University of Groningen

Oxidoreductase fusions

Aalbers, Friso

DOI:
[10.33612/diss.127076658](https://doi.org/10.33612/diss.127076658)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Aalbers, F. (2020). *Oxidoreductase fusions: engineering enzymes for coupled reactions and stability*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.
<https://doi.org/10.33612/diss.127076658>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

rijksuniversiteit
groningen

Oxidoreductase fusions

Engineering enzymes for coupled reactions and stability

by

Friso Sybren Aalbers

Cover design and thesis layout by: Nina Lindhout (ninaontwerp.nl)

The cover is an illustration of a fusion enzyme of an alcohol dehydrogenase (TbADH, *Thermoanaerobacter brockii*, PDB: 1YKF) with a Baeyer-Villiger monooxygenase (PAMO, *Thermobifida fusca*, PDB: 2YLR), with the nicotinamide ring (in blue) and FAD isoalloxazine ring (in red) highlighted.

Printed by: Ipskamp Printing

ISBN (printed version): 978-94-034-2602-0

ISBN (electronic version): 978-94-034-2603-7

Copyright © Friso Aalbers 2020, Groningen, The Netherlands

All rights reserved. No part of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means without prior permission of the author.

The research described in this thesis was carried out at the Groningen Biotechnology and Biomolecular Sciences Institute of the University of Groningen, the Netherlands.

The research project was financially supported by European Union (EU) project ROBOX (grant agreement n° 635734) under EU's horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1.

The views and opinions expressed in this thesis are only those of the author(s), and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein.

rijksuniversiteit
 groningen

Oxidoreductase fusions

Engineering enzymes for coupled reactions and stability

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 19 juni 2020 om 9.00 uur

door

Friso Sybren Aalbers

geboren op 13 september 1991
te Tytsjerksteradiel

Promotores

Prof. dr. M.W. Fraaije

Prof. dr. D.B. Janssen

Beoordelingscommissie

Prof. dr. D. J. Slotboom

Prof. dr. G. J. Poelarends

Prof. dr. S. Kara

Table of contents

<u>Section 1</u>	<u>Introduction</u>	
Chapter 1	Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes	7
Chapter 2	Baeyer-Villiger monooxygenases: tunable oxidative biocatalysts	27
<u>Section 2</u>	<u>Fusions of oxidoreductases</u>	
Chapter 3	Coupled reactions by coupled enzymes: alcohol to lactone cascade with alcohol dehydrogenase–cyclohexanone monooxygenase fusions	47
Chapter 4	Design of artificial alcohol oxidases: alcohol dehydrogenase – NADPH oxidase fusions for continuous oxidations	65
Chapter 5	What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monooxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis	79
<u>Section 3</u>	<u>Stability engineering</u>	
Chapter 6	Approaching boiling-point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering	97
<u>Section 4</u>	<u>Conclusions</u>	
Chapter 7	Summary and conclusions	121
	Supporting information	127
	Nederlandse samenvatting	158
	Fryske gearfetting	160
	Curriculum vitae	162
	List of publications	163
	Acknowledgements	165

Section 1

Introduction

Chapter 1: Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes

Friso S. Aalbers and Marco W. Fraaije

This chapter is based on a published article: Aalbers, F. S., & Fraaije, M. W. (2019). Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes. *ChemBioChem*, 20(1), 20-28. <https://doi.org/10.1002/cbic.201800394>

Abstract

One approach to bring enzymes together for multi-enzyme biocatalysis is genetic fusion. This enables production of multifunctional enzymes, which can be used for whole-cell biotransformations or for *in vitro* (cascade) reactions. In some cases and in some aspects, such as expression and conversions, the fused enzymes outperform the combination of the individual enzymes. On the other hand, some enzyme fusions are greatly compromised in activity and/or expression. In this chapter, we give an overview of studies on fusions between two or more enzymes that were used for biocatalytic applications, with a focus on oxidative enzymes. Typically, the enzymes are paired to facilitate cofactor recycling or co-substrate supply. In addition, different linker designs are briefly discussed. Although enzyme fusion is a promising tool for some biocatalytic applications, future studies could benefit from integrating the findings of previous studies, to improve reliability and effectiveness.

Introduction

1 Finding new and efficient ways to synthesize specific compounds is a central endeavor for both academic and industrial chemists. One particular approach involves the combination of multiple enzymes, which catalyze highly selective and sequential reactions in one pot.^[1] This multi-enzyme biocatalysis approach is attracting a steady increase in interest.^[1] Biocatalytic processes are non-toxic, can be operated in mild aqueous conditions, and can potentially be applied on an industrial scale.^[2] The characterization of more and more enzymes over the years, with diverse substrate scopes, enables researchers to devise novel and elegant reaction combinations. In addition, tailoring and optimizing biocatalysts through enzyme engineering is becoming faster and more efficient.^[3] The multi-enzyme strategy is deemed to be a critical stepping stone on the way to large-scale industrial application of biocatalysis.

One advancing aspect of multi-enzyme biocatalysis is the approach of fusing enzymes.^[1e,f] Primarily, enzyme fusion is a convenient way of producing two or more enzymes in one cell, allowing either whole-cell biotransformations, or co-purification. By combining enzymes in this fashion, a single multifunctional biocatalyst can be produced that can catalyze a cascade of reactions. Remarkably, in various studies evidence was found that the covalently tethered enzymes outperform the classic combination of the separate enzymes in some features, including: enhanced expression,^[18, 22] higher conversions of a cascade reaction,^[18, 20, 24, 32, 37] improved stability,^[37a,c, 54] and improved catalytic activity.^[23, 30, 37c, 54] However, these promising observations are inconsistent across studies, and generic methods to predictably design an effective bifunctional biocatalyst are still lacking. Moreover, some studies find a decrease in activity as a consequence of enzyme fusion, showing that fusing enzymes can be delicate.

Enzyme fusions can be made by genetically placing multiple genes together, without stop codon between the genes. The translation would lead to a single polypeptide with multiple enzymes covalently fused. Alternatively, it is possible to create enzyme fusions post-translationally using pairs of tags or scaffolds, of which there are several elegant examples,^[4] though this type of approach is beyond the scope of this chapter. To ensure proper folding and protein stability of fused enzymes, it is common to introduce a sequence coding for a peptide linker between the genes. This linker region can affect the orientation of the two enzymes. Another term often used for an enzyme fusion is “chimeric enzyme”, though this should concern the combination of particular

parts/domains of different enzymes, rather than full-length enzymes, like the name suggests.

The relative improvement of catalytic efficiency of a two-step reaction with a fusion enzyme has been attributed to the phenomenon of substrate channeling: direct migration of the product of one enzyme to the active site of the second enzyme, faster than random diffusion through the bulk solution. A recent review on compartmentalized cascade reactions elaborated on this topic. The authors state that proximity only provides an advantage if the intermediate concentration remains low, and that substrate channeling is only enabled when the fusion enzymes form larger clusters.^[1e] Substrate channeling is quite beneficial for cascade reactions, as it increases the rate of the overall reaction, and decreases intermediate life-time, which can be important for reactive and/or labile intermediates.^[5] Some researchers thoroughly investigated substrate channeling in their systems, and provided evidence for presence or absence thereof.^[5, 6]

In this chapter we focus on recent studies of enzyme fusions for biocatalytic applications, and compare the approaches and findings. In particular, studies concerning fusions consisting of two enzymes and/or catalytic domains are selected. This excludes studies about enzymes fused to peptides or protein domains, such as: affinity tags, carbohydrate-binding modules, expression-boosting domains, antibodies, and other catalytically-inactive proteins, which are reviewed elsewhere.^[7a] In addition, we included only a few studies on carbohydrate-active and biomass-degrading enzyme fusions, as they have been reviewed elsewhere.^[7b]

First, we give an overview of recent studies in which enzyme fusions were created and applied for biocatalytic (cascade) reactions, in particular involving oxidative enzymes (Section 2). General linker design, in terms of flexibility, length and orientation, have been addressed to some extent in reviews. However, the degree to which linkers affect activity and expression has not been reviewed recently, to our knowledge. Based on the studies we discuss in section 2, we provide an overview of the linker design of these fusions, and the effects of different linkers (Section 3). Lastly, we point out the gaps of knowledge, challenges, and we consider the advantages and disadvantages of the approach of fusion enzyme engineering (Section 4).

Enzyme fusions in biocatalysis

The first examples of fusion enzymes were found as natural fusions. Various organisms evolved gene organizations that resulted in fused enzymes. Some

1 known examples are tryptophan synthase,^[8] carbamoyl phosphatase synthase,^[9] and cytochrome P450 BM3.^[10] Given the fact that such enzyme fusions evolved and are frequently encountered, it suggests that enzyme fusion can provide an evolutionary advantage for some metabolic functions. In at least two studies the evolution of a genetic fusion took place: in one case as a result of selection for glycerol production^[11a], and in another case a rapid evolution method was used with selection for growth on guaiacol.^[11b] Related to this, in one study two enzymes were fused and produced *in vivo* to direct metabolic flux towards sesquiterpene production, which shows that enzyme fusion can affect the growth and phenotype of an organism.^[11c] However, it is not universally beneficial, since natural fusions are fairly rare, considering the total number of operons that contain multiple genes. Likewise, studies on enzyme fusions found improvements with some combinations of enzymes, and not with all types of enzymes.

Inspired by nature, various researchers began exploring artificial fusions of two enzymes, and compared these bifunctional enzymes to the separate enzymes. One of the first papers describes a fusion of a histidinol dehydrogenase with an aminotransferase.^[12] Another important early study was done on beta-galactosidase fusion with a galactose dehydrogenase, which showed improvement in the two step conversion from lactose to galactone.^[13] From then on, many more enzyme fusions were produced and studied, of which some have been reviewed before.^[7, 14] This chapter highlights some recent developments in fusing enzyme for biocatalytic applications.

Baeyer-Villiger monooxygenase (BMO) fusions

BMOs are an interesting class of biocatalysts, as they can catalyze Baeyer-Villiger reactions in mild conditions, transforming ketones to esters or lactones, often in a highly regio- and enantioselective manner. Interestingly, genome sequence analysis has revealed that some natural fusions exist: BMOs fused to an esterase, which can hydrolyze a lactone or ester product from the Baeyer-Villiger reaction.^[15] BMOs contain a flavin cofactor (FAD) as prosthetic group. For catalysis, the FAD cofactor is first reduced by NADPH, which enables the reduced FAD to react with dioxygen and form the oxygenating peroxyflavin intermediate. The reliance on NADPH is one of the greatest challenges when applying BMOs as biocatalysts. Because it is an expensive cofactor, efficient recycling is needed to make any BMO-based biocatalytic process feasible. A typical approach for recycling is to use a cofactor-recycling enzyme, which can oxidize a sacrificial substrate to transform NADP⁺ to

NADPH. Some typical examples of such enzymes are: glucose dehydrogenase (GDH), formate dehydrogenase (FDH), and phosphite dehydrogenase (PTDH).

Scheme 1. Dehydrogenase fusion with a Baeyer-Villiger monooxygenase (BVMO), in which the dehydrogenase provides NADPH that is needed for the BVMO reaction. a) A BVMO can also be paired with PTDH, which uses phosphite as sacrificial substrate to regenerate NADPH.^[16, 17] b) Fusion of alcohol dehydrogenase (ADH) with a BVMO for self-sufficient cascade reaction, from an alcohol substrate to ester or lactone.^[18, 19]

Of the latter enzyme, PTDH from *Pseudomonas* sp., Pazmiño and coworkers looked into the effects of fusing such a cofactor-recycling enzyme to BVMOs, to provide direct recycling of NADPH.^[16] It was found that PTDH-BVMO fusions enabled good expression of such bifunctional biocatalysts. By adding phosphite and only minor amounts of NADP^+ , the bifunctional enzymes could catalyze continuous Baeyer-Villiger oxidations (Scheme 1). Three distinct BVMOs were initially selected for probing the fusion approach, with varying substrate scope and enantioselectivity: cyclohexanone monooxygenase (CHMO), phenylacetone monooxygenase (PAMO) and cyclopentanone monooxygenase (CPMO). The bifunctional biocatalysts were found to largely retain the catalytic properties of the original enzymes, such as regio- and stereoselectivity, and kinetic parameters. For PTDH, there was a small increase in K_M for NADP^+ and phosphite, whereas the BVMOs had a small decrease in K_M for the ketone model substrate. Using either whole-cells or cell extracts from cells expressing the fusion enzymes, complete conversion was demonstrated for a large set of substrates. Conveniently, these conversions could even be performed without addition of the expensive cofactor NADP^+ , as

1 the cell extracts contained sufficient nicotinamide cofactor. In a follow-up study the PTDH fusion partner was optimized for its stability and expression in *E. coli* and the developed expression vector was shown to be successful for producing a wide range of PTDH-BVMOs.^[17]

Still, for a large-scale reaction the sacrificial substrate can constitute a significant cost contribution, as it also gives a by-product. An alternative to a sacrificial substrate for regenerating NADPH is a cascade reaction starting from the alcohol rather than the ketone (Scheme 1). An alcohol dehydrogenase can use NADP⁺ to oxidize the alcohol to a ketone to produce NADPH, and the ketone and NADPH can then be used by the BVMO. Recently, ADH-BVMO fusions were designed and expressed in *E. coli*, and the whole-cells were used in reactions with different hydroxy fatty acid substrates, to convert these in a cascade reaction to esters.^[18] As comparison, cells co-expressing the separate ADH and BVMO were included. Relative to those cells, the cells expressing the ADH-BVMO fusion with a glycine-rich linker showed greater cell activity, and higher conversions for all substrates tested. The authors ascribe this improvement primarily to higher expression of the fusion. Another notable observation was the difference in ratio of normal to abnormal ester product between different linkers, which suggests that linker design can influence this ratio, possibly through slight conformational changes. The authors linked the improved mass balance that was found for the fusions to the co-localization of the active sites, considering the hydrophobicity of the substrate and intermediate. This would be an interesting advantage for such fusion enzymes, and is reminiscent of the substrate channeling in natural fusion enzymes, e.g. tryptophan synthase.^[8]

Another study explored the same combination of enzyme classes to facilitate a cascade reaction from cyclohexanol to caprolactone. Three different ADH enzymes were selected, and a thermostable cyclohexanone monooxygenase (TmCHMO) was chosen as BVMO (**Chapter 3**).^[19] Inspired by the previous study on ADH-BVMO fusions, the glycine-rich linker was chosen, and all fusions were made in both orientations. Notably, the two short-chain dehydrogenase (SDR) ADHs were found to be inactive as N-terminal fusions (ADH-CHMO), and were also found to be a monomer, rather than a dimer/tetramer. It is likely that the fusion at the C-terminus of SDRs interferes with the oligomerization of the enzymes, which is necessary for activity. Other studies found a similar effect with a C-terminal fusion of other enzymes to an SDR, and with a C-terminal His-tag.^[20] For the other ADH and for TmCHMO no substantial change in activity was observed when fused. The most active fusion was used in a small-scale conversion of 200 mM cyclohexanol, which

reached 99% conversion, whereas the separate enzymes reached only 41% conversion. This study showed again that it is difficult to predict whether a protein can be used as a fusion partner. Even if inspection of crystal structures reveals an accessible N- and C-terminus, protein-protein interactions may be detrimental for correct folding or oligomerization.

Another interesting exercise in producing fusions with CHMO was recently reported by Peters et al. It was attempted to bring together three enzymes in one fusion protein: an ADH, an ene-reductase (ER), and a CHMO.^[20] As the group demonstrated prior to this work, through the combination of these three enzymes one can convert unsaturated cyclic alcohols to enantiopure chiral lactone products. Starting from an alcohol, the ADH would bring partial redox balancing, as the ER and CHMO both need the reduced nicotinamide cofactor. Their strategy was to first combine the ER and CHMO, and found that this successfully yielded a bifunctional enzyme. Unfortunately, the authors found that any fusion including the studied ADHs was not expressed. Still, the cascade reaction catalyzed by the ER-CHMO fusion outperformed the same reaction with the separate enzymes.

Flavin reductase (FR) fusions

Similar to BVMOs, styrene monooxygenases (SMOs) use a reduced flavin cofactor (FADH₂) to activate oxygen, though they catalyze a different reaction. SMOs bind styrene in such a way that the activated oxygen reacts with the unsaturated bond of styrene, forming one enantiomer of styrene oxide (Scheme 2). However, unlike BVMOs, this class of enzymes does not bind FAD tightly, and cannot bind an electron donor like NAD(P)H to reduce FAD. Therefore, SMOs need to receive the reduced flavin from another enzyme: NADH-dependent flavin reductase (FR), which is in many genomes either in the same operon (i.e. co-expressed with the SMO), or fused to the SMO.^[21] The flavin reductase is often referred to as ‘styrene monooxygenase reductase’ or StyB, and the oxygenase that catalyzes the oxidation is denoted as StyA. Recently, several groups have been artificially fusing FRs to SMOs, and investigated the effects of different linkers.

Scheme 2. Concept of fusing a flavin reductase (FR) to an $FADH_2$ -dependent enzyme. Another enzyme (not in scheme) facilitates regeneration of NAD^+ to $NADH$ (such as formate dehydrogenase). a) Fusion of SMO (StyA) with FR (StyB) for efficient coupling of reduced flavin for styrene oxidation.^[22, 23] b) Flavin halogenase (FH) can also be fused with an FR, to couple the two reactions.^[24]

In 2016, the groups of Gassner and Tischler have carefully investigated the effect of fusing the reductase with the epoxidase component.^[22] The fusions were evaluated based on activity on indole and derivatives thereof, to produce various dyes. Foremost, the authors argue that having the two enzymes fused together is convenient for engineering, expressing and performing oxidations. An important improvement that was observed, compared to the use of separate enzymes, is a higher coupling efficiency, which is the degree to which reduced FAD leads to oxidation versus formation of hydrogen peroxide (referred to as uncoupling). Through fusion, the enzymes are in a strict 1:1 ratio, which prevents having excess reductase that could lead to excessive uncoupling. In addition, the expression of the reductase domain individually results in inclusion bodies, whereas expression of the fusion of the epoxidase and reductase gave soluble and active bifunctional enzymes. However, the epoxidation activity was significantly decreased, compared to the separate enzymes. In contrast, another group that studied the same enzymes as a fusion found it had a similar activity to the combination of separate StyA/StyB in a 1:1 ratio.^[23] This activity was higher than the naturally fused StyA2B, and the fusion enzymes addressed above.^[22] The authors suspect that this difference is due to the glycine-rich flexible linker from their SMO, compared to the shorter and rigid linkers in previous SMO fusions. Both studies emphasize that the fusion of the two enzymes is advantageous in terms of expression, purification, and applying the enzymes.

Another class of enzymes that relies on an FR for reduced flavin are flavin-dependent halogenases (FHs). This extraordinary class of enzymes can catalyze regioselective halogenations of aromatic substrates (Scheme 2). Similar to the FR-SMO fusions addressed above, a group studied fusions of various FRs with FHs, to study the effects of this fusion approach, in particular for whole-cell applications.^[24] A decrease in activity was observed *in vitro*, though when expressing the fusions for whole-cell biotransformations, higher product titers were reached compared to cells co-expressing the separate enzymes. Again, as with the studies above, the authors indicate that the fusion approach simplifies the studying and engineering of the system.

From these examples it seems that for the combination of a flavin reductase with an enzyme that requires reduced flavin (SMO, FH), enzyme fusion is providing notable advantages. This is also a type of fusion that is encountered in several genomes, such as natural FR-SMO fusions and reductase-P450 fusions. In that sense it is quite important to see that there are examples where it does not work that well, to identify the important factors and prerequisites for creating functional fusions, such as linker design (which will be discussed in section 3).

Heme-containing enzymes: cytochrome P450 and peroxidase fusions

In biocatalysis, an extensively studied cytochrome P450 monooxygenase is the natural fusion P450 BM3 from *Bacillus megaterium*.^[13] It can catalyze enantio- and regioselective hydroxylations, and it consists of two catalytic domains: the CPR, which is a NADPH-dependent FAD- and FMN-containing reductase domain, and a monooxygenase heme domain. In some studies various artificial reductase-monooxygenase fusions were made, either to improve stability^[25] and/or to improve electron coupling efficiency.^[26] Sabbadin et al. developed a cloning vector, which contains the gene coding for the P450 reductase domain RhF-Red, that enables seamless ligation-independent cloning of any P450 heme domain fused to RhF-Red.^[27] This vector was used in a study in which 23 genes coding for heme domains from *Rhodococcus jostii* RHA1 were fused to RhF-Red, to screen the P450 fusions for activity on various pharmaceutical compounds.^[28]

Like the aforementioned monooxygenases, P450 monooxygenases also rely on reduced NAD(P)H for catalysis. In order to establish a biocatalytic process utilizing this class of enzymes, an NAD(P)H recycling system is needed. Like with the BVMOs mentioned above, Beyer and coworkers fused PTDH to P450 BM3, and studied this multifunctional self-sufficient enzyme.^[29] Remarkably, the PTDH-P450 fused enzyme showed a slight improvement in activity, and in

several other aspects performed better than the combination of the two separate enzymes. Even though the fusion is rather large (155 kDa), the expression is not compromised. The authors demonstrate the utility of the PTDH-P450 by converting several pharmaceutical compounds to hydroxylated drug-metabolites.^[29, 30] A similar study was done with a heterotrimeric P450 from *Pseudomonas putida*, where it was fused to PTDH for self-sufficient hydroxylations.^[31]

Scheme 3. Oxidase-Peroxygenase and Oxidase-Peroxidase fusions. a) By combining Alditol oxidase (AldO) with OleTJE, decarboxylations of myristic acid can be catalyzed with the *in situ* formed peroxide, at the cost of glycerol.^[32] b) The conversion from vanillyl alcohol by Eugenol oxidase (EUGO) leads to formation of vanillin and hydrogen peroxide. The peroxidase (DyP) can use peroxide to react create a radical form of vanillin, which can react to with another divanillin.^[34]

Enzyme fusion can be a great way to generate a cofactor or cosubstrate for a particular enzyme reaction. An example of cosubstrate production is achieved by the fusion of alditol oxidase with a cytochrome P450 (Scheme 3a). The particular P450 monooxygenase from *Jeotgalicoccus* sp., named OleT_{JE}, can act as a peroxygenase, and primarily catalyzes oxidative decarboxylation of fatty acids. In its mode as peroxygenase, the P450 merely requires hydrogen peroxide to perform oxygenations. In order to prevent peroxide induced enzyme inactivation, it is attractive to have *in situ* formation of hydrogen peroxide when employing a peroxygenase. By tethering a peroxide-dependent enzyme with an oxidase (generating hydrogen peroxide) as a fusion, reactions can be catalyzed with the *in situ* produced hydrogen peroxide.^[32] The choice for alditol oxidase allowed the use of glycerol as sacrificial substrate, which is

very cheap and abundant. The fusion enzyme was able to decarboxylate myristic acid to produce tridecene with the help of glycerol (Scheme 3a). To examine the effect of the fusion, the authors designed a linker that can be cleaved with a specific protease, and then directly compared the fusion cleaved and non-cleaved. They discovered that the non-cleaved fusion enzyme had a higher conversion of myristic acid, suggesting that there is a beneficial proximity effect. Interestingly, fusion of OleT_{JE} with the flavin reductase from BM3 produces a self-sufficient decarboxylase, yet with higher activity and broader substrate scope.^[33] It relies on oxygen and NADPH rather than hydrogen peroxide, similar to the PTDH-P450 BM3 mentioned above.^[29] The study demonstrates that the activity of this OleT_{JE} P450 can be altered with different types of electron donor-producing fusion partners.

An oxidase, which produces hydrogen peroxide, and an enzyme that uses hydrogen peroxide as substrate would form a perfect match. A recent study explored fusions of oxidases with a dye decolorizing peroxidase (DyP), which is a heme-containing enzyme that uses hydrogen peroxide to oxidize phenolic compounds and dyes (Scheme 3b).^[34] Four oxidases were selected and fused, without a linker, to the bacterial peroxidase SviDyP: alditol oxidase (HotAldO), 5-hydroxymethylfurfural oxidase (HMFO), chitooligosaccharide oxidase (ChitO), and eugenol oxidase (EugO). All four fusions could be expressed and purified, with a high degree of heme and flavin incorporation. The oxidase components of the fusions lost some activity, which could be due to the absence of a suitable linker. One challenging aspect of this combination is that most oxidases have a pH optimum around 7-8, while DyPs are most active around pH 4-5. Nevertheless, the authors were able to demonstrate successfully several coupled reactions, in which the fusions function as biosensors or as bifunctional biocatalysts for performing cascade reactions, in which both the hydrogen peroxide and the product from the oxidase are used by the peroxidase.

Alcohol dehydrogenase and transaminase fusions

Some other conceptually intriguing fusions include two examples with an alcohol dehydrogenase (ADH). Like in the examples mentioned in the section with BVMOs, ADHs can perform alcohol oxidations by using NAD(P)⁺. However, this reaction is thermodynamically less favorable than the reverse reaction. In order to use ADHs for complete alcohol oxidations, it is necessary to recycle NAD(P)⁺.^[35] To enable efficient recycling, an ADH can be combined with an NAD(P)H-oxidase (NOX). Such FAD-containing enzymes can oxidize NAD(P)H, and produce water or hydrogen peroxide as by-product. One study

describes a fusion between a glycerol dehydrogenase (GlyDH) and a NOX, to produce a self-sufficient enzyme that converts glycerol to dihydroxyacetone.^[36] However, the activity of the enzymes as fusion was significantly decreased. This was possibly due to the absence of a linker, and/or the orientation of GlyDH-NOX, which could interfere with the quaternary structure of the GlyDH.

Scheme 4. Fusion of an alcohol dehydrogenase with an aminotransferase (AT) enables a cascade reaction starting from an alcohol and producing a chiral amine, at the expense of NAD^+ and alanine, and producing NADH and pyruvate.^[39]

Asymmetric reductions of ketones to chiral alcohols can also be catalyzed by alcohol dehydrogenases. This reverse reaction relies on NAD(P)H , like the reactions catalyzed by BVMOs and P450s. Similar to how for those oxidative enzymes were fused to NAD(P)H -regenerating enzymes, a number of studies explored fusions of ADHs. Three studies fused formate dehydrogenase with a specific ADH^[37], and in one study glucose dehydrogenase was used.^[38] Even though fusing such dehydrogenases with one another can be more challenging, due to the typical quaternary structures of these enzymes, these studies reported on successful examples of active enzyme fusions with alcohol dehydrogenases.

Alcohol dehydrogenases can also serve in a cascade reaction with transaminases, as the latter can act on a ketone substrate, which can be produced by an ADH. Fusions of an ADH with an AT were designed and used to catalyze a cascade reaction to produce amines from alcohols (Scheme 4).^[39] While taking into account the quaternary structures of both enzymes, and to make space for proper folding, three linkers of different length were used: 20, 40, and 60 amino acids. The linkers consisted of PAS sequences: combinations of proline, alanine, and serine, which form disordered, uncharged and highly soluble spacers.^[40] Each of the linkers had a particular effect: PAS60 resulted in the highest soluble expression, PAS40 retained most activity, while PAS20

had higher conversion for the coupled reaction, with about 2-fold higher conversion compared to the separate enzymes. By measuring the initial rate of the second step, with different concentrations of the first substrate, it was shown that the fused system displays a beneficial proximity effect. However, only low conversions were performed, as the NADH was not recycled to NAD⁺ in this system. A similar yet redox-neutral combination would be the fusion of an ADH with an amine dehydrogenase, using alcohols and ammonia to produce amines.^[41]

Fusion design and linker design

When fusing two genes to produce a fusion enzyme, there are at least two things to consider: in which order to place the genes, and which linker to choose. In some cases the effect of the arrangement is not drastic, like for the PTDH-BVMO fusions, for which both enzymes were also active in the BVMO-PTDH orientation.^[16] However, other enzymes are inactive and/or fold improperly when another protein is attached to their N- or C-terminus, as was observed for SDR enzymes with C-terminal fusions (**Chapter 3**).^[19] Similar problems were observed when an improper linker was chosen, or in absence of a linker. In other words, linker design can be critical for the generation of an active fusion enzyme.

Broadly speaking, there are two aspects of the linker to consider: the composition (in terms of flexibility – rigidity, and hydrophilicity – hydrophobicity) and the length. Linkers that contain predominantly glycine are flexible, whereas prevalence of alpha-helix associated amino acids, such as alanine and lysine, form a rigid tether.^[42] To investigate the nature of this dichotomy in linkers, Li and coworkers performed simulations, and constructed a linker library consisting of varying amounts of flexible and rigid parts.^[43] By measurements on variants of this library using FRET, which is a perfect indicator of proximity, their simulations could be validated. Their work provides guidance in controlling the flexibility of a linker. With regards to the flexibility of glycine-containing linkers, another group investigated linkers with varying amounts of glycine and varying length.^[44] Their findings showed that length and a low percentage of glycines in the linker correlated with lower FRET frequency. Even though these two studies used protein fusions rather than enzyme fusions, the knowledge of linker design should be generally applicable.

An extensive review on loops and linkers elaborates on linker design in great detail, and emphasizes how linkers are not merely ‘connectors’ but have

an important effect on the microenvironment and orientation of the fusion.^[45] It turns out that the orientation between the two enzymes, as a consequence of the linker, can significantly impact the efficiency of the reaction,^[46] although in practice this is difficult to control.^[47] Some studies point out that by computational simulations and/or linker databases, a more consistent and less arbitrary design can be established,^[45, 48] as exemplified by the studies mentioned above^[43, 44], though up to now this has hardly been integrated with enzyme fusions. In one study, simulations were used to decide the gene order of a fusion enzyme of formate dehydrogenase with leucine dehydrogenase.^[37c]

Although the studies on linker design help to narrow down the selection of a linker, there is no clear consensus on both composition and length. It seems to be case dependent, as there is variation in the N- and C-termini of the enzymes, in dynamics (i.e. conformational movements), and in the tertiary and quaternary structure. The fusions discussed in this introductory chapter are listed in Table 1, alongside their respective linkers. Strikingly, there is quite some variation in composition and length of linkers. A commonly used and relatively successful linker is a glycine-rich linker, which forms a disordered loop that in theory provides freedom for folding and other conformational movements. The latter in particular might be an underestimated factor, since for some enzymes conformational changes play an important role in catalysis, including BVMOs.^[49, 50]

Some studies designed and produced fusions with different linkers, and proceed with the most suitable linker based on the initial results. For instance, with the FR-SMO fusions four different linkers were tested, from which two linkers lead to insoluble inclusion bodies. For the two linkers that did show soluble expression (StyAL1B, StyAL2B, Table 1), a decrease in activity was observed compared to the separate enzymes.^[22] However, when another group fused the same enzymes (Fus-SMO, Table 1) with a 30-amino acid glycine-rich linker, the level of activity was unaffected, and the expression level was higher (40 mg l⁻¹ compared to 12-15 mg l⁻¹ for the StyAL1B and StyAL2B).^[23] In a recent study of a natural FR-SMO fusion from *Variovorax paradoxus* EPS, the original linker (AREAV) was replaced with 6 distinct linkers.^[51] For each variant the expression was lower, and some catalytic properties were altered. Remarkably, one linker (AAAAA) displayed a lower K_M for FAD (from 33 μ M to 1.8 μ M) and higher monooxygenase activity.

While some studies show different consequences for different linkers, in others the linker hardly makes a difference. In the second study on PTDH-BVMO fusions,^[17] the PTDH-PAMO fusion was produced with four different linkers (Table 1). The authors found no difference in specific activity and

thermostability between the linkers, and conclude that for these two enzymes in particular the termini of the enzymes are accommodating, in terms of flexibility. However, in the study on the ER-CHMO fusions, in which the same linkers were used, the tryptophan linker performed significantly worse in conversions.^[20]

Although some studies listed used linkers of different length, there are a few studies that rigorously investigated the effect of linker length. In one of these studies, a reductase was fused to CYP153 from *Marinobacter aquaeolei*, and the linker length was varied between 11 and 32 residues.^[52] Three variants were found to have improved activity, of which one variant (with a linker of 18 residues) also showed improved stability and coupling efficiency, although expression was decreased. In another study, in which a P450 was fused to a flavodoxin shuttle protein, a library of linkers was designed, ranging from 1 to 16 amino acids. The variants were compared based on whole-cell conversion, and the most optimal linker was found to contain 10 amino acids.^[53] Strong differences were also found in a β -glucanase – xylanase fusion with different linker lengths, with greatly improved activities.^[54] Based on these three studies it seems that, in addition to linker composition, the linker length can have a pronounced effect on the properties of the enzymes. It would be interesting to see whether this is also the case for other enzyme systems, aside from the ones studied so far. To that end, a recent study reported a straightforward PCR method to generate linker libraries for any fusion construct.^[55]

Table 1 – List of enzyme fusions

Fusion name(s)	Enzyme types	Linker	Utility of fusion	Ref
TmCHMO-ADHA TmCHMO-ADHMi TbADH-TmCHMO	ADH, BVMO	Glycine-rich linker (13): SSGSGSGSGGSAG	Cascade reaction cyclic alcohol to lactone	Chapter 3, [19]
ADH-Gly-BVMO ADH-FOM-BVMO ADH-BVMO	ADH, BVMO	L1: (12) SGGSGSGSGGSAG L2: (30) SASNCLIGLFLNDQEL KKKAKVYDKIAKDV L3: (0)	Cascade reaction alcohol to ester	[18]

pCRE2-BVMO	PTDH, BVMO	L1: (6) SRSAAG L2: (13) SSGSGSGSGGSAG L3: (12) SSATGSATGSAG L4: (1) W	NADPH-recycling system	[16,17]
pCRE2-P450-BM3	PTDH, P450	Short-linker (6): SRSAAG	NADPH-recycling system	[29, 30]
StyAL1B StyAL2B StyAL3B StyAL4B	Styrene mono-oxygenase (StyA), Flavin reductase (StyB)	L1: (3) WYH L2: (6) WYHHHH L3: (6) WYHAAA L4: (3) WYH* (-3 aa from StyB)	Electron transfer for epoxidation of styrene	[22]
Fus-SMO	Styrene mono-oxygenase (StyA), Flavin reductase (StyB)	Flexible linker (30): ASGGGGSGGGSGGG GSGGGSGGGSGGAS	Electron transfer for epoxidation of styrene	[23]
FH-FR	Flavin-dependent Halogenase, Flavin reductase	L1: (10) PSPSTDQSPS L2: (16) VLHRHQPVTIGEPAAAR L3: (22) VLHRHQPVSPIHSRTIGEPAAAR	Electron transfer for halogenation	[24]
ADH-AT	ADH, Amino-transferase	L1: <u>PAS</u> linker: (20) ASPAAPAPAS PAAPAPSAPA L2: (40) <u>PAS</u> x2 L3: (60) <u>PAS</u> x3	Cascade reaction alcohol to amine, stabilization through linker	[39]
OleT _{JE} -AldO	P450, Alcohol Oxidase	L1: (18) GSG <u>LEVLFOG</u> PGSG GGGG L2: (45) A(EAAAK) ₄ -LEA-(EAAAK) ₄ A	Hydrogen peroxide supply for decarboxylation reaction, cleavable linker (underlined)	[32]
P-EugO P-HFMO P-ChitO P-HotAldO	Peroxidase, Alcohol Oxidase	None	Hydrogen peroxide supply for peroxidase, cascade reactions, biosensor	[34]

XenB-CHMO	Ene reductase, BVMO	L1: (13) SSGSGSGSGSAG L2: (12) SSATGSATGSAG L3: (1) W	Cascade reaction unsaturated cyclic alcohols to chiral lactones	[20]
GlyDH-NADH oxidase	ADH, NOX	None	Recycling of NAD ⁺ for glycerol oxidations	[36]
PTS-FPPS	Synthases	None	Metabolic flux redirection towards sesquiterpene production	[11b]

Conclusions and outlook

In the emerging platform of multi-enzyme biocatalysis, enzyme fusion can be a useful tool for simplification and optimization of a multi-enzyme system, in particular for enzyme production. In addition, expression of fused enzymes can enable cascade reactions *in vivo*, which in some cases outperform the cascade reactions with co-expressed enzymes. In particular, the tool seems well suited for cascade systems that rely on cofactors or co-substrates, such as: NAD(P)H, FADH₂, and H₂O₂. While in some cases the production of a bifunctional enzyme is primarily a matter of convenience, in other cases the pairing of two enzymes provides an advantage in terms of expression, catalytic activity, and stability.

From the overview of linkers from this set of biocatalytic fusion studies (Table 1) it seems that part of the studies chose none or a generally safe linker variant (the glycine-rich linker), and the other part explored different linkers. Even though linker design up to now is treated in a somewhat arbitrary fashion, as is signified by this set of studies, the choice of linkers in future studies could benefit from the collection of linkers in databases and computational simulations, as well as from the ground work that has been covered here.

This chapter primarily covered enzyme fusions for (oxidative) biocatalytic reactions, though there are also extensive developments in the field of carbohydrate-active enzyme fusions.^[7b] From there and from the other developing areas of enzyme fusion described in this chapter, inspiration can be drawn for novel investigations and applications.

This thesis

The following introductory chapter describes biotechnological application of BVMOs in general, with particular focus on the current challenges (**Chapter 2**). Then, concerning novel investigations, this thesis contains three research chapters that investigate the utility of the fusion approach in three different systems (Section 2: **Chapter 3, 4 and 5**). First, fusions between ADHs and a BVMO were studied, to enable redox-balanced cascade reactions from a cyclic alcohol to lactones (**Chapter 3**). This study was also briefly discussed in the current chapter. The second study describes fusions of different ADHs with an NADPH-oxidase, to facilitate regeneration of NADP⁺, which enables continuous alcohol oxidations with ADHs, essentially creating oxidases (**Chapter 4**). The final chapter on enzyme fusions investigates three different NADPH-recycling enzymes that are fused to a BVMO and an ADH, to facilitate NADPH for respectively Baeyer-Villiger oxidations and ketone reductions (**Chapter 5**). The fusions were directly compared in biotransformations-conversions on various compounds, and with different cosolvents. The final research chapter of this thesis describes the stabilization of an ADH through computational predictions, with the FRESCO method (Section 3: **Chapter 6**). The engineered ADH mutant is much more robust, and therefore more suited for biotechnological application.

Acknowledgements

This work has received funding from the European Union (EU) Project ROBOX (Grant Agreement No. 635734) under EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. The views and opinions expressed in this chapter are only those of the authors, and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein.

References

- [1] a) J. M. Sperl, V. Sieber, *ACS Catal.* **2018**, *8*, 2385–2396; b) J. H. Schrittwieser, S. Velikogne, W. Kroutil, *Catal.* **2018**, *8*, 205; c) J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev.* **2017**, *118*, 270–348; d) S. Wu, Z. Li, *ChemCatChem* **2018**, *10*, 2164–2178; e) K. S. Rabe, J. Müller, M. Skoupi, C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2017**, *56*, 13574–13589; f) M. B. Quin, K. K. Wallin, G. Zhang, C. Schmidt-Dannert, *Org. & Biomol. Chem.* **2017**, *15*, 4260–4271. g) S. P. France, L. J. Hepworth, N. J. Turner, S. L. Flitsch, *ACS Catal.* **2016**, *7*, 710–724.
- [2] R. A. Sheldon, J. M. Woodley, *Chem. Rev.* **2017**, *118*, 801–838.
- [3] a) R. A. Sheldon, P. C. Pereira, *Chem. Soc. Rev.* **2017**, *46*, 2678–2691; b) G. Li, J. Wang, M. T. Reetz, *Bioorg. Med. Chem.* **2018**, *26*, 1241–1251; c) J. L. Porter, R. A. Rusli, D. L. Ollis, *ChemBioChem* **2016**, *17*, 197–203.
- [4] a) H. Hirakawa, T. Nagamune in *Methods Mol. Biol.* (Eds.: J. C. Samuelson), Humana Press, **2013**, pp. 149–163. b) S. Schulz, D. Schumacher, D. Raszkowski, M. Girhard, V. B. Urlacher, *Front. Bioeng.*

- Biotechnol.* **2016**, 4, 57. c) Z. Yang, H. Wang, Y. Wang, Y. Ren, D. Wei, *ACS Synth. Biol.* **2018**, 7, 1244–1250. d) G. Zhang, M. B. Quin, C. Schmidt-Dannert, *ACS Catal.* **2018**, 8, 5611–5620.
- [5] I. Wheeldon, S. D. Minter, S. Banta, S. C. Barton, P. Atanassov, M. Sigman, *Nat. Chem.* **2016**, 8, 299–309.
- [6] C. You, Y. H. P. Zhang, *ACS Synth. Biol.* **2013**, 3, 380–386.
- [7] a) H. Yang, L. Liu, F. Xu, *Appl. Microbiol. Biotechnol.* **2016**, 100, 8273–8281; b) S. Elleuche, *Appl. Microbiol. Biotechnol.* **2014**, 99, 1545–1556.
- [8] C. Yanofsky, *Bacteriol. Rev.* **1960**, 24, 221.
- [9] H. Nyunoya, K. E. Broglie, C. J. Lusty, *Proc. Natl. Acad. Sci.* **1985**, 82, 2244–2246.
- [10] L. O. Narhi, A. J. Fulco, *J. Biol. Chem.* **1986**, 261, 7160–7169.
- [11] a) I. M. Salles, N. Forchhammer, C. Croux, L. Girbal, P. Soucaille, *Metab. Eng.* **2007**, 9, 152–159; b) M. Tumen-Velasquez, C. W. Johnson, A. Ahmed, G. Dominick, E. M. Fulk, P. Khanna, S. A. Lee, A. L. Schmidt, J. G. Linger, M. A. Eiteman, et al., *Proc. Natl. Acad. Sci. U. S. A.* **2018**, 115, 7105–7110. c) L. Albertsen, Y. Chen, L. S. Bach, S. Rattleff, J. Maury, S. Brix, J. Nielsen, U. H. Mortensen, *Appl. Environ. Microbiol.* **2010**, 77, 1033–104.
- [12] J. Yourno, T. Kohno, J. R. Roth, *Nature* **1970**, 228, 820–824.
- [13] L. Bülow, P. Ljungcrantz, K. Mosbach, *Nat. Biotechnol.* **1985**, 3, 821–823.
- [14] R. O. Conrado, J. D. Varner, M. P. DeLisa, *Curr. Opin. Biotechnol.* **2008**, 19, 492–499.
- [15] J. B. van Beilen, F. Murlane, M. A. Seeger, J. Kovac, Z. Li, T. H. M. Smits, U. Fritsche, B. Witholt, *Environ. Microbiol.* **2003**, 5, 174–182.
- [16] D. E. Torres Pazmiño, R. Snajdrova, B. J. Baas, M. Ghobrial, M. D. Mihovilovic, M. W. Fraaije, *Angew. Chemie* **2008**, 120, 2307–2310.
- [17] D. E. Torres Pazmiño, A. Riebel, J. de Lange, F. Rudroff, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* **2009**, 10, 2595–2598.
- [18] E. Y. Jeon, A. H. Baek, U. T. Bornscheuer, J. B. Park, *Appl. Microbiol. Biotechnol.* **2015**, 99, 6267–6275.
- [19] **Chapter 3:** F. S. Aalbers, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2017**, 101, 7557–7565.
- [20] C. Peters, F. Rudroff, M. D. Mihovilovic, U. T. Bornscheuer, *Biol. Chem.* **2017**, 398, 31–37.
- [21] E. W. van Hellemond, D. B. Janssen, M. W. Fraaije, *Appl. Environ. Microbiol.* **2007**, 73, 5832–5839.
- [22] T. Heine, K. Tucker, N. Okonkwo, B. Assefa, C. Conrad, A. Scholtissek, M. Schlömann, G. Gassner, D. Tischler, *Appl. Biochem. Biotechnol.* **2016**, 181, 1590–1610.
- [23] M. L. Corrado, T. Knaus, F. G. Mutti, *ChemBioChem* **2018**, 19, 679–686.
- [24] M. C. Andorfer, K. D. Belsare, A. M. Girlich, J. C. Lewis, *ChemBioChem* **2017**, 18, 2099–2103.
- [25] S. Eiben, H. Bartelmäs, V. B. Urlacher, *Appl. Microbiol. Biotechnol.* **2007**, 75, 1055–1061.
- [26] S. M. Hoffmann, M. J. Weissenborn, L. Gricman, S. Notonier, J. Pleiss, B. Hauer, *ChemCatChem* **2016**, 8, 1591–1597.
- [27] a) F. Sabbadin, R. Hyde, A. Robin, E. M. Hilgarth, M. Delenne, S. Flitsch, N. Turner, G. Grogan, N. C. Bruce, *ChemBioChem* **2010**, 11, 987–994; b) F. Sabbadin, G. Grogan, N. C. Bruce, in *Methods Mol. Biol.*, Humana Press, **2013**, pp. 239–249.
- [28] J. K. Kulig, C. Spandolf, R. Hyde, A. C. Ruzzini, L. D. Eltis, G. Grönberg, M. A. Hayes, G. Grogan, *Bioorg. Med. Chem.* **2015**, 23, 5603–5609.
- [29] N. Beyer, J. K. Kulig, A. Bartsch, M. A. Hayes, D. B. Janssen, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2016**, 101, 2319–2331.
- [30] N. Beyer, J. K. Kulig, M. W. Fraaije, M. A. Hayes, D. B. Janssen, *ChemBioChem* **2018**, 19, 326–337.
- [31] H. Watanabe, H. Hirakawa, T. Nagamune, *ChemCatChem* **2013**, 5, 3835–3840.
- [32] S. Matthews, K. L. Tee, N. J. Rattray, K. J. McLean, D. Leys, D. A. Parker, R. T. Blankley, A. W. Munro, *FEBS Lett.* **2017**, 591, 737–750.
- [33] C. Lu, F. Shen, S. Wang, Y. Wang, J. Liu, W. J. Bai, X. Wang, *ACS Catal.* **2018**, 8, 5794–5798.
- [34] D. I. Colpa, N. Loncar, M. Schmidt, M. W. Fraaije, *ChemBioChem* **2017**, 18, 2226–2230.
- [35] G. Rehn, A. T. Pedersen, J. M. Woodley, *J. Mol. Catal. B Enzym.* **2016**, 134, 331–339.
- [36] F. B. Fang, W. Jiang, Q. Zhou, S. Wang, *PLoS One* **2015**, 10, e0128412.
- [37] a) B. Li, Y. Li, D. Bai, X. Zhang, H. Yang, J. Wang, G. Liu, J. Yue, Y. Ling, D. Zhou, et al., *Sci. Rep.* **2015**, 4, 6750. b) I. Sührer, M. Haslbeck, K. C.-P. Biochemistry, U. **2014**, Elsevier 2014. c) Y. Zhang, Y. Wang, S. Wang, B. Fang, *Eng. Life Sci.* **2017**, 17, 989–996.
- [38] T. Sun, B. Li, Y. Nie, D. Wang, Y. Xu, *Bioresour. Bioprocess.* **2017**, 4, 21.
- [39] A. Lerchner, M. Daake, A. Jarasch, A. Skerra, *Protein Eng. Des. Sel.* **2016**, 29, 557–562.
- [40] M. Schlapschy, U. Binder, C. Borger, I. Theobald, K. Wachinger, S. Kislign, D. Haller, A. Skerra, *Protein Eng. Des. Sel.* **2013**, 26, 489–501.
- [41] F. G. Mutti, T. Knaus, N. S. Scrutton, M. Breuer, N. J. Turner, *Science* **2015**, 349, 1525–1529.
- [42] X. Chen, J. L. Zaro, W. C. Shen, *Adv. Drug Deliv. Rev.* **2013**, 65, 1357–1369.
- [43] G. Li, Z. Huang, C. Zhang, B.-J. Dong, R.-H. Guo, H.-W. Yue, L.-T. Yan, X.-H. Xing, *Appl. Microbiol. Biotechnol.* **2016**, 100, 215–225.

