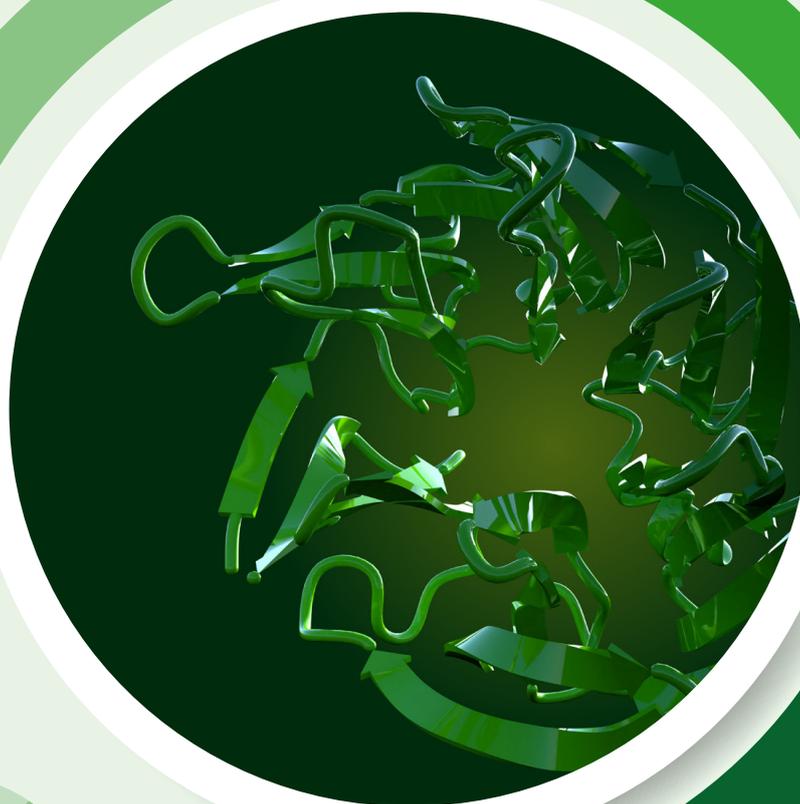


BIOTRANS 2021 • GRAZ

BOOK OF ABSTRACTS



BIOTRANS
GRAZ | 2021



15th International Symposium on Biocatalysis and Biotransformations
Graz | Austria | Europe | Worldwide online

July 19–22, 2021

Organized by



Design by Verena Resch (LuminousLab)

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COMMITTEES

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WELCOME TO BIOTRANS 2021!

It is a great honor and pleasure to welcome you to Biotrans 2021. 28 years after the very first Biotrans was held in 1993 in Graz, the 15th International Symposium on Biocatalysis and Biotransformations (Biotrans 2021) is for the first time hosted as an online conference by the University of Graz, Austria. In an exciting new format without borders, the Biotrans 2021 symposium provides the unique chance to bring the recent advances in biocatalysis research to every location in the world. Experimentalists as well as theoreticians joining from both academia and industry are presenting their recent findings covering the following fields:

- Artificial enzymes
- Biocatalytic synthesis and applications
- Cascades
- Computational approaches
- Enzyme engineering
- Mechanism and structure
- Natural pathways and metabolic engineering
- Novel enzymes, novel enzymatic reactions
- Reaction engineering and process design

The program is built around 4 keynote and 9 invited lectures, featuring 22 lectures and two presentations given by our Biotrans Award laureates. Approximately 380 poster presentations including short video poster pitches complete the program.

We would like to thank all participants for their contributions and express our appreciation to the national scientific advisory board and the Biotrans steering committee for their support and advice. Furthermore, we are grateful for the financial support provided by our sponsors.

Finally, we are looking forward to meeting you all and we wish you a scientifically fruitful, lively and enjoyable Biotrans 2021!

Wolfgang Kroutil (chair)
Mélanie Hall
Maryia Sumann
Christoph Winkler

Biotrans 2021 core team

DETAILED CONFERENCE PROGRAM

MONDAY JULY 19TH

TIME	all times are CET	
09:00 – 09:30	INTRODUCTION TO THE ONLINE PLATFORM given by our platform provider, DGM	
09:30 – 10:30	OPENING OF THE 15TH BIOTRANS Welcome by: <ul style="list-style-type: none"> - Vice-Rector Dr. Peter Riedler (University of Graz) - Dr. Günter Riegler, member of the city council of the City of Graz - Acknowledging the founders of the Biotrans series and chairs of Biotrans 1993: Prof. Herfried Griengl, Prof. Walter Steiner - Looking back to 14 previous Biotrans editions <ul style="list-style-type: none"> - Music by the band “Gimpelinsel Saitenmusi” performing violin music typical of the region “Ausseerland” located in the north western part of Styria - Graz/Styria/Austria: the host of Biotrans 2021 Wolfgang Kroutil, Mélanie Hall and Christoph Winkler (University of Graz) 	
SESSION 1 Chair: Wolfgang Kroutil		
10:30 – 11:15	<u>KEYNOTE</u> NICHOLAS TURNER » University of Manchester/UK The golden age of biocatalysis	
11:15 – 11:45	<u>INVITED LECTURE</u> MARCO FRAAIJE » University of Groningen/NL On the functionally flexible flavin cofactor: recent examples of resurrection and redesign of flavoenzymes	
11:45 – 12:15	<u>INVITED LECTURE</u> FRANCESCA PARADISI » University of Bern/CH Applications and evolution of a very versatile acetyl transferase	
12:15 – 13:00	MEET & MINGLE: NETWORKING (12:15 - 13:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms. Meet the speakers of the previous session in the following rooms: Nick Turner » English Pub Marco Fraaije » Beer Garden Francesca Paradisi » Clocktower Rooftop Bar	STAY FIT (12:25 – 12:40) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).
13:00 – 13:40	BREAK	
SESSION 2 Chair: Sergio Riva		
13:40 – 14:00	<u>LECTURE</u> FELIX KASPAR » Technische Universität Berlin/DE Diversification of nucleoside analogues by nucleoside phosphorylases	
14:00 – 14:20	<u>LECTURE</u> MARIIA BELIAEVA » University of Basel/CH <i>In vitro</i> production of ergothioneine isotopologues	

14:20 – 14:40	<u>LECTURE</u> BERNHARD HAUER » University of Stuttgart/DE Active-site loop variations adjust activity and selectivity of the cumene dioxygenase	
14:40 – 15:00	<u>LECTURE</u> ANIA FRYSZKOWSKA » Merck Sharp & Dohme/US Site-selective functionalization of native proteins using engineered enzymes	
15:00 – 15:30	<u>INVITED LECTURE</u> ELINA SIIROLA » Novartis/CH Learning to learn: Imine reductase improvement <i>via</i> (machine) directed evolution	
15:30 – 16:00	MEET & MINGLE: NETWORKING (15:30 – 16:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms. Meet the speakers of the previous session in the following rooms: Felix Kaspar » Beer Garden Mariia Beliaeva » Murinsel Café Bernhard Hauer » Wine Bar Ania Fryszkowska » Juice Bar Elina Siirola in the » Schloßberg Terrace	STAY FIT (15:40 – 15:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).
SESSION 3 Chair: Christoph Winkler		
16:00 – 16:30	POSTER PITCHES Presentation of the first seven of the 21 selected poster video pitches. For the live voting for the Poster Video Pitch Award, use link provided in the online program.	
16:30 – 16:50	<u>LECTURE</u> SOUMITRA ATHAVALE » California Institute of Technology/US Enantioselective, intermolecular amidation of benzylic C-H bonds with engineered P411 enzymes	
16:50 – 17:10	<u>LECTURE</u> DAVID PALMER » University of Saskatchewan/CA Repurposing an aldolase for heterocycle synthesis	
17:10 – 17:30	<u>LECTURE</u> ZHEN LIU » California Institute of Technology/US Enantioselective carbene N-H insertion reactions with P411 enzymes	
17:30 – 18:00	MEET & MINGLE: NETWORKING (17:30 – 18:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms. Meet the speakers of the previous session in the following rooms: Soumitra Athavale » Clocktower Rooftop Bar David Palmer » Cocktail Bar Zhen Liu » Beer Garden	STAY FIT (17:40 – 17:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).
18:00 – 20:00	RECEPTION Hosts: Wolfgang Kroutil, Mélanie Hall, Mathias Pickl, Christoph Winkler (University of Graz) <u>Main stage:</u> - Welcome address - Music by the band “Gimpelinsel Saitenmusi” - Biotrans Quiz <u>Meet & Mingle:</u> Continue meeting colleagues and biocatalysis enthusiasts in the „Meet & Mingle“ rooms	

TUESDAY JULY 20TH

TIME	all times are CET	
	SESSION 4 Chair: Wolf-Dieter Fessner	
09:00 – 09:45	<u>KEYNOTE</u> TOBIAS ERB » Max Planck Institute for Terrestrial Microbiology/DE How to capture CO ₂ : Designing new-to-nature enzymes, reaction cascades and artificial cells for a sustainable biocatalysis	
09:45 – 10:30	POSTER PITCHES Presentation of the remaining 14 of the 21 selected Poster Video Pitches. For the live voting for the Poster Video Pitch Award, use link provided in the online program.	
10:30 – 11:00	MEET & MINGLE: NETWORKING (10:30 - 11:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms. Meet the speaker of the previous session in the following room: Tobias Erb » Tea House	STAY FIT (10:40 – 10:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).
	SESSION 5 Chair: Sabine Flitsch	
11:00 – 11:30	<u>INVITED LECTURE</u> GIDEON GROGAN » University of York/GB Enzyme-catalyzed amidations by ATP-dependent amide ligases	
11:30 – 11:50	<u>LECTURE</u> AMIT MONDAL » Centre of Biomedical Research (CBMR)/IN Chemoenzymatic total synthesis of (–)-Luteoskyrin and (–)-Deoxyluteoskyrin	
11:50 – 12:10	<u>LECTURE</u> THOMAS THORPE » University of Manchester/GB A multi-functional biocatalyst that catalyses both conjugate reduction and reductive amination	
12:10 – 12:30	<u>LECTURE</u> HELEN HAILES » University College London/GB Enzymatic synthesis of novel halogenated alkaloids using a ‘parallel cascade’ strategy <i>in vitro</i>	
12:30 – 13:15	MEET & MINGLE: NETWORKING (12:30 - 13:15) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms. Meet the speakers of the previous session in the following rooms: Gideon Grogan » English Pub Amit Mondal » Schloßberg Terrace Thomas Thorpe » Coffeeshop Helen Hailes » Tea House	STAY FIT (12:40 – 12:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).
13:15 – 14:00	BREAK	
	SESSION 6 Chair: Vicente Gotor-Fernández	
14:00 – 14:30	<u>INVITED LECTURE</u> SILVIA OSUNA » Universitat de Girona & ICREA/ES Conformational regulation in enzyme design and evolution	
14:30 – 15:00	<u>INVITED LECTURE</u> EMILY BALSUS » Harvard University/US Chemical discovery in the microbial world	

15:00 – 15:45	<p><u>KEYNOTE</u> JIŘÍ DAMBORSKÝ » Masaryk University/CZ Web-based computational enzymes design for biocatalysis</p>	
15:45 – 16:15	<p>MEET & MINGLE: NETWORKING (15:55 – 16:10) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms.</p> <p>Meet the speakers of the previous session in the following rooms:</p> <p>Silvia Osuna » Juice Bar Emily Balskus » Murinsel Café Jiří Damborský » Beer Garden</p>	<p>STAY FIT (15:45 – 16:15) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).</p>
16:15 – 18:15	<p>POSTER SESSION 1 You may use the platform search function to find posters about the topics you like. For a good overview, see the digital book of abstracts. Make use of the “Group Video Chat” for a live discussion or the “Ask a question” feature for a text-based interaction. Use this chance to interact with others and make it a lively poster session!</p>	

WEDNESDAY JULY 21ST

TIME	all times are CET
09:00 – 11:00	<p>POSTER SESSION 2 You may use the platform search function to find posters about the topics you like. For a good overview, see the digital book of abstracts. Make use of the “Group Video Chat” for a live discussion or the “Ask a question” feature for a text-based interaction. Use this chance to interact with others and make it a lively poster session!</p>
	<p>SESSION 7 Chair: Mélanie Hall</p>
11:00 – 11:45	<p><u>KEYNOTE</u> MAGALI REMAUD-SIMEON » Toulouse Biotechnology Institute/FR Understanding and controlling alpha-glucan synthesizing enzymes</p>
11:45 – 12:05	<p><u>LECTURE</u> FRANCESCO MUTTI » University of Amsterdam/NL Protein engineering and applications of amine dehydrogenases</p>
12:05 – 12:25	<p><u>LECTURE</u> MIQUEL ESTÉVEZ-GAY » Universitat de Girona/ES Computational exploration and design of new halohydrin dehalogenase variants</p>
12:25 – 12:45	<p><u>LECTURE</u> MACIEJ SZALENIEC » Jerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Science/PL Tungsten Aldehyde Oxidoreductase – a new type of hydrogenase</p>
12:45 – 13:30	<p>MEET & MINGLE: NETWORKING Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms.</p> <p>Meet the speakers of the previous session in the following rooms:</p> <p>Magali Remaud-Simeon » Murinsel Café Francesco Mutti » Schloßberg Terrace Miquel Estévez-Gay » Wine Bar Maciej Szaleniec » Clocktower Rooftop Bar</p>
13:30 – 14:15	BREAK
14:15 – 14:45	<p><u>EXHIBITION EPPENDORF</u> NINA SCHRAND Feed automation in a <i>Pichia pastoris</i> bioprocess based on constant respiratory quotient Chair: Silvia Glueck</p>
14:45 – 15:45	<p>STAY FIT Join an extended „stay fit“ session to shake off the conference stiffness and power yourself with positive energy. Today our coach Doris Plank will help you recharge physically through a series of vitalizing exercises.</p> <p>Please, get a ball (Tennis ball) or similar, or a filled 0.5 L bottle. Please, prepare to get barefoot - if possible</p>
15:45 – 17:45	<p>EXCURSION: A TOUR OF GRAZ Professional tourist guide: Kerstin Hillsberg Host: Somayyeh Gandomkar</p> <p>Enjoy a virtual visit to Graz. A professional tourist guide will present the City of Graz in a live tour. You may ask questions on the run. In case of bad weather in Graz the guide will visit GrazMuseum.</p>

17:00 – 18:00	<p>CAREER PATHS IN BIOCATALYSIS 1 In the open panel discussion about career paths in biocatalysis you can meet and interact with scientists at different stages of their careers.</p> <p><u>Career in academia 1:</u> In this session, get a perspective from established seniors: Nicholas Turner (University of Manchester/UK) and Kurt Faber (University of Graz/AT) Host: Willem Breukelaar</p> <p><u>Women in academia:</u> In this session, meet two female PIs: Emily Balskus (Harvard University/USA) and Francesca Paradisi (University of Bern/CH) Host: Elisa Lanfranchi</p> <p><u>Career in industry:</u> In this session, meet two researchers from chemical industry: Elina Siirola (Novartis/CH) and Doru Roiban (GSK/UK) Host: Sarah Bierbaumer</p> <p><u>Founding a company:</u> In this session, meet founders Christian Gruber (founder of Innophore/AT) and Marco Fraaije (professor at University of Groningen and founder of Gecco Biotech/NL) Host: Mathias Pickl</p>
18:00 – 19:00	<p>CAREER PATHS IN BIOCATALYSIS 2 In the open panel discussion about career paths in biocatalysis you can meet and interact with scientists at different stages of their careers.</p> <p><u>Career in academia 2:</u> In this session, get a perspective from recently promoted PIs: Elaine O'Reilly (University College Dublin/GB) and Todd Hyster (Cornell University/USA) Host: Stefan Payer</p> <p><u>From academia to industry:</u> In this session, meet researchers who recently moved from academia to industry: Stefan Lutz (Codexis/USA) and Bettina Nestl (Innophore/AT) Host: Somayyeh Gandomkar</p> <p><u>Career as editor:</u> In this session, meet two journal editors from the Wiley publishing house: Charlotte Gers-Panther (ChemCatChem) and Ruben Ragg (ChemBioChem) Host: Jörg Schrittwieser</p>

THURSDAY JULY 22ND

TIME	all times are CET	
	SESSION 8 Chair: László Poppe	
09:00 – 09:30	<u>INVITED LECTURE</u> BYUNG-GEE KIM » Seoul National University/KR Unfinished story of Polyphenol Oxidases	
09:30 – 09:50	<u>LECTURE</u> DIRK TISCHLER » Ruhr-Universität Bochum/DE Sequence-function relation in flavin-dependent epoxidases	
09:50 – 10:10	<u>LECTURE</u> DAIJUN ZHENG » Toyama Prefectural University/JP Enantioselective cleavage of N-O bond: a cyanide-free strategy accessing to chiral β -hydroxy nitriles from olefins	
10:10 – 10:30	<u>LECTURE</u> LAURINE DUCROT » Université Paris-Saclay/FR Expanding the substrate scope of amine dehydrogenases by protein engineering	
10:30 – 11:00	<p>MEET & MINGLE: NETWORKING (10:30 – 11:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms.</p> <p>Meet the speakers of the previous session in the following rooms:</p> <p>Byung-Gee Kim » Schloßberg Terrace Dirk Tischler » Beer Garden Daijun Zheng » Murinsel Café Laurine Ducrot » Tea House</p>	<p>STAY FIT (10:40 – 10:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).</p>
	SESSION 9 Chair: Thomas Ward	
11:00 – 11:30	<u>INVITED LECTURE</u> GERARD ROELFES » University of Groningen/NL Designer enzymes featuring non-canonical amino acids as catalytic residue	
11:30 – 11:50	<u>LECTURE</u> LOTHAR ELLING » RWTH Aachen University/DE Enzyme cascades for the synthesis of hyaluronic acid	
11:50 – 12:10	<u>LECTURE</u> ELIF ERDEM » Aix Marseille University/FR Engineering NADPH dependent oxyfunctionalization in cyanobacteria	
12:10 – 12:30	<u>LECTURE</u> EDUARDO MACEDO DE MELO » Forschungszentrum Juelich/DE An approach for temporal regulation of one-pot multienzymatic cascades by local magnetic heating	
12:30 – 13:15	<p>MEET & MINGLE: NETWORKING (12:30 – 13:15) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms.</p> <p>Meet the speakers of the previous session in the following rooms:</p> <p>Gerard Roelfes » Murinsel Café Lothar Elling » Clocktower Rooftop Bar Elif Erdem » Juice Bar Eduardo Macedo De Melo » Wine Bar</p>	<p>STAY FIT (12:40 – 12:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).</p>

13:15 – 14:00	BREAK	
	SESSION 10 Chair: Laurence Hecquet	
14:00 – 14:20	<u>LECTURE</u> PASCAL SCHNEIDER » Heinrich-Heine University/DE Biocatalytic C-3 indole methylation – A useful tool for natural product synthesis	
14:20 – 14:40	<u>LECTURE</u> MARTIN GRININGER » Goethe University Frankfurt/DE Custom synthesis of new-to-nature fatty acids and polyketides by transferase design	
14:40 – 15:00	<u>LECTURE</u> JULIE DUCHARME » McGill University/CA Investigation of the conformational dynamics involved in cytochrome P450 3A4 allostery	
15:00 – 15:30	<u>INVITED LECTURE</u> CHRISTOPHER PRIER » Merck & Co./US Development of a flow process using an immobilized enzyme for the transamination of cyrene	
15:30 – 16:00	<p>MEET & MINGLE: NETWORKING (15:30 – 16:00) Interact with other conference participants <i>via</i> video chat in one of the “Meet & Mingle” rooms.</p> <p>Meet the speakers of the previous session in the following rooms:</p> <p>Pascal Schneider » Cocktail Bar Martin Grininger » Wine Bar Julie Ducharme » Tea House Christopher Prier » Coffeeshop</p>	<p>STAY FIT (15:40 – 15:55) Join our coach (Doris Plank) for stretching exercises at the main stage (no equipment required).</p>
	SESSION 11 Chairs: Ania Fryszkowska + Vladimír Křen	
16:00 – 16:30	<u>acib BIOTRANS JUNIOR AWARD</u> TODD HYSTER » Cornell University/US Photoenzymatic catalysis - using light to reveal new enzyme functions Award ceremony, followed by the Junior Award lecture	
16:30 – 17:00	<u>acib BIOTRANS SENIOR AWARD</u> KURT FABER » University of Graz/AT Finding enzymes by serendipity Award ceremony, followed by the Senior Award lecture	
17:00 – 17:15	POSTER PRIZES & PITCH TALK PRIZES	
17:15 – 17:45	CLOSING Laurence Hecquet (University Clermont Auvergne/FR) Wolfgang Kroutil, Mélanie Hall, Christoph Winkler (University of Graz/AT)	

STAY FIT @ BIOTRANS



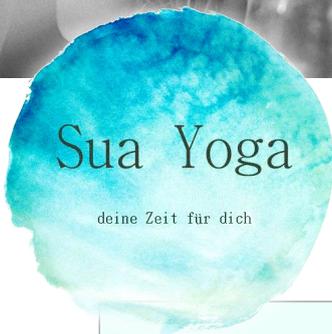
Dr.ⁱⁿ Doris Plank
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ABOUT ME

Next to my interests in chemistry and science, I developed my enthusiasm for movement very early on. Physical exercise, spiritual pursuits and travelling the world have taught me that movement is crucial for quality of life and well-being. Characteristics of my sessions are simple and accessible movements for everyone, everywhere.

Keep it simple sessions are great for corporate events, seminars and retreats.

Feel free to contact me so I can put together the perfect program for your next event!



REQUIREMENTS	<p>Space: just put your chair to the side.</p> <p>Plank Challenges and Wednesday: we will also be on the floor – maybe get a towel, yoga mat or similar.</p> <p>For your own comfort don't wear your tightest clothes, just something in which you can move a bit and take off your shoes.</p>
MONDAY	
1 st Session 12:25-12:40	Welcome & Body activation Simple mini flow for an all over body activation.
2 nd Session 15:40- 15:55	Stabilize and Center – Plank Challenge I Enter this standing mini flow – to keep a stable core.
3 rd Session 17:40-17:55	Stretch & Relax Mini flow designed for shoulders and neck wellness.
TUESDAY	
1 ST Session 10:40-10:55	Morning Power Dynamic mini flow - movements to energize and boost you.
2 ND Session 12:40-12:55	Balance & Focus – Plank Challenge II Mini flow to enhance your balance skills.
3 RD Session 15:55-16:10	Twist & Unwind Twisted mini flow – you will find yourself in some bound versions of simple poses.
WEDNESDAY	
14:45-15:45	Keep it simple! Surprise hour – please, get a ball (Tennis ball) or similar, or a filled 0.5 L bottle. Please, prepare to get barefoot - if possible.
THURSDAY	
1 st Session 10:40-10:55	Feet on the Ground Stabilizing mini flow with focus on your feet. Please, prepare to get barefoot - if possible.
2 nd Session 12:40-12:55	Stabilize and Center – Plank Challenge III Enter this standing mini flow – to keep a stable core – 2nd edition
3 rd Session 15:40-15:55	Close your Eyes Relaxing mini flow with calming breathing and exercises for your eyes.



One for All

SciVario® twin - Innovative, Intuitive, Intelligent

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In the EU project *PhotoBioCat* 12 international doctoral students under expert guidance use light as a “fuel” to accelerate enzymatic reactions by means of cyanobacteria. It is hoped that this will make the biocatalytic production of chemicals considerably more sustainable.

The project is coordinated by a team led by Robert Kourist, head of the University of Graz’s Institute of Molecular Biotechnology. The University of Graz’s Institute of Chemistry is also on board. The project is part of the NAWI Graz network, in one of Austria’s oldest and largest university systems.

PhotoBioCat has two main areas of focus. In one area, the use of cyanobacteria as biocatalysts for light-driven biotechnological applications is being examined and tested in a range of industrially relevant model reactions. Chemicals for polymers, cosmetics and medicines are being increasingly technologically produced using enzymes to accelerate reactions. However, up to now the enzymes have had to be driven using reducing equivalents — very complex molecules which are very expensive to synthesize.

The second area of focus of the project will be on raising the efficiency by which light energy is harvested and can be passed on to enzymatic reactions (in vitro, in other words without living carrier organisms, such as cyanobacteria).



PhotoBioCat’s Early-Stage Researchers

More information about the project: <http://www.photobiocat.eu>



This project has received funding from the European Union’s Horizon 2020 MSCA ITN-EJD program under grant agreement No 764920.



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esib / 2022
14TH–16TH NOVEMBER

Don't miss our upcoming networking event, the European Summit of Industrial Biotechnology.

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Company profile

Biotech company

with global technology leadership in enzyme engineering and bioprocess development for regulated markets such as the food and pharmaceutical industries

Visionary management team

with a strong and experienced second management level

Technology platform

for the development of new enzymes based on the principles of natural evolution

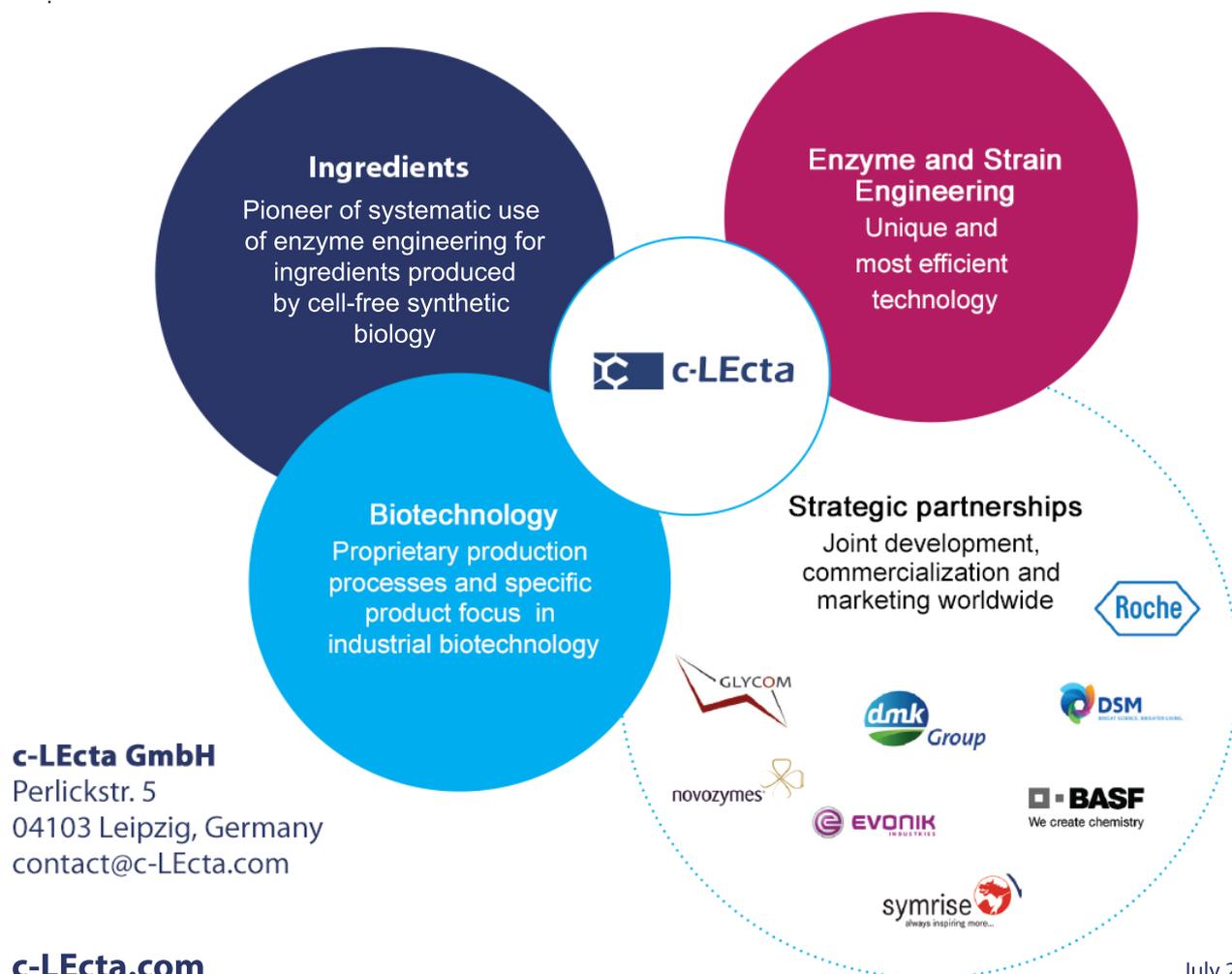
Megatrends

Participation in global megatrends in the areas of health, nutrition and Well-being

50 enzyme engineering projects

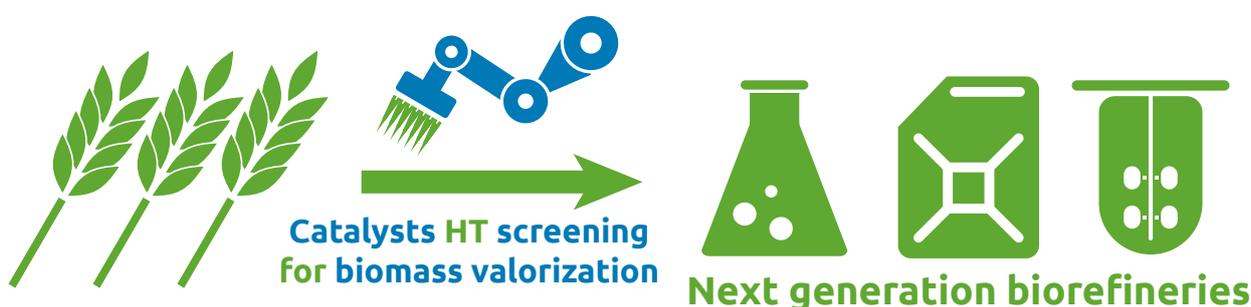
in the last 10 years with a success rate of over 90%, more than 15 years on the market

Business model



Brings catalysis over lightspeed

REALCAT

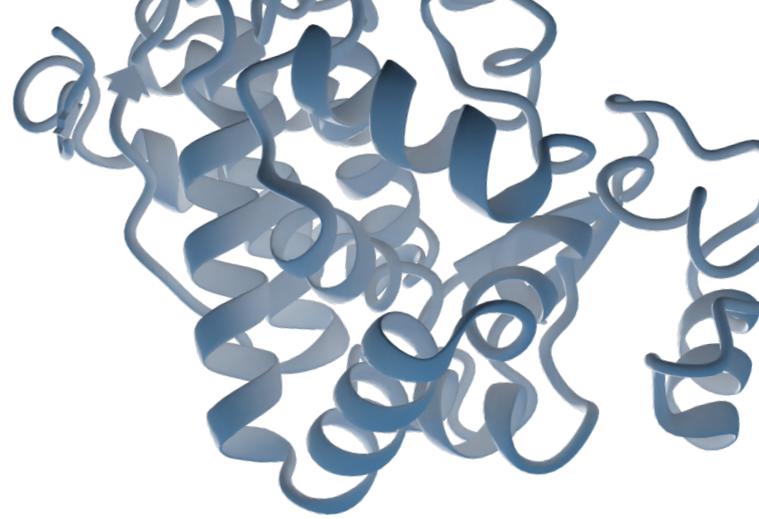


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KEYNOTE LECTURES



The Golden Age of Biocatalysis

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The true power of biocatalysis for chemical synthesis is now starting to be realised. Major technological developments in the past 10 years have transformed the way that we, as a community, are able to discover new enzymes, improve them so that they are fit for purpose, and then apply these engineered biocatalysts for target molecule synthesis. Enabling tools such as metagenomics, bioinformatics, protein engineering, directed evolution, *in silico* design etc. have led to a rapid expansion of the biocatalytic toolbox. This toolbox can now be more widely applied for organic synthesis, opening up new opportunities for retrosynthetic pathway design, and increasingly the use of multi-enzyme cascades for complex molecule synthesis. This lecture will draw on past and present experiences in the field and also look forward to try to anticipate what the future might bring [1].

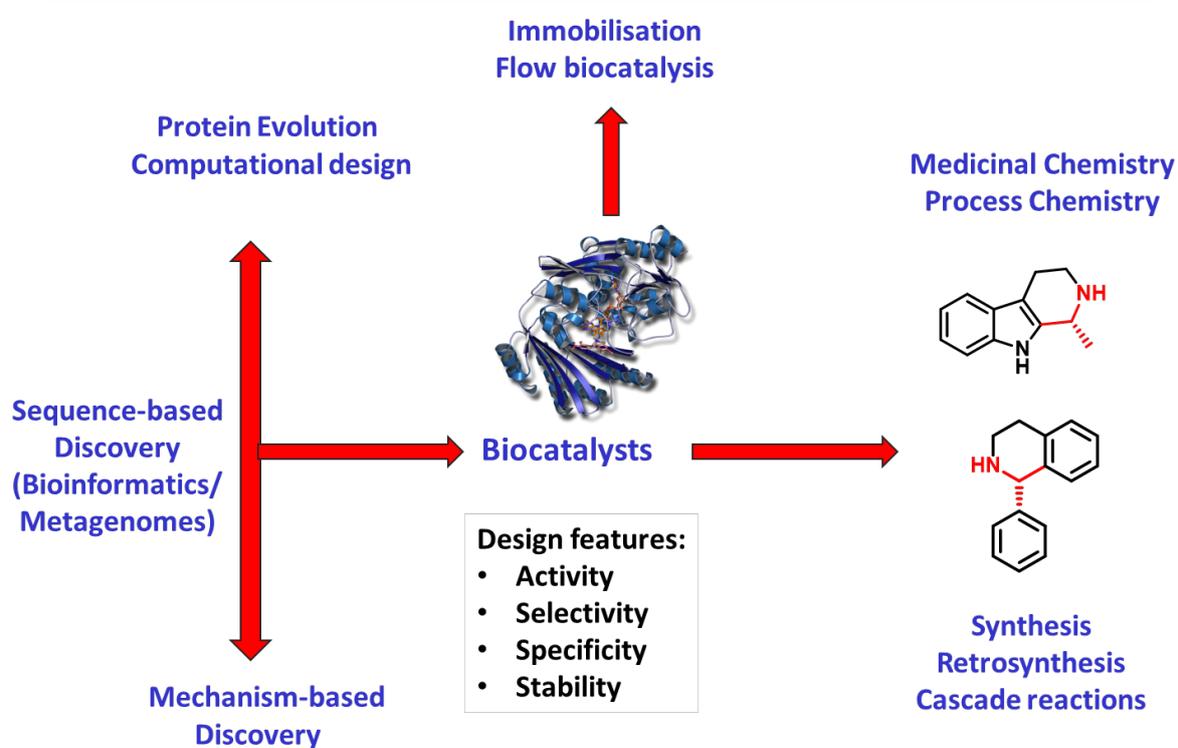


Figure 1. Current trends in biocatalysis.

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How to capture CO₂: Designing new-to-nature enzymes, reaction cascades and artificial cells for a sustainable biocatalysis

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Carbon dioxide (CO₂) is a potent greenhouse gas that is a critical factor in global warming. At the same time atmospheric CO₂ is a cheap and ubiquitous carbon source. However, synthetic chemistry lacks suitable catalysts to functionalize atmospheric CO₂, emphasizing the need to understand and exploit the CO₂ fixation mechanisms offered by Nature. In my talk I will discuss the evolution and limitation of naturally existing CO₂ fixing enzymes and present strategies how to discover and engineer novel enzymes and pathways for the fixation of CO₂ [1,2]. I will exemplify how these enzymes can be used to realize efficient catalytic networks for the conversion of CO₂ that outcompete those of natural photosynthesis [3]. Finally, I will talk about the challenges of transplanting these synthetic networks into natural and synthetic cells to create novel cell factories and catalytic systems for the capture and conversion of CO₂ into value-added compounds [4,5].

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Web-based Computational Enzyme Design for Biocatalysis

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Natural enzymes evolved for catalysis in an aqueous environment under mild conditions and often do not meet requirements for practical applications. Improvements in the catalytic activity, substrate specificity, or enantioselectivity of enzymes are traditionally achieved by modification of their active sites [1,2]. We have proposed that enzyme engineering endeavors should target both active sites as well as access tunnels and channels [3]. Using the haloalkane dehalogenases as model enzymes, we have demonstrated that engineering the access tunnels can significantly improve catalytic properties [4] and stability [5]. User-friendly software tools such as [Caver](#) [6], [Caver Analyst](#) [7], [CaverDock](#) [8] and [Caver Web](#) [9] have been developed for the computational design of protein tunnels and channels (**Figure 1**). Others, as [FireProt](#) [10] and [HotSpot Wizard](#) [11], aim for the automated design of stabilizing mutations and smart libraries. Using these tools, we were able to introduce a new tunnel [12] and flexible loop [13] to a protein structure and modify its conformational dynamics. We envisage that the next generation of enzyme design tools will combine structural bioinformatics with machine learning [14].



Figure 1. Protein Engineering Portal provides easy access to various web tools developed by the Loschmidt Laboratories (<https://loschmidt.chemi.muni.cz/portal/>). A future version of the portal will support submissions of jobs to several different tools simultaneously. Training on the proper use of the tools is provided by the Hands-on Computational Enzyme Design Courses (<https://loschmidt.chemi.muni.cz/academy/>).

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Understanding and controlling α -glucan synthesizing enzymes

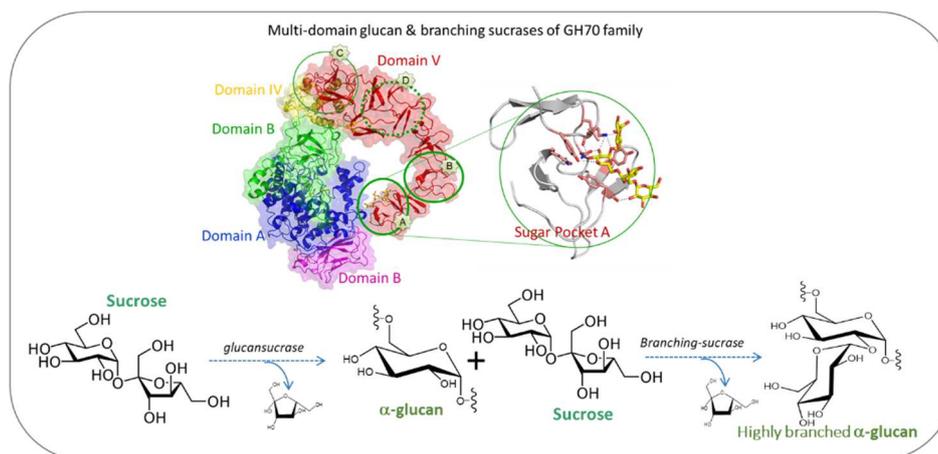
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Since autotrophic microorganisms learned to fix carbon dioxide in carbohydrates by photosynthesis, an immense variety of structures has spread in all areas of life. Oligosaccharides, polysaccharides or glycoconjugates have innumerable biological functions, and they have found applications in many industrial sectors. Nowadays, the demand for well-defined structures with specific biological and physicochemical properties is increasing in the food, feed, health, cosmetics, bulk and fine chemicals sectors. This is a great opportunity for glycan synthesizing enzymes and in particular for transglycosylases using low cost substrates. In the past few years and with the aim of tracing new routes to glycoproducts of interest, we have been searching for glucansucrases and branching sucrases in natural diversity [1,2].

These enzymes catalyse α -glucan, oligosaccharide and/or glycoconjugate synthesis via transglucosylation from sucrose substrate to various types of acceptors.

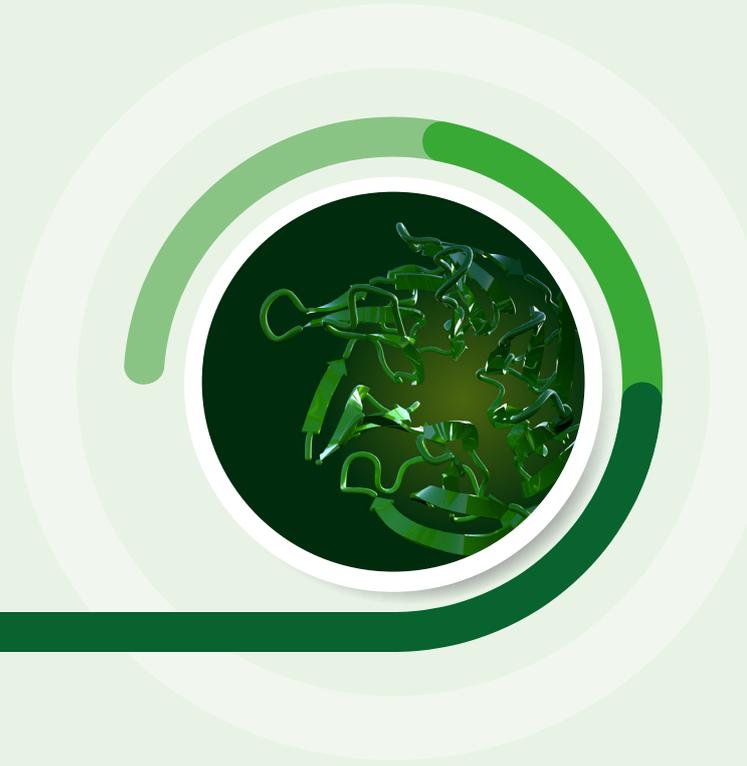


Focusing on solving new three-dimensional structures, we have thoroughly investigated how these incredibly versatile enzymes function at the molecular level. We have identified key structural determinants involved in the control of polymer size, in linkage specificity, or in the unnatural acceptor recognition [2,3]. We have applied protein engineering technologies to extend their catalytic potential. We have made them work in enzymatic or chemoenzymatic cascades. We have generated complex glyco-structures (block copolymers, glyco-co-polymers, glycosurfactants, glycosidic haptens, etc.) [4,5]. The presentation will provide an overview of these recent advances and open the discussion on challenges ahead.

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INVITED LECTURES



On the functionally flexible flavin cofactor: recent examples of resurrection and redesign of flavoenzymes

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Since the discovery of the canonical flavin cofactors, FMN and FAD, in the 1930s, biochemical studies have revealed that many redox and non-redox reactions are catalyzed by flavoenzymes. [1] The impressive list of reactions catalyzed by flavin-dependent enzymes suggests that the flavin cofactor is the most flexible natural cofactor. Detailed structure-function studies have disclosed that flavoenzymes can tune their specificity and reactivity by optimizing their active sites, including their entry and exit pathways. In most flavoenzymes, an FMN or FAD flavin cofactor is tightly but non-covalently bound. Yet, in a significant number of flavoenzymes, the flavin cofactor has been modified and/or covalently tethered to the protein in order to support a special catalytic feature [3]. Several of such modified flavins cofactors were only discovered in the last decade, such as the prenylated FMN and flavin-N5-oxide [3,4].

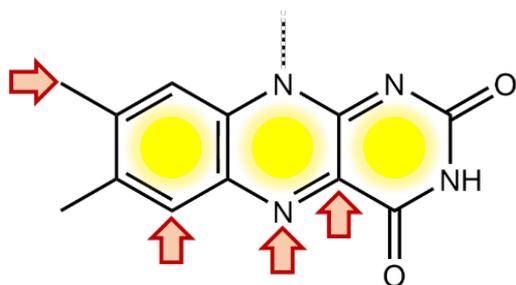


Figure 1. Isoalloxazine part of canonical flavin cofactors. The arrows indicate the most common sites for modifications in natural flavin cofactors.

In this lecture I will highlight recent discoveries of ‘new’ flavoenzymes with a focus on:

- (1) discovery and ancestral reconstruction of deazaflavin (F₄₂₀) cofactor-dependent enzymes for selective reductions and oxidations [5-8],
- (2) redesign of flavoprotein oxidases for selective oxidations of biomass-derived compounds [9,10],
- (3) new approaches for flavin cofactor and flavoenzyme immobilisations [10,11].

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Applications and Evolution of a very versatile Acetyl Transferase

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The acetyl transferase from *M. smegmatis* (MsACT) has been characterised for its highly hydrophobic active site and excellent synthetic performance in the preparation of esters and amides in water. [1-5]

This power of this enzyme has been exploited in our group first through covalent immobilization on a solid support which has enabled its use in continuous systems for the rapid production on a multi gram scale of melatonin and flavor esters [6,7] and then in the generation of a more active variant which opened the path also to the synthesis of a large range of thioesters, including acetyl CoA. [8] Here, the milestones of the discovery process that has accompanied the evolution (in terms of applications as well as protein engineering) to unlock the potential of this biocatalyst will be presented (Figure 1).

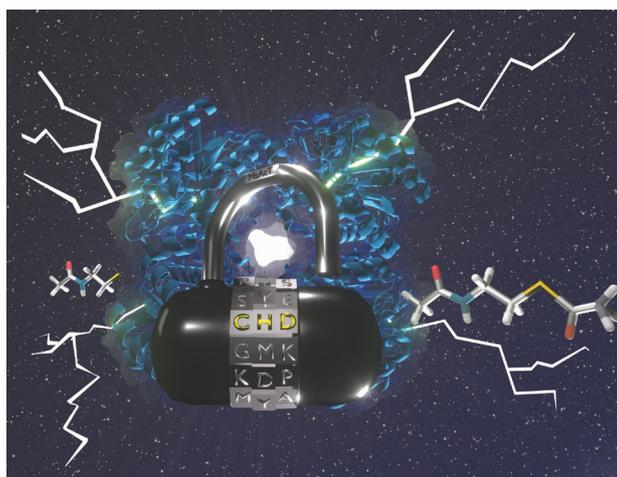


Figure 1. Unlocking the power of an acetyl transferase through applications and engineering.

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Learning to learn: Imine reductase improvement via (machine) directed evolution

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Imine reductases (IREDs) are valuable biocatalysts in pharmaceutical industry due to their high stereoselectivity in reductive aminations towards secondary and tertiary amines. Many pharmaceutical companies have genome mined their own wild-type selections of IREDs[1] and their applicability in intensified process conditions have been studied.[2, 3a] Enzyme engineering reports on imine reductases have also started to emerge.[3]

Working closely with drug discovery teams at Novartis, we have seen that we often find highly selective hits from our genome mined wild-type collection, however, to deliver even a gram of selected product is often challenging due to the low activity and stability of our IREDs. We therefore wanted to study how to quickly engineer these enzymes to suit our needs and the tight timelines of low-scale, medicinal chemistry projects. To study our methods, we applied reductive amination towards ZPL389[5] as our test reaction (Figure 1).

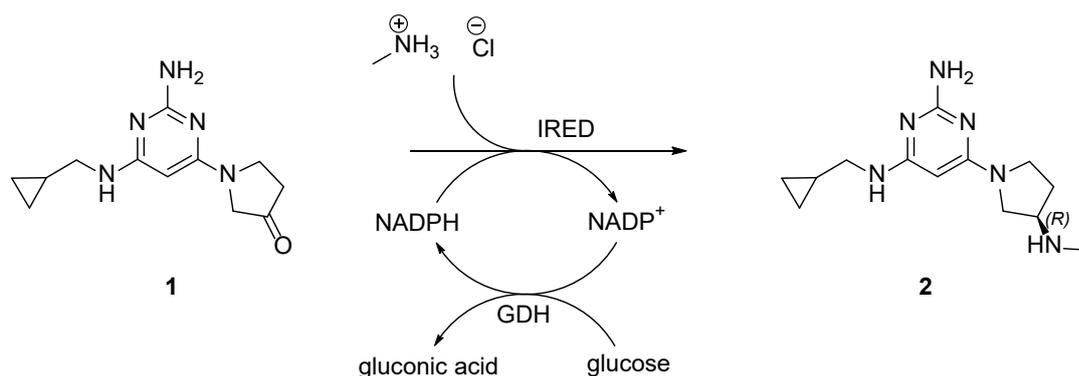


Figure 1. IRED catalysed (*R*)-selective reductive amination towards ZPL389 (**2**).

We compared classical directed evolution (error-prone PCR) and machine-directed evolution strategies.[6] In this talk we discuss those approaches as a tool to quickly evolve an enzyme for drug discovery projects.

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Enzyme-Catalyzed Amidations by ATP dependent Amide Ligases

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The acylation of amines to form amides is one of the most widely conducted reactions in synthetic medicinal chemistry, yet traditional methods often involve coupling agents resulting in processes that are far from atom efficient. There has hence been substantial interest in biocatalytic synthesis of amides [1], prompting successful studies on lipases and penicillin acylases, and more recently enzymes that are dependent upon ATP for amide ligation. We have evaluated the potential of ATP-dependent amide ligases from natural product pathways for the synthesis of pharmaceutical-type amides. The enzyme McbA, from the biosynthetic pathway to the marinacarbolines [2], was found to catalyse the ATP dependent coupling of carboxylic acids and amines in aqueous medium, and at low (1-5) equivalents of amine. McbA also displayed surprisingly promiscuous activity towards a range of carboxylic acid substrates [3], and also amines [4], suggesting potential for the synthesis of a range of valuable amide compounds from readily available precursors.

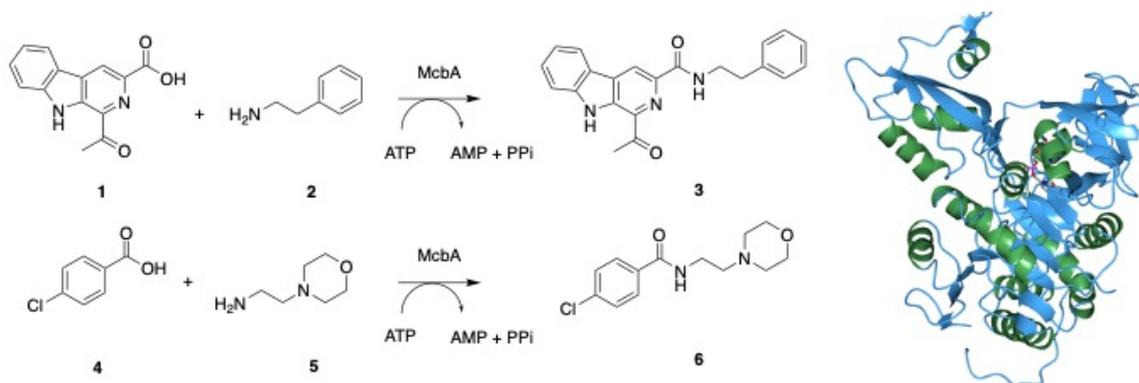


Figure 1. Coupling of acid **1** and amine **2** to form marinacarbolone amide **3** and synthesis of the anti-depression agent moclobemide **6** from **4** and **5** using McbA and ATP; **B**: Structure of McbA.

In addition to evaluating the specificity of McbA, we have determined its structure, in order to shed light on the determinants of specificity and reaction mechanism, and to act as a platform for rational engineering of the enzyme for expanded substrate range. We have also identified a number of homologs, some of which display significantly different catalytic properties.

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Conformational regulation in enzyme design and evolution

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Enzymes exist as an ensemble of conformational states, whose populations can be shifted by substrate binding, allosteric interactions, but also by introducing mutations to their sequence. Tuning the populations of the enzyme conformational states through mutation enables evolution towards novel activity.[1] A common feature observed in many laboratory-evolved enzymes, is the introduction of remote mutations from the catalytic center, which often have a profound effect in the enzyme catalytic activity. [2] As it happens in allosterically regulated enzymes, distal mutations regulate the enzyme activity by stabilizing pre-existing catalytically important conformational states.

In this talk, our new computational tools based on inter-residue correlations from microsecond time-scale Molecular Dynamics (MD) simulations and enhanced sampling techniques are applied in Tryptophan synthase (TrpS) complex. TrpS is composed of TrpA and TrpB subunits, which allosterically activate each other and have no activity when isolated. [3,4] We show how distal mutations introduced in TrpS resuscitate the allosterically-driven conformational regulation and alter the populations and rates of exchange between multiple conformational states, which are essential for the multistep reaction pathway of the enzyme.[3] The exploration of the conformational landscape of TrpS is key for identifying conformationally-relevant amino acid residues of TrpB distal from the active site.[4] We predict positions crucial for shifting the inefficient conformational ensemble of the isolated TrpB to a productive ensemble through intra-subunit allosteric effects. The experimental validation of the new conformationally-driven TrpB design demonstrates its superior stand-alone activity in the absence of TrpA, comparable to those enhancements obtained after multiple rounds of experimental laboratory evolution. Our work evidences that the current challenge of distal active site prediction for enhanced function in computational enzyme design can be ultimately addressed.

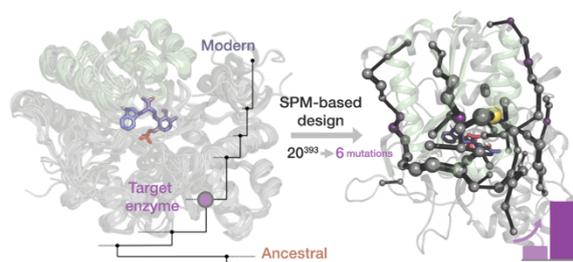


Figure 1. Scheme of the computational protocol used for the rational design of conformationally-driven stand-alone TrpB variants. Ancestral sequence reconstruction is combined with the correlation-based tool SPM.

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Chemical Discovery in the Microbial World

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Microbes have amazing chemical capabilities, performing reactions unprecedented in organic synthesis and producing complex, biologically active molecules not easily accessed via other approaches. Recent advances in DNA sequencing technologies have delivered a wealth of microbial genomes that encode novel metalloenzymes. The availability of this genomic data thus represents an unprecedented opportunity for the discovery of enzymes that have the potential to reveal new principles of catalysis and inspire the development of synthetic methodology. This talk will discuss our recent progress deciphering the chemistry of enzymes that mediate unusual C–X, C–C, and N–N bond forming reactions in microbial biosynthetic pathways. Functional and mechanistic characterization of these enzymes is uncovering reactivity with potential applications in biocatalysis and metabolic engineering.

Unfinished Story of Polyphenol Oxidases

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Polyphenol oxidase comprises a large groups of enzymes, such as tyrosinase, laccase, catecholase, etc. Among them, our group has mainly studied tyrosinase(TYR), which is a di-copper containing mono/dioxygenase which can do ortho-hydroxylation reaction for various poly-phenolic compounds collected from natural resources, especially plants. Although tyrosinase is a well-documented enzyme, interesting stories are still remained and unexplored if new concepts and molecular biology techniques are applied.

In this talk, the most recent detailed reaction mechanism of metal mediated catalysis will be reviewed. Then, based on the reaction mechanism, several examples of TYRs utilized in other interesting applications will be compared. To generate more diverse and specific tyrosinases, circular permutation(CP) of TYR and random screening based on phylogenetic trees were carried out, and their results were summarized. CP-derived TYR(BmTYR) could be converted into a catechol oxidase, and a new TYR, called *B. thailandensis* TYR, functional only at acidic pH was identified and their structure and reaction mechanism will be discussed.

Such tyrosinases can be not only used for ortho-hydroxylation of poly-phenol compounds but also used as a protein modification reagent through ortho-hydroxylation of tyrosine residues, hence *in situ* dopa generation. Above mentioned interesting properties can be utilized for protein-carbohydrate hydrogel formation using crosslinking of gelatin and hyaluronic acid in tissue engineering. Such gel-formation reaction can be controlled by photo-activatable enzyme design by introduction of non-natural amino acids in the structure, and tyrosinase active at acidic pH can be used for soluble melanin synthesis for biomedical material applications. This study will lead you to a different story such that TYR will be a very interesting enzyme worth of reinvestigation drawing our attention.

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[

Designer enzymes featuring non-canonical amino acids as catalytic residue

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Biocatalysis is recognized as a key component in the transition towards a more sustainable and “greener” chemistry. Yet, compared to the toolbox of chemists, enzymes have a limited chemical repertoire. Our research aims at creating enzymes for reactions that have no equivalent in nature. For this, we have developed new designer enzymes based on the transcription factor LmrR (Lactococcal multidrug resistance Regulator), by using biosynthetic incorporation of a catalytically active unnatural amino acid using expanded genetic code methodology.[1,2] Here, I will discuss our recent results on the design, directed evolution and application in catalysis of these novel designer enzymes.[3,4,5]

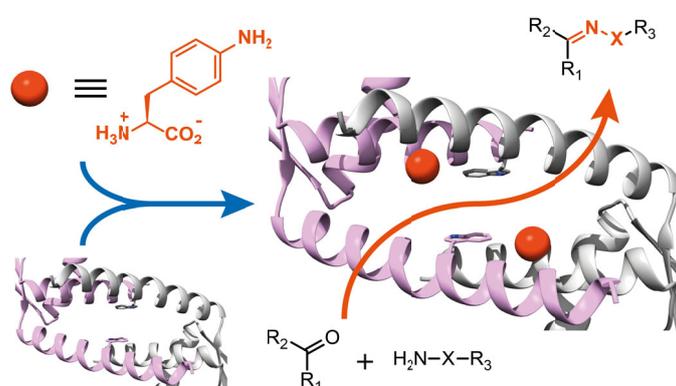


Figure 1. Example of an abiological reaction catalysed by an LmrR-based designer enzymes featuring a non-canonical p-aminophenylalanine residue as catalytic residue.

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Development of a Flow Process using an Immobilized Enzyme for the Transamination of Cyrene

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Biocatalysis, protein engineering, and flow chemistry are enabling technologies for process chemistry that together can be used to create highly efficient and sustainable manufacturing processes. While many industrial processes have been developed using free enzymes in aqueous solutions, immobilization of enzymes on a solid support can have many advantages, including improved reaction efficiency, improved enzyme stability, the ability to perform reactions in non-aqueous media, and simplified separation of products from catalysts.[1,2] This presentation will describe the development of a process using an immobilized transaminase for the reductive amination of cyrene, a bio-based feedstock chemical derived from cellulose. The product of this transformation, which we term cyrene amine, is a key chiral amine required for the synthesis of an experimental Bruton's tyrosine kinase inhibitor for the treatment of B-cell cancers.

To achieve a synthesis of cyrene amine, we identified a transaminase for the amination of cyrene with isopropylamine, and subsequently engineered the enzyme by directed evolution for improved activity, diastereoselectivity, and thermostability. Resins were then identified which allowed for efficient enzyme immobilization while retaining the enzyme's activity in water-saturated organic solvents. Performing the reaction in predominantly organic media enabled simplified isolation of the product via crystallization as the tosylate salt, as well as enabled facile separation of the enzyme from the amine product. Further streamlining, process improvements, and process risk reduction were achieved by performing the reaction in flow, passing the stream of reagents through a packed bed reactor containing the immobilized enzyme. The final process combines two renewable, bio-based solvents (cyrene and 2-MeTHF) with isopropylamine and an immobilized transaminase catalyst to efficiently produce a pharmaceutical intermediate.

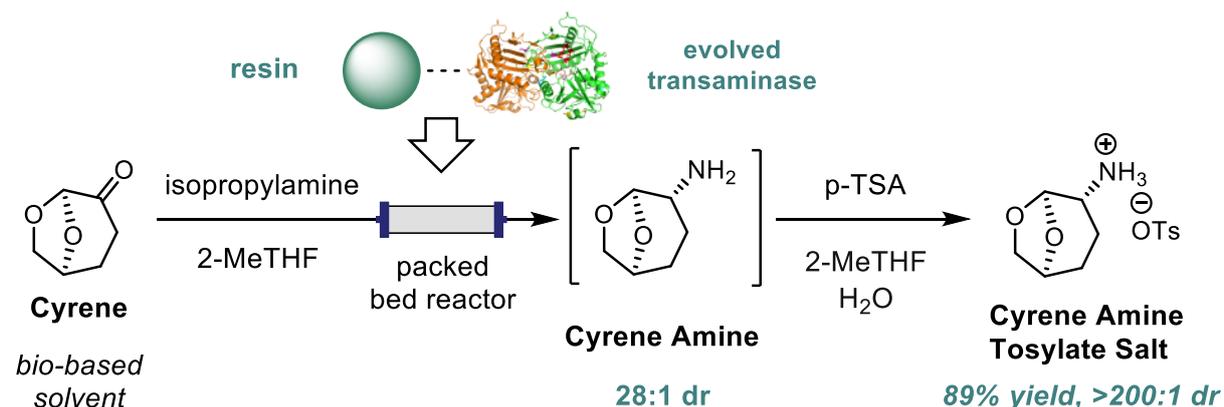


Figure 1. Immobilization of an evolved transaminase enables streamlined production and isolation of a key chiral amine building block from the renewable solvent cyrene.

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LECTURES



Diversification of Nucleoside Analogues by Nucleoside Phosphorylases

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Nucleoside analogues have become indispensable as molecular biology tools and pharmaceuticals for the treatment of various cancers and viral infections. In this context, a variety of sugar-modified nucleosides have gained renewed traction for the treatment of acute Covid-19 infections. Despite the great demand for such nucleoside analogues, their synthesis is still regarded as challenging [1] and available approaches typically exhibit a pronounced lack of divergence. As such, the introduction of desired substitutions at the nucleobase (e.g. for biological evaluation and screening purposes) often requires complete or partial re-synthesis of the target compound, making the overall endeavour laborious and inefficient. We addressed this synthetic challenge through the application of nucleoside phosphorylases. These enzymes enable the direct glycosylation of a variety of nucleobases as well as transglycosylations enabling a straightforward one-pot diversification of nucleoside analogues. To this end, we established high-throughput methodologies [2], thermodynamic frameworks [3,4] and robust bioprocesses employing extremely thermostable enzymes [5,6]. Collectively, our work has laid the foundation for the rapid development of biocatalytic nucleoside diversification platforms that deliver molecular diversity with perfect selectivity and high yields which can be predicted and optimized through principles of thermodynamic control [7].

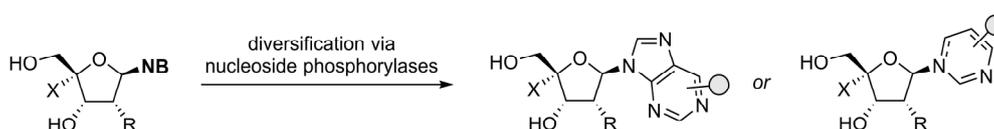


Figure 1. One-pot diversification of nucleoside analogues by nucleoside phosphorylases enables rapid access to molecular diversity.

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Active-site loop variations adjust activity and selectivity of the cumene dioxygenase

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Rieske non-haem dioxygenases (ROs) are enzymes achieving selective oxidation of a plethora of substrates. Nature has two different strategies to achieve this selectivity either by size and shape of the active site or substrate orientation, e.g. by anchoring amino acids. Part of this specificity arises from a sieve - like structure called tunnel that hampers the access of certain molecules to the active site of an enzyme. These tunnels are usually formed by highly flexible loop-like structures, especially at the entrance to the active site. In the cumene dioxygenase (CDO) from *Pseudomonas fluorescens* IP01, two loops in the periphery of the active site determine the access tunnels. Remarkably, these loops are barely conserved among the ROs, whereas the amino acids of the active pocket are highly similar within the enzyme family.

In our study, we have been replacing, introducing and deleting several amino acid residues to modulate these loops. However, the active site as well as the core of the protein structure is untouched. These drastic modifications emulate the specificity of biological oxidations and result in various optimized enzymatic properties, ranging from altered regioselectivity to strongly increased activity as well as a broadening substrate scope.

