ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

Whole-Cell-Based CYP153A6-Catalyzed (*S*)-Limonene Hydroxylation Efficiency Depends on Host Background and Profits From Monoterpene Uptake via AlkL

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ABSTRACT: Living microbial cells are considered to be the catalyst of choice for selective terpene functionalization. However, such processes often suffer from side product formation and poor substrate mass transfer into cells. For the hydroxylation of (*S*)-limonene to (*S*)-perillyl alcohol by *Pseudomonas putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500), containing the cytochrome P450 monooxygenase CYP153A6, the side products perillyl aldehyde and perillic acid constituted up to 26% of the total amount of oxidized terpenes. In this study, it is shown that the reaction rate is substrate-limited in the two-liquid phase system used and that host intrinsic dehydrogenases and not CYP153A6 are responsible for the formation of the undesired side products. In contrast to *P. putida* KT2440, *E. coli* W3110 was found to catalyze perillyl aldehyde reduction to the alcohol and no oxidation to the acid. Furthermore, *E. coli* W3110 harboring CYP153A6 showed high limonene hydroxylation activities (7.1 U g_{CDW}^{-1}). The outer membrane protein AlkL was found to enhance hydroxylation activities of *E. coli* twofold in aqueous single-phase and fivefold in two-liquid phase biotransformations. In the latter system, *E. coli* harboring CYP153A6 and AlkL produced up to 39.2 mmol (*S*)-perillyl alcohol L_{tot}^{-1} within 26 h, whereas no perillic acid and minor amounts of perillyl aldehyde (8% of the total products) were formed. In conclusion, undesired perillyl alcohol oxidation was reduced by choosing *E. coli*'s enzymatic background as a reaction environment and co-expression of the *alkL* gene in *E. coli* represents a promising strategy to enhance terpene bioconversion rates.

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KEYWORDS: cytochrome P450 monooxygenase; whole-cell biocatalysis; C–H-oxyfunctionalization; substrate uptake; outer membrane protein AlkL; two-liquid phase biotransformations

Introduction

Selective C–H-bond functionalization is one of the most challenging reactions in organic chemistry and a key research area to which enzymatic catalysis contributes considerably (Lewis et al., 2010). Cytochrome P450 (CYP) monooxygenases constitute a group of enzymes able to oxyfunctionalize C–H-bonds selectively under mild reaction conditions using molecular oxygen as oxidizing agent (Bernhardt, 2006). However, handling CYPs on a technical scale remains challenging and implementation of CYP-based bioprocesses in industry is hampered by low space-time yields (Julsing et al., 2008; Leak et al., 2009).

Undesired side product formation is a phenomenon often observed with enzymatic oxidations and results in reduced product titers, space-time yields, and selectivity. For CYPs, it is often related to molecular oxygen activation by reduced active site home. Such partially reduced active site oxygen can leave the active site (uncoupling) and lead to the formation of reactive oxygen species and/or cause side product formation by substrate oxidation at multiple sites or overoxidation at one specific site, i.e., the formation of aldehydes, ketones, and/or acids (Bühler and Schmid, 2004; Guengerich et al., 2011; Julsing et al., 2008). In case whole-cell catalysts are used, interaction of the enzymatic

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background with the target product may also lead to side product formation or product degradation (Meyer et al., 2005; van Beilen et al., 2003). Nevertheless, (recombinant) microbial cells are generally favored over isolated CYPs for technical applications, because whole cells can cope with oxidative stress caused by reactive oxygen species, eliminate the need for protein purification, and allow easy cofactor recycling and co-expression of auxiliary electron transport proteins (Duetz et al., 2001; Leak et al., 2009). These factors increase process stability and productivity and reduce costs.

Perillyl alcohol is a valuable compound because of its anticarcinogenic properties (Lebedeva et al., 2008), its application in cosmetics (Opdyke, 1981), and its use as a synthetic precursor (Marshall and Van Devender, 2001). Extraction of the terpenoid from plant material and chemical synthesis from limonene can both be regarded as inefficient and environmentally unfriendly (Jensen and Sharpless, 1975; Lee et al., 2000; Sakuda, 1969). Biocatalytic limonene oxidation using CYP153A6 provides a promising alternative method (Cornelissen et al., 2011; van Beilen et al., 2005). During two-liquid phase bioconversions of (*S*)-limonene to (*S*)-perillyl alcohol with recombinant *Pseudomonas putida* KT2440 harboring CYP153A6 from *Mycobacterium* sp. strain HXN-1500, undesired perillyl aldehyde and perillic acid made up 26% of the total amount of reaction products, thus affecting space-time yield and complicating downstream processing. The presence of plasmid pGEc47ΔB, harboring the alkane degradation gene cluster (*alk*) of *P. putida* GPo1 except *alkB* (van Beilen et al., 1992), was necessary for an efficient conversion (19-fold higher resting cell activity), but may contribute to side product formation. Recently, co-expression of *alkL* encoding an outer membrane protein was shown to facilitate uptake and improve the oxygenation of alkanes and fatty acid methyl esters using *E. coli* cells containing the alkane monooxygenase AlkBGT (Julsing et al., 2012b).

The aim of this study was to establish an efficient limonene hydroxylation process with reduced side product formation and a high space-time yield for the target product (*S*)-perillyl alcohol. For this purpose, factors limiting the conversion rate and causes of side product formation with *P. putida* were investigated. Approaches for process intensification included the use of *E. coli* W3110 as alternative host and *alkL* co-expression as a strategy to improve terpene mass transfer into cells in the absence of pGEc47ΔB.