- [44] M. van Rosmalen, M. Krom, M. Merckx, *Biochemistry* **2017**, *56*, 6565–6574.
- [45] E. Papaleo, G. Saladino, M. Lambrugh, K. Lindorff-Larsen, F. L. Gervasio, R. Nussinov, *Chem. Rev.* **2016**, *116*, 6391–6423.
- [46] S. I. Lim, B. Yang, Y. Jung, J. Cha, J. Cho, E. S. Choi, Y. H. Kim, I. Kwon, *Sci. Rep.* **2016**, *6*, 39587.
- [47] Y. T. Lai, L. Jiang, W. Chen, T. O. Yeates, *Protein Eng. Des. Sel.* **2015**, *28*, 491–500.
- [48] K. Yu, C. Liu, B. G. Kim, D. Y. Lee, *Biotechnol. Adv.* **2015**, *33*, 155–164.
- [49] R. Orru, H. M. Dudek, C. Martinoli, D. E. Torres Pazmiño, A. Royant, M. Weik, M. W. Fraaije, A. Mattevi, *J. Biol. Chem.* **2011**, jbc.M111.255075.
- [50] B. J. Yachnin, T. Sprules, M. B. McEvoy, P. C. K. Lau, A. M. Berghuis, *J. Am. Chem. Soc.* **2012**, *134*, 7788–7795.
- [51] D. Tischler, R. Schwabe, L. Siegel, K. Joffroy, S. Kaschabek, A. Scholtissek, T. Heine, *Molecules* **2018**, *23*, 809.
- [52] S. M. Hoffmann, M. J. Weissenborn, L. Gricman, S. Notonier, J. Pleiss, B. Hauer, *ChemCatChem* **2016**, *8*, 1591–1597.
- [53] K. D. Belsare, A. J. Ruff, R. Martinez, A. V Shivange, H. Mundhada, D. Holtmann, J. Schrader, U. Schwaneberg, *Biotechniques* **2014**, *57*, 13–20.
- [54] P. Lu, M. G. Feng, *Appl. Microbiol. Biotechnol.* **2008**, *79*, 579–587.
- [55] J. L. Norris, R. M. Hughes, *FEBS Open Bio* **2018**, *8*, 1029–1042.

Chapter 2: Baeyer-Villiger monooxygenases: tunable biocatalysts for oxidative chemistry

Maximilian J. L. J. Fürst, Alejandro Gran-Scheuch, **Friso S. Aalbers** and Marco W. Fraaije

This chapter is based on a published article: Fürst, M. J. L. J., Gran-Scheuch, A., Aalbers, F. S., & Fraaije, M. W. (2019). Baeyer-Villiger monooxygenases: tunable oxidative biocatalysts. ACS Catalysis. <https://doi.org/10.1021/acscatal.9b03396>

Author contribution Friso S. Aalbers: section '**Biotechnological application**', which is the only section included in this chapter, aside from the abstract. This publication was also used for the thesis "Robust Monooxygenase Biocatalysts Discovery and Engineering by Computational Design" by Maximilian J. L. J. Fürst. Graphical abstract designed by Nina Lindhout

Abstract

Pollution, accidents, and misinformation have earned the pharmaceutical and chemical industry a poor public reputation, despite their undisputable importance to society. Biotechnological advances hold the promise to enable a future of drastically reduced environmental impact and rigorously more efficient production routes at the same time. This is exemplified in the Baeyer-Villiger reaction, which offers a simple synthetic route to oxidize ketones to esters, but application is hampered by the requirement of hazardous and dangerous reagents. As an attractive alternative, flavin-containing Baeyer-Villiger monooxygenases (BVMOs) have been investigated for their potential as biocatalysts for a long time, and many variants have been characterized. After a general look at the state of biotechnology, we here summarize the literature on biochemical characterizations, mechanistic and structural investigations, as well as enzyme engineering efforts in BVMOs. With a focus on recent developments, we critically outline the advances towards tuning these enzymes suitable for industrial applications.

Obstacles of biotechnological application of BVMOs

The application of BVMOs is characterized as troublesome due to a number of important limiting factors, including: enzyme expression,^[1] enzyme stability,^[2] NADPH-dependence,^[3-4] oxygen-dependence,^[5] and substrate and product inhibition.^[6] However, depending on the specific BVMO, there will be different obstacles, e.g. some BVMOs have good expression, yet poor stability, or vice versa. In this first subsection, we will discuss each of these limitations, and refer to studies that have addressed them.

First of all, the application of BVMOs can be carried out in four different forms: with isolated enzymes, with immobilized enzymes, with crude/cell-free extract, or with whole cells. Of these, the most used is whole cells, as it provides a number of advantages: (1) improved stability of the enzymes due to the cell environment,^[7] (2) no addition of NADP(H) is needed, (3) through co-expression of other enzymes cofactor recycling or cascade reactions can be facilitated, (4) no cell lysis and enzyme purification steps are needed, and (5) it allows for continual expression of the enzyme(s). However, there are also some disadvantages with whole cells, such as: (1) mass balance issues and product removal,^[8] (2) troubled oxygen supply to the cells,^[5] (3) plasmid stability with requirement of antibiotic,^[9-10] and (4) difficult transportation of some substrates/products in and out of the cell.^[11] In addition, a study on a cascade reaction *in vivo*, where a kinetic model was used to analyse performance, discovered that cofactor concentration in the cell was limiting the reaction rate.^[12] Possibly, this challenge could be addressed through metabolic engineering, or use of a different host. Still, each of the ways to apply BVMOs has trade-offs, and it will be case specific whether one is more suitable than the other. A recent mini-review addresses some of these aspects that are relevant for the development a biocatalytic (industrial) process.^[13]

Industrial demand, TTN and stability

Most studies on BVMOs describe reactions on small lab-scale, yet to meet the demands of an industrial process, the limiting factors presented above need to be addressed. Specifically, to produce low-priced compounds, such as building blocks for polymers, a ratio of 2000-10000 g product / g (immobilized) enzyme (also referred to as 'biocatalyst loading') should be met in order to be an economically viable process.^[7] To illustrate, assuming a 100 g mol⁻¹ product and a 50 kDa enzyme, 20-100 mol product / g enzyme, the demanded ratio translates to 1·10⁶ – 5·10⁶ total turnovers (TTN) per enzyme. Due to these numbers many BVMOs are still excluded from industrial application, unless the

target product is of high value, such as pharmaceuticals, or great effort is invested to improve the biocatalyst and the process. In particular, improvement of the stability of the biocatalyst is needed in many cases, as is discussed in the subsection of 'Enzyme engineering' of this review (see full publication). Moreover, an important issue for both the metric 'total turnover number' (TTN) and 'stability' is their inconsistent use in studies so far. Many studies refrain from determining the TTNs, and stability is often described in different ways, such as melting point and half-life, which makes it difficult to compare data. In addition, the stability of an enzyme in an industrial setting could be different compared to the lab setting. One aspect of this issue is that many studies on the application of BVMOs use whole cells, and avoid describing enzyme characteristics such as (operational) stability and TTNs. One reason for the low extend of BVMO application is the absence of clear and reliable data on stability and expression. What could help the field of BVMO biocatalysis in general is if studies provide data for these characteristics, or at least a clear description of biocatalyst loading, because this metric gives an impression of the efficiency and stability of the biocatalyst, whether it is whole cells or isolated enzymes.

NADPH recycling

While expression and stability are more related to enzyme engineering, the efficient use of NADPH is primarily determined by the way the BVMO is applied. The dependence of BVMOs on NADPH is an important challenge, since the cofactor is expensive; around 1800\$ per 5 g, compared to 280\$ / 5 g of NADH.^[14] Therefore, it is necessary to minimize the amount of NADPH that is used. One way to address this challenge is to devise a set of reactions that are in redox balance. This can be achieved by combining an oxidation reaction, in which the reduced cofactor is formed, with the BVMO reaction, in which the reduced cofactor is oxidized again.^[15] A typical example of such a redox-neutral reaction is the combination of a BVMO with an enzyme that can catalyse the oxidation of a sacrificial substrate, e.g. glucose dehydrogenase.^[16-17] Alternatively, whole cells can be used for internal cofactor regeneration,^[18-19] as well as non-enzymatic ways.^[20] Nicotinamide cofactor regeneration strategies have been extensively reviewed elsewhere.^[21] Another type of a redox-balanced pair of reactions are cascade reactions, where the product of the first reaction is the substrate for the second reaction.^[22] One advantage of cascade reactions is that the isolation of intermediate products is not needed, as can be the case in other synthesis routes. In general, there are quite well-studied solutions to the challenge of cofactor recycling, with examples given

in the next subsection. Still, it is another degree of complexity for the application of BVMOs.

Oxygen supply

Another degree of complexity of industrial application is oxygen supply. Since a stoichiometric amount of oxygen is needed for the typical BVMO reaction, and aqueous solutions contain about 0.27 mM of oxygen at 25 °C, effective oxygenation of the reaction mixture is needed to convert higher concentrations of substrate. However, supplying pure oxygen directly can also be very dangerous, due to risk of explosion. Studies that describe the oxygen limitation for biocatalytic oxidations include a study on whole cells expressing AcCHMO, to convert bicyclo[3.2.0]hept-2-en-6-one.^[5] The authors observed a limitation of oxygen at a particular cell density ($> 2 \text{ g}_{\text{cdw}} / \text{L}$), above which the reaction rate was decreased. In this regard it seems that use of whole cells can be a disadvantage compared to isolated enzymes, since cells seem inefficient at taking up oxygen.^[5] To improve cellular concentrations of oxygen and study the effects, BVMO reactions were tested in whole cells co-expressing a bacterial hemoglobin gene.^[23] It turned out that the presence of the bacterial hemoglobin gave a 43% improvement compared to the control, conversion of cyclohexanone by CHMO. To address the limitation of oxygen for larger scale reactions, one can employ devices that can effectively transfer oxygen to a reactor.^[24, 25] A recent study described a strategy to monitor the oxygen concentration of a reaction, providing accurate values, despite the presence of multiple phases (such as biphasic systems).^[26] Although typically the subject of oxygen limitation is addressed at the process stage of a BVMO application, oxygen monitor technologies can help identify oxygen limitation at an early stage, such that a solution can be prepared before scaling up.

Product and substrate inhibition

Since product inhibition is a general issue for CHMO, a few studies addressed this specific aspect. In one case, CHMO was subjected to mutagenesis to make variants with less product inhibition, and the mutants were screened in a high-throughput, automated fashion.^[27] Through the robotic platform that was employed, a large number of mutants could be generated and screened. For the CHMO product inhibition, 4200 clones were screened, resulting in 6 hits, with the best mutant displaying a 2-fold higher resistance compared to wild-type CHMO at 600 mM ϵ -caprolactone. Engel et al. recently characterized a BVMO from *Aspergillus flavus*, which had no sign of inhibition from substrate or product using concentrations up to 100 mM, and compared conversions with a

few CHMO variants.^[28] In other studies,^[12, 29] the authors sought to minimize inhibition by optimizing cascade reactions through computational simulations, combined with *in vitro* experiments. The computational simulations incorporate many variables, such as the kinetic properties of the enzymes, and then predict the course of the reaction. In principle, the use of a cascade reaction with an alcohol dehydrogenase, starting from cyclohexanol, will keep the cyclohexanone concentrations low, thereby addressing some substrate inhibition. Still, to optimize all the parameters, such as the rate of substrate feeding, was shown to be a delicate task, and reliable data of kinetic parameters is very important in order to make accurate predictions. When substrate and/or product inhibition are observed, the approach of kinetic modelling could be valuable to pinpoint bottlenecks, and help to choose the right solution.

Scaled-up applications

Until now, only a few examples of industrial-scale application of BVMOs are known (Table 1). One actual industrial process involves a CHMO variant for the enantioselective sulfoxidation of pyrimetazole, to produce esomeprazole.^[30] Initially, the wild-type CHMO from *Acinetobacter* had barely any activity on the substrate. After 19 rounds of evolution, partially random and partially rational, the biocatalyst had several improved features: high activity (~140 000-fold improvement in productivity over wild-type), enantioselectivity (>99% ee), low percentage of overoxidation to sulfone, and high cofactor efficiency (less NADP⁺ was needed). Then, after process optimization, reactions at 50 g/L substrate could be performed, resulting in a yield of 87%, with 99.9% ee and 99% purity (based on HPLC). Although total turnover numbers are moderate (Table 1), for such a high-value product this is very acceptable. The takeaway from this example is that most research groups and companies do not have the means (in terms of equipment and/or financial) to perform 19 rounds of evolution, and to do this kind of process optimization. Although studies are finding promising results, the academic route of a biocatalyst is comparatively slow, which partially explains the lack of industrial application of BVMOs.

Table 1 – Preparative-scale reactions with BVMOs

Product	Product concentration (g/L) + isolated yield	Enzyme	Biocatalyst yield	Total turnover number (TTN)	Reference
Esomeprazole	50 (~151 mM) 87% yield (28.7 g)	AcCHMO variant	50 (g _{product} /g _{enzyme})	8841	[30]
bicyclo[3.2.0]hept-2-en-6-one lactone	4.5 (~41 mM) 55% yield (0.49 kg)	AcCHMO	3 (g _{product} / g _{cdw})*	n.a. (whole cells)	[31]
(Z)-11-(heptanoyloxy)undec-9-enoic acid	41 (132 mM) 68 % yield (75 g)	<i>Pseudomonas putida</i> BVMO (E6-BVMO _{C302L})	1.6 (g _{product} / g _{cdw})*	n.a. (whole cells)	[42]
3,3,5-trimethylcaprolactone	24.4 (~156 mM) 76% yield (1.9 kg)	TmCHMO	0.6 (g _{product} / g _{cww})*	n.a. (whole cells)	[51]
6-hydroxy-hexanoic acid	20 (~151 mM) 81% yield (8.1 g)	AcCHMO (C376L/M400L/T415C/A463C)	0.7 (g _{product} / g _{cww})*	n.a. (whole cells)	[64]
Lactone of (2R, 5R, 6R)-6-methylidihydrocarvone	0.82 (4.5 mM) 90% yield (49 mg)	CHMO_Phi1	6.7 (g _{product} /g _{enzyme})	2250	[83]
Precursor of Nylon-9	8 (70 mM) 70% yield (33 g)	CPDMO	2.3 (g _{product} / g _{cdw})*	n.a. (whole cells)	[45]

* g_{product} / g_{cdw} = gram product per gram cell dry weight, and g_{product} / g_{cww} = gram product per gram cell wet weight. TTN = total turnover number, representing the number of molecules converted per molecule of enzyme.

One application that has been studied to bring to industrial scale is the regioselective oxygenation of long-chain fatty acids into esters. These ester products can be subsequently hydrolyzed to obtain valuable medium-chain acid products, including: ω -hydroxycarboxylic acids, α,ω -dicarboxylic acids,^[32] and ω -aminocarboxylic acids (Scheme 2a).^[32] Interestingly, activity on such substrates is the primary function of many BVMOs in nature, as they are often co-expressed with esterases that catalyze subsequent hydrolysis of the esters. Several BVMOs have been identified that can act on long-chain fatty acids, in particular BVMOs from *Pseudomonas* species,^[33–35] such as PaBVMO from *Pseudomonas auruginosa*, and PpKT2440-BVMO from *Pseudomonas putida*.^[33] The PaBVMO was recently characterized, and is able to produce the abnormal ester products from long-chain aliphatic keto acids (C16–C20), which—after hydrolysis—yielded α,ω -dicarboxylic acids that are difficult to produce.^[35] The PpKT2440-BVMO could be applied in combination with an ADH from *Micrococcus luteus* to convert ricinoleic acid to (Z)-11-(heptanoyloxy)undec-9-enoic acid (Scheme 2a). However, this BVMO was difficult to express in *E. coli*, and to improve expression several strategies were explored, including: use of chaperones^[36, 37], enzyme fusion^[37, 38], polyionic fusion tag engineering, and use of constitutive promoter^[39]. At this point, through the polyionic tag and constitutive promoter, the reaction could reach a product concentration of 21.9 g/L on a 70 L scale. To further improve the whole cell application for the conversion of ricinoleic acid, and other long chain fatty acids, more strategies were applied: co-expression with fatty acid transporter FadL,^[37, 40, 41] stability engineering of the BVMO,^[42, 43] glucose feeding for improved metabolic stability,^[42] and use of a stable plasmid system.^[43] Through the stability engineering and glucose feeding, a product concentration of 41 g/L (132 mM) within 8 hours could be achieved (on 3 L scale). Recently, an overview of enzymatic fatty-acid transformations was published, including many cases that involve BVMOs.^[44]

These studies illustrate the work that is needed to bring lab-scale biocatalysis of BVMOs to an industrial bioprocess. Key obstacles in the case of PpKT2440-BVMO were expression and stability. This explains in part the limited number of industrial applications so far; if a BVMO can catalyze a desired reaction, there is typically a long road ahead of optimizing expression and stability, which is likely too costly and risky for industry. Although several BVMOs are characterized, they are by default not suited to be applied in industrial settings.

As some BVMOs are able to produce lactones from cyclic ketones, which can be used to make various polyester materials, studies have looked into

2 scaling up the production of lactones with BVMOs. The Mihovilovic group could demonstrate the production of a Nylon-9 monomer on 40 g scale, employing CPDMO in whole cells, and carefully addressing substrate and product inhibition, through substrate feeding and product removal (Table 1).^[45] An incomplete conversion of around 75% could be reached, which was attributed to the particular reactor at hand, which could not ensure proper mixing of the ketone substrate. The authors recommend future studies to monitor the reaction through off-gas measurements, similarly to Meissner et al.^[26]

To explore the synthesis of branched polyesters from biobased sources, Delgove et al. investigated the biotransformation of a set of seven substituted cyclic ketones with three self-sufficient BVMOs.^[46] The abnormal lactone products, which can be formed by some BVMOs, could represent novel building blocks for polyester synthesis. Conversion was demonstrated for four of the cyclic ketones, two of which resulted in mixtures of normal and abnormal product. In subsequent work, the authors upscaled the transformation of 3,3,5-trimethylcyclohexanone. TmCHMO was used as biocatalyst due to its stability and solvent tolerance, and was paired with PTDH as a fusion for cofactor recycling.^[47] Crucially, some strategies were used to overcome substrate inhibition and product solubility. By employing slow substrate feeding, methanol as cosolvent, and a biphasic system with toluene as second phase to sequester the product, a respectable space-time yield (STY) of $1.2 \text{ g L}^{-1} \text{ h}^{-1}$ could be reached. In a similar study, TmCHMO was used in combination with a separate GDH as recycling enzyme, and in a setup with continuous substrate feeding, a STY of $1.35 \text{ g L}^{-1} \text{ h}^{-1}$ was reached.^[48]

One approach to greatly enhance the total turnover number of a biocatalyst is through immobilization, and re-use of the immobilized biocatalyst. The same target reaction was also studied with immobilized TmCHMO.^[49] GDH was either co-immobilized or individually immobilized and added separately to the reactions. The co-immobilized enzymes could be reused while maintaining full activity for five rounds, and beyond that gradually decreased, obtaining 60% conversion after 14 reuses. Immobilization of enzymes for this conversion was recently optimized, by applying a different GDH, and testing different supports. Compared to the soluble TmCHMO and GDH, the immobilized forms had a 3.6-fold and 1.9-fold improvement respectively, in terms of the biocatalyst yield (37.3 g g^{-1} for TmCHMO and 474.2 g g^{-1} for GDH).^[50]

To investigate the relevance of these biocatalytic studies on 3,3,5-trimethylcyclohexanone conversion in terms of applicability, a life-cycle analysis (LCA) was done.^[51] The biocatalytic process^[47] was compared with a

chemical synthesis route, and no clear difference in climate change impact was found between the two routes. However, the environmental impact of the biocatalytic process would be lower compared to the chemical route when solvents and enzyme are recycled. The study details what factors to consider when developing a biocatalytic process, and the LCA approach can provide useful insight into the feasibility of a potential process.^[51] A very recent study looked to scale up the reaction on 3,3,5-trimethyl cyclohexanone, with TmCHMO and GDH. First, the authors tested four different formulations: cell-free extract, whole cells, fermentation broth, and sonicated fermentation broth. For TmCHMO the untreated fermentation broth was chosen for performance, and least amount of costs, and GDH was added separately. On a 100 L scale, the 2.58 kg obtained material contained 84.5% product, and the final yield was 76%.^[52] Although the biocatalyst yield (gram product per gram enzyme/cells) seem much lower compared to the same reaction with immobilized enzyme,^[50] it is actually difficult to compare these values, since a lot of costs go into the preparation and immobilization of the enzymes, in contrast to the fermentation broth used in this study.^[52] This demonstration is a great example of how to reach large scale production with a BVMO reaction by addressing important challenges, in particular the oxygen limitation through careful supply of pure oxygen. Despite the success, it would be interesting to see whether the low levels of cofactor in the cells affected this particular reaction, as was described by Milker et al.^[12]

The low number of preparative-scale applications (Table 1) can be explained by considering the obstacles that arise from upscaling. Oxygen limitation and substrate inhibition have been successfully addressed in recent demonstrations,^[45, 52] although one could argue that oxygen supply remains suboptimal with respect to using whole cells.^[5] On the other hand, obstacles such as enzyme stability and product inhibition are not easy to solve in any general way, since these will be case specific due to the use of different BVMOs and different products. A review on preparative scale biosynthesis with redox enzymes, states that greater characterization of BVMOs, together with addressing oxygen mass transfer, will bring more application in the future.^[53] Yet, we want to emphasize that the characterization of BVMOs, or development of BVMO variants, that meet the requirements of an industrial application (high expression, high stability, low inhibition, high enantioselectivity) is a major challenge. From the examples (Table 1), we can conclude that scaling up a BVMO reaction typically demands (1) resources, such as time to engineer the biocatalyst^[30, 42] and the process, and (2) facilities, such as proper reactors with equipment to control oxygen levels.^[45, 52] These demands for scaling up

reactions with BVMOs typically cannot be met by academic groups, and probably bear too much risk for companies. The disjointed nature of the problem, with on the one hand the biocatalyst engineering, and on the other the process design, calls for more collaboration between groups with different expertise and facilities.

2 Examples of cascade strategies and novel BVMO applications

Over the past decades, some progress was made in optimizing large-scale reactions, employing strategies such as biphasic systems,^[54] whole cell conversions,^[55] and enzyme immobilization.^[49, 56-57] Reviews focusing on biocatalysis with BVMOs from prior years are referred to for a broader overview.^[58-61] Alongside these developments, several groups have explored different reactions and combinations of reactions with BVMOs, of which we present an overview, focused on studies from recent years. In particular, these combinations of reactions include cascades, as well as chemoenzymatic routes.

To facilitate cofactor recycling, an elegant strategy is to use a cascade reaction. For BVMOs, a frequently looked-at example is the cascade reaction with CHMO and an alcohol dehydrogenase (ADH), starting from cyclohexanol (Scheme 1, blue box). The alcohol oxidation generates NADPH and cyclohexanone, which is then oxidized by CHMO to ϵ -caprolactone. Several groups investigated and developed this cascade reaction.^[62-64] Initially, troubles were found with substrate and product inhibition. Higher levels of conversions could be achieved by keeping the substrate concentration low, through slow feeding, and removal of the lactone product by a subsequent polymerization/hydrolysis using a lipase such as CAL-A (Scheme 1).^[65] This biosynthesis route was recently applied in whole cells with co-expression of CHMO and ADH on a 0.5 L scale, feeding of cyclohexanol, and addition of a lipase for hydrolysis of caprolactone to 6-hydroxyhexanoic acid (Table 1).^[66] After optimization, the process at 0.5 L scale could reach a product titre of 20 g L⁻¹, with an isolated yield of 81% of 6-hydroxyhexanoic acid.

Scheme 1. Overview of biocatalyst combinations for cascades involving cyclohexanol and CHMO. Blue box: redox-neutral cascade from cyclohexanol to ϵ -caprolactone. Red box: ADH conversion of 1,6-hexanediol, which can be combined with cyclohexanone conversion by CHMO to recycle NADPH.^[67] A cascade starting from cyclohexane involving a P450 was described.^[68] Unsaturated cyclic alcohols or unsaturated cyclic ketones can be used with ene-reductase (ER) cascades, to make chiral lactones.^[69, 70]

To address the cofactor balance, a different kind of cascade reaction was developed by Hollmann and Kara.^[67] With the production of lactones in mind, an alcohol oxidation reaction of a linear diol was run in parallel in one pot with a Baeyer-Villiger reaction on a cyclic ketone catalyzed by CHMO (Scheme 1, red box).^[67] As alcohol oxidation by an alcohol dehydrogenase (ADH) depends on NAD(P)^+ and produces NAD(P)H , combining this reaction with a BVMO or FMO reaction brings a redox balance. When one alcohol group of a linear diol becomes oxidized, the molecule undergoes cyclization to the hemiacetal or lactol. This lactol can be oxidized again to form a lactone (Scheme 1, red box). However, since the ADH generates two molecules of NAD(P)H in the conversion of one diol to one lactone, the substrate concentrations should be 2:1 of FMO substrate to ADH substrate. This approach was termed convergent cascade, since two different substrates converge to the same product; the lactone. The Kara group later made an analogous combination, to produce γ -butyrolactones using RjFMO-E (Scheme 2b).^[71] An interesting aspect of that study is that the FMO that was used to perform the Baeyer-Villiger reactions could accept NADH, making it a more feasible process compared to an NADPH-dependent reaction, considering the higher cost of NADPH^[72] and its inferior stability.^[73]

A related strategy is to create a genetic fusion of a cofactor recycling enzyme with a BVMO. This approach enables co-expression of both enzymes, and simplifies purification, whole cell conversions, and co-immobilization.

Enzyme fusions with BVMOs have been reviewed recently (**Chapter 1**).^[74] and thus will only briefly be discussed here. As it is a promising strategy to address cofactor recycling, a few recent studies investigated the possibilities. One study looked at fusions of three cofactor-regenerating enzymes with TmCHMO: glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH) and formate dehydrogenase (FDH) (**Chapter 5**).^[75] These were compared in conversions and tested with various substrates and cosolvents, including a deep eutectic solvent (DES). One particularly efficient setup consisted of a natural DES (NADES) with glucose and the GDH-TmCHMO fusion, in which the NADES enables higher substrate loading, while also containing excess glucose to push NADPH recycling by GDH. Recently, following up on the convergent cascade (Scheme 2b),^[71] fusions of the ADH and FMO were created to produce γ -butyrolactone in an unusual setup: organic solvent.^[76] Studies in the past have found that enzymes can actually be more stable and active in organic solvent, though the use was limited to lipases and esterases.^[77] However, the ADH-FMO reaction is more challenging as it relies on NADH, which is why the authors chose to fuse the two enzymes. Cell-free extract from cells expressing the enzyme fusion was lyophilized, and subsequently added to organic media with 5% (v/v) water, to which the two substrates (diol and cyclic ketone) were added.^[76] Although the yield was limited (27%), the fusion enzyme was able to perform the cascade reaction in this micro-aqueous media, and outperformed the combination of the separate enzymes. Moreover, no external NADH was added, which is appealing in terms of application.

The approach of enzyme fusion is also very suitable for multi-enzyme cascade reactions. In some cases, the fusion outperforms the combination of separate enzymes, which is linked to an effect of the proximity of the enzymes called substrate channelling.^[78-80] In 2013, Jeon et al. developed fusions of ADHs with BVMOs to convert hydroxy fatty acids into esters, in whole cells expressing the fusion enzyme (Scheme 2a).^[38] The authors could demonstrate that the fused enzyme had a higher level of conversion for the cascade reaction. A similar pair of ADH with TmCHMO was fused to produce ϵ -caprolactone from cyclohexanol (**Chapter 3**).^[81] Although the fusion was more productive than the separate enzymes, substrate feeding and product removal through a lipase were needed to obtain full conversions, as was described previously (Scheme 1).^[65] This fusion of an ADH with TmCHMO was also applied for a process to convert 2-butanol from methyl propionate (Scheme 2c), which is a precursor for the plastic feedstock methyl methacrylate.^[82] Prior to that study, TmCHMO was engineered to give higher conversion and a higher ratio of the abnormal product.^[83] Another study fused CHMO with an ene-reductase, to

enable cascades starting from an unsaturated cyclic ketone, or unsaturated cyclic alcohol by including an alcohol dehydrogenase as third enzyme, to make chiral lactones (Scheme 1 and Scheme 2d).^[70]

Scheme 2. Examples of cascade reactions involving BVMOs. a) Conversion of hydroxylated long-chain fatty acids to produce esters.^[36-43] The cascade could also start from an unsaturated precursor with a hydratase to make the hydroxyl group,^[37] or with a P450 to perform hydroxylation. b) Convergent cascade analogous to the reaction displayed in scheme 1, red box.^[67] This particular cascade relies on NADH, through the use of RjFMO-E.^[71] The same reaction was also used with fused enzymes in organic solvent.^[76] c) Cascade from 2-butanol to methyl propionate (the first product, abnormal), a precursor for methyl methacrylate.^[82, 83] d) Various monoterpenoid ketones can be transformed to obtain chiral lactones, through ene-reduction by an ene-reductase (ER, or old-yellow enzyme, OYE) followed by lactonization by CHMO.^[85-87] e) A chemoenzymatic route, that starts with Baeyer-Villiger oxidation, which is enantioselective (>99% ee), and is followed by a SmI₂-H₂O mediated radical cyclization reaction.^[90] The cyclization is completed by the third step, which is an alcohol oxidation by DMP (Dess-Martin periodinane) in dichloromethane. The two chemical steps maintained the chirality of the CHMO product, and final products could be obtained with >99% ee.

Other groups have also explored the potential of applying BVMOs for the production of lactones, in this case from monoterpenoid ketones, and followed-up with the polymerization of the products. In one study, a novel CHMO was

2 applied, after characterization, crystallization, and engineering to steer the regioselectivity.^[84] The CHMO_Phi1 structure was then used to perform modeling and simulations to explain the change in regioselectivity of the mutant. For menthone and dihydrocarvone, biosynthesis could be demonstrated as well as polymer synthesis. In another study, analogues of (*R*)-(-)-carvone were used as precursors for a reduction reaction with Old-Yellow Enzymes (OYE), and subsequent Baeyer-Villiger oxidation by CHMO_Phi1 (Scheme 2d).^[85] Reactions could be scaled up to 60-100 mg, demonstrating preparative biosynthesis of chiral caprolactones (Table 1). Dihydrocarvone was also produced through a whole cell biosynthesis by incorporating a BVMO and OYE, alongside a limonene synthesis pathway.^[86] This proof-of-principle study demonstrated the production of a renewable bioplastic monomers starting from glucose, through precise expression of the required enzymes. An important aspect of the conversion of monoterpenoid ketones by BVMOs is the chirality of the substrate and/or product. This was demonstrated in a study which used combinations of enoate reductases and various BVMOs in cascade reactions to convert (+)- and (-)-carvone to six different carvo-lactone stereoisomers (Scheme 2d).^[87] In a study by Stamm et al., a specific polyester with a ring was targeted, and using retrobiosynthesis, a chemoenzymatic route was devised to obtain the needed lactone from pinene.^[88] For the BVMO step in that route, the biocatalyst was engineered in order to accept the substrate. Recently, an overview of (chemo)enzymatic routes for (lactone) monomer production was published, including several examples of BVMO reactions.^[89]

Since the BVMO-catalyzed Baeyer-Villiger oxidation is often very selective, it can be a synthetically useful way to access chiral precursors for various synthesis routes. One example is a chemoenzymatic approach where chiral lactones are produced with AcCHMO, which are then converted to cycloheptanols and cyclooctanols through a radical cyclization reaction involving SmI₂-H₂O (Scheme 2e).^[90] The products from this approach contain structural components that are present in certain anti-cancer and antibacterial drugs. In another chemoenzymatic approach, Zhang et al. devised a cascade reaction starting with a photo-catalyzed reaction, of which the product was subsequently converted with BVMOs, among other enzymes.^[91] However, the two catalysts were incompatible, and higher conversions were obtained when the two steps were done separately. The challenges for applying such chemoenzymatic cascade reactions were discussed in a recent minireview.^[92] Another chemoenzymatic route involving a BVMO to produce (*R*)-Taniguchi lactone was recently studied.^[93] Two novel BVMOs were used, and the authors describe several other cyclic ketones that can be converted with these

biocatalysts, with varying degrees of regio- and enantioselectivity. These recent examples show that BVMOs can be useful in particular synthesis routes, and elude to a broader range of possible applications with these biocatalysts.

The development of strategies, like enzyme fusion, use of cosolvents, and cascade reactions have shown to be meaningful steps on a path toward biotechnological application. However, it is a path that still needs further exploration in order to meet the demands of an industrial process. The studies from the recent years show the variety of products that can be accessed through BVMOs. Given the limited turnover numbers that are reached in these studies, we conclude two things: (1) with the current state of BVMOs, any industrial application can only be economically feasible if the products are of high value (such as the esomeprazole^[30]), and/or through thorough optimization of the biocatalyst and process. (2) For the application of BVMOs for bulk chemicals (e.g. monomers) there are some examples^[45, 52], though more work needs to be done with respect to biocatalyst loading (in other words: operational stability and activity). So far, some studies have moved in the direction of biotechnological application, and shown to apply BVMOs for the biosynthesis of various compounds (Table 1). As BVMOs become more suited, reliable, and recognized for biocatalytic application, it is likely that more groups and companies will look to harness the utility that these biocatalysts can provide. Though, to realize scaled-up applications, joint efforts will be needed that bring together different expertise, ranging from enzyme engineering to process development, to effectively tackle the specific challenges.

References

- [1] Seo, J.-H.; Kim, H.-H.; Jeon, E.-Y.; Song, Y.-H.; Shin, C.-S.; Park, J.-B. Engineering of Baeyer-Villiger Monooxygenase-Based *Escherichia coli* Biocatalyst for Large Scale Biotransformation of Ricinoleic Acid into (Z)-11-(Heptanoyloxy) Undec-9-Enoic Acid. *Sci. Rep.* **2016**, 6, 28223.
- [2] Opperman, D. J.; Reetz, M. T. Towards Practical Baeyer-Villiger-Monooxygenases: Design of Cyclohexanone Monooxygenase Mutants with Enhanced Oxidative Stability. *ChemBioChem* **2010**, 11, 2589-2596.
- [3] Hummel, W.; Gröger, H. Strategies for Regeneration of Nicotinamide Coenzymes Emphasizing Self-Sufficient Closed-Loop Recycling Systems. *J. Biotechnol.* **2014**, 191, 22-31.
- [4] Wang, X.; Saba, T.; Yiu, H. H.; Howe, R. F.; Anderson, J. A.; Shi, J. Cofactor NAD (P) H Regeneration Inspired by Heterogeneous Pathways. *Chem* **2017**, 2, 621-654.
- [5] Baldwin, C. V.; Woodley, J. M. On Oxygen Limitation in a Whole Cell Biocatalytic Baeyer-Villiger Oxidation Process. *Biotechnol. Bioeng.* **2006**, 95, 362-369.
- [6] Staudt, S.; Bornscheuer, U. T.; Menyes, U.; Gröger, H. Direct Biocatalytic One-Pot-Transformation of Cyclohexanol with Molecular Oxygen into Varepsilon-Caprolactone. *Enzyme Microb. Technol.* **2013**, 53, 288-92.
- [7] Tufvesson, P.; Lima-Ramos, J.; Nordblad, M.; Woodley, J. M. Guidelines and Cost Analysis for Catalyst Production in Biocatalytic Processes. *Org. Process Res. Dev.* **2011**, 15, 266-274.
- [8] Doig, S. D.; Avenell, P. J.; Bird, P. A.; Gallati, P.; Lander, K. S.; Lye, G. J., ... & Woodley, J. M. (2002). Reactor operation and scale-up of whole cell Baeyer-Villiger catalyzed lactone synthesis. *Biotechnology progress*, 18(5), 1039-1046.

- [9] Sieben, M., Steinhorn, G., Müller, C., Fuchs, S., Chin, L. A., Regestein, L., & Büchs, J. (2016). Testing plasmid stability of *Escherichia coli* using the continuously operated shaken BIOreactor system. *Biotechnology progress*, 32(6), 1418-1425.
- [10] Kadisch, M., Willrodt, C., Hillen, M., Bühler, B., & Schmid, A. (2017). Maximizing the stability of metabolic engineering-derived whole-cell biocatalysts. *Biotechnology journal*, 12(8), 1600170.
- [11] Doig, S. D., Simpson, H., Alphand, V., Furstoss, R., & Woodley, J. M. (2003). Characterization of a recombinant *Escherichia coli* TOP10 [pQR239] whole-cell biocatalyst for stereoselective Baeyer-Villiger oxidations. *Enzyme and microbial technology*, 32(3-4), 347-355.
- [12] Milker, S.; Fink, M. J.; Oberleitner, N.; Ressmann, A. K.; Bornscheuer, U. T.; Mihovilovic, M. D.; Rudroff, F. Kinetic Modeling of an Enzymatic Redox Cascade in vivo Reveals Bottlenecks Caused by Cofactors. *ChemCatChem* 2017, 9, 3420-3427.
- [13] Woodley, J. M. Accelerating the Implementation of Biocatalysis in Industry. *Appl. Microbiol. Biotechnol.* 2019, 1-7.
- [14] Faber, K., *Biotransformations in Organic Chemistry*. Springer: 1992; Vol. 4.
- [15] Xue, R.; Woodley, J. M. Process Technology for Multi-Enzymatic Reaction Systems. *Bioresour. Technol.* 2012, 115, 183-195.
- [16] Hummel, W.; Gröger, H. Strategies for Regeneration of Nicotinamide Coenzymes Emphasizing Self-Sufficient Closed-Loop Recycling Systems. *J. Biotechnol.* 2014, 191, 22-31.
- [17] Wang, X.; Saba, T.; Yiu, H. H.; Howe, R. F.; Anderson, J. A.; Shi, J. Cofactor NAD (P) H Regeneration Inspired by Heterogeneous Pathways. *Chem* 2017, 2, 621-654.
- [18] Wachtmeister, J.; Rother, D. Recent Advances in Whole Cell Biocatalysis Techniques Bridging from Investigative to Industrial Scale. *Curr. Opin. Biotechnol.* 2016, 42, 169-177.
- [19] Dascier, D.; Kambourakis, S.; Hua, L.; Rozzell, J. D.; Stewart, J. D. Influence of Cofactor Regeneration Strategies on Preparative-Scale, Asymmetric Carbonyl Reductions by Engineered *Escherichia coli*. *Org. Process Res. Dev.* 2014, 18, 793-800.
- [20] Hollmann, F.; Arends, I. W.; Buehler, K. Biocatalytic Redox Reactions for Organic Synthesis: Nonconventional Regeneration Methods. *ChemCatChem* 2010, 2, 762-782.
- [21] Wang, X.; Saba, T.; Yiu, H. H.; Howe, R. F.; Anderson, J. A.; Shi, J. Cofactor NAD (P) H Regeneration Inspired by Heterogeneous Pathways. *Chem* 2017, 2, 621-654.
- [22] Muschiol, J.; Peters, C.; Oberleitner, N.; Mihovilovic, M. D.; Bornscheuer, U. T.; Rudroff, F. Cascade Catalysis-Strategies and Challenges En Route to Preparative Synthetic Biology. *Chem. Comm.* 2015, 51, 5798-5811.
- [23] Lee, W. H., Park, E. H., & Kim, M. D. (2014). Enhanced production of ϵ -caprolactone by coexpression of bacterial hemoglobin gene in recombinant *Escherichia coli* expressing cyclohexanone monooxygenase gene. *Journal of microbiology and biotechnology*, 24(12), 1685-1689.
- [24] Bolivar, J. M., Mannsberger, A., Thomsen, M. S., Tekautz, G., & Nidetzky, B. (2019). Process intensification for O₂-dependent enzymatic transformations in continuous single-phase pressurized flow. *Biotechnology and bioengineering*, 116(3), 503-514.
- [25] Gomes, M. D., Moiseyenko, R. P., Baum, A., Jørgensen, T. M., & Woodley, J. M. (2019). Use of image analysis to understand enzyme stability in an aerated stirred reactor. *Biotechnology progress*.
- [26] Meissner, M. P., Nordblad, M., & Woodley, J. M. (2018). Online Measurement of Oxygen-Dependent Enzyme Reaction Kinetics. *ChemBioChem*, 19(1), 106-113.
- [27] Dörr, M.; Fibinger, M. P.; Last, D.; Schmidt, S.; Santos-Aberturas, J.; Böttcher, D.; Hummel, A.; Vickers, C.; Voss, M.; Bornscheuer, U. T. Fully Automatized High-Throughput Enzyme Library Screening Using a Robotic Platform. *Biotechnol. Bioeng.* 2016, 113, 1421-1432.
- [28] Engel, J.; Mthethwa, K. S.; Opperman, D. J.; Kara, S. Characterization of New Baeyer-Villiger Monooxygenases for Lactonizations in Redox-Neutral Cascades. *Molecular Catalysis* 2019, 468, 44-51.
- [29] Scherkus, C.; Schmidt, S.; Bornscheuer, U. T.; Groger, H.; Kara, S.; Liese, A. Kinetic Insights into ϵ -Caprolactone Synthesis: Improvement of an Enzymatic Cascade Reaction. *Biotechnol. Bioeng.* 2017, 114, 1215-1221.
- [30] Bong, Y. K., Song, S., Nazor, J., Vogel, M., Widegren, M., Smith, D., ... & Mijts, B. (2018). Baeyer-Villiger Monooxygenase-Mediated Synthesis of Esomeprazole as an Alternative for Kagan Sulfoxidation. *The Journal of organic chemistry*, 83(14), 7453-7458.
- [31] Baldwin, C. V., Wohlgemuth, R., & Woodley, J. M. (2008). The first 200-L scale asymmetric Baeyer-Villiger oxidation using a whole-cell biocatalyst. *Organic Process Research & Development*, 12(4), 660-665.
- [32] Song, J. W., Lee, J. H., Bornscheuer, U. T., & Park, J. B. (2014). Microbial synthesis of medium-chain α , ω -dicarboxylic acids and ω -aminocarboxylic acids from renewable long-chain fatty acids. *Advanced Synthesis & Catalysis*, 356(8), 1782-1788.

