Cytochrome P450 monooxygenases: a study of the synthesis of industrial relevant aliphatic ω-hydroxy products

Cytochrome P450 Monooxygenasen: Eine Studie über die Herstellung von industriell bedeutenden aliphatischen ω-Hydroxyverbindungen

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List of publications

Part of this work has already been published or submitted for publication in the form of scientific article (I – III) or patent (IV):

I D. Scheps, S. Honda Malca, H. Hoffmann, B. M. Nestl, B. Hauer. Regioselective omega-hydroxylation of medium-chain alkanes and primary alcohols by CYP153 enzymes from *Mycobacterium marinum* M. and *Polaromonas* sp. JS666. *Org Biomol Chem* 2011, 9, 6727.

II S. Honda Malca, D. Scheps, L. Kühnel, E. Venegas-Venegas, A. Seifert, B. M. Nestl, B. Hauer. Bacterial CYP153A monooxygenases for the synthesis of omega-hydroxylated fatty acids. *Chem Commun* 2012, 48, 5115.

III D. Scheps, S. H. Malca, S. Richter, K. Marisch, B. M. Nestl, B. Hauer. Production of ω-hydroxy dodecanoic acid based on an engineered CYP153A fusion construct. Microbial Biotechnology 2013, 6, 694.

IV D. Scheps, B. Nebel, M. Breuer, H.-G. Wagner, B. Breitscheidel, B. Hauer. Process for the bioconversion of *n*-butane to 1-butanol. BASF SE. Patent filed.

The following publication has not been included in this work:

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Abstract

The ω-regioselective hydroxylation of aliphatic compounds like alkanes or fatty acids with different chain lengths is a longstanding problem in chemistry. Chemo-catalyzed reactions suffer from subterminal hydroxylation and overoxidation which can only be solved by harsh reaction conditions. Biocatalysis offers interesting tools for these complex questions.

In the present thesis several biocatalysts were investigated with the focus on cytochrome P450 monooxygenases (CYP or P450). These widely spread enzymes accept a variety of substrates and perform C–H hydroxylations with high regio- and stereospecificity. Particularly members of the bacterial CYP153A subfamily show interesting abilities in substrate specificity (alkanes) and terminal hydroxylation selectivity. Several CYP153A candidates were characterized *in vitro* towards alkanes, primary alcohols, mono (un)- and saturated fatty acids to determine the substrate specificity of the biocatalysts. Suitable enzymes have been selected to oxidize *n*-butane as well as dodecanoic acid. Further enzyme improvements were achieved by employing optimization techniques like rational protein design, directed evolution experiments and establishment of self-sufficient fusions.

CYP153A$_{P.\ sp.}$ from *Polaromonas* sp. and CYP153A$_{M.\ aq.}$ from *Marinobacter aquaeolei* were selected for a detailed *in vitro* analysis. CYP153A$_{P.\ sp.}$ was identified as predominant alkane ω-hydroxylase which hydroxylates C_5-C_{12} alkanes combined with ω-regioselectivity of up to 91 %. In contrast CYP153A$_{M.\ aq.}$ showed predominantly fatty acid ω-hydroxylase activity with a broad substrate spectrum ($C_{8:0}$-$C_{20:0}$ and 9(*Z*)/9(*E*)-$C_{14:1}$-$C_{18:1}$).

For the purpose of applying CYP153A$_{M.\ aq.}$ and CYP153A$_{P.\ sp.}$ in a bacterial whole cell system, a rational design approach was used to identify positions which are important for substrate selectivity and activity. CYP153A$_{M.\ aq.(G307A)}$ and CYP153A$_{P.\ sp.(G254A)}$ showed up to 10-fold higher activity against smaller substrates with further increased ω-regioselectivity (more than 95 %). To optimize the coupling efficiency as well as protein expression, self-sufficient fusion constructs were established. The heme domain of the monooxygenase was fused to the reductase domain (CPR) of P450 BM3 from *Bacillus megaterium*. The measured coupling efficiency (more than 70 %) with the test substrate dodecanoic acid (C12-FA) was higher for CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ in comparison to the use of single redox proteins (ca. 20 %).

Abstract

Different accessible biocatalysts for the hydroxylation of gaseous *n*-butane to liquid 1-butanol were compared. These experiments resulted in final concentrations of 0.74-0.88 g per liter butanol after 24 h (more than 90 % ω-regioselectivity) for CYP153A$_{P. sp.}$ and CYP153A6-BMO1 (CYP153A6-butane monooxygenase 1) applied in a *E. coli* resting cell experiment with their natural redox partners. After the improvements of enzymes *via* fusion establishment and optimization of the reaction conditions by a high-pressure tank, product concentrations of up to 4 g per liter after 24 hours were achieved with a cell mass of 18.7 g_{cdw}. For further enzyme optimization, directed evolution with a SeSaM-library was applied. This strategy was combined with a viability *in vivo* screening based on *Pseudomonas putida* KT2440. This strain offers the opportunity to select mutants with the ability to hydroxylate alkanes at the terminal position because they are able to grow on primary alcohols as carbon source. The screening with butane enabled the identification of two new hotspots (Ala184 and Thr300) in the used enzyme CYP153A$_{P. sp.}$. With a solid method in hand for the oxidation of gaseous *n*-butane in whole cells we turned our attention towards dodecanoic acid as substrate. After initial shaking flask experiments, the performance of a non-engineered non-solvent adapted *E. coli* resting cell system was increased in a small scale bioreactor (1 L). After first experiments with C12-FA yielding 1.2 g per liter ω-hydroxylated product in 30 h using a cell mass of 15.1 g_{cdw}, dodecanoic acid methyl ester was used as substrate for bioconversion experiments. An additional outer membrane transporter in form of AlkL from *P. putida* was coexpressed to overcome the transfer limitation of the substrates. Parallel feeding with a glucose/glycerol-mix and fine regulation of fermentation parameters (*e.g.* pO_2) lead to maximum production concentrations of 4 g per liter in 28 h using 18.2 g_{cdw} cell mass.

The present studies successfully demonstrated new possibilities to optimize different CYP153A enzymes *via* rational design, directed evolution and fusion experiments. With CYP153A- CPR$_{BM3}$ a new promising way for the production of 1-butanol based on butane was shown. The flexibility of the biocatalyst was proven with the highest ever reported end concentrations for the synthesis of ω-dodecanoic acids with bacterial production hosts.

Zusammenfassung

Die ω-regioselektive Oxidation von Alkanen, Fettsäuren und anderen aliphatischen Verbindungen, ist ein größtenteils ungelöstes Problem im Bereich der Chemie. Speziell die Vermeidung von subterminaler Oxidation sowie Überoxidation kann bei klassischen, chemischen Verfahren nur mithilfe von sehr energieintensiven Verfahren gewährleistet werden. Die Biokatalyse bietet einige interessante Ansätze um diese herausfordernde Fragestellung zu lösen

In der vorliegenden Arbeit wurden verschiedene Biokatalysatoren untersucht mit dem Schwerpunkt auf Cytochrom P450 Monooxygenasen (CYP oder P450). Diese in der Natur weit verbreiteten Enzyme akzeptieren unterschiedliche Substrate und sind in der Lage eine regio- und stereospezifische Hydroxylierung einer C-H Bindung durchzuführen. Speziell die Mitglieder der CYP153A Unterfamilie (aus Bakterien) zeigen eine bemerkenswerte Substratspezifität gegenüber Alkanen gepaart mit einer hohen Regioselektivität für die Oxyfunktionalisierung terminaler Positionen. Die Substratspezifität von verschiedenen CYP153A Enzymen wurden *in vitro* gegenüber Alkanen, primären Alkoholen, (un)gesättigten und gesättigten Fettsäuren getestet. Vielversprechende Enzyme wurden für die Oxidation von *n*-Butan und Dodecansäure ausgewählt. Verschiedenen Techniken wie rationales Design, gerichtete Evolution und die Synthese von Fusionsproteinen wurden verwendet, um die entsprechenden Enzyme zu optimieren.

Neben CYP153A$_{P.\ sp.}$ aus *Polaromonas* sp. JS666 wurde auch CYP153A$_{M.\ aq.}$ aus *Marinobacter aquaeolei* VT8 für detaillierte Studien in dieser Untersuchungsreihe ausgewählt. CYP153A$_{P.\ sp.}$ ω-hydroxyliert bevorzugt mittelkettige Alkane (C_5-C_{12}) mit einer ω-Regioselektivität von bis zu 91 %. Im Gegensatz dazu kann CYP153A$_{M.\ aq.}$ als Katalysatoren bezeichnet werden, welcher Fettsäuren ($C_{8:0}$-$C_{20:0}$ und 9(*Z*)/9(*E*)-$C_{14:1}$-$C_{18:1}$) als Substrate bevorzugt.

Mit dem Ziel CYP153A$_{M.\ aq.}$ und CYP153A$_{P.\ sp.}$ in einem bakteriellen Ganzzellsystem zu nutzen, wurde rationales Design verwendet um Aminosäurepositionen zu identifizieren, welche für die Substratselektivität und Aktivität wichtig sind. CYP153A$_{M.\ aq.(G307A)}$ und CYP153A$_{P.\ sp.(G254A)}$ zeigten bis zu 10-fach höhere Aktivität gegenüber kleineren Substraten mit zusätzlich erhöhter ω-Regioselektivität (mehr als 95 %). Um sowohl die Expression als auch die Elektronenübertragungseffizienz der Proteine zu verbessern, wurden Fusionsproteine

hergestellt. Die ausgewählten Häm-Domänen wurden mit einer Reduktase-Domäne (CPR) aus P450 BM3 aus *Bacillus megaterium* fusioniert. Die Elektronenübertragungseffizienz für Dodecansäure (C12-FA) war mit mehr als 70 % von CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ deutlich höher als mit einzelnen Proteinen (ca. 20 %).

Zunächst wurden unterschiedliche verfügbare Biokatalysatoren für die Oxidation von gasförmigem *n*-Butan zu flüssigem 1-Butanol verglichen. CYP153A$_{P.\ sp.}$ und CYP153A6-BMO1 (CYP153A6 Butan Monooxygenase) erzielten nach 24 Stunden Endkonzentrationen zwischen 0.74-0.88 g/L Butanol mit ruhenden *E. coli* Zellen und deren natürlichen Redoxpartnern. Nach der Optimierung der Enzyme mittels Proteinfusion (CYP153A$_{P.\ sp.(G254A)}$-CPR) und Verbesserung der Reaktionsbedingungen in Form eines Druckreaktors, konnten nach 24 h Produktkonzentrationen von bis zu 4 g/L mit einer Zellmasse von 18.7 g_{Ztm} gemessen werden. Des Weiteren wurde zur weiteren Enzymoptimierung eine gerichtete Evolution mit Hilfe einer SeSaM-Mutantenbibliothek durchgeführt. Diese Strategie wurden mit einem Wachstumsassay untersucht, welcher auf *Pseudomonas putida* KT2440 basiert. Dieser Stamm bietet die Möglichkeit Mutanten zu selektionieren, welche die Fähigkeit besitzen Alkane terminal zu oxidieren. Dies ist gegeben, da der Stamm in der Lage ist primäre Alkohole als Kohlenstoffquelle zu nutzen. Durch diese Versuchsreihe konnten für CYP153A$_{P.sp.}$ zwei neue „hot spots" (Ala184 und Thr300) für eine verbesserte Butanoxidation identifiziert werden. Nachdem ein System für die Oxidation von *n*-Butan in ganzen Zellen etabliert werden konnte, konnte dieses Wissen bei ähnlichen Systemen für die Hydroxylierung von Dodecansäure genutzt werden. Nach ersten Experimenten in Schüttelkolben wurde die Umsetzungsrate der nicht-Lösungsmittel adaptierten, nicht-metabolisch veränderten *E. coli* Zellen in einem Kleinfermenter (1 L) gesteigert. Mit C12-FA als Substrat konnte mit 15.1 g_{Ztm} Zellmasse nach 30 Stunden 1.2 g/L ω-hydroxyliertes Produkt erhalten werden. In nachfolgenden Experimenten wurde Dodecansäuremethylester als Substrat verwendet. Ein Transporter für den Substrattransfer über die äußere bakterielle Membran wurde zusätzlich genutzt, um diesem limitierenden Faktor entgegenzuwirken. Eine kontinuierliche Fütterungsstrategie mit einem Glukose/Glycerin-Mix und eine Optimierung der Fermentationsparameter (z.B. pO_2), führten zu maximalen Produktausbeuten von bis zu 4 g/L nach 28 Stunden Reaktionszeit mit 18.2 g_{Ztm} Zellmasse.

Die vorliegende Studie zeigt neue Möglichkeiten auf, um unterschiedliche Mitglieder der CYP153A Enzymfamilie mittels Methoden des rationalen Designs und gerichteter Evolution zu optimieren. Zudem konnten mit den CYP153A-CPR$_{BM3}$ Katalysatoren ein vielversprechender Weg für die Herstellung von 1-Butanol ausgehend von *n*-Butan demonstriert werden. Die Vielseitigkeit des Biokatalytischen Systems wurde mit den höchsten bisher veröffentlichten Endkonzentrationen bei der Synthese von ω-Hydroxy Dodecansäure mit einem bakteriellen Produktionssystem bestätigt.

1. Introduction

1.1. Industrial biocatalysis

Enzymes are biocatalysts, which are indispensable to life due to their ability to lower the activation energy of a high variety of biochemical reactions. Their most important characteristic is a high catalytic efficiency that leads to remarkable catalytic improvements of up to 10^{19}-fold compared to the corresponding uncatalyzed reactions.[1]

Several enzymes accept – next to their natural substrates – a wide range of complex natural and unnatural molecules as substrates. This substrate promiscuity is shown by enzymes with relaxed/broad substrate specificity. Another form of promiscuity is based on the catalytic mechanism, which describes that one enzyme performs different kinds of chemical reactions, without affecting the organisms fitness.[2] Hence these enzymes are interesting catalysts for industrial processes. Biocatalysts can work under different conditions like high temperature, unusual pH, and the presence of organic solvents.[3] But the most significant property of enzymes for industrial application is their high specificity and selectivity. Among others, industrial usage is based on (semi)purified, immobilized enzymes, and whole cell applications. Biocatalysis, which includes metabolic engineering approaches is used for the production of agrochemicals, food ingredients, pharmaceuticals, but also for the production of fine and bulk chemicals, which are traditionally made by chemocatalysis.[4]

One of the first modern processes, the synthesis of lactic acid, has been conducted in the United States in 1880.[5] In 1894, Jokichi Takamine published the first enzyme-based patent. He described the usage of an amylase mix and started the commercial usage of microbial enzymes.[6] More than 100 years ago, biocatalysis was employed to do chemical transformations on non-natural organic compounds. It was in 1908 when Rosenthaler and co-workers published the synthesis of *R*-(+)-mandelonitrile from benzaldehyde and hydrogen cyanide by an enzymatic preparation using a plant extract (including hydroxynitrile lyase).[7] This pioneered achievement represented one of the first descriptions of an asymmetric enzyme based reaction. In the following years, biocatalysis was further settled by different successful examples as synthetic tool for the production of valuable products. Neuberg and Hirsch showed the condensation of benzaldehyde with acetaldehyde

by a pyruvate decarboxylase from *Saccharomyces cerevisiae* to the optically active 1-hydroxy-1-phenyl-2-propanone. It was later used as precursor for a chemical conversion to L-(-)ephedrine in 1930.[8] This is one of the first examples of a promiscuous catalytic activity of an enzyme. In addition, the microbial hydroxylation by *Rhizopus* sp. had a huge impact in transforming progesterone to 11α-hydroxyprogesteron in 1952. It is still seen as the antecedent in the synthesis of corticosteroids (anti-inflammatory activity).[9] A breakthrough for the daily use in private households — especially regarding laundry detergents in the early 1960s — was the establishment of proteases, which catalyze the cleavage of peptide bonds in other proteins by hydrolysis.[10] The first usage of an immobilized enzyme process for the synthesis of chemicals was established in the mid 1970ies. It was meant to isomerize glucose into fructose to increase the requested sweetness. Due to the fact that fructose is sweeter than the starting product, it is especially the food industry, which profits from this process.[11] Fine chemicals in form of chiral products were predominantly targeted in biocatalytical applications over the last 20 years. An example is the hybrid chemical/biocatalytical processes for synthesis of (*R*)-1-phenylethylamine with a high enantiomerically purity *via* an acylation of the racemate by a lipase. Followed by separation of the (*S*)-enantiomer through distillation lead to the (*R*)-product by the use of a NaOH based hydrolysis reaction.[12] One of the still rare success stories of applied metabolic engineering processes concerning a bulk chemical was set up by Genencor and DuPont by developing the synthesis of 1,3-propanediol (PDO) in *E. coli*. In accordance with changes in central metabolism with the introduction of a new metabolic pathway, while combining four new genes from other bacterial strains to produce PDO with a high efficiency.[13] These prominent examples give the opportunity to emphasize the fact of biocatalysis being a powerful tool for the chemical and pharmaceutical industry to save energy requirements and use cheap (sometimes renewable) precursors. Often biocatalysis minimizes the amount of pollutants like waste stream and organic solvents and provides access to new products *via* smart synthesis routs.[14, 15]

1.2. Bulk and fine chemicals

There is no general accepted classification, which clearly defines bulk or fine chemicals.[16] Compounds often change their classification, because production volumes or prices defining

factors like raw materials and processing costs change permanently. Bulk chemicals or commodity chemicals include a broad spectrum of different inorganic chemicals, fertilizer, polymers and bulk petrochemicals and intermediates.[17] They are produced in extensive amounts (more than 10.000 – 20.000 t/p.a.) to relatively low costs (less than 10 $/kg). The beneficial price results from the fact that the required purity is usually expected to be lower than one of a fine chemical product. In contrast to a fine chemical process, the performed processes are highly adjusted, continuous, and excel due to their long lifecycle.[17, 18] In the field of bulk chemicals further subclassifications into "classical bulk" chemicals and "bulk-performance" chemicals can be made. They are distinguished in terms of their characteristic performance and they are mostly formulated before selling them to the market. Typical examples for "classical bulk" chemicals would be acetone, terephthalic acid or 1-butanol. As examples the other group polymers (propylene), surfactants (alkylbenzenesulfonates) or amino acids (glutamate) can be mentioned.[19]

In comparison to bulk chemicals, fine chemicals are sold at a high price (more than 10 $/kg) and relatively small amounts (less than 5.000 – 10.000 t/p.a.). Among others agrochemicals, fragrances ,flavors, pharmaceuticals and food additives belong to this classification.[20, 21] Fine chemicals are generally more expensive due to a higher structural complexity. Fine chemicals are complex, single and pure chemical substances. Multistep processes are often used to synthesize the product or biotechnological processes. They are sold for more than 10 $/kg in high purity. Examples for biotechnological produced fine chemicals on a more than 100 t/pa scale are amines ((*R*)-1-phenylethylamine), hydroxylated heterocyclic compounds (6-chloropicolinic acid) or special amino acids (6-aminopenicillanic acid).[12, 22]

Today, naphtha (crude oil) is the key commodity available to the chemical industry from oil refineries to produce bulk chemicals.[17, 23] Next to the essential aromatics compounds xylene, toluene or benzene, important precursors for synthesis by classical chemocatalysis like ethylene, propene or butadiene are derived *via* thermal naphtha cracking. Moreover, syngas (synthesis gas), which contains hydrogen and carbon monoxide in various proportions as well as methane, are crucial basic materials for the synthesis of acetic acid, ammonia or different/other oxo-products.[23]

An alternative way, especially to implement new raw materials, can be the application of biotechnology. The use of biotechnology for the synthesis of bulk chemicals, however, is in most cases challenging. Apart from long established amino acids processes, only the synthesis of 1,3 propanediol is a biotechnological successful story.[13] In this context it has to be mentioned that further fermentation plants – *e.g.* for the production of succinic acid – are currently being constructed.[24] Depending on the used biocatalyst, different limitations can appear. New production plants have to be taken into account, which requires capital investment and engineering knowledge. Required is furthermore enzyme stability for essential recycling procedures, high activities of the biocatalyst and cofactor dependence.[3, 25]

1.2.1. 1-Butanol: past and present

1-Butanol (*n*-butanol or butyl alcohol) is a colorless and flammable liquid. It has a typical banana-like odor and a boiling point of 118 °C.[26, 27] 1-Butanol is a versatile chemical intermediate or raw material used as plasticizer and solvent for paints, coatings and varnishes. It also provides an innovative product for a multitude of industrial applications, such as the production of plastics, textiles, cosmetics, drugs, antibiotics, vitamins, hormones and brake fluids. The worldwide 1-butanol production accounted for more than 2.8 million tons in 2008.[26] The primary alcohol can be converted into more valuable chemicals such as butyl acrylate (homo- and copolymers, surface coating) and methacrylate esters (resins, oil additive, enamels, paper production). Other important derivatives are glycol ethers and butyl acetates (paints and coatings). The bulk chemical is also processed into a vast number of chemical compounds such as pesticides (thiocarbazides), solvents and detergents.[28]

At the moment we are faced with a discussion about the usage of 1-butanol as next generation of biofuels. Produced by a fermentative approach (*e.g.* based on Clostridia) it could be possible to reduce oil dependency and greenhouse emission.[29] In contrast to already used alternative fuels like ethanol, 1-butanol or derivatives of it like isobutanol have higher energy contents. It can be blended into gasoline without the limitation/disadvantage of water adsorption and thus, transported and sold throughout established infrastructure.[29]

1.2.2. Chemical synthesis of 1-butanol

The leading production of 1-butanol is based on three synthetic routes. One is the hydroformylation process (oxo-synthesis), which contains the addition of hydrogen and carbon monoxide to carbon-carbon double bond of the steam cracker product propene using transition metal catalysts like Co, Rh or Ru (Figure 1 - 3a).[30, 31] Aldehyde mixtures in form of butyraldehyde and isobutyraldehyde are converted *via* a hydrogenation reaction (3b). Different reaction isomers of butanol (mixture of 1-butanol and isobutanol) are obtained by varying pressure and temperature conditions.[17] Next to other oxo-synthesis, the 'Shell-process' is the only application, in which hydroformylation of alkenes and hydrogenation of the intermediate aldehydes take place in the same reactor simultaneously. This process is mainly established to produce C_{12} - C_{15} alcohols.[17] 50 % of the butyraldehyde is also used for the synthesis of 2-ethylhexanol.[27] This property makes the hydroformylation reaction effective and selling of the important C_4-aldehydes can release important economic synergies.[27] These smart interactions are not possible with the Reppe-process, in which propene, carbon monoxide and hydrogen react in presence of a tertiary ammonium salt of ferrum carbonyle hydrides to 1-butanol and isobutanol in one step (5). The crotonaldehyde hydrogenation process provides an alternative petrochemical route for butanol production (4). Due to the fact that ethanol is used during the reaction, this process is realized exclusively in Brazil, where this alcohol can be cheaply obtained from biomass.[17, 31] *Via* combining aldol condensation along with an acidification (4a) and hydrogenation reactions (4b) the C_4 primary alcohol butanol can be catalytically synthesized.[17]

1.2.3. Biotechnological synthesis of 1-butanol

In the early 1920s, Chaim Weizmann developed the first commercial production of acetone and alcohols from renewable resources by a mixed culture. *Clostridium acetobutylicum* primarily carries out this fermentation process, which naturally produces acetone, butanol and ethanol in a ratio of 3:6:1.[32, 33] The first production plants for the ABE fermentation were developed because there was an exceeding demand of acetone for the cordite manufacture during the world wars, but butanol was only seen as an unwanted byproduct.[34, 35] After the

war, however, 1-butanol became more important. The acetone-butanol fermentation using *C. acetobutylicum* was the most important production process of these solvents during the first part of the last century, but also uneconomical as a result of the cheaper petrochemical production of butanol.[26] Remarkably, the industrial and scientific efforts increased concerning the *clostridia*-research. Reasons for this effect are the recent interest in renewable resources. Metabolically improved *C. acetobutylicum*-production systems have been generated, which produce 238 mM (17.6 g/L) butanol in 28 g/L total solvents.[36] As a consequence, in 2008, BP and DuPont intently announced to reestablish — in close cooperation — an industrial scale *clostridia*-process in Europe as well as to increase their efforts in fermentation processing (7).[37, 38] In addition Cathay Industrial Biotech and Songyuan Laihe Chemical in China plan amongst others to reestablish the ABE fermentation with a capacity of >1 million t/pa.[39, 40] At the moment the first semi continuous plants are based on corn starch, but as soared corn prices are not able to compete with petrochemical-processes in respect to final price further improvements have to been done in this field.[39] At the moment these companies try to concentrate on a corn stover based production process.[27]

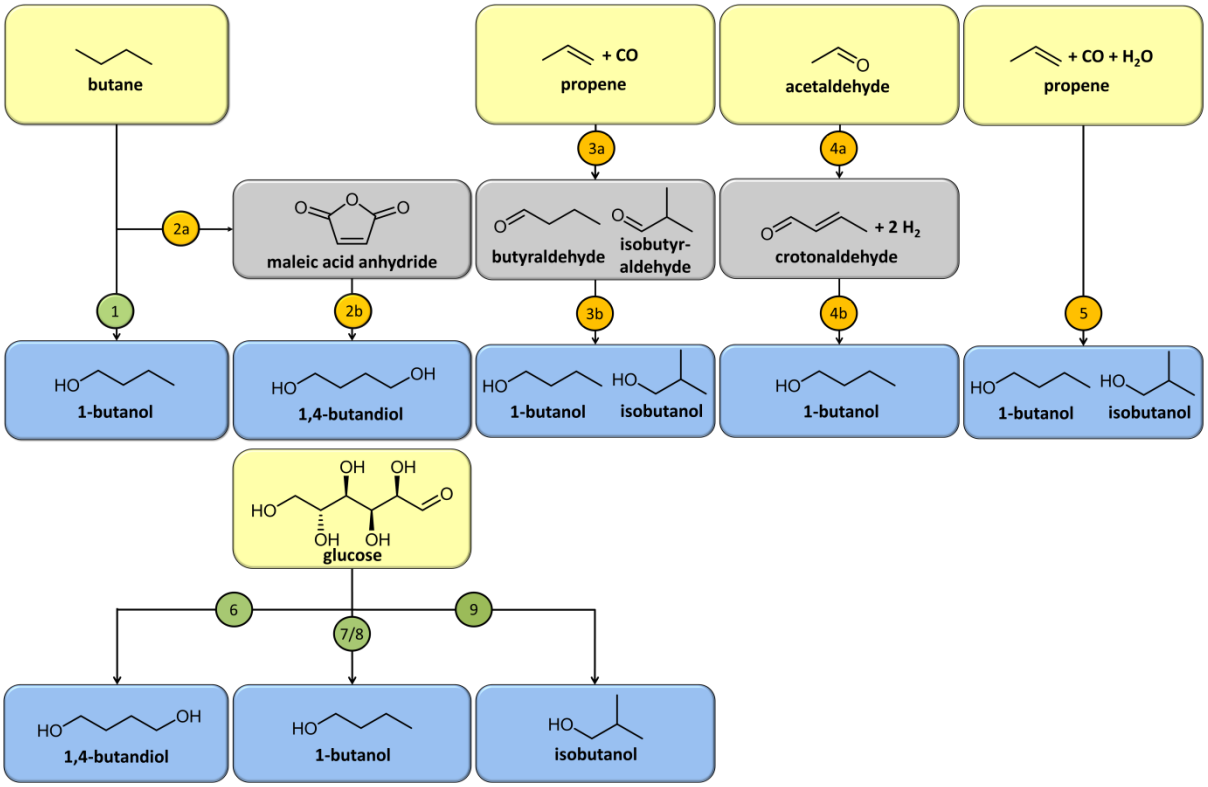

Figure 1: Production of C_4 chemicals: (1) Biocatalytic hydroxylation by CYP153A or alkB; (2) Synthesis of 1,4-BDO; (3) Hydrofromylation process and hydrogenation; (4) Aldol condensation along with dehydration and hydrogenation reactions; (5) Reppe process; (6) *E. coli* based fermentation process to gain 1,4-BDO; (6) Metabolic engineering approach with *E. coli*; (7) ABE fermentation with *Clostridia*; (8) yeast based fermentation to produce isobutanol.

Another interesting production pathway is based on metabolic engineering in *E. coli*. One example is therefore the integration of a highly active amino acid biosynthetic pathway for butanol production. Atsumi and co-worker hence combined the 2-ketoacid decarboxylase with an alcohol dehydrogenase for the transformation of common 2-keto acids. Titers of 300 mM (22 g/L) of isobutanol and 8 mM (0.6 g/L) of 1-butanol could be achieved in first studies.[41, 42] An alternative route to synthesize 1-butanol is the functional reversal of the β-oxidation cycle in *E. coli* that can be used as a metabolic platform (8) for the synthesis of alcohols like butanol with titers up to 188 mM (14 g/L).[43] In 2011 was reported that *E. coli* can produce 30 g/L with an integrated clostridial 1-butanol pathway (8).[44] Genomatica modify *E. coli* strains with an optimized oxidative tricarboxylic acid cycle for the synthesis of 1,4-butanediol (5).[45-47]

Engineering approaches in yeast (*Saccharomyces cerevisiae*) have recently entered the focus of several research groups. At present yeast based fermentation processes are operating for the production of isobutanol as biofuel by GEVO, USA, with a capacity of 18 million gallon/p.a. (9).

1.2.4. Biotechnological synthesis of 1-butanol based on *n*-butane

A promising feedstock for industrial processes, next to the classical crude oil-refinery, is *n*-butane. This small alkane is inexpensive and available in high amounts. Currently *n*-butane is mainly manufactured during crude oil production as byproduct, but mainly burned and only partially collected due to minimal industrial demand. *n*-Butane is in principal a component of natural gas and additionally available in commercial "Shell gas" (Liquefied petroleum gas (LPG)). "Shell gas" is of special interest since the United States and other countries like Iraq increase the efforts to make new petrochemical feedstocks accessible.[48] It consists primarily of propane and *n*-butane and offers therefore another cheap feedstock for the synthesis of products of higher value. One important *n*-butane application is the production of 1,4-butanediol. Direct operation is problematic, since selective hydroxylation of terminal non-activated C-H bonds remains a major challenge for classical chemocatalysis. There are several methods available based on metal catalysts, but these reactions are not selective or they rely on harsh reaction conditions and represent high energy-consuming processes due to high temperatures and high pressure conditions. Therefore, the synthesis of maleic acid anhydride with a vanadium pyrophosphate catalyst is applied as an intermediate (2a).[49, 50] Maleic acid anhydride can further be used in a next step *via e.g.* Davy process for the synthesis of 1,4-butandiol (2b). However, the lack of selective *n*-butane oxidation catalysts has strongly restricted further utilization of this raw material. An interesting possibility to use this raw material is the direct enzymatic conversion from *n*-butane to 1-butanol (1). The utilization of these inert substrates is also in biocatalysis challenging. At present only some microorganisms are known, which can metabolize aliphatic alkanes.[51] Consecutively several enzyme families could be identified, capable of hydroxylating alkanes to the corresponding alcohols (terminal and subterminal hydroxylation).[51] These are the first steps in hydrocarbon metabolism. For their biotechnological potential, recently different enzymatic system, which are able to

hydroxylate alkanes, have been studied. Alkane ω-hydroxylases are enzymatic systems, which show broad substrate specificity and are able to hydroxylate several substrates in respect to different chain length **(**Figure 2).

Short-chain alkanes (C_1–C_4) are hydroxylated by methane, propane and butane monooxygenases (MMO, PMO or BMO).[52-55] Two different types of MMOs exist in nature, whereas the first one is a soluble MMO (sMMO), the other one is a membrane-bound MMO (pMMO).[56, 57] Soluble MMOs show the ability to hydroxylate more than 50 different substrates including several aromatic compounds.[54, 56] They display a characteristic carboxylate bridged di-iron center, whereas the pMMOs possess an active site with a di-copper center.[58] Solved crystal structures of MMOs lead to experiments, which try to minimize the protein surface and establish a biomimetic enzyme core to perform hydroxylation reactions. But the reported activity against alkanes as substrates was low.[59, 60] While methanotrophes and the corresponding enzymatic systems are targets of different scientific studies, industrial applications are still missing. One limiting fact is that they exist only as multi-protein complex, which is necessary for the electron transfer.[56] Nearly all hydroxylases require interactions with a redox system, which consists of one or two additional redox proteins to provide a continuous electron transfer. These systems obtain reducing equivalents from NAD(P)H to their catalytic sites to perform the hydroxylation reaction. Alkane ω-hydroxylases therefore form together with the corresponding redox proteins (2-3 proteins) complexes, which build in the cell a functional unit. None of these four enzyme types (sMMO, pMMO, PMO or BMO) could be heterologous expressed, which drastically limit their handling for optimization experiments. Medium-chain alkanes (C_5–C_{16}) are oxidized by integral-membrane non-heme di-iron monooxygenases (pAH1 or alkB) or alternatively by P450 monooxygenases.[51, 61] AlkB has been identified in bacteria and fungi utilizing C_5–C_{16} alkanes as their sole carbon source**.** Functional characterization of genes are involved in alkane oxidation. The best studied alkane hydroxylase was isolated from *P. putida* GPo1.[62] This three component system contains the membrane bound oxygenase (alkB), a soluble rubredoxin (alkG) and a soluble rubredoxin reductase (alkT).[61-63] AlkB can be functionally expressed in *E. coli*.[64] Detailed information about reaction mechanism and structures of the catalytic center of MMOs and AlkB were reported by Austin and Groves.[65] In contrast to the di-iron proteins cytochrome P450s possess a thiolate-ligated

heme-prosthetic group in their active sites. This offers the opportunity for catalytic oxygen insertion into C-H bonds (details about P450s in section 1.3.).

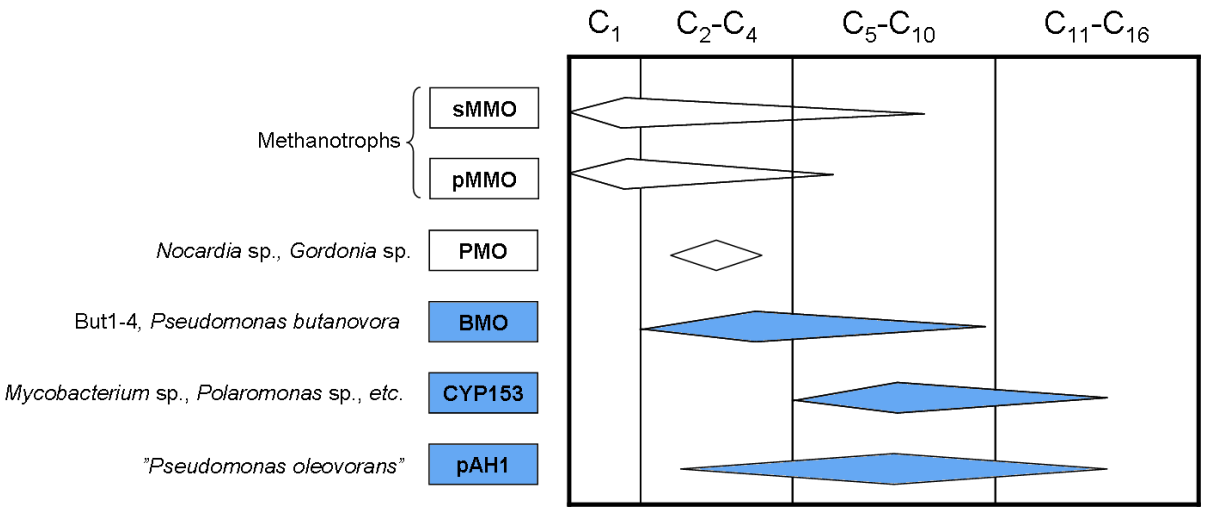

Figure 2: Overview of the natural substrate range of bacterial alkane hydroxylases with a focus on *n*-butane. Soluble methane monooxygenase (sMMO); Membrane-bound methane monooxygenase (pMMO); Propane monooxygenases (PMO); Butane monooxygenases (BMOs); Soluble medium-chain-length P450 monooxygenase (CYP153A). (pAH1 or alkB) Medium-chain-length integral membrane alkane hydroxylase.[52] (Adapted from van Beilen & Funhoff *et al.*, with permission from Springer)

1.2.5. ω-Hydroxy fatty acids: versatile and valuable products

ω-Hydroxy fatty acids (ω-OHFAs) are versatile fine chemicals with a broad range of application. They can be used as building blocks or ingredients in lubricants, adhesives, corrosion inhibitors, dermopharmaceuticals, cosmetics or anticancer products.[66-68] The biggest part, however, is used for the synthesis of polymers (*e.g.* poly(ω-hydroxy fatty acids)).[16] The further oxidation to α,ω-dicarboxylic acids (α,ω-DCAs) is possible. These diacids are applied *i.e.* in different polymers and polyketide antibiotics.[69]

The majority of ω-OHFAs and α,ω-DCA is produced *via* chemical processes. Especially compounds with a chain length of less than 6-8 can be made with high efficiency by oxidation with a Pd(II) catalyst under rough reaction conditions (high pressure and more than 130 °C).[70] Furthermore oxidative or reductive conversion of the double bond of

monounsaturated fatty acids can be used for the synthesis of ω-OHFAs.[71] Middle chained ω-OHFAs can be synthesized from their corresponding lactones by saponification and subsequent acidification.[68] Problematic is that all synthesis routs generate by-products and therefore connected with a cost intensive downstream procedure.[72]

An interesting alternative can be offered with a biocatalytical based process to overcome these limitations. CYP — belong to the yeast CYP52 subfamily — are known to oxidize alkanes and/or fatty acids to ω-OHFAs and α,ω-DCAs.[73] Cathay Biotech was alreadyable to establish a production process for the synthesis of C_{11}-C_{16} DCAs in 2003.[39]

1.3. Cytochrome P450 monooxygenases

1.3.1. Function and nomenclature

Cytochrome P450 monooxygenases (CYPs) represent due to the fact that more than 11.000 gene sequences are known, one of the biggest enzymes-superfamilies and they are distributed over various organisms in the whole tree of life (even viruses).[74, 75] P450 biocatalysts (EC 1.14.x.x) are regarded as the biggest class of enzymes in plants because of their crucial role in secondary metabolism.[76] They accept different other substrates in the cell, which includes steroids, xenobotics as well as lipids.[77] P450 enzymes show a wide spectrum of different reaction types. These include regio- and stereospecific C–H hydroxylations at different positions, dealkylation reactions and among others the epoxidation of C=C double bonds. This diversity explains the high physiological impact on live of the concerned organisms.[78] The name was derived from characteristic spectral properties of the heme-unit in the active center.[75] This heme is able to form a reduced iron/carbon monoxide complexes, which is responsible for the typical absorption shift from ~ 420 nm to ~ 450 nm (Soret-peak).[77] The 450 nm peak depicts the functional and active form of the enzyme. The P420 form results from the weakening or distortion of the thiolate bond of the cysteine residue that is the fifth ligand of the heme.[77] The common classification is based on the work of Nelson and co-workers, which is mainly dependent on sequence similarity and divides the enzyme-superfamily in families (more than 40 % similarity) and subfamilies (more than 55 % similarity).[75] For analysis and comparison of the high numbers

of different sequences and structures several bioinformatics tools are available *e.g.* the CYP450 Engineering Database (http://www.cyped.uni-stuttgart.de/).[79]

1.3.2. Structure

The P450 structure is unique, because no other protein structure show the same folding like this enzyme. However, in the different subfamilies a substantial difference in the positioning of structural elements can be detected. Although the general fold is always the same. Especially close to the heme center — in the core of the protein where the reaction itself takes place — the rate of conservation increases.[77] This particularly regards helices I and L, which are often targeted by mutation studies.[80] No more than three absolutely conserved amino acids (aa) exist in P450s.[81] To these aa belong the Glu and the Arg in an E-X-X-R-motif in the so called K-helix, which presumably is involved in stabilizing the center of the enzyme.[82] The third conserved aa is a Cys, which seems to be absolutely essential for the axial configuration of the heme. A further conserved aa is the Thr in a G-X-X-T motif in the I-helix, which often occurs in bacteria and which is important for the proton transfer step.[83] In addition, a consensus sequence (A/G-G-X-D/E-T) is noticeable, which seems to be crucial for the activation of oxygen.[84]

