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Total number of authors: 20

Published in:
Metabolic Engineering

Link to article, DOI:
10.1016/j.ymben.2020.10.001

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Production of moth sex pheromones for pest control by yeast fermentation

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ARTICLE INFO

Keywords:
Lepidoptera
Yarrowia lipolytica
Fatty alcohols
Fatty acyl-CoA desaturase

ABSTRACT

The use of insect sex pheromones is an alternative technology for pest control in agriculture and forestry, which, in contrast to insecticides, does not have adverse effects on human health or environment and is efficient also against insecticide-resistant insect populations. Due to the high cost of chemically synthesized pheromones, mating disruption applications are currently primarily targeting higher value crops, such as fruits. Here we demonstrate a biotechnological method for the production of (Z)-hexadec-11-en-1-ol and (Z)-tetradec-9-en-1-ol, using engineered yeast cell factories. These unsaturated fatty alcohols are pheromone components or the immediate precursors of pheromone components of several economically important moth pests. Biosynthetic pathways towards several pheromones or their precursors were reconstructed in the oleaginous yeast Yarrowia lipolytica, which was further metabolically engineered for improved pheromone biosynthesis by decreasing fatty alcohol degradation and downregulating storage lipid accumulation. The sex pheromone of the cotton bollworm Heliothis armigera was produced by oxidation of fermented fatty alcohols into corresponding aldehydes. The resulting yeast-derived pheromone was just as efficient and specific for trapping of H. armigera male moths in cotton fields in Greece as a conventionally produced synthetic pheromone mixture. We further demonstrated the production of (Z)-tetradec-9-en-1-yl acetate, the main pheromone component of the fall armyworm Spodoptera frugiperda. Taken together our work describes a biotech platform for the production of commercially relevant titles of moth pheromones for pest control via yeast fermentation.

1. Introduction

Mating disruption (MD) with sex pheromones is an efficient, safe and environmentally friendly method for pest control instead of using toxic insecticides (Reddy et al., 2010; Benelli et al., 2019). Mating disruption occurs when synthetic pheromones are released into the air of the fields, where they even in modest amounts prevent males from locating females, which disrupts insect reproduction and thereby also the insect infestation. Although the method has been around for over four decades, its usage is still very limited, with about 750,000 ha being treated with MD worldwide, which makes only 0.05% of the total arable and permanent crops area. Most of the MD-treated crops are high-value crops, such as apples, grapes, citruses (Ioriatti and Lucchi, 2016). The method is currently too expensive to be applied on the lower-value row crops, such as corn, soybean, cotton, and sorghum.

The majority of identified moth (Lepidoptera) sex pheromone...
components are unsaturated fatty alcohols, alcohol acetates, or aldehydes. These compounds are derived from the insect fatty acid metabolism and have 10–18 carbon-long skeletons. They are called Type I moth pheromone components and constitute approximately 75% of all known moth sex pheromone components (Ando et al., 2004; Lofstedt et al., 2016). The chemical diversity is to a large extent produced by the combined action of specific fatty acyl-CoA desaturases, fatty acyl-CoA reductases, and by chain-shortening (Bjostad and Roelofs, 1983). Lepidopteran desaturases and reductases are embedded in the endoplasmic reticulum. Desaturases act by introducing an oxygen atom into a specific location of a fatty acyl-CoA chain and then removing a water molecule to generate a double bond of cis- or trans-configuration (Sperling et al., 2003). The remaining oxygen atom from the molecular oxygen is reduced using the electrons obtained from NADH through cytochrome b5 reductase and cytochrome b5. Lepidopteran fatty acyl-CoA reductases use two NADPH molecules to directly generate fatty alcohol (Matsumoto et al., 1996). Desaturase and reductase activities are typically assayed by expressing them in heterologous systems, e.g., in S. cerevisiae devoid of the native elongase Elo1p and desaturase Ole1p activities, in insect cells, or in plant Nicotiana benthamiana (Petkevicius et al., 2020).

There is a potential in engineering oil plants to accumulate pheromone precursors in the form of lipids. Proof-of-concept production of several pheromone precursors has been established in Nicotiana species,

![Diagram](https://example.com/diagram.png)
but the content was too low for commercial exploitation (Ding et al., 2014; Xia et al., 2020). In another study, a model oil plant Camelina sativa was genetically modified to express a palmitoyl-acyl carrier protein-specific cholesterase together with a Δ11 desaturase. The content of (Z)-hexadec-11-en-1-ol (Z11-16Acid) in the seed oil was at 20% of the total fatty acids, which corresponds to around 40 g Z11-16Acid per kg seeds (Ortiz et al., 2020). Recombinant production of pheromone precursors in plants is complicated by the long development times of stable plant lines, costly regulatory procedures, and containment measures for growing genetically modified plants. In contrast, microbial cell factories can be readily engineered and cultured at large scale in contained bioreactors. So far, there has been only a single previous study, where insect pheromone pathway was expressed in a microbe with the purpose of establishing a cell factory for pheromone production. Specifically, a Δ11 fatty acyl-CoA desaturase and reductase from the turnip moth Agrotis segetum was expressed in S. cerevisiae (Hagström et al., 2013). However, as no metabolic engineering had been performed on the host, the titers of the product (Z)-hexadec-11-en-1-ol was only at ca. 0.2 mg/L, which is four-five orders of magnitude lower than what is required for economical pheromone manufacturing on scale.

