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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Accumulation of prenyl alcohols by terpenoid biosynthesis inhibitors in various microorganisms

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Abstract Squalene synthase inhibitors significantly accelerate the production of farnesol by various microorganisms. However, farnesol production by *Saccharomyces cerevisiae* ATCC 64031, in which the squalene synthase gene is deleted, was not affected by the inhibitors, indicating that farnesol accumulation is enhanced in the absence of squalene synthase activity. The combination of diphenylamine as an inhibitor of carotenoid biosynthesis and a squalene synthase inhibitor increases geranylgeraniol production by a yeast, *Rhodotorula rubra* NBRC 0870. An ent-kauren synthase inhibitor also enhances the production of farnesol and geranylgeraniol by a filamentous fungus, *Gibberella fujikuroi* NBRC 30336. These results indicate that the inhibition of downstream enzymes from prenyl diphosphate synthase leads to the production of farnesol and geranylgeraniol.

Keywords Farnesol · Farnesyl diphosphate · Geranylgeraniol · Prenyl alcohol · Terpenoid

Introduction

Farnesol (FOH) and geranylgeraniol (GGOH) so called as prenyl alcohols are known as major fragrance components in essential oils. Recently, they were also noted to be remarkable industrial materials for various terpenoid production for human health (Wang et al. 2005). For synthetic organic chemistry, FOH and GGOH are useful starting materials for the synthesis of the isoprenoid side chain moieties of tocopherol, menaquinone-4 and ubiquinone-10. Although it is difficult to selectively introduce multiple trans carbon double bonds into the isoprenoid structure by means of organic synthesis, all living organisms accomplish this with extreme precision through enzymatic condensation of a dimethylallyl diphosphate (DMAPP) molecule and several ones of isopentenyl diphosphate (IPP; Radetich and Corey 2002). The successive head-to-tail condensation of a DMAPP molecule and two IPP ones leads to farnesyl diphosphate (FPP), which is a precursor for further transformation to FOH, steroids, and various sesquiterpenes, while that of a DMAPP molecule and three IPP ones yields geranylgeranyl diphosphate (GGPP) as the precursor for GGOH and diterpenoids such as carotenoids and gibberellins (Po-Huang et al. 2002; Fig. 1).

Numerous biosynthetic pathways to terpenoids originate from only two intermediates, FPP and GGPP, in this manner. But the biosynthesis is strictly regulated, and the majority of organisms are not able to accumulate large amounts of the metabolites, the exception being a few microorganisms: a budding yeast, *Saccharomyces cerevisiae*, accumulating ergosterol in the cell membrane; a filamentous fungus, *Gibbrella fujikuroi*, producing gibberellin; and *Rhodotorula rubra*, producing carotenoids. These strains must have a highly potent terpenoid synthesis pathway.

On the other hand, specific inhibitors have been designed for the enzymes involved in the biosynthesis of these terpenoids. For instance, squalestatin 1 and zaragozic acid B strongly inhibit squalene (SQ) synthase by blocking the condensation of two molecules of FPP (Hasumi et al.

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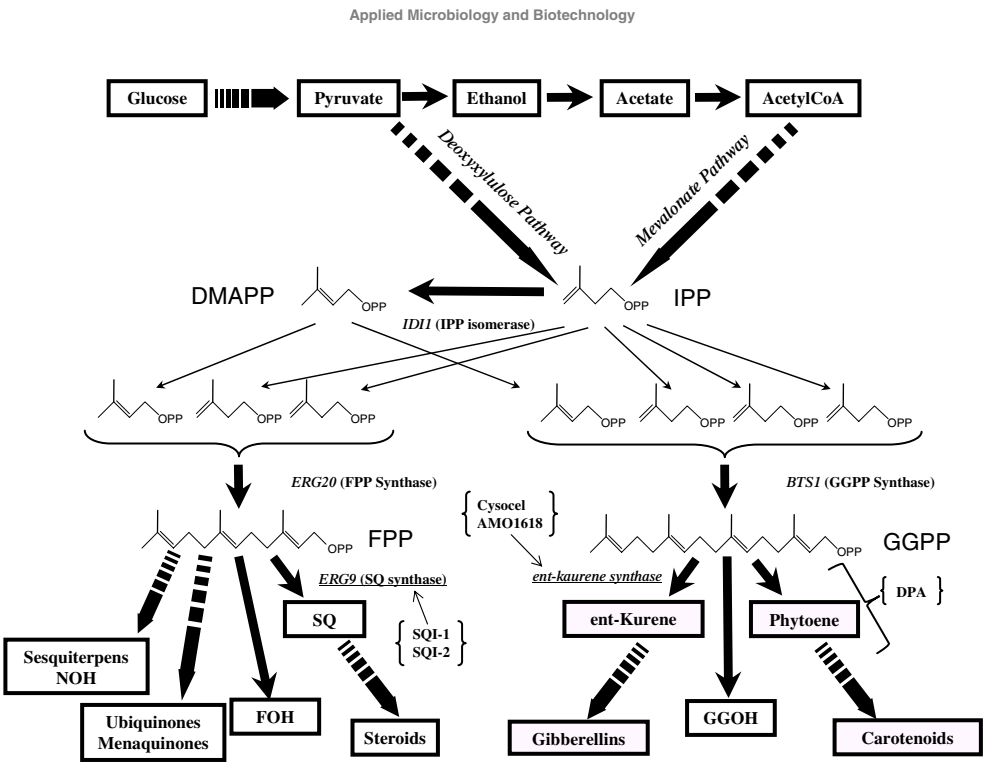

