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Author: Christina A. Müller Alexander Dennig Tim Welters Till Winkler Anna Joelle Ruff Werner Hummel Harald Gröger Ulrich Schwaneberg

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Whole-cell double oxidation of *n*-heptane

Christina A. Müller[a1], Alexander Dennig[a2], Tim Welters[a], Till Winkler[b], Anna Joelle Ruff[a], Werner Hummel[b], Harald Gröger[c], Ulrich Schwaneberg[a,*]

[a] Institute of Biotechnology, RWTH Aachen, Worringerweg 3, 52074 Aachen, Germany
Tel.: +49 241 80 24170, Fax: +49 241 80 22387, e-mail: u.schwaneberg@biotec.rwth-aachen.de

[b] Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, 52426 Jülich, Germany

[c] Faculty of Chemistry, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany

Co-author contribution:
C. A. Müller: Planning and performing experiments, discussion and interpretation of results, writing and editing manuscript
A. Dennig: Idea of artificial operon, design of primers for artificial operon, discussion, writing
T. Welters: Preliminary experiments with whole-cells, fermentation of artificial operon constructs
T. Winkler, W. Hummel: Cloned and provided ADHs (RE-ADH, Lb-ADH), discussion
A. J. Ruff: Discussion and interpretation of results, writing
H. Gröger: Concept of double oxidation reaction and whole-cell catalysis, discussion
U. Schwaneberg: Concept of double oxidation reaction and whole-cell catalysis, discussion, writing

Permanent address:

[1] Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria

[2] Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, 8010 Graz, Austria

*Corresponding author: Prof. Dr. Ulrich Schwaneberg

RWTH Aachen University
Lehrstuhl für Biotechnologie
Worringerweg 3
52074 Aachen
Tel.: +49 241 80 24170
Fax: +49 241 80 22387
E-mail: u.schwaneberg@biotec.rwth-aachen.de

Abstract

Biocascades allow one-pot synthesis of chemical building blocks omitting purification of reaction intermediates and expenses for downstream processing. Here we show the first whole cell double oxidation of *n*-heptane to produce chiral alcohols and heptanones. The concept of an artificial operon for co-expression of a monooxygenase from *Bacillus megaterium* (P450 BM3) and an alcohol dehydrogenase (RE-ADH) from *Rhodococcus erythropolis* is reported and compared to the widely used two-plasmid or Duet-vector expression systems. Both catalysts are co-expressed on a polycistronic constructs (single mRNA) that reduces recombinant DNA content and metabolic burden for the host cell, therefore increasing growth rate and expression level. Using the artificial operon system, the expression of P450 BM3 reached 81 mg g^{-1} cell dry weight. In addition, *in situ* cofactor regeneration through the P450 BM3/RE-ADH couple was enhanced by coupling to glucose oxidation by *E. coli*. Under optimized reaction conditions the artificial operon system displayed a product formation of 656 mg L^{-1} (5.7 mM) of reaction products (heptanols + heptanones), which is 3-fold higher than the previously reported values for an *in vitro* oxidation cascade. In conjunction with the high product concentrations it was possible to obtain *ee* values of >99 % for (*S*)-3-heptanol. Coexpression of a third alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH) in the same host yielded complete oxidation of all heptanol isomers. Introduction of a second ADH enabled further to utilize both cofactors in the host cell (NADH and NADPH) which illustrates the simplicity and modular character of the whole cell oxidation concept employing an artificial operon system.

Keywords: Cascade reaction, double oxidation, monooxygenase, alcohol dehydrogenase, directed evolution, alkanes

1. Introduction

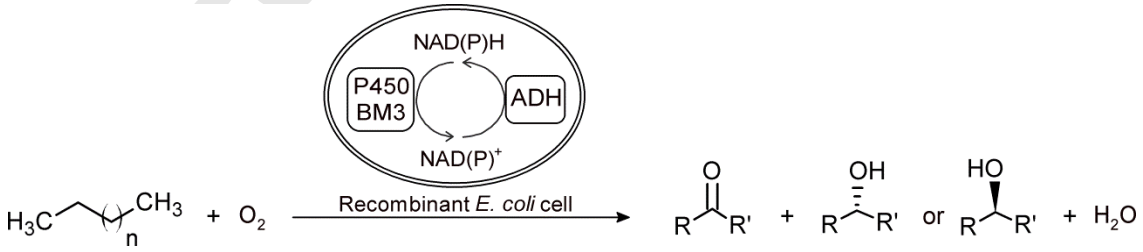
Molecular oxygen can be introduced into non-activated carbon atoms under mild reaction conditions in a selective manner employing biocatalysts (Hollmann et al., 2011). 'Operational stability' of biocatalysts and cofactor regeneration during oxidative biocatalysis are challenging tasks (Hollmann et al., 2011). Limitations in the performance of enzymes under non-natural conditions are often solved by protein engineering (Ruff et al., 2013; Tee and Schwaneberg, 2007). Simple and cost-effective solutions are favored since cofactors expenses often have a high impact on reaction economics.

Biocatalytic processes for the functionalization of alkanes have numerous advantages over traditional synthetic routes (Hollmann et al., 2011; Schrewe et al., 2013). For example, chemo-catalysts often are limited with respect to regio- and stereoselectivity and commonly perform with lower turnover numbers (Bordeaux et al., 2012). Additionally, synthetic efforts can yield expensive catalysts (metal complexes or metal-oxides) that pose challenges in waste disposal and often require harsh reaction conditions such as elevated temperatures (>200 °C) (Crabtree, 2001; Shilov and Shul'pin, 1997).

One-pot double oxidation of alkanes in cell-free systems has recently been published employing P450 BM3 monooxygenase variants and alcohol dehydrogenases (Müller et al., 2013; Staudt et al., 2013) with product titers up to 0.8 g L^{-1} and 0.2 g L^{-1} and TONs of 11641 and 3591 for cyclooctane and *n*-heptane as substrates respectively. P450 BM3 is a self-sufficient industrially attractive monooxygenase that is capable of hydroxylating a broad range of substrates, ranging from alkanes to steroids (Dennig et al., 2012; Whitehouse et al., 2012). Major limitations for a commercial application are relatively low stabilities and cofactor dependency of monooxygenases (Urlacher and Eiben, 2006) that are often associated with low coupling efficiencies for non-natural substrates (Bernhardt, 2006; Müller et al., 2013; Whitehouse et al., 2012).

Scheme 1 shows the developed whole cell double oxidation system which overcomes the main limitations of the *in vitro* one pot double oxidation by employing resting cells with expressed oxidoreductases as catalysts in a buffered medium, with supplemented glucose for cofactor regeneration, oxygen as oxidant and *n*-heptane as model substrate. Oxidation reactions that require regeneration of a cofactor or multi-enzymatic pathways can be operated efficiently in whole cells (Schrewe et al., 2013). In this way, the cell metabolism ensures intracellular availability and regeneration of reduction equivalents that are then employed as cofactors in the oxidative catalysis (Blank et al., 2010). A parallel resolution and separation of products (e.g. employing a two-liquid-phase system) (Lilly, 1982) was employed to minimize purification efforts and reduce process expenses.