First analysis of these enzyme variants points towards an altered geometry of already existing tunnels and the formation of completely new ones. The modification of loops as rarely addressed structural features promises a high potential for future enzyme engineering strategies and chemist might find inspiration in designing novel catalysts.

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Site-selective functionalization of native proteins using engineered enzymes

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The emergence of new biological therapeutic modalities requires the development of complementary tools for their efficient syntheses [1,2]. Availability of methodologies for site-selective modification of biomolecules, such as proteins, remains a significant and longstanding challenge, given their inherent complexity and the presence of repeating residues that bear functional groups with similar reactivity profiles.

Enzymes are a powerful extension to existing chemical approaches as they operate with high specificity under the mild reaction conditions required for protein functionalization. Herein, we describe a novel enzymatic strategy to selectively modify native peptides (Figure 1). Penicillin G acylases were engineered to distinguish among free amino moieties of insulin (two at the N-termini and an internal lysine). The evolved biocatalysts in a programmable manner manipulated cleavable phenylacetamide groups, providing selectively protected insulin derivatives. This enabled highly-specific chemical ligation to access homogenous insulin conjugates. Our ability to functionalize residues in native biomolecules opens up new avenues in the synthesis of novel biological drugs or probes.

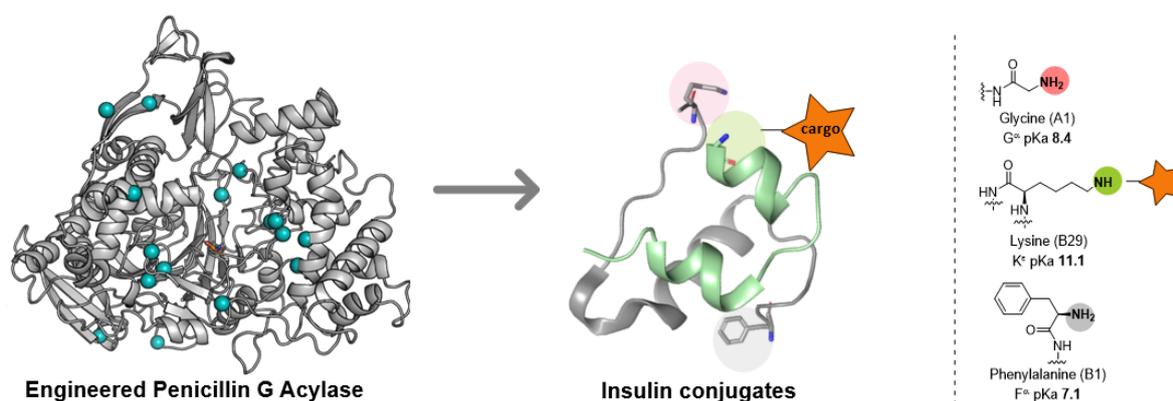


Figure 1. A novel biocatalytic strategy for well-defined conjugation of native proteins enables an efficient synthesis of insulin therapeutics.

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Enantioselective, intermolecular amidation of benzylic C-H bonds with engineered P411 enzymes

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Directed evolution of heme proteins has opened access to new-to-nature enzymatic reactivity [1]. Among these, reactions resulting from active site, iron-nitrene intermediates present a powerful strategy to forge C-N bonds with high site- and stereo-selectivity [2]. Herein, we report the development of a biocatalytic, intermolecular benzylic C-H amidation reaction operating at mild and scalable conditions. With hydroxylamine derivatives as nitrene precursors, feedstock aromatic compounds can be converted to chiral amides with excellent enantioselectivity (up to >99 % ee) and high yields (up to 87 %) (Figure 1). Kinetic isotope effect studies combined with active site modeling reveal mechanistic details of the amidation process.

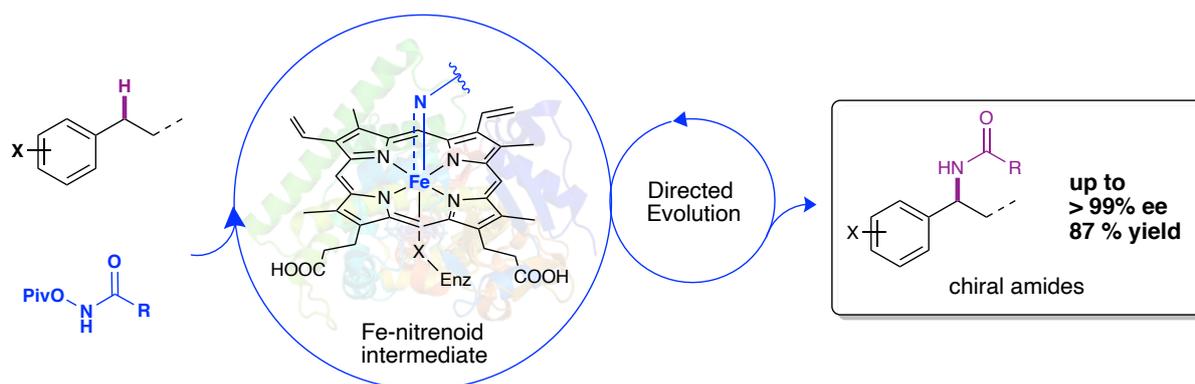


Figure 1. Biocatalytic, enantioselective benzylic C-H amidation.

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Repurposing an aldolase for heterocycle synthesis

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Aldolases catalyze carbon-carbon bond-forming reactions under mild conditions, but are capable of a variety of non-canonical reactions as well. We have been studying a type 1 aldolase, NahE, that is known to catalyze the scalable synthesis of unsaturated 2-oxo acids,[1] which are versatile chemical building blocks. Recently it has been shown that the enzyme can also be used for the stereoselective generation of aldol products.[2,3] Based on our understanding of homologous enzymes, we predicted that this enzyme might also catalyze heterocycle synthesis. Here we report on the substrate scope of the enzyme, including the unprecedented aldolase-catalyzed synthesis of substituted quinolines.

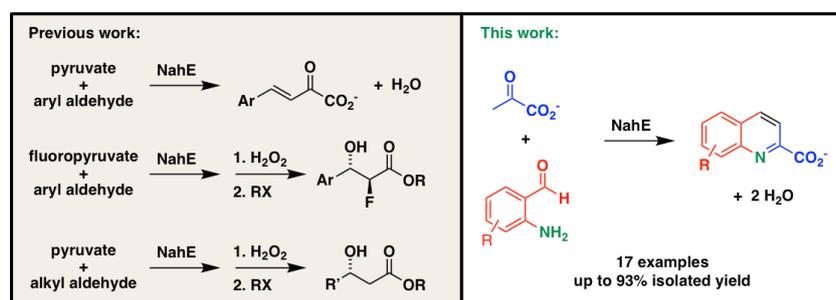


Figure 1. The aldol reactions catalysed by the aldolase NahE, and our new application of the same enzyme to quinoline synthesis.

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Enantioselective Carbene N–H Insertion Reactions with P411 Enzymes

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We developed an enzymatic platform for highly enantioselective carbene N–H insertion reactions to furnish a set of biologically relevant α -amino lactone products [1]. The engineered P411 (P450 with the axial cysteine ligand replaced by serine) enzymes acted as dual-function biocatalysts that promoted the transfer of the lactone carbene to amines and exerted excellent stereocontrol in the subsequent protonation step. Computational studies elucidated the detailed mechanism of this fascinating process, explaining the critical role of the serine residue at position 264 for achieving high activity and selectivity. The engineered active site controls the conformation of the lactone carbene, yielding to an enantiospecific *N*-nucleophilic attack for the ylide formation; it also precisely positions water molecules for rapid and stereoselective proton rearrangement before product release. Furthermore, we demonstrated that this enzymatic system can accept a broad range of amines for the desired amination reactions with high activity and enantioselectivity (up to >99% yield and 98% e.e.). The enzyme was shown to be robust, achieving high turnover numbers (e.g., 31,700 TTN with *p*-anisidine) and catalyzing these reactions in preparative scale, including preparation of a key intermediate for synthesis of (*S*)-ofurace [2]. We envision that this highly efficient system can be applied to the preparation of bioactive chiral amines for synthetic chemistry and drug discovery.

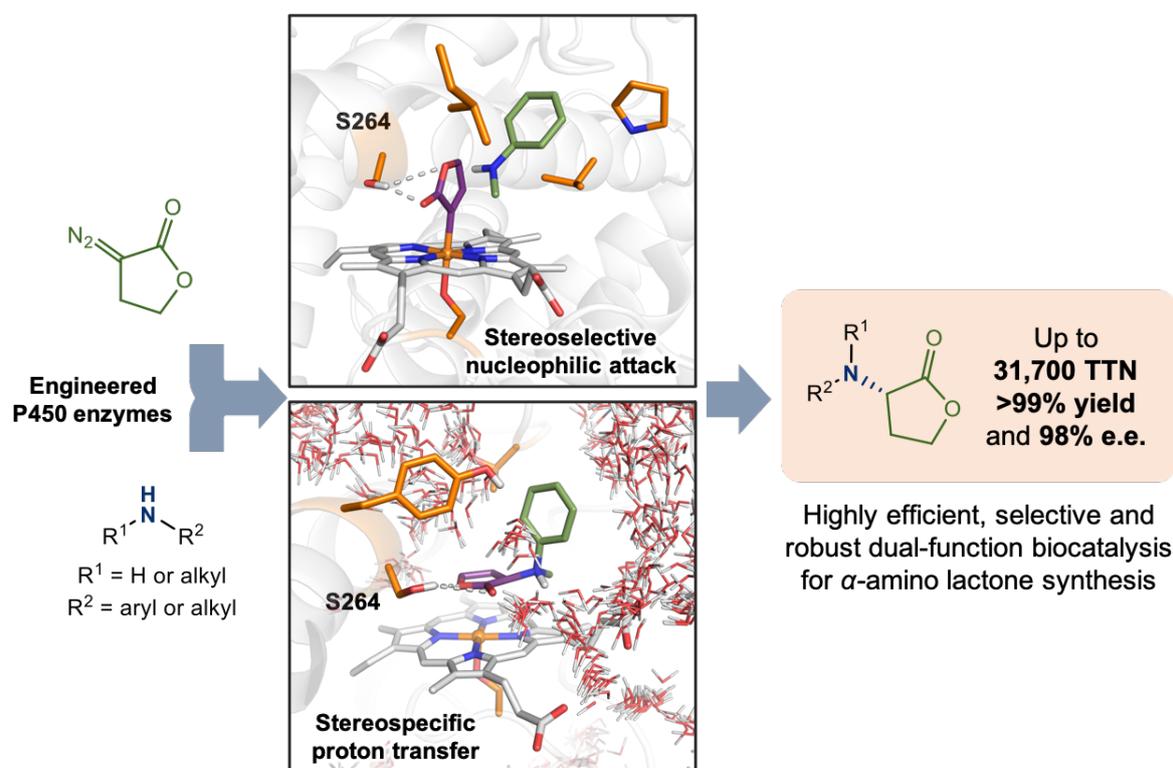


Figure 1. Enantioselective carbene N–H insertion of amines catalyzed by P411 enzymes.

References

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Chemoenzymatic Total Synthesis of (–)-Luteoskyrin and (–)-Deoxyluteoskyrin

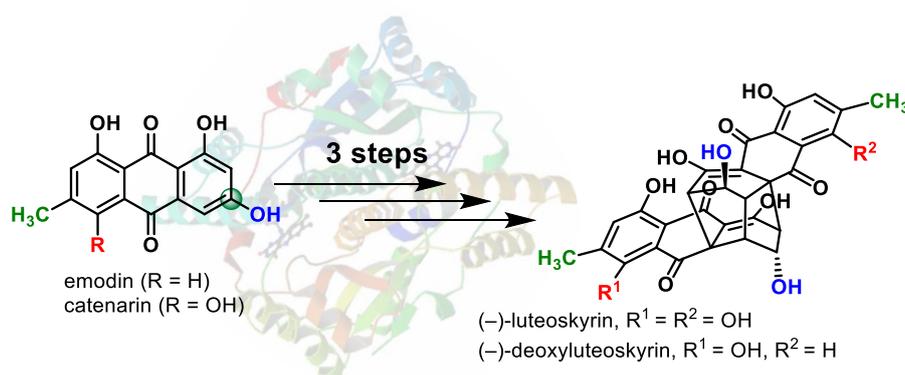
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Keywords: Biocatalysis, chemoenzymatic synthesis, natural product, bisanthraquinones.

Bisanthraquinones is a large family of polyketidic natural products, isolated from a number of fungi and lichen species.¹ They possess a highly complex molecular structure, with multiple stereogenic centres and display a diverse range of potent biological activities. Inspired by their proposed biosynthesis,² we have developed a biomimetic, chemoenzymatic strategy to achieve a long-standing synthesis of homodimeric, (–)-luteoskeyin and heterodimeric, (–)-deoxyluteoskeyin in just three steps. This required us to find routes to several putative biosynthetic intermediates never isolated or synthesized before. By reductive dearomatization of anthrol precursors with NADPH-dependent anthrol reductase of *Talaromyces islandicus* (ARti)³, we could synthesize chemically sensitive putative biosynthetic intermediates, under benign conditions, which are oxidized and dimerized to obtain (–)-luteoskyrin and (–)-deoxyluteoskyrin for the first time (*unpublished results*).



Scheme 1: Chemoenzymatic approach towards the synthesis of modified bisanthraquinones using anthrol reductases.

The involvement of new monomeric and dimeric intermediates (obtained during the execution of our biomimetic approach), in the biosynthesis of these complex natural products, is validated by their presence in the fungal culture of *Penicillium islandicum* NRRL 1036. This implies similar biogenesis of these natural products as our chemoenzymatic synthesis.

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A multi-functional biocatalyst that catalyses both conjugate reduction and reductive amination

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The prevalence of chiral amines in active pharmaceutical ingredients (APIs) and other high value chemicals [1] has led to an overarching goal in organic synthesis to develop efficient new catalytic methods for their preparation. [2] In this context, reductive amination (RA) is one of the most widely used and powerful methodologies in pharmaceutical and medicinal chemistry for forming C-N bonds via the reductive coupling of carbonyls and amines. [3,4] Furthermore, valuable amino-compounds often contain multiple stereogenic centres, however total control of their asymmetry is more challenging, resulting in less efficient multistep syntheses or more complex tandem catalyst systems. In this work, we report the discovery, characterisation and application of a new multi-functional oxidoreductase, which operates using an unprecedented conjugate reduction-reductive amination (CR-RA) mechanism (Figure. 1.). This enzyme enables the coupling of a broad selection of α,β -unsaturated carbonyls with amines for the efficient preparation of enantioenriched amine diastereomers. Moreover, employing a racemic substrate partner or conjugated dienyl-ketone provides a means of controlling additional stereocentres using the single catalyst. In addition, structural and preliminary mechanistic studies offer insight into key reaction intermediates found in the CR-RA catalytic cycle.

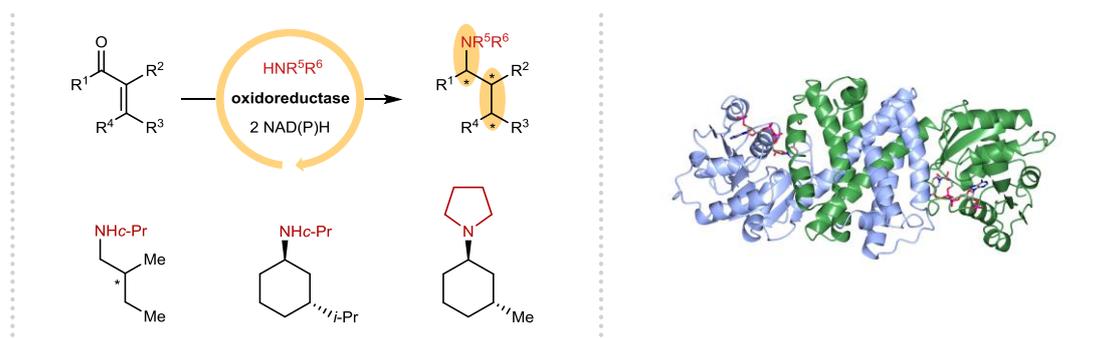


Figure 1. Conjugate reduction-reductive amination (CR-RA) catalysed by a single oxidoreductase.

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Enzymatic synthesis of novel halogenated alkaloids using a ‘parallel cascade’ strategy *in vitro*

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The tetrahydroisoquinoline (THIQ) ring system is present in a large variety of structurally diverse natural products exhibiting a wide range of biological activities. This has encouraged the development of strategies, particularly aimed at the enantioselective synthesis of non-natural THIQs with different substitutions at C-1. Routes to mimic the biosynthetic pathways to such alkaloids, by building cascade reactions *in vitro*, represents a successful approach and offers better stereoselectivities than traditional synthetic methods. In recent work biocatalytic strategies towards novel THIQs have been developed but applications towards halogenated THIQs are more challenging.[1-5]

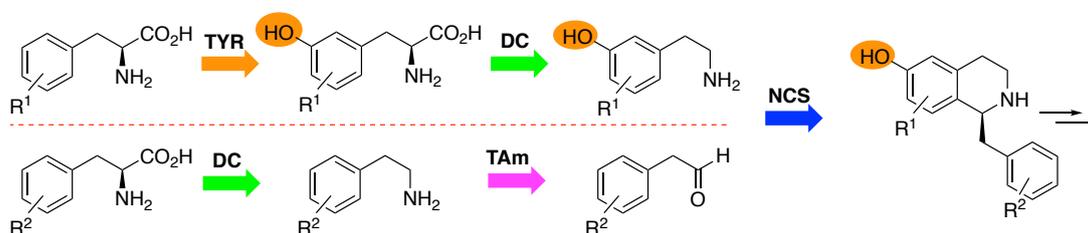


Figure 1. The ‘parallel cascade’ strategy to halogenated THIQs.

Here, the use of enzymes including tyrosinases (TYRs), a tyrosine decarboxylase (DC), transaminase (TAm) and norcoclaurine synthase (NCS) were combined in a ‘parallel cascade’ strategy in order to generate novel halogenated THIQs in high yields and optical purities. Notably, mutagenesis studies were applied to generate tyrosinase mutants, to enhance the acceptance of halogenated tyrosines for use in the cascades developed.

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Protein engineering and applications of amine dehydrogenases

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Amine dehydrogenases (AmDHs) are enzymes that catalyse the reductive amination of carbonyl compounds at the sole expense of ammonia and a hydride source (e.g., formate), (Fig. 1a). However, the diversity of substrate scope and activity among AmDHs was constrained.¹⁻³ With the aim of addressing this limitation, we created a new family of AmDHs that can aminate pharmaceutically relevant aromatic ketones with high activity and excellent stereoselectivity to mainly give (*R*)-configured amines.⁴ Interestingly, the AmDHs were created from the enzyme scaffold of an ϵ -deaminating L-lysine dehydrogenase (LysEDH) that does not operate any asymmetric transformation in its natural reaction. The best variant LE-AmDH-v1 is highly thermostable (*T*_m of 69 °C) and retains almost entirely its catalytic activity upon incubation up to 50 °C for several days. We could also access (*S*)-configured amines by performing the kinetic resolution of racemic amines.⁵ This was possible by combining the LE-AmDH-v1 with a NADH-oxidase (NOx). Using these optimal conditions, the kinetic resolution of a number of pharmaceutically relevant aromatic amines was complete (i.e., optimal conversion of ca. 50% with up to >99% ee). We also recently observed that LysEDH was capable of reducing aromatic aldehydes into primary alcohols. Therefore, we harnessed the promiscuous alcohol dehydrogenase (ADH) activity of LysEDH to create new variants that exhibited enhanced catalytic activity for the reduction of substituted benzaldehydes and arylaliphatic aldehydes to primary alcohols. Notably, these novel engineered dehydrogenases also catalysed the reductive amination reaction, thus exhibiting a dual AmDH/ADH activity.⁶ The catalytic bi-functionality of these enzymes was applied for a one-pot 'hydride-borrowing' cascade to convert benzyl alcohol to benzylamine using a single enzyme, thereby providing the first examples of enzymes showing 'alcohol aminase' activity (Fig 1b). In fact, the 'hydride-borrowing' amination cascade was previously developed by using an ADH and an AmDH that operate in tandem.^{7, 8} Although this transformation was initially conceived to be highly chemo- and stereo-selective, we have later introduced the capability of distinguishing between different hydroxyl functions within the same molecule (i.e., regioselectivity).⁹ The versatility of the biocatalytic alcohol amination process was also demonstrated in a work in which ADH-AmDH genes were introduced into *E. coli* to carry out this transformation *in vivo*.¹⁰

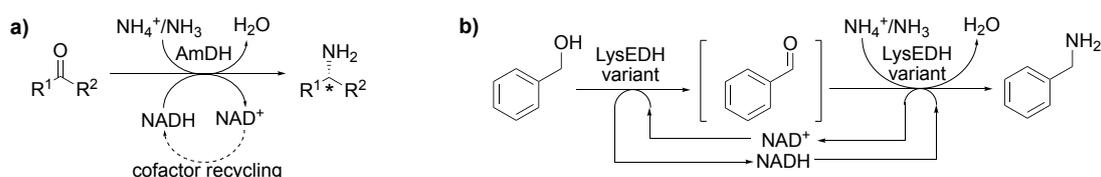


Figure 1. AmDHs applied for asymmetric reductive amination of ketones (a) and 'hydride-borrowing' amination of alcohols

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Computational Exploration and Design of new Halohydrin Dehalogenase variants

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Many enzymes present in Nature could be potentially used to synthesize chemically relevant chiral intermediates for drugs. One example is the enzyme family halohydrin dehalogenases (HHDH)[1], which catalyse the enzymatic conversion of ethyl (S)-4-chloro-3-hydroxybutyrate (ECHB) into the corresponding epoxide. This enzyme in the presence of a chloride ion can also catalyse the epoxide ring opening reaction yielding an ethyl (R)-4-cyano-3-hydroxybutyrate (HN), which is a precursor of Lipitor, a drug used for lowering the levels of cholesterol in blood. Within this enzyme family, different subclasses of HHDH have been identified showing a big spectrum of beneficial properties like thermal and organic solvent stability, activity, enantioselectivity and expanded substrate scope[2].

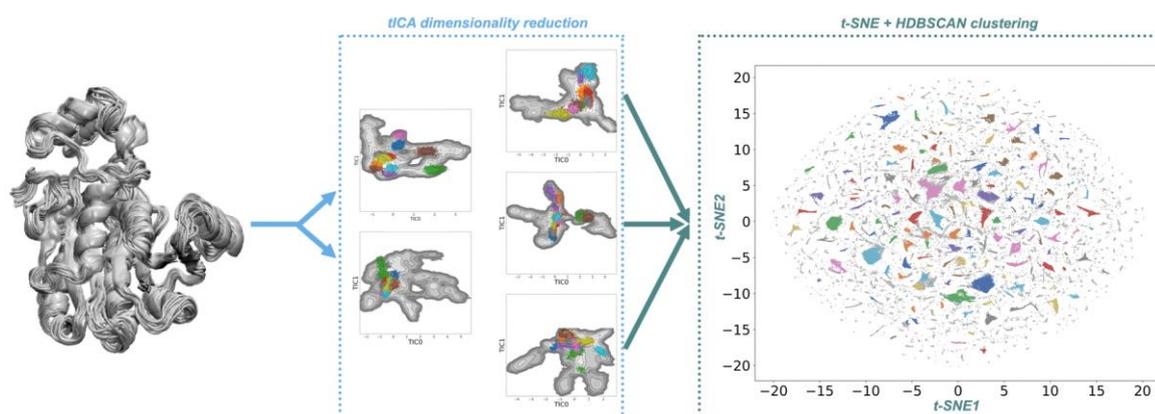


Figure 1. Computational protocol used to reconstruct the conformational landscapes of the different HHDH subclasses.

The computational exploration of the conformational landscape of HHDH can potentially reveal the key residues and conformations that grant these enzymes the properties mentioned. In this talk, Molecular Dynamics (MD) simulations coupled to machine learning and correlation-based tools are used to explore and analyze the conformations of different HHDH subclasses [3,4]. This is coupled with the analysis of the available active site tunnels for substrate binding on each HHDH conformation, which provides key information of the protein ability to accept bulky substrates, and thus for expanding the HHDH natural substrate and reaction scope. With this knowledge, a rational *in silico* design protocol of some HHDHs was conducted to expand the range of accepted nucleophiles for epoxide ring opening reactions, avoiding non-rational and expensive directed evolution strategies.

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Tungsten Aldehyde Oxidoreductase – a new type of hydrogenase

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The W-dependent aldehyde oxidoreductase from the betaproteobacterium *Aromatoleum aromaticum* (AOR_{Aa}) catalyses the oxidation of aldehydes to carboxylic acids and the respective reverse reaction, reduction of non-activated carboxylic acids to aldehydes. The AOR_{Aa} incorporates in its active center a tungsten atom, the heaviest element with biological function, in the form of metallopterin cofactor and exhibits a more complex structure (hypothetical $\alpha_2\beta_2\gamma_2$) than those encountered in archaeal AORs (α_2). The α subunit contains the tungsten cofactor and one FeS cluster, the β subunit contains a chain of four FeS clusters which links the tungsten cofactor with an FAD in the γ subunit. The oxidation of aldehydes can be coupled to NAD⁺ or benzyl viologen reduction. Notably, AOR_{Aa} is much more resistant to inactivation by O₂ after air exposure than archaeal AORs with a half-life time of 1h for the purified enzyme [1].

All known AORs exhibit activity for the reduction of carboxylic acids to aldehydes when coupled to strong reducing agents (such as tetramethyl viologen or Ti(III) complexes) [2]. Unexpectedly, the AOR from *A. aromaticum* turned out to be also a hydrogenase, capable to efficiently utilize electrons from H₂ oxidation for the reduction of carboxylic acids. Furthermore, this hydrogenase activity may be also used to reduced NAD⁺ to NADH, thus providing a new NADH-recycling system.

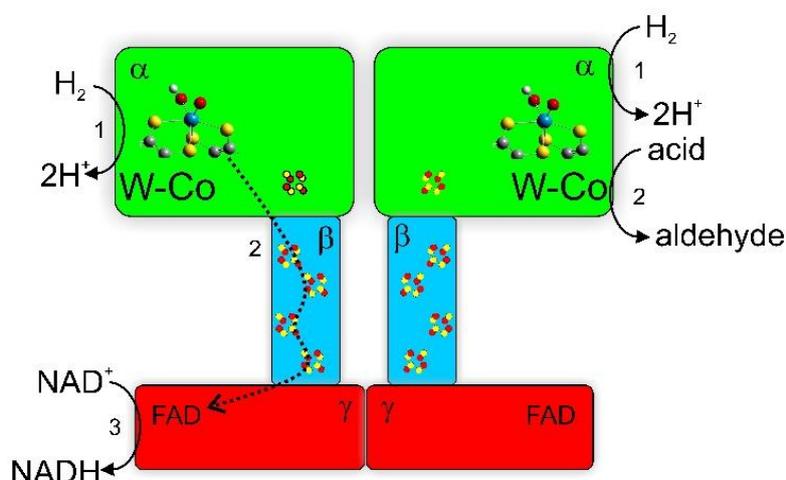


Figure 1. Two biotechnological applications of AOR_{Aa} for NADH recycling or acid reduction with H₂ as a reductant.

In the study, we use recombinant AOR_{Aa} produced in *Aromatoleum evansii*. The enzyme is able to reduce a wide range of substrates i.e., aromatic, heterocyclic, alkylaromatic and aliphatic carboxylic acids. The reactions were conducted under an inert gas atmosphere containing H₂ (0.1%-100%) and the enzyme was also active with a model syngas mixture (CO, N₂, H₂ 59/40/1 v/v) for up to 48 h. The initial enzyme activity in the reduction of benzylic acid was 0.5 U/mg (for 2.5 % H₂ in the headspace). We also characterized kinetic parameters of the enzyme which exhibits an apparent K_m for H₂ of 0.6 μM for 1 mM NAD⁺. The applicability of AOR_{Aa} for NADH recycling was demonstrated by reducing acetophenone to (*R*)-1-phenylethanol using an enantiomer-specific alcohol dehydrogenase.

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Sequence-function relation in flavin-dependent epoxidases

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Several flavoprotein monooxygenases (FPMOs) activate molecular oxygen to perform (enantio)selective epoxidations.[1] Especially, the group of styrene and indole monooxygenases (SMOs, IMOs) represent a powerful tool towards aryl-substituted and aliphatic epoxides. These are two-component systems, while an NADH-dependent flavin reductase fuels the epoxidase subunit with reduced FAD (Fig. 1). The catalytic cycle is defined and substrates undergo an electrophilic oxygenation by the C4a-hydroperoxy-FAD. While product is formed, the hydroxy-FAD releases water and both, product and oxidized FAD, are released for another cycle. Based on the 3D-structure of VpIndA1 (Fig. 1) in combination with process optimization we improved the catalysts towards non-favoured substrates.[2]

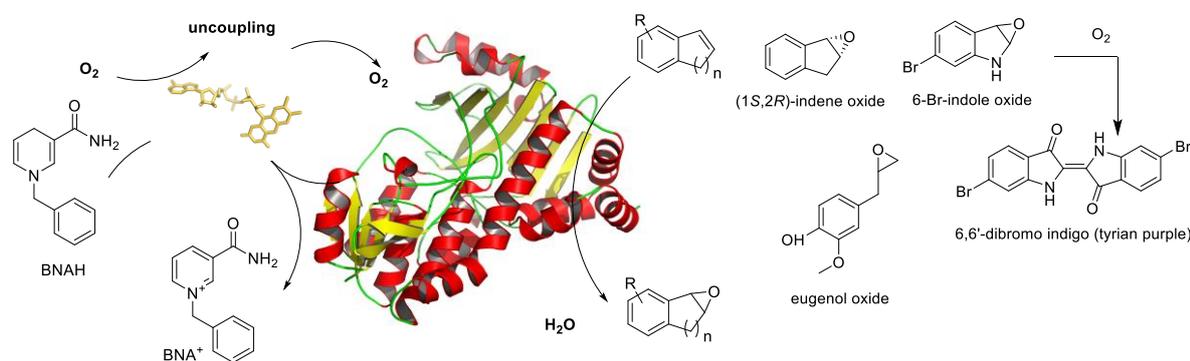


Figure 1. Structural insights in indole monooxygenase VpIndA1 from *Variovorax paradoxus* EPS. For simplicity drawn without substrate and cofactor in the active site. $n = 0$ to 2. R can be halogen, methyl, methoxy or hydroxyl-group. BNAH = 1-benzyl-1,4-dihydronicotinamide. On the right we show selected products of interest from biocatalytic point of view.

Styrene derivatives with strongly activating substituents in *p*-position are hardly accepted by FPMOs and also the conversion of indene, as representative of bicyclic aromatic compounds, is limited.[1-3] The addition of co-solvents (e.g. acetone) supports substrate availability and reductive power of the supplied nicotinamide mimetics. These mimetics are poorly soluble in water-based systems but represent a cost efficient route to efficiently reduce the FAD cofactor. We improved the activity towards indene by the factor of 2 and were able to show for the first time that lignin monomers like eugenol can be converted among other styrene derivatives. This IMO and related FPMOs produce mostly *S*-epoxides while a recent study identified *R*-selective enzymes.[3] This confirms our recent finding on fingerprint motifs which allows distinguishing SMO/IMO and predicting their selectivity.[4] On base of the VpIndA1 structure comprising bound substrate and cofactor it is now possible to specify the sequence-function in more detail and to improve the prediction of selectivity by the presence of important amino acid residues. In conclusion, by means of structural data, homology modelling and process optimization, we were able to improve the activity towards bicyclic substrates by rational design, towards *p*-hydroxy aromatic substrates by reaction engineering, to identify key residues for protein and thus process stability, and to provide efficiently electrons from various nicotinamide mimetics. This allows to demonstrate in combination with nicotinamide biomimetics to apply FPMOs in a cell-free biocatalytic process. Further, this study improves the understanding of amino acid sequence and function relationship among those epoxidases which simplifies further enzyme engineering approaches.

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Enantioselective cleavage of N-O bond: a cyanide-free strategy accessing to chiral β -hydroxy nitriles from olefins

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Aldoxime dehydratase (oxd) is a family of Heme-containing enzyme discovered about 20 years ago by Asano group, [1] which can catalyse the dehydration of aldoxime to nitriles in microorganism. This aldoxime-nitrile pathway provides insight into the biosynthesis of nitrile compounds in microorganism, which is different with the discovery in plant that to exploit Heme-containing enzyme P450s to synthesize nitrile compounds from corresponding amino acids. [2] With the dehydration function of aldoxime dehydratases (oxds), a cyanide-free platform to synthesize chiral nitriles has been established utilizing aldoxime precursors (**Figure 1a**). [3] Then, we demonstrated it's also a rare natural example to catalyse the Kemp elimination reaction via a -Fe-N- complex catalytic pathway (**Figure 1b**), [4] which is different to the classic acid-base mechanism. Furthermore, by combination of the cyanide-

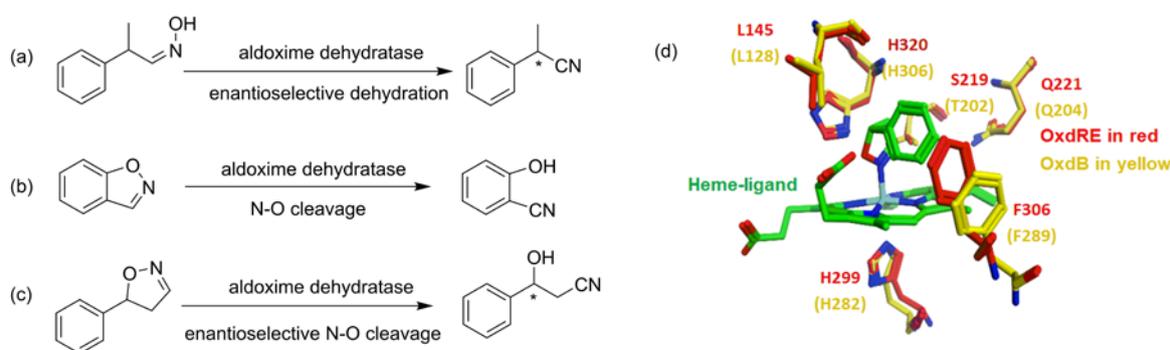


Figure 1. (a) Enantioselective dehydration catalysed by aldoxime dehydratases. (b) The Kemp elimination reaction catalysed by aldoxime dehydratases. (c) Asymmetric ring-opening of 4,5-dihydroisoxazole catalysed by aldoxime dehydratases. (d) Docking simulation of 5-phenyl-4,5-dihydroisoxazole to aldoxime dehydratases.

free synthesis of chiral nitriles and the Kemp elimination reaction catalysed by aldoxime dehydratases, we developed a new application of aldoxime dehydratase in the asymmetric ring-opening of 5-sub-4, 5-dihydroisoxazoles to synthesize chiral β -hydroxy nitriles (**Figure 1c**) with broad substrate scope, excellent enantioselectivity (up to 99% *ee*), and good turnover number (up to 11 s^{-1}). Upon simple isolation and treatment with an alkaline reagent, the remaining chiral 5-sub-4, 5-dihydroisoxazoles can be easily transformed into their corresponding β -hydroxy nitriles. Using site-directed mutagenesis, a ferrous Heme-containing active site (**Figure 1d**) was confirmed and two possible deprotonation pathways were proposed. [5]

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Expanding the substrate scope of Amine Dehydrogenases by protein engineering

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Facing the great demand of (chiral) amine intermediates in pharmaceutical and agrochemical industries^[1], biocatalytic direct reductive amination of carbonyl-containing compounds is a transformation that arouses the interest of many teams. Several enzyme families are now available to catalyse this reaction such as engineered Opine Dehydrogenases (OpDHs), Imine reductases (IREDs), Reductive Aminases (RedAms) and Amine Dehydrogenases (AmDHs)^[2]. The latter, previously restricted to engineered Amino Acid Dehydrogenases, has grown in recent years due to the discovery of genes coding for native AmDHs (nat-AmDHs) and now contains enzymes catalysing the formation of imines from ketones and ammonia and their subsequent reduction by NAD(P)H cofactor.^{[3][4]} This family has also been extended by the exploration of metagenomics data that led to the discovery of promising new enzymes.^[5]

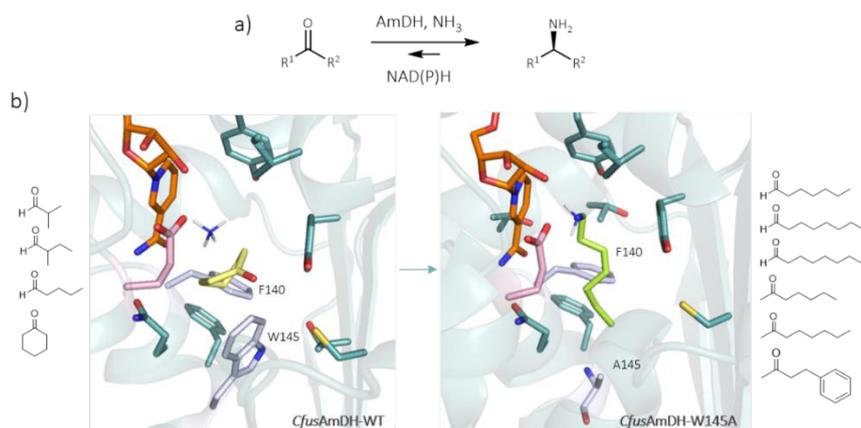


Figure 1. a) Transformation catalysed by Amine Dehydrogenases. b) Catalytic pockets of *CfusAmDH*-WT (left, model of association of NADP⁺, NH₄⁺ and cyclohexanone in *CfusAmDH* crystal structure) and *CfusAmDH*-W145A (right, model of association of NADP⁺ and heptylamine in *in silico* mutated *CfusAmDH* crystal structure). Side chain carbon atoms of NADP⁺, carbonyl substrate, ammonia and amine product are coloured respectively in orange, yellow, blue and green.

Using solved structures of some representatives and 3D-models, we described here some protein engineering work based on the exploration of the active site diversity within this family. A structural study allowed us to find some interesting target residues of the catalytic pocket to expand the substrate scope of these native AmDHs, currently mainly restricted to small aliphatic aldehydes and ketones (<C6). Mutations of two hindered aromatic amino acids into smaller amino acids in *CfusAmDH* and other nat-AmDHs allowed some enzymes to accept longer aliphatic aldehyde and ketone substrates up to C10, as well as aromatic compounds. Description of the strategies and results will be presented, including potential crystallisation and *in silico* analysis to explain an interesting switch of cofactor.

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Enzyme cascades for the synthesis of hyaluronic acid

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Hyaluronic acid (hyaluronan, HA) is a linear polysaccharide composed of repeating disaccharide units of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), (4GlcA β 1,3GlcNAc β 1-). As non-immunogenic, natural biopolymer HA has a wide range of applications in the medical and cosmetic industries. Bifunctional plasma membrane-integrated (Class I) or peripheral (Class II) HA synthases (HAS) are involved in HA biosynthesis and utilize UDP-GlcA and UDP-GlcNAc as monosaccharide donors.[1] Class II *Pasteurella multocida* HAS (PmHAS) was utilized for *in vitro* synthesis of size-defined monodisperse HA preparations.[1] However, nucleotide sugar substrates are considered as expensive. We here present novel enzyme module systems (EMs) for the *in vitro* one-pot synthesis of high molecular weight (HMW) HA with *in situ* production and regeneration of nucleotide sugars starting from sucrose or GlcA and GlcNAc (Scheme 1).[2,3]

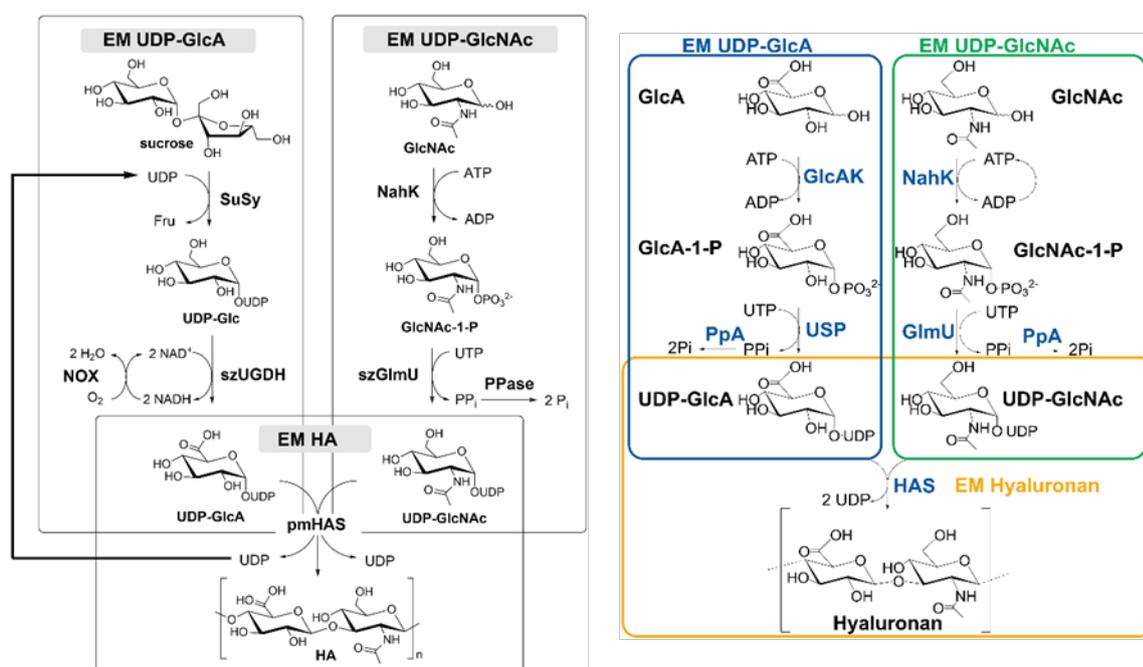


Figure 1. Enzyme cascades for one-pot HA synthesis.

A comprehensive analysis of system parameters was performed by Multiplexed-capillary electrophoresis. The mixture of cofactors, the PmHAS kinetics, and substrate ratio influence the polymerization rate and MW of HA. UDP-GlcA regeneration by sucrose synthase proved to be favourable for kinetic and economic reasons. With optimized reaction conditions, HA with a MW > 2 MDa and low dispersity was synthesized.[2] Starting from GlcA and GlcNAc, Mg²⁺ concentration and pH were defined as key parameters to produce HMW HA (> 3 MDa) with low dispersity.[3]. The presented EMs represent a cost-efficient HA synthesis from cheap and renewable starting materials.

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Engineering NADPH-dependent oxyfunctionalization in cyanobacteria

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Oxidoreductases, with their high selectivity, are tremendously attractive candidates for industrial applications. Type I Baeyer-Villiger Monooxygenases (BVMOs) [1] are flavin-dependent enzymes that utilize oxygen and NADPH for selective Baeyer-Villiger oxidations. However, NADPH is a limiting factor for large-scale studies despite the endogenous recycling in bacteria, since the stoichiometric amount of sacrificial hydrogen donors, such as glucose, is required. On the other hand, cyanobacteria provide a high amount of NADPH, and the recycling of the cofactor is carried out *via* the reactions that occurred during photosynthesis namely water oxidation. Therefore, the recombinant cyanobacteria carry a huge potential to be utilized as a catalyst for various oxidoreductions and can present an excellent alternative for NADPH driven Baeyer-Villiger oxidations. However, side reactions have been shown to hinder complete oxidation of BV.[2]

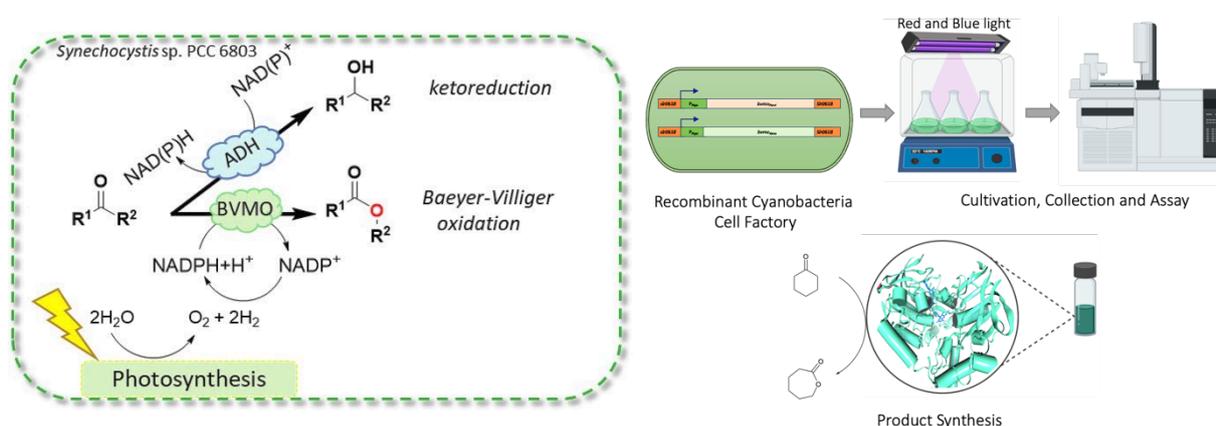


Figure 1. Baeyer-Villiger oxygenation in engineered *Synechocystis* PCC 6803.

In this study, a highly active BVMO_{Parvi} [3], and a newly discovered BVMO_{Xeno} were cloned into in *Synechocystis* sp. PCC 6803 genome under the control of two natural light inducible promoters. [2,4] Cell growth and reaction conditions were determined to stimulate enzyme production and increase the productivity of biotransformation. Thus, we were able to set up a successful bio-oxidation of cyclohexanone to caprolactone with a low level of side reactions (reduction, hydrolysis) and a high productivity, meanwhile expanding the substrate scope by using linear ketones, 4- to 7-membered cyclic ketones, as well as substituted cyclohexanones and unsaturated cyclic ketones.

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An approach for temporal regulation of one-pot multienzymatic cascades by local magnetic heating

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The *Hotzymes* consortium (<https://www.hotzymes.eu>) aims to regulate in time one-pot multienzymatic cascades of economic relevance by means of local magnetic heating (LMH). Although one-pot multienzymatic systems can be highly efficient processes, their implementation still faces a few challenges, like (i) coordination of reaction conditions for optimal/balanced activity of the cascade biocatalysts, and (ii) avoiding cross-reactivity in the system to reduce by-product formation. Previously,^[1] we observed that the activity of enzymes immobilized on magnetic nanoparticles can be remotely regulated by an alternating magnetic field (AMF). If different enzymes are immobilized on nanoparticles featuring different properties (shape, size, etc.), each biocatalyst will respond differently to the applied AMF. Therefore, by tuning AMF parameters (frequency, field) we are able to coordinate the activity of each biocatalyst on-demand by targeted local heating. The specific objective of our work-package is to sequentially regulate a one-pot, three-step, multienzymatic cascade towards the production of tetrahydroisoquinolines,^[2] an important group of pharmaceutical drugs. To achieve our objective, we will employ the immobilized enzymes (carbonylase, transaminase and norcochlorine synthase) in an optimized AMF reactor running at 4 °C to keep the biocatalysts in an inactive state and then sequentially activate each biocatalyst on-demand by tuning the AMF parameters. Heat transfer generated locally on the nanoparticle (when exposed to an AMF) drives the activation of the target enzyme. So far, we have investigated the immobilization of two tetrameric enzymes, an (*R*)-selective his-tagged *Acetobacter pasteurianus* pyruvate decarboxylase variant (*ApPDC-E469Q*) and the (*S*)-selective *Bacillus megaterium* amino-transaminase (*BmTA*), on different batches of iron oxide magnetic nanoparticles produced by our consortium partners. We observed that enzymes bound to nanoparticles functionalized with Co²⁺ instead of Cu²⁺ presented higher residual activity (~70%). The immobilisates also presented higher stability and processability than its free counterparts. As a challenge, aggregation events occurring at different processing and application stages were observed, suggesting cross-linking of immobilisates due to the multimeric nature of the studied enzymes and other attractive forces (magnetism, Van der Waals). To address these challenges, which reduces LMH, we currently modified the surface chemistry of the nanoparticles to increase their electrosteric stability and carried out a process optimization.

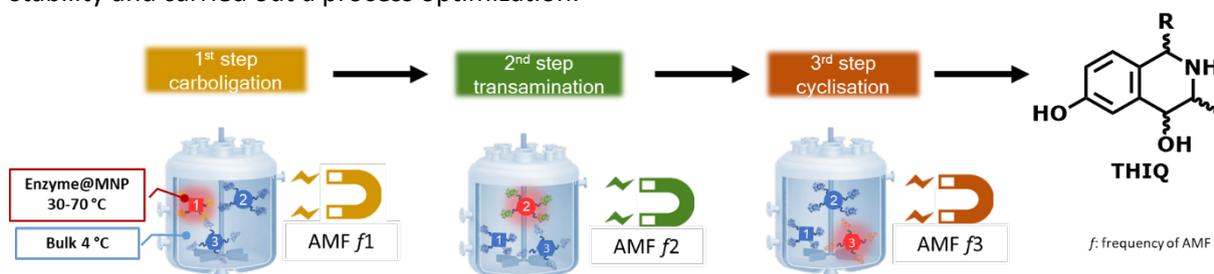


Figure 1. A one-pot multienzymatic cascade towards tetrahydroisoquinolines (THIQ) regulated by local magnetic heating.

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Biocatalytic C-3 indole methylation – A useful tool for natural product synthesis

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The indole alkaloid physostigmine is recently used for the treatment of glaucoma and pre-treatment to organophosphate poisoning based on its reversible inhibition of the acetylcholinesterase. A unique feature is the methylated quaternary stereogenic centre, introduced *via* a regio- and enantioselective SAM-dependent methyltransferase.^[1] Introducing this stereogenic centre in the correct absolute configuration is vital for the biological activity of physostigmine, depicting the importance of the methyl group and pointing out the recent demand for selective methylation reactions.^[2] Even though the chemical toolbox accessing quaternary, methylated carbons has been enlarged lately, direct methylation of indoles is often hampered by poor stereoselectivity.^[3] Thus, rather long and inefficient total syntheses are necessary to circumvent this bottleneck. Our objective is to tackle this problem by using the methyltransferase PsmD and prove its applicability towards biocatalytic, regio- and enantioselective late stage methylation of various indole alkaloids precursors providing a shortened access towards biologically active indole alkaloids.

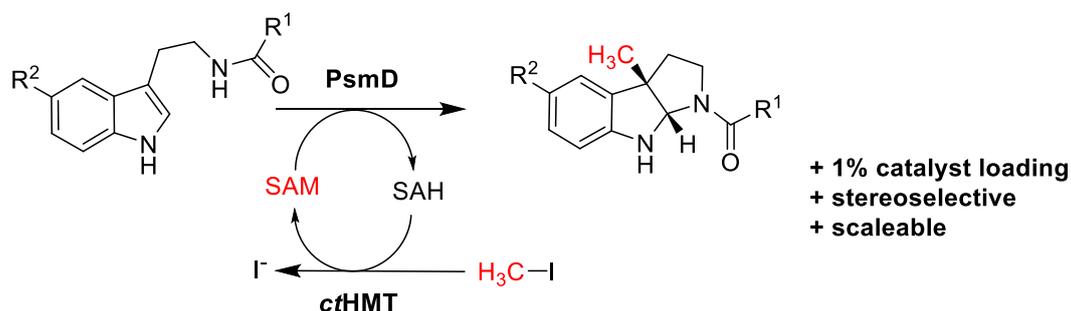


Figure 1. General procedure for the PsmD catalysed enantioselective synthesis of various hexahydropyrrolo[2,3-*b*]indoles.

In summary, we have expressed, isolated and characterized the C-3 indole methyltransferase PsmD. We further optimized the activity towards the natural substrate and determined its promiscuity. Furthermore, the relative configuration of this transformation was elucidated and PsmD was used for a natural product synthesis on 50 mg scale by consuming only catalytic amounts of SAH employing a SAM recycling system.^[4] C-3 indole alkaloid methyltransferases show a high potential with regard to direct regio- and enantioselective methylation of different indoles under mild conditions. This gives us the opportunity to access biologically active indole and novel hexahydropyrrolo[2,3-*b*]indoles which have not been accessible and produced previously.

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Custom synthesis of new-to-nature fatty acids and polyketides by transferase design

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Multienzyme type I fatty acid (FASs) and polyketide synthases (PKSs) provide compartmentalized reaction space for C-C bond formation. Compartmentalization is largely achieved by the transacylation function of FASs and PKSs, i.e. the enzymatic reaction responsible for selecting substrates from the bulk cytoplasm, and further relies on a specific protein architecture as well as the shuttled distribution of substrates between the enzymatic domains. Controlling the transacylation function of FASs and PKSs is a powerful mean to modulate the product output of these proteins ^[1,2], giving access to technologically and medicinally relevant compounds.

Two examples for transacylation-based engineering will be given in this talk: (i) The fungal FAS can be employed for the custom synthesis of commodity products, among them short-chain fatty acids, methylketones and a lactone ^[3-5]. The lactone is synthesized by two FAS constructs, which are controlled in sequence by transferase design. (ii) The transferase domain of the mammalian FAS features high transacylation rates for a wide range of substrates ^[2]. Built into modular PKSs, this approach enables the *in vitro* semisynthesis of new polyketides, including fluorinated polyketides with a macrolide structure of the next-generation antibiotic solithromycin.

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Investigation of the conformational dynamics involved in cytochrome P450 3A4 allostery

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P450 enzymes (CYPs) consist of a large family of hemoproteins catalyzing monooxygenation reactions and exhibiting complex allosteric and cooperative behaviors.^{1,2} Notably, the activity of the most important human drug-metabolizing enzyme, CYP3A4, is modulated by the simultaneous binding of multiple substrates in its active site – an atypical allosteric phenomenon stemming from its broad substrate specificity and structural flexibility. CYP3A4 allostery is well-documented, but the location of its allosteric site remains a matter of debate, and the underlying allosteric mechanisms are poorly defined. To investigate these questions, a new approach was developed combining small-molecule bioconjugation and hydrogen-deuterium exchange mass spectrometry (HDX-MS). The location of the allosteric site was probed using the bioconjugation of a progesterone derivative at various positions on the enzyme.³ These bioconjugates allowed to narrow down the position of the allosteric site and permanently reach the enzyme allosterically enhanced conformational state. The impact of bioconjugation on the conformational dynamics of CYP3A4 was characterized using HDX-MS.⁴ The data further defined the position of the allosteric site and revealed regions of the protein directly involved in enzyme activation. These results present a new perspective on the mode of action of CYP3A4 and contribute to the advancement of the fundamental understanding of protein allostery.

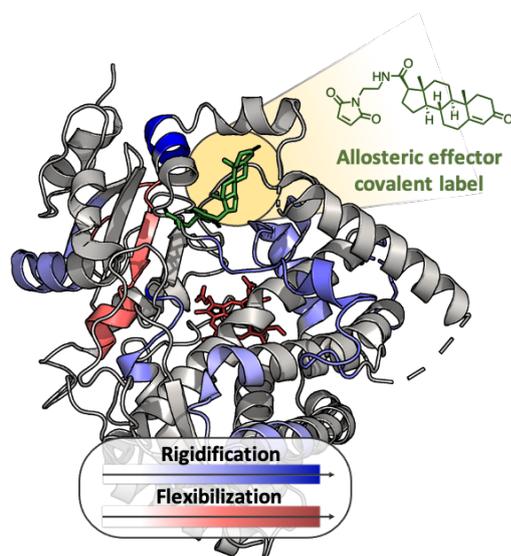


Figure 1. Graphical abstract

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Feed automation in a *Pichia pastoris* bioprocess based on constant respiratory quotient

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For optimizing the production yield of a *P. pastoris* culture, one of the most important factors is the feeding strategy. One of the leading strategies for protein production-oriented feeding is based on respiratory quotient (RQ). It ensures that the respiratory metabolism of glucose/glycerol are optimized for protein production purposes and the formation of by-products are limited [1].

Here we demonstrate the feasibility of constant RQ-based feeding for protein production-oriented yeast fermentation using the DASbox Mini Bioreactor System. RQ is calculated from the ratio of CO₂ produced by the microorganism to O₂ consumed by the microorganism. To determine the RQ in real-time, we integrated an exhaust analyzer with the bioprocess control system. The analyzer calculated RQ based on information on the gas composition and flow into the bioreactor and the exhaust composition (Figure 1). The RQ value was fed into a software script, which activated the feed pump, if RQ dropped below 1. We compared constant RQ-based feeding with a feeding strategy based on the dissolved oxygen concentration in the medium. Our study highlights the benefits of constant RQ control in *P. pastoris* fermentation as well as its effect on the amount of feed used and the microbial growth.

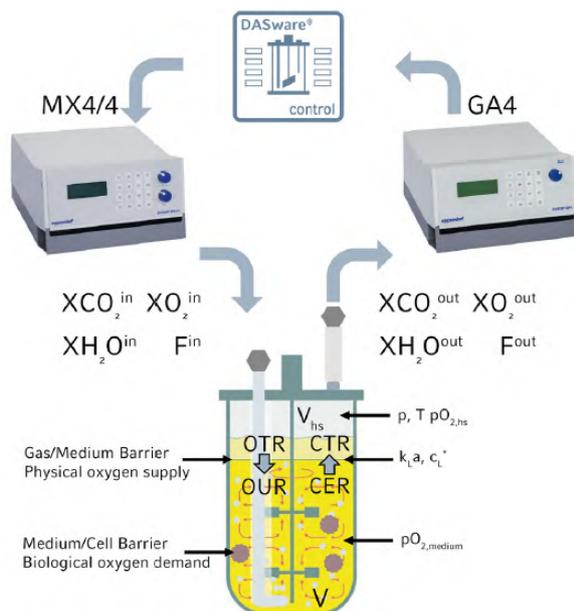


Figure 1. Determination of the respiratory quotient RQ. The exhaust analyzer GA4 calculates the oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) based on the compositions and flow rates of supplied gas and exhaust gas. RQ is calculated as the ratio of CTR and OTR.

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Photoenzymatic Catalysis – Using Light to Reveal New Enzyme Functions

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Enzymes are exquisite catalysts for chemical synthesis, capable of providing unparalleled levels of chemo-, regio-, diastereo- and enantioselectivity. Unfortunately, biocatalysts are often limited to the reactivity patterns found in nature. In this talk, I will share my groups efforts to use light to expand the reactivity profile of enzymes (Figure 1). In our studies, we have developed novel photoexcitation mechanisms involving common biological cofactors, such as NADH and FMN, to facilitate electron transfer to substrates bound within enzyme active sites. Alternatively, proteins can be used to electronically activate substrates for reduction by exogenous photoredox catalysts enabling radical formation to be localized to the protein active site. The resulting radicals can engage in a variety of inter- and intramolecular reactions with high levels of enantioselectivity. These approaches enable biocatalysts to solve long-standing selectivity challenges in chemical synthesis.

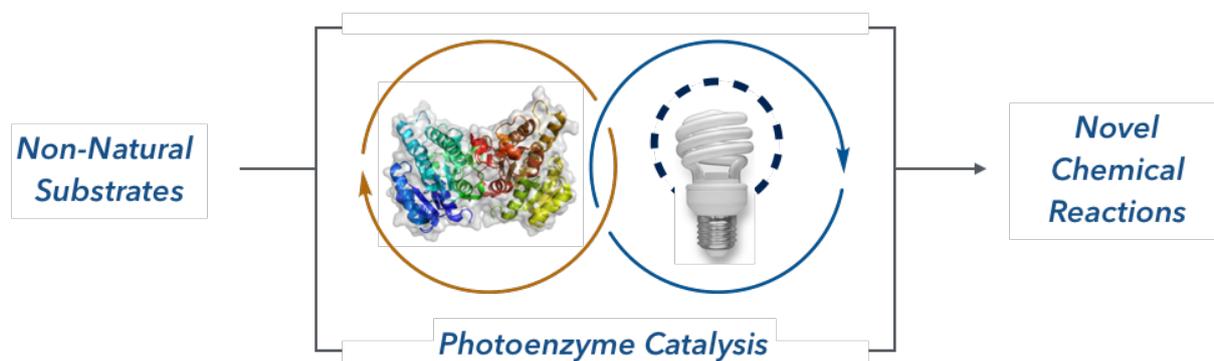


Figure 1. General Schematic for Photoenzymatic Catalysis

Finding Enzymes by Serendipity

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The overarching goal in biocatalysis is finding novel enzymes, which can be accomplished by several strategies:

— *Fast but treacherous*: Searching gene/protein databases yields candidates suggested by algorithms. You get approximately what you expect and you won't find surprising activities.

— *The needle in the haystack*: To find truly novel activities, you screen (preselected) cultures using a (surrogate) substrate with simple analytics [1]. The path from whole-cell activity to the novel protein is painstaking, but rewarding and you find novel activities, such as ω -TAs or imine-reductases.

— *The Lego-Construction Kit*: Take a well-known protein scaffold and convert its catalytic machinery to enable reactions unknown to Nature, such as C-Si-bond formation, metathesis, etc. [2].

— *Serendipity lurks*: Over the past years, we accidentally discovered a number of unexpected enzyme activities, which turned out quite successful. The inevitable influence of chance is highlighted by the following episodes: (i) An unexpected epoxide hydrolase activity in a nitrilase-preparation led to discovery of microbial epoxide hydrolases [3]. (ii) During a screening for sulfatases, an annoying organic-solvent stable ADH-activity led to a breakthrough in NAD^+ /NADH-regeneration [4]. (iii) The failed reduction of aliphatic nitro-compounds by ene-reductases revealed a biocatalytic Nef-reaction [5]. (iv) An attempted carboxylation of styrene derivatives by phenolic acid decarboxylases gave an asymmetric C=C-hydration [6].

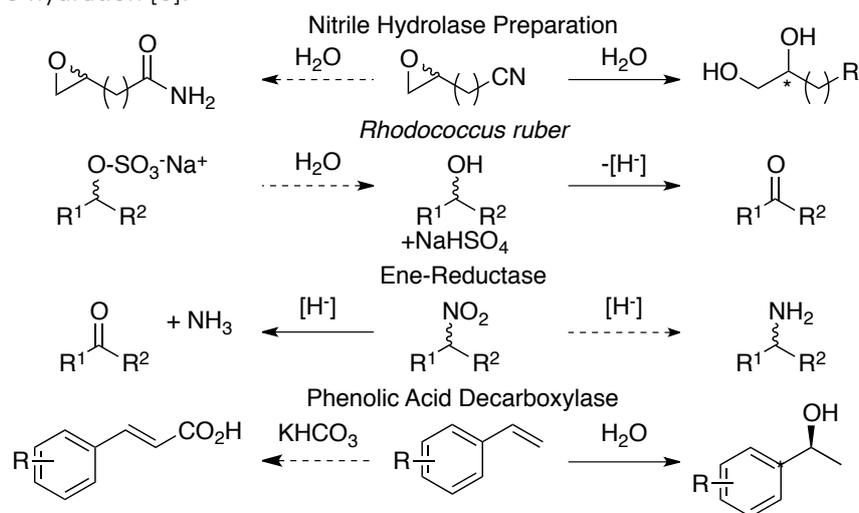


Figure 1. Expected (dashed arrows) and unexpected (solid arrows) enzyme activities.

