

# Oxygenation of Linear Alkanes with a Biosynthetic, Self-Sufficient, Selective, and Soluble Hydroxylase

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## Abstract

Selective oxygenation of C–H bonds carried by hydrophobic molecules is a challenging reaction. Numerous chemical and biological catalysts have been developed during the past decades to perform this difficult task. Chemical methods generally involve toxic or dangerous reagents and are not very selective. Thus, they are far from ideal and not fully compatible with the principles of green chemistry. On the road to biocatalytic and selective oxygenation of C–H bonds, we have produced and fully characterized a biosynthetic self-sufficient biocatalyst for alkane activation. To do so, we have fused a cytochrome P450 from *Alcanivorax borkumensis* SK2 with a Fe<sub>2</sub>S<sub>2</sub>/FMN reductase from *Rhodococcus* sp. NCIMB 9784. With this original enzyme, thereafter referred to as “A13-Red,” the highly selective and efficient hydroxylation of alkanes from *n*-hexane to *n*-decane on the terminal methylic position was enabled under mild conditions. This enzyme was the first member of a novel family of biosynthetic omega hydroxylases. Detailed protocols for A13-Red construction, purification, and optimized reaction conditions for the *in vitro* hydroxylation of its preferred substrate (*n*-octane) are described in detail into this chapter.

**Keywords:** Biosynthetic self-sufficient hydroxylase, C–H bond activation, Cytochrome P450, Green chemistry, Linear alkanes

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## 1 Introduction

Nature provides a large variety of hydrocarbons [1], including paraffins (alkanes, iso-alkanes, and alkenes), cycloparaffins (cycloalkanes, naphthenes), and aromatics, as a result of the metabolic activities of animals, plants, and microorganisms and by the physicochemical action of decay of organic matter. They are most evident in the form of crude oil and gas deposits. There is a body of opinion that petroleum (crude oil) deposits are too valuable to expend as fuel and should be conserved for the production of chemicals alone. Considering their numerous advantages (i.e., versatility, regio-, chemo-, enantioselectivity, environmental-friendly processes, etc.) [2], the role of microorganisms in a scenario of optimal use of such valuable raw materials would be central.

Indeed, a very large number of microorganisms have the ability to convert hydrocarbons from crude oil into biomass and useful intermediates or products such as polyhydroxyalkanoates, wax esters, or triacylglycerols [3, 4]. These microbes are the foundations of hydrocarbon biotechnology. Understanding the underlying biological mechanisms of these conversions is of fundamental and practical interest. Several groups of researchers have worked to elucidate these biochemical mechanisms for several decades [5, 6], but current economic uncertainties limit the amount of research activity and the implementation of emerging technologies.

One of the most widespread catabolic pathways in hydrocarbon metabolism among microorganisms is initiated by the primary enzymatic attack of the hydrocarbon molecule. This is generally an oxyfunctionalization yielding an alcohol. For alkanes in particular, Nature has developed a great diversity of enzymatic hydroxylation systems [2]. Most of them share common features: they use dioxygen from air as the oxidizing agent, they use reduced nicotinamide dinucleotides (NAD(P)H) as electron suppliers, they are metalloenzymes based on copper and/or iron, and they operate under mild conditions [2, 6].

Among all these enzymatic systems involved into alkane hydroxylation, cytochromes P450 (P450) from the CYP153 family attracted our attention because they are monomeric soluble enzymes that hydroxylate alkanes [7–11]. Despite their attractiveness, every CYP153 enzyme needs at least one supplementary protein (also called a redox partner) to shuttle electrons from NAD(P)H to the active center of the P450 responsible for the oxidation reaction [12–17]. The requirement for these redox partners could be seen as a disadvantage, but in fact, it has been shown through different examples that P450s can be artificially fused with either their natural partner or with a foreign partner [18–20]. The resulting biosynthetic proteins are usually enzymatically active and self-sufficient. Using this strategy, we have fused a cytochrome P450 from *A. borkumensis* SK2 (CYP153A13a) with a Fe<sub>2</sub>S<sub>2</sub>/FMN reductase from *Rhodococcus* sp. NCIMB 9784 (RhFred) [21, 22]. The resulting polypeptide (thereafter A13-Red) was reliably expressed in *Escherichia coli* using high-cell-density cultivation (up to 100 g of dry-cell weight per liter of culture medium) [23]. In this chapter, we detail the construction of the gene encoding for A13-Red, our two-step purification strategy to yield A13-Red approximately 90% homogeneous and its use for the regioselective hydroxylation of *n*-octane into optimized conditions.