- [33] Rehdorf, J., Kirschner, A., & Bornscheuer, U. T. (2007). Cloning, expression and characterization of a Baeyer-Villiger monooxygenase from *Pseudomonas putida* KT2440. *Biotechnology letters*, 29(9), 1393-1398.
- [34] Kirschner, A., Altenbuchner, J., & Bornscheuer, U. T. (2007). Cloning, expression, and characterization of a Baeyer-Villiger monooxygenase from *Pseudomonas fluorescens* DSM 50106 in *E. coli*. *Applied microbiology and biotechnology*, 73(5), 1065-1072.
- [35] Yu, J. M.; Liu, Y. Y.; Zheng, Y. C.; Li, H.; Zhang, X. Y.; Zheng, G. W.; Li, C. X.; Bai, Y. P.; Xu, J. H. Direct Access to Medium-Chain α , Ω -Dicarboxylic Acids by Using a Baeyer-Villiger Monooxygenase of Abnormal Regioselectivity. *ChemBioChem* **2018**, 19, 2049-2054.
- [36] Baek, A. H., Jeon, E. Y., Lee, S. M., & Park, J. B. (2015). Expression levels of chaperones influence biotransformation activity of recombinant *Escherichia coli* expressing *Micrococcus luteus* alcohol dehydrogenase and *Pseudomonas putida* Baeyer-Villiger monooxygenase. *Biotechnology and bioengineering*, 112(5), 889-895.
- [37] Sudheer, P. D., Yun, J., Chauhan, S., Kang, T. J., & Choi, K. Y. (2017). Screening, expression, and characterization of Baeyer-Villiger monooxygenases for the production of 9-(nonanoyloxy) nonanoic acid from oleic acid. *Biotechnology and Bioengineering*, 22(6), 717-724.
- [38] Jeon, E. Y., Baek, A. H., Bornscheuer, U. T., & Park, J. B. (2015). Enzyme fusion for whole-cell biotransformation of long-chain sec-alcohols into esters. *Applied microbiology and biotechnology*, 99(15), 6267-6275.
- [39] Seo, J.-H.; Kim, H.-H.; Jeon, E.-Y.; Song, Y.-H.; Shin, C.-S.; Park, J.-B. Engineering of Baeyer-Villiger Monooxygenase-Based *Escherichia coli* Biocatalyst for Large Scale Biotransformation of Ricinoleic Acid into (Z)-11-(Heptanoyloxy) Undec-9-Enoic Acid. *Sci. Rep.* **2016**, 6, 28223.
- [40] Jeon, E. Y., Song, J. W., Cha, H. J., Lee, S. M., Lee, J., & Park, J. B. (2018). Intracellular transformation rates of fatty acids are influenced by expression of the fatty acid transporter FadL in *Escherichia coli* cell membrane. *Journal of biotechnology*, 281, 161-167.
- [41] Sudheer, P. D.; Seo, D.; Kim, E.-J.; Chauhan, S.; Chunawala, J.; Choi, K.-Y. Production of (Z)-11-(Heptanoyloxy) Undec-9-Enoic Acid from Ricinoleic Acid by Utilizing Crude Glycerol as Sole Carbon Source in Engineered *Escherichia coli* Expressing BVMO-Adh-FadL. *Enzyme Microb. Technol.* **2018**, 119, 45-51.
- [42] Woo, J.-M.; Jeon, E.-Y.; Seo, E.-J.; Seo, J.-H.; Lee, D.-Y.; Yeon, Y. J.; Park, J.-B. Improving Catalytic Activity of the Baeyer-Villiger Monooxygenase-Based *Escherichia coli* Biocatalysts for the Overproduction of (Z)-11-(Heptanoyloxy) Undec-9-Enoic Acid from Ricinoleic Acid. *Sci. Rep.* **2018**, 8, 10280.
- [43] Seo, E. J., Kang, C. W., Woo, J. M., Jang, S., Yeon, Y. J., Jung, G. Y., & Park, J. B. (2019). Multi-level engineering of Baeyer-Villiger monooxygenase-based *Escherichia coli* biocatalysts for the production of C9 chemicals from oleic acid. *Metabolic engineering*, 54, 137-144.
- [44] Kim, S.-K.; Park, Y.-C. Biosynthesis of Ω -Hydroxy Fatty Acids and Related Chemicals from Natural Fatty Acids by Recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2019**, 103, 191-199.
- [45] Milker, S., Fink, M. J., Rudroff, F., & Mihovilovic, M. D. (2017). Non-hazardous biocatalytic oxidation in Nylon-9 monomer synthesis on a 40 g scale with efficient downstream processing. *Biotechnology and bioengineering*, 114(8), 1670-1678.
- [46] Delgove, M. A.; Fürst, M. J. L. J.; Fraaije, M. W.; Bernaerts, K. V.; de Wildeman, S. M. Exploring the Substrate Scope of Baeyer-Villiger Monooxygenases with Branched Lactones as Entry Towards Polyesters. *ChemBioChem* **2018**, 19, 354-360.
- [47] Delgove, M. A.; Elford, M. T.; Bernaerts, K. V.; De Wildeman, S. M. Application of a Thermostable Baeyer-Villiger Monooxygenase for the Synthesis of Branched Polyester Precursors. *J. Chem. Technol. Biotechnol.* **2018**.
- [48] Delgove, M.; Elford, M.; Bernaerts, K.; De Wildeman, S. Towards Upscaled Biocatalytic Preparation of Lactone Building Blocks for Polymer Applications. *Org. Process Res. Dev.* **2018**.
- [49] Delgove, M. A.; Valencia, D.; Solé, J.; Bernaerts, K. V.; De Wildeman, S. M.; Guillén, M.; Álvaro, G. High Performing Immobilized Baeyer-Villiger Monooxygenase and Glucose Dehydrogenase for the Synthesis of E-Caprolactone Derivative. *Applied Catalysis A: General* **2019**, 572, 134-141.
- [50] Solé, J.; Brummund, J.; Caminal, G.; Schürman, M.; Álvaro, G.; Guillén, M. Trimethyl-E-Caprolactone Synthesis with a Novel Immobilized Glucose Dehydrogenase and an Immobilized Thermostable Cyclohexanone Monooxygenase. *Applied Catalysis A: General* **2019**, 117187.
- [51] Delgove, M. A.; Laurent, A. B.; Woodley, J. M.; De Wildeman, S. M.; Bernaerts, K. V.; van der Meer, Y. A Prospective Life Cycle Assessment (Lca) of Monomer Synthesis: Comparison of Biocatalytic and Oxidative Chemistry. *ChemSusChem* **2019**.
- [52] Solé, J., Brummund, J., Caminal, G., Alvaro, G., Schürmann, M., & Guillén, M. (2019). Enzymatic synthesis of trimethyl-ε-caprolactone: process intensification and demonstration at 100 liter scale. *Organic Process Research & Development*.

- [53] Prier, C. K., & Kosjek, B. (2019). Recent preparative applications of redox enzymes. *Current opinion in chemical biology*, 49, 105-112.
- [54] Schulz, F.; Leca, F.; Hollmann, F.; Reetz, M. T. Towards Practical Biocatalytic Baeyer-Villiger Reactions: Applying a Thermostable Enzyme in the Gram-Scale Synthesis of Optically-Active Lactones in a Two-Liquid-Phase System. *Beilstein J. Org. Chem.* **2005**, 1, 10.
- [55] Wachmeister, J.; Rother, D. Recent Advances in Whole Cell Biocatalysis Techniques Bridging from Investigative to Industrial Scale. *Curr. Opin. Biotechnol.* **2016**, 42, 169-177.
- [56] Valencia, D.; Guillén, M.; Fürst, M. J. L. J.; López-Santín, J.; Álvaro, G. An Immobilized and Highly Stabilized Self-Sufficient Monooxygenase as Biocatalyst for Oxidative Biotransformations. *J. Chem. Technol. Biotechnol.* **2017**, n/a-n/a.
- [57] Krajčovič, T.; Bučko, M.; Vikartovská, A.; Lacík, I.; Uhelská, L.; Chorvát, D.; Neděla, V.; Tihlaříková, E.; Gericke, M.; Heinze, T. Polyelectrolyte Complex Beads by Novel Two-Step Process for Improved Performance of Viable Whole-Cell Baeyer-Villiger Monooxygenase by Immobilization. *Catalysts* **2017**, 7, 353.
- [58] Bucko, M.; Gemeiner, P.; Schenkmyerova, A.; Krajcovic, T.; Rudroff, F.; Mihovilovic, M. D. Baeyer-Villiger Oxidations: Biotechnological Approach. *Appl. Microbiol. Biotechnol.* **2016**.
- [59] de Gonzalo, G.; Mihovilovic, M. D.; Fraaije, M. W. Recent Developments in the Application of Baeyer-Villiger Monooxygenases as Biocatalysts. *ChemBioChem* **2010**, 11, 2208-2231.
- [60] Catucci, G.; Gao, C.; Sadeghi, S. J.; Gilardi, G. Chemical Applications of Class B Flavoprotein Monooxygenases. *Rendiconti Lincei* **2017**, 28, 195-206.
- [61] Torres Pazmiño, D. E.; Dudek, H. M.; Fraaije, M. W. Baeyer-Villiger Monooxygenases: Recent Advances and Future Challenges. *Curr. Opin. Chem. Biol.* **2010**, 14, 138-44.
- [62] Staudt, S.; Bornscheuer, U. T.; Menyes, U.; Hummel, W.; Groger, H. Direct Biocatalytic One-Pot-Transformation of Cyclohexanol with Molecular Oxygen into Varepsilon-Caprolactone. *Enzyme Microb. Technol.* **2013**, 53, 288-92.
- [63] Mallin, H.; Wulf, H.; Bornscheuer, U. T. A Self-Sufficient Baeyer-Villiger Biocatalysis System for the Synthesis of Varepsilon-Caprolactone from Cyclohexanol. *Enzyme Microb. Technol.* **2013**, 53, 283-7.
- [64] Sattler, J. H.; Fuchs, M.; Mutti, F. G.; Grischek, B.; Engel, P.; Pfeffer, J.; Woodley, J. M.; Kroutil, W. Introducing an in Situ Capping Strategy in Systems Biocatalysis to Access 6-Aminohexanoic Acid. *Angew. Chem. Int. Ed. Engl.* **2014**, 53, 14153-7.
- [65] Schmidt, S.; Scherkus, C.; Muschiol, J.; Menyes, U.; Winkler, T.; Hummel, W.; Groger, H.; Liese, A.; Herz, H. G.; Bornscheuer, U. T. An Enzyme Cascade Synthesis of Epsilon-Caprolactone and Its Oligomers. *Angew. Chem. Int. Ed. Engl.* **2015**, 54, 2784-7.
- [66] Srinivasamurthy, V. S.; Böttcher, D.; Bornscheuer, U. T. A Multi-Enzyme Cascade Reaction for the Production of 6-Hydroxyhexanoic Acid. *Z. Naturforsch. C Bio. Sci.* **2019**, 74, 71-76.
- [67] Bornadel, A.; Hatti-Kaul, R.; Hollmann, F.; Kara, S. Enhancing the Productivity of the Bi-Enzymatic Convergent Cascade for ε-Caprolactone Synthesis through Design of Experiments and a Biphasic System. *Tetrahedron* **2016**, 72, 7222-7228.
- [68] Pennec, A.; Hollmann, F.; Smit, M. S.; Opperman, D. J. One-Pot Conversion of Cycloalkanes to Lactones. *ChemCatChem* **2015**, 7, 236-239.
- [69] Oberleitner, N.; Peters, C.; Rudroff, F.; Bornscheuer, U. T.; Mihovilovic, M. D. In vitro Characterization of an Enzymatic Redox Cascade Composed of an Alcohol Dehydrogenase, an Enoate Reductases and a Baeyer-Villiger Monooxygenase. *J. Biotechnol.* **2014**, 192, 393-399.
- [70] Peters, C.; Rudroff, F.; Mihovilovic, M. D.; Bornscheuer, U. T. Fusion Proteins of an Enoate Reductase and a Baeyer-Villiger Monooxygenase Facilitate the Synthesis of Chiral Lactones. *Biol. Chem.* **2017**, 398, 31-37.
- [71] Huang, L.; Romero, E.; Ressmann, A. K.; Rudroff, F.; Hollmann, F.; Fraaije, M. W.; Kara, S. Nicotinamide Adenine Dinucleotide-Dependent Redox-Neutral Convergent Cascade for Lactonizations with Type II Flavin-Containing Monooxygenase. *Adv. Synth. Catal.* **2017**, 359, 2142-2148.
- [72] Faber, K., *Biotransformations in Organic Chemistry*. Springer: **1992**; Vol. 4.
- [73] Wu, J. T.; Wu, L. H.; Knight, J. A. Stability of NADPH: Effect of Various Factors on the Kinetics of Degradation. *Clin. Chem.* **1986**, 32, 314-319.
- [74] **Chapter 1:** Aalbers, F. S.; Fraaije, M. W. Enzyme Fusions in Biocatalysis: Coupling Reactions by Pairing Enzymes. *ChemBioChem* **2018**, 0.
- [75] **Chapter 5:** Mourelle-Insua, Á.; Aalbers, F. S.; Lavandera, I.; Gotor-Fernández, V.; Fraaije, M. W. What to Sacrifice? Fusions of Cofactor Regenerating Enzymes with Baeyer-Villiger Monooxygenases and Alcohol Dehydrogenases for Self-Sufficient Redox Biocatalysis. *Tetrahedron* **2019**.
- [76] Huang, L.; Aalbers, F. S.; Tang, W.; Röllig, R.; Fraaije, M. W.; Kara, S. Convergent Cascade Catalyzed by Monooxygenase-Alcohol Dehydrogenase Fusion Applied in Organic Media. *ChemBioChem* **2019**.
- [77] Klibanov, A. M. Improving Enzymes by Using Them in Organic Solvents. *Nature* **2001**, 409, 241.

- [78] Castellana, M.; Wilson, M. Z.; Xu, Y.; Joshi, P.; Cristea, I. M.; Rabinowitz, J. D.; Gitai, Z.; Wingreen, N. S. Enzyme Clustering Accelerates Processing of Intermediates through Metabolic Channeling. *Nat. Biotechnol.* **2014**, 32, 1011.
- [79] Kuzmak, A.; Carmali, S.; von Lieres, E.; Russell, A. J.; Kondrat, S. Can Enzyme Proximity Accelerate Cascade Reactions? *Sci. Rep.* **2019**, 9, 455.
- [80] Wheelodon, L.; Minter, S. D.; Banta, S.; Barton, S. C.; Atanassov, P.; Sigman, M. Substrate Channelling as an Approach to Cascade Reactions. *Nature chemistry* **2016**, 8, 299.
- [81] **Chapter 3:** Aalbers, F. S.; Fraaije, M. W. Coupled Reactions by Coupled Enzymes: Alcohol to Lactone Cascade with Alcohol Dehydrogenase–Cyclohexanone Monooxygenase Fusions. *Appl. Microbiol. Biotechnol.* **2017**, 101, 7557–7565.
- [82] Pereira, J. P.; van der Wielen, L. A.; Straathof, A. J. Perspectives for the Microbial Production of Methyl Propionate Integrated with Product Recovery. *Bioresour. Technol.* **2018**, 256, 187–194.
- [83] van Beek, H. L.; Romero, E.; Fraaije, M. W. Engineering Cyclohexanone Monooxygenase for the Production of Methyl Propanoate. *ACS Chem. Biol.* **2017**, 12, 291–299.
- [84] Messiha, H. L.; Ahmed, S. T.; Karuppiyah, V.; Suardíaz, R.; Ascue Avalos, G. A.; Fey, N.; Yeates, S.; Toogood, H. S.; Mulholland, A. J.; Scrutton, N. S. Biocatalytic Routes to Lactone Monomers for Polymer Production. *Biochemistry* **2018**, 57, 1997–2008.
- [85] Issa, I. S.; Toogood, H. S.; Johannissen, L. O.; Raftery, J.; Scrutton, N. S.; Gardiner, J. M. C3 and C6 Modification-Specific Oye Biotransformations of Synthetic Carvones and Sequential BVMO Chemoenzymatic Synthesis of Chiral Caprolactones. *Chemistry–A European Journal* **2019**, 25, 2983–2988.
- [86] Ascue Avalos, G. A.; Toogood, H. S.; Tait, S.; Messiha, H. L.; Scrutton, N. S. From Bugs to Bioplastics: Total (+)-Dihydrocarvide Biosynthesis by Engineered *Escherichia coli*. *ChemBioChem* **2019**, 20, 785–792.
- [87] Iqbal, N.; Stewart, J. D.; Macheroux, P.; Rudroff, F.; Mihovilovic, M. D. Novel Concurrent Redox Cascades of (R)- and (S)-Carvones Enables Access to Carvo-Lactones with Distinct Regio- and Enantioselectivity. *Tetrahedron* **2018**, 74, 7389–7394.
- [88] Stamm, A.; Biundo, A.; Schmidt, B.; Brücher, J.; Lundmark, S.; Olsén, P.; Fogelström, L.; Malmström, E.; Bornscheuer, U. T.; Syrén, P.-O. A Retrobiosynthesis-Based Route to Generate Pinene-Derived Polyesters. *ChemBioChem* **2019**, 0.
- [89] Ahmed, S. T.; Leferink, N. G.; Scrutton, N. S. Chemo-Enzymatic Routes Towards the Synthesis of Bio-Based Monomers and Polymers. *Molecular Catalysis* **2019**, 467, 95–110.
- [90] Morrill, C.; Jensen, C.; Just-Baringo, X.; Grogan, G.; Turner, N. J.; Procter, D. J. Biocatalytic Conversion of Cyclic Ketones Bearing α -Quaternary Stereocenters into Lactones in an Enantioselective Radical Approach to Medium-Sized Carbocycles. *Angewandte Chemie* **2018**, 130, 3754–3758.
- [91] Zhang, W.; Fueyo, E. F.; Hollmann, F.; Martin, L. L.; Pesic, M.; Wardenga, R.; Höhne, M.; Schmidt, S. Combining Photo-Organic Redox- and Enzyme Catalysis Facilitates Asymmetric C-H Bond Functionalization. *Eur. J. Org. Chem.* **2019**, 2019, 80–84.
- [92] Schmidt, S.; Castiglione, K.; Kourist, R. Overcoming the Incompatibility Challenge in Chemoenzymatic and Multi-Catalytic Cascade Reactions. *Chemistry–A European Journal* **2018**, 24, 1755–1768.
- [93] Rudroff, F.; Fink, M. J.; Pydi, R.; Bornscheuer, U. T.; Mihovilovic, M. D. First Chemo-Enzymatic Synthesis of the (R)-Taniguchi Lactone and Substrate Profiles of Camo and Otemo, Two New Baeyer-Villiger Monooxygenases. *Monatsh Chem* **2017**, 148, 157–165.

Section 2

Fusions of Oxidoreductases

Chapter 3: Coupled reactions by coupled enzymes: alcohol to lactone cascade with alcohol dehydrogenase–cyclohexanone monooxygenase fusions

Friso S. Aalbers and Marco W. Fraaije

This chapter is based on a published article: Aalbers, F. S., & Fraaije, M. W. (2017). Coupled reactions by coupled enzymes: alcohol to lactone cascade with alcohol dehydrogenase–cyclohexanone monooxygenase fusions. *Applied microbiology and biotechnology*, 101(20), 7557–7565. <https://doi.org/10.1007/s00253-017-8501-4>

Abstract

The combination of redox enzymes for redox-neutral cascade reactions has received increasing appreciation. An example is the combination of an alcohol dehydrogenase (ADH) with a cyclohexanone monooxygenase (CHMO). The ADH can use NADP⁺ to oxidize cyclohexanol to form cyclohexanone and NADPH. Both products are then used by CHMO to produce ϵ -caprolactone. In this study, these two redox-complementary enzymes were fused, to create a self-sufficient bifunctional enzyme that can convert alcohols to esters or lactones. Three different alcohol dehydrogenase (ADH) genes were fused to a gene coding for a thermostable cyclohexanone monooxygenase (CHMO), in both orientations (ADH-CHMO and CHMO-ADH). All six fusion enzymes could be produced and purified. For two of the three ADHs, we found a clear difference between the two orientations: one that showed the expected ADH activity, and one that showed low to no activity. The ADH activity of each fusion enzyme correlated with its oligomerization state. All fusions retained CHMO activity, and stability was hardly affected. The TbADH-TmCHMO fusion was selected to perform a cascade reaction, producing ϵ -caprolactone from cyclohexanol. By circumventing substrate and product inhibition, a >99% conversion of 200 mM cyclohexanol could be achieved in 24 hours, with >13000 turnovers per fusion enzyme molecule.

Introduction

The use of enzymes for industrial scale production of chemicals has great potential. Enzymatic reactions allow the production of specific compounds without the use of harsh chemicals, organic solvents or extreme conditions, such as high temperature and high pressure. With multi-step enzymatic reactions, also referred to as cascade reactions, complex compounds can be produced in a single setup, with minimal input and without the need of isolating intermediates (Muschiol et al. 2015; Xue and Woodley 2012).

There are several problems preventing the large-scale industrial application of enzymatic reactions (Woodley 2013). One important hurdle is stability; enzymes evolved to catalyse reactions at the level of a living cell, and cannot maintain production for long periods of time, with co-solvents, and at higher temperatures. Another frequently encountered issue is insufficient functional expression, in particular when the enzyme which presents the rate-limiting step in a cascade has a limited level of expression. Furthermore, general availability of enzymes can be an issue, and to improve expression of enzymes individually is comparatively costly.

An approach that could address some of these difficulties is producing catalytically complementary enzymes fused to each other. Fusions are generally created by organizing a single open reading frame coding for two or more enzymes, from which the translation would result in a multifunctional enzyme. Some studies on enzyme fusions have found various advantages, including improved catalytic efficiency, expression, folding, and stability (Chen et al. 2013; Yang et al. 2016). In some cases, as the fused enzymes are in close proximity, a product molecule from one enzyme could directly migrate to the active site of the second enzyme. This phenomenon is referred to as substrate channelling (Wheeldon et al. 2016). In this way a cascade reaction with fused enzymes can be more efficient (Iturrate et al. 2010). Although some studies have found particular benefits from fused enzymes, it is not clear how to design an efficient and stable fusion. The current state of the art of fusions has been reviewed recently (Elleuche 2015; Yang et al. 2016).

The aim of this study is to investigate fusion engineering as an approach to co-express and improve biocatalysts in terms of stability and catalytic efficiency. We fused alcohol dehydrogenases (ADHs, EC 1.1.1) to a Baeyer-Villiger monooxygenase (BVMO, EC 1.14.13), as the two types of enzymes can perform various cascade reactions together. ADHs can oxidize alcohols to ketones, whereby NADPH is formed, and a BVMO can then use the NADPH and oxygen to oxidize the ketones to lactones or esters. The cascade reaction

we aimed to optimize with this approach is the biocatalytic production of ϵ -caprolactone from cyclohexanol (Fig. 1), which has been targeted in various recent studies (Mallin et al. 2013; Staudt et al. 2013; Sattler et al. 2014; Schmidt et al. 2015).

Figure 1 – Cascade reaction from cyclohexanol to ϵ -caprolactone, involving an alcohol dehydrogenase (ADH) and a cyclohexanone monooxygenase (CHMO).

The BVMO that was used for each fusion is the recently characterized cyclohexanone monooxygenase from *Thermocristum municipale* (TmCHMO) (Romero et al. 2016). The CHMO is more thermostable and has higher solvent tolerance than the commonly used AcCHMO from *Acinetobacter calcoaceticus* NCIMB 9871. Therefore, the TmCHMO is a promising biocatalyst for industrial application. Three ADH enzymes were chosen as fusion partners for the TmCHMO, based on their preference for NADP⁺ and thermostability. Two are known thermostable enzymes: TbADH from *Thermoanaerobacter brockii* (Lamed et al. 1981) and ADHA from *Pyrococcus furiosus* (Van der Oost et al. 2001). The other one is a newly discovered alcohol dehydrogenase: ADHMi from *Mesotoga infera*, which was found based on sequence similarity with annotated cyclohexanol dehydrogenases, and was selected based on origination from a mesophile, and predicted acceptance of NADP⁺ using Cofactory (Geertz-Hansen et al. 2014).

Fusions between the ADHs and TmCHMO were made in both orientations; with the TmCHMO either at the C-terminus or at the N-terminus of the ADH (Table 1). In this way, six fusion constructs were created in total. The rationale for this approach is that either of the enzymes could be hindered by its fusion partner in one orientation. In particular, ADHs form oligomers, and the oligomerization may be obstructed, which could decrease their stability (Korkhin et al. 1999), and could prevent their activity (Breiter et al. 1994). The two enzymes are connected by a glycine-rich linker, which in a previous study on fusions was found to be the most favourable among the tested linkers (Jeon et al. 2015).

We intended to create a fusion that can convert cyclohexanol to ϵ -caprolactone in vitro, which is stable for a long period of time at elevated

temperatures, leading to high total turnover numbers. Activity of both enzymes of the fusions was determined, and their stability and oligomerization were studied. The best candidate was used to perform conversions of cyclohexanol into ϵ -caprolactone.

Results

Expression and purification of fusion enzymes

After cloning of each fusion construct (Table 1), transformed cells were grown for expression of the fusion enzymes. Expression was induced simultaneously with inoculation of the main culture, and this culture was incubated for 40 hours at 24 °C. Purification of the yellow fusion enzymes was achieved through nickel affinity chromatography (Fig. S1). The imidazole was removed by applying the yellow fraction to a desalting column, and subsequent elution with buffer. The purification yielded substantial protein amounts, which varied from 120 to 320 mg per litre culture. Similar amounts were obtained from expression and purification of the single ADH enzymes and TmCHMO, which suggests that the expression of the fusions is at least just as good (no data shown). In practice, expression with 50 mL cultures yielded a sufficient amount for biochemical experiments.

Table 1. Fusion constructs

Enzyme	N-terminal	Linker	C-terminal	Mw (kDa)
A-Tm	ADHA	Glycine-rich	TmCHMO	90
Tm-A	TmCHMO	Glycine-rich	ADHA	90
Mi-Tm	ADHMi	Glycine-rich	TmCHMO	91
Tm-Mi	TmCHMO	Glycine-rich	ADHMi	91
Tb-Tm	TbADH	Glycine-rich	TmCHMO	102
Tm-Tb	TmCHMO	Glycine-rich	TbADH	102

Each fusion construct also contained a His-tag at the N-terminal side. Molecular weight of each fusion is based on predicted size and SDS-PAGE. Glycine-rich linker amino acid sequence: SGGSGGSGGSAG.

The degree of FAD cofactor in the fusion enzymes can be verified by measuring the absorption at 441 nm and at 280 nm. Incorporation of this cofactor could be affected by the level of expression, as there could be insufficient FAD in the cell, and by hindrance from the fusion partner. Bound FAD is a sign of correctly folded flavoprotein. FAD ratios (A280/A441) of the fusions were found to range

from 14-17, which indicates that the majority of each fused TmCHMO has FAD bound after purification (Table S2). From here on, the fusions will be abbreviated as follows: A-Tm (ADHA-TmCHMO), Mi-Tm (ADHMi-TmCHMO), and Tb-Tm (TbADH-TmCHMO) for the N-terminal ADH fusions (ADH-CHMO), and the fusions with a C-terminal ADH (CHMO-ADH) inversely (Tm-A, Tm-Mi, and Tm-Tb).

Activity screening

To determine the activity of each fusion enzyme, change in absorption at 340 nm was followed, as described in the Materials and methods section. The ADH oxidation rate was determined with 10 mM cyclohexanol as substrate. Cyclohexanone is also a substrate for the ADHs, for the reduction reaction with NADPH. Therefore, the BVMO activity of the fusion enzymes was measured using thioanisole (0.25 mM) as substrate (Table 2). The activity of TmCHMO on thioanisole is very similar to its activity on cyclohexanone (Romero et al. 2016).

It was gratifying to note that every fusion retained activity of the TmCHMO fusion partner. Overall, the BVMO activity was fairly consistent, aside from a 2-fold decrease with Mi-Tm, and some fusions having slightly higher rates. Conversely, the alcohol oxidation activities of the fusions varied more severely. Neither A-Tm nor Tm-A showed any cyclohexanol oxidation activity. Interestingly, for the fusion Mi-Tm no activity was observed, whereas Tm-Mi did show clear oxidation rates. With the fusions of TbADH the reverse was observed: Tb-Tm has a wild-type level of activity, whereas Tm-Tb had a 4-fold lower activity.

Previous studies on the cascade reaction from cyclohexanol to ϵ -caprolactone (Fig. 1) have used AcCHMO, and pointed out that it is inactivated and inhibited by cyclohexanol, and by ϵ -caprolactone (Mallin et al. 2013; Staudt et al. 2013). It is important to know whether this also applies to TmCHMO, since inhibition could negatively affect the productivity of the cascade. Inhibition of TmCHMO was studied at several concentrations of inhibitor, and thioanisole was used as substrate to determine the activity. The single TmCHMO as well as the Tm-A fusion were tested. Regarding product inhibition, a 50% reduction of activity was observed in the presence of 66 mM of ϵ -caprolactone, and an 80% reduction at 133 mM. Results with cyclohexanol showed that presence of 2 mM was sufficient to cause a decrease in activity of 75% (data not shown). Like AcCHMO, TmCHMO also suffers from inhibition, though it is more robust.

Table 2. Fusion activity screening

Enzyme	ADH oxidation k_{obs} (s^{-1})	BVMO oxidation k_{obs} (s^{-1})
TmCHMO	-	1.05
ADHA	0.013	-
A-Tm	< 0.01	1.4
Tm-A	< 0.01	1.8
ADHMi	0.11	-
Mi-Tm	< 0.01	0.5
Tm-Mi	0.26	1.1
TbADH	1.35	-
Tb-Tm	1.08	1.9
Tm-Tb	0.28	1.6

Change in absorption at 340 nm measured at 25 °C in 50 mM Tris/HCl pH 8.0. Final substrate concentrations used: 10 mM cyclohexanol and 0.25 mM thioanisole. Cofactor concentration: 100 μM NADP⁺ or NADPH respectively. For ADHMi and its fusions 100 μM NAD⁺ was used for the alcohol oxidation. Reaction rates were calculated with protein concentrations determined from absorbance at 441 nm, using the extinction coefficient of TmCHMO ($\epsilon_{441} = 14.0 \text{ mM}^{-1} \text{ cm}^{-1}$).

Oligomerization of fusion enzymes

Alcohol dehydrogenases typically form dimers or tetramers, which in most cases is needed for stability and/or activity. Two techniques were used to investigate the oligomerization behaviour of the fusion enzymes: Blue-Native PAGE (BN-PAGE) combined with zymography, and gel filtration chromatography.

After BN-PAGE, the gel was subjected to zymography, which causes active ADHs to show a dark purple band (Fig. 2 and Fig. S2). The following enzymes showed no activity: both of the ADHA fusions, and Tm-Tb. Minor activity was seen for Mi-Tm, whereas the other enzymes showed clear dark bands (Tm-Mi, and Tb-Tm). Coomassie staining revealed that, aside from the two ADHA fusions, every inactive fusion also had a higher electrophoretic mobility compared to their active counter-parts, which suggests possible structural changes. When comparing two fusions consisting of identical proteins, e.g. Mi-Tm with Tm-Mi, it seems that each active fusion has lower electrophoretic mobility, indicative of distinct structural organization.

Protein size was estimated using gel filtration chromatography (Fig. S3). In accordance with results from the BN-PAGE, oligomers above 400 kDa were determined for the fusions A-Tm, Tm-A, Tm-C, Tm-Mi, and Tb-Tm, which supports the impression that these fusion enzymes form tetramers. The Tm-C fusion seems to be partly tetrameric and partly monomeric, based on gel filtration experiments. Each individual ADH had an elution time corresponding

to homodimers. The fusion enzymes C-Tm and Tm-Tb have a main peak at their monomer size, while for the Mi-Tm fusion a trimer size of about 280 kDa was observed.

Figure 2 Blue native-PAGE stained with coomassie and zymography simultaneously. Ladder values are given in kDa. The dark, purple stain results from the zymography, whereas the lighter, blue stain is caused by the coomassie blue G-250 treatment.

Enzyme stability

Since the fusion enzymes consist of a single polypeptide chain, the stability and the tendency to unfold could be changed compared to the individual enzymes. For each fusion enzyme the melting point was determined by using ThermoFluor and ThermoFAD (Table S2). With the latter, the release of the FAD from TmCHMO upon unfolding could be detected, while with ThermoFluor any protein unfolding gives a signal by binding of a dye (SYPRO orange). The more stable ADHs transfer some stability to TmCHMO (ΔT_m of up to +3 °C). The results from ThermoFluor have values similar to those from ThermoFAD. This is due to the lower melting point of TmCHMO compared to the ADHs.

The fusions Mi-Tm, Tm-Mi, and Tb-Tm display a second melting peak in the ThermoFluor assay (Fig. S4). In each case the second peak is some degrees lower than the single ADH melting point (T_m). For instance, the T_m of the single ADHMi is 69 °C, while the second peak of the Tm-Mi fusion is at 65 °C. This suggests that in those fusions each fusion partner unfolds more independently than the individual ADH.

To evaluate long-term stability, Tb-Tm was incubated at 37 °C for 42 hours (Fig. S6). Residual ADH and TmCHMO activity was determined by monitoring

the change in absorption at 340 nm after addition of substrate and nicotinamide cofactor. The long-term incubation was also carried out for TbADH and TmCHMO as separate enzymes. The fusion retained slightly more activity: after 42 hours, the fusion had 92% of the ADH and BVMO activity, whereas the separate TbADH and TmCHMO retained 88% and 82% activity, respectively.

Conversions

The most active and stable fusion was selected to perform small-scale conversions with cyclohexanol. Based on the results from the other experiments, the Tb-Tm fusion was selected. Biocatalytic production of ϵ -caprolactone was performed by combining the Tb-Tm fusion with NADP⁺ and cyclohexanol (Table 3). The total volume of each conversion was 0.5 mL. After conversion for 24 hours at 37 °C, the reaction sample was extracted three times with ethyl acetate, dried with magnesium sulphate, and then analysed using GC-MS (HP-5 column) (Fig. S5). The percentage of conversion is defined by depletion of substrate (cyclohexanol) and intermediate (cyclohexanone), compared to the control with enzyme.

Initial experiments (15 μ M Tb-Tm, 200 μ M NADP⁺, 24 hours, 37 °C) on low (10 mM) substrate concentration gave 100% conversion, while high (>100 mM) substrate loadings gave near to 0% conversion. The latter result can be explained by the TmCHMO inhibition by cyclohexanol. Therefore, substrate (1 M concentration) was fed into the reaction with a syringe pump at 5 μ L/hour (Syringe Pump NE-4000) to attain more conversion with higher substrate loading. By this, a conversion of 64% could be achieved in 24 hours. To investigate whether product inhibition was the main cause of the incomplete conversion, CAL-A lipase was included in the reactions, as described in a previous study (Schmidt et al. 2015). This lipase can convert ϵ -caprolactone to oligo-caprolactone, thereby reducing product inhibition. After incubation of the reaction including CAL-A (10 mg/mL) for 24 hours, the conversion reached 80.5%. The pH of the reaction drastically decreased during the conversion; from pH 8.0 to pH 5.0 after 24 hours, which is likely due to hydrolysis by the CAL-A lipase. To maintain pH, experiments were continued in a high concentration of potassium phosphate buffer (0.5 M, pH 8.0), which was tested with each enzyme for activity and stability (data not shown). This enabled us to get >99% conversion of 200 mM cyclohexanol within 24 hours. If a turnover is defined as the transformation of one molecule of cyclohexanol to one molecule of ϵ -caprolactone, then each fusion molecule performed 13 333 turnovers (TON), and NADP⁺ 1000 turnovers. Intriguingly, a parallel reaction was performed

with the TbADH and TmCHMO as separate enzymes, which gave a conversion of only 42%.

Table 3. Conversions

Enzyme(s)	Substrate (mM)	Time	Temperature	Conversion	TON
Tb-Tm (15 μ M)	200	24 hours	37 °C	>99 %	13 333
TbADH (15 μ M) + TmCHMO (15 μ M)	200	24 hours	37 °C	42 %	5 600
Tb-Tm (5 μ M) + TbADH (5 μ M)	200	24 hours	37 °C	95.8 %	19 160
Tb-Tm (5 μ M) + TmCHMO (5 μ M)	200	24 hours	37 °C	>99 %	20 000
Tb-Tm (20 μ M)	500	48 hours	30 °C	41.5 %	10 375
TbADH (20 μ M) +TmCHMO (20 μ M)	500	48 hours	30 °C	33 %	8 250

Conversions included substrate feeding (cyclohexanol, 1 M) at 5 μ l/hour, and 10 mg/ml CAL-A lipase, in 0.5 M potassium phosphate buffer pH 8.0. Conversion is calculated based on the depletion of cyclohexanol (and intermediate, cyclohexanone), from the GC-MS measurements. TON = Turnover number, per fusion molecule, regarding the transformation of cyclohexanol to ϵ -caprolactone as one turnover.

To investigate which of the two enzymes is rate-limiting, conversions with the fusion (5 μ M) and an additional amount of either the TbADH or TmCHMO (5 μ M) were made, such that the ratio between the two enzymes was 2:1 or 1:2 (Table 3). The reaction with additional TbADH reached 95.8% conversion after 24 hours, whereas the one with TmCHMO reached >99%, showing that the TmCHMO is the slowest fusion partner.

Discussion

In this chapter we designed ADH – TmCHMO fusion constructs, expressed the genes in *E. coli* cells, and then purified the fusion enzymes. Good amounts of FAD-containing fusion enzymes could be produced and purified. For studies on multi-enzyme reactions, the required enzymes are typically produced individually. By enzyme fusion, a single expression and purification yields bifunctional enzymes, thereby greatly reducing the effort and possibly improving the expression. Moreover, in some cases a fusion can perform successive reactions more efficiently, as found in various studies so far (Iturrate et al. 2010; Jeon et al. 2015; Lerchner et al. 2016; Peters et al. 2017).

However, we have found that this co-expression does not always produce fully active fusion enzymes. In particular, some of the fusions lacked ADH activity, while the TmCHMO retained activity in each of the six fusions. One

of the ADHs that was fused is the ADHA from *Pyrococcus furiosus*, which has extraordinary thermostability with a reported half-life of 22.5 hours at 90 °C (Van der Oost et al. 2001). Yet, its catalytic rates are relatively poor, in particular at lower temperatures. Considering this fact, it was not surprising to find that ADHA and its fusions were unable to oxidize cyclohexanol at 25 °C. Unlike this lack of activity of ADHA and its fusions, the reason for the inactivity of Mi-Tm is more obscure. The individual ADHMi enzyme, and its fusion Tm-Mi, are capable of oxidizing cyclohexanol. Therefore, the lack of activity is probably caused by the orientation, i.e. the fusion of the BVMO at the C-terminal side of the ADH. Incidentally, ADHMi is a short-chain dehydrogenase (SDR superfamily, with 250 amino acids on average). A recent study pointed out that for *Lactobacillus kefir* RADH, also an SDR, a C-terminal His-tag was detrimental for its activity (Peters et al. 2017). Furthermore, levodione reductase from *Leifsonia aquatica* had 40-70% decrease in activity with a C-terminally fused aminotransferase, depending on the linker length (Lerchner et al. 2016). The findings point to a common trend for SDR enzymes, where a fusion tag or enzyme at the C-terminal side of the SDR is disruptive for its activity.

In addition to lack of activity, the fusion Mi-Tm showed a smaller oligomeric size based on gel filtration, indicating that it forms a trimer. A plausible explanation for this aberration is that the region at the C-terminal side of the ADHMi is part of the oligomerization interface. The association between four ADH subunits would be hindered by the BVMO domains that are located at this interface. Consequently, the ADH is unable to form a particular oligomer, which could be needed for stability and activity (Breiter et al. 1994). On the other hand, the Tm-Mi fusion shows a tetramer size with the gel permeation experiments, and activity with zymography and spectrophotometric assay.

For the fusions with TbADH, a medium-chain reductase (MDR) enzyme, the situation is reversed. In this case, the fusion Tb-Tm has wild-type activity, while Tm-Tb has a four-fold lower ADH activity. The effect is less drastic compared to that of the ADHMi. The finding is in line with a study on phosphite dehydrogenase (PTDH)-BVMO fusions (Torres Pazmiño et al. 2008). In that study, the PAMO-PTDH fusion has 1.5-fold lower PTDH activity compared to the PTDH-PAMO fusion. Possibly, MDR enzymes favor the latter orientation in general, while showing a decrease in activity in the reverse orientation. As with Mi-Tm, the low activity of Tm-Tb correlates with a different oligomeric state, indicating a monomer, while the more active Tb-Tm seems to form a tetramer.

