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Metabolic engineering of *Saccharomyces cerevisiae* for linalool production

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Abstract
Objectives To engineer the yeast *Saccharomyces cerevisiae* for the heterologous production of linalool. *Results* Expression of linalool synthase gene from *Lavandula angustifolia* enabled heterologous production of linalool in *S. cerevisiae*. Downregulation of *ERG9* gene, that encodes squalene synthase, by replacing its native promoter with the repressible *MET3* promoter in the presence of methionine resulted in accumulation of 78 μg linalool l^{-1} in the culture medium. This was more than twice that produced by the control strain. The highest linalool titer was obtained by combined repression of *ERG9* and overexpression of *tHMG1*. The yeast strain harboring both modifications produced 95 μg linalool l^{-1}. *Conclusions* Although overexpression of *tHMG1* and downregulation of *ERG9* enhanced linalool titers threefold in the engineered yeast strain, alleviating linalool toxicity is necessary for further improvement of linalool biosynthesis in yeast.

Keywords *Lavandula angustifolia* · Linalool · Linalool synthase · Metabolic engineering · Monoterpene · *Saccharomyces cerevisiae*

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Introduction

The isoprenoid family, with over 55,000 compounds, represents the largest and most diverse class of metabolites (Wang et al. 2014; Zhao et al. 2013). Despite their diversity and structural complexity, all isoprenoids are composed of the simple five-carbon building block of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphateisoprene (DMAPP) (Maury et al. 2005; Zhao et al. 2013). Monoterpenes are a subfamily of isoprenoids consisting of two isoprene units and ten carbon atoms. Many of monoterpenes are principal components of essential plant oils and have found numerous applications as flavors and fragrances, biofuels, and feedstock for the

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synthesis of pharmaceuticals and industrial products (Behrendorff et al. 2013).

Many attempts have been made to use microbial cell factories as sustainable resources for the production of monoterpenes (Fischer et al. 2011; Herrero et al. 2008; Jongedijk et al. 2015; Liu et al. 2013; Oswald et al. 2007; Pardo et al. 2015; Rico et al. 2010; Yang et al. 2013; Zhou et al. 2014). In most of the studies either the bacterium *Escherichia coli* (Yang et al. 2013; Zhou et al. 2014) or the yeast *Saccharomyces cerevisiae* (Fischer et al. 2011; Herrero et al. 2008; Jongedijk et al. 2015; Liu et al. 2013; Oswald et al. 2007; Pardo et al. 2015; Rico et al. 2010) were chosen as hosts. However, these microorganisms are unable to accumulate sufficient titers of monoterpenes to support a viable industrial process. Therefore, there is a need to engineer the pathways involved in the synthesis of these compounds.

The yeast *S. cerevisiae* employees the mevalonate (MVA) pathway for the synthesis of isoprenoid precursors. Conversion of 3-hydroxy3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate catalyzed by HMG-CoA reductase is considered as one of the rate limiting steps for the biosynthesis of isoprenoid precursors in the MVA pathway. Overexpression of the catalytic domain of HMG-CoA reductase (*tHMG1*) has been shown to enhance the biosynthesis of sesquiterpenes (Asadollahi et al. 2009, 2010; Scalcinati et al. 2012a, b; Westfall et al. 2012) and monoterpenes (Rico et al. 2010). The farnesyl diphosphate (FPP) branch point comprises another regulatory site in the MVA pathway. Several studies (Asadollahi et al. 2008; 2010; Scalcinati et al. 2012a, b; Ro et al. 2006) have shown that repression of *ERG9* leads to the enhanced production of sesquiterpenes. However, the impact of *ERG9* repression on the biosynthesis of monoterpenes has not been reported.

This study has used *S. cerevisiae* as host for the production of monoterpenes. Linalool was chosen as a model monoterpene. Linalool is an acyclic monoterpene alcohol common to the floral scents of numerous plant species and is extensively used in perfumes, cosmetics, soaps and foods. In plants, linalool synthase catalyzes the conversion of geranyl diphosphate (GPP) to linalool. Several genes encoding for linalool synthase have been isolated and cloned from different plant species such as lavender (Landmann et al. 2007), kiwifruit (Chen et al. 2010), *Clarkia breweri* (Dudareva et al. 1996), *Arabidopsis thaliana* (Chen et al. 2003), rice (Yuan et al. 2008), and grape berries (Zhu et al. 2014). In this study, the *LIS* gene (encoding for linalool synthase) from lavender was heterologously expressed in *S. cerevisiae*. The effects of *tHMG1* overexpression and *ERG9* repression on linalool production were further investigated.

