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RESEARCH ARTICLE

Biotechnological production of the European corn borer sex pheromone in the yeast *Yarrowia lipolytica*

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Abstract

The European corn borer (ECB) *Ostrinia nubilalis* is a widespread pest of cereals, particularly maize. Mating disruption with the sex pheromone is a potentially attractive method for managing this pest; however, chemical synthesis of pheromones requires expensive starting materials and catalysts and generates hazardous waste. The goal of this study was to develop a biotechnological method for the production of ECB sex pheromone. Our approach was to engineer the oleaginous yeast *Yarrowia lipolytica* to produce (Z)-11-tetradecenol (Z11-14:OH), which can then be chemically acetylated to (Z)-11-tetradecenyl acetate (Z11-14:OAc), the main pheromone component of the Z-race of *O. nubilalis*. First, a C14 platform strain with increased biosynthesis of myristoyl-CoA was obtained by introducing a point mutation into the α -subunit of fatty acid synthase, replacing isoleucine 1220 with phenylalanine (Fas2p^{I1220F}). The intracellular accumulation of myristic acid increased 8.4-fold. Next, fatty acyl-CoA desaturases (FAD) and fatty acyl-CoA reductases (FAR) from nine different species of Lepidoptera were screened in the C14 platform strain, individually and in combinations. A titer of 29.2 ± 1.6 mg L⁻¹ Z11-14:OH was reached in small-scale cultivation with an optimal combination of a FAD (Lbo_PPTQ) from *Lobesia botrana* and FAR (HarFAR) from *Helicoverpa armigera*. When the second copies of FAD and FAR genes were introduced, the titer improved 2.1-fold. The native *FAS1* gene's overexpression led to a further 1.5-fold titer increase, reaching 93.9 ± 11.7 mg L⁻¹ in small-scale cultivation. When the same engineered strain was cultivated in controlled 1 L bioreactors in fed-batch mode, 188.1 ± 13.4 mg L⁻¹ of Z11-14:OH was obtained. Fatty alcohols were extracted

Abbreviations: BioPhe, biologically-derived pheromone blend; E/Z11-14:OAc, (E/Z)-11-tetradecenyl acetate; E/Z11-14:OH, (E/Z)-11-tetradecenol; EAG, electroantennography; ECB, the European corn borer; FAD, fatty acyl-CoA desaturase; FAME, fatty acid methyl ester; FAR, fatty acyl-CoA reductase

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from the biomass and chemically acetylated to obtain Z11-14:OAc. Electroantennogram experiments showed that males of the Z-race of *O. nubilalis* were responsive to biologically-derived pheromone blend. Behavioral bioassays in a wind tunnel revealed attraction of male *O. nubilalis*, although full precopulatory behavior was observed less often than for the chemically synthesized pheromone blend. The study paves the way for the production of ECB pheromone by fermentation.

KEYWORDS

fatty acyl-CoA desaturases, fatty acyl-CoA reductases, fatty alcohols, insect pheromones, *Yarrowia lipolytica*

1 | INTRODUCTION

Crop damage caused by insects is a severe problem in agriculture. Moths (Lepidoptera) are major insect pests.^[1-3] The European corn borer (ECB) *Ostrinia nubilalis* is the main pest of maize *Zea mays* in Europe.^[4] It is estimated that in the absence of treatment, up to 20% of the crop may be lost due to the damage by *O. nubilalis* larvae.^[5] Like other moth species, females of *O. nubilalis* produce and release a fatty acid-derived sex pheromone, which attracts conspecific males for mating.^[6] ECB is polymorphic with respect to its pheromone communication system and two pheromone races are recognized: the Z-race insects use a 97:3 blend of (Z)-11-tetradecenyl acetate (Z11-14:OAc) to (E)-11-tetradecenyl acetate (E11-14:OAc), while E-race insects use a 1:99 blend of the same components.^[6,7] On maize in Europe, the Z-race is most prevalent.^[8]

Mating disruption has been proven an effective and environmentally friendly solution for crop protection against moths. For mating disruption, a more or less species-specific pheromone blend is applied to the fields or orchards to disrupt mating partner detection and, in this way, decrease the propagation of the pest species.^[9,10] Currently, pheromones for pest management are produced synthetically from petrol-derived chemicals.^[11-13] Chemical synthesis typically comprises multiple steps, uses toxic chemicals and solvents, and expensive catalysts (Figure S1). Biotechnological production of several insect pheromone components has already been established in plants and yeasts.^[14-18] Oleaginous yeast *Yarrowia lipolytica* is particularly suitable for production of lepidopteran pheromones due to its naturally high level of fatty acid biosynthesis. Previously, we produced the precursor of the major pheromone component of *Helicoverpa armigera* (Z11-16:OH) at 2.5 g L⁻¹ in *Y. lipolytica* and proved the effectiveness of yeast-derived insect pheromone in field trapping experiments.^[16]

In this study, we aimed to produce Z11-14:OAc, the major pheromone component of the ECB Z-race, using engineered *Y. lipolytica* as a host and demonstrate the activity of yeast-derived pheromone on *O. nubilalis* males.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction

Plasmids were constructed according to Holkenbrink et al., 2018.^[19] Integration and guide RNA (gRNA) vectors were used to introduce gene expression constructs into characterized genome sites of *Y. lipolytica*.^[19] The design of integrative plasmids is illustrated in Figure S2. The plasmids contain two 500-bp-long homology arms. In-between these arms, there is a USER-cloning site flanked by two terminators. The gene of interest was cloned along with *TEF1*intron promoter into the USER-site to generate an expression cassette.^[19] Correct cloning was confirmed by Sanger sequencing. The plasmids were linearized with *SmaI* to generate linear integration fragments. The integration fragments were transformed into *cas9*-expressing *Y. lipolytica* along with a single guide RNA (gRNA) vector targeting the given integration site. The expression of gRNA was controlled by RNA polymerase III promoter and *RPR1* terminator. Additionally, gRNA vectors contained hygromycin or nourseothricin resistance cassettes for selection of yeast transformants. Cas9 protein bound to the gRNA induced double-strand break in the genomic DNA and the integration cassette was introduced into the given site by homologous recombination (Figure S2).

Primers, synthetic DNA, BioBricks, and plasmids used in this study are listed in Tables S1, S2, S3, and S4, respectively. BioBricks were amplified by PCR using Phusion U polymerase (Thermo Fisher Scientific) with the following thermal program: 98°C for 5 min, 30 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 30 s kb⁻¹), and 72°C for 4 min. After DNA electrophoresis on 1% agarose gel, BioBricks were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The integration vectors were digested with FastDigest *SfaI* (Thermo Fisher Scientific) and nicked with *Nb.BsmI* (New England BioLabs). BioBricks with compatible overhangs and nicked integration vectors were assembled and transformed into *Escherichia coli* strain DH5 α via Uracil-Specific Excision Reaction (USER) cloning. Clones containing the correct assembly were verified by PCR and Sanger sequencing.

2.2 | Strain construction

All strains constructed in this study are derived from the *Y. lipolytica* strain ST6629, described in Holkenbrink et al.^[16] The strain expresses *cas9* gene from *Streptococcus pyogenes*. The gene is codon-optimized for *Y. lipolytica* and is integrated into *KU70* locus, effectively inactivating *KU70* gene involved in DNA repair via the non-homologous end joining pathway. The strain hence has an increased rate of DNA repair via homologous recombination, which facilitates targeted genome editing. Additionally, this strain has modifications related to decreased degradation of fatty acids/alcohols and an increased pool of fatty acyl-CoAs. *Y. lipolytica* strain ST4840, which was obtained from Agricultural Research Service (NRRL, USA), served as a source for genomic DNA (gDNA). gDNA was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The entire list of the strains is provided in Table S5.

