Chapter 8

Production of Aromatic Plant Terpenoids in Recombinant Baker's Yeast

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Abstract

Plant terpenoids are high-value compounds broadly applied as food additives or fragrances in perfumes and cosmetics. Their biotechnological production in yeast offers an attractive alternative to extraction from plants. Here, we provide two optimized protocols for the production of the plant terpenoid *trans*-nootkatol with recombinant *S. cerevisiae* by either (I) converting externally added (+)-valencene with resting cells or (II) cultivating engineered self-sufficient production strains. By synthesis of the hydrophobic compounds in self-sufficient production cells, phase transfer issues can be avoided and the highly volatile products can be enriched in and easily purified from *n*-dodecane, which is added to the cell broth as second phase.

Key words Plant terpenoids, Yeast, *S. cerevisiae*, Cytochrome P450 enzymes, Biotransformation, (+)-Valencene synthase, t*HMG1*, Metabolic engineering

1 Introduction

Terpenes or terpenoids are primary constituents of essential oils of many types of plants and flowers and, hence, are of large industrial interest. Their isolation from natural resources is subjected to seasonal variations and often suffers from difficult removal of matrix-associated impurities. To date, some terpenoids are mainly produced via chemical synthesis that frequently involves toxic heavy metals, highly flammable compounds, or strong oxidants [1, 2]. Therefore, attention is being paid to the development of safe methods and innovative biotechnological solutions to reduce the ecological footprint [3–5]. The microbial synthesis of plant terpenoids has become an easy, comparably cheap, and viable alternative [6].

Terpenoids can be produced upon hydroxy-functionalization of cheap precursor terpenes by using highly specific membrane-anchored cytochrome P450 enzymes (CYPs) [7, 8]. CYP activity is tightly linked to a finely balanced system of NADPH cofactor recycling, oxygen supply, correct integration of heme iron into the active site of the CYP, and perfect interaction with the corresponding

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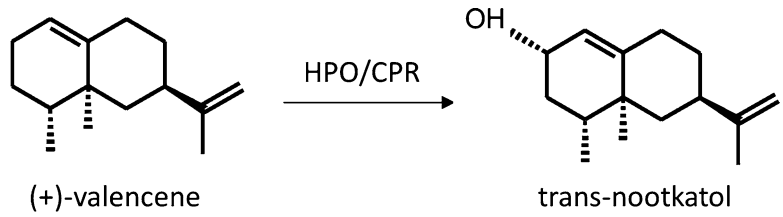

Fig. 1 Conversion of (+)-valencene to *trans*-nootkatol catalyzed by HPO and CPR

cytochrome P450 reductase (CPR) that functions as electron donor [9, 10]. Based on the system's complexity, the use of whole cellular systems is favored over cell lysates or purified enzymes to support cofactor supply and enable close interaction of the CYP enzyme with its corresponding reductase [11].

We have established and optimized protocols for the production of the model terpenoid *trans*-nootkatol in baker's yeast from (+)-valencene. We used a recombinant*S. cerevisiae* strain co-expressing CYP *Hyoscyamus muticus p*remnaspirodiene *o*xygenase (HPO) and the *c*ytochrome *P*450 *r*eductase from *Arabidopsis thaliana* (CPR) to hydroxylate the C2 atom of (+)-valencene for the production of the high-value terpenoid compound *trans*-nootkatol [12–14] (Fig. 1). Phase transfer issues represent a considerable problem of whole-cell biotransformations of hydrophobic substrates. Thus we constructed an efficient *S. cerevisiae* strain capable of producing relevant amounts of (+)-valencene intracellularly [13].

