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A single-host fermentation process for the production of flavor lactones from non-hydroxylated fatty acids

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1. Introduction

Lactones are cyclic carboxylic esters. Lactones with five-(γ)- or six-(δ)-membered rings and eight-to sixteen-carbons have fruity and milky aromas and are used in food and fragrance industries (Gaffield et al., 1997). The rising consumer demand for natural flavors and changes in legislations (EU Flavouring Regulation (EC) No 1334/2008, United States’ Code of Federal Regulation Title 21) made biological processes for lactone production more attractive. Due to the low abundance (ppm levels (Gaffield et al., 1997; Eduardo et al., 2013; Sánchez-Sevilla et al., 2014)) of lactones in natural sources, extraction of natural lactones is not economically feasible. Commercial flavor lactones are produced by chemical synthesis or by enzymatic or microbial conversion of hydroxy fatty acids (HFA). The HFAs are extracted from plants (Gaffield et al., 1997; Vandamme and Soetaert, 2002; Krings and Berger, 1998), which poses challenges as low yields, supply instability, and the presence of plant toxins in some plant materials (Krings and Berger, 1998; Milani et al., 2013) As an example, γ-decalactone can be made from ricinoleic acid, derived from castor seed oil. The content of ricinoleic acid in castor oil is up to 90%, translating into a yield of up to 1200 kg ricinoleic acid per ha (Milani et al., 2013). Despite low production cost compared to other plant oils (Gui et al., 2008), for farmers in many areas, profitability of castor oil production is still low and supply stability is affected by climate change (Milani et al., 2013). Furthermore, castor seeds contain up to 32 mg of ricin, a lethal phytotoxin, per gram of seeds (Sousa et al., 2017). The production of lactones from castor oil requires meticulous detoxification.

Microbial hosts capable of lactone biosynthesis from fatty acids, sugars or glycerol, would enable a cheaper and more sustainable lactone production. Several studies have demonstrated the production of lactones on complex medium with fungi Ashbya gossypii (Ravasio et al., 2014) and Aureobasidium pullulans (Luepongpatana et al., 2017). A. gossypii produces a broad-spectrum of lactones. Due to the similar physical properties of these lactones, it may be difficult and costly to isolate individual lactones. A. pullulans has been reported to produce over 5 g/L massoia lactone in the patent literature (Alphand et al., 2015; Ji and NGOH, 2017). The lactone is derived from the secreted polyol lipids containing several hydroxide acid moieties (Kurosawa et al., 2014). However, production of lactone other than massoia lactone has not been reported using this fungus. Another alternative is to produce hydroxylated fatty acids using microbes (Oh et al., 2015; Beopoulos et al., 2014) and then convert them into lactones with the aid of enzymes or other microbes in a two-step or single-pot process (Farbod et al., 1994; An et al., 2013; Kang et al., 2016). This would require the development and operation of two processes, resulting in extra capital and running costs.

We propose a method for single-step lactone production from non-hydroxylated fatty acid using oleaginous yeast Yarrowia lipolytica. Y. lipolytica is a GRAS organism, well suited for large-scale fermentation and genetic manipulation (Holkenbrink et al., 2018; Darvishi et al., 2014). The lactonase Y. lipolytica produces a broad-spectrum of lactones. Due to the similar physical properties of these lactones, it may be difficult and costly to isolate individual lactones. A. pullulans has been reported to produce over 5 g/L massoia lactone in the patent literature (Alphand et al., 2015; Ji and NGOH, 2017). The lactone is derived from the secreted polyol lipids containing several hydroxide acid moieties (Kurosawa et al., 2014). However, production of lactone other than massoia lactone has not been reported using this fungus. Another alternative is to produce hydroxylated fatty acids using microbes (Oh et al., 2015; Beopoulos et al., 2014) and then convert them into lactones with the aid of enzymes or other microbes in a two-step or single-pot process (Farbod et al., 1994; An et al., 2013; Kang et al., 2016). This would require the development and operation of two processes, resulting in extra capital and running costs.

We propose a method for single-step lactone production from non-hydroxylated fatty acid using oleaginous yeast Yarrowia lipolytica. Y. lipolytica is a GRAS organism, well suited for large-scale fermentation and genetic manipulation (Holkenbrink et al., 2018; Darvishi et al., 2014).
Oleic acid and linoleic acid were selected as example substrates because they are major constituents in various renewable low-value fatty feedstocks, such as used cooking oil, oil press cakes, olive oil distillates, and animal fats (Cruz et al., 2016; Bhatti et al., 2008). The proposed process enables lactone production from cheap feedstocks by a single microbial host.

