ARTICLE

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Synthesis of chiral 2-alkanols from *n*-alkanes by a *P. putida* Whole-Cell Biocatalyst

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ABSTRACT: The cytochrome P450 monooxygenase CYP154A8 from *Nocardia farcinica* was previously found to catalyze hydroxylation of linear alkanes (C_7–C_9) with a high regio- and stereoselectivity. The objective of this study was to integrate CYP154A8 along with suitable redox partners into a whole-cell system for the production of chiral 2-alkanols starting from alkanes. Both recombinant *Escherichia coli* and *Pseudomonas putida* whole-cell biocatalysts tested for this purpose showed the ability to produce chiral alkanols, but a solvent tolerant *P. putida* strain demonstrated several advantages in the applied biphasic reaction system. The optimized *P. putida* whole-cell system produced ~16 mM (*S*)-2-octanol with 87% *ee* from octane, which is more than sevenfold higher than the previously described system with isolated enzymes. The achieved enantiopurity of the product could further be increased up to 99% *ee* by adding an alcohol dehydrogenase (ADH) to the alkane-oxidizing *P. putida* whole-cell systems. By using this setup for the individual conversions of heptane, octane or nonane, 2.6 mM (*S*)-2-heptanol with 91% *ee*, 5.4 mM (*S*)-2-octanol with 97% *ee*, or 5.5 mM (*S*)-2-nonanol with 97% *ee* were produced, respectively. The achieved concentrations of chiral 2-alkanols are the highest reported for a P450-based whole-cell system so far.

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KEYWORDS: CYP154A8; *P. putida*; 2-octanol; ADH; P450; alkanes

Introduction

Selective oxidation of non-activated C-H bonds is one of the most challenging tasks in synthetic chemistry (Roduner et al., 2013). Cytochrome P450 monooxygenases (CYPs or P450s) are enzymes capable of catalyzing this reaction at ambient temperature and pressure, and present therefore an attractive alternative to chemical oxidation (Bernhardt and Urlacher, 2014; Lewis et al., 2011; O'Reilly et al., 2011; Roiban and Reetz, 2015). An important reaction catalyzed by certain P450s is the hydroxylation of (linear) alkanes (Roduner et al., 2013). Alkanes represent a cheap and easily available resource and their regio- and stereoselective hydroxylation can directly lead to valuable chiral building blocks for the chemical and pharmaceutical industry (Nolte and Urlacher, 2015; van Beilen and Funhoff, 2005, 2007). Wild type and "engineered" P450s have extensively been exploited for alkane hydroxylation. Terminal hydroxylation activity leading to primary alcohols was mainly described for the members of the CYP52 and CYP153 families as well as for engineered CYP102A1 (P450 BM3) variants (Craft et al., 2003; Funhoff et al., 2006; Koch et al., 2009; Kubota et al., 2005; Lundemo et al., 2016; Scheps et al., 2011), while subterminal regio- or stereoselective hydroxylation could be achieved by engineered P450 BM3 and CYP153A7 (P450pyrSM1) (Glieder et al., 2002; Meinhold et al., 2006; Nodate et al., 2006; Peters et al., 2003; Weber et al., 2011; Yang et al., 2014) as well as CYP154A8 from *Nocardia farcinica* studied in our group (von Bühler et al., 2013). CYP154A8 enables the production of chiral (*S*)-2-alcohols from the inert C_7–C_9-alkanes heptane, octane, and nonane with remarkably high regio- and stereoselectivity for a wild type enzyme (von Bühler and Urlacher, 2014). Apart from the oxidation of inert alkanes using P450s, chiral alcohols can also be produced by the reduction of prochiral keto-functionalized alkanes by alcohol dehydrogenases (ADHs) (Goldberg et al., 2007a,b). For example, ADH from *Lactobacillus brevis* (LB-ADH) catalyzes the reversible enantioselective reduction of ketones to the corresponding (*R*)-alcohols and requires NADPH as cofactor (Hummel, 1997; Leuchs and Greiner, 2011). The limited availability and higher costs of selective keto-functionalized alkanes compared to non-functionalized alkanes illustrates the relevance of P450 enzymes for the production of chiral alcohols.

One aspect, which must be taken into consideration when using P450s, is the choice of suitable redox partners. Most P450 enzymes require redox partner proteins which transfer electrons from NAD(P)H to the heme-containing catalytic center (Hannemann et al., 2007). In order to avoid laborious efforts to individually produce and isolate a P450 and its dedicated redox partners, whole-

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cell biocatalysts can be used instead. Whole-cell biocatalysts offer in addition other advantages such as a continuous supply of the required cofactor NAD(P)H, which is produced by the host cell metabolism, as well as an enhanced enzyme stability provided by the cellular environment (Schrewe et al., 2013). Various aspects related to whole-cell biocatalytic systems, including their limitations and advantages have been reviewed recently, and a variety of strategies for the optimization of cofactor dependent redox reactions has been described in detail (Bayer et al., 2015; Blank et al., 2010; Kell et al., 2015; Ladkau et al., 2014; Schrewe et al., 2013; Willrodt et al., 2015).

