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Production of Plant Sesquiterpenes in *Saccharomyces cerevisiae*: Effect of *ERG9* Repression on Sesquiterpene Biosynthesis

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Abstract

The yeast *Saccharomyces cerevisiae* was chosen as a microbial host for heterologous

biosynthesis of three different plant sesquiterpenes, namely valencene, cubebol and patchoulol.

The volatility and low solubility of the sesquiterpenes were major practical problems for

quantification of the excreted sesquiterpenes. *In situ* separation of sesquiterpenes in a two phase

fermentation using dodecane as the secondary phase was therefore performed in order to enable

quantitative evaluation of different strains. In order to enhance the availability of the precursor

for synthesis of sesquiterpenes, farnesyl diphosphate (FPP), the *ERG9* gene which is responsible

for conversion of FPP to squalene was downregulated by replacing the native *ERG9* promoter

with the regulatable *MET3* promoter combined with addition of 2 mM methionine to the medium.

This strategy led to a reduced ergosterol content of the cells and accumulation of FPP derived

compounds like target sesquiterpenes and farnesol. Adjustment of the methionine level during

fermentations prevented relieving *MET3* promoter repression and resulted in further improved

sesquiterpene production. Thus, the final titer of patchoulol and farnesol in the *ERG9*

downregulated strain reached 16.9 and 20.2 mg/L, respectively. The results obtained in this study

revealed the great potential of yeast as a cell factory for production of sesquiterpenes.

INTRODUCTION

Isoprenoids with more than 40,000 described compounds (Withers and Keasling, 2007) are the

largest and most structurally diverse group of plant metabolites. Isoprenoids play many different

biological roles in plants. Some of them play essential functional roles in photosynthesis

(chlorophylls, plastoquinone, and carotenoids), photoprotection (carotenoids), growth regulation

(gibberellic acid, abscisic acid, cytokinins, and brassinosteroids) and membrane structure and

function (phytosterols) (Chappell, 1995; McGarvey and Croteau, 1995; Wanke et al., 2001). However, most of the plant isoprenoids are not essential for viability and cell growth and are considered as secondary metabolites.

Isopentenyl diphosphate (IPP) which is the universal biological precursor of all isoprenoids (basic C_5 isoprene unit) can be obtained either through the mevalonate (MVA) or the 2-methylerythritol 4-phosphate (MEP) pathways.

Depending on the number of isoprene units, isoprenoids can be classified into several groups, such as monoterpenes, sesquiterpenes and diterpenes (respectively 2, 3 and 4 C_5 units). Sesquiterpenes are the most diverse class of isoprenoids with more than 300 identified carbon skeletons (Cane, 1999) and more than 7,000 characterized compounds (Connolly and Hill, 1991). From an industrial point of view, sesquiterpenes are interesting compounds because of their potent anticancer, antitumor, cytotoxic, antiviral and antibiotic properties as well as their characteristic flavours and aromas. Most sesquiterpenes are constituents of plant essential oils, but extraction from plants is not a suitable method for large scale production of many sesquiterpenes. Furthermore, using plants as resources for industrial production of sesquiterpenes include slow growth, varying composition and concentration depending on the geographical position and climate conditions. There is therefore much interest in using microorganisms as cell factories for the production of isoprenoids. The intracellular pools of isoprenoid precursors in microorganisms appear, to be however, not enough to provide high level production. It may therefore be necessary to deregulate the pathways involved in the biosynthesis of isoprenoid precursors in order to improve production (Kim and Keasling, 2001, Martin et al., 2003; Pitera et al., 2007). In the last decade numerous studies have been conducted with regard to engineer microorganisms for the production of different isoprenoids (For review see Maury et al., 2005).

In most studies *Escherichia coli* has been used as the host of interest for metabolic engineering of isoprenoid production (Alper et al., 2005; Carter et al., 2003; Martin et al., 2001). However, in some cases the MVA pathway in yeasts has been deregulated to improve the biosynthesis of different isoprenoids (DeJong et al., 2006; Jackson et al., 2003; Miura et al., 1998a; Miura et al., 1998b; Ro et al., 2006; Shimada et al., 1998; Yamano et al., 1994). In *Saccharomyces cerevisiae* the MVA pathway is the only pathway involved in the biosynthesis of isoprenoid precursors. This pathway leads to the formation of ergosterol as the major end product of the pathway, the content of which in *S. cerevisiae* can reach 5% of the dry weight (Lamačka and Šajbidor, 1997). Yeast therefore has a high inherent capacity for the biosynthesis of isoprenoid precursors that may be directed to the production of heterologous compounds.