The double oxidation of aliphatic alkanes enables direct synthesis of ketones and/or chiral alcohols, using molecular oxygen as sole oxidant in aqueous solution (see Scheme 1). The oxidation cascade products, ketones and chiral alcohols, are important building blocks for the pharmaceutical, agrochemical and food industry (Breuer et al., 2004). For instance, aliphatic ketones are employed in the production of flavors and fragrances (Weissermel and Arpe, 2003). The developed double oxidation process for *n*-heptane represents to our best knowledge the first one in a whole cell system.



Scheme 1: Principle of the whole-cell double oxidation of aliphatic alkanes catalyzed by P450 BM3 monooxygenase variants and *R*/*S*-selective alcohol dehydrogenases.

2. Material and Methods

2.1 Chemicals and reagents

All chemicals used were of analytical grade or higher and were purchased, if not stated otherwise from Sigma Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), TCI Europe (Eschborn, Germany), Merck (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). Enzymes and dNTPs were purchased from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Frankfurt, Germany). All employed HPLC-purified oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany).

2.2 Strategies for coexpression of P450 BM3 and ADHs

Different coexpression strategies for the heterologous expression of P450 BM3 variants (WT^{NADH}, 19A12, $19A12^{NADH}$, CM1, $CM1^{NADH}$) and ADHs (RE-ADH and Lb-ADH) in *Escherichia coli* (*E. coli*) cells were investigated. An overview of the generated coexpression strains can be found in Supplementary Data (Table S1). For generation of strains containing two plasmids chemically competent *E. coli* BL21(DE3) Gold $lacI^{Q1}$ cells (Blanusa et al., 2010) were transformed with 50 ng of each plasmid (pAlXtreme-1a and pKA1) simultaneously and were spread on agar plates containing both antibiotics (50 µg mL^{-1} kanamycin, 34 µg mL^{-1} chloramphenicol). Construction of the coexpression systems with the commercial vector pACYCDuet-1 from Merck (Darmstadt, Germany) was performed by restriction cloning. The RE-ADH gene was cloned employing restriction sites *NdeI* and *EcoRV* in the second multiple cloning site (MCS2). A restriction site *EcoRV* was introduced at the end of the RE-ADH gene-sequence by PCR (40 ng pKA1 RE-ADH as template, 1 U of Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.4 µM of each primer, final volume of 50 µL). PCR conditions: 98 °C for 2 min, 25 cycles (98 °C for 30 s, 57 °C for 30 s, 68 °C for 30 s), 72 °C for 10 min, employing forward primer (RE-ADH_F_*NdeI*) 5'-GGAGATATACATATGAAGGCAATCCAG-3' and reverse primer (RE-ADH_R_*EcoRV*) 5'-GCAGCCGATATCTTACAGACCAGGGACCACAACC-3'. The P450 BM3 monooxygenase variant $CM1^{NADH}$ was cloned into MCS1 using restriction sites *EcoRI* and *NcoI*. The resulting plasmid was transformed into chemically competent *E. coli* BL21 (DE3) Gold cells.

Construction of the artificial operon was performed using the PLICing (Phosphorothioate-based ligase-independent gene cloning) method (Blanusa et al., 2010). Four phosphorothioated oligonucleotide primers (Table 1) were designed for amplification of the P450 BM3 monooxygenase gene together with the pALXtreme-1a vector backbone (no. 1-2, larger fragment; vector DNA) and the RE-ADH gene (no. 3-4, smaller fragment; insert DNA).

Table 1: Oligonucleotide primers for construction of artificial operon plasmids by PLICing (Blanusa et al., 2010)

No.	Target DNA	Primer identifier	Sequence (5´-3´)[a]
1	pALXtreme-1a P450 BM3 (vector)	BM3 Fwd Op PTO	gctaacaaagccCGAAAGGAAGCTGAGTTGGC
2	pALXtreme-1a P450 BM3 (vector)	BM3 Rev 1046S Op PTO	gaacagtggatcATGATTACTAGTGGGTTACCCAGCGCTCACGTCTTTTG
3	RE-ADH (insert)	RE-ADH Fwd Op PTO	gatccactgttcAATAAGGAGGTATACCATGAAGGCAATCCAAGTACACG
4	RE-ADH (insert)	RE-ADH Rev Op PTO	ggctttgttagcTTACAGACCAGGGACCACAACCGC

[a] Lower case letters indicate phosphorothioated bonds

Using the forward primer for amplification of the RE-ADH gene (Table 1, no. 3) an additional ribosome binding site (RBS) for translation of the ADH in the artificial operon construct was introduced upstream of the ADH gene. The RBS contains the Shine-Dalgarno sequence (SD: UAAGGAGGU) (Ringquist et al., 1992; Shine and Dalgarno, 1975) and an optimal sequence spacing of 5 nucleotides (Chen et al., 1994) between SD and translational initiation codon (ATG). A schematic representation of the generated artificial operon can be found in Supplementary Data, Figure S1.

The vector pALXtreme-1a containing the respective P450 BM3 (WT^{NADH}, $19A12^{NADH}$, $CM1^{NADH}$) was used as template DNA for amplification of the monooxygenase together with the vector backbone. Prior to amplification it was linearized by digestion using *EcoRI*. RE-ADH was amplified directly from the circular pKA1 plasmid. A two-stage PCR was employed (Wang and Malcolm, 1999). The PCR mixture (50 µL) contained template DNA (20 ng), 1 U Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.2 µM forward or reverse primer. Following temperature program was used for the first amplification step: Initial denaturation at 98 °C for 2 min, followed by 5 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 9 min (P450 BM3) or 2 min (RE-ADH). Subsequently, 25 µL of forward and 25 µL of reverse amplified reaction products were mixed together and 0.5 U of Phusion DNA polymerase were added. PCR program for the second amplification step: Initial denaturation at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 6 min (P450 BM3) or 1 min (RE-ADH), and a final elongation step at 72 °C for 5 min.

2.3 Cultivation of *E. coli* in shake flasks

Cultivation and expression in shake flask of the *E. coli* constructs containing only one P450 BM3 monooxygenase variant (CM1 and P450 BM3 $CM1^{NADH}$) was performed as previously described (Staudt et al., 2012). Coexpression of two or three proteins (P450 BM3, RE-ADH, LB-ADH) in the generated *E. coli* strains (see Supplementary Data, Table S1), was achieved with the same protocol with minor modifications. The corresponding antibiotics, depending on the plasmid(s) present (50 µg mL^{-1} kanamycin for pALXtreme-1a; 34 µg mL^{-1} chloramphenicol for pKA1 or

pACYCDuet-1) were supplemented to the culture media. In addition, for expression of RE-ADH, the main culture was supplemented with 1 mM $ZnCl_2$ directly after induction with IPTG. Cell pellets were stored at -20 °C until further use.

