Supplementary Information

Novel P450-based biocatalyst for the selective production of chiral 2-alkanols

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Supplementary Materials and Methods

1. Chemicals and enzymes

The following chemicals were used: *n*-heptane (VWR), *n*-octane (Sigma-Aldrich), *n*-nonane (Alfa Aesar), *n*-decane (Alfa Aesar), 2-heptanol (TCI), 2-octanol (Sigma-Aldrich), 2-nonanol (Alfa Aesar) 2-decanol (TCI), and enantiomerically pure 2-(*S*)-heptanol (Alfa Aesar), 2-(*S*)-octanol (Merck), 2-(*S*)-nonanol (Sigma-Aldrich), 2-(*R*)-decanol (Sigma-Aldrich) and were of at least 97 % purity. Bovine serum albumin (BSA) and catalase from bovine liver was obtained from Sigma-Aldrich (Schnelldorf, Germany). NADPH and $NADP^+$ were commercially available from GERBU Biotechnik GmbH (Heidelberg, Germany).

2. Expression and quantification of the purified proteins

Enzymes were expressed and purified as described elsewhere.[1] Concentration of CYP154A8 was determined according to the method of Omura and Sato.[2] FdR and YkuN were quantified via UV/VIS spectroscopy using their published absorption coefficients.[3]

3. Biotransformations

3.1. Homogeneous aqueous reaction setup

The standard homogeneous reactions were performed in 500 ml reaction volume in 2 ml plastic reaction tubes. A master mix containing 0.675 mM CYP154A8, 6.75 mM YkuN, 0.675 mM FdR, 5 U GDH, 100 mM glucose, 10 mg/ml BSA, 600 U catalase and 200 mM $NADP^+$ was prepared and aliquoted to the reaction vessels containing *n*-alkane solution from a solution in ethanol to a final concentration of 4 mM. The reactions were performed at 25 °C for 24 h in a rotator (20 rpm). To ensure efficient cofactor regeneration the amount of glucose was set to 100 mM. Thus it is guaranteed that the cofactor regeneration is not rate limiting to the catalyst itself and additionally the low coupling of P450 with non-natural substrates does not preclude high product concentrations.

3.2. Biphasic reaction setup

The standard biphasic reactions were performed in 500 ml reaction volume in 2 ml plastic reaction tubes or 10 ml round bottoms flasks. A master mix containing 0.675 mM CYP154A8, 6.75 mM YkuN, 0.675 mM FdR, 5 U GDH, 100 mM glucose, 10 mg/ml BSA, 600 U catalase, 1 vol-% cosolvent (ethanol except otherwise stated) and 200 mM $NADP^+$ was prepared and aliquoted to the plastic tubes. The reaction was started by adding 100 µl of pure *n*-alkane (substrate) as second phase and was performed at 25 °C in either a rotator (20 rpm) or on a magnetic stirrer for 24 h.

3.3. Reaction setup for micro titer plate (MTP) measurements

For the determination of the substrate oxidation rate and coupling, reactions in 96 well plates were performed. A master mix containing 0.675 mM CYP154A8, 6.75 mM YkuN, 0.675 mM FdR, 10 mg/ml BSA, 600 U catalase, 1 vol-% ethanol and 1 mM substrate was prepared. Experiments in quadruplicates were done in 200 µl volume in a Tecan Infinite M200Pro UV/VIS MTP spectrophotometer. The measurements were started by injection of 20 µl of an NADPH solution. The concentration of the NADPH concentration was increased from 320 µM until the amount of product was high enough to be detected via GC/MS. By following the absorbance at 340 nm the concentration of NADPH in the reaction was monitored during the reaction and from the absorbance differences between the beginning and the end of the experiment the precise amount of NADPH was determined and used for subsequent calculations. After the end of the reaction 100 µl 1N HCl was injected to stop the reaction. After addition of 200 µM internal standard (ITSD) (s. section 4) each well was extracted separately with 500 µl toluene and subjected to GC/MS analysis as described in section 4.1.

4. Product analysis

Products were analysed on a GC/MS QP-2010 Plus instrument (Shimadzu, Tokyo, Japan) with helium as carrier gas. For all substrates except *n*-octane, 1-octanol was used as an internal standard. For *n*-octane the internal standard was 1-decanol. The reactions were stopped by the additions of 50 µl 6N HCl and subsequently the ITSD was added to a final concentration of 2 mM prior to extraction (2 × 500 µl toluene).

