# Biocatalytic allylic oxidation with bacterial P450 monooxygenases

Von der Fakultät für Chemie der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

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Tag der mündlichen Prüfung: 20.10.2023

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2023

**STATUTORY DECLARATION**

I hereby affirm that I have produced the present work independently and sousing the resources and literature provided.

Stuttgart, 19.06.2023 _____

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**Acknowledgment**

With immense joy, I express my gratitude on the first page of my doctoral dissertation. Without your support, assistance, and inspiration, it would not have been possible to reach this significant milestone in my academic career.

Firstly, I would like to thank Prof. Vlada Urlacher, Prof. Bernhard Hauer and Prof. Rolf Schmid who have guided my doctoral thesis and provided me with valuable advice and suggestions. Thanks to your professionalism, your knowledge, and your experience, my work has become more comprehensive and valuable.

I cannot forget the people with whom I have worked on publications that have contributed to the development of my academic work. Thanks to Prof. S. Laschat, Prof. M. C. Flickinger, Prof. J. Pleiss, Dr. A. Busch, Dr. Y. Khatri, Dr. Clemens Buhler. Thanks to you, I was able to gain valuable experiences and knowledge that benefitted my doctoral thesis. I would also like to thank my colleagues special Dr. Marco Girhard, Dr. Evelyne Weber, Dr. Demet Sirim, Dr. Alexander Seifert. Once again, thank you for your support and above all, for your time and kindness. I am proud of the achievements that I have attained thanks to you, and hope that our collaboration will continue in the future.

I would like to express my heartfelt gratitude to all of you for your tremendous support and assistance during my doctoral journey. I would like to extend a special thank you to my son Kuba Botan Bogazkaya, who has been a constant source of encouragement and motivation.

To my parents, Alicja and Jan Romankiewicz and brother Pawel Romankiewicz, cousin Kuba Ostwald. I am forever grateful for your unwavering belief in me and for providing me with the guidance and wisdom that have shaped my academic pursuits. Your love and support have been invaluable throughout this challenging process.

**Abbreviations**

| | |
|---|---|
| A | absorbance |
| APS | ammonium persulfate |
| bp | base pair(s) |
| CIAP | calf intestinal alkaline phosphatase |
| CYP | cytochrome P450 monooxygenase |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | dexynucleoside-5'-triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide, also known as riboflavin |
| GC | gas chromatography |
| GC/MS | gas chromatography/ mass spectroscopy |
| IPTG | isopropyl-β-D-thiogalactoside |
| Kb | kilobase |
| kDa | kilo Dalton |
| KPi | potassium phosphate buffer |
| LB | Luria-Bertani |
| M | mole per liter |
| mg | milligram |
| min | minute |
| ml | milliliter |
| mol | mole |
| NADH | β-nicotine adenine dinucleotide |
| NADPH | β-nicotine adenine dinucleotide phosphate |
| nm | nanometer |
| OD | optical density |
| PCR | polymerase chain reaction |
| PDB | Protein Data Bank |
| *p*NCA | *para*-nitrophenoxycarboxylic acid |
| $R_f$ | retardation factor |
| rpm | revolutions per minute |
| RT | retention time |

| | |
|---|---|
| sec | second |
| SDS | sodium dodecyl sulfate |
| TAE | Tris/acetate/EDTA buffer |
| TEMED | N, N, N’, N’-tetramethyl-ethylendiamine |
| TLC | thon layer chromatography |
| Tris | tris-(hydroxymethyl)-aminomethane |
| UV | ultraviolet |
| $^{o}C$ | degree Celsius |
| λ | wavelength |
| µg | microgram |

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# 1. Zusammenfassung

Diese Studie beschäftigt sich mit Cytochrom P450 Monooxygenasen, einer einzigartigen Enzymgruppe, die komplexe chemische Reaktionen katalysiert, darunter die Oxidation von zyklischen und azyklischen (Poly-)enen sowie Terpenen. Ihre Besonderheit liegt in der Fähigkeit, diese Reaktionen auf regio-, chemo- und stereoselektive Weise auszuführen, was die gezielte Oxidation spezifischer Molekülteile, die Durchführung bestimmter Reaktionstypen und die Erzeugung spezifischer räumlicher Anordnungen von Atomen oder Gruppen in Molekülen ermöglicht.

Die Herausforderung liegt darin, dass trotz der leichten Verfügbarkeit und günstigen Kosten der Ausgangsstoffe (Substrate), die Herstellung der oxidierten Produkte durch traditionelle chemische Verfahren schwierig und teuer ist. Die Cytochrom P450 Monooxygenasen bieten hier eine effiziente und kosteneffektive Lösung. Sie können eine breite Palette organischer Verbindungen zu Alkoholen oxidieren, indem sie atmosphärischen Sauerstoff verwenden. Bemerkenswert ist ihre Fähigkeit zur allylischen Hydroxylierung von Terpenen, einer hochwertigen chemischen Transformation (Abbildung 1-1).

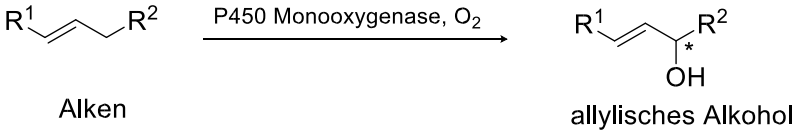



**Abbildung 1-1**: Enzymatische Hydroxylierung von Alkenen

Diese Forschungsarbeit konzentriert sich auf die Identifizierung und Charakterisierung geeigneter Cytochrom P450 Monooxygenasen (CYPs) in *Streptoyceten*. Durch eine Dünnschichtchromatographie (DC)-Methode wurden zwei vielversprechende *Streptomyces*-Stämme identifiziert. Aus diesen Stämmen wurden die Enzyme CYP105A1, CYP105B1, CYP105D5 und CYP170A1 kloniert und in *E. coli* zur weiteren Untersuchung exprimiert. Da die natürlichen Redoxpartner dieser CYPs unbekannt sind, wurde ein heterologes Redoxsystem aus *Pseudomonas putida* verwendet. Diese Arbeit liefert wichtige Einblicke für die industrielle Anwendung dieser Enzyme in der biokatalytischen Oxidation.

Interessanterweise zeigten die Studienergebnisse, dass drei der ausgewählten CYPs

(CYP105B1, CYP105D5 und CYP170A1) zyklische Modell Substrate wie α- oder β-Ionon auf eine regioselektive Weise zu Allylalkoholen hydroxylierten. Diese Reaktion erzeugt spezifisch Allylalkohole, was eine hohe Regioselektivität zeigt - das heißt, die Enzyme zielten gezielt auf bestimmte Teile der Moleküle ab, um sie zu oxidieren.

Andererseits führte die Oxidation von azyklischen Substraten mit diesen Enzymen zu einer Mischung von regioisomeren Epoxiden. Dies zeigt eine Vielseitigkeit der Enzyme, aber auch eine Variation in der Produktverteilung, abhängig von der Struktur des Ausgangssubstrats (Abbildung 1-2).

Diese Ergebnisse tragen dazu bei, das Verständnis für die Funktionsweise und die potenzielle Anwendung von CYPs in der Synthese von spezifischen und wertvollen Produkten zu erweitern. Sie unterstreichen das Potenzial von CYPs als leistungsstarke Werkzeuge in der biotechnologischen und pharmazeutischen Industrie, wo selektive und effiziente Oxidationsmethoden dringend benötigt werden.

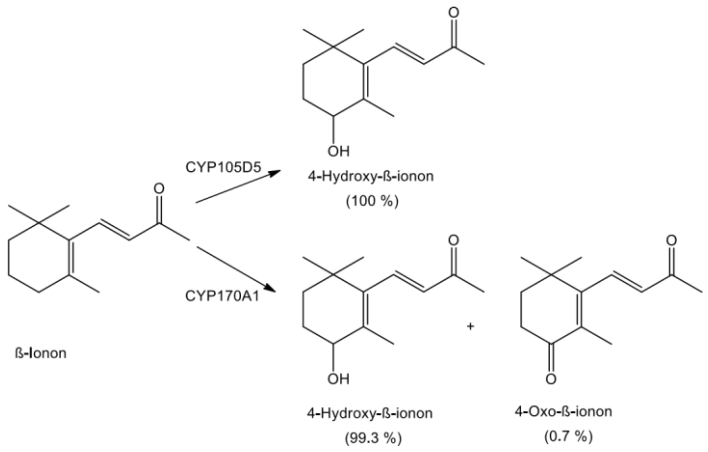



**Abbildung 1-2**: Oxidation von β-Ionon durch CYP105D5 and CYP170A1 aus *S. coelicolor A3 (2)*

Im Streben nach P450 Monooxygenasen (CYPs), die bei der selektiven allylischen Hydroxylierung von Terpenen aktiv sind, wurde eine erweiterte Suche in Zusammenarbeit mit der japanischen Firma Kirin Holdings durchgeführt. Hierbei wurde eine bakterielle CYP-Bibliothek durchforstet, um geeignete Kandidaten aus *Streptomyceten* zu identifizieren. *E. coli*-basierte Ganzzellkatalysatoren wurden zur Bestimmung der Enzymaktivität genutzt. Aus einer Bibliothek von 213 bakteriellen CYPs wurden zwei Vertreter der CYP154-Familie ausgewählt: CYP154A aus *Nocardia farcinica* und CYP154E1 aus *Thermobifida fusca YX*. Beide zeigten Monooxygenasenaktivität mit azyklischen Modellsubstraten. CYP154A zeigte die Fähigkeit, Nerol zu 8-Hydroxynerol und zwei weiteren noch unbekannten Produkten zu oxidieren. Bei der Reaktion mit

Nerylaceton entstanden 11-Hydroxy-, 12- Hydroxynerylaceton und ein 9,10-Epoxid. CYP154E1 zeigte eine einzigartige Fähigkeit: Es konnte azyklische cis/trans-Isomere sowohl chemoselektiv als auch regioselektiv an der terminalen allylischen Position hydroxylieren. Bei der Umsetzung von Geraniol und Nerol erzeugte CYP154E1 die terminalen Hydroxyprodukte 8-Hydroxygeraniol und 8-Hydroxynerol. Die Arbeit hebt die erstaunliche Diversität und Potenzial der CYPs in Bezug auf die selektive Oxidation von Substraten hervor. Diese Forschung unterstreicht weiterhin die Rolle von *Streptomyceten* als wertvolle Quelle für neuartige und effiziente biokatalytische Werkzeuge (Abbildung 1-3).

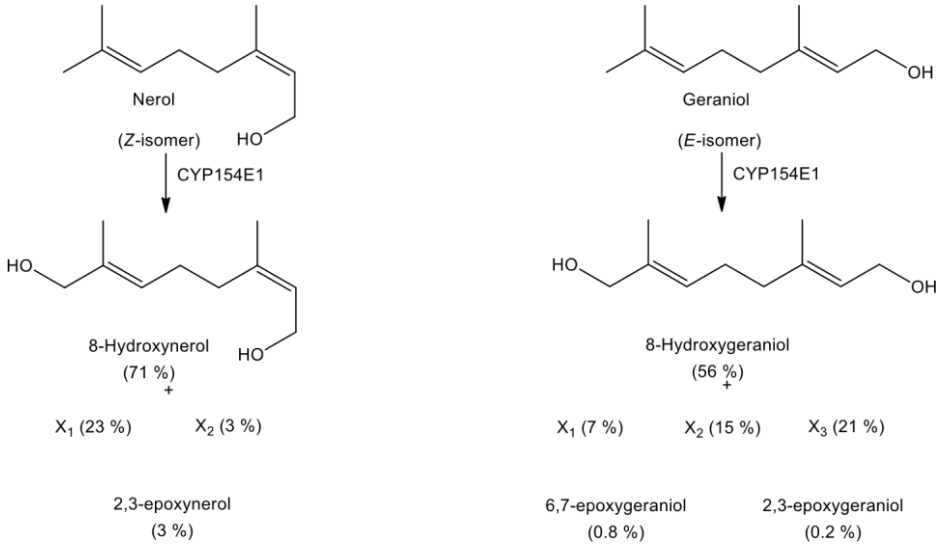



**Abbildung 1-3**: Oxidation von Nerol und Geraniol durch CYP154E1

Die Konversionen bei den durch CYPs katalysierten Prozessen waren bemerkenswert hoch (über 99 %). Bei der *in vivo*-Umsetzung von Geranylaceton wurden hauptsächlich Alkohole produziert: 8-Hydroxy-, 12-Hydroxy- und 11-Hydroxygeranylaceton, mit nur geringen Mengen an 9,10-Epoxygeranylaceton. Eine ähnliche Produktverteilung wurde bei der Umsetzung von Nerylaceton beobachtet, wobei 8-Hydroxy-, 12-Hydroxy- und 11-Hydroxynerylaceton entstanden. Hier wurde nur eine geringe Menge an Epoxidprodukt (etwa 1%) detektiert. Diese Ergebnisse unterstreichen die beeindruckende Effizienz und Selektivität der Cytochrom P450 Monooxygenasen bei der Oxidation von Terpenen. Mit fast vollständigen Umsätzen und der Fähigkeit, eine Vielzahl von Hydroxyderivaten zu erzeugen, zeigen diese Enzyme ihr Potenzial als äußerst wirksame biokatalytische Werkzeuge. Dies könnte erhebliche Auswirkungen

auf industrielle Anwendungen haben, insbesondere in Bereichen, in denen die selektive Funktionalisierung von Molekülen eine zentrale Rolle spielt, wie beispielsweise in der Pharmazie und der Feinchemie (Abbildung 1-4).

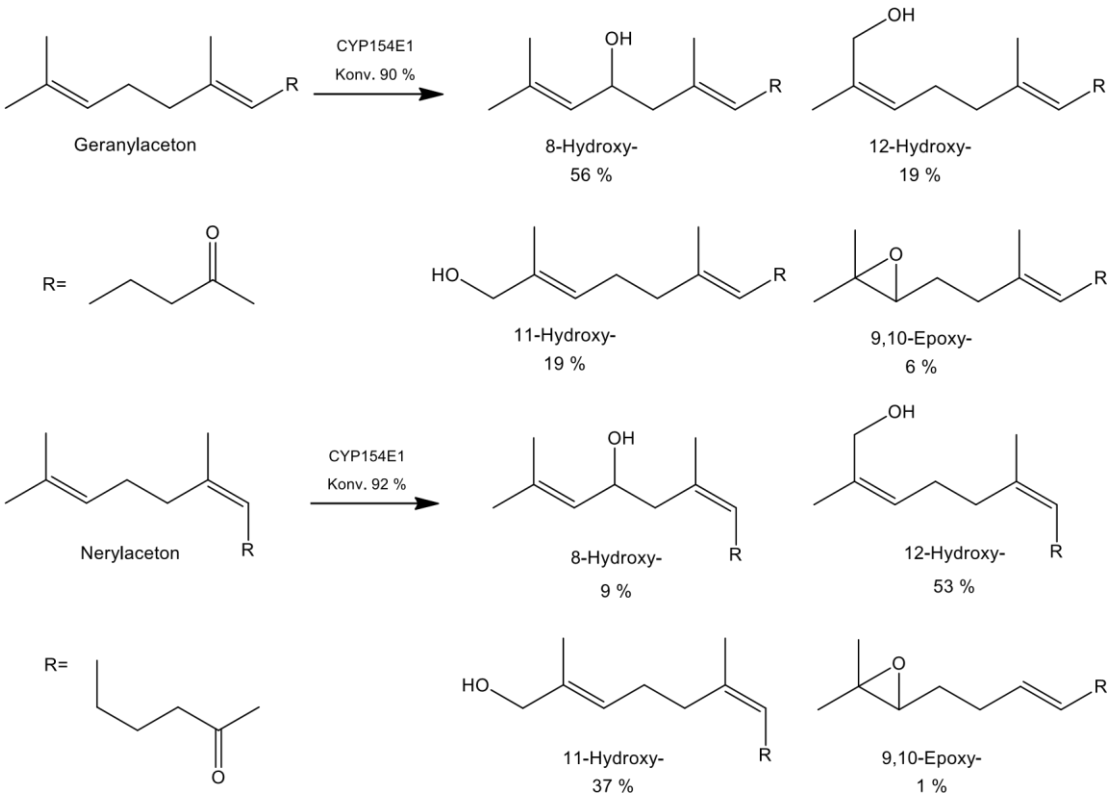



**Abbildung 1-4**: Oxidation *in vivo* von Geranylaceton und Nerylaceton

Durch den Einsatz von Proteindesign-Techniken wurde es möglich, Varianten des Enzyms CYP154E1 zu erstellen, die eine veränderte Selektivität bei der Oxidation azyklischer Modellsubstrate zeigten. Dies wurde durch eine Zusammenarbeit mit der Gruppe von Prof. Dr. J. Pleiss erreicht, in deren Rahmen Homologie-Modelle erstellt und molekulardynamische Simulationen durchgeführt wurden. Drei der vorgeschlagenen Mutanten, CYP154E1_V286L, CYP154E1_V286A und CYP154E1_V286F, zeigten dabei bemerkenswerte Unterschiede in ihrer Chemoselektivität. Die Variante CYP154E1_V286L beispielsweise oxidierte Geraniol und Nerol zu einer Mischung aus 6,7-Epoxy- und 2,3,6,7-Diepoxygeraniol, wobei im Gegensatz zum Wildtyp keine Alkohole detektiert wurden. Die Mutante CYP154E1_V286F zeigte eine hohe Chemoselektivität und oxidierte Geranylaceton zu einer Mischung von Allylalkoholen mit Hydroxygruppen an den Positionen C8, C12 und C11. Diese Befunde deuten auf ein hohes Potenzial für die maßgeschneiderte

Optimierung der P450 Enzyme hin, um die Effizienz und Selektivität für spezifische Reaktionen zu erhöhen. Darüber hinaus zeigte CYP154E1 eine beeindruckende Breite in der Substratspezifität, da es nicht nur Alkene, sondern auch *n*-Alkane und aliphatische Alkohole oxidieren konnte. Zum Beispiel, *n*-Heptan und *n*-Octan wurden zu einer Mischung aus ihren entsprechenden Alkoholen und Ketonen umgesetzt. Interessanterweise zeigte das Enzym eine deutlich erhöhte Aktivität bei der Umwandlung sekundärer Alkohole in die entsprechenden Ketone, verglichen mit der Hydroxylierung des Ausgangsalkans. Diese Arbeiten unterstreichen das außergewöhnliche Potenzial von Cytochrom P450 Enzymen in der Biokatalyse und die Möglichkeit ihrer gezielten Anpassung durch Proteindesign. Diese Fähigkeit zur gezielten Modifikation und Optimierung könnte es ermöglichen, leistungsfähige biokatalytische Werkzeuge für eine breite Palette von industriellen Anwendungen zu entwickeln.

Zur Vereinfachung der Anwendung dieser Ganzzellkatalysatoren in industriellen Prozessen wurde ein innovativer Ansatz zur Immobilisierung dieser Zellen in einer Latex SF091-Schicht erarbeitet, dank der Zusammenarbeit mit Prof. Michael Flickinger von der NC State University, USA. Nach erfolgreicher Immobilisierung der Zellen zeigten die Reaktionen eine bemerkenswerte Produktselektivität: Bei der Oxidation von Nerol mit CYP154E1 wurden 97% 8-Hydroxynerol und nur 3% 2,3Epoxynerol erzeugt. Diese Ergebnisse zeigen, dass die Immobilisierungstechnik nicht nur die Handhabung und Wiederverwendung der Ganzzellkatalysatoren erleichtert, sondern auch einen wesentlichen Einfluss auf die Produktselektivität hat. Die Verwendung von Immobilisierungstechniken wie dieser kann die wirtschaftliche Rentabilität biokatalytischer Prozesse erheblich steigern, da sie die Wiederverwendung der Katalysatoren ermöglichen und die Produktreinigung erleichtern. Darüber hinaus kann die verbesserte Selektivität, die durch die Immobilisierung erzielt wird, die Notwendigkeit kostspieliger Trenn- und Reinigungsverfahren reduzieren, was zur Senkung der Produktionskosten beiträgt.

In dieser Arbeit wird auch ein biokatalytisches System vorgestellt, in dem das organische Lösungsmittel sowohl das Substrat beherbergt als auch die in situ Rückgewinnung der Reaktionsprodukte ermöglicht. Das System nutzt rekombinante *E. coli* Zellen, die in der wässrigen Phase zurückgehalten werden, während die Produkte kontinuierlich aus der organischen Phase isoliert werden können. Mehrere

organische Lösungsmittel, wie n-Octan, iso-Octan, Hexadecan und Di-'Isonyl'phthalat (DINP) mit unterschiedlichen log P-Werten, wurden getestet, zusammen mit der wasserunlöslichen ionischen Flüssigkeit 1-Butyl-3-methylimidazolium-hexafluorphosphat (BMIM [PF6]), jeweils in verschiedenen Verhältnissen zur wässrigen Phase.

# 2. Abstract

This study focuses on cytochrome P450 monooxygenases, a unique group of enzymes that catalyze complex chemical reactions, including the oxidation of cyclic and acyclic (poly)enes and terpenes. Their distinctive feature is the ability to carry out these reactions in a regio-, chemo-, and stereoselective manner, which allows the targeted oxidation of specific moieties, the performance of specific reaction types, and the generation of specific spatial arrangements of atoms or groups in molecules. The challenge is that despite the easy availability and low cost of the starting materials (substrates), the production of the oxidized products by traditional chemical processes is difficult and expensive. Cytochrome P450 monooxygenases offer an efficient and cost-effective solution here. They can oxidize a wide range of organic compounds to alcohols using atmospheric oxygen. Of note is their ability to allylic hydroxylate terpenes, a high-value chemical transformation (Figure 2-1).

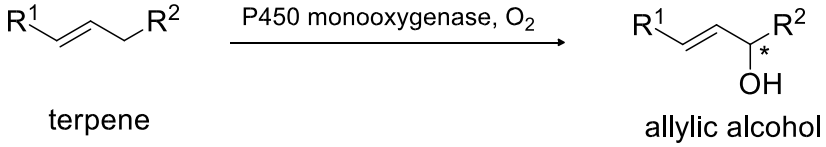



**Figure 2-1**: Biocatalytic hydroxylation of terpenes

This research focuses on the identification and characterization of suitable cytochrome P450 monooxygenases (CYPs) in Streptomyces. Two promising Streptomyces strains were identified by a thin layer chromatography (TLC) method. From these strains, the CYP105A1, CYP105B1, CYP105D5, and CYP170A1 enzymes were cloned and expressed in *E. coli* for further study. Since the natural redox partners of these CYPs are unknown, a heterologous redox system from *Pseudomonas putida* was used. This work provides important insights for the industrial application of these enzymes in biocatalytic oxidation.Interestingly, the

study results showed that three of the selected CYPs (CYP105B1, CYP105D5, and CYP170A1) hydroxylated cyclic model substrates such as α- or β-ionone to allyl alcohols in a regioselective manner. This reaction specifically generates allylic alcohols, demonstrating high regioselectivity that is, the enzymes targeted specific parts of the molecules for oxidation. On the other hand, oxidation of acyclic substrates with these enzymes resulted in a mixture of regioisomeric epoxides. This shows a versatility of the enzymes, but also a variation in the product distribution depending on the structure of the starting substrate (Figure 2-2). These results contribute to the understanding of the functioning and potential application of CYPs in the synthesis of specific and valuable products. They highlight the potential of CYPs as powerful tools in the biotechnological and pharmaceutical industries, where selective and efficient oxidation methods are urgently needed.

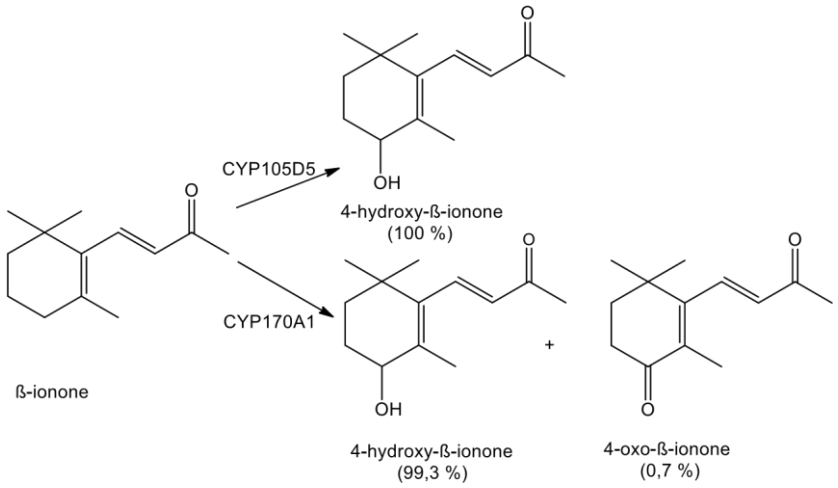



**Figure 2-2:** Oxidation of β-ionone by CYP105D5 and CYP170A1 from *S. coelicolor A3 (2)*

In pursuit of P450 monooxygenases (CYPs) active in the selective allylic hydroxylation of terpenes, an expanded search was conducted in collaboration with the Japanese company Kirin Holdings. Here, a bacterial CYP library was screened to identify suitable candidates from *Streptomyces*. *E. coli* based whole cell catalysts were used to determine enzyme activity. Two representatives of the CYP154 family were selected from a library of 213 bacterial CYPs: CYP154A from *Nocardia farcinica* and CYP154E1 from *Thermobifida fusca YX*. Both showed monooxygenase activity with acyclic model substrates. CYP154A showed the ability to oxidize nerol to 8-hydroxynerol and two other yet unknown products. Reaction with nerylacetone gave 11-hydroxy-, 12-hydroxynerylacetone and a 9,10-epoxide. CYP154E1 showed a unique ability: it could

hydroxylate acyclic cis/trans isomers both chemoselectively and regioselectively at the terminal allylic position. In the conversion of geraniol and nerol, CYP154E1 generated the Terminal hydroxy products 8-hydroxygeraniol and 8-hydroxynerol. The work highlights the amazing diversity and potential of CYPs in selective oxidation of substrates. This research further highlights the role of *Streptomyces* as a valuable source of novel and efficient biocatalytic tools (Fig. 2-3).

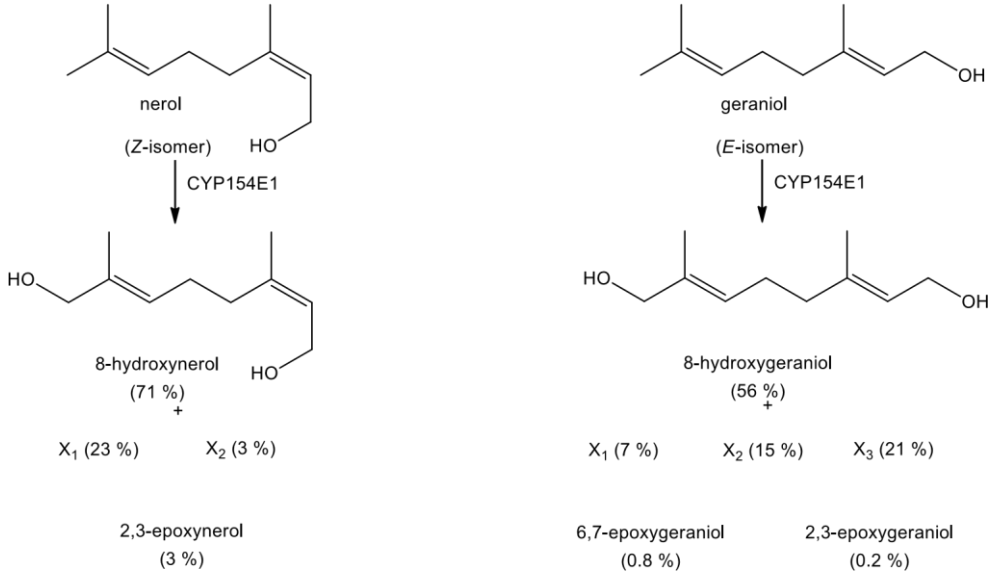



**Figure 2-3**: Oxidation of nerol and geraniol using CYP154E1.

Conversions in processes catalyzed by CYPs were remarkably high (above 99%). The *in vivo* conversion of geranylacetone produced mainly alcohols: 8-hydroxy-, 12-hydroxy-, and 11-hydroxygeranylacetone, with only minor amounts of 9,10epoxygeranylacetone. A similar product distribution was observed in the reaction of nerylacetone, giving 8-hydroxy-, 12-hydroxy-, and 11-hydroxynerylacetone. Here, only a small amount of epoxide product (about 1%) was detected. These results highlight the impressive efficiency and selectivity of cytochrome P450 monooxygenases in the oxidation of terpenes. With nearly complete conversions and the ability to generate a wide range of hydroxy derivatives, these enzymes demonstrate their potential as highly effective biocatalytic tools. This could have significant implications for industrial applications, especially in areas where selective functionalization of molecules plays a key role, such as pharmaceuticals and fine chemicals (Figure 2-4).

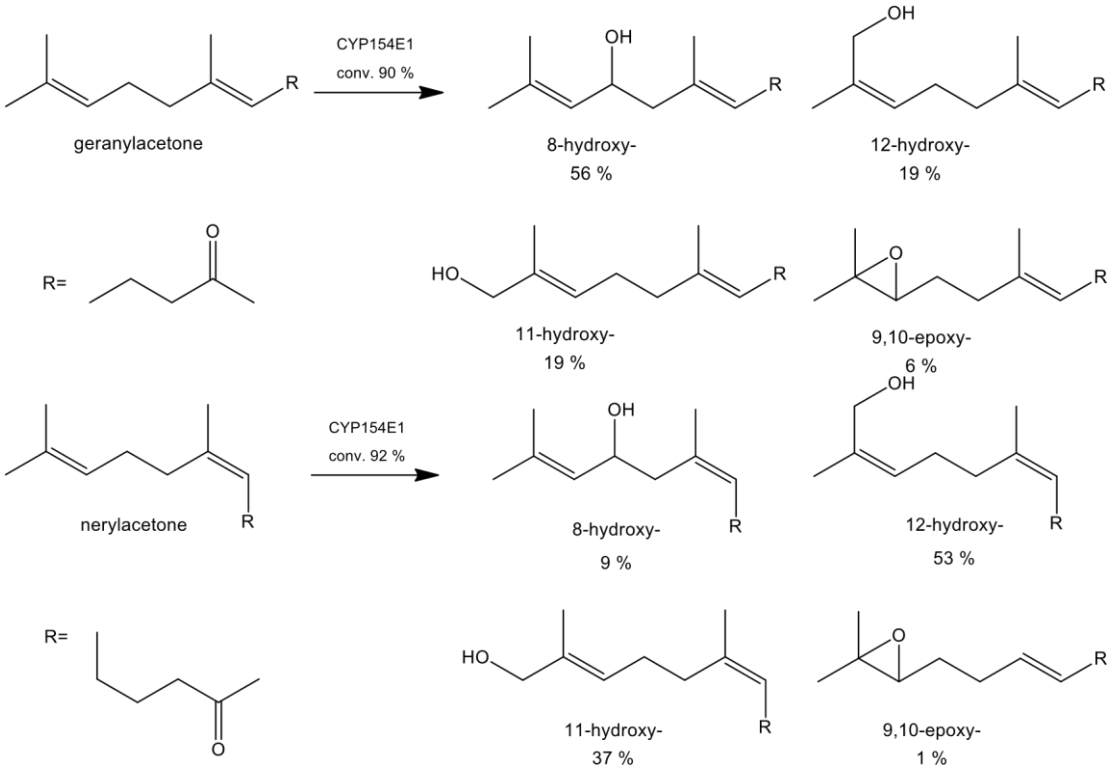



**Figure 2-4**: Oxidation *in vivo* of geranylacetone und nerylacetone

By using protein design techniques, it was possible to create variants of the CYP154E1 enzyme that showed altered selectivity in the oxidation of acyclic model substrates. This was achieved through a collaboration with the group of Prof. Dr. J. Pleiss, in which homology models were created and molecular dynamics simulations were performed. Three of the proposed mutants, CYP154E1_V286L, CYP154E1_V286A and CYP154E1_V286F, showed remarkable differences in their chemoselectivity. For example, the CYP154E1_V286L variant oxidized geraniol and nerol to a mixture of 6,7-epoxy- and 2,3,6,7-diepoxygeraniol, with no alcohols detected, in contrast to the wild type. The CYP154E1_V286F mutant showed high chemoselectivity and oxidized geranylacetone to a mixture of allyl alcohols with hydroxyl groups at positions C8, C12, and C11. These findings suggest a high potential for tailored optimization of P450 enzymes to increase efficiency and selectivity for specific reactions. Moreover, CYP154E1 showed impressive breadth in substrate specificity, as it could oxidize not only alkenes but also *n*-alkanes and aliphatic alcohols. For example, *n*-heptane and n-octane were converted to a mixture of their corresponding alcohols and ketones. Interestingly, the enzyme showed significantly increased activity in converting secondary alcohols to their corresponding ketones compared to hydroxylation of the parent alkane. This work highlights the extraordinary potential of cytochrome P450

enzymes in biocatalysis and the possibility of their targeted adaptation through protein design. This ability for targeted modification and optimization could enable the development of powerful biocatalytic tools for a wide range of industrial applications. To facilitate the application of these whole cell catalysts in industrial processes, an innovative approach to immobilize these cells in a latex SF091 layer was elaborated, thanks to the collaboration with Prof. Michael Flickinger from NC State University, USA. After successful immobilization of the cells, the reactions showed remarkable product selectivity: oxidation of nerol with CYP154E1 generated 97% 8-hydroxynerol and only 3% 2,3-epoxynerol. These results indicate that the immobilization technique not only facilitates the handling and reuse of whole-cell catalysts, but also has a significant impact on product selectivity. The use of immobilization techniques such as this can significantly increase the economic viability of biocatalytic processes by enabling catalyst reuse and facilitating product purification. In addition, the improved selectivity achieved by immobilization can reduce the need for costly separation and purification procedures, helping to lower production costs.

This work also presents a biocatalytic system in which the organic solvent both accommodates the substrate and enables in situ recovery of the reaction products. The system utilizes recombinant *E. coli* cells that are retained in the aqueous phase, while the products can be continuously isolated from the organic phase. Several organic solvents, such as *n*-octane, iso-octane, hexadecane, and di-'isonyl' phthalate (DINP) with different log P values, were tested, along with the water-insoluble ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM [PF6]), each at different ratios to the aqueous phase.

# 3. Introduction

## 3.1. Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases known as CYP, P450 or cytochromes P450 have their name after an absorption band at 450 nm of their carbon-monoxide bound form. P450s (E.C.1.14.14.1) are oxidative enzymes and they belong to class monooxygenases (E.C.1.14.x.y.) with heme as the prosthetic group. The major future is conserved cysteine setting as the fifth axial ligand to heme-iron allowed is responsable the unusual spectral properties.

Molecular oxygen is used by P450 monooxygenases to catalyze oxidation reactions. One oxygen atom is inserted into an organic substrate while another oxygen atom is reduced to the water. For iron reduction, most P450s, require compatible redox partner to deliver electrons from the reduced nicotinamide cofactor NADH or NADPH. Redox partners are represented by flavin-containing reductases, ferredoxin or flavodoxins. P450s build a larg superfamily of enzymes found in almost all organisms including plants, animals, fungi and prokaryotes. Human genome codes 57 *cyp* genes and plant *Arabidopsis thaliana* genome codes over 250 P450s while *E. coli* has no P450s. They support vital processes like biosynthesis of hormones, carcinogenesis, and production of antibiotics. P450s play a central role in detoxification of extern xenobiotics and drugs through degradation. drug metabolism. In eukaryotes P450s are bound to the endoplasmic reticulum of liver (in animals) and In prokaryotes P450s are soluble cytosolic enzymes.

Cytochrome P450 monooxygenases (CYP or P450) offer an attractive alternative to other oxidants and oxidases for several reasons.

First, their mode of action is remarkably versatile. P450 enzymes are known to process a wide range of substrates and perform various types of oxidation reactions, including hydroxylations, epoxidations, dealkylations, and more. Their ability to catalyze different types of reactions greatly increases their potential application in various fields.

Another important advantage of P450 enzymes is their selectivity. Not only can they oxidize specific bonds in complex molecules (regioselectivity), but they can also control the nature of the reaction (chemoselectivity) and influence the stereocenter of the resulting product (stereoselectivity). This precise control over the oxidation reaction is

often not achievable with chemical catalysts or other oxidases. In addition, P450 enzymes operate under mild conditions and use molecular oxygen from the air - an environmentally friendly and cost-effective oxidation source. In contrast, many traditional chemical oxidants require more stringent reaction conditions and can produce hazardous byproducts. Finally, P450 enzymes have a remarkable ability to oxidize molecules that are often difficult for other oxidases to access. They can oxidize substrates with complex structure or high stability, making them particularly useful for the synthesis of complex molecules and active pharmaceutical ingredients.In summary, cytochrome P450 monooxygenases are an attractive alternative to other oxidants and oxidases due to their versatile functionality, high selectivity, environmentally friendly and mild conditions, and ability to oxidize substrates that are difficult to access.

### 3.1.1. Cytochrome P450 superfamily

P450s were discovered in 1955 in the endoplasmic reticulum of the rat (Axelrod, 1955). In the name “P450” the letter “P” represents the word “pigment” and the number “450” describes maximum absorption wavelength while the enzyme is reduced and complexes with CO. This reaction is used in practice as a quantitative method for P450 detection (Omura and Sato 1964a, b). All genes of this large superfamily are the result of duplication and evolution of divergence of developed copies what gives a wide-range substrate specificity (Stryer 2005). There are 2252 CYP families, 41048 classified, 306707 unclassified sequences (database sequences January 2018; Urlacher et al. 2019).

In the official nomenclature “CYP” is a signature of genes encoding CYP enzymes followed by the number of gene families. Capital letter is the inscription of the superfamily and the next number is for the individual gene.

However, some P450s have names not corresponding to the CYP nomenclature . For example, CYP51A1 (lanosterol 14-α-demethylase), not officially abbreviated to **LDM** according to its substrate **L**anosterol and activity **D**e**M**ethylation (Waterman et. al., 1996).

According to CYP nomenclature, enzymes with 40 % amino acid identity belong to the same CYP family and with >55 % amino acid identity to the same subfamily (http://drnelson.uthsc.edu/CytochromeP450.html, http://www.cypalleles.ki.se/).

### 3.1.2. Challenges in the Identification of Physiological Redox Partners and Substrates for P450 Enzymes

The identification of physiological redox partners and substrates for P450 enzymes is a complex and challenging task. Several factors contribute to the difficulty of this process. Firstly, P450 enzymes are highly diverse and exist in many isoforms, each with distinct substrate preferences and catalytic activities. The human genome, for example, encodes for more than 50 different P450 enzymes. This diversity makes it challenging to predict the specific substrates and redox partners for each individual enzyme.

