Appl Microbiol Biotechnol (2009) 82:837–845
DOI 10.1007/s00253-008-1807-5

Overexpression of the gene encoding HMG-CoA reductase in *Saccharomyces cerevisiae* for production of prenyl alcohols

Chikara Ohto · Masayoshi Muramatsu · Shusei Obata · Eiji Sakuradani · Sakayu Shimizu

Received: 6 October 2008 / Revised: 19 November 2008 / Accepted: 29 November 2008 / Published online: 16 December 2008

Abstract To develop microbial production method for prenyl alcohols (e.g., (*E,E*)-farnesol (FOH), (*E*)-nerolidol (NOH), and (*E,E,E*)-geranylgeraniol (GGOH)), the genes encoding enzymes in the mevalonate and prenyl diphosphate pathways were overexpressed in *Saccharomyces cerevisiae*, and the resultant transformants were evaluated as to the production of these alcohols. Overexpression of the gene encoding hydroxymethylglutaryl (HMG)-CoA reductase was most effective among the genes tested. A derivative of *S. cerevisiae* ATCC 200589, which was selected through screening, was found to be the most suitable host for the production. On cultivation of the resultant transformant, in which the HMG-CoA reductase gene was overexpressed, in a 5-liter bench-scale jar fermenter for 7 d, the production of FOH, NOH, and GGOH reached 145.7, 98.8, and 2.46 mg/l, respectively.

Keywords Farnesol · Nerolidol · Geranylgeraniol · Prenyl alcohol · *Saccharomyces cerevisiae* · Production

Introduction

So called prenyl alcohols, such as (*E,E*)-farnesol (FOH), (*E*)-nerolidol (NOH), and (*E,E,E*)-geranylgeraniol (GGOH), are alcohol derivatives of (*E,E*)-farnesyl diphosphate (FPP) and (*E,E,E*)-geranylgeranyl diphosphate (GGPP), and are fragrance components of essential oils. They are used as the ingredients of perfumes, and are important as starting materials for the synthesis of hydrophobic vitamins and pharmacological agents (Benford et al. 1999; Hyatt et al. 2002). Among geometric isomers, (all-*E*)-isomers are significant because of their biological activities (Ogura and Koyama 1998).

Most FOH and NOH on the market are produced through chemical synthesis, small amounts of them being also prepared from essential oils as natural products. Chemically synthesized FOH and NOH are usually obtained as mixtures of (*E*)- and (*Z*)-isomers with double bond geometry. Although means of chemical synthesis of (all-*E*)-prenyl alcohols and their derivatives are now being developed (Negishi et al. 2002; Yu et al. 2005), a fermentative process can allow simultaneously a great number of reactions including specific ones for producing particular isomers and has greater potential for the efficient production of (all-*E*)-prenyl alcohols.

FPP, which is essential for steroid biosynthesis in the prenyl diphosphate pathway, is provided via the mevalonate pathway in eukaryotic cells of yeast *Saccharomyces cerevisiae* (Fig. 1). Since 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is one of the key enzymes for the ergosterol biosynthesis in the mevalonate pathway, enhancement of the reductase activity results in the accumulation of squalene (SQ) in yeast cells (Bard and Downing 1981; Donald et al. 1997; Polakowski et al. 1998; Servouse and Karst 1986). Chambon et al. (1990; 1991) reported that a SQ synthase gene deficient-yeast, as a sterol auxotroph, accumulated 0.74–1.3 mg of FOH per liter of culture. Neither NOH nor GGOH was reported to be accumulated by the sterol-auxotroph. The addition of an inhibitor of SQ

C. Ohto · M. Muramatsu · S. Obata
Bio Research Lab., Toyota Motor Corporation,
1 Toyota-cho,
Toyota 471-8572, Japan

E. Sakuradani · S. Shimizu (✉)
Division of Applied Life Sciences,
Graduate School of Agriculture, Kyoto University,
Kitashirakawa-oiwakecho, Sakyo-ku,
Kyoto 606-8502, Japan
e-mail: sim@kais.kyoto-u.ac.jp

