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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Saccharomyces cerevisiae whole cell biotransformation for the production of aldehyde flavors and fragrances

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Abstract

9-Carbon aldehydes such as (2*E*)-nonenal, (3*Z*)-nonenal, and (2*E*,6*Z*)-nonadienal are important melon and cucumber fragrance compounds. Currently, these molecules are produced either synthetically, which faces consumer aversion, or through biotransformation using plant-extracted enzymes, which is costly and inefficient. In this study, we constructed a *Saccharomyces cerevisiae* platform for the whole cell biotransformation of polyunsaturated fatty acids (PUFAs) to 9-carbon aldehydes. Heterologous expression of lipoxygenase (LOX) from *Nicotiana benthamiana* and hydroperoxide lyase (HPL) from *Cucumis melo* (melon) in *S. cerevisiae* enabled the production of (*2E*)-nonenal from readily available polyunsaturated fatty acid substrates. A 5.5-fold increase in (2*E*)-nonenal titer was then achieved utilizing genetic and reaction condition enhancement strategies. The highest titer of (2*E*)-nonenal was more than 0.11 mM, with about 9% yield. This platform can potentially be used to produce a variety of other aldehyde products by customizing with LOX and HPL enzymes of different regio-selectivities.

Key points

- *Establishment of a S. cerevisiae whole-cell biotransformation platform for cost-efficient, high-yield conversion of PUFAs into high value 9-carbon aldehyde compounds*
- *5.5-Fold improvement of (2E)-nonenal titer to > 0.11 mM achieved by enhancing reaction conditions and gene expression levels of LOX and HPL*

Keywords Natural flavor and fragrances · Whole cell biotransformation · Lipoxygenase · Hydroperoxide lyase · Aldehydes · Enzyme engineering

Introduction

Flavor and fragrance compounds play important roles in our sensory experience of food, beverages, perfumes, and consumer care products. For example, 9-carbon aldehydes derived from polyunsaturated fatty acids impart characteristic flavors and fragrances. In particular, (3*Z*)-nonenal imparts melon fragrance, (2*E*)-nonenal produces aged flavor in cereal products including rice and beer, and (2*E*,6*Z*)-nonadienal imparts cucumber and violet leaf fragrances (Huang and Schwab 2011). In addition, the 9-carbon alcohols, (3*Z*)-nonenol and (3*Z*,6*Z*)-nonadienol, are also odor-active and produce melon and cucumber flavors, respectively (Berndt and Feussner 2001). Furthermore, (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal have anti-microbial properties and are potential candidates for the control of mites in food and feed commodities (Hubert et al. 2008).

Currently, these compounds can be obtained from plant extracts, but this is a costly and low-yielding process. Chemical synthesis offers an alternative but faces challenges with complex, multi-step, and non-stereospecific reactions leading to racemic mixtures, which require costly downstream purification (Longo and Sanromán 2019). Importantly, consumers are increasingly averse to chemically synthesized compounds, and there is a correspondingly strong consumer demand for naturally derived products. Thus, there

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is a strong commercial interest in harnessing enzymatic or microbial routes to produce these compounds.

Industrial processes using plant extracts as sources of lipoxygenase (LOX) and hydrogen peroxide lyase (HPL) to produce aldehydes have been developed. An advantage of this is that enzymes are regio- and stereospecific, thus resulting in fewer side products. However, this approach requires the LOX and HPL reactions to be carried out in separate steps and is prone to fluctuations in plant (enzyme) supplies. An alternative approach is to develop engineered microorganisms such as bacteria or yeast to heterologously express the enzymes for aldehyde production (Huang and Schwab 2011). These microbial cell factories have the potential to utilize cheap and renewable feedstocks or side-streams for growth, thus lowering the cost and environmental impact of aldehyde production (Tsuneyoshi and Itoh 1988; Goers et al. 1989; Muller et al. 1995).

Aldehyde accumulation in common laboratory bacteria causes toxicity, resulting in inhibition of growth and changes in morphology (Zaldivar et al. 1999). This impedes large-scale production of flavor and fragrance compounds in workhorse strains such as *Escherichia coli*. Here, we report the development of a *Saccharomyces cerevisiae* platform for aldehyde production, using the 9-carbon aldehydes (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal as proof-of-principle. We show that by heterologous expression of LOX and HPL, zero-growth fermentation, and tuning the reaction conditions, the reaction can be improved so that the aldehyde products are obtained in relatively high concentration. Our *S. cerevisiae* platform can be easily tailored to produce other high-value aldehydes for flavor and fragrance and other applications.

Materials and methods

Strains and culture media

S. cerevisiae strain BY4741 (4,040,002, ATCC) was maintained in YPD solid medium (yeast extract 1%, peptone 2%, dextrose 2%, and agar 2%; all w/v) and cultured in YPD liquid medium (yeast extract 1%, peptone 2%, and dextrose 2%; all w/v) at 30 °C. Transformants were selected at 30 °C on synthetic defined (SD) drop-out media which is either histidine-deficient (His^-) (6.7 g/L yeast nitrogen base without amino acids (Sigma Y0626), 1.92 g/L yeast synthetic drop-out media supplements without histidine (Sigma Y1751), 2% (w/v) glucose, and 2% (w/v) agar) or leucine-deficient (Leu^-) (6.7 g/L yeast nitrogen base without amino acids (Sigma Y0626), 1.6 g/L yeast synthetic drop-out media supplements without leucine (Sigma Y1376), 2% (w/v) glucose, and 2% (w/v) agar). For liquid media, all components were kept the same, except agar was omitted. For galactose induction, media was supplemented with 2% (w/v) galactose and 2% (w/v) raffinose in place of glucose.

Recombinant plasmid construction

Codon optimized genes *9-lox* from *Nicotiana benthamiana* (Fig. S1A, GenBank accession number X84040) and a *9-hpl* from *Cucumis melo* (Fig. S1B, GenBank accession number AF081955) were generated by Integrated DNA Technologies. The *Pichia pastoris* pGAP promoter (Fig. S1C) from the pGAPZ A vector (Invitrogen) was used as a template for gene expression under GAP promoter.

Cloning vectors pESC-HIS and pESC-LEU (Agilent) which contain galactose-inducible GAL1 and GAL10 promoters in opposing directions were used. The pESC-Nb-9S-LOX plasmid (Fig. S1D) expresses an N-terminus HIS_6–tagged *9-lox*, driven by either pGAP or pGAL10 promoter. The pESC-Cm-HPL plasmid (Fig. S1E) expresses a C-terminal HIS_6–tagged *hpl* driven by either pGAP or pGAL10 promoter. To test the effect of the auxotrophic selection marker, HIS3 amplified from pESC-HIS was used to replace the LEU2 sequence in pESC-Cm-HPL. To generate the pG72 plasmid (Fig. S1F), the GAL promoter sequences in the pESC-HIS vector were first replaced with GAP promoter sequences and then assembled with the *9-lox* and *9-hpl* coding sequences. To facilitate library creation, a backbone plasmid, pG88, was constructed from pG72 by relocating the *hpl* gene, along with its GAP promoter and ADH1 terminator, to upstream of the HIS3 selection marker. With the GAP promoters at distinct regions, various promoters were then cloned upstream of *lox* and *hpl* in different permutations to create a promoter library.

