

Multi-enzymatic cascade reactions for oxyfunctionalization of alkanes employing P450 BM3 monooxygenases

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*Para mis padres, mi hermana, mis
chiquitos y mi otro chiquito, Alex.*

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Abstract

Selective oxyfunctionalization of hydrocarbons is a major challenge in industrial organic synthesis. The biocatalytic activation of alkanes under mild reaction conditions, using molecular oxygen as benign oxidant, has emerged as an interesting alternative synthesis route. In this regard, cytochrome P450 monooxygenases represent a promising class of catalysts for the selective hydroxylation of non-activated carbon atoms. Successful development of biocatalysts that meet the process requirements can be achieved by combination of enzyme engineering, as well as reaction and process engineering.

A novel process concept was developed based on the multi-enzymatic double oxidation of alkanes to alkanones in a one-pot synthesis, employing a cell free as well as a whole cell catalytic system that were benchmarked against each other. The enzymatic component of the catalytic cascade are a P450 BM3 monooxygenase and (S)- or (R)-selective alcohol dehydrogenases (RE-ADH and Lb-ADH). As model compounds *n*-heptane and cyclooctane were employed. Additionally, for *n*-heptane as substrate, a strategy for chiral resolution of (S)- or (R)-heptanols was developed. Initially, by means of protein engineering the monooxygenase variant P450 BM3 CM1^{NADH} was generated, showing a high coupling efficiency for NADH as cofactor (44%) and a high turnover frequency with the substrate *n*-heptane (600 min⁻¹).

The initial step of the biocatalytic oxidation cascade consists in the hydroxylation of the alkyl chain, catalyzed by a P450 BM3 monooxygenase under consumption of NAD(P)H to produce (R/S)-heptanols or cyclooctanol. In the second oxidation step and depending on the selectivity of the employed ADH, the oxidation of (S)- or (R)-heptanol or complete oxidation of cyclooctanol to the corresponding ketones is achieved. In addition, the ADH-catalyzed sequential oxidation allows the direct regeneration of the cofactor. As further extension, the complete oxidation of *n*-heptane employing both ADHs in a three enzyme system was achieved, allowing a shift of the reaction equilibrium completely to the ketone products.

Whole cells catalysis with the engineered P450 BM3 monooxygenases in the described multi-enzymatic oxidation process yielded up to 4-fold higher product concentrations compared to the *in vitro* system. For the conversion of *n*-heptane, employing a biphasic reaction system, a total product concentration of 5.7 mM (656 mg L⁻¹) was obtained. The conversion of cyclooctane yielded 94% of cyclooctanone with a concentration of 5.2 mM (652 mg L⁻¹). The concept of the double oxidation cascade with P450 BM3 monooxygenases and ADHs was successfully proven and enables further extension to other catalysts and substrates. The enzymatic components can be employed in a modular manner, allowing the synthesis of enantiomerically enriched alcohols or ketones, which represent important building blocks in the manufacturing of pharmaceuticals, agrochemicals, food additives and synthetic polymers.

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1 Introduction

1.1 Oxyfunctionalization of hydrocarbons: Current challenges

Petrochemical feedstocks are currently used as main raw materials for the production of bulk and fine chemicals. A selective oxyfunctionalization of hydrocarbons remains one of the most challenging tasks in chemistry, especially with regard to simple alkanes, arenes and benzylic positions^[1] (Figure 1-1), mainly due to a low reactivity of the C-H bonds^[2] and the poor chemo- and stereoselectivity of most chemocatalysts^[3].

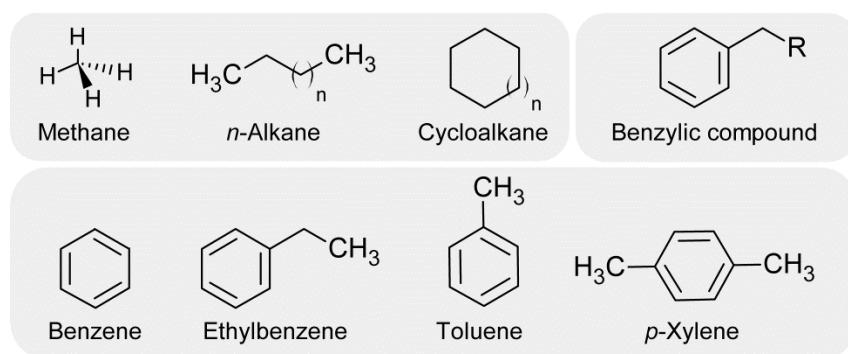


Figure 1-1: Some examples of representative hydrocarbons: alkanes, arenes, benzylic compounds.

The non-activated C-H bond is thermodynamically very strong ($\sim 400 \text{ kJ mol}^{-1}$) and is usually classified as kinetically inert at room temperature and towards mild oxidizing reagents.^[2] In fact, alkanes can readily react with oxygen in air, but only at elevated temperatures (deep oxidation or burning), to produce the thermodynamically stable products water and carbon dioxide.^[2] Alkanes such as methane and ethane, main components of natural gas, are used for example mainly as fuel for combustion in industry and private households.^[4] However, the combustion of alkanes from valuable petrochemical sources is not interesting from a synthetic point of view.^[2]

Traditional synthetic routes for functionalization of hydrocarbons involve the use of harsh reaction conditions with temperatures above 100°C and/or strong oxidizing agents or acids. The autooxidation of alkanes can be promoted by application of elevated temperatures and a chain initiator resulting in the formation of free radicals in a chain process. Transition metal ions can serve as catalyst for this autooxidation process.^[2] A great number of oxidation reactions are also carried out using stoichiometric amounts of inorganic oxidation agents, mainly dissolved salts of redox-active metals such as permanganate, manganese dioxide, and

chromium(VI) reagents, or other oxidizing agents like periodate, halides, peroxides or ozone.^[5] A main problem of these processes is the low selectivity due to the high exothermic nature of the reaction, resulting in multiple byproducts.

The aerobic oxidation of C-H bonds represents a “dream reaction”, since molecular oxygen is a highly abundant, non-toxic and clean oxidant.^[1] Nevertheless, the activation of the dioxygen represents a further hurdle, since O₂ in the ground or triplet state (two unpaired electrons with parallel spin) is rather unreactive.^[6] To circumvent the spin restriction the triplet state has to change (spin inversion), a kinetically impeded reaction, leading to the formation of more reactive oxygen species (e. g. hydroxyl radicals, peroxides).^[7] To facilitate the activation of oxygen and the C-H bond, transition metal complexes, containing copper, iron or manganese can be used. The Wacker oxidation^[8] catalyzed by PdCl₂ represents one of the most efficient chemical methods for oxyfunctionalization of olefins and enables the synthesis of aldehydes and ketones^[9].

Recent developments in the aerobic oxidation of alkanes using organometallic complexes in homogeneous or heterogeneous catalyzed oxidations have been reported. For heterogeneous catalyzed alkane functionalization transition metals such as Mn, Co or Pd are introduced within a defined framework consisting of crystalline nanoporous aluminophosphates (AlPOs)^[10], microporous zeolites^[11] or nanoporous carbon materials^[12]. This type of catalyst resembles a metal substituted molecular sieve.

In the field of homogeneous catalysis, research has focused mainly on the use of hydrogen peroxide as oxidizing agent. When oxygen is used as oxidant, activation is achieved by mean of an artificial reducing agent or an active metal complex with a porphyrin ligand.^[1] A well-known example of such a biomimetic metal-complex for the oxidation of alkanes to ketones is the ‘Gif System’, which consists of an iron catalyst, metallic zinc as reductant and acetic acid (or other acids) as proton source.^[13] This system displays however an undesired oxidation selectivity for branched alkanes (order secondary > tertiary > primary) and no selectivity for oxidation of arenes and olefins. Other biomimetic approaches using metallo-porphyrins with diverse ligands are inspired by the reaction mechanisms of cytochrome P450 monooxygenases and of the methane monooxygenase^[14]. Satisfactory results have been achieved using Mn^{III}-porphyrin,^[15] Fe^{II}-porphyrin^[16] or Ru-porphyrin^[17] complexes in the presence of ascorbic acid, hydrogen sulfide or acetaldehyde. In most of the cases, a compromise between selectivity and catalytic activity takes place. Simultaneous regio- and chemoselectivity are often not met by these catalysts.^[4] The most striking advances in the field of selective oxidation with molecular

oxygen are summarized in a comprehensive way in the recent reviews by Roduner *et al.*^[1] and Bordeaux *et al.*^[4]

Nonetheless, the stoichiometric need of prohibitive transition metal reagents^[18], or large quantities of organic solvents and acids, and the laborious separation and recycling steps for products, solvents and catalysts renders most of the currently used chemical synthesis routes unfavorable in regard to environmental impact and sustainability.^[5a] Despite of great advances in the development of novel processes for the organic synthesis, there is still the necessity to find more environmental friendly and sustainable solutions, especially in the field of selective oxidations. Environmental acceptable processes and products should fulfill certain guidelines that are defined as principles of ‘Green Chemistry’.^[19] Some of these principles are: efficient use of preferably renewable raw materials, atom efficiency, prevention of waste, less hazardous/ toxic chemical, and higher energy efficiency among others.^[5a] Therefore, the application of biocatalysis in the organic synthesis offers promising alternatives to meet the goals of ‘Green Chemistry’.

1.2 Biocatalysis in industrial synthesis

Biocatalysis can be defined as the application of enzymes and microbes in the synthetic chemistry, usually employing catalysts from natural sources for new purposes.^[20] In industrial biocatalysis, isolated enzymes or whole cells are used for the desired biotransformation of a target compounds to a value-added product. The portfolio of industrially relevant products that can be synthesized by biocatalytic routes covers active pharmaceuticals, food ingredients, intermediates for agrochemicals and pharmaceuticals, as well as bulk chemicals.^[21]

The origin of biocatalysis goes back to the ancient cultures of Mesopotamia, China and Japan where it was applied for the manufacture of foods and beverages. One of the first studies on the biocatalytic action of enzymes was developed in France by Payen and Persoz (1833), who described the isolation of diastase, a mixture of amylases from the active extract of germinating barley for the hydrolytic conversion of starch to dextrin and sugars.^[22] It was first in 1835 when the Swedish chemist Berzelius designated such a process as a catalytic reaction. Between 1835 and 1897, further investigation by the German scientists Schwann, who discovered Pepsin, Kühne who proposed the term ‘enzyme’ and the Buchner brothers, who demonstrated that alcoholic fermentation did not require whole cells but only the cell-free extract of yeast, contributed to the further development of biocatalysis as an independent research field. The first company to commercialize standardized enzyme preparations for industrial production,

namely isolated rennet for cheese making, was founded in 1874 by the Danish chemist Christian Hansen.^[23]

One substantial breakthrough to achieve a more widespread application of enzymes in industrial processes came along with the development of immobilization techniques to overcome stability and recyclability issues. Major processes that uses immobilized enzymes in a product scale of more than 10,000 tons per year are the production of high fructose corn syrup with a glucose isomerase, the acrylamide production catalyzed by the nitrile hydratase and the synthesis of 6-aminopenicillanic acid as building block for semisynthetic penicillin and cephalosporin antibiotics employing a penicillin amidase.^[23b]

The next crucial breakthrough in biocatalysis was achieved by introduction of DNA technologies for engineering of enzymes from 1970 upwards. Amylases, glucose isomerases, subtilisin (in washing powders) and penicillin amidases are used currently as recombinant enzymes in some of the most important industrial processes.^[24] Further development of protein engineering techniques has enabled the synthesis of non-natural intermediates, mainly for the pharmaceutical and the fine chemical industry. Nowadays, optimization of process stability and development of novel activities towards non-natural substrates are some of the main challenges, which are tackled by enzyme engineering approaches in the search for new or alternative synthetic processes.^[20]

In the last decades, the use of biocatalysis in industrial manufacturing process has settled down as a key technology next to more traditional organic synthesis routes. Up to 2006 Straathof estimated that more than 150 biotransformation processes had started on an industrial scale, most of them involving hydrolases or redox biocatalysts.^[25] Judging from the number of established processes, biocatalysis is applied at the moment mainly in the production of fine chemical that are employed in the development of pharmaceuticals.^[26]

In the last years growing interest also in the application of biocatalytic processes for production of bulk chemicals has emerged, not only within the academic but also the industrial research areas. In part, this turn is a result of a more widespread awareness of the environmental impact that current production processes have in our society. It is now accepted that more environmental friendly and sustainable technologies are required in the chemical industry.^[5a]

Some of the striking advantages of enzymes over organo or metallo-catalysts used in chemical synthesis are their remarkably high chemo-, regio- and stereoselectivity and the capability of accepting a varied range of substrates, consisting of simple or complex structures. Enzymatic reactions are performed in regard to temperature and pressure usually under mild reaction

conditions. Toxic reagents or higher amount of solvents are in general not required.^[27] These are intrinsic characteristics of enzymes due to their incidence in natural environments, where they carry out very specific tasks in a complex regulated and fine-tuned network of metabolic pathways. Another relevant aspect is the fact that biomass can be produced in a much simpler and cost-effective way in larger amounts and short periods of time. As the biocatalysts themselves are biodegradable, the environmental impact can be reduced.^[27]

1.2.1 Stereo-chemical synthesis of alcohols

Most of the produced optically active chemicals, such as amines, alcohols and carboxylic acids are used in the pharmaceutical, agrochemical and food industry. In the pharmaceutical and agrochemical industries an estimated share of 15% of the market accounts for chiral intermediates, which are used as chemical building blocks, auxiliaries or advanced intermediates.^[28] In this regard, the synthesis of optically pure compounds is partly imposed by the strict regulations of the US Food and Drug Administration (FDA) and the European Committee for Proprietary Medicinal Products for registration of new active compounds.^[28-29]

The production of chiral compounds can be classified in three different groups that comprise the classical methods, such as chemical racemic resolution or chromatographic separation, the asymmetric (chemical) resolution and the biotechnological (biocatalytic) processes.^[30] As an example, the synthesis of chiral alcohols starting from prochiral ketones and olefins is often achieved by asymmetric reduction using either chiral boron reagents^[31] and chiral phosphorous ligands^[32] or by enzymatic ketone reduction^[33].

Biotechnological processes offer in most cases an economical attractive^[33] and environmentally preferable access to optically active compounds.^[24, 34] Therefore, the number of industrial processes employing biocatalytic methods has increased significantly in the last years.^[35]

Biocatalytic processes which involve the generation of chiral centers during the catalyzed reaction employ mainly kinetic resolution and asymmetric synthesis.^[26] Hydrolases, especially lipases, are the most commonly employed enzymes in industrial processes, catalyzing the hydrolytic cleavage of carbon-oxygen single bonds to produce acids and alcohols. Upon addition of an acyl donor, kinetic resolution of the produced racemic mixture is achieved, as shown in Figure 1-2.^[24, 27] Lyases are another enzyme class which is usually employed for the synthesis of chiral alcohols. They catalyze the formation of C-C bonds, enabling upon addition of ketones or aldehydes as substrate the production of the corresponding alcohols.^[36]

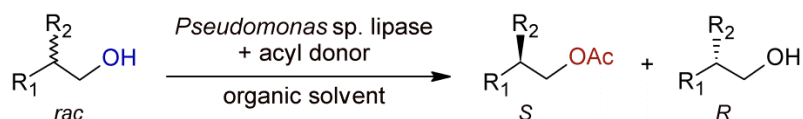


Figure 1-2: Kinetic resolution of primary alcohols by means of a lipase from *Pseudomonas* sp. The reaction is carried out in an organic solvent (e. g. benzene or CHCl_3) using an acyl donor such as vinyl acetate or acetic anhydride. Redrawn from Faber.^[27]

Oxidoreductases catalyze redox reactions (electron transfer from or to substrates) and are usually cofactor-dependent. Among the class of oxidoreductases the alcohol dehydrogenases (ADHs) play up to date the most relevant role in an industrial scale. The oxidation of chiral secondary alcohols with an enantioselective ADH enables the accumulation of one enantiomer through kinetic resolution, but a maximal yield of 50% is achieved when the starting material is a racemate (Figure 1-3 A).^[27]

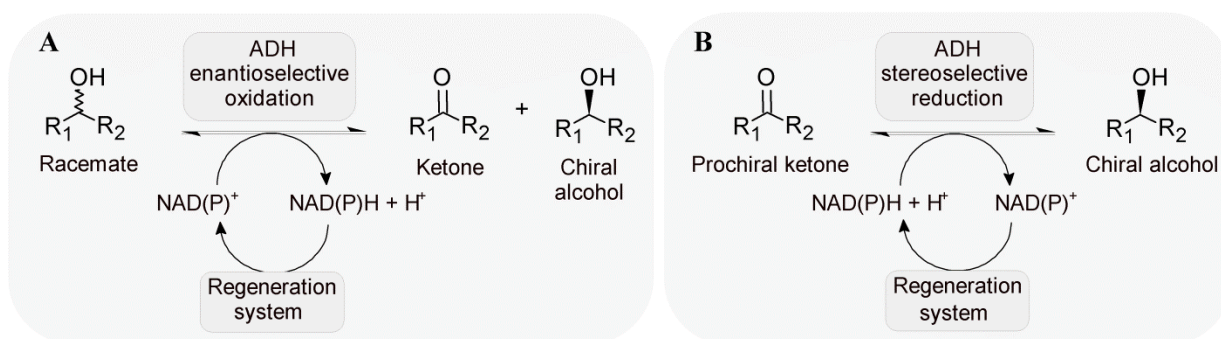


Figure 1-3: Alcohol dehydrogenase-catalyzed synthesis of chiral alcohols: Kinetic resolution (A) and asymmetric reduction of prochiral ketones (B). Modified from Faber.^[27]

Stereoselective asymmetric reduction of prochiral ketones using oxidoreductases is often the preferred route to chiral alcohols over kinetic resolution, since higher product yields can be achieved (theoretically up to 100%; Figure 1-3 B).^[33]

An interesting approach to circumvent the limiting yield of 50% in kinetic resolutions is the ADH-catalyzed deracemization of alcohols. Using two ADHs with complementary stereoselectivity and differing cofactor dependency the stereoinversion of one enantiomer is carried out, facilitating the enrichment of the desired enantiomer (Figure 1-4).^[37]

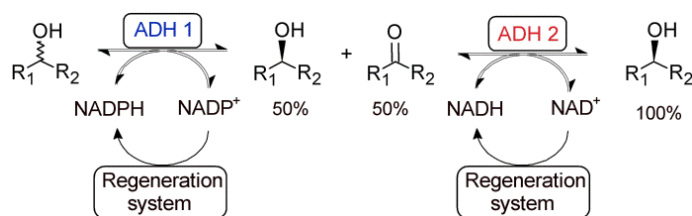


Figure 1-4: Deracemization of alcohols using enantio-complementary ADHs in a bi-enzymatic one-pot redox reaction. Redrawn from Hollmann *et al.*^[38]

Using this multi-enzymatic cascade reaction the entire potential of redox-catalysis can be utilized to achieve higher atom efficiencies.^[38]

Monooxygenases comprise a further group of oxidoreductases that are able to produce chiral alcohols, in this case by selective oxidation of a non-activated C-H bond. This synthetic approach is still not widely applied, with only a few applications in industrial processes, mostly based on the modification of a complex and large framework such as steroids (e.g. 11-deoxycortisol, progesterone) for pharmaceutical applications.^[27, 39] A further example is the production of the cholesterol-lowering drug pravastatin by hydroxylation of compactin with whole cells of *Streptomyces* sp. containing CYP105A3.^[40] In recent years much attention has been attracted to the advantageous use of cheaper substrates (e.g. alkanes) and the broad scope of available monooxygenase-catalyzed reaction for stereo-chemical hydroxylation reaction.^[41] The direct biocatalytical activation of alkanes is an interesting and more cost-effective alternative to the use of prochiral ketons in stereo-chemical synthesis, making the enzymatic oxyfunctionalization a key factor for the future applications of oxygenases.^[42] A more detailed introduction to this type of monooxygenase-catalyzed stereoselective enzymatic oxidation reactions can be found in chapter 1.3.

1.2.2 Production of ketones

Ketones are important building blocks in the chemical industry. Cycloalkanones are used in large scale industrial manufacturing of synthetic polymers like polyamides.^[43] The Bashkirev oxidation^[44] represents one traditional chemical synthesis route for the production of cycloalkanols employing molecular oxygen as oxidizing agent in presence of boric acid. Cycloalkanones are obtained by a further catalyzed dehydration reaction of the alcohols.^[45] In this multi-step process stoichiometric amounts of boric acid are required, as well as multiple catalysts and only low conversions with modest selectivities are achieved.^[43] Therefore, alternative synthesis routes to cycloalkanones using biocatalysis with a lower environmental impact and higher productivities are desirable.

Aliphatic ketones and aldehydes are usually used industrially for the production of flavors and fragrances.^[46] The generation of flavors by means of microbial biotransformation processes has one of the longest traditions, being employed in the preparation of food and beverages such as cheese, bread, wine and beer for many centuries. Accordingly, the biotechnological production of flavors is a well-established procedure in the chemical industry. Some fungal species can produce methylketones in aerobic fermentation processes under growth limiting conditions as a result of an overflow metabolism in the β -oxidation of fatty acids.^[46] 2-Heptanone and 2-nonanone are for instance important for the characteristic taste of Roquefort cheese. The production of C5 to C9 methylketones by transformation of the corresponding C6 to C10 fatty acids using *Penicillium roquefortii* spores in solid-state fermentation of buckwheat seeds has been reported. This process can yield up to 75 g L⁻¹ of 2-heptanone and 60 g L⁻¹ 2-nonanone.^[47]

1.2.3 Biocatalytic functionalization of alkanes

Enzyme-catalyzed oxidation of non-activated alkanes is in general achieved with higher selectivities than with chemocatalysts.^[38] Several enzymes that catalyze alkane oxygenation reactions occur naturally. The main classes are oxygenases and peroxidases.^[39] Peroxidases catalyze mainly oxygen transfer reactions, such as olefin epoxidations and sulfoxidations. Recently, the peroxygenase from *Agroclybe aegerita* was reported as first peroxidase to hydroxylate aliphatic chains (from propane to hexadecane) using H₂O₂ as oxidizing agent.^[48] A few oxidases and dehydrogenases are also capable of catalyzing hydroxylations. One example is the nicotinate dehydrogenase from *Bacillus niacini*, which uses water as oxygen donor.^[49] Oxygenases comprise a more diverse catalytic family due to their intrinsic physiological role in detoxification, synthesis of secondary metabolites or hydrocarbon degradation.^[39] Some of the most relevant classes of oxygenases that catalyze the oxidation of alkanes are shown in Table 1-1. Despite of differences in the catalytic mechanism all the depicted natural alkane oxygenases can be classified as transition-metal organocatalysts, using strictly iron or copper as metal. They share common features such as the availability to hydroxylate alkanes as a result of natural evolution, the use of molecular oxygen from air as sole oxidizing agent and the use of reduced nicotinamide dinucleotides (NAD(P)H) as electron suppliers.

Nevertheless, industrial applications of oxygenases are often hindered, mainly due to their low reaction rates, stabilities and cofactor-dependency.^[41a]

Table 1-1: Enzyme classes involved in the oxidation of alkanes. Adapted from van Beilen and Funhoff.^[50]

Enzyme class	Source	Features	Substrate range	Ref
Soluble methane monooxygenase	Methanotrophic bacteria	Dinuclear iron; soluble reductase	C ₁ -C ₈ (halogenated)-alkanes, alkenes, cycloalkanes	[51]
Particulate methane monooxygenase	Methanotrophic bacteria	Mononuclear or dinuclear copper; membrane bound hydroxylase	C ₁ -C ₅ (halogenated)-alkanes, alkenes	[51]
AlkB-related alkane hydroxylases	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Pseudomonas</i> , etc.	Dinuclear iron rubredoxin; Membrane hydroxylase	C ₅ -C ₁₆ alkanes, fatty acids, alkylbenzenes, cycloalkanes, etc.	[52]
Eukaryotic P450 (CYP52, class II)	<i>Candida maltosa</i> , <i>Candida tropicalis</i> , <i>Yarrowia lipolytica</i>	Microsomal oxygenase; P450 heme reductase	C ₁₀ -C ₁₆ alkanes, fatty acids	[53]
Bacterial P450 oxygenase system (CYP153, class I)	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Rhodococcus</i> , etc.	P450 oxygenases; heme ferredoxin + ferredoxin reductase, FAD	C ₅ -C ₁₀ alkanes, (cyclo)-alkanes, alkylbenzenes, etc.	[54]
Dioxygenase	<i>Acinetobacter</i> sp.	Homodimer; copper	C ₁₀ -C ₃₀ alkanes	[55]

A further constraint for an *in vitro* application of most of the oxygenases in Table 1-1 is the multicomponent and membrane associated character. The difficult recombinant expression and preparation of multicomponent, membrane-bound enzymes remains so far a limiting factor. A possible solution to overcome most of these challenges is their use as whole cell catalytic systems for synthetic applications.^[41b] Very promising is also the investigation of the recently discovered CYP153 family, whose members despite of the multicomponent character are soluble bacterial P450s.^[56]

Besides of the natural occurring alkane hydroxylating oxygenases described above, other cytochrome P450 enzymes have been engineered by means of protein evolution to accept alkanes as substrate. Most of the evolution studies have been performed with cytochrome P450 BM3 from *Bacillus megaterium* (*B. megaterium*; CYP102A1) and P450cam from *Pseudomonas putida* (CYP101). P450 BM3 is a soluble and self-sufficient medium- to long-chain fatty acid hydroxylase^[57] that has been evolved by different research groups towards a broad scope of substrates (reviewed by Whitehouse *et al.*^[58]) and especially by the research group of Arnold to a highly active alkane monooxygenase^[59]. As P450 BM3 is the scope of this thesis a more detailed introduction to this enzyme class is found in chapter 1.3.1. P450cam is a further soluble but multicomponent bacterial monooxygenase from *Pseudomonas putida*,^[60] which have been evolved successfully towards the hydroxylation of ethane^[61] instead of its natural substrate camphor. Bell *et al.* achieved this substrate shift through progressive adaption and shrinking of the substrate pocket by replacement of some of the original amino acids with more hydrophobic and larger residues.^[61] The biocatalytic

oxyfunctionalization of alkanes, combined with the possibility to tailor biocatalysts by protein evolution, offers a wide applicability of oxygenases for alternative processes in the oxidative chemistry.

1.2.4 Cofactor regeneration

The activity of oxidoreductases is dependent on the use of cofactors. Most oxygenases require the rather expensive nicotinamide cofactors NADH or NADPH as electron suppliers. An application of stoichiometric amounts of such cofactors for synthetic processes is economically not feasible. Therefore, an *in situ* regeneration of the oxidized and reduced nicotinamide cofactors is indispensable for a successful synthetic application.

To overcome the so called “cofactor challenge” in cell-free biocatalysis two basic strategies have been investigated: the use of enzymatic regeneration systems^[62] and regeneration by chemical, electrochemical or photochemical setups^[63]. The enzymatic regeneration strategy is currently the most efficient approach and commonly employed in industry and academia. It can be classified in two different types, substrate-coupled and enzyme-coupled regeneration^[62], depending on the reaction concept that is employed as shown in Figure 1-5.

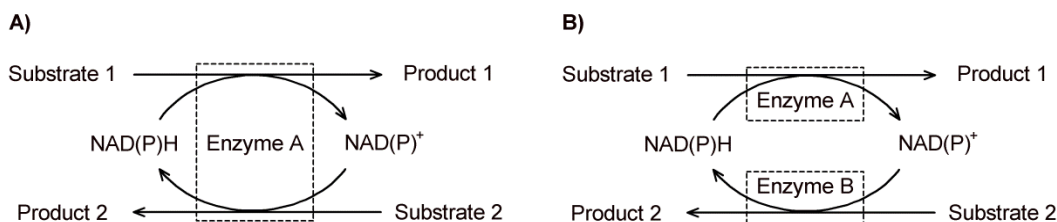


Figure 1-5: Different strategies for the regeneration of nicotinamide cofactors. A) The substrate-coupled system employs only one enzyme for the reduction of the substrate (main reaction) and for cofactor regeneration. **B)** In the enzyme-coupled approach the regeneration reaction is catalyzed by a second enzyme. Adapted from Weckbecker *et al.*^[62]

In the substrate-coupled system the same enzyme catalyzes the main reaction as well as the cofactor regenerating reaction. This approach is mainly applied with alcohol dehydrogenases, where a cheap alcohol is employed as sacrificial substrate for cofactor regeneration. Nevertheless, in this approach the selection of a suitable auxiliary substrate is restricted to the specificity of the biocatalyst.^[64] Isopropanol, commonly applied as cosubstrate in the production of (*R*)-alcohols from prochiral ketones, is oxidized to acetone with alcohol dehydrogenases like those from *Lactobacillus kefir* and *Lactobacillus brevis*.^[62]

In the enzyme-coupled strategy two enzymes and two substrates are required. The cofactor regeneration is carried out by the second enzyme independently from the main reaction. This system has a wide applicability not only for alcohol dehydrogenases as main catalyst, but also for other enzyme classes, since the selection of a regenerating enzyme and a cosubstrate is more flexible. Formate dehydrogenase (FDH) is often applied as regenerating enzyme in view of several advantages over other enzymes.^[65] FDH catalyzes the oxidation of formate, a cheap cosubstrate, to carbon dioxide. Formate and CO₂ are almost innocuous to almost all enzymes. Since this oxidation is irreversible the reaction equilibrium can be shifted to the product side. A disadvantage is its low specific activity (4-6 U mg⁻¹) compared to other dehydrogenases like alcohol or glucose dehydrogenase.^[62]

Glucose dehydrogenase (GDH) is also applied in lab- and industrial-scale for the enzyme-coupled regeneration of both types of reduced cofactors, NAD⁺ and NADP⁺, through oxidation of β -D-glucose to D-glucono-1,5-lactone, which hydrolyzes spontaneously to gluconic acid. The hydrolysis rate depends on the employed pH in a buffered reaction. A GDH can be produced in recombinant form, is commercially available, highly active and stable. The GDH from *B. megaterium* can achieve a specific activity of up to 550 U mg⁻¹ and glucose conversion rates in the range of 10-100 mM h⁻¹ have been determined.^[62, 66] General disadvantages in the use of isolated enzymes for cofactor regeneration are the higher cost in the preparation (isolation and purification), as well as the limited stabilities of the catalysts.

In whole cell catalysis with metabolic active cells (growing or resting cells), the regeneration of cofactors is achieved intrinsically in the cell by the cells metabolism (catabolic activity) using a carbon and energy source such as glucose. The glucose metabolism in *Escherichia coli* (*E. coli*) resting cells was simulated by *Blank et al.* to estimate the NADH regeneration capacity. The net NADH formation was calculated using an optimal glucose uptake rate of 2.4 mmol g_{CDW}⁻¹ h⁻¹ and taking into account the energy required (ATP) for cell maintenance. A maximal yield of 10.3 mol NADH per mole glucose was calculated, which results in a NADH formation rate of 22 mmol g_{CDW}⁻¹ h⁻¹.^[67] For redox biocatalysis, assuming a stoichiometric relation between product formation and cofactor consumption, this regeneration rate corresponds to a catalytic activity of 367 U g_{CDW}⁻¹.^[68]

Another strategy for cofactor regeneration in the host cell is the heterologous expression of a regenerating enzyme (e.g. GDH, FHD or ADH), similar to the described cell-free enzyme-coupled regeneration system using a sacrificial cosubstrate. In this way a metabolism-independent catalyst is generated. The external addition of cofactor is in most cases not necessary, since the intracellular cofactor pool is sufficient for the regeneration to proceed.^[69]

Traditionally, whole cells of *Saccharomyces cerevisiae* (*S. cerevisiae*) have been used for cofactor regeneration in industrial processes using the cellular metabolism, mainly for the reduction of ketones.^[70] Other organisms have also been implemented successfully, for example the resting cells of *Lactobacillus kefir* containing an alcohol dehydrogenase for the stereoselective reduction of 2,5-hexanedione to (2*R*,5*R*)-hexanediol.^[71]

Different recombinant whole cell catalytic systems have been designed, so-called designer bugs expressing the main catalytic enzyme and an auxiliary enzyme for cofactor regeneration.^[69, 72] This system has also been applied for multi-step reactions.^[73]

1.2.5 *In vitro* and whole cell catalysis

Biocatalytic processes can be performed using either isolated enzymes for *in vitro* biotransformation processes or whole cells containing a naturally compartmentalized form of the catalytic active enzyme. A further classification of these two basic formulations is dependent on the preparation of the biocatalyst as described below.

Isolated enzymes can be used as crude cell lysate or in a purified form. Purification of the catalysts is more expensive but can contribute to avoid side reactions and contaminations with other cell compounds.^[74] The low stability of most isolated enzymes is a challenging task, tackled in industrial processes mainly through immobilization techniques and nowadays also through protein engineering. Up to date, the majority of published papers on enzymatic biocatalysis report the use of isolated enzymes.^[74] In contrast, the majority of industrial biotechnological processes for synthetic applications in a large scale make use of whole cells.^[25] This demonstrates that within the academic research field a well-defined system, such as the isolated enzyme itself, is essential for a thorough investigation and characterization of the biocatalytic process, but in industrial manufacturing processes the whole cell as biocatalytic entity seem to meet the process requirements in a more economical fashion.

Whole cells can be used as living, metabolically active organisms or as inactive (dead) cells. In either case the cell provides a natural and therefore stabilizing environment for the catalytic enzyme.^[75] Metabolic active cells are needed in fermentative processes, in biotransformations with growing or resting cells that require cofactor regeneration, mainly for oxidoreductase-catalyzed reactions, and in biotransformations that are directly linked to the central carbon metabolism.^[76] For application of whole cells several parameters, besides of the main enzymatic activity, affect the performance of biotransformations. The most important parameters are mass transfer (of substrates, products, and oxygen), protein synthesis, cellular

metabolism, compartmentalization, cell growth and cell inactivation. Therefore, in whole cell catalysis the cell and not the enzyme is defined as the catalytic unit.^[76]

An economical advantage of biocatalysis over chemical catalysis is the simpler production of the highly regio- and enantioselective biocatalyst through fermentation. High-cell density fermentations yielding more than 100 g_{CDW} L⁻¹ have been reported for various microorganisms.^[77]

One striking drawback in whole cell processes is the possible contamination with byproducts as a result of other metabolic activities of the cell. In some cases, lower optical purities are yielded as a result of overlapping substrate specificities from native enzymes with a differing selectivity.^[64] Nowadays, the development of ‘designer cells’ for overexpression of the desired enzyme or coexpression of multiple enzymes is achieved using genetic engineering techniques.^[62] The possibility to avoid competing activities of other undesired enzymes can be achieved by gene knockout or disruption of entire metabolic pathways in the host organism.^[78] The recent developments in metabolic engineering offer the possibility of optimizing the metabolic flux, for example in regard to availability of reducing equivalents, and detecting possible limiting factors.^[23b, 67]

The limiting mass transfer in whole cells is another parameter that attracts much attention. The cell membrane can affect transfer of substrates and products, which on their part have a strong influence on the membrane integrity and fluidity. In the application of metabolically active cells target substrates or products are often toxic to the cell.^[75] In the case of organic solvents like short-chain aliphatic alkanes, that are small hydrophobic molecules, accumulation in the cytoplasmic membrane can occur. The loss of membrane integrity is considered to be a principal cause of toxicity in whole cell processes.^[79] Hence, the final disintegration of the membrane will result in the loss of proteins and cofactors. The partition of substrates and products between a membrane and an aqueous medium ($P_{m/aq}$) correlates to the partitioning coefficient of the same in an octanol-water mixture ($\log P_{o/w}$). As a rule of thumb, high cell toxicity is ascribed to compounds with a $\log P_{o/w}$ value between 1 and 4 (e.g. phenol, 1-octanol or chloroform).^[79b, 80] With a $\log P_{o/w}$ below 1 a compound is too hydrophobic to be able to enter the cell membrane. Above a $\log P_{o/w}$ of 4 the solubility of the compound is too low to be available in the aqueous phase.^[79b] However, the toxic effect depends strongly on the organism. The mass transfer coefficient of these compounds will depend not only on properties like size and hydrophobicity, but also on the availability of uptake or excretion mechanism in the cell.^[76] On the one hand the availability of substrates has to be assured to achieve sufficient reaction rates; on the other hand the long term stability of the cell is vital for the process

performance, for example in processes using catabolic cofactor regeneration. A technical solution to this dilemma is the use of organic/aqueous two-liquid phase system^[81] as shown in Figure 1-6.

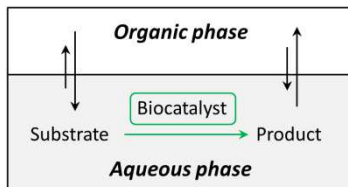


Figure 1-6: Organic/aqueous two-liquid phase system in biocatalysis. Partitioning of the substrate and the biotransformation product takes place between organic and aqueous phase. Thereby, the organic phase serves as substrate source and product sink, reducing inhibitory or toxic effects. Adapted from Heipieper *et al.*^[79b]

The application of a two-liquid phase system can optimize the availability of low-water soluble compounds (hydrophobic substrates) because it serves as substrate and product sink.^[81] Accumulation of products in the organic phase, so-called *in situ* product removal, enables the presence of only low concentrations of toxic compounds in the aqueous phase, protecting so the biocatalysts (whole cell or isolated enzyme).^[82] The selection of a suitable organic solvent as liquid phase has to take into account several parameters such as solvent capacity, toxicity, biocompatibility, flammability and availability.^[81] Solvents that have a $\log P_{o/w}$ value higher than 4 are water-immiscible and will probably not disturb the water shell around the biocatalyst.^[80] However, the biocompatibility of the solvent has to be evaluated for each case empirically. Some of the compounds that have been utilized as organic phase are: short- to medium-chain alkanes (i.e. *n*-heptane, *n*-octane, decane, dodecane)^[83], MTBE^[84] or bis-(2-ethylhexyl)-phthalate^[85]. It has been observed that whole cells are less sensitive to inactivation at the liquid-liquid interface than isolated enzymes.^[76] The *in situ* product removal also supports a direct down-stream processing of products.^[81] Under appropriate circumstances the substrate could also serve as bulk organic phase. This was demonstrated by Julsing *et al.* for the ω -oxyfunctionalization of fatty acids methyl esters.^[86]

Under some circumstances the low substrate uptake can still limit the reaction rates. To circumvent this problem permeabilization of the cell membrane has been applied successfully. Freeze-thawing, sonication and application of detergents and solvent represent some of the methods for permeabilization of cells.^[87] The engineering of the host cell, for example by deletion of lipopolysaccharides or lipoproteins in the outer cell membrane to facilitate substrate uptake has been described.^[88] In a similar way, the incorporation of an outer membrane protein for the passive transport of substrates, for example the AlkL membrane protein of

Pseudomonas sp. which facilitates alkane uptake has been reported. This strategy was demonstrated for the hydroxylation of (S)-limonene to (S)-perillyl alcohol.^[89]

Both isolated enzymes and whole cells can be immobilized in or on a carrier material to afford an easier recovery/re-use of the catalyst and higher process stabilities. The immobilization of whole cells involved in oxygen-dependent redox biocatalysis is however not recommended, since slow oxygen diffusion will rather limit the reaction rates.^[25] Depending on the targeted biotransformation reaction the application of the most suitable process strategy has to be studied in detail.

1.2.6 Multi-enzymatic cascades

One surpassing advantage of enzymes as natural components of living cells is their compatibility with each other under similar operational conditions. This property makes the use of multi-enzymatic cascade processes feasible, by coupling two or more biocatalytic reactions in a single flask (one-pot) as shown in Figure 1-7 A.^[27, 90] Such a process resembles the mode of action of cell metabolic pathways.

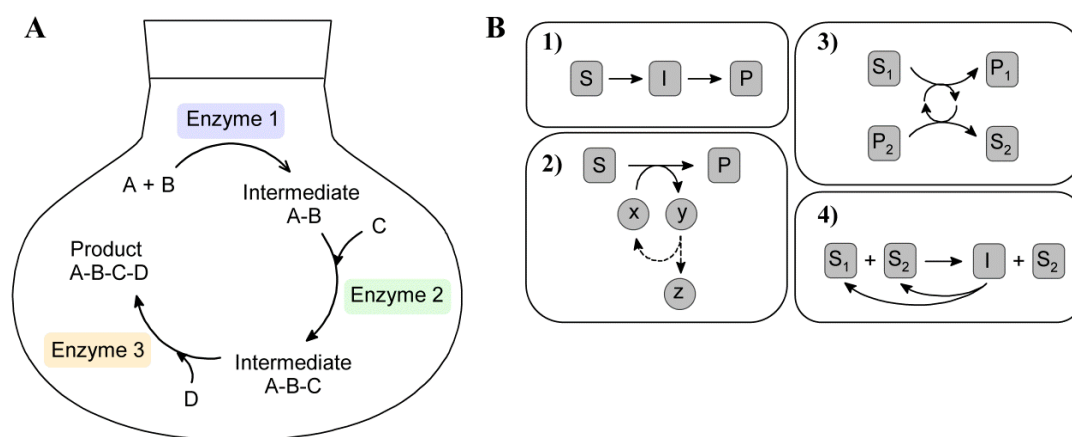


Figure 1-7: Multi-enzymatic cascade reactions. (A) Representation of a biocatalytic one-pot (multi-step) cascade reaction comprising 3 steps. Redrawn from Wohlgemuth.^[91] (B) Classification of enzymatic cascades according to process design: 1) linear cascade, 2) orthogonal cascade, 3) parallel cascade, 4) cyclic cascade. Redrawn from Ricca *et al.*^[90]

Growing interest has focused in the investigation of biocatalytic processes, which involve multi-enzymatic, chemo-enzymatic and enzyme-initiated cascades, and the application of either isolated enzymes or whole cell catalysts. The main benefits of a multi-enzymatic system are the reduction or omission of time-consuming isolation and purification steps, as well as the

possibility to shift the reaction equilibrium to the target product by selection of a suitable reaction sequence.^[91] This also means that the amount of waste can be minimized due to the lower requirement of solvents and working volume as well as the higher volumetric and space-time yield that can be achieved.^[92] Bottlenecks such as low compatibility of the enzymatic components or the reaction conditions can be circumvented by immobilization strategies, reactor design or directed protein evolution.^[92] Since combination of enzymes and metal catalysts is feasible, several possibilities for the design of new synthetic approaches are available (e.g. in the application of dynamic kinetic resolution).^[93]

Multi-enzymatic processes are usually classified based on the type of intermediate that is formed.^[90] The term “domino reaction” is employed when an unstable intermediate is generated and a spontaneous reaction leads to the stable product. “Tandem reactions” are those processes consisting of multiple biotransformation steps with concomitant formation of stable intermediates and products. A further classification of biocatalytic cascades into 4 main groups is based on the process design (Figure 1-7 B)^[90]: In linear cascades (1) a single substrate is converted to a single product after formation of the reactions intermediate. In orthogonal cascades (2) the main catalytic reaction is coupled to a second auxiliary reaction, either for regeneration of cofactors/cosubstrates or elimination of byproducts. Similar to the orthogonal approach, the parallel cascade (3) couples two main reactions for production of two products *via* an auxiliary reaction, which in turn involves the regeneration of cofactors or cosubstrates. The cyclic cascade (4) enables the accumulation of one target compound from a mixture of substrates (e.g. racemic mixtures) after iterative (cyclic) transformation of the unwanted substrate into an intermediate and subsequent transformation of the intermediate back to the starting racemic material. Depending on the process demands combination of different types of cascades is possible. Orthogonal cascades are classically used in processes that involve regeneration of cofactors as described in chapter 1.2.4, for example in the application of oxidoreductases.

Recently, several artificial multi-enzyme cascades have been developed for the production of optically active alcohols, amines and amino acids. Dehydrogenases and transaminases have been widely used for this purpose. An overview of this type of cascade reactions for synthesis of optically enriched compounds is given in Table 1-2.

One novel approach represent the deracemization and stereo-inversion of secondary alcohols using an ADH-catalyzed tandem reaction^[37, 94] as shown in Table 1-2, entry 1 (see also chapter 1.2.1, Figure 1-4). Another novel strategy is the biocatalytic amination of primary alcohols by means of two sequential, coupled reaction steps.^[95] The first step comprises the ADH-

catalyzed oxidation of the primary alcohol leading to production of the aldehyde, which is aminated in the second step by means of a ω -transaminase. The amide donor L-Alanine is regenerated from pyruvate by an L-alanine dehydrogenase, which in turn regenerates the nicotinamide cofactor NADH for the first reaction step. The described approach represents therefore a so called redox self-sufficient biocatalysis network (Table 1-2, entry 7). To broaden the concept, the authors developed a double amination tandem process for production of 1, ω -diols, involving in total four sequential reactions and two cofactor regeneration reactions.

