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Research Report

Microbial Production of Isoprenoid Compounds

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ABSTRACT Isoprenoid compounds have recently received increased attention as precursors of drugs, chemicals, and renewable advanced biofuels. Geranylgeraniol (GGOH) is one such valuable compound. In the yeast *Saccharomyces cerevisiae*, GGOH is synthesized through the sequential reactions of farnesyl diphosphate synthetase (encoded by the *ERG20* gene), geranylgeranyl diphosphate synthase (*BTS1*), and some endogenous phosphatases. We found that diacylglycerol diphosphate phosphatase (*DPP1*) gene overexpression could promote GGOH production. We also demonstrated that overexpression of a *BTS1-DPP1* fusion gene was more efficient for producing GGOH than co-expression of these genes separately. Overexpression of the hydroxymethylglutaryl-CoA reductase (*HMG1*) gene, which encodes the rate-limiting enzyme of the mevalonate pathway, resulted in overproduction of squalene (191.9 mg l^{-1}) rather than GGOH (0.2 mg l^{-1}). Co-expression of *BTS1-DPP1* and *BTS1-ERG20* fusion genes along with the *HMG1* gene resulted in an almost complete shift of the flux to GGOH production (228.8 mg l^{-1} GGOH and 6.5 mg l^{-1} squalene). Finally, we constructed a diploid prototroph strain co-expressing these genes from multicopy-integration vectors. This strain attained 3.31 g l^{-1} GGOH production in a ten-liter jar-fermentor. The use of bifunctional fusion genes that encode sequential enzymes in a metabolic pathway is an effective method for metabolic engineering.

KEYWORDS Isoprenoid compound, Geranylgeraniol, Squalene, Yeast, *Saccharomyces cerevisiae*, Fusion gene, Fusion enzyme, Metabolic engineering, Microbial production, Fermentation

1. Introduction

Isoprenoids (terpenoids) comprise one of the most structurally diverse groups of natural products, and they have a number of different and essential functions in living cells.[1,2] Recently, microbial production of isoprenoids, such as the antimalarial drug precursor artemisinic acid[3] and precursors to the anticancer drug Taxol,[4] has been reported. In the automotive field, isoprenoids have received much attention as precursors of renewable advanced biofuels.[5]

In this study, we established microbial production of a C20-isoprenoid of (*E, E, E*)-geranylgeraniol (GGOH). GGOH can be used as an important ingredient for perfumes, and as a desirable raw material for synthesizing vitamins A and E.[6,7] It is also known to induce apoptosis in various cancer and tumor cell lines.[8,9] GGOH is the dephosphorylated derivative of (*E, E, E*)-geranylgeranyl diphosphate (GGPP) (**Fig. 1**). In the yeast *Saccharomyces cerevisiae*, GGPP is synthesized by GGPP synthase (GGPS), encoded by the *BTS1* gene, that catalyzes the condensation of farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP). Biologically synthesized GGOH comprises (*all-E)*-geometric structures, and only the (*all-E)*-isomer has significant biological activities.[10] The chemically synthesized form is usually obtained as mixtures of (*E)*- and (*Z)*-isomers, and thus has lower potency than the native one. Therefore, there is a greater possibility of attaining efficient production of (*all-E*)-GGOH through fermentative production.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R), encoded by the *HMG1* gene, has been shown to be the major rate-limiting enzyme in the mevalonate pathway in *S. cerevisiae*.[11] Overproduction of HMG-R in *S. cerevisiae* strains results in accumulation of squalene rather than GGOH.[12] Squalene is synthesized through the

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condensation of two molecules of FPP catalyzed by squalene synthase (SQS), encoded by the *ERG9* gene in *S. cerevisiae* (Fig. 1). The addition of SQS inhibitors to cultures of *S. cerevisiae* strains results in the production of considerable amounts of farnesol (FOH; ~ 77.5 mg l^{-1}), and relatively low amounts of GGOH (~ 2.2 mg l^{-1}).[13] It has also been reported that SQS-deficient (Δ*erg9*) *S. cerevisiae* strains, being sterol-auxotrophic, accumulate FPP in their cells,[14] and excrete 1.3 mg l^{-1} of FOH into the culture medium.[15] Therefore, inactivation of SQS seems to enhance FOH rather than GGOH production. This is probably because of the low GGPS activity in *S. cerevisiae.* Our group previously found that GGOH production could be enhanced by overexpression of the *BTS1* gene in *S. cerevisiae* without SQS inhibition. In addition, co-expression of a fusion of the *BTS1* gene and farnesyl diphosphate synthetase gene (*ERG20*) along with the *HMG1* gene resulted in the production of 138.8 mg l^{-1} of GGOH and 60.0 mg l^{-1} of squalene.[16]