Previous studies on biocatalytic ϵ -caprolactone production have used AcCHMO, which was found to be inhibited by cyclohexanol and by ϵ -caprolactone. We found that, compared to AcCHMO, the TmCHMO, though more thermostable, is also prone to inhibition. This property is unfortunate for the cascade reaction with Tb-Tm, as the K_M for cyclohexanol of the TbADH (7 mM) is higher than the estimated K_I for cyclohexanol (< 2 mM) of TmCHMO. Based on the conversions in which either enzyme was added in a 2:1 ratio, the BVMO was shown to be the bottleneck of the cascade reaction. Therefore, the TmCHMO inhibition is an important aspect to investigate in future studies, in order to improve productivity of cascade reactions with these fusion enzymes.

The fusion Tb-Tm was selected to perform the cascade reaction to produce ϵ -caprolactone, as it showed the highest activity on cyclohexanol. Multiple adjustments had to be made in order to get more conversion. Inhibition of TmCHMO by substrate and product needed to be addressed, as well as the decrease in pH, which is probably the result of ϵ -caprolactone hydrolysis by CAL-A, as it would form 6-hydroxyhexanoic acid. The decrease in pH reduces the stability and the rates of the enzymes. In particular, the oxidation rate of ADHs is dependent on pH, as the deprotonation of the alcohol is favoured at higher pH, which promotes oxidation of the alcohol. With substrate feeding, addition of CAL-A lipase, and by using a strong buffer, >99% of 200 mM cyclohexanol could be converted in 24 hours. This result shows that the fusion can continuously perform oxidations by recycling of the cofactor, with a turnover number (TON) of >13 000 for the fusion enzyme. In a study with a similar cascade, in which AcCHMO was used, the enzyme reached a TON of approximately 5800 (Bornadel et al. 2016), which suggests that the superior stability of TmCHMO enables higher turnover numbers. The fusion seems to perform better than the combined individual enzymes. Several factors could play a role in this difference: slightly worse stability of the non-fused TmCHMO (Table S2, Fig. S6), higher activity of the TmCHMO in the Tb-Tm fusion (Table 2), and possibly a benefit of the co-localization of the two enzymes, as the cyclohexanone and NADPH formed by the ADH could directly migrate to the active site of the TmCHMO.

Conversions with relatively high final substrate concentrations showed difficulties due to solubility, enzyme stability, and inhibition. When the activity of the TmCHMO decreases, while the substrate feeding rate remains equal, the process will eventually stop due to complete inhibition of TmCHMO by the accumulated cyclohexanol. This effect also explains why factors, such as small differences in stability and activity, combined could lead to incomplete conversions, as was observed with the separate enzymes. It might be possible

to convert more substrate if the substrate feeding flow is decreased in proportion to the decrease of active enzyme, or if additional enzyme is added. The intricate balancing of the kinetic parameters and stability of the three enzymes involved in the cyclohexanol to 6-hydroxyhexanoic acid cascade reaction was recently investigated (Scherkus et al. 2017). The authors used computational simulations, as well as experiments, to find the most favourable approach and conditions for large-scale conversions. In a similar cascade, involving an enoate reductase in addition to ADH and CHMO, kinetic modelling was applied to identify bottlenecks for in vivo biotransformations (Milker et al. 2017). The kinetic models described could be applied to the robust Tb-Tm fusion presented herein, to further optimize the cascade towards an industrial process for ϵ -caprolactone production. Notably, as both the ADH and CHMO can accept a broad range of substrates, the fusions can be used for the production of various interesting esters or lactones through analogous cascade reactions.

Materials and methods

Materials, strains and culture media

Oligonucleotide primers for cloning and mutagenesis were ordered from Sigma-Aldrich. For amplification of the gene inserts, PfuUltra II HotStart PCR master mix was used, purchased from Agilent Technologies. Other chemicals were purchased from Sigma-Aldrich and Acros Organics. Precast native-PAGE gradient gels were ordered from Bio-Rad. CAL-A lipase was ordered from c-LEcta. As host strain for recombinant DNA, *Escherichia coli* NEB® 10-beta (New England Biolabs) was used. Precultures were grown in lysogeny broth (LB), and the subsequent main cultures in terrific broth (TB).

Cloning of fusion constructs

The TmCHMO gene was amplified from an in-house plasmid (pBAD-His-Tag-SUMO-TmCHMO, (Romero et al. 2016)) using PCR with two primer sets that have different flanking regions which contain restriction sites (Table S1). After PCR purification, the two obtained fragments were separately cloned into a pBAD vector. This vector has a region coding for a glycine-rich linker (SGGSGGSGGSAG), which is between two cloning sites, and the vector has an N-terminal His-tag. One PCR fragment was inserted into the NdeI-XhoI site of one vector, and the other between the PvuII and HindIII sites of another vector, using restriction and ligation. In this way, two vectors were obtained: one containing the TmCHMO gene at the N-terminal side of the glycine-rich linker, and one at the C-terminal side. To multiply the two vectors, the recombinant plasmids were used to transform chemically competent *E. coli* NEB® 10-beta cells. To identify colonies that had the desired insert, colony PCR was done, using a gene-specific forward and a vector-specific reverse primer. Colonies that gave PCR product were subsequently analysed using Sanger sequencing (GATC Biotech). Cells harbouring the correct plasmids were stored at -80 °C in 25% glycerol. For subsequent subcloning of the three ADH genes, the two vectors that contain the TmCHMO gene were used as backbones. The Tbadh (X64841.1) gene was obtained from an in-house plasmid (pBad::HheC::AdhT (Szymanski et al.

2010)); a plasmid containing the *adhA* gene was previously received from the laboratory of microbiology at Wageningen University (Van der Oost et al. 2001); *adhMi* (CCU83614.1) was ordered as a codon-optimized synthetic gene (MF276894), and was subcloned from the supplied standard vector (GenScript, USA). The genes were amplified using two primer sets (Table S1), and all the other steps described above were repeated for each ADH gene, with a few exceptions. To avoid digestion in the middle of either gene of one construct, first, *adhA* was cloned into the starting vector at the *PvuII*-*Apal* site, and subsequently the *TmCHMO* gene was inserted at the *NdeI*-*XhoI* site. Thus, six fusion constructs were cloned that have either the ADH gene or *TmCHMO* gene at the N-terminal side, and the other gene at the C-terminal end. For expression of the individual *adh* genes, fusion constructs with the *adh* upstream of the linker region were mutated with QuickChange primers, thereby introducing a stop codon at the end of each *adh* gene.

Expression and protein purification

E. coli cells harbouring the plasmids containing a gene fusion were grown overnight at 37 °C in 5 mL lysogeny broth (LB) and 50 µg/mL ampicillin. The precultures were inoculated from the -80 °C glycerol stocks. The next day the precultures were used to inoculate 50 mL of terrific broth (TB), containing 50 µg/mL ampicillin and 0.02% (w/v) L-arabinose, in baffled flasks. The 50 mL cultures were incubated at 24 °C for 40 hours. The cells were then harvested (3000 x g, 4 °C), and then resuspended in 10 mL of 50 mM Tris/HCl pH 8.0. Resuspended or pelleted cells were stored at -20 °C.

To purify the expressed proteins, the cell suspension was subjected to sonication for 10 minutes after thawing, and the lysate was centrifuged for 45 minutes at 29 100 x g (JA-17 rotor, Beckman, 4 °C). The supernatant was filtered through a membrane (0.45 µm pore size), and the filtrate was mixed with 1 mL of Ni²⁺ Sepharose resin (GE Healthcare), which was pre-equilibrated with 50 mM Tris/HCl pH 8.0. The mixture was incubated in closed gravity-flow column for 60 minutes at 4 °C while rotating. After the liquid was let through the column, the resin was washed with 6 column volumes of three solutions consisting of buffer (50 mM Tris/HCl pH 8.0) and varying concentration of imidazole: 0 mM, 5 mM, and 10 mM. Subsequently the bound proteins were eluted by applying 500 mM imidazole in 50 mM Tris/HCl pH 8.0. The obtained yellow elute was then applied to a PG-10 desalting column which was pre-equilibrated with 50 mM Tris/HCl pH 8.0, and the protein was eluted with the same buffer.

Protein analyses

From the fractions obtained during purification (cell-free extract, flow-through, wash, purified), 10 µL samples of 2 mg/mL protein were taken. After addition of SDS loading dye and incubation at 95 °C for 5 minutes, the samples were spun down at 13.000 x g for 1 minute, and then loaded onto an SDS-PAGE gel (GenScript, USA) and run according to the recommendations of the gel supplier. A protein ladder (PageRuler pre-stained, Thermofisher) was also loaded. The gels were run in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad), and current was applied using a PowerPac™ HC High-Current Power Supply (Bio-Rad), set at 120 V. When the blue front of the loading dye reached the bottom of the gel, the gel was removed from the chamber, rinsed with water, and stained with Coomassie InstantBlue™ (Fig. S1). Absorption spectra from 200 to 700 nm were taken of each purified fusion protein, diluted in buffer in a quartz cuvette (V-330 Spectrophotometer, JASCO). Using the obtained values at 280 nm and 441 nm, the protein concentration ($\epsilon_{441} = 14.0 \text{ mM}^{-1} \text{ cm}^{-1}$) and FAD ratio could be calculated.

Activity assays

Kinetic measurements were done by following the formation or depletion of NADPH at 340 nm. After mixing enzyme ($\leq 0.1 \mu\text{M}$) with substrate in buffer (50 mM Tris/HCl pH 8.0), 200 μM NADP or NADPH was added, briefly mixed in a cuvette, and then the reaction was followed (V-330 Spectrophotometer, JASCO). For ADH activity cyclohexanol (10 mM final concentration) was used as substrate, and for CHMO activity thioanisole (0.25 mM final concentration). The slopes of the initial 20 seconds were used to calculate the activity rates. The obtained slope value is expressed in absorption change per minute (Abs/min). This value was then divided by the extinction coefficient of NADPH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), in accordance with Lambert-Beer law, giving a value in mM per minute. By dividing this value by the protein concentration in the reaction, the k_{obs} values were obtained. All measurements were done in duplicates or triplicates.

Blue native PAGE and zymography

Native PAGE with all purified enzyme fusions was performed as described (Wittig et al. 2006), with some modifications. The cathode buffer consisted of 50 mM tricine, 7.5 mM imidazole, 0.002% w/v coomassie blue G-250 (pH 7.0), the anode buffer of 25 mM imidazole (pH 7.0), and the sample loading dye of 50% glycerol and 0.5% ponceau S (w/v). Samples were prepared by mixing 5 μL of fusion enzyme with 5 μL of loading dye, and were loaded onto the gel (4-15% Mini-PROTEAN® TGX™, Bio-Rad). The gel electrophoresis was started at 60 V (10 mA, 1 W) at 4 °C, the voltage was increased by 10 every 30 minutes, and after 2 hours the gel was removed. The gel was washed with water, and then incubated with a zymography reaction solution (2 mM phenazine methosulfate (PMS), 0.3 mM nitro-blue tetrazolium (NBT), 0.5 mM NADP⁺, 50 mM cyclohexanol, in 50 mM Tris/HCl pH 9.0) for 20 minutes at 25 °C. The reaction mixture was removed when clear purple spots appeared in the gel, and the gel was subsequently stained with 0.02% (w/v) coomassie blue G-250 in fixing solution (methanol:acetic acid:dH₂O 40:10:50), and finally destained with 8% acetic acid solution.

Gel filtration

Purified enzyme fusion samples were applied to a gel filtration column (Superdex 200 10/300 GL, GE Healthcare), which was equilibrated with buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5) and mounted onto an ÄKTA pure system (GE Healthcare), and the flow rate was set at 0.6 mL/min of the same buffer. Through detection at 280 nm and 450 nm, the elution of the fusion enzymes could be followed in time. A gel filtration standard (Bio-Rad) was applied in the same way, and based on the elution times a calibration curve was made, which was used to calculate the molecular weight of the fusion enzyme complexes.

Melting point determination and stability

To determine the melting temperature of each fusion and the individual enzymes, samples were analysed using the ThermoFluor® and ThermoFAD method (Forneris et al. 2009). Samples of 20 μL , containing 10 μM of enzyme, were incubated in a real-time PCR machine with a temperature gradient (20-99 °C, +0.5 °C/min) while measuring fluorescence every 0.5 °C step. For the ThermoFluor® method, the dye SYPRO® orange (ThermoFisher) was added to the samples. The determined value of melting temperature is the average value of two measurements. Long-term stability was evaluated by incubating 10 μM of enzyme in potassium phosphate buffer (100 mM, pH 8.0) at 37 °C, and measuring the activity rates at various time points. For activity rate determination of the alcohol dehydrogenase, 10 mM of cyclohexanol and 0.20 mM NADP⁺ were used, and for the cyclohexanone monooxygenase activity 0.25 mM of thioanisole with 0.20 mM NADPH were used. All measurements were performed in duplicate.

Conversions

Small scale biotransformations were performed in 2 mL Eppendorf tubes, with 0.5 mL of reaction mixture. The mixture consisted of fusion enzyme (5–20 μ M), cofactor (200 μ M NADP⁺), CAL-A lipase (10 mg/mL) and buffer (50 mM Tris/HCl pH 8.0 or 0.5 M potassium phosphate buffer pH 8.0). A control without fusion enzyme was run in parallel. The tubes with the reactions were incubated (37 °C, 600 RPM, ThermoMixer C, Eppendorf). During the incubation, substrate (stock: 1 M cyclohexanol in buffer) was fed into the tubes with a syringe pump at a rate of 5 μ L/hour (Syringe Pump NE-4000). In later experiments, 10 mg/ml CAL-A lipase (c-LEcta) was included in the reactions. The samples were extracted three times with equivalent volume of ethyl acetate, including 1.0 mM of acetophenone as external standard. The pooled extract (1.5 mL) was dried with magnesium sulphate, and then analysed using GC-MS (HP-5 column, injection temperature: 250 °C, oven temperature: 40 °C – 130 °C, 5 °C/min).

Submission of sequence

The codon-optimized nucleotide sequence coding for ADHMi from *Mesotoga infera* was submitted to GenBank according to the instructions of the website of GenBank. The amino acid sequence was originally obtained from ncbi (GenBank accession: CCU83614.1). To order the corresponding synthetic gene, the amino acid sequence was submitted to GenScript, which produced the codon-optimized gene for expression in *E. coli*.

Acknowledgements

This work was supported by the EU project ROBOX (grant agreement no. 635734) under the EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1.

Supporting information

For supporting information, see pages 128–132.

References

- Bornadel, A., Hatti-Kaul, R., Hollmann, F., & Kara, S. (2016). Enhancing the productivity of the bi-enzymatic convergent cascade for ϵ -caprolactone synthesis through design of experiments and a biphasic system. *Tetrahedron*, 72(46), 7222–7228.
- Breiter, D. R., Resnik, E., & Banaszak, L. J. (1994). Engineering the quaternary structure of an enzyme: construction and analysis of a monomeric form of malate dehydrogenase from *Escherichia coli*. *Protein Sci*, 3(11), 2023–2032.
- Chen, X., Zaro, J. L., & Shen, W. C. (2013). Fusion protein linkers: property, design and functionality. *Adv Drug Deliv Rev*, 65(10), 1357–1369.
- Elleuche, S. (2015). Bringing functions together with fusion enzymes—from nature's inventions to biotechnological applications. *Appl Microbiol Biotechnol*, 99(4), 1545–1556.
- Forneris, F., Orru, R., Bonivento, D., Chiarelli, L. R., & Mattevi, A. (2009). ThermoFAD, a ThermoFluor®-adapted flavin ad hoc detection system for protein folding and ligand binding. *FEBS J*, 276(10), 2833–2840.
- Geertz-Hansen, H. M., Blom, N., Feist, A. M., Brunak, S., & Petersen, T. N. (2014). Cofactory: Sequence-based prediction of cofactor specificity of Rossmann folds. *Proteins: Struct., Funct., Bioinf.*, 82(9), 1819–1828.

Iturrate, L., Sánchez-Moreno, I., Oroz-Guinea, I., Pérez-Gil, J., & García-Junceda, E. (2010). Preparation and characterization of a bifunctional aldolase/kinase enzyme: a more efficient biocatalyst for C-C bond formation. *Chem Eur J*, 16(13), 4018–4030.

Jeon, E. Y., Baek, A. H., Bornscheuer, U. T., & Park, J. B. (2015). Enzyme fusion for whole-cell biotransformation of long-chain sec-alcohols into esters. *Appl Microbiol Biotechnol*, 99(15), 6267–6275.

Korkhin, Y., KALB, J., Peretz, M., Bogin, O., Burstein, Y., & Frolow, F. (1999). Oligomeric integrity—the structural key to thermal stability in bacterial alcohol dehydrogenases. *Protein Sci*, 8(6), 1241–1249.

Lamed, R. J., Keinan, E. J. G. Z., & Zeikus, J. G. (1981). Potential applications of an alcohol-aldehyde/ketone oxidoreductase from thermophilic bacteria. *Enzym Microb Technol*, 3(2), 144–148.

Lerchner, A., Daake, M., Jarasch, A., & Skerra, A. (2016). Fusion of an alcohol dehydrogenase with an aminotransferase using a PAS linker to improve coupled enzymatic alcohol-to-amine conversion. *Protein Eng Des Sel*.

Mallin, H., Wulf, H., & Bornscheuer, U. T. (2013). A self-sufficient Baeyer–Villiger biocatalysis system for the synthesis of ϵ -caprolactone from cyclohexanol. *Enzym Microb Technol*, 53(4), 283–287.

Milker, S., Fink, M. J., Oberleitner, N., Ressmann, A. K., Bornscheuer, U. T., Mihovilovic, M. D., & Rudroff, F. (2017). Kinetic modeling of an enzymatic redox cascade in vivo reveals cofactor-caused bottlenecks. *ChemCatChem*.

Muschiol, J., Peters, C., Oberleitner, N., Mihovilovic, M. D., Bornscheuer, U. T., & Rudroff, F. (2015). Cascade catalysis—strategies and challenges en route to preparative synthetic biology. *Chem Comm*, 51(27), 5798–5811.

Peters, C., Rudroff, F., Mihovilovic, M. D., & T Bornscheuer, U. (2017). Fusion proteins of an enoate reductase and a Baeyer–Villiger monooxygenase facilitate the synthesis of chiral lactones. *Biol Chem*, 398(1), 31–37.

Romero, E., Castellanos, J., Mattevi, A., & Fraaije, M. W. (2016). Characterization and crystal structure of a robust cyclohexanone monooxygenase. *Angew Chem*, 128(51), 16084–16087.

Sattler, J. H., Fuchs, M., Mutti, F. G., Grischek, B., Engel, P., Pfeffer, J., Woodley, J. M., & Kroutil, W. (2014). Introducing an in situ capping strategy in systems biocatalysis to access 6-aminohexanoic acid. *Angew Chem Int Ed*, 53(51), 14153–14157.

Scherkus, C., Schmidt, S., Bornscheuer, U. T., Gröger, H., Kara, S., & Liese, A. (2017). Kinetic insights into ϵ -caprolactone synthesis: improvement of an enzymatic cascade reaction. *Biotechnol Bioeng*.

Schmidt, S., Scherkus, C., Muschiol, J., Menyes, U., Winkler, T., Hummel, W., Gröger, H., Liese, A., Herz, H., & Bornscheuer, U. T. (2015). An enzyme cascade synthesis of ϵ -caprolactone and its oligomers. *Angew Chem Int Ed*, 54(9), 2784–2787.

Staudt, S., Bornscheuer, U. T., Menyes, U., Hummel, W., & Gröger, H. (2013). Direct biocatalytic one-pot-transformation of cyclohexanol with molecular oxygen into ϵ -caprolactone. *Enzym Microb Technol*, 53(4), 288–292.

Szymanski, W., Postema, C. P., Tarabiono, C., Berthiol, F., Campbell-Verduyn, L., de Wildeman, S., de Vries, J. G., Feringa, B. L. & Janssen, D. B. (2010). Combining Designer Cells and Click Chemistry for a One-Pot Four-Step Preparation of Enantiopure β -Hydroxytriazoles. *Adv Synth Catal*, 352(13), 2111–2115.

Torres Pazmiño, D. E., Snajdrova, R., Baas, B. J., Ghobrial, M., Mihovilovic, M. D., & Fraaije, M. W. (2008). Self-sufficient Baeyer–Villiger monooxygenases: effective coenzyme regeneration for biooxygenation by fusion engineering. *Angew Chem*, 120(12), 2307–2310.

Van der Oost, J., Voorhorst, W. G., Kengen, S. W., Geerling, A., Wittenhorst, V., Gueguen, Y., & de Vos, W. M. (2001). Genetic and biochemical characterization of a short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur J Biochem*, 268(10), 3062–3068.

Wheeldon, I., Minter, S. D., Banta, S., Barton, S. C., Atanassov, P., & Sigman, M. (2016). Substrate channelling as an approach to cascade reactions. *Nat Chem*, 8(4), 299-309.

Wittig, I., Braun, H. P., & Schagger, H. (2006). Blue native PAGE. *Nat Protoc*, 1(1), 418.

Woodley, J. M. (2013). Protein engineering of enzymes for process applications. *Curr Opin Chem Biol*, 17(2), 310-316.

Xue, R., & Woodley, J. M. (2012). Process technology for multi-enzymatic reaction systems. *Bioresour Technol*, 115, 183-195.

Yang, H., Liu, L., & Xu, F. (2016). The promises and challenges of fusion constructs in protein biochemistry and enzymology. *Appl Microbiol Biotechnol*, 100(19), 8273-8281.

Chapter 4: Design of artificial alcohol oxidases: alcohol dehydrogenase – NADPH oxidase fusions for continuous oxidations

Friso S. Aalbers and Marco W. Fraaije

This chapter is based on a published article: Aalbers, F. S., & Fraaije, M. W. (2019). Design of Artificial Alcohol Oxidases: Alcohol Dehydrogenase–NADPH Oxidase Fusions for Continuous Oxidations. *ChemBioChem*, 20(1), 51-56. <https://doi.org/10.1002/cbic.201800421>

Abstract

With the aim of expanding the arsenal of industrially-applicable oxidative enzymes, fusions of alcohol dehydrogenases with an NADPH-oxidase were designed. In total three different alcohol dehydrogenases (LbADH, TbADH, ADHA) were expressed with a thermostable NADPH-oxidase fusion partner (PAMO C65D), and purified. The resulting bifunctional biocatalysts retained the catalytic properties of the individual enzymes, and act essentially like alcohol oxidases: transforming alcohols to ketones using dioxygen as mild oxidant, while merely requiring a catalytic amount of NADP⁺. In small-scale reactions the purified fusion enzymes show good performances, with 69-99% conversions, 99% *ee* with a racemic substrate, and high cofactor and enzyme total turnover numbers. As the fusion enzymes essentially act as oxidases, we found that commonly used high-throughput oxidase-activity screening methods can be used. Therefore, if needed, the fusion enzymes can be easily engineered to tune their properties.

Introduction

Alcohol oxidations are vital for the synthesis of various carbonyl compounds.^[1,2] In particular, a catalyst that features strict enantioselectivity can be used for kinetic resolution of alcohols.^[3] Enzymes can catalyze highly selective alcohol oxidations, and such biocatalytic oxidations have a relatively low environmental impact compared to chemically catalyzed oxidations.^[3-6]

The two main classes of enzymes that catalyze alcohol oxidations are: alcohol oxidases (EC 1.1.3), and alcohol dehydrogenases (ADHs, EC 1.1.1.1). Although oxidases are attractive biocatalysts, because they only depend on molecular oxygen as electron acceptor, there are not many alcohol oxidases available. On the other hand, there is a large array of characterized ADHs, with varying substrate specificities. The dehydrogenation reaction that the ADHs catalyze typically involves oxidized nicotinamide adenine dinucleotide (phosphate) (NAD(P)^+) as electron acceptor and an alcohol substrate, and transforms these into NAD(P)H and an aldehyde or ketone product. Inversely, the enzymes can also catalyze ketone reductions by using a reduced nicotinamide cofactor.

A major challenge concerning applying ADHs for alcohol oxidations is their dependence on the nicotinamide cofactor. Since the cofactor is too expensive to be applied in stoichiometric amounts, a recycling system is necessary to enable alcohol oxidations in an economically feasible manner.^[6,7] In addition, alcohol oxidation with NAD(P)^+ is thermodynamically less favorable than the reverse reaction, thus efficient recycling is needed to push against the equilibrium. One typical NAD(P)^+ -recycling approach is the addition of a sacrificial ketone substrate in excess, like acetone, which is readily reduced by the same ADH. This approach keeps the system simple, though it has some drawbacks such as difference in pH optima for the two reactions, occupation of active sites by the different substrates which leads to inhibition, and poor atom efficiency due to the excess of sacrificial substrate. Moreover, another downside is the inhibition caused by the product from the sacrificial ketone. Alternatively, an NAD(P)H oxidase (NOX, EC 1.6.3) can be used to regenerate NAD(P)^+ .^[6, 7] NOXs typically contain a tightly bound flavin cofactor and efficiently oxidize NAD(P)H using molecular oxygen, thereby forming hydrogen peroxide (type 1 NOX), or water (type 2 NOX).

Another type of flavin-containing enzyme is the Baeyer-Villiger monooxygenase (BVMO, EC 1.14.13), which can also bind NAD(P)H and oxygen, but which are used to catalyze Baeyer-Villiger oxidations or other oxygenations.^[8] One property of this class of enzymes is that after binding of

NAD(P)H and oxygen, and formation of the peroxyflavin intermediate, an uncoupling reaction can occur. During this reaction the reactive peroxyflavin shunts back to the oxidized state, H_2O_2 is formed, and NADP^+ is released. A mutant of a BVMO, phenylacetone monooxygenase (PAMO), was found to have a greatly enhanced uncoupling rate, thereby acting as an NADPH oxidase.^[9] Despite the profound change in activity, the stability of the C65D mutant was found to be as good as the wild-type PAMO, which is one of the most thermostable BVMOs characterized, with a melting temperature of 60 °C. With the favorable stability and pH optimum of the PAMO mutant (pH 8.0) compared to some other natural NOXs^[7], it is an attractive biocatalyst to apply for NADP^+ recycling.

Considering the combination of an ADH with a NOX for performing alcohol oxidations, we explored the approach of fusing these two enzymes together (Scheme 1). With this approach the enzymes can be produced and purified on one go, and, as the enzymes are co-localized, the NOX could support rapid regeneration of NADP^+ . In recent years the possibilities and advantages of enzyme fusions have been explored for various enzyme types, including fusions of redox enzymes.^[10-12] For instance, to enable recycling for NADPH-dependent enzymes, various BVMOs and a P450 monooxygenase were fused to phosphite dehydrogenase.^[13-15] Rather than using a sacrificial substrate like phosphite, some studies showed that it is also possible to fuse ADHs with a cyclohexanone monooxygenase (CHMO), to enable cascade reactions from alcohols to esters^[16], or from cyclohexanol to caprolactone^[17], for which the fusions were more efficient than the separate enzymes. Another recent example of enzyme fusions is the combination of oxidases with a peroxidase, producing fusions that can be used for cascade reactions, as the hydrogen peroxide that is produced by the oxidase can directly be used by the fused peroxidase.^[18]

Scheme 1. Alcohol dehydrogenases (ADHs) can catalyse alcohol oxidations and ketone reductions. By fusing an ADH with a NOX enzyme, which can oxidize the reduced nicotinamide cofactor NADPH using oxygen, the equilibrium is driven toward catalysing alcohol oxidations. In essence, the fusion of the two enzymes acts like an alcohol oxidase: an alcohol substrate is converted at the cost of oxygen, and hydrogen peroxide is produced.

The aim of this study is to investigate whether combining an ADH with a NOX as fusion produces a bifunctional enzyme that can be applied for dioxygen-driven alcohol oxidations, by facilitating regeneration of NADP⁺ (Scheme 1). Fusions were made by pairing the PAMO C65D mutant (NOX) with three NADP⁺-dependent alcohol dehydrogenases: LbADH (*R*-selective) from *Lactobacillus brevis*, TbADH from *Thermoanaerobacter brockii*, and a commercial ADH (ADHA).

The organization of the fusions was inspired by our previous study on ADH/CHMO fusions, in which we found a clear difference in ADH activity depending on the orientation: ADH-CHMO or CHMO-ADH (Chapter 3).^[17] The findings from that study indicate that short-chain dehydrogenases/reductases (SDRs) lose activity as N-terminal fusion (ADH-BVMO), possibly through perturbation of the dimer/tetramer formation of the SDRs. Although LbADH was not investigated in that study, other studies found that a C-terminal His-tag was detrimental for the activity.^[19] For ADHA we have similar evidence (unpublished results). Considering their classification as SDRs, we presumed that they would be active as C-terminal fusion (BVMO-ADH). Therefore, we designed the following fusion enzymes: NOX-A, NOX-L, and T-NOX (Table 1), each with an N-terminal His-tag.

Table 1. Alcohol dehydrogenase – NADPH-oxidase fusions produced

Enzyme	N-terminal	Linker	C-terminal	Mw (kDa)
NOX-A	PAMO C65D	SGSAAG	ADHA	90.3
NOX-L	PAMO C65D	SGSAAG	LbADH	90.5
T-NOX	TbADH	SGSAAG	PAMO C65D	101.4

A, ADHA; L, LbADH from *L. brevis*; T, TbADH from *T. brockii*; NOX, PAMO mutant C65D.

Results and discussion

The three fusion constructs were first cloned, and then transformed to *Escherichia coli* for recombinant expression. The expression levels were found to be similar to those of the individual ADH and NOX enzymes, based on SDS-PAGE with samples from the cell-free extracts (Figure S1, S2) and the amount of purified enzyme. After affinity chromatography purification, 40-150 mg of fusion enzyme per liter culture could be obtained. For wild-type PAMO, 40 mg of purified enzyme per liter culture was reported.^[20]

The UV/Vis absorbance spectra of the fusion enzymes were different compared to the spectrum of the single NOX. In the typical spectrum of oxidized FAD in NOX, the two absorbance maxima at 350-385 nm and at 440-

460 nm have roughly the same height, whereas for the fusions the 350-385 nm peak was more pronounced. This an indication for the presence of some fully reduced and/or semiquinone flavin. It is difficult to pinpoint the cause of this change; possibly the cells experienced more oxidative stress during the expression of these fusions, which affected the oxidation state of the NOX. To fully oxidize the flavin cofactor in the purified fusion enzymes, they were incubated with 10 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) overnight, which was subsequently removed through gel filtration chromatography. After this treatment, the spectra from the fusion enzymes resembled that of the individual NOX, indicating that the FAD is in oxidized state (Figure S3).

As mentioned before, the fusion of a protein to the N- or C-terminus of an enzyme could greatly influence the activity of that enzyme. Therefore, we determined the kinetic parameters of the ADH-NOX fusions and the single enzymes (Table 2). For the alcohol oxidation activity, cyclohexanol was chosen as model substrate, as it is a known substrate for each of the three ADHs. The alcohol oxidation results show minor differences in k_{cat} and K_M values between the fused and non-fused ADH enzymes; at most 1.5- to 2-fold differences in kinetic parameters. This indicates that the activity of the ADH enzymes was unaffected by the fusion. For the NOX activity, the differences were in a similarly small range, though there was a remarkable increase in K_M for NADPH of the NOX-A fusion. Still, the $K_{M,NADPH}$ for NOX-A is in the μM range. Overall, we found that the fused enzymes largely retained their catalytic properties, and proceeded to investigate their utility as biocatalysts.

Table 2. Alcohol and NADPH oxidation kinetics of the fusion enzymes.^[a]

Enzyme	Cyclohexanol oxidation			NADPH oxidation		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat} / K_M ($s^{-1} M^{-1}$)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} / K_M ($s^{-1} mM^{-1}$)
NOX	-	-	-	5.0 ^[b]	3.5 ^[b]	1400
ADHA	0.26	19	14	-	-	-
NOX-A	0.56	10	56	5.1	27	190
LbADH	2.2	31	71	-	-	-
NOX-L	2.0	29	69	4.4	5.8	760
TbADH	8.3	3.7	2200	-	-	-
T-NOX	5.7	5.8	980	2.8	5.7	490

[a] Kinetics determined by measuring change in absorbance at 340 nm at various concentrations of substrate (5-10 different concentrations, in duplicate or triplicate, Figures S4 and S5). Alcohol oxidation rates were measured in 20 mM KPO_4 pH 7.5, and NADPH oxidation was measured in 50 mM Tris/HCl pH 8.0, both at 25 °C. [b] Data taken from reference Brondani et al. 2014 (in 50 mM Tris/HCl pH 7.5)

To assess the applicability of the fusion enzymes for alcohol oxidations, conversion of cyclohexanol by NOX-A was tested. The reactions consisted of the following components: fusion enzyme, buffer, substrate, and NADP⁺. Initially, only moderate conversions could be achieved, ranging from 32% to 76% (data not shown). Possibly, the hydrogen peroxide that is formed during the reaction was inactivating one or both enzymes. Another complication could be the loss of FAD from the NOX fusion. To evaluate whether the addition of catalase or FAD could improve the level of conversion, each additive was separately added (Table 3). Both additives significantly improved the level of conversion. For the catalase, the reasons for the improvement are fairly straightforward: removing the hydrogen peroxide prevents damage to the enzymes while it regenerates oxygen that can be used by the NOX. The beneficial effect of additional FAD may lay in the stabilizing effect by forcing the enzyme to remain in its holo (FAD-bound) state. A recent study found that addition of FAD indeed improves stability of FAD-dependent mono-oxygenases.^[22] In that study, also superoxide dismutase (SOD) was found to give an improvement in biocatalyst performance. Yet, when we added 40 U of SOD, no improvement was found.

Table 3. Effect of additives on conversion with NOX-A

Enzyme	Additive	Conversion	TTN (enzyme)	TTN (cofactor)
NOX-A	-	75 %	39 500	395
NOX-A	FAD	93 %	46 500	465
NOX-A	catalase	89 %	44 500	445
NOX-A	FAD + catalase	95 %	47 500	475

TTN stands for total turnover number (amount of substrate converted per fusion enzyme). Reaction conditions: 50 mM cyclohexanol with 1 μ M of NOX-A in 50 mM Tris/HCl pH 8.5, 100 μ M NADP⁺, 64 hours at 24 °C, 500 RPM (ThermoMixer Eppendorf). Experiments were performed in duplicate. Amount of additives: 10 μ M FAD, 1000 U catalase.

Based on the results with FAD and catalase, these two additives were included in all subsequent conversions of cyclohexanol and 1-phenylethanol. As additional test substrate, we also included 1-phenylethanol to explore kinetic resolutions of this chiral alcohol (Scheme 2). Since TbADH is not active towards 1-phenylethanol, the Tb-NOX fusion was not tested for this substrate.

The results clearly show that the fusions can be used for effective alcohol oxidations, with high total turnover numbers (TTN) for both the enzyme and the cofactor (Table 4). Both metrics are of interest from an industrial perspective, as the biocatalyst and the cofactor have high cost contributions.^[7] However, the performance of T-NOX was considerably poorer when compared

with the other two fusion enzymes. Even though the T-NOX displayed the best kinetic parameters for cyclohexanol oxidation (Table 2), it showed the worst conversion (69%) of the three fusions enzymes, with only 23,000 turnovers (Table 4). The conversion was significantly better when compared to the conversion without FAD and catalase (only 51% conversion). This suggests that this fusion in particular suffered from the formation of hydrogen peroxide. Despite the addition of a substantial amount of catalase, still some hydrogen peroxide can accumulate because the affinity of catalase towards hydrogen peroxide is rather poor ($K_M > 10$ mM). TbADH is a medium-chain dehydrogenase that features a cysteine-coordinated zinc that is involved in catalysis, which could make it more sensitive to peroxide-induced inactivation.

Scheme 2. Kinetic resolution of *rac*-1-phenylethanol with the NOX-ADH fusions. The reaction would ideally yield 50% acetophenone, and 50% of 99% ee (*R*)- or (*S*)-1-phenylethanol.

For the conversions of 1-phenylethanol, it was gratifying to note that NOX-L retained the strict enantioselectivity of the native LbADH, with 50% conversion to acetophenone and yielding 99% ee of the (*S*)-1-phenylethanol. On the other hand, NOX-A showed a strong preference for the (*S*)-substrate. Depending on the duration of the reaction one could achieve (44%) (*R*)-1-phenylethanol of 99% ee, or primarily acetophenone (94%).

Table 4. Conversions of cyclohexanol and racemic 1-phenylethanol with ADH/NOX fusions

Enzyme	Substrate	Con-version	ee ^[a]	TTN (enzyme)	TTN (cofactor)
NOX-A	cyclohexanol	95 %	n.a.	31 666	475
NOX-L	cyclohexanol	99 %	n.a.	33 000	495
T-NOX	cyclohexanol	69 %	n.a.	23 000	345
NOX-A	<i>rac</i> -1-phenylethanol	94 %	99% (<i>R</i>)	31 333	470
NOX-A	<i>rac</i> -1-phenylethanol	56 % ^[b]	99% (<i>R</i>)	28 000	140
NOX-L	<i>rac</i> -1-phenylethanol	50 %	99% (<i>S</i>)	16 666	250

Reaction conditions: 50 mM substrate with 1.5 μ M of fusion enzyme in 50 mM Tris/HCl pH 8.5, 100 μ M NADP⁺, 64 hours at 24 $^{\circ}$ C, 500 RPM (ThermoMixer Eppendorf). Reactions with *rac*-1-phenylethanol include 2% DMSO. Amount of additives: 10 μ M FAD, 1000 U catalase. [a]: enantiomeric excess of the remaining alcohol substrate (Figure S6), [b]: 0.5 μ M NOX-A, 25 mM substrate, 100 μ M NADP⁺, 50 mM CHES (N-cyclohexyl-2-aminoethanesulfonic acid) pH 9.0 for 24 hours at 24 $^{\circ}$ C.

With the developed fusion approach, dioxygen-driven self-sufficient alcohol dehydrogenases were generated that can be applied for selective alcohol oxidations. Essentially, as the overall reaction only consumes molecular oxygen and produces hydrogen peroxide, these fusion enzymes can be regarded as artificial alcohol oxidases. With that in mind, we were interested to see whether such ‘alcohol oxidases’ are also suitable for established oxidase-based activity screening methods. One commonly used method for screening activity with oxidases is the horseradish peroxidase (HRP)-coupled assay.^[21] When the oxidases transform alcohols and concomitantly produce hydrogen peroxide, the HRP can use the peroxide to oxidize fluoro- or chromogenic substrates, by which an easily-detectable product is formed.

First, it was tested whether oxidase activity could be measured using a commonly used HRP-based method. The assay included 4-aminoantipyrine (AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) which, upon peroxidase-catalyzed oxidation, form a stable pink product. Using cell-free extract (after growth and expression of NOX-A) and 30 mM cyclohexanol, the reaction mixture turned pink after a few minutes, while the control reactions, that excluded any one of the components, remained colourless (Figure 1). One should know that no NADP⁺/NADPH was added to the assay mixture, which shows that the amount of nicotinamide cofactor in the extract was enough to support catalysis by NOX-A. *E. coli* cells contain roughly 100 μM of NADP⁺/NADPH.^[23] Even though activity of ADHs can be monitored through detection of NAD(P)H formation, this peroxidase-based assay offers a cheap (no cofactor needed), facile and rapid method to measure or detect ADH/NOX oxidase activity.

Figure 1. With an HRP-coupled assay, the alcohol oxidation activity of the NOX-A fusion can be detected, without addition of NADP⁺. The reaction included: buffer (50 mM Tris/HCl pH 7.5), HRP (0.8 U), AAP (0.1 mM) and DCHBS (1 mM), cell-free extract containing NOX-A (10 % v/v) and 30 mM cyclohexanol. Controls: (1) no substrate and (2) no cell-free extract.

Aside from screening activity of cell-free extracts, another peroxidase-based method has been developed that allows screening of oxidase activity in colonies.^[24] Such high-throughput approach is extremely suited for enzyme

engineering strategies. When making large mutant libraries, this approach is highly appealing, as the selection of positive mutants can be done based on the colour of the colonies. We have explored this option with the created NOX-A fusion. After transforming cells with a construct for expression of the NOX-A fusion, the cells were plated onto a permeable membrane, which was on top of a layer of LB agar containing arabinose for expression. After growing for 24 hours at 30 °C, the membrane with the colonies was transferred to an empty plate, frozen at -20 °C, thawed for partial lysis, and submerged in an assay mix. Colonies that expressed the NOX-A fusion quickly turned intensely blue, but not the colonies that express only NOX (Figure 2) or the ADH (not shown). The blue colonies could be picked and used for growth and plasmid isolation, relying on the cells that survived the freeze-thawing step.

Figure 2. The fusion of an ADH with a NOX enables the detection of alcohol oxidation activity in colonies. The plates contain colonies that expressed NOX-A (A) or NOX (B). Only the colonies that produce the fusion enzyme turn dark (blue) after addition of the assay mix.

This approach can be valuable when engineering an ADH/NOX towards a new substrate, as colonies would only turn blue when it has activity toward the substrate that is in the assay mix. In previous studies that used this method for screening of oxidase mutants, it was shown to be extremely useful for identifying improved variants, for example in case of engineering amine oxidase variants.^[24]

The designed NOX-ADH fusions presented in this study are not only suitable for performing alcohol oxidations with high total turnover numbers, the fusion of the two enzymes also enabled oxidase-based activity screening. All three ADH enzymes were active in fusion with a NOX, and each enzyme retained its catalytic properties and level of expression. Although the hydrogen peroxide-

forming NOX may not be suitable for some ADHs, very good conversions could be attained with addition of catalase. In that regard, ADH fusions with a water forming NOX could be more appealing from an industrial perspective.^[7] An alternative approach could be a triple fusion by adding a catalase as fusion partner. However, catalase was found to be problematic as fusion enzyme, prohibiting expression of catalase-fusion proteins (unpublished results). Considering that current screening of ADH mutants is quite labour-intensive and costly, it is noteworthy that the NOX fusion partner opens doors to a rapid and cheap qualitative activity screening, and a convenient qualitative colony-based screening. The NOX-ADH fusion approach can be a valuable tool for the development of useful and robust biocatalysts.

Materials and methods

Chemicals, reagents, enzymes and strains

Chemicals, media components, and reagents were obtained from Sigma(–Aldrich), Merck, Acros Organics, Alfa Aesar, Thermo Fisher, and Fisher Scientific. Oligonucleotides, superoxide dismutase (bovine SOD, recombinant), catalase (*Micrococcus lysodeikticus*), and horseradish peroxidase (HRP) were obtained from Sigma. T4 ligase and restriction enzyme BsaI were ordered from New England Biolabs. The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies. *E. coli* NEB® 10-beta (New England Biolabs) chemically competent cells were used as host for cloning of the recombinant plasmids, and for protein expression. Precultures were grown in glass tubes with lysogeny broth (LB), and for the subsequent main culture terrific broth (TB) was used in baffled flasks.

Golden Gate cloning

All fusion constructs were cloned with the Golden Gate cloning approach. Before commencing the amplification and assembly of the genes into the vector, the *pamo* gene (TFU_RS07375) was mutated through QuikChange to remove a BsaI site, and to introduce the C65D mutation. Henceforth, the mutated *pamo* gene will be referred to as *nox*. Primers were designed that contain a flanking region coding for the BsaI restriction site. These primers were used for PCR to amplify the *nox* and three alcohol dehydrogenase genes: *Tbadh* (X64841.1), *Lbadh* (AJ544275.1), and *adhA*. In addition to the BsaI sites, linker regions are added to reverse primers of the first gene and the forward primer of the second gene of a construct. These introduced linker regions together code for a short peptide linker (SGSAAG), after ligation of the PCR products. The primers were designed such that the PCR products from one ADH gene and the *nox* gene can be inserted into a pBAD vector. The pBAD vector contains two BsaI restriction sites, with an upstream region coding for an N-terminal His-tag (6xHis), an AraC promoter, and an ampicillin resistance-gene. The fusion constructs were produced by incubating together: two PCR products (an *adh* gene and *nox*), Golden Gate pBAD vector, BsaI restriction enzyme, T4 ligase, ligation buffer, and sterile miliQ water. The incubation temperature alternated between 16 °C (for 5 minutes) and 37 °C (for 10 minutes) for 30 cycles, then was set to 55 °C for 10 minutes, and finally to 80 °C for 20 minutes to inactivate the enzymes. To transform host cells with the fusion constructs, 3 µL of the Golden Gate reaction was added to chemically competent *E. coli* cells, and a heat shock was applied at 42 °C for 30 seconds. After overnight growth on an

lysogeny broth (LB) agar plate with ampicillin, colonies were picked, grown in liquid LB, and the plasmids were isolated and sent for sequencing (GATC, Germany) to confirm correct ligation of the genes.

