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Special Issue Article

Capturing of the monoterpene olefin limonene produced in *Saccharomyces cerevisiae*

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Abstract

Monoterpene olefins such as limonene are plant compounds with applications as flavouring and fragrance agents, as solvents and potentially also in polymer and fuel chemistry. We engineered baker's yeast *Saccharomyces cerevisiae* to express a (−)-limonene synthase from *Perilla frutescens* and a (+)-limonene synthase from *Citrus limon*. Both proteins were expressed either with their native plastid targeting signal or in a truncated form in which the plastidial sorting signal was removed. The yeast host strain for expression was AE9 K197G, which expresses a mutant Erg20 enzyme. This enzyme catalyses the formation of geranyl diphosphate, which is the precursor for monoterpenes. Several methods were tested to capture limonene produced by the yeast. Extraction from the culture medium by pentane, or by the addition of $CaCl_2$ followed by solid-phase micro-extraction, did not lead to detectable limonene, indicating that limonene is rapidly lost from the culture medium. Volatile terpenes such as limonene may also be trapped in a dodecane phase added to the medium during fermentation. This method resulted in recovery of 0.028 mg/l (+)-limonene and 0.060 mg/l (−)-limonene in strains using the truncated *Citrus* and *Perilla* synthases, respectively. Trapping the headspace during culture of the limonene synthase-expressing strains resulted in higher titres, at 0.12 mg/l (+)-limonene and 0.49 mg/l (−)-limonene. These results show that the volatile properties of the olefins produced require specific methods for efficient recovery of these molecules from biotechnological production systems. Gene Bank Nos were: KM015220 (*Perilla* limonene synthase; this study); AF317695 (*Perilla* limonene synthase; Yuba *et al*., 1996); AF514287.1 (*Citrus* limonene synthase; Lucker *et al*., 2002).

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Introduction

Monoterpenes are C10 compounds that consist of two isoprene units. In 2012, about 900 different types of monoterpenes had been identified in nature (Dictionary of Natural Products, 2012). They may occur as hydrocarbon olefins or may be modified by oxidation to monoterpene alcohols or acids. The majority of natural monoterpenes are found in plants where they form the main constituent(s) of essential oils. In plants, monoterpenes are produced in plastids in which the methylerythritol phosphate (MEP) pathway supplies the two isoprene units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Figure 1). In the plastids, these two isoprene units are combined, by GPP synthase, to form geranyl diphosphate (GPP). GPP is the universal precursor for the biosynthesis of all monoterpenes, and its conversion to the monoterpene skeleton is mediated by monoterpene synthases (Degenhardt *et al*., 2009).

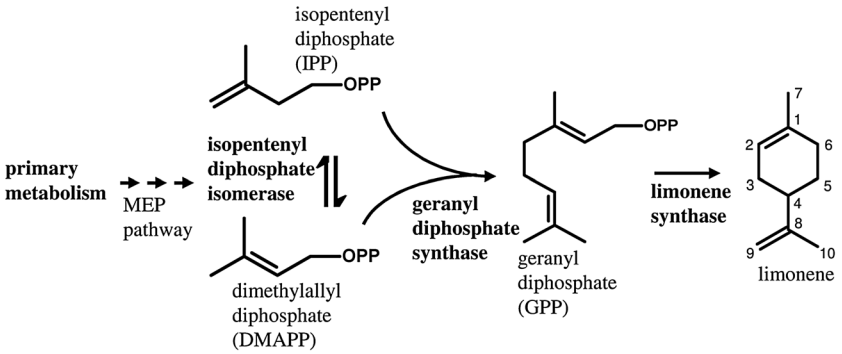

Figure 1. Biochemical pathway of limonene biosynthesis in plants

Limonene is a monoterpene olefin that is well known for its citrus-like olfactory properties. It has a well-documented antimicrobial activity (Brennan *et al.*, 2013; Zhang *et al.*, 2014) and is therefore often included in detergents and soaps. Limonene may be oxidized to several products of interest, including menthol, carvone and *Perilla* alcohol, which have wide applications as flavouring agents and preservatives (Duetz *et al.*, 2003; Mars *et al.*, 2001). Due to its unsaturated cyclic structure limonene has potential as a precursor for biopolymers (Wilbon *et al.*, 2013). Limonene has been proposed as an additive for diesel (Tracy *et al.*, 2009) and as a component for jet fuel replacements (Brennan *et al.*, 2012).

Natural sources of limonene include orange oil, derived from *Citrus* spp., in which it is present as the (+)-enantiomer, and oils from Lamiaceae, such as *Mentha* spp. and *Perilla frutescens*, in which the (−)-enantiomer is present (Duetz *et al.*, 2003). Limonene is mainly produced as a by-product of citrus juice processing, with a production volume of ~50 000 tonnes/year (Braddock, 2013). The limonene value may increase when new potential applications in the polymer and fuel industries lead to a higher demand that cannot solely be met by the citrus industry. Moreover, essential oils produced from citrus materials may contain significant amounts of pesticides (Nichkova *et al.*, 2009), which limits the application in food and household products. Such problems could be circumvented by an industrial-scale production of limonene in yeast.

In recent years, novel concepts for the production of terpenes for applications such as flavouring, pharmaceuticals and fuel have been explored. In particular, sesquiterpenes (C15) have been produced in fermentation systems, with high yields (Scalcinati *et al.*, 2012; Westfall *et al.*, 2012). Sesquiterpenes differ from monoterpenes in several respects. Sesquiterpenes usually have a relatively low toxicity towards microorganisms and do not rapidly evaporate from fermentation systems (Heeres *et al.*, 2014). Moreover, they can easily be separated from an organic fermentation phase, such as dodecane, by distillation. Monoterpenes, on the other hand, are highly volatile, which complicates their recovery from fermentation systems. Partition of solutes over the watery phase and culture headspace is defined by their Henry's law constant (K_H). The K_H of monoterpene olefins is low; e.g. at 25°C the K_H of limonene is only 0.048 M/atm (Leng *et al.*, 2013), indicating that 95% would evaporate to the headspace and thus be lost in an aerated shaker flask. Moreover, limonene is poorly tolerated by microorganisms such as yeast (Brennan *et al.*, 2013). Limonene has a low minimum inhibitory concentration (MIC) of 0.44 mM for *S. cervisiae* in a solvent-free system (Brennan *et al.*, 2012). Therefore, limonene may need to be continuously removed from a culture system in order to sustain growth.