Fig. 1 Biosynthesis pathway for terpenoids. The inhibitors are shown in the *braces*. *DMAPP* Dimethylallyl diphosphate, *DPA* diphenylamine, *FOH* farnesol, *FPP* farnesyl diphosphate, *GGOH* geranylgeraniol, *GGPP* geranylgeranyl diphosphate, *IPP* isopentenyl diphosphate, *NOH* nerolidol, *SQ* squalene, *SQI-1* 3-phenoxy α-phosphobenzene sulfonic acid tri-potassium salt, *SQI-2* 4-{4-(4-methoxybenzoyl) pyperizino} butylidene-1, 1-phosphonic acid tetra ethyl ester

1993; Bergstrom et al. 1993). Ent-kaurene synthase inhibitors, which block the cyclization of GGPP in the early step of gibberellin biosynthesis, have been used as agrochemicals for the repression of plant growth, as well as uniconazole-P, which inhibits the oxidation of ent-kaurene (Rademacher 2000). Diphenylamine (DPA) especially inhibits carotenoid biosynthesis during carotenoid biosynthesis (Gerhard 1997). These inhibitors possibly cause high production of prenyl alcohols which generate from the accumulated prenyl diphosphates by rapid hydrolysis of a specific allyl phosphatase in microorganisms (Bansal and Vaidya 1994; Jacob et al. 2003).

In this paper, we show that SQ synthase inhibitors, an ent-kaurene synthesis inhibitor and a carotenoid biosynthesis inhibitor increase the production of FOH and GGOH by various microorganisms. The inhibition of these enzymes is considered to be an effective strategy for prenyl alcohol production. We describe the microbial FOH and GGOH production by using the inhibitors related with the biosynthesis of the derivatives from FPP and GGPP, such as carotenoids, gibberellins, and steroids.

Materials and methods

Materials

Adekanol LG-109 was purchased from ADEKA Co. (Tokyo, Japan). Yeast extract, bactopeptone, malt extract, and LB broth were from Becton, Dickinson and Company (NJ, USA). Cysocel, ancymidol, inabenfide, and uniconazol were obtained from GL Science Co. (Tokyo, Japan). AMO1618, DPA, and prohexadione were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Oat meal was purchased from Morinaga, Co., Ltd. (Tokyo, Japan). 3-Phenoxy α-phosphobenzene sulfonic acid tri-potassium salt (SQI-1) and 4-{4-(4-methoxybenzoyl) pyperizino} butylidene-1, 1-phosphonic acid tetra ethyl ester (SQI-2) were from by Eisai Co., Ltd. (Tokyo, Japan). The other chemicals were from Nacalai Tesque, Inc. (Kyoto, Japan).