One of the major issues when applying recombinant bacterial cells in reactions like alkane oxidation, is the toxicity caused by the substrate and the products formed (Schrewe et al., 2013). For the P450-catalyzed hydroxylation of octane, *Pseudomonas putida* as well as *E. coli* whole-cell biocatalysts have been employed (Fujii et al., 2006; Fujita et al., 2009; Grant et al., 2011; Gudiminchi et al., 2012; Lonsdale et al., 2015; Nodate et al., 2006; Pennec et al., 2015; Vallon et al., 2013). In contrast to *E. coli* some *P. putida* strains are well known for their tolerance against a variety of organic solvents (Poblete-Castro et al., 2012). The general mechanisms of solvent tolerance in bacteria often include energy consuming processes (Ramos et al., 2015; Segura et al., 2012), which in *P. putida* are thought to be (partially) compensated by an increased energy metabolism (Blank et al., 2008). The ability to generate sufficient NAD(P)H in the presence of toxic organic solvents makes *P. putida* a potential host for redox-dependent whole-cell biocatalysis (Mi et al., 2014; Poblete-Castro et al., 2012). In addition to toxicity, low substrate solubility and impaired substrate uptake by a whole-cell catalyst often limit the biocatalytic activity. Addition of solubilizing agents (Bar, 1989; Bracco et al., 2013), water miscible organic solvents or carrier solvents (Schewe et al., 2009), as well as cell permeabilization or co-expression of an alkane transporter (Grant et al., 2014; Julsing et al., 2012; Olaofe et al., 2013) have been shown to improve the availability and uptake of hydrophobic substrates.

The aim of this study was to integrate CYP154A8 and suitable redox partners into *E. coli* and *P. putida* and to compare the applicability of these two whole-cell biocatalysts for the production of chiral 2-alkanols. In addition, a combined P450-alcohol dehydrogenase (ADH) system was established to improve the enantiopurity of the target 2-alcohols (Fig. 1).

Materials and Methods

Plasmids, Enzymes, Strains, and Chemicals

The primers, plasmids, and strains used or constructed during this study are listed in the supplementary information (SI, Table SI–III). For cloning purposes *E. coli* DH5α (Clontech/Heidelberg, Germany) was used. For whole-cell conversions the strains *P. putida* DSM 12264 (kindly provided by Prof. Jens Schrader, DECHEMA Forschungsinstitut DFI, Frankfurt, Germany) and *E. coli* JM109 (Lucigen/Middleton, WI) were used. Heptane, octane, and nonane were obtained from Sigma–Aldrich (St. Louis, MO) with high purity (>99%). All other chemicals and reagents were of analytical grade and purchased from commercial sources.

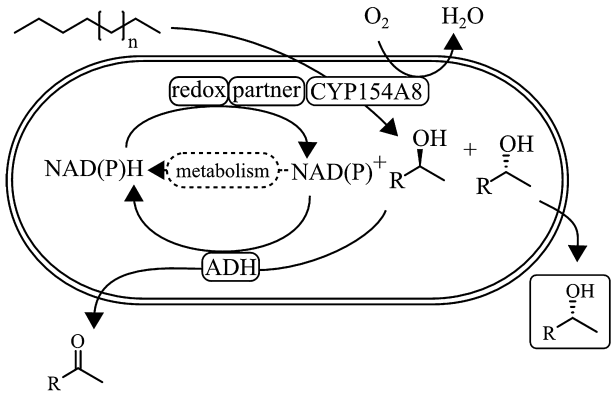

Figure 1. Whole-cell conversion of alkanes to (*S*)-2-alcohols. Regio- and stereoselective hydroxylation of alkanes is catalyzed by CYP154A8. Improved stereoselectivity is achieved by applying LB-ADH, which oxidizes the unwanted (*R*)-2-alcohol to the 2-ketone, additionally supporting the regeneration of the cofactor NADPH. Electron transfer to CYP154A8 is realized via the redox partners FdR (*E. coli*) and YkuN (*B. subtilis*) or PdR and Pdx (*P. putida*).

