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Various Oils and Detergents Enhance the Microbial Production of Farnesol and Related Prenyl Alcohols

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The object of this research was improvement of prenyl alcohol production with squalene synthase-deficient mutant *Saccharomyces cerevisiae* ATCC 64031. On screening of many kinds of additives, we found that oils and detergents significantly enhanced the extracellular production of prenyl alcohols. Soybean oil showed the most prominent effect among the additives tested. Its effect was accelerated by a high concentration of glucose in the medium. The combination of these cultivation conditions led to the production of more than 28 mg/*l* of farnesol in the soluble fraction of the broth. The addition of these compounds to the medium was an effective method for large-scale production of prenyl alcohols with microorganisms.

[**Key words:** farnesol, farnesyl diphosphate, geranyl geraniol, prenyl alcohol, terpenoid]

Farnesol [3,7,11-trimethyl-(all-E)-2,6,10-dodecatrien-1-ol (FOH)] has been known as a major fragrance component in the flowers of *Tilia vulgaris*, *Tilia europoea*, *Cananga odorata*, *Jasminum officinale* and *Rose damascena*, and the leaves of *Cymbopogon martini*. The essential oils extracted from these plant materials have been used as fragrances for cosmetics, soaps, aromatic oils and perfumes. FOH has also been utilized as starting materials for synthetic pharmaceuticals for stomach ulcers and vitamins. Recently, it was noted that derivatives of FOH are candidate anti-cancer reagents, because they induce apoptosis in various tumor cell lines (1–3). Except for the application of ingredients in natural fragrances, most FOH preparations on the market are obtained by means of chemical processes. Although chemical synthesis is generally advantageous for mass production, it is economically not feasible in the case of FOH synthesis. The low yield on stereoselective synthesis is a significant matter as to the introduction of all three trans carbon double bonds into the isoprenoid structure. There is no available procedure for separating these *cis*/*trans* isomers at low cost (4).

On the other hand, (all-E) farnesyl diphosphate, the phosphorylated form of FOH, is synthesized in many organisms by high stereoselective enzymes. Farnesyl diphosphate is a common intermediate for several essential components of living organisms, such as sterols, dolichols and quinones related to the mitochondrial respiratory chain, as a coenzyme. However, only a few instances of FOH production in mammalians and microorganisms have been reported, as opposed to in plants, which exhale more or less all trans FOH together with another component of essential oils. It was reported that FOH is detected in the broth of squalene synthase-deficient *Saccharomyces cerevisiae* ATCC 64031 (5). The administration of a squalene synthase inhibitor has also been found to increase the FOH level in rat serum (6), and the production of FOH by *Candida albicans* (7). However, the productivities were too low for practical large-scale production.

We screened many kinds of additives to improve the prenyl alcohol production with *S. cerevisiae* ATCC 64031. FOH is also a hydrophobic compound like oils and detergents. Especially, we hypothesized that the oils and the detergents are utilized for the FOH production as carbon sources or accelerates the release of FOH to the medium. In this report, we describe the enhancing effects of detergents and oils on the extracellular production of FOH. The addition of these compounds is considered to be an effective method for large-scale production of prenyl alcohol with microorganisms.

MATERIALS AND METHODS

Materials Yeast extract, bactopeptone and malt extract were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA). Adekanol LG-109 was from ADEKA (Tokyo). The other chemicals were purchased from Nacalai Tesque (Kyoto).

Microorganism culture *S. cerevisiae* (ATCC 64031) was maintained on YM agar plates (1% glucose, 0.3% yeast extract, 0.5% bactopeptone, 0.3% malt extract, 0.002% ergosterol and 1.5% agar). A 1000× ergosterol stock solution was prepared by dissolving 20 mg of ergosterol powder in 1 ml of ethanol containing 50% tergitol melted in a hot water bath. To examine the effects of various additives on the FOH production, 2 ml cultures in test tubes were incubated at 28°C with inoculation of 1% of the pre-culture. Ten liter jar-fermentors (Marubishi Bioengineering, Tokyo) with a 5 *l* working volume were seeded with 100 ml aliquots of cultures in

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Erlenmeyer flasks at 28°C with rotary shaking (120 rpm) for 30 h. The temperature, agitation speed, flow rate of germ-free air adjusted, and pH were controlled at 28°C, 300 rpm, 1 vvm, and 7.0 with 4 N NaOH and 4 N H_2SO_4, respectively. During fermentation, 2 ml aliquots of each culture were collected periodically to measure the amounts of FOH and glucose. Cell density was determined from the O.D. at 660 nm of the broth as turbidity. *Debaryomyces vanrijiae* JCM 2169, *Hanseniaspora valbyensis* IFO 0115 and *Candida glabrata* IFO 0005 were cultivated at 30°C in the YM medium supplemented with 1% soybean oil and 5% glucose for 3 d as described above.