2. Material and methods

2.1. Strain construction and cultivation

All strains in this study were derived from *Y. lipolytica* Y-63746 (MaTa, *Y. lipolytica* W29, ATCC® 20460™). *Y. lipolytica* Y-63746 was a kind gift from ARS Culture Collection, NCAUR, USA. Complete list of strains, plasmids, biobricks (DNA fragments for cloning), and primers is provided in the supplementary information (Tables S1–S5). Unless mentioned otherwise, cells were grown in YPD medium (20 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) at 30 °C and 250 rpm (ThermoFisher Scientific MaxQ8000) for preculture and cryostock preparation. YPD agar plates contained 15 g/L agar (Sigma 05040). To enable CRISPR-Cas9 mediated strain engineering, *Y. lipolytica* W29 was transformed with linearized vector pCB3634, which inserted Cas9 expression cassette into the KU70 locus (Holkenbrink et al., 2018), resulting in *aku70* strain ST6512. ST6512 was used as a reference and parental strain for the creation of other strains in this study. Plasmid constructions, gene insertions, and gene deletions were performed as described previously (Holkenbrink et al., 2018). Heterologous genes were codon optimized for *Y. lipolytica* and obtained as synthetic DNA fragments (GeneArt, Invitrogen). Deletions of POX1–6 genes were performed using 90-bp double-stranded oligos (IDT DNA) as repair templates, while other deletions were performed using DNA fragment containing 250–500 bp upstream and downstream homology arms (500–1000 bp in total). Guide RNA (gRNA) vectors and integrative vectors for gene expression were sequence-verified using Sanger sequencing (Eurofins). Yeast transformations were performed according to PEG/ssDNA/LiAc transformation protocol as described previously (Holkenbrink et al., 2018) using nourseothricin resistance (NatMX) as a selection marker. NatMX selection plate was prepared with standard YPD agar supplemented with 250 mg/L nourseothricin (Jena Bioscience, AB-101).

2.2. Spot assay

For pre-cultures, cells from cryostock were streaked in YPD agar for 2 days and further propagated in 14 mL tubes (Greiner™ 187262) with 1 mL YNB-AA medium (6.7 g/L Yeast Nitrogen Base without Amino Acids (Sigma, Y0626), 20 g/L glucose, 12 g/L KH2PO4, adjusted to pH 6) at 30 °C, 250 rpm overnight. The cells were centrifuged at 3000 g for 5 min and were resuspended in 10 mL ice-cold Wash Bu™ 20 °C. The cells were washed two times and incubated (30 °C, 250 rpm) until reaching exponential phase (OD600 between 0.1 to 0.5 and cultured (30 °C, 250 rpm) until reaching exponential phase (OD600 of 10–70). The cells were washed two times with 25 mL sterile deionized water to remove residual glucose. The cells were resuspended in sterile deionized water to obtain an OD600 of 10, and used to prepare ten-fold serial dilutions. 10 μL of diluted cells were spotted on fatty acid agar plates. Fatty acid agar plates contained 6.7 g/L YNB-AA, 15 g/L bacto agar, and 5 g/L of a fatty acid compound as the sole carbon source: oleic acid (Sigma, 364525), ethyl palmitate (Et–C16, Sigma, W245100), methyl myristate (Me–C14, Sigma, W272205), methyl laurate (Me–C12, Sigma, W271500), methyl decanoate (Me–C10, Sigma, W505501), and methyl octanoate (Me–C8, Sigma, W278209).

2.3. Preparation of cell extracts for enzymatic assay

The cell extracts were prepared as following. The cells from cryostock were propagated in 14 mL tubes with 1 mL YPD at 30 °C, 250 rpm overnight. The whole overnight culture was transferred into 25 mL YNB-AA medium in 250 mL shake-flasks and incubated at 30 °C and 250 rpm overnight. Cells were washed 2 times and inoculated at OD 0.5 into 50 mL YNBD-AA medium in 500 mL shake-flasks, incubated at 30 °C, and harvested at mid-exponential phase (OD600 between 8 to 12, Implen NanoPhotometer P300). Following this, ST6512 and ST6852 strains were centrifuged at 3000 x g for 5 min and resuspended in 50 mL sterile water. This step was repeated one more time to wash away residual glucose. Washed cells were resuspended in YNB-AA medium supplemented with 1 g/L Ethyl Palmitate (Sigma W245100) and 1 g/L Methyl Oleate (Sigma 311111) and incubated in 500 mL shake flask at 30 °C, 250 rpm for induction. Cells were harvested after 6 h. For other strains, cells from YNBD-AA were harvested without induction since heterologous acyl-CoA oxidases were expressed under a constitutive promoter. The harvested cells were washed twice with Wash Buffer (10 mM KH2PO4, pH 7.2). In each washing step, cells were first centrifuged at 6000 g, 4 °C for 5 min, resuspended in 50 mL Wash Buffer, and finally centrifuged again. Cells were then resuspended in 10 mL ice-cold Wash Buffer, and stored as two 5 mL-aliquots at −20 °C.

For the preparation of the cell extract, 5 mL cells were thawed at room temperature, washed two times with 5 mL ice-cold Enzyme Assay Buffer (50 mM KH2PO4, pH 7.4) and resuspended in 750 μL Enzyme Assay Buffer. The suspension was transferred into 2 mL-microtubes (Sarsted, 72.694.006) pre-filled with 700 mg of glass beads (Sigma, G1277), and homogenized in Precellys® 24 (Bertin Instruments) for five cycles of 20 s shaking at 5000 rpm with 5-min pauses between cycles, where the samples were placed on ice. Cell debris was removed by centrifugation at 21,000 x g for 10 min at 4 °C. If needed, the centrifugation step was repeated or extended up to 20 min until a clear supernatant was obtained.

