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ORIGINAL PAPER

Efficient hydroxylation of 1,8-cineole with monoterpenoid-resistant recombinant *Pseudomonas putida* GS1

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Abstract In this work, monoterpenoid hydroxylation with *Pseudomonas putida* GS1 and KT2440 were investigated as host strains, and the cytochrome P450 monooxygenase CYP176A1 ($P450_{cin}$) and its native redox partner cindoxin (CinC) from *Citrobacter braakii* were introduced in *P. putida* to catalyze the stereoselective hydroxylation of 1,8-cineole to (1*R*)-6β-hydroxy-1,8-cineole. Growth experiments in the presence of 1,8-cineole confirmed pseudomonads' superior resilience compared to *E. coli*. Whole-cell *P. putida* harboring $P450_{cin}$ with and without CinC were capable of hydroxylating 1,8-cineole, whereas coexpression of CinC has been shown to accelerate this bioconversion. Under the same conditions, *P. putida* GS1 produced more than twice the amount of heterologous $P450_{cin}$ and bioconversion product than *P. putida* KT2440. A concentration of 1.1 ± 0.1 g/L (1*R*)-6β-hydroxy-1,8-cineole was obtained within 55 h in shake flasks and 13.3 ± 1.9 g/L in 89 h in a bioreactor, the latter of which corresponds to a yield $Y_{P/S}$ of 79 %. To the authors' knowledge, this is the highest product titer for a P450 based whole-cell monoterpene oxyfunctionalization reported so far. These results show that solvent-tolerant *P. putida* GS1 can be used as a highly efficient recombinant whole-cell biocatalyst for a P450 monooxygenase-based valorization of monoterpenoids.

Keywords Biotransformation · 1,8-Cineole · Hydroxylation · Monoterpene · $P450_{cin}$ · *Pseudomonas putida* · Whole-cell biocatalysis

Introduction

Terpenes, a structurally and functionally diverse group of natural compounds comprising various number of isoprene (C_5) units, can be found in all kingdoms of life (Davis and Croteau 2000). Monoterpenoids consisting of two isoprene units are often hydrophobic and volatile. They constitute the major fraction of most essential oils (>90 %), imparting their characteristic scent and are therefore extensively used as flavoring agents and perfumes (Mahmoud and Croteau 2002; Bakkali et al. 2008). Moreover, they often show antibacterial, antifungal and anticarcinogenic activities which is why their application is considered in different fields, e.g. as herbicides and pesticides (Mahmoud and Croteau 2002), cosmetic (Choi 2012) or pharmaceutical ingredients (Patel et al. 2007), among others. Whereas some monoterpenoids such as α-pinene, *R*-limonene or 1,8-cineole are major components of different essential oils and therefore easily accessible in nature, their further oxyfunctionalized derivatives often possess desired specific bioactivities but usually occur in low amounts or in traces rendering their extractive isolation uneconomically (Schrader 2007). One such an example of industrial interest is (1*R*)-6β-hydroxy-1,8-cineole [(1R,4S,6S)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane-6-ol]: whereas its natural precursor 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) is abundantly available as the major component of eucalyptus oil, the product occurs only in traces in nature, e.g. as a metabolite of mammals and insects from dietary 1,8-cineole (Miyazawa et al. 1989; Southwell et al. 1995; Southwell et al. 2003) or as the

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alcoholic part of aroma esters found in the galingale rhizome (Someya et al. 2001). Hydroxylated 1,8-cineole can be used as chiral synthon in organic chemistry, as precursor for antimicrobials (Miyazawa and Hashimoto 2002) and in aroma compositions (Rodriguez et al. 2006).

As the regio- and stereoselective oxyfunctionalization of precursor hydrocarbons such as monoterpenoids by chemical means is usually difficult and costly, microbial conversion has been investigated over decades as a potential alternative. However, one major challenge using microbes for bioconversion or de novo production of monoterpenoids is their toxicity (Schewe et al. 2011; Alonso-Gutierrez et al. 2013). Different genetic approaches to enhance tolerance to toxic organic solvents in conventional microbial systems like *E. coli* and *S. cerevisiae* were attempted so far (Dunlop et al. 2011; Doukyu et al. 2012; Oh et al. 2012; Zingaro and Papoutsakis 2012; Chen et al. 2013; Dragosits and Mattanovich 2013; Watanabe and Doukyu 2014). As a technical solution to avoid toxic effects of terpenoids on producing microbes, different in situ product removal (ISPR) approaches were established (Schewe et al. 2015), causing an additional expense factor. Conversely, using a naturally solvent-tolerant microbe and adjusting its metabolic capacity to produce a compound of interest is another approach to establish a resilient microbial platform.

Such a promising candidate is *Pseudomonas putida* (Molina et al. 2013), a fast-growing, metabolically versatile Gram-negative bacterium well-known to resist various toxic compounds, such as heavy metals, hydrocarbons, antibiotics and xenobiotics (Saxena et al. 2002; Fernández et al. 2009, 2012; Udaondo et al. 2012), depending on the particular strain. The laboratory strain *P. putida* KT2440, the fully-sequenced (Nelson et al. 2002) and best-described *P. putida* strain so far, has already been used as a microbial host for in vivo bioconversion of monoterpenoids with heterologously expressed cytochrome P450 monooxygenases (van Beilen et al. 2005; Cornelissen et al. 2011, 2013), though this strain is not particularly known to have a pronounced solvent tolerance. As an example for a solvent-tolerant *P. putida*, *P. putida* GS1 (alias DSM 12264) isolated from wastewater sludge by enrichment culture with limonene as sole carbon source (Speelmans et al. 1998) will be focused on in this work. This strain has already been applied as a wild type for the oxidation of the monoterpene hydrocarbon limonene to perillic acid (Speelmans et al. 1998; Mars et al. 2001; Mirata et al. 2009). In the bioreactor, *P. putida* GS1 withstands limonene in two-digit gram per liter scale illustrating its pronounced resilience to the monoterpene hydrocarbon (Mirata et al. 2009). Recently, fast oxidation of the monoterpene alcohol geraniol to geranic acid by wild type *P. putida* GS1 was reported (Mi et al. 2014). Furthermore, de novo production of a monoterpenoid in *P. putida* was demonstrated for the first time by genetically engineering *P. putida* GS1 to produce geranic acid (Mi et al. 2014).