Oligonucleotide primers in Table S1 were synthesized by Integrated DNA Technologies. Restriction enzymes, Gibson Assembly Master Mix (E2611), and T4 DNA ligase (M0202) were from New England Biolabs. PCR reactions were carried out with iProof High-Fidelity DNA Polymerase (Cat no 1725301, Bio-Rad), according to standard protocols. The dNTPs were purchased from INtRON Biotechnology (25,022). PCR products were agarose gel purified using the E.Z.N.A Gel Extraction Kit (D2500, Omega Bio-Tek). Sequencing was performed by 1st Base, Singapore. Plasmids in *E. coli* were purified using the E.Z.N.A Plasmid Mini Kit I (D6942, Omega Bio-Tek). Transformation of plasmids into *E. coli* was performed by heat-shock method. *S. cerevisiae* electroporation was carried out following an established protocol (Adams et al. 1997).

Preparation of crude *S. cerevisiae* extracts

Two mL of *S. cerevisiae* culture was grown in SD drop-out liquid media for 2 days at 30 °C and 250 rpm. The starter culture was used to create a fresh culture in YPD liquid

media or SD drop-out liquid media at $OD_{600nm} = 1$. After another 2 days of growth, the cells were harvested by centrifugation. The cells were washed once with breaking buffer (100 mM potassium phosphate, pH 7.0) and resuspended in 0.5 mL of ice-cold lysis buffer (100 mM potassium phosphate, pH 7.0, containing 1 X cOmplete™ Mini EDTA-free protease inhibitor cocktail (Roche, 11,836,170,001)). Cells were disrupted using 0.5 mL of acid-washed glass beads (425–600 µm, G8772, Sigma) in a homogenizer (Bertin Precellys 24 Homogenizer) using 6 cycles of 20 s of vortexing at 6500 rpm and 5 min of incubation on ice. Lysates were centrifuged (10 min, 16,873 × g, 4 °C), and the supernatant was aliquoted into 100 µL, flash frozen in liquid nitrogen, and stored at − 80 °C. Total protein concentration in crude *S. cerevisiae* extracts was measured using the bicinchoninic acid assay (23,225, Pierce™ BCA Protein Assay Kit).

Determination of LOX and HPL activity in *S. cerevisiae* lysate by UV spectrophotometry

The activities of LOX and HPL enzymes in crude *S. cerevisiae* lysates were measured by detecting the formation or the depletion of the intermediate 9-hydroperoxyoctadecadienoic acid (9-HPOD), respectively. To measure LOX activity, crude *S. cerevisiae* extracts containing 33 µg of total protein were mixed with 400 µL of 100 mM sodium phosphate buffer (pH 7) and made up to 800 µL with water in a quartz cuvette. The reaction was started by adding 200 µL of master mix containing 100 µL of 100 mM sodium phosphate buffer (pH 7), 86 µL of water, 4 µL of 25% (v/v) Tween-20 (P1379 Sigma), and 10 µL of 60 mM substrate. To measure HPL activity, crude *S. cerevisiae* extracts containing 10 µg of total protein were mixed with 300 µL of 100 mM sodium phosphate buffer (pH 7) and made up to 600 µL with water in a quartz cuvette. The reaction was started by adding 400 µL of master mix containing 200 µL of sodium phosphate buffer (pH 7), 192.5 µL of water, and 7.5 µL of 6.4 mM 9-HPOD (12–1851-1, Larodan). For both LOX and HPL tests, the UV absorbance at $\lambda = 234$ nm was taken every 20 s for 2 min using a spectrophotometer. Mixing was achieved using a magnetic stirrer. The rate of increase or decrease in absorbance was converted to units of enzyme activity, where one unit (U) is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of 9-HPOD per minute. To overcome saturation of the rate curves, either the amount of crude *S. cerevisiae* lysate added to the reaction was lowered, or only the initial linear portion of the curves was used for rate calculations.

S. cerevisiae whole cell biotransformation

To perform whole cell biotransformation, a starter culture of *S. cerevisiae* cells was grown in shake flasks in SD drop-out liquid media at 30 °C and 250 rpm for 2 days. Next, the starter culture was used to create a fresh culture in YPD liquid media at $OD_{600nm} = 1$. After growing for 2 days at 30 °C and 250 rpm, cells were harvested by centrifugation at 2100 × g for 5 min, washed three times with 100 mM sodium phosphate buffer (pH 7), resuspended in the same wash buffer at required densities, and used directly for biotransformation. For cell permeabilization experiments, the washed cells were subject to various permeabilization treatments before they were washed three times and used for biotransformation. Biotransformations were performed at a 1 mL scale which typically composed of 400 µL of 100 mM sodium phosphate buffer (pH 7), 100 µL of cells ($OD_{600nm} = 100$, resuspended in pH 7, 100 mM sodium phosphate buffer), 486 µL of water, 4 µL of 25% (v/v) Tween-20 (P1379 Sigma), and 10 µL of 600 mM substrate linoleic acid (LA, L1376 Sigma-Aldrich) or alpha-linolenic acid (LNA, L2376 Sigma-Aldrich). The reaction was incubated at 25 °C for 4 h with 250 rpm shaking. For reaction condition enhancement experiments, the amount of cells and the substrate concentration were varied.

Permeabilization of *S. cerevisiae* cells

For permeabilization, cells were resuspended in various concentrations of N,N-dimethyltetradecylamine (DMA, 41,653 Sigma-Aldrich) suspension and incubated for various durations at 25 °C with mixing at 250 rpm. Following permeabilization, cells were centrifuged, washed three times with 100 mM sodium phosphate buffer (pH 7), resuspended in the same buffer, and used for biotransformation.

Optimization of permeabilization method was carried out through design of experiments (DoE) using JMP software (SAS, Cary, NC, USA). A software-determined fractional factorial design of 13 reactions was performed.

GC–MS analysis of products

After biotransformation, 2 mL of ACS-grade ethyl acetate (23,882.321 VWR) was added as extraction solvent to the cultures and mixed vigorously using a vortex mixer for 1 min to improve extraction. The samples were centrifuged for 5 min at 2100 × g to separate the aqueous and organic layers. About 500 µL of the upper organic phase was collected, filtered with a 0.2 µm PTFE filter (5190–5265 Agilent), and transferred to 1.5 mL vials. The extracts were analyzed using a 7890B GC system coupled to 5977B MS Detector (Agilent Technologies, USA). The system was equipped with a polar DB-WAX UI column (30 m × 0.25 mm I.D. × 0.25 µm; Agilent Technologies, USA) and a split/splitless injector. One µL extract was injected in splitless mode, with injector temperature set at 250 °C. The initial oven temperature was held for 5 min at 40 °C, increased to 115 °C at 25 °C/

min, followed by 5 °C/min to 145 °C and finally 80 °C/min to 240 °C and held for 15 min. Helium gas was used as carrier gas at a constant flow of 1.2 mL/min. The MS detector was operated in electron ionization mode at 70 eV with a source temperature of 230 °C, transfer line temperature of 250 °C, and a full scan mode within the range of m/z 28 to 300 at an acquisition rate of 3.6 scans/s. Four-minute solvent delay was set to offload the ethyl acetate peak. Product identification was carried out by comparing mass spectra with commercially available standards. The acquired data was analyzed using MassHunter Workstation software (version B.07.00, Agilent Technologies, Inc. 2014). A calibration curve was prepared for the determination of (2*E*)-nonenal concentrations (Fig. S2). The curve was prepared in ethyl acetate and included five concentration points, serially diluted from 0.125 mM to 0.0078 mM. Undecanal, 0.5 mM, was used as an internal standard. A second calibration curve was prepared to determine the (2*E*,6*Z*)-nonadienal concentrations (Fig. S3). Six concentration points, serially diluted from 0.250 mM to 0.0078 mM, were prepared in ethyl acetate and 0.5 mM Undecanal as an internal standard. All chemical standards were purchased from Sigma.

