ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Enhancement of Farnesyl Diphosphate Pool as Direct Precursor of Sesquiterpenes Through Metabolic Engineering of the Mevalonate Pathway in *Saccharomyces cerevisiae*

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ABSTRACT: The mevalonate pathway in the yeast *Saccharomyces cerevisiae* was deregulated in order to enhance the intracellular pool of farnesyl diphosphate (FPP), the direct precursor for the biosynthesis of sesquiterpenes. Overexpression of the catalytic domain of *HMG1*, both from the genome and plasmid, resulted in higher production of cubebol, a plant originating sesquiterpene, and increased squalene accumulation. Down-regulation of *ERG9* by replacing its native promoter with the regulatable *MET3* promoter, enhanced cubebol titers but simultaneous overexpression of *tHMG1* and repression of *ERG9* did not further improve cubebol production. Furtheremore, the concentrations of squalene and ergosterol were measured in the engineered strains. Unexpectedly, significant accumulation of squalene and restoring the ergosterol biosynthesis were observed in the *ERG9* repressed strains transformed with the plasmids harboring cubebol synthase gene. This could be explained by a toxicity effect of cubebol, possibly resulting in higher transcription levels for the genes under control of *MET3* promoter, which could lead to accumulation of squalene and ergosterol.

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KEYWORDS: metabolic engineering; *Saccharomyces cerevisiae*; sesquiterpene; mevalonate pathway; farnesyl diphosphate; squalene

Introduction

Sesquiterpenes are the most diverse class of isoprenoids with more than 7,000 characterized compounds (Connolly and Hill, 1991). Several properties of sesquiterpenes such as their potent anticancer, antitumor, cytotoxic, antiviral, and antibiotic properties as well as their characteristic flavors and aromas have made them industrially relevant and interesting compounds. Microbial production of sesquiterpenes has been investigated in the last decade by several groups (for review see Maury et al., 2005). The yeast *Saccharomyces cerevisiae* has been successfully used for heterologous production of sesquiterpenes (Asadollahi et al., 2008, 2009; Jackson et al., 2003; Kirby et al., 2008; Lindahl et al., 2006; Maury et al., 2008; Ro et al., 2006; Shiba et al., 2007; Takahashi et al., 2007). Although expression of heterologous sesquiterpene synthase genes in yeast allows production of the corresponding sesquiterpenes, titers are not sufficient to support a viable industrial process. It is therefore necessary to increase the supply of the precursor for their production. Sesquiterpenes are derived from farnesyl diphosphate (FPP), which is an intermediate of the sterol pathway. The first part of the sterol pathway, which is

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also called the mevalonate pathway, leads to the production of FPP but is also responsible for providing intermediates required for the biosynthesis of essential constituents of the cell such as heme A, ubiquinone, dolichols, farnesylated, and geranylgeranylated proteins (Kennedy et al., 1999; Maury et al., 2005). Regulation of the mevalonate pathway in eukaryotes is complex, but HMG-CoA reductase and the FPP branch-point represent two key regulation sites in the pathway (Maury et al., 2005).

HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate, is a highly regulated enzyme that is generally considered to represent the main flux controlling step in the pathway (Basson et al., 1987; Donald et al., 1997; Polakowski et al., 1998). *S. cerevisiae* possesses two isozymes of HMG-CoA reductase, Hmg1p and Hmg2p, encoded by *HMG1* and *HMG2*, respectively. Under aerobic conditions Hmg1p has the highest activity (Hampton et al., 1996). Over-expression of the region of the gene encoding the catalytic domain of Hmg1p leads to enhanced isoprenoid production in yeast (Engels et al., 2008; Jackson et al., 2003; Kirby et al., 2008; Ro et al., 2006; Verwaal et al., 2007).

The FPP branch-point represents another regulatory site in the mevalonate pathway where several enzymes compete for FPP as substrate. However, under normal growth conditions most of the FPP is used for sterol biosynthesis and by minimizing the flux towards sterols more FPP will be available for other enzymes of the branch-point, for example, heterologously expressed sesquiterpene synthases.

We have successfully reported production of three plant sesquiterpenes, namely valencene, cubebol, and patchoulol, in yeast by expression of the corresponding sesquiterpene synthase genes (Asadollahi et al., 2008). Minimizing the carbon flux towards sterols through attenuating the expression of *ERG9*, which encodes squalene synthase, was found to stimulate sesquiterpene production in yeast. This was obtained by replacing the endogenous *ERG9* promoter with a regulatable *MET3* promoter that is repressed in the presence of methionine (Gardner and Hampton, 1999). In this study, we further studied cubebol production in yeast. The mevalonate pathway in the cubebol producing yeast strain was deregulated by over-expression of the truncated *HMG1* (*tHMG1*) both from the genome and using plasmid based expression under control of strong promoters. The over-expression of *tHMG1* was combined with down-regulation of *ERG9* in order to further improve cubebol production. The constructed yeast strains were characterized in batch fermenters and the effect of the modifications was studied. We also analyzed the endogenous pathway for the engineered strains by measuring the intracellular pools of ergosterol and squalene.

Materials and Methods

Plasmid Construction

The plasmid pIP025 was constructed by cloning GFTpsC (a sesquiterpene synthase isolated from *Citrus paradisi* and encoding for a cubebol synthase) in a pESC-URA vector (Stratagene) under control of the *GAL1* promoter using *Bam*HI and *Xho*I restriction sites. Over-expression of the catalytic domain of HMG-CoA reductase (*tHMG1*) was performed using gap repair technique taking advantage of high efficiency homologous recombination in yeast. *tHMG1* was amplified from the genomic DNA of *S. cerevisiae* CEN.PK113-7D using the primers 5′-tctggcgaagaattgttaa-ttaagagctcaTTAGGATTTAATGCAGGTGACGGAC-3′ and 5′-cactaaagggcggccgcactagtatcgatgACT***ATG***GACCAATTG-GTGAAAACTG-3′. The lower case letters in the primers show the homologous overhang used for gap repair. The truncated coding region encodes for the HMG-CoA reductase lacking the 530 N-terminal residues. pESC-URA plasmid was digested with *Cla*I and *Sac*I restriction enzymes. Homologous recombination of PCR product into the digested expression vector after transformation of CEN.PK113-5D with both fragments resulted in pIP026 plasmid.

