Received Date : 20-Dec-2013

Revised Date : 06-Jun-2014

Accepted Date : 07-Jun-2014

Article type : Research Paper

Editor : Guenther Daum

A newly identified fatty alcohol oxidase gene is mainly responsible for the oxidation of long chain ω-hydroxy fatty acids in *Yarrowia lipolytica*

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Abstract

Nine potential (fatty) alcohol dehydrogenase genes and one alcohol oxidase gene were identified in *Yarrowia lipolytica* by comparative sequence analysis. All relevant genes were deleted in *Y. lipolytica* H222ΔP which is lacking β-oxidation. Resulting transformants were tested for their ability to accumulate ω-hydroxy fatty acids and dicarboxylic acids in the culture medium. The deletion of eight alcohol dehydrogenase genes (*FADH*, *ADH1-7*) which may be involved in ω-oxidation led only to a slightly increased accumulation of ω-hydroxy fatty acids whereas the deletion of the fatty alcohol oxidase gene (*FAO1*), which has not been described yet in *Y. lipolytica*, exhibited a considerably higher effect. The combined deletion of the eight (fatty) alcohol dehydrogenase genes and the alcohol oxidase gene further reduced the formation of dicarboxylic acids.

These results indicate that both (fatty) alcohol dehydrogenases and an alcohol oxidase are involved in ω-oxidation of long chain fatty acids whereby latter plays the major role. This insight marks the first step towards the biotechnological production of long chain ω-hydroxy fatty acids with the help of the non-conventional yeast *Y. lipolytica*. The overexpression of *FAO1* can be further used to improve existing strains for the production of dicarboxylic acids.

Keywords: *Yarrowia lipolytica*; alcohol dehydrogenase; alcohol oxidase; ω-oxidation; long chain fatty acid, *ADH*, *FADH*, *FAO*, *FAO1*, ω-hydroxy fatty acid, dicarboxylic acid

1. Introduction

1.1. The non-conventional yeast Yarrowia lipolytica

Yarrowia lipolytica is a non-conventional, dimorphic, ascomycetous yeast. It can be isolated from dairy products (Guerzoni *et al.*, 1993; Barth & Gaillardin, 1997; Vasdinyei & Deak, 2003) and sausages (Gardini *et al.*, 2001) as well as from soils, sewages and oil polluted environments (Barth & Gaillardin, 1997; Kim *et al.*, 1999; Schmitz *et al.*, 2000; Zinjarde & Pant, 2002). *Y. lipolytica* is obligatory aerobic and not able to grow at temperatures above 35 °C (Barth *et al.*, 2003). These characteristics are the basis for its apathogenicity (Holzschu *et al.*, 1979; Groenewald *et al.*, 2013) and the GRAS (*Generally Regarded As Save*) classification by the american Food and Drug Administration.

The genome of the *Y. lipolytica* strain E150 has been sequenced and annotated (Dujon *et al.*, 2004). The haploid genome of *Y. lipolytica* consists of six chromosomes (A-F) with a summarized size of 20.4 Mb (Casaregola *et al.*, 1997; Dujon *et al.*, 2004). The availability of advanced genetic tools was an important fundament for the increasing importance of *Y. lipolytica* in both general and applied science (Nicaud, 2012).

1.2. Utilizing of hydrophobic substrates

Y. lipolytica is able to utilize a various range of carbon sources, like glucose, glycerol, alcohols, acetate and a high range of hydrophobic substrates like plant oils, fats, fatty acids and *n*-alkanes (Ishikura & Foster, 1961; Nyns *et al.*, 1967; Klug & Markovetz, 1968; Barth & Gaillardin, 1997). A review of hydrophobic substrate utilization in *Y. lipolytica* - including the related pathways, enzymes and corresponding genes - is given by Fickers *et al.* (2005) and Thevenieau *et al.* (2010).

To metabolize hydrophobic substrates, *Y. lipolytica* secretes extracellular lipases, which convert triglycerides to glycerol and free fatty acids, and liposan - a bioemulsifier.

Furthermore protrusions are formed on the cell surface, to which the hydrophobic substrates are bound (Mlickova *et al.*, 2004). The hydrophobic substrates are brought into the cells with the help of membrane transporters (Kohlwein & Paltauf, 1984). *Y. lipolytica* is able to store free fatty acids as triglycerides and steryl esters into special intracellular compartments called *lipid bodies*.

After the import of *n*-alkanes into the cell, the yeast oxidizes them to fatty acids with the same chain length during the primary alkane oxidation (Fig. 1). Then the fatty acid is degraded to acetyl-CoA (and propionyl-CoA if it has an odd chain length) during β-oxidation. Acetyl-CoA is metabolized via the tricarboxylic acid and glyoxylate cycle afterwards.

Additionally to the peroxisomal β-oxidation of fatty acids (Kunau & Hartig, 1992), ω-oxidation occurs in the ER. This diterminal oxidation of fatty acids is normally much less important than β-oxidation (Mauersberger *et al.*, 1996). During ω-oxidation of fatty acids the C_ω-position (carbon with highest distance to carboxylic group of a fatty acid) is oxidized by a cytochrome P450-containing fatty acid ω-hydroxylase (FAH) to the corresponding ω-hydroxy fatty acid. This ω-hydroxy fatty acid is converted to the fatty aldehyde and the fatty aldehyde to the dicarboxylic acid. Probably these two steps are catalyzed by the same enzymes that catalyze the equivalent steps during primary alkane oxidation.

1.3. Alcohol dehydrogenases and oxidases in Y. lipolytica

While the corresponding genes for the most enzymes involved in the degradation of hydrophobic substrates in *Y. lipolytica* could be identified, it seems to be more difficult to do so for the (fatty) alcohol dehydrogenases and oxidases.

It is known that the oxidation of long chain alkanols is mainly catalyzed by H_2O_2-producing fatty alcohol oxidases located in peroxisomes (Mauersberger *et al.*, 1992; Ilchenko *et al.*, 1994). Until now it was not possible to identify corresponding genes by using the sequences of known alcohol oxidase genes of different organisms (e.g. *Candida*-species; (Vanhanen *et al.*, 2000)) as reference.

As early as 1979, Barth and Künkel identified one NAD^+-dependent and at least three $NADP^+$-dependent alcohol dehydrogenases (Barth & Künkel, 1979). Nine putative *ADH*-genes were identified in consequence of the *Y. lipolytica* genome sequencing project (see 3.1) (Dujon *et al.*, 2004). In 2005 Matatiele identified one alcohol dehydrogenase which may be involved in converting long chain fatty alcohols to the respective aldehyde by sequence analysis (Matatiele, 2005). But the expression of this fatty alcohol dehydrogenase gene

(*FADH*, *YALI0F09603g*) occurred to be pretty low when growing the cells in medium with *n*-alkanes as carbon source.

1.4. Biotechnological production of dicarboxylic and ω-hydroxy fatty acids

There are different yeasts (e.g. *Candida tropicalis* and *Y. lipolytica*) which are able to convert long chain alkanes or fatty acids to the respective α,ω-dicarboxylic acids with the general structure HOOC-$(CH_2)_n$-COOH (Hill *et al.*, 1986; Picataggio *et al.*, 1992; Smit *et al.*, 2005). To enable the conversion of hydrophobic substrates to dicarboxylic acids, β-oxidation has to be blocked. This can for example be realized by the deletion of the acyl-CoA oxidase-coding *POX* genes (Picataggio *et al.*, 1992; Smit *et al.*, 2005), what results in increased ω-oxidation of fatty acids (Fickers *et al.*, 2005).

For the biotechnological production of ω-hydroxy fatty acids instead of α,ω-dicarboxylic acids, the genes coding for enzymes that are responsible for the conversion of ω-hydroxy fatty acids to fatty aldehydes during ω-oxidation have to be deleted. This has already been successfully performed in *C. tropicalis* (Lu *et al.*, 2010). ω-Hydroxy fatty acids can be used as monomers for the production of biobased plastics and as valuable components in lubricants, adhesives, cosmetic ingredients and anticancer therapeutics (Lu *et al.*, 2010).

2. Material and methods

2.1. Microorganisms and cultivation

Escherichia coli DH10b (Invitrogen) was used as host organism for molecular cloning purposes. Media and growth conditions for *E. coli* were described by Sambrook and Russell (2001).

Y. lipolytica H222-S4 (Mauersberger *et al.*, 2001) was used as host strain for transformation and *Y. lipolytica* H222 (Barth & Gaillardin, 1996) as reference strain (negative control). The genotype of all *Y. lipolytica* strains used in this study is shown in Tab. 1. All strains are part of the collection of the department of microbiology of the TU Dresden. Cultivation conditions for *Y. lipolytica* were described by Barth and Gaillardin (1996).