1.3.3. Redox systems and classification

The catalytic cycle depends on the efficient delivery of two electrons to the heme center of the enzyme. Nearly all CYPs require interaction with their redox partners to provide electrons either as separate proteins or as fusion proteins. In most cases electrons are provided by reducing equivalents such as NADH or NAD(P)H.[85]

Consistent effort over the last years has defined a classification, which groups theses multi protein complexes in different classes depending on a topology of different proteins that are embedded in the electron transfer to the heme-iron. Bernhard and co-workers established an overall scheme that includes ten different classes (shown in in Figure 3).[85] The first two classes (I and II) cover the most of the now known enzymes. It has to be mentioned that some of the defined classes contain only one or two members (class III, IV and IX). Accordingly, not all classes will be explained in detail in the following part. Class I includes

nearly all bacterial and mitochondrial P450s from eukaryotes. All members of this class own three different components. That is why they are able to build one functional unit. The NAD(P)H dependent flavin adenine dinucleotide (FAD) containing reductase and the smaller [2Fe-2S] ferredoxin are required for the electron transfer to the catalytic unit. For example in bacteria all tree proteins are soluble, whereas in eukaryotic mitochondria only the ferredoxin is not membrane bound. In class II the enzymatic units as well as the redox system are typically membrane bound. The corresponding NAD(P)H cytochrome P450 reductase (CPR) includes two cofactors, which are FAD as well as flavin mononucleotide (FMN). Members of this class can be found in the membrane of the endoplasmic reticulum. It is reported that electrons generated by the redox partner — which are supposed to be supplied to the heme center for the oxidation process — are not transferred effectively.[83] Not surprisingly, nature evolves this multiprotein complex to respond to this problem. In class VII and VIII the P450 enzyme is located in combination with the redox system on a single polypeptide chain. In class VII the reducing equivalents from NADPH are transferred *via* a FMN and Fe/S containing reductase partner in a fused arrangement linked *via* the *N*-terminus to the enzyme.[86] A CPR reductase consists of a FAD containing region, which is related to a ferredoxin reductase and a FMN binding section similar to a flavodoxin. This redox complex is fused in class VIII *N*-terminal to the enzymatic unit. One member of this family, CYP102A1 (P450 BM3), is the fastest known bacterial P450 system.[87] As a catalytic self-sufficient fusion complex the enzyme displays almost lossless coupling efficiency and a high catalytic turnover number.[88, 89]

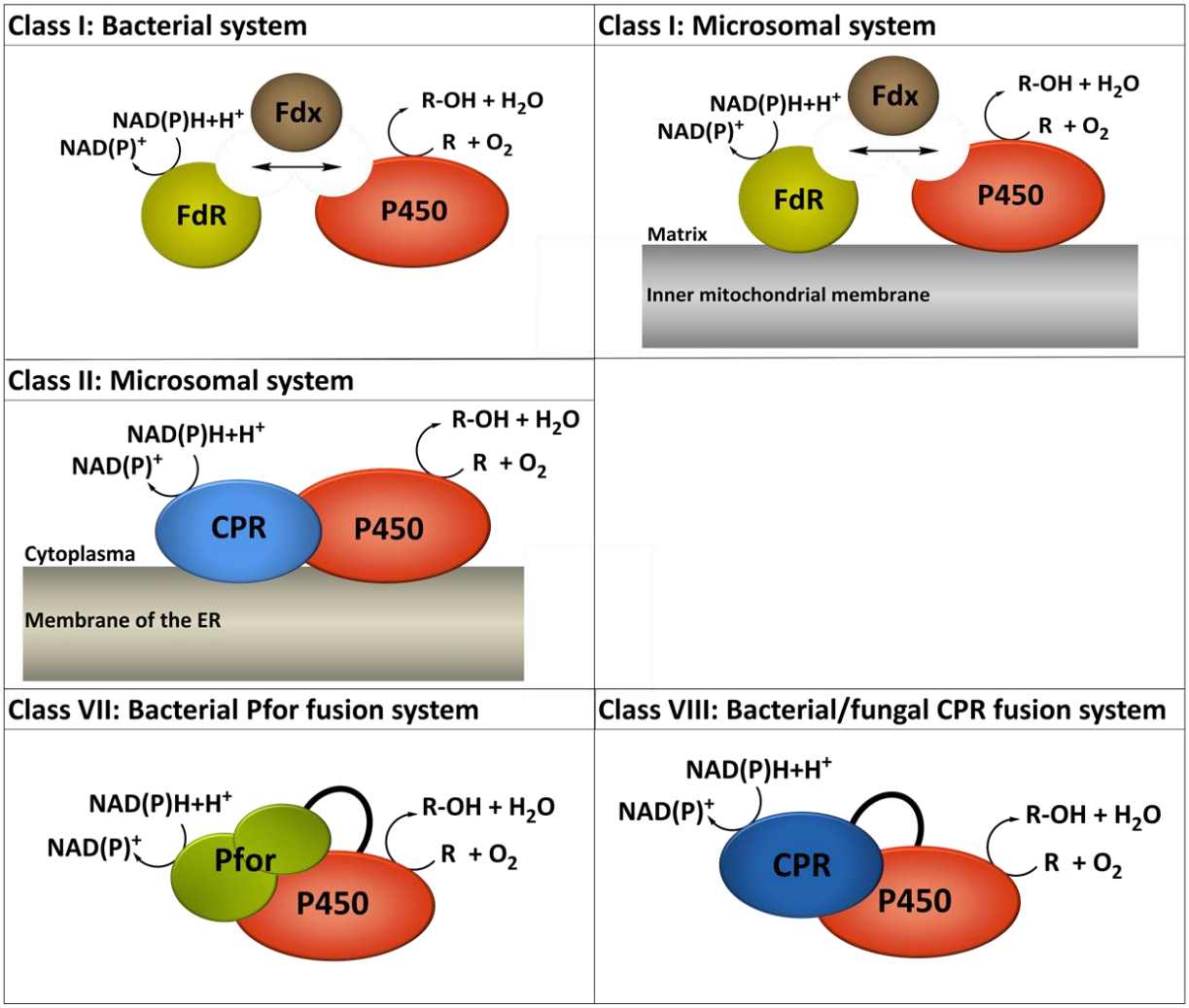

Figure 3: Schematic organization of different P450 systems in respect to their redox system.

Class I, bacterial system with three soluble proteins; (2) class I, mitochondrial system including membrane bound reductase and heme-domain and a soluble ferredoxin; (3) class II microsomal system with two membrane bound proteins; (4) class VII, soluble bacterial P450-PFOR fusion construct; (5) class VIII, soluble bacterial P450-CPR fusion system.[85] (Adapted from Hannemann *et al*., 2007, with permission from Elsevier)

1.3.4. Catalytic mechanism

Common substrate conversion by P450s follows a catalytic mechanism (Figure 4). Initial binding of a suitable substrate causes the displacement of H_2O that is linked in the resting

state as a ligand to the iron in the heme center **(1)**. Through the loss of H_2O the heme-iron shifts from a low-spin to a high-spin state, which offers the reduction **(3)** of the heme-iron to its ferrous form (Fe^{II} → Fe^{III}) by the transfer of one electron **(2)**. P450s generally utilize reducing equivalents from NAD(P)H cofactors to transfer electrons *via* an individual redoxsystem to the heme-center. Afterwards, the reduced iron is able to bind O_2 in form of a ferrous superoxo-complex **(4)**. Transfer of the essential second electron, originated from the redoxsystem, the iron complex is converted into a ferric peroxo anion **(5)**.

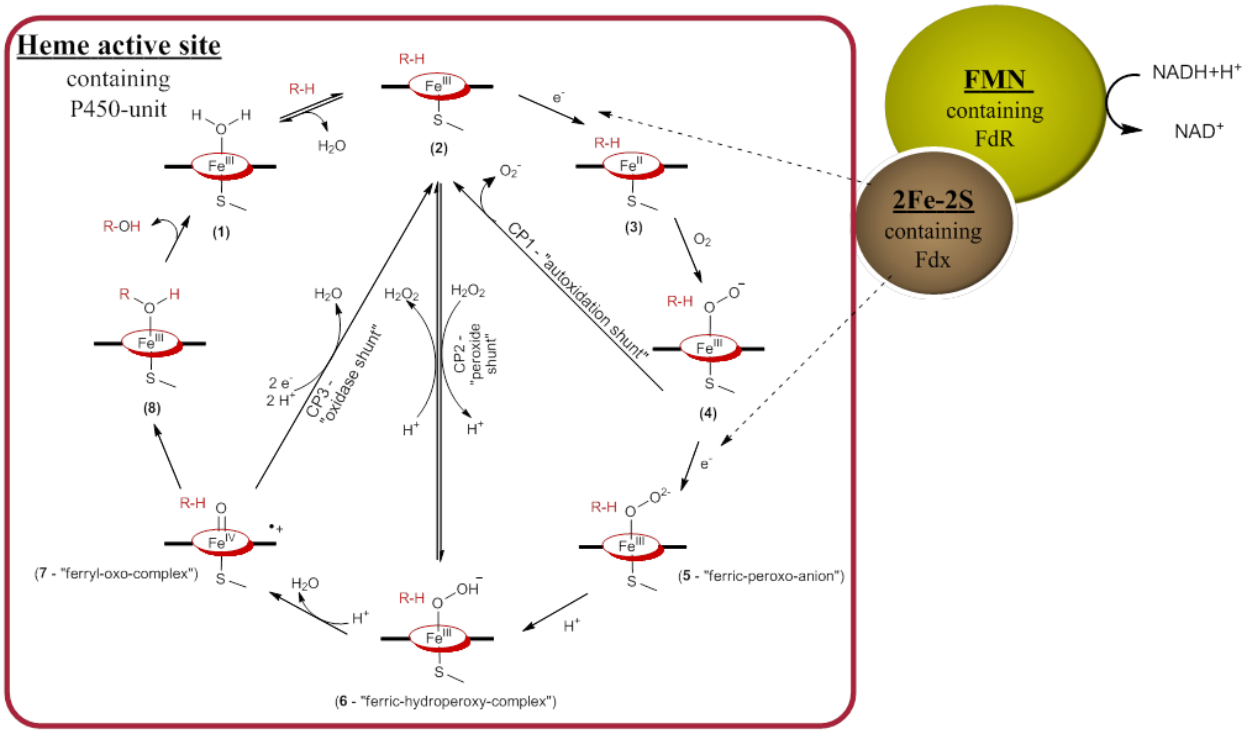

Figure 4: The characteristic catalytic cycle for P450 monooxygenases in hydroxylation reactions. Electron transfer by a class I system derived from NADH (hydride transfer) *via* redox partners to the heme active site (Adapted from Denisov *et al.*, 2005, with permission from ACS Publications).[83]

Via two protonation steps, first a ferric hydroperoxy-complex **(6)** followed by a ferryl-oxo-complex **(7)** in combination with the release of another H_2O a reactive intermediate is formed. In the successive step it is assumed that the reactive ferryl-oxo-complex transfers the single oxygen atom to the present substrate **(8)**. Finally the hydroxylated substrate is released.

Bypass mechanisms ("uncoupling-pathways") appear under three different conditions. **(CP1)** The second electron is not transferred into the iron to build a ferrous superoxy-complex ("autoxidation shunt"). **(CP2)** While the ferric hydroperoxy-complex **(6)** breaks down H_2O_2 can be formed ("peroxide shunt"). **(CP3)** The collapse of the ferryl-oxo-complex **(7)** leads to the release of H_2O ("oxidase shunt"). These uncoupling pathways could decrease the efficiency of the hydroxylation system enormous. On the other hand the byproducts could damage the enzyme or the whole cell system.[83]

1.3.5. Industrial impact of P450s

Specific oxo-functionalization of non-activated C-H bonds is one of the most desired chemical reactions, as it is a limitation in many synthetic routes. Due to the inert character of the sp^3 C-H bond a selective functionalization is difficult in chemical synthesis. Enzymes can be used to functionalize such inert compounds.[90] With P450, biocatalysis offers interesting candidates for these reaction types, because they show a broad substrate spectrum. They are rather evolvable and often target of mutation experiments to adjust the substrate specificity.[80] Despite this requirement, only a small number of P450 processes have been applied. Besides low stability, little turn over numbers and the need for redox systems, however the main limitation for industrial applications is the dependency on the expensive cofactor NAD(P)H.[91] Only the usage of whole cell processes could be achieved so far as a cheap and efficient cofactor-regeneration system, but the alternative has not been available so far.[92] Also the coupling (in)efficiency, which leads to reactive oxygen species, is often problematic. Due to their reactive functional group (*e.g.* hydroxyl or aldehyde groups), the formed products are mostly able to damage the enzymatic system of the host, which influences host stability.[93] Nevertheless, in several synthetic routes concerning fine chemicals — especially high price pharma-products — it was possible to overcome these limitations. Especially for extensive syntheses (>10 steps) P450s can offer a biocatalytic alternative.[92] Different prominent examples for successful P450 application are shown in Figure 5. The production of pravastatin, a therapeutic agent for hypercholesterolemia, by P450 catalyzed oxidation from *Streptomyces carbophilus* (P450sca-1,-2) is an example of a large scale application by microbial oxidation. The high stereoselectivity makes this process particularly suitable for chemical hydroxylation of aromatic compounds which usually lead

to regioisomers (Figure 5: 2).[94, 95] The fungal strain *Beauveria bassiana* Lu 700 was identified to hydroxylate selective (*S*)-2-phenoxypropionic acid to (*S*)-2-(4′-hydroxyphenoxy)propionic acid (Figure 5: 5).[96] Classical mutation experiments implying UV-light and *N*-methyl-*N*′-nitrosoguanidine-treatment helped to increase product yields. In the field of P450 research, a lot of progress was achieved concerning heterologous expression of P450s in typical bacterial hosts like *E. coli* or *P. putida*. Particularly in classical eukaryotic systems like *Saccharomyces cerevisiae* (yeast), P450s were successfully applied.[97] Especially the expression of eucaryotic P450s like *e.g.* CYP71A from O*ryzae sativa,* is much more promising in a eucaryotic hosts.[98] Recently several impressive examples of smart combinations of metabolic engineering strategies and heterologous expression of a suitable biocatalyst (P450) have led to new biosynthetic routes based on simple carbon sources. One example is the total synthesis of hydrocortisone by recombinant *Saccharomyces cerevisiae.*[99] This proofs the applicability of a complex P450 based pathway from higher eucaryotic cells into a yeast production host.[100] The used yeast cells harboring a number of foreign genes — including four heterologouse — expressed P450s (CYP11A1, CYP11B1, CYP17A1 and CYP21A1) enable the synthesis of a complex organic compound *via* a single organism (Fig.5: 7).[99] Keasling and co-workers used a *Saccharomyces cerevisiae* strain, which produced artimisinic acid (artimisinin precursor) on an industrial scale *via* successful metabolic engineering, primary the mevalonate pathway, and heterologous expression of different engineered CYP71A1 variants (Figure 5: 9).[98] It is conspicuous that no example of a production capacity more than 10.000 t/p.a. can be mentioned here. So far only fine chemicals in form of high price products — especially when protecting steps are necessary in classical organic synthesis — are synthesized by P450 processes. Next to these microbial processes it was further possible to apply P450s (CYP75A) in plants in form of genetic engineering techniques to produce delphinidin, which gives the flower a blue colour.[92] Despite the progress through enormous scientific efforts in the last two decades concerning the P450 research, it is still difficult to bear down the limitations of this enzyme class on a large scale process.

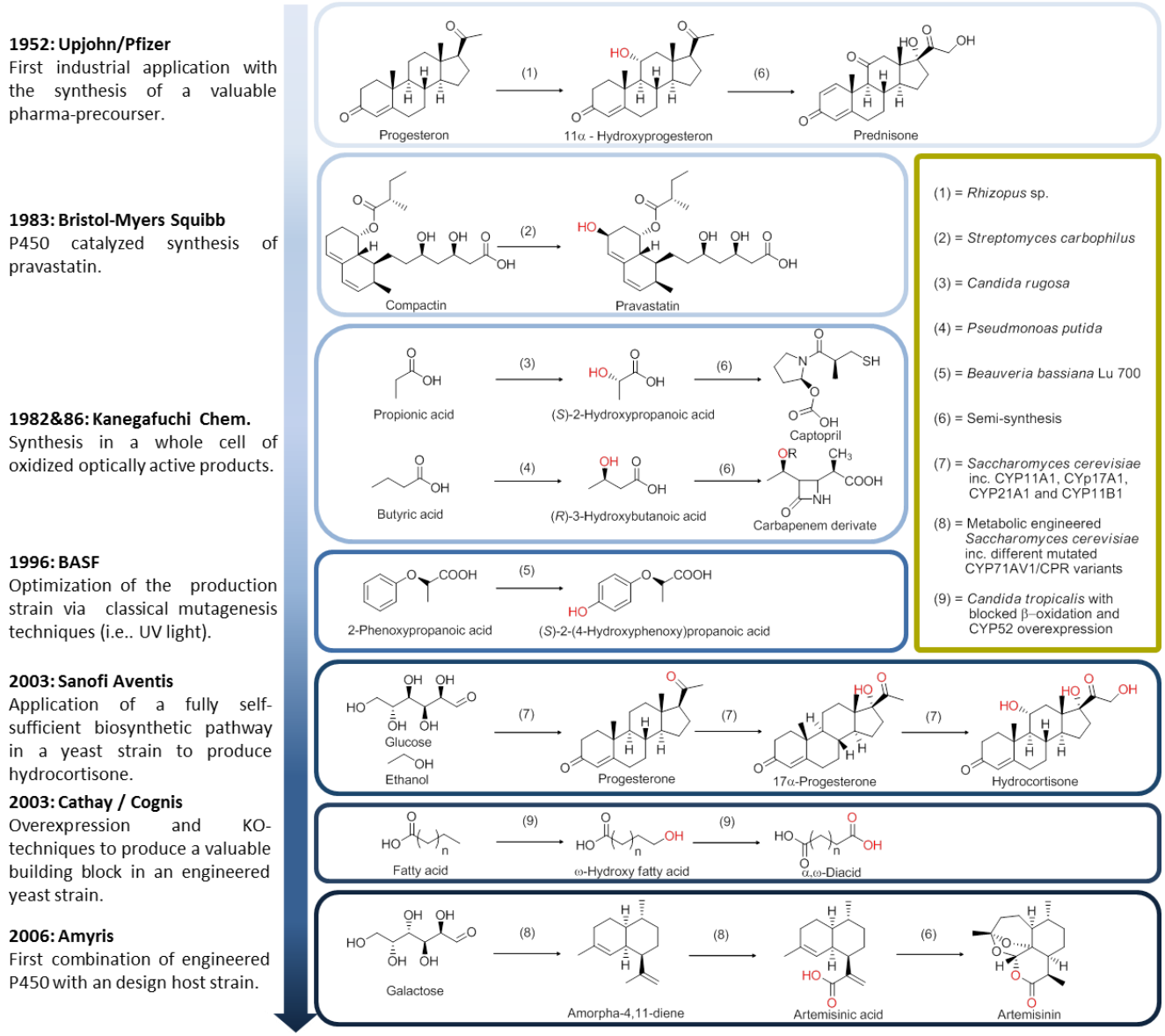

Figure 5: P450 monooxygenases in biotechnology – Prominent examples of industrial important products synthesized by P450 monooxygenases.

(1) 11α-Hydrocortison synthesis with a *Rhizopus* sp. strain from 1952;[9] (2) Selective oxidation of compactin to the pharma product pravastatin; [94, 95] (3, 4) Stereoselective hydroxylation of isobutyric acid and butyric acid to (*S*)-2-hydroxypropanoic acid and (*R*)-3-hydroxybutanoic acid *via a Candida* or *Pseudomonas* strain.[101-103] (6) (*S*)-2-(4′-hydroxyphenoxy)propionic acid production *via* an optimized *Beauveria bassiana* Lu 700 strain. [96] (7) The steroid pathway from Sanofi-Aventis, which includes fully self-sufficient pathways with 4 different P450 enzymes.[99, 100] (8) Cathay Biotech and Cognis producing different middle chained α,ω-diacids with an engineered yeast strain and CYP52A.[39, 104] (9) Production of the antimalarial drug precursor artimisinic acid *via* different engineered CYP71A variants in a metabolic reengineered yeast host.[98]

1.3.6. CYP153A family

CYP153 enzymes are class I enzymes, which operate as multi protein-complex, comprising next to the CYP two additional redox proteins (iron-sulfur ferredoxin and FAD containing ferredoxin reductase). They are interesting biocatalysts due to their high regioselectivity for the ω-position of aliphatic compounds as well as the fact that they can be expressed in soluble form. CYP153A1 from *Acinetobacter* sp. EB104 was the first cloned and characterized member of this subfamily.[105] CYP153A6 was used in whole cell biotransformations among others towards *n*-octane, 1-octene, and limonene (Figure 6).[106-109] Further bioconversions experiments (CYP153A6 + natural redox partners) in *E. coli* with *n*-octane as substrate resulted in 8.7 g/L 1-octanol after 48 h using 20 % (v/v) substrate and 11 g_{cdw} resting cells.[110] It was also shown that CYP153A13a is able to selectively oxidize different aromatic compounds including methyl ether moieties or phenolic compounds.[111]

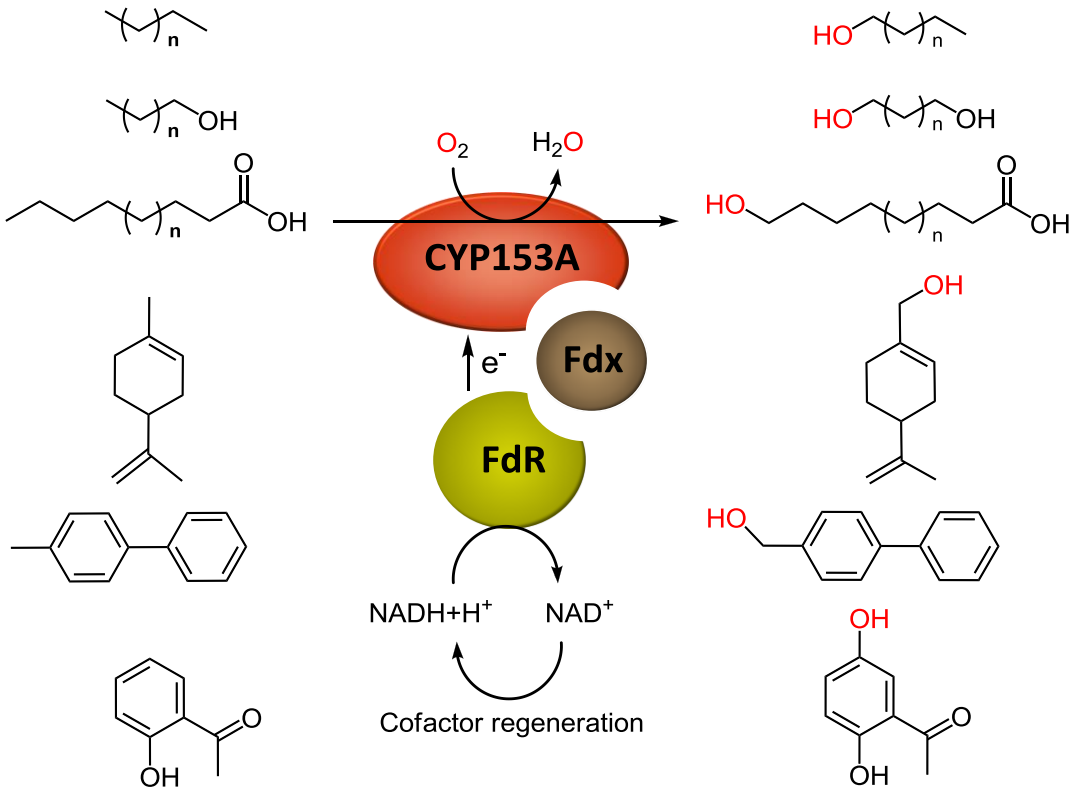

Figure 6: Schematic representation of the hydroxylation of aliphatic, cyclic and aromatic compounds catalyzed by CYP153A enzymes using their natural redox system for electron (e^-) transfer and an NADH regenerating.[112] (Adapted from Scheps & Honda et al., 2011, with permission from RSC)

Structural information has been provided by a homology model of CYP153A6, which allows the visualization of 11 active site residues. This model verifies the predominantly hydrophobic nature of the binding pocket. Recently the crystal structure of CYP153A7, in a substrate-free state, has been published.[108] Directed evolution experiments on CYP153A have been performed by Arnold *et al*. With the application of an *in vivo* screening, the substrate range could be shifted towards shorter alkanes *via* a single point mutation at position V97.[64]

1.4. Protein engineering

Due to an increasing requirement to use alternative sources, producing modern fuels (isobutanol, farnesol), materials (polyesters, ω-hydroxylated fatty acids) or bulk precursors (1-butanol, 1,4-butanediol), enzymes with novel functions and properties are needed. Although nature is known for its incredible biodiversity, not all biocatalytic demands can be addressed.[109] Mutation approaches, however, provide the opportunity to modify enzymes for the creation of proteins with the desired function to address these problems. These engineered enzymes show improvements like increasing thermostability above 65 °C, high organic solvent tolerance or high enantio-, regio- and chemoselectivity.[113-115] Different laboratory evolution methods (rational design and directed evolution) are well established to obtain suitable biocatalysts on a reasonable timescale. Well-known reactions like the ruthenium based metathesis (Grubbs-catalyst) or the palladium dependent Mizoroki-Heck cross-coupling reaction, however, remain challenging for biocatalytic approaches, since nothing comparable is found in nature.[116-118]

1.4.1. Directed evolution

In the early 1990s, Arnold and Stemmer adapted Darwin's principles of evolution on *in vitro* and *in vivo* designed experiments – the directed evolution. As a result, enzymes with new or optimized functions could be developed.[115, 119] Directed evolution has always faced difficulties to use the advantages of natural evolution by repeated rounds of (i) generating a gene library by mutagenesis, (ii) expressing different genes, and (iii) selecting (or screening) of corresponding libraries until the desired biocatalyst has been developed.[120] Many cycles

of directed evolution are necessary to create the desired protein. Classical random mutagenesis methods are based on x-ray, chemicals (polycyclic aromatic hydrocarbons), or mutator strains.[4] Nowadays, several well established gene mutagenesis methods like DNA-shuffling, error-prone polymerase chain reaction (epPCR) or sequence saturation mutagenesis (SeSaM) are used to mutate selected gene sequences.[115, 119, 121] The biggest challenge in putting the Darwinian approach into practice is still the essential screening step for mutant selection to continue the directed evolution process in the next level. Automation of high-throughput screening, faster mass spectroscopy based methods and smarter *in vivo* screening methods offer interesting solutions. [109, 120] In nature, the reengineering process has evolved over a long time span. [122]

1.4.2. Rational design

To confine screening efforts, the creation of smaller but smarter mutant libraries is desirable. To respond to these questions, different techniques have to be applied. Even if crystal structures of several enzyme families are nowadays available, a systematic analysis of similar sequences and structures is necessary. Especially because the number of potential substrate interacting sites (hotspots) is rather high and requires an exhaustive analysis of possible cooperative effects.[123] Sequence studies of various enzymes can be used to change the properties and functions of an enzyme to a considerable extent.[82] The usage of bioinformatic tools, like substrate docking simulations and MD-experiments can be useful for identifying amino acids, that control particular enzyme behaviors and demonstrating mechanisms for the diversification of catalytic functions in nature.[124] Although the target enzyme is well characterized this might not be true for the molecular basis for the desired function. Therefore, a focused mutant library which targets a small number of potential hotspots are often used to influence the function of the used enzyme.

1.5. Objectives of the present thesis

The selective terminal hydroxylation of aliphatic compounds with different chain lengths is a complex and demanding problem in synthetic chemistry, due to the fact that energy of the terminal position is ca. 15 kJ/mol higher than the subterminal position.[90] Classical chemocatalysis possesses problems like subterminal hydroxylation, overoxidation and harsh reaction conditions. Biocatalysis offers some alternatives for terminal hydroxylation. In most cases the identified enzymes possess interesting abilities but limitations that lead to low productivity. Due to this reasons the chosen biocatalyst has to be shaped *via* protein engineering approaches to cover the complete spectrum of requirements. The most enzymes like cytochrom P450 monooxygenases (CYP or P450) can perform C-H activation with a high regioselectivity. To achieve high product yields and an economic suitable process the usage of a whole cell system is necessary.

The aim of this study was to develop a stable recombinant bacterial strain harboring a ω-regioselective alkane hydroxylase. It is supposed to catalyze the terminal hydroxylation of gaseous *n*-butane to liquid 1-butanol and a model fatty acid (solid) to the corresponding ω-hydroxy fatty acid. The following goals were set:

Goal 1: Comparison of suitable biocatalysts and strains for the hydroxylation reaction of *n*-butane to 1-butanol. Proposed methodology: CYPED-driven search, first screening experiments to evaluate suitable candidates.

Goal 2: Determine of suitable expression and protein purification conditions to produce adequate amounts of the chosen biocatalyst for further investigations.

Goal 3: Detailed characterization of the biocatalyst *in vitro* with different aliphatic compounds.

Goal 4: Optimization of the enzymatic system in terms of electron coupling efficiency or protein engineering studies to tailor the enzyme for specific substrates.

Goal 5: Functional expression of the improved candidates in a bacterial production host and establishing of a reproducible biotransformation system to achieve the hydroxylation of *n*-butane and fatty acids.

Goal 6: Investigation of different technical parameters concerning the conversion of the gaseous substrate *n*-butane.

Goal 7: Analysis and optimization of the *in vivo* bioconversions of a model fatty acid in a small-scale bioreactor to identify limitations in the process.

In summary, this ambitious project should help to identify new biocatalysts, which can be used in an industrial application to produce important hydroxylated products.

2. Material and Methods

2.1. Strains and Plasmids

Strains and plasmids used in this work

Strain	Description / Use	Available	ITB No.
E. coli BL21(DE3)_pET28a(+) *M.aq.*-Enz (N-Term)	Expression of CYP153A$_{M. aq.}$ enzyme in *E. coli* BL21 (DE3)	Yes	ITB372
E. coli BL21 (DE3)_ pET28a(+) *M.aq.*-Red (C-Term)	Expression of CYP153A$_{M. aq.}$ reductase in *E coli* BL21 (DE3)	Yes	ITB373
E. coli BL21 (DE3) pET28a(+) *M.aq.*-Ferr (N-Term)	Expression of CYP153A$_{M. aq.}$ ferredoxin in *E coli* BL21 (DE3)	Yes	ITB374
*E. coli BL21 (DE3) pET28a(+) P.*sp.-Enz (N-Term)	Expression of CYP153A$_{P. sp.}$ enzyme in *E. coli* BL21(DE3)	Yes	ITB376
E. coli BL21 (DE3) pET28a(+) *P.*sp.-Red (C-Term)	Expression of CYP153A$_{P. sp.}$ reductase in *E. coli* BL21(DE3)	yes	ITB377
E. coli BL21 (DE3) pET28a(+) *P.*sp.-Ferr (N-Term)	Expression of CYP153A$_{P. sp.}$ ferredoxin in *E. coli* BL21(DE3)	Yes	ITB378
E. coli BL21 (DE3) pET28a(+) *M.aq.*-Operon	Expression of CYP153A$_{M. aq.}$ operon in *E. coli* BL21(DE3)	Yes	ITB375
E. coli BL21 (DE3) pET28a(+) *P.*sp.-Operon	Expression of CYP153A$_{P. sp.}$ operon in *E. coli* BL21(DE3)	Yes	ITB379
E. coli BL21 (DE3) pCom8 CYP153A6	Expression of CYP153A6 operon in *E. coli* BL21(DE3)	No	ITB380*
E. coli BL21 (DE3) pCom8 AlkBFG	Expression of AlkBFG in *E. coli* BL21(DE3)	No	ITB382*
But2	Predicted to be: *Ematobacter* sp.	No	ITB400**
But3	Predicted to be: *Azoareus* sp.	No	ITB401**
But4	Predicted to be: *Variovorax* sp.	No	ITB402**
But5	Predicted to be: *Mycobacterium* sp.	No	ITB403**
E. coli DH5_ CYP153A16-Pfor	Expression and amplification of CYP153A16-Pfor in *E. coli*	Yes	ITB405
E. coli DH5a - pCola-Deut-1: CYP153A *M.aq.*(G307A)-CPR$_{BM3}$ + AlkB	Expression and amplification of CYP153A$_{M\ aq.}$(G307A)-CPR$_{BM3}$ and AlkL in *E. coli* BL21(DE3)	Yes	ITB407

E. coli HMS174 (DE3) pET28(a)+ CYP153A *P*. sp. (V253L/G254A)-CPR_{BM3}	Expression of $CYP153A_{P.\ sp.(V253L/G254A)}$-$CPR_{BM3}$ in *E. coli* HMS174	Yes	ITB409
E. coli HMS174 (DE3) pET28(a)+ CYP153A *M.aq.*(V306L/G307A) - CPR_{BM3}	Expression of $CYP153A_{M.\ aq.(V306L/G307A)}$-CPRBM3 in *E. coli* HMS174	Yes	ITB439
E. coli HMS174 (DE3) - pET28(a)+ CYP153A *P*. sp.-CPR_{BM3}	Expression of $CYP153A_{P.\ sp.}$-CPR_{BM3} in *E coli* HMS174	Yes	ITB413
E. coli BL21 (DE3) pET28(a)+ CYP153A *M.aq.*-Pfor	Expression of $CYP153A_{M.\ aq.}$-Pfor fusion in *E. coli* BL21(DE3)	Yes	ITB418
P. putida KT 2440 pCom10 CYP153A*P*.sp.-Operon	Expression of $CYP153A_{P.\ sp.}$-Operon in *P. putida* KT 2440 (alkane induced)	Yes	ITB422 = ITB456
P. putida KT 2440 pJoe CYP153A*P*.sp.-Operon	Expression of $CYP153A_{P.\ sp.}$-Operon in *P. putida* KT 2440 (rhamnose induced)	Yes	ITB423 = ITB453
P. putida KT 2440 pCom10 CYP153A*P*.sp.-Operon + *Not*I RS	Expression of $CYP153A_{P.\ sp.}$-Operon in *P. putida* KT 2440 (alkane induced) used for SeSaM exchange	Yes	ITB430
E. coli DH5a pET28(a) + CYP153A *M.aq*.(G307A) CPR_{BM3}	Expression and plasmid amplification of $CYP153A_{M\ .aq.(G307A)}$-CPR_{BM3} in *E. coli*	Yes	ITB431
P. putida KT 2440 SeSaM RGA Butanol Screen	1. SeSaM-Screening with pCom10 $CYP153A_{P.\ sp.}$-Operon under "butane pressure"	Yes	ITB428
P. putida KT 2440-SeSaM FGA Butanol Screen	2. SeSaM-Screening with pCom10 $CYP153A_{P.\ sp.}$-Operon under "butane pressure"	Yes	ITB429
P. putida KT 2440_pCom8_CYP153A6	Expression of CYP153A6 operon in *P. putida*	Yes	ITB419
P. putida KT 2440_pCom8_AlkBFG	Expression of AlkBFG in *E. coli* BL21(DE3) or *P. putida*	Yes	ITB421
E. coli DH5α pCom10_PspAlk	Plasmid amplification of pCom10_PspAlk in *E. coli*	Yes	ITB461
KT2440_pCom10_MaqAlk	Expression of $CYP153A_{M\ .aq.}$-Operon in *P putida*	Yes	ITB455
E. coli BL21(DE3) pJOE CYP153A *M.aq.*-CPR_{BM3}	Expression of $CYP153A_{M.\ aq.}$-CPR_{BM3} in *E coli*	Yes	ITB468

E. coli BL21 (DE3) CYP116B3-Pfor	Expression of codon optimized CYP116B3-Pfor in *E. coli*	Yes	ITB406
Plasmid	**Description / Use**	**Available**	**ITB No.**
pMa-T: alkL (Gene Art)	Cloning and amplification of alkL from *P. putida*	Yes	pITB717
pET28(a)+:CYP153A6-CPR$_{BM3}$	Cloning and expression of CYP153A6-CPR$_{BM3}$ self-sufficient fusion	Yes	pITB727

***** provided by the lab of Prof. F. H. Arnold (Caltech, Pasadena, United States), ****** provided by the lab of Prof. K. H. Engesser (University of Stuttgart, Stuttgart, Germany)

2.2. *In vitro* substrate characterization of CYP153A

Materials and methods including the cloning strategy to create plasmids and strains for the reported *in vitro* experiments are published in reference I and II.[112, 125] The experimental procedure for the *in vitro* substrate characterization of the different CYP153A enzymes against alkanes, primary alcohols and fatty acids was already published in the corresponding references.[112, 125]

2.3. Protein expression and purification

Expression of the CYP153A enzymes, the corresponding natural redox partners and the artificial redox partners (CamA and CamB) were performed in shaking flasks reported in reference I, II and III.[112, 125] For the self-sufficient fusion constructs five liter fed-batch fermentation was applied to obtain higher protein yield which is described in 2.9.2. Purification of the proteins was carried out by different strategies. Affinity chromatography by metal ion exchange chromatography was used to isolate the single enzymes or redox proteins (His-tagged proteins) reported in reference I and II. For the purification of the self-sufficient fusion proteins a combination of anionic exchange chromatography and size exclusion separation was applied to avoid the coexpression of a purification tag. Both techniques are described in reference III.[112]

2.4. Quantification of proteins

For the determination of the P450 concentrations the carbon monoxide assay with cell free extract was used.[126, 127] The concentration determination from the artificial redox partners CamA and CamB were described elsewhere.[112]

2.5. Mutants identified *via* rational design

2.5.1. Site-directed mutagenesis

E. coli expression vector pET28a(+) harboring CYP153A$_{P.\ sp.}$-CPR or CYP153A$_{M.\ aq.}$-CPR was mutated using the common QuikChange protocol. Chemical competent *E. coli* HMS174 (DE3) cells were used for transformation reactions with the *Dpn*I-treated QC-PCR mixtures. The used primers are listed in Table 1. The created mutants were verified via sequencing (GATC-Biotech, Konstanz, Germany).

Table 1: Primers for site-directed mutagenesis experiments used in rational design approaches.

Name of variant	Primer	Sequence
CYP153A*P. sp.*(G254A)-CPR	F	CTGGGCAACCTCATTTTGCTGATCGTCGCGGGCAATGACACGACCCGC
	R	GCGGGTCGTGTCATTGCCCGCGACGATCAGCAAAATGAGGTTGCCCAG
CYP153A*P. sp.*(V253L/I/F)-CPR	F	GAGTTTCTGGGCAACCTCATTTTGCTGATCWTSGGCGGCAATGACACGAC
	R	GGTCGTGTCATTGCCGCCSAWGATCAGCAAAATGAGGTTGCCCAGAAACT
CYP153A*P. sp.*(V253A/N)-CPR	F	GAGTTTCTGGGCAACCTCATTTTGCTGATCGKNGGCGGCAATGACACGAC
	R	GAGTTTCTGGGCAACCTCATTTTGCTGATCGKNGGCGGCAATGACACGAC
CYP153A*P. sp.*(V253L/G254A)-CPR	F	CTGGGCAACCTCATTTTGCTGATCCTG GCGGGCAATGACACGACCCGC
	R	GCGGGTCGTGTCATTGCCCGCCAGGATCAGCAAAATGAGGTTGCCCAG
CYP153A*M. aq.*(G307A)-CPR	F	Reference III
	R	
CYP153A*M. aq.*(V306L)-CPR	F	Reference III
	R	
CYP153A*M. aq.*(V306L/G307A)-CPR	F	CGGTAATTTGACGCTGCTCATACTGGCGGGCAACGATACGACGCGC
	R	GCGCGTCGTATCGTTGCCCGCCAGTATGAGCAGCGTCAAATTACCG

2.6. Establishing of self-sufficient CYP153A constructs

A multi-step cloning strategy was used to create different self-sufficient CYP153A variants. CYP153A*M. aq.*-3xGGS-CPR$_{BM3}$ in following part also called CYP153A*M. aq.*-CPR$_{BM3}$ consisted of CYP153A*M. aq.* fused to the reductase domain (CPR$_{BM3}$) of CYP102A1 or P450 BM3 from *Bacillus megaterium* with an additional 3xGGS tandem region in the linker sequence. CYP153A*M. aq.*-Pfor$_{116B3}$ was comprised by CYP153A*M. aq.* and the native reductase domain (Pfor$_{116B3}$) of CYP116B3 from *Rhodococcus ruber* DSM 44319. CYP153A*P. sp.*-CPR$_{BM3}$ was built by fusing CYP153A*P. sp.* with CPR$_{BM3}$.