2.4. Acyl-CoA oxidase enzymatic assay

Acyl-CoA oxidase enzymatic assay mixture was prepared as described previously (Pagot et al., 1998; Shimizu et al., 1979). Enzyme assay mixture contained 50 mM KH2PO4, 0.825 mM 4-amoanitopyrine (Sigma A4283), 10.6 mM phenol (Sigma P1037), 0.01 mM FAD (Sigma P2665), 0.1 mM acyl-CoA, and 30 IU/mL horseradish peroxidase (Sigma P8250), and cell extracts (0.07–0.1 mg total-protein/mL assay mix). The following substrates were used: oleoyl-CoA (Sigma O1012), palmitoyl-CoA (Sigma P9716), myristoyl-CoA (Sigma M4414), lauroyl-CoA (Sigma D5269), decanoyl-CoA (Sigma L2659), and octanoyl-CoA (Sigma O6877). All chemicals were dissolved in Enzyme Assay Buffer as separate reagents and warmed up to 30 °C before mixing. 200 μL assay mix in 96-well plate (Greiner™ 655101) was incubated at 30 °C in a plate reader (BioTex Synergy™Mx), and absorbance at 500 nm was monitored. The activity was determined by using an extinction coefficient of 12.78 mM−1 cm−1 and pathway length of 5.9 mm (mathematically calculated). Reported numbers are means of two biological replicates measured with at least two technical replicates.

2.5. Lactone production in tube-cultures

Strains from cryostock or agar plates were grown in 2 mL YPD for 48 h in a 24 deep-well plate (VWR AXYGP-DW10ML24C) at 30 °C and 250 rpm shaking. This culture was used to inoculate 5 mL YPD in a Corning mini bioreactor (Sigma, CLS431720) with an initial OD600 between 0.1 to 0.5 and cultured (30 °C, 250 rpm) until reaching exponential phase (OD600 of 10–70). The cells were washed two times with 25 mL sterile deionized water (centrifugation at 3000 g for 5 min) and used to inoculate 2 mL of lactone production medium to an OD600 of 1 in rimless glass tubes (VWR, 212–0326) closed with Labocap lid (VWR, 391–590). The lactone production (LP) medium was mineral medium without glucose (Jensen et al, 2014) supplemented with 10 g/L yeast extract and 30 g/L of 90% oleic acid (Sigma, 364525) or 97% linoleic acid (TCI Europe, L0124) as carbon source. The fatty acids were dissolved in 99% ethanol to 60% w/v and then added to the medium. The tubes were incubated at 30 °C with 250 rpm shaking for 48 h.
2.6. Bright-field and GFP fluorescence image acquisition

The cells from cryostock were propagated in 14 mL tubes with 1 mL YPD at 30 °C, 250 rpm overnight. Cells were washed two times with 2 mL of 10 mM KH2PO4 pH 6 (centrifugation at 3000 g and 250 rpm). Cells were resuspended in 1 mL of 10 mM KH2PO4 pH 6, and loaded onto microscope slides. Bright-field and fluorescence images were taken at 100 × magnification in a Leica DFC300 FX microscope equipped with Leica EL600 external light source. All images were taken with the same acquisition settings.

2.7. Fed-batch bioreactor cultivation

Cells from cryostock were inoculated into 1 mL YPD in a 10 mL preculture tube followed by inoculation in 25 mL YPD in a sterile 250 mL baffled shake-flask overnight. 10 mL inoculum was transferred into two 1-L bioreactors (Biostat Q Plus, Sartorius, Gottingen, Germany) each contained 400 mL starter medium. Starter medium was mineral medium (Jensen et al, 2014) supplemented with 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L glycerol, and 5 mL of Antifoam 204 (Sigma A6426). Cells were grown in the starter medium until carbon source depletion. During the growth phase, dissolved oxygen level (DO) was maintained above 40% with minimum stirring and aeration speed of 300 rpm and 0.5 standard-liter per min (SPLM), respectively.

After the growth phase in starter media, 30, 60, and 80 mL of 800 g/L oleic acid solution in ethanol was fed at three time points for biotransformation. Equal volumes of concentrated YP solution (67 g/L yeast extract, 133 g/L peptone) and 5, 20, and 20 mL of Antifoam 204 were fed along with the oleic acid solution. Stirring speed and aeration rate were adjusted to 600 rpm and 1.3 SPLM, respectively, at the start of the cultivation. Data from two bioreactors is presented.