## 2 Materials

### 2.1 Construction of pA13-Red

1. Authentic sample of *Alcanivorax borkumensis* SK2 strain (DSM 11573) ([www.dsmz.de](http://www.dsmz.de))
2. Medium 809, composition for 1 L: NaCl 23.00 g, MgSO<sub>4</sub> × 7 H<sub>2</sub>O 5.80 g, MgCl<sub>2</sub> × 2 H<sub>2</sub>O 6.16 g, CaCl<sub>2</sub> × 2 H<sub>2</sub>O 1.47 g, Na<sub>2</sub>HPO<sub>4</sub> × 7 H<sub>2</sub>O 0.89 g, NaNO<sub>3</sub> 5.00 g, FeSO<sub>4</sub> × 7 H<sub>2</sub>O 0.03 g, sodium pyruvate 10.00 g, pH 7.0–7.5
3. Qiagen Gentra PureGene Yeast/Bacteria kit ([www.qiagen.com](http://www.qiagen.com))
4. pET28b(+) plasmid, Novagen ([www.merckmillipore.com](http://www.merckmillipore.com)) previously digested by *EcoRI* and *NotI* enzymes
5. pET28b(+)-pikC-RhFred plasmid [19] (generous gift from Dr D. Sherman, University of Michigan, USA)
6. KOD Hot Start Master Mix, Novagen
7. Qiagen PCR Purification kit
8. Primers 153A13a-fw and 153A13a-rv for amplification of CYP153A13a, primers Eco887-fw and Eco887-rv for removal of the *EcoRI* restriction site at position 887 of CYP153A13a, and primers RhFred-fw and RhFred-rv for amplification of RhFred (*see* Table 1 for primer sequences)
9. Restriction enzymes: *DpnI*, *NdeI*, *EcoRI*, and *NotI*, New England Biolabs ([www.neb.com](http://www.neb.com))
10. T4 DNA ligase, New England Biolabs
11. *Escherichia coli* strains: DH5 $\alpha$  and BL21 Star<sup>TM</sup> (DE3), Life Technologies ([www.lifetechnologies.com](http://www.lifetechnologies.com))
12. Kanamycin sulfate antibiotic, Sigma-Aldrich ([www.sigmaaldrich.com](http://www.sigmaaldrich.com))

**Table 1**

**Sequences of the primers used for the amplification of CYP153A13a and RhFred and for the removal of *EcoRI* restriction site at position 887 in CYP153A13a**

Primer	Sequence
153A13a-fw	GTAGCTCATATGTCAACGAGTTCAGT
153A13a-rv	TACAGCGAATTCTTTTTTAGCCGTCAA
Eco887-fw	ATCAATCGTCCTTTGGAGTTCATTGGTAATCT
Eco887-rv	GAGATTACCAATGAACTCCAAAGGACGATTG
RhFred-fw	ACGGCTAAAAAAGAATTCGTGCTGCACCGG
RhFred-rv	TAATGCGGCCGCTCAGAGTCGCA

13. Growth media: Luria Bertani (LB) and Terrific Broth (TB), Sigma-Aldrich
14. Qiagen Plasmid Purification kit

**2.2 Purification of A13-Red on Nickel-NTA Column**

1. *E. coli* strain BL21 Star™ (DE3)/pA13-Red (fresh or frozen wet-cell pellet)
2. Lysis buffer: Tris-HCl 25 mM, NaCl 500 mM, glycerol 10% (v/v), triton X-100 1% (v/v), lysozyme 0.25 mg/mL, PMSF 1 mM, DNase I 5 mg/mL, pH 7.5
3. Binding buffer: NaCl 800 mM, Tris-HCl 50 mM, pH 7.5
4. Washing buffer: NaCl 800 mM, Tris-HCl 50 mM, glycine 250 mM, pH 7.5
5. Elution buffer: NaCl 800 mM, Tris-HCl 50 mM, L-histidine 80 mM, pH 7.5
6. HisPrep FF 16/10 prepacked column (GE Healthcare)
7. Amicon Centrifugal Ultrafiltration Units (50-kDa cutoff)
8. Conservation buffer: Tris-HCl 25 mM, NaCl 500 mM, glycerol 20%, pH 7.5