A standard PCR protocol was used for gene amplification. Primers were designed with restriction sites (underlined) and overlapping sections (bold letters). For CYP153A*M. aq.*-CPR$_{BM3}$, the heme domain was created by PCR amplification with the forward

primer 5′- GGT CCA TGG GTA TGC CAA CAC TGC CCA GAA CAT TTG ACG AC -3′ and the reverse primer 5′- **CTG TTC AGT GCT AGG TGA AGG AAT GCT GCC GCC GCT GCC GCC GCT GCC GCC** ACT GTT CGG TGT CAG TTT GAC CAT CAA CC-3′. The CPR_{BM3} component was amplified by PCR with the forward primer 5′- **GCG GCA GCA TTC CTT CAC CTA GCA CTG AAC AG**T CTG CTA AAA AAG TAC GCA AAA AGG CAG AAA ACG CTC ATA ATA CGC CGC TGC -3′ and the reverse primer 5′- CAT CTC GAG TTA CCC AGC CCA CAC GTC TTT TGC GTA TC -3′. For Pfor1 ($CYP153A_{M.\,aq.}$-$Pfor_{116B3}$), the heme domain was amplified with the forward primer 5′- GGT CAT ATG ATA TCA TTA ATG CCA ACA CTG CCC AGA ACA TTT GAC G -3′ and the reverse primer 5′- **CTC GCC GAT G**GT GAC GGG ATG CTG CCG TTG CAG CAC ACT GTT CGG TGT CAG TTT GAC CAT CAA CCT GG -3′. The $Pfor_{116B3}$ domain was PCR-amplified with the forward primer 5′- **GCA GCA TCC CGT CAC CAT CGG CGA GCC CTC CAC CCG G**TC GGT GTC ACG CAC CGT CAC CGT CG -3′ and the reverse primer 5′- CAT AAG CTT TCA GAG TCG GAG GGT CAG TCG GTC G -3′.

The enzymatic part for the $CYP153A_{P.\,sp.}$-CPR constructs was created by PCR amplification with oligonucleotide 5′- GGT GCT AGC ATG AGT GAA GCG ATT GTG GTA AAC AAC CAA AAC G -3′ and 3′- TAG CAG ACT GTT CAG TGC TAG GTG AAG GAA TAG CGT TGA TGC GGA CGG GCA GCG ACT CAT AGC -5′ for the construct with the original linker sequence. The $CYP153A_{P.\,sp.}$-CPR redox system was amplified by PCR with oligonucleotides 5′- CAC TGA ACA GTC TGC TAA AAA AGT ACG CAA AAA GGC AGA AAA CGC TCA TAA TAC GCC GCT GC -3′ and 3′- CAT CTC GAG TTA CCC AGC CCA CAC GTC TTT TGC GTA TC -5′.

In a following step the matching amplified products were assembled at their described overlapping sections by PCR and ligated into pET-28a(+). The resulting plasmids were used to transform competent *E. coli* DH5*α* cells *via* heat shock. The success of cloning was verified by automated DNA-sequencing (GATC-Biotech, Köln, Germany).

2.6.1. Determination of coupling efficiency of P450s

The coupling efficiency was measured *via* two combined methods. Next to the photometrical determined NADPH consumption (at 340 nm) the concentration of the formed products during an *in vitro* biotransformation reaction were analyzed in addition. This method was described in reference III.

2.7. Sequence saturation mutagenesis-Tv (SeSaM)

The SeSaM-Tv method, including transversion mutations, to randomize the CYP153A$_{P.\ sp.}$ was done as described by Schwaneberg and co-workers.[121, 128, 129]

2.7.1. Purpald-assay

Cell free extracts were prepared as mentioned in reference I. Screening for activity against hexyl methyl ether (HME) was made like described elsewhere.[130] Potassium phosphate buffer (120 µL, 0.1 M, pH 7.5) and 2-4 µL of 400 mM HME in ethanol was added to 60 µL of cell free *E. coli* extracts. After 5 min of incubation at room temperature, NADPH (50 µL, 2 mM) was added to the reaction mix. Purpald solution (250 mM purpald salt in 2 M NaOH) was pipetted into the reaction mixture, exactly 20 min after the procedure was started, to build the purple product with the synthesized formaldehyde. The purple color was quantified at 550 nm using a photometer (Figure 7).

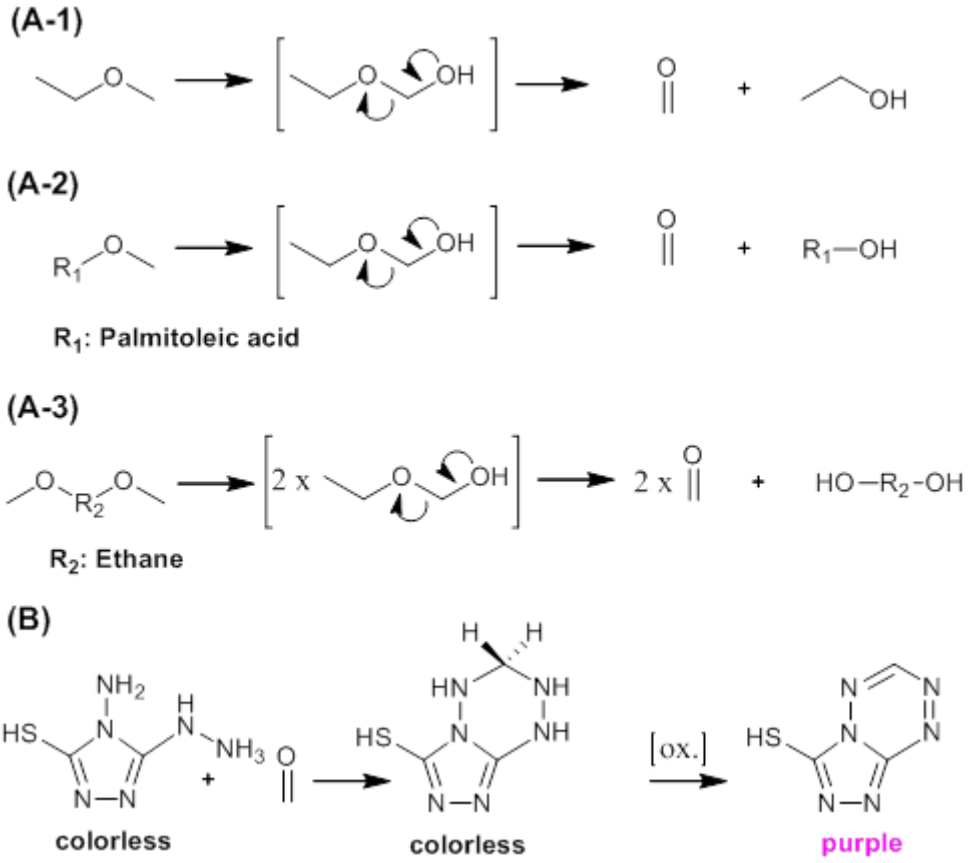

Figure 7: Screening for terminal hydroxylation *via* purpald-assay.

Terminal ω-hydroxylation of methoxy ethane (A-1), (*Z*)-16-methoxyhexadec-9-enoic acid (A-2), and 1,2-dimethoxyethane (A-3), results in formaldehyde. It might react with purpald and form a purple color. (B) Under aerobe conditions purpald reacts with formaldehyde to produce a purpald adduct, which shows a characteristic purple color and a significant peak at 550 nm.

2.7.2. Cell viability assay

CYP153*P. sp.*-operon carrying pCom10 (pCom10_Psp-Alk) expression vector (*n*-alkane-inducible) was modified by QuikChange to insert a *Not*I restriction site between enzyme and ferredoxin reductase (Table 2, Figure 8 (A)).[131] This step offers the opportunity to replace the SeSaM modified fragments with the wild type enzymes (Figure 8 (B)). Additionally an `ATG` start codon was inserted into the ferredoxin reductase to increase protein expression levels in *P. putida* KT2440.

Table 2: Primer for viability assay

Name of variant	Primer	Sequence
SeSaM amplification primer F	F	**5**`GGT CAT ATG AGA TCA TTA ATG AGT GAA GCG ATT GTG G **3**´
SeSaM amplification primer R	R	**5**´ATT GCG GCG CTC AAG CGT TGA TGC GGA CGG G **3**´
CYP153A$_{P.\ sp.}$_*Not*I_operon_F	F	**5**´GTC CGG ATC AAC GCT TGA AGC GGC CGC GAT CAT GAG CGA AAC TGT G **3**´
CYP153A$_{P.\ sp.}$_*Not*I_operon_F	R	**5**´CAC AGT TTC GCT CAT GAT CGC GGC CGC TTC AAG CGT TGA TGC GGA C **3**´

After heat shock procedure the *Pseudomonas* cells were washed three times with screening minimal medium (Figure 8, Table 3). SeSaM-libraries of *P. putida* KT2440 strains expressing CYP153A$_{P.\ sp.}$ mutants were cultivated in the screening minimal medium with antibiotics (kanamycin and irgasan) without carbon source and then enriched by sub cultivation (OD_{595nm} > 0.3) through continuous growth in *n*-butane as the sole carbon source to obtain optimized strains. Mutants were identified by automated DNA-sequencing (GATC-Biotech, Konstanz, Germany).

Table 3: Screening minimal media

Screening minimal media	
Chemicals	**Concentration**
Potassium dihydrogenphospate	2 g/L
Ammonium sulfate	1 g/L
Sodium chloride	0.1 g/L
Magnesium sulfate	0.2 g/L
Calcium chloride	0.02 g/L
Yeast extract	0.025 g/L
M12-Trace elements*	10 mL/L
M12-Vitamin solution*	10 mL/L
Kanamycin solution*	1 mL/L
Irgasan solution*	1 mL/L
ad 1L ddH_2O	

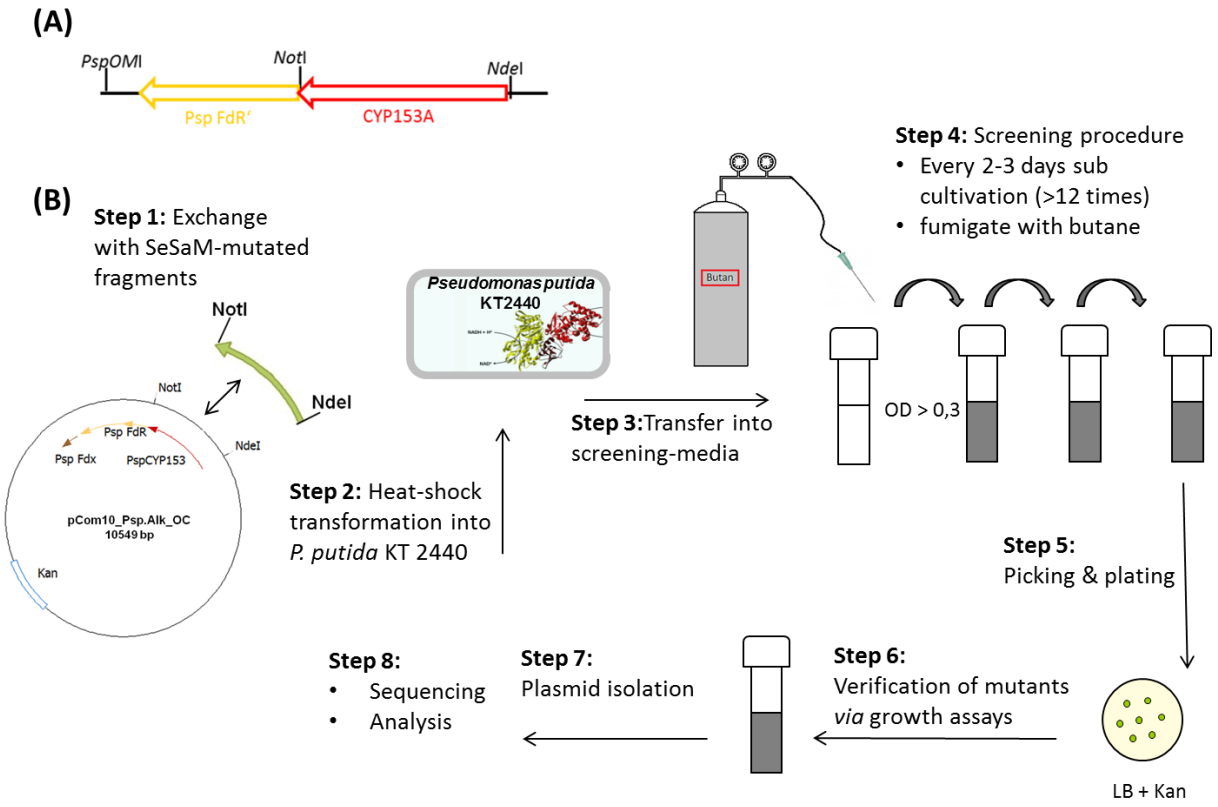

Figure 8: (A) *Not*I restriction site insertion into the CYP153A$_{P. sp.}$–operon. (B) Cell viability assay to identify improved butane monooxygenases.

2.8. Resting cell based conversion of *n*-butane to 1-butanol

2.8.1. 1-Butanol measurement by GC-MS headspace or HPLC-RI

To overcome time consuming extraction procedures a GC/MS headspace method for 1-butanol analysis was established. Samples were analyzed *via* a GC/MS QP-2010 instrument (Shimadzu, Japan) equipped with a FS-Supreme-5-column (30 m x 0.25 mm x 0.25 µm) in combination with a CombiPal Sampler operated in headspace mode and with a 2.5 mL heated headspace syringe. Helium as carrier gas (flow rate 0.69 mL/min) was applied with a split-ratio of 15:1. Electron impact (EI) ionization was used and the mass range from 20 to 200 *m/z* was detected. Both detector interface and injector temperature were set at 250 °C. One millilitre of the biotransformation mix was filled into a 20 mL headspace vial. If necessary, to overcome detector saturation, the biotransformation culture was diluted with the appropriate buffer medium. 100 µl of the internal standard solution (10 mM hexanol)

was added, the glas vials were sealed tightly. Temperature program: start 40 °C, hold 5 min, 5 °C/min to 85 °C, hold 1 min, 60 °C/min to 300 °C. For quantification of 1-butanol, the system was calibrated with the internal standard hexanol. Standard samples with different concentrations (0.01 – 2 mM of 1-butanol and 2-butanol) in 100 mM potassium phosphate buffer or in eM9 media were measured *via* GC/MS.

The concentration of 1-butanol was further analyzed by HPLC-RI in the aqueous biotransformation mix using 5 mM sulfuric acid as mobile phase. Cells from the fermentation fractions were separated from the supernatant by centrifugation at 20.000 × g for 1 minute (Centrifuge 5417 C, Eppendorf, Germany). The supernatant was transferred into pre-cooled glas vials, mixed with the internal standard xylitol (1M stock solution) to a final concentration of 10 mM and prepared for analysis by sterile filtration. HPLC analysis was carried out on an Agilent System (1200 series) using a cation exchange resin column Aminex HPX-87H (300 × 7.8 mm, Bio-Rad, USA) at 50-60 °C and a flow rate of 0.5 mL/min. The substrates and products were quantified using the corresponding standards and a refractive index detector (Agilent 1200series, G1262A). In contrast to the GC/MS headspace method the sensitivity with a minimal detection concentation of 1 mM was significantly lower.

2.8.2. Cultivation of CYP153A cells

1 µL plasmid was used to transform 10 µL competent *E. coli* BL21 (DE3) cells for the *in vivo* experiments. After 60 min regeneration in 190 µL SOC-media, 100 µL were used to start the 5 mL LB preculture, which was cultivated at 37 °C and 180 rpm. One milliliter preculture was used to inoculate the main culture. Cultivations for whole cell bioconversions were carried out in 1 L Erlenmeyer shake flasks containing 200 mL TB or eM9Y-media supplemented with the appropriate antibiotics. The growth was carried out in a shaker to an OD_{600} of 1.5 - 2. Expression was induced by the addition of 0.25 mM IPTG. The culture was supplemented with 8 g L^{-1} glycerol, 0.5 mM 5-aminolevulinic acid (δ-ALA) and 100 mg $FeSO_4$. The *E. coli* cells were incubated for 24 hours at 28 °C and 180 rpm and harvested by a centrifugation step at 4.000 x *g* and 4 °C for 30 min. Due to variations in the expression level of the different CYP153A variants, 3 further cultures were prepared to assure a high enzyme concentration. The pellets were washed with 100 mM potassium phosphate buffer (pH 7.4)

or eM9 media. After this procedure the cells were concentrated into 100 mL eM9 media (Table 4 (C) or 100 mM potassium phosphate buffer pH 7.5 to an end concentration of 30-50 g_{cww} L^{-1}. After the cells were provided with 1 % glycerol (*v*/*v*) and 20 mM glucose as carbon source, the gaseous substrate was added to the reaction mixture. Samples were taken after 1, 2, 4, 8 and 24 h reaction time.

Table 4: eM9(Y)-media

eM9(Y)-media	
Chemicals	**Concentration**
5 x M9 salts	200 mL
Magnesium sulfate (1M)	2 mL
Calcium chloride (1M)	100 µL
M12-Trace elements*	10 mL/L
M12-Vitamin solution*	10 mL/L
Yeast extract[a]	15 g/L
Glycerol (99 %)[b]	15 g/L
Kanamycin solution*	1 mL/L
Ad 1L ddH_2O	

[a] used for eM9Y; [b] only used for growth experiments, not during biotransformations

2.8.3. Conversion of *n*-butane to 1-butanol

Biotransformations were carried out with resting cells (ca. 15 g_{cdw} / 30 g_{cww}) in 100 mM potassium phosphate buffer pH 7.5 or eM9-media. It could be detected that an adaption with other alkanes (1 mM hexane) during the normal growth process is necessary for an efficient product formation. For the quantification of the formed C_4-product the amounts of 1-butanol and 2-butanol in the bioconversion flasks and downstream flasks were combined. The total amount of hydroxylated *n*-butane is named "butanol all up" in the following text. For biotransformation of gaseous *n*-butane, 100 mL of cell suspension including biocatalyst and 10-20 µL of antifoam 204 (Sigma-Aldrich) were stirred in a 250 mL Schott flask at room temperature (25 °C). The substrate was added continuously to the bioconversion mix with different inlet gas ratios of 1 – 20 % *n*-butane and 80 – 99 % synthetic air. The butane /air flow

rate was also varied from 10 - 50 L/h by using a Bronkhorst mass flow unit in order to determine the optimum conditions. Gas supply into the reaction mix was performed through a sparger after mixing in a dispenser nozzle. After defined time points samples from the bioreactor flask as well as the wash flasks were taken. The downstream installed washing flasks assure complete product discharge. The sample were sealed tightly and analyzed by GC/MS-headspace chromatography or HPLC.

2.8.4. CYP153A based 1-butanol production under pressure

The terminal hydroxylation of the gaseous C_4-substrate was also made in a high pressure reactor. The cells were produced as previously described and after a washing procedure mixed in 100 mM potassium phosphate buffer pH 7.5. 10 g of cold liquid *n*-butane was added in excess and built a second phase at a temperature of -5 °C (Figure 9). Afterwards the pressure tank (Carl Roth, high-pressure autoclave II) was sealed and connected *via* high pressure lines to a synthetic air gas cylinder. This setup offers the opportunity to apply a selected pressure between 1-100 bar to the reaction mixture (1-20 bar was applied). This step ensures also the supply of sufficient oxygen for the reaction. The (de)compression step at the beginning and during every sampling step was made as slowly as possible.

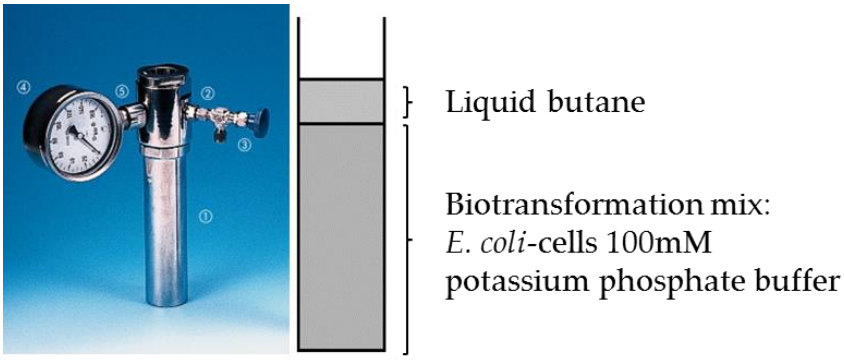

Figure 9: Experimental setup for the hydroxylation reactions with liquid *n*-butane.

2.9. Bacterial whole cells biotransformation for the production of ω-hydroxylated fatty acids

2.9.1. Construction of a dual expression vector with alkL and CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$

In order to construct a plasmid for the co-expression of alkL and CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$, a synthetic gene with the sequence of alkL (Geneart, Regensburg, Germany) and restriction sites *Bgl*II and *Xho*I was used. The CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ part was created *via* PCR amplification with the forward primer 5'- GG TCC ATG GGT ATG CCA ACA CTG CCC AGA ACA TTT GAC GAC-3' and the reverse primer 5'-CAT GAG CTC TTA CCC AGC CCA CAC GTC TTT TGC GTA TCG GCC -3'. Both gene components were cloned into the dual expression system pCOLADuet-1 using *Bgl*II and *Xho*I or *Nco*I and *Sac*I as restriction enzymes. The correct construction of pColaDuet-1:: CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ comprising alkL was confirmed by sequencing (GATC-Biotech, Köln, Germany).

2.9.2. Five liter fed-batch cultivation

A 5 L (operating volume) bioreactor (Infors AG, Bottmanning, Switzerland) containing 3.5-4 L of TB$_{Kan}$ medium (per liter deionized water: 12.0 g tryptone, 24.0 g yeast extract, 4.0 mL (*v*/*v*) glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4). 5 mL LB-medium was inoculated with freshly transformed *E. coli* HMS174 (DE3) with the corresponding CYP153A-CPR$_{BM3}$ fusion constructs. This preculture was used to inoculate a 1 L Erlenmeyer flask containing 250 mL of TB$_{Kan}$ medium and incubated at 37 °C and 180 rpm overnight on an orbital shaker until an OD_{600} of 3-5 was reached. Cultivation in the bioreactor was started by inoculation from the second preculture with necessary volume to a start OD_{600} of 0.05–0.1. The pH was maintained at 7.2 throughout the process using 28 % (*v*/*v*) NH_4OH and 10 % (*v*/*v*) H_3PO_4. The temperature was controlled at 37 °C during growth and 25 °C after induction of the expression with 0.1 mM IPTG. The dissolved oxygen content of the culture broth was regulated by variation of the airflow and the agitation speed and set to approximately 25 % during the growth and expression processes.

The expression was started upon reaching an OD_{600} of 9 - 10. Feeding was kept constant using an 80 % (*v*/*v*) glycerol solution and a speed of 30 g/h [132]. Additionally, the medium was supplemented with 5 mL of a 1 M $MgSO_4$ x $6H_2O$ solution, 500 µL of a 1 M δ-aminolevulinic acid solution, 50 g $(NH_4)_2PO_4$, 4 mL of a trace element solution [190 mg $CaCl_2 \cdot 2H_2O$, 90 mg $ZnSO_4 \cdot 7H_2O$, 90 mg $CoCl_2 \cdot 6H_2O$, 75 mg $CuSO_4 \cdot 5H_2O$, 50 mg $MnSO_4 \cdot H_2O$, 11.1 mg Na_2-EDTA$\cdot 2H_2O$, and 8.35 mg $FeCl_3 \cdot 6H_2O$ in 500 mL of ddH_2O] and 2 mL thiamine (100 g/L).

2.9.3. Biotransformations of dodecanoic acid or dodecanoic acid methyl ester by resting *E. coli* cells

Biotransformations in 1 L (operating volume) bioreactors (Infors AG, Bottmanning, Switzerland) were carried out with 450 mL resting cells solution (50 g_{cww}/L) in 200 mM K_iPO_4 buffer pH 7.4. The biotransformation phase was initiated by addition of 90 mL C12-FAME. Alternatively, 4.5 g C12-FA were solved in DMSO like described before and added to the biotransformation mix for the reactions with the free fatty acid substrate. The pH was adjusted to 7.4 at the beginning and controlled with an autoclavable amperometric probe (Mettler–Toledo GmbH, Schwerzenbach, Switzerland), but not regulated during the reaction. Likewise, the dissolved oxygen (pO_2) was not adjusted but monitored during the biotransformation process with an autoclavable amperometric probe (Mettler–Toledo GmbH, Schwerzenbach, Switzerland). Stirrer velocity was set up from 200 (C12-FA) to 800 rpm (C12-FAME). Temperature and aeration rate were maintained at 30 °C and 1.5 L/min, respectively.

2.9.4. Analysis of substrates and formed products

Conversions were stopped with 30 µl 37 % HCl, followed by the addition of decanoic acid as internal standard in a final concentration of 1 mM. The reaction mixtures were extracted twice with 2 volumes of diethyl ether. The organic phases were collected, dried with $MgSO_4$ (anhydrous) and evaporated. Samples were resuspended in 60 µl methyl *tert*-butyl ether (MTBE), followed by the addition of 60 µl of 1 % trimethylchlorosilane in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and incubation at 75 °C for 30 min for derivatization.

Samples were analyzed on a GC/FID instrument (Shimadzu, Japan) equipped with a DB- 5 column (30 m × 0.25 mm × 0.25 μm, Agilent, Germany) and with hydrogen as carrier gas (flow rate, 0.8 mL/min; linear velocity 30 cm/s). The injector and detector temperatures were set at 25 0°C and 310 °C, respectively. The column oven was set at 130 °C for 2 min, raised to 250 °C at a rate of 10 °C/min, held isotherm for 3 min, and then raised to 300 °C at 40 °C/min. A GC/MS QP-2010 instrument (Shimadzu, Japan) equipped with a DB-5 MS column (30 m × 0.25 mm × 0.25 μm, Agilent, Germany) and helium as carrier gas was used to identify the products in some characteristic samples. The injector and detector temperatures were set at 250 °C and 285 °C, respectively. The column oven temperature program was the same as that of the GC/FID. Mass spectra were collected using electrospray ionization (70 eV). Reaction products were identified by their characteristic mass fragmentation patterns. Substrate and product conversions were quantified from the GC/FID peaks using calibration curves estimated from a series of standard solutions C12-FA, C12-FAME, ω-OHC12 and α,ω-DCA, treated in the same manner as the samples. ω-Regioselectivities were estimated from the total hydroxylated product.

2.9.5. Determination of glycerol, glucose and acetate by HPLC analysis

Cells from the fermentation fractions were separated from the supernatant by centrifugation at 20.000 × g for 1 minute (Centrifuge 5417 C, Eppendorf, Germany). The supernatant was transferred into a new plastic tube, mixed with the internal standard xylitol to a final concentration of 10 mM and filtered (0.45 μm). HPLC analysis was carried out on an Agilent System (1200 series) using an Aminex HPX-87H Ion Exclusion Column (300 × 7.8 mm, Bio Rad, USA) at 60 °C, a mobile phase of 5 mM H_2SO_4, and a flow rate of 0.5 ml/min. The analytes were detected and quantified using the corresponding standards on a refractive index detector (Agilent 1200series, G1262A) set at 35 °C.

2.9.6. Determination of hydrogen peroxide formation in cell crude extracts

Quantitation of total (intracellular and extracellular) H_2O_2 was performed by the horseradish peroxidase/phenol/4-aminoantipyrine spectrophotometrical assay as described elsewhere (Xu, Bell et al, 2007). Reaction mixtures were prepared in 50 mM Tri-HCl buffer pH 7.5 to a

final volume of 800 µL and contained 100 µl cell culture, 10 µl BugBuster™ 10X Protein Extraction Reagent (Novagen, Wisconsin, USA), 12.5 mM phenol, 1.25 mM 4-aminoantipyrine and 0.1 mg/L horseradish peroxidase. The absorbance of each sample was set at zero before adding the peroxidase. Hydrogen peroxide concentrations were calculated from a calibration curve with known concentrations of H_2O_2 (2 - 80 µM) that yielded absorbances in the linear range. In order to exclude any potential interference of BugBuster™ with the enzymatic assay, the reagent was added to all standard solutions.

3. Results and Discussion

3.1. Enzyme selection

The following section comprises goal 1 which further includes the comparison of suitable biocatalysts and strains for the hydroxylation reaction of *n*-butane to 1-butanol. The methodology used for this project is based on a literature and CYPED database-driven search to identify suitable biocatalysts for the hydroxylation of aliphatic compounds. First screening experiments have thus been performed to evaluate suitable candidates that can be expressed at high levels in a prokaryotic host as enzymatically active protein possessing high ω-regioselectivity, good catalytic activity as well as high stability.

3.1.1. ω-Alkane hydroxylases for short and middle chain length aliphatic substrates

Generally, short chain alkanes (C_1–C_4) in nature are hydroxylated by methane, propane and butane monooxygenases[52-55], while medium-chain alkanes (C_5–C_{16}) are oxidized by integral-membrane non-heme di-iron monooxygenases (AlkB) or alternatively by P450 monooxygenases from the CYP153A subfamily (Fig.2.).[51, 61] The majority of methane monooxygenase (MMO)-like enzymes which also include butane monooxygenases BMO, display relatively wide substrate specificities, but unfortunately also indicate limiting factors especially due to their binuclear metallic reaction center. Unfortunately, as the majority of these hydroxylases (1) function as a part of large enzyme complexes, (2) are membrane-associated, (3) exhibit low stability, (4) as well as strong product inhibition phenomena and (5) limited expressions in alternative host strains, their potential for industrial applications is limited.[52, 56, 58] For these reasons further potential enzymatic systems such as AlkB, CYP153A and BMO have been analyzed in detail in *in vivo* oxyfunctionalizations using *n*-butane as substrate. In this sense, the alkane monooxygenase AlkBGT from *Pseudomonas putida* GPo1 and the cytochrome P450 monooxygenase ($CYP153A_{P.\ sp.}$) from *Polaromonas sp.* constitute versatile enzyme systems for the ω-oxyfunctionalization of short to medium chain-length alkanes. In the effort to produce 1-butanol from *n*-butane, we screened four different strains all described to carry

presumably BMOs or CYP153A-like monooxygenases: But2 from *Ematobacter* sp., But3 from *Azoareus* sp., But4 from *Variovorax* sp. and But5 from *Mycobacterium* sp. This set of But-strains used in this study was provided by the lab of Prof. K. H. Engesser (University of Stuttgart, Stuttgart, Germany). Recombinant *Pseudomonas putida* KT2440 expressing either alkBGT or the natural operon of CYP153A$_{P.\ sp.}$ have been tested as whole cell catalysts for this regioselective biooxidation. AlkB is a membrane-associated oxygenase able to hydroxylate a broad substrate spectrum of aliphatic compounds at the terminal position.[133, 134] Recently, substrate access limitations and the overoxidation of the test substrate 1-dodecanol to dodecanoic acid have been identified as the most important limitations to be addressed by this enzyme.[134, 135] These reports are consistent with our results as we could detect next to the product 1-butanol significant amounts of the corresponding overoxidation product butyric acid. The strains But2, But3, But4 and But5 were able to grow on *n*-butane as carbon source but we were unable to detect any 1-butanol products. These have been found to be responsible for the degradation of *n*-butane and thus, it seems more likely that they are able to efficiently metabolize the hydroxylated products. Due to the difficult inhibition or knockout of enzymes responsible for metabolizing the alcohol products, investigations were not further extended using these strains. The use of the CYP153A$_{P.\ sp.}$-operon comprising the monooxygenase and its natural redox partners expressed in *P. putida* resulted in the formation of 1-butanol with a high ω-regioselectivity (>95 %). The easy handling of soluble monooxygenases is another advantage with regard to the level of expression and option to further optimize the system by creating e.g., self-sufficient fusion enzymes.[91] In addition, evolving strategies for enzyme engineering by rational design or directed evolution have given access to candidates of further interest. The availability of new genetic engineering techniques allowed change the activity of the two bacterial cytochrome P450 monooxygenases CYP102A1 (P450 BM3) from *Bacillus subtilis* and CYP101A1 (P450cam) from *P. putida*, both described as subterminal hydroxylases, towards non-natural alkanes.[130, 136-139] P450cam variants with eight different mutation points were created to address short-chained alkanes.[139-141] In the case of P450 BM3 more than ten iterative rounds of random gene diversification, screening and selection were necessary in order to identify mutants displaying substrate affinity and coupling of cofactor consumption rivaling those of the natural P450s with their preferred substrates.[140] However, compared to the wild type

enzyme mutations resulted in variants with lower thermostability and ω-regioselectivity as the subterminal position is energetically easier accessible.[136, 138]

Due to these limitations an application as ω-hydroxylase seems to be not suitable and thus, indicates that they still lag behind a natural P450 alkane hydroxylase in terminal hydroxylations of small alkanes.

3.1.2. Selection of CYP153A candidates

In order to identify enzymes that are potential targets for oxidation of aliphatic compounds, we utilized a protein multiple sequence alignment approach based on collected data in the Cytochrome P450 Engineering CYPED database.[79] We identified potential candidates through this sequence alignment such as the P450 CYP153A$_{P.\ sp.}$ from *Polaromonas* sp. JS666, CYP153A$_{R.\ pa.}$ from *Rhodopseudomonas palustris* BisB18, CYP153A7, CYP153A8, CYP153A11 (all three from *Sphingopyxis macrogoltabida*), CYP153A16 from *Mycobacterium marinum* M and CYP153A6 from *Mycobacterium* sp. HXN-1500.[142] CYP153A$_{M.\ aq.}$ from *Marinobacter aquaeolei* VT8 was chosen because it showed the biggest difference in this multiple amino acid sequence alignment in comparison to other CYP153A candidates, which were included in this analysis. Such a high sequence diversity is often associated with differences in the catalytic selectivity of a cytochrome P450 subfamily.[143] Furthermore, based on reports on the directed evolution of CYP153A6-BMO1 showing an increased activity of CYP153A6-BMO1 towards *n*-butane attributed to a single amino acid substitution Ala97Val located on a loop close to the active site[64], we were able to identify CYP153A$_{P.\ sp.}$ which possess naturally a valine at position 95 being equal to an alanine residue at position 97 in CYP153A6-BMO1.

3.1.3. Expression and *in vitro* bioconversions of CYP153A enzymes

The *in vitro* activities and regioselectivities of three different CYP153A ω-hydroxylases against C_5-C_{12} alkanes, C_6-C_{12} primary alcohols as well as $C_{8:0}$-$C_{20:0}$ and 9(*Z*)/9(*E*)-$C_{14:1}$-$C_{18:1}$ fatty acids as substrates were investigated with the view to further pursue the goals 2 and 3 of this Ph.D. thesis. Gaseous substrates such as propane or butane have not been included in this first analysis, due to a difficult handing of these in a small scale (0.5 mL) reactions. The results for the *in vitro* biotransformations with CYP153A$_{P.\ sp.}$ and CYP153A16 in this study

were prepared in collaboration with S. Honda Malca as demonstrated in reference I and II. The supply of suitable CYP153A enzymes and their characterization were divided between Sumire Honda Malca (CYP153A16) and myself (CYP153A$_{P.\ sp.}$). Activities of the monooxygenases CYP153A$_{P.\ sp.}$, CYP153A$_{M.\ aq.}$ and CYP153A16 were reconstituted with an artificial redox system. This artificial electron transfer system consists of putidaredoxin reductase (CamA) and putidaredoxin (CamB) from *P. putida* which show an amino acid similarity of more than 60 % with the natural redox partners. Recently it was reported that the redox proteins from *Pseudomonas* can be used to reconstitute P450 activity, when the natural redox partners are difficult to synthesize by *E. coli* in a functional form.[144] Both the three CYP153 genes and the non-natural redox proteins CamA and CamB were expressed in soluble form in *E. coli* and purified *via* affinity chromatography (Figure 10). It was possible to detect significant changes corresponding to the level of CYP153A enzyme expression in *E. coli* BL21 (DE3). Protein concentrations of CYP153A$_{P.\ sp.}$ and CYP153A$_{M\ .aq.}$ with 32 mg/L and 21 mg/L active protein, respectively, were much higher than that of CYP153A16 with 1.25 mg/L (Table 6). The most optimal P450-CamA-CamB *in vitro* ratio was analyzed and determined to be 1:5:10 which leads to the conclusion that the ratio between the CYP153A enzymes and its natural redox system is unequally distributed in living cells.

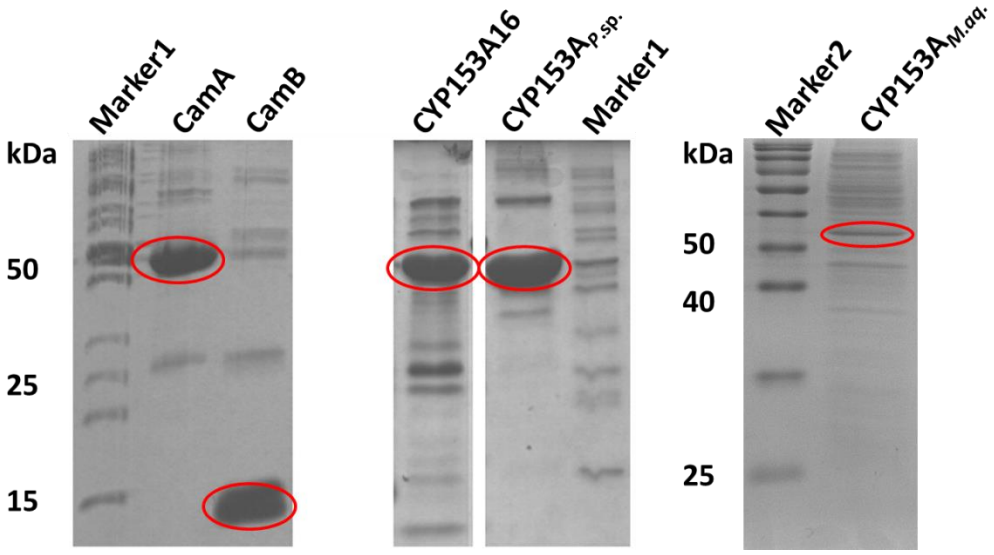

Figure 10: SDS-PAGE of purified proteins. The SDS gels show CamA (47 kDa); CamB (13 kDa); CYP153A16 (54 kDa); CYP153A$_{P.\ sp.}$ (49 kDa); CYP153A$_{M.\ aq.}$ (54 kDa); Marker1, PageRulerTM Unstained Protein Ladder; Marker2, PageRulerTM Prestained Protein Ladder. Protein solutions were analyzed on 15 % acrylamide.

3.1.4. *In vitro* bioconversions with aliphatic compounds

Similar to previously reported CYP153A enzymes, octane was also the preferred alkane for our monooxygenases CYP153A16, CYP153A*M. aq.* and CYP153A*P. sp.*.[107, 108, 142] Although their substrate ranges covers alkanes from C_5 - C_{12}, different activity models for each of the three CYP153As have been revealed. Compared to CYP153A16 or CYP153A*M. aq.*, CYP153A*P. sp.* exhibits higher terminal hydroxylase activity, but lower to inexistent α,ω-hydroxylase activity towards C_8-C_{12} alcohols. Therefore, CYP153A*P. sp.* has produced larger amounts of primary alcohols from alkanes than CYP153A16 or CYP153A*M .aq.*, but the two other ones formed considerably higher concentrations of α,ω-diols than CYP153A*P. sp.*. The corresponding α,ω-diols have been the product of the subsequent oxidation of the primary alcohol intermediate (Table 5). CYP153A*P. sp.* has shown diterminal hydroxylase activity only towards its most preferred substrates octane and nonane. However, the yields for 1,8-octanediol or 1,9-nonanediol have been lower than those obtained with CYP153A16 by 2- and 7-fold, respectively. The accumulation of α,ω-diols suggests that CYP153A16 or CYP153A*M .aq.* show a higher affinity towards C_8-C_{12} primary alcohols than CYP153A*P. sp.*. This, however, needs to be confirmed by spin-state shift experiments and the determination of substrate binding constants.