Media and growth conditions

Yeasts, bacteria, and archaea were cultivated in the following media: YMO (6% dextrose, 0.3% yeast extract, 0.5% bactopeptone, 0.3% malt extract, and 1% soybean oil), LBO (LB broth supplemented with 6% dextrose and 1% soybean oil), and NaClO (6% dextrose, 15.6% NaCl, 1.3% $MgCl_2 \cdot 6H_2O$, 2% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, 0.4% KCl, 0.02% $NaHCO_3$, 0.05% KBr, 0.5% yeast extract, and 1% soybean oil, pH 7.0) medium, respectively. YM is YMO medium containing 1% glucose without soybean oil. These media were supplemented with 0.002% ergosterol using a 1,000× stock solution. The 1,000× ergosterol solution was prepared by dissolving 20 mg of ergosterol powder in 1 mL of an ethanol solution

containing 50% tergitol melted in a hot water bath. After autoclaving, an SQ synthase inhibitor (SQI-1 or SQI-2) was added to the medium using a 100× solution sterilized with a 0.2 mm filter (Millipore Co., MA, USA). Each culture (2 mL) was carried out in a test tube at an appropriate temperature (25–30°C) with incubation of 1% of preculture broth incubated overnight, unless stated otherwise.

The cultivation profile of *Candida glabrata* NBRC (NITE Biological Resource Center, Chiba, Japan) 0005 was analyzed by using 10 L jar fermenters (Marubishi Bioengineering Co., Tokyo, Japan) with a working volume of 5 L. For inoculation (2%) into the fermenters, YM cultures in Erlenmeyer flasks were incubated at 30°C with rotary shaking (120 rpm) for 30 h. The temperature, agitation speed, flow rate of germ-free air, and pH were controlled to 7.0 adjusted with 4 N NaOH or 4 N H_2SO_4 at 30°C, 400 rpm, and 1 vvm. During the fermentation, 2 mL aliquots of the culture were collected periodically for analyses. Cell density was measured as either the O.D. at 660 nm as turbidity or by counting cells in appropriately diluted broth on a hematoplate under a phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany).

Gibbrella fujikuroi NBRC 30336 was cultured in medium comprising 8% glucose, 1.2% NH_4NO_3, 0.5% KH_2PO_4, 0.5% yeast extract and 1.5% oat meal. A filter-sterilized gibberellin biosynthesis inhibitor solution (15 mg/L) was added aseptically to the medium. The culture was performed at 30°C for 10 days with rotary shaking at 150 rpm.

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 mL of an internal standard solution (0.1% *v*/*v* undecanol in ethanol) had been placed in advance. Nerolidol (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, hold 7 min). The amount of a prenyl alcohol was calculated from the ratio of to selected ion peak area (69 mass fragment) to that of undecanol. For extraction from the culture of *G. fujikuroi*, several modifications of the above procedure were made. Namely, 20 mL of the broth was subjected to 10 min centrifugation (5,500×*g*) at 4°C to separate the extracellular fraction from the whole broth. The resultant cell pellets was transferred to the 50 mL vessel of BeadBeater (BioSpec Products Inc., OK, USA), and suspended in 20 mL of 0.5 mm glass beads and 20 mL of 0.25 M acetate buffer (pH 4.7) containing 0.025% Triton X-100. After the cells had been disrupted for 3 min with cooling on ice, the whole cell extract and the former extracellular fraction were combined. The prenyl alcohols were extracted with 30 mL of a solvent system (pentane/methanol=2:1 *v*/*v*), dried under a stream of air, and then dissolved in 200 mL of pentane. All data obtained in the present study are means of three independent determinations (SD is less than 7%).

Analysis of glucose and ethanol

The concentrations of glucose and ethanol in the broth were measured with an F-Kit (J. K. International Inc., Tokyo, Japan) based on the hexose kinase method and the enzyme assay involving the combination of alcohol dehydrogenase with acetaldehyde dehydrogenase, respectively.

Results

Effects of SQ synthase inhibitors on prenyl alcohol production in *C. glabrata*

C. glabrata NBRC 0005 was found as a potential producer of prenyl alcohols through our screening study. The strain produced both FOH (0.12 mg/L) and GGOH (0.05 mg/L) simultaneously in YM medium under similar conditions given in Table 1. The effects of SQ synthase inhibitors on the production of FOH and GGOH with this strain grown in YMO medium are shown in Table 1. The amounts of NOH and SQ are also measured and shown in the same table because the precursors (DMAPP and IPP) to FOH and GGOH are also used for the formation of NOH and SQ. As expected, these inhibitors (SQI-1 and SQI-2) specifically decreased the SQ level and caused elevation of both FOH and NOH productions. The amounts of FOH reached 14.98