4.1. Achiral GC/MS

Toluene extracts were measured using an FS-Supreme-5 30 m × 0.25 mm × 0.25 µm (Chromatographie Service GmbH, Langerwehe, Germany) fused silica. The oven temperature was 80 °C for 6 min, ramped to 140 °C at 10 K s^{-1} and subsequently heated with 50 K s^{-1} to 300 °C and held isothermal for 5 min.

4.2. Chiral GC/MS

Toluene extracts were dried over anhydrous $MgSO_4$ and for derivatisation incubated in GC/MS vials for 2 h at 90 °C with acetic anhydride. After the derivatisation took place the samples were washed twice with 500 µl water to remove excess acetic acid. Chiral analysis was done using an FS-CYCLODEX beta-I/P (Chromatographie Service GmbH, Langerwehe, Germany) column. The GC was held at 100 °C for 17 min, ramped to 200 °C at 10 K s^{-1} and held isothermal for 2 min. By comparison of an authentic sample of the racemic 2-alcohols

with an authentic sample of a single enantiomer the identity of the formed products was verified.

4.3. Determination of coupling and product formation rate

Based on the via GC/MS determined amount of 2-alcohol the substrate oxidation rate was calculated as:

$$r^{ox.} = \frac{n^{Prod}}{n^{P450} * t}$$

Further, coupling was calculated as:

$$coupling = \left(\frac{n^{Prod.}}{n^{NADPH}}\right) * 100$$

using: oxidation rate, $r^{ox.}$; amount of product, n^{Prod}; amount of NADPH, n^{NADPH}; time until depletion of NADPH, t; and amount of P450, n^{P450}.

4.4. Determination of partition coefficients

In order to determine the distribution of the formed 2-alcohols between the aqueous and the organic phase in the reaction system, partition coefficients have been determined. 5 ml potassium phosphate buffer (100 mM, pH 7.5) with ethanol as cosolvent and 10 mg/ml BSA were overlaid with 1 ml of *n*-alkane containing the corresponding 2-alcohol. To obtain comparable data, concentrations of all 2-alcohols were set to 2 mM (referring to the aqueous phase). Phases were equilibrated for 45 min on a rotator at 100 rpm. Subsequently, further equilibration and phase separation took place for 30 min without agitation. Finally, the samples have been centrifuged for 15 min at 3500 g and 20 °C. 500 µl aliquots of the organic and the aqueous phase have been taken and ITSD was added. Samples from the organic phase were analysed directly by GC/MS. Aqueous samples were extracted with toluene and analysed by GC/MS. Values from the aqueous phase have been normalized using the ITSD. Peak areas of the 2-alcohol in both phases have been used to calculate log*P* values.

$$\log P = \frac{A^{aq.}_{norm.}}{A^{org.}}$$

Using $A^{aq.}_{norm.}$: normalized peak area of the 2-alcohol in the aqueous phase and $A^{org.}$: peak area of the 2-alcohol in the equilibrated organic phase. Measurements were performed in triplicates.

Supplementary Results

5. Partition Coefficients

Table S1: Partition coefficients of the 2-alkanols determined for the respective biphasic buffer/alkane-reaction systems

Solute/ org. solvents	2-heptanol / *n*-heptane	2-octanol/ *n*-octane	2-nonanol / *n*-nonane	2-decanol / *n*-decane
$\log P^{alkane/buffer}$	1.0 ± 0.03	1.36 ± 0.02	1.36 ± 0.03	1.42 ± 0.1