**2.3 Desalting of A13-Red Fractions After Ni-NTA Column**

1. Desalting buffer: Tris-HCl 25 mM, NaCl 25 mM, glycerol 20%, pH 7.5
2. HiTrap desalting 5-mL prepacked columns (GE Healthcare)

**2.4 Purification of A13-Red on Q Sepharose Column**

1. Buffer A: Tris-HCl 25 mM, NaCl 80-mM glycerol 20%, pH 7.5
2. Buffer B: Tris-HCl 25 mM, NaCl 200 mM, glycerol 20%, pH 7.5
3. Q Sepharose XL prepacked column (1 mL) (GE Healthcare)

**2.5 Enzymatic Hydroxylation of n-Octane**

1. Wheaton 5-mL V-Vials with a magnetic stirrer
2. Reaction buffer: potassium phosphate buffer (100 mM, pH 7.4), glycerol 10%
3. *n*-Octane (Sigma-Aldrich; puriss. p.a., ≥99.0% GC)
4. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich, 25-mM stock solution into reaction buffer)
5. Bovine serum albumin (BSA) (Sigma-Aldrich, 100 g/L stock solution into reaction buffer)
6. NADP-dependent isocitrate dehydrogenase from *Bacillus subtilis* (BS-iDH, Sigma-Aldrich, 186 U/mL)
7. D/L-isocitrate (Sigma-Aldrich, 100-mM stock solution into reaction buffer)

8. MgCl<sub>2</sub> (Sigma-Aldrich, 500-mM stock solution into reaction buffer)
9. Catalase from bovine liver (Sigma-Aldrich, stock solution at 50 U/mL into reaction buffer)
10. Purified A13-Red (11.6- $\mu$ M solution into Buffer B)
11. Dodecane (Sigma-Aldrich) 400-mM solution into absolute ethanol (Sigma-Aldrich)

### 3 Methods

The expression vector was designed for generating a fusion between the respective genes encoding for a 6 $\times$ His-tag (N-Term), CYP153A13a from *A. borkumensis* SK2 and the P450 reductase domain (RhFred) of P450 RhF from *Rhodococcus* sp. NCIMB 9784 (C-Term) (Fig. 1) [22]. This construction enables the expression of A13-Red protein which can be captured by

10	20	30	40	50	60
MGSSHHHHH	SSGLVPRGSH	MMSTSSSTSN	DIQAKIINAT	SKVVPMHQLI	KALKNLMKVK
70	80	90	100	110	120
RKTIGTSRPQ	VHFVETDLPD	VNDLAIEDID	TSNPFLYRQG	KANAYFKRLR	DEAPVHYQKN
130	140	150	160	170	180
SAFGPFWSVT	RYEDIVFVDK	SHDLFSAEPQ	IILGDPPEGL	SVEMFIAMDP	PKHDVQRRAV
190	200	210	220	230	240
QGVVAPKNLK	EMEGLIRKRT	GDVLDLPLD	TPFNWVPVVS	KELTGRMLAS	LLDFPYDERE
250	260	270	280	290	300
KLVGWSDRLS	GASSATGGEF	TNEDEVFFDDA	ADMAWAFSKL	WRDKEARQKA	GEEPFGDLIS
310	320	330	340	350	360
MLQSNEDTKD	LINRPLEFIG	NLALLIVGGN	DTTRNSMSGG	VLALNQFPEQ	FEKLLKANPKL
370	380	390	400	410	420
IPNWSLKYSL	ATPLAYMRRV	AKQDVELNGQ	TIKKGDRVLM	WYASGNQDER	KFENPEQFII
430	440	450	460	470	480
DRKDTRNHVS	FGYGVHRCMG	NRLAELQLRI	LWEELLPRFE	NIEVIGEPER	VQSNFVRGYS
490	500	510	520	530	540
KMMVKLTAKK	<u>EFVLRHQQPV</u>	<u>TIGEPAAARAV</u>	SRTVTVERLD	RIADDVLRVL	LRDAGGKTLF
550	560	570	580	590	600
TWTPGAHIDL	DLGALSQYYS	LCGAPDAPSY	EIAVHLDPEP	RGGSRYSIHEQ	LEVGSPLRMR
610	620	630	640	650	660
GPRNHFALDP	GAEHYVFVAG	GIGITPVLAM	ADHARARGWS	YELHYCGRNR	SGMAYLVERA
670	680	690	700	710	720
GHGDRAALHV	SEEGTRIDLA	ALLAEPAPGV	QIYACGPGRL	LAGLEDASRN	WPDGALHVEH
730	740	750	760	770	780
FTSSLAALDP	DVEHAFDLEL	RDSGLTVRVE	PTQTVLDALR	ANNIDVPSDC	EEGLCGSCEV
790	800	810	820		
AVLDGEVDHR	DTVLTKAERA	ANRQMMTCCS	RACGDRLALR	L	