My cordial thanks go to the numerous students, PhDs, postdocs, colleagues & friends I had the pleasure to work with, your inspiration and enthusiasm enriched my life!

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POSTERS



Direct electron transfer between a redox-active anchor of an artificial metalloenzyme and a multi-walled carbon nanotube electrode

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Artificial metalloenzymes (ArMs) represent an exciting and rapidly expanding field of biocatalysis. The integration of an organometallic catalyst into a protein scaffold opens up opportunities for carrying out new-to-nature chemical transformations in a biocompatible environment. An ArM based on a non-covalent interaction between an anchor-catalyst conjugate and the protein scaffold, comprising an iron-siderophore bound to an iridium-based imine reduction catalyst and a periplasmic binding protein of *C. jejuni*, CeuE, has been developed.[1] The design exploits the reversible iron-siderophore binding mechanism for the recycling of the protein through the dissociation of the anchor-catalyst conjugate from the CeuE scaffold, facilitated by the reduction of the iron(III) centre to iron(II).

Herein, we outline our investigation into the feasibility of electrochemical reduction for the release of the redox-active anchor through the direct adsorption of a His-tagged CeuE protein scaffold onto an electrode modified with multiwalled carbon nanotubes (MWCNTs). CNTs have been shown to facilitate direct electron transfer between proteins and electrodes and can enable the incorporation of the ArM in flow biocatalysis, utilising recently developed carbon nanotube columns.[2,3] We employed protein film electrochemistry to show that a direct electrochemical connection can be established between the iron(III) centre of the anchor and the modified electrode. However, the cyclic voltammetry data suggests a high affinity of the siderophore anchor towards the CNT support which is proposed to facilitate an unfavourable orientation of the protein upon immobilisation. The consequences of this interaction on the application of this methodology for the immobilisation of the ArM are discussed.

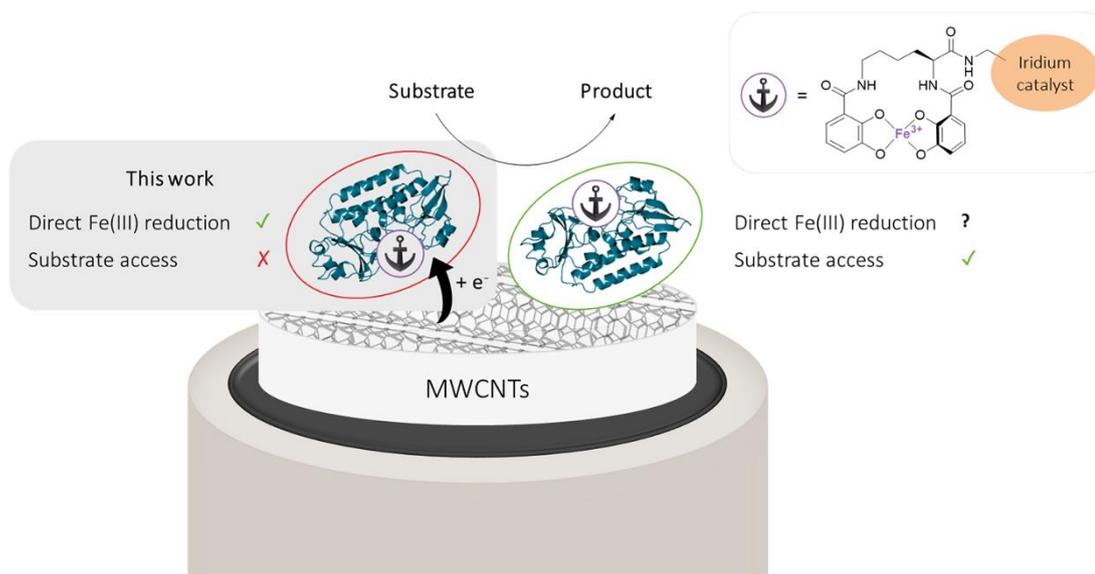


Figure 1. A schematic representation of the possible orientations of the ArM on the electrode surface modified with multi-walled carbon nanotubes (MWCNTs), in relation to the position of the anchor-catalyst conjugate. The consequence of each orientation on the substrate access for the catalytic reduction of imine is highlighted.

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PadR proteins as bioscaffolds for artificial enzyme design

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Incorporation of unnatural catalytic moieties into protein scaffolds allows us to create artificial enzymes capable of catalyzing non-natural transformations and as such is a promising means of expanding the reach of biocatalysis [1]. While the choice of catalytic moiety and incorporation strategy are major aspects in the design of artificial enzymes, the choice of bioscaffold is just as important as different scaffolds can provide different chemical environments. LmrR is a protein from the PadR-family that forms an open pore at its dimeric interface that exhibits promiscuous binding capabilities [2]. Using different catalytic moieties and incorporation strategies, a range of LmrR-based artificial enzymes have been created, showcasing the versatility of this protein as scaffold for the design of artificial enzymes [3,4].

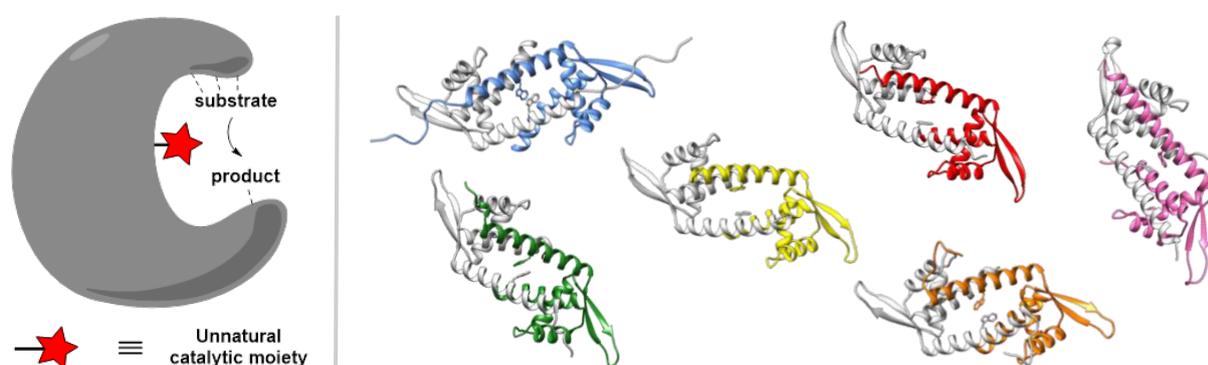


Figure 1. Left: Schematic representation of the principle of an artificial enzyme created by incorporation of an unnatural catalytic moiety into a protein bioscaffold. Right: Homology models of the PadR proteins studied in this work.

Inspired by the success of LmrR, we wondered if we could find structurally similar proteins to expand our library of bioscaffolds for the design of artificial enzymes. Therefore, in this work, a bioinformatics study was performed to discover and select six LmrR-like PadR proteins as potential new scaffolds for artificial enzyme design. The new PadR proteins were subsequently tested as scaffolds in artificial enzyme design by incorporation of unnatural catalytic moieties using either genetic code expansion or a supramolecular approach.

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LmrR-based artificial enzymes towards a “greener” chemistry

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Enzymes have shown to be powerful tools for the catalysis of reactions that occur in nature and preserve life and are considered fundamental for the drive to a more sustainable and “greener” chemical synthesis. Inspired by the activity of these biological catalysts, artificial enzymes with the ability of accelerating new-to-nature reactions have been created [1].

The Roelfes group selected the Lactococcal multidrug resistance regulator protein (LmrR) as a basis for different kinds of artificial enzymes. What makes the protein a suitable scaffold is its large and promiscuous binding pocket, that can interact with various compounds through hydrophobic interactions and its flexible structure that can adapt easily when hosting guest molecules [2]. Emulating the strategies that Nature itself uses to expand the “reaction space” of natural enzymes, it is possible to install abiotic and catalytic functionalities in LmrR’s pocket. The supramolecular binding of abiological metal cofactors, like copper-phenanthroline, in the LmrR pocket mimics the recruitment of cofactors in Nature [3] and the introduction of non-canonical amino acids, like para-amino-phenyl-alanine (pAF), through the stop codon suppression methodology simulates the natural post-translational modifications [4].

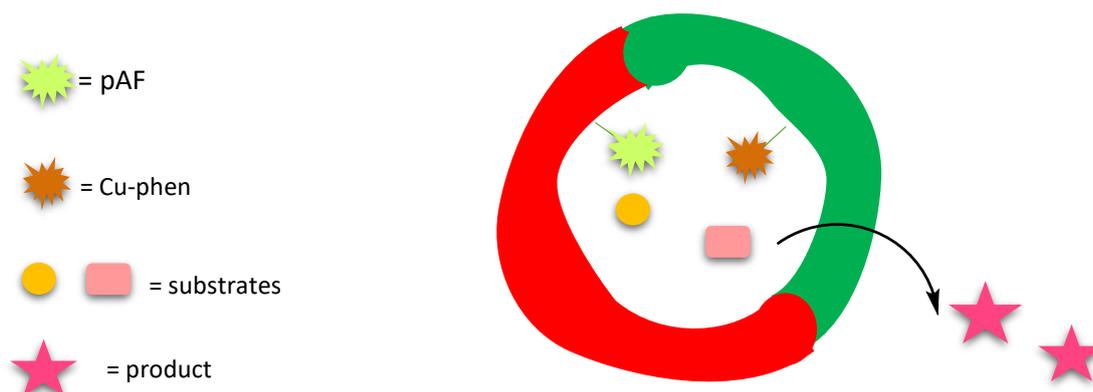


Figure 1: Schematic representation of the synergistic system installed in LmrR.

It is also possible to combine these two abiotic catalytic functionalities in the LmrR pocket to create a synergistic catalyst, which activates both the electrophile and the nucleophile. The synergism of pAF and copper-phenanthroline in LmrR was demonstrated in the enantioselective Michael addition reaction (up to >99% e.e.) [5]. This work aims to expand the application of the synergistic system for other C-C bond forming reactions, such as cycloaddition.

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Structural Investigation of Artificial Metalloenzymes

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The field of biocatalysis is welcoming the design of novel biocatalysts to enable reactions not seen before in Nature. Artificial metalloenzymes (ArMs) are designed to provide a platform for biocatalysis of unnatural reactions with the catalytic activity of transition metals and selectivity of enzymes. [1]

Research in the Jarvis group has been previously focused on the design and construction of ArMs using steroid carrier protein (SCP) as the protein scaffold and Cu(II) bound to bipyridine as the catalytic centre. [2, 3, 4] The resulting ArMs proved to be effective in catalysing an enantioselective Friedel-Crafts reaction (Figure 1). However, optimizing the novel catalyst has been challenging. While directed evolution has been shown to be a powerful approach for optimizing enzymes, it is more complicated to use in the process of ArM optimisation. Here we use a structural approach to improve our understanding of the active site of these ArMs and rationally design improved catalysts.

The aim of this work was to improve the understanding of ArMs using a well-known reaction and pave the way for 2,2'-bipyridine as a general ligand in ArMs.

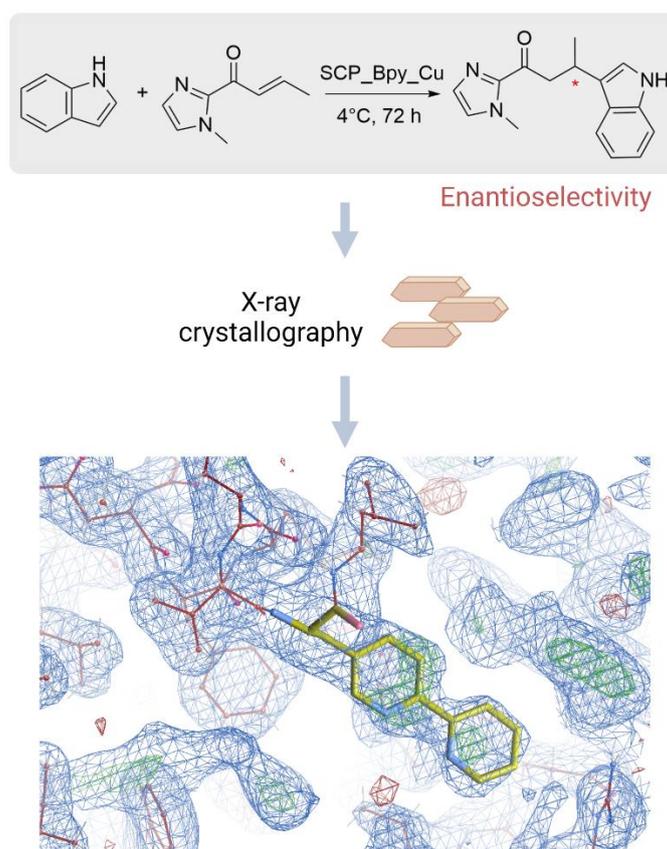


Figure 1. Workflow of Cu-ArMs design for the Friedel-Crafts reaction.

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Revisiting the role of asparagine in the active site of “classical” Old Yellow Enzymes

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The Old Yellow Enzyme (OYE) family is a group of ene-reductases that uses NADPH as co-factor to reduce activated C=C bonds. They have a TIM barrel fold and bind flavin mononucleotide (FMN) in their active site.[1,2] There are historically two types of OYE family members, “thermophilic-like” OYEs with a conserved histidine pair, and “classical” OYEs with a histidine and asparagine in the active site. These residues play a role in positioning the substrate by coordinating the electron-withdrawing group.[3]

In one of the seminal studies by Vincent Massey, the effect of interchanging these residues was examined by creating three mutants, namely OYE1_H191N, OYE1_N194H, and a double mutant OYE1_H191N_N194H. Unfortunately, the OYE1_N194H mutant could not be isolated in this study. Compared to the wild-type, the other two mutants had decreased phenolic affinity, NADH oxidation, and activity with 2-cyclohexanone.[4] Here, we investigated the “missing” mutant N194H by site-directed mutagenesis using OYE3. The active site of the new mutant (OYE3_N194H) replicates the double histidine residues found in other natural “thermophilic-like” OYE enzymes.

The crystal structure of the mutant was solved and showed that H194 adopted a similar position as N194 (Figure 1) in the wild-type enzyme.

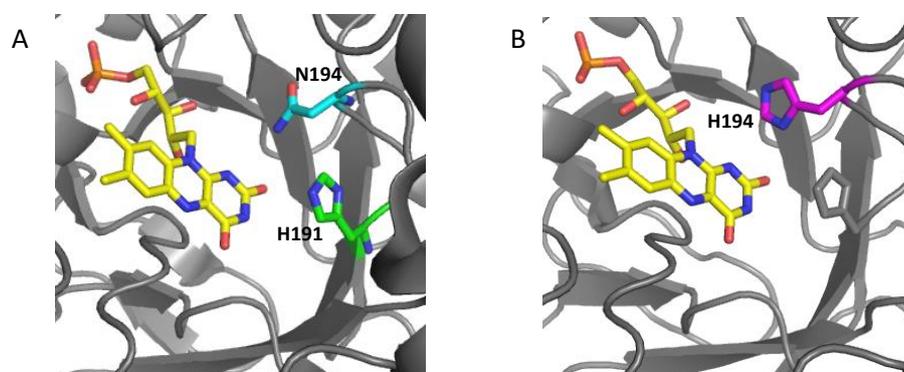


Figure 1. Active sites of A) OYE3 wild-type (PDB=5V4V) and B) OYE3_N194H mutant. FMN is shown in yellow.

Activity assays were performed with purified protein by measuring the rate of NADPH oxidation by the wild-type OYE3 and OYE3_N194H to compare activity. In addition, biotransformations using purified enzyme with a range of substrates were performed, and conversions were analysed by GC. Structural and activity analysis revealed that although the histidine occupies the equivalent position as the asparagine, activity was still lower with the OYE3_N194H mutant than the wild-type, and thus follows a similar trend to the study that focused on the OYE1_H191N and OYE1_H191N_N194H mutants.

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Unlocking Iminium Catalysis in Artificial Enzymes to Create a Friedel-Crafts Alkylase

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Enzymes are renowned for their extraordinary catalytic power under mild operating conditions, and great leaps are being made in their application for the preparation of societally important molecules utilising unnatural biotransformations. However, the array of chemistries facilitated by biocatalysts still cannot meet all demands of the synthetic chemist. The design and engineering of artificial enzymes containing abiological catalytic components is an important route towards expanding the biocatalytic repertoire[1]. Our group previously showed that biosynthetic incorporation of the unnatural amino acid para-aminophenylalanine into the multidrug-resistance regulatory protein LmrR yields an artificial enzyme (LmrR_pAF) for the hydrazone formation reaction, where the aniline sidechain participates in a mechanism reminiscent of organocatalysis[2].

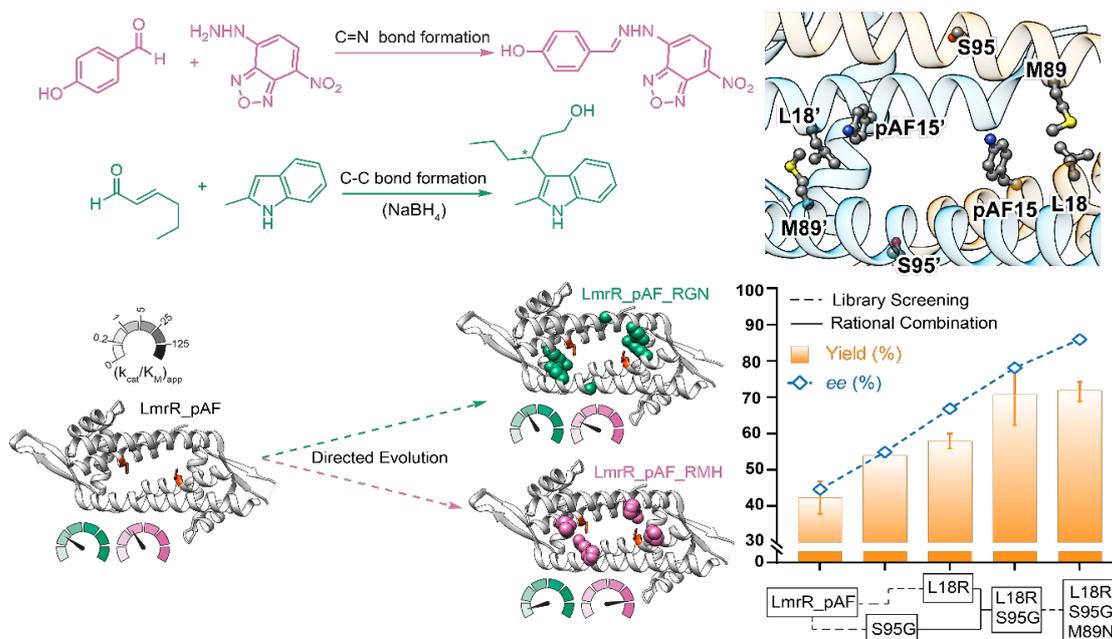


Figure 1. Left: promiscuous activities in an artificial enzyme emerge via divergent pathways. Right: Friedel-Crafts alkylation activity was increased after two rounds of directed evolution.

Here we show that LmrR_pAF can also catalyse a vinylogous Friedel-Crafts alkylation of indoles, a transformation unknown for natural enzymes, and a reaction class which is thus-far under-represented in nature[3]. We improved the yields and stereoselectivities of this transformation through low-throughput directed evolution informed by alanine-scanning. The resulting triple mutant displays improved yields and enantioselectivities for a variety of indole and enal substrates. Kinetic investigation of mutants obtained by directed evolution of LmrR_pAF shows that promiscuous activities emerge through divergent pathways and hence specialisation of the artificial enzymes, using unnatural catalytic machinery to recapitulate evolutionary effects seen in nature[4].

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De novo biosynthesis of a nonnatural cobalt porphyrin cofactor in *E. coli* and incorporation into hemoproteins

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Enzymes that bear a nonnative or artificially introduced metal center can engender novel reactivity and enable new spectroscopic and structural studies. However, in the case of metal-organic cofactors, such as metalloporphyrins, no general methods exist to build and incorporate new-to-nature cofactor analogs *in vivo*. We report here that *E. coli* BL21(DE3), a common laboratory expression strain, can both biosynthesize a cobalt protoporphyrin IX (CoPPiX) cofactor and incorporate this cofactor into several different hemoproteins. [1] We hypothesize that *de novo* biosynthesis of this cofactor is enabled by a promiscuous ferrochelatase enzyme. We use a variety of analytical methods to show that the resulting artificial metalloenzymes can be produced in reasonable yields, bear the canonical metal coordination ligands in most cases, and that CoPPiX makes up at least 95% of the total porphyrin content of each enzyme. Because this method is easy to implement, efficient, and amendable to whole cell and cell lysate preparations, we hope that this advancement will facilitate spectroscopic studies and the development of enzymes for CoPPiX-mediated biocatalysis.

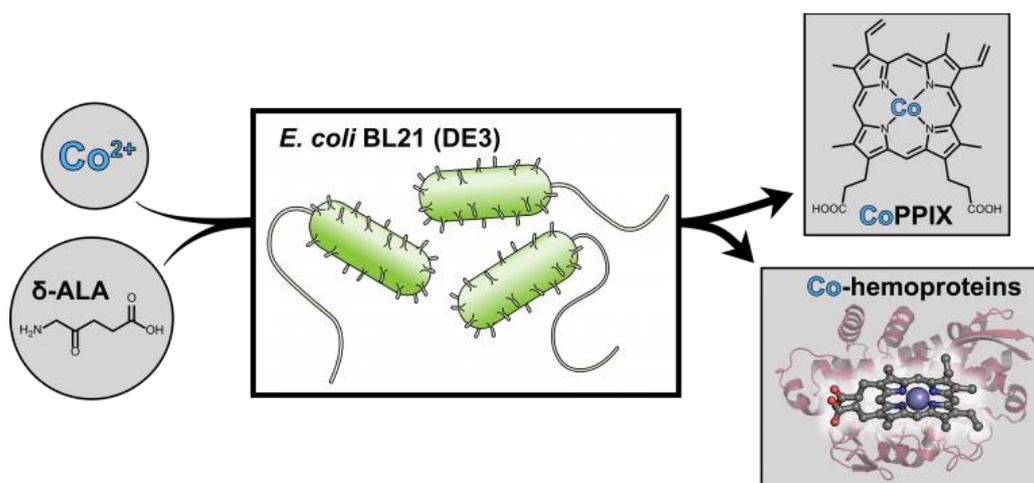


Figure 1. A straightforward procedure for the biosynthesis and *in vivo* incorporation of CoPPiX cofactors into hemoproteins.

[1] Perkins, L.J.; Weaver, B.R.; Buller, A.R.; Burstyn, J.N. PNAS. 2021

Engineering of artificial metalloenzymes for new-to-nature reactions

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Artificial metalloenzymes (ArMs) combine the broad reaction scope of organometallic catalysis with the exceptional catalytic performance, selectivity, and mild reaction conditions of enzymes. Therefore, they have a great potential to enable sustainable synthetic routes to various compounds of interest and, if functional in a cellular environment, open up new possibilities for metabolic engineering and synthetic biology [1]. Among the numerous strategies for creating ArMs, one of the most versatile approaches relies on the biotin-streptavidin technology. This strategy uses the high affinity of the homotetrameric protein streptavidin (Sav) for the vitamin biotin to non-covalently anchor biotinylated metal complexes (referred to as cofactors in this context) within the Sav protein. Using this approach, artificial enzymes have been created for a broad range of reactions.

However, most ArMs reported to date are only moderately active and barely function in living cells. To change this, we have established a whole-cell screening platform for ArMs that relies on periplasmic catalysis in *Escherichia coli* (Figure 1) [2]. In order to enable the rapid discovery of active ArMs for ideally any reaction in interest, we automated crucial parts of the screening procedure and created a sequence-defined Sav library that is rich in variants with high activity. This was achieved by simultaneously diversifying two crucial amino acid positions in close vicinity to the catalytic metal. With this platform in hand, we were able to discover substantially improved ArMs for five bio-orthogonal reactions [3]. Among these are the first gold-harboring ArMs for hydroamination and hydroarylation reactions. This platform provides an attractive starting point for ArM engineering. Moreover, it will serve as the basis of larger screening campaigns that target more positions in the enzyme. In this case, exhaustive screening quickly becomes impossible, but our observations suggest that machine learning can guide ArM engineering and enable the efficient optimisation of these promising biocatalysts.

We are convinced that this novel, systematic approach to ArM engineering will bring numerous applications in biocatalysis and metabolic engineering within reach.

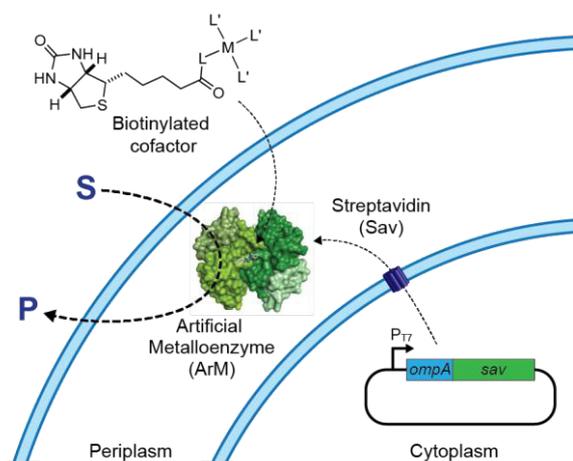


Figure 1. Periplasmic ArM catalysis in *E. coli*. Sav is secreted to the periplasm, where it binds an externally added biotinylated cofactor consisting of a catalytic metal M and ligands L and L' to afford an ArM.

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Synergistic Catalysis of Tandem Michael Addition / Enantioselective Protonation Reactions by an Artificial Enzyme

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Tertiary carbon stereocenters are ubiquitous in biologically active natural products and pharmaceuticals. Enantioselective protonation is conceptually one of the most efficient methods for generating a tertiary carbon stereocenter. However, enantioselective transfer of a proton presents tremendous challenges, especially in aqueous solvents: protons are difficult to control due to their small size, protons in water are highly mobile, proton transfer is generally very fast and the products can be prone to racemization. Nature has evolved several efficient enzymes such as decarboxylases and esterases that catalyse enantioselective protonation reactions. Approaches for non-enzymatic enantioselective protonation mainly rely on the stereoselective protonation of prochiral enolates. [1]

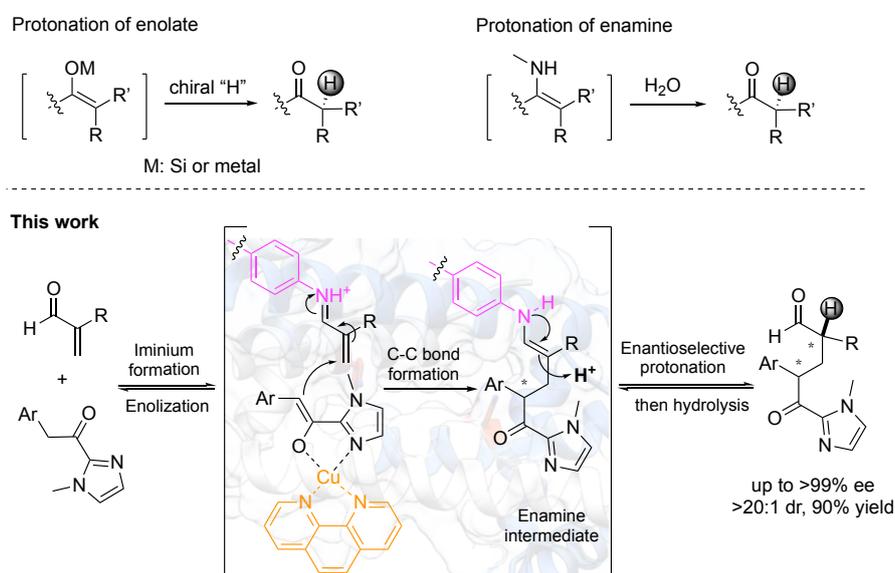


Figure 1. Tandem Michael addition and enantioselective protonation catalyzed by an LmrR-based artificial enzyme via synergistic combination of two catalytic sites: an unnatural pAF residue that activates the α -substituted enal via iminium ion formation and a Lewis acid Cu(II) complex that catalyzes the enolization of the Michael donor. After Michael addition reaction, the resulting enamine intermediate undergoes enantioselective protonation.

Herein, we report an artificial enzyme catalyzed tandem Michael addition /enantioselective protonation reaction of α -substituted acroleins with 2-acyl imidazole derivatives in water. The artificial enzyme uses a synergistic combination of two abiological catalytic sites: a genetically encoded non-canonical p-aminophenylalanine residue and a Lewis acid Cu(II) complex. The exquisite stereochemical control achieved in the protonation of the transient enamine intermediate generated by conjugate addition of the Michael donor is illustrated by the up to >20:1 dr and >99% ee obtained for the products. These results illustrate the potential of exploiting synergistic catalysis in artificial enzymes for challenging reactions.[2,3,4]

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Enantioselective Sequential Aldol Reaction Catalyzed by 2-Deoxy-D-Ribose -5-Phosphate Aldolase (DERA)

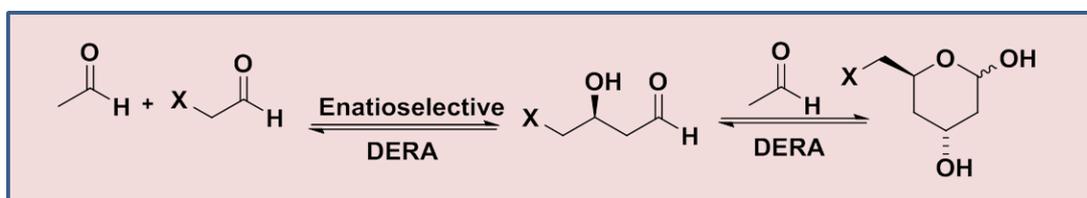
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Enantioselective aldol reactions are important methods to synthesize β -hydroxy carbonyl compounds in optical pure form. 2-deoxy-d-ribose-5-phosphate aldolase (DERA) is a class 1 aldolase that offers access to several building blocks for organic synthesis by catalyzing a stereoselective C-C bond formation between acetaldehyde and other aldehydes such as chloroacetaldehyde, propionaldehyde or ketones like acetone and fluoroacetone [1,2].

Here, we describe our studies with the acetaldehyde tolerant DERA Ib [3]. The gene encoding DERA Ib from *Lactobacillus brevis* ECU8302 was expressed in *Escherichia coli* and a combination of two mutations E78K and C42M was introduced to improve the acetaldehyde resistance and thermostability of the enzyme. The structures and activities of the two enzymes were characterized and compared with those of *E. coli* DERA. The double mutant showed much greater activity in the sequential aldol condensation reaction (using acetaldehyde and 2-substituted acetaldehydes as substrates) than the *E. coli* ortholog. At 25 °C and 1.4 M acetaldehyde, the chiral lactol intermediate was obtained in good yield. This intermediate is a synthon for the preparation of a large variety of optically pure super-statins, such as Rosuvastatin and Pitavastatin.

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Enzymatic Degradation of PET Plastic by Mechanical Agitation

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Due to the rising problem of plastic pollution, it is becoming necessary to develop new methods of removing plastic waste from the natural environment. As a plastic generally used for single use purposes, polyethylene terephthalate (PET) is produced on an annual scale of over 50 million tonnes.[1] A large proportion of the PET produced ends up as plastic waste in the environment, despite it being fully recyclable. In 2016, an enzyme able to degrade PET by using it as its sole carbon source, was discovered, providing a potential solution to the plastic waste problem.[2] In this work, known and novel PETase enzymes have been used in whole cell form to degrade PET substrates by both traditional biocatalytic methods and by mechanical agitation. Using HPLC analysis and SEM imaging, it has been found that optimising the time length of mechanical agitation and aging periods improved the degradation of PET substrates, achieving higher yields of breakdown products than seen previously. Offering scope for molecular recycling, a reduction in solvent used compared with traditional approaches combined with enzymatic methods, this is a sustainable option for PET breakdown.

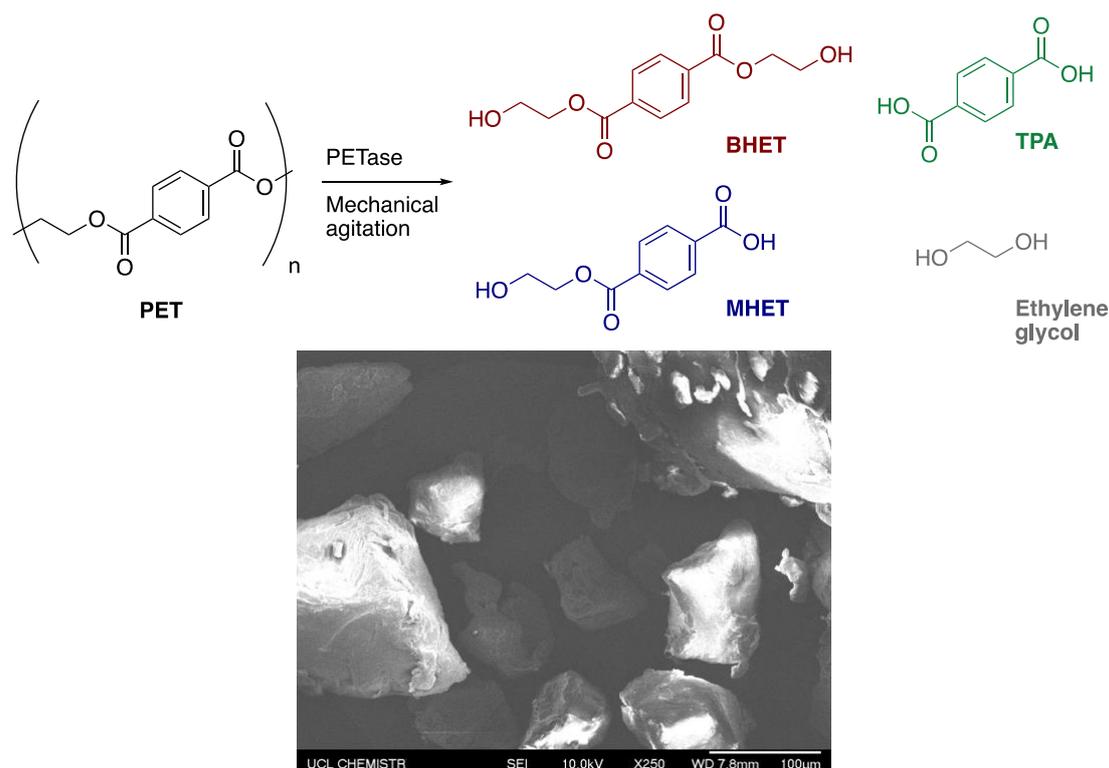


Figure 1. Scheme showing PET breakdown into monomers: bishydroxyethyl terephthalate (BHET), mono(2-hydroxyethyl) terephthalic acid (MHET), terephthalic acid (TPA) and ethylene glycol (EG). SEM imagery showing PET powder prior to enzymatic breakdown.

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Feruloyl esterase-based synthesis of bioactive esters based on phenolic compounds derived from halophyte *Salicornia* spp.

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Salicornia is a genus of succulent halophyte flowering plants that frequently occur in saline areas and comprise the most salt-tolerant land plant. The valuable properties of *Salicornia* species have been long known with displayed applications in folk medicine for treatment of bronchitis, hepatitis and diarrhea [1]. The important biological properties of *Salicornia*, such as their antioxidant, anti-inflammatory, hypoglycemic and cytotoxic activities, are owed to the numerous bioactive phenolic compounds found in their extracts [2]. Among them, hydroxycinnamic acids (HCAs), such as ferulic acid (FA), sinapic acid (SA), p-coumaric acid (p-CA) and caffeic acid (CA) are detected in high amounts [3]. This class of naturally occurring compounds shows strong antioxidant activity together with anti-aging, skin regenerative, anti-inflammatory and skin whitening properties among others [4] and has great potential for application in formulation of bioactive products, such as functional feed/ food and cosmetics. However, a significant limitation for their application in such formulations is often their low solubility in both aqueous and oil media. A common strategy to overcome this issue is to stabilize HCAs by an esterification reaction (Figure 1).

In this work, we screened various commercial enzymatic preparations having side-feruloyl esterase activity for their specificity towards HCAs and derivatives commonly found in *Salicornia* extracts. The most promising enzyme candidates were employed as biocatalysts for the esterification of HCAs aiming to improving their solubility in aqueous or oil media. Enzymatic stabilization offers a green and sustainable alternative to classic esterification methods that involve use of strong acids or expensive and toxic reagents as catalysts, high temperatures (150-250°C), long reaction times and low yields [5]. The approach could enable the application of HCAs and *Salicornia* extracts in a variety of bioactive products, based on highly selective esterification reactions. The work was supported by the Horizon 2020 funded project AQUACOMBINE (Grant agreement ID: 862834).

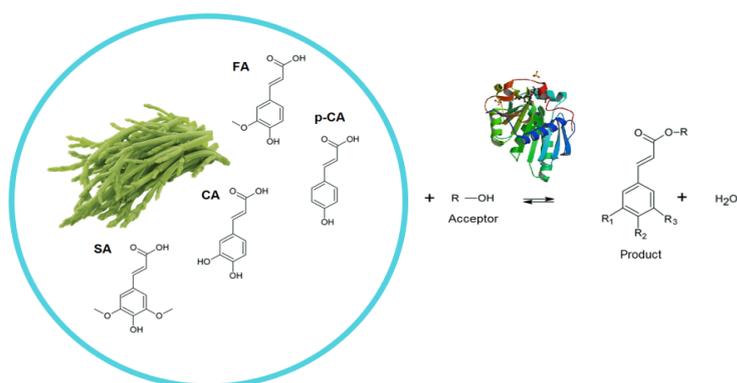


Figure 1. Enzyme catalysed esterification of HCAs.

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An Irreversible Chemo-Biocatalytic Cascade for the Synthesis of Substituted Pyrroles

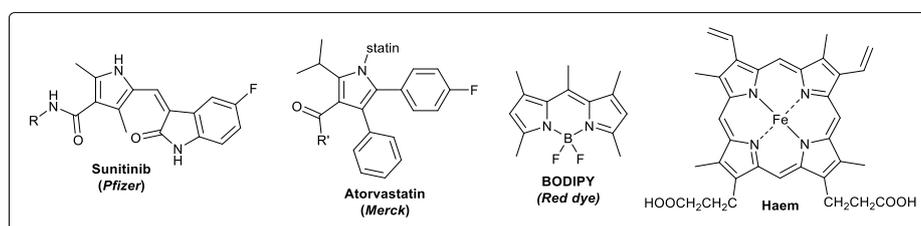
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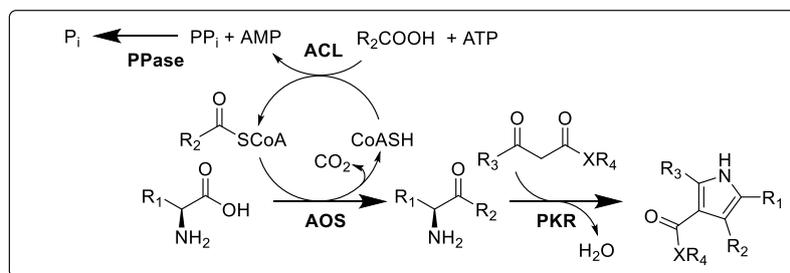
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The pyrrole moiety is an important scaffold in medicinal, polymer, dye and natural product chemistry. Pyrrole appears in the structure of the cholesterol-lowering blockbuster drug atorvastatin, the promising anticancer compound sunitinib and other biologically-useful molecules. One historical, but underutilised, route towards substituted pyrroles is the Paal-Knorr reaction (PKR), in which an α -oxoamine and a β -keto ester irreversibly condense together with elimination of H_2O .¹⁻³ The α -oxoamine precursor can be prepared with another long-known, but underutilised class of biocatalysts, the pyridoxal 5'-phosphate (PLP)-dependent α -oxoamine synthases (AOS). These catalyse the irreversible condensation of an amino acid with an acyl-CoA thioester. Here we couple the chemical PKR with the biocatalyst AOS in a one-pot process.



The selected enzyme, *ThAOS* is a promiscuous and thermophilic AOS enzyme reported in 2007.⁴ These properties suggested that it would be an ideal biocatalyst since the broad substrate range allows for the generation of a variety of substituted pyrroles, and the natural thermostability allows the biocatalytic reaction to run at the optimal elevated temperature of the PKR. To replenish the acyl-CoA thioester substrate within the system, an ATP-dependent, acyl-coenzyme A ligase (ACL) biocatalyst is included.



We have demonstrated the utility of *ThAOS* as a biocatalyst by exploiting the promiscuity for both of its substrates and optimised its coupling with the chemical PKR. This has resulted in the generation of a small panel of substituted pyrrole molecules from amino-acid, acyl-CoA-thioester and β -ketoester building blocks. The cascade is completed by the addition of a highly efficient pyrophosphatase (PPase) which reduces the inhibition of the ACL and allows good % conversion of fatty acids through to pyrroles. Finally, we have determined the x-ray structure of the *ThAOS* at high resolution which paves the way for engineering of this versatile biocatalyst and enable further expansion of its synthetic utility.

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Immobilization of Levansucrases and their Application for the Bioconversion of Lactose By-Products into Prebiotics

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There is an ever-increasing interest in exploring the catalytic action of levansucrase (LS, EC 2.4.1.10) for the synthesis of novel prebiotics that promote intestinal health. LSs from different microbial sources differ with respect to their reaction selectivity (hydrolysis/transfructosylation) and acceptor/product specificity. Modulating LS's microenvironments through immobilization is expected to not only modulate the reaction selectivity towards transfructosylation, but also to promote enzyme stabilization [1]. Focusing on lactose by-products, there is an undeniable increase in their production volume, one of which is a green-yellow liquid known as whey resulting from cheese or casein production from milk [2]. In the present study, the catalytic efficiency of selected LSs to catalyse the transfructosylation of lactose and sucrose into lactosucrose and fructooligosaccharides (FOSs) was assessed. The targeted lactosucrose and FOSs have garnered increased attention and demand due to their prebiotic and technofunctional properties [3].

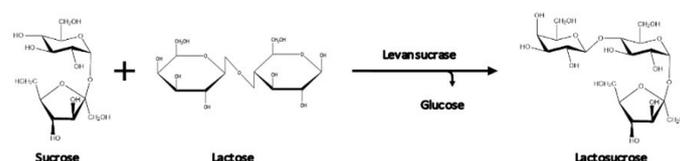


Figure 1. Synthesis of lactosucrose from sucrose and lactose using levansucrase

Four LS strains from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Burkholderia graminis* C4D1M (LS4) were selected and utilized in three different reactions that combine sucrose with either lactose powder, whey permeate, or milk permeate. By examining the catalytic efficiency on sucrose/lactose for all four LSs, it was found that almost all of those reactions demonstrated a higher transfructosylation activity as opposed to hydrolysis, with the sole exception of LS2 in the presence of milk permeate as a substrate. Furthermore, the bioconversion efficiency of lactose/sucrose into lactosucrose and FOSs exhibited varying time courses and end-product profiles depending on the type of LS and starting materials used. LS2 resulted in the highest bioconversion yields (87-100%), giving rise to 328 g/L and 251 g/L of lactosucrose with lactose powder and whey permeate, respectively. A complete bioconversion of lactose present in milk permeate into lactosucrose was achieved with LS1, LS2, and LS4 biocatalysts, whereas only 5% of bioconversion yield was obtained with LS3. Contrary to other LSs, LS3 showed a higher product specificity toward the synthesis of FOSs, in particular kestose, nystose, and fructosyl-nystose, in all investigated reaction systems.

Selected functionalised epoxy supports were investigated for the immobilization of LS1 and LS2 by multipoint covalent attachment. The highest immobilization yield of 94% and 87% and retained activity of 55% and 98%, respectively, were achieved upon the immobilisation of LS1 and LS2 on ReliZyme™ EP403/S functionalized with iminodiacetic acid/Cu (IDA/Cu) as compared to other supports such as Sepabeads® EC-EP/S-IDA/Cu and glyoxyl agarose-IDA/Cu. Additionally, a greater thermal stabilisation was obtained for both immobilised enzymes, especially with LS2 with a factor of 53 at 50°C. The increase in product specificity of immobilised LSs was attributed to the microenvironment effect and to the change in the 3D-structure upon immobilisation.

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Combining Chromoselective Photocatalysis with Biocatalysis

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Catalytic reactions can be controlled by varying the catalyst/coordinated ligands, directing groups or by tuning external parameters. The outcome of photochemical reactions may vary with different wavelengths, but examples using visible-light photocatalysis are rare.

The product obtained with the heterogeneous carbon nitride (CN) photocatalyst can be changed by the wavelength [1,2]. Using blue light results in electron holes with a higher oxidation potential than irradiation with green light, thus CN-OA-m species with different oxidation potentials shall be generated only through the choice of wavelength. This enabled the following reactions: photo-oxidation of ethylbenzene to acetophenone was performed in aqueous solution in the presence of blue light. Using green light, ethylbenzene did not react and only H₂O₂ was formed (**Figure 1**) [3].

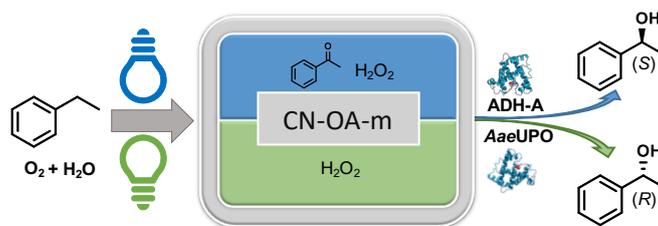


Figure 1. Chromoselective control of the stereochemical outcome of a photo-chemo-enzymatic reaction

This reactivity was combined with two enantioselective biocatalysts. The chromoselective activation of CN-OA-m with green light leading to the formation of H₂O₂ was combined with an unspecific peroxygenase from *A. aegerita* (*AaeUPO*). This allowed enantioselective hydroxylation of ethylbenzene to give (*R*)-1-phenylethanol (99 % *ee*). The outcome was highly influenced by the buffer used in the reaction. Green light conditions increased the stability of UPO compared to blue light, which allowed an extension of the substrate scope of *AaeUPO*.

In the presence of blue light a photocatalytic oxidation to acetophenone occurred. The latter was stereoselectively reduced by the alcohol dehydrogenase (ADH-A) from *Rhodococcus ruber* to produce (*S*)-1-phenylethanol (93 % *ee*).

This project received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764920. K.B. acknowledges the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement BioBased ValueCircle (956621). S.B. acknowledges the Austrian Science Fund (FWF, CATALOX DOC 46-B21). S.R. and B.P. acknowledge the Max-Planck Society and the German Chemical Industry Fund (Liebig Fellowship, Fonds der Chemischen Industrie, FCI). B.P. thanks the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2008 – 390540038 – UniSysCat. T.M. was funded by a studentship awarded by the industrial affiliates of the Centre of Excellence for Biocatalysis, Biotransformations and Biomanufacture (CoEBio3).

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Unspecific Peroxygenase can be Tuned for Oxygenase or Halogenase Activity by Controlling the Reaction pH

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Unspecific peroxygenases (UPOs) are heme proteins secreted by filamentous fungi as part of a collection of enzymes targeted towards lignin degradation. UPOs have gained significant interest due to their ability to catalyse the selective oxygenation of a large range of organic substrates at the expense of only hydrogen peroxide.[1]

UPOs are phylogenetically related to the heme-containing enzyme, chloroperoxidase (CPO).[2] CPO catalyses the oxidation of chloride and bromide to the corresponding hypohalous acids that can effect the halogenation of a number of organic substrates.[3,4] UPOs and CPOs both rely on the iron (IV) oxo complex, Compound I and given the similarities between these enzymes, it could be expected that UPOs also perform halogenations through the generation of hypohalous acid. Ullrich and Hofrichter demonstrated the halogenating capability of the UPO from *Agrocybe aegerita* (*AaeUPO*) with the bromination of phenol to form 2- and 4-bromophenol although the phenomenon was not extensively explored.[5]

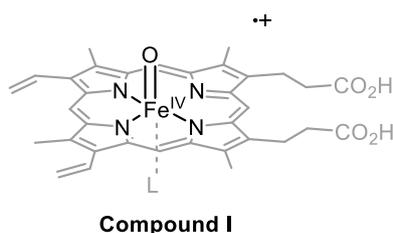
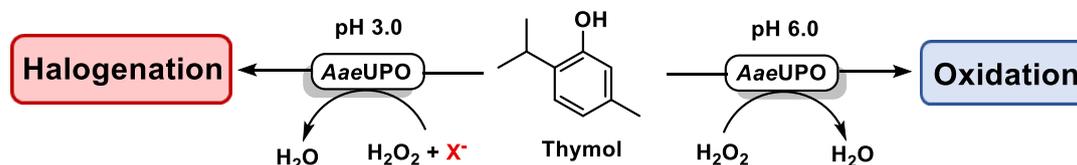


Figure 1. Compound I, the active catalytic species utilised by chloroperoxidase and unspecific peroxygenases

Whilst investigating the halogenating activity of *AaeUPO* we discovered that this enzyme can be tuned for oxygenase or halogenase activity of the same substrate by simply controlling the pH. We show that *AaeUPO* will catalyse either the bromination or oxygenation of the monoterpene thymol, dependent upon the pH of the solution, and that reaction conditions can be engineered to favour the optimal outcome for each product type.



Scheme 1. By controlling the pH of the solution, the unspecific peroxygenase from *Agrocybe aegerita* can be tuned for either oxygenase or halogenase activity.

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Enzymatic synthesis of bio-based polyesters containing levoglucosan units.

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In recent years, lignocellulosic biomass has emerged as an important renewable source for the production of green chemicals and materials [1]. As one of the recently most attractive use for biomass, biopolymers have been developed as environmentally friendlier alternative to petroleum-based polymers, presenting similar properties to their oil-based counterparts and a broad range of potential applications in food, cosmetic and pharmaceutical industries. Thus, new polyesters and polyamides have been researched from biomass-derived monomers, such as itaconic acid, furan-2,5-dicarboxylic acid, bio-olefins, terpenes, and so on [2].

In this context, Levoglucosan (1,6-anhydro- β -D-glucopyranose; LGA) – an anhydrous sugar obtained from the fast pyrolysis of cellulose, the major component of lignocellulosic biomass – has been identified as an attractive platform chemical [3,4]. LGA has a peculiar structure, having three hydroxyl groups disposed in *trans*-positions on a pyranose ring, which might confer unique properties to a new biopolymer. As far as we know, levoglucosan has not been yet employed as a monomer for polymers production. In this work, we report the production of terpolymers containing levoglucosan units *via* lipase-catalyzed polycondensation reactions.

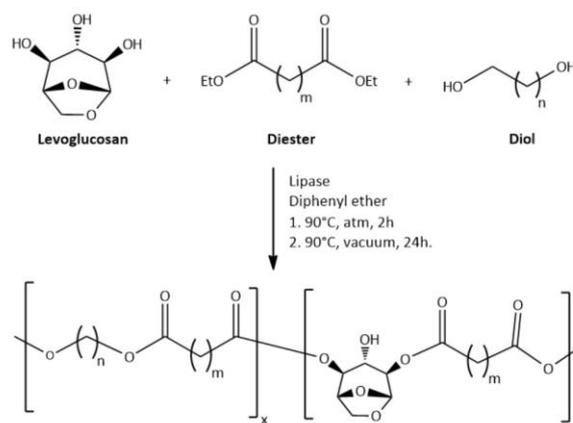


Figure 1. General schematic representation for lipase-catalyzed synthesis of polymers containing levoglucosan units.

Reactions were carried out using a two-stage method, where monomers were oligomerized under atmospheric pressure in the presence of the catalyst during at least 2 hours. Then, the polymerization step was initiated by applying vacuum in order to remove by-products and mixture was stirred for more 24 hours. Finally, solvent was removed and polymers obtained after precipitation in methanol. Different concentrations of monomers were evaluate in order to increase levoglucosan amount in the polymer structure.

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LuxAB-based microbial cell factories for the sensing, manufacturing and transformation of industrial aldehydes

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The application of genetically encoded biosensors enables the detection of small molecules in living cells and has facilitated the characterization of enzymes, their directed evolution and the engineering of (natural) metabolic pathways. [1] In this work, the LuxAB biosensor system from *Photobacterium luminescens* was implemented in *Escherichia coli* to monitor the enzymatic production of aldehydes from primary alcohols and carboxylic acid substrates. A simple high-throughput assay revealed the capability of the bacterial luciferase to detect structurally diverse aldehyde intermediates at low concentrations ($\geq 100 \mu\text{M}$) including aromatic and monoterpene aldehydes. The assay was used to extend the substrate scopes of three prokaryotic oxidoreductases: an alcohol dehydrogenase (*Pseudomonas putida*), a choline oxidase variant (*Arthrobacter chlorophenolicus*) and a carboxylic acid reductase (*Mycobacterium marinum*). Consequently, value-added aldehydes such as cinnamaldehyde, citral, and citronellal could be accessed *in vivo* in up to 80% yield. Furthermore, the dual role of LuxAB as sensing device and monooxygenase, emitting bioluminescence through the oxidation of aldehyde intermediates to the corresponding carboxylates, promises application in artificial enzyme cascades for the synthesis of carboxylic acids. [2] These findings advance the bio-based detection, preparation and transformation of industrially important aldehydes in living cells.

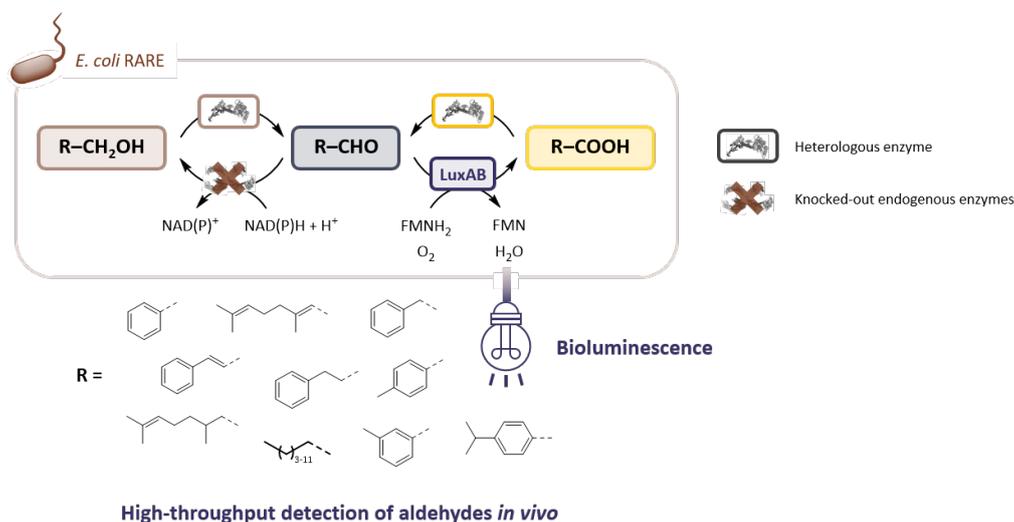


Figure 1. LuxAB-based microbial cell factory. Aldehydes can be produced from primary alcohols or carboxylates by heterologously expressed enzymes (cofactors omitted for clarity) in the highly engineered *E. coli* RARE strain. [3] The production of structurally diverse aldehydes can be monitored by LuxAB, which emits bioluminescence by the oxidation of aldehydes to the corresponding carboxylates. [4]

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Investigation of the Chymotrypsin-catalyzed Kinetic Resolution of Baclofen

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Baclofen, an artificial GABA receptor agonist, is a frequently used drug to treat muscle spasticity. [1] Although baclofen is conventionally manufactured as a racemic mixture, only the (*R*)-baclofen enantiomer actually mediates a therapeutic effect. [2] In this study we investigate the kinetic resolution of racemic baclofen, which is based on a three-step process (Figure 1), patented in 2007. [3] Baclofen is first esterified (**A.**), followed by the chymotrypsin-catalyzed kinetic resolution (**B.**) and a final hydrolysis step to (*R*)-baclofen (**C.**).

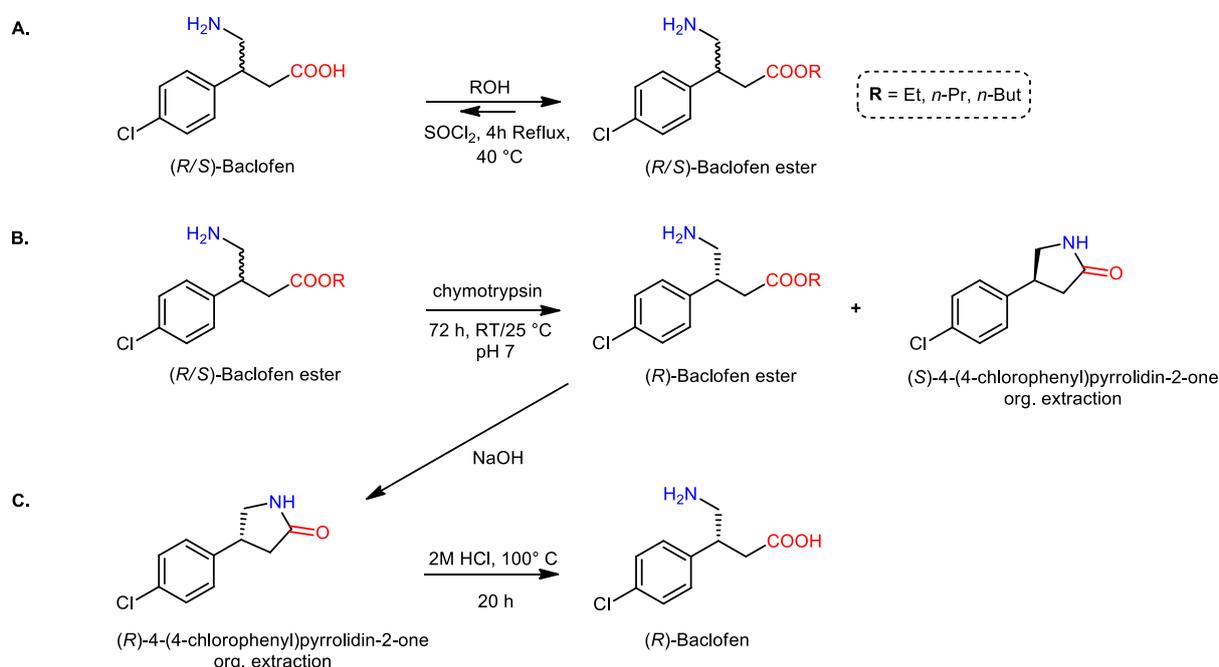


Figure 1: Conceptual schematic of the kinetic resolution of racemic Baclofen, consisting of esterification of the racemic baclofen (**A.**), kinetic resolution of the racemic baclofen ester (**B.**) and final hydrolysis of the enantiopure *R*-baclofen derivate (**C.**). [3]

Over the course of this study we could confirm the high enantioselectivity of this process, reaching enantiomeric excess values of up to 99.8 % for (*R*)-baclofen. The reaction parameters were scaled up, amounting to 2 g of racemic ester educt in step **B.** (see Figure 1). Furthermore, mild and facile reaction conditions were adapted and the existing process was improved. The instability of baclofen esters in basic milieu was also investigated in detail and successfully accommodated for product isolation.

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Structure and properties of the native amine dehydrogenase MATOUAmDH2

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Chiral amines are one of the most important building blocks in small molecule drug design. There is a pressing need for new alternatives to more traditional but unsustainable asymmetric reductive amination, commonly used in synthetic chemistry. NAD(P)H-dependent amine dehydrogenases (AmDHs) are capable of reducing unfunctionalized carbonyl centres and have been engineered from a range of enzyme families (including AADHs, transaminases and IREDs).^[1-4] Recently, interests have driven the search for native AmDHs (nat-AmDHs) that are capable of carrying out asymmetric reductive amination.^{[5][6]} We aim to structurally and biochemically characterise nat-AmDHs discovered from non-redundant sequence driven approaches.

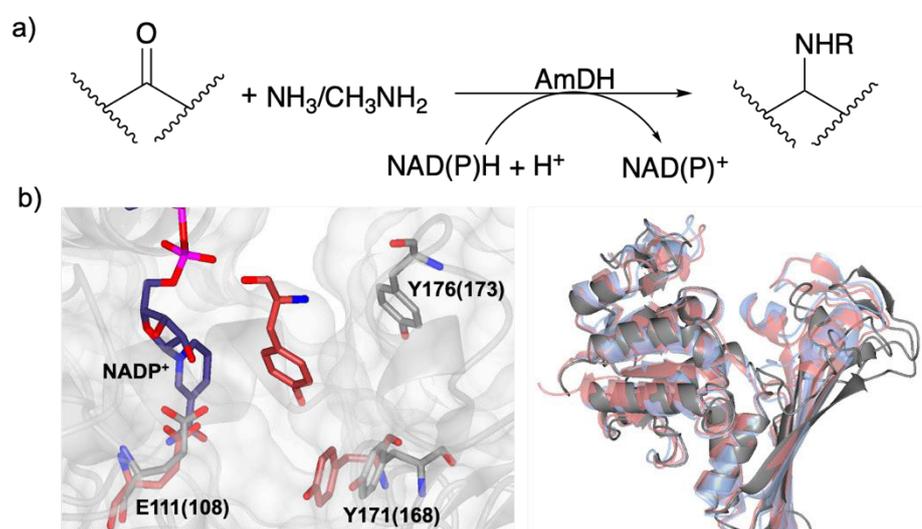


Figure 1. a) Reductive amination typically carried out by NAD(P)H dependent AmDHs. b) *Left panel:* Binding site residues from MATOUAmDH2 (grey) in complex with NADP+ (purple) superimposed with CfusAmDH PDB: 6IAU (red). *Right panel:* SSM superimposition of chains A from MATOUAmDH2 (grey), CfusAmDH, PDB: 6IAU (red) and MsmeAmDH, PDB: 6IAQ (blue).

Herein we report the structural and chemical properties of MATOUAmDH2 and the structural basis for the accommodation of larger substrates by using a range of techniques such as X-ray crystallography, GC and UV spectrophotometry. These findings highlight a larger binding site when compared to other similar AmDHs. MATOUAmDH2 is an interesting target for probing further mutagenesis studies that allow us to study its reductive amination properties. The findings discussed here affirm these nat-AmDHs as ideal enzymatic candidates for the reductive amination of pharmaceutically relevant substrates.

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From carbonyls to enantiomerically enriched alcohols: exploiting the substrate promiscuity of HSDHs for asymmetric catalysis

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Bacterial hydroxysteroid dehydrogenases (HSDHs) are NAD(P)H-dependent enzymes that belong to the superfamily of short-chain dehydrogenases/reductases. These enzymes display peculiar features that make them attractive for industrial applications. Indeed, they catalyse the reversible and regioselective oxidoreduction of the hydroxyl/oxo moieties of steroidal compounds recognizing the different positions of the steroidal skeleton (e.g., at C-3, C-7, and C-12). Additionally, HSDHs usually display high stereoselectivity, discriminating the hydroxyl group above the plane of the steroid molecule (β configuration) from the one below (α configuration).[1]

Although these enzymes have been thoroughly investigated during the last years, little is currently known regarding their substrate promiscuity in the biotransformation of alcoholic or ketonic substrates that differ from steroids.[2]

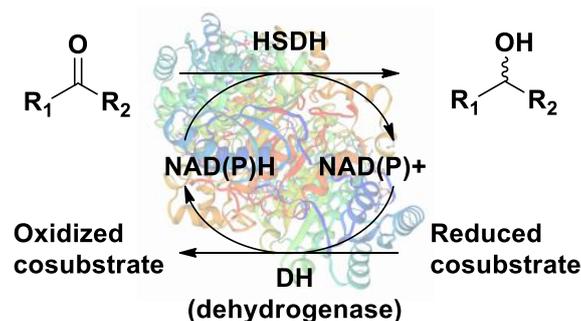


Figure 1. stereoselective reduction of ketonic moieties catalyzed by HSDHs.