Secondly, P450 enzymes often exhibit promiscuity, meaning they can catalyze the oxidation of multiple structurally diverse compounds. This broad substrate specificity further complicates the identification of physiological substrates, as there may be a wide range of potential molecules that can interact with a given P450 enzyme. Additionally, the redox partners of P450 enzymes are typically electron transfer proteins or coenzymes, such as cytochrome P450 reductase. These redox partners play a crucial role in delivering electrons to the P450 enzyme during the catalytic cycle. However, the specific redox partners for each P450 isoform may vary, and their identification requires extensive experimental investigation.

Moreover, the physiological substrates and redox partners for P450 enzymes are often endogenous compounds found in complex biological systems. Their identification requires a comprehensive understanding of the metabolic pathways, biochemical interactions, and physiological conditions in which the enzymes operate. This necessitates the use of advanced analytical techniques, such as mass spectrometry, metabolomics, and proteomics, which can be technically demanding and time-consuming.

In summary, the identification of physiological redox partners and substrates for P450 enzymes is a challenging task due to the diversity of P450 isoforms, their promiscuity, the complexity of endogenous biological systems, and the need for sophisticated experimental approaches. However, understanding these interactions is crucial for elucidating the biological functions and potential applications of P450 enzymes in various fields, including drug metabolism, biotechnology, and environmental sciences.

### 3.1.3. Functions in biological systems

P450 s are involved in degradati on and biosynthesis in archea, bacteria, fungi, plants as well as in mammals. In mammals P450s have a fundamental role in steroid hormone homeostasis, like synthesis and metabolism of testosterone and estrogens, cholesterol and vitamin D. Another function of P450s is to metabolize potentially toxic exogenous and endogenous compounds like bilirubin. In humans P450s like CYP3A4 and CYP2D9 are involved in 75 % of drug metabolism (Guengerich, 2008). Furthermore, many substances are bioactivated by P450s to their active form.

In prokaryotes P450s are able to detoxify xenobiotics, catabolize substances as carbon source. They play a role in biosynthesis of antibiotics and fatty acid metabolism (Pylypenko and Schlichtling, 2004). For example, CYP101 (known as $P450_{cam}$) from *Pseudomonas putida* and it was very important as a model for cytochromes. This enzyme is a part of a camphor-hydroxylating catalytic cycle and is composed of two electron transfer steps: from NADH via FAD-containing putidaredoxin reductase (PdR) and 2Fe-2S cluster-containing putidaredoxin (Pdx). Another interesting bacterial representitive is cytochrome $P450_{eryF}$ known as well as CYP107A1 from actinomycete bacterium- *Saccharopolyspora erythraea.* This P450 is essential for the biosynthesis of the antibiotic erythromycin by C6hydroxylation of the macrolide 6-deoxyerythronolide B. CYP102A1 known as P450 BM-3 from the soil bacterium *Bacillus megaterium* catalyzes NADPH- dependent hydroxylation of many fatty acids at the ω-1 through ω-3 positions (Fulco et al, 1986). BM-3 is a natural fusion protein unlike most other P450s and can be very useful in biotechnical applications (Munro et al. 2006).

### 3.1.4. Catalytic cycle of P450

The first proposed catalytic P450 cycle, which described the fundamental role of the protein, heme-iron, and oxygen, was reported in 1968 (Griffin B. W. et al., 1979; Gunsalus I. C. et al., 1975). This early work laid the foundation for understanding the mechanism of P450 enzymes. An updated form of the catalytic cycle is depicted in Figure 3-2.

In the early 1970s, it was discovered that both bacterial CYP101, known as P450cam, and microsomal P450 enzymes require two electrons for the reduction of heme iron from

$Fe^{3+}$ to $Fe^{2+}$ (Katagiri M. et al., 1968; Hedegaard J. et al., 1965; Conrad H. E. et al., 1965). These electrons are delivered sequentially, one after another, and multiple intermediates are involved in the process (Estabrook R. W. et al., 1971).

These early studies provided important insights into the electron transfer processes and the redox properties of P450 enzymes, laying the groundwork for further investigations into their catalytic mechanisms and applications in various fields of research. Substrate binds to a resting state of the low-spin (LS) heme $Fe^{3+}$ group (**1**) opposite to the axial thiolate and the conformation of the active site changes. Water molecule, coordinated as a sixth ligand as placeholder, is often displaced by a substrate molecule which changes the spin state from low-spin (LS) to the high-spin (HS) substrate-bound complex $Fe^{3+}$ (**2**). Iron is far away from porphyrin ring (out-of-plane structure). Substrate is bound on a hydrophobic site of protein. Redox potential changed from -300 mV up to -173 mV. Binding of the substrate induces transfer of one single electron ($e^-$) form NAD(P)H via redox partners. The HS has more positive reduction potential and is effortlessly reduced to ferrous state $Fe^{2+}$ (**3**). Molecular oxygen is bound to the heme center at the distal axial position, giving a relatively stable oxy-low-spin complex Fe-$O_2$ (**4**) (in-plane structure). A second single electron ($e^-$) transferred from redox partners, reduces dioxygen adduct Fe-$O_2$ adduct (**4**) and gives a short-lived peroxo-ferric intermediate (**5a**), its protonated form hydro-peroxo-ferric $Fe^{3+}$ complex (**5b**); second protonation at the distal oxygen atom results in heterolysis of O-O bond, releases a water molecule and highly reactive Compound I (**6**) (was isolated in 2010 (Rittle J., Green MT (November 2010). Finally, oxygenation of substrate occurs to form a product (**7**).

Proposal reaction can have procedure via free radical oxygen rebound mechanism, which shows that Compound I (**6**) that is formed by activation of molecular oxygen abstracts a hydrogen from the substrate and presents a carbon radical intermediate ($R^{\cdot}$). That intermediate recombines with the equivalent of iron-bound hydroxyl radical Fe (IV)-OH to give alcohol as a product (Fig. 3-1).

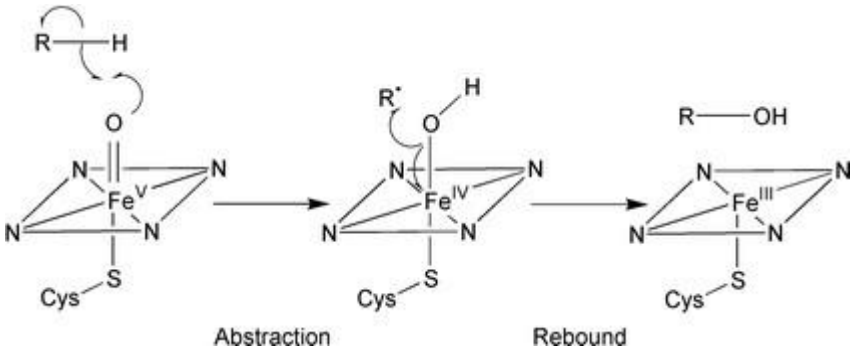



**Figure 3-1:** Rebound Mechanism

The P450 reaction cycle contains three branch points, where side reactions are possible. These three majors shunt reactions are:

-Autoxidation of the oxy-ferrous enzyme (**4**) with production of superoxide anion $O_2^-$ and return to (**2**).

-peroxide shunt where hydroperoxide dissociates from **5b** forming **2**. The most unproductive (when it goes about the substrate turnover) two-electron reduction of oxygen.

-oxidase uncoupling, where Compound I (**6**) is oxidized to water instead of oxygenation of the substrate, results in four-electron reduction of dioxygen forming two molecules of water.

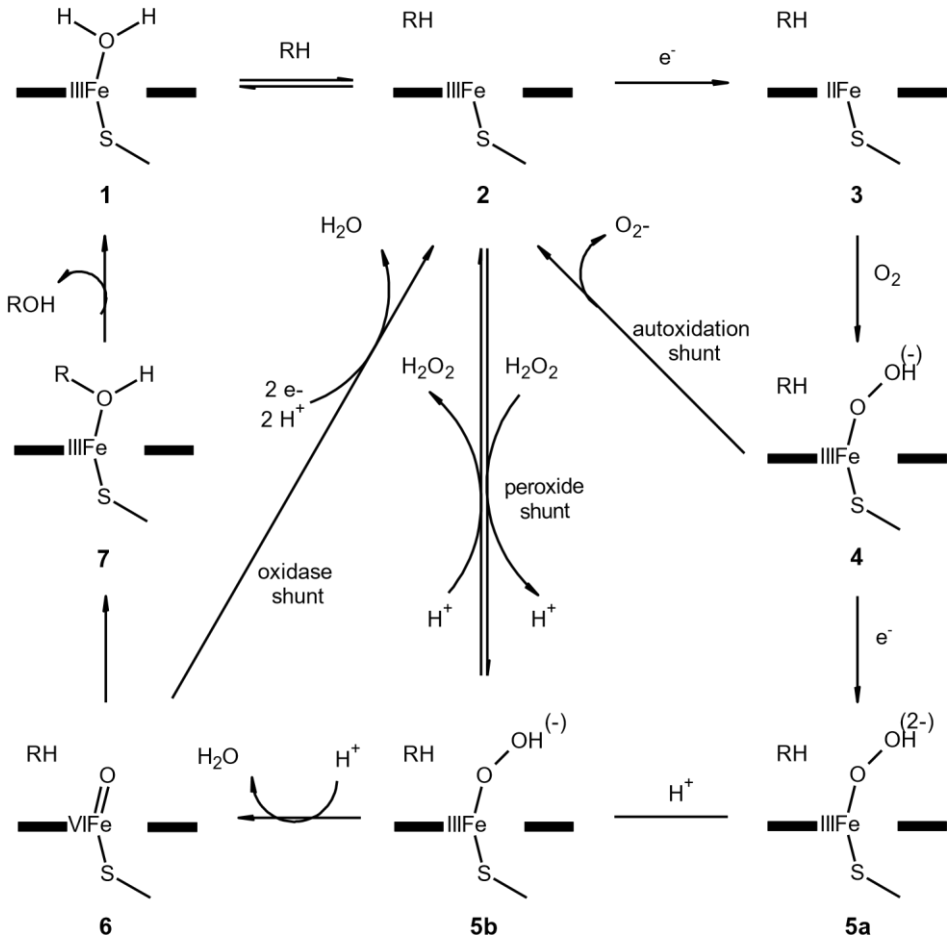



**Figure 3-2:** P450 catalytic cycle.

### 3.1.5. Classes of P450s

According to their redox partners P450s can be classified in several classes.

Class I contains CYPs that receive electrons from NAD(P)H via ferredoxin and ferredoxin reductase (Fig. 3-3). There are few studies about this redundancy such as for *Streptomyces coelicolor A3 (2)* CYP105D5 (Rodriguez-Saiz M. Moreno MA, 2001). This class also includes P450cam from *P. putida* and membrane bound human CYP11A.

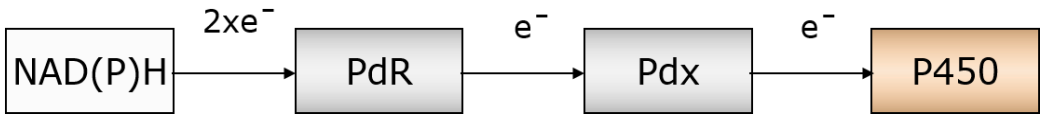



**Figure 3-3:** Class I

Class II belongs to most eukaryotic P450s that are localized in membranes of endoplasmic reticulum via N-terminal anchor and are related to separate NADPH-CPR (Cytochrome P450 reductase).

Class III is till now almost bacterial and used redox partners: FMN (flavin mononucleotide) dependent reductase and flavodoxin (FAD) like in CYP176A1 (P450cin) that metabolizes cineol (Hawkes DB et al., 2002).

Class IV: this class is found in an acidothermophilic archeon. Here CYPs can become electrons from reductase that is a non NADP(H) dependent on and is active with high pressure and temperature. CYP119 from *Sulfobus solfataricus* can use this kind of system (Puchkaev AV, et al. 2005).

To Class V belongs CYP51 from *M. capsulatus* as a first bacterial sterol biosynthetic protein. Contains a C-terminal fusion via a short linker to a 3Fe-4S domain (Jacson CJ et. al, 2002).

CYP177A1 represents class VI of P450s. This CYP was found in *Rhodococcus rhodochrous* strain 11Y and encodes a natural fusion: an N-terminal flavodoxin with a C-terminal haem that allows bacterium to obtain nitrogen from a high-explosive RDX (Seth-Smith SM et. al, 2002).

Class VII are fusion proteins like CYP116B2 (P450RhF) from *Rhodococcus* sp. NCIMB 9784. The system that is being discussed here is a C-terminal reductase FMN and NADPH binding motif with ferredoxin like an N-terminal haem linked to the C-terminus via a linker (Roberts GA et. al, 2002). Class VIII is a eukaryotic-like CPR-like domain on the C-terminus fused to N-terminal haem. To this class belongs P450 BM-3 from *B. megaterium* that metabolizes fatty acids, and a similar fungal system presents CYP505 (*Fussarium oxysporum*) (Kitazume T et. al., 2000).

Class IX CYP55 (P450nor) from *F. oxysporum* is a nitric oxide reductase and soluble eukaryotic CYP. Electrons are delivered from NADH (Tomura D. et. al, 1994). This Class X includes animal and plant CYPs. This type of catalysis reaction proceeds without a redox partner. Here we have examples like thromboxane or divinyl ether synthase (S. Kelly and D. E. Kelly 2013).

### 3.1.6. Reactions catalyzed by P450s

P450 is responsible for oxidation of many different compounds in many organisms. They catalyze a wide variety of chemical reactions. The main function is to convert substrates into more polar metabolites, which can be easily eliminated from the body directly or in the conjugated form, depending on the activity of phase II enzymes of xenobiotic metabolism (Cederbaum, 2006). P450s degrade substances involved in the process of cancer formation, among others such as polycyclic aromatic hydrocarbons,

aromatic or heterocyclic amines, pesticides, herbicides and most drugs (H. Raunio et al. 2015). P450s catalyze the following reactions.
hydroxylation of an aliphatic or aromatic carbons (propanolol - CYP1A2)
double bond epoxidation (carbamazepine - CYP3A4) oxidative S -
dealkylation (6-methylthiopurine - CYP2D6) reductive dehalogenation
azo- and nitro-reduction isomerization oxidative deamination
(amphetamine - CYP2D6) oxidative denitrification (aminophenazone -
CYP3A4) oxidative desulfurylation (thiopental - CYP2D6) oxidation of
alcohols and aldehydes (metronidazole - CYP3A4) oxidative O -
dealkylation (codeine - CYP2D6) oxidative N - dealkylation (diazepam -
CYP2C19) oxidative dehalogenation (halothane - CYP2E1) oxidative
cyclization (pyroxicam - CYP2C9)
aromatization of alicyclic substrates (nifedipine - CYP3A4, CYP3A3)
N- oxidation (mexiletine - CYP2D6)
S- oxidation (thioridazine - CYP2D6, omeprazole - CYP3A4)
oxidative decyclization (alprazolam - CYP3A4) oxygen group
transfer ester cleavage dehydrogenation.

Bacterial P450 monooxygenases, a type of enzyme, can catalyze a variety of reactions that are of great interest to industry, particularly in the context of allyl alcohols. One of the most important reactions catalyzed by P450 enzymes is the oxidation of organic compounds, including allyl alcohols. These enzymes can introduce an oxygen atom from molecular oxygen into the substrate, converting the allyl alcohol into an aldehyde or ketone. This reaction is especially valuable in the pharmaceutical, agrochemical, and biofuel industries, where such transformation could be used to produce high-value compounds.

The advantage of using bacterial P450 monooxygenases lies in their ability to perform this oxidation under mild conditions, without the need for harsh chemicals or high temperatures. In addition, these enzymes are capable of acting selectively, targeting specific positions within the molecule and allowing for control over the stereochemistry of the product. Moreover, the use of P450 monooxygenases is also considered environmentally friendly, as the only by-product of the reaction is water. This contrasts with some traditional methods of oxidation, which can result in the production of harmful by-products (Guengerich, F. P. 2011).

In summary, the ability of bacterial P450 monooxygenases to catalyze the oxidation of allyl alcohols can have significant implications for industrial processes, offering a more sustainable, selective, and efficient alternative to traditional methods.

how bacterial P450 monooxygenases have been used in industry, particularly in relation to allyl alcohols.

Pharmaceutical Industry: Many drugs are modified versions of natural products or have complex structures that are hard to synthesize from scratch. For example, Taxol, an important anti-cancer drug, contains several hydroxyl groups, some of which are introduced through oxidation of an allyl alcohol precursor. The use of P450 monooxygenases allows for the oxidation of these specific positions in a highly selective and efficient manner (Guengerich, F. P. 2011).

Biofuel Production: Biofuels such as biodiesel often involve the production of fatty acid methyl esters. The process can start with an allyl alcohol which can be oxidized by P450 monooxygenases to produce a carboxylic acid, which is then esterified to form the biofuel. The efficiency and selectivity of P450 enzymes can significantly improve the yield and quality of the biofuel produced (Schifrin, A., & Khatri, Y. 2016). Chemical Synthesis: In the manufacture of certain chemicals, allyl alcohols can be converted into more complex structures. One example is the production of fragrances. Some allyl alcohol derivatives, when oxidized, can create compounds with unique smells. P450 enzymes can provide a green and sustainable method for producing these chemicals, contributing to a more environmentally friendly industry (Munro, A. W., Girvan, H. M., & McLean, K. J. 2007).

Agrochemical Industry: Some pesticides and herbicides contain structures that can be derived from the oxidation of allyl alcohols. P450 monooxygenases can provide a method to create these compounds in a selective and sustainable manner (Urlacher, V. B., & Eiben, S. 2006).

These examples demonstrate the versatility and potential of bacterial P450 monooxygenases in various industrial applications. As research continues, it is expected that new uses for these enzymes will be discovered, further enhancing their value to industry.

## 3.2. *Streptomyces*

*Streptomyces* is the largest genus of the Actinobacteria phylum and belongs to the *Streptomycetaceae* family. Actinobacteria, including *Streptomyces*, are known for producing more than 10,000 bioactive compounds (Berdy et al., 2005). To date, over 576 species of Streptomyces bacteria have been identified, and this number continues to grow (Euzeby, 2008; Labeda et al., 2011). In 2002, the complete genome of *Streptomyces coelicolor A3(2)* was published (Bentley et al., 2002).

Streptomyces bacteria are prolific producers of antibiotics, accounting for two-thirds of clinically useful antibiotics such as neomycin, streptomycin (named after *Streptomyces*), chloramphenicol, and grisemycin (Kieser et al., 2000; Bibb et al., 2013). Moreover, *Streptomyces* species produce a wide range of bioactive compounds, including antifungal, antibacterial, antiparasitic drugs, and immunosuppressants (Watwe et al., 2001).

These properties hold immense importance in the clinical, veterinary, and agricultural fields. *Streptomyces* species have been recognized as a major source of numerous pharmaceuticals, including antibiotics and other therapeutic agents. The extensive research and understanding of *Streptomyces* bacteria's bioactive compound production capabilities have paved the way for their application in various industries, benefiting human and animal health, as well as agricultural practices. Chloramphenicol from *S. venezuelae* (Akagawa et al., 1975)

- Neomycin *S. fradiae* (Dulmage et al., 1953)
- Streptomycin *S. griseus* (Distler et al.,1987)
- Fosfomycin *S. fradiae* (Woodyer RD et al., 2006*)*

Streptomyces bacteria have found applications in medical treatments as well. For instance, certain compounds derived from *Streptomyces* species have been utilized as anticancer drugs. One example is migrastatin, which is derived from *S. platensis*. Another example is bleomycin, derived from *S. verticillus*, which has also been used as an anticancer agent. Additionally, boromycin from *S. antibioticis* has shown activity against the HIV-1 strain of HIV. These examples highlight the therapeutic potential of bioactive compounds produced by *Streptomyces* bacteria in the field of medicine. P450s from *Streptomyces coelicolor* (Myung et al., 2019):

- CYP105N1 Hydroxylation (Lim et al. 2012)

- CYP158A1 Biaryl ring coupling (Zhao et al., 2005, 2007)
- CYP158A2 Biaryl ring coupling (Zhao et al., 2005, 2007)
- CYP170A1 Hydroxylation (Zhao et al., 2009)
- CYP105D5 Hydroxylation (Chun et al. 2006, 2007)

In 2015 Omura and Campbell received a Nobel Prize in Physiology for discovery of the avermectin from *S. avermitilis* (Ikeda et al., 2017).

### 3.2.1. *Streptomyces* and importance in allylic hydroxylation

Streptomyces is a genus of bacteria that are well-known for their ability to produce a wide range of bioactive compounds, including antibiotics and other secondary metabolites. These bacteria have been extensively studied and utilized in the field of biotechnology and pharmaceutical research.

One of the notable features of *Streptomyces* bacteria is their production of enzymes, including cytochrome P450 monooxygenases, which play a crucial role in their ability to perform allylic hydroxylation reactions. Allylic hydroxylation refers to the addition of a hydroxyl group (-OH) to an allylic carbon, which is a carbon adjacent to a carboncarbon double bond.

The allylic hydroxylation reaction catalyzed by *Streptomyces* enzymes is highly valuable in the synthesis of bioactive compounds and natural products. By selectively introducing hydroxyl groups at specific positions in organic molecules, these enzymes can modify and diversify the chemical structures of compounds, leading to the production of new derivatives with altered biological activities.

This process is particularly important in drug discovery and development. Many pharmaceutical compounds derived from natural products undergo allylic hydroxylation as a key step in their synthesis or biosynthesis. The introduction of hydroxyl groups can enhance the compound's solubility, stability, or affinity to its target, thereby improving its therapeutic properties.

Furthermore, the ability of *Streptomyces* and their allylic hydroxylating enzymes to perform selective and regio-specific hydroxylation reactions is highly valuable in the synthesis of chiral compounds. By introducing a hydroxyl group at a specific position, the enzymes can create stereocenters, resulting in the production of enantiomerically pure compounds, which are crucial in the pharmaceutical industry.

Overall, Streptomyces bacteria and their allylic hydroxylating enzymes have significant importance in the field of biotechnology and pharmaceutical research. Their ability to catalyze allylic hydroxylation reactions enables the synthesis of diverse chemical compounds, including bioactive molecules, pharmaceuticals, and chiral intermediates, thereby contributing to the development of new drugs and therapeutic agents.

According to Huijbers, the use of P450 monooxygenases from Streptomyces and other sources is discussed for allylic alcohol oxidations, highlighting their importance and potential applications. They state, "P450 monooxygenases play a crucial role in the selective oxidation of allylic alcohols, allowing the introduction of hydroxyl groups at specific positions within the molecules" (Huijbers et al., 2014). In line with this, Gao and Ellis (2014) discuss the relevance of *Streptomyces* enzymes in selective allylic C-H bond oxidation reactions. They note, "The utilization of *Streptomyces* enzymes for allylic C-H bond oxidation has shown great promise in synthetic chemistry, enabling the development of efficient and selective synthetic methodologies" (Gao & Ellis, 2014). Furthermore, Tanaka explores the involvement of *Streptomyces* enzymes in the biosynthesis of hydroxylated mycotoxins and their role in the pathogenicity of filamentous fungi. They mention, "Allylic hydroxylation reactions catalyzed by Streptomyces enzymes are key steps in the biosynthesis of hydroxylated mycotoxins, contributing to the virulence of plant pathogenic fungi" (Tanaka et al., 2019, p. 230). In a related context, Awakawa and Fujii (2013) discuss the biosynthesis of fungal meroterpenoids, which often involve allylic hydroxylation reactions catalyzed by *Streptomyces* enzymes. They state, "*Streptomyces* enzymes play a crucial role in the allylic hydroxylation steps during the biosynthesis of fungal meroterpenoids, leading to the generation of structurally diverse and biologically active compounds" (Awakawa & Fujii, 2013).

## 3.3. Model substrates

Terpenes, also known as isoprenoids, are a class of organic compounds composed of multiple isoprene units $(C5H8)_n$. Terpenoids, on the other hand, are a subclass of terpenes that contain additional functional groups (Chemistry, International Union of Pure and Applied. IUPAC Compendium of Chemical Terminology). Terpenes and terpenoids collectively give rise to approximately 80,000 compounds, as noted by Christianson et

al. (2017). These compounds account for approximately 60% of known natural products with pharmaceutical bioactivity and are of significant interest to the fragrance and medicinal industries (Fim, 2010; Ashour et al., 2010). It is worth mentioning that terpenes and terpenoids are often chiral, meaning they exist as mirror images with different odors or toxicities, adding to their chemical diversity and biological properties.

Terpenes play vital roles in various biological processes and are widely distributed in nature. They contribute to the characteristic scents and flavors of many plants and are utilized in industries such as fragrance, pharmaceuticals, and agriculture. The structure of terpenes is based on repeating isoprene units, which are five-carbon building blocks. By combining multiple isoprene units, terpenes can vary in size and complexity, ranging from small molecules like monoterpenes to larger molecules like sesquiterpenes and diterpenes. Terpenoids, with their additional functional groups, exhibit diverse chemical properties and biological activities. The presence of functional groups such as hydroxyl (-OH), carbonyl (C=O), or carboxyl (-COOH) allows for a wide range of reactions and modifications, enabling the synthesis of various bioactive compounds.

Monoterpenoids for example geraniol consist of two isoprene units (Fig.3-4). Monoterpenoids are composed of two isoprene units, resulting in a 10-carbon structure. They are often represented as $C_{10}H_{16}$. The basic structure of monoterpenoids consists of a linear arrangement of four interconnected carbon rings, with a double bond between the two middle carbon atoms. Monoterpenoids can have various functional groups attached to their carbon skeleton, such as hydroxyl (-OH) or carbonyl (C=O) groups. This variability leads to the wide range of monoterpenoid compounds found in nature, each with unique chemical and biological properties. inctive floral and citrus aromas.

**Geraniol** ($C_{10}H_{18}O$) has a rose-like scent and is found in essential oils derived from plants such as roses, geraniums, and citronella. It has a slightly sweet and fruity aroma and is used as a fragrance ingredient in perfumes, soaps, and personal care products. Geraniol is also known for its potential insect-repellent properties. **Nerol** (C10H18O) is another monoterpene alcohol that is found in essential oils, particularly in orange blossom, neroli, and lemon oils. It has a sweet, floral, and citrusy fragrance. Nerol is commonly used as a flavoring agent in the food and beverage industry and is also employed in perfumes and aromatherapy products. Both geraniol and nerol are versatile compounds and can be further modified or used as precursors for the synthesis of other natural or synthetic compounds. Their pleasant scents make them popular ingredients

in the fragrance industry, while their potential biological activities have attracted attention in various research fields.

Diterpenoids: Diterpenoids are composed of four isoprene units, resulting in a 20carbon structure. They are often represented as $C_{20}H_{32}$. The basic structure of diterpenoids consists of two fused isoprene units, forming a bicyclic arrangement of four interconnected carbon rings. Like monoterpenoids, diterpenoids can have various functional groups attached to their carbon skeleton, contributing to their diverse chemical nature. Examples of diterpenoids include taxol and gibberellins, which play important roles in medicinal and plant growth regulation, respectively.Triterpenoids: Triterpenoids are composed of six isoprene units, resulting in a 30-carbon structure. They are often represented as $C_{30}H_{48}$. The basic structure of triterpenoids consists of three fused isoprene units, forming a polycyclic arrangement of six interconnected carbon rings. Triterpenoids are widely distributed in nature and exhibit a wide range of biological activities. They serve as precursors for various important compounds, including sterols, saponins, and triterpenoid glycosides. Examples of triterpenoids include oleanolic acid and betulinic acid, which possess significant medicinal properties.

It's important to note that the structures of tetra- and pentaterpenoids follow similar patterns, with eight and ten isoprene units, respectively, resulting in larger and more complex structures.

These terpenoid classes (monoterpenoids, diterpenoids, and triterpenoids) encompass a vast array of natural compounds found in plants, fungi, and other organisms. The specific structures and functional groups attached to these terpenoids contribute to their diverse biological activities and make them valuable sources of pharmaceuticals, flavors, fragrances, and other applications.

Geranylacetone and nerylacetone are both ketones derived from terpenoids, specifically from geraniol and nerol, respectively.

**Geranylacetone** (also known as geranyl methyl ketone or GMA) is derived from geraniol by the introduction of a carbonyl group (C=O) at the terminal carbon. Its chemical formula is $C_{12}H_{20}O$. Geranylacetone has a characteristic fruity, floral, and slightly spicy aroma. It is commonly used as a fragrance ingredient in perfumes, soaps, and cosmetic products. Additionally, it is employed as a flavoring agent in the food and beverage industry, providing a fruity note to various products.

**Nerylacetone** (also known as neryl methyl ketone or NMA) is derived from nerol by the introduction of a carbonyl group (C=O) at the terminal carbon. Its chemical formula is $C_{12}H_{20}O$. Nerylacetone has a sweet, floral, and citrusy scent, similar to nerol. It is often used as a fragrance component in perfumes and personal care products. Due to its pleasant aroma, it is also utilized in the flavor industry for adding a citrus-like note to food and beverage formulations.

Both geranylacetone and nerylacetone are important compounds in the fragrance and flavor industries. Their characteristic scents make them valuable ingredients for creating various aromatic compositions and enhancing the sensory experience of consumer products.

**Alpha-ionone** and **beta-ionone** are both sesquiterpenoids, specifically norisoprenoids, derived from the oxidative cleavage of carotenoids. They are responsible for the characteristic aroma and flavor of violets and are commonly used in the fragrance and flavor industries.

Alpha-ionone has the chemical formula $C_{13}H_{20}O$ and is characterized by a distinct violet-like scent. It is used extensively as a fragrance ingredient in perfumes, soaps, and cosmetics, adding a floral and powdery note to various products. It is also employed in the flavor industry to impart a violet-like flavor to food and beverages.

Beta-ionone, with the chemical formula $C_{13}H_{20}O$, is another norisoprenoid compound responsible for the violet-like aroma. It is widely used in the fragrance industry to create violet scents in perfumes, as well as in the flavor industry to provide a violet-like flavor to food and beverages.

Both alpha-ionone and beta-ionone are valuable compounds known for their pleasant and distinctive violet-like fragrances and flavors. Their presence in various consumer products adds a touch of floral elegance and contributes to the overall sensory experience.

Beta-ionone belongs to the group of terpenoids. It is a specific type of terpenoid known as a norisoprenoid. Norisoprenoids are derived from the oxidative cleavage of carotenoids, which are a class of tetraterpenoids. Beta-ionone is formed from the cleavage of the carotenoid beta-carotene and is responsible for its characteristic aroma, often described as floral or violet-like.

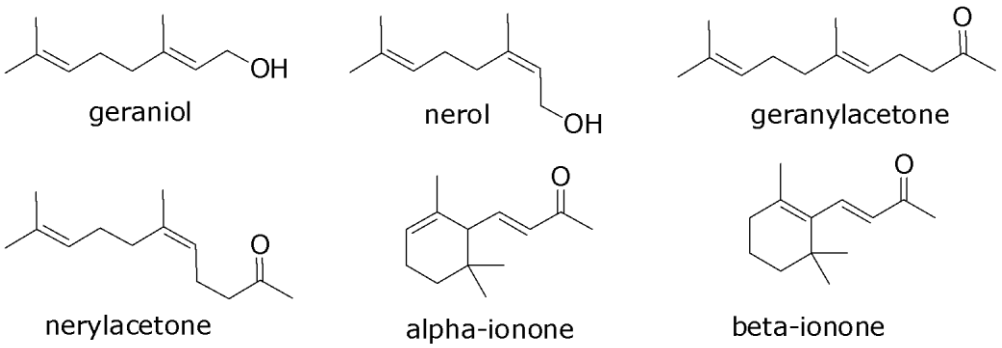



**Fig.3-4:** Model terpenes as substrates example

In summary, terpenes are a class of organic compounds composed of isoprene units, while terpenoids refer to terpenes that contain additional functional groups. These compounds play essential roles in nature and have significant applications in industries such as fragrance, pharmaceuticals, and agriculture.

### 3.3.1. Terpenoids: Functions and application

Terpenes and terpenoids, found abundantly in plants such as pine, camphor, and eucalyptus trees, contribute to the vast diversity of compounds in the chemical industry. They encompass various subclasses, including monoterpenes, sesquiterpenes, and diterpenes. For instance, gum turpentine derived from pines contains α-pinene and β-pinene as its major components.

China possesses rich species resources and has developed mature processes for terpene extraction, making it a significant exporter in the global market. Natural terpene ingredients exhibit low human toxicity and find application as biopesticides (https://foreverest.net/solution/biobased-chemicals/terpene-monomers-for-industrialapplications). Limonene, among other terpenes, can be utilized as a solvent, while αTerpineol has applications in the electronic industry (Foreverest.net). In the flavor and fragrance industry, terpenes with high purity, such as isocitronellene or isolongifolene, are sought after for their distinctive scents.

These examples highlight the wide-ranging industrial applications of terpenes and terpenoids, from biopesticides to solvents, and from electronic industries to flavor and fragrance applications. Their natural abundance, diverse properties, and low toxicity make them valuable resources in the chemical industry.

Pharmacological industry

Terpenes and terpenoids are natural compounds that have widespread applications in the pharmaceutical industry, and they play a key role in plant growth (Yang et al., 2020). These compounds exhibit a diverse range of biological activities, including antitumor, anti-inflammatory, antibacterial, antiviral, antimalarial effects, as well as promoting transdermal absorption, preventing and treating cardiovascular diseases, and possessing hypoglycemic activities (Yang et al., 2020).

Geraniol, a specific terpene, has shown promising activity against various types of cancer. It has been found to possess anti-lung cancer (Galle et al., 2014), colon cancer (Carnesecchi et al., 2001), prostate cancer (Kim et al., 2011), pancreatic cancer (Burke et al., 1997), and liver cancer activity (Ong et al., 2006; Cho et al., 2016; Kim et al., 2012).

These findings highlight the significant potential of terpenes and terpenoids, such as geraniol, as valuable agents in the treatment and prevention of various diseases, including cancer. Their wide range of bioactive properties makes them important targets for further research and development in the pharmaceutical industry.

*Food industry*

Alpha and beta-Ionones (Fig. 1) are aroma compounds commonly found in rose oil and widely utilized by the flavor industry (Pickenhagen et al., 1981). These compounds have significant applications in the food industry, particularly in the production of retinol. Alpha-ionone, for instance, is used in combination with liquorice to create candy products (Pickenhagen et al., 1981).

The economic value of ionones is evident, as their production is patented and subject to commercial interest (Yamamoto et al., 2009; Dobler et al., 2006). These patents highlight the industrial significance and proprietary nature of the processes involved in producing these compounds.

The utilization of alpha and beta-ionones in the food industry underscores their importance as valuable aroma compounds. Their presence in rose oil and their use in flavoring and fragrance applications contribute to the distinctive characteristics of various food products (Pickenhagen et al., 1981; Yamamoto et al., 2009; Dobler et al., 2006)

## 3.4. Allyl alcohols

Allylic alcohols are a class of organic compounds that contain an allyl group ($CH_2CH=CH_2$) bonded to a hydroxyl group (-OH) on the adjacent carbon atom. In other words, allylic alcohols have the hydroxyl group attached to a carbon atom that is directly bonded to a carbon-carbon double bond. The allyl group, which consists of three carbons and a double bond, imparts unique reactivity to allylic alcohols. This reactivity arises from the presence of the allylic carbon-carbon double bond, which can undergo various chemical transformations such as allylic substitution, oxidation, reduction, and functional group interconversion. Allylic alcohols find extensive applications in organic synthesis due to their ability to participate in diverse chemical reactions. They serve as versatile building blocks for the synthesis of complex organic compounds, including natural products, pharmaceuticals, and other valuable molecules. The allylic alcohol functionality can be selectively modified or transformed, allowing for the introduction of different functional groups and the creation of stereocenters.

Moreover, allylic alcohols exhibit distinct chemical properties and reactivity compared to other types of alcohols. The presence of the allylic double bond makes allylic alcohols susceptible to various reactions, such as allylic oxidation, allylation, and allylic rearrangements.

Overall, allylic alcohols are valuable compounds in organic chemistry due to their unique reactivity and versatility. Their involvement in numerous synthetic strategies and their role as intermediates in the synthesis of biologically active molecules make them important building blocks in the field of organic synthesis.

### 3.4.1. Applikations of allylic alcohols

Allyl alcohols, such as allyl alcohol itself or crotyl alcohol, are of great interest in organic chemistry. They are versatile and can serve as building blocks for a variety of chemical reactions and syntheses. Here are some reasons why they might be of interest as target compounds:

1. synthesis of plastics and polymers: Allyl alcohols can be used to make plastics and polymers. Their ability to polymerize easily makes them valuable starting materials in the plastics industry.

2. pharmaceutical applications: Allyl alcohols can serve as starting materials for the synthesis of pharmaceuticals. They can be modified to produce a variety of biologically active molecules.
3. production of fragrances and flavors: Some allyl alcohols have pleasant odors and can therefore be used in the perfume industry. They can also be used to produce flavoring agents for food and beverages.
4. organic synthesis: due to their reactive allyl group, allyl alcohols are useful reagents in organic synthesis. They can be used in a variety of reactions, including allylation of carbonyl compounds, preparation of epoxides, and participation in cycloaddition reactions.

It should be noted that allyl alcohols, especially allyl alcohol itself, are toxic and must be handled with care.

Application of Allyl Alcohols in Organic Synthesis

Allyl alcohols play a crucial role as versatile precursors in organic synthesis, particularly in the construction of complex organic compounds, including natural products and drugs. Their unique reactivity and functional group properties make them valuable building blocks for the creation of intricate molecular structures. In the realm of natural product synthesis, allyl alcohols serve as key starting materials for the assembly of complex molecules. They can undergo various transformations, such as allylation, oxidation, reduction, and functional group interconversion, enabling the introduction of diverse functional groups and stereochemistry into the target molecules. This flexibility allows for the synthesis of natural products with intricate frameworks and diverse biological activities.

Moreover, allyl alcohols find applications in the synthesis of pharmaceutical compounds and drug development. Their functional group versatility allows for the selective modification of molecular scaffolds, enabling the creation of analogues and derivatives with improved pharmacological properties. Allyl alcohols can be utilized in key steps, such as carbon-carbon bond formation, protecting group manipulations, and stereoselective transformations, to construct drug-like molecules with desired biological activities.

The application of allyl alcohols in organic synthesis offers a powerful toolbox for chemists to access complex structures, natural products, and potential drug candidates.

Their strategic use in various synthetic transformations contributes to the advancement of chemical synthesis and drug discovery efforts (Nicolaou, K. C., Montagnon, T., & Baran, P. S. 2002).

Studies on Biological Activity and Potential Medical Applications

Studies on the biological activity and potential medical applications of allyl alcohols have revealed their diverse pharmacological properties, including antibacterial, anticancer, antiviral, and other effects. Research in this field involves investigating the potential therapeutic applications of these compounds for the treatment of various diseases.

