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Abstract We have recently described the biocatalytic characterization of a self-sufficent biosynthetic alkane hydroxylase based on CYP153A13a from *Alcanivorax borkumensis* SK2 (thereafter A13-Red). Despite remarkable regio- and chemoselectivity, A13-Red suffers of a difficult-to-reproduce expression and moderate operational stability. In this study, we focused our efforts on the production of A13-Red using high-cell-density cultivation (HCDC) of recombinant *Escherichia coli*. We achieved 455 mg (5,000 nmol) of functional enzyme per liter of culture. Tight control of cultivation parameters rendered the whole process highly reproducible compared with flask cultivations. We optimized the purification of the biocatalyst that can be performed in either two or three steps depending on the application needed to afford A13-Red up to 95 % homogeneous. We investigated different reaction conditions and found that the total turnover numbers of A13-Red during the in vitro hydroxylation of *n*-octane could reach up to 3,250 to produce 1-octanol (1.6 mM) over a period of 78 h.

Keywords Alkane hydroxylation · Cytochromes P450 · High-cell-density cultivation · Green chemistry

Dedicated to the memory of Diane de Girval (Nov 1988–Dec 2013), a highly appreciated colleague and friend, who contributed to this work with passion.

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Introduction

One of the strategy to enable the notoriously difficult oxyfunctionalization of alkanes is based on the use of cytochrome P450 monooxygenases (CYPs) (Bernhardt 2006; Bordeaux et al. 2012; Grogan 2011). These enzymes, widely distributed from archae to mammals, are indeed able to introduce an atom of oxygen into a large range of hydrophobic compounds having unactivated C–H bonds (Bernhardt 2006; Grogan 2011; McLean et al. 2005; Meunier et al. 2004; Shaik et al. 2010; Urlacher and Girhard 2012). Enzymes from the CYP153 family were found to naturally hydroxylate linear C5 to C12 alkanes to their corresponding primary alcohols, opening a new pathway for creating interesting (soluble) alkane hydroxylases (van Beilen et al. 2006; Funhoff et al. 2007; Funhoff et al. 2006).We have recently described the purification and complete characterization of a self-sufficient biosynthetic CYP where CYP153A13a from *Alcanivorax borkumensis* SK2 is fused with RhFred, the flavin mononucleotide (FMN)/Fe2S2-containing reductase domain from P450 RhF isolated from *Rhodococcus* sp. NCIMB 9784 (Kubota et al. 2005; Nodate et al. 2006). This strategy yielded a functional enzyme (hereafter A13-Red) able to selectively hydroxylate the terminal position of alkanes under mild conditions in vivo and in vitro (Bordeaux et al. 2011).

Biocatalytic oxyfunctionalization of hydrocarbons is of potentially great interest for developing greener processes in

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synthetic chemistry but still suffers of several drawbacks (van Beilen et al. 2003; Bernhardt 2006; Bordeaux et al. 2012; Bühler and Schmid 2004; Grogan 2011; Urlacher and Girhard 2012). Achieving easy and reliable production of the biocatalyst, high activity, high specificity, and cofactor recycling are identified as important bottlenecks to be solved for rendering CYPs more suitable into in vitro applications (Bordeaux et al. 2012; Fasan et al. 2007; Grogan 2011; Maurer et al. 2003, 2005; Urlacher and Girhard 2012).

In this article, we describe our results concerning the optimized preparation of A13-Red using high-cell-density cultivation (HCDC) of recombinant *Escherichia coli* to yield a purified and functional enzyme. Taken together, these results represent a significant step toward a more practical usage of this self-sufficient biosynthetic alkane hydroxylase.

Materials and methods

Microorganism and media

E. coli BL21 Star™ (DE3) (Life Technologies) was transformed with the plasmid pETA13-Red encoding for CYP153A13a (A13a) from *A. borkumensis* SK2 fused to the FMN/Fe_2S_2-containing reductase from *Rhodococcus* sp. NCIMB 9784 (thereafter A13-Red) under the control of isopropyl β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter (Bordeaux et al. 2011). Media used for flask cultivations were Luria Bertani broth (LB) (Sigma-Aldrich), terrific broth (TB) (Sigma-Aldrich), or 2xYT (for 1 L bactotryptone 16 g, bacto yeast extract 10 g, and NaCl 5 g). The medium used for HCDCs was previously described by Riesenberg et al. (1990) where, except for glycerol, all other substrates are in excess (see Supporting Information for details). Kanamycin sulfate was added at a final concentration of 50 μg/mL.