To fill this gap, a library of thirteen 7α -, 7β -, or 12α -HSDHs (either already described or recently identified from metagenomic collections) was tested for the stereoselective reduction of a panel of carbonyl substrates. The screened compounds include selected ketones that partially resemble the structural features of steroids, α -ketoesters and α -diketones of pharmaceutical interest.[3,4]

Nearly all the tested HSDHs showed excellent activity and stereoselectivity towards these compounds, as it has been recently partially reported by us. [3,4]

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Exploiting Light for Photosensitized Sulfoxidation in Cyclic Deracemisation for the Production of Chiral Sulfoxides

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Non-racemic sulfoxides constitute valuable structural features which occur in many active pharmaceutical ingredients (API), particularly in proton pump inhibitors such as omeprazole® and its derivatives as well as in flavour and fragrance compounds [1]. Deracemisation strategies to facilitate the production of optically pure sulfoxides are much in demand [2]. In here we present a cyclic deracemisation process combining an enzyme-catalysed enantioselective sulfoxide reduction with an unselective photosensitized sulfide oxidation to yield the desired targets.

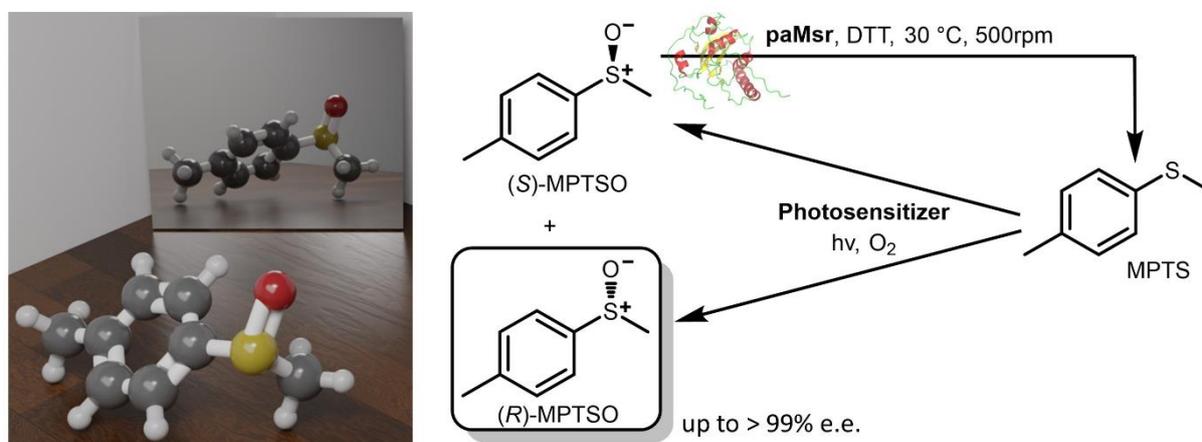


Figure 1. The (S)-MPTSO is mirrored in the cyclic one-pot deracemisation process by a selective biocatalytic reduction and subsequent photosensitized oxidation.

Starting from the *rac*-mixture of our model substrate methyl-*p*-tolyl sulfoxide (MPTSO), enantioselective sulfoxide reduction was achieved by an (*S*)-selective methionine reductase from *Pseudomonas alcaliphila* (paMsr) [3], to yield the corresponding methyl-*p*-tolyl sulfide (MPTS). Subsequent oxidation applying various photosensitizers revealed successful deracemisation towards the (*R*)-MPTSO with enantiomeric excesses (e.e.) in the range of 56-99%. The use of the biosynthesised photosensitizer protochlorophyllide (pchlide) [4] led to the best results in the deracemisation process yielding optically pure (*R*)-MPTSO (e.e. >99%) and low amounts of MPTS (<10%) after optimisation. Detailed investigation of the overall process identified the reduction step as significantly faster than the oxidation. The use of purified paMsr instead of cell free extract with pchlide as photosensitizer revealed the necessity of a detergent to enable high e.e.'s of the desired product.

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Synergy between nitroreductases and vanadium unlocks biocatalysed nitroreduction

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Anilines are valuable synthons in pharmaceuticals and agrochemicals and they are commonly produced by chemocatalytic reduction of the corresponding nitrobenzene precursors. Nonetheless, known synthetic methods often lack sufficient activity or selectivity, which results in low yields or the formation of a variety of undesired side products [1].

We envisaged biocatalysis as a promising alternative, which enables excellent selectivities under mild reaction conditions, even though no enzymatic approach has been described for the complete reduction of nitrobenzene to the corresponding aniline.

FMN-dependent nitroreductases (NRs) are enzymes capable of reducing a broad range of nitroaromatic compounds, however they have received limited attention in the synthetic organic chemistry community because their reduction of nitro compounds is limited to the generation of hydroxylamine [2]; further progression to the desired aniline is rare and results from spontaneous but slow disproportionation [3].

Herein, we demonstrate for the first time that the combination of NRs with vanadium overcomes this limitation and enables the full conversion of aromatic nitro compounds to the desired aromatic amines. NR reduces the nitroaromatic to the corresponding hydroxylamine, while vanadium actively disproportionates two hydroxylamine molecules to the respective amine and nitroso compounds [4]. This technology was proved to be versatile, as a wide selection of substrates was reduced with high yields and purity, and scalable, with up to 70 g/L substrate concentration and enzyme loading as low as 3.6 % w/w [5].

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Unique chemo-enzymatic synthesis of multivalent glycomimetics for scavenging of tumorigenic galectin-3

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Galectin-3 plays a crucial role in cancerogenesis, and its targeting is prospective for cancer diagnostics and therapy. Multivalent presentation of glycans can strongly increase the affinity to galectin-3, and further strengthening of interaction may be reached through aryl substitutions in the carbohydrate molecule.[1] We established a new, as yet undescribed chemoenzymatic method to produce selective C-3-substituted *N,N'*-diacetyllactosamine glycomimetics and coupled them to human serum albumin. The β -*N*-acetylhexosaminidase from *Talaromyces flavus* had the unique ability to efficiently synthesize the C-3-propargylated disaccharide, which was further conjugated with various aryl residues via click chemistry. Coupling to human serum albumin afforded multivalent neo-glycoproteins with up to 21 000-fold increased inhibitory potency compared to the lactose standard. Surface plasmon resonance brought further information on the kinetics of galectin-3 inhibition. The potential of prepared neo-glycoproteins to target galectin-3 was demonstrated on colorectal adenocarcinoma cells. The neo-glycoproteins efficiently scavenged exogenous galectin-3 in the microenvironment of cancer cells, inhibiting its interaction with the cell surface, and protecting T-lymphocytes against galectin-3-induced apoptosis.[2] Due to their straightforward synthesis, selectivity, non-toxicity, and high efficiency for targeting exogenous galectin-3, these neo-glycoproteins are prospective for application in the immunomodulatory treatment of galectin-3-overexpressing cancers.

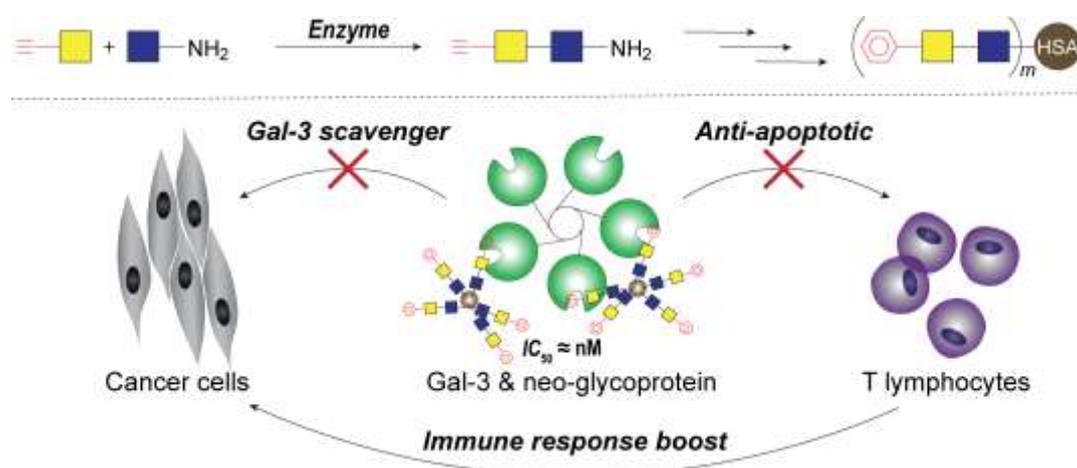


Figure 1. Chemo-enzymatic synthesis and biological impact of glycomimetic-carrying neo-glycoproteins.

Support from the Czech Science Foundation (grant project no. 20-00215S) and from mobility project LTC19038 (COST Action CA18103) by the Ministry of Education, Youth and Sports of the Czech Republic is gratefully acknowledged.

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Bacterial Biotransformation of Oleic Acid: New Findings on the Formation of γ -Dodecalactone and 10-Ketostearic Acid in the Culture of *Micrococcus luteus*

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Microbial conversion of oleic acid to form value-added industrial products has gained increasing scientific and economic interest. So far, the production of natural lactones with flavor and fragrance properties from fatty acids by non-genetically modified organisms (non-GMO) involves whole cells of bacteria catalyzing hydration of unsaturated fatty acids as well as yeast strains responsible for further β -oxidation processes [1,2]. Developing of non-GMO process, involving a sole strain possessing both enzymatic activities, significantly lowers the costs of the process and constitutes a better method from the customers' point of view regarding the bio-safety issue.

Twenty bacteria from the genus of *Bacillus*, *Comamonas*, *Dietzia*, *Gordonia*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, and *Streptomyces* were screened for oxidative functionalization of oleic acid [3]. *Micrococcus luteus* PCM525 was selected as the sole strain catalyzing the one-pot transformation of oleic acid into natural valuable peach and strawberry-flavored γ -dodecalactone used in the food, beverage, cosmetics, and pharmaceutical industry. Based on the identified products formed during the progress of biotransformation, we established the metabolic pathway. Moreover, three other strains (*Rhodococcus erythropolis* DSM44534, *Rhodococcus ruber* PCM2166, *Dietzia* sp. DSM44016) with high concomitant activities of oleate hydratase and alcohol dehydrogenase were identified as efficient producers of 10-ketostearic acid, which can be used in lubricant and detergent formulations.

Considering the prevalence of γ -dodecalactone and 10-ketostearic acid applications and the economic benefits of sustainable management, microbial bioconversion of oleic acid is an undeniably attractive approach. Our research contributes to the ongoing discussion on how renewable raw materials rich in fatty acids, such as fats and oils can be managed. This is an example of a bio-based conversion of oleic acid, which is a cheap and abundant compound in oleoindustry by-products (oil cakes, spent cakes, soapstocks). It could be used to produce expensive and valuable compounds used in a wide range of industries.

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From Waste to Value – Direct Utilization of α -Angelica Lactone as a Non-conventional Irreversible Acylating Agent in a Chromatography-free Lipase-catalyzed KR Approach Towards *sec*-Alcohols

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Although the classical enzymatic kinetic resolutions (EKRs) are among the most important reactions in biocatalysis, the requirement of application of chromatographic purification technique, which is responsible for generation of large amounts of waste organic solvents, is its major drawback. To minimize the environmental impact of such attempts and to address the current sustainability challenges, we decided to develop a novel EKR methodology, which relies on the usage of cheap, fully renewable, non-toxic, and irreversible acyl group donor reagent, namely α -angelica lactone. The employed activated enol lactone-based acyl donor proved to be a versatile reagent enabling efficient and highly enantioselective (*E*-value up to 483) lipase-catalyzed resolution of a set of racemic *sec*-alcohols with up to >99% ee and near to quantitative isolation yields according to conversions achieved during the respective EKR procedure. Moreover, α -angelica lactone provided in this case an ability of simple and fast separation of the enzymatic reactions' products via chromatography-free reactive liquid-liquid extraction (LLE) work-up using either saturated aqueous sodium bisulfate in DMF or Girard's P reagent in a mixture of EtOH/AcOH (90:10, v/v), respectively. The NaHSO₃/DMF LLE work-up and the subsequent re-isolation of the respective levulinate from the aqueous layer turned out to be less efficient than a separation with Girard's P reagent. The selective LLE of optically active levulinate-Girard P-hydrazones from the respective alcohols and further hydrolysis of the respective hydrazides with diluted HCl_{aq}, could be performed with high a level of recovery of both EKR products isolated usually without loss of enantiomeric purity.

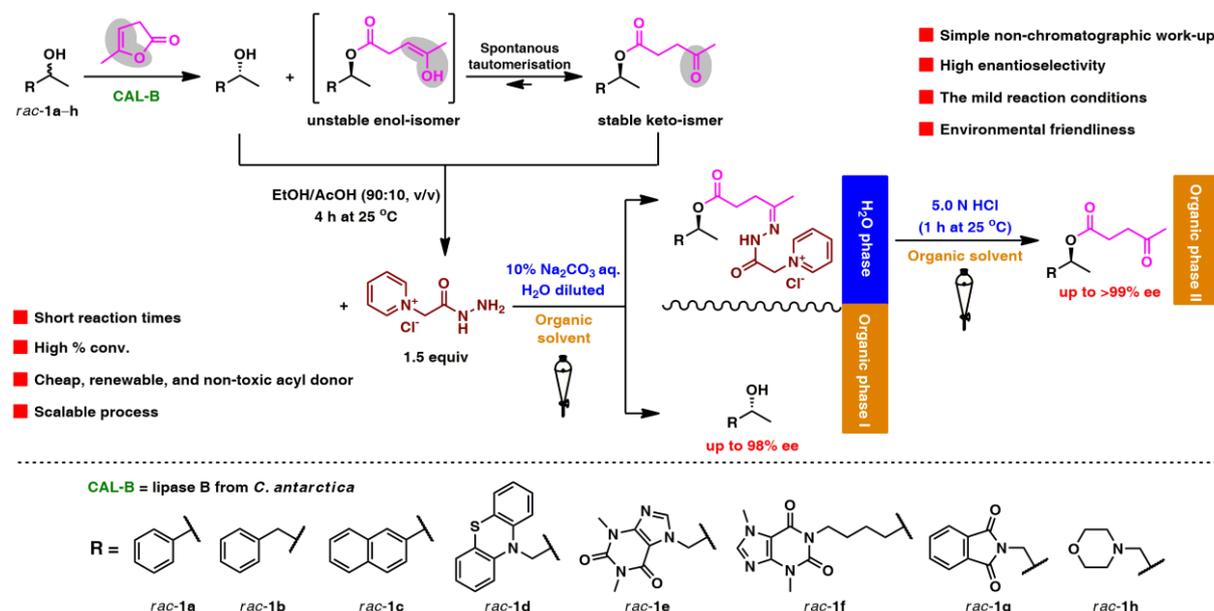


Figure 1. Chromatography-free lipase-catalyzed KR of racemic alcohols using α -angelica lactone and Girard's P reagent strategy.

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Biocatalytic Asymmetric Synthesis of *N*-Arylated Aspartic Acids and Pyrazolidin-3-ones

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N-arylated α -amino acids and pyrazolidin-3-ones are widely found in the core structures of various pharmaceutically active compounds and agrochemicals.[1] In this work, we developed an efficient and sustainable biocatalytic methodology for the synthesis of various chiral *N*-arylated aspartic acids and pyrazolidin-3-ones using ethylenediamine-*N,N'*-disuccinic acid lyase (EDDS lyase). The enzyme, which naturally catalyzes the reversible two-step sequential additions of ethylene diamine to two molecules of fumarate to provide (*S,S*)-EDDS, was shown to have a remarkably broad nucleophilic scope.[2-4] Here we show that EDDS lyase accepts a wide variety of anilines for the hydroamination of fumarate with high conversions, yielding optically pure (*ee* > 99%) (*S*)-*N*-arylated aspartic acids in good isolated yields. Furthermore, a two-step chemoenzymatic method was developed for the synthesis of chiral pyrazolidin-3-ones. EDDS lyase catalyzes the addition of various substituted arylhydrazines to fumarate, yielding the corresponding chiral *N*-(arylamino)aspartic acids. After acid-catalyzed cyclization, the desired corresponding pyrazolidin-3-ones were obtained without racemization (*ee* > 99%) and in good overall yields. In addition, the EDDS lyase catalysed biotransformation was successfully combined with the acid-catalyzed cyclization in one pot, providing a simple chemoenzymatic route for the synthesis of optically pure pyrazolidin-3-ones.[5]

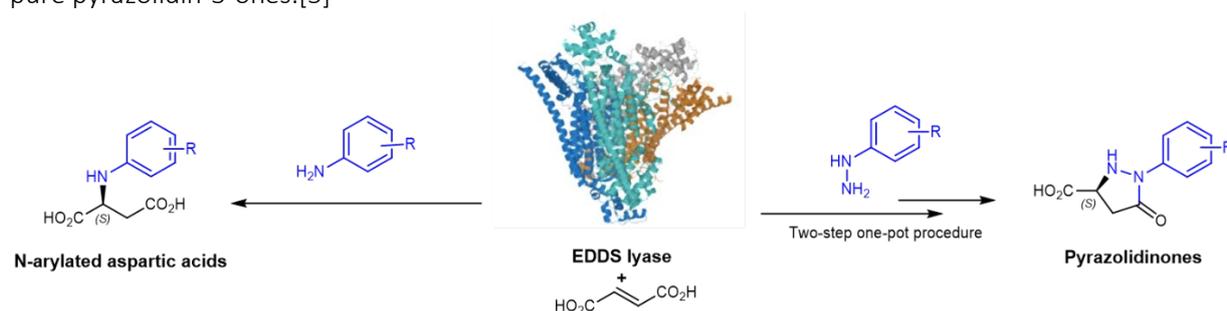


Figure 1. Biocatalytic strategy towards *N*-arylated aspartic acids and pyrazolidin-3-ones

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Old dog, new trick: reduction of oximes to amines using ene reductases

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Flavin-dependent ene-reductases (EREDs), such as those of the Old Yellow Enzyme (OYE) family, are a well described and studied class of enzymes, mostly applied for the enantioselective reduction of activated C=C double bonds (Figure 1, top). While a wide substrate scope has been established,^[1-3] the enzymes are not known for the reduction of C=heteroatom bonds. Recent work in our group has shown that several ene-reductases reduce the oxime functionality of β -keto- α -oximo esters very efficiently, yielding tetrasubstituted pyrazines from non-enzymatic cyclisation and oxidation of the product formed in biotransformation (Figure 1, middle).^[4]

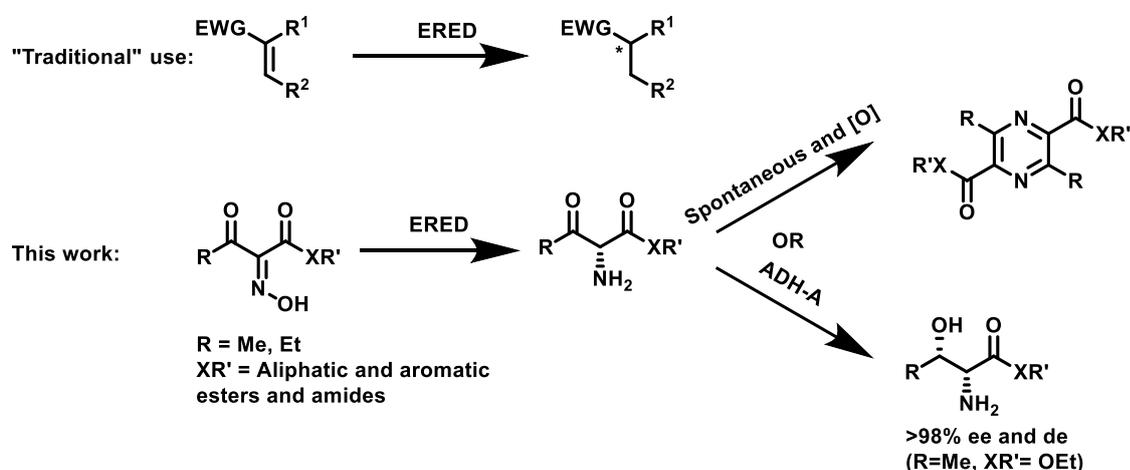


Figure 1. General reaction schemes of the well described “traditional” use of EREDs for the reduction of activated C=C double bonds (top) and the outline of this work, ERED-mediated reduction of an activated oxime to an amine intermediate, followed by non-enzymatic cyclisation and oxidation to tetrasubstituted pyrazines (middle), or followed by ADH-mediated ketone reduction to a threonine derivative (bottom).

The oxime reduction has been tested using a small substrate library (8 oximes) and six EREDs. This has shown that various oximes are transformed with high efficiency (up to 77% product formation within 24 hours), and that the experiments can easily be scaled up to preparative amounts. Typical experiments using 2 mmol oxime yield 150-250 mg pyrazine, corresponding to isolated yields of up to 62%.^[2] Furthermore, the biotransformation is highly enantioselective, as shown by a cascade using ADH-A in addition to the ene-reductase. This yields a threonine ester in high ee and de (Figure 1, bottom). Currently, we are exploiting this unusual reactivity by synthesising and testing a library of oximes derived from asymmetric malonic esters. Preliminary experiments have shown substrates of this description to be accepted by the enzyme and transformed into the corresponding primary amine. This work will be expanded further by establishing a substrate library and quantitative determination of product formation and enantiomeric excess. Additionally, we are testing more β -keto ester derived oximes in cascade biotransformations to gain insight on the putative influence of the substrates' side chains on the enantioselectivity of the reduction.

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Lactate oxidase immobilization for optical biosensing: a systematic screening of different solid carriers

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Clinical state of intensive health care patients can unpredictably worsen and a constant control is needed in these occasions. The increasing of **L-lactate** concentration in blood flow is correlated to oxygen depletion in muscles [1].

Currently, the market is dominated by electrochemical lactate sensors, which are simple, fast, cheap, and portable, but they can only perform single-point measurements. The aim of the project is to develop a **robust biocatalyst** that can be used for continuous monitoring of L-lactate in an **optical biosensor** set-up.

Lactate oxidase (LOx) from *Aerococcus viridans* is a well-characterized flavoenzymes that belongs to the α -hydroxy acid oxidase family. It assembles as a homotetramer of about 160 kDa and it catalyses the oxidation of lactate to pyruvate thanks to the presence of one FMN molecule retained non-covalently in the $(\alpha/\beta)_8$ - barrel structure of each subunit [2]. The reaction proceeds with a Ping-Pong mechanism, where the coenzyme is first reduced by lactate, then re-oxidised by a molecule of O_2 , which is converted to H_2O_2 [3].

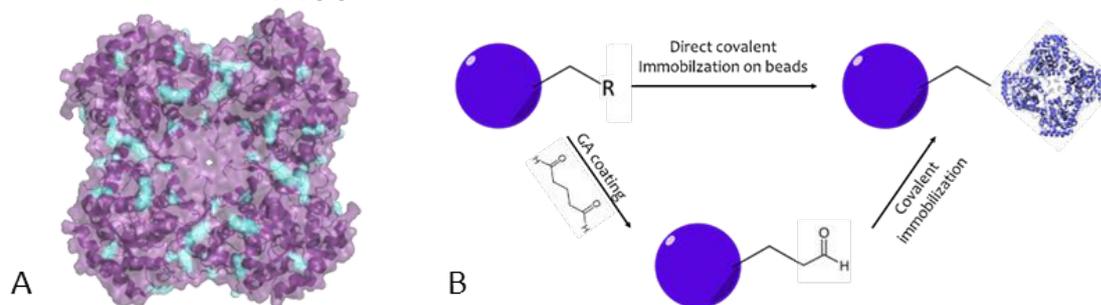


Figure 1. A) Structure of Lactate oxidase from *Aerococcus aureus* (pdb: 2j6x). Lysines on the surface are coloured in cyan. B) Scheme of immobilization strategy. Glutaraldehyde (GA) coating was performed with amine functionalized beads.

To make LOx compatible with the detector device, protein insolubilization on solid carrier is performed. The **immobilization** is mainly performed taking advantage of lysines ϵ - NH_2 groups located on the surface, which are able to react with a plethora of functional groups. Other immobilization methods (*e.g.* adsorption) are evaluated if the case.

A systematic screening of resins having different material and/or chemistry binding is in progress to understand the most favourable conditions for the preparation of a highly active immobilizate. Features of hydrophobic (*i.e.* acrylic and methacrylate polymer) and hydrophilic (*i.e.* agarose and chitosan) carriers are being tested. For most of them, amine, epoxy and azlactone groups are available, allowing us to evaluate various immobilization strategies.

Immobilizates showing the best **effectiveness factor** are selected for further optimization, considering that particle size has a big impact on mass transfer [4].

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Towards proteins shape and size effects in the immobilization on magnetic nanoparticles

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Enzyme immobilization is a technique that allow to easy separate the products from the biocatalysts, thus facilitating the enzyme reuse. However, immobilization also poses some challenges, among them the conservation of the original 3D structure and the protein orientation that guarantees a good activity of the immobilized variant. One the most widely used strategy to control enzyme orientation and avoid structural changes is the use of His-tag recombinant variants: the protein could be modified by the addition of a 6 histidine residues tail at N or C-terminus and the imidazole side chain could be coordinated by divalent transition metal ions on the surface of magnetic nanoparticles thanks to a chelating agent, the nitriloacetic acid, NTA. [1]

In this study the immobilization of His-tagged modified proteins, with different quaternary structure, to magnetic nanoparticles is explored. Monomeric fragment of E-Cadherin, monomeric Green Fluorescent Protein (GFP), dimeric Alcohol Dehydrogenase (ADH) from *T. thermophilus* and tetrameric Alcohol Dehydrogenase from *B. stearothermophilus* [2] are conjugated to magnetic nanoparticles surface. The conjugation is studied using different amounts of Co⁺² and Ni⁺², that present different affinity and specificity for the histidine imidazole side chain. Besides, we evaluate how the use of one transition metal, along with proteins with different quaternary structure affect, first the immobilization yield and the binding specificity and, later, the activity of the enzymes bound to the MNPs. In this context, proteins shape and size play a central role in the binding yield, specificity of the binding and particles colloidal stability, in association with the amount of available metal site, as our preliminary results suggest.

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Biocatalytic synthesis of non-standard amino acids by a decarboxylative aldol reaction

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The formation of carbon-carbon bonds lies at the heart of organic chemistry, but relatively few C-C bond forming enzymes have found their way into the biocatalysis toolbox. We report that the enzyme UstD^{1,2} performs a highly selective decarboxylative aldol addition with diverse aldehyde substrates to make non-standard, γ -hydroxy amino acids. We increased the activity of UstD through three rounds of classic directed evolution and an additional round of computationally-guided engineering. The enzyme that emerged, UstD^{2.0}, is very efficient in a whole-cell biocatalysis format and readily crystallizes. The X-ray crystal structure of UstD^{2.0} at 2.25 Å reveals the active site and empowers future studies. The utility of UstD^{2.0} was demonstrated via the stereoselective gram-scale syntheses of non-standard amino acids.

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Direct Conversion of Hydrazones to Amines using Transaminases

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Transaminase enzymes (TAMs) have been widely used to reversibly transform a ketone or aldehyde group into an amine, often resulting in optically pure products.[1] In this work, transaminases were reacted with hydrazones in a novel approach to form amine products. Several substrates were investigated, including those with furfuryl and phenyl moieties. It was determined that the amine yields increased when an electrophile was used, suggesting that they can sequester the hydrazine released in the reaction. Pyridoxal 5'-phosphate (PLP), a cofactor for transaminases, and polyethylene glycol (PEG) resins with pendant aldehydes were therefore found to increase the yield of amine formed. In particular, the amination of a (*S*)-(-)-1-amino-2-(methoxymethyl)pyrrolidine (SAMP) hydrazone gave promising results as a method to form chiral β -substituted amines.

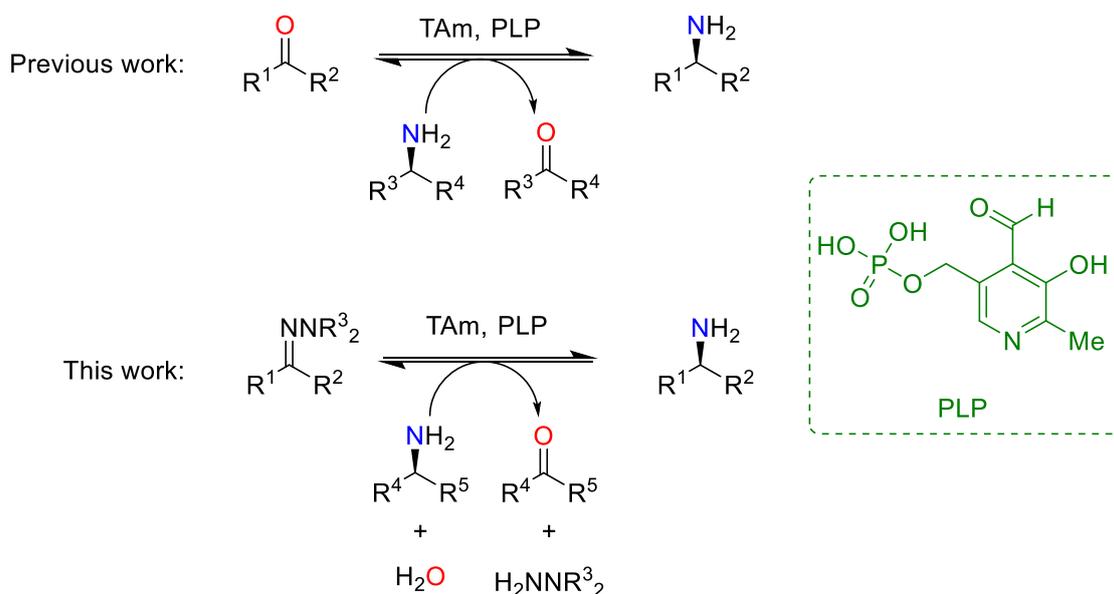


Figure 1. Previous work involves the amination of aldehydes and ketones using transaminases.[1] This work investigates the reaction of transaminases with hydrazones to form amines.

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Evolved thermostable transketolase for the valorization of vegetable oils

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The production of biosourced aliphatic aldehydes from vegetable oils, renewable raw material, offers many applications in the fields of cosmetics, detergents, polymeric materials and additives. Chemical processes by oxidative cleavage or in the presence of organocatalysts such as analogs of thiamine have been reported.[1,2] These processes require very high temperature, organic solvents and generate toxic by-products. The goal of this study is to develop a novel ecofriendly enzymatic C-C bond cleavage process catalyzed by a thiamine diphosphate (ThDP) dependent enzyme, transketolase (TK) (Figure 1).

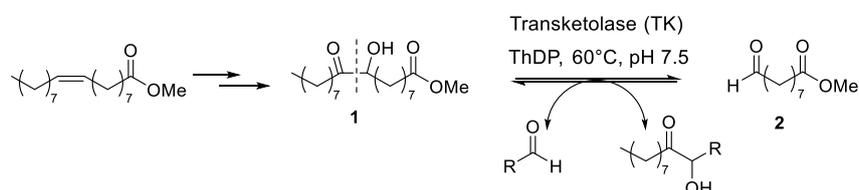


Figure 1. Main route to fatty aldehydes **2** from a modified methyl oleate derivative **1**

Transketolase is commonly used for the stereoselective formation of a C-C bond in the presence of hydroxypyruvate as donor substrate, rendering the reaction irreversible, and an aldehyde as acceptor substrate allowing the valorization of ketose products.[3] Our project aims to exploit the reverse TK-catalyzed reaction for the C-C bond cleavage of a modified fatty acid **1** derived from methyl oleate in order to generate highly valuable aldehydes **2**.

For this study, the thermostable TK from *Geobacillus stearothermophilus* (TK_{gst}) showing high activity and stability at 60°C will be used.[4] Based on our knowledge of TK_{gst} active site and our recent advances showing improved activities of TK_{gst} variants toward aliphatic substrates,[5-7] we applied semi-rational mutagenesis (SSM) on some targeted positions identified by molecular modeling. The TK_{gst} variant libraries were screened against a panel of hydroxyketones of increasing complexity from ketoses (TK_{gst} natural substrates) to methyl oleate derivative **1**. Finally, different enzymatic strategies will be investigated to shift the equilibrium of TK-catalyzed-reaction. The different step of the strategy and the results will be presented and discussed.[8]

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Accelerating biocatalysis screening workflows for medicinal chemistry through miniaturization

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This poster describes the development of miniaturized 384 well plate screening workflows including reaction preparation and workup, fast chiral analysis, data capture and interrogation. This workflow has accelerated the screening of a variety of enzyme classes (ketoreductases, ene-reductases, hydrolases, transaminases...) with a major reduction in substrate demand, critical for support of medicinal chemistry programs where often only tens or hundreds of mgs of substrate are available.

From single steps to multienzymatic catalysis. Upgrading biomass furans using laccases and amine transaminases

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Stepwise synthetic strategies require significant efforts in terms of energy, time, necessity to isolate chemical intermediates and optimization of the downstream processes. A key step for improving the 'pot-economy' and reducing the waste impact of the global process is the use of biocatalytic approaches.[1] Enzymes are amongst the most effective catalysts, displaying excellent chemo-, regio- and stereoselectivity, working under mild reaction conditions, and being compatible with other reagents including metal complexes and organocatalysts.[2] Despite of all these benefits, full implementation of these multiple transformations has not reached yet its complete maturity in the chemical industry due to different issues such as catalyst cost, stability, productivity, scaling-up and economic impact.[3] To overcome these limitations, enzyme engineering techniques, computer modelling strategies and high-throughput screening tools are strategic trends for making more convincing their applicability and paving the way towards actual sustainable chemical approaches.[4]

Herein, the design of a sequential system is presented towards the amination of furfuryl alcohol, as furan-based benchmark model compound produced from C5 sugar units of hemicellulose fraction during the manufacturing of agricultural biomass, searching for the production of furfuryl amine, relevant as monomer in biopolymers synthesis (Figure 1).[5] First promising results have been achieved studying the individual (chemo)enzymatic oxidation and transamination steps using laccases (and laccase mediator systems, LMS) and amine transaminases (ATAs).

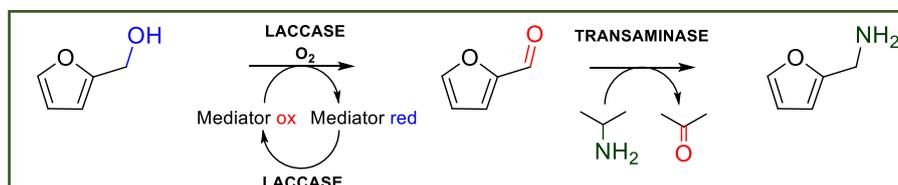


Figure 1. Production of furfuryl amine in a sequential cascade involving the use of laccases and ATAs.

Laccase-mediated oxidation reaction of furfuryl alcohol using commercial laccase from *Trametes versicolor* and 2,2,6,6-tetramethylpiperidin-1-yl)oxyl radical (TEMPO) as mediator has been investigated, studying different reaction conditions and parameters, satisfyingly achieving quantitative conversions at 100 mM of substrate. The biotransamination reaction step has also been examined selecting isopropylamine (IPA) as amine donor. The results of the two separate catalytic stages on both model substrates, ideally corresponding to the "multi-step" process, have demonstrated to be encouraging to be next applied to the HMF derivatives.

This research activity is part of a broader interdisciplinary project named INTERfaces, which received fundings by the European Union's Horizon 2020 program under Marie Skłodowska-Curie and aimed at building „Heterogeneous Multi-step Biocatalytic Reaction Cascades“.[6]

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Novel approaches to oligonucleotide synthesis using enzymes

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Biocatalysis has transformed chemical synthesis of small molecule APIs in recent years by shortening routes, lowering waste and by reducing costs. Enzymes are now seeing applications beyond small molecules, such as exploited in oligonucleotide synthesis. This strong technology base can be applied to develop enzymatic methods for oligonucleotide synthesis resulting in improved yields and purity profiles compared to traditional methods. Biocatalytic approaches are alleviating the pressures on existing solid phase capacity and resulting in more convergent syntheses.

Therapeutic oligonucleotides are a class of drug based on the structure of DNA and RNA. By selectively binding genetic material such as RNA, they influence the expression of targeted proteins to elicit a beneficial therapeutic effect. These oligonucleotides are rapidly degraded in the body and this has driven the need for chemical innovation to modify and stabilise these structures, which are typically oligomers comprising 20–30 nucleotide residues.[1] The synthesis of these molecules, combining natural and unnatural residues, has therefore presented many challenges during the discovery and development process, particularly when scaling-up oligonucleotide synthesis. Further chemical modifications and tagging strategies are required to target the oligonucleotides to their required site of action.[2]

This presentation will show case how enzymes can be used in both single and double stranded oligonucleotide synthesis with unnatural and natural nucleotides as well as carrying structural modifications.

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Reductive Photo-enzymatic Strategy for the Chemo-divergent Synthesis of Bromhydrin and Epoxides

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In Photobiocatalysis, the suitable orchestration of photo- and biocatalytic reactions has allowed access to new and more sustainable strategies in organic synthesis.[1]

Most of the established photobiocatalytic strategies rely on photooxidation of a substrate and further biocatalytic transformation[2] and photorecycling of redox cofactors for well-established biocatalytic processes.[2] However, some asymmetric photoreductive reactions have been recently accomplished using enzymes.[3] With this in mind, here we present a sequential combined stereoselective bioreduction/selective photocatalytic one electron reduction in order to convert α,α -dibromoketones into valuable bromhydrins or epoxides with high optical purity.

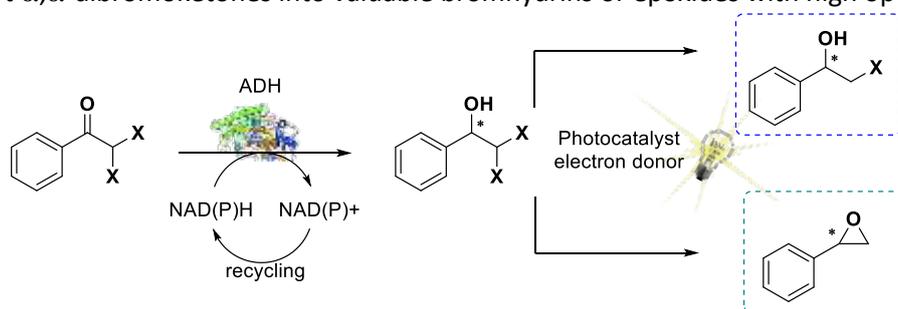


Figure 1. Chemodivergent synthesis of bromhydrins and epoxides by means of photobiocatalytic process.

The ADH-catalysed bioreduction of α,α -dibromoketones into the corresponding dibromhydrins was conducted with excellent conversion and enantiomeric excesses, as reported earlier.[4]

The photoreduction step starting from the racemic dihalohydrin was optimised regarding photocatalyst, sacrificial electron donor, solvent composition, atmosphere and reaction time.

From these experiments, two scenarios were recognized: on the one hand, selective monodebromination was observed when 2-PrOH was employed as solvent, thus affording the corresponding bromhydrin. On the other hand, monodebromination followed by spontaneous ring closure was evident in hydroalcoholic media giving rise to the corresponding epoxide.

For the one pot sequential design, the bioreduction was in a sealed glass vial purged with Ar. Once the ketone has been consumed, photocatalyst, electron donor dissolved in a cosolvent is added under inert atmosphere. Then, the vial is irradiated with blue LED for 10-24 h with magnetic stirring. Full conversion and yields up to 80 % were obtained for the chiral bromhydrin and 65% for the styrene oxide.

In this way, it is possible to direct the process to the formation of either the halohydrin or the epoxide by careful tuning of the reaction conditions. For the chemoselectivity, the sacrificial electron donor and solvent composition play a critical role.

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Enzymatic Asymmetric Reduction of Unfunctionalized C=C Bonds

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Current approaches for the asymmetric reduction of olefins are based on transition metal catalysis, e.g. ruthenium for Noyori-type reductions or iridium as described by the Pfaltz group.^[1] Future problems of the supply and waste treatment of those reactions encourage investigations on alternative synthesis routes.^[2] For now, biocatalytic approaches are limited to activated C=C bonds, conjugated to an electron withdrawing group, by ene-reductases proceeding via Michael-type addition of hydride.^[3] In contrast to activated C=C bonds, unfunctionalized olefins are electron rich and cannot be reduced by the same mechanism. Thus, new enzymes must be found for the reduction of this type of double bonds.

Geranylgeranyl reductases (GGRs) in archaea reduce all four C=C bonds in geranylgeranyl side chains of membrane lipids.^[4] The protein accepts the side chain in most stages within biosynthesis, e.g. as phosphate, glyceryl ether, or several phospholipid derivatives.^[4,5] Recently, GGRs were shown to reduce geranylgeraniol and farnesol as well.^[6] We investigated the substrate scope of archaeal GGRs from *Archaeoglobus fulgidus* and *Sulfolobus acidocaldarius* to show the possible use as biocatalyst for the asymmetric reduction of unfunctionalized C=C bonds.

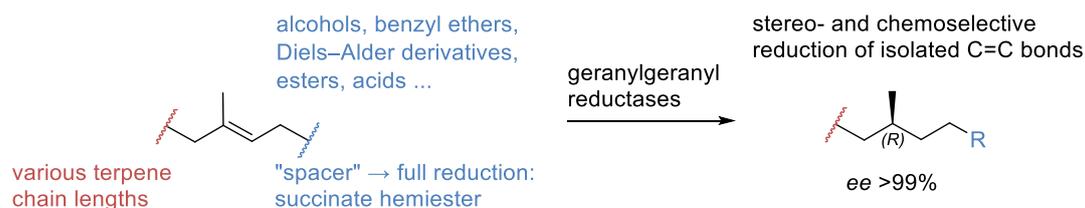


Figure 1. Asymmetric reduction unfunctionalized C=C bonds by GGRs.

We showed the reduction of smaller terpenes and the improved reduction by substrate engineering. Adding succinic acid hemiester as cleavable “spacer” leads to increased conversion and acceleration towards complete reduction of the substrate.^[7] Additionally, GGRs accept benzyl- and acetate-protected alcohols. Diels–Alder products of myrcene were accepted as well showing selective reduction of acyclic C=C bonds in presence of cyclic ones. Z-double bonds were not reduced and remained unaffected in mixtures of E,Z-isomers. The reaction occurs strictly (R)-selective with enantiomeric excess >99%.^[7] Our results show the possible use of GGRs for the asymmetric, chemo- and stereoselective reduction of isolated C=C bonds in methyl-branched olefins with divers head groups.

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A Structural View on the Stereospecificity of Plant Borneol-Type Dehydrogenases

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The development of sustainable processes for the valorization of by-products and other waste streams remains an ongoing challenge in the field of catalysis. Racemic borneol, isoborneol and camphor are currently produced from α -pinene, a side product from the production of turpentine. The pure enantiomers of these monoterpenoids are valuable as they have numerous applications in health formulations and act as reagents for asymmetric synthesis. However, their current method of production via extraction from plant essential oils has numerous disadvantages in terms of energy demand and waste accumulation, which makes an enzymatic route for their separation into optically pure enantiomers a desirable goal. To date, known short-chain borneol-type dehydrogenases (BDHs) from plants and bacteria lack the required specificity, stability or activity for industrial utilization. Prompted by reports on the presence of pure (-)-borneol and (-)-camphor in essential oils from rosemary [1] and partial purification of a BDH in sage [2] we set out to investigate dehydrogenases from the genus *Salvia*. One of the discovered enzymes, SrBDH1, presented high specificity and activity for borneol and isoborneol. Compared to other specific dehydrogenases [3], the one reported here shows higher stability, which was exploited to obtain the first three-dimensional structure of an enantiospecific BDH. This, together with docking studies, led to the identification of a hydrophobic pocket in the enzyme that plays a crucial role in the stereo discrimination of bornane-type monoterpenoids.

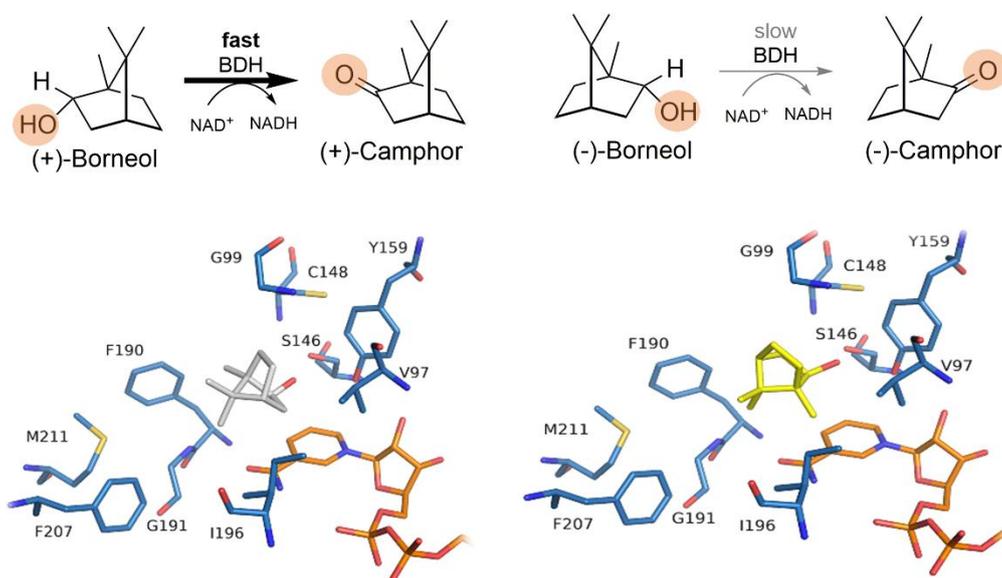


Figure 1. Oxidation of (+) and (-)-borneol catalyzed by a selective BDH and docking results showing substrates position in the active site of SrBDH1.

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Fungal prenyltransferases to produce antimicrobial prenylated phenolics

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In nature, prenyltransferases (PTs) catalyse the transfer of isoprenoid (prenyl) moiety from a prenyl donor to a prenyl acceptor substrate. Likely due to their increased hydrophobicity, the resulting prenylated products often shows a higher biological activity, such as antimicrobial activity of prenylated phenolics in plants' defense mechanism [1]. Interestingly, the compounds are also active against human pathogens, thus they possess potential as natural food preservatives or lead compounds for the development of new antibiotics [2]. Plants, however, produce too limited quantities of prenylated phenolics needed for a feasible industrial application.

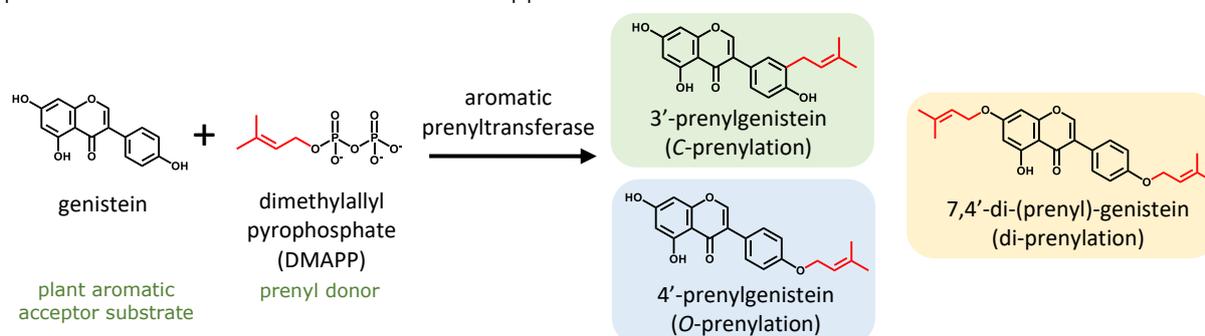


Figure 1. Prenylation of plant phenolics by an aromatic prenyltransferase, demonstrated here with the isoflavonoid genistein as the prenyl acceptor substrate and dimethylallyl pyrophosphate (DMAPP) as the prenyl donor substrate. Three potential prenylated products with various types of prenylation are shown.

Therefore, in this study we aim to explore the potential of recombinant PTs as biotechnological tools to efficiently produce antimicrobial prenylated phenolics. Three fungal PTs from the DMATS superfamily were selected due to their expected substrate promiscuity, distinct *C*- and *O*- prenylation, and/ or thermotolerance and organic solvent stability. The three selected PTs were: a predicted *O*-PT from *Sparassis crispa* (ScPT), a predicted 7-DMATS from thermophilic *Rasamsonia emersonii* CBS 393.64 (RePT), and a known PT from *Aspergillus terreus* A8-4 (AtaPT) [3]. We characterized their substrate scope with a wide variety of phenolic substrates and found that all three PTs were able to prenylate multiple subclasses of plant phenolics. ScPT predominantly catalysed *O*-prenylation on (iso)flavonoids and stilbenoids, specifically with substrate conversion up to 80% on isoflavonoids. RePT possessed a more narrow substrate specificity than ScPT and AtaPT, but showed a tolerance for prenylation at elevated temperatures ($\geq 50^\circ\text{C}$) and in the presence of organic solvent content ($\geq 20\%$ methanol). In accordance with literature, AtaPT had a broad substrate scope, including (iso)flavonoids and stilbenoids, and catalysed *C*- and *O*- prenylation as well as multiple prenylation (di- and tri-prenylation). Based on our findings, we conclude that the novel prenyltransferases ScPT and RePT are promising biocatalysts with potential applications in producing valuable bioactive prenylated phenolics for the food, cosmetic, and pharmaceutical industries.

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Biocatalytic stereoselective imine reduction towards (*R*)-reticuline, a key compound within the morphine biosynthesis

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Reticuline is an alkaloid, which occurs in the opium poppy flower and serves as a key compound for the biosynthesis of morphine-like structures. The poppy produces (*R*)-reticuline from its (*S*)-enantiomer *via* an epimerization including an oxidation towards the imine species followed by a stereoselective reduction [1]. More recently, it has been discovered that these two redox steps are catalyzed by a single protein, namely the naturally occurring fusion protein reticuline epimerase (REPI or STORR)[2]. This chimeric protein contains a N-terminal P450 (CYP) domain for oxidation and a C-terminal aldo-keto reductase for the subsequent reduction towards the tertiary amine (*R*)-reticuline. The latter enzyme is also called 1,2-dehydroreticuline reductase (*Ps*DRR) and is nicotinamide-dependent [1].

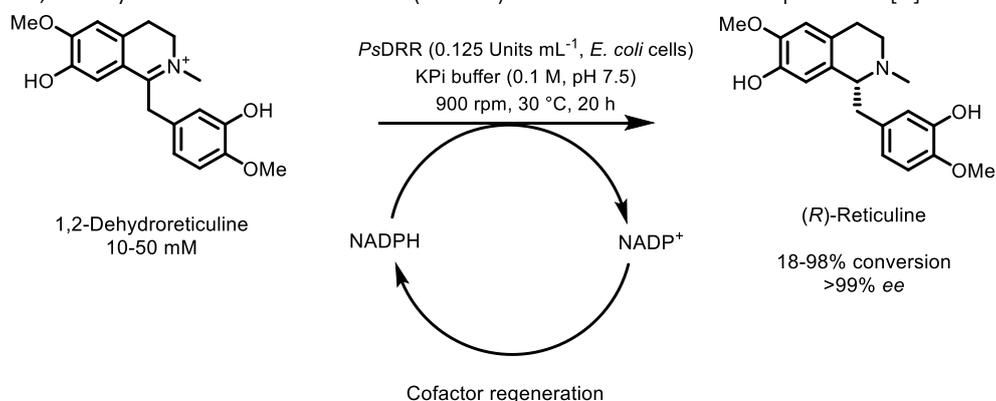


Figure 1. Asymmetric synthesis of (*R*)-reticuline employing the enzyme *Ps*DRR.

Interestingly, the two domains of the fusion protein can be heterogeneously overexpressed separately by conserving their functionality. Herein freeze-dried *E. coli* cells containing *Ps*DRR are used in an *in vitro* process exploiting an artificial cofactor regeneration system affording optically pure (*R*)-reticuline (Figure 1). The focus of this work is to investigate the preparative potential of the enzyme. The enzyme activity was determined *via* an end point assay and 0.125 Units mL⁻¹ were used for the optimization studies. A time study showed that the reaction is completed (i.e. quantitative HPLC yield) after 4 hours at 10 mM substrate concentration yielding the optically pure target compound (>99% *ee*). Since the focus lies on a high product titer, the substrate concentration was varied which revealed that substrate concentrations of 20 and 30 mM lead to the same product concentration, while at 20 mM substrate still quantitative conversion was observed. As a next step, 30 mM was chosen as the starting point for further optimization experiments. Comprehensive studies on pH-optima and buffer salt, on temperature profile and the effect of different cofactor regeneration systems were conducted. In summary, it was shown that the production of (*R*)-reticuline was achieved in a biocatalytic *in vitro* process employing whole lyophilized *E. coli* cells combined with an artificial cofactor regeneration system. Even though it was achieved to access significant productivity (about 100-fold compared to the literature [2]), additional optimization studies will be performed in order to further improve the reaction. This approach could serve as crucial step for the production of morphine-like compounds.

The University of Graz and the Field of Excellence BioHealth are acknowledged for financial support.

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Cloning, expression and characterization of hydroperoxide lysases for synthesis of 12-oxododecenoic acid

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In recent years, the impact of bio-based polymers has been rising and new monomers derived from renewable resources are needed to establish a future carbon-neutral polymer industry. An interesting precursor is 12-oxododecenoic acid, which may be further transformed into useful bi-functional monomers for biopolymer synthesis. In our work, we aim to create an enzyme cascade towards the oxo-acid including the enzymes lipase, lipoxygenase (LOX) and hydroperoxide lyase (HPL). The 13S-regiospecific soybean LOX1 catalyses the peroxidation of linoleic acid, which in turn is cleaved into 12-oxododecenoic acid and hexanal through a 13-specific HPL. While the green leaf volatile hexanal is already utilized commercially in the flavour and fragrance industry, 12-oxododecenoic needs further research to be established as novel intermediate in the chemical industry.

Four different hydroperoxide lyases from *Carica papaya*, *Hordeum vulgare*, *Psidium guajava* and *Sorghum bicolor* were cloned, expressed in *E. coli* and purified through metal affinity chromatography. Since solubility and hence catalytic activity of the HPLs was very low initially, the hydrophobic, non-conserved N-terminal sequences were deleted to improve the enzyme's solubility. Comparison of the truncated enzymes revealed that HPL from papaya exhibited the highest activity in UV-photometric analysis and thus, the papaya enzyme was chosen for further experiments to synthesize 12-oxododecenoic acid from 13-S-hydroperoxyoctadecadienoic acid (13-HPODE).

Linoleic acid was prepared from safflower oil either by enzymatic hydrolysis with lipases or by chemical hydrolysis and enrichment through urea fractionation. Commercially available LOX preparations from soybeans were used for the synthesis of the 13-HPODE regioisomer. To monitor hexanal, 12-oxododecenoic acid and 13-HPODE simultaneously, a gas-chromatographic assay was developed including extraction of the compounds, reduction with borohydride and silylation of the reduced hydroxylated metabolites. Time resolved studies with the papaya HPL revealed that the 12-oxododecenoic acid is highly reactive and less stable than the co-product hexanal. Thus, the development of product isolation strategies is needed.

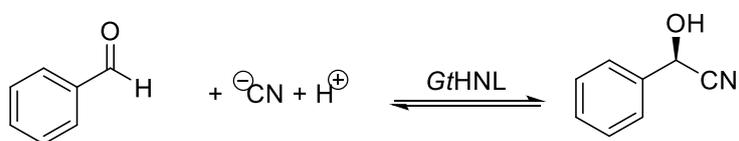
Probing batch and continuous flow reactions in organic solvents: *Granulicella tundricola* hydroxynitrile lyase (GtHNL)

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Granulicella tundricola hydroxynitrile lyase (GtHNL) is a manganese dependent enzyme [1] which catalyses the enantioselective synthesis of (*R*)-cyanohydrins (scheme 1). In this study, we used the immobilized triple mutant GtHNL-A40H/V42T/Q110H on Celite R-633.[2] All the reactions were performed in organic solvent (MTBE) saturated with an acidic buffer. This suppresses the chemical background reaction ensuring the synthesis of enantiopure cyanohydrins.[3,4]



Scheme 1. GtHNL catalysed hydrocyanation of benzaldehyde to (*R*)-mandelonitrile

The performance of a Spinchem rotating bed reactor (RBR) was compared to a continuous flow reactor (CFR) for the synthesis of (*R*)-mandelonitrile. A small scale batch reaction (BR) was used as a reference. RBR, a device designed to boost reactions rates due to the enhanced mass transfer, displayed similar conversions, recyclability and space-time-yield ($\sim 12 \text{ g h}^{-1} \text{ L}^{-1}$) as compared to the reference batch reaction. By switching to continuous flow with the CFR, good conversions were obtained within minutes with excellent enantioselectivity and high operational stability under all conditions evaluated. Remarkably, the CFR reached a STY of $784 \text{ g h}^{-1} \text{ L}^{-1}$ at 0.1 mL min^{-1} . This represents 65 times more product than both batch systems evaluated in this study and at least 14 fold increase as compared with earlier reports. [5] Overall, GtHNL-A40H/V42T/Q110H immobilized on Celite R-633 is an excellent biocatalyst for the synthesis of (*R*)-mandelonitrile with a great potential for industrial applications.

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***Micrococcus luteus* mediated oxidation of *threo*-9,10-dihydroxystearic acid – a key step in the conversion of oleic acid into pelargonic and azelaic acids**

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The limited availability of non-renewable petroleum-based sources and the increasing drive to reduce waste and energy consumption have prompted the scientific community to move towards the optimization of greener manufacturing processes. The use of agro-waste/bio-based renewable feedstocks, the development of catalytic systems, the reduction of solvents, avoiding toxic and harmful chemicals, are key features of ongoing research. A definite warning comes from the European Union to increase environmental sustainability, help with the transition to climate neutrality, recover unavoidable waste and promote the market of secondary raw materials and by-products.[1]

Following these considerations, we recently focused our attention on the exploitation of waste materials obtained by the refining processes of vegetable oils. The aim of the research was to establish an alternative route to the ozonolysis of commercial oleic acid [2] and obtain value from waste, i.e. azelaic acid for pharmaceutical and cosmetic industry and pelargonic acid for flavor and agrochemical products from soapstock, using chemo-enzymatic processes.

A sequential one-pot chemoenzymatic procedure for the conversion of *threo*-9,10-dihydroxystearic acid, easily obtained from oleic acid, into pelargonic and azelaic acids will be described. The oxidation of *threo*-9,10-dihydroxystearic acid into a mixture of the corresponding regioisomeric hydroxyketones was promoted by an alcohol dehydrogenase from *Micrococcus luteus*, selected from an extensive screening of commercial enzyme kits and strain collections. The stereochemistry of the oxidation process of chiral *threo*-9,10-dihydroxystearic acid is also investigated. The hydroxyketones obtained by the biocatalyzed oxidation were submitted to further oxidative cleavage by a mild treatment with aqueous NaClO in a biphasic mixture, affording pelargonic and azelaic acids in high yield (76 and 71%, respectively) with no need of column chromatography.[3]

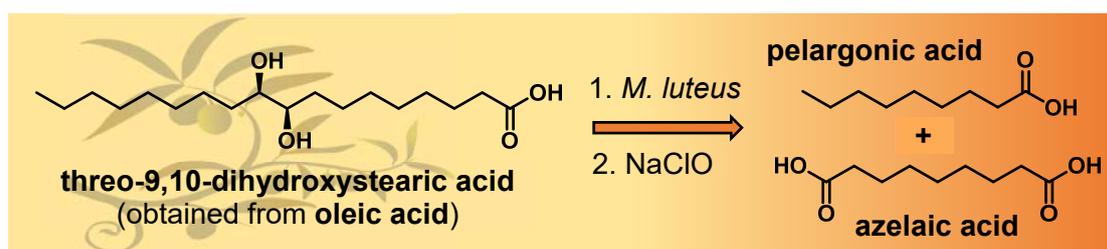


Figure 1. Chemoenzymatic procedure for the conversion of *threo*-9,10-dihydroxystearic acid to pelargonic and azelaic acids.

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Protein Immobilization by Sortagging

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Covalent, site-specific protein immobilization is a major challenge due to its robustness and control of protein orientation, which directly affects its functionality. Sortases are enzymes occurring in the cell wall of Gram-positive bacteria and are involved in the generation of long filamentous structures known as pili or fimbriae that extends from their surfaces interacting with their environment. Concretely, Sortase A (SrtA), the best studied sortase class, plays a key role in anchoring proteins with the recognition sequence LPXTG covalently to oligoglycine units of the bacterial cell wall. This sortase-mediated transpeptidation, known as sortagging, has been successfully used in protein bioconjugation, labeling and immobilization in solid supports as gold (Fig.1), silica or graphene oxide nanoparticles. [1,2]

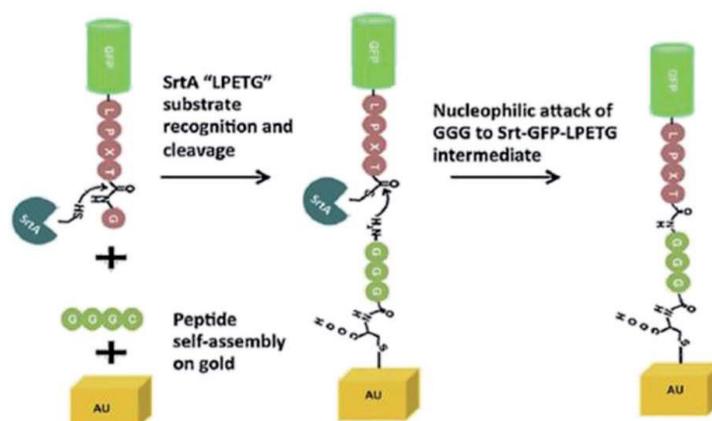


Figure 1. Immobilization of LPETG-GFP onto GGG-functionalized gold surface via sortagging

In this work we use the canonical *Staphylococcus aureus* Sortase A (SaSrtA) for the immobilization of GFP containing the LPETG domain in agarose supports activated with different nucleophiles as a proof of concept of the versatility of this strategy for protein immobilization. By fluorescence single-particle analysis we determine that GFP was immobilized at similar low yields onto agarose supports activated with primary amines as ethylenediamine or polyethyleneimine whereas no immobilization was detected in the case of agarose activated with poly(allylamine). On the other hand, the same supports activated with diglycine exhibited a clear increase in the immobilization yield, being the poly(allylamine) diglycine agarose the most efficient one, reaching values up to 25% of immobilized GFP.