Construction of Expression Vectors

For gene expression in *P. putida* the L-rhamnose-inducible expression vectors pJeM1-a, pSEVA44rha and pSEVA42rha were generated (SI, Table SII). pJeM1-a was obtained by replacing the eGFP gene from pJeM1 (Jeske and Altenbuchner, 2010) with a *Pme*I restriction site for subcloning. The required restriction sites for *Nsi*I and *Hind*III and modifications downstream of the L-rhamnose promotor system were introduced by PCR using primers rhaRSP-F1 and rhaRSP-R1 (SI, Table SI).

To generate pSEVA44rha, three DNA fragments were joined by ligation (SI, Scheme 1): (i) the L-rhamnose promotor system from pJeM1-a amplified by PCR using the primers rhaRSP-F2 and rhaRSP-R2 (SI, Table SI) after cleaved with *Pac*I and *Spe*I; (ii) the replication origin module (pRO1600/ColE1) from pSEVA247C after restriction with *Pac*I and *Psh*AI; and (iii) the antibiotic marker module (Sm/Sp) from pSEVA438 after restriction with *Spe*I and *Psh*AI. The co-expression plasmid pSEVA42rha was cloned in the same manner after restriction of pSEVA438 and pSEVA227R (SI, Scheme 2) with the corresponding endonucleases.

For co-expression of CYP154A8 with suitable redox partner proteins, artificial operons containing the desired redox genes were generated and cloned into pJeM1-a. The plasmid construction was carried out by DNA assembly (Gibson et al., 2009). First, pJeM1-a was cut at the *Pme*I restriction site. Next, the genes of CYP154A8 and fragments containing bicistronic operons of *yku*N and *fdr* genes or *cam*AB (*pdx* and *pdr*) were amplified in separate PCR reactions. The resulting PCR products contained overlapping sequences, which were introduced by the assembly primers (SI, Table SI). The overlapping sequences allowed the assembly of DNA fragments and the pJeM1-a backbone forming expression vectors with the tricistronic expression operon. The vectors carrying the genes for CYP154A8:Pdx:PdR and CYP154A8:YkuN:FdR were designated as pJeM1-a-A8PP and pJeM1-a-A8YF, respectively. For the co-expression with CYP154A8 and redox partners, the *lb-adh*

gene from *Lactobacillus brevis* (Hummel, 1997) was cloned in the same manner using pSEVA44rha and the assembly primers LB-F and LB-R (SI, Table SI). The constructed co-expression vector was designated as pSEVA44rha-LB. The co-expression plasmid pSEVA42rha-GDH harboring a *gdh* gene form *Bacillus megaterium* was obtained by similar cloning procedures. All vectors constructed (SI, Table SII) by ligation or DNA assembly reactions were confirmed by DNA sequencing. Cells expressing CYP154A8 and redox partners but no ADH carry the additional empty vector pSEVA44rha and are designated with "EV2."

Bacterial Cultivation and Protein Expression

Transformation of *P. putida* cells was carried out by electroporation (Choi et al., 2006). Transformation of *E. coli* JM109 cells was performed by heat-shock transformation of chemically competent cells. For selection of recombinant *P. putida* and *E. coli* cells (SI, Table SIII) kanamycin (30 μg/mL) and streptomycin (200 μg/mL for *P. putida*, 100 μg/mL in case of *E. coli*) was added to the liquid cultures or LB agar. For protein expression TB medium (400 mL) was inoculated from an overnight culture to an OD_{600} of 0.005 and cultivated at 180 rpm and 30°C in case of *P. putida* or 37°C in case of *E. coli*. At OD_{600} of ~0.6, 5′-aminolevulinic acid (40 μg/mL) and $FeSO_4$ (100 μM) were added and gene expression was induced by the addition of L-rhamnose (0.2% [w/v]). Thereafter, cultures were cultivated for further 36 h at 25°C and 160 rpm.

Whole-Cell Conversions of Alkanes

After expression, cells were harvested by centrifugation (8,000*g* for 20 min at 4°C). The cell pellet was suspended in potassium phosphate buffer (200 mM, pH 7.5) and adjusted to a cell wet weight (cww) of 100 g/L. Samples from this suspension were taken to determine: (i) cell dry weight (cdw); (ii) concentration of the P450 enzyme by CO-difference spectroscopy (Omura and Sato, 1964); and (iii) volumetric activity of ADH via NADPH conversion applying (*S*)-2-octanol as substrate in a spectrophotometric assay (SI, SII).