Culture growth and protein purification

Fusion enzymes were produced and purified as described previously.^[17] The *E. coli* cells harbouring the plasmids were grown in a 5 mL LB with 50 µg/mL ampicillin (37 °C, 135 RPM, 16-24 hours). From this preculture 2 mL (4 % v/v) was used to inoculate 50 mL of terrific broth (TB) media, which contained 50 µg/mL ampicillin and 0.02% filtered L-arabinose. The cells were grown in a 250-mL baffled flask (Sigma) at 24 °C, 135 RPM, for 40 hours. After harvesting (3000 x g, 4 °C, 20 minutes), the cells were stored at -20 °C for several days. To purify the enzymes, the cells were first resuspended in 10 mL of buffer (50 mM Tris/HCl pH 8, 5 mM imidazole) supplemented with FAD (10 µM final concentration) and PMSF (phenylmethylsulfonyl fluoride, 100 µM). Then the cell suspension was cooled in ice-water, subjected to sonication (5 seconds on/off, 10 minutes), and subsequently the lysate was centrifuged for 45 minutes at 18514 x g, 4 °C. The supernatant was filtered (pore size 0.45 µm) into a gravity flow column containing 1 mL of Ni²⁺ Sepharose resin (GE Healthcare), which was then closed and incubated for 60 minutes at 4 °C on a rocking table. The flow-through was collected, and the resin was washed with five column volumes of two solutions consisting of buffer (50 mM Tris/HCl pH 8.0) and imidazole: 10 mM and 20 mM. Subsequently, the bound proteins were eluted by applying 500 mM imidazole in 50 mM Tris/HCl pH 8.0. The obtained yellow elute was then incubated with 100 µM KFe(CN)₆ overnight (16 hours) at 4 °C on a rocking table, to oxidize any reduced FAD. The yellow solution was applied to a PG-10 desalting column, which was pre-equilibrated with 50 mM Tris/HCl pH 8.0, and the fusion enzyme was eluted with the same buffer. Purified protein was analysed with spectrophotometry (200-700 nm) and SDS-PAGE.

SDS-PAGE and UV-Vis spectra

During the purification, small samples were taken before each purification step. To these samples SDS loading dye was added, the mixture was incubated at 95 °C for 5 minutes, and subsequently centrifuged at 13000 x g for 1 minute. The samples were loaded onto a precast SDS-PAGE gel (GenScript, USA) and ran according to the supplier recommendations. The gels were run in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad), and current was applied using a PowerPac™ HC High-Current Power Supply (Bio-Rad), set at 120 V. When the blue front of the loading dye reached the bottom of the gel, after about 70 minutes, the gel was removed from the chamber, and stained with Coomassie InstantBlue™ (Expedeon, US). Of each purified fusion protein an UV/VIS absorption spectrum was taken, from 200 to 700 nm (V-330 Spectrophotometer, JASCO, USA), in a 1:10 dilution in buffer (50 mM Tris/HCl pH 8) in a quartz cuvette (Hellma® Analytics, Germany). Using the values at 441 nm, the protein concentration ($\epsilon_{441} = 12.4 \text{ mM}^{-1} \text{ cm}^{-1}$)^[20] was calculated.

Activity measurements and determination of kinetic parameters

Kinetic measurements were done by following the formation or depletion of NADPH at 340 nm. After mixing enzyme ($\leq 0.1 \text{ µM}$) with substrate in buffer (50 mM Tris/HCl pH 8.0), 100 µM NADP or NADPH was added, briefly mixed in a cuvette, and then the reaction was followed (V-330 Spectrophotometer, JASCO). For ADH activity cyclohexanol was used as substrate. For the NOX activity only NADPH was used, in different concentrations. The slopes of the initial 20 seconds were used to calculate the activity rates. The obtained slope value is expressed in absorption change per minute (Abs/min). This value was then divided by the extinction coefficient of NADPH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), in accordance with Lambert-Beer law, giving a value in mM per

minute. With that value and the enzyme concentration, the activity rate was calculated. All measurements were done in duplicates or triplicates. Activity data was fitted with GraphPad Prism 6.0 (GraphPad, US) (Figure S4, S5).

Conversions

Small scale biotransformations were performed in 2 mL Eppendorf tubes, with 0.5 mL of reaction mixture. The mixture consisted of fusion enzyme (1–5 μM), cofactor (200 μM NADP⁺), substrate, and buffer (50 mM Tris/HCl pH 8.5, unless stated otherwise). A control without fusion enzyme was run in parallel. The tubes with the reactions were incubated (24 °C, 600 RPM, ThermoMixer C, Eppendorf). The samples were extracted three times with equivalent volume of ethyl acetate. The pooled extract (1.5 mL) was dried with magnesium sulphate, and then analysed using GC-MS (HP-5 column, injection temperature: 250 °C, oven temperature gradient: 40 °C – 130 °C, 5 °C/min), or using chiral GC (Hydrodex β -TBDAC column (Aurora Borealis, The Netherlands), injection temperature: 250 °C, oven temperature gradient: 60 °C – 190 °C, 5 °C/min).

Oxidase activity assay

After expression of the NOX-A in *E. coli*, the cells were harvested, dissolved in buffer, and lysed by sonication. Only a small volume (e.g. 500 μL) of solubilized cells is needed for this assay. The insoluble fraction was separated by centrifugation (12000 RPM, 30 min, 4 °C). In a well of a 96-well plate, the following components were combined: soluble fraction after lysis (20 μL), 1.0 mM AAP (20 μL), 10 mM DCHBS (20 μL), 4 U HRP (4 μL), substrate, and buffer to a total volume of 200 μL . Since the lysate will contain NADP⁺/NADPH, it is not needed to add the cofactor. A pink product will form as a result of peroxide production, which can be followed in time to obtain a reaction rate ($\epsilon_{515} = 26 \text{ mM}^{-1} \text{ cm}^{-1}$).

Colony screening assay

The assay has been performed as described,^[24] with some adjustments. Cells harbouring the plasmid for expression of NOX-A were plated onto a porous-membrane (nitrocellulose, Amersham, UK), onto an LB agar plate that contains 50 $\mu\text{g}/\text{mL}$ ampicillin and 0.02% arabinose. After 40 hour-growth at 30 °C, or when the colony-size large enough to pick multiple times, the membrane is transferred to an empty Petri dish, and incubated at -20 °C for 1 hour. After thawing the membrane with the colonies at room temperature for 1 hour, the assay mix (20 mL) was gently poured onto the membrane, containing: 10 mL of melted agarose (2 %), 10 mL of 100 mM potassium phosphate buffer pH 7.5, 100 U HRP, 2 mM 4-chloro-1-naphthol, 50 mM cyclohexanol (substrate), 100 μM NADP⁺. The plate was incubated at 30 °C, and after minutes – hours the colonies with active ‘oxidases’ develop a dark blue colour.

Acknowledgements

The research for this work has received funding from the European Union (EU) project ROBOX (grant agreement n°635734) under EU’s Horizon 2020 Program Research and Innovation actions H2020-LEIT BIO-2014-1. The views and opinions expressed in this article are only those of the authors and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein.

Supporting information

For supporting information, see pages 133–138.

References

- [1] T. Mallat, A. Baiker, *Chem. Rev.* **2004**, *104*, 3037–3058.
- [2] R. A. Sheldon, *Catal. Today* **2015**, *247*, 4–13.
- [3] a) F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmeyer, B. Bühler, *Green Chem.* **2011**, *13*, 226–265.
b) J. Dong, E. Fernández-Fueyo, F. Hollmann, C. E. Paul, M. Pesic, S. Schmidt, Y. Wang, S. Younes, W. Zhang, *Angew. Chemie Int. Ed.* **2018**, *57*, 9238–9261.
- [4] R. D. Schmid, V. Urlacher in *Modern biooxidation: enzymes, reactions and applications* (Eds.: R. D. Schmid, V. Urlacher), John Wiley & Sons, **2007**, pp. XIII–XIV.
- [5] M. Pickl, M. Fuchs, S. M. Glueck, K. Faber, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 6617–6642.
- [6] J. Liu, S. Wu, Z. Li, *Curr. Opin. Chem. Biol.* **2018**, *43*, 77–86.
- [7] G. Rehn, A. T. Pedersen, J. M. Woodley, *J. Mol. Catal. B Enzym.* **2016**, *134*, 331–339.
- [8] D. E. Torres Pazmiño, H. M. Dudek, M. W. Fraaije, *Curr. Opin. Chem. Biol.* **2010**, *14*, 138–144.
- [9] P. B. Brondani, H. M. Dudek, C. Martinoli, A. Mattevi, M. W. Fraaije, *J. Am. Chem. Soc.* **2014**, *136*, 16966–16969.
- [10] R. J. Conrado, J. D. Varner, M. P. DeLisa, *Curr. Opin. Biotechnol.* **2008**, *19*, 492–499.
- [11] S. Elleuche, *Appl. Microbiol. Biotechnol.* **2014**, *99*, 1545–1556.
- [12] H. Yang, L. Liu, F. Xu, *Appl. Microbiol. Biotechnol.* **2016**.
- [13] D. E. Torres Pazmiño, A. Riebel, J. de Lange, **2009**.
- [14] D. E. Torres Pazmiño, R. Snajdrova, B. J. Baas, M. Ghobrial, M. D. Mihovilovic, M. W. Fraaije, *Angew. Chemie* **2008**, *120*, 2307–2310.
- [15] N. Beyer, J. K. Kulig, A. Bartsch, M. A. Hayes, D. B. Janssen, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2016**, *101*, 2319–2331.
- [16] E. Y. Jeon, A. H. Baek, U. T. Bornscheuer, J. B. Park, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 6267–6275.
- [17] **Chapter 3:** F. S. Aalbers, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 7557–7565.
- [18] D. I. Colpa, N. Lončar, M. Schmidt, M. W. Fraaije, *ChemBioChem* **2017**, *18*, 2226–2230.
- [19] C. Peters, F. Rudroff, M. D. Mihovilovic, U. T. Bornscheuer, *Biol. Chem.* **2017**, *398*, 31–37.
- [20] M. W. Fraaije, J. Wu, D. P. H. M. Heuts, E. W. van Hellemond, J. H. L. Spelberg, D. B. Janssen, *Appl. Microbiol. Biotechnol.* **2005**, *66*, 393–400.
- [21] A. R. Ferrari, Y. Gaber, M. W. Fraaije, *Biotechnol. Biofuels* **2014**, *7*, 37.
- [22] L. C. P. Goncalves, D. Kracher, S. Milker, M. J. Fink, F. Rudroff, R. Ludwig, A. S. Bommarius, M. D. Mihovilovic, *Adv. Synth. Catal.* **2017**, *359*, 2121–2131.
- [23] B. D. Bennett, E. H. Kimball, M. Gao, R. Osterhout, S. J. Van Dien, J. D. Rabinowitz, *Nat. Chem. Biol.* **2009**, *5*, 593–599.
- [24] M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, *Angew. Chemie* **2002**, *114*, 3309–3312.

Chapter 5: What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monooxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis

Ángela Mourelle-Insua, **Friso S. Aalbers**, Iván Lavandera, Vicente Gotor-Fernández, Marco W. Fraaije

Authors Ángela Mourelle-Insua and Friso S. Aalbers contributed equally to this work. This chapter is not part of another thesis.

This chapter is based on a published article: Mourelle-Insua, Á., Aalbers, F. S., Lavandera, I., Gotor-Fernández, V., & Fraaije, M. W. (2019). What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monooxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis. *Tetrahedron*, 75(13), 1832-1839.

<https://doi.org/10.1016/j.tet.2019.02.015>

Abstract

A collection of fusion biocatalysts has been generated that can be used for self-sufficient oxygenations or ketone reductions. These biocatalysts were created by fusing a Baeyer-Villiger monooxygenase (cyclohexanone monooxygenase from *Thermocrispum municipale*: TmCHMO) or an alcohol dehydrogenase (alcohol dehydrogenase from *Lactobacillus brevis*: LbADH) with three different cofactor regeneration enzymes (formate dehydrogenase from *Burkholderia stabilis*: BsFDH; glucose dehydrogenase from *Sulfolobus tokodaii*: StGDH, and phosphite dehydrogenase from *Pseudomonas stutzeri*: PsPTDH). Their tolerance against various organic solvents, including a deep eutectic solvent, and their activity and selectivity with a variety of substrates have been studied. Excellent conversions and enantioselectivities were obtained, demonstrating that these engineered fusion enzymes can be used as biocatalysts for the synthesis of (chiral) valuable compounds.

Introduction

Biocatalysis can be used to synthesize chiral building blocks, such as monomers for polymer materials, and precursors for pharmaceuticals.^[1–4] Enzymes are very suitable for catalyzing reactions with high enantioselectivity to obtain chiral products. For instance, alcohol dehydrogenases (ADHs, EC 1.1.1.X) – also known as carbonyl reductases or ketoreductases – depend on NAD(P)H to catalyze the asymmetric reduction of ketones to either (*R*)- or (*S*)-alcohols in excellent enantiomeric excess (*ee*).^[5] These enzymes, among others, have been applied for syntheses of pharmaceutical precursors.^[2,4] Another group of redox enzymes that depend on NAD(P)H are the Baeyer-Villiger monooxygenases (BVMOs) (EC 1.14.13.X). These FAD-containing enzymes can catalyze regio- and enantioselective transformations of ketones to esters or lactones, using dioxygen and NADPH. Interest in the application of BVMOs has grown, in particular for the transformation of substituted cyclic ketones to chiral lactones, for branched polyesters.^[6–9] Specifically, the recent discovery of robust BVMOs, such as the thermostable cyclohexanone monooxygenase from *Thermocrisipum municipale* (*Tm*CHMO), has led to great interest for exploring these monooxygenases for industrial applications, as most of the previously reported BVMOs were quite unstable.^[10]

Both ADHs and BVMOs rely on the cofactor NAD(P)H for catalysis, which is too expensive to apply in stoichiometric amounts, and therefore should be regenerated. There are a number of different approaches to recycle NAD(P)H.^[5,11,12] One approach is to apply another enzyme which can use the oxidized NAD(P)⁺ and a sacrificial cheap cosubstrate to regenerate the reduced nicotinamide cofactor: the so-called “coupled-enzyme” approach. Three commonly used coenzyme regenerating enzymes are formate dehydrogenase (FDH), glucose dehydrogenase (GDH), and phosphite dehydrogenase (PTDH), all using relatively cheap substrates.^[12] Instead of producing and adding these enzymes separately, the recycling enzyme can also be covalently fused to the NADPH-dependent enzyme through enzyme engineering (Scheme 1). In this way, a bifunctional and self-sufficient fusion biocatalyst is produced enabling conversion using merely one biocatalyst. Moreover, some studies on enzyme fusions provided evidence that tethering of two enzymes can improve the productivity of a multi-enzyme system (**Chapter 1**).^[13] In 2008 Torres Pazmiño et al. developed a platform for expressing BVMOs fused to PTDH for efficient cofactor regeneration.^[14,15] Later, a few other studies reported enzyme fusions with FDH,^[16] GDH,^[17] or PTDH,^[18–20] though no study yet has compared a single biocatalyst with different regenerating enzymes.

Scheme 1. Representation of self-sufficient ADHs and BVMOs, with either GDH, FDH or PTDH as fusion partner to recycle the nicotinamide cofactor NADPH.

The aim of this work is to explore different recycling enzymes as fusion partner for two oxidoreductase enzymes (BVMO and ADH), and to compare their strengths and weaknesses for biocatalytic applications. We fused three different regenerating enzymes to a BVMO (*Tm*CHMO, from *Thermocrisperm municipale*)^[10] and an (*R*)-selective alcohol dehydrogenase (*Lb*ADH, from *Lactobacillus brevis*)^[21–23], to generate a panel of self-sufficient biocatalysts. The three recycling enzymes are: FDH from *Burkholderia stabilis* (*Bs*FDH)^[24], GDH from *Sulfolobus tokodaii* (*St*GDH)^[25], and an engineered PTDH from *Pseudomonas stutzeri* (*Ps*PTDH).^[26,27] The fused enzymes were produced and purified, and applied for biotransformations. Both enzymes act on ketone substrates to form interesting products, though many of such organic substrates have low solubility in water. Therefore, the enzyme fusions were tested with a wide range of solvents. In particular, the tolerance of these biocatalysts to deep-eutectic solvents (DES) was evaluated, which at present has not been studied to a great extent.

Results and Discussion

Cloning, expression and purification

The cloning approach was similar to that of previous fusion cloning work.^[14,15] As linker region a variation of the short-linker from previous work was used, which was found to be optimal for fusions with BVMOs.^[15] For ease of denoting, *Tm*CHMO is named B (BVMO) while the *Lb*ADH is named A (ADH), see Table 1. The cofactor regeneration enzymes are also denoted in single letter codes: F for FDH, G for GDH and P for PTDH. After cloning of the six constructs (Table 1), the plasmids were used to transform competent *E. coli*

NEB 10b cells. These cells were then used for recombinant expression and subsequent enzyme purification. Only one of the six fusion constructs had an inadequate level of soluble expression: G-A. Although it was used for some of the initial experiments, in later experiments this fusion enzyme was omitted. The other fusion enzymes could be produced in high quantities (>50 mg/L culture). After lysis of the cells and protein purification through affinity column chromatography, the enzyme fusions were used for further testing and biotransformations.

Table 1. Fusion constructs

Enzyme Fusion	N-terminal	Linker	C-terminal	Mw (kDa)
F-B	<i>Bs</i> FDH	SGSAAG	<i>Tm</i> CHMO	104
G-B	<i>St</i> GDH	SGSAAG	<i>Tm</i> CHMO	102
P-B	<i>Pst</i> PTDH	SRSAAG	<i>Tm</i> CHMO	99
F-A	<i>Bs</i> FDH	SGSAAG	<i>Lb</i> ADH	71
G-A	<i>St</i> GDH	SGSAAG	<i>Lb</i> ADH	69
P-A	<i>Pst</i> PTDH	SGSAAG	<i>Lb</i> ADH	65

Reaction optimization

The first step was to establish optimal reaction conditions. The reactions were set up using cyclohexanone (5 mM) as model substrate for the *Tm*CHMO constructs, and acetophenone (5 mM) for the *Lb*ADH constructs (see Supporting Information, Section 2.1). The optimal reaction conditions for each biocatalyst system were similar. All fusions were able to fully convert the substrate under mild reaction conditions: phosphate buffer 100 mM pH 8.5 for *Tm*CHMO fusions, and phosphate buffer 100 mM pH 7.5 for *Lb*ADH constructs, at 30 °C in most cases. Previous studies have shown that PTDH is not inhibited by phosphate buffer.^[28] The only exception was G-B that afforded a better selectivity towards the formation of caprolactone at 24 °C, since at 30 °C a significant amount of cyclohexanol was observed due to the undesired activity of *St*GDH with cyclohexanone.

The biotransformations were monitored over time (Figure 1). The reactions catalyzed by *Bs*FDH and *Pst*PTDH constructs were mostly completed after 16 h, while the *St*GDH fusions needed 24 h to achieve full conversions. The conversion with the *Tm*CHMO fusions resulted in a linear increase of product in time, whereas the *Lb*ADH fusions showed a higher initial rate up to 70% conversion, and then it decreased until full conversion. This was probably due to the relatively high K_M value for acetophenone (2.8 mM) of *Lb*ADH and/or product inhibition.

Figure 1. Monitoring of the conversions in the self-sufficient *TmCHMO* and *LbADH*-catalyzed enzymatic biotransformations.

Assuming that the *TmCHMO* and *LbADH* enzymes had roughly the same catalytic properties (activity and K_M) for the three different fusions, the only difference concerning the kinetic performance of the fusion enzymes is due to the recycling enzyme. The amount of sacrificial substrate (10 mM glucose, 50 mM sodium formate or 20 mM sodium phosphite) was decided based on the reported K_M values of each enzyme, ensuring maximal activity (V_{max}) throughout the whole reaction. The k_{cat} values for the three recycling enzymes PTDH, FDH and GDH with $NADP^+$ are, respectively: 6.5 s^{-1} (at 25°C),^[15] 4.75 s^{-1} (at 30°C),^[24] and $\sim 3.8 \text{ s}^{-1}$ (at 35°C , derived from reported activity and temperature optimum).^[25] This was in agreement with the time course of the biotransformations (Figure 1): PTDH fusions were the fastest, while FDH fusions were the second. From this initial experiment it was clear that GDH constructs had the weakest performance. The respective biotransformations were the slowest, maybe due to the fact that the reaction with G-B worked better at 24°C while the *StGDH* has a relatively high temperature optimum. Furthermore, G-A showed also a poor expression.

Enzymatic preparations

The need for NADPH is a cost factor when considering the utilization of biocatalysts at larger scale. In this context, Torres Pazmiño et al. published interesting results using cell-free extract preparations of different self-sufficient BVMOs without external addition of the nicotinamide cofactor.^[14] This approach was also tested for our fusions (Table 2, entries 1, 4, 7, 10, 13 and 16), but resulted in good results only for G-B and P-B fusions. F-B and *LbADH* fusions had a significant lower activity. Apart from that, and still in order to

make the process economically feasible, each biotransformation using the purified enzyme was set up adding just 0.5 mM NADPH (Table 2, entries 3, 6, 9, 15 and 18). High conversions and excellent *ee* values were found, though the reactions with *Lb*ADH (entries 15 and 18) could not reach full conversion. Although the K_M value for NADPH is 40 μ M for the native *Lb*ADH, possibly the K_M of the respective fusion enzymes is a bit higher, as was observed for other PTDH-BVMO fusions.^[14] This could slow down the reaction, in particular in combination with the aforementioned slower reaction rates after 70% conversion, due to the high K_M for acetophenone (Figure 1).

Table 2. Biotransformations mediated by self-sufficient *Tm*CHMOs and *Lb*ADHs using different enzymatic preparations and different amounts of cofactor.

Entry	Fusion	Protein preparation	[NADPH] (mM)	Conv. (%) ^b	<i>ee</i> (%) ^{b,c}
1	G-B	Cell-free extract ^a	-	92	-
2	G-B	Purified	1	>99	-
3	G-B	Purified	0.5	96	-
4	F-B	Cell-free extract ^a	-	10	-
5	F-B	Purified	1	>99	-
6	F-B	Purified	0.5	99	-
7	P-B	Cell-free extract ^a	-	>99	-
8	P-B	Purified	1	>99	-
9	P-B	Purified	0.5	96	-
10	G-A	Cell-free extract ^a	-	13	81 (<i>R</i>)
11	G-A	Purified	1	>99	>99 (<i>R</i>)
12	G-A	Purified	0.5	n.d.	n.d.
13	F-A	Cell-free extract ^a	-	68	96 (<i>R</i>)
14	F-A	Purified	1	>99	>99 (<i>R</i>)
15	F-A	Purified	0.5	74	>99 (<i>R</i>)
16	P-A	Cell-free extract ^a	-	42	>99 (<i>R</i>)
17	P-A	Purified	1	>99	>99 (<i>R</i>)
18	P-A	Purified	0.5	86	>99 (<i>R</i>)

^a Cell-free extract preparations were prepared as described in the Materials and methods.

^b Measured by GC analysis. ^c Caprolactone is not an optically active molecule so no *ee* value is associated to *Tm*CHMO constructs results. For the reactions with the purified enzyme, 2 μ M of *Tm*CHMO fusion or 0.2 mg/mL (approx. 2.5 μ M) of *Lb*ADH fusion were used.

Cosolvent screening

At this point, the model reaction for each fusion was fully studied. However, in order to perform reactions with high substrate concentrations, the low water solubility of many ketone substrates must be addressed. One of the common

strategies is the employment of an organic cosolvent. For this reason, the enzyme fusions were first tested with a wide range of organic solvents (1% v/v, Figure 2), including methyl *tert*-butyl ether (MTBE), methanol (MeOH), ethanol (EtOH), 1,4-dioxane and acetonitrile (MeCN). First of all, it must be pointed out that not a single cosolvent affected the selectivity of *Lb*ADH fusion enzymes (data not shown), thus providing the enantiopure (*R*)-alcohol. MTBE and acetonitrile revealed to be the best cosolvents for all enzyme fusions, as they led to high conversions (>90%) in all cases. Methanol and ethanol showed very good results with most of the constructs, with the exception of P-B using MeOH and G-A using EtOH; in these cases the conversion values dropped down to 51% and 69%, respectively. Finally, 1,4-dioxane caused a huge drop of activity (90% decrease) with G-B and F-B fusions, and a 40% decrease for G-A.

These results are surprising, as it seems that particular combinations of enzymes can be sensitive to a cosolvent, even though other combinations containing one of those enzymes are tolerant. For instance, with 1,4-dioxane and F-B, it is not simply that *Tm*CHMO was intolerant, since P-B did give full conversion. The same was true for *Bs*FDH, as F-A was able to afford 90% conversion. Based on those two results, one would expect F-B to give high conversion as well, yet it was only 10%. In other words, it was observed that particular combinations of enzymes can be more tolerant to organic solvents than other combinations with the same enzyme. It could be that certain enzymes can transmit tolerance for a particular cosolvent when fused to its partner enzyme, e.g., PTDH in P-B for 1,4-dioxane.

Taking all these data into account, we decided to test the fusions behavior in a biphasic system using larger amounts of MTBE, as it was the best cosolvent among the tested ones (see Supporting information, Section 2.2). The *Tm*CHMO fusions were able to catalyze the Baeyer-Villiger oxidation of cyclohexanone with full conversion using up to 10% v/v of MTBE. An amount of 15% v/v of MTBE led to full conversion using *Bs*FDH and *Ps*PTDH constructs, but caused a drop in the *St*GDH fusion activity, indicating again that this fusion was less stable. On the other hand, F-A was capable to convert acetophenone into (*R*)-1-phenylethanol using up to 40% v/v of MTBE, while P-A showed a small drop of activity at 20% v/v of MTBE. Overall, it was clear that for this cosolvent *Bs*FDH was the most tolerant NADPH-regenerating enzyme.

Figure 2. Biotransformation of model substrates using different organic cosolvents (1% v/v).

Deep eutectic solvents (DES) represent a promising new generation of environmentally-friendly solvents.^[29] We evaluated the use of DES as cosolvents for the biotransformations catalyzed by the engineered fusion biocatalysts. Concentrations of 10% to 30% v/v of DES derived from choline chloride and glycerol (ChCl:Gly 1:2 mol/mol) were employed (see Supporting information, Section 2.3). G-B and F-B appeared as the most robust enzymes as they could achieve full conversion of caprolactone using up to 20% v/v of DES. Besides, F-A showed better results than P-A. As with the results with MTBE, *BsFDH* was the most tolerant cofactor regenerating enzyme in non-conventional media. Furthermore, a glucose-based DES (ChCl:glucose at 1.5:1 mol/mol) was tested both as cosolvent and cosubstrate for producing caprolactone by G-B (see Supporting information, Section 2.4). The corresponding results are very interesting as the biotransformation (without adding external glucose) led to full conversions.

Substrate screening

In order to check the synthetic versatility of the engineered fusion biocatalysts, other substrates were tested (see Figure 3). *TmCHMO* constructs were employed to catalyze the Baeyer-Villiger oxidation of substrates differing on the ring substitution and the ring size (substrates **2-4a**, Table 3). In this manner, 4-methylcyclohexanone (**2a**) was converted into (*S*)-4-methyl- ϵ -caprolactone (**2b**) in full conversion and excellent enantioselectivity (Table 3, entries 4-6). Cyclopentanone (**3a**) and cyclobutanone (**4a**) were also tested as substrates, but unfortunately, they were not converted at all (Table 3, entries 7-12). This was quite surprising, as the native *TmCHMO* has been reported to be active with both substrates.¹⁰ The only reported difference compared to substrate **1a**

was that **3a** and **4a** showed some substrate inhibition. Perhaps the fusion gives an even stronger substrate inhibition, or somehow the active site is slightly altered through having a fusion partner, disabling *Tm*CHMO to accept **3a** or **4a**.

Looking for a further exploitation of these enzyme fusions, thioanisole (**5a**) was tested as substrate (Table 3, entries 13–15). Mixtures of enantiopure methyl phenyl sulfoxide (**5b**) and the corresponding sulfone (**5c**) were obtained, as was observed in a recent study using another robust BVMO.^[30] A time-point study performed with the G-B confirmed the early formation of **5b** and its subsequent oxidation to **5c** (see Supporting Information, Section 2.5).

Figure 3. Panel of substrates studied in this chapter. Substrate **5a** for *Tm*CHMO can be oxidized to phenyl sulfoxide (**5b**) or to (methylsulfonyl)benzene (**5c**).

Keeping in mind previous studies on ketone reductions with ADHs,^[23] some acetophenone derivatives were chosen as alternative substrates for the *Lb*ADH fusions (substrates **7–12a**, Table 3). The lengthening of chain R^2 from a methyl group (acetophenone, **6a**; Table 3, entries 16 and 17) to an ethyl group (propiophenone, **7a**; Table 3, entries 18 and 19) caused a loss of activity, although the stereoselectivity remained perfect. As expected,^[23] when bioreductions were set up using butyrophenone (**8a**; Table 3, entries 20 and 21) or 2-methoxy-1-phenylethanone (**9a**; Table 3, entries 22 and 23) as substrates, a dramatic loss of activity was observed. At this point, we decided to evaluate the influence of the aromatic pattern substitution at different positions. For this purpose, we studied various chlorine-substituted acetophenone derivatives (**10–12a**). *Bs*FDH fusion behaved reasonably well with *p*-chloroacetophenone (**10a**; Table 3, entry 24) and *m*-chloroacetophenone (**11a**; Table 3, entry 26) as substrates. On the contrary, although *Ps*PTDH construct maintained excellent selectivity, it afforded low conversions for the same substrates (Table 3, entries 25 and 27). Finally, the biotransformation of *o*-chloroacetophenone (**12a**)

catalyzed by the *Bs*FDH fusion was set up leading to low conversions (Table 3, entry 28).

Table 3. Substrate scope of the engineered self-sufficient TmCHMO and LbADH fusion biocatalysts.

Entry	Fusion	Substrate	Conv. (%) ^a	ee (%) ^{a,b}
1	G-B	1a	>99	-
2	F-B	1a	>99	-
3	P-B	1a	>99	-
4	G-B	2a	>99	>99 (<i>S</i>)
5	F-B	2a	>99	>99 (<i>S</i>)
6	P-B	2a	>99	>99 (<i>S</i>)
7	G-B	3a	<1	-
8	F-B	3a	<1	-
9	P-B	3a	<1	-
10	G-B	4a	<1	-
11	F-B	4a	<1	-
12	P-B	4a	<1	-
13	G-B	5a	75% 5b + 21% 5c	>99 [(<i>R</i>)- 5b]
14	F-B	5a	26% 5b + 74% 5c	>99 [(<i>R</i>)- 5b]
15	P-B	5a	51% 5b + 47% 5c	>99 [(<i>R</i>)- 5b]
16	F-A	6a	>99	>99 (<i>R</i>)
17	P-A	6a	>99	>99 (<i>R</i>)
18	F-A	7a	85	>99 (<i>R</i>)
19	P-A	7a	55	>99 (<i>R</i>)
20	F-A	8a	<1	n.d.
21	P-A	8a	<1	n.d.
22	F-A	9a	10	>99 (<i>S</i>) ^c
23	P-A	9a	2	n.d.
24	F-A	10a	90	>99 (<i>R</i>)
25	P-A	10a	10	>99 (<i>R</i>)
26	F-A	11a	88	>99 (<i>R</i>)
27	P-A	11a	15	>99 (<i>R</i>)
28	F-A	12a	16	>99 (<i>R</i>)

^a Determined by GC analysis. ^b Enantiomeric excess values of product **5b** were determined by chiral HPLC analysis. ^c Change in Cahn-Ingold-Prelog priority. n.d. not determined.

Scaled-up biotransformations

At this point, we aimed to show the applicability of the enzyme fusions by scaling-up some selected biotransformations. Before that, the effect of the substrate concentration and the amount of biocatalyst were studied (Supporting information, Section 2.6) in order to identify the best conditions for each preparation. Once optimized, several semi-preparative biotransformations were set up using the best conditions for each fusion

without observing the formation of any by-products. Hence, for P-B 25 mg of cyclohexanone (10 mM) were transformed into caprolactone at pH 8.5 with full conversion but in moderate isolated yield (51%) after purification by extraction and centrifugation of the combined organic phases. Suspecting that part of the caprolactone had been hydrolyzed, the biotransformation was set up using a phosphate buffer, pH 7.5. In this case, it was possible to obtain the caprolactone in high isolated yield (90%). Afterwards, the same Baeyer-Villiger oxidation were performed using F-B and G-B. Again, full conversions and high isolated yields (89% and 80%, respectively) were attained for caprolactone after an extraction protocol. The *Lb*ADH constructs were also tested in semi-preparative biotransformations. However, F-A revealed worse results than those obtained at small scale. Using 15 mg of acetophenone (20 mM), it was transformed into (*R*)-1-phenylethanol in 65% conversion. After separation of the remaining ketone, the desired enantiopure alcohol was obtained in 51% isolated yield. On the other hand, the result obtained in the same bioreduction catalyzed by P-A was as good as that obtained at small scale, and the corresponding alcohol was produced in full conversion, with excellent enantioselectivity and high isolated yield (85%).

Conclusions

In this study we have developed and investigated three NADPH-regenerating fusion partners with two different enzyme systems: a BVMO and an ADH. With the exception of one fusion construct (*St*GDH with *Lb*ADH), all fusion enzymes resulted in good soluble expression as well as fully functional as self-sufficient biocatalysts. The fusion biocatalysts displayed tolerance to various organic cosolvents, including DES. From the different fusion partners, FDH was the most tolerant to organic solvents, fully converting cyclohexanone for the BVMO fusion at 15% v/v MTBE, and acetophenone for the ADH fusion even at 40% v/v MTBE. We also demonstrate that the fusion enzymes can be used as enantioselective biocatalysts for a large variety of reactions by retaining almost all catalytic characteristics of the native non-fused enzymes. We demonstrated the applicability of three different NADPH regenerating fusions for these two enzyme systems, which broadens the choice of regenerating enzymes for future applications of these systems.

Materials and methods

General materials and methods

Oligonucleotide primers were ordered from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). T4 ligase and restriction enzyme BsaI were ordered from New England Biolabs. The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies. *Escherichia coli* NEB® 10-beta (New England Biolabs) chemically competent cells were used as host for cloning of the recombinant plasmids, and as host for protein expression. All acetophenones, cyclohexanones, cyclopentanone, cyclobutanone and sulfides were purchased from Sigma-Aldrich-Fluka. NADPH as enzyme cofactor and all the other chemical reagents were obtained with the highest quality available from Sigma-Aldrich-Fluka (Steinheim, Germany). NMR spectra were recorded on a Bruker AV300 MHz spectrometer (Bruker Co., Faellanden, Switzerland). All chemical shifts (δ) are given in parts per million (ppm) and referenced to the residual solvent signal as internal standard. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) analyses were performed for conversion and enantiomeric excess measurements. GC analyses were performed on an Agilent HP6820 GC chromatograph equipped with an FID detector. HPLC analyses were carried out in a Hewlett Packard 1100 chromatograph UV detector at 210 nm. For more details, see Section 1 in the Supporting Information. Thin-layer chromatography (TLC) was conducted with Merck Silica Gel 60 F254 precoated plates and visualized with UV and potassium permanganate stain. Column chromatography was performed using Merck Silica Gel 60 (230-400 mesh).

Cloning and purification

Cloning of fusion constructs: Three fusion constructs with the *lbadh* gene were cloned into a pBAD vector through the Golden Gate method. The pBAD vector contains a region coding for an N-terminal His-tag (6xHis) upstream from the first BsaI restriction site, an AraC promoter, and an ampicillin-resistance gene (*bla*). Primers were designed to have a non-overlapping region coding for the BsaI restriction site (GGTCTCNNNNNN), such that the vector and PCR product have a four base-pair overlap after digestion with BsaI. These primers were used for PCR to amplify the *lbadh* gene (accession: Q84EX5) from an in-house plasmid, *fdh* (accession: EU825923.1) and *gdh* (accession: Q96ZY7) synthetic codon-optimized genes were ordered from Genscript (NJ, USA), and *ptdh* x12 mutant was also cloned from an in-house plasmid. Primers were designed such that the *lbadh* would be the second gene of the fusion; at the C-terminal side of the fusion enzyme. In addition to the BsaI sites, an additional 'linker' region was added to the reverse primers of the first gene and the forward primer of the second gene of a construct. These introduced 'linker' regions together code for a short peptide linker (SGSAAG), after ligation of the PCR products. The fusion constructs were produced by incubating together: two PCR products (*lbadh* gene and either *fdh*, *gdh* or *ptdh*), pBAD vector containing BsaI sites, BsaI restriction enzyme, T4 ligase, ligation buffer, and sterile miliQ water (total volume of 20 μ L). The incubation temperature alternated between 16 °C (for 5 minutes) and 37 °C (for 10 minutes) for 30 cycles, then was set to 55 °C for 10 minutes, and finally to 80 °C for 20 minutes to inactivate the enzymes. To transform host cells with the fusion constructs, 3 μ L of the Golden Gate reaction was added to chemically competent *E. coli* cells, and a heat shock was applied at 42 °C for 30 seconds. After overnight growth on a lysogeny broth (LB) agar plate with ampicillin, colonies were picked, grown in liquid LB with appropriate antibiotic, and the plasmids were isolated and sent for sequencing (GATC, Germany) to confirm correct ligation of the genes. For the three constructs with *TmCHMO*, traditional restriction-digestion cloning was used.

All enzymes were expressed using NEB 10- β *Escherichia coli* cells. The freshly-prepared strains were cultivated in TB medium supplemented with 50 μ g/mL ampicillin at 37 °C. Protein expression was induced by adding L-arabinose (0.02% w/v) when A600 reached 0.5. G-B and F-B were incubated overnight at 30 °C, P-A and P-B were incubated at 24 °C for 48 hours and G-A and F-A were incubated at 17 °C for 72 hours. After this time, the cells were harvested by centrifugation. The cells were resuspended in 20 mM phosphate buffer pH 7.5 (*Tm*CHMO fusions were implemented with FAD) and lysed in an iced bath by ultrasonication (cycles of 20s on/20s off for 7 min). After centrifugation, the supernatant was used either for protein purification using Ni-sepharose material or as cell-free extract preparations for biotransformations. For lyophilization purposes, the cells obtained after expression were resuspended in the minimum amount of 20 mM phosphate buffer pH 7.5 and lyophilized using an Alpha 2-4 DLplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

Conversion of cyclohexanone and derivative, cyclopentanone, cyclobutanone and thioanisole using self-sufficient *Tm*CHMOs

Purified self-sufficient *Tm*CHMOs (2 μ M or 4 μ M) were employed for biotransformation of cyclohexanone (5-25 mM), 4-methylcyclohexanone (5 mM), cyclopentanone (5 mM), cyclobutanone (5 mM) and thioanisole (5 mM) at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm. All reactions contained 1 or 0.5 mmol NADPH, the co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 100 mM phosphate buffer pH 8.5. Additionally, G-B fusion needs the external addition of magnesium chloride (1 mM). In some cases, a cosolvent (1% v/v 1,4-dioxane, 1% v/v acetonitrile, 1% v/v ethanol, 1% v/v methanol, 1-50% v/v tert-butyl methyl ether) or ChCl:Gly (1:2 mol/mol) was added. In any case, the final volume of the reaction was adjusted up to 0.5 mL in a 2 mL eppendorf tube.

For cell-free extract biotransformations, 5 mM cyclohexanone was used as substrate and the reaction was implemented with the corresponding co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 1 mM of MgCl₂·6H₂O when G-B was employed. Cell-free extract preparations were used at 8 mg/mL concentration and the final volume was adjusted with 100 mM phosphate buffer pH 8.5 up to a 0.5 mL in a 2 mL eppendorf tube. The reactions were incubated at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm for 24 hours.

When using lyophilized preparations (10 mg), cyclohexanone (5 mM) was used as substrate and reactions were implemented with NADPH (1 mM), the corresponding co-substrate (10 mM glucose for G-B, 50 mM sodium formate for F-B and 20 mM sodium phosphite for P-B) and 1 mM of MgCl₂·6H₂O when G-B was employed. The final volume was adjusted up to 0.5 mL with 100 mM phosphate buffer pH 8.5 in a 2 mL eppendorf tube and the reactions were incubated at 30 °C (F-B and P-B) or 24 °C (G-B) and 250 rpm for 24 hours.

After incubation, all reactions were extracted with ethyl acetate (2 x 0.2 mL) and the organic layers were combined and dried over anhydrous Na₂SO₄. The results were analyzed using GC and/or HPLC.

Preparative biotransformation of cyclohexanone into caprolactone using *Tm*CHMO fusions

G-B (2 μ M) was employed to convert 30 mg of cyclohexanone (5 mM) into caprolactone. The reaction media was composed of 100 mM phosphate buffer pH 8.5 implemented with NADPH (1 mM), glucose (10 mM) and MgCl₂·6H₂O (1 mM). The reaction was set up in a 50 mL Falcon tube and incubated at 24 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with ethyl acetate (3 x 20 mL), the organic layers separated by centrifugation (5 min, 4900 rpm),

combined and finally dried over anhydrous Na₂SO₄. Conversion was determined by GC. Caprolactone was obtained in 80% isolated yield (>99% conv.).

F-B (4 μ M) was used to convert 30 mg of cyclohexanone (15 mM) into caprolactone in 100 mM phosphate buffer pH 8.5 in a 50 mL Falcon tube. The reaction was implemented with NADPH (1 mM) and sodium formate (50 mM) and incubated at 30 °C for 24 hours. Afterwards, the mixture was extracted with ethyl acetate (3 x 20 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous Na₂SO₄. Conversion was determined by GC. Caprolactone was obtained in 89% isolated yield (>99% conv.).

P-B (2 μ M) was employed to convert 30 mg of cyclohexanone (10 mM) into caprolactone. The reaction media (100 mM phosphate buffer pH 7.5) was implemented with NADPH (1 mM) and sodium phosphite (20 mM) and the reaction was incubated at 30 °C for 24 hours. After this time, the mixture was extracted with ethyl acetate (3 x 20 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous Na₂SO₄. Conversion was determined by GC. Caprolactone was obtained in 90% isolated yield (>99% conv.).

General procedures for conversion of acetophenone and derivatives using self-sufficient *Lb*ADHs

Purified self-sufficient *Lb*ADHs (0.2 mg/mL or 4 mg/mL) were employed for biotransformation of acetophenone (5-25 mM) and derivatives (5 mM) at 30 °C and 250 rpm. All reactions contained 1 or 0.5 mmol NADPH, the co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A), MgCl₂·6H₂O (1 mM) and 100 mM phosphate buffer pH 7.5. In some cases, a cosolvent (1% v/v 1,4-dioxane, 1% v/v acetonitrile, 1% v/v ethanol, 1% v/v methanol, 1-50% v/v tert-butyl methyl ether), ChCl:Gly (1:2 mol/mol) or ChCl:Glu (1.5:1 mol/mol) was added. In any case, the final volume of the reaction was adjusted up to 0.5 mL in a 1.5 mL eppendorf tube.

