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Selective Water Addition

Investigations of hydratases from the genus *Rhodococcus*

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Investigations of hydratases from the genus *Rhodococcus*

Dissertation

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‘*Step by step…’*

To my parents

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Summary

Water addition reactions to (un)-activated double bonds are very rewarding reactions as they elegantly introduce a hydroxyl-group thereby often adding value to the generated product by establishing a novel stereocentre in tertiary, chiral alcohols. However, performing selective water addition reactions is an extremely challenging task using classical, chemical approaches. Next to overall unfavourable reaction equilibria, the unreactive water molecule is a poor nucleophile and therefore requires activation. Furthermore, due to its small size, a controlled, stereo- and regioselective addition is difficult to achieve. Consequently, establishing straightforward processes with a preferably high selectivity under reaction conditions as environmentally benign as possible is of high interest to both industry and academia.

The application of hydratases, enzymes capable of adding water to (un)-activated substrate groups, is thereby the prime alternative. Multiple substrate classes like for example unsaturated fatty acids (fatty acid hydratase), carotenoids (carotenoid-1,2-hydratases), monoterpenes (linalool (de)-hydratase-isomerase or limonene hydratase) as well as fumaric (fumarase) or maleic acid (malease) were already shown to be successfully converted. The use of hydratases is of such great benefit due to their high regio- and stereoselectivity and their mild reaction conditions without any harsh acid- or base-catalysed activation step required.

The genus *Rhodococcus* has proven itself as a useful tool in the development of novel biocatalysts due to hosting a large number of versatile enzymes. Reactions catalysed with Rhodococcus species or enzymes derived from this microorganism are widely applied and range from e.g. redoxreactions over hydrolysis reactions to lyase-catalysed addition reactions (**Chapter 2**). The research completed for this thesis had the aim to deepen the understanding of the hydration potential of *Rhodococcus* species in particular towards two different groups of substrates: activated α,β-unsaturated Michael acceptors as well as unactivated, unsaturated fatty acids. Here, the focus was laid each on the identification of the responsible hydratase-encoding genes as well as the establishment of the respective hydratase-catalysed water addition reactions using both whole-cells and purified enzymes thereby putting the genus *Rhodococcus* in the spotlight for future research.

Chapter 3 deals with the in-depth re-investigation of the Michael-addition of water to a large number of α,β-unsaturated Michael acceptors catalysed by *Rhodococcus* species. Based on contradictions and inconsistencies in previous reports, this chapter aimed at a final clarification of the Michael addition reaction including revisions of the reported substrate structure, the final stereochemistry of the product, the accepted substrate range as well as the stereochemical course of the reaction. First, the previously assumed substrate structure was corrected using 1- and 2-D-NMR spectroscopy experiments and the configuration of the final water addition product was shown to be (*R*)-configurated. During the examination of the substrate range, two coexisting effects were found responsible for the hydration reaction catalysed by *Rhodococcus* species. The majority of substrates was hydrated in an oxygen-independent manner by free amino acids, while the water addition to the revised model substrate was Michael hydratase (Mhy) catalysed and oxygen-dependent which was proven with reactions catalysed by Mhy-negative *Escherichia coli* (*E. coli*) cells as well as under anaerobic reaction conditions. $^{18}O_2$- and D_2O-labelling studies confirmed a true water addition to both groups of substrates and ruled out any oxidative process. A subsequent E2-elimination step of the deuterated product showed that the water addition was performed in *syn*-fashion. Combined, this chapter therefore described a thorough inspection of the hydration processes catalysed by the genus *Rhodococcus* and confirmed the presence of a novel O_2-dependent hydratase which is less promiscuous than previously stated.

After establishing the presence of the Michael hydratase in two *Rhodococcus* strains in the previous chapter, **Chapter 4** serves to expand the knowledge about the abundance of the Michael hydratase within the genus *Rhodococcus* and methodically investigate the microbial Mhy activity. In total, 17 strains from the *Nocardiacae* family were found to inherit the Mhy actvity. A screening of four different growth media as well as a statistical growth medium optimisation using a ‘Design of Experiments’ (DoE) approach of one of the four tested media showed that the Mhy activity was strongly dependent on the chosen growth medium composition. Using this statistical approach, the Mhy activity towards the model substrate was tripled. To lay the groundwork for the identification of the Mhy-encoding gene, the whole-genome of four undescribed Mhy active strains was sequenced. Additionally, in the quest of isolating the Mhy, a

subcellular fractionation showed that the desired enzyme was associated with the plasma membrane therefore establishing for the first time that this oxygen-dependent hydratase was a membrane protein.

Following the results, **Chapter 5** was designated to identifying the Michael hydratase. For this, a combination of different approaches was used. First, the isolation of the membrane protein of interest was tried using solubilisation protocols with both a number of detergents and styrene-maleic acid copolymers. After all solubilisation trials failed, it was attempted to identify the responsible Mhy encoding gene through comparative genomics. The whole-genome sequences of 25 *Rhodococcus* strains were obtained, translated into their respective proteomes, annotated and processed with an orthologous matrix (OMA) algorithm thereby categorising 17880 identified proteins ranked by highest abundances throughout the tested strains. Simultaneously, the isolated membranes of two highly active strains were measured using a shotgun proteomics approach. The results of both the membrane proteomics and comparative genomics were combined which led to the identification of three Mhy candidates. The three candidates were subcloned and heterologously expressed in an *E. coli* host system. Two candidates were successfully overexpressed, but neither showed the desired Mhy activity which ultimately led to the discontinuation of the Mhy identification process. So the Michael hydratase remains as an elusive yet highly interesting hydratase with rare properties.

The OMA algorithm set up for the Mhy identification was also shown to be of use in the search for other enzymes. In **Chapter 6**, it was used to identify an enzyme capable of water addition reactions to unactivated fatty acids: the oleate hydratase (Ohy). Upon expanding the OMA algorithm to 43 *Rhodococcus* strains, 20 Ohys were recognised throughout the genus. Subsequent phylogenetic analysis disclosed that, in total, the 20 Ohys belonged to three different so-called hydratase families (HFams). Specific species clustered together forming three clades with each clade belonging to one HFam. Sequence analysis revealed specific amino-acid patterns throughout the single HFams indicating different reaction mechanisms for each HFam. Representatives of two HFams were selected, cloned and heterologously expressed in a *E. coli* host system. A fatty acid screening with both representatives showed a complementary

hydration behaviour towards sterically demanding or multiply unsaturated fatty acids. With one of the two representatives being a completely novel and undescribed Ohy, a characterisation was carried out thereby expanding the toolbox of oleate hydratases available.

Samenvatting

Water additiereacties op (niet)-geactiveerde dubbele bindingen zijn zeer lonende reacties omdat ze op elegante wijze een hydroxylgroep introduceren, waardoor vaak waarde aan het gegenereerde product wordt toegevoegd door een nieuw stereocentrum in tertiaire, chirale alcoholen tot stand te brengen. Het uitvoeren van selectieve water additiereacties is echter een uiterst uitdagende taak met behulp van klassieke, chemische methodieken. Naast het algehele ongunstige reactie-evenwicht is het niet reactieve watermolecuul een slecht nucleofiel en vereist daarom activering. Bovendien is een gecontroleerde, stereo- en regioselectieve toevoeging vanwege zijn kleine formaat moeilijk te bereiken. Derhalve is het van groot belang voor zowel de industrie als de academische wereld om eenvoudige processen te ontwikkelen met een bij voorkeur hoge selectiviteit en onder milieuvriendelijke reactieomstandigheden.

De toepassing van hydratasen, enzymen die in staat zijn water toe te voegen aan (niet)-geactiveerde substraatgroepen, zijn daarbij het belangrijkste alternatief. Van meerdere substraatklassen, zoals bijvoorbeeld onverzadigde vetzuren (vetzuurhydratase), carotenoïden (carotenoïde-1,2-hydratasen), monoterpenen (linalool (de)-hydratase-isomerase of limoneenhydratase), evenals fumaarzuur (fumarase) en maleïnezuur (malease) is al bekend dat ze succesvol kunnen worden omgezet. Het gebruik van hydratasen is van een dergelijk groot belang vanwege hun hoge regio- en stereoselectiviteit en hun milde reactieomstandigheden, zonder dat een door zuur of base gekatalyseerde activeringsstap noodzakelijk is.

Het geslacht *Rhodococcus* heeft zichzelf bewezen als een nuttig hulpmiddel bij de ontwikkeling van nieuwe biokatalysatoren vanwege de beschikbaarheid van een groot aantal veelzijdige enzymen. Reacties gekatalyseerd door *Rhodococcus* soorten of enzymen afgeleid van dit micro-organisme worden breed toegepast en variëren van b.v. redoxreacties, hydrolysereacties tot aan door lyase gekatalyseerde additiereacties (**hoofdstuk 1**). Het onderzoek dat voor dit proefschrift is uitgevoerd, had tot doel het begrip van het potentieel van hydratatiereacties van *Rhodococcus* soorten te verdiepen, met name naar twee verschillende groepen substraten: α,β-onverzadigde Michael acceptoren en niet-geactiveerde, onverzadigde vetzuren. Hier werd de nadruk

gelegd op de identificatie van de verantwoordelijke hydratase coderende genen, evenals de vaststelling van de respectieve hydratase gekatalyseerde wateradditiereacties met behulp van zowel hele cellen als gezuiverde enzymen waardoor het geslacht *Rhodococcus* in de schijnwerpers werd gezet voor toekomstig onderzoek.

Hoofdstuk 2 behandelde het diepgaande heronderzoek van de Michael-additie van water aan een groot aantal α,β-onverzadigde Michael acceptoren gekatalyseerd door *Rhodoccous* soorten. Op basis van tegenstrijdigheden en inconsistenties in eerdere rapporten, werd er in dit hoofdstuk gestreefd naar een definitieve opheldering van de Michael-additie reactie inclusief herziening van de gerapporteerde substraatstructuur, de uiteindelijke stereochemie van het product, het geaccepteerde substraatbereik en het stereochemische verloop van de reactie. Allereerst werd de eerder aangenomen substraatstructuur gecorrigeerd met behulp van 1- en 2-D-NMR spectroscopie-experimenten en bleek de configuratie van het uiteindelijke watertoevoegingsproduct (*R*)-geconfigureerd te zijn. Tijdens het onderzoek van het substraatbereik werden twee naast elkaar bestaande effecten gevonden die verantwoordelijk zijn voor de hydratatiereactie die wordt gekatalyseerd door *Rhodococcus* soorten. Het grootste deel van de substraten werd op zuurstofonafhankelijke wijze gehydrateerd door vrije aminozuren, terwijl de toevoeging van water aan het herziene modelsubstraat Michael hydratase gekatalyseerd en zuurstofafhankelijk was, hetgeen werd bewezen met reacties gekatalyseerd door Mhy-negatieve *Escherichia coli* cellen evenals onder anaërobe reactiecondities. $^{18}O_2$- en D_2O-labelingsstudies bevestigden een echte toevoeging van water aan beide groepen substraten en sloten elk oxidatief proces uit. Een daaropvolgende E2-eliminatiestap van het gedeutereerde product toonde aan dat de toevoeging van water op een *syn* wijze werd uitgevoerd. Samenvattend, beschrijft dit hoofdstuk daarom een grondige inspectie van de hydratatieprocessen gekatalyseerd door het geslacht *Rhodococcus* en bevestigt het de aanwezigheid van een nieuwe zuurstofafhankelijke hydratase die meer specifiek is dan eerder vermeld.

Na het vaststellen van de aanwezigheid van de Michael hydratase in twee *Rhodococcus* stammen in het vorige hoofdstuk, diende **hoofdstuk 3** om de kennis over de talrijkheid van de Michael hydratase binnen het geslacht *Rhodococcus* uit te

breiden en systematisch microbiële Mhy-activiteit te onderzoeken. In totaal bleken 17 stammen uit de *Nocardiacae*-familie de Mhy-activiteit te vertonen. Een screening van vier verschillende groeimedia en een statistische optimalisatie met behulp van de 'Design of Experiments' (DoE) benadering van een van de vier media toonde aan dat de Mhy-activiteit sterk afhankelijk was van de gekozen samenstelling van het groeimedium. Met behulp van deze statistische benadering werd de Mhy-activiteit met het modelsubstraat verdrievoudigd. Om de basis te leggen voor de identificatie van het Mhy-coderende gen, werd het hele genoom van vier onbeschreven Mhy actieve stammen gesequenced. Bovendien, in de zoektocht naar het isoleren van de Mhy toonde een subcellulaire fractionering aan dat het gewenste enzym geassocieerd was met het plasmamembraan, waardoor voor het eerst werd vastgesteld dat deze zuurstofafhankelijke hydratase een menbraaneiwit was.

Na de resultaten werd **hoofdstuk 4** toegewijd om de Michael hydratase te identificeren. Hiervoor werd een combinatie van verschillende benaderingen gebruikt. Eerst werd geprobeerd het van belang zijnde membraaneiwit te isoleren met behulp van protocollen om de oplosbaarheid te verhogen met zowel detergentia als styreen-maleïnezuurcopolymeren. Nadat alle oplosbarheidstesten faalden, werd geprobeerd het verantwoordelijke Mhy-coderende gen te identificeren door middel van vergelijkende genoomanalyse. De gehele genoomsequenties van 25 *Rhodococcus* soorten werden verkregen, getransleerd in hun respectievelijke proteomen, geannoteerd en verwerkt met een Orthologe Matrix (OMA) algorithme, waardoor 17880 geïdentificeerde eiwitten werden gerangschikt volgens de hoogste aanwezigheid in de geteste stammen. Tegelijkertijd werden de geïsoleerde membranen van twee zeer actieve stammen gemeten met behulp van een shotgun proteomics benadering. De resultaten van zowel de membraan-proteomica als de comparatieve genomica werden gecombineerd, wat leidde tot de identificatie van drie Mhy-kandidaten. De drie kandidaten werden gesubkloneerd en heteroloog tot expressie gebracht in een *E. coli* gastheer. Twee kandidaten werden met succes tot overexpressie gebracht, maar geen van beide vertoonde de gewenste Mhy-activiteit. Dit leidde uiteindelijk tot de stopzetting van het Mhy-identificatieproces. Zo blijft de Michael hydratase een ongrijpbare maar toch ook zeer interessante hydratase met zeldzame eigenschappen.

Het OMA algoritme dat is opgezet voor de Mhy-identificatie was ook geschikt bij het zoeken naar andere enzymen. In **hoofdstuk 5** werd het gebruikt om een enzym te identificeren dat in staat is om wateradditiereacties te katalyseren aan niet-geactiveerde vetzuren: de oleaathydratase (Ohy). Bij het uitbreiden van de OMA algorithme tot 43 *Rhodococcus* stammen, werden 20 Ohy's door het gehele geslacht herkend. De daaropvolgende fylogenetische analyse onthulde dat de 20 Ohy's in totaal tot drie verschillende zogenaamde hydratase-families (HFams) behoorden. Specifieke soorten clusteren samen tot drie klades waarvan elke klade behoort tot een HFam. Sequentie analyse openbaarde specifieke aminozuurpatronen in de afzonderlijke HFam's die verschillende reactiemechanismen aangeven. Vertegenwoordigers van twee HFam's werden geselecteerd, gekloneerd, en heteroloog tot expressie gebracht in een *E. coli* gastheer. Een onderzoek naar de conversie van een groot aantal verschillende vetzuren met beide representatieve enzymen toonde een complementair hydratatiegedrag ten opzichte van sterisch veeleisende of meervoudig onverzadigde vetzuren. Met een van de twee enzymen, volledig nieuw en niet beschreven, werd een volledige karakterisering uitgevoerd waardoor de gereedschapskist van beschikbare oleaathydratasen werd uitgebreid.

2

Rhodococcus as versatile biocatalyst in organic synthesis

The application of purified enzymes as well as whole-cell biocatalysts in synthetic organic chemistry is getting more and more popular and both academia and industry are keen on finding and developing novel enzymes capable of performing otherwise impossible or challenging reactions. The diverse genus *Rhodococcus* offers a multitude of promising enzymes which therefore makes it one of the key bacterial hosts in many areas of research. This review focuses on the broad utilisation potential of the genus *Rhodococcus* in organic chemistry thereby especially highlighting the specific enzyme classes exploited and the reactions they catalyse. Additionally, close attention is paid to the substrate scope that each enzyme class covers. Overall, a comprehensive overview of the applicability of the genus *Rhodococcus* is provided which puts this versatile microorganism in the spotlight of further research.

This chapter is based on

H. Busch, P.-L. Hagedoorn, U. Hanefeld

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2.1 Introduction

The genus *Rhodococcus* belongs to the phylum Actinobacteria and its members are aerobic, gram-positive, non-motile bacteria showing high GC-contents.[1] Numerous members have been isolated from copious sources such as soil, groundwater, marine sediments, internal organs of insects, diseased and healthy animals or plants - to name just a few.[2] While the vast majority is considered harmless, few species showed pathogenic properties leading to foal pneumonia (*R. equi*) or leafy gall disease in plants (*R. fascians*). Starting in the 1980s, their application as (whole-cell) biocatalyst came to the fore and since then numerous reports of successful bioconversions were published with an increasing trend.[3] The longstanding synthesis of acrylamide on a multi-ton scale run at several production sites worldwide is hereby considered as the most outstanding example of a rhodococcal whole-cell process.[4-6]

Rhodococcus members are diverse catalysts that degrade a variety of both natural organic and xenobiotic compounds.[7] Amongst others, *Rhodococcus* species showed biodegradation potential against short- and long-chain alkanes, halogenated and nitro-substituted aromatic, heterocyclic and polycyclic compounds.[8] Next to physiological attributes such as a high general tolerance to substrates and solvents,[8,9] the metabolic diversity of the genus *Rhodococcus* can be explained by the (*i*) presence and mobility of large, linear plasmids, (*ii*) the multiplicity of catabolic genes, (*iii*) the high redundancy of biosynthetic pathways and (*iv*) sophisticated regulatory networks of their genomes.[1,3,10] This brands the genus *Rhodococcus* as the strong biocatalytic powerhouse as what it is seen for today. The usage of whole biosynthetic pathways from *Rhodococcus* strains in the bioremediation of organic pollutants derived from petroleum such as *o*-xylene was intensively investigated[11,12] and shown successful as was *e.g.* the lignin degradation catalysed by *R. jostii* RHA1.[13-15] Here, vanillin, a valuable chemical for food flavouring, was mainly produced.[13] The same strain also displayed biodegradation activity against polychlorinated biphenyls (PCBs).[16] Additionally, *Rhodococcus* strains were used in desulphurisation reactions able to degrade sulphur-containing compounds produced in the treatment of fossil fuels like benzothiophene (BT) or dibenzothiophene (DBT).[10,17]

As shortly displayed, the implementation of whole pathways to degrade numerous compounds is a promising tool with strong application potential in the future.[7,18] Nevertheless, this review will only focus on *Rhodococcu*s as useful tool in organic synthesis concentrating on defined enzyme reactions catalysed by purified enzymes or whole-cells primarily leading to enantioenriched products. It should serve as comprehensive overview of the state-of-the-art biocatalysis that is feasible with *Rhodococcus* concentrating on the reaction diversity and the respective substrate scope of each enzyme class described.

2.2 Promiscuous redox-reactions in *Rhodococcus*

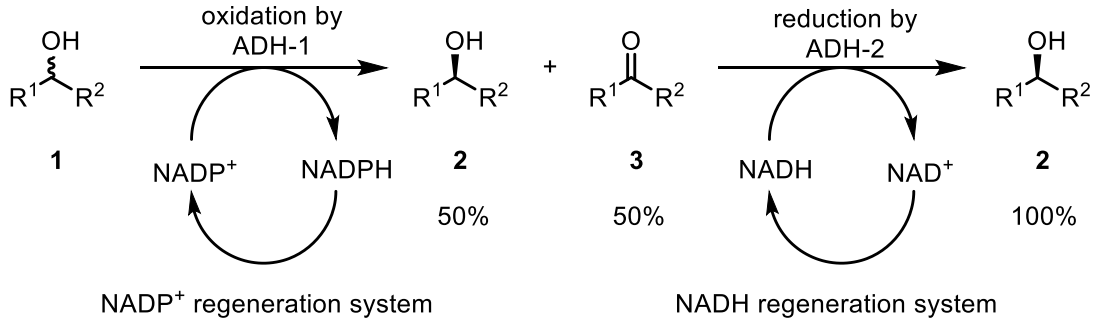
Oxidoreductases (EC 1) catalyse the electron transfer from one molecule (reductant) to another molecule (oxidant) thereby enabling oxidation-reduction reactions often under the requirement of cofactors like NAD(P)H serving as electron donor or acceptor. They make one of the biggest groups and as such, an impressive range of redox-reactions is feasible. Also the genus *Rhodococcus* harbours a large amount of oxidoreductases some of which are already well-established systems running on even industrial scale while others are still being thoroughly investigated and developed.

2.2.1. Alcohol dehydrogenases/keto reductases

Alcohol dehydrogenases (ADHs, EC 1.1.x) catalyse the transformation of primary and secondary alcohols to aldehydes and ketones. A common feature of an ADH-catalysed reaction is the requirement for a primary oxidant within the active site of the enzyme which oxidises the alcohol. This process generally occurs *via* a hydride abstraction. Nicotinamide cofactors (NAD+, NADP+), pyrroquinoline quinones or flavins are typical prosthetic groups acting as hydride acceptors which need to be regenerated during the reaction for the overall process to be a success.[19] A nicotinoprotein from *R. erythropolis* DSM 1069 showed activity towards aliphatic and aromatic primary and secondary alcohols whereby primary alcohols were the preferred substrates yielding aldehydes.[20] Another application of ADHs in an oxidative sense is the kinetic resolution of secondary alcohols leading to both ketones and enantiopure alcohols. When applying whole-cells of *R. ruber* DSM 44541, only the (*S*)-enantiomer of racemic mixtures of alcohols was oxidised leaving behind the unreacted (*R*)-alcohol. This proof-of-principle was successfully shown for the substrate sulcatol as well as aromatic and

aliphatic alcohols.[21] A *R. erythropolis* mediated kinetic resolution of methyl-nonactate yielded the two enantiomers (+)- and (-)-nonactate in excellent enantiomeric purity.[22] Fascinatingly, the stereoselectivity of the main product was influenced by switching the conditions from aerobic to anaerobic. (+)- and (-)-nonactate are desired building blocks for the macrotetrolide nonactin.

To overcome the limitation of only 50% theoretical yields in kinetic resolutions, a two-ADH system was developed resolving racemic alcohols (**1**).[23] First, one ADH (ADH-1) catalyses the oxidation of one enantiomer to the corresponding ketone (**3**) leaving one alcohol-enantiomer unreacted (**2**). A second, stereocomplementary ADH (ADH-2) is afterwards applied that reduces the obtained ketone (**3**) to the wanted alcohol-enantiomer (**2**) thereby enabling a theoretical yield of 100% (Scheme 1). Each step is catalysed by two different ADHs each requiring another cofactor.



Scheme 1: Deracemisation of racemic alcohols using a two-enzyme system with stereocomplementary ADHs.[23]

The (*S*)-selective ADH from *R. ruber* DSM 44541 (termed ADH-A) was part of this study leading, in combination with (*R*)-selective ADH from *Lactobacillus kefir* (LK-ADH), to the formation of (*S*)-alcohols in excellent yields and *ee*. A complete stereoinversion of chiral secondary alcohols was also achieved by coupling two stereocomplementary ADHs: while the first ADH oxidised the starting alcohol to the ketone intermediate, a second ADH reduced the ketones to the other alcohol enantiomers, resulting in a complete inversion of the stereochemistry.[24]

ADHs from *Rhodococcus* were also employed in an oxidative manner in whole-cell biotransformations for straightforward one-pot multistep reactions.[25] Two representative examples demonstrate the broad applicability of ADHs from

Rhodococcus within biocascades: the double oxidation of *c*-octane was achieved by a combination of three enzymes namely a monooxygenase P450 BM3, *Lactobacillus brevis* ADH (*Lb*ADH) and *R. erythropolis* ADH (*Re*ADH) including a cofactor regeneration system whereby all enzymes were coexpressed in a single host cell.[26] The monooxygenase first oxyfunctionalises the non-activated *c*-octane to *c*-octanol which is further oxidised by the two stereocomplementary ADHs. In a similar fashion, four recombinant enzymes (a selfsufficient P450 monooxygenase, two stereocomplementary ADHs (*Lb*ADH and *R*eADH) as well as a ω-transaminase (ATA-117)) were co-expressed in *E. coli* catalysing the chiral amination of benzylic compounds such as (substituted)-ethylbenzene in a one-pot two-step process.[27]

ADHs that catalyse the reverse reaction are so-called ketoreductases (KREDs). Through carbonyl reduction, the generation of (chiral) alcohols is thereby enabled. As the introduction of chiral centres is seen as more valuable than the loss of enantiomeric centres, this reverse reaction is more often applied in synthetic chemistry.

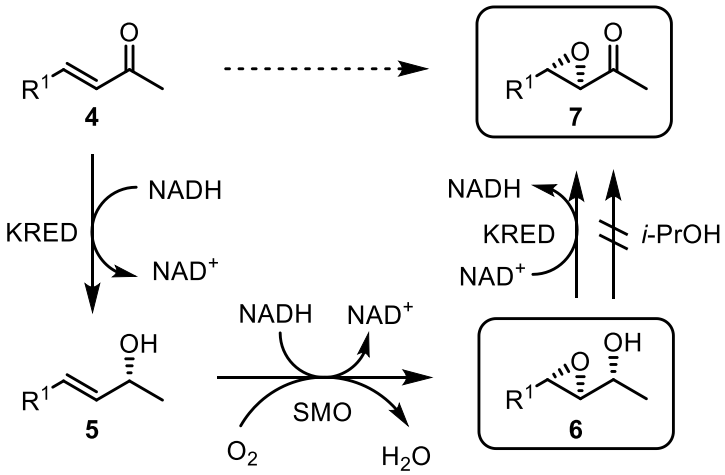
The reduction of aldehydes is hereby less often reported due to the fact that this reduction does not invoke a new stereocentre but leads to primary alcohols. However, a KRED from *Rhodococcus* sp. was employed in the multi-step chemo-enzymatic synthesis of Guerbet alcohols.[28] It was used to catalyse the final step by reducing an aldehyde to form a primary alcohol. The use of enzymes in this example led to an improvement of the overall reaction conditions reducing the temperature, the applied pressure and increasing the selectivity compared to alternative routes.

While most of the following research is based on the use of KREDs identified from *R. erythropolis* (*Re*ADH) and *R. ruber* (ADH-A), a newly characterised ketoreductase from *R. jostii* TMP1 was applied in the synthesis of chiral alcohols showing a broad substrate acceptance with increased affinity towards aliphatic 2,3-diketones, butan-3-one-2-yl alkanoates and acetoin as well as the respective derivatives.[29]

The substrate scope of a recombinantly expressed KRED from *R. erythropolis* was evaluated showing that a multitude of ketones was eligible for KRED-mediated reduction forming the (*S*)-selective alcohols.[30] Next to mono-, di- and tri-substituted acetophenones, also aliphatic ketones have been implemented in a biphasic reaction medium using an integrated cofactor-recycling system (formate dehydrogenase

system). The synthesis of 1-[3,5-*bis*(trifluoromethyl)phenyl]ethanol, another di-substituted acetophenone which acts as key intermediate in the synthesis of NK-1 receptor antagonists, was achieved with the same enzyme coupled to another cofactor-recycling system (glucose dehydrogenase system).[31] In a stirred-tank reactor, space-time yields of 260 g/L per day were achieved.

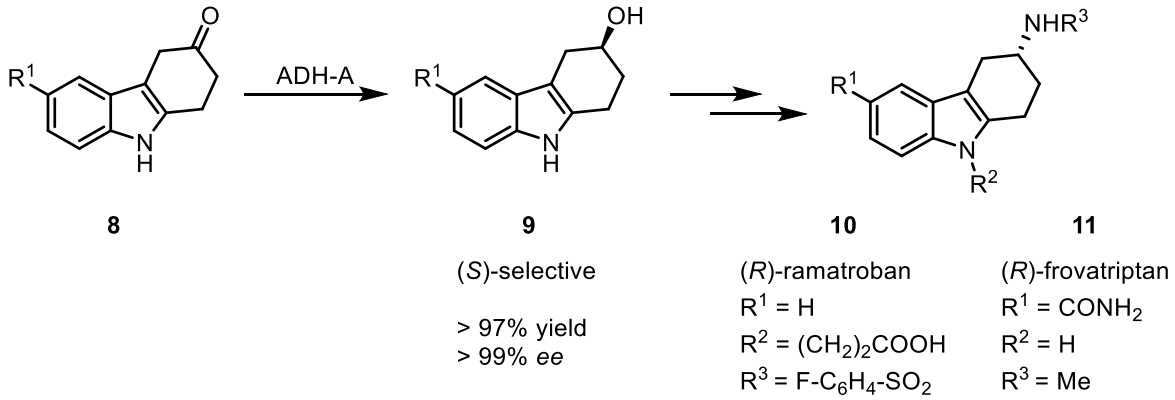
Interestingly, electron-deficient α,β-unsaturated ketones (**4**) were treated in a tandem reduction-epoxidation-dehydrogenation cascade involving two enzymes, namely an AHD from *R. erythropolis* DSM 43297 and a styrene monooxygenase both co-expressed in *E.coli*.[32] While aiming for the synthesis of chiral epoxy ketones (**7**), it was found that allylic epoxy alcohol intermediates (**6**) also form valuable synthons due to the presence of three contiguous stereocenters. Through adaption of the reaction conditions (addition of isopropanol which caused high concentrations of NADH, thereby blocking the dehydrogenation reaction), the major product could easily be switched from an epoxy ketone (**7**) to an allylic epoxy alcohol (**6**) thereby providing two useful synthons with only one reaction set-up (Scheme 2).



Scheme 2: Enzymatic cascade for selective synthesis of epoxy ketones and allylic epoxy alcohols.[32]

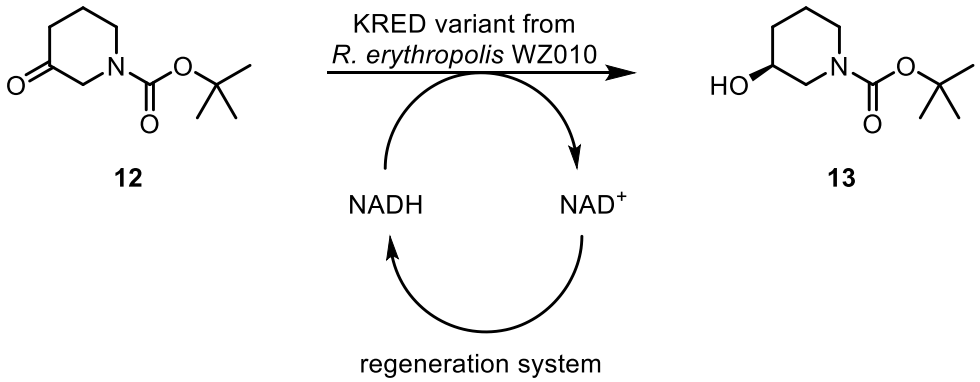
Additionally, trifluoroacetyl-acetophenones lead to chiral fluorinated hydroxyketones when converted by *Re*ADH.[33] The conversion of phenylacyl halides, here especially fluorides, was performed with recombinantly expressed ADH-A yielding fluorohydrins with high yields and enantiomeric purity.[34] ADH-A was also applied in the synthesis of pharmacologically active compounds (*R*)-Ramatroban (**10**), a thromboxane receptor

and protaflandin DP2 receptor antagonist, and (*R*)-Frovatriptan (**11**), a 5-hydroxytryptamine (serotonin) receptor antagonist, treating allergic rhinitis and asthma or migraine headaches, respectively.[35,36] In both total syntheses, the KRED was used to install an (*S*)-alcohol (**9**) which was subsequently inverted following a S_N2-mechanism to an (*R*)-amine. Afterwards, the selective functionalisation of the amine allowed generation of both desired compounds (Scheme 3).



Scheme 3: KRED catalysed ketone reduction in multistep chemo-enzymatic reaction sequence to pharmacologically active compounds (*R*)-ramatroban and (*R*)-frovatriptan.[35,36]

The same enzyme (ADH-A) was applied in the production of *syn*-α-alkyl-β-hydroxy amides through means of dynamic kinetic resolution. Various acyclic α-alkyl-β-keto amides were reduced yielding the (*S*)-selective products high yields and excellent *ee*.[37] Enantiopure 3,4-dihydroisocoumarins were easily accessible *via* a one-pot dynamic reductive kinetic resolution process catalysed by ADH-A[38] as was the asymmetric synthesis of (*S*)-*N*-Boc-3-hydroxypipridines (**13**) using a variant (Y54F) of a KRED from *R. erythropolis* WZ010.[39] *N*-Boc-3-piperidone (**12**) was selectively reduced thereby forming an important intermediate of ibrutinib, an inhibitor of Bruton's tyrosine kinase. The enzymatic process enhances the yield significantly compared to the chemical route (Scheme 4).



Scheme 4: Asymmetric bioreduction of *N*-Boc-3-piperidone (**12**) catalysed by KRED.[39]

The discussed examples show the broad applicability of ADHs/KREDs in organic synthesis by opening up the possibility of introducing new stereocenters through reduction processes or (dynamic) kinetic resolutions.

2.2.2. Oxidases

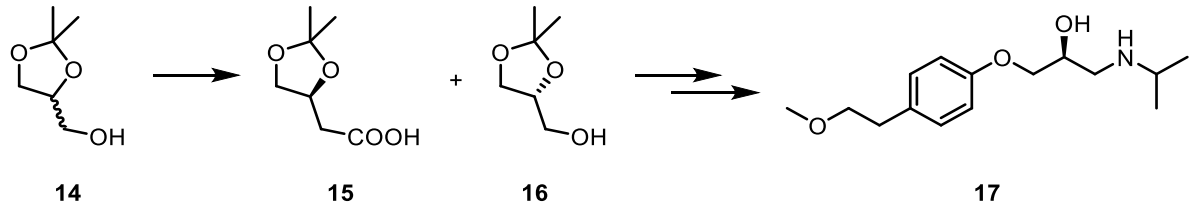
Another class of enzyme, alcohol oxidases, also catalyse the oxidation of alcohols to aldehydes, ketones and sometimes carboxylic acids. In comparison to alcohol dehydrogenases that catalyse this reaction *via* an electron-transfer to an organic cofactor, alcohol oxidases transfer electrons to molecular oxygen forming hydrogen peroxide as side-product. Using oxygen as cheap and readily available oxidant makes oxidases more interesting than alcohol dehydrogenases as they require expensive cofactors in either stoichiometric amounts or respective cofactor regeneration systems.[40]

Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is known to catalyse the oxidation of many 4-hydroxybenzylic alcohols. A homologue of this enzyme was found in *R*. sp. RHA1 which was termed eugenol oxidase (EUGO).[41] The enzyme was shown to catalyse the oxidation of vanillyl alcohol more efficiently than VAO from *P. simplicissimum*, but 4-alkylphenols as well as 4-(methoxymethyl)phenol were only poorly converted.

Cholesterol oxidases (ChoX) on the other hand catalyse the oxidation of the C3 hydroxyl-group of cholesterol and an isomerisation reaction ultimately yielding cholest-4-en-3-one.[40] Additionally to cholesterol, a cholesterol oxidase discovered in *R. erythropolis* also showed activity towards non-steroidal compounds such as smaller

cyclic allylic alcohols with good stereo- and enantioselectivity.[42] In an attempt to produce high-quality cholest-4-en-3-one the use of an aqueous/organic biphasic system was explored which simplifies the production process by improving separation and purification.[43] This led to final product with a purity of 99.78% which makes this new process design more approachable for industrial applications.

A *Rhodococcus* oxidase catalysed reaction already running on industrial scale is a kinetic resolution through the oxidation of racemic iso-propylideneglycerol (**14**) yielding both (*R*)-iso-propylideneglyceric acid (**15**) and (*R*)-iso-propylideneglycerol (**16**).[44,45] The use of this biocatalytic process simplifies the synthesis of desired (*R*)-iso-propylideneglycerol (**16**) compared to chemical processes. Whole-cells of *R. erythropolis* are employed in a fed-batch reactor leading to 50% maximum yield with high *ee* values. The product is a valuable C3 synthon in the synthesis of β-blockers like (*S*)-metoprolol (**17**) (Scheme 5).



14 **15** **16** **17**

Scheme 5: *R. erythropolis* oxidase catalysed process to produce (*R*)-iso-propylideneglycerol (**16**) which is used as C3-synthon for (*S*)-metoprolol (**17**).[44]

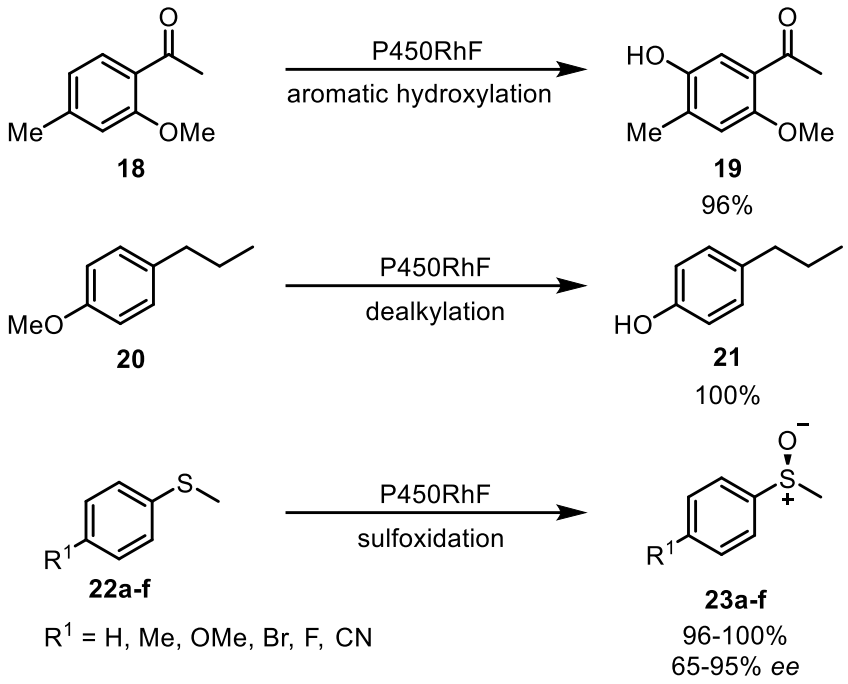
2.2.3 Oxygenases in *Rhodococcus*

Oxygenases utilise molecular oxygen as both substrate and electron acceptor, while the (above) discussed dehydrogenases catalyse the oxidation *via* hydrogen transfer reactions. Oxygenases can be classified into two groups: while monooxygenases introduce only one oxygen-atom, dioxygenases catalyse the introduction of two oxygen atoms from molecular oxygen. This type of reaction is particularly useful in synthetic chemistry as a selective activation of chemically inert C-H bonds is otherwise extremely difficult to achieve using classical chemical procedures.