Both P450s CYP153A16 and CYP153A*P. sp.* were able to oxidize pentane and hexane to the corresponding primary alcohols with excellent ω-regioselectivity. Considering both primary alcohols and α,ω-diols as the overall terminally hydroxylated products, excellent ω-regioselectivities for C_7-C_9 compounds were observed (90 – 96 % for CYP153A16 and 83 -95 % for CYP153A*P. sp.*) with the highest activity towards 1-octanol/1,8-octanediol. A major part of the formed byproducts were secondary alcohols (up to 37 % of the hydroxylated product with respect to longer alkanes). In contrast to CYP153A*P. sp.*, CYP153A16 and CYP153A*M. aq.* showed higher ω-regioselectivities and tend to further oxidize the ω-hydroxylated. For example, with CYP153A16 less than 5 % of the total formed hydroxy compounds was composed of secondary alcohols, while the concentration of aldehydes and fatty acids increased (representing a maximum of 32 % of the total product) with the increase of the carbon chain length. The oxidation pattern of CYP153A*M .aq.* was not analyzed in detail. Compared to CYP153A16, CYP153A*P. sp.* gave smaller proportions of further oxidized

products (Figure 11). Other byproducts than aldehydes or fatty acids have not been observed in bioconversions with CYP153A$_{P. sp.}$.

Table 5: Concentrations of terminally hydroxylated products (primary alcohols (C_n-1ol) and α,ω-diols) obtained from alkane oxidations catalyzed by CYP153A enzymes. (Adapted from Scheps & Honda et al., 2011, with permission from RCS Publications)

Substrate (1 mM)	CYP153A16		CYP153A$_{P. sp.}$		CYP153A$_{M. aq.}$	
	α,ω-diol [μM]	C_n-1ol [μM]	α,ω-diol [μM]	C_n-1ol [μM]	α,ω-diol [μM]	C_n-1ol [μM]
n-Pentane	—	34 ± 3	—	30 ± 2	N.D.	N.D.
n-Hexane	—	24 ± 1	—	62 ± 5	—	40± 6
n-Heptane	—	81 ± 8	—	103 ± 3	N.D.	N.D.
n-Octane	30 ± 3	120 ± 9	16 ± 1	165 ± 13	52 ± 8	113 ± 9
n-Nonane	113 ± 6	35 ± 2	16 ± 1	114 ± 3	N.D.	N.D.
n-Decane	65 ± 3	16 ± 1	—	99 ± 4	72 ± 7	33± 7
n-Undecane	N.D. (59%)†	< 10.0	—	< 10.0	N.D.	N.D.
n-Dodecane	< 10.0	< 10.0	—	< 10.0	N.D.	N.D.

Substrate (0.2 mM)	CYP153A16	CYP153A$_{P. sp.}$	CYP153A$_{M. aq.}$
	α,ω-diol [μM]	α,ω-diol [μM]	α,ω-diol [μM]
1-Hexanol	—	—	N.D.
1-Heptanol	—	—	N.D.
1-Octanol	39 ± 3	18 ± 0.2	51 ± 7
1-Nonanol	93 ± 4	16 ± 1	N.D.
1-Decanol	80 ± 6	—	88 ± 9
1-Undecanol	N.D. (74%)†	—	N.D.
1-Dodecanol	43 ± 2	—	N.D.

— not detected; N.D. not determined; † Percentage relative to total product estimated from the GC peak areas. CYP153A16 produced more 1-pentanol than 1-hexanol in a reproducible manner; The amount of1,11-undecanediol obtained was not quantified as no standard was available.

To further investigate the substrate range of CYP153A16, CYP153A$_{M. aq.}$ and CYP153A$_{P. sp.}$, C_6-C_{12} primary alcohols have been used as substrates. No hydroxylation activity against 1-hexanol and 1-heptanol could be observed with all three enzymes. CYP153A16 was able to ω-hydroxylate primary alcohols ranging from C_8 to C_{12}, displaying highest activity for

1-nonanol which is consistent with the results obtained using alkane substrates. Similarly, CYP153A$_{P.\ sp.}$ could only oxidize 1-octanol and 1-nonanol to the corresponding α,ω-diol products, but in significantly lower yields than CYP153A16 (Table 5).

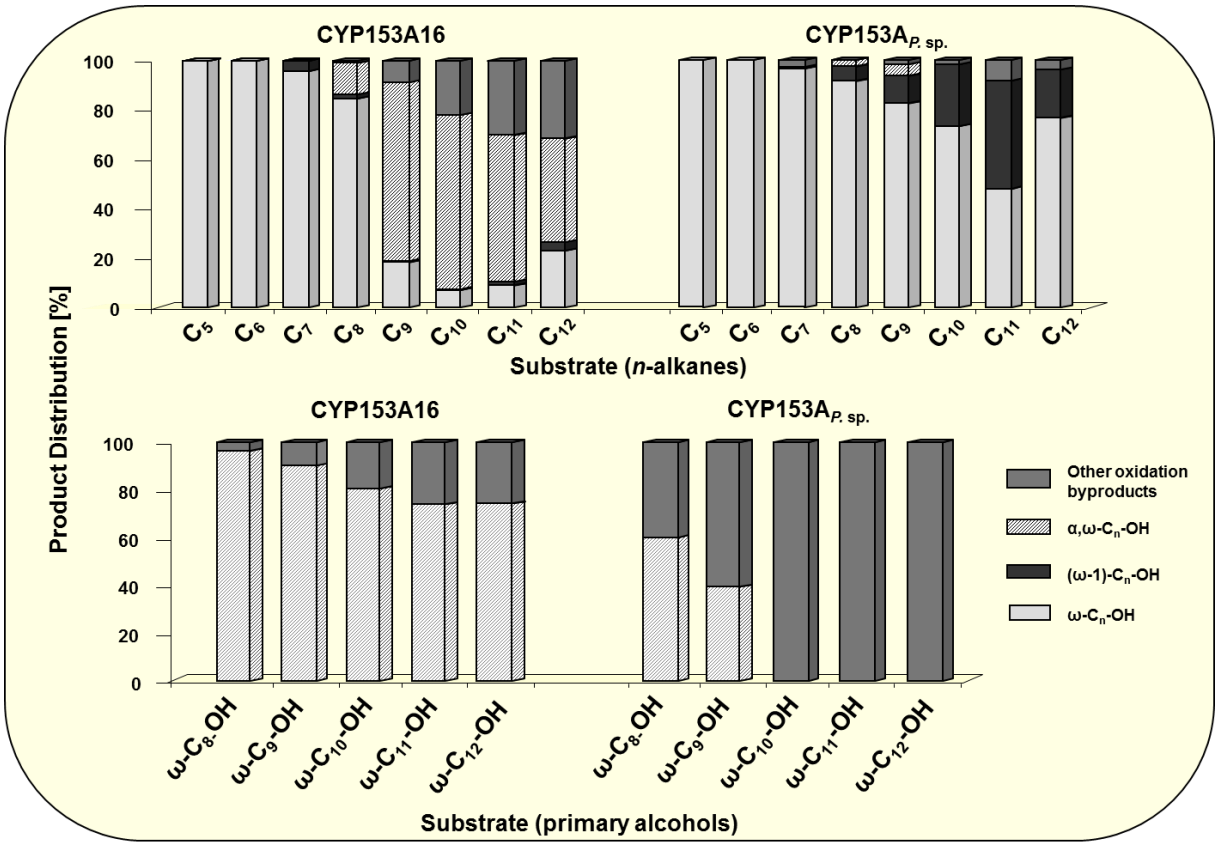

Figure 11: Product distribution using primary alcohols (C$_n$-1ol) as substrates. (Adapted from Scheps & Honda et al., 2011, with permission from RCS Publications)

The product profiles observed with each primary alcohol substrate are shown in Figure 11. With CYP153A16, 74 – 96 % of the total product obtained from the C$_8$-C$_{12}$ primary alcohols was converted into the corresponding α,ω-diol product, while the rest comprised aldehydes, fatty acids and the byproducts mentioned in the previous section. No α,ω-diacids could be detected in these experiments. Another result observed with CYP153A16 includes the formation of larger proportions of α,ω-diols from the C$_{11}$ and C$_{12}$ primary alcohols than from the hydroxylation using the corresponding alkanes. The concentrations of 1,11-undecanediol and 1,12-dodecanediol obtained from the primary alcohols were 1.2- and 4-fold higher,

respectively, than those obtained from the corresponding alkane substrates. When using alkanes as substrates, the alkanes and the formed primary alcohols presumably compete for binding in the active site of the enzyme, therefore lower yields of α,ω-diols are obtained. On the other hand, substrate competition no longer occurs using primary alcohols which would result in syntheses of higher α,ω-diol products. With respect to CYP153A$_{P.\ sp.}$, only 40 – 58 % of the total product corresponded to the C_8 and C_9 α,ω-diol with the remaining products comprising aldehydes and fatty acids. Even though CYP153A$_{P.\ sp.}$ has been shown to bind C_{10}-C_{12} ω-alcohols, it showed low catalytic activity towards these substrates. These alcohols have been exclusively converted to aldehydes.

Saturated fatty acids have been oxidized by CYP153A$_{P.\ sp.}$ in very low yields (less than 5 % conversion), whereas CYP153A16 and CYP153A$_{M.\ aq.}$ have been more active, displaying maximum activities towards the fatty acids $C_{13:0}$ (92 % conversion) and $C_{14:0}$ (83 % conversion), respectively. CYP153A16 has been active towards $C_{10:0}$-$C_{16:0}$ fatty acids, while CYP153A$_{M.\ aq.}$ showed a broader scope ($C_{9:0}$-$C_{20:0}$). The substrate range of CYP153A$_{P.\ sp.}$ has been slightly shifted towards shorter compounds ($C_{8:0}$-$C_{13:0}$). Monounsaturated fatty acids have been only oxidized by CYP153A16 and CYP153A$_{M.\ aq.}$, however, from these results it cannot be excluded that CYP153A$_{P.\ sp.}$ might be able to perform these reactions with a higher enzyme concentration or over a longer time course. CYP153A$_{M.\ aq.}$ was more active towards these compounds, showing a preference for 9(*E*)-$C_{16:1}$ (93 % conversion). Maximum substrate conversions of 35 % have been obtained with CYP153A16. CYP153A16 and CYP153A$_{M.\ aq.}$ have been more active towards the monounsatured 9(*Z*)/(*E*)-$C_{16:1}$-$C_{18:1}$ fatty acids than towards the saturated ones $C_{16:0}$-$C_{18:0}$. In terms of regioselectivity, CYP153A$_{P.\ sp.}$ catalyzed hydroxylations exclusively on the ω-position of fatty acids which might be attributed to the low activity towards these. CYP153A16 and CYP153A$_{M.\ aq.}$ showed also a high preference for this position, but their specificity varied depending on the CYP applied and the chain length of the substrate (Figure 12). CYP153A$_{M.\ aq.}$ has been highly ω-regioselective (more than 91 % ω-OHFA), while CYP153A16 produced up to 47 % (ω-1)-OHFA using the 9(*E*)-$C_{16:1}$ fatty acid. Surprisingly, CYP153A16 has shown the ability to α- and β-hydroxylate $C_{12:0}$-$C_{14:0}$ fatty acids (less than 3 % of the total product). Because of the fact that the α- and β-carbon atoms are located close the carboxyl group, it seems to be likely that no substrate separation occurs during the transfer to the active center. The iron-catalyzed oxygenation on such positions can

only be possible if the substrate entered the active site with its carboxyl moiety coordinated towards the heme centre. CYP153A16 and CYP153A$_{M.\ aq.}$ converted $C_{12:0}$-$C_{14:0}$ and 9(Z)-$C_{14:1}$ ω-OHFAs into α,ω-DCAs, though in low yields. Aldo-/ketoacids or epoxy products which might have arisen from the 9(Z)-monoenoic fatty acids were not detected. CYP153A16 produced more α,ω-DCAs (max. 11 % of the total product) than CYP153A$_{M.\ aq.}$. It has been furthermore observed that using an ω-hydroxylated fatty acid as starting material (ω-OH-$C_{12:0}$) did not lead to higher α,ω-DCA yields (data not shown).

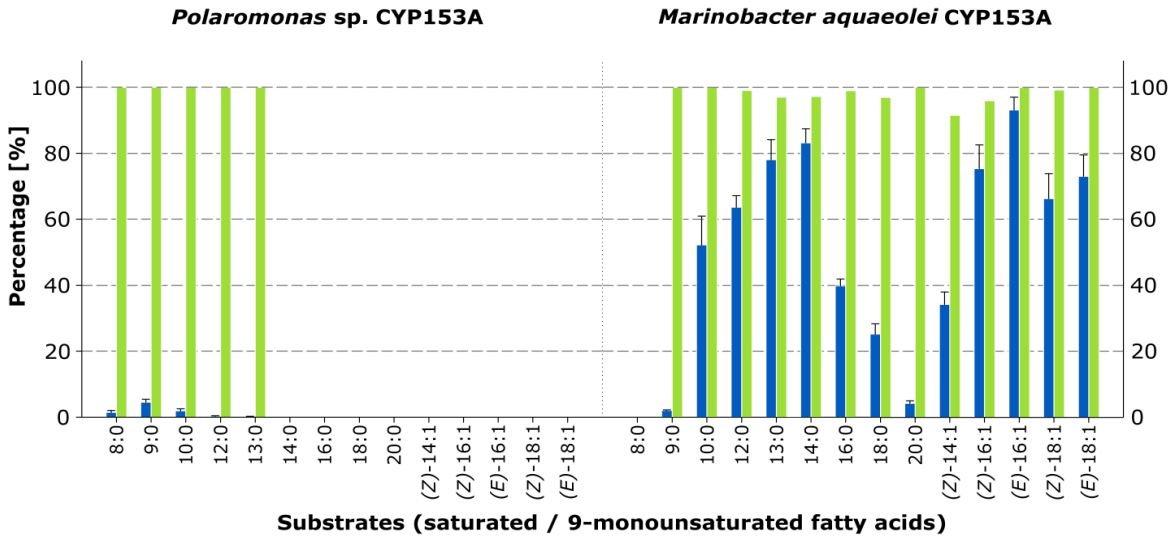

Figure 12: Substrate conversions (blue) and ω-regioselectivities (green) of fatty acid oxidation reactions catalyzed by the two CYP153A monooxygenases from *Polaromonas* sp. and *Marinobacter aquaeolei*. The initial substrate concentration was 0.2 mM and the reaction time 4 h. ω-Regioselectivities were estimated from the total hydroxylated product. (Published in Honda, Scheps et al., 2012, with permission from RCS Publications)

These findings identified during the *in vitro* experiments give the possibility to categorize the activities of these enzymes according their physiological roles. Besides the common β-oxidation pathway, microbial cells can also oxidize fatty acids to α,ω-DCAs by the ω-oxidation pathway. This ability was so far only characterized in eukaryotic yeast cells, but not in prokaryotes.[145] ω-Oxidation offers the opportunity to gain higher energy yields *via* di-terminal hydroxylase activity than by a direct utilization of fatty acids. The terminal hydroxylation of fatty acids is rate limiting. In two further enzymatic reactions, a terminal

carboxyl-group is formed *via* a fatty alcohol oxidase and a fatty aldehyde dehydrogenase.[104] Thus, the carboxyl-groups can be further activated to the corresponding acyl-coenzyme-A ester, which can be used in the β-oxidation of the corresponding organism like *Marinobacter aquaeolei* VT8.

CYP124A1 from *Mycobacterium tuberculosis* has been reported as ω-hydroxylase, which is able to convert both methyl-branched and linear fatty acids.[146] A low catalytic activity as well as *ω*-regioselectivity makes it debatable whether this enzyme is responsible for this reaction in nature.[146] AlkB as well as fatty acid hydroxylases (CYP153A16 and CYP153A$_{M.\ aq.}$) from the CYP153A subfamily can be due to their catalytic properties viewed as suitable candidates for the first step in the bacterial ω-oxidation pathway[125, 147]. Especially *P. putida* has shown a broad metabolic capability to utilize a wide substrate spectrum of diverse carboxylic acids.[148] Similar properties have been reported for *Marinobacter aquaeolei* VT8, which can survive under various conditions, due to its enormous metabolic flexibility.[149] These facts indicate that *ω*-oxidation is present in prokaryotic cells. *Marinobacter aquaeolei* VT8 is further able to produce wax esters, where fatty acids are linked by an ester bond to a fatty alcohol. These waxes can be used as an energy-storage for organisms.[150] Next to the published pathway by reducing fatty acyl-CoA to fatty alcohols like ω-hydroxy fatty acids, the direct oxidation of fatty acids offers a further possibility to synthesize these products.[151, 152]

Table 6: *In vitro* characterization of CYP153A monooxygenases

Biocatalyst	Expression [mg/L]	Stability [%][e]	Substrate (0.2 mM)	Conversion [%]	Product distribution [%] ω-OH	α,ω[b]	others[c]
CYP153A*P. sp*[a] - classified in group I	32	>90	*n*-octane	++	91	3	6
			1-octanol[d]	++	n.a.	60	40
			nonanoic acid	+	100	-	-
			MUFA	-	-	-	-
CYP153A*M. aq*[a] – classified in group II	21	>80	*n*-octane	++	85	12	3
			n-nonane	++	18	73	9
			1-nonanol[d]	+++	n.a.	90	10
			tetradecanoic acid	++++	88	5	7
			9(*Z*)-tetradecenoic acid	+++	66	7	27
CYP153A16[a] – classified in group II	1.25	5	*n*-octane	++	85	14	1
			n-nonane	++	74	25	1
			1-nonanol[d]	+++	n.a.	92	8
			dodecanoic acid	++++	97	2	1
			9(*Z*)-tetradecenoic acid	+++++	100	-	-

– (no conversion/not detected), + (1-5 %), ++ (5 – 30 %), +++ (31 – 60 %), ++++ (61 – 90 %), +++++ (> 90 %).[a] CYP153A from *Polaromonas* sp. (*P. sp.*), *Mycobacterium marinum* (A16) and *Marinobacter aquaeolei* (*M. aq.*).[b] α,ω-products: α,ω-diols from *n*-alkanes and 1-alcohols; α,ω-DCAs from fatty acids.[c] Other products: 2-alcohols, aldehydes and fatty acids from alkanes; aldehydes and fatty acids from 1-alcohols; hydroxylated regioisomers from fatty acids.[d] As reported in reference 1 or similar. Abbreviations: MUFA monounsaturated fatty acid; n.a. not applicable.[e] Tested at room temperature without conversion activity over 2 h.[f] 2 mM substrate was used for all biotransformation.

3.1.5. Classification of CYP153A$_{P.\ sp.}$, CYP153A$_{M.\ aq.}$ and CYP153A16

CYP153A$_{P.\ sp.}$ exhibited higher terminal hydroxylase activity, but lower di-terminal hydroxylase activity towards C_8-C_{12} alkanes. In comparison to CYP153A16 or CYP153A$_{M.\ aq.}$, CYP153A$_{P.\ sp.}$ has produced higher amounts of primary alcohols from alkanes with concomitant high ω-regioselectivity. CYP153A$_{P.\ sp.}$ has shown only low di-terminal hydroxylase activity towards octane and nonane with the concentration of products obtained (1,8-octanediol and 1,9-nonanediol) being significantly lower than with *e.g.* CYP153A16. The covered substrate spectrum against fatty acids is much smaller ($C_{8:0}$-$C_{13:0}$) than the one of the other investigated CYPs.

CYP153A16 and CYP153A$_{M.\ aq.}$ have produced considerably higher concentrations of α,ω-diols than CYP153A$_{P.\ sp.}$. These products have been the result of subsequent hydroxylation of the primary alcohol products. The enrichment of α,ω-diols supports the fact that these two enzymes have a clearly higher affinity towards the investigated primary alcohols ($C_{8\text{-}OH}$ to $C_{12\text{-}OH}$). Highest activity was obtained with 1-nonanol. Moreover, these two CYP153A candidates were capable to oxidize fatty acids selectively at the terminal position. CYP153A$_{M.\ aq.}$ could be identified as a predominantly fatty acid ω-hydroxylase with a broad substrate range. $C_{12:0}$-$C_{14:0}$ and $C_{16:1}$-$C_{18:1}$ were terminal functionalized with a high ω-regioselectivity (more than 95 %) and a high efficiency (up to 93 %).

In summary, two different oxidation patterns in relation to aliphatic compounds have been obtained by the three CYP153A enzymes reported here: (1) predominantly ω-hydroxylase activity with a focus on alkanes, (2) both ω-hydroxylase and α,ω-dihydroxylase activity using fatty acids. These results suggest classifying them into two different groups concerning activity and substrate specificity. Amino acid sequence alignments have shown that CYP153A$_{P.\ sp.}$ and CYP153A6 belong to group one and thus being alkane ω-hydroxylases.[107] By analyzing activities as well as protein similarities, CYP153A$_{M.\ aq.}$, CYP153A16, CYP153A13a from *Alcanivoras borkumensis* SK2 and CYP153A from *Acinetobacter* sp. OC4 were allocated to group both ω-hydroxylase and α,ω-dihydroxylase activity using fatty acids as substrates.[106, 153] The potential of P450s for production of high-value chemicals has long be recognized and research in these area aimed at commercial exploitation of these enzymes has

increased considerably in recent years. Engineering and optimization of P450s as well as efforts in host development in the coming years will undoubtedly lead to further exploitation of these versatile enzymes for high-value oxychemical synthesis.[12, 154] Based on these *in vitro* positive results we have chosen the CYP153A$_{P.\ sp.}$ as ideal candidate for the hydroxylation of small chain alkanes such as *n*-butane. For the synthesis of valuable ω-hydroxy fatty acids, CYP153A$_{M.\ aq.}$ seems to be the most promising candidate.

3.2. Optimization of CYP153A enzymes

3.2.1. Establishment of self-sufficient fusion constructs

This chapter deals with the optimization of the enzymatic system in terms of electron coupling efficiency and the establishment of protein engineering studies to tailor the enzyme for specific substrates (goal 4).

CYP153s belong to the bacterial class I P450s, which operate as three component systems. They comprise a catalytic unit (P450 domain) and two redox proteins. These redox proteins constitute an iron-sulfur electron carrier (ferredoxin) and a FAD-containing reductase (ferredoxin reductase), which are necessary for the transfer of electrons from NAD(P)H to the active site. The genes comprising the CYP153A$_{M.\ aq.}$ alkane hydroxylase operon (FAD-containing oxidoreductase, the CYP enzyme and [2Fe-2S] ferredoxin) were cloned with a His-tag to facilitate protein purification by immobilized metal ion affinity chromatography. The expression of the CYP153A$_{M.\ aq.}$ was successful in *E. coli* as described in section 3.1.3.4.[155] It has been further observed that the ferredoxin was expressed in functional form. However, the FAD-containing oxidoreductase was expressed as an apoflavoprotein. Even if there was a clear band with the right molecular weight on the SDS gel, the absence of the FAD cofactor was evidenced visually as well as by spectrophotometric analysis. The whole operon was expressed in *E. coli* and its activity measured in reactions with *n*-octane. Low substrate conversion levels were detected (data not shown). It was reported in reference I that CYP153A enzymes can also interact with natural *E. coli* redox partners. Therefore it is not surprising that small concentrations of formed hydroxy products could be measured.[156] For the sake of completeness, the natural redox partners of CYP153A$_{M.\ aq.}$ have not been applied in further whole cells biotransformations resulting from low conversion numbers.

Especially the electron transfer from the reductase domain to the catalytic domain is often slow and one of the rate-determining factors in many CYP systems.[157] An improvement in electron transfer to the heme domain is important for the efficient conversion of substrate to product by cytochrome P450 enzymes. In an optimal system all electrons provided by the cofactors (*e.g.* NAD(P)H) should be used in the formation of the hydroxylated product and must not be lost in uncoupling reactions.

To address the problems of low catalytic efficiency, inefficient electron transfer, complicated inner cellular arrangement of three single proteins and non-functional redox partners, a functional self-sufficient CYP153A$_{M.\ aq.}$ complex was developed.[91] The construct has been developed *via* a *C*-terminal peptide connection between the reductase domains (CYP102A1 CPR module from *Bacillus megaterium* and CYP116B3 Pfor module from *Rhodococcus ruber* DSM 44319) to the catalytic heme-unit of CYP153A$_{M.\ aq.}$ (Figure 13). Both CYP153A$_{M..\ aq.}$-Pfor$_{116B3}$ and CYP153A$_{M.\ aq.}$-CPR$_{BM3}$ have been successfully constructed. The measured kinetic parameters with the artificial redox partners (CamA and CamB) were rather low (< 8 min^{-1}). In the light of the results described elsewhere for the P450cam heme domain from *P. putida* using a triple fusion protein for hydroxylation reactions and their low activities by doing so, a triple fusion comprising putidaredoxin reductase-putidaredoxin-CYP153A was not discussed and generated.[158] Nevertheless, we have converted a bacterial class I protein to a class VII and class VIII system, respectively, following the P450 classification, which was introduced above (section 1.3.3.).

The first class VII self-sufficient cytochrome P450 has been discovered in *Rhodococcus* sp., the starting point for other candidates that have been discovered.[86, 159] The reductase domain has three different parts: the FMN and [2Fe–2S] unit as well as a cofactor (NAD(P)H) binding domain. In contrast to other P450 systems, the flavin cofactor is not FAD but instead FMN. Several examples have been described in the literature, where a bacterial P450 domain was successfully fused with a Pfor reductase domain (P450cam, P450PikC and P450balk).[160-162] The first self-sufficient fusion has been created using the soluble bacterial heme-domain from CYP153A$_{M.\ aq.}$ and the reductase domain of P450 BM3 CPR$_{BM3}$. In comparison to the CYP153A$_{M.\ aq.}$-Pfor$_{116B3}$ construct, CYP153A$_{M.\ aq.}$-CPR$_{BM3}$ elucidates better results with respect to activity and efficiency.

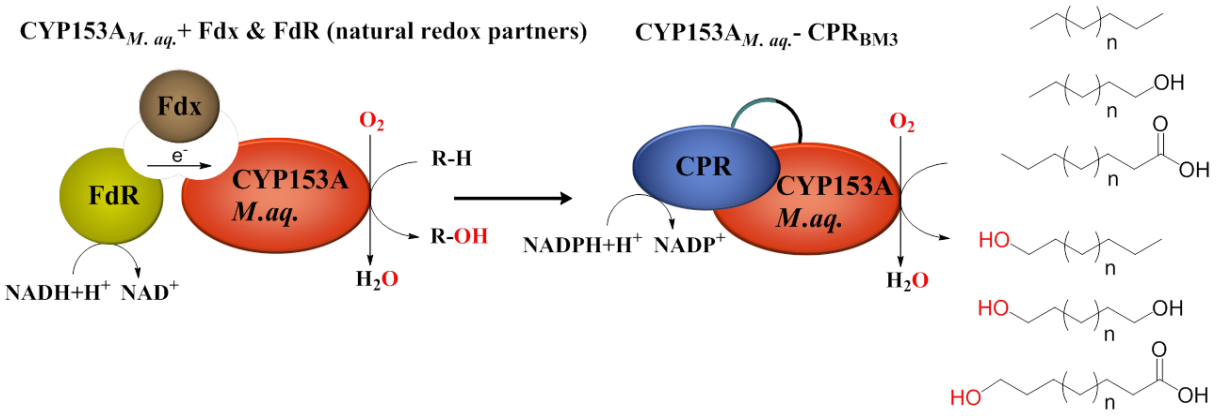

Figure 13: Establishing a self-sufficient fusion complex. (1) Construction of a functional self-sufficient fusion protein with a CPR$_{BM3}$ redox protein from the CYP153A$_{M.\ aq.}$ natural multiple protein complex.

The determined coupling efficiency with CYP153A$_{M.\ aq.}$-CPR$_{BM3}$ has been higher in comparison to the CYP153A$_{M.\ aq.}$-Pfor$_{116B3}$ (>65 % *vs.* >40 %). This effect results in a lower formation of H_2O_2 or superoxide radicals (Table 7). Since P450 applications are mostly performed in whole cell systems, these reactive byproducts can damage the P450 enzyme and thus, negatively influence the biocatalytic process. In this sense, a continuous regeneration of reducing equivalents (NAD(P)H+H$^+$) in the cell is essential for an effective hydroxylation process. Small disturbances in this cycle can have negative consequences.

Table 7: Coupling efficiencies[1] [%] of three different CYP153A self-sufficient fusion constructs and the artificial redox system CamA and CamB.

Substrate	CamA + CamB	Pfor	CPR	CPR$_{Mut}$
		Coupling [%][1]		
n-Octane	16	32	52	56
Octanoic acid	n.d.	-	22	n.d.
Dodecanoic acid	18	41	67	73

[1] The coupling efficiency is defined as the ratio between the product-formation rate and the NADPH-oxidation rate. n.d., not determined; -, not detected; Pfor, CYP153A$_{M.\ aq.}$-Pfor$_{116B3}$; CPR, CYP153A$_{M.\ aq.}$-CPR$_{BM3}$; CPR$_{Mut}$, CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$

The reductase domain CPR contains FAD and FMN cofactors, arranged close with a distance of only ~ 4 Å and only separated by a loop.[163] The electron flow appears at rates of ~50 s^{-1}.[164] Therefore, electron transport is fast enough not to hinder the overall reaction. A control mechanism regulates the reaction in the CPR which needs a conformational change to allow the electron transfer.[165] Two structural states have been predicted. The "open" conformation shows a rather short inter-flavin-distance , whereas the "closed" status shows a larger distance between the two cofactors.[165] A connecting loop is necessary, which is used as a hinge to convert the different conformations.[166] This leads to the conclusion that a tight binding to the heme domain is not beneficial, as a limitation of the conformation might influence the function of the CPR. The exact interaction of the different components in the electron transfer systems remains unclear. It has been reported that highly active P450 BM3 always consists of a dimeric conformation. Only in this form an inter-subunit electron transfer occurs between the involved CPR and P450 domain (FMN-to-heme).[167] At low concentrations of the catalyst, a decrease in activity is detectable.[168]. At this point in time, we assume that also a dimeric form might be involved in our hydroxylation reactions using the described CYP153A-CPR man made fusions.

To obtain a better understanding of the created fusion construct, we analyzed RIS1 and RIS2 (reductase interaction site 1 and 2) of the heme-domains of P450 BM3 (natural P450-CPR fusion protein), P450cam (natural P450-Pfor fusion protein), CYP153AaciA (exist as chimeric CYP153A-Pfor$_{116B2}$ fusion), CYP153A13a (exist as chimeric CYP153A-Pfor$_{116B2}$ fusion), CYP153A$_{M.\ aq.}$ and CYP153A$_{P.\ sp.}$ by using the CYPED (P450 engineering database). Counting the number of amino acids in each CYPED sequence for RIS1 revealed two peaks in the RIS1 length distribution. This allowed defining two classes. Proteins having short RIS1 with less than 10 residues make up 17.5 % of all protein entries. According to the result of the length analysis of RIS1 of the structural alignment, they comprise class I CYPs. Proteins having long RIS1 with more than 15 residues make up 81 % of all protein entries. According to the result of the length analysis of RIS1 of the structural alignment, they comprise class II CYPs. Only 1 % of all protein entries cannot reliably be assigned by RIS1 length since their length is in between 10 and 15 amino acids.[79, 81] The results have been listed in section 7.1.3. With respect to the protein sequence length, RIS1 is consists in all cases of 6-7 amino acid residues. On the other hand, RIS2 of P450 BM3 does not fit well with the section in sequence of the

other five enzymes. RIS2 of P450 BM3 shows 11 amino acids, whereas in the other case four to five are noticed. Nonetheless, CYP153A*M. aq.* and CYP153A*P. sp.* are able to interact with the natural reductase modules with the first amino acid in all six RIS2 parts to be an arginine, which is assumed to form hydrogen bridges to bind the reductase to the heme-domain.[79, 81] All four CYP153A enzymes possess—next to arginine at the start of the sequence—also an arginine at the last position of RIS2, which potentially offers a higher flexibility by forming hydrogen bridges with alternative redox systems. Even though these two reductase interaction sites were easy to determine in sequence and structure, unfortunately, a closer look into RIS1 and RIS2 does not offer a simple solution to predict suitable partners for the synthesis of chimeric fusion proteins.

3.2.2. Rational Protein Design

Rational design is a smart strategy to affect the substrate specificity of an enzyme and to form an optimized conversion tool.[4] Instead of progressively replacing different amino acids to change the natural substrate specificity by directed evolution, a rational protein design approach can be applied. Additionally to bioinformatics-tools including docking simulations, a focused mutant library can be designed by screening and analyzing protein sequences.[123, 169] Amino acids at position 307 in CYP153A*M. aq.*, which is located in the ´GGNDT´ motif, has been proposed as mutation hotspot in order to alter the substrate specificity and activity for terminal hydroxylation reactions against fatty acids (Figure 14).[170, 171] More detailed information about this hotspot identification and mutant analysis by kinetic analysis, established by Sumire Honda Malca, can be found in Honda, Scheps *et al.*, 2012 (reference II). Based on these results, also the corresponding amino acid residue in CYP153A*P. sp.*, Gly254 has been mutated. We wanted to investigate whether it was possible to transfer the improved substrate specificity towards fatty acids also to alkanes (Table 8). Wong and colleagues reported that a similar position in P450cam influenced the capability to hydroxylate alkanes at the terminal as well as subterminal positions.[172, 173] They further demonstrated that the position next to G254 in CYP153A*P. sp.* and G307 in CYP153A*M. aq.*, respectively, can affect the ability to hydroxylate short aliphatic compounds.[139] For the same reasons, position V253 in CYP153A*P. sp.* and V306 in CYP153A*M. aq.*, respectively, have been exchanged. Based on suggestions in literature from M. T. Reetz, phenylalanine, leucine,

isoleucine, methionine comprising amino acids with hydrophobic side chains as well as glycine have been chosen using degenerated codons with the intention to reduce the space in the active site to limit the possibility of substrate movement.[120] Furthermore, double mutants have been created to explore the cooperative effects of the introduced mutants.[120]

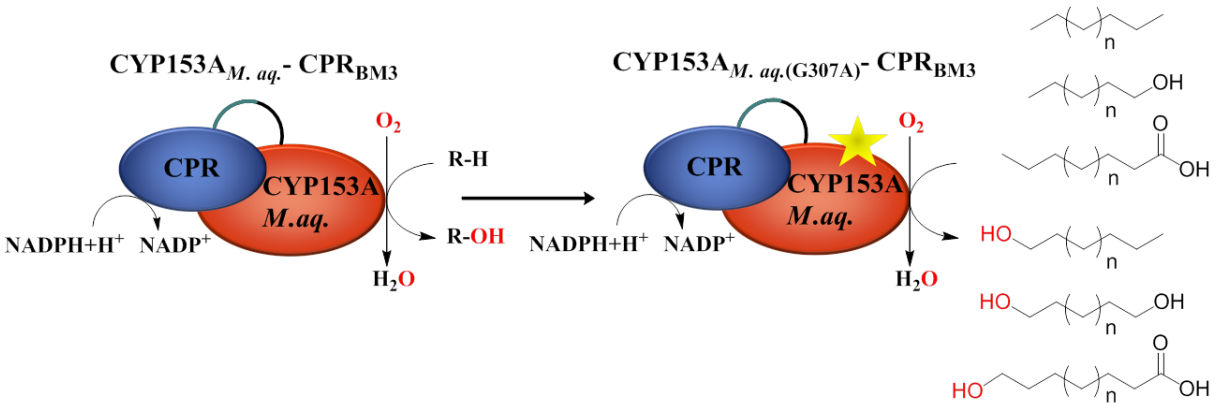

Figure 14: Establishing an optimized self-sufficient fusion complex comprising mutation G307A for altered substrate specificity and activity. Conversion of the self-sufficient CYP153A$_{M.\ aq.}$-CPR$_{BM3}$ into an optimized CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ with a change in a substrate spectrum against shorter compounds.

3.2.3. *In vitro* characterization of different fusion constructs

With the aim to confirm the functionality and to evaluate the substrate spectrum of the created self-sufficient fusion constructs, different variants have been analyzed by *in vitro* screening. Table 8 shows the conversion rates (activity) and ω-regioselectivities observed for the newly established CYP153A fusion systems. To develop uniform testing methods for reliable and comparable results, 1 µM of catalyst has been used for all bioconversions with the reaction being stopped after 1 h. The results obtained were consistent with those obtained in earlier studies and support that the introduction of an alanine at that position shift the substrate spectrum towards substrates of shorter chain length.[155] The regioselectivity of the CYP153A$_{P.\ sp.}$-CPR$_{BM3}$-catalyzed hydroxylations have been maintained with only small amounts of byproducts determined (2-alcohols, aldehydes and fatty acids). By replacing the glycine residue at position 254 with alanine (G254A), the ω-regioselectivity could be further increased (from 91 to 95 %). Moreover, in contrast to the use of the three single-proteins CamA, CamB and CYP153A monooxygenase, which form a multiple-protein

complex, the conversion rates could be further increased using our fusion construct. However, when considering the activity of the wild type known to be the fastest bacterial P450 (P450 BM3), the conversion rates determined herein are still low.[87] Furthermore we were able to demonstrate that the tested mutants at position V253 impact the conversion rates negatively in contrast to what was reported for P450cam.[139] Our results indicate that highest activity could be obtained with V253L and that G254A increased the overall oxidation activity. Conversion rates in a double mutant with V253L did not prove synergistic effects.

Table 8: *In vitro* characterization of CYP153A self-sufficient fusion constructs

Biocatalyst	Substrate[c]	Conversion [µM]	Product distribution [%]	
			ω-OH	Others[b]
CYP153A$_{P.\ sp.}$[a], CamA, CamB (artificial redox partners)	*n*-hexane	35	100	0
	n-octane	72	91	9
CYP153A$_{P.\ sp.}$-CPR[a]	*n*-hexane	78	100	0
	n-octane	175	92	8
CYP153A$_{P.\ sp.(V253L)}$-CPR[a]	*n*-hexane	20	100	0
	n-octane	41	95	5
CYP153A$_{P.\ sp.(G254A)}$-CPR[a]	*n*-hexane	118	100	0
	n-octane	210	94	6
CYP153A$_{P.\ sp.(V253L/G254A)}$-CPR[a]	*n*-hexane	35	100	0
	n-octane	60	100	0
CYP153A$_{M.\ aq.}$-CPR[a]	dodecanoic acid	125	n.d.	n.d.
CYP153A$_{M.\ aq.(V306L)}$-CPR[a]		41	n.d.	n.d.
CYP153A$_{M.\ aq.(G307A)}$-CPR[a]		155	n.d.	n.d.
CYP153A$_{M.\ aq.(V306L/G307A)}$-CPR[a]		50	n.d.	n.d.

[a] CYP153A from *Polaromonas* sp. (*P. sp.*), [b] Other products: 2-alcohols, aldehydes and fatty acids from alkanes; hydroxylated regioisomers from fatty acids, [c] 1 mM from alkanes and 0.2 mM from fatty acids.

3.2.4. Sequence Saturation Mutagenesis (SeSaM)

Rational design is a smart strategy to affect the substrate specificity of an enzyme and to form an optimized tool for the conversion of chemical compounds.[4] Functional specialization of a biocatalyst against one target needs a couple of rounds of random mutagenesis and selection.[80] Only this strategy offers the opportunity to find interesting positions on the surface of the protein, which are not related to the active center. There have been many ways developed to generate genetic diversity into a target sequence. One common method is to prepare a combinatorial library by error-prone PCR (epPCR).[174] This method is generally used, as it is easy and cheap to perform in a one-step procedure. Nevertheless epPCR includes two major limitations: (1) homogenous distribution of mutations throughout the complete gene is hard to achieve. Common DNA-polymerases incorporate nucleotides always at preferential sites, which results in a limited diversity. (2) DNA-polymerases are biased towards transitions, meaning that more than 40 % of all detected mutations are A→T and T→A substitutions.[121] However, SeSaM is capable to create a high-quality mutant library and to overcome the mentioned limitations. SeSaM is a conceptually novel and practically simple method that truly randomizes a target sequence at every single nucleotide position. A broad chemically diverse amino acid substitution makes this mutation approach a suitable alternative for the synthesis of random mutant libraries.