In this study, we chose *S. cerevisiae* as a host cell for heterologous production of three different plant sesquiterpenes namely valencene, cubebol and patchoulol. These three compounds are derived directly in a single enzymatic step from farnesyl diphosphate (FPP), the biological precursor for all sesquiterpenes. In the case of ergosterol, FPP is converted to squalene by squalene synthase, which is encoded by the *ERG9* gene. In order to increase the level of FPP and direct flux towards sesquiterpenes an obvious strategy is to attenuate the expression of the *ERG9* gene. Since ergosterol is vital for yeast growth and yeast cells are unable to assimilate exogeneous ergosterol during aerobic growth conditions, this gene can not be completely deleted. We therefore controlled the *ERG9* expression by using the *MET3* promoter (Gardner and Hampton, 1999; Mountain et al., 1991) to drive the expression of *ERG9*. This strategy has been successfully reported to increase two-fold the final titer of amorphadiene in an engineered yeast strain (Ro et al., 2006).

Since sesquiterpenes are highly volatile compounds loss of produced sesquiterpenes during fermentation through the off-gas is a major problem for characterization of the engineered strains (Martin et al., 2001; Martin et al., 2003). To circumvent this problem *in-situ* separation of released sesquiterpenes was performed in a two-phase fermentation using an organic solvent as the secondary phase.

MATERIALS AND METHODS

Cloning of *MET3* Promoter

The *MET3* promoter was amplified from genomic DNA of *S. cerevisiae* CEN.PK 113-7D using the primers ggactagtcctTGGTATAAGGTGAGGGGGTCCACAG and tccccgcggggaGAATACCACCGTGAGGAGCAGGCATG. The PCR conditions were in accordance with the Expand High Fidelity standard conditions (Roche Applied Science). Subsequently, the PCR fragments were digested by the restriction enzymes SpeI and SacII. In parallel, pUG6 plasmid (Güldener et al., 1996) was digested by the same couple of restriction enzymes. The DNA fragments were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen).

In vitro ligation of the digested plasmid with the digested PCR products was performed as the standard procedure given for T4 DNA ligase (Roche Applied Science). The resulting ligation mix was used to transform chemically competent *E. coli* cells (DH5α) (Inoue et al., 1990). Transformants were selected on LB medium supplemented with ampicillin (50 mg/L). The plasmid obtained was named pIP007.

ERG9 Promoter Replacement by MET3 Promoter

In order to replace the *ERG9* promoter by the *MET3* promoter, fusion PCR and a bipartite gene

targeting method (Erdeniz et al., 1997) were applied. Four fragments were separately amplified

before fusing them together in pairs. First, two fragments containing the *MET3* promoter and the

KanMX selection cassette were amplified from pIP007 in two separate, but overlapping,

fragments using the 2 couples of primers:

gatccccgggaattgccatgACGCTGCAGGTCGACAACCC/CCATGAGTGACGACTGAATCCGG

(Fragment A) and

CTATCGATTGTATGGGAAGCCCG/caatgccaattgtaatagcttcccatGTTAATTATACTTTATTCT

TGTTATTATTATAC (Fragment B). Furthermore, 500 bp upstream of the *ERG9* promoter in the

genome of *S. cerevisiae* were amplified using primers AGCCTCAGTACGCTGGTACCCG and

catggcaattcccgggggatcTGGGCTATGAAATGTACTGAGTCAG (Fragment C). The first 500 bp

of the *ERG9* ORF were as well amplified using primers

ATGGGAAAGCTATTACAATTGGCATTG and GTCGTAGTCGTGGACGGTTTGC

(Fragment D).

The resulting 4 PCR fragments were gel purified using the High Pure PCR Product Purification

kit (Roche Applied Science) and subsequently fused together in pairs using fusion PCR. Fused

fragments A and C were obtained after a fusion PCR using primers

AGCCTCAGTACGCTGGTACCCG and CCATGAGTGACGACTGAATCCGG while fused

fragments B and D were obtained after a fusion PCR using primers

CTATCGATTGTATGGGAAGCCCG and GTCGTAGTCGTGGACGGTTTGC. The two final

fusion PCR fragments were gel purified with the High Pure PCR Product Purification kit (Roche

Applied Science).

Strain Construction

Strain *S. cerevisiae* YIP-0V-01, obtained by transformation of strain YIP-00-03 by the plasmid pIP031 (plasmid obtained by cloning GFTpsD in pYX212 using EcoRI and NheI sites) was transformed with the obtained fusion PCR fragments to result in strain YIP-MV-02.