Table 1-2: Examples of multi-enzyme cascade reactions for the production of optically enriched compounds.

Entry	Substrate	Product	Type of reaction	Enzymes ^[a]	Ref
Optically active alcohols					
1	Racemic <i>sec</i> -alcohols	Pure stereoisomers of <i>sec</i> -alcohols	Deracemization <i>via</i> stereoinversion	Two ADHs with opposite enantioselectivity (Prelog, anti-Prelog) and opposite cofactor specificity; two independent CRS	[94]
2	Cholic acid	12-Ketourso-deoxycholic acid	Sequential oxidation and reduction (stereoinversion)	Three hydroxysteroid dehydrogenases (7 α -HSDH, 7 β -HSDH with opposite cofactor specificity; 12 α -HSDH); two independent CRS	[96]
3	Racemic alcohols, sulfides	Alcohols, sulfoxides	Concurrent kinetic resolution of alcohols and asymmetric synthesis of sulfoxides	ADH, Baeyer-Villiger monooxygenase (BVMO)	[97]
Amino acids					
4	α -Hydroxy acids	L-Amino acids	Asymmetric synthesis by coupled oxidation and reductive amination (redox-neutral)*	ADH and amino acid dehydrogenase (AADH); same cofactor specificity	[98]
5	Racemic α -amino acids	L-Amino acids	Deracemization by coupled oxidation and asymmetric reductive amination	D-Amino acid oxidase (D-AAO), leucine dehydrogenase (LeuDH); FDH (for cofactor regeneration); catalase (for degradation of H ₂ O ₂)	[99]
Amines					
6	Ketones	α -Chiral primary amine	Asymmetric synthesis, removal of by-product pyruvate	ω -Transaminase (TA), lactate dehydrogenase (LDH), GDH (for cofactor regeneration)	[100]
7	Primary alcohols	Primary amines	Amination of primary alcohols (coupled oxidation and amination)	ADH, ω -TA, L-Alanine dehydrogenase (for recycling of alanine and cofactor)	[95]

^[a] CRS: Cofactor regeneration system

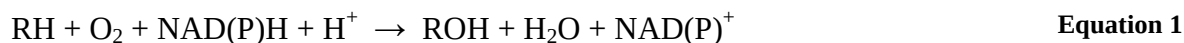
* Redox-neutral cascades are those that do not require external oxidation or reduction equivalents.

Another major focus of investigation has been the synthesis of complex molecules from simpler and cheap substrates as starting material using carbon-carbon bond forming enzymes like aldolases and nitrile hydratases.^[90] Besides, the synthesis of complex glycoconjugates is also achieved by multi-enzyme cascades by combination of pathway or pathway-unrelated enzymes.^[101]

Apart from the utilization of multi-enzymatic cascades in cell free systems, their application in whole cell approaches is very promising. Metabolic engineering^[102] can be employed for optimization of such *in vivo* catalysts. For utilization of a native production host the pathway flux can be enhanced by over-expression of rate-limiting metabolic genes using promoter engineering^[103], or by disruption of competing genes/pathways^[78]. An alternative is the engineering of heterologous expression hosts like *E. coli* or *S. cerevisiae*, which are most likely the best characterized production platforms so far. In this way, a new metabolic (artificial) route can be introduced in whole cells for synthesis of the target compound.^[104] To identify competing pathways or rate-limiting steps metabolic modeling or random approaches can be applied.^[104]

1.3 Cytochrome P450 monooxygenases

Oxidoreductases are one class of enzymes characterized by the catalytic availability to transfer electrons to the substrate, so called redox reactions.^[105] The oxidoreductases are divided into four main groups: oxygenases, peroxidases, oxidases and dehydrogenases. Oxygenases have a ubiquitous occurrence in all classes of living organisms, from eukaryotic to prokaryotic cells.^[67] They are capable of introducing oxygen into chemically inert C-H bonds.^[106] The catalytic mechanism of the different oxygenases is very diverse, some employ copper or iron as catalytic metal, others are for instance flavin-dependent. Oxygenases are further divided into monooxygenases and dioxygenases, catalyzing the specific oxyfunctionalization of carbon atoms by insertion of 1 or 2 oxygen atoms respectively.^[106] With exception of peroxidases and some dioxygenases, the oxidoreductase activity is dependent on redox cofactors, typically the nicotinamide cofactors NAD(P)H and NAD(P)⁺ that act as electron donors or acceptors.^[67] Cytochrome P450 monooxygenases are one class of heme-containing oxygenases, capable of oxidizing non-activated C-H bonds.^[107] The name cytochrome P450 is originated from its properties as a hemoprotein and its characteristic spectrum with an absorption maximum at 450 nm upon reduction and binding of carbon monoxide (CO).^[108] The general P450-catalyzed reaction is shown in Equation 1^[107]:



The heme is a prosthetic group of the type protoporphyrin IX that contains an iron nucleus, which is directly responsible for the activation of O₂. One atom of molecular dioxygen is often incorporated stereo- and regioselectively into the substrate, while the second atom is reduced to water.^[109] Aliphatic and aromatic hydroxylations are commonly catalyzed, but also other reactions such as epoxidations, dehydrogenations, deamination, dealkylation, as well as carbon-carbon bond formation or cleavage were reported.^[110] P450 monooxygenases are involved in diverse physiological reactions that are coupled to anabolic or catabolic pathways. They are responsible for hormone synthesis, drug metabolism and xenobiotic detoxification in mammalian and insect cells, as well as for herbicide resistance in plant cells.^[107] Microbial P450s are usually related to metabolic and synthetic pathways, for example for the synthesis of antibiotics or the degradation of hydrocarbons.^[58]

Cytochrome P450s use external electron donors for the activation of oxygen and a respective electron-supporting system, mostly from the microsomal or mitochondrial/bacterial type. P450s can be further classified based on the type of electron transport system used. Up to now 10 different classes of electron transport systems have been described, being the most frequently encountered the class I (bacterial and mitochondrial P450s) and the class II (bacterial and microsomal P450s).^[111] Class I P450 systems consist of three components, a FAD-containing reductase, a ferredoxin and the hemoprotein (P450). In bacterial P450s all three components are soluble, whereas in mitochondrial P450s the hemoprotein and the reductase are bound to the inner mitochondrial membrane, while the ferredoxin is present as a soluble protein in the mitochondrial matrix. Class II P450 systems are composed of two components, the cytochrome P450 and a microsomal NADPH-reductase that contains FAD and FMN. In the microsomal type the two components are membrane-associated. The Class VIII comprises self-sufficient P450s (e.g. P450 BM3 from *Bacillus megaterium*) that share the same components of Class II (reductase and P450 hemoprotein), fused as a single peptide and in soluble form. In microsomal and in self-sufficient cytochrome P450s the electron transfer takes place from NAD(P)H to the heme iron *via* the electron transfer partners.^[107, 111]

The catalytic cycle of P450 monooxygenases have been studied extensively^[109, 112] and an actual representation is shown in Figure 1-8. The porphyrin is embedded in the protein core, through coordination of the ferric iron atom by the thiolate ligand from cysteine, the four equatorial nitrogen ligands and a water ligand in the axial position, proximal to the thiolate (six-coordinated).^[113]

In the substrate free form the iron atom Fe^{III} retains in the low-spin state (I). Upon binding of a viable substrate the axial water ligand is displayed, causing a shift in the ferric spin state from low-spin to high-spin (II). The redox potential of the heme changes, facilitating the transfer of one electron supplied by NAD(P)H from the redox partner to the heme, with concomitant reduction of Fe^{III} to Fe^{II} (III). Dioxygen can bind now leading to formation of the oxy-complex (IV), whereupon a second electron is shuttled to the heme, generating the ferric peroxy complex (V).

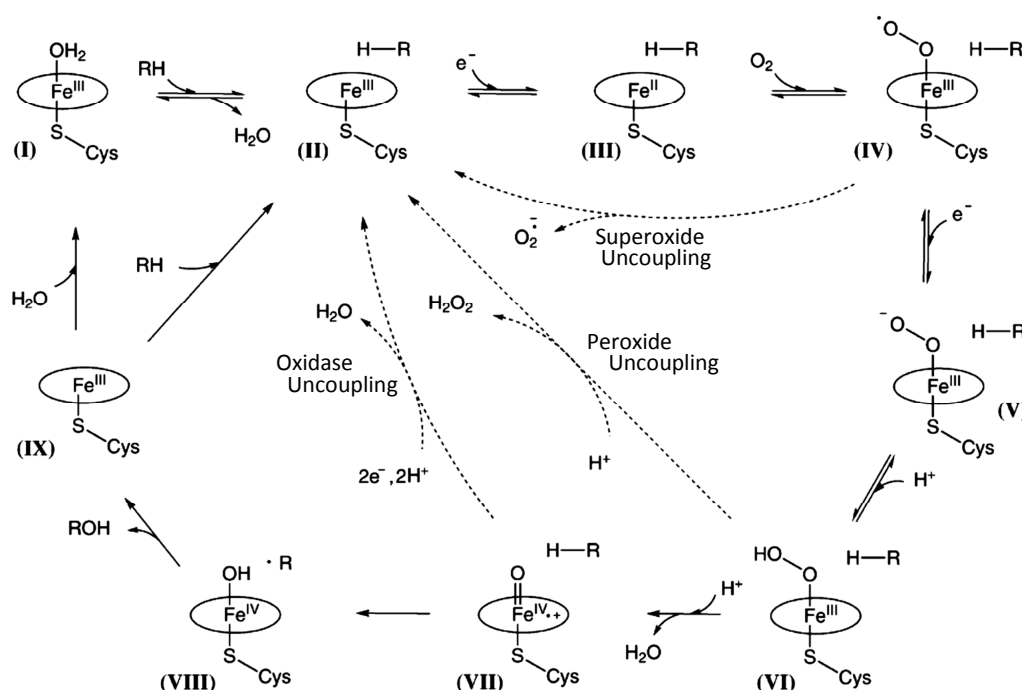


Figure 1-8: The catalytic cycle of P450 Monooxygenases. Since NAD(P)H consumption is not fully coupled to the product formation, possible uncoupling pathways are also depicted (dashed line). Modified from Whitehouse *et al.*^[58]

Protonation of the terminal oxygen atom leads to formation of the hydroperoxy compound (VI, Compound 0). This adduct is protonated a second time triggering the hydrolytic cleavage of the O-O bond and the release of water under generation of the Compound I (VII), which is considered as the active oxygen species responsible for the oxygenation of the substrate. Compound I is two oxidation states above the resting ferric state.^[109] In hydroxylation reactions the substrate is oxygenated *via* a radical rebound mechanism (VIII), where the hydrogen from the substrate is first abstracted ($\text{R}^{\cdot}$; VIII) by compound I. The generated radical

combines with oxygen to form the oxygenated product. The product is then released from the active site allowing the ferric atom to return to the fifth-ligand coordinated resting state (IX).^[58]

Most monooxygenases display a certain degree of uncoupling between the consumption of NAD(P)H as cofactor and the product formation. This means, the consumption of cofactor is not stoichiometric in relation to the product formation. For the natural enzyme's substrate the coupling efficiency is higher, as in the case of P450cam and camphor.^[114] Thus, three different uncoupling reactions can possibly take place at different stages during the catalytic cycle. A hindered binding of dioxygen (IV) can result in the loss of superoxide, probably due to a too slow electron transfer (superoxide uncoupling). If the substrate is loosely bound, water release could be inhibited and protonation of the wrong oxygen atom in the hydroperoxy compound (VI) would lead instead to the release of hydrogen peroxide (peroxide uncoupling). Finally, if the positioning of the substrate is not convenient for the abstraction of hydrogen to take place (VII), the reactive oxygen atom (compound I) will be reduced to water (oxidase uncoupling).^[58, 109, 112a]

Some examples of industrially employed monooxygenase-catalyzed biotransformations are given in Table 1-3. The production of dicarboxylic acids by *Candida tropicalis* is one of the most representative industrial processes catalyzed by a cytochrome P450 enzyme, where a combination of protein and process engineering was applied to generate a tailor-made biocatalyst. This process allows a significantly higher product concentration ($>100 \text{ g L}^{-1}$) and productivity ($1.9 \text{ g L}^{-1} \text{ h}^{-1}$).^[41b]

Table 1-3: Industrial biotransformations with cytochrome P450 enzymes. Adapted from Julsing *et al.*^[41b]

Reaction	Catalyst	Product titer	Company/ Ref
11-Deoxycortisol → Hydrocortisone	P450 _{lin} (<i>Curvulara lunata</i>)	n.a	Pfizer Inc./ Bayer Schering Pharma AG ^[115]
Amorpha-4,11-dien → Artemisinic acid	CYP71AV1 (<i>Saccharomyces cerevisiae</i>)	0.12 g L ⁻¹	Amyris Biotechnologies Inc. ^[116]
Compactin → Pravastin	CYP105A3 (<i>Streptomyces</i> sp. Y-110)	1.0 g L ⁻¹	Sankyo Comp./ Bristol Myers Squibb Comp. ^[40]
<i>n</i> -Tridecane → α,ω -Tridecanedioic acid	CYP52A1 (<i>Candida tropicalis</i>)	166 g L ⁻¹	Cognis Inc. ^[117]

1.3.1 Cytochrome P450 BM3 from *Bacillus megaterium*

Cytochrome P450 BM3 (CYP102A1) was isolated as third P450 from *Bacillus megaterium* by Fulco *et al.*^[118] in the 1980s. The enzyme was expressed as recombinant protein in *E. coli*^[57] and sequenced^[119] some years after identification. P450 BM3 is particularly interesting for *in*

vitro applications and synthetic processes since it is a soluble and redox self-sufficient monooxygenase with the FAD-FMN-reductase and hemoprotein domain on one polypeptide chain.^[57] A representation of this natural P450-reductase fusion system is shown in Figure 1-9. Characterization of the enzyme revealed a molecular weight of 119 kDa, consisting of the 55 kDa heme-domain and the 65 kDa reductase-domain. The reductase consists of two prosthetic flavin groups, FAD and FMN, each of them present in an equimolar ratio.^[57] The natural fusion system offers several advantages over multi-component systems, such as higher expression levels and easier isolation. Besides, through close proximity of the hemoprotein and the reductase partners the electron transfer is very fast, making of P450 BM3 the monooxygenase with the highest reported activities so far.^[58] The electrons are shuttled within one reductase monomer (FAD to FMN) and from the FMN domain of one monomer to the heme-domain of the second monomer. Hence, the catalytically functional and predominant form is a dimer. Dimerization is most likely dependent on the protein concentration.^[120]

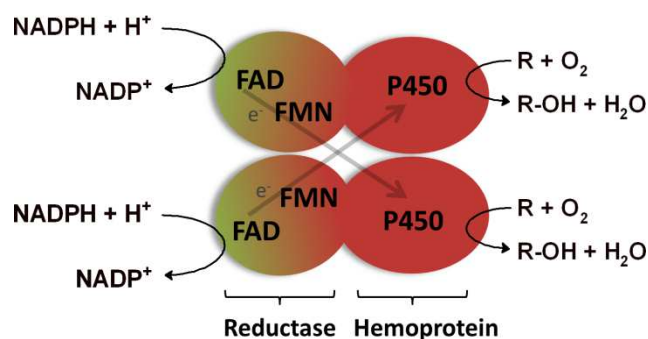


Figure 1-9: Schematic representation of catalytically active cytochrome P450 BM3 dimer. The cytochrome P450 from *Bacillus megaterium* is a soluble and self-sufficient monooxygenase, containing the FAD-reductase and the hemoprotein domain on one polypeptide chain. Adapted from Bernhardt^[107] and Girvan *et al.*^[121]

The physiological role of P450 BM3 is still not fully clear. For many years it was assumed that straight-chain saturated fatty acids (e.g. lauric acid) were the natural substrate of the enzyme. However, these straight-chain fatty acids do not induce the enzymes expression in *B. megaterium*^[122] and their oxidation is not fully coupled to the product formation.^[58] Some studies give evidence on the utilization of branched-chain fatty acids as natural substrates^[123], since they are major components of membrane lipids in bacteria such as *B. megaterium*.^[124] The hydroxylation of linear and branched medium- to long-chain fatty acids (C₁₂-C₂₀) takes place in subterminal positions, at the ω-1, ω-2 and ω-3 carbon atoms, with high turnover frequencies (up to 3500 min⁻¹) and coupling efficiencies between 88 and 98%.^[118a, 118b, 123, 125] Several crystal structures of the P450 BM3 heme-domain in the substrate-free and various

substrate-bound forms (mainly fatty acids as substrate) have been resolved.^[126] Alignment of the two forms demonstrated that significant conformational changes in the hemoprotein take place upon substrate binding. Re-orientation of side-chains from some residues in the active site makes the substrate pocket narrower. These changes reduce the accessibility of solvent molecules (e.g. water) to the active site and alter the ligation of the heme iron. The sixth coordination of the iron to the axial water ligand is lost allowing the binding of molecular oxygen.^[126c] The crystal structure of the heme domain revealed a long and mainly hydrophobic substrate access channel, containing at the entrance the amino acid residues R47 and Y51, which are regarded as gate keeper residues, restricting the entrance of substrates to the active site.^[127] These two residues regulate the binding of fatty acid substrates, mainly by interaction with the carboxyl group of the substrate with R47, and also the accession of water and cosolvents such as DMSO.^[128] Another key residue in P450 BM3 is F87, which is positioned close to the heme iron. In the substrate free structure, F87 is oriented perpendicular to the iron center, but rotates almost 90° to reach close proximity to the iron when the substrate is bound. The displacement of F87 is probably responsible for the loss of the water-iron interaction.^[126c] The crystal structures of the FMN-domain and very recently of the FAD-domain were resolved.^[126a, 129] However, the complete structure of P450 BM3 as fusion of reductase- and hemoprotein-domain is not yet available.

1.3.2 Evolution of cytochrome P450 BM3

The sole identification and isolation of a specific enzyme as biocatalyst for its implementation in a certain synthetic process is not always sufficient. Very often the process requirements are not directly met by the wild type (WT) enzyme.^[130] In this respect, some intrinsic limitations of cytochrome P450 monooxygenases are the rather low stabilities and reaction rates, the cofactor-dependency, and in most cases the lacking specificity for an industrial relevant product.^[131] The development of a tailor-made biocatalyst for industrial applications is nowadays feasible using molecular biology methods combined with a medium or high throughput screening system. In this way, the protein function is manipulated at the amino acid sequence level. Several properties of biocatalysts can be improved using protein evolution techniques and a screening (selection) system. Cytochrome P450 BM3 has been engineered extensively to broaden the substrate spectra, to enhance reaction activities and rates, to increase thermal or process stability, and to accept alternative electron transfer systems.^[58, 132] For this purpose random mutagenesis, as well as focused mutagenesis which comprises rational and semi-rational protein design have been applied effectively.^[132a]

The rational design of proteins is based on a detailed bioinformatic analysis (*in silico*). Therefore, the DNA or amino acid sequence, the protein structure as well as information on structure-function relationships are required. In some cases the amino acid sequence and a good homology model are sufficient for preliminary studies. Using *in silico* analysis the identification of specific amino acid positions for focused mutagenesis is possible. Whole plasmid amplification with mutagenic primers, for example by Site-Directed Mutagenesis (SDM), is the most commonly applied method to introduce site specific mutations of the selected amino acid residues. The main advantage of the rational protein engineering is the generation of rather small libraries, thereby reducing the screening effort. In addition, it allows the rational analysis of the structure-function relationship for a mutated residue. In a similar manner, the semi-rational protein design allows randomization of a selected residue to all 20 possible canonical amino also known as Site-Saturation Mutagenesis (SSM). In this way, the best amino acid exchange at the selected position can be detected.^[133] To limit the size of a library degenerated oligonucleotide primers are commonly employed. NNK-degenerated primers code for a total of 32 codons, covering all 20 amino acids exchanges. NDT-oligonucleotide primers, which code for 12 distinctive amino acids, can also be used for a further reduction of the library size.^[134] To investigate the synergistic effect of neighbored amino acid positions simultaneous saturation can be performed (up to 5 target amino acids), using more recently developed methods such as CASTing^[135], TrimerDimer^[136] or OmniChange^[137].

In contrast to rational design the concept of directed evolution of biocatalysts does not require information on structure and function of the protein. Substitutions of single amino acids are generated randomly by introducing mutations in a target DNA sequence. The main requirements for successful protein evolution are the functional expression of the protein in a microbial host and the availability of a high-throughput screening system to sample the targeted property.^[138] Mutagenesis and screening can thereby be repeated in iterative cycles.^[132a] The direct evolution strategy is illustrated in Figure 1-10.

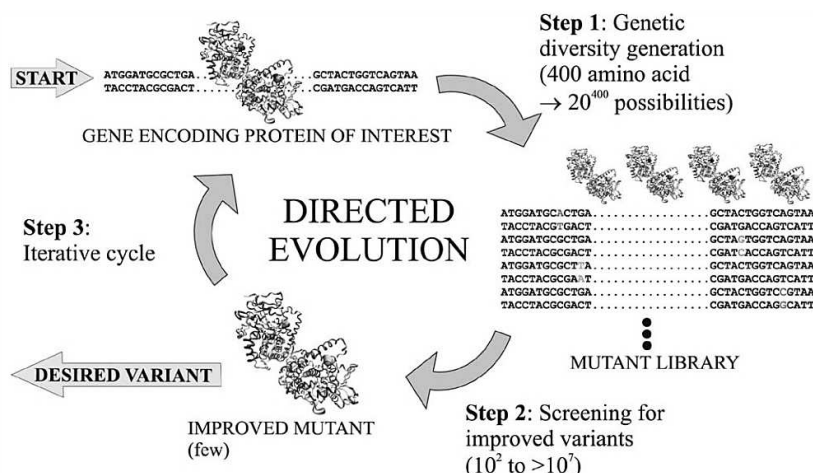


Figure 1-10: Scheme of a directed evolution experiment. Three steps are usually employed during the evolution of a biocatalyst: 1) Generation of diversity, 2) Screening, 3) Iterative cycles of evolution. Taken from Tee and Schwaneberg.^[132a]

A direct evolution experiment comprises three steps^[132a]: 1) generation of genetic diversity by introduction of random mutations, 2) identification of improved variants by means of a reliable screening system and 3) isolation of the improved variant followed by iterative cycles of diversity generation and screening. The accumulation of beneficial mutations can lead to significant improvement of a desired property and also to identification of essential amino acid positions in the protein structure, which are referred to as ‘hot spot’ residues. These residues can be further randomized by SSM to find the best amino acid exchange for a specific residue.^[133] Directed evolution also contributes largely to understand structure-function relationship of enzymes that cannot be explained by rational approaches.^[139] The generation of diversity is most commonly achieved by mutagenesis methods such as error-prone polymerase chain reaction (epPCR).^[140] In an ideal case the introduction of mutations occurs with an even frequency, equal distribution and unbiased introduction of mutations throughout the entire gene sequence.^[139, 141] The commonly employed epPCR method is robust and easy to handle but has the disadvantage of a biased mutational spectrum, displaying a higher frequency of transitions, a lack of consecutive mutations, and high dependency on the degeneracy of the genetic code.^[139, 142] In average, an exchange of only 6 amino acids out of 20 will occur.^[143] To circumvent this limitation the development of alternative methods for diversity generation is ongoing. One example is the Sequence Saturation Mutagenesis method (SeSaM) for randomization of a gene of interest at virtually every amino acid position, independently of the polymerase bias.^[141-142]

Most products that are attractive for an industrial application of P450 monooxygenases are not natural substrates of the enzyme. Therefore, great effort has been applied in enzyme engineering to achieve broader substrate spectra. P450 BM3 variants showing hydroxylation activities towards non-natural substrates as diverse as pharmaceuticals and complex biomolecules, precursors for flavors, fragrances and pheromones, aliphatic hydrocarbons, and aromatic substrates have been evolved in the last two decades.^[58]

Table 1-4: Evolved P450 BM3 variants towards medium- and short-chain alkanes

Variant	Mutagenesis method	Preferred substrate	TTN ^[a]	Features	Ref
139-3	Random/ Recombination	<i>n</i> -heptane	2100	Enlarged hydrophobic pocket, better alkane binding (improved exclusion of substrate from solvent); broadened substrate scope (e.g. terpenes), changed in regioselectivity.	[144]
35E11	DNA-shuffling/ SSM/ Recombination	<i>n</i> -hexane	6350	Restriction and compartmentalization of the active site cavity (better positioning of the alkane chain); reduced stability due to accumulation of mutations.	[145]
19A12	SDM/ Random	propane	10550	Changed conformation of helices (shorter distance between helix F and helix B'), significant gain in catalytic activity towards short-chain alkanes (i.e. propane).	[146]
P450 _{PMO} R2	SSM/ SDM/ Recombination	propane	45800	Further restriction of the substrate channel, exchange to polar residues in the substrate channel; highest activity on alkanes reported so far.	[146]

^[a] TTN: Total Turnover Number ($\mu\text{mol}_{\text{Total product}} \mu\text{mol}_{\text{P450}}^{-1}$).

In particular, P450 BM3 mutants with a changed substrate specificity and selectivity for medium- and short-chain alkanes were successfully engineered.^[59] Some of the engineered P450 BM3 monooxygenase variants and the corresponding substrate preferences are displayed in Table 1-4. The P450 BM3 wild type (WT) enzyme hydroxylates C₆-C₁₀ alkanes with low activities, but does not convert short-chain alkanes (<C₆).^[59] In contrast, the engineered variant 19A12 prefers short chain alkanes (C₃-C₇) as substrates. This variant hydroxylates propane with a NADPH-oxidation rate of 420 min⁻¹ and a total turnover number (TTN) of 10550. Employing *n*-heptane as substrate a TTN of 5360 ± 1020 was reached.^[146] The representation of the heme-domain structure of P450 BM3 in Figure 1-11 highlights a total of 18 amino acid residues in the P450 BM3 WT structure that were exchanged in the heme domain of the 19A12 variant. The substitutions in P450 BM3 variant 19A12 are: R47C, L52I, V78F, A82S, K94I, P142S, T175I, A184V, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, I366V.^[146]

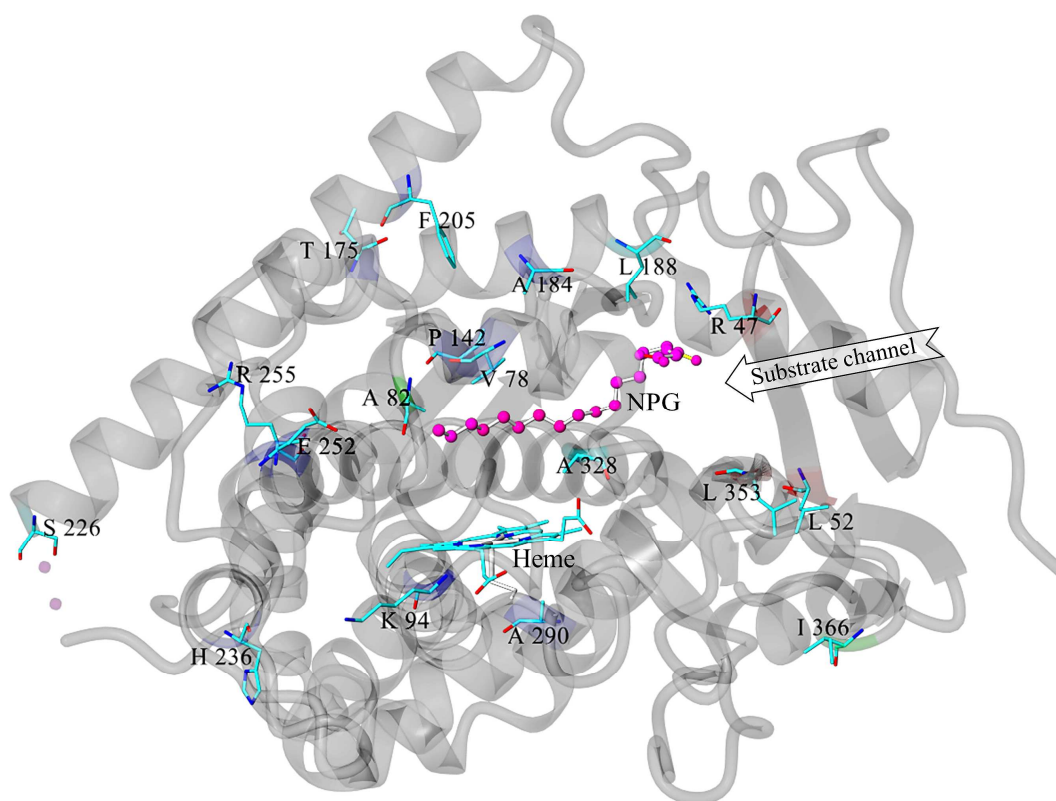


Figure 1-11: Structure of the substrate-bound heme-domain of cytochrome P450 BM3 (PDB code 1JPZ)^[126c]. Eighteen amino acid residues that were mutated in the P450 BM3 variant 19A12^[146] are displayed together with the heme in stick representation. The co-crystallized substrate *N*-palmitoyl glycine (NPG) is shown in pink.

Only two of the mutated amino acid residues, A82S and A328F are directly positioned in the active site cavity. The remaining residues are pointing towards the substrate channel (R47, V78, L188, A184) or are buried within the protein structure. The mutations along the substrate channel and the active site, especially A328F, allow the compartmentalization of the enzymes access channel into an inner heme pocket and a more solvent accessible substrate channel.^[59] The inner heme pocket has thereby a reduced cavity volume.^[59] Furthermore, Fasan *et al.* proposed that the substitution L188P represents the so called ‘turning point’ in the substrate specificity along the P450 BM3 ‘evolutionary lineage’ towards a higher catalytic activity with short-chain alkanes. The variant 19A12 displays only poor activities with long-chain alkanes and fatty acids.^[59, 146]

Engineering of P450 BM3 monooxygenases has also focused on the search for alternative electron transfer systems and alteration of the cofactor specificity, since the use of the wild type enzyme is preferentially towards the NADPH cofactor.^[147] Maurer *et al.* and Neeli *et al.*

identified that mutagenesis of W1046 in the reductase domain of P450 BM3 results in a switch of cofactor specificity from NADPH to NADH.^[147-148] Introducing a second mutation R966D into the variant W1046S showed a more than 100-fold increased k_{cat} value compared to the separately introduced mutations into the WT enzyme.^[149] Figure 1-12 shows the interaction of the two key positions W1046 and R966 with the phosphorylated form of the nicotinamide cofactor NADP⁺.

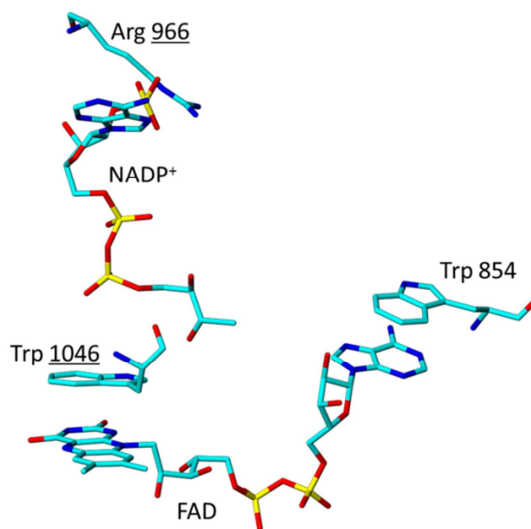


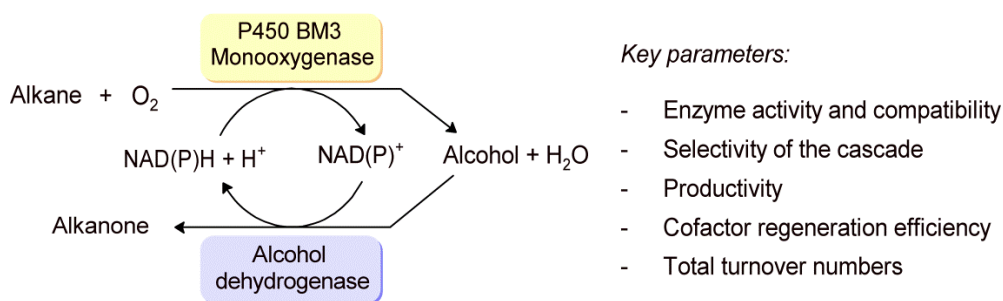
Figure 1-12: Representation of FAD binding to the ligand NADP⁺ in the P450 BM3 FAD/NADPH-binding domain. Some of the amino acid residues that interact with FAD are shown (W854, R966, W1046). The aromatic side chain of W1046 faces the isoalloxazine ring of FAD and acts as a hydrophobic ‘lid’ controlling the electron transfer.^[129] The crystal structure of FAD/NADP⁺-binding domain of P450 BM3 (PDB code 4DQL)^[129] was used for visualization.

The conserved aromatic residue W1046 acts as a hydrophobic ‘lid’ over the FAD in the reductase domain. Upon binding of NADPH the electron transfer will only occur when the side chain of tryptophan moves away.^[129] This ‘gate keeper’ residue is therefore important for discrimination between the phosphorylated and non-phosphorylated cofactors NADPH and NADH.^[147, 150] If W1046 is substituted to alanine, histidine or serine, the aromatic interaction is reduced together with the specificity for NADPH.^[147]

2 Aim of the Thesis

Selective oxyfunctionalization of alkanes remains a major challenge in the field of organic chemistry. In this regard, the implementation of biocatalytical processes under mild reaction conditions allows efficient and environmentally sustainable solutions in the oxidative chemistry.

The main goal of this study is the development and the validation of a double oxidation cascade making use of monooxygenases for oxyfunctionalization of alkanes in cell free and whole cell biotransformation processes according to the reaction Scheme 1.



Scheme 1: Biocatalytic direct oxidation of alkanes to alkanones.

The proposed double oxidation concept (Scheme 1) represents a novel synthesis route to obtain alkanones and/or chiral alcohols directly from non-activated alkanes in a regio and stereo selective manner. In contrast to most chemical oxyfunctionalization reaction, the use of biocatalysts allows introducing the desired selectivity in the catalytic process. A comprehensive validation of this synthesis concept will contribute to the development of an emerging technology platform, based on the application of multi-enzymatic redox processes.

A P450 monooxygenase from *Bacillus megaterium* (P450 BM3), catalyzing the initial hydroxylation of the alkyl chain, and an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) for a subsequent oxidation of the *in situ* produced alcohol were selected to perform the catalytic reaction. *n*-Heptane was selected as model compound for the preliminary evaluation of the described double oxidation concept.

P450 BM3 monooxygenase has an intrinsically lower activity than most alcohol dehydrogenases. Therefore, the P450-catalyzed hydroxylation of the alkane could be the rate limiting step in the coupled oxidation reaction. The dependency of monooxygenase-catalyzed

reactions on the regeneration of the nicotinamide cofactor is a further constraint for a biocatalytic application. In the described concept, the use of an alcohol dehydrogenase for the second oxidation step can afford simultaneously an enzyme coupled regeneration of the cofactor NAD(P)H. By means of protein engineering, fine tuning of the monooxygenase to achieve improved catalytic activity at a low mutational load was intended. The regio- and enantioselectivity as well as the cofactor compatibility of the P450 BM3 enzyme should be engineered to meet the requirements of the multi-enzymatic cascade process. In this way, a suitable cofactor regeneration strategy will be developed. A detailed characterization of the engineered monooxygenases as well as a thorough evaluation of the coupled oxidation reaction in terms of product formation and yields will give better insight into the process performance and the potential for optimization of the oxidation cascade.

Essential parameters that affect the catalytic performance and the product yields such as catalyst or substrate concentration and cofactor regeneration capacity should be investigated and optimized. By comparing the performance of *in vitro* and *in vivo* biotransformations, the catalytic efficiency of the system can be evaluated and further optimized for key parameters such as product yields and turnover numbers. Furthermore, a proof of the broad applicability of the synthesis concept should be pursued by expanding the system to other enzymatic components (e.g. other alcohol dehydrogenases) and other substrate types.

3 Experimental

3.1 Chemicals, reagents and kits

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), New England Biolabs (Frankfurt, Germany) and Sigma Aldrich (Steinheim, Germany). All employed HPLC-purified oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). The kits “NucleoSpin Plasmid kit” and “NucleoSpin Gel and PCR Clean-up” for purification of plasmid DNA and amplified DNA were purchased from Macherey Nagel (Düren, Germany).

3.2 Devices

5 L-Bioreactor Biostat MD	Sartorius, Göttingen, Germany
Äktaprime plus/ UV detector	GE Healthcare, München, Germany
Bench top shaker Certomat	Sartorius, Göttingen, Germany
BioPhotometer Plus	Eppendorf, Hamburg, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Centrifuge rotors A-4-81, A-4-62-MTP	Eppendorf, Hamburg, Germany
Centrifuge Sorvall RC-6	Thermo Fischer Scientific, Wilmington, USA
CO-lecture bottle station	Sigma Aldrich, Steinheim, Germany
Drying cabinet FP 53	Binder GmbH, Tuttlingen, Germany
DNA electrophoresis chamber	Bio-Rad, München, Germany
Evaporator IKA RV 10 basic	IKA-Werke, Staufen, Germany
GC-FID 2010 and 2010-Plus	Shimadzu GmbH, Duisburg, Germany
GC-MS QP2010	Shimadzu GmbH, Duisburg, Germany
Gel documentation U:Genius	Syngene, Cambridge, UK
Glass column TAC15/125PE5-AB-2	YMC Europe GmbH, Dinslaken, Germany
High Pressure Homogenizer	Avestin, Ottawa ON, Canada

Lyophilizer Alpha 1-2 LD plus	Christ, Osterode am Harz, Germany
Microtiter plate reader Tecan Sunrise	Tecan Group AG, Männendorf, Switzerland
Microtiter plate shaker Microtron	INFORS, Heinsbach, Germany
Mini-Protean Tetra Cell system	Bio-Rad, München, Germany
Multipoint magnet stirrer Variomag 15	Thermo Fischer Scientific, Wilmington, USA
NanoDrop Spectrophotometer 1000	Thermo Fischer Scientific, Wilmington, USA
PCR thermocycler Mastercycler pro S	Eppendorf, Hamburg, Germany
Polarimeter 241 MC	Perkin Elmer, Waltham, USA
Power supply Consort EV265	Consort bvba, Turnhout, Belgium
Sonicator Vibra-Cell VCX130	Sonics & Materials, Newton, USA
Spectrophotometer Varian Cary 50 UV	Agilent Technologies, Darmstadt, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Vortex Genie 2	Scientific Industries Inc., New York, USA

3.3 Culture media, additives and buffers

All media were sterilized by autoclaving at 120 °C for 20 min and were stored at room temperature until further use. The media additives and the antibiotics were sterilized by filtration through a 0.2 µm sterile polyvinylidene difluoride (PVDF) filter (Carl Roth, Karlsruhe, Germany) and stored at -20 °C if not stated otherwise.

LB medium (Lysogeny Broth) contained 10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl; pH 7.5 adjusted with 1 M HCl or NaOH. For the preparation of agar plates the medium contained additionally 20 g L⁻¹ agar-agar. The medium was supplemented with the appropriate antibiotic depending on the employed *E. coli* strain (refer to Table 3-2 for plasmids and antibiotic resistance).

SOC medium (Super Optimal broth with Catabolite repression) contained 20 g L⁻¹ tryptone, 2 g L⁻¹ NaCl, 5 g L⁻¹ yeast extract and 2.5 g L⁻¹ KCl. 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose were added as a sterile solution after autoclaving.

TB medium (Terrific Broth) contained 12 g L⁻¹ peptone, 24 g L⁻¹ yeast extract, 4 g L⁻¹ glycerol (solution A; 800 mL), 2.31 g L⁻¹ KH₂PO₄ and 12.54 g L⁻¹ K₂HPO₄ (solution B; 200 mL). The solutions A and B were prepared separately and mixed together after autoclaving. The following medium additives were used for expression of P450 BM3 monooxygenases: Trace Elements Solution (0.64 µM CuSO₄, 0.69 µM ZnSO₄, 0.72 µM MnSO₄, 0.76 µM CoCl₂, 59.43

μM $\text{Na}_2\text{-EDTA}$, $61.79 \mu\text{M}$ FeCl_3 ; stored at 4°C after autoclaving and sterile filtration), 5-Aminolevulinic acid hydrochloride (ALA; 0.5 mM), Isopropyl- β -D-thiogalactopyranoside (IPTG; 0.1 mM), Thiamine hydrochloride (0.1 g L^{-1}) and the respective antibiotic (see Table 3-2). The additives were added to the TB medium from a 1000x concentrated stock solution.

***E. coli* Minimal Medium** (EMM) contained 7 g L^{-1} K_2HPO_4 , 3 g L^{-1} KH_2PO_4 , 0.57 g L^{-1} sodium citrate dihydrate, 0.205 g L^{-1} MgSO_4 heptahydrate, 1 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, 50 mM Tris (pH 7.4).

Potassium phosphate buffer (KPi-buffer) was prepared in a concentration of 0.1 M and varying pH-values (7.0, 7.5 and 8.0) using stock solutions of 1 M K_2HPO_4 and 1 M KH_2PO_4 . The stock solutions were stored at 4°C until further use.

Tris/HCl buffer for purification of P450 BM3 monooxygenase contained 0.1 M Tris/HCl, pH 7.8 (12.11 g L^{-1} Tris, pH adjusted by titration with 37% (v/v) HCl). **Tris/HCl elution buffer** contained additionally 1 M NaCl. The buffers were freshly prepared and filtered using a glass filter.

3.4 Strains, vectors and preparation of chemically competent cells

The strains used in this work (Table 3-1) are *E. coli* DH5 α for sub-cloning and storage, and *E. coli* BL21 Gold (DE3) and *E. coli* BL21 Gold (DE3) lacI^{Q1} for recombinant gene expression. The vectors used in this work are listed in Table 3-2.

Table 3-1: Strains and their genotype

Strain	Genotype	Origin/ Ref
<i>E. coli</i> DH5 α	F- $\phi 80\text{lacZ}\Delta\text{M15 } \Delta(\text{lacZYA-argF})\text{U169 recA1 endA1 hsdR17(rk-, mk+)} \text{ phoA supE44 thi-1 gyrA96 relA1 } \lambda\text{-}$	[151]
<i>E. coli</i> BL21Gold (DE3)	F- $\text{ompT gal dcm lon hsdSB(rB- mB-)} \lambda(\text{DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]})$	Novagen [152]
<i>E. coli</i> BL21Gold (DE3) lacI^{Q1}	Same as <i>E. coli</i> BL21Gold (DE3); additionally pGRG36- lacI^{Q} was employed for site-specific integration of <i>lacI</i> into the chromosome	[153]

Chemically competent *E. coli* cells for plasmid transformation were prepared based on a standard protocol^[154] with some modifications. In the first washing step cells were resuspended in TFB-solution 1 (30 mM potassium acetate, 50 mM MnCl_2 , 100 mM RbCl_2 , 10 mM CaCl_2 , 15% (w/v) glycerol; pH 6.8), in the second washing step cells were resuspended in TFB-

solution 2 (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15% (w/v) glycerol; pH 6.8). With this method a transformation efficiency of 5×10^6 - 1×10^7 cfu μg^{-1} pUC19 was yielded.

Table 3-2: Vectors for recombinant protein expression

Vector	Promoter	Antibiotic resistance/ Concentration	Replicon	Origin/ Ref
pET28a(+)	T7	Kanamycin (Kan)/ 50 $\mu\text{g mL}^{-1}$	ColE1 (pBR322)	Novagen (Merck) ^[154]
pALXtreme-1a	T7	Kanamycin (Kan)/ 50 $\mu\text{g mL}^{-1}$	ColE1 (pBR322)	Derived from pET28a(+), ΔlacI^Q ^[153]
pACYCDuet-1	T7	Chloramphenicol (Cm)/ 34 $\mu\text{g mL}^{-1}$	P15A (pACYC184)	Novagen (Merck)
pKA1	T7	Chloramphenicol (Cm)/ 34 $\mu\text{g mL}^{-1}$	P15A (pACYC184)	Derived from pACYC18 ^[155]

3.5 Molecular biology methods

3.5.1 Cloning

Two different cloning techniques were used for construction of the protein expression vectors for the single expression and the coexpression of the enzymes used in this study (P450 BM3 monooxygenases and RE-ADH).