Allyl alcohols have demonstrated antibacterial activity against different strains of bacteria, making them potential candidates for the development of new antibacterial agents. Their ability to inhibit bacterial growth or disrupt bacterial biofilms has been investigated in several studies.

Furthermore, allyl alcohols have shown promising anticancer effects, exhibiting cytotoxicity towards various cancer cell lines. These compounds have been evaluated for their potential as anticancer agents, either as standalone treatments or in combination with other therapeutic approaches. Studies have explored their mechanisms of action, such as induction of apoptosis, inhibition of cell proliferation, or interference with cellular signalling pathways.

In addition, allyl alcohols have displayed antiviral activity against certain viral infections. Research has focused on their ability to inhibit viral replication or interfere with viral entry into host cells, suggesting their potential as antiviral agents.

These biological activities of allyl alcohols make them intriguing candidates for further exploration in the field of medicinal chemistry and drug development. Continued research is necessary to elucidate their mechanisms of action, optimize their efficacy, and evaluate their safety profiles for potential medical applications.

It is important to note that specific studies investigating the biological activities and medical applications of allyl alcohols can be found in scientific literature. Consulting research articles, reviews, and relevant publications in the field of medicinal chemistry and pharmacology will provide more comprehensive and detailed information on the potential therapeutic uses of these compounds.

### 3.4.2. Allyl alcohols of interest

Specifically, 8-hydroxynerol and 8-hydroxygeraniol, along with 11-hydroxynerylacetone, 11-hydroxygeranylacetone, 12-hydroxynerylacetone, and 12-hydroxygeranylacetone, are notable allyl alcohols with distinct characteristics.

8-Hydroxynerol and 8-Hydroxygeraniol:

8-Hydroxynerol and 8-hydroxygeraniol are terpenoid allyl alcohols derived from the oxidation of nerol and geraniol, respectively. They possess a hydroxyl group at the 8th position of the carbon chain. These compounds have been studied for their fragrance properties and potential applications in the fragrance and flavor industries.

11-Hydroxynerylacetone and 11-Hydroxygeranylacetone:

11-Hydroxynerylacetone and 11-hydroxygeranylacetone are allyl alcohol derivatives with a hydroxyl group at the 11th position of the carbon chain. These compounds have been investigated for their biological activities, including their potential as bioactive agents in various applications.

12-Hydroxynerylacetone and 12-Hydroxygeranylacetone:

12-Hydroxynerylacetone and 12-hydroxygeranylacetone are allyl alcohols with a hydroxyl group at the 12th position of the carbon chain. These compounds have been studied for their unique chemical properties and potential applications, particularly in the fragrance and flavor industries.

Overall, allyl alcohols such as 8-hydroxynerol, 8-hydroxygeraniol, 11-hydroxynerylacetone, 11-hydroxygeranylacetone, 12-hydroxynerylacetone, and 12-hydroxygeranylacetone represent a subset of compounds within the allyl alcohol class. They possess distinct structural features and may exhibit various biological activities, making them of interest in different fields, including fragrance, flavor, and potential bioactive applications.

4-Hydroxy-beta-ionone is a compound belonging to the class of norisoprenoids. It is derived from the oxidative cleavage of carotenoids and is known for its pleasant aroma. The compound has a hydroxyl group (-OH) attached to the beta-ionone backbone, resulting in the "4-hydroxy" designation. 4-Hydroxy-beta-ionone is often found in various natural sources such as fruits, flowers, and spices. It contributes to the aroma of many

fruits, including apricots and raspberries, and is also responsible for the characteristic scent of certain flowers, such as violets and roses. The compound is widely used in the fragrance industry as a perfume ingredient due to its floral and fruity fragrance profile.

## 3.5. Immobilization of biocatalysts

### 3.5.1. General aspects of enzyme immobilization

Immobilization methods are of great interest due to their ability to anchor enzymes or other biologically active molecules onto a solid support matrix. This technique offers several advantages:

Stability: Immobilization enhances the stability of the immobilized molecules, particularly against pH and temperature fluctuations, as well as chemical or mechanical stresses. This enables their prolonged and efficient use in industrial applications and biotechnological processes (Sheldon, 2007).

Reusability: Immobilization allows for the repeated use of the immobilized molecules, reducing costs and improving sustainability. After the reaction or application, they can be easily separated from the support matrix and reused for further processes (Mateo et al., 2007).

Enhanced Selectivity: Immobilization can improve the selectivity of the immobilized molecules by selectively influencing their interaction with substrates or reaction partners. By employing suitable support materials and immobilization methods, catalytic activity and specificity can be optimized (Liu et al., 2016).

Simplified Separation: Immobilization enables easy separation of the immobilized molecules from reaction mixtures or products, simplifying purification and separation processes. This allows for more efficient process operation and improved product purity (Sheldon, 2007).

### 3.5.2. Immobilization methods

Various immobilization methods exist, including:

Adsorption: Immobilized molecules are bound to the surface of a support material through physical adsorption. This is a simple and cost-effective method, but the stability of immobilization may be limited (Sheldon, 2007).

Covalent Binding: Immobilized molecules are covalently bound to the support material through chemical reactions. This method offers strong binding, but often requires specific functionalization steps (Mateo et al., 2007).

Encapsulation in Porous Materials: Immobilized molecules are encapsulated within the pores or channels of porous materials. This allows for high catalyst loading and improved stability (Mateo et al., 2007).

Encapsulation in Latex Particles: Latex particles present an attractive option for immobilization due to their high stability, ease of handling, and flexible surface modification. Immobilized molecules can be embedded within latex particles or adsorbed/covalently bound to their surface. The choice of immobilization method depends on various factors, including the nature of the molecule, desired stability, application, and available resources. Immobilization in latex particles offers an appealing option due to the aforementioned advantages, including ease of handling and versatile surface modification (Mateo et al., 2007).

### 3.5.3. Immobization in latex

The latex immobilization technique not only facilitates the handling and reuse of whole-cell catalysts, but also has a significant impact on product selectivity. The use of immobilization techniques such as this can significantly increase the economic viability of biocatalytic processes by enabling catalyst reuse and facilitating product purification. In addition, the improved selectivity achieved by immobilization can reduce the need for costly separation and purification procedures, helping to lower production costs.

The utilization of latex coatings for biocatalysis in living organisms can be traced back to the pioneering studies conducted by Lawton, Bunning, and Flanagan (Lawton et al., 1985; Bunning et al., 1988; Flanagan et al., 1990). These researchers explored the application of latex coatings as a means of encapsulating and protecting biocatalytic systems.

In subsequent research, the effectiveness of latex coatings for various applications has been demonstrated. Whole-cell biosensors, oxidations, and reductions have been successfully implemented using coatings composed of a 30 µm to 50 µm thick layer of bacteria combined with a latex layer and covered by a porous coating (Fig. 35) (Swope, Flickinger, 1996). This approach provides an effective means of immobilizing and preserving living organisms for biocatalytic processes.

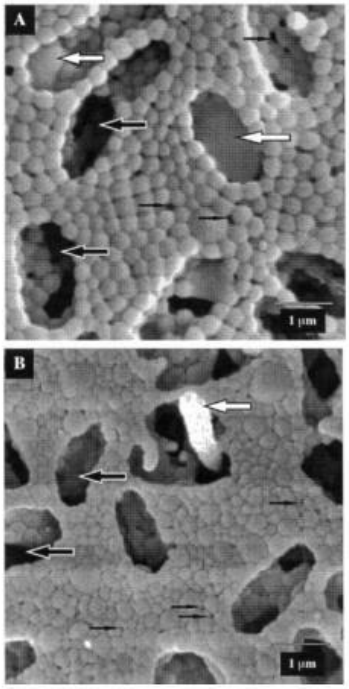



**Fig. 3-5:** Living cells permanently entrapped, surrounded by nanopores by polymer particles (acrylic/vinyl acetate copolymer latex. (Pictures from Publications of Flickinger et. Al. 2007). Cryo-SEM images of coating voids (large black arrows) created by entrapment of E. coli and pore spaces between polymer particles (small arrows). White arrows: E. coli. (A) Coating generated with monodispersed acrylate/vinyl acetate copolymer with sucrose (SF091) and glycerol. (B) Coating generated with polydispersed acrylate/vinyl acetate copolymer (RES661) plus glycerol (Reproduced with permission from ref 49. Copyright 1999 Elsevier).

Formulation and drying methods have been developed to generate permeability but not to kill the cells (M.C. Flickinger et al. 1999). These methods stabilize living organisms and are used more efficiently as biocatalysts in the chemical process industry. Thinness, microstructure, diffusion properties of latex coatings and high entrapped cell density are interesting for large industry scale. For these reasons industrial coating and printing technology offer the possibility to expand the uses of engineered microorganisms as biocatalysts (M.C. Flickinger et al.).

## 3.6. Two phase systems

### 3.6.1. Types of two phase systems

There are different types of two-phase systems that are used in the biotechnological industry. Two of the most well-known ones are the aqueous-organic two-phase system

and the solid-liquid two-phase system (Griffin B. W. et al. 1979, Gunsalus I. C. et al.1975).

The aqueous-organic two-phase system consists of an aqueous phase and an organic phase that are immiscible with each other. This type of system is commonly used to immobilize enzymes or microorganisms and enhance their catalytic activities. By immobilizing enzymes in an organic phase, they can operate in a stable environment while ensuring better solubility of substrates. This often leads to increased enzyme activity and improved reaction conditions (Katagiri M. et al. 1965, Conrad H. E. et al. 1965).

The solid-liquid two-phase system is based on the use of a solid support that is wetted with a liquid. This system is frequently used to immobilize cells or enzymes and achieve high cell density. The solid support can be porous and provide a large surface area to ensure high cell density and facilitate the diffusion of substrates and products (Conrad H. E. et al. 1965, Estabrook R. W. et al. 1971).

The use of two-phase systems in the biotechnological industry offers several advantages. Firstly, they allow for easy separation of enzymes or microorganisms from products and substrates, facilitating purification and product recovery. Secondly, they provide better control of reaction conditions and enable higher stability of enzymes or microorganisms. Thirdly, they can enhance catalytic efficiency and enable more selective reactions (Griffin B. W. et al. 1979, Gunsalus I. C. et al.1975, Hawkes DB 2002, Puchkaev AV, Ortiz Montellano PR 2005).

The use of two-phase systems is therefore of great interest in the biotechnological industry. They enable the efficient utilization of enzymes or microorganisms for biotechnological processes and contribute to the development of more sustainable and effective production methods. Furthermore, they provide opportunities for optimizing reaction conditions and achieving more selective reactions, which is valuable to produce high-quality products (Hawkes DB 2002, Puchkaev AV, Ortiz Montellano PR 2005).

### 3.6.2. Two phase system used in this work

Two-phase systems in biochemistry refer to reaction systems consisting of two immiscible phases in which biocatalytic reactions can take place. These systems offer several advantages, including improved substrate and product separation, stability of enzymes and substrates, and increased reaction rates. The use of two phase systems

enables the immobilization of enzymes or the use of soluble enzymes in combination with specialized phase transfer catalysts. This leads to increased reactivity, improved substrate availability, and facilitates the separation of products and catalysts (Fig. 3-6).

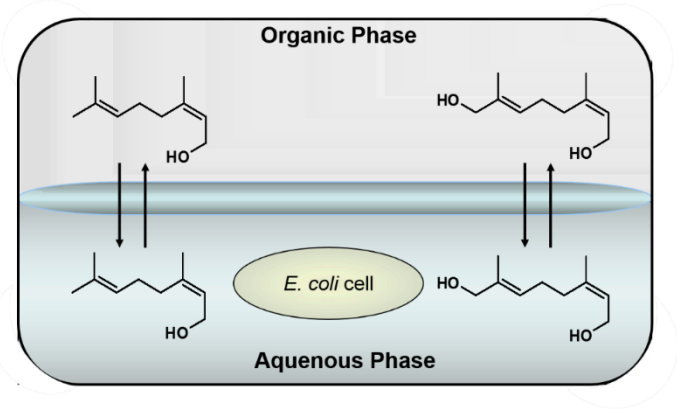



**Figure 3-6:** Scheme for biooxidation of nerol as a model substrate using recombinant *E. coli* in a two phase system.

A two phase system is not typically considered an immobilization method in the strict sense. It refers to a system consisting of two immiscible phases, often an aqueous phase and an organic phase, in which reactions can occur at the interface between the phases. While immobilization methods involve fixing enzymes or other biologically active molecules onto solid support matrices, two-phase systems utilize the presence of multiple phases to facilitate specific reactions.

Two phase systems offer advantages in biocatalysis by providing a suitable environment for enzyme activity and facilitating the separation of products from the reaction mixture. These systems create an interface where reactants can come into contact with the immobilized enzyme, allowing for efficient catalysis (Drauz and Waldmann 1999). The use of two phase systems in biocatalytic reactions, emphasizing their ability to enhance mass transfer and substrate availability. Selection of appropriate solvent systems and process conditions can optimize the efficiency of enzymatic reactions in two phase systems (Schmid et al. 2001). While two phase systems are not considered traditional immobilization methods, they offer unique advantages in biocatalysis, such as enhanced mass transfer and reaction rates. These systems can be employed alongside immobilization techniques to further improve the performance and efficiency of biocatalytic processes.

## 3.7. Aim of this work

The aim of this work is to enhance the understanding of P450 monooxygenases and their unique catalytic capabilities for industrial applications. The challenging nature of identifying physiological redox partners and substrates for these enzymes is acknowledged, and this research seeks to improve that understanding. By focusing on the allylic hydroxylation of terpenes, a specialized class of organic compounds, the study aims to elucidate the potential of P450 enzymes in the production of valuable products such as antibacterial agents, pheromones, and pharmaceutical intermediates.

Specifically, the research focuses on the oxidation of short-chain substrates, including $C_7$-$C_{12}$ alkanes and terpenoids, which have significant interest in the pharmaceutical and perfume industries. By identifying, isolating, cloning, and expressing functional P450 monooxygenases with the appropriate redox system, the study aims to characterize bacterial P450 enzymes capable of allylic hydroxylation. The immobilization of these enzymes on latex systems and the use of two-phase systems expands the potential applications of microorganisms as biocatalysts and provides alternatives for selective product formation.

Overall, this work aims to contribute to the development of efficient biotechnological solutions that can make chemical production more sustainable and resilient. By harnessing the unique catalytic abilities of P450 enzymes and understanding the significance of redox partners and concentration ratios, this research has the potential to pave the way for novel approaches in industrial biocatalysis and expand the applications of these enzymes in various sectors.

# 4. Material und Methods

## 4.1. Instruments

| ***Instrument*** | ***Characteristics*** | ***Supplier*** |
|---|---|---|

| | | |
|---|---|---|
| **Agarose gel electrophoresis apparatus** | DNA Sub Cell ™ | Bio Rad |
| | Mini Sub ™ DNA Cell | Bio Rad |
| | Mini Sub ™ Cell GT | Bio Rad |
| | Video Copy Processor P66E | Mitsubishi |
| | BWM 9X Monitor | Javelin Electronics |
| | UV-lamp table | MWG-Biotech |
| **Balances** | Basic, MCI Research RC 210 D | Sartourius |
| | Precision Advanced | OHAU® |
| **Centrifuges** | Eppendorf Centrifuge 5417 C | Eppendorf |
| | Eppendorf Centrifuge 5417 R | Eppendorf |
| | Eppendorf Centrifuge 5810 R | Eppendorf |
| | Universal 30 F | Hettich |
| | KR 22 I (Rotor: AK 500-11, 155 Jouan mm) Jouan G 412 | Du Pont |
| | Sorvall RC – 5B (Rotor: SA 600) | Instruments |
| **Electroporation** | Gene Pulser®, Pulse controller | Bio Rad |
| **Fluorimeter** | Fluostar | BMG |
| **Incubators** | Certomat R Incubator | Braun |
| | HT Incubator | Infors AG |
| | UM 500 | Memmert |
| | WTE | Binder |
| **Microwave oven** | Micro-Chef FM A935 | Moulinex |
| **PCR-Cycler** | Master Cycler Gradient | Eppendorf |
| | Robocycle Gradient 40 | Stratagene® |
| **pH-Meter** | Digital pH Meter pH525 | WTW |
| **Polyacrylamide gel electrophoresis apparatus** | Minigel-Twin G42 | Biometra® |
| | Model 583 Gel Dryer | BioRad |
| **Evaporator** | Rotavapor R-134 | Büchi |

| | | |
|---|---|---|
| **Sonifier** | Sonifier 250 | Branson |
| | Sonorex Super RK 514 H | Brandelin |
| **Spectrophotometer** | Ultrospec 3000 | PharmaciaBiotech |
| **UV/VIS** | Cary 3E UV-VIS | PharmaciaBiotech |
| **Thermomixer** | Thermomixer 5436 | Eppendorf |
| **Vacuum concentrator** | Speed Vac Concentrator 5301 | Eppendorf |
| **Vortex** | Vortex Genie 2 | ScientificIndustries |
| **Water bath** | Water bath B3 | Haake-Fisons |

## 4.2. Chemical and enzymes

| ***Supplier*** | ***Material*** |
|---|---|
| **ARK scientific GmbH, Darmstadt** | Oligonucleotides |
| **Applichem, Darmstadt** | Isopropyl-β-D-thiogalactopyranosid (IPTG) |
| **BIO-RAD Laboratories, Richmond, USA** | LMW protein standard |
| **MBI Fermentas, St. Leon-Rot** | Restriction endonucleases, T4-DNA ligase, Calf intestinal alkaline phosphatase (CIAP), *Pfu* DNA-polymerase |
| **DIFCO-laboratories, Detroit, USA** | Yeast extract, Bactotryptone, Bactoyeast extract |
| **FlukaChemie, Busch, Schwitzerland** | Agar, 5-Aminolevouline acid (5-ALA), Carbenicillin(Car), Chloramphenicol (Cam), Lyzozyme, NaCl, SDS, $Na_2HPO_4$, $NaH_2PO_4$, $K_2HPO_4$, $KH_2PO_4$, NaOH, HCl, Ethyl acetate, Petrol ether, |

| | |
|---|---|
| | Sodium dithionide, Rubidium chloride, Diethylether, Glucose, Magnesium sulfate anhydrous, Manganese chloride, Glycerol (99%, 87%), Dimethyl sulfoxide (DMSO), Geraniol (G), Nerol (N), Geranylacetone (GA), Nerylacetone (NA), TLC plates (0.2 mm, Silica 60 $F_{254}$) |
| **GIBCO BRL GmbH, Eggenstein** | Agarose, 1 kb-Ladder (DNA standdard) |
| **MWG, Ebersberg** | DNA sequencing |
| **Qiagen GmbH, Hilden** | QIAprep Mini Prep kit, QIAquick Gel Extraction kit |
| **Carl Roth GmbH, Karlsruhe** | Acrylamide (30%), Ampicilin (Amp), Coomassie Brilliant Blue R250, Ethylenediaminetetraacetic acid (EDTA), Ethidium bromide, Kanamycin (Kan), phenol/ chloroform/ isoamylalcohol (25: 24: 1; v /v /v) |
| **Riedel-de-Haen, Seelze, Germany** | Acetone, Methanol, Dichlomethane (DCM) |
| **Rohm and Haas, Spring House, PA** | Rovace latex SF091 |

## 4.3. Primers

| ***Name*** | ***Sequence 5' to 3'*** |
|---|---|
| **ARO_SU1_NdeI_F** | GGTAATTCCATATGACCGATACCGCC |
| **ARO_SU1_BamHI_R** | CTAGGATCCCTATTCCGTGTCCTCG |
| **ARO_SU2_NdeF** | GGAATTCCATATGACGACCGCAGAAC |
| **ARO_SU2_BamR** | CTAGGATCCTCAGTCGGTCACCGTG |
| **Aro_fdx_red_F15_coelic_NcoI_F** | TATGCCATGGTTATGCCCCGCCCTCT |
| **F_Kma_105d5_NdeI** | GCCCCCCATATGACGGACACCGACACGACG |

| | |
|---|---|
| **R_Kma_105d5_BamHI** | CGCGGATCCTCAGCCGACCCGCTCCGAGAC |
| **F_Kma_170A1_NdeI** | GCCCCCCATATGACCGTCGAGTCCGTCAACCC |
| **R_Kma_170A1_BamHI** | CGCGGATCCTCACCGCGCCACGGGCCTGACCAG |
| **Aro_fdx_red_F15_coelic_NdeI_R** | GAGAATTCCATATGTCAGGCGCCGCTCTC |
| **F_NdeI_154E1_Aro** | GGAATTCCATATGGGACAGTCCCGC |
| **R_EcoRI_154E1_Aro** | CAGTGAATTCTCAGGGTTTCGGGCG |
| **F_NdeI_CamB_Aro** | GGGAATTCCATATGTCTAAAGTAGTGTAT |
| **R_EcoRI_CamB_Aro** | CCGGAATTC TTACCATTGCCTATCGGG |
| **F_NdeI_CamA_Aro** | GGAATTCCATATGAACGCAAACGAC |
| **R_EcoRI_CamA_Aro** | GATCGAATTCTCAGGCACTACTCAG |
| **ARO_V286L_F** | CTTCGAATCAGCGCTGGTCATGCTGCCGTTC |
| **ARO_V286L_R** | GAACGGCAGCATGACCAGCGCTGATTCGAAGCG |
| **ARO_V286F_F** | CTTCGAATCAGCGTTCGTCATGCTGCCGTTC |
| **ARO_V286F_R** | GAACGGCAGCATGACGAACGCTGATTCGAAGCG |
| **ARO_V286A_F** | CTTCGAATCAGCGGCGGTCATGCTGCCGTTC |
| **ARO_V286A_R** | GAACGGCAGCATGACCGCCGCTGATTCGAAGC |
| **T7-Promotor** | TAATACGACTCACTATAGGG |
| **T7-Terminator** | GTTAGTTATTGCTCAGCGC |

## 4.4. Microorganisms and plasmids

| ***Microorganism*** | ***Strain*** |
|---|---|
| ***Escherichia coli*** | DH5α |
| ***Escherichia coli*** | BL21(DE3) |
| ***Escherichia coli*** | BL21(DE3)pLysS |

| | |
|---|---|
| ***Escherichia coli*** | BL21(DE3) Rosetta |
| ***Escherichia coli*** | BL21(DE3) Codon Plus RP |

| | |
|---|---|
| ***Streptomyces rimosus*** | DSM 40260 |
| ***Streptomyces venezuelae*** | DSM 40230 |
| ***Streptomyces lividans*** | Tübingen collection |
| ***Streptomyces antibioticus*** | Tübingen collection |
| ***Streptomyces ambofaciens*** | DSM 40053 |
| ***Streptomyces griseus*** | DSM 40236 |
| ***Streptomyces albus*** | DSM 40313 |
| ***Streptomyces nitrozolus*** | Tübingen collection |
| ***Streptomyces coelicolor*** | |
| ***Streptomyces pavulus*** | DSM 40722 |
| ***Streptomyces Tü2353*** | |
| ***Streptomyces griseolus*** | DSM 40854, ATCC 11796 |
| ***Streptomyces avermitilis*** | DSM 46492 |
| ***Streptomyces coelicolor A3(2)*** | DSM 40783 |
| ***Pseudomonas putida*** | ATCC 17453 |
| ***Thermobifida fusca YX*** | ATCC BAA-629 |

## 4.5. Vectors

| *Vector* | *Size [kb]* | *Resistance* | *Supplier* |
|---|---|---|---|
| **pET28a+** | 5.4 | Kanamycin | Novagen |
| **pET22b+** | 5.5 | Ampicilin | Novagen |
| **pET11a+** | 5.5 | Carbenicillin | Mercian Corporation, Japan (Novagen) |

## 4.6. General media, buffers and solutions

### 4.6.1. Common buffers and media

| *Buffers* | *Compound* | *Amount* |
|---|---|---|

| | | | |
|---|---|---|---|
| ***Potassium phosphate buffer*** **Added water to final volume 1 litre and pH adjusted to 7.5** | 1M $KH_2PO_4$ | 10 | ml |
| | 1M $K_2HPO_4$ | 40 | ml |
| ***Tris HCl buffer*** **Added water to final volume 1 litre and pH adjusted to 7.5 with HCl** | Tris | 6.1 | g |
| ***10 x TB phosphate buffer*** **Added water to final volume 0.1 litre** | $KH_2PO_4$ | 2.31 | g |
| | $K_2HPO_4$ | 12.5 | g |
| ***5 x M 9 salts*** **Added water to final volume 1 litre and pH adjusted to 7.0 with 5 N NaOH** | $Na_2HPO_4$ | 33.9 | g |
| | $KH_2PO_4$ | 15 | g |
| | NaCl | 2.5 | g |
| | $NH_4Cl$ | 5 | g |
| ***CV2 solution*** **Added water to final volume 0.2 litre** | 100 mM KBP | | |
| | 40 % Glycerol | 100 | ml |
| | 50 mg / ml Carbenicillin | 10 | ml |
| | 0.1M IPTG | 0.2 | ml |
| | | 0.2 | ml |
| ***TFB I solution*** **Added water to final volume 0.2 litre and pH adjusted to 5.8 with ethyl acetate** | Kalium acetate | 0.59 | g |
| | RbCl | 2.42 | g |
| | $CaCl_2$ | 0.29 | g |
| | $MnCl_2 \cdot 4\ H_2O$ | 2 | g |
| | Glycerol | 30 | ml |

| | | | |
|---|---|---|---|
| ***TFB II solution*** | MOPS | 0.21 | g |
| **Added water to final volume 0.1 litre and pH adjusted to 6.5 with NaOH** | $CaCl_2$ | 1.1 | g |
| | RbCl | 0.12 | g |
| | Glycerol | 15 | ml |
| ***L-1 solution*** | Tris HCl | 25 | mM |
| **Added water to final volume 0.1 litre and pH adjusted to 8 with KOH** | EDTA | 25 | mM |
| | Saccpharose | 300 | mM |

| ***Medium*** | ***Compound*** | ***Amount*** | |
|---|---|---|---|
| ***LB medium*** | Bacto-Tryptone | 10 | g |
| **Added water to final volume 1 litre.** | Hefe Extract | 5 | g |
| | NaCl | 5 | g |
| | Agar (plates) | 15 | g |
| ***TB medium*** | Bacto-Tryptone | 12 | g |
| **Added water to final volume 0.9 litre** | Yeast Extract | 24 | g |
| | Glycerol | 4 | ml |
| ***65 medium (M 65)*** | Malt Extract | 10 | g |
| **Added water to final volume 1 litre and pH adjusted to 7.2 with KOH.** | Yeast Extract | 4 | g |
| | Glucose | 4 | g |
| | $CaCO_3$ (plate) | 2 | g |
| | Agar (plate) | 12 | g |
| ***4 medium (M 4)*** **Added water to final volume 1 litre and pH adjusted to 7.5 with KOH** | Soya Peptone | 20 | g |
| | D-Mannitol | 20 | g |

| | | | |
|---|---|---|---|
| ***Preculture M 9 medium*** **Added water to final volume 0.1 litre.** | 50 mg / ml Carbenicillin | 100 | µl |
| | 5 x M 9 salts | 20 | ml |
| | 10 % Casamino acid | 10 | ml |
| | 20 % Glucose 50 | 2 | ml |
| | mM $CaCl_2$ | 200 | µl |
| | 2.5 mM $MgCl_2$ | 40 | µl |
| | 2 mg / ml Thymine | 1 | ml |
| | 1 M $FeSO_4$ | 10 | µl |
| ***Mainculture M 9 medium*** **Added water to final volume 0.1 litre.** | 50 mg / ml Carbenicillin | 100 | µl |
| | 5 x M 9 salts | 20 | ml |
| | 10 % Casamino acid | 10 | ml |
| | 50 mM $CaCl_2$ | 200 | µl |
| | 2 mg / ml Thymine | 1 | ml |
| | 1 M $FeSO_4$ | 10 | µl |
| | 80 mg / ml 5-ALA | 100 | µl |
| | O/N A.I.S. Solution I | 2 | ml |
| | O/N A.I.S. Solution II | 5 | ml |
| | O/N A.I.S. Solution III | 100 | µl |

### 4.6.2. Buffers for mini preparation of plasmid DNA

| ***Buffer*** | ***Compound*** | ***Amount*** | |
|---|---|---|---|
| ***Resuspension buffer*** | Tris/HCl | 100 | mM |
| | EDTA | 10 | mM |
| | RNase I | 250 | µg/ml |
| ***Lysis buffer*** | NaOH | 200 | mM |
| | SDS | 1 | % (w/v) |
| ***Neutralization buffer*** | Postassium acetate | 29.4 | g |
| | Acetic acid | 11.5 | ml |
| | $H_2O$ | 28.5 | ml |

### 4.6.3. Buffers and solutions for agarose gel electrophoresis

| *Buffer* | *Compound* | *Amount* |
|---|---|---|
| ***1 % agarose*** | Agarose | 4 g 400 ml |
| | 1 x Tris-acetate (TAE) | |
| ***50 x TAE buffer*** | Tri's base | 242 g/L |
| | Glacial acetic acid | 57 ml |
| | EDTA (0.5 M, pH 8.0) | 100 ml |
| ***6 x DNA loading buffer*** | Glycerol | 30 % (w/v) |
| | Bromphenol blue | 0.2 % (w/v) |
| | EDTA (pH 7.5) | 5 mM |

### 4.6.4. Buffers and solutions for polyacrylamide gel electrophoresis

| *Buffer* | *Compound* | *Amount* |
|---|---|---|
| ***4 x Lower Tris (200 ml)*** | Tris base | 36.46 g 0.8 g |
| | SDS | pH 8.8 |
| ***4 x Upper Tris (200 ml)*** | Tris base | 12.11 g 0.8 g |
| | SDS | pH 6.8 |
| ***Resolving gel (12.5 %)*** | Acrylamide bisacrylamide 37.5:1 (30 %) *4 x Lower Tris* | 3.33 ml 2 ml |
| | $H_2O_{bidest.}$ | 2.67 ml |
| | TEMED | 3 µl |
| | Ammonium persulfate (10 % (w/v)) | 40 µl |

| | | | |
|---|---|---|---|
| ***Stacking gel (3.9 %)*** | 4x Upper Tris | 1.0 | ml |
| | Acrylamide bisacrylamide 30% (v/v) | 0.52 | ml |
| | $H_2O_{bidest.}$ | 2.47 | ml |
| | Ammonium persulfate 10 % (m/v) | 40 | µl |
| | TEMED | 4 | µl |
| ***Staining solution*** | Coomassie Brilliant Blue | 1 | g |
| | Glacial acetic acid | 100 | ml |
| | Methanol | 300 | ml |
| | $H_2O_{bidest.}$ | 600 | ml |
| ***Destaining solution*** | Methanol | 300 | ml |
| | Glacial acetic acid | 100 | ml |
| | $H_2O_{bidest.}$ | 600 | ml |

### 4.6.5. Antibiotics and others

| ***Compound*** | ***Stock solution*** | ***Solvent*** | ***Final concentration (FC)*** |
|---|---|---|---|
| **Ampicilin (Amp)** | 100 mg / ml | $H_2O$ bidest. | 100 µg / ml |
| **Carbenicillin (Car)** | 50 mg / ml | $H_2O$ bidest. | 50 µg / ml |
| **Kanamycin (Kan)** | 30 mg / ml | $H_2O$ bidest. | 30 µg / ml |
| **Chloramphenicol (Cam)** | 34 mg / ml | Ethanol | 34 µg / ml |
| **IPTG** | 1 M | $H_2O$ bidest. | 1 mM |
| **PMSF** | 100 mM | Ethanol | 0.1 mM |
| **5-ALA** | 1 M | $H_2O$ bidest. | 1 mM |
| **FeCl3** | 1 M | $H_2O$ bidest. | 0.5 mM |

### 4.6.6. Commercial kits

| ***Kit*** | ***Supplier*** |
|---|---|
| **QIAquick Gel Extraction kit** | Qiagen GmbH |

# 4.7. Microbiological methods

## 4.7.1. Transformation of E. coli strains by heat shock

Preparation of *E. coli* competent cells by rubidium chloride method:

A fresh 500 µl of overnight culture of bacteria was used to inoculate 50 ml LB medium and incubated at 37 °C with sha king (180 rpm) till $OD_{600}$ = 0.4 – 0.5. The cells were incubated on ice for 15 min and centrifuged at 4 °C, 4000 rpm for 10 min in 50 ml falcon tubes. After centrifugation, cells were resuspended in a 20 ml TFB I buffer and incubated on ice for the next 15 min. To centrifuge at 4000 rpm for 10 min pellets, 2 ml of TFB II buffer was added and the cells were carefully resuspended and incubated on ice for 15 min. For long term storage, cells were aliquot (200 µl), frozen immediately in liquid nitrogen and stored at – 80 °C.

*E. coli* transformation procedure:

First, 1 µl plasmid DNA or 20 µl ligation mixture was added to 100 µl *E. coli* cells, mixed gently and incubated on ice for 30 – 40 minutes. Heat shock was carried out at 42 °C and for 45 seconds. After heat shock the tube was transferred onto ice for two minutes and 800 µl LB medium were added into the tube and incubated one hour at 37 °C and 450 rpm. Cells were centrifuged at 5000 rpm for 5 minutes and supernatant was discarded. The cells were resuspended in 50 µl of supernatant and spread on LB agar plate with appropriate antibiotics. Finally, the plate was incubated overnight at 37 °C.

## 4.7.2. Transformation of E. coli cells by electroporation

Preparation of *E. coli* competent cells for electroporation:

A fresh 500 µl overnight culture of bacteria was used to inoculate 50 ml LB medium and incubated at 37 °C with shaking (180 rpm) till $OD_{600}$ = 0.4 – 0.5. The cells were chilled on ice for 15 min and centrifuged at 4 °C, 4000 rpm for 10 min in 50 ml falcon

tubes. After centrifugation cells were resuspended in 25 ml ice-cold dd $H_2O$ and centrifuged again. The pellets were resuspended in 12.5 ml of autoclaved 10 % glycerol, centrifuged in the same conditions and resuspended in 2.5 ml of 10 % glycerol. Centrifuged cells were at the end resuspended in 250 µl of 10 % glycerol.
For long term storage, cells were aliquoted (60 µl), frozen immediately in liquid nitrogen and stored at – 80 °C.

Electroporation:

This method was used for efficient transformation of *E. coli* with several plasmids.
0.5 µl of plasmid 1 DNA (Amp) and 0.5 µl of plasmid 2 DNA (Kan) with different antibiotic resistance were transferred to a chilled cuvette and added to 60 µl of electro-component cells and then gently mixed. The cuvette was placed in the electroporation chamber and the electrical pulse was started. 940 µl of LB medium was added, transferred to the tube and incubated for one hour at 37 °C. Finally, the cells were resuspended in 50 µl of supernatant and spread on LB agar plate with appropriate antibiotics. The plate was incubated overnight at 37 °C.

| ***Conditions for E. coli electroporation*** | |
|---|---|
| **Volt** | 2.5 kv |
| **Electric field strength** | 25 µF |
| **Resistance** | 200 Ω |

### 4.7.3. Storage and cultivation of bacteria

Storage of *E. coli* and *Streptomyces* strains:

For storage 0.5 ml of autoclaved 87 % glycerol was added to 0.5 ml of overnight cultures ($OD_{600}$~2) and stored at – 80 °C. To recover the bacteria, the surface of frozen glycerol stock was scraped with a sterile needle onto an LB or M65 (for *Streptomyces* strains) agar plate with or without of appropriate antibiotics. Cultivation of *E. coli* in Erlenmeyer flasks

A single colony was picked from LB agar plate and inoculated in 5 ml LB medium with appropriate antibiotics and grown at 37 °C and 180 rpm. An overnight culture was used to inoculate the main culture medium and incubated at 30 °C and 140 - 160 rpm.

Cultivation of *Streptomyces* strains in Erlenmeyer flasks:

A colony was picked from an M65 agar plate and inoculated in a 5 ml M65 medium and grown at 37 °C and 180 rpm. After two days 5 ml M65 preculture was used to inoculate 50 ml main culture medium and incubated at 30 °C and 140 rpm for three days.

All strains were grown on M65 agar plates following the recommendations provided by DSMZ. After one week of incubation at 30 °C, a single colony was selected and used to inoculate 5 ml of liquid M65 medium. After two days, the preculture was used to inoculate a 50 ml main culture, which was then incubated for an additional three days. A model substrate at a final concentration of 0.9 mM (prepared as a stock solution in DMSO) was added to the culture and incubated for three more days. Samples of 1.5 ml were taken and extracted with diethyl ether, followed by drying with magnesium sulfate. The extracted samples were then subjected to analysis.

### 4.7.4. Heterologous protein expression in *E. coli*

Heterologous expression of P450-CamAB system for biotransformation *in vivo*:

Protein expression was efficient in E. coli BL21 (DE3). Over-expression was controlled by the T7 promoter. For co-expression of a P450 of interest together with Cam A (putidaredoxin reductase) and Cam B (putidaredoxin), 25 ml of M9-mixampicillin medium (6.78 g/L $Na_2PO_4$, 3 g/L $KH_2PO_4$, 0.5 g/L NaCl, 1 g/L $NH_4Cl$, 1 % casamino acid, 0.4 % D-glucose, 0.1 mM $CaCl_2$, 0.1 mM $FeSO_4$, 20 µg/ml thymine and 50 µg/ml ampicillin) was inoculated with 250 µl of the overnight culture in the same medium and shaken at 30 °C and 160 rpm for 24 hours. Finally, the culture was centrifuged (3000 rpm, 5 min., 4 °C) and resuspended in CV2 buffer for further investigations.
The Overnight Express™ Autoinduction System was used for high-level protein expression with the pET IPTG-inducible bacterial expression system without monitoring cell growth. Addition of these Overnight Express components to glucosefree complete media, allows auto induction, high cell densities, and increased yields of

soluble target proteins. Finally, the culture was centrifuged (3000 rpm, 5 min., 4 °C). Cells were resuspended in 5 ml of CV2 buffer (2 % glycerol, 50 µg/ml ampicillin, 0.1 mM IPTG, and 50 mM phosphate buffer adjust to pH 7.5).

This System contains three solutions (O/N A.I.S. Solution I, II and III). Solution I is a blend of carbon sources optimized for regulated uninduced growth to high cell density, followed by induction with lactose and continued growth. Solution 2 is a concentrated buffer that maintains pH throughout metabolic acid production and supplies the additional nitrogen necessary to support increased protein synthesis. Solution 3 provides critical magnesium for maximum cell density. Addition of these Overnight Express components to glucose-free complete media, high cell densities, and increased yields of soluble target proteins.