The plasmid pIP028 was obtained by cloning GFTpsC in pIP026 plasmid under control of the *GAL1* promoter using *Bam*HI and *Xho*I restriction sites. The plasmids pIP031 and pIP032 have been described previously (Asadollahi et al., 2008).

Integration of *tHMG1* Into Genome

For genomic integration of *tHMG1* under control of *TPI1* promoter a plasmid bearing a truncated version of *HMG1*, associated with a selective marker, *Kl URA3* (*Kluyveromyces lactis URA3*), was constructed. *tHMG1* was amplified from *S. cerevisiae* CEN.PK 113-7D genomic DNA by PCR using primers containing overhangs with *Eco*RI restriction site on one side (5′-accggaattcACT***ATG***GACCAATTGGTGAAAA-CTG-3′) and *Nhe*I restriction site on the other side (5′-tct-agctagcCACATGGTGCTGTTGTGCTT-3′). After digestion with *Eco*RI and *Nhe*I, the PCR fragments were ligated into pYX212 plasmid, in between the two restriction sites *Eco*RI and *Nhe*I. *Kl URA3* was further cloned into pYX212 bearing *tHMG1* at the *Nhe*I restriction site. *Kl URA3* flanked by two direct repeats was first PCR amplified from the plasmid pWJ1042 (Reid et al., 2002) using the primers 5′-tctagctagcTTCGGCTTCATGGCAATTCCCG-3′ and 5′-tctagctagcTAACGCCAGGGTTTTCCCAGTCAC-3′. After digestion with *Nhe*I, the PCR product was ligated into the pYX212 plasmid bearing *tHMG1* and resulted in pTTU plasmid.

In order to integrate the P_{TPI1}-*tHMG1-Kl URA3* construct into the *URA3* locus, fusion PCR and the bipartite gene targeting method (Erdeniz et al., 1997) were used. The construct was amplified from the plasmid pTTU in two separate, but overlapping, fragments (Fig. 1) using the primers listed in Table I. Furthermore, 500 bp upstream and downstream of *URA3* on the genome of *S. cerevisiae* were amplified. The four resulting PCR fragments were fused together in pairs by fusion PCR (Fig. 1). *S. cerevisiae* strain

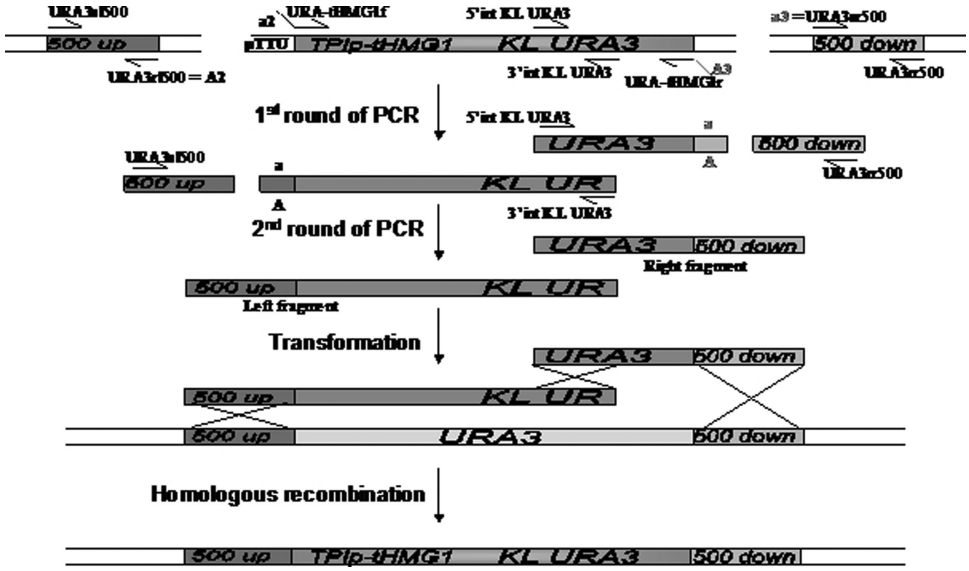

Figure 1. Insertion of P_{TPI1}-*tHMG1-KI URA3* into the genomic *URA3* locus of *S. cerevisiae*. The P_{TPI1}-*tHMG1-KL URA3* construct is amplified from pTTU in two separate, but overlapping, reactions using primers at the extremities with adaptamers homologous to the insertion site. 500 bp up- and downstream from the insertion site are amplified as well. After a second round of PCR, where the four resulting fragments are fused together in pairs, the two fusion fragments are transformed into *S. cerevisiae*.

CEN.PK 113-5D was subsequently transformed with the fusion PCR fragments. Transformants were selected on SC-ura medium after incubation at 30°C for 2–4 days. Correct integrants were identified by PCR using both external primers (URA3sl500 and URA3rr500) and a combination of external and internal primers (5′int KL URA3 and URA3rr500). Subsequently, correct transformants were restreaked on a medium containing 5-fluoroorotic acid (5-FOA) for the loop out of the *Kl URA3* marker and the reuse of uracil auxotrophy (Reid et al., 2002). Thus, the strain YIP-M0-03 was obtained.

Strain Construction

The native promoter of *ERG9* gene was previously replaced with a regulatable *MET3* promoter providing the strain YIP-M0-04 (Asadollahi et al., 2008).