Results

Expression and optimization of LOX and HPL activities in *S. cerevisiae*

In the plant 9-lipoxygenase pathway, a 9-lipoxygenase (9-LOX) enzyme catalyzes the addition of molecular oxygen to the ninth carbon of a polyunsaturated fatty acid (PUFA) such as linoleic acid (LA) or alpha-linolenic acid (LNA) to form the peroxide intermediates 9-HPOD or 9-HPOT, respectively. This is followed by cleavage of the intermediates 9-HPOD or 9-HPOT by a hydrogen peroxide lyase (HPL) enzyme to form the aldehyde products (3*Z*)-nonenal or (3*Z*,6*Z*)-nonadienal, respectively (Fig. 1). (3*Z*)-nonenal or (3*Z*,6*Z*)-nonadienal can isomerize spontaneously or via enzymatic conversion to (2*E*)-nonenal or (2*E*,6*Z*)-nonadienal

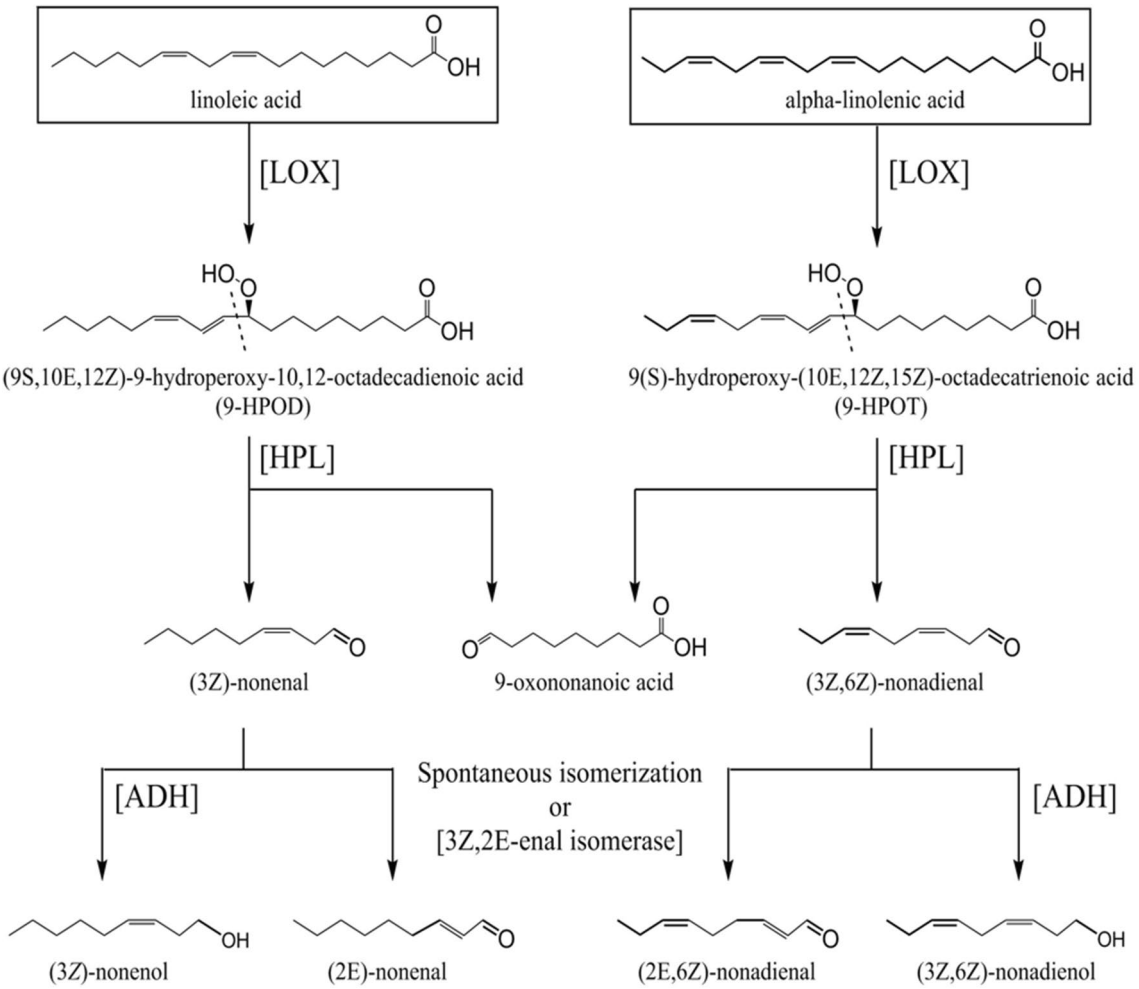


Fig. 1 9-Lipoxygenase reaction scheme. Enzyme abbreviations are LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase

respectively, or alternatively be reduced by enzymes such as alcohol dehydrogenases (ADH) to the corresponding alcohols (3*Z*)-nonenol or (3*Z*,6*Z*)-nonadienol (Fig. 1).

We cloned and expressed a previously characterized plant 9-LOX from *Nicotiana benthamiana* (Huang and Schwab 2011) and a 9/13-HPL from *Cucumis melo* (Tijet et al. 2001) separately in *S. cerevisiae* to optimize their activities using different plasmid backbones, promoters, and growth conditions. Our results show that expressing LOX or HPL on a high copy (2 μ) plasmid under control of the strong galactose-inducible (GAL)-promoter resulted in a LOX or HPL activity of ~ 0.5 or ~ 0.25 U/mg of total protein respectively upon addition of the inducer galactose, but there was no detectable LOX/HPL activity in the absence of the inducer (Fig. 2). Expressing LOX or HPL under control of the constitutive *P. pastoris* GAP promoter produced comparable or higher levels of activity compared to the GAL promoter in the presence of the inducer (Fig. 2). Thus, we selected the constitutive GAP promoter, which eliminates the need for expensive inducer molecules and reduces overall process costs, for further studies. In addition, cultivation of *S. cerevisiae* for 2 days in synthetic defined (SD) media, followed by