2.4 Fermentation of *E. coli* for heterologous protein expression

Fermentation of *E. coli* BL21 (DE3) Gold $lacI^{Q1}$ strains containing the artificial operon (pALXtreme-1a P450 BM3 WT^{NADH}-RE-ADH, $19A12^{NADH}$-RE-ADH, and $CM1^{NADH}$-RE-ADH) was carried out in a 5 L-bioreactor Biostat MD from Sartorius (Göttingen, Germany). The cultivation in the fermentor was analogue to the cultivation in shake flask using a working volume of 5 L TB medium supplemented with kanamycin (50 µg mL^{-1}) and trace elements (0.64 µM $CuSO_4$, 0.69 µM $ZnSO_4$, 0.72 µM $MnSO_4$, 0.76 µM $CoCl_2$, 59.43 µM Na_2-EDTA, 61.79 µM $FeCl_3$9). A constant aeration rate of 0.5 vvm (2.5 L min^{-1}) and stirrer speed of 400 rpm were used. The initial pH of 7.1 was maintained throughout the fermentation using 15% (v/v) ammonia and 30% (v/v) phosphoric acid solutions. If necessary, foam formation was regulated by addition of polyethylene glycol. The cultivation was started by inoculation with an overnight culture of the respective *E. coli* construct (pALXtreme-1a P450 BM3 WT^{NADH}-RE-ADH, $19A12^{NADH}$-RE-ADH, and $CM1^{NADH}$-RE-ADH) in 100 mL LB medium (50 µg mL^{-1} kanamycin; 37 °C for 16 h, 250 rpm). The temperature was maintained at 37 °C until an optical density (OD_{600}) of 1 was achieved. At this point protein expression was induced by addition of IPTG (0.1 mM) and supplemented with 5-aminolevulinic acid hydrochloride (0.5 mM) and thiamine hydrochloride (0.3 mM). After induction the temperature was decreased to 25 °C and was kept constant for 24 to 26 h until cell harvest. Cells were collected by centrifugation (15 min, 4500 g at 4 °C) and cell pellets were washed in 0.1 M KPi buffer (pH 8.0) and stored at -20 °C until further use.

2.5 Correlation of optical density, cell dry weight and P450 content

P450 content and cell dry weight were correlated for each strain to a specific optical density (OD_{600}). Cells were resuspended in 0.1 M KPi-buffer (pH 8.0) to obtain homogeneous mixtures with an approximated OD_{600} of 30, 40, 50 and 60 (corresponding to approx. 6, 8, 11 and 13 g_{CDW} L^{-1}). One mL of resuspended cells (OD_{600} of 30, 40, 50 and 60) was used to measure the active P450 BM3 concentration in the lysate by CO-difference spectroscopy using a standard procedure (Omura and Sato, 1964). Therefore, crude cell lysate was prepared by sonication as previously described (Staudt et al., 2012). The cell dry weight (CDW; in g_{CDW} L^{-1}) was determined by centrifugation of 1 mL of resuspended cells (21000 g, 2 min), followed by storage in a drying cabinet FP 53 (Binder GmbH, Tuttlingen, Germany) at 95 °C for 48 h and subsequent weighing.

2.6 Whole cell conversion of *n*-heptane and GC analytics

Optimized reactions for conversion of *n*-heptane with resting cells were carried out in a two-liquid phase system. The aqueous phase contained the corresponding *E. coli* cells in 1 mL 0.1 M KPi-buffer (pH 8.0) at a final OD_{600} of 40 (if not stated otherwise), supplemented with 10 g L^{-1} D-glucose for cofactor regeneration. The organic phase consisted of 200 µL pure *n*-heptane. The aqueous phase was pipetted into a 5 mL-screw top vial (CS Chromatographie, Langerwehe, Germany) and was incubated with D-glucose for 5 min at RT, prior to addition of *n*-heptane forming the organic layer. The reaction mixture was stirred at 600 rpm and RT with a stirring bar (3 mm x 10 mm, Carl Roth, Karlsruhe, Germany) on a multipoint magnet stirrer Variomag 15 (Thermo Fischer Scientific, Wilmington, USA). After 48 h, the biotransformation mixture was transferred to a reaction vessel containing 100 µL HCl to quench the reaction. Reaction products were extracted with 0.4 mL MTBE containing 10 mM decane as internal standard (0.2 % v/v) and 0.4 mL of *n*-heptane to achieve a final volume of 1 mL organic solvent. In this way, product concentrations are referring to the volume of the aqueous phase (c_{Aq}). The organic layer was dried over $NaSO_4$ and the reaction products were analyzed by GC as described before (Müller et al., 2013).

3. Results and Discussion

A whole cell double oxidation process for synthesis of heptanone and resolution of (*R*)- and (*S*)-heptanol was investigated. Initially three different coexpression systems (2-plasmid, pACYCDuet-1 and artificial operon) for the simultaneous expression of a P450 BM3 monooxygenase ($CM1^{NADH}$) and an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) were evaluated. Finally, the artificial operon system was selected as coexpression system for strains containing different P450 BM3 variants and an alcohol dehydrogenase (P450 BM3 WT^{NADH}-RE-ADH, P450 BM3 $19A12^{NADH}$-RE-ADH, P450 BM3 $CM1^{NADH}$-RE-ADH) and biomass and monooxygenase production was established in a fermentor setup. Subsequently, several parameters for the double oxidation of *n*-heptane such as reaction medium, cell mass and substrate concentrations were investigated and optimized. Employing the optimized conversion parameters the double oxidation cascade was benchmarked against the corresponding single hydroxylation of *n*-heptane. Finally, the process concept was extended to a third catalyst, the alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH), for the stereoselective synthesis of (*S*)-heptanols or synthesis of heptanones as main reaction products with high selectivity.

3.1 Whole cell systems for coexpression of P450 BM3 and RE-ADH

Coexpression of multiple proteins in a single strain allows producing of a whole cell catalyst containing all enzymatic components that are required for a double oxidation cascade. Initially, three different coexpression systems were investigated, namely a 2-plasmid-system, a commercial pACYCDuet-1 expression system, and a novel artificial operon system, in which both oxidoreductases were coexpressed with an artificial artificial operon containing two

ribosome binding sites (RBS) (Figure 1). The P450 BM3 monooxygenase variant CM1^{NADH} (Müller et al., 2013) was chosen together with the alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) (Abokitse and Hummel, 2003) as target genes for coexpression. Superscripted NADH indicates altered cofactor specificity (substitutions R966D, W1046S)(Maurer et al., 2005), so that CM1 accepts NADH and NADPH as reduction equivalents.

