Recovery of *E,E*-Farnesol from Cultures of Yeast *erg9* Mutants: Extraction with Polymeric Beads and Purification by Normal-Phase Chromatography

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Saccharomyces cerevisiae erg9 *mutants blocked at squalene synthase require ergosterol for growth and produce E,E-farnesol. Typically, at least half the amount of farnesol remains cell associated. Practically insoluble in water, farnesol can be extracted from production cultures of the* erg9 *mutants using either methanol/hexane or poly(styrene-co-divinylbenzene) beads. The first method consumes more solvents and requires centrifugation to clear an interface emulsion. The second method uses 50% less solvent and the beads can be used repeatedly for extraction. The solvent-free crude extract from the beads extraction contained higher concentration of farnesol (76–77%) than that from the solvent extraction (61–65%). Farnesol was obtained after normal-phase chromatography in high overall recovery (94%) and purity (99%).*
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Introduction

The isoprenoid pathway is present in all living organisms, providing a wide array of metabolites for essential cellular functions. There is a great deal of interest in the metabolic engineering of this pathway[1] to produce compounds with commercial values such as farnesol and geranylgeraniol,[2] coenzyme Q_{10} (CoQ10),[3-5] mevalonate,[6] antimalarial drug artemisimic acid,[7] and numerous carotenoids.[8-13] The central part of the isoprenoid pathway (Scheme 1) involves the synthesis of farnesyl diphosphate (FPP) via the sequential addition of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP). Unlike wild type *S. cerevisiae*, mutants blocked at squalene synthase (*ERG9* gene) required ergosterol for growth and accumulated both FPP and *E,E*-farnesol.[14] Potential applications for farnesol include its antifungal activity[15] and as a precursor in the chemical syntheses of perfume Ambrox[16,17] and polyprenols.[18]

It is a common practice to extract hydrophobic products from a production culture with organic solvents. Reported here is a method of extracting farnesol from whole culture broth of yeast *erg9* mutants with synthetic beads and subsequent purification by normal-phase chromatography. Compared with the solvent extraction, the beads method reduced the consumption of organic solvents and resulted in higher purity of farnesol in the solvent free extract. Additionally, the beads can be reused for farnesol extraction without special treatment.

Materials and Methods

Reagents

All solvents used were of analytical grade. Triton X-100, ergosterol, and *E,E*-farnesol were purchased from Sigma. Poly(styrene-co-divinylbenzene) resin (300–800 μm) and *E*-nerolidol were from Aldrich. *E,E*-Farnesoic acid was from Echelon Biosciences (Salt Lake City, Utah).

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Measurement of farnesol in culture samples

Methanol, hexane, and a whole culture broth (1:1:1 v/v/v) were mixed with a vortex mixer for 1.5 min and centrifuged. The top hexane layer was analyzed using gas chromatography-mass spectrometry (GC-MS).

Thin layer chromatography (TLC)

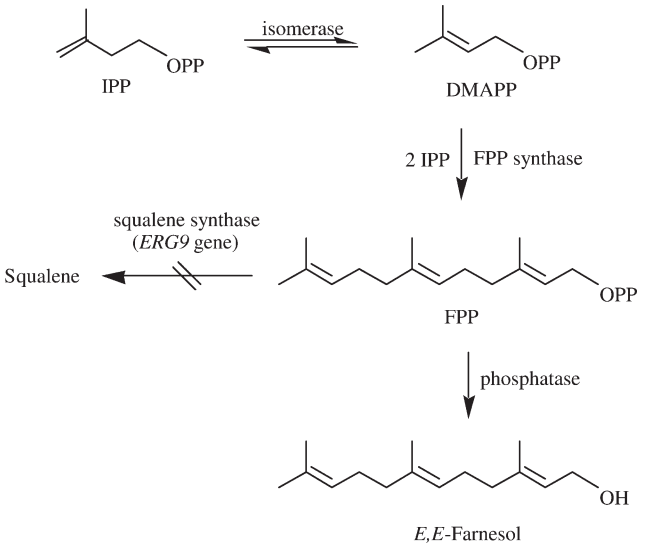
Silica gel plates were developed with 8:2 (v/v) hexane/acetone and visualized with iodine vapor. *E,E*-farnesol showed an R_f value of 0.32.