The aim of this study was to optimize the production of unsaturated fatty alcohols in yeast and to validate the biological activity of the yeast-derived moth pheromone components. We have engineered oleaginous yeast Yarrowia lipolytica as the cell factory and demonstrated production of two common moth pheromone pheromone components, (Z)-hexadec-11-en-1-ol and (Z)-tetradec-9-en-1-ol, which are the immediate precursors of the corresponding, economically important, aldehyde and acetate pheromone components.

2. Results and discussion

2.1. Establishing pathways towards moth pheromones in yeast

To establish pathways towards moth pheromone compounds in yeast, we first investigated a range of fatty acyl-CoA desaturases and reductases for the production of (Z)-hexadec-11-en-1-ol (Z11-16OH) (Fig. 1A) by expressing the enzymes in combinations in baker’s yeast Saccharomyces cerevisiae. The fermented Z11-16OH can be chemically oxidized to (Z)-hexadec-11-enal (Z11-16Al), which is the main sex pheromone component of several row crop pests, such as the cotton bollworm Helicoverpa armigera, the striped rice stembomer Chilo suppressalis, and the yellow rice stemboomer Scirpophaga incertula (El-Sayed, 2014). The combination of a desaturase from Amylophylos transilieni and a reductase from H. armigera resulted in the highest titre of 1.7 ± 0.4 mg/L Z11-16OH (Fig. 1B), which was an order of magnitude enhancement in comparison to the previous study (Hagstrom et al., 2013). The improvement was likely due to the utilization of a desaturase variant with a higher activity in yeast and due to expression of the genes from constitutive promoters using constructs stably integrated into the yeast genome (Jensen et al., 2014).

Next, we wanted to achieve the biosynthesis of (Z)-tetradec-9-en-1-yl acetate (Z9-14Ac), which is the main sex pheromone component of the fall armyworm Spodoptera frugiperda, a rising pest with a high occurrence of insecticide resistance (Binning et al., 2014; Xia et al., 2020). For this, we searched for a Δ9-desaturase with a higher activity and specificity towards tetradecanoyl-CoA than to hexadecanoyl-CoA (Fig. 1C). The activities of six heterologous desaturase candidates were tested in a S. cerevisiae ole1aol1 strain devoid of the native desaturation and elongation activities. The cells were cultivated with supplementation of methyl tetradeconate (14Me) and the total lipids were analysed to determine the desaturated fatty acids (Fig. 1D). The strain expressing the desaturase from Drosophila melanogaster resulted in the highest concentration of 3.67 ± 0.99 mg/L methyl (Z)-tetradec-9-enate (Z9-14Me) and in the highest Z9-14Me to methyl (Z)-hexadec-9-enoate (Z9-16Me) ratio, indicating a higher specificity towards the tetradecanoyl-CoA substrate. To establish the complete pathway towards Z9-14Ac in the yeast S. cerevisiae, we expressed the D. melanogaster Δ9 desaturase together with the H. armigera reductase and S. cerevisiae AF11 known to catalyse acetylation of fatty alcohols (Ding et al., 2016). The resulting strain produced 7.3 ± 0.2 mg/L of Z9-14Ac in comparison to 1.4 ± 0.4 mg/L in an analogous strain lacking the heterologous Δ9 desaturase (Fig. 1E).

2.2. Optimization of the oleaginous yeast Yarrowia lipolytica for moth pheromone production

We rationalized that an oleaginous yeast should be a more suitable cell factory for production of fatty alcohol-based moth pheromones than the baker’s yeast that has a low content of the fatty acid precursor acetyl-CoA in the cytosol and can only accumulate small amounts of intracellular lipids. In contrast, the oleaginous yeast Yarrowia lipolytica has a naturally high fatty acid metabolism and has been engineered for commercial production of polyunsaturated omega-3 fatty acids (Xue et al., 2013) and for production of lipids (Shaw et al., 2016). Robust genetic tools, including the CRISPR/Cas9 method, have recently been developed for Y. lipolytica, and allow the rapid engineering of yeast species (Holkenbrink et al., 2018; Stoviczek et al., 2015).

The first hurdle we encountered when co-opting Y. lipolytica for the production of pheromones, was the prevention of endogenous degradation of the target fatty alcohols Z11-16OH and (Z)-tetradec-9-en-1-ol (Z9-14OH). We deleted one by one and in combination the genes encoding the enzymes potentially implicated in fatty alcohol degradation: fatty aldehyde dehydrogenases Hfd1p and Hfd4p (Iwama et al., 2014), as well as fatty alcohol oxidase Fao1p (Iwama et al., 2015) (Fig. 2A). Moreover, we deleted pexio10-mediated biogenesis factor Pex10p, thus interrupting the correct assembly of peroxisomes and preventing acyl-CoA degradation. Single deletions of HFD1/HFD4/FAO1/PEX10 genes increased the titre of Z11-16OH two-to-three-fold, while the combination of four deletions resulted in a 19-fold titre increase (Fig. 2B). The quadruple deletion strain (ST5789) produced 14.9 ± 3.6 mg/L of Z11-16OH in comparison to 0.8 ± 0.1 mg/L produced by a reference strain only expressing the biosynthetic pathway towards Z11-16OH (ST3844).