In brief, yeast harbors the mevalonate pathway, which is the essential metabolic pathway necessary for the production of aroma components in plants. The yeast mevalonate pathway can be engineered to produce terpenes by implementing the corresponding terpene synthase, e.g., (+)-valencene synthase from Alaska cedar heartwood (*Callitropsis nootkatensis*) [15]. ValS [(+)-valencene synthase] forms (+)-valencene from farnesyl pyrophosphate (FPP). In order to increase the FPP pool in *S. cerevisiae*, a truncated version of *HMG1* was over-expressed. In numerous related projects, over-expression of t*HMG1* has successfully enhanced production of terpenoids [16–19]. Self-sufficient production of *trans*-nootkatol was performed in biphasic systems using *n*-dodecane for trapping the synthesized terpenoids [13]. This elegant method prevents toxicity or inhibitory effects of high concentrations of terpenoids on yeasts providing an online extraction step [16, 18, 20], because high concentrations have recently been described to hamper (+)-valencene biohydroxylation in *S. cerevisiae* [21]. Several other groups have published *S. cerevisiae* strains that self-sufficiently produce (+)-valencene and *trans*-nootkatol [15, 22]. Inspired by the cultivation protocol for *P. pastoris* [14], we optimized cultivation conditions by introducing a prolonged growth phase followed by milder induction conditions. This seemed to strengthen cells, allowing them to reach higher initial densities, followed by redirection of the metabolic flux to terpenoid products after induction.

2 Materials

2.1 Conversion of Externally Added Terpenes with S. cerevisiaeResting Cells

1. *S. cerevisiae* strain: W303 co-expressing CYP and CPR, e.g., HPO and CPR from a (+)-galactose inducible pYES2 co-expression vector harboring a uracil selection marker [13].
2. Amino acid drop-out powder (DOP): Grind equal amounts of adenine, lysine, tyrosine, histidine, leucine, and tryptophan with mortar and pestle to generate a homogeneous powder (*see* **Note 1**).
3. Synthetic defined-uracil plates (SD-ura plates): Dissolve 6.7 g of Difco™ yeast nitrogen base w/o amino acids (YNB) and 1 g of DOP in 900 mL of ddH_2O (*see* **Note 2**) and add 20 g of agar. Dissolve 20 g of glucose in 100 mL of ddH_2O. Autoclave both solutions and combine as soon as they are cooled to ~45 °C to pour plates.
4. Synthetic defined-uracil 2 % growth medium (SDG 2 % medium): Dissolve 6.7 g of Difco™ yeast nitrogen base w/o amino acids (YNB) and 1 g of DOP in 900 mL of ddH_2O (*see* **Note 2**). 20 g of glucose is dissolved in 100 mL ddH_2O. Autoclave and combine the solutions when they are cooled to room temperature.
5. Synthetic defined-uracil induction medium (SDI medium): Dissolve 6.7 g of Difco™ yeast nitrogen base w/o amino acids (YNB) and 1 g of DOP in 900 mL of ddH_2O (*see* **Note 2**). Dissolve 20 g of galactose and 7 g of raffinose (*see* **Note 3**) in 100 mL ddH_2O, sterile filter, and combine solutions.
6. Sterile 300 mL baffled flasks covered with aluminum foil.
7. Eppendorf BioPhotometer®.
8. 50 mL Falcon® tubes.
9. 50 mM potassium phosphate buffer, pH 7.4.
10. PYREX® tubes.
11. 100 mM (+)-valencene stock solution (prepare fresh before use): Weigh one drop of (+)-valencene (M = 204.35 g/mol) in a PYREX® tube and add DMSO to reach a final concentration of 100 mM (e.g., add 1 mL of DMSO to 2.04 mg of (+)-valencene). Add 1 % Triton®X-100 (*see* **Note 4**).
12. Ethyl acetate.
13. VXR basic Vibrax®.
14. Eppendorf 5810R centrifuge.
15. GC vials, inserts, crimp caps, and crimper.
16. GC-FID: Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID), HP-5 column (cross-linked 5 % Ph-Me Siloxane; 10 m × 0.10 mm × 0.10 μm).