2.2.3.1. Monooxygenases

2.2.3.1.1 P450 monooxygenases

The so-called cytochrome P450s (P450s, CYPs) monooxygenases contain a haem prosthetic group with an Fe(III)-ion embedded in a porphyrin ring with a cysteine sulphur as axial ligand to the iron. They activate molecular oxygen for the hydroxylation of organic compounds. In this process, the second oxygen is reduced to water. P450s are NAD(P)H dependent enzymes. The for the redox-reaction required electrons are transferred from the cofactor to the haem through one or two ‘electron transport enzymes’ or ‘redox partners’.[46,47] This makes P450s multicomponent enzymes. The involved components can either be free or directly linked to each other with the latter being called a self-sufficient enzyme. One of these self-sufficient P450s was discovered in *R*. sp. NCIMB 9784 where the reductase partner (RhFRed) containing the FMN- and NADPH binding motif and a Fe_2S_2-ferredoxin-like component is directly linked to the oxidase part.[48,49] The natural substrate of this enzyme has not yet been discovered, but it showed a promiscuous substrate scope mediating dealkylation reactions, aromatic hydroxylation, epoxidation and asymmetric sulfoxidation (Scheme 6).[50]



Scheme 6: Representative bioconversion catalysed by P450RhF monooxygenase showing aromatic hydroxylation, dealkylation and sulf-oxidation potential.[50]

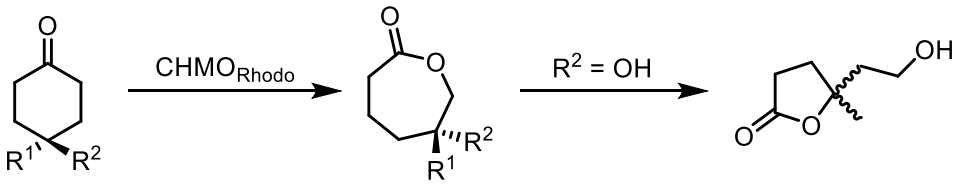
The reductase unit of this P450 is, however, more often employed. The haem domain of P450RhF can be swapped with other haem domains thereby fusing the reductase domain RhFRed to a number of different enzymes.[47,51] One successful example is given by the hydroxylation of testosterone by a chimeric fusion protein consisting of the haem-domain from CYP154 from *Nocardia farcinca* IFM 10152 and the reductase domain RhFRed.[51]

2.2.3.1.2 Baeyer-Villiger monooxygenases

The Baeyer-Villiger reaction involves the oxidation of a carbonyl compound ultimately leading to an ester or lactone. So-called Baeyer-Villiger monooxygenases (BVMOs) catalyse this reaction under milder reaction conditions compared to harsh chemical procedures.[52] Flavin-containing enzymes require electrons from a reduced cofactor. The flavin-cofactor reacts with molecular oxygen thereby forming a reactive peroxyflavin intermediate which performs the nucleophilic attack on the carbonyl function. Upon a rearrangement, the respective ester or lactone is formed.[52]

Prochiral *c*-butanones with alkyl- or aromatic substituents in 3-position were converted by the two *c*-hexanone monooxygenases discovered earlier in *Rhodococcus*[53]

(CHMO$_{Rhodo1}$ and CHMO$_{Rhodo2}$) to yield (*S*)-selective butyrolactones.[54] Both enzymes displayed especially high activity towards bulky substituent piperonyl and aromatic residues with substituents in *m*- and *p*-position. The same enzymes were employed in an activity screening towards 4-4-disubstituted *c*-hexanones (**24**) to obtain the respective caprolactones (**25**).[55] In the special case of 4-hydroxy-4-methyl-*c*-hexanone (**24c**), a caprolactone (**25c**) was generated which spontaneously formed a five-membered ring (**26**) (Scheme 7).

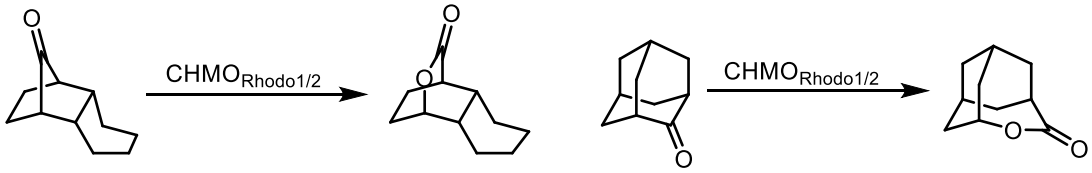


24a-c **25a-c** **26**

R^1 = Me
R^2 = Me, Et, OH

Scheme 7: *Rhodococcus* Baeyer-Villiger monooxygenase catalysed oxidation of 4,4-disubstituted *c*-hexanones(**24**).[55]

Additionally, the same set of enzymes was used in another screening towards a number of bridged *c*-ketones.[56] Both tested *Rhodococcus* enzymes readily converted almost all tested bridged ketones (**27**,**29**) with high yields and good to excellent stereoselectivities yielding bicyclic lactones (**28**,**30**) (Scheme 8).



27 **28** **29** **30**

100%
99% (-)

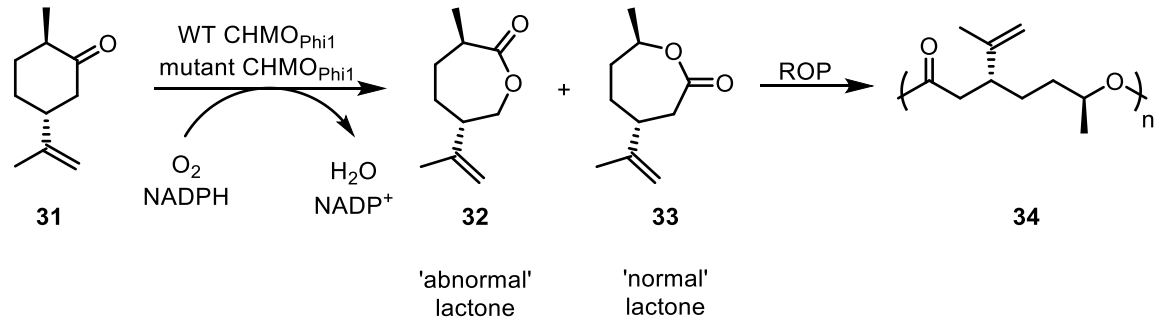
100%

Scheme 8: Representative Baeyer-Villiger oxidations of bridged c-ketones (**27**,**29**) catalysed by CHMOs from *Rhodococcus*.[56]

The ability to resolve *N*-protected β-amino ketones was investigated using the two *c*-hexanone monooxygenases from *Rhodococcus* amongst other bacterial BVMO.[57] CHMO$_{Rhodo1+2}$ both showed the ability to resolve linear(-branched) aliphatic and aryl-aliphatic 4-amino-2-ketones with a strong preference for middle-chain 4-amino-2-

ketones. Longer substrates (C12) were, however, not converted. No activity was detected for 5-amino-3-ketones. Next to the more intensively studied *c*-hexanone monooxygenases from *Rhodococcus* ($CHMO_{Rhodo1+2}$), several novel BVMOs have been identified in various strains. Increasing the number of BVMOs leads to an expansion of the application potential of this useful enzyme class.

Through a kinetic resolution of 2-(3-penten-1-yl)-*c*-hexanone catalysed by a *c*-dodecanone monooxygenase from *R. ruber* SC1 (CDMO)[58] the formation of a homologue of the jasmine lactone was achieved with excellent stereoselectivity.[59] Preparation of the jasmine lactone, a desired compound in fragrance industry, was accomplished with a CHMO from *Arthrobacter* in the same study. The species *R. jostii* RHA1 is particularly known for a high abundance of oxidative enzymes[60,61] which gave rise to a comprehensive investigation of the presence of BVMOs in this strain.[62] Following a genome mining approach, a total number of 22 novel BVMOs was identified, expressed and tested on a diverse set of 39 substrates ranging from linear and cyclic aliphatic ketones to aromatic amines, sulphides and ketones. Here, especially six of the identified BVMOs stood out due to their high substrate promiscuity converting at least 10 and up to 29 of the tested substrates. Furthermore, the same microorganism was shown to also host eight new flavin-containing monooxygenases (FMOs) three of which were successfully applied in Baeyer-Villiger oxidations.[63] Interestingly, the novel enzymes did not favour a specific coenzyme (NADH or NADPH) and their potential as useful biocatalyst was shown by successful conversions of both an aromatic and a bicyclic ketone. A novel CHMO from *Rhodococcus* sp. Phi1 ($CHMO_{Phi1}$) was used in an attempt to chemo-enzymatically produce lactone monomer dihydrocarvide (**32**/**33**) from monoterpenoid starting materials (**31**).[64] A subsequent metal-assisted ring opening polymerisation (ROP) led to the generation of polydihydrocarvide (**34**), a polymer used as thermoplastic elastomer. Depending on whether the wild-type enzyme or a triple mutant was employed, the synthesis of so-called abnormal (**32**) and normal lactone (**33**) were favoured, respectively (Scheme 9).



Scheme 9: 'Semisynthetic' production of polydihydrocarvide (**32**/**33**) using a triple mutant of $CHMO_{Phi1}$.[64]

In a similar fashion, a *c*-pentadecanone monooxygenase from *Pseudomonas* sp. HI-70 led to the formation of polymenthide in the same study. Lastly, a novel BVMO from *R. pyridinivorans* DSM 44555 with extraordinary resistance towards high substrate loading and good stability was shown to convert a number of linear aliphatic ketones.[65] Both 2- and 3-ketones and respective derivatives were converted. Particularly interesting was the production of 3-acetoxypropionate from methyl levulinate with a space-time-yield (STY) of 5.4 g/L per day thereby more than doubling the highest STY reported thus far.

2.2.3.1.3 Styrene and indole monooxygenases

While the BVMOs are built up by a single-component, the styrene (SMO) and indole monooxygenases (IMO) form their own subgroup within the two-component flavin-dependent monooxygenases.[66] Reduced FAD which binds in the active site of the monooxygenase, is used for the activation of molecular oxygen and is delivered by a NAD(P)H-dependent flavin reductase. While SMOs consist of a monooxygenase (StyA) and a reductase component (StyB), IMOs can either be built up in the same manner (two-component system with monooxygenase 'IndA' and reductases 'IndB') or as a self-sufficient fusion protein (ImoA2B) associated with an additional monooxygenase (IndA1).

Styrene monooxygenases catalyse the conversion of styrene and its derivatives and also showed activity against aryl alkyl sulfides. The epoxidation reaction as well as the sulfoxidation reaction solely yielded the respective (*S*)-enantiomers using rhodococcal SMOs with different regeneration systems.[67-70] Furthermore, the SMO from *R*. sp. ST-10 was used to convert aliphatic alkenes including terminal, internal, unfunctionalised

as well as di- and tri-substituted alkenes thereby generating (*S*)-epoxyalkanes.[71] The same gene was overexpressed in *Kocuria rhizophila* DC2201, a strain with exceptionally high tolerance against organic solvents.[72] This led to an increased conversion yield, thereby making this system a suitable biocatalyst for the environmentally milder production of (*S*)-epoxyalkanes in high purity.

Indole monooxygenases (IndA1 and IndA2B-systems) from *Rhodococcus* species also act on styrene derivatives and catalyses epoxidation and sulfoxidation reactions.[67,73,74] Additionally, SMOs and IMOs were shown to produce indigoid dyes without the formation of byproducts like indirubin.[66,75]

2.2.3.2 Dioxygenases

Aromatic dioxygenases carry out the *cis*-dihydroxylation to arene substrates thereby generating valuable vicinal *cis*-dihydrodiols. The aromatic ringhydroxylating dioxygenases are non-haem iron-dependent enzymes containing a mononuclear iron-active site and a Rieske type [FeS] cluster for electron transfer. These enzymes also require a ferredoxin and a flavin containing reductase that reacts with NADH. To date, mainly toluene and naphthalene dioxygenases from *Pseudomonas putida* (mutants) have been exploited, but next to those also naphthalene dioxygenases[76] and novel *o*-xylene degrading *Rhodococcus* strains were discovered.[77]

In particular, an increased interest on the isolation of the strain *R*. sp. DK17 arose when it was shown to replicate on *o*-xylene and utilise several aromatic compounds (benzene, alkylbenzene, phenol, phthalate) thereby displaying its high degradation potential.[11] Until now, subsequent studies primarily dealt with the deeper understanding of its unique reaction mechanism including the point of initial attack in the arene substrate.[77] In the course of this in-depth investigation, several substrates were already shown to be modified by either the wild-type or engineered enzyme.[78] Amongst others, the *o*-xylene-3,4-dioxygenase from *R*. sp. DK17 showed for example activity against *m*- and *p*-xylene[79] as well as larger substrates such as naphthalene, indan, tetralin and indene whereby in all cases the respective *cis*-dihydrodiols were generated.[80-82]

R. sp. I24 was found to be a strain that oxidises indene *via* three different enzyme activities: next to a monooxygenase and a dioxygenase both inducible with naphthalene, a toluene inducible dioxygenase is present.[83,84] Great attention has been paid to the bioconversion of indene to *cis*-(1*S*,2*R*)-indandiol which is a known precursor for (-)-*cis*-(1*R*,2*R*)-1-aminoindan-2-ol, a key chiral synthon for the HIV protease inhibitor Crixivan. Whole-cell experiments with *R*. sp. I24 carried out in a batch- or fed-batch manner struggled with low yields due to the numerous side-reactions catalysed by the other oxygenases present.[83,85] Therefore the toluene inducible dioxygenase (TID) was heterologously expressed in *E. coli* and investigated.[86] The desired *cis*-(1*S*,2*R*)-indandiol was produced with an enantiomeric excess of 45.2% over *cis*-(1*R*,1*R*)-indandiol.

Several other *Rhodococcus* species have been investigated for their degradation potential towards the group of BTEXS (benzene, toluene, ethylbenzene, xylene isomers, styrene) aromatics. For example, the conversion of benzoate was catalysed by a benzoate dioxygenase with a narrow substrate scope from *R. opacus* 1CP.[87] 2,3-dihydroxybiphenyl-1,2-dioxygenases from *Rhodococcus* have been recombinantly expressed showing activity towards a number of catechols with 2,3-dihydroxybiphenyl being the best accepted substrate[88,89] and several catechol-1,2-dioxygenases were shown to cleave (alkyl-substituted and halogenated) catechols.[90,91]

However, to the best of our knowledge, their use as biocatalysts in synthetic chemistry has yet been limited and not been exploited to its full potential.

2.2.4 Miscellaneous oxidation potential

As already mentioned, *Rhodococcus* strains show impressive degradation behaviour towards a multitude of compounds. Several monooxygenases present in these biodegradation pathways have been identified and implemented in biocatalytic applications. To further showcast the hydroxylation potential of enzymes isolated from *Rhodococcus* as well as whole-cells, several examples are discussed.

With the responsible enzymes staying elusive in some cases, a number of different terpenoids were described to be transformed by whole-cells of *Rhodococcus*. As

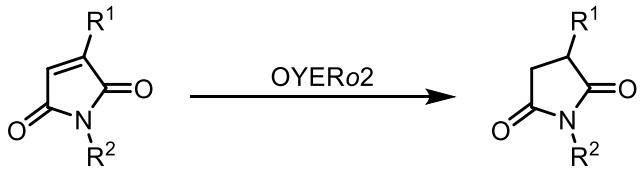
example, **D**-limonene was oxidised to (+)-*trans*-carveol by *R. opacus* PWD4[92] and β-myrcene to geraniol.[93]

A 3-ketosteroid-9α-hydroxylase was identified in *R. erythropolis* SQ1 which the 9α-hydroxylation of compounds 4-androstene-3,17-dione and 1,4-androstadiene,3-17-dione.[94] *R. equi* ZMU-LK19 was applied in the asymmetric hydroxylation and diastereoselective oxidation of (+)-2-substituted tetrahydroquinolines generating chiral 2-substituted-1,2,3,4-tetrahydroquinoline-4-ols and chiral 2-substituted-2,3-dihydroquinolin-4(1H)-ones.[95]

2.2.5 C=C-bond reductases

The selective reduction of C=C-double bonds, especially in α,β-unsaturated carbonyl compounds, is seen as valuable reaction in the production of chiral building blocks and is catalysed by flavin-dependent ene-reductases (EREDs, EC 1.3.1.31) or also called Old Yellow Enzymes (OYE).[96] Like many other reported organisms, also *Rhodococcus* showed reduction-potential towards a diverse number of substrates. The reactivity towards seven chalcone-derivatives was screened for using *R*. sp. DSM 364 amongst other microorganisms.[97] Whole-cells of *R*. sp. DSM 364 catalysed the reduction of all seven substrates, including all derivatives with both electron withdrawing and electron donating groups, exclusively to the respective dihydrochalcones while leaving the carbonyl moiety unreacted. Several *Rhodococcus* strains (*R. erythropolis* and *R. rhodochrous*) were used in an investigation on their reduction potential towards activated ketones, an aldehyde, an imide and nitro-compounds.[98] Based on the conversion of ketoisopherone to levodione, stereoselectivity studies have been performed: all so far reported ene-reductases found in plants, yeasts, bacteria and parasites only gave access to the (*R*)-configurated product. However, in this study, whole-cell bioconversions of six out of seven strains led to the formation of (*S*)-product. The same reaction was carried out with purified ene-reductases which led to the (*R*)-product. It was therefore proposed that the whole-cells produce a mixture of both (*R*)- and (*S*)-levodione from which only the (*R*)-enantiomer is further converted by the other enzymes present in the whole-cell mixture leaving the (*S*)-levodione as main product unreacted.[98] A (*R*)-selective 'thermophilic-like' ene-reductase from *R. opacus* 1CP obtained by genome mining was

heterologously expressed in *E. coli* and subsequently characterised.[99] Based on sequence similarity, this enzyme was categorised as a member of the 'thermophilic-like' (YqjM-like) OYE group, but it only showed a temperature optimum of 37 °C instead of higher temperatures of ≥ 70 °C which are usually described for thermophilic enzymes. It showed the highest activities towards (substituted) maleimides (**35**) leading to the corresponding succinates (**36**) (Scheme 10).



35a-d **36a-d**

R^1 = H, R^2 = H	100%
R^1 = H, R^2 = Methyl	100%
R^1 = H, R^2 = Ethyl	100%
R^1 = Methyl, R^2 = Phenyl	> 99%, > 99% *ee*

Scheme 10: Ene-reductase catalysed reduction of α,β-unsaturated maleimides (**35**).[99]

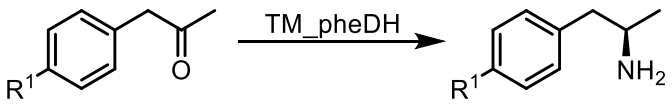
2.2.6 Amino acid and amine dehydrogenase

Amino acid (AADH, EC 1.4.1.x) and amine dehydrogenases (AmDH) catalyse the reductive amination of α-keto acids and ketones yielding α-amino acids and amines, respectively, using NAD(P)H as cofactor. Ammonia is mostly chosen for as nitrogen source.

R. sp. M4 hosts a phenylalanine dehydrogenase (PheDH) which primarily converts phenylpyruvate to **L**-phenylalanine *via* a reductive amination process[97]. Additionally, the enzyme was also shown to accept other (sterically demanding) α-keto acids such as 4-(methylsulfanyl)-2-oxobutanoic acid, 2-oxo-4-phenylbutanoate or 2-oxo-5-phenylpentanoate making it interesting for its broad substrate tolerance.[100,101]

The amine dehydrogenases (AmDH) are a recently developed group of enzymes that derive from amino acid dehydrogenases.[102] They act on prochiral ketones opening up a new synthetic route towards chiral amines. A new (*R*)-selective AmDH (TM_pheDH) was engineered from the *Rhodococcus* phenylalanine dehydrogenase by directed evolution.[103] With this new enzyme, it was possible to reduce phenylacetone (**37a**)

and 4-phenyl-2-butanone leading to (*R*)-amphetamine (**38a**) and (*R*)-1-methyl-3-phenylpropylamine with excellent *ee* values (> 98%). The immobilisation of this AmDH on magnetic nanoparticels (MNP) increased the productivity and stability compared to the free enzyme.[104] The enzyme was also found to be active towards *o*-methoxyphenylacetone derivatives (**37b**), aliphatic ketones and so-called 'bulky-bulky' ketones such as 1-phenylbutan-2-one or 1-phenylpentan-3-one (Scheme 11).[105]



37a-b **38a-b**

R^1 = H, OMe

Scheme 11: Reductive amination of representative ketones catalysed by AmDH engineered from *R*. sp. M4.[103,105]

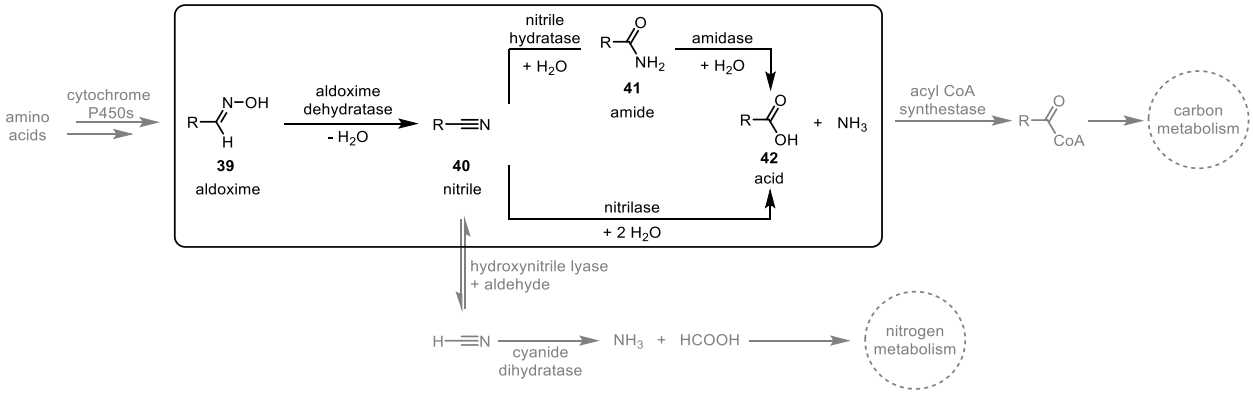
2.2.7 Desaturase

A Δ6-desaturase from a *R*. sp. KSM-B-MT66 mutant was applied in a two-phase system to catalyse a *cis*-desaturation of low-cost saturated starting material.[106] Next to unsaturated acyl fatty acids, chloroalkanes and simple alkanes were accepted and the dehydrogenation always took place 9-C-atoms away from the terminal methyl group. This reaction was used to generate intermediates for the preparation of substituted fatty acids used in the dermatological pharmacy and was run with a space-time-yield of 16.8 g/L per day. Recently, novel Δ6-desaturase enzymes were identified in *R*. sp. and their use to produce *cis*-6-hexadecenoic acid were patented for future applications.[107,108]

3. Enzymes from the aldoxime-nitrile pathway

Enzymes present in the aldoxime-nitrile pathway catalyse both the synthesis and decomposition of nitrogen-containing organic compounds thereby playing a key role in the carbon and nitrogen metabolism of microbes and plants (Scheme 12).[109-111] Through oxidation and decarboxylation of amino acids, aldoximes (**39**) are generated[112,113] which are subsequently dehydrated to give nitriles (**40**). These nitriles can either undergo a hydroxynitrile lyase-catalysed decomposition reaction yielding hydrogen cyanide and aldehydes or they can be converted to carboxylic acids (**42**) *via*

two possible routes: a one-step reaction catalysed by nitrilases or *via* an amide intermediate (**41**) catalysed by a coupled nitrile hydratase and amidase system.[109,110] The resulting acids and ammonia are afterwards consumed in the carbon and/or nitrogen metabolism.



Scheme 12: General aldoxime-nitrile pathway in microbes with synthetically used enzymes from *Rhodococcus* highlighted.[109]

Various *Rhodococcus* species use the aldoxime-nitrile pathway and therefore the respective enzymes (aldoxime dehydratase, nitrilase, nitrile hydratase and amidase) are present in many strains.[111,114] The versatile use of these enzymes in synthetic organic chemistry from a laboratory 'proof-of-principle' to multi-ton scale for industrial applications showcases the strength of *Rhodococcus* as biocatalyst in this area.

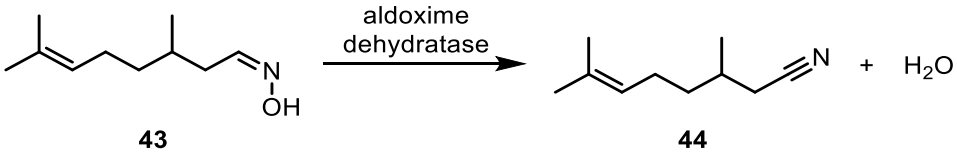
2.3.1 Aldoxime dehydratase

Nitriles are valuable starting points in the synthesis of both bulk chemicals and chiral pharmaceuticals.[96,112] Their synthesis, however, either requires high temperatures (ammoxidation) or the use of highly toxic hydrogen cyanide as reagent. Aldoxime dehydratases (Oxd, EC 4.99.1.5) are a recently discovered group of enzymes found in bacteria.[115,116] They offer an environmentally friendly, cyanide-free alternative to produce nitriles starting from easily accessible aldoximes which can be produced by a condensation reaction of aldehydes with hydroxylamine.[117]

Several studies examined the substrate scope of aldoxime dehydratases from *Rhodococcus* for non-chiral aldoximes[116, 118-120]: while arylaliphatic aldoximes were generally better converted by other organisms[121], strain *Rhodococcus* sp. YH3-3 was the only organism showing activity against substituted aromatic and furan-derived

aldoximes. It also displayed higher activities towards a number of substrates with heteroaromatic moieties compared to other Oxds thereby showing unique properties[112,116]. Linear and branched aliphatic aldoximes with a chain length of C2 to C6 are accepted and converted to their respective nitrile by a number of ROxd.[116,118,119] A comprehensive study compared the activity of five heterologously expressed aldoxime dehydratases of which two originate from *Rhodococcus* strains (*R. globerolus* A-4, OxdRG; *R*. sp. N-771, OxdRE) towards chiral aldoximes including arylaliphatic, heteroaromatic aliphatic, cyclic aliphatic and acyclic long-chain aliphatic aldoximes with an particular interest in the stereochemical course of the reaction.[120] Interestingly, in some cases it was shown that depending on the choice of isomeric structure of the substrate (*E*- or *Z*-aldoxime), the enantiopreference in the final nitrile (*R*- or *S*-configuration) could be influenced.

A recent example displaying the growing industrial importance of this enzyme class can be seen in a filed patent from BASF which describes the conversion of a number of terpenes - important odoriferous compounds in the fragrance industry. One example is the biocatalytic production of citronellyl nitrile (**44**) which is known to have a rose-like fragrance (Scheme 13).[122]



43
citronellal oxime

44
citronellyl nitrile

Scheme 13: Patented biocatalytic citronellyl nitrile (**44**) production by dehydration of citronellal oxime (**43**) catalysed by aldoxime dehydratase.[122]

2.3.2 Nitrile hydratase

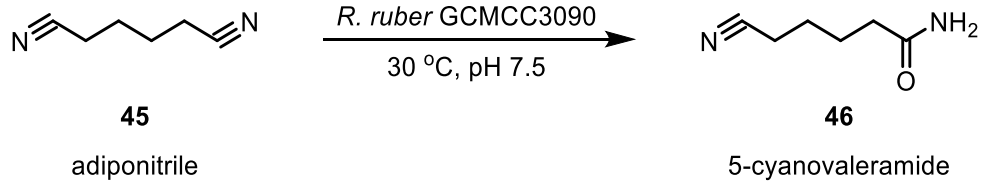
While aldoxime dehydratases form valuable nitriles, nitrile hydratases (NHase, EC 4.2.1.84), which are mononuclear iron- or cobalt-dependent enzymes, belong to the group of nitrile-degrading enzymes yielding amides through hydration reactions. This reaction is often followed by an amidase-catalysed hydrolytic step when applying *Rhodococcus* whole-cells which converts the formed amides to their respective

carboxylic acids. This section will summarise both the use of NHase as single catalyst and systems using both enzymes as a coupled two-step system.

2.3.2.1 NHase as single biocatalyst

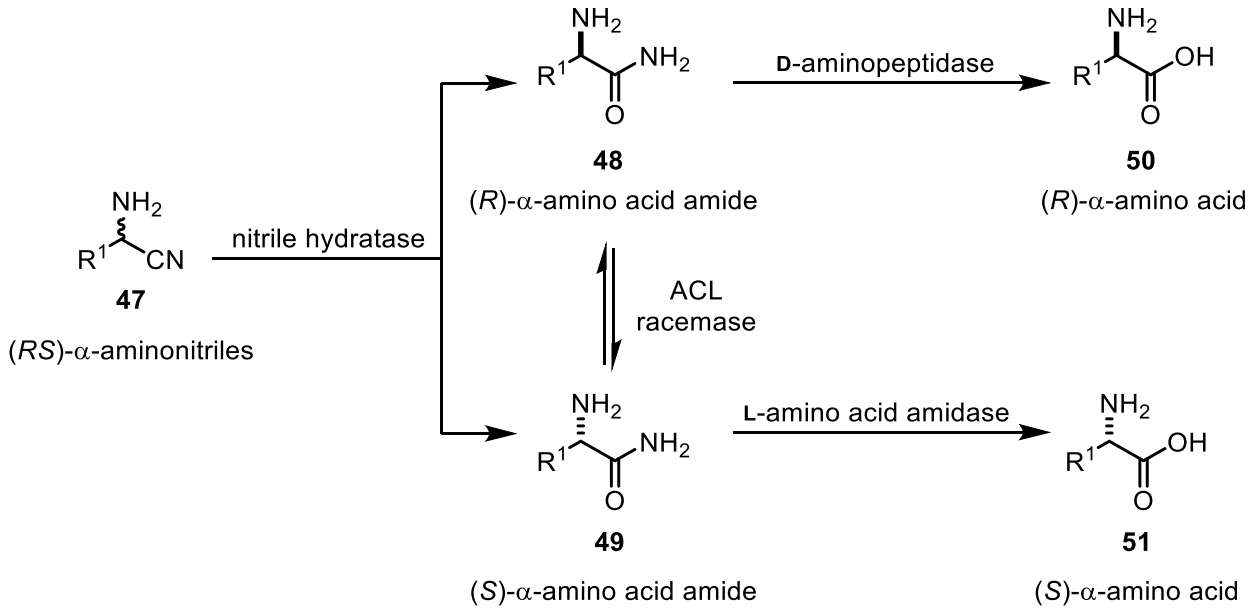
The production of amides from nitriles has become crucial to industry and therefore the commercial interest in nitrile-degrading enzymes has gained immense attention as many excellent reviews display.[109,114,123,124] *Rhodococcus* strains are industrially used to prepare amides essential for humankind such as acrylamide (*R. rhodochrous* J1, > 400000 t/a, Nitto Chemical Industry[5,6]) or nicotinamide (*R. rhodochrous* J1, > 11500 t/a, Lonza AG[45,125]). In this case, the nitrile-hydratase of industrially important strain *R. rhodochrous* J1 was shown to be cobalt-dependent.[126] Main products acrylamide and its polymers are used as coagulators in the leather and textile industry while nicotinamide is one of the two forms of vitamin B3 which is used in cosmetic industry and animal feed supplementation.[109,127]

NHases are versatile enzymes that accept a broad range of different nitriles. The hydrolysis of aromatic and arylalkyl nitriles was intensively studied and proven successful for *e.g.* pyridyl-, pyrazinyl-, (substituted) benzyl-, furyl- and thionyl-moieties[128-133] as well as trans-2,3-epoxy-3-aryl-propannitriles[134] or *rac*-mandelonitrile.[135] *R. boritolerans* FW815 was shown to have a strong 2,2-dimethyl-c-propanecarbonitrile (DMCPCN) hydratase activity in absence of amidase activity leading to an enrichment of 2,2-dimethyl-c-propanecarboxamide (DMCPCA) – an important precursor for the drug cilastatin which is an inhibitor of a renal peptidase that is involved in the metabolism of other drugs thereby making these other, combined drugs more effective.[136] Dinitriles are also accepted substrates: whole-cells of *Rhodococcus* sp. were shown to convert fluorinated aromatic dinitriles[137] and *R. rhodochrous* IFO 15564 was active towards alicyclic mono- and dinitriles affording the products in low to moderate yields.[138] Resting cells of *R. ruber* CGMCC3090 converted the aliphatic adiponitrile to selectively give 5-cyanovaleramide (5-CVAM) which is used in the synthesis of caprolactam, a common precursor for Nylon 6 (Scheme 14).[139]



Scheme 14: Nitrile hydratase catalysed bioconversion of adiponitrile (**45**) yielding 5-cyanovaleramide (**46**) using whole-cells of *R. ruber* GCMCC3090.[139]

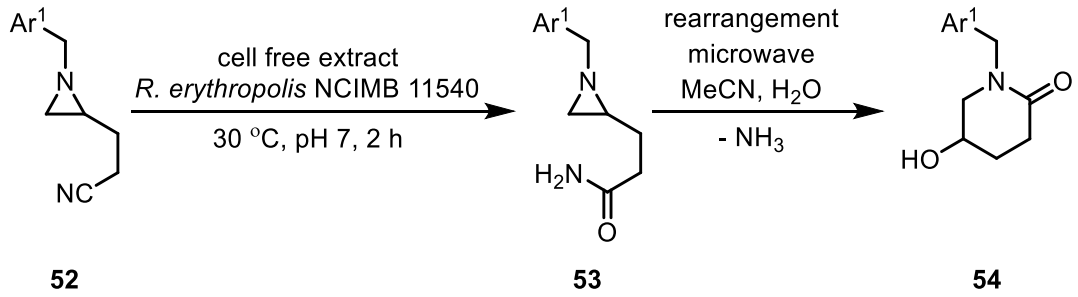
Prochiral substrates such as α- or β-substituted nitriles were used in bioconversions as well: starting from α-racemic aminonitriles (**47**), respective (*R*)-(**50**) and (*S*)-selective α-amino acids (**51**) were produced each using a three-enzyme cascade reaction.[140] In both reaction pathways, a NHase from *R. opacus* 71D was first applied to give a racemic mixture of α-amino acid amides (**48**/**49**). A dynamic kinetic resolution catalysed by ACL racemase and subsequently either **D**-aminopeptidase ((*R*)-amino acid (**50**)) or **L**-amino acid amidase ((*S*)-amino acid (**51**)) yielded the final products (Scheme 15).



Scheme 15: Dynamic kinetic resolution of rac-aminonitriles to produce chiral α-amino acids (**50**,**51**). The first step is catalysed by a nitrile hydratase from *R. opacus* 71D.[140]

On the other hand, a partially purified NHase was successfully applied in the conversion of β-substituted nitriles such as 3-oxonitriles, 3-hydroxynitriles and 3-(acyloxy)nitrile yielding the corresponding amides in moderate to good yields.[141] Whole-cells of *R. erythropolis* NCIMB 11540 on the other hand were used in the

synthesis of piperidin-2-ones (**54**) starting with a NHase-catalysed hydration reaction of 1-(arylmethyl)-2-(2-cyanoethyl)aziridines (**52**) (Scheme 16).[142]

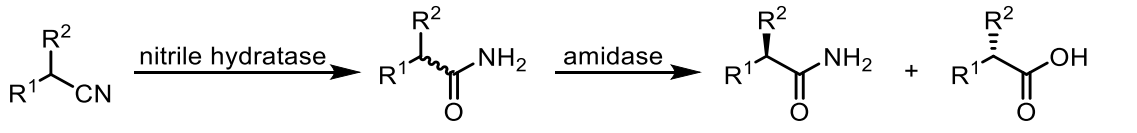


52 **53** **54**

Scheme 16: Chemo-enzymatic cascade reaction to synthesise piperidin-2-ones (**54**). Enzymatically catalysed step is performed by soluble nitrile hydratase from *R. erythropolis* NCIMB 11540.[142]

2.3.2.2 Two-enzyme systems

Most of the *Rhodococcus* strains used in these bioconversions express both NHase and amidase which can lead to the direct follow-up reaction catalysed by amidases yielding valuable carboxylic acids. Nitrile hydratases generally show low stereoselectivities. The amidase hydrolysis reaction, however, is usually very stereospecific leading to the (*S*)-carboxylic acid (**58**) and leaving the unreacted (*R*)-amide (**57**) behind (Scheme 17).[143,144] Many examples successfully used the presence of both enzymes to achieve the production of both selective amides and carboxylic acids by kinetic resolution.



55 nitrile **56** *rac*-amide **57** (*R*)-amide **58** (*S*)-carboxylic acid

Scheme 17: General scheme of NHase/amidase catalysed reaction.

When starting with prochiral α-substituted nitriles, the synthesis of optically active (*R*)-amides and (*S*)-carboxylic acids was achieved. Several 2-aryl-alkylnitriles were accepted substrates[145,146] as was *rac*-naproxen nitrile which directly formed the

desired and biologically active drug (*S*)-naproxen.[147] Also, enantioenriched 3-aryl-3-hydroxy-2-methylene carboxylic acids and amides were prepared from the respective nitriles using whole-cells of *R. rhodochrous* AJ270.[148]

Multiple examples of dinitriles were applied as well: incubation of *R. erythropolis* AJ270 cells with *meso-c*-pentane-1,3-dinitriles yielded optically active amide-products in high yields.[149] *Via* an amidase-catalysed kinetic resolution step and following treatment with CH_2N_2, monocyano amides were resolved into (-)-amide and (-)-ester. Additionally, it was shown that the amidase also catalyses the desymmetrisation of *meso-c*-pentane-1,3-dicarboxamides affording enantiopure pentanecarboxylic acids.

Malononitriles are α,α-substituted dinitriles which form malonic diamides and malonic acid monoamides upon incubation with whole-cells of *R. rhodochrous* IFO 15564.[150] Next to that, cyanohydrins (α-hydroxy nitriles) were also successfully hydrolysed leading to enantiopure α-hydroxy carboxylic acids like *e.g.* the pharmaceutical intermediate (*R*)-chloromandelic acid on gram-scale[151] as was a large variety of aminonitriles ranging from α-aryl-, α-alkyl-substituted glycine nitriles[152-154] to aziridine-2-carbonitriles[154], azetidine-2-carbonitriles and 4-oxoazetidine-2-carbonitriles.[156,157]

Besides α-substituted nitriles, also β-substituted nitriles are suitable substrates for the NHase/amidase system yielding interesting building blocks with more remote stereogenic centres. Next to several studies focusing on 3-substituted glutaronitrile derivatives,[158-160] 3-hydroxy-arylpropanenitriles,[161] 3-hydroxy-4-aryloxybutanenitriles[161,162] and 3-(benzoyloxy)pentanenitrile[163] were also converted using *Rhodococcus* cells. A number of *Rhodococcus* strains was also shown to be active on 3-benzyl-oxy- and 3-benzoyloxypentanedinitriles[88,159-161,164] which opens up a new synthetic route to the drug Atorvastatin (trade name Lipitor by Pfizer), an HMG-CoA reductase inhibitor for lowering blood cholesterol.[165]

The above mentioned examples all successfully exploit the presence of both enzymes to obtain amides and carboxylic acids. However, the amidase also causes unwanted side-product formation in other reactions when the intermediate amide is the desired final product. To avoid the amidase-catalysed follow-up reaction in the preparation of acrylamide, an amidase-negative mutant was designed using a genetic knock-out

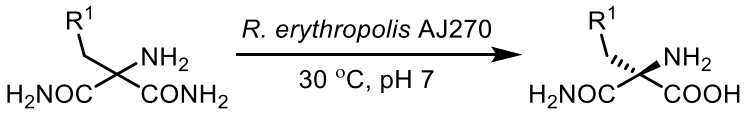
technique. This approach increased the acrylamide yield by 25 % compared to the wild-type strain.[166]

2.3.3 Amidase

Amidases (EC 3.5.1.4) belong to the group of amidohydrolases and have been widely researched due to the biological relevance of amides in nature. They generally show a broad substrate scope and good enantioselectivity.[167] The examples discussed in the above chapter already display the versatility of *Rhodococcus* amidases and their synthetic potential in combination with nitrile hydratases. However, this chapter highlights specific examples achieved with single-amidase systems.