Gas chromatography-mass spectrometry

Gas chromatography was performed using a Hewlett Packard 5890 chromatograph equipped with a 5970 mass selective detector. An Rtx-5MS column (Restek, 0.25 mm ID, 15 m, 1 μm film) was kept at 80°C for a min and heated to 300°C at 15°C/min. Column head pressure was 10 psi. Injector port was at 220°C and the detector was at 300°C. Retention time for *E,E*-farnesol was 9.8 min.

HPLC measurement of Triton X-100 and E,E*-farnesol*

A Luna C18 column (Phenomenex, 4.6 × 250 mm, 5 μm) was employed using 95:5 (v/v) methanol/water as mobile phase at 0.6 mL/min flow rate. The elution was monitored with a photodiode array detector and chromatograms were displayed at 206 nm. The retention time for Triton X-100 and *E,E*-farnesol was 7.58 and 8.45 min, respectively. Ergosterol did not elute in 40 min.



Scheme 1. Biosynthetic pathway of *E,E*-farnesol in *S. cerivisiae erg9* mutants.

HPLC for detecting ergosterol

Ergosterol was detected using the same column but methanol as the mobile phase. The retention time was 20 min at 0.6 mL/min flow rate. Triton X-100 and *E,E*-farnesol eluted at 6.05 and 6.53 min, respectively. Another alternative was to use ethanol as the mobile phase and ergosterol was eluted at 8.6 min.

Preparative extraction of E,E-*farnesol from whole culture broth with methanol/hexane*

Solid sodium hydroxide was added to the sample to adjust pH to 8.1. This step was to remove most of the farnesoic acid. Same volume of methanol was added and mixed. Hexane (also the same volume as the culture broth) was added and the mixture was hand shaken for 2 min. The bottom layer was discarded and some of the clear hexane layer was transferred to a clean glass container. The remaining sample with the interface emulsion was centrifuged at 250*g* for 5 min in glass tubes. The combined hexane extract could be directly applied to a silica gel column for purification. Alternatively, hexane was removed using a rotary evaporator (35–40°C). The sample was kept on the evaporator for an extra 4 min after no visible solvent condensation could be observed to afford a yellowish brown liquid as the crude farnesol sample.

With poly(styrene-co-divinylbenzene) beads

One liter fermentation culture containing up to 3 g farnesol and 75 g poly(styrene-co-divinylbenzene) beads were mixed in a 2 L flask on a shaker for 16 h at room temperature. The residual farnesol in the beads-free cell suspension was found to be 0.0085 g/L (methanol/hexane extraction and GC-MS analysis). The cell suspension was discarded and the beads were washed with water (3 × 1 L). A pipet was used to remove as much water as possible from the bed of the beads. The beads were extracted repeatedly with acetone (2 × 167 mL), hexane (2 × 250 mL), and acetone again (167 mL). In each extraction the solvent was allowed to be in contact with the beads for 5 min with occasional shake mixing. After each extraction, the flask was carefully placed in a slanted position and as much solvent was removed as possible from the bed of the beads with a pipet. The acetone and hexane extracts were combined (total volume 1 L) and 1 L water was added. The mixture was manually shaken for 1.5 min and kept still for phase separation. The volume of the clear top layer should be roughly a third the volume of the bottom layer. The top layer was separated and solvent was removed using a rotary evaporator at 35–40°C. The sample was dried on the evaporator for additional 4 min after no visible solvent condensation could be observed. A yellow liquid (3.57 g) was obtained as the crude extract sample that contained about 75% (w/w) farnesol as measured by GC-MS.