2.8. Cell dry-weight measurement

To remove the oil phase, 1 mL (CDW < 100 g/L) or 0.5 mL (CDW ≥ 100 g/L) bioreactor sample was washed two times by pelleting at 18,000 x g for 5 min and resuspending the pellet with 1 mL deionized water. The suspension was loaded into a Whatman™ cellulose nitrate membrane filters disc with 0.45 μm pore size (VWR 7184-004) and washed with 1 mL deionized water through vacuum. Before use, filters were dried in a microwave at 390 Watt for 10 min and weighed. After loading the cells, the filters were microwaved for 20 min at 390 Watt. Dry weight was determined by the difference of dry filter weight before and after loading the sample. Dry weight for each bioreactor was measured in duplicates. The presented data is average values from two bioreactors.

2.9. Lactone extraction

For quantification of lactones produced in tube-cultures, 200 μL of absolute ethanol containing 1 g/L of internal standard (IS) γ-undecalactone (Sigma, 89985) was added directly into the cultivation tube (final IS concentration 100 mg/L). For quantification of lactones in bioreactors, 2 mL sample was transferred into a cell-free glass tube, and 1 mL of an ethanol containing internal standard at 4.75 g/L concentration was added (final IS concentration 237.5 mg/L). The tube was vortexed in a multi-vortexer (VWR, DVX-2500) in pulse-mode at 1500 rpm for 45 s. Then, three mL of n-hexane (Merck Life Science, 1.04391.1000) was added and the tube was vortexed in the multi-vortexer in pulse-mode at 1500 rpm for 5 min to extract lactones into the hexane phase. Water and hexane phase were separated by centrifugation at 1000 x g for 5 min (room temperature). The hexane phase was transferred to a 4 mL glass vial (Mikrolab Aarhus, ML33134) filled with a small amount of Na2SO4 (Sigma, 798592) to remove residual moisture. 120 μL of the hexane phase was loaded into 1 mL HPLC glass vial with 200 μL insert (Mikrolab Aarhus ML 33117) and sent for GC-MS analysis.

2.10. GC-MS analysis

The GC-MS analysis was performed on a Bruker Daltonics Scion GC436-MS instrument equipped with Agilent HP-Innowax column (30m × 0.250mm × 0.25-μm). One microliter of the sample was injected in Split/splitless injector set at 250 °C initially in splitless mode and after 2 min with a split ratio of 50. The helium gas flow was 1.0 mL/min. The temperature of the column oven was set to the following: 50 °C for 1 min, ramp 10 °C/min to 210 °C, 210 °C for 5 min, ramp 10 °C/min to 300 °C, 300 °C for 5 min. Samples were ionized with EI (70 eV) in full scan mode from 50-350 Da. Transfer line and ion source were set to 250 °C and 200 °C, respectively, and the collection delay was set to 4 min. Ion-85 count and the sum of ion-99 and 71 counts were used to quantify γ-dodecalactone and δ-dodecalactone, respectively. A calibration analysis using lactone standards was performed to determine correlations between peak areas of these quantifying ions with an area of ion-85 of γ-undecalactone. The obtained correlations were used to determine lactone concentration based on internal standard concentration. Lactone standards were mixtures of γ-undecalactone, γ-dodecalactone (Sigma, 777991), and δ-dodecalactone (Sigma, 74026) at 6.25–400 mg/L concentration in n-hexane.

3. Results

3.1. Controlled chain-shortening through engineering of β-oxidation pathway

Production of lactones from abundant fatty acids required the engineering of controlled chain-shortening and of fatty acid hydroxylation pathway (Fig. 1a). Controlled chain-shortening was achieved by manipulating the substrate specificity of peroxisomal acyl-CoA oxidase (POX), which catalyzes the first step of β-oxidation cycle. During β-oxidation, the β-carbon of an acyl-CoA is oxidized in four steps, which give off one acetyl CoA and one acyl-CoA with 2-carbon shorter. Different chain-length specificities of several oxidases have been reported, especially in plants and yeasts (Arent et al., 2008; Hooks et al., 1996, 1999; Picataggio et al., 1991; Wang et al., 1999a)

We aimed to establish a chain-shortening module that terminates peroxisomal fatty acid β-oxidation at 10- (C10) and 12-carbons (C12), which are the most common chain-lengths of commercial flavor lactones (Fig 1b and c). *Y. lipolytica* has six POX enzymes encoded by genes POX1-6. These POXes have different chain-length specificities of several oxidases have been reported, especially in plants and yeasts (Arent et al., 2008; Hooks et al., 1996, 1999; Picataggio et al., 1991, 1999a).

We started by deleting the six acyl-CoA oxidase coding genes (POX1-6) in *Y. lipolytica* strain ST6512 (ku70::Cas9), resulting in strain ST6852 (apo1-6). Next, we selected several long-chain-specific POXes (LCPOXes) that may be suitable for obtaining our targeted chain-lengths of C10 and C12. We chose a putative long-chain POX from moth *Agrotis segetum* (AsePOX). The transcript of AsePOX was present in *A. segetum* pheromone glands (Ding and Löfstedt, 2015). As the major constituent of this moth’s pheromone is derived from C12-C14 fatty acid (Löfstedt et al., 1985), we selected this POX as a candidate. We also chose two plant acyl-CoA oxidases, ACX2 from *Arabidopsis thaliana* (AtACX2) and a long-chain POX from a cucurbit cultivar (*Cucurbita*), which have been previously characterized in *vito* and showed specificity towards long-chain fatty acids (Hooks et al., 1996; Hayashi et al., 1998). By performing BLASTp search (Altschul et al, 1997) using protein sequence of AtACX2 and CucLACO as separate queries, putative
long-chain acyl-CoA oxidase from *Rhinolophus sinicus* (Dong et al., 2017) (*RsAcox2*) was identified. Lastly, we included POX2 from *Y. lipolytica* (*YlPOX2*) as its long-chain specificity for C10 fatty acid or longer has been previously demonstrated (Waché et al., 2002). The long-chain POXes used in this study are summarized in Table 1 and Supplementary Information 2.