An amidase from *R*. sp. MTB5 showed good activity towards a large number of aliphatic, aromatic and heterocyclic amides.[168] Diamides and specific amino acids were, however, only poorly accepted. α-alkyl phenylacetamides have been kinetically resolved using an amidase present in *R*. sp. AJ270.[169] (*S*)-naproxen can be produced starting from both racemic naproxen nitrile using the two-enzyme system[147] as well as from racemic naproxen amide using cells of *R. erythropolis* MP50.[170] In a similar manner, *R. erythropolis* AJ270 catalysed the enantioselective hydrolysis of racemic ibuprofen amide leading to the biologically active compound.[171]

In the production of (*R*)-malonamic acids, amidases from *Rhodococcus* have proven to be successful tools. Whole-cells of *R*. sp. CGMCC 0497 converted aromatic α,α-disubstituted malonamides with substituents in *ortho*-, *meta*- and *para*-position with high yields and excellent enantioselectivities as well as dialkyl-substituents with slightly lower *ee*-values.[172] The amidase present in whole-cells of *R. erythropolis* AJ270 transformed a number of prochiral malonamides yielding a range of carbamoylacetic acid products.[173] α-substituted α-amino-malonamides (**59**) were transformed to highly functionalised tetrasubstituted α-amino acids (**60**) with high yields with excellent *ee*-values by the same organism (Scheme 18).[174]

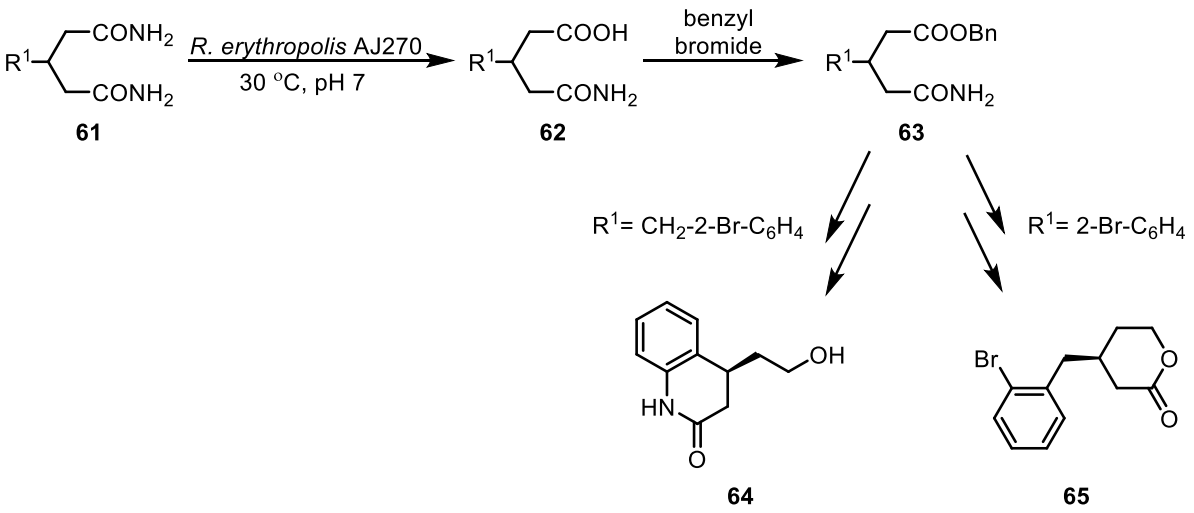


59 **60**

R^1 = alkyl, alkenyl, Ph

Scheme 18: Synthesis of enantioenriched functionalised α-tetrasubstituted α-amino acids (**60**) through amidase-catalysed hydrolysis.[174]

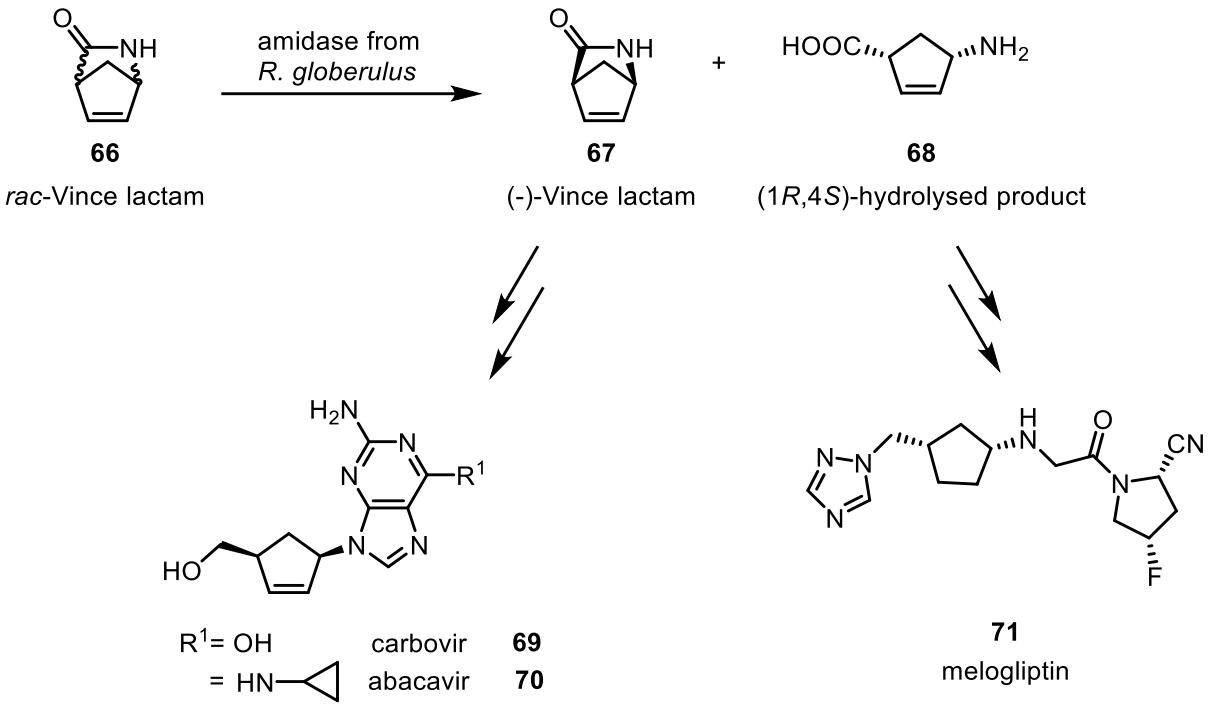
Interestingly, this organism was also used to catalyse the desymmetrisation of both five-membered *meso*-*N*-heterocyclic- and *meso*-*c*-pentane dicarboxamides to afford functionalised enantiopure derivatives of pyrrolidine, dihydropyrrole and piperidine as well as *c*-pentanecarboxylic acids.[175,176] The same amidase was reported to also hydrolyse a palette of prochiral 3-aryl- and 3-arylmethyl glutaramides (**61**) leading to highly enantioselective glutaric acid monoamide derivatives (**62**).[177] This amidase-catalysed hydrolysis paved the way for a straightforward chemo-enzymatic synthesis of dihydroquinolinone (**64**) and a δ-lactone (**65**) thereby showcasing the many synthetic applications of this biocatalyst (Scheme 19).



Scheme 19: Chemo-enzymatic syntheses of dihydroquinolinone (**64**) and δ-lactone (**65**).[177]

Lactams, cyclic analogues of amides, are generally more stable and their hydrolysis therefore often requires harsher reaction conditions leading to the respective amino acids.[167] Nonetheless, certain amidases i.e. lactamases from *Rhodococcus* were effectively used in the hydrolysis reaction. The hydrolysis of Vince-lactams (**66**) (2-azabicyclo[2.2.1]hept-5-en-3-one) and their derivatives received increased attention due to the fact that the two resulting products are valuable synthons in the pharmaceutical industry for the manufacture of antiviral agents carbovir (**69**) and abacavir (**70**) or for the antidiabetic melogliptin (**71**).[178] *R. equi* NCBI 40312 showed

activity against Vince-lactam[179] also an amidase from *R. globerulus* presented excellent enantioselectivities for the obtained amino acid products (Scheme 20).[180]



Scheme 20: Kinetic resolution of Vince-lactam using an amidase from *R. globerulus* with subsequent follow-up chemistry of enantiopure products leading to antiviral compounds carbovir (**69**) and abacavir (**70**) and antidiabetic melogliptin (**71**).[180]

Strains *R*. sp Oct1 and *R*. sp U224 were recently shown to act on various ω-lactams with the enzyme present in *R*. sp Oct1 having a more relaxed substrate scope while the enzyme from *R*. sp U224 only accepted ω-octo- and ω-laurolactam.[181,182]

2.3.4 Nitrilase

While all above mentioned enzymes from *Rhodococcus* present in the aldoxime-nitrile pathway were intensively studied and applied in many different processes and reactions, nitrilases (EC 3.5.5.1, or nitrile aminohydrolase) from *Rhodococcus* are less prominent and other microorganisms (*Synechocystis*, *Alcaligenes*, *Pseudomonas* etc.) clearly dominate this area of research. However, a small number of reactions were described using *Rhodococcus* whole-cells or purified enzymes.

Bioconversions of acrylonitrile with *R. rhodochrous* J1 yielded acrylic acid with exceptional yields of 390 g/L[183] which was even further improved with a mutant strain of *R. rhodochrous* tg1-A6 leading to 414.5 g/L.[184]

The production of ammonium acrylate by two *Rhodococcus* isolates was further investigated and compared.[185] While *R. erythropolis* 3843 expressed a nitrile hydratase/amidase system, *R. ruber* NCIMB 40757 expressed a nitrilase. In this comparative study, the single-enzyme nitrilase-system was superior to the two-enzyme carboxylic acid production due to favourable reaction kinetics and because the NHase and amidase do not share the same temperature optimum.

R. rhodochrous PA-34 was shown to hydrolyse α-aminonitriles to obtain optically active **L**-amino acids (amongst others **L**-leucine, **L**-valine and **L**-methionine) as well as **D**-alanine.[186] Furthermore, strains *R. rhodochrous* J1 and R. sp. NDB 1165 were used to generate nicotinic acid, also called niacin, starting from 3-cyanopyridine with yields of 172 and 196.8 g/L, respectively.[187,188] Nicotinic acid is, next to nicotinamide, a form of vitamin B3 and its synthesis is therefore commercially interesting.

It is worth mentioning that even though only a small number of nitrilase-catalysed reactions with *Rhodococcus* were described, this nitrile-degrading enzyme class in general is well-studied and applied in a multitude of reactions which can be followed-up on in several comprehensive reviews and books.[114,189-191]

2.4 Hydrolase activity in *Rhodococcus*

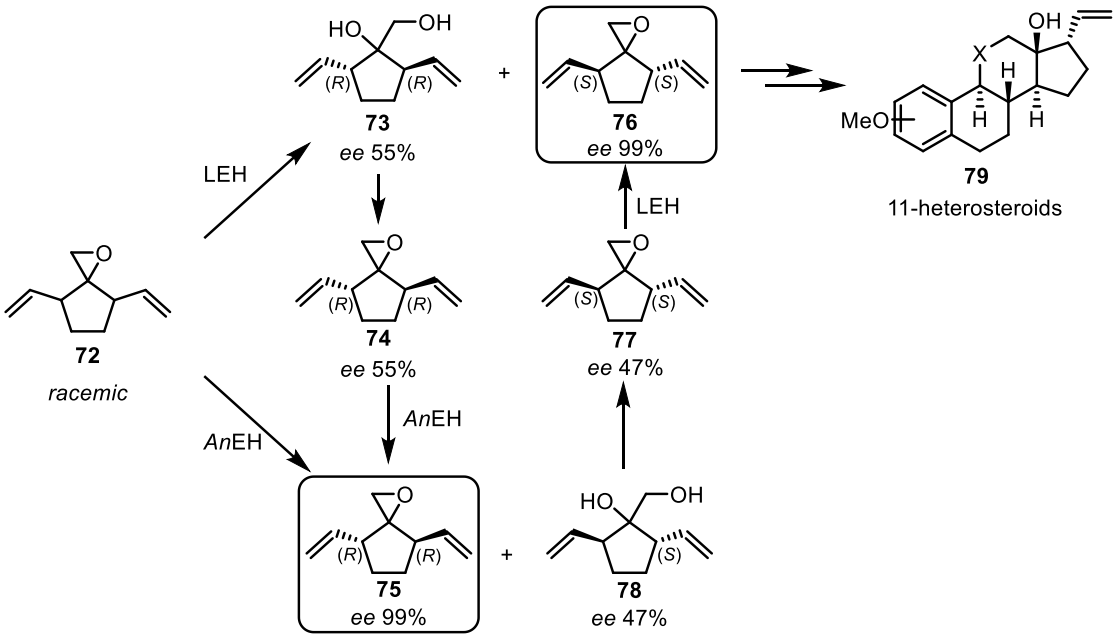
2.4.1 Epoxide hydrolases (EC 3.3.2.x)

Epoxide hydrolases (EHs) catalyse the ring-opening reaction of oxirane rings leading to vicinal diols and enantiopure epoxides as final products. Most valuable are EHs that either show high enantioselectivities or a low enantiopreference with high levels of enantioconvergence. Many studies focused on the asymmetric hydrolysis of geminal (2,2-disubstituted) oxiranes using *Rhododoccus* as biocatalyst whereby all investigated EHs hydrolysed the (*S*)-enantiomer forming an (*S*)-diol and leaving the (*R*)-epoxide behind.[192] The investigated substrate scope includes linear alkyl-, alkenyl-, alkynyl- or benzylsubstituted epoxides. While *R. ruber* DSM 43338 acted on 2-methyl-2-(aryl)alkoxyoxiranes,[193] *R*. sp. NCIMB 11216 was shown to convert (±)-2-

methyl-2-alkyl-epoxides.[194] Oxiranes bearing an alkene or alkyne function were particularly well transformed using the same strain.[195] Additionally, two *Rhodococcus* strains isolated from marine sediments were used in the hydrolysis of styrene oxide and its chlorinated derivatives.[196]

R. erythropolis DCL14 bears an epoxide hydrolase that holds a particular position within the family of EHs. While most of the described EHs belong to the α,β-hydrolase fold superfamily, the so-called 'limonene-1,2-epoxide hydrolase' (LEH) shows a 3-dimensional structure that is dissimilar to the others.[197] Consequently, it follows a different reaction mechanism which was proposed as a single step 'push-pull' mechanism rather than a multiple-step mechanism described for members of the α,β-hydrolase fold superfamily.[192] Initial investigations showed a narrow substrate range for the wild-type enzyme. Only the natural substrate limonene-1,2-epoxide was converted with excellent stereoselectivity while several alicyclic and few 2-methyl-1,2-epoxides showed only reasonable stereoselectivities.[198,199] With an iterative saturation mutagenesis strategy, three LEH variants were developed with an expanded substrate range and opposite stereoselectivities making this enzyme more attractive for future applications.[200]

A preparative hydrolytic kinetic resolution of *rac-trans*-spiroepoxides was first achieved with EHs from *Aspergillus niger* (*An*EH) and wild-type LEH from *R. erythropolis* DCL14. In the kinetic resolution of *rac-trans*-spiroepoxides (**72**), the two employed EHs exhibit opposite enantioselectivities and by applying both enzymes in a combined two-step process, both enantiomers (**75**,**76**) were generated with excellent enantiomeric excess values and in high yields: the racemic spiroepoxide (**72**) was *e.g.* first hydrolysed by LEH leading to the (*R*),(*R*)-diol (**73**) and (*S*),(*S*)-epoxide (**76**). Through a chemical recyclisation the (*R*),(*R*)-diol (**73**) was transformed into (*R*),(*R*)-epoxide (**74**) keeping the moderate enantiopurity. The spiroepoxide (**74**) was then subsequently resolved by the enantiocomplementary EH from *A. niger*. Enantiopure spiroepoxides are seen as valuable synthons in the synthesis of 11-heterosteroids (Scheme 21).[201]



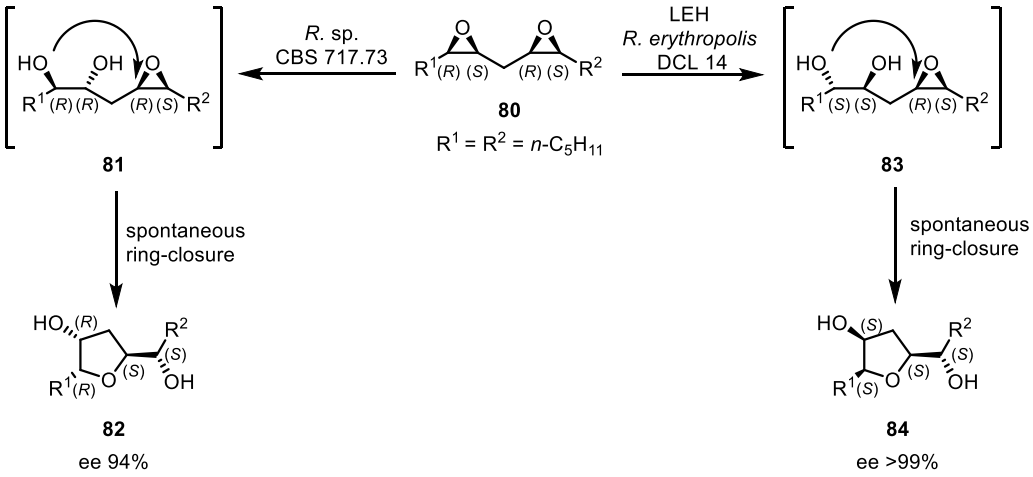
Scheme 21: Optimised two-enzyme system for hydrolytic kinetic resolution of trans-spiroepoxide (**72**) using *An*EH and LEH. Enantiopure spiroepoxides (**75**,**76**) can be used in the synthesis of enantiopure 11-heterosteroids (**79**).[201]

The asymmetric hydrolysis of (±)-2,3-*cis*- and *trans*-disubstituted oxiranes was performed by *Rhodococcus ruber* DSM 43338 with excellent enantioselectivities reaching 95% and 86% *ee*, respectively.[202]

Next to geminal oxiranes, tri-substituted epoxides were successfully converted as well. In an enantioconvergent fashion, whole-cells of *R. ruber* SM 1789 catalysed the hydrolysis of trialkyl-oxiranes achieving high yields of the respective (*R*)-configured vicinal diols with high *ee* values.[203] Expanding the epoxide hydrolase system to substrates bearing olefinic side-chains enabled the straightforward asymmetric synthesis of natural compounds myrcenediol and 7,7-dimethyl-6,8-dioxabicyclo[3.2.1]octane which is known as a volatile contributor to the aroma of beer in a chemoenzymatic reaction procedure.[204] In another enantioconvergent asymmetric synthesis using an EH from *Rhodococcus*, it was possible to synthesise the monoterpenoid coumarin (*R*)-(+)-marmin with 95% *ee* under anaerobic reaction conditions.[205]

The biocatalytic desymmetrisation of *meso*-epoxides with EHs was the focus of other studies: an EH from *R. ruber* ML-0004 was expressed in *E. coli* which showed a

selective activity towards *cis*-epoxysuccinate producing enantiopure **L**-(+)-tartaric acid.[206] An enzyme-triggered hydrolysis-cyclisation reaction was observed when transforming methylene-interrupted meso-*bis*-epoxide (**80**) (6,7:9,10-*bis*(epoxy)pentadecane) with whole-cells of *R*. sp. CBS717.73 (Scheme 22). With this reaction, in total four stereocenters were established in the derived THF-derivative product (**82**).[207] Expansion of this study showed that four different THF-derivatives (representative **82**,**84**) each with excellent *ee* and *de* values were achieved by using EHs from multiple organisms (*e.g*. *Rhodococcus* species, *Solanum tuberosum*, *Aspergillus niger*, *Mycobacterium tuberculosis*). The stereospecificities of the enzymes thereby determined the final stereoselectivities of the products while the cyclisation reaction follows Baldwin's rule without the operation of a cyclase.[208]



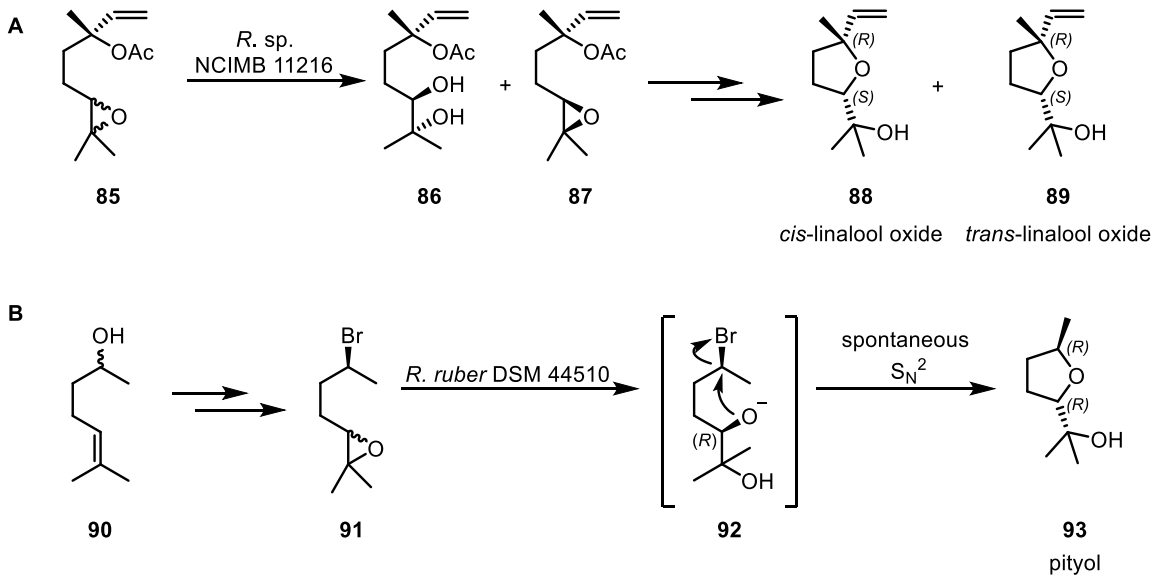
Scheme 22: Stereochemical course of hydrolysis/cyclisation reaction catalysed by two *Rhodococcus* representatives.

The three aforementioned variants of LEH obtained by an iterative saturation mutagenesis approach were applied in the hydrolytic desymmetrisation of several *meso*-epoxides and *cis*-1,2-homodesubstituted *meso*-epoxides forming both the (*R*,*R*)- and (*S*,*S*)-diols with very good *ee* values.[200]

The resolution of *rac*-2-methylglycidyl benzyl ether, a versatile building block, was achieved through a whole-cell bioconversion of *R. ruber* SM 1789 yielding (*R*)-vicinal diol.[209] Following this study, several other strains were tested on the same substrate. While most *Rhodococcus* strains showed (*S*)-selectivity and acted with retention of the stereoselectivity, the limonene epoxide hydrolase from *R. erythropolis* showed an

inversion of the substrate leading to a homochiral mixture.[210] *R. ruber* CBS 717.73 catalysed the hydrolysis of 2-benzyloxymethyl-2-methyloxirane, another protected epoxy-alcohol, and was applied in a chemoenzymatic route to produce (*S*)-chromanemethanol, an important building block in the Vitamin E synthesis, in 26% overall yield and 99% *ee.*[211]

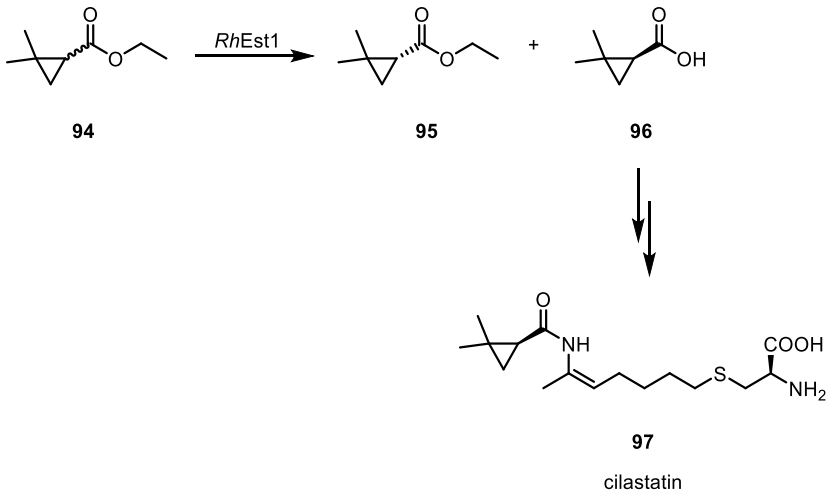
As portrayed, epoxide hydrolases from *Rhodococcus* are biocatalysts forming valuable synthons that can be used in a multitude of follow-up reactions. Next to the described application potential, *Rhodococcus* was also utilised in the production of other natural products such as linalool oxides (**88**,**89**) or pityol (**93**). *Cis*-(**88**) and *trans*-linalool (**89**) oxides were obtained *via* a chemo-enzymatic route starting from (3*RS*,6*R*)-2,3-epoxylinalyl acetate (**85**) with the *R*. sp NCIMB 11216 mediated resolution of the diastereomeric mixture of the starting compound as key step (Scheme 23A).[212] (2*R*,5*R*)-pityol (**93**), a bark beetle pheromone, was synthesised starting from (±)-sulcatol (**90**) *via* an lipase-catalysed deracemisation and a subsequent EH-catalysed diastereoconvergent hydrolysis of a haloalkyl oxirane (**91**) (Scheme 23B).[213]



Scheme 23: Natural product synthesis of (A) *cis*-(88) and *trans*-linalool oxides (89) and (B) pityol (93) using *Rhodococcus* whole-cell biocatalysts.

2.4.2 Esterase activity

Few reports concentrate on the application of other hydrolases from *Rhodococcus*. Amongst them, an esterase from *R*. sp. ECU1013 (RhEst1) was shown to hydrolyse *rac*-ethyl-2,2-dimethyl-*c*-propanecarboxylate (**94**, rac-DMCPCM) to give (*S*)-(+)-2,2-dimethyl-c-propylcarboxylic acid (**96**, (*S*)-DMCPCA) – a valuable precursor in the synthesis of the drug cilastatin (**97**) (Scheme 24).[214,215] This shows an alternative route to cilastatin compared to the previously mentioned nitrile hydratase-catalysed process starting with 2,2-dimethyl-c-propanecarbonitrile.[136]



Scheme 24: Enantioselective resolution of a racemic mixture of DMCPCM (**94**) to yield (*S*)-DMCPCA (**96**) – a precursor for the drug cilastatin (**97**).[214]

The production of both (*S*)- and (*R*)-linalool was achieved with two (partially) purified enzymes ((*S*)- and (*R*)-linalyl acetate hydrolase, respectively) from *R. ruber* DSM 43338 *via* the hydrolysis of the corresponding acetate esters.[216] An urethane hydrolase from *R. equi* TB-60 hydrolysed a diverse range of compounds including anilides, amides and esters such as toluene-2,4-dicarbamic acid butyl ester (TDCB).[217]

2.5 Hydratase activity

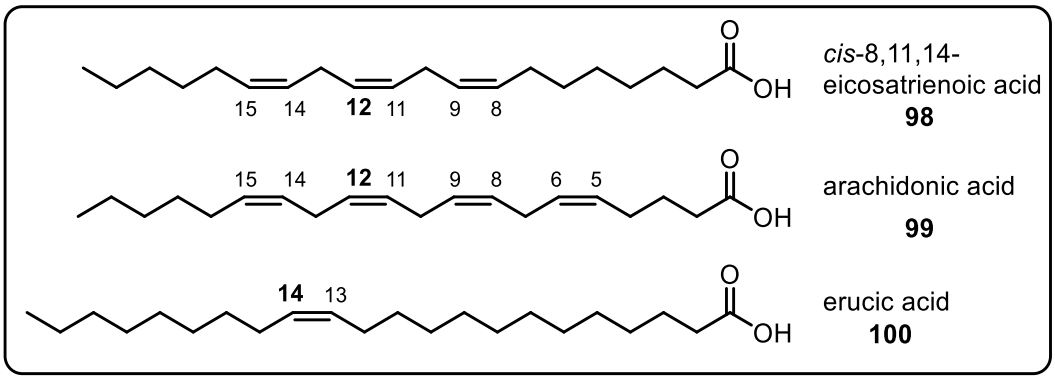
Hydratases catalyse the addition of water to (un)-activated double bonds to form valuable enantiopure alcohols. It can be stated that nitrile hydratases from *Rhodococcus* have been profoundly investigated and were the centre of research regarding hydratase activity. Yet, several *Rhodococcus* strains showed an intriguing

hydration potential towards other diverse compounds and their development is on the rise.

2.5.1 Oleate hydratase

Recently, a novel oleate hydratase (Ohy, EC 4.2.1.53) was isolated from *R. erythropolis* CCM 2595 and characterised.[218] Structural analysis showed that this Ohy structurally differs from previously described bacterial Ohys. This enzyme was shown to be an active monomer while all other characterised Ohys showed dimeric structures instead.[219-221] The purified Ohy was shown to accept a small range of unsaturated fatty acids adding water exclusively in 10-position while more complex lipids were not converted.[218] *In-silico* analysis of the occurrence and phylogenetic relationship of annotated oleate hydratases in 43 *Rhodococcus* strains revealed that distinct *Rhodococcus* clades show Ohy potential, thereby discovering 19 novel oleate hydratases (**Chapter 6**).[222] Two representatives, Ohys from *R. erythropolis* PR4 and *R. pyridinivorans* DSM 20415 sharing a sequence similarity and identity of 46% and 30%, respectively, were heterologously expressed and tested on (multiple) unsaturated fatty acids. While both Ohys converted smaller fatty acids in the same manner, they showed a complementary substrate scope towards sterically demanding and longer fatty acids (Figure 1).

***Re*Ohy (*R. erythopolis* PR4)**



***Rp*Ohy (*R. pyridinivorans DSM 20415*)**

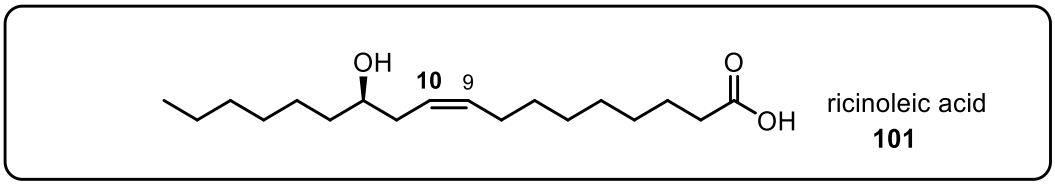


Figure 1: Exemplary display of complementary substrate scope of two Ohy from *Rhodococcus*. The bold number indicates the location of the hydroxy-group in the final hydrated product.[222]

A recently filed patent involves the Ohy-catalysed conversion of free fatty acids, such as oleic acid, derived from renewable feedstock of bio-based oils.[223] This is one example of the successful use of a novel Ohy from *Rhodococcus* that further improves the potential application of *Rhodococcus* as useful biocatalyst.

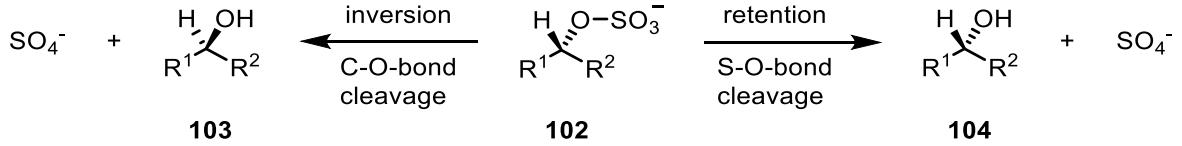
2.5.2 Michael hydratase

Whole-cells of *R. rhodochrous* ATCC 17896 were shown to catalyse a so-called Michael addition of water to α,β-unsaturated carbonyl compound (*E*)-4-hydroxy-3-methylbut-2-enoic acid and its respective ethyl-derivative.[224] Upon water addition, an internal nucleophilic attack leads to the formation of useful synthon (*R*)-4-hydroxy-4-methyldihydrofuran-2(3H)-one. The stereochemical course of the reaction was investigated showing that the water-addition takes place in *syn*-fashion (**Chapter 3**).[225,226] Interestingly, this hydratase demonstrated unusual behaviour as it requires oxygen for higher activities, but labelling studies with D_2O and $^{18}O_2$ proved a true water addition instead of an oxidative process.[226] Unfortunately, this membrane-associated enzyme has not been isolated and the coding gene remains elusive up to this point **Chapters 4** and **5**).

2.6 *Rhodococcus* acting on sulphur-containing compounds

2.6.1 Sulfatases

Sulfatases (EC 3.1.6.x) are a group of enzymes that catalyse the hydrolytic cleavage of sulfate esters by releasing inorganic sulfate and the corresponding alcohols.[227] Fascinatingly, sulfatases can achieve an enantio-convergent transformation of racemic sulfate esters (**102**) into only one stereoisomeric secondary alcohol yielding up to 100 % yield theoretically thereby surpassing traditional kinetic resolution processes. Sulfatases thereby determine not only the enantioselectivity but also the stereoselectivity with respect to retention (**104**) or inversion (**103**) of configuration (Scheme 25).[228]



Scheme 25: Retention or conversion during enzymatic sulfate ester hydrolysis catalysed by sulfatase.

The hydrolysis of an alkyl-sulfate through the breakage of the S-O-bond by nucleophilic attack leads to a retention while the breakage of the C-O-bond on the other hand leads to inversion of configuration at the carbon atom bearing the centre of chirality.[228] *R. ruber* DSM 44541 was shown to have a secondary alkyl sulfatase, called RS2, which acts through strict inversion. Methyl- and ethyl-(alkyl)sulfate esters with a varying chain-length were shown to be hydrolysed.[229,230] Addition of Fe^{3+}-ions led to an increase in activity which, however, was not suitable for preparative scale reactions.[231,232]

While most of the investigations concentrated on secondary alkyl-sulfates, few reports about cyclic sulfates have come forward. Growing cells of *R*. sp. CCZU10-1, however, were used to transform four cyclic sulfates: 1,3-propanediol cyclic sulfate (1,3-PDS), 1,2-propanediol cyclic sulfate (1,2-PDS), ethylene sulfate and glycol sulfate.[227] All four cyclic sulfates were hydrolysed which makes this the first time that a *Rhodococcus* species acted on sulfate or sulfite thereby generating diols.

2.6.2 Sulfide monooxygenase

Chiral sulfoxides are key synthons in chiral drug production and used as versatile auxiliary compounds, as chiral ligands or catalysts. The asymmetric oxidation of prochiral sulphides catalysed by whole-cell biocatalysts is superior to classical chemical routes.[233] Phenyl methyl sulphide (PMS) was oxidised into (*S*)-phenyl methyl sulfoxide (PMSO) by whole-cells of *R*. sp. ECU0066[234,235] and *R*. sp. CCZU10-1.[233] Both studies showed the expansion of substrate scope to a small set of PMS derivatives. A sulfide/sulfoxide flavin-dependent monooxygenase from *R*. sp. IGTS8 was produced in *E. coli* and investigated in detail.[236] It was shown that the enzyme oxidises DBT in two consecutive steps yielding DBT sulfone.

2.7 Conclusions

Enzymes present in *Rhodococcus* strains cover a broad field of diverse reactions ranging from redox-reactions and hydrolysis reactions of epoxides or esters to hydration of fatty acids or Michael acceptors. The most prominent and best exploited enzymes in *Rhodococcus* are most of the enzymes present in the aldoxime-nitrile pathway with the nitrile hydratase being the prime example of a successful biocatalyst being run on a multi-ton industrial scale process. On the other hand, enzymes that have only recently been discovered like the aldoxime dehydratase or enzymes acting on sulphur-containing compounds have the potential for further development to the industrial biocatalysts of the future.

Genome mining as well as the isolation of more *Rhodococcus* strains especially from rare sites open up the chance to identify new enzymes with interesting properties such as an extraordinarily broad substrate scope, higher enantio- or stereoselectivities for defined reactions or better overall stabilities at extreme temperatures or unusual reaction media. Additionally, the engineering of known enzymes to enhance their function also increases the number of possible chemical reactions. One such example is the evolution of amine dehydrogenase from amino acid dehydrogenase which led to a straightforward synthesis of (*R*)-selective amines which had been a major challenge in the past.

Few reactions were carried out with purified enzymes while the majority of reactions shown were catalysed by either *Rhodococcus* whole-cells (wild-type or mutants) or *E. coli* whole-cell reactions with heterologously expressed enzymes from *Rhodococcus*. It was shown that enzymes from *Rhodococcus* are valuable resources in the design of novel biocascades which will attract only more attention in the near future. Here, the combination of enzymes in well-established expression hosts is theoretically boundless given that the present enzymes share reaction condition ranges (*e.g.* pH, temperature, solvent) and act on the same desired compounds. In summary, the genus *Rhodococcus* truly deserves to be termed ‘biocatalytic powerhouse’: its enzymatic diversity and overall robustness make this microorganisms one of the key players in many areas of biocatalysis and will continue to do so.

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3

Re-investigation of hydration potential of *Rhodococcus* whole-cell biocatalysts towards Michael acceptors

The implementation of a stereoselective Michael addition with water as substrate is still a major challenge by classical, chemical means. Inspired by nature's ability to carry out this attractive reaction with both high selectivity and efficiency, the interest in hydratases (EC 4.2.1.x) to accomplish a selective water addition is steadily rising. The gram-positive bacterial genus *Rhodococcus* is known as biocatalytic powerhouse and has been reported to hydrate various Michael acceptors leading to chiral alcohols. This study aimed at the in-depth re-investigation of the hydration potential of *Rhodococcus* whole-cells towards Michael acceptors. Here, two concurrent effects responsible for the hydration reaction were found: while the majority of substrates was hydrated in an oxygen-independent manner by free amino acid catalysis, an enzyme-catalysed water addition to (*E*)-4-hydroxy-3-methylbut-2-enoic acid was proven to be oxygen-dependent. $^{18}O_2$-labelling studies showed that no $^{18}O_2$ was incorporated in the product. Therefore, a novel O_2-dependent hydratase distinct from all characterised hydratases so far was found.

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3.1 Introduction

The selective addition of water to (un)-activated double bonds is known to be a very appealing yet chemically challenging reaction. Though water as a reactant provides benefits regarding sustainability and atom-efficiency, it is both a poor electro- and nucleophile and therefore often first requires activation by *e.g.* strong acids. Additionally, unfavourable reaction equilibria often impede a profitable reaction.[1-3] While chemists still struggle to find activating conditions without diminishing the stereoselectivity, nature developed a way to circumvent these problems by the use of enzymes. Their unique 3-dimensional structures provide ways to activate the water molecule as well as to stabilise transition states during the reaction.[1,4] Therefore, using microbial activities or applying purified enzymes in biocatalytic reactions to achieve a selective water addition to double bonds is nowadays often seen as advantageous.[5-7] Hydratases (EC 4.2.1.x) catalyse the water addition to activated as well as isolated double bonds. However, especially enzymes from the primary metabolism exhibit a narrow substrate scope which confines the applicability of these enzymes to their natural substrates.[4] While the use of hydratases to convert natural substrates already offers a huge advantage over classical chemical routes and has already been applied in industry,[8-11] the identification of hydratases with a broader substrate scope is highly desirable. A biocatalytic hydration of two non-natural, α,β-unsaturated substrates using whole-cells of *Rhodococcus rhodochrous* ATCC 17895 was first reported in 1998.[12] Further research to develop a straightforward approach to produce β-hydroxy carbonyl compounds catalysed by a presumed 'Michael hydratase' followed in 2015. It was described that whole-cells of *Rhodococcus* additionally add water to small ring-closed organic molecules like *c*-hexenone or *c*-pentenone thereby improving future applications of an attractive water addition system.[13]

Rhodococcus is a genus of a gram-positive bacteria that has been described to host a variety of enzymes and degradation pathways with high potential for the use in industrial processes.[8,14] Its metabolic diversity often stems from large, linear plasmids that carry the protein-encoding genes for degradative enzyme systems.[15] Abundant horizontal gene-transfer events occurring *via* these linear plasmids as well as a high

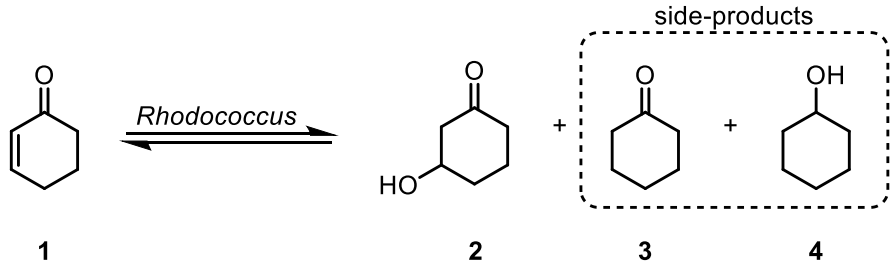
multiplicity of catabolic enzymes contributed to the catabolic versatility of *Rhodococcus* genus members.[15-17]

To study the described water addition in-depth, the identification and isolation of the responsible enzyme were first aimed for. Due to complications in the protein isolation, whole-cell systems were further investigated to explore the substrate range. During this process inconsistencies and contradictions within the earlier reports became evident.[12,13] Therefore, the aim of this study was to assess and extend those reports. In addition to a re-evaluation of described substrate structures, the main focus was on the clarification of the reaction type. Labelling studies with D_2O and $^{18}O_2$ with subsequent high resolution liquid chromatography-mass spectrometry (HRLC-MS) analysis were used to identify whether the net microbial hydration activity involves a true water addition or an oxidative process.