To create a SeSaM library, four independent experimental steps are necessary: Step 1: creating a pool of DNA fragments with a random size distribution. Step 2: enzymic elongation of the DNA fragments with a universal base. Step 3: full-length gene synthesis. Step 4: universal base replacement by standard nucleotides. In this sense, target gene sequences were amplified followed by the synthesis of different DNA fragments of random length. Through agarose-gel analysis, the successful amplification of the gene could be confirmed (Figure 15: A) and an additionally automated gel-electrophoresis gives the opportunity to investigate complex mixtures of ssDNA fragments (Figure 15: B). Phosphor imager analyses ensures (Figure 15: C) that more than one additional nucleotide was inserted at the end of the DNA strand and a high genetic diversity was generated. A final sequencing

approach of 20 randomly picked candidates demonstrated the effectiveness and quality of this generated library (only one wild type sequence could be found).

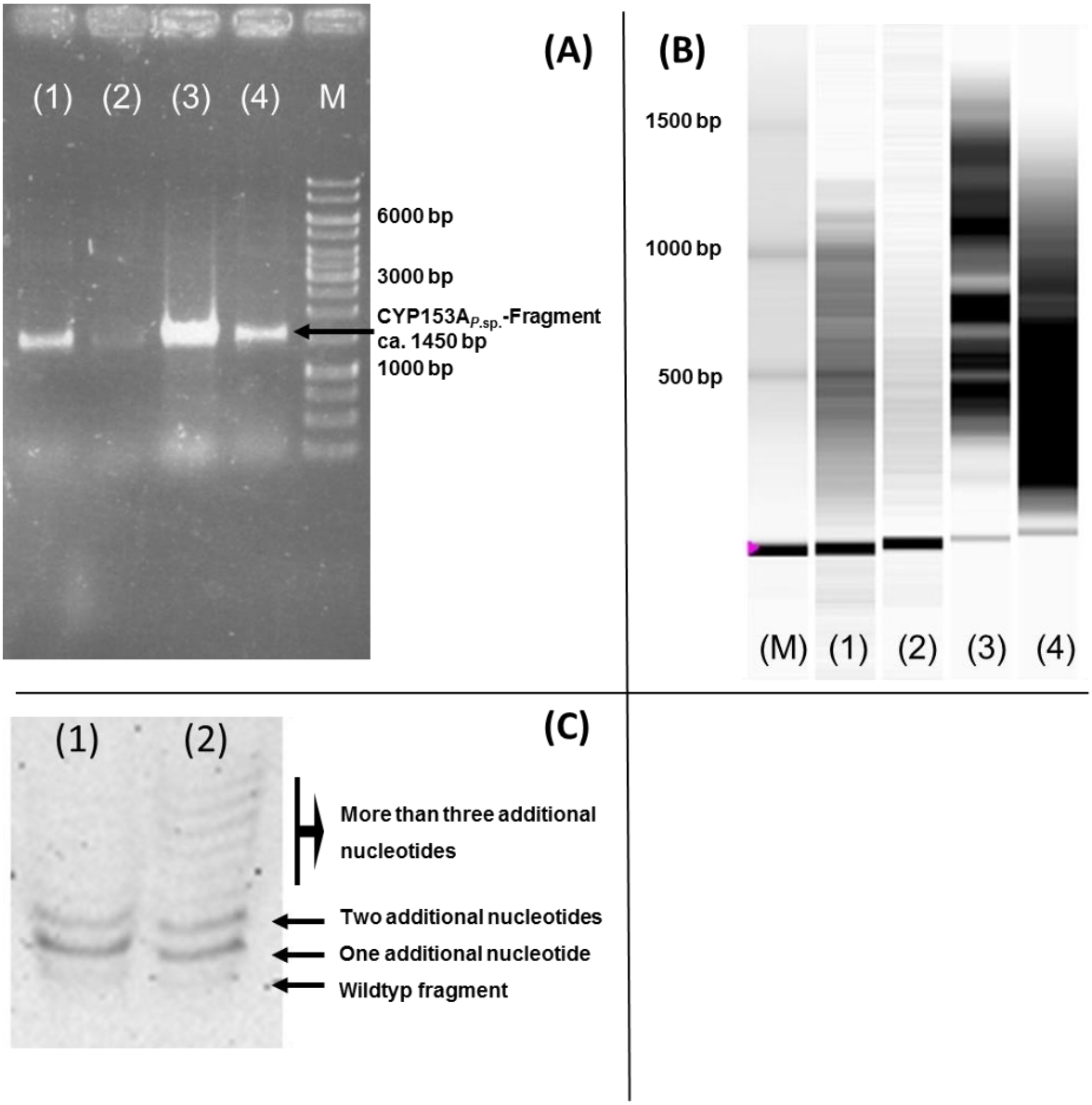

Figure 15: Synthesis of the SeSaM library (A) Agarose gel to determine the amplification of the SeSaM target gene (CYP153A$_{P. sp.}$) in (pre)step 1. (B) Fragment distribution analysis of CYP153A$_{P. sp.}$ analyzed by automated gel electrophoresis after SeSaM step 1. (C) Phosphor imager analyses of 3′-tailed FITC-oligonucleotides, separated on 25 % acrylamide gels.

3.2.5. Screening of the SeSaM mutants through cell viability/growth assays

Measurement of cell viability and proliferation forms the basis for numerous assays of a cell population's response to external factors. Before addressing the specifics of a given method, one needs to revisit, in a generic sense, the 'essential' question being asked by any experimental model which purports to serve as a surrogate for 1-butanol. The analysis of the large mutant library for *n*-butane hydroxylation is challenging as the formed 1-butanol is difficult to detect at low concentrations and does not show other specific characteristics (colorless) for an alternative analytical system. The classical NAD(P)H oxidation, however, may also not provide an accurate measure of hydroxylase activity (>1000 variants) because reducing equivalents from NAD(P)H can be diverted into forming reduced oxygen intermediates, such as H_2O or H_2O_2. With these undesired side reactions, NAD(P)H consumption measurements are difficult to assess.[137] Therefore, in an initial attempt we intended to screen our SeSaM library using a microtiter plate assay (purpald–assay), sensitive to product formation. This assay offers the opportunity of a high-throughput screening combined with a specific photometric analysis. The product formation of terminal oxidized products can be detected with a screen based on a demethylation reaction of a surrogate substrate (dimethyl ether, Figure 7: A).[130, 175] Instead of *n*-butane, methoxy ethane can be used in this assay. The alternative substrate shares most of the physical properties of *n*-butane including shape, size and solubility. The hydroxylated ether spontaneously decomposes to ethanol and formaldehyde. The so formed formaldehyde is able to react with the assay compound purpald and air to a purple compound, which can be specifically detected at 550 nm (Figure 7: B).

An alternative microtiter plate assay for a library screening is based on the utilization of a *p*-nitrophenooxy-derivative which does form an unstable intermediate in the hydroxylation reactions. This intermediate dissociates to form the corresponding aldehyde while *p*-nitrophenolate is released into the assay solution (yellow color). However, this assay is not suitable for our approach due to our focus on terminal hydroxylation reactions.[136, 176] Although, both microtiter plate assays are labour and time intensive, they were applied from Arnold and co-workers with the aim to convert P450 BM3 into a short alkane hydroxylase. Using the described colorimetric screen with a *p*-nitrophenooxy-derivative and parallel

measurements of NADPH consumption, various alkane hydroxylating mutants have been identified.[136] As the most active candidates have shown activity towards octane, the selection to shift the substrate spectrum towards shorter alkanes (propane) with dimethyl ether demethylation in high-throughput screening manner was successful.[138]

The establishment of an *in vivo* screening assay represents a compact alternative to the already described and laborious microtiter plate assays. The application of viability/growth-assay, which is based on the ability of *P. putida* KT2440 to grow with 1-butanol as carbon source (unpublished data) offers an interesting opportunity. The corresponding 1-butanol pathway including the identification of a 1-butanol dehydrogenase enzyme has been already.[55, 177] We proved in previous experiments that this host is able to grow on *n*-butane, when it is complemented by a plasmid-encoded alkane hydroxylase (CYP153A$_{P.\ sp.}$-operon). Continuous growth under selection pressure with *n*-butane gas, gives the opportunity to enrich more active terminally butane-hydroxylating enzymes in a given culture.[64]

Finally, three reasons led to the decision to choose the viability assay instead of the purpald assay: (1) The surrogate substrates for the purpald assay, like the dimethyl ether, are similar to the desired substrate *n*-butane, which should be attained. However, there is still a noticeable difference, which can significantly influence the selection process of the library. (2) The numbers of screened mutants is lower for the purpald assay through the 96 format microtiter plate dependency. (3) The screening process using the purpald assay is more time intensive, because every mutant must be examined individually. However, noteworthy are the facts that the purpald assay is not only applicable to screen increased conversion rates against short primary alcohols (Figure 7: A-1).

However, a continuative screening for higher production rates of ω-hydroxylated fatty acids like industrial relevant ω-hydroxy oleic acid is possible by using the purpald assay, which cannot be easy applied in the mentioned viability-assay (Figure 7: A-2). It is imaginable that with the synthesis of two molecules of formaldehyde *via* the hydroxylation of 1,2-dimethoxyethane a selection of α,ω-diol producing enzymes is feasible. That is due to higher absorption intensity (purple).

3.2.6. Analysis of the SeSaM library using the cell viability assay

After twelve sub cultivation steps of the starting culture, ten randomly selected strains were plated out on LB_{Kan} plates using the last enrichment culture. After isolation of the plasmids the candidates were analyzed *via* growth experiments (after retransformation) against *P. putida* KT2440 carrying a plasmid coding for the wild type $CYP153A_{P.\ sp.}$-operon (reference strain). Four variant strains showed at least a 2-fold faster growth rate in comparison with the reference strain. After plasmid isolation and DNA sequencing, the protein sequences of the $CYP153A_{P.\ sp.}$ variants were aligned with CYP153A7 from *Sphingopyxis macrogoltabida*, whose crystal structure (PDB ID 3RWL) is available. PyMOL and the CYPED database were utilized to analyze the position of the SeSaM-hotspots. Results are shown in Table 9 and Figure 16. Not surprising to us was the observance that the higher growth rate was not only obtained by beneficial mutations in the hydroxylase, but also by adaptations of the host and vector. To make sure that the identified hotspots really affect the oxidation of *n*-butane to 1-butanol, *in vitro* experiments should performed as a next step.

Table 9: Identified SeSaM-hotspots in $CYP153A_{P.\ sp.}$.

Position of mutation	Location in the structure
N220	Protein surface
A184, T185, A186	C-terminus of the F-helix
N274	Protein surface
T300	Located in SRS5; position 4 after the ExxR motif.

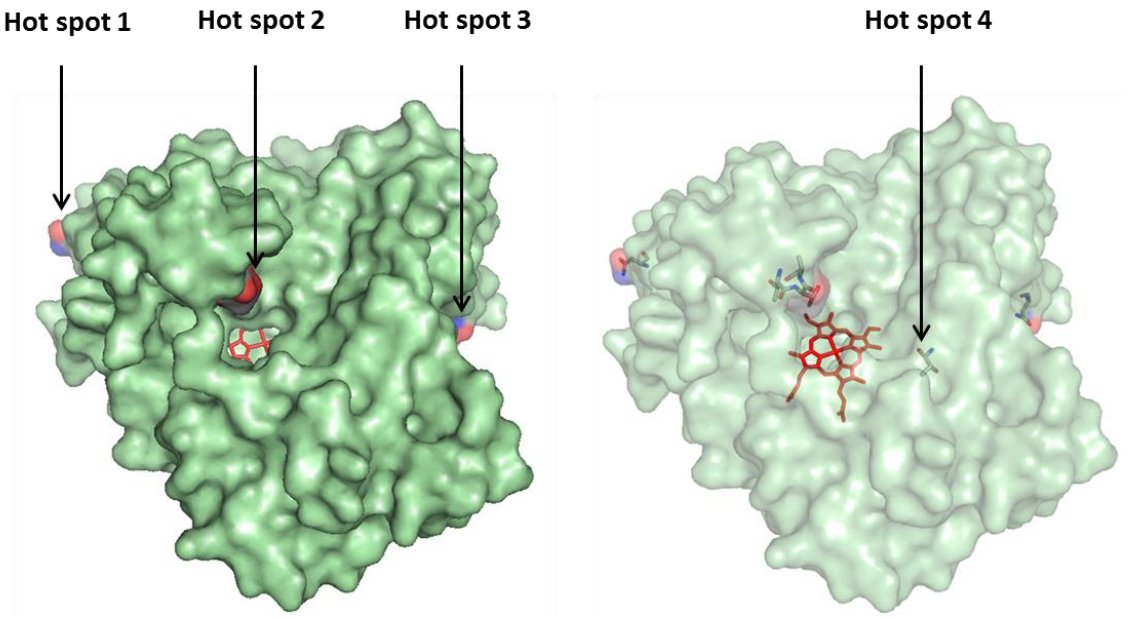

Figure 16: Representation of the structure of CYP153A*P. sp.*. The substrate-free crystal structure of CYP153A7 from *Sphingopyxis macrogoltabida* was used as model. CYP153A7 shares 79 % protein similarity with CYP153A*P. sp.* The images were generated by PyMOLs. Hotspots 1-4 were identified by an *in vivo* screening of the SeSaM mutant library.

To gain a better insight into our mutagenesis study, we compared our mutated CYP153A*P. sp.* enzyme with the structural related monooxygenase P450cam which is not a natural alkane hydroxylase. However, Wong and co-workers reported the transformation of P450cam into an alkane hydroxylase *via* an intensive mutation study (more than 8 different mutation points). Table 10 shows a comparison of established CYP153A hotspots like G307 and L354 for CYP153A*M. aq.* and G254 for CYP153A*P. sp.* with P450cam mutants. In comparison to the wild type enzyme improved K_m and k_{cat} values could be determined for G307 in CYP153A*M. aq.* *via* measurements of the kinetic parameters. In contrast to V247 for P450cam the similar positions G307 and for CYP153A*M. aq.* and G254 for CYP153A*P. sp.* has a negative impact on terminal hydroxylation (section 3.2.2.). Xu and co-workers proved that mutation L244M (I-helix) in P450cam, which is equivalent to residues L303 in CYP153A*M. aq.* and L250 in CYP153A*P. sp.*, has a positive effect on the hydroxylation of alkanes. This position is located on the I-helix like V247 or G248. Therefore this position could also be an interesting objective for further mutation studies. The substitution of leucine with an amino acid with bulkier and more hydrophobic side chain like methionine seems to be promising. A184 is one of the identified SeSaM hotspots for CYP153A*P. sp.* and is located at the C-terminus of the F-helix

next to the entrance tunnel of the active center. This position shows similarity to T185M in P450cam which represent a proven hotspot for alkane hydroxylation.[139] Due to these facts this position offers an interesting target for further mutation experiments. Another identified SeSaM-hotspot (T300 for CYP153A$_{P.\ sp.}$) is located at a short distance to L354 for CYP153A$_{M.\ aq.}$ (known to influence the ω-regioselectivity) on substrate recognition site 5. Additionally, this position is located on substrate recognition site 5, which often influence the substrate specificity of P450 enzymes.[82, 123] Due to the unknown function of the remaining two hotspots (Table 9) which are located on the surface of the protein, the main focus of future engineering tasks will be on the verification of this mutagenesis results in particular on the influence of amino acid residues at position A184 and P300 on butane hydroxylation.

Table 10: Rational analysis of CYP153A$_{M.\ aq.}$ and CYP153A$_{P.\ sp.}$ and comparison with P450cam.

P450cam			CYP153A$_{M.\ aq.}$				CYP153A$_{P.\ sp.}$			
Amino acid	Position	Verified function	Amino acid	Position	Verified function	Predicted function	Amino acid	Position	Verified function	Predicted function
L244M[a]	I –Helix	W	L303[a]	I –Helix	n.d.	X	L250[a]	I –Helix	n.d.	X
V247L[a]	I –Helix	W	V306L[a]	I –Helix	X	-	V253L[a]	I –Helix	X	-
G248A[a]	I –Helix	W	G307A[a]	I –Helix	X	-	G254A[a]	I –Helix	X	-
V295	SRS-5	W	L354[a]	SRS-5	X	-	L301[a]	SRS-5	n.d.	X
L294M	SRS-5	n.d.	P353[b]	SRS-5	n.d.	X	P300[b]	SRS-5	n.d.	X
T185M	n.d.	W	A229[a,b]	C-terminus of the F-helix	n.d.	Y	A184[a,b]	C-terminus of the F-helix	n.d.	Z / X

n.d. not determined; `-` not prediction possible; SRS-5 substrate recognition site; (W) Influence the terminal oxidation activity against alkanes; (X) Influence the terminal oxidation activity against aliphatic compounds; (Y) Decrease of terminal hydroxylation activity against positively charged aliphatic compounds; (Z) Increase of terminal hydroxylation activity against positively charged aliphatic compounds; [a] identified by rational design or sequence analysis; [b] identified by SeSaM-library screening for terminal butane oxidation.

3.3. Conversion of 1-Butanol

The conversion of *n*-butane to 1-butanol is of special interest, because the primary alcohol is an interesting precursor for the chemical industry. For economic reasons the possibility to synthesize a chemical product or an easy to handle liquid fuel from natural gas is a worthwhile pursuit. The selective terminal hydroxylation of small inert alkanes is a complex challenge, which is only possible with a reactive ionic compound or radical species. Additionally overoxidation to the associated aldehyde or fatty acid is a problem that occurs due to the fact that the inserted electron withdrawing group attenuates the corresponding C-H bond. Unfortunately, chemical methods for the selective terminal oxidation of alkanes are rare, only few transition-metal-based catalysts do this type of conversion under very harsh reaction conditions. Furthermore the reagents and byproducts are hazardous to the environment.[178]

3.3.1. Setup for *in vivo* biotransformation

At the beginning an assembly was set up to examine the ability of different strains to oxidize *n*-butane (section 3.1.2.) under defined and reproducible conditions, which cover goal 5 in the objectives (Figure 17). This experimental setup gave us the possibility to evaluate different process parameters: *n*-butane gas / air ratio, gas flow, biotransformation media, implementation of product removal, product toxicity analysis, energy sources for cofactor regeneration and aeration improvement *via* sparger. Molecular oxygen has to be present during the reaction in the medium in order to provide the necessary oxygen. Therefore the safety regulations were followed exactly. In the process a hydroxyl group is inserted into *n*-butane by an enzymatic oxidation. For an effective regeneration of the used cofactor (NAD(P)H) by the metabolism of the host cells oxygen is also necessary.

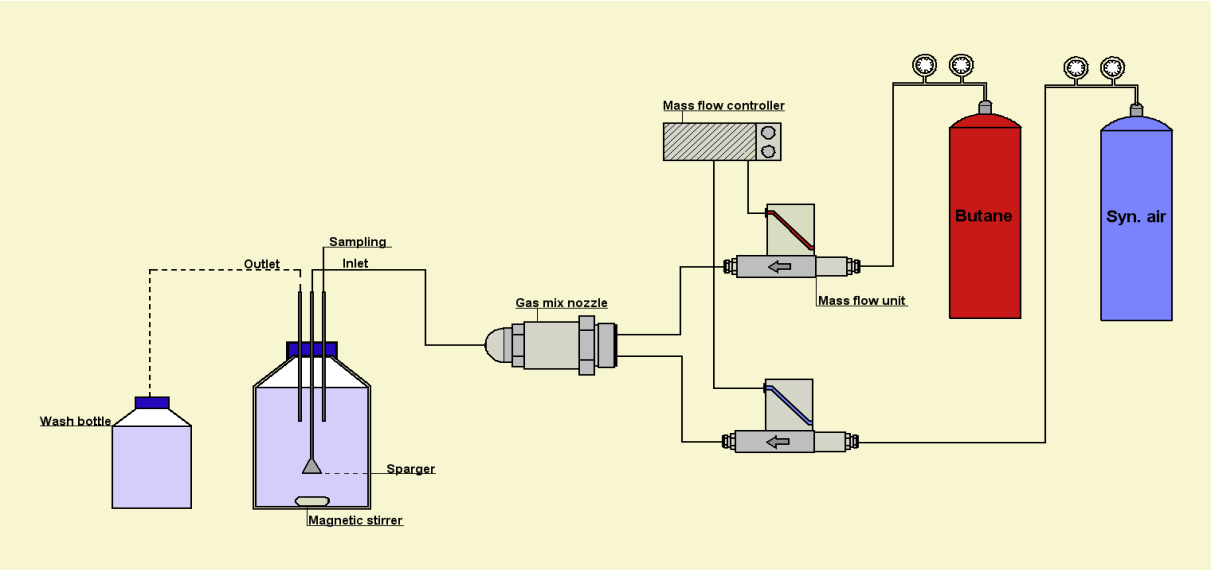

Figure 17: Fermentation assembly for 1-butanol synthesis from *n*-butane.

E. coli resting cells were applied for the bioconversion experiments. A whole cell process in comparison to isolated enzymes offers possibilities for biocatalysis to regenerate cofactors and run enzymatic systems, which are linked to the capability of an effective and continuous regeneration system of the needed cofactors.[179] Whole cells confer a high stability of the monooxygenase by providing a safe cell compartment with optimal surrounding conditions and possess the ability to scavenge reactive oxygen species originating from uncoupling reactions, which could damage the structure and function of the proteins in the cell or even inactivate the biocatalyst.[180]

The exposure of whole cells to 1-butanol over long time periods negatively influenced the total product yields obtained in our experiments. Without implementation of product removal, a total product concentration of 70 % (7.8-8.2 mM) was achieved. A fast and reliable product removal enables constant 1-butanol production by preventing cell damage and cell death due to an accumulation of polar products in the cell membrane.[181, 182] Furthermore the use of wash bottles helps avoiding product loss through evaporation. Also the addition of different glycerol/glucose mixtures as carbon source, reported to have a beneficial effect on cell function and nicotinamide cofactor regeneration, were investigated.[110] Due to the fact that glycerol is known to be a driving force for cofactor regeneration in whole cell-mediated redox biocatalysis,[183] media containing either 0.05-0.3 % glucose, 0.5-2 % glycerol or a

mixture of glucose/glycerol were tested. In the absence of glycerol or glucose butanol concentrations less than 0.5 mM were detected. A mixture of 20 mM glucose and 1 % glycerol was found to be the most efficient carbon source concentration for butanol synthesis. In this case carbon source depletion was not observed. In respect to gas flow, best conversion results could be achieved with a gas flow of 25 L/h and a mixture of 5/95 % (*n*-butane / synthetic air).

3.3.2. *In vivo* synthesis of *n*-butane to 1-butanol at atmospheric pressure

The improved reaction parameters described above were used for biotransformations with the CYP153A6-BMO1-operon and the CYP153A$_{P.\ sp.}$-operon in *E. coli* resting cells. The conversions of gaseous *n*-butane to liquid 1-butanol by a genetically improved CYP153A6-BMO1-operon during the first 4 hours of the reaction was more efficient in eM9 reaction-media (10.7 mM butanol per 30 g_{cww}) than in 100 mM potassium phosphate media (7 mM butanol per 30 g_{cww}). Mainly with the goal to prevent amino acid catabolized repression all experiments were not performed in the fermentation medium eM9Y which contains a nitrogen source (yeast extract). The experiments with the CYP153A$_{P.\ sp.}$-operon results in 9 mM butanol per 30 g_{cww} in eM9 reaction-media after four hours and 5.4 mM butanol per 30 g_{cww} in 100mM potassium phosphate at the same time (Figure 18). CYP153A$_{P.\ sp.}$ is showing a noticeable slower production rate (up to 25 %) compared to CYP153A6-BMO1. From this obtained data, we think that the reaction-media composition impacts the cofactor regeneration system of the whole cell system positively. Furthermore it seems to be likely that adaption effects lead to a longer lacking phase with regard to hydroxylation activity in the potassium phosphate media. Resting cells for biotransformations in 100 mM potassium phosphate medium were grown prior in terrific broth medium which comprise a rich medium and thus might achieve positive overall effects which results in higher product concentrations. The expression of the biocatalysts in the eM9Y media was in comparison to the TB-media ca. 20 % lower for both candidates. Gudiminchi and co-workers reported that lower CYP153A concentration can lead to higher product concentration in respect to octane oxidation experiments.[184] It is conceivable that we obtain similar effects. CYP153A6-BMO1 produced a maximum of 12.1 mM 1-butanol (29 mg 1-butanol per g_{cww} resting cells) after 8 hours in 100 mM potassium phosphate

biotransformation-media. In comparison, the product yield in eM9 reaction-media reached a maximum of 10.3 mM 1-butanol (25 mg 1-butanol per g_{cww} resting cells) after 4 hours reaction time. Thereafter a strong decrease in productivity was detected over time (Figure 18). Experiments using the natural operon of CYP153A$_{P. sp.}$ resulted in maximum product yields of 9 mM 1-butanol in eM9 and 10.4 mM 1-butanol in 100 mM potassium phosphate bioconversion-media, respectively, equivalent to 19.3 mg and 22.2 mg 1-butanol per g_{cww} resting cells. In comparison to CYP153A6-BMO1, CYP153A$_{P. sp.}$ displayed approximately 10 % lower *n*-butane conversion with a ω-regioselectivity of 86 % (90 % ω-regioselectivity of CYP153A6-BMO1).[64] By using CYP153A6-BMO1, we obtained a yield of 0.9 g 1-butanol/L, being similar to the activity reported for an engineered P450 BM3 variant (15 mM with 4 g_{cdw}/L in 4 hours).[140] The latter enzyme is known to hydroxylate propane and higher alkanes primarily at the more energetically favourable subterminal positions (ω-1, ω-2, ω-3),[138, 185] whereas enzymes of the CYP153A subfamily offer preferred ω-regioselectivities. In terms of productivity, conversions in eM9 medium resulted in concentrations of 495 mmol 1-butanol/(g_{cww})/ h for CYP153A6-BMO1 and 315 mmol for CYP153A$_{P. sp.}$, respectively. In contrast, 119 mmol 1-butanol/(g_{cww})/h were obtained with the best engineered P450 BM3 variant under similar media conditions.[140] Another attractive feature of these hydroxylation reactions is that they are very selective and products do not suffer from overoxidation, as no oxidation to butanal or butanoic acid and further reaction to 1,4-butanediol was detected. However, after having monitored the presence of these byproducts *in vitro*, the formation of intermediates cannot be excluded. These byproducts might be utilized by the whole cells as carbon or energy sources.[156]

It has been well defined that in the presence of non-native substrates the mechanisms controlling efficient catalysis in monooxygenase enzymes are disrupted. This causes the formation of reactive oxygen species and thus, rapid enzyme inactivation.[186] For both used catalysts, we determined only a minor impact in apparent monooxygenase concentration after 2 hours reaction time during the reactions with *n*-butane. These values are consistent with data from section 3.4., which shows that during hydroxylations using a CYP153A and fatty acids as substrates the concentration of the biocatalyst is only decreasing up to 20 % after ca. 24 hours. Similar losses in concentration of enzyme 25- 35 % were also published for other P450 reaction longer than 24 hours.[64]

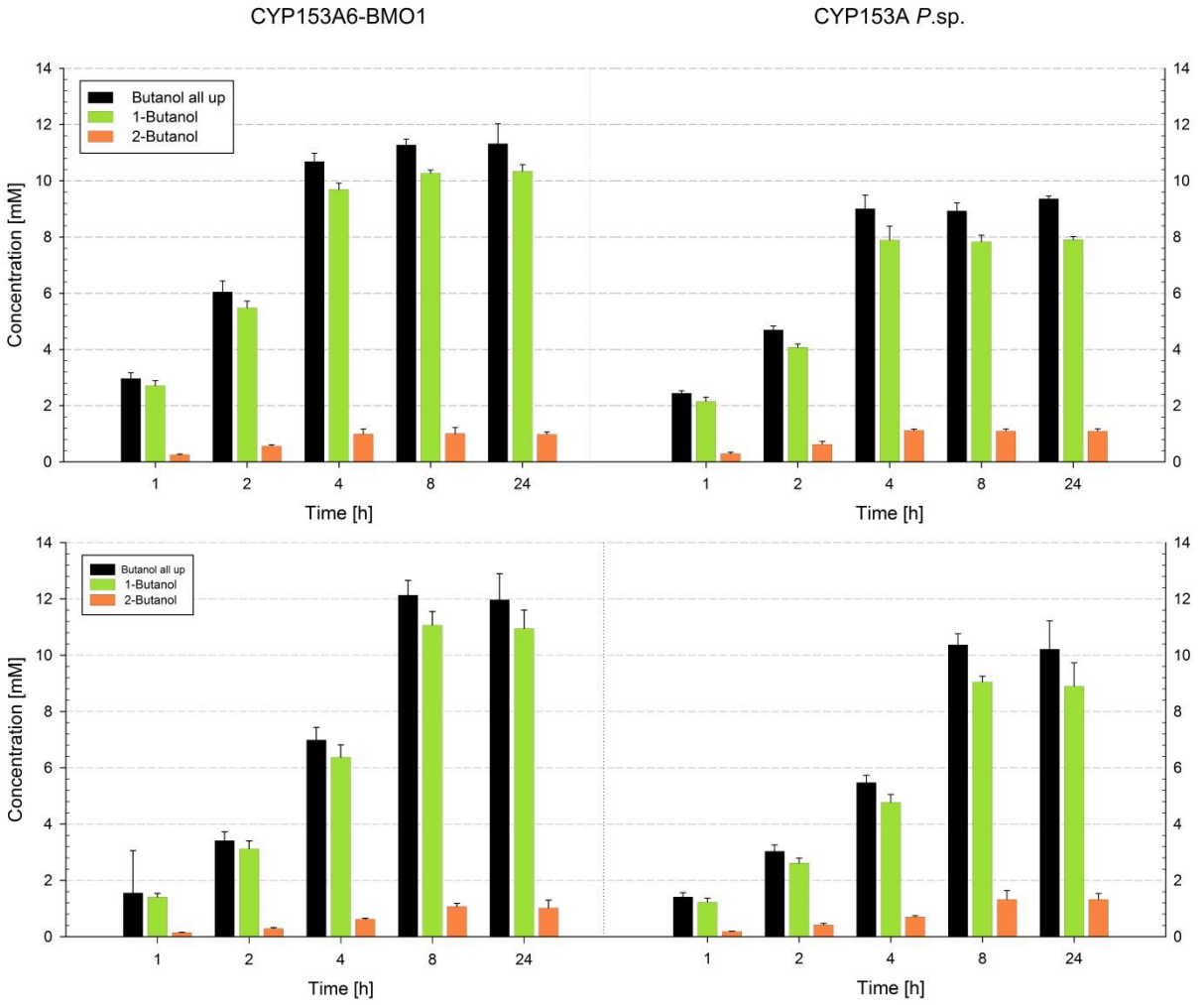

Figure 18: Total butanol synthesis in resting *E. coli* BL21 (DE3) cells with two different enzymatic systems (CYP153A6-BMO1 and CYP153A*P.* sp.).

(A) Biotransformations were performed in eM9 media with glucose/glycerol as carbon source after cultivation in eM9Y. (B) Cells were resuspended in 100 mM potassium phosphate buffer with glucose/glycerol as carbon source after cultivation in TB.

Koch and coworkers created next to the CYP153A6-BMO1 another improved *n*-butane monooxygenases. AlkB-BMO2 (15.7 U/g_{cdw}) showed in comparison to the wild typ (6.1 U/g_{cdw}) a significant higher productivity, but the limitation of overoxidizing the synthesized primary alcohol could not be overcome.[64, 135] The here applied CYP153A6-BMO1 showed a 75 % increased turnover rate of 49 min^{-1} in comparison to the wild typ CYP153A6. Furthermore the selectivity for the terminal position could be improved from 78 % to 89 % of

butanol all up.[64] The variances between the two different *ω*-hydroxylases (CYP153A6-BMO1 and CYP153A$_{P.\ sp.}$), which were investigated at the beginning of the project, were quite small, but in the ongoing work it was decided to focused on CYP153A$_{P.\ sp.}$ as enzymatic construct to further explore an unknown enzyme of the P450 subfamily.

It was reported before that the application of a self-sufficient fusion construct for CYP153A13-red (CYP153A13 –Pfor$_{116B2}$) is beneficial in respect to conversion of alkanes.[162] Kinetic data like coupling efficiency for the hydroxylation of octane to 1-octanol confirm this assumption.[187] As a result of this CYP153A$_{P.\ sp.}$-CPR$_{BM3}$ and CYP153A$_{P.\ sp.(G254A)}$-CPR$_{BM3}$ were established to take advantage of this knowledge (further details in section 3.2.1.). CYP153A$_{P.\ sp.(G254A)}$-CPR$_{BM3}$ concentrations in *E. coli* HMS174 (DE3) reached an average of 60-75 mg/g$_{cdw}$. The G254A variant was expressed in similar levels than the wild type enzyme. When expressing the natural operon of CYP153A6 protein concentrations of 0.5-1 μmol P450/g$_{cdw}$ were reported with IPTG induction as well as auto-induction with a pET-vector system in *E. coli*.[184] These protein yields are higher than for CYP153A$_{P.\ sp.(G254A)}$-CPR$_{BM3}$. In fact the expression for the self-sufficient fusions was performed in a 5L fed-batch process which offers different opportunities to further increase to protein levels.[184] As it is shown in Figure 19 there is a clearly higher amount of butanol all up with CYP153A$_{P.\ sp.(G254A)}$-CPR$_{BM3}$ in comparison to the use of the natural operon (CYP153A$_{P.\ sp.}$ + natural redox partners). Already the new developed self-sufficient fusion constructs show a clear optimization of the productivity *in vivo*. These reactions were performed with 50 g$_{cww}$ *E. coli* resting cells (17-18g$_{cdw}$).

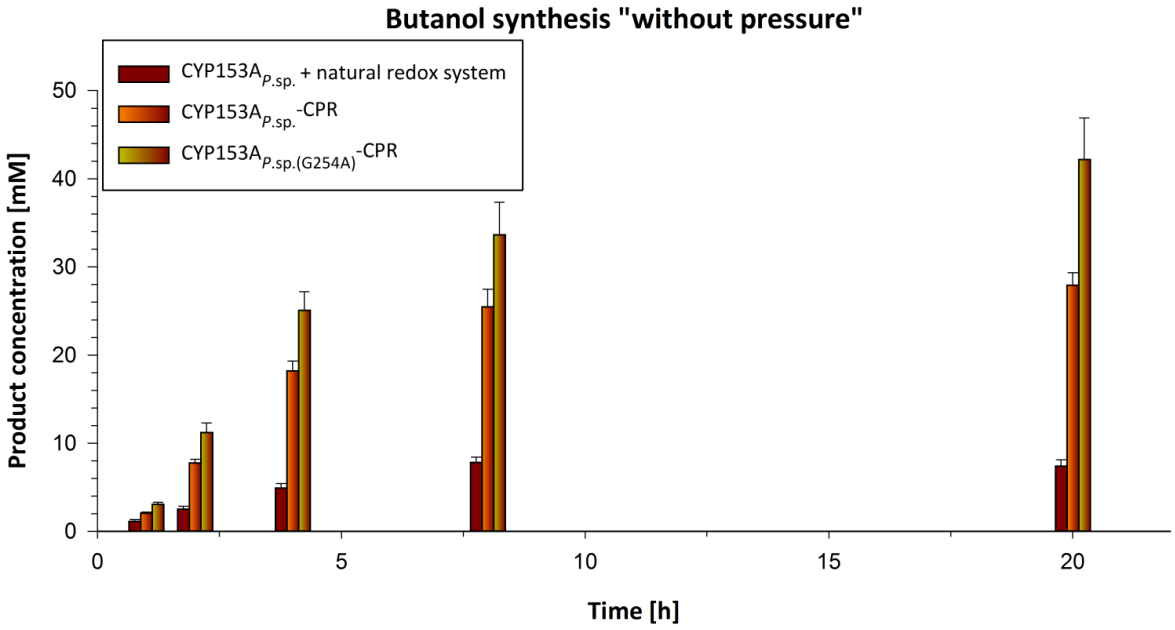

Figure 19: Comparison of butanol production with different CYP153A$_{P. sp.}$ systems.

Total butanol synthesis in resting *E. coli* HMS174 (DE3) cells with three different types of one enzymatic system (CYP153A$_{P. sp.}$ + natural redox partners, CYP153A$_{P. sp.}$-CPR, CYP153A$_{P. sp.(G254A)}$-CPR). Cells were resuspended in 100 mM potassium phosphate reaction media with glucose/glycerol as carbon source after cultivation in TB medium.

3.3.3. 1-Butanol production "under pressure"

The solubility of the inert substrate *n*-butane (61 mg/L at 20 °C) in water or other aqueous biotransformation media is and more than 1000 times lower in comparison to 1-butanol (79 g/L at 20 °C).[188] This fact constitutes a critical parameter for the biocatalytic process. In an attempt to enhance substrate availability, *in vivo* experiments under pressure conditions were performed using a high pressure reactor tank, which cover goal 6 in the objectives section . Elevated *n*-butane pressure means that the overall pressure in the reaction system is above the atmospheric pressure. The overall pressure in the reaction system is caused by *n*-butane and oxygen needed for the hydroxylation reaction. Preferably a mixture of *n*-butane and synthetic air is used to the reaction system with a selected pressure between 1 and 20 bar. The highest product yields could be obtained at 15 bar pressure (Table 11).

Table 11: *In vivo n*-butane oxidation yields of CYP153A$_{P. sp.}$-operon with different pressure conditions

CYP153A$_{P. sp.}$	
Pressure [bar]	Butanol [mM]
Atmospheric pressure	10.4 ± 1.0 (11)
5	13.8 (10)
10	15.9± 2.7 (9)
15	17.8 ± 2.1 (9)
20	12.73 ± 1.3 (9)

Values in parentheses are the percentage of 2-butanol formed during hydroxylations. Only 1- and 2-butanol were analysed in detectable amounts.

The biotransformation in 100 mM potassium phosphate reaction-media increased under pressure (Figure 20) yielded from 42 mM after 20 h to 61 mM butanol all up. This product yield could be obtained by using the self-sufficient monooxygenases (CYP153A$_{P. sp.(G254A)}$-CPR$_{BM3}$) and a cell mass of 50 g$_{cww}$ *E. coli* resting cells (18.7g$_{cdw}$), equivalent to 1.12 g pure biocatalyst. In former studies, Reetz and co-workers showed the application of perfluoro fatty acids in combination with the subterminal hydroxylase P450 BM3 for the oxidation of short alkanes without intensive mutation experiments. Using *n*-butane as substrate 8.4 mM 1-butanol and 2-butanol could be produced. Since the co-substrates, which are responsible for the filled binding pocket and indirect for the oxidation reaction itself, are expensive and only hard to recycle, this method is not applicable in an industrial scale. In this study also a self-build low-pressure reactor was applied, but not a detailed analysis of the pressure parameters could be made.