Codon-optimized genes encoding selected LCPOXes were expressed under the control of a strong constitutive TEF promoter with intron (Holkenbrink et al., 2018; Tai and Stephanopoulos, 2013) in ST6852 (*Δpox1-6*) strain. The native sequence was used for *YlPOX2*. Although all of the four heterologous LCPOXes contained the N-terminal peroxisome-targeting signal 1 (PTS1, Supplementary Information 1), we still appended the yeast PTS1 (SKL) at the C-terminal of the proteins. No PTS1 sequence was added to *YlPOX2*. To determine the cellular localization of the LCPOXes, hrGFP-tag was attached to the N-termini of heterologous LCPOX and inserted into ST6511 (*Δpox1,3,4,5,6*). The fluorescence pattern was typical for peroxisomal localization for all the LCPOXes, with exception of AsePOX, for which the fluorescence signal was low (Fig. 2a–d). The low fluorescence may be due to low expression level or protein misfolding. Notably, the GFP intensities of AtACX2 and CuLACO strains were considerably higher than that of *RsAcox2* (Fig. 2b–d).

To assess the chain-length specificity of five LCPOXes in vivo, the LCPOX genes were individually expressed without a GFP tag under TEFintron promoter in ST6852 (*Δpox1-6*) strain. The strains were plated on oleic acid or fatty acid methyl esters (FAMEs) of different chain-lengths as sole carbon source (Fig. 3a). After 5 days of incubation, the control strain ST6512 (*POX1-6*) grew well on all tested substrates except methyl octanoate. Because ST6512 has a significant enzymatic activity on octanoyl-CoA (Fig. 3b), the lack of growth of ST6512 in octanoate plate might be the result of either toxicity of octanoic acid or insufficient induction of POXes by octanoic acid. ST6852 (*Δpox1-6*) did not grow on any of the tested substrates except for C16, where it grew poorly. Nevertheless, growth on C16 may be attributed to the activity of four putative mitochondrial acyl-CoA dehydrogenases (ACADs), identified by BLAST search with *Rhodosporidium toruloides* ACAD protein sequence (Zhu et al., 2012) as query (Supplementary Information 3). ST7384 (*Δpox1-6 AsePOX*) showed only a slightly better growth than ST6852 (*Δpox1-6*) on oleic acid and on C14 substrate, which is consistent with the low expression of AsePOX (Fig. 2a). ST7386 (*Δpox1-6 CuLACO*) grew on C16 and only slightly on C14. ST7385 (*Δpox1-6 AtACX2*) and ST7581 (*Δpox1-6 YlPOX2*) grew on C18, C16, C14, and C12 fatty acid, but not on C10, which makes these strains applicable for the production of C10 lactones. ST7387 (*Δpox1-6 RsAcox2*) grew well on C18, C16, and C14 fatty acids, but only weakly on C12 fatty acid, suggesting selectivity towards C14 fatty acids and higher. This makes ST7387 (*Δpox1-6 RsAcox2*) a suitable chassis for the production of C12 lactones.
3.2. Analysis of chain-length specificity of heterologous POXes by enzymatic assay

We measured the oxidase activities of the crude extracts of engineered strains on different acyl-CoA substrates (Fig. 3b and c). For the preparation of extracts, strains ST6512 and ST6852 (Δpox1-6) were cultivated with supplementation of ethyl palmitate and methyl oleate to induce expression of POXes. The rest of the strains did not require induction, because LCPOXes were expressed from the constitutive TE-Fintron promoter.

Oxidase activity of ST6852 (Δpox1-6) was below the detection limit for all acyl-CoA tested, which correlated with the lack of growth of this strain on fatty substrates. The activities of heterologous LCPOXes (Fig. 3c) were about two orders of magnitude lower than YIPOX2 in ST7581 (Δpox1-6 YIPOX2) (Fig. 3b). This low oxidase activity was nevertheless sufficient to support the growth of the strains on fatty substrates (Fig. 3a), indicating that the growth may be limited by other factors, e.g., the uptake and transport of the fatty substrates in the cells. It could also be that the in vitro enzymatic assay does not reproduce the in vivo conditions well. It is possible that in vivo, the concentration of oxidase substrates are well below the Km values and the native enzymes have much lower activities than in in vitro assays with high concentrations of substrates.