3.2 Results and Discussion

3.2.1 *Rhodococcus* whole-cell catalysis

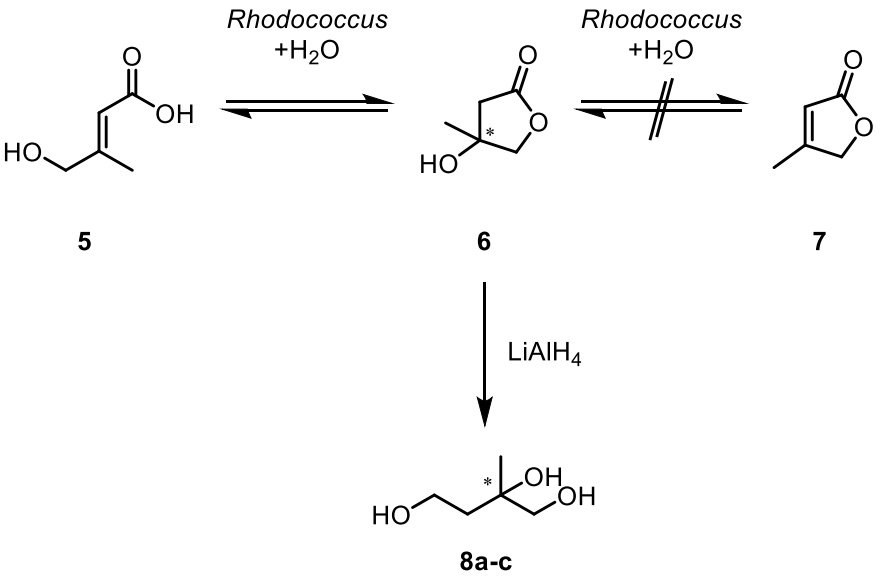
Whole-cells of *Rhodococcus rhodochrous* ATCC 17895 were described to catalyse the Michael-addition of water to several substrates.[12,13] However, due to the presence of alcohol dehydrogenases and ene-reductases in *Rhodococcus* whole-cells, which act on small organic molecules like *c*-hexenone, several side-reactions were observed leading to the formation of unwanted side-products (Scheme 1).[18]



Scheme 1: Whole-cell biotransformation of *c*-hexenone with *Rhodococcus* whole-cells leads to desired water addition product and unwanted side-reactions.

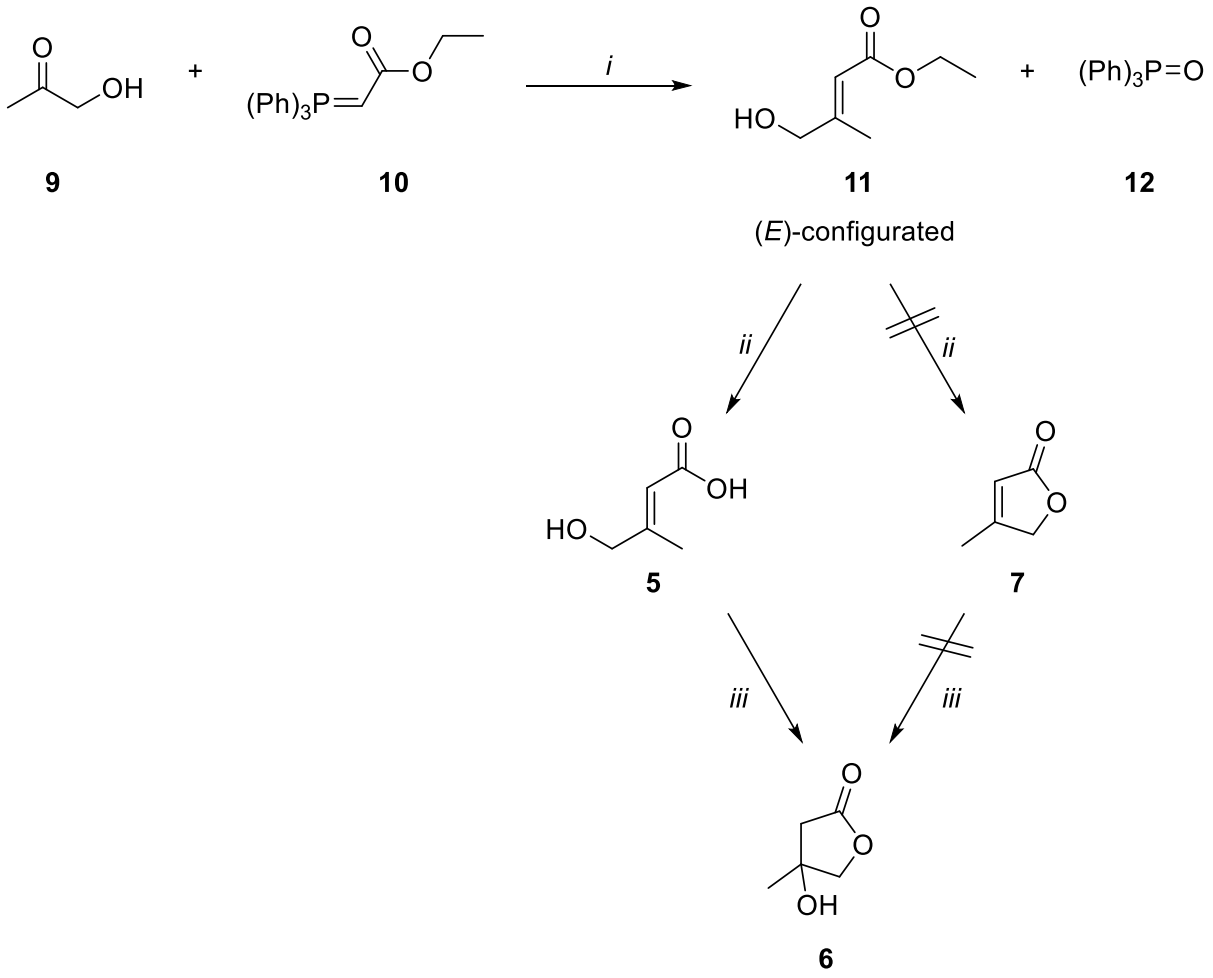
3.2.2 Substrate re-evaluation

In the original report Holland *et al.* stated that 4-methyl-furan(*5H*)-one (**7**) is hydrated by whole-cells from *Rhodococcus* to give the respective 4-hydroxy-4-methyldihydrofuran-2(3H)-one (**6**) (Scheme 2).[12] However, bioconversions with commercially available **7** showed no expected product formation. Upon closer investigation of the chemical structure, it became evident that previous reports[12,13] assumed a wrong substrate structure.



Scheme 2: Bioconversions using whole-cells from *Rhodococcus* using substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**, confirmed substrate, left side) and 4-methyl-furan(*5H*)-one (**7**, not converted, right side). A subsequent reduction of lactone **6** led to triol **8** to confirm the stereochemistry.

In those reports, the synthesis of the biotransformation substrate was carried out as a two-step procedure involving a Wittig-reaction followed by a basic hydrolysis (**Scheme 3**).[12,13]



Scheme 3: Two-step model substrate synthesis with subsequent *Rhodococcus*-catalysed water addition: (*i*) Wittig reaction, toluene, 120 °C, 4 h; (*ii*) basic hydrolysis reaction, MeOH, 0 °C to room temperature, 16 h; (*iii*) biocatalysed water addition: 100 mg/ml resuspended whole-cells, 100 mM KPi buffer, pH 6.2, 16 hours.

The Wittig-reaction, the reaction of aldehydes or ketones with phosphonium ylides, isolated or generated *in-situ* from phosphonium salts, leads to (*E*)- or (*Z*)-alkenes under formation of phosphine oxides.[19] The stereochemistry of the resulting alkene thereby depends on the ylide used: stabilised ylides provide (*E*)-alkenes while unstabilised ylides tend to give (*Z*)-alkenes. While the reaction mechanism to form (*Z*)-alkenes is still controversially discussed,[20,21] the straightforward formation of (*E*)-alkenes using stabilised ylides is widely agreed on in the chemical community. Therefore, the Wittig reaction of hydroxyacetone (**9**) and stabilised phosphonium ylide (carbethoxymethylene)triphenylphosphorane (**10**) leads, contrary to previously published data,[12,13] to (*E*)-configurated alkene **11** and triphenylphospine oxide (**12**).

The subsequent basic hydrolysis gives (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) in moderate yields instead of reported 4-methyl-furan-2(*5H*)-one (**7**).

1- and 2-dimensional NMR spectroscopy of the product confirmed the absence of ring-closed product **7**. Nuclear Overhauser effect spectroscopy (NOESY) experiments of synthesised compound (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) revealed a through-space correlation between the vinylic proton and the methylene group but did not show any correlation with the methyl group (Figure 1).

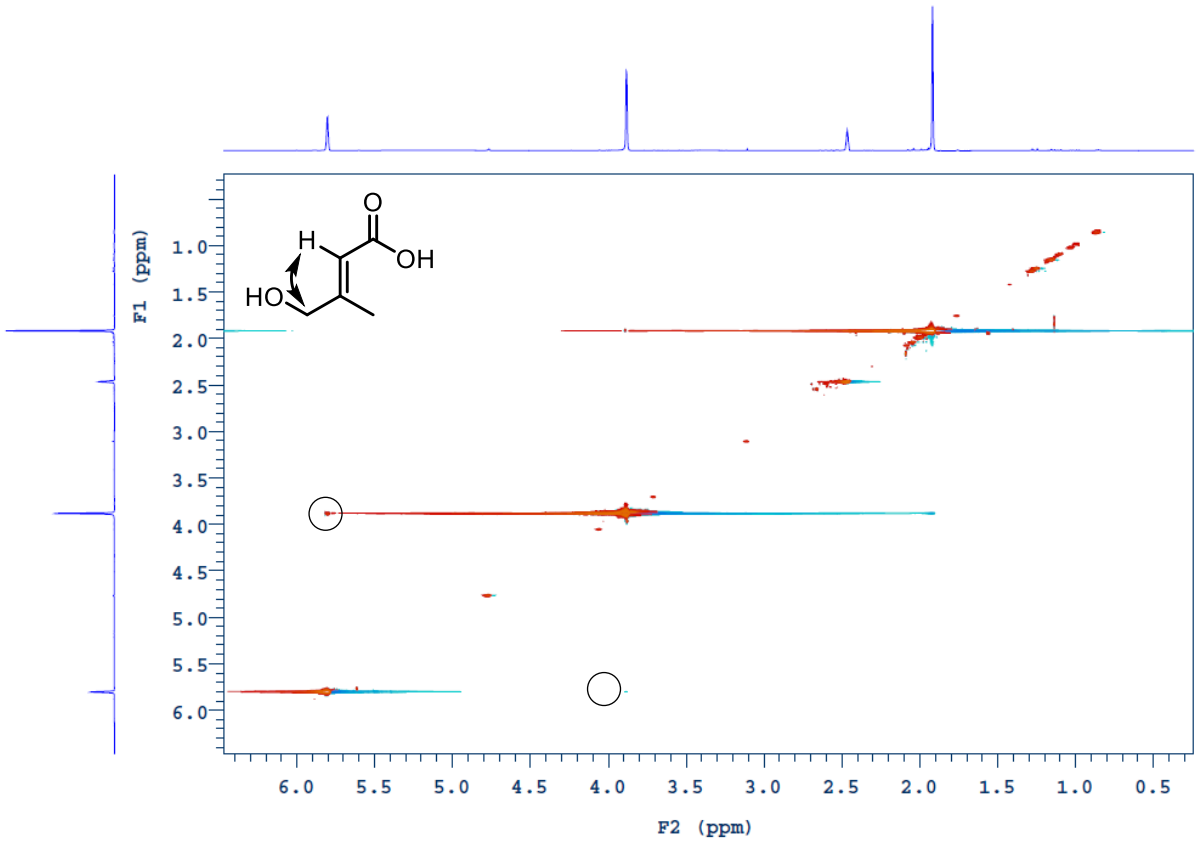
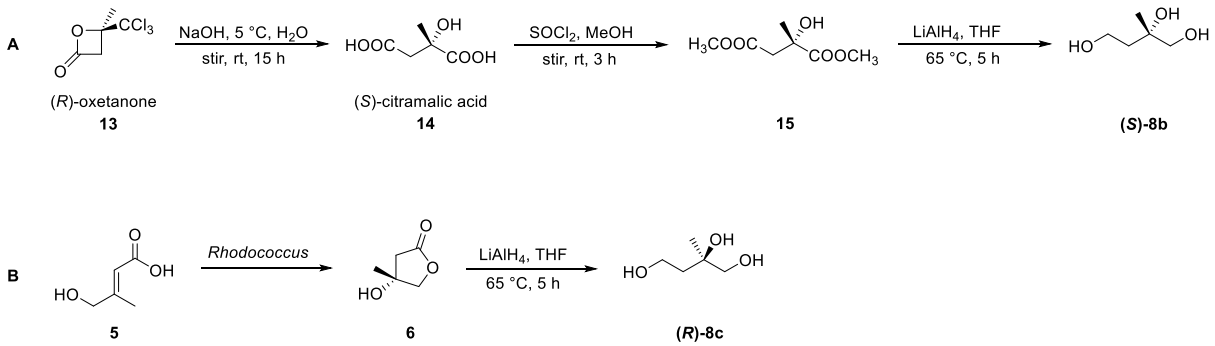


Figure 1: Nuclear Overhauser effect spectrum (NOESY) of biotransformation substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**).

Attempts to obtain the corresponding open-chain (*Z*)-isomer through basic hydrolysis of commercially available 4-methyl-furan(*5H*)-one (**7**) were unsuccessful.

Previous reports were contradictory in assigning the stereochemistry of final lactone **6**: (*R*)-configurated in the first report and the opposite (*S*)-configuration in the second study, both based on optical rotation.[12,13] To ultimately confirm one of the two hypotheses, the stereochemistry of reduced triol **8** (Scheme 2) was compared to

reference compounds using chiral gas chromatography: (*S*)-triol **8b** was obtained *via* a three-step chemical route (Scheme 4A): (*S*)-citramalic acid (**14**) was prepared from (*R*)-oxetanone **13** and was subsequently esterified with thionyl chloride to yield dimethyl-(*S*)-2-hydroxy-2-methyl-succinate (**15**, 60% yield).[22,23] Treatment of the ester **15** with an excess of LiAlH4 in tetrahydrofuran gave (*S*)-2-methylbutane-1,2,4-triol (**8b**).[22] While racemic triol **8a** was commercially available, lactone **6** was obtained from a large-scale biotransformation with *R. pyridinivorans* DSM 20415 and afterwards reduced with $LiAlH_4$ to give triol **8** (Scheme 4B). Comparative chiral gas chromatography analysis of all three triol samples (**8a-c**) revealed that the triol obtained from a large-scale biotransformation with *R. pyridinivorans* DSM 20415 was (*R*)-configurated (**8c**, Figure 2).



Scheme 4: Synthetic routes to 2-methylbutane-1,2,4-triols. A: chemical synthesis towards (*S*)-triol **8b** in three steps; B: biocatalytic generation of lactone **6** with subsequent reduction to (*R*)-triol **8c**.

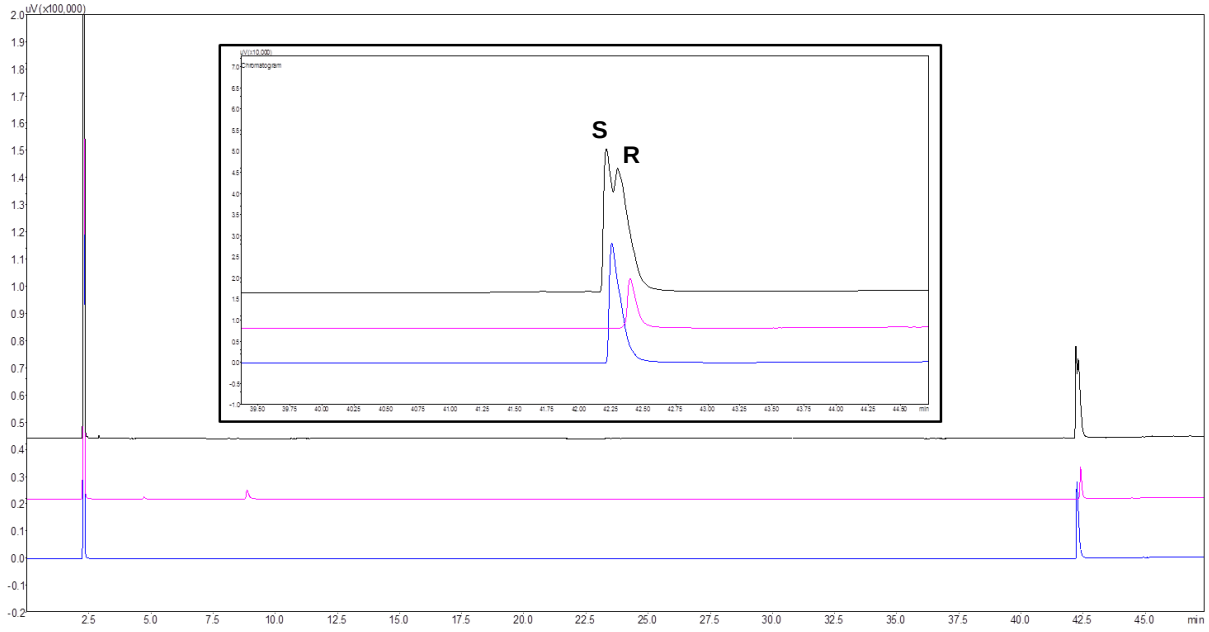


Figure 2: Gas chromatograms of triols **8a-c**. From top to bottom: racemic triol **8a** (black line, commercially available), enzymatically obtained triol (*R*)-**8c** (pink), synthetically obtained (*S*)-triol **8b** (blue).

Following the revision of the substrate structure, the Mhy activity was (re)-evaluated on a number of other Michael acceptors that have been reported previously.[12,13] The conversion of *c*-hexenone (**1**), *c*-pentenone, and *c*-heptenone yielded indeed hydroxylated product in low yields while methyl crotonate was not accepted.

3.2.3 Oxygen-dependency

Whole-cell optimisation experiments with substrate **5** surprisingly showed an improved product yield with an increasing headspace to reaction-mixture ratio (Figure 3). These findings indicate an oxygen-dependency which was not reported earlier. On the contrary, previous reports stated that both the reaction of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and *c*-hexenone (**1**) were as efficiently catalysed under N_2-atmosphere as under air. With this experiment the possibility of an oxidative process was previously excluded.[13] To resolve these apparently conflicting findings, the oxygen-dependency of the water addition reaction was re-investigated. Here, numerous experiments using substrate **5** under N_2-atmosphere did not lead to any product formation while *c*-hexenone (**1**) was still converted and gave similar results as in aerobic trials (Figure 3). The contradicting results of water addition activity under anaerobic conditions

depending on the substrate class led to the question whether both substrate classes are converted by the same reaction mechanism and biocatalyst. Consequently, control reactions with whole-cells of *Escherichia coli* TOP 10 containing an empty pBADHisA expression vector were carried out. No product formation in the case of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) was observed while *c*-hexenone (**1**) showed comparable product formations with the *E. coli* control system.

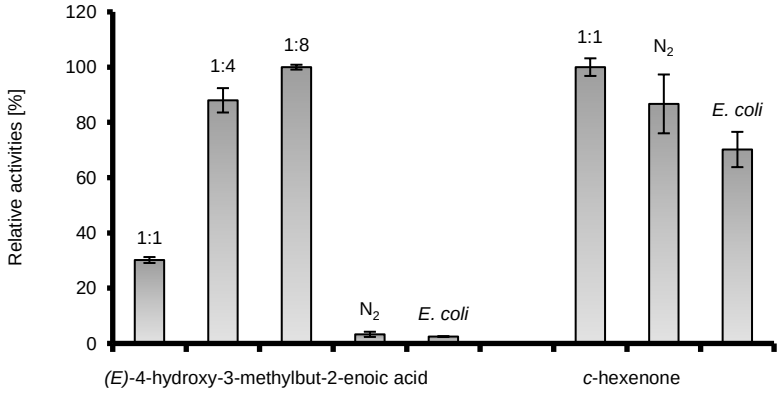


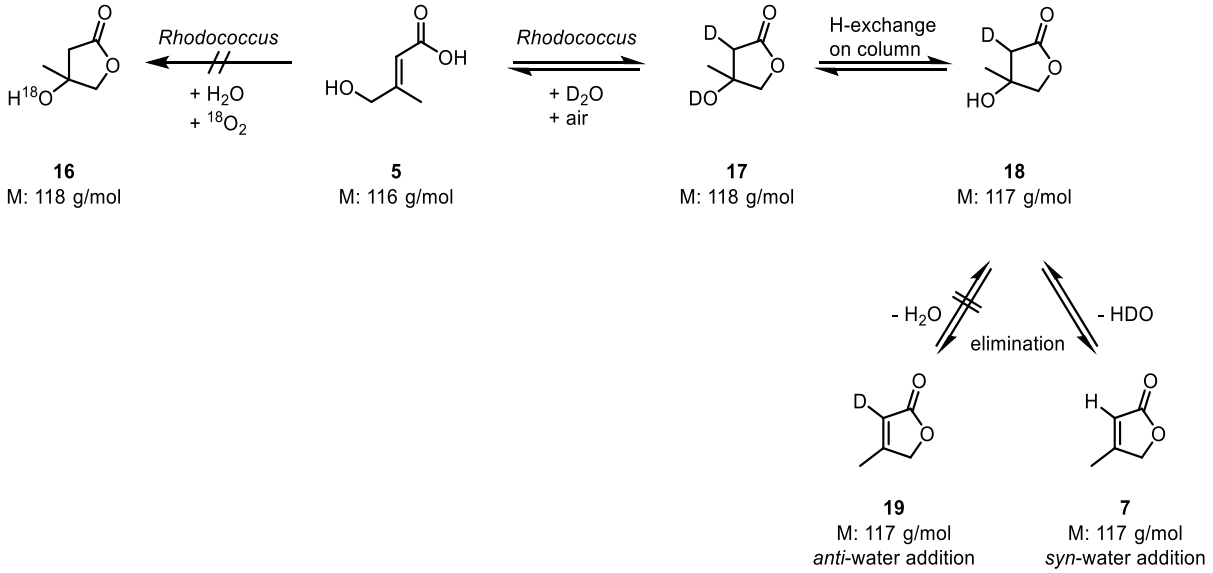
Figure 3: Investigation of oxygen-dependency of bioconversions with substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**, left) and *c*-hexenone (**1**, right) using different headspace-reaction mixture ratios [1:1, 1:4, 1:8 (reaction mixture:air)], N_2 and *E. coli* reactions. Relative activities normalised on highest activity achieved (100% with ratio 1:8 for substrate **5** and 100% for the ratio 1:1 for substrate **1**).

These results indicate the presence of two different effects acting on the two substrate classes. One possible explanation for the latter observation is a water addition catalysed by free amino acids. In the past, amino acids were already shown to add water to Michael acceptors like *c*-hexenone (**1**) or *c*-pentenone without any oxygen requirement. **L**-lysine obtained the highest formation of *rac*-3-hydroxy-*c*-hexanone with a yield of 21%.[24] It is therefore likely that the amino acids present in both *E. coli* and *Rhodococcus* catalyse the reaction on *c*-hexenone regardless of the presence or absence of oxygen in our system as well.

3.2.4 Oxidation or water addition?

To exclude the possibility of an oxidative process instead of a water addition reaction, a number of labelling experiments were carried out. The conversion of **1** and **5** was

catalysed both by cells of *R. rhodochrous* ATCC 17895 and *R. pyridinivorans* DSM 20415 under $^{18}O_2$ atmosphere in H_2O as well as under air in both D_2O and H_2O. The results were subsequently analysed with high resolution liquid chromatography-mass spectrometry (HRLC-MS). In case of an oxidative process, the product will show a higher mass due to the incorporated ^{18}O-atom (**16**) (Scheme 5). On the other hand, a water addition will lead to the incorporation of two deuterium-atoms (**17**) of which one is readily exchangeable thus **18** will be detected. A subsequent chemical elimination step will reveal whether the deuterated water was added in *syn*- (**7**) or *anti*-fashion (**19**) as the chemical E2-elimination selectively takes place in *anti*-fashion.



Scheme 5: Schematic labelling studies with $^{18}O_2$ and D_2O on (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) with a subsequent elimination step including the molecular weights of respective intermediates.

Biotransformations with both cells and both substrates **1** and **5** did not incorporate any labelled ^{18}O-atom thereby excluding any oxidative process. Comparative HRLC-MS analysis of reactions run under air with D_2O and H_2O showed a clear formation of compound **18** for substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and the respective deuterated product for *c*-hexenone indicating the incorporation of D_2O and thereby confirming a water addition for both *Rhodococcus* strains. Control reactions without cells did not show any significant water addition activity (Figures 4 and 5).

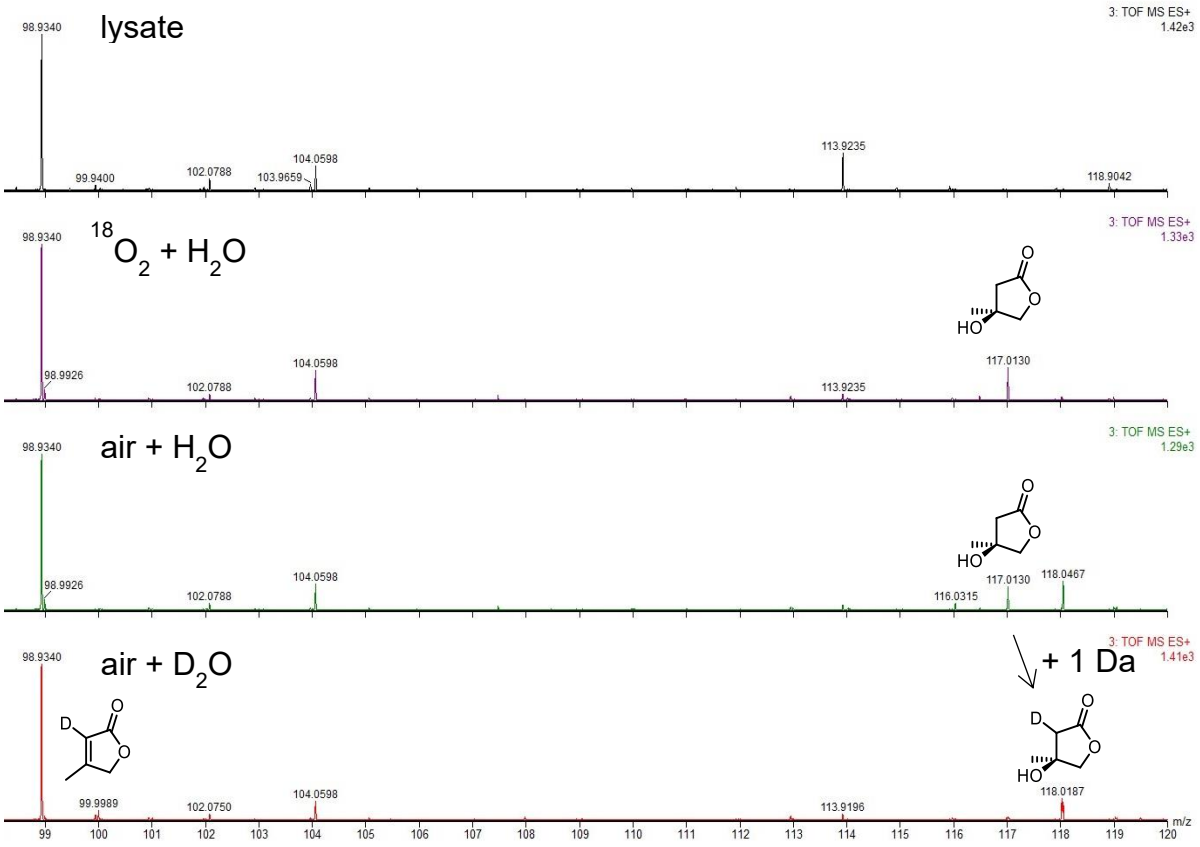


Figure 4: Mass spectrum of labelling studies using substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) converted by *Rhodococcus rhodochrous* ATCC 17895 whole-cells showing the molecular masses in Da of the investigated compounds.

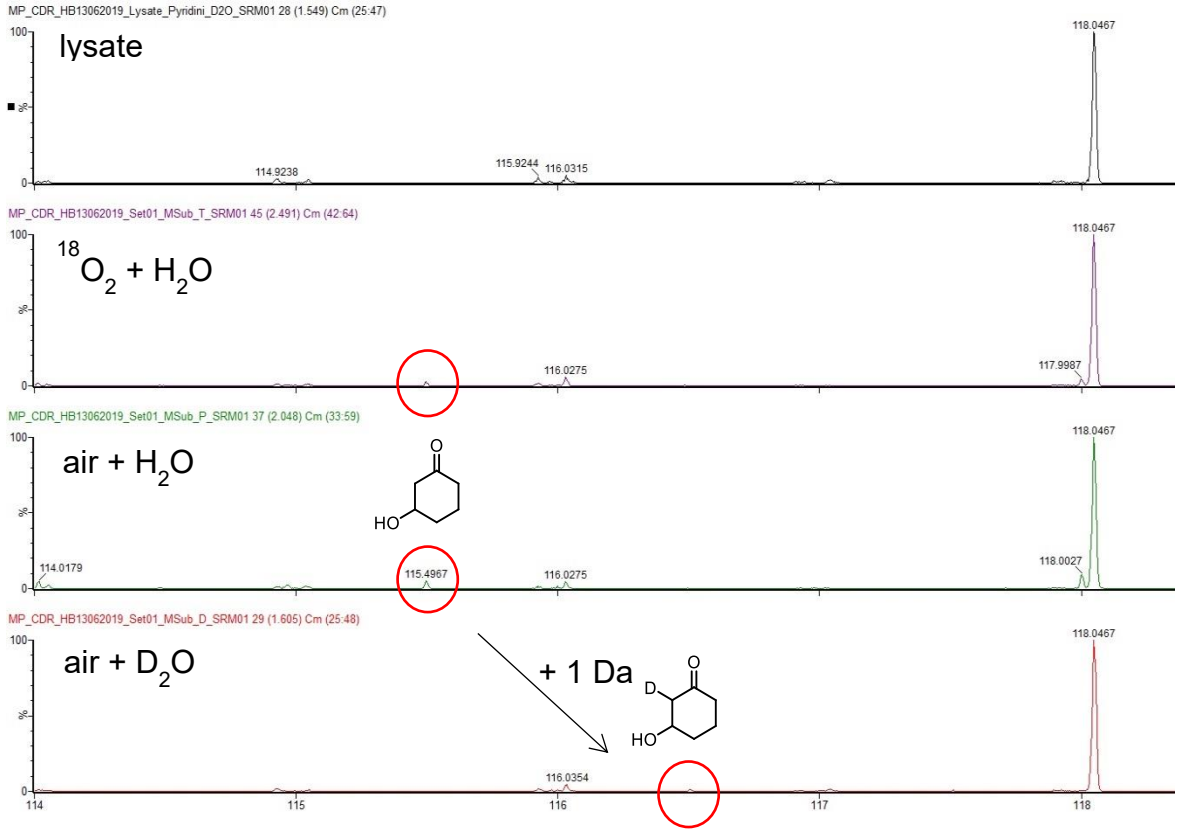


Figure 5: Mass spectrum of labelling studies using substrate *c*-hexenone (**1**) converted by *Rhodococcus pyridinivorans* DSM 20415 whole-cells showing the molecular masses in Da of the investigated compounds.

To review the course of the water addition (*syn*- versus *anti*-addition) purified deuterated compound **18** was obtained in a large-scale biotransformation with *Rhodococcus rhodochrous* ATCC 17895 whole-cells (Figure 6). A subsequent chemical *anti*-elimination was performed yielding exclusively the undeuterated product **7** (Figure 7).

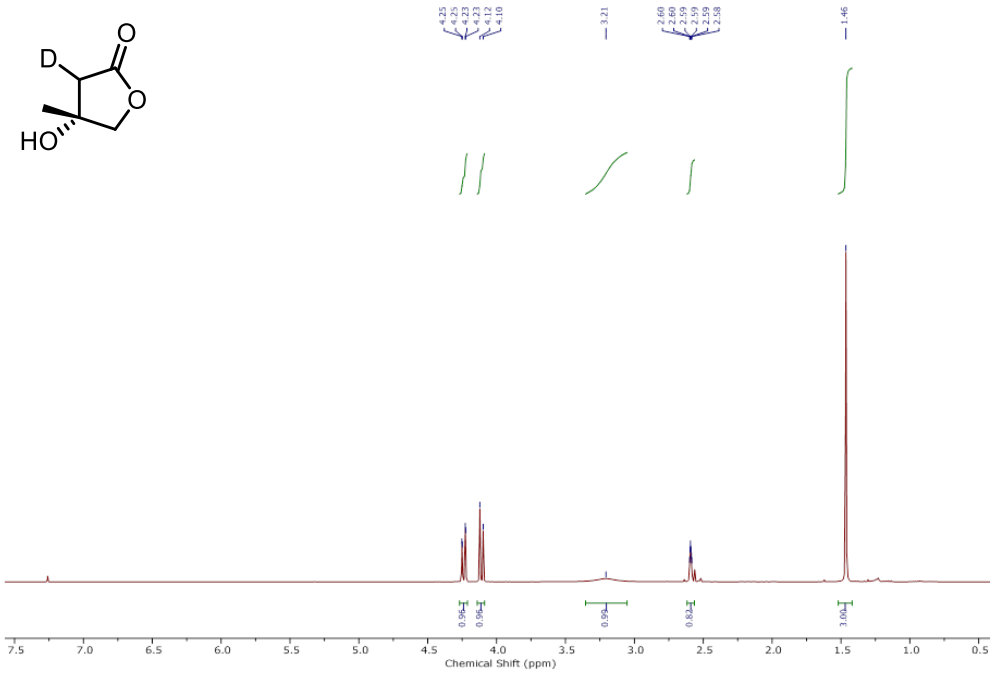


Figure 6: ^{1}H-NMR spectrum of 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one-3-*d* (**18**).

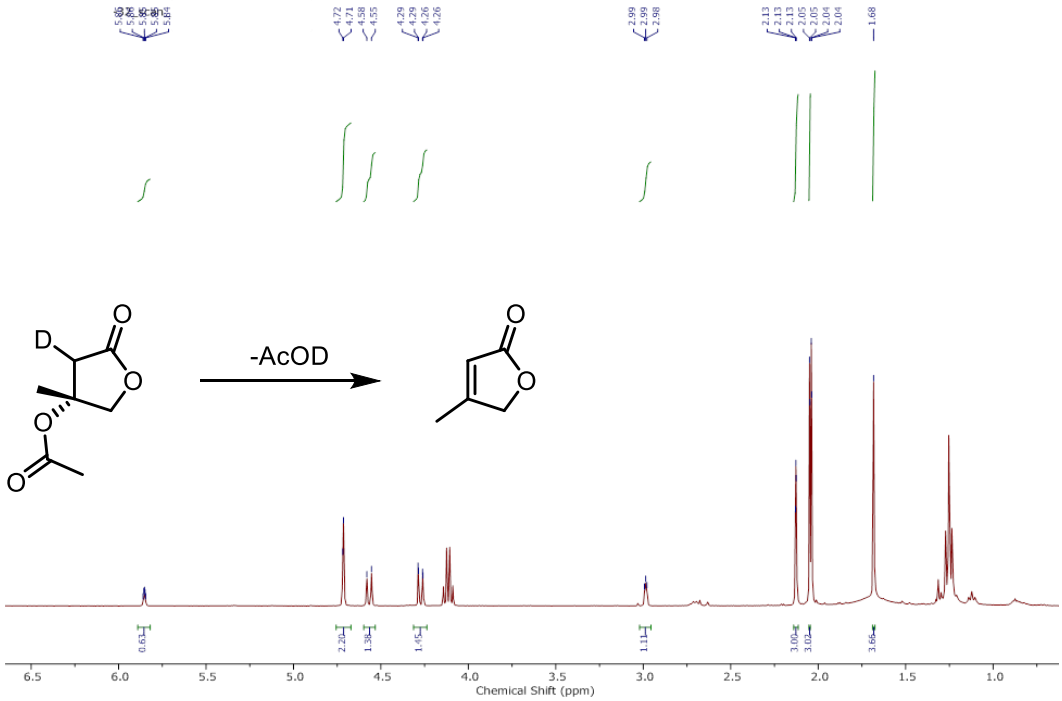


Figure 7: ^{1}H-NMR spectrum of crude elimination mixture. Elimination product 4-methyl-furan-2(*5H*)-one (**7**): ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.13 (s, 3H), 4.72 (s, 2H), 5.84 (S, 1H). In accordance with literature.[25]

This experiment thus confirms earlier results,[13] but due to the revised substrate structure which is an open-chain rather than a ring-closed molecule, the water addition catalysed by *Rhodococcus* whole-cells consequently occurs in *syn*-fashion.

Biotransformations carried out under nitrogen atmosphere proved that the presence of oxygen is required for the conversion of **5** to take place. This was further sustained by the fact that more lactone **6** was produced under pure $^{18}O_2$-atmosphere than under air-conditions with less oxygen present (Figure 3). Additional experiments with D_2O under nitrogen atmosphere revealed no product formation.

The difference to the conversion of *c*-hexenone (**1**), however, is the remaining activity in the absence of oxygen as well as comparable activities achieved with *E. coli* cells. The obtained results from the labelling study further confirm the earlier theory that this conversion is an amino-acid catalysed process for ring-closed α,β-unsaturated carbonyl compounds like *c*-hexenone, *c*-pentenone and *c*-heptenone. This leaves compound **5** and its ethyl-derivative (*E*)-4-hydroxy-3-ethylbut-2-enoic acid[12] to be the sole substrates requiring oxygen for a water addition. Due to high enantiomeric excess values of the final lactones which were shown in earlier studies,[13] the high substrate specificity and the fact that the activity is associated with the membrane fraction, we propose an enzyme-catalysed process rather than a metal-assisted or amino-acid catalysed water addition.

The described O_2-dependent behaviour of a presumed hydratase is, however, surprising and atypical compared to other identified hydratases as they are either known to be oxygen-independent or negatively affected by trace amounts of oxygen. The latter enzymes often hold metal-containing cofactors or cysteine residues that lead to the sensitivity.[2] One example of this group is the linalool (de)hydratase-isomerase which catalyses the water addition and isomerisation to unactivated monoterpenes like (*S*)-(+)-linalool to yield β-myrcene and geraniol.[2,26,27] Interestingly, *Rhodococcus erythropolis* MLT1 cells were shown to catalyse a similar reaction converting β-myrcene to geraniol.[2,28] In this whole-cell system, however, oxygen is required for the reaction and anaerobic conditions lead to no product formation.[28] This phenomenon is similar to our findings and, to the best of our knowledge, the only other report of an oxygen-dependent formal hydration reaction. The substrates, however, differ as our

substrates belong to the group of activated Michael acceptors while in β-myrcene an unactivated double bond is hydrated. Nonetheless, it is remarkable that this phenomenon was observed only twice and only in *Rhodococcus* cells.