Analysis of FOH and nerolidol (NOH) Supernatants (extracellular fractions) were obtained by centrifugation of 2 ml of each culture at 200×*g* for 10 min at room temperature. After washing the precipitates (cellular fractions) twice with physiological saline and suspending them in 0.75 ml of physiological saline, the cells were disrupted with 0.5 mm glass beads using a multi-bead shocker (Yasui Kikai, Osaka) for 20 min. The prenyl alcohols were extracted separately from the extracellular and cellular fractions with a 1.6 volume of extraction solvent (methanol: pentane, 3:5 v/v). After centrifugation of the mixture at 200×*g*, the upper organic layer was transferred for 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. NOH, FOH and geranylgeraniol (GGOH) were analyzed with an Agilent 5973 GC/MS system (column, HP-5MS, 0.25 mm id×30 m; Agilent Technologies, CA, USA; column temperature, 115°C, hold 1.5 min, raise at 70°C/min to 250°C, hold 2 min, and raise at 70°C/min to 300°C, hold 7 min). The amounts of prenyl alcohols were calculated from the ratio of the selected ion peak areas (69 mass fragment) to that of undecanol. All data obtained in the present study are means of three independent determinations (SD is less than 7%).

Analysis of glucose The ethanol and glucose concentrations in broth were determined with an F-Kit (J.K. International, Tokyo) based on the methods involving alcohol oxidase and hexose kinase, respectively.

RESULTS

Effect of tergitol on the production of prenyl alcohols

Figure 1 shows the effect of tergitol on the production of prenyl alcohols by squalene-synthase deficient mutant *S. cerevisiae* ATCC 64031 (5). After 3 d cultivation, the extracellular concentration of FOH had significantly increased

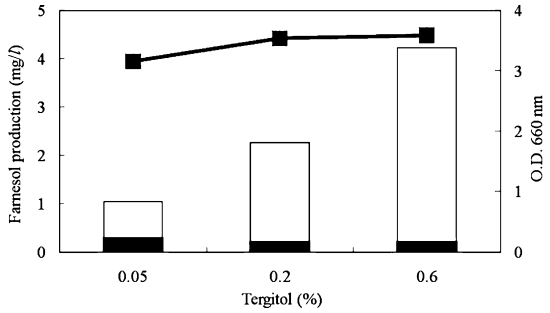

FIG. 1. Effect of tergitol on the FOH production by a squalene synthase-deficient mutant of *S. cerevisiae*. *S. cerevisiae* ATCC 64031 was cultivated at 28°C in YM medium containing 4 mg/*l* of ergosterol and various concentrations of tergitol for 3 d. After separation by centrifugation and cell disruption, FOH production in the cell and extracellular fractions were measured individually. Open boxes, FOH in extracellular fraction; closed boxes, FOH in cellular fraction; closed squares, cell density determined as optical density at 660 nm.

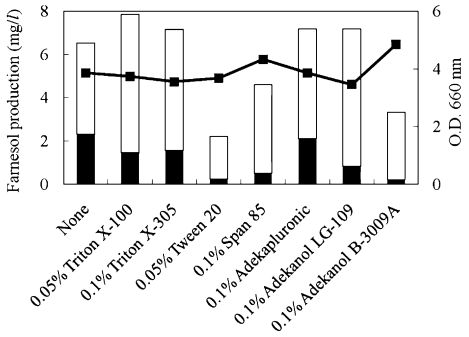

FIG. 2. Effects of detergents on FOH production by a squalene synthase-deficient mutant of *S. cerevisiae*. *S. cerevisiae* ATCC 64031 was cultivated in YM medium containing 4 mg/*l* of ergosterol, 0.05% tergitol and various detergents for 3 d. Open boxes, FOH in extracellular fraction; closed boxes, FOH in cellular fraction; closed squares, cell density determined as optical density at 660 nm.

with increasing tergitol content in the medium. No alteration in the intracellular concentration FOH was observed with any concentration of tergitol. As shown by the O.D. at 660 nm of the broth, there was not much difference in the growth between 0.05% and 0.6% of tergitol.