For **restriction cloning** endonucleases from Fermentas (St. Leon-Rot Germany) or New England Biolabs (Frankfurt, Germany) were used according to manufacturer instructions. Insert and vector DNA (1-2 μg) were cut for 1 h with 1 U of the corresponding restriction endonuclease. The linearized DNA fragments were purified either directly with a DNA clean up kit or if necessary by gel extraction and subsequent clean up (NucleoSpin[®] Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany). In case of the pACYCDuet-1 vector additional incubation of 60 min at 37 °C with 0.5 U of alkaline phosphatase (Calf Intestinal Phosphatase, New England Biolabs, Frankfurt, Germany) per μg vector DNA was performed to reduce background by removal of the 5'phosphate groups. The ligation of insert and vector DNA in a molar ratio 1:10 was performed using T4 DNA Ligase (Fermentas, St. Leon-Rot Germany) following the suppliers recommendations (incubation of 1 h at RT or overnight at 16 °C). The ligation reaction (4 μL) was subsequently transformed into chemically competent *E. coli* cells (chapter 3.5.2).

As alternative to restriction cloning the ligase-independent cloning method **PLICing** (Phosphorothioate-based ligase-independent gene cloning) was used.^[153] In the first step the vector backbone and the target gene were amplified in a polymerase chain reaction (PCR), using oligonucleotide primers containing twelve complementary phosphorothioated nucleotides and a high-fidelity DNA polymerase. A standard PCR contained the template DNA (10-30 ng), 1 U Phusion DNA polymerase, 0.2 mM dNTPs, 1 x Phusion high fidelity buffer and 0.2 μ M of each primer in 50 μ L of final volume. Following temperature program was used for amplification in a PCR cycler Mastercycler pro S (Eppendorf, Hamburg, Germany): initial denaturation at 98 °C for 2 min, 25 cycles of denaturation at 98 °C for 30 s, annealing at 50-60 °C for 1 min and elongation at 72 °C for 1 min kb^{-1} . A final elongation step at 72 °C for 5 min was performed. The annealing temperature was chosen according to the employed primers as listed in Table 6-1. Subsequently, the PCR products were analyzed by agarose gel electrophoresis and purified using a PCR cleanup kit. Agarose gel electrophoresis was performed as described by Sambrook and Russell^[156] using a 0.8% (w/v) agarose gel and Roti-Safe GelStain from Carl Roth (Karlsruhe, Germany) for visualization of DNA. For identification of DNA size the GeneRuler 1 kb DNA Ladder from Fermentas (St. Leon-Rot, Germany) was used. The template DNA was digested by incubation at 37 °C for 16 h using 20 U of *DpnI* endonuclease.

In the second step chemical cleavage of the introduced phosphorothioated nucleotides was achieved by incubation of the amplified DNA with an iodine/ethanol solution. The cleavage reaction contained 0.12 pmol vector DNA or 0.2 pmol insert DNA, 6 mM iodine, 6% (v/v) ethanol, 50 mM Tris-HCl buffer (pH 9.0) in a final volume of 5 μ L. The reaction mix was incubated for 5 min at 70 °C. In the last step, the hybridization of the linearized vector and insert DNA is performed by mixing equal volumes of the cleavage reactions (typically 5 μ L of vector and 5 μ L of insert) prior to incubation at RT (25 °C) for 5 min. The hybridization reaction (commonly 4 μ L) was subsequently transformed into chemically competent *E. coli* cells (chapter 3.5.2).

3.5.2 Transformation of DNA into chemically competent *E. coli* cells

For transformation of DNA into chemically competent *E. coli* cells, 4 μ L of the ligation or hybridization reaction or 30 to 50 ng of plasmid DNA were employed. The DNA was added to 100 μ L frozen competent *E. coli* cells followed by incubation for 20 min on ice. DNA uptake was achieved by heat-shock at 42 °C for 45 s, followed by incubation for 2 min on ice. For regeneration 0.9 mL SOC medium were added to the cells followed by incubation at 37 °C for

45 min and 250 rpm. The cells were spread on LB agar plates containing appropriate antibiotics. The plates were incubated at 37 °C overnight.

3.5.3 Verification of clones

The obtained clones after transformation were verified by colony PCR or by linearization of the isolated plasmid DNA using appropriate restriction endonucleases (following the supplier instructions) and subsequent visualization by agarose gel electrophoresis as described in chapter 3.5.1. For colony PCR a single colony was picked from the agar plate, resuspended in 15 µL ddH₂O and incubated at 98 °C for 15 min to achieve cell lysis and solubilization of plasmid DNA. The PCR mixture contained 5 µL of colony DNA, 5 U of *Taq* DNA polymerase, 0.2 mM dNTPs, 1x *Taq* buffer and 0.4 µM each forward and reverse primer in 15 µL. Following temperature program was used: initial denaturation at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 1 min kb⁻¹. After cyclic amplification a final elongation at 72 °C for 10 min was performed.

3.5.4 Construction of heterologous protein expression strains

3.5.4.1 Expression of P450 BM3 monooxygenases and alcohol dehydrogenases

Recombinant gene expression of the employed P450 BM3 monooxygenase variants was performed using the pET28a(+)-derived pALXtreme-1a vector^[153], which is a smaller version of the pET vector lacking the *lacI* gene (appendix, Figure 6-1). To reconstitute regulation of the *lac*-operon the *E. coli* strain BL21 Gold (DE3) *lacI*^{Q1} was used. An overview of the generated strains is shown in the appendix section (**Error! Reference source not found.**).

The cytochrome P450 BM3 wild type (WT) gene from *B. megaterium* (CYP102A1) was cloned into pALXtreme-1a by PLICing (see chapter 3.5.1). Phosphorothioated oligonucleotide primers were used for this purpose (see Table 6-1, Nr. 1-4). For construction of the P450 BM3 19A12 expression vector the heme-domain of the P450 BM3 19A12 variant^[146] was purchased as synthetic DNA (Life Technologies, Darmstadt, Germany) and subcloned by restriction cloning (chapter 3.5.1) using endonucleases *NcoI* and *MssI* (*PmeI*) into the pALXtreme-1a vector harboring the P450 BM3 WT gene, replacing the wild type heme domain by the synthetic 19A12 heme domain. The P450 BM3 variant CM1 was generated by SSM and screening (chapter 3.5.7 and 3.9) using oligonucleotide primers for position R255 (Nr. 13,

Table 6-4). The P450 BM3 mutants containing the single mutations R255P and P329H were constructed using SDM and the corresponding primers (Nr. 1-4, Table 6-3).

The expression vector pKA1, harboring the (S)-alcohol dehydrogenase from *Rhodococcus erythropolis* (*R. erythropolis*, RE-ADH)^[157], and the (R)-alcohol dehydrogenase gene from *Lactobacillus brevis* (*L. brevis*, Lb-ADH)^[158] were kindly provided by Prof. Dr. W. Hummel (Institute of Molecular Enzyme Technology, H.-H.-University, Research Centre Jülich, Germany). The Lb-ADH gene was cloned into the expression vector pKA1 by restriction cloning (chapter 3.5.1) using the restriction sites *NdeI* and *BamHI*. The expression vectors were transformed into chemically competent *E. coli* BL21 Gold (DE3) cells (vector map in appendix, Figure 6-2).

3.5.4.2 Construction of NADH-dependent P450 BM3 mutants

To switch cofactor specificity of P450 BM3 variants the mutations T1046S and R966D were introduced in the P450 BM3 genes WT, 19A12 and CM1 by SDM (chapter 3.5.6) using the corresponding forward and reverse primers (Nr. 5-8, Table 6-3).

3.5.4.3 Coexpression strategies for P450 BM3 and ADHs

Three different coexpression strategies for the heterologous expression of P450 BM3, RE-ADH and Lb-ADH in the same *E. coli* host were tested. An overview of the generated coexpression strains is given in the appendix section (Table 6-8). The first strategy consisted of transformation of two plasmids in one *E. coli* strain. For expression of two proteins, the plasmids pALXtreme-1a, harboring the corresponding P450 BM3 gene and pKA1, harboring the RE-ADH or the Lb-ADH gene were chosen. For expression of three proteins, the pALXtreme-1a plasmid containing the operon-fusion P450 BM3-RE-ADH (see below) and the pKA1 plasmid containing the Lb-ADH were used (see vector maps, appendix Figure 6-1 and Figure 6-2). Chemically competent *E. coli* BL21(DE3) Gold lacI^{Q1} cells were transformed with 50 ng of each plasmid simultaneously and were spread on agar plates containing both antibiotics (Chloramphenicol, Kanamycin) for selection.

The second strategy is based on the coexpression using the commercial vector pACYCDuet-1 from Merck (Darmstadt, Germany), which contains two multiple cloning sites (MCS; vector map, appendix Figure 6-3).^[159] Cloning of both target genes was performed by restriction cloning (chapter 3.5.1). First, the RE-ADH gene was cloned in the multiple cloning site MCS2 using the restriction sites *NdeI* and *EcoRV*. The restriction site *EcoRV* was introduced at the

end of the RE-ADH gene-sequence by PCR using oligonucleotide primers containing the corresponding mutation (Nr. 1-2, Table 6-2). The PCR contained 40 ng pKA1 RE-ADH as template, 1 U of Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.4 μ M each forward and reverse primer in a final volume of 50 μ L. Following temperature program was used: initial denaturation at 98 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 57 °C for 30 s and elongation at 68 °C for 30 s, as well as a final elongation at 72 °C for 10 min. The resulting pACYCDuet-1 plasmid containing the RE-ADH was used for cloning of the P450 BM3 monooxygenase variants into the multiple cloning site MCS1 using restriction sites *EcoRI* and *NcoI*, followed by transformation into *E. coli* BL21 (DE3) Gold cells.

The third coexpression strategy is based on the generation of a synthetic operon-fusion for simultaneous expression of both proteins from a single T7 promoter in the pALXtreme-1a vector. Construction of the synthetic operon-fusion was performed using the PLICing methodology. Hence, four phosphorothioated oligonucleotide primers (No. 5-8, Table 6-1) were design for amplification of the RE-ADH gene (smaller fragment; insert DNA), and the P450 BM3 monooxygenase gene together with the pALXtreme-1a vector backbone (larger fragment; vector DNA). A schematic representation of the generated operon-fusion containing plasmid is shown in Figure 3-1.

By means of the forward primer for the RE-ADH gene (RE-ADH Fwd_Op_PTO) a second ribosome binding site containing the Shine-Dalgarno sequence (SD: UAAGGAGGU)^[160] and an optimal sequence spacing^[161] between SD and translational initiation (ATG) of 5 nucleotides was introduced. PLICing was performed as described in chapter 3.5.1 with some modifications.

The vector pALXtreme-1a P450 BM3 was used as template DNA for amplification of the vector backbone. Prior to amplification the plasmid was linearized by digestion using the *EcoRI* restriction site. RE-ADH was amplified directly from the circular pKA1 plasmid. A two-step PCR was used as described in chapter 3.5.6. In the first step, 5 cycles of linear amplification with one single primer were carried out with an elongation time of 9 min for P450 BM3 and of 2 min for RE-ADH). For the second exponential amplification step with both primers 25 cycles and an elongation time of 6 min for P450 BM3 and 1 min for RE-ADH were used. The resulting amplified products for PLICing are shown in the appendix section (Figure 6-7).

PLICing, transformation in chemically competent *E. coli* BL21(DE3) Gold lacI^{Q1} cells and verification of the generated coexpression clones was performed as previously described in chapter 3.5.2.



Figure 3-1: Plasmid map of the expression vector operon-fusion pALXtreme-1a P450 BM3-RE-ADH. Nucleotide sequences containing the PTO-primer annealing sites for PLICing (Phosphorothioate-based ligase-independent gene cloning) are indicated below (red). Generation of an operon-fusion between P450 BM3 and RE-ADH is achieved by introducing a second ribosome binding site between both gene, consisting of the Shine-Dalgarno (SD) sequence and a spacing of 5 nucleotides between SD and translation initiation codon (ATG).

3.5.5 DNA sequencing

The generated plasmids for single expression and coexpression of recombinant proteins used in this work were verified by sequencing of the target genes. Isolation and purification of plasmid DNA was performed using the NucleoSpin Plasmid kit from Macherey Nagel (Düren, Germany) following the supplier instructions. Concentration of DNA was determined with the NanoDrop spectrophotometer 1000 (Thermo Scientific, Wilmington, USA). Plasmid DNA

containing P450 BM3 was sent for sequencing at Eurofins MWG Operon (Ebersberg, Germany) using appropriate oligonucleotide primers (sequencing primers Nr. 1 to 4, appendix Table 6-6). Sequencing of SSM clones of P450 BM3 was done partially covering gene fragments containing the targeted codon. In this case sequencing was performed in 96-well MTPs at GATC Biotech AG (Konstanz, Germany) using primers Nr. 1 or 2 (appendix Table 6-6).

3.5.6 Site-Directed Mutagenesis

The introduction of point mutations in the target genes was achieved using a modified two-stage^[162] Site-Directed Mutagenesis (SDM) protocol based on the QuikChange SDM System developed by Stratagene (La Jolla, CA).

In the first PCR-step linear amplification of the target DNA with a single oligonucleotide primer is performed (forward or reverse), avoiding in this way primer dimerization. In the second PCR-step equal parts of amplified products from the first step are mixed together followed by exponential DNA amplification. Both forward and reverse oligonucleotide primers containing the desired mutation were designed based on the recommendations of the QuikChange Site-Directed Mutagenesis manual. The employed oligonucleotide primers are listed in the appendix section, Table 6-3.

The PCR mixture for SDM contained template DNA (10-20 ng), 1 U Phusion DNA polymerase, 0.2 mM dNTPs, 1x Phusion buffer and 0.2 μ M forward or reverse primer in 50 μ L final volume. Following temperature program was used for the first amplification step: initial denaturation at 98 °C for 2 min, followed by 3 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min kb⁻¹. Subsequently, 25 μ L of forward and 25 μ L of reverse reaction were mixed together and 0.5 U of Phusion DNA polymerase was added. The second amplification step was analogous to the first one, using 15 amplification cycles instead of 3 and followed by a final elongation step at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis and purified using a PCR cleanup kit. Template DNA was digested by addition of 20 U of DpnI endonuclease followed by incubation at 37 °C for 16 h. The mutated plasmid (30 to 50 ng) was transformed into chemically competent cells (chapter 3.5.2).

3.5.7 Site-Saturation Mutagenesis

Site-saturation Mutagenesis (SSM) is used to introduce a randomized codon at a specific amino acid position in the gene of interest. The employed procedure is analogue to SDM except for the use of so called wobble primers, which contain instead of a single point mutation a randomized (wobble) codon. Degenerated NNK-oligonucleotides were used, where N codes for G, A, T and C, and K codes for G and T, enabling the introduction of all 32 base triplets into a gene sequence. The targeted codon is therefore randomized to all 20 canonical amino acids. The design of oligonucleotide primers and the mutagenic procedure were performed as described for the SDM protocol (chapter 3.5.6).

The oligonucleotide primers used for generation of randomized mutant libraries of P450 BM3 wild type (WT) as starting template are listed in Table 6-4. Employing P450 BM3 variant 19A12 as template the primers in Table 6-5 were used for SSM. In both cases the smaller vector pALXtreme-1a was used, allowing for a higher transformation efficiency and higher number of obtainable colonies for generation of the mutant library. The mutated plasmid (30 to 40 ng) was transformed into competent *E. coli* BL21 (DE3) Gold lacI^{Q1} cells, following cultivation on agar plates containing kanamycin.

3.5.8 Error-prone PCR using P450 BM3 variant 19A12 as template

Error-prone PCR (epPCR) is a modified version of the standard PCR procedure to increase the natural error rate of the employed DNA polymerase, typically a *Taq* DNA polymerase. One possible way to enhance the error rate is to supplement the PCR with altered concentrations of MnCl_2 , modified dNTPs ratios or the use a highly error-prone polymerase.^[163]

In this study varying concentrations of MnCl_2 (0, 10, 20, 40 and 60 nM) were tested, along with the use of an optimized error-prone-*Taq* DNA polymerase, to achieve an inactivation ratio of 50%. The mutation frequency in an epPCR library can be estimated on the basis of the inactivation ratio (fraction of inactive clones) by sampling a small number of clones, commonly 90 clones in a MTP. In previous experiments an inactivation ratio of 50% showed to be beneficial when screening for enhanced activity of P450 BM3 monooxygenases.^[164] Therefore, an inactivation ratio of 50% was pursued to identify clones with improved catalytic performance.

Phosphorothioated oligonucleotide primers 1 and 2 (appendix Table 6-1) were used for amplification of the target gene DNA (P450 BM3 19A12) and primers 3 and 4 (appendix Table 6-1) for vector DNA (pALXtreme-1a). The epPCR mixture (50 μL) for amplification of the

P450 BM3 gene contained 30 ng of template DNA, 5 U *epTaq* DNA polymerase, 0.2 mM dNTPs, 1x *epTaq* buffer, 0-60 nM MnCl_2 and 0.4 μM each forward and reverse primer. Following temperature program was used: initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 68 °C for 1.5 min. After exponential amplification, a final elongation at 68 °C for 10 min was performed. The vector DNA was amplified under the same conditions, in the absence of MnCl_2 and using the high fidelity Phusion DNA polymerase (1 U) instead of the *epTaq* DNA polymerase. Cloning and transformation of the randomly mutated gene for generation of a mutant library was carried out using PLICing as described in chapter 3.5.1 and 3.5.2.

3.6 Cultivation of *E. coli* for heterologous protein expression in shake flasks

3.6.1 Expression of recombinant P450 BM3 monooxygenases

The cultivation of recombinant *E. coli* strains for heterologous expression of the different P450 BM3 monooxygenase variants took place in a volume of 50 to 400 mL, using 0.25 to 2 L Erlenmeyer flasks. A preculture of 4 mL LB medium, supplemented with the respective antibiotic (Table 3-2), was prepared by inoculation from a 25% (v/v) glycerol stock of the corresponding strain and subsequent incubation at 37 °C and 250 rpm for 16 h overnight. The main culture in TB medium (see chapter 3.3), supplemented with the respective antibiotic and trace elements was inoculation with 1% (v/v) preculture and was incubated at 37 °C and 250 rpm until an OD_{600} of 0.8 to 1 was reached (typically after 2.5 h). Protein expression was induced by addition of 0.1 mM IPTG, 0.5 mM ALA and 0.1 g L^{-1} thiamine and the flask was further incubated at 30 °C and 250 rpm for 20 h.

In the course of this study optimization of the expression temperature was conducted and a temperature of 25 °C was selected for enhanced expression of P450 BM3 monooxygenase variants. *E. coli* cells were harvested by centrifugation (10 min, 4500 g at 4 °C) and the cells pellets were washed in 0.1 M KPi buffer (pH 8.0) if not stated otherwise. The cell pellets were stored at -20 °C until further use.

3.6.2 Expression of recombinant RE-ADH

The RE-ADH was expressed and partially purified according to a published protocol^[155] with minor modifications. An *E. coli* BL21 Gold (DE3) pKA1 RE-ADH preculture was prepared in LB medium containing chloramphenicol (Cm; 34 $\mu\text{g mL}^{-1}$; 37 °C, 250 rpm, 16 h overnight).

The main culture (50 mL LB medium/Cm) was inoculated with 1% (v/v) of the preculture and was cultivated at 37 °C and 250 rpm. After reaching an OD₆₀₀ of 0.5 the expression was induced by addition of 25 µM IPTG and 1 mM ZnCl₂ followed by cultivation for 20 h at 30 °C and 150 rpm. Cells were harvested by centrifugation (10 min, 4500 g at 4 °C) and the cell pellet was frozen until further use.

3.6.3 Coexpression of P450 BM3 monooxygenase and alcohol dehydrogenase

For coexpression of two proteins, a P450 BM3 monooxygenase and an alcohol dehydrogenase (RE-ADH or Lb-ADH), or coexpression of three proteins, the P450 BM3 and two ADHs in one strain (RE-ADH and Lb-ADH), the established protocol for expression of P450 BM3 was used (chapter 3.6.1), taking into account the addition of the corresponding antibiotics. The coexpression strains used in this study, together with the corresponding antibiotic resistance are summarized in the appendix section (Table 6-8). In addition, if the RE-ADH gene was present in the employed genetic construct, the main culture in TB-medium was supplemented with 1 mM ZnCl₂ directly after induction of the expression with IPTG.

3.7 Cultivation of *E. coli* for heterologous expression in Microtiter Plates

3.7.1 Expression of a P450 BM3 monooxygenase mutant library

Generation of mutant libraries for activity screening was carried out by cultivation of a single clone in each well of a flat bottom 96-well microtiter plate (MTP) (Greiner bio-one, Frickenhausen, Germany) after the mutagenic procedure (SSM or epPCR).

First a master plate was generated, which serves for long term storage and for inoculation of expression cultures. In each well of the MTP 150 µL LB medium, supplemented with kanamycin (50 µg mL⁻¹) were pipetted. Single colonies were picked from a transformation agar plate using sterile toothpicks and were transferred to the corresponding well for inoculation. The MTP was cultivated for 16 h in a MTP-shaker (Microtone INFORS, Heinsbach, Germany) at 37 °C, 900 rpm and 70% air humidity. After cultivation 100 µL of 50% (v/v) glycerol were added to each well for storage of the plate at -80 °C. As reference samples the *E. coli* strains containing the template plasmid pALXtreme-1a P450 BM3 WT or P450 BM3 19A12 (starting activity control) and the empty vector (no activity control) were cultivated in triplicate in the same MTP. When the WT enzyme was used as starting material for mutagenesis the variant

19A12 served as positive control. A schematic representation of the layout of a master plate is shown in Figure 3-2.

In total 2 plates with 174 clones each per saturated amino acid position were generated. The randomized library statistics were calculated using the web-interface GLUE-IT (<http://guinevere.otago.ac.nz/cgi-bin/aef/glue-IT.pl>).^[165] Using a library size of 174 clones and NNK-degenerated primers a completeness of 99.76% and a probability of 95.3% for all amino acids to be present in the library is achieved.

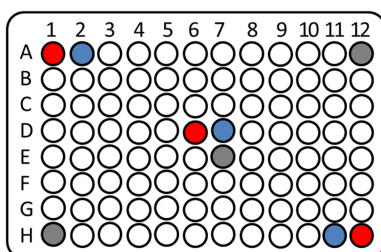


Figure 3-2: Layout of a master plate for cultivation and long term storage of a P450 BM3 mutant library. The reference samples P450 BM3 WT (red), P450 BM3 19A12 (blue) and the empty vector strain (gray) are cultivated in triplicate and all other wells contain one single mutagenesis clone.

Protein expression of the mutant library took place in 96-well 2 mL-deepwell microtiter plates (Eppendorf, Hamburg, Germany). After thawing the master plate was first replicated in a new flat bottom MTP containing LB medium ($50 \mu\text{g mL}^{-1}$ kanamycin) using a 96-pin replicator. This preculture plate was incubated as described above and was further used for inoculation (1% v/v) of 600 μL TB medium, supplemented with trace elements (1x), kanamycin ($50 \mu\text{g mL}^{-1}$), ALA (0.5 mM), IPTG (0.1 mM) and thiamine hydrochloride (0.1 g L^{-1}), per well of the deep well MTP (chapter 3.3). The plate was covered with a sterile lid and sealed with parafilm for incubation at 30°C , 900 rpm and 70% air humidity for 20 h in the MTP-shaker. The cells were harvest by centrifugation at 3480 g and 4°C . The supernatant was discarded and the plate containing the cell pellets was stored at -20°C overnight or until further use for screening.

3.7.2 Preparation of P450 BM3 crude cell lysates in MTP format

Cell pellets from the expression in MTP (chapter 3.7.1) were thawed for 20 min prior to resuspension in 0.1 M KPi buffer (pH 8.0), using a volume of 350 μL in the case of P450 BM3 WT or 150 μL for P450 BM3 19A12. Thereafter, 250 μL freshly prepared lysozyme solution (5 g L^{-1} , 0.1 M KPi buffer pH 8.0) was added to each well and the plate was incubated at 37°C

for 1 h in the MTP-shaker (900 rpm, 70% humidity). The crude cell lysate was gained by centrifugation for 10 min (3480 g, 4 °C).

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

The fermentation of *E. coli* strains for heterologous expression of the different P450 BM3 monooxygenase variants and production of higher amounts of cell mass 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 shaking flask using a working volume of 5 L TB medium supplemented with kanamycin (50 µg mL⁻¹) and trace elements (chapter 3.3).

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. To regulate foam formation (if necessary) polyethylene glycol was used. The cultivation was started by inoculation with an overnight culture of the respective *E. coli* strain in 100 mL LB medium (50 µg mL⁻¹ kanamycin; 37 °C for 16 h, 250 rpm). The temperature was controlled at 37 °C until an OD₆₀₀ of 1 was achieved. At this point the protein expression was induced by addition of IPTG (0.1 mM), as well as ALA (0.5 mM) and thiamine hydrochloride (0.1 g L⁻¹) as additives, and the temperature was decreased to 25 °C and was kept constant for the next 24 to 26 h until the cell harvest.

In the course of the fermentation the dissolved oxygen content (pO₂), the temperature, the pH and the stirrer speed, as well as the pumping volumes of acid, base and antifoam were measured online. For monitoring cell growth by measurement of OD₆₀₀ and protein expression, samples of culture broth were taken regularly over the first 8 h and at the end of the fermentation. The protein expression was evaluated by SDS-PAGE and the P450 BM3 concentration determined by CO-difference spectroscopy (chapter 3.10.1). For cell harvest the cells were separated from the culture broth by centrifugation (15 min, 4500 g at 4 °C). Cell pellets were washed in 0.1 M KPi buffer (pH 8.0) and stored at -20 °C until further use.

3.9 Screening of mutant libraries with the NADPH depletion assay

Screening for activity towards *n*-heptane was achieved following a published procedure.^[132b] The reaction mixture for measuring NADPH-depletion^[166] in flat bottom MTPs contained if not stated otherwise *n*-heptane (1 mM), DMSO as cosolvent (4% v/v), crude cell lysate (50 µL; chapter 3.7.2) and NADPH (0.2 mM) in a final volume of 250 µL KPi buffer (0.1 M; pH 8.0). Before addition of NADPH the mixture containing cell lysate and substrate was incubated for

5 min at RT. In the meantime, the blank value of the entire plate (for each well) was recorded. The reaction was started by addition of the NADPH and immediately thereafter absorption at 340 nm over 10 min in 9 s intervals (60 cycles) was measured using a MTP reader Tecan Sunrise (Tecan Group AG, Männedorf, Switzerland). A plate without addition of substrate but with DMSO (cosolvent) as control reaction was prepared and measured in an analogous manner.

The activity of each P450 BM3 clone towards hydroxylation of *n*-heptane was correlated to the depletion of the NADPH cofactor during the reaction. NADPH-depletion values were calculated as slope of the absorption decrease during the first 60 s (linear range). For each well the corresponding blank value was subtracted from the absolute absorption value to correct for the background signal. The relative activity of each clone was defined as ratio between the NADPH-depletion values of the respective clone and the average value of the starting activity control (P450 BM3 WT or P450 BM3 19A12). A plate containing in 93 wells the parent strain, and in the 3 remaining wells the empty vector strain as a negative control, was screened in an analogous way to determine the standard deviation of the assay. Clones displaying an enhanced activity in comparison to the parent template were selected for a rescreening using the above mentioned procedure. Based on the relative activity and the sequencing results (MWG Eurofins DNA, Ebersberg, Germany) detection of improved P450 BM3 variants was performed.

3.10 Protein preparation and analytics

3.10.1 Evaluation of protein expression by SDS-PAGE

Protein expression was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook and Russell.^[156] 10% SDS-PAGE gels were used for electrophoresis for 75 min at 30 mA in a Mini-Protean Tetra Cell system from Bio-Rad (München, Germany). As a marker 4 µL of Page Ruler Prestained Protein Ladder from Fermentas (St. Leon-Rot, Germany) were used. For protein visualization gels were stained in a Coomassie Brilliant Blue solution (0.25% v/v coomassie, 45.5% v/v methanol, 9.2% v/v acetic acid) for 20 min, followed by destaining in 10% (v/v) acetic acid solution overnight.

3.10.2 Quantification of P450 concentration by CO-difference spectroscopy

The concentration of active P450 BM3 monooxygenase in purified samples or lyophilized crude cell lysates was determined from the CO-difference spectra of the reduced heme-iron (absorption maximum at 450 nm) using a standard procedure by Omura and Sato^[108].

Lyophilized enzyme samples from purified protein or cell lysate were resuspended in 0.1 M KPi buffer (pH 8.0). The CO-difference spectra was recorded with a Varian Cary 50 spectrophotometer (Agilent Technologies, Darmstadt, Germany) after reducing the sample by addition of sodium dithionite and gassing with carbon monoxide for 1 min using a lecture bottle station (Sigma Aldrich, Steinheim, Germany). The solely reduced sample served as reference to subtract the baseline signal.

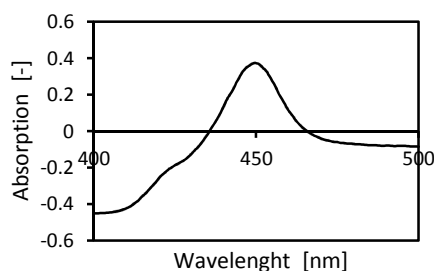


Figure 3-3: Typical CO-difference spectrum of P450 BM3 WT for quantification of P450 monooxygenase concentrations.

Figure 3-3 shows the typical CO-difference spectrum of a P450 BM3 monooxygenase. For calculation of the P450 BM3 concentration the Lambert-Beer law equation and an extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ were used.

3.10.3 Preparation of crude cell lysates

For preparation of P450 BM3 crude cell lysates frozen cell pellets were resuspended in 0.1 mM KPi buffer (pH 8.0) to obtain a cell-suspension of 20-25% (w/v). In case of the RE-ADH the frozen cell pellet was resuspended in 0.1 M KPi buffer (pH 6.0) to obtain a cell-suspension of 1.3 g wet cells per 20 mL. Cell disruption was achieved using either 3 cycles of sonication with a Vibra-Cell VCX130 (Sonics & Materials, Newton, USA; 40% amplitude, 30 s/cycle, 30 s pause between cycles) for volumes up to 5 mL or an Avestin EmulsiFlex-C3 high pressure homogenizer (Ottawa, ON, Canada) for larger volumes by application of three cycles of 1500 bar.

The lysates were centrifuged (30 min, 24500 g at 4 °C) in a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Rockford, IL, USA) and further clarified by filtration through a 0.45 µm filter (Roth, Karlsruhe, Germany). For preparation of a non-purified and lyophilized enzyme sample, the crude cell lysate was shock-frozen in liquid nitrogen, lyophilized for 48 h at -54 °C in an Alpha 1-2 LD plus Freeze dryer (Christ, Osterode am Harz, Germany) and stored at -20 °C until further use.

3.10.4 Purification of P450 BM3 variants

Expression of the NADH-dependent monooxygenase mutants and preparation of crude cell lysates for further purification were performed as described above in a 400 mL-culture, except that cell pellets were resuspended in the purification buffer (0.1 M Tris/HCl, pH 7.8). The fresh cell lysates were subsequently purified by means of anion exchange chromatography using a Toyopearl DEAE 650S matrix (Tosoh Bioscience, Stuttgart, Germany) and an established protocol making use of a two-step elution with sodium chloride with small modifications.^[167]

An Äktaprime Plus system with UV-detector (GE Healthcare, München, Germany) and a packed glass column (TAC15/125PE5-AB-2 Kronlab, now YMC Europe GmbH, Dinslaken, Germany) were used for purification and collection of the eluate. The column was initially equilibrated with 0.1 M Tris/HCl buffer (pH 7.8), followed by loading of the cell lysate and binding of the protein to the matrix at a constant flow rate of 5 mL min⁻¹. In the first washing step with 14% of the elution buffer (140 mM NaCl; conductivity 17 mS cm⁻¹) weaker bounded proteins were eluted. In a second step using a gradient between 14 and 25% of elution buffer (140-250 mM NaCl; conductivity 17-22 mS cm⁻¹) the P450 BM3 monooxygenase was eluted and collected in 5 mL fractions. A 10% SDS-PAGE gel was used for visualization of the purity of the eluted proteins. The fractions with highest purity were pooled together. The selected fractions were concentrated using an Amicon Ultra-4 centrifugation tube (30 kDa cut-off; Millipore, Schwalbach, Germany), followed by desalting and buffer exchange in KPi buffer (0.1 M; pH 8.0) using a PD-10 gel-filtration column (GE Healthcare, München, Germany). A purity of 80 to 90% was estimated after electrophoresis in a 10% SDS-PAGE^[168] gel.

The purified monooxygenase mutants were shock-frozen in liquid nitrogen, lyophilized for 48 h at -54 °C in an Alpha 1-2 LD plus Freeze dryer (Christ, Osterode am Harz, Germany) and stored at -20 °C until further use.

3.10.5 Characterization of P450 BM3 monooxygenases in cuvette

Spectrophotometric activity assay: To assay activity of the P450 BM3 mutants NADH or NADPH depletion was monitored in a cuvette at 340 nm using a Varian Cary 50 spectrophotometer (Agilent Technologies, Darmstadt, Germany). The concentration of active, P450 BM3 monooxygenase (purified or as crude cell lysate) was first determined as described in chapter 3.10.2. The standard reaction mixture consisted of *n*-heptane (10 mM), ethanol as cosolvent (2% v/v), and P450 BM3 (0.2 μ M) in KPi buffer (950 μ L, 0.1 M; pH 8.0) if not stated otherwise. After 5 min of pre-incubation the reaction was started by addition of NADH or NADPH from a stock solution (50 μ L, 8 mM). When the cofactor was almost consumed the reaction was quenched by addition of concentrated HCl (100 μ L, 37% v/v) and was directly extracted for quantification of products via GC (see chapter 3.13). The initial oxidation rate (in $\mu\text{mol}_{\text{cofactor}} \mu\text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$ or abbreviated min^{-1}) of the corresponding P450 BM3 monooxygenase was calculated for the first 20 s of the catalyzed hydroxylation reaction using the slope (in min^{-1}) of the resulting regression curve. Quantification of the exact amount of consumed NAD(P)H (in μmol) was accomplished using the extinction coefficient $\epsilon_{\text{NAD(P)H}}$ of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and the Lambert-Beer law equation.

Determination of Turnover Frequency, Coupling Efficiency and Specific Activity: The maximal initial product formation rate or turnover frequency (TOF; in $\mu\text{mol}_{\text{product}} \mu\text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$ or abbreviated as min^{-1}) of the corresponding P450 BM3 monooxygenase was also calculated for the first 20 s of the reaction, based on the quantified total product amount *via* GC and the coupling efficiency determined for the entire reaction. Coupling efficiencies (in %) for the hydroxylation of *n*-heptane were calculated for each monooxygenase as ratio between total product concentration and consumed cofactor in the course of the reaction (started by NAD(P)H addition until quenching). The specific activity (in $\text{U g}_{\text{P450}}^{-1}$) was calculated using the corresponding turnover frequency value and a molecular weight of P450 BM3 of 119 kDa ($0.119 \text{ g } \mu\text{mol}^{-1}$).

3.10.6 Purification of RE-ADH

Since the RE-ADH is highly thermo-stable (up to 65 °C) it can be partially purified using one step of heat treatment as described by Abokitse *et al.*^[157] The freshly prepared crude cell lysate was incubated for 15 min at 65 °C. The removal of precipitated proteins was performed by centrifugation (30 min, 24500 g at 4 °C). The supernatant was further clarified by filtration through a 0.45 μm filter (Roth, Karlsruhe, Germany) and stored at 4 °C. A purity of nearly 80% was estimated according to SDS-PAGE visualization.^[168]

3.10.7 RE-ADH and GDH activity determination

The activity of the heat-treated (S)-alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) and of the commercially available glucose dehydrogenase (GDH) from *Pseudomonas sp.* (Sigma Aldrich, Steinheim, Germany) was assayed spectrometrically with a Varian Cary 50 (Agilent Technologies, Darmstadt, Germany) by measuring the increase in absorption of NADH at 340 nm. Volumetric activities (in U mL⁻¹) were calculated using the Lambert-Beer law equation.

For RE-ADH: The reaction mixture (999 μ L; cuvette) contained the corresponding alcohol (1-, 2-, 3- or 4-heptanol; 10 mM) and NAD⁺ (1 mM) in KPi buffer (0.1 M; pH 8.0). The reaction was started by addition of RE-ADH (1 μ L; partially purified). One unit was defined as the amount of enzyme that reduces 1 μ mol of NAD⁺ per minute.

Total protein concentration was determined by the method of Bradford using bovine serum albumin as standard.^[169] A control reaction with ethanol (4% v/v), which is used in the cascade reaction as cosolvent, was also recorded and revealed no activity of RE-ADH towards ethanol. The K_M-value of the RE-ADH for the cofactor NAD⁺ using 2-heptanol as substrate was determined in an analog manner, varying the NAD⁺ concentration (1 to 4000 μ M).

For GDH: the reaction mixture contained KPi buffer (1 mL, 0.1 M; pH 8.0), D-glucose (10 mM) and NAD⁺ (1 mM). The reaction was started by addition of GDH (1 μ L). The K_M-value of the GDH for the cofactor NAD⁺ using D-glucose as substrate was determined in an analogous manner, varying the NAD⁺ concentration (3 to 2000 μ M).

For determination of K_M-values the slope of each absorption curve was plotted against the NAD⁺ concentration and the resulting data was fitted using a Michaelis-Menten plot and Origin 8.6 software (OriginLab Corporation, Northampton, MA, USA).

3.11 *In vitro* conversion of *n*-heptane

3.11.1 Hydroxylation of *n*-heptane by P450 BM3 monooxygenases

To determine product formation the *in vitro* conversion of *n*-heptane was monitored over the first 24 h (1 mL, stirred screw top vial, room temperature). A typical reaction mixture contained, if not stated otherwise, *n*-heptane (7 μ L, 50 mM) and ethanol (13 μ L, 1.3% v/v) as cosolvent for enhanced solubility of the organic substrate, P450 BM3 (0.5 μ M), and catalase (600 U, to remove traces of hydrogen peroxide) in KPi buffer (1 mL, 0.1 M; pH 8.0). After an incubation period of 5 min the reaction was started by addition of NADH (2 mM). After a

reaction time of 10, 30, 60, 120, 240 min and 24 h, the reaction mixture (1 mL) was transferred to a reaction vessel containing HCl (100 μ L) to stop the reaction and the aqueous phase was directly extracted as described in chapter 3.13.1. Quantification of the produced heptanol and heptanone regioisomers was carried out by GC analysis (chapter 3.13.2). The catalytic performance of the system was evaluated in terms of total turnover numbers (TTN). This TTN value refers to the P450 BM3-catalyzed hydroxylation, calculated for a 24 h reaction time as ratio between total product concentration (in μ M) and concentration of the P450 BM3 catalyst (in μ M).

3.11.2 One-pot double oxidation of *n*-heptane by P450 BM3 and RE-ADH

The biocatalytic double oxidation of *n*-heptane was evaluated using the P450 BM3 monooxygenase variants together with the RE-ADH in a one-pot reaction. The reaction was carried out in an analogue manner as the sole hydroxylation reaction describe in chapter 3.11.1. The only modification consisted in the addition of 10 U of RE-ADH (referring to the activity determined with 2-heptanol) to the reaction mix. Sampling of the reaction was performed in parallel to the sole P450 reaction.

For the reaction engineering experiments an incubation of 24 h and a varying concentration of the different reaction compounds was used. The TTN values of the double oxidation cascade were calculated as described in chapter 3.11.1. The yield for the enzymatic resolution of (*R*)-heptanol in the second RE-ADH-catalyzed oxidation step are calculated for each (*R*)-heptanol as product, starting from the enantiomeric mixture of (*R*)/(*S*)-2-heptanol or (*R*)/(*S*)-3-heptanol (and not *n*-heptane) as substrate. For the calculation of yields conversion of the non-chiral 1- and 4-heptanol was not considered. The products were quantified by chiral GC analysis as mentioned in chapter 3.13.4.

3.11.3 One-pot double oxidation of *n*-heptane with supplementation of glucose dehydrogenase

In parallel to the double oxidation reaction of *n*-heptane described in chapter 3.11.1, a second oxidation reaction containing a supporting NADH-regeneration system was performed. In this case glucose dehydrogenase (1 U) and D-glucose (2 mM) were added to the reaction mixture. The reaction samples were treated in an analogue manner as described in chapter 3.11.2 for quantification of products and calculation of TTNs.

3.12 Whole cell catalysis for double oxidation cascades

The whole cell conversion of *n*-heptane and cyclooctane was carried out using *E. coli* strains expressing a single P450 BM3 monooxygenase or co-expressing the monooxygenase and an alcohol dehydrogenase (RE-ADH). The employed strains are specified in the appendix section (**Error! Reference source not found.** and Table 6-8).

Selected *E. coli* strains were cultivated and expressed either in shaking flasks (chapter 3.5.6) or in a 5 L-bioreactor (chapter 3.8). After cultivation, the frozen cell pellets were thawed and used for the conversion reactions. P450 content and cell dry weight were determined for each batch of cells used in the biotransformation experiments.

3.12.1 Correlation of optical density to cell dry weight and P450 content

To characterize recombinant *E. coli* cells expressing a distinctive P450 BM3 monooxygenase from a single cultivation batch, the P450 content and the cell dry weight were correlated to a specific optical density at a wavelength of 600 nm (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. For each adjusted OD_{600} value 1 mL of resuspended cells was used to obtain the crude cell lysate by sonication (see chapter 3.10.3) and to measure the P450 BM3 concentration in the lysate by CO-difference spectroscopy (see chapter 3.10.2).

For determination of the corresponding cell dry weight (CDW; in $g_{CDW} L^{-1}$) 1 mL of resuspended cells was centrifuged (21000 g, 2 min) followed by drying of the cell pellet in a drying cabinet FP 53 (Binder GmbH, Tuttlingen, Germany) at 95 °C for 48 h and subsequent weighing of the dried cell pellet.

3.12.2 Whole cell conversion of *n*-heptane and cyclooctane

The optimized reaction for conversion of *n*-heptane with resting cells was 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) for a final OD_{600} of 40, supplemented with 10 $g L^{-1}$ D-glucose for cofactor regeneration. The organic phase consisted of 0.2 mL pure *n*-heptane. The aqueous phase was pipetted into a 5 mL-screw top vial (CS Chromatographie, Langerwehe, Germany) and was incubated together with 25 μL D-glucose that was added from a 400 $g L^{-1}$ stock solution, for 5 min at RT, prior to the addition of *n*-heptane forming a second layer. The reaction mixture was stirred at 600 rpm 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). For monitoring product formation over 48 h the reaction mixture (aqueous and organic phase) was transferred at a determined time point to a reaction vessel containing 100 μL HCl to quench the reaction.

The subsequent extraction procedure was performed as described in chapter 3.13.1. Quantification of the synthesized heptanol and heptanone regioisomers was carried out by GC analysis (chapter 3.13.2). The catalytic performance of the system was evaluated in terms of product concentration and product yield. Product yield on catalyst $Y_{p/x}$ is defined as ratio between the product concentration and the amount of catalyst as cell dry weight in the reaction (in $\text{mg}_{\text{product}} \text{g}_{\text{CDW}}^{-1}$). To evaluate the expression level of recombinant P450 BM3 protein in the cells the P450 yield $Y_{\text{P450}/x}$ was calculated based on the intracellular P450 concentration of a specific amount of cells (in $\text{mg}_{\text{P450}} \text{g}_{\text{CDW}}^{-1}$).

The biotransformation of cyclooctane using *E. coli* resting cells was performed analogue to the procedure described above for *n*-heptane. Instead of a two-liquid-phase system the substrate cyclooctane was applied directly from a stock solution in ethanol (20 μL ; 500 mM) to the aqueous phase containing the resuspended cells for a final concentration of 10 mM cyclooctane and 2 % (v/v) ethanol. Extraction and quantification of products by GC analysis was performed as described in chapter 3.13.

3.13 Product analysis by Gas Chromatography

3.13.1 Extraction of products for GC analysis

The standard extraction procedure consisted in the addition of MTBE (2-Methoxy-2-methylpropane) as solvent containing an internal standard (decane) that serves for correction of pipetting errors or evaporation of samples during the work out. In case of the *in vitro* conversion of *n*-heptane (chapter 3.11) 0.4 mL MTBE containing 1 mM decane (0.02% v/v) was used as solvent. For whole cell catalysis biotransformations containing a second organic phase of 0.2 mL *n*-heptane (chapter 3.12) the reaction products were extracted by addition of 0.4 mL MTBE with 10 mM decane as internal standard (0.2 % v/v) and additionally 0.4 mL of *n*-heptane to achieve a final volume of 1 mL organic solvent. In this way product concentrations are related to the volume of the aqueous phase (c_{Aq}). The extraction of whole cell cyclooctane biotransformations was performed using 0.4 mL MTBE and 10 mM (0.2 % v/v) decane as internal standard.