AsePOX activity was only measurable on methyl hexadecanoate (C16), which also correlates that the strain could only grow on C16 substrate (Fig. 3a). The oxidase activity profiles of ST7581 (Δpox1-6 YIPOX2) and control strain ST6512 (POX1-6) were in agreement with the previous reports (Wang et al., 1999b; Luo et al., 2002a). Notably,
there was a discrepancy between the in vitro activity and in vivo growth for oxidases RsAcox2, AtACX2, and YlPOX2. ST7387 (Δpox1-6 RsAcox2) extracts had a high activity on C12 substrate, but the strain only grew on substrates longer than C12. Similarly, AtACX2 and YlPOX2 were active on C10 in vitro, but the cells did not grow on C10 substrates. A possible explanation could be that liberation of a single acetyl-CoA molecule per substrate molecule cannot provide the cell with sufficient energy for substrate uptake and activation, transport into peroxisomes, the export of chain-shortened product, and for the growth. While if more than two acetyl-CoA molecules are released per substrate molecule, the export of chain-shortened product, and for the growth becomes feasible. Furthermore, based on the low activities on C8-CoA, it is likely that this and shorter acyl-CoAs are not processed effectively and may accumulate in CoA form or as free fatty acids and this can elicit some toxicity effects (Borrull et al., 2015) as suggested by our spot assay result on Me-C8 (Fig. 3a). This could explain why despite the high in vitro activity on C10 and C12, strains ST7581 (Δpox1-6 YlPOX2) and ST7387 (Δpox1-6 RsAcox2) did not grow on C10 and 12 substrates, respectively.

Based on the in vivo and in vitro results, we selected YlPOX2 oxidases for the production of C10 lactones, and RsAcox2 oxidase for C12 lactones. While the strain with CuLACO ST7386 (Δpox1-6 CuLACO) grew well on C14 and C16 substrates, its growth on oleic acid was poor. As we intended to use oleic acid as one of the substrates for lactone production, we did not choose CuLACO oxidase for the further work.

3.3. Lactone production through fatty acid hydratases and controlled-chain shortening

To obtain γ- and δ-lactones with 10- to 12-carbons via chain-shortening, hydroxy group needs to be located in Δ8 and Δ13 positions of C16-fatty acid or in Δ10 and Δ13 positions of C18-fatty acids. Hydroxylation can be achieved by the action of hydratase, hydroxylase, lipoxygenase, or epoxy-hydrolase (Tressl et al., 1996; Schwab et al., 2008). Compared to alternative pathways such as fatty acid (oleate) hydroxylase in fungi and plants or fatty acid lipoxygenase and perox-ygenase in fruits, fatty hydratases route takes only one step towards fatty acid hydroxylation (Volkov et al., 2010; Bevers et al., 2009). Fatty hydroxylase requires incorporation of fatty acid through the long Kennedy pathway prior to hydroxylation (Lin et al., 1998). Lipoxygenase and epoxy-hydrolase routes require at least two steps from free fatty-acid and form unstable intermediates (Schwab et al., 2008; Schöttler and Boland, 1996). Conversely, the known hydratases act on free fatty acids as substrates (Volkov et al., 2010). We chose hydratases because free fatty acids are easier to generate in the cell in comparison to the substrates of other hydroxylation enzymes.

Oleate 10-hydratase (OHY) generates 10-hydroxyoctearic acid from oleate. We chose the variant from Streptophomonas maltophilia (Joo et al., 2012) (SmOHY, Table 1). Linoleate 13-hydratase (LHY) makes 13-hydroxyoctearic acid from linoleate. LHY gene was sourced from Lactobacillus acidophilus (Park et al., 2015) (LaLHY, Table 1). We designed to express OHY in the strain that performs chain shortening to 12 carbons to obtain γ-dodecalactone from oleic acid (Fig. 1b). LHY was to be expressed in the strain with chain shortening to 10 carbon atoms, so δ-dodecalactone is produced from linoleic acid (Fig. 1c). The SmOHY and LaLHY genes were codon-optimized for Y. lipolytica and expressed with C-terminal hGFP tags from TEFintron promoter to check the expression and localization. Both enzymes were expressed in the cytosol (Fig. 2e and g). We also wanted to express these enzymes in peroxisomes. To achieve this, we expressed them with a C-terminal PTS1 signal (SKL) and an N-terminal hGFP tag. The PTS1 signal was either fused directly to the protein or was spaced by a 2xGGGS linker. The variants with the linker localized into peroxisomes as expected (Fig. 2f and h), while SmOHY variant without the linker showed in the cytosolic localization (Supplementary Information 4).