Heterogonous expression of CYP154E1 from *Thermobifida fusca YX* in *E. coli* BL21(DE3) for *in vitro* reactions:

Preculture was inoculated from a single colony into 5 mL Luria-Bertani (LB)-medium supplied with kanamycin (30 µg/mL) and grown at 37 °C with shaking at 180 rpm. This culture was used to inoculate 400 ml TB-medium supplied with kanamycin (30 µg/mL). The cells were grown at 37 °C with shaking 180 rpm to an until $OD_{600}$ reach ~0.8 and expression of the gene was induced by addition of isopropyl-β-Dthiogalactopyranoside (IPTG) to 1 mM, heme-precursor 5-aminolevulinic acid (5- ALA) to 1 mM and $FeSO_4$ to 0.5 mM. After a further 19 h of growth at 30 °C with shaking at 160 rpm the cells were harvested by centrifugation (20 min, 8000 rpm, 4°C). The cell pellet was then resuspended in loading buffer (50 mM potassium phosphate buffer, 0.1 mM PMSF, pH 7.5) and lysed by sonication on the ice (4 x 1 min, output control = 4, duty cycle 40 %; BransonSonifier W250, Dietzenbach, Germany), followed by centrifugation at 18000 rpm, 4 °C for 40 min.

Heterogonous expression of redox partners CamA (PdR) and CamB (Pdx) in *E. coli* BL21 (DE3) for *in vitro* reactions:

For heterogonous expression of separate CamA and CamB genes, Luria-Bertani (LB)-medium was used.

Preculture was inoculated from a single colony into 5 mL Luria-Bertani (LB)-medium supplied with kanamycin (30 µg/mL) and grown at 37 °C with shaking at 180 rpm for 3-4 hours afterwards used to inoculate 400 ml LB-medium supplied with kanamycin (30 µg/mL). The cells were grown at 37 °C with shaking 180 rpm to an until $OD_{600}$ reach ~0.6 and expression of the gene was induced by addition of IPTG to 0.1 mM, $FeSO_4$ to 0.1 mM (for putidaredoxin Cam B) and 50 µL of trace element solution. After a further 16 h of growth at 25 °C with shaking at 120 rpm the cells were harvested by centrifugation (20 min, 8000 rpm, 4 °C). The cell pellet was then resuspended in loading buffer (50 mM potassium phosphate buffer, 0.1 mM PMSF, pH 7.5) and lysed by sonication on the ice, followed by centrifugation at 18000 rpm, 4 °C for 40 min.

Heterogonous expression of CYP154E1 mutants (V286L, V286A, and V286F) in *E. coli* BL21 (DE3):

Preculture was inoculated from a single colony into 5 mL Luria-Bertani (LB)-medium supplied with kanamycin (30 µg/mL) and grown at 37 °C with shaking at 180 rpm for 3-4 hours afterwards used to inoculate 400 ml TB-medium supplied with kanamycin (30 µg/mL). The cells were grown at 37 °C with shaking 180 rpm to and until $OD_{600}$ reached ~0.7 and expression of the gene was induced by addition of IPTG to 1 mM and 50 µL of trace element solution. After a further 16 h of growth at 25 °C with shaking at 120 rpm the cells were harvested by centrifugation (20 min, 8000 rpm, 4 °C). The cell pellet was then resuspended in loading buffer (50 mM potassium phosphate buffer, 0.1 mM PMSF, pH 7.5) and lysed by sonication on the ice, followed by centrifugation at 18000 rpm, 4 °C for 40 min.

## 4.8. Molecular biological methods

### 4.8.1. Polymerase chain reaction (PCR)

The polymerase chain reaction is used for enzymatic replication of a short fragment of DNA which lies between two parts of a known sequence. Two oligonucleotides are used as primers to determine the DNA fragment to be amplified. The DNA template is

denatured by heating to break the hydrogen bonds between the DNA strands. When the solution cools to the annealing temperature, the primers bind to their target sequence, and DNA polymerase extends the annealed primers by joining the free nucleotide bases to the primers. The product can be used as a template for the next amplification, when the process is repeated, resulting in an exponential increase of the desired DNA product.

Procedure:

Genomic DNA and plasmid DNA was used as template DNA. Original polymerase buffer and *Pfu* DNA polymerase was used.

| ***Component*** | ***Volume [µl]*** | ***Final concentration*** |
|---|---|---|
| **10 x buffer with MgSO4** | 5 µl | 1 x |
| **dNTPs (10 mM)** | 4 µl | 0.8 mM |
| **DMSO** | 2.5 µl | 5 % |
| **Primer Forward (100 pM)** | 1 µl | 2 pM |
| **Primer Reverse (100 pM)** | 1 µl | 2 pM |
| **DNA** | 1 µl | 70 – 144 ng |
| ***Pfu* DNA polymerase** | | 1 U |
| **Added d H2O to final volume of 50 µl** | 1 µl | |

Program:

| | ***Temperature*** | ***Time [min]*** |
|---|---|---|
| **Initial denaturation** | 96 °C | 4 |
| **Denaturation** | 96 °C | 1 |
| **Annealing** | 54-58 °C | 1 |
| **Extension** | 72 °C | 2 min 30 sec |

| | | |
|---|---|---|
| **Go to denaturation step** | **repeat 30 times** | |
| **Final extension** | 72 °C | 4 |
| **Hold time** | 8 °C | |

### 4.8.2. Isolation of plasmid DNA from *E. coli*

*Minipreparation with a spin-column*

This method was used to isolate highly pure plasmid DNA for sequencing or enzymatic digestion. Spin Miniprep kit from Qiagen or Sigma was used. It is also based on alkaline lysis, followed by purification of the DNA by ion-exchange chromatography.

Procedure:

2 ml overnight culture was harvested by centrifugation at 14000 rpm. The plasmid DNA isolation was performed according to the manual of the manufacturer.

### 4.8.3. DNA digestion with restriction endonucleases

Restriction endonuclease cuts double-stranded DNA at specific position, known as recognition sequence. Many in laboratory used restriction enzymes produce sticky ends of DNA molecules, which can be further ligated by T4 ligase to other DNA molecules with the same sticky ends.

Procedure:

Purified DNA (50 ng/ µl) was mixed with 5 µl 10 x restriction enzyme buffer and two restriction endonucleases (10 U) and water was added to a final volume of 50 µl, mixed gently and short spins centrifuged. The reaction mixture was then overnight incubated 2 h - overnight at 37 °C. The digested DNA was purified by agarose gel extraction afterwards.

### 4.8.4. Dephosphorylation of DNA

Recircularization of vector without insertion of foreign DNA during ligation decreases cloning efficiency. Because DNA ligation absolutely needs a 5’ phosphate group, the self ligation can be blocked by removing the 5’ phosphate group from the vector with

calf intestinal alkaline phosphatase (CIAP). This treatment was used to decrease vector background in cloning.

Procedure:
After digestion of vector 1 U of alkaline phosphatase (CIAP) was added, gently mixed, short spin centrifuged and incubated 1 hour at 37 °C. After dephosphorylation reaction mixture was being incubated at 80 °C for 15 min for CIAP denaturation. The DNA was purified by agarose gel extraction to remove undigested plasmid, salts and proteins for further usage.

### 4.8.5. Ligation of DNA with T4 DNA ligase

DNA ligase joins two DNA molecules by forming a covalent phosphodiester bond between 3' hydroxyl end and 5' phosphate end of DNA, using ATP as energy donor, resulting in a new DNA molecule.

Procedure:

| ***Component*** | ***Volume [µl]*** | ***Final concentration*** |
|---|---|---|
| **Vector** | 3 µl | 24 ng |
| **Insert gene** | 1 µl | 32 – 52 ng |
| **T4 Ligase** | 1 µl | 1 U |
| **10 x T4 Ligase buffer** | | 10 x |
| **Added d H2O to final volume of 20 µl** | 2 µl | |

The ligation mixture was gently mixed, short spin centrifuged and incubated overnight at 16 °C.

### 4.8.6. Agarose gel electrophoresis

Electrophoresis uses a mechanism like sifting objects through a sieve. In the case of DNA-based gel electrophoresis, an electric field is used to push negatively charged DNA molecules through a gel matrix towards the anode. The migration rate is affected by several parameters. The concentration of agarose, voltage, conformation of DNA and size of DNA are the most important parameters. Under the same condition, larger

and or DNA molecules move more slowly than smaller or supercoiled DNA because of stronger frictional drag. Ethidium bromide, a fluorescent dye, causes DNA to move slower, because ethidium bromide intercalates and uncoils DNA to some extent.

Procedure:

Ethidium bromide was added to warm 1 % agarose solution (5 µl to 50 ml of 1 % agarose solution). The warm agarose was put into a rack with a comb. After the gel was polymerised, the comb was removed, and the gel was transferred to an electrophoresis tank with 1 x TAE buffer. DNA samples were mixed with a DNA loading buffer (3 µl of DNA + 5 µl of loading buffer). 1 kb- ladder was used as a slide standard. Electrophoresis was performed at 120 V for 30 min and 400 mA. The gel was examined by UV light and documented.

### 4.8.7. Isolation of DNA from agarose gel

The band corresponding to the desired DNA fragment was cut using scalpel under UV light - table. To avoid damage of DNA by UV have to be done very rapidly and the DNA fragment was treated as described in the QIAquick Gel

### 4.8.8. Quantitation of DNA

The DNA concentration can be determined by spectrophotometric measurement of the absorption in ultraviolet range. The readings were taken at 260 nm and 280 nm. The OD at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to 50 µg/ ml for double stranded DNA, 40 µg/ ml for single stranded DNA. The ratio of $OD_{260}$/ $OD_{280}$ gives an estimate of the purity of the DNA. Pure DNA preparations are from 1.8. Pure RNA is 2.0. If there is contamination of protein or phenol the ratios will be much lower than 1.8 for DNA.

### 4.8.9. Quick -change™ for site-directed mutagenesis

*In vitro* site-directed mutagenesis was carried out with the QuickChange™ SiteDirected Mutagenesis Kit (Strategene). The basic procedure utilizes a circular, double stranded DNA vector with an insert and two synthetic complementary oligonucleotide primers

containing the desired mutation. By elongation of the annealed oligonucleotide primers a mutated plasmid is generated using temperature cycling and *Pfu* DNA polymerase. Following the temperature cycling the product is treated with *DpnI.* This endonuclease, specific for methylated and hemimethylated DNA, is used to digest template DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore, in contrast to the newly synthesized strands, susceptible to *DpnI* digestion.

Procedure:
Plasmid DNA (Spin Mini Prep) was used as a DNA template. The components of the mixture were pipetted together according to the following general scheme:

| ***Component*** | ***Volume [µl]*** | ***Final concentration*** |
|---|---|---|
| **10 x *Pfu* buffer** | 5 | 1x |
| **DMSO** | 5 | 10 % (v/v) |
| **dNTP Mix (containing 10 mM of each dNTP)** | 1 | 200 µM of each dNTP |
| **Primer F** | 1 | 0.022 µM |
| **Primer R** | 1 | 0.022 µM |
| ***Pfu* DNA polymerase** | 1 | 2.5 U/µl |
| **Template DNA** | 1 | 5-50 ng |
| **Added dd$H_2O$ to final volume of 20 µl- 50 µl** | | |

PCRs were performed in a thermocycler (Eppendorf) using following program:

| | ***Temperature*** | ***Time [min]*** |
|---|---|---|
| **Initial denaturation** | 96 °C | 1 |
| **Denaturation** | 96 °C | 1 |
| **Annealing** | 54-58 °C | 1 |
| **Extension** | 72 °C | 4 |
| **Go to denaturation step a** | **d repead 30 times** | |
| **Final extension** | 72 °C | 4 |

| | |
|---|---|
| **Hold time** | 8 °C |

## 4.9. Biochemical methods

### 4.9.1. Cell disruption by sonication

Cells were being centrifuged at 8000 rpm for 20 min and resuspended in 8 ml 50 mM Potassium phosphate buffer pH 7.5 or 50 mM Tris HCl buffer pH 7.5 containing 1 mM phenylmethane-sulphonylfluoride (PMSF). For this purpose Sonifier 250 sonicator was used. The cells were chilled on ice with water and a small amount of NaCl and sonicated for 1 min (output 35 W). After every 2 minutes the procedure was 3 times repeated. The cell extract was being centrifuged at 4 °C, 8000 rpm for 1 hour. The cell lysate was removed to a new falcon tube.

### 4.9.2. Protein purification via IMAC

In order to facilitate enzyme purification a $His_6$-tag (Hengen et al. 1995) was added at the N-terminus of the recombinant protein. The separation is based on the affinity of His-tagged proteins towards two-valent metal ions like nickel ions.

Buffers:

| ***Buffer*** | ***Compound*** | ***Concentration*** |
|---|---|---|
| **Loading buffer** | KPi | 50 mM |
| | KCl | 300 mM |
| **Wash buffer** | KPi | 50 mM |
| | KCl | 300 mM |
| | Imodazole | 20 mM |
| | PMSF | 0.1 mM |
| **Elution buffer** | KPi | 50 mM |
| | KCl | 300 mM |
| | Imodazole | 200 mM |
| | PMSF | 0.1 mM |
| **All the buffers were adjust 7.5.** | | |

Procedure:

The N-terminally attached $His_6$-tag allowed purification by immobilized metal chelate affinity chromatography (IMAC), utilising a Ni-NTA-resin (Qiagen, Germany). Since the recombinant protein is the only component with a $His_6$-tag, the $His_6$-tagged protein is bound to the resin whereas all other proteins pass through the column. The column-bound enzyme was washed with a 50 mM potassium phosphate buffer (pH 7.5) containing 500 mM sodium chloride and 20 mM imidazole. Elution was performed by increasing the imidazole concentration to 200 mM. Imidazole competes with the $His_6$-tagfor nickel binding. Imidazole and sodium chloride were removed by dialysis against 50 mM potassium puffer (pH 7.5) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) overnight. The purity of the enzyme was estimated by SDS-PAGE, using 12.5 % polyacrylamide running gels (Laemmli 1970). Enzyme samples were stored at -20°C until use.

### 4.9.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The sodium dodecyl sulphate polyacrylamide gel electrophoresis is used to separate proteins according to their molecular weight to check the purity and molecular mass. SDS denatures and binds to the proteins, and therefore, SDS gives them a negative charge proportional to the mass. Proteins migrate to the anode of gel in an electric field. Polyacrylamide gel works here as a molecular sieve, big molecules run slower through the gel than small molecules due to stronger frictional drag.

| ***LMW standard (Bio-Rad)*** | |
|---|---|
| **Protein** | Molecular mass [kDa] |
| **Phosphorylase B** | 97.4 |
| **Serum albumin** | 66.2 |
| **Ovalbumine** | 45.0 |
| **Carbonic anhydrase** | 31.0 |
| **Trypsin inhibitor** | 21.5 |
| **Lysozyme** | 14.0 |

Procedure:

After assembling two glass plates the resolving gel was poured into the gap between the glass plates. The gel was overlaid with isopropanol to ensure a flat surface and to exclude air. Isopropanol was washed off with water after the gel had polymerized. The stacking gel was poured onto the polymerized resolving gel and a comb was inserted in the stacking gel solution. After the stacking gel had polymerized, the comb was removed and the gel was put in an electrophoresis chamber, which was filled with an electrophoresis buffer. The protein samples were mixed with a loading buffer and heated at 95 °C for 5 min. After that the samples were loaded on stacking gel with the low molecular weight (LMW) standard as reference. For each gel the electrophoresis was performed at 10 mA for 10 min and 25 mA for approximately 50 min. After electrophoresis the gel was covered with staining solution on a shaker overnight and distained three times with destaining solution. Finally the gel was dried at 80 °C in vacuum between filter paper and cellophane foil to be stored.

### 4.9.4. Spectroscopic analysis

Concentration of cytochrome P450 monooxygenase:

Binding spectra were recorded at 25 °C on an Ultrospec 3000 UV/vis spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Carbon monoxide can bind ferrous P450 and it is possible to get a shift of the maximum absorbance of heme to 450 mn. Omura and Santo calculated as first the P450 concentration in solution.

$$[P450] = (A_{450} – A_{490}) \times dil. \times 1000 / \varepsilon$$

ε: coefficient = 91 $mM^{-1}cm^{-1}$ $A_{450}$:
absorbance at 450 nm

$A_{490}$: absorbance at 490 nm

The sample containing the P450 solution was divided into two cuvettes. One of the aliquots was treated with carbon monoxide. Rapidly before the measurement was started a couple crystals of sodium dithionite were added and directly measured.

The absorption of the sample in the region between 400 and 500 nm was measured using as a reference sample without carbon monoxide.
The cells were yielded and resuspended in 10 ml 50 mM potassium phosphate buffer pH 7.5 containing 1 mM PMSF and disrupted by sonication using a Sonifier 250 sonicator, kept on ice and sonicated for 1 min with output 40 W, 40 % work interval and 1 min break. This process was repeated 4 times. The cell extract was centrifuged at 18000 rpm for 20 min at 4 °C. The cell lysate was carefully removed in a new tube, filtrated by 0.45 µm filters and concentrated on centrifugal filter devices *Centricon* YM-30 membranes approved for diagnostic *in vitro*. For spectroscopic characterization and oxidation activity measurements a crude filtered protein was used.

Reduction of CYP154E1 via non natural redox partners Pdx and PdR from *P. putida* ATCC17453:

The redox partners PdR and Pdx are known to support foreign bacterial cytochrome P450 activity. The two proteins were used as the P450 reductase system in the conversion of terpenoids and alkanes by CYP154E1. To check if they are able to transfer electrons from NADH to the P450 the following experiment was performed:

Procedure:
The 10 µM CYP154E1 with two redox partners 50 µM Pdx and 50 µM PdR from *P. putida* were resuspended in 50 mM phosphate buffer adjusted to pH 7.5 with 1 mM EDTA and mixed. For determination of CO difference spectra 0.1 mM of model substrate (30 mM stock solution in DMSO) was added and mixed for 30 min under nitrogen. Afterwards to the mixture 1 mM NADH was added and divided into two quartz cuvettes via 1 ml. One of the cuvettes was saturated about 3 min with CO at a rate of about 1 bubble per second and after 10 min the absorbance difference between reduced P450 and P450 bound to CO between 350 and 500 nm was measured (Maurer Dissertation 2006).

| ***Component*** | ***Working concentration*** |
|---|---|
| **P450** | 10 µM |
| **Substrate** | 200 µM |

| | |
|---|---|
| **PdR** | 50 µM |
| **Pdx** | 50 µM |
| **NADH** | 1 mM |
| **KPi buffer adjust to pH 7.5** | 50 mM |
| **Total volume** | 1 ml |

Monitoring of substrate binding affinities of wild-type CYP154E1 and mutants (V286L, V286A, V286F):

The UV spectral changes which accompany substrate interactions with P450s have been used to indicate certain binding characteristics, or so called types of ligand binding. There are three categories: type I, type II and reverse type I. These changes, which occur in the UV spectra following ligand-P450 complexion reactions, represent a method for classifying varieties of P450 active site interactions (Schenkman et al., 1981). Specifically type I spectral change entails a reduction in the soret absorption at 422 nm and a concomitant increase in the 392 nm absorption, which may be regarded as a shift in the hemoprotein equilibrium from low- to highspin ferric P450. Substrates which exhibit a type II spectral change appear to act primarily as inhibitors of the enzyme by ligating the heme iron, forming a relatively stable, tightly bound complex. The type II spectral change is characterized by a decrease in absorption at around 390-405 nm accompanied by an increase at 422435 nm. The third variety of spectral change is essentially a mirror image of the type I case. Consequently, in reverse type I there is an increased absorption at 420 nm and a decrease at 390 nm. Substrates that displace the six-coordinated solvent in the resting P450 usually induce a shift to the blue which reflects a low- to high-spin transition of the iron. Differential spectroscopy is used to monitor binding of substrates. For determination of Kd values, the reciprocal values of the absorption difference between 392 nm and 422 nm were plotted against the reciprocal values of the substrate concentration.

A solution of substrates in DMSO was subsequently added to produce a final substrate concentration of between 10 to 600 µM. A reference sample containing enzyme solution and the appropriate amount of DMSO was used.

Procedure:
Potential substrates and purified enzymes were pipetted together according to the following general scheme and spectral changes were monitored between 300 and 500 nm.

| ***Component*** | ***Working concentration*** |
|---|---|
| **P450** | 10 µM |
| **Substrate** | 10-600 µM |
| **KPi buffer adjust to pH 7.5** | 50 mM |
| **Total volume** | 1 ml |

Determination of concentration of putidaredoxin reductase (PdR) and putidaredoxin (Pdx):

Concentration of the FAD-containing putidaredoxin reductase (PdR; 46 kDa; EC 1.18.1.3) from *P. putida* was calculated using extinction coefficient of 10.4 $mM^{-1}$ $cm^{-1}$ at 455 nm, respectively (Gunsalus and Wagner 1978).
Concentration of the Pdx (camB) was calculated using reduced Pdx prepared by adding aliquots of a sodium dithionite solution to a quartz cuvette containing oxidized Pdx under nitrogen. The scan in which the 325-, 415-, and 455-nm absorbances were minimized was taken as the best representation of fully reduced Pdx. Dilution factor corrections due to the added volume of dithionite were made (Holden and Vilker 1997).

Activity of putida redoxin reductase (PdR):

Tests for reductase domain-dependent electron transfer to exogenous electron acceptor cytochrome *c* were performed at room temperature in 50 mM potassium phosphate buffer (pH 7.5) as described by Gustafsson (Gustafsson, Roitel et al.

2004).

## 4.10. Biocatalytic methods

### 4.10.1. Biotransformation *in vitro*

The 0.5 ml reaction was carried out in 2 ml microtube preparation at 30 °C and 450 rpm. Reaction mixtures consisted of 0.2 mM substrate, 0.2 mM NADH, 2 % ethanol, 50 mM potassium phosphate buffer adjust to pH 7.5, 10 µM of CYP154E1, and 50 µM of PdR and 50 µM of Pdx were prepared and NADH regeneration system was used. After 4h, reaction mixture was extracted with 500 µl ethyl acetate, diethyl ether or dichloromethane and concentrated up to 150 µl and measured on GC/MS. For NADH-regeneration each reaction was supplemented with 4 mM glucose-6phosphate, 4 U/mL G-6-P dehydrogenase, and 0.2 mM $NAD^+$, in a final 0.5 ml reaction volume, according to Wong and Whitesides from 1980.

In the following experiments crude enzyme solutions were used. A model substrate was added as a 30 mM stock solution in DMSO to a final concentration of 0.2 mM. Negative controls were the same composition except enzymes. An addition of cofactor NADPH (0.6 mM) initiated a reaction and after 1.5 hours incubation at room temperature, mixture was extracted with DCM. After centrifugation, the organic phase was dried over magnesium sulfate anhydrous and analyzed on GC/MS.

*Derivatization and enantioselectivity determination:*

To permit detection of compounds not directly amenable to analysis like short chain alkenes derivatization was used. It is the process of chemically modifying a compound to produce a new compound which has properties that are suitable for analysis using a GC.

For enantioselectivity determination, short chain alkenes were derivatized with acetic anhydride and analysed using GC with a chiral column.

*Procedure:*

The 1 ml reaction mixture described above was extracted with 2 ml of dichloromethane (DCM), vaporised and 550 µl toluol with 500 µl acetic anhydride was added. After 3 hours of incubation at 80 °C, mixture was washed with 2 ml of water and organic phase was analysed on GC equipped with a chiral column FS-

Cyclodex beta -I/P (0.25 mm x 50 m, 0.25 µm, CS-Chromatography Service, Langerwehe, Germany).

## 4.11. Analytics

### 4.11.1. Product extraction and a GC/MS-analysis

For qualitative and quantitative product analysis gas chromatography, gas chromatography-mass spectrometry (GC/MS) and TLC was used.

GC/MS was performed on a Shimazu GC/MS QP2010 (Kyoto, Japan) equipped with a FS Supreme-5 column (0.25 mm x 30 m, 0.25 µm, CS-Chromatography Service, Langerwehe, Germany).

*GC/MS temperature programs:*

| ***Substance*** | ***Temperature program*** |
|---|---|
| **Geraniol**<br>**Nerol**<br>**Geranylacetone**<br>**Nerylacetone** | 120 °C (3 min) - 165 °C (5 °C $min^{-1}$) - 280 °C (30 °C $min^{-1}$) -285°C |
| **α-Ionone**<br>**β-Ionone** | 150 °C (1 min) - 250 °C (20 °C $min^{-1}$) - 250 °C (5 min) -280°C |
| **(-)- α -Pinene** | 90 °C (5 min) - 250 °C (15 °C $min^{-1}$) - 250 °C (1 min) |
| **R-(+)-Limonene** | 80 °C (2 min) - 180 °C (10 °C $min^{-1}$) - 250 °C (30 °C $min^{-1}$) |
| **Valencene** | 150 °C (5 min) - 250 °C (10 °C $min^{-1}$) - 250 °C (5 min) - 300 °C (50 °C $min^{-1}$) - 300 °C (1 min) |

| ***Substance*** | ***Temperature program*** |
|---|---|
| ***n*-pentane**<br>***n*-hexane 2-hexanol** | 40 °C (1 min) - 80 °C (2 °C $min^{-1}$) - 80 °C (1 min) - 200 °C (30 °C $min^{-1}$) |

| | |
|---|---|
| ***n*-heptane 3-heptene-5-ol 1-heptanol *n*-octane 1-octanol 2-octanol** | |

**3-octanol**
**4-octanol**
**1-octene-3-ol 2-**
**octene-1-ol *n*-**
**decane 1-decanol**
**1,2-dihydroxydecane**

---

Chiral analysis GC was performed on a FS-Cyclodex beta -I/P column (0.25 mm x 50 m, 0.25 µm, CS-Chromatography Service, Langerwehe, Germany.

*Reaction conditions:*
GC (chiral column) temperature program:

| ***Substance*** | ***Temperature program*** |
|---|---|
| ***n*-octane** | 90°C (2 min) - 175°C |

### 4.11.2. Thin Layer Chromatography (TLC)

The general practices of TLC are widely used (Bobbitt, 1963). Silica gel 60 $F_{254}$ thinlayer aluminum sheets 5-10 cm (Fluka) were used. Chromatographic solvent system was represented by a mixture of petroleum ether (PE) and ethyl acetate (EE) at ratio 2:1 (v/v). Solvent mixture had been deposited in the bottom of the development chamber approximately 20 min before use. About 10 ml of each reaction sample as well as authentic standard sample solutions in diethyl ether (1 mg/ml) were applied on the silica gel sheets about 1 cm from the base. Sheets were then placed into the development chamber. The developer was allowed to move up 5–7 cm above the solvent surface. The chromatograms were air dried at room temperature and evaluated first under $UV_{254nm}$ light for the identification of allylic alcohols. Afterwards the substrates and products spots were visualized using two different visualizers (a) potassium permanganate mixture ($K_2CO_3$: $KMnO_4$: $H_2O$ ¼ 0.15: 1: 15 (v/v/v), or (b) aqueous solution of Fast Blue RR Salt.

## 4.12. Immobilization of recombinant E. coli BL21 (DE3) on latex material

For oxidation experiments with latex entrapped *E.coli,* coatings contained 5 mL of 50 mM potassium phosphate buffer pH 7.2 supplemented with 2 mM final concentration of nerol (300 mM stock solution in DMSO) small petri dish bioreactors and incubated at 30°C and 90 rpm for 4 days. After five days of incubation 1 mL of a sample was extracted with 500 µl of ethyl acetate and a small amount of sodium chloride and then dried with magnesium sulphate. For analysis of product formation thin-layer chromatography (TLC) method was used. For different experiments different concentrations of nerol were used.

### 4.12.1. Latex toxicity to E. coli

The contact toxicity of SF091 latex emulsions (Rohm and Haas) were investigated using the CV2-washed cell paste of *E. coli* expressing CYP154E1, grown in M9 medium. The cell-coating mixtures were prepared according to the method described above using latex emulsion at pH 3.6 and pH 6.95 and a third one as a control with adding 2 mM nerol (in DMSO) solution instead of the latex emulsion. After incubation (30˚C, 60 min), the material in each tube was resuspended in 1ml CV2 plus 2 mM nerol and plated for colony forming units (CFU) on LB agar plates.

### 4.12.2. Preparation of nano-porous latex coatings SF091

Polymer emulsion mixture for immobilization of *E. coli* in biocatalytic coating was formulated by using Rovace SF091 (Rohm and Haas, Spring House, PA). SF091 is a monodispersed copolymer vinyl acetate-co-acrylic latex emulsion (average particle size of 300 nm, solids content of 54.9 % (w/w), glass transition temperature, $T_g$ about 10 °C ) adjusted to pH 7.2. The emulsion was adopted with 1.3 g wet cell paste, 402 µl filter-sterilized sucrose solution (0.58 g/mL), 162.5 µl neat glycerol, and 1083 µl of SF091 latex.

Production of coatings was performed by using the method of Lyngberg et al. [1999]. Template was prepared by the following methods:

*Procedure 1:*

Punching nine 12.7 mm diameter holes through 77 µm thick pressure sensitive vinyl adhesive film (Con-Tact, Stamford, CT) and applied 123 µm thick polyester (DuPont Melinex 454, Tekra Corporation, New Berlin, WI) or photo paper with a rubber roller.
A #26 and #0 (better for *S. coelicolor*) Mayer rod (Paul N. Gardner Co., Pompano Beach, FL) was used with a constant pressure drawdown, placed in a humidified acrylic chamber at 23.7 °C and 51.3 % relative humidity, RH monitored by a calibrated digital temperature and relative humidity sensor (model TM125, Dickson, Addison, IL). After drying for 60 min the patches were punched out and ready to use.
The dry thickness of coatings was measured using a digital micrometer (Model IDC112GEB, Mitutooyo USA Corporation, Plymouth, MI; convex tip surface area 0.44 $mm^2$, 1.1 MPa pressure exerted by the tip).

*Procedure 2:*

Punching nine 50 mm diameter holes through 153 µm thick pressure sensitive film and applied 123 µm thick polyester (DuPont Melinex 454, Tekra Corporation, New Berlin, WI) or photo paper with a rubber roller. A #0 (better for *S. coelicolor*) Mayer rod (Paul N. Gardner Co., Pompano Beach, FL) was used with a constant pressure drawdown, placed in a humidified acrylic chamber at 23.7 °C and 51.3 % relative humidity, RH monitored by a calibrated digital temperature and relative humidity sensor (model TM125, Dickson, Addison, IL). After drying for 60 min the patches were punched out and ready to use. The dry thickness of coatings was measured using a digital micrometer (Model ID-C112GEB, Mitutooyo USA Corporation, Plymouth, MI; convex tip surface area 0.44 $mm^2$, 1.1 MPa pressure exerted by the tip).

*Procedure 3:*

Cute 50 mm diameter holes through 153 µm thick pressure sensitive film and applied 123 µm thick polyester (DuPont Melinex 454, Tekra Corporation, New Berlin,
WI). Bacteria were painted on PE and placed in a humidified chamber at 23.7 °C and 51.3 % relative humidity. After drying for 60 min the patches were cut out and ready to use. The dry thickness of coatings was measured using a digital micrometer (Model ID-C112GEB, Mitutooyo USA Corporation, Plymouth, MI; convex tip surface area 0.44 $mm^2$, 1.1 MPa pressure exerted by the tip).

For immobilization of recombinant *E. coli* cells on latex SF091, procedure 3 was preferred.

# 5. Results

## 5.1. New assay for determination of allylic alcohols

Oxidation of terpenes and terpenoids by cytochrome P450 monooxygenases may result in both epoxides and allylic alcohols. These substances can easily be separated and identified by GC or GC-MS. This approach is, however, time- and material-consuming. For the fast and exact identification of oxidation products a simple, fast and sensitive thin-layer chromatography (TLC) assay was required. This method can be used to monitor chemical and biochemical reactions within a short timeframe. Visualisation of the spots on silica under different conditions will distinguish either between different epoxides or epoxides and allylic alcohols and therefore will enable a quite rapid screening of oxygenases with different regio- and chemoselectivity or allow reliable microbial screening.

### 5.1.1. Assay development

Seventeen standards were used for optimization of the TLC method: geranylacetone (1) and its oxidized derivatives 5,6-epoxy-(1), 9,10-epoxy-(1), 5,6;9,10-bisepoxide(1), 11-hydroxy-(1), 7- or 8-hydroxy-(1) (the position of oxidation could not be identified exactly); nerylacetone (2) and its oxidized derivatives 5,6-epoxy-(2), 9,10epoxy-(2), 5,6;9,10-bisepoxide-(2) and 11- hydroxy-(2); geraniol (3) and its derivatives 2,3-epoxy-(3) and 6,7-epoxy- (3), nerol (4) and its derivatives 2,3-epoxy(4) and 6,7-epoxy-(4) Table 5-1. As a more appropriate mobile solvent phase for TLC for all compounds, a mixture of petroleum ether (PE) and ethyl acetate (EE) was chosen. What is more, well-defined spots were obtained at all tested ratios, but a mixture PE with EE at ratio 2:1 (v/v) was optimal. In the beginning, potassium permanganate mixture ($K_2CO_3$: $KMnO_4$: $H_2O$ = 0.15:1:15 (v/v/v)) was tested for visualization of the spots on silica gel. Potassium permanganate can be used in TLC as a mixture with sulphuric acid for detection of, for example, carboxin and oxycarboxin (Tripathi et al. 1973) or phosphine oxide (Ertel et al. 1962). Aqueous solution of potassium permanganate (1–2 %) is routinely used for visualization of unsaturated aliphatic compounds (Prochazka et al.

1989). As to our knowledge, however, there is no example in the literature describing any visualizing reagent suitable for separation of oxygenated terpenoides e.g., epoxides and allylic alcohols. Almost all tested compounds, including epoxides and allylic alcohols migrated to the Rf values given in Table 1 and appeared on the sheets as yellow spots on a magenta background. Both bisepoxides gave only very weak signals, indicating a much higher detection limit than those of other standards. The lower limits for all other compounds were appr. 700–1000 ng.

Geranylacetone and its oxidized derivatives (epoxides and allylic alcohols) could also be identified using Fast Blue RR Salt solution with subsequent heating at 100 °C for 5 min. This bifunctional reagent is widely used for detection of phenols, pyrone derivatives, and some other aromatic compounds, which are able to build reddish dyes with Fast Blue RR Salt (Jork et al. 1989). Acyclic terpenoids had not been tested with Fast Blue RR Salt previously and therefore the mechanism of the interaction is not clear. Anyway, the procedure resulted in grey spots on a yellow background. Other standard compounds were not tested with Fast Blue RR Salt. Interestingly, the exposure to ultra-violet light (254 nm) without any previous colour development of chromatograms allows seeing only the fluorescent hydroxylated compounds (allylic alcohols), but not the epoxides (Fig. 5-1, A).

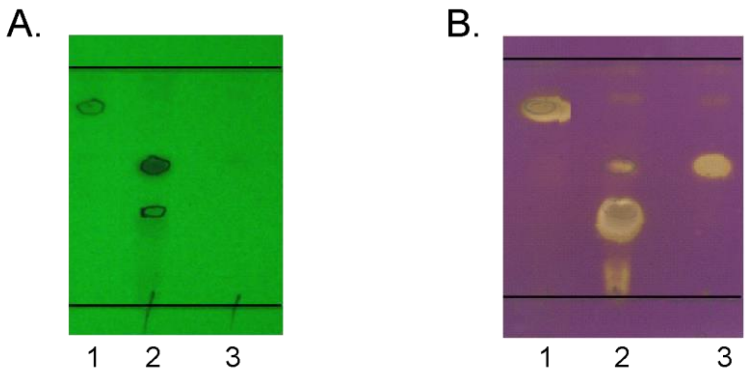



**Figure 5-1: A**: TLC-plate under UV light at 254 nm; **B**: TLC-plate coloured with aqueous solution of potassium permanganate; **1**: geranylacetone; **2**: 11-hydroxy- and 8-hydroxygeranylacetone; **3**: 9,10epoxygeranylacetone.

**Table 5-1:** $R_f$ values calculated for some terpenoids and their oxidized derivatives after TLC development and visualization with potassium permanganate

| *Compounds* | *$R_f$ values* |
|---|---|
| **Geranylacetone** | 0.88 |

| | |
|---|---|
| **9,10-epoxygeranylacetone** | 0.56 |
| **7- or 8-hydroxygeranylacetone** | 0.60 |
| **5,6-epoxygeranylacetone** | 0.65 |
| **5,6;9,10-diepoxygeranylacetone** | 0.35 |
| **11-hydroxygeranylacetone** | 0.38 |
| **Nerylacetone** | 0.91 |
| **9,10-epoxynerylacetone** | 0.57 |
| **5,6-epoxynerylacetone** | 0.67 |
| **5,6; 9,10-diepoxynerylacetone** | 0.39 |
| **11-hydroxynerylacetone** | 0.27 |
| **Geraniol** | 0.77 |
| **6,7-epoxygeraniol** | 0.39 |
| **2,3-epoxygeraniol** | 0.58 |
| **Nerol** | 0.60 |
| **6,7-epoxynerol** | 0.22 |
| **2,3-epoxynerol** | 0.57 |

### 5.1.2 Analytical verification

To verify the TLC method described here, we used a triple mutant of P450 BM-3 from *Bacillus megaterium*, capable of oxidation of geranylacetone. The reaction was performed as described in the experimental section and the products were extracted and then applied on a silica gel sheet. After development of the chromatogram in the chamber, spots visualization has been carried out using all three methods described above. In order to visualize only the fluorescent allylic alcohols, but not the epoxides, the chromatogram was observed under ultraviolet light (254 nm). Small signals corresponding to 7- or 8-hydroxylated products (the exact structure of the reference substances was not clear) and 11-hydroxy-geranylacetone, were visible on the chromatogram (Table 5-2). The following visualization using potassium permanganate or Fast Blue RR salt revealed presence of 9,10-epoxy-geranylacetone a part of the alcohols, (Table 5-2). The same extract of the reaction mixture has been applied on the GC column. GC analysis confirmed the preliminary data, obtained by TLC. Triple

mutant of P450 BM-3 produces up to 94 % of 9,10-epoxygeranylacetone, 4 % of 7- or 8-hydroxylated geranylacetone and 2 % of 11-hydroxygeranylacetone (Fig. 5-2).