Production and capture of monoterpenes by expressing plant terpene synthases in yeast has hardly been explored, because yeast does not naturally produce monoterpenes or its precursor GPP. Yeast mediates its isoprenoid biosynthesis by the mevalonate (MVA) pathway, which, like the MEP pathway in plants, also produces IPP and DMAPP. In yeast these building blocks are then converted to the C15 molecule farnesyl pyrophosphate (FPP) by activity of FPPsynthase Erg20. Recently, a modified Erg20 enzyme, K197G, has been shown to produce partly GPP instead of FPP, and a strain in which wild-type Erg20 has

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been replaced by this mutant Erg20 enzyme has been employed for the production of geraniol and linalool by a geraniol synthase from basil (Fischer *et al*., 2011).

In this study we explored the capture of limonene from a monoterpene-producing yeast strain. Using the *ERG20* mutant strain AE9 K197G, with plant genes encoding a (+)-limonene synthase from *Citrus limon*, and a (−)-limonene synthase from *P. frutescens*, yeast was engineered to produce both enantiomers of limonene. We compared different methods for capturing limonene from these strains, and demonstrated that optimization of the capture method for a volatile product contributed considerably to its effective yield during production in a fermentation system.

Materials and methods

Chemicals

(−)-Limonene was purchased from Merck–Schuchardt (Hohenbrunn, Germany). Pentane and ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). (±)-linalool and *cis*-nerolidol were purchased from Fluka Chemika (SanktGallen, Switzerland). Geranyl acetone, *n*-dodecane and geraniol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of plasmids and strains

The *C. limon* (+)-limonene synthase was amplified and subcloned in the pGEMTeasy vector (Promega) from the pBlueC62 plasmid previously described (Lucker *et al*., 2002), with primers from the start and stop codons, ClLS-F and ClLS-R (Table 1), and transformed to *E. coli* DH5*α*.

P. frutescens (−)-limonene synthase was amplified from cDNA from young leaves of a green *P. frutescens* variety, purchased from Chiltern Seeds (Wallingford, UK). The *P. frutescens* plants had a perillyl aldehyde chemotype (Tabata, 2000). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from the RNA using a SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The limonene synthase candidate cDNA was amplified with primers overlapping the start and stop codons, PfLS-F and PfLS-R (Table 1), ligated in the pGEMTeasy vector and transformed to *E. coli* DH5*α*. Positive clones for ClLS and PfLS were analysed by sequencing (Macrogen Europe, The Netherlands). The cloned gene encoded a PfLS protein with 96% amino acid identity to the *Perilla frutescens* limonene synthase gene published by Yuba *et al*. (1996), with GenBank Accession No. AF317695 (see supporting information, Figure S1). The PfLS protein contained the RRX_8W and DDXXD motifs, typical for monoterpene synthases (Bohlmann *et al*., 1998; Williams *et al*., 1998).

For the *in vitro* assay, the plastid targeting signal of the proteins was removed, by using a new forward primer for ClLS and PfLS, starting from the RRX_8W motif (see supporting information, Figure S1) (Lucker *et al*., 2002). These truncated versions of the monoterpene synthases (CltLS and PftLS) were amplified with restriction site primers CltLS-SalF, PftLS-SalF, CltLS-NotR and PftLS-NotR (Table 1) and cloned in the pCDF–duet (Novagen, Madison, USA) expression vector, in-frame with the HIS-tag under the T7 promoter, using restriction enzymes *Sal*I and *Not*I. The presence of the insert was confirmed by sequencing. For expression, plasmids pCDF–duet, pCDF–duet–PftLS and pCDF–duet–CltLS were transformed to the *E. coli* BL21RIL strain (Stratagene, Santa Clara, CA, USA).

For expression in yeast, constructs expressing full-length terpene synthase proteins or expressing truncated proteins (without plastid targeting) were prepared in the pESC–HIS–CPR plasmid. This plasmid contains the NADPH–cytochrome P450 reductase gene *AtCPR1* from *Arabidopsis thaliana*, which has been amplified from plasmid pUDE172 (Koopman *et al*., 2012), using primers with *Sal*I/*Xho*I restriction sites CPR1–SalF and CPR1–XhoR (Table 1). The gene was cloned in the commercially available pESC–HIS (Agilent, Santa Clara, CA, USA) plasmid under control of the Gal1 promoter. The *P. frutescens* and *C. limon* limonene synthase genes were amplified using primers with *Not*I/*Pac*I restriction sites PfLS–NotF, PfLS–PacR, ClLS–PacR and ClLS–NotF for full-length versions and PftLS–NotF and CltLS–NotF for truncated versions (Table 1) from plasmids pGEMT–PfLS and pBlueC62, and introduced into the *Not*I and *Pac*I restriction sites present following the GAL10 promoter of pESC–HIS–CPR, to yield pESC–HIS–CPR1–PfLS, pESC–

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Table 1. Overview of strains, plasmids and oligonucleotides

