Whole-cell (+)-ambrein production in the yeast Pichia pastoris

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A B S T R A C T

The triterpenoid (+)-ambrein is a natural precursor for (-)-ambrox, which constitutes one of the most sought-after fragrances and fixatives for the perfume industry. (+)-Ambrein is a major component of ambergris, an intestinal excretion of sperm whales that is found only serendipitously. Thus, the demand for (-)-ambrox is currently mainly met by chemical synthesis. A recent study described for the first time the applicability of an enzyme cascade consisting of two terpene cyclases, namely squalene-hopene cyclase from Alcellobacillus acidocaldarius (AuSHC D377C) and tetraprenyl-β-curcumene cyclase from Bacillus megaterium (BmeTC) for \textit{in vitro} (+)-ambrein production starting from squalene. Yeasts, such as \textit{Pichia pastoris}, are natural producers of squalene and have already been shown in the past to be excellent hosts for the biosynthesis of hydrophobic compounds such as terpenoids. By targeting a central enzyme in the sterol biosynthesis pathway, squalene epoxidase Erg1, intracellular squalene levels in \textit{P. pastoris} could be strongly enhanced. Heterologous expression of AuSHC D377C and BmeTC and, particularly, development of suitable methods to analyze all products of the engineered strain provided conclusive evidence of whole-cell (+)-ambrein production. Engineering of BmeTC led to a remarkable one-enzyme system that was by far superior to the cascade, thereby increasing (+)-ambrein levels approximately 7-fold in shake flask cultivation. Finally, upscaling to 5 L bioreactor yielded more than 100 mg L\textsuperscript{-1} of (+)-ambrein, demonstrating that metabolically engineered yeast \textit{P. pastoris} represents a valuable, whole-cell system for high-level production of (+)-ambrein.

1. Introduction

The triterpenoid (+)-ambrein is a major component of ambergris, an intestinal excretion of sperm whale that represents one of the most valuable animal resources for perfume production. Apart from (+)-ambrein, ambergris also contains several choleseterol-type sterols (Ohloff, 1982). Upon exposure to sea water, sun light and air, (+)-ambrein undergoes oxidative degradation, yielding (-)-ambrex and several other odor-active compounds (Sell, 2006). This natural process can be simulated by reacting pure (+)-ambrein with singlet oxygen, thereby yielding several photo-oxidation products, including ambrox, γ-coronal, α-ambrinol and dehydroambraxoid (Ohloff, 1990). Beyond application in the perfume industry, animal studies have also demonstrated the potential anti-nociceptive (Taha, 1992) and aphrodisiac (Taha et al., 1995) properties of (+)-ambrein as well as possible effects on the cardiovascular system (Raza et al., 1999) and on smooth muscle response (Taha et al., 1998). As the natural ambergris supply is highly limited, total or partial syntheses (reviewed by Zerbe and Bohlmann, 2015) have been developed for production of (+)-ambrein, (-)-ambrox and related compounds. A recent study by Ueda et al. (2013) described the possibility to produce (+)-ambrein from squalene applying only two enzymes (Fig. 1). Squalene-hopene cyclase variant D377C from Alcellobacillus acidocaldarius (AuSHC D377C) produces 3-deoxyachilleol (Sato and Hoshino, 1999), which can be converted to (+)-ambrein by a second enzyme, a versatile tetraprenyl-β-curcumene cyclase from Bacillus megaterium (BmeTC) first described by Sato et al. (2011).

For the generation of (+)-ambrein, the authors incubated \textit{Escherichia coli} cell-free extracts expressing the aforementioned terpene cyclases with...
squalene or 3-deoxychaledil, respectively. The latter had been purified in between the two conversion steps. This, and the relatively low yield rendered the described approach not immediately feasible for industrial approaches (Ueda et al., 2013). Moreover, employing squalene as a substrate significantly increases process costs. In contrast to E. coli, yeasts naturally produce triterpenoid precursors, such as squalene or 2,3-oxidosqualene via their intrinsic mevalonate and sterol biosynthesis pathway. Furthermore, yeasts can easily be genetically manipulated and, for these reasons, represent ideal hosts for terpenoid production as reviewed, for example, by Wriessnegger and Pichler (2013) or Leavell et al. (2016).

Although most studies addressing terpenoid biosynthesis in yeast focus on sesquiterpenoids (C15) or carotenoids (C40), a few have also been successful in establishing yeast, especially Saccharomyces cerevisiae, as a production platform for triterpenoids (C30). This concerns mainly ginsenosides, through metabolic engineering (Dai et al., 2013; Kirby et al., 2008; Madsen et al., 2011), or cell engineering approaches (Arendt et al., 2017) and modified cultivation procedures (Moses et al., 2013). Though not yet as well-studied as S. cerevisiae, where metabolic engineering of the mevalonate pathway for terpenoid precursor production is described (Ro et al., 2006), the methylotrophic yeast Pichia pastoris exhibits some properties that render it highly interesting as a production platform. The success of recombinant membrane protein expression in P. pastoris has been shown numerous times (reviewed by Byrne, 2015; Emmerstorfer et al., 2014). Its ability to grow to very high cell densities, i.e. > 100 g/L cell dry weight in bioreactors, makes it very attractive for industrial purposes (Cerovigno et al., 2002). Furthermore, its applicability for terpenoid production has been demonstrated in several studies (Liu et al., 2015; Wriessnegger et al., 2016, 2014; Zhao et al., 2016). To our knowledge, the work by Liu et al. (2015) is the only one to date that describes metabolic engineering of P. pastoris for heterologous triterpenoid production. In brief, expression of ERG1 (squalene epoxidase) was increased while ERG7 (lanosterol synthase) expression, the next protein in ergosterol biosynthesis pathway, was downregulated to accumulate 2,3-oxidosqualene, the precursor for dammaraneol-I. Furthermore, cultures were supplemented with squalene, which significantly enhanced productivity. In contrast to this approach, to generate sufficient amounts of squalene for (+)-ambrein synthesis, our strategy aimed at downregulating ERG1 expression and activity. In S. cerevisiae, Erg1 activity had been successfully decreased in several studies utilizing the inhibitor terbinfine, which resulted in clearly enhanced levels of squalene (Garaiová et al., 2014; Han et al., 2018; Klobučníková et al., 2003), while this study focuses on the first analysis of the effects of terbinfine on Erg1p in P. pastoris. In addition to supplementing terbinfine, also the possibility to downregulate expression of ERG1 was evaluated. Therefore, the native promoter of ERG1 was exchanged for the regulatable PISI promoter, which can be partially repressed using zinc or inositol (Delic et al., 2013). On top of converting 3-deoxychaledil to (+)-ambrein, BmeTC can convert squalene to 8α-hydroxypolypoda-13,17,21-triene and, subsequently, 14β-hydroxynor- cera-8(26)-ene and onoceren oxide (Fig. 1) (Ueda et al., 2013). To ensure that sufficient amounts of 3-deoxychaledil can be formed from squalene by AsHSC D377C while reducing the formation of 8α-hydroxypolypoda-13,17,21-triene, a sequential cultivation and expression strategy was developed. The first phase was dedicated to cell growth and squalene accumulation. At the beginning of the second phase, expression of AsHSC D377C controlled by the FLD1 promoter (Shen et al., 1998) was induced with methanol. During the third and last phase of cultivation, both AsHSC D377C and BmeTC were expressed employing the AOX1 promoter (Tschopp et al., 1987) and using methanol (MeOH) as inducer. Another essential part of this study was to develop analytical methods that allowed us to detect and quantify the different triterpenoids extracted from engineered P. pastoris strains as the GC-MS method described by Ueda et al. (2013) cannot be used to separate the highly similar compounds such as squalene and 3-deoxychaledil, or 8α-hydroxypolypoda-13,17,21-triene and (+)-ambrein. Following these approaches, together with engineering of BmeTC towards a bifunctional enzyme, we managed to establish P. pastoris as the first eukaryotic host for whole-cell production of (+)-ambrein with yields that render it highly interesting for future industrial applications.