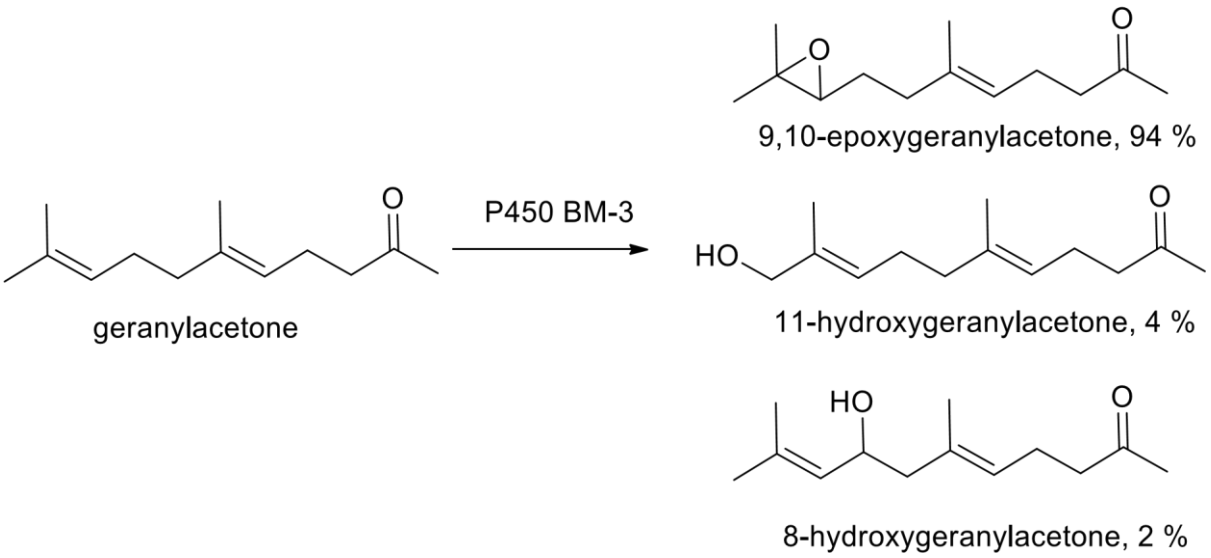



**Figure 5-2:** Oxidation of geranylacetone by P450 BM-3 monooxygenase.

**Table 5-2:** Identification of the products from two biooxidations.

| $R_f$ Values/TLC | Products of biooxidation |
|---|---|
| **Geranylacetone with P450 BM-3** | |
| **0.60** | 7- or 8-hydroxygeranylacetone |
| **0.56** | 9,10-epoxygeranylacetone |
| **0.38** | 11-hydroxygeranylacetone |
| **Nerylacetone with *S. griseolus*** | |
| **0.67** | 5,6-epoxynerylacetone |
| **0.57** | 9,10-epoxynerylacetone |

## 5.2. Primary screening of *Streptomyces* strains

Monooxygenation, a key reaction in various biological processes, is commonly catalyzed by flavin monooxygenases and cytochrome P450 enzymes. It is wellestablished in the literature that numerous P450 monooxygenase genes are present in many Streptomyces strains. These enzymes exhibit diverse catalytic

activities and play crucial roles in the biosynthesis of natural products and the metabolism of xenobiotics (chemical substances foreign to an organism). The abundance of P450 monooxygenase genes in Streptomyces highlights the significance of these enzymes in various biological and biochemical pathways (D. P. OKeefe and P.A. Harder 1991). Twelve different bacterial *Streptomyces* strains were investigated *in vivo* for their oxidation activity towards acyclic monoterpenes and derivatives (Table 5-3).

**Table 5-3:** Summary of all tested bacterial strains in this study: (+) indicates activity, (-) indicates no activity.

| ***Strains*** | ***Activity towards model acyclic monoterpenes*** |
|---|---|
| ***Streptomyces rimosus*** **DSM 40260** | - |
| ***Streptomyces venezuelae*** **DSM 40230** | - |
| ***Streptomyces lividans*** | - |
| ***Streptomyces antibioticus*** | - |
| ***Streptomyces ambofaciens*** **DSM 40053** | - |
| ***Streptomyces griseus*** **DSM 40236** | - |
| ***Streptomyces albus*** **DSM 40313** | - |
| ***Streptomyces nitrozolus*** | - |
| ***Streptomyces pavulus*** **DSM 40722** | - |
| ***Streptomyces griseolus*** **DSM 40854** | + |
| ***Streptomyces avermitilis*** **DSM 40854** | - |
| ***Streptomyces coelicolor A3 (2)*** **DSM 46492** | + |

All Streptomyces strains were cultured in M65 medium (M and M 4.3.3.) for the specified period. To screen the activities of P450 monooxygenases, a combination of fast TLC method and GC analysis was employed. Among the tested strains, *S. griseolus* DSM 40854 and *S. coelicolor A3 (2)* exhibited promising results, prompting further investigations. Notably, S. griseolus displayed the ability to utilize nerylacetone as a substrate. After the completion of the reaction, product extraction, and TLC analysis, the resulting chromatogram was developed and evaluated using visualization

techniques. Intriguingly, no signals were detected under ultraviolet light, indicating the absence of any alcohols in the analyzed mixture (Romankiewicz et al., 2006).

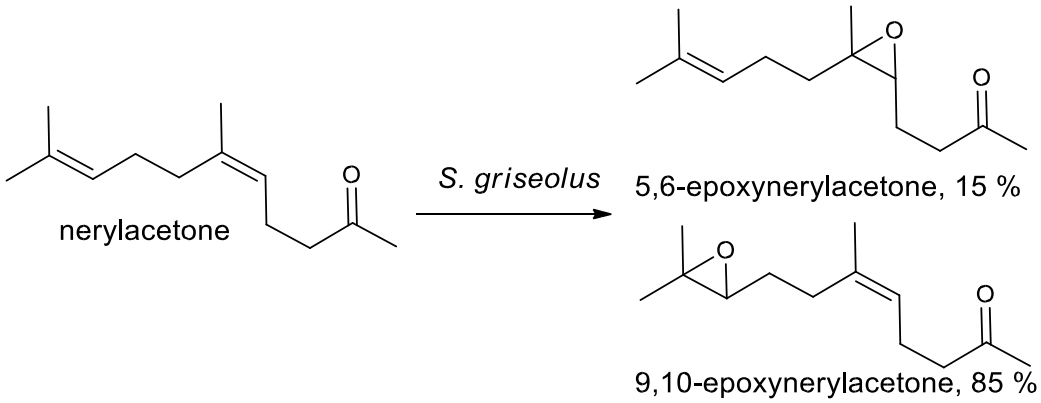



**Figure 5-3:** Oxidation of nerylacetone by *S. griseolus* DSM 40854

Potassium permanganate staining during TLC analysis revealed the presence of two yellow spots, identified as 5,6-epoxy-nerylacetone and 9,10-epoxy-nerylacetone (Table 5-3). The quantification of the results was performed using GC analysis. When *S. griseolus* cells were incubated with pure nerylacetone, it was observed that 15% of the product was 5,6-epoxy-nerylacetone, while 85% was 9,10-epoxy-nerylacetone. Notably, no alcohols or bisepoxides were detected in the reaction mixture (Fig. 5-3). In the case of geranylacetone, *S. griseolus* converted it into a mixture of epoxides and an alcohol. On the other hand, nerylacetone was oxidized by *S. griseolus* to yield two epoxides. Furthermore, geraniol was more selectively oxidized by S. griseolus to produce 6,7-epoxy- and 2,3-epoxygeraniol, compared to nerol (Fig. 5-4).

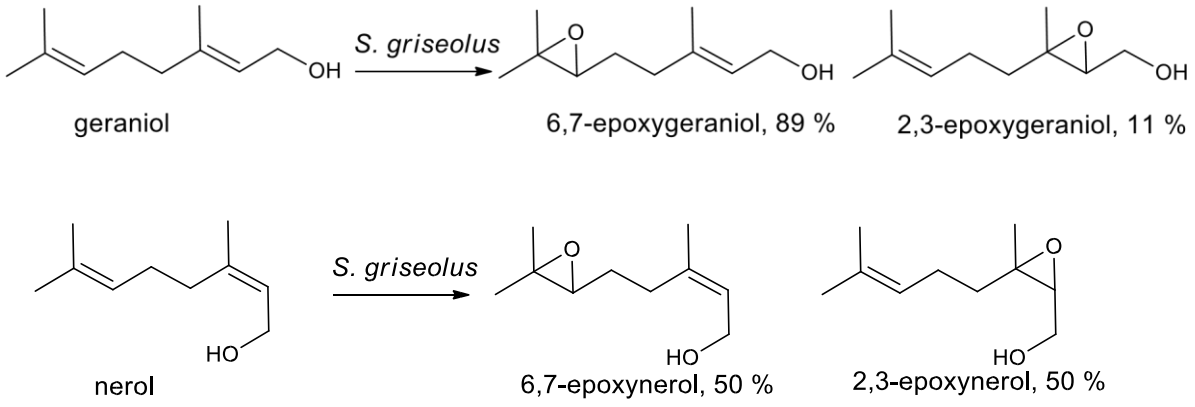



**Figure 5-4:** Oxidation of geraniol and nerol by *S. griseolus* DSM 40854

*S. coelicolor A3 (2)* DSM 46492 was not able to convert geranylacetone but nerol. TLC revealed two new spots (Fig. 5-5).

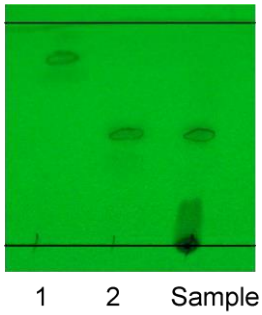



**Figure 5-5:** TLC-plate under UV at 254 nm; **1**: nerol; **2**: 8-hydroxynerol reference; **sample**: oxidation of nerol by *S. coelicolor A3 (2)* strain (spot respond to 8-hydroxynerol reference).

GC-MS analysis identified 8-hydroxynerol as a product, the second product could not be identified exactly, but its MS fragmentation corresponds to an epoxide (Fig. 5-6).

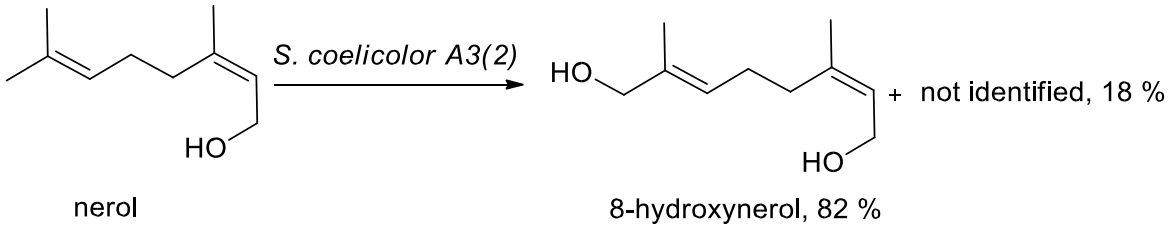



**Figure 5-6:** Oxidation of nerol by *S. coelicolor A3 (2)* DSM 46492

We examined the product ratio during eight days of the biotransformation. We monitored the product ratio over an eight-day period. After two days, the percentage of 8-hydroxynerol reached 39%, while the mixture of epoxides accounted for 42% of the total products. Additionally, an unknown product was detected at a ratio of 19%. Notably, no other peaks were observed during the entire eight-day examination. By the end of the eight-day period, the allylic alcohol content increased to 82%, while the unknown product decreases and eventually disappeared, constituting 18% of the total products (Fig. 5-7).

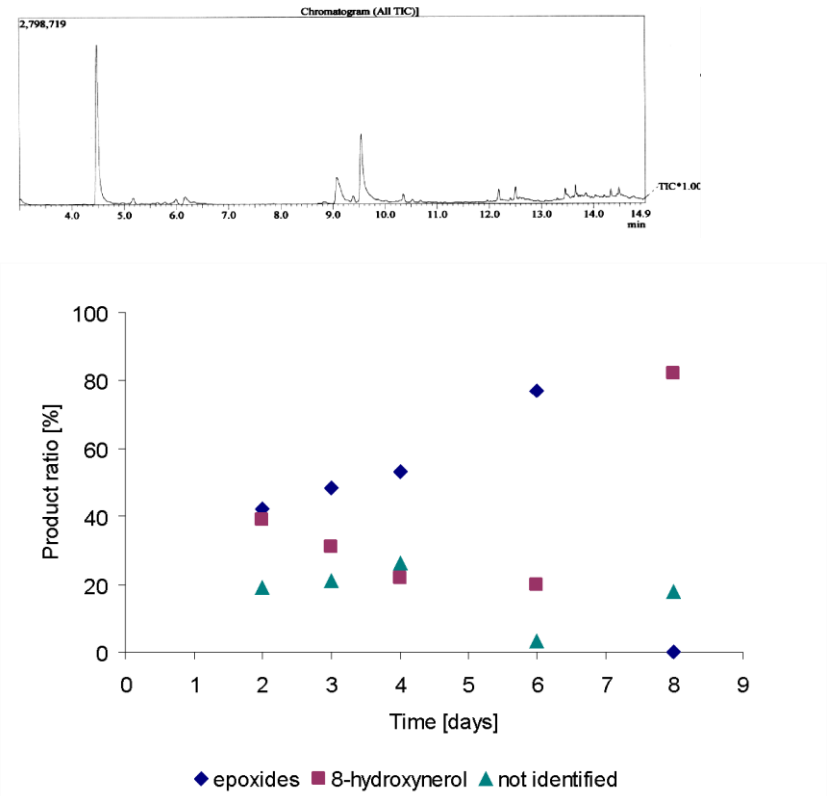



**Figure 5-7:** Biotransformation of nerol by *S. coelicolor A3 (2)* DSM 46492 strain

## 5.3 Cloning and characterization of recombinant CYPs from *Streptomyces*

Monooxygenation is generally catalyzed by flavin monooxygenases or cytochrome P450s. From the literature it is known that many *Streptomyces* strains have numerous P450 monooxygenase genes. A newly characterized cytochrome P450 monooxygenase from *Streptomyces coelicolor* shows great potential in the oxidative modification of natural products. Through thorough enzymatic analyses, this P450 monooxygenase exhibits impressive catalytic capabilities, allowing for the modification of various natural compounds. This discovery opens exciting possibilities for the development of biocatalytic processes, enabling the synthesis of valuable products utilizing this versatile enzyme. In the field of *Streptomyces* research, have proposed methods and strategies for identifying and characterizing P450 monooxygenase genes within *Streptomyces* genomes. These approaches facilitate the discovery of novel enzymes with promising applications in biocatalysis and natural product synthesis.

Furthermore, Kim et al. (2017) have made significant contributions by identifying and characterizing novel cytochrome P450 monooxygenase genes from Streptomyces peucetius. These enzymes play a crucial role in the biosynthesis of anthracycline antibiotics, a medically important class of compounds. Their research highlights the importance of P450 monooxygenases in the production of bioactive natural products, offering valuable insights into their potential applications in drug development and synthesis. The soil bacterium S. griseolus DSM 40854 harbors two P450 monooxygenases, namely P450 SU1 (CYP105A1) and P450 SU2 (CYP105B1), which have been previously reported to oxidize terpenes (Ward et al., 2003; Celik et al., 2005). *Streptomyces coelicolor A3 (2)* DSM 46492, on the other hand, possesses a total of eighteen different P450 enzymes. Among these, CYP105D5 has been identified as a P450 that tightly binds fatty acids and generates hydroxylated products when coupled with heterologous redox systems (Lamb et al., 2007). Another noteworthy P450 monooxygenase is CYP170A1, also known as albaflavenone synthase, which plays an essential role in the biosynthesis of the antibiotic albaflavenone in the soil bacterium. CYP170A1 is involved in the catalytic hydroxylation of terpenoids and exhibits terpene synthase activity (Waterman et al., 2009). Given the background information on these P450 monooxygenases, we designed primers and cloned the selected P450 genes from *S. griseolus* DSM 40854 and *Streptomyces coelicolor A3 (2)* DSM 46492 for expression in *Escherichia coli.*

### 5.3.1 Cloning of CYP105A1 and CYP105B1 from S. griseolus in pET22b+ and expression

Genomic DNA isolated from *S. griseolus* DSM 40854 using phenol-isopropanol extraction was used as template for amplifying two genes by PCR: CYP105A1 (P450 SU1, 1451 base pairs (bps) and CYP105B1 (P450 SU2, 1452 bps) with their cognate ferredoxins (Fd1 and Fd2). The concentration of genomic DNA was 144 ng/ µl (Fig.

5-8, lane 1).

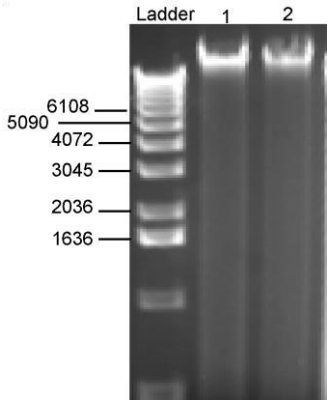



**Figure 5-8:** Genomic DNA of *Streptomyces griseolus* DSM 40854 (lane 1) and *S. coelicolor A3 (2)* DSM 46492 (lane 2). A ladder 1 kb was used.

The *NdeI* and *BamHI* restriction sites were introduced by specific primers to clone two single P450 genes CYP105A1 and CYP105B1 from *S. griseolus* DSM 40854 with their ferredoxins into the multiple cloning site of pET22b+ expression vector. The pET22b+ vector is IPTG inducible, and the expression is under the control of the strong bacteriophage T7 promoter, which benefits a high yield expression of target proteins. The PCR product and the vector were digested with the endonucleases *NdeI* and *BamHI* and purified. Because of the high transformation efficiency of *E. coli* DH5α, the ligation product was transformed into E. *coli* DH5α competent cells at first. After transformation and the isolation of plasmid DNA, positive clones were identified by analytical restriction analysis with *NdeI* and *BamHI* (Fig. 5-9). The insert of positive clones was sequenced, in order to exclude the presence of undesired mutations. One clone for each P450 respectively was selected for further experiments.

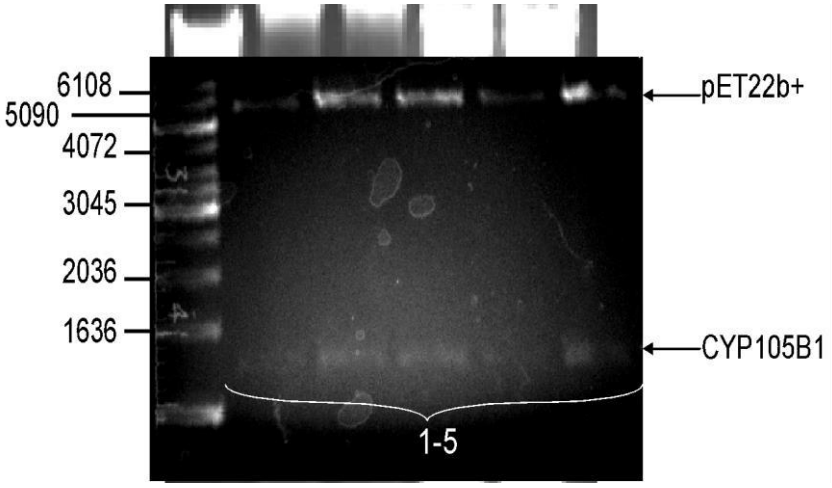



**Figure 5-9:** Exemplary, test digestion of the pET22b*-CYP105B1 (P450 SU2) with *NdeI* and *BamHI*..

Lane 1-5: expected bands' sizes 5482 and 1452 bp. A ladder 1 kb Ladder was used.

Because T7 promoter requires T7 RNA polymerase and *E. coli* DH5α lacks the T7 RNA polymerase, the expression construct was transformed in *E. coli* BL21 (DE3) afterwards for protein expression (P450 SU1 and SU2). The transformants were grown in 400 ml TB medium and induced at $OD_{600}$=0.8 and 25 °C. The expression level of CYP105A1 after 0, 1, 2, 11, 24 and 30 hours after induction was analyzed by SDS-PAGE (Fig. 5-10). Already after one hour of induction a new band was visible at 54 KDa which corresponded to the target P450 protein (Fig. 5-35, lane 2). The maximal overexpression was reached after 30 hours for both P450s. Yield of the active P450 monooxygenase, calculated from the CO-difference spectrum, amounted to 9.45 mg $l^{-1}$ for CYP105A1 and 14.2 mg $l^{-1}$ for CYP105B1.

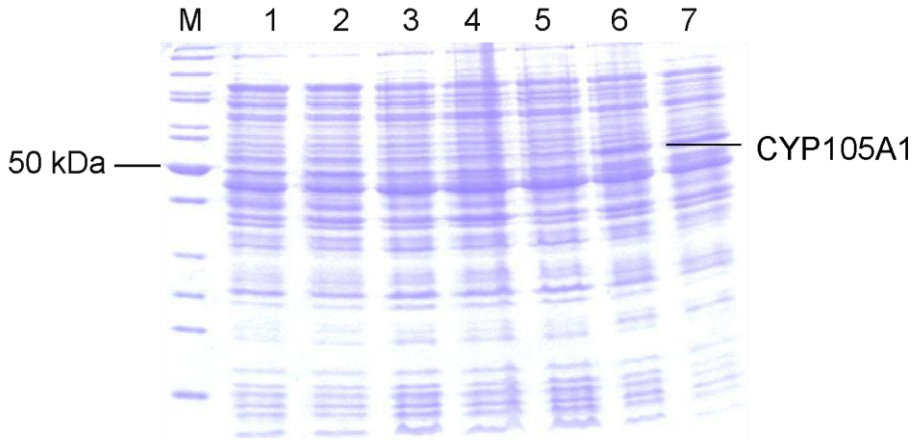



**Figure 5-10:** Exemplarily SDS-PAGE for the expression of P450 SU1 (CYP105A1) from *Streptomyces griseolus* DSM 40854. The overexpressed protein is visible at 54 kDa after induction with IPTG. Lane 1: before induction; lane 2: 1 hour after induction; lane 3: 2 hours after induction; lane 4: 11 hours after induction; lane 5: 24 hours after induction; lane 6: 30 hours after induction; M: molecular weight standard.

### 5.3.2 Cloning and expression of CYP105D5 and CYP170A1 from *S. coelicolor*

Genomic DNA was isolated from *Streptomyces coelicolor A3 (2)* DSM 46492 and used as template for amplifying two genes: CYP105D5 (1452 base pairs) with its cognate ferredoxin and CYP170A1 (1386 bps) (Guengerich et al. 2007, Waterman et al. 2009). Samples were further used for performing PCR. The use of the genomic DNA concentration was 109 ng/ µl (Fig. 5-11, lane 2).

The *NdeI* and *BamHI* restriction sites were introduced by specific primers to clone two single P450 genes CYP105D5 with their ferredoxin and CYP170A1 from *S. coelicolor A3 (2)* into the multiple cloning site of pET 22b+ expression vector. The PCR products (Fig. 5-36) and the vector were digested and purified.

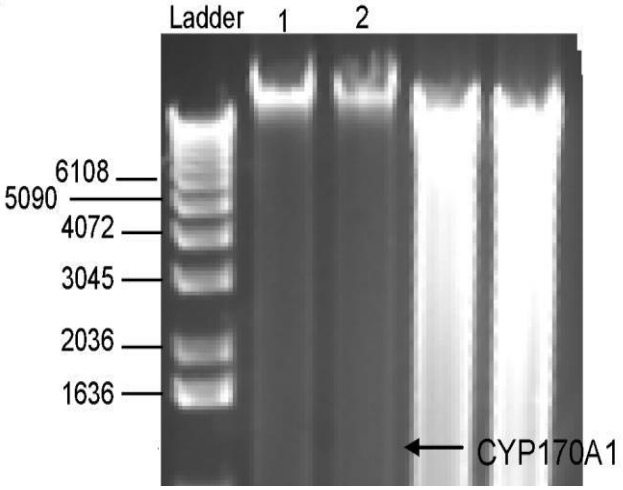



**Figure 5-11:** Amplification of CYP170A1 gene by PCR. As ladder 1 kb Ladder was used, PCR product expected band 1386 bp (lane 1).

The ligation product was transformed into E. *coli* DH5α competent cells at first for both P450s. After transformation and the isolation of plasmid DNA, positive clones were identified by analytical restriction analysis using *NdeI* and *BamHI* (Fig. 5-12). The insert of positive clones was sequenced, in order to exclude the presence of undesired mutations. One clone was selected for further experiments.

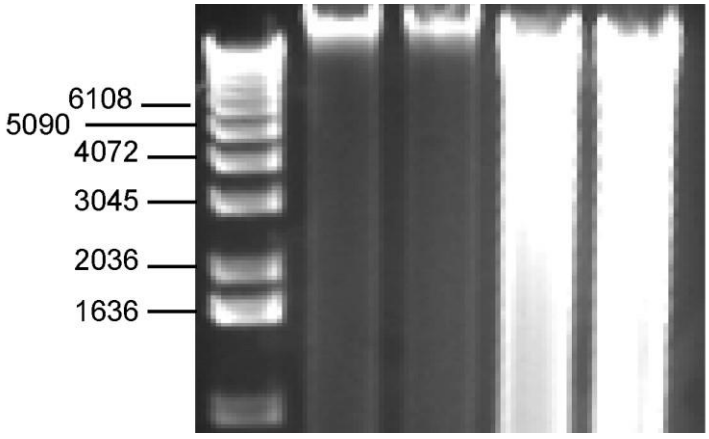



**Figure 5-12:** Here exemplary, test digestion of the pET22b*-CYP105D5 with *NdeI* and *BamHI*.. Lane 1-2: expected bands' sizes 5482 and 1239 bp. A ladder 1 kb Ladder was used.

The expression construct was transformed in *E. coli* BL21 (DE3) pLys for protein expression of CYP105D5 and CYP170A1. The transformants were grown in 400 ml TB medium and induced at $OD_{600}$=0.8 and 30 °C. The expression levels of both P450s were analyzed by SDS-PAGE. After induction a new band was visible at 54 KDa which corresponded to the theoretical weights of the target CYP105D5 protein, and in case of CYP170A1 band at 51 kDa. The maximal overexpression was reached after 28 hours for both P450s. Yield of active P450 monooxygenase, calculated from the CO-difference spectrum, amounted to 12.78 mg $l^{-1}$ for CYP105D5 and 2.68 mg $l^{-1}$ for CYP170A1 (sensitive on reduction with sodium dithionite, maximal absorption at 420 nm).

### 5.3.3. Cloning of P450s in pCYP-CamAB vector and expression for biotransformations *in vivo*

The search for suitable redox partners for P450 monooxygenases can be a challenging task. While E. coli serves as a convenient host for heterologous expression of bacterial P450 enzymes due to the absence of endogenous P450 genes, identifying compatible redox partners for these enzymes is not always straightforward. P450 monooxygenases require specific redox partner proteins, such as ferredoxins and reductases, to transfer electrons during their catalytic cycle. The challenge lies in the fact that the redox partners for a particular P450 enzyme are not always located near the P450 gene itself. Therefore, it is necessary to search for suitable redox partners that can effectively interact with the P450 enzyme to enable its proper function. This search involves investigating potential interactions between various redox partners and the P450 enzyme of interest.

One approach is to explore cross-activities between P450 enzymes and "foreign" redox partners. For example, certain P450s have been found to exhibit functional compatibility with redox partners from other organisms. One such example is the putidaredoxin reductase PdR and putidaredoxin Pdx from Pseudomonas putida ATCC17453, which have shown cross-activity with multiple P450 enzymes.

Overall, the difficulty in finding appropriate redox partners for P450 monooxygenases stems from the need for specific interactions between these components to ensure efficient electron transfer and catalytic activity. The plasmid pCYP-camAB, kindly provided by Prof. A. Arisawa from Kirin Company, Japan contained CamA and CamB from *P. putida* genes. This construct, schematically demonstrated in Fig. 5-13, is a useful system for the co-expression of various P450 genes with redox partners' genes from *P. putida* in recombinant *E. coli.*

The *NdeI* and *SpeI* restriction sites were introduced by specific primers to clone P450 gene of interest into the multiple cloning site of pCYP-CamAB expression vector.The ligation product was transformed into E. *coli* DH5α competent cells. After transformation and the isolation of plasmid DNA, positive clones were identified by analytical restriction analysis using *NdeI* and *SpeI*. The insert of positive clones was sequenced, in order to exclude the presence of undesired mutations. Finally, one clone for each of the three combinations was selected for further experiments. The expression constructs were inserted into *E. coli* BL21 (DE3) for protein expression. The transformants were grown overnight in the main medium and 250 µl of the overnight culture were inoculated in the same medium and shaken at 30 °C for 24 hours. The expression strategy is schematically demonstrated in Fig. 5-13.

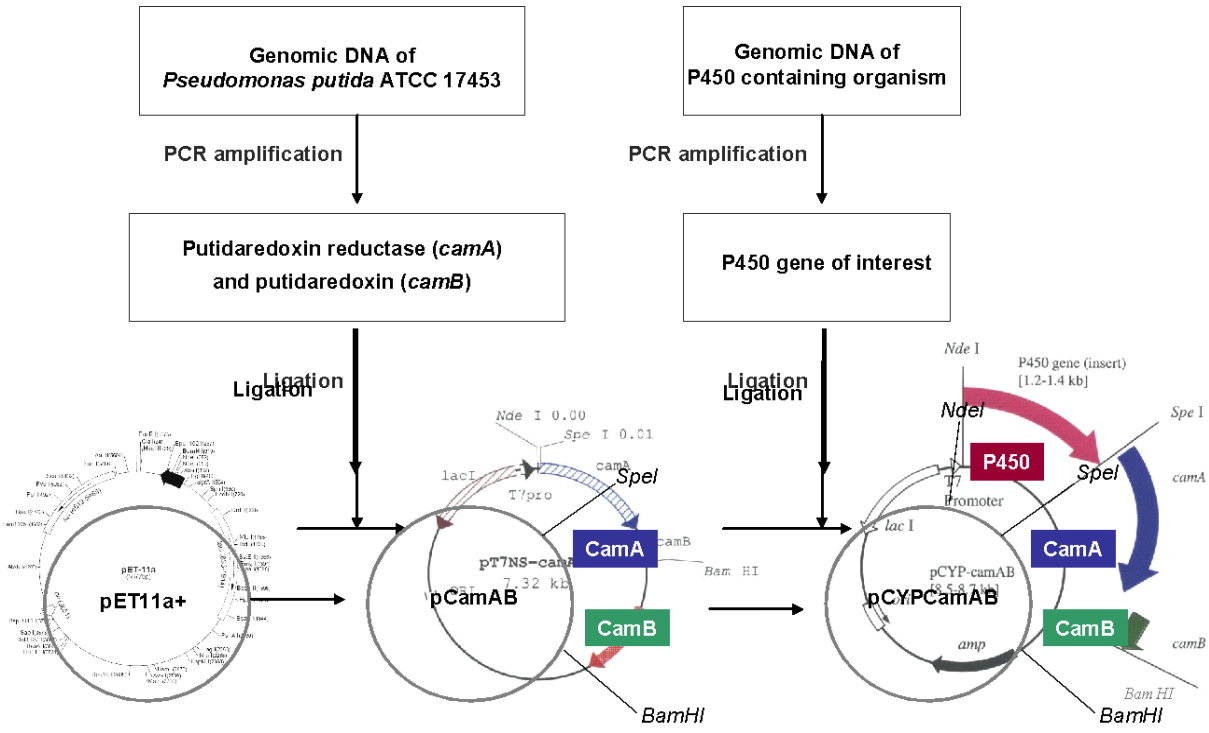


**Figure 5-13:** Plasmid map of pET11a+, carrying P450 of interest, PdR (CamA) and Pdx (CamB) from *P. putida* ATCC 17453 (as constructed at Kirin Corporation).

The yield of the active putidaredoxin (Pdx) was measured at 43.2 mg per liter, corresponding to a concentration of 3.6 millimoles per liter. The production of active putidaredoxin reductase (PdR) resulted in a yield of 95.18 mg per liter, equating to a concentration of 2.02 millimoles per liter.

### 5.3.4 Spectral properties of P450s from *Streptomyces*

The absorption spectra of the oxidized CYP105B1 (SU2) demonstrated characteristic heme Soret bands at approximately 420 nm, indicative of cytochromes. Upon addition of carbon monoxide (CO) and subsequent reduction with sodium dithionite, a shift in the Soret band from 418-423 nm to 449-454 nm was observed, indicating the formation of a Fe2+-CO complex, as expected for cytochrome P450 enzymes. To further analyze the P450 monooxygenases, CO-difference spectra were obtained by subtracting the spectrum of reduced P450 from the ferrous-CO complex spectrum. Figure 5-14 illustrates the CO-difference spectra of CYP105A1, CYP105B1 from *S. griseolus* DSM 40854, CYP105D5 from S. coelicolor A3 (2) DSM 46492, and
CYP170A1. CYP105A1 exhibited a main peak at 452 nm and a smaller Soret band at 422.5 nm, suggesting potential sensitivity of the protein to sodium dithionite, which could impact its folding and activity. CYP105B1 displayed a main peak at 449.5 nm and a smaller peak at 421 nm. CYP105D5 showed a main peak at 448 nm and a smaller peak at 423.5 nm. Notably, CYP170A1 exhibited only a peak at 420 nm, which could indicate a misfolding issue during expression and may require further investigation through activity experiments. These findings provide valuable insights into the absorption characteristics of the studied P450 monooxygenases and highlight the importance of understanding their spectral properties for subsequent functional analyses. All proteins (CYP105A1, CYP105B1, CYP105D5, and CYP170A1) were successfully expressed in TB medium, with CYP105A1 reaching a concentration of 8.8 mg/L, CYP105B1 at 14.18 mg/L, and CYP105D5 at 10.13 mg/L. However, due to the absence of a peak at 450 nm, the concentration of CYP170A1 could not be accurately determined. It should be noted that the in vitro conversion results using the ferredoxin

reductase from *S. coelicolor* were below 1-2 %, and therefore, these results were not presented in this study.

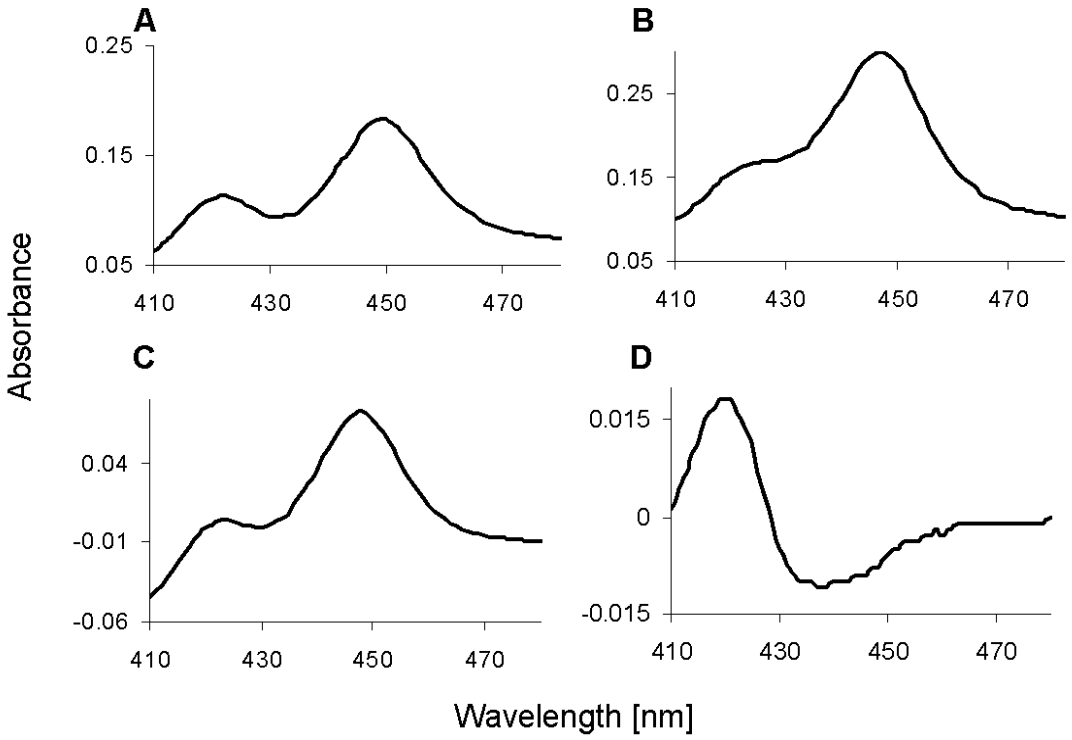



**Figure 5-14:** CO-difference spectra of expressed P450 monooxygenases reduced with sodium dithionite: A: CYP105A1, B: CYP105B1, C: CYP105D5, D: CYP170A1.

### 5.3.5 *In vivo*- catalysis with P450s from *Streptomyces* in pCYP-CamAB

To increase the production of hydroxylated products, we implemented a whole-cell oxidation system using recombinant *E. coli* cells. This system involved the coexpression of the target P450 enzymes (CYP105B1, CYP105D5, and CYP170A1) along with putidaredoxin and putidaredoxin reductase from *Pseudomonas putida*. Unfortunately, due to time constraints, the SU1 gene was not tested in this system.

To construct a plasmid containing all three genes (P450 gene of interest, CamA, and CamB), the CYP105B1, CYP105D5, and CYP170A1 genes were amplified following the procedures outlined in chapters 5.3.1 and 5.3.2. To assess the activity of the SU1 enzyme, we conducted experiments using the ferredoxin reductase FdR15 as a potential redox partner. However, these tests did not demonstrate any activity for SU1, suggesting that it may not function effectively in conjunction with FdR15. As a result, further investigations with other redox partners were not pursued, and the results obtained with FdR15 are not presented in this study.

CYP105B1 oxidized acyclic terpenoid geraniol to 6,7-epoxygeraniol (66 %) as a main product and to mixture of two allylic alcohols: 8- (17 %) and 9-hydroxygeraniol (17 %) with 35 % conversion (Table 5-4). Nerol was converted by this monooxygenase to 6,7-epoxynerol (64 %) and to the allylic alcohol 8-hydroxynerol (36 %, conversion 22 %). Geranylacetone was converted in 61 % to the mixture of four compounds: epoxide 9,10-epoxygeranylacetone (31 %) and three alcohols in positions 8-, 9-, and 11- (24 %, 31 %, 14 %). *Z* isomer nerylacetone was oxidized to mixture of epoxy- compounds: 9,10-epoxy- (62 %) and 5,6-epoxygeranylacetone (38 %) with 26 % conversion. CYP105B1 *S. griseolus* showed activity towards α- and β-ionone and both substrates are selective hydroxylized to allylic alcohols: 3-hydroxy- -ionone (conversion of 76 %) and to 4-hydroxy-β-ionone (conversion of 54 %) (Table 5-5). CYP105B1 oxidized (+)-valencene to mixture of products like *Z*- and *E*-nootkatol (29 %, 14 %) and to further hydroxylation product (+)-nootkatone (57 %) with 12 % of conversion (Table 5-5). The sesquiterpenoid nootkatone is a major contributor to the aroma of grapefruit and it is present in commercial flavourings. Consequently, its synthesis has attracted a considerable interest. (+)-α-Pinene was converted by CYP105B1 in 47 % to mixture of 18 % pineneoxide, verbenol as main product (59 %) and further oxidation product of verbenol, verbenone (23 %). Interesting is that no further oxidation activity towards pineneoxide was detected.