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Characterization of a new bacterial galactose oxidase from the bacterium *Arthrobacter siccitolerans*

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Keywords: Biocatalysis, Copper radical oxidases, peroxidase-forming enzymes; lignin-degrading auxiliary enzymes

Galactose oxidases (GalOxs, D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9) are mononuclear copper-radical oxidases (CROs) that catalyze a two-electron regioselective oxidation of primary alcohols into the corresponding aldehydes, with the concomitant reduction of O₂ to H₂O₂. [1] The oxidation is performed without requiring organic cofactors and instead use an intrinsic metalloradical complex of Cu(II) which renders these enzymes suitable for biotechnological applications. [2] GalOxs have shown potential in the production of renewable building blocks for polymer industry, valuable chemicals and materials from plant biomass within lignocellulose biorefineries. [1] In this work, we have cloned and successfully overexpressed in *E. coli* a GalOx from the bacterium *Arthrobacter siccitolerans*. AsGalOx shows pHop at 7.5 and Top at 25°C using the HRP coupled assay. The highest enzymatic activity was measured for the oxidation of D-Gal, but AsGalOx also exhibited activity for a range of mono and disaccharides such as D-Raf, L-Ara, Lac, D-Mel, D-Glu. Work is currently in progress to increase the titers of soluble enzyme in cellular extracts of recombinant *E. coli* cells. Future work aims at providing catalytic, stability and structural fingerprints of AsGalOx and set-up bioconversion processes for the synthesis of useful bio-products.

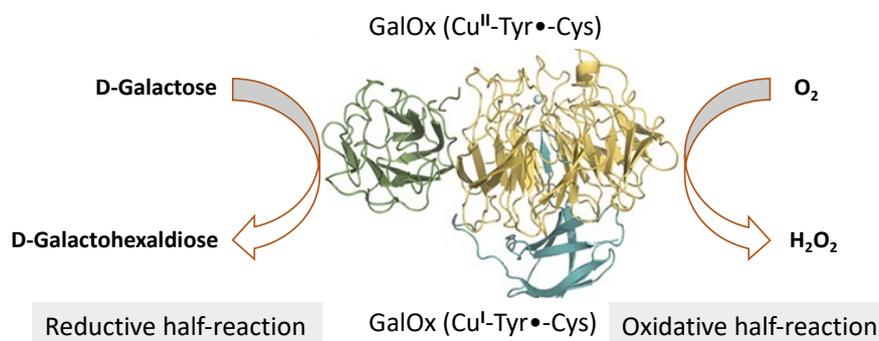


Figure 1. Catalytic mechanism of copper-radical galactose oxidase (GalOx).

Acknowledgments: This work is supported by the grant B-Ligzymes (GA 824017) from the European Union's Horizon 2020 Research and Innovation Program.

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Easy and scalable affinity immobilization for batch and continuous processes

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Biocatalytic processes are gaining importance in industry and often they require a minimum of downstream processes to concentrate or isolate the enzyme(s) of interest. A commonly used approach is to fuse a histidine tag to the protein and purify it by IMAC prior to its immobilization. When the process is scaled up, a cost-effective, scalable, regenerable resin will offer an increase in value and productivity.

In this work, we present a newly developed methacrylic iminodiacetic acid-functionalized resin, that allows an effortless capture / immobilization and biocatalytic process using a His-tagged ketoreductase. The study confirms the possibility to immobilize the enzyme in batch or flow immediately after fermentation, perform biocatalytic reactions and remove the enzyme once exhausted. Moreover, the entire process offers the opportunity for an easy scale-up, which can be difficult and expensive in other cases.

The resin attains metal ion (Ni^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Fe^{2+}) loadings of 0.25 mmol metal/g and high affinity towards His-tagged proteins. The affinity immobilization of the His-tagged ketoreductase, was compared to various other methacrylic resin-enzyme interactions, i.e. covalent, ionic and hydrophobic. The results showed that the His-tag purification/immobilization provides up to 4 folds increase in the specific activity compared to other immobilizations, which is directly related to the selectivity of the resin employed. Additionally, the immobilized ketoreductase showed excellent stability over 5 cycles in both aqueous and organic media, and it was possible to regenerate the resin up to 5 times without any lost in enzyme binding.

The ketoreductase immobilization and biocatalysis were not only studied in batch, but also in flow using a packed column. The enzyme was immobilised directly in column and then the biocatalysis was carried out in a continuous process. Several substrate concentrations were studied reaching a maximum space time yield (STY) of almost 90 g/L/h (more than 2 kg/L/day).

Biomass-derived, renewable carriers for enzyme immobilization

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Biocatalyst immobilization is crucial for many application, as it allows continuous processing reducing both process cost and production of waste. However, traditional, fossil-based enzyme carriers are unsuitable for application in a renewable and bio-based application context. In this project we aim to develop new immobilization methods, using cheap, renewable carriers as well as green immobilization procedures.

Rice husk is a widely available and cheap byproduct of the food industry, and has previously demonstrated to be a suitable carrier for enzyme immobilization.[1],[2] It has been studied with various enzymes, including Lipase B from *Candida antarctica*[1],[2] (CaLB), Invertase from *Saccharomyces cerevisiae*,[2] Thermolysin from *Geobacillus sp.*[2] and two commercial asparaginases.[1] CaLB on rice husk has proven to be more efficient than CaLB immobilized on commercial, fossil based carriers for applications in viscous, low-water content media.

Rice husk has been characterized spectroscopically to assess its structural characteristics. Moreover, glucoamylase from *Aspergillus niger*, an industrially relevant enzyme used in starch processing, was the subject of a computational study to determine possible immobilization strategies on the novel carrier.

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Citric Acid Production from Wood Sugars by the Yeast *Yarrowia lipolytica*

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The need to abandon the fossil based linear economy, pushes for a search of alternative carbon-based materials [1]. For this purpose, different alternatives for petroleum-derived plastics are currently being researched. An intermediate to consider in polymer development is citric acid (CA) which can be efficiently produced from raw agricultural materials. However, the use of first-generation biomass feedstocks is not desirable as it competes with food production. Sugars derived from lignocellulose, on the other hand, present an opportunity to utilize lower quality wood that is not suitable for construction or paper production. Citric acid is industrially produced from glucose by microbial fermentation. Conventionally, the microorganism of choice is filamentous white rot fungus *Aspergillus niger* [2]. It is able to produce CA in high amounts and ferment a variety of cheap materials. However, the use of yeasts for CA production has not been as extensively investigated. The interest in yeasts, especially species *Yarrowia lipolytica*, over moulds, can be foreseen due to a number of advantages, such as better tolerance of metal ions and high substrate concentrations, ease of cultivation and higher rates of fermentation [3].

The production and accumulation of CA is strongly influenced by the composition of the growth medium. There are many factors to consider, but the most crucial aspect is the carbon source. A variety of agricultural waste products containing pectins, cellulose and starch can be utilized by microorganisms for CA production [4]. Using wood sugars from lignocellulosic materials has not been thoroughly investigated, nevertheless, some insight into possible use of xylan, xylan hydrolysate and sugars present in hemicellulose hydrolysate has been made [5]. However, the lack of research into possible utilization of such substrates by yeast strains presents an opportunity for new discoveries. The aim of this study is to develop a fermentative process for CA production by yeasts *Y. lipolytica*, employing wood sugars as a carbon source. A variety of strains were screened, including commercially available and wild type ones. The importance of different growth media components and additives were tested to create optimal conditions for high CA yield. The results will be presented in more detail during the poster session.

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Dynamic Kinetic Resolution of heterobiaryl aldehydes employing alcohol dehydrogenases

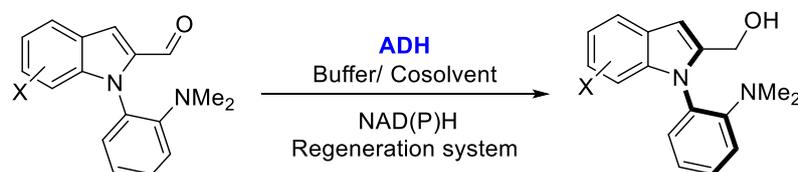
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Axially chiral biaryl compounds are widely spread in natural products and bioactive substances, with several applications in catalyst preparation and material science [1]. By this reason, their enantioselective preparation is becoming a field of interest [2]. Several methodologies based on metal-catalysis and organocatalytic procedures have been developed, but only few examples employing biocatalysts have been described, being the preparation of axially chiral biaryl *N*-oxides by dynamic kinetic resolution (DKR) using alcohol dehydrogenases one of the last examples [3].

Recently, our research group has developed a strategy for the atroposelective synthesis of heterobiaryl alcohols by DKR. The strategy relies on the labilization of the stereogenic axis that takes place thanks to the Lewis acid-base interaction (LABI) [4]. In our ongoing strategy to carry out the atroposelective synthesis of C-N containing molecules, we have designed a set of indole-based heterobiaryl aldehydes, featuring a similar LABI, that have been atroposelectively reduced to the corresponding chiral alcohols in a DKR process by commercially available alcohol dehydrogenases. By optimizing the biocatalytic procedure, it is possible to obtain the desired alcohols with excellent conversions and optical purities in nearly all cases, thus representing a novel methodology for the synthesis of this type of structures.



12 examples

X: H, OMe, Me, F, Cl, CF₃.

Figure 1. Dynamic Kinetic Resolution of heterobiaryl aldehydes to atropisomeric alcohols employing alcohol dehydrogenases.

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Systematic analysis of carrier-bound immobilization strategies for unspecific peroxygenase

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Unspecific peroxygenases (UPOs) (EC1.11.2.1) represent the best alternative to the traditional catalysis for the hydroxylation of organic compounds. This enzyme family, is today well known for catalyzing the regio- and stereospecific hydroxylation of non-activated C-H bonds at the expense of H₂O₂. [1] The main goal of this study is to develop an efficient light-driven heterogeneous biocatalytic cascade by combining a photocatalyst and a stereosepecific UPO to synthesize (*R*)-1-phenyl ethanol, an interesting chiral alcohol widely used as pharma intermediate and in the perfumery industry (**Figure 1**).

Among the different enzymes belonging to the UPOs family, the *Agroclybe aegerita* UPO (*AaeUPO*) was chosen for the project thanks to its high activity. [2] Actually, its recombinant counterpart PaDa-I is the biocatalyst employed in the experiments, since it shows an optimal activity, selectivity and stability. [3] When it comes to the photocatalytic part of the system, the choice is made on the nitrogen-doped carbon nanodots, which permit the *in situ* production of the H₂O₂ and to overcome one of the main challenges related to the UPOs. In fact, on the one hand, the hydrogen peroxide is the oxidant agent needed for the substrate conversion, on the other hand, an excess of it easily inactivates *AaeUPO*.

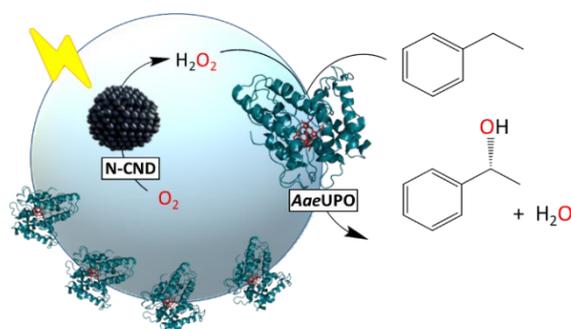


Figure 1. Illustration of biocatalytic conversion of ethylbenzene into (*R*)-1-phenyl ethanol by the unspecific peroxygenase from *Agroclybe aegerita* (*AaeUPO*) immobilized on a carrier material. Different carriers were investigated, the most promising are the amino carrier from Purolite Life Sciences and Opal EziG™ carrier from EngineZyme A.B.

In order to make their use feasible on industrial scale, some difficulties, such as narrow operational conditions, solubility issues and downstream processing, need to be overcome; all these disadvantages have a common solution: Enzyme immobilization. [4] In this perspective, both carrier-bound and carrier free methods are under investigation within this study and among the different materials evaluated for the carrier-bound methods, amino carriers (furnished by Purolite Life Sciences) and porous glass materials EziG™ (furnished by EnginZyme A.B.) seem to be highly promising.

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Chemoenzymatic, Biomimetic Synthesis of (–)-Flavoskyrins and its (Bio)synthetic Implications

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Isolated nearly 70 years ago by Howard and Raistrick from *Penicillium islandicum* Sopp NRRL 1175, the dimeric natural product, (–)-flavoskyrin (**3a**, R¹ = R² = CH₃) has been found to possess a unique molecular scaffold, with a C-C and C-O bond between the two monomeric anthraquinones.¹ The role of (–)-flavoskyrin (**3a**) as a key biosynthetic intermediate was established by its cascade conversion to a caged modified bisanthraquinone natural product, (–)-rugulosin, in the presence of pyridine.² This inspired us to investigate the biogenesis of **3a** and led us to develop a first two steps, biomimetic, chemoenzymatic method for the synthesis of (–)-flavoskyrin (**3a**) starting from emodin (**1a**, R = CH₃) using an anthrol reductase of *Talaromyces islandicus* (ARTi).³ Further, optimization allowed us to use molecular oxygen instead of Pb(OAc)₄ for oxidation of enzymatic product, (*R*)-configured dihydroanthracenone (**2a**, R = CH₃), followed by dimerization to (–)-flavoskyrin (**3a**) in a catalyst free condition.

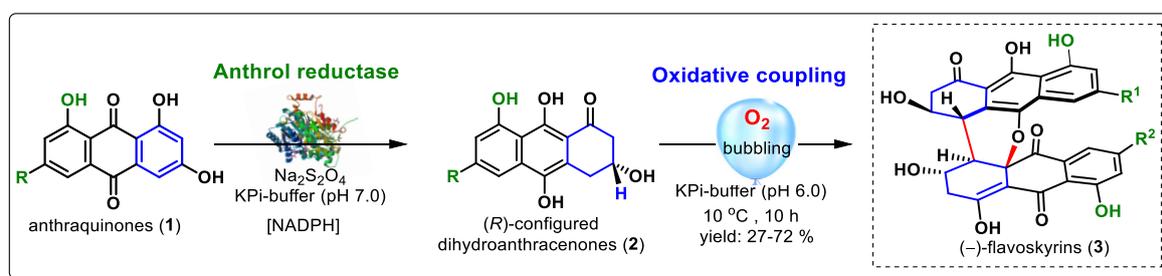


Figure 1. Synthesis of (–)-flavoskyrins by catalyst-free oxidation of chemoenzymatically synthesized (*R*)-configured dihydroanthracenones.

The method is being applied to synthesize a number of homo- and heterodimeric (–)-flavoskyrins (**3**) in just two steps, starting from anthraquinones in 27-72% yield.³ The work implies for the role of such a new type of homodimerized (R¹ = R²) and heterodimerized (R¹ ≠ R²) flavoskyrins as intermediates in the (bio)synthesis of modified bisanthraquinones which have been isolated from nature or are yet to be isolated.

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GlycoProFit: Cost-efficient synthesis of health-promoting glycobioses

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The GlycoProFit project aims to establish the “Cost-efficient synthesis of health-promoting glycobioses” using classical table sugar (sucrose) as substrate and enzymes as green biocatalysts. After the synthesis of a variety of healthier alternatives, such as kojibiose (α -1,2-glucobiose) [1,2], the new disaccharides and analogues will be subjected to detailed analysis to determine their characteristics in food applications (incl. cariogenic effect, caloric value and prebiotic properties). The most promising candidates will then be produced at a larger scale to enable their taste and texture profiling. The GlycoProFit project is a collaborative research project where five different partners, academic research groups, combine their strengths and expertise in order to create and analyse new biocatalytic production routes for glycobioses with health-promoting properties.

The engineering of Sucrose Phosphorylase (SP) resulted in two dedicated biocatalyst for kojibiose and nigerose productions. The selectivity was improved via enzyme engineering towards both sugars [1,3]. Process optimization allowed kojibiose production at the kilogram scale, and simple but efficient downstream processing, using a yeast treatment and crystallisation, resulted in more than 3 kg of highly pure crystalline kojibiose. These amounts allowed a deeper characterisation of its potential in food applications and comparison of the bulk functionality of highly pure kojibiose to that of sucrose, hereby mapping its potential as a new sweetener in confectionery products [2].

In the case of nigerose, the final mutant showed to have an increased catalytic efficiency (k_{cat}/K_M) and was put to use for producing nigerose on 0.5 L scale, leading to a multi-grammes scale. The product was isolated by means of enzymatic degradation of the contaminating sugars and subsequent preparative liquid chromatography [3].

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Single-particle enzyme kinetics: an in-operando tool to elucidate the functionality of cofactor dependent immobilized biocatalysts.

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Multidimensional kinetic analysis of immobilized enzymes is essential to understand the enzyme functionality at the interface with solid materials. These studies are even more interesting for ready-to-use self-sufficient heterogeneous biocatalysts where a cofactor-dependent enzyme is co-immobilized with its corresponding cofactor on porous microbeads.[1] However, spatiotemporal kinetic characterization of heterogeneous biocatalysts at a microscopic level and under operando conditions has been rarely approached. Inspired by single-cell fluorescence microscopy studies, we performed single-particle measurements under operando conditions to comprehend the microscopic dynamics of self-sufficient heterogeneous biocatalysts.[2] Harnessing the auto-fluorescence of industrially relevant phosphorylated cofactors (NADH, FAD and PLP), we perform time-lapse fluorescence microscopy and analyze the enzyme activity confined within porous microbeads.

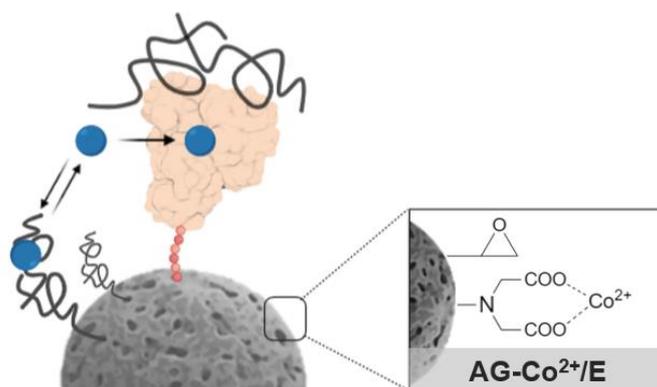


Figure 1. Schematic illustration of agarose beads functionalized with cobalt chelates and epoxy groups for the site-selective enzyme immobilization and the assembly of the cationic polymers for the subsequent cofactor co-immobilization.

We study the spatiotemporal performance of heterogeneous biocatalysts to better understand linkage between enzyme kinetic parameters and enzyme crowding and spatial organization. We exploit image processing of time-lapse fluorescence microscopy to determine intraparticle kinetics of self-sufficient heterogeneous biocatalysts made of His-tagged enzymes co-immobilized with their corresponding phosphorylated cofactors on agarose microbeads. The resulting biocatalysts are analyzed under static and in operando conditions to investigate the cofactor binding thermodynamics, enzyme density and enzyme apparent Michaelis-Menten kinetics at a single-particle level. We elucidate relationships between cofactor binding thermodynamics and apparent kinetics while we show that particle size and enzyme concentration are the major sources of functional variability. Finally, we highlight that intraparticle kinetics are significantly affected by the enzyme spatial organization through studies at the sub-micrometric level.

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Transformation of a library of enzymatically synthesized coenzyme A conjugates into natural and new-to-nature phenolics

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Coenzyme A (CoA) serves as a carrier and activator for carboxylic acids in more than 100 types of biochemical reactions, e.g. in the reduction of acids to aldehydes, in hydroxylation of aromatic acids, or in acylation of small molecules and proteins. In this study, a library of CoA conjugates was prepared by ligation of CoA with using a promiscuous plant ligase (Fig. 1). This library comprised twenty thioesters of cinnamates, benzoates and short-chain fatty acids with different functionalization [1]. It was exploited in subsequent enzymatic transformations yielding three types of valued plant natural products:

- (I) the coupled retro-aldol-type conversion into aroma benzaldehydes (Fig. 1) by a lyase [1],
- (II) the oxidative cyclization into coumarins catalysed by dioxygenases (Fig. 1),
- (III) acylation / cyclization by a polyketide synthase-like enzyme producing antifungal 5-alkylpyrones.

In all cases, we employed enzymes with relaxed substrate specificity, which were able to generate naturally occurring phenolics but also new-to nature derivatives via these biorthogonal reactions. Finally, we identified amino acid residues that seem to be important for the promiscuity of the enzymes using modelling and substrate docking.

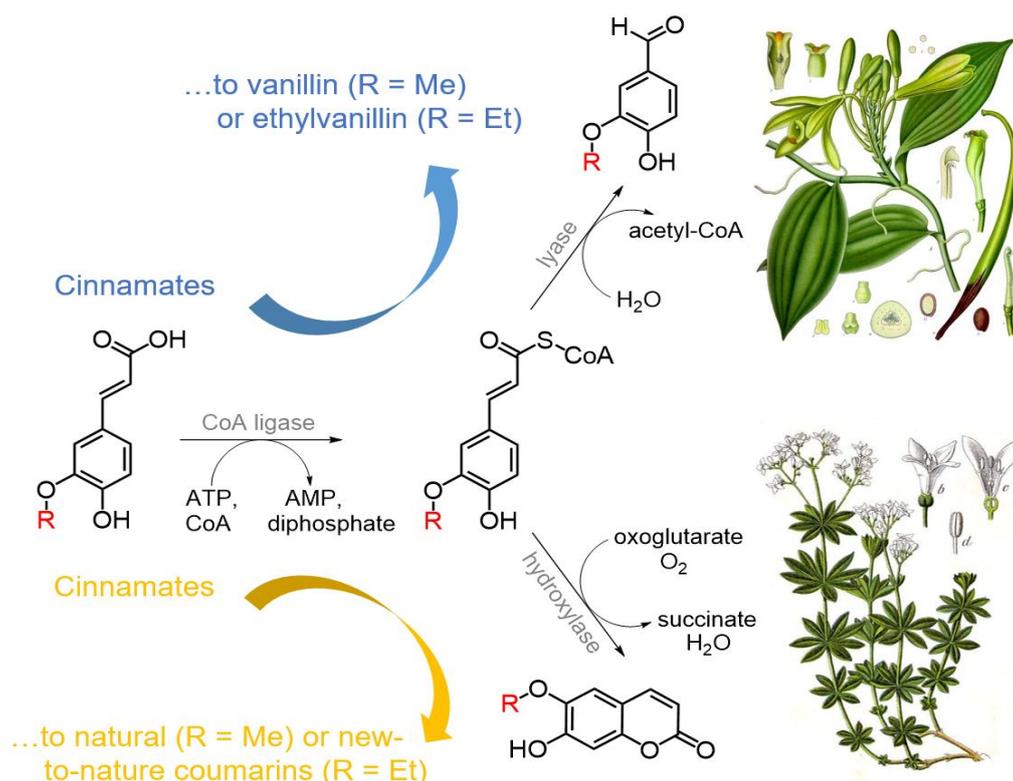


Figure 1. Formation of natural products and derivatives from cinnamates. After conjugation to CoA by a promiscuous ligase, the cinnamoyl thioesters are converted either to benzaldehydes (top) or coumarins (bottom), which are important aroma compounds in e.g. Vanilla or sweet woodruff, respectively. Historic plant illustrations were taken from reference [2].

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Isaria fungi - powerful tools for flavonoid glycosylation

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Flavonoids, secondary plant metabolites, show many biological activities, including preventing the development of lifestyle diseases, autoimmune diseases (multiple sclerosis among others) [1], acting as prebiotics [2]. Finally, by activating various anti-inflammatory pathways, flavonoids could be used to treat SARS-CoV-2 induced inflammatory storm [3].

However, the potential of flavonoids is not fully utilized due to their low oral bioavailability resulting, *i.a.*, from low solubility in water. One way to improve the solubility and stability of flavonoids is their glycosylation [4,5]. Several methods are known for attaching a glycosidic unit to a flavonoid aglycone. Among the biotechnological processes, whole-cell biotransformations are still of great importance.

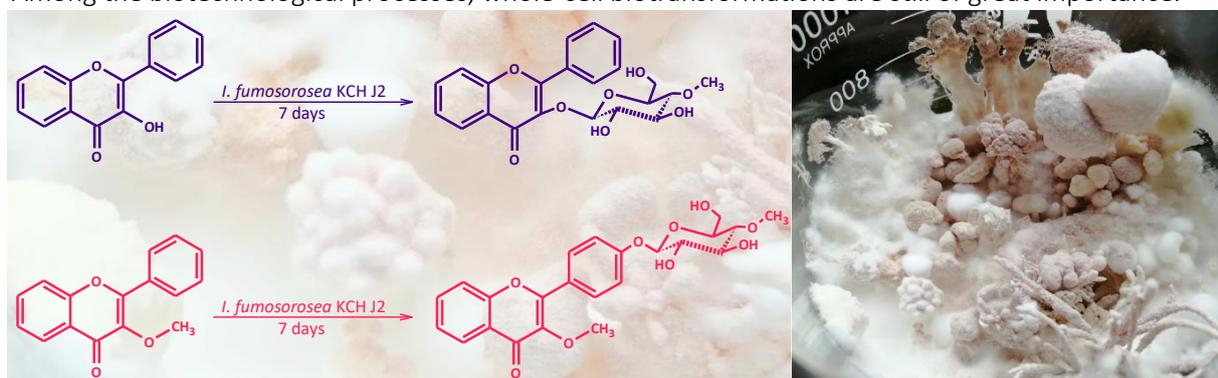


Figure 1. *Isaria fumosorosea* KCH J2 (right) has the ability to glycosylate hydroxy-, and methoxyflavonoids [6].

For several years, our team has been using entomopathogenic filamentous fungi to glycosylate variously substituted flavonoids [6-8]. Thanks to the use of pTLC for the separation of biotransformation products, we use minimal amounts of organic solvents. The structures of the purified compounds are determined using NMR (^1H , ^{13}C , HMBC, HSQC) and confirmed by mass spectrometry.

Isaria fungi effectively attach the 4-*O*-methylglucopyranose to both aglycons with and without hydroxyl substituents (Figure 1). We were the first to describe the glycosylation of flavonoid aglycones with methyl- and methoxy- substituents, as well as an unsubstituted flavone. The great advantage of the biotransformations we carry out is their simplicity, low cost and mild conditions, so they can be considered environmentally friendly methods.

We have created a library of several dozen flavonoid glycosides, and their number is constantly growing.

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***E. coli* or yeast – who is the better host for microbial carboxylate reduction?**

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The choice of the expression host is often determined by the availability of genetic tools, growth speed, achievable cell densities and strain stability. Also, the ability to carry out certain post-translational modifications or targeting events may influence the decision for a certain host. Academic labs – more often than not – start investigations of new enzymes in *E. coli*. This bacterium is well understood, easy to manipulate and handle, fast growing and a standard workhorse in many labs who are active in the field of biocatalysis. Other labs prefer yeasts like *Saccharomyces cerevisiae* or *Komagataella phaffii* (alias *P. pastoris*) for reasons like their ability to secrete proteins to the culture supernatant, to perform glycosylations or to promote disulfide bridge formation.

Carboxylic acid reductases (CARs) are enzymatic tools for the direct reduction of carboxylic acids to their respective aldehydes and follow up products [1]. CARs consist of three domains and require post-translational phosphopantetheinylation [2]. CARs can be found in both bacteria and fungi. Several groups have investigated bacterial CARs in yeasts (*S. cerevisiae*, *K. phaffii*, *Y. lipolytica* etc) while others studied fungal CARs in *E. coli*. The general rule of thumb to choose a yeast for fungal target enzymes and *E. coli* for bacterial enzymes was not applied in case of CARs, apparently.

In this study, we aimed to understand whether *E. coli* or *K. phaffii* is the best host for the production, activation and use of CARs in a whole cell system to produce aldehydes from their respective carboxylic acid precursors. Specifically, the CAR and PPTase from *Mycobacterium marinum* (MmCAR/MmPPTase) were co-expressed in both organisms to allow a possibly fair and direct comparison. The resulting biocatalysts have been evaluated with different substrates (aromatic and aliphatic).

Acknowledgements

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Setting up a toolbox for biocatalytic C4-prenylation of tryptophan derivatives by 4-dimethylallyltryptophan synthase

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4-Dimethylallyltryptophan synthases (4-DMATSs) have been identified in different ergot fungi as responsible for the selective C4-prenylation of L-tryptophan to obtain 4-dimethylallyltryptophan (4-DMAT). This compound constitutes a conserved intermediate in the general quite diverse ergot alkaloid pathways and is transformed to chanoclavine-I after oxidation via an FAD-dependent (EasE) and a heme-dependent oxidoreductase (EasC) [1,2,3]. First characterization of a DMATS from *A. japonicus* (DmaW) revealed L-tryptophan and L-abrine (*N*-methyl-L-tryptophan) to be preferred substrates for prenylation. By optimization of reaction conditions and expansion of the DMATS toolbox using alternative DMATSs and enzyme engineering, the acceptance towards different tryptophan derivatives should be increased (Figure 1). Homologous enzymes from *C. purpurea* and *T. benhamiae* gave better acceptance of substituted tryptophans, like 1-methyl-DL-tryptophan and various C5-substituted analogues. Conversion of the latter substrates could be increased even further by site-directed mutagenesis at position Y195 and T108 to serine. Thereby, optimization and implementation of different enzyme catalysts allowed scale-up of enzymatic C4-prenylation and first preparative applications.

Since the chemical synthesis of prenylated C4-tryptophans would require several steps including protection strategies, the biocatalytic prenylation offers here a unique option not possible with chemical methods in a single step. Additionally, the prenylation of substituted tryptophan introduces a starting point for the synthesis of different new ergot alkaloid derivatives.

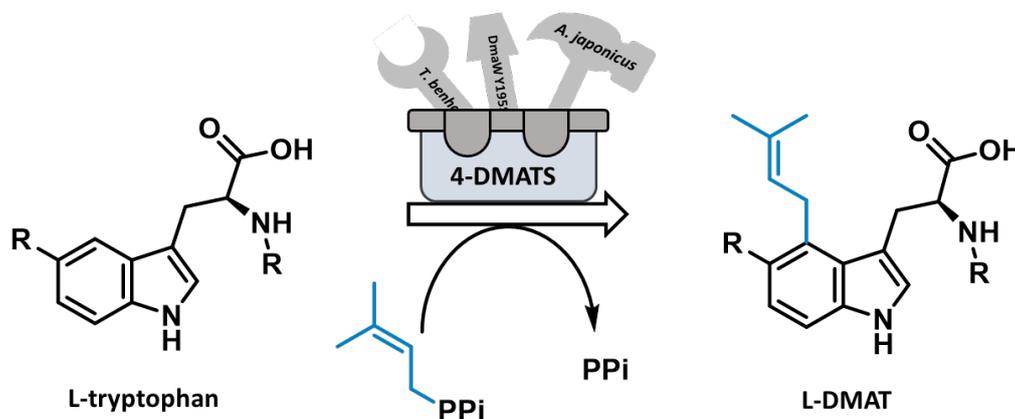


Figure 1. Enzymatic C4-prenylation of substituted L-tryptophan derivatives by different wild-type and engineered 4-DMATSs and DMAPP as prenyl donor

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Discovery of a bacterial eugenol oxidase by structure-guided genome mining

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Bacterial eugenol oxidases (EUGOs) form together with their fungal homologues vanillyl alcohol oxidases (VAOs) an enzyme family of versatile biocatalysts.[1,2] They catalyse a variety of different reaction types on a broad substrate scope.[3] The oxidation of alkanes or alcohols is the characteristic reaction of both clades including the oxidation of eugenol and vanillyl alcohol.[4,5] Both, alcohols, and ketones are important building blocks in the chemical industry. Nevertheless, the bacterial clade of this enzyme family received little scientific attention so far. Thus, our goal is to explore bacterial eugenol oxidases in order to identify novel biocatalysts.

Along these efforts, the bacterial eugenol oxidase *NspEUGO* from *Nocardioides* sp. YR527 was discovered in a sequence-based genome mining approach which was guided by homology modelling. The enzyme was selected for characterization based on natural amino acid exchanges in the catalytic centre. For two amino acid alterations, an activity increasing effect as single mutations was already described by Ewing *et al.*[6]

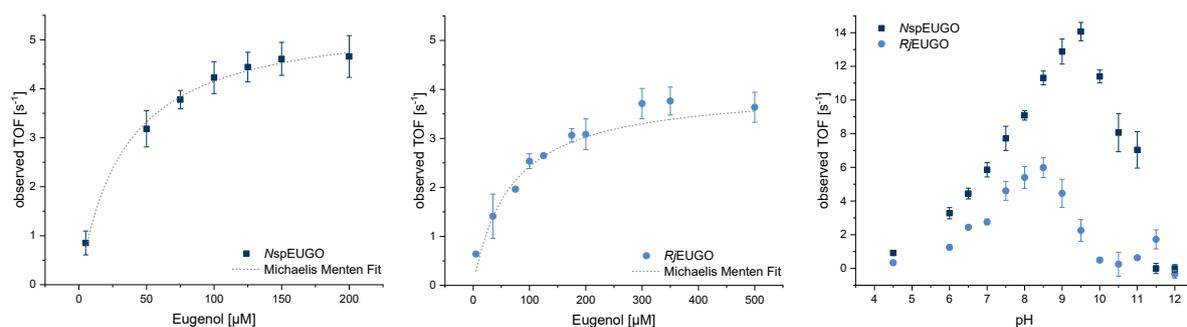


Figure 1. The novel bacterial eugenol oxidase *NspEUGO* (dark blue) was investigated and compared to the best described member of the respective family, *RhjEUGO* (light blue). *NspEUGO* was found to catalyse the oxidation of eugenol at a 30% percent increased frequency at standard conditions. Variation of the pH revealed a remarkably increase of >200% in catalytic frequency under alkaline conditions.

For *NspEUGO*, a catalytic frequency of $5.53 \pm 0.19 \text{ s}^{-1}$ under standard conditions (50 mM potassium phosphate buffer, pH 7.5, 25°C) on eugenol was found which can be increased to a catalytic frequency of $15.95 \pm 0.32 \text{ s}^{-1}$ under optimal conditions in glycine NaOH buffer at pH 9.5. This catalytic frequency is more than doubled in comparison to the best described member of this enzyme family, *RhjEUGO*, under optimal conditions.[5] In order to utilize this highly active biocatalyst, *NspEUGO* was immobilized on different carrier materials and found to produce $>2 \text{ g L}^{-1} \text{ h}^{-1}$ vanillin by oxidation of vanillyl alcohol in a test scale of 250 mL.

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Use of 2-deoxyribose-5-phosphate aldolase for the synthesis of precursors of pharmacologically relevant compounds

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In recent years, alternative processes involving enzymes or microorganisms as biocatalysts have been applied for the synthesis of pharmaceutical, industrial and agricultural chemicals, demonstrating the advantages that biocatalysis has over traditional chemical procedures. Aldol additions biocatalyzed by aldolases can yield enantiomerically pure products from non-chiral substrates. Particularly, in our laboratory, we identified microorganisms with 2-deoxyribose-5-phosphate aldolase (DERA; EC 4.1.2.4) activity useful for preparing intermediates for antivirals and anticholesterolemic drugs synthesis (Figure 1). The natural reaction of DERA is the aldol addition between acetaldehyde and glyceraldehyde-3-phosphate (G3P) generating deoxyribose-5-phosphate (DR5P). DR5P can be used as intermediate in the preparation of deoxynucleosides, compounds widely used as antiviral drugs. Another important characteristic of this enzyme is its ability to catalyze a double aldol addition affording 2,4,6-trideoxyhexoses. These compounds are intermediates in the synthesis of statins, drugs used to control blood cholesterol levels. However, DERA is inhibited at high aldehyde concentrations, which is a problem for large-scale reactions. To solve this drawback, an acetaldehyde resistant *Pectobacterium atrosepticum* strain, has been selected [1]. This enzyme has been cloned, expressed and genetically modified to improve its catalytic efficiency. Employing recombinant DERA whole cells, the complete conversion to DR5P using G3P as substrate was obtained. In addition, when acetaldehyde was used as donor and acceptor, the corresponding 2,4,6-trideoxyhexose was obtained in 50% yield [2].

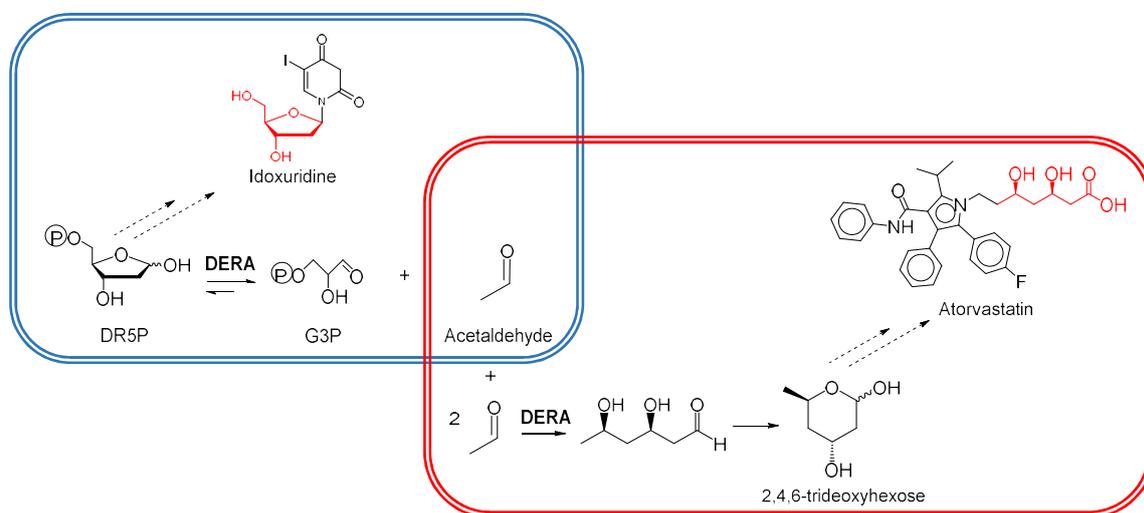


Figure 1. Aldol additions biocatalyzed by DERA to prepare intermediates compounds for antivirals and anticholesterolemic drugs synthesis.

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Enzymatic alternatives to current permanent hair straightening methods

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Within the cosmetic industry, hair care has been a leader in consumption for many years with a market share expected to reach \$100 billion by 2024.¹ Hair straightening is amongst the most popular hair treatments for reasons including easier daily hair maintenance and beauty standards. However, current methods rely on the use of harsh chemicals, such as sodium hydroxide and formaldehyde with associated environmental and user risks. These include hair loss and breakage, as well as serious scalp disorders. In this way, the recent development of biotechnology in cosmetics opens the way to more sustainable and safer alternatives to harsh chemical relaxers.

This project explores the feasibility of using enzymes as active agents in permanent hair straightening treatments. Indeed, the chemistry involved in hair cosmetic treatments (*i.e.* disulfide bond reduction and crosslinking reinstatement) is prevalent in natural enzymatic activities. In an initial approach, the focus was placed on two enzymes with potential hair disulfide bond reducing activity: keratinase and protein disulfide reductase. The former belongs to the family of serine proteases, able to selectively cleave peptide bonds within the keratin structure and widely used in a range of industries (cosmetic, poultry, leather etc.).²⁻⁴ The latter, protein disulfide reductases, catalyse thiol-disulfide exchange reactions and are highly selective for exposed disulfide bonds in folded proteins.⁵⁻⁸ To this date, various proteins belonging to the protein disulfide oxidoreductase family have been identified and characterised, including thioredoxins, glutaredoxins and protein disulfide isomerases.^{9,10}

A particular focus was placed on keratinase from *Bacillus licheniformis* and thioredoxin enzymes both from *Escherichia coli* and *Bacillus subtilis*.¹¹⁻¹⁶ These enzymes were analysed as potential disulfide bond reducing candidates using a variety of substrates (casein, insulin, keratin azure and human hair) and assays including Ellman's assays, keratin azure assays and insulin assays. The associated genes were also designed, cloned and expressed in *Escherichia coli* BL21 (DE3) cells.^{11,16} In this way, an enzyme capable of reducing hair keratin disulfide bonds will be selected. The ability of enzymes, such as lanthionine synthetases, to reinstate crosslinks between hair fibres will then be assessed. The final stage of my project will involve developing enzymatic hair straightening formulations showing a desired permanent straightening effect at reasonable costs with sufficient shelf-life and known allergenicity.

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Small ring synthesis via ene-reductase mediated reductive cyclization

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Small carbocycles like cyclopropanes, -butanes and -pentanes are structural elements that are frequently occurring in valuable products like active pharmaceutical ingredients. Thus, efficient methods for their preparation are in high demand, especially when they manage to meet high standards of sustainability. Since biocatalysis is always at the forefront of methods when it comes to sustainability, it was our desire to develop an ene-reductase based method for the synthesis of small rings.

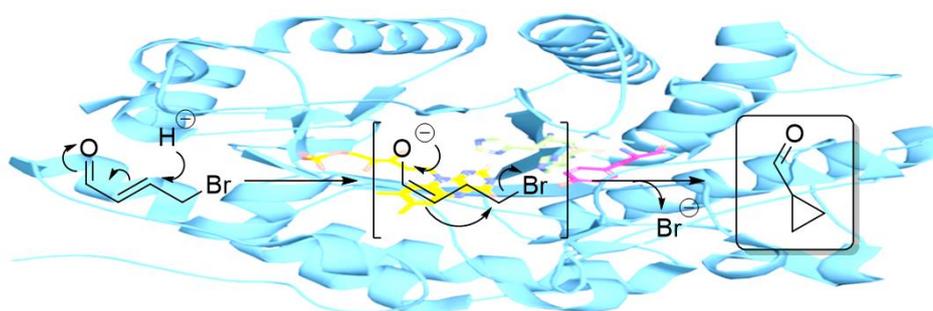


Figure 1. Working principle of ene-reductase catalyzed reductive cyclization [1].

To utilize ene-reductases for the biosynthesis of small cycloalkanes, substrate as well as enzyme engineering is required. In terms of substrates, halogenated enals were used. The substrates should be converted by ene-reductase variants, which lack protic residues in their active sites. Due to missing protic side chains, the enolate intermediate of the commonly accepted ene-reductase mechanism is not protonated as fast as it would happen in the wild-type enzyme. The higher persistence of the enolate intermediate within the active site of the ene-reductase variants, leads to the possibility of a nucleophilic attack at the terminal alkyl halide of the utilized substrate. This alternative trapping route of the enolate intermediate leads to halide displacement accompanied by the formation of a new, ring constructing C-C bond. Overall modified ene-reductases were intended to convert halogenated enals to cyclic carbaldehydes via a mechanism that can be described as reductive cyclization [1].

So far the concept of ene-reductase mediated cyclization could be successfully employed for the biosynthesis of cyclopropane-carbaldehyde derivatives in good diastereo- and excellent enantioselectivities. Currently the expansion of this method to four- and five-membered rings is under investigation.

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Ground-State Electron Transfer as an Initiation Mechanism for Biocatalytic C–C Bond Forming Reactions

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ABSTRACT: Developing non-natural reaction mechanisms is an attractive strategy for expanding the synthetic capabilities of substrate promiscuous enzymes. Here, we report an 'ene'-reductase catalyzed asymmetric hydroalkylation of olefins using α -bromoketones as radical precursors. Radical initiation occurs via ground-state electron transfer from the flavin cofactor located within the enzyme active site, an underrepresented mechanism in flavin biocatalysis. Four rounds of site saturation mutagenesis were used to access a variant of the 'ene'-reductase from *Zymomonas mobilis* (NCR) capable of catalyzing a cyclization to furnish β -chiral cyclopentanones with high levels of enantioselectivity. Additionally, wild-type NCR can catalyze intermolecular couplings with precise stereo-chemical control over the radical termination step. This report highlights the utility for ground-state electron transfers to enable non-natural biocatalytic C–C bond forming reactions.[1]

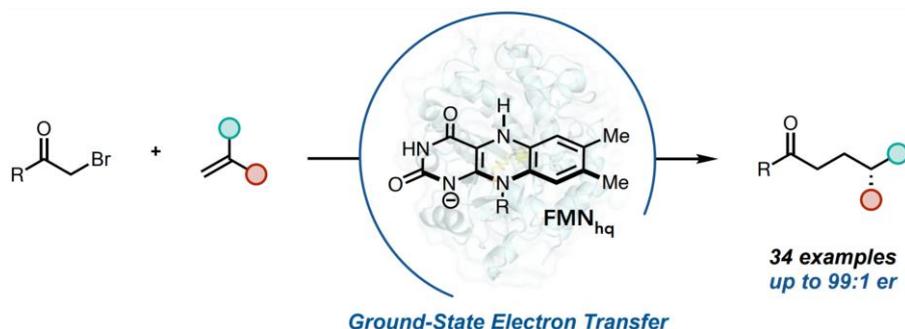


Figure 1. Ground-state electron transfer as an initiation mechanism for C–C bond formation.

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A novel colorimetric assay for transketolase activity via generation of *N*-aryl hydroxamic acids

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Transketolase (TK) *in vivo* reversibly transfers a two-carbon hydroxyacetyl unit from a phosphorylated ketose to a phosphorylated aldose. The thermostable TK from *Geobacillus stearothermophilus* (TK_{gst}) [1] has been improved by directed evolution to also accept non-phosphorylated and non-hydroxylated alkyl, and even highly hydrophobic arylalkyl aldehyde acceptors.[2] Such reactions become practically irreversible if 2-oxoacids such as hydroxypyruvate or pyruvate are used as ketol donors.[3]

Analogous to the TK_{gst} catalyzed acyloin formation from benzaldehydes and 2-oxoacids,[2] reaction of nitrosobenzenes yields so-called hydroxamic acids (HA) as products. The medicinal properties of this compound class,[4] originating from their propensity for forming strong chelate complexes to metal dependent enzymes, render them highly interesting for the pharmaceutical industry. Using variously substituted nitrosobenzenes as electrophiles, and either hydroxypyruvate or pyruvate as nucleophilic substrates in the presence of TK_{gst} and cofactors, more than a dozen of HAs could be successfully prepared (Fig. 1). TK_{gst} was found to be particularly suited for the conversion of non-polar nitrosobenzene substrates with considerable tolerance towards non-ordinary media, allowing the use of co-solvents such as DMSO or acetone up to 20% of the total reaction volume. [2]

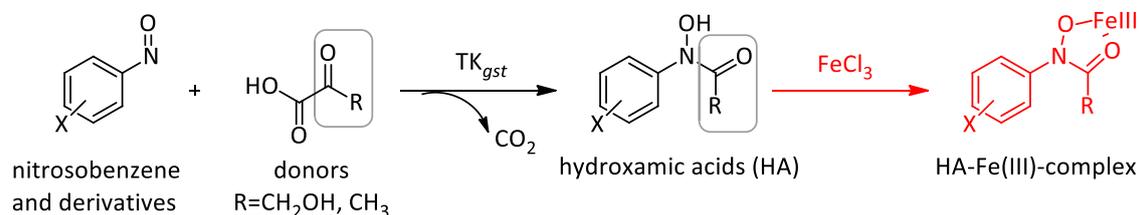


Figure 1. General TK_{gst} catalyzed carbonylation reaction leading to *N*-aryl hydroxamic acids and formation of colored HA-Fe(III)-complex by reaction with iron (III) chloride.

In the presence of iron (III) ions, HA give rise to a deeply red-colored HA-Fe(III)-complex. This allows not only for an immediate visible detection of HA products but also for sensitive spectrophotometric quantification in high-throughput format. Detailed results of the assay principle, its development and application to the screening of enzyme libraries for engineering will be presented.

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Biotransformation of 3-*n*-butylidenephthalide to 3-hydroxy-3-butylphthalide. The fungistatic activity against *Candida* strains

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Phthalides are bioactive secondary metabolites of plants especially belonging to the *Apiaceae* family [1]. They exhibit various pharmacological properties, for instance anti-inflammatory, neuroprotective, antioxidative, antihyperglycemic and fungistatic [2-4]. It has been estimated that nearly 80% of antifungal agents are not investigated due to adverse effects [5]. Therefore, there is a necessity to evaluate the metabolism of novel treatments. Small quantities of compounds formed during *in vivo* metabolism may pose a problem with their determination. Whole-cells biotransformations may facilitate the identification of metabolites [6].

The aim of the work was to examine the possibility of application of the whole-cell of microorganisms to obtain the 3-hydroxy-3-butylphthalide (2) – the exact mammalian metabolite of bioactive 3-*n*-butylidenephthalide (1). Additionally, we proposed the mechanism of the process, as well as conducted lipophilicity assays, connected with absorption, distribution, metabolism, and excretion of phthalides. We also examined the antifungal activity of compounds against selected *Candida* strains to check if there is any lipophilicity- or structure-activity relationship.

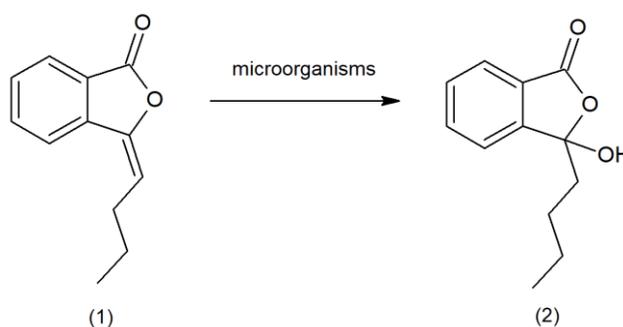


Figure 1. Biotransformations of 3-*n*-butylidenephthalide (1) to 3-hydroxy-3-butylphthalide (2).

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Synthesis of enantiopure 1-(het)aryl-2,2-difluoroethanamines by transaminase-catalysed reactions

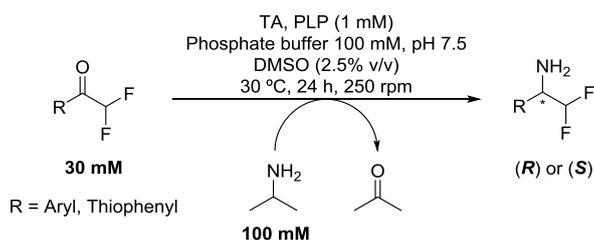
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In the last years, fluorine chemistry has grown into an area of multidisciplinary research because of the applications that organofluorine compounds offer. In fact, diverse synthetic strategies have been developed for the introduction or modification of fluorinated organic compounds,[1,2] but more specifically the pharmaceutical industry has driven a steady increase in the number of drug candidates containing fluorine atoms.[3] Thus, nowadays it is recognized as the second preferred heteroatom in biologically active organic compounds, and the reason is simple, as the presence of fluorine atoms in a target can modify the biophysical and bioactive properties of the molecule with the objective to improve its efficacy and its metabolic stability, among other properties.[4]

Herein, inspired by a recent work developed by Prof. Grogan in collaboration with our group, in which difluorinated amines were obtained employing reductive aminases,[5] and following the studies regarding the reactivity of fluorinated compounds with transaminases (TAs),[6] we present a biocatalytic alternative procedure where different commercial and overexpressed TAs are capable of performing the transamination reaction on a series of 2,2-difluorinated ketones (Scheme 1).



Scheme 1. Transamination reaction of 2,2-difluorinated ketones.

A library of enantioenriched 1-aryl-2,2-difluoroamines has been obtained with different substituents at the phenyl ring. The transamination reaction also proceeds with other heteroaromatic α,α -difluoro ketones, like 2,2-difluoro-1-(2-thiophenyl)-1-ethanone. Among the different TAs tested, the best results are obtained with TAs from *Arthrobacter* sp. and its mutant MutR11, both overexpressed in *E. coli*, giving rise to the (S)-enantiomers with *ee* >99% and excellent yields in the presence of a small excess of the amine donor (2-PrNH₂).

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Bioactive lipids from borage oil enriched in gamma-linolenic acid by immobilized lipases

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In the present work, borage oil (*Borago officinalis*) was used as the main source of gamma linolenic acid (GLA) to obtain ethyl esters by ethanolysis. The use of enzymes as an alternative to chemical oil modification as well as the use of solvent-free systems help to achieve the objectives set in Green Chemistry, with great interest at industrial level as this will be the global trend in the forthcoming years. Besides, enzyme immobilization has become more important over the last decades to allow reutilization of biocatalysts [1,2].

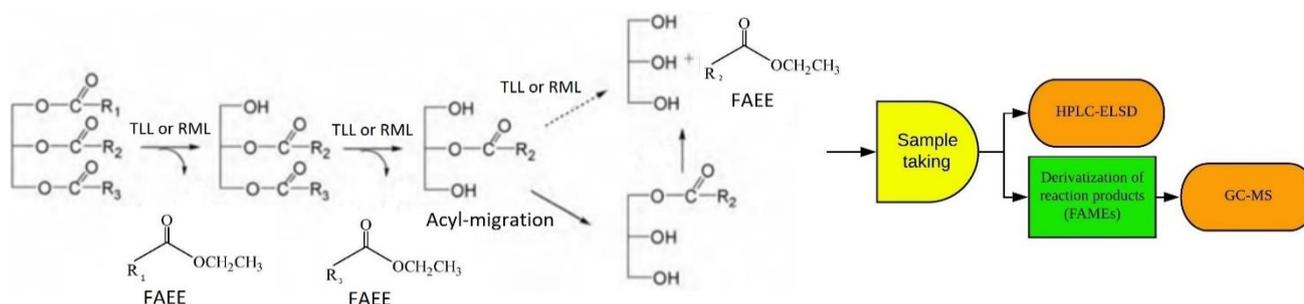


Figure 1. RML (or TLL)-catalysed TAG ethanolysis.

The chemical ethanolysis of borage oil in alkaline medium was compared to the enzymatic reaction using immobilized lipases. Two types of commercially immobilized lipases were studied, *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML). In addition, TLL was immobilized on hydrophobic porous support (Octadecyl-Sepabeads®). After a preliminary study, fatty acid ethyl ester (FAEE) yields of both reactions were compared under the same conditions (25°C and 200 rpm). The derivative which achieved the best yield was used to carry out reactions in a solvent-free system at different temperatures (25°C and 40°C). Additionally, ethyl esters were derivatized by methylation and its profile were analyzed by gas chromatography coupled to mass spectrometry (GC-MS).

Results shown that lipases adsorbed on hydrophobic resins in the laboratory were more active than immobilized commercial enzymes. The conversion yield for borage oil ethanolysis catalyzed by TLL immobilized on C18-Sepabeads® supports were similar to the chemical pathway (93.4% and 99.5%, respectively). Surprisingly, when this derivative was used in a solvent-free system (at 40°C and 200 rpm), it was possible to obtain a high FAEE yield of 84.26% in the first 24 hours of reaction. Moreover, it was possible to re-use the immobilized derivative for the performance of five reaction cycles maintaining 68% of its initial activity.

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Surface display of laccases on *E. coli* for lignin depolymerisation

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Lignin is one of the most abundant aromatic polymers on earth. The use of this raw material for the production of new value added products is of great interest. However, due to the strong bonds within the lignin polymer, degradation remains difficult [1]. One possible way to degrade the lignin is the usage of laccases. Laccases are copper-containing oxidoreductases capable of oxidizing various phenolic and non-phenolic compounds leading to bond-cleavage in lignin [2].

The aim of this study was to construct a laccase whole cell biocatalyst for an eco-friendly and economically feasible degradation of lignin. For this, two different types of laccases were evaluated: The monomeric laccase CotA from *Bacillus coagulans* and the trimeric small laccase (SLAC) from *Streptomyces coelicolor*. Both laccases were separately displayed as Maximized Auto Transporter mediated Expression (MATE) fusion proteins on the surface of *Escherichia coli* Δ *cueO* [3], which is lacking *E. coli*'s chromosomal encoded laccase CueO (Figure 1). Both constructed whole cell biocatalysts showed activity with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. By limiting the oxygen supply during gene expression the activity of the laccase whole-cell biocatalysts could be increased by a factor of 11 and 29 for cells displaying CotA-MATE and SLAC-MATE, respectively. This resulted in an activity of 2.9 U/mL⁻¹_{OD1} (CotA-MATE) and 0.7 U/mL⁻¹_{OD1} (SLAC-MATE).

To analyse whether the fusion proteins CotA-MATE and SLAC-MATE are able to convert lignin, the whole-cell biocatalysts were incubated with kraft lignin and ABTS as a mediator. Subsequently, the samples were analysed by LC-qToF-MS. The amount of each detected compound in the samples incubated with cells exhibiting the laccases was compared to the amount of that compound in the samples incubated with cells lacking the indicated laccase. It was shown that many different low molecular weight lignin compounds are formed when the kraft lignin is incubated with the CotA-MATE fusion proteins. Therefore, we can conclude, that the CotA-MATE containing whole cell biocatalysts can be used for the depolymerisation of lignin.

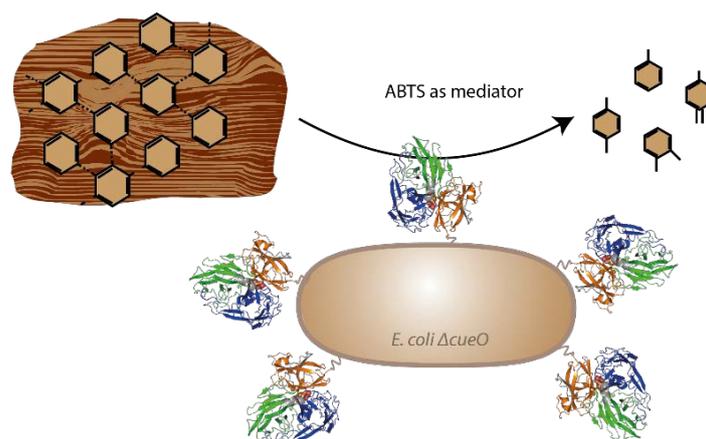


Figure 1. Schematically processes for lignin degradation by laccase whole cell biocatalysts. *E. coli* Δ *cueO* cells present the laccase CotA of *B. coagulans* on the surface, which can then degrade the heterogeneous polymer lignin by using ABTS as a mediator.

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Chemoenzymatic synthesis of valuable chiral intermediates: β -hydroxy- α -amido esters and β -hydroxy- α -cyanoalkyl esters

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Stereoselective reduction of α -amido and α -cyanoalkyl- β -keto esters leads to the formation of important chiral intermediates such as optically pure β -hydroxy- α -amido esters and β -hydroxy- α -cyanoalkyl esters, respectively. These compounds are important building blocks for the synthesis of a variety of natural and pharmaceutical products [1]. β -Hydroxy- α -cyanoalkyl esters are promising precursors for the synthesis of optically active α -substituted γ -butyrolactams and δ -butyrolactams.

Our continuing interest on the enzymatic reductions of α -substituted- β -keto esters using ketoreductases as the biocatalysts [2], led us to investigate the stereoselectivity of the enzymatic reduction of α -amido- β -keto esters as well as α -cyanoalkyl- β -keto esters [3].

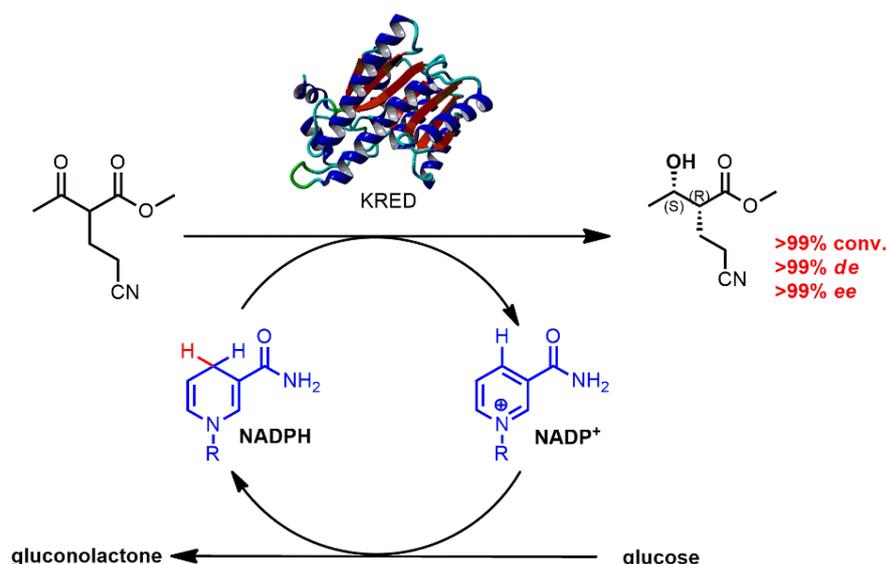


Figure 1. Stereoselective reduction of α -cyanomethyl β -keto ester catalyzed by NADPH-dependent ketoreductases.

In the present work we show that NADPH-dependent ketoreductases can catalyze these transformations with excellent activity and high stereoselectivity. In particular, *tert*-butyl 2-acetamido 3-hydroxy-4 methylpentanoate, a valuable chiral intermediate for the synthesis of natural product lactacystin [4], was obtained in high yield and excellent *anti*-diastereoselectivity, as well as the synthesis of β -hydroxy- α -cyanoalkyl esters was accomplished in high yield and optical purity leading to one stereoisomer out of four (> 99 % *de*, > 99 % *ee*, >99 % conversion).

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Acknowledgments

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Enzymatic rutinoylation of phenolic compounds by 6-O- α -rhamnosyl- β -glucosidase from *Sarocladium strictum* and evaluation of their antitumoral effects

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6-O- α -L-rhamnosyl- β -D-glucosidases are diglycosidases that catalyze the cleavage of the glycosidic bond between the aglycone and the disaccharide rutinose (α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranose) [1]. Retaining 6-O- α -L-rhamnosyl- β -D-glucosidases exhibit transglycosylation activities which provide an approach for obtaining novel rutinoylated molecules with enhanced capacities. These rutinoides could exhibit novel biological and biomedical activities [2]. Specifically, the presence of rhamnose as terminal glycosidic unit can change the pharmacokinetic properties of therapeutic agents [3]. Rhamnose-capped molecules appear to be resistant to hydrolysis in human tissues due to the absence of endogenous rutinoides and α -L-rhamnosidases. Thus, the rhamnose-capped molecules can be targeted where L-rhamnose receptors are located in order to enhance the pharmacological effects [3].

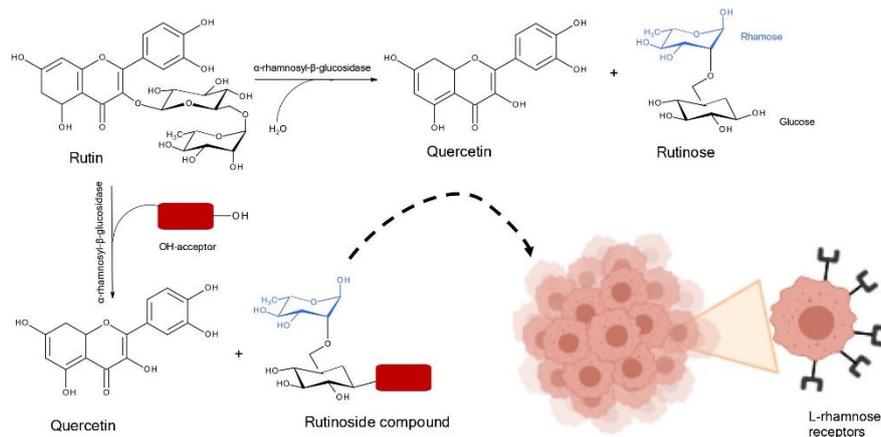


Figure 1. Biocatalytic pathways proposed for the synthesis of rutinoides compounds and their antitumor application.

