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ORIGINAL PAPER

Cell physiology rather than enzyme kinetics can determine the efficiency of cytochrome P450-catalyzed C–H-oxyfunctionalization

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Abstract Cell physiology is a critical factor determining the efficiency of reactions performed by microbial biocatalysts. In order to develop an efficient biotransformation procedure for the hydroxylation of (*S*)-limonene to (*S*)-perillyl alcohol by recombinant *Pseudomonas putida* cells harboring the cytochrome P450 monooxygenase CYP153A6, physiological parameters were optimized. The previously reported synthesis of (*S*)-perillyl alcohol by *P. putida* GPo12 was based on complex and sensitive octane feeding strategies (van Beilen et al. in Appl Environ Microbiol 71:1737–1744, 2005), indicating the pivotal role of cell physiology. In contrast to previous findings, the screening of different carbon sources showed that glycerol and citrate are suitable alternatives to octane allowing high specific limonene hydroxylation activities. The use of *P. putida* KT2440 as an alternative host strain and citrate as the carbon source improved practical handling and allowed a 7.5-fold increase of the specific activity (to 22.6 U g_{CDW}^{-1}). In two-liquid-phase biotransformations, 4.3 g of (*S*)-perillyl alcohol L_{tot}^{-1} were produced in 24 h, representing a sixfold improvement in productivity compared to previously reported results. It is concluded that, for selective cytochrome P450-based hydrocarbon oxyfunctionalizations by means of living microbial cells, the relationship between cell physiology and the target biotransformation is crucial, and that understanding the relationship should guide biocatalyst and bioprocess design.

Keywords Cytochrome P450 monooxygenase · Microbial physiology · Whole-cell biocatalysis · C–H-oxyfunctionalization · *Pseudomonas putida*

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Introduction

Although selective oxidation reactions of unactivated C–H-bonds are of considerable importance for the chemical industry, they often remain inaccessible or difficult to accomplish by chemical means. As such reactions are ubiquitous in nature, it is reasonable to exploit the potential of natural catalysts, for example cytochrome P450 monooxygenases (CYP), for selective C–H-oxyfunctionalizations [3, 14, 22].

Living (recombinant) microorganisms expressing the desired genes are often favored over purified enzymes for applications of CYPs on a technical scale, because living cells are self-renewable and provide a constant environment, which stabilizes the oxygenase of interest. CYPs usually depend on redox proteins that shuttle electrons from a nicotinamide co-factor (NAD(P)H) to the heme–iron center of the CYP. Co-expression of the genes encoding such electron transfer proteins is more straightforward than in vitro reconstitution of the multicomponent system. Moreover, living cells can regenerate NAD(P)H via cell metabolism, which circumvents the need for separate addition of expensive co-factors or co-factor regeneration systems [12, 25, 56].

When whole cells are applied, the reaction efficiency (addressed in terms of productivity, yield, product titer, and stability [47]) is not only determined by intrinsic properties of the enzyme catalyzing the desired reaction (e.g., activity, selectivity, coupling efficiency), but also by its direct environment, the microbial cell. Critical factors related to cell physiology include gene expression levels, co-factor regeneration, mass transfer, interaction with electron transfer proteins, and side product formation [8].

In the field of oxygenase biocatalysis, many research efforts focus on the targeted manipulation of intrinsic oxygenase properties. Intriguing examples include expansion of the substrate spectrum, allowing the hydroxylation of unnatural substrates, and the improvement of catalytic turnover rates and coupling efficiencies [11, 13, 18]. Although the implementation of these engineered enzymes in biocatalytic processes is often envisaged, cell physiological aspects are rarely addressed [22]. This is remarkable because optimization of intrinsic enzyme properties to improve overall process efficiency only makes sense if full exploitation of the enzyme's potential in a bioreactor is guaranteed. In other words, it makes sense in case enzyme properties rather than cell physiological parameters are limiting the desired reaction. However, for reactions catalyzed by CYP-containing microorganisms, physiological aspects can be expected to constrain the cell's catalytic efficiency.

In this study, we investigated in detail which physiological parameters limit the hydroxylation of (*S*)-limonene to (*S*)-perillyl alcohol catalyzed by whole *Pseudomonas* cells containing CYP153A6 (Fig. 1) with the aim to develop an efficient biotransformation procedure.

Perillyl alcohol is of considerable pharmaceutical interest because of its antibacterial and antifungal properties [10]. It is currently being tested in clinical trials for the prevention (e.g., as a dietary supplement) and for treatment of various kinds of cancer [19, 26]. Perillyl alcohol is also applied in cosmetics [32] and as synthetic precursor [28, 29, 43]. Because of its low natural abundance, extraction of perillyl alcohol from plant tissues only yields small amounts of the desired terpenoid [27]. Chemical oxidation

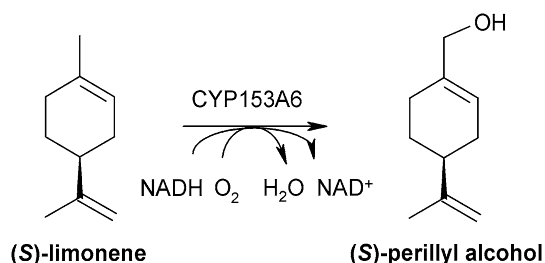

Fig. 1 Regioselective hydroxylation of (*S*)-limonene to (*S*)-perillyl alcohol by recombinant microbial cells containing CYP153A6

of readily accessible limonene gives a complex mixture of products with low yields of perillyl alcohol [21, 40]. In contrast, soluble CYP153A6 from *Mycobacterium* sp. strain HXN-1500 has been found to exclusively oxidize limonene at the carbon atom at position 7 [15].