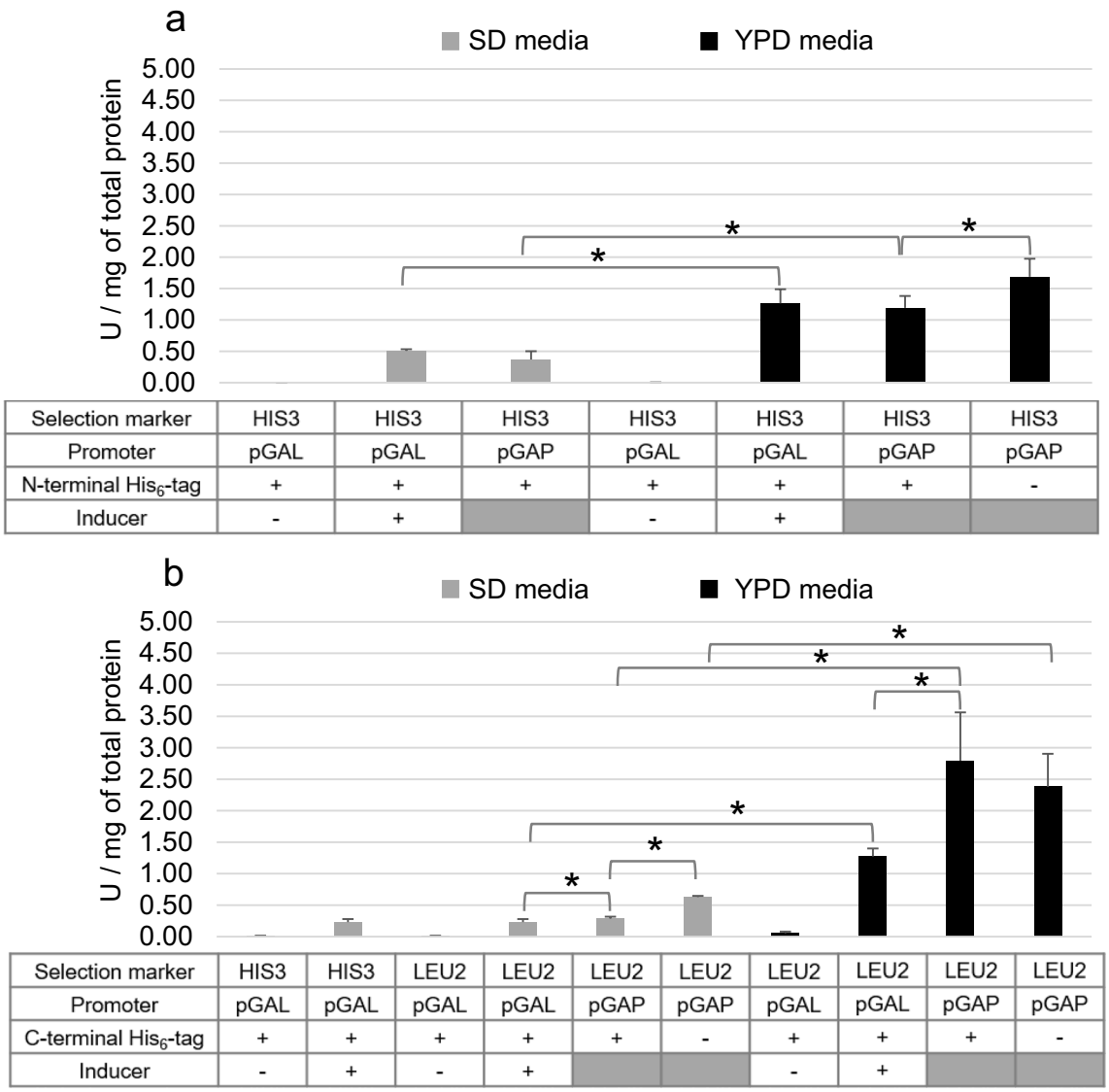


Fig. 2 Activity of LOX and HPL in crude extracts of *S. cerevisiae* as measured by UV assay. Bars represent the average of $n=3$ biological replicates; $n=2$ for non-induced controls. *X*-axis labels indicate selection marker on the plasmid used to express LOX/HPL (HIS3 or LEU2), media used promoter driving expression of LOX/HPL (pGAP or pGAL), presence (+) or absence (−) of a HIS_6 tag attached to LOX/HPL, presence (+) or absence (−) of inducer for inducible pGAL promoter only. Activity of **a** LOX and **b** HPL. Cells were grown in SD media only (■ SD media) or in SD media followed by YPD media (■ YPD media). Error bars represent standard error. Data was analyzed using *t*-test: $^*P<0.05$

2 days in YPD media, more than tripled LOX activity when compared to cells grown in SD media for 4 days (Fig. 2a). This effect was greater for HPL, with the enzyme activity increasing by up to ~ 10 times in YPD compared to SD media (Fig. 2b). The presence of a histidine (HIS_6) tag on the N-terminus of LOX decreases activity of the enzyme compared to the untagged form (Fig. 2a). This may be because the N-terminus of LOX is required for membrane-association and consequently its function (Chen and Funk 2001; Hammarberg et al. 2000; Shibata and Axelrod, 1995). On the other hand, HIS_6 tag on the C-terminus of HPL did not significantly affect its activity when cultivated in YPD (Fig. 2b). Thus, we chose to express untagged LOX and HPL in *S. cerevisiae* under control of the constitutive GAP promoter and cultivate the cells in YPD to maximize the activities of both enzymes.

S. cerevisiae cells as one-pot whole cell biocatalysts for aldehyde production

To produce the 9-carbon aldehydes in a one-pot reaction using a single yeast strain, we cloned LOX and HPL into a single plasmid (pG72) for co-expression (Fig. S1F) and transformed the plasmid into *S. cerevisiae* to yield the G72 strain. Since aldehydes are inhibitory to cell growth, we envisioned performing the reaction using zero-growth fermentation (Huang et al. 2011; Julsing et al. 2011). Furthermore, it has been shown previously that the addition of the substrate LNA to growing cells expressing soybean 13-LOX and watermelon HPL did not lead to detectable product formation, whereas the addition of the substrate to resting cells (washed and resuspended in potassium phosphate buffer) resulted in green note aldehyde formation (Buchhaupt et al. 2012). Our results show that the desired aldehyde (2*E*)-nonenal is produced when 6 mM LA is added to resting cells co-expressing LOX and HPL, thus establishing proof-of-principle that we had built a functional whole cell biocatalyst for 9-carbon aldehyde production (Fig. 3).

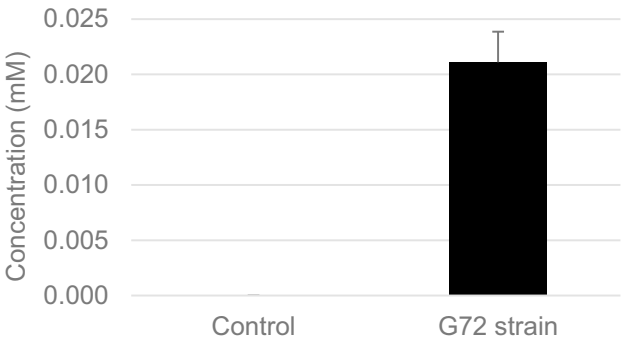


Fig. 3 Production of (2*E*)-nonenal using whole *S. cerevisiae* cells (G72 strain). Biotransformation was performed at 1 mL scale for 4 h at 25 °C with 250 rpm shaking. A final concentration of 6 mM LA was used as substrate. Control refers to a reaction vial containing all reagents except cells. Bars represent average of $n = 3$ biological replicates. Error bars represent standard error