Effects of detergents on the production of prenyl alcohols Various kinds of detergents other than tergitol were examined as shown in Fig. 2. The extracellular production of FOH was essentially high even in the control tubes, because 0.05% tergitol was added to the medium to suspend ergosterol, which is required for growth of the strain. Most compounds showed a similar effect to that of tergitol. Adekapluronic (polyoxyethylene polyoxypropylene glycol) had no effect on the FOH production. Tween 20 (polyoxyethylene sorbitane monolaurate), Span 85 (sorbitane trioleate), and Adekanol B-3009A (polyglycerol fatty acid ester) induced remarkable extracellular production of FOH but decreased the sum of the intra-and-extracellular production of FOH. Although Triton X-100 and X-305 (polyethylene glycol tert-octylphenyl ether) relatively well increased the extracellular production, these compounds foamed up vigorously in a jar-fermenter and thus prevented the control of the cultivation. An anti-foaming reagent, Adekanol LG109 (polyglycerol fatty acid ester), had preferable effect on FOH production.

Effects of vegitable oils on the production of prenyl alcohols Since the above effects could be attributed to separation of FOH from the surface of the cell membrane by detergents, we further examined fatty acid triglycerides, which are inexpensive for utilization for a large-scale process (Fig. 3). The extracellular ratio in the total FOH amount was high even in the control tubes, because not only the addition of 0.05% tergitol prepared for the ergosterol solution but also the longer cultivation time (5 d) accelerated the release of FOH to the medium. The extracellular production of FOH was enhanced by the addition of various triglycerides to the cultures. Soybean oil (1%, v/v) increased FOH production four times without enhancement of the cellular FOH level. Although soybean and olive oils increased the turbidity of the broth in comparison with almond and fish oils, the cell densities measured under a microscope were almost the same for the various oils. The differences could arise from the tendency to form an emulsion.

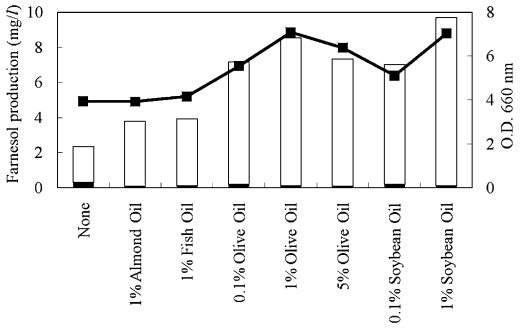

FIG. 3. Effects of edible oils on FOH production by a squalene synthase-deficient mutant of *S. cerevisiae*. *S. cerevisiae* ATCC 64031 was cultivated in YM medium containing 0.05% tergitol, 4 mg/*l* of ergosterol and various edible oils for 5 d. Open boxes, FOH in extracellular fraction; closed boxes, FOH in cellular fraction; closed squares, cell density determined as optical density at 660 nm.

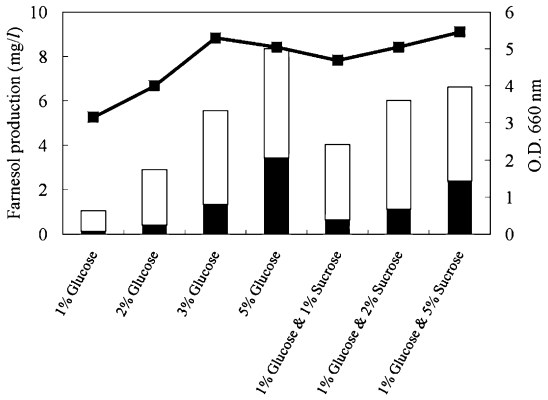

FIG. 4. Effect of carbohydrates on FOH production by a squalene synthase-deficient mutant of *S. cerevisiae*. *S. cerevisiae* ATCC 64031 was cultivated in YM medium containing 0.05% tergitol, 4 mg/*l* of ergosterol and various concentrations of carbohydrates for 5 d. The glucose concentration includes the glucose content of the YM medium. Open boxes, FOH in extracellular fraction; closed boxes, FOH in cellular fraction; closed squares, cell density determined as optical density at 660 nm.