Materials and Methods

Bacterial Strains, Plasmids, and Microbiological Methods

All chemicals used for this study were purchased from Sigma–Aldrich (Steinheim, Germany) or Carl–Roth (Karlsruhe, Germany) at the highest purity available. (*S*)-limonene was routinely used at 99% purity except for two-liquid phase reactor experiments, in which 95%-pure (*S*)-limonene was applied. The strains and plasmids used are listed in Table I. *P. putida* cells were either grown on Luria–Bertani (LB) broth (Sambrook and Russell, 2001), E2 medium (Lageveen et al., 1988), or, for bioreactor experiments, aqueous batch medium (ABM) (Preusting et al., 1993). *E. coli* cells were either grown in LB broth, M9 medium (Sambrook and Russell, 2001), or, for bioreactor experiments, in Riesenberg medium (Riesenberg et al., 1991) supplemented with US* trace elements (Panke et al., 1999). E2 and ABM medium were supplemented with 0.5 and 1% (w/v) citric acid, respectively. M9 and Riesenberg medium were supplemented with 0.5 and 1.5% (w/v) glucose, respectively. The appropriate antibiotics (50 mg L^{-1} gentamycin or kanamycin, 12.5 mg L^{-1} tetracycline) were added to all media. Cultures were incubated in baffled Erlenmeyer flasks at 30°C. Cell densities were monitored by measuring the optical density at 450 nm (OD_{450}) as described elsewhere (Cornelissen et al., 2011). Recombinant

Table I. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Refs.
E. coli DH1	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A69 *thi*-1 *rel*A1	Hanahan (1983), Meselson and Yuan (1968)
E. coli DH5α	*sup*E44 Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan (1983)
E. coli W3110	F^-, λ^-, *rph*-1, IN(*rrnD–rrnE*)1	Bachmann (1972)
P. putida KT2440	*P. putida* mt-2 cured of the TOL plasmid, solvent-sensitive	Bagdasarian et al. (1981)
pGEc41	Contains *alkBFGH* and *alkST* genes in broad-host-range vector pLAFR1, Tc^r	Eggink et al. (1987)
pGEc47	Contains genes necessary for growth on alkanes (*alkBFGHJKL* and *alkST*) in broad-host-range vector pLAFR1, Tc^r	Eggink et al. (1987)
pGEc47ΔB	pGEc47 without gene for the alkane monooxygenase AlkB, Tc^r	van Beilen et al. (1992)
pCom8-PFR1500	Contains genes for CYP153A6, ferredoxin, and ferredoxin reductase from *Mycobacterium* sp. strain HXN-1,500 in broad-host-range vector pCom8, Gm^r	van Beilen et al. (2005)
pCom8-PFR1500L	pCom8-PFR1500 with *alkL* gene, Gm^r	This study
pCom10-AlkL	pCom10 with *alkL* gene, Km^r	Julsing et al. (2012a,b)
pLAFR3	Broad-host-range vector pLAFR1 with 454 bp *Hae*II fragment of pUC8 containing multiple cloning sites and α-complementation for β-galactosidase activity, Tc^r	Staskawicz et al. (1987)
pLAFR3-AlkL	pLAFR3 containing *alk* promoter and *alkL* gene, Tc^r	This study

Tc, tetracycline; Gm, gentamycin, and Km, kanamycin.

strains were obtained by introducing plasmids via electroporation (2,500 V, Equibio EasyjecT Prima, Ashford, UK) or heat shock treatment (Sambrook and Russell, 2001).

Construction of pCom8-PFR1500L and pLAFR3-AlkL

Restriction enzymes, T4 ligase, and thermostable alkaline phosphatase were purchased from Fermentas GmbH (St. Leon-Rot, Germany). High-fidelity DNA polymerase was obtained from Finnzymes Oy (Espoo, Finland). All enzymes were used according to the supplier's recommendations. The primers used in this study are listed in Table II. For the construction of pCom8-PFR1500L, the *alkL* gene was amplified by PCR using pGEc47 as template and primers F1 and R1. The 757 bp PCR-product and pCom8-PFR1500 were digested with *Hin*dIII. Then, pCom8-PFR1500 was treated with phosphatase, ligated with the digested PCR-product, and transformed into *E. coli* DH5α. For the construction of pLAFR3-AlkL, the *alkL* gene together with *alk* promoter and ribosomal binding site was amplified by PCR using pCom10-AlkL as template and primers F2 and R2. The 908 bp PCR-product and pLAFR3 were digested with *Hin*dIII and *Bam*HI, ligated, and transformed into *E. coli* DH5α. Correct plasmid constructions were confirmed by sequencing of the insert regions.

Determination of Intracellular CYP Concentration

CYP153A6 concentrations in whole cells were quantified by CO-difference spectra as described before (Cornelissen et al., 2011) using a molar extinction increment between 450 and 490 nm of 91 $mM^{-1}\,cm^{-1}$ (Omura and Sato, 1964).

Activity Determination With Resting Cells

Activities are given in U g_{CDW}^{-1}, with 1 U = 1 μmol product min^{-1}. Activities of resting cells at low cell densities (0.5 $g_{CDW}\,L^{-1}$) were determined as described before (Cornelissen et al., 2011). Whole-cell reaction kinetics was investigated by varying the limonene concentration in resting cell assays between 0.01 and 1.7 mM. Apparent kinetic parameters were determined by weighed nonlinear regression for enzyme kinetic data using the software OriginPro 7.5 (OriginLab Corporation, North Hampton, MA).

Table II. Primers used in this study.

Primer name	Sequence[a]	Restriction site
F1	5′-CCCAAGCTTCAACAAAACGAGGGTAGCAC-3′	*Hin*dIII
R1	5′-CCCAAGCTTCTGCGACAGTGACAGACCTG-3′	*Hin*dIII
F2	5′-CCCAAGCTTCATGCTTGCGGCCAGCTCGT-3′	*Hin*dIII
R2	5′-TCGACGGATCCTTAGAAAACATATGACGCA-3′	*Bam*HI

[a]Restriction sites are underlined.

For biotransformations with higher concentrations of resting cells (~10 $g_{CDW}\,L^{-1}$), bacteria were cultivated in a 3.1 L KLF 2000 bioreactor (Bioengineering, Wald, Switzerland) by adding a 0.1 L preculture to 0.9 L of fresh medium. Growth conditions were set to 30°C, 1,000 rpm, pH 7.4, and 1.5 vvm aeration. Cells were grown to a biomass concentration of 0.2 $g_{CDW}\,L^{-1}$ and induced by addition of 0.025% (v/v) di-cyclopropyl ketone (DCPK). For *E. coli* cultures, 0.5 mM δ-aminolevulinic acid (ALA) was added. Incubation was continued for 5 h, after which cells were harvested by centrifugation (20 min, 4,600*g*, 4°C) and resuspended to a concentration of ~10 $g_{CDW}\,L^{-1}$ in 50 mM potassium phosphate buffer (pH 7.4), containing 1% (w/v) of the energy source. Of this stock, 200 mL were transferred to a 0.5 L stirred tank RALF bioreactor (Bioengineering). Reaction conditions were set to 30°C, 1,500 rpm, and 5 (vvm) aeration. The reaction was started by substrate addition from 0.2 M stock solutions in ethanol. Terpene concentrations were determined by gas chromatography (GC): 2 mL samples were taken during the reaction and processed as described before (Cornelissen et al., 2011). For the determination of terpene concentrations by high performance liquid chromatography (HPLC), 0.5 mL samples were taken and added to 0.25 mL ice-cold acetonitrile (ACN). After vortexing for 1 min, samples were centrifuged (15 min, 17,000*g*, 4°C) and the supernatant was analyzed (see Analytical Methods Section).