We have recently described a new α -L-rhamnosyl- β -D-glucosidases from the fungus *Sarocladium strictum* DMci 093557. The enzyme was able to hydrolyze 7-O- β -rutinosyl- and 3-O- β -rutinosyl-flavonoids and it also exhibited transglycosylation activities, transferring rutinose from rutin onto a broad spectrum of alcoholic acceptors, primary, secondary and phenolic alcohols. Remarkably, the rutinoylation of short chain length alcohols (methanol, ethanol) was not detected, while butanol, Isoamyl alcohol, phenethyl alcohol and phenolic acceptors such as resveratrol, 4-methylumbelliferone (4MU), resorcinol and phloroglucinol, were rutinoylated with reasonable yields. The antitumoral effects of the phenolic synthesized rutinoides (rutinosyl-resveratrol, rutinosyl-4MU and rutinosyl-resorcinol) was assessed on cancer cell lines (Huh7, Hepa1.6 and Panc02).

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Biobased synthesis of metaraminol in an enzymatic cascade using microbially produced precursors from renewable feedstocks

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Chiral amino alcohols offer a great field of application as pharmaceutically active ingredients, precursors thereof or synthons for the fine chemical industry. Biocatalytic production of chiral amines can provide these valuable compounds from renewable resources under environmentally favourable process conditions. The amino alcohol metaraminol is an active pharmaceutical ingredient for hypotension treatment and can serve as precursor for more complex, bioactive compounds. The enzymatic production of metaraminol was shown previously in an enzymatic cascade starting from 3-OH-benzaldehyde using isopropylamine (IPA) as amine donor in the reductive amination step [1]. The drawback of this approach is the use of IPA in excess, consequently releasing large amounts of acetone. Pyruvate is introduced as another high-purity agent derived from fossil resources. Therefore, a more sustainable alternative is by using biobased pyruvate and alanine as shown in figure 1.

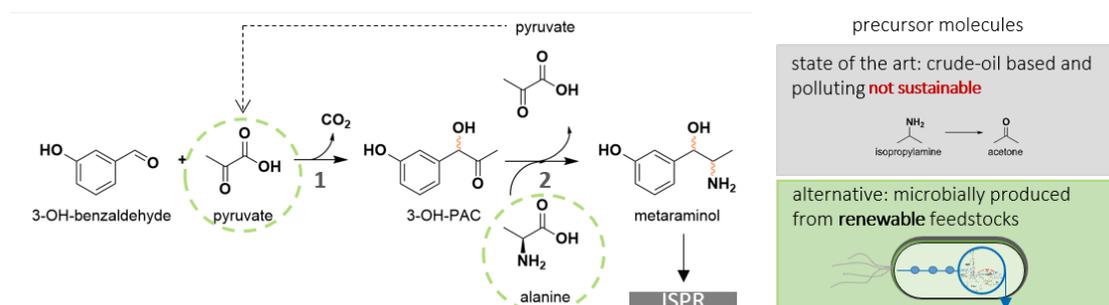


Figure 1. Two-step enzymatic cascade towards metaraminol consisting of a carboligation (1) and reductive amination (2) step. 3-OH-benzaldehyde and pyruvate are converted by a variant of the pyruvate decarboxylase variant ApPDC from *Acetobacter pasteurianus*. The intermediate (R)-3-OH-PAC is converted by the amine transaminase Cv2025 (from *Chromobacterium violaceum*) using alanine as the amine donor. The aliphatic precursors pyruvate and alanine can be produced microbially from second generation feedstocks. Pyruvate can be recycled within the cascade. *In-situ* product removal (ISPR) is used to shift the reaction equilibrium.

By iterative strain and bioprocess engineering the microbial production of pyruvate and alanine in a one-pot fermentation process was achieved. The final concentrations were sufficient for direct carboligation of 3-OH-benzaldehyde and biobased pyruvate to (R)-3-OH-phenylacetylcarbinol (-PAC) in the fermentation supernatant with good conversion and stereoselectivity. Furthermore, putative inhibitory effects from identified side products could be ruled out experimentally for both enzymes. The intermediate (R)-3-OH-PAC can be subsequently converted to metaraminol by an amine transaminase using biobased alanine as the amine donor. Due to an unfavourable reaction equilibrium, the spatial separation of the cascade steps was necessary. Extraction-based *in-situ* product removal was investigated as a method to increase product yields of the transamination reaction. The contribution elucidates the compatibility of biocatalytic conversions in complex reaction matrices and highlights implications for the integration of novel biobased hybrid processes.

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Broadening the scope of glycerol dehydrogenase: oxidation of amino substituted propanediols

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Nitrogen heterocycles are structures found in bioactive compounds and pharmaceuticals, and its structure, position of amino groups and stereochemistry, leads to different biological activities. In addition, these compounds are highly demanded building blocks in chemical and drug synthesis. Amino-substituted acetones are useful precursors for the synthesis of N-heterocycles, such as pyrroles and pyrazines [1]. Chemical synthesis approaches to access these valuable compounds, lacks in selectivity, yielding low conversion degrees and undesired by-products [2]. Enzymes are highly selective catalytic tools enabling the environmentally friendly production of this class of compounds, like aminated 3-hydroxyacetone derivatives.

In this work, we described the enzymatic oxidation of 3-aminopropane-1,2-diol (N00), 3-(methylamino) propane-1,2-diol (NM00) and 3-(dimethylamino) propane-1,2-diol (N2M00), to their respective aminated 3-hydroxy acetone derivatives, to further use them as substrates for N-heterocycles synthesis. To that aim, we engineer a glycerol dehydrogenase of *Bacillus stearothermophilus* (BsGlyDH) [3].

Firstly, we create 6 mutants through rational design (M1-M6) according to its role in the substrate binding [3]. Results of the oxidation of the substrates are shown in the fig. 1, where glycerol, the natural substrate was added as reference.

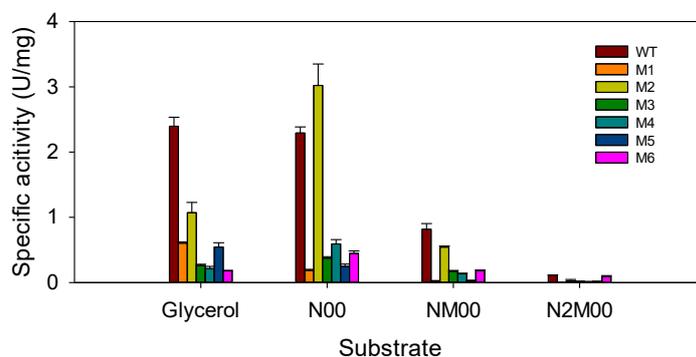


Figure 1. Oxidation profile of 1 amino propanediol derivatives by BsGlyDH variants. Reaction conditions: 100mM substrate, 1 mM of NAD⁺, in sodium phosphate buffer 25mM pH 7.0, at 30°C.

Native enzyme shown to be active with all substrates, in particular show to be statistically equally active over N00 and glycerol, suggesting no interference of the amino group on the active site. Mutant M2 shown to be the most active biocatalysts against substrate N00, 32% most active compared with the wild type enzyme, and 2.8-fold more active in comparison to glycerol. More sterically demanding substrates were not efficiently oxidized by the wt BsGlyDH or any of its variants. In fact, almost all BsGlyDH variants expressed lower activity than the wild type enzyme towards 3NM00 and 3N2M00, with the exception of the double mutant M6, which does not shown statistically difference against the oxidation of substrate N2M00.

Next investigations will focus on the saturated mutagenesis in this position to improve the oxidation rate towards more sterically demanding NM00 and N2M00.

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Production, Upscale and Application of Recombinant Whole-cell Styrene Monooxygenase

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Styrene monooxygenases (SMOs) are highly enantioselective flavoprotein monooxygenases that catalyse the epoxidation of alkenes to chiral epoxides. SMOs consist of two subunits—FAD-dependent monooxygenase (StyA) and NADH-dependent flavin-reductase (StyB)—encoded by *styA* and *styB* of *styABDE* gene cluster responsible for the degradation of styrene in native bacterial species. Initially, when the question of biocatalytic production of chiral epoxides arose, wild-type SMOs from *Pseudomonas* and *Rhodococcus* were widely studied and used. Since then, many other bacterial species expressing styrene degradation pathways have been described. Such discoveries, together with the expansion of recombinant technologies, metagenomics, and protein engineering, have led to the generation of novel SMOs with improved properties [1-3].

Chiral compounds containing oxirane ring or products of their hydrolysis comprise a group of important building blocks and precursors in organic synthesis in the pharmaceutical industry. However, biocatalysis still suffers from the low availability of enzymes in large quantities and is often replaced by chemical catalysis on an industrial scale. Chiral epoxides production usually involves Sharpless and Jacobsen oxidation that demand extreme reaction conditions and suffer from poor enantioselectivity [4, 5]. SMOs, on the other hand, operate under mild conditions, are highly enantioselective, and exhibit an affinity towards a broad substrate spectrum. Thus, the establishment of cost-effective upscaled SMO production and further improvement of their catalytic properties could remarkably reduce the costs of chiral epoxides production and eliminate the environmental burden brought on by their chemical synthesis.

The genes of StyA and StyB originating from *Marinobacterium litorale* encoding SMO were selected by genome mining and designed for fusion via the linker peptide L2 recently published in [2]. SMO was then expressed as a fused protein (StyAL2StyB) in *Escherichia coli* BL21(DE3) under the control of IPTG inducible *lacT7* promoter. Firstly, high cell density fermentation was optimised on the 1.5 L scale reaching 35 g_{DCW}/L. SMO was further purified characterised, and tested for epoxidation of 34 structurally different alkenes. It was found, that with increasing purity of enzyme, the catalytic activity decreases dramatically. Whole-cell SMO was therefore proven to be the most suitable biocatalyst and chosen for upscale epoxidation of 5 selected alkenes. Eventually, (*S*)-4-chlorostyrene oxide, (*S*)-allylbenzene oxide, (2*R*,5*R*)-1,2:5,6-diepoxyhexane, 2-(3-bromopropyl)oxirane, and (*S*)-4-(oxiran-2-yl)butan-1-ol were produced in excellent enantiopurity (ee > 99%, 95%, 97%, 99%, and 99%, respectively) [1].

Acknowledgment

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Designing multienzymatic cascades for the biosynthesis of acyl-CoA derivatives from low-cost precursors.

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Acyl-CoA derivatives are fundamental molecules that could be found in the metabolism of different biomolecules, such as lipids, proteins or carbohydrates. In which they play a special central role as a major donor source of useful metabolic energy, either catabolic or anabolic routes [1,2]. Nonetheless, the examples of synthetic routes through these molecules in fine chemistry are scarce. Herein, we report two different routes to synthesize acyl-CoA derivatives from different low-cost aldehydes or carboxylic acids, such as propionaldehyde or acetic acid.

For this synthesis, we used two rarely exploited enzymes: an acetyl-CoA synthetase (ACS) and a CoA-acylating aldehyde dehydrogenase (PduP), both enzymes were characterized and incorporated on two different multienzymatic cascades. The first one, the PduP, catalyzes the production of acyl-CoA derivatives from aldehydes consuming NAD^+ and CoASH. The second one, the ACS, catalyzes the biotransformation of carboxylic acids, in presence of ATP and CoASH, into acyl-CoA derivatives. Both cascades also include an *in situ* cofactor regeneration needed for both enzymes (NAD^+ and ATP, respectively). We also studied the substrate scope of those cascades; several chain length and ramifications of the substrates were tested, demonstrating the high applicability and complementarity of this novel enzymatic systems. These novel multienzymatic cascades allows the development of new strategies in fine chemistry, thanks to the *in situ* production of these low stable but high-cost molecules.

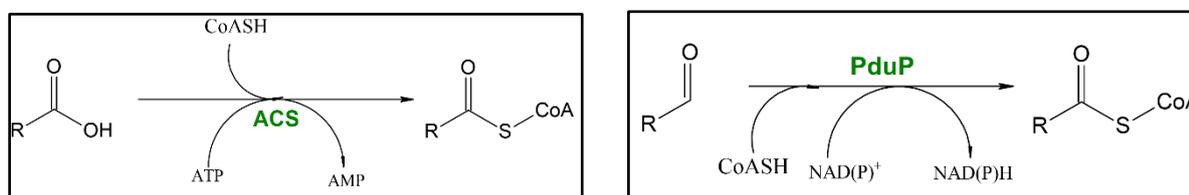


Figure 1. Enzymatic routes scheme for the synthesis of Acyl-CoA derivatives.

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The Potential of Selective Fucosyltransferases

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Fucosyltransferases are crucial for the synthesis of fucosylated epitopes. In eukaryotes, FucTs produce human milk oligosaccharides, Lewis epitopes, or blood group antigens that are crucial for immunity or recognition processes. In bacteria, FucTs produce these antigens in the form of lipopolysaccharides to undergo the host immune response. Furthermore, pathogenic biomolecules (toxins, viruses, etc.) bind to fucose-presenting cell surfaces. The large-scale production of fucosylated structures provides a variety of epitopes suitable for investigation of the above-mentioned processes.

For the synthesis of fucosylated glycans, suitable recombinant enzymes are necessary. Bacterial enzymes present the most promising group since the production in *Escherichia coli* is feasible. *Helicobacter pylori* strains contain a variety of FucTs, comprising α 2-, α 3-, and α 3/4FucTs [1]. We produced three *H. pylori* FucTs from literature [2-4] and tested their synthetic capability with a variety of novel substrates derived from poly-LacNAc. The substrate library comprised various lactosamine derivatives and their H-antigen analogues (Figure 1). The enzymes showed an interesting and yet unprecedented behaviour with more complex structures such as *N*-acetyllactosamine tetrasaccharides. Furthermore, terminally attached fucose had impact on the site of further fucosylation.

Investigation of other bacterial FucTs with these new substrates might pave the way to a library of enzymes enabling a regioselective fucosylation of complex glycans without employing more complicated approaches (e.g. protection of reactive groups).

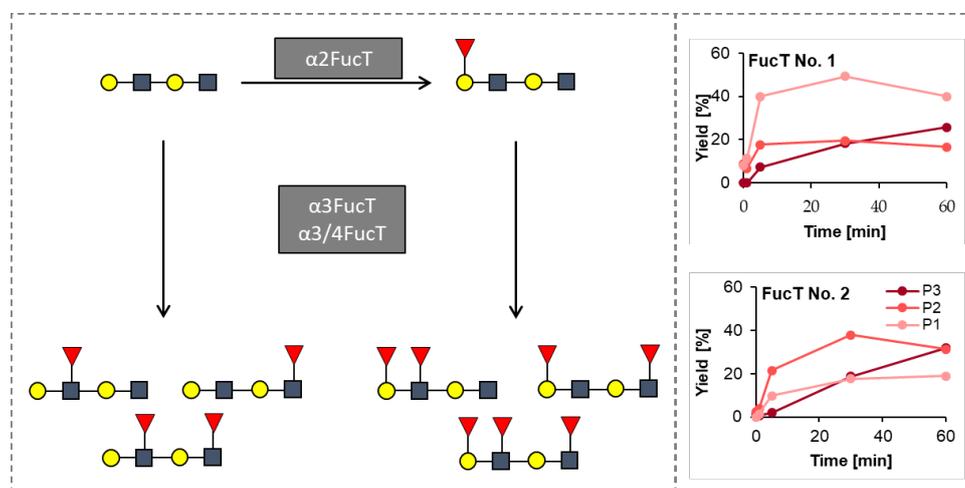


Figure 1. Left: Sample FucT reactions with an *N*-acetyllactosamine tetrasaccharide and the corresponding H-antigen analogue. Right: Products (P1-P3) resulting from exemplary FucT reactions catalyzed by two different enzymes.

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Support from the Czech Science Foundation (grant project no. 20-00215S) and the Collaborative Research Center (CRC) grant SFB 985 project C3 from DFG (Deutsche Forschungsgemeinschaft) is gratefully acknowledged.

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Bacterial Whole Cells Synthesis of Whisky Lactones in a Solid-State Fermentation Bioreactor Prototype

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Lactones are an industrially important aroma compounds, which are often used as a fragrance ingredients in various foods such as sweets, drinks, baked goods. One of them is whisky lactone, which is an essential component of aged alcoholic beverages such as whisky, cognac, and brandy beverages [1]. *Cis*-whisky lactone isomers are described as earthy and woody fragrances, while *trans*-isomers are reminiscent of celery [2]. Obtaining whisky lactone with the use of inexpensive medium such as by-products of the agro-food industry is an additional advantage of sustainable management of agriculture residues.

Agro-industrial side streams such as linseed, rapeseed and primrose cakes were used as a medium in solid-state fermentation (SSF) for microbial oxidation of *anti*- and *syn*-3-methyl-octane-1,4-diols to obtain corresponding *trans*- and *cis*-whisky lactones [3]. In preliminary screening transformations, a wide range of whole bacterial cells were tested on the basis of oxidation activity. Among the different oil cakes tested, biotransformations carried out on linseed cake were characterized by the highest conversion and stereoselectivity. Bacteria of the *Rhodococcus*, *Gordonia*, *Dietzia*, and *Streptomyces* genera carried out transformations with complete conversion after 3 days. Several preparative-scale oxidations performed in a self-constructed SSF bioreactor catalyzed by *Rhodococcus erythropolis* DSM44534, *Rhodococcus erythropolis* PCM2150, and *Gordonia rubripertincta* PCM2144 afforded optically active *trans*-(+)-(4*S*,5*R*), *cis*-(+)-(4*R*,5*R*) and *cis*-(-)-(4*S*,5*S*) isomers of whisky lactones, respectively (**Figure 1**). Various extraction methods were applied for the isolation of the products, and among them, the combination of steam distillation with simple extraction were the most efficient. Preparative biotransformations were conducted under precise control of conditions in a bioreactor based on a Raspberry Pi Zero W. The proposed low-cost (ca. USD 100) bioreactor is a standalone system that is fully autoclavable and easy to use.

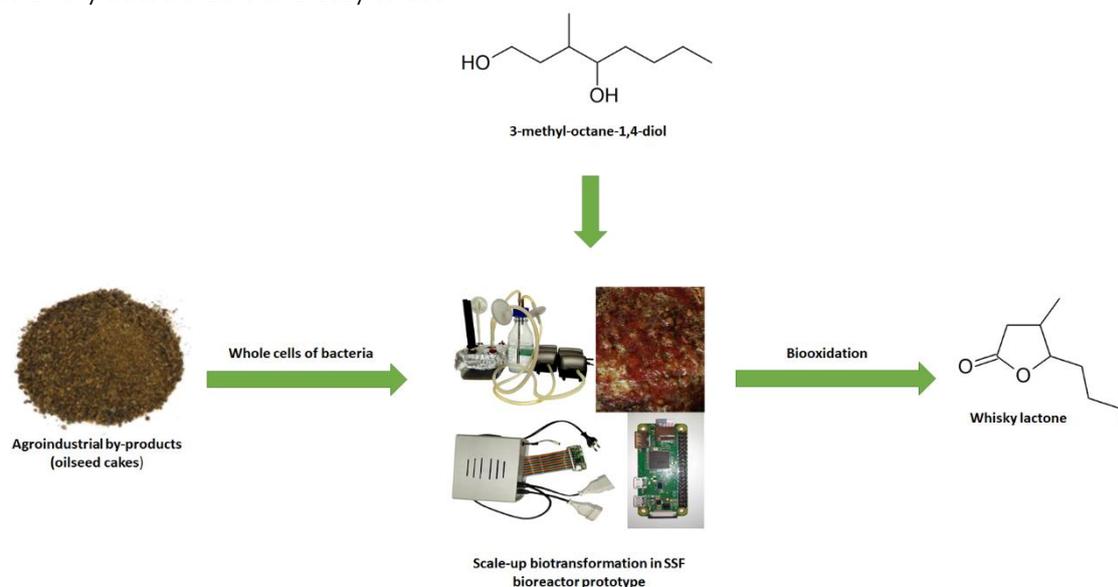


Figure 1. Preparative scale biotransformations in prototype SSF bioreactor.

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Toward application of unspecific peroxygenases in the presence of high concentrations of water-miscible organic co-solvents.

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Organic co-solvents are used in biocatalysis to increase concentrations of typically hydrophobic substrates in aqueous reaction systems. In this regard, the application of water-miscible solvents such as acetonitrile or methanol amongst others is a straight-forward approach as this allows to operate in a simple one-phase system.[1] However, effects on enzyme stability, activity and selectivity have to be carefully evaluated also with regard to the type and concentration of the respective co-solvent [2,3].

Herein, we report the systematic investigation of water-miscible organic co-solvents on the activity and selectivity of the recombinant unspecific peroxygenase from *Agrocybe aegerita* (rAaeUPO). Unspecific peroxygenases (UPO, EC 1.11.2.1) are fungal heme-thiolate enzymes that are particularly attractive for application as they rely on simple H₂O₂ as co-substrate and can achieve high activities in range of total turnover numbers (TTN) of $\geq 10^5$. [4]

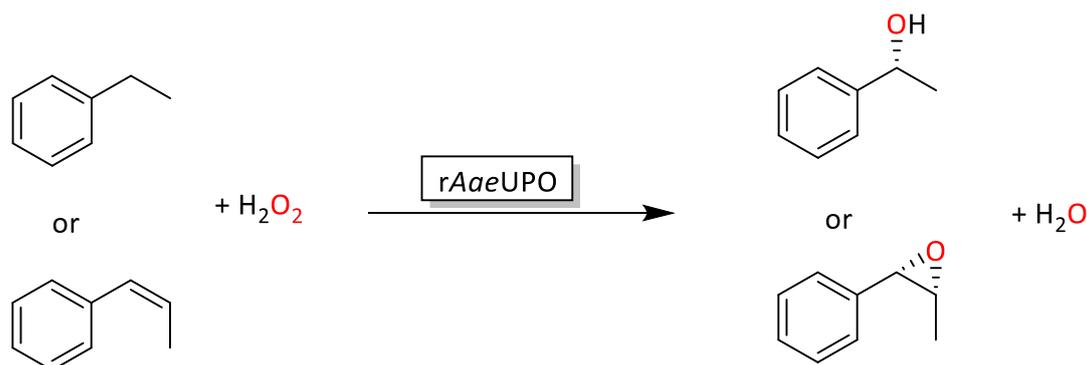


Figure 1. AaeUPO-mediated oxidations used as model reactions studying the effect of organic co-solvents.

Testing a number of common water-miscible co-solvents in oxidation reactions of various UPO model substrates (Figure 1) followed a trend that revealed not only the most suitable solvent but also evidence of activity against certain co-solvents. rAaeUPO showed promising activities in presence of high concentrations of ≥ 50 vol% co-solvent, which enabled to use high substrate concentrations on a larger scale. Taking together all findings, we highlight the potential of UPOs for large-scale applications in presence of high concentrations of organic co-solvents by envisioning a general hand-on guide for usage of peroxygenases.

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Parameters influencing Lipase-Catalyzed Glycolipid Synthesis in Deep Eutectic Solvents

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Glycolipids are surfactants with a wide field of applications. In contrast to petrochemical derived surfactants, they are less harmful to the environment and they can be produced by enzymatic synthesis from carbohydrates and fatty acids in organic solvents under nearly water free conditions. Growing environmental awareness leads not only to a quest for environmentally friendly products, but also to a more sustainable and green chemistry. Here, deep eutectic solvents (DES) represent a new alternative solvent system, being non-flammable, non-volatile, non-toxic and biodegradable. Enzymatic synthesis of glycolipids has already been successfully performed in hydrophilic deep eutectic solvents, e.g. consisting of choline chloride in combination with carbohydrates, which can serve as substrates at the same time. However, mechanisms and limiting factors for glycolipid synthesis in DES are not well understood at the moment. Therefore, an external mass transfer limitation test was applied to two hydrophilic DES and the influence of fatty acid concentration and fatty acid distribution were investigated. As polarity likely has an influence on transesterification reactions in organic solvents and ionic liquids, we also evaluated a hydrophobic DES as new medium for enzymatic glycolipid synthesis. Besides, the influence of enzyme concentration, sugar concentration and water content on glycolipid synthesis were analysed in the newly introduced hydrophobic DES.

It was shown that no external mass transfer limitation is existing in such a system, but that the fatty acid accessibility is a limiting factor in glycolipid synthesis in DES [1]. In hydrophobic DES, reaction velocity and product yields are significantly enhanced compared to hydrophilic DES [2]. As a further advantage, the enzyme can be easily removed from the hydrophobic DES and used for further syntheses. Polarity of the applied deep eutectic solvents were shown to influence reaction velocity and yields even though lipases are active in both hydrophilic and hydrophobic deep eutectic solvents.

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WHOLE CELL PRODUCTION OF FATTY ALDEHYDES BY *MYCOBACTERIUM MARINUM* CARBOXYLATE REDUCTASE

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Fragrance and flavour industries could not imagine business without aldehydes.[1] Up-to-date, processes used for commercial production raise environmental and ecological concerns.[2] To fulfil the demand of a mild and selective reduction of carboxylic acids as well as the utilization of renewable feedstocks to aldehydes, carboxylic acid reductases (CARs) are gaining importance.[3] CAR enzymes (E.C.1.2.1.30, CAR) exhibit a broad substrate scope for the conversion of organic acids selectively to the respective aldehydes, a truly valuable addition to the toolbox of biocatalysis and chemical biology.[4] It was demonstrated that especially bacterial CARs show high acceptance for medium- to long-chain fatty substrates.[5]

Within this study, CAR enzymes were used to determine their substrate scopes for the reduction of saturated, unsaturated and branched short- to long-chain fatty acids. Due to the preference of *Mm*CAR for medium- to long-chain fatty acid, octanoic acid was chosen as model substrate for *in vivo* production of octanal due to rather low yields in *de novo* studies.[6] *In vivo* limitations such as product toxicity and cofactor supply, were optimized by using ISPR⁵ and an *in vitro* regeneration system[7] for NADPH and ATP. After 41 h, 89.1 mM of octanoic acid were converted, which correlates to a productivity of $\sim 11.42 \text{ g L}^{-1}$. [8] The results of this study demonstrate the scope of potential to develop bioprocesses for substituting the petroleum-based products in industry.

Acknowledgements

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An RBS library in cyanobacteria to boost biocatalytic activity

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Cyanobacteria are promising and, quite literally, the “greenest” candidates for microbial biocatalysts. These bacteria offer a truly sustainable production system by using water, carbon dioxide and sunlight as their source of carbon and energy. With our interest in redox-enzymes as catalysts, their photoautotrophic metabolism equips them with unique advantages [1]. During photosynthesis (i) they produce high amounts of high-energy cofactor NADPH, which can be used directly to drive enzymatic reactions [2]. Secondly, (ii) oxygen is produced as by-product, which can feed oxygen-driven reactions [3].

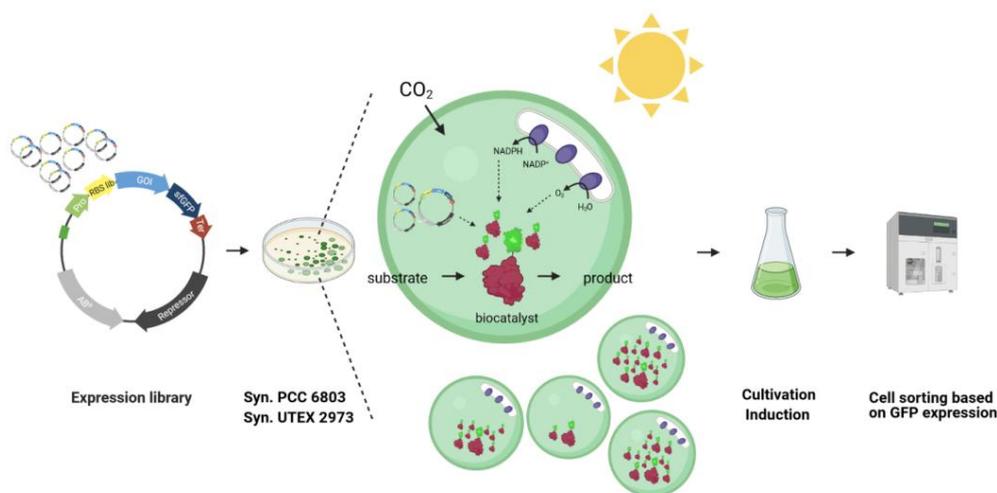


Figure 1. Synthetic tool-box allows a fast and simple screening for diverse expression levels of biocatalysts in cyanobacteria. (Created with BioRender.com)

Despite these great advantages for biocatalysis, progress within this research field is limited by the remaining challenges. On the one hand, one big challenge is still to obtain sufficient expression levels of recombinantly expressed genes within the cyanobacterial host. On the other hand, high throughput screenings are limited by the special cyanobacterial cultivation conditions.

Based on these major limitations, we developed a strategy, which aims to tackle both bottlenecks. First, (i) we built an RBS library together with a cloning approach, compatible with the CyanoGate cloning kit [4], that allows a fast and simple assembly of a plasmid-based expression library of a specific gene of interest (GOI). To enable high throughput screening (ii), we fuse GFP C-terminally to our GOIs. This enables high throughput screening for diverse expression levels. Applying this strategy, we aim to realize and improve biotransformations in the cyanobacterial strains *Synechocystis sp.* PCC 6803 and *Synechococcus elongatus* UTEX 2973. As enzymes, we investigate an alcohol dehydrogenase (RR-ADH) [5], a ketoreductase (LfSDR1M50) [6], an enoate reductase (YqjM) [7], and a Baeyer-Villiger monooxygenase (CHMO_{Acineto}) [5]. By comparing the biocatalytic activity of different expression strains, we aim to find high performing cyanobacterial whole-cell biocatalysts.

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Accelerated production and characterization of catalytically active inclusion bodies via automated workflows

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As a novel enzyme immobilization approach, overexpression of enzymes in *Escherichia coli* in combination with a linker and an aggregation tag generates catalytically active inclusion bodies (CatIBs). CatIBs are known for their high stability as well as their easy and low cost production. [1]

However, since their discovery a few years ago, only a few CatIB variants were described in literature. Although a substantial number of specific aggregation tags and linker sequences are known, there is not enough structural understanding to predict a suitable combination of aggregation tag and linkers to successfully design a CatIB for a selected target protein. One reason is the required time and resources to perform manual cloning as well as the characterization workflows of different target genes in combination with a variety of linker and aggregation tags. [1]

The aim of the project is to automate the molecular biology construction workflows as well as the production and characterization processes to accelerate the generation of successful CatIB variants. As prototypic examples, the lysine decarboxylase (*EcLDCc*) from *E. coli*, the alcohol dehydrogenase (*LbADH*) of *Lactobacillus brevis* and the glucose dehydrogenase (*BsGDH*) of *Bacillus subtilis* are used as models for CatIB formation.

Therefore, an automated workflow was established to generate the CatIB variants via Golden Gate cloning. The variants were cultivated in a BioLector and an automated at-line microscopic analysis was performed to determine the amount as well as morphology of the inclusion bodies inside the cells. After cell disruption, the CatIBs were purified and the enzymatic activities of the variants were tested via activity measurement.

The developed automated workflows enabled a fast generation, production, purification and analysis of successful CatIB variants. Microscope pictures of the variants showed different morphologies of the inclusion bodies dependent on linker as well as aggregation tag structures. Moreover, the activity analysis also revealed different residual CatIB activities of the variants. The experimental data give rise to the conclusion that linker/aggregation tag combinations clearly influence the ability to successfully generate performant CatIBs.

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Bioelectrosynthesis of aldonic acids using cellobiose dehydrogenase as a platform

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Aldonic acids are the byproducts of lactose, cellobiose, or glucose oxidation by cellobiose dehydrogenase (CDH). Aldonic acids are useful as chelating and antimicrobial compounds and gain attention in pharmaceutical, cosmetic, and detergent applications.[1] CDH is a flavocytochrome secreted by fungi that has two domains, a catalytic FAD-dehydrogenase domain (DH) connected to a direct electron transferring *b*-type cytochrome domain (CYT) by a flexible linker peptide. The substrate oxidation is performed by the DH domain, which leads to the two-electron reduction of the FAD. Subsequently, one electron will then be transferred to the CYT domain that acts as an electron acceptor. The direct electron transfer properties of CDH can be used to produce various aldonic acids in a bioelectrocatalytic process.[2] In this research, we investigated whether the isolated CYT domain could be employed to improve the direct electron transfer in the bioelectrosynthesis of aldonic acids. The full-length CDH and its isolated CYT were recombinantly produced in *P. pastoris* and purified. 50 μ M CDH was electrochemically recycled when immobilize on a thioglycerol-modified gold electrode. This bioelectrosynthetic process was used to convert 100 mM cellobiose into cellobionic acid. We introduced different concentrations of the CYT domain (0, 50, 100, 200, and 500 μ M) to support bioelectrosynthesis and to study its impact on the direct electron transfer in the protein layer. Results show the increase of catalytic currents with the additionally added CYT domain. Concentrations of the formed product were calculated. Our investigation shows an alternative approach for the production of aldonic acids.

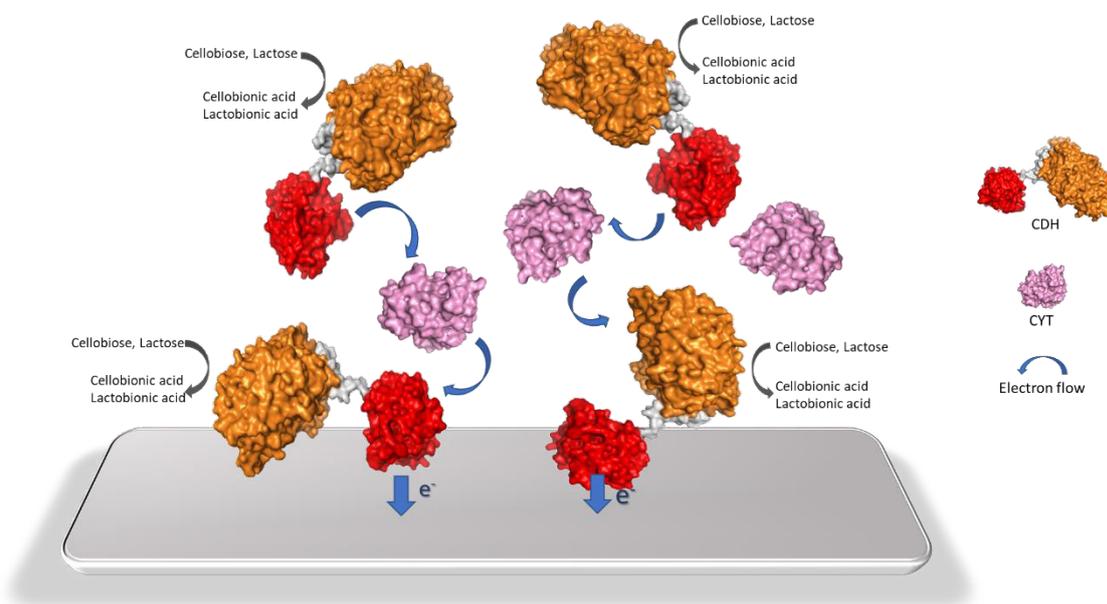


Figure 1. Schematic representation of CDH and CYT multilayers for the bioelectrosynthesis of aldonic acids. The CYT domain facilitates the electron transfers from CDH to the electrode.

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Microbial deracemization of *rac*-benzoin catalyzed by Blossom Protect™ agent

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The development of innovative and economically viable methodologies for the synthesis of chiral active pharmaceutical substances is one of the most important challenges of modern science, as most of the approved drugs have a stereogenic structure. Enantiomers are distinguished by biological systems, may have different pharmacokinetic properties (absorption, distribution, biotransformation and excretion) and pharmacologic effects which can directly influence the effectiveness of the therapy.

The use of biocatalytic systems of natural bioreagent cells in the enzymatic desymmetrization of prochiral/*meso* compounds and in the kinetic resolution of racemates seems to be an effective method of synthesizing chiral biologically active compounds, for example alcohols with a defined chirality.

The bioreagent cells, in comparison to the isolated enzymes, contain a whole set of enzymes capable of mutual converting the hydroxyl and carbonyl groups of many organic compounds. The stereoselectivity and, thus, enantiomeric purity of the products obtained depend on the substrate structure and chirality of the oxidoreductases, which the microorganism is equipped with. Modifications of the reagent structure and the change of the bioprocess conditions can give the opportunity to receive products with a specific configuration. [1,2]

Herein, we report the stereoselective deracemization of *rac*-1,2-diphenyl-1-hydroxyethanone (**1**) in the presence of Blossom Protect™ to (*S*)-**1** with good-to-excellent conversion and enantioselectivity (up to 99% ee). Blossom Protect™ is an antifungal agent used in fruit orchards to combat white mold, containing two strains of *Aureobasidium pullulans* DSM 14940 and DSM 14941.

In this study, the influence of reaction parameters on the selectivity of microbial deracemization of *rac*-**1** with the use of natural metabolic pathways of *A. pullulans* was investigated.

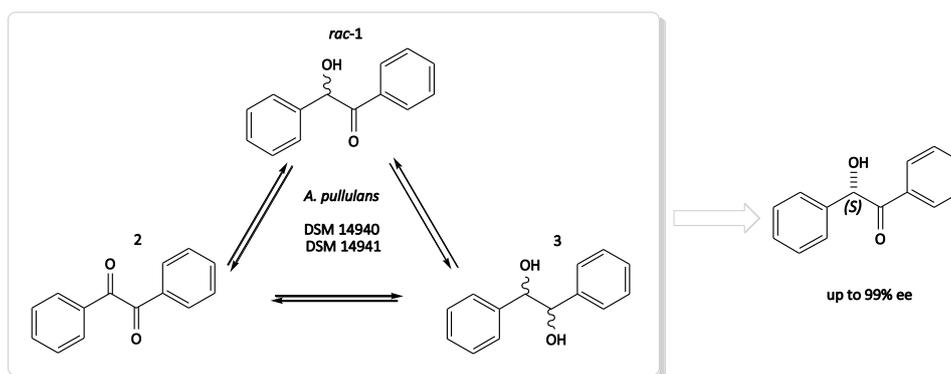


Figure 1. Microbial deracemization of *rac*-benzoin.

Microbial biocatalysis results show that it is possible to obtain chiral building blocks of defined configuration under mild conditions, without the need of using often toxic and expensive chemical catalysts. The biocatalytic protocol is simple, economical, and does not require the cultivation of the bioreagent.

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Synthesis and kinetic resolution of propargylic epoxides

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Halohydrin dehalogenases (HHDHs) are versatile biocatalysts that facilitate the conversion between halohydrins and epoxides in both directions. Their ability to catalyze enantioselective epoxide ring-opening reactions with different nucleophiles (azide, cyanide, cyanate, thiocyanate etc.) can be used in synthesis of optically active epoxides, β -substituted alcohols and heterocyclic compounds. Further transformation (hydrolysis, reduction, intermolecular click reactions) gives rise to valuable building blocks (e. g. amino alcohols, aziridines, triazoles, oxazolidinones) in synthesis of pharmaceutical and natural compounds.[1]

Propargylic epoxides and alcohols, owing to the presence of a triple bond, undergo various intermolecular and intramolecular reactions.[2,3] Unfortunately, there are few described methods for enantioselective synthesis of these compounds, and those available require expensive or custom-made catalysts. Using propargylic epoxides as substrates of HHDHs, enantiomerically pure starting compounds for triple bond and/or nucleophile transformations, such as intramolecular click reactions, can be obtained. Therefore, the synthesis of substituted propargylic epoxides and subsequent enantioselective ring opening by halohydrin dehalogenases (HheA-N178A and HHeC) was described (Figure 1).

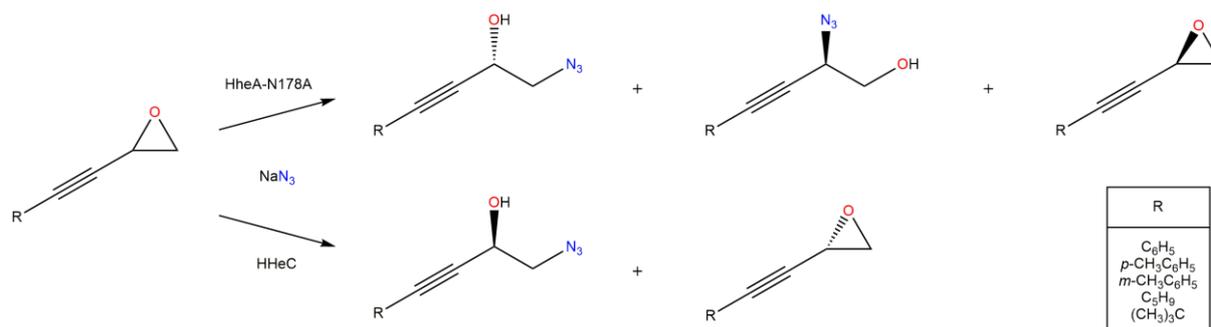


Figure 1. Kinetic resolution of synthesized propargylic epoxides with sodium azide catalysed by halohydrin dehalogenases HheA-N178A and HHeC

Phenyl-, *tert*-butyl- and cyclopentyl- substituted epoxides were synthesized from the corresponding terminal acetylenes by introduction and epoxidation of a double bond. Also, *p*- and *m*-tolyl derivatives were synthesized in a similar reaction sequence, starting from the corresponding iodotoluene and trimethylsilylacetylene.

Kinetic resolution reactions in the presence of sodium azide were catalyzed by two HHDHs with opposite stereopreference. With both enzymes reactions yielded enantiomerically pure secondary azido alcohols (e.e. > 99%, E > 200). While HheA-N178A gave mostly (*S*)- β -azido alcohol (β : α ratio between 90:10 and 54:46), HHeC yielded almost exclusively (*R*)- β -azido alcohol (up to to 99:1).

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Aryl-alcohol oxidases: Promising biocatalysts for the production of flavours, fragrances and precursors for biopolymers

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Aryl-alcohol oxidases (AAOs) are FAD-dependent oxidoreductases oxidizing primary benzylic and aliphatic (poly)unsaturated alcohols to the corresponding aldehydes while reducing molecular oxygen to hydrogen peroxide. These enzymes are predominantly secreted as glycoproteins by wood-decaying fungi and are promising biocatalysts for biotechnological applications due to their broad substrate spectrum. AAOs have been applied for the synthesis of valuable building blocks, flavours and fragrances and in dye decolorization and biobleaching.[1] By exploiting AAOs as supplier of hydrogen peroxide for peroxide-dependent peroxidases or peroxygenases their scope of application can be further expanded. However, their often quite low expression in recombinant hosts is a crucial challenge for their application at industrial scale. We have expressed different fungal AAOs at high levels in the methylotrophic yeast *Pichia pastoris*. For example, *PeAAO2* from *Pleurotus eryngii* was produced in *P. pastoris* with a yield of 315 mg/l in a fed-batch fermentation process which is one of the highest yields reported so far.[2] The enzyme oxidized typical AAO substrates like *p*-anisyl alcohol, veratryl alcohol and *trans,trans*-2,4-hexadien-1-ol and several compounds that have not been described as AAO substrates so far like cumic alcohol and piperonyl alcohol (Fig. 1). Piperonal, the oxidation product of piperonyl alcohol, is used in the flavour and fragrance industry and is an important precursor for the synthesis of pharmaceuticals and agrochemicals. An AAO from *Moesziomyces antarcticus* was shown to oxidize 5-hydroxymethylfurfural (HMF) and its oxidized derivatives (Fig. 1) and is thus quite interesting for the synthesis of the renewable building block 2,5-furandicarboxylic acid (FDCA), which can be used as precursor for the production of bio-based polyesters.

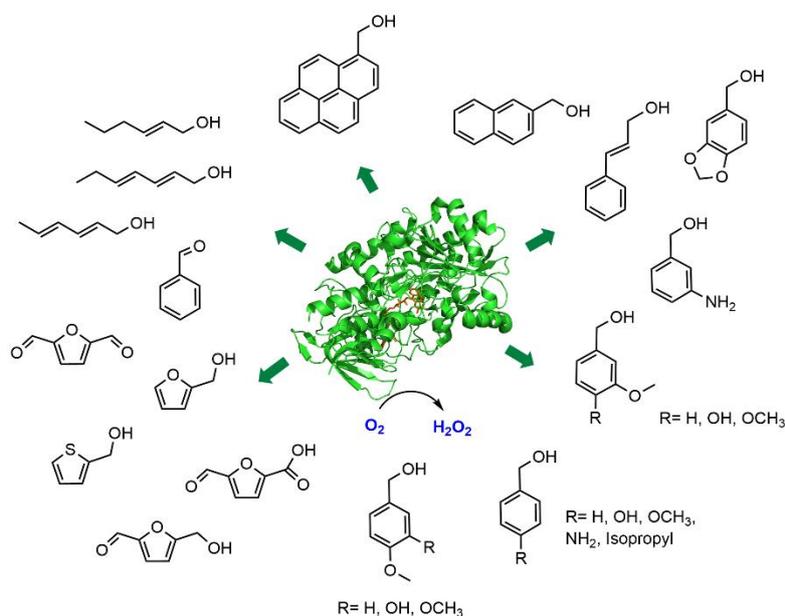


Figure 1. Substrates accepted by the investigated AAOs.

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Biotransformation of 2'-hydroxy-2-methylchalcone in culture of *Beauveria bassiana* KCH J1.5 to obtain new glycosylated dihydrochalcones

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Flavonoids are widespread in nature, and consist of many subgroups of biologically active compounds. Among them worth attention are dihydrochalcones, which can be found inter alia in apples as phloretin and its 2'-O-glucoside – phloridzin exhibiting antidiabetic, anti-inflammatory, antioxidant, antihyperglycemic, antimicrobial, cardio- and hepatoprotective potential. Structure-antioxidant capacity relationship studies of dihydrochalcone compounds in *Malus* revealed variety of mechanisms engaged in their antioxidant activity, with necessity of 2'-hydroxyl moiety presence. [1,2,3]

Physicochemical and biological properties of flavonoids are modulated by a presence of sugar and methyl moiety, which are responsible for increase of their stability and bioavailability. [4,5]

In order to obtain new, potentially biological active dihydrochalcone glycosides with methyl moiety attached, we employed combination of chemical and biotechnological methods. [6]

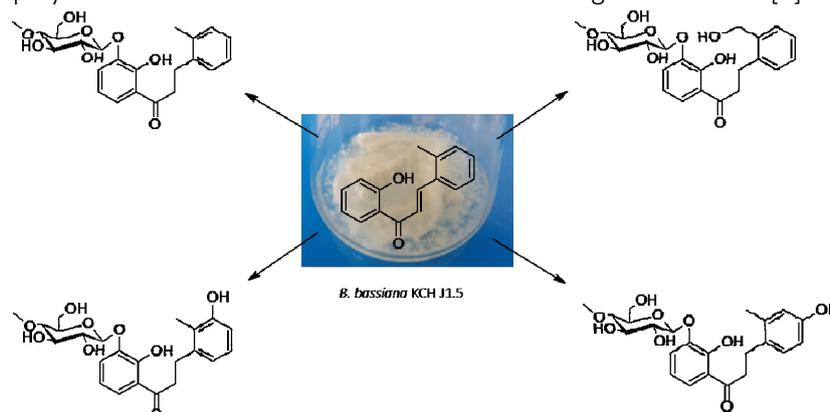


Figure 1. Biotransformation of 2'-hydroxy-2-methylchalcone in culture of *Beauveria bassiana* KCH J1.5.

After synthesis of 2'-hydroxy-2-methylchalcone in the Claisen-Schmidt condensation reaction, we used entomopathogenic filamentous fungi strain *Beauveria bassiana* KCH J1.5 as biocatalyst, and we obtained four glycosylated dihydrochalcones that have not been described in the literature so far. The compounds were separated by the means of preparative thin layer chromatography (TLC), and their structures were determined based on spectroscopic methods (^1H NMR, ^{13}C NMR, COSY, HSQC, HMBC).

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Amino Benzamidoxime (ABAO)-Based Assay to Identify Efficient Aldehyde-Producing *Pichia pastoris* Clones

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The chemoselective synthesis of aldehydes is a challenging task. Nature provides carboxylic acid reductases (CARs) as elegant tools for the direct reduction of carboxylic acids to their respective aldehydes. The discovery of new CARs and strains that efficiently produce these enzymes necessitates a robust high-throughput assay with selectivity for aldehydes. We recently reported a simple assay that allows the substrate independent and chemoselective quantification of aldehydes (irrespective of their chemical structure).[1] The assay utilized amino benzamidoxime (ABAO), which forms UV-active and fluorescent dihydroquinazolines. The assay works well in the presence of *E. coli* cells and was used for the engineering of CAR from *Nocardia iowensis* (NiCAR) to improve its activity.[2]

In this study, we adapted the ABAO assay for the identification and comparison of *Pichia pastoris* clones with the ability to produce aldehydes from carboxylic acid. Specifically, the CAR and PPTase from *Mycobacterium marinum* (MmCAR/MmPPTase) were co-expressed using different bidirectional promoters (BDPs).[3] A library of 598 clones was screened for piperonal production with the ABAO assay and the results were validated by HPLC-UV quantification.

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Exploration of ThDP–dependent transferase SucA substrate specificity

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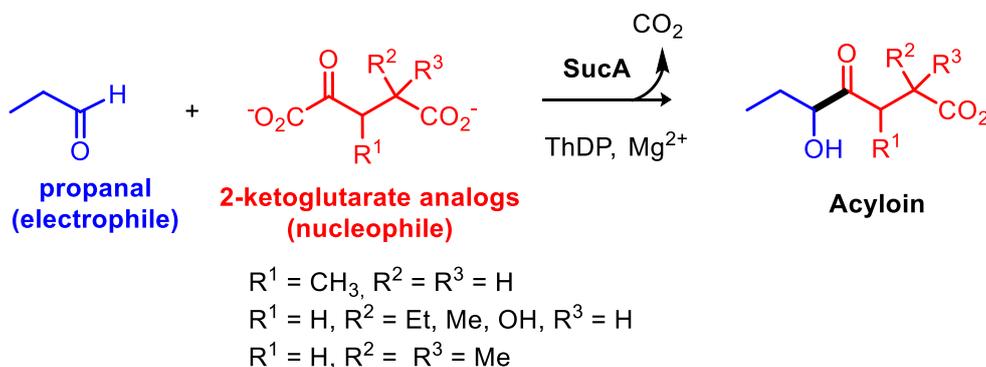
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The ThDP-dependent transferase SucA from *E. coli* is the subunit E1 from the multienzymatic complex in charge to transform 2-ketoglutarate (KG) into succinyl CoA.[1] SucA catalyses the decarboxylation of KG, to form the succinic semialdehyde (SSA) ThDP adduct. *In vitro* conditions, SucA transfers the succinyl moiety onto aldehydes, allowing the formation of acyloins. M. Beigi *et al* demonstrated its potential to catalyse stereoselective C-C bond formation starting from KG and alkyl or aromatic aldehydes.[2] Recently, SucA was also involved to generate (*R*)-acetoin from acetaldehyde, this aldehyde having both donor and acceptor roles.[3]

Since little is known on this enzyme, we decided to explore its substrate specificity by varying the donor structures. The ability of SucA to catalyse decarboxylation and acyl transfer on propanal was tested with several 2-ketoglutarate analogs, substituted at position 3 or 4, that were previously synthesized in our lab.[4] This work relates the assay of these alternatives substrates of SucA and then the small scale synthesis of the corresponding acyloins. Noteworthy, 4-hydroxy-KG was *in situ* generated through an enantioselective pyruvate aldolase catalyzed reaction. This is how a one-pot one-step cascade was developed, combining a pyruvate aldolase and SucA.



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Modification of globular proteins, what laccase can do?

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Laccase has shown great potential in the cross-linking or conjugation of protein, contributing to the structuralization, texturization and functional modulation of the protein-containing biopolymer matrix. This study investigated different biocatalytic mechanisms of laccase (Fig. 1) in the modification of globular proteins, including egg white lysozyme, ovalbumin and potato proteins (patatin and potato protease inhibitors). Laccase-catalyzed oxidation of protein is favored by the exposure of tyrosine residue; higher catalytic efficiency was shown towards patatin model substrates (k_{cat}/K_m , 0.010-0.92 $\text{mM}^{-1}\text{S}^{-1}$) than the other protein substrates. Cross-linking of potato proteins was achieved by laccase alone or by laccase-ferulic acid system; the latter contributed to higher cross-linking extent (5.7-31.4% VS 17.2-45.5%). The formation of potato protein-polysaccharide conjugates was also achieved by laccase-catalyzed reaction, resulting in a conjugation extent of 5-43 %. Lysozyme and ovalbumin showed low reactivity as substrates for laccase due to the low accessibility of their tyrosine and their compact structure. Surface phenolic grafting of protein substrates improved their cross-linking by laccase leading to the formation of oligomerized and polymerized proteins. Compositional, structural and functional characterization of selected modified proteins were carried out to understand the structure-function relationships associated with laccase-catalyzed modification and to explore the applications of fungal laccase in functional enhancement of protein ingredients.

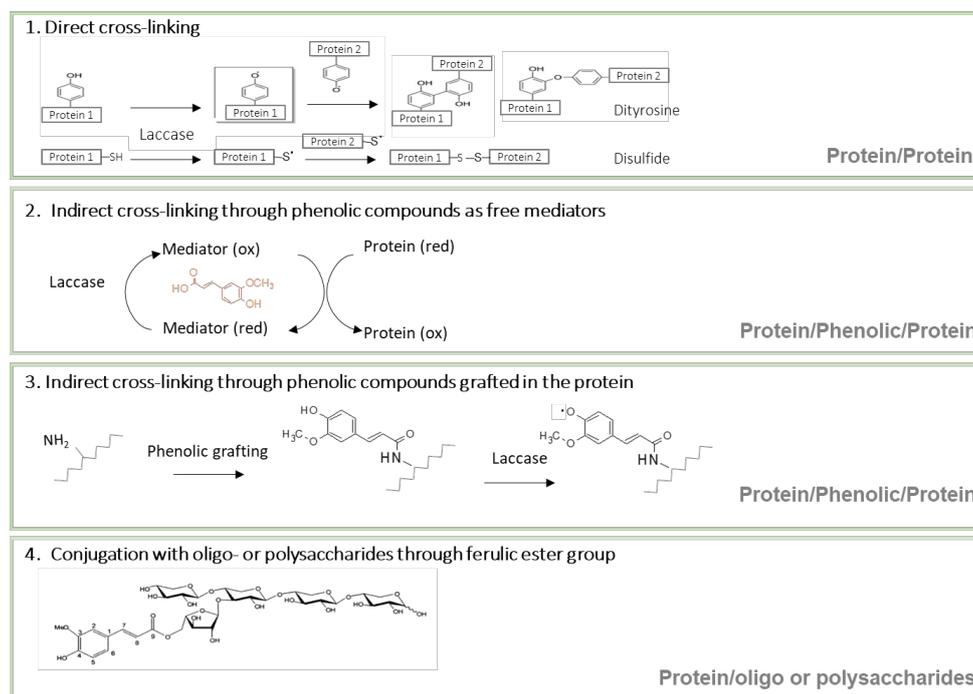


Figure 1. Different laccase biocatalytic mechanisms in protein modification

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One-pot biocatalytic route from cycloalkanes to nylon monomer by designed *Escherichia coli* consortia

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Adipic acid (AA) and 1,6-hexanediol (HDO) are vital and widely used nylon monomers that are currently produced by energy-intensive, multistage chemical oxidations that are hazardous to the environment^[1-4]. Therefore, the development of environmentally friendly, safe, neutral routes to AA and HDO is important^[5]. Firstly, we reported an *in vivo* artificially designed biocatalytic cascade process for biotransformation of cycloalkanes to AA (figure 1(1)). To reduce protein expression burden and redox constraints caused by multi-enzyme expression in a single microbe, the biocatalytic pathway is divided into three basic *E. coli* cell modules. The modules possess either redox-neutral or redox-regeneration systems and are combined to form *E. coli* consortia for use in biotransformation. The designed consortia of *E. coli* containing the modules efficiently convert cycloalkanes or cycloalkanols to AA and other aliphatic α, ω -dicarboxylic acids (DCAs) without addition of exogenous coenzymes^[6]. Meanwhile, a biocatalytic cascade process for biotransformation of cyclohexane to HDO under mild conditions in a one-pot-one-step manner was also constructed (figure 1(2)). The engineered *E. coli* consortia, which contained the corresponding cell modules, efficiently converted not only cyclohexane or cyclohexanol to HDO, but also other cycloalkanes or cycloalkanols to related dihydric alcohols^[7]. Thus, this developed biocatalytic process provides a promising alternative to the current industrial process for manufacturing nylon monomers.

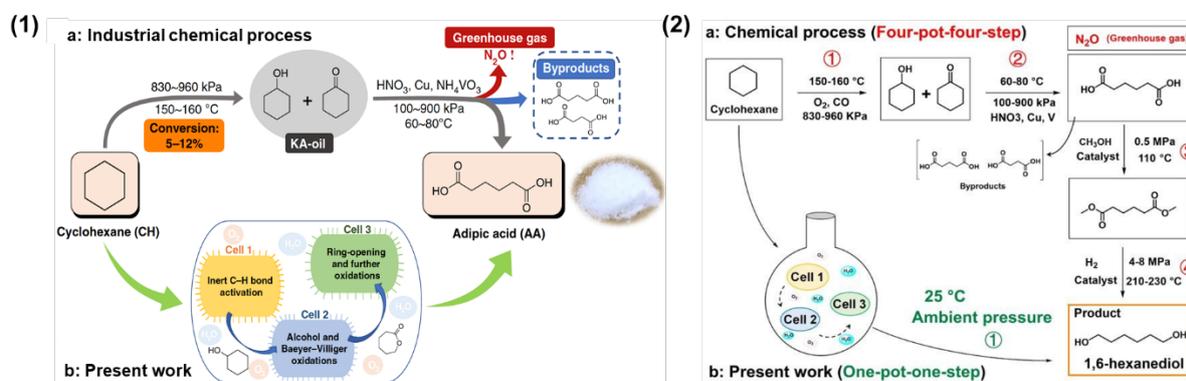


Figure. 1 Industrial chemical and designed biocatalytic processes for AA and HDO production.

(1): (a) Current industrial process for synthesis of AA by multistage chemical oxidation from cyclohexane. (b) Designed one-pot biocatalytic route for synthesis of AA from cyclohexane using an *E. coli* consortium, composed of three *E. coli* cell modules.

(2): (a) Current industrial process for synthesis of HDO by four-pot-four-step multistage chemical reactions. (b) Concept of design of a one-pot-one-step biocatalytic cascade for synthesis of HDO with an *E. coli* consortium.

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Glycosyltransferase co-immobilization for the synthesis of the nothofagin

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Nothofagin, the 3'-C-β-D-glucoside of phloretin, is a prominent antioxidant that are widely applicable for food additives, cosmetic ingredients therapeutics, Pharmaceuticals as well as nutraceuticals.[1] the glycosyltransferase cascade reaction has been successfully reported for the synthesis of nothofagin, involving a C-glycosyltransferase from rice (*Oryza sativa*; *OsCGT*) and sucrose synthase from soybean (*Glycine max*; *GmSuSy*), utilizing sucrose synthase (*GmSuSy*) reacts with uridine 5'-diphosphate to generate uridine 5'-diphosphate (UDP)-glucose as used for C-glycosyltransferase (*OsCGT*) catalyze highly selective 3'-C-β-D-glucosylation of the dihydrochalcone phloretin to synthesize the flavonoid C-glucoside of nothofagin.[2] Our aim is to make a fundamental improvement in reaction efficiency with a recyclable catalyst.

Immobilization is an important method from the general applied bio-catalysis toolbox for the enzyme recycling. Generally, co-immobilization (the colocalizing immobilization of multiple enzymes on the same solid carrier) can benefit the efficiency of heterogeneously catalyzed enzymatic cascade reactions by exploiting the effects of spatial proximity.[3]

Z_{basic2} module, which is an engineered arginine-rich variant of the Z domain, obtained from a 7kDa three-helix bundle from the B domain of staphylococcal proteins.[4] The additional positive charge of the Z_{basic2} tag at neutral pH provides a convenient driving force for the electrostatic adsorption on an anionic support.[4]

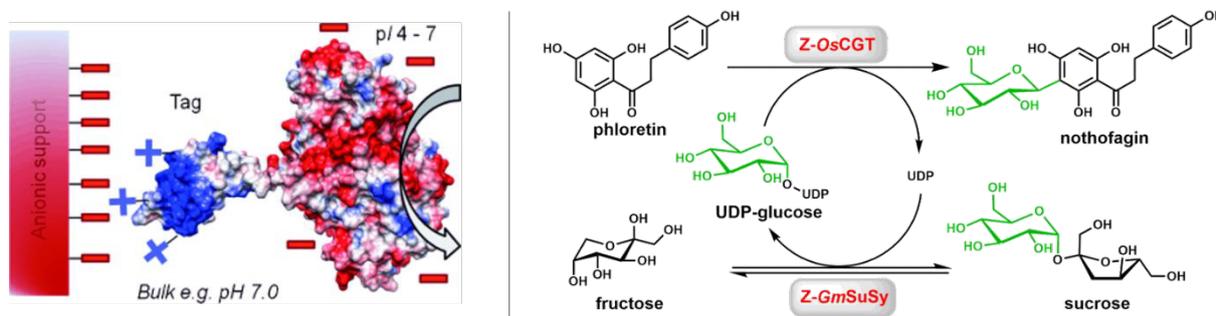


Figure 1. Synthesis of nothofagin through enzymatic C-glycosylation of phloretin from sucrose via UDP-glucose. C-glycosyltransferases from rice (*OsCGT*) and sucrose synthase from soybean (*GmSuSy*) are immobilized with Z_{basic2} module.

In our study, we present an efficient and flexible strategy for the co-immobilization of the rice (*Oryza sativa*) C-glycosyltransferase (*OsCGT*) and the soybean (*Glycine max*) sucrose synthase (*GmSuSy*). Building on surface tethering via the Z_{basic2} module, this strategy is novel to the class of Leloir glycosyltransferases; and it opens up new important opportunities for development of a glycosyltransferase-based process technology for nothofagin synthesis.[5]

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A Novel One-Pot Enzyme Cascade for the Biosynthesis of Non-Natural Deoxyribonucleotides

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Non-natural deoxyribonucleotides are an important class of compounds with applications ranging from synthetic biology to medical science. Synthesis of these compounds is challenging due to the complex nature of the molecule and its phosphorylation. While different biocatalytic approaches have been proposed for the biosynthesis of non-natural ribonucleotides [1, 2], novel strategies will be required for an efficient biosynthesis of non-natural deoxyribonucleotides.

In this study, we established a novel one-pot enzyme cascade for the biosynthesis of non-natural deoxyribonucleotides, consisting of three well-characterized but so far unrelated nucleotide-modifying enzymes. An adenine phosphoribosyltransferase couples the non-natural nucleobase to the ribose moiety, followed by polyphosphate kinase catalysed phosphorylation and reduction by a ribonucleotide reductase (Figure 1). As showcase reaction, we describe the biosynthesis of cladribine triphosphate, the active metabolite of the anti-cancer and multiple sclerosis drug cladribine.