Yeast transformations were performed as described in Holkenbrink et al.^[19] with the modifications described below. *Y. lipolytica* strains were streaked on Yeast Peptone Dextrose (YPD) plates (20 g L⁻¹ glucose, 10 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, 15 g L⁻¹ agar) and grown for 24 h at 28°C. A small patch of biomass was taken with an inoculation loop and re-streaked on YPD plates containing 0.7 g L⁻¹ complete supplement mixture (Formedium). After 24 h incubation at 28°C, the cells were scraped off, resuspended in 1 mL 0.5 M sterile sucrose solution, and centrifuged for 5 min at 3000 × g at room temperature. The supernatant was discarded, the cells resuspended in 0.5 M sucrose solution, and a volume corresponding to OD₆₀₀ 2.6 was transferred to a sterile tube for transformation. Tubes were centrifuged for 5 min at 3000 × g at room temperature, and the supernatant was removed. 500–1000 ng of gRNA plasmid and integration plasmid, which previously was linearized with SmaI (Thermo Fisher Scientific) and gel-purified prior transformation, were added to the pellet. The mixture was resuspended in 100 µL of transformation mix (Table S6) and incubated at 39°C for 1 h. After the heat shock, the tubes were centrifuged, the supernatant removed, and the pellet was resuspended in 500 µL liquid YPD medium. The cells were incubated for 2 h at 28°C 300 rpm shaking. The pellet was again collected by centrifugation, resuspended in 100 µL of 0.5 M sterile sucrose solution, and plated on YPD plates containing Hygromycin B (200 mg L⁻¹) (Carl Roth) or Nourseothricin (250 mg L⁻¹) (Jena Bioscience) for selection. After 2–3 days of incubation at 28°C, single colonies appeared, which were tested for correct integration by PCR using vector-specific primers and primers complementary to genomic loci close to the integration site (Table S1).

2.3 | Small scale cultivations

Y. lipolytica strains were inoculated from a YPD agar plate to an initial OD₆₀₀ of 0.2 into 2.5 mL YPG medium (10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, 40 g L⁻¹ glycerol) in 24 well-plate (EnzyScreen). The plate was incubated at 28°C and 300 rpm for 22 h. The plate was centrifuged at 3500 g for 5 min at 20°C, the medium was removed and

the cells were resuspended in 1.25 mL production medium (50 g L⁻¹ glycerol, 5 g L⁻¹ yeast extract, 4 g L⁻¹ KH₂PO₄, 1.5 g L⁻¹ MgSO₄, 0.2 g L⁻¹ NaCl, 0.265 g L⁻¹ CaCl₂·2H₂O, 2 mL L⁻¹ trace elements solution: 4.5 g L⁻¹ CaCl₂·2H₂O, 4.5 g L⁻¹ ZnSO₄·7H₂O, 3 g L⁻¹ FeSO₄·7H₂O, 1 g L⁻¹ H₃BO₃, 1 g L⁻¹ MnCl₂·4H₂O, 0.4 g L⁻¹ Na₂MoO₄·2H₂O, 0.3 g L⁻¹ CoCl₂·6H₂O, 0.1 g L⁻¹ CuSO₄·5H₂O, 0.1 g L⁻¹ KI, 15 g L⁻¹ EDTA). The plate was incubated at 28°C and 300 rpm for 28 h. Each strain was cultivated in triplicate.

2.4 | Bioreactor fed-batch cultivations

Bioreactor fed-batch mode cultivations were carried out in biological triplicates on initial YPG medium (50 g L⁻¹ glycerol, 10 g L⁻¹ yeast extract (Carl Roth), 10 g L⁻¹ peptone) in controlled stirred bioreactor vessels with 1.0 L total aerated end working volume (Infors Minifors 2 systems, Infors AG, Switzerland) at 28°C. All reactors were equipped with pH and optical dissolved oxygen probes (Hamilton AG, Switzerland) and off-gas analyzers (BlueSens GmbH, Germany). pH was controlled at 4.5 ± 0.1 with automated addition of a 4 M solution of NaOH. The dissolved oxygen control was set at a minimum threshold of 20% with a cascade control by gradually increasing the stirrer speed of two six-blade Rushton turbines and the aeration rate using Eve fermentation control software (Infors AG). The initial aeration rate was set to 1 L min⁻¹, with stirring at 400 rpm. The CO₂ (%) and O₂ (%) in the off-gas were monitored continuously during the fed-batch cultivations. Bioreactor cultures of strain *Y. lipolytica* ST9253 were inoculated from shake flask precultures in the exponential growth phase (250 mL baffled shake flasks, 40 mL cultivation volume, YPG 40 g L⁻¹ glycerol, 10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone) to a starter fermentation volume of 620 mL. After the depletion of the initially supplied glycerol carbon source, the cultures were fed with a nutrient-rich feed solution (700 g L⁻¹ glycerol, 10 g L⁻¹ yeast extract, 10 g L⁻¹ (NH₄)₂SO₄). The composition of the feed solution was set to facilitate the production of the target compounds after an initial biomass buildup phase. Off-line samples were taken regularly to analyze residual glycerol concentration (Megazyme Inc. assay kit), optical density at 600 nm (Genesys photometer, Thermo Fischer Scientific), cell dry weight, and fatty alcohol concentrations.

2.5 | Extraction and derivatization of lipids

1 mL of cultivation broth was sampled into 4 mL glass vials and centrifuged at 3500 g for 5 min at 20°C. The supernatant was removed, and 1 mL of 1 M HCl in anhydrous methanol was added to the pellet. Samples were vortexed for 20 s and incubated at 70°C for 2 h for methanolysis reaction to proceed. Samples were cooled down to room temperature, 1 mL of 1 M NaOH in methanol, 500 µL of saturated NaCl solution in water, 1 mL of hexane, and 10 µL of internal standard (2 g L⁻¹ of methyl nonadecanoate (19:Me) in hexane) were added sequentially. Vials were vortexed for 10 s, centrifuged at 3500 g for 5 min at 20°C, and the upper organic layer was taken for analysis.

2.6 | Extraction of fatty alcohols from fermentation samples

In small-scale screening experiments, 1 mL of cell culture was sampled into 4 mL glass vials and centrifuged at 3500 g, 5 min, 21°C. The supernatant was removed, and 1 mL of ethyl acetate:ethanol mixture (85:15, v/v) was added to the pellet together with 10 µL of internal standard (2 g L⁻¹ of methyl nonadecanoate (19:Me) in ethyl acetate). Vials were vortexed for 20 s and incubated for 1 h, followed by 5 min vortexing. 300 µL of water was added, samples vortexed for 10 s, centrifuged at 3500 g, 5 min, 21°C, and the upper organic layer was taken for analysis.

Samples from bioreactors were processed as follows. 100 µL of cell culture was taken, and the total broth was processed in the same way as for small-scale samples except that the first centrifugation step was omitted.

2.7 | Extraction and work-up of fatty alcohols from bioreactor cultivation for acetylation reaction

The 2.4 L of broth from the bioreactor cultivation was centrifuged at 3000 g for 5 min at room temperature, and the supernatant was separated from the pellet. Fatty alcohols were extracted from the supernatant by adding 2.2 L of ethyl acetate and incubating on a multi-vortexer for 1 h. Organic phase was decanted. To extract fatty alcohols from the pellet, 100 mL of ethyl acetate was added to the pellet, and the mix was shaken on a multi-vortexer for 6 h. The organic phase was decanted, 100 mL of fresh solvent was added to the biomass pellet, and the mixing repeated for 1 h. The organic phase was decanted. All ethyl acetate fractions from the supernatant and pellet extraction were combined and dried with anhydrous sodium sulfate. The solvent was removed in a rotary evaporator.

The extracted crude fatty alcohol mixture was passed through a silica gel column, which was prepared as described in.^[20] The silica was washed with hexane and then subsequently with a gradient of hexane/ethyl acetate at the proportion of 95:5 to 40:60 (% v/v). Purest fractions based on thin-layer chromatography were collected, and the solvent was evaporated in a rotary evaporator.

2.8 | Acetylation of fatty alcohols

The purified fatty alcohol mixture was acetylated by using a 1.2 molar equivalent of acetic anhydride and 0.1 equivalent of sodium acetate anhydrous (equivalents were based on 14:OH). The reaction mixture was allowed to stir for 1 h at 80°C in a water bath and then transferred to a separatory funnel where it was diluted with ethyl acetate and washed with a saturated solution of sodium bicarbonate. The organic phase was dried with anhydrous sodium sulfate, and the solvent evaporated in rotary evaporator.