Two-Liquid Phase Biotransformations

Two-liquid phase biotransformations with *E. coli* were performed as described previously (Kuhn et al., 2010). In short, a feed solution (500 g L^{-1} glucose and 13.4 g L^{-1} $MgSO_4 7H_2O$) was used for fed-batch cultivation after the batch phase (1 L) was finished (indicated by a steep pO_2 increase). An exponential feeding strategy was applied to sustain a growth rate of 0.2 h^{-1}. When the biomass concentration reached 15 $g_{CDW}\,L^{-1}$, 460 mL bis(2-ethylhexyl) phthalate (BEHP), 40 mL (*S*)-limonene, 1.4 mL DCPK, and 66 mg ALA were added to start the biotransformation, during which a constant feed of 0.15 g glucose min^{-1} was applied. Samples for GC and HPLC analysis were taken as described above.

Partition Coefficients in Two-Liquid Phase Systems

The partition coefficient (K_p) of perillyl aldehyde in a BEHP/medium system was determined as described before (Cornelissen et al., 2011). K_p is defined as the ratio of organic and aqueous phase concentrations.

Analytical Methods

Limonene concentrations were determined by GC as described previously (Cornelissen et al., 2011). Perillyl alcohol and aldehyde concentrations were determined by the same GC method or by reversed phase HPLC. Perillic acid concentrations were determined by HPLC.

Commercially available standards were used to identify and quantify the terpenes by GC or HPLC. A LaChrom Elite system (VWR, Darmstadt, Germany) equipped with a LiChroCART 250-4 LiChrospher 100 RP-18 e (5 μm) column and LiChroCART 4-4 LiChrospher 100 RP-18 e (5 μm) precolumn (Merck KGaA, Darmstadt, Germany) was used for HPLC analyses. Perillyl alcohol and aldehyde were detected at 208 nm using an L-2450 UV-diode array detector (VWR). The oven temperature was set to 40°C and the flow rate to 1.1 mL min^{-1}. The mobile phase consisted of ACN and distilled water (dH_2O), which were mixed according to the following profile: from 0 to 2 min constant ratio dH_2O:ACN = 72:28, linear gradient from 2 to 2.9 min to reach dH_2O:ACN = 44:56, constant ratio for 0.2 min, linear gradient from 3.1 to 4.1 min to reach dH_2O:ACN = 42:58, constant ratio for 0.5 min, linear gradient from 4.6 to 5.6 min to reach dH_2O:ACN = 22:78, constant ratio for 0.5 min, linear gradient from 6.1 to 13 min to reach dH_2O:ACN = 3:97, constant ratio for 1 min, linear gradient from 14 to 20 min to reach dH_2O:ACN = 72:28. For perillic acid quantification, the same system and settings were used with the following modifications: perillic acid was measured at 215 nm and the mobile phase consisted of ACN and 50 mM ammonium acetate buffer (pH 5.0) mixed according to the following profile: from 0 to 8 min constant ratio buffer:ACN = 64:36, switch to buffer:ACN = 3:97, constant ratio for 2 min, switch to buffer:ACN = 64:36, constant ratio for 7 min.

Results

Substrate Availability Limits Limonene Hydroxylation in Two-Liquid Phase Bioconversions

The two-liquid phase concept with BEHP as organic carrier solvent was followed to prevent toxic effects of substrate and product and to enable in situ product extraction (Cornelissen et al., 2011; van Beilen et al., 2005). In two-liquid phase bioconversions, specific limonene hydroxylation activities of citrate-grown *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) have been found to be 5.8-fold lower than in aqueous resting cell assays. The high partition coefficient of limonene in a BEHP/medium system ($K_p = 19{,}000$) causes low limonene concentrations in the aqueous phase, which may lead to a kinetic limitation of limonene hydroxylation. In order to verify this hypothesis, apparent aqueous reaction kinetics was investigated by varying the limonene concentration in resting cell activity assays and several two-liquid phase biotransformations were performed with varying amounts of limonene in the organic phase.

Kinetic analyses showed that limonene hydroxylation by *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) follows Michaelis–Menten-type kinetics involving uncompetitive substrate inhibition: $V = V_{max} \times [S]/(K_s + [S] \times (1 + [S]/K_i))$ with $V_{max} = 25.3 \pm 0.6$ U g_{CDW}^{-1}, $K_s = 238 \pm 8$ μM, and $K_i = 2.44 \pm 0.17$ mM, whereas K_s is the substrate uptake constant for whole cells. Based on these kinetic parameters and the partition coefficient for limonene, theoretical maximum activities in the two-liquid phase system were estimated as a function of the substrate concentration in the organic BEHP phase, assuming uptake from the aqueous phase only and non-limiting mass transfer from the organic into the aqueous phase (solid line in Fig. 1A). In two-liquid phase biotransformations, both maximum activities and product formation patterns depended on the limonene concentration (Fig. 1). Measured maximum activities agreed well with the theoretical maximum activities (Fig. 1A). These results are clear evidence for substrate limitation during (*S*)-limonene hydroxylation with *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) growing in a BEHP-based two-liquid phase system.