**Fig. 1** Complete protein sequence of A13-Red. The construction was designed to obtain a 6 $\times$ His-tag (residues from 5 to 10) merged with CYP153A13a from *A. borkumensis* SK2 (residues from 21 to 490) and the P450 reductase domain (RhFred) of P450 RhF from *Rhodococcus* sp. NCIMB 9784 (residues from 509 to 821). P450 and reductase domains are separated by the native peptidic linker (residues from 491 to 508) found in P450 RhF from *Rhodococcus* sp. NCIMB 9784. The underlined sequence corresponds to a characteristic peptide identified by nanoLC/MS/MS analysis

immobilized metal affinity chromatography (IMAC). After high-cell-density cultivation, cells of BL21 Star™ (DE3)/pA13-Red can be harvested by centrifugation and frozen at  $-20^{\circ}\text{C}$  until the purification process described below.

### 3.1 Construction of pA13-Red

Construction of pA13-Red was performed in three steps. The first step is the cloning of *rhfred* gene (accession number AAM67416) into pET28b(+) between *EcoRI* and *NotI* to yield the intermediate plasmid pETRed. The second step is the amplification of *cyp153a13a* gene (accession number AY505118) from *A. borkumensis* SK2 genomic DNA and its modification in order to remove the *EcoRI* restriction site. Finally, the third step is the cloning of this modified gene into pETRed to yield pA13-Red.

#### (a) Step 1: Cloning of *rhfred* gene into pET28b(+)

1. The RhFred part of the construction should be amplified using RhFred-fw and RhFred-rv primers. The PCR reaction conditions are  $95^{\circ}\text{C}$  for 5 min; 30 cycles of  $95^{\circ}\text{C}$  for 20 s,  $60^{\circ}\text{C}$  for 10 s, and  $70^{\circ}\text{C}$  for 30s; and a final extension at  $72^{\circ}\text{C}$  for 5 min. The final reaction volume is 50  $\mu\text{L}$ .
2. Purify the PCR product using the Qiagen cleanup kit.
3. Digest the PCR fragment with *EcoRI* and *NotI* enzymes for 2 h at  $37^{\circ}\text{C}$  followed by gel extraction or PCR cleanup.
4. Clone the digested RhFred insert into pET28b(+) plasmid previously digested by *EcoRI* and *NotI* enzymes by standard ligation procedure and transform it into *E. coli* DH5 $\alpha$  competent cells following manufacturer's protocol.
5. The success of the cloning should be verified by colony PCR using RhFred-fw and RhFred-rv primers and sequencing.
6. Prepare plasmid pETRed using Qiagen MiniPrep kit and following manufacturer's instructions for cultivation.