The present work, finally, demonstrates that *P. putida* GS1 is also a well-suited host for heterologous expression of a P450 monooxygenase and its electron-shuttling partner protein. Furthermore, the exploitation of the recombinant *P. putida* GS1 as a robust whole-cell biocatalyst in a bioreactor for stereoselective monoterpenoid hydroxylation was explored. For this purpose, cytochrome P450 monooxygenase CYP176A1 ($P450_{cin}$) and cindoxin (CinC), its native redox partner protein from *Citrobacter braakii* (Hawkes et al. 2002) was used as the model system. $P450_{cin}$ catalyzes the regio- and stereo-selective hydroxylation of 1,8-cineole (also known as eucalyptol) to (1*R*)-6β-hydroxy-1,8-cineole (Hawkes et al. 2002; Azerad 2014) (Scheme 1). Throughout the publication, the simplified denominations for substrate and product, as proposed by Azerad (2014), will be used.

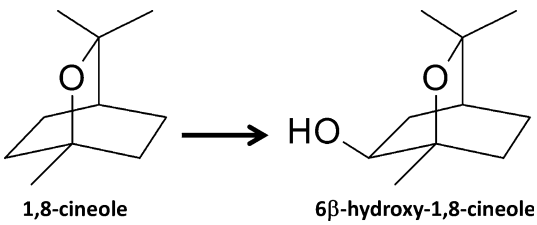
Materials and methods

Chemicals and media

All commercially available chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Carl-Roth (Karlsruhe, Germany).

For cultivation of *E. coli* and *P. putida*, respectively, lysogeny broth (LB) or terrific broth (TB) medium was used as complex medium. For cultivation of *P. putida* in minimal medium, E2 medium (Mirata et al. 2009) was used. For solid media, 15 g/L agar–agar was added. Kanamycin was added from a sterile stock solution of 30 mg/mL to a final concentration of 30 µg/mL, if required.

For in vitro synthesis of a sufficient amount of (1*R*)-6β-hydroxy-1,8-cineole for application as an analytical standard, 5 mL LB containing kanamycin were used as precultures of recombinant *E. coli* BL21(DE3) strains expressing $P450_{cin}$, CinC (Zengin Çekiç et al. 2010) and *E. coli*-derived ferredoxin reductase Fpr, respectively. Cell



Scheme 1 $P450_{cin}$-driven regio- and stereoselective hydroxylation of 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) to (1*R*)-6β-hydroxy-1,8-cineole [(1R,4S,6S)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ol; adapted from (Hawkes et al. 2002)]

cultures were cultivated overnight at 37 °C and 180 rpm. A volume of 300 mL TB containing kanamycin in 2 L-Erlenmeyer flasks without baffles were inoculated with 3 mL preculture and incubated at 37 °C and 180 rpm. Gene expression was started 3.5 h after inoculation by addition of 300 µL 1 M IPTG and temperature was lowered to 25 °C. Cells were harvested 20 h after induction of gene expression in 50 mL volumes by centrifugation at 4000×*g*, 4 °C for 30 min. Pellets were each washed in 10 mL phosphate buffer (pH 7, 50 mM) and stored at −20 °C until use. Cell disruption was performed using an ultrasonic cell disruptor (W-250D, Branson, 5 mm diameter tip, 30 % amplitude, 1 s pulse with 2 s pause for 3 min in total) on ice. Lysed cells were centrifuged at 4000×*g*, 4 °C and 30 min and supernatant was used for (1*R*)-6β-hydroxy-1,8-cineole synthesis. Synthesis was performed in 300 mL-Erlenmeyer flasks with screw cap at 30 °C, 150 rpm overnight in a total reaction volume of 50 mL comprising 4.8 mg glucose dehydrogenase (c-LEcta Leipzig, Germany), 400 µL catalase (no. 60460, Sigma-Aldrich, Taufkirchen, Germany), 1 mL supernatant of lysed cells expressing $P450_{cin}$, 2 mL supernatant of lysed cells expressing CinC, 3 mL supernatant of lysed cells expressing episomal Fpr, 80 µL 1,8-cineole, 80 µL 100 mM $NADP^+$, 8 mL 500 mM glucose, 3.36 mL 1 M H_2KPO_4, 4.64 mL 1 M HK_2PO_4, 27.5 mL ddH_2O. Each 50 mL reaction solution was mixed with 50 mL diethylether and organic phase was dried to give (1*R*)-6β-hydroxy-1,8-cineole for application as an analytical standard.

Strains, genotypes, plasmids and oligonucleotides

All strains, genotypes, plasmids and oligonucleotides used in this study are listed in Table 1.

Plasmid construction and transformation

pJeM1 (Jeske and Altenbuchner 2010) was digested with *Nde*I and *Hin*dIII to cut out *egfp*. Plasmid pJeM1-CinA was constructed by cloning of codon-optimized (*E. coli*) *cinA* (Zengin Çekiç et al. 2010) using primer 1 and 2 (see Table 1) at *Nde*I and *Hin*dIII sites of *egfp*-less pJeM1 backbone. Plasmid pJeM1-CinAC was then constructed by cloning of codon-optimized (*E. coli*) *cinC* (Zengin Çekiç et al. 2010) using primer 3 and 4 at *Hin*dIII and *Bsu*36I sites of pJeM1-CinA. Resulting constructs were verified by sequencing. Plasmids were then transformed via electroporation into *E. coli* (Dower et al. 1988) and *P. putida* (Choi et al. 2006).

Growth and metabolization assays

Growth assays were performed to investigate the influence of 1,8-cineole on cell growth of the tested microorganisms. A volume of 6 mL LB was inoculated with *E. coli* JM109, *P. putida* KT2440 or GS1 and cultivated overnight at 180 rpm and 37 °C for *E. coli* and 30 °C for both pseudomonads. Cultivations were performed in 100 mL-Erlenmeyer flasks without baffles using 20 mL LB inoculated with precultures to an optical density (OD_{600}) of 0.1. The corresponding amount of 1,8-cineole, depending on the experiment carried out, was added directly after inoculation. All 1,8-cineole concentrations given here and throughout are referred to medium volume irrespective of its solubility to maintain consistent indication [water solubility of 1,8-cineole at 30 °C is 2.7 g/L corresponding to 17.5 mM (Yalkowsky et al. 2010)]. *E. coli* JM109 was cultivated at 37 °C and 180 rpm, *P. putida* GS1 and KT2440 were cultivated at 30 °C, 180 rpm. Cell growth was monitored by OD_{600} measurements (WPA CO8000, Biochrom) every hour. Maximum specific growth rate of the strains in the presence of various concentrations of 1,8-cineole (μ_{max}) was compared to the maximum specific growth rate of control cultures without monoterpene (μ_{max0}) by comparing slopes of linear logarithmic curve $\ln(OD_{600})$ during exponential growth phase.