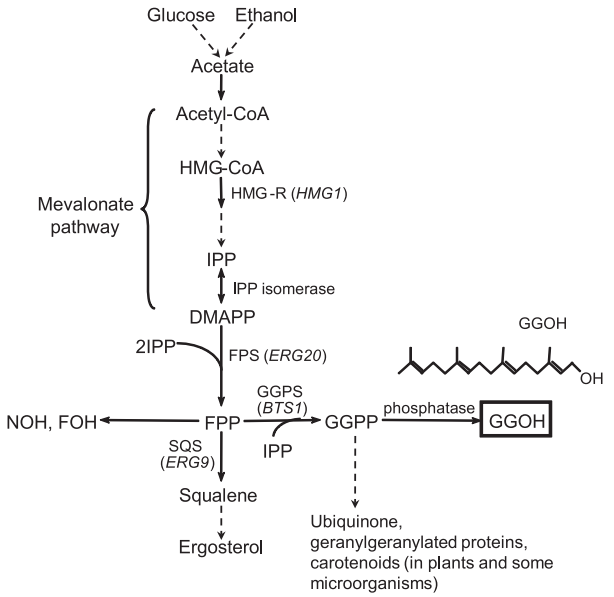

Fig. 1 The biosynthetic pathway for GGOH in *Saccharomyces cerevisiae*. Solid arrows indicate the one-step conversions in the biosynthesis, and the dashed arrows the several steps. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; FOH, farnesol; NOH, nerolidol; GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol. Enzymes (genes): HMG-R, HMG-CoA reductase (encoded by the *HMG1* gene); FPS, FPP synthase (*ERG20*); GGPS, GGPP synthase (*BTS1*); SQS, squalene synthase (*ERG9*).

These results suggest that GGOH can be produced from GGPP through some endogenous phosphatase activities when GGPP synthesis is enhanced. We therefore hypothesized that enhancement of the phosphatase activity could increase the productivity of GGOH. However, it is not clear what kind of phosphatase enhances GGOH production. It has been reported that the products of the diacylglycerol diphosphate phosphatase gene (*DPP1*) and lipid phosphate phosphatase gene (*LPP1*) account for most of the FPP and GGPP phosphatase activities in a particulate (membrane-associated) fraction of *S. cerevisiae*.[17] In this study, we found that GGOH production could be enhanced by overexpression of these phosphatase genes. We also demonstrated that overexpression of the *BTS1-DPP1* and *BTS1-ERG20* fusion genes along with the *HMG1* gene further increased GGOH production. Finally, we constructed a high-level GGOH-producing yeast available for industrial processes involving so-called multicopy-integration vectors. The productivity of GGOH was evaluated in test tube cultures and a ten-liter jar-fermentor.

2. Materials and Methods

2. 1 Strains and Media

The yeast strains used in this study are listed in **Table 1**. Synthetic dextrose (SD) medium and SD agar plates were used for transformant selection and precultures of recombinant yeasts harboring episomal plasmids.[18] YM broth and YPD medium were used for fermentation experiments in test tubes.[19] YMP medium was used for the precultures in the jar-fermentation experiments. YMP medium comprised 5 g l^{-1} yeast extract, 5 g l^{-1} malt extract, 10 g l^{-1} peptone, and 5 g l^{-1} glucose (pH was not adjusted). The main culture medium for jar-fermentations comprised 1 g l^{-1} glucose, 0.85 g l^{-1} KH_2PO_4, 1.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 5 g l^{-1} $(NH_4)_2SO_4$, 0.35 g l^{-1} $CaCl_2 \cdot 2H_2O$, and 1.5 g l^{-1} of an antifoaming agent (Adekanol LG-109; ADEKA Co., Tokyo, Japan), and corn steep liquor adjusted to pH 5.5 with aqueous ammonia. The bacterial cultures were grown at 37ºC in LB broth containing 100 μg ml^{-1} ampicillin when appropriate.

2. 2 Construction of Yeast Episomal Plasmids

The plasmids pRS434GAP, pRS435GAP, and pRS436GAP[20] were used as 2μ-based yeast episomal plasmids. The genes cloned into these plasmids were constitutively expressed under the control of the strong *TDH3* promoter. The *DPP1* and *LPP1* genes were cloned into pRS436GAP and named pRS436DPP1 and pRS436LPP1, respectively. The *BTS1* gene was cloned into pRS435GAP and named pRS435BTS1. A *BTS1-DPP1* fusion gene was constructed by fusing the *DPP1* gene to the 3' end of the *BTS1* gene and cloned into pRS436GAP and named pRS436GGDP. The *HMG1* gene was cloned into pRS434GAP and named pRS434HMG1. The episomal plasmid for overexpression of the *BTS1-ERG20* fusion gene (pRS435GGF) was constructed previously.[16]

2. 3 Construction of Multicopy-integration Vectors

The *HMG1* gene was cloned into the multicopy-integration vectors pRS504GAP and pRS524GAP **(Fig. 2)**; these are targeted to rDNA loci, and named pRS504HMG1 and pRS524HMG1, respectively. The *BTS1-ERG20* fusion gene was cloned into the multicopy-integration vector pRS515GAP (Fig. 2), which is targeted to rDNA loci, to yield pRS515GGF. The *BTS1-DPP1* fusion gene was cloned into the multicopy-integration vector pDI626GAP (Fig. 2), which is targeted to Ty δ loci, and named pDI626GGDP . Yeasts were transformed as previously described.[18]

2. 4 Phosphatase Activity

The cell free extracts used in the phosphatase activity assays were prepared as follows. Cells were grown at

Table 1 Plasmids, vectors and yeast strains used in this study.