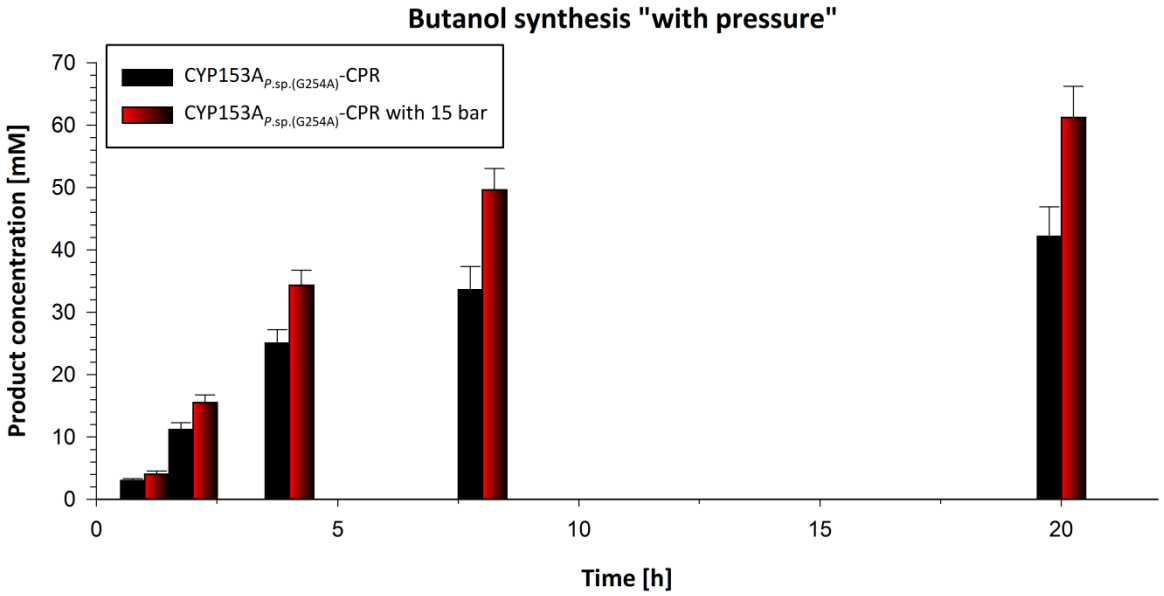

Figure 20: Comparison of butanol synthesis with CYP153A$_{P. sp.(G254A)}$-CPR under pressure.

Total butanol production based on *n*-butane in resting *E. coli* HMS174 (DE3) cells with CYP153A$_{P. sp.(G254A)}$-CPR as enzymatic system. Cells were resuspended in 100 mM potassium phosphate buffer with glucose/glycerol as carbon source after cultivation in TB medium. One reaction was performed under ambient conditions and the other one at 15 bar pressure

Via cell forming unit (CFU) experiments show that the number of living cells in the pressure experiments significantly dropped (> 80 %). This fact limits the possibility to recycle the cells for other biotransformation experiments. Following reasons were investigated, which can lead to inactivation of the cells:

- Loading the autoclave with pressure
- Pressure release after reaction (15 bar)
- High difference in temperatures between aqueous and liquid *n*-butane phase

Table 12: Cell forming unit experiments

Inactivation by pressurizing		Inactivation by (de)pressurizing		Inactivation by pressurizing + liquid *n*-butane	
Pressure	**active cells [%]**	**Pressure / sampling**	**active cells [%]**	**Pressure**	**active cells [%]**
0 bar	**96**	**0 bar**	**97,5**	**0 bar without *n*-butane**	**95**
5 bar	**94**	**15 bar / 1st sample**	**93**	**0 bar with *n*-butane**	**48**
10 bar	**94**	**15 bar / 6th sample**	**82**	**15 bar with *n*-butane**	**22**
20 bar	**92**	-	-	-	-

`-´, not determined

To sum up the results from (Table 12), the greatest negative effect on the resting cells could be obtained by combining pressure (15 bar) and liquid *n*-butane. Nevertheless a negative effect on the whole cells could be also obtained by just pressurizing and covering the cells with a liquid *n*-butane layer in comparison to the sample at atmospheric pressure without liquid *n*-butane. Reason for the low number of CFU using pressure and liquid *n*-butane can be the high difference in temperature between aqueous phase (room temperature) and liquid *n*-butane phase (~ -10 °C). Due the fact that during reaction the mixture was stirred continuously, permanently fresh whole cells were transported to the *n*-butane layer. It is known that whole cells in aqueous system will be damaged by freezing them. The reduced CFU number using only increased pressure can be explained by the fact that especially during pressure release high physical sheering force lead to significant cell damage. This effect can be reduced by lowering the filling and flushing velocity (volume gas per minute).

To overcome the temperature problem the substrate *n*-butane has to be filled into the pressure reactor as gas phase at ambient temperature. In fact the reaction in the pressure tank was a mixed resting cell/cell lysate reaction, according to the cell forming unit experiments. Interestingly biocatalytic activity at low temperature (lesser than -0.5 °C) with a P450 BM3 enzyme to hydroxylate *n*-butane to 2-butanol was published in 2012.[189] These results were achieved with an *in vitro* setup including a cofactor regeneration system similar to the setup described in section 3.1.3. and show that the P450 enzyme remain active even at low temperatures (0 °C).[189] Nonetheless is the loss of the intact cells problematic, because an efficient cofactor regeneration is not ensured and a recycling of the biocatalyst not possible.[179] On account of the fact that *E. coli* cells lack tolerance against higher titer of 1-butanol, which limits growth already significant at concentration of ca. 10 g/L.[190]

In comparison to other synthetic routes – next to the petrochemical ones—like the transfer of the clostridial 1-butanol pathway into *E. coli* (anaerobic process) which achieved ca. 30 g/L butanol or the reversal of the β-oxidation which end up in ca. 14 g/L butanol the gained 4 g/L butanol with the used CYP153A is significantly lower.[43, 44] However, it should be emphasized that none of the other pathways is using directly *n*-butane as substrate, moreover they are based on sugar compounds. Therefore these examples show in contrast to the *n*-butane to 1-butanol process a distinct decreased atom economy. An efficient electron transfer by an alternative system like an electrode surface could offer another interesting opportunity to make this biotransformation process industrially applicable.[191]

3.4. Synthesis of *ω*-hydroxy dodecanoic acid

3.4.1. *In vivo* synthesis of *ω*-hydroxy dodecanoic acid in a small scale bioreactor

ω-Hydroxy dodecanoic acid (ω-OHC12) represents a product of economic interest, because it can be used as building block for polymer synthesis as described in section 1.2.2 or reference II. The work undertaken in this section covers goal 7 of this Ph.D. thesis.

Here it is noteworthy mentioning that significant advances on the bio-based production of ω-hydroxy fatty acids have recently been accomplished. Gross and coworkers reported an engineered *Candida tropicalis* strain able to produce 174 g/L ω-hydroxy tetradecanoic (ω-OHC14) from 200 g/L tetradecanoic acid methyl ester (C14-FAME) in 148 h, the highest yield reported in the literature so far.[73] A work-intensive strain optimization approach, which included the elimination of 16 genes (i.e., native CYP52 enzymes, alcohol oxidases and alcohol dehydrogenases), was the basis of the reported yeast-based bioconversion experiments.[73] A disadvantage concerning the *Candida* strain utilized in the reported study is its classification as a pathogen, which makes it difficult to transfer this technology into the industry (at least in Europe), due to stringent regulations regarding S2 microorganisms.[192] Another relevant work consist of the use of the selective bacterial ω-hydroxylase system AlkBGT towards fatty acid methyl esters, with reported rates of 104 Ug_{CDW}^{-1} for nonanoic acid methyl ester.[147] However, the substrate spectrum of the AlkBGT system is not broad, as it only includes fatty acids with a chain length ranging from C_5 to C_{12}.[125, 147] Furthermore, overoxidation to the corresponding α,ω-diacids seems to be likely, since these products have been reported as the final products of AlkB-mediated oxidation of alkanes of similar size.[135] For the selective ω-hydroxylation of industrial relevant products longer than 12 carbon atoms like dodecanoic acid (C12-FA), an alternative biocatalyst in a bacterial host strain could help to tackle the issues indicated above.

The project stage "synthesis of ω-hydroxy fatty acids" has been carried out in a gradual manner. After the detailed *in vitro* substrate characterization of three CYP153A enzymes and rational design of the best candidate (described in Sections 3.1. and 3.2.), the results were transferred into an *in vivo* system. In this context, the established functional self-sufficient fusion constructs described in section 3.2.1. were first applied in shake flask experiments

with resting cells (S. Honda Malca, Ph.D. thesis). It was demonstrated therein that *P. putida* KT2440 and *E. coli* BL21 (DE3) harboring CYP153A$_{M.\ aq.(G307A)}$–CPR$_{BM3}$ produce around 0.1 g/L and 0.5 g/L ω-OHC12 from 1g/L C12-FA, respectively (Reference III). In addition, it was observed that without any strain engineering efforts, *E. coli* performed as a more suitable production host in terms of total product yields. At this point, bioconversions were scaled up to 1 L stirred-tank bioreactors using recombinant *E. coli* HMS74 (DE3). This setup offered the opportunity to measure different biotransformation parameters in parallel.

Transferring the experimental conditions used in shake flasks to bioreactors was not a trivial task, mainly because of concerns related to substrate solubility and foaming during the bioprocess. Producing higher amounts of ω-OHC12 implied the use of a significantly higher concentration of the fatty acid substrate. In contrast with the 1 g/L C12-FA used in shake flasks, at least 5 - 10 g/L C12-FA were utilized in the bioreactors. Foaming is related to aeration rates and the pH of the fermentation medium. Because strong foaming leads to the loss of the fermentation medium and disturbs the bioprocess, pH regulation by the addition of a base was switched off. Under these conditions, biotransformations of 10 g/L C12-FA yielded 1.2 g/L of ω-OHC12 acid after 30 hours (Figure 21). From the total products, 92 % represented the corresponding ω-OHFA, 1 %, the (ω-1)-OHFA product, and 7 %, the corresponding α,ω-DCA.

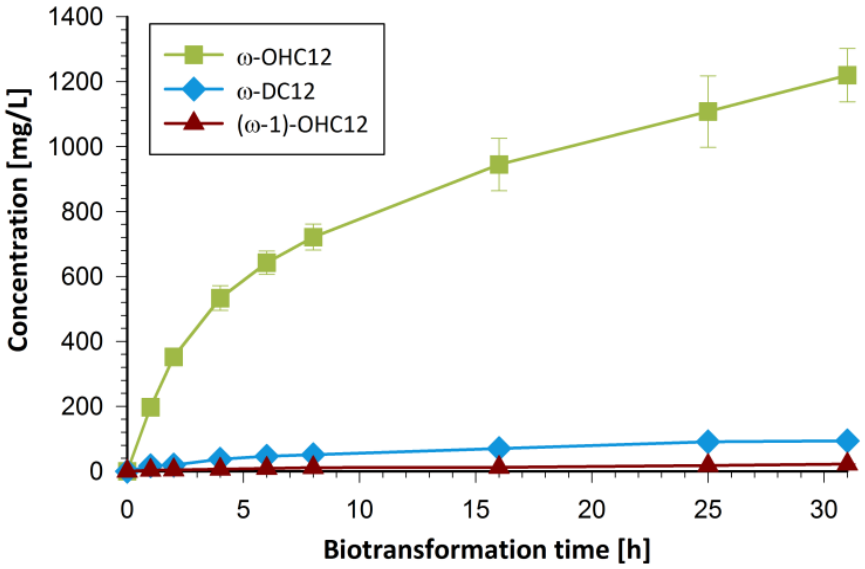

Figure 21: Whole cell biotransformations of dodecanoic acid with resting *E. coli* cells in a 1 L stirred-tank bioreactor. Recombinant *E. coli* HMS174 (DE3) strains contained pET28a(+)-CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$. Abbreviations: (ω-1)-OHC12, 11-hydroxy dodecanoic acid; ω-OHC12, 12-hydroxy dodecanoic acid; α,ω-DC12, 1,12-dodecanedioic acid.

To address the operational difficulties observed in the previous biotransformation setup, several changes were made in the next experimental stage: (1) Use of C12-FAME as substrate in a two-phase system. This configuration offers, on one hand, the opportunity to constantly extract the formed hydroxylated products – with toxic and inhibitory effects – into the organic layer and, on the other hand, the advantage of reducing foam formation. (2) Coexpression of the transport system AlkL to optimize the substrate transfer rate into the microbial cell. It has been described before that this protein can significantly increase the transport of hydrophobic aliphatic substrates across the membrane.[193] These two improvements led to obtain a yield of 4.0 g/L ω-OHC12 acid from C12-FAME after 24 hours (Figure 22). The ω-regioselectivity (including ω-OHC12 and α,ω-DC12) of the system was higher than 98 %. More than 91 % of the total hydroxylated product consisted of the corresponding ω-OHFA.

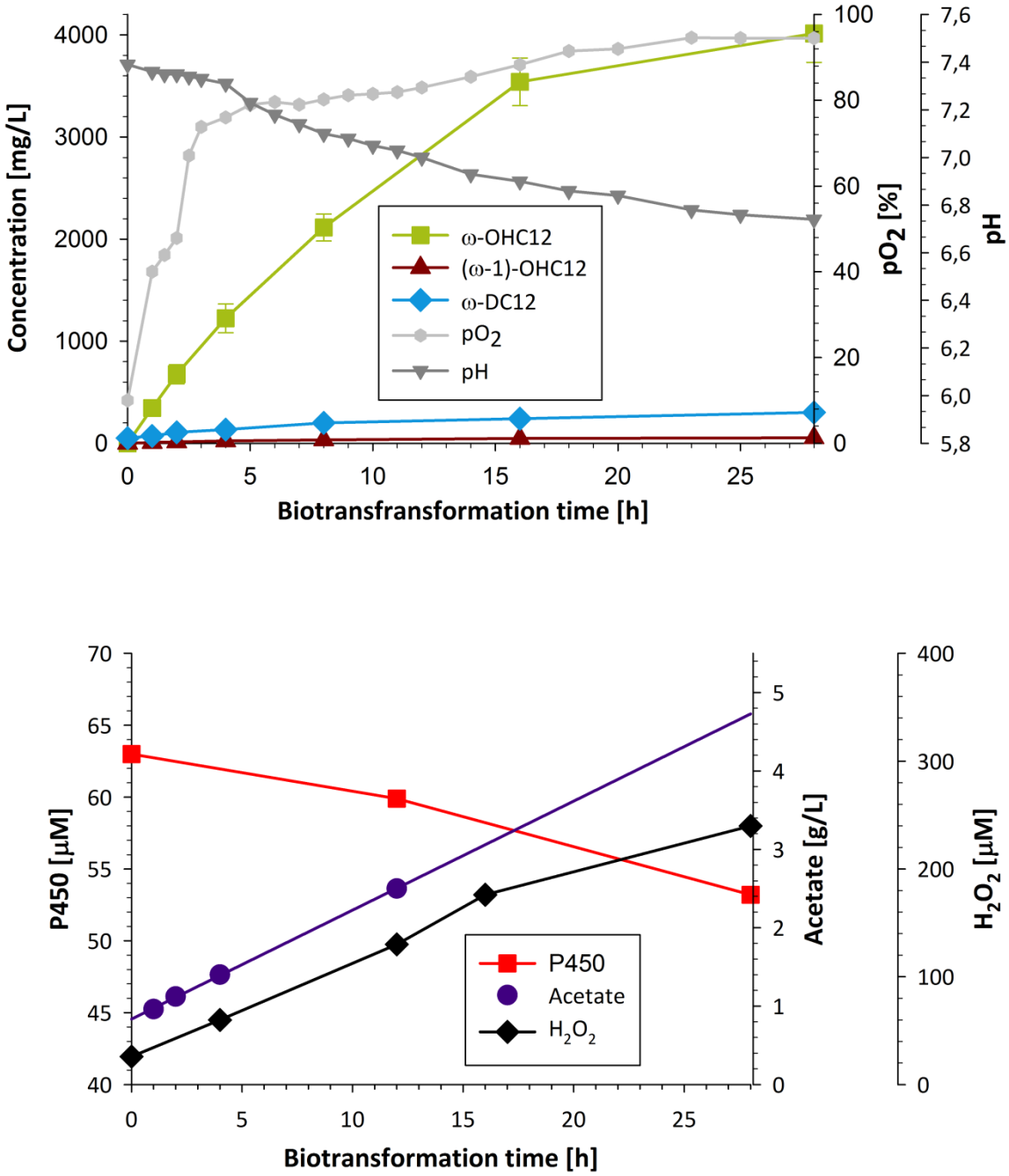

Figure 22: Whole cell biotransformations of dodecanoic acid methyl ester with resting cells in a 1 L stirred-tank bioreactor (above). Acetate, hydrogen peroxide and P450 concentrations (below). Recombinant *E. coli* HMS174 (DE3) strains contained pET28a(+)-CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$. Abbreviations: (ω-1)-OHC12, 11-hydroxy dodecanoic acid; ω-OHC12, 12-hydroxy dodecanoic acid; α,ω-DC12, 1,12-dodecanedioic acid; pO_2, dissolved oxygen; P450, P450 concentration in 1 g_{cdw}.

Permanent neutralization of the pH has been shown to be required for optimal yields in the production of hydroxylated fatty acids during P450-based processes.[194] In addition, optimal aeration rates are essential to ensure efficient NADPH regeneration in the resting cells.[93] Due to the technical limitations of the used bioreactor, no further improvements could be applied to overcome these issues. However, in a following scaling-up step it should be considered that different technical solutions are possible.[195, 196] [197] [180]

Similarly to the *in vivo* bioconversions of C12-FA, ω-OHC12 formation rates decreased over time (Figure 22). However, the product plateau was reached with C12-FAME as substrate after 16 h of biotransformation, i.e., 8 h later than that observed in the experiments with C12-FA. This might be attributed to the two-phase system configuration, as the permanent extraction of the intermediates or products should minimize enzyme inhibition or cell toxicity. Despite this substantial advantage, accumulation of hydrogen peroxide and acetate are still limitations during the bioprocess. Hydrogen peroxide reached a concentration of up to 240 μM after 28 h. Hydrogen peroxide originates from oxidative stress and as a side product from electron uncoupling during the P450 catalytic cycle.[198] Even though *E. coli* can tolerate small hydrogen peroxide concentrations (50 μM) owing to its DNA repair machinery,[199] higher hydrogen peroxide titers could lead to the loss of P450 activity (10 - 20 %) during biotransformations. P450s generally lose their essential heme group when they are exposed to similar hydrogen peroxide concentrations.[200] Another explanation for the detected product plateau is the high concentration of formed acetate (2.2 g/L after 12 hours, Figure 22). It is known that acetate concentrations higher than 2.4 g/L can destabilize inner cellular proteins and lead to a decrease of biomass.[201]

Although our prokaryotic system produced 7-fold lower ω-OHFA yields compared to the highest reported in the literature (28 g/L ω-hydroxy tetradecenoic acid in 24 h by an engineered eukaryotic strain),[73] it should be considered that the recombinant *E. coli* strain used in this work underwent neither metabolic engineering nor solvent-adaptation. Furthermore, a more accurate comparison with the previous work is difficult to perform, since detailed information on the applied cell biomass and CYP52A expression levels are missing.[73] The measured product formation rate after 2 hours reaction time was 17 mg/L of ω-OHC12 per gram cell dry weight. With a scenario of a constant product formation rate, it is possible to estimate a maximum product yield of 7.5 g/L in 24 hours (Figure 23). On one

hand, it can be assumed that yeast CYP52A enzymes have higher activities or stabilities than bacterial CYP153A enzymes. On the other hand, there are further implications intrinsic to the eukaryotic system used. Microorganisms with a natural ω-oxidation capability like the reported *Candida tropicalis* strain should be more robust, as they have evolved to adapt themselves to grow on alkanes and fatty acids, making them more suitable for a reaction process. Other genetic and metabolic factors might be related to the higher efficiency observed in the yeast production host as well. In this regard, it is remarkable to see that nearly all industrial P450 processes are based on eukaryotic systems, especially fungal ones (see section 1.3.5.).

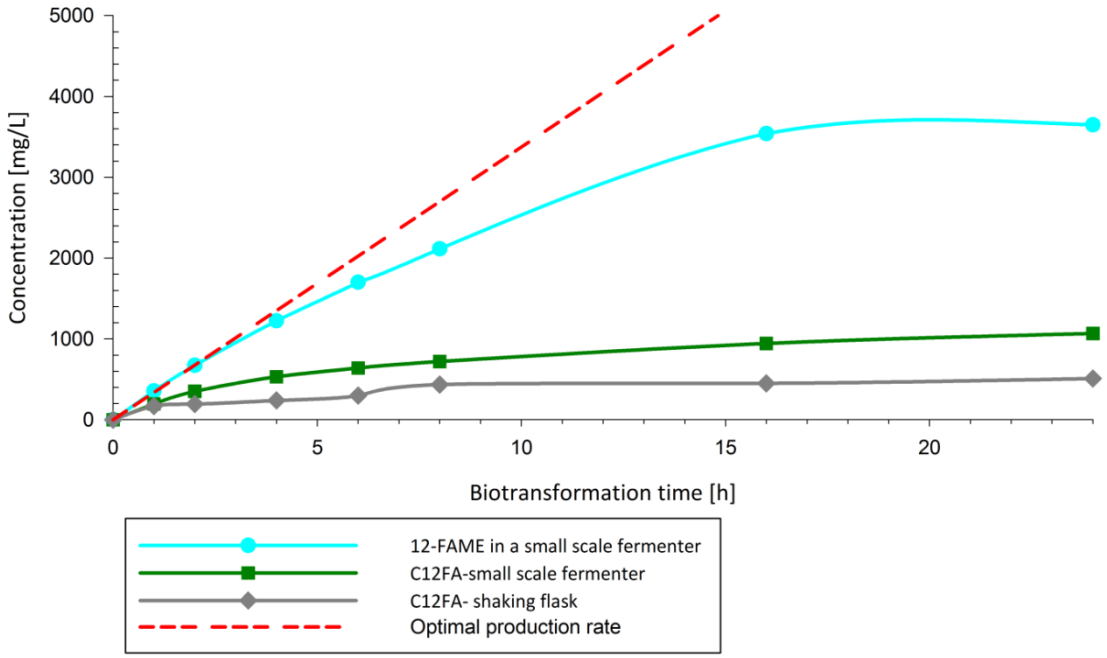

Figure 23: Whole cell biotransformations of dodecanoic acid (C12-FA) or dodecanoic acid methyl ester (C12-FAME) by a CYP153A-based system in shake flasks or in a small scale fermenter. Recombinant *E. coli* strains containing pCola Duet1-CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ + AlkL were used for the conversions with C12-FAME. pJOE-CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ were used for the conversions with C12-FA in shaking flasks. Recombinant *E. coli* strains contained pET28a(+)-CYP153A$_{M.\ aq.(G307A)}$-CPR$_{BM3}$ were used for the conversions with C12-FA in a small scale fermenter.

3.4.2. Opportunities for the optimization of ω-OHC12 production

The results presented in the *in vivo* biotransformation experiments have been assessed in more detail in order to identify different limitations and to suggest what could be a target of further research in this field. A list of goals is indicated below and the possible strategies to achieve them are presented in Figure 24.

- To increase the turnover number of the used CYP153A
- To increase the coupling efficiency of the applied self-sufficient fusion construct
- To reduce H_2O_2 and acetate levels
- To minimize product overoxidation
- To minimize undesired substrate and product depletion

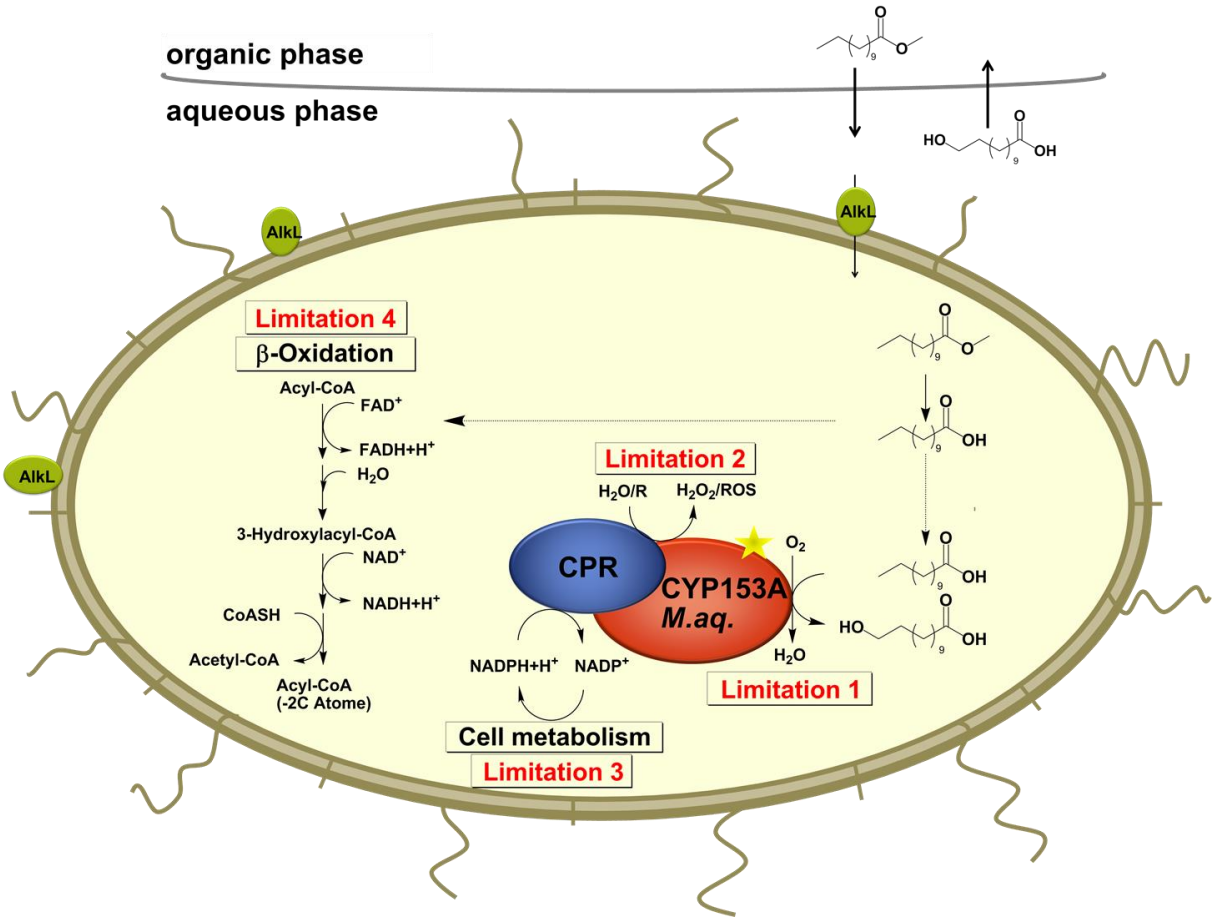

Figure 24: Limitations in ω-hydroxy fatty acid production by CYP153A in *E. coli* in a two phase system. (1) CYP153A improvements *via* engineering approaches to increase the activity and stability of the biocatalyst. (2) Optimization of the coupling efficiency of the self-sufficient fusion construct and reduction of the formation of reactive oxygen species (ROS) by co-expressing ROS-scavenging enzymes (e.g., catalase). (3a) Ensurance of sufficient supply of cofactor with an additional NAD(P)H regeneration system or increase of the flux of NAD(P)H-producing reactions. (3b) Restriction in the production of harmful acetate formation by the selection of a suitable C-source feeding strategy or by strain engineering approaches. (3c) Elimination of oxidoreductases (alcohol dehydrogenases/oxidases and aldehyde dehydrogenases) responsible for the overoxidation of the formed ω-hydroxylated products to e.g., α,ω-diacids. (4) Elimination of β-oxidation genes to avoid substrate and product depletion (this figure was adapted from C. Grant et al., 2012).[202]

4. Conclusion and Outlook

4.1. *In vitro* characterization and optimization of CYP153A enzymes

In this thesis, the first detailed substrate characterization of three different CYP153A enzymes from *Polaromonas* sp. JS666, *Marinobacter aquaeolei* VT8 and *Mycobacterium marinum* M. (CYP153A$_{P. sp.}$, CYP153A$_{M. aq.}$ and CYP153A16) with different aliphatic substrates in combination with a product formation analysis has been reported (reference I and II). The characterization has been made based on *in vitro* biotransformation experiments with the artificial redox system CamA and CamB from *P. putida*, which were expressed in soluble form in *E. coli* like the investigated enzymes. It has to be considered that such artificial system can be used to determine the substrate spectra of the enzymes, but higher turnover numbers could be only expected with the natural redox partners.[162]

The substrate conversion levels and formed products of different alkanes (C_5-C_{12}), primary alcohols (C_6-C_{12}) and fatty acids ($C_{8:0}$-$C_{20:0}$ and 9(*Z*)/9(*E*)-$C_{14:1}$-$C_{18:1}$) were investigated. It was reported that CYP153A enzymes were active towards the aliphatic substrates, which were hydroxylated mainly to the ω-OH-product. As a result of the *in vitro* biotransformation experiments and sequence analyses, it was possible to identify two groups in the CYP153A subfamily. A monooxygenase with predominantly alkane ω-hydroxylase was CYP153A$_{P. sp.}$ This enzyme shares high protein sequence similarity with CYP153A6, which has never been reported to ω-hydroxylate primary alcohols nor fatty acids.[107] CYP153A$_{P. sp.}$ exhibited activity and high ω-regioselectivity towards C_5-C_{12} alkanes, with a preference for octane. This biocatalyst can be used as a suitable candidate for the selective ω-hydroxylation of alkanes.

Empirical data and protein similarity analyses also indicated that CYP153A$_{M. aq.}$, CYP153A16, CYP153A13a and CYP153A from *Acinetobacter* sp. OC4 show both ω-hydroxylase and α,ω-dihydroxylase activity and can be classified into a second group.[106, 153] It could be shown that CYP153A$_{M. aq.}$ and CYP153A16 show substrate conversion levels of up to 93% towards fatty acids. Due to its higher expression levels, stability and broader substrate range, CYP153A$_{M .aq.}$ is the most promising candidate to terminally hydroxylate medium- to

long-chained fatty acids, including $C_{8:0}$-$C_{20:0}$ saturated and $C_{14:1}$-$C_{18:1}$ monounsaturated substrates.

One of the limitations in using P450 enzymes is electron transport and coupling efficiency. Since the functional expression of the natural redox partners of CYP153A$_{M.\ aq.}$ in *E. coli* is problematic, self-sufficient CYP153A-reductase fusion proteins were designed and applied in this study. In order to compare different electron transfer systems, the FAD/[2Fe 2S]-containing reductase domain (Pfor$_{116B3}$) of CYP116B3 and the FAD/FMN-containing reductase domain (CPR$_{BM3}$) of P450 BM3 were fused to CYP153A$_{M.\ aq.}$. The latter fusion construct showed a higher coupling efficiency and a higher catalytic activity than the former. The influence of two different heme-reductase linker regions in CYP153A$_{M.\ aq.}$-CPR$_{BM3}$ on enzyme activity was also evaluated, but no significant difference was found. For the first time a class I (ferredoxin-interacting) heme domain fused to a flavin reductase was demonstrated to be functional. This finding shows the versatility of the CPR system and encourages its application to increase the coupling efficiencies and, consequently, the oxidation activities of other class I CYPs.

To further optimize the coupling efficiency of the fusion constructs and get a deeper understanding of the factors determining optimal heme-reductase interactions, different strategies could be applied: (1) Diversification of the polypeptide length, which is added to the natural linker region, is proven to influence the activity and coupling efficiency of a man-made fusion chimera.[160, 203] More possibilities of movement increase the chance to bind in the required conformation. (2) Mutations, especially in the enzyme pocket, can help to stabilize the substrate and increase the binding affinity, preventing uncoupling effects.[125, 139, 204] (3) Identification of interaction structures between the reductase and the CYP. Modeling studies can play a crucial role in the understanding of protein-protein interactions. Using docking techniques, Wade and coworkers identified hotspots involved in the interaction of P450cam and CamB.[205, 206] However, large conformational changes in both heme and reductase domains during catalysis makes it difficult to find critical positions for these interactions.[207]

Rational design studies on CYP153A$_{M.\ aq.}$ resulted in the identification of a variant (G307A) that showed higher activity and shifted substrate range towards shorter substrates. Here the

equivalent position in CYP153A*P. sp.*, G254, was subjected to the same amino acid substitution, resulting in an enzyme variant able to convert short alkanes like *n*-butane to ω oxofunctionalized products. Furthermore, mutations on a second active site hotspot, V306 in CYP153A*M. aq.* and V253 in CYP153A*P. sp.*, influenced the terminal hydroxylation activities of the enzymes, though in a negative way. A further achievement of this study was the creation of a SeSaM mutant library of CYP153A*P. sp.* and the comparison of different selection and screening strategies to identify mutants with a higher ω-hydroxylation activity towards *n* butane. Comparative cell growth was applied to identify two CYP153A*P. sp.* variants with mutations on residues Ala184 and Thr300 that exhibited increased ω-hydroxylation activity towards *n*-butane; however, the reason of their positive effects cannot be explained yet. It seems thus noteworthy to establish a small focused mutant library using these positions to understand their influence on enzyme activity. In addition to the hotspots randomly identified in section 3.2.6, another position, L303 in CYP153A*M. aq.* and L250 in CYP153A*P. sp.*, was proposed to be a target for substitution in order to modify the substrate specificity of the enzymes.

Given that hydrocarbons are mainly obtained from fossil fuels, an alternative biosynthetic route to obtain these compounds from inexpensive renewable raw materials has been described. Schirmer and coworkers explained the use of an acyl-ACP reductase and an aldehyde decarbonylase to synthesize alkanes from fatty acids in living organisms.[208] Renewable feedstocks are normally the source of fatty acids of an even number of carbon atoms. These fatty acids can be reduced to the corresponding even-numbered primary alcohols *via* an acyl-ACP reductase and an aldehyde reductase. However, an odd-numbered primary alcohol could be obtained from an even-numbered fatty acid by adding a CYP153A enzyme downstream the aldehyde decarbonylase (Figure 25). This pathway would give the opportunity to synthesize commercially valuable primary alcohols that are traditionally obtained from non-renewable resources.

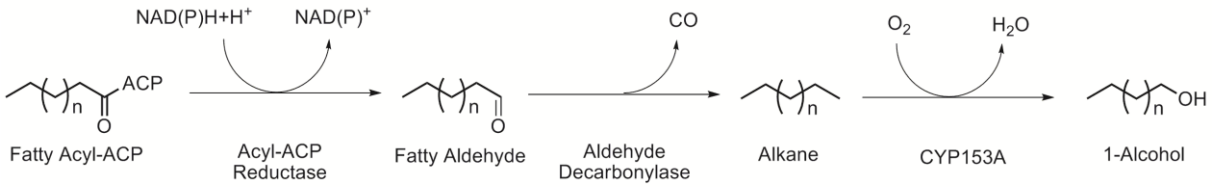

Figure 25: Biocatalytic pathway to produce renewable primary alcohols based on fatty acids.

4.2. Synthesis of 1-butanol

One of the aims of this work was to evaluate an efficient route to produce 1-butanol from *n*-butane. In this context, it was necessary to identify and further improve an enzymatic system able to catalyze the selective oxofunctionalization of the terminal non-activated C-H bond of gaseous short-chained alkanes. A group of monooxygenases, comprised by MMOs, BMOs, AlkB and CYP153A enzymes, were included in the list of potential suitable biocatalysts. After establishing a reproducible *in vivo* biotransformation system to compare different *n*-butane oxidizing strains, CYP153A$_{P.\ sp.}$ was selected on the basis of its solubility, high expression levels in *E. coli*, high ω-regioselectivity and suitable substrate spectrum which included alkanes of short to medium chain length. Following the substrate characterization of the enzyme candidate, efforts were oriented to optimize the conditions of a whole cell-based bioprocess. Improvements on reaction media, carbon sources, gas flow, *n*-butane/air ratios and product removal were performed in this study.

Due to the fact that low turnover numbers are reported for CYP153A enzymes, one of the main challenges faced in this work was to increase the hydroxylation activity of CYP153A$_{P.\ sp.}$. A combination of protein engineering techniques already described in section 4.1, including rational design and the construction of fusion constructs, led to obtain a more efficient biocatalyst. Resting *E. coli* cells containing the improved monooxygenase were tested in biotransformations under different conditions. In order to increase the amount of *n*-butane gas in the reaction medium, a stirred tank pressure reactor system was assembled. After the final optimization step, a product concentration of ca. 4 g/L could be achieved after

24 h with 18.7 g_{cdw} under a pressure of 15 bar with the CYP153A$_{P.\ sp.(G254A)}$–CPR construct. This is the highest titer reported for an enzymatic system used in the direct hydroxylation of *n*-butane to 1-butanol. Nevertheless, the system is only viable for 16 - 24 h. The exposure to cold liquid *n*-butane caused cell death throughout the process, which severely limits the recycling capability of the biocatalyst. The toxicity of the formed 1-butanol product is another factor responsible for this effect.[209]

It is evident that further improvements on the biocatalytic efficiency of the production host should consider strategies to tackle substrate and product toxicity. Solvent tolerance is still a longstanding problem in biotechnology, even though certain prokaryotes are known to possess the machinery to withstand relatively high concentrations of organic solvents. Product toxicity can be minimized by the overexpression of proteins involved in efflux pumps.[181, 210] However, this represents only a partial solution, since 1-butanol is not only problematic within the cell, but it also damages the cell membrane when it is present in the surrounding media. Classical mutagenesis techniques based on UV-light or chemical mutagens in combination with stepwise enrichment cultures of tolerant cells in a chemostat-bioreactor have been applied to increase the solvent tolerance of different strains.[211, 212] Furthermore, transcriptional engineering *via* cyclic AMP has also led to increased 1-butanol tolerance.[190] A different reaction setup, which offers the addition of gaseous butane under pressure, can help to overcome this problem. Another interesting advantage of the use of CYP153A enzymes in butane hydroxylation reactions is the subsequent ω-oxidation of 1-butanol to 1,4-butanediol. CYP153A$_{M.\ aq.(G307A)}$-CPR could be an interesting enzyme for this application.

4.3. Production of ω-hydroxy fatty acids

Hydroxylation reactions with the optimized fusion variant CYP153A$_{M.\ aq.(G307)}$-CPR$_{BM3}$ were applied in a first bioprocess step in a small scale (1L) reactor to investigate biotransformation parameters and synthesize ω-OHC12. The experiments were made in *E. coli* as production host without further strain optimizations. After a reaction time of 30 h, a product concentration of 1.2 g/L could be obtained with 15.1 g_{cdw} from 10 g/L C12-FA as substrate. Due to saponification problems in combination with a strong foam formation, C12-FAME

was determined to be a more suitable substrate compared to C12-FA. As it was reported that substrate import through the outer membrane lipopolysaccharide layer into the cytosol of *E. coli* is problematic, an additional importer was necessary. To increase substrate uptake by the cells, the outer membrane transporter AlkL from *P. putida* Gpo1 was co-expressed. Another process-related optimization step was the control of dissolved oxygen in the reaction media, which is essential not only for the hydroxylation process itself, but for an efficient cofactor regeneration process in the cell. The successful production of industrially relevant ω-OHC12 in a g/L scale (4.0 g/L, 28 h and 18.2 g_{cdw}) was reported in this study. In contrast to classical petrochemical routes, a new biocatalytic pathway based on renewable substrates was reported.

Different limitations which influence the yields of ω-hydroxy fatty acids have been discussed in section 3.4. A tighter control of metabolic pathways responsible for unwanted substrate or product depletion and the prevention of acetate and hydrogen peroxide formation seem to be essential for higher product yields. In a next step, the flexibility of the hydroxylase system, which was already proven *in vitro*, (section 3.1.) should be tested *in vivo.* This implies using fatty acids of different chain lengths such as hexanoic acid, nonanoic acid or oleic acid for the synthesis of other commercially attractive ω-OH-fatty acids. However, short-chained fatty acids like hexanoic acid are less hydrophobic and more toxic to the cells than the C12 fatty acid. Long-chained fatty acids like oleic acid are less harmful than the C12 fatty acid, but expected to be less accessible to the cells due to their low solubility. In addition, certain fatty acids could be preferred as carbon or energy sources over others by the cells. Therefore, the type of strategy will depend on the fatty acid substrate used. In this sense, it is important to evaluate when it is necessary to work with β-oxidation knock-out strains, how to improve substrate solubility in the reaction media and to determine if the AlkL transport system still contributes to substrate uptake.

5. Reference

[1] R. Wolfenden, M. J. Snider, *Acc Chem Res* **2001**, *34*, 938.

[2] I. Nobeli, A. D. Favia, J. M. Thornton, *Nat Biotechnol* **2009**, *27*, 157.