To test 1,8-cineole as the sole carbon source for *P. putida* GS1 and KT2440, 100 µL of 5 mL LB overnight cultures (30 °C, 200 rpm) were plated out on E2 agar without glycerol. *p*-Cymene was used as the positive control. The tested hydrocarbon was sufficiently provided as vapor phase (Scheme 2). Agar plates were incubated at 30 °C for 9 days and formation of colonies was observed.

To investigate bioconversion of 1,8-cineole by wild type *P. putida* GS1 and KT2440, 20 mL LB precultures were cultivated in 100 mL-Erlenmeyer flasks with baffles overnight at 30 °C and 200 rpm. A volume of 10 mL LB was inoculated in 100 mL-Erlenmeyer flask without baffles with precultures to an OD_{600} of 0.2 and cultivated at 30 °C and 200 rpm for 24 h. 1,8-Cineole was added to the cultures directly after inoculation to a final concentration of 60 mM. For qualitative gas chromatography-mass spectrometry (GC–MS) analysis, cell suspension was extracted with 3 mL ethyl acetate. Cells, aqueous and organic phase were separated by centrifugation and the organic phase was dried over Na_2SO_4 and organic phase was analyzed via GC–MS.

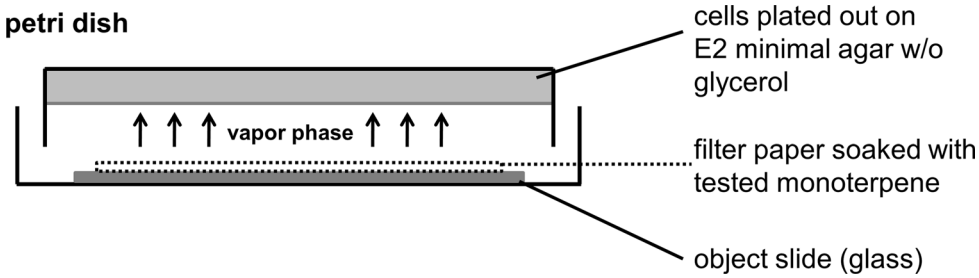
1,8-Cineole biotransformation

For simultaneous 1,8-cineole biotransformation and $P450_{cin}$ measurements, first precultures of 6 mL LB containing kanamycin were cultivated in tubes at 30 °C and 180 rpm overnight. A volume of 10 mL LB containing kanamycin (second precultures) were inoculated with first precultures to an OD_{600} of 0.5 in 100 mL-Erlenmeyer flasks without baffles. Recombinant cells were induced with sterile filtered

Table 1 Bacterial strains and genotypes, plasmids and oligonucleotides used in this study

Strains, plasmids and oligonucleotides	Description/genotype/sequence	Source
Plasmid		
pJeM1	expression plasmid derived from pBBR1MCS-2; elements of the rhamnose expression system: promoter *rhaP*$_{BAD}$ and activator genes *rhaR, rhaS*; *mob*, *kan*^{R}, *eGFP*	Jeske and Altenbuchner (2010)
pJeM1-CinA	*egfp*-less pJeM1 backbone with *cinA* (codon-optimized for *E. coli*)	this work
pJeM1-CinAC	*egfp*-less pJeM1 backbone with *cinA cinC* (both codon-optimized for *E. coli*)	this work
Strain		
E. coli NEB 5-alpha	DH5α derivative, *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB GmbH, Frankfurt am Main, Germany
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F́ *tra*Δ36, *pro*AB, *laq*I^{q}ZΔM15]	Promega, Mannheim, Germany, (Yanisch-Perron et al. 1985)
P. putida KT2440	*P. putida* mt-2 derivative cured of the TOL plasmid	DSMZ, Braunschweig, Germany, (Bagdasarian et al. 1981)
P. putida GS1	alias DSM 12264, wild type *P. putida* isolated from sludge of wastewater treatment system in the Netherlands	DSMZ, Braunschweig, Germany, (Speelmans et al. 1998)
Oligonucleotides		
Primer 1	5′-TAGCTACATATGGGCACCGCGACCGTG-3′	This work
Primer 2	5′-GGCCGGAAGCTTGTTTAAACTTATTATTCGCTCAGACG-3′	This work
Primer 3	5′-GCCGAAGCTTAAGAAGGAGA ATGAATGCCCTGATTTTATATGGCAC-3′	This work
Primer 4	5′-TATACCTCAGGTCAGAGCTCTCAACTAACTGCCGGAGTTGC-3′	This work

Recognition sites of restriction endonucleases are underlined



Scheme 2 Schematic of the experimental setup to investigate 1,8-cineole and *p*-cymene as the sole carbon source for *P. putida* wild type strains KT2440 and GS1

aqueous 100× *L*-rhamnose to a final concentration of 0.2 % (w/v) 4 h after inoculation and cultivated overnight at 30 °C and 180 rpm. A volume of 120 mL LB containing kanamycin was inoculated with second precultures to an OD_{600} of 0.07 in 300 mL-Erlenmeyer flasks without baffles and cultivated at 30 °C and 180 rpm. Gene expression was induced directly after inoculation using *L*-rhamnose as described above and the substrate 1,8-cineole was added to a final concentration of 10 mM.

For 1,8-cineole biotransformation in different media, first precultures were cultivated as described above. A volume of 10 mL LB containing kanamycin (second precultures) were inoculated with first precultures to an OD_{600} of 0.1 in 100 mL-Erlenmeyer flasks without baffles and cultivated overnight at 30 °C and 180 rpm. A volume of 20 mL of LB, TB and E2, respectively, containing kanamycin was inoculated with second preculture to an OD_{600} of 0.1 in 100 mL-Erlenmeyer flasks without baffles and cultivated at 30 °C and 180 rpm. Gene expression was induced directly after inoculation using *L*-rhamnose as described above. A final concentration of 4 mM 1,8-cineole was added in 12 h-intervals for 36 h.

Samples were taken over time for determination of cell growth, (1*R*)-6β-hydroxy-1,8-cineole (OH-cineole) concentration and $P450_{cin}$ content, if required. For quantitative GC–MS analysis, 500 µL cell culture were extracted with 500 µL diethyl ether containing 0.8 mM thymol as the internal standard. Cells, aqueous and organic phase were separated by centrifugation and the organic phase was dried over Na_2SO_4 and organic phase was analyzed via GC–MS.