Biotransformation experiments were carried out in triplicate in shaking flasks closed with pulp stoppers (100 mL) containing cell suspension (5 mL) and an aqueous reaction solution (5 mL), resulting in a final cell concentration of 50 g/L (cww). Cell concentration of 50 g/L was chosen as a compromise between sufficient oxygen supply and biocatalyst concentration (data not shown). The aqueous reaction solution contained the following ingredients in different compositions: glycerol (200 mM) or glucose (100 mM), the solubilizing agent methyl-β-cyclodextrin (M-β-CD; 4% [w/v]) and $MgCl_2$ (1 mM) in the case of experiments in which LB-ADH was co-expressed. The reactions were started by the addition of either heptane or octane or nonane (1 mL, forming a second phase), which corresponds to ~9.1% (v/v) and performed up to 48 h at 25°C and 200 rpm. To exclude energy source depletion as a limiting factor, the glucose concentration was monitored semi-quantitatively by test strips (Quantofix) from Machery-Nagel (Düren, Germany). When glucose concentrations dropped to approximately 20 mM (after 12 and 24 h), additional glucose (100 mM) was added. Similarly, additional doses of glycerol (200 mM) were added to the corresponding biotransformation at the same time points as was done in the experiments with glucose.

Product Extraction and Chromatographic Analysis

For each time point, the reaction in each flask was stopped by addition of hydrochloric acid (37%, 50 μL). As internal standard 1-decanol (2 mM) from a stock solution in toluene was used. Extraction of each sample was carried out with toluene (10 mL). Products were analyzed on achiral and chiral columns by gas chromatography coupled to mass spectrometry (GC/MS) as described previously (von Bühler and Urlacher, 2014). Product concentrations were calculated using achiral GC/MS analytics and on the basis of internal standard calibrations of 2-alcohol and 2-ketone. All concentrations given in the manuscript refer to the amounts of products in the total reaction volume. The enantiomeric excess was calculated via peak area integration of the two 2-alcohol enantiomers after derivatization and separation by chiral GC/MS analysis.

Results

Effect of Energy Source and Methyl-β-Cyclodextrin on Whole-Cell Conversions

In order to generate a whole-cell biocatalyst for regio- and stereoselective alkane hydroxylation, the genes coding for CYP154A8 from *N. farcinica* and the NADH dependent redox partner system consisting of putidaredoxin and putidaredoxin reductase from *P. putida* (Pdx/PdR) were co-expressed in *P. putida* DSM 12264 (PA8PP) and *E. coli* JM109 cells (EA8PP). The concentration of CYP154A8 was substantially higher in *P. putida* cells as compared to *E. coli* cells (1,224 nmol/g_{cdw} vs. 53 nmol/g_{cdw}, respectively) suggesting that *P. putida* is the better host for expression. In initial biotransformation experiments with resting cells, glucose or glycerol was added as energy source to support cofactor supply through the cellular metabolism. Oxidation of octane was observed at the C2 position with 98% regioselectivity. Besides the main product 2-octanol, formation of 2-octanone, and minor amounts of 3-octanol were observed. A similar product distribution and a preference for the *S*-enantiomer has previously been observed in a biphasic reaction system operating with the corresponding isolated enzymes (von Bühler and Urlacher, 2014). For both *E. coli* and *P. putida* whole-cell conversions, higher product concentrations were always obtained in the presence of glucose (Fig. 2). This effect was however less pronounced with *E. coli* (Fig. 2). After 4 h of conversion the *E. coli* system produced 1.1 mM (*S*)-2-octanol with 88% *ee*, whereas with *P. putida* 1.4 mM (*S*)-2-octanol with 88% *ee* was obtained. Therefore, glucose was chosen as energy source for further experiments, unless stated otherwise.

Next, we investigated whether product concentrations could be increased by extending the conversion time. Indeed, after 6 h conversion both *P. putida* and *E. coli* produced up to 2.0 mM (*S*)-2-octanol with 90% *ee* (Fig. 3). However, whereas no further product accumulation was observed after 24 h with *E. coli*, product concentration with *P. putida* reached 4.1 mM (Fig. 3). It is of note that, independent of the energy source used, cell aggregation was

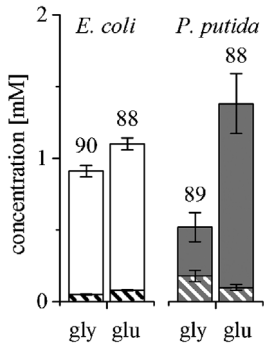

Figure 2. CYP154A8-mediated whole-cell conversions of octane with *E. coli* and *P. putida* after 4 h, using glycerol (gly) or glucose (glu) as energy source. In all cases, Pdx/PdR were used as redox partners. Filled bars: (*S*)-2-octanol with *ee*-value in %, dashed bars: 2-octanone. Resting cells: *E. coli* 10.2 g_{cdw}/L (P450: 53 nmol/g_{cdw}), *P. putida* 7.4 g_{cdw}/L (P450: 1,224 nmol/g_{cdw}).