Plasmids	***Inserted genes***
pCDF–duet	
pCDF–duet–PftLS	Truncated *P. frutescens* (−)-limonene synthase (T7 promoter)
pCDF–duet–ClLS	Truncated *C. limon* (+)-limonene synthase (T7 promoter)
pESC–HIS	
pESC–HIS–CPR1	Cytochrome P450 reductase (*CPR1*) (*Gal1* promoter)
pESC–HIS–CPR1–PfLS	*CPR1* (*Gal1*) and full-length *P. frutescens* (−)-limonene synthase (*Gal10*)
pESC–HIS–CPR1–ClLS	*CPR1* (*Gal1*) and full-length *C. limon* (+)-limonene synthase (*Gal10*)
pESC–HIS–CPR1–PftLS	*CPR1* (*Gal1*) and truncated *P. frutescens* (−)-limonene synthase (*Gal10*)
pESC–HIS–CPR1–CltLS	*CPR1* (*Gal1*) and truncated *C. limon* (+)-limonene synthase (*Gal10*)
S. cerevisiae strains	*Genotype*
AE9 K197G	*Mat α, his3Δ, leu2Δ0, ura3-, trp1Δ63, YJL167W::kanMX4* (*pLB41erg20K197G*) (Fischer *et al.*, 2011)
Sc-pESC	AE9 K197G with pESC–HIS (this study)
Sc-CPR1	AE9 K197G with pESC–HIS–CPR1 (this study)
Sc-PfLS	AE9 K197G with pESC–HIS–CPR1–PfLS (this study)
Sc-ClLS	AE9 K197G with pESC–HIS–CPR1–ClLS (this study)
Sc-PftLS	AE9 K197G with pESC–HIS–CPR1–PftLS (this study)
Sc-CltLS	AE9 K197G with pESC–HIS–CPR1–CltLS (this study)
Oligonucleotides	*Sequence*
PfLS–F	ATGTATACCGGTGTAATAATGC
PfLS–R	TTACAACCATTGCTCGAACAAG
ClLS–F	ATGTCTTCTTGCATTAATCCCTCAACCTTGGTTACCT
ClLS–R	TCAGCCTTTGGTGCCAGGAGATGC
PftLS–SalF	ACTGTCGACCGACGATCCGGAAACTACAGTCCTT
PftLS–NotR	TGCGGCCGCTTACAACCATTGCTCGAACAAGATGTCTGTCATCT
CltLS–SalF	ACTGTCGACAGGAGATCAGCAAACTACCAACCTTCA
CltLS–NotR	TGCGGCCGCTCAGCCTTTGGTGCCAGGAGATGC
PfLS–NotF	TAGCGGCCGCTATGTATACCGGTGTAATAATGCATATGGCGATTCCT
PfLS–PacR	ACTTAATTAATTACAACCATTGCTCGAACAAGATGTCTGTCATCT
ClLS–NotF	TAGCGGCCGCTATGTCTTCTTGCATTAATCCCTCAACCTTGGTTACCT
ClLS–PacR	ACTTAATTAATCAGCCTTTGGTGCCAGGAGATGC
PftLS–NotF	ACTGCGGCCGCATGGCACGACGATCCGGAAACTACAGTCCTT
CltLS–NotF	ACTGCGGCCGCATGGCAAGGAGATCAGCAAACTACCAACCTTCAATT
CPR1–SalF	TACGTCGACATGACTTCTGCTTTGTACGCTTCTGACTTGTT
CPR1–XhoR	TATGCTCGAGTTACCAAACGTCTCTCAAGTATCTACCTTCAGTTT

HIS–CPR1–PftLS, pESC–HIS–CPR1–ClLS and pESC–HIS–CPR1–CltLS, respectively.

The yeast strain *Saccharomyces cerevisiae* AE9 K197G (Fischer *et al.*, 2011) was kindly provided by Dr Fischer from Strasbourg University and was maintained on SD medium [1.7 g/l yeast nitrogen base (without amino acids, Difco), 5 g/l $(NH_4)_2SO_4$, 20 g/l D-glucose, 20 g/l purified Bacto agar] supplemented with uracil, histidine and leucine. The strain was transformed with plasmids pESC–HIS–CPR1, pESC–HIS, pESC–HIS–CPR1–PfLS, pESC–HIS–CPR1–ClLS, pESC–HIS–CPR1–PftLS and pESC–HIS–CPR1–ClLS, using lithium acetate (Gietz and Schiestl, 2007), resulting in strains *Sc*-CPR1, *Sc*-pESC, *Sc*-PfLS, *Sc*-ClLS, *Sc*-PftLS and *Sc*-ClLS, respectively (Table 1). After transformation, cells were selected on synthetic medium for 3–4 days at 30°C on SD minimal medium supplemented with uracil and all amino acids except histidine.

In vitro assay terpene synthases

Starter cultures (5 ml) of recombinant BL21 with pCDFduet, pCDFduet–PftLS and pCDFduet–ClLS were grown overnight at 37°C, 250 rpm in LB medium with 1% glucose supplemented with spectinomycin (50 µg/ml) and chloramphenicol (50 µg/ml); 250 µl starter culture was diluted in 25 ml 2 × YT medium with antibiotics and incubated at 37°C and 250 rpm until the optical density at 600 nm was 0.6–0.8. After addition of 25 µl 1 M IPTG, the cultures were incubated overnight at 18°C and

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250 rpm. The cultures were centrifuged for 15 min at 3400 rpm and the supernatants discarded. The pellets were resuspended in 1 ml ice-cold buffer A (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1.4 mM β-mercaptoethanol). About 0.2 g zirconium/silica beads 0.1 mm (Biospec Products, Bartlesville, USA) were added to each pellet and the cells lysed by shaking for 10 s in a FastPrep machine (FP120 Bio101 Savant) at speed 6.5. After cooling of the tubes on ice for 2 min, they were shaken again for 10 s. Subsequently, the lysates were centrifuged for 10 min at 13 000 × *g* and 4°C and the supernatants were immediately used for enzyme assays. A mix of 32.5 µl 100 mM Tris, pH 7.4, 800 µl assay buffer (15 mM MOPSO, 12.5% v/v glycerol, 1 mM ascorbic acid, 0.1% Tween 20, 1 mM $MgCl_2$ and 2 mM DTT, pH 7.0) and 20 µl 250 mM sodium orthovanadate was prepared in a glass tube, and 100 µl of the cell supernatant and 5 µl 10 mM geranyl diphosphate were added. The mix was immediately covered with an overlay of 1 ml pentane and incubated at 30°C for 1 h under gentle agitation. The tubes were vortexed well and centrifuged for 5 min at 3400 rpm. The pentane phase was collected, dried over anhydrous Na_2SO_4 and injected into a 7890A gas chromatograph (Agilent) equipped with a mass selective detector (Model 5975C, Agilent), scanning in the range 45–450 *m/z*. Splitless injection of 1 µl sample was performed at 250°C on a Zebron ZB-5MS column (30 m × 0.25 mm, 0.25 µm thickness; Phenomenex) at a helium flow rate of 1 ml/min. The temperature programme was 2.25 min at 45°C, then at 40°C/min to 300°C, then 3 min at 300°C. When using dodecane the detector was switched off at room temperature for 10 min to prevent saturation. Compounds were identified by comparing the retention times and mass spectra with a mix of authentic standards, including terpinolene, γ-terpinene and (−)-limonene..