Plasmids, vectors and yeast strains	Relevant features	Reference or source
Plasmids and vectors[a]		
pRS434GAP	YEp (2μ), *TRP1* marker, DDBJ accession No. 304854	20
pRS435GAP	YEp (2μ), *LEU2* marker, DDBJ accession No. 304858	20
pRS436GAP	YEp (2μ), *URA3* marker, DDBJ accession No. 304862	20
pRS504GAP	multicopy-integration vector targeted to rDNA loci, *TRP1d* marker	this study
pRS524GAP	multicopy-integration vector targeted to rDNA loci, *TRP1d* marker	this study
pRS515GAP	multicopy-integration vector targeted to rDNA loci, *LEU2d* marker	this study
pDI626GAP	multicopy integration vector targeted to Ty δ loci, *URA3d* marker	this study
Yeast strains		
YPH499	*MAT* **a** *lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ura3-52*	Stratagene
YPH500	*MAT* **α** *lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ura3-52*	Stratagene
Strains with episomal plasmids		
LPP1/YPH499	pRS436LPP1/YPH499	this study
DPP1/YPH499	pRS436DPP1/YPH499	this study
BTS1/YPH499	pRS435BTS1/YPH499	this study
LPP1/BTS1/YPH499	pRS436LPP1/pRS435BTS1/YPH499	this study
DPP1/BTS1/YPH499	pRS436DPP1/pRS435BTS1/YPH499	this study
GGDP/YPH499	pRS436GGDP/YPH499	this study
GGF/GGDP/HMG1/YPH499(YEp)	pRS434HMG1/pRS435GGF/pRS436GGDP/YPH499	this study
Strains with multicopy-integration vectors		
HMG1/YPH499	pRS504HMG1/YPH499	this study
GGDP/HMG1/YPH499	pDI626GGDP/pRS504HMG1/YPH499	this study
GGF/GGDP/HMG1/YPH499	pRS515GGF/pDI626GGDP/pRS504HMG1/YPH499	this study
GGF/GGDP/HMG1/YPH499-H	GGF/GGDP/HMG1/YPH499 *HIS3*	this study
GGF/GGDP/HMG1/YPH500	pDI626GGDP/pRS524HMG1/YPH500	this study
GGF/GGDP/HMG1/YPH500-AK	GGDP/HMG1/YPH500 *ADE2 LYS2*	this study
5X10	Diploid prototroph genarated by mating GGF/GGDP/HMG1/YPH499-H to GGF/GGDP/HMG1/YPH500-AK	this study

[a] All the plasmids and vectors contained the *TDH3* promoter and *CYC1* terminater from *S. cerevisiae* for gene expression.

30°C in selective SD medium for 24 h with shaking (130 rpm). Cells were harvested, washed, and then resuspended in extraction buffer (120 mM citrate, pH 4.3) supplemented with protease inhibitors. Cells were mixed with glass beads (425-600 microns, acid-washed; SIGMA, MO, USA) and disrupting by vortexing vigorously for 10 min at 4°C. The suspensions were centrifuged at 500 × *g* for 5 min to remove the unbroken cells and the supernatants were used for the phosphatase assays. All the extraction procedures were carried out at 4°C. The phosphatase activity toward geranylgeranyl diphosphate (GGPPase) was assayed as the formation of geranylgeraniol from geranylgeranyl diphosphate using a modification of the method of Bansal and Vaidya.[21]

2. 5 Production of Prenyl Alcohols in Test Tube Cultures

The production of prenyl alcohols [FOH, nerolidol (NOH), and GGOH] and squalene was evaluated in test tube cultures. The precultures were grown in selective SD medium for two days at 30°C, followed by inoculation (1/100 vol/vol) into 2.5 ml of YM broth or 2 ml of YPD medium in test tubes equipped with stainless molten caps, and cultivated at 30°C for 4 days with shaking (130 rpm). The cell density at 600 nm (OD_{600}) was measured after 4 days cultivation. Prenyl alcohols were extracted from whole cells with n-pentane, followed by determination of their amounts with a GC/MS system as previously described.[20] For determination of the squalene content with prenyl alcohols, cells were disrupted before extraction because squalene cannot be extracted efficiently without cell disruption.

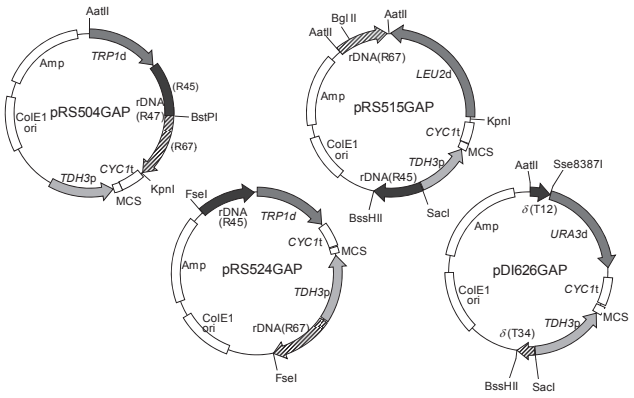

Fig. 2 Structures of multicopy-integration vectors. MCS, multicloning site.

2. 6 Jar Fermentation

Yeast precultures were prepared in Erlenmeyer flasks at 30°C with rotary shaking (120 rpm) for 30 h. The main culture medium (initial volume, 3.5 liters) in a ten-liter jar-fermentor (Model MSJ-U2W; Marubishi Bioengineering Co., Tokyo, Japan) was seeded with a 100 ml aliquot of the preculture. The temperature, agitation speed, flow rate of germ-free air, and pH were controlled at 33°C, 900 rpm, 1 vvm, and 5.5 with 4 N NaOH and 4 N H_2SO_4. A glucose solution (50% wt/vol) was fed from 0 to 24 h, and the feed rate was increased with the consumption. After 24 h, a mixed solution of glucose and ethanol (25% wt/vol and 50% vol/vol, respectively) was fed at a feed rate of 5.8 g h^{-1}. During the fermentation, duplicate aliquots of culture (2 ml each) were collected periodically to determine the amounts of prenyl alcohols without cell disruption as mentioned above.