2.9 | Gas chromatography-mass spectrometry (GS-MS) analysis

Analysis was carried out on an Agilent GC 7820A coupled to a MS 5977B, equipped with a split/spitless injector and a DB-Fatwax UI column (30 m x 250 µm x 0.25 µm). The operation parameters were: 1 µL injection, split ratio 20:1 for FAMES and alcohol samples and 10:1 for acetates, injector temperature 220°C, constant flow 1 mL min⁻¹ of helium, oven ramp 80°C for 1 min, 20°C min⁻¹ to 150°C, then 1°C min⁻¹ to 200°C, then 20°C min⁻¹ to 230°C. MS was scanning between *m/z* 30 and 350. For compound identification, reference standards were purchased from Pherobank, and retention times together with mass spectra were compared to the sample of interest. Quantification was made based on 6 points calibration curves. Visual inspection of chromatograms was performed using MassHunter Qualitative Analysis Navigator B.08.00 (Agilent), while quantification was performed using MS Quantitative Analysis (Quant-My-Way) (Agilent).

2.10 | Electrophysiological responses of male *Ostrinia nubilalis*

Antennal responses of male ECB adults to BioPhe were evaluated by electroantennography (EAG) using a commercially available electroantennographic system (Ockenfels Syntech GmbH, Buchenbach, Germany). The antenna of a virgin two-to-three-day old male adult was excised from the head using micro-scissors and attached between two stainless-steel wire electrodes with the assistance of electrically conductive gel (G008, Fiab, Italy). The base of the antenna was connected to the indifferent electrode, whilst the distal end was connected to the recording electrode after cutting off a few segments of the tip. The signal was amplified 10X by a Universal AC/DC pre-amplifier probe connected to the recording electrode, and the analog signal was amplified and detected with a data acquisition controller (IDAC-4, Ockenfels Syntech GmbH).

Test stimulus was the BioPhe containing 7.2% Z11-14:OAc, according to GC-MS analysis. Commercially available Z11-14:OAc, the major sex pheromone of the *O. nubilalis* Z-race, was used as a reference compound (96% purity, Sigma-Aldrich, Germany). The BioPhe blend, as well as commercially available pheromone compounds, were diluted in pentane. For each stimulus, a 10 µL aliquot of the solution was pipetted to a piece of filter paper (7 x 30 mm, Whatman no. (1), and the solvent was allowed to evaporate before being inserted into a Pasteur pipette for odor delivery. Control stimulus consisted of a clean Pasteur pipette with a filter paper impregnated with solvent (pentane). Stimuli were provided as 0.3 s air puffs into a continuous flow of filtered and humidified air. The airflow, at 25 cm³ s⁻¹ rate, tube diameter 1 cm, was generated by an air stimulus controller (CS-55, Syntech, The Netherlands).

The reference stimulus, consisting of a filter paper carrying 100 ng of Z11-14:OAc, was provided at regular intervals during each recording session. The EAG response to each reference stimulus was defined

as 100%, and all responses to test stimuli between adjacent references were normalized in % relative to the references. At least 1 min for weak and moderate stimuli and 5 min for strong stimuli were allowed between successive stimulations to let the antenna recover. The BioPhe blend was tested at a 100 ng dose (containing ~7 ng Z11-14:OAc), as well as at a 1 μ g dose (i.e., 70 ng Z11-14:OAc), in order to reach an amount of major sex pheromone comparable to the 100 ng dose of the Z11-14:OAc stimulus.

The minor sex pheromone, E11-14:OAc, was also tested, as well as both isomers at a 97:3 Z:E ratio, based on the ratio released from the female moth gland as reported in the literature for ECB Z-race.^[7,21] In addition, considering the high amount of tetradecyl acetate (14:OAc) in the BioPhe blend (~50%), the EAG response to 50 ng of this compound was also evaluated (for consistency reasons 50 ng is approx. the amount of 14:OAc contained in the 100 ng BioPhe test dose). All aforementioned stimuli were tested on a total of 20 antennal preparations.

2.11 | Behavioral bioassays

Behavioral studies were performed in a dark room under red light (2.5–3 lux) at $21 \pm 2^\circ\text{C}$ and $70 \pm 5\%$ relative humidity. Male moths were tested in a wind tunnel as described in.^[22] For odor delivery, a piece of triangular-shaped filter paper (2.5 cm base, 4 cm height) was pipetted with appropriate volume of the test solution and, following solvent evaporation, was hung from the suction hook in the tunnel.

Pheromone standards and BioPhe samples were dissolved in pentane. The major and minor sex pheromone components Z11-14:OAc and E11-14:OAc, respectively, were mixed at a 97:3 ratio and were used as a positive control at a 50 μ g dose, which, through preliminary testing, was concluded to be the minimum amount eliciting positive courtship behavior, that is, source-oriented flight pattern, abdominal hair pencils display and abdomen curling upon contact. Additionally, to evaluate potential antagonistic effects of BioPhe blend contaminants on the males' responses, 14:OAc (most prominent component ~50% of BioPhe blend) and Z9-16:OAc (~9% of BioPhe blend) have been tested.

A dose of 700 μ g BioPhe was used in the bioassays (containing 50 μ g Z11-14:OAc and 350 μ g 14:OAc) and a dose of 350 μ g 14:OAc was added to 50 μ g pheromone standard (97:3) to emulate abundance and ratio in the BioPhe. Also a dose of 50 μ g pheromone standard (97:3) with the addition of 4.5 μ g of Z9-16:OAc (9% present in the BioPhe blend) was tested, as to our knowledge the role of Z9-16:OAc has not been investigated as antagonist to male response. Other components of BioPhe, such as Z9-14:OAc have already been reported in the literature as antagonist to male response.^[23,24] Behavioral tests were conducted between the 3rd and 5th h in the scotophase.^[24,25] 2 h before testing, 2–5 days old male moths were transferred in individual 400 mL clear plastic cups covered with perforated lids and left in the conditions of the dark room to acclimate. For each test, the odor source was attached to the tunnel ceiling, and after 30 s, an individual male was released in the tunnel, and its behavior was recorded for 10 min.

The following sequences of behavior were recorded: close approach (less than 10 cm) and contact with the source (landing). In addition, the approach and landing steps were further analyzed into four grades, and each grade was assigned its corresponding value. For the landing step, grades were discriminated as follows: grade (1) brief contact with the source (no landing), grade (2) landing on source for 1–2 s, grade (3) landing on source for more than 2 s, and grade (4) landing on source accompanied by hairpencils display and/or abdomen curling (copulation attempt). Similarly, for the approach step were discriminated as: grade (1) close approach to source for 1–2 s, grade (2) close approach with zig-zag patterned flight for >2 s, grade (3) close approach with zig-zag patterned flight and hairpencils display for 1–2 s, grade (4) close approach with zig-zag patterned flight and hairpencils display for >2 s.

Between treatments, the flight tunnel inner walls and suction hook were wiped with acetone and left to aerate for 10 min before the next treatment. For each treatment, 30 males were tested. Males were used once and were discarded after testing.

2.12 | Statistical analyses

The electrophysiological and behavioral data were subjected to analysis of variance (ANOVA) (SAS Institute, 2000). The means of electrophysiological data and the response grades were separated using the Tukey (honestly significant difference, HSD) test at $p = 0.05$.

3 | RESULTS

3.1 | Screening of fatty acyl-CoA desaturases

In *O. nubilalis*, a $\Delta 11$ desaturase acts on myristoyl-CoA (14:CoA), which in turn is generated by β -oxidation of the abundant fatty acid synthesis product hexadecanoyl-CoA (16:CoA), and generates a mixture of E/Z11-14:CoA.^[6,26] In *Y. lipolytica*, myristic acid is a minor component of the total fatty acid pool, comprising only about 0.25% of the total fatty acids.^[27] To increase the supply of 14:CoA in *Y. lipolytica*, we substituted isoleucine 1220 of the fatty acid synthase ketoacyl synthase domain to phenylalanine (Fas2p^{I1220F}). This mutation was proposed to hinder the binding of longer acyl-CoAs in the active site tunnel of the FAS complex and was reported to result in the production of shorter chain acyl-CoAs.^[27] The mutation has already been proven to be effective in the production of Z9-14:OH, where it increased the titer 15-fold.^[16] In this study, we introduced the FAS mutation into *Y. lipolytica* strain ST6629, which was previously developed for decreased degradation of fatty alcohols and decreased storage lipid synthesis.^[16] The resulting ST7982 showed 8.4-fold increase in myristic acid production compared to the parental strain (Figure S3).