When strains ST3844 and ST5789 were incubated with externally supplied 1 g/L Z11-16OH and 1 g/L Z9-14OH each, strain ST3844 largely depleted the supplied alcohols, with only 6.2 ± 3.8 mg/L Z11-16OH left at the end of the cultivation. In contrast to that, strain ST5789 showed a remaining concentration of 630.9 ± 137.1 mg/L Z9-14OH and 620.3 ± 73.9 mg/L Z11-16OH. Less than 1 g/L of fatty alcohols were recovered probably due to evaporation and some losses during the recovery procedure. A control, which contained only cultivation medium and externally supplied fatty alcohols, showed a remaining concentration of 500.7 ± 135.7 mg/L Z9-14OH and 536.9 ± 166.1 mg/L Z11-16OH (Fig. S1). The experiment confirmed that the degradation rate of fatty alcohols was much lower in the strain with deletion of HFD1, HFD4, PEX10, and FAO1 genes.

Another challenge with Y. lipolytica as a host was to reduce the channelling of fatty acyl-CoAs, the fatty alcohol precursors, into storage lipids. We hence downregulated the expression of the gene encoding glycerol-3-phosphate acyltransferase (GPAT), which catalyses the first committing step towards glycerolipid- and glycerophospholipid biosynthesis. The downregulation was achieved by truncating the GPAT promoter to 100 base pairs and confirmed by qRT-PCR (Fig. S2.A). The downregulation of GPAT improved the titre of Z11-16OH from 14.9 ± 3.6 mg/L to 20.6 ± 5.4 mg/L (Fig. 2B). At the same time, the total fatty acid content of the cells was reduced by 53% (Fig. S2.B, C). The combination of Y. lipolytica genome edits that reduce the fatty alcohol degradation and lipid accumulation thus resulted in a basic platform chassis, where various moth pheromone pathways can be inserted.

The strain, however, predominantly produced fatty alcohols of 16-carbon chain length. In order to enable the production of 14-carbon
pheromones, we introduced a mutation into fatty acid synthase subunit Fas2p\textsuperscript{I1220F}, which was previously reported to benefit the biosynthesis of tetradecanoyl-CoA (Rigouin et al., 2017). We expressed the pathway towards \(Z_9\)-14OH in the engineered \(Y.\) lipolytica strains and obtained 4.9 ± 1.4 mg/L titre in the basic platform chassis and 73.6 ± 16 mg/L \(Z_9\)-14OH in the platform chassis with additional Fas2p mutation (Fig. 3B). The mutation thus resulted in a 15-fold improvement of a 14-carbon product and should be beneficial for producing also other pheromones derived from tetradecanoyl-CoA.

To further improve the production of \(Z_11\)-16OH, we integrated additional copies of desaturase and reductase genes to pull the flux towards pheromone biosynthesis. Integration of the second copy of the
pathway increased the titre 4.6-fold. The strain with three copies of the pathway produced 169 ± 14 mg/L Z11-16OH, a 9.7-fold increase in comparison to the single copy strain (Fig. 3A). When the optimized yeast strain was fermented in a 10L-bioreactor, 2.57 g/L of the target product Z11-16OH was obtained. The fatty alcohols were extracted from the yeast biomass using organic solvents and purified on a silica column. The eluted fractions with a high content of the product were pooled and oxidized into corresponding aldehyde using tetrakisacetonitrile copper (I) triflate/TEMPO catalyst system (Hoover and Stahl, 2011). The composition of the aldehyde preparation was Z11-16Ald, hexadecanal (16Ald), and (Z)-hexadec-9-enal (Z9-16Ald) in ratio 82:13:5 (Fig. S3, S4). The Z11-16Ald is the major and Z9-16Ald is the minor pheromone component in H. armigera and C. suppressalis, where the reported ratios between the two pheromone components in H. armigera were from 99:1 to 93:7 (Dunkelblum et al., 1980; Nesbitt et al., 1980; Zhang et al., 2012), in C. suppressalis the reported ratio is 90:10 (Tatsuki et al., 1983).16Ald is also present in the pheromone glands of both insect species, but it does not elicit a behavioural response. The composition of the yeast-derived pheromone may thus be close enough and well suited for trapping and mating disruption of these insect species with Z11-16Ald as a major and Z9-16Ald as a minor pheromone component. The biologically produced pheromone mix was subsequently subjected to activity tests on H. armigera in the laboratory and field.