6. Gas chromatograms of 2-alkanols from chiral GC/MS analysis

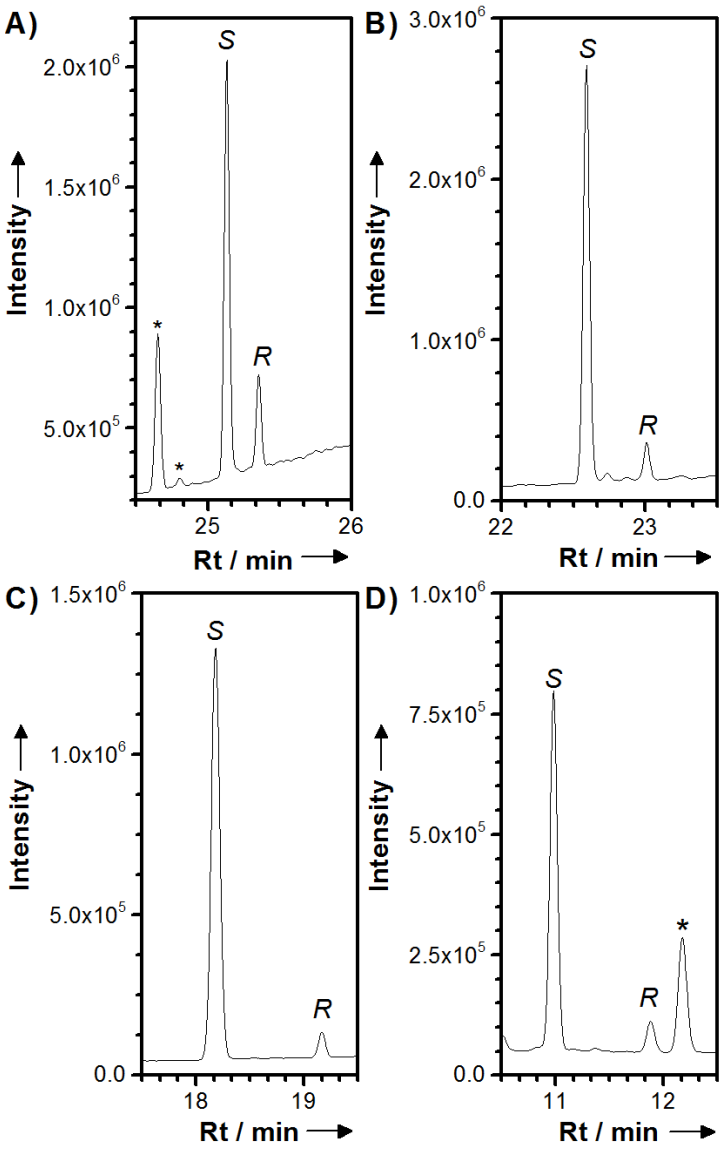

Figure S1: Chromatograms of the chiral GC/MS analysis of the 2-alkanols produced by CYP154A8 from: **A)** 2-decanol **B)** 2-nonanol **C)** 2-octanol **D)** 2-heptanol. Peaks are labelled with the stereodescriptors assigned with the help of authentic standards. An asterisk (*) denotes background peaks.