Next, seven $\Delta 11$ desaturases with previously documented E/Z11-14:CoA activity were tested in strain ST7982 (Figure 1, Figure S4). These were the desaturase Lbo_PPTQ from the grapevine moth *Lobesia botrana*,^[28] Onu11 from the European corn borer *O. nubilalis*,^[26] EpoE11 from the light brown apple moth *Epiphyas postvittana*,^[29]

FIGURE 1 Characterization of $\Delta 11$ desaturases. (A) Production of *E/Z*11-14:acid and (B) Z11-16:acid by *Y. lipolytica* containing different moth fatty acyl-CoA desaturases. Quantification of fatty acids was done after derivatization into methyl esters. Results are obtained from three biological replicates. Error bars indicate standard deviations

CroZ11 from the oblique banded leafroller moth *Choristoneura rosaceana*,^[30] CpaE11 from the spotted fireworm moth *Choristoneura parallela*,^[31] Hzead11 from the corn earworm *H. zea*,^[32] and Msexad11 from the tobacco hornworm *Manduca sexta*.^[33] The resulting strains were cultivated, their lipids methanolized into FAMES, and analyzed on GC-MS. Lbo_PPTQ expression resulted in the highest content of Z11-14:acid. The Z/E isomer ratio was 9:2. For the strain expressing the OnuE11 desaturase, no new compounds could be detected, indicating that this desaturase is most likely not well expressed or active in *Y. lipolytica*. Likewise, no activity was detected for the desaturases EpoE11 and CpaE11, which were previously reported to produce E11-14:acid in another yeast, *Saccharomyces cerevisiae*, albeit in minuscule amounts.^[29,31] The strain expressing CroZ11 produced a mixture of *E/Z*11-14:acid with an excess of the Z isomer; however, the amounts of both isomers were lower compared to the strain expressing Lbo_PPTQ. The desaturase Hzead11 appeared to be a promiscuous $\Delta 11$ desaturase with a high preference towards 16:acid (Figure 1). Apart from Z11-16:acid, we also detected *E/Z*11-14:acid and some other unsatu-

rated fatty acids, which remain to be identified. (Figure S4). The Msexad11 desaturase-expressing strain produced *E/Z*11-14:acid in ratio 1:1, where the amount of the E isomer was the highest among the tested desaturases.

In summary, Lbo_PPTQ resulted in the highest content and purity of Z11-14:acid and was therefore chosen for establishing the biosynthetic pathway towards ECB pheromone in *Y. lipolytica*.

3.2 | Screening of fatty acyl-CoA reductases

We further screened four FARs to identify the reductase with the highest activity and selectivity towards Z11-14:acid. The four reductases were: HarFAR from cotton bollworm *H. armigera*,^[34] SlitpgFARII from African cotton leafworm *Spodoptera littoralis*,^[35] and two reductases from *O. nubilalis*, OnuFAR_E and OnuFAR_Z.^[36] First, we expressed the four reductases individually in the strain ST7982. The resulting strains were cultivated as follows: first, the cells were grown for

FIGURE 2 Characterization of reductases. (A) Profiles of *E/Z*11-14:OH produced by *Y. lipolytica* containing different reductases when cultivation media was supplemented with a mixture containing equal amounts (500 mg L^{-1}) of *E/Z*11-14:Me. (B) Fatty alcohol profiles of *Y. lipolytica* strains expressing Lbo_PPTQ together with different reductases. The control strain has no reductase introduced. Results are obtained from three biological replicates. Error bars indicate standard deviations

biomass propagation for 22 h, followed by a production phase in media supplemented with equal amounts of *E* and Z11-14:Me (500 mg L^{-1} of each isomer) lasting for 28 h. Fatty alcohols were extracted from the broth and quantified on GC-MS. HarFAR and SlitpgFARII expressing strains produced around 40 and 14 mg L^{-1} respectively of each isomer, E11-14:OH and Z11-14:OH, from the corresponding methyl esters, and did thus not exhibit a preference towards any isomer. OnuFAR_E produced very low amounts ($<1 \text{ mg L}^{-1}$) of *E/Z*11-14:OH, which were below the quantification limit. However, manual inspection of the chromatograms showed that this reductase had a bias towards *E* isomer, as expected. No fatty alcohols were detected in the strain expressing OnuFAR_Z (Figure 2, Figure S5).

Next, for de novo production of Z11-14:OH, we expressed the four reductases in the strain ST9992, which contained Fas2p^{1220F} mutation and expressed desaturase Lbo_PPTQ. Interestingly, while HarFAR resulted in 2.8-fold higher product titer than SlitpgFARII in the feeding assay described above, in this experiment with de novo production of

unsaturated fatty acid, a higher Z11-14:OH titer was obtained in strain with SlitpgFARII, $37.7 \pm 2.6 \text{ mg L}^{-1}$ versus $29.2 \pm 1.6 \text{ mg L}^{-1}$ for HarFAR strain. SlitpgFARII and HarFAR likely have different kinetic parameters, such as substrate affinity, maximum velocity, and potential inhibition by substrate or product. The intracellular concentrations of substrate and product are likely different under conditions when the substrates are taken up from the medium or biosynthesized internally and hence one condition may benefit SlitpgFARII and *vice versa*.

However, the strain expressing SlitpgFARII additionally produced $402.5 \pm 17.3 \text{ mg L}^{-1}$ of 14:OH and $54.7 \pm 4.0 \text{ mg L}^{-1}$ of Z9-16:OH, which is 2.2- and 1.8-fold higher compared to HarFAR, respectively (Figure 2B). Very high activity towards tetradecanoate of SlitpgFARII was also observed by Antony et al.,^[35] who reported that among a wide variety of saturated, mono-, and di-unsaturated fatty acyl substrates, tetradecanoate was converted into alcohol most efficiently.

We chose HarFAR for further strain engineering due to its high activity and better selectivity towards Z11-14:CoA than SlitpgFARII.

FIGURE 3 Production of Z11-14:OH in small scale cultivations and in 1 L fed-batch fermentation. (A) Metabolic engineering of *Y. lipolytica* for improved production of Z11-14:OH. High myristate producing strain ST7982, containing mutation in α -subunit of the fatty acyl synthase complex (Fas2p^{L1220F}) was used as background strain. Results are obtained from three biological replicates. (B) Fed-batch fermentation results of ST9253. Data obtained for optical density (OD), cell dry weight (DW), glycerol, and Z11-14:OH concentration are represented as averages from three bioreactors and standard deviations are calculated which are shown as error bars

3.3 | Metabolic engineering for improved production of Z11-14:OH

After selecting Lbo_PPTQ and HarFAR as the biosynthetic enzymes for Z11-14:OH production, we proceeded with further metabolic engineering strategies to increase the product titer and specific yield (Figure 3A). First, we introduced two gene copies of HarFAR into the high myristate producing strain ST7982 to enable biosynthesis of fatty alcohols. The resulting strain ST8225 produced 318.3 ± 52.4 mg L⁻¹ total fatty alcohols, among which 14:OH constituted 67.5% and was the most abundant fatty alcohol. In order to produce Z11-14:OH,

we expressed Lbo_PPTQ in ST8225 leading to ST8373, which gave 21.5 ± 2.2 mg L⁻¹ of the target compound, with a specific yield of 0.8 mg Z11-14:OH per g DW. Compared to the parental strain, this strain produced 46.7% more total fatty alcohols. Then we introduced a second copy of Lbo_PPTQ and this led to a further 3-fold improvement of Z11-14:OH titer and specific yield.