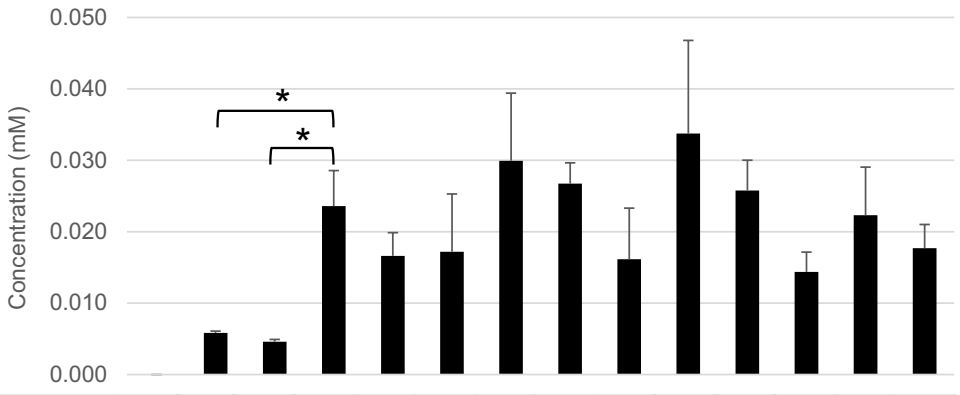
Permeabilization of *S. cerevisiae* cells prior to biotransformation improves aldehyde yield

As LOX and HPL are intracellular membrane–associated proteins, the free fatty acid substrate must first enter the cell for the reaction to occur. Previously, several reports have explored the use of various permeabilization agents (e.g., ethanol, isopropanol, or N,N-dimethyltetradecylamine (DMA)) to enhance substrate or product transport in or out of the cell, respectively, or to release soluble intracellular proteins into the extracellular space (Shepard et al. 2002; Trawczyńska and Wójcik 2015; Liu et al. 2000). We selected DMA as the permeabilization agent of choice as we found that the concentrations needed for cell permeabilization (~ 0.01% (v/v)) are three orders of magnitude smaller than that for the alcohols (~ 40% (v/v)) (data not shown), leading to lower costs. We also explored physical methods such as freeze–thaw and electroporation to permeabilize the cells prior to biotransformation (data not shown), which although successful on a small scale, are not readily scalable to large volumes of cells. Thus, we selected chemical methods of permeabilization (particularly DMA) for ease and consistency, especially during scale-up.

We optimized the concentration of DMA, cell densities, and duration of permeabilization using a design of experiment (DoE) approach, where the variables were cell densities ($OD_{600nm} = 50$, 100, or 200), final DMA concentration (0.2%, 1%, and 2%; all v/v), and duration of permeabilization (30 min, 60 min, and 120 min) (Fig. 4). To reach comparable titers of (2*E*)-nonenal, cells at OD_{600nm} 50 require at least 60 min of permeabilization (Fig. 4). However, permeabilizing OD_{600nm} 100 and 200 cells for the various durations and DMA concentration resulted in similar titers of (2*E*)-nonenal. Our results show that 30 min of permeabilization with 0.2% (v/v) DMA, using cell densities of either OD_{600nm} 100 or 200 is sufficient to produce high titers of (2*E*)-nonenal (Fig. 4).

Enhancement of reaction conditions—cell-to-substrate ratio

To further increase titer, we sought to improve the reaction conditions of the biotransformation step. Previous studies have demonstrated the effects of LA on *S. cerevisiae* metabolism and survival (Casu et al. 2018; Ferreira et al. 2011). Hence, we sought to optimize the cell-to-substrate ratio by varying the final substrate concentration and the final cell density in the biotransformation step. We started by testing a broad range of 4 substrate concentrations (6 mM,



OD_{600nm}	Ctrl	50	50	50	50	50	100	100	100	200	200	200	200	200
v/v % DMA		1	2	0.2	0.2	2	0.2	1	2	0. 2	0.2	1	2	2
Duration (mins)		30	30	60	120	120	30	60	120	30	120	120	30	60

Fig. 4 Concentration of (2*E*)-nonenal achieved when G72 cells were permeabilized with varying cell densities, DMA concentrations (v/v%), and duration. After permeabilization, cells were washed and resuspended at $OD_{600nm} = 100$. Then, 100 µL of cell suspension was used for the biotransformation. A final concentration of 6 mM LA was used as substrate. Control (Ctrl) refers to a reaction vial containing all reagents except cells. Bars represent average of $n = 2$ biological replicates. Error bars represent standard error. Data were analyzed using *t*-test: $^*P < 0.05$

3 mM, 1.5 mM, and 0.75 mM) and 4 cell-to-substrate ratios (Fig. 5a, b).

We found that as the cell-to-substrate ratio decreased, the titer of (2*E*)-nonenal generally decreased (Fig. 5a). Six mM LA produced the highest (2*E*)-nonenal titer across all cell-to-substrate ratios (Fig. 5a), but the reaction was less efficient as the percentage yields were lowest (Fig. 5b). The reaction was most efficient with a cell to substrate ratio of 1.667 and LA concentration of 0.75 mM (highest percentage yield of 3.75%) (Fig. 5b), but the corresponding titer was low at < 0.03 mM (Fig. 5a). To further improve both the titer and percentage yields, we explored a wider range of cell to substrate ratios (6.665 to 0.400) together with 3 substrate concentrations (1.23 mM, 1.125 mM, and 0.75 mM). We found that 1.23 mM LA and a cell-to-substrate ratio of 3.332 produced the highest titer of (2*E*)-nonenal (0.09 mM) (Fig. 5c) with a percentage yield of > 7% (Fig. 5d). Single experiments were conducted for each of the conditions to facilitate the rapid identification of cell-to-substrate loading combination that yields high product titer, and subsequent experiments using this selected condition were carried out in duplicates to ensure reliability.

S. cerevisiae promoter library to fine-tune LOX and HPL ratios

It has been shown that the peroxide intermediate, 9-HPOD, is toxic to the cell and can deactivate or inhibit HPL (Tran Thanh et al. 2007; Matsui et al. 1992; Evans et al. 1998). Hence, it is imperative that the expression levels of LOX and HPL are fine-tuned such that 9-HPOD does not accumulate to high levels in the cell. We hypothesized that using a strong promoter for LOX and a weak promoter for HPL would cause an accumulation of the toxic 9-HPOD, resulting in low levels of (2*E*)-nonenal (Fig. 6a). Conversely, using a weak promoter for LOX and a strong promoter for HPL would increase the yield of (2*E*)-nonenal (Fig. 6a). To test this, we used a promoter library to fine-tune the expression levels of LOX and HPL in the cell. Previously, a synthetic promoter library based on the constitutive GAP promoter has been constructed and shown to display activities ranging from ~ 0.6 to 2000% of the wild-type GAP promoter when expressing the reporter gene yeast-enhanced green fluorescent protein in *Pichia pastoris* (Qin et al. 2011). We selected 7 promoter variants (8 including wild-type pGAP) for library construction, with G1 (Mut 1) being the strongest and G7 (Mut 7) being the weakest in *P. pastoris*. The strength of the wild-type pGAP was between G4 (Mut 4) and G5 (Mut 5). As these were *P. pastoris*-based promoters, their activities might differ in *S. cerevisiae*. Thus, to assess the effect of host and gene-dependent context, we determined the activity of the synthetic promoters on the LOX and HPL genes in *S. cerevisiae* separately. The order of the promoter strengths was vastly different in *S. cerevisiae* compared to *P. pastoris*, and this order was also different between LOX and HPL (Fig. 6b, c), making it challenging to rationally optimize the expression of LOX and HPL together. Thus, we generated pG88 plasmids expressing selected combinations of the promoters to drive the expression of LOX and HPL (Fig. 6d). The WT-LOX, Mut 5-HPL combination produced the highest titer of (2*E*)-nonenal of more than 0.11 mM (Fig. 6e) when 1.23 mM LA and a cell to substrate ratio of 3.332 was used.