For cell-free extract biotransformations, 5 mM acetophenone was used as substrate and the reaction was implemented with the corresponding co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A) and MgCl₂·6H₂O (1 mM). Cell-free extract preparations were used at 8 mg/mL concentration and the final volume was adjusted with 100 mM phosphate buffer pH 7.5 up to a 0.5 mL in a 1.5 mL eppendorf tube. The reactions were incubated at 30 °C and 250 rpm for 24 hours.

When using lyophilized preparations (10 mg), acetophenone (5 mM) was used as substrate and reactions were implemented with NADPH (1 mM), the corresponding co-substrate (10 mM glucose for G-A, 50 mM sodium formate for F-A and 20 mM sodium phosphite for P-A) and MgCl₂·6H₂O (1 mM). The final volume was adjusted up to 0.5 mL with 100 mM phosphate buffer pH 7.5 in a 1.5 mL eppendorf tube and the reactions were incubated at 30 °C and 250 rpm for 24 hours. After incubation, all reactions were extracted with ethyl acetate (2 x 0.2 mL) and the organic layers were combined and dried over anhydrous Na₂SO₄. The results were analyzed using GC and/or HPLC.

Preparative biotransformation of acetophenone into (R)-1-phenylethanol using *Lb*ADH fusions

F-A (0.4 mg/mL) was used to convert 30 mg of acetophenone (20 mM) into (R)-1-phenylethanol. The reaction media contained NADPH (1 mM), sodium formate (50 mM), MgCl₂·6H₂O (1 mM) and 100 mM phosphate buffer pH 7.5. This mixture was incubated at 30 °C for 24 hours and, afterwards, it was extracted with ethyl acetate (3 x 20 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous Na₂SO₄.

Conversion and enantiomeric excess were determined by GC. (R)-1-phenylethanol was obtained in 51% isolated yield (65% conv.) after purification by column chromatography (Hex: EtOAc 3:1).

P-A (0.2 mg/mL) was employed to convert 30 mg of acetophenone (10 mM) into (R)-1-phenylethanol. The reaction media (100 mM phosphate buffer pH 8.5) was implemented with NADPH (1 mM), sodium phosphite (20 mM) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 mM) and the reaction was incubated at 30 °C for 24 hours. After this time, the mixture was extracted with ethyl acetate (3 x 20 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over anhydrous Na_2SO_4 . Conversion and enantiomeric excess were determined by GC. (R)-1-phenylethanol was obtained in 85% isolated yield (>99% conv.).

Acknowledgements

The research for this work has received funding from the European Union (EU) project ROBOX (grant agreement n°635734) under EU's Horizon 2020 Program Research and Innovation actions H2020-LEIT BIO-2014-1. The views and opinions expressed in this article are only those of the authors and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein. Financial support from the Spanish Ministry of Economy and Competitiveness (MEC, Projects CTQ2013-44153-P and CTQ2016-75752-R) is gratefully acknowledged. Á.M.-I thanks MEC for a predoctoral fellowship inside the FPI program.

Supporting information

For supporting information, see pages 139-145.

References

- [1] Pollard DJ, Woodley JM. *Trends Biotechnol.* 2007; 25: 66–73.
- [2] Sun H, Zhang H, Ang EL, Zhao H. *Bioorg Med Chem.* 2018; 26: 1275–1284.
- [3] Sheldon RA, Woodley JM. *Chem Rev.* 2018; 118: 801–838.
- [4] Albarrán-Velo J, González-Martínez D, Gotor-Fernández V. *Biocatal Biotransf.* 2018; 36: 102–130.
- [5] Hollmann F, Arends IWCE, Holtmann D. *Green Chem.* 2011; 13: 2285–2313.
- [6] Delgove MAF, Fürst MJLJ, Fraaije MW, Bernaerts KV, De Wildeman SMA. *ChemBioChem.* 2018; 19: 354–360.
- [7] Delgove MAF, Elford MT, Bernaerts KV, De Wildeman SMA. *J Chem Technol Biotechnol.* 2018; 93: 2131–2140.
- [8] Delgove MAF, Elford MT, Bernaerts KV, De Wildeman SMA. *Org Process Res Dev.* 2018; 22: 803–812.
- [9] Messiha HL, Ahmed ST, Karuppiah V, Suardiaz R, Ascue Avalos GA, Fey N, Yeates S, Toogood HS, Mulholland AJ, Scrutton NS. *Biochemistry.* 2018; 57: 1997–2008.
- [10] Romero E, Castellanos JRG, Mattevi A, Fraaije MW. *Angew Chem - Int Ed.* 2016; 55: 15852–15855.
- [11] Wu H, Tian C, Song X, Liu C, Yang D, Jiang Z. *Green Chem.* 2013; 15: 1773–1789.
- [12] Hummel W, Gröger H. *J Biotechnol.* 2014; 191: 22–31.
- [13] **Chapter 1:** Aalbers FS, Fraaije MW. *ChemBioChem.* 2018. doi: 10.1002/cbic.201800394.
- [14] Torres Pazmiño DE, Snajdrova R, Baas BJ, Ghobrial M, Mihovilovic MD, Fraaije MW. *Angew Chem - Int Ed.* 2008; 47: 2275–2278.
- [15] Torres Pazmiño DE, Riebel A, de Lange J, Rudroff F, Mihovilovic MD, Fraaije MW. *ChemBioChem.* 2009; 10: 2595–2598.
- [16] Zhang Y, Wang Y, Wang S, Fang B. *Eng Life Sci.* 2017; 17: 989–996.
- [17] Sun T, Li B, Nie Y, Wang D, Xu Y. *Bioresour Bioprocess.* 2017; 4: 21.

- [18] Watanabe H, Hirakawa H, Nagamune T. *ChemCatChem*. 2013; 5: 3835–3840.
- [19] Beyer N, Kulig JK, Bartsch A, Hayes MA, Janssen DB, Fraaije MW. *Appl Microbiol Biotechnol*. 2016; 101: 2319–2331.
- [20] Beyer N, Kulig JK, Fraaije MW, Hayes MA, Janssen DB. *ChemBioChem*. 2018; 19: 326–337.
- [21] Niefind K, Müller J, Riebel B, Hummel W, Schomburg D. *J Mol Biol*. 2003; 327: 317–328.
- [22] Leuchs S, Greiner L. *Chem Biochem Eng Q*. 2011; 25: 267–281.
- [23] Rodríguez C, Borzęcka W, Sattler JH, Kroutil W, Lavandera I, Gotor V. *Org Biomol Chem*. 2014; 12: 673–681.
- [24] Hatrongjit R, Packdibamrung K. *Enzyme Microb Technol*. 2010; 46: 557–561.
- [25] Ohshima T, Ito Y, Sakuraba H, Goda S, Kawarabayasi Y. *J Mol Catal B Enzym*. 2003; 23: 281–289.
- [26] Vrtis JM, White AK, Metcalf WW, van der Donk WA. *Angew Chem*. 2002; 114: 3391–3393.
- [27] Johannes TW, Woodyer RD, Zhao H. *Appl Environ Microbiol*. 2005; 71: 5728–5734.
- [28] Costas AMG, White AK, Metcalf WW. *J Bio Chem*. 2001; 20: 17429–17436.
- [29] Müller CR, Lavandera I, Gotor-Fernández V, Domínguez de María P. *ChemCatChem*. 2015; 7: 2654–2659.
- [30] de Gonzalo G, Fürst MJLJ, Fraaije MW. *Catalysts*. 2017; 7: 288.

Section 3

Stability Engineering

Chapter 6: Approaching boiling-point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering

Friso S. Aalbers, Maximilian J. L. J. Fürst, Stefano Rovida, Milos Trajkovic, J. Rubén Gómez Castellanos, Sebastian Bartsch, Andreas Vogel, Andrea Mattevi, and Marco W. Fraaije

This chapter is based on a published article: Aalbers, F. S., Fürst, M. J. L. J., Rovida, S., Trajkovic, M., Castellanos, J. R. G., Bartsch, S., Vogel, A., Mattevi, A. & Fraaije, M. W. (2020). Approaching boiling point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering. *Elife*, 9, e54639. <https://doi.org/10.7554/eLife.54639>

This chapter is not part of another thesis.

Abstract

Enzyme instability is an important limitation for the investigation and application of enzymes. Therefore, methods to rapidly and effectively improve enzyme stability are highly appealing. In this study we applied a computational method (FRESCO) to guide the engineering of an alcohol dehydrogenase. Of the 177 selected mutations, 25 mutations brought about a significant increase in apparent melting temperature ($\Delta T_m \geq +3$ °C). By combining mutations, a 10-fold mutant was generated with a T_m of 94 °C (+51 °C relative to wild type), almost reaching water's boiling point, and the highest increase with FRESCO to date. The 10-fold mutant's structure was elucidated, which enabled the identification of an activity-impairing mutation. After reverting this mutation, the enzyme showed no loss in activity compared to wild type, while displaying a T_m of 88 °C (+45 °C relative to wild type). This work demonstrates the value of enzyme stabilization through computational library design.

Introduction

The oxidation of alcohols and reduction of ketones in nature is primarily governed by alcohol dehydrogenases (ADHs) (EC 1.1.1.1) (Kavanagh et al., 2008; Oppermann et al., 2003). There is a vast diversity of ADHs that can transform a large range of substrates, often with high regio- and enantioselectivity. Most ADHs employ nicotinamide cofactors for catalysis. In particular, ketone reductions catalysed by ADHs are used in industry to synthesize chiral alcohols (Nealon et al., 2015; Zheng et al., 2017). Although ADHs have been mainly applied for ketone reductions, recent studies show promising results as biocatalysts for selective oxidations (Solé et al., 2019).

NAD(P)-dependent ADHs catalyse the transfer of a hydride from the reduced nicotinamide coenzyme to reduce ketones to alcohols, or the transfer of a hydride from alcohols to reduce NAD(P)⁺. A large subset of alcohol dehydrogenases belongs to the family of short-chain reductases/dehydrogenases (SDRs) (Kavanagh et al., 2008). Enzymes from this family encompass around 250 amino acid-long sequences, typically form tetramers, use NAD(H) or NADP(H) as cofactor, and mainly act on secondary alcohols and ketones. The characteristic catalytic triad in SDRs is a combination of Ser-Tyr-Lys (Bhatia et al., 2015). These residues are located close to the C4 of the nicotinamide ring of the cofactor, at which the hydride is located or is transferred to. The catalytic triad, along with several residues in its vicinity, form a proton-relay system that either assists in the deprotonation of an alcohol to initiate alcohol oxidation, or facilitates the protonation of a ketone substrate with concurrent hydride transfer, resulting in ketone reduction.

Most enzymes in nature have evolved to catalyse reactions within cells, in crowded, mild and aqueous conditions, and only for limited periods of time (DePristo et al., 2005; Goldenzweig and Fleishman, 2018). Moreover, enzymes that can be easily degraded after some time enable greater metabolic adaptability for the organism, for instance when a local resource depletes, or a signaling event occurs (DePristo et al., 2005; Parsell and Sauer, 1989). Therefore, many enzymes are only moderately stable. When considering enzymes for applications, this can lead to various difficulties in heterologous expression, degradation upon isolation, and poor biocatalyst performance. Enzymes that have the potential to be applied as biocatalyst are often insufficiently stable (Bommarius and Paye, 2013; Woodley, 2019). One such enzyme is ADHA, an alcohol dehydrogenase from *Candida magnoliae* DSMZ 70638. This enzyme was isolated for its ability to reduce several ketones with excellent enantioselectivities (Table 1). These catalytic features make ADHA

attractive for industrial applications. Although heterologous expression of ADHA is good, the melting temperature of ADHA is moderate, with an apparent T_m of 43 °C. If stability of ADHA could be improved, it would become a very attractive biocatalyst for selective oxidations or reductions.

Table 1 – Initial measurements of ADHA WT activity and selectivity with several ketones.

Substrate (concentration)	k_{obs}^* (U/mg)	Enantiomeric excess product (<i>ee</i>)
Ethyl acetoacetate (100 mM)	4.1	n.d.
Cyclohexanone (50 mM)	3.2	n.a.
Ethyl 4-chloro-3-oxobutanoate (COBA) (50 mM)	0.3	> 99% (<i>R</i>)
4-Chloroacetophenone (4-CAP) (50 mM)	2.4	97.2% (<i>S</i>)

* k_{obs} values are averages based on 2–3 replicates and for each average the error was smaller than 5%.

Several studies over the past two decades have developed strategies to stabilize proteins and enzymes. A subset of these strategies specifically targeted the charge of the protein or enzyme surface or interface (Björk et al., 2004; Gribenko et al., 2009). More recently, the field has mostly aimed at the development of computational methods to predict mutations that improve the stability of proteins (Malakauskas and Mayo, 1998; Murphy et al., 2012; Shah et al., 2007) and/or enzymes (Bednar et al., 2015; Borgo and Havranek, 2012; Korkegian et al., 2005; Moore et al., 2017). Considering the large improvements in stability that were found using these methods with minimal screening work in the lab, such computational approaches are highly appealing.

Our goal was to improve the stability of ADHA through enzyme engineering, guided by a computational method. The requirement for this endeavour was to obtain a crystal structure of the enzyme. The structure is needed as input for our in-house developed method: ‘framework for rapid enzyme stabilization by computational libraries’ (FRESCO) (Wijma et al., 2018). This computational protocol incorporates two algorithms to calculate folding free energy (ΔG^{Fold} , expressed in kJ mol⁻¹), and compares these calculations ($\Delta\Delta G^{Fold}$) between the wild-type structure and variants with a single mutation. The calculations are performed for all possible single mutations. The variants with the best scores are simulated with molecular dynamics, to inspect the flexibility and conformational stability. In the last round of enzyme engineering, mutations that improve the stability are combined to obtain a final robust variant. The main motivation to apply

FRESCO is that it is relatively fast, because it greatly reduces the required screening effort compared to other enzyme engineering approaches.

Here, we present the results of the application of FRESCO on ADHA. This has led to a variant which has been mutated at 10 positions and displays a drastically increased thermostability ($T_m = 94.5\text{ }^{\circ}\text{C}$, $\Delta T_m = +51\text{ }^{\circ}\text{C}$). However, it was found that this engineered ADHA displayed very little activity. Elucidation of its crystal structure disclosed the structural basis for the inactivation: one particular mutation, in combination with other introduced mutations, resulted in a reorientation of a loop region through which binding of the nicotinamide cofactor is hampered. By reverting this mutation, a highly stable ($T_m = 88\text{ }^{\circ}\text{C}$) and active ADHA variant was obtained, demonstrating the power of using computationally predicted mutations in combination with structural analysis to engineer robust enzymes.

Results

Characterization of ADHA

Characterization of ADHA immediately indicated a high reductive activity towards a broad panel of ketones (Table 1) with high enantioselectivity ($> 97\%$ *ee*). This result illustrated that the enzyme could be applied for the synthesis of various chiral alcohols. Therefore, we concluded that ADHA has great potential for the application as a biocatalyst. However, we soon realized that most of the enzyme activity is lost in reactions of 8 h or longer, even at moderate temperatures such as $25\text{ }^{\circ}\text{C}$ or $30\text{ }^{\circ}\text{C}$ (data not shown). Therefore, we decided to employ FRESCO to generate a more robust variant.

Crystal structure of wild-type ADHA

A prerequisite for FRESCO is the availability of a crystal structure of the target protein. Therefore, already in the early stages of the project, the untagged ADHA was tested for crystallization trials. The purified enzyme crystallized in many conditions and the enzyme three-dimensional structure was solved in the apo and NADP⁺-bound state at $2.0\text{ }\text{\AA}$ and $1.6\text{ }\text{\AA}$ resolution, respectively (Figure 1, Supplementary file 1A). ADHA is a homotetramer as typically observed in most SDRs (Kavanagh et al., 2008). A search for homologous structures in the Protein Data Bank showed that the closest structural homologue is another alcohol dehydrogenase from *Candida magnoliae* (PDB entry 5MLN [Tavanti et al., 2017]; root-mean-square deviation of $1.0\text{ }\text{\AA}$ for 238 C α atoms with 64% sequence identity; Figure 1). The structure of ADHA bound to NADP⁺ unveils, apart from a disordered nicotinamide moiety, a well-defined

catalytic pocket with a size suited for small cyclic compounds such as cyclohexanol, a good substrate of the enzyme.

Figure 1 - The crystal structure of ADHA. The figure highlights the final weighted 2Fo-Fc map for NADP⁺ bound to a subunit of the wild-type ADHA (subunit A, contour level 1.2 σ). The nicotinamide moiety of the cofactor is disordered and was not included in the final model.

FRESCO computational predictions

The obtained crystal structure of the wild-type tetrameric ADHA allowed us to predict stabilizing mutations through a structure-based computational approach using FRESCO (Wijma et al., 2018). A detailed description of the procedure is available as a step-by-step protocol suitable for biochemists with minimal computational experience and all the scripts are deposited online (Wijma et al., 2018). Licenses for the ΔG^{fold} -determining software components (Rosetta and FoldX) are free for academic purposes, whereas our preferred software for modelling and MD simulations, YASARA, needs to be purchased. Depending on the size of the protein, a computer cluster may be necessary for the calculation-intensive parts of the predictions. After FRESCO's *in silico* screening, a final selection of mutants is expressed, purified and tested for stability *in vitro*. The experimental part has also been described in great detail (Fürst et al., 2018) and requires readily purchasable consumables and equipment commonly available in most labs.

Because the energy prediction algorithms Rosetta and FoldX do not accept non-proteinogenic residues and because the ligand-free enzyme represents the

physiologically more relevant form, we used the crystal structure of ADHA without NADP⁺ for the stabilizing mutation predictions. While FRESCO usually excludes residues of ligand-binding sites, we decided to include such residues for predicting stabilizing mutations. Because the enzyme is relatively small, we could still easily discard activity-impairing mutations in the experimental phase. We did not include the residues that form the canonical catalytic triad (T148, Y161, and K165), which are highly conserved residues and essential for enzyme activity. Curiously, unlike ADHA, the majority of SDRs have a serine at the position of T148. In fact, the catalytic triad of SDRs is usually designated as SYK (serine, tyrosine, lysine). Clearly, a catalytic threonine can also support redox catalysis in an SDR-type alcohol dehydrogenase, and such an alternative catalytic triad has also been observed before (King et al., 2007). The calculations for the 4503 possible mutations were ranked and mutations with a $\Delta\Delta G^{\text{fold}} \leq -5 \text{ kJ mol}^{-1}$ were briefly simulated with molecular dynamics (MD) simulations to probe the flexibility of the variant. The resulting 478 computer-generated mutant structures were visually inspected to remove chemically undesirable mutations (clashes, exposed hydrophobic residues, loss of hydrogen bonds), and highly flexible mutants (based on the short MDs) (Wijma et al., 2018). In addition, several positions (> 20) had more than four suggested beneficial mutations. In those cases, we chose two or three distinct suggested mutations. Finally, we selected 177 single mutations for experimental screening.

In vitro analysis of mutant library

The mutant library was prepared using the QuikChange method following the published protocol (Fürst et al., 2018). After growth of the cells harbouring the genes with each mutation and inducing protein expression, the cells were lysed and the His-tagged ADHA variants were purified by affinity chromatography. The purified samples were desalted and the apparent T_m was measured in an RT-PCR machine in duplicate. 26 mutants repeatedly did not give a clear signal, which may have been caused by insufficient expression. Of the 151 mutants for which a melting temperature could be measured, 52 had a similar T_m when compared to wild-type ADHA ($-1 \leq \Delta T_m \leq +1 \text{ }^\circ\text{C}$), 43 were destabilizing ($\Delta T_m < -1 \text{ }^\circ\text{C}$), and 56 were stabilizing ($\Delta T_m > +1 \text{ }^\circ\text{C}$) (Figure 2). Of the 56 stabilizing mutants, 25 had a $\Delta T_m \geq +3 \text{ }^\circ\text{C}$. However, when measuring activity, 11 of these 25 mutants displayed very poor or no activity (Supplementary file 1B). For the next phase of the engineering project, we focused on 10 mutations that retained most of the activity and resulted in a significantly higher T_m (labelled red in Figure 2).

Figure 2 — Difference in T_m for 151 FRESCO-predicted ADHA mutants. The average of two measurements is given and the standard error. The T_m of wild-type ADHA is 43 °C (set as 0). The 10 stabilizing mutations with a red bar were combined. Melting curves of wild type and the final mutant (M9*) are depicted in Figure 2—figure supplement 1.

Figure 2—figure supplement 1 — Melting curves. Apparent melting temperatures of wild type (in grey) and M9* variant. RFU = relative fluorescence units. M9* displays a first melting peak at 79 °C, second peak at 88 °C. One curve is shown from three technical replicates.

Combining mutations

The most stabilizing mutations were ranked according to their thermostability and combined by consecutively introducing them one by one (Table 2). The combined mutants (named MX, with X = the number of mutations) were made using the plasmid of the previous mutant (e.g. M4 was made from the plasmid of M3). Only in the case of the V193L and T194V mutations, the mutations were introduced in one step as these residues were next to each other (going from M7 to M8). Interestingly, the combined mutants showed superior expression compared to wild-type ADHA, and could be expressed at higher temperatures for shorter periods of time, such as 37 °C overnight (Supplementary file 2A). Beyond M4, the enzyme gave two melting points in the ThermoFluor measurements (indicated by the values in parentheses, Table 2), of which the first curve appears 10–11 °C before the second. The first curve is typically significantly lower in intensity compared with the second (major) curve (Figure 2—figure supplement 1) and may reflect a mild unfolding event such as the dissociation of the tetrameric enzyme into (active) dimers.

Gratifyingly, we observed that the mutations were additive, as they incrementally increased the enzyme thermostability. The additive effect is not perfect, as the theoretical maximal additive effect would be 56.5 °C, while M9 displays an improvement of 51.5 °C. This may indicate that some of the mutations are part of the same structural “soft spot” that triggers early unfolding events in the wild type. Still, an increase of > 50 °C by introducing 10 mutations was highly gratifying. Adding more hits (S25A, G41N, G75L) from the library beyond the 10th mutation did not increase the apparent melting temperature any further. This prompted us to investigate the M9 mutant in more detail. We found that the M9 enzyme retained catalytic activities, though with a 100-fold higher K_M for NADP⁺ (1.0 mM, compared to 9.5 μM) and a 4-fold decreased k_{cat} (0.25 s⁻¹, compared to 1.1 s⁻¹) (Figure 3). We suspected that this would be the result of a shift in optimal temperature for activity, due to the presumed higher rigidity of this highly stabilized mutant. But even at higher temperatures (≥ 40 °C), the activity of the M9 mutant did not drastically change. Clearly, the multiple mutations did result in a highly stable but catalytically impaired enzyme and it was essential to elucidate the specific structural cause of this.

Table 2 – ADHA mutants with highest ΔT_m and retained activity. In parentheses the temperature of the first unfolding event (minor peak) is given. Michaelis-Menten kinetics for wild type and M9 are shown in Figure 3.

Single mutants	k_{obs}^* (U/mg)	$T_{m,app}$ (°C)	ΔT_m (°C)
Wild type	0.6	43.0	-
L150F	0.5	57.25 $\pm$ 0.25	14.0 $\pm$ 0.25
G149A	0.4	52.5 $\pm$ 0	9.5 $\pm$ 0
M157K	0.4	51.0 $\pm$ 0	8.0 $\pm$ 0
S197E	0.5	49 $\pm$ 1	7.5 $\pm$ 1
V238L	0.4	50.0 $\pm$ 0	7.0 $\pm$ 0
G159A	1.2	48.5 $\pm$ 0	5.5 $\pm$ 0
N212R	1.1	48.0 $\pm$ 0	5.0 $\pm$ 0
T194V	n.d.	47.0 $\pm$ 0	4.0 $\pm$ 0
V193L	n.d.	46.5 $\pm$ 0	3.5 $\pm$ 0
V217P	n.d.	46.0 $\pm$ 0	3.0 $\pm$ 0
Combination mutants	k_{obs}^* (U/mg)	$T_{m,app}$ (°C)	ΔT_m (°C)
M2 (L150F + M157K)	0.4	64.0 $\pm$ 0	21.0 $\pm$ 0
M3 (M2 + S197E)	0.1	69.0 $\pm$ 0	26.0 $\pm$ 0
M4 (M3 + V238L)	0.2	72 (62) $\pm$ 0.25	29.0 $\pm$ 0.25
M5 (M4 + N212R)	0.2	75.5 (64) $\pm$ 0	32.5 $\pm$ 0
M6 (M5 + G149A)	0.1	81.0 (69) $\pm$ 0	38.0 $\pm$ 0
M7 (M6 + G159A)	0.1	85.0 (74) $\pm$ 0	42.0 $\pm$ 0
M8 (M7 + V193L + T194V)	0.05	90.0 (81) $\pm$ 0.25	47.0 $\pm$ 0.25
M9 (M8 + V217P)	0.03	94.5 (84) $\pm$ 0	51.5 $\pm$ 0
M9* (M9 - S197E)	0.8	88.0 (78.5) $\pm$ 0	45.0 $\pm$ 0

* k_{obs} values are averages based on 2–3 replicates and for each average the error was smaller than 5% (between $\pm$ 0.0015–0.04 U/mg). Cyclohexanol was used as substrate.

Figure 3 – Michaelis-Menten plots for kinetics with NADP^+ . (A) ADHA wild type (B) M9 mutant. Note that the X-axis scaling is different. The inset of B presents the same data with a different Y-axis scaling. Plots are fitted with Michaelis-Menten in GraphPad prism 6.07.

Crystal structure of the M9 mutant

To investigate the structural alterations caused by the stabilizing mutations and their effect on enzyme catalysis, we decided to crystallize mutant M9. As with the wild-type ADHA, several conditions gave crystals of the M9 enzyme, enabling determination of its structure at 2.6 Å resolution. Although the crystallization conditions contained 1.0 mM NADP^+ , there was no electron density for any ligand in the M9 structure. This was not surprising, considering the high K_M (1.0 mM) for NADPH of this mutant. When inspecting the M9 structure, it is worth noting that all the introduced mutations are in the same area of the enzyme three-dimensional structure. Specifically, the mutations are located centrally on each of the two flat faces of the homo-tetramer, where two opposing subunits come together (Figure 4A). Most mutations are located close to the tetramer centre (Figure 4B) and many are surface-exposed, or surface-near.

Superposition of the wild-type ADHA and M9 tetramers revealed a very close structural similarity with a root-mean-square deviation of 0.58 Å for 956 C α atoms. The only considerable backbone shift (up to 3.1 Å) concerns loop 196–214 (Figure 4B,C). Residues 196–198 in this loop are involved in binding the NADP^+ pyrophosphate group as shown by the crystal structure of the wild-type ADHA bound to NADP^+ . The observed structural alteration in this particular loop immediately provides an explanation for the increase of K_M for this cofactor in M9: the introduced E197 seems to occupy the NADP^+ pyrophosphate binding site.

Figure 4 – Structure of the M9 mutant of ADHA with mutated residues highlighted. (A and B) quaternary structure of M9. The tetramer is organized such that the N-termini are on the outside (on the edge of the top-down view of A and B), whereas the C-termini all point inwards; which is where most and the most stabilizing mutations were found. (A) M9 structure with all atoms represented as balls. The four monomers are shaded in various colours, highlighting the particular clustering of the observed stabilizing mutations. (B) The structure as ribbon model, superimposing the mutant (blue ribbon, red spheres indicate mutated residue) and the wild type (cyan ribbon, yellow spheres). (C) Colour scheme as in B. The loop (196-214) that is dislocated as a result of the S197E mutation, compared to the structure of wild-type ADHA. The shift is accompanied by a flip of R18 into the NADP-binding pocket, likely due to an electrostatic attraction from the mutant glutamate. As a result, the cofactor (green carbons) is only bound in the wild type, while absent from the mutant structure.

Along with the shift of the loop, the carboxylate moiety of the S197E mutation induces an interaction with the guanidinium of R18 (Figure 4C) – a residue that crucially binds the 2' phosphate of NADP⁺ in the wild-type structure. While the favourable electrostatics of this new interaction is a likely explanation for the mutation's stability effect, it also explains the lower affinity for NADP⁺ (and the absence of the cofactor in the mutant's crystals), as the salt bridge neutralizes R18 and occupies the NADP⁺ binding pocket.

Closely located to the S197E mutation are several other introduced mutations: G149A, L150F, V193L, T194V, and V217P. These mutations cause rather small alterations by exchanging a hydroxyl for a methyl group or slightly increasing the bulkiness of aliphatic side chains. These residues are part of a cluster of hydrophobic residues adjacent to the loop 196–214 and involve several aliphatic (I20, P191, M198, I215, I220) and aromatic (F207, W214, Y244) side chains. G149A, L150F (the most stabilizing mutation of all), V193L, T194V, and V217P all contributed substantially to thermostability, with up to 14 °C increase in the T_m values at the single mutant level (Table 2). The structural analysis clearly shows that improved side-chain packing and hydrophobic interactions are the likely source of their stabilizing effect.

Another mutation causing improved hydrophobic packing is G159A (T_m increase of 5.5 °C) that locates close to I102, F171, and L175. V238L (+7 °C) is a unique case, promoting more extended interaction of the subunits within the tetramer (Figure 5). Curiously, the mutation does so by allowing a hydrophobic interaction with itself at the dimer interface. V217P is a seemingly conservative mutation at the beginning of an α -helix and yet caused a significant effect (+3 °C; Table 2 and Figure 4C). Prolines often occur at 'helical capping' positions by restraining the backbone conformation and positioning a substituted backbone amino group that is unable to form H-bonding interactions (Richardson and Richardson, 1988). The V217P mutation follows this trend. Among the identified mutations, M157K is the only one that replaces a hydrophobic residue with a hydrophilic and charged side chain (Figure 4C). It has a large (+8 °C; Table 2) effect on protein stability, which very likely arises from the removal of a solvent-exposed hydrophobic group and its replacement with the charged and hydrophilic amine of a lysine residue.

Lastly, N212R is possibly the most puzzling mutation. It positions an arginine side chain in a fully solvent-exposed location (Figure 4C) and its conspicuous (+5 °C) stability can be tentatively attributed to the more pronounced polarity of an arginine side chain as compared to an asparagine (Szilágyi and Závodszky, 2000).

Figure 5 – Dimer interface with the V238L mutation ($\Delta T_m = 7\text{ }^{\circ}\text{C}$). (A and B) indicate the different monomers in the ADHA tetramer.

In summary, the detailed structural analysis of the mutation sites reveals a consistent pattern concerning the stabilizing effects: all stabilizing mutations of M9 promote hydrophobic packing of side chains or install highly polar and charged groups on fully solvent-exposed positions on the protein surface. The crystal structure of M9 also provided a clue to explain the rather low activity of M9.

Rescue of activity

Since we observed and suspected that the S197E mutation recruits R18 into the cofactor binding pocket and thereby impairs NADP⁺ binding (Figure 4C), we reverted this mutation. The 9-fold M9* mutant (M9 without S197E) was purified and characterized. As expected, we observed a decrease of 6.5 °C in T_m for M9* compared to M9 (Table 2). Yet, it was satisfying that M9* displayed proper enzyme activity: the K_M for NADP⁺ of M9* has the same value as wild type (9.6 μM), and its k_{cat} at 25 °C was only slightly lower (Table 3, Figure 6). With this final stabilized and active variant at hand, some more characterization experiments were carried out to probe its biocatalytic properties.

By measuring the alcohol oxidation activity at various temperatures, a largely shifted and wider temperature optimum was found for M9* (Figure 7A). The enzyme shows highest activity at 55–60 °C. Only at temperatures > 75 °C, its activity decreases significantly. Still, the enzyme is also active at 85 °C, whereas wild-type ADHA displays hardly any activity > 40 °C. These data suggest that only the major unfolding event (at a relatively high temperature) observed by ThermoFluor results in inactivation of M9*. The apparent melting temperature was also measured in the presence of 0.5 mM NADPH. The M9 mutant showed a +3 °C increase, which is much smaller than the drastic +9 °C improvement in the T_m of the wild type upon NADP⁺ binding. We

suspect that the regions that are stabilized through binding NADPH in the wild-type ADHA are partially stabilized by the FRESCO mutations, hence the relatively small gain in stability with NADPH as ligand for M9*.

Figure 6 – Michaelis-Menten plots for kinetics with NADP⁺. ADHA wild type (grey, triangles) and M9* mutant (M9 with S197E reverted) (black, diamonds). Plots are fitted with the Michaelis-Menten equation in GraphPad prism 6.07.

Table 3 – Characteristics of wild-type, M9, and M9* ADHA.

Enzyme	<i>T</i> _m (°C)	<i>k</i> _{cat} (s ⁻¹)	<i>K</i> _{M,NADP⁺} (μM)	<i>k</i> _{cat} / <i>K</i> _{M,NADP⁺} (s ⁻¹ mM ⁻¹)	Conversion and <i>ee</i> (COBA)	Conversion and <i>ee</i> (4-CAP)
ADHA	43.0	1.1	9.5	116	> 99%	> 99%
WT	± 0	± 0.04	± 1.2	± 35	> 99% <i>ee</i> (<i>R</i>)	97.2% <i>ee</i> (<i>S</i>)
M9	94.5	0.27	1040	0.26	n.d.	n.d.
	± 0	± 0.02	± 108	± 0.2		
M9*	88.0	0.7	9.6	73	> 99%	> 99%
	± 0	± 0.01	± 0.7	± 14.5	> 99% <i>ee</i> (<i>R</i>)	98% <i>ee</i> (<i>S</i>)

Activity measurements were performed at 25 °C in duplicate or triplicate and the respective Michaelis-Menten plots are depicted in Figure 6. Melting curves are depicted in Figure 2—figure supplement 1. Conversions were performed with 5 μM of ADHA and 10 mM of prochiral ketone substrate: ethyl 4-chloro-3-oxobutanoate (COBA) and 4-chloroacetophenone (4-CAP). Details and chromatograms are provided in Supplementary file 4.

To evaluate the stability over time, wild type and M9* ADHA were incubated at 37 °C, and their oxidation activity was measured at several time points (Figure 7B). As wild-type ADHA has a *T*_m at 43 °C, it was losing activity steadily over the first couple of hours, with a half-life of around 2 hr. After 22 h at 37 °C, the remaining activity of wild-type ADHA was only 8.7%, whereas

the M9* still displayed full activity. Only after 11 days at 37 °C, the M9* ADHA lost 50% of its activity (Figure 7—figure supplement 1). The stability of the mutant was largely retained also in the presence of co-solvents, with isopropanol showing the largest ΔT_m (−21 °C; Table 4). This tolerance to the presence of 20% cosolvent is quite useful, as it enables higher concentrations of hydrophobic substrates.

Figure 7 – Properties of wild-type and M9* ADHA. (A) Temperature-activity profile using cyclohexanol as substrate. The dashed lines indicate the T_m of the wild type (at 43 °C) and apparent melting temperatures of M9* (78.5 °C and 88 °C) (B) Enzyme activity monitored over time at 37 °C (buffer composition: 50 mM Tris-HCl pH 7.5). Figure 7—figure supplement 1 depicts the enzyme activity over time for 18 days.

Figure 7—figure supplement 1 – Long-term stability. Long-term stability at 37 °C of the wild-type protein (in grey, triangles) compared to M9* (in black, diamonds). Plot fitted with one-phase decay in GraphPad prism 6.07.

Table 4 – Final melting temperatures (T_m) of M9* in various cosolvents (at 20% v/v). Values were determined in duplicates.

Cosolvent	T_m (°C)	ΔT_m (°C)
-	88	-
Methanol	76	-12
Ethanol	71.5	-16.5
Isopropanol	67	-21

Discussion

With the aim of creating a robust ADHA variant, we applied FRESCO and reached a 9-fold mutant that shows a significantly improved thermostability with largely retained activity (Table 3). This study achieved the highest improvement of melting temperature of all FRESCO studies to date. Up to now, there have been six studies that applied FRESCO, and it has shown effective improvements in stability with T_m differences up to +35 °C (Table 5). In particular, enzymes with higher order quaternary structures (e.g. dimeric and tetrameric enzymes) gained significant improvements of +28 °C and +35 °C (Table 5). Dimers and tetramers may gain a lot of stability from having subunit-subunit interfaces that stabilize hydrophobic regions of the enzyme. In contrast, enzymes containing cofactors (oxidases, Baeyer-Villiger monooxygenases) showed relatively moderate improvement: the FAD-containing HMF oxidase and cyclohexanone monooxygenase were improved by only 12–13 °C (Table 5). This may due to the fact that the employed computational algorithms (Rosettaddg and FoldX) cannot take into account the flavin-protein interactions (Wijma et al., 2018).

The initial FRESCO calculations and predictions for ADHA had a good hit rate: of the tested 177 mutations, 39 had a stabilizing effect of > 2 °C (22%). This is in line with previous uses of FRESCO, in which 20–40% of the FRESCO-predicted mutations were found to be stabilizing (Fürst et al., 2018; Wijma et al., 2018). To see if the calculated energy values correlate with the observed ΔT_m , the data were plotted and statistically analysed (Supplementary file 3). The Rosetta calculations bore no relation to the measured melting temperatures, whereas for the FoldX values some correlation was found (Pearson $r = 0.3$, $p < 0.02$). This somewhat better predictive quality of FoldX as compared to Rosetta has been described before (Buß et al., 2018). Still, 4/9 of the selected mutations were not found by FoldX, despite being highly stabilizing (Supplementary file 3A). Clearly, the combination of both

calculations, together with the MD simulations in the FRESCO workflow, results in a satisfactory rate of predicted stabilizing mutations.

Table 5 – Studies that have applied FRESCO for stabilization of enzymes, to date.

Enzyme	Abbreviation	Size (aa)	Quaternary structure	ΔT_m	Reference
Limonene epoxide hydrolase	LEH	149	Dimer	+35 °C	(Wijma et al., 2014)
Haloalkane dehalogenase	LinB	250	Monomer	+22 °C	(Floor et al., 2014)
Hydroxymethyl furfural oxidase	HMFO	525	Monomer	+12 °C	(Martin et al., 2018)
Peptide amidase	PAM	508	Monomer	+23 °C	(Wu et al., 2016)
Halohydrin dehalogenase	HheC	254	Tetramer	+28 °C	(Arabnejad et al., 2017)
Cyclohexanone monooxygenase	CHMO	529	Monomer	+13 °C	(Fürst et al., 2019)
Glucose oxidase	GOX	605	Dimer	+8.5 °C	(Mu et al., 2019)
ω -Transaminase	ω -TA	455	Dimer	+23 °C	(Meng et al., 2020)
Short-chain dehydrogenase	ADHA	246	Tetramer	+45 °C	This work

It could be argued that for the computational predictions the residues in close contact with the NADP⁺ should be excluded, to avoid the complications that were found with the S197E mutation. During the visual screening of the single mutations with MD simulations the introduced glutamic acid was pointing outwards into the solvent, giving the impression of a somewhat harmless, surface-exposed mutation. The interaction it would have with R18, when in the context of the other introduced mutations, was impossible to predict. This newly introduced interaction was blocking binding of the nicotinamide cofactor. Yet, the 10-fold mutant (M9) is still active and could be of use when the enzyme needs to be used under challenging conditions. It also shows the power of combining FRESCO with a thorough structural inspection of the generated thermostable mutant. By structural analysis, the effects of individual mutations can be evaluated and, when needed, alternative structure-inspired mutations can be prepared. In this study, it led to the 9-fold M9* mutant of ADHA, which is highly thermostable, solvent tolerant, and active.

This study demonstrates how a computational method can give guidance for a path from a moderately stable enzyme to a highly robust enzyme, suitable for industrial settings with long reaction times and cosolvents. Although

FRESCO requires some screening effort, the method greatly reduces the time and screening amount compared to directed evolution or semi-rational approaches. Moreover, since individual stabilizing mutations are combined, there is more certainty and freedom compared to computational methods that suggest stable variants with multiple mutations (Goldenzweig et al., 2016; Musil et al., 2017).

Conclusions

A highly robust ADH was engineered through computational library design. It is the highest improvement in stability obtained with this method so far, with an improvement of $\Delta T_m +45$ °C. Although some mutations might be avoided in the future, through careful consideration of cofactor binding, the nine selected mutations caused no significant decrease in activity or NADP⁺ binding. This method can be effective to stabilize enzymes for industrial applications, in particular if the enzyme has a higher order quaternary structure.

Materials and methods

Materials, strains and chemicals

Oligonucleotide primers for cloning and mutagenesis were ordered from Sigma-Aldrich. For amplification of the gene inserts or to perform QuikChange mutations, PfuUltra II HotStart PCR (Polymerase) master mix was used, purchased from Agilent Technologies. Other chemicals were purchased from Sigma-Aldrich and Acros Organics. Precast native PAGE gradient gels were ordered from GenScript. As host strain for recombinant DNA and for protein expression, *Escherichia coli* NEB® 10-beta (New England Biolabs) was used. Precultures were grown in lysogeny broth (LB), and the subsequent main cultures in terrific broth (TB) or YJM505 medium (Studier, 2005).

Cloning, expression and purification

The gene of ADHA was identified in a PCR-based genome screening for new ketoreductases from *Candida magnoliae* DSMZ 70638 using degenerate PCR primers. A gene fragment of a new ketoreductase was identified. By using a refined PCR-primer the full open reading frame of the previously unknown ketoreductase ADHA was identified. The gene encoding for ADHA was cloned into c-Lecta's proprietary expression vector pLE1A17 (a derivative of pRSF-1b, Novagen). After transformation of electrocompetent *E. coli* BL21 (DE3) cells with the pLE1A17-ADHA vector, the cells were cultivated in YJM505 medium (Studier, 2005), supplemented with kanamycin (50 mg/L) at 37 °C. Expression was induced at logarithmic phase by IPTG (0.1 mM) and carried out at 30 °C for 16-18 hours. Cells were harvested by centrifugation (3220 x g, 20 min, 4 °C) and disrupted with cell lysis buffer (50 mM Tris-HCl pH 7.0; 2 mM MgCl₂, 1x CellLytic B (Sigma-Aldrich); DNA nuclease 0.02 U, lysozyme 0.5 mg/mL). The crude extracts were separated from cell debris by centrifugation (3220 x g 30 min, 4 °C). The supernatant was sterile-filtrated over a 0.2 µm-membrane filter and subsequently freeze dried. A brownish powder was

obtained. This sample was purified by anion-exchange (Q Sepharose Fast Flow, GE Healthcare) and gel-permeation chromatography (Superdex 200, GE healthcare) in 50 mM Tris-HCl pH 7.5.

The genes encoding for the mutant enzymes were cloned into a vector with a cleavable tag (ADHA M9*: N-His-SUMO in pBAD vector). In this way, the proteins could be obtained in high purity, and, after cleavage of the tag, in the native form. A preculture inoculated with a single colony of *E. coli* NEB 10-beta harboring the pBAD vector was grown overnight in LB (5 mL) with ampicillin (50 mg/L) at 37 °C. This preculture was used to inoculate TB (5 v/v %) and was simultaneously induced with 0.02% L-arabinose. The cells were grown at 24 °C for another 38 hours. Then the cells were spun down (3000 x g, 10 minutes, 4 °C), resuspended in buffer (50 mM Tris-HCl pH 7.5), and sonicated (5 seconds on/off, 70% amplitude) on ice for cell lysis. Debris was spun down (21000 x g, 45 minutes, 4 °C), and the supernatant was applied to an IMAC column (GE Healthcare). After washing with buffer, and buffer with 5 mM imidazole, the His-tagged protein was eluted with 300 mM imidazole in buffer. The sample was subsequently subjected to a gravity-flow desalting column (PD-10, GE healthcare), to remove the imidazole. In case of SUMO-tagged protein, the protein was then incubated overnight at 4 °C with SUMO protease. The next day, the cleaved protein was separated from the SUMO-protease by IMAC and further purified by gel-permeation chromatography (Superdex 200, GE healthcare) in 50 mM Tris-HCl pH 7.5. The protein samples were analysed by SDS-PAGE (**Supplementary file 2**).