After transferring the aqueous phase to a reaction vessel containing 0.1 mL concentrated HCl, the solvent was added carefully and the sample was mixed vigorously for 1 min. By centrifugation at 21000 g for 2 min separation of both liquid layers was achieved. The organic layer was transferred to a fresh reaction vessel, dried over NaSO₄ by vigorous mixing, centrifuged again (21000 g, 2 min) and filled in a GC vial.

3.13.2 GC Quantification by means of a calibration curve

Quantification of the reaction products was performed using a Shimadzu GC-2010 gas chromatograph coupled to a flame ionization detector (FID) (Shimadzu, Japan). For quantification the GC was fitted with a column FS-Supreme-5ms (5% Phenylpolysilphenylen-siloxane; 30 m x 0.25 mm x 0.25 µm film thickness; CS-Chromatographie, Germany). A typical column oven temperature program for separation of the heptanol regioisomers consisted of following steps: 40 °C for 19.5 min, 20 °C min⁻¹ to 100 °C, 3 min at 100 °C.

For each corresponding alcohol and ketone a 7-point calibration curve was generated using authentic standards and a defined amount of decane (1 mM or 10 mM) as internal standard. A characteristic GC-chromatogram and a calibration curve, exemplarily for 2-heptanol, are shown in the appendix section (Figure 6-4 and Figure 6-5). The authentic standards 1-heptanol and 2-heptanol (Merck, Darmstadt, Germany), 2-heptanone, 3-heptanol, and 4-heptanone (Sigma Aldrich, Steinheim, Germany), 3-heptanone, and 4-heptanol (TCI Europe, Eschborn, Germany) had all the purity >98%. Retention times for 1-, 2-, 3-, 4-heptanol, 2-, 3-, and 4-heptanone were respectively: 13.7, 15.3, 15.9, 16.5, 17.2, 18.0, 22.7 min.

The separation of cyclooctane conversion products with the same column FS-Supreme-5ms was achieved using following oven temperature program: 80 °C, 2 °C min⁻¹ to 110 °C, 30 °C min⁻¹ to 200 °C, 40 °C min⁻¹ min to 300 °C for 1 min. For quantification of cyclooctane, cyclooctanol and cyclooctanone 4-point calibration curves were generated using authentic standards (purity >98%) and 10 mM decane as internal standard. Retention times for cyclooctane, cyclooctanol and cyclooctanone respectively: 3.4, 9.13, 8.2 min.

3.13.3 Determination of regio- and stereoselectivity of the P450 BM3 variants

The enzymatic hydroxylation of *n*-heptane, used in this study as model substrate, leads to generation of all possible regioisomers 1-, 2-, 3- and 4-heptanol. Additionally, in two of this regioisomers, 2- and 3-heptanol, chiral centers can be generated. The distribution of the regio- and stereoisomers upon conversion of *n*-heptane with the selected P450 BM3 monooxygenases

was analyzed *via* gas chromatography (GC). In terms of regioselectivity, the product distribution was normalized to 100% and was calculated based on GC quantification of each regioisomers as described in chapter 3.13.2. Stereoselectivity was calculated as enantiomeric ratio (*er*; percentage of each enantiomer) or as enantiomeric excess (*ee*; percentage of excess of one enantiomer compared to the other).

3.13.3.1 Determination of the absolute configuration of (*S*)- and (*R*)-3-heptanol

Pure enantiomeric standards of 3-heptanol were not commercially available but racemic 3-heptanol could be separated on a chiral GC column into both enantiomer peaks. Therefore, the absolute configuration of each GC peak was assigned based on polarimetry measurement and correlation to the optical rotation (or sign of the rotation) of each enantiomer according to following literature values: 4.9° for (*R*)-(-)-3-heptanol^[170] and +5.8° for (*S*)-(+)-3-heptanol^[171].

Samples of each enantiomer in excess were obtained through conversion of (*rac*)-3-heptanol, either with the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH)^[158] or the (*S*)-specific RE-ADH^[157]. The Lb-ADH reaction was performed using recombinant *E. coli* whole cells (OD₆₀₀ of 22) in KPi-buffer (25 mL, 0.1 M, pH 6.0), containing acetone (2 mL, 8% v/v), MgSO₄ (4.25 mg), NADP⁺ (2 mM) and 3-heptanol (0.4 M), for 24 h at room temperature. *E. coli* whole cells containing the recombinant Lb-ADH were kindly provided by Prof. Dr. Hummel (FZ Jülich, Germany). The products were extracted twice by addition of chloroform (10 mL). After centrifugation (2 min at 21000 g) the organic layers were combined, dried over NaSO₄ and concentrated to 1 mL using an evaporator IKA RV 10 basic (IKA-Werke, Staufen, Germany). Subsequently, the extracted reaction was used for polarimetric measurement with a Perkin Elmer 241 MC Polarimeter (Waltham, USA) and for chiral GC analysis as described in chapter 3.13.4. The polarimeter was first blanked against chloroform and the optical rotation was determined at a wavelength of 589.3 nm and 25 °C.

3.13.3.2 Lipase-catalyzed resolution of (*S*)-2-heptanol

The 2-heptanol enantiomers could not be separated on a chiral GC column, therefore determination of the stereoselectivity was based on the lipase-catalyzed resolution of the (*S*)-2-heptanol enantiomer as described by Patel *et al.* with some modifications.^[172] In this process the (*R*)-heptanol products are acetylated by Lipase, leaving the (*S*)-heptanol enantiomers unreacted.

The products of the sole P450 BM3-catalyzed hydroxylation of *n*-heptane (chapter 3.11.1) were extracted in 1 volume of *n*-heptane (1 mL) and split in two equal volumes of 0.4 mL. One volume was treated with Lipase B for acylation of the (*R*)-2-heptanol [20 U, 4 mg of Lipase from *Candida Antarctica*/acrylic resin (Sigma Aldrich, Steinheim, Germany)] and succinic anhydride (0.2 mg), one volume without the addition of Lipase as reference reaction. Both samples were incubated in parallel at 35 °C and 600 rpm for 24 h. The samples were analyzed by gas chromatography as described in chapter 3.13.4 to determine the area of the 2-heptanol peak in the acetylated sample and the reference sample. Comparison to the reference reaction without Lipase allows to calculate the total amount of 2-heptanol and the corresponding percentage of reacted (*R*)-heptanol and to determine the enantiomeric ratio.

3.13.4 Quantification of chiral products

For chiral separation and quantification of (*R*)- and (*S*)-3-Heptanol, as well as the remaining (*S*)-2-heptanol after acylation of the (*R*)-enantiomer, a GC-2010 Plus with a FID-detector (Shimadzu, Japan) was fitted with a column FS-Hydrodex β -TBDAC (heptakis-(2,3-di-O-acetyl-6-O-*t*-butyldimethylsilyl)- β -cyclodextrin; 25 m x 0.25 mm; Macherey-Nagel GmbH, Düren, Germany). Following oven temperature program was used: 50 °C, 5 °C min⁻¹ to 95 °C, 9.5 min. Retention times for (*R*)- and (*S*)-3-heptanol respectively: 13.00, 13.28 min; for (*S/R*)-2-heptanol: 14.46 min.

4 Results and Discussion

The results and discussion section is divided in three main chapters. In the first chapter the protein engineering of P450 BM3 monooxygenase by focused mutagenesis (10 positions for P450 BM3 19A12; 17 positions for P450 BM3 WT) and random mutagenesis (630 clones for P450 BM3 19A12 as parent) is described to improve the enzymes activity for the subterminal hydroxylation of *n*-heptane. Additionally, this chapter includes the switch in cofactor specificity from NADPH to NADH of selected P450 BM3 variants by mean of focused mutagenesis and the detailed characterization of the variants in terms of catalytic performance and selectivity. The second chapter comprises the ‘proof of concept’ for the *in vitro* one-pot double oxidation of *n*-heptane in a biocatalytic cascade, employing the engineered NADPH-dependent P450 BM3 monooxygenase variants and the (S)-selective alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). In the third chapter an extension of the aforementioned catalytic process to the application of whole cell catalysts is described. It includes a comparison of different strategies for coexpression of P450 BM3 variants and alcohol dehydrogenases (RE-ADH and ADH from *Lactobacillus brevis* - Lb-ADH), as well as an evaluation of the catalytic performance for the whole cell system employing *n*-heptane and cyclooctane as model substrates.

4.1 Protein engineering of P450 BM3 monooxygenases

This chapter is focused on the engineering of P450 BM3 monooxygenases to achieve highly active mutants for the selective oxyfunctionalization of medium-chain alkanes (C₇-C₈). The model substrate *n*-Heptane was selected due to the easier handling compared to shorter-chain alkanes which are gaseous at room temperature. As a first approach, the monooxygenase P450 BM3 19A12^[146] was selected as the starting variant for focused and random mutagenesis towards increased activity for the subterminal hydroxylation of *n*-heptane. Later on, the focus of engineering was shifted to the P450 BM3 wild type enzyme as starting template to engineer mutants with a reduced mutational load, but displaying a high activity on *n*-heptane and improved coupling efficiency. Furthermore, the change in cofactor specificity from NADPH to NADH of the selected P450 BM3 monooxygenase variants was investigated in order to achieve cofactor-compatibility with the NADH-dependent RE-ADH^[157]. In addition, NADH is cheaper and more stable than NADPH making an application in cell free catalysis more suitable.^[173] Finally, a detailed characterization of the engineered P450 BM3 variants in terms of catalytic performance, as well as regio and stereoselectivity was performed.

4.1.1 Focused and random mutagenesis of P450 BM3 19A12

The monooxygenase variant P450 BM3 19A12 was generated by Fasan *et al.* in previous studies and displayed a higher catalytic performance compared to P450 BM3 WT with short and middle-chain alkanes, mainly from *n*-propane to *n*-heptane (TTN of 10500 and 5360 respectively).^[59, 146] P450 BM 19A12 contains in total 18 substitutions (R47C, L52I, V78F, A82S, K94I, P142S, T175I, A184V, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, and I366V; see chapter 1.3.2). Recently, the substrate spectrum of the P450 BM3 variant 19A12 has been further expanded from linear alkanes to cyclic alkanes such as cyclohexane, cyclooctane and cyclodecane.^[174]

The heme-domain of the P450 BM3 variant 19A12 was ordered as synthetic DNA and was subcloned into the expression vector pALXtreme-1a (see chapter 3.5.4.1). The expression of soluble protein from P450 BM3 19A12, together with the wild type (WT) and the empty vector control was analyzed by SDS-polyacrylamide gel electrophoresis (chapter 3.10.1) and the P450 concentration was determined by CO-difference spectroscopy (chapter 3.10.2). The accumulation of 18 mutations in the P450 BM3 19A12 variant resulted in a significant decrease in expression, lower than the in the WT (Figure 4-1).

The concentration of active monooxygenase in the cell extracts was 3 μ M for 19A12 and 51 μ M for WT, when expressed in parallel shaking flasks under the same incubation conditions (30 °C at 250 rpm for 20 h after induction). Under this incubation conditions the variant 19A12 achieved only 6% of active protein expression as compared to the WT.

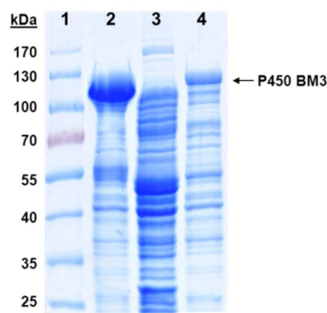


Figure 4-1: Expression level of P450 BM3 wild type (WT) and BM3 19A12. Protein visualization of crude cell lysates on a 10% SDS-PAGE gel: (1) Molecular weight standard (PageRuler, Fermentas); (2) P450 BM3 WT; (3) empty vector control; (4) P450 BM3 19A12. The molecular weight of P450 BM3 WT and variants is 119 kDa. Taken from Müller *et al.*^[175]

For directed evolution of P450 BM3 monooxygenase an appropriate high throughput screening assay was required. A product-based high throughput detection system for alcohols, such as

heptanol, is in practice difficult to realize. On the one hand, the gas chromatographic detection of alcohols is simple and can give both quantitative and qualitative values, however, only with a low to medium throughput. On the other hand, for chemical detection of alcohols the use of toxic heavy metals or harsh pH conditions is required.^[176] Therefore, one feasible alternative to assay monooxygenase activity is the direct measurement of the cofactor-consumption during the hydroxylation reaction. The NAD(P)H-depletion assay has been used with success in the last decade for several protein evolution studies^[144, 177]. An established protocol is also available.^[166] The reported NADPH-depletion assay was adapted for P450 BM3 19A12 as starting template, using crude cell lysates after cultivation and expression in deep-well MTP format (chapter 3.7). The assay was optimized in regard to substrate concentration, as well as DMSO and cell lysate volumes. The best result was achieved with 0.2 mM *n*-heptane, 4% (v/v) DMSO and 50 μ L lysate.

A standard deviation of 21% for the optimized NADPH-depletion assay with *n*-heptane as substrate was determined after simultaneous screening 90 single P450 BM3 19A12 clones as shown in Figure 4-2.

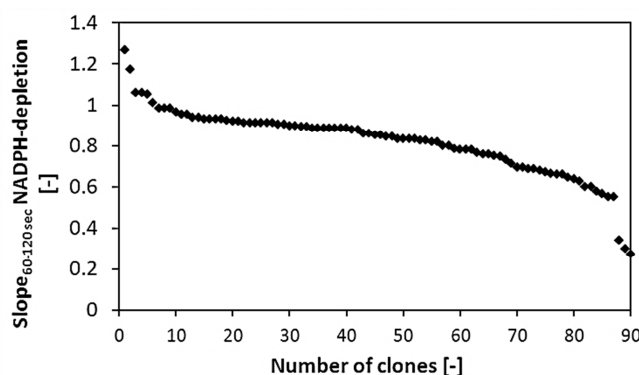


Figure 4-2: Determination of the standard deviation of the NADPH-depletion assay: *n*-heptane hydroxylation by P450 BM3 19A12. The calculated standard deviation is 21%.

Initially, we pursued not only the detection of NADPH-consumption, but also the subsequential production of hydrogen peroxide as a measure for the uncoupling of the hydroxylation reaction (peroxide uncoupling)^[178]. The ABTS-assay (2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) was employed for this purpose.^[179]

Detection of hydrogen peroxide was performed under the reaction conditions used for the NADPH-consumption assay, with and without addition of *E. coli* crude cell lysate. Lysates from two different P450 BM3 variants, 19A12 and 139-3^[144] were used. Variant BM3 139-3 has a highly uncoupled electron transfer system (8% coupling efficiency for *n*-propane)^[59] and

therefore a higher production of hydrogen peroxide during conversion of *n*-heptane is expected. Nevertheless, detection of hydrogen peroxide was not possible when crude cell lysate was present in the reaction mixture as shown in Figure 4-3.

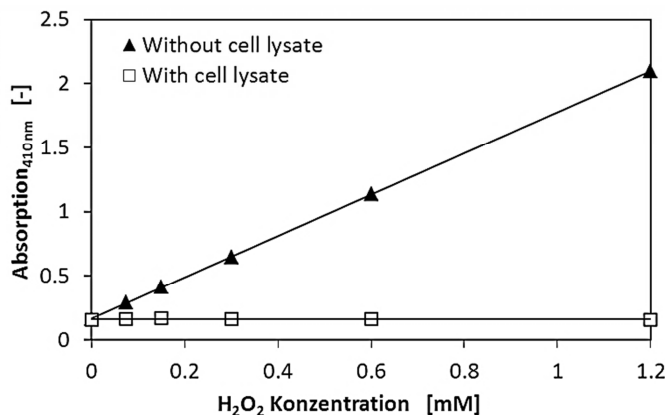


Figure 4-3: ABTS-assay for detection of hydrogen peroxide. A concentration of 0-1.2 mM H₂O₂ was used with and without supplementation of *E. coli* crude cell lysate. The reaction contained: 20 U mL⁻¹ horse radish peroxidase, 2.6 mM ABTS, 40 µL cell lysate or buffer, varying concentration of H₂O₂ (0-1.2 mM) in KPi buffer (250 µL, 0.1 M, pH 8.0).

Based on this result it was assumed that the cell lysate contains native catalase (hydroperoxidase), which will degrade the hydrogen peroxide added to the reaction. It is well known that *E. coli* cells can produce hydroperoxidases (HPI and HPII) as adaptive response to oxidative stress, mainly during the stationary phase of cultivation.^[180] Hence, an application of the ABTS assay in the expected format for a high throughput screening with cell lysates is not feasible.

The screening of P450 BM3 mutants proceeded using only the NADPH-depletion assay. For the detection of clones with an improved activity compared to the parent template (P450 BM3 19A12), the activity of the parent was set to 100% and the threshold was determined as 120% based on the standard deviation of the assay. Based on literature research^[126b, 181] and on a prescreening of available P450 BM3 libraries 10 amino acid positions, which may influence substrate binding and conversion of substrates in the active site cavity of P450 BM3 were selected.

The amino acid positions V26, A74, F87 and V184 were separately saturated, while residues R47-Y51, I236-E267 and A264-T268 were saturated simultaneously using for each pair a single oligonucleotide primer containing both randomized positions. The variant P450 BM3 19A12 was used as template as described in chapter 3.5.7. For all positions, amplification of

the template DNA with the mutagenic oligonucleotide primers yielded the correct amplification product. For each single position, 180 clones were screened using the established NADPH-depletion assay (chapter 3.9). In the case of two simultaneous saturated positions, 360 clones were screened. The clones that showed a significantly higher activity than the parent P450 BM3 19A12 (>121 %) were selected for rescreening.

From screening all constructed SSM libraries, only those at positions A74 and V184 showed an improved *n*-heptane hydroxylation activity under the screening conditions. From both libraries 10 clones were selected and cultivated in shaking flask for rescreening with the NADPH-consumption assay. The sequencing results revealed that the best two mutants harbored the substitution A74E, and V184S/P188Q, being P188Q a side amino acid substitution. The single amino acid substitutions V184S and P188Q were subsequently introduced into P450 BM3 19A12 by SDM (chapter 3.5.6) to elucidate the individual effect of each mutation.

After expression in shaking flask and determination of the P450 concentration (chapter 3.10.2) the activity of the mutants was determined by using equal amounts of enzyme (normalized P450 concentration of 20 nM) for the NADPH-depletion assay. Afterwards, the activity was calculated based on the slope of NADPH-depletion and in relation to the activity of the variant 19A12 (100%) as shown in Figure 4-4. The single substitutions A74E, V184S and P188Q enhanced the activity of variant 19A12 in the NADPH-assay up to 43%. The absolute product formation in the hydroxylation of *n*-heptane was further evaluated for these mutants with normalized concentrations of P450 BM3 monooxygenase (chapter 3.10.5).

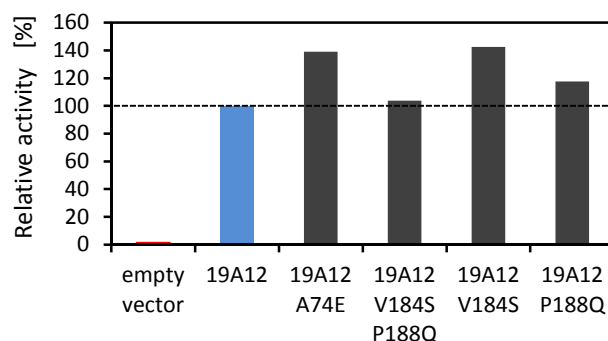


Figure 4-4: Relative activity of P450 BM3 19A12 mutants in the NADPH-depletion screening. The activity of the parent variant P450 BM3 19A12 (blue) as reference is set to 100%.

None of the selected mutants A74E, V184S and P188Q showed an increased product formation rate compared to the parent variant 19A12 when equal amounts of P450 monooxygenase were employed in the reaction. The improved activities observed during NADPH-depletion

screening were most likely a result of increased expression levels of the mutant or higher uncoupling rates. The low and unstable expression level of P450 BM3 19A12 in MTP format was determined to be a bottleneck for further engineering of the protein. Based on the quantification of active P450 after the shake flask expression experiments it was observed, that mutation P188Q lead to a significant rise in the P450 concentration compared to the parent variant 19A12 (up to 2-fold; 4.5 μ M vs. 2 μ M). Therefore, during screening the side mutation P188Q led to an apparent improvement in activity caused only by an increased expression level. This result is not surprising since the destabilizing effect of the mutation L188P, despite of its significant benefit towards activity in BM3 19A12, was reported previously by Fasan *et al.*^[59] The authors introduced the mutation L188P in the P450 BM3 variant 35E11 and observed a perturbation in the heme environment as indicated by CO-difference spectroscopy (appearance of a 420-nm peak accounting for 40% of the total enzymes concentration). They also investigated the impact of accumulated mutations in P450 BM3 variants on the enzyme's thermostability. The half-denaturation temperature T_{50} as a benchmark of stability decreased gradually with a growing number of accumulated mutations. The T_{50} value of P450 BM3 19A12 was estimated as 46 °C, 9 °C lower than the value of the WT (55 °C).^[59]

Random mutagenesis was used as a second strategy for evolution of P450 BM3 19A12 to improve *n*-heptane hydroxylation activity. An error-prone (ep) PCR library containing an inactivation ratio of 50% was generated (chapter 3.5.8). Different concentrations of MnCl_2 , in combination with an *ep*Taq DNA polymerase were tested and for each concentration 96 clones were screened using the NADPH-depletion assay. The ratio of active clones, compared to the parent P450 BM3 19A12 as reference, and inactive clones, compared to the empty vector strain, were calculated (Figure 4-5).

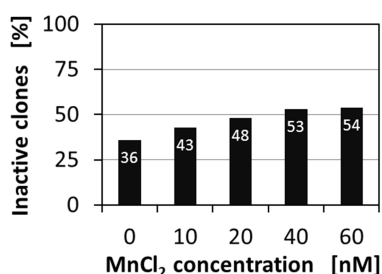


Figure 4-5: Inactivation ratio in epPCR libraries of P450 BM3 19A12 in correlation to the concentration of MnCl_2 .

A MnCl_2 concentration of 40 nM with an apparent inactivation rate of 53% was chosen for generation of the library. In total 630 clones were subjected to a first round of screening and out of this 49 were selected for rescreening in replicates (5-times). In the rescreening, none of

the selected clones displayed significantly increased activity compared to the parental P450 BM3 variant 19A12 as shown in Figure 4-6. The relative activity of most clones did not exceed the activity of 19A12 within the standard deviation range of the assay (21%). As a result of both semi-rational and random mutagenesis approaches no improved variants were found based on the NADPH-depletion assay.

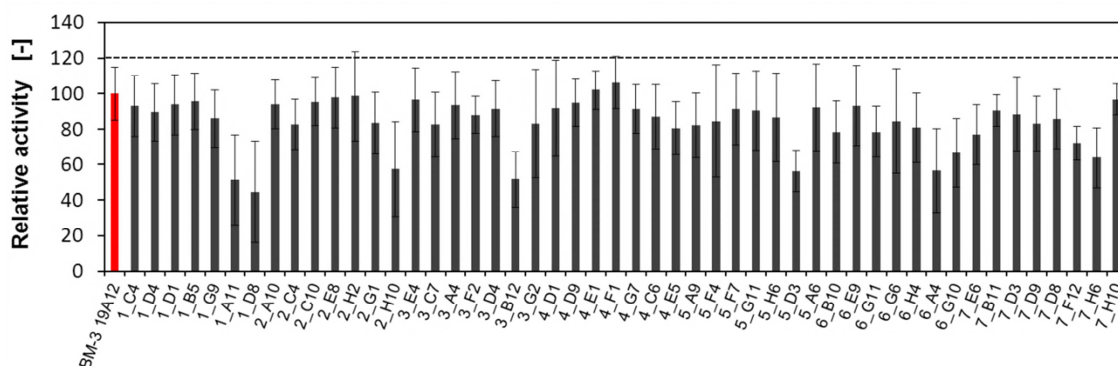


Figure 4-6: Rescreening of a P450 BM3 19A12 epPCR-library with *n*-heptane as substrate. In total 49 clones were screened with the NADPH-depletion assay (average of 5 replicates each). Activity of 19A12 was set to 100%.

Both the low stability of the 19A12 variant due to a high mutational load (in total 18 amino acid substitutions) and the low expression rate as compared to the wild type enzyme (6%), represent major bottlenecks for further evolution of the enzyme. Together with a high standard deviation of 21% in the NADPH-depletion assay, the low or unstable expression of the parental enzyme reduced the probability of finding optimized mutants.

In previous studies from Arnold's research group on directed evolution, especially of P450 BM3 monooxygenases, the authors pointed out that most of the activating substitutions had also a destabilizing effect on the enzyme.^[59, 182] In order to assure the evolvability of the P450 BM3 mutant 35E11 the authors had to introduce two neutral but stabilizing substitutions (L52I and I366V) leading to the variant ETS8. In a subsequent round of mutagenesis the improved variant 19A12 was generated by introduction of the substitution L188P. However, this substitution had again a destabilizing effect on the enzyme. It has been reported, that during protein engineering most mutations are deleterious and therefore the preservation of fold and function of the protein decreases exponentially with a growing number of random mutations.^[183] For achieving a further improved activity of P450 BM3 19A12 towards hydroxylation of *n*-heptane a change in the selection pressure, also in regard to an increased protein stability, would have been required. Therefore, a screening based on direct product detection combined with a selective pressure for stability would be advantageous.

In summary, the P450 BM3 wild type enzyme with a significantly higher expression level (17-fold) and thermostability represents a more robust and promising starting point for directed evolution than the variant 19A12.

4.1.2 Focused mutagenesis of P450 BM3 WT

As described in chapter 4.1.1 the expression and stability of P450 BM3 19A12 is significantly low compared to the WT. By means of directed evolution and rational protein design of the 19A12 variant it was not possible to yield a mutant with further improved activity towards *n*-heptane. Hence, new emphasis was placed on the investigation of single mutations responsible for the higher catalytic activity towards *n*-heptane, having into account the expression level of the protein. The expression level of the monooxygenase in the host cell is a significant factor for future application of the catalysts in whole cell biotransformation processes. Large part of the work in this chapter 4.1.2 was performed by Beneeta Akkapurathu within the frame of her master thesis.^[184]

To elucidate the contribution of each single amino acid substitutions, the 18 previously reported mutated positions in the P450 BM3 19A12 variant were saturated individually in BM3 WT using degenerated NNK-oligonucleotides. In this way all possible amino acid substitutions at each position can be investigated. For each of these ‘hot spot’ residues a SSM-library consisting of 180 clones was generated (chapter 3.5.7) and was screened with the NADPH-depletion assay (in total 3460 clones; chapter 3.9). Due to an error in the synthesis of the oligonucleotide primers for position K94, this library was not taken into account. From each saturated position, the 10 best clones showing the highest relative activities were chosen for a rescreening in triplicate in MTP-format using the NADPH-depletion assay. For determining which substitutions were the most beneficial at each respective position, all rescreening clones were partially sequenced (covering the saturated site). Figure 4-7 displays the relative activity from the rescreening results for each position, taking into account the substitution found by sequence analysis of the corresponding clone.

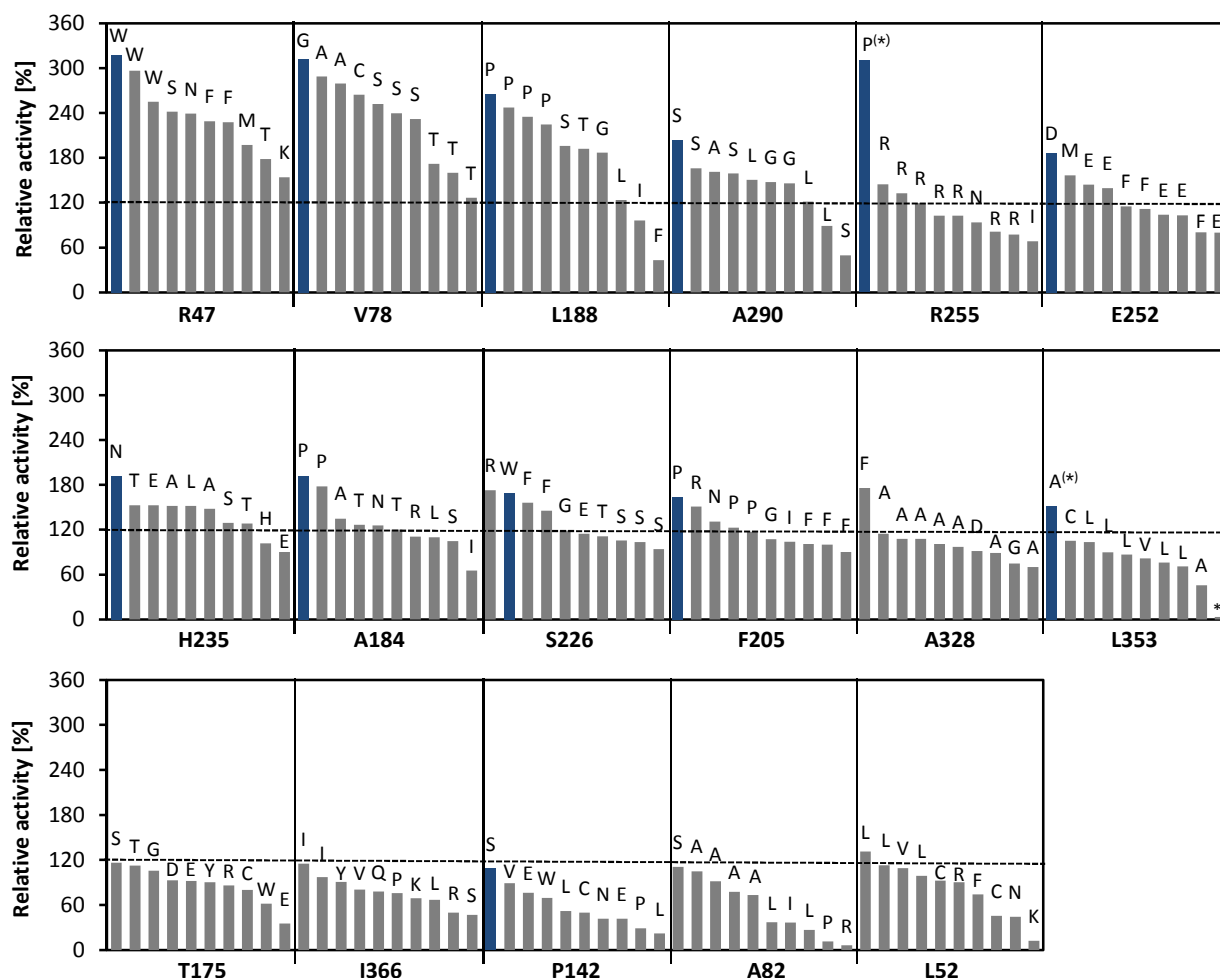


Figure 4-7: Rescreening of 10 clones for 17 SSM libraries of P450 BM3 using *n*-heptane and NADPH-depletion assay. The activity of the WT was set to 100% and the threshold value of the assay at 120% (dashed line). For each clone the corresponding mutation is depicted in the graph ((*): contains an additional mutation).

The rescreening results in Figure 4-7 show that mainly substitutions of the amino acid residues R47, V78, L188 and to a lesser extent E252 and A290 had a significant effect on the activity towards the screened substrate. For the three positions 47, 78 and 188 out of 10 clones at least eight showed an improved activity of up to 318%. In these cases, more than one beneficial substitution was found. The three most active clones were R47W, V78G and R255P-P329H, all showing more than 3-fold improved activity in the assay. For the positions 226, 236, 252, and 290 also more than one clone with a distinctive substitution led to an enhanced activity. In regard to positions 184, 255, 328 and 353, only one distinctive substitution in each case displayed an enhanced activity. The positions 52, 82, 142, 175, and 366 did not show any significant improvement. In some positions also a high incidence of the wild type codon was found. This was the case for R255 and A328, displaying 7-times the WT codon, and for L353

and E252 5-times. Sequence analysis revealed, for 7 clones, additional mutations at a different site than the saturated one, in most of the cases the mutation being located at the oligonucleotide primer site (appendix Table 6-9). Interestingly, two double mutants, containing an additional mutation, R255P-P329H and L353A-I254V, were the most active clone for each corresponding saturated position (Figure 4-7, blue*). Using a threshold of 150%, which represents a 1.5-fold increase in activity, the best clone per position was selected for further characterization. In addition, also the clone P142S, which was not significantly improved, was chosen as a control (selected clones are depicted in blue, Figure 4-7).

To elucidate the effect of each single amino acid substitution in the significantly improved double mutant R255P-P329H, the wild type gene was subjected to SDM to generate the single mutants P450 BM3 R255P and P450 BM3 P329H. For simplification reasons in the following part of this work the double mutant R255P-P329H is abbreviated as CM1. The selected clones were expressed in shaking flask and characterized in terms of NADPH-oxidation (or initial oxidation rate), product formation (or turnover frequency, TOF) and coupling efficiency using crude cell lysates with normalized monooxygenase concentrations (see chapter 3.10.5). The characterization values are specified in Table 4-1. Figure 4-8 shows the improvement in productivity for each P450 BM3 variant compared to the WT as reference.

The highest initial oxidation rate of 1788 min^{-1} was measured for the control variant P450 BM3 19A12. However, this variant has a very low coupling efficiency for NADPH (14%), resulting in an inefficient utilization of the cofactor and thereby in a lower product formation. 19A12 shows a 19-fold improved activity in contrast to the WT, which shows a low TOF of 13 min^{-1} . Turnover frequencies (TOF) and coupling efficiencies are more representative values than NADPH-oxidation rates, because they correlate directly to the catalytic performance and the efficiency of cofactor uptake during the hydroxylation reaction. In this regard, the single mutant R255P and the double mutant CM1 achieve the highest TOF of 420 and 658 min^{-1} respectively. The gain in product formation is proportional to the higher coupling efficiency in both variants (38% and 45% respectively).

Table 4-1: Characterization of best P450 BM3 variants from SSM^[a]

P450 BM3		Initial oxidation rate [min ⁻¹] ^[c]	TOF [min ⁻¹] ^[d]	Coupling efficiency [%] ^[e]
Reference	WT	68	13	20
Variants	P329H	<u>23</u>	<u>7</u>	32
	A290S	77	17	22
	F205P	115	28	25
	P142S	110	30	27
	L353A	157	31	20
	V78G	258	34	<u>13</u>
	R252D	143	43	30
	A184P	227	55	24
	L188P	281	77	27
	H236N	320	78	24
	S226W	291	85	29
	R47W	525	131	25
	R255P	1115	420	38
	CM1 (R255P-P329H)	1455	658	45
Control	19A12 ^[f]	1788	248	<u>14</u>

^[a] Characterization was performed with crude cell lysates; the reaction contained *n*-heptane (10 mM), ethanol as cosolvent (2% v/v), and P450 BM3 (0.25 μ M) in KPi buffer (950 μ L, 0.1 M; pH 8.0). The highest values are highlighted in bold; lower values than WT are underlined.

^[c] Initial oxidation rates in $\mu\text{mol}_{\text{NADPH}} \mu\text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$ from spectrophotometric measurements.

^[d] For turnover frequency (in $\text{mol}_{\text{product}} \text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$) products were quantified via GC.

^[e] Coupling efficiency (in %) as ratio between initial oxidation rate and TOF.

^[f] Reaction contained 0.02 μ M P450.

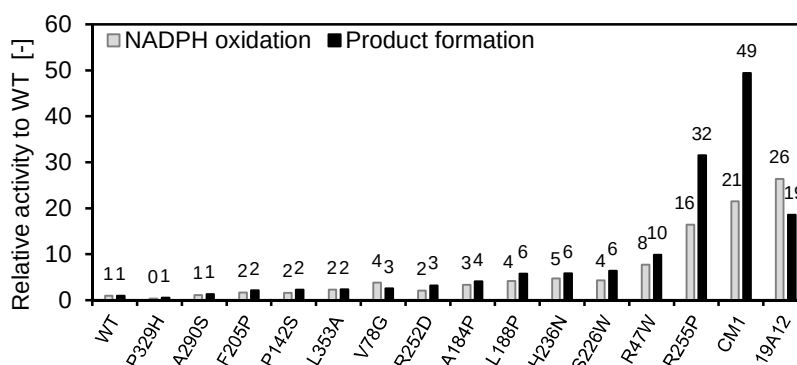


Figure 4-8: Relative activity of selected P450 BM3 mutants. Initial NADPH oxidation and product formation are displayed as ratio to the wild type (WT) activity. Modified from Müller *et al.*^[175]

With exception of the variants V78G, L353A and 19A12, all mutants show an enhanced coupling efficiency for *n*-heptane as substrate when compared with the wild type enzyme (20%). The overall performance of the variant CM1 is significantly improved compared to the WT, showing a 49-fold higher product formation (Figure 4-8). The best single substitution leading to a higher hydroxylation activity is R255P. To a lesser extent mainly the substitutions R47W, S226W, H236N and L188P contribute individually to an optimized *n*-heptane hydroxylation with a 6 to 10-fold improved product formation. The single mutation P329H,

which is present in the CM1 variant, shows an even lower activity than the WT, but an improved coupling efficiency of 32%. Furthermore, variant CM1 shows 56% higher TOF and 18% higher coupling efficiency than the single amino acid substitutions R255P. It can be assumed that amino acid substitutions R255P and P329H contribute in CM1 in a synergistic manner, enabling a further enhanced catalytic performance. This single substitutions display individually a significantly higher coupling efficiency (38% for R255P and 32% for P329H) and together in the variant CM1 a further improvement to 45%.

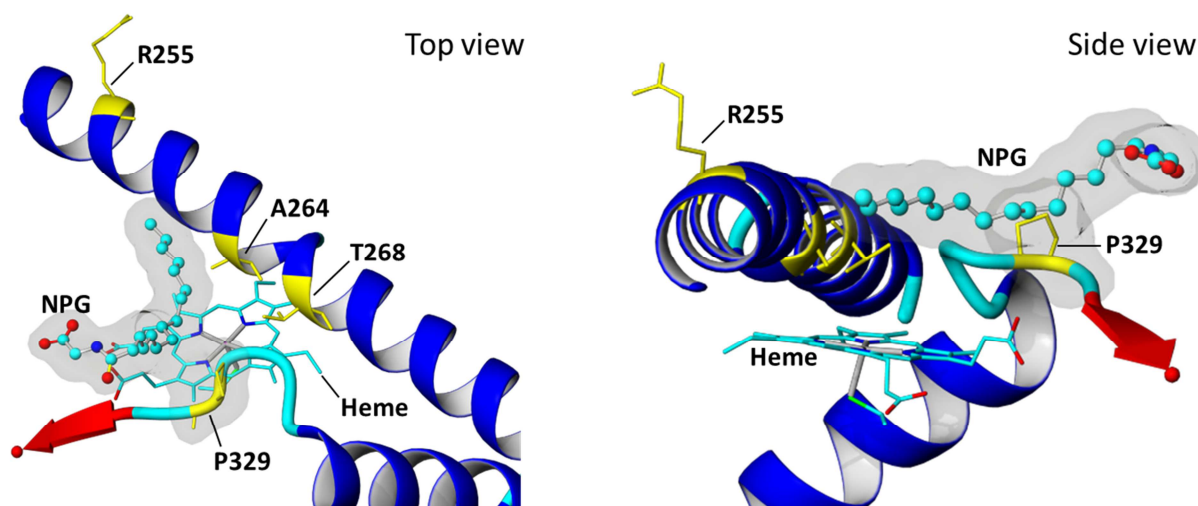


Figure 4-9: Substrate-bound conformation of P450 BM3 WT highlighting residues R255 and P329. The I-Helix and K-helix from the NPG-bound structure of P450 BM3 WT (PDB code 1JPZ)^[126c] are displayed together with the heme and the substrate (ball & stick).

It is well known that there are conformational changes in specific regions of P450 BM3, induced after substrate binding.^[58, 126c] One of these regions is the I-helix, which is located adjacent to the active site, above the heme. The rearrangement of the I-helix was reported to be essential for the displacement of the axial water molecule that ligands the heme-iron, which causes the shift from low-spin to high-spin in the initial part of the catalytic cycle.^[126c] Residue R255 is located in the I-helix as shown in Figure 4-9. It can also be noticed that the residue proline at position 329 is located in close proximity to the substrate in the active site cavity, making an interaction feasible. Both residues, R255 and P329, are highly conserved in the CYP102A sub-family.^[58] However, mainly the exchange of arginine at position 255 to proline results in a higher catalytic performance towards *n*-heptane hydroxylation. It can be therefore assumed, that the substitution to proline affects the conformation of the I-helix. In the wild type structure of P450 BM3, a salt bridge between R255 and D217 is described, which is extended upon substrate binding and is in part responsible for the rearrangement of the I-helix.^[58] This

salt bridge cannot be formed in the SDM mutant R255P as well as in the variant CM1, since proline - in contrast to arginine - is uncharged. The single substitution P329H showed on the one hand a positive effect on the coupling efficiency, but on the other a negative effect on the product formation. Histidine is a more bulky residue than proline. The exchange to histidine at position 329 could therefore interfere with the entrance and positioning of the substrate in the active site cavity, leading to the observed decrease in hydroxylation activity. However, the combination of both mutations (R255P-P329H) in CM1 leads to a 50-fold higher catalytic activity (TOF of 658 min⁻¹; Table 4-1), inferring a synergistic effect of both mutations. R255P can probably compensate the negative effect of P329H on substrate binding through rearrangement of the I-helix, enabling an even better and tighter positioning of the substrate.

The highly active P450 BM3 CM1 mutant was chosen for evaluation of the *in vitro* and *in vivo* double oxidation of *n*-heptane, together with selected alcohol dehydrogenases in a biocatalytic cascade reaction. The P450 BM3 monooxygenases 19A12 and WT were also used as reference catalysts to compare and expand the catalytic concept.

4.1.3 Change in cofactor specificity of P450 BM3 variants

An efficient biocatalytic cascade reaction requires the compatibility of the used enzymatic components. In this study, the coupling of two enzymes - a P450 BM3 monooxygenase and an alcohol dehydrogenase from *R. erythropolis* (RE-ADH) - is intended. In this way an oxidation cascade can take place, with a first P450-catalyzed hydroxylation and therefore activation of an alkyl chain followed by a second ADH-catalyzed oxidation of the introduced hydroxyl group. These two catalysts have, however, different cofactor specificities. P450 BM3 has a high preference for NADPH over NADH, whereas RE-ADH is NADH specific.^[147, 157] In previous reports from Maurer *et al.* and Neeli *et al.* the cofactors specificity of P450 BM3 was successfully exchanged by introducing mutations in the reductase domain of the enzyme (see chapter 1.3.2).^[147-148]

The previously reported substitutions W1046S and R966D^[148], which lead to a switch in cofactor preference, were introduced in the P450 BM3 wild type, 19A12 and CM1 by SDM (chapter 3.5.6). In this way, the three NADH-dependent P450 BM3 variants WT^{NADH}, 19A12^{NADH}, and CM1^{NADH} were generated. For simplification efforts, the abbreviation “NADH” indicates the introduction of mutations W1046S and R966D in the corresponding enzyme.

The generated NADH-dependent variants were characterized for both cofactors NADH and NADPH using purified enzymes. Anion-exchange chromatographic purification of the P450

BM3 variants (chapter 3.10.4) using a DEAE matrix and an elution gradient from 140 to 250 mM sodium chloride yielded a purity of > 90 %. The purity was estimated by visualization in a SDS-PAGE gel as shown for 19A12^{NADH} and CM1^{NADH} in Figure 4-10 (appendix Figure 6-6).

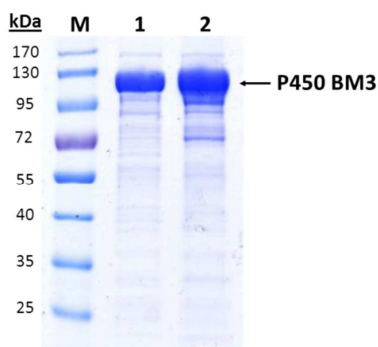


Figure 4-10: Purified monooxygenases P450 BM3 19A12^{NADH} (1) and CM1^{NADH} (2). Proteins were visualized on a 10% SDS-PAGE gel. Molecular weight of P450 BM3 is 119 kDa. An estimated purity of >90% was achieved; (M) Molecular weight standard (Prestained PageRuler, Fermentas).