### 2. Material & methods

#### 2.1. Vector and strain construction

![Fig. 1. Generation of (+)-ambrein from squalene using AsHSC D377C and BmeTC according to Ueda et al. (2013).](image)

E. coli TOP10F′ (F′<lacIq Tn10(teh5)>) merA Δ(mrr-hsdRMS-mcrBC) p80lacZΔM15 lacX74 nupG recA araD139 Δ(ara-leu) 7697 galU galK rpsL(Str<sup>R</sup>) endA1 λ<sup>+</sup>) from life technologies, Vienna, Austria was used for vector construction and amplification. P. pastoris strains constructed in this study were based on strains CBS7435 his4 and CBS7435 his4 ku70 (Näätsaari et al., 2012). Plasmid backbones employed for strain constructions in P. pastoris had been described in the same work. All strains described in this work are listed in Table 1.

`Flusion` High Fidelity DNA polymerase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) was used for gene amplification according to the recommended PCR protocol (for primer sequences see Table S1). To exchange the native promoter of ERG1 for the PBI promoter, an integrative expression plasmid containing the following elements was assembled: PISI promoter (primers 5′ & 6′), ERG1 coding sequence (GenBank number: LT962478.1, bases 1999855–2001333), 5′ (primers 1 & 2) and 3′ (including ERG1 gene; primers 7′ & 8′) untranslated regions of the ERG1 locus for homologous integration were all amplified from genomic DNA of strain CBS7435 his4. HIS4 selection marker.

### Table 1

| Strain | Description | Source |
|--------|-------------|--------|
| Wildtype (WT) | CBS7435 his4 | Näätsaari et al. (2012) |
| WT ku70 | CBS7435 his4 ku70 | Näätsaari et al. (2012) |
| **<sup>P<sub>ISI</sub></sup>** ERG1 WT ku70 SHC | CBS7435 his4, pPpHIS4 [P<sub>PISI</sub>-ERG1<sup>WT</sup>] | This study |
| WT TC | CBS7435 his4, pPpFIZE [BmeTC] | This study |
| **<sup>P<sub>ISI</sub></sup>** ERG1 SHC | CBS7435 his4, pPpHIS4 [P<sub>PISI</sub>-ERG1<sup>WT</sup>], pPpKan[AsHSC D377C]<sup>SHC</sup> | This study |
| **<sup>P<sub>ISI</sub></sup>** ERG1 TC | CBS7435 his4, pPpHIS4 [P<sub>PISI</sub>-ERG1<sup>WT</sup>], pPpFIZE [BmeTC] | This study |
| **<sup>P<sub>ISI</sub></sup>** ERG1 SHC | CBS7435 his4, pPpHIS4 [P<sub>PISI</sub>-ERG1<sup>WT</sup>], pPpKan[AsHSC D377C], pPpFIZE [BmeTC] | This study |
| **<sup>P<sub>ISI</sub></sup>** ERG1 TC | CBS7435 his4, pPpHIS4 [P<sub>PISI</sub>-ERG1<sup>WT</sup>], pPpKan[AsHSC D377C], pPpFIZE [BmeTC] | This study |

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(primers 3 & 4) was amplified from pPhHIS4 and origin of replication and kanamycin resistance cassette (primers 13 & 14) were amplified from pET-28. These parts including overlapping sequences were amplified by PCR and were joined by Gibson assembly. Prior to P. pastoris transformation, the plasmid was digested with selected restriction enzymes (Thermo Scientific, St. Leon-Rot, Germany) to generate an integration cassette flanked by homologous sequences for targeted integration into the ERG1 locus (Fig. S1). Correct integration was confirmed through colony PCR (primer pairs 9 & 10 and 11 & 12). AscSH D377C (GenBank number of native gene: AB007002.1) and BmeTC (GenBank number of native gene: CP001982.1, 2130781–2132658) were manually codon-harmonized for expression in P. pastoris. Therefore, the frequency of each codon occurring in the respective gene was analyzed for P. pastoris as well as for the originating organism (A. acidocaldarius or B. megaterium) using the gcua tool (http://gen.schoedl.de/) (Fuhrmann et al., 2004) in combination with codon usage tables provided by http://www.kazusa.or.jp (Nakamura et al., 2000). For frequently used codons in the originating organism, frequently occurring codons in P. pastoris were chosen while codons with medium and low frequency were substituted with ones that occur with medium frequency in P. pastoris. These synthetic genes were purchased from GeneArt® (Fig. S2). For expression of AscSH D377C, the AOX1 promoter of the pPpKan expression vector was exchanged for the FLDI promoter (primers 15 & 16; amplified from CBS7435 his4 genome) by subcloning with SsuI and SpeI. The synthetic gene encoding AscSH D377C was amplified (primers 17 & 18) to encode an N-terminal FLAG-tag sequence and to add SpeI and NotI restriction sites for integration into the expression vector as well as a Kozak sequence (GGAAGC). For expression of BmeTC, a novel expression named pFZE (Fig. S3) was designed by amplifying AOXI promoter (primers 23 & 24) and terminator (primers 25 & 26) from pPpKan, 3’AOXI sequence (primers 29 & 30) from pAAHSea (Almad et al., 2014) and the flippase cassette with Zeocin resistance (primers 27 & 28) from pPpKCI (Mudassar Almad, manuscript in preparation). Subsequently, all parts were assembled through Gibson cloning. The synthetic BmeTC gene was amplified (primers 19 & 20) to add Ascl and PucI sites as well as Kozak sequence and to encode an N-terminal myc-tag sequence. To generate and express BmeTC D377C variant, the BmeTC sequence including Kozak sequence and myc-tag was first amplified from pPpFZE[BmeTC] with primers 21 & 22 containing SpeI and NotI restriction sites for subcloning into pPpKan_pFLD. For amino acid exchange D377C, a slightly modified protocol of Stratagene’s QuickChange site-directed mutagenesis kit was applied using Phusion High Fidelity DNA polymerase. In brief, 25 µL of two separate mutagenesis PCR mixtures were prepared, that contained either the forward or the reverse primer (primers 31 & 32). After five cycling steps were amplified and the two PCR reactions were performed according to the Stratagene manual, the two PCR reactions were combined and PCR was continued for another 13 cycles (Edelheit et al., 2009). In general, all cloned and modified sequences were checked by sequencing (Micsynth AG, Balgach, Switzerland). Expression vectors were linearized with SmaI (pPpKan_pFLD) or SmaI (pPpFZE) and were transformed into electrocompetent P. pastoris cells according to the protocol of Lin-Cereghino et al. (2005). Aliquots were plated on histidin-free minimal media or on YPD plates containing either 25 µg L⁻¹ Zeocin™ (InvivoGen, Vienna, Austria) or 300 mg g L⁻¹ geneticin sulfate (Formedium™, Norfolk, United Kingdom), respectively.

