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

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ABSTRACT

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 *Alicyclobacillus acidocaldarius* (*Aa*SHC D377C) and tetraprenyl-β-curcumene cyclase from *Bacillus megaterium* (*Bme*TC) for *in vitro* (+)-ambrein production starting from squalene. Yeasts, such as *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 *P. pastoris* could be strongly enhanced. Heterologous expression of *Aa*SHC D377C and *Bme*TC and, particularly, development of suitable methods to analyze all products of the engineered strain provided conclusive evidence of whole-cell (+)-ambrein production. Engineering of *Bme*TC 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^{-1} of (+)-ambrein, demonstrating that metabolically engineered yeast *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 the sperm whale that represents one of the most valuable animal resources for perfume production. Apart from (+)-ambrein, ambergris also contains several cholestenol-type sterols (Ohloff, 1982). Upon exposure to sea water, sun light and air, (+)-ambrein undergoes oxidative degradation, yielding (-)-ambrox 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 dehydroambraoxid (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 *Alicyclobacillus acidocaldarius* (*Aa*SHC 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* (*Bme*TC) first described by Sato et al. (2011).

For the generation of (+)-ambrein, the authors incubated *Escherichia coli* cell-free extracts expressing the aforementioned terpene cyclases with

*Abbreviations: Aa*SHC, *Alicyclobacillus acidocaldarius* squalene-hopene cyclase; AOX1, alcohol oxidase; *Bme*TC, *Bacillus megaterium* terpene cyclase; BSM, basal salt medium; CDW, cell dry weight; FLD1, formaldehyde dehydrogenase 1; HRP, horse radish peroxidase; PTM1, Pichia trace metals; YNB, yeast nitrogen base; YPD, yeast extract peptone dextrose medium

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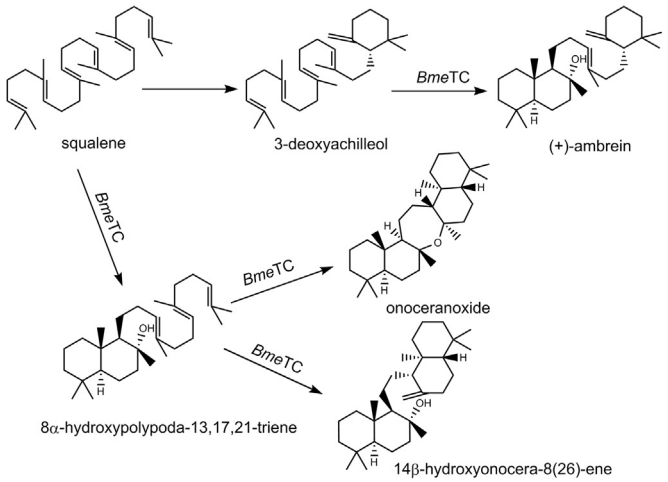

Fig. 1. Generation of (+)-ambrein from squalene using *Aa*SHC D377C and *Bme*TC according to Ueda et al. (2013).

squalene or 3-deoxyachilleol, respectively. The latter had been purified in between of 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 (C_{15}) or carotenoids (C_{40}), a few have also been successful in establishing yeast, especially *Saccharomyces cerevisiae*, as a production platform for triterpenoids (C_{30}). 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 well-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 (Cereghino 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 dammarenediol-II. 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 terbinafine, which resulted in clearly enhanced levels of squalene (Garaiová et al., 2014; Han et al., 2018; Klobučníková et al., 2003), while this study represents the first analysis of the effects of terbinafine on Erg1p in *P. pastoris*. In addition to supplementing terbinafine, also the possibility to downregulate expression of *ERG1* was evaluated. Therefore, the native promoter of *ERG1* was exchanged for the regulatable *PIS1* promoter, which can be partially repressed using zinc or inositol (Delic et al., 2013). On top of converting 3-deoxyachilleol to (+)-ambrein, *Bme*TC can convert squalene to 8α-hydroxypolypoda-13,17,21-triene and, subsequently, 14β-hydroxyonocera-8(26)-ene and onoceranoxide (Fig. 1) (Ueda et al., 2013). To ensure that sufficient amounts of 3-deoxyachilleol can be formed from squalene by *Aa*SHC 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 *Aa*SHC D377C controlled by the *FLD1* promoter (Shen et al., 1998) was induced with methylamine. During the third and last phase of cultivation, both *Aa*SHC D377C and *Bme*TC 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-deoxyachilleol, or 8α-hydroxypolypoda-13,17,21-triene and (+)-ambrein. Following these approaches, together with engineering of *Bme*TC 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

E. coli TOP10F′ (F′[*lac*I^q Tn10(tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 λ$^-$) 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.

Phusion® 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 *PIS1* promoter, an integrative expression plasmid containing the following elements was assembled: *PIS1* 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

Table 1
Strains used in this study.

Strain	Description	Source
Wildtype (WT)	CBS7435 *his4*	Näätsaari et al. (2012)
WT ku70	CBS7435 *his4 ku70*	Näätsaari et al. (2012)
P_{PIS1}-*ERG1*	CBS7435 *his4*, p*Pp*HIS4[P_{PIS1}-*ERG1*]	This study
WT ku70 SHC	CBS7435 *his4 ku70*, p*Pp*Kan[*Aa*SHC D377C]	This study
WT TC	CBS7435 *his4*, p*Pp*FZE[*Bme*TC]	
P_{PIS1}-*ERG1* SHC	CBS7435 *his4*, p*Pp*HIS4[P_{PIS1}-*ERG1*], p*Pp*Kan[*Aa*SHC D377C],	This study
P_{PIS1}-*ERG1* TC	CBS7435 *his4*, p*Pp*HIS4[P_{PIS1}-*ERG1*], p*Pp*FZE[*Bme*TC]	
P_{PIS1}-*ERG1* SHC TC	CBS7435 *his4*, p*Pp*HIS4[P_{PIS1}-*ERG1*], p*Pp*Kan[*Aa*SHC D377C], p*Pp*FZE[*Bme*TC]	This study
P_{PIS1}-*ERG1* TC D373C	CBS7435 *his4*, p*Pp*Kan[*Bme*TC D373C]	This study