For enzyme characterization, the initial oxidation rates, the turnover frequencies and the coupling efficiencies were determined as described in chapter 3.10.5. The TOF was determined by GC-quantification of all regioisomers (chapter 3.13.2) and was calculated by taking into account the overall amount of products (1-, 2-, 3-, and 4-heptanol). Figure 4-10 shows an overview of the characterization data.

WT^{NADH} displays a low activity towards *n*-heptane with both cofactors (A: 132 min⁻¹ for NADH) and a moderate coupling efficiency (C: 18% for NADPH). The variants 19A12^{NADH} and CM1^{NADH} prefer NADH over NADPH in contrast to WT^{NADH} (A and B). The oxidation rate of NADH with variant 19A12^{NADH} is 9% higher than with the variant CM1^{NADH} (A). Nevertheless, CM1^{NADH} shows a 24% higher turnover frequency than 19A12^{NADH} (B), which can mainly be attributed to its higher coupling efficiency (C: 44% vs. 32%). Using NADH as cofactor a TOF of 485 min⁻¹ for 19A12^{NADH} and 604 min⁻¹ for CM1^{NADH} was determined. The corresponding specific activities are 4075 U g_{P450}⁻¹ and 5073 U g_{P450}⁻¹ respectively (using a size of 119 kDa per active site).

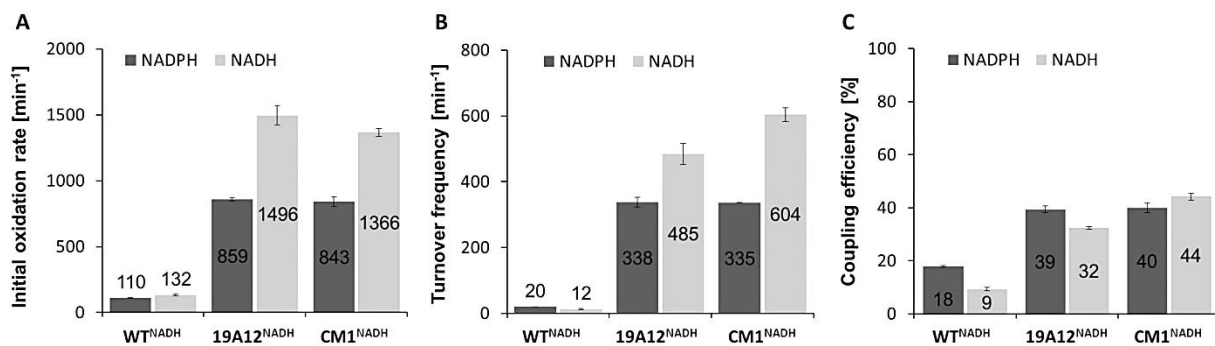


Figure 4-11: Initial oxidation rates, turnover frequencies and coupling efficiencies of WT^{NADH}, 19A12^{NADH}, and CM1^{NADH} monooxygenases with the cofactors NADPH and NADH. All three BM3 variants contain the substitutions W1046S and R966D for switching the cofactor preference from NADPH to NADH. Figures A-C show: (A) Initial cofactor oxidation ($\text{mol}_{\text{cofactor}} \text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$) determined via cofactor consumption, (B) Turnover frequency ($\text{mol}_{\text{product}} \text{mol}_{\text{P450}}^{-1} \text{min}^{-1}$) quantified by GC, and (C) Coupling efficiency (%) as ratio between product formation and cofactor oxidation. Taken from Müller *et al.*^[175]

The characterization results show that for all three P450 BM3 variants the cofactor preference was successfully switched from NADPH to NADH. In the case of variants 19A12^{NADH} and CM1^{NADH} not only the cofactor preference changed but also higher product yields were achieved using NADH as cofactor instead of NADPH (43% improvement for 19A12^{NADH} and 80% for CM1^{NADH}). The catalytic performance of the NADPH-dependent variant BM3 CM1 using NADPH as cofactor (chapter 4.1.2, Table 4-1; 658 min⁻¹, 45%) and the NADH-dependent variant CM1^{NADH} with NADH (Figure 4-11 B, 604 min⁻¹, 44%) is very similar, with values in the same range. The catalytic values of the variants 19A12 (NADPH; 248 min⁻¹; 14%) and 19A12^{NADH} (NADH; 485 min⁻¹; 32%) differ strongly. It has to be noticed, that in the case of the NADPH-dependent variants the characterization was performed with non-purified cell lysates and therefore differences in the catalytic performance can be expected.

The catalytic characterization of BM3 CM1^{NADH} showed also a comparably high activity with *n*-heptane (604 min⁻¹ or 10 s⁻¹ with NADH) as found for other engineered P450 BM3 variants for the oxidation of similar non-natural substrates like *n*-octane (variant 53-5H, TOF of 660 min⁻¹ using NADPH)^[145]. In this regard, a fine compilation of k_{cat} values for P450 BM3 can be found in the review by Whitehouse *et al.*^[58]

4.1.4 Determination of selectivities of NADH-dependent P450 BM3 variants

In synthetic approaches, mainly with regard to oxidations, one of the most remarkable advantages of enzymatic catalysts over chemical catalysts is the intrinsically high selectivity of enzymes.^[38] This is predominantly the case for the selective biotransformation of native

substrates. For engineered enzymes with a broadened substrate scope, as in the case of P450 BM3 with non-natural substrates, the hydroxylation selectivities can differ significantly from the wild type enzyme. After catalytic characterization of the engineered NADH-dependent P450 BM3 variants WT^{NADH}, 19A12^{NADH} and CM1^{NADH}, also the regio- and the stereo-selectivity were determined as described in chapter 3.13.3.

All possible regioisomers resulting from the biocatalytic conversion of *n*-heptane were analyzed in a qualitative and quantitative manner by gas chromatographic separation (chapter 3.13.2). Separation of the regioisomers 1-, 2-, 3- and 4-heptanol was achieved using a column FS-Supreme-5ms as shown in Figure 4-12.

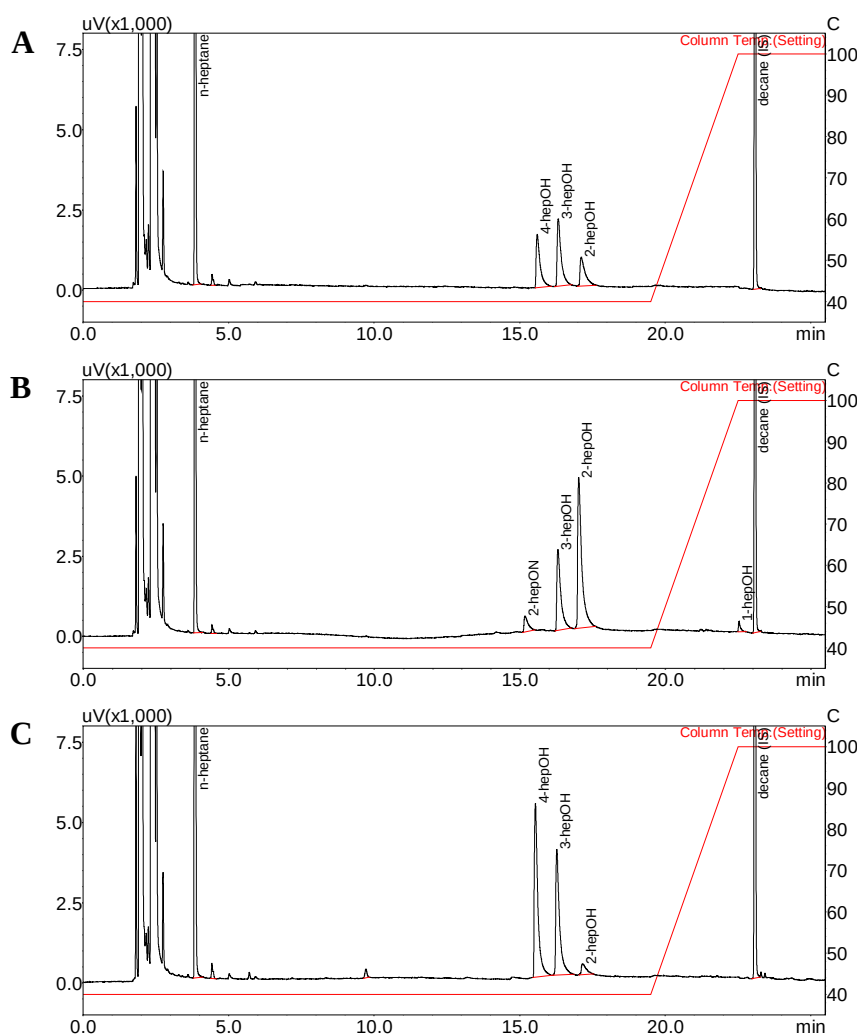


Figure 4-12: Identification of heptanol regioisomers by gas chromatography. Single hydroxylation of *n*-heptane catalyzed by P450 BM3 variants WT^{NADH} (A), 19A12^{NADH} (B) and CM1^{NADH} (C). Separation was achieved using a column FS-Supreme-5ms (30 m x 0.25 mm x 0.25 μ m film thickness; CS-Chromatographie, Germany) fitted in a Shimadzu GC-2010 gas chromatograph with a flame ionization detector (FID) (Shimadzu, Japan). The column oven temperature program was 40 $^{\circ}$ C for 19.5 min, 20 $^{\circ}$ C min⁻¹ to 100 $^{\circ}$ C, 3 min.

Using GC quantification data the regioselectivity was calculated as shown in Figure 4-13. WT^{NADH} is a subterminal hydroxylase with a similar regioselectivity (2-, 3- or 4-heptanol) to the hydroxylation pattern of fatty acids^[58]. Following the same pattern, WT^{NADH} produces all 3 subterminal hydroxylated heptanol regioisomers in amounts higher than 24%. In contrast to the WT, P450 BM3 monooxygenases 19A12^{NADH} and CM1^{NADH} have a modified regiospecificity and a significant hydroxylation preference for two instead of three subterminal carbon atoms. 19A12^{NADH} hydroxylates mainly the C2-atom (64% 2-heptanol) followed by C3 (33% 3-heptanol). CM1^{NADH} produces preferentially 4-heptanol (48%) followed by 3-heptanol (42%) and 2-heptanol (9%).

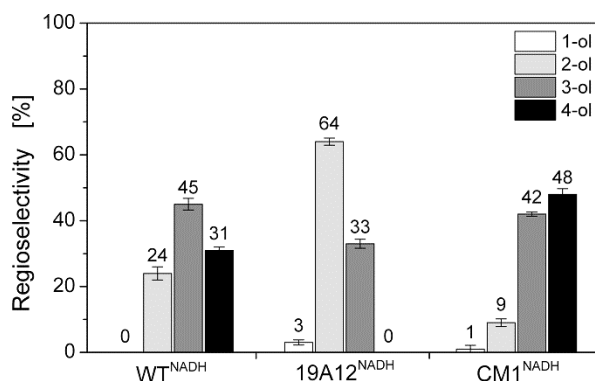


Figure 4-13: Regioselectivity of the *n*-heptane hydroxylation by P450 BM3 monooxygenase WT^{NADH}, 19A12^{NADH} and CM1^{NADH}. The product distribution was calculated for each variant based on GC-quantification of the regioisomers (1-, 2-, 3- and 4-heptanol) and was normalized to 100%. Taken from Müller *et al.*^[175]

The stereoselectivity was determined likewise using a GC-based methodology. The pure enantiomeric standards of 3-heptanol were not commercially available, but racemic 3-heptanol could be separated on the chiral GC column FS-Hydrodex β -TBDAC (for details refer to chapter 3.13.4). The chromatographic separation of the possible hydroxylation and double-oxidation products using the abovementioned chiral GC column is shown in Figure 4-14 A. The absolute configuration of the chiral GC peaks of 3-heptanol was assigned by measuring the polarimetry of each enantiomer in excess. Using the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH)^[158] and the (*S*)-specific ADH from *Rhodococcus erythropolis* (RE-ADH)^[157] for resolution of 3-heptanol, it was possible to obtain an excess of the (*S*)- and (*R*)-enantiomer respectively, as shown in Figure 4-14 B.

For both conversions the enantiomeric excess (*ee*) was calculated and the absolute configuration was confirmed by polarimetry measurements. In the Lb-ADH-catalyzed

resolution of 3-heptanol a high enantiomeric excess of 90.3% was obtained and a rotatory power value of +0.96 was determined (chloroform as solvent, 589.3 nm; 25 °C).

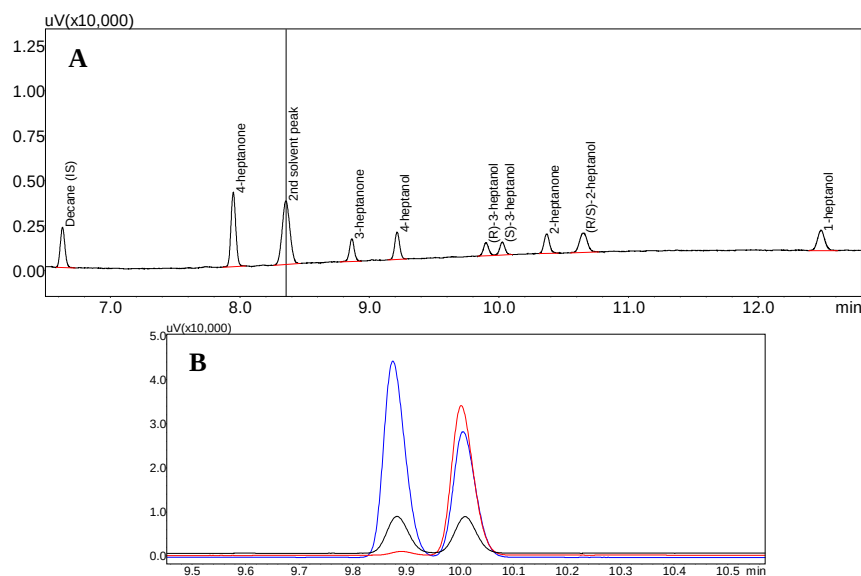


Figure 4-14: Separation of possible *n*-heptane conversion products via GC and absolute configuration of (*R/S*)-3-heptanol. A) Authentic standards of 1-, (*rac*)-2-, (*rac*)-3-, 4-heptanol and 2-, 3-, 4-heptanone. **B)** Chiral resolution of racemic-3-heptanol (black) was performed either with the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis* (Lb-ADH)^[158] (red curve; (*S*)-3-heptanol, 10.0 min, *ee* 90.3%) or the (*S*)-specific ADH from *Rhodococcus erythropolis* (RE-ADH)^[157] (blue curve; (*R*)-3-heptanol, 9.87 min, *ee* 19.8%). Taken from Müller *et al.*^[175]

Through the RE-ADH-catalyzed resolution, an enantiomeric excess of only 19.8% was achieved, corresponding to a rotatory power of -0.01 (chloroform; 589.3 nm; 25 °C). Correlation to the sign of the optical rotation data from literature (-4.9° for (*R*)-(-)-3-heptanol^[170] and +5.8° for (*S*)-(+)-3-heptanol^[171]) revealed, that the first peak (retention time of 9.88 min corresponds to (*R*)-3-heptanol and the second peak (10.01 min) to (*S*)-3-heptanol. The lower optical rotatory values compared to those from literature can be attributed to the lower concentrations obtained by the enzymatic resolution approach and to the lower enantiomeric excess used for the polarimetry measurement. Quantification of the peak areas was used for determination of the enantiomeric ratio (*er*) or excess (*ee*) of the catalyzed hydroxylation reaction (see 3.13.4).

In contrary to 3-heptanol, the 2-heptanol enantiomers could not be separated on the chiral GC column. The enantioselectivity of the P450 BM3-catalyzed hydroxylation of *n*-heptane was determined by a subsequent lipase-catalyzed resolution of the (*S*)-2-heptanol enantiomer. Patel *et al.*^[172] published a method for resolution of racemic 2-heptanol based on the enantioselective

acetylation of the (*R*)-2-heptanol enantiomer by means of incubation with Lipase B and succinic anhydride as acetylating agent. The reaction products were (*R*)-2-heptyl hemisuccinate and (*S*)-2-heptanol, the latter with an *ee* of 99.3%. This protocol was used with some modifications for resolution of 2-heptanol as a product from the P450 BM3-catalyzed hydroxylation reaction, as shown in Figure 4-15. Comparison of the products from the acetylation reaction and a reference reaction without Lipase (no acetylation) allowed the determination of the enantiomeric ratio (*er*) and the enantiomeric excess (*ee*) for each P450 BM3 variant.

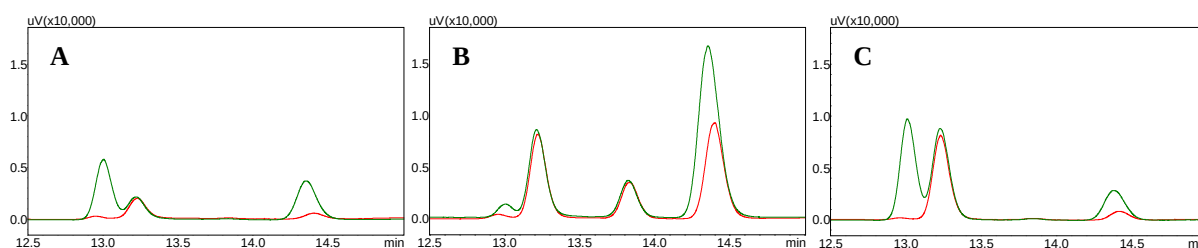


Figure 4-15: Lipase-catalyzed acetylation of (*R*)-2-heptanol. The GC spectra for the P450 BM3 variants WT^{NADH} (A), 19A12^{NADH} (B) and CM1^{NADH} (C) show the products after acetylation of (*R*)-2-heptanol (14.46 min) and (*R*)-3-heptanol (13.0 min) leaving the (*S*)-heptanol enantiomer unreacted (red line). A reference reaction without Lipase (green line) allows for calculation of the enantiomeric ratio. Taken from Müller *et al.*^[175]

The stereoselectivity of P450 BM3 NADH-dependent variants in terms of enantiomeric ratio (*er*) and enantiomeric excess (*ee*) is given in Table 4-2.

Table 4-2: Stereoselectivity of P450 BM3 WT^{NADH}, 19A12^{NADH} and CM1^{NADH} in the hydroxylation of *n*-heptane.

P450 BM3 variant	2-Heptanol		3-Heptanol	
	<i>ee</i> [%]	<i>er</i> (S:R)	<i>ee</i> [%]	<i>er</i> (S:R)
WT ^{NADH}	74 ± 0.1 (<i>R</i>)	13:87	50 ± 3 (<i>R</i>)	25:75
19A12 ^{NADH}	4 ± 3 (<i>S</i>) ^[a]	52:48	86 ± 2 (<i>S</i>)	93:7
CM1 ^{NADH}	46 ± 0.2 (<i>R</i>)	27:73	12 ± 1 (<i>S</i>)	56:44

^[a] High deviation is caused by low differences in the values of enantiomer peaks. Taken from Müller *et al.*^[175]

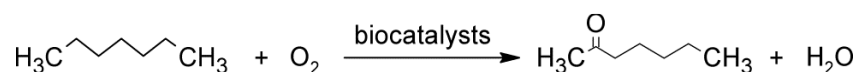
Remarkably, the stereo-preferences for chiral 2- and 3-heptanol differ significantly between WT^{NADH}, 19A12^{NADH}, and CM1^{NADH}. WT^{NADH} produces preferentially (*R*)-heptanols with an *ee* of 49% for (*R*)-3-heptanol and 74% for (*R*)-2-heptanol, whereas 19A12^{NADH} favors the synthesis of (*S*)-3-heptanol in a higher excess of 86% and produces a nearly racemic mixture of 2-heptanol (*ee* 3.9% for (*S*)-2-heptanol). CM1^{NADH} shows also a slight excess of (*S*)-3-heptanol (*ee* 12%) and a clear preference for (*R*)-2-heptanol (*ee* 46%).

In conclusion, WT^{NADH} is (*R*)-selective towards 2- and 3-heptanol, whereas 19A12^{NADH} and CM1^{NADH} are (*S*)-selective with respect to 3-heptanol. In the case of CM1^{NADH}, only two substitutions R255P and P329H are required to partially invert the enzymes stereoselectivity. Additionally, 19A12^{NADH} is poorly stereoselective in the production of 2-heptanol in contrast to the (*R*)-stereoselective WT^{NADH} and CM1^{NADH}.

4.2 *In vitro* double-oxidation of *n*-heptane with direct cofactor regeneration

Monooxygenases are rarely employed in industrial scale, despite their synthetic potential for oxyfunctionalization of alkanes. C-H bond activation with O₂ as clean oxidant has been described as a dream reaction in organic chemistry.^[5a]

Enzymatic cascade reactions for the oxyfunctionalization of alkanes are interesting as an alternative to chemical synthesis routes in case that challenges in cofactor supply, insufficient coupling rates, and stability issues can be addressed successfully. In this regard, significant progress has been achieved to improve stabilities and coupling rates of monooxygenases by rational design and/or directed evolution.^[132b, 146, 185] The concept of the double oxidation cascade, as shown in Scheme 2, using molecular oxygen as sole oxidant and direct regeneration of the cofactor NADH, is synthetically attractive since use of additional cosubstrates can be omitted or at least be reduced.



Scheme 2: Double oxidation of *n*-heptane. Reaction scheme of the biocatalytic direct-oxidation of *n*-heptane to 2-heptanone in aqueous medium, using a monooxygenase-dehydrogenase cascade and oxygen as sole oxidizing agent. Taken from Müller et al.^[175]

For a proof of concept of the biocatalytic one-pot double oxidation cascade, *n*-heptane was selected as substrate. As enzymatic components a P450 BM3 monooxygenase and a (*S*)-enantioselective, NADH-dependent alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) were chosen. Three different P450 BM3 variants were selected to evaluate the concept of the double oxidation cascade. To ensure cofactor compatibility the NADH-dependent P450 BM3 monooxygenases WT^{NADH}, 19A12^{NADH}, CM1^{NADH} were employed. These NADH-dependent variants were previously purified and characterized in detail with regard to activity, to coupling efficiency and to selectivity for the catalyzed hydroxylation of *n*-heptane to 1-, 2-, 3-, and 4-heptanol (see chapter 4.1.3 and 4.1.4). For the variants

19A12^{NADH} and CM1^{NADH} not only the cofactor preference was changed, but also higher product yields using NADH as cofactor were achieved. In the case of 19A12^{NADH} 43% higher product formation was observed; for CM1^{NADH} up to 80%. Subsequently, the activity of the alcohol dehydrogenase RE-ADH towards the oxidation of heptanol-isomers was determined. For a proof of concept, the influence of biotransformation parameters such as the substrate load, the use of cosolvent and additional cofactor regeneration via glucose dehydrogenase (GDH) was studied. To achieve a good performance of the cascade reaction these parameters were optimized. Finally, the catalyzed double oxidation of *n*-heptane was monitored over 24 h to characterize the reaction in terms of absolute product concentrations and total turnover numbers.

4.2.1 Determination of RE-ADH activity

The (S)-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) was selected for the oxidation of 2-heptanol due to its high activity and selectivity.^[158] A protocol for heterologous protein expression in *E. coli* and purification of RE-ADH are published.^[157] The RE-ADH is characteristic for its high thermo-stability up to 65 °C. Therefore, a simple and efficient purification by heat treatment is feasible. A good expression level of RE-ADH was achieved under the described conditions (chapter 3.6.2) and purification by heat treatment was performed (chapter 3.10.6), yielded protein with an estimated purity of >90% as shown in Figure 4-16. The protein content in the purified sample was measured by the method of Bradford using a calibration curve with Bovine Serum Albumin (BSA) as protein standard. A concentration of 8.3 mg mL⁻¹ was calculated.

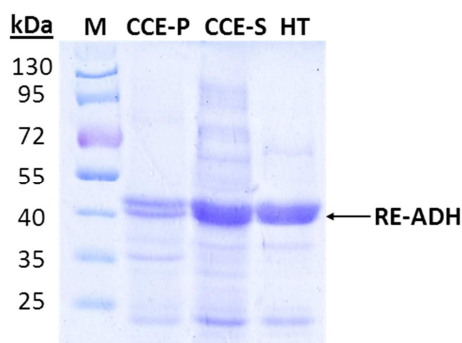


Figure 4-16: Expression of RE-ADH and purification by heat treatment. RE-ADH visualized on a 10% SDS-PAGE gel as soluble protein fraction after expression in *E. coli*. (M): Molecular weight standard (Prestained PageRuler, Fermentas); (CCE-S): crude cell extract, soluble fraction; (CCE-P): Crude cell extract, pellet fraction. After heat treatment (HT) the soluble protein had an estimated purity of >90%.

To investigate the substrate acceptance and preferences of the RE-ADH towards the four heptanol isomers 1-, 2-, 3- and 4-heptanol, the volumetric and specific activity of the purified enzyme were determined photometrically (Table 4-3). The photometric activity measurement was performed at a pH of 8.0 using 0.1 M KPi-buffer, conditions which were used later for the double oxidation cascade reaction. In case of 2- and 3-heptanol the racemic compound was employed for the measurements, whereupon only the (S)-enantiomers are converted by the selective RE-ADH.

Table 4-3: Activity of purified RE-ADH towards heptanol isomers

Substrate	Volumetric activity [U mL ⁻¹] ^[a]	Specific activity [U mg ⁻¹] ^[b]
1-heptanol	11 ± 1	1.3
2-heptanol (racemate)	387 ± 34	46.7
3-heptanol (racemate)	56 ± 2	5.8
4-heptanol	7 ± 0.3	0.9

^[a] The reaction contained 10 mM substrate, 1 mM NAD⁺, 0.1 µL RE-ADH in 0.1 M KPi-buffer (pH 8.0).

^[b] A concentration of 8.3 mg mL⁻¹ was used for calculations.

RE-ADH displays a high preference for (S)-2-heptanol over the other heptanol regioisomers with a specific activity of 47 U mg⁻¹. (S)-3-heptanol is converted to a lesser extent, showing an 8-fold lower specific activity of 6 U mg⁻¹. The activities for the non-chiral isomers 1- and 4-heptanol are 36- and 52-fold lower than with the preferred substrate 2-heptanol. RE-ADH accepts a broad range of substrates, from aliphatic and aromatic ketones or alcohols (also halogenated acetophenones) to β-keto esters. The previously reported specific activities for the reduction of ketones as substrate, for example with 2-heptanone and 2-decanone reach >100 U mg⁻¹, and for *p*-Cl-acetophenone an activity of 846 U mg⁻¹ was determined.^[155, 157] The activities for the oxidation reaction are usually lower, in the case of 2-heptanol displaying a 2-fold decrease. A low *K_M* value for the RE-ADH-catalyzed reduction of 2-heptanone was reported (0.48 mM), representing a high affinity for this substrate.^[186] The *K_M* value for the oxidation of 2-heptanol is not determined yet. However, the same authors reported a *K_M* value for the reduction of *p*-Cl-acetophenone of 0.82 mM, which is higher than the value for the oxidation of *p*-Cl-phenylethanol (0.38 mM), suggesting a higher affinity of the RE-ADH for the alcohol compared to the ketone. These findings could be investigated for 2-heptanol in future studies.

Besides, to characterize more in detail the capacity of the cofactor regeneration by RE-ADH in the double oxidation cascade, the *K_M* and *V_{max}* values for the reduced cofactor form NAD⁺ were determined (Figure 4-17).

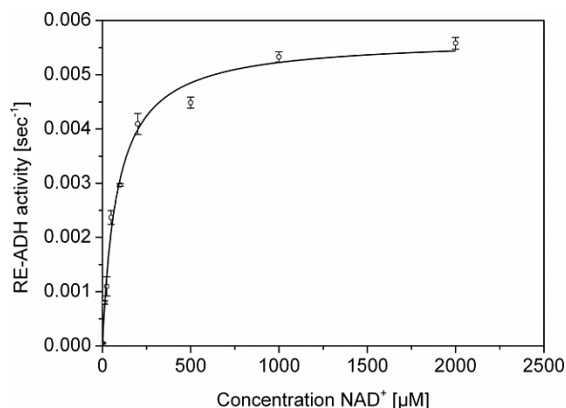


Figure 4-17: Determination of K_M and V_{max} values of the alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) for the cofactor NAD^+ . 2-heptanol was used as substrate (10 mM in 0.1 M KPi-buffer, pH 8.0; 0.18 U RE-ADH). Data was fitted using a Michaelis-Menten plot and Origin 8.6 software (OriginLab Corporation, Northampton, MA, USA). Taken from Müller *et al.*^[175]

The K_M value of the RE-ADH for NAD^+ using 2-heptanol as substrate is $84 \pm 9 \mu\text{M}$ and V_{max} reaches 0.005 s^{-1} . Abokitse measured a K_M value of $90 \mu\text{M}$ for the reduction of NAD^+ with *p*-Cl-phenylethanol as substrate using also the recombinant RE-ADH from *E. coli*.^[186] This value is in good accordance to the K_M value determined for 2-heptanol in this study.

4.2.2 Reaction engineering of the double oxidation cascade

First approaches for the double oxidation of *n*-heptane in one-pot proved that this novel synthesis concept could be successfully applied with the selected enzymatic components (P450 BM3 and RE-ADH) and the selected substrate *n*-heptane. The biocatalytic production of heptanone starting from *n*-heptane was detected by GC. However, total product concentrations with all testes P450 BM3 monooxygenases were low. It was observed, that quantification of absolute products concentrations instead of substrate conversion was more accurate due to possible evaporation of *n*-heptane during the reaction and the sample work-up (boiling point of 98.4°C). To minimize evaporation of *n*-heptane and to ensure the substrate availability in the course of the reaction, tightly closed screw top vials (containing silicon/PTFE sealing disks) were used for each individual reaction sample.

Varying concentrations of RE-ADH from 10 to 40 U mL^{-1} were tested to achieve an optimal ketone to alcohol ratio. A RE-ADH concentration of 10 U mL^{-1} yielded the best result (data not shown). In further experiments the influence of substrate and P450 BM3 concentrations, as well as the use of ethanol as cosolvent were investigated.

Figure 4-18 shows the product concentrations and the corresponding conversion (quantified over product and not over substrate) for the in vitro double oxidation cascade with varying concentration of *n*-heptane as starting substrate. Concentrations from 0.5 mM to 1 M were employed.

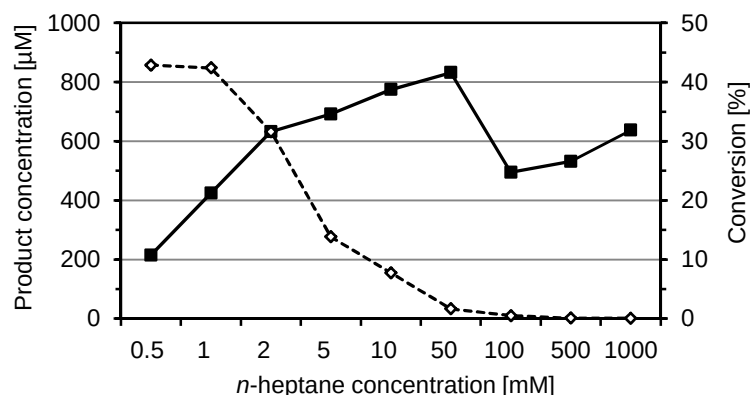


Figure 4-18: Effect of the *n*-heptane concentration on product formation and conversion rates. The total product concentration is the sum of total heptanol and heptanone isomers via GC. The conversion was calculated on the basis of quantified product (ratio of total product to starting concentration of substrate). The reaction contained 0.1 M KPi buffer (1 mL; pH 8.0), 0.2 μM P450 BM3 19A12^{NADH}, 600 U catalase, 10 mM D-glucose, 3 U GDH, 2 mM NADH and varying *n*-heptane concentrations in ethanol (0.5-2% v/v) as cosolvent. For 500 and 1000 mM *n*-heptane no cosolvent was used applying *n*-heptane directly to the reaction mix.

A concentration of 50 mM *n*-heptane yielded the highest product concentration, but only a conversion of 1.7%. A maximal conversion of 43% was achieved with the lowest tested *n*-heptane concentration of 0.5 mM. The biocatalytic system shows up to 50 mM an inverse proportionality between product formation and substrate conversion. For higher concentrations of *n*-heptane (0.1 to 1 M) the product formation declines. In the case of 0.1 M *n*-heptane only 0.5% (v/v) ethanol was used as cosolvent and for the concentrations 0.5 and 1 M no cosolvent was used. The solubility of *n*-heptane in water, with a $\log P_{o/w}$ value of 4 is extremely low (3.4 mg L⁻¹ at 25 °C).^[187] Therefore, a reduced product formation could result from insufficient substrate availability caused by the poor *n*-heptane solubility in the aqueous systems if only low amounts or no cosolvent are used.

The effect of ethanol as cosolvent, to ensure higher substrate solubility in the aqueous reaction phase was investigated further. Product concentrations after hydroxylation of *n*-heptane were compared with and without supplementation of ethanol (1.3% v/v) in the reaction as shown in Table 4-4. The addition of D-glucose and glucose dehydrogenase (GDH) to achieve regeneration of the cofactor NADH during the hydroxylation reaction was also tested.

Table 4-4: Effect of ethanol as cosolvent in the P450 BM3 19A12^{NADH}-catalyzed hydroxylation of *n*-heptane.

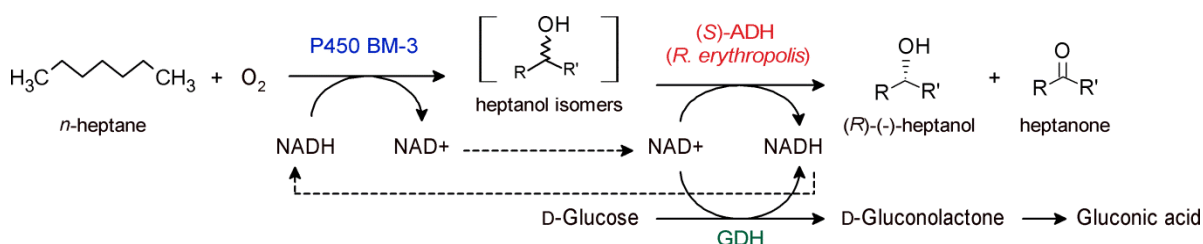
Components ^[a]	Product concentration [μ M]	
	No cosolvent	1.3% (v/v) ethanol
P450 ^[a]	301	519
P450 + GDH ^[b]	461	641

^[a] The reaction contained 50 mM *n*-heptane, 1.3% (v/v) ethanol, 0.2 μ M P450 BM3 19A12^{NADH}, 600 U catalase, 2 mM NADH in 0.1 M KPi buffer (1 mL; pH 8.0) and ^[b] additionally 10 mM D-glucose, 3 U GDH.

The use of ethanol as cosolvent enhances the product formation by 72% when sole P450 BM3 is used and by 39% when the cofactor is additionally regenerated over GDH. Therefore, an amount of 1.3% (v/v) ethanol was selected for the double oxidation cascade.

4.2.2.1 Additional cofactor regeneration using glucose dehydrogenase

In order to explore if the cofactor regeneration is a limiting factor in the double oxidation cascade an additional NADH-regeneration strategy over D-glucose/glucose dehydrogenase (GDH)^[24, 188] was investigated as shown in Scheme 3.



Scheme 3: Double oxidation of *n*-heptane using a P450 BM3 monooxygenase and an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) with supporting cofactor regeneration via glucose dehydrogenase (GDH). In the first oxidation step *n*-heptane is converted by the P450 monooxygenase to heptanol under NADH consumption yielding NAD⁺. In the second oxidation step RE-ADH catalyzes the chiral resolution of the *in situ* produced (*R*)-heptanols and the synthesis of the corresponding ketones under reduction of NAD⁺ to NADH (cofactor regeneration). The conversion of D-glucose to D-gluconolactone enables also the reduction of NAD⁺ to NADH, as a supporting cofactor recycling system. R: CH₃, C₂H₅ or C₃H₇; R': C₅H₁₁, C₄H₉ or C₃H₇. Taken from Müller *et al.*^[175]

Additional cofactor regeneration over glucose/GDH can improve the regeneration capacity and can compensate for cofactor losses as a result of the low coupling efficiencies of P450 monooxygenases, where NADH is utilized for undesired (uncoupling) reactions. GDH regenerates the oxidized cofactor NAD⁺ to NADH by oxidation of D-glucose to glucono-1,5-lactone, which hydrolyzes spontaneously, in dependence of the applied pH and buffer to gluconic acid.^[66] The reaction equilibrium will therefore be shifted to the product side (Scheme

3). The beneficial effect of using GDH in the double oxidation cascade for additional cofactor regeneration was previously confirmed in chapter 4.2.2, Table 4-4. It is important to take into account, that in the presence of RE-ADH and GDH two NAD^+ -consuming reactions run in parallel. The main goal was to achieve complete conversion of the targeted heptanol isomers by the alcohol dehydrogenase, accompanied by sufficient regeneration of the oxidized cofactor NADH. The effect of additional cofactor regeneration is shown in Figure 4-19, where three different systems consisting of the P450 BM3 monooxygenase ($19\text{A}12^{\text{NADH}}$) in combination with the RE-ADH (1), the GDH (2) or both RE-ADH and GDH (3) were analyzed. The sole regeneration of NADH over D-glucose/GDH yields a 1.4-fold higher product concentration (567 μM ; 2) compared to the double oxidation reaction with RE-ADH (402 μM ; 40% heptanol, 60% heptanone; 1). In the coupled reaction consisting of all three enzymatic components – P450 BM3, RE-ADH and GDH – a similar product concentration is achieved (551 μM ; 3). However, in this case, the RE-ADH-catalyzed oxidation of heptanols to the corresponding ketones is nearly suppressed, yielding only a 15 times lower concentration of heptanone (4%) than in the reaction without GDH.

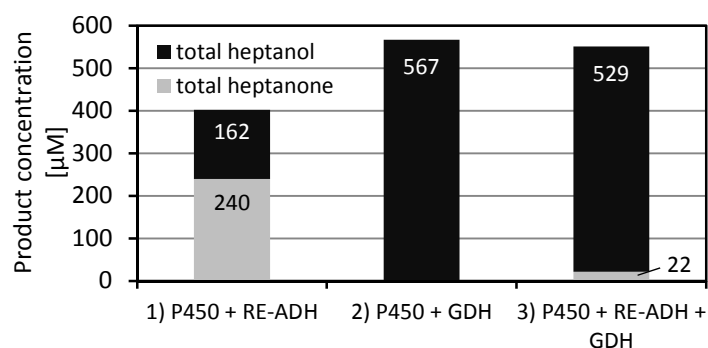


Figure 4-19: Evaluation of glucose dehydrogenase for cofactor regeneration. The reaction contained 10 mM *n*-heptane, 0.1 μM P450 BM3 $19\text{A}12^{\text{NADH}}$, 2.5 U catalase, 10 mM D-glucose and 2 mM NADH in 0.1 M KPi buffer (1 mL; pH 8.0); additionally in (1) 0.2 U RE-ADH, in (2) 0.2 U GDH and in (3) 0.2 U RE-ADH and 0.2 U GDH.

Evaluation of the coupled enzymatic approach with supporting cofactor regeneration over GDH lead to the assumption, that both dehydrogenases compete for the available amount of reduced cofactor NAD^+ . Hence, in order to compare the affinity of GDH and RE-ADH for NAD^+ the corresponding K_M and V_{max} values of GDH were determined in an analogue manner to the determination of the kinetic values for the RE-ADH (see Figure 4-20).

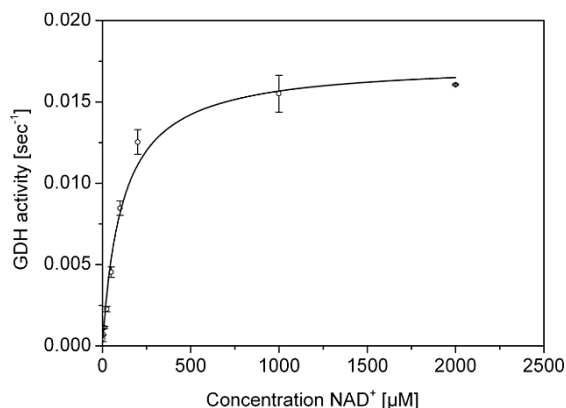


Figure 4-20: Determination of K_M and V_{max} values of the glucose dehydrogenase (from *Pseudomonas* sp.) for the cofactor NAD^+ . D-Glucose was used as substrate (10 mM) in 0.1 M KPi-buffer, pH 8.0; 0.16 U GDH. Data was fitted using a Michaelis-Menten plot and Origin 8.6 software (OriginLab Corporation, Northampton, MA, USA). Taken from Müller *et al.*^[175]

The GDH from *Pseudomonas* sp. shows a K_M of $113 \pm 15 \mu M$ and a V_{max} of $0.017 s^{-1}$ for NAD^+ , when D-glucose is used as substrate. The determined K_M value for NAD^+ is in a similar range to the previously reported value for the GDH from *Bacillus* sp. (Amano Enzyme) of $182 \mu M$ at a pH of 8.0. At a lower pH of 6.4 also a lower K_M value ($77 \mu M$) was measured.^[66]

Compared to the kinetic data of RE-ADH for the oxidation of 2-heptanol (K_M $84 \pm 9 \mu M$, V_{max} $0.005 s^{-1}$, chapter 4.2.1) the GDH displays a slightly lower affinity for NAD^+ but achieves a nearly 3-fold higher oxidation velocity of the cofactor. If a higher concentration of glucose than of 2-heptanol is available, NAD^+ will be most likely consumed faster by the GDH than the RE-ADH. In other words, depending on the substrates availability, the GDH can out-compete the RE-ADH leading to activity loss in the second oxidation step of the enzymatic cascade reaction. Therefore, the effect of different D-glucose concentrations in the cascade reaction was further investigated as shown in Figure 4-21. The graph shows the dependency of product yields and product distribution (total heptanols and heptanones) on the employed concentration of glucose.

Up to a glucose concentration of 2 mM the ratio of heptanone to heptanol remains nearly constant ($71:29\% \pm 1\%$) and a comparably high concentration of total product (1.12 mM) is achieved. A slightly higher product concentration is yielded using 5 mM glucose (1.15), but the overall amount of heptanone is reduced. Higher glucose concentrations (10 to 20 mM) repress entirely the RE-ADH-catalyzed oxidation of heptanols to heptanones.

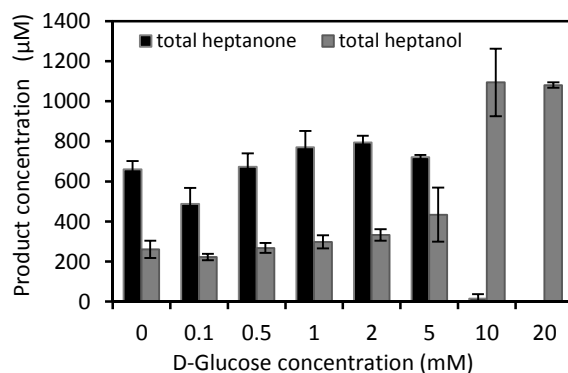


Figure 4-21: Effect of D-glucose concentrations on the double oxidation reaction of *n*-heptane under supplementation of glucose dehydrogenase (GDH from *Pseudomonas* sp.). Concentrations of total heptanol (black) and total heptanone (gray) are displayed as sum of the corresponding regioisomers based on GC-quantification. The reaction contained *n*-heptane (50 mM) in 1 mL 0.1 M KPi buffer (pH 8.0), NADH (2 mM), catalase (600 U), P450 BM3 19A12^{NADH} (0.2 μM), RE-ADH (10 U), GDH (3 U) and D-glucose (varying concentrations from 0.1 to 20 mM). Modified from Müller *et al.*^[175]

These results prove that fine-tuning of the glucose concentration represents a key parameter to ensure sufficient cofactor supply for the enzymatic counterparts. In order to maximize the product formation and shift the reaction equilibrium towards completion of the RE-ADH catalyzed oxidation, a concentration of 2 mM was finally selected for the enzymatic cascade reaction.