Shaking flask cultivations were performed in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] or minimal medium with various carbon sources [3 % (v/v) glycerol and/or 1 % (v/v) *n*-alkane (DD: dodecane, PD: pentadecane, HD: hexadecane) or 1 % (w/v) fatty acid (DDA: dodecanoic acid, PDA: pentadecanoic acid, HDA: hexadecanoic acid), 17.3 g l^{-1} KH_2PO_4, 1.35 g l^{-1} K_2HPO_4 x 3 H_2O, 3 g l^{-1} $(NH_4)_2SO_4$, 0.7 g l^{-1} $MgSO_4$ x 7 H_2O, 0.5 g l^{-1} NaCl, 0.4 g l^{-1} $Ca(NO_3)_2$ x 4 H_2O, 0.5 mg l^{-1} H_3BO_3, 0.04 mg l^{-1}

$CuSO_4$ x 5 H_2O, 0.1 mg l^{-1} KI, 0.4 mg l^{-1} $MnSO_4$ x 4 H_2O, 0.2 mg l^{-1} Na_2MoO_4 x 2 H_2O, 0.4 mg l^{-1} $ZnSO_4$ x 7 H_2O, 6 mg l^{-1} $FeCl_3$ x 6 H_2O, 0.3 mg l^{-1} thiamine hydrochloride] at 28 °C and 220 rpm. The pH was adjusted to 5.5-6.0 by the addition of NaOH or 20 g l^{-1} $CaCO_3$. In liquid medium cultures 0.5 % (v/v) glycerol was fed once after 48 h.

Cultivation in a 600 ml fermenter (Multifors, INFORS HT) was performed in minimal medium with glucose as carbon source [5 % (w/v) glucose, 1 g l^{-1} KH_2PO_4, 0.16 g l^{-1} K_2HPO_4 x 3 H_2O, 3 g l^{-1} $(NH_4)_2SO_4$, 0.7 g l^{-1} $MgSO_4$ x 7 H_2O, 0.5 g l^{-1} NaCl, 0.4 g l^{-1} $Ca(NO_3)_2$ x 4 H_2O, 0.5 mg l^{-1} H_3BO_3, 0.04 mg l^{-1} $CuSO_4$ x 5 H_2O, 0.1 mg l^{-1} KI, 0.4 mg l^{-1} $MnSO_4$ x 4 H_2O, 0.2 mg l^{-1} Na_2MoO_4 x 2 H_2O, 0.4 mg l^{-1} $ZnSO_4$ x 7 H_2O, 6 mg l^{-1} $FeCl_3$ x 6 H_2O, 0.3 mg l^{-1} thiamine hydrochloride] at 28 °C. Oxygen saturation was held above 55 %. The cells were inoculated with an OD_{600} of 1 and grown at pH 5.5 for 24 h. Then 15 g l^{-1} DD was added and the pH was adjusted to 8.0. Every 24 h glucose was fed to a final concentration of 5 % (w/v).

All used microorganisms were stored in 25 % (v/v) glycerol at -80 °C.

2.2. Nucleic acids and plasmid construction

For the construction of the gene disruption cassettes, the promoter and the terminator regions were amplified by PCR using the *pXXX*_fw/*pXXX*_rv and *XXXt*_fw/*XXXt*_rv primers (Tab. 2), respectively (*XXX* stands for the disrupted gene).

For the construction of the deletion cassettes for the *POX* genes, an I-*Sce*I restriction site was introduced at the end of the promoter and the beginning of the terminator region. Promoter and terminator fragment were fused by overlap PCR using the primers *pXXX*_fw and *XXXt*_rv. The overlap fragment was then ligated into the pJET1.2/blunt vector (Fermentas), cut with I-*Sce*I (restrictases purchased from Fermentas) and the loxP-*URA3*-loxP cassette, which was cut from pJMP113 plasmid (Fickers *et al.*, 2003) by using I-*Sce*I restriction enzyme, was inserted.

For the construction of the deletion cassettes for the genes *ADH1-ADH6* and *FAO1*, a *Bam*HI restriction site was introduced at the end of the promoter and the beginning of the terminator region. Additionally, a *Hin*dIII restriction site was added to the beginning of the promoter region and an *Nde*I (*ADH1*: *Not*I, *FAO1*: *Eco*RI) restriction site was added to the end of the terminator region. The fragments were ligated into the pJET1.2/blunt or the pUCBM21 (Roche) vector. The resulting plasmids were cut with *Bam*HI and the URA blaster (TcR-*URA3*-TcR cassette), which was cut out from pUC-Lys2-DK2 plasmid (S. Leubner, C. Otto, unpublished) by using *Bam*HI and *Bgl*II, was inserted.

For the construction of the *ADH7* deletion vector, the complete gene (including promoter and terminator region) was amplified via PCR using the primers *pADH7*_fw and *ADH7t*_rv and cloned into the pJET1.2/blunt vector. The *ADH7* ORF was removed by restriction with *San*DI and *Nsi*I and the URA blaster, which was cut out from pUC-Lys2-DK2 plasmid by using *San*DI and *Nsi*I, was inserted.

The gene disruption cassettes were gained by PCR or restriction. The successful disruption of a gene was verified by PCR using the primers *pXXX*_fw/*XXXt*_rv and *pXXX*_fw/*XXXt*_rv2 or by southern blot analysis. *XXXt*_rv2 binds in the genomic area of the deleted gene (outside of the deletion cassette).

For the construction of the *FAO1* overexpression cassette, the *FAO1* ORF was amplified from genomic DNA of *Y. lipolytica* H222 using the primers *pTEF_FAO1*_ol_fw and *FAO1*_rv_*Sph*I. Additionally, the 3' end of the *TEF1* promoter was amplified using the primers *pTEF_Spe*I_fw3 and *pTEF_FAO1*_ol_rv. Both fragments were fused by overlap PCR using the outer primers. The overlap PCR product was cut with *Sph*I and *Spe*I and inserted in the similarly cut vector pIntB-HMG1 vector (Matthäus *et al.*, 2013). A new integration site with a central *Asc*I restriction site was identified on chromosome C and gained by PCR (primers: INT_*Asc*I_fw_*Kpn*I and INT_*Asc*I_rv_*Kpn*I). The integration site was cut with *Kpn*I and ligated into the *Kpn*I cut vector pIntB-*FAO1*. The resulting vector pIntC-*FAO1* was linearized with *Asc*I before transformation. The homologous integration was confirmed by PCR using the primers INT_*Asc*I_fw_out and INT_*Asc*I_rv_out.

2.3. Transformation of Y. lipolytica

0.2-1 µg of the gene disruption or overexpression cassettes were transformed into the desired uracil auxotroph *Y. lipolytica* recipient strain according to Barth and Gaillardin (1996).

2.4. Marker rescue

To regain uracil auxotrophy, the Cre-lox recombination system according to Fickers *et al.* (2003) and 5-FOA selection according to Boeke *et al.* (1984) were used.

For Cre-lox recombination the cells were transformed with 200 ng of the pUB4-CRE plasmid, which carries a gene for the Cre recombinase and a hygromycin B resistence gene. The transformed cells were plated on solid YPD medium containing 0.6 g l^{-1} hygromycin B (Roth).

For 5-FOA selection a loop of cells was overnight grown in minimal medium and different dilutions (10^4 - 10^6 cells) were plated on solid minimal medium containing 2 % (w/v) glucose, 20 mg l^{-1} uracil and 1 g l^{-1} FOA (Fermentas).
All plates were incubated at 28 °C for several days.

2.5. Isolation of genomic DNA

10 ml of yeast culture were pelleted and the cell pellet was resuspended in 500 µl lysis buffer (100 mM Tris pH 8.0, 50 mM EDTA, 1 % (w/v) SDS in H_2O) and cell lysis was performed in FastPrep FP120A-230 (Thermo Fisher Scientific) after adding of 400 µl of glass beads (0,75-1 mm, Roth). 275 µl 7 M ammonium acetate pH 7.0 were added to the supernatant and incubation was performed at 65 °C and on ice for 5 min, respectively. Then 500 µl chloroform were added, centrifugation was performed and the upper phase was precipitated with 1 ml isopropanol and centrifuged. The DNA pellet was washed with 500 µl ice cold 70 % (v/v) ethanol, dried and dissolved in 50 µl dH_2O. RNA was eliminated by adding 0.2 g l^{-1} RNase A (Fermentas) and incubation at 37 °C for 4 h.

2.6. Southern blot analysis

Southern blot analysis was performed to verify homologous integration of the disruption cassettes according to Southern (1975). Therefore 1 µg of genomic DNA was digested with *Bgl*II, separated by agarose gel electrophoresis and vacuum blotted on a Hybond-N nylon membrane (Amersham). The promoter region of the deleted genes was prepared by PCR and used as DNA probe. Probe labeling was done with the *Random Primed DNA Labeling Kit* (Roche). Detection was performed with the help of the *ECL Plus Western Blotting Detection Systems Reagents* (GE Healthcare) and the *FluorChem SP Imaging System* (Alpha Innotech).