Strain YIP-M0-04 was obtained after exclusion of plasmid pIP031 from strain YIP-MV-02 by selection on plates containing 5-fluoroorotic acid (5-FOA). Strains YIP-MC-02, YIP-MV-01 and YIP-MP-01 were obtained after transformation of strain YIP-M0-04 by either pIP032 (obtained by cloning GFTpsC into pYX212 using EcoRI and NheI sites), pIP027 (obtained by cloning GFTpsD into pESC-URA using AgeI and SacII sites) or pIP029 (obtained by cloning PatTps177 into pESC-URA using BamHI and XhoI sites), respectively.

Transformation of strain YIP-00-03 by either pIP032, pIP013 (obtained by cloning of GFTpsC into pESC-TRP by Gap repair using the 2 primers ATACTTTAACGTCAAGGAGAAAAAACCCCGGATCCCGTTatggcacttcaagattcaga and TCTTCTTCGGAAATCAACTTCTGTTCCATGTCGACGCTtcaaaagggaacaggcttct), pIP027 or pIP029 led to strains YIP-0C-01, YIP-0C-02, YIP-0V-02 and YIP-0P-02, respectively. Table 1 demonstrates the strains used in this study.

Sequences of the genes GFTpsD (valencene synthase), GFTpsC (cubebol synthase) and PatTps177 (patchoulol synthase) can be obtained from Genbank: accession numbers CQ813508, CQ813505 and AY508730, respectively.

Media for Shake Flasks

Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used for preparing pre-cultures and also for investigating the effect of different organic solvents on growth. The shake flasks contained

100 ml medium with the following composition: 7.5 g/L $(NH_4)_2SO_4$; 14.4 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 2 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam (Sigma). The pH of mineral medium was adjusted to 6.50 by adding 2M NaOH and autoclaved separately from the carbon source solution. Vitamin solution was filter sterilized and aseptically added to the medium after autoclaving. Shake flasks were incubated in a shaking incubator at 30 ºC and 150 rpm.

Media for Batch Cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of either glucose or galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 5 g/L $(NH_4)_2SO_4$; 3 g/L KH_2PO_4; 0.5 g/L $MgSO_4.7H_2O$; 1 ml/L trace metal solution; 1 ml/L vitamin solution and 50 µl/L synperonic antifoam.

Glucose and galactose were autoclaved separately from the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization. Expression of *ERG9* was repressed by supplementing media with 2 mM filter sterilized methionine.

Batch Fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2M NaOH. The temperature was kept constant at 30 ºC. The air flow was 4 L/min (1 vvm) and was sterilized by filteration and the off gas passed through a condenser.

Agitation was adjusted to maintain the dissolved oxygen tension above 20% of air saturation. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Denmark). Batch fermentors were inoculated to an initial OD_{600} of 0.02 from a liquid pre-culture. 300 ml dodecane was added aseptically to the media at OD_{600} 0f 1 ± 0.1.

OD and Dry Weight Determinations

The OD of samples was determined at 600 nm in duplicate by using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved by using 0.45 µm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

Determination of Ergosterol Content

Ergosterol content of yeast cells was determined as described by Breivik and Owades (1957) with slight modifications. 500 ml baffled, cotton-stopped, shake flasks containing 100 ml minimal medium and 20 g/L glucose as carbon source were inoculated with one colony of different yeast strains and incubated for 24 h in a shaking incubator at 30 ºC and 150 rpm. A certain volume of culture media corresponding to approximately 100 mg of dry cells was harvested by centrifuging at 3000 rpm for 5 min. The cell pellet was washed with distilled water and the cell suspension was centrifuged for another 5 min at 3000 rpm. 10 ml of 25% alcoholic potassium hydroxide solution (dissolving 25 g KOH in distilled water to 40 ml and then brought to 100 ml with absolute ethanol) was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to 100 ml round bottom flasks. The flasks were connected to a water cooled

condenser and samples were saponified in a water bath for 3 h at 90 ºC. Following incubation

flasks were allowed to cool to room temperature. The contents of flasks were transferred to new

glass tubes and the non-saponified sterols were extracted by adding 10 ml heptane followed by

vigorous vortex mixing for 2 min. For each set of experiments a fresh blank was prepared by

adding 10 ml heptane to 10 ml of alcoholic potassium solution and vortex mixing for 2 min. After

30 min when the heptane layer had clarified, 0.5 ml of heptane layer in both sample and blank

was diluted ten-folds in 4.5 ml absolute ethanol. The absorbance of all samples was read against

blank at 230 and 281.5 nm. The ergosterol content was calculated as milligram ergosterol per

gram dry weight using the following equation:

$$Ergosterol(mg\,/\,gDW) = \left(\frac{OD_{281.5nm}}{290} - \frac{OD_{230}}{518} \right) \times F$$

where F is a correction factor for dilutions and sample sizes and 290 and 518 are $E^{(1\%,\,1\,cm)}$ of

crystalline ergosterol and 24(28)-dehydroergosterol, respectively.