Materials and methods

Plasmid construction

The plasmid pHMG1 was constructed by cloning *tHMG1* in a pESC-URA vector (Stratagene) under control of *GAL10* promoter. The *tHMG1* gene was amplified from the genomic DNA of *S. cerevisiae* CEN.PK 113-7D using the primers hmg1F and hmg1R (see Supplementary Table 1). PCR was performed using *pfu* DNA polymerase (Thermo Scientific) in an reaction mixture containing genomic DNA, deoxynucleotides, reaction buffer and primers. The PCR product was then digested with enzymes *Cla*I and *Sac*I and ligated using T4 ligase into pESC-URA (Stratagene) as expression vector after linearization with *Cla*I and *Sac*I. The ligation mix was used to transform competent *E. coli* cells (DH5). Transformants were selected on LB medium supplemented with ampicillin (50 mg l^{-1}) and verified by sequencing.

The coding sequences of the *LIS* gene from lavender (*Lavandula angustifolia*) (GenBank accession no. DQ263741.1) was previously isolated and cloned in pGEX-4T-1 resulting in construct pGEX-4T-1-LIS (Landmann et al. 2007). The *LIS* gene from lavender was inserted into pHMG1 and pESC-URA plasmids under control of *GAL1* promoter using gap repair technique taking the advantage of high efficiency of homologous recombination in yeast. The PCR was performed using *pfu* DNA polymerase in a reaction mixture containing template plasmid (pGEX-4T-1-LIS), deoxynucleotides, reaction buffer and lisF and lisR primers (see Supplementary Table 1). The plasmids pESC-URA and pS10H were then double digested with *Xho*I and *Bam*HI. The digested plasmids and DNA fragments obtained by PCR were transformed into the competent cells of *S. cerevisiae* CEN.PK 113-5D thus allowing gap repair by homologous recombination. Transformants were selected on SC-URA plates after incubation at 30 °C for 2–3 days. Insertion of the *LIS* gene into pESC-URA and pS10H

plasmids using gap repair technique resulted in pLIS and pHLIS plasmids, respectively.

Strain construction

Table 1 lists yeast strains used in this study. The strains YLIS and YERG_LIS were obtained by transforming pLIS plasmid into the yeast strains CEN.PK 113-5D and YERG, respectively. Overexpression of *tHMG1* in the linalool producing yeast strains was performed by transforming pHLIS plasmid into CEN.PK 113-5D and YERG strains resulting in YHLIS and YERG_HLIS strains, respectively (Table 1).

Production of linalool in shake-flask cultures

The four strains harboring the *LIS* gene on plasmid as well as the control strain (Table 1) were grown in baffled, 500 ml Erlenmeyer flasks. A defined minimal medium (Verduyn et al. 1992) was used. Galactose at 20 g l^{-1} was used as sole source of carbon. The flasks inoculated with the yeast cells were incubated at 30 °C with shaking (180 rpm). The *ERG9* expression in the YERG_LIS and YERG_HLIS strains was repressed by supplementing the media with 2 mM of filter-sterilized methionine.

Quantification and identification of linalool

Cells from stationary phase were harvested by centrifugation (5000×*g* for 5 min). The supernatant was passed through a SPE cartridge (C18, 6 ml 500 mg; Machery-Nagel) pre-equilibrated first with methanol (3 × 10 ml) and then with distilled deionized water (3 × 10 ml). Linalool was eluted with 5 ml dichloromethane. After drying with anhydrous Na_2SO_4, the solvent was evaporated under N_2. Dichloromethane, 0.5 ml, was added to each sample and samples were stored at −20 °C until further analysis.

GC was used for the analysis of linalool and was performed with an HP-5 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). He at 1.5 ml min^{-1} was used as carrier gas. A split/splitless injector was used in the splitless mode. The injector and FID detector were set to 200 and 250 °C, respectively. The oven was initially at 50 °C and, after 1 min, was increased to 75 °C at 10 °C min^{-1} and subsequently increased to 90 °C at 3 °C min^{-1}. The oven was finally increased to 250 °C at 10 °C min^{-1} and then held for 5 min. Linalool concentrations were estimated using standard solutions and are given as the averages of results from at least three independent cultures.