Culturing yeast for production

Single colonies were inoculated in 5 ml SD-galactose medium (1.7 g/l YNB, 5 g/l $(NH_4)_2SO_4$, 20 g/l galactose) to generate precultures and grown in a shaking incubator at 300 rpm 30°C for 48–72 h. The cultures were started by inoculating 50 ml SD-galactose with the preculture to a final optical density at 600 nm of 0.05 in 250 ml Erlenmeyer flasks, and were grown at 30°C and 300 rpm for the indicated times. For all culture experiments, the optical density at 600 nm was between 6 (headspace set-up) and 10 (dodecane set-up) at the final time of sampling.

Collecting volatiles

In one set of experiments (Figure 2a), 50 ml cultures were transferred from the 250 ml Erlenmeyer flasks to a separation funnel after 3 days of growth, and extracted using 5 ml pentane by vigorous shaking for 2 min and separation of pentane in the funnel, followed by centrifugation of the pentane phase (5 min, 3500 × *g*) to further clarify the pentane phase. Pentane was further prepared for GC–MS analysis as described above.

In a second set of cultures (Figure 2b), the 50 ml SD-galactose medium was overlayed with 5 ml *n*-dodecane during fermentation in the 250 ml Erlenmeyer flasks, as described by Cankar *et al*. (2011).

A third set of 50 ml cultures were grown (Figure 2c) and at regular intervals 0.5 ml samples were taken from the 250 ml Erlenmeyer flasks and immediately transferred to GC–MS vials. To each vial, 0.5 ml of 5 M $CaCl_2$ solution was added and the vial was immediately capped. Each sample was incubated at 50°C for 10 min and was subsequently automatically extracted and injected into the GC–MS, using a Combi PAL autosampler (CTC Analytics AG). Headspace volatiles were extracted by exposing a 65 µm polydimethylsiloxane-divinylbenzene SPME fibre (Supelco, Bellefonte, USA) to the vial headspace for 20 min without agitation and heating at 50°C. The fibre was inserted into a GC 8000 (Fisons Instruments) injection port (with a 1 ml liner) and volatiles were desorbed for 1 min at 250°C (splitless). Chromatography was performed on an HP-5 (50 m × 0.32 mm × 1.05 µm) column, with helium as the carrier gas (37 kPa). The GC interface and MS source temperatures were 260°C and 250°C, respectively. The GC temperature programme began at 80°C (2 min), was then raised to 150°C at a rate of 5°C/min, raised to 250°C at 18°C/min and finally held at 250°C for 2 min. Mass spectra in the 45–400 *m/z* range were recorded on an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans/s, a solvent delay of 2 min and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using Xcalibur software (http://www.thermo.com).

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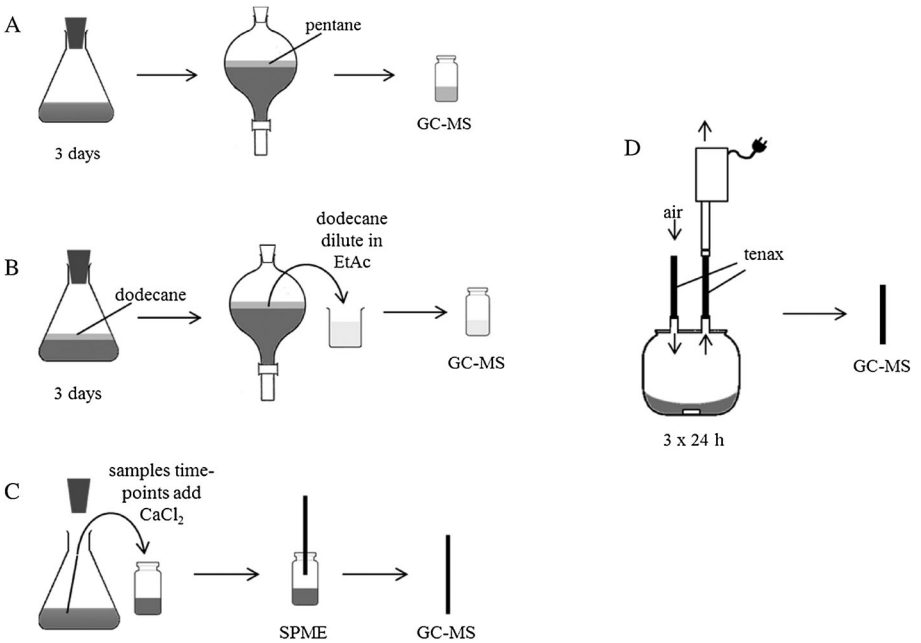

Figure 2. Schematic workflow of tested limonene collection approaches: (A) pentane extraction; (B) dodecane overlay; (C) solid-phase micro-extraction; (D) headspace trapping

A fourth set of cultures (Figure 2d) was used for trapping volatiles, using the method for trapping plant volatiles described in van Herpen *et al.* (2010). Precultures were diluted in 50 ml SD-galactose medium to OD_{600} 0.05 and were cultured in Rundrand-Glas 100 cuvettes of 500 ml (Weck), equipped with inlets and outlets for gas, in a 30°C chamber. The cultures were stirred at 700 rpm, using a Teflon stir bar. During cultivation, air was constantly refreshed through Steel sorbent cartridges (89 × 6.4 mm o.d.; Markes) containing 200 mg Tenax TA 20/35 for collection of volatiles. Tenax cartridges were conditioned for 60 min at 285°C under a nitrogen flow of 30 psi, using a TC-20 multitube conditioner, and were kept airtight with brass caps until use. Air was sucked through the containers at a flow rate of 100 ml/min for the indicated times through the Tenax cartridges to trap culture-produced volatiles. Incoming air was purified with a second Tenax cartridge. Before GC–MS analysis, the cartridges were dried for 15 min at room temperature with a nitrogen flow of 15 psi.

Headspaces of cultures trapped on Tenax cartridges were analysed using a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before thermodesorption, the traps were flushed with helium at 30 ml/min for 3 min to remove moisture and oxygen. After flushing, the collected volatiles were desorbed from the Tenax cartridges at 220°C (Ultra; Markes, Llantrisant) for 10 min at a helium flow of 30 ml/min. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant) at a temperature of 35°C. Volatiles were injected on the analytical column (ZB-5MSI, 30 m × 0.25 mm i.d., 1.0 µm film thickness; Zebron, Phenomenex), injecting at split ratio 62 by ballistic heating of the cold trap to 290°C for 3 min. The temperature programme started at 40°C (3.5 min hold) and rose at 10°C/min to 280°C (2.5 min hold). The column effluent was ionized by electron impact (EI) ionization at 70 eV. Mass scanning was done in the range 35–400 *m/z*, with a scan time of 0.3 s. The eluted compounds were identified using Xcalibur software (Thermo, Waltham, USA) by comparing the mass spectra with those of authentic reference standards.