3.3 Conclusions

Michael additions with the unreactive water-molecule as substrate are highly attractive yet chemically very challenging reactions. *Rhodococcus* cells were shown to have microbial activity towards a number of Michael acceptors leading to β-hydroxy carbonyl compounds. This study serves to expand the knowledge about these water addition reactions and a presumed 'Michael hydratase' was investigated in detail.

Though trials to isolate or identify the responsible hydratase failed, whole-cell and membrane systems were employed to examine the substrate scope. Here, previously described substrates were re-visited and corrected regarding their chemical structure using 1- and 2D-NMR analysis. Experiments under nitrogen atmosphere revealed two different effects being responsible for the water addition depending on the substrate used. The main group of substrates was proposed to be amino-acid catalysed as the same hydration activity was found in the presence and absence of oxygen as well as with *E. coli* cells. (*E*)-4-hydroxy-3-methylbut-2-enoic acid, however, was shown to be oxygen-dependently converted only by *Rhodococcus* cells and presumed to be enzyme-catalysed. Complete absence of oxygen led to no hydration. Labelling studies with D_2O and $^{18}O_2$ exposed a true water addition in *syn*-fashion and excluded any oxidative process. The described microbial hydration activity therefore remains highly attractive yet still elusive. A complete understanding of the oxygen dependency, a probable reaction mechanism, the finding of its natural substrate as well as an expansion of its substrate scope for future applications will only be possible upon identification of the responsible membrane protein (-complex) and successful heterologous expression in a suitable host system.

3.4 Experimental Data

3.4.1 Chemicals

Unless stated otherwise, all commercial chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification. Petroleum ether

was purchased from VWR International (Amsterdam, The Netherlands) and distilled before utilisation. Hydroxyacetone was purchased from Alfa Aesar (Kandel, Germany) and *rac*-2-methyl-1,2,4-triol was purchased from Chemspace (Riga, Latvia).

3.4.2 Chemical synthesis procedures

Compounds (*E*)-ethyl-4-hydroxy-3-methylbut-2-enoate (**11**) and (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) were synthesised as described earlier and results are in accordance with literature.[29,30] *Rac*-3-hydroxy-*c*-hexanone was synthesised following described protocol and data is in accordance with literature.[24] Bioconversion product (*R*)-4-hydroxy-4-methyldihydrofuran-2(*3H*)-one was also in accordance with literature.[31]

(*S*)-citramalic acid (**14**)

A solution of sodium hydroxide (10 M, 3 ml) was added dropwise to a suspension of (*R*)-oxetanone **13** (1.017 g, 5 mmol) in water (5 ml) at 5 °C. The mixture was stirred at room temperature for 15 h, acidified to pH 1 with concentrated hydrochloric acid (10 M) and evaporated under reduced pressure. The crude acid was extracted from the residue with warm ethyl acetate (4 × 5 mL) and the crude product (yellow oil) was used without further purification (67%, 0.5 g, 3.35 mmol). ^{1}H-NMR (400 MHz, Methanol-*d*4) δ: 2.74 (d, *J* = 16.1 Hz, 1H), 2.44 (d, *J* = 16.2 Hz, 1H), 1.25 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-*d*4) δ: 177.3, 172.7, 71.9, 43.7, 25.3. NMR data is in accordance with literature.[23,32]

Dimethyl-(*S*)-2-hydroxy-2-methylsuccinate (**15**)

(*S*)-citramalic acid (**14**) (296 mg, 2 mmol) was dissolved in methanol (1 mL) and thionyl chloride (2 equiv.) was added dropwise under cooling. The reaction mixture was stirred at room temperature for 3 h and followed by TLC. The solvent was evaporated and the crude product (yellow oil) was used without further purification (97%, 340 mg, 1.94 mmol). ^{1}H-NMR (400 MHz, Methanol-*d*4) δ: 3.74 (s, 3H), 3.64 (s, 3H), 2.92 (d, J = 15.7 Hz, 1H), 2.66 (d, J = 15.7 Hz, 1H), 1.41 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-d4) δ: 176.9, 172.3, 73.8, 52.9, 52.1, 45.4, 26.6. NMR data is in accordance with literature.[22]

(*S*)- and (*R*)-2-methylbutane-1,2,4-triol (**8b**+**8c**)

Triols **8b** and **8c** were independently synthesised following the same reaction conditions but using different starting materials: for the synthesis of (S)-**8b** a solution of dimethyl-(*S*)-2-hydroxy-2-methylsuccinate (**15**) (120 mg, 0.7 mmol) in tetrahydrofuran (0.5 ml) was prepared and for synthesis of (R)-**8c** a solution of bioconversion product 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one (**6**) (90 mg, 0.77 mmol) in tetrahydrofuran (0.5 ml) was prepared. The solutions were each added dropwise to a solution of lithium aluminium hydride (85.1 mg, 2.3 mmol) in tetrahydrofuran (3.5 mL). After formation of hydrogen gas stopped, the mixtures were heated at reflux for 5 h, cooled to 0°C and the excess reducing agent was neutralised by the successive addition of water (2 mL) and aqueous sodium hydroxide (2.5 M, 2 mL). After stirring for 1 h at room temperature, the mixtures were filtered and the filter cakes were washed with tetrahydrofuran (3 mL) and absolute ethanol (3 mL). The filtrate and washings were combined and evaporated. The residues were passed through a short column of silica gel with DCM:Methanol (1:9) as eluent. (*S*)-selective triol **8b** was obtained as yellow oil with a yield of 70% (59 mg, 0.49 mmol) while (*R*)-selective triol **8c** was obtained as yellow oil in 55% yield (58 mg, 0.42 mmol). ^{1}H-NMR for (*S*)-**8b** (400 MHz, Methanol-*d*4) δ: 3.72 (m, 2H), 3.37 (d, *J* = 4.9 Hz, 2H), 1.84 – 1.64 (m, *J* = 6.9 Hz, 2H), 1.17 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-*d*4) δ: 73.4, 70.5, 59.2, 41.5, 24.4. ^{1}H-NMR for (*R*)-**8b** (400 MHz, Methanol-*d*4) δ: 3.79 – 3.66 (m, 2H), 3.37 (d, *J* = 4.7 Hz, 2H), 1.82 – 1.65 (m, 2H), 1.17 (s, 3H). ^{13}C-NMR (101 MHz, Methanol-*d*4) δ: 73.4, 70.5, 59.2, 41.5, 24.3. NMR data are in accordance with literature.[22]

3.4.3 Labelling studies

Whole-cells of *R. pyridinivorans* DSM 20415 and *R. rhodochrous* ATCC 17895 were resuspended to a final cell content of 100 mg/mL in either deuterated (100 mM, pD 6.2 ~ pH 6.6) or standard (100 mM, pH 6.2) KPi buffer. Substrates (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) and c-hexenone (**1**) were added to a final concentration of 10 mM. Reactions were either carried out under $^{18}O_2$-atmosphere or under air (headspace-reaction mixture ratio 3:1). Reactions were run overnight at 28 °C and the

supernatant was analysed by high resolution liquid chromatography-mass spectrometry (HRLC-MS).

3.4.4 Elimination studies

Whole-cells of *R. rhodochrous* ATCC 17895 were resuspended to a final cell content of 100 mg/mL in deuterated KPi buffer (100 mM, pD 6.2 ~ pH 6.6). Substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) was added to a final concentration of 15 mM. The reaction was run for 48 hours at 28 °C. The whole-cell mixture was extracted with EtOAc and concentrated upon flash column purification (eluent EtOAc : heptane 1:1, 99 mg, 0.85 mmol, 19%). ^{1}H-NMR for (*R*)-6 (400 MHz, chloroform-*d*) δ: 4.24 (d, *J* = 9.7 Hz, 1H), 4.11 (d, *J* = 9.7 Hz, 1H), 3.21 (s, 1H), 2.59 (m, 1H), 1.46 (s, 3H). 13C NMR (101 MHz, chloroform-*d*) δ 176.7, 79.8, 74.5, 43.2, 24.8. 30 mg of purified 4-hydroxy-4-methyldihydrofuran-2(*3H*)-one-3-*d* (**18**) was dissolved in 1 mL EtOAc and 35 µL acetic anhydride and 120 µL trimethlamine were slowly added to the solution followed by 50 µL 4-dimethylaminopyridine (DMAP, 3 mg/mL solution in EtOAc). The reaction was stirred at room temperature for 24 hours. Afterwards, 1 mL H_2O was added and the organic layer was separated and dried over $MgSO_4$. The crude mixture was measured by NMR-spectroscopy.

3.4.5 Bacterial strains and microorganisms

R. pyridinivorans DSM 20415 was purchased from the German Collection of Microorganisms and Cell Culture (Leibniz Institute DMSZ) while *R. rhodochrous* ATCC 17895 was bought from the American Type Culture Collection (Manassas, Virginia, US).

3.4.6 Microbiological protocols

The organisms were maintained at 4 °C on a nutrient agar plate (3 g beef extract, 5 g peptone, 15 g agar dissolved 1 L de-ionised water and autoclaved at 121 °C) and were regularly sub-cultured. The growth medium used consisted of 1 L de-ionised water with 6.59 g glucose, 9.2 g peptone, 1.84 g yeast extract, 7 mM K_2HPO_4 (1.2 g), 3 mM KH_2PO_4 (0.4 g) with a final pH of 6.8 and was autoclaved at 110 °C. A preculture was inoculated with a single colony and grown overnight at 28 °C. The preculture (2 mL) was used to inoculate 1 L of culture medium grown in 5 L flasks. The culture was

incubated for 72 hours at 28 °C with 180 rpm orbital shaking. The cells were harvested by centrifugation (17.696 *xg*, 15 min, 4 °C). The cells were washed with potassium phosphate buffer (100 mM, pH 6.2) and stored at -20 °C.

Whole-cells of *Rhodococcus* were resuspended in 100 mM KPi buffer (pH 6.2) to a final cell content of 100 mg/mL. Substrates (10 mM) were added to the reaction volume of 500 µL and incubated 24 hours at 28 °C at 1000 rpm. Small scale samples were extracted twice with EtOAc (2 x 250 µL), dried with Na2SO4 and analysed on GC-FID.

3.4.7 NMR-spectroscopy

^{1}H, ^{13}C-NMR and NOESY spectra were recorded on an Agilent (400 MHz and 101 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H-NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = duplet, dd = double duplet t = triplet, q = quartet, m = multiplet), coupling constant, integration. Data for ^{13}C-NMR are reported in terms of chemical shift.

3.4.8 Gas-chromatography (GC)

Achiral GC-FID analysis was performed with a Shimadzu type GC-2010 Plus equipped with a CP Wax 52 CB column (50 m x 0.53 mm x 2.0 µm) using N_2 as carrier gas. The following conditions were used for the separation using direct injection: injector 280 °C, detector (FID) 280 °C, column flow rate 1.43 mL/min: (*i*) (*E*)-4-hydroxy-3-methylbut-2-enoic acid temperature programme: start at 90 °C, hold time 3 min, rate 5 °C/min to 250 °C hold time 1 min; (*ii*) *c*-hexenone temperature programme: start at 80 °C, hold time 2 min, rate 10 °C/min to 125 °C, hold time 5 min, 20 °C/min to 250 °C, hold time 1 min. Aliphatic, unsaturated aldehydes were measured on the same GC equipment with using a split-injection: split ratio 30:1, column flow rate: 0.96 mL/min. Temperature programme: start at 80 °C, hold time 3.5 min, rate 10 °C/min to 110 °C, hold time 1 min, rate 10 °C/min to 125 °C, hold time 1 min, rate 10 °C/min to 175 °C, hold time 1 min, rate 10 °C/min to 205 °C, hold time 1 min, rate 20 °C/min to 250 °C, hold time 3 min.

Chiral GC-FID analysis was performed with a Shimadzu type GC-2010 Plus equipped with a Chirasil Dex CB column (25 m x 0.32 mm x 0.25 µm) using He as carrier gas. The following conditions were used for the separation using a split-injection: injector

250 °C, detector (FID) 275 °C, column flow rate 1.14 mL/min: (*rac*/*R*/*S*)-2-methylbutane-1,2,4-triol temperature programme: start at 120 °C, hold time 40 min, rate 25 °C/min to 230 °C hold time 3 min.

3.4.9 High resolution liquid chromatography – mass spectrometry (HRLC-MS)

HRLC-MS data was obtained using a Waters® acquity UPLC system and a Waters® Q-Tof PremierTM mass spectrometer. Supernatants of the samples from labelling studies were directly used for injection. A mobile phase of acetonitrile/water with 0.1 % formic acid was used following a gradient method. All spectra were obtained in positive ion mode.

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4

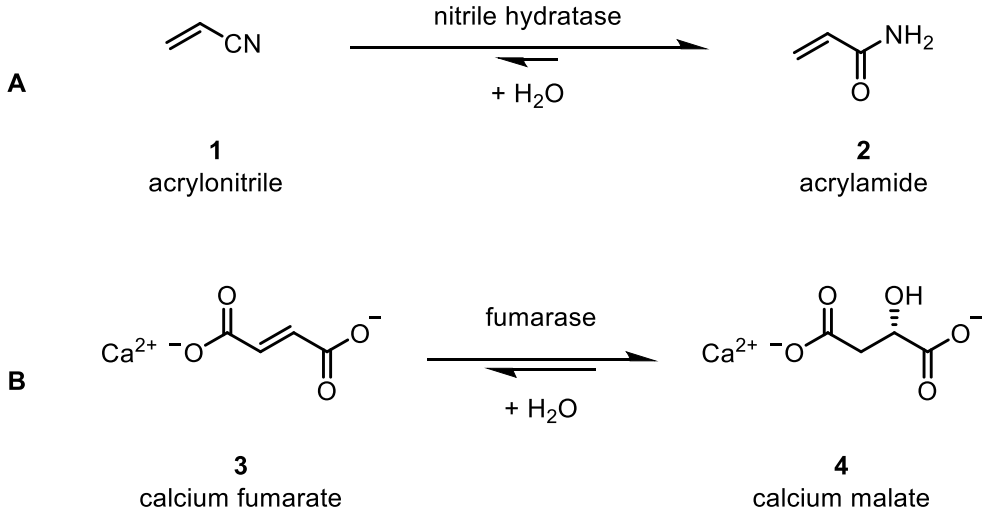
Optimisation and expansion of microbial Michael hydratase activity in the genus *Rhodococcus*

The gram-positive *Rhodococcus* serves as rich resource for novel biocatalysts due to their presence of large numbers of catabolic genes. One example of such an intriguing enzyme is the ‘Michael hydratase’ which selectively adds water to α,β-unsaturated carbonyl compounds. In this chapter, the microbial activity of *R. rhodochrous* ATCC 17895 towards (*E*)-4-hydroxy-3-methylbut-2-enoic acid is examined and optimised using a statistical ‘Design of Experiments’ approach. Furthermore, the presence of the desired Mhy in several *Rhodococcus* strains is investigated. Finally, the location within the cells was determined leading to the discovery that the Mhy responsible protein(-complex) is membrane-associated.

4.1 Introduction

Chemists challenge themselves by formulating a palette of so-called ‘dream reactions’ which are troublesome or impossible to carry out with existing tools and techniques. Here, often the identification of novel catalysts is a helpful tool to explore new synthetic routes thereby opening up new alternatives to existing processes. One of these ‘dream reactions’ that was formulated in the past is the hydroxyfunctionalisation of (un)-activated olefins to give secondary and especially tertiary alcohols.[1-3] This reaction in particular was shown to be difficult to be fulfilled with classical chemical reactions,[4,5] but is easier accessible by using hydratases (**Chapter 3**). As explained in depth in **Chapter 2** of this thesis, the genus *Rhodococcus* gained immense interest, industrially as well as academically, for presenting a broad variety of novel and useful biocatalysts. These bacterial systems can either be exploited as whole-cell catalysts or as a source of enzymes with exceptional properties which can be heterologously expressed in host cells as *Escherichia coli* (*E. coli*) or *Pichia pastoris* (*P. pastoris*).[6-8]

To demonstrate the industrial application of hydratases, the nitrile hydratases serve as prime example: the usage of nitrile hydratases from *Rhodococcus rhodochrous* J1 (*R. rhodochrous* J1) yields more than 400.000 tons of acrylamide (**2**) per year[9,10] and is thereby the first petrochemical industry process that uses a biocatalyst to produce a bulk chemical (Scheme 1A).[11,12] Another example of the production of a chiral compound on industrial scale using an hydratase is the production of **L**-malic acid (**4**) which is used in food, cosmetic and pharmaceutical industries. An amount of 2000 tons of **L**-malic acid (**4**) is produced by the bioconversion of fumaric acid (**3**) catalysed by a fumarase present in suspended whole-cells of *Corynebacterium glutamicum* every year by the Amino GmbH, Germany.[11,13,14] In this process, only **L**-malic acid (**4**) is produced with no traces of **D**-malic acid (Scheme 1B).

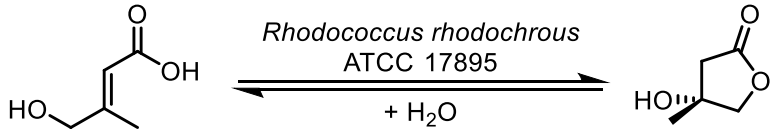


Scheme 1: Industrially used hydratases. A: nitrile hydratase catalysed acrylamide production; B: fumarase catalysed water addition to calcium fumarate.

But even though this biocatalytic approach provides the product with an excellent stereoselectivity and in very good yields, it also serves as example for the downside of enzymatic processes. In many cases enzymes are very substrate-specific due to the unique 3-dimensional structure in their active sites leading to only a limited number of possible reactions. This is particularly true for many described hydratases which are often part of the primary metabolism. To proceed with the above mentioned example, fumarases are able to add water to fumarate, (di-)fluorofumaric acid as well as chlorofumaric acid at sufficient reaction rates. However, converting the bromo-, iodo- and methylderivates causes a significant decrease in reaction rates. Additionally, both (*Z*)-configurated butenedioic acid as well as monoacids are not accepted as substrates.[15-17] The transition state theory assumes that the active site of an enzyme is designed and shaped in such a way that the transition state of the natural substrate structure is maximally stabilised. Therefore, already a small change in the substrate structure can cause less stabilisation and subsequently less sufficient reaction rates or eventually lead to unfeasible reactions. Hence, enzymes are successfully applied in the synthesis of highly specific products, but at the same time they often only convert a small number of substrates. Protein engineering approaches offer a way to adapt the 3-dimensional structure of the protein to *e.g.* broaden the range of accepted substrates. The active site of the enzyme is then re-designed in such a way to stabilise more or other, different unnatural substrates in their respective transition states. An

alternative to changing the properties of known proteins, however, is the identification of new biocatalysts with undescribed characteristics and the ability to convert new substrates.

Holland *et al.* were first to report of a novel Michael hydratase activity with resting whole-cells of *Rhodococcus rhodochrous* ATCC 17895 acting on (*E*)-4-hydroxy-3-methylbut-2-enoic acid (Scheme 2, revised substrate structure).[18,19] Although the substrate scope was smaller than previously reported, the Michael hydratase still represents an intriguing biocatalyst for the generation of tertiary alcohols.[19]



5 **6**

Scheme 2: Stereoselective water addition to (*E*)-4-hydroxy-3-methylbut-2-enoic acid catalysed by *Rhodococcus rhodochrous* ATCC 17895.

The aim of this chapter was to establish and optimise a *R. rhodochrous* ATCC 17895 whole-cell reaction system for the bioconversion of (*E*)-4-hydroxy-3-methylbut-2-enoic acid in terms of shorter preparation times, less chemicals used and overall lower production costs of the whole-cell biocatalyst. With this, the correlation between the growth conditions and the final product concentration was investigated. In order to identify a more active biocatalyst, 16 additional members of the *Nocardiacae* family were screened under various growth conditions. To subsequently lay the groundwork for the identification of the Mhy encoding gene, four uncharacterised strains were sequenced. Additionally, the location of the responsible protein within the cell was determined by subcellular fractionation experiments.

4.2 Results

4.2.1 Growth medium optimisation

Previous reports stated that a resting cell suspension of whole-cells from *R. rhodochrous* ATCC 17895 converts substrate **5** to give final product **6** (Scheme 2).[18] To investigate possible protein candidates responsible for the Mhy activity, it was first tried to increase the amount of expressed Mhy hydratase. For this, the growth conditions were optimised as well as the influence of the addition of model substrate **5** during bacterial growth was studied.

Metabolic processes are tightly regulated at the control of transcription, translation and even by allosteric and post-translational processes. If an enzyme coding gene is under transcriptional control by its substrate or product or another metabolite, the expression can be induced and enhanced by the addition of the specific compound to the growth medium. These mostly small organic molecules are so-called 'inducer' molecules which can be added to the bacterial culture during the growth phase. The expression of the oleate hydratase from *Elizabethkingiia meningoseptica* was *e.g.* shown to be upregulated by the supplementation of the substrate oleic acid.[20] Another example is given by the β-galactosidase from *E. coli*: upon addition of succinate, the cells produced 25 % of the soluble cell protein as β-galactosidase while the addition of other carbon-sources like glycerol, glucose or lactose resulted in significantly lower levels of β-galactosidase.[21] It was therefore tried to increase the amount of expressed Michael hydratase by adding 0.67 mM of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) to a growing culture of *R. rhodochrous* ATCC 17895. However, while the untreated cells showed a yield of 34 % in the subsequent bioconversion of **5**, the substrate-induced cells only showed a yield of 1.9 % leading to the conclusion that the addition of model substrate **5** is not beneficiary but detrimental to this system.

Holland *et al.* reported the use of a rich growth medium to grow Mhy active *Rhodococcus* cells. This medium required next to peptone, yeast extract and glucose the addition of $MgSO_4$ and $Fe(II)SO_4$ with especially the unconventionally high concentration of required Fe^{2+}-ions (1.97 mM) standing out.[18] Iron is known to be toxic for the cell if added in too high concentrations.[22] Other reports involving the addition of iron to the growth medium of *Rhodococcus* cells fall in the range of 0.5 – 3.6 µM

which is nearly 550 times lower than the herein described amount.[23-25] Due to this, the influence of iron on this system is especially curious and was therefore investigated in detail. Subsequently, the influence of different ratios between the medium components $MgSO_4$, glucose, yeast extract and peptone on the Mhy whole-cell activity was explored using a Design of Experiments (DoE) approach.[26]

Varying concentrations of Fe^{2+}-ions (0 - 3.96 mM) were added to the growth medium while all other growth medium components were kept identical. The harvested cells were afterwards used in standardised biotransformations (Scheme 2) to evaluate the activity of the cells. Here, no advantage of adding high concentrations of Fe^{2+} was discovered (data not shown). On the contrary, adding no Fe^{2+}-ions to neither pre- and main-culture nearly doubled the product concentration which led to the decision to avoid the addition of $Fe(II)SO_4$ henceforth (Figure 1). This example serves to show that an excessive iron supplementation was more harmful than beneficial in growing more active *R. rhodochrous* ATCC 17895 cells due to the toxicity of iron towards the cell.

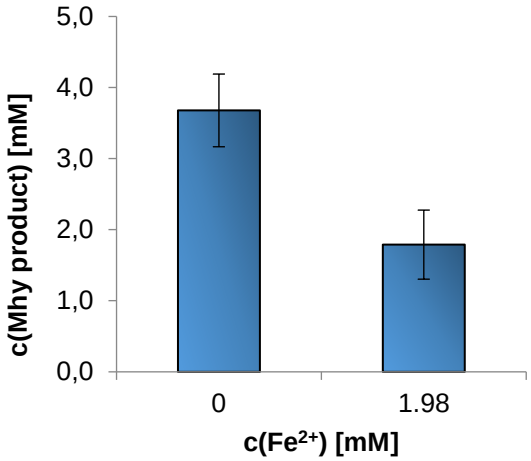


Figure 1: Influence of Fe^{2+}-supplementation to growth medium (pre- and main culture) on the final product concentration obtained *via* biotransformation catalysed by *R. rhodochrous* ATCC 17895 whole-cells. Reactions were normalised based on wet cell weight. *Reaction conditions:* 100 mg/ml cell content, 10 mM substrate concentration, 24 hours, 26 °C.

To investigate the influence of the other medium components (glucose, peptone, yeast extract and $MgSO_4$), two rounds of growth medium optimisation have been carried out using a statistical approach (DoE).[26] The first screening revealed that the glucose concentration in particular has a large influence on the final product formation. A higher

ratio led to lower product concentrations while a lower ratio of glucose to the other medium components showed higher final product formations. Additionally, it was shown that the concentration of $MgSO_4$ (investigated concentration ranged from 0 – 8.12 mM) had an insignificant influence on the final product production and the addition was therefore further on refrained from (data not shown). The second screening round concentrated on the optimal ratio of the three main components peptone, yeast extract and glucose. Again, smaller amounts of glucose led to higher product concentrations while the increase of both peptone and yeast extract yielded more final product. In order to further analyse the interactions of peptone and yeast extract while keeping the glucose concentration at the minimal tested concentration (6.59 g/L), a response surface analysis was designed (Figure 2A) with a predicted local maximum at a peptone concentration of 9.2 g/L and yeast extract concentration of 1.84 g/L. Experimental confirmation of the calculated local maximum resulted in a three-times higher product concentration compared to standard conditions described by Holland *et al.*[18] To order to extrapolate the data, a global maximum analysis was modelled with a global maximum at 12.2 g/L peptone and 2.78 g/L yeast extract (Figure 2B). An experimental validation of the postulated global maximum was carried out. Here, compared to the original growth medium composition described by Holland *et al.* the activity could not be enhanced by choosing the global optimum growth conditions.

The estimated global maximum did therefore not lead to higher product concentrations, but yielded on the contrary lower amounts than the local maximum. This shows that the optimisation process is only valid within a certain range when working with biological systems as informatics cannot fully predict the biological interactions. However, the determination of the local maximum led to a more active biocatalyst and simultaneously decreased the amount of chemical waste and preparation time.

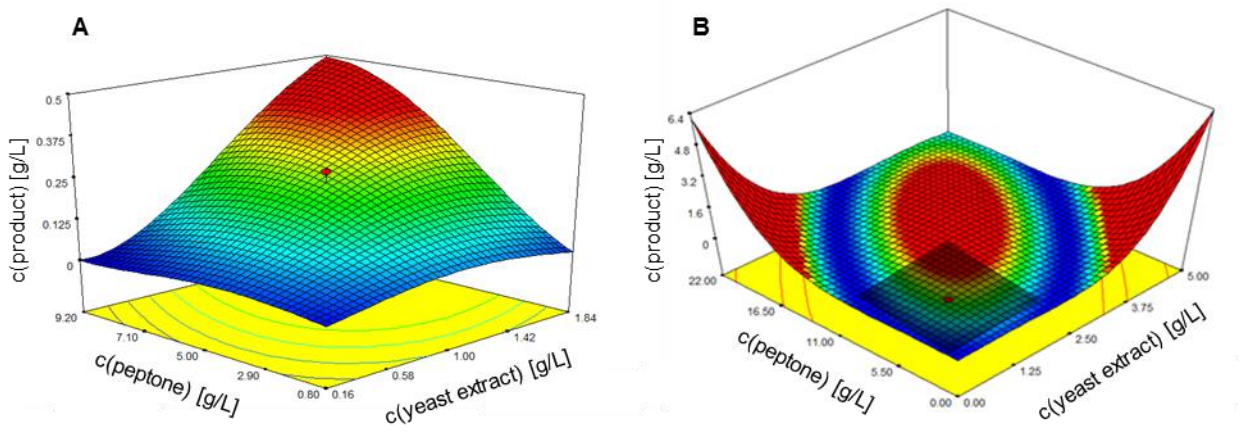


Figure 2: Response surface analysis of product concentration in function of peptone and yeast extract concentrations in growth medium. Glucose concentration was kept stable at 6.59 g/L. A: Local maximum with concentrations within the range of the experimental screening; B: Global maximum modelled by extrapolation, highlighted plane corresponds to area shown in A.

The overall results show that the Mhy activity of resting cells strongly depends on the composition of the growth medium and that the expression of Mhy can be engineered by varying the concentrations of medium components.

4.2.2 Vast spectrum of Michael hydratase active wild-type strains

To further investigate the presence of the Michael hydratase within the whole *Nocardiaceae* family, a broad range of family members has been cultivated under different conditions and tested for Mhy activity. While mostly exploring *Rhodococcus* strains from different species, the genera of *Nocardia*, *Williamsia* as well as *E. coli* were subjects of investigation as well (Table 1).

Table 1: List of investigated bacterial wild-type strains sub-divided into respective species.

#	Family	Genus	Species	Strain
1	*Nocardiaceae*	*Rhodococcus*	*erythropolis*	PR4
2	*Nocardiaceae*	*Rhodococcus*	*fascians*	DSM 20669
3	*Nocardiaceae*	*Rhodococcus*	*fascians*	DSM 43673
4	*Nocardiaceae*	*Rhodococcus*	*opacus*	DSM 43205
5	*Nocardiaceae*	*Rhodococcus*	*pyridinivorans*	DSM 44555

6	*Nocardiaceae*	*Rhodococcus*	*pyridinivorans*	DSM 20415
7	*Nocardiaceae*	*Rhodococcus*	*qingshengii*	DSM 46766
8	*Nocardiaceae*	*Rhodococcus*	*qingshengii*	DSM 45257
9	*Nocardiaceae*	*Rhodococcus*	*rhodochrous*	ATCC 17895
10	*Nocardiaceae*	*Rhodococcus*	*rhodochrous*	DSM 101666
11	*Nocardiaceae*	*Rhodococcus*	*rhodochrous*	DSM 43241
12	*Nocardiaceae*	*Rhodococcus*	*rhodochrous*	DSM 43273
13	*Nocardiaceae*	*Rhodococcus*	*ruber*	DSM 45280
14	*Nocardiaceae*	*Rhodococcus*	*ruber*	DSM 43338
15	*Nocardiaceae*	*Rhodococcus*	*sp.*	R312
16	*Nocardiaceae*	*Nocardia*	*seriolae*	DSM 44129
17	*Nocardiaceae*	*Williamsia*	*sterculiae*	DSM 45741
18	*Enterobacteriaceae**	*Escherichia*	*E. coli*	TOP10

**E. coli* TOP10 cells inherit an empty pBADHisA expression vector.

All strains have first been grown under the optimised custom rich conditions (6.59 g/L glucose, 9.2 g/L peptone, 1.84 g/L yeast extract). However, not all strains revealed the desired Mhy activity which led to the decision to test all strains under three additional growth medium conditions recommended by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). All strains were grown under the same conditions (time, temperature, rpm) and the results of the whole-cell bioconversions were afterwards normalised based on a total protein concentration of 3 mg/ml determined *via* standard BCA assays (Figure 3).

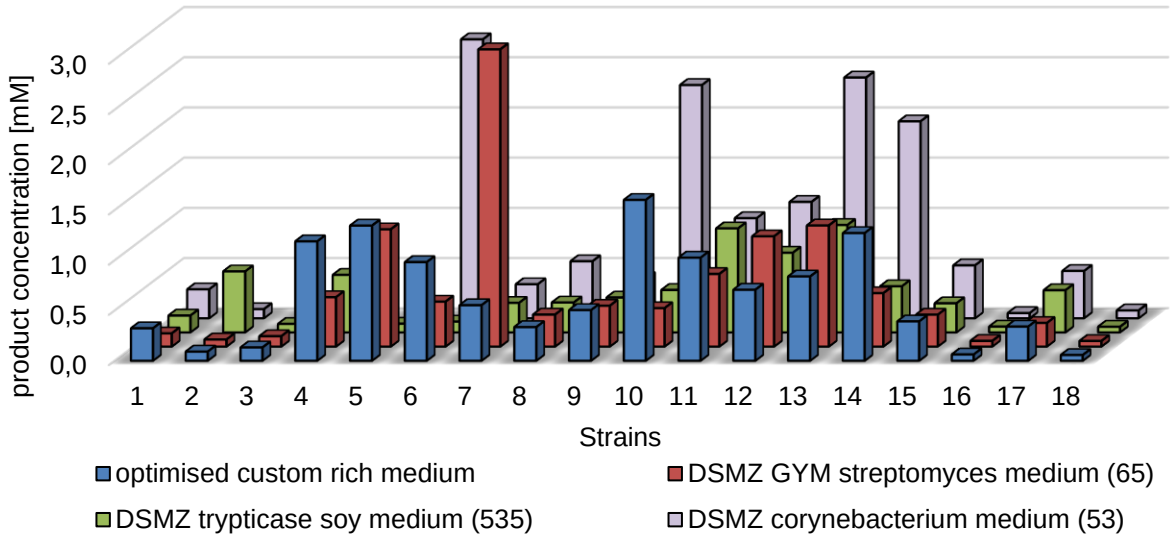


Figure 3: Growth medium screening with 18 strains grown under four conditions: optimised custom rich medium (blue), DSMZ GYM Streptomyces medium (65, red), DSMZ trypticase soy medium (535, green), DSMZ corynebacterium medium (53, violet). All reactions have been performed in triplicates. *Reaction conditions:* 3 mg/ml total protein concentration, 10 mM substrate concentration, 24 h, 28 °C.

E. coli was shown to be inactive towards substrate **5** and was hereafter used as negative control system. All other strains did, however, show at least a small Mhy activity under any given growth condition. The productivity of *N. seriolae* DSM 44129 (Figure 3, strain 16) was further increased with longer incubation times (> 5 days, data not shown). While some strains presented a comparable Mhy activity under any given growth condition (*e.g*. strains 1, 8, 12, 15, 17), the Mhy activity of other strains was clearly enhanced with specific growth media (*e.g*. strains 6, 7 or 13). This screening clearly shows the relevance of the chosen growth medium which is highly specific for each strain. An overall trend, however, can be seen in comparably lower activities when using the trypticase soy medium and *vice versa* higher activities with corynebacterium medium. The latter includes a high concentration of peptone with was shown to have a beneficial influence on the Mhy activity in the optimised custom rich medium in the growth medium optimisation trials while trypticase soy medium includes tryptone and an enzymatic digestion of soy bean. Presumably, this change of the prevalent nitrogen source causes the differences in activity.

Originally, it was intended to identify a Mhy inactive strain that is in close phylogenetic relation to an active Mhy strain. With this, the two strains could be compared to filter out the protein-encoding genes only present in the Mhy active strain thereby decreasing the number of possible Mhy candidates significantly. However, as all tested *Rhodococcus* cells showed Mhy activity, it was decided to analyse and compare all active strains to later on identify Mhy candidates.

4.2.3 Genomic characterisation of Mhy active strains

To be able to link the Michael hydratase activity to genomic information, whole-genome sequences had to be obtained for undescribed strains. While 14 of the investigated strains were already sequenced and the data made publically available on NCBI (National Center for Biotechnology Information),[27] the genomic DNA (gDNA) of the non-sequenced strains was extracted and subsequently sequenced (Table 2).

Table 2: Sequence properties of in-house sequenced *Rhodococcus* strains annotated by RAST.[28]

	R. fascians DSM 43673	*R. pyridinivorans* DSM 20415	*R. rhodochrous* DSM 101666	*N. seriolae* DSM 44129
Genome size [kb]	5,512,432	5,275,644	6,120,791	8,046,074
GC content [%]	64.6	67.7	67.8	68.0
N50	894,937	204,034	5,517,475	8,046,074
L50	2	7	1	1
number of contigs (> 1kbp)	19	174	3	1
number of subsystems	426	426	318	323
number of coding sequences	5330	5018	5767	9798
number of RNAs	56	67	67	73

All three *Rhodococcus* strains show an expected genome size (5.2 – 6.1 Mbp) while the *Nocardia* strain has a larger size of 8 Mbp. The GC content was as expected for

Nocardiacae family members in the range of 64 – 68 %. While the strains *R. fascians* DSM 43673 and *R. pyridinivorans* DSM 20415 showed a larger amount of contigs (19 and 174, respectively), *R. rhodochrous* DSM 101666 yielded three contigs and *N. seriolae* DSM 44129 only one. The latter strain is therefore likely to only consist of only one large chromosome while *R. rhodochrous* DSM 101666 consists of a large chromosome (5.52 Mbp) and two plasmids (441 kB and 162 kB) with at least the latter being circular.

4.2.4 Location of Mhy in cells from *Rhodococcus*

In order to establish where the desired Michael hydratase is located within the *Rhodococcus* cell, a subcellular fractionation was performed. Here, cullular components are separated from each other by procedures including *e.g.* selective centrifugation.[29] First, whole-cells were disrupted to separate cell pellet from cell free extract (CFE). A subsequent ultra-centrifugation step of the cell free extract yielded the isolated membrane removed as a pellet separated from the soluble proteins. Each fraction was applied to a bioconversion of model substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**5**) to confirm the respective Mhy activity (Figure 4).

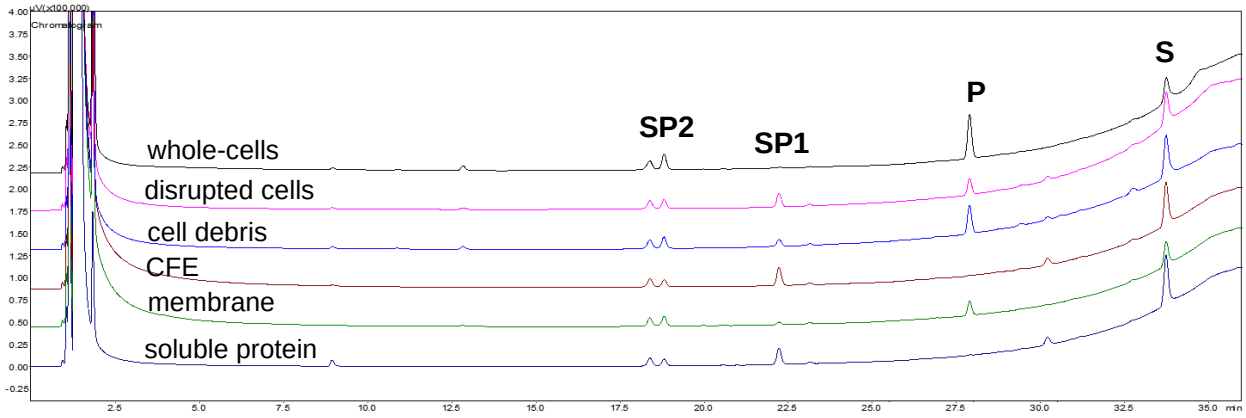


Figure 4: Gas chromatograms showing bioconversions with fractions obtained in subcellular fractionation. *From top to bottom*: whole-cells (black), disrupted cells (pink), cell debris (blue), CFE (brown), membranes (green), soluble protein (purple). *Retention times*: 33.5 min (**S**, substrate **5**), 27.7 min (**P**, product **6**), 22.3 min (**SP1**, competing side-product), 18.6 min (**SP2**, water elimination side-product methylfuranone). *Reaction conditions*: 100 mg/mL cell content for solid fractions/1 mL of obtained liquid fractions, 10 mM substrate concentration, 24 h, 26 °C.

Whole-cells, disrupted cells as well as the obtained cell debris showed the expected product **6**. The cell debris still showed Mhy activity due to the presence of unbroken cells and membranes that were not suspended in the CFE as the disruption of the thick cell walls of gram-positive bacteria like *Rhodococci* is difficult to achieve and was therefore insufficient. The CFE only showed minor product formation which can be explained with a higher dilution factor used for liquid fractions causing lower total protein concentrations used in the bioconversion. Upon separating the membranes from the soluble protein, only the membranes showed the formation of desired product **6** with no detectable Mhy activity located in the liquid soluble protein fraction. Even a subsequent concentration of the soluble protein fraction to increase the total protein concentration did not lead to any desired product formation (data not shown). This experiment therefore clearly proved that the Mhy was associated with the plasma membrane and was not a soluble protein.