Analysis of Sugars and Extracellular Metabolites

To determine the concentration of sugars and extracellular metabolites in the culture media, 2 ml

samples were withdrawn from the fermentor and immediately filtered through a 0.45 μm pore-

size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was stored at -20 ºC

until HPLC analysis. Glucose, galactose, glycerol, pyruvate, succinate, acetate, and ethanol

concentrations were determined in a Waters 717 plus Autosampler HPLC system equipped with a

Bio-Rad Aminex HPX-87H reverse phase column (Biorad, Hercules, CA) at 60 ºC using 5 mM

H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Glucose, galactose, glycerol, succinate, and

ethanol were detected refractometrically (Waters 410 Differential Refractometer Detector,

Millipore Corp., Milford, MA). Pyruvate and acetate were determined spectrophotometrically with a Waters 490E Programmable Multiwavelength Detector set at 210 nm

Analysis of Sesquiterpenes in the Organic Layer

Samples from organic layer were centrifuged for 5 min at 3500 rpm and subsequently analyzed by GC-MS to determine the level of sesquiterpenes during the course of fermentation. GC-MS analyses were run on a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer. Analytes from 1 µl samples were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) using helium as carrier gas at the flow rate of 1.2 ml/min. A split/splitless injector was used in the splitless mode. The initial oven temperature was 80 ºC and injector temperature was 250 ºC. After 1 min the oven temperature was increased to 120 ºC at the rate of 10 ºC/min and subsequently increased to 160 ºC at the rate of 3 ºC/min. The oven temperature was finally increased to 270 ºC at the rate of 10 ºC/min and held for 5 min at this temperature.

Quantification of compounds was carried out using standard curves generated after each analysis run.

Identification of Sesquiterpenes by Solid Phase Microextraction (SPME) Method

Identification of sesquiterpenes in the yeast strains transformed with heterologous sesquiterpene synthases was performed by extracting volatile compounds from the head-space of the samples on 100 µm polydimethyl siloxane (PDMS) fibers (Supelco, Bellefonte, PA, USA). Fibers were conditioned according to the suppliers' instruction. Two ml samples from the culture media were immediately frozen and stored in 4 ml screw cap glass vials at -20 ºC until analysis. The contents

of vials were thawed on ice and allowed to equilibrate at room temperature. Extraction was

performed for 25 min at 60 ºC while mixing the sample with a small magnet. After extraction the

analytes were thermally desorbed at 250 ºC from SPME fiber into the injector of gas

chromatogram in the splitless mode. The oven temperature was held initially at 45 ºC for 1 min,

then raised to 130 ºC by a ramp of 10 ºC/min followed by a ramp of 3 ºC/min to 160 ºC. The

oven temperature was further raised to 250 ºC at 10 ºC/min and maintained at this temperature for

5 min to equilibrate.

Analysis of Methionine

The concentration of methionine in the media was measured by HPLC by the method of Barkholt

and Jensen (1989).

RESULTS

Sesquiterpene Synthase Genes are Functional in the Engineered Yeast Strains

Yeast strains were transformed with individual plasmids bearing one of the three sesquiterpene

synthase cDNAs under control of *TPI* or *GAL* promoters: a valencene synthase (GFTpsD) and a

cubebol synthase (GFTpsC) from grapefruit and a patchoulol synthase (PatTps177) from

patchouli. To confirm the functionality of these plasmids the transformed strains were grown in

shake flasks containing 100 ml minimal medium and 20 g/L galactose as carbon source. Volatile

compounds were adsorbed on SPME fibers and analyzed by GC-MS. All the transformed strains

were able to synthesize sesquiterpenes. In the case of valencene synthase, valencene was clearly

the major product (Fig. 1-a) in accordance with the relative high product specificity observed

with this enzyme in *in-vitro* assay (data not shown). However, the patchoulol synthase and the

cubebol synthase are multiple product enzymes. The patchoulol synthase produces, in *in-vitro* assays, 14 different sesquiterpenes with patchoulol as major product (Deguerry et al, 2006). Similarly, the cubebol synthase produces mainly cubebol besides at least 14 additional products (unpublished data). Analysis of the volatiles produced by yeast cells transformed with either the patchoulol or cubebol synthase cDNA revealed the similar sesquiterpene profiles as observed in the *in-vitro* assays. Figures 1-b and 1-c illustrate the range of sesquiterpene products produced by the cubebol synthase from grapefruit and patchoulol synthase from patchouli, respectively.