Cultivation conditions

All fermentations were repeated two times. All cultivations (400-mL initial volume) were performed one pot into a 1-L bioreactor equipped with standard measuring and control units (temperature, pH, dissolved oxygen (xO_2), and stirrer speed). Stirring speed was maintained between 1,200 and 1,900 rpm, and the pH was controlled at 7.1 with 16 % NH_4OH. The concentration of dissolved oxygen was maintained above 20% of saturation. Aeration rate from 1.5 to 4 vvm was achieved by airflow controller. HCDCs were divided into a phase of biomass production followed by a phase of recombinant protein expression. The biomass production phase was performed at 37 °C and postinduction phase at 25 °C. Unlimited growth on glycerol (30 g/L) was achieved during two initial batch modes. Fed-batch mode was performed using exponential feeding with the feeding solution highly concentrated in glycerol (see Supporting Information). Growth rate was set to 0.1/h applying the equations described by Korz et al. (1995). After this biomass production phase, IPTG (0.1, 1, or 5 mM) and 5-aminolevulinic acid (300 μM) were added to start the induction phase. Exponential feeding was replaced by pH-stat feeding by programing the pump controler to add the feeding solution if pH value goes above 7.5 and until it reaches back 7.1. Evolution of A13-Red concentration was monitored over time by regular sampling and CO-binding analysis. Induction phase was stopped after approximately 24 h. Evolution of cell concentration during cultivation was monitored by measuring optical density at 600 nm (OD600) using the appropriate dilution before measurement. Cell dry weight (CDW) was measured after incubation of a 10-mL culture sample at 120 °C for 72 h.

Two-step purification of A13-Red produced by HCDC

The whole process of purification was performed at 4 °C. Cells from HCDC were pelleted by centrifugation (7,000 rpm, 20 min, 4 °C) and frozen at −20 °C until use. For immobilized metal affinity chromatography (IMAC) capture, a fraction of the cell pellet (30 g) was thoroughly resuspended in lysis buffer (60 mL) and incubated on ice for 30 min. Centrifugation (8,000 rpm, 20 min, 4 °C) was performed two times to remove cell debris. Supernatant was filtered on 0.45 μm and loaded on 5-mL HisTrap column (GE Healthcare Life Sciences) at 2.5 mL/min. Binding buffer was applied at 5 mL/min until OD280 reached baseline, followed by washing buffer. Elution was performed with elution buffer at 2.5 mL/min. The different fractions were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Elution buffer was exchanged by ultrafiltration against conservation buffer and CYP samples conserved at −20 °C. For ion-exchange chromatography, before loading, a sample (1 mL) from IMAC was thawed and desalted by loading onto 5-mL HiTrap Desalting column (GE Healthcare Life Sciences) using buffer A. For NaCl linear gradient, the desalted sample (5 mL) was loaded onto a 1-mL Q Sepharose XL column (GE Healthcare Life Sciences) using buffer A until OD280 reaches baseline. A linear gradient was applied from 100 % buffer A to 75 % A+25 % B within 40 min at 1 mL/min. Fractions (1 mL) were collected and analyzed by SDS-PAGE. For NaCl step gradient, the desalted sample (5 mL) was loaded onto 1-mL Q Sepharose XL column using buffer A until OD280 reaches the baseline. Buffer C was applied until OD280 reaches the baseline followed by buffer D, both at 1 mL/min. Fractions (1 mL) were collected and analyzed by SDS-PAGE, those containing A13-Red were pooled, and elution buffer was exchanged by ultrafiltration against conservation buffer and CYP samples conserved at −20 °C.

Octane hydroxylation assays

Every reaction was performed into Wheaton 5-mL V-Vials at 25 °C under gentle magnetic stirring, repeated three times, and errors were estimated to be 5–10 %. For one-phase reaction medium, a solution of *n*-octane into absolute ethanol (400 mM) was added into potassium phosphate buffer (100 mM, pH 7.4). The enzyme A13-Red (500 nM) was preincubated 1 min before reduced nicotinamide adenine dinucleotide phosphate (NADPH, 500 μM) was added to start the reaction performed in a total volume of 1 mL. For two-phase reaction media, aqueous phase (500 μL) was built up with the appropriate additives (see Table 2 for details) added to potassium phosphate buffer (100 mM, pH 7.4). Additive concentrations were as follows: Triton X-100 (5 %*v*/*v*), bis(2-ethylhexyl) sulfosuccinate sodium (5 %*w*/*v*), bovine serum albumin (BSA) (10 g/L), NADP-dependent isocitrate dehydrogenase from *Bacillus subtilis* (5 U), D/L-isocitrate (20 mM), $MgCl_2$ (5 mM), and catalase from bovine liver (0.5 U). Organic layer (*n*-octane, 500 μL) was added dropwise to the aqueous layer under gentle magnetic stirring. Once the addition was done, A13-Red (500 nM) was preincubated 1 min before NADPH (500 μM) was added to the emulsion to start the reaction performed in a total volume of 1 mL. One-phase reactions were quenched by the addition of butyl acetate (100 μL). For two-phase reactions, a 100-μL sample was taken from the emulsified solution. For both conditions, after vortexing (1 min) and centrifugation (2 min, 10,000*g*, at room temperature), the organic layer was collected and analyzed by gas chromatography (GC)/mass spectrometry (MS) using dodecane as the internal standard (see Supporting Information for details).