Van Beilen and co-workers recently expressed the respective genes in *Pseudomonas putida* GPo12 and, during two-liquid-phase biotransformations, achieved (*S*)-perillyl alcohol formation at a productivity of 0.03 g l^{-1} h^{-1} [53]. The described reaction required the use of octane as carbon source and the presence of two plasmids: (i) pCom8-PFR1500, which contains the genes for CYP153A6 and its electron transfer partners, enabling the conversion of octane to octanol and (ii) pGEc47ΔB, which contains all genes necessary for growth on octanol and other medium-chain length alkanols.

In the present study, physiology-related aspects, such as carbon source, host strain, and growth control were optimized, enabling a significant improvement of (*S*)-perillyl alcohol production with respect to productivity and product concentrations. It was shown that, irrespective of intrinsic properties of the enzyme itself, the efficiency of a cell-based biocatalytic reaction is largely determined by the physiology of the applied microbial cells.

Materials and methods

Chemicals, bacterial strains, plasmids, and microbiological methods

All chemicals used for this study were purchased from Sigma–Aldrich (Germany) or Carl-Roth (Germany) with the highest purity available. (*S*)-limonene was routinely used at 99% purity except for reactor experiments, in which technical grade (95%) (*S*)-limonene was applied. The strains and plasmids used in this study are listed in Table 1. Strain *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) was a kind gift of Dr. J. B. van Beilen (Université de Lausanne, Switzerland) and Prof. Dr. B. Witholt (ETH Zurich, Switzerland).

Cells were either grown on Luria–Bertani (LB) broth [41], E2 medium [24] or, for bioreactor experiments, aqueous batch medium (ABM) [36]. All media were supplemented with the appropriate antibiotics (50 μg ml^{-1} gentamycin, 12.5 μg ml^{-1} tetracycline). E2 medium was supplemented with 0.5% (w/v) acetate, citrate, glucose, glycerol, or succinate and ABM medium was supplemented with 1% (w/v) citric acid or glycerol. Cultures were incubated in screw-capped, baffled Erlenmeyer flasks in horizontal shakers at 30°C. Octane vapor as carbon source was supplied by evaporation from a small solvent container inside the flask.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
Strains		
P. putida GPo12	*P. putida* GPo1 cured of the OCT plasmid, solvent-sensitive	[45]
P. putida KT2440	*P. putida* mt-2 cured of the TOL plasmid, solvent-sensitive	[1]
P. putida S12	Solvent-tolerant	[20]
P. putida DOT-T1E	Solvent-tolerant	[38]
Pseudomonas sp. strain VLB120	Solvent-tolerant, styrene degrader	[33]
Plasmids		
pGEc47ΔB	Contains all genes necessary for growth on alkanols (*alkFGHJKL* and *alkST*) in broad-host-range vector pLAFR1, Tc^r	[55]
pCom8-PFR1500	Contains genes for CYP153A6, ferredoxin, and ferredoxin reductase from *Mycobacterium* sp. strain HXN-1500 in broad-host-range expression vector pCom8, Gm^r	[53]

Cell densities were monitored by measuring the optical density at 450 nm (OD_{450}), using a spectrophotometer. The correlation factor between OD_{450} and cell dry weight (CDW) concentration was determined as described before [4], whereby 1 OD_{450} unit was found to correspond to 0.223 g_{CDW} l^{-1}.

Recombinant strains were obtained by introducing plasmids pCom8-PFR1500 and pGEc47ΔB into different *Pseudomonas* strains by electroporation (2,500 V, Equibio EasyjecT Prima, Ashford, UK) [41]. Plasmid transformation was verified by antibiotic resistance and growth on octane as sole carbon source.

Activity determination with resting cells and cell extracts

Pseudomonas cells harboring pCom8-PFR1500 and pGEc47ΔB were grown in E2 medium to a biomass concentration of 0.1 g_{CDW} L^{-1}. Then, cells were induced by the addition of 0.1% (v/v) octane or 0.025% (v/v) dicyclopropylketone (DCPK) (both induction strategies led to the same expression behavior) and incubation was continued for 5 h. Subsequently, cells were harvested by centrifugation (10 min, 4,600×*g*, 4°C) and resuspended in 50 mM potassium phosphate buffer (pH = 7.4), containing 1% (w/v) of the carbon source (1% (w/v) glucose was added when octane was used as carbon source), to a cell concentration of 0.5 g_{CDW} l^{-1}. This cell stock was distributed as 2-ml aliquots in Pyrex tubes with Teflon caps. After 5 min of incubation in a rotary shaker at 30°C and 300 rpm, 2 mM (*S*)-limonene from an ethanol stock was added. The reaction was carried out in triplicate for 10 min and subsequently stopped by the addition of 2 ml ice-cold diethyl ether containing 0.2 mM dodecane. After adding a saturating amount of NaCl and vortexing for 30 s, the organic and aqueous phases were separated by centrifugation (5 min, 4,600×*g*, 4°C). The organic phase was dried over anhydrous Na_2SO_4 and analyzed by gas chromatography (GC).

For activity measurements in cell extracts, cells were grown, induced, and harvested as described for resting cell assays. Then, cells were resuspended in 50 mM potassium phosphate buffer (pH = 7.4; 5% (v/v) glycerol, 1 mM DTT) to obtain a cell stock with a biomass concentration of 15 g_{CDW} L^{-1}. Cells were disrupted by three passages through a pre-cooled French press (5.5 MPa, SLM-Aminco, Rochester, NY, USA). Membranes and cell debris were separated from the extracts by ultracentrifugation (20 min, 150,000×*g*, 4°C). After the addition of 2.5 mM NADH, 1 ml of supernatant was used for activity assays as described above. Product formation over time was normalized to the biomass concentration of the cell stock obtained before cell disruption to allow for a comparison with whole-cell activities. A specific activity of 1 U g_{CDW}^{-1} is defined as 1 μmol min^{-1} g_{CDW}^{-1}.