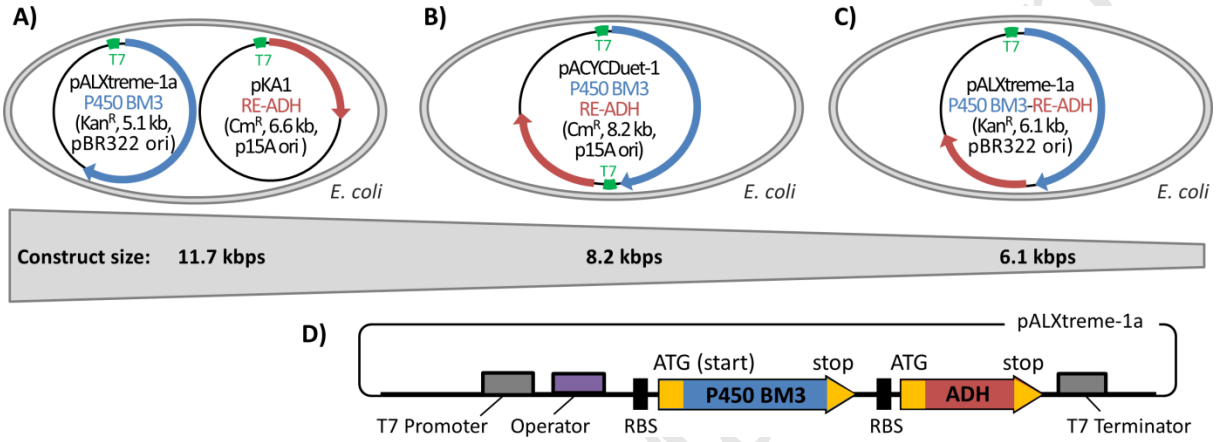


Figure 1: Different strategies for coexpression of two genes of interest: a P450 BM3 monooxygenase (CM1^{NADH}) and an alcohol dehydrogenase (RE-ADH). The three investigated systems are: (**A**) 2-plasmid-system, (**B**) commercial pACYCDuet-1 system, (**C**) artificial operon system. The amount of recombinant DNA transformed in an *E. coli* cell as expression host is given in kbps. (**D**) Schematic representation of the artificial operon vector with two RBSs: ribosome binding sites.

The first expression system consisted of two expression plasmids (pALXtreme-1a and pKA1) in an *E. coli* cell. The plasmids pALXtreme-1a, harbors the P450 BM3 CM1^{NADH} gene and pKA1, harbors RE-ADH. The second system employs the commercial vector pACYCDuet-1 (Merck, Darmstadt, Germany). pACYCDuet-1 enables expression of two enzymes, each under the control of a T7 promotor/lac operator and a separate ribosome binding site (RBS) (Tolia and Joshua-Tor, 2006). The third coexpression system is based on a synthetic artificial operon or polycistronic plasmid (Tan, 2001) using the PLICing methodology for cloning (Blanusa et al., 2010). In the latter case the simultaneous expression of both proteins is regulated as operon consisting of one T7 promoter, one operator and one terminator. Induction of protein expression with IPTG leads to the transcription of a single polycistronic mRNA that contains the information for both enzymes (the selected P450 monooxygenase and the selected ADH). The polycistronic mRNA harbors two ribosome binding sites for translation of the individual proteins, each of them upstream of a translation initiation codon (ATG). A reported optimal sequence-spacing for *E. coli* of five nucleotides between the Shine-Dalgarno sequence and ATG was used (Chen et al., 1994). The described artificial operon resembles therefore a simplified bacterial operon (Jacob et al., 2005).

The catalytic activities of monooxygenases (0.01-10.0 U/mg) are in general significantly lower compared to other enzyme classes such as hydrolyses (Lehmann et al., 2012; Shivange et al., 2012). RE-ADH shows an evidently higher activity of 47 U mg^{-1} for the oxidation of 2-heptanol compared to an activity of 5 U mg^{-1} of P450 BM3 $CM1^{NADH}$ for the hydroxylation of *n*-heptane (Müller et al., 2013). Therefore, it is important to ensure a high concentration of P450 monooxygenase in the whole-cell catalyst to avoid a rate limitation in the initial oxidation step. A rather balanced over-expression of P450 BM3 $CM1^{NADH}$ and RE-ADH was finally achieved with best results for the artificial operon strain (pALXtreme-1a P450 BM3-RE-ADH) (see Supplementary Data, Figure S2). To determine the intracellular concentration of P450 BM3 and variants in each coexpression strain the titer of active P450 BM3 protein (in $mg_{P450}\ g_{CDW}^{-1}$) was calculated. Product yields on catalyst ($Y_{p/x}$) (Schrewe et al., 2013) for the double oxidation of *n*-heptane, as well as RE-ADH activities (in U g_{CDW}^{-1}) and P450 content are shown in Figure 2. These values were calculated for each strain after expression in shake flasks, as well as for one selected strain (artificial operon P450 BM3 $CM1^{NADH}$-RE-ADH) after expression in a fermentor under controlled growing conditions.

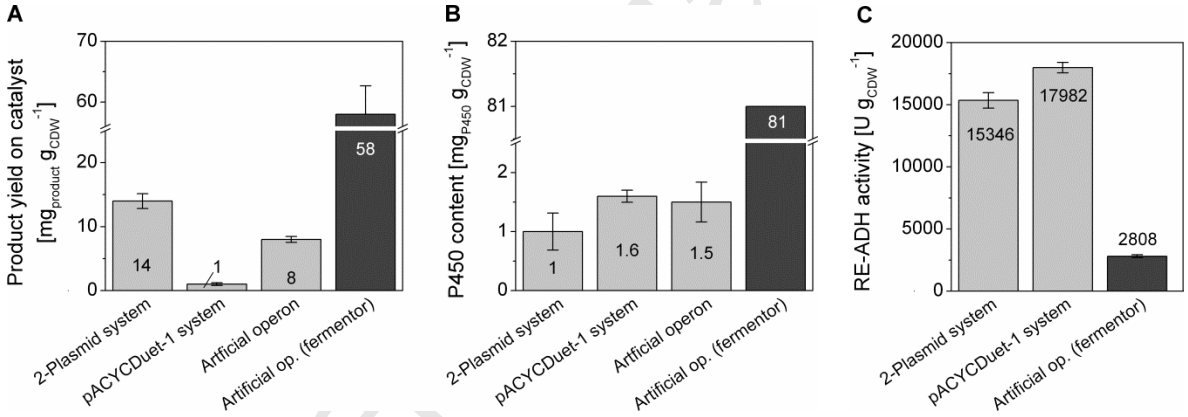


Figure 2: Comparison of three different coexpression strategies for P450 BM3 $CM1^{NADH}$ and RE-ADH in *E. coli* cells. Expression in shake flasks (light gray) and in a fermentor (dark gray); Product yields on catalyst (**A**), P450 BM3 $CM1^{NADH}$ content (**B**) and RE-ADH activities (**C**) were calculated in respect to the cell dry weight. Reaction conditions: Resting cells in 0.1 M KPi buffer pH 8.0 (1 mL; OD_{600} 40: 8.5 $g_{CDW}\ L^{-1}$ for 2-plasmid and artificial operon system (fermentor), 10.7 $g_{CDW}\ L^{-1}$ for pACYCDuet and artificial operon system), 10 g L^{-1} D-glucose, 200 µL *n*-heptane, 51 h stirring at RT.