Strains YIP-MV-07 and YIP-MC-13 were obtained by transforming YIP-M0-03 with the pIP031 and pIP032 plasmids, respectively. Strains YIP-0C-04, YIP-MC-05, YIP-MC-07, YIP-MC-08, YIP-MC-09, and YIP-MC-10 were constructed by transforming their corresponding backgrounds with either pIP025 or pIP028 plasmids. Table II lists the strains used in this study.

Media for Shake Flasks

Baffled, cotton-stopped, 500 mL Erlenmeyer flasks were used for preparing precultures and also for preparing samples for ergosterol measurement and HMG-CoA reductase assays. The composition of media has been described previously (Asadollahi et al., 2008).

Media for Batch Cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of galactose as the sole carbon

Table I. Primers for amplification of fusion PCR fragments.

Primer	Sequence
URA-tHMG1s	atgtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgtGAAAAGTGCCACCTGACGTC
URA-tHMG1r	ttgctggccgcatcttctcaaatatgcttcccagcctgcttttctgtaacgTGTAAAACGACGGCCAGTGAG
5′int KL URA3	CTTGACGTTCGTTCGACTGATGAGC
3′int KL URA3	GAGCAATGAACCCAATAACGAAATC
URA3sl500	AAACGACGTTGAAATTGAGGCTACTGCG
URA3rl500	GGACTAGGATGAGTAGCAGCACGTTCC
URA3sr500	GGGAAGCATATTTGAGAAGATGCGGC
URA3rr500	GGAAACGCTGCCCTACACGTTCGC

The sequence of the primer used for amplification of the DNA fragments is given in capital letters, while the overhangs used in fusion PCR are in small letters.

Table II. Strains used in this study.

Strain	Genotype	Plasmid	Plasmid description	Reference
YIP-00-03 (CEN.PK113-5D)	*MATa MAL2-8^c SUC2 ura3-52*	None		Peter Kötter[a]
YIP-0V-01	*MATa MAL2-8^c SUC2 ura3-52*	pIP031	pYX212 2μ *URA3* P_{TPI1}-GFTpsD	Asadollahi et al. (2008)
YIP-0C-01	*MATa MAL2-8^c SUC2 ura3-52*	pIP032	pYX212 2μ *URA3* P_{TPI1}-GFTpsC	Asadollahi et al. (2008)
YIP-0C-04	*MATa MAL2-8^c SUC2 ura3-52*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-M0-04	*MATa MAL2-8^c SUC2 ura3-52, erg9::P_{MET3}-ERG9*	none		Asadollahi et al. (2008)
YIP-M0-03	*MATa MAL2-8^c SUC2, ura3::P_{TPI1}-tHMG1*	none		This study
YIP-MV-07	*MATa MAL2-8^c SUC2, ura3::P_{TPI1}-tHMG1*	pIP031	pYX212 2μ *URA3* P_{TPI1}-GFTpsD	This study
YIP-MC-13	*MATa MAL2-8^c SUC2, ura3::P_{TPI1}-tHMG1*	pIP032	pYX212 2μ *URA3* P_{TPI1}-GFTpsC	This study
YIP-MC-05	*MATa MAL2-8^c SUC2 ura3-52*	pIP028	pESC-URA 2μ *URA3* P_{GAL1}-GFTpsC P_{GAL10}-*tHMG1*	This study
YIP-MC-07	*MATa MAL2-8^c SUC2 ura3-52, erg9::PMET3-ERG9*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-08	*MATa MAL2-8^c SUC2 ura3-52, erg9::P_{MET3}-ERG9*	pIP028	pESC-URA 2μ *URA3* P_{GAL1}-GFTpsC P_{GAL10}-*tHMG1*	This study
YIP-MC-09	*MATa MAL2-8^c SUC2, ura3::P_{TPI1}-tHMG1*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-10	*MATa MAL2-8^c SUC2, ura3::P_{TPI1}-tHMG1*	pIP028	pESC-URA 2μ *URA3* P_{GAL1}-GFTpsC P_{GAL10}-*tHMG1*	This study

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source was used for all batch fermentations. 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. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture. Three hundred milliliters of 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).

Analysis of Galactose and Ethanol

Analysis of sugars and extracellular metabolites in the culture media was performed using HPLC as described (Asadollahi et al., 2008).

Analysis of Sesquiterpenes in the Organic Layer

Samples from organic layer were centrifuged for 5 min at 3,500 rpm and subsequently analyzed by GC–MS to determine the level of sesquiterpenes during the course of fermentation as described (Asadollahi et al., 2008).

Extraction of Sterols

A certain volume of overnight culture medium corresponding to approximately 100 mg of dry cells was harvested by centrifuging at 5,000 rpm for 10 min. After washing with distilled water, the cell pellet was re-suspended in 4 mL HCl 0.2 N and heated in a water bath set at 85°C for 1 h and then allowed to cool to room temperature. After centrifuging for 10 min at 5,000 rpm and removing the supernatant, the cell pellet was resuspended in 2 mL methanol containing 0.2% (w/v) pyrogallol and 1 mL KOH 4 N and transferred to a 14 mL glass vial sealed with a PTFE lined screw cap, heated again for 2 h in a water bath set at 85°C for saponification. Following incubation, glass vials were allowed to cool to room temperature. Sterols were then extracted by addition of 5 mL heptane followed by vigorous vortex mixing for 2 min. After 2 h when the heptane layer had clarified, it was transferred to a new glass vial for HPLC and GC–MS analyses.

Analysis of Ergosterol

Quantitative determination of ergosterol was carried out by reverse-phase HPLC (Hewlett-Packard HP 1090 chromatograph series II with built in diode array detector and autoinjector). Sterols were separated on a Develosil column (C30-UG-5, Nomura Chemicals, Aichi, Japan) with a mobile phase consisting of methanol and acetonitrile. The analysis was performed at 40°C and it was monitored at 280 nm. The amount of ergosterol was determined using absolute calibration curves obtained after each analysis run.