Analytical procedures

Using commercially available standards, substrate and products were identified and quantified by GC (Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA). The chromatograph was equipped with a 30-m FactorFour capillary column VF-5 ms (Varian, Palo Alto, CA, USA) and a programmed temperature vaporizer injector run in splitless mode. The oven was programmed with the following temperature profile: from 90 to 100°C at a rate of 2°C min^{-1}, from 100 to 140°C at a rate of 20°C min^{-1}, and from 140 to 300°C at 40°C min^{-1}. Dodecane (0.2 mM)

was added as an internal standard to the diethyl ether used for extraction.

Determination of cellular CYP concentration

Active CYP153A6 in whole cells was quantified by CO-difference spectra [31]. Induced cells were harvested as described above and resuspended in 100 mM potassium phosphate buffer (pH = 7.4) to obtain a cell concentration of 3.3 g_{CDW} l^{-1}. Then 0.5 ml of a 20 mM sodium dithionite solution was added to 0.5 ml of this stock and a baseline was recorded with a UV–visible spectrophotometer (Varian, Type CARY 300, Palo Alto, CA, USA). Subsequently, the sample was gassed with carbon monoxide (Linde AG, Munich, Germany) for 30 s and a CO-difference spectrum was recorded between 400 and 600 nm. The CYP-concentration was calculated using a molar extinction increment between 450 and 490 nm of 91 mM^{-1} cm^{-1} [31].

Partition coefficients in two-liquid-phase systems

The partitioning behavior of (*S*)-limonene and (*S*)-perillyl alcohol was determined in a biphasic system consisting of 1 ml organic solvent and 1 ml ABM medium (1% (w/v) citrate) in screw-capped Pyrex tubes at varying terpene concentrations. After vigorous shaking for 5 min, the samples were allowed to further equilibrate for 24 h under continuous shaking in a rotary shaker at 30°C and 300 rpm. The phases were separated by centrifugation (5 min, 4,600×*g*, 4°C). The organic and aqueous phases were diluted in or extracted with diethyl ether, respectively. For the aqueous phase, a saturating amount of NaCl was added to improve the extraction efficiency. Subsequently, the samples were dried over anhydrous Na_2SO_4 and analyzed by GC. The partition coefficient (K_p) is defined as the concentration in the organic phase divided by the concentration in the aqueous phase.

Two-liquid-phase biotransformations in a bioreactor

For biotransformations, a 3.1-l stirred tank reactor (KLF 2000, Bioengineering, Switzerland) equipped with two Rushton turbine impellers was used. Batch cultivation was started by adding 100 ml of *P. putida* grown in ABM medium to 900 ml of fresh ABM medium. The pH was regulated at 7.1 by addition of 30% (w/v) phosphoric acid when citrate was used as a carbon source. When glycerol was used, no regulation of the pH was necessary. Batch cultures were stirred at 1,400 rpm and aerated with 1.8 vvm sterile-filtered air until the carbon source was completely consumed, indicated by a sudden increase of the pO_2-value. Then, 2 ml of ABM microelement stock solution [36] was added and fed-batch cultivation was started, applying an exponential feeding strategy that sustained growth at a predetermined growth rate (μ) of 0.1 h^{-1} (citrate) or 0.05 h^{-1} (glycerol). The citrate-feed solution contained per liter 500 g citric acid, 7.6 g $MgSO_4 \cdot 7H_2O$, and 90.4 ml of a 25% NH_3 solution in H_2O. The glycerol-feed solution contained per liter 500 g glycerol, 6.9 g $MgSO_4 \cdot 7H_2O$, and 72.7 g $(NH_4)_2SO_4$. The pO_2-value was maintained above 30% of oxygen saturation by increasing the stirrer speed. When a biomass concentration of 15 g_{CDW} l^{-1} was reached, the stirrer speed was increased to 2,000 rpm and the biotransformation was started by the addition of an organic phase consisting of 460 ml bis(2-ethylhexyl)phthalate (BEHP), 40 ml technical grade (95%) (*S*)-limonene, and 1.4 ml DCPK. Foam formation was limited by the addition of antifoam 204 (Sigma–Aldrich, Schnelldorf, Germany).

Results

Carbon source selection: citrate and glycerol allow good biocatalyst performance and simple process control

The use of octane as a carbon source for *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500), as applied in former studies [15, 53], has several disadvantages for the development of an efficient biotransformation procedure for the synthesis of perillyl alcohol: (a) Cells growing on octane vapor display low growth rates and their practical handling is difficult (e.g., instability of cells, explosion risk). (b) With octane as carbon source and inducer of the *alk* regulatory system (controlling gene expression from pCom8-PFR1500 and pGEc47ΔB), growth and enzyme synthesis can not be uncoupled. (c) CYP153A6 is involved in growth as well as the desired biotransformation. The competition of octane and limonene for the same active site is expected to negatively affect biocatalyst performance. Hence, more conventional carbon sources were tested as alternatives to octane. The specific hydroxylation activity of resting cells was used as an indicator for biocatalyst performance (Table 2).