For determination of $P450_{cin}$ concentrations, 10 mL cell culture were centrifuged at 4000×*g* for 10 min and washed in 2.5 mL 20 mM Tris–HCl (pH 7.4). Cells were chilled on ice and disrupted using ultrasonic cell disruptor (W-250D, Branson, 5 mm diameter tip, 20 % amplitude, 0.5 s pulse and 0.8 s pause for 10 min in total). A volume of 2 mL disrupted cell suspension was centrifuged at 14,000×*g* for 10 min. Supernatant was filtered through 0.22 µm-PVDF syringe filter and filtrate was used for photometric measurements.

Analytical methods

Qualitative and quantitative analyses by GC–MS were conducted using Shimadzu GC-17A Ver. 3 GC, QP5050 MS (ValcoBond VB-5 column, 30 m × 0.25 mm × 0.25 µm) and helium as the carrier gas. Qualitative analyses were performed using temperature program as follows: starting temperature at 50 °C, 3 °C/min to 161 °C. Carrier gas: 26 kPa column inlet pressure, 0.7 mL/min column flow, 30 cm/s linear velocity, 51:1 split ratio, 36.3 mL/min total flow. Quantitative analyses were performed using temperature program as follows: starting at 80 °C, 25 °C/min to 140 °C with 2.1 min holding, then −10 °C/min to 100 °C and 50 °C/min to 325 °C. Carrier gas: 64 kPa column inlet pressure, 1 mL/min column flow, 36.7 cm/s linear velocity, 11:1 split ratio and 14 mL/min total flow. Using this program, retention time for (1*R*)-6β-hydroxy-1,8-cineole was 4.9 min. Mass spectrum data (listed in the form of major fragment ions and relative intensities in parentheses): (1*R*)-6β-hydroxy-1,8-cineole 170 (M^+), 126 (60), 111 (34), 108 (76), 93 (32), 83 (23), 71 (61), 69 (39), 55 (24), 43 (100), 41 (34); 6-oxo-1,8-cineole (ketocineole) 168 (M^+), 140 (26), 111 (10), 83 (15), 82 (100), 69 (21), 67 (24), 55 (18), 43 (57), 41 (29), 39 (13). Discrimination of the diastereomers (1*R*)-6α- and (1*R*)-6β-hydroxy-1,8-cineole using ValcoBond VB-5 was previously demonstrated (Zengin Çekiç et al. 2010).

$P450_{cin}$ content was calculated by using CO difference spectrum (Omura and Sato 1964).

Fed-batch fermentation

In vivo hydroxylation of 1,8-cineole on bioreactor scale was performed using a DASGIP Bioblock system (DASGIP, Jülich, Germany). Applied bioreactors were each equipped with one two-bladed pitched-blade impeller and a gas sparger with gas outlets made from sintered metall. DO and pH were monitored online by Mettler Toledo electrodes (InPro 6820 and 405-DPNAS, respectively). DO was controlled by automatic incremental adaption of stirrer speed from 300 to 1200 rpm and maintained at 30 % once the cultures entered the exponential growth phase and showed elevated O_2 uptake. The aeration rate was kept constant at 20 L/h of pressurized air, the equivalent of 0.1 vvm (gas volume flow per unit of liquid volume per minute). The pH was controlled by addition of 12.5 % NH_4OH solution via an automatically controlled pump and maintained at 7. The preculture in 20 mL LB containing 30 µg/mL kanamycin was cultivated overnight in 100 mL-Erlenmeyer shake flask and used as seed train to inoculate main cultures in 300 mL TB containing 30 µg/mL kanamycin to an initial OD_{600} of 0.1 (t = 0 h). Main cultures were cultivated for 24 h at 30 °C and a stirrer speed of 300 rpm. A sterile feed solution composed of 612 g/L (w/v) glycerol and 10 g/L $MgSO_4$ was added at a constant flow rate of 2 mL/h starting 9 h after inoculation. The feed was stopped 95.5 h after inoculation. Twenty hours after inoculation and 4 h before induction of gene expression, 1,8-cineole was added to the cultures to a final aqueous concentration of 4 mM to pretreat cells with the monoterpenoid substrate. Recombinant gene expression was induced 24 h after inoculation by addition of 100× sterile filtered aqueous *L*-rhamnose to a final concentration of 0.2 % (w/v). Two hours after induction of recombinant gene expression and during biotransformation, aeration rate was kept between 10 and 20 L/h. A 1,8-cineole feed with a constant rate of 0.1 mL/h was started 6 h after gene expression (t = 0 h on respective product formation graphs). Samples were taken over time for OD_{600} and (1*R*)-6β-hydroxy-1,8-cineole concentration determination.

Results

Utilization and bioconversion of 1,8-cineole by *P. putida* wild type strains

To investigate *P. putida* as an alternative recombinant microbial host for $P450_{cin}$-driven hydroxylation of 1,8-cineole, growth and metabolization assays were carried out using *P. putida* GS1 and KT2440 wild type strains to validate their expected resilience to 1,8-cineole and to preclude utilization of the monoterpenoid as carbon source.

For experiments concerning utilization of 1,8-cineole as the sole carbon source, cells were plated out on E2 minimal agar lacking glycerol, and 1,8-cineole was applied as vapor phase to prevent agar dissolving effects by the monoterpenoid. *p*-Cymene was used as the positive control, since *P. putida* GS1 is known to grow on this volatile, aromatic compound as the sole carbon source (Speelmans et al. 1998). No growth was observed after 9 days for both pseudomonads in the presence of 1,8-cineole, whereas a thick cell layer was visible for *P. putida* GS1 grown with *p*-cymene (data not shown).

Both *P. putida* strains were then grown in the presence of 1,8-cineole in complex medium to investigate background bioconversion that may hamper the application of the microorganisms for selective whole-cell 1,8-cineole hydroxylation with heterologous $P450_{cin}$. The cells grew to stationary phase and no conversion products were detected by qualitative GC–MS analyses (data not shown).