2.2. Media and strain cultivation

P. pastoris cultures were grown in YPD containing 1% yeast extract, 2% peptone (both obtained from Becton, Dickinson and Company, Schwechat, Austria) and 2% glucose (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Minimal dextrose (MD) plates (1.34% Difco™ yeast nitrogen base w/o amino acids (YNB, from Becton, Dickinson and Company, Schwechat, Austria), 4 × 10⁻²% biotin, 2% dextrose) were used for selection of strains containing the pPhHIS4 expression vector. E. coli was cultivated in LB medium (Lennox) purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Media for plates were solidified by addition of agar to 1.5%. Pre-cultures were grown in YPD medium at 28 °C and 130 rpm overnight. Main cultures of 50 mL YPD in baffled 300 mL shake flasks covered with cotton cloth were inoculated to an OD₆₀₀ of 0.1 and were cultivated at 28 °C and 130 rpm. After 24 h of growth, for induction of PFDL1 but not of PAOXI, 12.5 µL of a 40% methylamine solution in H₂O (Sigma-Aldrich®, Vienna, Austria) were added to the culture. Alternatively, to induce PFDL1 and/or PAOXI, methanol was added to a final concentration of 0.75% every 12 h. To increase intracellular squalene accumulation, 5 µL of 1 mg L⁻¹ terbutamine hydrochloride (Sigma-Aldrich®, Vienna, Austria) solution in ethanol was added to the cultures in the beginning and every 48 h of cultivation.

2.3. Expression analysis

Sample preparation for SDS–PAGE was carried out according to the method of Riezman et al. (1983), with slight modifications. In brief, 3 OD₆₀₀ units taken after 48 h of induction were transferred to 1.5 mL reaction tubes, were centrifuged at 2000 rpm for 5 min and the supernatants were removed. Cell pellets were resuspended in 300 µL of 1.85 M NaOH (7.5% β-mercaptoethanol) and were incubated on ice for 10 min. Then, 300 µL of 50% (w/v) trichloroacetic acid were added and following incubation on ice for 1 h, the samples were centrifuged at maximum speed for 5 min at 4 °C. After removing the supernatants, the cell pellets were washed with 500 µL of ddH₂O, resuspended in 50 µL of loading dye (NuPAGE®) and were incubated for 10 min at 70 °C. Cell debris was spun out and 15 µL aliquots were loaded onto an SDS–PAGE gel. Protein levels were checked by immunoblotting using primary antibodies against FLAG- or c-myc-tags (F1804 and C3956 from Sigma-Aldrich®, Vienna, Austria). HRP-conjugated secondary antibodies (A4416 and A9169 from Sigma-Aldrich®, Vienna, Austria) and enhanced chemiluminescent signal detection (SuperSignal™, Pierce Biotechnology, Rockford, IL) were used to visualize immunoreactive bands. SDS–PAGE and immunoblotting were performed according to the manual of the NuPAGE® SDS–PAGE Gel System (life technologies, Vienna, Austria). For determination of intracellular localization of heterologous proteins, cells were harvested after 24 h of induction and cell lysis and fractionation was performed as described by Geier et al. (2012). Fifteen µg of protein of total cell lysate, cytosolic fraction and microsomal fraction was precipitated with trichloroacetic acid and was subsequently loaded onto SDS–PAGE. Protein expression levels were analyzed by immunoblotting as described above.

2.4. Quantification of triterpenoids and sterols by GC-MS and GC-FID

Sterol extraction of cell culture volumes corresponding to 10 OD₆₀₀ units was performed essentially as described by Hirz et al. (2013). In brief, cells were resuspended in 0.6 mL of methanol, 0.4 mL of 0.5% pyrogallol in methanol, and 0.4 mL of 60% KOH. Five µL of a 2 mg mL⁻¹ cholesterol solution in ethanol were added as internal standard. After heating the samples for 2 h at 90 °C, saponified lipids were extracted twice with 1 mL of n-heptane. Dried extracts were dissolved in 10 µL of pyridine and were derivatized with 50 µL of N, O-bis(trimethylsilyl)trifluoroacetamide. Samples were diluted with 200 µL of ethyl acetate and analyzed by gas chromatography–mass spectrometry (GC-MS) or gas chromatography - flame ionization detector (GC-FID). Quantification of analytes was performed by correlating the peak area of the internal standard cholesterol to the peak area of the respective compound.
2.4.1. GC-MS method

A 7.5 m OPTIMA® delta-6 column (Macherey-Nagel; polysiloxane phase with autoselectivity 0.10 mm × 0.10 µm) was used on a ShimadzuQP2010 plus GCMS system equipped with a single quadrupole mass filter with electron impact ionization (EI 70 eV). Sample aliquots of 1 μL were injected in split mode (split ratio 15:1) at 270 °C injector and 300 °C detector temperatures with hydrogen as carrier gas at constant flow rate of 60 cm s⁻¹. The oven temperature program was as follows: 70 °C for 1 min, 30 °C min⁻¹ ramp to 320 °C (3 min). MSD was operated in a mass range of 50–550 amu with 6.6 scans/s and at electron multiplier voltage of 1.10 kV.

2.4.2. GC-FID method

For routine analysis, a GC-FID method was developed. Therefore, a OPTIMA® delta-6 column (Macherey-Nagel; polysiloxane phase with autoselectivity; 7.5 m × 0.10 mm×0.10 µm) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID) was used. Sample aliquots of 1 μL were injected in split mode (split ratio 30:1) at 250 °C injector temperature and 320 °C detector temperature with hydrogen as carrier gas and a flow rate set to 0.4 mL min⁻¹ in constant flow mode (58 cm s⁻¹ linear velocity). The oven temperature program was as follows: 70 °C for 1 min, 30 °C min⁻¹ ramp to 310 °C (1 min).