2.7. Dry cell weight measurement

10 ml of the yeast culture were harvested, washed with 1 % (v/v) Tween 80 and distilled water and dried overnight at 100 °C in a pre-weighted 15 ml reaction tube. The tubes were cooled to room temperature and then weighted to calculate dry cell weight.

2.8. Cell lysis

1 ml of cells with an OD_{600} of 50 was harvested in a non-transparent 2 ml reaction tube. Then 1 ml 50 mM Tris-HCl pH 8.5 buffer and 1 ml glass beads (0.5 – 0.75 mm in diameter) were added. The mixture was homogenized in FastPrep FP120A-230 (Thermo Fisher Scientific) 4

times for 30 s at maximum speed and then centrifuged at 500 x g and 4 °C for 10 min. The whole procedure was performed on ice and the cell extract was protected from light.

2.9. Determination of total protein amount

The total protein amount was determined with the help of the Bradford protein assay (Bradford, 1976). 160 µl of the diluted probe (1:20 to 1:200) was mixed with 40 µl of Bradford reagent (Bio-Rad) in a 96-well microtitre plate and the absorption at λ = 595 nm was measured. Distilled water was used as blank and different concentrations of BSA were used to obtain a standard curve.

2.10. Fatty alcohol dehydrogenase and oxidase enzyme assay

The fatty alcohol dehydrogenase and oxidase enzyme assay was performed as described by Matatiele (2005). Brief: The fatty alcohol dehydrogenase assay [50 mM Tris-HCl pH 8.5, 1.3 mM dodecan-1-ol in DMSO, 2 mM NAD^+, 2 mM NAP^+, 1.5 % (v/v) cell extract] was measured with a recording spectrophotometer at 30 °C and λ = 340 nm and the enzyme activity was calculated from the measured slope ($\varepsilon_{NAD(P)H}$ = 6.3 mM^{-1} cm^{-1}). The fatty alcohol oxidase assay [50 mM glycine-NaOH pH 9.0, 0.35 mM dodecan-1-ol in DMSO, 0.013 % (w/v) peroxidase (150 U/mg), 0.044 % (w/v) ABTS, 6 mM sodium acide, 1 - 5 % (v/v) cell extract] was measured with a recording spectrophotometer at λ = 405 nm and the enzyme activity was also calculated from the measured slope (ε_{ABTSox} = 18.4 mM^{-1} cm^{-1}).

2.11. Extraction and quantification of alkanes and fatty acids

The hydrophobic substances were extracted from 1 ml of the culture probe by using 1 ml of diisopropyl ether. Aquatic and solvent phase were separated by centrifugation and the upper phase was transferred into a new glass vial, dehydrated with magnesium sulfate and 200 µl of silylation reagent (10 % (v/v) TMCS in MSTFA, Macherey-Nagel) was added. The probe was incubated at 80 °C for 1 h and then stored at 4 °C until GC analysis was performed.

GC analysis was performed with 1 µl of the silylated reagents using a *GC-17A* gas chromatograph (Shimadzu) with a *OPTIMA® 5* - 0.25 µm 30 m (0.25 mm ID) capillary (Macherey-Nagel). Nitrogen was used as carrier gas with a flow rate of 0.9 ml min^{-1} at 80 °C. The temperature of the oven was raised from 80 °C to 320 °C with a rate of 10 K per minute. The temperature of the injector block was 250 °C and the split ratio was 25:1. The Probes

were detected using a flame ionization detector (FID), which used synthetic air (pressure: 50 kPa) and hydrogen (60 kPa). The temperature of the detector block was 280 °C.

The chromatograms were analyzed by using the software *ClassVP 4.3a* or *GCSolution 2.41* from Shimadzu.

3. Results

3.1. Identification of potential (fatty) alcohol dehydrogenase and oxdidase genes by comperative sequence analysis

The *Y. lipolytica* genome database (*http://www.genolevures.org/yali.html*) lists nine (putative) alcohol dehydrogenase genes (Tab. 3), which are called *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, *ADH6*, *ADH7*, *ADH8* and *FADH* hereinafter. The enzyme coded by *ADH8* is probably not involved in the bioconversion of fatty alcohols because it is a putative aryl alcohol dehydrogenase. It is not clear whether *ADH4* and *ADH5* are coding for alcohol dehydrogenases because the similarity of their protein sequences to the corresponding sequences of *Saccharomyces cerevisiae* is pretty low (aa identities: 24 % and 25 %). A comparison of the known *ADH* genes of *C. tropicalis* with the putative genes of *Y. lipolytica* reveals similarities to the genes *ADH1*, *ADH2*, *ADH3*, *ADH6*, *ADH7* and *FADH*.

A putative fatty alcohol oxidase gene was identified by using the protein domain-based BLAST method *Domain enhanced lookup time accelerated* (*DELTA*)-*BLAST* (Boratyn *et al.*, 2012). The three known alcohol oxidase genes of *C. tropicalis* (*FAO1*, AY538780; *FAO2a*, AY538781; *FAO2b*, AY53878; (Eirich *et al.*, 2004)) were blasted against the whole genome of the *Y. lipolytica* strain E150 (Dujon *et al.*, 2004). All three *C. tropicalis* genes showed some similarity to the *Y. lipolytica* gene *YALI0B14014g* (aa identities: 13 %, 15 % and 15 %) which is called *FAO1* hereinafter.

3.2. Strain construction

To block β-oxidation and enable ω-oxidation of fatty acids all six Acyl-CoA oxidase-coding *POX* genes were consecutively deleted in *Y. lipolytica* H222-S4. β-oxidation of fatty acids was not fully blocked until all six *POX* genes were deleted. The resulting *POX1-6* deletion strain (*Y. lipolytica* H222ΔP) was not able to grow in medium with fatty acids as single carbon source. *Y. lipolytica* H222ΔP was used as basis to construct strains which are additionally lacking selected *ADH* genes and/or *FAO1* (Tab. 4).

In *Y. lipolytica* H222ΔP eight *ADH*-genes (*FADH, ADH1-7*) were deleted and the resulting strain was called *Y. lipolytica* H222ΔPΔA. Additionally, a strain lacking the *POX*-genes and

FAO1 (*Y. lipolytica* H222ΔPΔF) and a strain lacking *POX1-6* and both all relevant *ADH* genes and *FAO1* (*Y. lipolytica* H222ΔPΔAΔF) was constructed.

Furthermore, the *FAO1* gene was overexpressed in *Y. lipolytica* H222ΔP by the insertion of an additionally gene copy controlled by the strong constitutive promoter of the *TEF1* gene. The respective strain was called *Y. lipolytica* H222ΔPoF.

The transformants were checked for homologous integration of the inserted cassette via PCR and/or southern blot. There was no significant difference of the growth rate between the constructed strains and the wild type during growth in medium with glucose or glycerol as carbon source (data not shown).

3.3. Influence of ADH and FAO genes on ω-oxidation

To determine whether the (fatty) alcohol dehydrogenase genes (*FADH*, *ADH1-7*) or the fatty alcohol oxidase gene (*FAO1*) are mainly responsible for the oxidation of ω-hydroxy fatty acids in *Y. lipolytica*, the strains H222ΔP, H222ΔPΔA and H222ΔPΔF were tested for their ability to accumulate dicarboxylic acids and ω-hydroxy fatty acids. For this purpose the strains were cultivated in minimal medium with glycerol and dodecane (DD), dodecanoic acid (DDA), pentadecane (PD), pentadecanoic acid (PDA), hexadecane (HD) or hexadecanoic acid (HDA). During cultivation glycerol was normally consumed after 48 h when 0.5 % (v/v) glycerol was fed. The respective dicarboxylic acids and ω-hydroxy fatty acids were successively accumulated at the end of cultivation.

The products were mainly found in the culture supernatant and not in the cells. A microscopic analysis of the cells revealed that they formed large lipid bodies which increased in size during the first 72 h of cultivation (data not shown).

Y. lipolytica H222ΔP produced relatively large amounts of dicarboxylic acids after 96 h of cultivation and only very small amounts of ω-hydroxy fatty acids with ratios from 5:1 (DDA) to 259:1 (DD) (Fig. 2).

The deletion of eight putative *ADH* genes led only to a slightly reduced production of the respective dicarboxylic acid and a slightly increased accumulation of the ω-hydroxy fatty acid. In contrast to this the deletion of *POX1-6* and *FAO1* resulted in an increased accumulation of ω-hydroxy fatty acids and a notably reduced production of the particular dicarboxylic acid. Finally product ratios from 2:5 (DDA) to 13:1 (HDA) could be realized.

Surprisingly, alkanes (DD, PD and HD) could be used as substrates for bioconversion as well as fatty acids (DDA, PDA and HDA) since the conversion rate was only in case of the use of PD slightly reduced.