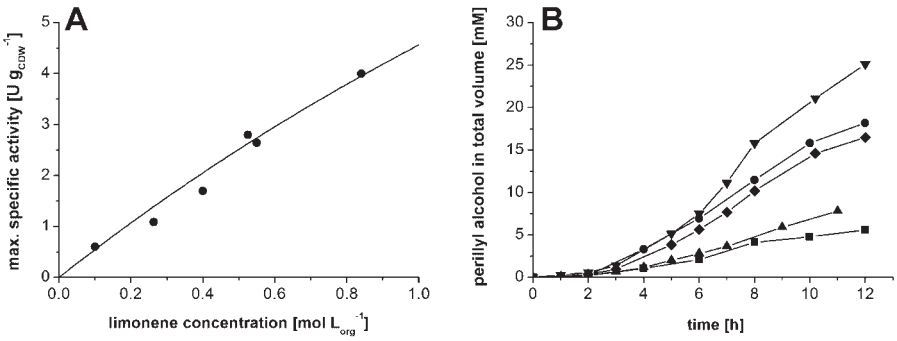

Figure 1. **A**: Correlation between the limonene concentration in the organic phase (phase ratio 1:3) and the maximum specific hydroxylation activities of *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) during two-liquid phase bioconversions. Both measured (closed symbols) as well as theoretical activities (line) are plotted. Theoretical values were calculated using the parameters of the aqueous kinetics and the partition coefficient of limonene in a BEHP/medium system (see text). **B**: Time course of perillyl alcohol formation during two-liquid phase biotransformations with different initial limonene concentrations in the organic phase (0.1 M (■), 0.25 M (▲), 0.5 M (◆), 0.6 M (●), and 0.9 M (▼)). The same growth conditions were applied allowing a similar course of cell concentrations.

Side Product Formation by *P. putida*

During such two-liquid phase bioconversions of (*S*)-limonene to the target product (*S*)-perillyl alcohol, perillyl aldehyde and perillic acid accumulated as side products (Cornelissen et al., 2011). No side products have been detected during activity assays with low cell densities ($0.5\,g_{CDW}\,L^{-1}$), which may be due to a kinetic limitation by the small amount of alcohol produced ($\leq$0.36 mM) and side product concentrations close to the detection limit ($\sim$0.01 mM by HPLC). In order to characterize side product formation by *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500), resting cells were applied at a high cell density ($8.6\,g_{CDW}\,L^{-1}$). A bioreactor was used to prevent oxygen limitation during the reaction, which was started by limonene addition to a total concentration of 6 mM (Fig. 2). In the first 2 h, limonene evaporation, as confirmed in a system without cells, led to a rapid decrease in the sum of terpene concentrations. In total, 65% of the limonene was lost by evaporation, while the remaining 35% were oxidized with a maximum specific activity of $6.5\,U\,g_{CDW}^{-1}$, which agrees well with the $7.2\,U\,g_{CDW}^{-1}$ expected at a limonene concentration of 6 mM considering the kinetic parameters introduced above. Perillyl alcohol was further oxidized to perillyl aldehyde and perillic acid (Fig. 2). The aldehyde was found in trace amounts, suggesting that alcohol oxidation is the rate-limiting step ($\leq 1.1\,U\,g_{CDW}^{-1}$) during conversion of limonene to perillic acid. The acid was not further converted nor degraded by *P. putida* KT2440 (data not shown). Thus, in absence of a carrier solvent, resting *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) produce perillic acid from limonene with perillyl alcohol and aldehyde as intermediates.

To elucidate whether host intrinsic enzymes are responsible for side product formation, activity assays were performed with *P. putida* strains harboring pCom8-PFR1500 and pGEc47ΔB, pGEc47ΔB only, or no plasmid. Perillyl alcohol and perillyl aldehyde were used as substrates. *P. putida* KT2440 converted perillyl aldehyde to the acid at a rate of $2.4\,U\,g_{CDW}^{-1}$ (Fig. 3A) with a similar conversion pattern as found for *P. putida* KT2440 (pGEc47ΔB) (pCom8-PFR1500) ($2.5\,U\,g_{CDW}^{-1}$, data not shown). This indicates that host intrinsic enzymes are responsible for the conversion of perillyl aldehyde to perillic acid.

P. putida KT2440 (pGEc47ΔB)(pCom8-PFR1500), *P. putida* KT2440 (pGEc47ΔB), and *P. putida* KT2440 converted the alcohol directly to the acid at different specific rates of 0.9, 5.2, and $0.4\,U\,g_{CDW}^{-1}$, respectively (Fig. 3B–D). Thus, although host intrinsic enzymes are capable of oxidizing perillyl alcohol at low rates, a pGEc47ΔB-encoded enzyme is mainly responsible for perillyl alcohol oxidation. The results show that side product formation is caused by the enzymatic background of *P. putida* KT2440 harboring pGEc47ΔB.

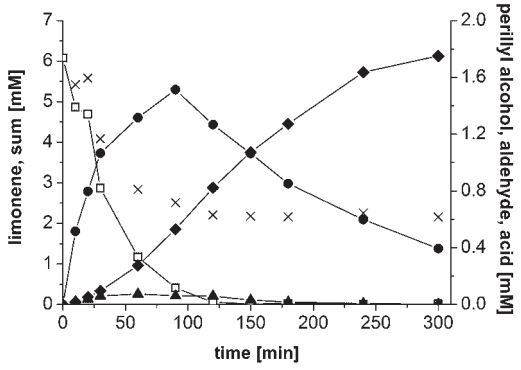

Figure 2. Time course of limonene hydroxylation by resting cells of *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500). (*S*)-limonene was added to a cell suspension with a biomass concentration of $8.6\,g_{CDW}\,L^{-1}$. Concentrations of (*S*)-limonene (□), (*S*)-perillyl alcohol (●), (*S*)-perillyl aldehyde (▲), (*S*)-perillic acid (◆), and the sum of all terpenes (×) are given. Perillic acid concentrations were determined by HPLC, all other terpene concentrations were determined by GC.

Roles of AlkL and AlkJ in Limonene Oxidation

Plasmid pGEc41 is identical to pGEc47ΔB, but does not encode genes for the alcohol dehydrogenase AlkJ, the acetyl-CoA synthase AlkK, and the outer membrane protein AlkL. The plasmid was used to investigate the possible involvement of AlkJ in side product formation and the influence of AlkL on the hydroxylation rate. This plasmid carries all genes encoding the alkane monooxygenase AlkBGT (Eggink et al., 1987), which does not accept limonene as a substrate (data not shown). *P. putida* KT2440 (pGEc41)(pCom8-PFR1500) showed a much lower limonene hydroxylation activity than *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) (0.9 instead of $16.3\,U\,g_{CDW}^{-1}$), comparable to that found for *P. putida* KT2440 (pCom8-PFR1500) ($0.8\,U\,g_{CDW}^{-1}$). The results do not allow conclusions on the role of AlkJ in side product formation, but indicate that AlkL is necessary for efficient limonene oxidation.