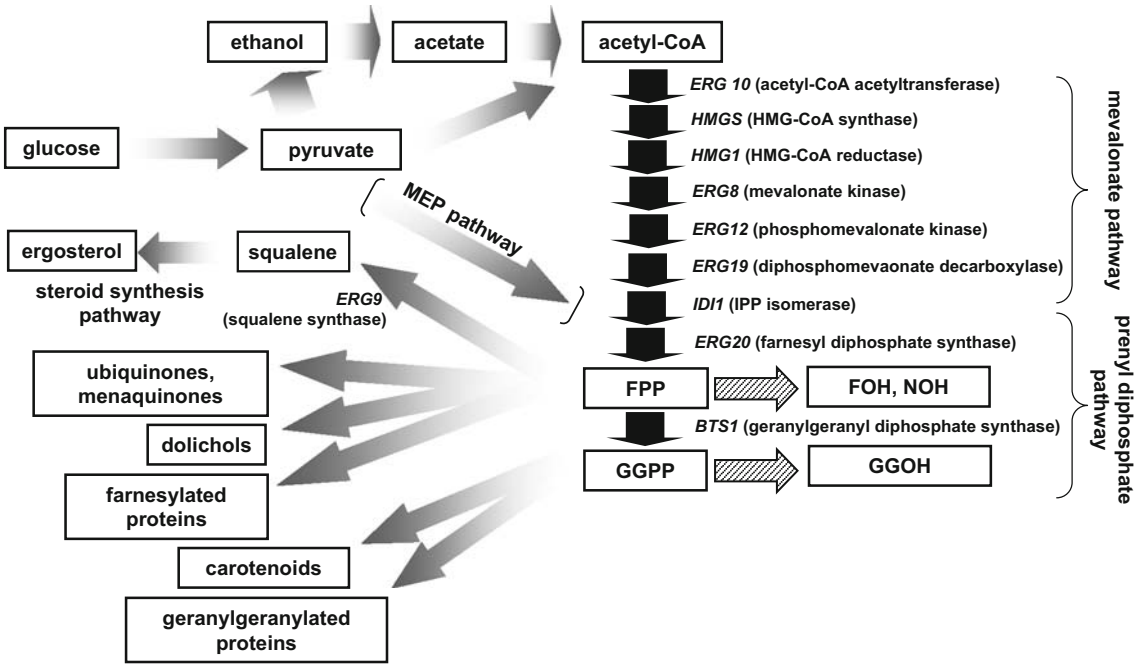

Fig. 1 Isoprenoids synthesis pathway. *Black arrows* indicate the reactions and the enzymes that are encoded by the genes overexpressed in this study. The MEP (methylerythritol phosphate) pathway and mevalonate pathway are known for isopentenyl diphosphate (C5 isoprenoid) synthesis. The MEP pathway is a prokaryotic pathway in bacteria and some unicellular eukaryotes. The mevalonate pathway is a eukaryotic pathway that *S. cerevisiae* has. FPP, (*E,E*)-farnesyl diphosphate; GGPP, (*E,E,E*)-geranylgeranyl diphosphate; FOH, (*E,E*)-farnesol; NOH, (*E*)-nerolidol

synthase to fungal cultures was also shown to exhibit potential for production of FPP and FOH, the main product being FPP and a small amount of FOH being detected in this case (Bergstrom et al. 1993).

The present paper describes evaluation of the enzyme genes involved in the mevalonate pathway for the overproduction of prenyl alcohols using *S. cerevisiae* overexpressing these genes, and selection of suitable host cells for the overexpression and optimization of the culture conditions for the overproduction with the resultant transformant cells. The results clearly demonstrate that *S. cerevisiae* overexpressing the HMG-CoA reductase gene (*HMG1*) is useful for the overproduction of these alcohols.

Materials and methods

Yeast strains

S. cerevisiae strains (ATCC 200589, 76625, 76626, 208352, and 201238) and other strains were obtained from the American Type Culture Collections, Rookville, USA. Their genotypes and other properties can be found in the ATCC catalog. Recombinant yeast AURGG101 is an ATCC 200589 recombinant having *AUR1-C* instead of *AUR1* for resistance to aureobasidin (Hashida-Okado et al. 1998).

Construction of yeast expression vectors

YEp (yeast episomal plasmid) type expression vectors were constructed from pRS vectors (Stratagene, La Jolla, CA, USA) and pYES2 (Invitrogen, Carlsbad, CA, USA) according to Mumberg et al. (1995). YCp (yeast centromeric plasmid) type expression vectors were constructed from a 4.1 kb *Nae*I-*Pvu*II fragment containing *TRP1* in pRS414 (Stratagene), and a 1.0 kb *Bam*HI fragment containing an *ADH1* transcription promoter and terminator in pAUR123 (Takara Bio Inc., Tokyo, Japan). The resultant vectors were pRS414PTadh having ADH1p-ADH1t in the same direction as *TRP1* and pRS414TPadh having ADH1p-ADH1t in the opposite direction to *TRP1*. Information on the constructed vectors is presented in Table 1.

Cloning of genes encoding the enzymes involved in the prenyl alcohol synthesis

All the primers designed for the cloning of genes encoding the enzymes involved in prenyl alcohol synthesis are shown in Table 2 (see Fig. 1 for enzyme names). Based on the information on GenBank accession no. M22002, the *S. cerevisiae HMG1* 3.2 kb DNA fragment was amplified using *HMG1* primers from the ATCC 44773 cDNA library (Clontech, Mountain View, CA, USA), and cloned into pT7Blue-T vector (Novagen, Merck KGaA, Darmstadt,

Table 1 Yeast expression vectors

Vector	Type	Marker (strand)[a]	Promoter and terminator (strand)[a]	Replication origin and direction[b]	Accession no.
pRS414PTadh	YCp	*TRP1* (+)	*ADH1* and *ADH1* (+)	ARS4 and CEN6	AB363801
pRS414TPadh	YCp	*TRP1* (+)	*ADH1* and *ADH1* (−)	ARS4 and CEN6	AB363802
pRS434ADH	YEp	*TRP1* (+)	*ADH1* and *CYC1* (−)	2 μ (+)	AB304853
pRS434GAP	YEp	*TRP1* (+)	*TDH3* and *CYC1* (−)	2 μ (+)	AB304854
pRS434PGK	YEp	*TRP1* (+)	*PGK1* and *CYC1* (−)	2 μ (+)	AB304855
pRS434TEF	YEp	*TRP1* (+)	*TEF2* and *CYC1* (−)	2 μ (+)	AB304856
pRS435GAP	YEp	*LEU2* (−)	*TDH3* and *CYC1* (−)	2 μ (+)	AB304858
pRS444ADH	YEp	*TRP1* (+)	*ADH1* and *CYC1* (−)	2 μ (−)	AB304865
pRS444GAP	YEp	*TRP1* (+)	*TDH3* and *CYC1* (−)	2 μ (−)	AB304866
pRS444PGK	YEp	*TRP1* (+)	*PGK1* and *CYC1* (−)	2 μ (−)	AB304867
pRS444TEF	YEp	*TRP1* (+)	*TEF2* and *CYC1* (−)	2 μ (−)	AB304868
pRS445GAP	YEp	*LEU2* (−)	*TDH3* and *CYC1* (−)	2 μ (−)	AB304870

[a] (+), opposite strand from an ampicillin resistant gene; (−) same strand as ampicillin resistant gene.
[b] (+), same direction as in pYES2 for YEp vectors; (−), opposite direction from in pYES2 for YEp vectors.

Germany) to obtain pT7HMG1. The cloned DNA exhibits the following substitutional differences in nucleotide sequence from M22002; C203T (S68F), T426C, T1026C, T1167C, T1348C, G1557A, A1605G, T1820C (L607S), A2451G and A2726G (H909R). This *HMG1* having substitutions was designated as *HMG1′*. A 3.2 kb *Apa*LI-*Sal*I fragment containing *HMG1′* was inserted into the *Sma*I-*Sal*I sites of pALTER-1 (Promega, Madison, WI, USA), and change the substitutions of C203T, T1820C and A2726G into the corresponding original nucleotides, based on the provided protocol to obtain pALHMG106.