Under the applied resting cell assay conditions guaranteeing an efficient supply of oxygen and energy source, the specific activity of octane-grown *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) was determined at 23.6 U g_{CDW}^{-1}, which is 7.9 times higher than the reported value for this strain [53]. In contrast to previously reported results [53], glycerol and citrate were found to be suitable alternative carbon sources. Whereas glycerol enabled similar specific activities as octane, citrate is more attractive in terms of achievable growth rate, enabling a 40% lower specific

Table 2 Growth rate (μ) and specific hydroxylation activity of resting cells of two *P. putida* strains utilizing different carbon sources

Carbon source	*P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500)		*P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500)	
	Growth rate[a] (h^{-1})	Specific activity[b] (U g_{CDW}^{-1})	Growth rate[a] (h^{-1})	Specific activity[b] (U g_{CDW}^{-1})
Acetate	0.29	4.0 ± 0.5	0.34	5.4 ± 1.1
Citrate	0.57	13.8 ± 1.8	0.57	16.3 ± 2.1
Glucose	0.54	6.7 ± 0.6	0.45	7.1 ± 0.7
Glycerol	0.38	22.2 ± 3.2	0.36	22.6 ± 0.4
Octane	0.24	23.6 ± 1.6	0.30	18.1 ± 0.4
Succinate	0.57	1.7 ± 0.1	0.68	0.8 ± 0.4

CDW, cell dry weight

[a] Growth rate of uninduced cells

[b] 1 U g_{CDW}^{-1} is defined as 1 μmol min^{-1} g_{CDW}^{-1}

Table 3 Specific hydroxylation activities of whole cells and cell extracts of citrate-grown *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500)

	Specific activity (U g_{CDW}^{-1})	CYP concentration (nmol g_{CDW}^{-1})	Turnover number (nmol min^{-1} $nmol^{-1}$)[b]
Whole cells	13.8 ± 1.8	152 ± 15	92 ± 17
Cell extracts[a]	4.7 ± 0.2	190 ± 1	25 ± 1

[a] Activity and CYP concentration of cell extracts were normalized to the cell dry mass applied for cell lysis via French press

[b] Turnover numbers are given in nmol product formed per min per nmol CYP153A6

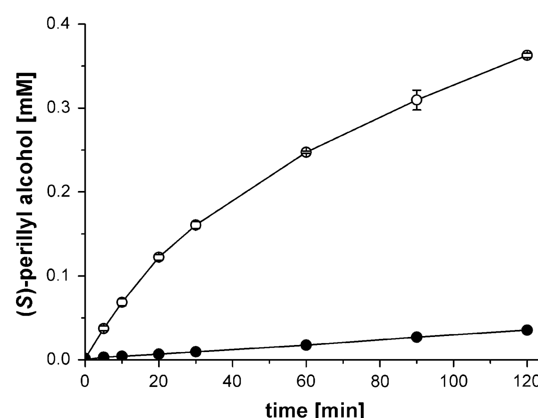

Fig. 2 Time course of (*S*)-perillyl alcohol formation with resting cells of *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) (*open symbols*) and *P. putida* GPo12 (pCom8-PFR1500) (*closed symbols*) using citrate as carbon source. The applied cell and substrate concentrations were 0.5 g_{CDW} l^{-1} and 2 mM (*S*)-limonene, respectively

activity. These two carbon sources were investigated in more detail by evaluating their effect on CYP gene expression. The concentration of heme-containing CYP153A6 enzymes in whole cells was determined by means of whole-cell CO-difference spectra (see Materials and methods section for details) and found to be 152 and 121 nmol g_{CDW}^{-1} for citrate- and glycerol-grown cells, respectively. For octane-grown cells, the CYP153A6-concentration amounted to 132 nmol g_{CDW}^{-1}. Uninduced *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) showed neither CYP production nor activity towards (*S*)-limonene. This confirmed that all three carbon sources allow efficient CYP153A6 gene expression. The fact that CYP concentrations do not directly correlate with observed activities indicates that CYP gene expression does not limit limonene hydroxylation.

CYP153A6 catalysis was found to be more efficient in intact cells supplemented with 1% (w/v) citrate than in cell-free extracts supplemented with 2.5 mM NADH (Table 3), confirming that living cells provide a well-suited environment for the CYP system used.

Pseudomonas putida GPo12 (pGEc47ΔB)(pCom8-PFR1500) growing on octane requires enzymes encoded by pGEc47ΔB to convert octanol (formed by CYP153A6 from octane) to octanoyl-CoA, a compound that can be degraded by β-oxidation [54]. By using citrate or glycerol as a carbon source, plasmid pGEc47ΔB is no longer required for growth. However, citrate-grown cells harboring only pCom8-PFR1500 displayed a dramatically reduced ability to produce perillyl alcohol (Fig. 2, specific activity reduced by a factor of 19), although the active CYP153A6 concentration was found to be in a similar range (at 106 nmol g_{CDW}^{-1}) as in the two-plasmid-containing strain. The reason for this observation remains unclear and is the topic of further investigations. However, the use of citrate or glycerol as a carbon source for *P. putida* GPo12 harboring pGEc47ΔB as well as pCom8-PFR1500 allows circumvention of some major disadvantages of the original biotransformation procedure.

Host strain selection: *P. putida* KT2440 as the most promising host

The host strain defines the microenvironment for the enzyme of interest and determines many factors critical for productive whole-cell biocatalysis, including the complexity of process handling, gene expression, protein folding, substrate mass transfer across the cell membrane, co-factor regeneration, specificity, and product degradation. Recombinant *E. coli* GEc137 (pGEc47ΔB), containing CYP153A6, was found to show very low limonene hydroxylation activities (0.1 U g_{CDW}^{-1}) [53]. Thus, the use of *E. coli* strains was considered not to be feasible for productive (*S*)-perillyl alcohol synthesis. Here, in addition to *P. putida* GPo12, the sequenced GRAS strain *P. putida* KT2440 [30, 37] and the three solvent-tolerant strains *P. putida* S12, *P. putida* DOT-T1E, and *Pseudomonas* sp. strain VLB120 were evaluated with respect to their suitability for (*S*)-limonene hydroxylation.

In bioreactor experiments, Funhoff et al. observed that cultivation of *P. putida* GPo12 (pGEc47ΔB) on octane, expressing different CYP153 genes from pCom8 and pCom12 vectors, was only successful after an initial adaptation time at low stirring speeds (650 rpm; no aeration) [16]. In agreement with these observations, *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) did not grow on citrate at an initial stirrer speed of 1,000 rpm and an aeration rate of 2 vvm. This indicates a high sensitivity of this strain to high oxygen tension and/or shear stress. *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) showed more stable cultivation characteristics and similar specific activities as compared to the GPo12 recombinant, with lower activities when grown on acetate, glucose, or succinate and high activities when grown on octane, glycerol, or citrate (Table 2). With citrate as carbon source the KT2440 recombinant even showed 1.2-fold higher activities than the GPo12 recombinant.