Comparison of cell growth between *E. coli* and *P. putida* in the presence of 1,8-cineole

Cell growth of *E. coli* JM109, *P. putida* GS1 and *P. putida* KT2440 was monitored in the presence of various 1,8-cineole concentrations to compare the tolerance of *E. coli* and *P. putida*. Determined μ_{max}/μ_{max0} values are shown in Fig. 1. All strains showed minor growth impairment at 5 mM 1,8-cineole. At 10 mM 1,8-cineole, cell growth of *E. coli* JM109 was distinctly lowered, while cell growth of *P. putida* GS1 and KT2440 did not change significantly with both strains, reaching an OD_{600} of ~2.7 after 4 h and ceased at 20 mM and 40 mM. At these elevated concentrations, μ_{max} of *P. putida* GS1 and KT2440 are obviously reduced. At 20 mM 1,8-cineole, μ_{max} of *P. putida* KT2440 was reduced to 75.5 ± 9.6 % of μ_{max0} and μ_{max} of *P. putida* GS1 to 57.6 ± 4.2 %. At 40 mM, μ_{max} of *P. putida* KT2440 was reduced to 68.3 ± 11.4 % and μ_{max} of *P. putida* GS1 to 64.1 ± 10.8 %. Concurrently, corresponding lag-phases were slightly extended (2-4 h longer, data not shown).

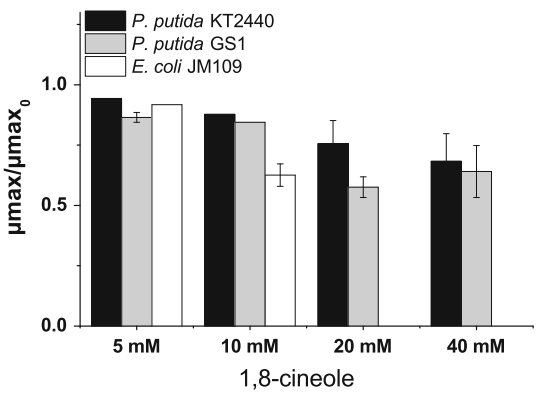


Fig. 1 Growth inhibition of *P. putida* KT2440 (*black bars*), *P. putida* GS1 (alias DSM 12264; *gray bars*) and *E. coli* JM109 (*white bars*) by different concentrations of 1,8-cineole. Ratios of maximum specific growth rate at given 1,8-cineole concentration (μ_{max}) to maximum specific growth rate without monoterpenoid (μ_{max0}) are depicted for each organism. All experiments were performed in shake flasks with aluminum caps. Samples were taken for OD_{600} measurements to calculate μ_{max}. The average of duplicates and corresponding standard deviations are shown

Comparison of *P. putida* KT2440 and GS1 as P450 expression hosts

In the following experiment, the bicistronic construct comprising the genes for the monooxygenase $P450_{cin}$ and its native redox partner CinC was introduced into *P. putida* KT2440 and GS1 to compare its performance in both pseudomonads, especially since both strains appeared to tolerate similar 1,8-cineole concentrations (Fig. 1). We added 10 mM 1,8-cineole directly after inoculation, on the one hand, to prevent substrate depletion and, on the other hand, to stay below the strong growth-inhibiting concentration, as determined for *P. putida* wild types in Fig. 1. Although both wild type pseudomonads tolerate 10 mM 1,8-cineole with only minor growth impairment, the corresponding recombinant strains were distinctly affected at the same concentration which was expressed primarily in filamentous and slow growth. Nevertheless, both strains showed similar growth behavior but differed in $P450_{cin}$ concentration and, hence, in product formation rate (Fig. 2). The recombinant *P. putida* GS1 strain reached a final concentration of 940 ± 33 μM (1*R*)-6β-hydroxy-1,8-cineole after 24 h, whereas the corresponding *P. putida* KT2440 strain reached 408 ± 18 μM product in the same period of time. Concurrent $P450_{cin}$ concentrations were 0.098 ± 0.01 μM for *P. putida* GS1 and 0.036 ± 0.05 μM for KT2440. Additional coexpression of the native reductase CinB did not lead to significantly higher product concentrations (data not shown).

Production of (1*R*)-6β-hydroxy-1,8-cineole by recombinant *P. putida* GS1 in different media

P. putida GS1 was used for further experiments of in vivo hydroxylation of 1,8-cineole in shake flasks, since this strain was shown to produce more than twice the amount of (1*R*)-6β-hydroxy-1,8-cineole under the same conditions compared to *P. putida* KT2440, as shown above (Fig. 2). Product concentrations of *P. putida* harboring solely $P450_{cin}$ and the strain harboring $P450_{cin}$ and its native redox partner cindoxin CinC were compared to validate the positive effect of CinC on the in vivo biotransformation of 1,8-cineole, as previously shown for heterologous expression in *E. coli* (Slessor et al. 2012). Two complex media, namely LB and TB medium, and the minimal medium E2 were tested for cultivation and (1*R*)-6β-hydroxy-1,8-cineole production. To maintain an adequate concentration of substrate and to avoid its growth-inhibiting effects, 4 mM 1,8-cineole were added every 12 h in the first 36 h. Cells were induced and substrate was added directly after inoculation. Samples were taken over time for determination of OD_{600} and product concentration. Figure 3 shows the OD_{600} and the corresponding (1*R*)-6β-hydroxy-1,8-cineole

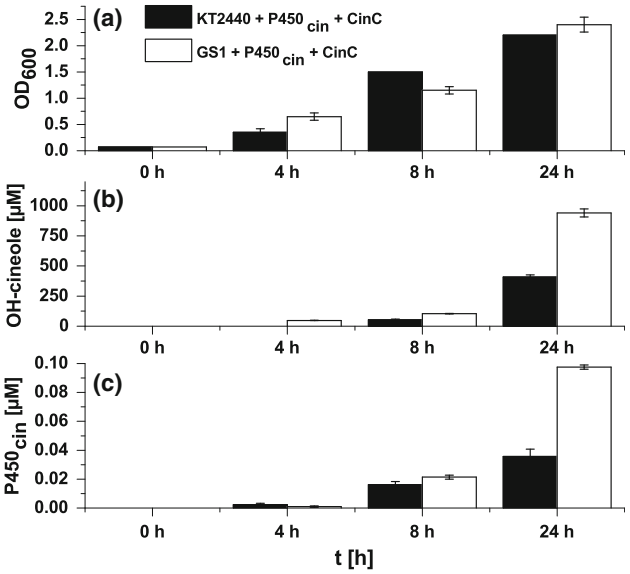


Fig. 2 Comparison of *P. putida* KT2440 (*black bars*) and GS1 (alias DSM 12264; *white bars*) as the microbial hosts for in vivo hydroxylation of 1,8-cineole via the $P450_{cin}$ system. Bicistronic construct comprising the genes encoding the monooxygenase $P450_{cin}$ (CYP176A1) and its native redox partner cindoxin (CinC) was introduced into both pseudomonads. OD_{600} (**a**), (1*R*)-6β-hydroxy-1,8-cineole (OH-cineole) (**b**) and $P450_{cin}$ concentrations (**c**) were compared over 24 h in the presence of an initial substrate concentration of 10 mM. All experiments were performed in shake flasks with aluminum caps. Samples were taken for all measurements simultaneously. The average of duplicates and corresponding standard deviations are shown