Interestingly, further analysis of the subcellular fractionation GC data revealed the presence of an additional side-product (Fig. 4, **SP2**) with a retention time of 22.5 min. This new side-product, whose structure stayed elusive, was detected in all fractions except in the unbroken whole-cells. This led to the conclusion that substrate **5** was also a substrate for another enzyme which was present in the cytoplasm. With this result, it was assumed that substrate **5** could not reach the cytoplasm through the unbroken whole-cells, which became only accessible after disrupting the whole-cells. Furthermore, it was shown that the isolated membrane fraction only showed a minor side-product **SP2** formation compared to the other fractions. This further confirms the presence of two competing enzymes acting on substrate **5** which could also explain the low product **6** production when using the CFE.

Subcellular fractionations were carried out for two additional strains with high activity to confirm that the Mhy is located in the membrane as well. For this, *R. pyridinivorans* DSM 20415 and *R. ruber* DSM 45280, which displayed high Mhy activities, were chosen and treated under the same optimised subcellular fractionation conditions. Also here, respective whole-cells displayed Mhy activity as well as the disrupted cells (data not shown). Furthermore, even with highly concentrated soluble protein fractions, no desired product was formed. This result undoubtedly established the presence of the

Michael hydratase in the membrane fraction which means that the protein under investigation is incorporated or attached to the plasma membrane of *Rhodococcus* species.

4.3 Conclusion

This chapter demonstrates the stepwise optimisation of the growth conditions for *R. rhodochrous* ATCC 17895 to generate a more active whole-cell biocatalyst for the selective water addition to Michael acceptor (*E*)-4-hydroxy-3-methylbut-2-enoic acid. The addition of Fe^{2+} in high concentrations was shown to be detrimental while the addition of Mg^{2+} only had negligible effects on the overall performance. By excluding unnecessary components from the growth medium, resources like time and costs for the overall process were reduced. The combination and ratio of the three main components glucose, yeast extract and peptone was shown to affect the Mhy activity significantly with lower glocuse concentrations resulting in cells with the highest Mhy activity. To evaluate the abundance of the desired Mhy within the *Nocardiacae* family, 17 family members and one *E. coli* strain were grown with four different growth media and tested for Mhy activity. Here, all *Nocardiacae* family members showed the desired Mhy activity. However, the activity was highly dependent on the chosen growth medium. *E. coli* on the other hand was not able to form product under any of the chosen conditions. Finally, this chapter includes the assessment of the location of the desired Michael hydratase within the cell. A subcellular fractionation led to the conclusion that the protein(-complex) is membrane-associated.

4.4 Experimental

4.4.1 General information

Unless reported otherwise, all commercial chemicals were purchased from Sigma Aldrich. Calcium carbonate was bought from Acros Organics. Hydroxyacetone was purchased from Alfa Aesar. Peptone and yeast extract were bought from BD Becton, Dickinson and Company. Sodium hydroxide was purchased from J.T. Baker while glucose was bought from Fluka Analytical. Chloroform-*d*, D_2O (99.9%) and dimethylsulfoxide-d_6 were bought from Euriso-Top. Petroleum ether (PE) was purchased from VWR International and distilled before utilisation.

Enzymes ribonuclease A from bovine pancreas and deoxyribonuclease I from bovine pancreas were from Sigma-Aldrich as well as lysozyme and the rOche complete protease inhibitor tablets.

4.4.2 Statistical software

The growth medium optimisation was carried out using the software Design Expert ver. 7.0.0 (Stat-Ease Inc., Minneapolis, MN). For all designs, the concentrations of product (g/L) were used as responses which were analysed using the analysis of variance (ANOVA) combined with the Fisher's test to evaluate if a given variable had a significant effect for a confident interval of 5 % ($p < 0.05$).

4.4.2.1 Screening experiments

A 2^{4-1} factorial fractional design with 1 generator (p=1), resolution IV, was used in order to study the effect of four numeric factors: glucose (A), peptone (B), yeast extract (C) and magnesium sulphate heptahydrate (D) as well as their respective interactions. Using one replicate, one block and four central points, a matrix of 12 experiments (eight factorial points plus four central points) was developed, choosing empirically numeric values for the two levels (+, -) of the four factors (A, B, C, D). The central points contained the factors at reference levels (reference culture medium composition described by Holland *et al*.[18]). The factorial points were randomised by the software, generating together with the central points a matrix of experiments. The matrix of experiments in terms of coded factors (-, 0, +) as well as the compositions of each culture media (g/L) are shown below (Table 3).

Table 3: Matrix of experiments showing coded factors (-, 0, +) and specific concentrations of medium components (g/L).

	Glucose (A)	**Peptone (B)**	**Yeast extract (C)**	**Magnesium sulphate heptahydrate (D)[a]**
1	0 (15)	0 (1)	0 (5)	0 (1)
2	+ (30)	- (0.5)	- (2.5)	+ (2)
3	- (7.5)	- (0.5)	- (2.5)	- (0)
4	0 (15)	0 (1)	0 (5)	0 (1)
5	0 (15)	0 (1)	0 (5)	0 (1)

6	+ (30)	+ (2)	- (2.5)	- (0)
7	+ (30)	+ (2)	+ (2.5)	+ (2)
8	+ (30)	- (0.5)	+ (2.5)	- (0)
9	+ (30)	+ (2)	+ (2.5)	- (0)
10	- (7.5)	- (0.5)	+ (2.5)	+ (2)
11	- (7.5)	+ (2)	- (2.5)	+ (2)
12	0 (15)	0 (1)	0 (5)	0 (1)

[a] Magnesium heptahydrate concentrations in (g/L) correspond to 0 mM (coded factor -), 4.06 mM (coded factor 0) and 8.12 mM (coded factor +).

4.4.2.2 Response surface methodology experiments

A full circumscribed central composite design (CCD) was developed. The numeric factors chosen were glucose (A), peptone (B), yeast extract (C). Each numeric factor was varied over five levels: '+α and –α' (axial points), '+ and –' (factorial points) and a central point (0). The total number of runs was 20, consisting of six repeated central points, six axial points and eight factorial points. The numeric values for the levels of the factorial points and axial points were chosen empirically. The central points contained the factors at reference levels (reference culture medium composition described by Holland *et al.*[18]) and the factorial points were randomised by the software generating, together with the axial points and the central points, a matrix of experiments. The matrix of experiments in terms of coded factors (-α, -, 0, +, +α) together with the respective compositions of each culture media (g/L) are shown below (Table 4).

Table 4: Matrix of experiments showing coded factors (-α, -, 0, +, + α) and specific concentrations of medium components (g/L).

	Glucose (A)	**Peptone (B)**	**Yeast Extract (C)**
1	0 (15)	0 (5)	0 (1)
2	0 (15)	0 (5)	0 (1)
3	+α (23.4)	0 (5)	0 (1)
4	- (10)	- (2.5)	- (0.5)
5	- (10)	- (2.5)	+ (1.5)
6	+ (20)	+ (7.5)	- (0.5)
7	+ (20)	- (2.5)	+ (1.5)

8	0 (15)	+α (9.2)	0 (1)
9	- (10)	+ (7.5)	- (0.5)
10	0 (15)	0 (5)	+α (1.84)
11	0 (15)	0 (5)	0 (1)
12	+ (20)	- (2.5)	- (0.5)
13	0 (15)	-α (0.8)	0 (1)
14	0 (15)	0 (5)	-α (0.16)
15	0 (15)	0 (5)	0 (1)
16	0 (15)	0 (5)	0 (1)
17	+ (20)	+ (7.5)	+ (1.5)
18	-α (6.59)	0 (5)	0 (1)
19	0 (15)	0 (5)	0 (1)
20	- (10)	+ (7.5)	+ (1.5)

4.4.3 Analytical methods

4.4.3.1 NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Agilent (400 MHz and 100 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H-NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = duplet, dd = double duplet t = triplet, q = quartet, m = multiplet), integration. Data for ^{13}C-NMR are reported in terms of chemical shift.

4.4.3.2 Gas chromatography (GC)

Achiral GC analysis of the bioconversions of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (X) was performed with a Shimadzu type GC-2010 Plus equipped with a CP Wax 52 CB column (50 m x 0.53 mm x 2.0 µm) using N_2 as carrier gas. The following conditions were used for the achiral separation using direct injection: injector 280 °C, detector (FID) 300 °C, column flow rate 1.43 mL/min, linear velocity 37.0 cm/sec, pressure 98.5 kPa, temperature program: start at 90 °C, hold time 3 min, rate 5 °C/min to 250 °C hold time 1 min.

4.4.4 Chemical synthesis procedures

The syntheses of compounds (*E*)-ethyl-4-hydroxy-3-methylbut-2-enoate, (*E*)-4-hydroxy-3-methylbut-2-enoic acid and (*R*)-4-hydroxy-4-methyldihydrofuran-2(*3H*)-one have been described earlier (Chapter 2) and are in line with literature.[30-32]

4.4.5 Microorganisms and microbiological protocols

4.4.5.1 Microorganisms and cell cultures

4.4.5.1.1 Cultivation of *Rhodococcus, Nocardia, Williamsia* and *Escherichia coli* wild-type strains

Rhodococcus rhodochrous ATCC 17895 (#10) was purchased from ATCC (American Type Culture Collection, Manassas, USA). Strains 1-9 and 11-17 were purchased at DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and *E. coli* TOP 10 cells (#18) were obtained from Fisher Scientific (Landsmeer, The Netherlands).

The organisms were maintained at 4 °C on a nutrient agar plate (3 g beef extract, 5 g peptone, 15 g agar dissolved 1 L de-ionised water and autoclaved at 121 °C) and were regularly sub-cultured. The optimised custom rich growth medium used consisted of 1 L de-ionised water with 6.59 g glucose, 9.2 g peptone, 1.84 g yeast extract, 7 mM K_2HPO_4 (1.2 g), 3 mM KH_2PO_4 (0.4 g) with a final pH of 6.8 and was autoclaved at 110 °C. The Corynebacterium medium consisted of 1 L de-ionised water with 10 g of casein peptone, tryptic digest, 5 g yeast extract, 5 g glucose, 5 g NaCl (and 15 g agar for Corynebacterium agar) and was autoclaved at 110 °C. The GYM Streptomyces medium consisted of 1 L de-ionised water with 4 g glucose, 4 g yeast extract, 10 g malt extract (and 2 g $CaCO_3$ and 15 g agar for GYM Streptomyces agar) and was autoclaved at 110 °C. The trypticase soy broth consisted of 1 L de-ionised water with 30 g trypticase soy broth (and 15 g agar for trypticase soy broth agar) and was autoclaved at 121 °C.

Table 5: Overview of medium components of the four tested growth media (g/L).

Components	Custom rich medium	Corynebacterium Medium (53)	GYM Streptomyces medium (65)	Trypticase soy medium (535)
glucose	6.59	5	4	--
peptone	9.2	--	--	--
casein peptone (tryptic digest)	--	10	--	--
malt extract	--	--	10	--
trypticase soy broth	--	--	--	30
yeast extract	1.84	5	4	--
NaCl	--	5	--	--
K_2HPO_4	1.2	--	--	--
KH_2PO_4	0.4	--	--	--
pH	6.8	7.2-7.4	7.2	7.3

For large scale growth experiments, a preculture was inoculated with a single colony and grown overnight at 28 °C. The preculture (2 mL) was used to inoculate 1 L of culture medium grown in 5 L flasks. The culture was incubated for 72 hours at 28 °C (or 120 h for *Williamsia sterculiae*) with 180 rpm orbital shaking. The cells were harvested by centrifugation (17.696 x g, 15 min, 4 °C) with a subsequent removal of the supernatant. The culture was washed with potassium phosphate buffer (100 mM, pH 6.2) and centrifuged again (17.696 x *g*, 15 min, 4 °C). The supernatant was discarded, the pellet stored at -20 °C.

For the growth medium screening experiment, 5 mL of respective medium grown in 15 mL falcon tubes, was inoculated with a single colony. The culture was then incubated for 72 hours at 28 °C with 180 rpm orbital shaking. The harvest, washing step and storage were carried out as described above.

4.4.5.1.2 Small scale biotransformations and extraction procedures

Whole-cells of *Rhodococcus, Nocardia* and *Williamsia* and *E. coli* were resuspended in 100 mM KPi buffer (pH 6.2) to either a final cell content of 100 mg/ml or total protein

concentration of 4 mg/ml (determined with standard BCA assay). The substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (1) (10 mM) was added to the reaction volume of 1 ml and incubated 24 hours at 28 °C at 1000 rpm. Small scale samples have been extracted twice with EtOAc (2 x 500 µL), dried with Na_2SO_4 and injected on GC.

4.4.5.1.3 Subcellular fractionation

The harvested cells were resuspended in 50 mM KPi buffer (pH 7.4, 10% glycerol, 2 mM $MgSO_4$, 300 mM NaCl). A spatula tip of DNAse and RNAse as well as 1 mM phenylmethylsulfonyl fluoride (PMSF) and a rOche complete protease inhibitor tablet were added and incubated at room temperature for 60 minutes. The cells were disrupted using a cell disrupter (2.00 kbar, 2 cycles). The lysate was centrifuged at 10.000 rpm (17.696 x *g*), 15 min at 4 °C. The pellet containing (un)broken cells was collected and stored at -20 °C. The cell free extract containing soluble proteins and membranes was collected and ultracentrifuged at 45.000 rpm (158.000 x *g*) for 1 h at 4 °C. The pelleted membranes were resuspended in KPi buffer (pH 7.4, 10% glycerol, 2 mM $MgSO_4$, 300 mM NaCl), homogenised using a Potter-Elvehjem homogeniser and subsequently added dropwise to liquid nitrogen. The membrane was stored at - 80 °C. The supernatant containing the soluble proteins was collected.

4.4.5.2 Genomic DNA extraction

The genomic DNA of strains *R. pyridinivorans* DSM 20415, *R. fascians* DSM 43673, *R. rhodochrous* DSM 101666 and *N. seriolae* DSM 44129 were isolated from a cell pellet (50 – 100 mg) using the UltraClean®Microbila DNA Isolation Kit following the procedure recommended by the manufacturer.

4.4.5.3 Whole-genome sequencing and *de-novo* assembly

4.4.5.3.1 Sequencing

Strains *R. pyridinivorans* DSM 20415, *R. fascians* DSM 43673 and *R. rhodochrous* DSM 101666 were in-house sequenced on a MiSeq sequencer (Illumina, San Diego, CA) to obtain an 300 cycle paired-end library with an insert-size of 550 bp using PCR-free library preparation. For the nanopore sequencing of strain *N. seriolae* 44129, an 1D sequencing library (SQK-LSK108) was loaded onto an FLO-MIN106 (R9.4) flowcell, connected to the MinION Mk1B. MinKNOW software (Oxford Nanopore) was

used for quality control of active pores and for sequencing. Raw files generated by MinKNOW were base called, on a local compute server (HP ProLiant DL360 G9, 2x XEON E5-2695v3 14 Cores and 256 RAM), using Albacore (version 2.3.1; Oxford Nanopore). Reads, in fastq format, with minimum length of 1000 bp were extracted, yielding 718 Megabase sequence with an average read length of 7.44 kb.

4.4.5.3.2 *De-novo* assembly

De-novo assembly of strains *R. pyridinivorans* DSM 20415 and *R. fascians* DSM 43673 were performed using SPAdes (version 3.9.0).[33] The *de-novo* assembly of strains *R. rhodochrous DSM 101666* and *N. seriolae DSM 44129* were performed using Canu (v1.4, settings: genomesize=6m).[34] *R. rhodochrous* DSM 101666 produced a 6.12 Megabase genome with three contigs: the largest with a length of 5.52 Megabase and two smaller with lengths of 441.03 and 162.29 kilobase, respectively. Paired-end Illumina library was aligned to the assembly using BWA[35] and the resulting BAM file (Binary alignment map file) was processed by Pilon[36] for polishing the assembly (for correcting assembly errors) using a correction of only SNPs and short indels (–fix bases parameter). *N. seriolae* DSM 44129 produced a single contig with a length of 8 Megabase. For correcting assembly errors, the raw nanopore reads were mapped to the 8 MB contig using minimap2[37] and converted with samtools[38] to Binary alignment map (BAM). The resulted BAM file was processed with Nanopolish[39] for the final polishing (error correcting).

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5

The attempted identification of a novel Michael hydratase from *Rhodococcus*

The isolation or identification of a novel Michael hydratase (Mhy) from *Rhodococcus* was aimed for by a combined approach of (*i*) enzyme solubilisation using various detergents or styrene-maleic acid copolymers, (*ii*) comparative genomic studies as well as (*iii*) membrane proteomic studies and (*iv*) in-silico analysis of identified candidates. While all isolation efforts failed, the combination of results obtained from the –omics studies led to the identification of potential Michael hydratase candidates. The identified membrane proteins were cloned and heterologously expressed in *Escherichia coli* (*E. coli*) and tested for the desired water addition activity towards (*E*)-4-hydroxy-3-methylbut-2-enoic acid. Unfortunately, none of the tested candidates showed the desired activity leaving the Mhy behind as a yet elusive membrane-associated hydratase.

5.1 Introduction

In the course of identifying novel proteins responsible for catalysing attractive reactions, the location of the protein within the cell has to be determined first. Soluble proteins can be easily purified while membrane proteins are considered as more troublesome due to their interaction with the plasma membrane. The plasma membrane of bacteria is formed by a bilayer of phospholipids and incorporates a multitude of proteins. The amphiphilic phospholipids are organised in such a way that the polar heads face outwards while the hydrophobic tails point inwards thereby establishing a two-layer lipid system. Naturally, proteins can interact with this bilayer system in three different ways resulting in the classification of membrane proteins (MP) into the subclasses of integral, peripheral and amphitropic (lipid-anchored) membrane proteins (Figure 1).[1]

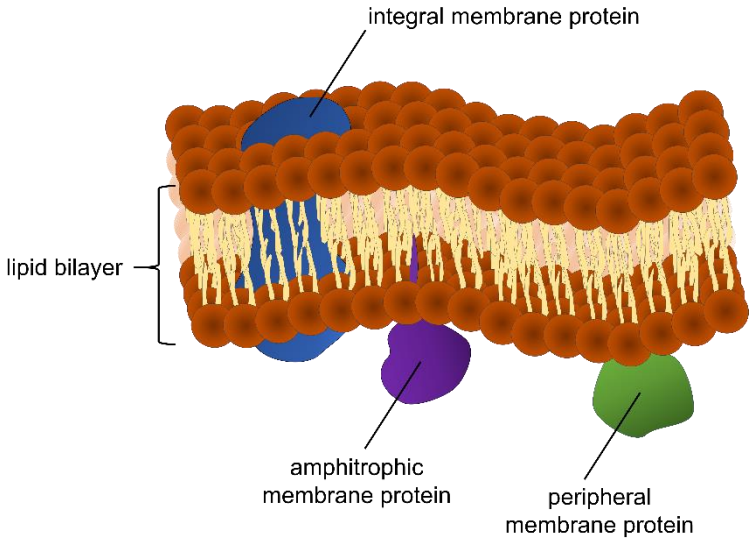


Figure 1: Model of plasma membrane with different types of membrane proteins (MPs).

Peripheral MPs interact with the lipid-bilayer only non-covalently *via* electrostatic interactions or hydrogen bonds while integral membrane proteins show stronger interactions with the hydrophobic moieties of the phospholipid bilayer.[2] In order to fully characterise and understand the function of membrane proteins, it is important to find conditions to stabilise them dissolved from the lipid-bilayer in a soluble form.

While peripheral membrane proteins can often be separated by changing the ionic interactions using highly concentrated salt solutions, integral membrane proteins often require harsher solubilisation conditions using *e.g.* detergents. Recent reviews summarised novel approaches to overcome the solubilisation limitations by for example substituting the exterior lipid facing hydrophobic residues of integral membrane proteins for hydrophilic ones using a 'computational redesign' approach or by fusing them to soluble fusion proteins.[3,4] However, this has only been applied to a small number of MPs until now. For most of the characterised membrane proteins detergents have been used to solubilise and stabilise the membrane protein. This, however, often requires an extensive detergent screening to find the right balance between stability and solubilisation as no universal detergent exists.[5]

Detergents typically consist of an hydrophilic head group attached to an hydrophobic tail. They can be classified into three groups dependening on the charge of the head group of the amphiphilic molecule: ionic (cationic or anionic), non-ionic or zwitterionic. Ionic detergents such as sodium dodecyl sulfate (anionic) or cetyltrimethylammonium bromide (cationic) are charged and tend to denature proteins. On the other hand, non-ionic detergents are uncharged and often aid in the extraction of functional proteins due to their smaller tendency to denature proteins. Zwitterionic detergents, however, contain head groups which are both negatively and positively charged. This type of detergent shows even smaller degrees of protein denaturation while inhibiting protein-protein interactions.[6]

An alternative to the classical detergents is provided by the use of styrene maleic acid (SMA) copolymers. Styrene maleic acid copolymers are recently discovered tools in the membrane protein solubilisation.[7-9] They offer a new approach by the spontaneous formation of discoidal particles, so-called lipid nanodiscs, due to self-insertion upon addition to the membrane-bound proteins as has lately been described in various review articles.[10-13]

The successful solubilisation of membrane domains directly from intact gram-positive bacteria *e.g.* the penicillin-binding protein complex PBP2/PBP2a from *Staphylococcus aureus* has been published recently.[14] This serves to show the powerful potential of SMA copolymers in the membrane solubilisation also from natural plasma membranes.

Since with this procedure the membrane protein will retain its original lipid environment in the solubilised particles, the protein is expected to retain its structure and function. MP solubilised with detergents or SMAs are therefore stabilised differently (Figure 2).

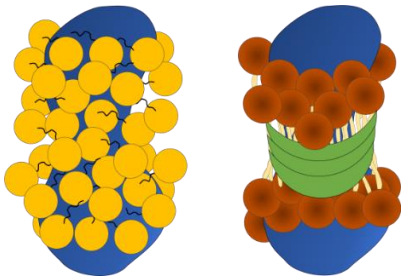


Figure 2: Schematic membrane proteins stabilised by detergent molecules (A) and styrene-maleic acid copolymers (B).

The rapidly developing fields of genomics as well as proteomics give valuable insight into specifics of the investigated organism on a DNA- or protein level, respectively. While the comparison of *e.g.* whole-genome sequences of organisms (comparative genomics) gives valuable information about the presence of certain genes linked to desired properties, the field of proteomics provides detail about the totality of proteins produced by the organism of interest under particular conditions.

The aim of this chapter is to demonstrate the steps that were taken to identify Michael hydratase candidates as well as the subsequent examination of possible candidates. The Mhy candidates were ascertained using a combined approach of microbiological techniques such as growth medium optimisation (Chapter 4) and solubilisation experiments as well as comparative genomics, membrane proteomics and *in-silico* analyses. The most promising Michael hydratase candidates were afterwards cloned and heterologously expressed in an *E. coli* host system. The Mhy activity was probed using the conversion of model substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid.

5.2 Results

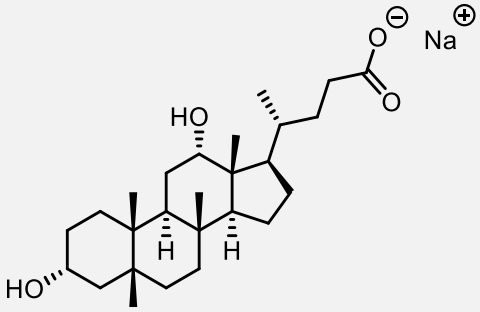
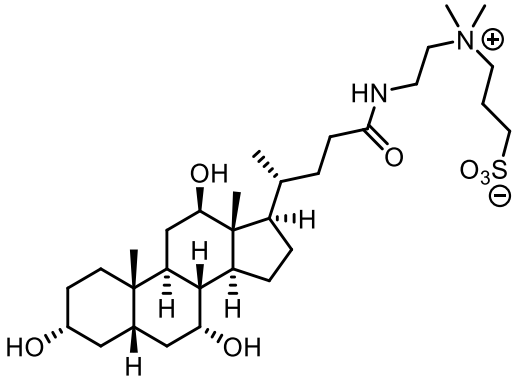
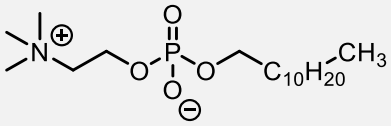
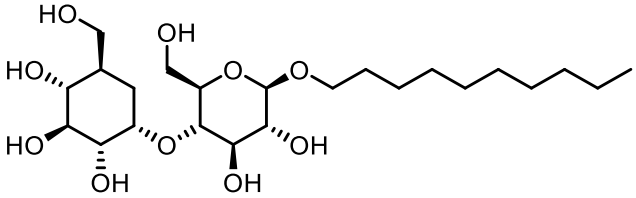
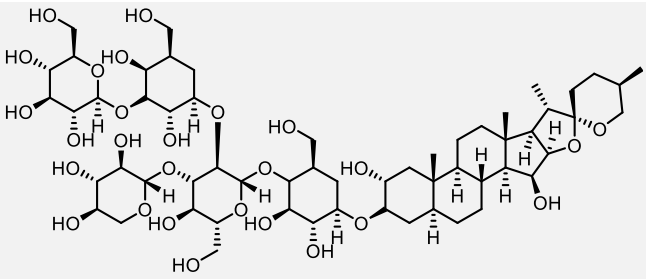
5.2.1 Michael hydratase – a novel membrane protein?

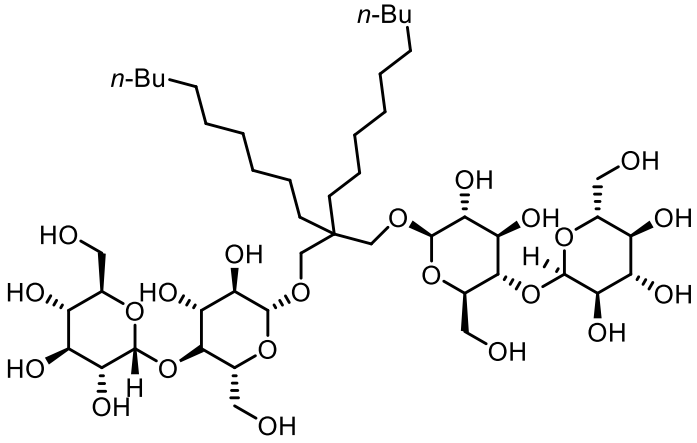
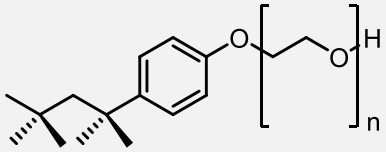
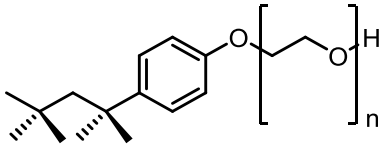
As was described in **Chapter 4**, the subcellular fractionation showed an increased Mhy activity associated with the plasma membrane fraction of *Rhodococcus* cells. Normally, it is beneficial to collect as much information about the physical properties of the membrane protein as possible beforehand to adapt the choice of detergents and

minimise the screening efforts because parameters such as the chosen buffer system, temperature or pH all have an influence on the performance of the detergent.[15] Unfortunately, however, the desired Michael hydratase was never isolated before and its coding sequence was unknown and therefore no type of detergent could be excluded. To first determine whether the Michael hydratase behaves like a peripheral or integral membrane protein, solubilisation trials using highly concentrated salt solutions have been carried out which disrupt the electrostatic interactions between peripheral MP and the membrane. After incubation of the membrane with highly concentrated salt solution, all Mhy activity remained in the membrane pellet and no activity was recovered in the soluble fraction. This led to the conclusion that the desired Mhy is not a peripheral, but an integral or lipid anchored MP having stronger connections to the membrane.

Therefore, a number of detergents ranging from anionic to zwitter- and non-ionic was tested on the plasma membrane fraction of *R. rhodochrous* ATCC 17895 (Table 1) at different pH-values. Membrane samples were incubated with the respective detergent and a sample of the mixture of re-suspended and solubilised membranes was taken after a given time. Subsequently, the remaining membrane was separated from the supernatant and all three samples were analysed by their activity towards (*E*)-4-hydroxy-3-methylbut-2-enoic acid.

Table 1: Detergents utilised in Michael hydratase solubilisation trials.

#	Name	Structure	Remark
1	sodium deoxycholate		anionic
2	chaps		zwitter-ionic
3	FOS-CHOLINE-12		zwitter-ionic
4	*n*-dodecyl β-**D**-maltoside (DDM)		non-ionic
5	digitonin		non-ionic

7	LMNG		non-ionic
8	Triton X-100	 n= 9-10	non-ionic
9	Triton X-114	 n= 7-8	non-ionic

Unfortunately, however, the addition of any of the detergents in table 1 led to a complete loss of activity in all three samples (treated sample, membrane and supernatant; Figure 3).

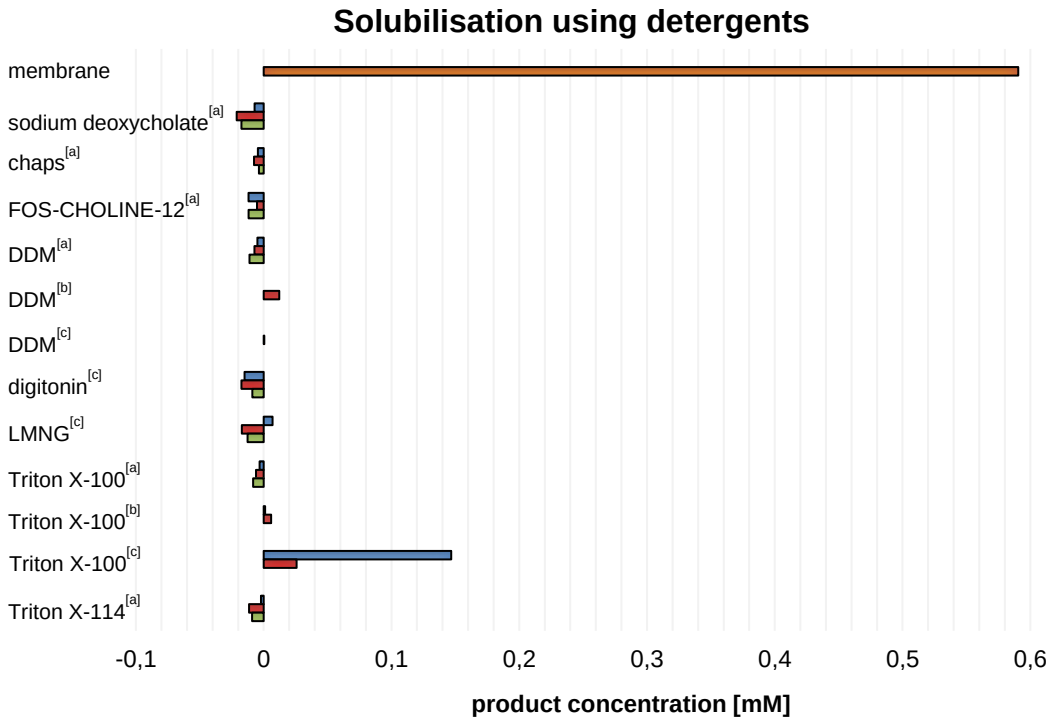


Figure 3: Results of the detergent solubilisation trials with isolated membrane from *R. rhodochrous* 17895. Positive control: untreated membrane sample (orange); treated samples (blue); supernatant (red); membrane (green); Solubilisation experiments run at different pH-values: [a]pH 6.2; [b]pH 7.4; [c]pH 8.0.

Therefore, none of the tested detergents was useful to solubilise the desired protein. Instead, the remaining membrane as well as the mixture did not show any activity towards the substrate while the positive control, unsolubilised membranes, was active. This shows that the detergent molecules interfered with the desired MP and could *e.g.* mean that the Mhy is a complex membrane protein consisting of multiple subunits from which only single subunits were solubilised or that the Mhy was indeed successfully solubilised but that the non-membrane environment resulted in an inactivated form of the protein.

To assess whether it was possible to solubilise the Mhy with styrene-maleic acid copolymers in which the Mhy would be in a membrane-like environment, three different SMAs with different ratios of styrene and maleic acid were also tested on membrane samples of *R. rhodochrous* ATCC 17895 at different temperatures. In a similar manner,

membranes were incubated with the SMAs and samples were analysed as described previously. Here, a substantially different activity pattern was detected (Figure 4).

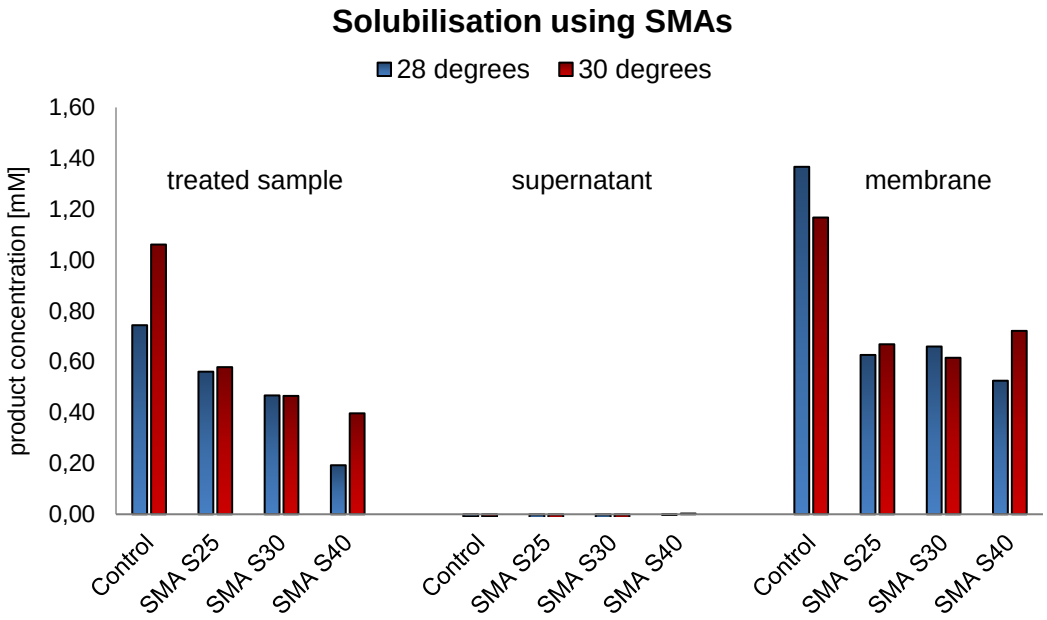


Figure 4: Results of the SMA solubilisation trials with isolated membrane from *R. rhodochrous* 17895 at two different temperatures: 28 °C (blue) and 30 °C (red).

With all three SMAs, it was not possible to solubilise the desired Mhy as no activity was recovered in the supernatant fraction. However, different to the detergent trials, Mhy activity remained both in the treated and the membrane sample showing that the SMA did not interfere with the activity of the membrane protein. By analysing the total protein content with a standard BCA assay, it was shown that circa 1/3 of the proteins present in the treated samples was later on found in the supernatant while circa 2/3 remained in the membrane sample. The SMA trials did therefore not lead to a successful solubilisation of the Mhy but did, however, open up the opportunity to enrich the Mhy in the membrane sample by solubilising other MPs from the natural membrane.

As all enzyme isolation methods did not lead to a successful solubilisation of the desired Mhy, it was decided to concentrate on the identification of the responsible gene or protein using genomic and proteomic studies and discontinue the solubilisation trials.

5.2.2 Comparative genomics

While the isolation of the Michael hydratase was discontinued, alternative ideas had to be developed to still identify the responsible Mhy-encoding gene. First, several *Rhodococcus* strains were tested in an effort to identify two closely related strains with one being active towards (*E*)-4-hydroxy-3-methylbut-2-enoic acid and one being inactive. An overlay of both whole-genome sequences would then deliver a limited number of possible Mhy candidates. It was, however, revealed that all tested *Rhodococcus* strains were Mhy active in at least one of the four tested cultivation conditions (Chapter 4). Therefore, it was decided to compare the genomic information of all 25 Mhy active strains to filter out the proteins which are present in every strain instead.

A total number of 25 Mhy active *Rhodococcus* strains were used in this comparative genomic study. The whole-genome sequences were either obtained by in-house sequencing (Chapter 4) or made publically available *via* the National Centre for Biotechnology Information (NCBI).[16] All used strains were translated into their respective proteomes and annotated using Prokka (version 1.12).[17] Based on these annotated proteomes, a comparison tool was developed: an optimised orthologous matrix (OMA) algorithm was used (standalone version 2.2.0) to first group all present orthologous proteins and afterwards examine the presence of each orthologous protein in every strain.[18] The identified proteins were ordered by their abundance within all tested strains leading to a total number of 17880 recognised, orthologous proteins.

5.2.3 Membrane proteomics

As described earlier, the Mhy activity in *Rhodococcus* is associated to the membrane fraction (Chapter 4). Therefore membranes of specific strains were analysed using protein mass spectrometry to establish which proteins are present in chosen membrane samples. Overlaying the membrane proteomes of different strains as well as comparing these results with the genomic analysis of all strains was believed to decrease the amount of possible Mhy candidates.

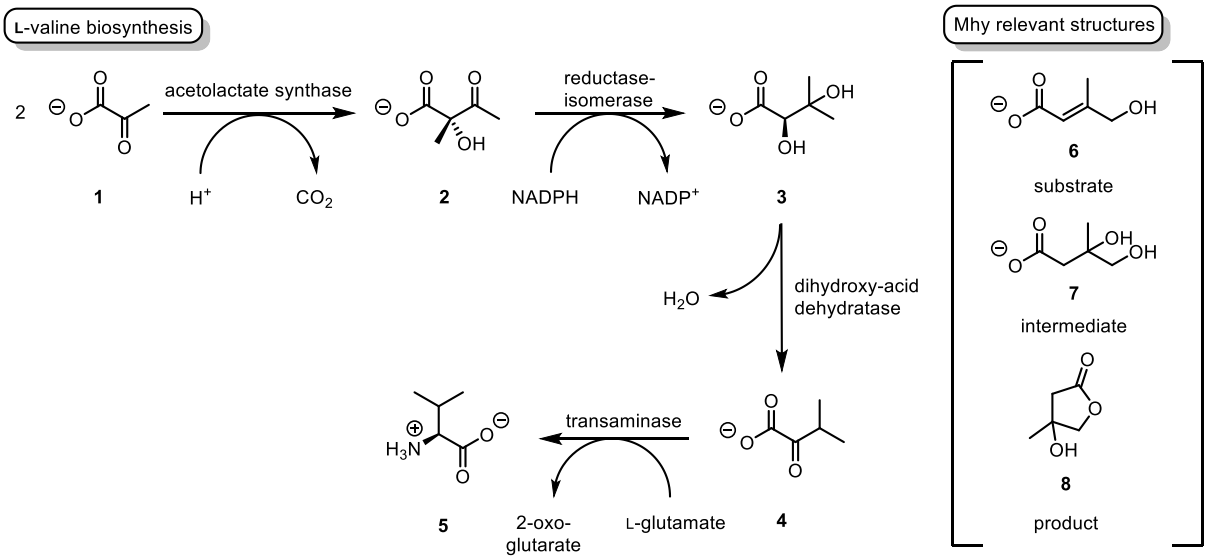
Membranes of two *Rhodococcus* strains (*R. pyridinivorans* DSM 20415 and *R. ruber* DSM 45280) with high activities were isolated from the respective whole-cells and treated with an SMA 25-solution as described before to enrich the Mhy in the membrane. The enriched membranes were afterwards lysed with an urea lysis buffer. Lysis buffers generally have denaturing and reducing properties to disrupt the intra- and intermolecular interactions as well as solubilising properties. The used urea lysis buffer consists of a chaotrope (urea), detergent (here CHAPS), reducing agents, carrier ampholytes and inhibitors of *e.g*. proteases.[19] The lysed membrane samples were afterwards trypsin-digested and measured on a reverse phase ESI-MS/MS using a Q-TOF mass spectrometer. The identified peptides were subsequently assembled and compared to the full proteomes of *R. pyridinivorans* DSM 20415 and *R. ruber* DSM 45280 which were previously obtained by the translation of the whole-genome sequence.