Analysis of Squalene

Samples from sterol extraction were injected into a Thermo Finnigan Focus GC coupled to a Focus DSQ quadrupole mass spectrometer for squalene analysis and determination. Sterols were separated on a SLB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA) 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°C and injector temperature was 250°C. After 1 min the oven temperature was increased to 270°C at the rate of 10°C/min and was then held for 20 min at this temperature to equilibrate.

HMG-CoA Reductase Activity

Fifty milliliters of overnight culture medium containing 20 g/L of glucose as carbon source was harvested by centrifuging at 5,000 rpm for 10 min. The cell pellets were washed twice with 25 mL of 50 mM phosphate buffer (pH 7.0). After removing the supernatant, cells were resuspended in 300 μL of lysis buffer (100 mM phosphate buffer, pH 7.0; 1 mM Na_2EDTA, pH 8.0; 5 mM DTT). The suspension was then transferred to a 2 mL FastPrep® tube and precooled glass beads (0.25–0.50 mm) were added to reach the meniscus. The cells were broken by vortexing in FastPrep® instrument (FP 120, BIO 101, Savant, Holbrook, NY) at the speed set to 5.0 m/s for 3 × 20 s separated by periods of cooling on ice. An additional 300 μLl of lysis buffer was added to assist the recovery of the extract. The extract was transferred to a new Eppendorf tube and Nonidet P-40 was added to 0.5% (w/v) and incubated on ice for 1 h. The extract was centrifuged at 10,000*g* for 2 min at 4°C. Activity assays were modified from the procedure of Quain and Haslam (1979). For the assay, a reaction buffer containing 100 mM KPO_4 buffer, pH 7.0, 0.5% (w/v) Nonidet P-40, 5 mM DTT was prepared. Stock solutions of NADPH and HMG-CoA in KPO_4 buffer, 3 mM, pH 7.0, were prepared in separate tubes. Assays were done in 1 mL (1 cm path length) quartz cuvettes by adding reaction buffer and 150 μM NADPH. The cuvette temperature was kept constant at 30°C by circulating water. Different amounts of cell extract (50, 100, and 150 μL) were added to the cuvette. After 5 min of incubation at 30°C to stabilize the endogenous oxidation of NADPH, 150 μM HMG-CoA was added to the cuvette as substrate. The initial change in absorbance at 340 nm due to oxidation of NADPH was recorded.

The protein content of cell extract was determined using the Lowry method (Lowry et al., 1951) using BSA as standard.

Results

Over-Expression of *tHMG1* From the *S. cerevisiae* Genome

Enzymatic activity of HMG-CoA reductase was assayed for CEN.PK113-7D and YIP-M0-03 strains based on the ability of cell extracts to oxidize NADPH in the presence of HMG-CoA as substrate. Over-expression of *tHMG1* from the genome increased the specific activity of this enzyme from 4.04 ± 0.49 in CEN.PK113-7D to 5.82 ± 0.29 U/(mg protein) in the YIP-M0-03 strain where U is defined as nmol NADPH oxidized min^{-1} (data not shown). The effect of *tHMG1* over-expression on sesquiterpene biosynthesis was examined by measuring sesquiterpenes produced by the strains YIP-MV-07 and YIP-MC-13 in shake flask. These two strains produced approximately 40% more sesquiterpene compared to their corresponding control strains, YIP-0V-01 and YIP-0C-01, respectively (data not shown). As, the 45% increase in the HMG-CoA reductase specific activity after *tHMG1* over-expression led to approximately 40% increase in the final titer of sesquiterpenes, the flux control coefficient (FCC) (Nielsen, 1997) for HMG-CoA reductase in the mevalonate pathway is calculated to be close to one using the method of large deviations (Small and Kacser, 1993). Thus, this enzyme exerts a major flux control in the mevalonate pathway.

Over-Expression of *tHMG1* From an Episomal Expression Vector

The specific enzyme activity of HMG-CoA reductase was further enhanced by expressing *tHMG1* under the control of a strong promoter in a high copy plasmid. For this, the plasmid pIP028 was constructed. The plasmid pIP025 was also constructed as a control by cloning only GFTpsC into the pESC-URA plasmid under control of the *GAL1* promoter. The strains over-expressing *tHMG*1 were characterized in 5 L batch two-phase fermentations for both growth (Fig. 2) and cubebol production (Fig. 3). For all the strains, over-expression of *tHMG1* resulted in a decrease in the specific growth rate (Fig. 2) probably as a consequence of squalene accumulation which could be toxic for cells at high concentrations (Donald et al., 1997).

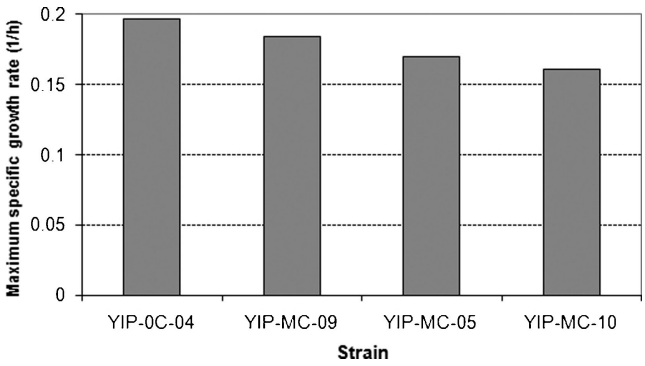

Figure 2. Effect of *tHMG1* over-expression on the maximum specific growth rate of yeast strains. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