7. Time dependence of n-octane conversion

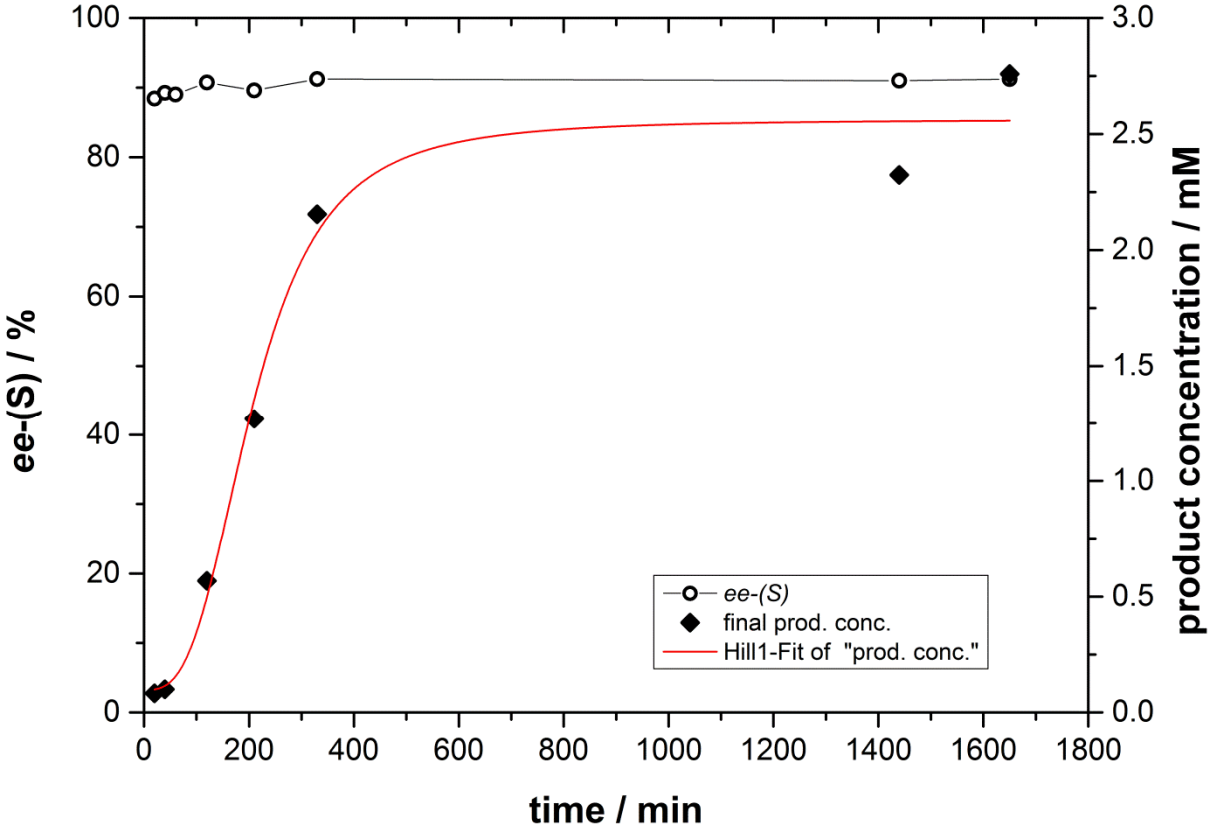

Figure S2: Monitoring of *n*-octane conversion and the *ee* value of 2-(*S*)-octanol over time. A common master mix (MM, composition as given in section 3.2) was prepared for all time points. 500 µl of the MM were aliquoted into separate 2 ml plastic reaction tubes. After the given time the reaction mixture was extracted to determine the amount of product and the enantiomeric excess via GC/MS.