2.3. Electrophysiological responses of male H. armigera

We measured the electroantennographic responses of male H. armigera adults to the yeast-derived pheromone blend (Bio-Ald), standard compounds, and mixtures of the standards (Fig. 4). Ald mix #1 contained Z11-16Ald, Z9-16Ald, tetradecanal (14Ald), and pentadecanal (15Ald) (80:5:5:5, respectively). Ald mix #2 contained equal volumes of each of the same components as Ald mix #1 (25:25:25:25 ratio).

Bio-Ald elicited the same magnitude of response on the male antenna as Ald mix #1 and significantly higher to that of the equimolal Ald mix #2 and to Z9-16Ald, the secondary compound of the H. armigera pheromone. The major sex pheromone compound, Z11-16Ald, yielded a high antennal response, whereas the minor sex pheromone, Z9-16Ald, induced a considerably lower response. The significantly lower response to Ald mix #2 is a clear indication that the antennal response is mainly attributed to Z11-16Ald and when its quantity in the mixture is lowered, the antennal response also drops. These results indicate that biologically produced Z11-16Ald induces the same magnitude of sensory stimulation as the chemically synthesized Z11-16Ald, the major compound of the moth’s native pheromone.

2.4. Monitoring of H. armigera flight in the field

The pheromone blend was further tested by trap catches of the cotton bollworm H. armigera in two cotton fields in Northern Greece. The cotton-growing areas have moderate moth populations with occasional regional and temporal outbursts (Milonas et al., 2016). Mean weekly male catches in traps baited with yeast-derived pheromone (Bio-Ald) and synthetic pheromone (Z11-16Ald: Z9-16Ald at 97:3 ratio, Control) dispensers are shown in Fig. 5 for two independent trials. Capture data
from the traps indicated that the flight peak occurred on the 2nd week of July (24.7 ± 2.7 males/trap/week for Bio-Ald traps and 22.3 ± 1.8 for the Control traps at Thermi and 19.3 ± 3.0 males/trap/week for Bio-Ald traps and 17.0 ± 3.5 for the Control traps at Lamia). In both regions, the total number of males trapped with the different lures were not significantly different (Bio-Ald: 80.3 ± 4.3 males/trap, Control: 72.0 ± 4.0 males/trap at Thermi and Bio-Ald: 60.3 ± 4.3 males/trap and Control: 57.0 ± 3.6 males/trap at Lamia). It is apparent that the biologically produced pheromone was equally effective under field conditions as the commercially available chemically synthesized one.

3. Conclusions

In summary, we have demonstrated biological production of practically and commercially relevant titers of several lepidopteran sex pheromone components or their precursors in yeast cell factories. A biocatalytic production is particularly advantageous for the production of chemicals for which isomeric composition is critical, such as moth pheromones (Löfstedt et al., 2016). The enzymes can deliver the required stereoisomers, while in chemical synthesis, a mix of isomers is often obtained and may be difficult to separate especially in large quantities. Furthermore, the fermentation is carried out in a cheap medium with glycerol as the sole carbon source, using yeast cells as the only catalyst. This is in contrast to chemical synthesis that will typically require special starting material, expensive catalysts, and several synthesis steps (Yadav and Reddy, 1988; Herbert et al., 2013). Reduced production costs and lower environmental impact of the biotech route in comparison to the chemical synthesis have been established for multiple chemicals, particularly for natural products (Pellis et al., 2018; Jullesson et al., 2015). As an additional advantage, major and minor pheromone components can be produced in a single process in a ratio that is suitable for the target insect. The work creates the foundation for the production of pheromones at a lower cost enabling pheromone-based pest control in row crops, such as rice, cotton, and maize.

4. Materials and methods

4.1. DNA assembly and yeast strain construction

Heterologous genes were codon-optimized for S. cerevisiae or Y. lipolytica and synthesized (GeneArt, Thermo Scientific). The vectors for gene expression and knock-outs were assembled and transformed into yeast according to the published methodologies (Jensen et al., 2014; Stovicek et al., 2015; Holkenbrink et al., 2018; Jessop-Fabre et al., 2014; Stovicek et al., 2015; Holkenbrink et al., 2018; Jessop-Fabre et al., 2014; Jullesson et al., 2016). The sequences of the genes and primers, the schemes for gene amplification, cloning and strain assembly are provided in Tables S1-5. The diagrams on Fig. S5 illustrate the workflow for the construction of engineered yeast strains. Yeast strain Y. lipolytica GB20 was a kind gift of Volker Zickermann (Goethe-University Frankfurt am Main, Germany). Yeast strain S. cerevisiae CEN.PK102–SB was obtained from Peter Kötter (Goethe-University Frankfurt am Main, Germany). Strain Y. lipolytica Y-17536 was received from the Agricultural Research Service (NRRL, USA). For removal of selection markers from the genome of Y. lipolytica, we used CreA recombinase gene obtained from plasmid pSH66 (EUROSCARF selection).

4.2. Chemicals and media

All chemicals were purchased from Sigma-Aldrich. Pheromone standards were purchased from WERNER BioAgents.

Cultivation of yeast strains.