[3] A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258.

[4] N. J. Turner, *Nat Chem Biol* **2009**, *5*, 567.

[5] E. R. Stanley Falkow, Karl-Heinz Schleifer, Erkko Stackerbrandt, *The Prokaryotes, Vol. 3*, **2006**.

[6] J. Takamine, US 525,823, **1894**.

[7] L. Rosenthaler, *Biochemische Zeitschrift* **1908**, *14*, 238.

[8] C. Neuberg, J. Hirsch, *Biochemische Zeitschrift* **1921**, *115*, 282.

[9] D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister, H. M. Leigh, *Journal of the American Chemical Society* **1952**, *74*, 5933.

[10] E. Smulders, P. Krings, H. Verbeek, *Tenside Surfactants Detergents* **1997**, *34*, 386.

[11] A. M. Yoshimasa Fujita, Isao Kawakami, Tadashi Hishida, Akira Kamata, Yusuke Maeda, *U.S. 4078970*, **1978**.

[12] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Kesseler, R. Sturmer, T. Zelinski, *Angew Chem Int Ed Engl* **2004**, *43*, 788.

[13] C. E. Nakamura, G. M. Whited, *Curr Opin Biotechnol* **2003**, *14*, 454.

[14] J. C. Warner, *Abstracts of Papers of the American Chemical Society* **2011**, *241*.

[15] J. C. Warner, *Abstracts of Papers of the American Chemical Society* **2011**, *242*.

[16] B. Kamm, P. R. Gruber, M. Kamm, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**.

[17] K. Weissermel, H.-J. Arpe, *Industrial organic chemistry*, Wiley-VCH, Weinheim, **2003**.

[18] K. Sutherland, *Filtration & Separation* **2008**, *45*, 12.

[19] R. D. Schmid, *Taschenatlas der Biotechnologie und Gentechnik, Vol. 2. Aufl.*, Wiley-VCH, Weinheim, **2006**.

[20] S. Serra, C. Fuganti, E. Brenna, *Trends Biotechnol* **2005**, *23*, 193.

[21] A. A. Desai, *Angew Chem Int Ed Engl* **2011**, *50*, 1974.

[22] A. Kiener, EP 0442430, **1991**.

[23] G. Alfke, W. W. Irion, O. S. Neuwirth, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**.

[24] H. Kapp, K-H. Rieser, J. van Harten, S. Nuijten, **2012**. http://www.basf.com/group/pressemitteilungen/P-12-444. Accesion date: 2.2.2013,

[25] B. M. Nestl, B. A. Nebel, B. Hauer, *Curr Opin Chem Biol* **2011**, *15*, 187.

[26] E. M. Green, *Curr Opin Biotechnol* **2011**, *22*, 337.

[27] S. Y. Lee, J. H. Park, S. H. Jang, L. K. Nielsen, J. Kim, K. S. Jung, *Biotechnol Bioeng* **2008**, *101*, 209.

[28] R. Cascone, *Chem Eng Prog* **2008**, S4.

[29] P. Durre, *Ann N Y Acad Sci* **2008**, *1125*, 353.

[30] J. Falbe, H. Tummes, H. D. Hahn, *Angewandte Chemie-International Edition* **1970**, *9*, 169.

[31] H. Zimmermann, R. Walzl, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**.

[32] C. Weizmann, US 1,315,585, **1919**.

[33] D. T. Jones, D. R. Woods, *Microbiol Rev* **1986**, *50*, 484.

[34] H. Bahl, P. Dürre, in *Biotechnology Set*, Wiley-VCH Verlag GmbH, **2008**, pp. 285.

[35] P. Dürre, *Curr Opin Biotechnol* **2011**, *22*, 331.

[36] L. M. Harris, L. Blank, R. P. Desai, N. E. Welker, E. T. Papoutsakis, *J Ind Microbiol Biotechnol* **2001**, *27*, 322.

[37] M. Reardon, V. Corr, **2012**. http://www.bp.com/genericarticle.do?categoryId=9024973&contentId=7046879. Accesion date: 2.2.2013, **2008**.

[38] T. Lutke-Eversloh, H. Bahl, *Curr Opin Biotechnol* **2011**, *22*, 634.

[39] E. Waltz, *Nat Biotechnol* **2012**, *30*, 480.

[40] Y. Ni, Z. Sun, *Appl Microbiol Biotechnol* **2009**, *83*, 415.

[41] S. Atsumi, T. Hanai, J. C. Liao, *Nature* **2008**, *451*, 86.

[42] C. R. Shen, J. C. Liao, *Metab Eng* **2008**, *10*, 312.

[43] C. Dellomonaco, J. M. Clomburg, E. N. Miller, R. Gonzalez, *Nature* **2011**, *476*, 355.

[44] C. R. Shen, E. I. Lan, Y. Dekishima, A. Baez, K. M. Cho, J. C. Liao, *Appl Environ Microbiol* **2011**, *77*, 2905.

[45] R.M.R. Feldman, U. Gunawardena, J. Urano , P. Meinhold, A.A. Aristidou, C.A. Dundon, C. Smith, US 8,018,375 B2, **2008**.

[46] R. Haselbeck, J.D. Trawick, W. Niu, A.P. Burgard, *Vol. EP2488652*, **2012**.

[47] H. Yim, R. Haselbeck, W. Niu, C. Pujol-Baxley, A. Burgard, J. Boldt, J. Khandurina, J. D. Trawick, R. E. Osterhout, R. Stephen, J. Estadilla, S. Teisan, H. B. Schreyer, S. Andrae, T. H. Yang, S. Y. Lee, M. J. Burk, S. Van Dien, *Nat Chem Biol* **2011**, *7*, 445.

[48] E. Watkins, *Oil & Gas Journal* **2011**, *109*, 19.

[49] K. Lohbeck, H. Haferkorn, W. Fuhrmann, N. Fedtke, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**.

[50] G. S. Patience, R. E. Bockrath, *Applied Catalysis a-General* **2010**, *376*, 4.

[51] J. B. van Beilen, E. G. Funhoff, *Curr Opin Biotechnol* **2005**, *16*, 308.

[52] J. B. van Beilen, E. G. Funhoff, *Appl Microbiol Biotechnol* **2007**, *74*, 13.

[53] N. Hamamura, R. T. Storfa, L. Semprini, D. J. Arp, *Appl Environ Microbiol* **1999**, *65*, 4586.

[54] J. Colby, D. I. Stirling, H. Dalton, *Biochem J* **1977**, *165*, 395.

[55] D. J. Arp, *Microbiology* **1999**, *145 (Pt 5)*, 1173.

[56] M. Merkx, D. A. Kopp, M. H. Sazinsky, J. L. Blazyk, J. Muller, S. J. Lippard, *Angew Chem Int Ed Engl* **2001**, *40*, 2782.

[57] H. H. Nguyen, S. J. Elliott, J. H. Yip, S. I. Chan, *J Biol Chem* **1998**, *273*, 7957.

[58] R. Balasubramanian, S. M. Smith, S. Rawat, L. A. Yatsunyk, T. L. Stemmler, A. C. Rosenzweig, *Nature* **2010**, *465*, 115.

[59] A. Gunay, K. H. Theopold, *Chem Rev* **2010**, *110*, 1060.

[60] S. Friedle, E. Reisner, S. J. Lippard, *Chem Soc Rev* **2010**, *39*, 2768.

[61] D. J. Owen, G. Eggink, B. Hauer, M. Kok, D. L. McBeth, Y. L. Yang, J. A. Shapiro, *Mol Gen Genet* **1984**, *197*, 373.

[62] T. H. Smits, B. Witholt, J. B. van Beilen, *Antonie Van Leeuwenhoek* **2003**, *84*, 193.

[63] T. Ueda, M. J. Coon, *J Biol Chem* **1972**, *247*, 5010.

[64] D. J. Koch, M. M. Chen, J. B. van Beilen, F. H. Arnold, *Applied and Environmental Microbiology* **2009**, *75*, 337.

[65] R. N. Austin, J. T. Groves, *Metallomics* **2011**, *3*, 775.

[66] A. Abe, K. Sugiyama, *Anticancer Drugs* **2005**, *16*, 543.

[67] A. V. Rawlings, *Int J Cosmet Sci* **2003**, *25*, 63.

[68] M. M. S. Stephan, B. Mohar, *Organic Process Research & Development* **2006**, *10*, 481.

[69] S. Huf, S. Krugener, T. Hirth, S. Rupp, S. Zibek, *European Journal of Lipid Science and Technology* **2011**, *113*, 548.

[70] R. Giri, J. Q. Yu, *Journal of the American Chemical Society* **2008**, *130*, 14082.

[71] S. P. Fore, T. L. Ward, F. G. Dollear, *Journal of the American Oil Chemists Society* **1963**, *40*, 30.

[72] S. Picataggio, T. Rohrer, K. Deanda, D. Lanning, R. Reynolds, J. Mielenz, L. D. Eirich, *Biotechnology (N Y)* **1992**, *10*, 894.

[73] W. H. Lu, J. E. Ness, W. C. Xie, X. Y. Zhang, J. Minshull, R. A. Gross, *Journal of the American Chemical Society* **2010**, *132*, 15451.

[74] D. W. Nebert, D. R. Nelson, M. J. Coon, R. W. Estabrook, R. Feyereisen, Y. Fujii-Kuriyama, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, et al., *DNA Cell Biol* **1991**, *10*, 1.

[75] D. R. Nelson, *Hum Genomics* **2009**, *4*, 59.

[76] D. Werck-Reichhart, A. Hehn, L. Didierjean, *Trends Plant Sci* **2000**, *5*, 116.

[77] O. d. Montellano, *Cytochrome P450: Structure, Mechanism, and Biochemistry, Vol. 3rd ed*, Springer, **2005**.

[78] H. S. Astrid Sigel, Roland K. O. Sigel, *The Ubiquitous Roles of Cytochrome P450 Proteins, Vol. Volume 3*, New York: Wiley, **2007**.

[79] D. Sirim, F. Wagner, A. Lisitsa, J. Pleiss, *BMC Biochem* **2009**, *10*, 27.

[80] S. T. Jung, R. Lauchli, F. H. Arnold, *Curr Opin Biotechnol* **2011**, *22*, 809.

[81] D. Sirim, M. Widmann, F. Wagner, J. Pleiss, *BMC Struct Biol* **2010**, *10*, 34.

[82] A. Seifert, J. Pleiss, *Curr Drug Metab* **2012**, *13*, 197.

[83] I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chemical Reviews* **2005**, *105*, 2253.

[84] S. Graham-Lorence, J. A. Peterson, *Faseb J* **1996**, *10*, 206.

[85] F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim Biophys Acta* **2007**, *1770*, 330.

[86] G. A. Roberts, G. Grogan, A. Greter, S. L. Flitsch, N. J. Turner, *J Bacteriol* **2002**, *184*, 3898.

[87] M. A. Noble, C. S. Miles, S. K. Chapman, D. A. Lysek, A. C. MacKay, G. A. Reid, R. P. Hanzlik, A. W. Munro, *Biochem J* **1999**, *339 (Pt 2)*, 371.

[88] A. W. Munro, D. G. Leys, K. J. McLean, K. R. Marshall, T. W. Ost, S. Daff, C. S. Miles, S. K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, P. L. Dutton, *Trends Biochem Sci* **2002**, *27*, 250.

[89] A. W. Munro, S. N. Daff, K. L. Turner, S. K. Chapman, *Biochem Soc Trans* **1997**, *25*, S628.

[90] A. E. Shilov, G. B. Shul'pin, *Chemical Reviews* **1997**, *97*, 2879.

[91] A. W. Munro, H. M. Girvan, K. J. McLean, *Biochim Biophys Acta* **2007**, *1770*, 345.

[92] R. Bernhardt, *J Biotechnol* **2006**, *124*, 128.

[93] V. B. Urlacher, M. Girhard, *Trends Biotechnol* **2012**, *30*, 26.

[94] N. Serizawa, S. Serizawa, K. Nakagawa, K. Furuya, T. Okazaki, A. Terahara, *J Antibiot (Tokyo)* **1983**, *36*, 887.

[95] T. Matsuoka, S. Miyakoshi, K. Tanzawa, K. Nakahara, M. Hosobuchi, N. Serizawa, *Eur J Biochem* **1989**, *184*, 707.

[96] C. Dingler, W. Ladner, G. A. Krei, B. Cooper, B. Hauer, *Pesticide Science* **1996**, *46*, 33.

[97] J. X. Heinisch, C. P. Hollenberg, in *Biotechnology Set*, Wiley-VCH Verlag GmbH, **2008**, pp. 469.

[98] D. K. Ro, E. M. Paradise, M. Ouellet, K. J. Fisher, K. L. Newman, J. M. Ndungu, K. A. Ho, R. A. Eachus, T. S. Ham, J. Kirby, M. C. Chang, S. T. Withers, Y. Shiba, R. Sarpong, J. D. Keasling, *Nature* **2006**, *440*, 940.

[99] F. M. Szczebara, C. Chandelier, C. Villeret, A. Masurel, S. Bourot, C. Duport, S. Blanchard, A. Groisillier, E. Testet, P. Costaglioli, G. Cauet, E. Degryse, D. Balbuena, J. Winter, T. Achstetter, R. Spagnoli, D. Pompon, B. Dumas, *Nat Biotechnol* **2003**, *21*, 143.

[100] C. Duport, R. Spagnoli, E. Degryse, D. Pompon, *Nat Biotechnol* **1998**, *16*, 186.

[101] J. Hasegawa, M. Ogura, S. Hamaguchi, M. Shimazaki, H. Kawaharada, K. Watanabe, *Journal of Fermentation Technology* **1981**, *59*, 203.

[102] J. Hasegawa, M. Ogura, H. Kanema, N. Noda, H. Kawaharada, K. Watanabe, *Journal of Fermentation Technology* **1982**, *60*, 501.

[103] T. Iimori, M. Shibasaki, *Tetrahedron Letters* **1986**, *27*, 2149.

[104] D. L. Craft, K. M. Madduri, M. Eshoo, C. R. Wilson, *Appl Environ Microbiol* **2003**, *69*, 5983.

[105] T. Maier, H. H. Forster, O. Asperger, U. Hahn, *Biochem Biophys Res Commun* **2001**, *286*, 652.

[106] N. Fujita, F. Sumisa, K. Shindo, H. Kabumoto, A. Arisawa, H. Ikenaga, N. Misawa, *Biosci Biotechnol Biochem* **2009**, *73*, 1825.

[107] E. G. Funhoff, J. Salzmann, U. Bauer, B. Witholt, J. B. van Beilen, *Enzyme and Microbial Technology* **2007**, *40*, 806.

[108] E. G. Funhoff, U. Bauer, I. Garcia-Rubio, B. Witholt, J. B. van Beilen, *J Bacteriol* **2006**, *188*, 5220.

[109] F. H. Arnold, *Nature* **2001**, *409*, 253.

[110] R. K. Gudiminchi, C. Randall, D. J. Opperman, O. A. Olaofe, S. T. Harrison, J. Albertyn, M. S. Smit, *Appl Microbiol Biotechnol* **2012**.

[111] T. Otomatsu, L. M. Bai, N. Fujita, K. Shindo, K. Shimizu, N. Misawa, *Journal of Molecular Catalysis B-Enzymatic* **2010**, *66*, 234.

[112] D. Scheps, S. H. Malca, H. Hoffmann, B. M. Nestl, B. Hauer, *Organic & Biomolecular Chemistry* **2011**, *9*, 6727.

[113] W. Augustyniak, A. A. Brzezinska, T. Pijning, H. Wienk, R. Boelens, B. W. Dijkstra, M. T. Reetz, *Protein Sci* **2012**, *21*, 487.

[114] M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, K. E. Jaeger, *Angewandte Chemie-International Edition* **1997**, *36*, 2830.

[115] J. C. Moore, F. H. Arnold, *Nat Biotechnol* **1996**, *14*, 458.

[116] R. H. Grubbs, T. M. Trnka, in *Ruthenium in Organic Synthesis*, Wiley-VCH Verlag GmbH & Co. KGaA, **2005**, pp. 153.

[117] T. Mizoroki, K. Mori, A. Ozaki, *Bulletin of the Chemical Society of Japan* **1971**, *44*, 581.

[118] R. F. Heck, J. P. Nolley, *Journal of Organic Chemistry* **1972**, *37*, 2320.

[119] W. P. Stemmer, *Nature* **1994**, *370*, 389.

[120] M. T. Reetz, *Angew Chem Int Ed Engl* **2011**, *50*, 138.

[121] T. S. Wong, K. L. Tee, B. Hauer, U. Schwaneberg, *Nucleic Acids Res* **2004**, *32*, e26.

[122] P. C. Babbitt, J. A. Gerlt, *Adv Protein Chem* **2000**, *55*, 1.

[123] A. Seifert, J. Pleiss, *Proteins* **2009**, *74*, 1028.

[124] A. Warshel, P. K. Sharma, M. Kato, Y. Xiang, H. Liu, M. H. Olsson, *Chem Rev* **2006**, *106*, 3210.

[125] S. H. Malca, D. Scheps, L. Kuhnel, E. Venegas-Venegas, A. Seifert, B. M. Nestl, B. Hauer, *Chemical Communications* **2012**, *48*, 5115.

[126] T. Omura, R. Sato, *J Biol Chem* **1964**, *239*, 2370.

[127] T. Omura, R. Sato, *J Biol Chem* **1964**, *239*, 2379.

[128] T. S. Wong, K. L. Tee, B. Hauer, U. Schwaneberg, *Analytical Biochemistry* **2005**, *341*, 187.

[129] H. Mundhada, J. Marienhagen, A. Scacioc, A. Schenk, D. Roccatano, U. Schwaneberg, *Chembiochem* **2011**, *12*, 1595.

[130] M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *Journal of the American Chemical Society* **2003**, *125*, 13442.

[131] T. H. Smits, M. A. Seeger, B. Witholt, J. B. van Beilen, *Plasmid* **2001**, *46*, 16.

[132] S. Pflug, S. M. Richter, V. B. Urlacher, *J Biotechnol* **2007**, *129*, 481.

[133] J. B. van Beilen, D. Penninga, B. Witholt, *J Biol Chem* **1992**, *267*, 9194.

[134] O. Favre-Bulle, E. Weenink, T. Vos, H. Preusting, B. Witholt, *Biotechnol Bioeng* **1993**, *41*, 263.

[135] C. Grant, J. M. Woodley, F. Baganz, *Enzyme Microb Technol* **2011**, *48*, 480.

[136] A. Glieder, E. T. Farinas, F. H. Arnold, *Nat Biotechnol* **2002**, *20*, 1135.

[137] P. Meinhold, M. W. Peters, M. M. Y. Chen, K. Takahashi, F. H. Arnold, *Chembiochem* **2005**, *6*, 1765.

[138] P. Meinhold, M. W. Peters, A. Hartwick, A. R. Hernandez, F. H. Arnold, *Advanced Synthesis & Catalysis* **2006**, *348*, 763.

[139] F. Xu, S. G. Bell, J. Lednik, A. Insley, Z. Rao, L. L. Wong, *Angew Chem Int Ed Engl* **2005**, *44*, 4029.

[140] R. Fasan, M. M. Chen, N. C. Crook, F. H. Arnold, *Angew Chem Int Ed Engl* **2007**, *46*, 8414.

[141] C. J. C. Whitehouse, S. G. Bell, L. L. Wong, *Chemical Society Reviews* **2012**, *41*, 1218.

[142] J. B. van Beilen, E. G. Funhoff, A. van Loon, A. Just, L. Kaysser, M. Bouza, R. Holtackers, M. Rothlisberger, Z. Li, B. Witholt, *Appl Environ Microbiol* **2006**, *72*, 59.

[143] F. P. Guengerich, *Chem Biol Interact* **1997**, *106*, 161.

[144] M. Girhard, K. Machida, M. Itoh, R. D. Schmid, A. Arisawa, V. B. Urlacher, *Microb Cell Fact* **2009**, *8*, 36.

[145] I. N. Van Bogaert, S. Groeneboer, K. Saerens, W. Soetaert, *Febs J* **2010**, *278*, 206.

[146] J. B. Johnston, P. M. Kells, L. M. Podust, P. R. Ortiz de Montellano, *Proc Natl Acad Sci U S A* **2009**, *106*, 20687.

[147] M. A. Schrewe M., Willrodt C., Bühler B. and Schmid A., *Advanced Synthesis and Catalysis* **2011**, *353*, 3485 – 3495.

[148] K. E. Nelson, C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzez, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. A. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, C. M. Fraser, *Environ Microbiol* **2002**, *4*, 799.

[149] E. Singer, E. A. Webb, W. C. Nelson, J. F. Heidelberg, N. Ivanova, A. Pati, K. J. Edwards, *Appl Environ Microbiol* **2011**, *77*, 2763.

[150] U. Wolfmeier, H. Schmidt, F.-L. Heinrichs, G. Michalczyk, W. Payer, W. Dietsche, K. Boehlke, G. Hohner, J. Wildgruber, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**.

[151] B. D. Wahlen, W. S. Oswald, L. C. Seefeldt, B. M. Barney, *Appl Environ Microbiol* **2009**, *75*, 2758.

[152] R. M. Willis, B. D. Wahlen, L. C. Seefeldt, B. M. Barney, *Biochemistry* **2010**, *50*, 10550.

[153] T. Fujii, T. Narikawa, F. Sumisa, A. Arisawa, K. Takeda, J. Kato, *Biosci Biotechnol Biochem* **2006**, *70*, 1379.

[154] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185.

[155] S. Honda Malca, D. Scheps, L. Kuhnel, E. Venegas-Venegas, A. Seifert, B. M. Nestl, B. Hauer, *Chem Commun (Camb)* **2012**, *48*, 5115.

[156] D. Scheps, S. H. Malca, H. Hoffmann, B. M. Nestl, B. Hauer, *Org Biomol Chem* **2011**, *9*, 6727.

[157] F. P. Guengerich, *Biol Chem* **2002**, *383*, 1553.

[158] O. Sibbesen, J. J. De Voss, P. R. Montellano, *J Biol Chem* **1996**, *271*, 22462.

[159] L. Liu, R. D. Schmid, V. B. Urlacher, *Appl Microbiol Biotechnol* **2006**, *72*, 876.

[160] A. Robin, G. A. Roberts, J. Kisch, F. Sabbadin, G. Grogan, N. Bruce, N. J. Turner, S. L. Flitsch, *Chem Commun (Camb)* **2009**, 2478.

[161] S. Li, L. M. Podust, D. H. Sherman, *J Am Chem Soc* **2007**, *129*, 12940.

[162] M. Nodate, M. Kubota, N. Misawa, *Appl Microbiol Biotechnol* **2006**, *71*, 455.

[163] M. Wang, D. L. Roberts, R. Paschke, T. M. Shea, B. S. S. Masters, J. J. P. Kim, *Proceedings of the National Academy of Sciences of the United States of America* **1997**, *94*, 8411.

[164] A. Gutierrez, M. Paine, C. R. Wolf, N. S. Scrutton, G. C. Roberts, *Biochemistry* **2002**, *41*, 4626.

[165] N. G. Leferink, C. R. Pudney, S. Brenner, D. J. Heyes, R. R. Eady, S. Samar Hasnain, S. Hay, S. E. Rigby, N. S. Scrutton, *FEBS Lett* **2012**, *586*, 578.

[166] D. Hamdane, C. Xia, S. C. Im, H. Zhang, J. J. Kim, L. Waskell, *J Biol Chem* **2009**, *284*, 11374.

[167] R. Neeli, H. M. Girvan, A. Lawrence, M. J. Warren, D. Leys, N. S. Scrutton, A. W. Munro, *FEBS Lett* **2005**, *579*, 5582.

[168] T. Kitazume, D. C. Haines, R. W. Estabrook, B. Chen, J. A. Peterson, *Biochemistry* **2007**, *46*, 11892.

[169] E. Weber, A. Seifert, M. Antonovici, C. Geinitz, J. Pleiss, V. B. Urlacher, *Chem Commun (Camb)*, *47*, 944.

[170] A. Seifert, S. Tatzel, R. D. Schmid, J. Pleiss, *Proteins* **2006**, *64*, 147.

[171] J. Mestres, *Proteins* **2005**, *58*, 596.

[172] S. G. Bell, E. Orton, H. Boyd, J. A. Stevenson, A. Riddle, S. Campbell, L. L. Wong, *Dalton Transactions* **2003**, 2133.

[173] S. G. Bell, J. A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton, L. L. Wong, *Chemical Communications* **2002**, 490.

[174] R. C. Cadwell, G. F. Joyce, *PCR Methods Appl* **1992**, *2*, 28.

[175] C. H. Lee, C. E. Frasch, *Anal Biochem* **2001**, *296*, 73.

[176] F. H. A. a. G. Georgiou, *Screening and selection methods. Methods in molecular biology, Vol. 230*, Humana Press, **2003**.

[177] A. S. Vangnai, D. J. Arp, *Microbiology* **2001**, *147*, 745.

[178] Y. Ishii, S. Sakaguchi, T. Iwahama, *Advanced Synthesis & Catalysis* **2001**, *343*, 393.

[179] M. K. Julsing, S. Cornelissen, B. Buhler, A. Schmid, *Curr Opin Chem Biol* **2008**, *12*, 177.

[180] J. M. Woodley, *Adv Appl Microbiol* **2006**, *60*, 1.

[181] S. Isken, J. A. de Bont, *Extremophiles* **1998**, *2*, 229.

[182] E. J. Steen, R. Chan, N. Prasad, S. Myers, C. J. Petzold, A. Redding, M. Ouellet, J. D. Keasling, *Microb Cell Fact* **2008**, *7*, 36.

[183] L. M. Blank, B. E. Ebert, B. Buhler, A. Schmid, *Biotechnol Bioeng* **2008**, *100*, 1050.

[184] R. K. Gudiminchi, C. Randall, D. J. Opperman, O. A. Olaofe, S. T. Harrison, J. Albertyn, M. S. Smit, *Appl Microbiol Biotechnol* **2012**, *96*, 1507.

[185] R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, *J Mol Biol* **2008**, *383*, 1069.

[186] S. Kadkhodayan, E. D. Coulter, D. M. Maryniak, T. A. Bryson, J. H. Dawson, *J Biol Chem* **1995**, *270*, 28042.

[187] M. Bordeaux, A. Galarneau, F. Fajula, J. Drone, *Angew Chem Int Ed Engl* **2011**, *50*, 2075.

[188] T. Chemistry, Thieme Chemistry, **2012**.

[189] S. Staudt, C. A. Muller, J. Marienhagen, C. Boing, S. Buchholz, U. Schwaneberg, H. Groger, *Beilstein Journal of Organic Chemistry* **2012**, *8*, 186.

[190] H. Zhang, H. Chong, C. B. Ching, H. Song, R. Jiang, *Appl Microbiol Biotechnol* **2012**, *94*, 1107.

[191] A. Fantuzzi, Y. T. Meharenna, P. B. Briscoe, F. Guerlesquin, S. J. Sadeghi, G. Gilardi, *Biochim Biophys Acta* **2009**, *1787*, 234.

[192] B. d. Justiz, in *BGBI.I, Vol. 2012*, **1999**.

[193] M. K. Julsing, M. Schrewe, S. Cornelissen, I. Hermann, A. Schmid, B. Buhler, *Appl Environ Microbiol* **2012**, *78*, 5724.

[194] Z. Su, J. H. Horner, M. Newcomb, *Chembiochem* **2012**, *13*, 2061.

[195] A. Knoll, J. Buechs, E. Heinzle, A. P. Biwer, C. L. Cooney, in *Development of Sustainable Bioprocesses*, John Wiley & Sons, Ltd, **2007**, pp. 155.

[196] E. Heinzle, A. P. Biwer, C. L. Cooney, in *Development of Sustainable Bioprocesses*, John Wiley & Sons, Ltd, **2007**, pp. 11.

[197] S. Katoh, F. Yoshida, in *Biochemical Engineering*, Wiley-VCH Verlag GmbH & Co. KGaA, **2012**, pp. 185.

[198] E. Cabiscol, J. Tamarit, J. Ros, *Int Microbiol* **2000**, *3*, 3.

[199] B. Demple, J. Halbrook, *Nature* **1983**, *304*, 466.

[200] L. Z. Karpetz, N. V. Adrianov, Karuzina, II, S. Dzhuzenova Ch, A. I. Archakov, *Biull Eksp Biol Med* **1988**, *105*, 547.

[201] A. Siriphongphaew, P. Pisnupong, J. Wongkongkatep, P. Inprakhon, A. S. Vangnai, K. Honda, H. Ohtake, J. Kato, J. Ogawa, S. Shimizu, V. B. Urlacher, R. D. Schmid, T. Pongtharangkul, *Appl Microbiol Biotechnol* **2012**, *95*, 357.

[202] C. Grant, A. C. Pinto, H. P. Lui, J. M. Woodley, F. Baganz, *Biotechnol Bioeng* **2012**, *109*, 2179.

[203] A. Robin, V. Kohler, A. Jones, A. Ali, P. P. Kelly, E. O'Reilly, N. J. Turner, S. L. Flitsch, *Beilstein J Org Chem* **2011**, *7*, 1494.

[204] S. Kille, F. E. Zilly, J. P. Acevedo, M. T. Reetz, *Nature Chemistry* **2011**, *3*, 738.

[205] R. C. Wade, D. Motiejunas, K. Schleinkofer, Sudarko, P. J. Winn, A. Banerjee, A. Kariakin, C. Jung, *Biochim Biophys Acta* **2005**, *1754*, 239.

[206] L. Rui, S. S. Pochapsky, T. C. Pochapsky, *Biochemistry* **2006**, *45*, 3887.

[207] S. Modi, M. J. Sutcliffe, W. U. Primrose, L. Y. Lian, G. C. Roberts, *Nat Struct Biol* **1996**, *3*, 414.

[208] A. Schirmer, M. A. Rude, X. Li, E. Popova, S. B. del Cardayre, *Science* **2010**, *329*, 559.

[209] J. Ruhl, A. Schmid, L. M. Blank, *Appl Environ Microbiol* **2009**, *75*, 4653.

[210] M. J. Dunlop, Z. Y. Dossani, H. L. Szmidt, H. C. Chu, T. S. Lee, J. D. Keasling, M. Z. Hadi, A. Mukhopadhyay, *Mol Syst Biol* **2011**, *7*, 487.

[211] M. Hermann, F. Fayolle, R. Marchal, L. Podvin, M. Sebald, J. P. Vandecasteele, *Appl Environ Microbiol* **1985**, *50*, 1238.

[212] Y. L. Lin, H. P. Blaschek, *Appl Environ Microbiol* **1983**, *45*, 966.

6. Appendix

6.1. Abbreviations

× g	gravitational acceleration
°C	degrees Celsius
µl	microliter
µm	micrometer
µM	micromolar
α,ω-DC12	1,12-dicarboxylic dodecanoic acid
aa	amino acid
AlkB	alkane-1-monooxygenase from *Pseudomonas putida* Gpo1
AlkG	rubredoxin from *Pseudomonas putida* Gpo1
AlkT	rubredoxin reductase from *Pseudomonas putida* Gpo1
ATTC	Amercian Type Culture Collection
BMO	Butane monooxygenase
BDO	Butanediol
bp	base pair
C12-FA	dodecanoic acid
C12-FAME	dodecanoic acid methyl ester
CamA	putidaredoxin reductase from *Pseudomonas putida*
CamB	putidaredoxin from *Pseudomonas putida*
CFU	Cell forming units
cdw	cell dry weight

CPR	cytochrome P450 reductase
cww	cell wet weight
CYP	cytochrome P450 monooxygenase
CYP153A6	CYP153A monooxygenase from *Mycobacterium* sp. HXN 1500
CYP153A16	CYP153A monooxygenase from *Mycobacterium marinum* M.
$CYP153A_{M.\ aq.}$	
or CYP153A *M. aq.*	CYP153A monooxygenase from *Marinobacter aquaeolei* VT8
$CYP153A_{P.\ sp.}$	
or CYP153A *P.* sp.	CYP153A monooxygenase from *Polaromonas* sp. JS666
CYP153A-operon	CYP153A + natural redox partners
DAD	diode array detector
DCA	dicarboxylic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	DL-dithiothreitol
E. coli	*Escherichia coli*
eM9	*Escherichia coli* M9 minimal salt media
eM9Y	*Escherichia coli* M9 minimal salt media + yeast extract
FAD	flavin adenine dinucleotide
FdR	NAD(P)H-dependent ferredoxin oxidoreductase
Fdx	ferredoxin
FMN	flavin mononucleotide

g	gram
g_{cww}	pro gramm cell wet weight
g_{cdw}	pro gramm cell dry weight
g_{Ztm}	pro Gramm Zelltrockenmasse
GC	gas chromatography
GC/FID	gas chromatography coupled to flame ionization detector
GC/MS	gas chromatography coupled to mass spectrometry
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
h	hour
HPLC	high-performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
L	liter
LB	Luria-Bertani broth
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MMO	Methane monooxygenase
OCT	octane-degrading plasmid in *Pseudomonas putida* Gpo1
OD_{595}	optical density measured at 595 nm
OHFA	hydroxylated fatty acid

p.a.	per anno / per year
P. putida	*Pseudomonas putida*
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
PspFdR	ferredoxin reductase from *Polaromonas* sp. JS666
PspFdx	ferredoxin from *Polaromonas* sp. JS666
QC	Site directed mutagenesis
RID	refractive index detector
RIS2	reductase interaction site
ROS	reactive oxygen species
rpm	rounds per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	terrific broth
t/p.a.	tons per anno
WT	wild type
ω-OHC12	12-hydroxy dodecanoic acid
(ω-1)-OHC12	11-hydroxy dodecanoic acid

6.2. Genes and Proteins

Stop and start codons are underlined.

PspAlk gene cluster from ***Polaromonas* sp. JS666**

(CYP153A*P. sp.*→ CYP153A*P. sp.*-FdR→ CYP153A*P. sp.*-Fdx)

ATGAGTGAAGCGATTGTGGTAAACAACCAAAACGACCAAAGCAGGGCATACGCGA
TCCCGCTTGAGGACATTGATGTAAGCAATCCGGAGCTGTTTCGCGACAATACGATGT
GGGGTTATTTTGAGCGTCTGCGCCGCGAAGACCCCGTGCATTACTGTAAGGACAGCT
TGTTTGGTCCGTACTGGTCGGTGACCAAGTTCAAGGACATCATGCAGGTGGAGACCC
ATCCGGAGATATTTTCATCCGAGGGCAATATCACCATCATGGAGTCCAATGCGGCGG
TAACCCTGCCGATGTTCATTGCGATGGATCCGCCCAAGCACGACGTGCAGCGCATGG
CGGTCAGTCCGATCGTGGCGCCGGAGAACCTCGCCAAGCTCGAAGGTCTGATCCGC
GAGCGTACCGGTCGTGCGCTGGATGGCCTGCCGATCAACGAGACCTTTGACTGGGTC
AAGCTCGTTTCGATCAACCTGACGACGCAGATGCTGGCGACGCTGTTTGATTTCCCTT
GGGAAGACCGTGCCAAGCTGACGCGCTGGTCGGATGTCGCGACGGCGCTGGTCGGC
ACGGGCATTATTGATTCGGAAGAGCAGCGCATGGAGGAGCTCAAGGGGTGCGTGCA
ATACATGACCCGGCTGTGGAACGAGCGCGTCAATGTGCCACCGGGCAATGATCTGA
TATCGATGATGGCGCACACCGAGTCCATGCGCAACATGACGCCGGAAGAGTTTCTG
GGCAACCTCATTTTGCTGATCGTCGGCGGCAATGACACGACCCGCAACTCGATGACC
GGCGGCGTGCTGGCGCTCAACGAAAATCCGGACGAATACCGCAAGCTGTGCGCCAA
CCCGGCGCTGATCGCCTCCATGGTGCCGGAGATCGTTCGTTGGCAGACACCGCTGGC
GCACATGCGGCGTACCGCGCTGCAGGACACCGAGCTCGGCGGCAAGTCCATTCGCA
AGGGTGACAAGGTCATCATGTGGTATGTCTCCGGCAACCGTGATCCCGAAGCGATTG
AAAATCCGGACGCGTTCATCATTGATCGCGCCAAGCCGCGCCATCACCTCTCGTTCG
GTTTCGGCATTCACCGCTGCGTGGGCAACCGTCTCGCCGAGTTGCAGCTGCGCATCGT
TTGGGAGGAGTTGCTCAAGCGCTGGCCCAATCCAGGTCAGATCGAGGTCGTTGGCGC
GCCCGAGCGCGTGCTGTCGCCCTTTGTGAAGGGCTATGAGTCGCTGCCCGTCCGCAT
CAACGCTTGA

GTGAGCGAAACTGTGATTATTGCCGGCGCCGGTCAGGCGGCCGGCCAGGCGGTTGC
GAGCCTGCGGCAAGAGGGATTCGACGGGCGCATCGTGCTGGTCGGCGCCGAGCCGG
TGTTGCCGTATCAGCGCCCGCCGCTGTCGAAGGCATTTTTGGCGGGCACCTTGCCGCT
GGAGCGATTGTTCCTGAAGCCGCCGGCATTCTACGAGCAGGCGCGTGTGGACACGCT
GCTCGGGGTGGCCGTCACCGAACTTGATGCCGCCCGGCGGCAGGTGAGGCTGGACG
ATGGCCGCGAACTGGCGTTTGATCATCTGCTGCTGGCGACTGGCGGGCGTGCCCGTC
GGCTTGACTGCCCGGGTGCCGACCATCCGCGCCTGCACTATCTGCGCACCGTGGCTG
ATGTTGACGGCATTCGTGCCGCTCTGCGTCCCGGGGCCCGGCTGGTGCTGATCGGCG
GCGGCTACGTCGGACTCGAGATCGCCGCCGTGGCCGCCAAACTGGGGCTCGCGGTG
ACCGTGCTGGAAGCGGCGCCGACGGTGCTGGCGCGTGTCACTTGTCCGGCCGTGGCG
CGCTTCTTCGAAAGCGTGCACCGGCAGGCGGGCGTGACGATCCGCTGCGCGACGAC
GGTCTCCGGCATCGAGGGCGATGCTTCGCTGGCGCGGGTCGTGACCGGCGATGGCGA
ACGCATTGACGCCGACCTGGTCATTGCCGGCATCGGTCTGCTGCCGAACGTCGAGTT
GGCGCAGGCCGCGGGTCTGGTCTGCGACAACGGCATCGTCGTCGACGAGGAATGCC
GGACCTCTGTGCCCGGCATTTTCGCGGCTGGCGACTGCACGCAGCATCCGAACGCGA

TCTACGACAGTCGGCTGCGTCTCGAATCGGTGCACAACGCCATTGAGCAGGGCAAG
ACGGCGGCGGCGGCCATGTGTGGCAAGGCCAGGCCGTATCGGCAGGTGCCGTGGTT
CTGGTCCGATCAGTACGACCTCAAGTTACAAACCGCGGGACTCAACCGCGGCTATG
ACCAGGTCGTGATGCGGGGCAGTACCGACAACCGTTCGTTTGCGGCGTTCTACCTGC
GCGACGGGCGATTGCTTGCCGTCGATGCGGTCAACCGCCCGGTCGAGTTCATGGTGG
CCAAAGCGCTGATTGCGAACCGCACCGTCATCGCGCCCGAGCGGCTCGCCGACGAG
CGTATCGCAGCGAAGGACCTGGCCGGCTGA

ATGACAAAAGTTACTTTTATTGAACACAATGGTACGGTCCGCAACGTGGACGTCGAC
GACGGCCTGTCGGTGATGGAGGCCGCCGTCAACAACCTGGTGCCGGGCATCGATGG
CGACTGCGGTGGCGCCTGCGCCTGCGCCACCTGCCATGTGCACATCGACGCCGCCTG
GCTGGACAAGTTGCCGCCGATGGAGGCGATGGAAAAGTCGATGCTTGAGTTTGCCG
AGGGCCGCAACGAAAGCTCGCGCCTGGGTTGTCAGATCAAGCTCAGCCCCGCGCTT
GACGGCATTGTGGTGCGCACGCCGCTCGGCCAGCACTGA