2.2 In Vivo Terpenoid Production with a Self-Sufficient S. cerevisiae Strain

1. Yeast strain: W303 expressing terpene synthase (+/− mevalonate pathway engineering), e.g., harboring integrated copies of ValS and t*HMG1*, and driving HPO/CPR expression from a co-expression vector [13].
2. Amino acid drop-out powder (DOP): Grind equal amounts of adenine, lysine, tyrosine, histidine, leucine, and tryptophane with mortar and pestle to generate a homogeneous powder.
3. Synthetic defined-uracil plates (SD-ura plates): Dissolve 6.7 g of Difco™ yeast nitrogen base w/o amino acids (YNB) and 1 g of DOP in 900 mL of ddH_2O (*see* **Note 2**) and add 20 g of agar. Dissolve 20 g of glucose in 100 mL of ddH_2O. Autoclave both solutions and combine as soon as they are cooled to ~45 °C to pour plates.
4. Synthetic defined-uracil 1 % growth medium (SDG 1 % medium): Dissolve 6.7 g of Difco™ yeast nitrogen base w/o amino acids (YNB) and 1 g of DOP in 900 mL of ddH_2O (*see* **Note 2**). Dissolve 10 g of glucose in 100 mL ddH_2O for a final glucose concentration of 1 %. Autoclave and combine the solutions as soon as they are cooled to room temperature.
5. Sterile 100 mL flasks covered with aluminum foil.
6. Eppendorf BioPhotometer®.
7. *n*-Dodecane.
8. Induction solution: 20 mg Galactose and 7 mg raffinose dissolved per mL of ddH_2O (*see* **Note 5**).
9. Supplementary solution: 0.167 g Yeast nitrogen base and 25 mg DOP dissolved per mL of sterile ddH_2O (*see* **Note 5**).
10. 50 mL Falcon® tubes.
11. Eppendorf 5810 R centrifuge.
12. GC vials, inserts, crimp caps, and crimper.
13. GC-FID: Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID), HP-5 column (cross-linked 5 % Ph-Me Siloxane; 10 m × 0.10 mm × 0.10 μm).

3 Methods

3.1 Conversion of Externally Added Terpenes with Resting Cells (Fig. 2)

1. Freshly grow the *S. cerevisiae* strain harboring the cytochrome P450 and reductase co-expression plasmid on an SD-ura plate for 2–3 days [13].
2. Take a cell pellet about the size of a pinhead and inoculate 5 mL of SDG medium containing 2 % glucose in a 50 mL Falcon® tube. Shake cultures overnight at 170 rpm and 30 °C for 15–20 h.