Expression in shake flask resulted for the pACYCDuet-1 and the artificial operon system in a higher production of functional P450 BM3 $CM1^{NADH}$ per cell dry weight (1.5-1.6 $mg_{P450}\ g_{CDW}^{-1}$). Surprisingly the product yields (on catalyst) were lower (1 and 8 mg g_{CDW}^{-1}) than with the 2-plasmid system (14 mg g_{CDW}^{-1}). Due to the high overexpression of RE-ADH in the 2-plasmid and pACYCDuet systems dehydrogenase activities up to 17900 U g_{CDW}^{-1} were determined. However, the growth of *E. coli* strains containing two plasmids was significantly slower (2x) compared to the strains containing only one plasmid and the amount of produced biomass was halved likely due to expression of antibiotic resistance genes which increases the metabolic load

(Diaz Ricci and Hernandez, 2000; Glick, 1995). In the case of the pACYCDuet-1 system only a poor product yield on catalyst (1 mg g_{CDW}^{-1}) was achieved, even though the activity of RE-ADH was highest, so that finally the artificial operon construct was selected for expression in a fermentor. Coexpression of proteins using the described artificial operon system has several advantages over the other two coexpression methods. Several genes can be expressed using only one plasmid, reducing thereby the metabolic burden on the host cell (Birnbaum and Bailey, 1991). The small size of the employed vector pALXtreme-1a is also advantageous since it reduces the DNA synthesis load of the host and increases cell density (Glick, 1995). Furthermore the employed ligase-independent cloning (PLICing) avoids the time-consuming introduction of restriction sites into the artificial operon construct.

Through controlled fermentation it was possible to enhance the content of P450 BM3 $CM1^{NADH}$ (artificial operon) by 54-fold compared to cultivation in shake flasks, by lowering the expression of RE-ADH (activity of 2800 U g_{CDW}^{-1}). A P450 BM3 $CM1^{NADH}$ product yield on catalyst of $Y_{P450/x}$ of 81 mg_{P450} g_{CDW}^{-1} was achieved (Figure 2 B). The value represents a specific P450 BM3 $CM1^{NADH}$ formation of 683.5 $nmol_{P450}$ g_{CDW}^{-1} and is slightly higher than the reported value by Pflug *et al*. of 644 $nmol_{P450}$ g_{CDW}^{-1} for expression of P450 BM3 WT in *E. coli* (BL21(DE3) pET28a(+)-system) in a fed-batch fermentation process, with a cell mass concentration of 19.8 g_{CDW} L^{-1}.(Pflug et al., 2007). Normalized to the cell mass a product yield on catalyst (artificial operon P450 BM3 $CM1^{NADH}$-RE-ADH) of 58 mg g_{CDW}^{-1} was obtained. The increase in product on catalyst (7.25-fold) is accompanied by the increase in P450 production (81 mg_{P450} g_{CDW}^{-1}; 54-fold improved) (Figure 2 B). This result highlights the importance of optimizing the heterologous expression of the monooxygenase to achieve higher specific activities in whole cell catalysis. Duetz et al. (2001) estimated that theoretical specific activities of intact cells containing NAD(P)H-dependent oxygenases with high k_{cat} values (5 to 75 s^{-1}) could reach 150 to 4500 U g_{CDW}^{-1}. The authors pointed out that activities significantly higher than 500 U g_{CDW}^{-1} have experimentally not been reached so far. Therefore, other bottlenecks such as product inhibition or mass transfer rate limitations have to be addressed and integrated into process development (Duetz et al., 2001).

3.2 Reaction engineering for whole cell biotransformation of *n*-heptane

Using the artificial operon system three different constructs were generated for expression in *E. coli*, each containing a different P450 BM3 variant (WT^{NADH}, $19A12^{NADH}$, $CM1^{NADH}$) together with RE-ADH. Biotransformation conditions for the whole cell double oxidation of *n*-heptane were optimized in respect to the reaction medium (*E. coli* minimal medium, KPi buffer), the pH-value (7.0 to 8.0) and the glucose concentration (0 to 10 g L^{-1}). Best results were obtained in KPi-buffer at pH 8.0 and a glucose concentration of 10 g L^{-1}. The application of glucose facilitates the intracellular cofactor regeneration of the reduced cofactors [NAD(P)H], compensating in this way the loss of reduction equivalents as a result from the low coupling efficiencies of monooxygenases.

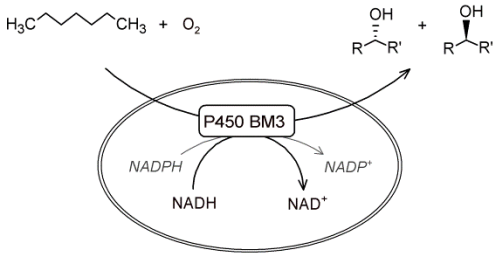
The influence of substrate concentrations (from 1 to 1045 mM) on product formation was also investigated. Product concentrations (heptanols + heptanones) of up to 2 mM were measured for a substrate concentration between 440 and 1045 mM, which corresponded to a volumetric content of 7 and 17% (v/v) *n*-heptane in an aqueous-organic two-liquid-phase system. A main advantage of a two-phase biotransformation system for the double oxidation of *n*-heptane is the use of an organic carrier solvent which acts simultaneously as substrate reservoir and product sink (León et al., 1998; Ortiz de Montellano, 2005). Besides, further cosolvents such as DMSO or ethanol are not required. Solvents with log $P_{ow} \geq 4$ are regarded as biocompatible (León et al., 1998; Ortiz de Montellano, 2005) and *n*-heptane has a log P_{ow} value of 4.66 (at 25 °C) (Schantz and Martire, 1987; Tewari et al., 1982). The results of this study show that *n*-heptane is compatible as organic carrier with *E. coli* whole cells. Reaction products (heptanols and heptanones) are more polar than *n*-heptane and have log P_{ow} values of 2.4 and 1.8 (Ortiz de Montellano, 2005). However, heptanols and heptanones will preferentially accumulate in the organic phase (*n*-heptane) rather than in the aqueous phase, enabling an *in situ* product removal. Two-phase systems in biotransformations have in general a 'simpler' downstream processing by separation of phases and recycling of the organic phase for further biotransformations (Lilly, 1982). The low price of *n*-heptane as bulk chemical renders an application as organic carrier solvent possible. The use of *n*-heptane and other alkanes like cyclohexane, *n*-hexane, *n*-octane and isooctane as organic solvent in biphasic biotransformations have being described for cell free enzymes (Gröger et al., 2003; Maurer et al., 2005) as well as for whole-cell processes (Andersson et al., 1998; Favre-Bulle et al., 1991; Mouri et al., 2006; Wubbolts et al., 1996).