Table 1 Effects of squalene synthase inhibitors on prenyl alcohol production by *Candida glabrata* NBRC 0005

Inhibitor		NOH (mg/L)	FOH (mg/L)	GGOH (mg/L)	SQ (mg/L)
Name	Conc. (mg/L)				
None	0	0.00	0.57	0.26	15.64
SQI-1	1	0.07	0.83	0.29	14.32
	40	0.07	7.60	0.18	5.82
SQI-2	1	0.02	4.32	0.19	9.38
	20	0.05	13.87	0.18	8.92
	40	0.13	14.98	0.19	8.37

C. glabrata NBRC 0005 was cultivated in YMO medium, consisting of 6% dextrose, 0.3% yeast extract, 0.5% bactopeptone, 0.3% malt extract, and 1% soybean oil, in the presence of an inhibitor at 30°C for 3 days.

mg/L on addition of 40 mg/L of SQI-2. This value was approximately 50 times higher than that in the control YMO medium (0.57 mg/L).

Cultivation profile of *C. glabrata* in the presence of SQI-2

For optimization of the production of prenyl alcohols, the cultivation profile of strain NBRC 0005 in the presence of 20 mg/L of SQI-2 was analyzed (Fig. 2). Glucose in the medium was consumed with increasing cell density and completely consumed after 90 h. The growth of this strain steeply increased until 22 h after inoculation, and then reached a plateau level. The accumulation of FOH started at 22 h after cessation of cell proliferation and reached 119 mg/L at 92 h, i.e., inversely proportional to ethanol consumption.

Effects of SQ synthase inhibitors on the production of prenyl alcohols in various microorganisms

Various yeasts (337 strains) and bacteria (53 strains) that we stock were cultivated with 20 mg/L of SQI-2. FOH and GGOH increased in all the strains of yeasts tested, whereas enhanced production was observed in only a few bacterial strains with low level of SQI-2 (0.2 mg/L). The yeasts listed in Table 2 accumulated less than 1.0 mg/L of FOH and 0.5 mg/L of GGOH on cultivation without SQI-2 (data not shown). Table 2 shows the production of FOH and GGOH by several representative microorganisms. More than 20 mg/L of SQI-2 exhibited the effect on the accumulation of the prenyl alcohols; on the other hand, 40 mg/L of SQI-2 caused the growth inhibition of some microorganisms. *Candida albicans* NBRC 1060, *Phaffia rhodozyma* ATCC 66270, and *Filobacidium capsuligenum* NBRC 1185 were the best three FOH-producing strains. The FOH accumulation level with each of these strains was 1.5 times higher than that with *C. glabrata* NBRC 0005. These strains exhibited GGOH production, but only approximately 1/20 that of FOH. On the other hand, *Rodotorula rubra* NBRC 0870, *Bullera pseudoalba* NBRC 10179, and *Sporobolomyces salmonicolor* NBRC 0374 were the best three strains as to GGOH production; the values reached about 15 mg/L or more with a ratio of GGOH to FOH below 1/3. GGOH production was also high in an archaea, *Haloferax volcanii* NBRC 14742, of which the cell membrane comprises of isoprenoids. But the prokaryotic organisms produced only a little FOH (<0.2 mg/L), despite that the bacterial cell membrane comprises hopanoids

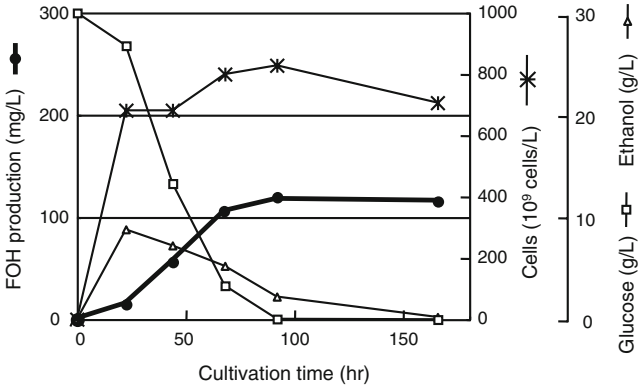

Fig. 2 Cultivation profile of *C. glabrata* in the presence of SQI-2. *C. glabrata* NBRC 0005 was cultivated at 30°C in YMO medium containing 20 mg/L of SQI-2. The temperature, agitation speed, flow rate of germ-free air, and pH were controlled at 30°C, 400 rpm, 1 vvm, and 7.0, adjusted with 4 N NaOH or 4 N H_2SO_4