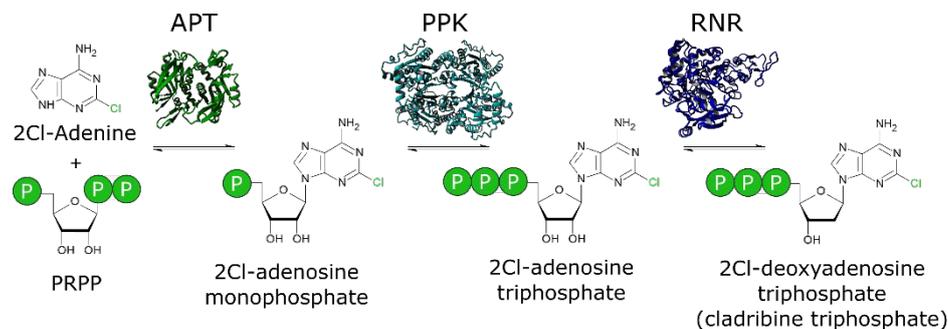


Figure 1. Novel cascade for the biosynthesis of the non-natural deoxyribonucleotide cladribine triphosphate, consisting of the enzymes adenine phosphoribosyltransferases (APT), polyphosphatekinase (PPK) and ribonucleotide reductase (RNR).

The cascade was prepared by first testing the compatibility of the single enzymes in terms of reaction conditions and interference between the respective reactions. Common reaction conditions were established and no major detrimental effects of the respective reaction components on the other reactions were observed. The cascade was assembled and tested with the natural substrate adenine, where it proved capable of the production of deoxyadenosine triphosphate. After adjustments to the applied enzyme concentrations, also the conversion of 2Cl-adenine was detected. The main product 2Cl-adenosine triphosphate (cladribine triphosphate) was produced in 90 min with a reaction yield of 80%, the only major side product being 2Cl-adenosine diphosphate (20%). Product concentrations and yields are comparable to previous methods for the biosynthesis of non-natural nucleotides [1, 2]. In conclusion, the novel cascade established in this study is a feasible tool for the biosynthesis of the non-natural deoxyribonucleotide cladribine triphosphate. Considering the natural substrate promiscuity of its enzymes, the cascade may be developed into a platform technology for the biosynthesis of other members of this important class of compounds.

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Cell-free biotransformations of polyols using self-sufficient and multifunctional heterogeneous biocatalysts.

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Combination of several enzymes in one-pot opens a very attractive pathway to access more complex synthetic schemes to manufacture high added-value products in a more sustainable manner¹. Nowadays, chemical biomanufacturing is dominated by microbial fermentations that use native pathways to mainly produce alcohols, acids or aminoacids. In the last decade, cell-free multi-enzyme systems have emerged as an alternative to whole cell biotransformations in chemical manufacturing since cell-free systems can work towards non-natural substrates under non-physiological conditions often demanded by industrial processes¹. However, enzyme isolation faces some challenges (productivity, robustness and costs) for implementing cell-free multi-enzyme systems into the industrial context. In particular, the valorization and upgrading of renewable polyols (glycerol, bio-diols...) into aminoalcohols and ether derivatives is highly attractive in applied biocatalysis. These biotransformation often demand cofactor-dependent enzymes that pose challenges for the implementation of these cell-free systems as they demand for exogenous cofactors that hamper the downstream processing and limit their economic feasibility of the process.

Inspired by the spatial organization and the molecular confinement of metabolic pathways within the living cells, our group has exploited immobilization techniques to spatially organized a variety of multi-enzyme systems (oxidoreductases, oxidases, transaminases) confined with their corresponding cofactors (NAD(P)H, PLP, FAD...) across the surface of synthetic porous carriers^{2,3}. Our work aims at creating *in vitro* and confined enzymatic pathways as self-sufficient and multi-functional heterogeneous biocatalysts, where cofactors and enzymes are successfully recycled and reutilized during several operational cycles to increase their total turnover numbers (TTN).

To illustrate this concept, several multi-functional heterogeneous biocatalysts were validated for upgrading bio-diols and glycerol to manufacture aminoalcohols⁴ and alkyl/alkoxy glyceryl ethers⁵. In this communication, we will disseminate the benefits of co-immobilizing enzymes and cofactors but also the limitations of this strategy. Finally, the performance of these immobilized multi-enzyme systems in flow-biocatalysis has been tested accompanied by some metrics that quantify the sustainability of the processes.

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Chemoenzymatic, Asymmetric Total Synthesis of Biologically Active Nodulone C and D

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Nodulones is a class of polyketidic natural products, which have been isolated from a number of endophytic fungi in recent years.^{1, 2} They possess dihydronaphthalenone as a common structural scaffold, which is difficult to synthesize because of the presence of chemically sensitive β -hydroxyketone group. We have been interested in a biomimetic synthesis of nodulone C (**1**) and *trans*-nodulone D (**2**), reportedly isolated from orchid associated endophytic fungi, *Daldinia eschscholzii* of *Paphiopedilum exul*.^{1, 2} Nodulone C (**1**) shows antimicrobial and anti-proliferative activity against HUVEC and K-562 cell lines¹, while nodulone D (**2**) acts as an immunosuppressant². Inspired by the enzymatic reduction of polyhydroxynaphthalenes and hydroxynaphthoquinones by tetrahydroxynaphthalene and trihydroxynaphthalene reductase (T₄HNR/T₃HNR) of *M. grisea*,³ we planned a late-stage reduction of putative biosynthetic substrates for the synthesis of nodulone C and D.

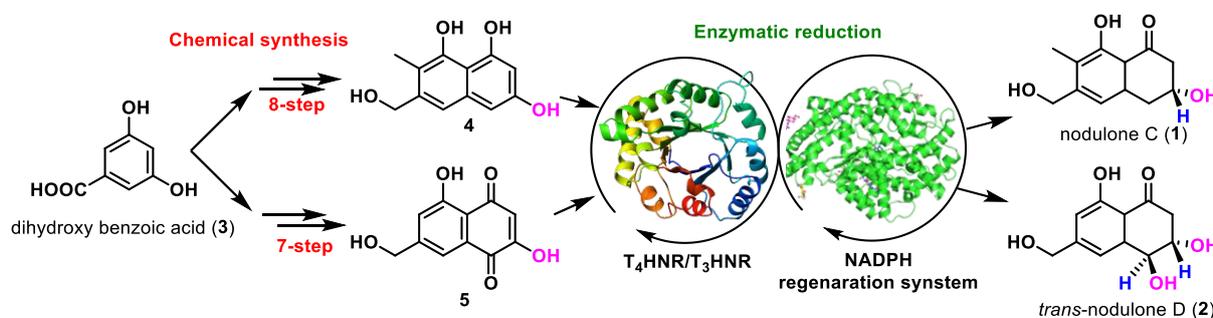


Figure 1. Chemoenzymatic synthesis of nodulone C (**1**) and nodulone D (**2**) using NADPH-dependent T₄HNR/T₃HNR.

With the successful total synthesis of probable substrates (**4** and **5**) in 7-8 steps, followed by asymmetric reduction using T₄HNR or T₃HNR, herein we report the first chemoenzymatic, asymmetric synthesis of nodulones C and D. This implies for similar biosynthesis of these natural products and the involvement of T₄HNR related enzyme(s) in the reduction of putative substrates.

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Modifying chitosan molecular weight using promiscuous enzymes

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Natural polymers have emerged as a potent solution for replacing petroleum-based polymeric materials; in this context, chitin and chitosan are the second most abundant biopolymers on the earth after cellulose, so that the sustainable production and employment of chitinous biomass for various industrial applications and for biomedical usage has become part of global development strategies [1]. The enzymatic hydrolysis of chitosan to produce chitooligosaccharides, which display a plethora of biological activities [2], is mediated in nature by chitosanases [3]. These enzymes, although very specific, are very expensive, so that the employ of other cheaper hydrolases would be highly beneficial from an economic point of view. This "promiscuous" activity, depicted in Figure 1, is favoured by the GlcNAc moiety *via* a mechanism termed substrate-assisted catalysis (SAC), as this functional group in the substrate contributes to enzyme catalysis, resulting in the retention of the end monomeric unit configuration [4].

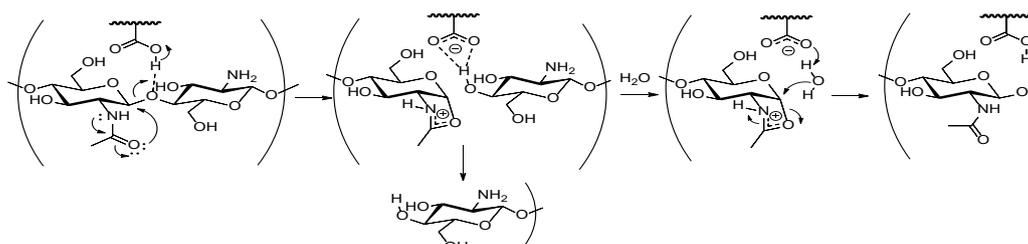


Figure 1. Substrate-assisted catalysis (SAC) for the hydrolysis of chitosan

In this communication, we will present the results obtained upon the use of different commercial lipases (from Novozymes) to hydrolase chitosan. Reactions were carried out at acid pH (AcOH/AcNa 0.3/0.2 M) at 50°C and followed by GPC using a dextran calibration curve; Mw data were compared to the control chitosan. Reactions without enzyme showed that the observed hydrolysis is solely due to the enzyme since no spontaneous depolymerization of the polymer was observed under the experimental conditions. Best results were obtained with Novocor (CALA lipase) and Lipozyme TL 100 (*Thermomyces lanuginosus*), which proved to be very efficient. Reaction with Novocor was faster (22% reduction of Mw at 0.25 h), although the highest reduction at 24h (97%) was observed using Lipozyme TL 100. A detailed report of the resulting products shows the different profile of polymeric fractions obtained.

Acknowledgements

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Halohydrin dehalogenase-catalysed synthesis of fluorinated aromatic chiral building blocks

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Halohydrin dehalogenases (HHDHs) catalyse enantioselective formation and conversion of epoxides. They can be applied in the kinetic resolution of racemic epoxides by utilising a range of anionic nucleophiles such as azide, cyanide, cyanate or nitrite.[1,2] The ring-opening products and remaining epoxides are versatile and important synthetic intermediates for fine chemicals. In this work HHDH was employed for the transformation of epoxides bearing fluoroaryl groups (Figure 1). Fluoroaromatic compounds are widely used as starting materials for chemical syntheses, especially pharmaceuticals and agrochemicals. Introduction of fluorine atoms into the molecule usually increases target effectiveness, biological half-life, bioabsorption *etc.* The growing interest in fluorinated organics makes the development of synthetic procedures leading to such compounds desirable.

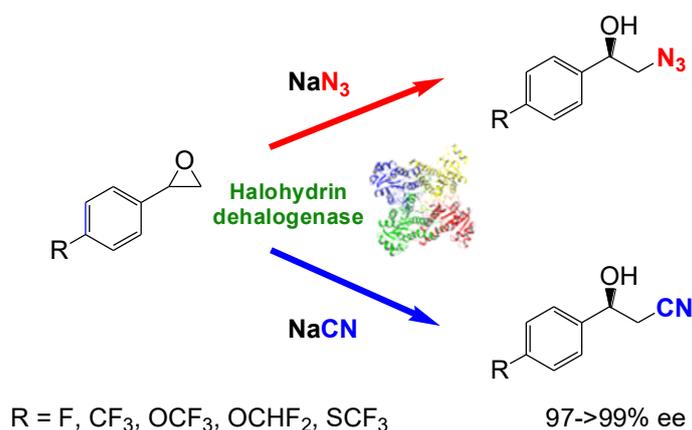


Figure 1. Kinetic resolution of fluorine-substituted styrene oxide derivatives was catalysed by halohydrin dehalogenase.

Kinetic resolution of a series of fluorinated styrene oxide derivatives was studied using the enzyme from *Agrobacterium radiobacter* AD1 (HheC) and a variant W249P.[3] A mutant HheC-W249P catalysed nucleophilic ring-opening with azide and cyanide ions with excellent enantioselectivity (*E*-values up to >200) and higher activity compared to the wild-type, which gives access to various enantiopure β -substituted alcohols and epoxides. It was found that the enzyme tolerates substrates in concentrations over 50 mM. However, different side reactions were observed at elevated concentrations and with prolonged reaction time. The biocatalytic azidolysis and cyanolysis of racemic 4-CF₃-styrene oxide were performed on preparative scale, affording (*R*)-2-azido-1-(4-trifluoromethylphenyl)-ethanol in 38% yield and 97% ee, and (*S*)-3-hydroxy-3-(4-trifluoromethylphenyl)-propionitrile in 30% yield and 98% ee.

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Engineering cyanobacteria for light-driven hydroxylation of steroids

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Although our daily life strongly relies on organic synthesis, which is easily upscalable but leads to high amounts of waste, the development of more sustainable ways to produce fine chemicals is mandatory. Redox reactions, require stoichiometric amounts of sacrificial electron donors, which have to be disposed of afterwards. Compared to conventional synthesis or biocatalysis in heterotrophic bacteria, biocatalysis in cyanobacteria represents an interesting alternative. In these organisms, the electrons fuelling these reactions can be provided by the light-dependent oxidation of water that occurs during the photosynthetic electron flow. Among oxidoreductases, cytochrome P450 monooxygenases are particularly suitable to be expressed in cyanobacteria, as they catalyze reactions consuming O₂, a by-product of photosynthesis, and they can accept electrons from endogenous ferredoxins naturally present in these hosts/chassis [1]. In this work, the enzyme CYP110D1 from *Nostoc* sp. PCC 7120 was expressed in the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803. CYP110D1 is known to catalyze the hydroxylation of the hormone testosterone, yielding 15-β-hydroxytestosterone, a valuable compound for the pharmaceutical industry [2]. The enzyme coding sequence was cloned into a self-replicative vector under the control of different promoters [3] and the generated constructs were used to transform *Synechocystis*. Once the presence of the enzyme within the recombinant strains was confirmed, bioconversion assays revealed that CYP110D1 converts testosterone to the desired product in a light-dependent way and without the need for sacrificial electron donors. Finally, the conditions for the bioconversion were then analyzed and optimized.

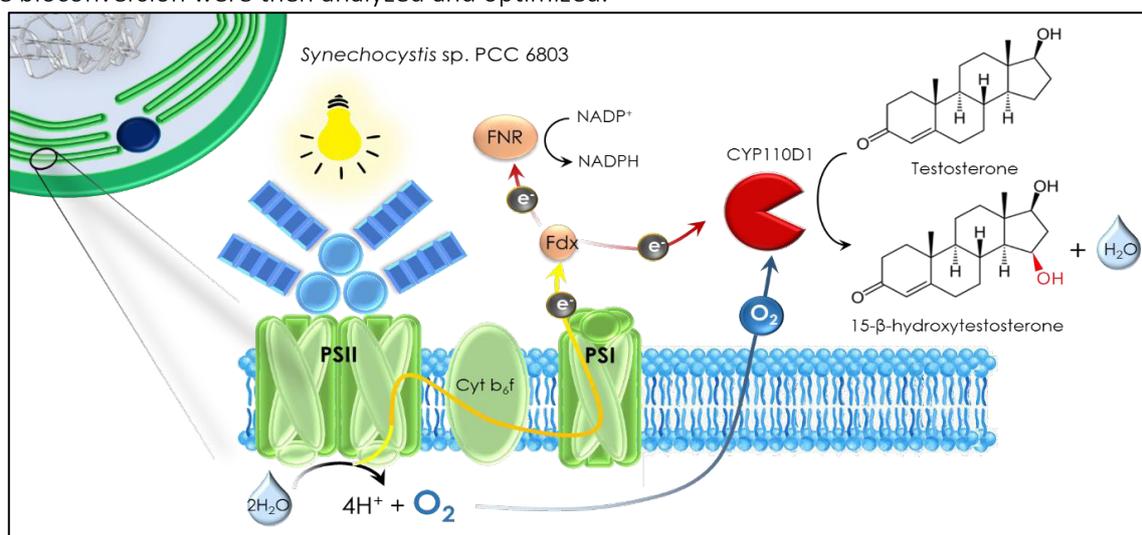


Figure 1. Schematic representation of light driven CYP110D1 biocatalysis in *Synechocystis* sp. PCC 6803. PSII, photosystem II; PSI, photosystem I; Cyt_{b₆f}, cytochrome _{b₆f}; Fdx, ferredoxin; FNR, ferredoxin reductase.

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Synthesis of human drug metabolites by unspecific peroxygenases in EvoEnzyme's multi-UPO screening kit -EVOKIT-

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A screening kit of evolved fungal unspecific peroxygenase (UPO) enzymes is under development for scalable production of human drug metabolites and fine chemicals. Emulating the role of human liver P450 monooxygenases, UPOs can perform large portfolio of selective oxyfunctionalizations on pharmacological compounds. But unlike P450s, peroxygenases employ simple H₂O₂ as oxidant, which makes them independent from expensive cofactors. The catalytic abilities of UPOs in the kit -EVOKIT-, that attract both the pharmaceutical and chemical sector, include alkyl and aromatic hydroxylations, aliphatic and aromatic epoxidations, O-dealkylations, N-dealkylations (including ester and ether cleavage), N-oxidations, S-oxidations and brominations [1]. All our UPO enzymes, which are coming from different fungal species, are cloned in recombinant eukaryotic hosts capable for producing them in larger quantities.

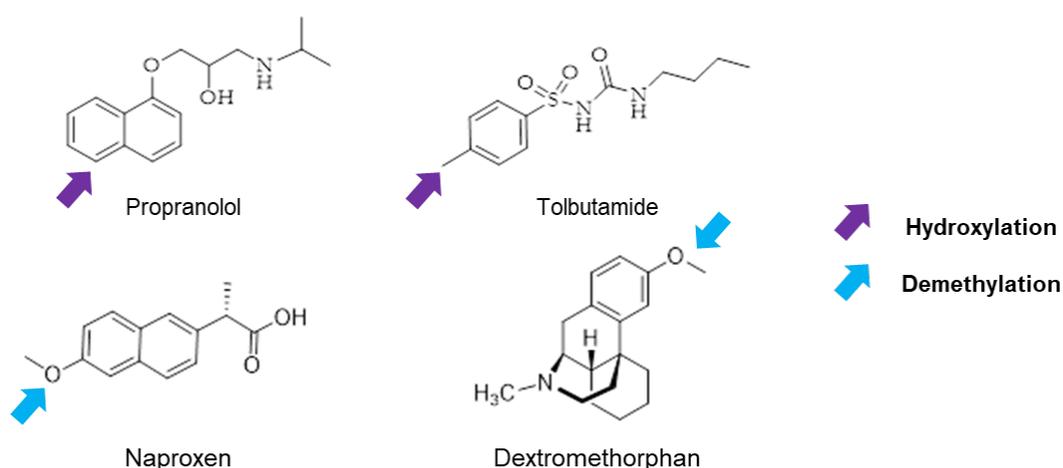


Figure 1. UPO activities on selected pharmaceutical agents.

We have tested several engineered UPO variants from the EVOKIT for their capacity to synthesize authentic human drug metabolites from four pharmaceutical compounds: dextromethorphan, naproxen, propranolol and tolbutamide, Figure 1. [2,3]. Dextromethorphan and naproxen were converted to corresponding human metabolites via demethylation reaction by all tested variants to the varying degree of conversion. In the case of propranolol and tolbutamide, UPOs hydroxylation generated human metabolites 5-hydroxypropranolol and 4-hydroxytolbutamide, respectively. Along with identification of metabolites and after the hit verification, reactions can be scaled up to produce larger quantities of desired metabolite for further analysis.

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Biocatalytic scope of halohydrin dehalogenase from *Mycobacterium* sp. GP1 (HheB2)

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Halohydrin dehalogenases (HHDHs) belong to a distinct group of enzymes catalyzing the removal of a halide ion and a proton from a vicinal halohydrin with formation of an epoxide.[1] Moreover, these enzymes can catalyze the epoxide ring-opening reactions with a range of anionic nucleophiles affording β -substituted alcohols as products.[2] Based on sequence similarities, they are divided into 7 phylogenetic groups: A, B, C, D, E, F and G. Activity, enantioselectivity and enantiopreference are dependent on the type of enzyme and the substrate structure.[3] Among all, the enzyme HheC from *Agrobacterium radiobacter* (group C) is the most studied because of its high enantioselectivity and wide nucleophile scope.[3] However, the major shortcoming of HheC is its narrow substrate tolerance, due to the relatively small active site. To expand the catalytic relevance of HHDHs we focus our attention to an enzyme from different organism. The HheB2 from *Mycobacterium* sp. GP1 (group B) has been previously characterised as non-enantioselective enzyme, and neglected as biocatalyst.[4,5] In this work HheB2 was investigated in the ring-opening reaction on a set of 20 structurally different aliphatic and aromatic epoxides using sodium azide as nucleophile (Figure 1).

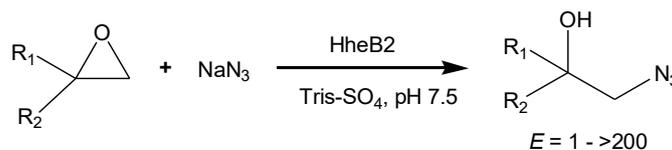


Figure 1. Nucleophilic ring-opening of epoxides catalysed by HheB2 from *Mycobacterium* sp. GP1

The screening confirmed low to moderate enantioselectivity of HheB2 towards monosubstituted epoxides, however high activity and enantioselectivity in conversion of 2,2-disubstituted (E -values up to >200). Similar to HheC, the large enhancement of enantioselectivity is obtained when a second substituent (methyl or ethyl) is present at the chiral centre. The results further extend the repertoire of enantioselective HHDHs and their application in the kinetic resolution of epoxides.

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Promiscuous lipases promote the synthesis of 2*H*-chromenones

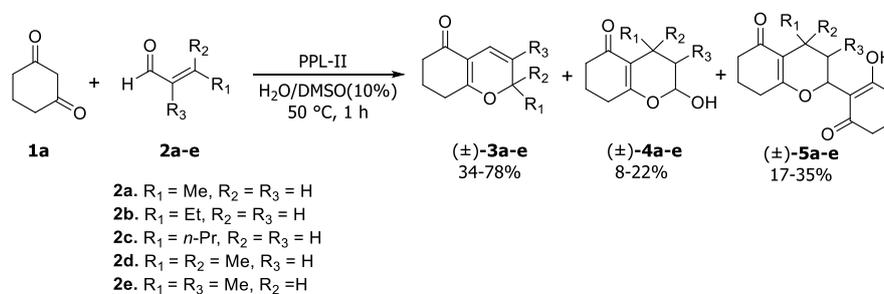
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From a synthetic point of view, the promiscuous enzymes represent an alternative as selectivity induction catalysts, and a sustainable methodology [1]. Lipases stand out for their broad tolerance to substrates different from triacylglycerols (their native substrates). In this study was explored a synthesis of 2*H*-pyran nucleus, which are important building blocks contained in a wide range of natural products such as arisugacin H, ferprenin, cannabichromene, huajiaosimuline [2].

The reaction using 1,3-cyclohexanodione **1a** and crotonaldehyde **2a** catalysed by Porcine Pancreatic Lipase (PPL-II). It was studied using a complete factorial design 2⁴ in order to identify the variables (PPL-II mass, molar equivalents of reagents **1a** and **2a**, percentage of water and temperature) that influenced the most in the yields and selectivities among 2*H*-chromenone products **3a**, **4a** and **5a** (Scheme 1). These products result from a 1,2-addition, 1,4- conjugate addition and both.



Scheme 1. Biocatalyzed reactions of diketone **1a** and α,β -unsaturated aldehydes **2a-e** by PPL-II.

The PPL-II mass and the temperature were the variables that affected the production of the compound 2-hydroxy-2*H*-chromenone **4a**, while no influence was noticed over the other two compounds (e.g., **3a** and **5a**).

The best condition to obtention of **4a** (22% yield) was 20 mg of PPL-II, 0.1 mmol of 1,3-cyclohexanedione **1a** and 50 °C. It was used to extent the scope of the reaction to other α,β -unsaturated aldehydes **2b-e** that resulted in 2*H*-chromenones **4b-e** with yields 8-22%, and favored the *anti* diastereoisomers, nonetheless without enantioselectivities.

In general, the yields of the compounds **4a-e** were not high due to the inherent reactivities of the reagents and intermediates, enabling a mixture of the three products.

It is noteworthy that compounds **3a-e** and **5a-e** were also interesting to be synthesized by the biocatalytic route using PPL-II, since they have functionalized structures.

Finally, the reactions when carried out in the absence of PPL-II provided a mixture of compounds **3a**, **4a** and **5a** it in low yields (13%, 3% and 12%), respectively.

To conclude, with this work was demonstrated the importance of using promiscuous lipases in organic synthesis in the carbon-carbon bond formation as an efficient biocatalytic methodology.

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Novel Transaminases For The Enzymatic Amination Of 5-Hydroxymethylfurfural

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5-hydroxymethylfurfural (HMF) is produced by the dehydration of biomass-derived carbohydrates and can thus be an attractive, renewable, and bio-based starting material [1]. It has attracted great interest in recent years as a result of the possibility to transform it further into high-value derivatives. Furfurylamines can be applied for instance as monomers in the biopolymer synthesis and in the pharmaceutical industry as pharmaceutical compounds such as diuretics, antihypertensives and antiseptic reagents [2]. Despite the large interest in HMF and the opportunity for a green synthesis of its amine derivative, only little research has been observed for the biocatalytic transformation into 5-hydroxymethylfurfurylamine (HMFA).

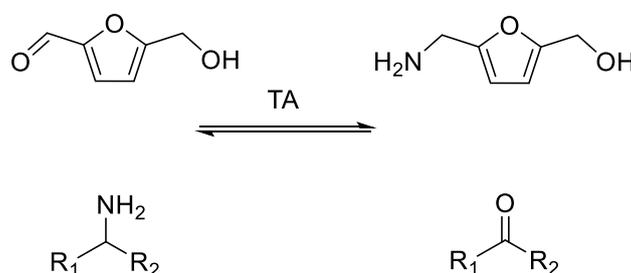


Figure 1. Reaction scheme of the transaminase-catalysed amination of 5-hydroxymethylfurfural

In this work we have tested 11 amine transaminases (ATAs) for their ability to produce HMFA using HMF as starting material. Four of those ATAs successfully produced HMFA within 24h. The ATAs from *Aspergillus oryzae* (Ao), *Silicibacter pomeroyi* (Sp) and *Chromobacterium violaceum* (Cv) all worked with isopropylamine (IPA) and alanine as amine donor. The ATA from *Aspergillus terreus* (At) only reacted with alanine. The best results were achieved with the Cv ATA at 97% conversion after 1h, and with SpATA at 89% after 4h. With this work our course towards a scalable and sustainable production process for HMFA was set.

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Activity and operational stability of halohydrin dehalogenases in aqueous-organic biphasic systems

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Halohydrin dehalogenases (HHDHs) are industrially relevant enzymes that show exceptional selectivity, activity, and versatility regarding accepting nucleophiles in epoxide ring-opening reactions.[1] HHDHs can be employed in the synthesis of important and versatile pharmaceutical building block (*S*)-2-(4-fluorophenyl)-3-hydroxypropanenitrile through kinetic resolution of *rac*-2-(4-fluorophenyl)oxirane.[1,2] The major limitations of this reaction are poor substrate solubility and its susceptibility to hydrolytic decomposition. Introduction of organic phase in the aqueous reaction media can have a positive effect both on the solubility and hydrolytic stability of epoxide, leading to higher volume productivity. However, possible side effects of the organic phase introduction are enzyme activity and operational stability decrease, hence they need to be experimentally investigated and quantified. In this work, we determined the activity of wild-type HheC from *Agrobacterium radiobacter* in the presence of a wide solvent/buffer ratio range. As a result, correlation between solvent logP values and concentration of half-inactivation (C_{50}) was observed, whereby C_{50} represents concentration of solvent that irreversibly reduces enzyme initial activity by half.[3]

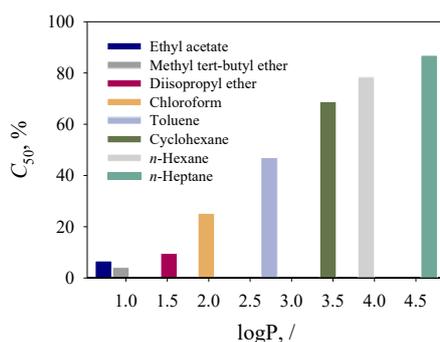


Figure 1. Dependence of concentration of half-inactivation (C_{50}) on partition coefficient between octanol and water (logP) for different solvents

In addition to affecting the initial enzyme activity, the organic solvent may also affect the enzyme activity during continuous use. Hence, enzyme stability in selected solvents and at different ratios during time was monitored. Besides HheC, we examined the co-solvent activity and stability of thermostable variant ISM-4 obtained with iterative saturation mutagenesis,[4] as resistance to high temperatures is often accompanied with enhanced co-solvent activity and stability.[5] Finally, based on the experiments performed on a model system, we investigated the co-solvent operational stability of ISM-4 during (*S*)-2-(4-fluorophenyl)-3-hydroxypropanenitrile synthesis. We continually monitored the enzyme activity during reaction and estimated the values of enzyme deactivation rate constant (k_d , min^{-1}).

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Development of laccase-functionalized graphene oxide for detecting 1,4-dihydroxy-N-methyl-N-nitroso-aniline

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In recent decades, immobilized enzyme-based biosensors have been widely developed for applications in various fields from the environment to biomedical applications [1]. Various immobilization strategies are used to improve the stability and reuse of enzymatic biosensors, including physical adsorption, entrapment, covalent bonding, and affinity immobilization [2]. Laccase is a type of blue multi-copper oxidase whose appealing is that copper atoms are in the active site of the enzyme and have certain advantages such as the ability to catalyse electron-transfer reactions and high stability over the experimental conditions [3].

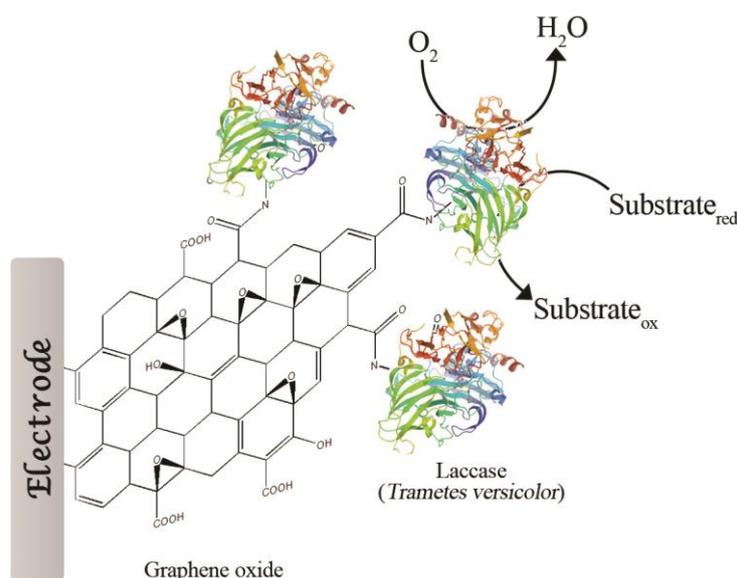


Figure 1. Schematic presentation of laccase-based biosensor for detecting nitrosamines.

To prepare the biosensor, the glass carbon electrode was sonicated in ethanol for 5 min and dried at room temperature. Then 0.5 mg of *Trametes versicolor* laccase was mixed with 1.0 mL of graphene oxide solution (0.3 mg/mL) in an Eppendorf tube. The electrochemical properties and morphology of the enzymatic biosensor were investigated by cyclic voltammetry and scanning electron microscopy, respectively. The prepared biosensor was applied to determine 1,4-dihydroxy-N-methyl-N-nitroso-aniline (dephostatin) as a nitrosamine sample. As a future prospect, it is strongly believed that graphene oxide can be used as an efficient immobilization matrix for oxidoreductase proteins.

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Laccase-catalysed removal of tramadol from model aqueous solution

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Concerns about the ecological hazards of pharmaceuticals released into the environment have improved methods of remediating toxic aromatic compounds with microorganisms or their enzymes. Enzymatic bioremediation is potentially a rapid and environmentally friendly technique for the removal of environmental toxic residues [1].

Tramadol [2-(dimethyl amino)-methyl)-1-(30-methoxyphenyl)cyclohexanol hydrochloride] is an opioid medication used to treat moderate to severe pain. Tramadol has been detected in various environmental matrixes and wastewaters with concentrations varying up to about 1116 ng L⁻¹ [2,3].

The present study describes the use of laccase from *Trametes versicolor* for biotransformation and bioremediation of Tramadol. Tramadol was removed under optimal conditions of laccase activity 1 U ml⁻¹, 45 °C, pH 4.5, and 1 mM 1-hydroxybenzotriazol. The use of 1-hydroxybenzotriazole, a laccase mediator, enhanced the removal yield. The effect of mediator at different concentrations on substrate removal was also investigated. The system is expected to be effective in the biotransformation of phenolic pharmaceuticals.

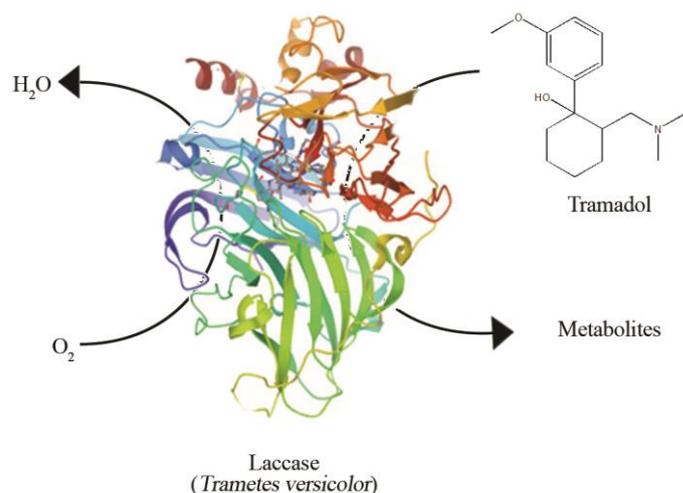


Figure 1. Schematic representation of laccase-catalysed removal of tramadol.

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The STAIRWAY Project: sustainable recovery and biocatalytical valorization of medicinal plants wastes

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The aim of the STAIRWAY research project is to develop sustainable recovery procedures of valuable natural compounds from wastes of industrial production processes of medicinal plants and, by applying biocatalyzed transformations, to further convert them into high-value derivatives for the nutraceutical, cosmetic and pharmaceutical industry.

The use of medicinal plants extracts is being steadily increased by the growing demand of natural bioactive products as dietary supplements in health foods and in personal care and cosmetic preparations, such as skin care products. In light of this constant global market grow, the development of sustainable and fully integrated manufacturing processes, from plants cultivation to wastes fate, is clearly of the utmost importance.

In this view, the STAIRWAY project aims at finding a different fate for two selected biomass wastes from the industry of medicinal plants extracts. Specifically, these residues are obtained after selective extraction of oils from *Serenoa repens* Small (saw palmetto) fruits and *Cucurbita pepo* L. (pumpkin) seeds (Figure 1), which are commercialized for the treatment of genito-urinary tract pathologies. Both starting plant biomasses are highly rich in valuable natural compounds such as fatty acids, fatty alcohols, phytosterols, β -carotene and its isomers, γ - and δ -tocopherol, flavonoids and their derivatives, and saponins, and it is very likely that several of them are still present in the industrial wastes.

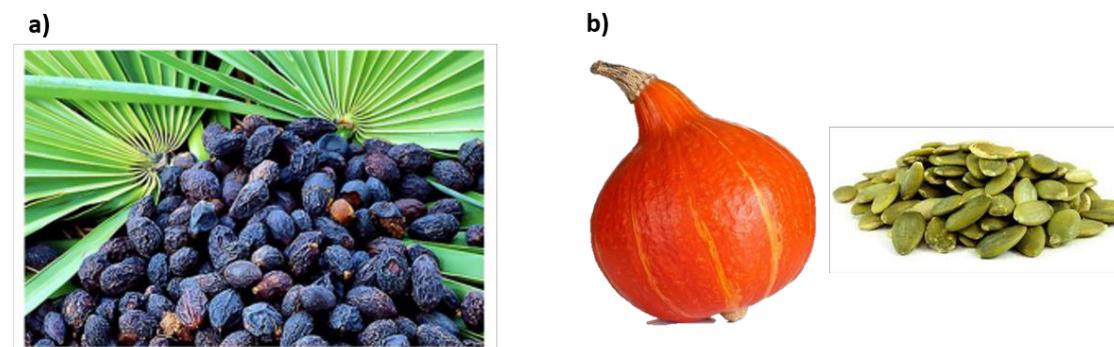


Figure 1. a) *Serenoa repens* Small (saw palmetto) fruits; b) *Cucurbita pepo* L (pumpkin) fruit and seeds.

Herein we will present the results of the STAIRWAY project in the definition of the structural richness still available in the biomass wastes after the industrial extraction processes. Extracts enriched in the main natural compounds have been collected through a “green” extraction technique, Supercritical Fluid Extraction (SFE), and then purified and analysed by chromatographic and spectroscopic methods. Biocatalytic procedures, based on the use of either whole cell microorganisms or isolated enzymes, are under development to selectively manipulate the biomass wastes or selected recovered natural compounds, for both food/nutraceutical and cosmetic/pharmaceutical applications.

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Cofactor regeneration strategies for cost efficient industrial biocatalysis

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A wide range of synthetically useful transformations are available via cofactor-dependent enzymes. Nevertheless, the cofactor requirement also poses economic and practical challenges for the application of these biocatalysts. Considerable research effort was focusing in the last decades on the development of reliable *in situ* regeneration systems for specifically NAD(P)⁺ and NAD(P)H. Application of those cofactors for use in redox biocatalysis on industrial scale is nowadays established, although challenges remain.

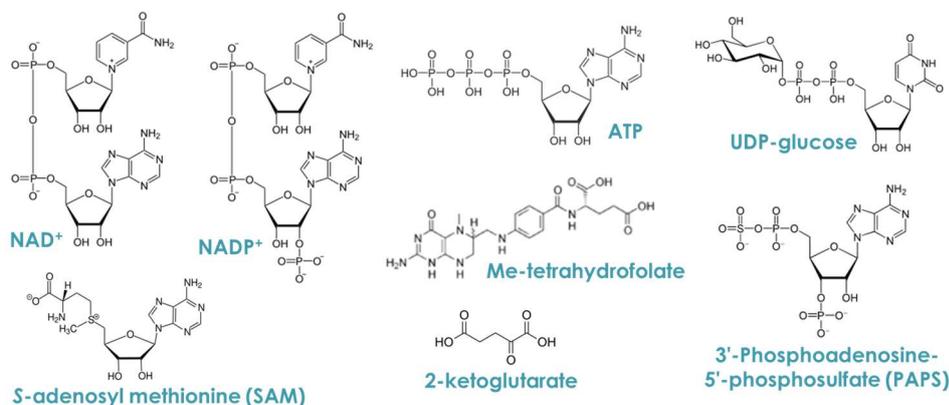


Figure 1. Various co-factors required by different enzyme classes.

In contrast, other cofactors and co-substrates remain difficult for cost-efficient biocatalysis. As a result, e.g. ATP dependent enzymes are basically solely used in fermentative processes and not for *in vitro* biocatalysis on industrial scale.

We will present InnoSyn's strategies for cost-efficient cofactor regeneration for *in vitro* biocatalysis on three examples under industrially relevant conditions. First, the regeneration of NADPH by glucose dehydrogenase for Baeyer Villiger Monooxygenase catalyzed reaction on pilot plant scale will be shown. Next, the technically and economically feasible *in vitro* regeneration of ATP, besides NAD(P)⁺ and NAD(P)H the most abundant cofactor in nature, will be presented. Finally, the application of glycosyl transferase technology for production of a valuable nutritional compound will be presented. The required co-factor UDP-glucose is efficiently recycled by a sucrose synthase enzyme. On a 1 liter scale reaction, this led to the production of ~10 g isolated, high value product.

A Diverse Library of Chiral Cyclopropane Scaffolds via Chemoenzymatic Assembly and Diversification of Cyclopropyl Ketones

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Biocatalysis has been rapidly growing as an attractive solution for chemical synthesis and manufacturing, in particular for the production of drugs, APIs, and other high-value compounds. This is motivated by enzymes' potential for high chemo- and stereoselectivity along with their ability to operate under mild aqueous conditions and their amenability to adopt improved or new functions through protein engineering.¹⁻² Our group and others have recently explored heme-based proteins to carry out 'abiological' carbene and nitrene transfer reactions and generate new C-C, C-N and C-X bonds with high efficiency and stereoselectivity.³ Previously, our laboratory engineered sperm whale myoglobin (Mb) into novel biocatalysts for the asymmetric synthesis of medicinally important motifs, such as optically active cyclopropanes and polycyclic heterocycles with high activity and stereoselectivity.⁴⁻⁵

We recently developed a chemoenzymatic strategy for the stereoselective assembly and structural diversification of cyclopropyl ketones, a highly versatile yet underexploited class of functionalized cyclopropanes. This was done by employing engineered Mb catalysts capable of producing enantiopure keto-functionalized cyclopropanes, coupled with multi-site chemical diversification of enzymatically generated products. As shown in **Figure 1**, we developed a Mb catalyst for the highly stereoselective cyclopropanation of diazoketones to generate a remarkable substrate scope of cyclopropyl ketones, obtaining 28 different products with excellent level of enantio- and diastereoselectivity (86-99% de, 91-99% ee). We further demonstrated that these enzymatically generated carbonyl cyclopropanes are amenable to chemical diversification to produce a wide array of valuable asymmetric building blocks. Specifically, we obtained ten different cyclopropane-containing motifs that are challenging to obtain via traditional synthetic methods and are of potentially high value for accessing new bioactive molecules. This work represents the first example of a highly stereoselective intermolecular cyclopropanation of diazoketones involving an iron-based catalyst. It also demonstrates the power of combining biocatalysis and organic synthesis to access a collection of synthetically useful optically active synthons. This work was recently published in *J. Am. Chem. Soc.*⁶

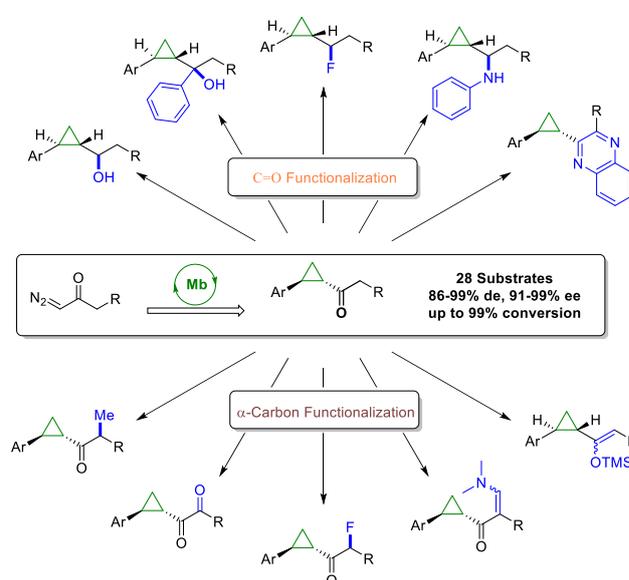


Figure 1. Chemoenzymatic Assembly of Cyclopropane Scaffolds via Mb-catalyzed Cyclopropanation and Chemical Diversification

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Mining of novel enzymes for biomass and plastic degradation in the Actinobacteria strain collection isolated from Trondheim's fjord, Norway

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Actinobacteria has been known as a rich repository of enzymes with diverse functions such as breaking down of biomass or plastic degradation, the two applications that greatly contribute to the development of bioeconomy and solution for plastic pollution[1]. Through the long-term collaborated bioprospecting activities between SINTEF and the Norwegian University of Science and Technology (NTNU), a Actinobacteria strain collection isolated from Trondheim fjord, Norway has been established in which more than 1200 samples have been sequenced.

Bacterial enzymes which can degrade lignin such as multicopper oxidases and peroxidases have been identified from species of the Actinobacteria phylum. A family of small two-domain laccases (SLAC) from Actinobacteria which was firstly described from *Streptomyces coelicolor* and later in other species have been shown to degrade lignin's model compounds.[2] Degradation of lignin substrates were also observed in various dye de-colourising (Dyp-type) peroxidases-from Actinobacteria species such as *Rhodococcus* spp., *Amycolatopsis* spp., and *Thermobifida* spp.[3] In the OXYMOD project funded by The Norwegian Research Council, to identify enzymes that can potentially degrade lignin into high value aromatic building blocks, the data mining approach was applied to the genomes of the strain collection using publicly available profiles Hidden Markov Model (profiles HMM) or newly generated from the sequences of Hfam39 family obtained from the BioCatNet Laccase and Multicopper Oxidase Engineering Database (LccED).[4] Various potential candidates have been identified from the mining including a thermostable SLAC-type multicopper oxidase and a Dyp-type peroxidase whose activities were confirmed. The enzymes showed activity against different phenolic substrates and lignin model compounds making them promising candidates for further investigation. Bio or enzymatic degradation of polyethylene terephthalate (PET) and other types of plastic has recently gained attention as an alternative or complementary approach for solving the issue of plastic pollution. In the publication of Danso et al. in 2018,[5] bioinformatic screening of public sequence databases provided an indication that many putative PET-degrading hydrolases (PETases) could potentially be found from the Actinobacteria phylum. The same data mining pipeline was carried out in the MarBioTech project funded by ERA-MBT using the PETase profile HMM provided by the University of Hamburg to identify potential PET degrading enzymes from the strain collection. Several candidates have been identified by narrowing down the data mining hits using the Sequence Similarity Network approach. The activities of the enzymes against PET and polycaprolactone (PCL) have been investigated which will then serve as a basis for further studies of plastic degradation by Actinobacteria species. In conclusion, Actinobacteria species from Trondheim's fjord have been shown to be a genetic repository with the great potential of finding new enzymes in the application of biomass and plastic degradation.

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Sulfuryl transfer catalysed by different arylsulfate sulfotransferases using alternative donor and acceptor substrates

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Sulfated compounds play an important role in biological functions such as signal transduction, extracellular interaction, molecular recognition, and detoxification. Furthermore, sulfated molecules are involved in the synthesis of a broad range of everyday chemical commodities like detergents, pigments, agrochemicals, etc. [1]. Unfortunately, chemical sulfurylation methods generally entail the use of harsh chemical conditions such as the presence of highly reactive compounds, acidic conditions, organic solvents or high temperatures [2]. Moreover, they often display low chemo- and/or regioselectivity and unwanted side reactions. Alternatively, the development of enzymatic-based strategies for sulfurylation of the desired compounds may have some advantages, since they could be performed under mild conditions and enzymes usually display high chemo- and regioselectivity [3]. In this sense, sulfotransferases are the obvious choice for this task since their natural role is to catalyse the transfer of a sulfuryl group from an activated donor to the hydroxy group of an acceptor.

The work presented here describes the application of several arylsulfate sulfotransferases (ASSTs) either (i) selected from the literature [*i.e.* ASSTA and ASSTB from *D. hafniense*, ASST from *H. ochraceum* (ASSTHoch), ASTIV from rat liver (*R. norvegicus*), ASST LipB and ASST Cpz4 from *Streptomyces sp* and STF9 from *M. avium*] or (ii) found by BLAST analysis using ASSTA as template [ASST from *D. dehalogenans* (ASSTDdeh), ASST from *D. formicoaceticum* (ASSTDfor), ASST from *D. hafniense* (ASSTDhaf03), ASST from *H. effluvia* (ASSTHeff) and ASST from *D. orientis* (ASSTDor)] [4]. For the novel ASSTs, a study on the influence of the temperature, pH and the presence of organic co-solvents on the activity was performed. The results obtained revealed that these enzymes were active between 25 and 45 °C, their optimum pH was pH 8.0-9.0 and their tolerance towards the presence of cosolvents strongly depends on the solvent used, being isopropanol the best tolerated. Additionally, the acceptance of alternative sulfuryl donors, such as *N*-hydroxysuccinimide sulfate (NHSS), SO₃-pyridine (PyrSO₃), SO₃-trimethylamine (Me₃NSO₃) or SO₃-trimethylamine (Et₃NSO₃), instead of *p*-nitrophenyl sulfate (*p*NPS), by ASSTs was investigated. All enzymes tested showed activity when using NHSS, with the exception of STF9. Concerning the other amine complexes employed, ASSTB, LipB, Cpz4, ASSTDfor, ASSTDhaf03 and ASSTDdeh were able to form catechol monosulfate when using Me₃NSO₃ and Et₃NSO₃. In contrast, no product formation was observed in the reactions containing PyrSO₃. Moreover, a study of the sulfuryl acceptor substrate scope was performed by measuring the ASST activity displayed toward 15 different compounds, including aromatic and aliphatic compounds bearing both hydroxyl and amine groups. In this regard, each ASSTs showed a characteristic substrate selectivity, most of them being able to sulfurylate both, aromatic and aliphatic compounds, although at lower rates for the later ones.

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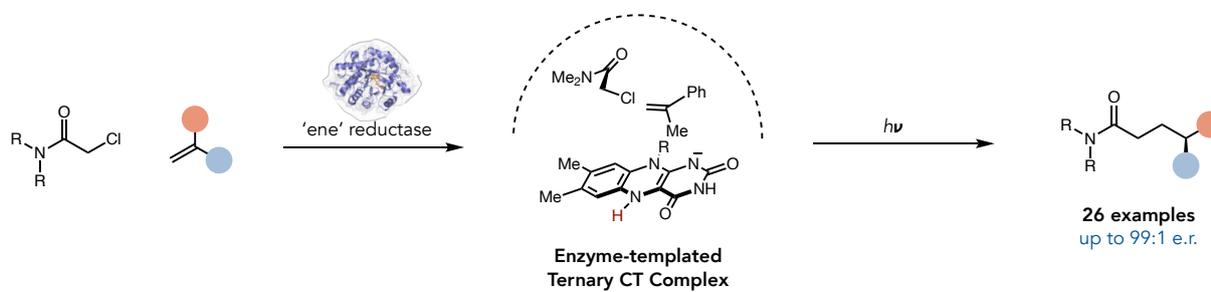
Light-enabled Enzymatic Intermolecular Hydroalkylation of Alkenes

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Intermolecular C-C bond forming reactions are highly desirable reactions in organic chemistry due to their ability to generate complexity quickly to form synthetically valuable products. Enzymes would be ideal catalysts to make C-C bonds due to their exquisite selectivity and control of reactive intermediates. Unfortunately, the types of intermolecular C-C bond forming reactions that can be performed by biocatalysts using their native mechanisms are extremely limited [1]. This poster demonstrates how flavin-dependent enzymes, 'ene'-reductases (EREDs), can catalyze an intermolecular coupling between α -chloroamides and a variety of alkenes to form C-C bonds [2]. Excitingly, the enzyme shows excellent control for setting a remote stereocenter and both enantiomers can be accessed by using two different EREDs. This reaction can be scaled up to the gram scale using cell-free lyophilized lysate. Mechanistic studies show the enzyme gates radical formation to occur only when both coupling partners are in the active site.



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Comparative study and optimization of lignocellulosic biomass pretreatment methods

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Lignocellulosic biomass is the most important renewable resource for the production of biochemicals and biofuels, serving as a viable alternative to fossil fuels. The main obstacle in the valorization of lignocellulosic biomass is the requirement of a pretreatment step in the process. [1,2]

Although many pretreatment methods were already investigated, most of them are high energy consumers and pollute the environment. For this reason, the present work proposes an innovative approach regarding biomass pretreatment and enzymatic hydrolysis, that can improve the exploitation of the biomass components. Five types of lignocellulosic biomass (hardwoods, softwoods, wheat straw, cardboard and mixture) were exposed to different physico-chemical pretreatment methods (hot water, ultrasounds, hot water and ultrasounds, concentrated acetic acid, and an unconventional solvent – the ionic liquid 1-ethyl-3-methylimidazolium acetate). The pretreated biomass was subjected to enzymatic hydrolysis with two commercially available cellulases (Accelerase 1000, and Cellic CTec2), under optimized reaction conditions.

Lignocellulosic biomass

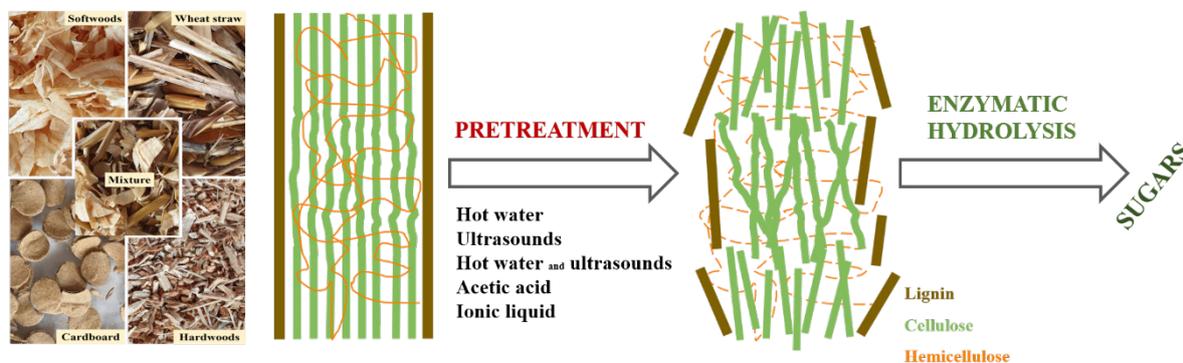


Figure 1. Schematic representation of lignocellulosic biomass pretreatment

Considering that among the selected types of lignocellulosic biomass, wheat straw is the largest biomass feedstock in Europe and the second largest in the world [3], we selected it to further optimize the ultrasound-assisted pretreatment by experimental design. Temperature, biomass loading, and pretreatment time were identified as major factors affecting the sugar conversion yield.

Acknowledgements

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Synthesis of Natural Products and Key Pharmaceuticals by 2-Oxoglutarate Dependent Dioxygenases

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2-Oxoglutarate dependent dioxygenases (2-ODDs) are non-heme Fe^{II}-containing dioxygenases that activate molecular oxygen via decarboxylation of 2-oxoglutarate to perform a wide range of oxidative reactions. However, transformations catalyzed by 2-ODDs are only rarely exploited for biocatalytic purposes to date. Recently, we could show that a 2-ODD biocatalyst can facilitate key-steps in a chemoenzymatic synthesis of pharmaceutically important lignan natural products.^[1] In this study, a 2-ODD from *Podophyllum hexandrum* was implemented for the stereoselective, oxidative cyclisation of the readily prepared yatein precursor to (deoxy- and *epi*-) podophyllotoxin. The biocatalytic C–C bond formation has been run on preparative-scale (up to 2 g) to yield the enantiomerically pure *epi*-podophyllotoxin in 32% overall yield over 4 steps.^[1]

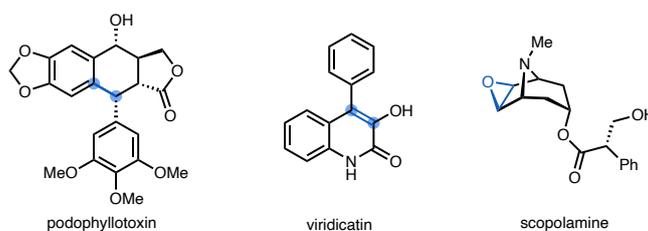


Figure 1. Natural products obtained via 2-ODDs biotransformations. The respective bonds and functional groups introduced by 2ODDs are highlighted in blue.

Currently, the focus of research has been put on other 2-ODDs, namely the hyoscyamine 6 β -hydroxylase (H6H, a key enzyme in the biosynthesis of scopolamine)^[2] and AsqJ from *Aspergillus nidulans* (involved in the biosynthesis of quinolone antibiotics like viridicatin).^[3] In addition to probing their scope of substrates we want to gain a deeper understanding of the underlying mechanistic principles of operation and deactivation in this class of enzymes (a common problem of 2-ODDs).^[4] The insight gained here may aid future implementation of 2-ODDs in chemoenzymatic syntheses and promote them as valuable biocatalysts for challenging (bio-)chemical transformations.

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Upgrading 5-(hydroxymethylfurfural) for the straightforward synthesis of 5-hydroxymethylfurfurylamine

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The synthesis of biomass-based platform chemicals has gained recent interest due to the increasing need for greener and sustainable alternatives to fossil-based industrial chemicals and products. In this context, 5-hydroxymethylfurfural (HMF) and its derivatives, such as 5-hydroxymethylfurfurylamine (HMFA), are some of the most studied and exploited bio-derived compounds and currently being considered as key platform molecules for the production of pharmaceuticals and commodity chemicals.[1,2]

It is worth noting that procedures already reported to afford HMFA are typically very low-yielding or require the use of unsustainable metal-based catalysts under harsh reaction conditions.[3] Amine transaminases provide a greener solution through straightforward transformations. Nevertheless, amine product isolation and the achievement of high conversion require an exhaustive study to implement an efficient HMFA synthesis.[4] Herein, the screening and application of different amine transaminases is described (Figure 1), rendering the desired HMFA with good conversion values in a very selective manner. Special attention has been paid to the optimization of the reaction conditions in terms of substrate concentration, amine donor source and enzyme loading.

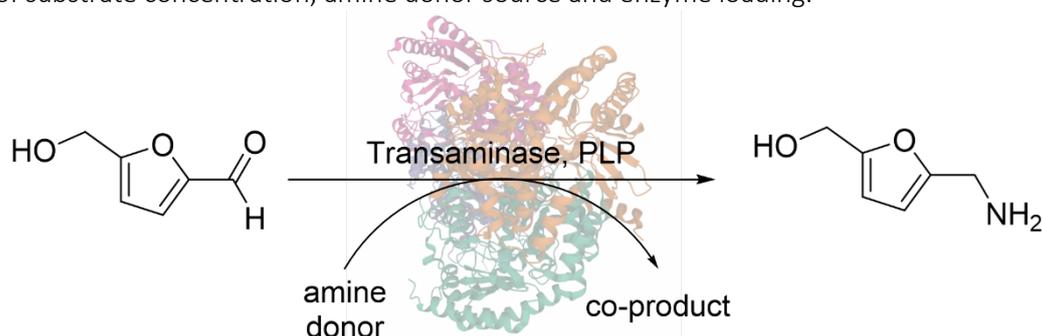


Figure 1. Transaminase-catalysed reaction of HMF towards HMFA.

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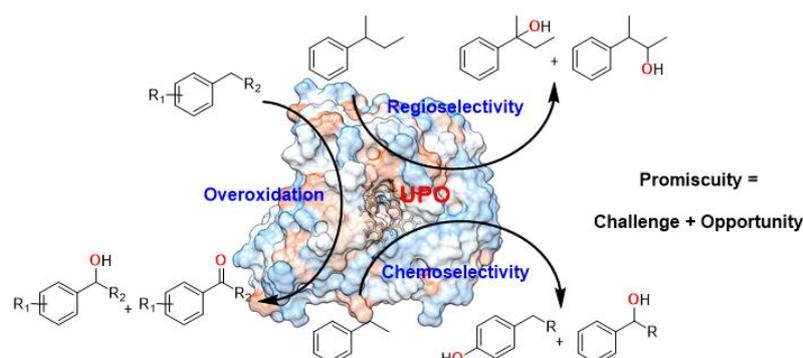
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Practical and Scalable Applications of the Unspecific Peroxygenase from *Agrocybe aegerita*

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The fungal unspecific peroxygenases (UPOs) were first identified in *Agrocybe aegerita* in 2004^[1]. UPOs are capable of catalysing versatile oxidations, including hydroxylation of non-activated C-H bonds, which are underrepresented among industrial biotransformations^[2]. Thanks to their robustness and efficient utilisation of hydroperoxides, UPOs are in the focus of intensive research, helped by the development of systems enabling the heterologous expression of these enzymes in *Pichia pastoris* host^[3].

The two major challenges facing UPO-catalysed transformations are the oxidative deactivation of the enzyme and the often poor selectivity due to promiscuous enzyme activity. The former could be addressed by reaction optimisation, whilst our current research endeavours are directed towards understanding the selectivity of a variant of *Agrocybe aegerita* UPO (rAaeUPO)^[3]. In an effort to do this, substrate screening was combined with the scaleup of promiscuous reactions, thus enabling the characterisation of major products.

The alcohol oxidation activity of rAaeUPO was chosen for in-depth study, as it remains a pervasive side-reaction during hydroxylation reactions and it is also a useful transformation in its own right. The enzyme was found to perform the oxidation of a wide range of primary and secondary alcohols. Detailed investigation of the kinetics and mechanism of these reactions also shone light on the basis of the curious reactivity trends across different substrate classes.

To demonstrate the selectivity and scalability of rAaeUPO-catalysed oxidations, the enzyme-catalysed Achmatowicz and *aza*-Achmatowicz reactions were performed on scale, often showing better performance than existing chemical or biocatalytic methods^[4]. These transformations even showed robust performance at substrate loadings as high as 0.4 M. The presented results thus highlight the utility of UPOs as versatile oxidative biocatalysts.

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Laccase-mediated synthesis of bioactive dyes

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The laccase-mediated biotransformation of simple organic chemicals can be an effective tool for environment-friendly synthesis of new products about new properties and new possible applications. New compounds about dyeing, antioxidative and antimicrobial properties can be obtained during action of laccase on phenolic compounds and aromatic amines in buffered conditions. The oxidation of precursors requires only molecular oxygen, the purest co-substrate of the reaction. The poor solubility of phenolic compounds and aromatic amines in water prompts the addition of small amounts of organic solvents, increases the number of compounds as potential laccase's substrates and allows the use of their higher concentrations. Additionally, a low concentration of organic solvents affects the number of specific interactions between reactive radicals of substrates and the formation of products. In the case of laccase-mediated catalysis, it is sometimes very difficult of direct formation of only one main product. This is because the final step of product synthesis, from reactive and unstable radical forms of substrate, occurs spontaneously and often unpredictably [1,2].

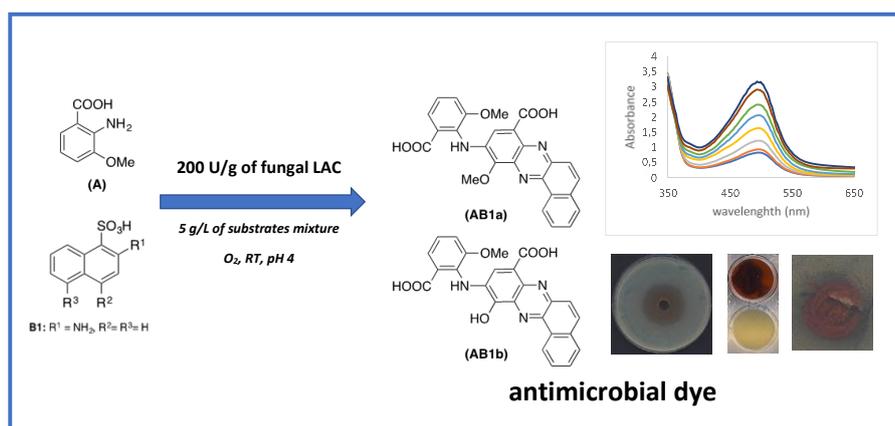


Figure 1. Scheme of the laccase-mediated synthesis of antimicrobial dyes [3].