Further optimization of the whole cell oxidation system was achieved by evaluation of the optimal cell mass concentration. A correlation between cell density (OD_{600})/ cell dry weight (CDW), monooxygenase concentration and product formation was determined (see Supplementary Data, Figure S3 A-B) for cell mass concentrations between 2 and 13 $g_{CDW}\ L^{-1}$. Product yields on catalysts for the three investigated artificial operon constructs (WT^{NADH}-RE-ADH, $19A12^{NADH}$-RE-ADH, $CM1^{NADH}$-RE-ADH) differed depending on the cell mass employed. Best results were achieved using 6 g L^{-1} CDW for the construct containing P450 BM3 $19A12^{NADH}$ (61 mg g_{CDW}^{-1}) and ~8 g L^{-1} for those containing P450 BM3 WT^{NADH} (44 mg g_{CDW}^{-1}) as well as $CM1^{NADH}$ (60 mg g_{CDW}^{-1}). This CDW values correspond to a P450 concentration of 0.16, 0.58 and 0.68 g L^{-1}, respectively. Interestingly, the obtained product yields for *n*-heptane oxygenation by each artificial operon construct are not proportional to the respective P450 concentration. Additional process analysis would be required to elucidate contributions of monooxygenase/dehydrogenase ratios, mass transfer limitations at higher cell mass concentrations (Baldwin and Woodley, 2006), and/or oxygen limitations (Duetz et al., 2001). These optimized reaction conditions were employed further to evaluate the performance of the double oxidation of *n*-heptane (Table 2, entry 3, 4 and 5).

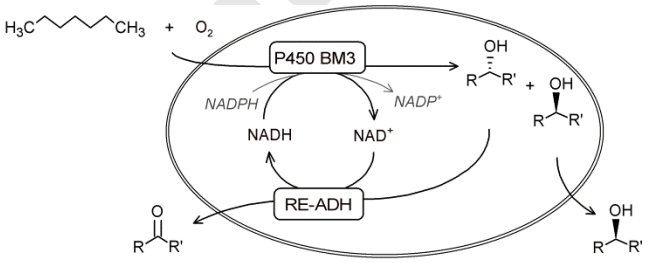
3.3 Single hydroxylation and double oxidation of *n*-heptane

Table 2 summarizes the performance of whole-cell catalysts for the single monooxygenase reaction (Table 1, left) and double oxidation employing an artificial operon system (Table 2, right). The single hydroxylation was investigated employing P450 BM3 variant CM1 (NADPH-dependent) and CM1^{NADH} (NADH-dependent) to provide a detailed comparison (Table 2, entry 1 and 2). P450 BM3 CM1^{NADH} utilizes both NADH (TOF of 604 min^{-1}) and NADPH (TOF of 335 min^{-1}) (Müller et al., 2013).

***Table 2*:** Comparison of process performance for the whole cell catalytic systems: single hydroxylation (monooxygenase P450 BM3 CM1 and P450 BM3 CM1^{NADH}; left) and double oxidation (P450 BM3 WT^{NADH}, 19A12 ^{NADH}, CM1^{NADH} and RE-ADH; right) of *n*-heptane using the artificial operon expression system.[a] The product concentration for the analogue double oxidation of *n*-heptane using *in vitro* biotransformation (Müller et al., 2013) is given as reference (entry 6; for 24 h reaction time). Regioselectivity of the whole cell conversion of *n*-heptane for the corresponding products (heptanols and heptanones) is given in Supplementary data, Table S2.



Single expression P450 BM3 variants



Artificial operon P450 BM3-RE-ADH

Entry	Multi-enzymatic system	Product concentration		Product yield on catalyst $Y_{p/x}$	P450 content
		[mM]	[mg L^{-1}]	[mg g_{CDW}^{-1}]	[mg_{P450} g_{CDW}^{-1}]
	Single expression (shake flask)				
1	P450 BM3 CM1	1.13 ± 0.11	132 ± 13	16 ± 2	53
2	P450 BM3 CM1^{NADH}	2.32 ± 0.07	269 ± 9	40 ± 1	58
	Artificial operon (fermentor)				
3	P450 BM3 WT^{NADH}-RE-ADH	2.95 ± 0.01	342 ± 31	44 ± 4	74
4	P450 BM3 19A12^{NADH}-RE-ADH	3.40 ± 0.07	390 ± 8	61 ± 1	25
5	P450 BM3 CM1^{NADH}-RE-ADH	5.67 ± 0.34	656 ± 40	76 ± 5	81
	In vitro system				
6	P450 BM3 CM1^{NADH} + RE-ADH	1.80	207	--	--

[a] Product concentrations are the sum of all heptanol and heptanone isomers quantified *via* GC with product standards in triplicate; the reaction mixture contained *E. coli* cells (OD_{600} 40 for P450 BM3 CM1 (8.1 g_{CDW} L^{-1}, CM1^{NADH} (6.7 g_{CDW} L^{-1}) and CM1^{NADH}-RE-ADH (8.5 g_{CDW} L^{-1}); OD_{600} 30 for WT^{NADH}-RE-ADH (7.7 g_{CDW} L^{-1}) and 19A12^{NADH}-RE-ADH (6.3 g_{CDW} L^{-1}) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L^{-1} D-glucose and 1045 mM (200 µL) *n*-heptane as organic phase; RT, 48 h, stirring.

P450 BM3 CM1 and CM1^{NADH} (P450 content of 53 and 58 mg_{P450} g_{CDW}^{-1} respectively) yielded a 2-fold difference in absolute product concentrations (1.13 vs 2.32 mM). This result demonstrates a direct benefit of employing the engineered P450 BM3 CM1^{NADH} monooxygenase in the whole cell system compared to the only NADPH consuming counterpart. NADH is involved mainly in

aerobic energy generation (catabolism), while NADPH is involved in anabolic pathways (biomass synthesis) (Blank et al., 2010). Therefore, a higher net formation of NADH in resting (i.e. non-growing but metabolically active) *E. coli* cells is expected compared to NADPH, when glucose is supplemented for intracellular cofactor regeneration. In fact, 7 NADH- and only 4 NADPH-dependent enzymes are involved in glucose catabolism (Blank et al., 2010). A theoretical maximal NADH yield of 10 mol $mol_{glucose}^{-1}$ has been estimated for resting *E. coli* cells (Blank et al., 2008). Therefore, P450 BM3 WT^{NADH}, 19A12 ^{NADH}, and $CM1^{NADH}$ variants were used for all artificial operon experiments.

In the double oxidation system with both oxidoreductases ($CM1^{NADH}$-RE-ADH) as artificial operon, a product yield of 76 mg g_{CDW}^{-1} was obtained, which is nearly doubled compared to the single monooxygenase reaction with P450 BM3 $CM1^{NADH}$ (Table 2, entry 5 vs. 2). The improved performance of the double oxidation system can be attributed to an optimized intracellular expression (P450 content of 81 vs. 58 mg_{P450} g_{CDW}^{-1}) and an improved ADH-coupled intracellular regeneration of NADH.