Unlike *P. putida* KT2440 and *P. putida* GPo12, solvent-tolerant *Pseudomonas* strains are known to adapt to the presence of organic solvents that are otherwise toxic to bacteria [57], thus having the potential to overcome toxic effects of limonene and perillyl alcohol. In growth experiments with citrate or glycerol as carbon source, induction of all three solvent-tolerant strains by octane resulted in a strong reduction of the growth rates and instable, slimy cell pellets after cell harvesting. This was most likely caused by partial cell lysis and/or the activation of solvent tolerance adaptation mechanisms upon addition of octane. After induction by DCPK, cells still showed a strongly reduced growth rate, but cell pellets were firm after centrifugation allowing reliable harvesting. Surprisingly, and in all cases, no evidence for perillyl alcohol formation was found in resting cell assays.

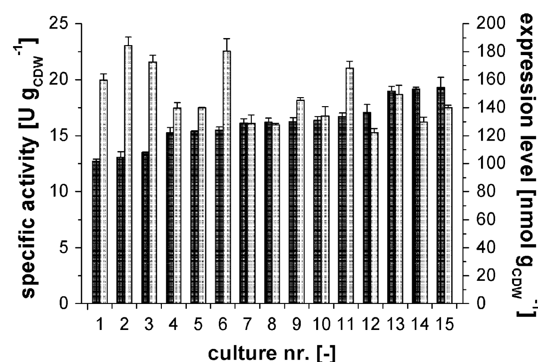

Fig. 3 Reproducibility of specific activity (*dark gray*) and CYP-gene expression level (*light gray*) of citrate-grown *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500). Both parameters were measured by growing 15 independent cultures originating from 15 randomly picked colonies from one E2 agar plate (0.5% (w/v) citrate)

For *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) grown on citrate, the reproducibility of the limonene hydroxylation activity and CYP153A6 expression level was evaluated using 15 independently grown cultures (Fig. 3). Average values and variations amounted to 16.3 ± 2.1 U g_{CDW}^{-1}, 148 ± 20 nmol g_{CDW}^{-1}, and 111 ± 24 nmol min^{-1} $nmol^{-1}$ for specific activity, CYP concentration, and turnover number, respectively. With 13.1 and 13.6%, the variations were somewhat higher than the experimental variation (2.3 and 2.8%) for activities and CYP concentrations, respectively. This points to a moderate biological variance.

CYP153A6 concentrations do not seem to correlate directly with the corresponding specific activities (Fig. 3). This indicates that the bioconversion is not limited by gene expression of the CYP system, but rather by other cell physiological parameters, such as cofactor regeneration or substrate mass transfer.

In summary, *P. putida* KT2440 is the most suitable host for CYP153A6 expression and catalysis and was therefore used for further investigations.

Toxicity of (*S*)-perillyl alcohol and (*S*)-limonene to *P. putida* KT2440

Compounds with log $P_{O/W}$-values between 1 and 4, such as perillyl alcohol (Table 4), are typically toxic to bacteria, whereas compounds with a log $P_{O/W}$-value between 4 and 5, such as limonene (Table 4), can be considered of intermediate toxicity. To investigate toxic effects of (*S*)-limonene and (*S*)-perillyl alcohol on *P. putida* KT2440, the growth rate reduction of exponentially growing cells, determined in dependence of the concentration of these terpenes, was measured (Fig. 4).

A minor reduction in growth rate was observed when cells were incubated with low concentrations (<10 mM) of

Table 4 Physical properties of limonene and perillyl alcohol

	Critical concentration[a] (mM)	Aqueous solubility at 25°C (mM)	Log $P_{O/W}$ (−)	Log K_p^b at 30°C (−)
Limonene	>62	0.10 [44]	4.46 [44]	4.28 ± 0.05
Perillyl alcohol	>4	0.75 [19]	2.38 [19]	2.27 ± 0.02

[a] The critical concentration is defined as the concentration at which severe toxic effects were observed. The term 'concentration' is also used when the aqueous solubility was exceeded and a second liquid phase was formed

[b] K_p is defined as the concentration of limonene or perillyl alcohol in the BEHP phase divided by the concentration of these compounds in the aqueous phase

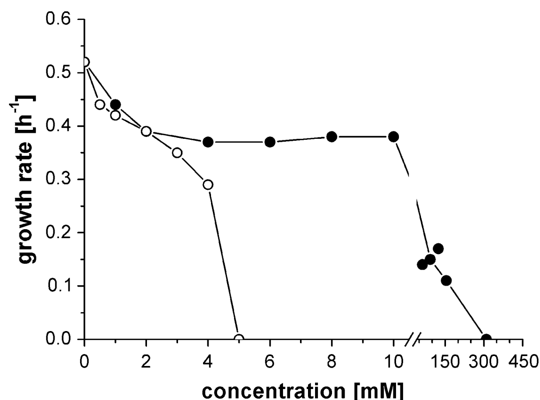

Fig. 4 Growth rate of *P. putida* KT2440 in E2 medium with different amounts of (*S*)-limonene (*closed symbols*) or (*S*)-perillyl alcohol (*open symbols*) at 30°C

(*S*)-limonene (although the aqueous solubility is exceeded, the term 'concentration' is used for explanatory reasons). However, after adding 1% (v/v) limonene, the growth rate was reduced to $\sim$0.14 h^{-1} and growth completely ceased after addition of 5% (v/v) limonene. Perillyl alcohol is toxic to *P. putida* KT2440 at lower concentrations. Complete cessation of growth was observed at 5 mM and above (Fig. 4).