Limonene Hydroxylation by Recombinant *E. coli*

With the aim to minimize side product formation, the influence of the enzymatic background of *E. coli* on perillyl alcohol formation was investigated. For the oxidation of substituted toluenes to corresponding alcohols, aldehydes, and acids, unspecific dehydrogenases from *E. coli* were found to counteract alcohol oxidation (Bühler et al., 2000; Meyer et al., 2005). Hence, *E. coli* may be an interesting host for perillyl alcohol synthesis. *E. coli* W3110 was chosen because it is well-described (Hayashi et al., 2006), suitable for *cyp* gene expression (Fasan et al., 2010), and not reported to contain enzymes acting on intermediates of limonene degradation pathways. Upon perillyl alcohol addition to *E. coli* W3110, no significant conversion was observed (Fig. 4A). Only trace amounts of perillyl aldehyde and no perillic acid were detected. Perillyl aldehyde as substrate was reduced almost completely to perillyl alcohol at a rate of

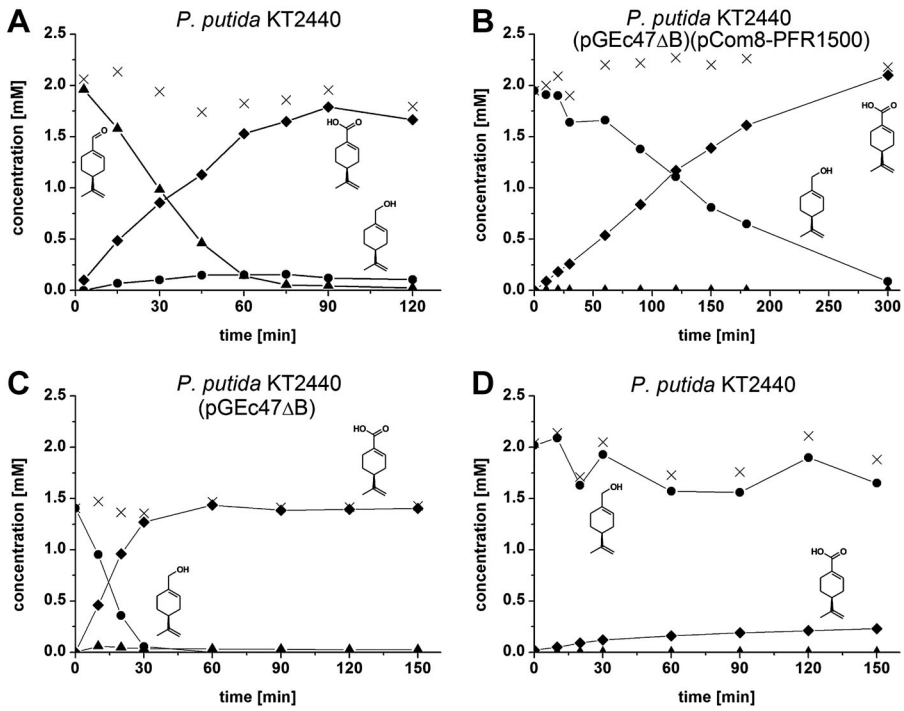

Figure 3. Perillyl alcohol and aldehyde oxidation by resting *P. putida* KT2440 cells in a 0.3 L bioreactor. **A**: (*S*)-perillyl aldehyde oxidation by *P. putida* KT2440, biomass concentration: 9.7 $g_{CDW}\,L^{-1}$. **B**: (*S*)-perillyl alcohol oxidation by *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500), biomass concentration: 10.6 $g_{CDW}\,L^{-1}$. **C**: (*S*)-perillyl alcohol oxidation by *P. putida* KT2440 (pGEc47ΔB), biomass concentration: 7.2 $g_{CDW}\,L^{-1}$. **D**: (*S*)-perillyl alcohol oxidation by *P. putida* KT2440, biomass concentration: 8.0 $g_{CDW}\,L^{-1}$. Concentrations of perillyl alcohol (●), perillyl aldehyde (▲), perillic acid (◆), and the sum of all terpenes (×) are given. All concentrations were determined by HPLC.

4.9 U g_{CDW}^{-1} (Fig. 4B). Thus, the enzymatic background of *E. coli* W3110 appears promising for perillyl alcohol synthesis.

E. coli GEc137, a *fadR*-deficient DH1 derivative, harboring pGEc47ΔB and pCom8-PFR1500 has been reported to enable a limonene hydroxylation activity of only 0.1 U g_{CDW}^{-1} (van Beilen et al., 2005). This suggests that *E. coli* is not suitable for limonene hydroxylation. To verify this, the activity of induced *E. coli* W3110 (pCom8-PFR1500) was determined in activity assays, and, surprisingly, high activities were found (Table III). This activity even doubled for *E. coli* additionally harboring pGEc47ΔB, correlating with a higher CYP153A6 expression level (Table III). Because these observations are in contradiction with the study using *E. coli* GEc137 (pGEc47ΔB)(pCom8-PFR1500) (van Beilen et al., 2005), activities of *E. coli* DH1 and *fadR*-

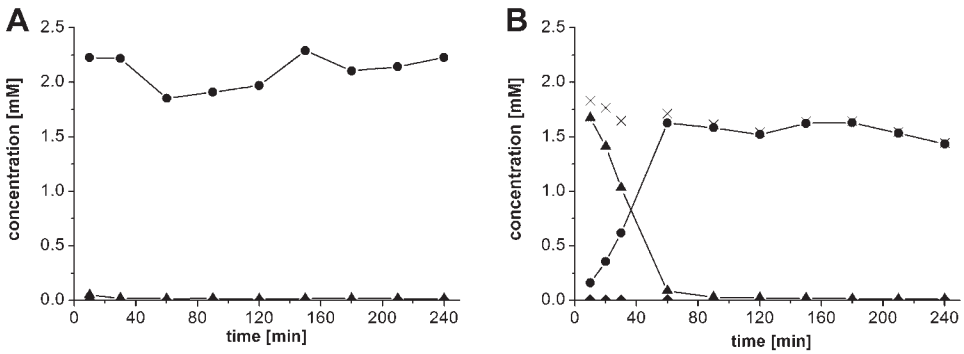

Figure 4. Perillyl alcohol and aldehyde conversion by resting *E. coli* W3110 cells in a 0.3 L bioreactor. **A**: (*S*)-perillyl alcohol tested as substrate for *E. coli* W3110, biomass concentration: 5.5 $g_{CDW}\,L^{-1}$. **B**: (*S*)-perillyl aldehyde reduction by *E. coli* W3110, biomass concentration: 5.5 $g_{CDW}\,L^{-1}$. Concentrations of perillyl alcohol (●), perillyl aldehyde (▲), perillic acid (◆), and the sum of all terpenes (×) are given. All concentrations were determined by HPLC.