8. Gas chromatograms of *n*-alkane conversion from achiral GC/MS analysis

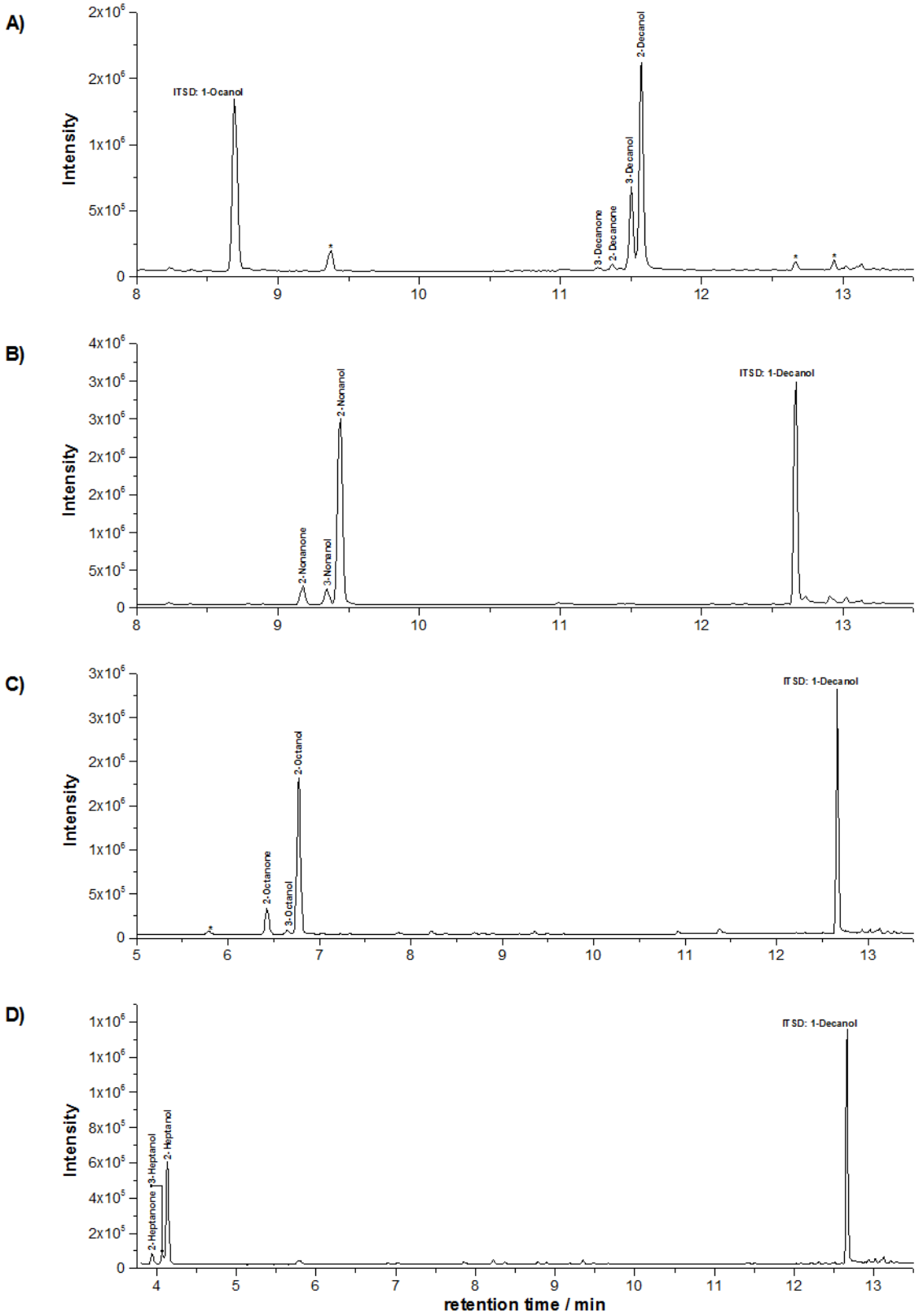

Figure S3 Chromatograms of the achiral GC/MS analysis of *n*-alkane conversions catalysed by CYP154A8: decane **A)**, nonane **B)**, octane **C)** and heptane **D)**. Peaks are labelled with the names of the corresponding products as well as internal standard (ITSD), and background peaks (*).

9. Optimization of the biphasic reaction system

In biphasic system several issues different to homogeneous reaction systems have to be addressed: Interfacial surface area is crucial for the mass transfer between aqueous and organic phase. This can be influenced by different mixing method.

With the standard biphasic reaction setup we tested: no mixing, shaking with a rotator and mixing with a magnetic stir bar in round bottom flasks. The results showed that the use of a rotator increased product formation by a factor of ~16 compared to the system without shaking (Figure S4 A). Mixing in a round bottom flask resulted however in lower amounts of products than in the system with a rotator. This indicated destabilisation and/or inactivation of the involved enzymes caused by shearing forces.

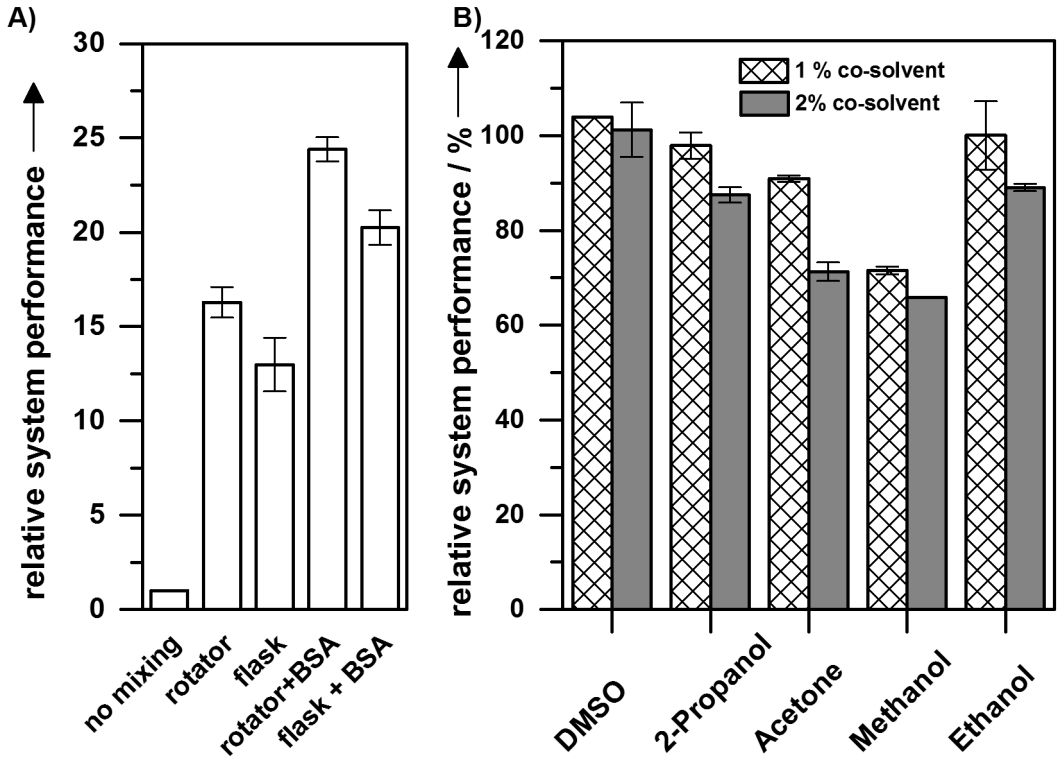

Figure S4. A) Octane oxidation catalysed by CYP154A8 in a biphasic system with different mixing techniques applied in order to increase the interfacial surface area as well as BSA to stabilise the enzymes. Values are normalized to "no mixing". **B**) Conversion of octane by CYP154A8 with different co-solvents tested in 1 vol-%and 2 vol-% concentration. Values are normalized to the value achieved with the default 1 vol-% ethanol. Where error bars cannot be seen, they are smaller than the line width.