3.4. Cultivation of all strains under production conditions

To determine the potential of all constructed strains for the production of ω-hydroxy fatty acids and dicarboxylic acids, they were cultivated in a 600 ml fermenter using glucose as carbon source and dodecane (DD) as substrate for bioconversion. DD was chosen as substrate because it is a suitable model substrate for the bioconversion of shorter and longer *n*-alkanes because of its medium chain length. The usage of *n*-alkanes as substrate for bioconversion is preferred to those of fatty acids because latter are more expensive, have to be emulsified and lead to strong foaming when used in a fermenter. After 24 h of cultivation, 15 g l^{-1} DD was added and the pH was adjusted to 8.0 as described by Thevenieau (2006).

Y. lipolytica H222ΔP produced about 6 g l^{-1} of dodecane dioic acid (DDDA) and nearly no ω-hydroxy dodecanoic acid (ω-HDDA) after four days of cultivation (Fig. 3).

About the same result was achieved during the cultivation of *Y. lipolytica* H222ΔPΔA. Only at the beginning of the production phase a slightly increased accumulation of ω-HDDA was observed compared to *Y. lipolytica* H222ΔP.

In contrast to this, *Y. lipolytica* H222ΔPΔF produced about 7 g l^{-1} of ω-HDDA and only 3 g l^{-1} of DDDA after four days of cultivation.

Surprisingly, the *POX*, *ADH* and *FAO* deleted strain *Y. lipolytica* H222ΔPΔAΔF was still able to convert DD to ω-HDDA and DDDA. Hereby the production of ω-HDDA was increased and those of DDDA decreased compared to *Y. lipolytica* H222ΔPΔF (about 9 g l^{-1} of ω-HDDA and 0.3 g l^{-1} of DDDA after three days of cultivation).

The overexpression of *FAO1* in the *POX* deleted strain *Y. lipolytica* H222ΔP led to an increased production of DDDA in comparison to *Y. lipolytica* H222ΔP (about 11 g l^{-1} after four days of cultivation).

In the middle of the production phase (after three days of cultivation) fatty alcohol dehydrogenase and oxidase activity was quantified using respective enzyme assays.

Surprisingly, there was no significant difference in the fatty alcohol dehydrogenase activity between all tested strains detectible (Fig. 4). Generally, the measured enzyme activity showed a strong deviation and varied from 1 to 10 U mg^{-1} between different probes.

As expected, fatty alcohol oxidase activity was only detectible in *Y. lipolytica* H222ΔP and H222ΔPΔA and not in the *FAO1* deletion strains *Y. lipolytica* H222ΔPΔF and H222ΔPΔAΔF. The measured enzyme activity was very low (about 0.03-0.04 U mg^{-1} protein). The detected fatty alcohol oxidase activity was more the tenfold increased by the overexpression of *FAO1* in H222ΔPoF.

4. Discussion

4.1. Identification of potential (fatty) alcohol dehydrogenase and oxdidase genes by comperative sequence analysis

In this study we identified nine potential (fatty) alcohol dehydrogenase genes (*FADH*, *ADH1-8*) whereby eight may be involved in the conversion of alkanes and fatty acids during primary alkane oxidation and ω-oxidation, respectively. These genes showed some similarities to respective genes in *S. cerevisiae* and *C. tropicalis*. It is still not clear which of these genes really codes for enzymes with alcohol dehydrogenase activity since Barth and Künkel identified four simultaneously expressed alcohol dehydrogenases with molecular weights of 70, 120 and 240 kDa during growth in glucose containing medium (Barth & Künkel, 1979). The molecular weight of the proteins predicted from the identified *ADH* genes varies from 37.0 to 53.2 kDa what indicates that the enzymes might occur as multimers in the cell. In *S. cerevisiae* Adh1p occurs as tetramer whose subunits have a molecular mass of 36 kDa (Veillon & Sytkowski, 1975; Klinman & Welsh, 1976).

Additionally to the *ADH* genes one potential fatty alcohol oxidase gene (*FAO1*) was identified, whose corresponding enzyme is involved in ω-oxidation. Although the gene has not been described yet, the corresponding enzyme was isolated in 1994 (Ilchenko *et al.*, 1994). The isolated enzyme showed maximum substrate specificity towards primary alcohols with chain lengths from C12 to C18 and had an isoelectric point (pI) of 5.5 and a molecular weight (MW) of 70 ± 2 kDa. These values are near to the ones which can be calculated from the nucleotide sequence of *FAO1* (pI: 5.31, MW: 67.6 kDa) what indicates that the determined gene is the one which has been searched for.

4.2. Strain construction

All six Acyl-CoA oxidase-coding *POX*-genes (*POX1-6*) had to be deleted to fully block β-oxidation in *Y. lipolytica*. This has already been observed before by Thevenieau (2006). In contrast to this, in *C. tropicalis* β-oxidation can be blocked by the deletion of *POX4* and *POX5* (Picataggio *et al.*, 1991).

It is further remarkable that the combined deletion of the *POX* and *ADH* genes as well as the *FAO1* gene was not lethal and did not significantly affect the growth of the respective strains. Different results were obtained with genetically engineered *C. tropicalis* strains; a similar strain in which among others all four identified *FAO* genes and seven of eight *ADH* genes were deleted was shown to be less healthy then the origin strain (Lu *et al.*, 2010).

4.3. Influence of ADH and FAO genes on ω-oxidation

The *Y. lipolytica* strains H222ΔP, H222ΔPΔA and H222ΔPΔF were cultivated in minimal medium with glycerol and different long chain *n*-alkanes and fatty acids as carbon sources. There was no observable difference of the growth rate between the production strains and the wild type. This might be explained with the fact that energy is mainly gained from the metabolization of glycerol and that even the acyl-CoA oxidase deficient strains stored high amounts of the hydrophobic substrates into intracellular *lipid bodies*.

The wild type strain *Y. lipolytica* H222 utilized the offered glycerol as well as the hydrophobic substrates continuously whereby neither dicarboxylic acids nor ω-hydroxy fatty acids were accumulated in the culture medium. In *Y. lipolytica* H222 β-oxidation is still intact and so the hydrophobic substrates are degraded to acetyl-CoA which is used in tricarboxylic acid cycle (see 1.2). In most alkane-assimilating yeasts β-oxidation is much more prominent than ω-oxidation of fatty acids (Mauersberger *et al.*, 1996).

The *POX* deletion strain *Y. lipolytica* H222ΔP was able to produce high amounts of dicarboxylic acids. The deletion of *FAO1* had a stronger effect in preventing the degradation of ω-hydroxy fatty acids then the deletion of eight *ADH* genes.

So it can be assumed that in *Y. lipolytica* both alcohol dehydrogenases and oxidases (Fao1p) are involved in the bioconversion of ω-hydroxy fatty acids to dicarboxylic acids during ω-oxidation whereby latter seem to play the major role. These results are different to the ones achieved with *C. tropicalis* where an accumulation of ω-hydroxy fatty acids was realized by the deletion of six cytochrome P450, four alcohol oxidase and six alcohol dehydrogenase genes whereby the deletion of alcohol dehydrogenase genes seemed to be most important (Lu *et al.*, 2010). In *C. tropicalis* it was also not possible to fully block the degradation of ω-hydroxy fatty acids because of the lethal effects of a deletion of the involved enzymes.

Neither the deletion of the *ADH* genes nor the deletion of *FAO1* had a noticeable effect on primary alkane oxidation since the bioconversion rates of the respective strains were not significantly smaller when *n*-alkanes were used as substrates instead of fatty acids.

Most of the detected amounts of ω-hydroxy fatty acids and dicarboxylic acids were found in the culture medium and not in the cell pellet. This indicates that both substances are secreted after their synthesis by the yeast cells. It is known that *Y. lipolytica* is able to secrete organic acids like citric acid (Barth & Gaillardin, 1996; Barth & Gaillardin, 1997). The intracellular fatty acids are probably bound to glycerol and stored as triglycerides.

4.4. Cultivation of all strains under production conditions

As expected, the productivity of all testes strains war increased during cultivation in fermenter. Similar to the shaking flask cultivations, *Y. lipolytica* H222ΔP and H222ΔPΔA accumulated high amounts of dodecane dioic acid (DDDA). In contrast to this *Y. lipolytica* H222ΔPΔF and H222ΔPΔAΔF mainly accumulated ω-hydroxy dodecanoic acid (ω-HDDA) underlining the hypothesis that *FAO1* is mainly responsible for ω-oxidation of long chain fatty acids in *Y. lipolytica*. The *POX*, *ADH* and *FAO1* deleted strain accumulated only small amounts of DDDA and can therefore be used as basis to construct a strain for the biotechnological production of ω-hydroxy fatty acids.