observed during the biotransformation with *P. putida*, (SI, Fig. S1) but not with *E. coli* cells. A similar phenomenon has previously been described as a cellular adaption mechanism in the presence of toxic organic solvents (Gaur and Khare, 2009). To increase product titers cyclodextrins are frequently used in whole-cell conversions (Bracco et al., 2013; He et al., 2015; Kang et al., 2015). Cyclodextrins are known to enhance solubility of hydrophobic compounds, while simultaneously reducing toxic effects (Singh et al., 2002). Indeed, the addition of methyl-β-cyclodextrin (M-β-CD) to the whole-cell conversions had a general positive effect on product formation (Fig. 3). In the presence of M-β-CD, *P. putida* exhibited a 2.5-fold increase in (*S*)-2-octanol formation after 6 h as compared to control reactions, whereas with *E. coli* a ~twofold increase was observed (Fig. 3). After 24 h, 2-octanol formation only slightly increased with

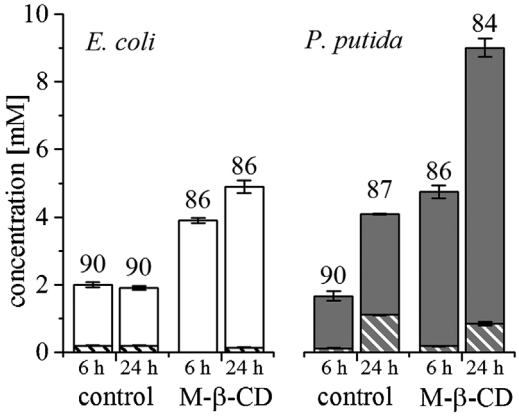

Figure 3. CYP154A8 mediated whole-cell conversions of octane with *E. coli* or *P. putida* after 6 h and 24 h using glucose as energy source. Conversions were carried out in the absence (control) or presence of methyl-β-cyclodextrin (M-β-CD). In all cases Pdx/PdR were used as redox partners. Filled bars: (*S*)-2-octanol with *ee*-value in %, dashed bars: 2-octanone. Resting cells: *E. coli* 8.9 g_{cdw}/L (P450: 230 nmol/g_{cdw}), *P. putida* 9.1 g_{cdw}/L (P450: 1,265 nmol/g_{cdw}).

E. coli (4.9 mM with 86% *ee*). In contrast, *P. putida* showed a twofold increased concentration of 9.0 mM (*S*)-2-octanol with 84% *ee*. Interestingly, *P. putida* cells in the presence of M-β-CD did not aggregate during octane conversions (SI, Fig. S1).

Additional co-expression of a glucose dehydrogenase (GDH) from *B. megaterium* aiming to support cofactor regeneration, did not lead to elevated product concentrations after 24 h, even though for *P. putida* the product formation was accelerated within the first 6 h of reaction (SI, Fig. S2). Taken together the data demonstrate that *P. putida* is the more suitable host for octane hydroxylation; therefore, this organism was chosen for further experiments.

Effect of the Redox Partners on Whole-Cell Conversions

As the physiological redox partners of CYP154A8 still remain unidentified, in addition to the non-physiological NADH-dependent redox partners Pdx/PdR described in the previous section, a mixed redox system consisting of the flavodoxin YkuN from *Bacillus subtilis* and the NADPH-dependent flavodoxin reductase from *Escherichia coli* (FdR) have also been described as suitable redox partners for CYP154A8 (Choi et al., 2010; von Bühler et al., 2013; von Bühler and Urlacher, 2014). In vitro the combination of YkuN with FdR supported the highest conversion with CYP154A8 (von Bühler et al., 2013). We therefore tested the influence of both redox partner pairs on the biotransformation of octane by the *P. putida* whole-cell biocatalyst in the presence of M-β-CD. For this purpose, YkuN/FdR or Pdx/PdR were co-expressed with CYP154A8 in *P. putida* (SI, Fig. S3) and the two resulting systems were compared.

Under identical growth and expression conditions, *P. putida* cells co-expressing YkuN/FdR with CYP154A8 ($A8YF^{EV2}$) produced a ~2.5-times lower amount of P450 than *P. putida* cells co-expressing CYP154A8 with Pdx/PdR ($A8PP^{EV2}$). Nevertheless, the first whole-cell biocatalyst produced higher concentrations of 2-octanol (Fig. 4), which was accompanied by a slight increase in amounts of the over-oxidation product 2-octanone (SI, Fig. S4). Obviously, lower P450 concentrations did not limit the productivity of the whole-cell system. In both systems (*S*)-2-octanol was produced while constantly maintaining up to 89% *ee* over time. Furthermore, the increased total turnover numbers (TTN) and initial 2-octanol formation rates indicated that the NADPH dependent YkuN/FdR whole-cell system is more efficient than the Pdx/PdR system in supporting the CYP154A8-catalyzed hydroxylation of octane (Table I).