As a result, a number of 154 proteins were positively identified in the membrane sample of *R. pyridinivorans* DSM 20415 while the membrane sample of *R. ruber* DSM 45280 gave 90 identified proteins. These proteins were classified as the most abundant membrane proteins identified on the basis of at least two distinctive peptides.

5.2.4 Identification of Michael hydratase candidates

5.2.4.1. Identification of abundant (de-)hydratases in *Rhodococcus* through genomic analysis

In order to process the obtained data of 17880 recognised proteins within all investigated *Rhodococcus* strains, the focus was initially on all annotated (de-)hydratases. The 25 most abundant have been inspected closely. Within those 25 hydratases, two annotated dihydroxy-acid dehydratases (DHADs) were found. The two different protein sequences only shared a sequence identity of 38.5% and sequence similarity of 53.9%. In nature, the dihydroxy-acid dehydratase is part of the valine- and isoleucine biosynthesis pathway (Scheme 1).



Scheme 1: Schematic L-valine biosynthesis pathway with Mhy relevant structures (substrate **6**, intermediate after water addition **7** and product **8**) displayed for comparison.

Here, two pyruvate molecules (**1**) are first coupled by an acetolactate synthase to give intermediate **2** which is afterwards reduced and isomerised by a NADPH-dependent reductase-isomerase. This leads to the formation of 2,3-dihydroxy-3-methylbutanoate (**3**) which is then dehydrated to give water and 3-methyl-2-oxobutanoate (**4**). This compound is finally converted to L-valine (**5**) by a valine transaminase. As 2,3-dihydroxy-3-methylbutanoate (**3**), the natural substrate of dihydroxyacid-dehydratases, shows a significant similarity (similar size, charge and position of functional groups) to model substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid (**6**, Scheme 1), these two (de-) hydratases have been investigated more thoroughly.

DHADs are known to have a homologous protein called 6-phosphogluconate dehydratase (6-PGDH) which converts larger substrates such as 6-phospho-D-gluconate and is part of the Entner-Douderoff pathway.[20] This glucose degrading pathway is predominantly found in eukaryotes, but has also been recently described in mostly gram-negative bacteria. However, some examples of gram-positive bacteria are also documented with *R. opacus* or *R. jostii* being two of them.[21,22] Searching all 25 sequenced genomes for the enzymes present in the ED-pathway gave reason to believe that also other *Rhodococcus* species use this pathway as most of them display at least one of the respective enzymes. Proving this would, however, need further examination.

5.2.4.2 *In-silico* analysis of Mhy candidates

To exclude the possibility that the second annotated dihydroxyacid dehydratase from the genomic analysis is in fact the 6-phosphogluconate dehydratase (PGDH), the presence of all three proteins of interest in one single strain has been investigated. Here, two strains (*R. ruber* DSM 45280 and *R. opacus* DSM 43205) indeed displayed both annotated DHADs as well as the 6-phosphogluconate dehydratase. An analysis of the genomic context of the three proteins from *R. ruber* DSM 45280 was carried out using the RAST annotation server (Figure 6).[23-25]

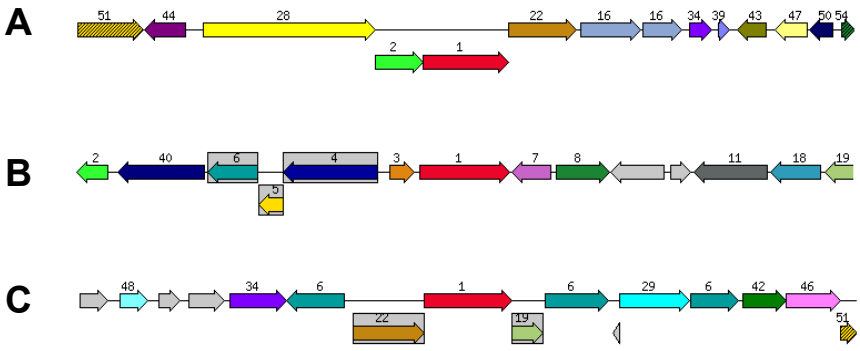


Figure 6: RAST alignment of Mhy candidates from *R. ruber* DSM 45280.[23-25] A – 1st annotated dihydroxyacid dehydratase, B – 2nd annotated dihydroxyacid dehydratase, C – annotated 6-phosphogluconate dehydratase. Protein 1 (red) is the respective protein of interest (DHAD/6-PGDH).

As can be seen, all three proteins are part of different pathways clustering with enzymes from different pathways. The first DHAD (A) was not surrounded by expected proteins from the valine biosynthesis pathway (nitrate/nitrite transporter (51), indolepyruvate ferredoxin oxidoreductase (28), **D**-3-phosphoglycerate dehydrogenase (2), **L**-lactate dehydrogenase (22) and multiple hypothetical enzymes (16, 39, 43, 50)). The second annotated DHAD (B), however, was found in close proximity to other proteins present in the valine-biosynthesis pathway (acetolactase synthase large subunit (4) and small subunit (5), ketol-acid reductoisomerase (6)) leading to the assumption that the second DHAD is indeed the correctly annotated DHAD. When comparing multiple *Rhodococcus* strains, the neighbouring proteins of the first DHAD were also found to be not cohesive opposed to the second DHAD. The 6-PGDH (C) clustered, as expected, with enzymes present in the Entner-Duderoff pathway

(glucose-6-phosphate 1-dehydrogenase (22), 4-hydroxy-2-oxoglutarate aldolase (19)).

All three sequences were analysed regarding their potential interaction with the membrane as the Mhy was shown to be membrane-associated. For this, the three protein sequences have been processed with two independent transmembrane online prediction tools.[26,27] Both webtools only predicted a small membrane interaction potential for one of the two DHAD sequences and no interaction for the (2 α-helices identified, Figure 7).

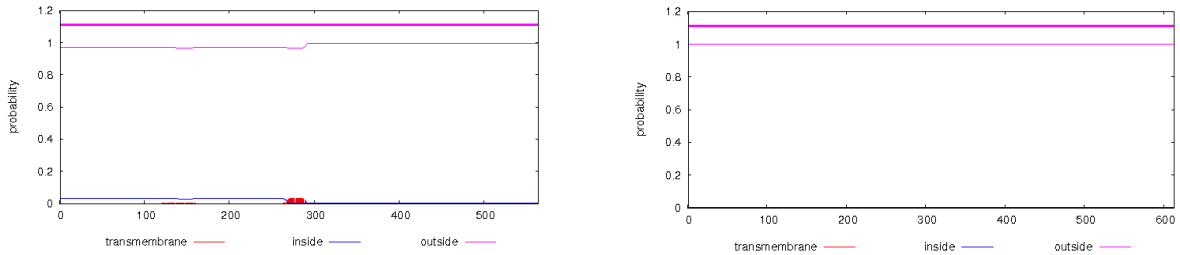


Figure 7: Probability for transmembrane interaction of the two annotated dihydroxy-acid dehydratases (left: small probability for membrane interaction, 1st DHAD; right: no predicted membrane interaction representative for 2nd DHAD and 6-PGDH).[27]

Due to this result, the two DHADs were thereupon named ‘memDHAD’ (1st DHAD, small membrane interaction, not part of the L-valine biosynthesis pathway) and ‘freeDHAD’ (2nd DHAD, no membrane interaction, part of the L-valine biosynthesis pathway gene cluster) for an easier distinction.

Based on existing structural information of a DHAD from *Arabidopsis thaliana*[28] homology models of the two DHADs candidates were constructed.[29-33] PGDH, on the other hand, was modelled after a phosphogluconate dehydratase from *Shewanella oneidensis*[34] (Figure 8).

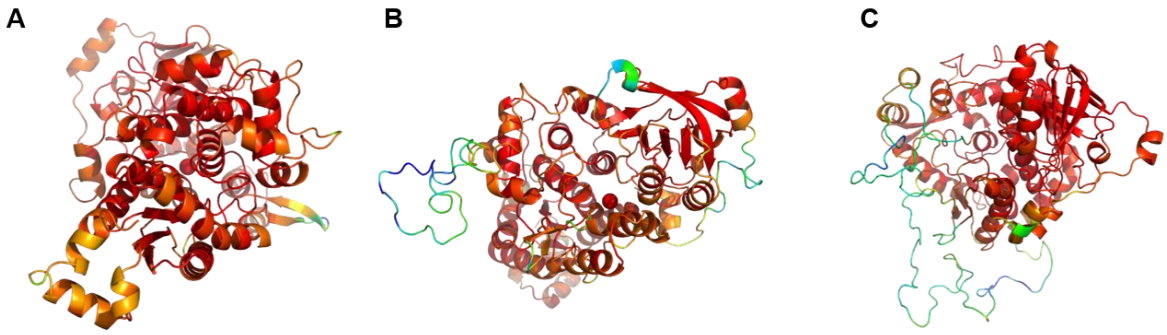


Figure 8: Swiss-homology models of memDHAD (A), freeDHAD (B) and PGDH (C). Proteins A and B were modelled after a DHAD from *A. thaliana* (5ZE4) and protein C after a PGDH from *S. oneidensis* (2GP4).[29-33]

Surprisingly, the memDHAD fit best to the model protein showing a sequence identity of 48.9% to the DHAD of *A. thaliana*. This result was unexpected, because the described DHAD from *A. thaliana* was soluble and had no membrane interaction. Therefore, a better fit with the freeDHAD was anticipated. Remarkably, exactly this protein showed one large unidentified loop in which it is distinctively different to any described structure. The same phenomena was found for the PGDH. In all three homology models an interaction with FeS-clusters was predicted whereby the two DHADs appeared to have similar interactions to an [4Fe4S]-cluster while the PGDH again differed.

5.2.4.3 Combination of results from membrane proteomics and genomic studies

To experimentally confirm the presence of the Mhy candidates in the tested membrane samples, the three protein sequences were cross-referenced and compared with the two membrane proteomes from *R. pyridinivorans* DSM 20415 and *R. ruber* DSM 45280.

The memDHAD protein was recognised amongst the 154 identified proteins in the membrane of *R. pyridinivorans* DSM 20415. However, it could not be detected within the 90 identified proteins in the membrane of *R. ruber* DSM 45280. Nevertheless, the presence of memDHAD in the whole-cells was already established earlier. FreeDHAD and PGDH were both not found in *R. pyridinivorans* DSM 20415. Interestingly, by lowering the quality requirement of two unique peptide hits down to only one unique

peptide hit showed that the PGDH was indeed found in the membrane of *R. ruber* DSM 45280.

The fact that only one Mhy candidate was present in only one of the two membrane samples minimised the probability for them being the desired Mhy candidates yet did not exclude the possibility completely. Factors in the sample preparation such an insufficient lysis or digestion could cause discrepancies in the final amount of detected proteins as well as an incompleteness of the obtained membrane proteome due to a potential low abundance of the Mhy.

Generally, both membranes shared only a small set of proteins (56) with more than two unique peptide hits from which only three proteins were termed 'hypothetical'. *In-silico* analysis did not reveal any specific characteristics which is why these were not further investigated.

As all three proteins each showed properties which were promising or unaccounted for (Table 2) considering for example their membrane interaction, predicted structure and genomic context, it was decided to test all three proteins in the whole-cell bioconversion of (*E*)-4-hydroxy-3-methylbut-2-enoic acid.

Table 2: Summary of properties analysed *in-silico* of Mhy candidates.

	memDHAD	**freeDHAD**	**PGDH**
Predicted membrane interaction	yes	no	no
Present in membrane proteome	yes (*R. pyridinivorans* DSM 20415)	no	no
Gene clusters (RAST)	Genomic context unclear	Part of valine biosynthesis gene cluster	Part of ED pathway gene cluster
Fit to model (SWISS model)	**Best fit** – highly similar to described DHAD sequence similarity of 90%	One large unidentified loop	Large unidentified loop

Predicted FeS cluster	Yes – [4Fe4S]	Yes – [4Fe4S]	Yes – different FeS cluster binding motif

[a]upon lowering quality requirements to 1 significant peptide hit instead of two.

5.2.5 Experimental validation of Michael hydratase candidates

The Michael hydratase candidates were cloned into a pBADHisA expression vector and E. coli Top10 was transformed using the obtained expression plasmid. The expression protocol was optimised and expression thereupon induced by the addition of **L**-arabinose with a final concentration of 0.02% (Figure 9).

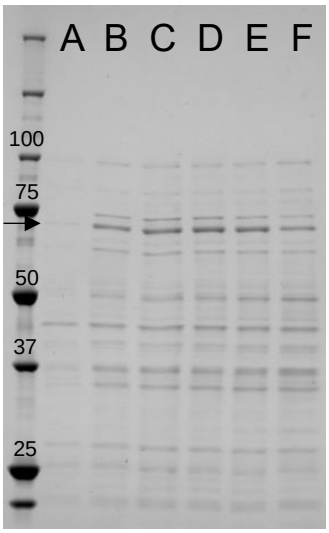


Figure 9: Representative SDS-PAGE analysis of 'freeDHAD' pilot expression in *E. coli* Top10 with different L-arabinose concentrations. Precision Plus Protein marker was used as standard (kDa, from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20). Protein concentration was normalised based on OD_{600} measurements. Lane A (before induction) and lanes B-F after 4 h at 37 °C: B (2%), C (0.2%), D (0.02%), E (0.002%), F (0.0002%) with L-arabinose concentrations displayed in brackets.

While the candidates memDHAD and freeDHAD show an overexpression with protein bands at 62 and 64 kDa, respectively, overexpression of PGDH was not achieved (63 kDa). To confirm whether the two DHADs catalyse the water addition to substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid, a whole-cell *E. coli* bioconversion was carried out using the standard reactions used with *Rhodococcus* whole-cells. It was, however,

displayed that neither of the two tested DHADs was active towards the substrate showing no difference to the silent background reaction by empty *E. coli* TOP 10 cells.

This result was the final result and closed investigations in the search for the desired Michael hydratase which therefore remains an elusive enzyme with interesting biochemical and biocatalytic properties.

5.3 Conclusion

To conclude, this study dealt with the various isolation and identification processes undertaken to describe the Michael hydratase in the genus *Rhodococcus*. Membrane solubilisation trials using detergents did not lead to any retained activity in the supernatant fraction. Treating the membranes with styrene-maleic acid copolymer solutions (SMA) led to an enrichment of Mhy in the membrane fraction yet no solubilisation was detected. After numerous attempts, the isolation efforts were therefore discontinued. The identification of the respective Mhy-encoding gene or protein was aimed for using the combination of a comparative genomics study (25 Mhy active *Rhodococcus* strains), membrane proteomic study (two *Rhodococcus* membranes) and the resulting *in-silico* analysis. Through this, three Michael hydratase candidates were identified two of which were annotated as dihydroxyacid-dehydratases and one as 6-phospho gluconate dehydratase. Both DHADs were successfully overexpressed in *E.coli* while the heterologous expression of PGDH failed. Activity tests of both heterologously expressed DHADs showed no activity towards substrate (*E*)-4-hydroxy-3-methylbut-2-enoic acid resulting in the final conclusion that the tested candidates were therefore not the desired Michael hydratases.

5.4 Experimental

5.4.1 General information

Unless reported otherwise, all commercial chemicals were purchased from Sigma Aldrich. FOS-CHOLINE-12 as well as lauryl maltoside were purchased from Anatrace Products. Serdolit-MB1 was purchased from Serva Electrophoresis GmbH, while Pharmalyte 3-10 was bought from Thermo Fischer.

5.4.2 Analytical methods

5.4.2.1 NMR spectroscopy

^{1}H and ^{13}C-NMR spectra were recorded on a Agilent (400 MHz and 100 MHz, respectively) instrument and were internally referenced to residual solvent signals. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = duplet, dd = double duplet t = triplet, q = quartet, m = multiplet), integration. Data for ^{13}C NMR are reported in terms of chemical shift.

5.4.2.2 Gas chromatography (GC)

Achiral GC analysis of the bioconversions of (*E*)-4-hydroxy-3-methylbut-2-enoic acid was performed with a Shimadzu type GC-2010 Plus equipped with a CP Wax 52 CB column (50 m x 0.53 mm x 2.0 µm) using N_2 as carrier gas. The following conditions were used for the achiral separation using direct injection: injector 280 °C, detector (FID) 300 °C, column flow rate 1.43 mL/min, linear velocity 37.0 cm/sec, pressure 98.5 kPa, temperature program: start at 90 °C, hold time 3 min, rate 5 °C/min to 250 °C hold time 1 min.

5.4.2.3 High-performance liquid chromatography (HPLC)

Samples of (*E*)-4-hydroxy-3-methylbut-2-enoic acid (3) and 4-hydroxy-4-methyl-dihydrofuran-2(*3H*)-one (4) were dissolved in 1 mL H_2O to a concentration of 5 µg/µL and further diluted to a concentration of 0.2 µg/µL. 5 µL of each sample were analysed using a (polar) RP-Q-TOF-MS system. The analysis was performed in ES+ and ES- mode. The data were analysed using MassLynx 4.1. The MS accurancy is +/- 0.05 Da.

5.4.2.4 Comparative Genomic studies

Whole-genome sequences of all investigated *Rhodococcus* species were annotated using Prokka (version 1.12).[17] Pairwise orthologues of the proteome of the annotated genome were computed using Orthologous Matrix (OMA) software (standalone version 2.2.0).[18]

5.4.2.5 Protein mass spectrometry (MS)

5.4.2.5.1 Sample preparation

Membrane vesicles of *R. pyridinivorans* DSM 20415 and *Rhodococcus ruber* DSM 45280 were resuspended to a final protein concentration of 5 mg/ml with 20 mM Tris.Cl buffer (pH 8, 150 mM NaCl, 10% glycerol) and incubated with 2.5 % (w/v) SMA-25 for 2 hours at room temperature under slow rotation (3 rpm). After the incubation, the remaining membrane pellet was separated from the solubilised proteins by centrifugation (2.5 h, 44.720 x *g*). Before, during and after the incubation samples were taken to determine the total protein concentration. Control experiments SMA-25 (diluted and undiluted), buffer only and membrane of *R. pyridinivorans* DSM 20415 without SMA-25 have been performed.

5.4.2.5.2 Trypsin digestion

Upon trypsin digestion, enriched membranes were lysed by incubation with an urea lysis buffer (9.5 M urea, 2% (w/v) CHAPS, 1% (w/v) DTT, 2% carrier ampholytes) at 37 °C for 15 minutes. The solubilised membrane proteins were brought to a final protein concentration of approximately 100 µg/µL. To 100 µL protein solution, 30 µL of a 10 mM DTT solution were added and incubated at 37°C for 1 hour. In the following, 30 µL of a freshly prepared 20 mM IAA solution was added and incubated in the dark for 30 minutes. The solution was diluted to below 1 M urea using 200 mM bicarbonate buffer and an aliquot of approximately 25 µg protein were digested using trypsin at 37°C overnight (using a trypsin to protein ratio of approximately 1:50).

5.4.2.5.3 Solid phase extraction

Peptides were desalted using an Oasis HLB 96-well plate (Waters) according to the manufacturer protocols. The purified peptide eluate was further dried using a speed-vac concentrator.

5.4.2.5.4 Large-scale shot-gun proteomics

The speed-vac tried peptide fraction was resuspended in H_2O containing 3% acetonitrile and 0.1% formic acid under careful vortexing. An aliquot corresponding to approx. 300 ng protein digest was analysed in duplicates using an one dimensional

shot-gun proteomics approach.[35] Briefly, 1 µL of sample were analysed using a nano-liquid-chromatography system consisting of an ESAY nano LC 1200, equipped with an Acclaim PepMap RSLC RP C18 separation column (50 µm x 150 mm, 2µm and 100A), and an QE plus Orbitrap mass spectrometer (Thermo). The flow rate was maintained at 300 nL/min over a linear gradient from 5% to 30% solvent B over 90 minutes, and finally to 75% B over 25 minutes. Solvent A was H_2O containing 0.1% formic acid, and solvent B consisted of 80% acetonitrile in H_2O and 0.1% formic acid. The Orbitrap was operated in data depended acquisition mode acquiring peptide signals form 400 -1200 m/z at 70K resolution, where the top 10 signals were isolated at a window of 1.6 m/z and fragmented using a NCE of 28. The AGC target was set to 5e4, at a max IT of 150 ms and 17.5K resolution.

5.4.2.5.5 Database search and data processing

Raw data were analysed using PEAKS Studio 8.5 (Bioinformatics Solutions Inc., http://www.bioinfor.com) allowing 20 ppm parent ion and 0.02 Da fragment mass error tolerance. Search conditions further included considering three missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q deamidation as variable modifications. Data were matched against a global *Rhodococcus* sequence database (Uniprot, Sept 2018, Tax ID 1827) and an in-house generated protein database translated form the genome sequence generated as described above. Database search included the GPM crap contaminant database (https://www.thegpm.org/crap/) and a decoy fusion for determining false discovery rates. Peptide spectrum matches were filtered against 1% false discovery rate (FDR) and protein identifications with two or more unique peptides were considered as significant hit. Relative protein abundances were correlated to combined peptide areas.

5.4.3 Microorganisms and microbiological protocols

5.4.3.1 Microorganisms and cell cultures

5.4.3.1.1 Cloning

All synthetic genes (memDHAD, freeDHAD, PGDH) were cloned in pBADHisA expression vectors and subsequently transformed with *E. coli* Top10 cells. Colonies

were maintained on LB-agar plates. Enzymes can be found under the accession codes KXF84314.1 (memDHAD), KXF87041.1 (freeDHAD) and KXF85555.1 (PGDH).

5.4.3.1.2. Mhy candidate expression

The Mhy candidates (memDHAD, freeDHAD, PGDH) were expressed in *E. coli* TOP10 cells grown in LB medium. Pre-cultures were inoculated with a single colony and grown overnight at 37 °C with orbital shaking (180 rpm). The main cultures (500 mL in a 2 L shake flask) were inoculated with 2 ml of pre-culture and grown to an optical density of 0.6 – 0.8 at 37 °C with orbital shaking (180 rpm). The protein expression was induced by adding **L**-arabinose to a final concentration of 0.02 %. After 16 hours of growing at 25 °C cells were harvested and resuspended in 20 mM Tris.Cl (pH 8) and kept at -20 °C.

5.4.3.2 Gel electrophoresis

5.4.3.2.1 Agarose gel

DNA was separated in 0.8 wt% agarose gel with 1x TAE buffer (pH 8.5). The genetic material was made visible with SYBR Safe DNA Staining (ThermoFischer). Gels were run at 100 V for 60 min.

5.4.3.2.2 SDS-Page

The proteins were separated in Criterion™ XT 4-12% Bis-Tris precast gels (Bio-Rad) with XT MOPS (3-(*N*-morpholino)-propanesulfonic acid) buffer (Bio-Rad). Gels were run for 60 min at 200 V (constant) and subsequently stained with SimplyBlue SafeStain (Novex). For the determination of the molecular weight of the proteins, the Precision Plus Protein™ Unstained Standard (Bio-Rad) was used.

5.4.3.2 Membrane solubilisation trials

5.4.3.2.1 Peripheral membrane solubilisation

The membrane vesicles were resuspended to a final protein concentration of 2 mg/ml in 1 M NaCl and homogenised. The suspension was kept on ice for 30 minutes and mixed by vortexing three times. The suspension was afterwards centrifuged for 60 minutes at 13.000 rpm at 4° C. The pellet was resuspended in KPi buffer (100 mM, pH

6.2). Both the supernatant and resuspended pellet were tested for activity following the small scale biotransformation protocol.

5.4.3.2.2 Integral membrane protein solubilisation - detergents

The membrane vesicles were resuspended to a final protein concentration of 5 mg/ml in either 100 mM KPi buffer (pH 6.2) or 20 mM Tric.Cl buffer (pH 8 or pH 7.4, 5 mM $MgSO_4$, 300 mM NaCl, 10% glycerol) and afterwards incubated at 4 - 30 °C for 30 - 120 minutes with gentle inverting with a final volume of 2 ml. 900 µl of the solubilisation mixture were taken to test the activity following the small scale biotransformation and extraction protocol. The remaining sample was centrifuged for 1 hour at 45.000 x *g*. The pellets were resuspended in 900 µl of the respective buffer. The supernatant (900 µl) and resuspended pellets were tested following the small scale biotransformation protocol. A control reaction with membranes (5 mg/ml) without added detergent was treated equally.

5.4.3.2.3 Integral membrane protein solubilisation – styrene maleic acid copolymers (SMAs)

10% (w/v) stock solutions (20 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol) of three pre-hydrolysed SMAs (XIRAN® SL40005 S40, XIRAN® SL30010 S30, XIRAN® SL25010 S25; kind gift of Polyscope, Geleen, The Netherlands) were prepared. Two different concentrations of membranes were tested using the SMA solubilisation buffer: first, the membrane vesicles were resuspended to a final protein concentration of 5 mg/ml in 2.5% (w/v) SMA buffer and afterwards incubated at 28 or 30 °C for 30 minutes in a waterbath with gentle inverting every 5 minutes with a final volume of 2 ml. Samples were taken and treated the same way as described for the detergent solubilisation trials.

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6

Exploring the abundance of oleate hydratases in the genus *Rhodococcus* – discovery of novel enzymes with complementary substrate scope

Oleate hydratases (Ohys, EC 4.2.1.53) are a class of enzymes capable of selective water addition reactions to a broad range of unsaturated fatty acids leading to the respective chiral alcohols. Much research was dedicated to improving the applications of existing Ohys as well as to the identification of undescribed Ohys with potentially novel properties. This study focuses on the latter by exploring the genus *Rhodococcus* for its plenitude of oleate hydratases. Three different *Rhodococcus* clades showed the presence of Ohys whereby each clade was represented by a specific oleate hydratase family (HFam). Phylogenetic and sequence analyses revealed HFam-specific patterns amongst conserved amino acids. Oleate hydratases from two *Rhodococcus* strains (HFam 2 and 3) were heterologously expressed in *E. coli* and their substrate scope investigated. Here, both Ohys showed a complementary behaviour towards sterically demanding and multiple unsaturated fatty acids. Furthermore, this study includes the characterisation of the newly discovered *Rp*Ohy.

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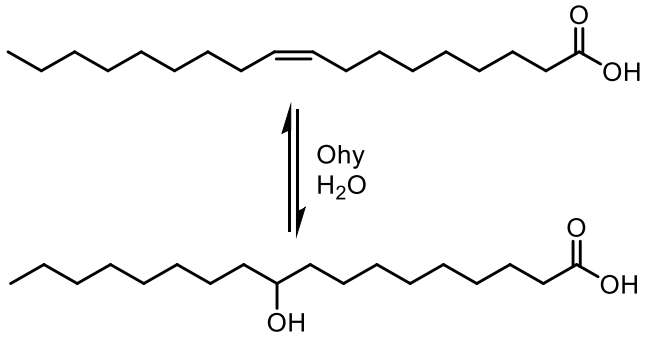
6.1 Introduction

Rhodococcus is a genus of aerobic, gram-positive bacteria and is known for its diverse biocatalytic activity towards a plethora of substrates.[1-3] Aliphatic, aromatic or heterocyclic compounds as well as alicyclic hydrocarbons, cholesterol, nitriles and lignin have been shown to be converted by members of the versatile *Rhodococcus* family.[2,3] The reason for their catabolic adaptability is explained by interacting factors such as their large genome sizes, the redundancy of biosynthetic pathways and the presence of large, linear plasmids often harbouring multiple copies of genes encoding degrading enzymes.[2-5]

The immense progress made in genomic studies enables a fast and extensive processing of bacterial genome information to designate gene functions to undescribed enzymes[5]. This helps identifying novel biocatalysts in *e.g. Rhodococcus* and therefore increases the biotechnological potential for this catalytic powerhouse. One enzyme family that receives increasing attention is the class of hydratases (E.C. 4.2.1.x) which belong to the group of lyases. They catalyse the reversible water addition to π-bond systems and can be categorised in two groups based on their substrate scope: isolated double bonds or conjugated systems.[6,7]

The oleate hydratase belongs to the first group acting on isolated double bonds in fatty acids like *e.g.* oleic acid to produce 10-hydroxystearic acid (Scheme 1).[7,8] Up until now, all characterised oleate hydratases are FAD-dependent. However, the precise role of FAD in the protein remains not fully understood.[7]



Scheme 1: Oleate hydratase catalysed water addition to oleic acid (18:1, *cis*-9) yielding 10-hydroxystearic acid.

With the newly developed ‘Hydratase Engineering Database’, it is now possible to distinguish between 11 homologous families (HFam) of fatty acid hydratases.[9] The sequence comparison of 2046 hydratase sequences exposed the presence of the 11 families (‘HFam 1 to 11’) whose members share an average sequence identity of 62%. With 1188 sequences, HFam2 makes the largest group followed by HFam1 and HFam3.[9]

Recently, the first oleate hydratase from *Rhodococcus* was characterised and the structure elucidated.[10] The protein belongs to HFam3 and thereby offers the first structural insights to a representative of this hydratase family. Unlike the other three crystallographically resolved oleate hydratases which were all shown to be homodimers,[11-13] this protein is described to be an active monomer in solution. The characterised Ohy*Re* from *R. erythropolis* CCM 2595 catalyses the conversion of a number of fatty acids exclusively in 10-position yielding the respective hydroxylated fatty acids.[10] This example again serves to show the rich abundance of diverse biocatalytic activities within *Rhodococcus* and demonstrates the possibilities of finding novel biocatalysts within this genus.

The aim of this study is to investigate the abundance of oleate hydratases within the genus *Rhodococcus*. Genome mining of 43 strains from different *Rhodococcus* families revealed 20 annotated oleate hydratases. Subsequently, the discovered Ohys were analysed phylogenetically and categorised based on their HFam affiliation. Thereby, overall three different groups of oleate hydratases have been distinguished (HFam 1 to 3) in *Rhodococcus*. One representative of each HFam2 and HFam3 was selected for heterologous expression and an extensive whole-cell substrate screening was carried out to investigate differences in substrate acceptance. Additionally, the thereby newly discovered oleate hydratase from *Rhodococcus pyridinivorans* DSM 20415 (HFam2) was characterised and the properties were compared to the earlier described Ohy*Re* (HFam3).[10]

6.2 Results and Discussion

6.2.1 Whole-genome alignment of genus *Rhodococcus*

High quality whole-genome sequences (WGS) from 43 *Rhodococcus* strains were either obtained by sequencing (**Chapter 4**) or were publicly available from the National Centre for Biotechnology Information (NCBI).[14] Whole-genome sequences were analysed with the Orthologous Matrix (OMA) software (standalone version 2.2.0) using an improved matrix algorithm to generate pairwise orthologues of the proteomes of the annotated genomes.[15,16] Within the 43 investigated strains, 20 oleate hydratases were identified. In total, three different HFams were recognised by the 'Hydratase Engineering Database': HFam 1, 2 and 3.[10]

All investigated *R. equi* strains display an oleate hydratase from HFam1. The largest group consists of 11 strains (including all *R. erythropolis* and all *R. qingshengii*, *R. enclensis* NIO-1009, *R. rhodochrous* DSM 101666 and *R. rhodochrous* ATCC 17895 as well as *Rhodococcus* R312) displaying an oleate hydratase from HFam3. On the other hand, all *R. pyridinivorans* strains as well as the *R. biphenylivorans* TG9 show an oleate hydratase from HFam2. Interestingly, one strain, *R. erythropolis* DSM 43066, displays two oleate hydratases with one belonging to HFam2 and the other one to HFam3.

To investigate the relation between the strains, a phylogenetic tree was created based on the 1% of most complete computed orthologous groups and visualised with MEGA (Figure 1).[16,17] Similar results were obtained when a phylogenetic tree was based on the 16S rRNA sequences (data not shown). As clearly visible, the three groups inheriting the same type of oleate hydratase cluster together. They form three independent clades from now on called '*erythropolis*-clade' (green), '*pyridinivorans*-clade' (blue) and '*equi*-clade' (orange).

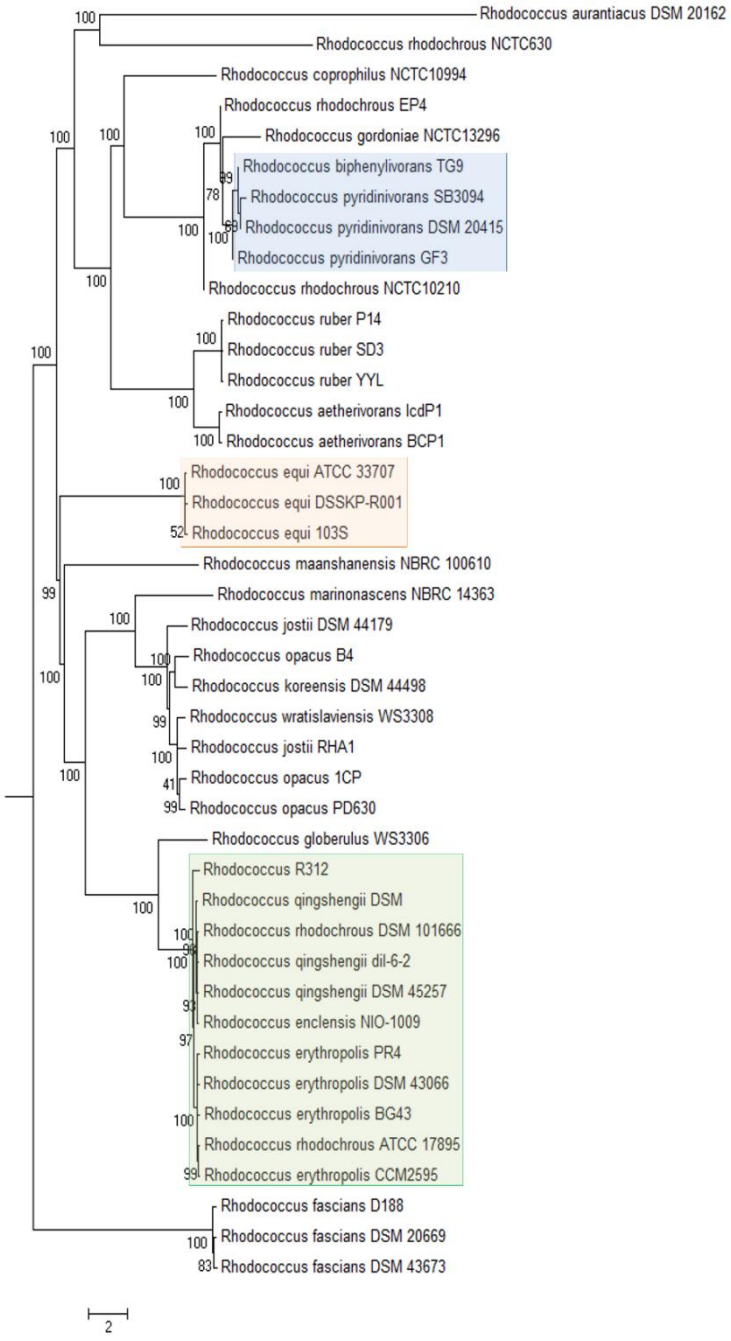


Figure 1: Phylogenetic tree of 43 investigated *Rhodococcus* strains based on the 1% of most complete computed orthologous groups.[16,17] '*R. pyridinivorans'*-clade highlighted in blue, '*R. equi*'-clade in orange and '*R. erythropolis*'-clade in green.

To analyse the relation between the annotated Ohys amongst the different families, a phylogenetic tree was generated. *R. erythropolis* DSM 43066 is the only strain with two different types of oleate hydratase present. While the '*R. erythropolis*'- and '*R. equi*'-

clade are built up uniformly, the Ohy from *R. erythropolis* DSM 43066 forms its own sub-group within the '*R. pyridinivorans*'-clade (Figure 2).