Table 2 Primer list for cloning of enzyme genes involved in prenyl alcohol synthesis

Primer name	Sequence
HMG1-F	5′-ATGCCGCCGCTATTCAAGGGACT-3′
HMG1-R	5′-TTAGGATTTAATGCAGGTGACGG-3′
HMGS-F	5′-ATGAAACTCTCAACTAAACTTTGTT-3′
HMGS-R	5′-GTTCAGCAAGATGCAATCGATGGGG-3′
ERG12-F	5′-AACTGCAGATGTCATTACCGTTCTTAACTTC-3′
ERG12-R	5′-CCGAGCTCTTATGAAGTCCATGGTA AATTCG-3′
ERG8-F	5′-AACTGCAGATGTCATTACCGTTCTAACTTC-3′
ERG8-R	5′-CCGAGCTCTTATGAAGTCCATGGTAAATTCG-3′
ERG19-F	5′-AACTGCAGATGACCGTTTACACAGCA TCCGT-3′
ERG19-R	5′-CGGAATTCTTATTCCTTTGGTAGACCAGTCT-3′
IDI1-F	5′-ATGACTGCCGACAACAATAGTAT-3′
IDI1-R	5′-TTATAGCATTCTATGAATTTGCC-3′
ERG20-F	5′-ATGGCTTCAGAAAAAGAAATTAG-3′
ERG20-R	5′-CTATTTGCTTCTCTTGTAAACTT-3′
BTS1-F	5′-ATGGAGGCCAAGATAGATGAGCT-3′
BTS1-R	5′-TCACAATCCGGATAAGTGGTCTA-3′
FPPS-F	5′-TGAGGCATGCAATTTCCGCAGCAACTCG-3′
FPPS-R	5′-TCAGAATTCATCAGGGGCCTATTAATAC-3′
ERG10-F	5′-TCCCCGCGGATGTCTCAGAACGTTTACATT GT-3′
ERG10-R	5′-TGCTCTAGATCATATCTTTTCAATGACAAT GGA-3′
idi-F	5′-TCCCCGCGGATGCAAACGGAACACGTCAT TTT-3′
idi-R	5′-TGCTCTAGATTATTTAAGCTGGGTAAATGC AGA-3′

In a similar manner, the genes encoding other enzymes in the mevalonate pathway were cloned from ATCC 44773 cDNA. 1.5 kb of *HMGS*, 1.3 kb of *ERG8*, 0.9 kb of *IDI1*, 1.0 kb of *BTS1*, and 0.9 kb of *ERG20* were amplified with *HMGS*, *ERG8*, *IDI1*, *BTS1*, and *ERG20* primers, and cloned into pT7Blue-T to obtain pT7HMGS, pT7ERG8, pT7IDI1, pT7BTS1, and pT7ERG20, respectively. 1.3 kb of *ERG12* and 1.2 kb of *ERG19* were amplified with *ERG12* and *ERG19* primers, digested with *Pst*I-*Sac*I and *Pst*I-*Eco*RI, and then cloned into the *Pst*I-*Sac*I and *Pst*I-*Eco*RI sites of pT7Blue-T to obtain pT7ERG12 and pT7ERG19, respectively. A *BST1* gene fragment derived from pT7BST1 through *Bam*HI-*Sal*I digestion was ligated into the *Bam*HI-*Xho*I sites of pYES2 to obtain pYESGGPS.

The *Escherichia coli* isopentenyl diphosphate-dimethylallyl diphosphate isomerase gene was used in the plasmid p3-47-11 (Hemmi et al. 1998). *E. coli* FPP synthase gene was amplified with the genome from JM109 and FPPS primers, digested with *Eco*RI-*Sph*I, and then cloned into *Eco*RI-*Sph*I sites of pALTER-Ex2 (Promega) to obtain pALispA4.

Construction of vectors for expression of the genes encoding the enzymes involved in the prenyl alcohol synthesis

The *HMG1* fragment derived on digestion of pT7HMG1 with *Bam*HI and *Sal*I was inserted into the *Bam*HI-*Xho*I

sites of pYES2 to obtain pYES-HMG1. The *HMG1* fragment derived on digestion of pALHMG106 with *Sma*I and *Sal*I was cloned into the *Sma*I-*Sal*I sites of pRS414PTadh, pRS414TPadh, pRS434GAP, pRS444GAP, pRS434TEF, pRS444TEF, pRS434PGK, and pRS444PGK to obtain pRS414PTadh-HMG1, pRS414TPadh-HMG1, pRS434GAP-HMG1, pRS444GAP-HMG1, pRS434TEF-HMG1, pRS444TEF-HMG1, pRS434PGK-HMG1, and pRS444PGK-HMG1, respectively.

A 1.2-kb fragment bearing *ERG10* (Hiser et al. 1994) was amplified directly from ATCC 76625 genomic DNA with *ERG10* primers, digested with *Sac*II and *Xba*I, and then cloned into the *Sac*II-*Xba*I sites of pRS435GAP and pRS445GAP to obtain pRS435GAP-ERG10 and pRS445GAP-ERG10, respectively.

The *HMGS* (*Bam*HI-*Sal*I), *ERG12* (*Sma*I-*Sal*I), *ERG8* (*Bam*HI-*Sal*I), *ERG19* (*Bam*HI-*Sal*I), *IDI1* (*Bam*HI-*Sal*I), *ERG20* (*Xba*I-*Bam*HI), and *BTS1* (*Bam*HI-*Mlu*I) fragments derived from pT7HMGS, pT7ERG12, pT7ERG8, pT7ERG19, pT7IDI1, pT7ERG20, and pYESGGPS were cloned into the corresponding sites of pRS435GAP and pRS445GAP to obtain pRS435GAP-HMGS, pRS445GAP-HMGS, pRS435GAP-ERG12, pRS445GAPERG12, pRS435GAP-ERG8, pRS445GAP-ERG8, pRS435GAP-ERG19, pRS445GAP-ERG19, pRS435GAP-IDI1, pRS445GAP-IDI1, pRS435GAP-ERG20, pRS445GAP-ERG20, pRS435GAP-BTS1, and pRS445GAP-BTS1, respectively.

pALispA4 was digested with *Sph*I and *Eco*RI, and then ligated with *Sph*I-*Sac*II linker DNA, 5′pTTTCCGCGGAA ACATG3′, and *Eco*RI-*Eco*52I linker DNA, 5′pAATTGAC GGCCGTC3′. The fragment was digested with *Sac*II and *Eco*52I, inserted into the *Sac*II-*Eco*52I sites of pRS435GAP and pRS445GAP, and designated as pRS435GAP-ispA and pRS445GAP-ispA, respectively.