Both limonene and perillyl alcohol seem to exhibit phase toxicity (toxicity as the result of the presence of an organic/aqueous interface [2]) to *P. putida* KT2440, because severe toxic effects of both compounds were observed only at concentrations well above their aqueous solubility. The observed toxic effects might also be the result of impurities present in the added limonene and perillyl alcohol, although this does not seem likely as these terpenes were used at purities of ≥99%.

The results show the necessity to apply an aqueous-organic two-liquid-phase system in a bioreactor in order to prevent toxic effects.

Two-liquid-phase biotransformations

To investigate if modifications with respect to host strain and carbon source translate into improved productivity under process conditions, two-liquid-phase biotransformations were performed on a 1.5-l scale, applying growing cells of *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) and either citrate (Fig. 5a) or glycerol (Fig. 5c) as carbon source. Batch cultivation gave biomass yields ($Y_{x/s}$) of 0.36 g_{CDW} $g_{citrate}^{-1}$ and 0.31 g_{CDW} $g_{glycerol}^{-1}$. After the batch phase had been completed, exponential feeding of additional carbon source was initiated to further increase the biomass concentration. Based on biomass yields on carbon, nitrogen, and magnesium sources [48], the feed composition was designed in such a way that cultures were carbon source-limited and the aqueous concentrations of nitrogen and magnesium in the reactor remained constant during fed-batch cultivation. For citrate-fed cultures, an acidic feed (using citric acid instead of tri-sodium citrate) enabled the use of the pH as an indicator for overfeeding.

During fed-batch cultivation, the biomass yield ($Y_{x/s}$) dropped to 0.23 g_{CDW} $g_{citrate}^{-1}$ or 0.18 g_{CDW} $g_{glycerol}^{-1}$ and high cell densities only were obtained when cells were grown at low rates ($\mu \leq 0.1$ h^{-1} and $\mu \leq 0.05$ h^{-1} for growth on citrate and glycerol, respectively).

By using the described feeding strategies, it was possible to increase the biomass concentration during fed-batch cultivation to 44 g_{CDW} l^{-1}, which led to limiting oxygen concentrations ($pO_2 < 10\%$ at an aeration rate of 5 vvm and a stirrer speed of 2,800 rpm). Due to the high oxygen requirements demanding high energy input, such a biomass concentration would not be feasible on a large scale and was therefore not applied in further experiments. Instead, biotransformations were initiated at a biomass concentration of 15 g_{CDW} l^{-1} by the addition of BEHP containing (*S*)-limonene and DCPK.

When citrate was used as the carbon source, organic phase concentrations of up to 13.0 g (*S*)-perillyl alcohol L_{org}^{-1} were obtained in 24 h. This corresponds to a product concentration in the total volume of 28.7 mM (4.3 g L_{tot}^{-1}; Fig. 5a) and a product yield on substrate consumed ($Y_{p/s}$) of 48% mol mol^{-1}. If only the first 14 h are considered, in which cells displayed a high productivity, a $Y_{p/s}$-value of 62% mol mol^{-1} was reached. The remainder of consumed (*S*)-limonene was evaporated from the reactor or converted to the side products perillyl aldehyde and perillic acid (Fig. 5a). During the biotransformation, a maximum productivity of 0.42 g L_{tot}^{-1} h^{-1} was achieved (Fig. 5b). Longer reaction times did not result in higher product concentrations.