For identification of the enantiomer of limonene produced, materials were eluted from Tenax cartridges by ethyl acetate, concentrated under a nitrogen flow and analysed on the 7890A gas chromatograph (Agilent; see above) equipped with an

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enantioselective column with stationary phase Heptakis (6-*O*-TBMDS-2,3-di-*O*-methyl)-*β*-cyclodextrin; 50% w/w in OV1701) of 25 m and i.d. 0.25 mm. The column was operated with a helium flow of 1.2 ml/min. For use with the enantioselective column, the oven temperature was programmed to 50°C for 12 min, followed by a temperature increase of 10°C/min to 170°C, where it was maintained for 2 min. Authentic standards of (−)-limonene and (+)-limonene were used for identification of the enantiomers.

Results

Construction of limonene-producing yeast strains

The construction of limonene-producing yeast strains was initiated by selection of two limonene synthases with different enantiomer product specificities. The PfLS from *P. frutescens* has been described as a (−)-limonene synthase (Yuba *et al*., 1996), while the ClLS from *C. limon* has been described as a (+)-limonene synthase (Lucker *et al*., 2002). Both limonene synthases were expressed in *E. coli* BL21, and the production of limonene was confirmed by *in vitro* enzyme assays, using GPP as a substrate, followed by GC–MS analysis of the products (see supporting information, Figure S2). For expression in yeast, either the full open reading frames (including plastid targeting signal) or the truncated version encoding only the mature enzyme, were introduced into plasmid pESC–HIS–CPR under control of the GAL10 promoter. The plasmids were introduced into the yeast strain AE9 K197G (Fischer *et al*., 2011) and maintained by auxotrophic selection on histidine. As controls, the strain *Sc*-pESC, containing an empty pESC–HIS, and the strain *Sc*-CPR1, containing an empty pESC–HIS–CPR, were run along. CPR1 was introduced for possible follow-up experiments involving cytochrome P450 enzymes. No difference could be observed between *Sc*-pESC and *Sc*-CPR1 in any of the experiments performed.

Collecting limonene produced by yeast cultures

Several options were tested for collecting limonene from the growing cultures, for which the different set-ups are shown in Figure 2. When the terpenes produced were collected with SPME or by pentane extraction, which both involve collecting culture samples at a fixed time point, limonene was not detected as a product of the yeast culture. Continuously collecting the produced terpenoids by a dodecane overlay or by continuous headspace trapping proved to be more successful. The results from each of the four methods are described below.

Pentane extraction

Yeast strains were grown for 3 days in flasks and were subsequently extracted with *n*-pentane (Figure 2A). The pentane layers were analysed by GC–MS. A number of monoterpene alcohols were found, including linalool and geraniol (see supporting information, Figure S3). The retention time and mass spectrum of limonene were established with a standard (see supporting information, Figure S5), but no limonene could be detected.

Solid-phase micro-extraction

During growth in a shaker flask, culture samples were taken for 3 days into glass vials (Figure 2C). Volatiles from the medium were released into the headspace by addition of a high concentration of $CaCl_2$. Volatiles were captured on an SPME fibre and analysed by GC–MS. In all cultures, several monoterpene alcohols could be observed at all time-points, including geraniol and linalool, as reported previously (Fischer *et al*., 2011) (Figure 3; see also supporting information, Figure S5). No limonene was observed. Combined, the results above suggest that the bulk of limonene escapes to the headspace before extraction.

Dodecane overlay

In order to capture volatiles during growth, the strains were grown for 3 days in flasks in the presence of an overlay of *n*-dodecane (Cankar *et al*., 2011) (Figure 2B). Dodecane layers were collected on day 3, diluted in EtAc and analysed by GC–MS. As dodecane and solvent contaminants (decane, undecane) have similar retention times to monoterpenes, analysis of the monoterpene alcohols and limonene produced was not straightforward. Still, a small quantity of limonene could be detected in the limonene synthase-expressing strains (see supporting information, Figure S4). Quantities of limonene of around 0.028 (±0.0095)

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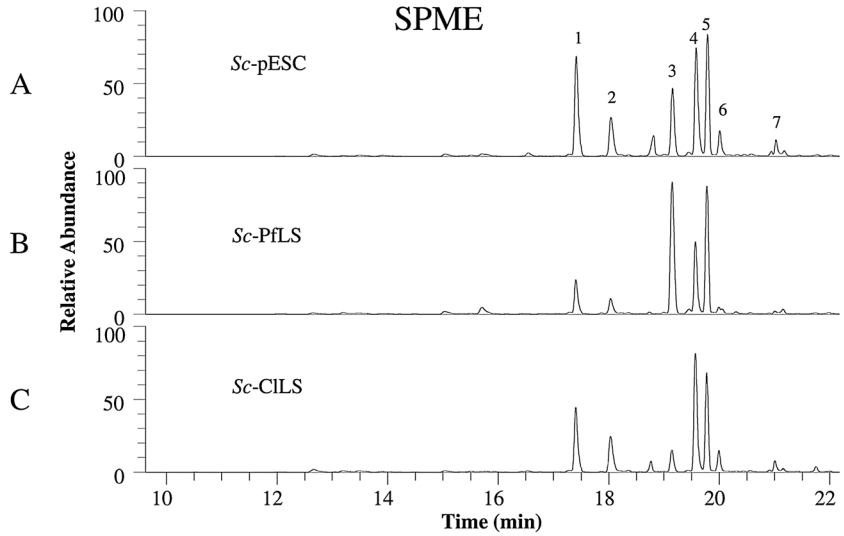

Figure 3. GC–MS chromatograms (total ion count, mass range 45–400 *m/z*) of solid-phase micro-extraction at 27 h (100% = 6e7 mass counts): (A) *Sc*-pESC; (B) *Sc*-PfLS; (C) *Sc*-ClLS. Peaks were identified as: 1, linalool; 2, phenylethyl alcohol; 3, camphor; 4, menthol; 5, alkane-like unidentified compound; 6, terpineol; 7, geraniol; camphor and menthol (peaks 3 and 4) were also present in SPME analysis of culture medium without yeast, indicating that they do not originate from yeast strain activity

mg/l (+)-limonene and 0.060 (±0.029) mg/l (−)-limonene were detected in cultures expressing the truncated *Citrus* and *Perilla* synthases, respectively. No monoterpene alcohols could be observed in the chromatograms, as these compounds overlap with the excess of dodecane (see supporting information, Figure S4). For cultures expressing the non-truncated limonene synthases, trace amounts of limonene were detected in the dodecane overlay.