The *idi* fragment was amplified from p3-47-11 with *idi* primers, digested with *Sac*II and *Xba*I, and then inserted into the *Sac*II-*Xba*I sites of pRS435GAP and pRS445GAP to obtain pRS435GAP-ORF182 and pRS445GAP-ORF182, respectively.

Transformation and cultivation

The desired DNAs were introduced into yeast cells using a Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA) according to the protocol recommended. The transformants were selected on agar plates of SD (BIO101, Vista, CA) medium lacking specified amino acids for the auxotrophic markers or YPD (1% yeast extract, 2% polypepton and 2% glucose) containing 1 mg/ml aureobasidin A at 30 °C. All yeast clones in the present study were stable. For prenyl alcohol production, 0.25 ml aliquots of saturated liquid cultures on the SD and YPD selection media were used as the pre-cultures for 2.5-ml main cultures in 18-mm diameter glass test tubes by rotary shaking for 130 rpm at 30°C in a type TXY incubator shaker (Takasaki Scientific Instruments Co., Kawaguchi, Japan). The media for the main cultures were Difco YM broth (Becton, Dickinson and Company, NJ, USA) for constitutive promoters, and a variation of the SD medium, in which glucose was replaced with 2% (*w*/*v*) galactose and 1% (*w*/*v*) raffinose, for GAL1 promoter. Each culture broth was diluted 30-fold with water to determine the cell growth by OD_{600}.

Preparation of HMG1 deletion mutants

To prepare a plasmid, designated as pYHMG044, without the transmembrane domain encoded by 1192–1266 bp of *HMG1*, desired portions together with pYES were amplified with pYES-HMG1 as a template, HMG(1191–1165) primer (5′AGAAGATACGGATTTCTTTTCTGCTTT3′), and HMG(1267–1293) primer (5′AACTTTGGTG-CAAATTGGGTCAATGAT3′), blunt-ended with Klenow enzyme, and then circularized again by self-ligation. The lack of shifts in the reading frames of *HMG1* mutants was confirmed by nucleotide sequencing around the junction boundaries.

Jar-fermenter cultivation

Recombinant yeast AURGG101 retaining pYHMG044 (pYHMG044/AURGG101) was inoculated from a slant into 200 ml of CSM-URA medium (MP Biomedicals, Irvine, CA) in a 500-ml Erlenmeyer flask with baffle plates, and cultured at 30°C for 2 days with shaking at 130 rpm. The resultant culture was centrifuged at 1,500×*g* for 5 min at 4°C, and washed with sterilized physiological saline three times. Cells were suspended in 200 ml of physiological saline, and 50 ml of the cell suspension was inoculated into 5 l of the fermenter medium and cultured under the following conditions. The fermenter medium comprised 5% galactose, amino-acid-containing Difco yeast nitrogen base (Becton, Dickinson and Company), 1% soybean oil (Nacalai Tesque, Kyoto, Japan), and 0.1% Adekanol LG109 (Adeka, Tokyo, Japan). The operational conditions were: cultivation apparatus, MSJ-U 10 L (Marubishi Bioengineering Co., Tokyo, Japan); cultivation temperature, 26°C; aeration rate, 1 vvm; agitation, 300 rpm; pH, proportional control with 4 N sodium hydroxide solution and 2 N hydrochloric acid solution.

Analysis of prenyl alcohols

For whole extraction of prenyl alcohols, methanol (1.2 ml) and pentane (2 ml) were added to 2 ml of broth in a test

tube, followed by thorough mixing. After brief centrifugation, the upper pentane layer was transferred for gas chromatography/mass spectroscopy (GC/MS) to a vial, in which 10 μl of an internal standard solution (0.1% *v*/*v* undecanol in ethanol) had been placed in advance. NOH, FOH, and GGOH were analyzed with an Agilent 5973 GC/MS system (column, HP-5MS, 0.25 mm id×30 m; column temperature, 115°C, hold 1.5 min, raise at 70°C/min to 250°C, hold 0 min, raise at 70°C/min to 300°C, and hold 7 min). For quantitative analysis of a prenyl alcohol, undecanol was used as an internal standard. The selected ion peak area (69 mass fragment) of a prenyl alcohol (1 mg/ml)/ that of undecanol (1 mg/ml) was defined as a standard ratio. The amount of a prenyl alcohol was calculated by comparison of the ratio in an unknown sample supplemented with 1 mg/ml of undecanol to the standard ratio. NOH (Sigma-Aldrich, St. Louis, MO, USA), FOH (Sigma-Aldrich), and SQ (Tokyo Chemical Industry, Tokyo, Japan) were used as authentic standards. GGOH was provided by Eisai Co. Ltd. (Tokyo, Japan). All data presented in the present paper are the means of three independent determinations (SD, less than 7%).

Results

Overexpression of the genes encoding enzymes in the prenyl alcohol synthesis

The genes encoding enzymes in the mevalonate and prenyl diphosphate pathways for the early steps of steroid biosynthesis (see Fig. 1 for genes and the corresponding enzymes) were overexpressed in *S. cerevisiae* ATCC 200589 and ATCC 76625 as host strains. The genes were introduced by means of multicopy episome vectors and expressed with the *TDH3* constitutive promoter, the ten resultant recombinants for each overexpression test were cultured in YM broth for 4 days at 30°C, and prenyl alcohol and SQ were quantified (Fig. 2). Overexpression of *HMG1* was the most effective among all other genes in both hosts for prenyl alcohol production. In addition, *ERG20* increased the production only in ATCC 200589. The *HMG1* expression in ATCC 200589 increased the production of SQ, NOH, FOH, and GGOH, whereas that in ATCC 76625 caused high SQ production, low production of FOH and GGOH, and no NOH production.