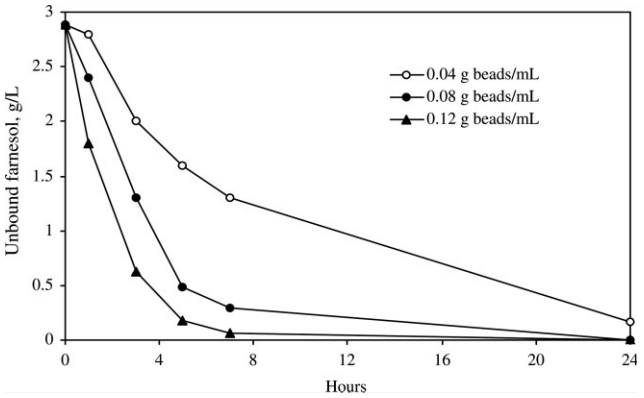


Figure 1. Effect of time and concentration of beads on the concentration of unbound farnesol (g/L).

Purification of E,E-*farnesol*

A silica gel SPE column (Fisher, 10 g silica, 40–45 μm, 60 Å pore size, 2.7 × 4 cm bed) was washed with 40 mL hexane (gravity flow). A crude extract (up to 1 g) in hexane containing up to 0.7 g *E,E*-farnesol was applied to the column. The inside of the column was rinsed with 5 mL hexane after sample application. A mixture of 100:1 (v/v) hexane/acetone was added. The first 110 mL eluent was discarded. Eight 5-mL fractions (#1–8) were then collected. The elution solvent was changed to 95:5 (v/v) hexane/acetone and ten 10-mL fractions (#9–18) were collected. The fractions were screened with TLC (1 μL) for *E,E*-farnesol. Normally fraction 8 contained mostly *E,E*-farnesol with trace amount of *trans*-nerolidol (R_f = 0.4). The amount of *E,E*-farnesol started to drop in fraction 18. Fractions containing *E,E*-farnesol (normally # 8 to 18) were combined. Hexane and acetone were removed using a rotary evaporator (35–40°C). The sample was kept on the evaporator for an extra 4 min after no visible solvent condensation could be observed. The recovery of *E,E*-farnesol was 94% or higher.

Determination of farnesol purity

A known amount of purified *E,E*-farnesol was dissolved in ethanol (0.5–1 g/L). The amount of *E,E*-farnesol was measured with both GC-MS (0.2 μL) and HPLC using Sigma *E,E*-farnesol (96% pure) as the standard. The purity was found to be 99% (w/w).

Results and Discussion

Farnesol is practically insoluble in water. Because of the presence of detergent Triton X-100 in the production culture,

typically less than half the amount of farnesol resides in the extracellular environment. Farnesol could be extracted effectively by mixing the whole culture broth with methanol and hexane in 1:1:1 (v/v/v) ratio.[2] Centrifugation was performed to clear the interface emulsion. Isoprenoid compounds *E*-nerolidol and *E,E*-farnesoic acid were identified among the impurities. Other known impurities included Triton X-100 and ergosterol. Treatment of the culture with sodium hydroxide before solvent extraction removed most of the farnesoic acid.

The solvent extraction method was effective in extracting farnesol from a whole culture broth, but the amount of organic solvents consumed was large and centrifugation had to be performed to clear the emulsion. The equilibrium of farnesol distribution between cells and the extracellular environment may be disturbed by the addition of hydrophobic beads. Theoretically, the extracellular farnesol should bind to the beads and as a result cell associated farnesol should partition into the extracellular environment. If sufficient beads are used, all the farnesol may eventually adsorb to the hydrophobic beads.

When mixed with a whole culture broth of a farnesol producer, poly(styrene-co-divinylbenzene) beads showed a dose- and time-dependent removal of farnesol from the cell suspension (Figure 1). For a given volume of culture and a given time period, higher concentration of beads removed farnesol at a faster rate as indicated by the measurement of residual farnesol in the beads-free cell suspension. More beads, however, would require more organic solvents in recovering farnesol from the beads. For convenience, a goal was set to use a minimum amount of beads to extract farnesol overnight in 16 h. For 1 L fermentation culture containing up to 3 g/L farnesol, 75 g of beads was found sufficient to completely remove farnesol in 16 h of mixing.