3. Results

3. 1 Effect of Overexpression of Phosphatase Genes on GGOH Production

Episomal plasmids pRS436LPP1 and pRS436DPP1 were introduced into YPH499, and the GGPPase activities in the cell free extracts of the resulting transformants (LPP1/YPH499 and DPP1/YPH499, respectively) were assayed. The specific GGPPase activity increased 2.0- and 3.1-fold with *LPP1* and *DPP1* gene overexpression compared with the wild type, respectively (data not shown).

To evaluate the effect on promotion of GGOH production by phosphatase overexpression, the *LPP1* and *DPP1* genes were co-expressed with the *BTS1* gene in YPH499 with plasmids pRS436LPP1, pRS436DPP1, and pRS435BTS1. The recombinants were incubated in YM broth in test tubes for 4 days followed by determination of the GGOH content (**Fig. 3**). Single *BTS1* overexpression led to GGOH production (0.11 mg l^{-1}). Overexpression of additional phosphatase genes had a positive effect, increasing GGOH production. The *DPP1* gene gave a better result than the *LPP1* gene, while single *DPP1* gene overexpression did not led to GGOH production.

In our previous study, the *BTS1-ERG20* fusion gene exhibited a good effect, promoting GGOH production. So in this study, we fused the *BTS1* gene with the *DPP1* gene. Single overexpression of the *BTS1-DPP1* fusion gene with plasmid pRS436GGDP further increased GGOH production (*GGDP*; Fig. 3). GGOH production was 2.9-fold higher with the fusion gene compared with co-expression of these genes separately (*DPP1/BTS1*). Squalene, FOH, and NOH were not detected in any of the strains shown in Fig. 3. These results suggest that overexpression of the *BTS1-DPP1* fusion gene is highly effective for promoting GGOH production

3. 2 Construction of a GGOH-producing Strain with Multicopy-integration Vectors

Although episomal plasmids attain high average copy numbers, they suffer from low segregational stability.[22] In contrast, vectors integrated into the genome are stable, but since general yeast integration vectors are integrated at only one to two copies per cell, the expression levels of the introduced genes are not so high. To increase the expression levels, multicopy-integration vectors targeted to repetitive chromosomal DNA sequences such as the ribosomal DNA (rDNA) clusters and long terminal repeats of the Ty element, known as δ sequences, have been developed.[23-26] We constructed multicopy-integration vectors targeted to rDNA and Ty δ loci to stably overexpress the genes that can promote GGOH production (Fig. 2).

First, the *HMG1* gene was overexpressed in order to enhance the mevalonate pathway. Multicopy-integration vector pRS504HMG1 was introduced into the rDNA loci of YPH499. A significant amount of squalene (191.9 mg l^{-1}) was produced by the resulting transformant (HMG1/YPH499; **Fig. 4**), while the host strain did not produce a detectable amount of squalene (data not shown). GGOH production was only 0.2 mg l^{-1} in this strain. The *BTS1-DPP1* fusion gene was then introduced into the δ sequences of strain HMG1/YPH499 with multicopy-integration vector pDI626GGDP. GGOH production was increased in the resulting transformant (GGDP/HMG1/YPH499), and conversely squalene production was decreased. Finally, the *BTS1-ERG20* fusion gene was introduced into the rDNA loci of strain GGDP/HMG1/YPH499

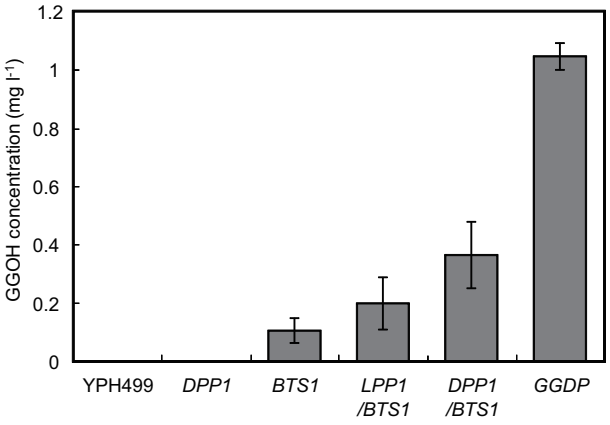

Fig. 3 GGOH production by recombinant strains overexpressing the *DPP1*, *LPP1*, and *BTS1* genes, and *BTS1-DPP1* (GGDP) fusion genes with episomal plasmids in test tube cultures. YPH499, host strain; *DPP1*, pRS436DPP1/YPH499; *BTS1*, pRS435BTS1/YPH499; *LPP1/BTS1*, pRS436LPP1/pRS435BTS1/YPH499; *DPP1/BTS1*, pRS436DPP1/pRS435BTS1/YPH499; GGDP, pRS436GGDP/YPH499. The data represent the averages ± standard deviations for more than three independent clones.