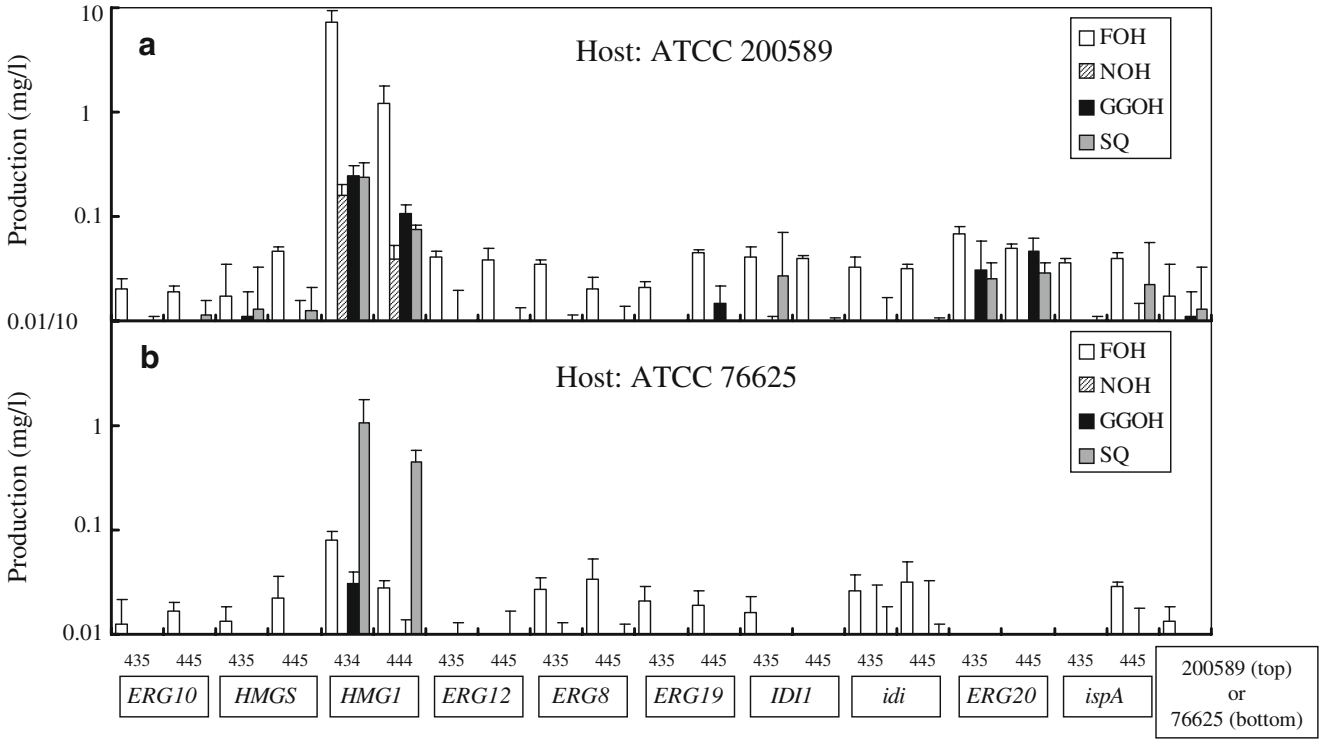

Fig. 2 Prenyl alcohol and squalene production by overexpression of mevalonate and prenyl diphosphate pathway genes in recombinant yeasts ATCC 200589 and ATCC 76625. The genes, except for *HMG1*, were introduced by means of pRS435GAP (434) and pRS445GAP (445) vectors, and *HMG1* was introduced by means of pRS434GAP (434) and pRS444GAP (444) vectors. The genes overexpressed by the vectors are shown in boxed letters. Prenyl alcohol and squalene (SQ) production by the ATCC 200589 (**a**) and ATCC 76625 (**b**) recombinants is indicated. *Boxed* 200589 and 76625 show the production by the corresponding host strains. Each *bar* represents the mean±SD of three independent experiments

Screening of host strains suitable for prenyl alcohol production

To select yeast host strains suitable for prenyl alcohol production like ATCC 200589, the *HMG1*-overexpressing episome (pRS434GAP-HMG1) were introduced into 36 type strains purchased from ATCC, and yeast recombinant AURGG101. Five recombinants for each host strain were cultured in YM medium for 4 days at 30°C, and prenyl alcohols and SQ were measured as shown in Table 3. Many strains, with overexpression of *HMG1*, exhibited high SQ production. In particular, ATCC 96029 and ATCC 201741 produced 9.5 and 10 mg/l of SQ, respectively. AURGG101 exhibited extremely higher production of prenyl alcohols, which was higher than that by ATCC 200589, as shown in Fig. 2.

Table 3 Prenyl alcohol and squalene production by HMG-CoA reductase overexpressing yeast strains

ATCC No.	Production (mg/l)			
	SQ	FOH	NOH	GGOH
200589	0.23	8.2	0.15	0.25
44769	0.016	0.018	–	–
44770	0.89	–[a]	–	–
44771	0.47	–	–	–
44773	0.82	0.013	–	–
44774	–	–	–	–
44809	–	–	–	–
76621	1.0	–	–	–
76622	0.23	–	–	–
90713	0.23	0.022	0.028	0.011
90833	4.2	–	–	–
90840	0.58	0.012	0.018	–
90844	0.22	–	0.011	–
90845	0.23	–	0.013	–
90850	0.12	–	–	–
90851	1.0	–	–	–
90852	0.78	–	–	–
90918	–	–	–	–
96029	9.5	0.021	–	–
96098	0.051	–	–	–
96099	0.13	–	–	–
96100	0.060	–	–	–
96519	0.22	–	–	–
200027	0.23	0.090	–	0.010
200028	0.021	–	–	–
200179	0.010	–	–	–
201180	2.8	–	–	–
201297	0.021	–	–	0.011
201392	0.095	–	–	–
201518	0.17	–	–	–
201566	0.19	–	–	–
201741	10.0	0.029	–	0.016
201871	0.062	–	–	–
204405	2.9	–	–	–
204802	0.78	–	–	–
204803	–	–	–	–
204804	1.0	–	–	–
AURGG101[b]	1.2	35.6	0.71	1.2

[a] – Not detected
[b] Yeast AURGG101 is an ATCC 200589 recombinant having *AUR1-C* instead of *AUR1* for resistance to aureobasidin

Screening of promoters suitable for prenyl alcohol production

An inducible promoter of the *GAL1* promoter was evaluated as well as constitutive promoters of the *PGK1*, *TDH3*, and *TEF2* genes by *HMG1* overexpression for the production of prenyl alcohols, using strains ATCC 200589 and other four strains (ATCC 208352, ATCC 201238, ATCC 76625 and ATCC 76626) as recombinant hosts (Fig. 3). The recombinants derived from all other hosts showed high SQ production and low prenyl alcohol production, compared with those of the recombinants from ATCC 200589. The recombinants produced high amounts of prenyl alcohols under the control of each promoter. In particular, the ATCC 200589 recombinant with the *GAL1* promoter exhibited the highest prenyl alcohol production (FOH, 72.7 mg/l; NOH, 36.8 mg/l; GGOH, 0.84 mg/l).