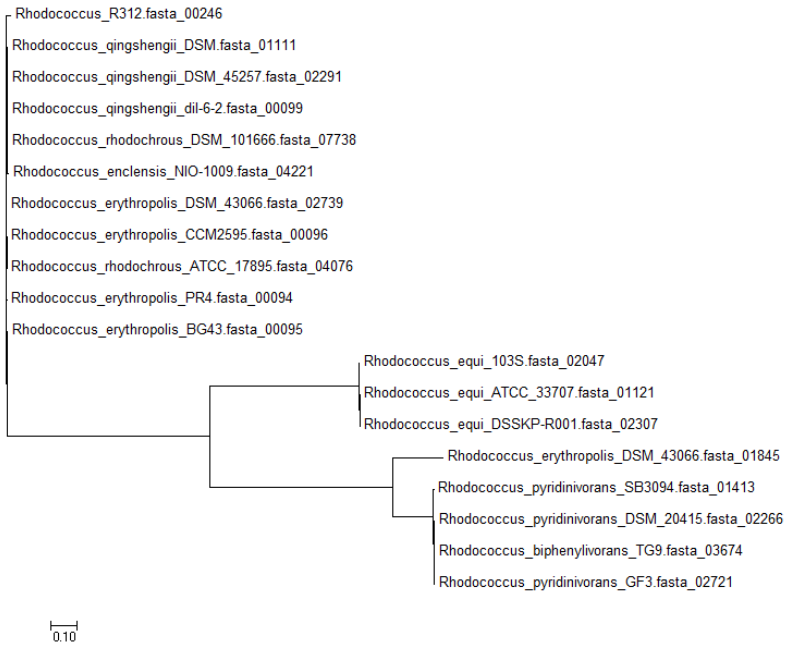


Figure 2: Phylogenetic tree of discovered oleate hydratases from *Rhodococcus* showing three clades: (*i*) *erythropolis*-clade, (*ii*) *equi*-clade, (*iii*) *pyridinivorans*-clade. Oleate hydratase protein sequences were aligned using muscle (version 3.8.31).[18] Distance-matrix was calculated by FastTree (version 2.1.9, JTT+CAT model) and then visualized by MEGA.[17,19]

To identify similarities and distinct differences between the three Ohy groups, a sequence alignment has been performed to study the specific conservation pattern. Schmid *et al*. described 80 highly conserved amino acids throughout all investigated oleate hydratases.[9] While the function of some conserved amino acids is already known, others remain unknown. Up until now, all oleate hydratases require FAD for a successful substrate conversion although the state of the cofactor is not changed during the reaction.[7] Momentarily, the common scientific belief is that the FAD cofactor only plays a structural role being essential for a correct positioning of amino acid in the active site.[10,11] One sensitive sequence motif for the FAD-cofactor binding region was identified as G69xG[LI][AG]x[LM][AS][AG]Ax[FY][LM][IV]R[DE][GA]x(3)Gxx[IV]x[IFVl][LFY]E96 (positions according to Em-OAH1).[9] While all identified oleate hydratases from HFam 1 (G14-E41) and HFam 3 (G13-G40) as well as HFam 2 Ohy from *R. erythopolis* DSM

43066 (G29-E56) share the same motif, the other Ohys in the '*pyridinivorans*'-clade differ distinctively in two of the conserved positions: T33 (instead of A (79%) or G (11%)[9]) and S37 (instead of A (71%), G (16%) or T (8%)) (positions 73 and 77 according to Em-OAH1[9], Figure 3). Especially threonine (T33) instead of Ala or Gly will increase the polarity.

```
Rhodococcus R312                          KLSHKAYMIGAGIGNLSAAVYLIRDGKWSGEDIIIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus enclensis NIO-1009            KLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus qingshengii DSM 46766         KLSHKAYMIGAGIGNLSAAVYLIRDGEWSGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus qingshengii DSM 45257         KLSHKAYMIGAGIGNLSAAVYLIRDGEWSGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus qingshengii dil-6-2           KLSHKAYMIGAGIGNLSAAVYLIRDGEWSGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus rhodochrous DSM_101666        KLSHKAYMIGAGIGNLSAAVYLIRDGEWSGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus erythropolis CCM2595          NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus rhodochrous ATCC 17895        NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus erythropolis BG43             NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus erythropolis DSM 43066 HFam3  NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus erythropolis PR4              NLSHKAYMIGAGIGNLSAAVYLIRDGEWNGEDITIMG-LDMHGANDGESAATFQHQYGHR    62
Rhodococcus equi 103S                     PKQAHIWIVGGGIAGMAAAVFAIRDAGVPGENVHILEQLDIEGGS----------LDGAR    54
Rhodococcus equi ATCC 33707               PKQAHIWIVGGGIAGMAAAVFAIRDAGVPGENVHILEQLDIEGGS----------LDGAR    54
Rhodococcus equi DSSKP-R001               PKQAHIWIVGGGIAGMAAAVFAIRDAGVPGENVHILEQLDIEGGS----------LDGAR    54
Em-OAH1                                   YDNSKIYIIGSGIAGMSAAYYFIRDGHVPAKNITFLEQLHIDGGS----------LDGAG    109
Rhodococcus erythropolis DSM 43066 HFam2  VDGKTAWFVGSGLASLAGAAFMIRDGQMAGNNITVLERLKLPGGA----------LDGIK    69
Rhodococcus pyridinivorans SB3094         VENKTAWFVGAGLTSMASAVFMIRDGQLSGDKITILERLDLPGGA----------LDGIK    69
Rhodococcus pyridinivorans GF3            VENKTAWFVGAGLTSMASAVFMIRDGQLSGDKITILERLDLPGGA----------LDGIK    69
Rhodococcus biphenylivorans TG9           VENKTAWFVGAGLTSMASAVFMIRDGQLSGDKITILERLDLPGGA----------LDGIK    69
Rhodococcus pyridinivorans DSM 20415      VENKTAWFVGAGLTSMASAVFMIRDGQLSGDKITILERLDLPGGA----------LDGIK    69
                                                :::*.*: .::.* : ***.   ...: .:  *.: *.           *
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Figure 3: CLUSTAL multiple sequence alignment of *Rhodococcus* oleate hydratases compared to *Em*-OAH1 in the range of conserved FAD-binding region (G_{69}-E_{96}).[20]

Additionally, amino acids R118-M123 (Em-OAH1) placed in a loop-region were shown to be involved in cofactor binding and catalysis.[11] While the HFam2 clade shows a 'RGGREM' motif like Em-OAH1 (HFam 11, Figure 4), *Rhodococcus* HFam1 and HFam3 Ohys all share a 'RGGRML' motif. In Em-OAH, E122 takes an important role in the water activation step[11] and based on the high motif similarity, it is likely that *Rhodococcus* HFam2 Ohys react in a similar way. The other two groups, however, do not share the same motif and previous studies claimed a different reaction mechanism distinct for HFam3 Ohys.[10] Due to the same amino acid pattern, it is likely that *Rhodococcus* HFam1 Ohys will react in the same way as HFam3 Ohys.

Figure 4: CLUSTAL multiple sequence alignment of *Rhodococcus* oleate hydratases compared to *Em*-OAH1 in the area of conserved R_{118}GGREM$_{123}$ (*Em*-OAH1) motif responsible for cofactor binding and presumably involved in catalysis.[20]

Finally, residues 436 and 438 (according to Em-OAH1) are involved in the binding of the carboxylate function of the substrate.[10,11] At both positions, the probabilities of four different amino acids were described to be 54% T, 31% V, 8% A and 5% S (residue 436 Em-OAH1) and 48% N, 22% H, 12% A and 9% P (residue 438 Em-OAH1).[9] Analysis of the two residues showed a distinct pattern depending on the chosen HFam (Figure 5).

Figure 5: CLUSTAL multiple sequence alignment of *Rhodococcus* oleate hydratases compared to *Em*-OAH1 in the area of conserved R_{118}GGREM$_{123}$ (*Em*-OAH1) motif responsible for cofactor binding and presumably involved in catalysis.[20]

While *Rhodococcus* Ohys from HFam1 all show the combination of residues valine and alanine, oleate hydratases in the *pyridinivorans*-clade all exhibit residues threonine and asparagine, respectively. HFam3, on the other hand, displays amino

acids valine and histidine. The latter residue was shown to be directly involved in the carboxylate binding in the case of Em-OAH1 as a single-point mutation from asparagine to alanine (N438A according to Em-OAH1) led to a reduced activity.[11] Presence of alanine in that position in the HFam1 oleate hydratases additionally hints at a different reaction mechanism for this group of enzymes. A recent study investigated the impact of these two amino acids on the final regio- and stereoselectivity and showed that selective mutations can lead to a complete reversal of activity with the example of the two fatty acid hydratases from *Lactobacillus acidophilus* (FA-HY1 and FA-HY2). Here, less bulky substituents in these two positions enabled the substrate to go deeper into the carboxylate end of the substrate channel.[21]

6.2.2 Fatty acid screening

A number of fatty acids has been tested for activity using *E. coli* whole-cells overexpressing *Rp*Ohy (DSM 20415), *Re*Ohy (PR4) as well as *E. coli* TOP 10 cells containing an empty pBADHisA expression vector as negative control.

Due to recent reports about the benefits of applying whole-cell systems over cell free extract or purified enzymes such as a higher operational stability, the prevention of time-consuming steps like protein purification and therefore an overall easier handling, all reactions have been carried out in a whole-cell system.[22,23] All reactions were performed for the duration of 6 days under the same standard conditions including the addition of 0.2 mM FAD, 5 mM DTT and 5 mM NADH in triplicate. The chosen conditions lead to an FAD reducing environment which was shown to have a beneficial influence on the Ohy activity which is reportedly more active in the presence of reduced FAD compared to oxidised FAD.[11,22] Additionally, each reaction was flushed with N_2 to avoid oxidation of unsaturated fatty acids. After 6 days, reactions were quenched, extracted and the crude mixture was derivatised upon gas chromatography-mass spectrometry analysis (Figures 6-9, representative chromatograms of bioconversions using ricinoleic acid and arachidonic acid as substrate).

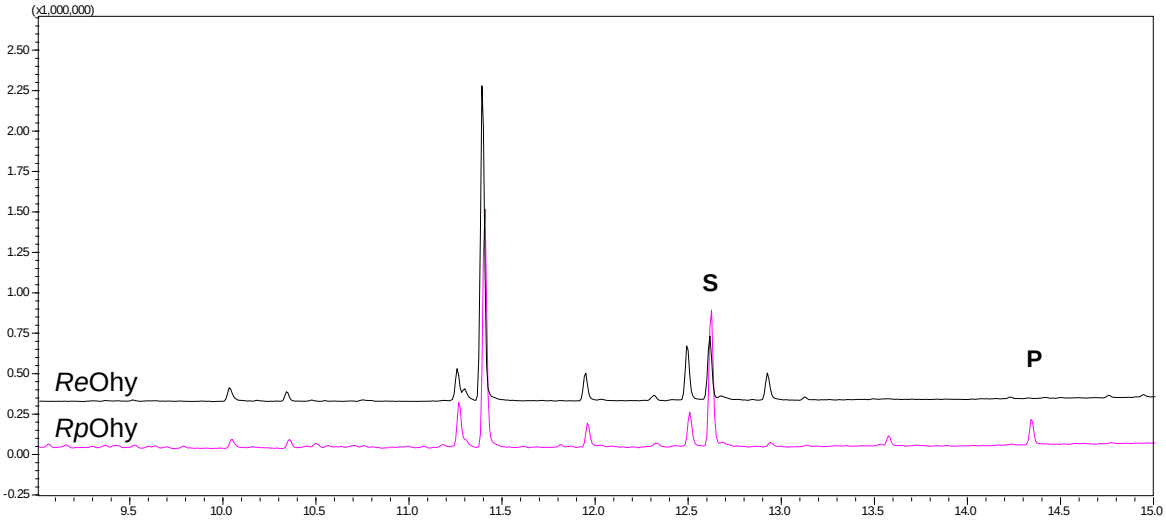


Figure 6: GC-MS chromatograms showing the products of *E. coli* whole-cell bioconversions of *Re*Ohy (black) and *Rp*Ohy (pink) of ricinoleic acid indicating substrate (S) and product (P) peaks.

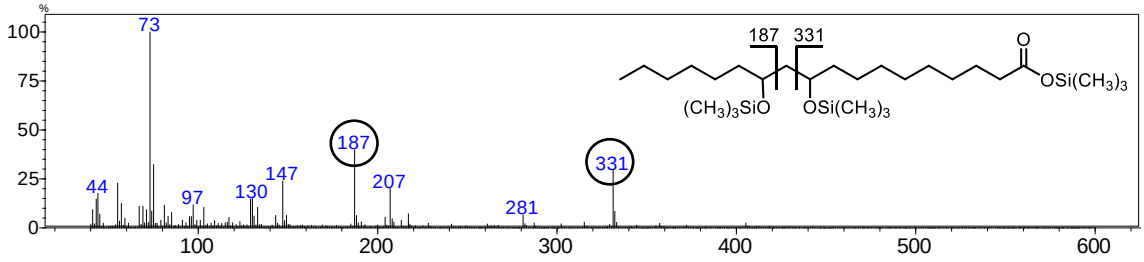


Figure 7: Mass spectrum of silylated, hydroxylated ricinoleic acid with significant fragment ions highlighted: *m/z* 187 and 331.

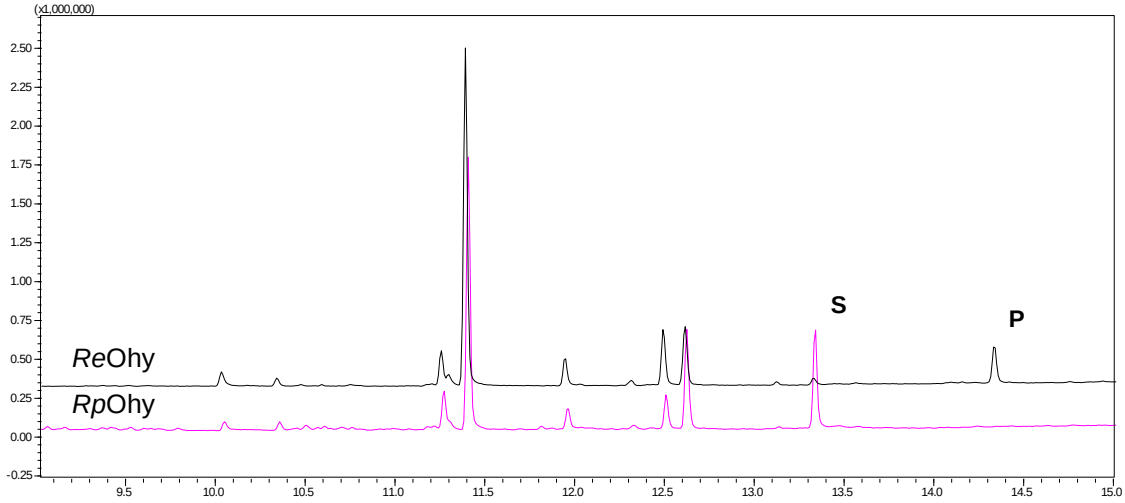


Figure 8: GC-MS chromatograms showing the products of *E. coli* whole-cell bioconversions of *Re*Ohy (black) and *Rp*Ohy (pink) of arachidonic acid indicating substrate (S) and product (P) peaks.

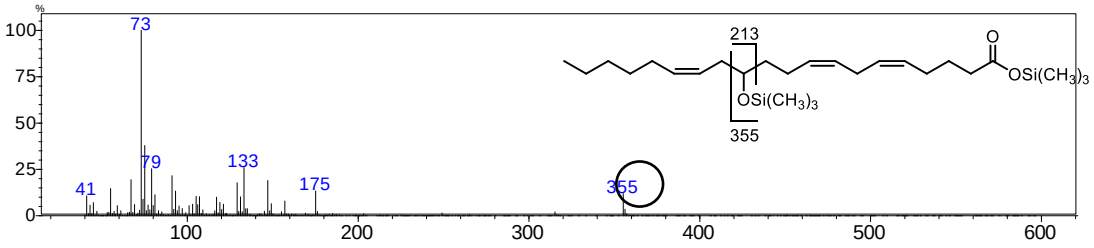
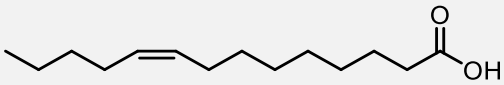
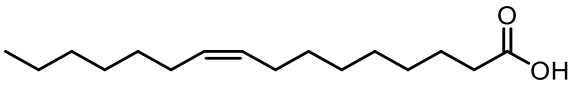
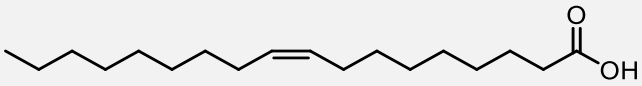
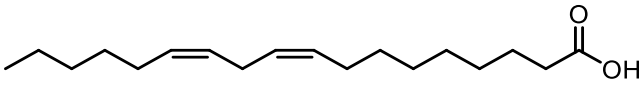
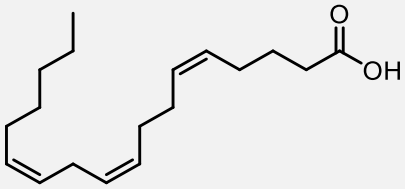
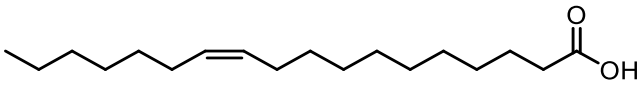
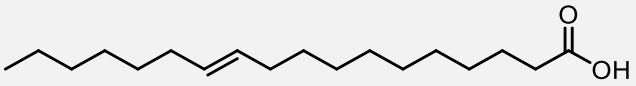
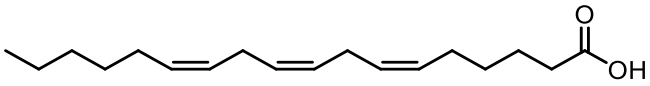
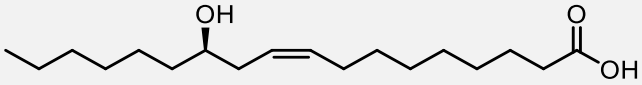
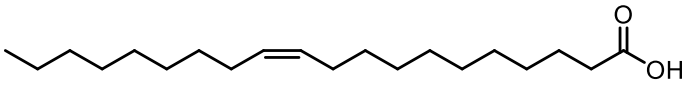
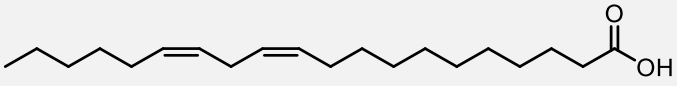
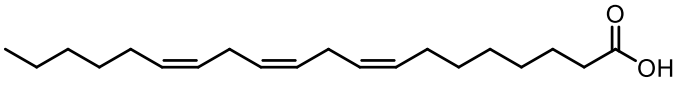
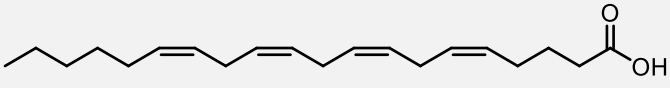
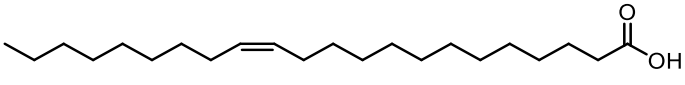
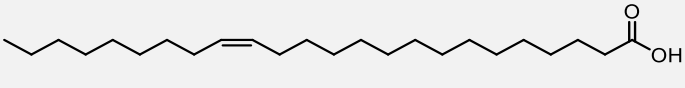


Figure 9: Mass spectrum of silylated, hydroxylated arachidonic acid with significant fragment ions highlighted: *m/z* 355. Ion 213 was not detected due to an re-arrangement following the allyl-position of the alcohol.

All reactions using *E. coli* Top 10 cells bearing the empty pBADHisA vector did not show any product formation ruling out background hydration activity. 15 fatty acids were tested with representatives from *R. pyridinivorans* DSM 20415 (*Rp*Ohy, HFam2) and from *R. erythropolis* PR4 (*Re*Ohy, HFam3, Table 1). If applicable, these whole-cell screening results were compared to the earlier described Ohy*Re* (CCM2595, HFam3) substrate screening using purified enzyme by Lorenzen *et al*.[10]

Table 1: Tested substrates and position of hydroxyl-group in final product of *Rp*Ohy (DSM 20415) and *Re*Ohy (PR4) whole-cell substrate screening. All reactions have been performed in triplicates.

#	Substrate	*Rp*Ohy (DSM 20415)[a]	*Re*Ohy (PR4)[a]	*Re*Ohy (CCM 2595)[b]
1	 myristoleic acid (14:1, *cis*-9)	10	10	--[c]
2	 palmitoleic acid (16:1, *cis*-9)	10	10	10
3	 oleic acid (18:1, *cis*-9)	10	10	10
4	 linoleic acid (18:2, *cis*-9,12)	10	10	10
5	 pinolenic acid (18:3, *cis*-5,9,12)	10	10	unknown[d]
6	 *cis*-vaccenic acid (18:1, *cis*-11)	--[c]	--[c]	--[c]
7	 *trans*-vaccenic acid (18:1, *trans*-11)	--[c]	--[c]	unknown[d]

8	 γ-linolenic acid (18:3, *cis*-6,9,12)	10	10	10
9	 ricinoleic acid (18:1, *cis*-9, (*R*)-12-OH)	10	--[c]	unknown[d]
10	 *cis*-11-eicosenoic acid (20:1, *cis*-11)	12	12	unknown[d]
11	 *cis*-8,11-eicosadienoic acid (20:2, *cis*-8,11)	--[c]	12	unknown[d]
12	 *cis*-8,11,14-eicosatrienoic acid (20:3, *cis*-8,11,14)	--[c]	12	--[c]
13	 *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid) (20:4, *cis*-5,8,11,14)	--[c]	12	--[c]
14	 erucic acid (22:1, *cis*-13)	--[c]	14	unknown[d]
15	 nervonic acid (24:1, *cis*-15)	--[c]	--[c]	unknown[d]

[a]Position of hydroxyl-group determined by GC-MS. [b]Position of hydroxyl-group and substrate acceptance determined by Lorenzen *et al.*[10] [c]no water addition and [d]not tested.

Three substrates were converted by neither of the tested oleate hydratases: nervonic acid (**15**) as well as *cis*- and *trans*-vaccenic acid (**6**,**7**). Nervonic acid (**15**) is likely to be

too long to fit in the active sites of the proteins and *trans*-vaccenic acid (**7**) does not reach the active site as the double-bond is required to be *cis*-configurated as was reported earlier.[11,22] Myristoleic acid (**1**) was converted by both *Re*Ohy (PR4) and *Rp*Ohy (DSM 20415) under the chosen whole-cell conditions. When using the purified enzyme with shorter incubation times, no water addition was observed by Lorenzen *et al*.[10] This example further shows the benefits of the more stable whole-cell system over the use of purified enzyme.

As expected, fatty acids palmitoleic acid (**2**) and oleic acid (**3**) were converted by both tested enzymes. Linoleic acid (**4**) as well as γ-linolenic acid (**8**) were also accepted by all Ohys and water addition was exclusively observed in 10-position. The results from this screening are in alignment with the results obtained by Lorenzen *et al*. for Ohy*Re* (CCM 2595).[10] The all-*cis*-configurated pinolenic acid (**5**) was a substrate for both tested *Rhodococcus* oleate hydratases. Here, the water addition was only observed in 10-position. Up to now, only two other oleate hydratases from *Lactobacillus acidophilus* (NTV001 (FA-HY1) and LMG 11470) were shown to convert pinolenic acid whereby both catalyse the water addition in 13-position.[7, 24,25] This shows a clear preference for the 10-position over the 13-position for both *Rhodococcus* oleate hydratases which can be explained by the two amino acids V393 and H395 (*Re*Ohy) and T390 and N392 (*Rp*Ohy) (corresponding to T436 and N438 in Em-OAH) following the results from recent studies.[21] In FA-HY1, these positions are occupied by two smaller serine amino acids meaning that the substrate is entering the substrate tunnel further leading to the water addition in 13-position.[21] In our example, however, histidine and asparagine are bulkier thereby directing a water addition into 10-position. In general, the addition in 13-position was not detected with any of the tested substrates.

While *Re*Ohy did not show any activity towards ricinoleic acid (**9**), *R*pOhy produced the 10,12-dihydroxylated fatty acid. Next to *Rp*Ohy, only two other hydratases (*Lactobacillus plantarum* Aku 1009a and *Lysinibacillus fusiformis*) were reported to show activity towards ricinoleic acid.[26,27] Its interesting emulsifying properties make 10,12-dihydroxystearic acid a potentially useful biosurfactant.[26] Fatty acids **10**-**13** all carry a *cis*-double bond in 11-position as does *cis*-vaccenic acid (**6**). While *Rp*Ohy only converted *cis*-11-eicosenoic acid (**10**) with low activity, *Re*Ohy showed activity towards

all four long, unsaturated fatty acids leading to the mono-hydrated 12-hydroxy fatty acids, exclusively. FA-HY1 (*Lactobacillus acidophilus*) was described to also convert fatty acids **12** and **13**. However, the water addition for fatty acid **13** occurred in 15-position while with fatty acid **12**, water addition took place in 12- and 15-position.[24] *Re*Ohy (PR4) is therefore the first oleate hydratase being able to selectively catalyse the water addition to long-chain, unsaturated fatty acids selectively in 12-position.

The tendency to convert large fatty acids could be explained by a larger active site in *Re*Ohy. In this screening, *Re*Ohy was shown to even convert erucic acid (**14**) in 14-position which, to the best of our knowledge, has not been reported for any other oleate hydratase. The active state as a monomer might have an influence on the ability to convert longer fatty acids. Surprisingly, both oleate hydratases were not able to convert *cis*-vaccenic acid (**6**) which has significant similarities with *cis*-11-eicosenoic acid but is two carbon-atoms shorter. The absence of these two carbon atoms is likely the reason why the shorter *cis*-vaccenic acid is not accepted.

This comprehensive substrate screening gives new insight into the capability of oleate hydratases from *Rhodococcus* to convert a number of fatty acids selectively. It was shown that especially with more complex and unsaturated fatty acids both investigated Ohys act complementary which can be further exploited for industrial applications. The described variety of the substrate scope of investigated Ohys is therefore consistent with the sequence variety among the different Ohy families and members. Chromatograms comparing conversions of *Rp*Ohy and *Re*Ohy as well as the GC-MS data of hydroxylated fatty acids can be found in the Supplementary Information.

6.2.3 *Rp*Ohy characterisation

The oleate hydratase from *R. pyridinivorans* DSM 20415 has a calculated protein weight of 67.8 kDa and consists of 601 amino acids. After heterologous expression in *E. coli* TOP10 cells, the N-terminally His_6-tagged enzyme was purified by Ni^{2+}-affinity chromatography and a subsequent size-exclusion chromatography (SEC). The purified protein had a yellow colour indicating that the FAD-cofactor was incorporated in the enzyme after the purification. The standard activity of *Rp*Ohy was measured by following the conversion of oleic acid to 10-hydroxystearic acid for five hours.

A beneficial effect of FAD addition to the reaction mixture was investigated in the range of 0 – 100 µM. Even without any addition of FAD, product formation (35%) was observed, but the addition of FAD increased the activity. However, the amount of added FAD (10 – 100 µM) did not have a significant impact on the product formation (51 - 57%) (Figure S30-32). Therefore, it was decided to add 20 µM to ensure a fully FAD-saturated protein in all bioconversions. The temperature tolerance for *Rp*Ohy was measured in a range from 15 - 60 °C (Figure 10). The highest activity was achieved at 25 °C (61%), but the protein remained active in a broad temperature range from 15 °C (47%) to 40 °C (18%). Higher temperatures, however, led to its deactivation.

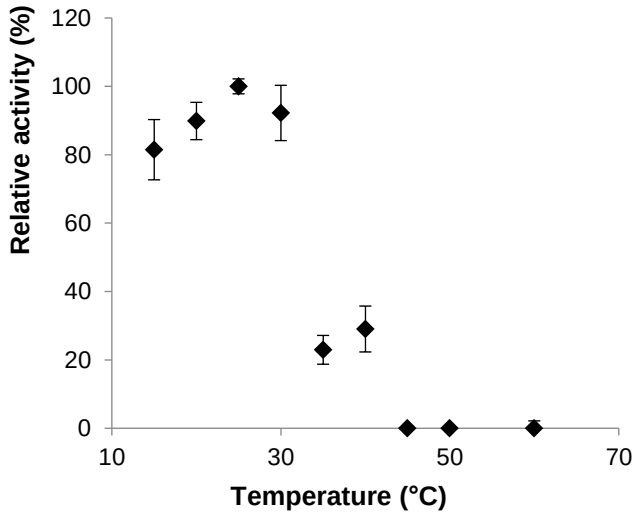


Figure 10. Temperature tolerance with temperature optimum of *Rp*Ohy. Reactions (triplicates) were carried out in 20 mM acetate buffer (pH 5) for five hours at respective temperatures.

The pH acceptance of *Rp*Ohy was investigated in the pH range of 3.6 – 10 (Figure 11). The highest activity was determined at pH 5 (acetate buffer, 20 mM). The protein remained active over a broad pH range from pH 5 – 8. These results for this *Rhodococcus* Ohy are comparable to the results obtained by Lorenzen *et al*. for Ohy*Re* who described a temperature optimum at 28 °C and a pH optimum of 7.2 with good conversion rates in the range from pH 5 – 8.[10]

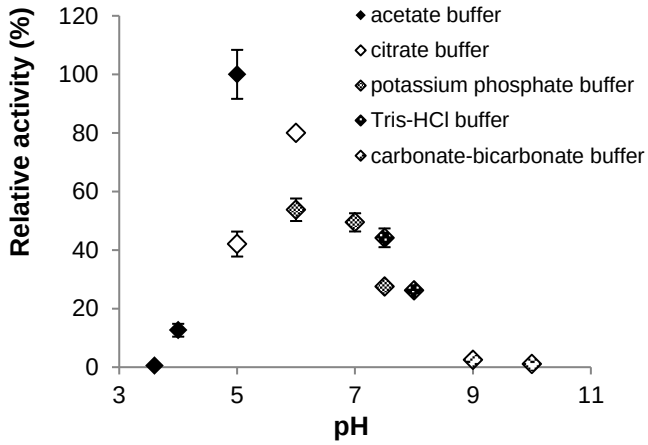


Figure 11. pH tolerance with pH optimum of *Rp*Ohy. Reactions (triplicates) were carried out at 25 °C for five hours at respective pH values.

Determination of the kinetic parameters for *Rp*Ohy with oleic acid as substrate have been carried out. Here, a K_M value of 15 ± 46 µM with a v_{max} of 7.8 ± 1.6 U/mg were found (Figure 12).

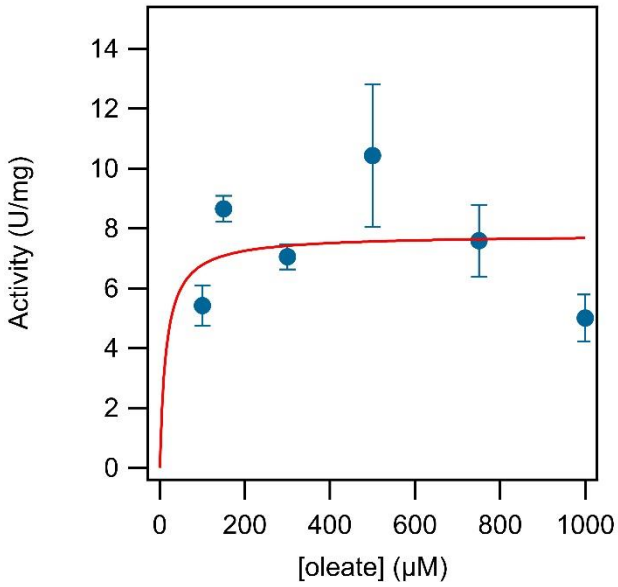


Figure 12: Michaelis Menten kinetics of *Rp*Ohy.

6.3 Conclusions and Outlook

Genome analysis showed sequence diversity among Ohys that can be exploited for the discovery of novel features and will broaden future applications. *Rhodococcus* cells host a large number of interesting catabolic enzymes and are known for their

extraordinary potential for bioremediation.[2,4] The genus *Rhodococcus* was investigated in detail for the presence of oleate hydratases using an orthologous algorithm matrix. From the analysis of 43 whole-genome sequences, 20 putative Ohys were identified clustered into three different clades. Sequence alignment and phylogenetic analysis revealed that each clade contained oleate hydratases from one specific family (HFam1-3). Family-specific amino acid patterns were elucidated. Two representatives from HFam2 and HFam3 were chosen for heterologous expression in *E. coli* and tested on a large number of fatty acids. The two tested oleate hydratases were found to act complementary with longer and sterically more demanding fatty acids. Finally, this study includes the characterisation of purified *Rp*Ohy which represents a novel member of the HFam2.

The family-specific patterns could hint at different reaction mechanisms and explain the differences in substrate recognition. Docking studies with structural models based on published crystal structures from *L. acidophilus* NCFM for *Rp*OHy and *R. erythropolis* CCM2595 for *Re*Ohy were inconclusive due to the absence of FAD in the crystal structures which resulted in the uncertainty of the position and configuration of the cofactor. The structure elucidation of *Rp*Ohy will give further insight in the HFam2 specific reaction mechanism as well as further explain the differences in substrate acceptance.

6.4 Experimental Data

6.4.1 Chemicals

All commercial chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). The UltraClean®Microbial DNA Isolation Kit was purchased from MOBIO Laboratories, Inc. (Uden, The Netherlands).

6.4.2 Bacterial strains and microorganisms

The *Rhodococcus pyridinivorans* DSM 20415 was purchased from the German Collection of Microorganisms and Cell Culture (Leibniz Institute DMSZ). Plasmid pBad/His A and *E. coli* TOP10 cells were obtained from Fisher Scientific (Landsmeer, The Netherlands). Genbank accession numbers of the original and codon-optimised

his-tagged nucleotide sequences of *Rp*Ohy are MN563120 and MN563121, respectively.

6.4.3 Genomic DNA extraction

The genomic DNA of strain *R. pyridinivorans* DSM 20415 was isolated from a cell pellet (50 – 100 mg) using the UltraClean®Microbila DNA Isolation Kit following standard procedure.

6.4.4 Whole-genome sequencing

Genomic DNA of *R. pyridinivorans* DSM 20415 was in-house sequenced on a MiSeq sequencer (Illumina, San Diego, CA) to obtain an 300 cycle paired-end library with an insert-size of 550 bp using PCR-free library preparation, yielding 2.6 Gigabases in total. De novo assembly was performed using SPAdes (version 3.9.0).[28] The assembled genome of *R. pyridinivorans* DSM 20415 were annotated by using Prokka (version 1.12).[29]

6.4.5 Orthologous Matrix (OMA) and phylogenetic analysis

Pairwise orthologues of the proteome of the annotated genome were computed using Orthologous Matrix (OMA) software (standalone version 2.2.0).[16] A species tree was inferred based on the 1% most complete computed orthologous groups. MEGA (version 7.0.21) was used for visualization. Oleate hydratase protein sequences were aligned using muscle (version 3.8.31).[18] Distance-matrix was calculated by FastTree (version 2.1.9, JTT+CAT model) and then visualized by MEGA.[19]

6.4.6 Cloning

The oleate hydratase coding genes from *Rhodococcus erythropolis* PR4 (*Re*Ohy) and from *Rhodococcus pyridinivorans* DSM 20415 (*Rp*Ohy) were used as templates for a codon-optimised gene-synthesis (BaseClear B.V., Leiden, NL). Both synthetic genes (*Re*Ohy and *Rp*Ohy) were sub-cloned in pBadHisA expression vectors and subsequently transformed into *E. coli* Top10 cells following standard procedures.

6.4.7 Protein expression and *Rp*Ohy purification

*Re*Ohy and *Rp*Ohy were expressed in *E. col*i TOP10 cells grown on LB medium. Pre-cultures were inoculated with a single colony and grown overnight at 37 °C with orbital shaking (180 rpm, Innova®44, New Brunswick Scientific). The main cultures (1 L in a 5 L baffled shake flask) were inoculated with 5 mL of pre-culture and grown to an optical density of 0.6 – 0.8 at 37 °C with orbital shaking (180 rpm). The protein expression was induced by adding L-arabinose with a final concentration of 0.2 %. After 16 hours of growing at 25 °C cells were harvested (15 min, 4 °C, 17,700 *xg*), washed with 20 mM Tris.Cl (pH 8) and kept at 20 °C. Frozen cell pellets of *Rp*Ohy were thawed, resuspended in 20 mM Tris.Cl (pH 8) and disrupted by high pressure homogenisation (Constant Systems Ltd., UK). Subsequent centrifugation (15 min, 4 °C, 17,700 xg) yielded cell debris and soluble protein fraction. The supernatant was applied on a Ni^{2+}-NTA His-trap column (HisTrap FF, GE Healthcare, flow rate 1 mL/min) and purified on a NGC™ chromatography system (Bio-Rad Laboratories, Inc., US). The purified protein solution was desalted using a PD10 desalting column. The histag-purified *Rp*Ohy was afterwards separately applied on a HiLoadTM 26/60 SuperdexTM 200 prep grade size exclusion column (flow rate 2 mL/min). Respective fractions were collected and concentrated.

6.4.8 Fatty acid screening

For the fatty acid screening, *E. coli* Top 10 whole-cells with overexpressed *Rp*Ohy, *Re*Ohy or with an empty pBADHisA expression vector were resuspended to a wet-cell content of 100 mg/mL in 50 mM Tris.Cl, pH 7.5, supplemented with 50 mM glucose. Fatty acid stocks were dissolved with co-solvent DMSO (1 % final concentration in reaction). To each reaction, 0.2 mM FAD, 5 mM DTT and 5 mM NADH were added. Reactions (500 µL) were started by substrate addition (500 µM) and were carried out with orbital shaking (800 rpm) at 30 °C for 6 days.

6.4.9 *Rp*Ohy characterisation

For the standard small-scale biotransformations with purified enzyme, *Rp*Ohy (5 µM) was diluted in 20 mM of acetate buffer, pH 5 (200 µL final volume) and 20 µM FAD were added. Reactions were started with the substrate addition (500 µM with 1 %

DMSO) and run for 5 hours at 25 °C with orbital shaking (800 rpm). Temperature optimum was determined in the range of 20 °C to 60 °C with reactions run in acetate buffer (20 mM, pH 5). The pH optimum was investigated in the pH range of 3.6 to 10 at 25 °C. The following buffers were used (20 mM): pH 3.6-5 (acetate buffer), pH 5-6 (citrate buffer), pH 6-7.5 (potassium phosphate buffer), pH 7.2-8 (Tris-HCl buffer) and pH 8-10 (carbonate-bicarbonate buffer). To determine the kinetic parameters, bioconversions using different concentrations (100 µM – 1 mM) and reaction times (1-60 min) were carried out.

6.4.10 Sample derivatisation

All reactions were quenched by the addition 20 µL of 1 M HCl after the respective reaction time. Reactions were extracted with an equal ethyl acetate (EtOAc) volume before derivatisation. 100 µL of extraction mixture was derivatised with 200 µL of derivatising agent (1:1 pyridine:N,O-bis(trimethylsilyl)trifluoroacetaminde (BSTFA) with 1 % trimethyl-silylchloride (TMSCl)) for 1 h at 60 °C.

6.4.11 Sample analysis

Achiral GC-FID analysis of the derivatised hydroxylated fatty acids was performed with a Shimadzu type GC-2014 equipped with a CP sil5 CB column (50 m x 0.53 mm x 1.0 µm) using N2 as carrier gas. The following conditions were used for the achiral separation using direct injection: injector 340 °C, detector (FID) 360 °C, column flow rate 20.0 mL/min, temperature program: start at 130 °C, hold time 4 min, rate 15 °C/min to 330 °C hold time 5 min.

Gas chromatography-mass spectrometry of derivatised hydroxylated fatty acids was performed with the Shimadzu GC-2010 system which is connected to the GCMS-QP2010s mass detector from Shimadzu. The column CP sil5 CB column (25 m x 0.25 mm x 0.4 µm) was used. Injections were performed with the autoinjector AOC-20i from Shimadzu. The injector temperature was kept at 250 °C. The injector was used in split-mode with a split ratio of 30:1 at a pressure of 51.2 kPa. Temperature program for fatty acids 1 and 3-15: start at 130 °C, hold time 4 min, rate 15 °C/min to 330 °C hold time 5 min. Temperature program for fatty acid 2: start at 130 °C, hold time 4 min, rate 5

°C/min to 325 °C hold time 7 min. Structure determination was based on the comparison of monomer peaks using external standards.

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Conclusion and Outlook

This thesis was aimed at the examination of the genus *Rhodococcus* as valuable source for biocatalysts and in particular hydratases – enzymes with the unique ability to add water to double bonds in a controlled and selective fashion. A large number of *Rhodococcus* species has already been shown to be successfully applied in a multitude of different reactions while the hydratase activity of *Rhodococcus* species in general, with the renowned nitrile hydratase being the exemption, have been only scarcely discussed.

An in-depth re-investigation of the hydration potential of various *Rhodococcus* species towards α,β-unsaturated Michael acceptors led to the discovery of the presence of a unique Michael hydratase (Mhy). Though the many attempts to both isolate and identify the enzyme were not successful, novel properties of this intriguing yet elusive enzyme were discovered: the Mhy was identified as the first oxygen-dependent hydratase and was shown to be associated with the plasma membrane in *Rhodococcus* species. The catalysed water addition was carried out in syn-fashion which is a rare feature amongst hydratases found in nature. Additionally, the enzyme of which the natural substrate stays unidentified, was proven to be not as promiscuous as previously assumed. All these findings further increase the importance of identifying this enzyme to develop a robust biocatalyst capable of performing this difficult reaction.

The orthologous matric (OMA) algorithm that was applied in the quest to identify the Mhy coding gene produced an extremely valuable searching tool or database that can be used in the pursuit of other interesting biocatalysts in the future. It classified all present orthologous proteins in the genus *Rhodococcus* and can easily be extended with further strains or species or reduced according to the search's demands. One successful example was shown in this Thesis as the OMA algorithm was applied in the investigation of the abundance of oleate hydratases (Ohys) in the genus *Rhodococcus*. Here, 20 oleate hydratases were identified amongst 43 investigated Rhodococcus strains. Two representatives were successfully produced in an E. coli host system as a proof of principle demonstrating the strength and broad applicability of the developed *Rhodococcus* database.

In doing this, a novel oleate hydratase from *R. pyridinivorans* was identified and subsequently characterised. A fatty acid substrate screening showed the rare ability of

this enzyme for converting the hydroxylated ricinoleic acid yielding a dihydroxylated product which has potential application as biosurfactant. A future expansion of the substrate scope will even broaden its applicability. At the same time, the fatty acid substrate scope was broadened for a previously described oleate hydratase from *R. erythropolis*. This enzyme displayed a large substrate tolerance with a high regioselectivity especially towards long and multiply unsaturated fatty acids making it interesting for future industrial applications.

In conclusion, this thesis was dedicated to identifying novel hydratases to achieve the challenging task of finding mild reaction conditions for a selective water addition. With these novel identified biocatalysts either in form of purified enzymes or whole-cell biocatalysts, chiral, tertiary alcohols were produced which are known as key synthons for a plenitude of compounds and are *e.g.* often applied in the production of pharmaceuticals or cosmetic products. This thesis therefore helps satisfying the demand for the development of environmentally benign alternatives to harsh, ecologically harmful, chemical procedures in the synthesis of some of these key intermediates and at the same time highlights the overall importance of biocatalysis as an essential tool in today’s society.

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'It is better to fail in originality than to succeed in imitation.'

Herman Melville

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Curriculum vitae and list of publications

Curriculum vitae

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List of publications

- '*Rhodococcus* as versatile biocatalyst in organic synthesis'
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- ‘Biocatalysis explained: From Pharmaceutical to Bulk Chemical Production’
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- ‘Hydroxynitrile lyases covalently immobilized in continuous flow microreactors’
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