4.2.2.2 Study on the inhibitory effect of products

A possible inhibition of the products from the *n*-heptane hydroxylation on the activity of selected P450 BM3 monooxygenase was evaluated. For this experiment the P450 BM3 variant 19A12^{NADH} was chosen. The effect of a pre-incubation step with the regioisomers 2-Heptanol and 2-heptanone, which are the main products of the 19A12^{NADH}-catalyzed double oxidation, was investigated. It was assumed, that the remaining heptanol and heptanone regioisomers will interact in a similar manner with the enzyme. The P450 BM3 enzyme was incubated for 5 min with 1 mM 2-heptanol or 2-heptanone prior to starting the hydroxylation reaction of *n*-heptane in a cuvette (1 mL) by addition of NADH. A reference reaction without a pre-incubation step was run in parallel. Each reaction was monitored photometrically at 340 nm until the cofactor was entirely depleted. The products were extracted for GC analysis (3.13.1 and 3.13.2) to enable comparison of the product formation with and without a pre-incubation step. Initial NADH-oxidation rates (calculated as described in chapter 3.10.5) and product concentrations are shown in Table 4-5.

Table 4-5: Effect of pre-incubation with the double-oxidation products 2-heptanol and 2-heptanone on the activity of P450 BM3 19A12^{NADH}.

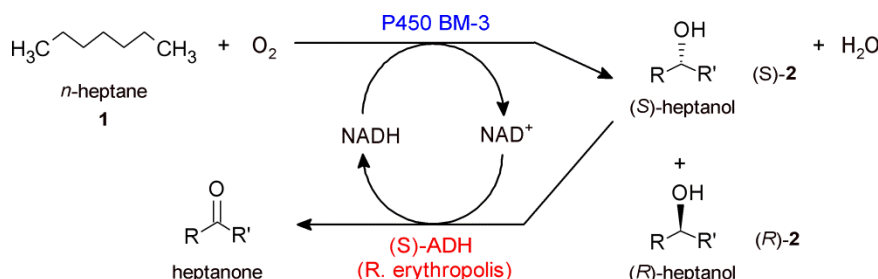
No.	Reaction type	Initial oxidation rate [min ⁻¹]	Product concentration [μM]	
			2-Heptanol	3-Heptanol
1	Standard ^[a]	1928 ± 3	74.3 ± 6.8	33.8 ± 2.7
2	Pre-incubation 2-heptanol	1909 ± 41	68.1 ± 6.2	33.7 ± 1.9
3	Pre-incubation 2-heptanone	1884 ± 21	72.3 ± 6.8	31.3 ± 2.4

^[a] The reaction contained 50 mM *n*-heptane, 1.3% (v/v) ethanol, 0.2 μM P450 BM3 19A12^{NADH} and 0.4 mM NADH in 1 mL 0.1 M KPi buffer (pH 8.0); additionally pre-incubation step with 1 mM 2-heptanol^[b] or 1 mM 2-heptanone^[c].

The product concentrations achieved in the standard reaction (entry 1), as well as in the reaction with a pre-incubation of P450 BM3 with 2-heptanol (entry 2) or 2-heptanone (entry 3) are within a standard deviations of <3.5%. The initial oxidation rates of all three reactions are within a standard deviation rate of less than 1%. Based on these results it can be assumed that a short term inhibition of the P450 BM3 monooxygenase by the *n*-heptane hydroxylation products 2-heptanol and 2-heptanone will not take place.

4.2.3 Double oxidation of *n*-heptane: conversion and total turnover numbers

The reaction in Scheme 4 shows the double oxidation of *n*-heptane, catalyzed by a P450 BM3 monooxygenase and the alcohol dehydrogenase RE-ADH.



Scheme 4: Double oxidation of *n*-heptane using P450 BM3 monooxygenases and an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) as cascade catalysts. In the first oxidation step *n*-heptane (1) is converted by a P450 BM3 monooxygenase (WT^{NADH}, 19A12^{NADH}, and CM1^{NADH}) to heptanol (enantiomeric mixture of (S)/(R)-2) under NADH consumption yielding NAD⁺. In the second oxidation step enzymatic resolution^[28] of the *in situ* produced (*R*)-heptanols ((*R*)-2) and synthesis of the corresponding ketones (3) is achieved under reduction of NAD⁺ to NADH (cofactor regeneration). R: CH₃, C₂H₅ or C₃H₇; R': C₅H₁₁, C₄H₉ or C₃H₇. Taken from Müller *et al.*^[175]

During the initial oxidation reaction *n*-heptane is hydroxylated by P450 BM3 WT^{NADH} or variants (19A12^{NADH}, CM1^{NADH}) to the heptanol isomers (product (S)/(R)-2). In the subsequent step the (S)-specific RE-ADH catalyzes the further oxidation of the preferred (S)-alcohols [(S)-2] to the corresponding ketones (product 3), thus enabling an *in situ* resolution of the

(*R*)-enantiomers (product (*R*)-2). The second oxidation is accompanied by the reduction of NAD^+ to NADH. The regenerated cofactor NADH can be reused by the monooxygenase for hydroxylation of *n*-heptane.

Figure 4-22 shows the performance of WT^{NADH} , $19\text{A}12^{\text{NADH}}$, and $\text{CM}1^{\text{NADH}}$ in the initial hydroxylation step (upper part) and in the double oxidation reaction (lower part).

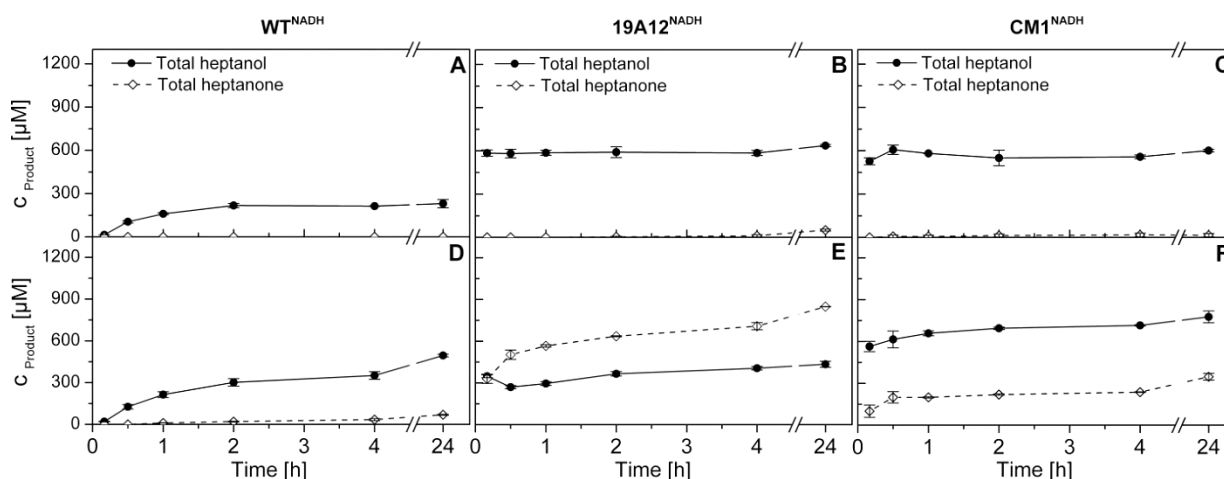


Figure 4-22: Conversion of *n*-heptane to heptanols (initial hydroxylation step; upper part) and conversion of *n*-heptane to heptanones (double oxidation; lower part). Initial hydroxylations are shown for (A) WT^{NADH} , (B) $19\text{A}12^{\text{NADH}}$ or (C) $\text{CM}1^{\text{NADH}}$ and the double oxidations in presence of the RE-ADH: (D) WT^{NADH} , (E) $19\text{A}12^{\text{NADH}}$, and (F) $\text{CM}1^{\text{NADH}}$. Total heptanol (●) and total heptanone (◇) concentrations are shown as sum of the corresponding regioisomers (1-, 2-, 3-, and 4-heptanol) which were quantified via GC after a reaction time of 0.17, 0.5, 1, 2, 4 and 24 h (for details on reaction conditions see chapter 3.11.3). Taken from Müller *et al.*^[175]

Conversion of *n*-heptane in the single hydroxylation reaction with WT^{NADH} , $19\text{A}12^{\text{NADH}}$, or $\text{CM}1^{\text{NADH}}$ stops nearly after 1 hour yielding product concentrations of 0.23, 0.69, and 0.62 mM heptanols respectively. In contrast, during the double oxidation reaction the product formation increases continuously over more than 4 hours. This can be attributed to the NADH cofactor recycling during the double oxidation. A detailed comparison in terms of total product concentrations shows an increase from 0.23 to 0.56 mM for WT^{NADH} (Figure 4-22 A→D), from 0.69 to 1.3 mM for $19\text{A}12^{\text{NADH}}$ (Figure 4-22 B→E), and from 0.62 to 1.1 mM $\text{CM}1^{\text{NADH}}$ (Figure 4-22 C→F).

RE-ADH determinates due to its specificity for the (*S*)-isomers the product ratio between heptanones and heptanols in the coupled reaction. This ratio differs significantly between $19\text{A}12^{\text{NADH}}$ and $\text{CM}1^{\text{NADH}}$. $19\text{A}12^{\text{NADH}}$ /RE-ADH produces twice the amount of heptanones (0.85 mM vs. 0.43 mM heptanol), whereas $\text{CM}1^{\text{NADH}}$ /RE-ADH produces twice the amount of heptanols (0.78 mM vs. 0.35 mM heptanone). $\text{BM}3 \text{ WT}^{\text{NADH}}$ achieves a product concentration

of approximately 0.56 mM when coupled to the alcohol dehydrogenase (Figure 4-22 **D**). Nevertheless, the concentration of heptanones achieved with BM3 WT^{NADH} is very low (69 μ M vs. 0.5 mM heptanol) and differs significantly from 19A12^{NADH} and CM1^{NADH}.

Notably, when starting from the alkane *n*-heptane the double oxidation concept also allows producing (*R*)-alcohols due to the selectivity of the RE-ADH. Table 4-6 summarizes the yields obtained on (*R*)-2- and (*R*)-3-heptanol in the double oxidation cascade. The yields from the enzymatic resolution experiment are calculated based on the overall amount of the chiral ((*R*)/(*S*)-2- and (*R*)/(*S*)-3-heptanol) and their non-chiral products (2- and 3-heptanone), which were set to 100% disregarding the conversion of 1- and 4-heptanol. The highest yields on (*R*)-heptanols were obtained for the BM3 variants WT^{NADH} (89% (*R*)-2-heptanol), followed by CM1^{NADH} (72% (*R*)-2-heptanol). Comparably low yields were achieved with 19A12^{NADH} (51% and 20% (*R*)-2- and (*R*)-3-heptanol respectively).

Table 4-6: Obtainable yields for the RE-ADH catalyzed resolution of (*R*)/(*S*)-heptanols in the 2nd step of the double oxidation cascade. Taken from Müller *et al.*^[175]

	Yield [%] ^[a]	
	(<i>R</i>)-2-Heptanol	(<i>R</i>)-3-Heptanol
P450 BM3 WT^{NADH}	89 $\pm$ 1	82 $\pm$ 2
P450 BM3 19A12^{NADH}	51 $\pm$ 3	20 $\pm$ 1
P450 BM3 CM1^{NADH}	72 $\pm$ 3	58 $\pm$ 3

^[a] See Table 4-2 for details on the theoretical composition (enantiomeric ratio *er*) of the heptanol mixture which serves as substrate. Conversion of (*S*)-2-heptanol and (*S*)-3-heptanol by RE-ADH was determined to be >99%.

The theoretical composition of the heptanol mixture from the initial hydroxylation step, which serves as substrate for the RE-ADH-catalyzed second oxidation, should be equivalent to the enantiomeric ratio (*er*) values in Table 4-2. However, the calculated yields for the resolution of (*R*)-heptanols in the cascade reaction are in most of the cases higher than the expected values from the single, non-coupled hydroxylation reaction (Table 4-6 vs. Table 4-2). The biggest differences are found for the resolution of (*R*)-3-heptanol in the cascade with 19A12^{NADH} and CM1^{NADH}. Here, 32% and 185% more (*R*)-heptanol was yielded respectively, compared to the enantiomeric ratios achieved in the single hydroxylation reaction. The differences are probably caused by a shift in the product distribution and reaction equilibrium that takes place during the double oxidation, due to the continuous regeneration and therefore higher availability of the cofactor.

Figure 4-23 shows the results of the double oxidation with WT^{NADH}, 19A12^{NADH}, and CM1^{NADH} in presence of the D-glucose/GDH-regeneration system. The produced NAD⁺ during

the initial oxidation step is utilized simultaneously by the RE-ADH and the GDH for the second coupled oxidation and regeneration step.

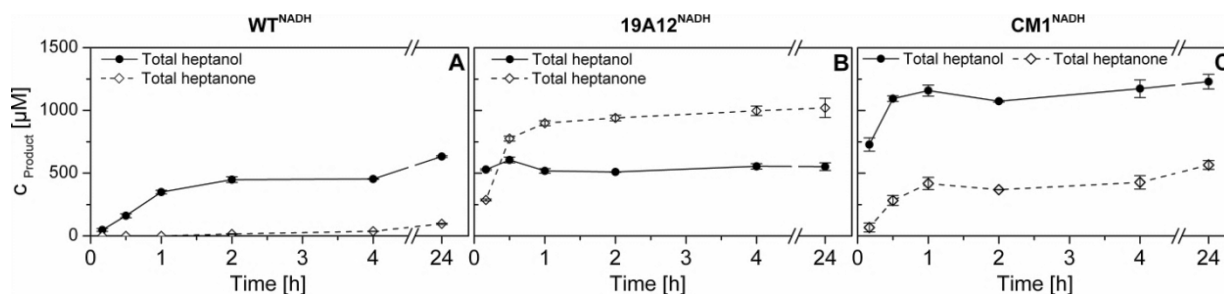


Figure 4-23: Double oxidation of *n*-heptane with a supporting regeneration system (D-glucose/GDH) for the cofactor NADH. Figures A-C show: (A) BM3 WT^{NADH}, (B) 19A12^{NADH} and (C) CM1^{NADH}. Total heptanol (●) and total heptanone (◇) concentrations are displayed as sum of the corresponding regioisomers which were quantified via GC after a reaction time of 0.17, 0.5, 1, 2, 4 and 24 h (for details on reaction conditions see chapter 3.11.3). Taken from Müller *et al.*^[175]

The total product concentrations in the double oxidation cascades with supporting cofactor regeneration (D-glucose/GDH) are significantly higher. In the case of BM3 WT^{NADH} a maximal product concentration of 0.73 mM is obtained (215% higher than without GDH; Figure 4-23 A). The variants 19A12^{NADH} and CM1^{NADH} produced 1.57 and 1.8 mM total product, which correspond to a 129% and 188% increased product formation (Figure 4-23 B and C). Product distribution patterns in the double oxidation cascades as well as conversion and yields in the second oxidation step remained within standard deviations in presence and absence of the D-glucose/GDH regeneration system.

Based on the quantified product concentrations after a reaction time of 24 hours, total turnover numbers (TTN) were determined for the single and the double oxidation reactions as well as for the double oxidation reactions with supporting cofactor regeneration (D-glucose/GDH) and are given in Table 4-7. The employed monooxygenases (WT^{NADH}, 19A12^{NADH}, CM1^{NADH}) displayed a >1.8-fold improved TTN (entry 2) in the double oxidation cascades compared to the single hydroxylation reaction in absence of RE-ADH (entry 1 and 2).

Supplementation of a NADH regeneration system with D-glucose as a cosubstrate and GDH yielded higher product concentrations for all three BM3 variants and consequently TTNs increased up to 3-fold (entry 1 vs. 3). 19A12^{NADH} and CM1^{NADH} achieved comparably high TTN values (up to 3591) which are under all tested reaction conditions more than 2-fold higher than the corresponding TTN-values of WT^{NADH}.

Table 4-7: Total turnover numbers of *n*-heptane oxidation with P450 BM3 WT^{NADH}, 19A12^{NADH} and CM1^{NADH}. Reaction in absence of RE-ADH (entry 1), in presence of RE-ADH (entry 2) and in presence of RE-ADH with additional NADH regeneration (GDH/D-glucose; entry 3). Taken from Müller *et al.*^[175]

No.	Components	WT ^{NADH}	TTN ^[a]	
			19A12 ^{NADH}	CM1 ^{NADH}
1 ^[b]	only P450	462 ± 56	1374 ± 2	1247 ± 30
2 ^[c]	P450 + RE-ADH	1128 ± 27	2514 ± 92	2196 ± 167
3 ^[d]	P450 + RE-ADH + GDH	1457 ± 24	3146 ± 213	3591 ± 186

^[a] TTN: Total Turnover Number ($\mu\text{mol}_{\text{Total product}} \mu\text{mol}_{\text{P450}}^{-1}$); total product is the sum of all quantified heptanol and heptanone isomers (24 h).

^[b] The reaction mixture contained *n*-heptane (50 mM) in KPi buffer (1 mL, 0.1 M; pH 8.0), ethanol (1.3% v/v), NADH (2 mM), catalase (600 U), P450 BM3 (0.5 μM).

^[c] Additionally to entry 1: RE-ADH (10 U).

^[d] Additionally to entry 1: RE-ADH (10 U), D-glucose (2 mM), GDH (1 U).

19A12^{NADH} was selected as reference based on its catalytic performance for the conversion of *n*-heptane (18 substitutions) showing a TTN of 5360 ± 1020 , which is to the best of our knowledge the highest reported value for biocatalytical hydroxylation of this compound.^[59] The total turnover numbers for 19A12^{NADH} reached in this study up to 3150. Differences to the previously reported TTN might be attributed to the altered cofactor specificity (NADH instead of NADPH) and the employed regeneration system (GDH vs. isocitrate dehydrogenase) as well as the reaction conditions.

The catalytic characterization of BM3 CM1^{NADH} showed a comparably high activity with *n*-heptane (TOF value of 604 min^{-1} or 10 s^{-1} with NADH; Figure 4-11) as found for other engineered P450 BM3 variants for the oxidation of non-natural substrates like octane (variant 53-5H, TOF of 660 min^{-1} using NADPH)^[145]. A fine compilation of k_{cat} values for P450 BM3 is reported in the review by Whitehouse *et al.*^[58]

The oxyfunctionalization of alkanes under mild conditions was summarized in a recent review.^[4] Oxidation of *n*-heptane to the corresponding alcohols and ketones was reported for a Mn(TDCPP)Cl-complex together with H₂O₂ as oxidant with a TTN of 202.^[4, 189] Total turnover numbers for 19A12^{NADH} and CM1^{NADH} are 15-fold higher (see Table 4-7) and the modular concept with a RE-ADH allows in addition to produce chiral alcohols.

Some general trends for the described double oxidation cascade transformations of *n*-heptane were identified. Coupling of two oxidation reactions with direct cofactor regeneration boosts the product formation by more than 1.8-fold (up to $1.3 \text{ mM}/150 \text{ mg L}^{-1}$ total product; Figure 4-22). The cofactor recycling during the second oxidation step enables an improved product formation, which is reflected in higher total turnover numbers (up to 2500) compared to the single hydroxylation reaction. The product formation can however be further increased (3-fold)

to 1.8 mM/ 207 mg L⁻¹ by means of a supporting cofactor regeneration system (Figure 4-23); in case of D-Glucose/GDH TTNs up to 3500 without changes in the stereo-selectivities were obtained (Table 4-7).

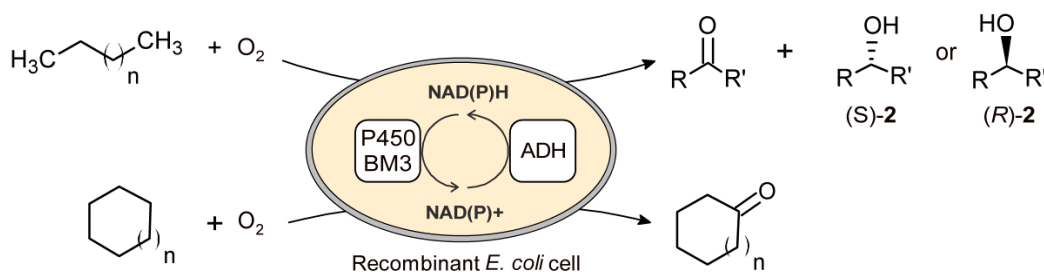
These results demonstrate that the direct regeneration of NADH through enzyme-coupling compensates the losses of the cofactor due to the uncoupling of the monooxygenase-catalyzed oxidation. The coupling efficiencies of P450 BM3 achieved in this work are comparable to those of previous evolutionary studies for non-natural substrates which are often below 80%.^[132b, 137, 177a, 190] Nevertheless, enhancing coupling efficiency of monooxygenases and thereby reducing the amount of generated hydrogen peroxide remains an important challenge to improve process stability and total turnover numbers of P450s in general. The coupling efficiency of P450 BM3 was significantly improved by protein engineering for NADH from 9 to 44% in this study, and up to 95% (NADPH) in others.^[146] The regioselectivity of P450 BM3 could also be improved significantly for non-native substrates like limonene^[191], steroids^[192], and 2-aryl-acetic acid esters^[193]. In this regard, tuning of P450 enzymes for non-native transformations was recently reviewed in a comprehensive way by Fasan.^[194]

The stereo-preferences of the employed ADH opens up the possibility of a non-racemic resolution of enantiomers starting from a *n*-alkane substrate and yielding (*R*)- or (*S*)-alcohols as value added products in double oxidation cascade reactions. The specificity of the RE-ADH ((*S*)-selective), which accepts only (*S*)-alcohols for NADH production reduces the amount of regenerated cofactor. In an ideal case, two enzymes with matching *K_M*-values and specificities, and a highly coupled cofactor oxidation would be sufficient to overcome constraints in the cofactor availability.

4.3 Whole cell double oxidation of alkanes by P450 BM3 monooxygenases and alcohol dehydrogenases

The use of whole cell catalytic approaches can enable a cost-effective and simpler route for the production of key-intermediates in organic synthesis.^[25] Compared to an *in vitro* reaction system the whole cell system provides through compartmentalization a natural environment for the enzyme under physiological conditions, resulting in a higher stability of the catalyst.^[75] A further striking advantage of whole cells over *in vitro* systems is the intracellular availability of redox equivalents and the possibility to regenerate cofactors by mean of the cells metabolism. In particular, oxidation reactions that require the regeneration of a cofactor or multi-enzymatic pathways can be operated very efficiently in whole cells.^[76] The aim of the following study was to establish and investigate a whole cell system for the one-pot double oxidation of

alkanes. In chapter 4.2 the coupled oxidation of *n*-heptane by means of a P450 BM3 monooxygenase and the alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) was described.^[175] The concept was extended by generating an *E. coli* production host for coexpression of both catalysts in a single cell. The generated whole cell catalyst should be employed in the multi-enzymatic double oxidation of alkanes as shown in Scheme 5.



Scheme 5: Whole cell double oxidation of alkanes using a multi-enzymatic cascade reaction.

In this alternative process only a few components are required: resting cells in an aqueous medium (catalyst), glucose for cofactor regeneration, oxygen as oxidizing agent and the target substrate. The double oxidation enables the direct synthesis of ketones or chiral alcohols that represent important building blocks for the pharmaceutical, agrochemical and food industry.^[28] For instance, aliphatic ketones are employed in the production of flavors and fragrances and cycloalkanones in the manufacture of synthetic polymers (polyamides).^[43] Different coexpression strategies were compared, among them a novel system for coexpression of two recombinant genes as a fused operon. The system was expanded to a second alcohol dehydrogenase, an (*R*)-selective NADPH-dependent ADH from *Lactobacillus brevis* (Lb-ADH)^[158] with complementary selectivity to the RE-ADH. By applying two stereo-complementary alcohol dehydrogenases a complete oxidation of the intermediate alcohols to the alkanones is strived for. In this way, the multi-enzymatic concept can be used similar to a biocatalytic tool-box, either for synthesis of chiral alcohols or for production of pure ketones. The biocatalytic cascade consisting of an initial oxyfunctionalization of the unsaturated hydrocarbons of *n*-heptane and cyclooctane as model substrates, and a second oxidation to the alkanones was evaluated using the described system.

4.3.1 Coexpression of NADH-dependent P450 BM3 monooxygenases and alcohol dehydrogenases

The heterologous coexpression of multiple proteins in a single host allows the production of a whole cell catalyst containing all enzymatic components that are necessary for a biocatalytic cascade. The P450 BM3 variant CM1^{NADH}, together with RE-ADH were chosen as target genes to determine the optimal coexpression strategy for the designed biocatalytic cascade (Scheme 5). Part of the preliminary work in chapter 4.3.1, 4.3.2 and 4.3.3 was accomplished by Tim Welters within the frame of his bachelor thesis.^[195]

Three distinctive coexpression strategies were tested. Figure 4-24 shows the properties of each generated vector construct that was used for coexpression of the two genes of interest.

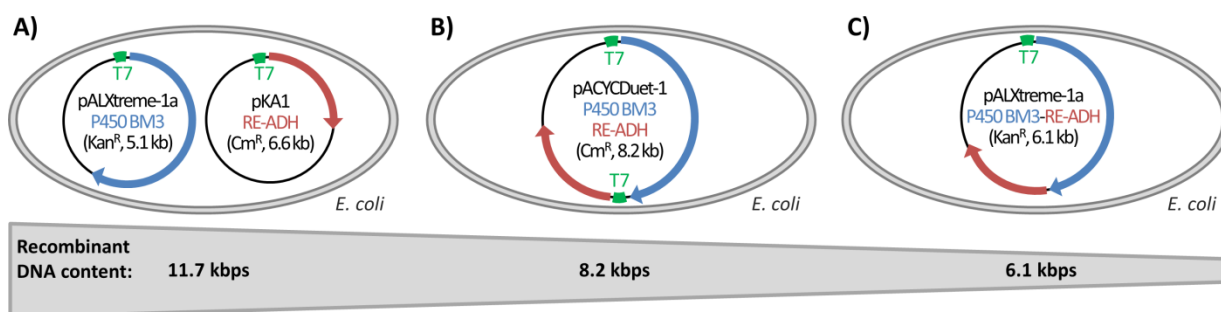


Figure 4-24: Different strategies for coexpression of two genes of interest (P450 BM3 monooxygenase and RE-ADH). The three investigated systems are: 2-plasmid-system (A), commercial pACYCDuet-system (B), operon-fusion system (C). The amount of recombinant DNA transformed in an *E. coli* cell as expression host is given in kbps.

A first strategy consisted in the transformation of two expression plasmids in *E. coli*. The plasmids pALXtreme-1a (containing P450 BM3) and pKA1 (containing RE-ADH) were selected (vector maps, appendix Figure 6-1 and Figure 6-2) as two-plasmid expression system. Compatibility of the selected vectors for coexpression in one host strain is ensured by the different origin of replication (replicon) and antibiotic resistance in both plasmids (see chapter 3.4, Table 3-2).

As a second strategy the commercial vector pACYCDuet-1 (Merck, Darmstadt, Germany) was employed.^[159] This vector comprises two expression regions each consisting of a T7 promotor/lac operator and a separate ribosome binding site (RBS), followed by a multiple cloning site (MCS1 and MCS2) and a terminator region for simultaneous expression of two proteins (vector map, appendix Figure 6-3).

The third coexpression strategy is based on the generation of a synthetic operon-fusion or polycistronic plasmid^[196], for simultaneous expression of both proteins regulated by a single T7 promoter, operator and terminator originated from the pALXtreme-1a vector (vector map, Figure 3-1). Figure 4-25 shows a simplified representation of the constructed operon-fusion vector.

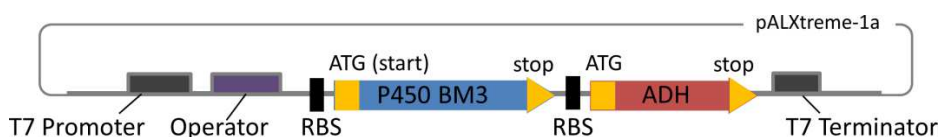


Figure 4-25: Schematic representation of the operon-fusion vector construct for coexpression of a P450 BM3 monooxygenase and an alcohol dehydrogenase. RBS: ribosome binding site.

The expression of all systems is induced by supplementation of IPTG, resulting in case of the operon-fusion construct (Figure 4-25) in the transcription of a single polycistronic mRNA that contains the information for both the P450 monooxygenase and the ADH. The polycistronic mRNA harbors two ribosome binding sites for translation of the single proteins, each of them upstream of the start codon in 6 bps distance. The described synthetic operon-fusion resembles therefore a simplified bacterial operon.^[197]

The generated strains were cultivated in shake flask and the whole cells were subsequently analyzed in regard to the expression level of P450 BM3 and ADH (Figure 4-26). Furthermore, the catalytic performance of the whole cell catalyst in the one-pot double oxidation of *n*-heptane was investigated (Figure 4-27).

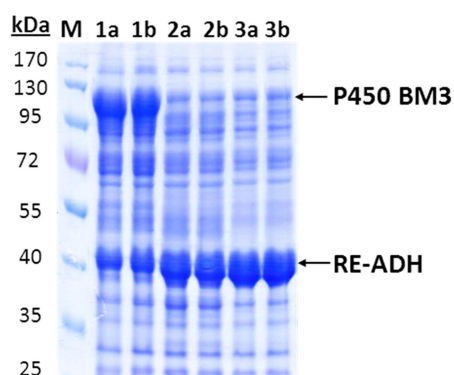


Figure 4-26: Coexpression of P450 BM3 CM1^{NADH} and RE-ADH. Protein expression from two independent shake flask experiments (a) and (b) visualized on a 10% SDS-PAGE gel (lysate from 0.6 mg cell wet weight per lane). Coexpression strategies: (1) operon-fusion, (2) pACYCDuet-1-vector system, (3) 2-plasmid system; (M) Molecular weight standard (Prestained PageRuler, Fermentas).

A rather balanced over-expression of P450 BM3 CM1^{NADH} and RE-ADH was achieved with best results using the operon-fused construct (pALXtreme-1a P450 BM3-RE-ADH) as shown in Figure 4-26 (1a and 1b). The ADH was expressed in higher amounts with the 2-plasmid and the pACYCDuet-1 system. However, this two vector constructs (2-plasmid system and pACYCDuet-1) showed a significantly lower expression of the threefold larger P450 BM3 monooxygenase (119 kDa) compared to RE-ADH (39 kDa). The catalytic activities of P450 monooxygenases are in general significantly low compared to other enzyme classes.^[41b] RE-ADH shows a considerably higher activity of 47 U mg⁻¹ for the oxidation of 2-heptanol compared to the P450 BM3 CM1^{NADH} activity of 5 U mg⁻¹ for the hydroxylation of *n*-heptane (chapter 4.2.1 and 4.1.3). Therefore, it was important to ensure a high concentration of the P450 monooxygenase in the whole cell catalyst to avoid a rate limiting initial oxidation step. To determine the intracellular concentration of P450 BM3 in each coexpression strain the yield of active P450 BM3 protein $Y_{P450/x}$ (in mg_{P450} g_{CDW}⁻¹; x = cell dry weight) was calculated. The product yields on catalyst ($Y_{p/x}$) for the double oxidation of *n*-heptane and the P450 yields ($Y_{P450/x}$) are shown in Figure 4-27. These values were calculated for each strain after expression in shake flask, as well as for one selected construct (P450 BM3 CM1^{NADH}-RE-ADH, operon-fusion) after expression in the fermentor under controlled growing conditions (see chapter 3.8).

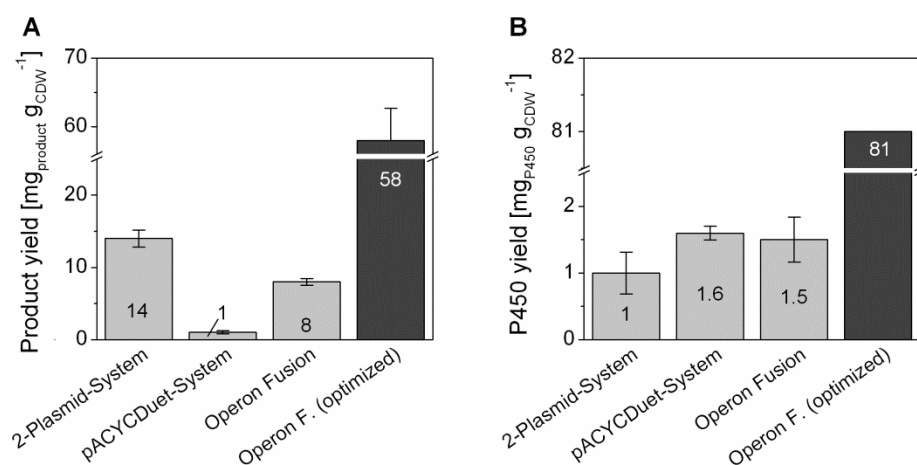


Figure 4-27: Comparison of three different coexpression strategies of P450 BM3 CM1^{NADH} and RE-ADH in *E. coli* cells. *E. coli* BL21(DE3) Gold (lacI^{Q1}) containing three different constructs (see Figure 4-24), expression in shake flask (light gray) and in Fermentor (operon-fusion, dark gray); Product yields (A) and P450 yields (B) were calculated in relation to the cell dry weight. Reaction conditions: Resting cells in 0.1 M KPi buffer pH 8.0 (1 mL; OD₆₀₀ 40), 10 g L⁻¹ D-glucose, 200 μL *n*-heptane, 51 h stirring at RT.

The expression in shake flask yielded for the pACYCDuet and the operon-fusion constructs higher P450 BM3 content per cell dry weight, as determined by quantification *via* CO-

difference spectroscopy (1.5 and $1.6 \text{ mg}_{\text{P450}} \text{ g}_{\text{CDW}}^{-1}$; Figure 4-27 B). On the contrary, the corresponding product yields of 1 and $8 \text{ mg g}_{\text{CDW}}^{-1}$ were lower than the yield of $14 \text{ mg g}_{\text{CDW}}^{-1}$ achieved with the 2-Plasmid system (Figure 4-27 A). Besides, the analysis by SDS-PAGE electrophoresis of expression for the operon-fusion construct P450 BM3 CM1^{NADH}-RE-ADH in shake flask did not correlate entirely with the measured concentration by CO-difference spectroscopy (Figure 4-26 vs. Figure 4-27 B). A possible reason for the differing result would be the occurrence of a high content of inactive (miss folded) monooxygenase, which would not be detected by the CO-spectroscopic measurement. The use of the 2-Plasmid system showed however differences compared to the operon-fusion system. It was observed, that the growth of the *E. coli* strain containing two plasmids was nearly twofold slower than the strains containing only one plasmid (pALXtreme-1a or pACYCDuet) and the amount of biomass produced was nearly 2-fold lower. For cultivation, expression and maintenance of both plasmids two antibiotics are required, not only increasing the metabolic burden of the host cell but also the production costs of the catalyst. The synthesis of high levels of antibiotic resistance genes along with the targeted genes requires considerable amounts of energy, debilitating the cell and lowering the cell density.^[198] The negative effects on growth of two co-transformed plasmids encoding each one antibiotic resistance genes on the growth of *Pseudomonas putida* cells was reported before.^[199] In the case of the pACYCDuet-vector construct the poor product yield achieved ($1 \text{ mg}_{\text{product}} \text{ g}_{\text{CDW}}^{-1}$, Figure 4-27 A) renders its application not feasible. This result is surprising since the P450 yield in the pACYCDuet-vector system and the operon-fusion system is very similar (1.6 vs. $1.5 \text{ mg}_{\text{P450}} \text{ g}_{\text{CDW}}^{-1}$; Figure 4-27 B). Other properties such as the concentration of active ADH or the effect of antibiotics on the cells metabolism could have a strong influence on the catalytic performance of each coexpression strain.

Coexpression of the target proteins or also other proteins using the described operon-fusion strategy has several advantages over other coexpression methods. Using the described ligase-independent cloning technology (PLICing)^[153] the insertion of target genes in the expression vector is not dependent on the availability or compatibility of restriction sites, as in the case of restriction cloning methods^[196]. In addition, multiple genes can be cloned in only one step while consecutive steps are required for restriction cloning. Moreover, several genes can be expressed using only one plasmid, thereby reducing the metabolic burden on the host cell. It was demonstrated, that a higher plasmid copy number increases the metabolic load and more energy is required for DNA maintenance.^[200] As an example, the relative specific growth rate of *E. coli* HB101 cells was correlated to the DNA content and plasmid copy number. The growth rate decreased from 1.0 to 0.92 upon transformation of one plasmid (12 copies) and to 0.91 when two plasmids were transformed (24 copies).^[201] It was also reported, that due to

higher plasmid content the cell increases significantly the concentration of several enzymes involved in the citric acid cycle to replenish the intermediates required for nucleotide synthesis.^[200] A small plasmid size, as in the case of the employed vector pALXtreme-1a, not only reduces the energetic burden on the host but also leads to higher cell density during cultivation^[198b]. The recombinant DNA content is in the case of the 2-plasmid-system nearly 2-fold higher (11.7 kbps) than the DNA content of the operon-fusion system (6.1 kbps; Figure 4-24). As surplus, higher transformation efficiencies are achieved using smaller plasmids for gene cloning.^[153, 202] This becomes more evident when more than three enzymes have to be clones in one vector. The lower metabolic burden and energy requirement could be from significant benefit for the application of operon-fusion systems in biotransformations with growing cells.

4.3.2 Fermentation of operon-fusion strains

The three *E. coli* BL21 (DE3) Gold lacI^{Q1} strains containing the different monooxygenases as operon-fusion vector system, P450 BM3 WT^{NADH}-RE-ADH, 19A12^{NADH}-RE-ADH and CM1^{NADH}-RE-ADH, were selected for cultivation and expression under controlled conditions in a bioreactor. During the fermentation important cultivation parameters such as dissolved oxygen (pO₂) and pH were monitored online (appendix, Figure 6-8). The pH was kept at all times constant at 7.1. Cell densities (measured as OD₆₀₀) and expression levels of P450 BM3 and RE-ADH by SDS-PAGE electrophoresis were determined during the entire fermentation process. In all three fermentations an OD₆₀₀ of 18 to 19 was achieved after 26 hours of induced protein expression (in total ~30 h run time). This value corresponds to a biomass concentration of nearly 4 g_{CDW} L⁻¹. After induction the expression of P450 BM3 increased gradually throughout the entire course of fermentation as monitored by SDS-PAGE analysis (appendix Figure 6-9). The expression level of RE-ADH was in contrast rather constant until 8 h after induction and was slightly higher at the end of the fermentation (21 h). The monooxygenase expression level in the operon-fusion strain CM1^{NADH}-RE-ADH was the highest of all three strains. A final cell concentration of 3.86 g_{CDW} L⁻¹ containing a P450 BM3 concentration of 0.31 g_{P450} L⁻¹ (2.64 μM) was determined for this strain. This represents a specific protein formation of 683.5 nmol_{P450} g_{CDW}⁻¹ (P450 yield Y_{P450/x} of 81.3 mg_{P450} g_{CDW}⁻¹). The specific protein formation that was determined for this strain is slightly higher (6%) than the value of 644 nmol_{P450} g_{CDW}⁻¹ that was reported by Pflug *et al.* for the heterologous expression of P450 BM3 WT in *E. coli* (BL21(DE3) pET28a(+)-system) in a fed-batch fermentation process.^[203] This is to best of our knowledge the highest previously reported production yield for this

enzyme. However, the batch-fermentations presented in this study yielded 5-fold lower final cell mass concentrations ($3.8 - 4 \text{ g}_{\text{CDW}} \text{ L}^{-1}$). In the reported fed-batch fermentation process the reported cell concentration of $19.8 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ was achieved by inducing protein expression at a significantly higher optical density and using an optimized feeding and aeration strategy.^[203]

Through controlled fermentation it was possible to enhance significantly the P450 BM3 content in the cells, in case of P450 BM3 CM1^{NADH}-RE-ADH by 54-fold compared to the shake flask cultivation as shown in Figure 4-27 A ($81 \text{ vs. } 1.5 \text{ mg}_{\text{P450}} \text{ g}_{\text{CDW}}^{-1}$). The translational coupling of both genes using a single operon is beneficial to achieve a high expression level of both enzymes, probably through a ribosome-mediated positive regulation of the translation event.^[204] This means that a positive effect can be observed when the translation of neighboring genes is tightly coupled. One possible mechanism for positive regulation would be that ribosomes terminating translation of the first gene may be able to re-initiate translation on the second gene.^[204b] An improved expression in the fermentor compared to the one in shake flask is probably the result of controlled pH and stirring, allowing to adjust a constant pH, as well as a homogenous mixing of the culture broth and a higher aeration rate.

Using the maximal specific activity of $5073 \text{ U g}_{\text{P450}}^{-1}$ for P450 BM3 CM1^{NADH} as reference value (purified enzyme, see chapter 4.1.3) it is possible to calculate the theoretical specific activities of the whole cell catalyst. The fermentation of the operon-fusion construct enabled the production of cells with a theoretical specific activity of $411 \text{ U g}_{\text{CDW}}^{-1}$. Compared to a theoretical specific activity of $7.6 \text{ U g}_{\text{CDW}}^{-1}$ for cells from shake flask expression, the gain in activity through overexpression of the protein by means of fermentation is impressive.

Using the cell mass from fermentation in the biotransformation of *n*-heptane the product yield was increased by 7-fold from 8 to $58 \text{ mg g}_{\text{CDW}}^{-1}$ (Figure 4-27 B). The increase in product yield $Y_{\text{p/x}}$ is nearly proportional to the increase in P450 yield $Y_{\text{P450/x}}$. This result highlights the importance of optimizing the heterologous expression of the monooxygenase in the employed production host to achieve higher specific activities. Duetz *et al.* estimated that the theoretical specific activities of intact cells containing NAD(P)H-dependent oxygenases with high k_{cat} values ($5\text{-}75 \text{ s}^{-1}$) could reach a value between 150 and $4500 \text{ U g}_{\text{CDW}}^{-1}$.^[75] In a recent study Blank *et al.* estimated an activity of $367 \text{ U g}_{\text{CDW}}^{-1}$ for redox-reactions in resting *E. coli* cells, under consideration of the maintenance energy demands and NAD(P)H regeneration via glucose with a maximal glucose uptake rate of $2.4 \text{ mmol g}_{\text{CDW}} \text{ h}^{-1}$.^[68] It was pointed out that in the practice activities significantly higher than $500 \text{ U g}_{\text{CDW}}^{-1}$ have not been reached so far.^[75-76] Therefore, further bottlenecks such as product inhibition or mass transfer rate limitations have to be addressed carefully and integrated in the process development.^[75]

4.3.3 Reaction engineering for whole cell biotransformations

In addition to protein and strain engineering this thesis emphasized also on reaction engineering to optimize important parameters that influence the operational performance of a whole cell P450-ADH catalyst in a multi-enzymatic reaction.

Key parameters such as glucose concentration, pH-value, reaction medium, substrate, and cell mass (catalyst) concentrations were evaluated and optimized to achieve higher product concentrations in the double oxidation of *n*-heptane to chiral heptanols and heptanones. The performance of resting cells was evaluated in phosphate buffer and *E. coli* Minimal Medium (EEM; pH of 7.0), which are widely applied in whole cell conversions.^[205] As further modification of the 0.1 M potassium phosphate buffer the pH value was varied from 7.0 to 8.0 and the supplementation of different D-glucose concentrations was investigated, as shown in Figure 4-28.

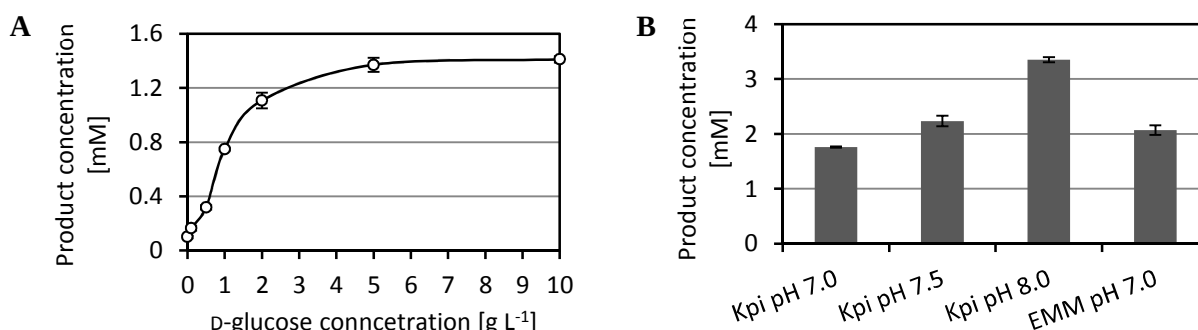


Figure 4-28: Effect of glucose and different reaction media on the whole cell double oxidation of *n*-heptane. Reaction conditions: BL21 Gold (DE3) *lacI*^{Q1} pALXtreme-1a P450 BM3 CM1^{NADH}-RE-ADH in (A) OD₆₀₀ 50 (11 g_{CDW} L⁻¹, 7.3 μM P450), 80 mM *n*-heptane in EEM pH 7.0 (1 mL), varying D-glucose concentration (0-10 g L⁻¹), 20 h at RT; in (B) OD₆₀₀ 70 (15 g_{CDW} L⁻¹, 10 μM P450), 10 g L⁻¹ D-glucose, 300 μL *n*-heptane, varying reaction medium (1 mL; 0.1 M KPi, pH 7.0, 7.5, 8.0 or EEM), 24 h at RT.