Minimum Methionine Concentration for *ERG9* repression

The minimum concentration of methionine for adjusting the expression of *ERG9* was determined by growing the *ERG9* repressed yeast cells in shake flasks supplemented with varying amounts of methionine (Fig. 2).

Repression of *ERG9* led to lower specific growth rates compared to the wild type strain and also reduced the final concentration of biomass in the medium presumably as a result of decreased flux towards ergosterol. Increasing the methionine concentration to 1 mM resulted in an elongation of the lag phase and a lower final biomass concentration. However, there was no further effect on growth profile at concentrations of methionine above 1 mM (Fig. 2). So the minimum required level of methionine should be 1 mM. However, to maintain the concentration of methionine at the minimum required level for *ERG9* repression during the course of fermentation, 2 mM methionine was chosen for running fermentations.

Repression of *ERG9* is likely to decrease the ergosterol content in the yeast strains. This was confirmed by determining the ergosterol content of the *ERG9* repressed strains and comparing to wild type. For the wild type the ergosterol content was found to be 18.75 ± 1.92 mg ergosterol/g

DW, whereas it was found to be 14.95 ± 2.91 mg ergosterol/g DW for YIP-MV-02 when there was no methionine added to the medium and 3.31 ± 0.04 mg ergosterol/g DW when 2 mM methionine was added to the medium.

Screening Different Solvents for *in situ* Separation of Sesquiterpenes

The volatility and low solubility of sesquiterpenes proved a major practical problem in characterizing the engineered sesquiterpene producing strains (data not shown). To circumvent this problem a two-phase fermentation using a biocompatible organic solvent was developed for the characterization of the constructed strains. Two-phase fermentation has been reported for *in situ* separation of amorphadiene produced by metabolically engineered *E. coli* (Newman et al, 2006).

Selection of an appropriate solvent is the key driver for successful operation of a two-phase fermentation. We first identified three different organic solvents (diisononyl phthalate, oleyl alcohol, and dodecane) which seemed to have suitable characteristics (biocompatibility, immiscibility, and low volatility) and investigated their applicability in two-phase fermentations in shake flasks with *S. cerevisiae*. None of these solvents had significantly detrimental effects on the specific growth rate (Fig. 3). However, phase separation was faster and clearer when dodecane was the organic solvent, and this solvent was therefore chosen for the subsequent experiments. The effect of dodecane on the growth was further examined in a 5 L batch fermenter, and it was found not to have any negative effects on cell growth (data not shown).

Two-Phase Fermentation: An Efficient Technique for Characterizing the Engineered Sesquiterpene Producing Yeast Strains

Yeast strains transformed with plasmids harboring each of the three sesquiterpene synthase genes under control of either *TPI* or *GAL* promoters were characterized in two-phase 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose. Samples were taken from the organic phase at different times and were analyzed for sesquiterpene content. Time-course analysis (Fig. 4) showed collectively accumulation of sesquiterpenes in the dodecane phase as cells were growing and proved the potential of this system for capturing the volatile, hydrophobic compounds. At the end of each fermentation a sample from the aqueous phase was analyzed by SPME to check the presence of sesquiterpenes in the aqueous phase. However no sesquiterpenes were detectable reflecting the high partition coefficient of these compounds into the dodecane phase.

Table 2 summarizes the final concentrations and yields of the sesquiterpenes for the yeast strains harboring synthase genes. However patchoulol constitutes only 37% of the total sesquiterpenes synthesized by the patchoulol synthase (Deguerry et al., 2006) and the total concentration and yields of this sesquiterpene (strain YIP-0P-02) are significantly higher than for the other strains indicating higher activity of patchoulol synthase than valencene and cubebol synthases. In the same way, cubebol represents only 28% of sesquiterpenes produced by the cubebol synthase (unpublished data) and the total quantities of sesquiterpenes produced by strains expressing this enzyme are higher than for strains expressing the valencene synthase indicating that cubebol synthase is also more active than valencene synthase. Total sesquiterpene concentrations were estimated based on the known percentage of the target sesquiterpenes produced by the different sesquiterpene synthases (28% and 37% for cubebol and patchoulol, respectively).

Replacement of *GAL* promoter with *TPI* promoter for controlling cubebol synthase gene reduced the efficiency of sesquiterpene production both in term of final concentration and yield when galactose was the carbon source (YIP-0C-01 vs. YIP-0C-02).

ERG9 Repression Diverts FPP towards Farnesol

Analysis of dodecane layer from two-phase batch fermentation of the *ERG9* repressed strain without any sesquiterpene synthase (YIP-M0-04) revealed formation of farnesol as an FPP-derived compound during fermentation .