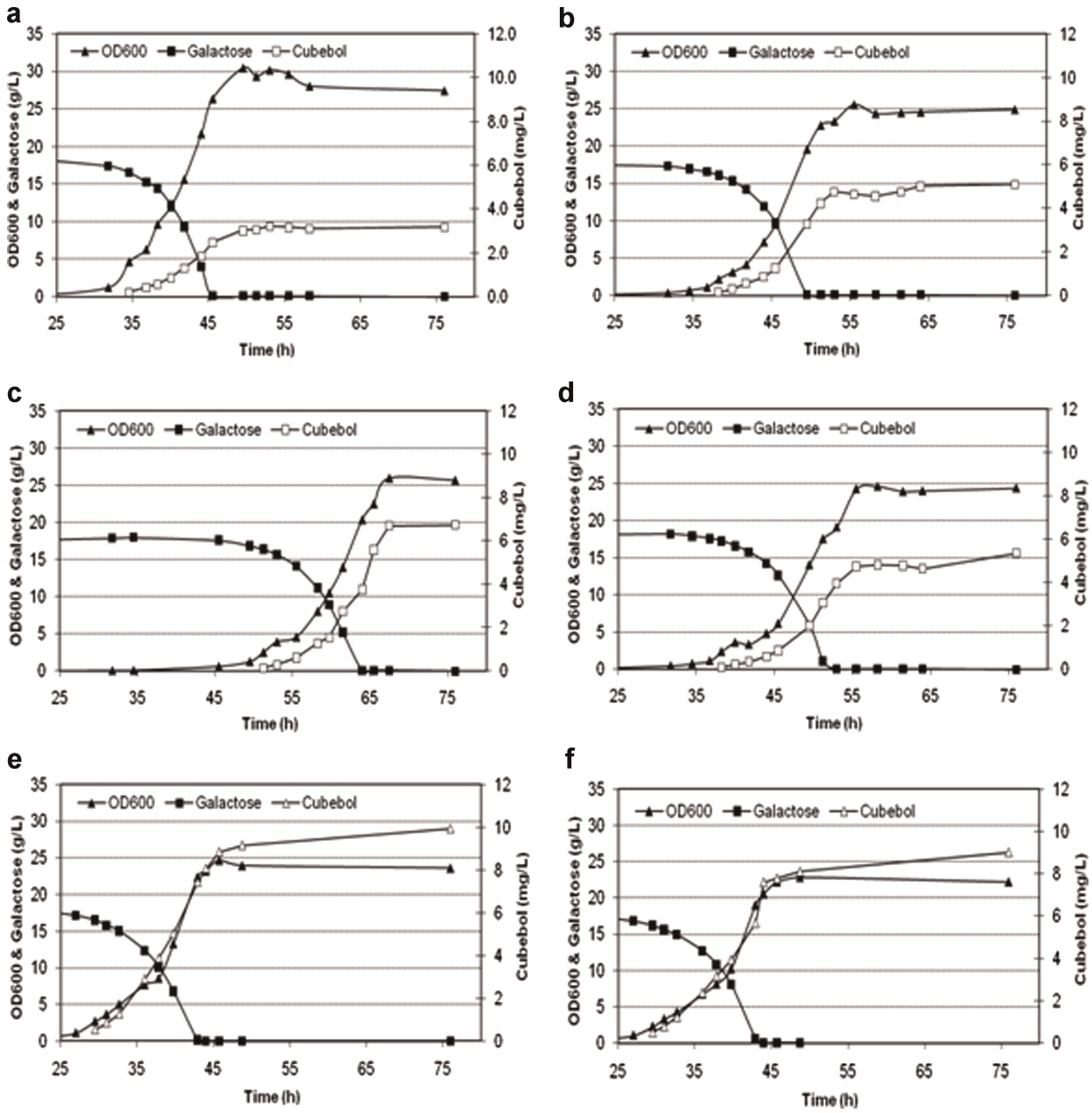

Figure 3. Cubebol, galactose, and biomass (OD_{600}) profiles as a function of time for the cubebol producing yeast strains (**a**) YIP-0C-04; (**b**) YIP-MC-09; (**c**) YIP-MC-05; (**d**) YIP-MC-10; (**e**) YIP-MC-07; (**f**) YIP-MC-08. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Over-expression of *tHMG1* led to increased final titers and yields of cubebol compared to the control strain, YIP-0C-04 (Table III and Fig. 3). In Table III, the total sesquiterpene concentration was calculated using the known percentage of cubebol in the mixture produced by the multi-product enzyme, cubebol synthase (Asadollahi et al., 2008). Over-expression of *tHMG1* from the genome or plasmid both resulted in increased cubebol production, but plasmid over-expression resulted in a larger increase. However, simultaneous over-expression of *tHMG1* from both plasmid and genome in a single strain (YIP-MC-10) did not further improve cubebol production.

Comparison of the yield of cubebol on galactose and on ethanol showed that ethanol was a more efficient carbon source for cubebol production (Table III).

Combining *tHMG1* Over-Expression and *ERG9* Down-Regulation

In an earlier study (Asadollahi et al., 2008), we found that repression of *ERG9* in a sesquiterpene producing yeast strain led to accumulation of the FPP derived compound, farnesol, indicating an increase in the intracellular pool of FPP due to

Table III. Final concentrations and yields of sesquiterpenes for the different yeast strains.