Headspace trapping

A set-up was built which allowed trapping of exhaust volatiles during growth (Figure 2D). Cultures were grown in a 500 ml closed cuvette under constant stirring and a continuous airflow through the cuvette headspace. Volatiles were sequestered from the outgoing air through a sampling cartridge containing a volatile absorbent and analysed by GC–MS (Figure 4). Sampled air of cultures expressing limonene synthases showed limonene (Rt = 10.3 min) as one of the predominant compounds, in addition to the monoterpene alcohols, which occurred in all cultures (Figure 4). Mass spectra and retention times of produced limonene were matching to the standard (Figure 4; see also supporting information, Figure S5).

Strong differences in productivity were observed between cultures expressing native and truncated limonene synthases. For the strains expressing the full-length *LS* gene, the *Sc*-PfLS strain produced up to 8 µg limonene/l culture/h, compared to 0.4 µg limonene/l culture/h by *Sc*-ClLS. However, the strains with truncated versions of the limonene synthases (*Sc*-PftLS and *Sc*-CLtLS) displayed higher productivity. The *Sc*-PftLS strain showed a maximal productivity of 24 µg limonene/l culture/h; for the *Sc*-CltLS strain, this was 3.6 µg limonene/l culture/h. Limonene formation was highest during day 1 (*Sc*-PftLS) or day 2 (*Sc*-ClLS, *Sc*-CLtLS, *Sc*-PfLS), but limonene levels reached a plateau value at day 3 (Figure 5A). Cumulatively, the amounts of limonene collected from the headspace over 3 days were 0.33 and 0.014 mg/l for the *Sc*-PfLS and *Sc*-ClLS strains, respectively. For truncated strains production levels were higher, reaching 0.49 (*Sc*-PftLS) and 0.12 mg/l (*Sc*-CltLS), respectively.

Limonene formation was compared to formation of the endogenous terpene alcohols at 45 h (Figure 5B). Linalool and geraniol production and formation of the sesquiterpene alcohol nerolidol were not significantly different in the *Sc*-PfLS or *Sc*-ClLS strains relative to the control strain.

The enantioforms produced by the *Sc*-ClLS and *Sc*-PfLS cultures were analysed using GC–MS

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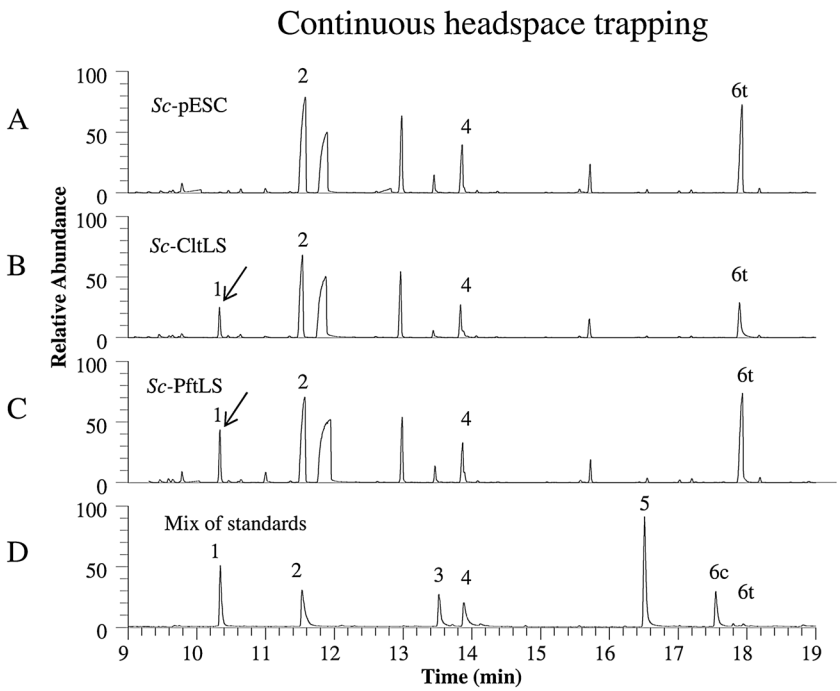

Figure 4. GC–MS chromatograms (total ion count, mass range 35–400 *m/z*) of continuous headspace trapping 21–45 h yeast cultures and standard mix: (A) *Sc*-pESC (100% = 2.4e9 mass counts); (B) *Sc*-ClLS (100% = 2.4e9 mass counts); (C) *Sc*-PftLS (100% = 2.4e9 mass counts); (D) mixture of standards (100% = 8.0e7 mass counts). Indicated peaks represent: 1, limonene; 2, linalool; 3, nerol; 4, geraniol; 5, geranyl acetone; 6c, *cis*-nerolidol; 6 t, *trans*-nerolidol; arrows, limonene peak in limonene-producing strains *Sc*-ClLS and *Sc*-PftLS

with an enantioselective column, and confirmed production of (+)-limonene by *Sc*-ClLS and (−)-limonene by *Sc*-PfLS (Figure 6).

Discussion

Capture method may affect limonene yield

In this study we compared ways to capture a highly volatile terpene from limonene-producing yeast cultures. To this end, we generated a set of yeast strains producing both enantiomers of the monoterpene olefin limonene. Our studies indicated that the highest yield for capturing of limonene was obtained when the produced monoterpene was continuously captured from the headspace during growth, using an absorbent material. No limonene could be captured from the yeast cultures by solvent extraction (see supporting information, Figure S3) or SPME (Figure 3), whereas monoterpene alcohols such as linalool were well detected by these methods (Figure 3). Differences in boiling point between the olefin limonene (176°C) and the alcohol linalool (198°C) do exist, but can hardly explain the strong differences in recovery from the culture media of the two compounds. More likely, the behaviour of the two compounds can be explained by big differences in partitioning between water and air, which are reflected in the Henry's law constant (K_H). For example, at 25°C the K_H of linalool is 21 M/atm (Leng *et al.*, 2013), while for limonene the K_H is 500-fold lower (0.048 M/atm), indicating that indeed linalool will mostly stay in the medium, while limonene would mostly evaporate and be lost to the headspace of an aerated shaker flask.