#### (b) Step 2: Amplification and modification of *cyp153a13a* gene

1. Freeze-dried sample of *A. borkumensis* SK2 (DSM 11573) is reactivated following provider instructions and cultivated at  $28^{\circ}\text{C}$  into Medium 809 for 48 h.
2. After cultivation, cells are harvested by centrifugation and genomic DNA is extracted using Qiagen Genra PureGene kit and following manufacturer's instructions.
3. For removal of *EcoRI* site at position 887 of CYP153A13a, overlap extension strategy is performed. Extracted genomic DNA was used as a template for fragment A and fragment B amplifications. Fragment A is amplified using 153A13a-fw and Eco887-rv primers. The PCR reaction conditions are  $95^{\circ}\text{C}$  for 2 min; 30 cycles of  $95^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 10 s, and  $70^{\circ}\text{C}$  for 20 s; and a final extension at  $72^{\circ}\text{C}$  for 5 min. Fragment B is amplified using Eco887-fw and 153A13a-rv

primers under the same PCR conditions. The final reaction volumes are both 50  $\mu$ L.

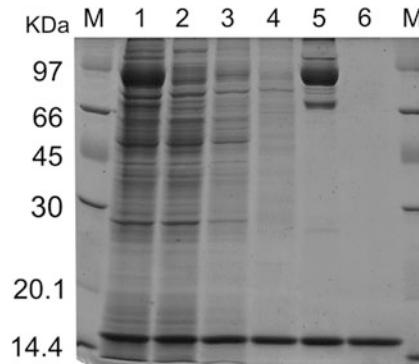
4. The crude PCR mixtures are digested by *DpnI* and PCR products purified with Qiagen PCR Purification Cleanup kit.
5. Fragments A and B are reassembled using 153A13a-fw and 153A13a-rv primers. The PCR reaction conditions are 95°C for 2 min; 30 cycles of 95°C for 20 s, 50°C for 10 s, and 70°C for 30 s; and a final extension at 72°C for 5 min. The final reaction volume is 50  $\mu$ L.
6. Purify the PCR product using the Qiagen cleanup kit.

(c) Step 3: Cloning of *cyp153A13a* gene into pETRed

1. Digest the PCR fragment with *NdeI* and *EcoRI* enzymes for 2 h at 37°C followed by gel extraction or PCR cleanup.
2. Clone the digested CYP153A13a insert into pETRed plasmid previously digested by *NdeI* and *EcoRI* enzymes by standard ligation procedure and transform it into *E. coli* DH5 $\alpha$  competent cells.
3. The success of the cloning should be verified by colony PCR using 153A13a-fw and RhFred-rv primers and sequencing.
4. Prepare plasmid pA13-Red using Qiagen MiniPrep kit and following manufacturer's instructions for cultivation (*see Note 1*).
5. Transform pA13-Red into BL21 Star<sup>TM</sup> (DE3) competent cells following manufacturer's protocol (*see Note 2*).

### 3.2 Purification of A13-Red on Nickel-NTA Column

1. Thoroughly resuspend the cell pellet in cold (4°C) lysis buffer (use approximately 2 mL for 1 g of pellet).
2. Incubate at 4°C for 30 min.
3. Centrifuge at 10,000 rpm, 30 min, 4°C.
4. Filter the supernatant through a 0.45- $\mu$ m membrane.
5. Before loading, equilibrate the HisPrep FF 16/10 column with 10 column volumes (CV) of binding buffer.
6. Load the cell-free extract on the column (2.5 mL/min).
7. Wash the column with binding buffer (5 mL/min) until optical density at 280 nm (OD280) reaches baseline.
8. Wash the column with washing buffer (5 mL/min) until OD280 reaches baseline.
9. Elute the protein with elution buffer (2.5 mL/min). Collect 1-mL fractions and pool protein containing fractions.
10. Analyze the captured A13-Red by SDS-PAGE (Fig. 2) (*see Note 3*).
11. Elution buffer can be exchanged by ultrafiltration against conservation buffer and samples stored at -20°C.