We anticipated that further improvement could be achieved by overexpressing native FAS1 from *Y. lipolytica*. Previous studies in *S. cerevisiae* showed that the activity of the FAS complex is enhanced when the copy number of FAS1 gene is increased.^[37] Indeed, overexpression of FAS1 (strain ST9253) resulted in 60.7% improvement in the total

FIGURE 4 GC-MS chromatogram representing fatty alcohol sample before (above) and after (below) acetylation. Numbers in red represent the amount of each fatty acetate ($\text{mg} \pm \text{SD}$) in the final sample. Measurements were performed in technical triplicates.

fatty alcohol titer and increased production of Z11-14:OH by 54.7%, resulting in $93.9 \pm 11.7 \text{ mg L}^{-1}$ of Z11-14:OH in small-scale cultivation. The increase of Z11-14:OH specific yield was 34%.

The introduced genome edits did not significantly affect the growth of the yeast strains. The final biomass concentrations were similar, ranging from 26.1 to 29.5 g DW L^{-1} .

3.4 | Production of Z11-14:OAc by fed-batch fermentation and alcohol acetylation

The engineered strain ST9253 (MATa *ku70Δ Cas9 hfd4Δ hfd1Δ pex10Δ fao1Δ hfd2Δ hfd3Δ GPAT_100bpPr Fas2p^{1220F} 2xLbo_PPTQ 2xHarFAR ↑FAS1*) was cultivated in controlled bioreactors in fed-batch mode. The initial batch phase served as a biomass propagation phase. After the depletion of the initial carbon source supplied in the batch phase, the composition of the fed-batch feed solution was set to facilitate the production of fatty alcohols. In the first 42 h of fermentation, the accumulation of biomass was targeted, and stationary phase was reached after 49 h (Figure 3B). The start of the fed-batch facilitated a shift towards nitrogen-limited conditions and thereby transition from the biomass build-up phase to production of fatty alcohols.

In the fatty alcohol production phase of the fermentation process, we observed a constant increase in specific yield. The specific produc-

tion yield of Z11-14:OH peaked at the very end of fermentation and reached $6.7 \text{ mg product g}^{-1} \text{ DW}$. At this time point, the titer of Z11-14:OH was $188.1 \pm 13.4 \text{ mg L}^{-1}$, while the cell dry weight reached $28.2 \pm 0.7 \text{ g L}^{-1}$ (Figure 3B). The specific yield in bioreactors was 2-fold higher than what was obtained in small-scale cultivation. This could be explained by improved aeration, pH control, and/or glycerol feed control. The titer of 14:OH reached $1,350 \pm 188 \text{ mg L}^{-1}$. It was the most abundant fatty alcohol in the fermentation broth.

After 120 h of fed-batch fermentation, fatty alcohols were extracted with organic solvent and purified on a silica column. The resulting fatty alcohol mixture containing approximately 250 mg of Z11-14:OH was acetylated with acetic acid anhydride. It resulted in full conversion, where $320.1 \pm 13.4 \text{ mg}$ of Z11-14:OAc was obtained, and no traces of alcohols were left at the end of the reaction (Figure 4). The resulting product is referred to as BioPhe, for biologically-derived pheromone.

3.5 | Electrophysiological responses of male *Ostrinia nubilalis*

We studied electrophysiological responses of male *O. nubilalis* insect antennas to pure chemical pheromone components, BioPhe product, and several blends. As expected, EAG responses of ECB Z-race male moths were higher for 100 ng Z11-14:OAc than for E11-14:OAc

FIGURE 5 Performance of biologically-derived *O. nubilalis* sex pheromone. (A) Electrophysiological responses of male *O. nubilalis* antennae to biologically-derived pheromone blend (BioPhe), standard compounds (Z11-14:OAc, E11-14:OAc and 14:OAc), and mixtures of the standard compounds. Response to 100 ng of Z11-14:OAc was defined as 100%, and all responses normalized in % relative to this reference. Means followed by the same letter are not significantly different ($p > 0.5$, Tukey's studentized range [HSD] test, $F = 51.650$, $df = 5$, $p = 0.000$). (B) Behavioral responses of *O. nubilalis* males to yeast-derived pheromone blend (BioPhe), standard compounds (Z11-14:OAc, E11-14:OAc, 14:OAc) and mixtures of the standard compounds. Phe 97:3 represents pheromone composition originally found in *O. nubilalis* Z-race and is generated from standard compounds Z11-14:OAc, E11-14:OAc in ratio 97:3. Means followed by the same letter are not significantly different ($p > 0.05$, Tukey's studentized range [HSD] test). Approach, $F = 4.579$ $df = 3$, $p = 0.005$, Landing, $F = 5.899$, $df = 3$, $p = 0.001$

(Figure 5A). Also, the 97:3 (Z11-14:OAc/E11-14:OAc) mixture at 100 ng dose elicited the same magnitude of response as the major pheromone component (Z11-14:OAc). The biologically-derived pheromone BioPhe at 100 ng elicited a significantly lower response, as expected, because Z11-14:OAc is present just at 7.2%, *ergo*, only about 7 ng of Z11-14:OAc were directed to the antenna. When the quantity of BioPhe was increased 10-fold, the EAG response was also increased. In this treatment, the amount of Z11-14:OAc present in the biologically-derived mixture was of the same order of magnitude as the reference (i.e., ~70 ng). However, it should be noted, that some of the other BioPhe components may also contribute to the augmented response recorded. In this context, we tested the effect of 14:OAc, given the fact that it comprises about 50% of BioPhe. At 50 ng dose, analogous to that contained in 100 ng BioPhe, it was

clearly detected by the antenna but to a much lesser degree than the reference compound.

3.6 | Behavioral bioassays

Male ECB moths responded with approach and landing to 50 μg of the pheromone blend corresponding to the Z-race (Figure 5B). As BioPhe contains just 7.2% Z11-14:OAc, a 700 μg stimulus of BioPhe was tested in lab bioassays (containing approx. 50 μg Z11-14:OAc). Significantly fewer source-oriented approaches and fewer landings were observed compared to the response elicited by the optimal Z-race pheromone, indicating the presence of compound(s) interfering with the insects' anticipated behavior.

In the BioPhe sample 14:OAc comprises almost 50% of the total amount. To emulate a BioPhe sample, 350 μg 14:OAc were added to the 50 μg pheromone blend (97:3). In this case, males scored significantly lower in both approach and landing. This is an indication that 14:OAc at this dose interferes with the precopulatory behavior of *O. nubilalis*.

Similarly, when 4.5 μg of Z9-16:OAc was added to the 50 μg pheromone blend (97:3), significantly fewer landings were recorded, but the frequency of approaches did not differ significantly from what was observed in response to the native pheromone.

4 | DISCUSSION

We have evaluated the performance of seven FADs and four FARs, which are involved in insect sex pheromone biosynthesis, to establish the production of Z11-14:OH in *Y. lipolytica*. Among FADs, Lbo_PPTQ showed the highest production of (Z)-11-tetradecenoate and was selected for further strain engineering. Several desaturases, namely, Onu11, EpoE11, and CpaE11, were not active. The desaturase Onu11 was previously assayed in other recombinant hosts, such as *S. cerevisiae* and *Nicotiana benthamiana* with divergent results. In *S. cerevisiae*, it generated E/Z11-14:acid in ratio 4:5 and also produced Z11-16:acid,^[26] while no activity of this enzyme was observed in *N. benthamiana*.^[14] The other two desaturases that were non-functional in our study, EpoE11 and CpaE11, were reported to act on 14:acid and produce E11-14:acid in *S. cerevisiae*, but the amounts were very low.^[29,31] One possible explanation is that the non-functional desaturases were poorly expressed or misfolded. A recent study by Buček et al. showed that different moth desaturases could have different expression levels.^[38] Codon-optimization could also have been suboptimal.^[39]