concentrations for *P. putida* GS1 solely expressing $P450_{cin}$ or $P450_{cin}$ in combination with CinC for all three tested media. Distinctly lower product concentrations were obtained using *P. putida* GS1 solely expressing $P450_{cin}$ than using the corresponding strain expressing $P450_{cin}$ and CinC. The production strain harboring the bicistronic construct reached a maximum OD_{600} of 4.7 ± 0.14 and a final product concentration of 3.3 ± 0.05 mM in LB medium, a maximum OD_{600} of 9.4 ± 0.42 and a final product concentration of 6.6 ± 0.32 mM in TB medium and a maximum OD_{600} of 4.3 ± 0.21 and a final product concentration of 2.4 ± 0.14 mM in E2 medium. It was observed that prolonged cultivation led to further oxidation of (1*R*)-6β-hydroxy-1,8-cineole to 6-oxo-1,8-cineole. To investigate whether an endogenous activity of the host itself was responsible for this delayed oxidation of (1*R*)-6β-hydroxy-1,8-cineole, *P. putida* GS1 wild type was cultivated in the presence of (1*R*)-6β-hydroxy-1,8-cineole and (1*R*)-6-oxo-1,8-cineole formation was monitored. Indeed, (1*R*)-6-oxo-1,8-cineole was formed in the aging culture, which confirms that *P. putida* GS1 is capable of oxidizing the secondary alcohol group of (1*R*)-6β-hydroxy-1,8-cineole to the corresponding keto group (Fig. S1).

Production of (1*R*)-6β-hydroxy-1,8-cineole by recombinant *P. putida* GS1 in a fed-batch bioreactor

Pseudomonas putida GS1 coexpressing $P450_{cin}$ and CinC was cultivated in a bioreactor to test its performance as a recombinant whole-cell biocatalyst for the regio- and stereoselective hydroxylation of 1,8-cineole under controlled conditions and at high cell density up to two-digit gram cdw per liter scale. Samples were taken for determination of OD_{600} and (1*R*)-6β-hydroxy-1,8-cineole concentration over time (Fig. 4). A maximum cell density of an OD_{600} of 107.5 ± 3.5 corresponding to 32.25 ± 1.05 g cdw per liter (Mirata et al. 2009) was reached after approximately 19 h of biotransformation. OD_{600} value decreased continuously after 2 days to an OD_{600} of 46.5 ± 7.8 corresponding to 13.95 ± 2.34 g cdw per liter after 89 h of biotransformation. A final (1*R*)-6β-hydroxy-1,8-cineole concentration of 78.2 ± 10.9 mM corresponding to 13.3 ± 1.9 g/L was obtained within 89 h. Oxidation of (1*R*)-6β-hydroxy-1,8-cineole to (1*R*)-6-oxo-1,8-cineole was found to occur later and became obvious at t = 113 h, whereas no further increase of (1*R*)-6β-hydroxy-1,8-cineole concentration was observed anymore. Product loss by evaporation in the fed-batch bioreactor had been found to be negligible under the applied conditions (10 % loss in 90 h; data not shown).

Discussion

With this work, *P. putida* was introduced as the microbial host for regio- and stereoselective hydroxylation of 1,8-cineole via heterologous expression of $P450_{cin}$ from *Citrobacter braakii*. Although pseudomonads are not commonly known to utilize 1,8-cineole as a carbon source, a bacterium closely related to *P. flava* was found to grow on this monoterpenoid (MacRae et al. 1979). To preclude natural utilization and degradation of 1,8-cineole by *P. putida*, cells were cultivated in the presence of the monoterpenoid. Both *P. putida* strains investigated, GS1 and KT2440, were verified to be unable to metabolize 1,8-cineole and, furthermore, were shown to be more tolerant to this monoterpenoid than *E. coli* by growth experiments in liquid medium (Fig. 1). The latter result corresponds to pseudomonads' well-described viability in the presence of many usually toxic substances (Saxena et al. 2002; Fernández et al. 2009, 2012; Udaondo et al. 2012). Slightly extended lag-phases observed at higher concentrations of 1,8-cineole were very likely caused by cellular adaption processes (Ramos et al. 2002). Therefore, both *P. putida* KT2440 and *P. putida* GS1 were investigated as potential

alternative microbial hosts for (1*R*)-6β-hydroxy-1,8-cineole production.

$P450_{cin}$ was previously reported to efficiently convert 1,8-cineole to (1*R*)-6β-hydroxy-1,8-cineole in shake flask experiments when coexpressed with its native redox partner cindoxin (CinC) in *E. coli*, presumably supported by endogenous flavodoxin reductase Fpr (Slessor et al. 2012). Thus, a bicistronic $P450_{cin}$/CinC expression construct was analogously applied to *P. putida* GS1 and KT2440 and the rate of in vivo hydroxylation of 1,8-cineole and $P450_{cin}$ content were compared (Fig. 2). Although both wild type strains were not considerably impaired in growth at the respective initial monoterpenoid concentration, induced recombinant strains were distinctly but similarly inhibited in cell growth (Fig. 2a), most probably due to elevated metabolic load by 1,8-cineole stress response in combination with plasmid maintenance, heterologous gene expression and exposure to the antibiotic. Nevertheless, $P450_{cin}$ content in *P. putida* GS1 was more than twice as high as in KT2440 under same conditions of cultivation, resulting in more than twice the amount of (1*R*)-6β-hydroxy-1,8-cineole after 1 day. Similar results were obtained for *P. putida* GS1 expressing single $P450_{cin}$ compared to the corresponding KT2440 in previous experiments (data not shown). The reason for the better performance of *P. putida* GS1 to express $P450_{cin}$ shown is currently unknown. Although both strains are designated as *P. putida*, they may differ a lot regarding their origin (Speelmans et al. 1998; Nakazawa 2002). Therefore, the same expression system may operate dissimilarly (e.g. codon usage, gene expression, heme production, etc.). Additionally, episomal expression of *gfp* in *P. putida* GS1 and KT2440 using pMiS1 was shown to be similar (paper under revision), which indicates that the difference in expression pattern found for $P450_{cin}$ is not a general characteristic. Since (1*R*)-6β-hydroxy-1,8-cineole formation was faster using recombinant *P. putida* GS1 than using KT2440, GS1 was applied for further studies.