Effects of the glucose concentration on the production of prenyl alcohol As a further experiment, we examined the effect of the carbohydrate concentration on FOH production (Fig. 4). Compared with media ranging with glucose concentrations from 1% to 5%, we found that the total FOH production and the proportion of FOH in the intracellular fraction linearly increased with increasing glucose concentration. The medium containing 5% glucose gave approximately five times higher production of FOH than that containing 1% glucose. The addition of sucrose caused a similar phenomenon (data not shown).

Batch-scale production of FOH under the optimized conditions The combination of 1% soybean oil and 6% glucose drastically increased the extracellular production of FOH in a 10-*l* jar-fermentor (Fig. 5). Increasing with microbial growth, the overall production of FOH in both cultures (A and B) reached approximately 25 mg/*l*, which is approximately 3 times higher than that on test tube cultivation. In the culture without soybean oil, the maximum FOH production was observed at 64.5 h (26 mg/*l*), 86% FOH accumulating in the cell fraction. The addition of 1% soybean oil reduced the intracellular FOH level to 3%, and increased the total production (28 mg/*l*, 85.5 h). The residual glucose steeply decreased within the initial 20 h. The cell mass increased from 20 h and reached the stationary state at 60 h. The increase in FOH production followed the growth curve and further increased after the stationary growth phase.

Effects of soybean oil on the production of prenyl alcohols by various microorganisms In order to confirm that the enhancement of prenyl alcohol production on the addition of soybean oil to the growth medium, we first searched, in preliminary experiments, for microorganisms producing prenyl alcohols among our culture collection species including 4 archae, 122 filamentous fungi, 98 yeasts, 35 actinomycetes and 65 bacteria. No prenyl alcohol production was observed for the archae or filamentous fungi. A low amount of nerolidol (<0.015 mg/*l*-broth) was found for only 3 actinomyces species. FOH was detected for 4 bacterial species (<0.01 mg/*l*-broth) and for 34 of 154 yeast species (<0.09 mg/*l*-broth). Thirty-three yeast species produced GGOH (<0.11 mg/*l*-broth), along with FOH in the case of some strains. The production of trace amounts of prenyl alcohols was detected

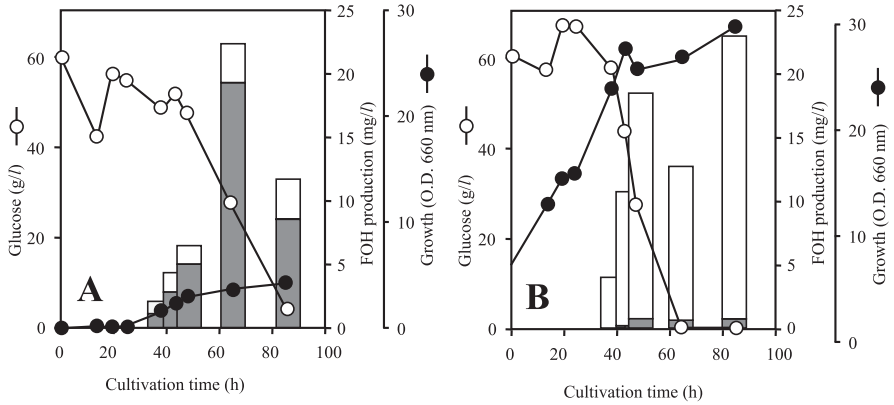

FIG. 5. Effect of soybean oil on FOH production by a squalene synthase-deficient mutant of *S. cerevisiae* in the presence of a high glucose concentration. *S. cerevisiae* ATCC 64031 was cultured at 26°C, 300 rpm and 1 vvm in YM medium containing 6% glucose (A), and the medium with 1% soybean oil added (B). Extracellular (open boxes) and intracellular (closed boxes) FOH were separately analyzed by GC/MS. Open circles show the glucose remaining in the medium. Closed circles indicate the growth curve of the cells.

TABLE 1. Prenyl alcohol production with three yeasts strains (mg/*l*)[a]

	Oil[b]	NOH		FOH		GGOH	
		Ext[c]	Cell[d]	Ext[c]	Cell[d]	Ext[c]	Cell[d]
Debaryomyces vanrijiae	−	0.0	0.0	73.7	14.3	0.0	6.2
JCM 2169	+	0.0	0.0	809.8	67.2	241.4	54.3
Hanseniaspora valbyensis	−	0.0	0.0	13.6	7.4	0.0	103.5
IFO 0115	+	0.0	0.0	254.9	25.2	493.6	76.6
Candida glabrata	−	0.0	0.0	35.7	4.5	5.4	28.3
IFO 0005	+	192.8	0.0	861.5	34.9	909.0	51.7