The yield of farnesol when cells were assimilating galactose as carbon source was 10.90 mg/g DW and the final titer was 22.6 mg/L. The concentration and yield of the compounds directly derived from FPP are higher when *ERG9* is repressed. Taken together with the reduced ergosterol content of the *ERG9* repressed strains, this confirms the possibility of using this strategy for enhancing FPP availability for the sesquiterpene synthases.

Combination of ERG9 Repression and Sesquiterpene Synthase Expression

ERG9 repression was shown to result in formation of FPP derived by-products and therefore it is likely that FPP availability for sesquiterpene synthases was improved. Hence, combination of *ERG9* repression and sesquiterpene synthase expression was expected to give rise to higher accumulation of sesquiterpenes. To investigate this hypothesis the YIP-M0-04 strain was transformed with plasmids each bearing one of the three sesqueterpene synthase genes and the constructed strains were characterized in 5 L batch fermenters. None of the sesquiterpene synthase genes were able to convert all the available FPP to the target sesquiterpene and again farnesol was observed as FPP derived by-product (Fig. 5).

Comparing the final concentrations of desired sesquiterpenes and farnesol produced by *ERG9* repressed strains and the corresponding strains where *ERG9* is under control of the endogenous promoter (Table 2 and Table 3) indicate that except for the patchoulol synthase, repression of *ERG9* did not lead to significantly higher amounts of sesquiterpenes but mainly resulted in the diverting of FPP to farnesol. This again shows that patchoulol synthase activity in the yeast cells is higher than the activities of the other two sesquiterpene synthases.

Replacing the *GAL* promoter in the YIP-MV-01 strain with *TPI* promoter (YIP-MV-02) resulted in lower amount of valencene and higher yield and concentration of farnesol.

Regulating Methionine Concentration Alters the Flux towards Sesquiterpenes

ERG9 downregulation was not concomitant with considerable improvement in the final concentrations of the target sesquiterpenes, but comparing the yields (Table 2 and Table 3) demonstrates that there was some increase in the yields. Plotting the sesquiterpene concentration versus dry weight of biomass or galactose concentration allowed calculation of yields and this revealed two different linear phases during the galactose consumption phase (Fig. 6) suggesting that the rate of sesquiterpene accumulation in the organic phase dropped at one time during the fermentation. It was speculated that methionine was metabolized by the cells and the subsequent reduced levels of methionine would relieve a true repression of the *ERG9* gene and consequently lowered the accumulation rate of sesquiterpenes. Analyzing the methionine concentration in the culture media confirmed that there was a total consumption of methionine (data not shown). To examine the effect of methionine concentration on the level of products we therefore ran fermentations with the YIP-MP-01 strain using higher amounts of methionine added to the medium at different times during fermentation. Maintaining the methionine concentration at

higher level during the fermentation resulted in a 47% increase in the final patchoulol titer and a 82% increase in the final farnesol titer. Thus, the final concentrations of patchoulol and farnesol reached to 16.9 and 20.2 mg/L, respectively.

Farnesol is Converted to other By-Products at the End of Fermentation

It was noticed that there is a drastic drop in the farnesol concentration for the *ERG9* repressed strains when all carbon sources had been depleted. This decrease could not be a result of evaporation of farnesol from the organic phase since the same pattern was not observed for other compounds. We speculated that at the end of fermentation and after depletion of all carbon sources, farnesol is further converted to other related compounds and appearance of a second peak during GC-MS analysis for FPP-derived compounds at the end of the fermentations supported this hypothesis (Fig. 7).

Galactose is Preferred Carbon Source for Biosynthesis of Sesquiterpenes

Galactose is an expensive carbon source and *S. cerevisiae* posses a lower specific growth rate on this carbon source. Therefore for industrial fermentation it would be desirable to use glucose as carbon source. We investigated the possibility of using glucose in the media by growing yeast cells expressing cubebeol and valencene synthases under control of the *TPI* promoter (YIP-MC-02 and YIP-MV-02, respectively) using glucose rather than galactose as carbon source. However, the results showed that both the final titer and the yield were lower when cells were grown on glucose (Table 3 and Table 4).

DISCUSSION

In this study we report heterologous production of three different plant sesquiterpenes in the yeast *S. cerevisiae*. The corresponding sesquiterpene genes for cubebol, valencene and patchoulol were expressed in yeast and were shown to be functional, and this is the first instance of a cubebol synthase being expressed for heterologous production of cubebol.