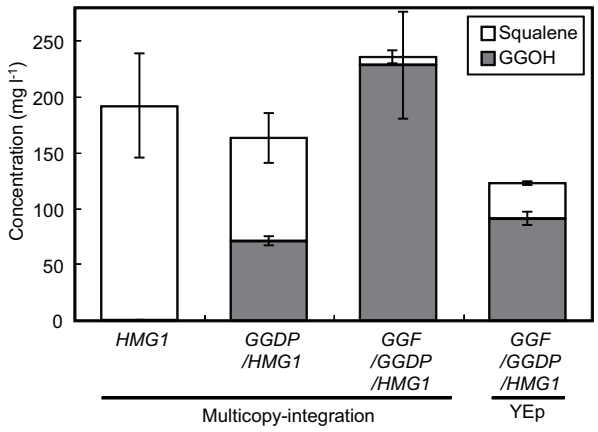

Fig. 4 Production of squalene and GGOH by recombinant strains overexpressing the *HMG1* gene, and *BTS1-DPP1(GGDP)* and *BTS1-ERG20 (GGF)* fusion genes in test tube cultures. *HMG1*, pRS504HMG1/YPH499; *GGDP/HMG1*, pDI626GGDP/pRS504HMG1/YPH499; *GGF/GGDP/HMG1*, pRS515GGF/pDI626GGDP/pRS504HMG1/YPH499; *GGF/GGDP/HMG1*(YEp), pRS434HMG1/pRS435GGF/pRS436GGDP/YPH499. The data represent the averages ± standard deviations for more than three independent clones.

with multicopy-integration vector pRS515GGF. The GGOH production by the resulting transformants (GGF/GGDP/HMG1/YPH499) was further increased and only slight amounts of squalene were produced. The average GGOH and squalene production by 43 independent clones was 228.8 mg l^{-1} and 6.5 mg l^{-1}, respectively. FOH and NOH (a positional isomer of FOH formed from FPP under acidic conditions[20,27,28]) production was low in all the transformants (less than 0.2 and 0.7 mg l^{-1}, respectively). In this way, we constructed a haploid strain that overproduces almost only GGOH.

3. 3 Construction of a GGOH-producing Strain with Episomal Plasmids

In comparison, we also attempted to co-express these three genes (*HMG1*, *BTS1-ERG20,* and *BTS1-DPP1*) in YPH499 with three episomal plasmids, pRS434HMG1, pRS435GGF, and pRS436GGDP, respectively. After successive introduction of these three plasmids, the resulting transformants (GGF/GGDP/HMG1/YPH499(YEp)) produced 91.0 mg l^{-1} of GGOH with 31.5 mg l^{-1} of squalene still being produced (Fig. 4). GGOH production with the episomal plasmids was less than half that of the multicopy-integration vectors.

3. 4 Construction of a Diploid Prototroph Strain

Diploids and prototrophs are industrially attractive because of their advantage of good growth in low cost medium. In order to complement auxotrophic markers, DNA fragments of the *HIS3*, *LYS2*, and *ADE2* coding regions were prepared by PCR from S288C genomic DNA. The *HIS3* fragment was introduced into GGF/GGDP/HMG1/YPH499, and a histidine prototroph was selected on a SD-His plate. The resulting transformant was named GGF/GGDP/HMG1/YPH499-H.

YPH500 was sequentially transformed with multicopy-integration vectors pRS504HMG1, pDI626GGDP, and pRS515GGF to produce a transformant, GGF/GGDP/HMG1/YPH500. This strain produced 152.1 mg l^{-1} and 2.5 mg l^{-1} of GGOH and squalene, respectively, in test tube cultures. The *LYS2* and *ADE2* fragments were introduced into this strain, followed by selection of a lysine and adenine prototroph on a SD-Lys-Ade plate. The resulting transformant was named GGF/GGDP/HMG1/YPH500-AK. Fresh cultures of GGF/GGDP/HMG1/YPH499-H and GGF/GGDP/HMG1/YPH500-AK were mixed and mated in YPD medium overnight, followed by selection on a SD plate without adenine and CSM. The resulting diploid prototroph strain was named strain 5X10. Diploid construction was confirmed by microscopic analysis of the spore formation by strain 5X10 on a sporulation plate.

3. 5 GGOH Production with the Diploid Prototroph Strain

The growth rate was improved for diploid prototroph strain 5X10 compared with haploid auxotrophic strains (data not shown). Strain 5X10 produced a higher amount of GGOH (283.1 mg l^{-1}) than the haploid strains with only slight amounts of FOH, NOH, and squalene in test tube cultivation. This strain was then cultivated in the ten-liter jar-fermentor under aerobic conditions as described in the Materials and Methods. A mixed solution of glucose and ethanol was fed, and this increased GGOH production up to 3.31 g l^{-1} after a 206 h incubation (**Fig. 5**). Squalene, FOH, and NOH production amounted to 0.11, 0.07, and 0.01 g l^{-1} respectively. In this way, we constructed a diploid prototroph strain that overproduces almost only GGOH. GGOH and squalene production by the recombinant yeasts examined in this study is summarized in **Table 2**.