Finally, a maximum of 15.7 mM (*S*)-2-octanol with 87% *ee* was produced by the CYP154A8 and YkuN/FdR expressing *P. putida* whole-cell biocatalyst within 24 h.

Effect of LB-ADH on Enantiopurity

Although high regio- and enantioselectivity of CYP154A8 was observed during octane oxidation, still minor amounts of (*R*)-2-octanol were produced. To increase the *ee*-value for (*S*)-2-octanol, we decided to introduce the stereospecific alcohol dehydrogenase (ADH) from *Lactobacillus brevis* in this system. In an ideal case LB-ADH converts the unwanted (*R*)-2-octanol to 2-octanone, while simultaneously supporting cofactor regeneration.

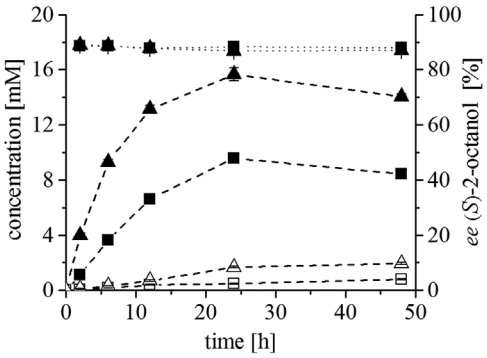

Figure 4. *P. putida* whole-cell conversions of octane after co-expression of CYP154A8 with either YkuN/FdR (triangles) or Pdx/PdR (squares). Conversions were carried out in the presence of M-β-CD and glucose as energy source. Dashed lines: concentrations [mM] of 2-octanol (filled symbols) and 2-octanone (open symbols); dotted lines: *ee*-values of (*S*)-2-octanol. Resting cells of *P. putida:* A8YF^{EV2} 11.9 g_{cdw}/L (P450: 298 ± 23 nmol/g_{cdw}) or A8PP^{EV2} 11.1 g_{cdw}/L (P450: 786 ± 22 nmol/g_{cdw}).

High overall ADH activity in the range of 1,700–2,500 U/g_{cdw} reflected successful co-expression of LB-ADH with CYP154A8 and the redox partners in *P. putida* (A8PP + LB and A8YF + LB, Table I). It is interesting to note that although the ADH activity was similar in the different whole-cell systems, the P450 content was markedly different. Independent of the energy source used, P450 amounts in whole-cell systems co-expressing YkuN/FdR were threefold to eightfold lower as compared to the systems with Pdx/PdR (Table I). Nevertheless, product concentrations obtained with YkuN/FdR as redox partners always exceeded those obtained with Pdx/PdR, which led to increased cell/P450-specific formation rates (Table I).

The cofactor dependency of the applied redox partners is different. Thus, in terms of the required cofactor in the resulting whole-cell systems the CYP154A8 catalyzed reaction was in the case of YkuN/FdR (NADPH) coupled to the $NADP^+$-dependent ADH reaction (hereinafter referred to as coupled system), and in the case of Pdx/PdR (NADH) uncoupled from the $NADP^+$-dependent ADH reaction (hereinafter referred to as uncoupled system).

In the presence of glucose, higher 2-octanol concentrations were obtained compared to glycerol, but only the coupled system showed an initial high *ee* of up to 95%. After 48 h, in both the uncoupled and coupled systems the *ee*-value for (*S*)-2-octanol dropped to 60–65% (Fig. 5A). This was in contrast to the corresponding systems in the absence of LB-ADH, which maintained an *ee* of ~87% throughout the whole-cell conversions (Fig. 4).

In the presence of glycerol, the coupled and uncoupled system clearly showed a different behavior. Whereas the uncoupled system showed a drop in *ee*-values to ~60% after 48 h, the coupled system maintained high *ee*-values of up to 99% (Fig. 5B). Finally, in the presence of glycerol, the coupled (YkuN/FdR) P450-ADH system produced 5.4 mM (*S*)-2-octanol with 97% *ee* after 48 h of conversion (Fig. 5B).

To demonstrate the broader applicability of the *P. putida* whole-cell catalyst for alkane hydroxylation, conversions of heptane, and nonane were also carried out. In all whole-cell conversions the *ee*-values of the formed (*S*)-2-alkanol could be increased by combining CYP154A8 with LB-ADH to form either 2.6 mM (91% *ee*) (*S*)-2-heptanol or 5.5 mM (*S*)-2-nonanol (97% *ee*) within 24 h, respectively (SI, Table SIV).