Crystallization and structure determination

Wild-type and mutant ADHA, purified as above, crystallized under several conditions. To probe crystallization conditions, several screens were preformed using an Oryx 8 robot (Douglas Instruments, UK) starting from PEG Suite, Ammonium Sulphate Suite and Classic Suite (Nextal). Preliminary crystallization experiments were performed in sitting drop using MRC SWISS CI plates, with a 1:1 protein:reservoir ratio and 0.4 - 1 mL crystallization droplets. Crystallization experiments were performed also with proteins incubated with 1 mM NADP⁺. The best crystals grew in 20% w/v PEG 3350, 0.2 M sodium citrate. These conditions were then used for all (wild-type and mutant) crystallization experiments. Crystals were harvested from crystallization droplets using nylon cryo-loops (Hampton Research/Molecular Dimensions), shortly soaked in a cryo-protectant solution containing 20% w/v PEG 3350, 0.2 M sodium citrate and 20-25% v/v PEG 400, and then flash-cooled in liquid nitrogen. Data collection was performed at ESRF ID23 beam line (European Synchrotron Radiation Facility, Grenoble, France) for the wild-type crystals, and at the Swiss Light Synchrotron (Paul Scherrer Institut, Villigen, SUI) for the M9 mutant. Wild-type crystals diffracted at 1.6 - 2.0 Å, while the resolution of diffraction by the mutant crystals was 2.6 Å. Data processing, structure determination by molecular replacement, and structure refinement were performed with XDS and programs of the CCP4 suited using standard protocols (Kabsch, 1993; Winn et al., 2011). The crystallographic statistics are listed in **Supplementary file 1A**.

Computational methods

Calculations for the FRESCO workflow to predict stabilizing mutations were carried out as described (Wijma et al., 2018). Although we also obtained a crystal structure of ADHA in complex with NADP, we chose the wild-type structure without the ligand for our calculations, because of two reasons: firstly, the FoldX and rosetta software only accept protein residues and the cofactor would have to be omitted for these parts in any case. Secondly, NADP is assumed to interact only transiently to the enzyme and does not bind permanently as a prosthetic group.

Thus, to realistically model the enzyme in its most relevant physiological condition, we did not consider the ligand-bound structure for the MD simulations either. In brief, we then prepared the structure using YASARA by adding hydrogens, predicted their optimal H-bonding network, and removed buffer and water molecules from the structure. The Rosettaaddg and FoldX algorithms were then applied as described, and the resulting list of energies was used to identify 478 mutations with a $\Delta\Delta G^{\text{fold}}$ improvement of $< -5 \text{ kJ mol}^{-1}$. We re-added the water molecules and furthermore solvated the protein in a charge-neutralized simulation cell stretching $7.5 \text{ \AA}$ around all atoms. MD simulations were carried out with YASARA using the Yamber3 force field. In five independently seeded simulations, the system was first energy minimized, heated to 298 K in 30 ps , equilibrated for 20 ps , and finally simulated in the production run for 50 ps . The MD trajectories' average structures were superimposed and compared with those of the wild type in a visual inspection step of all MD-simulated mutants. Taking into account both the predicted static, as well as the MD simulated structures, mutants were not considered for experimental verification if we observed an increased backbone or side-chain flexibility, hydrogen bond impairment, or hydrophobic exposure. The Peregrine high-performance computing cluster (University of Groningen, Groningen) was used to perform all calculations for Rosettaaddg, FoldX, and the molecular dynamics (MD) simulations. The MD simulations were carried out using the software Yasara Structure (YASARA Biosciences GmbH).

Mutagenesis and small-scale growth and expression

Following the published protocol (Fürst et al., 2018), mutant oligonucleotides were designed and ordered, and the mutant library was prepared via QuikChange. QuikChange was also used to prepare mutants in which several mutations were combined. The construct used for small-scale expression of the mutants was an L-arabinose-inducible pBAD vector, where the ADHA gene is fused to an N-terminal His-tag. The plasmid was used to transform chemically competent *E. coli* NEB 10 beta cells. Cell cultures were grown in four 96-deepwell plates, containing 1 mL per well. When the 1 mL TB medium was inoculated with an overnight preculture ($5\% \text{ v/v}$), arabinose was also added, and the 96-deepwell plates were incubated for 38 hours at $24 \text{ }^{\circ}\text{C}$. Then, the cells were centrifuged to create a single pellet for each mutant from 4 mL of culture broth. Cells were resuspended in 50 mM Tris-HCl pH 7.5 . After lysis as described (Fürst et al., 2018), His-tag purification and desalting, the samples were used for determining activity and thermostability melting point determination.

Melting point determination by ThermoFluor

For probing the thermostability of each mutant, the apparent melting temperatures were measured by the ThermoFluor method (Pantoliano et al., 2001). For this, purified enzyme was mixed with the SYPRO orange dye and the apparent T_m was measured in an RT-PCR (CFX96 Touch Real-Time PCR, BioRad, signal setting: FRET), in accordance with the described method (Cummings et al., 2006). All 177 enzyme mutants were screened in technical duplicate.

Activity assay

Activity was measured by monitoring the formation of NADPH at 340 nm . After mixing enzyme ($\leq 0.1 \text{ }\mu\text{M}$) with substrate (cyclohexanol, if not stated: at 70 mM) in buffer (50 mM Tris-HCl pH 7.5), $100 \text{ }\mu\text{M}$ NADP⁺ was added, briefly mixed in a cuvette, and then the reaction was followed (V-330 Spectrophotometer, JASCO). The slopes of the initial 20 seconds were used to determine the rates, in Abs/min . This value was then divided by the extinction coefficient of NADPH (ϵ_{340}

= $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), in accordance with the Lambert-Beer law, resulting in a rate in mM/min. By dividing this value by the protein concentration in the reaction, the k_{obs} values were obtained. All measurements were done in technical duplicates or triplicates.

Temperature stability

Dilutions of wild-type and mutant ADHA were made in buffer (50 mM Tris-HCl pH 7.5), to a concentration of 0.15–0.3 mg/mL. These were incubated in a water bath at 37 °C. At several intervals, samples were taken, mixed with buffer and substrate (70 mM cyclohexanol) that were prewarmed at 37 °C in the same water bath. To start the reaction, cofactor (200 μM NADP⁺) was added. Absorbance at 340 nm was monitored (V-330 Spectrophotometer, JASCO), and activity was determined based on the slope.

Conversions and enantiomeric excess

A solution of ethyl-4-chloro-3-oxobutanoate (COBE) in DMSO (50 μL , 200 mM) was added to a Tris-HCl buffer (950 μL , 50 mM, pH 7.5) containing 0.5 mM NADPH, 5 μM of ADHA (WT or M9*), 5 μM of PTDH and 50 mM sodium phosphite. The mixture was incubated for 24 h in an orbital shaker (150 rpm, 25 °C). The solution was extracted with ethyl acetate (EtOAc) (3 x 1 mL), centrifuging after each extraction (10000 x g, 2 min), and the combined organic solutions were dried over anhydrous Mg_2SO_4 , and analyzed by chiral GC. Enantiomeric excess determinations for COBE were measured by chiral GC-FID. GC-FID analyses were carried out with an Agilent Technologies 7890A GC system. In **Supplementary file 4** chromatograms and details are provided regarding the settings for the GC.

A solution of 4-chloroacetophenone (4-CAP) in DMSO (50 μL , 200 mM) was added to a Tris-HCl buffer (950 μL , 50 mM, pH 7.5) containing 0.5 mM NADPH, 5 μM of ADH, 5 μM of PTDH and 50 mM sodium phosphite. The mixture was incubated for 24 h in an orbital shaker (150 rpm, 25 °C). The solution was extracted with EtOAc (3 x 1 mL), centrifuging after each extraction (10000 x g, 2 min), and the combined organic solutions were dried over anhydrous Mg_2SO_4 . Organic solvent is evaporated under reduced pressure and residue is dissolved in 3 mL heptane/iso-propanol (v/v = 95:5) and analyzed by chiral HPLC. Enantiomeric excess determinations for 4-CAP were measured by normal phase (np) HPLC analysis (Chiralcel OD-H column) using UV-detection (Shimadzu SCL-10Avp). In **Supplementary file 4** chromatograms and details are provided regarding the settings for the HPLC.

Acknowledgements

This research received funding from the European Union (EU), project ROBOX (grant agreement no. 635734) under the EU's Horizon 2020 Program Research and Innovation actions H2020-LEIT BIO-2014-1 and from the Italian Ministry of Education, University and Research (MIUR), Dipartimenti di Eccellenza Program (2018–2022) – Department of Biology and Biotechnology “L. Spallanzani”, University of Pavia. The views and opinions expressed in this article are only those of the authors and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein.

Supporting information

For supporting information, see pages 146–155.

References

- Arabnejad H, Lago MD, Jekel PA, Floor RJ, Thunnissen AMWH, Terwisscha Van Scheltinga AC, Wijma HJ, Janssen DB. 2017. A robust cosolvent-compatible haloalcohol dehalogenase by computational library design. *Protein Eng Des Sel* **30**:175–189. doi:10.1093/protein/gzw068
- Bednar D, Beerens K, Sebestova E, Bendl J, Khare S, Chaloupkova R, Prokop Z, Brezovsky J, Baker D, Damborsky J. 2015. FireProt: Energy- and Evolution-Based Computational Design of Thermostable Multiple-Point Mutants. *PLoS Comput Biol* **11**. doi:10.1371/journal.pcbi.1004556
- Bhatia C, Oerum S, Bray J, Kavanagh KL, Shafqat N, Yue W, Oppermann U. 2015. Towards a systematic analysis of human short-chain dehydrogenases/reductases (SDR): Ligand identification and structure-activity relationships. *Chem Biol Interact* **234**:114–125. doi:10.1016/j.cbi.2014.12.013
- Björk A, Dalhus B, Mantzilas D, Sirevg R, Eijsink VGH. 2004. Large improvement in the thermal stability of a tetrameric malate dehydrogenase by single point mutations at the dimer-dimer interface. *J Mol Biol* **341**:1215–1226. doi:10.1016/j.jmb.2004.06.079
- Bommarius AS, Paye MF. 2013. Stabilizing biocatalysts. *Chem Soc Rev* **42**:6534–6565. doi:10.1039/c3cs60137d
- Borgo B, Havranek JJ. 2012. Automated selection of stabilizing mutations in designed and natural proteins. *Proc Natl Acad Sci U S A* **109**:1494–1499. doi:10.1073/pnas.1115172109
- Buß O, Rudat J, Ochsenreither K. 2018. FoldX as Protein Engineering Tool: Better Than Random Based Approaches? *Comput Struct Biotechnol J*. doi:10.1016/j.csbj.2018.01.002
- Cummings MD, Farnum MA, Nelen MI. 2006. Universal screening methods and applications of ThermoFluor®. *J Biomol Screen* **11**:854–863. doi:10.1177/1087057106292746
- DePristo MA, Weinreich DM, Hartl DL. 2005. Missense meanderings in sequence space: A biophysical view of protein evolution. *Nat Rev Genet*. doi:10.1038/nrg1672
- Floor RJ, Wijma HJ, Colpa DI, Ramos-Silva A, Jekel PA, Szymański W, Feringa BL, Marrink SJ, Janssen DB. 2014. Computational library design for increasing haloalkane dehalogenase stability. *ChemBioChem* **15**:1660–1672. doi:10.1002/cbic.201402128
- Fürst MJLJ, Boonstra M, Bandstra S, Fraaije MW. 2019. Stabilization of cyclohexanone monooxygenase by computational and experimental library design. *Biotechnol Bioeng* **116**:2167–2177. doi:10.1002/bit.27022
- Fürst MJLJ, Martin C, Lončar N, Fraaije MW. 2018. Experimental Protocols for Generating Focused Mutant Libraries and Screening for Thermostable Proteins. *Methods in Enzymology*. pp. 151–187. doi:10.1016/bs.mie.2018.04.007
- Goldenzweig A, Fleishman SJ. 2018. Principles of Protein Stability and Their Application in Computational Design. *Annu Rev Biochem* **87**:105–129. doi:10.1146/annurev-biochem-062917-012102
- Goldenzweig A, Goldsmith M, Hill SE, Gertman O, Laurino P, Ashani Y, Dym O, Unger T, Albeck S, Prilusky J, Lieberman RL, Aharoni A, Silman I, Sussman JL, Tawfik DS, Fleishman SJ. 2016. Automated Structure- and Sequence-Based Design of Proteins for High Bacterial Expression and Stability. *Mol Cell* **63**:337–346. doi:10.1016/j.molcel.2016.06.012
- Gribenko A V, Patel MM, Liu J, McCallum SA, Wang C, Makhatadze GI. 2009. Rational stabilization of enzymes by computational redesign of surface charge-charge interactions. *Proc Natl Acad Sci U S A* **106**:2601–2606. doi:10.1073/pnas.0808220106
- Kabsch W. 1993. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. *J Appl Crystallogr* **26**:795–800. doi:10.1107/S0021889893005588
- Kavanagh KL, Jörnvall H, Persson B, Oppermann U. 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families: The SDR superfamily: Functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell Mol Life Sci*. doi:10.1007/s00018-008-8588-y
- King JD, Harmer NJ, Preston A, Rejcek M, Field RA, Blundell TL, Maskell DJ. 2007. Predicting Protein Function from Structure-The Roles of Short-chain Dehydrogenase/Reductase Enzymes in Bordetella O-antigen Biosynthesis. *J Mol Biol* **374**:749–763. doi:10.1016/j.jmb.2007.09.055
- Korkegian A, Black ME, Baker D, Stoddard BL. 2005. Computational thermostabilization of an enzyme. *Science (80-)* **308**:857–860. doi:10.1126/science.1107387
- Malakauskas SM, Mayo SL. 1998. Design, structure and stability of a hyperthermophilic protein variant. *Nat Struct Biol* **5**:470–475. doi:10.1038/nsb0698-470

- Martin C, Ovalle Maqueo A, Wijma HJ, Fraaije MW. 2018. Creating a more robust 5-hydroxymethylfurfural oxidase by combining computational predictions with a novel effective library design. *Biotechnol Biofuels* **11**:56. doi:10.1186/s13068-018-1051-x
- Meng Q, Capra N, Palacio CM, Lanfranchi E, Otzen M, van Schie LZ, Rozeboom HJ, Thunnissen A-MWH, Wijma HJ, Janssen DB. 2020. Robust ω -Transaminases by Computational Stabilization of the Subunit Interface. *ACS Catal* 2915–2928. doi:10.1021/acscatal.9b05223
- Moore EJ, Zorine D, Hansen WA, Khare SD, Fasan R. 2017. Enzyme stabilization via computationally guided protein stapling. *Proc Natl Acad Sci U S A* **114**:12472–12477. doi:10.1073/pnas.1708907114
- Mu Q, Cui Y, Tian Y, Hu M, Tao Y, Wu B. 2019. Thermostability improvement of the glucose oxidase from *Aspergillus niger* for efficient gluconic acid production via computational design. *Int J Biol Macromol* **136**:1060–1068. doi:10.1016/j.ijbiomac.2019.06.094
- Murphy GS, Mills JL, Miley MJ, Machius M, Szyperski T, Kuhlman B. 2012. Increasing sequence diversity with flexible backbone protein design: The complete redesign of a protein hydrophobic core. *Structure* **20**:1086–1096. doi:10.1016/j.str.2012.03.026
- Musil M, Stourac J, Bendl J, Brezovsky J, Prokop Z, Zendulka J, Martinek T, Bednar D, Damborsky J. 2017. FireProt: web server for automated design of thermostable proteins. *Nucleic Acids Res* **45**:W393–W399. doi:10.1093/nar/gkx285
- Nealon CM, Musa MM, Patel JM, Phillips RS. 2015. Controlling Substrate Specificity and Stereospecificity of Alcohol Dehydrogenases. *ACS Catal*. doi:10.1021/cs501457v
- Oppermann U, Filling C, Hult M, Shafqat N, Wu X, Lindh M, Shafqat J, Nordling E, Kallberg Y, Persson B, Jörnvall H. 2003. Short-chain dehydrogenases/reductases (SDR): The 2002 updateChemico-Biological Interactions. pp. 247–253. doi:10.1016/S0009-2797(02)00164-3
- Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P, Salemme FR. 2001. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J Biomol Screen* **6**:429–40. doi:10.1177/108705710100600609
- Parsell DA, Sauer RT. 1989. The structural stability of a protein is an important determinant of its proteolytic susceptibility in *Escherichia coli*. *J Biol Chem* **264**:7590–7595.
- Richardson JS, Richardson DC. 1988. Amino acid preferences for specific locations at the ends of α helices. *Science (80-)* **240**:1648–1652. doi:10.1126/science.3381086
- Shah PS, Hom GK, Ross SA, Lassila JK, Crowhurst KA, Mayo SL. 2007. Full-sequence Computational Design and Solution Structure of a Thermostable Protein Variant. *J Mol Biol* **372**:1–6. doi:10.1016/j.jmb.2007.06.032
- Solé J, Brummund J, Caminal G, Schürman M, Álvaro G, Guillén M. 2019. Ketoisophorone Synthesis with an Immobilized Alcohol Dehydrogenase. *ChemCatChem*. doi:10.1002/cctc.201901090
- Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**:207–234. doi:10.1016/j.pep.2005.01.016
- Szilágyi A, Závodszy P. 2000. Structural differences between mesophilic, moderately thermophilic and extremely thermophilic protein subunits: results of a comprehensive survey. *Structure* **8**:493–504. doi:10.1016/S0969-2126(00)00133-7
- Tavanti M, Parmeggiani F, Castellanos JRG, Mattevi A, Turner NJ. 2017. One-Pot Biocatalytic Double Oxidation of α -Isophorone for the Synthesis of Ketoisophorone. *ChemCatChem* **9**:3338–3348. doi:10.1002/cctc.201700620
- Wijma HJ, Floor RJ, Jekel PA, Baker D, Marrink SJ, Janssen DB. 2014. Computationally designed libraries for rapid enzyme stabilization. *Protein Eng Des Sel* **27**:49–58. doi:10.1093/protein/gzt061
- Wijma HJ, Fürst MJLJ, Janssen DB. 2018. A Computational Library Design Protocol for Rapid Improvement of Protein Stability: FRESCOMethods in Molecular Biology. pp. 69–85. doi:10.1007/978-1-4939-7366-8_5
- Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AGW, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS. 2011. Overview of the CCP4 suite and current developments. *Acta Crystallogr Sect D Biol Crystallogr*. doi:10.1107/S0907444910045749
- Woodley JM. 2019. Accelerating the implementation of biocatalysis in industry. *Appl Microbiol Biotechnol*. doi:10.1007/s00253-019-09796-x
- Wu B, Wijma HJ, Song L, Rozeboom HJ, Poloni C, Tian Y, Arif MI, Nuijens T, Quaedflieg PJLM, Szymanski W, Feringa BL, Janssen DB. 2016. Versatile Peptide C-Terminal Functionalization via a Computationally Engineered Peptide Amidase. *ACS Catal* **6**:5405–5414. doi:10.1021/acscatal.6b01062
- Zheng YG, Yin HH, Yu DF, Chen X, Tang XL, Zhang XJ, Xue YP, Wang YJ, Liu ZQ. 2017. Recent advances in biotechnological applications of alcohol dehydrogenases. *Appl Microbiol Biotechnol*. doi:10.1007/s00253-016-8083-6

Section 4

Conclusions

Chapter 7: Summary and conclusions

Friso S. Aalbers

Preface

At the time of the start of my PhD there were not many studies that investigated enzyme fusions. There were a few studies that applied it to their system of enzymes, and found it worked reasonably well: the bifunctional enzymes typically retain their native activities, as described in this thesis, and can display better expression. What remains a somewhat surprising and puzzling effect is that some enzyme fusions can outperform the respective separate enzymes in a two-step reaction. Studies that report such results typically attribute this increased efficiency to ‘substrate channeling’, which means that the substrate from the one enzyme migrates directly to the second enzyme, rather than into the bulk solvent through diffusion. This particular effect is both highly appealing as well as quite obscure. The occurrence and validity of this effect is currently investigated and debated in several recent works.^[1-5]

From these recent works there are three key points, in my view: (1) for (natural) bifunctional enzymes with defined internal tunnels, through which the intermediate migrates to the other active site, it is clear that substrate channeling occurs. A well-known example is tryptophan synthase (Figure 1).^[6] In extension to that, I speculate that an important factor for such channeling is the hydrophobicity of the substrates/intermediates. Considering that the bulk solvent is water – hydrophilic, polar – a hydrophobic molecule probably prefers to migrate through or along enzyme subunits, or domains. This effect is also observed with (trans)membrane enzymes, like cytochrome P450s (CYPs) that are often membrane embedded and therefore bind hydrophobic molecules that migrate through/along the membrane. In fact, the role of these CYPs in our liver is to transform xenobiotics and drug molecules into more hydrophilic molecules, through hydroxylation.

Figure 1. Schematic representation of tryptophan synthase. The product of the first reaction, in the active site of the α -subunit (red), internally migrates to the β -subunit (yellow). Taken from [2].

Key point (2): the relative acceleration caused by proximity (closeness of the two active sites) seems to manifest only when the intermediate concentrations remain low (Figure 2).^[2] This happens when the first enzyme is slower than the second enzyme, and the overall reaction does not reach steady state (at which all enzymes convert substrate at a maximal rate). Lastly (3), the effect seems more pronounced in a crowded environment, such as inside cells.^[3-5] This sounds reasonable in the sense that the diffusion and migration of molecules is more obstructed in such a crowded and dynamic environment.

Figure 2. Schematic image, with enzyme 1 in red and enzyme 2 in yellow, depicting the mechanism through which the proximity of the enzymes can make a difference in rate (k), as shown on the right panel. The $k(\text{direct})$ indicates the rate from direct migration of the intermediate to the second enzyme. Image taken from [2].

Despite the interest in this ‘substrate channeling’ phenomenon, and its relevance for enzyme fusions, this thesis instead aimed to investigate the direct application of the enzyme fusions approach. We set out to explore the utility of enzyme fusion for particular systems involving oxidoreductases from a more pragmatic perspective: does it work (for these enzymes)? And; is it useful? (Does it improve overall rates, expression, stability?). This means that even though the main subject of this thesis is ‘enzyme fusion’, several questions

remain open: what factors decide whether substrate channeling occurs between two fused enzymes? Can one predictably make an enzyme fusion that displays substrate channeling? Are results from particular enzyme fusions translatable to other combinations of enzymes? Nevertheless, the exploration presented in this thesis can give inspiration and insight for both fundamental studies, as well as the biotechnological application of enzyme fusions.

- [1] Wheelodon, I., Minter, S. D., Banta, S., Barton, S. C., Atanasov, P., & Sigman, M. (2016). Substrate channelling as an approach to cascade reactions. *Nature chemistry*, 8(4), 299.
- [2] Rabe, K. S., Müller, J., Skoupi, M., & Niemeyer, C. M. (2017). Cascades in Compartments: En Route to Machine-Assisted Biotechnology. *Angewandte Chemie International Edition*, 56(44), 13574-13589.
- [3] Sweetlove, L. J., & Fernie, A. R. (2018). The role of dynamic enzyme assemblies and substrate channelling in metabolic regulation. *Nature communications*, 9(1), 1-12.
- [4] Kuzmak, A., Carmali, S., von Lieres, E., Russell, A. J., & Kondrat, S. (2019). Can enzyme proximity accelerate cascade reactions?. *Scientific reports*, 9(1), 455.
- [5] Ellis, G. A., Klein, W. P., Lasarte-Aragón, G., Thakur, M., Walper, S. A., & Medintz, I. L. (2019). Artificial Multienzyme Scaffolds: Pursuing in Vitro Substrate Channeling with an Overview of Current Progress. *ACS Catalysis*, 9, 10812-10869.
- [6] Yanofsky, C. (1960). The tryptophan synthetase system. *Bacteriological reviews*, 24(2), 221.

Summary and conclusions

To study an enzyme, or to apply an enzyme as catalyst for the synthesis of a product, ideally one can cheaply produce the enzyme in high quantities and with high purity. Nowadays, the production of enzymes through heterologous expression is a fundamental technology for the fields of enzymology (the study of an enzyme its role in cells and/or its mechanism) and biocatalysis (the application of enzymes as catalysts). If a particular process relies on the combination of two or more enzymes, then the total cost of the enzymes can be quite high. Rather than expressing a single enzyme, from an open-reading frame with a single gene, an interesting strategy is to clone two genes sequentially. The expression then results in a bifunctional fusion enzyme. Through this enzyme fusion strategy, it is possible to obtain two enzymes for the ‘cost’ of one enzyme (by ‘cost’ considering: cloning, growth of cells, expression of the genes, purification of the enzymes).

This approach can be very valuable for the application of multi-enzyme reactions. For instance, in cascade reactions one enzyme catalyzes a reaction of which the product is the substrate for a second enzyme, and possibly the product thereof a substrate for the third enzyme. These types of multistep reactions have several advantages: performing the reactions in one pot, keeping intermediate concentrations low (in case the intermediate is labile or reactive), and making use of by-products from the reactions. Ideally the cascade reaction is also self-sufficient with respect to redox balance, for instance if the first enzyme requires NAD(P)H, while the second enzyme uses

NAD(P)⁺ to generate NAD(P)H. In that case, no additional cofactor (except for a catalytic amount), or cofactor recycling system is required, and this is important because the cofactor is often the major cost-contributor. This is also relevant to multi-enzyme systems that employ enzymes as cofactor-regenerating enzymes; to minimize the use of valuable cofactor.

This thesis describes the current state and use of the enzyme fusion strategy, as well as the biotechnological application of oxidoreductases (alcohol dehydrogenases, Baeyer-Villiger monooxygenases, oxidases) in general. The introductory chapters give an overview of the current state of enzyme fusions (**Chapter 1**) and biotechnological application of Baeyer-Villiger monooxygenases (**Chapter 2**), which discusses several studies of enzyme fusions. The following chapters focus on enzyme engineering of oxidoreductases in two ways: (1) through the enzyme fusion approach (**Chapters 3, 4 and 5**), and (2) through computationally-guided mutagenesis (**Chapter 6**).

In **Chapter 3** of this thesis, three different alcohol dehydrogenases (ADHs) were fused to a Baeyer-Villiger monooxygenase (BVMO) to create a fusion that could perform the cascade reaction from cyclohexanol to ϵ -caprolactone. The two major findings in that chapter are: (1) the orientation of the fusion can be crucial for the activity of the enzyme(s), in particular for ADHs belonging to the short-chain reductase/dehydrogenases (SDRs) class, and (2) the fused system outperforms the combination of separate enzymes in the cascade reaction.

Continuing on the use of alcohol dehydrogenases (ADHs), **Chapter 4** describes the fusion of ADHs with an NADPH-oxidase (NOX) that can facilitate recycling of NADP⁺ with concurrent formation of hydrogen peroxide. The major finding from Chapter 3 helped with deciding the orientation of the fusions. Through the tethering of a NOX to an ADH, continuous alcohol oxidations could be performed with minimal cofactor supply. Here the key discoveries were: (1) differences in catalytic parameters such as k_{cat} and K_{M} do not necessarily translate to the same differences in conversions, and (2) since the fused ADH-NOX enzyme essentially acts like an alcohol oxidase, oxidase-based activity screening strategies can be used, which can enable rapid engineering of ADHs and/or NOXs.

In earlier work of the research group, fusions of the NADPH-regenerating phosphite dehydrogenase (PTDH) with Baeyer-Villiger monooxygenases (BVMOs) were studied (Torres Pazmiño et al.). **Chapter 5** expands on that work by adding glucose dehydrogenase (GDH) and formate dehydrogenase (FDH) as NADPH-regenerating fusion partners for a BVMO, as well for an ADH, to supply NADPH for ketone reductions. Altogether, with the PTDH,

GDH and FDH paired with a BVMO or an ADH, six fusion enzymes were created and applied for biotransformations. Takeaways from this study were: (1) tolerance as well as sensitivity to cosolvents can be somewhat transmitted through a fusion, (2) the use of a natural deep eutectic solvent (NADES) consisting of glucose and choline chloride can facilitate both glucose as substrate for the GDH, as well as solubilization of high concentrations of hydrophobic substrates. Lastly (3), although the different fusions can (negatively) affect substrate acceptance, most fusions retained activity and could be employed for gram-scale conversions.

Lastly, as general approach for the optimization of enzymes for biotechnological application, **Chapter 6** described the use of a computational strategy to guide the engineering of an alcohol dehydrogenase. The FRESCO approach was carried out, and after combining stabilizing mutations, a drastic improvement of thermostability was found, with a difference in melting temperature of + 51 °C (from 43 °C to 94 °C). Through crystallization, structure determination, and structural inspection thereof, an activity-impairing mutation was found and reverted. The final 9-fold mutant had almost no loss in activity compared to wildtype, while displaying a 130-fold increase in half-life (at 37 °C). This promising result shows that current methods can make a major difference for the application of enzymes for bioprocesses, since the vastly more stable enzyme can perform much better in an industrial setting.

The studies (**Chapters 3-6**) carried out for this thesis explored different strategies to optimize biotransformations with oxidoreductases. Although the fusion strategy can give some negative effects for particular enzymes, such as lower expression (**Chapter 5**, for GDH-ADH) and lower activity (**Chapter 3**, depending on the orientation of the fusion), in most cases described in this thesis the fusion was either beneficial or neutral. In other words, at the very least the strategy enabled successful production of two enzymes for the ‘cost’ of one. In the best case, it outperforms the combination of separate enzymes (**Chapter 3**), and gives utility for activity screening in enzyme engineering efforts (**Chapter 4**). In that sense, and considering the results presented herein, enzyme fusion is clearly a useful tool for some applications. Together with the results from **Chapter 6**, this thesis illustrates the potential of the engineering tools ‘enzyme fusion’ and ‘stability engineering’. As more theses and studies describe the development and use of such tools, the closer we get to truly industrial application of biocatalysis, with sustainable processes that require less energy and do not rely on fossil resources and harsh chemicals.

Supporting information

Supporting information

SI Chapter 3: Coupled reactions by coupled enzymes: alcohol to lactone cascade with alcohol dehydrogenase–cyclohexanone monooxygenase fusions

Table S1. Oligonucleotide primers for fusion cloning. Nucleotide order is from 5' to 3'. Fusions were made with either an *adhA*'s first gene ('begin'), with the *TmCHMO* gene downstream, or as second gene ('end'), after the *TmCHMO* gene.

begin_AdhA_NdeI_F	GGAATTCCATATGAAGGTTGCCGTAATTACTGGGG
begin_AdhA_XhoI_R	CTGACTCGAGTACTCAGGTTTTTGATAAATTGAGCG
end_AdhA_PvuII_F	GCTTCAGCTGGTATGAAGGTTGCCGTAATTACTGGGG
end_AdhA_ApaI_R	GACAGGGCCCTTAATACTCAGGTTTTTGATAAATTGAGCG
begin_AdhMi_NdeI_F	GGAATTCCATATGAAGCGTTTTGAGGGCAAGGTGG
begin_AdhMi_XhoI_R	CTGACTCGAGCCCTTCGCGGTATAACCACCATC
end_AdhMi_HindIII_R	CTCAAGCTTACTTCGCGGTATAACCACC
end_AdhMi_PvuII_F	GGAATTCCAGCTGGTATGAAGCGTTTTGAGGGCAAG
begin_AdhT_NdeI_F	GGAATTCCATATGAAAGGTTTCGCC
begin_AdhT_XhoI_R	CTGACTCGAGGCCAGAATAACAACCTGG
end_AdhT_PvuII_F	GCTTCAGCTGGTATGAAAGGTTTCGCCATG
end_AdhT_HindIII_R	CTCAAGCTTACGCCAGAATAACAACCTG
begin_TmCHMO_NdeI_F	GGAATTCCATATGAGCACCACCCAGACCC
begin_TmCHMO_XhoI_R	CTGACTCGAGGCCACCGCTTGCGCACGTTACCC
end_TmCHMO_PvuII_F	GCTTCAGCTGGTATGAGCACCACCCAGACCC
end_TmCHMO_HindIII_R	CTCAAGCTTACGCCACCGCTTGCGCACGTTCA

Figure S1. SDS-PAGE of the purification of 6 fusion constructs. Values of PageRuler sizes expressed in kDa. The 'eluted' fraction is the protein sample obtained after the elution with 500 mM imidazole.

Fig. S2 Blue native-PAGE, first stained with zymography (left tile of every fusion), and afterwards stained with Coomassie blue (right tile).

Fig. S3 Gel filtration chromatograms of the fusion enzymes, with elution volumes on the X-axis (mL) and the relative absorption at 280 nm on the Y-axis. Elution volumes lower than 11 mL correspond to a kDa $\geq$ 400, and volumes more than 13 mL correspond to molecular weights $\leq$ 100 kDa, based on calibration curve made from standards (BioRad). A: A-Tm (grey) and Tm-A (black). B: Mi-Tm (grey) and Tm-Mi (black). C: Tb-Tm (grey) and Tm-Tb (black).

Table S2. Fusion enzyme data

Enzyme	FAD ratio	Expression (mg/L culture)	Melting point [a] (°C)	Melting point [b] (°C)	Melting point [c] (°C)
ADHA	-	-	-	98.5	-
A-Tm	17.4	120	48.0	48.5	-
Tm-A	-	-	48.5	53.0	-
ADHMi	-	-	-	69.0	-
Mi-Tm	15.9	180	49.5	50.0	65.5
Tm-Mi	15.0	290	48.8	50.3	65.0
TbADH	-	-	-	93.0	-
Tb-Tm	16.2	320	48.8	51.5	85.3
Tm-Tb	14.4	320	49.0	49.5	-

Table S2. FAD ratio (A280/A441), expression, ThermoFluor® and ThermoFAD results. Melting point [a] is the ThermoFAD result, [b] is the ThermoFluor® value of the first peak, [c] is the ThermoFluor second peak.

Fig. S4 ThermoFluor® of the samples showing second peaks. Black line = Mi-Tm, dashed black line = Tm-Mi, grey line = Tb-Tm. The graphs represent the average of duplicate experiments.

Fig. S5 GC-MS result of the conversion of cyclohexanol to ϵ -caprolactone after 24 hours (Table 3, first row). Black = control, pink = TbADH with TmCHMO separately, blue = TbADH-TmCHMO fusion. The first peak, with a retention time of 4.8 minutes, corresponds to cyclohexanol. The second peak (5.0) is cyclohexanone. The external standard (acetophenone) has a peak at 7.7 min. The last peak, at 8.9 minutes, corresponds to ϵ -caprolactone.

Fig. S6 Stability of ADH activity (left) and CHMO activity (right) over time at 37 °C, 0.5 M KPi pH 8.0. The dashed lines represent the activity of the Tb-Tm fusion, the bold line the activity of the separate TbADH (left) or TmCHMO (right).

SI Chapter 4: Design of artificial alcohol oxidases: alcohol dehydrogenase – NADPH oxidase fusions for continuous oxidations

Table of contents:

Object	Page	Content
Table S1	111	List of primers
Figure S1	112	SDS-PAGE of cell-free extracts from all constructs
Figure S2	112	SDS-PAGE of purified fraction from all constructs
Figure S3	113	Absorbance (FAD) spectrum from ADH-NOX fusions before and after ferricyanide treatment
Figure S4	114	Activity curves fitted with Michaelis-Menten formula – ADH activity
Figure S5	115	Activity curves – NADPH oxidase activity
Figure S6	115	Chiral GC chromatograms – rac-1-phenylethanol conversions
Figure S7	116	Colony screening assay plates – after treatment

Table S1: List of primers: Primers used in this study are listed in the table below. The underlined part of the sequence is the BsaI recognition site, which is incorporated to allow Golden gate cloning. Gene1 indicates the gene coding for the enzyme that would be at the N-terminus side of the fusion, whereas Gene2 would be C-terminal.

Primer name	Primer sequence
Tbadh_gene1_fw	<u>CGGTCTC</u> GCCATATGAAAGGTTTCGCCATGC
Tbadh_gene1_rv	<u>CGGTCTC</u> TGCAGAGCCCGAGGCCAGAATAACAACCTGG
adhA_gene2_fw	<u>TAGGTCTC</u> GCTGCAGCTGGTATGACGTCCTCCTTCTCTCC
adhA_gene2_rv	CAAT <u>GGTCTCT</u> CAAGTTAGGCGCAGTAGCCGTTGTCTG
Lbadh_gene2_fw	<u>TAGGTCTC</u> GCTGCAGCTGGTATGAGCAACCGTCTAGATG
Lbadh_gene2_rv	<u>TGGTCTCT</u> CAAGTTATTGTGCCGTGTAGCC
Nox_gene1_fw	<u>CGGTCTC</u> GCCATATGGCCGGGCAGACGAC
Nox_gene1_rv	<u>CGGTCTCT</u> GCAAGAGCCCGAGGTGAGGACGAAACCTTCGTAGCCC
Nox_gene2_fw	<u>TGGTCTC</u> GCTGCAGCTGGTATGGCCGGGCAGACGAC
Nox_gene2_rv	<u>TGGTCTCT</u> CAAGTTAGGTGAGGACGAAACCTTCGTAG

QuikChange	Primer sequence
PAMO_C65D_fw	GGGGGCGCGGGACGACATCGAGAG
PAMO_C65D_rv	CTCTCGATGTCGTCCCGCGCCCCC
PAMO_bsai_fw	TGGTGTCTATCGAACAGCAC
PAMO_bsai_rv	ATAGACACCAGCATGTTGCTGAG

Figure S1 SDS-PAGE. Gel from the cell-free extract from each construct: 1. Ladder, 2. NOX, 3. ADHA, 4. LbADH, 5. TbADH, 6. NOX-A, 7. NOX-L, 8. T-NOX

Figure S2 SDS-PAGE purified fractions. Gel of each purified fraction: 1. Ladder, 2. ADHA, 3. NOX-A, 4. LbADH, 5. NOX-L, 6. TbADH, 7. T-NOX, 8. NOX

a) NOX-A before (blue) and after (green).

b) NOX-L before (blue) and after (green), with high and low concentration.

c) Tb-NOX before (green) and after (blue), with high and low concentration.

Figure S3 FAD spectra from NOX-ADH fusions. Absorption spectra taken from 200-700 nm, before and after treatment with ferricyanide.

Figure S4. Plots of initial rates (activity, y-axis) against cyclohexanol concentration (x-axis). Reaction conditions: 25 °C, 20 mM KPO₄ pH 7.5, 0.2 mM NADP⁺, 0.01-1 μ M of enzyme. Activity measurements were taken in duplicate or triplicate, with 7-10 different substrate concentrations. The 'in s' in the subtitles refers to the dimension on the Y-axis: in seconds. The data was plotted in GraphPad Prism 6, and fitted to the Michaelis-Menten equation using non-linear regression with least squares fit. Coefficient of determination ranged from $R^2 = 0.96$ to 0.98. Kinetic values obtained are presented in Table 2.

Figure S5. Plots of initial rates (activity, y-axis) against NADPH concentration (x-axis). Reaction conditions: 25 °C, 20 mM KPO₄ pH 7.5, varying concentrations of NADPH, 0.01-1 μM of fusion enzyme. Activity measurements were taken in duplicate or triplicate, with 6 different substrate concentrations. The data was plotted in GraphPad Prism 6, and fitted to the Michaelis-Menten equation using non-linear regression with least squares fit. Coefficient of determination: NOX-A $R^2 = 0.96$, NOX-L $R^2 = 0.93$, T-NOX $R^2 = 0.92$. Kinetic values obtained are presented in Table 2.

Figure S6 Chiral GC analysis. Results from kinetic resolution. Samples were extracted with ethyl acetate, dried over magnesium sulfate, and analyzed by chiral GC (Hydrodex β-TBDAC column (Aurora Borealis, The Netherlands)). The retention times were: acetophenone (10.5 min), (R)-1-phenylethanol (11.5 min), (S)-1-phenylethanol (11.6 min).

Figure S7: Colony based oxidase screening. a) Colonies expressing: ADHA (left), NOX-A (middle), NOX (right), then treated with assay mix (see Materials and methods). b) Replicate, with the three constructs on the same plate, plated in separate lanes (red striped lines).

SI Chapter 5: What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monooxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis

1. Analytical data

1.1. Panel of substrates studied in this work, and their respective products.

1.2. GC analyses for determination of conversions and enantiomeric excess.

The following columns were used: HP-1 (A), HP-5 (B), CP-Chirasil-Dex-CB (C) and Rt-bDEXse (D).

Compound	Column	Program ^a	Retention time (min)
1a	A	30/5/30/180/2	6.2
1b	A	30/5/30/180/2	8.7
2a	D	110/2.5/120/0/10/200/1	5.1
2b	D	110/2.5/120/0/10/200/1	11.8 (S), 12.1 (R)
3a	A	30/5/30/180/2	3.6
3b	A	30/5/30/180/2	6.8
4a	B	30/5/30/180/0	1.8
4b	B	30/5/30/180/0	7.1
5a	A	30/5/30/180/2	8.5
5b	A	30/5/30/180/2	9.9
5c	A	30/5/30/180/2	10.2
6a	C	110/0/2.5/120/0/10/200/1	4.8
6b	C	110/0/2.5/120/0/10/200/1	7.0 (R), 7.2 (S)
7a	C	120/2.5/140/5/170/10/200/1	5.3
7b	C	120/2.5/140/5/170/10/200/1	8.3 (R), 8.5 (S)
8a	C	110/2.5/140/10/200/1	8.5
8b	C	110/2.5/140/10/200/1	11.7 (R), 11.9 (S)
9a	C	110/0/2.5/120/0/10/200/1	7.8
9b	C	110/0/2.5/120/0/10/200/1	8.7 (S), 8.8 (R)
10a	C	130/5/10/180/3	6.8
10b	C	130/5/10/180/3	9.5 (R), 9.8 (S)
11a	C	130/5/10/180/3	6.2
11b	C	130/5/10/180/3	9.4 (R), 9.7 (S)
12a	C	110/0/2.5/120/0/10/160/2/20/180/3	6.7
12b	C	110/0/2.5/120/0/10/160/2/20/180/3	9.5 (R), 9.8 (S)

^a Program: initial temp. (°C)/time (min)/slope (°C/min)/temp. (°C)/time (min)/slope (°C/min)/temp. (°C)/time (min).

1.3. HPLC analyses for determination of enantiomeric excess.

For determination of the enantiomeric excess of sulfoxide **5b** the chiral HPLC column Chiralcel OD (25 cm x 0.46 cm) was employed.

- Flow rate: 0.8 mL/min.
- Eluent: *n*-hexane/2-propanol 90:10.
- Temperature: 25 °C.
- Retention times (±)-**5b**: t_R = 12.9 min and t_S = 16.5 min.

2. Biotransformations

2.1. Initial screening.

In preliminary studies, different reaction conditions were tested in order to determine the optimal conditions.