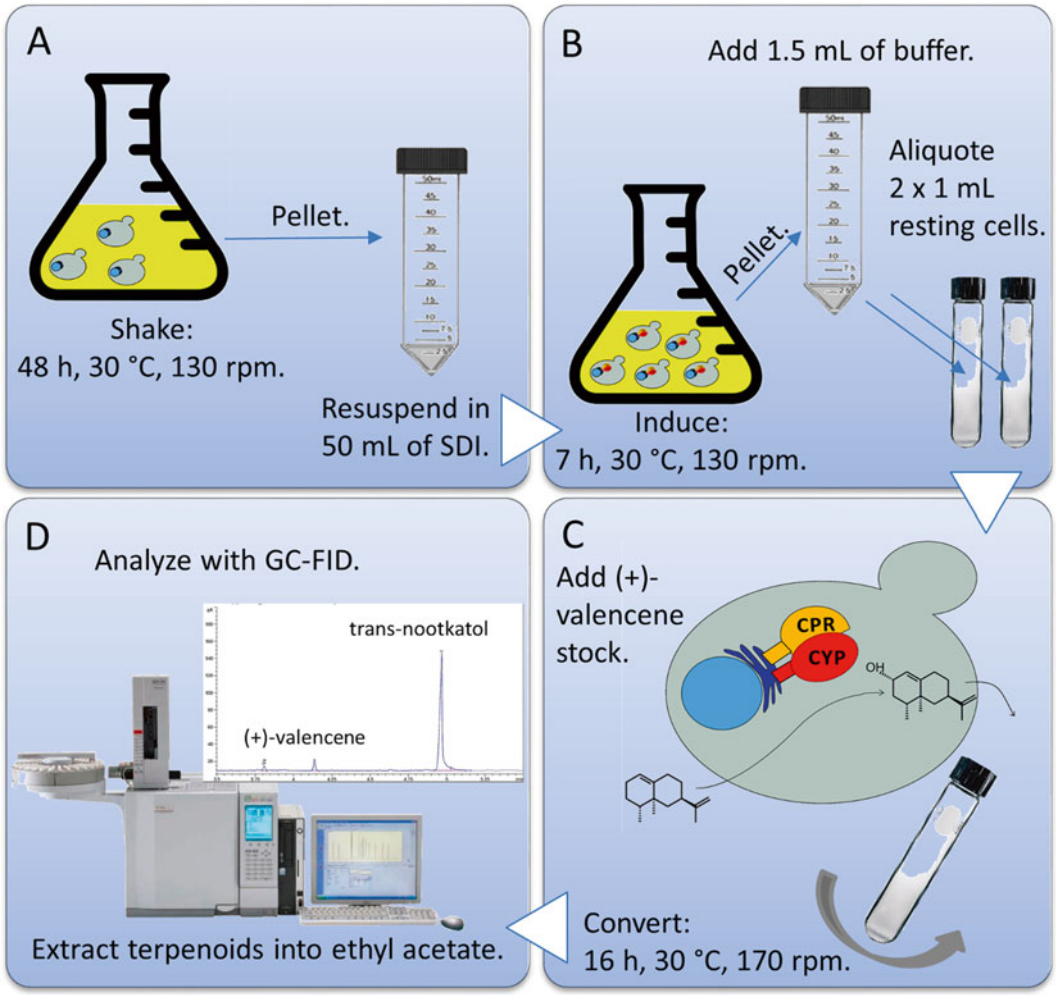

Fig. 2 Conversion of externally added terpenes with *S. cerevisiae* resting cells. After cultivating the yeast strain for 24 h in SDG 2 % medium, centrifuge the culture and resuspend the cell pellet in SDI medium (**A**). Induce co-expression of HPO and CPR by shaking cells for 7 h at 170 rpm and 30 °C. To generate resting cells, pellet 600 OD_{600} units of yeast cells and resuspend the pellet in 1.5 mL of 50 mM potassium phosphate buffer, pH 7.4, for two aliquots of 1 mL to be transferred to PYREX® tubes (**B**). Add 20 μL of 100 mM (+)-valencene stock solution each and let proceed bioconversions for 16 h (**C**). Extract terpenes and terpenoids with 500 μL of ethyl acetate on a VXR basic Vibrax® for 30 min. Separate phases by centrifugation and analyze organic phases by GC-FID (**D**)

3. Use overnight culture to inoculate 50 mL of SDG media in 300 mL baffled flasks to an OD_{600} of 0.1 and cultivate cells for 48 h at 130 rpm and 30 °C (*see* **Note 6**).
4. Transfer the cell suspension to a 50 mL Falcon® tube.
5. Spin down the cell suspension for 5 min at 1062 × *g* in an Eppendorf 5810 R centrifuge and carefully resuspend the cell pellet in 50 mL of SDI medium (*see* **Note 7**).

6. Transfer the cell suspension to sterile 300 mL baffled flasks and induce cells for 7 h at 130 rpm and 30 °C (*see* **Note 8**).
7. Determine OD_{600} of induced cells with an Eppendorf BioPhotometer® and calculate the volume required to obtain 600 OD_{600} units. For example, if the end-OD_{600} of induced cells is 15, divide 600 by 15 to get the mL of cell volume needed. In this case, 40 mL of induced cells would be used to prepare resting cells.
8. For preparing resting cells, transfer the volume of induced cells equaling 600 OD_{600} units to a 50 mL Falcon® tube.
9. Centrifuge for 5 min at 1062 × *g* in an Eppendorf 5810 R centrifuge and carefully resuspend the cell pellet in 1.5 mL of 50 mM potassium phosphate buffer, pH 7.4 (*see* **Note 9**).
10. Split the cell suspension into two equal aliquots in PYREX® tubes and add 20 μL (*see* **Notes 10** and **11**) of 100 mM (+)-valencene stock solution (*see* **Note 12**) to each aliquot.
11. Let biotransformations proceed for 16 h at 170 rpm and 30 °C (*see* **Note 13**).
12. Extract terpenoids into 500 μL of ethyl acetate with a VXR basic Vibrax® at room temperature and maximum speed for 30 min.
13. Separate phases by centrifugation at 2720 × *g* for 15 min.
14. Transfer 70 μL of the upper ethyl acetate phase to GC-crimp vials (*see* **Note 14**).
15. GC-FID measurement: 1 μL of samples are injected in split mode (split ratio 30:1) at 250 °C injector temperature and 320 °C detector temperature with H_2 as carrier gas and a flow rate set to 0.4 mL/min in constant flow mode (49 cm/s linear velocity). Oven temperature program: 100 °C for 1 min, 20 °C/min ramp to 250 °C, and 45 °C/min ramp to 280 °C (0.5 min) [13].
16. For quantification, measure standard solutions with known concentrations of terpenoids with GC-FID and plot a calibration curve from the obtained results. Scale the abscissa in ng/μL of the terpenoid standard solutions. The ordinate represents the signal intensity measured.