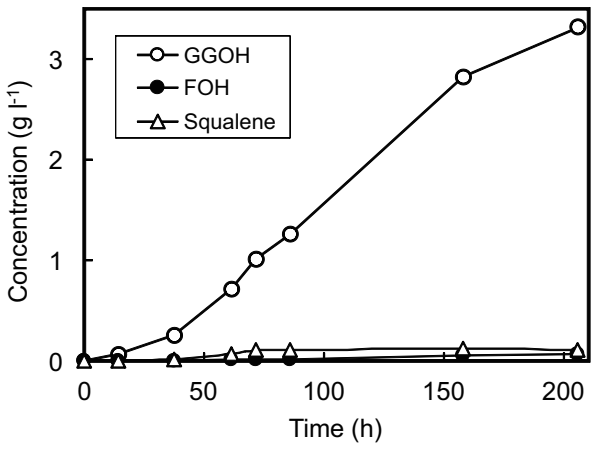

Fig. 5 Fermentation profile of diploid prototroph strain 5X10 in a ten-liter jar-fermentor. After glucose feeding was stopped, as shown in the Materials and Methods, a mixed solution of glucose and ethanol (25% w/v and 50% v/v, respectively) was fed after 24 h at a feed rate of 5.8 g h^{-1}. The data represent the averages for duplicate samples.

4. Discussion

In this study, we demonstrated the microbial production of isoprenoid compounds by metabolic engineering of the yeast *S. cerevisiae*. Enhancement of the mevalonate pathway by overproduction of HMG-R resulted in the accumulation of squalene rather than GGOH. Overexpression of several key enzymes enabled a "pull out" of the target compound (GGOH) from the metabolic flow. The phosphatase Dpp1p proved to be one of these key enzymes. We demonstrated that the overexpression of the *DPP1* gene enhanced GGOH production (Fig. 3). It has been reported that Dpp1p prefers GGPP as a substrate over its precursors such as IPP.[17] Such substrate preference is suitable for GGOH production because the precursors of GGOH tend not to be dephosphorylated by overproduced Dpp1p before their use for GGOH synthesis.

We also found that overexpression of the artificial fusion of the *BTS1* and *DPP1* genes was more efficient for producing GGOH than co-expression of these genes separately (Fig. 3). We think that the two sequential enzymatic reactions can be catalyzed more efficiently by one molecule of the bifunctional fusion protein than the separate two enzymes. Such a proximity effect and substrate channeling for fusion enzymes have been reported to increase the overall catalytic activity of the reaction *in vitro*.[29-32] We demonstrated that the use of bifunctional fusion genes was also highly effective for metabolic engineering.

Finally, we constructed a high-level GGOH-producing yeast that is available for industrial processes involving multicopy-integration vectors. We demonstrated that co-expression of the two fusion genes (*BTS1-DPP1* and *BTS1-ERG20*) along with the *HMG1* gene resulted in an almost complete shift of the metabolic flux from squalene to GGOH production (Fig. 4 and Table 2). Then, we constructed a diploid prototroph strain, 5X10. The GGOH productivity and growth rate of strain 5X10 were higher than those of the haploid strains. Using this diploid prototroph strain, we established a means of GGOH overproduction in a laboratory scale jar-fermentor, as shown in Fig. 5. Gradual feeding of a mixed solution of glucose and ethanol further increased GGOH production up to 3.31 g l^{-1}. This corresponds to a carbon conversion efficiency of 1.65% (GGOH produced per carbon sources consumed) and a GGOH content of 70.9 mg g^{-1} dry cell weight. These values are highly comparable with the production of carotenoids [33] and isoprenoids[2] such as lycopene (7.8 mg g^{-1}),[34] β-carotene (5.9 mg g^{-1}),[35] amorphadiene (149 mg l^{-1}),[3] and artemisinic acid (1.06 g l^{-1})[36] by recombinant yeasts. This study clearly demonstrates that high-level GGOH overproduction by a metabolically engineered *S. cerevisiae* strain may be suitable for industrial processes.

Table 2 Summary of GGOH and squalene production by the recombinant yeasts in test tube cultures and jar-fermenter.

Yeast strains	Concn (mg l^{-1}) (SD)[a]	
	GGOH	Squalene
YPH499	n.d.[b]	n.d.[b]
HMG1/YPH499	0.2 (0.2)	191.9 (0.2)
GGDP/HMG1/YPH499	71.1 (4.1)	91.8 (22.2)
GGF/GGDP/HMG1/YPH499	228.8 (47.6)	6.5 (5.4)
GGF/GGDP/HMG1/YPH499(YEp)	91.0 (5.6)	31.5 (1.5)
GGF/GGDP/HMG1/YPH500	152.1 (19.4)	2.5 (1.4)
5X10	283.1 (21.1)	0.08 (0.03)[c]
5X10 (jar-fermenter)	3,310.0	106.9[c]

[a] The data are averages (SD) for more than three independent clones in test tube cultures except for 5X10 (jar-fermenter), for which are the averages for two samples in a ten-liter jar-fermentor.
[b] n.d., not detected.
[c] Squalene concentrations might have been underestimated because the cells were extracted without cell disruption.