After farnesol bound to the beads, its removal with hexane turned out to be ineffective probably because the beads were wet and the contact with hexane was not sufficient. Extraction with acetone prior to hexane resulted in nearly quantitative farnesol recovery. Acetone removed water and some farnesol from the hydrophobic beads and promoted the contact between hexane and the beads in the subsequent extraction. In one experiment, the same batch of beads was repeatedly used in three extractions and no loss of extraction power was observed.

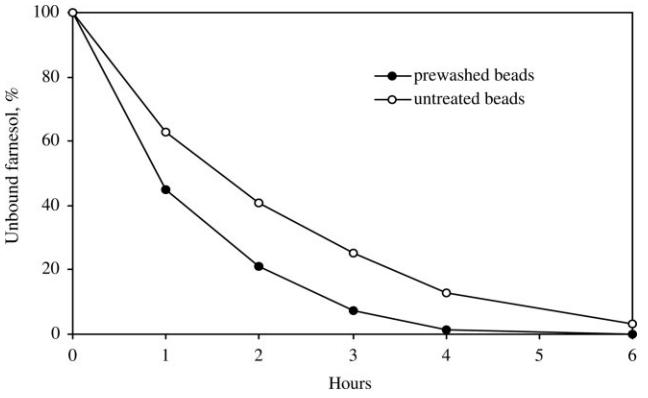


Figure 2. Comparison of rate of farnesol extraction (% unbound farnesol) between acetone prewashed beads and untreated beads (3.75 g beads and 50 mL culture).

If hexane was the last solvent used to elute farnesol from the beads, after vacuum drying the beads floated instead of sinking in the next application, probably due to the presence of residual hexane trapped in the pores of the resin. If farnesol elution was done in such a fashion that the last solvent was acetone, the beads after vacuum drying would sink when mixed with a fermentation culture. When the mixing was performed with a magnetic stir bar, some beads were ground to powder. Mixing on a shaker proved to be both effective and safe for the beads.

Two experiments, each consisting of two consecutive extractions using the same batch of beads, were performed to determine if the beads could be reused without vacuum drying. In the first experiment, the beads (last washed with acetone to elute farnesol) were directly used for a second extraction. Measurement of residual farnesol showed that the beads extracted farnesol from a fermentation culture as effectively the first time they were used. This demonstrated that the beads could be reused without the need of drying. In the second experiment, the beads (last washed with acetone to elute farnesol) were rinsed with water to remove residual acetone and used again for a second extraction. Determination of farnesol in the cell suspension showed complete removal of farnesol in 16 h, indicating that wet beads worked as effectively as dry ones.

As discussed previously, for a given ratio of sample to beads, the rate of farnesol extraction was time dependent. The slow binding of farnesol to the beads may be caused by inefficient contact between the aqueous sample and the hydrophobic beads. This contact could be improved by prewashing the beads with a water miscible organic solvent such as acetone or ethanol. Two experiments were carried out to compare the rate of farnesol extraction. Both employed 3.75 g beads and 50 mL fermentation culture broth, but one of them used beads that were prewashed with acetone followed by water. The prewashed beads extracted farnesol faster than the dry beads, removing 92 and 98% farnesol in 3 and 4 h, respectively. In comparison, the dry beads removed 75 and 87% farnesol in 3 and 4 h, respectively (Figure 2). In practice, the choice between using dry beads for overnight 16 h extraction and prewashed beads for 4 h extraction depends on convenience. It should be kept in mind that prewashing the beads consumes extra solvent, a cost factor to consider in large scale operations.

The two extraction methods are summarized in Table 1. Compared with the methanol/hexane extraction, the beads

Table 1. Comparison of the Two Farnesol Extraction Methods Using 100 mL Fermentation Culture (Two Experiments for Each Method)

Extraction Method	Methanol (mL)	Hexane (mL)	Acetone (mL)	Centrifugation	% FOH Recovery	% FOH* (w/w)
Solvent	100	100	0	Yes	96	65
					102	61
Beads	0	50	50	No	99	76
					100	77

FOH, *E,E*-farnesol. Seven grams of beads were mixed with the sample for 16 hours.
* In crude samples obtained after removal of solvent.

method showed comparable farnesol recovery with the following advantages: (1) it cuts the consumption of organic solvents by half; (2) it does not require centrifugation due to good phase separation; (3) the beads can be reused; (4) it yields a crude extract with higher farnesol content. The crude sample obtained using beads after the removal of solvents showed a yellow color while that from the solvent extraction was yellowish brown.