**Fig. 2** SDS-PAGE (12% polyacrylamide) analysis of ion metal affinity chromatography fractions. The soluble fraction after lysis (1) was applied to HisPrep 16/10 column at a 2.5 mL/min flow rate. The flowthrough fraction containing unbound proteins (2) was collected. Two steps of washing were performed: the first one is carried out with binding buffer and the second one with washing buffer. Both fractions (respectively, 3 and 4) were collected. Elution of A13-Red was performed using histidine (5). A final elution with 2-M imidazole (6) was performed as a control

### 3.3 Desalting of A13-Red Fractions After Ni-NTA Column

After Ni-NTA capture, A13-Red samples are contained into a salt-rich buffer (elution buffer). Prior to ion exchange chromatography, this salt-rich buffer has to be exchanged against desalting buffer for an optimal interaction between A13-Red and the stationary phase:

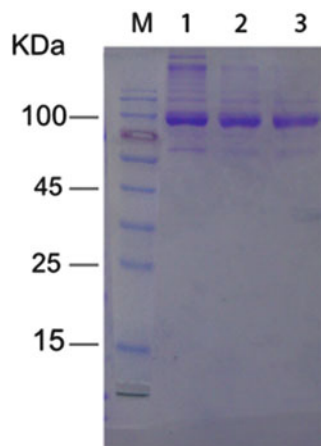
1. Before loading, two HiTrap desalting columns of 5 mL are connected in series and equilibrated with 10 CV of desalting buffer (*see Note 4*).
2. Load 1 mL of Ni-NTA purified A13-Red (1 mL/min).
3. Elute the protein with desalting buffer (1 mL/min). Collect 0.5-mL fractions; pool those containing the protein and having the desired conductivity.

### 3.4 Purification of A13-Red on Q Sepharose XL Column

A second purification step for removing the 70-kDa impurity was set up based on ion exchange. Since A13-Red has a calculated pI of 6.01, Q Sepharose stationary phase (strong cation) was preferred (*see Note 5*). The optimized protocol is described below:

1. Before loading, equilibrate the Q Sepharose XL 1-mL pre-packed column with Buffer A (10 CV).
2. Load the desalted A13-Red sample (up to 25 mL, 1 mL/min).
3. Wash the column with Buffer A (1 mL/min) until OD280 reaches baseline.
4. Elute A13-Red with Buffer B (1 mL/min). Collect 1-mL fractions and pool A13-Red containing fractions.





**Fig. 3** SDS-PAGE (12% polyacrylamide) analysis after ion exchange and size exclusion chromatography. Lane 1: A13-Red sample after Q Sepharose XL (strong cation) chromatography. Lanes 2 and 3: A13-Red samples after Superdex 75 16/60 chromatography (not described in this chapter)

5. Analyze the purified A13-Red by SDS-PAGE (Fig. 3) (*see* **Notes 6** and **7**).
6. Store the protein at 4°C. For prolonged storage (more than 1 week), it is preferable to store A13-Red in small aliquots at -80°C.

A13-Red from this two-step purification protocol can be used without further purification in hydroxylation reactions.

### 3.5 Enzymatic Hydroxylation of *n*-Octane

Every reaction was performed into Wheaton 5-mL V-Vials at 25°C under gentle magnetic stirring, repeated thrice, and errors were estimated to be 5–10%. The protocol described below is optimized for a 1-mL total reaction volume (aqueous phase 500 µL/organic phase 500 µL).

1. Add 279 µL of reaction buffer into a Wheaton 5-mL V-Vial.
2. Add 50 µL of BSA 100 g/L (*see* **Note 8**).
3. Add 5 U (25 µL in our case) of BS-iDH stock solution (*see* **Note 9**).
4. Add 100 µL of D/L-isocitrate 100 mM.
5. Add 5 µL of MgCl<sub>2</sub> 500 mM.
6. Add 0.5 U of catalase stock solution (10 µL in our case) (*see* **Note 10**).
7. Add *n*-octane (500 µL) drop wise under gentle magnetic stirring.
8. Add 21 µL of A13-Red stock solution (final concentration 500 nM) and incubate for 1 min.

9. Add 10  $\mu\text{L}$  of NADPH 25 mM to trigger the hydroxylation reaction.
10. Tightly screw the V-Vial cap and seal it with parafilm.