Volatility of the sesquiterpenes and the loss of a considerable amount of products through the off-gas was a bottleneck for characterization of the constructed strains. To overcome this problem, we demonstrated the *in situ* separation of the synthesized sesquiterpenes using a two-phase fermentation process, where the second phase was an organic solvent. We examined three organic solvents (dodecane, diisononyl phthalate and oleyl alcohol), and none of these solvents showed significantly detrimental effect on the growth rate. For further work we chose dodecane since phase separation was faster and more distinct for this solvent.

The availability of FPP which is the precursor for all sesquiterpene synthases was enhanced by replacement of the native *ERG9* promoter with a regulatable *MET3* promoter and repressing the promoter with the presence of 2 mM methionine. The ergosterol content was drastically reduced as a consequence of this repression. Nevertheless, this strategy did not improve appreciably the production of the target sesquiterpenes but resulted in further production of farnesol as an FPP-derived by-product. Consistent with this finding are accumulation of farnesol in *Candida albicans* (Buurman et al., 2004; Hornby et al., 2003) as a consequence of treatment with zaragozic acid, which is potent inhibitor of squalene synthase. Furthermore, deletion of *ERG9* gene in *S. cerevisiae* was reported to lead to accumulation of farnesol in *S. cerevisiae* (Song, 2003). Poor expression of the plant sesquiterpene genes may explain why sesquiterpene synthesis was not improved significantly after repression of *ERG9*. The use of codon optimized

sesquiterpene synthases may help in diverting more FPP towards the desired sesquiterpenes. The production of amorphadiene in yeast was significantly enhanced when the codon usage of the amorphadiene synthase was optimized (Martin et al., 2001; Martin et al., 2003).

Metabolism of methionine during the growth phase relieved the repression of *ERG9*, which was accompanied by a reduction in the rate of sesquiterpene synthesis. Further experiments showed that it was possible to improve the sesquiterpene production by supplying more methionine during fermentation. Under these conditions the final titer of patchoulol and farnesol reached to 16.9 and 20.2 mg/L, respectively. More precise regulation of methionine should improve further the efficiency of sesquiterpene generation.

The effect of replacing galactose as an expensive carbon source with glucose, which is less expensive and is faster metabolized by cells, on the sesquiterpene biosynthesis was investigated. However, higher final titer and yield were observed when cells were grown on galactose. Considering that the reported titers and yields have been achieved only by downregulating one gene in the pathway, this work demonstrates the capacity of yeast as a cell factory for the production of sesquiterpenes.

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Figure Legends

Figure 1. GC profile of the sesquiterpenes produced by yeast strains expressing (a) valencene synthase (YIP-0V-02) (b) cubebol synthase (YIP-0C-02) (c) patchoulol synthase (YIP-0P-02)

Figure 2. Effect of methionine on growth pattern of *ERG9* repressed yeast strain (YIP-MV-02) cultivated in shake flasks containing minimal medium and 20 g/L glucose. (Without methionine: □; 0.25 mM methionine: ■; 0.50 mM methionine: ▲; 1.0 mM methionine: △; 1.5 mM methionine: ◆; 2 mM methionine: ◇)

Figure 3. Growth pattern of YIP-0V-01 strain in the presence of 10% (v/v) different organic solvents; No solvent: ●; Diisononyl phthalate: ▼; Dodecane: ○; Oleyl alcohol: △

Cells were cultivated in shake flasks containing 100 ml minimal medium and 15 g/L glucose. Organic solvent was added aseptically at OD=1 ± 0.1.

Figure 4. Sesquiterpene concentration as a function of time for yeast strains expressing sesquiterpene synthases (a) YIP-0C-01 (b) YIP-0C-02 (c) YIP-0V-02 (d) YIP-0P-02

Figure 5. Target sesquiterpenes and farnesol concentrations as a function of time for *ERG9* repressed yeast strains expressing sesquiterpene synthases (a) YIP-MC-02 (b) YIP-MV-01 (c) YIP-MV-02 (d) YIP-MP-01; Valencene (◆) Cubebol (●) Patchoulol (■) Farnesol (△)

Figure 6. Target sesquiterpenes and farnesol concentrations as a function of galactose concentration for calculating yields on galactose. (a) YIP-MV-01 (b) YIP-MC-02 (c) YIP-MP-01; Valencene (♦) Cubebol (●) Patchoulol (■) Farnesol (△)

Figure 7. Farnesol degradation at the end of fermentation and appearance of an unknown compound. (a) YIP-MV-01 (b) YIP-MC-02; Farnesol (△) Unknown comound (●)

Table Legends

Table 1. Strains used in this study

Table 2. Final concentrations of target sesquiterpenes and total sesquiterpenes and yields of target sesquiterpenes (mg sesquiterpene per gram dry weight or gram consumed galactose in the galactose consumption phase) for yeast strains expressing sesquiterpene synthases. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Table 3. Final concentrations and yields of sesquiterpenes for the *ERG9* repressed yeast strains. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Table 4. Final concentrations and yields of sesquiterpenes and farnesol for the *ERG9* repressed yeast strains expressing sesquiterpene synthases under control of *TPI* promoter. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L glucose.