Table 2 Effects of a squalene synthase inhibitor (SQI-2) on prenyl alcohol production by various microorganisms

Class	Strain	FOH (mg/L)	GGOH (mg/L)
Yeast	Top 3 strains for FOH production		
	Candida albicans NBRC 1060	108.8	3.5
	Phaffia rhodozyma ATCC 66270	108.7	5.8
	Filobacidium capsuligenum NBRC 1185	106.6	3.6
	Control strain		
	Candida glabrata NBRC 0005	74.6	3.4
	Saccharomyces cerevisiae		
	Saccharomyces cerevisiae NBRC 0258	77.5	1.9
	Saccharomyces cerevisiae ATCC 9080	54.1	2.2
	Saccharomyces cerevisiae ATCC 76625	0.1	0.1
	Squalene-producing strains		
	Saccharomycopsis fibuligera NBRC 1665	0.1	4.4
	Saccharomycopsis fibuligera NBRC 1744	0.1	4.6
	Top 3 strains for GGOH production		
	Rhodotorula rubra NBRC 0870	4.4	24.4
	Bullera pseudoalba NBRC 10179	40.7	15.0
	Sporobolomyces salmonicolor NBRC 0374	35.7	14.4
Bacteria	Top 3 bacterial strains for prenyl alcohol production		
	Brevibacterium linens NBRC 12171	0.5	0.0
	Staphylococcus epidermidis NBRC 3762	0.4	0.0
	Exiguobacterium acetylicum NBRC 12146	0.1	0.0
Archaea	*Haloferax volcanii* NBRC 14742	57.7	2.5

ATCC 66270, NBRC 1185, and NBRC 10179 were cultivated at 24°C for 10 days, and the other strains at 30°C. YMO, LBO, and NaClO were used for yeasts, bacteria, and archaea, respectively. Squalene synthase inhibitor SQI-2 (20 mg/L) was added to the medium. After cultivation, prenyl alcohols were directly extracted with pentane–methanol as a solvent system.

synthesized from squalene. The susceptibility to the inhibitors was considerably diverse in the species. The commonly used strain as a recombinant host YPH499 in laboratories, *S. cerevisiae* ATCC 76625, showed low productivity of the prenyl alcohols, in comparison with the NBRC 0258 strain, previously called *Saccharomyces mangini*, and *Carlsbergensis* strain ATCC 9080, known as an ergosterol-producing strain. Similarly, *Saccharomycopsis fibuligera,* which has been known as an SQ over-producing strain, did not show high production of prenyl alcohols.

Effects of an SQ synthase inhibitor on the *erg* 9 mutant of *S. cerevisiae*

S. cerevisiae ATCC 64031, an *erg* 9-deficient mutant, shows a low SQ content inherently (0.23 mg/L), and it was scarcely affected by SQI-2 addition (Table 3). FOH production by the strain was originally high (19.65 mg/L), and not altered by the inhibitor (19.58 mg/L). Thus, neither the production of prenyl alcohols nor SQ by the *erg* 9-deficient mutant is affected by the inhibitor SQI-2. In contrast, the SQ content of the wild-type strain, *S. cerevisiae* NBRC 0538, was repressed from 16.22 to 0.81 mg/L on the addition of SQI-2, and FOH production was promoted approximately 100 times. Furthermore, the wild strain accumulated more amounts (40.05 mg/L) of FOH by using SQI-2.

Effects of carotenoid biosynthesis inhibitors on GGOH production

DPA is an inhibitor of the enzymes related with carotenoid biosynthesis DPA remarkably enhanced GGOH production by carotenoid-producing yeasts, *R. rubra* NBRC 0870 and *Rhodotorula minuta* NBRC 0715, on the addition of the SQ synthase inhibitor (Table 4). The addition of DPA together with SQI-2 also slightly increased the FOH and NOH production, compared with in the case of SQI-2 alone. These effects of DPA have never been observed without the SQ synthase inhibitor SQI-2.