Next, we expressed heterologous hydroxylases in a suitable chain-shortening yeast chassis (Fig. 4). Specifically, we inserted SmOHY and LaLHY without hGFP tag into strain ST6512 (POX1-6), resulting in strains ST6759 (POX1-6 SmOHY) and ST6760 (POX1-6 LaLHY) and cultivated them on mineral medium supplemented with 10 g/L yeast extract and 30 g/L of either oleic acid or linoleic acid. We observed production of 1.9 ± 0.8 mg/L of γ-dodecalactone in ST6759 with oleic acid as substrate (Fig. 5a) and 1.2 ± 0.4 mg/L of δ-dodecalactone in ST6760 with linoleic acid as substrate (Fig. 5b). When the hydratases were inserted into strain ST6852 (APOX1-6) lacking the peroxisomal β-oxidation capacity, no lactone production was detected (Fig. 5a and b). We further generated strain ST7417 (Δpox1-6 SmOHY RsAcox2) to specifically terminate β-oxidation at C12 and thereby avoid degradation 4-hydroxydodecanolic acid, which is the precursor of γ-dodecalactone. This strain produced 12 ± 6.4 mg/L of γ-dodecalactone, which proved the effectiveness of replacing the native POXes with RsAcox2 to control chain-shortening. Analogously, we constructed strains ST7584 (Δpox1-6 LaLHY YlPOX2) expressing YlPOX2 for chain shortening to ten carbons. The strains, however, did not produce detectable levels of δ-dodecalactone from linoleic acid (Fig. 5b). It could be that the oxidase activity was too high, when the oxidases were expressed from a very strong promoter and fine-tuning this could improve δ-dodecalactone production.

3.4. Metabolic engineering to improve lactone production

It has been reported previously that oleate 10-hydratase from
**Figure 5.** a) γ-dodecalactone and b) δ-dodecalactone titer after different gene deletions and insertions. 30 g/L oleic acid (γ-dodecalactone) or linoleic acid (δ-dodecalactone) was present at the start of cultivation. “-“ and “+” symbols indicate absence or presence of the corresponding genetic change, respectively. “++” indicates 2 copies of the corresponding gene are present. Results were obtained from at least three biological replicates. Error bars represent standard deviation. Statistical analysis was performed using Student’s t-test (two-tailed; \( P \leq 0.05 \), \( * * P \leq 0.01 \), \( ** * P \leq 0.001 \), n.s: not significant; two-sample unequal variance).

*S. cerevisiae* has been shown to reduce the oleate uptake (Zou et al., 2002). Introduction of this gene into ST8276 did not increase γ-dodecalactone production and therefore this strategy was not included in the following new strain designs.

We continued by examining whether expressing SmOHYp in peroxisomes would result in lactone production. We tested this by comparing production in a strain expressing a cytosolic SmOHY variant (SmOHYcyt, ST8276) or peroxisomal SmOHY variant (SmOHYper, ST8822) or both (SmOHYcyt+per, ST8824) in *apo1-6 Δfaa1 RsAcox2Δfaa1* background. Peroxisomal variant resulted in less lactone than the cytosolic variant. The addition of peroxisomal variant to the strain expressing a cytosolic variant did not significantly improve the titer (Fig. 5a). This implies that peroxisomal expression provided little to no benefit. Furthermore, there is also a possibility that lactone production in ST8822 occurred due to the presence of SmOHYp in the cytosol before the protein got imported into peroxisomes.

Following the above findings, we hypothesized that lactone production was limited by the low SmOHY activity. We expressed an additional copy of cytosolic SmOHY resulting in strain ST8896 (*apo1-6 Δfaa1 RsAcox2 2xSmOHYcyt SmOHYper*). ST8896 produced 74.6 ± 11.0 mg/L γ-dodecalactone, which is 3.6-fold higher than the parental strain (Fig. 5a).

We applied analogous engineering approaches to the strain ST7584 (*apo1-6 LaLHY YIPOX2*). Deletion of FAA1 resulted in δ-dodecalactone production at 0.17 ± 0.03 mg/L (Fig. 5b). ScFAT1DSOS6A and peroxisomal expression strategies did not give improvement, as was the case with γ-dodecalactone production. Additional copy of cytosolic LaLHY improved the δ-dodecalactone titer 10-fold to 1.74 ± 0.3 mg/L, indicating that low hydratase activity was limiting the flux towards lactone.

Lastly, we tried to use commercial olive oil instead of oleic acid and we obtained 1.62 ± 0.45 mg/L γ-dodecalactone with strain ST8896. Although the titer was inferior to that obtained on pure oleic acid, this result suggests that lactone production from plant oils is possible.

### 3.5. Lactone production in fed-batch bioreactor

The highest γ-dodecalactone-producer strain ST8896 (*apo1-6 Δfaa1 RsAcox2 2xSmOHYcyt SmOHYper*), was tested in fed-batch bioreactors. Previous studies have provided valuable insights on optimizing stirred-bioreactor conditions for γ-dodecalactone production from castor oil and methyl ricinoleate. Production of γ-dodecalactone from methyl ricinoleate was better at high aeration rate (600 rpm stirring, 3 vvm aeration rate), high methyl ricinoleate concentration in the medium (50 g/L) (Gomes et al., 2010), and at high cell densities (Braga and Belo, 2015). Comparison of media used by Moradi et al. (2013) (Moradi et al., 2013) and Braga and Belo (2015) (Braga and Belo, 2015) suggests that higher C/N ratio and repeated feeding could also benefit lactone production. We, therefore, designed our bioreactor cultivation conditions accordingly.