After examining FADs, we screened several FARs for increased specificity towards fatty acids with C14 chain length. Previously, HarFAR was shown to prefer (Z)-9-tetradecenoate over (Z)-11-hexadecenoate and (Z)-9-hexadecenoate.^[34] We hypothesized that this reductase should also be able to act on (E/Z)-11-tetradecenoate, which is structurally similar to (Z)-9-tetradecenoate. Indeed, a feeding experiment and co-expression with Lbo_PPTQ confirmed that HarFAR could produce E/Z11-14:OH from corresponding acids. (E/Z)-11-tetradecenoate was also converted into alcohol by *Y. lipolytica* expressing SlitpgFARII. This FAR previously produced the highest amounts of Z11-14:OH among the four tested reductases from *Spodoptera* spp.^[35] Reductases OnuFAR_E and OnuFAR_Z were shown to be selective for E and Z isomers of 11-tetradecenoate, respectively,^[36] however, in our study, OnuFAR_E showed little and OnuFAR_Z no activity. The same results were obtained when these enzymes were screened in the plant *N. benthamiana*.^[14] One possible explanation for the lack of functionality of these enzymes in yeast and plant expression hosts could be incorrect folding of the reductases in the ER membrane, which has a different lipid composition in yeasts and plants than in insects.^[40–43]

In order to improve the production of Z11-14:OH, we employed metabolic engineering strategies, such as integration of multiple copies of genes and enhancement of precursor supply by overexpression of

FAS1 subunit of *Y. lipolytica*. In small-scale cultivations of the engineered yeast strain, we achieved $93.9 \pm 11.7 \text{ mg L}^{-1}$ of Z11-14:OH. On the way of building the production strain (ST9253), we observed that introduction of desaturase Lbo_PPTQ not only contributed to the biosynthesis of Z11-14:OH, but it also improved the total fatty alcohol titer. It was previously shown that overexpression of the native OLE1 desaturase increased fatty acid biosynthesis in general, likely because the fatty acid synthase complex FAS is less inhibited by unsaturated fatty acyl-CoAs than by saturated fatty acyl-CoAs.^[44–46]

For collection of pheromone for EAG and behavioral tests, the engineered strain was fermented in controlled 1 L bioreactors in fed-batch mode. We used glycerol as the carbon source with a high carbon-to-nitrogen ratio to favor fatty alcohol production.^[47–52] We obtained $188.1 \pm 13.4 \text{ mg L}^{-1}$ of Z11-14:OH. The strain also produced large amounts (over 1.3 g L^{-1}) of the saturated by-product 14:OH, indicating a significant limitation of the desaturation step. This may be improved by further strain engineering and fermentation optimization in the future. To our knowledge, this is the first study showing the production of Z11-14:OH in a microbial host. Previously this pheromone precursor was recombinantly synthesized in plant *N. benthamiana* yielding 14 μg from 1 g leaf tissue.^[14] While *Y. lipolytica* has been engineered for the production of fatty alcohols in multiple other studies, the common products are naturally unsaturated or saturated fatty alcohols.^[48,49,53–56]

To convert the fermented alcohol into acetate, which is the active sex pheromone component of *O. nubilalis*, a chemical acetylation step was performed, which resulted in full conversion and yielded $320 \pm 13.4 \text{ mg}$ of Z11-14:OAc. Interestingly, based on the current knowledge about insect pheromone biosynthesis, this reaction should be catalyzed by acyltransferases in insects.^[6,57] However, until now, no enzymes from moths catalyzing this reaction have been found even though some gene candidates have been proposed and tested.^[58] To achieve in vivo production of fatty alcohol acetates, acetyltransferases from other organisms, such as Atf1p from *S. cerevisiae* or EaDAC from the burning bush *Euonymus alatus*, could be expressed.^[14,59] We previously demonstrated that (Z)-9-tetradecenyl acetate can be synthesized directly in *S. cerevisiae* strain overexpressing ATF1 gene (WO/2018/109167).

The yeast-derived pheromone blend caused a response of *O. nubilalis* males in electroantennogram experiments similar to what could be expected based on the responses to synthetic pheromone compounds. Furthermore, the blend was attractive to insects in behavioral bioassays in a wind tunnel, although to a lower level than the chemically synthesized pheromone blend. In addition, full precopulatory behavior was observed less often. In order to induce a complete the ECB precopulatory behavior, a higher purity or more optimal Z:E isomer ratio may be required. In our study, Z-race of ECB was explored in the EAG and behavioral assays. The strain uses a 97:3 blend of Z11-14:OAc to E11-14:OAc as sex pheromone.^[6] In the yeast-derived pheromone sample, the ratio of these compounds was 89:11, that is, E-isomer was in excess in comparison to the optimal ratio. Potentially, this ratio could be improved by engineering the stereospecificity of the desaturase.^[38,60] Notably, reduced precopulatory behavior is not necessarily a hindrance

and may even be a benefit for mating disruption. Therefore, activity studies in the field are warranted.

The effectiveness of managing ECB by mating disruption was previously proven in the USA, where pheromone-dispenser-treated areas had noticeably lower levels of mating compared to untreated control areas.^[61] Infestation of many other Lepidoptera pests, including the codling moth *Cydia pomonella* and the grapevine moth *L. botrana*, is also successfully reduced by mating disruption and has been applied commercially for over 20 years.^[10] The cost of mating disruption is higher than insecticide treatment,^[62] but it is used for high-value fruits due to other advantages, such as absence of toxic residues. For lower-value crops, for example, maize, mating disruption using chemical pheromones is too expensive.^[63] Prices of chemically synthesized pheromones range from several hundred to several thousand dollars per kg, therefore reduction of production costs is one of the major driving forces to create efficient microbial or plant factories for biosynthesis of these compounds.^[17,63,64] Economic viability of fermentation-based processes for production of specialty oleochemicals has already been shown, where compounds such as omega-3 fatty acids have been produced at commercially relevant titers reaching $\sim 10 \text{ g L}^{-1}$.^[65,66]

In summary, we have successfully employed yeast *Y. lipolytica* for production of *O. nubilalis* sex pheromone precursor Z11-14:OH and showed that the resulting yeast-derived pheromone was biologically active in modulating the behavior of *O. nubilalis* males.

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AUTHOR CONTRIBUTIONS

Karolis Petkevicius: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; validation; visualization; writing-original draft; writing-review & editing. Eleni Koutsoumpeli: Formal analysis; investigation; methodology; writing-review & editing. Petri Christina Betsi: Formal analysis; investigation; methodology; writing-review & editing. Bao-Jian Ding: Resources; validation; writing-review & editing. Kanchana Rueksomtawin Kildegaard: Investigation; methodology; writing-review & editing. Hilbert Jensen: Data curation; investigation; methodology; writing-review & editing.

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CONFLICT OF INTEREST

Irina Borodina, Carina Holkenbrink, Christer Löfstedt, Bao-Jian Ding are co-inventors on patent applications WO2016207339, WO2018109167, and WO2018109163. Karolis Petkevicius, Kanchana Rueksomtawin Kildegaard, Hilbert Jensen, Nora Mezo, Andrea Mazziotto, Anders Gabrielsson, Christina Sinkwitz, Bettina Lorantfy, Carina Holkenbrink, Irina Borodina have financial interest in BioPhero ApS. Dimitris Raptopoulos has financial interest in Novagrica SA.

DATA AVAILABILITY STATEMENT

Not applicable.