Table 3 Effects of a squalene synthase inhibitor (SQI-2) on prenyl alcohol production by *S. cerevisiae*

Strain no.	ERG9	SQI-2 (mg/L)	Production (mg/L)		
			FOH	GGOH	SQ
NBRC 0538	Wild	0	0.46	0.24	16.22
	Wild	40	40.05	0.49	0.81
ATCC 64031	Deficient	0	19.65	0.25	0.23
	Deficient	40	19.58	0.26	0.25

The wild-type strain (NBRC 0538) and the ERG9-deficient strain (ATCC 6401) were cultivated in YMO medium with squalene synthase inhibitor SQI-2 at 26°C for 7 days.

Table 4 Effects of a squalene synthase inhibitor (SQI-2) and a carotenoid biosynthesis inhibitor (DPA) on prenyl alcohol production by carotenoids-producing yeasts

Strain	DPA (mg/L)	SQI-2 (mg/L)	Production (mg/L)			
			NOH	FOH	GGOH	SQ
NBRC 0870	0	0	0.0	0.0	0.0	0.1
	20	0	0.0	0.0	0.0	0.1
	0	20	4.7	7.9	4.9	3.8
	20	20	8.2	8.9	53.7	0.8
NBRC 0715	0	0	0.0	0.0	0.0	0.1
	20	0	0.0	0.0	0.0	0.0
	0	20	4.0	9.7	14.5	1.1
	20	20	6.1	9.5	42.8	1.0

Rhodotorula rubra (NBRC 0870) and *Rhodotorula minuta* (NBRC 0715) were inoculated with 5% of the preculture broth incubated overnight and then cultivated in YMO medium at 30°C for 10 days.

Effects of gibberellin inhibitors on prenyl alcohol production

In order to investigate the effects of various gibberellin inhibitors on the prenyl alcohol production, *Gibberella fujikuroi* NBRC 30336 was cultivated with 15 mg/L of an inhibitor (Table 5). Cysocel and AMO1618, which inhibit both copalyl-diphosphate synthase and ent-kaurene synthase in the early steps of the gibberellin pathway, caused remarkable production of FOH and GGOH at 15 mg/L. Phosphon D, functioning as a similar inhibitor, showed a similar enhancement effect at more than 50 mg/L (data not shown). No prenyl alcohols were detected in the case of ancymidol, inabenfide, or uniconazol, which inhibit cytochrome P450-dependent monooxygenases associated with a

Table 5 Effects of gibberellin synthesis inhibitors on prenyl alcohol production by *Gibberella fujikuroi* NBRC 30336

Target enzyme	Inhibitor	FOH (mg/L)	GGOH (mg/L)
None	None	0.00	0.00
Ent-kaurene synthase	Cysocel	0.43	2.89
	AMO1618	0.25	2.71
P450-dependent monooxygenase	Ancymidol	0.00	0.00
	Inabenfide	0.00	0.00
	Uniconazol	0.00	0.00
3β-Hydroxylase	Prohexadione	0.00	0.00

The strain was cultivated in medium containing various inhibitors of gibberellin biosynthesis (15 mg/L) at 30°C for 10 days.

series of oxidative reactions following the biosynthesis of ent-kaurene. Prohexadione, an inhibitor of 3β-hydroxylase, which catalyzes the hydroxylation of gibberellin, GA20, in the late steps of the formation of active gibberellic acid, also has no effect on prenyl alcohol production. Essentially, the same results were obtained with other *G. fujikuroi* strains, such as NBRC 5268, NBRC 9977, NBRC 30336, NBRC 30337, and NBRC 31251 (data not shown).

Discussion

In this report, we have shown that several inhibitors of SQ synthase, ent-kaurene synthase, and carotenoid biosynthesis promote the production of FOH and GGOH by various microorganisms. These inhibitors repress the conversion of FPP and GGPP into various terpenoids, which could be hydrolyzed immediately to the corresponding prenyl alcohols by specific prenyl phosphatases (Nah et al. 2001). It has been reported that the level of FOH in rat serum increased on the administration of an SQ synthase inhibitor, squalestatin l, and FOH production on cultures of *C. albicans* was also enhanced in the presence of an SQ synthase inhibitor, zaragozic acid B (Bansal and Vaidya 1994; Bostedor et al. 1997; Jacob et al. 2003). Table 2 clearly shows similar enhanced production of FOH. FOH production being enhanced on suppression of SQ synthase activity is also the case in various kinds of microorganisms such as yeasts, bacteria, actinomyces, and archeae. In particular, most of the yeasts tested exhibited strongly increased FOH production on the addition of inhibitors.