For analysis, the emulsified solution was transferred into a 1.5-mL tube and dodecane was added (5  $\mu\text{L}$ , final concentration into the organic phase 4 mM, neglecting dodecane solubility into the aqueous phase). The sample was vortexed for 1 min and subjected to centrifugation (2 min, 10,000*g*, room temperature), and the organic layer was collected and analyzed by GC–MS (Shimadzu GC-2010 instrument) with an SLB-5MS column (30-m length; 0.25-mm diameter; Supelco). The injection and detection temperatures were both 250°C. For reactions with octane, the method used was an isotherm at 35°C for 5 min, followed by a temperature raise to 190°C at a rate of 10°C/min. The products were quantified by selected ion monitoring (SIM) program, and their concentrations were calculated from the corresponding area using dodecane as the internal standard (*see* **Note 11**).

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## 4 Notes

1. The resulting plasmid pA13-Red and the strain BL21 Star<sup>TM</sup>(DE3)/pA13-Red are freely available for academic and collaborative research under request.
2. Cultivation of microorganisms in flasks with a rich medium (LB, TB, or 2 $\times$ YT) 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 in average  $35 \pm 25$  mg of functional CYP per liter of culture with TB as described in references [22, 23]. 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 high-cell-density cultivation (HCDC) with instrumented fermenters as described in details in reference [23]. Using this protocol, up to 5,000 nmol of functional A13-Red can be obtained per liter of HCDC.
3. IMAC can be sufficient to obtain A13-Red with 95% purity after flask cultivation, but it was not true for A13-Red produced by HCDC where only 70–75% purity was reached by performing IMAC only (Fig. 2). Since A13-Red is irreversibly inhibited by imidazole, elution was performed using histidine. One of the most abundant contaminating proteins (approx. 70 kDa) was identified to be CYP153A13a by acrylamide gel extraction and trypsin digestion of the extract followed by NanoLC/MS/MS analysis (Fig. 1). We assumed that a portion

of A13-Red was subjected to proteolysis into the cytoplasm of the bacteria during expression.

4. For scaling up the desalting step on A13-Red samples, up to five columns can be connected in series. For sample volumes up to 15 mL, HiPrep 26/10 Desalting is available. Up to four HiPrep 26/10 Desalting columns can be connected in series without increased backpressure (up to 60-mL sample volume).
5. A first series of experiments with a linear gradient of NaCl showed that the 70-kDa impurity was eluted at 80-mM NaCl while A13-Red at 200 mM.
6. High molecular weight impurities (above 200 kDa) still remained present after this step (Fig. 3). It is likely that these contaminants are polymers of A13-Red since their concentration progressively increased over time into the conservation buffer at  $-80^{\circ}\text{C}$  into pure A13-Red samples. They can be eliminated by size exclusion chromatography using a Superdex 75 16/60 column (Fig. 3), but their presence had no significant impact on enzymatic hydroxylation activity.
7. For each purification step, functional P450 concentration is determined from the carbon monoxide difference spectra as previously described [24], using an extinction coefficient of  $91\text{ mM}^{-1}\text{ cm}^{-1}$  for the 450 minus 490-nm peak. Compared to the Bradford protein assay, CO-binding is a selective assay for P450. Moreover, it allows to determine the ration between functional (450 nm) and nonfunctional P450 (420 nm).
8. Stock solutions for the different additives should be prepared with reaction buffer (potassium phosphate buffer (100 mM, pH 7.4), glycerol 10%). For example, to prepare  $\text{MgCl}_2$  500-mM stock solution, solid  $\text{MgCl}_2$  should be dissolved into reaction buffer.
9. By definition, for BS-iDH, one unit (U) corresponds to the amount of enzyme which converts 1- $\mu\text{mol}$  D-isocitrate to  $\alpha$ -ketoglutarate per minute at pH 7.5 and  $37^{\circ}\text{C}$  (NADP as cofactor).
10. By definition, one unit of catalase will decompose 1.0  $\mu\text{mole}$  of  $\text{H}_2\text{O}_2$  per min at pH 7.0 at  $25^{\circ}\text{C}$ , while the  $\text{H}_2\text{O}_2$  concentration falls from 10.3 to 9.2 mM, measured by the rate of decrease of OD240.
11. Under these conditions, A13-Red remains active up to 78 h, and 1.6 mM of *n*-octanol is formed corresponding to a total turnover number about 3,250. No other regio-isomer or over-oxidation product was detected confirming the remarkable regio- and chemoselectivity of A13-Red.

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