Results

High-cell-density cultivation of recombinant *E. coli* overexpressing A13-Red

Cultivation of microorganisms in flasks with a rich medium (LB, TB, or 2xYT) is the most simple approach, but important parameters for reproducibility such as medium composition, aeration, or pH cannot be controlled tightly. Expression of A13-Red in recombinant *E. coli* using this simple approach yielded an average of 35±25 mg of functional CYP per liter of culture with TB (Bordeaux et al. 2011). We even witnessed unproductive cultivations. These large variations from batch to batch prompted us to develop a more reliable protocol to express A13-Red using HCDC with instrumented fermentors.

Our results of HCDC using the synthetic medium described by Riesenberg et al. (1990) with glycerol as the sole carbon source are shown in Fig. 1. The whole cultivation was performed one pot into the same fermentor and split into a phase of biomass production and a phase of recombinant protein expression. The biomass production phase was made of three successive substeps: batch mode number 1, batch mode number 2, and fed-batch mode (Fig. 1). Cell growth during the batches number 1 (16 h) and number 2 (2 hours) was unlimited and occurred at maximal rate (for batch number 1, μ_{max}=0.19/h and for batch number 2, μ_{max}=0.30/h). Consumption of initial glycerol and metabolic by-products (e.g., acetic acid) is indicated each by an increase of the dissolved oxygen concentration. Care was brought to keep the dissolved oxygen xO_2 above 20 % by adjusting both agitation and aeration rates. Each batch consumed 12 g of glycerol to finally yield approximately 18 g_{CDW}/L. During the fed-batch step, limited growth at fixed and constant rate (μ_{set}= 0.1/h) was achieved by exponential feeding with glycerol as described by Korz et al. (1995) (see Supporting Information for details). This growth rate appeared to be the best compromise between achievable biomass, cultivation time, and technical constraints related to optimal aeration of the medium. During 18 h, cell concentration increased to reach a final biomass of 105±5 g_{CDW}/L ($Y_{X/S}$=0.25g_{CDW}/$g_{glycerol}$).

At this point, the phase of recombinant protein expression was started by adding IPTG (0.1, 1, or 5 mM) and 5-aminolevulinic acid (300 μM) into the medium. Two important parameters were modified compared with the biomass production phase. First, the medium temperature was decreased from 37 to 25 °C. Second, cells were still fed with glycerol, but instead of exponential, pH-stat feeding was preferred. We studied the effect of initial IPTG concentration (0.1, 1, and 5 mM) on the titer of functional A13-Red over 24 h of induction. For each concentration of IPTG, we observed that after reaching a maximal titer, A13-Red concentration slowly decreased. Maximal titer appeared more rapidly with higher inducer concentration, but A13-Red concentration was not directly proportional to IPTG concentration (Table 1). For example, A13-Red concentration reached 320 mg/L after 15 h with 0.1-mM IPTG, while it reached 360 mg/L after 12 h with tenfold more IPTG. An important metabolic change can be visualized 7 h after IPTG addition (Fig. 1) when oxygen consumption rate and cell growth decreased significantly while glycerol was still consumed. Compared with flask cultivation conditions, these results represent up to 46-fold larger titers of functional A13-Red with a maximum of 455±5 mg (4,973 nmol) per liter of culture medium. It represented 23.0, 29.5, and 33.9 nmol A13-Red/g_{CDW} for 0.1, 1, and 5 mM, respectively.