Product yields on catalyst of 76 mg g_{CDW}^{-1} were obtained for the $CM1^{NADH}$-RE-ADH whole cell system, which also yielded the highest intracellular content of P450 BM3 $CM1^{NADH}$ (81 mg g_{CDW}^{-1}). Lower product yields and P450 expression levels were obtained for the artificial operon constructs WT^{NADH}-RE-ADH and $19A12^{NADH}$-RE-ADH (Table 2, entry 3 and 4 vs. 5). The artificial operon $19A12^{NADH}$-RE-ADH showed the lowest P450 content of 25 mg_{P450} g_{CDW}^{-1} despite of cultivation in a fermentor under similar conditions.

Finally a total product concentration of 5.7 mM (658 mg l^{-1}) was measured for the artificial operon system P450 BM3 $CM1^{NADH}$-RE-ADH after 48 hours, which consisted of 4.1 mM alcohols and 1.6 mM ketones. The conversion of *n*-heptane was monitored over time (see Supplementary Data, Figure S4) and a nearly linear increase in product formation was recorded within the first 8 hours, with a specific activity of up to 0.76 U g_{CDW}^{-1} (9.3 U g_{P450}^{-1}) and a space time yield (STY) of 45 mg l^{-1} h^{-1} after 4 hours. Deceleration in product generation was observed until the reaction was terminated after 48 h. The total turnover number after 48 h was determined to be 968 $mol_{product}$ mol_{P450}^{-1}.

Notably, the final product concentrations in all three double oxidation systems for *n*-heptane as substrate were considerably higher after 48 h (up to 3.2-fold) than in the *in vitro* biotransformation systems employing *n*-heptane for double oxidation with a monooxygenase and a dehydrogenase (24 h) (Müller et al., 2013). It has to be considered that for the *in vitro* oxidation system a shorter reaction time of 24 h was employed. However, the whole cell system yielded with P450 BM3 $CM1^{NADH}$-RE-ADH after 24 h already a 2.3-fold higher total product concentration than the corresponding *in vitro* system (4.13 mM vs. 1.8 mM total product; see Supplementary Data, Figure S4). In summary, higher product concentrations are achieved using the whole cell system compared to a cell free system (Table 2, entry 5 vs. 6).

3.4 Extension of the double oxidation cascade to a third catalyst: Lb-ADH

As a proof of concept to broaden the applicability of the whole-cell double oxidation concept, a second alcohol dehydrogenase, the NADPH-dependent ADH from *Lactobacillus brevis* (LbADH) (Hummel, 1997) was included as a second plasmid construct (pKA1 RE-ADH). The LbADH is (*R*)-selective and therefore complementary to the (*S*)-selective RE-ADH. In an ideal case a complete oxidation of the intermediate heptanols to the corresponding heptanones can be achieved. In addition, the proposed concept can be used as a biocatalytic tool-box for synthesis of chiral alcohols instead of ketones when one specific ADH is employed converting only the 'unwanted' alcohol to the corresponding ketone (Table 3 and 4).

Table 3 summarizes the results of shaking flask experiments in which Lb-ADH was introduced as third enzyme in the whole cell systems with and without RE-ADH. The performance is compared by employing for the initial oxygenation P450 BM3 19A12 and CM1 variants. All four whole cell systems were successfully expressed in shake flasks (see Supplementary Data, Figure S5) and *n*-heptane conversion was achieved.

Table 3: Process performance of the whole cell double oxidation of *n*-heptane with two- or three-enzyme catalysts: One P450 BM3 variant (19A12, 19A12^{NADH}, CM1, CM1^{NADH}) and one or two alcohol dehydrogenases (Lb-ADH, RE-ADH). Product concentrations were determined in duplicate.

Entry	Whole cell catalysts (shake flask expression)	Total product concentration[a]		TTN[b]	Product yield on catalyst $Y_{p/x}$	P450 content
		[mM]	[mg L^{-1}]		[mg g_{CDW}^{-1}]	[mg_{P450} g_{CDW}^{-1}]
1	BM3 19A12 + Lb-ADH	0.47	54	2930	8	3
2	BM3 CM1 + Lb-ADH	0.23	26	854	4	5
3	BM3 19A12^{NADH}-RE-ADH + Lb-ADH	0.85	97	1393	16	12
4	BM3 CM1^{NADH}-RE-ADH + Lb-ADH	0.29	33	534	6	11

[a] Product concentrations as sum of heptanol and heptanone isomers after GC quantification; the reaction mixture contained *E. coli* cells (OD_{600} 40: 6.9, 6.4, 5.9 and 5.7 g_{CDW} L^{-1} for entry 1, 2, 3 and 4 respectively) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L^{-1} D-glucose and 1045 mM (200 µL) *n*-heptane as second phase; 48 h at RT, stirring.
[c] TTN: Total Turnover Number ($\mu mol_{product}$ μmol_{P450}^{-1})

n-Heptane is converted more efficiently (~2-fold) with P450 BM3 19A12 and 19A12^{NADH} than with the corresponding CM1 variants in the Lb-ADH-catalyzed double oxidations (Table 3, entry 1 and 2). A further enhanced product formation of 80% for the variant 19A12^{NADH} and of 30% for the variant CM1^{NADH} was achieved using the whole cell systems with the second alcohol dehydrogenase RE-ADH (Table 3, entry 3-4 vs. 1-2). A main difference between the two and the three enzyme systems is the intracellular concentration of P450 monooxygenase. In the three enzyme systems the P450 content of 19A12^{NADH} and CM1^{NADH} is 4-fold and 2-fold higher (Table 3, entry 3 and 4; 12 and 11 mg_{P450} g_{CDW}^{-1} respectively) which corresponds well to the increased product concentrations and indicates that the monooxygenase reaction is limiting the product formation. In fact, the three-enzyme coexpression systems (P450^{NADH}-RE-ADH + Lb-ADH) display overall an up to 13-fold lower absolute product yield when compared with the

two-enzyme systems (artificial operon of P450 BM3^{NADH} and RE-ADH; Table 3, entry 4 and 5 *vs.* Table 2, entry 3 and 4). These results may be caused by the up to 7-fold reduced P450 BM3 content in the three- compared to the two-enzyme systems. Total turnover numbers (TTNs) for the monooxygenase are higher (up to 2930) with the P450 BM3 variants 19A12 and 19A12^{NADH} than for the corresponding CM1 variants.

Table 4 summarizes the region- and stereoselectivity of the four double oxidation systems in shake flask experiments.

Table 4: Selectivity of the whole cell double oxidation of *n*-heptane employing as monooxygenase a P450 BM3 variant (19A12, 19A12^{NADH}, CM1, CM1^{NADH}) and one or two alcohol dehydrogenases (Lb-ADH, RE-ADH).

