-Desat3 from *P .exessana* and Obr-TerDes from *O. brumata*. Sequence alignment was conducted using the PRALINE multiple alignment server (Simossis and Heringa, 2005). The green framed amino acid, may determine the length of bound acyl chains. Framed with a blue box are amino acid that could bind to the acyl-chain. The purple framed amino acid represents a highly conserved amino acid, which interaction may help stabilize the kink for double bond introduction. Grey boxes represent predicted transmembrane domains in Ole1p. Green areas represent the by Pfam database predicted cytochrome b5 domain (Finn et al., 2016).

Figure 41: Sequence alignment of desaturases from *M. musculus* (SCD1), *P. excessana* (Pex-Desat3), *O. brumata* (Obr-TerDes) and *S. cerevisiae* (Ole1p). The alignment was conducted using the PRALINE multiple alignment server (Simossis and Heringa, 2005). The amino acids framed with the green box, may determine the length of bound acyl chains. The purple marked amino acid represents a highly conserved amino acid, which interaction may help stabilize the kink for double bond introduction. Amin acids indicated with a black box, change the substrate specificity in mouse SCD3 (Bai et al., 2015). Grey boxes represent transmembrane domains as predicted in SCD1, while green highlight area represent by Pfam database predicted cytochrome b5 domain in Ole1p (Finn et al., 2016).

Table 31: Sequences similarities analysis of potential terminal desaturases. Values show the similarity between single sequences in %. All grey highlighted values are between 70 % and 99,9 %.

	Pex-Desat3	Obr-TerDes	EKV 44635.1	EKM 75648.1	ESK 92783.1	EFI 94599.1	EFI 94388.1	EPX 72095.1	EGO 03565.1	EOR 00207.1	EIM 20316.1	CAP 70780.1	EIM 91634.1
Pex-Desat3	100 %												
Obr-TerDes	76.35 %	100 %											
EKV 44635.1	41.15 %	40.8 %	100 %										
EKM 75648.1	41.33 %	40.98 %	99.47 %	100 %									
ESK 92783.1	44.3 %	42.03 %	80.91 %	81.08 %	100 %								
EFI 94599.1	42.9 %	41.85 %	79.68 %	79.85 %	80.91 %	100 %							
EFI 94388.1	44.3 %	43.95 %	77.4 %	77.58 %	78.8 %	83.36 %	100 %						
EPX 72095.1	39.4 %	37.12 %	59.89 %	59.36 %	57.96 %	58.84 %	60.42 %	100 %					
EGO 03565.1	38.17 %	36.07 %	80.21 %	80.38 %	76.88 %	74.95 %	71.62 %	60.59 %	100 %				
EOR 00207.1	28.19 %	25.91 %	57.09 %	57.44 %	58.84 %	59.54 %	57.09 %	50.96 %	59.01 %	100 %			
EIM 20316.1	29.94 %	28.19 %	59.54 %	59.89 %	61.12 %	61.82 %	59.71 %	51.31 %	58.84 %	83.36 %	100 %		
CAP 70780.1	49.03 %	45 %	61.12 %	61.47 %	64.97 %	63.92 %	64.97 %	56.56 %	57.79 %	47.63 %	51.13 %	100 %	
EIM 91634.1	47.98 %	45.18 %	63.57 %	63.92 %	65.49 %	65.32 %	67.25 %	54.11 %	60.77 %	50.61 %	52.88 %	68.12 %	100 %

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