Table III. Specific (*S*)-limonene hydroxylation activities of and CYP153A6 expression levels in *E. coli* W3110 and *P. putida* KT2440 harboring different plasmids.

	E. coli W3110		*P. putida* KT2440	
Plasmid(s)	Specific activity[a] ($U\,g_{CDW}^{-1}$)	Expression level ($nmol\,g_{CDW}^{-1}$)	Specific activity[a] ($U\,g_{CDW}^{-1}$)	Expression level ($nmol\,g_{CDW}^{-1}$)
pCom8-PFR1500	7.1 ± 1.8	156 ± 8	0.8 ± 0.1	63 ± 12
pCom8-PFR1500 and pGEc47ΔB	14.3 ± 0.5	249 ± 31	16.3 ± 2.1	148 ± 20
pCom8-PFR1500L	16.6 ± 1.9	166 ± 5	<0.5	34 ± 1
pCom8-PFR1500 and pLAFR3-AlkL	15.2 ± 2.6	146 ± 16	<0.5	20 ± 5

Product formation was measured by GC: $1\,U = 1\,\mu mol$ of product formed per min. CDW, cell dry weight.
[a]The activities given are based on perillyl alcohol formation within 10 min after adding 2 mM (*S*)-limonene to resting cells ($0.5\,g_{CDW}\,L^{-1}$).

deficient *E. coli* W3110 carrying the same plasmids were tested. Also for these two strains, high activities were observed (18.1 ± 1.9 and $15.5 \pm 1.1\,U\,g_{CDW}^{-1}$, respectively). Thus, neither the use of a *fadR*-deficient strain nor the use of *E. coli* W3110 instead of DH1 explains the discrepancy between previous observations and results presented here. However, *E. coli* W3110 (pCom8-PFR1500) constitutes an active biocatalyst and was used for further investigations.

AlkL Enhances Limonene Hydroxylation With *E. coli* But Not With *P. putida*

The presence of pGEc47ΔB in *E. coli* and *P. putida* enhances limonene hydroxylation by CYP153A6, but it was also found to be involved in alcohol oxidation (Fig. 3). In order to test whether the outer membrane protein AlkL is responsible for the activity enhancement of cells carrying pGEc47ΔB, the *alkL* gene was cloned into pCom8-PFR1500 (giving pCom8-PFR1500L) and into pLAFR3 under the control of the *alk* promoter (giving pLAFR3-AlkL). pLAFR3 is the basic vector of pGEc47ΔB, not containing sequences from the OCT plasmid of *P. putida* GPo1 (Staskawicz et al., 1987). *E. coli* W3110 carrying pCom8-PFR1500L or pLAFR3-AlkL and pCom8-PFR1500 showed 2.1- to 2.3-fold higher limonene hydroxylation activities than *E. coli* W3110 (pCom8-PFR1500) at similar CYP153A6 levels (Table III). This demonstrates that *alkL* expression enhances limonene hydroxylation by *E. coli* cells. However, this effect was not observed with *P. putida* (Table III). Interestingly, the presence of pGEc47ΔB enhanced CYP153A6 expression levels in *E. coli* and especially in *P. putida*, where only in this case reasonable expression levels were reached. Variations among the low expression levels in *P. putida* without pGEc47ΔB are considered to be of minor significance. Obviously, the OCT plasmid-derived fragment on pGEc47ΔB contains an additional factor/mechanism promoting gene expression under control of the *alk* regulatory system. This factor especially is relevant in *P. putida*, which may explain why the presence of *alkL* in recombinant *P. putida* did not restore substantial CYP153A6 activity levels. The high activity and the promising enzymatic background make *E. coli* W3110 (pCom8-PFR1500L) highly interesting for limonene hydroxylation.

Two-Liquid Phase Biotransformations With *E. coli*

To investigate whether these promising biocatalyst properties translate into high productivities and yields, two-liquid phase biotransformations were performed with growing *E. coli* W3110 harboring pCom8-PFR1500 or pCom8-PFR1500L and BEHP as carrier solvent. *E. coli* W3110 (pCom8-PFR1500) produced $56\,mmol\,L_{org}^{-1}$ ($18.9\,mmol\,L_{tot}^{-1}$) of perillyl alcohol within 31.5 h (Fig. 5A). During the biotransformation, $3.8\,mmol\,L_{tot}^{-1}$ of perillyl aldehyde were formed, while perillic acid formation was not detectable. With *E. coli* W3110 (pCom8-PFR1500L), fivefold higher activities (Table IV) and twofold higher product titers ($115\,mmol\,L_{org}^{-1}$ or $39.2\,mmol\,L_{tot}^{-1}$) were achieved within 26 h (Fig. 5B). In this period, no perillic acid and only $3.5\,mmol\,L_{tot}^{-1}$ perillyl aldehyde were formed (8% of the total amount of products). This translates into 1.4-fold higher product concentrations and 2.7-fold less side products as compared to *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) (Table IV), a considerable improvement in biocatalytic perillyl alcohol production.