Table 1.

Strain	Genotype	Plasmid	Plasmid description
YIP-00-03 (CEN.PK113-5D)	*MAT*a *MAL2-8^c SUC2 ura3-52*	none	
YIP-0C-01	*MAT*a *MAL2-8^c SUC2 ura3-52*	pIP032	pYX212 2μ *URA3* P$_{TPI1}$-GFTpSC
YIP-0C-02	*MAT*a *MAL2-8^c SUC2 trp1-289*	pIP013	pESC-TRP 2μ *TRP1* P$_{GAL1}$-GFTpSC
YIP-0P-02	*MAT*a *MAL2-8^c SUC2 ura3-52,*	pIP029	pESC-URA 2μ *URA3* P$_{GAL1}$- PatTps177
YIP-0V-01	*MAT*a *MAL2-8^c SUC2 ura3-52*	pIP031	pYX212 2μ *URA3* P$_{TPI1}$-GFTpSD
YIP-0V-02	*MAT*a *MAL2-8^c SUC2 ura3-52*	pIP027	pESC-URA 2μ *URA3* P$_{GAL1}$- GFTpSD
YIP-M0-04	*MAT*a *MAL2-8^c SUC2 ura3-52, erg9 ::P$_{MET3}$-ERG9*	none	
YIP-MC-02	*MAT*a *MAL2-8^c SUC2 ura3-52, erg9 ::P$_{MET3}$-ERG9*	pIP032	pYX212 2μ *URA3* P$_{TPI1}$-GFTpSC
YIP-MP-01	*MAT*a *MAL2-8^c SUC2 ura3-52, erg9 ::P$_{MET3}$-ERG9*	pIP029	pESC-URA 2μ *URA3* P$_{GAL1}$- PatTps177
YIP-MV-01	*MAT*a *MAL2-8^c SUC2 ura3-52, erg9 ::P$_{MET3}$-ERG9*	pIP027	pESC-URA 2μ *URA3* P$_{GAL1}$- GFTpSD
YIP-MV-02	*MAT*a *MAL2-8^c SUC2 ura3-52, erg9 ::P$_{MET3}$-ERG9*	pIP031	pYX212 2μ *URA3* P$_{TPI1}$-GFTpSD

Table 2.

Yeast strain	Promoter	Valencene (mg/L)	Cubebol (mg/L)	Patchoulol (mg/L)	Total sesquiterpenes (mg/L)	Y_{XP} (mg/g DW)	Y_{SP} (mg/g galactose)
YIP-0V-02	*GAL1*	2.0			2.0	0.33	0.08
YIP-0C-02	*GAL1*		2.0		7.1	0.33	0.07
YIP-0C-01	*TPI1*		1.5		5.4	0.21	0.04
YIP-0P-02	*GAL1*			6.7	18.1	1.21	0.31

Table 3.

Yeast strain	Sesquiterpene synthase	Promoter	Final titer of target sesquiterpene (mg/L)	Total sesquiterpenes (excluding farnesol) (mg/L)	Farnesol (mg/L)	Yield of target sesquiterpene on biomass (mg/g DW)	Yield of target sesquiterpene on galactose (mg/g galactose)	Yield of farnesol on biomass (mg/g DW)	Yield of farnesol on galactose (mg/g galactose)
YIP-MC-02	Cubebol	*TPI1*	1.6	5.7	29.9	0.39	0.23	13.15	5.30
YIP-MV-01	Valencene	*GAL1*	3.0	3.0	19.6	3.13	0.67	9.05	3.77
YIP-MV-02	Valencene	*TPI1*	0.6	0.6	34.6	0.38	0.14	14.93	5.99
YIP-MP-01	Patchoulol	*GAL1*	11.5	31.1	11.1	6.25	2.13	4.98	2.06
YIP-M0-04	----	----	----	0	22.6	0	0	10.90	1.76

Table 4.

Yeast strain	Valencene (mg/L)	Cubebol (mg/L)	Farnesol (mg/L)	Yield of corresponding sesquiterpene on glucose (mg/g glucose)	Yield of farnesol on glucose (mg/g glucose)
YIP-MC-02		1.5	20.0	0.05	0.82
YIP-MV-02	0.2		15.7	0.01	0.84

a)

b)

c)

a) b)