The disruption of the SQ synthase gene, ERG9, in *S. cerevisiae* caused FOH production (Chambon et al. 1990). ERG9-deficient *S. cerevisiae* ATCC 64031 need ergosterol for its growth, and synthesize no intermediates on the ergosterol biosynthetic pathway. Some important regulatory system caused by the intermediates may lack in *S. cerevisiae* ATCC 64031. As shown in Table 3, *S. cerevisiae* NBRC 0538 cultivated with SQ synthase inhibitor SQI-2 produced higher amounts of prenyl alcohols than *S. cerevisiae* ATCC 64031. In this case, it is thought that *S. cerevisiae* NBRC 0538 cultivated with SQI-2 synthesizes a small amount of the intermediates due to partial inhibition of ERG9. *S. cerevisiae* NBRC 0538 cultivated with SQI-2 may be more suitable for accumulation of FOH rather than ERG9-deficient *S. cerevisiae* ATCC 64031.

It is known that FOH causes the degradation of HMG-CoA reductase, a rate-limiting enzyme in the mevalonate pathway, followed by the inhibition of cell cycle progression of *S. cerevisiae* on the generation of reactive oxygen species in mitochondria (Meigs et al. 1996; Machida et al. 1999). Such regulation occurs with more than 30 mM FOH; however, *C. glabrata* NBRC 0005 produced over 1 mM FOH without growth inhibition (Fig. 2). In addition, approximately 0.5 mM FOH was also produced on test tube cultivation of various yeasts, such as *C. albicans* NBRC 1060, *P. rhodozyma* ATCC 66270, and *F. capsuligenum* NBRC 1185 (Table 2). These differences between *S. cerevisiae* and other yeast strains tested here are considered to be due to the influence of soybean oil, an ingredient of the medium. Most of the FOH secreted into the medium was present in the soybean oil fraction after centrifugation of the suspending broth (data was not shown). This finding suggests that down regulation of the mevalonate pathway by extracellular FOH is considerably low because of the separation of FOH from the cell surface on the incorporation into the small globules of soybean oil dispersed throughout the whole medium.

The accumulation of GGPP can occur on the inhibition of the enzymes converting GGPP into other terpenoids. The carotenoid-producing microorganism *Rhodotorula* also showed enhanced GGOH production on cultivation with 20 mg/L of DPA, which is an inhibitor of the enzymes related with carotenoid biosynthesis, and 20 mg/L of SQI-2 at one time (Table 4). DPA exerted its effect in *Rhodotorula* on the inhibition of ERG9 by SQI-2. Some metabolic regulation by the intermediates such as FPP, GGPP, and phytoene may exist in these strains. A gibberellin overproducing fungus, *G. fujikuroi*, exhibited the GGOH production of 3 mg/L on the addition of an ent-kauren synthase inhibitor that blocks the conversion from GGPP to kaurene (Table 5). These results suggest that the accumulation of GGPP enhances GGOH production.

Finally, it might be difficult to produce high amounts of GGOH with microorganisms. An ent-kauren synthase inhibitor, Cysocel, unexpectedly caused the production of FOH (0.5 mg/L) as well as GGOH (Table 5). DPA increased GGOH and FOH production in the presence of an SQ synthase inhibitor. One reason for this may be the difference in acceptability by the phosphatase of GGPP and FPP. Fujii et al. (1982) suggested that it is more difficult for a longer isoprenoid molecule to be hydrolyzed by phosphatase. Another reason may be that an increase in the GGPP level leads to feedback inhibition of GGPP synthase, the substrate, FPP, accumulating simultaneously. Rapid hydrolysis of FPP may be a convenient control mechanism for the synthesis of various second metabolites from FPP because FPP functions as a key intermediate for branching of the pathways to sterols, carotenoids, polyprenols, and gibberellins. FOH, which is formed from FPP through hydrolysis from FPP, functions as a trigger for the degradation of HMG-CoA reductase, a rate-limiting enzyme in the mevalonate pathway. When cells need up regulation of terpenoid biosynthesis, FOH can also be

converted back to FPP through two step kinase actions (Meigs et al. 1996; Ohnuma et al. 1996).

This study clearly demonstrated that the inhibition of enzymes downstream of FPP synthase causes over-production of the prenyl alcohols by various microorganisms.

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