2.5. Purification and NMR analysis of 3-deoxyachilleol

An ethyl acetate extract (40 mL) of metabolites from cell lysate - obtained from 700 mL of WT ku70 SHC fermentation broth using a Mereckenschlager (MSK) homogenizer (Sartorius, Goettingen, Germany) as described by Hirz et al. (2013) - was concentrated under reduced pressure and the residue purified via flash chromatography on silica gel (0.035–0.070 mm, 60 Å, Autos Organics) using cyclohexane as eluent. All fractions containing a pure compound at an Rf value of 0.39 (cyclohexane) were pooled and concentrated under reduced pressure. The isolated amount was 6.0 mg and the material confirmed as 3-deoxyachilleol by GC-MS and NMR analysis.

2.6. Bioreactor cultivation of strain \( P_{\text{PSI}}-\text{ERG1} \) TC D373C

For bioreactor cultivation, “Pichia Fermentation Process Guidelines” (Invitrogen) were followed, with some minor adjustments. Precultures were grown in 300 mL baffled shake flasks containing 50 mL of buffered complex glycerol medium, BMGY, composed of 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 40 g glycerol, 12 mL PTM1 (per liter 0.17 g CaSO₄·2H₂O, 2.86 g K₂SO₄, 0.64 g KOH, 14 g MgSO₄·7H₂O, 4.25 g H₃PO₄, 0.22 g NaCl, 40 g H₂O, 12 mL PTM1 (per liter 5.0 mL of H₂SO₄ (69%), 5.99 g CuSO₄·5H₂O, 1.18 g KI, 3.0 g MnSO₄·H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.02 g H₂BO₃, 0.92 g CoCl₂·6H₂O, 42.18 g ZnSO₄·7H₂O and 65.0 g dO₂ electrode. The inlet-gas flow mode (58 cm s⁻¹) was as follows: 70 °C for 1 min, 30 °C min⁻¹ ramp to 320 °C (3 min). MSD was operated in a mass range of 50–550 amu with 6.6 scans/s and at electron multiplier voltage of 1.10 kV.

3. Results and discussion

In order to generate a \( P. \text{pastoris} \) strain capable of producing (+)-ambrein, several engineering approaches were employed. First, intracellular levels of the precursor of (+)-ambrein, squalene, were significantly enhanced by reducing its flux towards ergosterol biosynthesis. Secondly, the enzyme cascade consisting of \( \text{AsHC D377C} \) and \( \text{BmeTC} \) was heterologously expressed. To analyze all products resulting from the activity of these two enzymes in whole cells, analytical methods including GC-FID and GC-MS were developed and refined that enabled separation of several of these highly similar compounds. Additionally, engineering of \( \text{BmeTC} \) (D373C) led to a novel, more versatile enzyme that is able to catalyze the conversion starting from squalene to 3-deoxyachilleol and further to (+)-ambrein far more efficiently than the previously described two-enzyme cascade. Finally, the potential of the resulting \( P. \text{pastoris} \) strain for (+)-ambrein production at larger scale was demonstrated in a 5L bioreactor cultivation.

3.1. Increasing squalene levels by targeting ERG1 expression and activity

Under standard cultivation conditions, squalene levels in wild type \( P. \text{pastoris} \) are usually below detection limit (Adelantado et al., 2017). To our knowledge, the only study on triterpenoid production in \( P. \text{pastoris} \) in which proteins involved in sterol biosynthesis were targeted focused on accumulation of 2,3-oxidosqualene (Liu et al., 2015). To increase intracellular squalene supply for (+)-ambrein biosynthesis, two strategies, both targeting Erg1p activity, were employed. First, the applicability of the Erg1p inhibitor terbinafine that has been described before to be beneficial for squalene accumulation in \( S. \text{cerevisiae} \) (Garairová et al., 2014) was tested on \( P. \text{pastoris} \). Initial tests revealed that concentrations of up to 6 µg mL⁻¹ of terbinafine did not impair growth of \( S. \text{cerevisiae} \), while \( P. \text{pastoris} \) growth was clearly reduced within the first 24 h of cultivation at terbinafine levels higher than 0.1 µg mL⁻¹ due to a prolonged lag-phase. Compared to the values obtained with 0.1 µg mL⁻¹, a concentration of 0.2 µg mL⁻¹ of terbinafine resulted in 50% reduced optical density while supplementation with 0.4 µg mL⁻¹ terbinafine reduced growth of \( P. \text{pastoris} \) by approximately 80% after 24 h of cultivation. Therefore, the concentration of 0.1 µg mL⁻¹ of terbinafine was used for all experiments. Quantification of squalene and ergosterol levels upon cultivation with or without terbinafine showed a strong effect of terbinafine on squalene levels, yielding 14 mg L⁻¹ after 24 h of cultivation (Fig. 2). Additionally, the native promoter of \( \text{ERG1} \) was replaced by the \( \text{PISI} \) promoter that can be regulated through zinc and inositol levels in cultivation medium (Delic et al., 2013). Significant levels of squalene could be detected in strain \( P_{\text{PSI}}-\text{ERG1} \) after 24 h of cultivation, although slightly lower than the amount obtained with terbinafine supplementation. Interestingly, these two approaches could be combined and yielded 58 mg L⁻¹ of squalene, which is markedly more than just
the effects of each of the two approaches added up (Fig. 2). Furthermore, for the combined approach an effect on sterol biosynthesis was visible as ergosterol levels were clearly decreased.

Apparently, the amounts of zinc or inositol present in yeast extract are suitable to achieve partial repression of the PIS1 promoter while not completely inhibiting sterol biosynthesis, which would likely impair viability and growth of the cells. These experiments also showed that after 48 h of cultivation, the growth-impairing effect of terbinafine (concentrations up to 0.4 µg mL⁻¹) was diminished and all cultures reached the same optical density at this time point irrespective of the tested terbinafine concentration. This might either be due to a possible instability of terbinafine at 28 °C over time or to the possibility that, despite a partial inhibition of ERG1, 48 h constituted a sufficient time range to synthesize the amount of sterols essential for cell growth. Thus, this time point was chosen to repeat supplementation with terbinafine during longer cultivations. To our knowledge, this is the first time that accumulation of high levels of squalene in P. pastoris was achieved.

3.2. Expression of AaSHC D377C and BmeTC in P. pastoris

The first step towards whole-cell (+)-ambrein production was to test if the two terpene cyclases described by Ueda et al. (2013), AaSHC D377C and BmeTC, could be expressed in yeast. Therefore, codon-harmonized genes were designed and protein levels after 48 h of induction of the respective protein were assessed by immunoblotting (Fig. 3). Both proteins could successfully be expressed in P. pastoris. While there was hardly any difference in AaSHC D377C amounts between strain Pₚₛᵢ₅-ERG1 SHC D377C and Pₚₛᵢ₅-ERG1 SHC D377C TC, expression levels of BmeTC were clearly lower in the strain co-expressing AaSHC D377C when the sequential expression strategy was employed.