Discussion

Limonene Overoxidation by *P. putida*

P. putida KT2440 (pGEc47ΔB)(pCom8-PFR1500) cells almost completely convert limonene to perillic acid in the absence of a carrier solvent (Fig. 2). Limonene oxidation to perillic acid may proceed via a three-step oxygenation catalyzed by CYP153A6. Such multistep oxidations are common for oxygenases (Bühler and Schmid, 2004; Guengerich et al., 2011) and examples include the oxidation of amorpha-4,11-diene to artemisinic acid by CYP71AV1 (Ro et al., 2006) and of *ent*-kaurene to *ent*-kaurenoic acid by CYP701A3 (Helliwell et al., 1999). Perillyl alcohol oxidation may also be catalyzed by strain-intrinsic enzymes, as it was reported, for example, for the geraniol dehydrogenase of *Castellaniella defragrans* (Lüddeke et al., 2012). Several *P. putida* strains have been reported to convert limonene to perillic acid, for example, *P. putida* PL (Dhavalikar and Bhattacharyya, 1966), *P. putida* GS1 (Speelmans et al.,

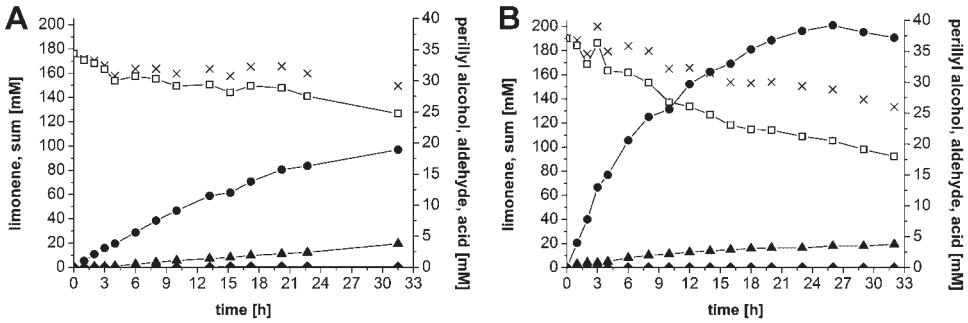

Figure 5. Two-liquid phase bioconversions (phase ratio 1:3) of (*S*)-limonene by whole cells of *E. coli* (pCom8-PFR1500) (**A**) and *E. coli* (pCom8-PFR1500L) (**B**) in a 3.1 L bioreactor. Concentration time courses (based on total volume): (*S*)-limonene (□), (*S*)-perillyl alcohol (●), (*S*)-perillyl aldehyde (▲), (*S*)-perillic acid (◆), and sum of terpenes (×). Terpene concentrations in the organic phase were determined by GC (limonene, perillyl alcohol, and perillyl aldehyde) and in the aqueous phase by HPLC (perillyl alcohol, perillyl aldehyde, and perillic acid).

1998), and *P. putida* DSM12264 (Mirata et al., 2009). For these strains, limonene degradation was attributed to the *p*-cymene pathway (Speelmans et al., 1998). The enzymes responsible for perillyl alcohol oxidation in *P. putida* PL have been identified as perillyl alcohol dehydrogenase (Ballal et al., 1966) and perillyl aldehyde dehydrogenase (Ballal et al., 1967). Such dehydrogenases also enable oxidation of perillyl alcohol to perillic acid in *P. putida* KT2440 (Fig. 3). Besides host intrinsic enzymes, pGEc47ΔB-encoded enzymes, with AlkJ as probable candidate, also seem to contribute to perillyl alcohol oxidation (Fig. 3C). Given the 5.8-fold lower alcohol oxidation activity of *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) compared to *P. putida* KT2440 (pGEc47ΔB), CYP153A6 gene expression appears to reduce pGEc47ΔB-encoded alcohol oxidation activity. However, this observation and the absence of aldehyde oxidation in *E. coli* containing active CYP153A6 (Fig. 5) indicate that CYP153A6 does not contribute to side product formation. Whereas aldehyde oxidation in *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) can be ascribed to the *P. putida* KT2440 host background, alcohol oxidation seems to be catalyzed by both host intrinsic and pGEc47ΔB-encoded enzymes.

P. putida as Host for Perillic Acid Formation

In a non-optimized system with 8.6 $g_{CDW}\,L^{-1}$ of resting *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500) and 6 mM limonene, 1.8 mM (*S*)-perillic acid (0.29 $g\,L^{-1}$) were produced within 5 h (Fig. 2). This represents a space-time yield of 58 $mg\,L^{-1}\,h^{-1}$, which is only 3.2-fold lower than that of a recently described bioprocess for (*R*)-perillic acid production by *P. putida* DSM12264 (185 $mg\,L^{-1}\,h^{-1}$) (Mirata et al., 2009). Optimization of the catalyst, for example, by overexpression of a suitable alcohol dehydrogenase gene, and the bioprocess, for example, by using higher cell densities, implementing a closed gas loop reactor to prevent limonene stripping (Pescheck et al., 2009), and applying limonene feeding and in situ product removal to prevent substrate and product toxicity (Mirata et al., 2009), are expected to considerably

Table IV. Process parameters of two-liquid phase limonene hydroxylation with *P. putida* KT2440 (pGEc47ΔB)(pCom8-PFR1500), *E. coli* W3110 (pCom8-PFR1500), and *E. coli* W3110 (pCom8-PFR1500L).

		Biotransformation		
Parameter	Unit	*P. putida* KT2440 (pGEc47ΔB) (pCom8-PFR1500)[a]	*E. coli* W3110 (pCom8-PFR1500)	*E. coli* W3110 (pCom8-PFR1500L)
Working volume	L	1.5	1.5	1.5
Phase ratio (V_{org}:V_{tot})	—	1:3	1:3	1:3
Reaction time	h	24	31.5	26
Maximal cell concentration	$g_{CDW}\,L_{aq}^{-1}$	20.1	27.8	29.3
Final perillyl alcohol conc.	$g\,L_{tot}^{-1}$	4.37	2.88	5.96
Final perillyl aldehyde conc.	$g\,L_{tot}^{-1}$	0.40	0.57	0.53
Final perillic acid conc.	$g\,L_{tot}^{-1}$	1.25	0	0
Product share of perillyl alcohol	mol%	74	83	92
Maximal specific perillyl alcohol formation rate	$U\,g_{CDW}^{-1}$	2.8	0.9	4.6
Overall productivity	$g\,L_{tot}^{-1}h^{-1}$	0.18	0.09	0.23

[a]Data from Cornelissen et al. (2011).

enhance space-time yields for perillic acid production. CYP153A6 allows both (*R*)- and (*S*)-limonene hydroxylation at similar rates (Duetz et al., 2003). Process optimization might therefore render an efficient approach that gives access to both (*R*)- and (*S*)-perillic acid, depending on the substrate fed. In summary, *P. putida* KT2440 is a suitable host in case perillic acid is the desired product.