Enzyme purification

The biosynthetic protein A13-Red harbors a hexahistidine tag in order to perform a preliminary capture step based on IMAC. Even though this step can be sufficient to achieve 95 % purity after flask cultivation, it was not true for A13-Red produced

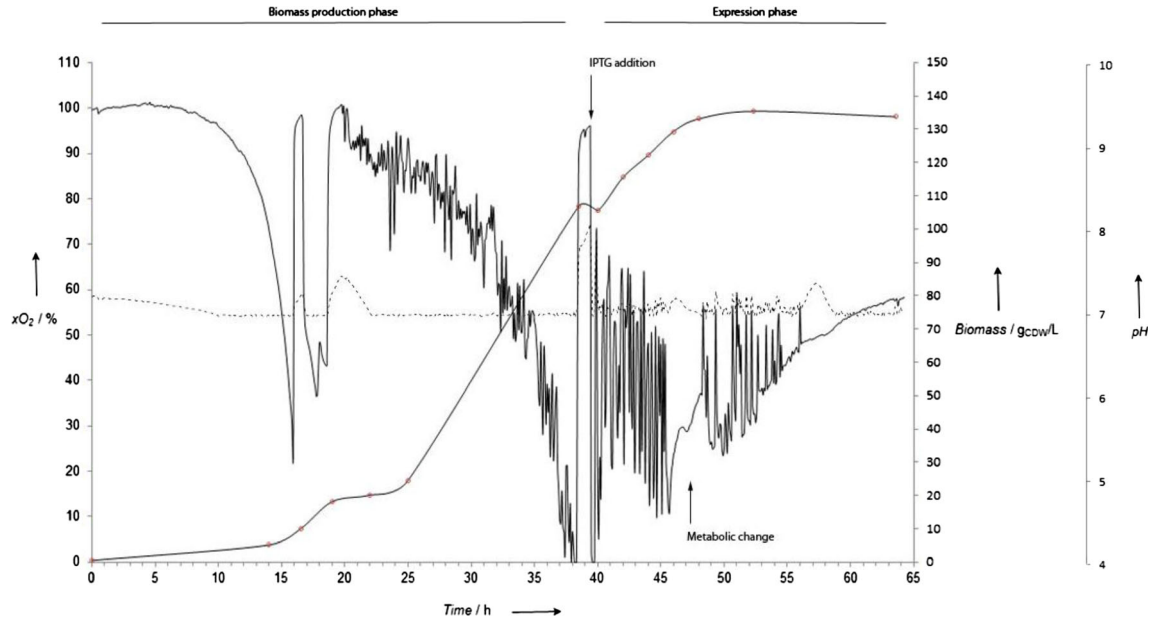

Fig. 1 High-cell-density cultivation of recombinant *E. coli* BL21 Star™ (DE3) expressing A13-Red in Riesenberg medium using glycerol as the sole carbon source. Biomass production phase was made of combining two batches at maximal growth rate followed by a fed-batch with a desired growth rate μ_{set}=0.1/h (exponential feeding). Consumption of the initial glycerol and metabolic by-products (e.g., acetic acid) are indicated each by an increase of the dissolved oxygen concentration. Induction phase was started by adding IPTG (*arrow*) and cells were fed on demand by a pH-stat feeding algorithm. Time course of dissolved oxygen (xO_2, %, *plain line*), cell concentration (g_{CDW}/L, *open circles*), and pH of the medium (*dashed line*) are represented

by HCDC where only 70–75 % purity was reached (Fig. 2). Since A13-Red is irreversibly inhibited by imidazole, elution was performed using histidine. One of the most abundant contaminating proteins (approximately 67 kDa) was identified to be CYP153A13a by trypsin digestion followed by nano LC/MS/MS analysis (see Supporting Information). Thus, we assumed that a portion of A13-Red is subjected to proteolysis into the cytoplasm of the bacteria during expression.

A second chromatography step was set up based on ion exchange. Since A13-Red has a calculated pI of 6.01, the Q Sepharose stationary phase (strong cation) was tested. The first series of experiments with a linear gradient of NaCl was designed to determine if A13a and A13-Red could be eluted at sufficiently different concentrations. It demonstrated that A13a was eluted at 80 mM NaCl while A13-Red at 200 mM. The second series of experiments with a two-step gradient of NaCl (step 1=80 mM and step 2=200 mM) was very efficient to separate A13a from A13-Red yielding the latter approximately 85–90 % homogeneous (Fig. S1). However, high molecular weight impurities (above 200 kDa) still remained present after this step.

Finally, the third step for removing high molecular weight contaminants based on size exclusion chromatography with a high resolution Superdex 75 stationary phase yielded A13-Red almost pure (95 % homogeneous judged by SDS-PAGE analysis) and properly folded. (Figs. S1 and S2).