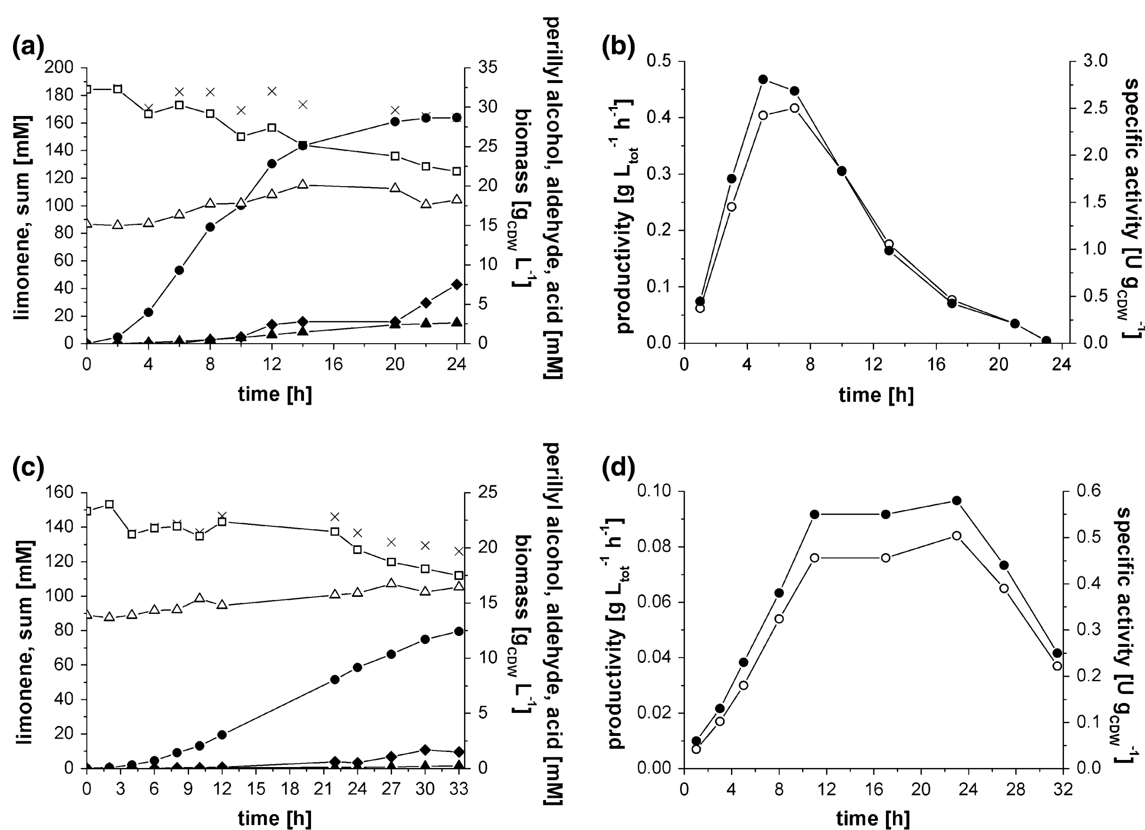

Fig. 5 Two-liquid-phase bioconversions of (*S*)-limonene by whole-cells of *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) growing on citrate (**a**, **b**) or glycerol (**c**, **d**). **a**, **c** show the concentration time courses (based on total volume) for the following compounds: (*S*)-limonene (*open square*), (*S*)-perillyl alcohol (*filled circle*), perillyl aldehyde (*filled triangle*), perillic acid (*filled diamond*), sum of terpenes (*times*), and biomass (*open triangle*). Terpene concentrations were determined by gas chromatography. **b**, **d** show the productivity (*open circle*) and specific activity (*filled circle*) profiles during the bioconversions

Remarkably, when glycerol was used as a carbon source, significantly lower concentrations of (*S*)-perillyl alcohol were obtained. The product concentration in the organic phase reached a maximum of 5.7 g L_{org}^{-1} after 33 h of bioconversion. This corresponds to a product concentration in the total volume of 12.4 mM (1.9 g L_{tot}^{-1}; Fig. 5c) and a product yield on substrate consumed ($Y_{p/s}$) of 33% mol mol^{-1}. A maximum productivity of 0.08 g L_{tot}^{-1} h^{-1} was achieved (Fig. 5d). Also in this case, longer reaction times did not result in higher product concentrations.

Independently repeated biotransformations for both carbon sources showed similar product formation patterns (data not shown). The accumulation of the side products perillyl aldehyde and perillic acid was observed in all biotransformations (Fig. 5a, c).

During the bioconversions with both carbon sources, the specific hydroxylation activities of cells sampled 5 h after biotransformation start were determined in separate small-scale resting cell assays as described in the Materials and methods section. The measured specific activity of citrate-grown cells was 17.8 U g_{CDW}^{-1}, which is similar to that found for cells grown in shake flasks. In contrast, the specific activity of glycerol-grown cells was 4.5 U g_{CDW}^{-1}, which is significantly lower than that found for cells grown in shake flasks (Table 2).

The use of *P. putida* KT2440 and optimizing growth and reaction conditions allowed for a sixfold improvement in productivity and a 1.9-fold higher product concentration as compared to the previously reported biotransformation [53].

Discussion

Specific activities and their dependence on physiological conditions

In order to exploit the full potential of a recombinant enzyme, conditions supporting optimal biocatalyst physiology have to be identified. A crucial parameter is the choice of the carbon source. In a previous study, a delicate balance between CYP153A6 apoprotein synthesis and heme synthesis was speculated to be achieved exclusively in cells that grow on alkanes [53]. However, under the optimized reaction conditions used in this study, glycerol- and citrate-grown cells also showed high hydroxylation activities (Table 2), which agrees with the finding that

citrate and glycerol do not repress the *alk* regulatory system of pCom vectors in *Pseudomonas* [46]. The use of citrate or glycerol as an alternative carbon source to octane offers two important advantages for the development of an efficient biotransformation procedure for limonene hydroxylation: (a) CYP153A6 is fully dedicated to the desired bioconversion and not involved in alkane catabolism. (b) The use of citrate or glycerol allows uncoupling of enzyme synthesis and cell growth as these carbon sources do not induce the *alk* regulatory system. This enables faster growth and simplified process handling.

The specific activity of octane-grown *P. putida* GPo12 (pGEc47ΔB)(pCom8-PFR1500) was determined to be 7.9 times higher than previously reported [53]. This difference may be explained by different assay conditions, which were optimized in this study with respect to oxygen availability (low cell concentrations, small reaction volumes, high shaking speed). Moreover, resting cells were always supplemented with an energy source (1% (w/v) of the carbon source) during the assay.

Living microbial cells displayed higher activities and enzyme-specific turnover numbers than cell extracts (Table 3). Lower activities and turnover numbers in extracts can be caused by partial denaturation of the essential electron transport proteins (ferredoxin and ferredoxin reductase) during preparation of the extracts. Another reason might be reduced interactions between CYP153A6 and the electron transport partners due to a lower degree of structural organization or lower local concentrations of the three proteins. Thus, entire cells harboring the CYP153A6 system are suitable catalysts for limonene hydroxylation because they provide a stabile and organized environment.

Overall, adapting carbon source, energy source, and oxygen availability as well as the choice for whole cells or cell extracts were identified as crucial factors determining the efficiency of biocatalytic limonene hydroxylation.

Specific activities during two-liquid-phase biotransformations

During two-liquid-phase bioconversions with growing cells, much lower specific activities (Fig. 5b, d) were obtained as compared to resting cell assays (Table 2). Resting cells need NADH for catalysis and maintenance, whereas growing cells require additional NADH for biomass formation. However, a limitation by intracellular NADH availability is unlikely as growing cells have been reported to support higher oxygenase activities [5, 9]. E.g., NADH availability was found not to be a limiting factor for styrene epoxidation by growing *Pseudomonas* sp. strain VLB120ΔC at rates up to 54 U g_{CDW}^{-1} [34].