Yeast strain	Cubebol (mg/L)	Total sesquiterpenes (excluding farnesol) (mg/L)	Farnesol (mg/L)	Yield of cubebol on biomass (mg/g DW)	Yield of cubebol on galactose (mg/g galactose)	Yield of cubebol on ethanol (mg/g ethanol)
YIP-0C-04	3.2	11.3		0.43	0.15	0.22
YIP-MC-09	5.1	18.2		0.52	0.17	0.55
YIP-MC-05	6.7	23.9		0.60	0.22	0.72
YIP-MC-10	5.3	18.9		0.58	0.17	0.46
YIP-MC-07	9.9	35.3	16.3	1.59	0.54	1.18
YIP-MC-08	9.0	32.1	18.4	1.44	0.54	1.06

Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

ERG9 down-regulation. Besides farnesol, this strategy also improved the target sesquiterpene production. Here we investigated the effect of combining *ERG9* down-regulation and *tHMG1* over-expression on cubebol production. Down-regulation of *ERG9* alone in a cubebol producing strain led to a threefold increase in cubebol titer but combination of *ERG9* down-regulation and *tHMG1* over-expression did not further improve cubebol production (Table III and Fig. 3).

Effect of *tHMG1* Over-Expression and *ERG9* Down-Regulated Strains on Ergosterol Content of the Cells

To assess the effect of *tHMG1* over-expression on ergosterol biosynthesis, which is the end product of the pathway, the ergosterol content of *tHMG1* over-expressed strains were determined and compared with the control (YIP-0C-04) and WT (CEN.PK113-7D) strains (Fig. 4). The increase in the ergosterol content of cells is not proportionally correlated with the increase in the HMG-CoA reductase specific activity and cubebol titer meaning that conversion of HMG-CoA to mevalonate is not the only flux controlling step in the complete ergosterol pathway and that there are other regulatory sites downstream in the pathway towards ergosterol (Polakowski et al., 1998).

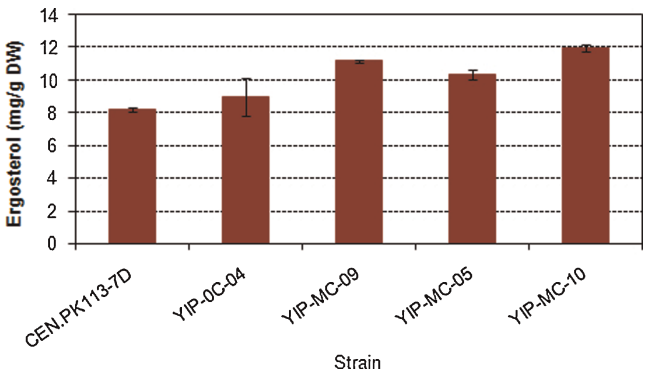

Figure 4. Influence of *tHMG1* over-expression on ergosterol content of yeast strains. Cells were grown in shake flasks containing 100 mL medium and 20 g/L galactose. Data are shown as mean ± SD of three independent experiments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

We previously observed reduced ergosterol content of cells upon down-regulation of *ERG9* by replacing its endogenous promoter with the promoter of *MET3*, which is repressed in the presence of methionine (Asadollahi et al., 2008). In the current study, we also determined the ergosterol content of all *ERG9* down-regulated strains expressing the cubebol synthase gene or no sesquiterpene synthase (Fig. 5). Surprisingly, while YIP-M0-04 strain is characterized by a lower ergosterol content, as expected, we observed that once this background strain is transformed with the plasmids harboring the cubebol synthase gene (pIP025 and pIP028), ergosterol biosynthesis is restored both on galactose and glucose as carbon sources.

Influence of *tHMG1* Over-Expression and *ERG9* Down-Regulation on Squalene Accumulation

Over-expression of *tHMG1* did not lead to a substantial increase in the ergosterol content indicating the existence of other regulatory steps in the pathway. One of these regulatory steps is conversion of squalene to squalene epoxide catalyzed by squalene epoxidase. Therefore an

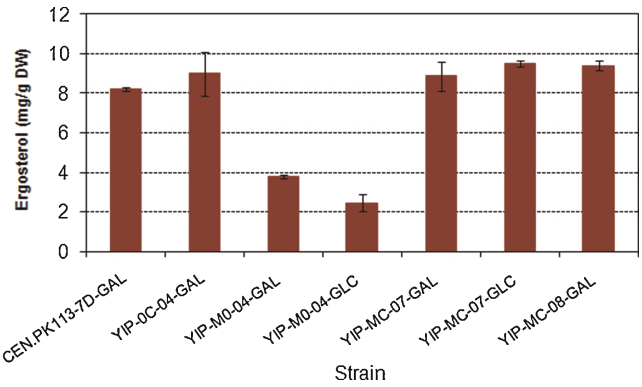

Figure 5. Influence of *ERG9* down-regulation and the combination of *ERG9* repression and *tHMG1* over-expression on ergosterol content of yeast strains. Cells were grown in shake flasks containing 100 mL medium and 20 g/L of either galactose (GAL) or glucose (GLC) as carbon sources. Repression of *ERG9* was induced by supplementing media with 2 mM methionine. YIP-M0-04 strain was also supplemented with 100 mg/L uracil as it had auxotrophy towards uracil. Data are shown as mean ± SD of three independent experiments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

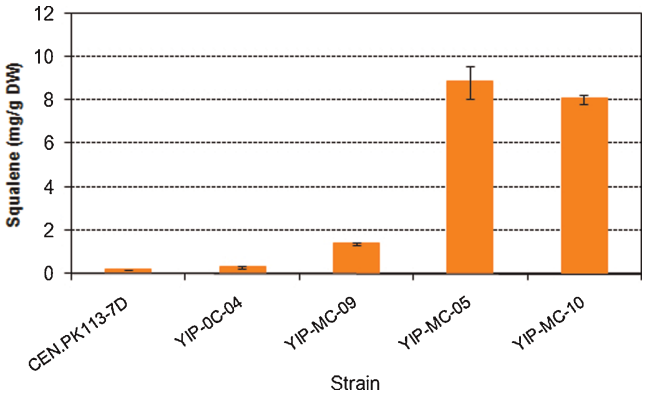

Figure 6. Influence of *tHMG1* over-expression on squalene accumulation in the yeast strains. Cells were grown in shake flasks containing 100 mL medium and 20 g/L galactose. Data are shown as mean ± SD of three independent experiments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

accumulation of squalene in the strains with higher HMG-CoA reductase activity is expected (Polakowski et al., 1998). Extraction and measurement of squalene in the *tHMG1* over-expressed strains revealed a dramatic change in the squalene concentration from trace amounts in the WT and control strains to several milligrams per gram of biomass for the mutants (Fig. 6).