Production of sesquiterpenes from culture broth often deploys a biphasic fermentation, using a dodecane overlay during culture (Scalcinati *et al.*, 2012). Such a system could also potentially sequester the produced limonene during the production (Anthony *et al.*, 2009). In our hands, headspace trapping showed a four- to eight-fold higher titre of limonene [0.12 mg/l (+)-limonene and 0.49 mg/l (−)-limonene] compared to the

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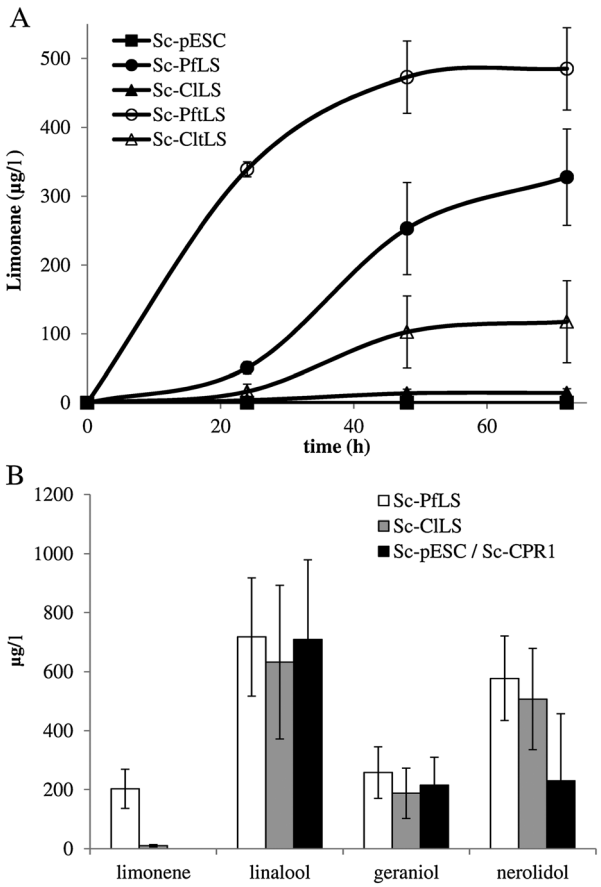

Figure 5. Monoterpene production by yeast strains, captured by continuous headspace trapping: (A) cumulative limonene production by yeast strains during 3 days; (B) production of limonene, linalool, geraniol and nerolidol by yeast strains on day 2 of fermentation. Indicated are the average values of three independent cultures; error bars indicate SD

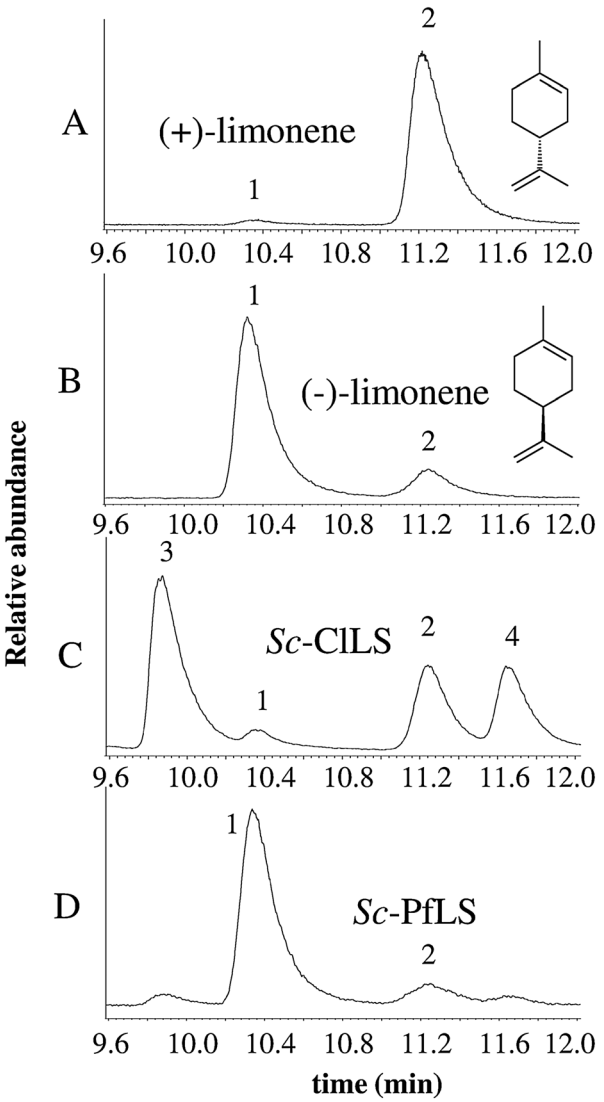

Figure 6. Analysis of chirality of limonene produced by different yeast strains using GC–MS on an enantioselective column: (A) (+)-limonene standard; (B) (−)-limonene standard; (C) products of *Sc*-ClLS; (D) products of *Sc*-PfLS. Indicated peaks represent: 1, (−)-limonene; 2, (+)-limonene; 3, hexanoic acid; and 4, ocimene; identities of peaks 3 and 4 were suggested by the NIST library

biphasic system [0.028 mg/l (+)-limonene and 0.060 mg/l (−)-limonene]. Possibly this is the result of the continuous aeration of the cultures for headspace trapping, which may lead to better growth and/or better monoterpene production. Alternatively, the presence of dodecane could inhibit limonene production. Dodecane itself is not known to have strong negative effects on the cell viability of yeast. However, contaminants such as decane are present in dodecane (see supporting information, Figure S4) and may have (mild) toxic properties (Williams *et al*., 1998). A clear advantage of headspace trapping over biphasic fermentation is that limonene is not mixed with compounds of similar polarity and boiling point, thus facilitating downstream processing. Still, for some applications there is no need for separation, e.g. if the overlay solvent is chosen in such a way that it can be used directly as a biofuel (Brennan *et al*., 2012). Trapping of headspace products from yeast cultures has not yet been applied on an industrial scale, and the laboratory set-up as explored here (Figure 2D) may not be suitable for up-scaling. However, devices based on cold-trap condensers

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could be suitable for trapping of monoterpene olefins on an industrial scale (Booth, 2013).