[a] Cells were grown at 30°C in a medium containing 1% glucose, 0.3% yeast extract, 0.5% bactopeptone and 0.3% malt extract for 3 d.
[b] Soybean oil (1%) was added to the medium (+) or not added (−).
[c] Prenyl alcohol production in extracellular fractions.
[d] Prenyl alcohol production in cellular fractions.

for almost 20% of the yeasts strains tested (data not shown). We examined the influence of 1% soybean oil on prenyl alcohol production in the most FOH producing strain, *D. vanrijiae* JCM 2169, the GGOH producing strain, *H. valbyensis* IFO 0115, and the strain producing both, *C. glabrata* IFO 0005. On the addition of 1% soybean oil to the medium, drastic stimulation of the extracellular production was observed for all of these strains. In particular, the secretion efficacy as to NOH and GGOH was prominent for *C. glabrata* (Table 1).

DISCUSSION

This paper demonstrates that any detergents and edible oils increase the extracellular production of prenyl alcohols with the *S. cerevisiae* mutant lacking squalene synthase and other various microorganisms. It is known that exogenous FOH causes accelerated degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is a significant rate-limiting enzyme for terpenoid biosynthesis by Chinese hamster ovary cells (8). In *S. cerevisiae*, 12.5 mM (2.8 mg/*l*) of FOH has a 32% static growth inhibitory effect by generating of toxic reactive oxygen species through the mitochondrial electron transport system (9). Moreover, the growth of halophilic archaeon *Haloferax volcanii* is also inhibited by 50% in rich medium by only 2 mM (0.4 mg/*l*) FOH (10). These phenomena indicate that over-production of prenyl alcohols has a deleterious effect on cells. Hence, the biosynthesis of prenyl alcohols is rigorously controlled, but it is difficult to discover over-producing cells in natural sources. We detected prenyl alcohols in 74 of 324 species of microorganisms, but the amounts were extremely low (below 0.1 mg/*l*). The addition of oils and detergents to the cultures improved the productivity of prenyl alcohols, increasing their production approximately 100 times regardless of the species. It was presumed that the added oils and detergents made the released FOH stable by formation of micelle which reduced the toxicity of FOH to the cells. These effects are more enhanced in an enrichment medium as to the carbon source, which is utilized for the biosynthesis of acetyl-CoA, an early intermediate in the mevalonate pathway. A high carbon source to nitrogen source ratio would lead to excess acetyl-CoA, because a lack of a nitrogen source is a rate-determining role in cell division, which requires a great deal of acetyl-CoA for the synthesis of proteins, nucleic acids and saccharides. It is considered that oils and detergents decrease the intracellular FOH level by removing it from the surface of the cellular membrane, and thereby reduce the regulation of FOH as to the enzyme.

Although a novel farnesyl diphosphatase and geranylgeranyl diphosphatase have been found in cell extracts of *C. albicans* and *S. cerevisiae*, respectively (11), these specific enzymes might not be essential for the production of prenyl alcohols from prenyl diphosphates. Enzymatic hydrolysis of prenyl diphosphates occurs with a quite common potato acid phosphatase (12). Our findings also indicate that FOH production by microorganisms is not a peculiar phenomenon. In a large number of species, farnesyl diphosphate might be easily hydrolyzed to FOH by non-specific phosphatases. Rapid hydrolysis of farnesyl diphosphate may be a convenient control mechanism for the synthesis of various second metabolites from farnesyl diphosphate, because it functions as a key intermediate for the branching to the pathways to numerous terpenoids. Temporarily accumulated FOH can be converted into farnesyl diphosphate by kinases (13). Oils and detergents facilitate the release of FOH from the cell membrane into the medium, thereby abolish the regulation of the prenyl alcohol production. As a matter of fact, the supplementation of 1% soybean oil facilitated the release of FOH from the cells to the medium as well as the cell growth as shown in Fig. 5B. After the glucose consumption, the cell growth gradually increased and the FOH production reached 28 mg/*l* at 85.5 h. It was considered that the part of the excess energy from the carbon sources was converted to FOH.

The present results may be useful for over-production by means of a biological process. In particular, our findings will possibly lead to a break-through as to the over-production of hydrophobic compounds, which has so far been with microorganisms. Tochopherol and carotenoids, which are useful materials in food and pharmaceutical areas, could be the next target materials.

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