Quantification of ergosterol for the *ERG9* down-regulated strains showed unexpectedly high levels of ergosterol for these strains whenever cubebol synthase was also present. Therefore, it would be interesting to investigate if expression of cubebol synthase in an *ERG9* repressed strain leads to accumulation of squalene as well. Figure 7 compares the squalene levels of the *ERG9* repressed strains with the wild type and control strain. Again, to our surprise, we observed high squalene levels for the *ERG9* repressed strains upon expression of cubebol synthase but in contrary to the results from the ergosterol analysis, the squalene level was very low when the YIP-MC-07 strain was grown on glucose as carbon source (Fig. 7).

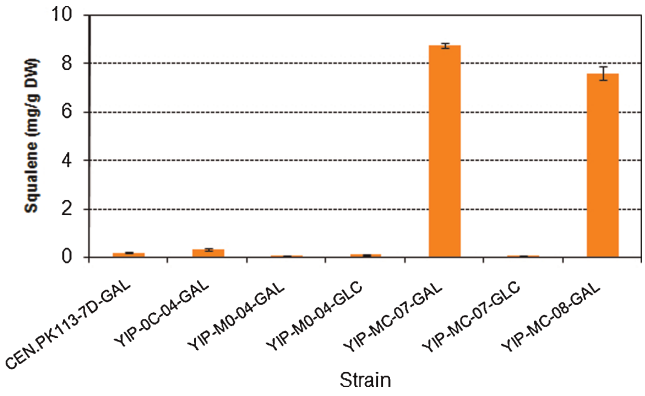

Figure 7. Influence of *ERG9* down-regulation and the combination of *ERG9* down-regulation and *tHMG1* over-expression on squalene accumulation in the different yeast strains. Cells were grown in shake flasks containing 100 mL medium and 20 g/L of either galactose (GAL) or glucose (GLC) as carbon sources. Repression of *ERG9* was induced by supplementing media with 2 mM methionine. YIP-M0-04 strain was also supplemented with 100 mg/L uracil since it had auxotrophy towards uracil. Mean ± SD of three independent experiments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Discussion

In this study, we investigated the effects of deregulating the mevalonate pathway in the yeast *S. cerevisiae* on the biosynthesis of cubebol. The effects of the manipulations on the endogenous pathway were also studied by analyzing the accumulation of squalene and ergosterol in the engineered strains. Deregulation of the mevalonate pathway was accomplished by constructing strains over-producing HMG-CoA reductase, which is responsible for the catalysis of the main flux controlling step in the mevalonate pathway. Over-expression of the truncated *HMG1* encoding the catalytic domain of HMG-CoA reductase by integrating *tHMG1* into the genome under control of the strong constitutive *TPI1* promoter enhanced the specific activity of the enzyme by approximately 45% and increased the biosynthesis of valencene and cubebol by about 40% in shake flask experiments. Over-expression of *tHMG1* from an episomal high copy plasmid under control of the strong inducible *GAL10* promoter was more efficient and the final concentration of cubebol in batch fermenter reached 6.7 mg/L compared to 5.1 mg/L when *tHMG1* was over-expressed by genomic integration. However, simultaneous over-expression of *tHMG1*, both from the genome and a high copy plasmid, did not further improve cubebol production. This is probably due to the slight changes in mRNA level of *tHMG1* after integrating a single copy of *tHMG1* in a background strain over-expressing *tHMG1* from a high copy plasmid.

Over-expression of *tHMG1* also resulted in the accumulation of squalene in the cells. Squalene accumulating organisms are scarce in the nature and the liver oil of deep-sea shark, which is traditionally used for squalene production (Donald et al., 1997), is one of the few exceptions, which is composed of up to 60% squalene by weight (Pietsch and Jaeger, 2007). Although the wild type strain contained trace amounts of squalene, the *tHMG1* over-expressed strains were able to accumulate up to 9 mg squalene/g DW. Accumulation of squalene as a result of *tHMG1* over-expression has been reported by other investigators as well (Donald et al., 1997; Polakowski et al., 1998) and proposes the reaction catalyzed by squalene epoxidase as a flux controlling step in the ergosterol biosynthetic pathway (Polakowski et al., 1998; Veen et al., 2003). Comparison of kinetic parameters of the enzymes squalene synthase and squalene epoxidase may explain why squalene expoxidase is a flux controlling step in the ergosterol pathway. The yeast squalene synthase has a K_m

value of 2.5 μM for its substrate FPP (LoGrasso et al., 1993) and the wild type specific activity for this enzyme was found to be 460 pmol min^{-1} mg^{-1} (Jennings et al., 1991) whereas the reported numbers for the yeast squalene epoxidase are 13.5 μM and 32.1 pmol min^{-1} mg^{-1}, respectively (Satoh et al., 1993). The higher K_m value of squalene epoxidase for its substrate and lower specific activity of this enzyme suggest that squalene epoxidase has lower capacity than squalene synthase and this could lead to accumulation of squalene if the flux through the pathway exceeds a certain limit, for example, when HMG-CoA reductase activity is increased. Expression of cubebol synthase does not result in a sufficiently high drain of FPP to avoid squalene accumulation. Typical K_m values of sesquiterpene synthases for FPP are in the range of 0.4–10 μM (Picaud et al., 2005), which is comparable with that of yeast squalene synthase. However, the reported catalytic activities for sesquiterpene synthases are much lower than the k_{cat} for the yeast squalene synthase. For instance, the k_{cat} values for amorpha-4, 11-diene synthase from *Artemisia annua* L. vary from 3.4×10^{-4} to 15.4×10^{-3} s^{-1} depending on pH and the divalent metal ions (Picaud et al., 2005) and germacrene D synthase from *Zingiber officinale* has a k_{cat} value of 3.34×10^{-3} s^{-1} (Picaud et al., 2006) whereas squalene synthase has a k_{cat} value of 0.53 s^{-1} (LoGrasso et al., 1993). These low values indicate that the conversion of FPP to sesquiterpenes by sesquiterpene synthases is less efficient than the condensation of two FPP molecules to squalene by squalene synthase.