Fig. 1b: One individual clone of each strain was inoculated into 2 mL medium (100 g/L glucose, 2 g/L yeast extract, 0.33 g/L (NH₄)₂SO₄, 1.33 g/L MgSO₄·7H₂O 1.33, 0.267 g/L NaCl, 2 mL/L trace metals solution (4.5 g/L CaCl₂·2H₂O, 4.5 g/L ZnSO₄·7H₂O, 3 g/L FeSO₄·7H₂O, 1 g/L H₂BO₃, 1 g/L MnCl₂·2H₂O, 0.4 g/L Na₂MoO₄·2H₂O, 0.3 g/L CoCl₂·6H₂O, 0.1 g/L CuSO₄ : 5H₂O, 0.1 g/L KI, 15 g/L EDTA), 8 mg/L thiamine, 0.67 mg/L biotin, 20 mg/L uracil, 380 mg/L leucine, 76 mg/L histidine, 100 mM potassium hydrogen phthalate buffer) in a 12-mL glass tube (Duran, Wertheim, Germany) with metal labocap lids (Lüdiswiss, Flawil, Switzerland) and incubated for 48 h at 30 °C with shaking at 250 rpm.

Fig. 2b: One individual clone of each strain was inoculated into 5 mL YPD medium with 8% glucose (10 g/L yeast extract, 20 g/L peptone, 80 g/L dextrose) in 12-mL glass tubes (Duran, Wertheim, Germany) with metal labocap lids (Lüdiswiss, Flawil, Switzerland) and incubated overnight at 30 °C with shaking at 250 rpm. The following day the overnight culture was centrifuged, the supernatant was discarded and the pellet was resuspended in 2 mL nitrogen-limited medium (2.9 g/L (NH₄)₂SO₄, 1.7 g/L YNB (without amino acids and ammonium sulphate), 380 mg/L leucine, 76 mg/L lysine, 20 mg/L uracil and 60 g/L glucose). The cultures were incubated for 48 h at 30 °C with shaking at 250 rpm.

Fig. 3a: Three individual colonies of strains expressing desaturases were inoculated into 1 mL selective medium (SC-Ura-Leu) and incubated at 30 °C and 300 rpm for 48 h. The cultures were diluted to an OD600 of 0.4 in 5 mL selective medium (SC-Ura-Leu) supplemented with 2 mM
CuSO₄ and the 0.5 mM methyl tetradecanoate (14Me) (Larodan Fine Chemicals, Sweden). The methyl tetradecanoate stock solution was prepared to a concentration of 100 mM in 96% ethanol. The yeast cultures were incubated at 30 °C at 300 rpm for 48 h.

Fig. 1c: Strains ST4854 and ST5290 were inoculated into 5 mL synthetic complete medium (SC-His-Leu-Trp supplemented with 20 mg/L uracil and 76 mg/L histidine) and cultivated in 12-mL glass tubes (Duran, Wertheim, Germany) with metal labocap lids (Ludiswiss, Flawil, Switzerland) overnight at 30 °C with shaking at 250 rpm. The following day the overnight culture was centrifuged, the supernatant was discarded and the pellet was resuspended in 2 mL of mineral medium, which had the composition as described elsewhere (Löfstedt et al., 2016). The medium was supplemented with 20 mg/L uracil and 76 mg/L histidine. The cultures were incubated at 30 °C with shaking at 250 rpm for 48 h.

Fig. 2c: One individual clone of each strain was inoculated into 3 mL mineral medium (14.5 g/L KH₂PO₄, 0.5 g/L MgSO₄, 2 mL/L trace metals solution (4.5 g/L CaCl₂-2H₂O, 4.5 g/L ZnSO₄7H₂O, 3 g/L FeSO₄7H₂O, 1 g/L H₃BO₃, 1 g/L MnCl₂-2H₂O, 0.4 g/L Na₂MoO₄·2H₂O, 0.3 g/L CoCl₂-6H₂O, 0.1 g/L CuSO₄·5H₂O, 0.1 g/L KI, 15 g/L EDTA), 1 g/L vitamin solution (50 mg/L biotin, 200 mg/L-aminobenzoic acid, 1 g/L nicotinic acid, 1 g/L L-carnitine, 1 g/L L-carnosine, 1 g/L pyridoxine HCl, 1 g/L thiamine HCl, 25 g/L myo-inositol), 6.9 g/L uracil, 50 g/L glycerol, 1.9 g/L leucine, 0.38 g/L uracil, 0.38 g/L L-lysine) in a 24 deep-well plate with air-permeable lid (EnzyScreen) and cultivated for 24 h at 30 °C with shaking at 250 rpm. The following day 3 mL of mineral medium were inoculated with the preculture in a 24 deep-well plate with air-permeable lid (EnzyScreen) and cultivated for 48 h at 30 °C with shaking at 250 rpm. After 24 h of cultivation, 119 μL of glycerol (corresponding to 50 g/L) were added to each well.