Effect of truncated HMG1 on prenyl alcohol production

To determine whether the putative transmembrane region in HMG-CoA reductase is troublesome or not for prenyl alcohol production, we mutated *HMG1* lacking the transmembrane regions for transformation of ATCC 200589 and AURGG101. Although the most mutated *HMG1* led to no production of prenyl alcohols in both yeasts (data not shown), plasmid pYHMG044 caused higher production of FOH in both yeasts (Table 4). The pYHMG044/AURGG101 recombinant yeast produced 8.6, 2.6 and 2.3 mg/l of FOH, NOH, and GGOH, respectively.

Bench-scale production with a jar fermenter

To allow sufficient aeration and glucose addition, we carried out a jar-fermenter cultivation for prenyl alcohol production using the pYHMG044/AURGG101 recombinant yeast that was supposed to be the best recombinant so far obtained to produce prenyl alcohols. Figure 4 shows the results of fermentation with pH control for 7 (Fig. 4 top) and without pH control (Fig. 4 bottom). After culturing for 125 h, we added a glucose solution to give a final 5% (*w*/*v*) glucose (dotted lines in Fig. 4). Without pH control, 122.7 mg/l FOH, 98.8 mg/l NOH, 2.46 mg/l GGOH and 0.79 mg SQ had been produced at the end of the culture.

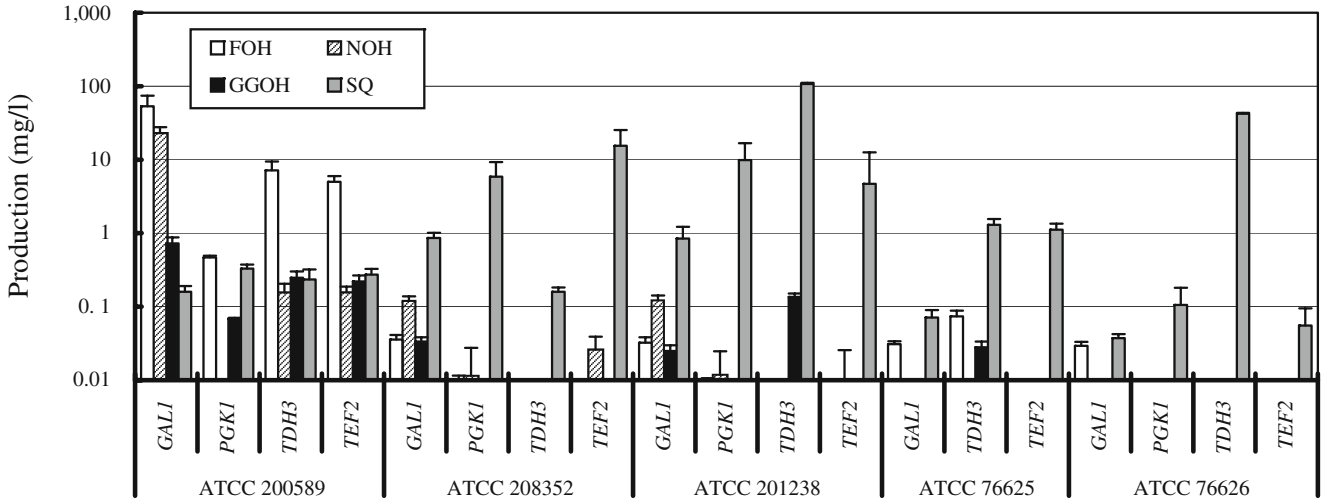

Fig. 3 Evaluation of promoters for *HMG1* overexpression to produce prenyl alcohols. *S. cerevisiae* ATCC 200589, ATCC 208352, ATCC 201238, ATCC 76625, and ATCC 76626 were used for overexpression of *HMG1* under the *GAL1*, *PGK1*, *TDH3*, or *TEF* promoter. The amounts of prenyl alcohols and SQ produced by the yeast recombinant were measured. Each *bar* represents the mean±SD of three independent experiments

With maintenance of pH 7, the production reached 145.7, 0.87, 1.79, and 1.33, respectively.

Discussion

This paper reports the overexpression of genes, promoters and hosts for the overproduction of prenyl alcohols in *S. cerevisiae.* The effects of expression of the genes involved in the mevalonate and prenyl diphosphate pathways, as shown in Fig. 1, were examined for prenyl alcohol production by means of Yep-type overexpression shuttle vectors. According to the data in Fig. 2, *HMG1* overexpression was the main positive effect for enhancement of the production. Essentially the same results were obtained on statistical analysis by quantification theory type I (Hayashi 1952). The specific direction of the gene overexpressed also had a positive effect in ATCC 200589 and ATCC 76625. But the main products differed between the two strains: it was FOH in the former and SQ in the latter. Moreover, *ERG20* overexpression had a positive effect on the GGOH production in ATCC 200589. This phenomenon indicates that *ERG20*, encoding FPP synthase, overexpression increases the supply of FPP, which is the allylic diphosphate substrate for GGPP synthase (Jiang et al. 1995), and increases GGOH production through hydrolysis of GGPP synthesized by the enzyme. The same direction of the genes overexpressed as the 2 μ replication origin in pYES2 had a positive effect on the production of FOH, whereas the different direction had a low effect. Although that reason was unclear, we must consider the direction of the target gene in the YEp type vector.