Two common complex media, LB and TB, and the minimal medium E2 that is used as the monoterpenoid production medium for GS1 (Mirata et al. 2009; Mi et al. 2014) were tested, as certain media compositions may benefit the desired biotransformation. Additionally, in vivo performance of $P450_{cin}$ in combination with its native redox partner CinC was compared to the in vivo performance of single $P450_{cin}$ in each medium (Fig. 3). In all cases, product formation was faster using the bicistronic construct, confirming the importance of the native redox partner CinC, as it was previously reported by in vitro experiments (Hawkes et al. 2010). In *P. putida*, endogenous proteins are able to take over the function of CinC, though at lower level: recombinant *P. putida* GS1 solely expressing the monooxygenase $P450_{cin}$ was also able to produce reasonable amounts (>1 mM in ~2 days) of (1*R*)-6β-hydroxy-1,8-cineole.

The highest cell density and product concentration was obtained with *P. putida* GS1 harboring the bicistronic construct using TB medium (Fig. 3b). As stated by Slessor and co-workers, in vivo hydroxylation of 1,8-cineole ceased after approximately 44 h in *E. coli* with TB medium, however 3.1 g (1*R*)-6β-hydroxy-1,8-cineole was purified from 3.5 L cell broth after 3 days of cultivation (Slessor et al. 2012). A concentration of 1.1 g/L was produced in 55 h using *P. putida* GS1 and the bicistronic construct in shake flasks, ranging in the same magnitude as the *E. coli* system. A total of 16 mM 1,8-cineole had been sequentially added to the culture and 6.6 ± 0.3 mM (1*R*)-6β-hydroxy-1,8-cineole was formed, which corresponds to a yield $Y_{p/s}$ of 41 % mol/mol. In the fed-batch bioreactor, a maximum cell density of OD_{600} ~ 100 and a final concentration of 78.2 ± 10.9 mM (1*R*)-6β-hydroxy-1,8-cineole were obtained within 89 h corresponding to 13.3 ± 1.9 g/L product (Fig. 4) and an overall yield $Y_{p/s}$ of 79 ± 11 % mol/mol. The distinctly higher yield compared to the value in shake flasks is most likely due to continuous glycerol and substrate feed at low rate avoiding their excess accumulation. This fed-batch set-up was applied to exploit the advantage of the pronounced monoterpenoid resilience of *P. putida* GS1, as indicated during the growth comparison experiments with *P. putida* and *E. coli* shown in Fig. 1. The fed-batch process allowed for an active product formation period of about 4 days (Fig. 4). Additionally, lower 1,8-cineole concentration during fed-batch cultivation might lead to decreased impairment of cell viability compared to batch approaches and therefore to reduced stress response benefiting bioconversion as well. However, the significant decrease of OD_{600} values beginning about 2 days after starting the 1,8-cineole feed nevertheless indicates increased cell lysis also for *P. putida* GS1. This may be the result of multiple effects, e.g. the continuous dosage of the toxic monoterpenoid substrate, the accumulation of the potentially toxic monoterpenoid product or the depletion of medium components essential for maintaining cell integrity. Nevertheless, compared to product concentrations and space–time yields reported for other recombinant P450-driven monoterpenoid biotransformations using *P. putida* (van Beilen et al. 2005; Cornelissen et al. 2011), (1*R*)-6β-hydroxy-1,8-cineole production with *P. putida* GS1 ranges in the same order of magnitude even without applying in situ product removal (ISPR) techniques.

Yet, it is not known whether this product concentration is toxic and inhibits cell growth and/or product formation, respectively, since (1*R*)-6β-hydroxy-1,8-cineole is not commercially available to be applied in toxicity studies and has first to be produced in larger quantities. Investigations

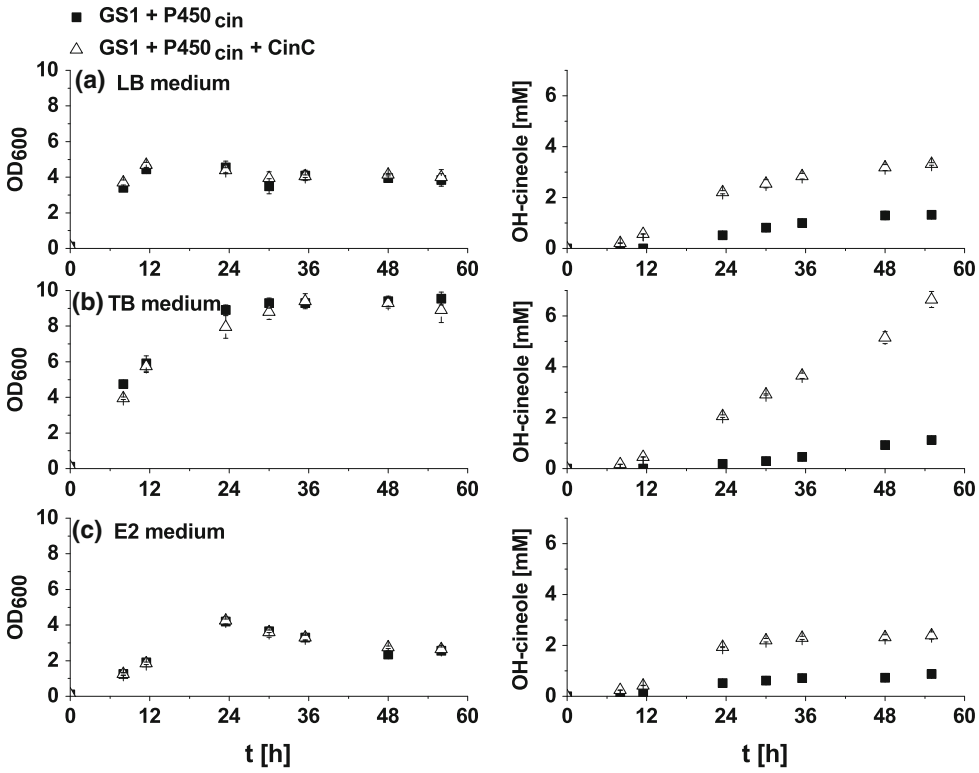


Fig. 3 Comparison of the time courses of OD_{600} and (1*R*)-6β-hydroxy-1,8-cineole (OH-cineole) concentration by recombinant *P. putida* GS1 (alias DSM 12264) expressing the monooxygenase $P450_{cin}$ (CYP176A1) solely (*squares*) and in combination with its native redox partner cindoxin (CinC) (*triangles*) in complex media LB (**a**) and TB (**b**) and minimal medium E2 (**c**). 4 mM 1,8-cineole was added every 12 h for the first 36 h. All experiments were performed in shake flasks with aluminum caps. The average of duplicates and corresponding standard deviations are shown