4.3 Metabolic extraction and analysis on GC/MS

Fig. 1b, e; Fig. 2b For extraction, 1 mL of culture was transferred into a 4-mL glass vial and 10 μL of internal standard solution (1 μg/μL methyl (Z)-heptadec-10-enoate in 100% ethanol) was added. The samples were freeze-dried in a freeze dry system (Freezone6 and Mx3005P (Agilent Technologies). Relative expression level was calculated using double delta method (ΔΔCt), where ΔΔCt = (ΔCtE - ΔCtC).

4.4 Fatty alcohol degradation analysis

Cells were cultivated according to the same method as described above for Fig. 2b with the exception that the preculture was incubated for 36 h (instead of overnight) and Z9-14OH and Z11-16OH were added to a final concentration of 1 g/L (indicated by + Alc). The concentration of fatty acids in the whole broth was determined after 48 h of incubation in the nitrogen-limited medium. Extraction of samples was performed as follows: 100 μL of broth was extracted with 1 mL of ethyl acetate:ethanol (85:15) and using 10 μL of methyl nonadecanoate (19Me. 2 mg/mL) as internal standard. The samples were vortexed for 20 s and incubated for 1 h at room temperature, followed by 5 min of vortexing. 300 μL of H₂O was added to each sample. The samples were vortexed and centrifuged for 5 min at 21 °C and 3000·g. The upper organic phase was analysed via gas chromatography-mass spectrometry (GC/MS). GC/MS analyses were performed on an Agilent 7890A GC coupled to 5977B mass selective detector. The GC was equipped with a split/spitless injector and a DB-Fatwax UI column (30 m × 0.25 mm × 0.25 μm). The operation parameters were: 1 μL split injection (30:1), injector temperature 220 °C and constant flow 1 mL/min helium. The oven temperature was set to 80 °C for 1 min, then increased at a rate of 15 °C/min to 210 °C, followed by a hold at 210 °C for 7 min, and then increased at a rate of 20 °C/min to 230 °C. Fatty acids were analysed in selected ion monitoring (SIM) mode using the following mass-to-charge-ratios for quantification: 55.1 and 74.1. Compounds were identified by comparison of retention times with those of the corresponding commercially available standards. Data were analysed by the Mass Hunter software B.08.00.

4.5 Analysis of GPAT expression by qRT-PCR

For qRT-PCR analysis, yeast strains ST5789 (control) and ST5791 (a strain with truncated GPAT promoter) were cultivated in triplicates according to method described above for Fig. 2b. The harvesting and pre-treatment before RNA extraction was as described in Dahlin et al. (2019). Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. First strand cDNA synthesis was performed using SuperScript™ I Reverse Transcriptase (ThermoFisher Scientific). 20 ng of cDNA was used for qRT-PCR analysis which was done using DyNaMo Flash SYBR Green qPCR Kit on a Stratagene Mx3005P (Agilent Technologies). Relative expression level was calculated using double delta method ($\Delta\Delta Ct$), where $\Delta\Delta Ct = (\Delta CtE - \Delta CtC)$.

4.6 Analysis of lipid content in strain with GPAT downregulation

Cells cultivated in triplicates according to method described above for Fig. 2b were subjected to FAMES analysis. 1 mL of broth was transferred to 4 mL glass vials and centrifuged at 3000 g for 5 min at room temperature, the supernatant discarded and the cell pellet treated with 1 mL of 1M HCl in methanol, vortexed and incubated at 80 °C for 2 h. After methanolysis, the mixture was neutralized with 1 mL of 1M NaOH in methanol, then 0.5 mL of saturated NaCl in water was added and 1 mL of hexane was added together with internal standard (19Me). Mixture was vortexed and centrifuged at 3000 g for 5 min at room temperature and then the upper phase recovered for GC analysis. GC/MS analysis was
done on Agilent 7820A GC system coupled to 5977B mass selective detector. The GC was equipped with DB-Fat wax UI column (30 m × 0.25 mm × 0.25 μm) and helium was used as carrier gas (average velocity 37 cm/s). The MS was scanning between m/z 30 and 350 and the injector was configured to split mode (split ratio 10:1) at 220 °C. The oven temperature was set to 80 °C for 1 min, then increased at a rate of 15 °C/ min to 210 °C, followed by a hold at 210 °C for 7 min. Then temperature increased at a rate of 20 °C/min to 230 °C. For injection 1 μL of sample was used. Compounds were identified by comparison of retention times with those of reference compounds and quantified based on internal standard. For dry cell weight (DW) measurements 1 mL of culture was taken, centrifuged for 5 min at 16000 g, supernatant discarded, washed with 1 mL sterile water, centrifuged once again and water discarded. Washed pellets were kept in an oven at 65 °C for 48 h and biomass weighted. Effect of GAP downregulation was evaluated based on ratio between FAMES and DW normalized to ST-5789. FAMES which were included in analysis can be seen in Fig. S2.