The highest product concentration (total product heptanol and heptanone) was achieved with a glucose concentration of 10 g L⁻¹ and 0.1 M KPi-buffer at a pH of 8.0. In the absence of glucose a very low product concentration is achieved (0.1 mM). Since no nicotinamide cofactor is added externally, the product formation without supplementation of glucose is probably facilitated by the remaining intracellular concentration of cofactors. A rather sigmoidal dependency between the glucose concentration and the product formation is observed. At a glucose concentration of 5 to 10 g L⁻¹ the reaction reaches saturation.

In the established oxidoreductase-catalyzed *in vivo* biotransformation approach, the essential cofactor NADH for the P450-catalyzed reaction is regenerated most likely *via* the glucose

metabolism of the *E. coli* host and in part by the artificial cascade for substrate coupled regeneration of NADH. Resting, but metabolically active cells are used, implying that redox cofactors and biomass precursors should not be consumed for the synthesis of biomass allowing a higher NADH yield.^[68] Yet, the required energy for cell maintenance has to be provided throughout the conversion reaction. The regeneration rate of NADH will partially depend on the regeneration capacity of the cells, which can represent a limiting factor in biocatalytic redox process.^[68, 206] The supplementation with glucose is therefore beneficial for supporting the necessary cofactor regeneration as confirmed by the results in Figure 4-28 A.

Blank *et al.* estimated the metabolic capacity for NADH regeneration in resting *E. coli* cells.^[68] For an optimal glucose uptake rate of $2.4 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ a theoretical NADH-formation rate of $22 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ was calculated. Under assumption of an equimolar stoichiometry between NADH and the biocatalytic product, the maximal biocatalytic activity for oxidoreductases would reach $367 \text{ U g}_{\text{CDW}}^{-1}$. At higher glucose uptake rates the oxidation of glucose will not be complete (overflow metabolism) resulting in the catabolic production of byproducts like acetate and CO_2 .^[68] Using 10 g L^{-1} glucose and a cell mass concentration of $11 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ (Figure 4-28 A), the available concentration of glucose can be calculated as $5.04 \text{ mmol g}_{\text{CDW}}^{-1}$. At this concentration the product concentration reaches a maximum of 1.41 mM which is slightly higher than for 5 g L^{-1} glucose. At a glucose concentration of 2.4 mM the effect on productivity is detrimental (overflow metabolism, altered glucose flux, pH shifts due to acid formation), therefore the whole cell becomes a “black box”.

Metabolic stress caused by high bioconversion rates or formation of reactive oxygen species as a result of uncoupling can also lead to production of acetate or other metabolites.^[206] Bühler *et al.* reported a strong pH-dependency on the specific activity of growing cells in the long-term two-liquid-phase oxidation of 3,4-dimethylbenzyl alcohol. At pH 7.4 a significantly higher activity was reached compared to pH 7.1. The authors concluded that a pH-influenced competition for NADH between the heterologous expressed catalyst (xylene monooxygenase) and the respiratory chain took place. In short term experiments of 5 min with resting cells this pH-effect was not significant. Long-term studies were however not conducted.^[206]

Based on the presented results from Figure 4-28 (A) it can be concluded, that the pH of the reaction medium has a strong effect on the biocatalytic activity, also when resting cells are used. A difference of 1.0 in the pH value (from 7.0 to 8.0) enhanced the product concentration by 2-fold. The enhanced productivity is probably caused by pH-influenced changes in the cell metabolism or/and in the interaction between the expressed oxidoreductases and the regeneration capacity of the cell. The higher pH value of 8.0 gives the system a better buffering

capacity against acidifying byproducts (e.g. acetate as product from overflow metabolisms) when higher glucose amounts for regeneration are employed. The higher pH is therefore beneficial for the bioconversion of *n*-heptane. A scale-up of the whole cell reaction process using a bioreactor and the possibility to monitor and control the pH would be desirable for further optimization of the reaction conditions.

To determine the correlation between substrate concentration and product yield different *n*-heptane concentrations ranging from 1 to 1045 mM were applied. Additionally, the use of *n*-heptane not only as substrate but also as organic carrier for a two-liquid-phase system was investigated. Concentrations of 93, 440 and 1045 mM are equivalent to an *n*-heptane volume of 15, 75 and 200 μ L and a volumetric content of the organic component in an aqueous-organic two phase system of 1.5, 7 and 17 % (v/v) respectively. In case of 200 μ L (1450 mM) *n*-heptane an aqueous-organic bilayer was formed. Product formation using different *n*-heptane concentrations, as well as the product yield based on substrate consumption $Y_{p/s}$ (equivalent to $\text{mol}_{\text{product}} \text{mol}_{\text{substrate}}^{-1} \times 100\%$) were determined as shown in Figure 4-29.

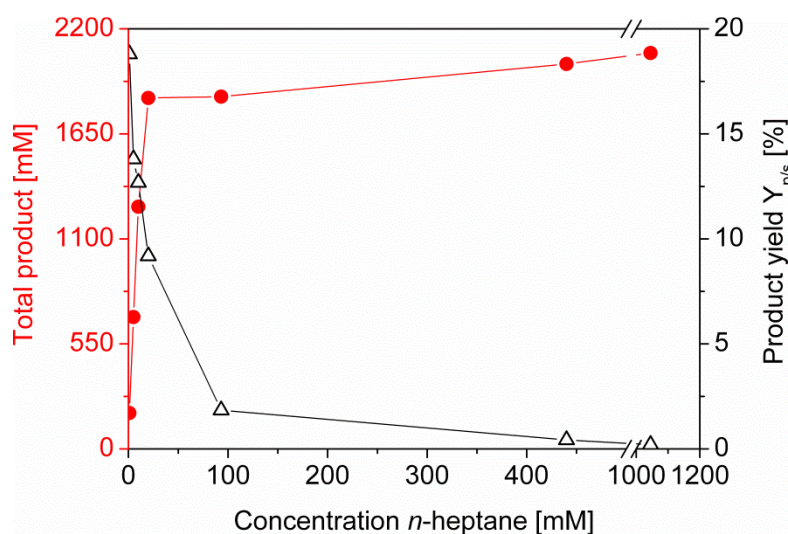


Figure 4-29: Effect of varying *n*-heptane concentrations on product concentration and yield. Total product concentrations (●) and product yields $Y_{p/s}$ over substrate (Δ) were calculated taking into account the sum of all heptanol and heptanone isomers. Reaction conditions: BL21 Gold (DE3) lacI^{Q1} pALXtreme-1a P450 BM3 CM1^{NADH}-RE-ADH in 0.1 M KPi buffer pH 8.0 (1 mL; OD_{600} 70, 15 $\text{g}_{\text{CDW}} \text{L}^{-1}$, 10 μM P450), 10 g L^{-1} D-glucose, varying *n*-heptane concentration; 24 h stirring at RT.

The highest product yield $Y_{p/s}$ (19%) was obtained employing 1 mM substrate. However, decreasing product yields ($Y_{p/s}$) are the result of the higher substrate load used in the remaining measurements. Higher product concentrations (up to 2 mM) were measured for the substrate concentrations 440 and 1045 mM, which correspond to a volumetric content of 7 and 17%

(v/v) *n*-heptane. Here an aqueous-organic two-phase reaction system is present. The advantage of this system lies on the use of *n*-heptane as organic carrier solvent, which serves simultaneously as substrate reservoir and product sink.^[207] It is generally accepted, that the biocompatibility of an organic solvent used as second phase in bicatalytic processes correlates to the $\log P_{o/w}$ value - the octanol/water partition coefficient - as a measure of the solvent hydrophobicity. Solvents with $\log P_{o/w} \geq 4$ are regarded as biocompatible, mainly for free enzymes in two-liquid-phase systems.^[207a] For whole cell systems this correlation depends strongly on the microorganism species.^[207b] In some cases a transition into a toxic range was observed for $\log P_{o/w}$ values from 3 to 5.^[1, 208] *n*-Heptane has a $\log P_{o/w}$ value of 4.66 (at 25 °C).^[187a, 187b] In fact, the $\log P_{o/w}$ value gives rather a precise correlation for the mutual solubility of organic solvents and water in water-immiscible systems.^[209] Taking into account the higher solubility of *n*-heptane in water than water in *n*-heptane, the use of a two-phase system should enable higher substrate availability throughout the biotransformation process. Heptanol and heptanone as main products of the cascade reaction, with $\log P_{o/w}$ values of around 2.22^[210] and 1.98^[187a] respectively, are more apolar than the substrate *n*-heptane. They will most likely accumulate in the organic phase, enabling an *in situ* product removal and avoiding a possible product inhibition of the catalyst. The results of this study imply that the use of *n*-heptane as organic carrier in the described approach for *E. coli* whole cells is feasible.

Other advantages of two-phase systems for biocatalytic transformations are besides the *in situ* product recovery a simpler work up by separation of the phases and a possible recycling of the organic phase for further biotransformations.^[81] The low price of *n*-heptane as bulk chemical make an application in a higher amount as organic liquid phase feasible, then it serves simultaneously as starting material for the synthesis of value-added products. Due to the fact that P450 BM3 hydroxylates a broad range of organic substrates^[58] the selection of a suitable organic solvent is strongly limited. Besides, the use of *n*-heptane and other alkanes like *n*-hexane, cyclohexane, *n*-octane and isooctane as organic solvent in biphasic biotransformations have being described previously for cell free enzymes^[83c] as well as whole cell processes^[148, 174, 211].

The biotransformation was further optimized in regard to cell mass concentrations for each P450 BM3 construct. The correlation between optical cell density (OD₆₀₀) and cell dry weight (CDW) was calculated for cells harboring the operon-fusion constructs P450 BM3 WT^{NADH}-RE-ADH, 19A12^{NADH}-RE-ADH and CM1^{NADH}-RE-ADH after expression in the fermentor. The corresponding P450 BM3 concentrations were determined by CO-difference spectroscopy (appendix Figure 6-10). The correlation between cell dry weight and P450 concentration is

linear (2 to 13 g_{CDW} L⁻¹). Based on this correlation the intracellular expression titer of P450 BM3 or simplified, the P450 yield ($Y_{P450/x}$), was calculated for each monooxygenase variant (see chapter 4.3.4, Table 4-8). The highest expression level of P450 BM3 monooxygenase was achieved for the construct CM1^{NADH}-RE-ADH with a P450 yield of 81 mg_{P450} g_{CDW}⁻¹.

The effect of different cell mass concentrations on the product yield was evaluated as well. The maximal product yields are shown in Figure 4-30.

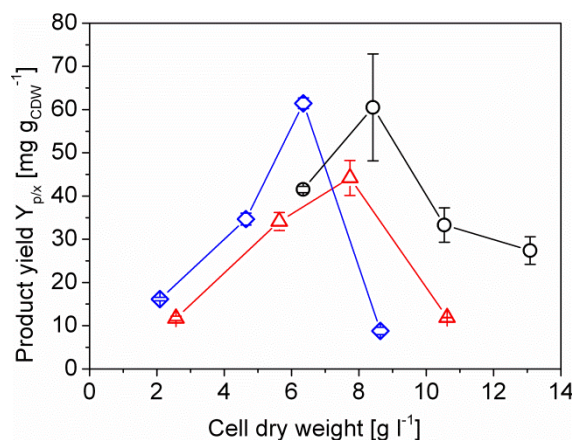


Figure 4-30: Product yields in the whole cell biotransformation of *n*-heptane using different operon-fusion strains. The strains are: BL21 Gold (DE3) Gold lacI^{Q1} pALXtreme-1a P450 BM3 WT^{NADH}-RE-ADH (red), 19A12^{NADH}-RE-ADH (blue) and CM1^{NADH}-RE-ADH (black). Reaction conditions: Resting cells in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L⁻¹ D-glucose, 200 μL *n*-heptane with varying cell concentrations (CDW from 2 to 13 g L⁻¹), 48 h stirring at RT.

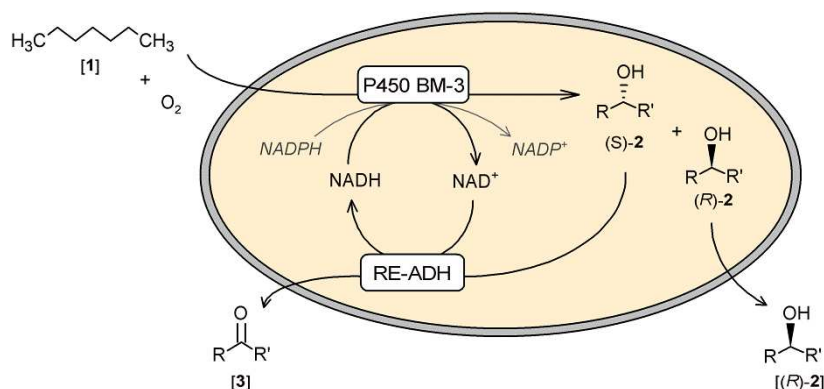
Product yields for the three selected P450 BM3 monooxygenases are strongly dependent on the concentration of cell mass employed in the reaction. Each batch of cells containing one of the P450 BM3 monooxygenase variants displays a distinctive optimal cell mass concentration. The highest product yields were achieved using 6.3 g_{CDW} L⁻¹ (OD₆₀₀ of 30) for the strain containing P450 BM3 19A12^{NADH}, 7.7 g_{CDW} L⁻¹ (for OD₆₀₀ of 30) for WT^{NADH} and 8.4 g_{CDW} L⁻¹ (OD₆₀₀ of 40) for CM1^{NADH}. This CDW values correspond to monooxygenase concentrations of 0.16, 0.58 and 0.68 g_{P450} L⁻¹ respectively. Interestingly, the product yield is not proportional to the amount of P450 BM3 monooxygenase employed in the reaction. This yield is most likely dependent on the intracellular concentration ratio between both cascade-enzymes (monooxygenase and dehydrogenase), but also on possible mass transfer limitations (substrate, products, oxygen) at high cell mass concentrations. In this regard, a limitation in the oxygen supply can lead to a reduce monooxygenase activity.^[75] The reduction of oxygenase activity caused by high cell concentrations was also observed by Baldwin and Woodley.^[212] The authors proposed that a heterologous expressed oxygenase, which have a lower affinity (higher

K_M value) for oxygen than the respiratory chain of the cell, will not be able to compete efficiently for the dissolved oxygen if the exogenous cell respiration exceeds a certain threshold. The P450 monooxygenase catalyzed reaction, affected by mass transfer limitations and by a lower catalytic activity, is probably slower than the cell metabolism.

The determined optimal cell mass concentrations for each coexpression strain were employed in the following biotransformation reactions.

4.3.4 Whole cell multi-enzymatic catalysis: double oxidation of *n*-heptane and cyclooctane

The biotransformation of *n*-heptane with whole cells co-expressing P450 BM3 and RE-ADH was performed under the optimized conditions described in chapter 3.12.2. The schematic representation of the cascade reaction catalyzed by recombinant *E. coli* cells is displayed in Scheme 6.



Scheme 6: Whole cell double oxidation of *n*-heptane catalyzed by a P450 BM3 monooxygenase variant and an ADH from *Rhodococcus erythropolis* (RE-ADH). In the initial cascade step *n*-heptane is hydroxylated by the P450 BM3 monooxygenase to the corresponding heptanol isomers. The second step is comprised by the oxidation of (S)-heptanol to the corresponding ketones, enabling the accumulation of the (R)-heptanol enantiomers and the concomitant regeneration of the cofactor NADH.

The multi-enzymatic cascade allows the production of heptanones and the chiral resolution of (R)-heptanols in a coupled activity of the recombinant expressed P450 BM3 monooxygenase and ADH in *E. coli*. The biotransformation was monitored over 48 hours using cells expressing P450 BM3 CM1^{NADH}-RE-ADH. The progress of the cascade reaction in terms of product concentrations is shown in Figure 4-31. An overview of the performance parameters in the double-oxidation reaction is given in Table 4-8 (entry 5).

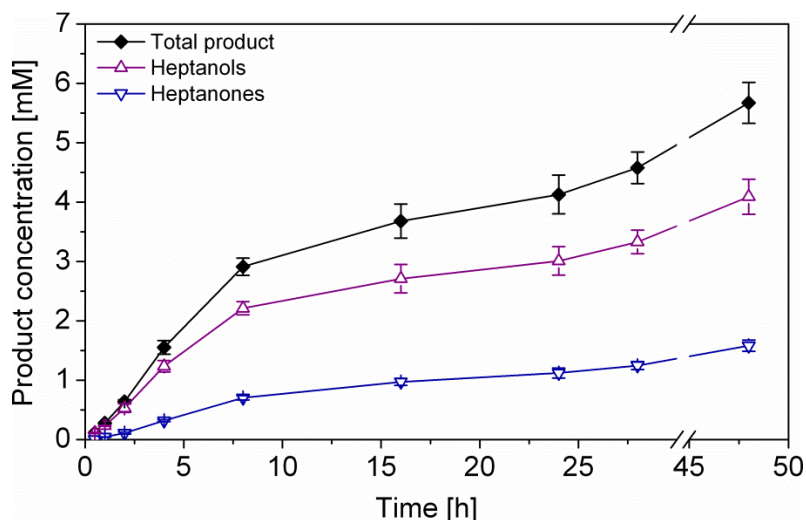


Figure 4-31: Whole cell conversion of *n*-heptane over 48 hours. Total product concentrations (black) consisting of heptanol (purple) and heptanone (blue), as sum of the corresponding regiomers (GC quantification), measured after a reaction time of 0.5, 1, 2, 4, 8, 16, 24, 28 and 48 h at RT. Reaction conditions: *E. coli* BL21 (DE3) Gold lacI^{Q1} pALXtreme-1a P450 BM3 CM1^{NADH}-RE-ADH (OD_{600} 40; 8.5 g_{CDW} L⁻¹, 5.9 μM P450) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L⁻¹ D-glucose, 200 μL *n*-heptane as second phase.

A total product concentration of 5.7 mM (658 mg L⁻¹) was measured after 48 hours, consisting of 4.1 mM alcohol and 1.6 mM ketone. The product formation rate was nearly linear until 8 hours, with the highest specific activity of 0.76 U g_{CDW}⁻¹ (9.3 U g_{P450}⁻¹) and a maximal space time yield (STY) of 45 mg L⁻¹ h⁻¹ after 4 hours. From 8 hours onwards the product formation rate decreases, but product accumulated constantly until the end of the reaction at 48 h. A total turnover number for the reaction time of 48 h was determined as 968 mol_{product} mol_{P450}⁻¹. The maximal specific activity reached with the system is 541-fold less than the theoretical specific activity of 411 U g_{CDW}⁻¹ that was calculated for whole cells expressing P450 BM3 CM1^{NADH} (refer to chapter 4.3.1). Different parameters such as mass transfer limitations, enzyme inactivation or loss of cell viability and cofactor regeneration capacity could lead to the lower specific activity reached with the multi-enzymatic system so far. It is worth mentioning, that the biotransformation process was performed in a small scale with a reaction volume of 1 mL and magnetic bar stirring. To investigate the bottlenecks of the system and optimize product yields a scale-up using a bioreactor with controlled process parameters like pH, stirring and aeration rate would be advantageous.

A direct comparison of the performance of the single hydroxylation as well as the double oxidation of *n*-heptane was carried out (Table 4-8). For this purpose the strains expressing the sole P450 BM3 variant CM1 (NADPH-dependent) and CM1^{NADH} (NADH-dependent) were employed under the optimized biotransformation conditions.

Table 4-8: Process performance for the whole cell single hydroxylation and the double oxidation of *n*-heptane with P450 BM3 and RE-ADH^[a]. The product concentration for the *in vitro* double-oxidation system is given as reference.

Entry	Multi-enzyme system	Product concentration		Product yield	P450 yield on
		[μM]	[mg L ⁻¹]	on catalyst Y _{p/x} [mg g _{CDW} ⁻¹]	catalyst Y _{P450/x} [mg _{P450} g _{CDW} ⁻¹]
Single expression (shake flask)					
1	P450 BM3 CM1	1133 ± 110	132 ± 13	16 ± 2	53
2	P450 BM3 CM1 ^{NADH}	2319 ± 74	269 ± 9	40 ± 1	58
Operon-fusion (Fermentation)					
3	P450 BM3 WT ^{NADH} -RE-ADH	2952 ± 6	342 ± 31	44 ± 4	74
4	P450 BM3 19A12 ^{NADH} -RE-ADH	3400 ± 69	390 ± 8	61 ± 1	25
5	P450 BM3 CM1 ^{NADH} -RE-ADH	5671 ± 344	656 ± 40	76 ± 5	81
In vitro system					
6	P450 BM3 CM1 ^{NADH} + RE-ADH	1800	207	--	--

^[a] Product concentrations are the sum of all quantified heptanol and heptanone isomers; the reaction mixture contained *E. coli* cells (OD₆₀₀ 40 for P450 BM3 CM1, CM1^{NADH} and CM1^{NADH}-RE-ADH; OD₆₀₀ 30 for WT^{NADH}-RE-ADH and 19A12^{NADH}-RE-ADH) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L⁻¹ D-glucose, 1045 mM (200 μ L) *n*-heptane as second liquid phase.

Despite of a similar P450 BM3 content in CM1 and CM1^{NADH} after shake flask expression (53 and 58 mg_{P450} g_{CDW}⁻¹ respectively), the product formation with the NADH-dependent variant is 2-fold higher and the product yield, normalized to the amount of cells in the reaction, even 2.5-fold improved (Table 4-8, entry 2 vs. 1). This values show the direct benefit of using the engineered NADH-dependent monooxygenase as whole cells catalyst over the native NADPH-dependent counterpart.

In the double oxidation cascade with cells containing CM1^{NADH}-RE-ADH the product yield of 76 mg_{product} g_{CDW}⁻¹ is nearly doubled compared to the reaction with cells containing only P450 CM1^{NADH} (Table 4-8, entry 5 vs. 2). The improved performance of this catalyst can be explained by the 1.4-fold optimized intracellular expression of the P450 monooxygenase (Table 4-8, 81 vs. 58 mg_{P450} g_{CDW}⁻¹).

The highest product yield of 76 mg_{product} g_{CDW}⁻¹ was measured for the strain containing CM1^{NADH}-RE-ADH, which also displayed the highest intracellular content of P450 BM3 (Table 4-8, 81 mg_{product} g_{CDW}⁻¹). Product yields as well as P450 BM3 expression levels were lower for WT^{NADH}-RE-ADH and 19A12^{NADH}-RE-ADH. A low P450 yield of 25 mg_{P450} g_{CDW}⁻¹ was achieved in the case of the 19A12^{NADH} operon-fusion compared to the other two expressed monooxygenase variants, despite of cultivation in the fermentor under optimized growing conditions (Table 4-8, entry 3, 4 and 5). The poor expression of P450 BM3 19A12^[175] is

probably caused by the low stability of this monooxygenase variant as previously discussed in chapter 4.1.1. These results highlight the importance of an elevated expression level besides of the enzymes activity to achieve the best catalytic performance. Especially for the strain containing the operon-fusion CM1^{NADH}-RE-ADH the significant benefit of a good expression was proven, obtaining a 54-fold improved production of the monooxygenase after expression in fermentor compared to the expression in shaking flask (Figure 4-27 B) and a 7-fold enhanced product yield $Y_{p/x}$ (Figure 4-27 A).

Final product concentrations in the double oxidation of *n*-heptane achieved with all three selected P450 BM3 monooxygenases are considerably higher using the *in vivo* instead of the *in vitro* biotransformation system. A comparison to the best results of the *in vitro* cascade reaction, under supplementation of cofactor regeneration *via* GDH (chapter 4.2.3), shows that with P450 BM3 WT^{NADH} a 4-fold, with CM1^{NADH} a 3-fold and with 19A12^{NADH} a 2-fold higher product concentration is achieved using whole cell catalysis (Table 4-8, entry 3-5 vs. Figure 4-22, panel D-F).

To proof a broad applicability of the whole cell double oxidation concept, the coexpression of the respective catalysts was extended to a third enzyme, a second alcohol dehydrogenase, the (*R*)-selective NADPH-dependent ADH from *Lactobacillus brevis* (Lb-ADH)^[158]. The Lb-ADH has a complementary selectivity to the (*S*)-specific RE-ADH^[157].

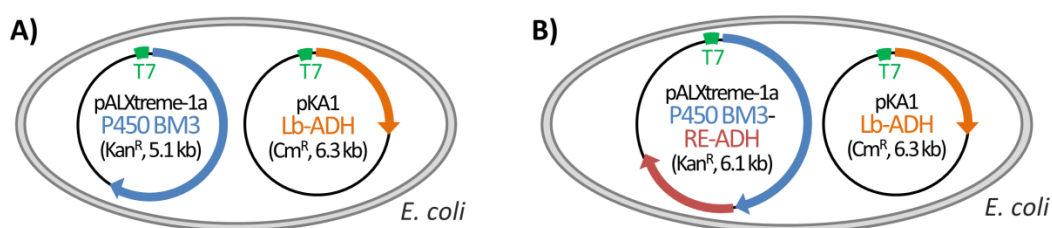
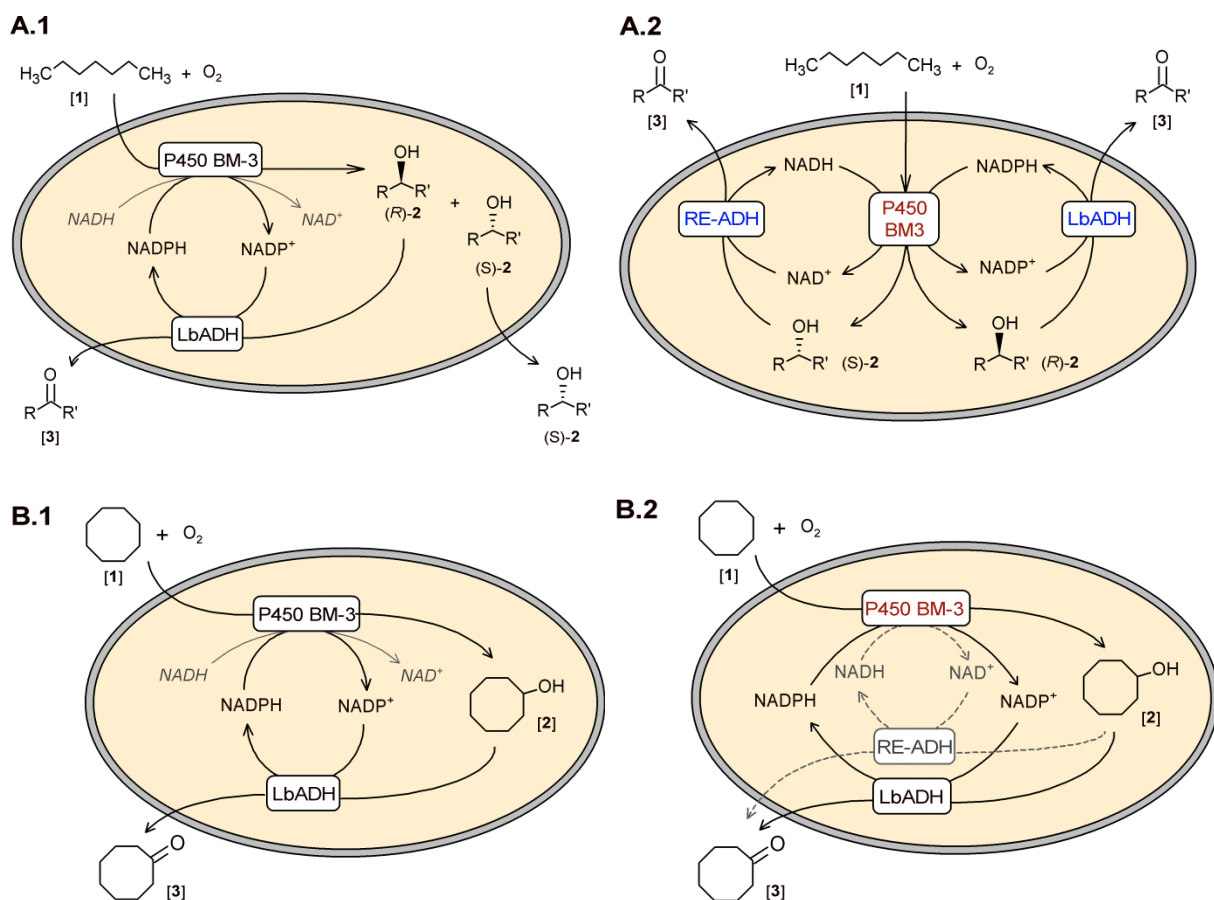


Figure 4-32: Coexpression of multiple enzymes in *E. coli* cells using a 2-plasmid-system. The (*R*)-selective, NADPH-specific Lb-ADH is expressed from a second plasmid (pKA1), together with P450 BM3 monooxygenase (NADPH-dependent) in a two-enzyme system (A), or with the operon-fusion between P450 BM3 monooxygenase (NADH-dependent) and (*S*)-selective NADH-specific RE-ADH in a three-enzyme system (B).

Coexpression strains containing two enzymes, the Lb-ADH and an NADPH-dependent P450 BM3 monooxygenases (19A12 or CM1), as well as strains containing three enzymes, two dehydrogenases - Lb-ADH, RE-ADH - and one P450 BM3 monooxygenase (NADH-dependent 19A12^{NADH} or CM1^{NADH}) were generated by transformation of the Lb-ADH using a second plasmid (pKA1 vector) as shown in Figure 4-32. These generated strains for the

coexpression of multiple enzymes are listed in the appendix section (Table 6-8). The strains were expressed in shake flask (chapter 3.6.3) and successful expression of all enzymatic components was evaluated by SDS-PAGE gel electrophoresis (appendix Figure 6-11). A very high expression level of the Lb-ADH was achieved in all generated strains, while the expression of P450 BM3 and RE-ADH were comparably low. Lb-ADH is encoded by the smallest of all three genes (765 bps) in a separate plasmid. It has been reported, that Lb-ADH is expressed in a highly efficient way and the stability of the enzyme is remarkably high compared to other ADHs.^[158, 213]



Scheme 7: Whole cell double oxidation of *n*-heptane (A) and cyclooctane (B) by multi-enzymatic expression in *E. coli*. Coexpression of a P450 BM3 monooxygenase variant and an alcohol dehydrogenases from *Lactobacillus brevis* (Lb-ADH) in a two-enzyme system (1) or additionally an alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH) in a three-enzyme system (2). With the multi-enzymatic system in **A.1** heptanone and (S)-heptanol are produced, whereas in **A.2** complete oxidation to heptanone is achieved. The system in **B.1** and **B.2** allows the synthesis of cyclooctanone.

n-Heptane and cyclooctane were selected as substrates for the whole cell multi-enzymatic cascade reaction. The double oxidation of cyclooctane using an *in vitro* system was proven

successfully in a previous report.^[174] A representation of the catalyzed reaction using a two-enzyme or a three-enzyme system consisting of a P450 BM3 monooxygenase and alcohol dehydrogenases Lb-ADH and RE-ADH is shown in Scheme 7.

The biotransformations were performed under the conditions described in chapter 3.12.2. The performance of the whole cell double oxidation cascade using Lb-ADH is summarized in Table 4-9. The product distribution patterns for each employed strain and substrate are given in Table 4-10.

Table 4-9: Process performance of the multi-enzymatic whole cell double oxidation of *n*-heptane and cyclooctane

Entry	Whole cell catalysts (shake flask expression)	Total product concentration		TTN ^[c]	Product yield on catalyst Y _{p/x} [mg g _{CDW} ⁻¹]	P450 yield Y _{P450/x} [mg _{P450} g _{CDW} ⁻¹]
		[μM]	[mg L ⁻¹]			
	Substrate: <i>n</i>-Heptane^[a]					
1	BM3 19A12 + Lb-ADH	471 ± 19	54 ± 2	2930 ± 121	8 ± 0.3	3
2	BM3 CM1 + Lb-ADH	230 ± 17	26 ± 2	854 ± 65	4 ± 0.3	5
3	BM3 19A12 ^{NADH} -RE-ADH + Lb-ADH	851 ± 80	97 ± 9	1393 ± 132	16 ± 2	12
4	BM3 CM1 ^{NADH} -RE-ADH + Lb-ADH	291 ± 6	33 ± 1	534 ± 11	6 ± 0.1	11
	Substrate: Cyclooctane^[b]					
5	BM3 19A12 + Lb-ADH	3916 ± 70	494 ± 9	23736 ± 426	72 ± 1.3	3
6	BM3 19A12 ^{NADH} -RE-ADH + Lb-ADH	5168 ± 74	652 ± 9	8303 ± 121	111 ± 1.6	12

^[a] Product concentrations as sum of heptanol and heptanone isomers; the reaction mixture contained *E. coli* cells (OD₆₀₀ 40) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L⁻¹ D-glucose, 200 μ L *n*-heptane as second phase.

^[b] Product concentrations as sum of cyclooctanol and cyclooctanone; the reaction mixture contained *E. coli* cells (OD₆₀₀ 40) in 0.1 M KPi buffer pH 8.0 (1 mL), 10 g L⁻¹ D-glucose, 10 mM cyclooctane, 2% (v/v) ethanol as cosolvent.

^[c] TTN: Total Turnover Number (μ mol_{Total product} μ mol_{P450}⁻¹)

In the biotransformation reaction of *n*-heptane through the coupled P450 BM3 and Lb-ADH catalyzed oxidations the product concentrations are 2-fold higher for the variant 19A12 compared to the variant CM1 (Table 4-9, entry 1 and 2). A further enhanced productivity of 80% for the variant 19A12^{NADH} and of 30% for the variant CM1^{NADH} was achieved using the strains containing the second alcohol-dehydrogenase RE-ADH (Table 4-9, entry 3-4 vs. 1-2). In this case the monooxygenase and the RE-ADH share the same cofactor preference for NADH, while Lb-ADH is NADPH dependent. A striking difference between the two and the three enzyme system is the concentration of P450 monooxygenase, calculated as P450 yield ($Y_{P450/x}$). In the three enzyme systems the P450 content is 4-fold and 2-fold higher (entry 3 and

4, 12 and 11 mg_{P450} g_{CDW}⁻¹ respectively). The higher P450 concentrations are in accordance to the higher product concentrations obtained with each system.

Table 4-10: Selectivity of the whole cell double oxidation of *n*-heptane and cyclooctane

Entry	Catalysts	Relative amount [% GC area]							
		<i>Heptanol isomers (ee of (S))</i>				<i>Heptanone isomers</i> ^[a]			
		1-	2-	3-	4-	2-	3-	4-	
1	P450 BM3 19A12 + Lb-ADH	0	26 /n.d.	15 (>99)	0	41	18	0	
2	P450 BM3 CM1 + Lb-ADH	0	0	11 (>99)	1	14	31	43	
3	P450 BM3 19A12 ^{NADH} -RE-ADH + Lb-ADH	0	0	0	0	67	31	2	
4	P450 BM3 CM1 ^{NADH} -RE-ADH + Lb-ADH	0	0	0	0	15	41	44	
		<i>Cyclooctanol</i>		<i>Cyclooctanone</i>		<i>Unidentified peak areas</i>			
5	P450 BM3 19A12 + Lb-ADH	0.3		96.6		3			
6	P450 BM3 19A12 ^{NADH} -RE-ADH + Lb-ADH	0.1		94.4		5.4			

^[a] Heptanal was not detected.

n.d.: not determined

Coupling the P450 monooxygenase with the (*R*)-enantioselective Lb-ADH leads to accumulation of the (*S*)-heptanol isomers, while the (*R*)-heptanols are oxidized to the corresponding heptanones (Table 4-10, entry 1 and 2). In the 19A12^{NADH}-Lb-ADH-catalyzed cascade reaction a relative amount of 59% ketone is obtained (2- and 3-heptanone; entry 1) and in case of CM1^{NADH}-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. In contrast to the two-enzyme system, employing three enzymes (P450 + RE-ADH + Lb-ADH) the intermediate (*R/S*)-heptanols that are produced during the initial P450 BM3-catalyzed hydroxylation step are completely oxidized to the corresponding ketones (Table 4-10, entry 3 and 4). In the latter case, the absolute amount of the heptanone-isomers corresponds precisely to the sum of the heptanol and heptanone regioisomers in the two-enzyme system. This is remarkable, since two ADHs are present that are capable to perform the reversible reaction.

The product distribution of the synthesized heptanones in the *in vivo* system is in accordance to the regioselectivity of each P450 BM3 variant that was determined *in vitro* (chapter 4.1.4). Cells containing the P450 BM3 variant 19A12^{NADH} produce predominantly 2-heptanone followed by 3-heptanone (Table 4-10, entry 3, 67 % and 31%). With the variant CM1^{NADH} higher amounts of 4- and 3-heptanone are produced (Table 4-10, entry 4, 44% and 41% respectively). The complete oxidation of the heptanol intermediates lead therefore to an improved availability of redox cofactors, which is demonstrated by the enhanced product

formation (Table 4-9, 1.8-fold entry 3 vs. 1; 1.3-fold entry 4 vs. 2). In addition, the 1.8-fold higher product concentration in case of 19A12^{NADH} can be explained by the rather (S)-selective character of this P450 variant as determined for the purified enzyme *in vitro* (chapter 4.1.4, Table 4-2). In presence of the (S)-enantioselective RE-ADH all produced heptanol is oxidized, driving the reaction equilibrium to the side of the ketones. Therefore, the enzyme-coupled cofactor regeneration during the second oxidation step is more efficient when the two stereo-complementary dehydrogenases -Lb-ADH and RE-ADH- are present.

Nevertheless, the three-enzyme coexpression system consisting of P450^{NADH}-RE-ADH + Lb ADH displays overall lower absolute product yields (up to 13-fold difference) when compared with the two-enzyme coexpression system consisting of P450^{NADH} and RE-ADH (Table 4-9, entry 4 and 5 vs. Table 4-8, entry 3 and 4). This may be caused by the up to 7-fold reduced P450 BM3 yield ($Y_{P450/x}$) in the three-enzyme system compared to the two-enzyme system. The lower expression is a result of the non-optimal cultivation in shake flasks employing a two-plasmid coexpression strategy.

The concept of double oxidation of alkanes was successfully transferred to a new substrate class, converting cyclooctane to cyclooctanone in the whole cell multi-enzymatic process. Cycloalkanones are important building blocks for the production of synthetic polymers, e.g. polyamide.^[43] A three component system consisting of P450 BM3 19A12^{NADH}-RE-ADH and Lb-ADH (Table 4-9, entry 6) yielded a very high product concentration of 5.2 mM (652 mg L⁻¹) and the highest product yield (over catalyst) of 111 mg g_{CDW}⁻¹. Compared to the system with two enzymes (Table 4-9, entry 6 vs. 5) a 32% improved product formation was achieved. As previously described for the multi-enzymatic double oxidation of *n*-heptane the difference in product formation correlates to the difference in P450 BM3 content. The cells containing three enzymes (P450 19A12^{NADH}-RE-ADH + Lb-ADH; Table 4-9 entry 6) had a 4-fold higher P450 BM3 content (12 mg_{P450} g_{CDW}⁻¹) than cells with two enzymes (P450 19A12 + Lb-ADH; Table 4-9 entry 5, 3 mg_{P450} g_{CDW}⁻¹). The higher P450-concentration led to an improved product formation during whole cell catalysis.

However, in terms of total turnover numbers (TTN) the two-enzyme system P450 BM3 19A12 + Lb-ADH displayed the highest value of 23736. The excellent TTN value is caused in this case by the relative high product concentration of 494 mg L⁻¹ in relation to a relatively low intracellular P450 BM3 concentration (3 mg_{P450} g_{CDW}⁻¹; Table 4-9, entry 5). This means, in this strain the cofactor regeneration is very effective for the low concentration of P450 available, allowing a high efficiency in the product formation. For the other strains with higher P450 content, the regeneration is limiting, resulting in lower TTN values. The TTN of 23736 is

6.6-fold higher than the best TTN of 3591 determined for the *in vitro* double oxidation of *n*-heptane with P450 BM3 CM1^{NADH}-RE-ADH and cofactor regeneration over D-glucose/GDH (chapter 4.2.3, Table 4-7). In addition, it represents one of the highest TTN reported for a P450 BM3 catalyzed reaction. Higher TTNs have been reported for the *in vitro* conversion of dodecanoic acid with the NADH-dependent P450 BM3 variant WT^{NADH} (substitutions R966D/W1046S; TTN of 66700) and for the conversion of propane with the P450 BM3_{PMO}R2 variant (TTN of 45800).^[146, 214]

The conversion of cyclooctane was performed in a highly effective way (Table 4-10) with 94 to 97% towards cyclooctanone as product. Only trace amounts of cyclooctanol as intermediate of the double oxidation cascade were detected (up to 0.3%). In addition, other product peaks were detected, probably as a result of overoxidation or secondary hydroxylations catalyzed by the P450 BM3 monooxygenase. These phenomena are commonly encountered in the application of oxygenases^[39, 41a], especially in the case of P450 BM3^[215]. However, the relative amount of other products did not exceed 5% and should not interfere with later purifications. Based on the GC-quantification it was observed that a significant loss of the cyclooctane substrate during the reaction or the work up occurred. An apparent yield of 52% on cyclooctanone for the P450 BM3 19A12^{NADH}-RE-ADH + Lb-ADH system (entry 6) was calculated based on an initial substrate concentration of 10 mM. An advantage of cyclooctane as substrate for the double oxidation directly to cyclooctanone is given by the fact, that stereo centers cannot be generated upon single hydroxylation of the molecule. Therefore, almost all intermediate cyclooctanol can be oxidized by the employed ADHs in a chemoselective way.

The cell toxicity of cyclooctane is similar to that of *n*-heptane with a slightly lower Log $P_{o/w}$ value of 4.45.^[216] Cyclooctanone as main product of the multi-enzymatic cascade has a predicted Log $P_{o/w}$ of 2.11^[216], which is significantly lower than the value of the substrate. In the biotransformation of cyclooctane 2% (v/v) ethanol was used as cosolvent. Accumulation of the more polar product cyclooctane in the cosolvent, in a similar way like the effect of *n*-heptane as organic carrier in a biphasic system, could lead to a beneficial product removal outside of the cell. In this case, a shift of the reaction equilibrium towards the double oxidation product cyclooctanone instead of the intermediate alcohol cyclooctanol would be expected. The use of a two-liquid-phase system for conversion of cyclooctane could ensure higher substrate availability and enhanced *in situ* product removal to achieve improved catalytic performance and product yields.

Furthermore, the enzyme-coupled cofactor regeneration with two recombinant ADHs instead of one seems to be beneficial for the catalytic performance in the cell. The employed ADHs are

not only stereo-complementary but display also an opposite cofactor specificity (NADH-dependent RE-ADH and NADPH-dependent Lb-ADH). The employed P450 BM3^{NADH} variants are able to use both phosphorylated and non-phosphorylated cofactors as shown by the catalytic characterization data with purified enzyme (chapter 4.1.3, Figure 4-11). In this way, all regenerated cofactors NADH and NADPH become available for the P450-catalyzed hydroxylation. The over-expression of the P450 BM3 monooxygenase in the host cell is beneficial to drive the reaction to completion towards the ketones or the pure chiral alcohols. Based on the product quantification results for both substrates *n*-heptane and cyclooctane, with more than 94% cyclooctanone and >99% heptanone as main products, it can be assumed that the concept of the double-oxidation cascade is therefore advantageous to shift the reaction equilibrium to the side of the ketones. The alcohol dehydrogenases can perform the reversible reductive reaction. However, in the developed system accumulation of the alcohols was negligible.

The benefits of the multi-enzymatic expression using the operon-fusion strategy were summarized in the previous chapter 4.3.1. For the two-enzyme system consisting of a P450 BM3 and an RE-ADH the advantage of expressing two catalysts as a fused-operon was demonstrated. In the same way, coexpression of all three enzymatic components – P450 BM3, RE-ADH and Lb-ADH – using an operon-fusion would be more advantageous to achieve improved protein and product yields.