Entry	Catalysts	Relative amount [% GC area]						
		Heptanol isomers (*ee of (S)*)				*Heptanone isomers*[a]		
		1-	2-	3-	4-	2-	3-	4-
1	P450 BM3 19A12 + Lb-ADH	<0.1	26 (n.d.)	15 (>99)	<0.1	41	18	<0.1
2	P450 BM3 CM1 + Lb-ADH	<0.1	<0.1	11 (>99)	1	14	31	43
3	P450 BM3 19A12^{NADH}-RE-ADH + Lb-ADH	<0.1	<0.1	<0.1	<0.1	67	31	2
4	P450 BM3 CM1^{NADH}-RE-ADH + Lb-ADH	<0.1	<0.1	<0.1	<0.1	15	41	44

[a] Heptanal was not detected.
n.d.: not determined

Coupling the P450 monooxygenases (19A12, 19A12^{NADH}, CM1, CM1^{NADH}) to the (*R*)-enantioselective Lb-ADH leads to accumulation of the (*S*)-heptanol isomers, while the (*R*)-heptanols are oxidized to the corresponding heptanones (Table 4, entry 1 and 2). In the 19A12-Lb-ADH-catalyzed cascade reaction a relative amount of 59% heptanones (2- and 3-heptanone; entry 1) is obtained and in case of CM1-Lb-ADH 88% (2-, 3- and 4-heptanone; entry 2). Chiral resolution of (*S*)-3-heptanol is achieved in an amount of 15% and 11% respectively. The three enzyme systems (Table 4, entry 3 and 4) convert in contrast to the two-enzyme systems (Table 4, entry 1 and 2) the intermediate (*R*/*S*)-heptanol products completely to the corresponding ketones. The product distribution of the synthesized heptanones in the *in vivo* system is in accordance to the reported regioselectivity of the P450 BM3 variants (19A12^{NADH}, CM1^{NADH}) in the *in vitro* system (Müller et al., 2013). Remarkably, a nearly quantitative conversion is obtained in the case of the three enzyme system (P450 BM3 19A12^{NADH}-RE-ADH + Lb-ADH, entry 3; P450 BM3 CM1^{NADH}-RE-ADH + Lb-ADH, entry 4), despite that ADHs are capable of performing the dehydrogenase reaction in a reversible manner.

Summary and Conclusion

A whole cell double-oxidation cascade concept was developed by coupling a monooxygenase- and an alcohol dehydrogenase-catalyzed reaction with integrated cofactor regeneration. Three

different coexpression strategies (2-plasmid, pACYCDuet-1 and artificial operon system) were evaluated for a P450 BM3 monooxygenase variant CM1^{NADH} and an alcohol dehydrogenase (RE-ADH) in *E. coli*. The artificial operon coexpression strategy P450BM3 CM1^{NADH}-RE-ADH yielded after several optimization steps a total product concentration of 5.7 mM (656 mg L^{-1}; (*R*)-heptanols and heptanones) and a product yield on catalyst of 76 mg g_{CDW}^{-1}. The latter whole cell double oxidation system, with a remarkably high intracellular content of P450 BM3 CM1^{NADH} (81 mg g_{CDW}^{-1}), yields an up to 3-fold improved product concentration compared to the corresponding *in vitro* system. A total turnover number (TTN) of 2930 was determined for the P450 BM3 variant 19A12 for oxidation of *n*-heptane in the two-enzyme system together with the Lb-ADH. Remarkably, the addition of a second alcohol dehydrogenase, the (*R*)-specific Lb-ADH, resulted in a nearly complete oxidation of *n*-heptane to the corresponding heptanones (>99 % selectivity).

The whole cell oxidation system of *n*-heptane was established as a two-liquid-phase system, consisting of an aqueous phase with resting *E. coli* cells (catalyst), glucose for cofactor regeneration, and the organic phase in which *n*-heptane is applied as organic carrier and substrate in a volumetric ratio of 17% (v/v). It represents a promising and cost-effective alternative to *in vitro* reaction systems for the industrial application of oxidoreductases, especially if one considers that engineered industrial production strains produce 5-10 times more enzyme than the employed *E. coli* strain.

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Highlights

Double oxidation of linear alkanes in a cascade reaction in water with oxygen as oxidant

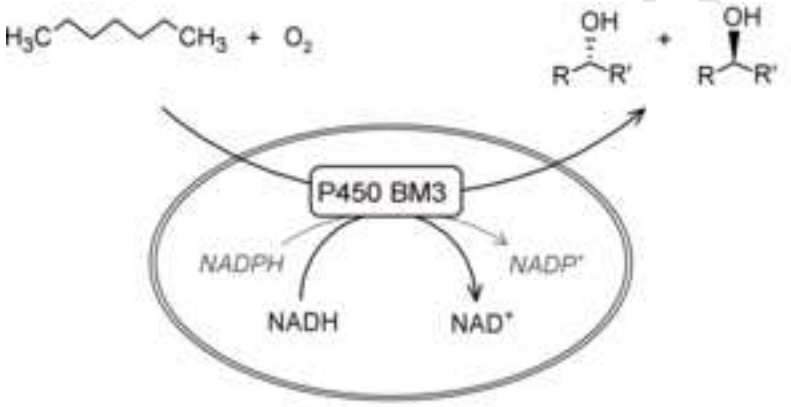
Whole cell catalysts for double oxidation of *n*-heptane

Design of artificial operons for efficient expression of two enzymes

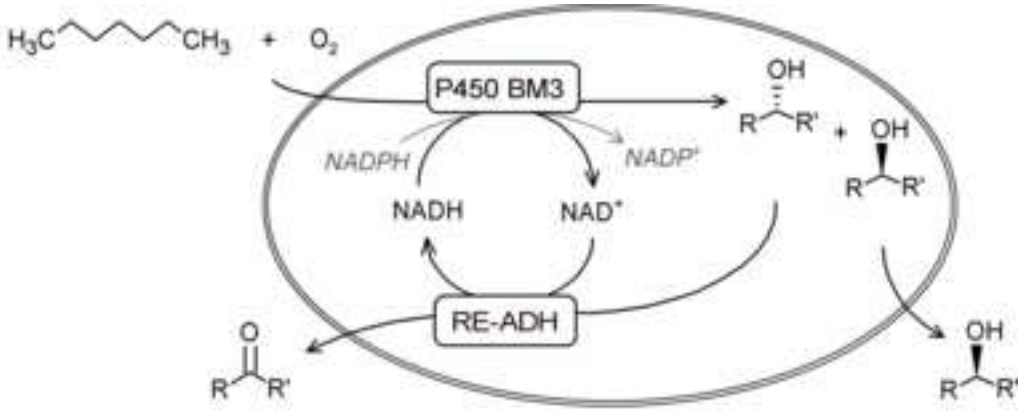
Coexpression of three oxidoreductases in *E. coli*

Double oxidation with coupled *in vivo* cofactor regeneration

Table 2 (figure)



Single expression P450 BM3 variants



Artificial operon P450 BM3-RE-ADH