4.7. Preparation of yeast-derived biological pheromone sample

The fermentations were carried out in a BioFlo 415 bioreactor (Eppendorf/New Brunswick Scientific), equipped with an in-situ sterilized 14 L stainless steel vessel (10 L max working volume), pH was controlled at 5.0 ± 0.1 with automated addition of a 1M solution of NaHCO₃ and a 4M solution of NaOH. Dissolved oxygen was measured using a polarographic electrode and automatically controlled at 20% saturation by changing the stirring speed of three 6-blade Rushton turbines. Strain Yarrowia lipolytica ST6379 was inoculated into 6 L of fermentation medium (2 g/L yeast extract, 13.4 g/L yeast nitrogen base, 0.76 g/L lysine, 0.76 g/L uracil, 0.024 mg/L thiamine, 0.002 g/L biotin, and 50 g/L glycerol). After 20 h of fermentation, the culture was supplemented with 750 mL nutrient-rich feed (composed of 16.2 g/L yeast extract, 108.6 g/L yeast nitrogen base, 0.2 g/L lysine, 0.02 g/L thiamine, and 326 g/L glycerol), followed by a pulse of glycerol to a concentration of 50 g/L in the reactor at 32 h. From 36 h, glycerol was fed continuously keeping a steady glycerol concentration of 20–30 g/L in the bioreactor. The fermentation lasted a total of 48 h. The concentration of Z11-16OH was 2.57 g/L.

Liquid-liquid extraction with ethyl acetate was performed on a total of 4.2 L fermentation broth. Fermentation broth was centrifuged at 4000 × g for 5 min. The supernatant was discarded and the remaining pellet was freeze dried and pulverized. 1 L ethyl acetate was added to the pulverized powder and incubated on a multi-vortexer for 8 h. After filtering off the solvent, the biomass cakes were re-extracted with 0.5 L ethyl acetate. All extracts were combined and the solvent was evaporated to dryness. The extract was resuspended in 25 mL ethyl acetate.

For purification, the crude extract (4.7 g) was passed through a plug of silica gel (approximately 100 g), in a filtration funnel. The silica was washed with hexanes, and then subsequently with a gradient of hexanes: ethyl acetate at the proportions of 95:5 (%) to 80:20 (%), with 5% increments. Fractions were analysed by TLC eluted with a mixture of hexanes:ethyl acetate in the proportion 80:20 and gas chromatography. The purest fractions were combined and the solvent was evaporated, initially by rotary evaporator and then by means of high vacuum pump. The total amount of purified material recovered was 1.8 g. A fraction of the purified material was transformed into the aldehyde, according to the following protocol: TEMPO (2,2,6,6-Tetramethyl-1-piperidinonyloxy, 26 mg) and 1-methyldiazole (28 mg) were added to a well-stirred suspension of the alcohol (800 mg), acetonitrile (3 mL), 2,2′-bipyridyl (26 mg) and tetrakisacetonitrile copper(I) triflate (62 mg). The mixture was stirred at room temperature and open atmosphere for 2 h and the completion of the reaction was verified by gas chromatography. The solvent was evaporated and the recovered material was extracted with hexanes, water and NaHCO₃ aqueous solution. The organic phase was dried with MgSO₄, filtered and the solvent was evaporated under reduced pressure. The resulting material (0.58 g) was dissolved in 10 mL ethyl acetate and analysed as follows. Three replicates 200 μL of the solution were transferred into 5-mL volumetric flasks, and ethyl acetate was added to complete the volume to the meniscus line. The contents of the flasks were mixed by swirling or inverting the flasks up and down several times and aliquots of 1.5 mL were transferred to autosampler vials for GC injection. Analysis was performed in an Agilent 7890 equipped with an FID detector and an HP-5 capillary column. The oven temperature program involved an initial temperature of 115 °C, increased at a rate of 40 °C/min to 162 °C and held for 3 min. The temperature was finally increased to 40 °C/min to 280 °C and held for 3 min. Three concentrations of technical grade Z11-16Ald in ethyl acetate were prepared and the solutions were also injected into the GC to create a calibration curve and the equation of the line was used for quantification of Z11-16Ald, according to Table S6. The concentration of Z11-16Ald was determined to be 35.33 mg/mL based on three replicates with the RSD of 0.72% between replicates.

4.8. Electrophysiological responses of male H. armigera

The antennal responses of Helicoverpa armigera male adults to the pheromone blend produced from yeast fermentation were evaluated by electroantennography (EAG) using a commercially available electroantennographic system (Syntech, The Netherlands). Antennae of a virgin, two-to-three-days-old male adult were used. The signal was amplified and detected with a data acquisition controller (IDAC-4, Syntech, The Netherlands).

Yeast-derived pheromone blend (Bio-Ald), standard compounds and mixtures of the standards were tested at a total of 39 antennal preparations. As standard compounds, the two pheromone components of H. armigera pheromone, Z11-16Ald and Z9-16Ald, were used. Two mixtures of the two monounsaturated aldehydes with tetradecanal and pentadecanal were also tested on grounds that presence of tetradecanal and pentadecanal has been verified in the pheromone blend produced by yeast fermentation (MS identification, data not shown). The two blends tested were Ald mix 1: Z11-16Ald, Z9-16Ald, 14Ald, 15Ald at 80:5:5:5 ratio and Ald mix 2: Z11-16Ald, Z9-16Ald, 14Ald, 15Ald at 1:1:1:1 ratio. Ald mix 1 blend constituents approximate the abundances found in the yeast-derived pheromone blend and notably the ratio of the two monounsaturated aldehydes (94:6) approximate the optimal pheromone blend ratio for H. armigera.