The benefit of employing stereo-complementary ADHs for the double oxidation of non-activated alkanes direct to the ketones was demonstrated, resulting in a complete oxidation of the intermediate alcohols and in an enhanced regeneration capacity. Using the whole cell multi-enzyme approach up to 650 mg L⁻¹ total product for the conversion of *n*-heptane as well as cyclooctane was achieved. This represents a more than 3-fold improved product concentration compared to the best results of the *in vitro* multi-enzymatic cascade (200 mg L⁻¹ for P450 BM3 CM1^{NADH} + RE-ADH + D-glucose/GDH; chapter 4.2.3). In the case of linear alkanes like *n*-heptane chiral resolution of the enantiomeric alcohols can be performed. The biotransformation of cyclic alkanes like cyclooctane directly to cyclooctanone is achieved with selectivity of 94%.

The described synthesis concept can be defined as a “modular” multi-enzymatic reaction system. It was demonstrated, that multiple enzymatic component can be coexpressed in a single *E. coli* cell to generate a whole cell catalyst. The enzymatic components can be exchanged according to the desired chemo- regio and enantioselectivity in the reaction. The selection of the biocatalysts, P450 monooxygenase and ADHs, as well as the target substrate

(aliphatic vs. cyclic alkane) showed a significant impact on the system's performance. Other monooxygenases and ADHs with different substrate selectivities could be implemented in the described process concept as well as any other beneficial catalyst.

5 Summary and Conclusion

Biocatalysis offers alternative routes for the cost-effective and environmental friendly use of hydrocarbons as raw material for the production of fine chemicals, pharmaceuticals and building blocks for the chemical industry. Therefore, the enzymatic functionalization of alkanes allows the sustainable use of petrochemicals for production of value-added compounds, instead of their constricted application in combustion processes. Cytochrome P450 monooxygenases are one class of enzymes, which have the remarkable capability of using molecular oxygen as sole oxidizing agent for alkane functionalization. This type of reaction is difficult to achieve by chemical synthesis. Some of the most striking advantages of enzymes over metallo- or organo-catalysts for the hydroxylation of non-activated C-H bonds are higher chemo- and stereoselectivities, mild reaction conditions, and usually higher turnover rates and numbers.

A novel strategy is the application of biocatalytic oxidation cascades using cytochrome P450 monooxygenases for the enzyme-catalyzed oxyfunctionalization of alkanes. In this work semi-rational protein engineering was used to design a P450 monooxygenase for its subsequent implementation in the whole cell multi-enzymatic double oxidation cascade. The coupled reaction should consist of a P450 monooxygenase-catalyst functionalization of the alkane, followed by an ADH-catalyzed oxidation of the intermediate alcohol. The second oxidation reaction serves also for efficient cofactor regeneration of the cofactor NAD(P)H.

Eighteen amino acid residues were rationally selected, saturated and screened for improved hydroxylation of *n*-heptane. Employing an optimized GC-FID protocol for separation of all heptanol isomers, rescreening and characterization of the best variants in regard to the regioselective hydroxylation of *n*-heptane was performed. A highly active P450 BM3 monooxygenase variant (CM1) was detected, which, under screening conditions, showed a 49-fold improved product formation rate compared to the wild type enzyme. The engineered variant P450 BM3 CM1 contains two unreported amino acid substitutions R255P and P329H, both located in the heme-domain and in close distance to the substrate binding pocket and active site cavity. It was proven in a SDM study, that both amino acids act in a synergistic manner to enable this remarkable catalytic activity despite of the low mutational load. Variant 19A12 contains eighteen mutations, which lead to a low monooxygenase expression level compared to the WT enzyme.

To design an economically more favorable process, the cofactor preference was switched from NADPH to NADH in the P450 BM3 WT, variant CM1, and variant 19A12. The altered cofactor specificity not only allows the use of the cheaper and more stable cofactor NADH, but also ensures the compatibility with NADH-dependent alcohol dehydrogenases for an enzyme coupled cofactor regeneration. To evaluate the influence of the switched cofactor selectivity a detailed catalytic characterization of the generated NADH-dependent monooxygenases was performed. The variants P450 BM3 CM1^{NADH} and 19A12^{NADH} showed enhanced hydroxylation activities for *n*-heptane with TOF values of 604 min⁻¹ and 485 min⁻¹ and coupling efficiencies for the cofactor NADH of 44% and 32%, respectively. All three engineered P450 BM3 variants WT^{NADH}, CM1^{NADH} and 19A12^{NADH} have a distinctive hydroxylation pattern when *n*-heptane is used as substrate. The regioselectivity of WT^{NADH} is similar to the one observed with fatty acids, where all three subterminal carbon atoms are hydroxylated in amounts higher than 24%. On the contrary, the variant CM1^{NADH} produces mainly 4-heptanol (48%) and 3-heptanol (42%), while 19A12^{NADH} shows a preference for 2-heptanol (64%) and 3-heptanol (33%). Furthermore every variant displayed a characteristic stereoselectivity profile. Interestingly, in P450 BM3 CM1^{NADH} the stereo-preferences for the hydroxylation of *n*-heptane were partially inverted by the substitutions R255P and P329H. This variant is (*S*)-selective with respect to 3-heptanol and (*R*)-selective in case of 2-heptanol.

The three engineered P450 BM3 monooxygenase variants were further used to evaluate the multi-enzymatic oxidation of *n*-heptane and cyclooctane. A double-oxidation cascade concept was developed by coupling a monooxygenase- and an alcohol dehydrogenase-catalyzed reaction with integrated cofactor regeneration. The use of three different P450 BM3 monooxygenase variants, displaying distinctive regio and stereoselectivities, and two stereo-complementary ADHs – the (*S*)-selective RE-ADH and the (*R*)-selective Lb-ADH - was evaluated by determination of catalytic parameters such as specific activity, product concentrations, enantiomeric excess, TTN, product yields (on catalysts, $Y_{p/x}$) and P450 yields ($Y_{P450/x}$). The P450 BM3 variant was used for the initial hydroxylation of the alkane, followed by the selective oxidation of the alcohol by means of the alcohol dehydrogenase. The biocatalytic double oxidation concept allowed the direct synthesis of alkanones from alkanes by simultaneous regeneration of the cofactor NAD(P)H. In addition, when *n*-heptane was employed as substrate, depending on the stereoselectivity of the employed ADH, the enzymatic resolution of (*S*)- or (*R*)-heptanols was achieved. Therefore, the synthesis concept offers a direct route from an inexpensive alkane to valuable high-priced synthons for the pharmaceutical, agrochemical and food industry. The described synthetic concept was

implemented in a cell free biotransformation (*in vitro*) as well as in a whole cell catalytic system (*in vivo*).

The *in vitro* conversion of *n*-heptane was performed under mild reaction conditions, i.e. at room temperature, in water and using molecular oxygen as sole oxidant which is superior to most chemical hydroxylation reactions. In the P450 BM3 CM1^{NADH}-RE-ADH catalyzed double oxidation a total product concentration of 1.3 mM (150 mg L⁻¹; (*R*)-heptanols and heptanone) was obtained. The product formation was 1.8-fold higher compared to the single P450 BM3-catalyzed hydroxylation of *n*-heptane employing the same amount of starting cofactor. A further increase in the system performance was achieved by supplementation of a supporting cofactor regeneration system containing D-glucose and GDH. Fine-tuning of the glucose concentration in the cascade reaction proved to be a key parameter to optimize cofactor regeneration and increase the product formation. In the multi-enzymatic cascade containing P450 BM3 CM1^{NADH}, RE-ADH and GDH a total product concentration of 1.8 mM (207 mg L⁻¹) and a TTN of 3500 were obtained, without changes in the stereoselectivity of the reaction. The TTNs of the P450 BM3 variants 19A12 and CM1 for the oxyfunctionalization of *n*-heptane outperforms state-of-the-art chemical catalysts by 15-fold.

Furthermore, the implementation of a multi-enzymatic cascade into a whole cell process was pursued. This would represent a more cost-effective alternative to *in vitro* reaction systems for an industrial application of oxidoreductases. In this regard, major advantages are that the addition of expensive external cofactors (NAD(P)H) and supplementary enzymes is not required. To extend the concept of the double oxidation of alkanes to whole cells catalysis, three coexpression strategies for P450 BM3 and ADH in *E. coli* were evaluated and optimized. The best coexpression strategy consisted in the generation of an operon-fusion between P450 BM3 and RE-ADH in the pALXtreme-1a vector. For the operon-fusion strategy that was developed in this thesis a remarkably high specific protein formation (protein yield) of 680 nmol_{P450} g_{CDW}⁻¹ (81 mg_{P450} g_{CDW}⁻¹) was obtained by fermentation under controlled cultivation conditions. This represents the highest obtained yield for expression of P450 BM3 in *E. coli* reported so far and allows further optimization, not only for P450 monooxygenases. Remarkably, the obtained P450 BM3 concentration was 54-fold improved compared to the standard cultivation in shake flask.

For whole cell conversion of *n*-heptane a two-liquid-phase process was established. The system consisted in an aqueous phase, containing *E. coli* cells (catalyst) and glucose for cofactor regeneration in buffer, and the organic phase - applying *n*-heptane both as organic carrier and substrate - in a volumetric ratio of 17% (v/v). Using the cells containing the operon-fusion

P450 BM3 CM1^{NADH}-RE-ADH 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 was obtained. Hence, the whole cell double oxidation with P450 BM3 and RE-ADH yielded up to 3-fold improved product concentrations compared to the *in vitro* system. A high expression of the P450 monooxygenase in the whole cell catalysts was identified as determining factor to achieve the best process performance. The cascade (P450 monooxygenase variants and (*S*)-specific RE-ADH) was extended by a third enzyme, the (*R*)-specific Lb-ADH. A complete oxidation of *n*-heptane to the corresponding heptanones with a chemoselectivity of >99% was achieved employing the three enzyme system, consisting of both stereo-complementary ADHs and P450 BM3.

As a final step, the multi-enzymatic whole cell double oxidation concept was expanded to cyclic alkanes, choosing cyclooctane as model compound. The conversion of cyclooctane with the three enzyme system P450 BM3 19A12^{NADH}-RE-ADH-Lb-ADH was highly chemoselective yielding 94% cyclooctanone with a product concentration of 5.2 mM (652 mg L⁻¹) and a product yield (on catalysts) of 111 mg g_{CDW}⁻¹. In addition, the third highest reported TTN for P450 BM3 monooxygenase of 23700 was reached using the two enzyme system containing P450 BM3 19A12 and Lb-ADH. It was successfully demonstrated that by means of the multi-enzymatic double oxidation cascade the reaction equilibrium could be shifted completely to the side of the ketone upon oxyfunctionalization of the alkanes.

In conclusion, a significantly high expression of the P450 BM3 monooxygenase turned out to be crucial to achieve a good performance in the whole cell catalysis. The reported multi-enzymatic bicatalytic cascade represents the first concept in which a chiral resolution of alcohols is achieved after functionalization of an alkane, including a product based cofactor regeneration in a one-pot reaction. Product ratios for alcohols and ketones can be fine-tuned by simple catalyst selection, overcoming the drawbacks of low selectivities of the employed P450 BM3 catalyst. The developed double oxidation concept can be optimized *in vitro* and directly be transferred to an *in vivo* process. The multi-enzymatic process is a tunable and modular system, in which the employed enzymes (monooxygenases and alcohol dehydrogenases) can be exchanged depending on the targeted specificity and selectivity. As a toolbox the multi-enzymatic system enables the synthesis of enantiomerically pure alcohols or ketones. The concept of the double oxidation cascade can further be expanded to other stereoselective oxidoreductases or further enzyme classes such as aminotransferases or lyases.

6 Appendix

6.1 Abbreviations

AADH	Amino acid dehydrogenase
ABTS	2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)
ADH	Alcohol dehydrogenase
ALA	Aminolevulinic acid
BM	<i>Bacillus megaterium</i>
BSA	Bovine Serum Albumin
BVMO	Baeyer-Villinger Monooxygenase
CCE	Crude cell extract
CDW	Cell dry weight
cfu	Colony-forming units
Cm	Chloramphenicol
CRS	Cofactor regeneration system
CYP	Cytochrome P450 monooxygenase
D-AAO	D-amino acid oxidase
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH ₂ O	Double-distilled water
DEAE	Diethylethanolamine
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate solution mix (equimolar concentration of dATP, dCTP, dGTP, dTTP)
dTTP	Deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	<i>exempli gratia</i> (for example)
<i>ee</i>	enantiomeric excess
EEM	<i>Escherichia coli</i> minimal medium
ep	error prone
<i>er</i>	enantiomeric ratio
<i>et al.</i>	<i>et alii</i> (and others)
FAD	Flavin adenine dinucleotide
FDH	Formate dehydrogenase
FID	Flame ionization detector
FMN	Flavin mononucleotide
Fwd	Forward
GC	Gas chromatography
GDH	Glucose dehydrogenase
h	hour(s)
HSDH	Hydroxysteroid dehydrogenase

HT	Heat treatment
HTS	High Throughput Screening
i.e.	<i>id est</i> (that is)
IPTG	Isopropyl β -D-thiogalactopyranosid
Kan	Kanamycin
kb	Kilobase(s)
K_M	Michaelis-Menten constant
KPi-buffer	Potassium phosphate (inorganic) buffer
<i>L. brevis</i>	<i>Lactobacillus brevis</i>
LB	Lysogeny broth
Lb-ADH	Alcohol dehydrogenase from <i>Lactobacillus brevis</i>
LDH	Lactate dehydrogenase
LeuDH	Leucine dehydrogenase
$\log P_{o/w}$	logarithm of the partition coefficient <i>P</i> of a solvent in a 1:1 mixture of octanol and water
MCS	Multiple cloning site
min	Minute(s)
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTBE	Methyl <i>tert</i> -butyl ether (2-Methoxy-2-methylpropane)
MTP	Microtiter plate
n.d.	Not determined
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NDT	Oligonucleotide triplet (N: G or A or C or T; D: G or A or T)
NNK	Oligonucleotide triplet (N: G or A or C or T; K: G or T)
NPG	<i>N</i> -palmitoyl glycine
OD ₆₀₀	Optical density at a wavelength of 600 nm
P450	Cytochrome P450 monooxygenase
PCR	Polymerase chain reaction
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
PLICing	Phosphorothioate-based ligase-independent gene cloning
PVDF	Polyvinyl idenfluoride
<i>R</i>	<i>Rectus</i> (right, clockwise)
<i>R. erythropolis</i>	<i>Rhodococcus erythropolis</i>
<i>rac</i>	racemic
RBS	Ribosome binding site
RE-ADH	Alcohol dehydrogenase from <i>Rhodococcus erythropolis</i>
Ref	Reference
Rev	Reverse
rpm	Revolutions per minute
RT	Room temperature
s	Second(s)
<i>S</i>	<i>Sinister</i> (left, counterclockwise)

<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	Shine-Dalgarno sequence
SDM	Site Directed Mutagenesis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>sec</i>	secondary
SOC	Super optimal broth with catabolite repression
sp.	Species
SSM	Site Saturation Mutagenesis
TA	Transaminase
TB	Terrific broth
<i>tert</i>	tertiary
TFB	Transformation buffer
TOF	Turnover frequency
Tris	Tris(hydroxymethyl)aminomethane
TTN	Total turnover number
UV	Ultraviolet
$V_{\max}$	maximal reaction rate
WT	Wild type

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6.5 List of primers

In this work oligonucleotide primers were used for the amplification and cloning of the target genes P450 BM3 (Table 6-1) and RE-ADH (Table 6-2).

Table 6-1: Oligonucleotide primers for cloning by PLICing

Nr.	Targeted DNA	Primer identifier	Sequence (5'-3')	T _{annealing} [°C]
1	P450 BM3 gene	Fwd_BM3 Insert-PLIC	catgggcatgacAATTAAAGAAATGC CTCAG	58
2	P450 BM3 gene	Rev-BM3 Insert-PLIC	cgacggagctcgAATTCTTATTACCC AGC	58
3	pET28a(+)-based vectors	Fwd_pET28-BM3-PLIC	cgagctccgtcgACAAGCTTGCG	58
4	pET28a(+)-based vectors	Rev_pET28-BM3-PLIC	gtcatgcccagGTATATCTCCTTC	58
5	pALXtreme-1a P450 BM3	BM3 Fwd_Op_PTO	gctaacaagccCGAAAGGAAGCTG AGTTGGC	55
6	pALXtreme-1a P450 BM3	BM3 Rev_1046S_Op_PTO	gaacagtggatcATGATTACTAGTGG GTTACCCAGCGCTCACGTCTTT TG	55

7	RE-ADH	RE-ADH Fwd_Op_PTO	gatccactgttcAATAAGGAGGTATA CCATGAAGGCAATCCAAGTAC ACG	55
8	RE-ADH	RE-ADH Rev_Op_PTO	ggctttgtagcTTACAGACCAGGGAC CACAACCGC	55

Table 6-2: Oligonucleotide primers for amplification of RE-ADH

Nr.	Primer identifier	Sequence (5'-3')
1	RE-ADH_F_ <i>NdeI</i>	GGAGATATACATATGAAGGCAATCCAG
2 ^[a]	RE-ADH_R_ <i>EcoRV</i>	GCAGCCG <u>ATAT</u> CTTACAGACCAGGGACCACAACC

^[a] The underlined nucleotides serve for introduction of the restriction site *EcoRV* (are not complementary).

Single mutations were introduced by SDM (Table 6-3).and the generation of different SSM libraries was achieved employing primers in Table 6-4 and Table 6-5. The generated mutants were then sequenced with gene- or vector-specific primers (Table 6-6).

Table 6-3: Oligonucleotide primers for Site-Directed Mutagenesis

Nr.	Amino acid exchange	Primer identifier	Sequence (5'-3')
1	Arg255Pro	SDM BM3 R255P_F	CGCTTGATGACGAGAACATTCCTTATCAAATTATTAC ATTC
2	Arg255Pro	SDM BM3 R255P_R	GAATGTAATAATTTGATAAGGAATGTTCTCGTCATC AAGCG
3	Pro329His	SDM BM3 P329H_F	GCTTATGGCCAACTGCTCATGCGTTTTCCCTATATGC
4	Pro329His	SDM BM3 P329H_R	GCATATAGGGAAAACGCATGAGCAGTTGGCCATAA GC
5	Arg966Asp	SDM BM3 R966D_F	GCTTCATACCGCTTTTTCTGACATGCCAAATCAGCC
6	Arg966Asp	SDM BM3 R966D_R	GGCTGATTTGGCATGTCAGAAAAAGCGGTATGAAGC
7	Trp1046Ser	SDM BM3 W1046S_F	GATACGCAAAAGACGTGAGCGCTGGGTAATAAGAA TTCG
8	Trp1046Ser	SDM BM3 W1046S_R	CGAATTCTTATTACCCAGCGCTCACGTCTTTTGCCTA TC

Table 6-4: Oligonucleotide forward-primers for Site-Saturation Mutagenesis of P450 BM3 WT

Nr.	Amino acid position	Primer identifier	Sequence (5'-3')
1	Arg47	SSM_R47_F	CGAGGCGCCTGGTNNKGTAACGCGCTACTTATC
2	Leu52	SSM_L52_F	CGTGTAAACGCGCTACNNKTCAAGTCAGCGTCT
3	Val78	SSM_V78_F	GTCAAGCGCTTAAATTTNNKCGTGATTTTGCAGGAGACG
4	Ala82	SSM_A82_F	GCTTAAATTTGTACGTGATTTTNNKGGAGACGGGTATTATAC
5	Pro142	SSM_P142_F	GATGAGCATATTGAAGTANNKGAAGACATGACACGTTTAACG
6	Thr175	SSM_T175_F	CAGCCTCATCCATTTATTNNKAGTATGGTCCGTGC
7	Ala184	SSM_A184_F	GTCCGTGCACTGGATGAANNKATGAACAAGCTGCAGCGAGC
8	Leu188	SSM_L188_F	GATGAAGCAATGAACAAGNNKAGCGAGCAAATCCAGACG
9	Phe205	SSM_F205_F	GATGAAAACAAGCGCCAGNNKCAAGAAGATATCAAGG
10	Ser226	SSM_S226_F	GCAGATCGCAAAGCANNKGGTGAACAAAGCGATG
11	His236	SSM_H236_F	CGATGATTTATTAACGNNKATGCTAAACGGAAGATC
12	Glu252	SSM_E252_F	GAGCCGCTTGATGACNNKAACATTCGCTATCAAATTATTAC
13	Arg255	SSM_R255_F	GCTTGATGACGAGAACATTNNKTATCAAATTATTACATTC
14	Ala290	SSM_A290_F	CCACATGTATTACAAAAANNKGCAGAAGAAGCAGCACG
15	Ala328	SSM_A328_F	GCTGCGCTTATGGCCAACTNNKCCTGCGTTTTCCCTATATG
16	Leu353	SSM_L353_F	GAAAAAGGCGACGAANNKATGGTTCTGATTCTCAGC
17	Ile366	SSM_I366_F	CTTCACCGTGATAAAACANNKTGGGGAGACGATGTGG

Table 6-5: Oligonucleotide forward-primers for Site-Saturation Mutagenesis of P450 BM3 19A12

Nr .	Amino acid position	Primer identifier	Sequence (5'-3')
1	Val184	SSM_V184_F	GTCCGTGCACTGGATGAANNKATGAACAAGCCGCA GCGAGC
2	Ala74	SSM_A74_F	CTTAAGTCAANNKCTTAAATTTTTCCGTGATTTTTTC AGG
3	Arg47/Tyr51	SSM_R47/Y51_F	CGAGGCGCCTGGTNNKGTAACGCGCANNKATATCAA GTCAGCG
4	Phe87	SSM_F87_F	CAGGAGACGGGTTANNKACAAGCTGGACGCATG
5	Val26	SSM_V26_F	GTTATTAAACACAGATAAACCGNNKCAAGCTTTGA TGAAAATTGC
6	Ile263/Glu267	SSM_I263/E267_F	CAAATTATTACATTCTTANNKGCGGGACACNNKAC AACAAGTGGTC
7	Ala264/Thr268	SSM_A264/T268_F	CATTCTTAATTNNKGGACACGAANNKACAAGTGGT CTTTTATC

Table 6-6: Oligonucleotide primers for the sequencing of P450 BM3

Nr.	Primer identifier	Sequence (5'-3')
1	T7 promoter (forward)	TAATACGACTCACTATAGGG
2	P450BM3_2.1	CTTTAACAGCTTTTACCGAGATCAGC
3	P450BM3_2-4	CGGAAAAGATCCAGAAAC
4	P450BM3_3-4	GCAACGCTTGATTCACAC
8	T7 terminator (reverse)	CTAGTTATTGCTCAGCGGT

6.6 Overview of generated *E. coli* strains

The generated *E. coli* strains are listed in Table 6-7 and 6-8.

Table 6-7: Overview of the *E. coli* BL21 (DE3) Gold lacI^{Q1} strains harboring the pALXtreme-1a vector for single expression of P450 BM3 monooxygenase variants.

Recombinant Protein	Substitutions (1-letter code)	Origin/ Ref.
P450 BM3 WT	-	[57]
P450 BM3 WT ^{NADH}	W1046S, R966D	SDM/ this work; [148]
P450 BM3 19A12	R47C, L52I, V78F, A82S, K94I, P142S, T175I, A184V, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, I366V	Heme fragment ordered as synthetic DNA; [146]
P450 BM3 19A12 ^{NADH}	18 mutations from 19A12; W1046S, R966D	SDM of 19A12; this work
P450 BM3 19A12 A74E	18 mutations from 19A12; additionally A74E	SSM of 19A12; this work
P450 BM3 19A12 V184S	18 mutations from 19A12; additionally V184S	SSM of 19A12; this work
P450 BM3 19A12 P188Q	18 mutations from 19A12; additionally P188Q	SSM of 19A12; this work
P450 BM3 CM1	R255P, P329H	SSM of WT; this work
P450 BM3 CM1 ^{NADH}	R255P, P329H, W1046S, R966D	SDM of CM1; this work
P450 BM3 R255P	R255P	SDM of WT; this work
P450 BM3 P329H	P329H	SDM of WT; this work

Table 6-8: Overview of *E. coli* strains for coexpression of multiple enzymes: P450 BM3 monooxygenase variants and alcohol dehydrogenases RE-ADH and Lb-ADH

<i>E. coli</i> strain	Plasmid and recombinant protein	Features
BL21 (DE3) Gold lacI^{Q1}	pALXtreme-1a P450 BM3 WT ^{NADH} + pKA1 RE-ADH pALXtreme-1a P450 BM3 19A12 ^{NADH} + pKA1 RE-ADH pALXtreme-1a P450 BM3 CM1 ^{NADH} + pKA1 RE-ADH	2-Plasmid-System (5.1 kb/ Kan ^R + 6.6 kb/ Cm ^R respectively); both contain T7 promoter/ <i>lac</i> operator
BL21 (DE3) Gold lacI^{Q1}	pALXtreme-1a P450 BM3 WT + pKA1 Lb-ADH pALXtreme-1a P450 BM3 19A12 + pKA1 Lb-ADH pALXtreme-1a P450 BM3 CM1 + pKA1 Lb-ADH	2-Plasmid-System (5.1 kb/ Kan ^R + 6.2 kb/ Cm ^R respectively); both contain T7 promoter/ <i>lac</i> operator
BL21 (DE3) Gold	pACYCDuet-1 P450 BM3 WT ^{NADH} (MCS1) + RE-ADH (MCS2) pACYCDuet-1 P450 BM3 19A12 ^{NADH} (MCS1) + RE-ADH (MCS2) pACYCDuet-1 P450 BM3 CM1 ^{NADH} (MCS1) + RE-ADH (MCS2)	Duet expression system (Novagen); 8.2 kb/ Cm ^R ; 2x T7 Promoter/ <i>lac</i> operator, 2x RBS

BL21 (DE3) Gold <i>lacI</i> ^{Q1}	pALXtreme-1a P450 BM3 WT ^{NADH} -RE-ADH	„Synthetic“ operon-fusion; 6.1 kb/ Kan ^R ; 1x T7 promoter/ <i>lac</i> operator, 2x RBS
	pALXtreme-1a P450 BM3 19A12 ^{NADH} -RE-ADH	
	pALXtreme-1a P450 BM3 CM1 ^{NADH} -RE-ADH	
BL21 (DE3) Gold <i>lacI</i> ^{Q1}	pALXtreme-1a P450 BM3 WT ^{NADH} -RE-ADH + pKA1 Lb-ADH	2-Plasmid-System, three enzymes (operon-fusion 6.1 kb/ Kan ^R + 6.2 kb/ Cm ^R); features see above
	pALXtreme-1a P450 BM3 19A12 ^{NADH} -RE-ADH + pKA1 Lb-ADH	
	pALXtreme-1a P450 BM3 CM1 ^{NADH} -RE-ADH + pKA1 Lb-ADH	

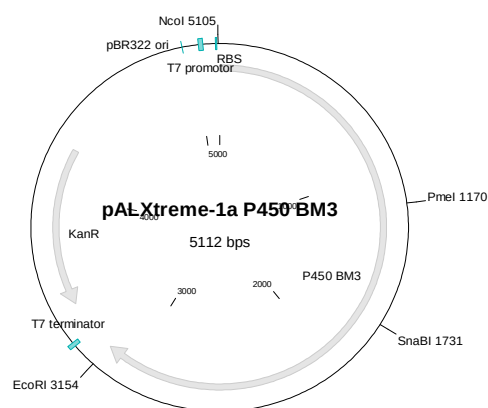


Figure 6-1: Plasmid map of the expression vector pALXtreme-1a containing the P450 BM3 monooxygenase. The P450 BM3 gene (insert DNA) was introduced by PLICing.

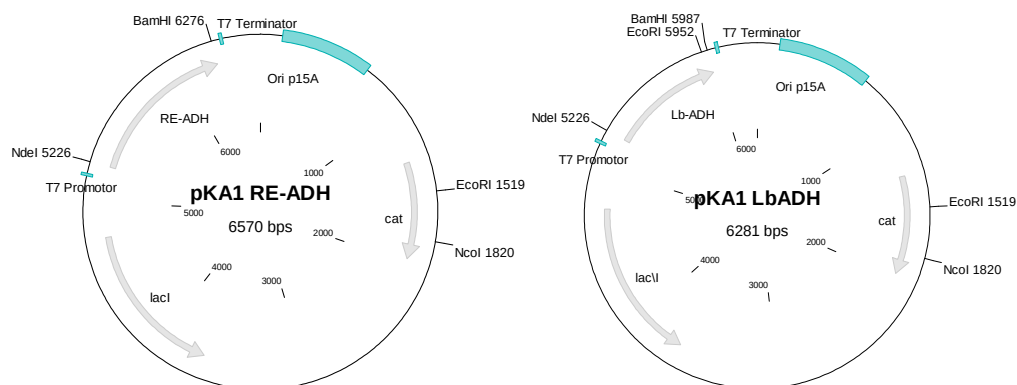


Figure 6-2: Plasmid map of the expression vector pKA1 containing the gene of the alcohol dehydrogenases RE-ADH (A) or Lb-ADH (B). The insert DNA was introduced by restriction cloning using *NdeI* and *BamHI*.

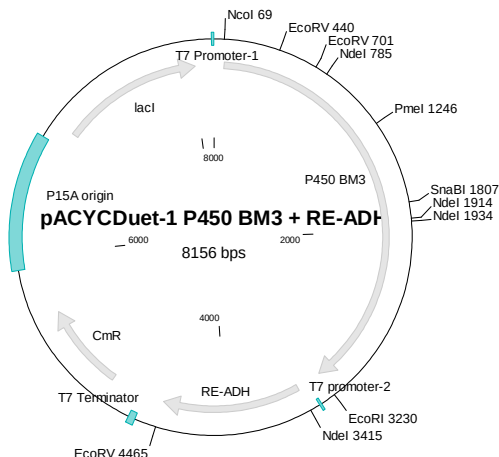


Figure 6-3: Plasmid map of the expression vector pACYCDuet-1 containing the P450 BM3 monooxygenase and alcohol dehydrogenase genes. Cloning was performed by restriction using for RE-ADH the endonucleases *NdeI* and *BamHI*.

6.7 Supplementary information

The analysis of reaction products was performed by gas chromatography as shown in Figure 6-4 and products were quantified using calibration curves for each regioisomers (Figure 6-5).

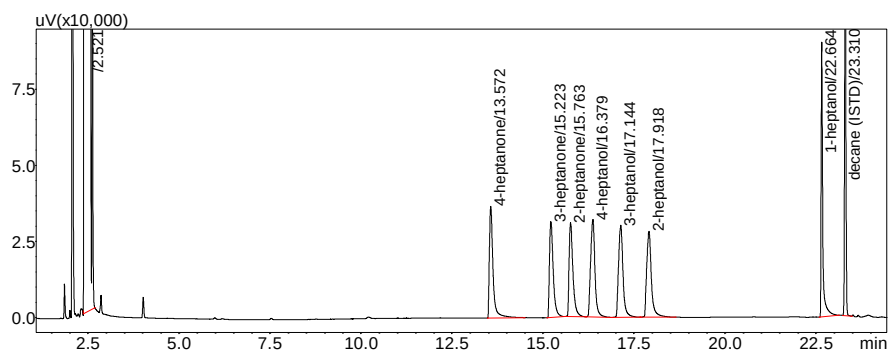


Figure 6-4: Identification of heptanol and heptanone regioisomers by gas chromatography using authentic standards. The graph shows as an example one point of the calibration curve used for quantification of product concentrations and regioselectivity of the enzymatic single or double oxidation of *n*-heptane. Separation of the regioisomers was achieved using a column FS-Supreme-5ms (30 m x 0.25 mm x 0.25 μ m film thickness; CS-Chromatographie, Germany) fitted in a Shimadzu GC-2010 gas chromatograph with a flame ionization detector (FID) (Shimadzu, Japan). Column oven temperature program: 40 $^{\circ}$ C for 19.5 min, 20 $^{\circ}$ C min $^{-1}$ to 100 $^{\circ}$ C, 3 min.

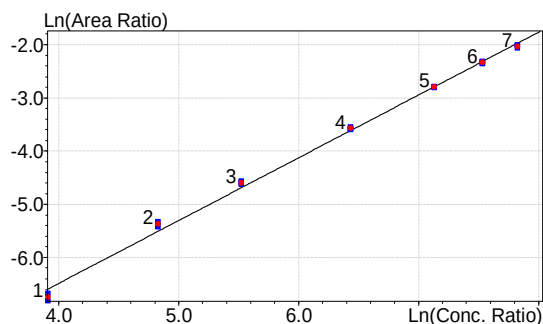


Figure 6-5: Calibration curve for 2-heptanol. A 7-point calibration curve, using authentic standards and an exponential fitting, was calculated for each heptanol and heptanone regioisomers in triplicate (RSD <10%; R^2 >0.99; Software GC Solutions, Shimadzu, Japan). The calibration curve for 2-heptanol is shown here exemplarily.

Partial sequencing of the 10 best mutants for 17 saturated amino acid residues in P450 BM3 WT revealed in eight clones additional mutations besides of a mutation at the targeted position. The additional mutations found are listed in Table 6-9.

Table 6-9: Additional mutations in P450 BM3 clones selected from rescreening

Amino acid position	Clone nr. (rescreening)	Substitution ^[a]	
		at targeted site	at other site
T175	6	T175E	F173I
L188	3	L188F	M185L
F205	5	F205R	D208E
H236	8	H236A	N240L
H236	9	H236T	M237L
R255	2	R255P	P329H
L353	6	L353A	I254V
I366	10	I366S8	A327T

^[a] Mutations detected by partially sequencing (900 bp, fragment containing the saturated site) of the P450 BM3 clones; the possibility of other mutations at not sequenced sites cannot be excluded.

The purification of P450 BM3 monooxygenases using anion-exchange chromatography (Toyopearl DEAE 650S matrix) was performed as shown in Figure 6-6.

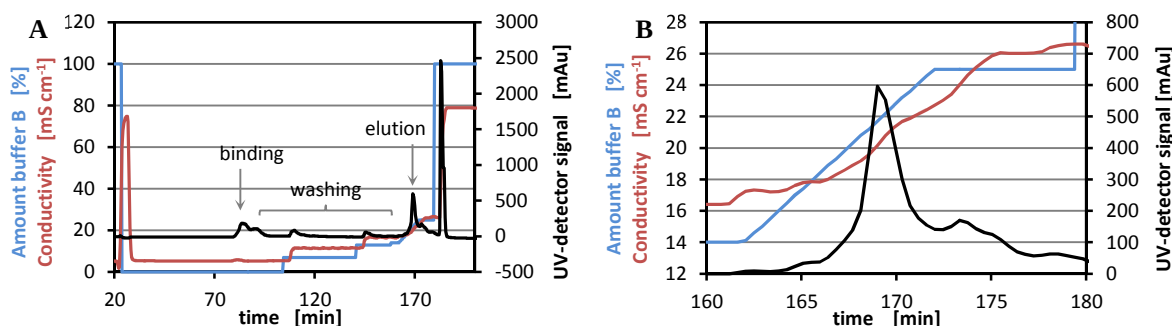


Figure 6-6: Purification profile of P450 BM3 CM1^{NADH} using anion-exchange chromatography. An entire purification run in (A) consist of following steps: loading of crude cell lysate and protein binding, washing of the column matrix and elution of bounded proteins. A detail representation in (B) shows the elution of the target protein P450 BM3 (165-172 min) using a gradient of elution buffer from 14 to 25% (equal to 140 to 250 mM NaCl) and a conductivity increase from 17 to 22 mS cm⁻¹. Elution fractions (5 ml) corresponding to the target peak were collected and analyzed by SDS-PAGE (10%).

Generation of the operon-fusion constructs in the vector pALXtreme-1a was achieved by PLICing^[153] after amplification of vector and insert DNA as shown in Figure 6-7.

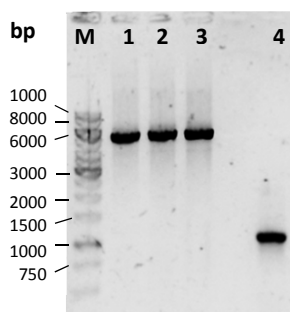


Figure 6-7: Amplification of pALXtreme-1a P450 BM3 and RE-ADH for generation of operon-fusion constructs by PLICing. Each pALXtreme-1a vector containing the P450 BM3 gene WT^{NADH} (line 1), 19A12^{NADH} (line 2) or CM1^{NADH} (line 3), as well as the insert gene RE-ADH (line 4), were amplified with sequence specific primers to generate upon chemical cleavage complementary overhangs for site specific hybridization.

During fermentation of *E. coli* BL21 (DE3) Gold lacI^{Q1} containing the operon-fusion plasmids pALXtreme-1a P450 BM3 WT^{NADH}-RE-ADH, 19A12^{NADH}-RE-ADH and CM1^{NADH}-RE-ADH different parameters were monitored online (pH, pO₂, temperature, stirrer speed, addition of acid and base) as shown exemplarily for 19A12^{NADH}-RE-ADH in Figure 6-8. The cell density (OD₆₀₀) was monitored offline by sampling culture broth before and after induction of the recombinant protein expression. The expression level of P450 BM3 and RE-ADH was analyzed by SDS-PAGE (exemplarily for 19A12^{NADH}-RE-ADH, Figure 6-9).

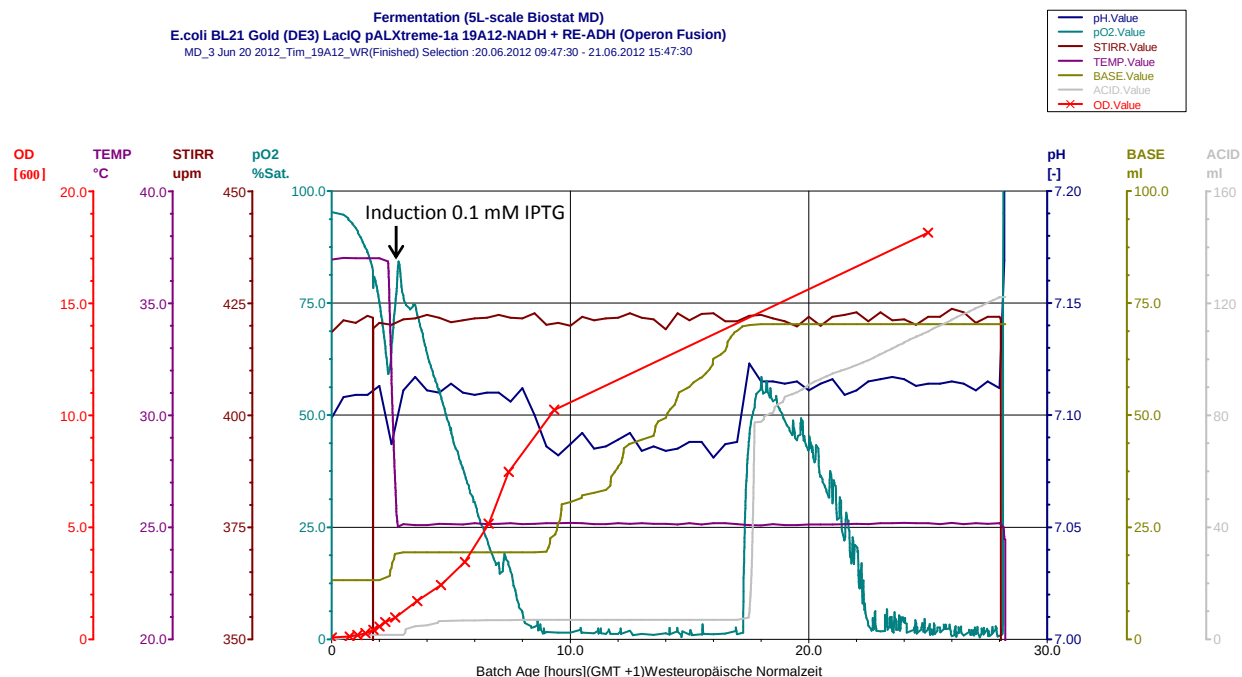


Figure 6-8: Fermentation of *E. coli* BL21 (DE3) Gold lacI^{Q1} pALXtreme-1a P450 BM3 19A12^{NADH}-RE-ADH (operon-fusion) in a 5 L-bioreactor.

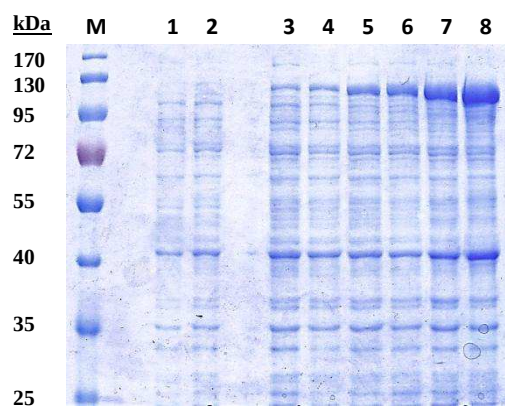


Figure 6-9: Protein expression level during fermentation of *E. coli* BL21 (DE3) Gold lacI^{Q1} pALXtreme-1a P450 BM3 19A12^{NADH}-RE-ADH. SDS-PAGE (10%) of cell lysates from fermentation before induction (1) and 0.5 h (2), 2.5 h (3), 3.5 h (4), 4.5 h (5), 5.5 h (6), 8 h (7) and 21 h (8) after induction of the recombinant protein expression by addition of IPTG.

Each fermentation batch of *E. coli* cells containing the operon-fusion constructs for coexpression of P450 BM3 monooxygenases (WT^{NADH}, 19A12^{NADH}, CM1^{NADH}) and RE-ADH was characterized in terms of P450 content per cell dry weight as shown in Figure 6-10. The correlation of P450 concentration to cell dry weight was linear in this OD range and was used

to calculate P450 yields $Y_{P450/x}$ and total turnover numbers TTN in the double oxidation cascades.

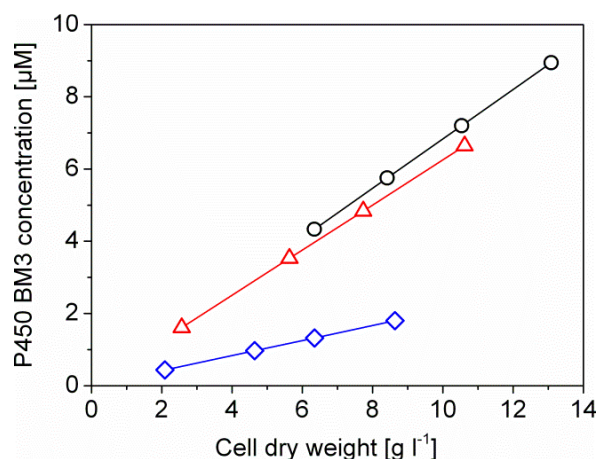


Figure 6-10: Correlation between P450 concentration and cell dry weight of operon-fusion strains from fermentation. The strains are BL21 Gold (DE3) Gold lacI^{Q1} pALXtreme-1a P450 BM3 WT^{NADH}-RE-ADH (red), 19A12^{NADH}-RE-ADH (blue) and CM1^{NADH}-RE-ADH (black).

Figure 6-11 shows the SDS-PAGE analysis of P450 BM3 monooxygenases, RE-ADH and Lb-ADH in the vector constructs for coexpression of two or three enzymes.

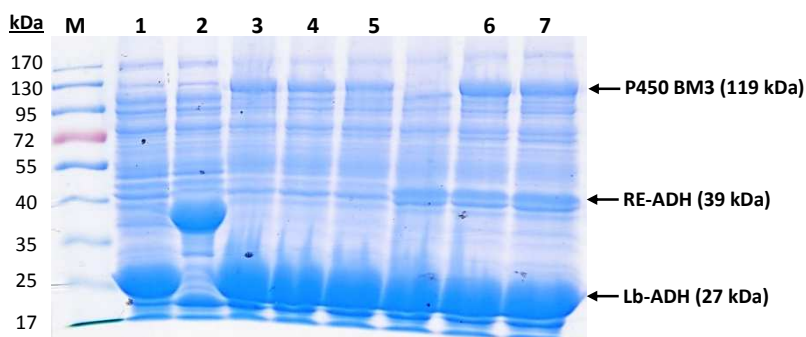


Figure 6-11: Single expression or coexpression of the alcohol dehydrogenase Lb-ADH, together with P450 BM3 and RE-ADH. Visualization on a 10% SDS-PAGE gel. Single expression of recombinant proteins Lb-ADH (1) and TE-ADH (2); Coexpression of two enzymes: P450 BM3 WT + Lb-ADH (3), P450 BM3 19A12 + Lb-ADH (4), P450 BM3 CM1 + Lb-ADH (5); Coexpression of three enzymes: P450 BM3 19A12^{NADH} + RE-ADH + Lb-ADH (6), P450 BM3 CM1^{NADH} + RE-ADH + Lb-ADH (7); (M) Molecular weight standard (Prestained PageRuler, Fermentas). All strains were expressed in shake flask.

7 References

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