Identification of compounds was by GC–MS. Samples were injected into the inlet liner of an Agilent 6890 N GC equipped with 5973 N mass selective detector. He at 1.5 ml min^{-1} was used as carrier gas. The direct capillary interface linking the GC to the mass spectrometer was maintained at 260 °C during the analytical run. The mass spectrometer was operated in electron impact ionization mode with the electron energy set at 70 eV. The ion source

Table 1 Yeast strains used in this study

Strain	Genotype	Plasmid	Plasmid description	References
CEN.PK 113-5D	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52*	None		Peter Kötter[a]
YERG	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52, erg9::P_{MET3}-ERG9*	None		Asadollahi et al. (2008)
Control	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52*	pESC-URA	pESC-URA 2µ *URA3*	This study
YLIS	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52*	pLIS	pESC-URA 2µ *URA3* P*GAL1*-LIS	This study
YHLIS	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52*	pHLIS	pESC-URA 2µ *URA3* P*GAL1*-LIS P_{GAL10}-*tHMG1*	This study
YERG_LIS	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52, erg9::P_{MET3}-ERG9*	pLIS	pESC-URA 2µ URA3 P*GAL1*-LIS	This study
YERG_HLIS	*MAT* ***a*** *MAL2-8^c SUC2 ura3-52, erg9::P_{MET3}-ERG9*	pHLIS	pESC-URA 2µ URA3 P*GAL1*-LIS P_{GAL10}-*tHMG1*	This study

[a] Institut für Mikrobiologie, der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

temperature was maintained at 230 °C. The column type and oven temperature program were similar to those used in GC analysis.

Results and discussion

Expression of *LIS* gene enables heterologous linalool biosynthesis

To confirm the functionality of the *LIS* gene, the yeast strain bearing pLIS plasmid (YLIS strain) was examined for linalool production. Whereas no peak for linalool was observed for the control strain, appearance of a peak at the retention time corresponding to that of authentic standard indicated heterologous production of linalool and functionality of the *LIS* gene (Fig. 1). The identity of the peak was further confirmed by GC–MS analysis and comparing the MS spectra with those of authentic standard. Linalool was the major product of the *LIS* gene which is in agreement with the results obtained by Landmann et al. (2007). Linalool synthase from *L. angustifolia* exclusively produces R-linalool (Landmann et al. 2007) which has a much lower odor threshold compared to S-linalool (Chen et al. 2010).

Expression of *LIS* gene from *L. angustifolia* gave linalool at 32 μg l^{-1} (Table 2) in the culture medium which is comparable with the amounts obtained by expression of *LIS* gene from *Clarkia breweri* in yeast (Rico et al. 2010). By contrast, Oswald et al. (2007) did not detect any linalool upon expression of *LIS* gene from *C. breweri* in their yeast strains.

Although expression of *LIS* gene from *L. angustifolia* enabled heterologous linalool production in *S. cerevisiae*, the titers are low and therefore further engineering of it to improve linalool production is needed.

Overexpression of *tHMG1* enhances linalool production

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyses the synthesis of mevalonate from HMG-CoA and is considered as one of the rate limiting steps in the MVA pathway. Augmented production of terpenoids can occur upon overexpression of *tHMG1* in yeast strains (Asadollahi et al. 2010; Rico et al. 2010; Scalcinati et al. 2012a, b; Westfall et al. 2012).