A limitation in substrate availability is a more probable explanation for the difference between activities observed in resting cell assays and two-liquid-phase biotransformations. In a BEHP-based two-liquid-phase system, growing *E. coli* JM101 (pSPZ3) catalyzing pseudocumene oxidation showed a maximum specific activity, which was ten times lower than the activity achieved with resting cells (initial substrate concentration in the BEHP-phase: 170 mM) [9]. This difference was attributed to a limitation in substrate availability with the specific activity depending on the substrate concentration in the organic phase [7]. Considering the high partition coefficient of limonene in the applied BEHP/medium system (Table 4), which is similar to the partition coefficient of pseudocumene (log $K_p = 4.39$) [7], substrate availability is likely to limit perillyl alcohol formation.

Limited substrate availability, however, does not explain the observed difference in productivity between cells growing on glycerol and citrate during carbon-limited fed-batch cultivation in a two-liquid-phase system (Fig. 5). Remarkably, growth under the former conditions led to fivefold lower resting cell activities (4.5 U g_{CDW}^{-1} as determined in separate assays) as compared to batch growth (22.6 U g_{CDW}^{-1}). With citrate as the carbon source, both conditions resulted in similar activities (17.8 and 16.3 U g_{CDW}^{-1}, respectively). One reason for the reduced activities during glycerol-limited growth might be the low feed rate (for a $\mu \leq 0.05$ h^{-1}), applied in order to achieve high cell densities. Low growth rates may result in low specific activities due to a reduced transcription of ribosomal rRNA, affecting (recombinant) gene expression [17]. Low growth rates also increase the relative maintenance requirements of microorganisms, leading to a reduced yield on carbon source ($Y_{x/s}$) [23]. This is consistent with the reduced biomass yields obtained during fed-batch cultivations with glycerol as well as citrate.

In summary, two-liquid-phase biotransformations appear to be affected, beside the above-mentioned physiological constraints, by substrate availability. Such a physical limitation may be alleviated by screening for alternative solvents. In such a screening, aspects like product extraction capacity, biocompatibility, and ease of product recovery or the co-expression of a substrate uptake system should also be considered.

Cells carrying plasmid pGEc47ΔB display enhanced hydroxylation activities

The presence of plasmid pGEc47ΔB in *P. putida* cells was shown to enhance specific hydroxylation activities by a factor of 19 (Fig. 2). Intracellular CYP153A6 concentrations remained largely unaffected by the presence of plasmid pGEc47ΔB and thus can be ruled out as a possible cause for the increased activity. A similar effect has been described for different recombinant *E. coli* strains performing CYP102A1-catalyzed pentadecanoic acid

hydroxylation [42]. *E. coli* strains carrying plasmid pGEc47 (encoding the same genes as pGEc47ΔB plus a gene for the non-heme alkane monooxygenase AlkB) displayed 1.9- to 2.9-fold higher specific hydroxylation activities than *E. coli* strains without this plasmid. It was speculated that this difference was due to a substrate uptake system encoded on pGEc47 [42]. This uptake system might also be responsible for the enhanced hydroxylation activities of *P. putida* carrying pGEc47ΔB. On the other hand, pGEc47ΔB also contains two genes encoding electron transport proteins (AlkG and AlkT), which are biochemically similar to the redox partners of CYP153A6, FdR and Fdx. Like FdR, AlkT contains a flavin adenine (FAD) redox center and accepts electrons from NADH [49, 51], whereas Fdx and AlkG are both electron transfer proteins containing iron-sulfur clusters [35, 51, 52]. AlkG and AlkT together were shown to be able to reduce cytochrome *c* [49] and thus might sustain electron transfer to the heme–iron center of CYP153A6. In addition, the gene for FdR on pCom8-PFR1500 is not preceded by a recognizable ribosome-binding site and overlaps with the CYP153A6 gene by 1 base pair [53], possibly leading to low FdR expression levels. This may limit limonene hydroxylation in *P. putida* cells harboring only pCom8-PFR1500.

Optimization of the electron transfer chain by screening for alternative redox partners can be an effective tool to further improve process performance. Such a screening has been performed for CYP106A2, converting testosterone to 15β-hydroxytestosterone, and has led to a 16-fold improvement in product yield [39]. The redox partners of the alkane monooxygenase AlkB (AlkG and AlkT) are interesting candidates for improvement of electron transfer to CYP153A6.

Yield, chemoselectivity, and productivity are affected by product degradation

During the two-liquid-phase bioconversions, small amounts of perillic acid and perillyl aldehyde were found as side products. The formation of these unwanted side products reduces the overall biotransformation yield and productivity and hinders efficient product isolation.

It remains to be investigated whether the small amounts of perillyl aldehyde and perillic acid are due to perillyl alcohol oxidation catalyzed by dehydrogenases or due to multiple oxidations catalyzed by CYP153A6. Multistep oxidations are common among CYP enzymes [50] and might be minimized by protein engineering. Alternatively, the reaction might be controlled kinetically using the two-liquid-phase concept as shown before for the multistep oxidation of pseudocumene [6, 7].

In the case where host intrinsic dehydrogenases are involved, multiple oxidations would strongly depend on the enzymatic background and thus host strain physiology. To circumvent unwanted product degradation by dehydrogenases, knock-out strains or alternative host strains lacking these enzymes, but still displaying high specific activities, could provide a solution.

Conclusions

Although CYP-based biooxidations have a high potential for industrial application, the productivities achieved are often too low to be of economic relevance [22]. In two-liquid-phase biotransformations, the conversion of (*S*)-limonene to (*S*)-perillyl alcohol was shown to be limited by cell physiology and not by intrinsic properties of CYP153A6. Careful host and carbon source selection allowed a 7.5-fold increase of the specific activity of resting cells. In two-liquid-phase biotransformations, the optimized physiological conditions and process control translated into a sixfold improvement in productivity compared to previously reported results. Further limitations identified include substrate availability and overoxidation. It can be expected that similar parameters constrain the reaction efficiency of many other CYP-based biotransformations. Therefore, it is strongly recommended to consider physiological effects for whole-cell catalyst design.

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