Biotransformation using LNA as substrate

To test the ability of our biotransformation platform to produce other aldehydes beyond (2*E*)-nonenal, we used a different substrate for biotransformation, LNA, which is expected to lead to production of (2*E*,6*Z*)-nonadienal (Fig. 1). While

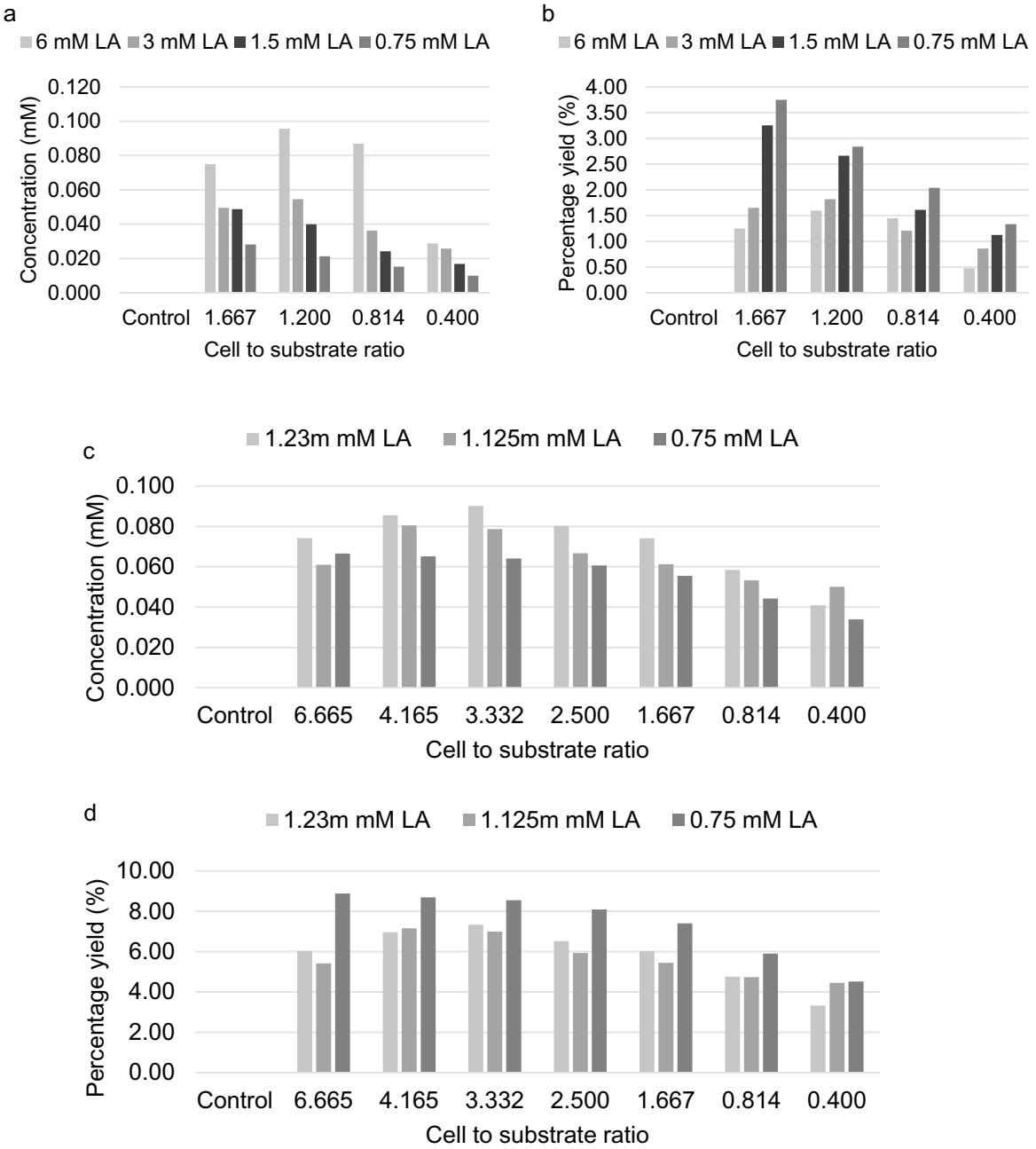
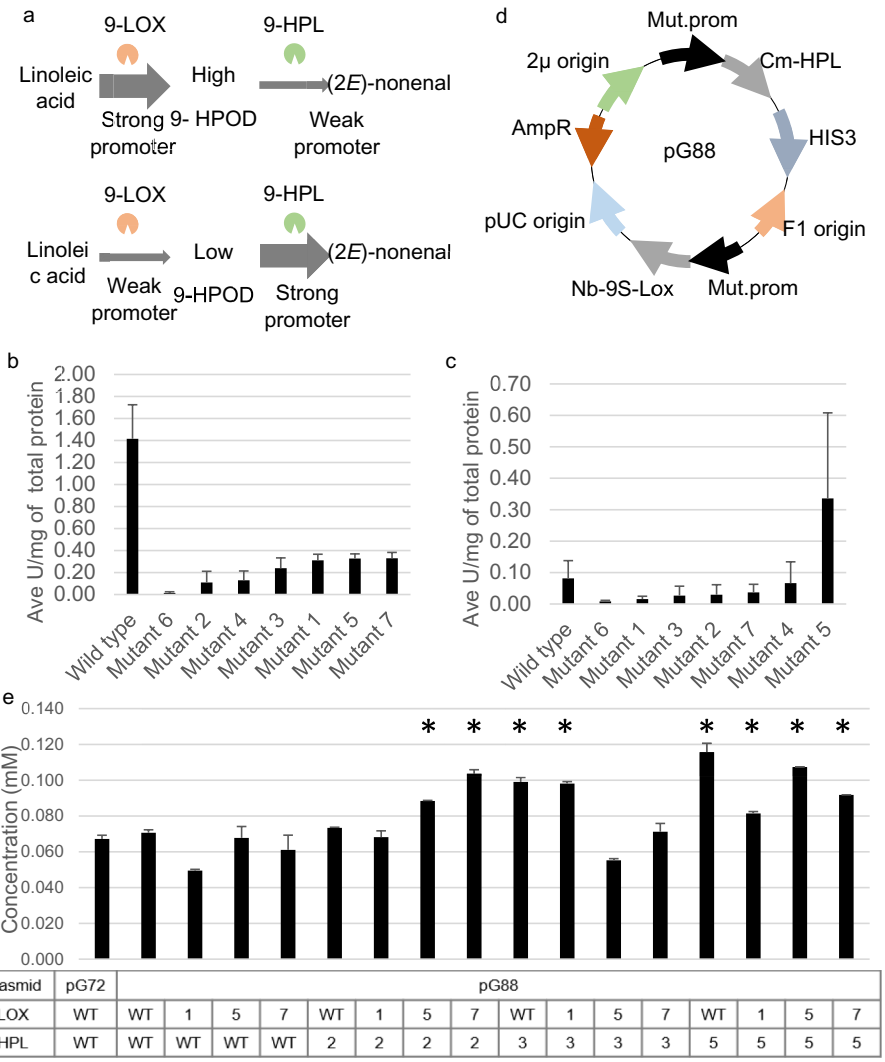