Since *HMG1* overexpression gave different profiles for prenyl alcohol and SQ production between ATCC 200589 and ATCC 76625, we studied these productivities with *HMG1* overexpression using other strains, as shown in Table 3. Almost all strains tended to produce mainly SQ and low amount of prenyl alcohols. Among these ATCC strains, we detected relatively large amounts of prenyl alcohols in the cultures of two recombinants (ATCC 201741 and ATCC 200027). But the amounts were quite low compared to the case of ATCC 200589 recombinant. These differences possibly depend on the difference in the SQ synthase activity. If the enzyme activity is weaker in ATCC 200589, the activation of the pathways will result in the accumulation of FPP, which is the substrate of the enzyme, and following production of FOH through hydrolysis of FPP. Recombinant AURGG101 derived from ATCC 200589 produced large amounts of prenyl alcohols. In particular, FOH in AURGG101 reached 35.6 mg/l (Table 3), which was approximately fourfold higher than that in ATCC 200589, as shown in Fig. 2. The *AUR1* is considered to be involved in synthesis of inositol phosphorylceramide (Kuroda et al. 1999). The *AUR1-C* is

Table 4 Prenyl alcohol production with a truncated *HMG1*

Host strain	Plasmid	Production (mg/l)		
		FOH	NOH	GGOH
ATCC 200589	pYES-HMG1	1.3	–[a]	0.1
	pYHMG044	2.2	0.7	–
AURGG101	pYES-HMG1	8.0	2.7	2.3
	pYHMG044	8.6	2.6	2.3

[a] – Not detected

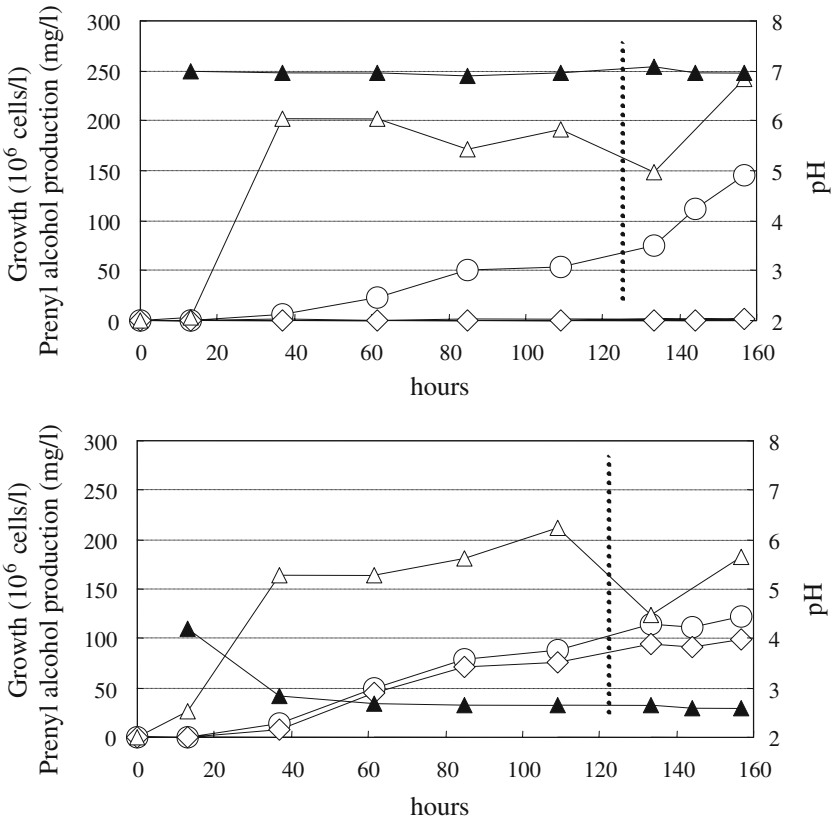

Fig. 4 Time courses of prenyl alcohol production by the recombinant yeast in 5-l jar fermenter. The pH of the culture medium was adjusted at 7 (*top*) and not adjusted (*bottom*). The glucose feeding (indicated by *vertical dotted line*) was done at 125 h with the final concentration of 5% (*w*/*v*). The FOH (*open circle*) and NOH (*open diamond*) production, pH (*closed triangle*) of the cultural medium, and cell growth (*open triangle*) were measured. The GGOH and SQ production was less than 3 mg/l during cultivation (data not shown)

replaced with two amino acid residues, causing the resistance to aureobasidin. The mutation of the *AUR1* to the *AUR1-C* in AURGG101 may change the lipid composition and metabolic flow relating with ceramide and other lipids, resulting in the accumulation of prenyl alcohols. AURGG101 is the most suitable host strain for developing a prenyl alcohol production system.

Screening for promoters suitable for *HMG1* overexpression for prenyl alcohol production among typical constitutive promoters of *TDH3*, *TEF2* and *PGK1*, and an inducible promoter of *GAL1* by using ATCC 200589 and several other host strains clearly demonstrated that the *GAL1* promoter was the best promoter for prenyl alcohol production in ATCC 200589, and the *TDH3* promoter was better for SQ production in the strains other than ATCC 200589 (Fig. 3). Between the *TDH3* and *GAL1* promoters, there was a difference in the carbon source in the medium (glucose or galactose/raffinose), and the *GAL1* promoter had no preferable effect in other strains except for ATCC 200589. These results indicate that ATCC 200589 cells bearing the episome by which *HMG1* was overexpressed with the *GAL1* promoter would have the greatest potential for prenyl alcohol production.

The deleted *HMG1* encoding the catalytic domain lacking the *N*-terminal transmembrane portion has often been used for overexpression (Jackson et al. 2003; Shimada et al. 1998; Szkopinska et al. 1997; Veen et al. 2003; Wang and Keasling 2002). We obtained various *HMG1*-deletion mutants and overexpressed them with the *GAL1* promoter in ATCC 200589 and AURGG101. Most of the obtained yeast recombinants with the transmembrane domain-deleted mutants decreased the production of prenyl alcohols significantly (data not shown). This finding indicates that the transmembrane domain of HMG-CoA reductase is necessary for its activity in the case of the yeast expression system in the present study. However, the pYHMG044/AURGG101 recombinant yeast without the transmembrane domain encoded by 1,192–1,266 bp of the *HMG1* gene exhibited a little higher production of the prenyl alcohols, compared with the control pYES-HMG1/AURGG101 yeast. We found that the transmembrane region encoded by 1,192–1,266 bp is not necessary for the activity, and the change in the HMG-CoA reductase conformation led to the accumulation of the prenyl alcohols, especially, FOH.