The overexpression of *FAO1* increased the production of DDDA in *Y. lipolytica* H222ΔPoF indicating that the oxidation of ω-hydroxy fatty acids is a rate limiting step during production of dicarboxylic acids.

Surprisingly, the conversion of dodecane was neither blocked nor reduced in *Y. lipolytica* H222ΔPΔAΔF. Also dodecan-1-ol was converted in all strains during alcohol dehydrogenase enzyme assay indicating that primary alkane oxidation was neither affected by the deletion of eight *ADH* genes nor by the deletion of *FAO1*. Unfortunately, the enzyme assay is not specific enough to tell whether the measured slope is a result of alcohol dehydrogenase or other enzymatic activity. This is because the detected NADH is not only produced by alcohol dehydrogenases but also by aldehyde dehydrogenases which are converting the accruing fatty aldehyde to the corresponding fatty acid. So it requires further investigation to elucidate whether dodecan-1-ol is oxidized by remaining alcohol dehydrogenase activity or by cytochrome P450 as it has been shown *in vitro* for *Candida maltosa* (Scheller *et al.*, 1998).

In contrast to this, fatty alcohol oxidase activity was eliminated in the *FAO1* deletion strains and enhanced in the respective overexpression strain. The detected activity was quite low, as described before by Matatiele (2005). This might be because of the instability and light and heat sensitivity of the enzyme (Kemp *et al.*, 1990). Our results indicate that in *Y. lipolytica* only one fatty alcohol oxidase exists although Ilchenko *et al.* (1994) predicted the existence of several FAO enzymes.

4.5. Outlook

This work provides only the first step towards the biotechnological production of ω-hydroxy fatty acids with the non-conventional yeast *Y. lipolytica*. To reach this aim, further research has to be done. Possible future strategies would for example be the reduction of *lipid body* formation which could be achieved by the deletion of the phosphatidic acid phosphatase gene

(*PAH1*, *YALI0D27016g*) and/or the acyl-CoA diacylglycerol acyltransferase gene (*DGA1*, *YALI0E32769g*) and by the overexpression of the NADPH-cytochrome P450 reductase gene (*CPR1*, *YALI0D04422g*) (Thevenieau, 2006; Athenstaedt, 2011). The further optimization of the cultivation conditions might also lead to an increased productivity.

Existing strains for the production of dicarboxylic acids (Thevenieau, 2006) cloud be improved by the overexpression *FAO1*.

Additionally to the biotechnological aspects of this work the created *ADH* and *FAO* deletion strains (*Y. lipolytica* H222ΔPΔA and H222ΔPΔF) could be used to further characterize the alcohol dehydrogenase and oxidase genes of *Y. lipolytica*. It also remains to be clarified why *POX*, *ADH* and *FAO* deletion strain *Y. lipolytica* H222ΔPΔAΔF is still able to oxidize dodecanol.

5. References

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Tables

Tab. 1 *Y. lipolytica* strains used in this study

***Y. lipolytica* strain**	**Genotype**	**Reference**
H222	*MATA* wild type	(Barth & Gaillardin, 1996)
H222-S4	*MATA ura3-302::SUC2*	(Mauersberger *et al.*, 2001)
H222ΔP	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6::URA3*	This work
H222ΔP *ura*$^-$	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6*	This work

H222ΔPΔA	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6 Δfadh Δadh1 Δadh2 Δadh3 Δadh5 Δadh6 Δadh7 Δadh4::URA3*	This work
H222ΔPΔA *ura*$^-$	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6 Δfadh Δadh1 Δadh2 Δadh3 Δadh4 Δadh5 Δadh6 Δadh7*	This work
H222ΔPΔF	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6 Δfao1::URA3*	This work
H222ΔPΔAΔF	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6 Δfadh Δadh1 Δadh2 Δadh3 Δadh4 Δadh5 Δadh6 Δadh7 Δfao1::URA3*	This work
H222ΔPoF	*MATA ura3-302::SUC2 Δpox1 Δpox2 Δpox3 Δpox4 Δpox5 Δpox6 FAO1::URA3*	This work

Tab. 2 Primers used in this study

Introduced restriction sites (RS) are underlined. Sequences which are complementary to the genomic DNA of *Y. lipolytica* are written in capital letters and non-complementary sequences in small letters.

Name	**Sequence (5'→3')**	**RS**
*pPOX1*_fw	TCCAGAAGCGCTACAAAGAG	
*pPOX1*_rv	attaccctgttatccctaTGAAGGTTGCAGTCGTAGTC	I-*Sce*I
*POX1t*_fw	tagggataacagggtaatTGCGATCTCGATGAGTGATG	I-*Sce*I
*POX1t*_r*v*	GCCCAGAAGATTGGAATGAC	
*pPOX2*_fw	atataccgcggGATTCCGCCAAGTGAGACTG	*Cfr*42I
*pPOX2*_rv	attaccctgttatccctaCGTCGAGGAAGTAGGTCATC	I-*Sce*I
*POX2t*_fw	tagggataacagggtaatGCGAGCTTGATGAGGAATAG	I-*Sce*I
*POX2t*_rv	atataccgcggCCTGACGCCAATTTGAAGAG	*Cfr*42I
*pPOX3*_fw	atataccgcggCTGGGCTGTTCGGTCGATAG	*Cfr*42I
*pPOX3*_rv	tagggataacagggtaatAGGACGCACAACGCCATCAC	I-*Sce*I
*POX3t*_fw	attaccctgttatccctaCGCTCCCATTGGAAACTA	I-*Sce*I
*POX3t*_rv	atataccgcggTCTCTTCGCTGTGGTCTAGG	*Cfr*42I
*pPOX4*_fw	atataccgcggTCCACCGTTCTCCTTCATAC	*Cfr*42I
*pPOX4*_rv	tagggataacagggtaatATGTCTCTAGGGTCGAAGTC	I-*Sce*I
*POX4t*_fw	attaccctgttatccctaTGGCAAGCCTCACTACTACG	I-*Sce*I
*POX4t*_rv	atataccgcggTGCGGCGGAACTACTGTATC	*Cfr*42I
*pPOX5*_fw	atataccgcggGGGATTCTCCGGGTTATTTG	*Cfr*42I
*pPOX5*_rv	tagggataacagggtaatACGTCTCGGACCTTGAATTG	I-*Sce*I
*POX5t*_fw	attaccctgttatccctaCCTTCAACCTGTCCGACTTC	I-*Sce*I
*POX5t*_rv	atataccgcggGAAGCGGTCCTCGTTGTATG	*Cfr*42I
*pPOX6*_fw	GTGTAGCAACTCGGATACAG	
*pPOX6*_rv	tagggataacagggtaatGGTCCATAAGCAGAGTGTTC	I-*Sce*I
*POX6t*_fw	attaccctgttatccctaACCCTCGACCTCCTTATTAC	I-*Sce*I