Fig. 5 Concentration and percentage yield of (2*E*)-nonenal achieved with varying cell-to-substrate ratios and substrate concentrations. **a** Absolute (2*E*)-nonenal concentration and **b** percentage yield for 4 different cells to substrate ratios with 4 different LA concentrations. **c** Absolute (2*E*)-nonenal concentration and (d) percentage yield with 6 different cell-to-substrate ratios and 3 different LA concentrations. Permeabilization was performed at $OD_{600nm} = 100$, with 0.2% (v/v) DMA, for 30 min with shaking at 250 rpm and at 25 °C. *X*-axis labels indicate the cell-to-substrate ratio. Control refers to a reaction vial containing all reagents except cells. Bars represent $n = 1$ biological replicate

the HPL used in this study has been reported to possess broad substrate specificity and is able to cleave the substrates 9-HPOD, 13-HPOD, 9-HPOT, and 13-HPOT (the latter two are intermediates produced from LNA), the activity of the LOX used in this study with other substrates besides LA is unknown. Our results show that permeabilized G72 strain could produce (2*E*,6*Z*)-nonadienal when provided with LNA as substrate, confirming that 9-LOX from *N. benthamiana* is active against LNA (Fig. 7). Furthermore, the production of (2*E*,6*Z*)-nonadienal was significantly (~ 3X) higher compared to (2*E*)-nonenal (Fig. 7). These results established a proof-of-principle that our platform can be readily applied to



Plasmid	pG72	pG88															
LOX	WT	WT	1	5	7	WT	1	5	7	WT	1	5	7	WT	1	5	7
HPL	WT	WT	WT	WT	WT	2	2	2	2	3	3	3	3	5	5	5	5

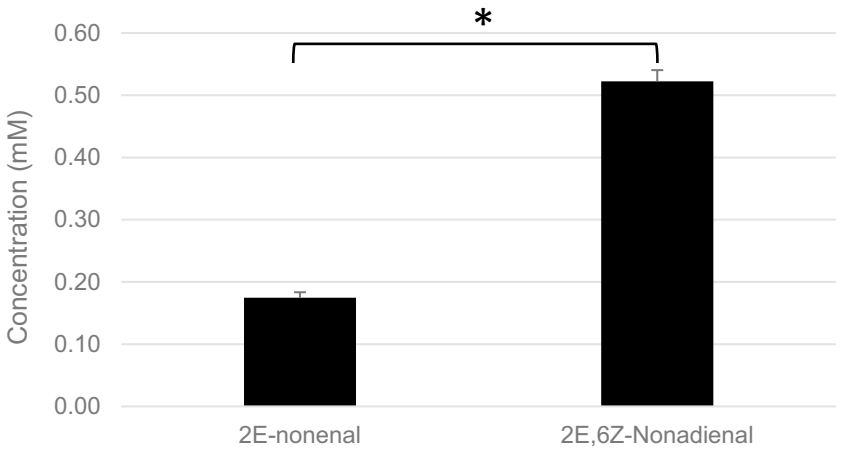


Fig. 6 Promoter library scheme. **a** Different promoter combinations to fine tune the levels of LOX and HPL can lead to different concentrations of the toxic intermediate 9-HPOD. Activity levels of **b** LOX and **c** HPL proteins in *S. cerevisiae* when driven by synthetic GAP promoters compared to the WT GAP promoter. **d** pG88 plasmid map. The different mutant promoters were cloned into the "Mut. prom" region to drive LOX and HPL expression. **e** Yield of (2*E*)-nonenal when LOX and HPL are driven by select promoters. WT refers to wild-type GAP promoter, while the numbers refer to the mutant GAP promoters. Permeabilization was performed at $OD_{600nm} = 200$, with 0.2%(v/v) DMA, for 30 min with shaking at 250 rpm at 25 °C. Biotransformation reaction was performed with 1.23 mM LA at 25 °C for 4 h with a cell-to-substrate ratio of 3.332. Products were detected using GC–MS. Bars represent average of $n = 2$ biological replicates. Error bars represent standard error. Data were analyzed using *t*-test: $^*P < 0.05$ for promoter combinations with (2*E*)-nonenal concentration more than pG72

Fig. 7 Concentrations of (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal achieved when LA and LNA are used as substrates, respectively. Permeabilization was performed at $OD_{600nm} = 200$, with 0.2% (v/v) DMA, for 30 min with shaking at 250 rpm and at 25 °C. Biotransformation reaction was performed with 6 mM LA or LNA at 25 °C for 4 h with a cell-to-substrate ratio of 1.667. Products were detected using GC–MS. Bars represent an average of $n = 3$ biological replicate. Error bars represent standard error. Data were analyzed using *t*-test: $^*P < 0.05$

produce other aldehydes beyond (2*E*)-nonenal, in this case by using a different substrate.

Discussion

A few enzymatic pathways have been proposed to produce medium-chain aliphatic aldehydes. One relies on carboxylic acid reductase (CAR) to reduce carboxylic acids to the corresponding aldehyde (Akhtar et al. 2013; Alibhai and Hu, 2012). Another pathway relies on alpha-dioxygenases (α-DOX), which convert carboxylic acid substrates to aldehydes, with the corresponding loss of one carbon as carbon dioxide (Sporleder et al. 2013). However, in both cases, the carboxylic acid substrate is not readily available from natural sources. We chose the lipoxygenase (LOX) pathway, where polyunsaturated fatty acids are first converted to fatty acid hydroperoxides by LOX, followed by conversion to the aldehydes by HPL (Fig. 1). This pathway is advantageous since the substrates are readily and cheaply derived from natural botanical sources (e.g., plant oils), which is also a pre-requisite for the product to be considered a natural flavor.

Other regulatory guidelines (EU) that must be satisfied for products to be labeled natural include a physical, enzymatic, or microbial production process, and products that are identifiable in nature. In this study, we have established an *S. cerevisiae* whole cell biotransformation platform to produce 9-carbon aldehydes, which in nature are the key compounds responsible for the flavor and fragrances of fruits such as melon and cucumber, thus satisfying the requirements of the natural label. We selected the *S. cerevisiae* as expression host for several reasons: many plant enzymes have been functionally expressed in it (Yesilirmak and Sayers 2009), it is a well-established model organism with many available genetic tools and large knowledge base in fermentation and biotransformation, and it is a Generally Regarded As Safe (GRAS) organism suitable for the synthesis of food and cosmetic ingredients.