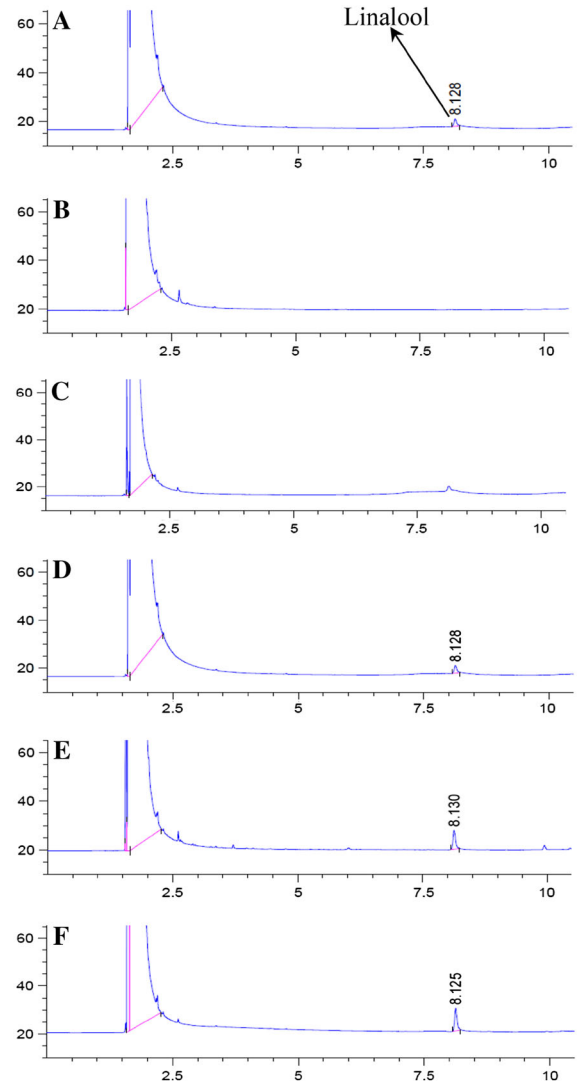

Fig. 1 GC profiles of samples (**a**) Standard (**b**) Control strain (**c**) YLIS strain (**d**) YHLIS strain (**e**) YERG_LIS strain (**f**) YERG_HLIS strain

Table 2 The amounts of linalool produced by each strain

Strain	Linalool (μg l^{-1})
Control	ND[a]
YLIS	32 ± 3.4
YHLIS	51 ± 4.8
YERG_LIS	78 ± 7.3
YERG_HLIS	95 ± 8.6

[a] Not detected

The yeast strain bearing *tHMG1* gene on plasmid (YHLIS) accumulated 51 μg linalool l^{-1} in the culture medium (Table 2). Hence overexpression of *tHMG1* enhanced linalool production by more than 50 %. [It should be noted that the higher production of linalool cannot be attributed to higher cell densities as cultivation of both YLIS and YHLIS strains gave rise to similar ODs in the early hours of stationary phase (data not shown)].

Repression of *ERG9* enhances the production of linalool

The MVA pathway ends with the formation of FPP. Most of the FPP is subsequently utilized for the synthesis of sterols. Ergosterol, the main sterol in *S. cerevisiae*, is responsible for structural membrane features such as fluidity and permeability, in a similar way as cholesterol is in human cells (He et al. 2003). Conversion of FPP to squalene is the first committed step in the synthesis of sterols. This reaction is catalyzed by squalene synthase encoded by *ERG9*. Since FPP is the direct precursors for the biosynthesis of sesquiterpenes, many attempts have been made to divert more FPP towards sesquiterpenes through attenuation of *ERG9* expression (Asadollahi et al. 2008, 2010; Ro et al. 2006; Scalcinati et al. 2012a, b). However, there is no report on the effect of *ERG9* repression on the biosynthesis of monoterpenes. Replacing the native promoter of *ERG9* gene with the repressible *MET3* promoter enables repression of *ERG9* in the presence of methionine. The results of this study revealed improved linalool production using the *ERG9*-repressed yeast strain (YERG_LIS). A linalool titer of 78 μg l^{-1} was obtained using this strain which was more than twice that produced by the YLIS strain. Repression of *ERG9* resulted in even more profound effect on linalool synthesis than did the overexpression of *tHMG1*.

Combined *tHMG1* overexpression and *ERG9* repression further improves linalool production

The effect of simultaneous overexpression of *tHMG1* and down-regulation of *ERG9* on linalool production was also investigated. This strategy improves the flux towards linalool precursors while reduces the flux towards ergosterol. Our previous results (Asadollahi et al. 2010) revealed that overexpression of *tHMG1* in an *ERG9* down-regulated yeast strain does not further improve cubebol production. However, Ro et al. (2006) reported a two-fold improvement in amorphadiene titer after repression of *ERG9* in a yeast strain overexpressing *tHMG1*. The results of this study also revealed that overexpression of *tHMG1* in the *ERG9* repressed yeast strain further improves linalool production. Thus, YERG_HLIS strain produced 95 μg linalool l^{-1}, which is almost 20 % more than that produced by YHLIS (Table 2).

Conclusion

Saccharomyces cerevisiae was engineered for heterologous linalool production. Expression of the *LIS* gene from *L. angustifolia* enabled biosynthesis of linalool though the titers were low. Overexpression of *tHMG1* and down-regulation of *ERG9* improved linalool production. Combination of *tHMG1* overexpression and *ERG9* down-regulation further enhanced linalool production and resulted in an engineered strain producing 95 μg linalool l^{-1}, an amount threefold higher than that produced by YLIS strain (Table 2).

Although several papers have reported heterologous production of monoterpenes using microbial hosts, the titers are still low compared to the titers obtained for other isoprenoids. This could be attributed to the toxicity of monoterpenes and therefore alleviating monoterpene toxicity is required to achieve high concentrations of monoterpenes using microbial hosts.

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Supporting Information Supplementary Table 1—Primers used for the PCR amplifications.

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