During the growth on the starter media, 12.9 ± 2.2 g/L of cell dry-weight (CDW) was measured. Oleic acid was then added in three pulses marked with black arrows on Fig. 6a. Upon the addition of oleic acid, the dissolved oxygen dropped to near zero and remained low in spite that we increased the agitation rate during the cultivation. Lactone concentration peaked at 109 mg/L ± 28 mg/L at 16 h and at 282 ± 75 g/L at 88 h (Fig. 6a). There was a sharp increase in lactone concentration between 62 and 83 h, which was likely due to the higher cell density. In fact, specific productivity in the 62-83 h period (0.10 gr-lactone/L/h per average CDW) was still lower than in the 0-16 h period (0.25 gr-lactone/L/h per average CDW).

Some degradation of lactone clearly occurred during the fermentation (e.g., between 20 and 60 h and after 83 h). This was not due to the lack of substrate, because oleic acid was present at all the tested time points (visible as a distinct top layer in the centrifuged samples). It has been previously reported that γ-dodecalactone produced from castor oil was degraded by strains that had intact POXes (Braga et al., 2015). The
observed degradation suggests that our strain could benefit from a more tight chain-shortening control in the future. Furthermore, significant improvements in lactone production could be achieved by optimizing the fermentation media, feeding profile, and fermentation conditions.

4. Discussion

We report, for the first time, a single-host microbial process for lactone production from abundant non-hydroxylated fatty acids. The host was oleaginous yeast *Y. lipolytica*, engineered to carry out fatty acid hydroxylation and a controlled chain shortening. We employed bacterial fatty acid hydratases, which hydroxylate fatty acids in a single step in contrast to alternative pathways, such as fatty acid (oleate) hydroxylase, lipoxygenase, or peroxygenase.

Controlled β-oxidation is the key step in lactone formation from hydroxy fatty acids (Gatfield et al., 1997). Previous studies in *Y. lipolytica* showed that by substituting all POXes with only the long-chain specific POX2, γ-decalactone production was improved. In the current study, we tested four heterologous acyl-CoA oxidases in addition to the native *YlPOX2*. The *RsAcox2*, which originates from a Chinese rufous horseshoe bat *Rhinolophus sinicus*, enabled us to improve production of γ-dodecalactone. Two different chain-length characterization methods were tested in selecting suitable POXes, namely growth assay and enzymatic assay. The combination of these methods guided the selection of suitable POXes.

We explored several metabolic engineering strategies for improving the lactone titer. We showed that increasing the copy number of hydratase genes eventually yielded the best improvement. This might be explained by the poor kinetic properties of fatty acid hydratases. The measured *kcat* and *Km* of *SmOhyp* are, respectively, 118 min⁻¹ and 38.9 μM on oleic acid (Kang et al., 2017). For *LaLhyp*, the *kcat* and *Km* values on linoleic acid are 553 min⁻¹ and 7 mM, respectively (Kim et al., 2015). As comparison, the reported values of *kcat* and *Km* of purified *YlPox2p* are 22.5 s⁻¹ (1350 min⁻¹) and 18 μM, respectively (Luo et al., 2002b). These values reflect how the kinetic performances of the hydratases are inferior relative to that of *YlPox2p*. Poor kinetics of *LaLhyp* could also explain the low titer δ-decalactone titer we obtained.

Although it has been shown that fatty acid hydratases cannot accept acyl-CoA as substrate, deletion of *FAA1*, which was expected to increase the pool of free fatty acids, did not yield a significant improvement. Since free fatty-acids are more hydrophobic than acyl-CoAs, their availability to the hydratases may be limited by the transport events. Therefore, instead of deleting *FAA1*, fusing the hydratase with a thioesterase might help tackling the limited free fatty-acids availability due to their hydrophobic nature. In this manner, the thioesterase would act like a shuttle that will provide the substrate from the cytosolic acyl-CoAs. Peroxisomal localization of hydratases was not beneficial either. This could be due to the highly-oxidative peroxisomal environment or due to some inhibitors, such as hydrogen peroxide and reactive oxygen species.

The fed-batch fermentation of the engineered γ-decalactone producing strain resulted in lactone titer of 282 ± 75 g/L at 88 h, however some degradation was observed during the fermentation.
process, indicating that further process optimization is needed.

5. Conclusion
We have established strains to produce γ-dodecalactone and δ-decalactone from non-hydroxylated fatty acids by engineering of controlled chain-shortening and hydroxylation modules in oleaginous yeast Y. lipolytica. By testing various metabolic engineering strategies, we concluded that boosting the expression and activity of fatty acid hydrolases would result in further strain improvement.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.pcyb.2019.08.009.

Author contributions
ER and IB conceived this project. ER, JD, GW, CH, and IB designed the metabolic engineering strategies and lactone production experiments. ER and JD performed the experiments. ER, MID, and CH designed the bioreactor experiments. ER and JtH carried out the bioreactor experiments. ERM, JD, GW, CH, and IB were involved in analyzing and interpreting the data.

Competing interest
IB and CH have financial interest in BioPhero ApS.

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