Localization studies (Fig. S4) revealed that both proteins were membrane-associated in A. acidocaldarius. With AaSHC D377C being expressed about 24 h earlier than TC, membrane space to accommodate BmeTC might be limited. This hypothesis was supported by immunoblot analysis (Fig. 3) of Pₚₛᵢ₅-ERG1 SHC D377C TC samples from cultivations during which expression of AaSHC D377C and BmeTC was induced simultaneously using MeOH. In this case, expression levels of both proteins were clearly lower compared to the strains expressing only one of the two enzymes.

3.3. Analysis of AaSHC D377C and BmeTC products in whole cells

After confirming expression of AaSHC D377C and BmeTC, the next step was to establish analytical methods that allowed separation of all triterpenoids that would be produced. First analyses of P. pastoris strains expressing AaSHC D377C with an established GC-MS method (Ueda et al., 2013) indicated that 3-deoxyachilleol was formed as the squalene peak showed a shift of its maximum. Our work confirmed the findings of Ueda et al. (2013) who stated that these two compounds cannot be separated easily due to their highly similar chemical structure. It should not go unnoticed that contradicting work was published recently (Ke et al., 2018). Consequently, a GC-MS method suitable to separate highly similar compounds such as squalene/3-deoxyachilleol and 8a-hydroxypolyopoda-13,17,21-triene/(+)-ambrein was of utmost importance and therefore established (Fig. 4). Squalene and ergosterol were identified using authentic standards. To confirm 3-deoxyachilleol production, the compound was purified by RP-HPLC and was subsequently identified by NMR (see section 3.3.1 and supplemental information). (+)-Ambrein was identified by mass spectrometry (Fig. S5) and comparison to published results (Rowland and Sutton, 2017), and by using an authentic ambergris standard to confirm (+)-ambrein retention time in GC-FID (Fig. S6).

Furthermore, Ueda et al., 2013, described that BmeTC produces 8a-hydroxypolyopoda-13,17,21-triene, 14β-hydroxynocore-8(26)-ene and onocerinoxide from squalene, which correspond - based on mass spectrometry (Fig. S5) – to peaks number 5, 8 and 9 in Fig. 4. A novel, as yet unidentified product was detected for the BmeTC D373C variant (Fig. 4C, peak 3). We suggest this compound to be a bicyclic derivative of squalene/3-deoxyachilleol based on mass spectroscopy and a strikingly consistent retention time shift observed for squalene (Fig. 4C, peak 1, no cyclic structure), 3-deoxyachilleol (Fig. 4C, peak 2, one cyclic structure) and the novel compound (Fig. 4C, peak 3, 2 cyclic structures proposed). Furthermore, the mass spectrum of this new product (Fig. S5) shows high similarity to bis-(6,11-cyclofarnes-2,7(14)-dien), that is a squalene-derived, symmetric triterpenoid with six-carbon rings at each end of the molecule (Behrens et al., 2000), which corresponds to 3-deoxyachilleol being cyclized also at the other terminus.

Analysis of mass spectra (Fig. S4) revealed that when N,O-bis(trimethylsilyl)trifluoroacetamide was used for derivatization, ergosterol and

**Fig. 2.** Intracellular squalene accumulation in strains WT and Pₚₛᵢ₅-ERG1 after cultivation for 24 h in YPD, without or with 0.1 µg mL⁻¹ of terbinafine. Mean values and standard deviations of biological triplicates are given.

**Fig. 3.** Immunoblot analysis using antibodies directed against FLAG-tag (FLAG-AaSHC D377C) and myc-tag (myc-TC and myc-TC D373C). Pₚₛᵢ₅-ERG1 TC was employed as negative control for α-FLAG detection while Pₚₛᵢ₅-ERG1 SHC D377C was used as negative control for α-myc detection. PonceauS stain of the nitrocellulose membranes was performed as control of transferred protein amount.

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the internal standard cholesterol were completely converted to corresponding trimethylsilyl (TMS) ethers while silylation of the tertiary alcohols \( \alpha \)-hydroxypolypoda-13,17,21-triene, (+)-ambrein and \( \beta \)-hydroxyonocera-8(26)-ene was not successful corresponding well to the findings of Rowland and Sutton (2017). For routine analyses, a GC-FID method was developed (Fig. S6).

### 3.4. Identification of 3-deoxyachilleol by NMR

\(^1\)H- and \(^{13}\)C NMR data of purified 3-deoxyachilleol (Figs. S8 and S9): 

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta = 5.12 \) (4H, m), 4.75 (1H, bs), 4.54 (1H, bs), 2.17–1.87 (14H, m), 1.82–1.70 (2H, m), 1.68 (3H, s), 1.60 (12H, s), 1.55 – 1.35 (5H, m), 1.30–1.10 (2H, m), 0.91 (3H, s), 0.83 (3H, s). \(^{13}\)C NMR (75 MHz, CDCl\(_3\), APT): \( \delta = 149.57, 135.86, 135.25, 135.05, 132.39, 124.58, 124.50, 124.45, 124.20, 108.95, 53.77, 39.93, 39.89, 38.40, 36.54, 35.04, 32.70, 28.60, 28.46, 28.42, 26.94, 26.84, 26.37, 25.84, 24.94, 23.91, 17.84, 16.28, 16.22, 16.16.

The data matches the reported values from literature for 3-deoxyachilleol (Sato and Hoshino, 1999).

### 3.5. Positive impact of \( P_{\text{PIS1}}\)-ERG1 on \( \text{AaSHC} \ D377C \) and \( \text{BmeTC} \) productivity

Considering the beneficial effect of terbinafine combined with exchanging the native ERG1 promoter on squalene accumulation, \( \text{AaSHC} \ D377C \) or \( \text{BmeTC} \) were expressed in this strain background. Triterpenoid and sterol levels were assessed after 72 h of induction in the presence of terbinafine. For \( \text{AaSHC} \ D377C \) (Fig. 5A), the effect of \( P_{\text{PIS1}}\)-ERG1 on 3-deoxyachilleol production levels was visible but not as pronounced as might have been anticipated based on the results in Fig. 2. Substantial amounts of squalene were still present and ergosterol levels were slightly elevated, indicating that squalene was

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**Fig. 4.** GC-MS total ion chromatograms of \( P. \ pastoris \) strains after 72 h of induction. A: strain \( P_{\text{PIS1}}\)-ERG1 \( \text{SHC D377C} \) B: strain \( P_{\text{PIS1}}\)-ERG1 \( \text{TC} \) C: strain \( P_{\text{PIS1}}\)-ERG1 \( \text{TC D373C} \). Compounds detected in cell pellet: squalene (1), 3-deoxyachilleol (2), presumably bicyclic squalene derivative (3), cholesterol-TMS ether (4, internal standard), 8α-hydroxypolypoda-13,17,21-triene (5), (+)-ambrein (6), ergosterol-TMS ether (7), 14β-hydroxyonocera-8(26)-ene (8), onoceranoxide (9).