Entry	Fusion ^a	Cosubstrate	pH ^b	T (°C)	Conv. (%) ^c	ee (%) ^c
1	G-B	Glucose (10 mM)	7.5	30	76d	-
2	G-B	Glucose (10 mM)	8.5	30	85d	-
3	G-B	Glucose (30 mM)	8.5	30	>99	-
4	G-B	Glucose (10 mM)	8.5	24	>99	-
5	F-B	Formate (10 mM)	7.5	30	13d	-
6	F-B	Formate (10 mM)	8.5	30	16d	-
7	F-B	Formate (50 mM)	8.5	30	>99	-
8	P-B	Phosphite (20 mM)	8.5	30	>99	-
9	G-A	Glucose (10 mM)	7.5	30	>99	>99 (R)
10	F-A	Formate (50 mM)	7.5	30	>99	>99 (R)
11	P-A	Phosphite (20 mM)	7.5	30	>99	>99 (R)

^a 2 μM of *Tm*CHMO constructs were employed and 0.2 mg/mL of *Lb*ADH fusions. Cyclohexanone (5 mM) and acetophenone (5 mM) were chosen as model substrates for *Tm*CHMOs and *Lb*ADHs, respectively. ^b In all cases, 100 mM phosphate buffer was employed. ^c Determined by GC analysis based on areas (%). Conversion values are referred to the formed products. ^d Cyclohexanol was obtained as by-product.

2.2. Influence of different amounts of MTBE in the biotransformations.

All biotransformations were set up using 2 μ M *Tm*CHMO construct or 0.2 mg/mL *Lb*ADH fusion. The reaction media was composed of 100 mM phosphate buffer pH 8.5 (*Tm*CHMOs) or pH 7.5 (*Lb*ADHs), 1 mM NADPH and the corresponding cosubstrate (10 mM glucose, 50 mM sodium formate or 20 mM sodium phosphite). Cyclohexanone (5 mM) and acetophenone (5 mM) were employed as substrates. The reactions were incubated for 24 hours at 250 rpm and 24 °C (G-B) or 30 °C (other constructs).

Enty	Fusion	%v/v MTBE	Conv. (%) ^a	ee (%) ^a
1	G-B	1	>99	-
2	G-B	2.5	>99	-
3	G-B	5	>99	-
4	G-B	10	>99	-
5	G-B	15	78	-
6	G-B	30	56	-
7	F-B	1	>99	-
8	F-B	2.5	>99	-
9	F-B	5	>99	-
10	F-B	10	>99	-
11	F-B	15	>99	-
12	F-B	30	90	-
13	P-B	1	>99	-
14	P-B	2.5	>99	-
15	P-B	5	>99	-
16	P-B	10	>99	-
17	P-B	15	>99	-
18	P-B	30	91	-
19	F-A	1	>99	>99 (R)
20	F-A	2.5	>99	>99 (R)
21	F-A	5	>99	>99 (R)
22	F-A	10	>99	>99 (R)
23	F-A	15	>99	>99 (R)
24	F-A	20	>99	>99 (R)
25	F-A	30	>99	>99 (R)
26	F-A	40	>99	>99 (R)
27	F-A	50	91	>99 (R)
28	P-A	1	>99	>99 (R)
29	P-A	2.5	>99	>99 (R)
30	P-A	5	>99	>99 (R)
31	P-A	10	>99	>99 (R)
32	P-A	15	>99	>99 (R)
33	P-A	20	91	>99 (R)
34	P-A	30	40	>99 (R)
35	P-A	40	10	>99 (R)
36	P-A	50	<1	n.d.

^a Measured by GC analysis based on areas (%). Conversion values are referred to the formed products. n.d.: not determined.

2.3. Suitability of deep eutectic solvent (DES) composed by choline chloride (ChCl) and glycerol (Gly) with the enzyme fusions.

A DES composed by ChCl:Gly (1:2 mol/mol) was tested as cosolvent in the biotransformations catalysed by self-sufficient enzymes. Amounts of DES from 10% v/v to 30% v/v were added to the reaction media.

Effect of different amounts of ChCl:Gly (1:2 mol/mol) in the biotransformations catalyzed for fusion enzymes.

2.4. NADES with G-B

Several experiments were performed in order to test the capability of a NADES (natural deep eutectic solvents) composed of choline chloride and glucose (1.5:1 mol/mol) as both cosolvent and cosubstrate of the biotransformation catalyzed by *G-B*.

Entry	Addition of glucose	t (h)	Cycle	Conv. (%) ^a
1	Yes (10 mM)	24	1	>99
2	No	24	1	>99
3	No	2	1	23
4	No	4	1	51
5	No	6	1	100
6	No	16 ^b	2	16

^a Measured by GC analysis based on areas (%). Conversion values are referred to the formed product. ^b The reaction media from entry 5 was used for a second biotransformation after the extraction of the caprolactone produced in the first cycle.

2.5. Time-point study of Baeyer-Villiger oxidation of thioanisole (**5b**) mediated by *G-B*.

Conversions were monitored in the *G-B* mediated transformation of thioanisole (**5b**) into mixtures of methyl phenyl sulfoxide (**5b**) and (methylsulfonyl)benzene (**5c**) in order to confirm the early formation of **5b**.

Monitoring of G-B conversions in the Baeyer-Villiger oxidation of thioanisole (5b).

Entry	t (h)	Conv. (%) ^a	Conv. (%) 5b ^a	ee (%) 5b ^b	Conv. (%) 5c ^a
1	2	20	20	>99	<1
2	4	33	33	>99	<1
3	6	52	52	>99	<1
4	10	99	75	>99	20
5	16	>99	75	>99	21
6	24	>99	75	>99	21

^a Measured by GC based on areas (%). Conversion values are referred to the formed product. ^b Measured by HPLC.

2.6. Influence of substrate concentration.

– Biotransformations using 2 μM or 0.2 mg/mL of enzyme.

Entry	Fusion	[Fusion]	[Substrate] (mM) ^a	Conv. (%) ^b	ee (%) ^b
1	G-B	2 μM	5	>99	-
2	G-B	2 μM	10	31	-
3	G-B	2 μM	15	28	-
4	G-B	2 μM	20	13	-
5	G-B	2 μM	25	7	-
6	F-B	2 μM	5	>99	-
7	F-B	2 μM	10	60	-
8	F-B	2 μM	15	17	-
9	F-B	2 μM	20	14	-
10	F-B	2 μM	25	9	-
11	P-B	2 μM	5	>99	-
12	P-B	2 μM	10	>99	-
13	P-B	2 μM	15	82	-
14	P-B	2 μM	20	47	-
15	P-B	2 μM	25	25	-
16	F-A	0.2 mg/mL	5	>99	>99 (R)
17	F-A	0.2 mg/mL	10	72	>99 (R)
18	F-A	0.2 mg/mL	15	69	>99 (R)
19	F-A	0.2 mg/mL	20	58	>99 (R)
20	F-A	0.2 mg/mL	25	60	>99 (R)
21	P-A	0.2 mg/mL	5	>99	>99 (R)
22	P-A	0.2 mg/mL	10	96	>99 (R)
23	P-A	0.2 mg/mL	15	94	>99 (R)
24	P-A	0.2 mg/mL	20	88	>99 (R)
25	P-A	0.2 mg/mL	25	84	>99 (R)

^a Cyclohexanone was used as substrate for TmCHMO fusions and acetophenone for LbADHs. ^b Measured by GC analysis based on areas (%). Conversion values are referred to the formed products.

– Biotransformations using 4 μM or 0.4 mg/mL of enzyme.

Entry	Fusion	[Fusion]	[Substrate] (mM) ^a	Conv. (%) ^b	ee (%) ^b
1	G-B	4 μM	5	>99	-
2	G-B	4 μM	10	38	-
3	G-B	4 μM	15	28	-
4	G-B	4 μM	20	20	-
5	G-B	4 μM	25	12	-
6	F-B	4 μM	5	>99	-
7	F-B	4 μM	10	99	-
8	F-B	4 μM	15	93	-
9	F-B	4 μM	20	42	-
10	F-B	4 μM	25	15	-
11	P-B	4 μM	5	>99	-
12	P-B	4 μM	10	>99	-
13	P-B	4 μM	15	82	-
14	P-B	4 μM	20	53	-
15	P-B	4 μM	25	39	-
16	F-A	0.4 mg/mL	5	>99	>99 (R)
17	F-A	0.4 mg/mL	10	97	>99 (R)
18	F-A	0.4 mg/mL	15	96	>99 (R)
19	F-A	0.4 mg/mL	20	96	>99 (R)
20	F-A	0.4 mg/mL	25	80	>99 (R)
21	P-A	0.4 mg/mL	5	>99	>99 (R)
22	P-A	0.4 mg/mL	10	90	>99 (R)
23	P-A	0.4 mg/mL	15	90	>99 (R)
24	P-A	0.4 mg/mL	20	67	>99 (R)
25	P-A	0.4 mg/mL	25	53	>99 (R)

^a Cyclohexanone was used as substrate for *Tm*CHMO fusions and acetophenone for *Lb*ADHs. ^b Measured by GC analysis based on areas (%). Conversion values are referred to the formed products.

– Biotransformations using lyophilized *E. coli* preparations.

Entry	Fusion	[Substrate] (mM) ^a	Conv. (%) ^b	<i>ee</i> (%) ^b
1	G-B	5	9	-
2	G-B	10	<1	-
3	G-B	15	<1	-
4	G-B	20	<1	-
5	G-B	25	<1	-
6	F-B	5	>99	-
7	F-B	10	53	-
8	F-B	15	29	-
9	F-B	20	18	-
10	F-B	25	14	-
11	P-B	5	>99	-
12	P-B	10	60	-
13	P-B	15	38	-
14	P-B	20	25	-
15	P-B	25	21	-
16	F-Ac	5	>99	>99 (R)
17	F-Ac	10	>99	>99 (R)
18	F-Ac	15	98	>99 (R)
19	F-Ac	20	97	>99 (R)
20	F-Ac	25	94	>99 (R)
21	P-Ac	5	>99	>99 (R)
22	P-Ac	10	>99	>99 (R)
23	P-Ac	15	>99	>99 (R)
24	P-Ac	20	91	86 (R)
25	P-Ac	25	82	79 (R)

^a Cyclohexanone was used as substrate for *Tm*CHMO fusions and acetophenone for *Lb*ADHs. ^b Measured by GC analysis based on areas (%). Conversion values are referred to the formed products. ^c 10% v/v of MTBE was added as cosolvent in order to maintain an excellent stereoselectivity.

SI Chapter 6: Approaching boiling-point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering

Supplementary file 1A: Crystallographic data of the ADHA crystal structures.

	Wild-type ADHA bound to NADP ⁺	Wild-type ADHA	Mutant M9
PDB Code			
Wavelength (Å)	0.972640	0.978994	0.999998
Resolution range (Å)	45.54-1.60	44.30-2.00	49.16-2.50
Space group	I2	P2 ₁	P2 ₁
Unit cell (Å)			
a, b, c (Å)	76.11,113.14,124.21	52.52,87.25,102.92	51.67,86.52,102.75
α , β , γ (°)	90,94.0,90	90,101.6,90	90,100.6,90
Unique reflections	134154 (6625)	61616 (4486)	29671 (3412)
Multiplicity	3.6 (3.5)	4.8 (4.5)	4.5 (4.8)
Completeness (%)	97.4 (97.5)	99.7 (98.6)	96.0 (98.2)
Mean I/sigma (I)	6.0 (2.0)	14.3 (1.9)	3.8 (1.2)
R-merge (%)	15.5 (98.0)	6.1 (61.2)	19.3 (111.7)
CC _{1/2}	0.982 (0.398)	0.999 (0.785)	0.979 (0.637)
R-work (%)	14.1	19.5	18.9
R-free (%)	19.7	25.0	28.9
Number of non-hydrogen atoms	7849	7427	7291
Protein residues	246	245	246
RMS (bonds) (Å)	0.027	0.017	0.008
RMS (angles) (°)	2.50	1.84	1.19
Ramachandran favoured (%)	97.16	95.57	93.32
Ramachandran allowed (%)	2.84	4.0	6.47

Supplementary file 1B: Stabilizing mutations that disrupt activity.

#	Mutation	ΔT_m (°C)	k_{obs} (U/mg)	Possible effect(s)
1	A155F	14.0	< 0.01	Blocking active site, disrupting catalysis
2	S92E	12.0	< 0.01	Electrostatic interaction with catalytic Lys
3	S39D	7.8	< 0.01	Disrupts NADPH-phosphate binding through negative charge repulsion
4	R18D	7.0	< 0.01	Disrupts NADPH-phosphate binding through negative charge repulsion
5	R40E	7.0	< 0.01	Disrupts NADPH-phosphate binding through negative charge repulsion
6	N232H	6.5	< 0.01	Affects / disrupts dimer interface
7	D222Q	5.0	< 0.01	-
8	A155Y	4.5	0.02	Blocking active site, disrupting catalysis
9	S17D	4.0	n.d.	Disrupts NADPH-phosphate binding through negative charge repulsion
10	R40P	3.8	n.d.	Negatively affects NADPH-phosphate binding
11	Q44E	3.5	< 0.01	Disrupts NADPH-phosphate binding through negative charge repulsion

Note that all mutations that probably disrupt NADPH-binding introduce a negative charge (aspartic or glutamic acid). Determined activities are given in units / mg / min.

Supplementary file 2A. Expression comparison wild-type ADHA and M9 at different temperatures, with biological duplicates. The expression at 37 °C was 16 hours (overnight), whereas the expression at 30 °C and 24 °C were for 38 hours. Protein ladder (first lane on the left) is pre-stained PageRuler (ThermoScientific, MA USA). The black arrow indicated the position of the ADHA on the gel. Crude = crude extract, after sonication and before centrifugation. CFE = cell-free extract, after spinning down cell-debris. E = elute, fraction collected after addition of imidazole, during IMAC purification.

Supplementary file 2B. Purification of His-SUMO-M9*. The left arrow indicates the size of SUMO-M9*, the arrow in the right side indicates the size of M9* after SUMO cleavage. 1. Ladder, 2. Cell-free extract, 3. Flow-through, 4. First elute, 5. Second elute, 6. First flow-through after SUMO cleavage (low concentration), 7. First flow-through after SUMO cleavage (high concentration), 8. Second flow-through after SUMO cleavage (low concentration), 9. Second flow-through after SUMO cleavage (high concentration), 10. and 11. Elute with imidazole after SUMO cleavage.

Supplementary file 3A. Predicted values (in Gibbs free energy $\Delta\Delta G$, kJ/mol) of hits and correlation to experimental data (apparent melting temperatures).

	Apparent melting T	Number of values	Average ($\Delta\Delta G$ (kJ))	Median ($\Delta\Delta G$ (kJ))	Standard deviation
FoldX	Selected ($\Delta T_m \geq 3^\circ\text{C}$)	5	-35	-36	± 5
FoldX	$\Delta T_m \geq 3^\circ\text{C}$	10	-38	-36	± 14
FoldX	$\Delta T_m \geq 2^\circ\text{C}$	16	-34	-33	± 17
FoldX	$\Delta T_m < 2^\circ\text{C}$	47	-22	-19	± 14
FoldX	$\Delta T_m \leq 0^\circ\text{C}$	29	-21	-18	± 12
Rosettaddg	Selected ($\Delta T_m \geq 3^\circ\text{C}$)	9	-17	-18	± 7
Rosettaddg	$\Delta T_m \geq 3^\circ\text{C}$	25	-15	-12	± 9
Rosettaddg	$\Delta T_m \geq 2^\circ\text{C}$	43	-16	-13	± 11
Rosettaddg	$\Delta T_m < 2^\circ\text{C}$	108	-14	-11	± 10
Rosettaddg	$\Delta T_m \leq 0^\circ\text{C}$	74	-14	-11	± 9

As with **Supplementary file 3B**, the FoldX values show better correlation with the experimental data. The lower the number of values for a particular correlation, the less valuable the correlation, due to low sample size.

Supplementary file 3B. Calculated values compared to observed melting temperatures, fitted with linear regression in GraphPad prism 6.07. The graph depicts the slope from the linear regression, with the dotted lines representing the 95% confidence intervals of the slopes. Rosetta (left pane, circles): Pearson correlation of $r = 0.09$; no significant correlation ($P = 0.25$) between apparent T_m measurements and calculated stabilization ($\Delta\Delta G^{\text{Fold}}$, more negative is more stable) FoldX (right pane, diamonds): Pearson correlation of $r = 0.3$; significant correlation ($P < 0.02$).

Supplementary file 4A. Settings for GC and HPLC

GC:

Column: Chirasil-DEX CB column (25 m $\times$ 0.25 mm)

Injector temperature: 250°C

Detector temperature: 250°C

Column temperature: 110°C kept for 2 min, 2°C/min increased to 126°C, kept for 5 min, and then 2°C/min increased to 160°C

Injection volume: 1 μL

Split ratio: 50

Retention times: ketone (8.363 min), (*R*)-alcohol (12.500 min), (*S*)-alcohol (12.563 min)

HPLC:

Column: Chiralcel OD-H column (4.6 mm $\times$ 250 mm)

Detector wavelength: 254 nm

Mobile phase: n-Heptane/isopropanol (95/5)

Flow rate: 1 mL/min

Injection volume: 10 μL

Retention times: ketone (5.140 min), (*R*)-alcohol (8.905 min), (*S*)-alcohol (8.179 min).

Supplementary file 4B. GC chromatograms for ethyl-4-chloro-3-oxobutanoate (COBA)

Order of chromatograms: CTRL – substrate in the buffer, **PTDH** – substrate with everything except ADH, **WT** – reaction with ADHA wildtype, **M9F** – reaction with ADHA M9*, **R-CHBE** – Ethyl (R)-(+)-4-chloro-3-hydroxybutyrate commercial standard, **S-CHBE** – Ethyl (S)-(-)-4-chloro-3-hydroxybutyrate commercial standard.

Overlaid GC chromatograms for ethyl-4-chloro-3-oxobutanoate (COBA) – WT (green), M9* (purple), R-CHBE (red) and S-CHBE (blue).

Supplementary file 4C. HPLC chromatograms for 4-chloroacetophenone (4-CAP)

Order of chromatograms: **A** – substrate in the buffer, **B** – substrate with everything except ADH, **C** – reaction with ADHA wildtype, **D** – reaction with ADH M9*, **E** – (R)-1-(4-Chlorophenyl)ethanol standard, **F** – (S)-1-(4-Chlorophenyl)ethanol standard, **G** – Racemic 1-(4-Chlorophenyl)ethanol standard.

A – Substrate in the buffer (control)

<Chromatogram>

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5,140	8494473	1256928	100,000			
Total		8494473	1256928				

B – Substrate with everything except ADH

<Chromatogram>

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Area%	Height	Conc.	Unit	Mark
1	5,142	10980498	100,000	1408987	100,000		
Total		10980498	100,000	1408987			

C – Reaction with ADHA wildtype

<Chromatogram>

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Area%	Height	Conc.	Unit	Mark
1	5,148	1615	0,495	292	0,495		
2	8,257	320854	98,377	33266	98,377		
3	8,992	3678	1,128	357	1,128		
Total		326147	100,000	33914			

D – Reaction with ADH M9*

<Chromatogram>

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Area%	Height	Conc.	Unit	Mark
1	5,141	1030	0,324	194	0,324		
2	8,249	314142	98,825	32654	98,825		
3	8,990	2707	0,851	279	0,851		
Total		317878	100,000	33127			

E – (R)-1-(4-Chlorophenyl)ethanol standard**<Chromatogram>**

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	7,304	11189	503	0,000			
2	8,180	6950	484	0,000			
3	8,905	209316	13323	0,000			
Total		227455	14310				

F – (S)-1-(4-Chlorophenyl)ethanol standard**<Chromatogram>**

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	5,124	313366	12083	0,000			
2	8,179	207303	13291	0,000			
3	8,906	6442	434	0,000			
Total		527111	25808				

G – Racemic 1-(4-chloro)-phenylethanol standard**<Chromatogram>**

mAU

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Area%	Height	Conc.	Unit	Mark
1	8,175	165596	51,746	9964	0,000		
2	8,899	154418	48,254	9628	0,000		V
Total		320014	100,000	19592	0,000		

Nederlandse samenvatting

Fryske gearfetting

Curriculum vitae

List of publications

Acknowledgements/ Dankwoord

Nederlandse samenvatting

Enzymen zijn eiwitten die chemische reacties kunnen versnellen, en daarom ook biokatalysatoren worden genoemd. Bij de reacties zetten ze een substraat om naar een product.

Enzymen zijn selectief: alleen stoffen die het enzym in kunnen, worden omgezet. Enzymen zijn ook heel selectief in welke reacties ze versnellen, omdat het substraat slechts op een beperkt aantal manieren in het enzym past. Dit komt goed uit want dat voorkomt ongewenste producten, die bij chemische reacties vaak wel worden gevormd. De toepassing van enzymen is zeer interessant, omdat deze kunnen worden ingezet als alternatief voor huidige chemische processen.

In dit proefschrift wordt beschreven in hoeverre enzymen aan elkaar geplakt (gefuseerd) kunnen worden, en in hoeverre dat de toepassing van enzymen verbetert. In het bijzonder is er nadruk op oxidoreductases. Dit zijn enzymen die oxidatie- of reductiereacties versnellen. Bij dergelijke reacties worden elektronen verplaatst. Voor deze speciale reacties worden door de enzymen zogenaamde cofactoren gebruikt. Veel voorkomende cofactoren zijn NAD(P)H en FAD. FAD is van vitamine B2 afgeleid en zit in bepaalde oxidoreductases hecht gebonden en daardoor kunnen die enzymen reacties met elektronen versnellen.

Deze klasse van enzymen is uiterst geschikt om te fuseren, omdat de enzymen elkaar kunnen aanvullen: het ene enzym neemt elektronen van NADPH voor de eerste reactie (en vormt daarbij NADP⁺), en het andere enzym geeft vervolgens juist elektronen terug aan NADP⁺ (en vormt weer NADPH).

Hoofdstuk 1 geeft een overzicht van fusies met oxidoreductases in de literatuur tot nu toe. **Hoofdstuk 2** geeft een overzicht van (industriële) toepassingen met een specifiek type enzym, Baeyer-Villiger mono-oxygenases, die in de rest van het proefschrift ook zijn bestudeerd als fusie met een ander enzym. Met name is er aandacht voor de huidige obstakels voor het toepassen van dergelijke enzymen.

In **hoofdstukken 3, 4, en 5** zijn respectievelijk drie verschillende fusie-systemen onderzocht en beschreven, met verschillende combinaties van oxidoreductases. In het bijzonder wordt gebruik gemaakt van alcohol dehydrogenases (die NADP⁺/NADPH kunnen gebruiken), en Baeyer-Villiger mono-oxygenases (die FAD bevatten en daarmee met NADPH en zuurstof

reageren). De fusies werden getest op het vermogen om veel reacties te versnellen in langdurige reacties met veel reactant.

Deze langdurige reacties worden conversies genoemd. Dit zijn belangrijke metingen, omdat hiermee kan worden bepaald of de fusie van twee enzymen zich anders gedraagt dan de combinatie van dezelfde twee enzymen los van elkaar.

In **hoofdstuk 3** bleek dat de fusie meer omzettingen deed dan de combinatie van twee losse enzymen, en dus beter werkte voor die toepassing.

In **hoofdstuk 4** bleek dat de fusies kunnen worden gebruikt voor de detectie van activiteit en hiermee kan eenvoudig een groot aantal soortgelijke enzymen getest worden.

In **hoofdstuk 5** werden drie verschillende typen enzymen, die alle drie NADPH kunnen genereren, met elkaar vergeleken in fusie met een ander enzym, om te kijken welke van die drie het meest geschikt is voor toepassing.

In **hoofdstuk 6** wordt een andere strategie gebruikt om enzymen te verbeteren voor toepassing: mutagenese, met behulp van computerberekeningen. Een mutatie zorgt voor een verandering van één van de vele aminozuren in enzym. Zo kan een ander aminozuur meer interacties maken met de naastgelegen aminozuren, waardoor het eiwit stabielere wordt.

Door deze strategie toe te passen, kon de stabiliteit van een alcoholdehydrogenase verbeterd worden. De variant die is verkregen is, is veel weerbaarder en kan voor langere tijd op hoge temperatuur reacties versnellen, daar waar het oorspronkelijke enzym het al snel begaf.

Fryske gearfetting

Enzymen binne proteïnen dy't gemyske reaksjes fersnelle kinne en wurde dêrom ek biokatalysts neamd. Yn 'e reaksjes konvertearje se in substraat yn in produkt.

Enzymen binne selektyf: allinich stoffen dy't passe yn it enzym kinne omboud wurde. Enzymen binne ek hiel selektyf yn hokker reaksjes se fersnelle, om't it substraat allinich yn in it enzym past op in beheind tal wizen. Dit wurket goed, om't it foarkomt dat net winske produkten ûntsteane, dy't oars faak foarme wurde by gemyske reaksjes. It gebrûk fan enzymen is hiel ynteressant, om't se brûkt wurde kinne as alternatyf foar hjoeddeistige gemyske prosessen.

Dizze dissertaasje beskriuwt yn hoefier enzymen oan inoar plakt wurde kinne (fuseare), en yn hoefier dit it gebrûk fan enzymen ferbettert. Benammen is der klam op oksidoreduktases.

Dit binne enzymen dy't oksidaasje- of reduksjereaksjes fersnelle. Elektronen wurde oerset yn sokke reaksjes. Enzymen brûke saneamde kofaktors foar dizze spesjale reaksjes.

Kofaktors binne meinammen NAD(P)H en FAD. FAD is ôflaat fan fitamine B2 en is strak bûn yn wiswiere oksido-reduktasen, en dêrom kinne dizze enzymen reaksjes mei elektronen fersnelle. Dizze klasse fan enzymen is ekstreem geskikt foar fusearjen, om't de enzymen inoar oanfolle kinne: it iene enzym nimt elektroanen fan NADPH foar de earste reaksje (wêrtroch NADP⁺ foarme wurdt), en it oare enzym jout dan elektroanen werom nei NADP⁺ (en foarmt wer NADPH).

Haadstik 1 jout in oersjoch yn 'e literatuer oant no ta fan fúzjes mei oksido-reduktasen.

Haadstik 2 jout in oersicht fan (yndustriële) tapassingen mei in spesifyk soarte enzym, Baeyer-Villiger mono-oxygenases, dy't yn it ferfolg fan it proefskrift ek bestudearre waarden as in fúzje mei in oar enzym. Yn it bysûnder wurdt omtinken jûn oan de hjoeddeistige obstakels foar it gebrûk fan sokke enzymen.

Yn **haadstikken 3, 4 en 5** waarden trije ferskate fúzjesystemen ûndersocht en beskreaun, mei ferskate kombinaasjes fan oxidoreduktasen. Benammen wurdt gebrûk makke fan alkoholdehydrogenases (dy't NADP⁺/NADPH brûke kinne), en Baeyer-Villiger mono-oxygenases (dy't FAD befetsje en sa reagearje mei NADPH en soerstof).

De fúzjes waarden hifke op it fermogen om in soad reaksjes te fersnellen yn langduorjende, hege reaktante reaksjes. Dizze langer duorjende prosessen wurde konvertearingen neamd. Dit binne wichtige mjittingen, om't se brûkt wurde kinne om te bepalen oft de fúzje fan twa enzymen him oars benimt as de kombinaasje fan deselde twa enzymen ûnôfhinklik fan inoar.

Yn **haadstik 3** die bliken dat de fúzje mear konvertearje koe as de kombinaasje fan twa skiede enzymen, en wurke dêrom better foar dy tapassing.

Yn **haadstik 4** die bliken dat de fúzjes brûkt wurde kinne foar de deteksje fan aktiviteit, en hjir mei kin in grut oantal fergelykbere enzymen maklik test wurde.

Yn **haadstik 5** wurde trije ferskate soarten enzymen, dy't alle trije NADPH generearje kinne, fergelike yn in fúzje mei in oar enzym, om te sjen hokker fan dy trije it meast geskikt is foar gebrûk.

Yn **haadstik 6** wurdt in oare strategy brûkt om enzymen te ferbetterjen foar in tapassing: mutagenese, mei gebrûk fan komputerberekeningen. In mutaasje yn it enzym feroarsaket in feroaring fan ien fan it grutte oantal aminosieren yn it enzym. Op dizze wize kin in oar aminosuer mear ynteraksjes meitsje mei de neistlizzende aminosieren, wêrtroch it proteïne stabiler wurd.

Troch dizze strategy ta te passen, koe de stabiliteit fan in alkohol-dehydrogenase ferbettere wurde. De fariant dy't krigen wurde, is folle stabiler en kin reaksjes by in hege temperatuer foar in langere perioade fersnelle, wêr't it orizjinele enzym it al gau bejoech.

Curriculum vitae

Personal information

First names: Friso Sybren

Last name: Aalbers

Date of birth: 13-09-1991

Place of birth: Wyns, the Netherlands

Education

(2013-2015) MSc Molecular biology and biotechnology – University of Groningen

- Thesis at NGBI (Nutrition-Gut-Brain Interactions research group) – Örebro University, Sweden

(2009-2013) BSc Chemistry – University of Groningen

- Minor Plant Biotechnology – Wageningen University
- Chemistry of life track

(2003-2009) High school (VWO) – Piter Jelles Montessori Leeuwarden

Experience

(2019-present) Postdoc at Structural Biology lab, Department of Biology and Biotechnology – University of Pavia, Italy

(2015-2019) PhD student at Molecular enzymology group, Groningen Biomolecular sciences and biotechnology (GBB) – University of Groningen

(2013) Student assistant practical course ‘Cell biology’ – University of Groningen

Workshops

(2019) Masterclass “Computational Enzyme Library design” – University of Groningen

(2018) Summer School “Technology Transfer in Life Sciences” – Dresden, Germany

(2018) Process Development for Biooxidations – DTU, Lyngby, Denmark

(2017) Summer School “Biotechnology as a Key Enabling Technology” – Siena, Italy

(2017) Applied Biocatalysis – University of Groningen

(2015) Symposium “Industrial Enzymes” – ACIB, Pavia, Italy

List of publications

- (Chapter 6) Aalbers, F. S., Fürst, M. J., Rovida, S., Trajkovic, M., Castellanos, J. R. G., Bartsch, S., Vogel, A., Mattevi, A., & Fraaije, M. W. (2020). Approaching boiling point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering. *eLife* 2020;9:e54639
- (Chapter 2) Fürst, M. J. L. J., Gran-Scheuch, A., Aalbers, F. S., & Fraaije, M. W. (2019). Baeyer–Villiger Monooxygenases: Tunable Oxidative Biocatalysts. *ACS Catalysis*, 9(12), 11207-11241.
- (Chapter 5) Mourelle-Insua, Á., Aalbers, F. S., Lavandera, I., Gotor-Fernández, V., & Fraaije, M. W. (2019). What to sacrifice? Fusions of cofactor regenerating enzymes with Baeyer-Villiger monooxygenases and alcohol dehydrogenases for self-sufficient redox biocatalysis. *Tetrahedron*, 75(13), 1832-1839.
- Huang, L., Aalbers, F. S., Tang, W., Röhlig, R., Fraaije, M. W., & Kara, S. (2019). Convergent cascade catalyzed by monooxygenase–alcohol dehydrogenase fusion applied in organic media. *ChemBioChem*, 20(13), 1653-1658.
- (Chapter 4) Aalbers, F. S., & Fraaije, M. W. (2019). Design of Artificial Alcohol Oxidases: Alcohol Dehydrogenase–NADPH Oxidase Fusions for Continuous Oxidations. *ChemBioChem*, 20(1), 51-56.
- (Chapter 1) Aalbers, F. S., & Fraaije, M. W. (2019). Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes. *ChemBioChem*, 20(1), 20-28.
- (Chapter 3) Aalbers, F. S., & Fraaije, M. W. (2017). Coupled reactions by coupled enzymes: alcohol to lactone cascade with alcohol dehydrogenase–cyclohexanone monooxygenase fusions. *Applied microbiology and biotechnology*, 101(20), 7557-7565.
- Aalbers, F. S., Turkenburg, J. P., Davies, G. J., Dijkhuizen, L., & Van Bueren, A. L. (2015). Structural and functional characterization of a novel family GH115 4-O-methyl- α -glucuronidase with specificity for decorated arabinogalactans. *Journal of molecular biology*, 427(24), 3935-3946.

Acknowledgements / Dankwoord

This thesis could only have been made with the support of a number of very important people.

Beste **Marco**, sinds onze eerste echte kennismaking tijdens mijn bachelor-project, ben ik anders gaan denken over wetenschap, maar bovenal anders gaan denken over mezelf. Door jouw inspirerende manier van begeleiden en projecten opzetten heb ik heel veel kunnen leren, bereiken, samenwerken, en groeien. Je stelt je heel beschikbaar en flexibel op, wat veel ruimte geeft voor probeersels en zoektochten, wat ik zeer op prijs stelde. Daarnaast hou je altijd een oogje in het zeil, waardoor uiteindelijk alles op z'n plek valt. Wat ik echt aan je bewonder is hoe je ondanks al je successen en je positie iedereen heel gelijkwaardig en hartelijk behandelt, en dat is overduidelijk bij de labuitjes/retreats en avonden die je georganiseerd hebt. Voor dat alles ben ik je heel erg dankbaar. Ik droom ervan om op een dag een groep te kunnen beginnen zoals jij dat hebt gedaan. Ik wens je het allerbeste en heel veel succes voor de toekomst!

Beste **Dick**, ook al was je niet mijn directe begeleider, heb ik heel veel van je geleerd tijdens deze 4 jaren. Met name tijdens werkbесprekingen geeft je commentaar veel inzicht, en is je kritische houding inspirerend. De combinatie van jouw groep met die van Marco geeft een zeer goede en fijne werksfeer, in de labs, kantoren, en in de koffiehoeke.

I would like to thank the reading committee: **Prof. dr. Slotboom**, **Prof. dr. Poelarends** and **Prof. dr. Kara**, for their time to read through and comment on my thesis. Additional thanks to **Kara** and **dr. Lei Huang** for an excellent and fruitful collaboration project during my PhD.

I would like to thank my hosts for two terrific exchanges during my PhD: **Prof. dr. Woodley**, **Mafalda Dias Gomes** and **Murray Meissner** in Copenhagen-Lyngby, and **Prof. dr. Schwaneberg**, **Anna Gärtner**, and **Alex Weingartner** in Aachen. In addition, I thank the whole **ROBOX consortium** for the incredible collaboration and achievements.

Dear **Andrea**, thanks a lot for the collaboration and the exchange during my PhD. Your open and curious attitude are really inspiring, and I am sure I will have a great time in your group in Pavia.

Dear **Hanna**, even though you left the group 2-3 months after I joined, your friendly and incredibly helpful attitude had a long-lasting impact, and thank you for helping out at the start, which is always a challenging time.

Beste **Willem**, ik ben je dankbaar voor je enthousiaste positieve houding en voor je hulp aan het begin van mijn PhD. Dat ik van jouw je proefschrift kreeg met daarin jouw woorden “Hey Friso, veel plezier met je PhD!” is me bijgebleven en heeft zeker aan bijgedragen dat ik er plezier uithaalde!

Dear **Cyntia**, I had a great time with you in the office, and it was an honor that I was your paranymph. I really liked your playful and gentle attitude.

Misun, it meant a lot to me that you invited me to join your office at the start of my PhD. I really enjoyed chatting and discussing with you about anything, and I felt that we both appreciated it a lot. I think in that time we started talking about a band, and getting a couch for the office. I am so grateful to you that we started the band, and we played together for so long. It was such a unique and joyful experience. I like your playful, youthful and enthusiastic attitude. Thanks also to **Martin**, you are a very fun and cheerful guy, and I really enjoyed the time with the two of you. I wish you both all the best!

Max, I also knew from the moment we met that it would work out. Even though one could say that our personalities are quite different, I really had terrific times with you, starting from the crazy adventure in Italy, playing in the band together, all the way to now finishing the last collaboration. I also learned quite a lot from you, and you were incredibly helpful with advice and for the projects on which we worked together. Thank you for all that Max. **Cora**, your joyfulness and enthusiasm are really contagious, thank you for all the great times! I wish you both all the best!

Elvira (Romero), you are a really fun and great colleague! I had great times with you, and I learned a lot from you. Thank you for everything!

Brenda, thank you for your kindness and for the conversations we had. It felt really easy and comfortable to talk with you about anything.

Antonija, thank you for being such a positive, warm and friendly person. I really enjoyed chatting with you and having you in the group.

Elisa, thank you for teaching me and helping me out in the lab, and for being so friendly and helpful.

Dana, ik vond het heel leuk en gezellig met jou in de groep. Ook vond ik het fijn om met jou Nederlands te kunnen praten, anders was ik het toen misschien verleerd. Ik wens je veel goeds voor de toekomst!

Cara **Caterina**, my paranymp, you are such a joyful and supporting friend. I am so grateful for your enthusiasm and honesty, and all the help you gave with organizing my defense. I wish you a great future!

Caro **Simone**, I really enjoy chatting with you about anything, and appreciate your thoughtfulness and support. You together with Caterina were definitely a factor for my decision to go to Italy, as being examples of great Italian people that I've met. Then again, perhaps you are exceptions, since you both left Italy, but nevertheless you both carry something about this country and culture with you that sparked my curiosity and enthusiasm. Thank you for all the help as my paranymp, the help with all the documentation for going to Italy, for the insightful discussions about work, and of course for all the good times! **Ivana**, thank you for your kindness, and being so involved with all the social activities. I wish you both the best!

Nikola, thank you for your comradery, cheerfulness and supportiveness. You taught me a lot about how to be a good scientist, but also how to have good times, of which we had many. Thank you for being there with advice and support when I needed it. Also, thanks a lot for taking such a huge part in helping the lab. **Lur**, thank you for being such a warm and kind person, and for all the good times. I look forward to visiting Basque country!

Mohamed, thanks for all the good times and help, especially at the start of our PhD, going to many movies and helping out a lot. I really appreciate your kind and relaxed attitude. I wish you the best for the future!

Hemant, it was fun to have you in the group, you are a very chill and kind guy. Thank you for the good times!

Sam, it was nice to have shared the lab with you; my neighbour from across the aisle. It was quite helpful to discuss experiments and results with you.

Marzena, thank you for your enthusiasm and joyfulness, and for introducing me to the choir, which I enjoyed a lot.

Alejandro, you are such a kind, funny and chill guy, and I really enjoyed working with you. It was very easy to be open with you, and having you around also outside work at parties always catalyzed a fun, relaxed and social environment. Thank you for all the good times! **Mel**, thanks for being so cheerful and enthusiastic! I enjoyed playing together with you in the band, and in your band before!

Milos, thank you for being such a kind and helpful colleague. It was really fun to work with you on the lab. I wish **Jasna**, **Sofia**, and you a great time in the Netherlands!

Hugo, bedankt voor je hulp en advies! Je komt vaak met handige ideeën en suggesties, en je kritische houding bij werkbesprekingen geeft vaak interessante discussies.

Nikolas, thanks for all the great times! I loved making music with you. You are a such a fun and chill guy, and I really appreciate your knowledge of music.

Ángela, thank you for your enthusiasm, kindness and the great collaboration. It was a pleasure to host you the lab.

Jeroen, ik vond het heel leuk en gezellig met jou op het kantoor. Ik kon altijd makkelijk met jou praten, of het even bijkletsen was of iets van werk te bespreken. Dank je daarvoor en ik wens je veel succes en plezier!

Christiaan, bedankt voor de gezellige tijden en de fijne samenwerking! Je was zeer behulpzaam tijdens mijn PhD. Dank voor alles!

Elvira (Bombino), thank you for all the help and the good times!

Dear colleagues **Gautier**, **Eduardo**, **Qinlong**, **Estela**, **Yapei**, **Yiming**, **Andrea** (Fabara), **Hein**, **Carlos**, thanks for all the good times! It was really fun and pleasant to work with all of you, and I wish you the best!

Beste **Henriëtte** en **Andy-Mark**, bedankt voor alle gezellig momenten in de koffiehoeke en voor jullie hulp.

Beste **Coen**, **Lennart**, **Yannick**, en **Yaël**, mijn studenten, bedankt voor jullie geweldige inzet en samenwerking. Het was leuk en heel leerzaam om jullie te begeleiden. Veel succes voor de toekomst!

Reuben, it was really fun to hang out with you, at and outside work. I really enjoyed chatting with you about anything, and I dig your thoughtfulness and humour. **Ginny**, your cheerfulness is really contagious. Thank you for your kindness and enthusiasm! Thank you both for all the good times and being there at the farewell drink. I wish you both the best!

Linda, thank you for your joyfulness and kindness. Best wishes for you and your family.

Titia, ik vond het heel gezellig met jou op de afdeling en buiten lab! Je bent zo tof en enthousiast. Ik hoop dat je een hele leuke tijd tegemoet gaat!

Rudy, it was great to have you around and hang out with you. Have a great time!

Ilias, I really enjoyed hanging out with you, playing music with you, and playing DnD with you. You are such a fun and compassionate character! The band and the DnD group were two great sources of joy for me for the past two years, so thank you so much for that. I wish you the best for the future!

Simone (Romanini), **Nadia**, **Kostas**, **Despina**, **Ciccio**, **Cosimo**, **Jorge**, thank you all for the amazing fun times. I really appreciate your humour, jokes and relaxed attitudes. I had such a blast playing DnD with you all, and having you around at other social events. Best wishes to you all!

Mathias, I had such a great time with you when you were in Groningen. It was so nice to hang out with you, my only Magic-playing and Dota-playing colleague! I wish you the best for the future!

Vakil, it was very nice to chat and hang out with you. Especially at the start of our PhD we had some great times together, then we grew a bit apart, but nevertheless, I really enjoyed catching up with you! I wish you and **Alisa** all the best for the future!

Bouke, ik vind onze vriendschap heel bijzonder en fijn. We go way back, eestejaars Scheikunde aan de uni, 2010? Het is altijd zo chill, leuk, makkelijk en gezellig met jou. Bedankt dat je er voor me was tijdens mijn PhD, en voor alle gezellige tijden!

Andrea (Caruso), I really enjoy hanging out with you. You are such a cool, chill, kind and open guy. You were another great Italian person to me, which was a small factor in my consideration to move to Italy, and I know completely that it is not a logical reasoning, but perhaps there is still some truth in it. I really appreciate our friendship, and you really helped me think differently about myself. Thanks for being there during that time. Tante belle cose!

Jasper, tja wat zal ik zeggen. Gewoon vet mooi dat je er altijd voor me bent en we altijd leuke en gezellig tijden samen hebben. Onze vriendschap was een steunpilaar tijdens mijn PhD. Bedankt baas!

Beste matties **Bram** (van der Veen), **Job**, **Casper**, **Sander** en **Bram** (Boonstra), jullie zijn absolute toppers. Gedurende m'n hele PhD en de jaren daarvoor hebben we zoveel leuke tijden beleefd. Met name alle Lauwersoog-avonturen heb ik heel hoog staan!

Lieve **Anda**, **Bauke**, **Ids en Rienk**, mijn lieve, fijne, gezellige en gekke (op de beste manier) familie. Dank jullie wel voor al jullie liefde en steun. Ik hoop dat ik jullie trots heb gemaakt!

Leave **Pake en Beppe**, a goeie! Tankewol foar jo leafde, en dat jullie zo met mij meeleven. Lieve **Syb en Tineke**, bedankt voor jullie steun en jullie fijne berichten.

Lieve **Nina**, mijn liefste, mijn ontwerper. Ik ben je zo dankbaar voor al je liefde en steun. Sinds het moment dat we begonnen te daten, in februari, was ik helaas voor nog maar 10 maanden in Groningen, maar van al mijn 10 jaar dat ik in Groningen ben geweest waren dat wel mijn 10 leukste maanden. Je hebt een prachtig boekje voor me gemaakt, en je hebt me heel veel gesteund en geholpen met alle dingen voor mijn verdediging. Je was werkelijk mijn derde paranymph. Ik ben zo gek op je. Bedankt voor alles, liefje! Op naar een prachtige toekomst!