Farnesol was purified using normal-phase chromatography on silica gel using crude extracts from both the solvent and beads extractions. Typically farnesol was obtained in 94% overall recovery and 99% purity.

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Literature Cited

1. Barkovich R, Liao JC. Metabolic engineering of isoprenoids. *Metab Eng*. 2001;3:27–39.
2. Millis JR, Maurina-Brunker J, McMullin TW. Production of farnesol and geranylgeraniol. US Patent 6,689,593 B2; 2004.
3. Zahiri HS, Yoon SH, Keasling JD, Lee SH, Kim SW, Yoon SC, Shin YC. Coenzyme Q_{10} production in recombinant *Escherichia coli* strains engineered with a heterologous decaprenyl diphosphate synthase gene and foreign mevalonate pathway. *Metab Eng*. 2006;8:406–416.
4. Park Y-C, Kim S-J, Choi J-H, Lee W-H, Park K-M, Kawamukai M, Ryu Y-W, Seo J-H. Batch and fed-batch production of coenzyme Q_{10} in recombinant *Escherichia coli* containing the decaprenyl diphosphate synthase gene from *Gluconobacter suboxydans*. *Appl Microbiol Biotechnol*. 2005;67:192–196.
5. Choi J-H, Ryu Y-W, Seo J-H. Biotechnological production and applications of coenzyme Q_{10}. *Appl Microbiol Biotechnol*. 2005;68:9–15.
6. Tabata K, Hashimoto S-I. Production of mevalonate by a metabolically-engineered *Escherichia coli*. *Biotechnol Lett*. 2004;26: 1487–1491.
7. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho, KA, Eachus RA, Ham TS, Kirby J, Chang MCY, Withers ST, Shiba Y, Sarpong R, Keasling JD. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*. 2006;440:940–943.
8. Wang C-W, Oh M-K, Liao JC. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol Bioeng*. 1999;62:235–241.
9. Smolke CD, Martin VJJ, Keasling JD. Controlling the metabolic flux through the carotenoid pathway using directed mRNA processing and stabilization. *Metab Eng*. 2001;3:313–321.
10. Shimada H, Kondo K, Fraser PD, Miura Y, Saito T, Misawa N. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. *Appl Environ Microbiol*. 1998;64:2676–2680.
11. Farmer WR, Liao JC. Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat Biotechnol*. 2000;18:533–537.
12. Yuan LZ, Rouviere PE, LaRossa RA, Suh W. Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. *Metab Eng*. 2006;8:79–90.
13. Mijts BN, Lee PC, Schmidt-Dannert C. Engineering carotenoid biosynthetic pathways. *Methods Enzymol*. 2004;388: 315–329.
14. Song L. Detection of farnesyl diphosphate accumulation in yeast *ERG9* mutants. *Anal Biochem*. 2003;317:180–185.
15. Ramage G, Saville SP, Wickes BL, Lopez-Ribot JL. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol*. 2002;68:5459–5463.
16. Tanimoto H, Oritani T. Practical synthesis of Ambrox? from farnesyl acetate involving lipase catalyzed resolution. *Tetrahedron: Asymmetry*. 1996;7:1695–1704.
17. Ishihara K, Ishibashi H, Yamamoto H. Enantio- and diastereoselective stepwise cyclization of polyprenoids induced by chiral and achiral LBAs. A new entry to (-)-Ambrox, (+)-podocarpa-8,11,13-triene diterpenoids, and (-)-tetracyclic polyprenoid of sedimentary origin. *J Am Chem Soc*. 2002;124: 3647–3655.
18. Radetich B, Corey EJ. A general stereocontrolled, convenient synthesis of oligoprenols that parallels the biosynthetic pathway. *J Am Chem Soc*. 2002;124:2430–2431.

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