3.2 Production of Terpenes and Terpenoids in a Self-Sufficient *S. cerevisiae* Strain

1. Freshly grow the *S. cerevisiae* strain harboring recombinant terpene synthase (+/− mevalonate pathway engineering), e.g., ValS, t*HMG1*, and CYP/CPR-co-expression plasmid [13], on an SD-ura plate for 2–3 days.
2. Inoculate 5 mL of SDG 1 % medium in a 50 mL Falcon® tube with a pin-head sized colony of the yeast strain. Shake overnight for 20 h at 25 °C and 170 rpm.

3. Determine OD_{600} and inoculate 50 mL of SDG 1 % medium (*see* **Note 15**) in 100 mL flasks to a starting OD_{600} of 0.1. Shake at 25 °C and 150 rpm for 24 h.
4. Add 1 mL of supplementary solution and 1 mL of induction solution (*see* **Note 16**) to the cell broth and directly overlay with 10 vol.-% (e.g., 5 mL) *n*-dodecane (Fig. 3). Cultivate at 25 °C and 150 rpm for another 24 h.
5. Add 1 mL of supplementary solution and 1 mL of induction solution and cultivate at 25 °C and 150 rpm for another 24 h.
6. Transfer the cell suspension including the organic phase to a 50 mL Falcon® tube.
7. Separate phases by centrifugation for 15 min at 2720×*g* Eppendorf 5810 R centrifuge.
8. Transfer 70 µL of the upper *n*-dodecane phase to a GC-crimp vial with insert (*see* **Note 17**).
9. GC-FID measurement: 1 µL of samples are injected in split mode (split ratio 30:1) at 250 °C injector temperature and 320 °C detector temperature with H_2 as carrier gas and a flow rate set to 0.4 mL/min in constant flow mode (49 cm/s linear velocity). Oven temperature program: 100 °C for 1 min, 20 °C/min ramp to 250 °C, and 45 °C/min ramp to 280 °C (0.5 min) [13] (*see* **Note 18**).
10. For quantification, measure standard solutions with known concentrations of terpenoids with GC-FID and plot a calibra-

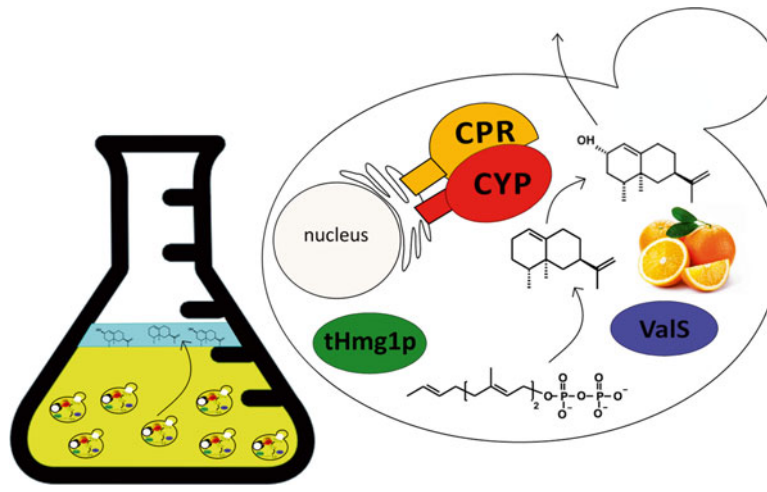

Fig. 3 In vivo terpenoid production with a self-sufficient *S. cerevisiae* strain. Upon over-expression of t*HMG1*, an increased intracellular pool of FPP is generated, which can be further converted to (+)-valencene with a highly specific valencene synthase (ValS). Subsequently, HPO/CPR convert the terpene to *trans*-nootkatol, which is enriched in *n*-dodecane overlaying the cell broth

tion curve from the obtained results. Scale the abscissa in ng/μL of the terpenoid standard solutions. The ordinate represents the signal intensity.