Table 1 Induction of A13-Red expression in high-cell-density cultivation of recombinant *E. coli* BL21 Star™ (DE3)

IPTG initial concentration (mM)	Cell concentration at induction time (g_{CDW}/L)	Maximal cell concentration after induction (g_{CDW}/L)	A13-Red maximal titer (mg/L)	Time to reach maximal titer of A13-Red (h)
0.1	88	142	320	15
1	103	135	360	12
5	112	147	455	10

IPTG isopropyl β-D-thiogalactopyranoside

In vitro hydroxylation activity of A13-Red produced by HCDC

Our objective was to evaluate the quality of A13-Red produced by HCDC through measurement of total turnover (TTN) during *n*-octane hydroxylation (moles of 1-octanol produced per mole of enzyme) into different reaction conditions. In our case, it clearly appeared that A13-Red hydroxylation activity was higher in a two-phase medium with water/octane 1:1 (TTN=233; Table 2, entry 5) rather than in a one-phase medium with ethanol as cosolvent (TTN=141; Table 2, entry 1). However, in a two-phase medium with water/isopropyl ether/octane, A13-Red activity was weak (TTN=46; Table 2, entry 2). Surprisingly, biotransformations in the presence of tensioactives [Triton X-100 or bis-(2-ethyl hexyl)-sulfosuccinate sodium (AOT)] consistently underperformed

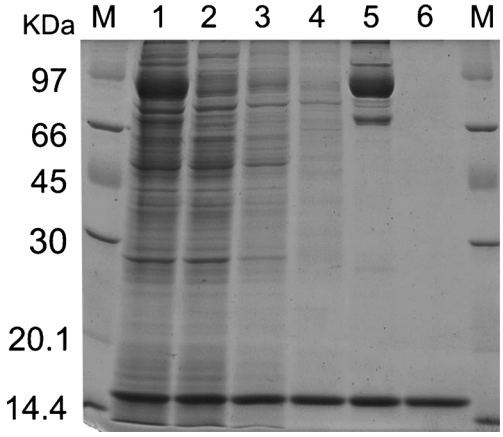

Fig. 2 SDS-PAGE (12 % polyacrylamide) analysis of ion metal affinity chromatography fractions. *Lane 1* lysis soluble fraction. *Lane 2* column flow through containing unbound proteins. *Lane 3* flow through with binding buffer. *Lane 4* flow through with washing buffer. *Lane 5* elution with elution buffer containing 80 mM histidine. *Lane 6* flow through with 2 M imidazole

(Table 2, entries 3, 4, 6, and 7). Into every two-phase condition, A13-Red irreversibly precipitated within 15 min, drastically limiting the TTN values. When reactions were performed in two-phase medium with water/octane 1:1, in the presence of BSA (10 g/L), isocitric dehydrogenase from *B. subtilis* (iCDH), D/L-isocitrate (20 mM), and catalase from bovine liver, the production of 1-octanol lasted up to 78 h with an ultimate TTN about 3,250 (Table 2, entry 8). No other regioisomer or overoxidation product was detected confirming the remarkable regio- and stereo-selectivity of A13-Red. Enzymatic activity of A13-Red when produced by HCDC and two-step purification was found identical to A13-Red produced by flask cultivation and one-step IMAC purification.

Discussion

High-cell-density is obtained when the concentration of cells during cultivation exceeds 100 g of CDW per liter of culture (g_{CDW}/L) (Riesenberg 1991). Here, we achieved final cell densities up to almost 150 g_{CDW}/L. Compared with flask cultivation, HCDC is a highly reproducible method for producing high titers of recombinant protein in a reduced volume (elevated volumetric productivity), at lower production costs (Choi et al. 2006; Lee 1996; Riesenberg 1991) and within short cultivation times (Faulkner et al. 2006; Korz et al. 1995). To the best of our knowledge, HCDC has never been applied to the production of a recombinant CYP enzyme. Only one

Table 2 In vitro hydroxylation activity of A13-Red on octane versus reaction conditions

Entry	Substrate concentration+additives[a]	TTN[b]	Reaction completion[c] (h)	1-Octanol produced (μM)
1	4 mM octane+ethanol[d]	141	0.25	71
2	4 mM octane+$(i\text{Pr})_2\text{O}$[e]	46	0.25	23
3	4 mM octane+$(i\text{Pr})_2\text{O}$[e]+Triton X-100[f]	56	0.25	28
4	4 mM octane+$(i\text{Pr})_2\text{O}$[e]+AOT[g]	5	0.25	2.5
5	Pure octane[h]	233	0.25	116
6	Pure octane[h]+Triton X-100[f]	88	0.25	44
7	Pure octane[h]+AOT[g]	13	0.25	6.5
8	Pure octane[h]+BSA[i]+iCDH[j]+catalase[k]	3,246	78	1,623

BSA bovine serum albumin

[a] All reactions were carried out in potassium phosphate buffer (100 mM, pH 7.4) containing glycerol (10 %*v*/*v*), with A13-Red (0.5 μM) and NADPH (500 μM) at 25 °C

[b] The total turnover number was determined by GC/MS analysis after completion of the reaction as (μmol 1-octanol)/(μmol A13-Red)