**Fig. 5.** Triterpenoid levels in strains \( \text{SHC (D377C)} \) and \( P_{\text{PIS1}}\)-ERG1 \( \text{SHC (D377C)} \) (A) or \( \text{TC} \) and \( P_{\text{PIS1}}\)-ERG1 \( \text{TC} \) (B) after induction for 72 h (48 h of induction for \( \text{BmeTC} \)) in media supplemented with 0.1 \( \mu \)g mL\(^{-1} \) of terbinafine. Mean values and standard deviations of biological triplicates are given.
not consumed by AaSHC D377C and was indeed converted to sterol. In contrast, for BmeTC based productivity, the \( P_{\text{ERG1}} \) background was highly beneficial, increasing 8a-hydroxypolypoda-13,17,21-triene yield by approximately four-fold. The efficient flux of squalene towards 8a-hydroxypolypoda-13,17,21-triene also resulted in decreased ergosterol levels. Collectively, this suggests limitations in the productivity of AaSHC D377C, since Sato and Hoshino (1999) reported that in vitro the AaSHC D377C variant exhibited a strongly decreased activity compared to the wild type enzyme.

3.6. Whole cell (+)-ambrein production with AaSHC -D377C and BmeTC

After establishing that both AaSHC D377C and BmeTC exhibited their expected activity in \( P. \text{pastoris} \), the enzymes were co-expressed in the same strain. To provide sufficient amounts of 3-deoxyachilleol as substrate for BmeTC and thereby improve the relative amounts of (+)-ambrein compared to 8a-hydroxypolypoda-13,17,21-triene, the expression of 3-deoxyachilleol and 8a-hydroxypolypoda-13,17,21-triene was induced for 24 h before expressing it in parallel with BmeTC. GC-FID analysis of the cultures revealed that the main products of strain \( P_{\text{ERG1}} \)-AaSHC D373C TC were 3-deoxyachilleol and 8a-hydroxypolypoda-13,17,21-triene. However, a small amount of (+)-ambrein, approximately 2 mg L\(^{-1}\), could also be detected.

3.7. Engineering of BmeTC

The results for strain \( P_{\text{ERG1}} \)-ERG1 SHC D377C TC showed that, while (+)-ambrein was produced to some extent, there seemed to be limitations regarding the efficiency of the enzyme cascade. Distinct amounts of squalene and, remarkably, also 3-deoxyachilleol were found in the cells. Therefore, engineering of BmeTC was considered a promising approach to eventually improve the conversion of 3-deoxyachilleol. Although there was no BmeTC structure available and the sequence homology to the closest-related structure, strikingly AaSHC, is only around 30%, certain amino acid stretches are conserved (Sato et al., 2011). Among these conserved regions we identified the DXDD motif, which has been previously shown to be of central importance for AaSHC-catalyzed reactions. Specifically the D\(^{377C} \) exchange led to a shift in the product spectrum from hopene to 3-deoxyachilleol (Sato and Hoshino, 1999). Therefore, this particular amino acid exchange was introduced forming the BmeTC D373C variant by site-directed mutagenesis. Analysis of triterpenoids of strain \( P_{\text{ERG1}} \)-ERG1 TC D373C (Fig. 4C and Table 2) surprisingly revealed that 3-deoxyachilleol was produced in significant amounts that were comparable to those of strain \( P_{\text{ERG1}} \) SHC D377C. Furthermore, also considerable 8a-hydroxypolypoda-13,17,21-triene levels were detected while 14β-hydroxyonocera-8(26)-ene and onoceranoxide were below detection limit. Remarkably, the strain expressing BmeTC D373C also produced clearly more (+)-ambrein, i.e. 15 mg L\(^{-1}\) in shake flask cultivation, than we achieved through co-expressing AaSHC D377C and BmeTC.

The DXDD motif, which was not only found in squalene-hopene cyclases but also in class II diterpene cyclases (Pristi et al., 2007), is described to be essential in stabilizing the carbocation during conversion of squalene to hopene. When the last aspartic acid in the motif was exchanged for cysteine, the main product of AaSHC became the monocyclic 3-deoxyachilleol instead of the pentacyclic hopene (Sato and Hoshino, 1999). We were highly surprised that by introducing this mutation into BmeTC, the enzyme produced 3-deoxyachilleol but still kept its original ability to generate the bicyclic 8a-hydroxypolypoda-13,17,21-triene. Moreover, it generated (+)-ambrein through its promiscuous activities. However, the tetra- and pentacyclic products 14β-hydroxyonocera-8(26)-ene and onoceranoxide were hardly detectable for the BmeTC D373C variant. This appears to correlate with the findings that the exchange of aspartic acid for cysteine at this position in AaSHC hindered the formation of multicyclic products due of instability of the cation (Sato and Hoshino, 1999). This hypothesis is also supported by the fact that, in contrast to squalene-hopene cyclases, the related enzyme class of oxidosqualene cyclases contains an asparagine at the position corresponding to D\(^{377C} \) in AaSHC while the subsequent amino acid is a cysteine instead of asparagine. Thereby, oxidosqualene cyclases are not able to protonate a carbon-carbon double bond (Gao et al., 2012). Initial studies on the catalytic mechanism of BmeTC applying homology modelling and subsequent testing of active site variants have been published very recently (Tenkovskaia et al., 2017). It was shown that despite a high similarity of the active site architecture between AaSHC and BmeTC, BmeTC exhibits a, so far, unique catalytic mechanism. Also, phylogenetic comparison of squalene-hopene cyclases sequences derived from a range of different bacteria with their homologues found in various Bacillus strains revealed that the second type forms an outgroup from all other bacterial SHCs (Bosak et al., 2008). Solving the BmeTC structure and additional mutational studies would clearly contribute to the detailed understanding of its reaction mechanism.

3.8. Cultivation of \( P_{\text{ERG1}} \)-ERG1 TC D373C in bioreactor

To evaluate the potential of our most advanced strain for industrial purposes, \( P_{\text{ERG1}} \)-ERG1 TC D373C was cultivated in bioreactor at 5 L scale. Pre-tests in shake flasks had shown that despite relatively high concentrations of zinc in the standard bioreactor cultivation medium for \( P. \text{pastoris} \), BSM, no growth inhibition of the engineered \( P_{\text{ERG1}} \)-ERG1 strain background was detected. Samples of the bioreactor culture were taken at different time points during the methanol induction phase and triterpenoid levels were assessed (Fig. 6A). Already before induction with methanol was started, marked amounts of 3-deoxyachilleol, 8a-hydroxypolypoda-13,17,21-triene and (+)-ambrein were observed. This finding correlates with data from Nakagawa et al. (2004) describing that \( P_{\text{FLD1}} \) promoter showed between 20% and 35% of activity when glycerol was used as carbon source instead of methanol. Furthermore, immunoblot analysis (Fig. 6B) confirmed that small amounts of BmeTC D373C were expressed already before induction. Upon induction with methanol, the relative amount of (+)-ambrein compared to 3-deoxyachilleol and 8a-hydroxypolypoda-13,17,21-triene increased significantly, indicating that - in the absence of detectable amounts of squalene – enhanced levels of BmeTC D373C were essential to confer both reactions unto the terpenoid substrates and form (+)-ambrein. After 74 h of induction, cultivation in 5 L bioreactors resulted in an (+)-ambrein production level of 105 mg L\(^{-1}\). Immunoblot analysis showed that BmeTC D373C levels were stable throughout the induction phase.