We have employed the strategy of whole cell biotransformation starting from an advanced substrate (i.e., free fatty acids), instead of simple sugars such as glucose. Although much work has been done to engineer polyunsaturated free fatty acid production in *S. cerevisiae*, which is not an endogenous producer, efforts so far have produced heterogeneous mixtures of fatty acids and low yields of linoleic/alpha-linolenic acids (Uemura 2012). Importantly, whole cell biotransformation allows for the reaction to be started upon the addition of the substrate, thus allowing for cell growth to be decoupled from production. This is important since the aldehyde products can negatively impact cell growth. Furthermore, the reaction occurs on a timescale of minutes. Whole cell biotransformation allows for rapid extraction of the aldehyde products before it is converted to other side products such as more stable isomers or alcohol (Fig. 1). Previously, *S. cerevisiae* whole cell biotransformation has been developed to produce 6-carbon green leaf aldehydes and alcohols, but the reaction yielded mixtures of 6-carbon aldehydes/alcohols and the pathway and reaction conditions were not optimized (Buchhaupt et al. 2012).

In this report, we have increased the product titer and yield of the reaction by enhancing the permeabilization conditions, biotransformation reaction conditions, and gene expression levels of LOX and HPL. Permeabilization increases the influx of the substrate and efflux of the product, thereby increasing product recovery. Following enhancement of the permeabilization conditions using DoE (Fig. 4), we improved the concentration and percentage yield of (2*E*)-nonenal by varying the cell-to-substrate ratio and substrate concentration (Fig. 5). Different strategies to reduce the levels of toxic 9-HPOD intermediate were explored. The strategies we tried included tuning the ratio of HPL to LOX in the cell via promoter libraries (Fig. 6) and ethanol-inducible promoters of varying strengths (data not shown) and synthetic HPL–LOX enzyme fusions to enhance substrate channeling (data not shown). In the latter case, bifunctional enzymes containing AOS-LOX (Boutand and Brash 1999), as well as HPL-LOX (Senger et al. 2005; Andrianarison et al. 1991; Liagre et al. 1996) activities, and even trifunctional AOS-HPL-LOX activities (Chen et al. 2015), have been found in nature. However, these enzymes display high substrate promiscuity and tend to generate a range of volatile products (Senger et al. 2005; Chen et al. 2015). By optimizing the permeabilization conditions, biotransformation reaction conditions, and fine-tuning the gene expression levels of LOX and HPL, we achieved steady and significant increases in both the concentrations and percentage yield of (2*E*)-nonenal to > 0.11 mM and ~ 9%, respectively (Fig. 8).

We also evaluated the effects of temperature and host system on the production of (2*E*)-nonenal. We hypothesized that a lower temperature could reduce the reactivity of the peroxide intermediate 9-HPOD, thereby reducing any side product formation and increasing (2*E*)-nonenal yield. However, we found that at 16 °C, there were non-specific products that were absent at 30 °C (Fig. S4). Furthermore, since plant LOX and HPL have an optimal temperature between 30 °C and 40 °C (Aanangi et al. 2016; Liburdi et al. 2021; Rabetafika et al. 2008), and a low temperature in a large-scale biotransformation process would incur additional cooling costs, 30 °C was selected as the reaction temperature. pH 7 was chosen as the pH of the reaction as plant LOX and HPL have optimal pH between pH 6 and 7 (Aanangi et al. 2016; Gardner 1991; Liburdi et al. 2021).

P. pastoris GAP promoters have been used for expression of recombinant proteins in *S. cerevisiae* (Vellanki et al. 2007, 2013). When evaluating host strains, we expressed the LOX and HPL in *P. pastoris* GS115 strain and *S. cerevisiae*

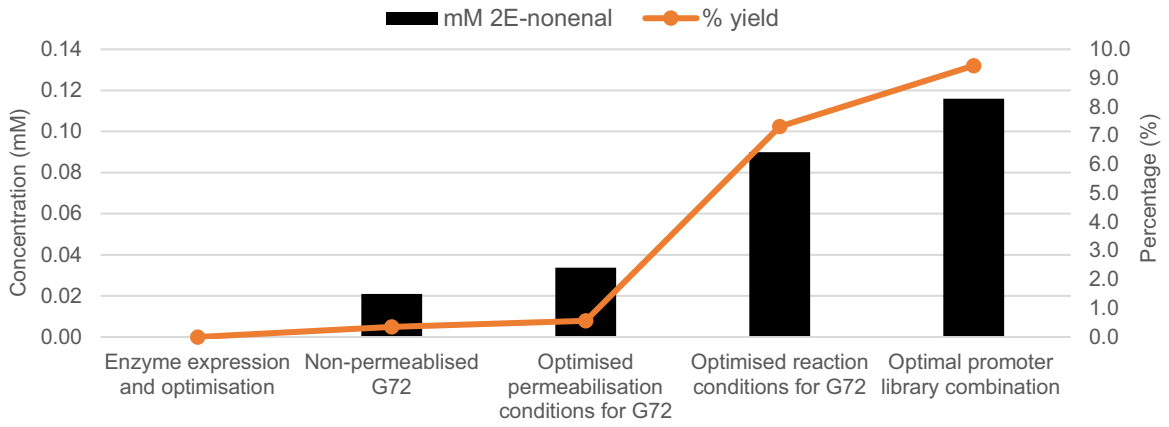


Fig. 8 Stepwise increase in (2*E*)-nonenal concentration and percentage yield. By optimizing permeabilization conditions, reaction conditions, and promoter library combination, high yields of (2*E*)-nonenal were achieved

BY4741 strain, under the same *P. pastoris* GAP promoter (Fig. S5). Although there was no significant difference in the activity of LOX expressed in the two host strains (Fig. S5a), we found much higher HPL activity in *S. cerevisiae* compared to *P. pastoris* (Fig. S5b). Hence, we selected *S. cerevisiae* as the host, keeping LOX and HPL under the control of the *P. pastoris* GAP promoter. Future studies can be performed using other strong constitutive promoters specific to *S. cerevisiae*.

When LNA was tested as a substrate, the G72 strain produced approximately 3 times more (2*E*,6*Z*)-nonadienal than (2*E*)-nonenal (Fig. 7). This could be due to the fact that the HPL used is more active with 9-HPOT (the intermediate formed from LNA) than 9-HPOD (Tijet et al. 2001), thus leading to rapid clearance of the intermediate and lower concentrations of reactive intermediates. Our results suggest that the LOX from *N. benthamiana* is also active with LNA as a substrate, which has not been shown previously (Huang and Schwab 2011).

Overall, we have established an *S. cerevisiae* cell platform for the whole cell biotransformation of polyunsaturated fatty acids to 9-carbon aldehyde flavors. By employing genetic strategies and tuning the permeabilization and biotransformation reaction conditions, we demonstrate control of the highly reactive peroxide pathway, increasing the concentration of (2*E*)-nonenal by > 5.5X and the percentage yield of (2*E*)-nonenal by > 20X compared to non-permeabilized G72 strain (Fig. 8). This platform can be harnessed to produce other aldehydes by using alternative substrates such as LNA, as well as by potentially expressing LOX and HPL enzymes with different regio-selectivities.

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Author contribution NVMAM designed research, conducted experiments, analyzed data, and wrote the manuscript. NYZ conceived and designed research, analyzed data, and wrote the manuscript. GWM designed research, conducted experiments, and analyzed data. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare no competing interests.

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