Discussion

P450s have been recognized as a powerful tool for regio- and stereoselective alkane hydroxylation. Once a suitable enzyme is selected, it might be used in the context of a whole-cell biocatalyst. In the present work, CYP154A8 from *N. farcinica* was chosen as a hydroxylase and co-expressed with redox partner proteins either in the solvent-sensitive *E. coli* JM109 strain or in the solvent-tolerant *P. putida* DSM 12264 strain.

When PdR and Pdx were co-expressed with CYP154A8 aiming at octane hydroxylation, *E. coli* yielded lower amounts of CYP154A8 compared to *P. putida*. However, this did not limit the whole-cell conversions at that stage, thus excluding the P450 content as a limiting factor under conditions used. Apart from P450

Table I. Summary of parameters obtained during *P. putida* CYP154A8-mediated whole-cell conversions of octane.

System	A8PP^{EV2}	A8PP + LB	A8PP + LB	A8YF^{EV2}	A8YF + LB	A8YF + LB
Energy source	Glucose	Glucose	Glycerol	Glucose	Glucose	Glycerol
Cell amount ($g_{cdw} \cdot L^{-1}$)	11.1	11.9	9.3	11.9	11.9	11.1
P450 content ($nmol \cdot g_{cdw}^{-1}$)	786 ± 22	925 ± 102	823 ± 6	298 ± 23	112 ± 16	176 ± 12
ADH activity (U/g_{cdw})[a]	–	1,670 ± 44	1,794 ± 34	–	2,477 ± 66	1,834 ± 25
Maximum 2-octanol concentration (mM)/after 24 h	9.6	11.1	3.0/2.2	15.7	13.7	5.4/4.7
Maximum 2-octanol yield ($g \cdot L^{-1}$)	1.2	1.4	0.4	2.0	1.8	0.7
ee-(*S*)-2-octanol (%)[b]	88	61	60	87	66	97
TTN ($nmol_{2\text{-octanol}} \cdot nmol_{P450}^{-1}$)[c]	1,100	1,000	400	4,400	10,300	2,750
Maximum 2-octanol per catalyst ($mg \cdot g_{cdw}^{-1}$)	110	120	40	170	150	60
Cell-specific formation rate ($mg \cdot g_{cdw}^{-1} \cdot h^{-1}$)[d]	6.7	8.1	4.3	22.4	19.8	9.3
P450-specific formation rate ($nmol_{2\text{-octanol}} \cdot min^{-1} \cdot nmol_{P450}^{-1}$)[d]	1.1	1.1	0.7	9.6	22.6	6.8

cdw, cell dry weight; U, μmol min^{-1}; – no ADH activity observed, all cells not expressing the ADH carry the empty vector pSEVA44rha (EV2).
[a]Determined via NADPH formation during oxidation of (*R*)-2-octanol to 2-octanone with cell lysate.
[b]After 48 h of conversion.
[c]Determined for highest observed 2-octanol concentration.
[d]Initial formation rate of 2-octanol determined after 2 h of conversion.

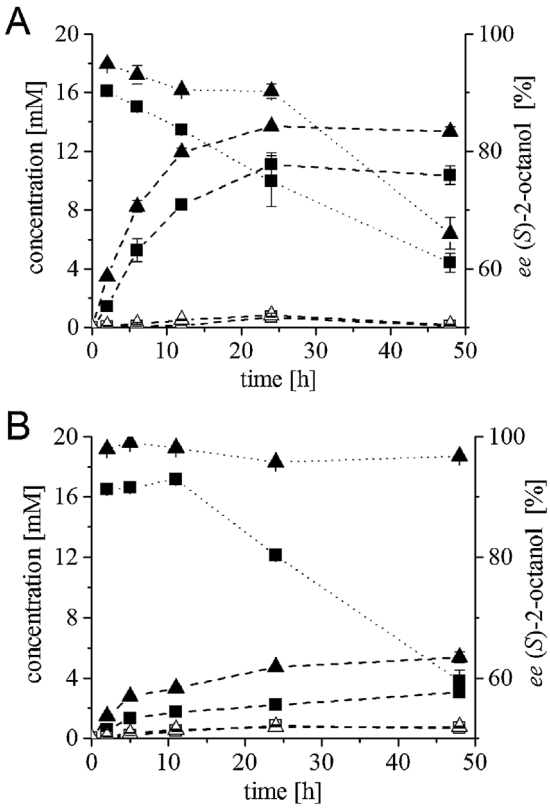

Figure 5. *P. putida* whole-cell conversions of octane after co-expression of CYP154A8 with YkuN/FdR and LB-ADH (triangles) or Pdx/PdR and LB-ADH (squares) in the presence of M-β-CD and glucose (**A**) or glycerol (**B**) as energy source. Dashed lines: concentrations of 2-octanol (filled symbols) and 2-octanone (open symbols), dotted lines: *ee*-values of (*S*)-2-octanol. Conversion parameters see also Table I.