**Table 5-4:** Oxidation of acyclic terpenes by different P450s in P450-CamAB system

| CYP | geraniol | nerol | geranylacetone | nerylacetone |
|---|---|---|---|---|
| 105B1 | 6,7-epoxy-, 8-hydroxy- | 6,7-epoxy-, 8-hydroxy- | 9,10-epoxy-, 8-hydroxy-, 9-hydroxy-, 11-hydroxy | 9,10-epoxy- |
| 105D5 | 6,7-epoxy-, 9-hydroxy- | 6,7-epoxy-, 8-hydroxy- | 9,10-epoxy-, 8-hydroxy-, 9-hydroxy-, 11-hydroxy | 9,10-epoxy- |
| 170A1 | n. d. | n. d. | 9,10-epoxy-, 11-hydroxy | 9,10-epoxy- |

Geraniol was converted by CYP105D5 from *S. coelicolor A 3 (2)* to two oxidation products: 6.7-epoxy- (77 %) as a main product and 9-hydroxygeraniol (23 %) with conversion of 62 % (Table 5-9). Nerol was converted in 14 % to one epoxide: 6,7epoxynerol (83 %) and to allylic alcohol 8-hydroxynerol (17 %) as byproduct. Resemble to CYP105B1 from *S. griseolus*, *E* isomer geranylacetone was oxidized by CYP105D5 to the mixture of 9,10-epoxygeranylacetone (51 %) as a main product and three alcohols 8-, 9-, and 11-geranylacetone (26 %,17 %, 6 %). Nerylacetone was converted in 37 % to epoxides: 9,10-epoxy- (79 %) and 5,6-epoxynerylacetone

(21 %). CYP105D5 shows selective hydroxylation activity towards cyclic α- and βionone. α-Ionone was converted to 3-hydroxy-α-ionone with 29 % conversion and regioisomer β-ionone in the reaction with CYP105D5 gave selective hydroxylation to only 4-hydroxy-β-ionone with conversion of 59 %. CYP105D5 oxidized (+)-valencene to mixtures of products like *Z*- and *E*-nootkatol (33 %, 11 %) and to further hydroxylation product (+)-nootkatone (56 %) with 19 % of conversion.

**Table 5-5:** Oxidation of cyclic terpenes by different P450s in P450-CamAB system

| CYP | α-ionone | β-ionone | (+)-α-pinene | valencene |
|---|---|---|---|---|
| 105B1 | ■ | ■ | ■ ■ ■ | ■ ■ ■ |
| 105D5 | ■ | ■ | ■ ■ ■ | ■ ■ ■ |
| 170A1 | n. d. | ■ | n. d. | n. d. |

■ 3-hydroxy-
■ 4-hydroxy-
■ *Z*-nootkatol
■ *E*-nootkatol
■ nootkatone
■ pineneoxide
■ verbenol
■ verbenone

(+)-α-Pinene was oxidized by CYP105D5 after 4 hours to a mixture of 9 % pineneoxide, 16 % verbenol, 69 % sorberol as the main product, borneol 2 % and 4 % verbenone (Fig.5-14).

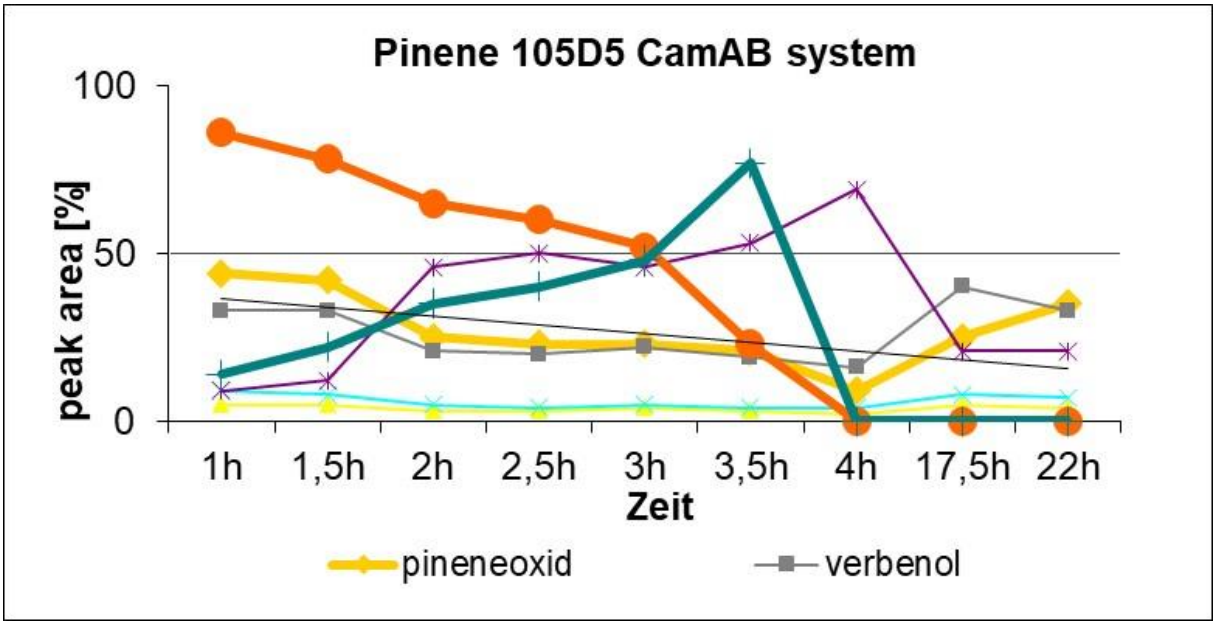



**Figure 5-14:** Oxidation of pinene by CYP105D5- CamAB system.

The results of a time-course experiment, indicating the conversion rates of various compounds over a given period. The conversion rates of pinene oxide, verbenol, borneol, verbenone, and sorberol were monitored at different time intervals (Fig. 514). At the start (time 0), the conversion rate was 86%. As the reaction progressed, the conversion rates fluctuated. After 1 hour, the conversion rate decreased to 78%. Subsequent time intervals showed further reductions, reaching a conversion rate of 52% at 3 hours. At 3.5 hours, the conversion rate decreased to 23%. Finally, at 4 hours and beyond, the conversion rate dropped to 0%. The specific conversion rates of each compound varied throughout the experiment. Notably, the conversion rate of sorberol showed a significant increase initially, reaching 77% at 3.5 hours, before decreasing to 0% at later time points. Other compounds, such as pinene oxide, verbenol, borneol, verbenone, exhibited fluctuating conversion rates over time. These results provide insights into the dynamic nature of the conversion process and highlight the time-dependent variations in compound conversions. Further analysis and experimentation are necessary to understand the underlying factors influencing the observed trends. CYP170A1 from *S. coelicolor A3 (2)*, in conjunction with putidaredoxin and putidaredoxin reductase, exhibited limited oxidation activity towards geraniol and nerol, and low activity towards geranylacetone and nerylacetone. The main product formed from the oxidation of geranylacetone by CYP170A1 was 9,10-epoxygeranylacetone (80%), with a minor product being the allylic alcohol 8-hydroxygeranylacetone (20%). The overall conversion rate for this reaction was 8%. In the case of nerylacetone, only 9,10-epoxynerylacetone was produced, albeit at a lower conversion rate. CYP170A1 selectively hydroxylated β-ionone to yield 4-hydroxy-β-ionone, but the conversion rate was only 4%.

Interestingly, no activity was observed towards its regioisomer, α-ionone. When E. coli cells expressing only the redox partners were used, no product formation was observed. Additionally, CYP170A1 did not exhibit activity towards (+)-valencene or (+)-α-pinene.

## 5.4. Screening of recombinant P450-library

The primary screening of CYP154E1 from *T. fusca YX* revealed a high catalytic activity for allylic hydroxylation of terpenoids. To further confirm and validate these findings, in vitro bioconversions were conducted using a purified mixture of CYP154E1,

putidaredoxin reductase (PdR), and putidaredoxin (Pdx). The CYP154E1 enzyme from *T. fusca* YX, along with PdR and Pdx from *P. putida*, were heterologously expressed in *E. coli*, subsequently purified, and thoroughly characterized for their enzymatic properties. These in vitro experiments aimed to provide additional evidence and insights into the catalytic capabilities of CYP154E1 for the allylic hydroxylation reactions.

During the oxidation of model substrates by CYP154A from *Nocardia farcinica*, recombinant *E. coli* coexpressing CYP154A with redox partners exhibited distinct oxidation patterns. The Z-isomer nerol was oxidized to a mixture of two unknown products, X1 (45%) and X2 (10%), along with the formation of the allylic alcohol 8hydroxynerol (45%). On the other hand, the *E*-isomer geraniol resulted in the formation of one unknown product X1 (7%) and the dominant product X3 (61%). Notably, the X2 product observed in the nerol conversion was not detected. Furthermore, the allylic alcohol of interest, 8-hydroxygeraniol, was identified as a byproduct with a relative ratio of only 32%.

Geranylacetone underwent oxidation to yield a mixture of unknown products: 8-hydroxy- (6%), unknown X3 product (3%), 12-hydroxy- (15%), as well as 30% of 9,10-epoxygeranylacetone and 35% of the allylic alcohol 11-hydroxygeranylacetone. The Z-isomer nerylacetone was converted to the epoxide 9,10-epoxynerylacetone, along with a mixture of two allylic alcohols: 71% of 11-hydroxy- and 15% of 12-hydroxynerylacetone. Negative controls without P450 were also included in the experiments.These results provide valuable insights into the substrate specificity and product profiles of CYP154A from *Nocardia farcinica* during the oxidation of model substrates.

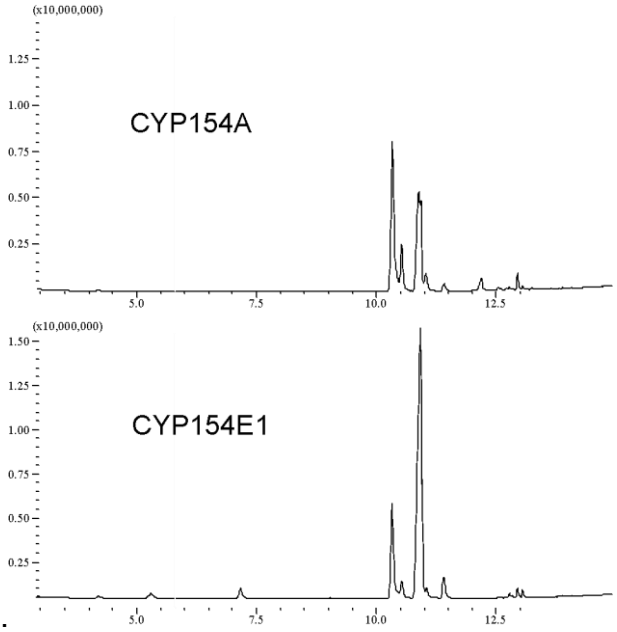



**Figure 5-15:** GC/MS spectra of the nerol oxidations products by 450 monooxygenases from the CYP154 family

The oxidation of model substrates by CYP154E1 from *Thermobifida fusca YX* in the whole-cell system of *E. coli* exhibited high efficiency and activity, leading to the conversion of acyclic terpenes such as nerol and geraniol. Nerol was transformed into a mixture of products, including 2,3-epoxynerol (3%), two unknown products X1 (23%) and X2 (3%), and the desired product of interest, 8-hydroxynerol, with a high yield of 71% (Fig. 5-16).

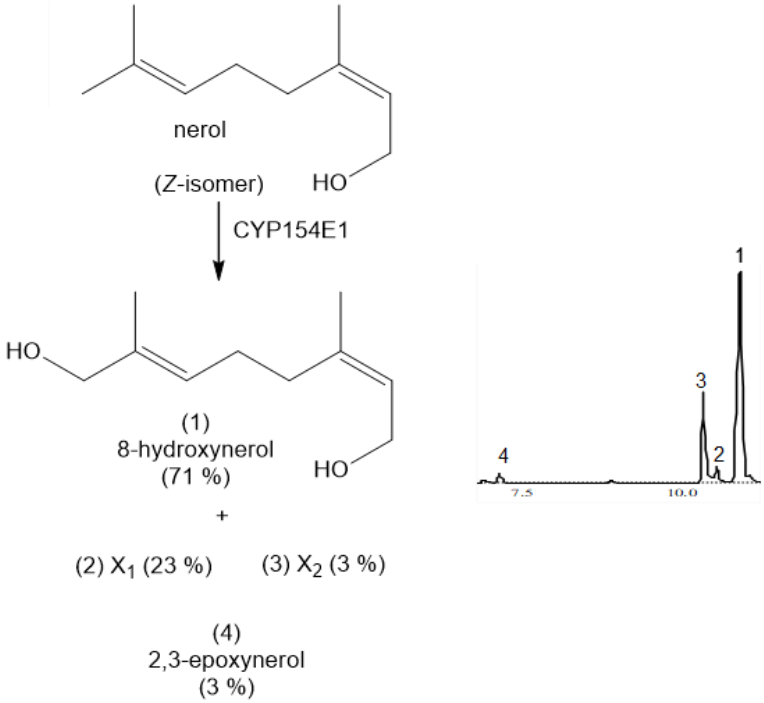


**Figure 5-16:** Oxidation of nerol by CYP154E1 and GC/MS spectra

The time-dependent conversion of the model substrate by the CYP154E1-CamAB system was investigated to determine the efficiency of the enzymatic reaction. Over the course of 24 hours, a remarkable 100% conversion of nerol was achieved. This indicates the high catalytic activity and effectiveness of the CYP154E1 enzyme in oxidizing the substrate. The successful and complete conversion of nerol highlights the potential of the CYP154E1-CamAB system as a powerful tool in biocatalysis and the synthesis of valuable products. These findings contribute to our understanding of the enzymatic capabilities of CYP154E1 and its potential applications in various industrial processes (Fig. 5-17).

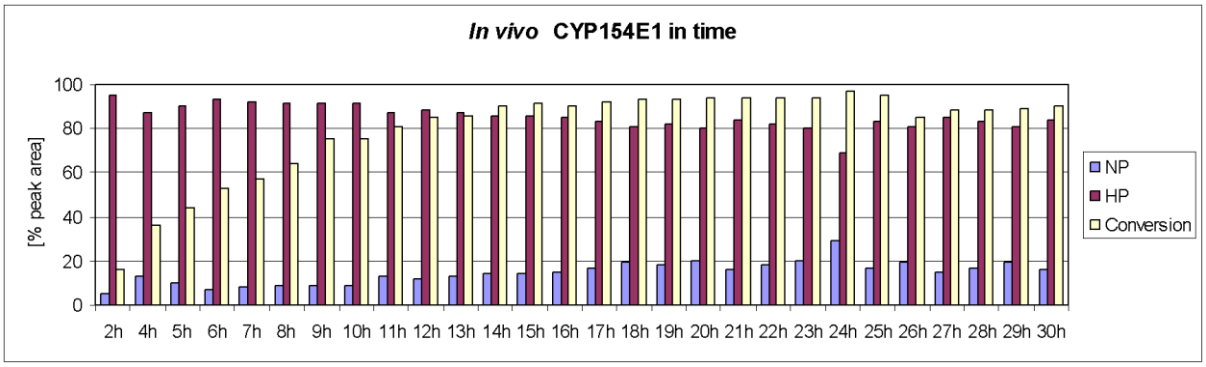



**Figure 5-17:** Oxidation of nerol by CYP154E1-CamAB system during 30 hours.

Acyclic geraniol was converted (98 %) to the product of interest: 8-hydroxygeraniol with 56 %, to three unknown products: X1 (7 %), X2 (14 %), X3 (22 %) and also to a mixture of 2,3-epoxy- (0.2 %) and 6,7-epoxygeraniol (0.8 %).

Geranylacetone was converted (90 %) to 6 % of 9,10-epoxygeranylacetone and a mixture of three alcohols: 8-hydroxy- (56 %), 12-hydroxy- (19 %) and 11-hydroxygeranylacetone (19 %). Conversion of the *Z*-isomer nerylacetone yielded to 9,10-epoxynerylacetone (1 %) and to two alcohols: 8-hydroxy- (9 %), 12-hydroxy- (53 %) and 11-hydroxygeranylacetone (37 %).

Isomers of ionone were tested and converted to hydroxyproducts. α-Ionone was oxidized to 3-hydroxy- α-ionone with 88 % conversion. CYP154E1 oxidized β-Ionone to 4-hydroxy-β-ionone with conversion of 73 %.

### 5.4.1. Characterization of CYP154E1

Cloning of CYP154E1 in pCYP-CamAB vector and expression for biotransformation *in vivo*:

The CYP154E1 gene from *Thermobifida fusca XY* was amplified from the p154E1-camAB vector, previously constructed at Kirin Corporation. The *NdeI* and *EcoRI* restriction sites were introduced by specific primers. After cloning into pET28a+, ligation, *E. coli* transformation and the isolation of plasmid DNA positives clones were identified by analytical restriction analysis using *NdeI* and *EcoRI* (Fig. 518).

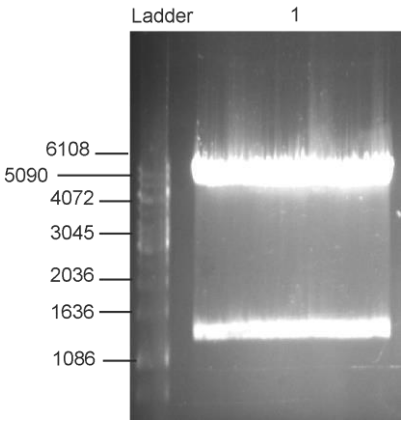



**Figure 5-18:** Test digest of the plasmid DNA isolated after ligation of the PCR product and pET 28a+-vector: Lane 1: 1 kb Ladder; Lane 2: CYP154E1 gene (1209 bps) and pET28a+ (5000 bp)

The insert of the positive transformants was sequenced, in order to avoid any replication error by DNA polymerase during PCR amplification. One clone was selected for further experiments.

The *E. coli* (DE3) transformants were grown in 400 ml TB medium and induced by 1 mM IPTG, 0.5 mM $FeCl_3$ at $OD_{600}$=0.8 and 25 °C. The expression levels of CYP154E1 were analyzed by SDS-PAGE (Fig. 5-7). After induction a new band was visible at 44.7 KDa which corresponded to the target CYP154E1 protein. The maximal overexpression was reached after 16 hours. Yield of active P450 monooxygenase, calculated from the CO-difference spectrum, amounted to 14.9 mg $l^{-1}$ (333 nmol $l^{-1}$).

$His_6$-tag allowed for an easy high-performance one-step purification of a protein by immobilized metal affinity chromatography on Ni-sepharose (Fig. 5-19). Purification experiments were performed at pH 7.5. Under this condition, the target enzyme binds to the column. Most cell proteins were not bound to the column or were washed out by

a wash buffer with a low concentration of imidazole (40 mM). The main band in lane 5 corresponds to the expected expression product at 44,700 Da that is identical to the molecular weight estimated from the protein sequence. The amount of purified CYP154E1 calculated from CO-difference spectra corresponded to 91% of the total protein concentration. This suggested effective heme incorporation during expression and its stability upon purification.

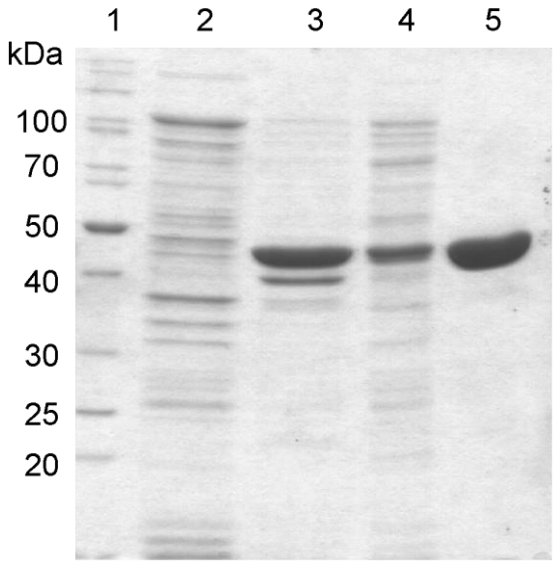



**Figure 5-19:** SDS-PAGE analysis of CYP154E1 purification (10 µg protein per lane); lane 1 molecular weight standard**:** PageRuler Unstained Protein Ladder**,** lane 2 cells for induction, lane 3 cells after induction, lane 4 cell extract, lane 5 purified CYP154E1

Spectral properties of CYP154E1:

The UV-vis spectroscopic characterization of CYP154E1 revealed characteristic features observed in P450 monooxygenases (Fig. 5-8). The absorption spectrum of the oxidized CYP154E1 exhibited a Soret band at 420 nm, a typical signature of heme-containing enzymes. The difference spectrum obtained by comparing the reduced form with the reduced CO-bound form (achieved using non-natural redox partners, PdR and Pdx) displayed a peak at 450 nm, consistent with the expected pattern for cytochrome P450 enzymes (Fig. 5-20). The heme reduction with heterologous redox partners resulted in an 86% reduction compared to sodium dithionite reduction.

Upon the addition of nerol to the oxidized enzyme, a shift in the Soret band was observed, transitioning from 420 nm to 392 nm. This spectral shift is characteristic of a Type I spectrum, which indicates a change in the spin state of the heme iron upon substrate binding. In this case, the shift from a higher wavelength to a lower wavelength suggests the conversion from a low-spin to a high-spin state of the heme iron, reflecting the interaction between the substrate and the active site of the enzyme. This conformational change is a key feature in the catalytic mechanism of cytochrome P450 enzymes.

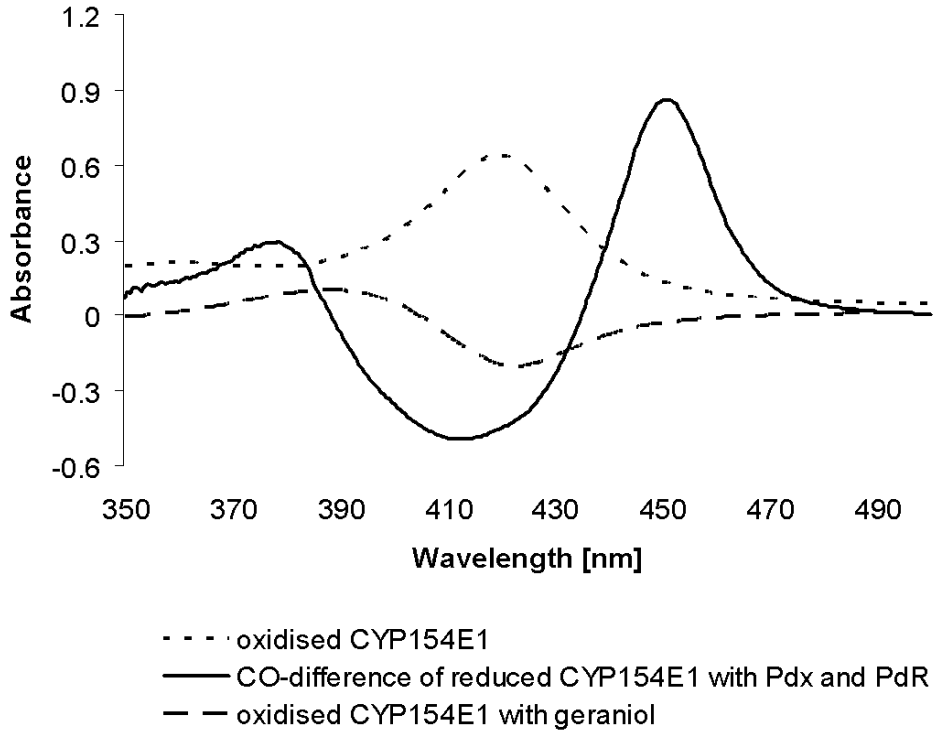



**Figure 5-20:** UV/Vis absorption spectra of CYP154E1: dash line oxidised CYP154E1, dotted line oxidised CYP154E1with geraniol, and solid line CO-difference spectrum of CYP154E1 reduced with non natural redox partner Pdx and PdR from *P. putida* ATCC 17453.

### 5.4.2. In vivo- catalysis with CYP154E1 with camAB system in E. coli

For further investigations the expression constructs were inserted in *E. coli* BL21 (DE3) for protein expression. The transformants were grown under the same conditions as in paragraph 5.3.4. The procedure of *in vivo* oxidation of terpenoids is demonstrated in the following schema (Fig. 5-21).

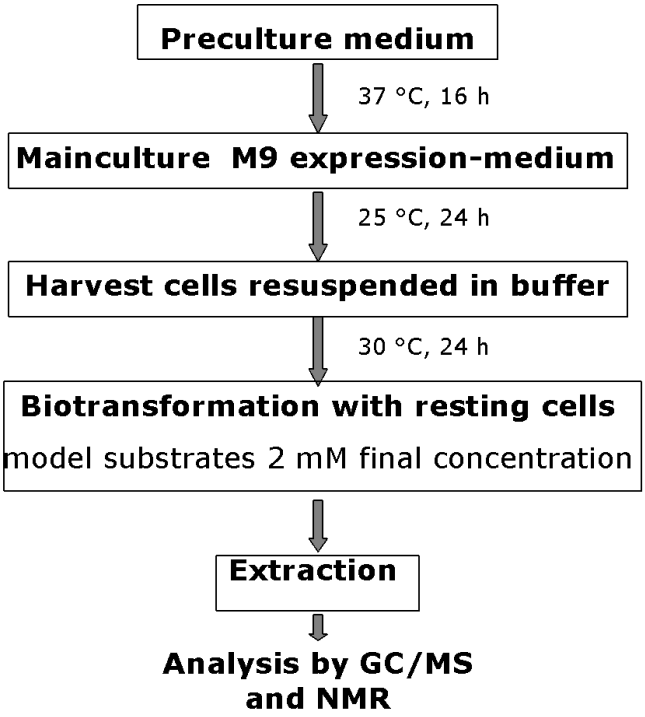



**Figure 5-21:** Procedure of *in vivo* biotransformation of terpenoids using bacterial P450 monooxygenases in P450-CamAB system.

### 5.4.3. *In vitro*- catalysis with CYP154E1

To explore the range of substances that can be converted by CYP154E1 different aliphatic compounds were tested in a reconstituted *in vitro* system with purified PdR and Pdx. Reaction conditions are described in M. and M. (Chapter 4.10.1.). The *E*- and *Z*-isomers geraniol and nerol were oxidized by the CYP154E1 wild type and the substrate conversion and product distribution were analyzed by GC/MS. Products could be identified using authentic samples synthesized at the Institute of Organic Chemistry, University of Stuttgart by Prof. Dr. Sabine Laschat.

*Geraniol and nerol*

98 % of geraniol was oxidized by wild type regio- and chemoselectively to a single product, which was identified as *E*-2,6-dimethylocta-2,6-diene-1,8-diol (8hydroxygeraniol) (Fig. 5-22). Conversion of nerol by the wild type enzyme reached 77 %, but was less selective than for geraniol, and resulted in an allylic alcohol (2*E*,6*Z*)-2,6-dimethylocta-2,6-diene-1,8-diol (8-hydroxynerol) (96 %) and *Z*-3,7Dimethyl-6,7-epoxy-2-octenol (6,7-epoxynerol, conversion achieved 4 %) as a byproduct.

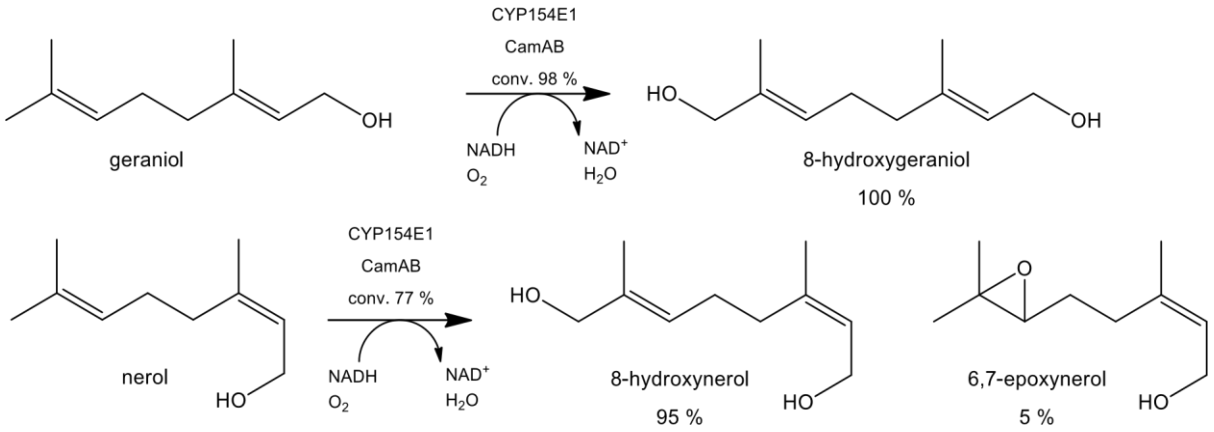



**Figure 5-22:** Oxidation of geraniol and nerol by CYP154E1 from *T. fusca YX*

*Geranylacetone and nerylacetone*

CYP154E1 also converted to geranylacetone, forming a mixture of alcohols and an epoxide (Fig. 5-23). Products of this oxidation reaction were alcohols 12hydroxygeranylacetone, 11-hydroxygeranylacetone and 7-hydroxygeranylacetone that reached 41 %, 42 % and 6 %, calculated from the peak areas and 9 % of an 9,10-epoxygeranylacetone was detected as an epoxide byproduct.

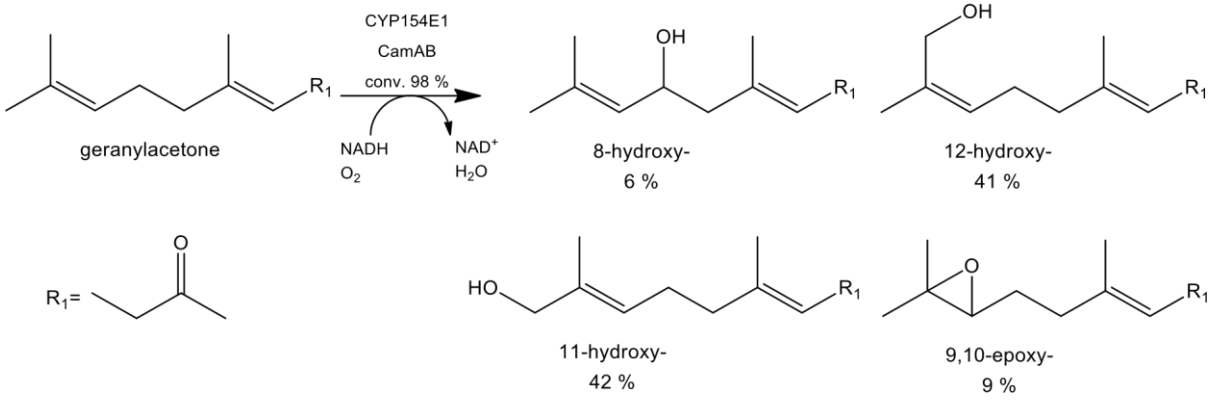



**Figure 5-23:** Oxidation of geranylacetone by CYP154E1 from *T. fusca YX*

Surprisingly the *Z*-isomer nerylacetone was oxidized by CYP154E1 wild type more selectively than the *E*-isomer geranylacetone (Fig. 5-24). Three products were identified as allylic alcohols in position C7 (12 %), C11 (32 %) and C12 (56 %) with respect to high, 98 % conversion of 0.2 mM nerylacetone.

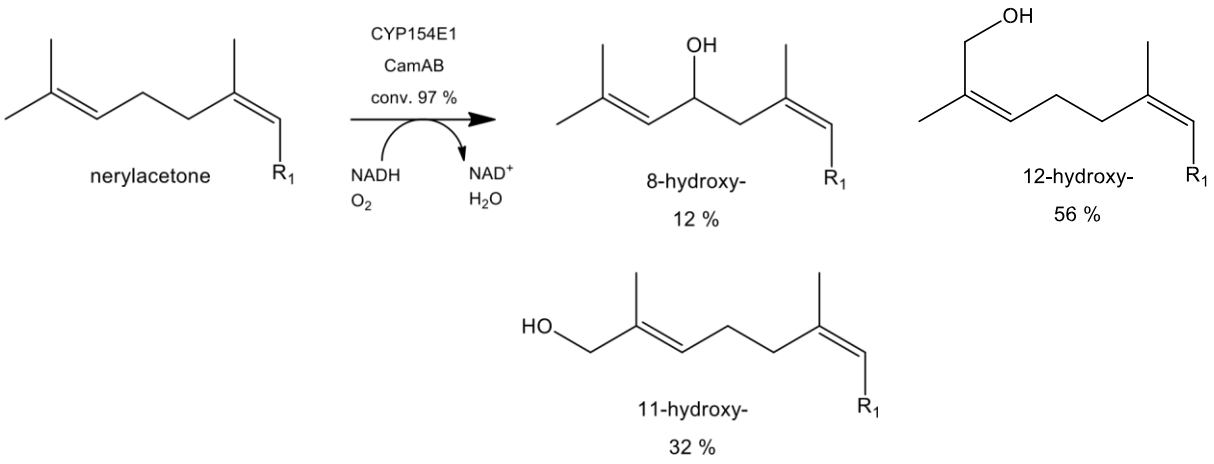



**Figure 5-24:** Oxidation of nerylacetone by CYP154E1 from *T. fusca YX*

*α-ionone and β-ionone*

CYP154E1 wild type oxidized the regioisomers α-ionone and β-ionon to allylic alcohols and partially further to the corresponding enones. Wild type converted αionone region- and chemoselectively to a single 3-hydroxy-α-ionone (100 %) with conversion of 71 %. Oxidation of β-ionone by CYP154E1 gave a mixture of two products 4-hydroxy- β-ionone (82 %) as a main product and 4-keto-β-ionone (18 %) (Tab. 5-6).

**Table 5-6:** Oxidation of cyclic terpenes using CYP154E1.

| ***Substrate*** | ***Products*** | | ***Conversion [%]*** |
|---|---|---|---|
| ***α*-ionone** | 3-hydroxy- | | 71 |
| ***β*-ionone** | 4-hydroxy- 82 % | 4-keto- 18 % | 15 |

CYP154E1 was subjected to a comprehensive substrate screening involving various short-chain alkanes and alkenes, as well as their derivatives. The enzyme demonstrated no activity towards *n*-pentane and n-hexane. However, *n*-heptane was efficiently converted to 2-heptanol (79%), 3-heptanol (11%), and further oxidized products such as 2-heptanone (7%) and 3-heptanone (3%). The conversion achieved for *n*-heptane was 22%. Similarly, 2-hexanol was selectively oxidized to the ketone 2hexanone with a conversion of 51%, while heptanol was transformed into the

aldehyde heptanal with a conversion of 25%. No activity was observed towards C10 alcohols, namely *n*-decanol and 1,2-decanediol, as well as *n*-decane.

In terms of alkenes, the short-chain C8 alcohols 1-octen-3-ol and 2-octene-1-ol were tested. CYP154E1 exhibited selective oxidation of 1-octen-3-ol to the ketone 1octene-3-one, achieving a conversion of 8%. However, no product formation was detected with 2-octene-1-ol as a substrate.

The binding affinities of CYP154E1 for oxidized substrates were determined spectrophotometrically. Titrating the oxidized substrates with the P450 monooxygenase resulted in a typical Type I binding spectrum, indicating displacement of the water coordinated to the heme iron in the resting enzyme. The tightest binding was observed for n-heptane (Kd=23 µM), n-octane (Kd=19 µM) and 1-octanol (Kd=19 µM). On the other hand, the substrate binding affinities decreased for 2-hexanol, 1-octene-3-ol, and 1-heptanol, with Kd values of 203 µM, 180 µM, and 118 µM, respectively. These substrates required higher concentrations to perturb the spin state equilibrium. The absorption differences showed a linear increase with increasing substrate concentrations (Table 5-7).

**Table 5-7:** Oxidation of alkanes catalyzed by CYP154E1.

| **Substrate** | **Products** | ***Conversion*** ***[%]*** | ***$K_d$*** ***[µM]*** |
|---|---|---|---|
| **2-hexanol** | 2-hexanone | 51 | 203 |
| ***n*-heptane** | 2-heptanol 79 %<br>3-heptanol 11 %<br>2-heptanone 7 %<br>3-heptanone 3 % | 22 | 23 |
| **1-heptanol** | Heptanal | 25 | 118 |
| ***n*-octane** | 2-octanol 24 %<br>3-octanol 6 %<br>2-octanone 60 %<br>3-octanone 10 % | 10 | 19 |

| | | | |
|---|---|---|---|
| **1-octanol** | 1-octanal | 5 | 19 |
| **2-octanol** | 2-octanone | 52 | 51 |
| **3-octanol** | 3-octanone | 47 | 40 |
| **4-octanol** | n.d. | n.d. | - |
| ***n*-decane** | n.d. | n.d. | - |
| **1-octen-3-ol** | 1-octen-3-one | 8 | 180 |
| **2-octene-1-ol** | n.d. | n.d. | - |

n.d.= not determined

Our interest focused on short chain alkane *n*-octane that was converted to a mixture of alcohols and their ketones with a strong regiopreferency for position ω-1. 24 % of 2-octanol and 6 % of 3-octanol were formed and further oxidized to their ketones: 60 % of 2-octanone and 10 % of 3-octanone. Conversion achieved 10 % after one hour when reaction was supported by cofactor regeneration. A significant aspect of this study was the evaluation of the enantioselectivity of the P450 monooxygenase in the oxidation reaction of *n*-octane. The products of this reaction were two alcohols: 2-octanol and 3-octanol, which were further oxidized to the corresponding ketones. The overall conversion achieved for this reaction was 10%.

To facilitate the detection of compounds that are not directly amenable to analysis, such as short-chain alkanes, product derivatization was employed. The reaction mixture was extracted and subsequently evaporated. Acetic anhydride and toluene were then added, followed by incubation at 85°C for 2 hours. The organic phase was analyzed using gas chromatography (GC) equipped with a chiral column. This allowed for the separation and identification of the derivatized products. Stereoselective hydroxylation of *n*-octane using CYP154E1 provided mixture of two alcohols: 2- and 3-octanol and enantiomeric excess (*ee*) for the *(S)*-2-octanol was 85 % and for the *(R)*-3-octanol of 90 % *ee*. In this reaction conversion of *n*-octane achieved 18 % (Fig. 5-25).