4 Notes

1. Depending on the size of mortar and pestle take 2–4 g of each amino acid for preparing the DOP.
2. Stir the solution containing DOP and YNB prior to autoclaving for at least 2 h to achieve complete solubility of poorly soluble components.
3. The addition of 0.7 % raffinose to the SDI medium is optional, but it has shown to reduce induction stress by providing a growth advantage without affecting the induction process [23, 24].
4. Triton®X-100 is highly viscous and hard to pipette. Carefully take up the detergent by slowly handling the pipette. Flush the pipette tip multiple times with 100 mM (+)-valencene stock solution to quantitatively transfer all of the Triton®X-100.
5. Depending on the total number of yeast cultures to induce, prepare 50–100 mL of induction and supplementary solution. The solutions can be stored at 4 °C for up to 3 months.
6. Prolongation of the growth phase before induction was shown to be essential for high-level expression of CYP/CPR combinations [13] and is the most innovative aspect of this protocol.
7. Preheat SDI medium to 30 °C in an incubator or water bath to avoid any cellular cold shock response.
8. It is advisable to analyze activities of cells harvested after different time points of induction to find out the ideal time point providing the most active recombinant protein.
9. Depending on the pellet size of induced cells, the volume of 50 mM potassium phosphate buffer needs to be adjusted. Add 1.5 mL of 50 mM potassium phosphate buffer to 600 OD_{600} units to reach a final volume of 2 mL of resting cells.
10. Due to the high volatility of terpenes it is recommended to use stock solutions directly after preparation to avoid significant variations of substrate amounts applied for conversions.
11. DMSO and 1 % Triton®X-100 act as solubilizers for poorly water-soluble substrates. However, terpenes may separate from DMSO and form a second phase by time. To achieve the application of equal amounts of substrate per PYREX® tube, it is advisable to vortex the substrate stock solution every time before transferring the aliquots to resting cells for conversion.

12. Prior to conversion with *S. cerevisiae* resting cells, a toxicity test is recommended, because especially mono- and sesquiterpenoids are toxic for yeast cells. For toxicity studies, simply incubate your recombinant strain with aliquots of stock solutions containing different substrate concentrations. Compare the growth of treated versus untreated cells to estimate cellular toxicity [25, 26].
13. For activity assays with resting cells, seal PYREX® tubes loosely with screw caps and fix the caps with seal tape to maintain a balance between oxygen supply and substrate volatilization during conversion. Additionally, fix the PYREX® tubes in a rather steep angle to guarantee maximal turbidity. Make sure to maintain the same angle for all conversions.
14. Sometimes, the centrifugation step needs to be prolonged due to formation of a massive protein interlayer between the aqueous and organic phase. If phases cannot be separated properly, the addition of and extraction with another 500 μL of ethyl acetate are recommended to increase the volume of the upper organic phase. The additional volume must be considered in calculating terpenoid concentrations.
15. The amount of glucose needs to be reduced from 2 to 1 % to ensure depletion of the carbon source within 24 h of growth. Otherwise galactose induction will suffer from repression by residual glucose.
16. We initially applied 2.5 times higher galactose concentrations for induction, but this yielded only 50 % of the terpenoid productivity compared to the method described here. Due to the fact that galactose has a relatively high market price, lowering the galactose concentration has an additional economic benefit.
17. For disposal of two-phase cultures, gather the rest of the cultivations in a 2 L flask and let phases separate overnight. Remove the upper organic phase quantitatively and discard appropriately. Ensure solvent-free autoclaving of the aqueous cell broth.
18. The retention time of the analytes enriched in *n*-dodecane will change compared to the GC-FID spectra of samples extracted with ethyl acetate as described in Subheading 3.1. *n-Dodecane* causes all the compounds to elute from the column belatedly.