Besides catalase, bovine serum albumin (BSA) was tested as well. In accordance with the data from Maurer et al. (2005) the protein stabilizing effect of BSA to the reaction mix led to an increase in product formation of about 50 %.[4] BSA may in addition act as sink for the substrate, because its natural function is to bind hydrophobic compounds.

Low substrate solubility is often a limiting factor for biocatalysis in biphasic systems, e.g. the solubility of octane in water is only 0.66 mg/l = 5.8 mM at 25 °C.[5] To increase the accessibility of alkanes for the enzyme in addition to ethanol which was used by default, a set of water miscible co-solvents in two different concentrations (1 vol-% and 2 vol-%) was tested: DMSO, 2-propanol, acetone and methanol. The results indicated that product formation was maximal with DMSO or ethanol (Figure S4 B), although the difference between 1 and 2 % co-solvent was minimal. Because DMSO was found to be oxidized by P450s to dimethyl sulfone we decided to use ethanol as co-solvent.

10. Results of the supplementation experiments

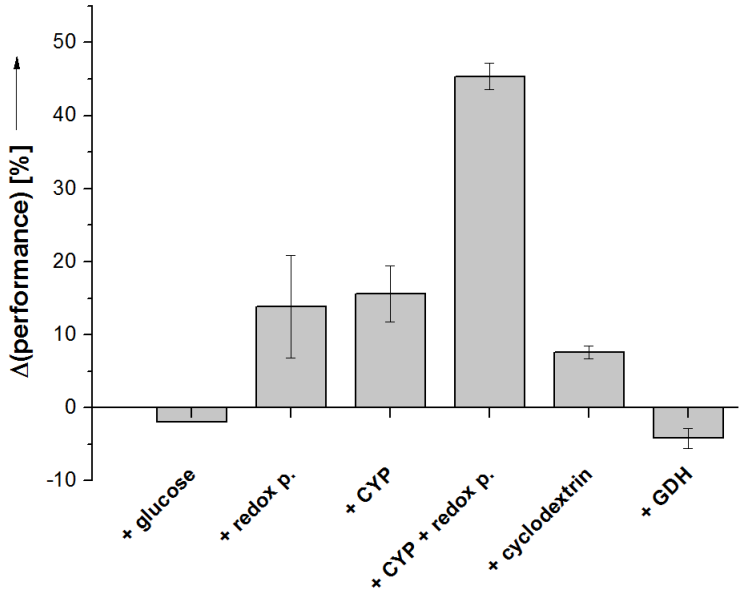

Figure S5: Results of the supplementation experiments: In order to prove the influence of enzyme stability on *n*-alkane conversion the following components were added to a standard biphasic reaction after 5,5 h of reaction: 1. glucose, 2. YkuN and FdR, 3. CYP154A8, 4. YkuN, FdR and CYP154A8, 5. methyl-β-cyclodextrin (to increase substrate/product solubility), 6. GDH. To evaluate the effect of the supplementation the samples were extracted after 24 h and product concentration analysed via GC/MS. Values are given as % relative to the system without supplementation.

SupplementalNotes and references

1 C. von Bühler, P. Le-Huu and V. B. Urlacher, *Chembiochem*, 2013, **14**, 2189–2198.

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3 Z.-Q. Wang, R. J. Lawson, M. R. Buddha, C.-C. Wei, B. R. Crane, A. W. Munro and D. J. Stuehr, *J. Biol. Chem.*, 2007, **282**, 2196–2202.

4 S. C. Maurer, K. Kühnel, L. A. Kaysser, S. Eiben, R. D. Schmid and V. B. Urlacher, *Adv. Synth. Catal.*, 2005, **347**, 1090–1098.

5 National Library of Medicine (US), Division of Specialized Information Services, *Hazardous Substances Data Bank,* available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.