[c] Delay before completion of the reaction defined as a stable concentration of 1-octanol in the reaction medium and determined by GC/MS analysis. For entry 1, reaction was estimated to stop after 5 min, but for homogeneity reasons, activity was still measured for 15 min

[d] Final concentration of ethanol was 1 % (*v*/*v*)

[e] Two-phase reaction medium (aqueous/organic=1:1) where di-isopropyl ether was used as the organic phase

[f] Final concentration of Triton X-100=5 % (*v*/*v*)

[g] Final concentration of bis(2-ethyl hexyl)sulfosuccinate sodium (AOT)=5 % (*w*/*v*)

[h] Two-phase reaction medium (aqueous/organic=1:1, *v*/*v*) where octane was used as the organic phase

[i] Final concentration of bovine serum albumin=10 g/L

[j] NADPH recycling system made of NADP-dependent isocitric dehydrogenase from *Bacillus subtilis* (iCDH, 5 U), D/L-isocitrate (20 mM), and $MgCl_2$ (5 mM)

[k] Catalase from bovine liver (0.5 U)

study reports the production of CYP102A1 but in medium-cell-density cultivation of recombinant *E. coli* (20 g_{CDW}/L) (Pflug et al. 2007). During our process, cultivation on glycerol was preferred than to glucose to limit the amount of acetate produced during the process. Indeed, it is well demonstrated that acetate production by *E. coli* is much lower when grown on glycerol than on glucose (Lee 1996). Furthermore, acetate is known to reduce the amount of recombinant protein produced in HCDC (Lee 1996).We employed a classical cultivation strategy where we split the phase of biomass production and the phase of recombinant protein expression (Luo et al. 2006). It allowed us to grow cells without metabolic stress due to recombinant protein production since A13-Red concentration remained below the detection limit of CO-binding analysis during preinduction. This indicated that under these conditions, basal level of A13-Red expression was kept very low before induction. It is very different compared with cultivations into TB medium where significant levels of A13-Red were detected before induction (unpublished results).

Expression of A13-Red was induced by the addition of IPTG. We tested three different initial concentrations corresponding to 1.1, 9.7, and 44.6 μmol IPTG/g_{CDW}. During postinduction, feeding was controlled by a pH-stat algorithm and xO_2 was kept at 40 % in average. This type of feeding avoids the accumulation of substrate in culture broth and most importantly fitting with the large metabolic changes occurring into cells overexpressing a recombinant protein (Luo et al. 2006). Pflug et al. used oxygen starvation during postinduction phase to maximize CYP102A1 expression levels (Pflug et al. 2007), but this strategy induced such a brutal change/stress to the cells in our case that it led to extremely low levels of A13-Red likely due to massive cell death. During postinduction, we also decreased cultivation temperature from 37 to 25 °C, because temperature lowering is usually performed to further control cell metabolism: nutrient uptake and growth rate can be reduced, thus reducing the formation of toxic by-products and the formation of inclusion bodies (Lee 1996). We observed that an initial concentration of 1 mM IPTG (9.7 μmol/g_{CDW}) was a good compromise regarding productivity (29.5 nmol A13-Red/g_{CDW}). Comparison with the results obtained by Pflug et al. clearly demonstrated that CYP102A1 is better expressed than A13-Red in *E. coli* (Table 3, entries 1 and 2).This is not surprising since CYP102A1 is well known for its high solubility. This trend can be illustrated by theoretical recombinant protein solubility predictions (Wilkinson and Harrison 1991). Indeed, CYP102A1 has 64 % chance of solubility when overexpressed in *E. coli* while A13-Red has only 41 % chance of solubility. It is a clear indication that getting reproducible, soluble, and functional amounts of A13-Red was a challenge. Scheps et al. have reported the construction and expression of chimeric proteins where CYP153A from *Marinobacter aquaeloei* was fused to the reductase domain of CYP102A1. They achieved up to 600 nmol CYP/g_{CDW} into 15 g_{CDW}/L cell density cultivations of *E. coli* HMS174 (DE3) with TB (Table 3, entry 3) (Scheps et al. 2013). Studies by Gudiminchi et al. and Olaofe et al. on CYP153A6 from *Mycobacterium* sp. HXN-1500 expressed in *E. coli* have reported 200 μmol CYP/g_{CDW} into low-cell-density cultivations with LB (Table 3, entry 4)(Gudiminchi et al. 2012; Olaofe et al. 2013). Anterior results published by Funhoff et al. were obtained with CYP153 enzymes including A6 and A13 using *Pseudomonas putida* as expression host. Despite the lack of comparable P450 concentrations, it appeared clearly that A6 was expressed at a higher level than A13 (Funhoff et al. 2007). In our hands, the biosynthetic fusion between A6 and RhFred always led to the irreversible accumulation of inclusion bodies (unpublished results). Even though expression levels are always protein-dependent, these comparisons show that A13-Red is a difficult protein and that our strategy based on HCDC allowed us to circumvent a low-expression level per cell by increasing the concentration of cells and to produce several hundred milligram of A13-Red per liter.