*POX6t*_rv	CTCTTCTTGACTGGCATAGC	
*pFADH*_fw	atataaagctTGCGGCTCGGCACGTGATCTG	*Hin*dII
*pFADH*_rv	atataggatccATCGTGCGTACGTCGCTAGTG	*Bam*HI
*FADHt*_fw	atataggatccCGACCGGCACGATCAATTGG	*Bam*HI
*FADHt*_rv	atatacatatgGGTGCATCTCAGCCCGACCTC	*Nde*I
*FADHt*_rv2	TCCCGAAACACAGAACTTCC	
*pADH1*_fw	atataaagctTGGTGGACGTTCCGGCAGACAG	*Hin*dII
*pADH1*_rv	atataggatccCTCCCAGGCATCTCCACACTC	*Bam*HI
*ADH1t*_fw	atataggatccCACTTACAGGCTTAGCAAGG	*Bam*HI
*ADH1t*_rv	atatagcggccgcGGAAATCACGCTTGATTCG	*Not*I
*ADH1t*_rv2	TAGGCGCTGGTACAGAAGAG	
*pADH2*_fw	atataaagctTGAGTACAGTAGGTGGTACTC	*Hin*dII
*pADH2*_rv	atataggatccAGTGGTGGTGGTGGTGGTAG	*Bam*HI
*ADH2t*_fw	atataggatccTTTACGTGCAACAGGAGGAG	*Bam*HI
*ADH2t*_rv	atatacatatgGCCTGTCTTGAGTTCTTTGG	*Nde*I
*ADH2t*_rv2	AGGGTCGTAGATAACGAGTC	
*pADH3*_fw	atataaagctTCACGTGGCTGCTGGGCCAACC	*Hin*dII
*pADH3*_rv	atataggatccCGCACGGTATCGGAGCATCG	*Bam*HI
*ADH3t*_fw	atataggatccCGCGGCTATTGACGCTGAGG	*Bam*HI
*ADH3t*_rv	atatacatatgCCCGTCAGCTCCATCGACGAGTG	*Nde*I
*ADH3t*_rv2	AGGTGTACTGTAGCCACCCTGAC	
*pADH4*_fw	atataaagctTCCGGCCAGCCGCTGGCAACG	*Hin*dII
*pADH4*_rv	atataggatccACACGACAGCTGCACCTGAC	*Bam*HI
*ADH4t*_fw	atataggatccCAGCCATGAGCCAGGCATTG	*Bam*HI
*ADH4t*_rv	atatacatatgGGCGCCAGCCACATTTGCCCTC	*Nde*I
*ADH4t*_rv2	AGCGATACAGCAGTTGACTC	
*pADH5*_fw	TCAGCCGTCTACTTGTAGAG	
*pADH5*_rv	atataggatccGTGGCTCGGATACTCCTGAC	*Bam*HI
*ADH5t*_fw	atataggatccAGCCGGAGGTCAGATCAAGC	*Bam*HI
*ADH5t*_rv	atatacatatgGCGCAATAGTTCGCCGGCCTG	*Nde*I
*ADH5t*_rv2	CTCGTGTTGTGCCTTTCTTG	
*pADH6*_fw	atataaagctTGCGCGACAACCCATAGCGATGGC	*Hin*dII
*pADH6*_rv	atataggatccGATAAGAGGGCGCTCTGACC	*Bam*HI
*tADH6*_fw	atataggatccGGCGTGACATCGAGTTTGG	*Bam*HI
*tADH6*_rv	atatacatatgCTACGTCTCGCCGCAGAGGG	*Nde*I
*tADH6*_rv2	AGCGAGAGGTTATACGGAAG	
*pADH7*_fw	CTCCTACAGCCTCTCAAGAC	
*tADH7*_rv	GTCTACAAGACAGCCCAGAG	
*tADH7*_rv2	CCGCTTGAGAAGAGCAATAC	
*pFAO1*_fw	atataaagctTCGCCACCTGTCCACGTCTCG	*Hin*dII
*pFAO1*_rv	atataggatccGCGAAGCGACGTGTGGTGAG	*Bam*HI
*FAO1t*_fw	atataggatccGCTGAGCACGCGAGTACACC	*Bam*HI
*FAO1t*_rv	atatagaattcGATCTGTCGTACAACTAAGG	*Eco*RI
*FAO1t*_rv2	CAGAAGTTACGACGCCAAGG	

*pTEF_FAO1*_ol_fw	ccttctgagtataagaatcattcaaAATGTCTGACGACAAGCACAC	
*FAO1*_rv_*Sph*I	gcaTGCTTAGATTCGAGGTCGGAGAT	*Sph*I
*pTEF_Spe*I_fw3	CTACGCTTGTTCAGACTTTG	
*pTEF_FAO1*_ol_rv	gtgtgcttgtcgtcagacatTTTGAATGATTCTTATACTCAGAAGG	
INT_*Asc*I_fw_*Kpn*I	ggtacCACGCACGGATAGTTTATCCA	*Kpn*I
INT_*Asc*I_rv_*Kpn*I	ggtacCCAAAGTCAACTAATGTCAAGTAAAG	*Kpn*I
INT_*Asc*I_fw_out	CCTCCAACGTGACTTTC	
INT_*Asc*I_rv_out	AGAGACCTCCCACAAAG	

Tab. 3 List of (putative) alcohol dehydrogenase and oxidase genes in *Yarrowia lipolytica*

Gene	Name	Amino acids / MW[1] [kDa]	Database entry[2]
YALI0F09603g	*FADH*	381 / 40.4 kDa	similar to uniprot|P32771 *Saccharomyces cerevisiae* YDL168w SFA1 long-chain alcohol dehydrogenase and DEHA0G06457g *Debaryomyces hansenii*
YALI0D25630g	*ADH1*	349 / 37.1 kDa	uniprot|Q9UW08 *Yarrowia lipolytica* Alcohol dehydrogenase 1
YALI0E17787g	*ADH2*	351 / 37.3 kDa	uniprot|Q9UW07 *Yarrowia lipolytica* Alcohol dehydrogenase 2
YALI0A16379g	*ADH3*	349 / 37.1 kDa	uniprot|Q9UW06 *Yarrowia lipolytica* Alcohol dehydrogenase 3
YALI0E15818g	*ADH4*	492 / 53.2 kDa	some similarities with uniprot|P10127 *Saccharomyces cerevisiae* YGL256w ADH4 alcohol dehydrogenase IV
YALI0D02167g	*ADH5*	346 / 37.3 kDa	some similarities with uniprot|P38113 *Saccharomyces cerevisiae* YBR145w ADH5 alcohol dehydrogenase V
YALI0A15147g	*ADH6*	348 / 37.0 kDa	similar to uniprot|Q9UW06 *Yarrowia lipolytica* Alcohol dehydrogenase 3
YALI0E07766g	*ADH7*	346 / 37.6 kDa	similar to uniprot|P07246 *Saccharomyces cerevisiae* YMR083w ADH3 alcohol dehydrogenase III
YALI0C12595g	*ADH8*	424 / 48.3 kDa	similar to uniprot|Q02895 *Saccharomyces cerevisiae* YPL088w putative aryl-alcohol dehydrogenase
YALI0B14014g	*FAO1*	609 / 67.6 kDa	weakly similar to uniprot|Q8NK56 *Cryptococcus neoformans* SMG1

[1] Molecular weight (MW) was calculated with the *Compute pI/Mw tool* (*http://web.expasy.org/compute_pi/*)

[2] *Yarrowia lipolytica* genome database (*http://www.genolevures.org/yali.html*)

Tab. 4 List of *Yarrowia lipolytica* deletion strains created in this study

Strain	*POX1-6*	*FADH*	*ADH1*	*ADH2*	*ADH3*	*ADH4*	*ADH5*	*ADH6*	*ADH7*	*FAO1*
H222ΔP	×									
H222ΔPΔA	×	×	×	×	×	×	×	×	×	
H222ΔPΔF	×									×
H222ΔPΔAΔF	×	×	×	×	×	×	×	×	×	×

Figure legends

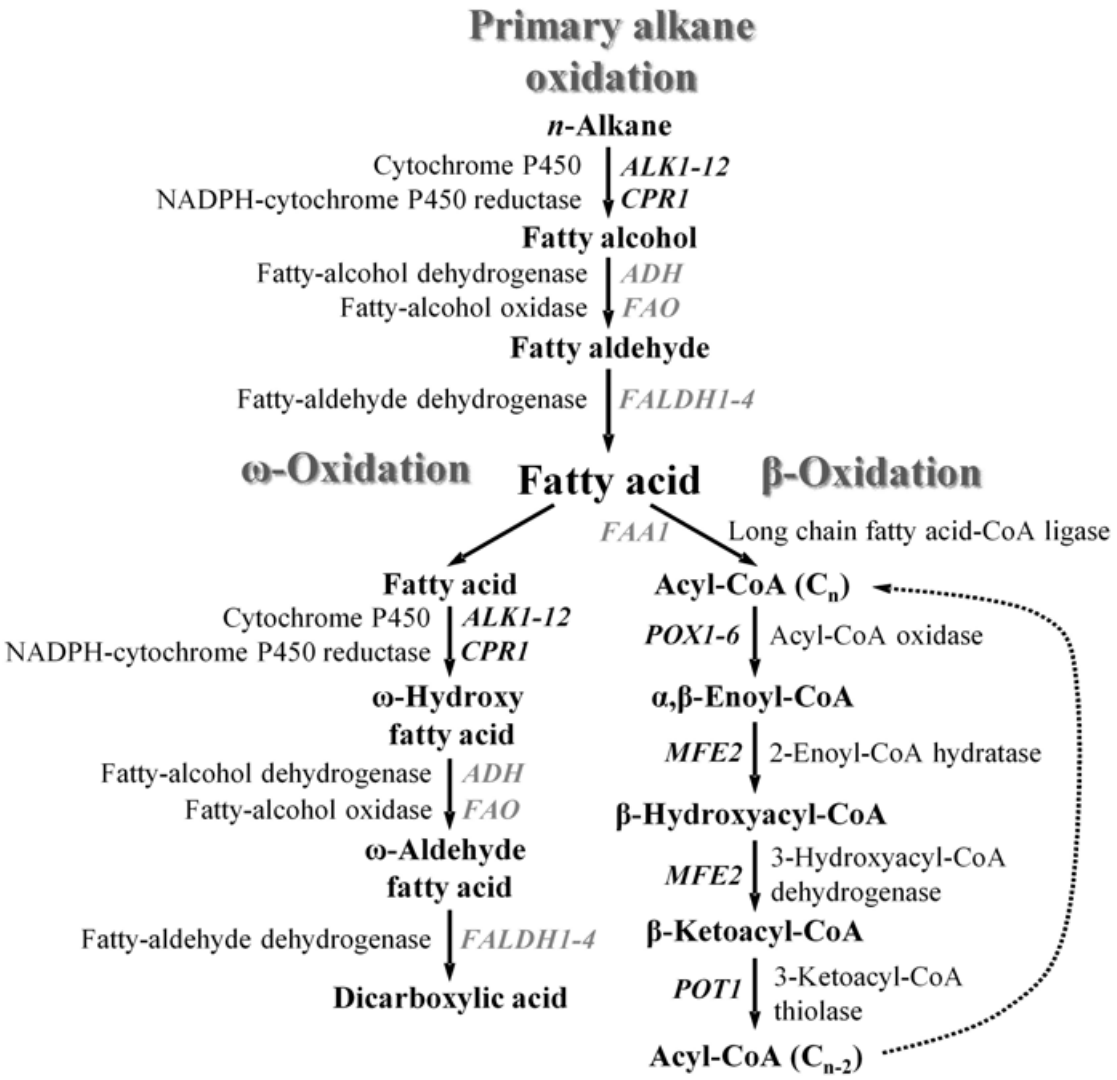
Fig. 1 Metabolic pathways for the degradation of hydrophobic substrates in *Yarrowia lipolytica*. *n*-Alkanes are taken up into the cell and then converted to the corresponding fatty acid by primary alkane oxidation. The fatty acid is then degraded to acetyl-CoA (or propionyl-CoA from alkanes with odd chain length) during β-oxidation. The diterminal oxidation of fatty acids (ω-oxidation) can also occur. The names of the formed chemical substances are given as well as the names of the involved enzymes and the abbreviation of the corresponding genes. Successfully identified genes are written in black and putative genes in grey. *ALK1*: *YALI0E25982g*, *ALK2*: *YALI0F01320g*, *ALK3*: *YALI0A20130g*, *ALK4*: *YALI0B13816g*, *ALK5*: *YALI0B13838g*, *ALK6*: *YALI0B01848g*, *ALK7*: *YALI0A15488g*, *ALK8*: *YALI0C12122g*, *ALK9*: *YALI0B06248g*, *ALK10*: *YALI0B20702g*, *ALK11*: *YALI0C10054g*, *ALK12*: *YALI0A20130g*, *CPR1*: *YALI0D04422g*, *ADH and FAO*: see chapter 1.3, *FALDH1*: *YALI0A17875g*, *FALDH2*: *YALI0E15400g*, *FALDH3: YALI0B01298g*, *FALDH4: YALI0F23793g*, *FAA1*: *YALI0D17864g*, *POX1*: *YALI0E32835g*, *POX2*: *YALI0F10857g*, *POX3*: *YALI0D24750g*, *POX4*: *YALI0E27654g*, *POX5*: *YALI0C23859g*, *POX6*: *YALI0E06567g*, *MFE2*: *YALI0E15378g*, *POT1*: *YALI0E18568g*.