Over-expression of *tHMG1* resulted in a reduction in the specific growth rate of yeast strains. This could be due to accumulation of squalene and its cytotoxic effects at high concentrations (Donald et al., 1997). Treatment of fungi with squalene epoxidase inhibitors such as terbinafine results in ergosterol depletion of fungal cell membrane and also high intracellular concentration of squalene which is believed to interfere with normal fungal membrane function and cell wall synthesis (Liu et al., 2004; Mukherjee et al., 2003). In contrary to the lower specific growth rate of squalene accumulating yeast strains observed by us and Donald et al. (1997), Veen et al. (2003) did not observe any adverse effect of squalene accumulation on cell growth.

Since most of the FPP is used for the biosynthesis of sterols, minimizing the flux of FPP towards sterols should enhance available FPP for other enzymes of the FPP branch-point including sesquiterpene synthases. We previously (Asadollahi et al., 2008) reduced the FPP utilization for sterols by replacing the endogenous *ERG9* promoter with a repressible *MET3* promoter. This strategy led to reduced ergosterol content of cells and enhanced sesquiterpene biosynthesis, but we also found that there was accumulation of farnesol as a major FPP derived by-product. Here, we combined over-expression of *tHMG1* and down-regulation of *ERG9* to both enhance the flux through the pathway and improve the availability of FPP for sesquiterpene synthases. Down-regulation of *ERG9* alone increased the cubebol titer more than threefold and farnesol was also accumulated. However, over-expression of *tHMG1* in this background did not further improve cubebol production. This does not accord with the results obtained by Ro et al. (2006) who reported a twofold increase in amorphadiene titer after down-regulation of *ERG9* in a *tHMG1* over-expressed yeast strain and a further 50% increase after integration of a second copy of *tHMG1* in this background.

Examination of the YIP-MC-07 and YIP-MC-08 strains for their sterol content revealed that these two strains were able to build up the same levels of ergosterol as the wild type strain during growth on both galactose and glucose as carbon source whereas the YIP-M0-04 strain had a lower ergosterol content. This means that the presence of cubebol synthase restored the ergosterol biosynthesis in these strains. Surprisingly, these strains also accumulated a substantial amount of squalene comparable to the amounts accumulated by the *tHMG1* over-expressed strains, but only when they were grown on galactose as carbon source. The essential oils from plants have been extensively applied in folk medicine to prevent infectious diseases and in recent years they have received a lot of attention as potent antimicrobial compounds (Kiran et al., 2008). It has been proven that the monoterpene and sesquiterpene components of the plant essential oils are mostly responsible for this antimicrobial activity (Parveen et al., 2004). The mechanism of toxicity of α-terpene (a cyclic monoterpene) was investigated by DNA microarray analysis of *S. cerevisiae* cells treated with this monoterpene. The mRNA levels of 793 genes were significantly affected. Several genes in the amino acid metabolism including the *MET3* gene were amongst the significantly up-regulated genes (Parveen et al., 2004). If cubebol or other sesquiterpenes synthesized by the multi-product enzyme, cubebol synthase, have a similar effect on the expression of *MET3* gene then the *ERG9* gene, which we put under regulation by the *MET3* promoter, might be up-regulated leading to accumulation of squalene and ergosterol. The fact that it is only the ergosterol level that is increased, and not the squalene level, during growth on glucose can be explained by low level expression of cubebol synthase by the *GAL1* promoter in the presence of glucose (Kristoffersen et al., 2000; Madeo et al., 1997). In contrast to our results, in a recent study on the production of amorphadiene by *ERG9* repressed yeast strains, addition of methionine to the medium reduced both ergosterol and squalene as expected (Paradise et al., 2008). This might be due to different influences of sesquiterpenes synthesized by amorphadiene synthase and cubebol synthase on the transcript levels of *MET3*.

Comparison of cubebol yields in different phases of growth showed that ethanol was more efficient as carbon source than galactose. The higher yield on ethanol could be due to a higher cytosolic pool of acetyl-CoA when yeast grows on ethanol as carbon source. Also during growth on ethanol, the SNF1 protein kinase is activated, which subsequently inhibits the activity of acetyl-CoA carboxylase (Mitchelhill et al., 1994; Woods et al., 1994). This subsequently reduces the acetyl-CoA consumption for fatty

acid biosynthesis and thus more acetyl-CoA can be directed to the mevalonate pathway. In addition, *ACS1*, which encodes acetyl-CoA synthetase is less repressed in the presence of ethanol compared to glucose (van den Berg et al., 1996) and probably galactose. Furthermore, metabolism of ethanol by its conversion to acetaldehyde and then acetate, which is subsequently converted to acetyl-CoA, is accompanied by generation of NADPH, which is required as a cofactor for HMG-CoA reductase.

In summary, the engineered yeast strains in this study produced up to approximately 10 mg/L cubebol or 52 mg/L of total sesquiterpenes. Taking into account, the accumulated squalene which is biosynthesized from the same precursor, the yeast strains were able to produce roughly 110 mg/L sesquiterpene equivalents, and the yeast strains hereby represent a suitable platform for further development of an industrial process for production of sesquiterpenes.

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