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REFERENCES

1. Zalucki, M. P., Shabbir, A., Silva, R., Adamson, D., Shu-Sheng, L., & Furlong, M. J. (2012). Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): Just how long is a piece of string? *Journal of Economic Entomology*, 4, 1115–1129.
2. Kassie, M., Wossen, T., Groote, H. D., Tefera, T., Sevgan, S., & Balew, S. (2020). Economic impacts of fall armyworm and its management strategies: Evidence from southern Ethiopia. *European Review of Agricultural Economics*, 47, 1473–1501.
3. Reay-Jones, F. P. F., Wilson, L. T., Reagan, T. E., Legendre, B. L., & Way, M. O. (2008). Predicting economic losses from the continued spread of the Mexican rice borer (Lepidoptera: Crambidae). *Journal of Economic Entomology*, 101, 237–250.
4. Franeta, F., Mikić, S., Milovac, Ž., Mitrović, B., Indić, D., & Vuković, S. (2019). Maize defence mechanisms against the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae). *International Journal of Pest Management*, 65, 23–32.
5. Siegfried, B. D., & Hellmich, R. L. (2012). Understanding successful resistance management. The European corn borer and Bt corn in the United States. *GM Crops & Food*, 3, 184–193.
6. Lassance, J. M. (2016). The European corn borer *Ostrinia nubilalis*: Exotic pest and model system to study pheromone evolution and speciation. In J.D. Allison, & R.T. Cardé (Eds.), *Pheromone Communication*

- in *Moths: Evolution, Behavior, and Application*. University of California Press, 233–244.
7. Kochansky, J., Cardé, R. T., Liebherr, J., & Roelofs, W. L. (1975). Sex pheromone of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), in New York. *Journal of Chemical Ecology*, 1, 225–231.
 8. Klun, J. A., COOPERATORS. (1975). Insect sex pheromones: Intraspecific pheromonal variability of *Ostrinia nubilalis* in North America and Europe. *Environmental Entomology*, 4, 891–894.
 9. Hummel, H. E., Langner, S., & Breuer, M. (2015). Lobesia mating disruption by pheromone mesofiber dispensers. *Acta Fytotechnica et Zootechnica*, 18, 151–153.
 10. Ioriatti, C., & Lucchi, A. (2016) Semiochemical strategies for tortricid moth control in apple orchards and vineyards in Italy. *Journal of Chemical Ecology*, 42, 571–583.
 11. Turczel, G., Kovács, E., Merza, G., Coish, P., Anastas, P. T., & Tuba, R. (2018). Synthesis of semiochemicals via olefin metathesis. *ACS Sustainable Chemistry & Engineering*, 7, 33–48.
 12. Chi, D. T., & Vang, L. V. (2018). Synthesis and field examinations of the sex pheromone of the diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) in the Mekong Delta of Vietnam. *Can Tho University Journal of Science*, 54, 1–6.F.
 13. Yadav, J. S., & Reddy, E. J. (2000). Synthesis of (3E,5Z)-3,5-Dodecadienylacetate, the sex pheromone of *Phtheochroa cranaodes* (Lepidoptera: Tortricidae). *Bioscience, Biotechnology, and Biochemistry*, 64, 1726–1728.
 14. Ding, B. J., Hofvander, P., Wang, H. L., Durrett, T. P., Stymne, S., & Löfstedt, C. (2014). A plant factory for moth pheromone production. *Nature Communications*, 5, 3353.
 15. Hagström, Å. K., Wang, H. L., Liénard, M. A., Lassance, J. M., Johansson, T., & Löfstedt, C. (2013). A moth pheromone brewery: Production of (Z)-11-hexadecenol by heterologous co-expression of two biosynthetic genes from a noctuid moth in a yeast cell factory. *Microbial Cell Factories*, 12, 125.
 16. Holkenbrink, C., Ding, B. J., Wang, H. L., Dam, M. I., Petkevicius, K., Kildegaard, K. R., Wenning, L., Sinkwitz, C., Lorántfy, B., Koutsoumpeli, E., França, L., Pires, M., Bernardi, C., Urrutia, W., Mafra-Neto, A., Ferreira, B. S., Raptopoulos, D., Konstantopoulou, M., Löfstedt, C., & Borodina, I. (2020). Production of moth sex pheromones for pest control by yeast fermentation. *Metabolic Engineering*, 62, 312–321.
 17. Petkevicius, K., Löfstedt, C., & Borodina, I. (2020). Insect sex pheromone production in yeasts and plants. *Current Opinion in Biotechnology*, 65, 259–267.
 18. Löfstedt, C., & Xia, Y. H. (2021). 3 – Biological production of insect pheromones in cell and plant factories. In J. Gary, G. J. Blomquist, & R. J. Vogt (Eds.), *Insect Pheromone Biochemistry and Molecular Biology* (Second Edition) (pp. 89–121). Academic Press.
 19. Holkenbrink, C., Dam, M. I., Kildegaard, K. R., Beder, J., Dahlin, J., Belda, D. D., & Borodina, I. (2018). EasyCloneYALI: CRISPR/Cas9-based synthetic toolbox for engineering of the Yeast *Yarrowia lipolytica*. *Biotechnology Journal*, 13, 1700543.
 20. Pedersen, D. S., & Rosenbohm, C. (2001). Dry column vacuum chromatography. *Synthesis*, 16, 2431–2434.
 21. Klun, J. A., Chapman, O. L., Mattes, K. C., Wojtkowski, P. W., Beroza, M., & Sonnet, P. E. (1973). Insect sex pheromones: Minor amount of opposite geometrical isomer critical to attraction. *Science*, 181, 661–663.
 22. Krokos, F. D., Ameline, A., Bau, J., Sans, A., Konstantopoulou, M., Frérot, B., Guerrero, A., Eizaguirre, M., Malosse, C., Etchepare, O., Albajes, R., & Mazomeno, B. E. (2002). Comparative studies of female sex pheromone components and male response of the corn stalk borer *Sesamia nonagrioides* in three different populations. *Journal of Chemical Ecology*, 28, 1463–1472.
 23. Gemenio, C., Sans, A., López, C., Albajes, R., & Eizaguirre, M. (2006). Pheromone antagonism in the European corn borer moth *Ostrinia nubilalis*. *Journal of Chemical Ecology*, 32, 1071–1084.
 24. Glover, T. J., Perez, N., & Roelofs, W. L. (1989). Comparative analysis of sex-pheromone-response antagonists in three races of European corn borer. *Journal of Chemical Ecology*, 15, 863–873.
 25. Roelofs, W., Glover, T., Tang, X. H., Sreng, I., Robbins, P., Eckenrode, C., Löfstedt, C., Hansson, B. S., & Bengtsson, B. O. (1987). Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 7585–7589.
 26. Roelofs, W. L., Liu, W., Hao, G., Jiao, H., Rooney, A. P., & Linn, C. E. J. (2002). Evolution of moth sex pheromones via ancestral genes. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 13621–13626.
 27. Rigouin, C., Gueroult, M., Croux, C., Dubois, G., Borsenberger, V., Barbe, S., & Bordes, F. (2017). Production of medium chain fatty acids by *Yarrowia lipolytica*: combining molecular design and TALEN to engineer the fatty acid synthase. *ACS Synthetic Biology*, 6, 1870–1879.
 28. Ding, B. J., Xia, Y. H., Wang, H. L., Andersson, F., Hedenström, E., Gross, J., Löfstedt, C. (2021). Biosynthesis of the sex pheromone (E,Z)-7,9-dodecadienyl acetate in the European grapevine moth, *Lobesia botrana*, involving $\Delta 11$ desaturation and an elusive $\Delta 7$ desaturase. *Journal of Chemical Ecology* (in press). <https://doi.org/10.1007/s10886-021-01252-3>.
 29. Liu, W., Jiao, H., Murray, N. C., O'Connor, M., & Roelofs, W. L. (2002). Gene characterized for membrane desaturase that produces (E)-11 isomers of mono- and diunsaturated fatty acids. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 620–624.
 30. Hao, G., O'Connor, M., Liu, W., & Roelofs, W. L. (2002). Characterization of Z/E11- and Z9-desaturases from the obliquebanded leafroller moth, *Choristoneura rosaceana*. *Journal of Insect Science (Online)*, 2, 26.
 31. Liu, W., Rooney, A. P., Xue, B., & Roelofs, W. L. (2004). Desaturases from the spotted fireworm moth (*Choristoneura parallela*) shed light on the evolutionary origins of novel moth sex pheromone desaturases. *Gene*, 342, 303–311.
 32. Rosenfield, C. L., You, K. M., Marsella-Herrick, P., Roelofs, W. L., & Knipple, D. C. (2001). Structural and functional conservation and divergence among acyl-CoA desaturases of two noctuid species, the corn earworm, *Helicoverpa zea*, and the cabbage looper, *Trichoplusia ni*. *Insect Biochemistry and Molecular Biology*, 31, 949–964.
 33. Buček, A., Matoušková, P., Vogel, H., Šebesta, P., Jahn, U., Weißflog, J., Svatoš, A., & Pichová, I. (2015). Evolution of moth sex pheromone composition by a single amino acid substitution in a fatty acid desaturase. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 12586–12591.
 34. Hagström, A. K., Liénard, M. A., Groot, A. T., Hedenström, E., & Löfstedt, C. (2012). Semi-selective fatty acyl reductases from four heliothine moths influence the specific pheromone composition. *PLoS One*, 7, e37230.
 35. Antony, B., Ding, B. J., Moto, K., Aldosari, S. A., & Aldawood, A. S. (2016). Two fatty acyl reductases involved in moth pheromone biosynthesis. *Scientific Reports*, 6, 29927.
 36. Lassance, J. M., Groot, A. T., Liénard, M. A., Antony, B., Borgwardt, C., Andersson, F., Hedenström, E., Heckel, D. G., & Löfstedt, C. (2010). Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. *Nature*, 466, 486–489.
 37. Wenz, P., Schwank, S., Hoja, U., & Schüller, H. J. (2001). A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene *FAS2* by the *FAS1* gene product. *Nucleic Acids Research*, 29, 4625–4632.
 38. Buček, A., Vazdar, M., Tupec, M., Svatoš, A., & Pichová, I. (2020). Desaturase specificity is controlled by the physicochemical properties of a single amino acid residue in the substrate binding tunnel. *Computational and Structural Biotechnology Journal*, 18, 1202–1209.