During purification process, we noticed the presence of a 67-kDa contaminant after IMAC capture. Further analysis unambigously identified a large portion of CYP153A13a. A13-Red is fused to a hexahistidine tag at its N-terminal end, and it is likely that if the nonstructured peptide linker between the CYP and the reductase domains is the target a protease present in the cytoplasm, the remaining fragment is able to be captured during IMAC. Using *E. coli* BL21 Star™ (DE3), which does not contain the lon nor OmpT proteases, did not significantly reduced this phenomenon. A13-Red purification was optimized, and the enzyme could be readily obtained by up to 90 % homogeneous over two simple and scalable chromatographic steps. This purity grade is sufficient for most in vitro enzymatic measurements. It is probable that high molecular weight contaminants are polymers of A13-Red since their concentration progressively increased over time in the conservation buffer at −80 °C into pure A13-Red samples. More demanding applications such as precise kinetic studies or X-ray crystallography should require the additional purification step developed here to yield A13-Red up to 95 % homogeneous (see Supporting Information for details).

Whole-cell biocatalysis is an attractive approach for the application of cofactor-dependent oxygenases (Duetz et al. 2001; Li et al. 2002). Very interesting results have been published recently using *E. coli* expressing CYP153 enzymes or CYP102A1 mutants (van Beilen et al. 2005; Fasan et al. 2007; Fujii et al. 2006; Funhoff et al. 2006; Gudiminchi et al. 2012; Honda Malca et al. 2012; Koch et al. 2009; Nodate et al. 2006; Olaofe et al. 2013; Scheps et al. 2013, 2011) to produce primary or secondary alcohols. In our previous report, we confirmed the results by Nodate et al. (2006) concerning the ability of *E. coli* resting cells expressing A13-Red to produce

Table 3 Selected results of recombinant CYP expression from literature

Entry	Enzyme	CYP concentration				Reference
		mg/L	nmol/L	mg/g_{CDW}	$nmol/g_{CDW}$	
1	CYP102A1[a]	795	6,794	56	483	(Pflug et al. 2007)
2	CYP102A1[b]	1,490	12,720	75	644	(Pflug et al. 2007)
3	$CPR2_{mut}$	Na	Na	55	600	(Scheps et al. 2013)
4	CYP153A6	Na	Na	9.5	200	(Gudiminchi et al. 2012; Olaofe et al. 2013)
5	A13-Red	455	4,973	3.1	33.9	This study

CYP cytochrome P450 monooxygenases, Na not applicable

[a] Cultivated on glucose as the sole carbon source

[b] Cultivated on glycerol as the sole carbon source

1-alcohol in vivo. However, cytotoxicity of the substrates and moderate efficiencies for coupling the product formation to cofactor consumption (50 % for octane oxidation) were assumed to be strong limitations in our case. Moreover, cell lysis drastically limited a practical recovery of the products by forming very stable micellar systems (Bordeaux et al. 2011). Despite the attractiveness of whole-cell biocatalysis, investigating the behavior of isolated CYPs is of fundamental interest. Substrate and cofactor availabilities as well as enzyme stability are well identified as key parameters directly affecting the catalytic efficiency of CYPs in vitro (van Beilen et al. 2003; Urlacher and Girhard 2012). This was also the case for octane hydroxylation catalyzed by A13-Red as suggested by our preliminary experiments (Bordeaux et al. 2011). Solubility of octane in water is poor (4 μM) which drastically limits its availability for the enzyme and the hydroxylation activity. Diverse solutions to overcome this difficulty have been proposed in the literature, such as the addition of a cosolvent (monophasic medium) (Peters et al. 2003), the use of a tensioactive (stabilized micellar medium) (Ryan and Clark 2008), or of a two-phase medium in which the organic phase can be either made of pure (Maurer et al. 2005) or of diluted substrate (Ryan and Clark 2008). Nevertheless, predicting activity and stability profiles of an enzyme versus different reaction conditions is merely unfeasible. Into a one-phase medium with ethanol as cosolvent, we previously reported 410 TTN using 150 nM A13-Red (61.5 μM 1-octanol), while here, we report only 141 TTN using 500 nM A13-Red (71 μM 1-octanol). Since TTN is a fair reflection of operational stability, this result indicates a very short lifetime of A13-Red into one-phase reaction medium (estimated to approximately 5 min). It surprisingly appeared that A13-Red stability was tripled into a two-phase medium with water/octane 1:1 (TTN=233) rather than in a one-phase medium. Our results into stabilized emulsions were in contradiction with those reported for $P450_{cam}$ where authors noticed an important