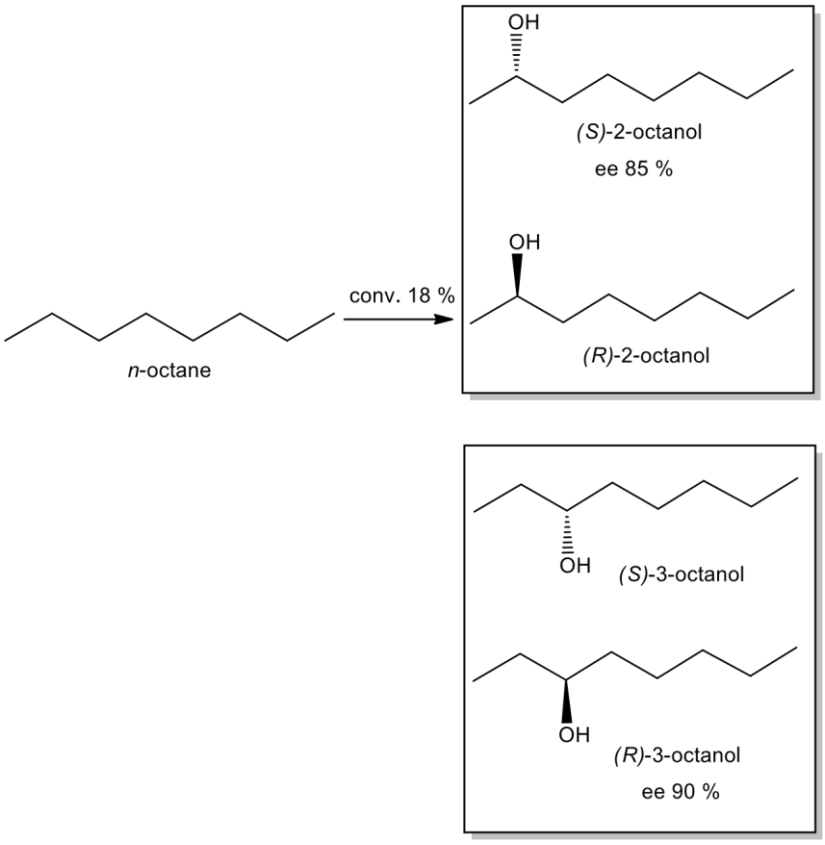



**Figure 5-25:** Enantioselectivity of CYP154E1 during *n*-octane oxidation

Nevertheless, when the secondary alcohols 2-octanol and 3-octanol was used as substrates, the corresponding ketones were formed with a much higher activity than oxidation of parental alkane (conversions of: 10 % vs 52 % vs 47 %). Although 1-octanol was not produced upon octane-oxidation, it was accepted as substrate by CYP154E1 and oxidized to a single aldehyde. However, activity was much lower, and conversion achieved only 5 %. Furthermore, by oxidation of 4-octanol no product formation was observed (Tab. 5-7).

To confirm the enantioselectivity of the oxidation reaction using CYP154E1, both enantiomers of 2-octanol, namely *(S)*-2-octanol and *(R)*-2-octanol, were individually used as starting materials (Fig. 5-26). The reaction of *(S)*-2-octanol resulted in the formation of the corresponding ketone, 2-octanone, with a conversion of over 99%. In contrast, no conversion was observed when *(R)*-2-octanol was used as the substrate. Additionally, when *(S)*-3-octanol was employed as the starting material, the corresponding ketone, 3-octanone, was observed with a conversion rate of 98%. Unfortunately, *(R)*-3-octanol was not commercially available at the time, of the measurements and was therefore not tested in this study.

These results clearly demonstrate the enantioselective nature of the oxidation reaction catalyzed by CYP154E1, as it selectively oxidizes the *(S)*-enantiomer of octanols while showing no activity towards the *(R)*-enantiomer.

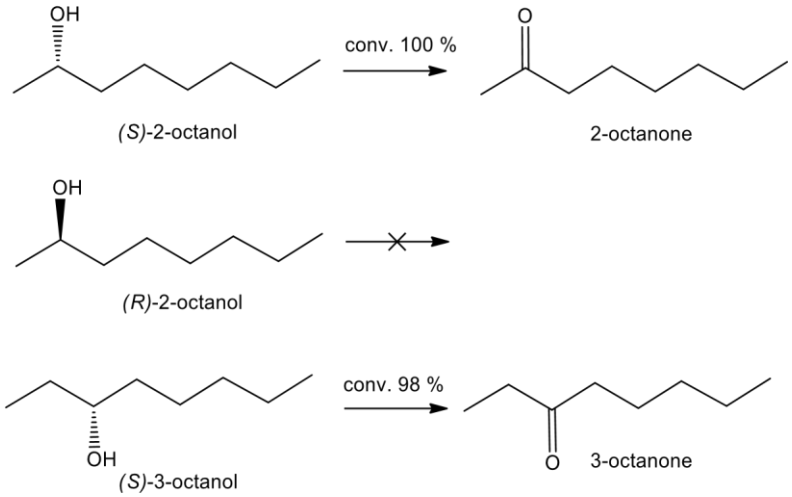



**Figure 5-26:** Oxidation of octanols using CYP154E1.

### 5.4.4. Rational design of CYP154E1

To improve activity of CYP154E1 to hydoxy products in allelic position, we tried to change peptides in position 5 after ExxR motiv. No structural information of this enzyme is available and the most similar P450s with known three-dimensional structure enzymes are CYP154A1 with only 39.9 % identity and CYP154C1 with 37.6 % identity. Previously a systematic analysis of 31 P450 crystal structures and more than 11.294 P450 sequences (Dr. Nelson P450 homepage, http://drnelson.uthsc.edu/P450.statsfile.html, Aug. 2009) allowed to derive rules on how to identify one position in the substrate binding cavity of a P450 monooxygenase which is preferentially involved in substrate binding and thus in regio- and chemoselectivity control (Seifert and Pleiss 2009). This position can easily be identified on sequence level based on its defined distance to the highly conserved ExxR motif, even in the absence of structure information. According to this rule, the residue at position 5 after the ExxR motif is closest to the heme centre and therefore putatively substrate interacting in CYPs that have a positively charged heme interacting residue

in position 9 to 11 (98.4 % of all CYPs). In CYP154E1 V286 corresponds to position 5 after ExxR (Fig. 5-27).

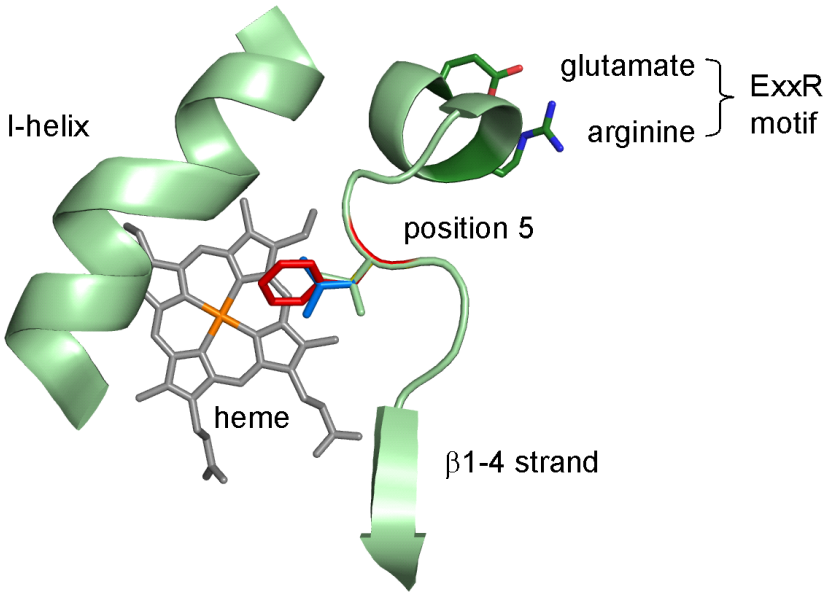



**Figure 5-27:** Immediate heme surroundings shown for the nearest relative of CYP154E1 with available crystal structure (CYP154A1, PDB entry 1odo). The residue in position 5 (V286 in CYP154E1) reaches close to the heme centre (orange) in almost all CYPs and was therefore substituted by phenylalanine (red), leucine (blue), and alanine (by courtesy of Dr. A. Seifert).

The analysis of the CYP154E1 enzyme structure revealed that the residue at position 286 is predominantly hydrophobic, as described in the study by Seifert and Pleiss (2009). In order to investigate the influence of this residue on substrate orientation near the heme center, we performed site-directed mutagenesis by substituting valine (V286) with alanine, leucine, and phenylalanine.

The choice of alanine and leucine was based on their shorter side chains compared to phenylalanine, which has a larger aromatic ring. This substitution aimed to modify the spatial arrangement of substrates near the heme center. The mutagenesis procedure was conducted using Quick-Change PCR, as outlined in the Materials and Methods section (Chapter 4.8.10).

The resulting CYP154E1 mutants, namely V286L, V286A, and V286F, were expressed in E. coli BL21 (DE3) and characterized using the same experimental approach as the wild-type enzyme. The electron transfer was facilitated by the redox partners PdR and Pdx, ensuring proper functionality and comparability of the mutants with the wild-type enzyme.

### 5.4.5. *In vitro* oxidation using CYP154E1 mutants.

The 0.5 ml reaction with CFE containing PdR, Pdx, P450 and 0.2 mM substrate (10 mM stock solution in DMSO) support with cofactor regeneration system was extracted, concentrated and measured on GC/MS. Products could be identified using authentic samples as references.

*Geraniol and nerol*

The *E*- and *Z*-isomers geraniol and nerol were oxidized by mutants of CYP154E1 and product of distribution was analyzed by GC/MS. Three mutants V286L, V286A and V286F showed unselective formation of epoxides with respect to geraniol and nerol. Geraniol was epoxydized with 11 % conversion by mutant CYP154E1_V286L to mixture of two epoxides , which were identified as 2,3,6,7-diepoxygeraniol (74 %) and 6,7-epoxygeraniol (26 %) (Fig. 5-28). CYP154E1_V286L oxidized nerol with 4 % conversion to epoxide 2,3,6,7-diepoxynerol (42 %) and to *Z*-3,7-dimethyl6,7-epoxy-2-octenol (6,7-epoxynerol) (58 %) (Fig. 5-29).

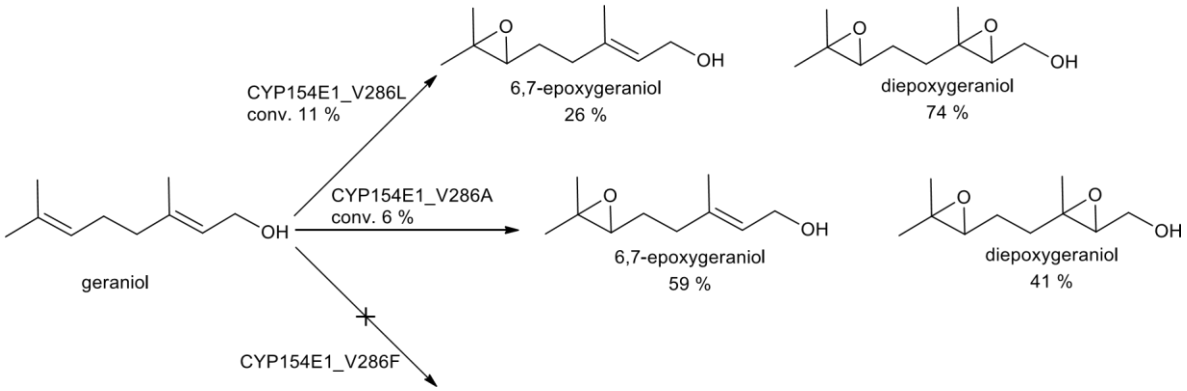



**Figure 5-28:** Biooxidation of geraniol using mutans variations of CYP154E1.

CYP154E1_V286A oxidized geraniol to a mixture of epoxides: 6,7-epoxy- with 59 % and 2,3,6,7-diepoxygeraniol with 41 % and 6 % conversion was observed. Its isomer nerol was oxidized as well though with 3 % conversion, and 49 % of 6,7epoxy- and 51 % 2,3,6,7-diepoxynerol were formed (Fig.5-29).

Mutant CYP154E1_V286F activity was not detectable on gas chromatography towards model terpenoids geraniol and nerol.

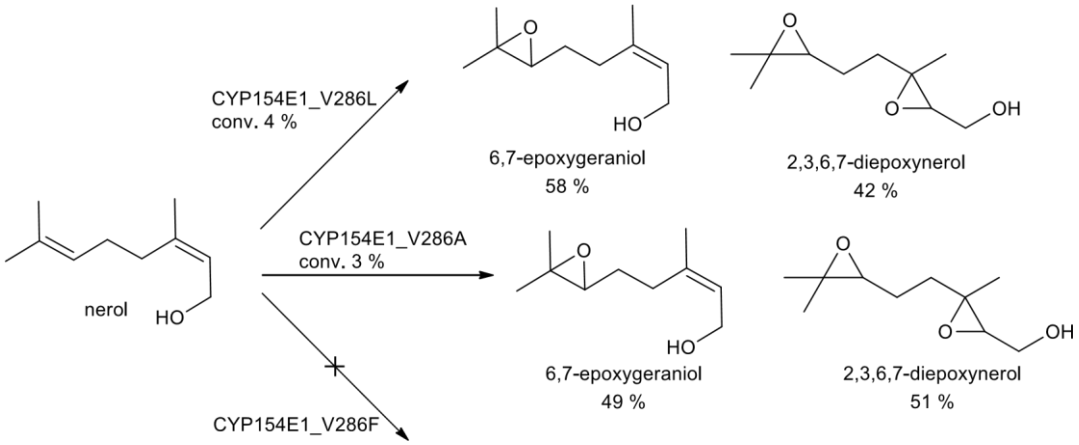



**Figure 5-29:** Oxidation of nerol using CYP154E1 mutants

*Geranylacetone and nerylacetone*

Regio- and chemoselectivity of the mutants towards geranylacetone and nerylacetone differ end from those of the wilde-type enzyme again. The product analysis showed a strong preference of all three mutants for the formation of hydroxyproduct in position C8 as a main compound.

The variant CYP154E1_V286L produced 8-hydroxygeranylactone as the main product (44 %) and further compounds, 12-hydroxy- and 11-hydroxy- with a percentage ratio of 35%: 18%. Oxidation to 3 % of 9,10-epoxygeranylacetone was detectable and conversion achieved 20 % (Tab. 5-8). Nerylacetone was converted up to 19 % to a mixture of alcohols 28 % of 8-hydroxy- 72 % of 12-hydroxygeranylacetone (Tab. 5-9).

**Table 5-8:** Oxidation of geranylacetone using mutants of CYP154E1.

| ***P450*** | ***Products of geranylacetone*** | | | ***Conversion [%]*** |
|---|---|---|---|---|
| **CYP154E1_V286L** | 9,10-epoxy- 3 % | 8hydroxy- 44 % | 12-hydroxy- 35 % | 11-hydroxy- 18 % | 20 |
| **CYP154E1_V286A** | 9,10-epoxy- 23 % | 8hydroxy- 46 % | 12-hydroxy- 15 % | 11-hydroxy- 16 % | 25 |

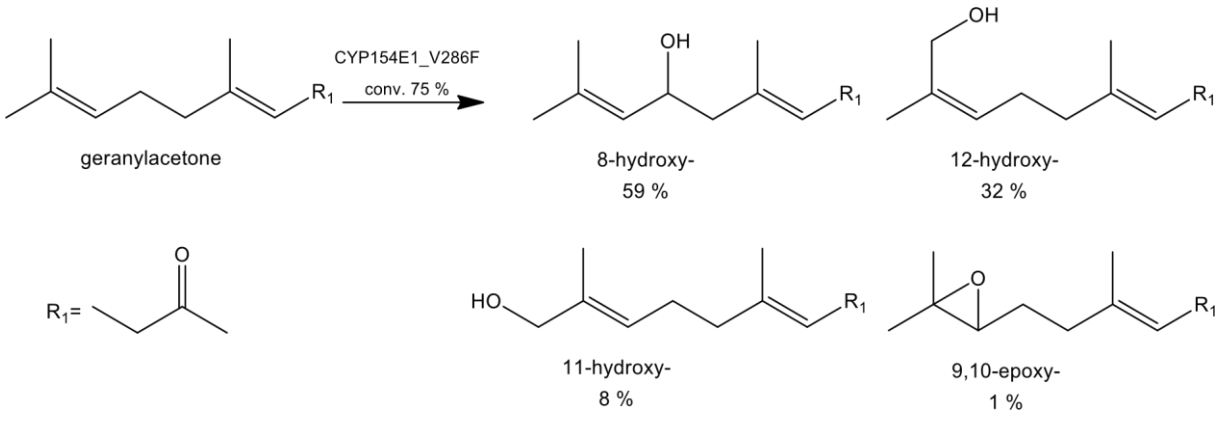
The variant CYP154E1_V286A hydroxylated 25% of geranylacetone to 8hydroxy- (46 %), 12-hydroxy- (15 %) and to 11-hydroxynerylacetone (16 %). Furthermore, 23 %

of 9,10-epoxygeranylacetone was produced as well. Surprisingly, the Z-isomer nerylacetone was oxidized in 42 % to only two alcohols, namely to 25 % of 8-hydroxy- and 75 % of 12-hydroxynerylacetone.

**Table 5-9:** Catalytic oxidation of nerylacetone using mutants of CYP154E1.

| ***P450*** | ***Products of nerylacetone*** | | ***Conversion [%]*** |
|---|---|---|---|
| **CYP154E1_V286L** | 8-hydroxy- 28 % | 12-hydroxy- 72 % | 19 |
| **CYP154E1_V286A** | 8-hydroxy- 25 % | 12-hydroxy- 75 % | 42 |

CYP154E1_V286F showed a strong shift in regio- and chemoselectivity and converted 75 % geranylacetone to a mixture of allylic alcohols with a preference for position C8 (59 %) over C12 (32 %) and C11 (8), with oxidation to epoxide less than >1 %. No epoxide formation was observed using CYP154E1_V286F mutant when nerylacetone was used as substrate. Mixture of alcohols was detected namely 8-hydroxy- (24 %), 12-hydroxy- as preferred product in 63 % and 11hydroxynerylacetone in 13 %, when conversion achieved 90 %. To my knowledge, for the first time a P450 catalyst is presented that oxidises nerylacetone preferentially at allylic position 12 to form 12-hydroxynerylacetone (Fig. 5-30).



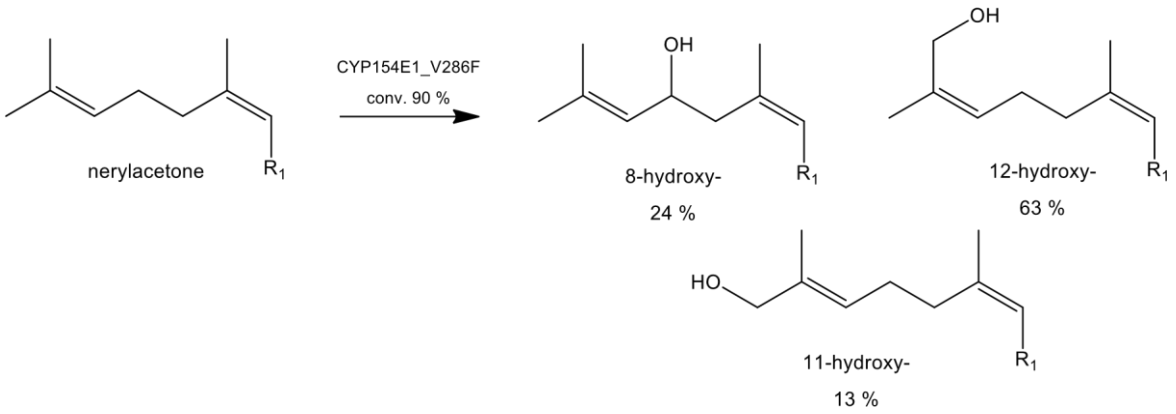



**Figure 5-30:** Oxidation of geranylacetone and nerylacetone using the mutant CYP154E1_V286F

*Short chains alkanes and alcohols*

Cyclic short chain compounds were investigated with the three CYP154E1 mutants as well. In this study $C_5$-$C_{10}$ alkanes were tested and surprisingly CYP154E1 mutants were capable of oxidizing chemo- and regioselectively the model substrates.

2-Hexanol was converted to 2-hexanone using all three mutants CYP154E1_V286L, _V286A and _V286F and conversion was 1%, 2% and 15%. Mutant CYP154E1_V286L oxidized *n*-octane to a single alcohol 3-octanol and conversion achieved 11%. An alcohol 2-octanol was converted to an aldehyde 1octanal (conversion of 2 %), oxidation of 3-octanol was not detectable and 4-octanol was oxidized to 4-octanone with conversion of 2%.

CYP154E1_V286A converted *n*-octane exclusively to an alcohol 3-octanol (conversion 10 %). The oxidation of 2-octanol using CYP154E1 resulted in the formation of the corresponding enone, 2-octanone, with a conversion of 6%. However, no detectable oxidation of 3-octanol was observed. Additionally, the oxidation of 4-octanol yielded 4-octanone with a conversion of only 2% (Table 5-10). Only V286F was able to oxidize 2-octanol to 2-octanone and the conversion achieved 48 %. From three mutant variants only V286F oxidized 3octanol to enone 3-octanone (100 %, conversion 2 %). Surprisingly, compared to the wild type, the three CYP154E1 mutants showed selective formation of 4octanone (100 %) from 4-octanol which was not found by the wild type. Oxidation products of *n*-decane and its alcohols: 1-decanol and 1, 2-decanodiol was not detected by any of the mutants.

**Table 5-10:** Oxidation of alkanes using mutants of CYP154E1.

| *Substrate* | *CYP154E1 mutants* | | | *Conversion* | | |
|---|---|---|---|---|---|---|
| | **V286L** | **V286A** | **V286F** | *[%]* | | |
| ***n*-octane** | 3-octanol | 3-octanol | 3-octanol | 11 | 10 | 13 |
| **2-hexanol** | 2-hexanone | 2-hexanone | 2-hexanone | 1 | 2 | 15 |
| **2-octanol** | 2-octanone | 2-octanone | 2-octanone | 2 | 6 | 48 |
| **3-octanol** | n.d. | n.d. | 3-octanone | n.d. | n.d. | 2 |
| **4-octanol** | 4-octanone | 4-octanone | 4-octanone | 2 | 2 | 1 |

n.d.= not detected

## 5.5. Immobilization of recombinant *E. coli* expressing CYP154E- CamAB on latex SF091

With the whole recombinant *E. coli* cells described in chapter XX, CYP154E1 from *T. fusca YX* oxidized nerol to the product of interest: 8-hydroxynerol. Along with 71% 8-hydroxynerol, 29 % two undesired unidentified by-products X2 and X3 were formed. To avoid byproducts' formation and received the product of interest selectively, we searched for different possibilities like mutations of wild type by changing position 5 after ExxR motive or by coating of recombinant cells in latex. This is a promising approach for immobilization of catalytic strains for biotransformations. This part of the PhD work was carried out in this group by Prof. Michael C. Flickinger from the Department of Microbiology at North Carolina State University in the USA and in the Laboratory in ITB Stuttgart.

We developed a novel 153 µm thick nanoporous bi-layer latex coating for preserving recombinant *E. coli* cells expressing heterologous CYP154E1 from *T. fusca YX*, putidaredoxin and putidaredoxin reductase from *P. putida*. Heterologous *E. coli* BL21 (DE3) cells were entrapped in acrylate/ vinyl acetate copolymer matrix (Tg~10 °C) and cast into 4 cm diameter patch coatings (cell coat) containing ~106 CFU covered by a nano-porous topcoat like shown in Fig. 5-31.

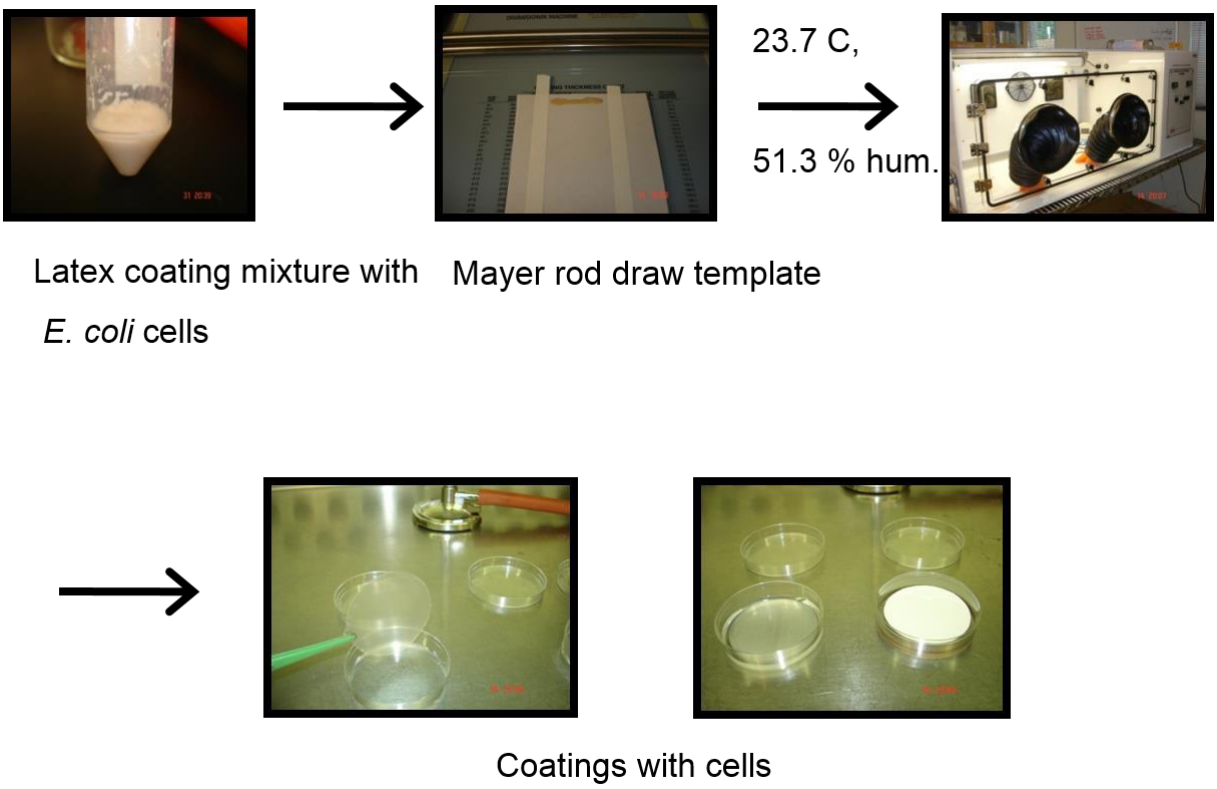



**Figure 5-31:** Template designed for latex coating using *E. coli* BL21 (*DE3*) cells expressing CYP154E1-CamAB system.

During the drying process of the latex emulsion, a combination of cell preservation through partial desiccation and the formation of pores occurs. This leads to the entrapment of living cells within the nanopores created by partially coalesced polymer particles. The presence of nanoporosity is crucial for maintaining the viability of microbial cells and enhancing the reactivity of the coating.

The contact toxicity tests revealed that SF091 with a biocide concentration of 30 ppm did not exhibit significant toxicity towards the entrapped E. coli cells. Hence, this concentration was selected for further experiments. The concentration of E. coli in the cell coat emulsion was determined to be (1.08 ± 0.27) x 10^6 colony forming units (CFU) per milliliter, as determined by serial dilution and plate count.

Several different conditions were investigated, and the optimal ones were selected and presented in Figure 5-32.

The best conditions:

1.3 g of wet cells and addition of glass pearls with vortex

402 µl of sucrose (5.8 g/L)

162.5 µl of glycerol (100 %)

Live mixture for 10 min and centrifuged (6000 rpm, 10 min)

1083 µl of latex SF091

Using PE and photo paper and big blank 142 in small Petri dishes with 5ml of 50 mM KPi buffer pH 7.2 and 1 mM FC of nerol (30 mM stock solution in DMSO) for 5 days ⟶ 1 ml sample extract with 500 µl EtOAc and NaCl

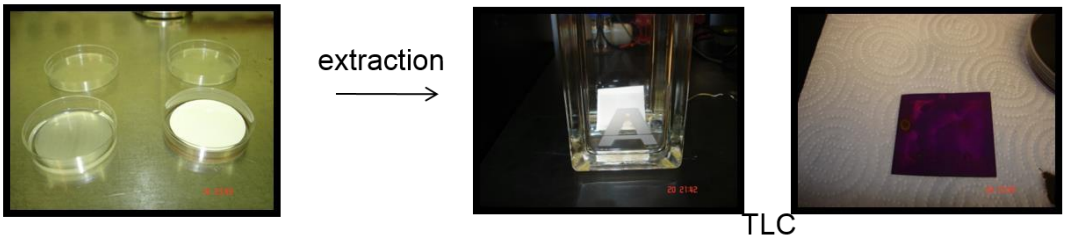



**Figure 5-32:** Optimi zation of latex coating for *E. coli* BL21 (*DE3*) cells expresse ing CYP154E1-CamAB system using model substrate nerol.

The catalytic properties of recombinant *E. coli* cells immobilized in latex were investigated using the oxidation of nerol as a model reaction. The oxidation reactions were carried out in Petri dishes containing 5 mL of 50 mM potassium phosphate buffer (pH 7.5) with 0.4-2 mM nerol dissolved in DMSO (with a concentration of 0.7% DMSO in the sample). The reaction mixture was incubated at 30 °C and 90 rpm for 5 days. After incubation, samples were extracted with ethyl acetate, dried, and analyzed using GC-MS.

The immobilized cell culture demonstrated superior performance in the degradation of by-products compared to the conventional free cell culture. The ratio of the desired product, 8-hydroxynerol, increased from 71% in the free cell system to 97% in the immobilized cell system, while maintaining a high substrate conversion rate of 62%. The ratio of the epoxy product, 2,3-epoxynerol, remained stable at 3% in both the immobilized and free cell systems. Notably, the immobilized cell system eliminated the presence of the byproducts X1 (23%) and X2 (3%) observed in the free cell system. The conversion rates for different nerol concentrations were as follows: 94% for 0.4

mM nerol, 86% for 1 mM nerol, 70% for 1.5 mM nerol, and 62% for 2 mM nerol. These resultsare summarized in Figure 5-33A.

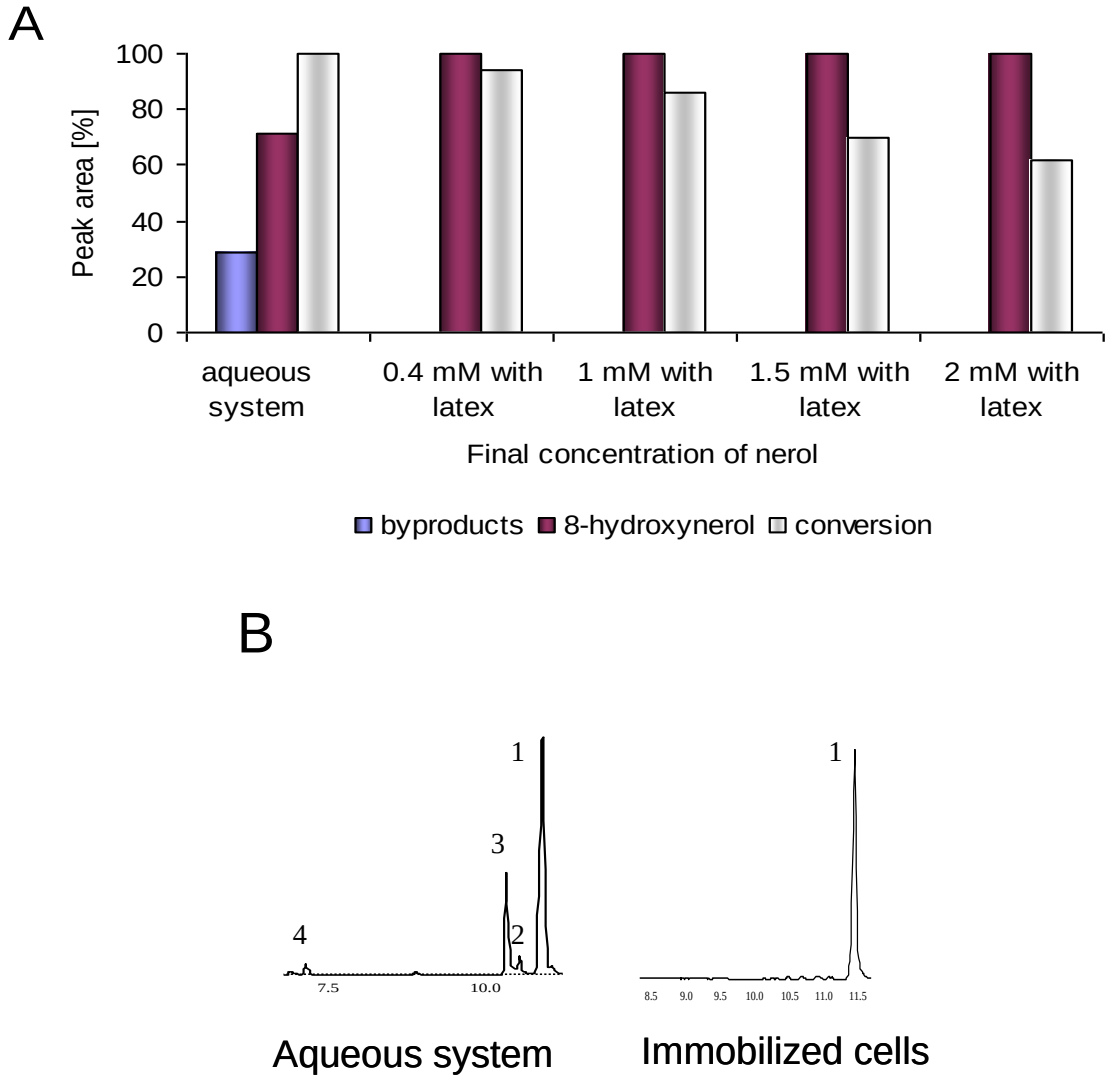



**Figure 5-33:** Oxidation of nerol by immobilized *E. coli* cells: A. at different substrate concentrations; B. GC-analysis of free whole cell system and immobilized cells (**1** is 8-hydroxynerol product of interest **2** is X3 unknown compound **3** is X2 unknown compound **4** is 2,3-epoxynerol).

This study represents the first report on the use of nerol as a substrate for the oxidation reaction using the immobilized E. coli cells expressing heterologous CYP154E1. The results demonstrate the effectiveness of the immobilized cell system in achieving high conversion rates and selective production of the desired product, 8hydroxynerol. Compared to the conventional free cell system, the immobilized cells showed improved degradation of by-products, resulting in the absence of X1 and X2 byproducts. These

findings highlight the potential of this immobilized cell system for the efficient and selective oxidation of nerol, offering new opportunities for biocatalytic applications. Detailed results can be found in Figure 5-33A.

## 5.6. In vivo catalysis with *E. coli* in two phase systems

To minimize the formation of degradation products, a two-liquid organic-aqueous phase system was employed, as depicted in Figure 5-34. This system offers improved control over reaction conditions and can enhance the selectivity and efficiency of the oxidation process. By utilizing this two-phase system, the aim is to optimize the production of desired products while minimizing unwanted by-products.

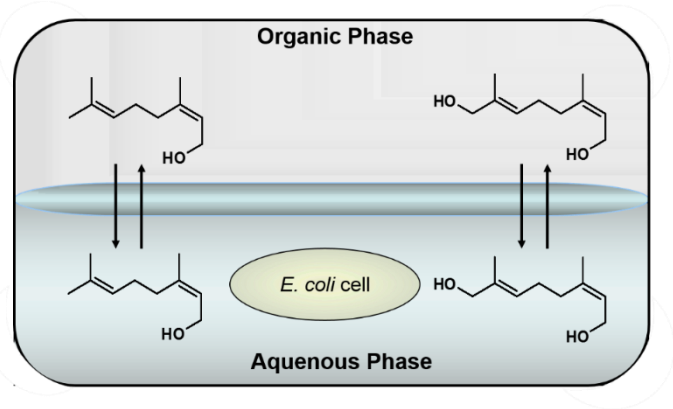



**Figure 5-34:** Scheme for oxidation of nerol using recombinant *E. coli* in a two phase system.

In this system, organic solvent serves as a reservoir for the substrate and for *in situ* recovery of the reaction products. The biocatalytic system containing recombinant *E. coli* cells retained in the aqueous phase while the products may continuously be isolated from the organic phase.

Several organic solvents *n*-octane, *iso*-octane, hexadecane, di-'isonyl'phthalate (DINP) with different log P values as well as a water-immiscible ionic liquid, 1-butyl-3methylimidazolium hexafluoro-phosphate (BMIM [$PF_6$]) in different percentage ratio to the aqueous phase have been tested (Fig. 5-35).

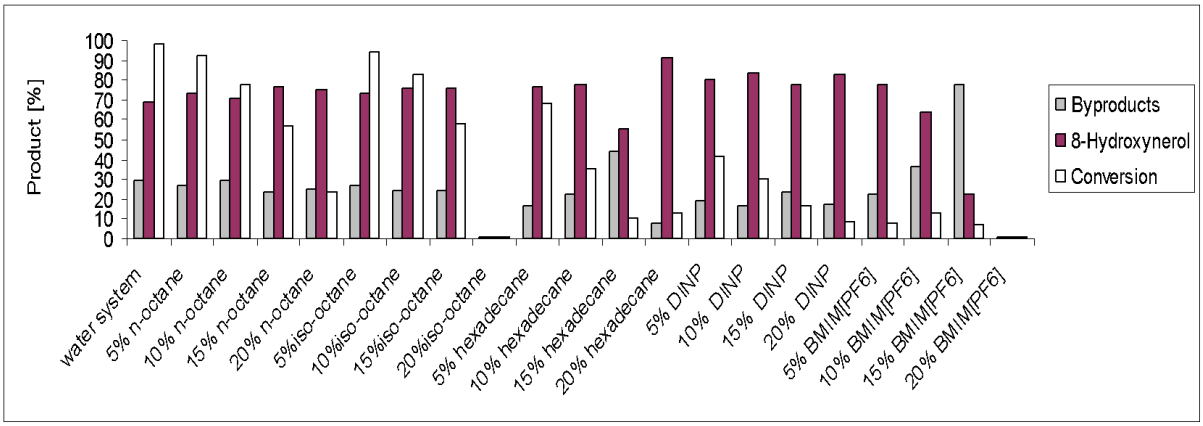



**Figure 5-35:** Product formation using different organic solvents in two phase systems with CYP154E1 CamAB system and nerol as a model substrate.

The diagram illustrates the results obtained from the oxidation of nerol using different organic-aqueous phase systems. Each system is characterised by a specific concentration of various organic compounds, including *n*-octane, *iso*-octane, hexadecane, DINP, and BMIM[PF6]. The diagram displays the number of byproducts, the production of 8-hydroxynerol (the desired product), and the overall conversion rate for each organic phase system. The concentrations of the organic compounds are represented on the X-axis, while the corresponding values for the byproducts, 8-hydroxynerol, and conversion rate are shown on the Y-axis.