4. Conclusion

In this study, we demonstrate for the first time the potential of \( P. \text{pastoris} \) for accumulating substantial amounts of squalene by targeting a central enzyme in sterol metabolism, \( \text{ERG1p} \). We established a yeast

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**Table 2**

Triterpenoid levels in strains \( P_{\text{ERG1}} \)-ERG1 SHC (D377C) TC and \( P_{\text{ERG1}} \)-ERG1 TC D373C after induction for 72 h in media supplemented with 0.1 \( \mu \text{g mL}^{-1} \) of terbinfine. Mean values and standard deviations of biological triplicates are given.

| Triterpenoid          | \( P_{\text{ERG1}} \)-ERG1 SHC (D377C) TC | \( P_{\text{ERG1}} \)-ERG1 TC D373C |
|----------------------|------------------------------------------|-----------------------------------|
| Squalene [mg L\(^{-1}\)] | 15.9 ± 4.6                                | 20.1 ± 4.9                        |
| 3-deoxyachilleol [mg L\(^{-1}\)] | 29.3 ± 7.4                                | 63.1 ± 4.6                        |
| 8α-hydroxypolypoda-13,17,21-triene [mg L\(^{-1}\)] | 68.2 ± 12.6                                | 108.2 ± 7.6                       |
| (+)-ambrein [mg L\(^{-1}\)] | 1.9 ± 0.2                                  | 14.9 ± 2.1                        |
| Ergosterol [mg L\(^{-1}\)] | 86.4 ± 7.6                                 | 58.9 ± 4.8                        |
whole-cell system for (+)-ambrein biosynthesis by heterologously expressing two terpene cyclases, AsSHC D377C and BmTC that had been previously applied for conversion of squalene to (+)-ambrein in a two-step in vitro approach with E. coli as host. Engineering of BmTC generated an enzyme that could catalyze the whole reaction from squalene to (+)-ambrein far more efficiently (factor of 7) compared to the previous two-enzyme system, yielding 15 mg L\(^{-1}\) cell culture of (+)-ambrein in shake flasks. Upscaling to 5 L bioreactors resulted in over 100 mg L\(^{-1}\) of (+)-ambrein, underlining the potential of this engineered \textit{P. pastoris} strain as triterpenoid production platform. Combining cell and enzyme engineering approaches, (+)-ambrein yields of our strain clearly exceed production levels of the only other whole-cell system, \textit{E. coli}, that had been reported so far (Ke et al., 2018). Metabolic and enzyme engineering approaches as well as adjusting cultivation and process conditions are highly promising and will further enhance (+)-ambrein yield.

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Competing interests

S.M, G.A.S, T.J.P, K.V. and H.P. have filed a patent application on the strains described in this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mec.2018.e00077.

References

Adelantado, N., Tarazona, P., Grillitsch, K., Garcia-Ortega, X., Monforte, S., Valero, F., Feussner, I., Daum, G., Ferrer, P., 2017. The effect of hypoxia on the liposome of recombinant \textit{Pichia pastoris}. Microb. Cell Fact. 16, 86. http://dx.doi.org/10.1186/s12934-017-0699-4.

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., 2014. Protein expression in \textit{Pichia pastoris}: recent achievements and perspectives for heterologous protein production. Appl. Microbiol. Biotechnol. 98, 5301–5317. http://dx.doi.org/10.1007/s00253-014-5732-5.

Arendt, P., Miettinen, K., Pollier, J., De Rycke, R., Callewaert, N., Goosens, A., 2017. An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metab. Eng. 40, 165–175. http://dx.doi.org/10.1016/j.mybemb.2017.02.007.

Behrens, A., Schaeffer, P., Bernasconi, S., Albrecht, P., 2000. Mono- and bicyclic squalene derivatives as potential precursors for porphyrin synthesis in lacustrine sulfur-rich sediments. Geochim. Cosmochim. Acta 64, 3327–3336. http://dx.doi.org/10.1016/S0016-7037(00)00423-3.

Bosak, T., Lesick, R.M., Pearson, A., 2008. A poly cyclic terpenoid that alleviates oxidative stress. Proc. Natl. Acad. Sci. USA 105, 6725–6729. http://dx.doi.org/10.1073/pnas.0800199105.

Byrne, B., 2015. \textit{Pichia pastoris} as an expression host for membrane protein structural biology. Curr. Opin. Struct. Biol. 32, 9–17. http://dx.doi.org/10.1016/j.jocsbi.2015.01.005.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. Production of \textit{Squalene epoxidase} in \textit{Pichia pastoris} cultures. Curr. Opin. Biotechnol. 13, 329–332. http://dx.doi.org/10.1016/S0958-1669(02)00330-0.

Dai, Z., Liu, Y., Zhang, X., Shi, M., Wang, B., Wang, D., Huang, L., Zhang, X., 2013. Metabolic engineering of \textit{Saccharomyces cerevisiae} for production of ginsenosides. Metab. Eng. 20, 146–156. http://dx.doi.org/10.1016/j.ymbemb.2013.10.004.

Delic, M., Mattanovich, D., Gasser, B., 2013. Repressible promoters - a novel tool to generate conditional mutants in \textit{Pichia pastoris}. Microb. Cell Fact. 12, 6. http://dx.doi.org/10.1186/1475-2859-12-6.

Edelheit, O., Hanukoglu, A., Hanukoglu, I., 2009. Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. BMC Biotechnol. 9, 61. http://dx.doi.org/10.1186/1472-6750-9-61.

Emmerstorfer, A., Wriessnegger, T., Hirz, M., Pichler, H., 2014. Overexpression of membrane proteins from higher eukaryotes in yeasts. Appl. Microbiol. Biotechnol. 98, 7671–7689. http://dx.doi.org/10.1007/s00253-014-5948-4.

Fuhrmann, M., Hausherr, A., Kernitz, L., Schödl, T., Heitzer, M., Hegemann, P., 2004. Monitoring dynamic expression of nuclear genes in \textit{Chlamydomonas reinhardtii} by using a synthetic luciferase reporter gene. Plant Mol. Biol. 55, 869–881. http://dx.doi.org/10.1023/B:PLBI.0000031003.84950.fc.