Table 4 Selected in vitro hydroxylation activities of CYPs

Entry	Catalyst	Substrate	Product (selectivity)	TTN	Reference
1	CYP153A[a]	*n*-Octane	1-Octanol (91 %)	55	(Scheps et al. 2011)
2	CYP102A1[b]	*n*-Octane	Octanols[c] (na)	2,200	(Maurer et al. 2005)
3	$P450_{PMO}$ R2	*n*-Propane	2-Propanol (90 %)	45,800	(Fasan et al. 2007)
4	$P450_{PMO}$ R2	Ethane	Ethanol (100 %)	2,450	(Fasan et al. 2008)
5	CYP102A1[d]	Cyclohexane	Cyclohexanol (100 %)	12,850	(Maurer et al. 2005)
6	$P450_{cam}$	Camphor	Hydroxycamphor[e] (na)	28,900	(Ryan and Clark 2008)
7	A13-Red	*n*-Octane	1-Octanol (>99 %)	141/410[f]	This study and (Bordeaux et al. 2011)
8	A13-Red	*n*-Octane	1-Octanol (>99 %)	3,246	This study

TN total turnover number (μmol product/μmol catalyst), *Na* not applicable

[a] CYP153A from *Polaromonas* sp. Strain JS666

[b] Mutant A174G/F87V/L188Q

[e] 2-, 3-, and 4-octanol as well as the corresponding octanones were produced

[d] Mutant R47L/Y51F

[e] Hydroxycamphor as well as 2,5-diketocamphane were produced

[g] We have reported 410 TTN when using 150 nM A13-Red, and we report here 96 TTN using 500 nM A13-Red (see text for explanations)

enzymatic activation in the presence of AOT for camphor hydroxylation (Ryan and Clark 2008) suggesting that A13-Red and $P450_{cam}$ behave very differently into stabilized emulsions. Rapid and irreversible precipitation of A13-Red was estimated to occur within 15 min into two-phase reaction media. This is a common trend for enzymes in the presence of large amounts of organic solvent (Serdakowski and Dordick 2008). This can be, at least partially, avoided by the addition of protectants such as glycerol or BSA (Hofstetter et al. 2004). Recycling of NADPH is necessary to avoid stoechiometric consumption of this expensive cofactor during the reaction. Substoechiometric amounts of NADPH that can be used when recycling is performed by a second enzyme that uses a (preferentially cheap) sacrificial substrate to selectively and efficiently reduce NADP+(Wichmann 2005; Zhao and van der Donk 2003). In our case, commercial iCDH proved to be an effective system. Similar efficiency of this recycling system was previously reported when used with CYP102A1 mutants (Fasan et al. 2007). Finally, poisoning of A13-Red by H_2O_2 can be highly reduced in the presence of catalase as shown with CYP102A1 (Maurer et al. 2005). The cumulated stabilizing effects of BSA and catalase on A13-Red were very close to that on CYP102A1 mutants into similar biphasic systems (Maurer et al. 2005). It confirmed that the use of isolated CYPs is a valuable strategy and that such a system is applicable to different CYPs.

Achieving terminal oxidation selectively and efficiently is typically a great challenge. We have observed 3246 TTN for the 99 %+selective production of 1-octanol from *n*-octane. So far, this is the highest value reported for in vitro octane hydroxylation which makes A13-Red the most efficient and selective octane ω-hydroxylase to date (Table 4, entries 1 and 2). However, this TTN value is still 1 order of magnitude lower than the highest TTN value reported for CYP102A1 mutants (Table 4, entries 3–5) and wild-type P450cam (Table 4, entry 6).

For the first time, we have demonstrated in this study that a CYP enzyme can be readily prepared using HCDC in high titers. A special focus on in vitro biotransformation has permitted to reach high-hydroxylation activities on octane. The octane layer continuously extracts the octanol produced and can be easily separated from the aqueous layer by centrifugation. Compared with whole-cell bioconversions, where extremely stable emulsions were observed, recovery of the product is potentially simpler and cytotoxicity problems are not occurring in vitro. Time-consuming and effort-intensive molecular engineering of A13-Red was avoided, even though it could likely yield more efficient variants. With this biocatalyst on one hand and optimized reaction conditions on the other hand, we provide a new tool for the in vitro oxyfunctionalization of medium-chain alkanes, a notoriously difficult reaction.

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