Fig. 2 Accumulation of dicarboxylic acids and ω-hydroxy fatty acids in *Yarrowia lipolytica* H222ΔP, H222ΔPΔA and H222ΔPΔF. The cells were grown in minimal

medium with glycerol and various *n*-alkanes and fatty acids (DD: dodecane; DDA: dodecanoic acid; PD: pentadecane; PDA: pentadecanoic acid; HD: hexadecane; HDA: hexadecanoic acid). The amounts of produced dioic acids and ω-hydroxy fatty acids (DDDA: dodecanedioic acid; ω-HDDA: ω-hydroxy dodecanoic acid; PDDA: pentadecanedioic acid; ω-HPDA: ω-hydroxy pentadecanoic acid; HDDA: hexadecanedioic acid; ω-HHDA: ω-hydroxy hexadecanoic acid) were determined by GC analysis after 96 h of cultivation.

Fig. 3 Cultivation of *Yarrowia lipolytica* H222ΔP (A), H222ΔPΔA (B), H222ΔPΔF (C), H222ΔPΔAΔF (D) and H222ΔPoF (E) under production conditions. The cells were grown in minimal medium with glucose in a fermenter. After 24 h of cultivation pH was adjusted to 8.0 and 15 g l^{-1} dodecane (DD) were fed. Every 24 h the amounts of produced dodecanedioic acid (DDDA) and ω-hydroxy dodecanoic acid (ω-HDDA) were determinded as well as the dry cell weight (DCW).

Fig. 4 Fatty alcohol dehydrogenase (A) and oxidase (B) activity in *Yarrowia lipolytica* H222ΔP, H222ΔPΔA, H222ΔPΔF, H222ΔPΔAΔF and H222ΔPoF during cultivation under production conditions. The cells were harvested and cell lysis was performed after three days of cultivation under production conditions. The respective enzyme activity was determined using an enzyme assay and set in relation to the total amount of protein.



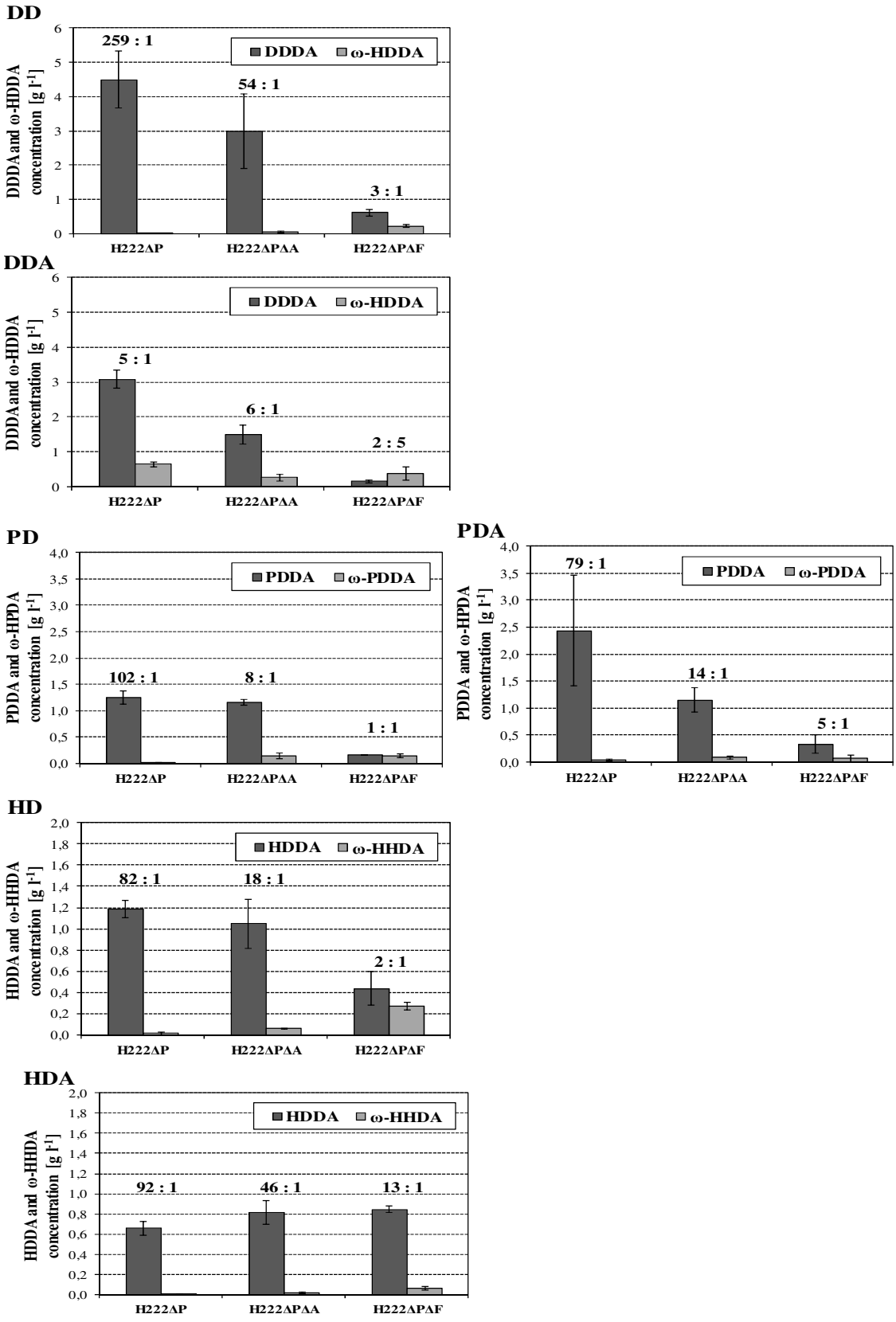
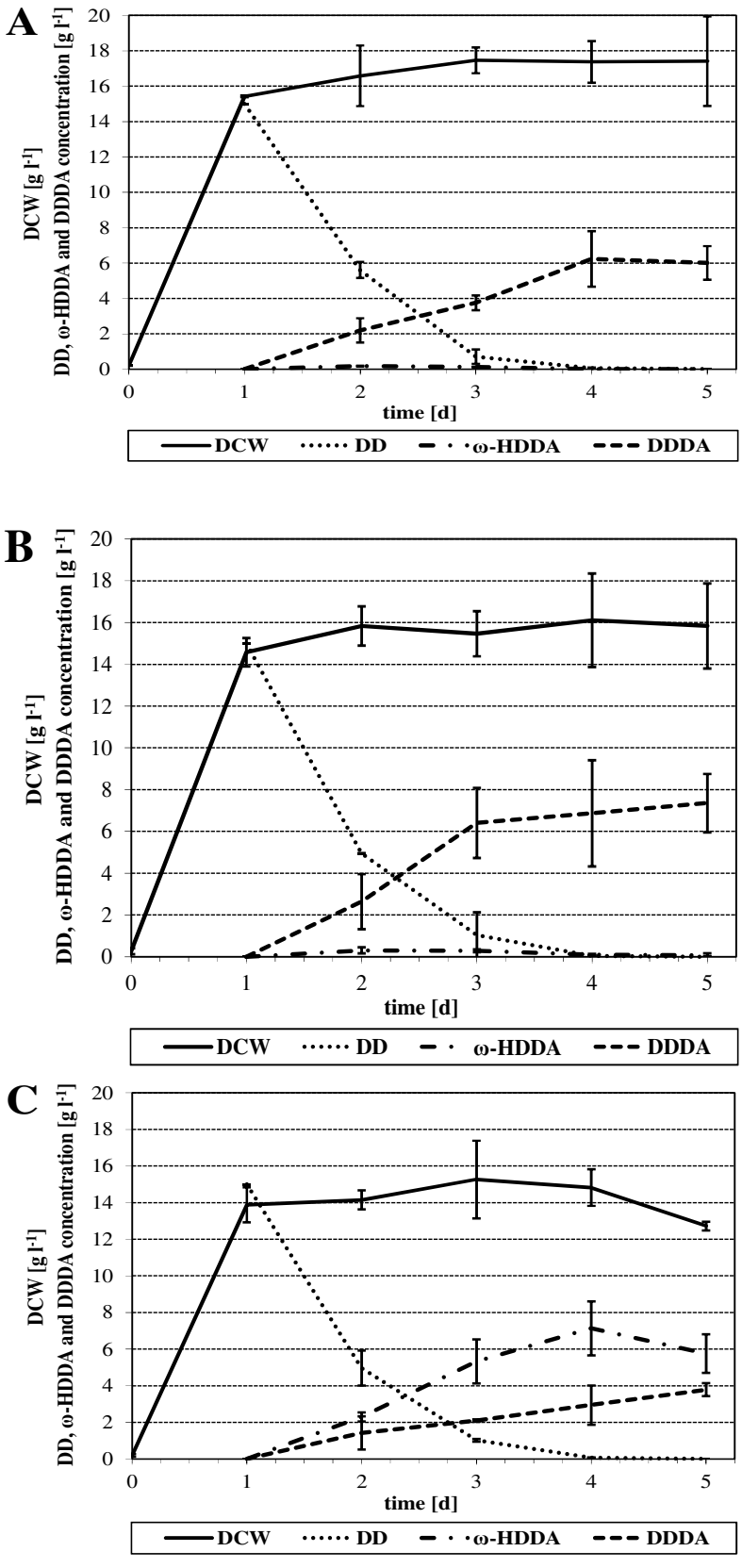