MaqAlk gene cluster from ***Marinobacter aquaeolei*** **VT8**

(CYP153A*M. aq.*-Fdx→ CYP153A*M. aq.*→ CYP153A*M. aq.*-FdR)

ATGGGCGGTCACGATGGGCCGGAATATGCACATGTCGCAGAATTCAAAGCTGGTTCC
TCGGTAATGCAAATCGCTGTTGATAGCGCCATTCCCGGTATCGACGGGGATTGTGGG
GGGGAGTGCGCCTGCGGTACCTGCCACGTTATCGTCACGAACGAATGGTTCAGCAAG
ACAGGCACGCCTGGCAATGAGGAAGAACAAATGCTGTCAATGACACCGGAGCGGG
CGAGCACTTCGCGCCTGGGCTGCCAGGTGGTACTGACTGATGAAATGGACGGCATG
ACCGTGCATTTACCCGAGTTCCAGATGTGA

ATGCCAACACTGCCCAGAACATTTGACGACATTCAGTCCCGACTGATTAACGCCACC
TCCAGGGTGGTGCCGATGCAGAGGCAAATTCAGGGACTGAAATTCTTAATGAGCGC
CAAGAGGAAGACCTTCGGCCCACGCCGACCGATGCCCGAATTCGTTGAAACACCCA
TCCCGGACGTTAACACGCTGGCCCTTGAGGACATCGATGTCAGCAATCCGTTTTTATA
CCGGCAGGGTCAGTGGCGCGCCTATTTCAAACGGTTGCGTGATGAGGCGCCGGTCCA
TTACCAGAAGAACAGCCCTTTCGGCCCCTTCTGGTCGGTAACTCGGTTTGAAGACAT
CCTGTTCGTGGATAAGAGTCACGACCTGTTTTCCGCCGAGCCGCAAATCATTCTCGGT
GACCCTCCGGAGGGGCTGTCGGTGGAAATGTTCATAGCGATGGATCCGCCGAAACA
CGATGTGCAGCGCAGCTCGGTGCAGGGAGTAGTGGCACCGAAAAACCTGAAGGAG
ATGGAGGGGCTGATCCGATCACGCACCGGCGATGTGCTTGACAGCCTGCCTACAGA
CAAACCCTTTAACTGGGTACCTGCTGTTTCCAAGGAACTCACAGGCCGCATGCTGGC
GACGCTTCTGGATTTTCCTTACGAGGAACGCCACAAGCTGGTTGAGTGGTCGGACAG
AATGGCAGGTGCAGCATCGGCCACCGGCGGGGAGTTTGCCGATGAAAATGCCATGT
TTGACGACGCGGCAGACATGGCCCGGTCTTTCTCCAGGCTTTGGCGGGACAAGGAGG
CGCGCCGCGCAGCAGGCGAGGAGCCCGGTTTCGATTTGATCAGCCTGTTGCAGAGCA
ACAAAGAAACGAAAGACCTGATCAATCGGCCGATGGAGTTTATCGGTAATTTGACG
CTGCTCATAGTCGGCGGCAACGATACGACGCGCAACTCGATGAGTGGTGGCCTGGTG
GCCATGAACGAATTCCCCAGGGAATTTGAAAAATTGAAGGCAAAACCGGAGTTGAT

TCCGAACATGGTGTCGGAAATCATCCGCTGGCAAACGCCGCTGGCCTATATGCGCCG
AATCGCCAAGCAGGATGTCGAACTGGGCGGCCAGACCATCAAGAAGGGTGATCGAG
TTGTCATGTGGTACGCGTCGGGTAACCGGGACGAGCGCAAATTTGACAACCCCGATC
AGTTCATCATTGATCGCAAGGACGCACGAAACCACATGTCGTTCGGCTATGGGGTTC
ACCGTTGCATGGGCAACCGTCTGGCTGAACTGCAACTGCGCATCCTCTGGGAAGAAA
TACTCAAGCGTTTTGACAACATCGAAGTCGTCGAAGAGCCCGAGCGGGTGCAGTCC
AACTTCGTGCGGGGCTATTCCAGGTTGATGGTCAAACTGACACCGAACAGTTA

ATGGTAAGCAAACGTAAAGAGAGGACGGTCATTGTTGGCGGTGGGCACGCAGCAGG
TGCCCTCCTGACAGCCTTACTCCAAAAAAAATATCAACATGAGGTCGTTCTGGTGGG
GAATGAACCTCATCCGCCCTACCATCGACCGCCGCTGTCCAAGAATTACCTGACAGG
AGACGTTGATCAGGAGTCGCTGTACCTGAAACCGCGCTCGGTATACGAGAACGCAG
GCCATCAGTTGCGGCTCGGTGTGCGCGTCGAACAAATTGATCGGGACAGTAGCACCA
TCAGCTTGTCGGATCAGAGCAGGCTGCAATACGATCGACTGGTCCTGGCCACCGGGT
CACACCTTCGACACCTGAACGCGCCCGGGGCTGACTTAAATGGCATTCATTACCTGC
ACGACATAGCTGATTCAGAGGTACTGCGTGAACAGTTAGTTGCTGGAAAGCGCCTGG
TCGTCGTGGGTGGTGGTTACATCGGCCTTGAGGTGGCGGCCAGTGCCAACAAAAAAG
GTGTTAATGTCACGGTGCTAGAAGCCGCCGAACGTCTTATGCAGCGCGTTACGGGCC
CGGAAATATCAGCGTTCCTTTACGACAAACACCGTGGCGCCGGCGTGGACGTACGTC
TGAACACAGCGGTAACCGGCTTCGAAGCGGGCGATCAGGGGCATGTGGCTGGCGTG
ACGTTGGCGGACGGAAGCACCGTACCGGCCGACATCGTCCTTGTGTCGATCGGCATT
ATCCCGGAAACCGCTCTGGCTAAGGACGCCGGCCTGCCCTGTGATAACGGTATTATT
GTTGACGAATTTACCCGTACCGAGGACCCCGCCATCTTGGCGATCGGTGACTGCACC
CGGCACCGGAATCTTTTCTTCGAGAAGATGCAACGACTCGAGTCTGTCGCCAATGCT
GTCGATCAGGCTCGTACAGCCGCGGCAACCCTGATGGGTGAGGAGAAACCCTATGA
TAGCGTTCCATGGTTCTGGTCAAACCAGTACGATGTTCGTCTGCAGATGGTAGGATTG
TCGCAAAATCATGATCAGCGAGTGGTTCGAGGCACCCCCGAGGATAAAGGATTTGC
CGTGTTCTATCTCCGCGAAGGCTGTGTTATTGCTGTTGACGCGGTCAACCTGCCCCTT
GCTTTTTTGGTAGGCAAGACACTCGTTCAACAACGCAGAACGATCAACCCGGAACTA
ATAGAGGATCCGGATACTGAACTGAAATCTTTGGTGAACGGAAGGCTCCAGAGTTG
A

MmAlk gene cluster ***Mycobacterium marinum*** **M**

(CYP153A16-Fdx→CYP153A16→ CYP153A16-FdR)

ATGGCAGTTGTCACATTTGTCTCCCACGGCGGCGAGAAGTATGAGGCGCCTCTCGAG
GAAGGTCAGTCACTGATGCGGGTCGCGACCAACAATGCGGTGCCCGGCATCGACGG
CGACTGCGGAGGCGAAGCCGCGTGCGGCACCTGCCATGTGATCGTCGATCCGCAAT
GGTCCGATCGGGTCGGCCTCTCCGGGGCCAATGAAGAGGAGATGCTCGCGATGAAC
CCCGAGCGTCAGCCGACCTCCCGGCTGTCCTGCCAGATGCAGGTCTCTGAGGCGTGG
GACGGTTTGATCGTCCATCTGCCCGAGTTCCAACTGTGA

GTGAGCAATATTCGCGAGGCAGTCACTGCCAAGGCTCAGGCAACAATTCCGATGGA
CCGAATAATCCAGGGCGCCCACCTCTACGACAGAACGCGGCGCTGGGTCACCGGCA
CCAACGGTGAAAAAATCTTCATCGAGCGACCGATCCCGCCGGCTGACGAGGTTGAA

CTGACCGACATCGACCTTAGCAATCCTTTCCTCTATCGTCAGGGTCGCTGGAAGTCCT
ATTACGAGCGCCTACGCAACGAGGCTCCCGTGCACTATCAGGCCCACAGCGCGTTCG
GCCCGTTCTGGTCGGTGACGCGGCATGCCGACATCGTGGCCGTCGACAAGAACCAC
GAGGTCTTCTCCTCCGAGCCGTTCATCGTCATCGGGAGCCCGCCGCGCTTCCTCGACA
TTGCGATGTTCATCGCGATGGACCCCCCAAAACACGACCGGCAACGGCAGGCTGTC
CAGGGTGTGGTCGCACCGAAGAACCTGCGTGAGATGGAGGGCCTCATCCGCGAGCG
GGTGGTAGACGTGCTCGACGCTCTGCCGCTTGGCGAACCGTTCAACTGGGTGCAGCA
CGTCTCGATCGAGCTAACCGCGCGCATGCTGGCCACGCTGCTGGACTTCCCGTTCGA
GCAGCGGCGCAAGCTCGTCCAATGGTCCGATCTCGCCACCTCCATGGAGCAAGCCA
ACGGTGGGCCCTCGGACAACGACGAGATATTTCGCGGCATGGTCGATATGGCTAGA
GGTCTCAGCGCTCACTGGCGGGACAAGGCAGCCCGGACAGCTGCCGGAGAGCTGCC
CGGCTTCGATCTGATCACCATGTTGCAGAGCGACGAGAGCACCAAGGACCTGATCG
ATCGCCCGATGGAGTTCTTGGGCAACTTGGTATTGCTGATCGTGGGTGGCAACGATA
CGACCCGCAATTCCATGAGCGGTGGTGTTCTGGCGCTGAACGAGTTCCCTGACCAGT
TTGAGAAGCTGAAGGCGAACCCCGAGCTGATCCCCAACATGGTCTCGGAGATCATC
CGGTGGCAAACCCCGCTCGCGCATATGCGCCGGATCGCCAAGGCCGACACTGTGCT
CAACGGGCAGTTCATCCGCAAGGGCGACAAGGTCCTGATGTGGTACGCCTCGGGCA
ACCGCGACGAGCGCGTGTTCGATCGGCCCGATGACCTGATTATCGATCGGGCCAACG
CCCGTAACCACATCTCCTTCGGTTTCGGCGTGCACCGCTGTATGGGTAACCGGCTGGC
CGAGATGCAGTTGCGGATCCTGTGGGAGGAGCTGCTTCCGCGGTTCGAGAACATCGA
GGTCGTCGGTGAGCCCGAGTACGTGCAGTCCAACTTCGTGAGGGGGATCAGTAAGCT
GATGGTCCGCCTCACCCCGAAAGGTGGCGC

ATGACCGTGCAGCGAGCGGTCATCGCGGGGGCCAGCCACGCGGGCACCCAGCTCGC
CGCCAGTCTTCGCCGAGAAGGGTGGGACGGCGAAATCGTCCTCGTCGGCGATGAGT
CGGCGTTGCCCTACCAGCGGCCCCCGCTGTCCAAGTCGTACCTGGCCGACAAATGCG
AACTGGCCGAACTCGCGATCCGCAACTCGGATTTCTACGCCAAGCAGCGGATCCGA
CTCCTGGATGCGACGGTGGCGGCGGTCGACCGCTCGGCTGGTCATGTCGTGCTGAGT
ACCGGCGACGCACTGCCCTACGACAAGCTCGCGCTGTGCACTGGCGCCCGGCCTCGT
CGGCTCCCCACCCCGGGAGCGGACCTGGCCGGAGTCTTCTACCTACGCACCGCCGCG
GACGGCGAGATGATCCGAGAGGCCGCCGGCCCCGGGCGTCGGGCGGTGATCGTCGG
CGGCGGCTACATCGGACTGGAGACAGCCGCCTCGTTGCGTGCACTGGGTCTGGAGGT
CACCCTGCTCGAGGCGACCGGGCGCGTCCTTGAACGGGTCACCGCCCCGGAGGTATC
GGAGTTCTTCGACCGGATCCACCGGGAGGAGGGCGTCAACATCCGGACGGGCACGC
TGGTCGAGGCTCTGTCCGGCGACGGCAGGGTCCGCGAAGTAATCCTGGCCGGTGGCG
AATCAATTCCCGCCGACCTCGTCATTGTCGGCATCGGCGTGGAGCCGAACACCGAGC
TCGCCGCCACCGCGGGCCTGGTCGTCGACAACGGCGTCGTGATCGACGATCAGGCCC
GGACTAGCGACCCCGACATCGTGGCCGCCGGGGACTGCGCCAGCCACGACATGGCC
CGTTACGGCCGTCGCATCCGCCTGGAGTCCGTGCCGAGCGCGGCCGAGCAGGCCAA
GGTCGCCGCCGCGACCGTCTGTGGGAAGTCCAAGAAGATAGCGGCCCTTCCATGGTT
CTGGTCAGATCAATACGACCTCAAGCTCCAGATCGCCGGTCTCAACACCGGGTACGA
CGAGGTCGTCCTCAGCGGCGACCCGACCCGGGAGCGCGACTTCACCTGCTTCTACCT
CCGTGCCGGCGAGCTTCTTGCCGCCGACTGCATCAACCGTCCCCGCGACTTCATGTTC
AGCAAGCGGGTCATCACGCAGCAAGTCGCCGTCGAACGGGCCGAACTGGTGCTCGC
CGGCTCGGACTGA

MspAlk gene cluster ***Mycobacterium*** **sp. HXN-1500**

(CYP153A6→ CYP153A6-FdR→ CYP153A6-Fdx)

ATGACCGAAATGACGGTGGCCGCCAGCGACGCGACGAACGCGGCGTACGGGATGG
CCTTGGAGGACATCGATGTGAGCAATCCCGTGCTGTTCCGGGACAACACCTGGCATC
CCTATTTCAAACGCCTGCGCGAAGAGGATCCGGTTCACTACTGCAAGAGCAGCATGT
TCGGCCCCTACTGGTCGGTGACCAAGTACCGCGACATCATGGCAGTCGAGACCAAC
CCGAAGGTGTTCTCGTCGGAGGCTAAAAGTGGCGGCATCACCATCATGGACGACAA
CGCCGCAGCATCGCTTCCCATGTTCATCGCCATGGATCCACCGAAACACGATGTCCA
GCGAAAGACCGTGAGCCCGATCGTGGCGCCCGAAAACCTTGCCACCATGGAATCGG
TGATTCGCCAGCGCACCGCGGACCTCCTCGACGGATTGCCGATCAATGAAGAGTTCG
ACTGGGTGCATCGGGTGTCGATCGAATTGACCACGAAGATGCTGGCGACGCTGTTCG
ATTTTCCCTGGGACGACCGCGCCAAGTTGACGCGCTGGTCGGACGTCACCACGGCGT
TGCCCGGTGGCGGGATCATCGATTCTGAAGAACAGCGCATGGCCGAGCTGATGGAG
TGCGCGACGTATTTCACCGAGCTGTGGAACCAGCGCGTGAATGCCGAACCCAAGAA
CGATCTCATCTCGATGATGGCCCATTCGGAGTCAACACGACACATGGCGCCCGAGGA
ATATCTCGGAAACATCGTGCTGCTGATCGTCGGCGGCAACGACACCACCCGCAACTC
GATGACCGGCGGTGTGTTGGCCCTGAACGAATTTCCCGACGAATACCGCAAACTGTC
CGCCAACCCGGCGTTGATCAGCTCTATGGTGTCGGAGATCATCCGGTGGCAAACACC
TCTTTCGCACATGCGTCGTACCGCATTGGAAGACATCGAGTTCGGCGGCAAGCACAT
CCGCCAGGGCGACAAAGTCGTGATGTGGTACGTGTCCGGCAACCGGGACCCCGAGG
CCATCGACAATCCCGACACATTCATCATCGATCGCGCCAAGCCCCGCCAGCACTTGT
CCTTCGGGTTCGGCATCCACCGCTGCGTCGGCAACAGACTCGCCGAACTACAGCTCA
ACATCCTGTGGGAAGAAATCCTCAAACGGTGGCCGGACCCACTGCAGATCCAGGTT
CTTCAAGAACCGACCCGCGTGCTCTCACCGTTCGTCAAGGGCTACGAATCGCTGCCC
GTGCGCATCAACGCCTGA

ATGATCCACACCGGCGTGACCGAAGCCGTTGTGGTGGTCGGTGCCGGCCAGGCTGGC
GCACAGACAGTCACCAGCCTTCGACAAAGAGGGTTCGAGGGGCAGATCACCCTGCT
CGGCGACGAGCCGGCGCTGCCCTATCAGCGCCCCCCACTGTCGAAAGCCTTCCTGGC
CGGCACCCTCCCGCTGGACCGCCTGTACCTACGCCCTGCGGCGTTCTACCAGCAAGC
CCACGTCGATGTCATGGTCGACACTGGGGTGAGCGAGCTCGACACCGAAAACAGAC
GCATCCGGCTCACCGACGGCCGCGCTATCAGCTTCGATCACCTAGTGCTGGCCACCG
GCGGCCGCCCCCCGCCCGCTGGCCTGCCCTGGTGCCGATCACCCCCGCGTCCACTACC
TGCGAACAGTGACCGACGTAGACAGGATCCGCTCCCAGTTCCATCCCGGGACACGG
CTGGTCCTGGTGGGCGGCGGTTACATCGGCCTCGAAATCGCCGCGGTAGCCGCAGAA
CTCGGGTTGACCGTGACCGTCCTCGAAGCACAAACCACCGTCTTGGCACGGGTCACC
TGTCCGACGGTGGCCCGCTTCTTCGAACACACCCACCGGCGAGCAGGAGTGACGATT
CGGTGCGCAACCACAGTCACACGCATCCACGACAGCTCGTCCACCGCACGCATTGA
ACTCGATAGCGGCGAGTACATCGACGCAGACCTCGTCATAGTTGGGATAGGATTGCT
CCCCAACGTCGACTTAGCCTCAGCAGCTGGGCTGACATGCGAAAGCGGCATCGTCGT
GGACAGCCGTTGCCAGACAAGCGCACCTGGCATCTACGCAGCCGGTGACTGCACCC
AGTACCCGAGCCCCATATACGGCCGACCACTTCACCTCGAGTCGGTGCACAACGCC
ATCGAACAGGCCAAAACGGCCGCCGCAGCGATCCTCGGCAGAGACGAGCCGTTCCG
TCAAGTGCCCTGGTTCTGGTCAGACCAGTACAACATCAAACTACAGACGGCCGGCGT
CAACGAAGGCTACGACGACGTGATCATCCGGGGTGATCCGGCCTCAGCATCGTTTGC

AGCCTTCTACCTGCGCGCCGGGAAACTGCTGGCCGTCGATGCAATCAACCGGCCGCG
CGAATTCATGGCGTCGAAAACCCTTATCGCCGAACGCGCAGAGGTAGACCCGACGC
AACTCGCCGACGAGAGCCTCCCCCCCACAGCCCTTGCGGCGGCGGTCAACGGCCCT
ACCCGCGCAACGTCCCCAACCTCCCTCTAA

ATGCCGAAGATCACCTACATCGACTACACCGGTACGAGCCGCTGCGTTGACGCCGA
AAACGGCATGTCACTGATGGAAATCGCCATCAATAACAACGTGCCAGGCATCGACG
GGGACTGCGGCGGGGAGTGCGCATGCGCGACATGCCATGTGCACGTCGATGCAGAC
TGGTTGGACAAACTGCCGCCCAGCAGCGACCAAGAGGTGTCAATGCTGGAATTCTGT
GATGGCGTAGACCACACATCCCGACTCGGCTGCCAAATCAAGATTTGCCCGACTTTG
GATGGCATCGTCGTACGGACACCAGCTGCACAACATTAG

AlkBGT gene cluster from ***Pseudomonas putida***

(AlkB → AlkG → AlkT)

alkB

ATGCTTGAGAAACACAGAGTTCTGGATTCCGCTCCAGAGTACGTAGATAAAAAGAA
ATATCTCTGGATACTATCAACTTTGTGGCCGGCTACTCCGATGATCGGAATCTGGCTT
GCAAATGAAACTGGTTGGGGGATTTTTTATGGGCTGGTATTGCTCGTATGGTACGGCG
CACTTCCATTGCTTGATGCGATGTTTGGTGAGGACTTTAATAATCCGCCTGAAGAAGT
GGTGCCGAAACTAGAGAAGGAGCGGTACTATCGAGTTTTGACATATCTAACAGTTCC
TATGCATTACGCTGCATTAATTGTGTCAGCATGGTGGGTCGGAACTCAGCCAATGTCT
TGGCTTGAAATTGGTGCGCTTGCCTTGTCACTGGGTATCGTGAACGGACTAGCGCTCA
ATACAGGACACGAACTCGGTCACAAGAAGGAGACTTTTGATCGTTGGATGGCCAAA
ATTGTGTTGGCTGTCGTAGGGTACGGTCACTTCTTTATTGAGCATAATAAGGGTCATC
ACCGTGATGTCGCTACACCGATGGATCCTGCAACATCCCGGATGGGAGAAAGCATTT
ATAAGTTTTCAATCCGTGAGATCCCAGGAGCATTTATTCGTGCTTGGGGGCTTGAGGA
ACAACGCCTTTCGCGCCGTGGCCAAAGCGTTTGGAGTTTCGATAATGAAATCCTCCA
ACCAATGATCATCACAGTTATTCTTTACGCCGTTCTCCTTGCCTTGTTTGGACCTAAGA
TGCTGGTGTTCCTGCCGATTCAAATGGCTTTCGGTTGGTGGCAGCTGACCAGTGCGAA
CTATATTGAACATTACGGCTTGCTCCGTCAAAAAATGGAGGACGGTCGATATGAGCA
TCAAAAGCCGCACCATTCTTGGAATAGTAATCACATCGTCTCTAATCTAGTGCTGTTC
CACCTTCAGCGGCACTCGGATCACCACGCGCATCCAACACGTTCTTATCAGTCACTT
CGGGATTTTCCCGGCCTGCCGGCTCTTCCGACGGGTTACCCTGGTGCATTTTTGATGG
CGATGATTCCTCAGTGGTTTAGATCAGTTATGGATCCCAAGGTAGTAGATTGGGCTG
GTGGTGACCTTAATAAGATCCAAATTGATGATTCGATGCGAGAAACCTATTTGAAAA
AATTTGGCACTAGTAGTGCTGGTCATAGTTCGAGTACCTCTGCGGTAGCATCGTAG

alkG

ATGGCTAGCTATAAATGCCCGGATTGTAATTATGTTTATGATGAGAGTGCGGGTAAT
GTGCATGAGGGGTTTTCTCCAGGTACGCCTTGGCACCTTATTCCTGAGGATTGGTGCT
GCCCCGATTGCGCCGTTCGAGACAAGCTTGACTTCATGTTAATTGAGAGCGGCGTAG

GTGAAAAGGGCGTCACCTCAACCCATACTTCGCCAAATTTATCCGAGGTTAGTGGCA
CAAGTTTAACTGCTGAAGCAGTGGTTGCGCCGACAAGCTTAGAGAAATTGCCTAGTG
CCGACGTTAAAGGCCAAGATCTATATAAAACTCAACCTCCAAGGTCTGATGCCCAA
GGCGGGAAAGCATACTTGAAGTGGATATGTATTACTTGTGGCCATATATATGATGAG
GCGTTGGGCGATGAGGCCGAGGGTTTTACTCCAGGTACTCGCTTTGAGGATATTCCTG
ATGACTGGTGCTGTCCGGATTGCGGGGCTACGAAAGAAGACTATGTGCTCTACGAGG
AAAAGTGA

alkT

CTAATCAGGTAATTTTATACTCCCTGCAAGCGCACCTTGACGTTTAGGCAAATTTATT
GTCTCAGTTGCAATCAGTCGCTCCTGCTTGTACGCAAGGACTTCTAGTTCAAGAGTTT
CGTTATTAATTGCAACAACGAGTTTATCGTAGTCCTTTAGAGCACCAAGTCCTTGCAG
CGCCATCCCTTTAAGATCAGACCAGAACCGTGGTGGGGTTGGTGCTGGTGTTGATGT
GCCACAGATGCTACTTGCGACAATTTGAGCGTGTGTAACCGCATTATGAATTGTCTCT
AAACGTACCATCGTTCCCCAAAAAGGATTTCTAGCCATTGCGCAGTCGCCGATTGCA
TATATACTTGTATCCGATGTACACATCTGATCATCGACCACAACACCATTACTCACTT
CAAGGGCCGCCTCAGTTGCCAGCTCTAGCTCTGGGATAGCACCGATTCCAACTACAA
TCAGATCCGCCTGAATTTCTTCTCCACTTTCAAGTACGCATTGTTCAACATGGCCATTC
CTGCCCTTTATAGACGTTAATTTCGCATTCAGCTTGAACTCAATTCCTTCAGCCTCCAG
GCGGGCTCTGACTAAGTTTGCTGCTGCCGGCGTAACCACGCGCGCCATTACACGCGG
GGTGGCTTCTATCACTGTGACCCTCTTCCCTAAGCCCACCGCAGCTGAGGCGACTTCA
AGCCCGATTACTCCGCCGCCCAACACAACAACAGACGCACTCTCCACAAGTTTCCTA
CGTAAATTTTTGGCGTCTTCCATACTGCGTAAATAGCAGACCCCAGACAGTTCAGAC
CCCTCGCAGGTTAACCTACGTGCGCTAGCAGGTGTTGCAAGAATCAATTTTTCATAC
GCGTATTCTTTTCCATCTTTAGAAGAAACTATCTTACGCCCCACGTCGATTGATACAA
TCGGTGTATTTAACGAAATGGTAATATTGTTATTCGTATAAAAACCTTCTGGCTTTAA
TGGCACTGCGGATTCTGCAATCTCACTTGTCAGAAAAGCCTTGGATAGAGGAGGCCG
CTGATAAGGCGCCACAGACTCCCTGCTAAAAATCCTAATTTCCCCTTTATAACCATAT
TGACGAAGCCAGAACGCAGCATTTACTCCAGCTGTACCAGCGCCAACAACAACGAT
TGCCAT

AlkL gene from ***Pseudomonas putida***

ATGAGTTTTTCTAATTATAAAGTAATCGCGATGCCGGTGTTGGTTGCTAATTTTGTTTT
GGGGGCGGCCACTGCATGGGCGAATGAAAATTATCCGGCGAAATCTGCTGGCTATA
ATCAGGGTGACTGGGTCGCTAGCTTCAATTTTTCTAAGGTCTATGTGGGTGAGGAGCT
TGGCGATCTAAATGTTGGAGGGGGGGCTTTGCCAAATGCTGATGTAAGTATTGGTAA
TGATACAACACTTACGTTTGATATCGCCTATTTTGTTAGCTCAAATATAGCGGTGGAT
TTTTTTGTTGGGGTGCCAGCTAGGGCTAAATTTCAAGGTGAGAAATCAATCTCCTCGC
TGGGAAGAGTCAGTGAAGTTGATTACGGCCCTGCAATTCTTTCGCTTCAATATCATTA
CGATAGCTTTGAGCGACTTTATCCATATGTTGGGGTTGGTGTTGGTCGGGTGCTATTTT
TTGATAAAACCGACGGTGCTTTGAGTTCGTTTGATATTAAGGATAAATGGGCGCCTG
CTTTTCAGGTTGGCCTTAGATATGACCTTGGTAACTCATGGATGCTAAATTCAGATGT
GCGTTATATTCCTTTCAAAACGGACGTCACAGGTACTCTTGGCCCGGTTCCTGTTTCT
ACTAAAATTGAGGTTGATCCTTTCATTCTCAGTCTTGGTGCGTCATATGTTTTCTAA

Pfor$_{116B3}$ gene fragment from ***Rhodococcus ruber*** **DSM 44319**

GTGCTGCAACGGCAGCATCCCGTCACCATCGGCGAGCCCTCCACCCGGTCGGTGTCA
CGCACCGTCACCGTCGAGCGCCTGGACCGGATCGTCGACGACGTGCTGCGCGTCGTC
CTACGGGCTCCTGCAGGAAATGCGTTGCCCGCGTGGACTCCTGGCGCCCACATCGAT
GTCGACCTCGGTGCGCTGTCGCGGCAGTACTCCCTGTGCGGTGCGCCCGACGCGCCC
ACCTACGAGATCGCCGTTCTGCTGGACCCCGAGAGCCGCGGTGGCTCGCGCTACGTC
CACGAACAGCTCCGGGTGGGGGGATCGCTCCGGATTCGCGGGCCCCGGAACCACTT
CGCGCTCGACCCCGACGCCGAGCACTACGTGTTCGTGGCCGGCGGCATCGGCATCAC
CCCCGTCCTGGCCATGGCCGACCACGCCCGCGCCCGGGGGTGGAGCTACGAACTGC
ACTACTGCGGCCGGAACCGTTCCGGGATGGCCTATCTCGAGCGGGTCGCCGGGCACG
GGGACCGCGCCGCCCTGCACGTCTCGGCGGAAGGCACCCGGGTCGACCTCGCCGCC
CTCCTCGCGACGCCGGTGTCCGGCACCCAGATCTACGCGTGCGGGCCCGGACGGCTG
CTCGCCGGACTCGAGGACGCGAGCCGGCACTGGCCCGACGGTGCGCTGCACGTCGA
GCACTTCACCTCGTCCCTCACGGCACTCGACCCGGACGTCGAGCACGCCTTCGACCT
CGACCTGCGCGACTCGGGACTCACCGTGCGGGTCGAGCCCACCCAGACCGTCCTCG
ACGCGTTGCGCGCCAACAACATCGACGTGCCCAGCGACTGCGAGGAAGGCCTCTGC
GGCTCCTGCGAGGTCACCGTCCTCGAAGGCGAGGTCGACCACCGCGACACCGTGCT
CACCAAGGCCGAGCGGGCGGCGAACCGGCAGATGATGACCTGCTGCTCGCGTGCCT
GCGGCGACCGACTGACCCTCCGACTCTGA

Codon optimized (geneart) Pfor$_{116B3}$ gene fragment from ***Rhodococcus ruber*** **DSM 44319**

GTTCTGCAGCGTCAGCATCCGGTTACCATTGGTGAACCGAGCACCCGTAGCGTTAGC
CGTACCGTTACCGTTGAACGTCTGGATCGTATTGTTGATGATGTTCTGCGTGTTGTTCT
GCGTGCACCGGCAGGTAATGCACTGCCTGCATGGACACCGGGTGCACATATTGATGT
TGATCTGGGTGCACTGAGCCGTCAGTATAGCCTGTGTGGTGCACCTGATGCACCGAC
CTATGAAATTGCAGTTCTGCTGGATCCGGAAAGCCGTGGTGGTAGCCGTTATGTTCAT
GAACAGCTGCGTGTTGGTGGTAGCCTGCGTATTCGTGGTCCGCGTAATCATTTTGCAC
TGGATCCGGATGCAGAACATTATGTTTTTGTTGCCGGTGGTATTGGTATTACACCGGT
GCTGGCAATGGCAGATCATGCACGTGCTCGTGGTTGGAGCTATGAACTGCATTATTG
TGGTCGTAATCGTAGCGGTATGGCATATCTGGAACGTGTTGCAGGTCATGGTGATCGT
GCAGCTCTGCATGTTAGTGCCGAAGGCACCCGTGTTGATCTGGCAGCACTGCTGGCA
ACACCGGTGAGCGGCACCCAGATTTATGCATGTGGTCCGGGTCGTCTGCTGGCAGGT
CTGGAAGATGCAAGCCGTCATTGGCCTGATGGTGCACTGCATGTTGAACATTTTACC
AGCAGCCTGACCGCACTGGATCCTGACGTTGAACATGCATTTGATCTGGATCTGCGT
GATAGTGGTCTGACCGTTCGTGTTGAACCGACCCAGACCGTTCTGGATGCACTGCGT
GCAAATAATATTGATGTTCCGAGCGATTGCGAAGAGGGTCTGTGTGGTAGCTGTGAA
GTTACCGTGCTGGAAGGTGAAGTTGATCATCGTGATACCGTTCTGACCAAAGCAGAA
CGTGCAGCAAATCGTCAGATGATGACCTGTTGTAGCCGTGCATGTGGTGATCGTCTG
ACCCTGCGTCTGTAA

CPR$_{BM3}$ reductase from ***Bacillus megaterium***

ATTCCTTCACCTAGCACTGAACAGTCTGCTAAAAAAGTACGCAAAAAGGCAGAAAA
CGCTCATAATACGCCGCTGCTTGTGCTATACGGTTCAAATATGGGAACAGCTGAAGG
AACGGCGCGTGATTTAGCAGATATTGCAATGAGCAAAGGATTTGCACCGCAGGTCG
CAACGCTTGATTCACACGCCGGAAATCTTCCGCGCGAAGGAGCTGTATTAATTGTAA
CGGCGTCTTATAACGGTCATCCGCCTGATAACGCAAAGCAATTTGTCGACTGGTTAG
ACCAAGCGTCTGCTGATGAAGTAAAAGGCGTTCGCTACTCCGTATTTGGATGCGGCG
ATAAAAACTGGGCTACTACGTATCAAAAAGTGCCTGCTTTTATCGATGAAACGCTTG
CCGCTAAAGGGGCAGAAAACATCGCTGACCGCGGTGAAGCAGATGCAAGCGACGA
CTTTGAAGGCACATATGAAGAATGGCGTGAACATATGTGGAGTGACGTAGCAGCCT
ACTTTAACCTCGACATTGAAAACAGTGAAGATAATAAATCTACTCTTTCACTTCAATT
TGTCGACAGCGCCGCGGATATGCCGCTTGCGAAAATGCACGGTGCGTTTTCAACGAA
CGTCGTAGCAAGCAAAGAACTTCAACAGCCAGGCAGTGCACGAAGCACGCGACATC
TTGAAATTGAACTTCCAAAAGAAGCTTCTTATCAAGAAGGAGATCATTTAGGTGTTA
TTCCTCGCAACTATGAAGGAATAGTAAACCGTGTAACAGCAAGGTTCGGCCTAGATG
CATCACAGCAAATCCGTCTGGAAGCAGAAGAAGAAAAATTAGCTCATTTGCCACTC
GCTAAAACAGTATCCGTAGAAGAGCTTCTGCAATACGTGGAGCTTCAAGATCCTGTT
ACGCGCACGCAGCTTCGCGCAATGGCTGCTAAAACGGTCTGCCCGCCGCATAAAGT
AGAGCTTGAAGCCTTGCTTGAAAAGCAAGCCTACAAAGAACAAGTGCTGGCAAAAC
GTTTAACAATGCTTGAACTGCTTGAAAAATACCCGGCGTGTGAAATGAAATTCAGCG
AATTTATCGCCCTTCTGCCAAGCATACGCCCGCGCTATTACTCGATTTCTTCATCACCT
CGTGTCGATGAAAAACAAGCAAGCATCACGGTCAGCGTTGTCTCAGGAGAAGCGTG
GAGCGGATATGGAGAATATAAAGGAATTGCGTCGAACTATCTTGCCGAGCTGCAAG
AAGGAGATACGATTACGTGCTTTATTTCCACACCGCAGTCAGAATTTACGCTGCCAA
AAGACCCTGAAACGCCGCTTATCATGGTCGGACCGGGAACAGGCGTCGCGCCGTTT
AGAGGCTTTGTGCAGGCGCGCAAACAGCTAAAAGAACAAGGACAGTCACTTGGAGA
AGCACATTTATACTTCGGCTGCCGTTCACCTCATGAAGACTATCTGTATCAAGAAGA
GCTTGAAAACGCCCAAAGCGAAGGCATCATTACGCTTCATACCGCTTTTTCTCGCAT
GCCAAATCAGCCGAAAACATACGTTCAGCACGTAATGGAACAAGACGGCAAGAAA
TTGATTGAACTTCTTGATCAAGGAGCGCACTTCTATATTTGCGGAGACGGAAGCCAA
ATGGCACCTGCCGTTGAAGCAACGCTTATGAAAAGCTATGCTGACGTTCACCAAGTG
AGTGAAGCAGACGCTCGCTTATGGCTGCAGCAGCTAGAAGAAAAAGGCCGATACGC
AAAAGACGTGTGGGCTGGGTAA

Putidaredoxin reductase (CamA) from ***Pseudomonas putida***

GTGAACGCAAACGACAGCGGCTGGGAAGGCAATATCCGGTTGGTGGGGGATGCGAC
GGTAATTCCCCATCACCTACCACCGCTATCCAAAGCTTACTTGGCCGGCAAAGCCAC
AGCGGAAAGCCTGTACCTGAGAACCCCAGATGCCTATGCAGCGCAGAACATCCAAC
TACTCGGAGGCACACAGGTAACGGCTATCAACCGCGACCGACAGCAAGTAATCCTA
TCGGATGGCCGGGCACTGGATTACGACCGGCTGGTATTGGCTACCGGAGGGCGTCCA
AGACCCCTACCGGTGGCCAGTGGCGCAGTTGGAAAGGCGAACAACTTTCGATACCT

GCGCACACTCGAGGACGCCGAGTGCATTCGCCGGCAGCTGATTGCGGATAACCGTCT
GGTGGTGATTGGTGGCGGCTACATTGGCCTTGAAGTGGCTGCCACCGCCATCAAGGC
GAACATGCACGTCACCCTGCTTGATACGGCAGCCCGGGTTCTGGAGCGGGTTACCGC
CCCGCCGGTATCGGCCTTTTACGAGCACCTACACCGCGAAGCCGGCGTTGACATACG
AACCGGCACGCAGGTGTGCGGGTTCGAGATGTCGACCGACCAACAGAAGGTTACTG
CCGTCCTCTGCGAGGACGGCACAAGGCTGCCAGCGGATCTGGTAATCGCCGGGATTG
GCCTGATACCAAACTGCGAGTTGGCCAGTGCGGCCGGCCTGCAGGTTGATAACGGC
ATCGTGATCAACGAACACATGCAGACCTCTGATCCCTTGATCATGGCCGTCGGCGAC
TGTGCCCGATTTCACAGTCAGCTCTATGACCGCTGGGTGCGTATCGAATCGGTGCCCA
ATGCCTTGGAGCAGGCACGAAAGATCGCCGCCATCCTCTGTGGCAAGGTGCCACGC
GATGAGGCGGCGCCCTGGTTCTGGTCCGATCAGTATGAGATCGGATTGAAGATGGTC
GGACTGTCCGAAGGGTACGACCGGATCATTGTCCGCGGCTCTTTGGCGCAACCCGAC
TTCAGCGTTTTCTACCTGCAGGGAGACCGGGTATTGGCGGTCGATACAGTGAACCGT
CCAGTGGAGTTCAACCAGTCAAAACAAATAATCACGGATCGTTTGCCGGTTGAACCA
AACCTACTCGGTGACGAAAGCGTGCCGTTAAAGGAAATCATCGCCGCCGCCAAAGC
TGAACTGAGTAGTGCCTGA

Putidaredoxin (CamB) from ***Pseudomonas putida***

ATGTCTAAAGTAGTGTATGTGTCACATGATGGAACGCGTCGCGAACTGGATGTGGCG
GATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCAATGGTATCTACGATATTGTCGGT
GATTGTGGCGGCAGCGCCAGCTGTGCCACCTGCCATGTCTATGTGAACGAAGCGTTC
ACGGACAAGGTGCCCGCCGCCAACGAGCGGGAAATCGGCATGCTGGAGTGCGTCAC
GGCCGAACTGAAGCCGAACAGCAGGCTCTGCTGCCAGATCATCATGACGCCCGAGC
TGGATGGCATCGTGGTCGATGTTCCCGATAGGCAATGGTAA

6.2.1. RIS1 and RIS2

Figure 26: RIS1 and RIS2 (reductase interaction site 1&2) analyses of heme-domains of different P450s (P450 BM3, P450cam, CYP153AaciA, CYP153A13a, CYP153A$_{M.aq.}$ and CYP153A$_{P.sp.}$) *via* the CYPED based on the suggestions of Sirim and coworkers.[79, 81]