Aliquots of 1 μg of each of the compounds (or mixtures) was presented to the male antenna. Stimuli were provided as 0.3 s air puffs into a continuous flow of filtered and humidified air. The air flow, at 25 cm²/s rate, tube diameter 1 cm, was generated by an air stimulus controller (CS-55, Syntech, The Netherlands). At least 1 min was allowed between successive stimulations in order to allow the antenna to recover. Control stimulus consisted of filter paper and solvent (n-pentane). A reference stimulus, consisting of a 1 μg of Z11-16Ald (the major sex pheromone of H. armigera), was provided at regular intervals during each recording session. The EAG response to each reference stimulus was defined as 100%, and all responses to the test stimuli between adjacent references were normalized in % relative to the references.

4.9. Monitoring of H. armigera flight in the field

Field trials were conducted in Thermi (northern Greece 40°32′11.6″N 23°00′08.0″E) and in Lamia (central Greece 38°87′64.1″N 22°36′81.3″E) on experimental pesticide-free cotton fields (planted area 1.5 ha each). The two discrete geographical regions having similar meteorological conditions (temperature 27-28 °C; rainfall 2-0.5 mm for July and August respectively).

Dispensers used as control were grey rubber septa (bromobutyl elastomers) loaded with 2 mg of H. armigera pheromone blend Z11-16Ald: Z9-16Ald at 97:3 ratio (provided by Novagrica Hellas SA). Treatment dispensers (Bio-Ald) were similarly bromobutyl elastomers.
loaded with 2 mg of the yeast-derived pheromone. BHT and bumer-
trizole were added as antioxidant and UV absorber respectively at 5% 
w/w. Six funnel traps (three dispensers loaded with commercially 
available pheromone, control, and three loaded with the yeast-derived 
pheromone (Bio-Ald) were installed at 1.2 m height. Baited traps were 
in operation from early July until early September, the traps were 
rotated clockwise weekly, and males captured were recorded once per 
week and removed. Pheromone dispensers were renewed every month 
(Kikionis et al., 2017).

4.10. Statistical analyses

The electrophysiological and field data were subjected to analysis of 
variance (ANOVA) (SAS Institute, 2000). The means of electrophysio-
logical data were separated using the Tukey (honestly significant dif-
erence, HSD) test at P = 0.05. The field data presented as means of male catches per trap per week.

Author contributions

IB and CL conceived the study. CH, BJD, HLW, MID, KP, KKK, LW, CS 
and BL performed the experiments on pheromone production in yeasts. 
LF, MP, and BF carried out fermentation in controlled bioreactors and 
extracted the pheromones. CB, WU, and AM-N purified and oxidized the 
sample for activity tests. EK, DR, and MK performed laboratory and field 
tests of pheromones. IB drafted the manuscript and all the authors have 
contributed to writing.

Declaration of competing interest

IB, CH, CL, BJD, MID, HLW are co-inventors on patent applications 
WO2016207339, WO2018109167, and WO2018109163. IB, CH, KKK, 
BL, KP, CS, and LW have financial interest in BioPhero ApS. BSF has 
financial interest in Biotrend SA. DR has financial interest in Novagricia 
SA. AM-N has financial interest in ISCA Technologies.

Acknowledgements

This project has received funding from the European Union’s Hori-
zon 2020 research and innovation programme under grant agreement 
No. 760798 (OLEFINE) and from the Bio Based Industries Joint Un-
tertaking (BBI JU) under the European Union’s Horizon 2020 research 
and innovation programme under grant agreement No: 886662 
(PHERA). IB and CL acknowledge the financial support from the Novo 
Nordisk Foundation under grant agreements No: NNF15CC016592 
and NNF10CC1016517 and from Region Sjælland Vækstforum, ViiRS. 
CL acknowledges funding from the Swedish Foundation for Strategic 
Research (No. RBP 14–0037, Oil Crops for the Future). KP acknowledges 
the funding from Innovationsfonden under grant agreement number 
8053-00179B. LW has received funding from the European Union’s 
Horizon 2020 research and innovation programme under the Marie 
Skłodowska-Curie grant agreement No 840491. MK and DR acknowl-
edge Dr. Stefanos Andreadis (Hellenic Agricultural Organization – 
Demeter) for providing experimental field. The authors thank Volker 
Zickermann (Goethe-University Frankfurt am Main, Germany), Peter 
Kötter (Goethe-University Frankfurt am Main), and the Agricultural 
Research Service (NRRL, USA) for the yeast strains. We thank Dr. Hamne 
Bjerre Christensen and Dr. Mette Kristensen for the assistance with GC-
MS analysis and Dr. Pep Carusati for help with preparation of pher-
omone sample for activity tests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. 