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References

1. Salvador JAR, Clark JH (2002) The allylic oxidation of unsaturated steroids by tert-butyl hydroperoxide using surface functionalised silica supported metal catalysts. Green Chem 4:352–356
2. Majetich G, Behnke M, Hull K (1985) A stereoselective synthesis of (+/-)-nootkatone and (+/-)-valencene via an intramolecular Sakurai reaction. J Org Chem 50:3615–3618
3. Fraatz MA, Berger RG, Zorn H (2009) Nootkatone—a biotechnological challenge. Appl Microbiol Biotechnol 83:35–41
4. Chang MCY, Keasling JD (2006) Production of isoprenoid pharmaceuticals by engineered microbes. Nat Chem Biol 2:674–681
5. Kirby J, Keasling JD (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 60:335–355
6. Emmerstorfer A, Wriessnegger T, Hirz M, Pichler H (2014) Overexpression of membrane proteins from higher eukaryotes in yeasts. Appl Microbiol Biotechnol 98:7671–7698
7. Zhao Y-J, Cheng Q-Q, Su P, Chen X, Wang X-J, Gao W, Huang L-Q (2014) Research progress relating to the role of cytochrome P450 in the biosynthesis of terpenoids in medicinal plants. Appl Microbiol Biotechnol 98:2371–2383
8. Kitaoka N, Lu X, Yang B, Peters RJ (2015) The application of synthetic biology to elucidation of plant mono-, sesqui-, and diterpenoid metabolism. Mol Plant 8:6–16
9. Laursen T, Jensen K, Møller BL (2011) Conformational changes of the NADPH-dependent cytochrome P450 reductase in the course of electron transfer to cytochromes P450. Biochim Biophys Acta 1814:132–138
10. Jensen K, Møller BL (2010) Plant NADPH-cytochrome P450 oxidoreductases. Phytochemistry 71:132–141
11. Bernhardt R, Urlacher VB (2014) Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. Appl Microbiol Biotechnol 98:6185–6203
12. Takahashi S, Yeo Y-S, Zhao Y, O'Maille PE, Greenhagen BT, Noel JP, Coates RM, Chappel J (2007) Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. J Biol Chem 282:31744–31754
13. Emmerstorfer A, Wimmer-Teubenbacher M, Wriessnegger T, Leitner E, Muller M, Kaluzna I, Schurmann M, Mink D, Zellnig G, Schwab H, Pichler H (2015) Overexpression of *ICE2* stabilizes cytochrome P450 reductase in *Saccharomyces cerevisiae* and *Pichia pastoris*. Biotechnol J 10:623–635
14. Wriessnegger T, Augustin P, Engleder M, Leitner E, Muller M, Schurmann M, Mink D, Zellnig G, Schwab H, Pichler H (2014) Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metab Eng 24:18–29
15. Beekwilder J, van Houwelingen A, Cankar K, van Dijk ADJ, de Jong RN, Stoopen G, Bouwmeester H, Achkar J, Sonke T, Bosch D (2014) Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant Biotechnol J 12:174–182
16. Asadollahi MA, Maury J, Møller K, Nielsen KF, Schalk M, Clark A, Nielsen J (2008) Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of *ERG9* repression on sesquiterpene biosynthesis. Biotechnol Bioeng 99:666–677
17. Farhi M, Marhevka E, Masci T, Marcos E, Eyal Y, Ovadis M, Abeliovich H, Vainstein A (2011) Harnessing yeast subcellular compartments for the production of plant terpenoids. Metab Eng 13:474–481
18. Scalcinati G, Knuf C, Partow S, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V (2012) Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquiterpene α-santalene in a fed-batch mode. Metab Eng 14:91–103
19. 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 (2006)

Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943

20. Girhard M, Machida K, Itoh M, Schmid RD, Arisawa A, Urlacher VB (2009) Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb Cell Fact 8:36
21. Gavira C, Höfer R, Lesot A, Lambert F, Zucca J, Werck-Reichhart D (2013) Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. Metab Eng 18:25–35
22. Cankar K, van Houwelingen AMML, Bosch HJ, Sonke T, Bouwmeester H, Beekwilder MJ (2011) A chicory cytochrome P450 monooxygenase CYP71AV8 for the oxidation of (+)-valencene. FEBS Lett 585:178–182
23. Sherman F (1991) Getting started with yeast. Methods Enzymol 194:3–21
24. Bergman LW (2001) Growth and maintenance of yeast. Methods Mol Biol 177:9–14
25. Liu J, Zhu Y, Du G, Zhou J, Chen J (2013) Exogenous ergosterol protects *Saccharomyces cerevisiae* from D-limonene stress. J Appl Microbiol 114:482–491
26. Brennan TCR, Turner CD, Krömer JO, Nielsen LK (2012) Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. Biotechnol Bioeng 109:2513–2522