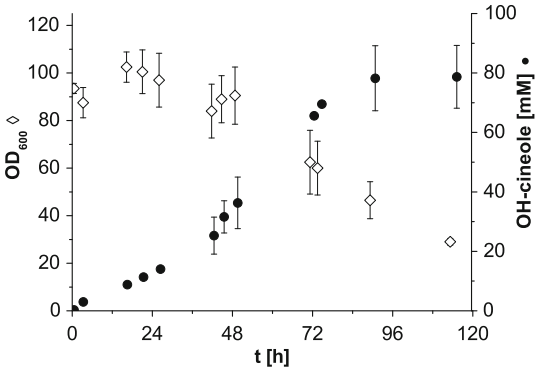


Fig. 4 Production of (1*R*)-6β-hydroxy-1,8-cineole (OH-cineole) by recombinant *P. putida* GS1 (alias DSM 12264) harboring $P450_{cin}$ and its native redox partner cindoxin CinC in a fed-batch bioprocess. OD_{600} (*open diamonds*) and product concentration (*closed circles*) were measured simultaneously. Cells were grown in TB medium for 24 h before induction of gene expression (t = 0 h). Glycerol and $MgSO_4$ feeding started 9 h after inoculation and was maintained for 86.5 h. Pure 1,8-cineole feeding was started 6 h after induction (0.1 mL/h). The average of duplicates and corresponding standard deviations are shown

of the (potentially synergistic) stress effects caused by the presence of substrate and/or product in combination with the heterologous plasmid-borne gene expression have to be still carried out in order to establish an optimized bioprocess. Moreover, kanamycin had to be applied as antibiotic resistance marker for the episomal $P450_{cin}$ and CinC expression system, hence, appropriate genomic integration of these genes would make antibiotic selection pressure unnecessary. Besides, the $P450_{cin}$ to CinC ratio has been reported to have a great impact on NADPH consumption in in vitro experiments, therefore, balancing of gene expression will presumably influence product formation rate in *P. putida* as well (Hawkes et al. 2010).

Furthermore, identification of cellular mechanisms of *P. putida* GS1 leading to improved 1,8-cineole (and perhaps (1*R*)-6β-hydroxy-1,8-cineole) tolerance may be promising for host optimization. Improved solvent tolerance in microbes, in fact, does not necessarily result in higher productivity (Woodruff et al. 2013) and it is often only envisioned to be beneficial (Doukyu et al. 2012; Kang and Chang 2012; Anfelt et al. 2013), nevertheless, resilience to toxic compounds was demonstrated to improve its production in several cases (Tomas et al. 2003; Dunlop et al. 2011; Lin et al. 2013; Volmer et al. 2014; Foo et al. 2014). Complementary, elimination of unnecessary or undesired traits by gene deletion would also benefit an application of *P. putida* GS1 as a microbial platform, as it was demonstrated by de Lorenzo and co-workers for *P. putida* KT2440 (Martínez-García et al. 2014a, b).

Further optimization of the process was not in the focus of this study but can be implemented in subsequent studies. For example, the temperature optimum of $P450_{cin}$ expression (in *E. coli*) has been reported below 28 °C (Hawkes et al. 2002). Since an expression temperature of 30 °C was utilized in this study, an adaption of expression temperature

might lead to higher expression levels and activity of $P450_{cin}$. Also, in situ product removal of the product by application of adsorbers (Bormann et al. 2012) might further increase product concentrations.

Bioprocess development like determination of the optimal substrate feed rate, optimal cultivation/expression temperature, usage of ISPR as well as comprehensive strain development were not the major objectives of the present work; however, respective optimization is expected to improve this monoterpenoid production accordingly and will be considered in future work.

De Voss and co-workers also denoted that a small amount of 6-oxo-1,8-cineole (ketocineole) was detected, which was presumably catalyzed by $P450_{cin}$ itself by overoxidation (Slessor et al. 2012). As overoxidation using *P. putida* GS1 was observed only in prolonged cultivation, it was checked whether the host itself may be responsible for this fast but delayed oxidation of (1*R*)-6β-hydroxy-1,8-cineole, in addition to the aforementioned overoxidation activity of $P450_{cin}$. Interestingly, wild type *P. putida* GS1 was indeed able to oxidize (1*R*)-6β-hydroxy-1,8-cineole to 6-oxo-1,8-cineole during prolonged cultivation, presumably due to changes of the gene expression pattern in the aging culture leading to the emergence of endogenous dehydrogenase(s) capable of catalyzing this reaction. Interestingly, natural oxidation of the monoterpene primary alcohols perillyl alcohol and geraniol by *P. putida* has been observed by different groups and in the authors' laboratory, respectively (Speelmans et al. 1998; van Beilen et al. 2005; Cornelissen et al. 2013; Mi et al. 2014), whereas the undesired oxidation of the secondary hydroxyl group of (1*R*)-6β-hydroxy-1,8-cineole occurred only during prolonged biotransformation. Although it was shown that (1*R*)-6β-hydroxy-1,8-cineole production with only minor 6-oxo-1,8-cineole formation is possible up to the double-digit gram per liter concentration range in the fed-batch bioreactor (Fig. 4), identification of responsible dehydrogenases catalyzing the further oxidation to 6-oxo-1,8-cineole may be beneficial for strain engineering leading to complete absence of the keto product. On the other hand, in monoterpenoid oxyfunctionalizations where the monoterpene ketone is favored over the alcohol, e.g. carvone over carveol or verbenone over verbenol, sooner expression of endogenous genes encoding those enzymes would benefit the yield of the ketone.

Conclusion

In conclusion, exemplified by the regio- and stereoselective hydroxylation of 1,8-cineole, *P. putida* GS1 has been proven to be an efficient heterologous host for in vivo monoterpenoid oxyfunctionalization reaching more than 13 grams per liter product under bioreactor conditions. To the authors' knowledge, this is the highest product titer obtained in a microbial P450-based monoterpene hydroxylation reported so far. Therefore, further investigations will focus on the potential of this strain as a microbial platform for monoterpenoid production in general.

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