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References

(1) Barkovich, R. and Liao, J. C., "Metabolic Engineering of Isoprenoids", *Metab. Eng.*, Vol.3, No.1 (2001), p.27.
(2) Kirby, J. and Keasling, J. D., "Metabolic Engineering of Microorganisms for Isoprenoid Production", *Nat. Prod. Rep.*, Vol.25, No.4 (2008), p.656.
(3) Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R. and Keasling, J. D., "Production of the Antimalarial Drug Precursor Artemisinic Acid in Engineered Yeast", *Nature*, Vol.440, No.7086 (2006), p.940.
(4) Ajikumar, P. K., Xiao, W. H., Tyo, K. E., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B. and Stephanopoulos, G., "Isoprenoid Pathway Optimization for Taxol Precursor

Overproduction in *Escherichia Coli*", *Science*, Vol.330, No.6000 (2010), p.70.
(5) Zhang, F., Rodriguez, S. and Keasling, J. D., "Metabolic Engineering of Microbial Pathways for Advanced Biofuels Production", *Curr. Opin. Biotechnol.*, Vol.22, No.6 (2011), p.775.
(6) Benford, H. L., Frith, J. C., Auriola, S., Monkkonen, J. and Rogers, M. J., "Farnesol and Geranylgeraniol Prevent Activation of Caspases by Aminobisphosphonates: Biochemical Evidence for Two Distinct Pharmacological Classes of Bisphosphonate Drugs", *Mol. Pharmacol.*, Vol.56, No.1 (1999), p.131.
(7) Hyatt, J. A., Kottas, G. S. and Effler, J., "Development of Synthetic Routes to D,L-α-tocopherol (Vitamin E) from Biologically Produced Geranylgeraniol", *Organic Process Research & Development*, Vol.6, No.6 (2002), p.782.
(8) Takeda, Y., Nakao, K., Nakata, K., Kawakami, A., Ida, H., Ichikawa, T., Shigeno, M., Kajiya, Y., Hamasaki, K., Kato, Y. and Eguchi, K., "Geranylgeraniol, An Intermediate product in Mevalonate Pathway, Induces Apoptotic Cell Death in Human Hepatoma Cells: Death Receptor-Independent Activation of Caspase-8 with Down-regulation of Bcl-xL Expression", *Jpn. J. Cancer Res.*, Vol.92, No.9 (2001), p.918.
(9) Ohizumi, H., Masuda, Y., Nakajo, S., Sakai, I., Ohsawa, S. and Nakaya, K., "Geranylgeraniol is a Potent Inducer of Apoptosis in Tumor-cells", *J. Biochem. (Tokyo)*, Vol.117, No.1 (1995), p.11.
(10) Ogura, K. and Koyama, T., "Enzymatic Aspects of Isoprenoid Chain Elongation", *Chem. Rev.*, Vol.98, No.4 (1998), p.1263.
(11) Hampton, R., Dimster-Denk, D. and Rine, J., "The Biology of HMG-CoA Reductase: The Pros of Contra-regulation", *Trends Biochem. Sci.*, Vol.21, No.4 (1996), p.140.
(12) Ohto, C., Muramatsu, M., Obata, S., Sakuradani, E. and Shimizu, S., "Overexpression of the Gene Encoding HMG-CoA Reductase in *Saccharomyces cerevisiae* for Production of Prenyl Alcohols", *Appl. Microbiol. Biotechnol.*, Vol.82, No.5 (2009), p.837.
(13) Muramatsu, M., Ohto, C., Obata, S., Sakuradani, E. and Shimizu, S., "Accumulation of Prenyl Alcohols by Terpenoid Biosynthesis Inhibitors in Various Microorganisms", *Appl. Microbiol. Biotechnol.*, Vol.80, No.4 (2008), p.589.
(14) Song, L., "Detection of Farnesyl Diphosphate Accumulation in Yeast *ERG9* Mutants", *Anal. Biochem.*, Vol.317, No.2 (2003), p.180.
(15) Chambon, C., Ladeveze, V., Oulmouden, A., Servouse, M. and Karst, F., "Isolation and Properties of Yeast Mutants Affected in Farnesyl Diphosphate Synthetase", *Curr. Genet.*, Vol.18, No.1 (1990), p.41.
(16) Ohto, C., Muramatsu, M., Obata, S., Sakuradani, E. and Shimizu, S., "Production of Geranylgeraniol on Overexpression of a Prenyl Diphosphate Synthase Fusion Gene in *Saccharomyces cerevisiae*", *Appl. Microbiol. Biotechnol.*, Vol.87, No.4 (2010), p.1327.
(17) Faulkner, A., Chen, X., Rush, J., Horazdovsky, B., Waechter, C. J., Carman, G. M. and Sternweis, P. C., "The *LPP1* and *DPP1* Gene Products Account for Most of the Isoprenoid Phosphate Phosphatase Activities in *Saccharomyces cerevisiae*", *J. Biol. Chem.*, Vol.274, No.21 (1999), p.14831.
(18) Tokuhiro, K., Ishida, N., Kondo, A. and Takahashi, H., "Lactic Fermentation of Cellobiose by a Yeast Strain Displaying β-glucosidase on the Cell Surface", *Appl. Microbiol. Biotechnol.*, Vol.79, No.3 (2008), p.481.
(19) Tokuhiro, K., Ishida, N., Nagamori, E., Saitoh, S., Onishi, T., Kondo, A. and Takahashi, H., "Double Mutation of the *PDC1* and *ADH1* Genes Improves Lactate Production in the Yeast *Saccharomyces cerevisiae* Expressing the Bovine Lactate Dehydrogenase Gene", *Appl. Microbiol. Biotechnol.*, Vol.82, No.5 (2009), p.883.
(20) Ohto, C., Muramatsu, M., Obata, S., Sakuradani, E. and Shimizu, S., "Overexpression of the Gene Encoding HMG-CoA Reductase in *Saccharomyces cerevisiae* for Production of Prenyl Alcohols", *Appl. Microbiol. Biotechnol.* (2008).
(21) Bansal, V. S. and Vaidya, S., "Characterization of Two Distinct Allyl Pyrophosphatase Activities from Rat Liver Microsomes", *Arch. Biochem. Biophys.*, Vol.315, No.2 (1994), p.393.
(22) Parekh, R. N., Shaw, M. R. and Wittrup, K. D., "An Integrating Vector for Tunable, High Copy, Stable Integration into the Dispersed Ty δ Sites of *Saccharomyces cerevisiae*", *Biotechnol. Prog.*, Vol.12, No.1 (1996), p.16.
(23) Lopes, T. S., Klootwijk, J., Veenstra, A. E., van der Aar, P. C., van Heerikhuizen, H., Raue, H. A. and Planta, R. J., "High-copy-number Integration into the Ribosomal DNA of *Saccharomyces cerevisiae*: A New Vector for High-level Expression", *Gene*, Vol.79, No.2 (1989), p.199.
(24) Sakai, A., Shimizu, Y. and Hishinuma, F., "Integration of Heterologous Genes into the Chromosome of S*accharomyces cerevisiae* Using a Delta Sequence of Yeast Retrotransposon Ty", *Appl. Microbiol. Biotechnol.*, Vol.33, No.3 (1990), p.302.
(25) Lopes, T. S., Hakkaart, G. J., Koerts, B. L., Raue, H. A. and Planta, R. J., "Mechanism of High-copy-number Integration of pMIRY-type Vectors into the Ribosomal DNA of *Saccharomyces cerevisiae*", *Gene*, Vol.105, No.1 (1991), p.83.
(26) Sakai, A., Ozawa, F., Higashizaki, T., Shimizu, Y. and Hishinuma, F., "Enhanced Secretion of Human Nerve Growth Factor from *Saccharomyces cerevisiae* Using an Advanced δ-integration System", *Biotechnology (N.Y.)*, Vol.9, No.12 (1991), p.1382.
(27) Nishino, T., Suzuki, N. and Katsuki, H., "Enzymatic Formation of Nerolidol in Cell-free Extract of *Rhodotorula Glutinis*", *J. Biochem.*, Vol.92, No.6