Previous studies of monoterpene production in yeast have deployed different recovery systems. For instance, production of the monoterpene alcohol linalool has been analysed by SPME (Rico *et al*., 2010), while production of geraniol has been analysed after extraction from the culture medium by solid-phase extraction, using C18 material (Fischer *et al*., 2011). Production of cineole in yeast has been monitored by SPME and liquid–liquid extractions using ethylacetate and hexane (Ignea *et al*., 2011). Very recently, yeast strains producing limonene or sabinene into a dodecane overlay have been reported (Behrendorff *et al*., 2013; Ignea *et al*., 2014). In this study, we compared four recovery systems using the same limonene-producing strains. Our results indicate that these studies may underestimate the productivity of the constructed strains.

Solvolysis, rather than pyrophosphatase activity, is responsible for the formation of monoterpene alcohols in the *ERG20* mutant

The yeast strain applied in this work carries a mutant FPP synthase and is capable of sustaining growth (indicating that FPP is still synthesized for ergosterol synthesis), but also produces geranyl diphosphate that is available for the synthesis of monoterpenes (Fischer *et al*., 2011). In strains carrying no monoterpene synthase, this leads to the formation of a number of monoterpene alcohols, such as linalool and geraniol (Chambon *et al*., 1990; Fischer *et al*., 2011) (Figures 3 and 4). These monoterpene alcohols could arise by the activity of pyrophosphatases, which may convert GPP into monoterpene alcohols, or could be the result of solvolysis of GPP in the mutant farnesyl diphosphate synthase (Chambon *et al*., 1990). When a pyrophosphatase would be a dominant factor in the formation of geraniol, one could speculate that the formation of limonene, as observed when expressing limonene synthases, would lead to competition for the GPP pool and, as a result, higher formation of limonene would lead to reduced formation of pyrophosphatase products. However, in none of the experiments reported in this study could a reduction of linalool or geraniol be observed in the high limonene-producing strains. Therefore, the present study supports an important role for solvolysis in the formation of monoterpene alcohols in the strain AE9 K197G. In a recent study, yeast expressing another *ERG20* mutant also showed formation of monoterpene and sesquiterpene alcohols, indicating that mutations in FPP synthase will promote solvolysis (Ignea *et al*., 2014).

Options for boosting volatile terpene production in yeast

Heterologous eukaryotic systems for the production of limonene derivatives have so far hardly been explored. In contrast to bacteria, both yeasts and plants are capable of functional expression of plant cytochrome P450s, which may be useful for the production of oxidized forms of limonene (e.g. Lucker *et al*., 2004). Baker's yeast is a well-known platform for the expression of plant cytochrome P450s and is used in the production of many food ingredients.

The titre of limonene strongly depends on the availability of GPP. The GPP concentration depends on the presence of GPP synthase activity, but also on the availability of the GPP building blocks IPP and DMAPP. As explained above, we and others have explored the potential of mutant Erg20 enzymes for supplying GPP (Ignea *et al*., 2014). Next to using a mutant Erg20, wild-type Erg20 can also be deployed to generate GPP. Recently, it was shown that medium composition and pH may trigger GPP formation in yeast, which was demonstrated by the formation of limonene upon ClLS expression (Behrendorff *et al*., 2013), particularly in rich medium. A third option to supply GPP would be to express a true GPP synthase in yeast. For example, the GPP synthase gene from *Picea abies* was co-expressed with the cineole synthase from *Salvia fruticosa*, which increased cineole production (Ignea *et al*., 2011). Possibly, co-expression of this GPP synthase with monoterpene olefin synthases, such as limonene synthase, may lead to an improved ratio between the olefin and the monoterpene alcohols, but this remains to be established.

Several strategies have been followed to increase the supply of precursors IPP and DMAPP. Behrendorff *et al*. (2013) showed that limonene can be produced to a titre of 1.5 mg/l limonene, using a yeast strain that expresses a truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR)

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and codon-optimized citrus (+)-limonene synthase. HMGR is the key regulatory point of the yeast mevalonate pathway, and tHMGR is a deregulated form of that enzyme. Overexpression of the isopentenyl diphosphate isomerase (IDI) that isomerizes IPP and DMAPP units in the precursor pathway has been used to increase sabinene titres in yeast three-fold (Ignea *et al*., 2014). Our AE9 K197G strain provides already a reasonable production of GPP by the mutated FPPS enzyme, but most likely a true GPP synthase in yeast, combined with a boosting of IPP production, may increase limonene production levels significantly. In *E. coli*, it has been shown that limonene production can be dramatically improved by overexpression of a heterologous mevalonate pathway (Alonso-Gutierrez *et al*., 2013; Willrodt *et al*., 2014), and such an approach may also be applicable to improve the yield of limonene in yeast (Paddon *et al*., 2013).

Yeast production of monoterpene olefins such as limonene from simple carbohydrates would be a significant contribution to the biotechnological production of flavour molecules, but also for the production of fuels and biopolymers as part of the transition to a biobased economy (Brennan *et al*., 2012). Capturing the products from a microbial system has advantages over the plant-based system, as limonene production could be more stable and food-grade. At the moment, limonene yields from yeast are not yet on a level to replace plant production (Braddock, 2013) and further strain optimizations are necessary to increase production (Brennan *et al*., 2012; Leng *et al*., 2013), while efficient capture of the volatile product is essential for optimal harvest of the volatile product.

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Author contributions

E.J. conceived and executed experiments and wrote the manuscript; K.C. and J.R. executed experiments and contributed to writing the manuscript; S.v.d.K. and H.B. contributed to writing the manuscript; and J.B. conceived experiments and wrote the manuscript.

Abbreviations

DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GC–MS, gas chromatography–mass spectrometry; GPP, geranyl diphosphate; IDI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; MEP, methylerythritol phosphate; rpm, rotations per minute; SPME, solid phase micro-extraction.

Conflict of Interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. Protein sequence alignment of the cloned *P. frutescens* limonene synthase KM015220 and GenBank Accession No. AF317695

Figure S2. GC–MS analysis (total ion count, mass range 45–450 *m/z*) of *in vitro* assays of limonene synthases

Figure S3. GC–MS chromatogram of pentane extract of yeast culture from strain *Sc*-PfLS

Figure S4. GC–MS analysis of dodecane culture overlays after dilution in EtAc

Figure S5. Mass spectra of GC–MS peaks from headspace trapping

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