The values in the diagram provide insights into the performance of each organic phase system in terms of byproduct formation, 8-hydroxynerol production, and the efficiency of the conversion process. By analysing the data, it is possible to identify trends and the addition of organic solvents and ionic liquids in the oxidation reaction of nerol resulted in a decrease in overall productivity of the whole cell system, albeit with reduced concentrations of byproducts. To strike a balance between productivity and byproduct concentration, the system with 10% iso-octane was chosen. In this system, the ratio of 8-hydroxynerol increased from 69% to 80%, while the levels of the byproducts X1 and X2 decreased. The substrate conversion rate remained high at 84%.

In further experiments, β-cyclodextrin (βCD) was tested in different ratios with CYP154E1 in the CamAB system using nerol as the model substrate. βCD, which is soluble in water but insoluble in organic solvents, was used to reduce compound solubility. The reaction of CYP154E1 with nerol and βCD in a 1:1 ratio led to a significant reduction in byproduct formation from 29% to only 8%. Additionally, 92% of

8-hydroxynerol, the desired product, was observed, while the conversion rate remained high at 90%. When the ratio of CYP154E1 to βCD was changed to 1:2, the byproduct formation decreased to 9%, and 91% of 8-hydroxynerol was obtained, with a conversion rate of 86%.

These findings highlight the potential of using β-cyclodextrin as an additive to improve the selectivity and efficiency of the oxidation reaction of nerol, reducing byproduct formation and increasing the yield of the desired product (Fig. 5-36).

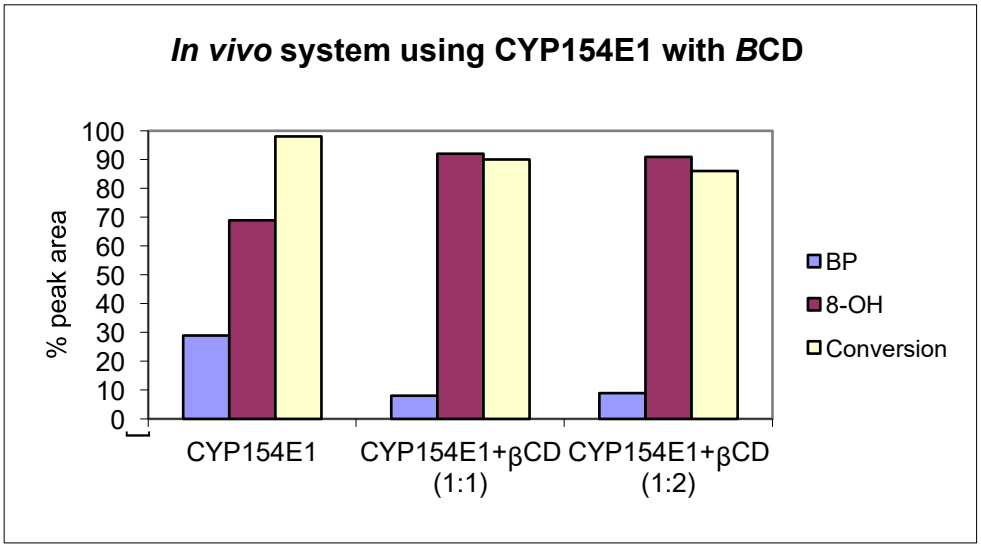



**Figure 5-36:** Product formation using β-cyclodextrines in two phase system with CYP154E1 CamAB system and nerol as a model substrate. BP is a byproduct.

# 6. Discussion

The research conducted in this dissertation on the CYP154 family has been further validated and confirmed in C. von Bühler's publication from November 2013. The findings presented in this study are of great importance for both industrial applications and future research.

Oxidation of terpenes and terpenoids by cytochrome P450 monooxygenases can yield a variety of products, including epoxides and allylic alcohols. While gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) can be used for their separation and identification, these methods are time-consuming and require substantial amounts of materials. To address this issue, a simple, fast, and sensitive thin-layer chromatography (TLC) assay was developed in this study to enable the rapid identification of oxidation products. The TLC assay offers several advantages

in monitoring chemical and biochemical reactions within a short timeframe. By visualizing the spots on a silica plate under different conditions, it becomes possible to distinguish between different epoxides or differentiate epoxides from allylic alcohols. This capability allows for a rapid screening of oxygenases with varying regio- and chemoselectivity or enables reliable microbial screening. The use of TLC as a screening tool in this study has proven to be valuable for the identification of oxidation products of terpenes and terpenoids. By employing this technique, the researchers were able to quickly assess the regio- and chemoselectivity of the cytochrome P450 monooxygenases under investigation. The method's simplicity and sensitivity make it a useful tool for initial screening and characterization of enzymes, especially in the context of identifying specific product profiles.

Furthermore, the TLC assay provides a cost-effective and time-efficient alternative to traditional GC-based methods, making it suitable for high-throughput screening of enzymatic reactions. Its application in this study allowed for the rapid identification and differentiation of epoxides and allylic alcohols, providing valuable insights into the reaction selectivity of the cytochrome P450 monooxygenases.

In conclusion, the development and utilization of the TLC assay in this study has demonstrated its efficacy as a fast and reliable screening tool for investigating the regio- and chemoselectivity of cytochrome P450 monooxygenases in the oxidation of terpenes and terpenoids. The method's simplicity, sensitivity, and cost-effectiveness make it an asset for future enzyme screening efforts, enabling efficient identification of potential biocatalysts and facilitating the optimization of enzymatic reactions for industrial applications.

The discussion highlights are the activity and substrate specificity of different cytochrome P450 monooxygenases (CYP105B1, CYP105D5, and CYP170A1) in oxidizing various terpenes and terpenoids. These enzymes were investigated in conjunction with putidaredoxin and putidaredoxin reductase as redox partners. The findings provide insights into the enzymatic capabilities and limitations of these systems.

CYP105B1 exhibited selective oxidation of geraniol, resulting in the formation of 6,7-epoxygeraniol as the main product. Conversion of geraniol to 8-hydroxygeraniol, an allylic alcohol, was also observed. The oxidation of geranylacetone by CYP105B1 led

to the formation of a mixture of compounds, including the epoxide 9,10-epoxygeranylacetone and three allylic alcohols. The regioselectivity and conversion rates varied depending on the substrate. Nerylacetone was selectively oxidized to 9,10-epoxynerylacetone and 5,6epoxygeranylacetone. The enzyme also demonstrated activity towards α- and βionone, producing selective hydroxylation products.

CYP105D5 from *Streptomyces coelicolor A3 (2)* exhibited similar substrate specificity, oxidizing geraniol to 6,7-epoxygeraniol and 9-hydroxygeraniol. Nerol was converted to 6,7-epoxynerol and 8-hydroxynerol. The oxidation of geranylacetone and nerylacetone resulted in the formation of epoxides and allylic alcohols. CYP154D5 also displayed selective hydroxylation activity towards α- and β-ionone.

In contrast, CYP170A1 from Streptomyces coelicolor A3 (2) showed limited oxidation activity towards the tested substrates. Geranylacetone was primarily converted to 9,10-epoxygeranylacetone, with a minor amount of 8-hydroxygeranylacetone. Nerylacetone was only oxidized to 9,10-epoxynerylacetone. CYP170A1 exhibited selective hydroxylation of β-ionone, while no activity was observed towards α-ionone. The enzyme did not show activity towards (+)-valencene or (+)-α-pinene.

The difficulty in finding compatible heterologous redox partners for cytochrome P450 monooxygenases is well-recognized in the field. The presence of putidaredoxin and putidaredoxin reductase from Pseudomonas putida has been shown to enhance the enzymatic activities and product profiles of certain P450 enzymes. These redox partners are known to interact with the P450 enzymes and facilitate the transfer of electrons during the catalytic cycle, which is essential for their function (1)(2).

However, the lack of significant activity observed in the presence of putidaredoxin and putidaredoxin reductase in this study suggests that additional factors may be at play in influencing the overall catalytic efficiency and specificity of the investigated cytochrome P450 monooxygenases. It is possible that these enzymes require specific co-factors or co-substrates that were not provided in the experimental setup.
Alternatively, the enzymes may have specific structural or conformational requirements that were not met under the tested conditions.

Further investigations are needed to fully understand the interplay between cytochrome P450 monooxygenases and their redox partners, as well as the factors that influence their catalytic efficiency and specificity. These findings highlight the complexity of enzyme systems and the importance of considering multiple factors when designing biocatalytic processes for industrial applications.

In addition to the putidaredoxin and putidaredoxin reductase from Pseudomonas putida, attempts were made to use other redox partners *in vitro*, including FdR15 from *Streptomyces coelicolor A3(2)*. However, the conversions observed with FdR15 were found to be too low to be considered significant. Further studies are needed to explore and optimize the redox system for these cytochrome P450 monooxygenases. Understanding the specific requirements and interactions between the enzymes and their redox partners is crucial for improving the catalytic performance and expanding the applications of these enzymes in various biotechnological processes.

The primary screening revealed a remarkable catalytic activity for the allylic hydroxylation of terpenoids. To further validate and strengthen these initial findings, in vitro bioconversions were performed using a purified mixture of CYP154E1, putidaredoxin reductase (PdR), and putidaredoxin (Pdx). The successful heterologous expression of CYP154E1 from *T. fusca YX*, along with the redox partners PdR and Pdx from *P. putida*, in *E. coli* allowed for their subsequent purification and comprehensive characterization of their enzymatic properties. These *in vitro* experiments aimed to provide additional evidence and deeper insights into the catalytic capabilities of CYP154E1 for allylic hydroxylation reactions.

During the oxidation of model substrates by CYP154A from Nocardia farcinica, the coexpression of recombinant *E. coli* cells carrying CYP154A and the required redox partners led to distinct oxidation patterns. The *Z*-isomer nerol underwent oxidation to yield a mixture of two unknown products, X1 (45%) and X2 (10%), in addition to the formation of the allylic alcohol 8-hydroxynerol (45%). Interestingly, the dominant product observed in the oxidation of the *E*-isomer geraniol was X3 (61%), while only a minor product, X1 (7%), was detected. Notably, the X2 product observed in the nerol conversion was not detected in the geraniol oxidation. Furthermore, the allylic alcohol of interest, 8-hydroxygeraniol, was identified as a byproduct with a relative ratio of only 32%.

The oxidation of geranylacetone resulted in a mixture of unknown products, including 8-hydroxy-, an unknown X3 product, and 12-hydroxy-, along with significant amounts of 9,10-epoxygeranylacetone (30%) and the allylic alcohol 11-hydroxygeranylacetone (35%). Similarly, the Z-isomer nerylacetone was converted to the epoxide 9,10epoxynerylacetone, along with a mixture of two allylic alcohols: 71% of 11-hydroxy- and 15% of 12-hydroxynerylacetone. Negative controls without P450 enzymes were included in the experiments to confirm the role of the enzymes in the observed oxidation reactions.

These findings provide valuable insights into the substrate specificity and product profiles of CYP154A from *Nocardia farcinica* during the oxidation of model substrates. The distinct oxidation patterns observed for different terpenoid substrates highlight the regioselectivity and product diversity of this enzyme. Furthermore, the successful heterologous expression and purification of CYP154E1 from *T. fusca YX*, along with the investigation of its catalytic properties in the in vitro bioconversions, contribute to our understanding of the enzymatic capabilities of CYP154E1 for allylic hydroxylation reactions.

Overall, this study expands our knowledge of the catalytic potential of CYP154A and CYP154E1 enzymes in the oxidation of terpenoid substrates, shedding light on their substrate selectivity, regioselectivity, and product profiles. These findings have implications for the development of biocatalytic processes and the production of valuable compounds in industries such as pharmaceuticals, flavors, and fragrances. Further research in this field can leverage these insights to optimize enzyme performance, engineer selectivity, and explore new applications in synthetic biology and biotechnology.

These findings have implications for industrial applications as they demonstrate the potential of the CYP154E1 enzyme in catalyzing selective oxidations of acyclic short chain compounds. The regio- and chemoselectivity exhibited by these enzymes can be harnessed to produce specific oxygenated compounds, which are of high value in various industries such as pharmaceuticals, flavors and fragrances, and fine chemicals. The ability to selectively convert substrates into desired products offers a more efficient and sustainable approach compared to traditional chemical synthesis methods.

Furthermore, the insights gained from this research contribute to the understanding of enzyme engineering and the rational design of biocatalysts. By investigating the influence of specific amino acid residues, such as V286, on the catalytic properties of the CYP154E1 enzymes, this study provides valuable information for the development of enzymes with enhanced selectivity and activity. These engineered enzymes can find applications in various biotechnological processes, including the synthesis of complex molecules and the production of bioactive compounds.

In terms of further research, the identification of key residues and their impact on enzyme selectivity opens new avenues for exploring the substrate space of cytochrome P450 enzymes. Future studies can focus on expanding the range of substrates tested and investigating the catalytic properties of other CYP154 family members. Additionally, structural studies and computational modeling can provide insights into the mechanistic details underlying the observed selectivity and guide further enzyme engineering efforts.

From an industrial standpoint, the discovery of CYP154E1 as a highly active and selective enzyme for the oxidation of acyclic terpenoids to allylic alcohols offers great potential to produce valuable compounds. Allylic alcohols have widespread applications in the fragrance, flavor, and pharmaceutical industries, serving as key building blocks for the synthesis of various products. The ability to efficiently produce these compounds using a biocatalytic system has several advantages over traditional chemical synthesis, including reduced environmental impact, milder reaction conditions, and potentially higher yields. The findings open new possibilities for the development of more sustainable and cost-effective processes in these industries.

This knowledge contributes to our understanding of enzymatic reactions involved in the oxidation of complex organic compounds, paving the way for further exploration and optimization of biocatalytic systems. Additionally, the development of the TLC Assay as a fast and reliable screening method demonstrates the potential for streamlining the discovery and characterization of novel enzymatic activities.

Overall, this study has implications for both industry and research, offering new opportunities to produce valuable allylic alcohol compounds and advancing our understanding of enzymatic transformations. It highlights the importance of biocatalysis as a powerful tool in synthetic chemistry and underscores the potential for the

development of more sustainable and efficient processes in the production of fine chemicals.

Specifically, position 286 in the enzyme was targeted, and valine was replaced with hydrophobic amino acids of different sizes to assess their impact on the enzyme's performance. The results showed that the V286A mutant exhibited increased epoxide formation from geraniol and nerol compared to the wild-type enzyme. However, with geranylacetone and nerylacetone, the mutant showed lower selectivity, resulting in the production of three allylic alcohols without any epoxide formation. On the other hand, the V286F mutant displayed higher chemo- and regio-selectivity, primarily forming 7-hydroxygeranylacetone and 12-hydroxynerylacetone, respectively. Interestingly, this study reports, for the first time, the successful engineering of P450 enzymes that exhibit allylic hydroxylation mainly towards 7-hydroxygeranylacetone and preferentially to 12-hydroxynerylacetone. It highlights the fact that regioselectivity and activity are not mutually exclusive properties and can be controlled through targeted mutations.

In conclusion, this work successfully identified position 286 in CYP154E1 as a critical determinant of its regioselectivity and catalytic activity towards acyclic terpenoids. The engineered mutants exhibited altered selectivity profiles, demonstrating the potential for tailoring P450 enzymes for specific biocatalytic applications. These findings contribute to our understanding of the structure-function relationship in P450 enzymes and provide a foundation for future studies aiming to further optimize the regioselectivity and activity of these enzymes. It was important to investigate the capabilities of the CYP154E1 mutants in oxidizing acyclic short chain compounds, specifically C5-C10 alkanes. Surprisingly, the mutants displayed chemo- and regioselectivity in oxidizing these substrates.

All three mutants, CYP154E1_V286L, _V286A, and _V286F, were able to convert 2hexanol to 2-hexanone. The V286L mutant selectively oxidized n-octane to 3-octanol, while 2-octanol was converted to 1-octanal, and 4-octanol was oxidized to 4octanone. The V286A mutant also converted n-octane exclusively to 3-octanol, with 2-octanol being oxidized to 2-octanone and 3-octanol to 3-octanone. The V286F mutant, on the other hand, was the only one capable of oxidizing 3-octanol to the enone 3-octanone. Interestingly, compared to the wilde-type enzyme, all three mutants showed selective formation of 4-octanone from 4-octanol, which was not observed in the wild type. None

of the mutants were able to detectably oxidize n-decane or its alcohols, 1-decanol and 1,2-decanediol.

These findings highlight the potential of the CYP154E1 mutants to selectively oxidize short chain alkanes, demonstrating their utility in the production of specific oxygenated compounds. The regioselectivity exhibited by the mutants offers a valuable tool for the synthesis of targeted products. However, further studies are needed to fully understand the structural basis and mechanistic details underlying the observed selectivity and activity of these mutants.

In conclusion, this work successfully demonstrated the chemo- and regioselective oxidation of acyclic short chain compounds by the CYP154E1 mutants. The mutants exhibited varying capabilities in oxidizing different substrates, providing valuable insights into the role of residue V286 in determining the enzyme's selectivity. These findings have significant implications for biocatalysis and the production of tailored oxygenated compounds, offering new opportunities for enzyme engineering and biotechnological applications.

The utilization of a two-phase system in this study, particularly with CYP154E1, has proven to be advantageous. Previous studies by M. Girhard in 2009 have utilized a similar approach with recombinant E. coli expressing CYP109B1 (M. Girhard et al., 2009). The introduction of an immiscible organic solvent, such as *n*-octane, isooctane, and hexadecane, in the two phase system significantly reduced the formation of byproducts. The results obtained in this study demonstrate the effectiveness of the two-phase system in improving the selectivity and productivity of the enzymatic oxidation reaction. Among the various organic solvents tested, 10% isooctane exhibited the best performance, resulting in a higher percentage (76%) of the desired product, 8-hydroxynerol, while maintaining a high conversion rate of 83%. Additionally, the formation of byproducts was significantly reduced from 29% to 13% when compared to a single-phase system.

The use of a two phase system offers several advantages. Firstly, it provides a suitable environment for the enzyme to perform its catalytic activity, as the immiscible organic solvent creates a distinct phase where the reaction can take place. This separation of phases helps to minimize unwanted side reactions and enhance the selectivity of the enzyme towards the desired product. Secondly, the presence of the organic solvent

can also aid in solubilizing hydrophobic substrates, thereby improving their accessibility to the enzyme and increasing the overall reaction efficiency.

The reduction in byproduct formation observed in the two-phase system is of great significance for industrial applications. By minimizing the formation of undesired byproducts, the overall process becomes more efficient and cost-effective. Additionally, the improved selectivity and productivity achieved in the two-phase system contribute to the development of more sustainable and environmentally friendly biocatalytic processes.

In conclusion, the implementation of a two-phase system in this study has proven to be a valuable strategy for enhancing the enzymatic oxidation of acyclic short chain compounds by CYP154E1. The improved selectivity, productivity, and reduced byproduct formation make this approach highly advantageous for both industrial applications and future research in the field of biocatalysis.

The use of latex coatings for biocatalysis, which was initially explored by Lawton, Bunning, and Flanagan, has proven to be a valuable approach (Lawton et al., 1985; Bunning et al., 1988; Flanagan et al., 1990). These coatings consist of a bacteriaplus-latex layer covered by a porous coating, allowing for the utilization of whole-cell biosensors, oxidations, and reductions. The development of formulation and drying methods has facilitated the generation of permeable coatings that preserve the viability of the cells, making them highly efficient biocatalysts in the chemical process industry.

The thinness, microstructure, and diffusion properties of latex coatings, coupled with the high cell density achieved through entrapment, make them particularly attractive for large-scale industrial applications. The utilization of industrial coating and printing technology further expands the potential uses of engineered microorganisms as biocatalysts (M.C. Flickinger et al., 2001).

In this study, we successfully applied a latex coating containing CYP154E1 entrapped in Latex 091 for the oxidation of nerol. Notably, we observed a significant increase in the ratio of the desired product, 8-hydroxynerol, from 71% in the free-cell system to 97% in the immobilized cell system, while maintaining a high substrate conversion rate of 62%. Moreover, the immobilized cell system exhibited a steady ratio of the epoxy product, 2,3-epoxynerol, compared to the free-cell system. The most remarkable

finding was the complete absence of the byproducts X1 (23%) and X2 (3%) when immobilized cells were used.

It is important to note that immobilized cells typically exhibit similar selectivity to suspended cells. However, in this system, we observed different selectivity under various conditions such as temperature, pH value, DMSO solvent, and different extraction chemicals. This highlights the influence of the immobilization method and the potential to modulate the selectivity of the biocatalytic system.

The successful application of the latex coating technique in this study offers several advantages. The immobilization of cells within the latex matrix provides protection and stability, allowing for repeated use and improved longevity of the biocatalyst. Additionally, the porous nature of the coating allows for the diffusion of substrates and products, facilitating efficient mass transfer and enhancing the overall performance of the biocatalytic system.

In conclusion, the utilization of latex coatings, such as the Latex 091 system, for biocatalysis offers significant advantages in terms of selectivity, stability, and scalability. The ability to manipulate the selectivity of the immobilized cells and the elimination of undesirable byproducts demonstrate the potential of this system for industrial applications. Further investigations into the optimization of process parameters and the exploration of other latex coating formulations will continue to advance the field of biocatalysis.

In summary, the aim of this work was to explore the catalytic properties of bacterial P450 monooxygenases for the oxidation of short-chain substrates, such as C7-C12 alkanes and terpenoids. By identifying and characterizing functional P450 enzymes and their redox partners, the study aimed to uncover their potential for allylic hydroxylation reactions. Additionally, the use of immobilization techniques on latex systems and the implementation of two-phase systems provided novel approaches to enhance the efficiency and selectivity of these biocatalytic processes.

The findings of this research hold significant promise for the pharmaceutical and perfume industries, where the oxidation of short-chain substrates is of great interest. The identification and isolation of specific P450 enzymes, along with the understanding of their redox properties and the utilization of appropriate immobilization techniques,

offer opportunities for the development of sustainable and efficient biotechnological solutions.

By expanding the applications of P450 enzymes and exploring alternative reaction systems, this work contributes to the advancement of industrial biocatalysis. The insights gained from this study can inform the design of future biotechnological processes, leading to more sustainable and resilient chemical production methods. Ultimately, this research paves the way for the development of innovative approaches in the field of biocatalysis and opens new possibilities for the utilization of P450 enzymes in various sectors.

# 7. Publications


1. Griffin B. W.; Peterson J. A.; Estabrook R. W. In Porphyrins; Dolphin D., Ed.; Academic Press: New York, 1979; Vol. 7, p 333.

2. Gunsalus, I. C., Pederson, T. C., & Sligar, S. G. (1975). Oxygenase-catalyzed biological hydroxylations. *Annual review of biochemistry*, *44*, 377-407.

3. Katagiri M, Ganguli BN, Gunsalus IC. A soluble cytochrome P-450 functional in methylene hydroxylation. *J Biol Chem.* **1968**, *243(12)*, 3543-3546.

4. Hedegaard J.; Gunsalus I. C., Mixed Function Oxidation: IV. An induced methylene hydroxylase in camphor oxidation, *J. Biol. Chem.* **1965**, *240*, 4038-4043.

5. Conrad HE, Dubus R, Namtvedt MJ, Gunsalus IC. Mixed Function Oxidation. II. Separation and properties of the enzymes catalizing camphor Lactonization. *J Biol Chem.* **1965**, *240*, 495-503.

6. Hildebrandt A, Estabrook RW. Evidence for the participation of cytochrome b 5 in hepatic microsomal mixed-function oxidation reactions. Arch *Biochem Biophys.* **1971**,*143 (1)*, 66-79.

7. Gunsalus IC, Pederson TC, Sligar SG. Oxygenase-catalyzed biological hydroxylations. *Annu Rev Biochem.* **1975**, *44*, 377-407.

8. Neu HC, Heppel LA. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem.* **1965**, *240 (9)*, 3685-3692.

9. Estabrook RW, Baron J, Peterson J, Ishimura Y. Oxygenated cytochrome P-450 as an intermediate in hydroxylation reactions. *The Biochemical Journal*. 125, 3P-4P.

10. Hawkes, David B. *et al.* “Cytochrome P450cin (CYP176A), Isolation, Expression, and Characterization*.” *J Biol Chem.* **2002**, *277*, 27725 - 27732.

11. Jackson CJ, Lamb DC, Marczylo TH, Warrilow AG, Manning NJ, Lowe DJ, Kelly DE, Kelly SL. A novel sterol 14alpha-demethylase/ferredoxin fusion protein (MCCYP51FX) from Methylococcus capsulatus represents a new class of the cytochrome P450 superfamily. *J Biol Chem.* **2002**, *277(49)*, 46959-46965.

12. Seth-Smith HM, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, Bruce NC. Cloning, sequencing, and characterization of the hexahydro-1,3,5-

Trinitro-1,3,5-triazine degradation gene cluster from Rhodococcus rhodochrous. *Appl Environ Microbiol.* **2002**, *68(10),* 4764-4771.

13. Roberts GA, Grogan G, Greter A, Flitsch SL, Turner NJ. Identification of a new class of cytochrome P450 from a Rhodococcus sp. *J Bacteriol.* **2002** ,*184(14)*, 3898-3908.

14. Kitazume T, Takaya N, Nakayama N, Shoun H. Fusarium oxysporum fatty-acid subterminal hydroxylase (CYP505) is a membrane-bound eukaryotic counterpart of Bacillus megaterium cytochrome P450BM3. *J Biol Chem.* **2000,** *275(50)*, 39734-3940.

15. Gidrol X, Lin AS, Dégousée N, Yip SF, Kush A. Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *Eur J Biochem.* **1994**, *224(1)*, 21-28.

16. Kelly SL, Kelly DE. Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? *Philos Trans R Soc Lond* B *Biol Sci.* **2013**, *368(1612)*, 20120476.

17. Cederbaum A., Cytochrome P450 2E1-dependent oxidant stress and upregulation of anti-oxidant defense in liver cells, *J. of Gastroenterology and Hepatology* **2006**, *21(3)*, 22-25.

18. Raunio H, Kuusisto M, Juvonen RO, Pentikäinen OT. Modeling of interactions between xenobiotics and cytochrome P450 (CYP) enzymes. *Front Pharmacol.* **2015**, *6*, 123.

19. Zuber R, Modrianský M, Dvorák Z, Rohovský P, Ulrichová J, Simánek V, Anzenbacher P. Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities. *Phytother Res.* **2002**, *16(7),* 632-638.

20. Tomaszewski P, Kubiak-Tomaszewska G, Pachecka J. Cytochrome P450 polymorphism--molecular, metabolic, and pharmacogenetic aspects. II. Participation of CYP isoenzymes in the metabolism of endogenous substances and drugs. *Acta Pol Pharm.* **2008**, *65(3),* 307-318.

28. Guengerich FP. Cytochrome p450 and chemical toxicology. *Chem Res Toxicol.* **2008**; *21(1)*, 70-83.

29. Schifrin, A., & Khatri, Y. P450 monooxygenases (P450ome) of the model white rot fungus Phanerochaete chrysosporium. *Critical reviews in microbiology* ***2016***, *42(6)*, 833-841.

30. Munro, A. W., Girvan, H. M., & McLean, K. J. Cytochrome P450–redox partner fusion enzymes. *Biochimica et Biophysica Acta (BBA)-General Subjects* **2007**, *1770(3)*, 345-359.

31. Urlacher, V. B., & Eiben, S. Cytochrome P450 monooxygenases: perspectives for synthetic application. *Trends in biotechnology* **2006**, *24(7)*, 324-330.

32. Huijbers, M. M., Montersino, S., Westphal, A. H., Tischler, D., van Berkel, W. J., & Janssen, D. B. Allylic alcohol oxidations with P450 monooxygenases. *Nature Communications* **2014**, *5*, 3604.

33. Gao, J., & Ellis, H. R. Recent advances in selective allylic C-H bond oxidation. *Tetrahedron Lett.* **2014**, *55(31)*, 4223-4233.

34. Tanaka, Y., Nishikawa, T., Miyawaki, T., Yoshida, H., & Fujii, I. Biosynthesis of hydroxylated mycotoxins by filamentous fungi and their roles in plant pathogenicity. *Journal of Natural Medicines*, **2019,** *73(2)*, 230-241.

35. Awakawa, T., & Fujii, I. Biosynthesis of fungal meroterpenoids. *Nature Product Reports*, **2013**, *30(2)*, 1139-1149.

36. Nicolaou, K. C., Montagnon, T., & Baran, P. S. Organic synthesis with allylic carbonates. *ChemComm* **2002**, *2*, 101-112.

37. Denmark SE, Fu J. Catalytic enantioselective addition of allylic organometallic reagents to aldehydes and ketones. *Chem Rev.* **2003**, *103(8)*, 2763-94.

38. Pellissier, H. Asymmetric allylic substitution reactions with organometallic reagents. *Chem. Rev.* **2007**, *107(8)*, 3250-3314.

39. Urlacher, V. B., & Girhard, M. Microbial Cytochrome P450 Monooxygenases in Biotechnology. *Applied Microbiology and Biotechnology*, **2012**, *86(2)*, 393-404.

40. Trost, B. M., & Brennan, M. K. Asymmetric allylic alkylation with metals and metalloids. *Chem. Rev.*, **2009**, *109(1)*, 119-171.

41. Drauz, K., & Waldmann, H. Enzyme Catalysis in Organic Synthesis—A Comprehensive Handbook. Wiley-VCH, **1999**.

42. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B. Industrial biocatalysis today and tomorrow. *Nature* **2001**;*409(6817)*, 258-268.

43. Helmchen, G., & Dahnz, A. Enantioselective allylic substitutions with unsaturated nucleophiles. *Chem. Rev.*, **2014**, *114(7)*, 3376-3406.

44. Zhang H, Li P, Ren S, Cheng Z, Zhao G, Zhao W. *Sc*CobB2-mediated Lysine Desuccinylation Regulates Protein Biosynthesis and Carbon Metabolism in *Streptomyces coelicolor*. *Mol Cell Proteomics* **2019**, *18(10)*, 2003-2017.

45. Conrad HE, Lieb K, Gunsalus IC. Mixed function oxidation. 3. An electron transport complex in camphor ketolactonization. *J Biol Chem.* **1965,** *240(10)*, 4029-4037.

46. Estabrook RW, Baron J, Hildebrandt A. A new spectral species associated with cytochrome P-450 in liver microsomes. *Chem Biol Interact.* **1971**, *3(4)*, 260-261.

47. Andrei V. Puchkaev, Paul R. Ortiz de Montellano, The Sulfolobus solfataricus electron donor partners of thermophilic CYP119: an unusual non-NAD(P)H-dependent cytochrome P450 system, *Archives of Biochemistry and Biophysics* **2005**, *434(1)*, 169-177.

48. Tao Weixin, Yang Anna, Deng Zixin, Sun Yuhui, CRISPR/Cas9-Based Editing of Streptomyces for Discovery, Characterization, and Production of Natural Products, *Frontiers in Microbiology* **2018**, Vol. 9.

49. Kim, S., Kim, D. H., & An, Y. J. Discovery of novel cytochrome P450 monooxygenase genes in *Streptomyces peucetius* and their involvement in the biosynthesis of anthracycline antibiotics. *Applied Microbiology and Biotechnology* ***2017***, *101(7)*, 2819-2833.

50. Liu, Z., Wei, D., & Zheng, L. Immobilization methods and applications of enzymes in industry. *Current Organic Chemistry* **2016**, *20(10)*, 1037-1053.

51. Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & FernandezLafuente, R. Improvement of enzyme activity, stability, and selectivity via immobilization techniques. *Enzyme and Microbial Technology* **2007**, *40(6)*, 1451-1463.

52. Sheldon, R. A. Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis* **2007**, *349(8-9)*, 1289-1307.

57. Parte AC, Sardà Carbasse J, Meier-Kolthoff JP, Reimer LC, Göker M. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol.* **2020**, *70(11)*, 5607-5612.

58. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (**2000**). Practical Streptomyces Genetics (2nd ed.). Norwich, England: John Innes Foundation.

59. Bibb MJ."Understanding and manipulating antibiotic production in actinomycetes". *Biochemical Society Transactions* **2013**, *41(6)*, 1355-1364.

60. Labeda DP. Multilocus sequence analysis of phytopathogenic species of the genus Streptomyces. *Int J Syst Evol Microbiol.* **2011**, *61(Pt 10)*, 2525-2531.

61. Urlacher VB, Girhard M. Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology. *Trends Biotechnol.* **2019**, *37(8)*, 882-897.

62. Bentley SD, Chater KF, Cerdeño-Tárraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* **2002**, *417(6885)*, 141-147.

63. Watve MG, Tickoo R, Jog MM, Bhole BD. "How many antibiotics are produced by the genus Streptomyces?". *Archives of Microbiology*. **2001**, *176 (5)*, 386–390.

Akagawa H, Okanishi M, Umezawa H. A plasmid involved in chloramphenicol production in Streptomyces venezuelae: evidence from genetic mapping. *J Gen Microbiol.* **1975**, *90(2)*, 336-346.

64. Dulmage HT. "The production of neomycin by Streptomyces fradiae in synthetic media". *Applied Microbiology* **1953**, *1(2)*, 103–106.

65. Distler J, Ebert A, Mansouri K, Pissowotzki K, Stockmann M, Piepersberg W. "Gene cluster for streptomycin biosynthesis in Streptomyces griseus: nucleotide sequence of three genes and analysis of transcriptional activity". *Nucleic Acids Research* **1987**, *15(19)*, 8041–8056.

66. Woodyer RD, Shao Z, Thomas PM, Kelleher NL, Blodgett JA, Metcalf WW, *et al.* "Heterologous production of fosfomycin and identification of the minimal biosynthetic gene cluster". *Chemistry & Biology* **2006**, *13(11)*, 1171–1182.

67. Zhao, B., Guengerich, F. P., Voehler, M. and Waterman, M. R. Role of active site water molecules and substrate hydroxyl groups in oxygen activation by cytochrome P450 158A2: a new mechanism of proton transfer. *J. Biol. Chem.* **2005**, *280*, 42188-42197.

68. Zhao, B., Lamb, D. C., Lei, L., Kelly, S. L., Yuan, H., Hachey, D. L. and Waterman, M. R. Different binding modes of two flaviolin substrate molecules in cytochrome P450 158A1 (CYP158A1) compared to CYP158A2. *Biochemistry* **2007**, *46*, 8725-8733.

69. Zhao, B., Lei, L., Vassylyev, D. G., Lin, X., Cane, D. E., Kelly, S. L., Yuan, H., Lamb, D. C. and Waterman, M. R. Crystal structure of albaflavenone monooxygenase containing a moonlighting terpene synthase active site. *J. Biol. Chem.* **2009**, *284*, 36711-36719.

70. Zhao, B., Moody, S. C., Hider, R. C., Lei, L., Kelly, S. L., Waterman, M. R. and Lamb, D. C. Structural analysis of cytochrome P450 105N1 involved in the biosynthesis of the zincophore, coelibactin. *Int. J. Mol. Sci.* **2012**, *13*, 8500-8513.

71. Chun, Y. J., Shimada, T., Sanchez-Ponce, R., Martin, M. V., Lei, L., Zhao, B., Kelly, S. L., Waterman, M. R., Lamb, D. C. and Guengerich, F. P. Electron transport pathway for a *Streptomyces* cytochrome P450: cytochrome P450 105D5-catalyzed fatty acid hydroxylation in *Streptomyces coelicolor A3(2)*. *J. Biol. Chem.* **2007**, *282*, 17486-17500.

72. Chun, Y. J., Shimada, T., Waterman, M. R. and Guengerich, F. P. Understanding electron transport systems of *Streptomyces* cytochrome P450. *Biochem. Soc. Trans.* **2006**, *34*, 1183-1185

73. Myung-A Cho, Songhee Han, Young-Ran Lim, Vitchan Kim, Harim Kim and Donghak Kim, *Streptomyces* Cytochrome P450 Enzymes and Their Roles in the Biosynthesis of Macrolide Therapeutic Agents. *Biomol Ther* **2019**, *27(2)*, 127-133.

74. Christianson, David W. (2017). "Structural and Chemical Biology of Terpenoid Cyclases". *Chem. Rev.* **2017**, *117 (17)*, 11570–11648.

75. Ashour, Mohamed; Wink, Michael; Gershenzon, Jonathan "Biochemistry of Terpenoids: Monoterpenes, Sesquiterpenes and Diterpenes". Biochemistry of Plant Secondary Metabolism **2010**, 258–303.

76. Wenqiang Yang1, Xu Chen1, Yanli Li1 , Shaofen Guo1, Zhen Wang1, and Xiuling Yu1, Advances in Pharmacological Activities of Terpenoids, *Natural Product Communications* **2020**, 15(3), 1-13.

77. Galle M, Crespo R, Kladniew BR, Villegas SM, Polo M, de Bravo MG. Suppression by geraniol of the growth of A549 human lung adenocarcinoma cells and inhibition of the mevalonate pathway in culture and in vivo: potential use in cancer chemotherapy. *Nutr Cancer.* **2014**, *66(5),* 888-895.

78. Carnesecchi S, Schneider Y, Ceraline J, et al. Geraniol, a component of plant essential oils, inhibits growth and polyamine biosynthesis in human colon cancer cells. *J Pharmacol Exp Ther.* **2001**, *298(1)*, 197-200.

79. Kim S-H, Bae HC, Park E-J, et al. Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways. *Biochem Biophys Res Commun.* **2011**, *407(1)*, 129-134.

80. Burke YD, Stark MJ, Roach SL, Sen SE, Crowell PL. Inhibition of pancreatic cancer growth by the dietary isoprenoid's farnesol and geraniol. *Lipids* **1997**, *32(2)*, 151-156.

81. Ong TP, Heidor R, de Conti A, Dagli MLZ, Moreno FS. Farnesol and geraniol chemopreventive activities during the initial phases of hepatocarcinogenesis involve similar actions on cell proliferation and DNA damage, but distinct actions on apoptosis, plasma cholesterol and HMGCoA reductase. *Carcinogenesis* **2006**, *27(6)*, 1194-1203.

82. Cho M, So I, Chun JN, Jeon J-H. The antitumor effects of geraniol: modulation of cancer hallmark pathways. *review. Int J Oncol.* **2016,** *48(5)*, 1772-1782.

83. Kim S-H, Park E-J, Lee CR, *et al.* Geraniol induces cooperative interaction of apoptosis and autophagy to elicit cell death in PC-3 prostate cancer cells. *Int J Oncol.* **2012**, *40(5)*, 1683-1690.

84. Yamamoto, T., Yagi, K., Ishida, K. Method for producing optically active alphaionone. US20090216039 (**2009**).

85. Dobler, W., Bahr, N., Breuer, K., Kindler, A. Continuous process for producing pseudoionones and ionones. US7141698 (**2006**).

86. Von Bühler C, Le-Huu P, Urlacher VB. Cluster screening: an effective approach for probing the substrate space of uncharacterized cytochrome P450s. *Chembiochem.* **2013**, *14(16)*, 2189-2198.

## *Lebenslauf*

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| | |
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