Gao, Y., Houzatkó, R.B., Peters, R.J., 2012. Terpenoid synthase structures: a so far incomplete view of complex catalysis. Nat. Prod. Rep. 29, 1153–1175. http://dx.doi.org/10.1039/c2np20059g.

Garajová, M., Zambová, V., Šimová, Z., Griač, P., Hapala, I., 2014. Squalene epoxidase as a target for manipulation of squalene levels in the yeast \textit{Saccharomyces cerevisiae}. PEMS Yeast Res. 14, 310–323. http://dx.doi.org/10.1186/1567-1364-12-107.

Geier, M., Braun, A., Emmerstorfer, A., Pichler, H., Glieder, A., 2012. Production of human cytochrome P450 ZD6 drug metabolites with recombinant microbes—a comparative study. Biotechnol. J. 7, 1346–1358. http://dx.doi.org/10.1002/biot.201200187.

Han, J.Y., Seo, S.H., Song, J.M., Lee, H., Choi, E.-S., 2018. High-level recombinant production of squalene using selected \textit{Saccharomyces cerevisiae} strains. J. Ind. Microbiol. Biotechnol. 1–13. http://dx.doi.org/10.1007/s10295-018-1556-4.

Hirz, M., Richter, G., Lettner, E., Wriessnegger, T., Pichler, H., 2013. A novel cholesterol-producing \textit{Pichia pastoris} strain is an ideal host for functional expression of human \textit{Na,K}-ATPase \textit{α3β1} isoform. Appl. Microbiol. Biotechnol. 97, 9465–9478. http://dx.doi.org/10.1007/s00253-014-5948-4.

Fig. 6. A: Triterpenoid levels in \textit{P. pastoris}-\textit{ERG1} TC D373C cultivated in bioreactor. Samples were taken at different time points during methanol induction phase and culture volume corresponding to 10 mg of CDW was prepared for GC-FID analysis. The displayed data represent duplicate measurements. B: Immunoblot analysis of BmTC D373C protein levels during MeOH induction phase and Ponceau\(\text{S}\) stain of the PVDF membrane.
Nakagawa, T., Ito, T., Fujimura, S., Chikui, M., Mizumura, T., Miyaji, T., Yurimoto, H., Madsen, K.M., Udatha, G.D.B.R.K., Semba, S., Otero, J.M., Koetter, P., Nielsen, J., Liu, X.-B., Liu, M., Tao, X.-Y., Zhang, Z.-X., Wang, F.-Q., Wei, D.-Z., 2015. Metabolic engineering of Pichia pastoris for the production of dammarenediol-II. J. Biotechnol. 216, 47–55. http://dx.doi.org/10.1016/j.jbiotec.2015.10.005.

Madsen, K.M., Udatha, G.D.B.R.K., Semb, S., Otero, J.M., Koetter, P., Nielsen, J., Elzinka, Y., Kushihiro, T., Panagiotou, G., 2011. Linking genotype and phenotype of Saccharomyces cerevisiae strains reveals metabolic engineering targets and leads to triterpenes in hyper-producers. PLoS One 6, e14763. http://dx.doi.org/10.1371/journal.pone.0014763.

Moses, T., Poeller, J., Thevelein, J.M., Goossens, A., 2013. Bioengineering of plant (tri)terpenoids: from metabolic engineering of plants to synthetic biology in vivo and in vitro. New Phytol. 200, 27–43. http://dx.doi.org/10.1111/nph.12325.

Näätänen, L., Mistilbeger, B., Ruth, C., Hajek, T., Hartner, F.S., Glieder, A., 2012. Deletion of the Pichia pastoris Kl70 homologue facilitates platform strain generation for gene expression and synthetic biology. PLoS One 7, e39720. http://dx.doi.org/10.1371/journal.pone.0039720.

Nakagawa, T., Ito, T., Fujimura, S., Chikui, M., Mizumura, T., Miyaji, T., Urimoto, H., Kato, N., Sakai, Y., Tomizuka, N., 2004. Molecular characterization of the KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. PLoS One 7, e39720. http://dx.doi.org/10.1371/journal.pone.0039720.

Ohlo, G., K. Riechstoß and Geruchssinn: die molekulare Welt der Düfte. Springer.

Prisic, S., Xu, J., Coates, K.M., Peters, R.J., 2007. Probing the role of the DXDD motif in class II diterpene cyclases. ChemBioChem 8, 869–874. http://dx.doi.org/10.1002/cbic.200700045.

Raza, M., Taha, S.A., El-Khawad, I., 1999. Studies on the cardiovascular effects of ambrein pretreatment in rats. Nat. Prod. Sci. 5, 25–32.

Riechstöß, H., Haase, T., van Loon, A.P., Grivell, L.A., Suda, K., Schatz, G., 1983. Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. EMBO J. 2, 2161–2168.

Rowland, S.J., Sutton, P.A., 2017. Chromatographic and spectral studies of jetsam and archived ambergris. Nat. Prod. Res. 31, 1752–1757. http://dx.doi.org/10.1080/14786419.2017.1290618.

Sato, T., Hoshino, H., Yoshida, S., Nakajima, M., Hoshino, T., 2011. Bifunctional triterpene/sesquiterpene cyclase: tetraprenyl-β-amyrinucumene cyclase is also squalene cyclase in Bacillus megaterium. J. Am. Chem. Soc. 133, 17540–17543. http://dx.doi.org/10.1021/ja2060019.

Sato, T., Hoshino, T., 1999. Functional analysis of the DIXDTA motif in squalene-hopene cyclase by site-directed mutagenesis experiments: initiation site of the polycyclization reaction and stabilization site of the carbocation intermediate of the initially cyclized A-ring. Biosci. Biotechnol. Biochem. 63, 2189–2198. http://dx.doi.org/10.1271/bbb.63.2189.

Seckler, B., Poralla, K., 1986. Characterization and partial purification of squalene-hopene cyclase from Bacillus acidocaldarius. Biochim. Biophys. Acta - Gen. Subj. 881, 356–363. http://dx.doi.org/10.1016/0304-4165(86)90027-9.

Sell, C., 2006. Ingredients for the Modern Perfumery Industry. In: The Chemistry of Fragrances. Royal Society of Chemistry, Cambridge, 52–131. http://dx.doi.org/10.1039/97818553344200002.

Shen, S., Sulter, G., Jeffries, T.W., Gregg, J.M., 1998. A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast Pichia pastoris. Gene 196, 93–102. http://dx.doi.org/10.1016/S0378-1119(98)00315-1.

Taha, S.A., 1992. Studies on the mode of action of ambrein as a new antinociceptive compound. Jpn. J. Pharmacol. 60, 67–71. http://dx.doi.org/10.1016/j.jjp.2016.02.004.

Wriessnegger, T., Pichler, H., 2013. Yeast metabolic engineering for the production of dammarenediol-II. J. Biotechnol. 216, 47–55. http://dx.doi.org/10.1016/j.jbiotec.2015.10.005.