The present study can provide several approaches for overproduction of FOH in *S. cerevisiae*. To summarize, the best strategy for FOH production is the expression of the modified *HMG1* dominated by the *GAL1* promoter in the ATCC 200589 strain with the *AUR-C* mutation

(pYHMG044/AURGG101). The highest FOH yield (145.7 mg/l) was obtained with pYHMG044/AURGG101 under the cultivation conditions of constant pH 7 (Fig. 4). Without pH control, the broth pH decreased below 2.5 after approximately 60 h incubation with an increase in the production of NOH, compared with that of FOH. FPP might be preferentially converted into NOH in an acidic environment, being a positional isomer of FOH.

Despite that yeast HMG-CoA reductase, which is a rate-limited enzyme in the mevalonate pathway, was feedback-regulated (Dimsterdenk et al. 1994), the recombinant overproduced FOH in the medium. This may be explained by that gene overexpression overcame the feedback-regulation, or the feedback-regulation by prenyl alcohol derivatives was not tightly controlled in ATCC 200589.

The present study is useful for the production of FOH with recombinant yeasts. In particular, ATCC 200589 is a quite peculiar strain of *S. cerevisiae* with regard to prenyl alcohol production.

Acknowledgments We thank Ms. Chiharu Kato and Ms. Yoshie Tsukahara for their technical assistance.

References

Bard M, Downing JF (1981) Genetic and biochemical aspects of yeast sterol regulation involving 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Gen Microbiol 125:415–420

Benford HL, Frith JC, Auriola S, Mönkkönen J, Rogers MJ (1999) Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol Pharmacol 56:131–140

Bergstrom JD, Kurtz MM, Rew DJ, Amend AM, Karkas JD, Bostedor RG, Bansal VS, Dufresne C, Vanmiddlesworth FL, Hensens OD, Liesch JM, Zink DL, Wilson KE, Onishi J, Milligan JA, Bill G, Kaplan L, Nallin Omstead M, Jenkins RG, Huang L, Meinz MS, Quinn L, Burg RW, Kong YL, Mochales S, Mojena M, Martin I, Pelaez F, Diez MT, Alberts AW (1993) Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc Natl Acad Sci USA 90:80–84

Chambon C, Ladeveze V, Oulmouden A, Servouse M, Karst F (1990) Isolation and properties of yeast mutants affected in farnesyl diphosphate synthetase. Curr Genet 18:41–46

Chambon C, Ladeveze V, Servouse M, Blanchard L, Javelot C, Vladescu B, Karst F (1991) Sterol pathway in yeast. Identification and properties of mutant strains defective in mevalonate diphosphate decarboxylase and farnesyl diphosphate synthetase. Lipids 26:633–636

Dimsterdenk D, Thorsness MK, Rine J (1994) Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol Biol Cell 5:655–665

Donald KAG, Hampton RY, Fritz IB (1997) Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 63:3341–3344

Hashida-Okado T, Ogawa A, Kato I, Takesako K (1998) Transformation system for prototrophic industrial yeasts using the *AUR1* gene as a dominant selection marker. FEBS LETT 425:117–122

Hayashi C (1952) On the prediction of phenomena from qualitative data on the quantification of qualitative data from the mathelective cervical lymph node dissection in patients ematico-statistical point of view. Annals of the Institute of Statistical Mathematics 3:69–98

Hemmi H, Ohnuma S, Nagaoka K, Nishino T (1998) Identification of genes affecting lycopene formation in *Escherichia coli* transformed with carotenoid biosynthetic genes: Candidates for early genes in isoprenoid biosynthesis. J Biochem 123:1088–1096

Hiser L, Basson ME, Rine J (1994) *ERG10* from *Saccharomyces cerevisiae* encodes acetoacetyl-CoA thiolase. J Biol Chem 269:31383–31389

Hyatt JA, Kottas GS, Effler J (2002) Development of synthetic routes to D,L-α-tocopherol (vitamin E) from biologically produced geranylgeraniol. Org Process Res Dev 6:782–787

Jackson BE, Hart-Wells EA, Matsuda SPT (2003) Metabolic engineering to produce sesquiterpenes in yeast. Org Lett 5:1629–1632

Jiang Y, Proteau P, Poulter D, Ferro-Novick S (1995) *BTS1* encodes a geranylgeranyl diphosphate synthase in *Saccharomyces cerevisiae*. J Biol Chem 270:21793–21799

Kuroda M, Hashida-Okado T, Yasumoto R, Gomi K, Kato I, Takesako K (1999) An aureobasidin A resistance gene isolated from *Aspergillus* is a homolog of yeast *AUR1*, a gene responsible for inositol phosphorylceramide (IPC) synthase activity. Mol Gen Genet 261:290–296

Mumberg D, Muller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122

Negishi E, Liou SY, Xu CD, Huo SQ (2002) A novel, highly selective, and general methodology for the synthesis of 1,5-diene-containing oligoisoprenoids of all possible geometrical combinations exemplified by an iterative and convergent synthesis of coenzyme Q(10). Org Lett 4:261–264

Ogura K, Koyama T (1998) Enzymatic aspects of isoprenoid chain elongation. Chem Rev 98:1263–1276

Polakowski T, Stahl U, Lang C (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol 49:66–71

Servouse M, Karst F (1986) Regulation of early enzymes of ergosterol biosynthesis in *Saccharomyces cerevisiae*. Biochem J 240:541–547

Shimada H, Kondo K, Fraser PD, Miura Y, Saito T, Misawa N (1998) Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680

Szkopinska A, Grabinska K, Delourme D, Karst F, Rytka J, Palamarczyk G (1997) Polyprenol formation in the yeast *Saccharomyces cerevisiae*: Effect of farnesyl diphosphate synthase overexpression. J Lipid Res 38:962–968

Veen M, Stahl U, Lang C (2003) Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res 4:87–95

Wang GY, Keasling JD (2002) Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. Metab Eng 4:193–201

Yu JS, Kleckley TS, Wiemer DF (2005) Synthesis of farnesol isomers via a modified Wittig procedure. Org Lett 7:4803–4806