(1982), p.1731.
(28) George-Nascimento, C., Pont-Lezica, R. and Cori, O., "Non Enzymic Formation of Nerolidol from Farnesyl Pyrophosphate in the Presence of Bivalent Cations", *Biochem. Biophys. Res. Commun.*, Vol.45, No.1 (1971), p.119.
(29) Seo, H. S., Koo, Y. J., Lim, J. Y., Song, J. T., Kim, C. H., Kim, J. K., Lee, J. S. and Choi, Y. D., "Characterization of a Bifunctional Enzyme Fusion of Trehalose-6-phosphate Synthetase and Trehalose-6-phosphate Phosphatase of *Escherichia coli*", *Appl. Environ. Microbiol.*, Vol.66, No.6 (2000), p.2484.
(30) Kim, G. J., Lee, D. E. and Kim, H. S., "Construction and Evaluation of a Novel Bifunctional *N*-carbamylase-D-hydantoinase Fusion Enzyme", *Appl. Environ. Microbiol.*, Vol.66, No.5 (2000), p.2133.
(31) Gilbert, M., Bayer, R., Cunningham, A. M., DeFrees, S., Gao, Y., Watson, D. C., Young, N. M. and Wakarchuk, W. W., "The Synthesis of Sialylated Oligosaccharides Using a CMP-Neu5Ac Synthetase/sialyltransferase Fusion", *Nat. Biotechnol.*, Vol.16, No.8 (1998), p.769.
(32) Liang, P. H. and Anderson, K. S., "Substrate Channeling and Domain-domain Interactions in Bifunctional Thymidylate Synthase-dihydrofolate Reductase", *Biochemistry (Mosc.)*, Vol.37, No.35 (1998), p.12195.
(33) Das, A., Yoon, S. H., Lee, S. H., Kim, J. Y., Oh, D. K. and Kim, S. W., "An Update on Microbial Carotenoid Production: Application of Recent Metabolic Engineering Tools", *Appl. Microbiol. Biotechnol.*, Vol.77, No.3 (2007), p.505.
(34) Shimada, H., Kondo, K., Fraser, P. D., Miura, Y., Saito, T. and Misawa, N., "Increased Carotenoid Production by the Food Yeast *Candida utilis* through Metabolic Engineering of the Isoprenoid Pathway", *Appl. Environ. Microbiol.*, Vol.64, No.7 (1998), p.2676.
(35) Verwaal, R., Wang, J., Meijnen, J. P., Visser, H., Sandmann, G., van den Berg, J. A. and van Ooyen, A. J., "High-level Production of Beta-carotene in *Saccharomyces cerevisiae* by Successive Transformation with Carotenogenic Genes from *Xanthophyllomyces Dendrorhous*", *Appl. Environ. Microbiol.*, Vol.73, No.13 (2007), p.4342.
(36) Ro, D. K., Ouellet, M., Paradise, E. M., Burd, H., Eng, D., Paddon, C. J., Newman, J. D. and Keasling, J. D., "Induction of Multiple Pleiotropic Drug Resistance Genes in Yeast Engineered to Produce an Increased Level of Anti-malarial Drug Precursor, Artemisinic Aid", *BMC Biotechnol.*, Vol.8 (2008), p.83.

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