Alcohol–Aldehyde Interconversion by *E. coli* Follows a Dehydrogenase-Type Equilibrium

Dehydrogenases catalyze reversible hydrogen transfer reactions, often using NAD(P/H) as co-factor (Blank et al., 2010). The estimated Gibbs free energy of primary alcohol oxidation mediated by NAD-dependent dehydrogenases lies around 4.8 kcal mol^{-1} (Mavrovouniotis, 1990). This translates into an equilibrium constant of $\sim 1 \times 10^{-3}$ (Bühler et al., 2000). The aldehyde–alcohol ratio at equilibrium depends on the intracellular NADH/NAD^+ ratio. Assuming a NADH/NAD^+ ratio of 0.3 for resting *E. coli* under aerobic conditions (Julsing et al., 2012a), an aldehyde–alcohol ratio of 3.3×10^{-3} can be expected. Considering that this value is an estimate neglecting pH effects, it matches well with the measured ratio in the range of 5.8–9.3 $\times 10^{-3}$ (Fig. 4). This suggests that, in *E. coli* W3110, perillyl aldehyde reduction is catalyzed by NAD(P)H-dependent dehydrogenase(s) not constrained by, for example, alcohol oxidase activities.

In two-liquid phase bioconversions, in situ extraction leads to perillyl aldehyde accumulation in the organic phase. Based on the alcohol and aldehyde concentrations in the organic phase and corresponding partition coefficients (188 for the alcohol and 1,314 for the aldehyde), the aqueous aldehyde–alcohol ratio after 6 h of biotransformation was determined to be 0.013 and remained constant for 24 h (Fig. 6). Based on this ratio, a NADH/NAD^+ ratio of 0.08 can be estimated for growing *E. coli* harboring AlkL, matching well with expected values of 0.05–0.2 for aerobically grown, glucose-limited *E. coli* (Julsing et al., 2012a; Snoep et al., 1993). This indicates that, after 6 h, a dehydrogenase-based alcohol–aldehyde equilibrium is established and maintained.

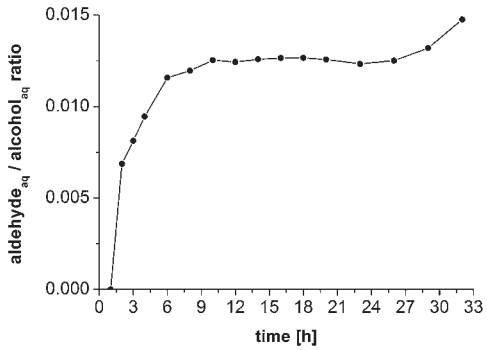

Figure 6. Perillyl aldehyde–perillyl alcohol ratio during two-liquid phase bioconversions of (*S*)-limonene by whole cells of *E. coli* (pCom8-PFR1500L) in a 3.1 L bioreactor. The aldehyde–alcohol in the aqueous phase was calculated from (molar) concentrations of perillyl aldehyde and perillyl alcohol in the organic phase and corresponding partition coefficients (see Results Section).

Selecting *E. coli* W3110 as alternative host enabled a significant reduction in side product formation and, by means of *alkL* co-expression, simultaneously led to an increase in cellular and volumetric productivity compared to the use of *P. putida* KT2440. In conclusion, *E. coli* W3110 is preferred over *P. putida* as host in case perillyl alcohol is the desired product. The knockout of alcohol dehydrogenases responsible for perrilyl alcohol oxidation in *E. coli* may further reduce side product formation.

Overexpression of *alkL*: A General Approach to Improve Whole-Cell Biocatalysis?

As was shown previously (Carter et al., 2003; Fontanille and Larroche, 2003), the outer membrane of gram-negative bacteria forms an effective barrier for monoterpenes. Strategies to circumvent mass transfer limitation over the outer membrane include the use of membrane permeable mutants (Ni and Chen, 2004), physical or chemical methods to enhance membrane permeability (Hanlon et al., 2007), or cell surface display strategies (Jose et al., 2002; Yim et al., 2010). The latter has the disadvantage that co-factor supply is critical and auxiliary proteins have to be co-displayed or added (Julsing et al., 2008). The other two methods increase cell permeability, are non-selective, and may affect cell viability and biocatalyst stability, for example, via leakage of essential ions, metabolites (e.g., NADH), and other macromolecules (Heipieper et al., 2007). This is undesired for oxygenase-based catalysis because only viable cells enable sustained co-factor regeneration, de novo synthesis of recombinant proteins and degradation of reactive oxygen species. Recently, *alkL* co-expression in *E. coli* W3110 containing AlkBGT, the membrane-bound alkane monooxygenase system from *P. putida* GPo1, was found to enhance the ω-hydroxylation of octane fourfold, nonane 40-fold, and dodecanoic acid methyl ester 28- and 62-fold in an aqueous single-phase and a two-liquid phase system, respectively (Julsing et al., 2012b). Here, with *E. coli* cells harboring a soluble CYP, AlkL was also found to improve monoterpene hydroxylation rates. This indicates a broad enzyme and substrate scope for AlkL.

Introduction of AlkL into *E. coli* enabled two- and fivefold increased limonene hydroxylation activities in aqueous single-phase and two-liquid phase systems, respectively (Tables III and IV). Thus, in the absence of AlkL, the reaction appears to be constrained by limonene transfer over the outer membrane. AlkL relieves this limitation in *E. coli* at least to some extent. As observed for dodecanoic acid methyl ester, the effect of AlkL was more prominent, when limonene was applied in a two-liquid phase system. In the case of limonene hydroxylation, substrate limitation, as it

was observed in the BEHP-based two-liquid phase system (Fig. 1), may have been reduced in the presence of AlkL as a result of favorable whole-cell kinetics (lower K_s for limonene).

AlkL also seems to be important for substrate uptake into *P. putida* cells as can be concluded from the 18-fold lower activity of cells carrying pGEc41 instead of pGEc47ΔB. However, for *P. putida* an additional pGEc47ΔB-encoded factor seems to be relevant, as AlkL alone did not enhance oxidation rates (Table III). Overall, co-expression of genes encoding outer membrane proteins/channels may develop to an important strategy to enable or improve whole-cell oxyfunctionalizations.

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