Figure 2



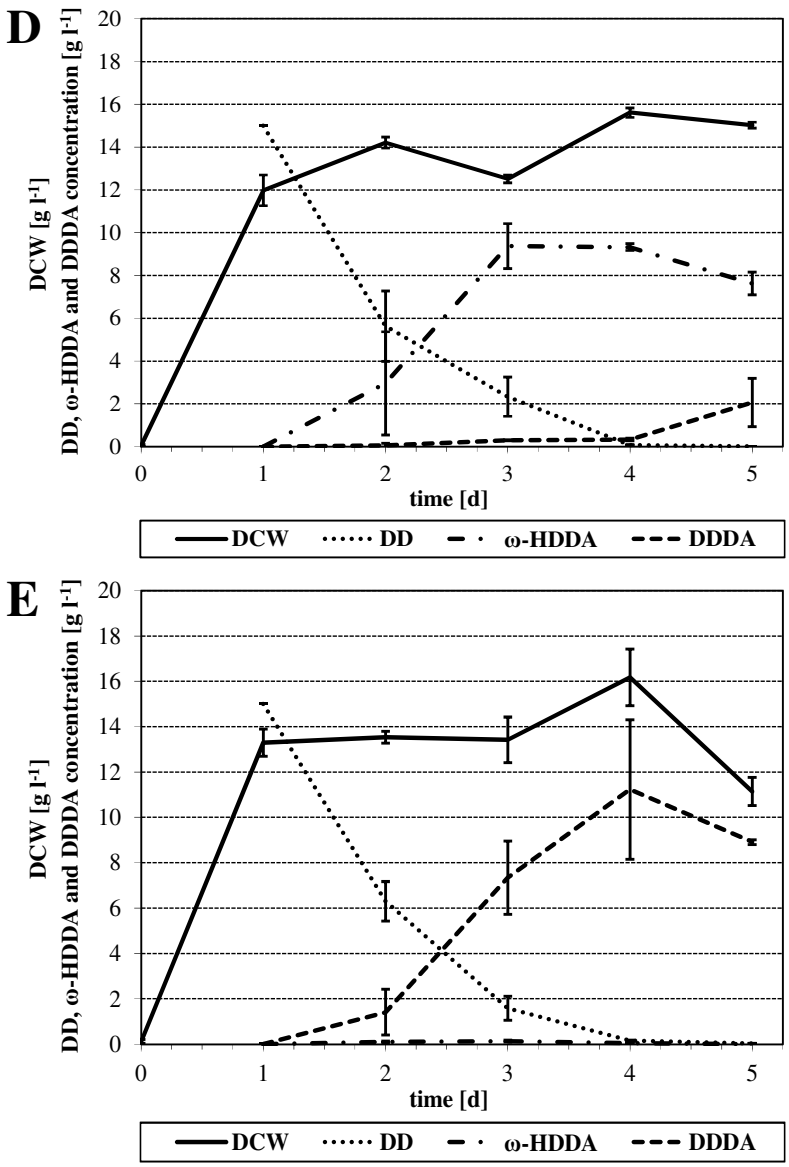


Figure 3

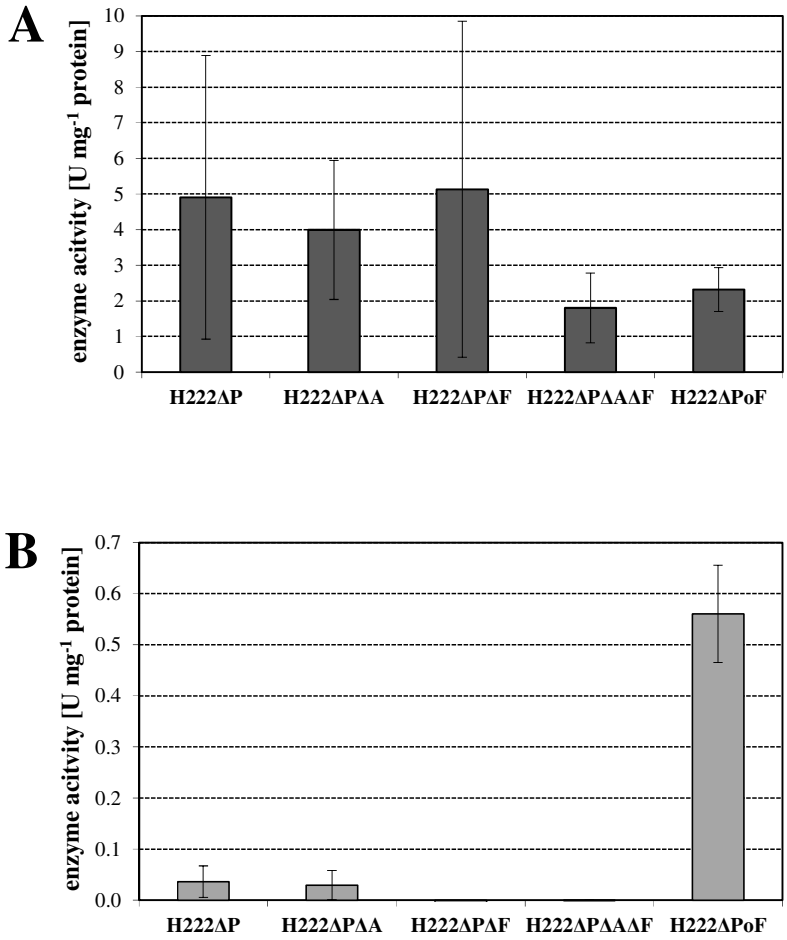


Figure 4