concentration, sufficient cellular NADH supply was reported to be crucial when PdR and Pdx were employed as redox partners (Mouri et al., 2006; Sevrioukova and Poulos, 2011). Our results demonstrated that cofactor supply in recombinant *P. putida* as well as *E. coli* was better when glucose was used as energy source instead of glycerol (Fig. 2).

Activity and stability of the applied whole-cell biocatalyst are often affected by a higher energy demand and adaption of cellular processes in the presence of organic solvents (Heipieper et al., 2007; Segura et al., 2012). Concerning this matter, fundamental differences have been described during styrene epoxidation with recombinant *P. putida* and *E. coli*. While *P. putida* cells increased glucose uptake and NAD(P)H regeneration rates in the presence of octanol to compensate the increased energy demand (Blank et al., 2008), *E. coli* cells lacked this ability (Bühler et al., 2008). In our experiments, growing concentrations of the product 2-octanol and the continuous exposure to octane obviously reduced the viability of the solvent-sensitive *E. coli* cells. In contrast to this, a higher productivity and longer-retaining catalytic activity of the solvent-tolerant *P. putida* confirmed its ability to compensate the increasing toxicity of organic solvents. The observed cell aggregation (Fig. S1) has been suggested as a protective mechanism of *P. putida* toward organic solvents (de Carvalho et al., 2004). However, cell aggregation can limit substrate uptake. To enhance octane solubility and thus ensure its increased uptake by cells, we used methyl-β-cyclodextrin (M-β-CD), which indeed resulted in improved conversions (Fig. 3). Besides, the complexation of formed products by methyl-β-cyclodextrin probably mediated a reduced toxicity and improved cell viability.

Furthermore, the choice of heterologous redox partners has a strong effect on the CYP154A8-based whole-cell system. When heterologous YkuN/FdR were co-expressed with CYP158A8 instead of Pdx/PdR, higher product concentrations were achieved although the P450 content was lower. This effect is probably due to the more efficient electron transfer via YkuN/FdR, although the expression levels and their intracellular stoichiometry might also have an effect. The co-expression of the cofactor-regenerating GDH did not sufficiently increase product concentration, demonstrating that reduced cofactors were not limiting under conditions used. Moreover, a possible limitation due to energy source depletion was excluded by maintaining their concentrations at high level during biotransformations. However, the productivity of the *P. putida* whole-cell biocatalyst could not be further improved. One possible reason for this is decreasing enzyme stability over time. In a cell free system the stability of the redox partner proteins and CYP154A8 turned out to have a major impact on substrate conversion (von Bühler and Urlacher, 2014). Nevertheless, the optimized *P. putida* whole-cell biocatalyst produced ~16 mM (*S*)-2-octanol with 87% *ee* starting from octane, which is, to the best of our knowledge, the highest 2-octanol production by a P450-system described so far.

In the next step, *ee*-values for the product (*S*)-2-octanol were increased by introducing the gene of the $NADP^+$-dependent alcohol dehydrogenase from *L. brevis* (LB-ADH) into the established whole-cell system. Interestingly, the highest *ee*-value up to 99% could only be achieved when LB-ADH was combined with the NADPH-dependent redox partner system YkuN/FdR and glycerol as an energy source (Fig. 5, Table I). Thus, both the chosen energy source and the specific cofactor requirement of the applied enzymes affected the enantiopurity of 2-alkanols.

The achieved 5.4 mM (*S*)-2-octanol with 97% *ee* is, to the best of our knowledge, still the highest concentration for regio- and stereoselective octane hydroxylation catalyzed by a P450-based whole-cell system. Additionally, general applicability of the YkuN/FdR-P450-LB-ADH system to improve product enantiopurity was demonstrated with heptane and nonane as further substrates (SI, Table SIV).

With all three alkanes, product enantiopurity could only be increased at the expense of productivity: threefold lower product concentrations were achieved in presence of glycerol compared to the best performing system with glucose. However, an improved enantiopurity, a higher productivity and an extended process time compared to the free enzyme-based system (von Bühler et al., 2013) show the superiority of the applied *P. putida* whole-cell system.

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