39. Wang, J. R., Li, Y. Y., Liu, D. N., Liu, J. S., Li, P., Chen, L. Z., & Xu, S. D. (2015). Codon optimization significantly improves the expression level of α -amylase gene from *Bacillus licheniformis* in *Pichia pastoris*. *BioMed Research International*, 2015, 248680.
40. Hagström, A. K., Walther, A., Wendland, J., & Löfstedt, C. (2013). Sub-cellular localization of the fatty acyl reductase involved in pheromone biosynthesis in the tobacco budworm, *Heliothis virescens* (Noctuidae: Lepidoptera). *Insect Biochemistry and Molecular Biology*, 43, 510–521.
41. Legmann, R. N., & Margalith, P. (1987). A comparative study of the lipid composition of yeasts with different fermentative capacities. *Applied Microbiology and Biotechnology*, 26, 49–54.
42. Marheineke, K., Grünewald, S., Christie, W., & Reiländer, H. (1998). Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. *FEBS Letters*, 441, 49–52.
43. Xia, Y. H., Ding, B. J., Wang, H. L., Hofvander, P., Sunesson, C. J., & Löfstedt, C. (2020). Production of moth sex pheromone precursors in *Nicotiana* spp.: A worthwhile new approach to pest control. *Journal of Pest Science*, 93, 1333–1346.
44. Chiralá, S. S. (1992). Coordinated regulation and inositol-mediated and fatty acid-mediated repression of fatty acid synthase genes in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 10232–10236.
45. Qiao, K., Imam Abidi, S. H., Liu, H., Zhang, H., Chakraborty, S., Watson, N., Kumaran Ajikumar, P., & Stephanopoulos, G. (2015). Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. *Metabolic Engineering*, 29, 56–65.
46. d'Espaux, L., Ghosh, A., Runguphan, W., Wehrs, M., Xu, F., Konzock, O., Dev, I., Nhan, M., Gin, J., Reider Apel, A., Petzold, C. J., Singh, S., Simmons, B. A., Mukhopadhyay, A., García Martín, H., & Keasling, J. D. (2017). Engineering high-level production of fatty alcohols by *Saccharomyces cerevisiae* from lignocellulosic feedstocks. *Metabolic Engineering*, 42, 115–125.
47. Workman, M., Holt, P., & Thykaer, J. (2013). Comparing cellular performance of *Yarrowia lipolytica* during growth on glucose and glycerol in submerged cultivations. *AMB Express*, 3, 58.
48. Wang, G., Xiong, X., Ghogare, R., Wang, P., Meng, Y., & Chen, S. (2016). Exploring fatty alcohol-producing capability of *Yarrowia lipolytica*. *Biotechnology for Biofuels*, 9, 107.
49. Dahlin, J., Holkenbrink, C., Marella, E. R., Wang, G., Liebal, U., Lieven, C., Weber, D., McCloskey, D., Ebert, B. E., Herrgård, M. J., Blank, L. M., Borodina, I., & Borodina, I. (2019). Multi-omics analysis of fatty alcohol production in engineered Yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. *Frontiers in Genetics*, 10, 747.
50. Rutter, C. D., & Rao, C. V. (2016). Production of 1-decanol by metabolically engineered *Yarrowia lipolytica*. *Metabolic Engineering*, 38, 139–147.
51. Beopoulos, A., Nicaud, J. M., & Gaillardin, C. (2011). An overview of lipid metabolism in yeasts and its impact on biotechnological processes. *Applied Microbiology and Biotechnology*, 90, 1193–1206.
52. Beopoulos, A., Cescut, J., Haddouche, R., Uribealarea, J. L., Jouve, C. M., & Nicaud, J.-M. (2009). *Yarrowia lipolytica* as a model for bio-oil production. *Progress in Lipid Research*, 48, 375–387.
53. Zhang, J. L., Cao, Y. L., Peng, Y. Z., Jin, C. C., Bai, Q. Y., Zhang, R. S., ... Yuan, Y. J. (2019). High production of fatty alcohols in *Yarrowia lipolytica* by coordination with glycolysis. *Science China Chemistry*, 62, 1007–1016.
54. Cordova, L. T., Butler, J., & Alper, H. S. (2020). Direct production of fatty alcohols from glucose using engineered strains of *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 10, e0e00105.
55. Xu, P., Qiao, K., Ahn, W. S., & Stephanopoulos, G. (2016). Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 10848–10853.
56. Wang, W., Wei, H., Knoshaug, E., Van Wychen, S., Xu, Q., Himmel, M. E., & Zhang, M. (2016). Fatty alcohol production in *Lipomyces starkeyi* and *Yarrowia lipolytica*. *Biotechnology for Biofuels*, 9, 227.
57. Yew, J. Y., & Chung, H. (2015). Insect pheromones: An overview of function, form, and discovery. *Progress in Lipid Research*, 59, 88–105.
58. Ding, B. J., & Löfstedt, C. (2015). Analysis of the *Agrotis segetum* pheromone gland transcriptome in the light of sex pheromone biosynthesis. *Bmc Genomics [Electronic Resource]*, 16, 711.
59. Ding, B. J., Lager, I., Bansal, S., Durrett, T. P., Stymne, S., & Löfstedt, C. (2016). The yeast ATF1 acetyltransferase efficiently acetylates insect pheromone alcohols: implications for the biological production of moth pheromones. *Lipids*, 51, 469–475.
60. Ding, B. J., Carraher, C., & Löfstedt, C. (2016). Sequence variation determining stereochemistry of a $\Delta 11$ desaturase active in moth sex pheromone biosynthesis. *Insect Biochemistry and Molecular Biology*, 74, 68–75.
61. Fadamiro, H. Y., Cossé, A. A., & Baker, T. C. (1999). Mating disruption of European corn borer, *Ostrinia nubilalis* by using two types of sex pheromone dispensers deployed in grassy aggregation sites in Iowa cornfields. *Journal of Asia-Pacific Entomology*, 2, 121–132.
62. Williamson, E. R., Folwell, R. J., Knight, A., & Howell, J. F. (1996). Economics of employing pheromones for mating disruption of the codling moth, *Carpocapsa pomonella*. *Crop Protection*, 15, 473–477.
63. Miller, J. R., & Gut, L. J. (2015). Mating disruption for the 21st century: Matching technology with mechanism. *Environmental Entomology*, 44, 427–453.
64. Sonenshine, D. E. (2017). Pheromones: Function and use in insect and tick control. In *Reference Module in Life Sciences*. Elsevier, 24–39.
65. Marella, E. R., Holkenbrink, C., & Siewers, V., Borodina, I. (2018). Engineering microbial fatty acid metabolism for biofuels and biochemicals. *Current Opinion in Biotechnology*, 50, 39–46.
66. Xie, D., Jackson, E. N., & Zhu, Q. (2015). Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: from fundamental research to commercial production. *Applied Microbiology and Biotechnology*, 99, 1599–1610.

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