The aim of this work was the transformation of different organic compounds into bioactive products about antimicrobial and antioxidative potential. Varying reaction conditions, such as the different pH, co-solvents presence or laccase and substrate concentrations, were optimized to achieve the high yield of bioactive compounds synthesis using fungal laccase as the catalyst.

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Smart methyl acceptors as game changers for methyltransferases

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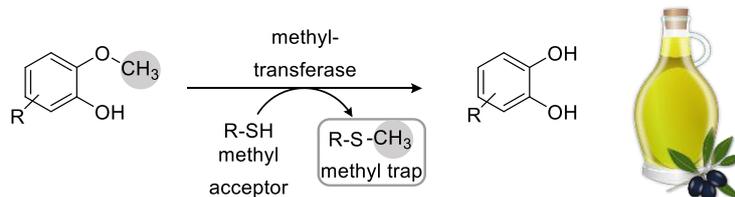
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Biocatalytic dealkylation of ethers is an attractive option to generate phenolics, an endemic functionality among bioactive compounds. Monooxygenases have previously been investigated, however, they require molecular oxygen, which may impact on the yield of the desired product due to unwanted oxidative side reactions [1]. Cobalamin methyltransferases shuttle methyl groups between structural related molecules, and they have successfully been applied for the demethylation of natural compounds [2,3]. The biotransformation is conveniently performed in anaerobic and mild conditions, yet the reaction equilibrium is the main limiting factor. We investigated thiols as alternative smart methyl donors. The methyl group is trapped in the form of a thio-ether co-product, thus shifting the equilibrium toward the demethylation of the guaiacol derivatives (Scheme 1). Using ethyl 3-mercaptopropionate a broad panel of methoxy aromatics was demethylated with conversions mostly above 90%. Among these, homovanillyl alcohol was efficiently demethylated to the valuable antioxidant hydroxytyrosol and 97% isolated yield was obtained from one-gram scale biotransformation. The process may be applied for the synthesis of the certified-natural equivalent.



Scheme 1. Oxygen-free biocatalytic demethylation of guaiacol using various thiols as methyl acceptor.

Acknowledgments

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Development of a Flow Process using an Immobilized Enzyme for the Transamination of Cyrene

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Biocatalysis, protein engineering, and flow chemistry are enabling technologies for process chemistry that together can be used to create highly efficient and sustainable manufacturing processes. While many industrial processes have been developed using free enzymes in aqueous solutions, immobilization of enzymes on a solid support can have many advantages, including improved reaction efficiency, improved enzyme stability, the ability to perform reactions in non-aqueous media, and simplified separation of products from catalysts.[1,2] This presentation will describe the development of a process using an immobilized transaminase for the reductive amination of cyrene, a bio-based feedstock chemical derived from cellulose. The product of this transformation, which we term cyrene amine, is a key chiral amine required for the synthesis of an experimental Bruton's tyrosine kinase inhibitor for the treatment of B-cell cancers.

To achieve a synthesis of cyrene amine, we identified a transaminase for the amination of cyrene with isopropylamine, and subsequently engineered the enzyme by directed evolution for improved activity, diastereoselectivity, and thermostability. Resins were then identified which allowed for efficient enzyme immobilization while retaining the enzyme's activity in water-saturated organic solvents. Performing the reaction in predominantly organic media enabled simplified isolation of the product via crystallization as the tosylate salt, as well as enabled facile separation of the enzyme from the amine product. Further streamlining, process improvements, and process risk reduction were achieved by performing the reaction in flow, passing the stream of reagents through a packed bed reactor containing the immobilized enzyme. The final process combines two renewable, bio-based solvents (cyrene and 2-MeTHF) with isopropylamine and an immobilized transaminase catalyst to efficiently produce a pharmaceutical intermediate.

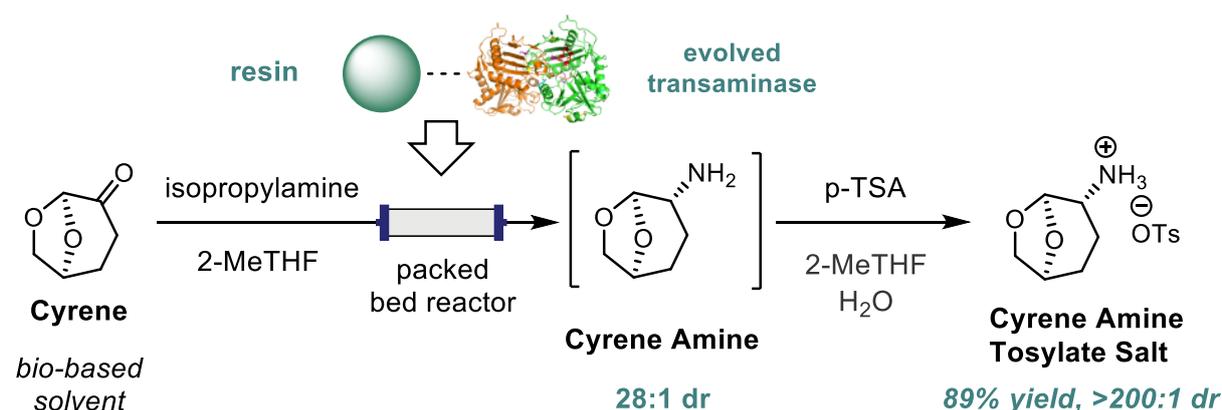


Figure 1. Immobilization of an evolved transaminase enables streamlined production and isolation of a key chiral amine building block from the renewable solvent cyrene.

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Scalable enzymatic synthesis of fragrant acetates

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In perfumery and flavour compositions, acetates are the most important aliphatic esters conferring pleasant organoleptic properties to the products. [1] Although most of these bioactive compounds are naturally sourced, their isolation often makes the process economically unfeasible. In order to fulfil the ever growing demands, numerous chemical procedures have been successfully developed. However, increasing health consciousness and environmental concerns spur the development of benign yet efficient biocatalytic protocols as viable green alternatives.

Lipase-catalysed reactions have received considerable attention due to the robust activity, remarkable selectivity, and stability of these enzymes in non-aqueous environments, particularly under solvent-free conditions. [2] In this regard, glycol-based solvents, which can also serve as an acetyl donors in transesterification reactions, represent a promising green alternative to the conventional reaction media. Among them, glycerol triacetate (GTA) was recently applied as both an esterification agent and solvent in enzymatic acetylation of a number of alcohols. [3] Due to some advantageous properties of the commercially available ethylene glycol diacetate (EGDA) compared to GTA - such as lower boiling point and viscosity, better recyclability, and regeneration - it can be considered as an interesting substitute.

Thus, we have investigated the lipase-mediated acetylation of structurally diverse alcohols in neat EGDA (*Figure 1*) generating a range of natural acetates for perfumery and/or flavour purposes in good yields and remarkable selectivity. [4]

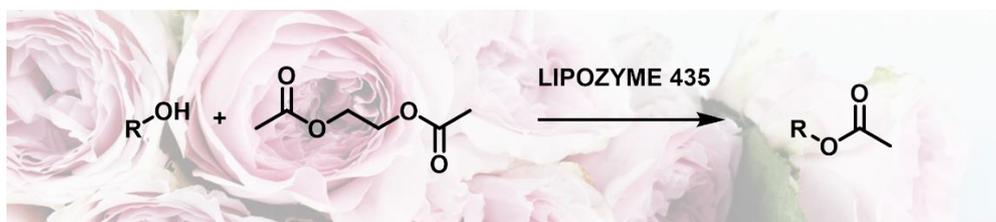


Figure 1. Lipase-catalysed acetylation of alcohols furnishing fragrant acetates.

Such enzymatic system exhibits high reactivity and chemoselectivity towards both activated (allylic) and non-activated (primary/secondary) alcohols. The scalability of the optimised conditions was demonstrated by multigram synthesis of fragrant (*Z*)-hex-3-en-1-yl acetate in 70% yield. The Lipozyme 435/EGDA system also enabled site-selective acetylation of (hydroxyalkyl) phenols as well as the kinetic resolution of chiral secondary alcohols. Finally, its discrimination power was illustrated by model competitive experiments of equimolar mixtures of two isomeric alcohols. As a result, the practical synthesis and isolation of pure 1-pentyl acetate as a single product in 68% yield (based on 1-pentanol) from the equimolar mixture of 1-pentanol and 3-pentanol was achieved.

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Stereoselective monoreduction of bulky 1,2-dicarbonyls catalyzed by a benzyl reductase from *Pichia glucozyma* (KRED1-Pglu)

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Enantiomerically enriched hydroxyketones are well-established intermediates for the synthesis of several bioactive compounds [1] and can be chemically obtained by stereoselective reduction of one of the carbonyl moieties of the corresponding diketones. However, enzymatic strategies are characterized by higher catalytic efficiency, milder reaction conditions, higher stereo- and regioselectivity, and fewer numbers of synthetic steps. Therefore, they can be chosen as convenient and environmentally friendly alternatives.[2]

A NADPH-dependent benzyl reductase from the non-conventional yeast *Pichia glucozyma* (KRED1-Pglu [3]) was over-expressed in *E. coli*, purified and exploited to catalyze the asymmetric monoreduction of bulky aromatic 1,2-dicarbonyl compounds (**Figure 1**). The cofactor was recycled by an enzyme-coupled system (glucose-glucose dehydrogenase (GDH) from *Bacillus megaterium*). The recombinant KRED1-Pglu showed a wide range of activity (24-97% conversion) and excellent stereoselectivity (*ee* \geq 96% in all but one case). On the contrary, it proved either inactive or very poorly active towards most 1,3- and 1,4-dicarbonyls tested as potential substrates. In order to understand this peculiar behavior, the enzyme was crystallized (1.77 Å resolution) and its active site was investigated to identify the recognition residues involved in the desymmetrization reaction. QM and classical calculations also allowed for a proposal of the catalytic mechanism, along with an *in silico* reactivity prediction.[4]

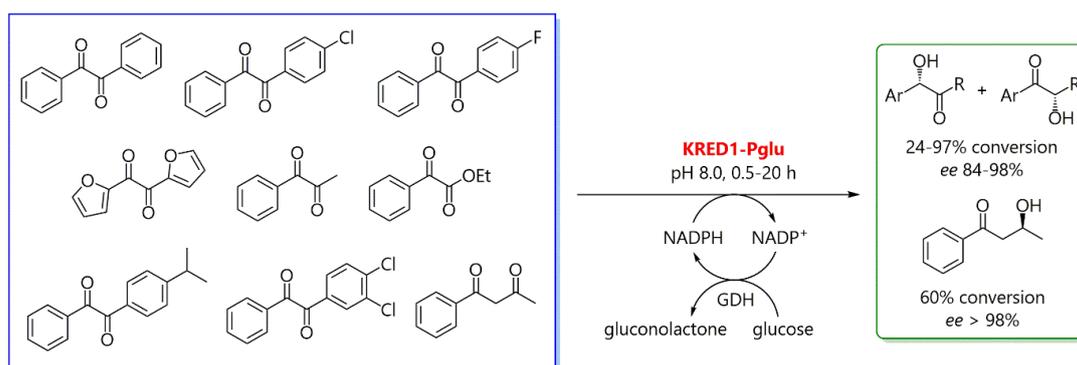


Figure 1. Stereoselective monoreductions of dicarbonyls catalyzed by KRED1-Pglu.

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Advanced morphological characterization of His-tagged immobilized enzymes by means of infrared microscopy

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The research is a part of the EU-ITN INTERfaces project and aims to develop novel methodologies for the advanced characterization and rational optimization of carriers for compartmentalized immobilization of enzymes (<http://www.h2020interfaces.eu>). This approach involves correlation of the morphological properties and physical-chemical characteristics of the carriers with the information on the structure and conformational behaviour of the enzyme.

EziG™ carriers (EnginZyme AB, Sweden) are a class of materials that have a controlled porosity glass (CPG) core and are coated with different organic polymers. They contain non-toxic chelated Fe³⁺ for the binding of His-tagged enzymes, which gives higher affinity and enables extensive reuse¹. A significant part of the project is focused on understanding how the structure and activity of proteins are modified after the immobilization. This approach requires detailed morphological characterization of the carriers, as well as an understanding of the interactions and conformational changes that occur at the carrier-enzyme interface². Using Infrared microscopy, the vibrational spectra are recorded for every single sample compared to a reference sample. The carriers are characterized spectroscopically by the Fourier transform FTIR. Once the enzyme is immobilized, FTIR microscopy offers information related to that the distribution of the enzyme at the micron scale (enzyme diffusivity)³.

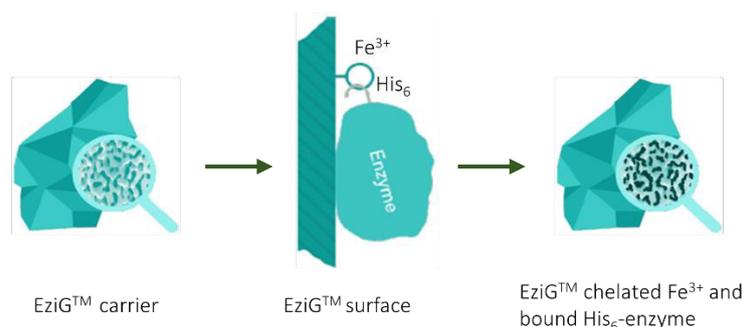


Figure 1. EziG™: immobilization of His-tag enzymes on their surface

The final goal will be the application of optimized immobilized enzymes in chemo-enzymatic cascade reactions in low water media, which will be exploited to transform the bio-based monomer, such as hydroxymethylfurfural (HMF), into other valuable chemical building blocks.



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Evaluation of P450 activity in lyophilized recombinant *E. coli* cells

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Cytochrome P450 monooxygenases (P450s or CYPs) are heme-thiolate proteins that catalyse several types of oxidation reactions and accept a wide range of substrates, including xenobiotics and steroids. Since the past three decades, the interest to this group of enzymes as potential candidates for biocatalysis has been increasing due to their ability to introduce oxygen into non-activated C-H-bonds, often at specific positions [1].

To perform oxidation reactions, P450s rely on redox equivalents that are usually derived from the nicotinamide cofactors NAD(P)H and transferred to the heme group via redox partner proteins. Often, the use of recombinant microbial cells expressing P450s allows to overcome the cofactor dependency issue. However, despite their potential, a broader use of P450 based whole-cell biocatalysts is often limited by insufficient uptake of substrates and efflux of products through the cell membrane [1].

In this work, an *E. coli* whole-cell biocatalyst harbouring CYP105D from *Streptomyces platensis* DSM 40041 was used as model system for the oxidation of testosterone to 2 β -hydroxytestosterone (Figure 1) [2]. The chosen redox partner system originates from *Pseudomonas putida* and involves the FAD-containing, NADH-dependent putidareductase (PdR) and the 2Fe-2S putidaredoxin (PdX) [3].

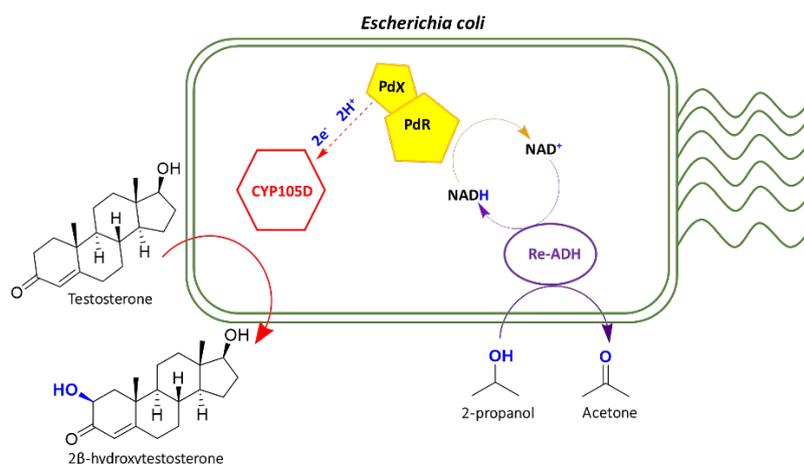


Figure 1. Schematic overview of the oxidation of testosterone using CYP105D from *S. platensis* in *E. coli*. Putidaredoxin reductase (PdR) and putidaredoxin (PdX) from *P. putida* are used as redox partners for CYP105D. The alcohol dehydrogenase (Re-ADH) from *R. erythropolis* was used for cofactor regeneration.

Testosterone conversion was first enhanced by optimized handling of resting *E. coli* cells. Further, the usage of lyophilized recombinant cells carrying the target enzymes has been found as an attractive alternative for testosterone oxidation. In this regard, while the co-expression of the alcohol dehydrogenase from *Rhodococcus erythropolis* (Re-ADH) did not affect P450 concentration in the cell, its presence was mandatory for NADH regeneration and thus for sufficient P450 activity with lyophilized recombinant cells. Optimization of the lyophilization procedure enabled conversions of testosterone comparable with resting cells.

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Copper Radical Alcohol Oxidases, tasteful biocatalysts

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Copper Radical Oxidases (CROs), have been for decades represented almost exclusively by the archetypal Galactose 6-Oxidase, concealing a vast family that starts to reveal an unsuspected diversity.[1,2] The recent discovery of a new phylogenetic group of CROs, so-called primary alcohol oxidases (CRO-AlcOx),[3] opens the way towards alternative routes to alcohol oxidation, a cornerstone reaction in fine and organic chemistry. In this study, we harnessed CRO-AlcOx as potent new catalysts for the production of aldehydes for the flavors and fragrances industry. We established the operating conditions for the production of odorant aromatic and fatty aldehydes using a CRO-AlcOx from the phytopathogenic fungus *Colletotrichum graminicola*. [4] Through biocatalytic conversions experiments combined to *in silico* modelling, we provided comprehensive insights into the mechanism of CRO-AlcOx oxidation of activated and unactivated alcohols, substrate-dependent overoxidation, and alkyl-gem-diol inhibition. We also demonstrated the applicability of the *C. graminicola* CRO-AlcOx in a bi-enzymatic cascade with an ene-reductase, for the enantioselective production of (*R*)-citronellal, a key intermediate in industrial route to (-)-menthol. Altogether, these results constitute a proof of concept of CRO-AlcOx as biocatalysts for flavors and fragrances applications and contribute to a better understanding and control towards the mastery of these promising enzymes.

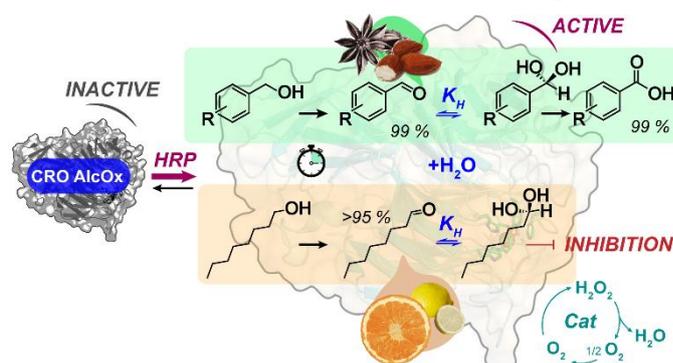


Figure 1. CRO-AlcOx oxidation of aromatic and aliphatic alcohols for flavors and fragrances applications.

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Enzyme-conjugates for diagnostics

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Nature provides us with an inspiring wealth of different proteins. Some biopolymers, for instance, have repetitive amino acid sequences, which give them special properties such as strength or elasticity. Others, such as transmembrane glycoproteins of viruses, may be used in diagnostics or as vaccines. Yet others can be applied as catalysts in drug design. Recombinant DNA-technologies offer viable alternatives to molecules of human or animal origin (i.e. human-recombinant trypsin), which are neither safe nor ethically acceptable, and provide unlimited access to natural compounds that are poorly accessible by extraction from plants, fungi and viral sources.

Our mission is to find solutions to produce protein-based biomaterials and enzymes using the yeast *Pichia pastoris*. The dramatic COVID-19 situation and the associated millions of deaths led to our first available product: the SARS-CoV-2 spike protein and variants.

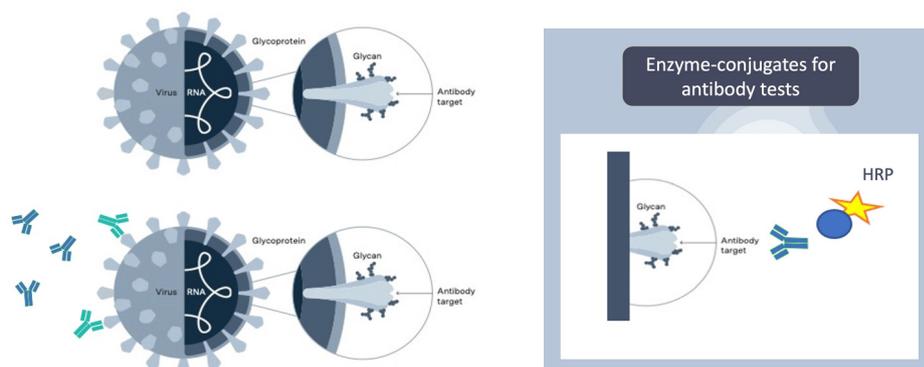


Figure 1. *P. pastoris* recombinant SARS-CoV-2 spike protein and protein-enzyme conjugates for diagnostics.

Our recombinant SARS-CoV-2 antigens find application in research, diagnostics or can even be tested as a vaccine. bisy BIOS also focuses on product applications, i.e. the development of novel strategies for antibody testing. We are aiming for an antibody test made of *Pichia* produced components only. Currently, we are evaluating different antigen variants and protein-enzyme conjugates.

Next to antigens we also target all kinds of enzymes for application in biocatalysis.

Development of a facile synthesis of L-theanine catalyzed by immobilized *B. subtilis* γ -glutamyl-transferase

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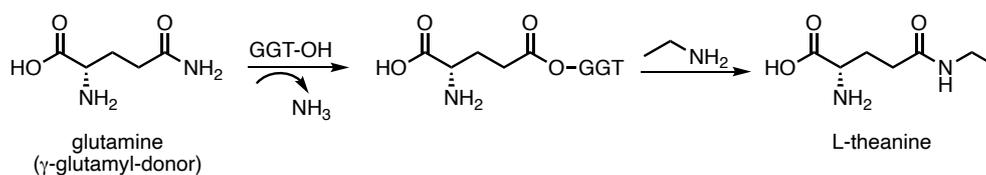
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L-Theanine (L-Th; γ -glutamylethylamide), a unique amino acid found in green tea, is a valuable FDA-approved nutraceutical product with recognized health benefits and umami taste properties.[1] To meet the increasing demand of L-Th from pharmaceutical and food industries, several methods for its production have been developed (*i.e.*, extraction from green tea leaves, chemical, or enzymatic synthesis). Extraction is wearisome and time consuming, whereas chemical routes are made troublesome by the extensive use of protective groups. Biocatalysis provides, instead, a more straightforward path: γ -glutamyl transferases (GGTs, E.C. 2.3.2.2) catalyze the transfer of the γ -glutamyl moiety of glutamine or other γ -glutamyl compounds (*e.g.*, glutathione) to natural or modified amino acids, thus producing γ -glutamyl derivatives (Scheme).[2]



Scheme. GGT-catalyzed synthesis of L-theanine.

The GGT from *Bacillus subtilis* (*BsGGT*), an organism generally recognized as safe (GRAS), was immobilized on glyoxyl-agarose (GLX-AG) resulting in high protein immobilization yield and activity recovery (>95%). The synthesis of L-Th was accomplished by simply mixing the reactants (L-glutamine and ethylamine) in the presence of *BsGGT*-GLX-AG at 25 °C; neither buffers, nor other additives were needed. Donor/acceptor ratio, pH, enzymatic units and reaction time were screened. Under optimized conditions, L-Th was obtained in 70% isolated yield in a very high purity.

The product was recovered through a simple purification protocol based on removal of the biocatalyst, distillation of the volatiles, concentration of the reaction mixture and precipitation with ethanol. Immobilized *BsGGT* was recovered and re-used for five consecutive reaction cycles and retained 100% activity after ten months at 4 °C. This procedure represents a green, easy, fast and scalable approach to L-Th synthesis.

Acknowledgements

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Iridium complex catalyzed *in solutio* reduction for flavin hydroquinone-dependent monooxygenases

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Harnessing abiotic reactions to drive enzymes and reveal their unique catalytic properties is the wrench to expand bio-catalytic reaction systems. Flavoproteins are destined to bridge the abiotic and the biotic redox sphere possessing flavin cofactors as efficient and versatile electron mediators and flavoprotein monooxygenases (FPMOs) are particularly interesting, as these enzymes activate O₂ as a green co-substrate and insert a single oxygen into their substrate, while the other oxygen atom is reduced to water. Structurally, there are two main types of FPMOs i) single-component and ii) two-component enzymes. Two-component flavoprotein monooxygenases (FPMOs) consist of a reductase and an oxygenase enzyme.[1] The proof of functionality of the latter without its counterpart has been demonstrated for some representatives of this group of flavin-dependent enzymes, such as recently for the 2,5-DKCMO, a FMNH₂-dependent type II Baeyer-Villiger monooxygenase.[2] In the natural system flavin is the hydride shuttle between the reductase and the oxygenase, which requires the flavin to be either in the fully oxidized (for the reductase activity), or the fully reduced (two-electron reduced) state (for the oxygenase activity), see **Figure 1a**. The independence of the oxygenase of two-component FPMOs from its reductase counterpart allows the implementation of novel methods for the hydride transfer (two-electrons) to recycle fully reduced from fully oxidized flavin and hence to recycle flavin in reductase-free systems. Herein, we present the implementation of [Cp*Ir(bpy-OMe)H]⁺, a pH-robust Iridium^{III} complex [3], known in e.g. photochemical formic acid dehydrogenation, or the reductive alkylation of proteins [4], but no hydride transfer to process enzymatic reactions, to drive the enzymatic reaction of FMNH₂- and FADH₂-dependent monooxygenases, as shown in **Figure 1b**.

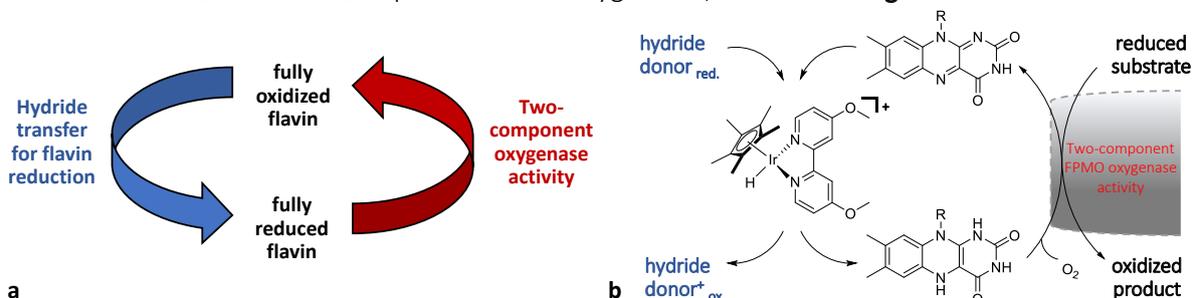


Figure 1. Flavin recycling for two-component FPMOs through the (abiotic) flavin reduction (in blue) and the (re)oxidation of the fully reduced flavin (in red) as the result of the flavin hydroquinone-dependent monooxygenase activity.

The Baeyer-Villiger oxidation by 2,5-DKCMO and the epoxidation of several styrene derivatives by StStyA, as (bio)catalytic model systems, remained processing for several hours. [Cp*Ir(bpy-OMe)H]⁺ did not only allow us to accelerate the nicotinamide-driven reaction setup, but also to apply formic acid as a co-substrate. We expect this methodology to be transferable to other flavin hydroquinone-dependent enzymes and to benefit as a future technology for *in situ* flavin reduction for any enzyme catalysis.

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Biocatalysis in green and blue: Cyanobacteria

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Cyanobacteria, ancient, photosynthesizing prokaryotes, have gained interest as host for sustainable production of fine and bulk chemicals. Yet, the photosynthetic metabolism shows also promising characteristics for whole cell catalysis, such as *in situ* production of oxygen, high NADPH/NADH ratio and the sustainable recycling of co-factors (e.g. ATP) by the photosynthesis apparatus [1].

An ideal sustainable process, does not only reduce (or even capture) atmospheric CO₂, but also valorises waste-products. Ferulic acid for example is an aromatic compound from lignocellulosic biomass degradation. We are planning to establish the transformation of ferulic acid, as a cheap waste-product, to Vanillin, a value-added compound, in a two-step enzyme reaction, with the Cyanobacterium *Synechococcus elongatus* PCC11901.

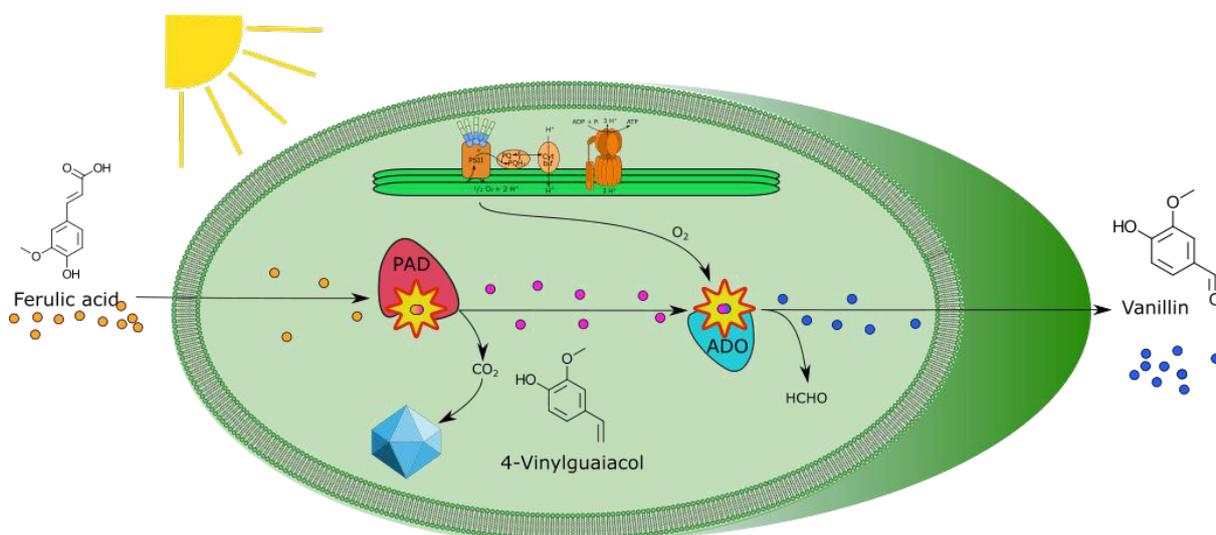


Figure 1. Conversion of Ferulic acid to Vanillin in a two-step reaction, while exploiting the photosynthesis apparatus.

Synechococcus elongatus PCC11901 is a recently discovered marine cyanobacterium, a close relative to *Synechococcus elongatus* PCC7002. One of its unique features is the fast growth and high biomass accumulation (highest cell density ever reported for any Cyanobacterium) [2], a major obstacle for large-scale usage of these microbes. Its fast growth and natural transformability renders it a highly promising strain for biotechnological applications.

The transformation of ferulic acid to vanillin has been proven in *E. coli*, using two heat-stable enzymes, a phenolic acid decarboxylase (PAD) and an aromatic dioxygenase (ADO) [3]. During this two-step enzymatic reaction, CO₂ is released, while O₂ is consumed. The potential with Cyanobacteria as host for this reaction lies within its ability to convert CO₂ into biomass, while toxic accumulation of cellular O₂ is averted. The build-up of other toxic compounds (formaldehyde), and the toxicity of substrate, intermediate and product will be considered.

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Chaperone assisted cell-free protein synthesis for screening of novel enzymes

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In vitro or cell-free protein synthesis (CFPS) has become an established tool for rapid transcription and translation [1]. CFPS can complement traditional *in vivo* protein synthesis to accelerate the discovery of novel enzymes or enzyme variants [2]. A common challenge is that proteins, when synthesized with heterologous systems, often do not fold properly and therefore become insoluble. Since correct three-dimensional protein folding is critical for full protein function, these insoluble proteins are usually inactive. Molecular chaperones prevent protein aggregation and promote protein folding. Exogenous addition of molecular chaperone proteins has effectively facilitated the synthesis of various soluble proteins in CFPS systems [3]. Another approach shifts the synthesis of chaperones back into the cell of the source organism of the cell extract for CFPS [4]. Therefore, an *Escherichia coli* strain is transformed with a plasmid encoding molecular chaperones. These are synthesized during growth of the strain, which serves as the basis for the cell extract. The extract thus contains not only everything for transcription and translation, but also chaperones that will support the synthesis of soluble protein.

Chaperone-enriched CFPS extracts were developed to synthesize α -ketoglutarate-dependent L-lysine dioxygenases (KDOs), which are difficult to express in soluble form in *E. coli*. Five different chaperones containing CFPS systems were prepared from *E. coli* strains and tested to screen novel and putative KDOs (Figure 1). The CFPS systems allowed efficient expression of soluble enzymes without the need for exogenous addition or co-expression of folding effectors. Subsequent activity assays demonstrated the successful hydroxylation of L-lysine to hydroxy-L-lysine for several KDOs, including four novel and previously biochemically uncharacterized and undescribed enzymes.

In this study, CFPS using chaperone-enriched cell extracts was shown to be an efficient and rapid method for the synthesis of enzymes that are rather difficult to express heterologously.

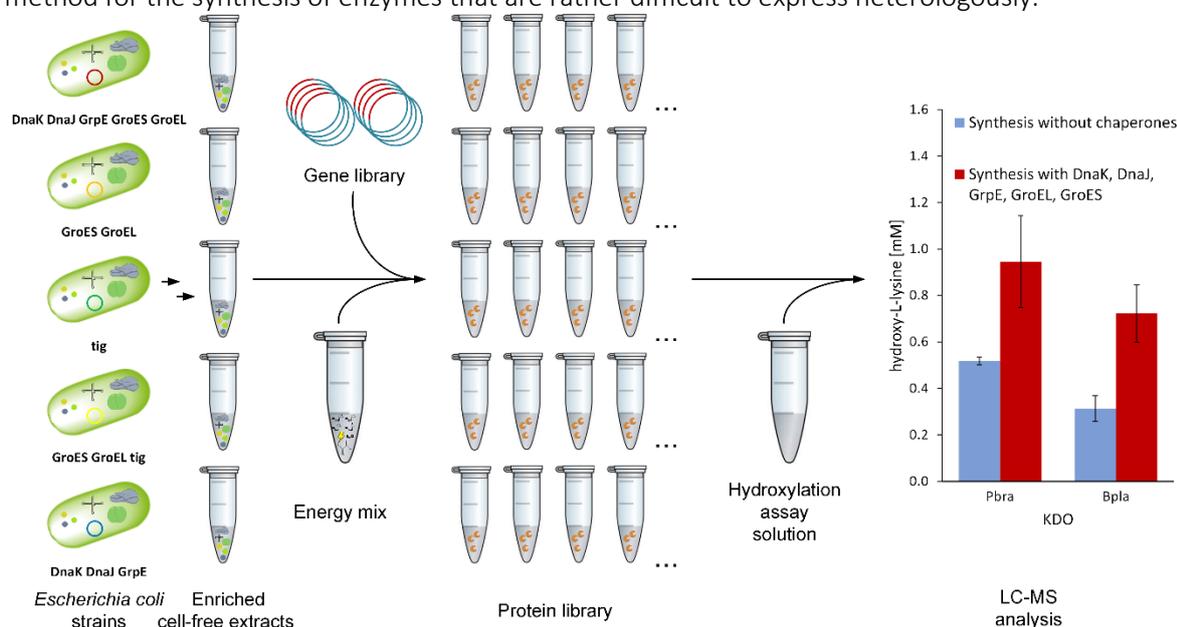


Figure 1. Schematic workflow for the screening of a gene library with chaperone assisted cell-free protein synthesis

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Amine group donor assessment in the pyrroline asymmetric synthesis by transaminases

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Pyrroline alkaloids are intermediate compounds in the synthesis of complex structures alkaloids. Their asymmetric synthesis is still a challenge encouraging the developments new chemical and biocatalytic synthesis method for obtaining enantiopure compounds. Consequently, transaminases (TAs) are used to catalyse the reversible reductive amination of carbonyl groups, providing chiral amines with excellent conversions and enantiomeric excess (*ee*).¹ During transamination reaction to obtain chiral amines an amino group donor is used. It is an important parameters since it is associated with the reaction equilibrium control, impacting the efficiency of the reaction.² In this research, alanine and isopropyl amine (IPA) were evaluated as amino group donors in the transamination reaction of 24 commercial TAs from CODEXIS®. In this way, donane-2,5,8-trione **1a** and dodecane-2,5,8,11-tetraone **1b** were used as substrates, and after transamination of their less hindered carbonyl group, a spontaneous cyclization produced pyrroline compounds **2a**, **2b**.

As a result, the biocatalytic process with alanine showed that *S*-selective TA (ATA-237) yielded **2a** with an excellent conversion >99% and *ee* of 80%. On the other hand, the *R*-selective TA (ATA-P1-F03) yielded **2a** with 83% of conversion and *ee* of 85%. In the case of **2b**, the *R*-selective TA (ATA-P2-A01) and *S*-selective TA (ATA-237) achieved an excellent conversion >99% and *ee* >99%, as shown in **Figure 1**.

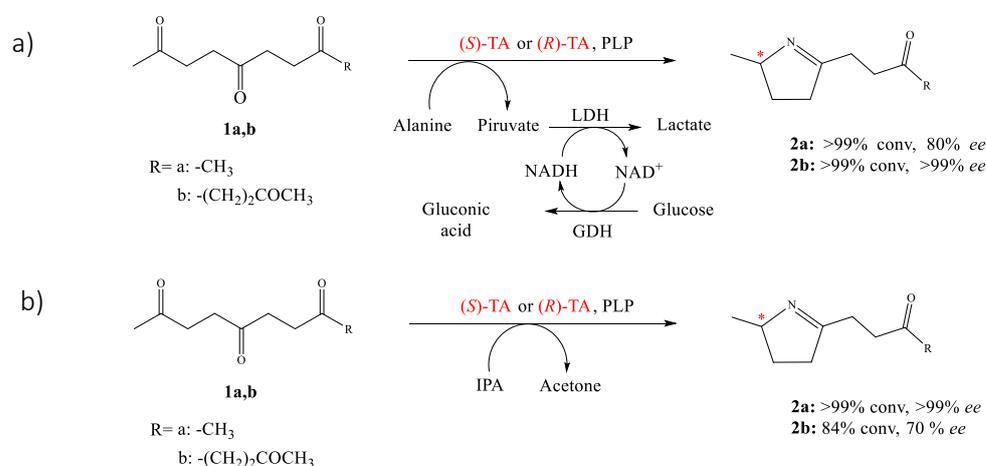


Figure 1. Biocatalysis reaction of substrates **1a**, **1b** by commercial TA a) Alanine as amino group donor b) IPA as amino group donor. **Reaction condition:** 2 mg mL⁻¹ of TA, 10 mM of substrate (10% DMSO), PLP, 100mM of IPA or 100 mM of alanine (LDH-lactate dehydrogenase, glucose, GDH-glucose dehydrogenase), 200 mM Tris-HCl, 30 °C by 24h.

Alternatively, when IPA was used instead of alanine, the *S*-selective TA(ATA-251) and *R*-selective TA (ATA-P2-B01) yielded **2a** with excellent conversion >99% and *ee* >99%. However, for compound **2b** the maximum conversion was 84% by *R*-selective TA(ATA-P1-F03). As a conclusion, using the method with IPA as amine group donor to obtain compound **2a**, excellent results were achieved, with the advantage of being an economic method. Meanwhile, using alanine as amine group donor yielded **2b** with excellent conversions and *ee*.

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Upscale of the recombinant production of myrosinase in *Pichia pastoris*

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Myrosinase (EC 3.2.1.147) is a plant defence enzyme which hydrolyses glucosinolates, a group of plant secondary metabolites, into a range of bioactive products. At neutral pH, isothiocyanates are formed [1]. This group of compounds has become of particular interest due to their neuroprotective and cancer-preventive effects. There is evidence showing their anti-tumorigenic action against different types of cancer, both *in vitro* and *in vivo*. Due to their low toxicity profile, ITCs are considered to be excellent chemotherapeutic candidates against tumour initiation and progression [2], which makes myrosinase a pharmaceutically interesting enzyme.

Myrosinase has been identified in plants, fungi, insects and even human gut microbes. However, the amount of active isothiocyanates generated from glucosinolates-rich diet in human colon is negligible. Therefore, the attention is given to exogenous production of isothiocyanates by highly active plant myrosinases. The purification of myrosinases from their native plant hosts is extremely challenging because plant myrosinases are generally encoded by a multigene family. Recombinant production of plant myrosinases proved to be a powerful tool in the production of a single myrosinase isoenzymes to facilitate their purification and characterisation. Previously, recombinant production of different plant myrosinases in *Pichia pastoris* was performed [3-5]. However, the production itself was not studied, optimised or upscaled.

We performed a high-cell density extracellular production of active TGG1 myrosinase from *Arabidopsis thaliana* in *Pichia pastoris* Mut^S strain under the control of AOX1 promoter. The production was upscaled from a shake flask to 0.35 and 3 litre laboratory fermenters for the first time. Optimisation of cultivation conditions increased the productivity 3-fold. Overall, a specific productivity of 4.1 U/L_{medium}/h was achieved, which represent a 102.5-fold increase in myrosinase productivity compared to flask cultivations. The produced recombinant AtTGG1 myrosinase exhibited similar catalytic properties as its native counterpart and showed excellent long-term stability. After 12 months of storage at -80°C, myrosinase retained all of its activity regardless of the use of a cryopreserving agent [6].

Acknowledgement

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Reactive Deep Eutectic Solvents (RDEs): a new tool for Phospholipase D-catalyzed preparation of polar head modified phospholipids

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In recent years, the development of new biocatalytic strategies aiming to achieve Sustainable Development Goals is a highly investigated topic. In this context, the use of new protocols involving biocatalysis as well as the investigation of greener suitable solvents in the chemical modification of organic materials fits perfectly the demand for an environmental sustainable research.

To this end, the use of Deep Eutectic Solvents (DESs) represents a valid greener alternative to the use of common organic solvents in the preparation of polar head modified phospholipids (PLs) with phospholipase D (PLD)-catalyzed biotransformations. PLs constitute the major components of all biological membranes and their amphiphilic structure makes them a suitable topic for many areas of biomedical research and for several industrial applications as surfactant, stabilizers and detergents. In particular, the biological activity of PLs mainly depends on the chemical identity of the polar head. For this reason the modification of PLs polar head for specific applications is of fundamental importance.[1]

In this work natural phosphatidylcholine (PC) has been submitted to PLD-catalyzed transphosphatidylation using new reaction media composed by tailored mixtures of DESs and buffer. These new solvents were able to solubilize PC, avoiding the use of other toxic organic solvents which were usually necessary in these bioconversions, as well as acting as nucleophilic reactants [2]. Furthermore, in these reaction conditions the secondary hydrolysis side-reaction was quite inhibited driving the conversion toward transphosphatidylation and making the final new PLs easier to recover. The presented results appeared to pave the way to the development of further studies on DES-mediated PLs preparations in a sustainable perspective.

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IREDFisher: Computational tool to access chiral amine precursors for pharmaceutical compounds

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Imine reductases (IREDs) have emerged as attractive biocatalysts for the synthesis of chiral amines, which are themselves prevalent building blocks in pharmaceutical compounds (API).[1,2] Recently large panels of IREDs have been established, e.g. by metagenomic mining, thus leading to a wide diversity of biocatalysts with the potential to catalyse reductive amination reactions of interest.[2] Extensive screening of these panels allows hits to be identified which subsequently can be engineered to meet the required operating conditions for a viable process. This approach results in large numbers of enzymes that require high-throughput screening (HTS) methods for their evaluation.[3] However, screening remains a major bottleneck in biocatalysis since it is highly time and money consuming.[2] Hence, an *in silico* enzyme screening to predict the active enzymes is highly desired to reduce the screening efforts.

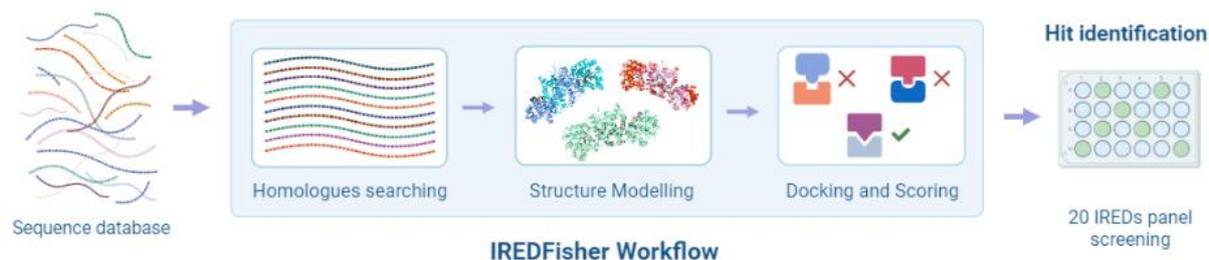


Figure 1. IREDFisher general workflow. Sequence databases are inspected and enzymes are ranked to facilitate hit identification to access chiral amines.

A computational four-step workflow was constructed to predict the active IREDs for any given substrate. IREDFisher performs an initial sequence inspection, structure modelling, molecular docking and subsequent scoring in order to rank the enzyme candidates.

The workflow was validated using a 384-enzyme panel and the top 20 predicted hits for four different carbonyl substrates, starting from readily acceptable to more demanding substrates, were screened. For the first three compounds more than half of predicted hits were found to be active, with some exhibiting excellent conversions without making reaction optimization necessary. Furthermore, the workflow was tested towards challenging substrate such as aldehydes containing a carboxyl group, a novel reaction that allows for further derivatisation. Four predicted enzymes exhibited moderate conversion towards the reductive amination of formylbenzoic acid. Now IREDFisher, along with IRED screening and engineering, can be applied to target industrially viable transformations for the synthesis of pharmaceuticals.

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Chemoenzymatic Deracemization of Lisofylline Catalyzed by (Laccase/TEMPO)-Alcohol Dehydrogenase System

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Asymmetric synthesis of non-racemic compounds is an important issue of current organic chemistry. Since great number of *sec*-alcohols constitute key chiral building blocks useful for the syntheses of high-value added products (i.e., pharmaceuticals, agrochemicals, flavours, fragrances etc.), it is of the utmost importance to develop practical, selective, scalable and environmentally benign methods of their preparation. Unfortunately, the vast majority of synthetic methods toward optically active alcohols suffer significant limitations mainly associated with low reaction yields and high cost of isolation and purification of the products. In this regard, the desymmetrization of prochiral- or *meso*-substrates is considered as one of the most synthetically useful transformations, whereas among the most efficient and selective biocatalysts capable of reducing ketones are alcohol dehydrogenases (E. C. 1.1.1) (ADHs). In the last few years, an innovative catalytic system composed of laccase from *Trametes versicolor* (LTv), oxy-radical TEMPO and ADH has been successfully applied in sustainable deracemization of various benzylic [1], allylic [2] and propargylic [3] alcohols. This method, despite having proven useful for deracemization of a broad spectrum of activated alcohols, is still underexploited toward non-activated aliphatic *sec*-alcohols. Therefore, an extension of the substrate scope for more demanding compounds in terms of oxidation of their hydroxyl groups remains challenging.

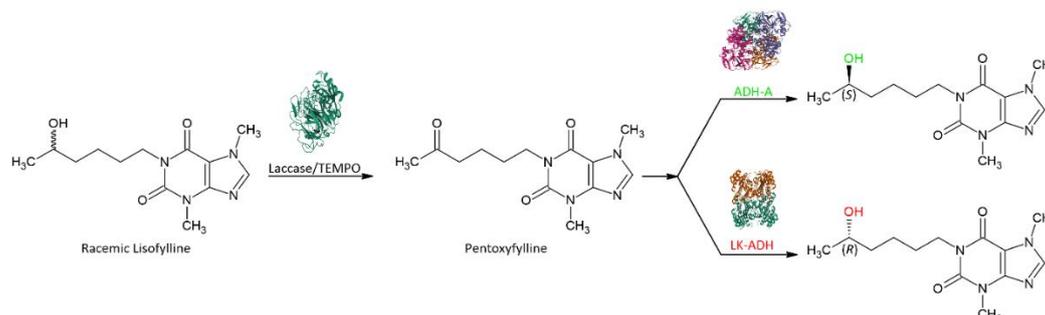


Figure 1. Chemoenzymatic stereo-complementary deracemization of lisofylline using (laccase/TEMPO)-ADH catalytic system.

In this study, we report the development of one-pot/two-step deracemization procedure for lisofylline (LSF) active agent. This task was accomplished *via* a tandem bi-enzymatic oxidation-reduction reaction sequence based on (LTv/TEMPO)-ADH catalytic system. LSF is synthetic methylxanthine exhibiting strong anti-inflammatory and immunomodulatory properties; therefore, it has been investigated as a promising drug candidate for various autoimmune disorders, including Type 1 diabetes [4]. During multi-step optimization procedure of LTv/TEMPO-catalyzed oxidation of LSF, we showed improvement of the conversion from initial 16% up to 95%, demonstrating by this high synthetic potency of this method when compared to traditional chemical reactions requiring toxic oxidants used in stoichiometric amounts. In turn, stereoselective reduction of pentoxifylline using recombinant ADHs from *Rhodococcus ruber* (ADH-A) and *Lactobacillus kefir* (LK-ADH) furnished both LSF enantiomers (>99% ee) with high 93–94% conv. The coupling of the above-mentioned steps are under investigation.

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Using the enzymatic toolbox for the synthesis of chiral iminocyclitols

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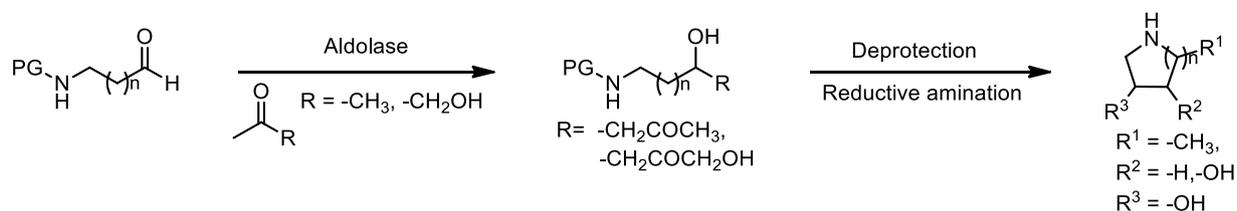
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Chiral iminocyclitols (“azasugars”) are bioactive natural products that constitute essential building blocks for pharmaceutical synthesis.[1] Different chemical routes have been developed to obtain stereochemically pure iminocyclitols. However, these routes often require multiple steps including laborious protective group manipulations, which adds expenses, can lead to undesired by-product formation, lowers atom economy and produces potentially toxic waste. As an alternative, enzymatic key steps often proceed with high stereoselectivity. Also, reactions catalyzed by enzymes are often more sustainable, as biocatalysts are nontoxic, biodegradable, and perform reactions in water under mild reaction conditions. Furthermore, enzymes are usually compatible with each other and can be used in multi-enzyme reaction cascades in a one-pot synthesis manner.

Recently, Roldán et al. have shown the synthesis of different chiral iminocyclitols using aldolase-catalyzed addition of simple aliphatic nucleophiles to Cbz-protected amino aldehydes followed by Pd-catalyzed reductive amination.[2] Here we present a complementary strategy using only enzyme-mediated protective group manipulations and inexpensive organic reagents. After formation of a new asymmetric C-C bond between a protected amino aldehyde and different nucleophiles catalyzed by specific carboligases, the resulting aldol products are enzymatically deprotected. Spontaneous cyclic imine formation is immediately followed by reductive amination, which is achieved chemically or biocatalytically, to generate the desired iminocyclitols with high yield.



Scheme 1. Synthesis of iminocyclitols by combining a carboligase, enzymatic deprotection and reductive amination.

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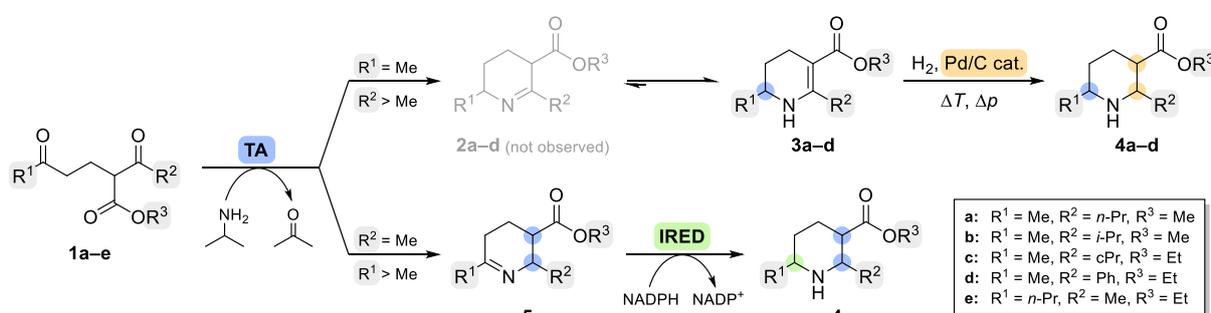
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Chemo-Enzymatic Asymmetric Synthesis of Trisubstituted Piperidines

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Piperidine rings form the core structure of more than 700 known alkaloids and are also a frequent structural motif in pharmaceutical drugs.[1–3] Asymmetric methods for the preparation of chiral, substituted piperidines are hence of considerable synthetic interest. We have recently developed a chemo-enzymatic asymmetric synthesis of the piperidine alkaloid dihydropinidine *via* biocatalytic transamination and imine reduction, in which the diketoester **1a** (Scheme 1) is an intermediate.[4] As a continuation of this work, we have investigated the transamination of several diketoesters **1** and the transformation of the resulting unsaturated heterocycles into trisubstituted piperidines **4**.



Scheme 1. Transamination of diketoesters **1** leads to cyclic enamines **3** or cyclic imines **5** that can be diastereoselectively reduced to trisubstituted piperidines **4**.

Substrates **1a-d**, in which R¹ is a methyl group, were aminated at the distal ketone moiety to give—after spontaneous cyclisation and tautomerisation—enamines **3a-d**, which proved remarkably resistant towards reduction. Catalytic hydrogenation required elevated temperature (100 °C) and pressure (80 bar) and was implemented in a flow setup (H-cube). Preparative-scale flow hydrogenations of three enamines **3** afforded amines **4** in fair yield (32–49%) and excellent optical purity (*ee* > 99%, *de* > 99%). In contrast, the transamination of substrate **1e** (R¹ = *n*-Pr, R² = Me) took place at the carbonyl group in β-position of the ester, leading to cyclic imine **5e** with excellent enantio- and moderate diastereocontrol (*ee* > 99%, *de* = 0–92%). The diastereomers of **5e** were separated by column chromatography and individually subjected to reduction by a panel of 14 IREDs, which afforded the corresponding trisubstituted piperidine **4e** in high diastereomeric purity (*de* > 99% in 15 of 28 reactions).

In summary, we have established concise chemo-enzymatic routes to optically pure, trisubstituted piperidines **4**, which might become synthetically useful as chiral building blocks.

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Lipase catalysed synthesis of functional polyesters and their application as binders in organic coatings

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Polyester-polyols with pendant hydroxyl groups in the chain are attractive materials which offer additional functionalization points in the polymer chain. In contrast to chemical polycondensation certain hydroxyl groups remain unaffected in enzymatic polyesterification due to the lipases regioselectivities. An aliphatic-aromatic polyester with two different classes of pending hydroxyl groups has been synthesized by lipase catalysis. Using an aromatic diol with pending phenolic groups in lipase catalyzed polycondensation with adipic acid required the addition of hexane diol as third monomer for polycondensation to take place. Reaction conditions were optimized to maximize the fraction of incorporated aromatic diol. Here, a low polymerization temperature of 50°C was realized. A 3D printed reactive agitator was used for optimized reactant dispersion and synthesis scale-up. Post-polymerization with glycerol at low temperature integrated additional aliphatic hydroxyl groups, reduced the polydispersity and optimized the end group functionality. The new functional material with enhanced mechanical properties was used as binder in organic coatings, combined with different hardeners and studied in-depth by application analysis.

Enantioselective synthesis of pharmaceutically relevant arylbutylamines by transaminases with an engineered binding pocket

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Scalable asymmetric synthesis of chiral amines from sterically demanding α -aryl ketones by the employment of amine transaminases (ATAs) remains to be a challenging task due to the poor water solubility of the substrates, thermodynamically unfavourable character of the reaction, stabilizing resonance effect of conjugated ketones and enzyme-substrate inhibition. To overcome these obstacles in the synthesis of bulky arylalkylamines, we employed a reaction engineering strategy using simultaneous optimization of enzyme characteristics and process configuration. Engineered ATAs having an enlarged small binding pocket were applied for the first synthesis of enantiomerically pure arylbutylamine (*R*)-**1**, a chiral component of human leukocyte elastase inhibitor DMP 777 (L-694,458), as well as for the asymmetric synthesis of pharmaceutically relevant (*S*)-**4**, a structural subunit of deubiquitinase inhibitor degrasyn (WP1130).

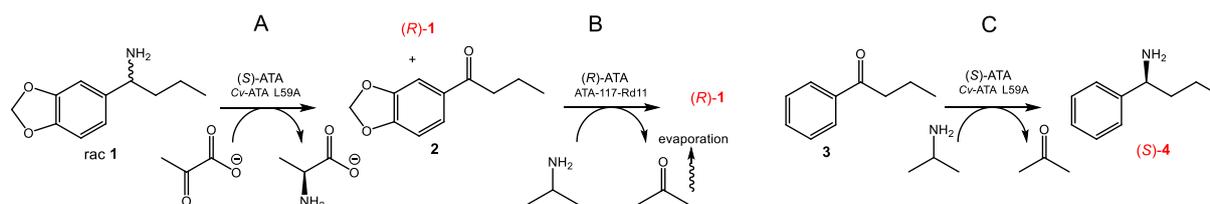


Figure 1. Kinetic resolution of rac **1** (A) and asymmetric synthesis of (*R*)-**1** (B) and (*S*)-**4** (C) by engineered ATAs.

Kinetic resolution of the racemic amine **1** was performed using the L59A variant of the (*S*)-selective ATA from *Chromobacterium violaceum* (Cv-ATA),[1] leaving the residual (*R*)-enantiomer in excellent yield and >99% ee. At moderate enzyme loading and absence of cosolvent, high volumetric productivity of 0.22 mol L⁻¹ h⁻¹ (42.5 g L⁻¹ h⁻¹) was achieved. Alternatively, we employed the engineered (*R*)-selective ATA-117-Rd11 [2] for an asymmetric synthesis of (*R*)-**1**. Under optimized conditions with removal of acetone co-product, 86% conversion of prochiral ketone **2** gave (*R*)-**1** with excellent enantiomeric excess (>99% ee).

We also evaluated the utility of Cv-ATA L59A for the stereoselective synthesis of (*S*)-**4**. The enzyme displayed activity similar to that reported for the triple mutant ATA from *Ochrobactrum anthropic* [3] and good tolerance to high concentrations of isopropylamine, producing enantiomerically pure (*S*)-**4**.

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Immobilization of aldehyde dehydrogenase employing cofactor regeneration for use in biocatalysis

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The use of enzymes in biochemical processes is of interest due to their ability to operate under mild conditions while attaining relatively high reaction rates. Additionally, utilisation of enzymes helps avoid the use of harsh reaction media and the production of hazardous waste materials. The use of enzymes in their soluble form is not desirable as it does not allow for re-use of the enzyme and can contaminate the product(s), with purification from reaction media often being difficult. Industrial processes have focussed on the use of enzymes such as esterases with less of an emphasis on redox enzymes. A limitation of the use of redox enzymes is the need to supply redox equivalents, either via direct oxidation or reduction of the enzyme or using cofactors such as NAD⁺. A challenge with using cofactors is that they are required in stoichiometric amounts relative to the substrate and given their cost, the use of NAD(P)⁺ dependent enzymes requires cofactor regeneration systems. This typically involves the use of a secondary enzyme and a stable, inexpensive substrate to recycle the cofactor. We describe the immobilization of a thermophilic aldehyde dehydrogenase (ALDH) for biocatalysis in a flow reactor, employing L-lactic dehydrogenase (L-LDH) as a secondary enzyme for the regeneration of NAD⁺ (Figure 1).

Screening of the activity of ALDH demonstrated that the enzyme is not substrate specific and can oxidise a range of aliphatic, aromatic and di-aldehydes. While the maximum catalytic rate was achieved using hexanal ($1.08 \pm 0.03 \text{ U mg}^{-1}$), reasonable rates of oxidation of benzaldehyde, terephthalaldehyde and *p*-tolualdehyde were also obtained. Simultaneous immobilization and purification of the his-tagged ALDH was successfully performed from crude cell extract of *E. coli* expressing the ALDH [1], resulting in a catalytically active ALDH assayed in a flow reactor system. This immobilization process significantly reduced the lengthy purification processes, eliminating a 2-day purification step by directly immobilizing via his-tag affinity interactions from cell lysate. L-LDH was utilised in a cofactor regeneration mechanism to enable continual supply of NAD⁺ to drive the ALDH synthesis of carboxylic acids.

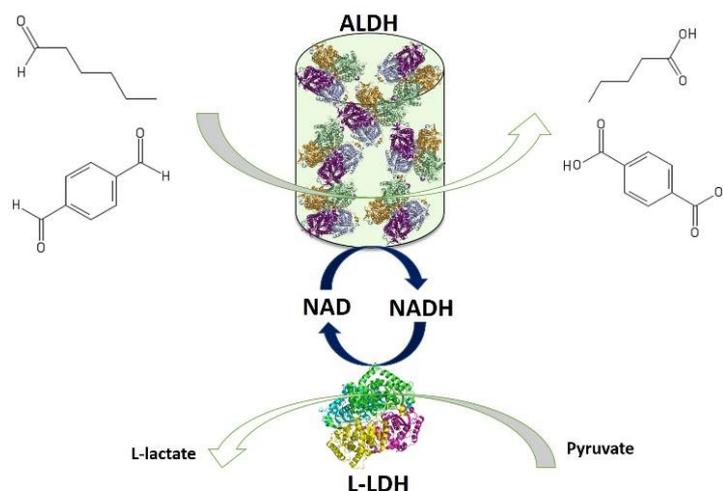


Figure 1. Graphical representation of biocatalytic mechanism. Immobilized ALDH is used to convert an aldehyde to carboxylic acid while employing cofactor regeneration of NAD cofactor through use of L-LDH.

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Enzyme mediated purification strategy following a continuous flow Curtius rearrangement

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The use of continuous flow as an enabling technology within the fine chemical and pharmaceutical industries continues to gain momentum [1-3]. The associated safety benefits with flow for handling of hazardous or highly reactive intermediates are often exploited to offer industrially relevant and scalable Curtius rearrangements. However, in many cases the Curtius rearrangement requires excess nucleophile for the reaction to proceed to high conversions. This can complicate work procedures to deliver high purity products. However, as this poster will highlight tandem processing and coupling of the Curtius rearrangement with an immobilized enzyme can elegantly facilitate chemoselective tagging of the residual reagent, resulting in a facile purification process under continuous flow [4].

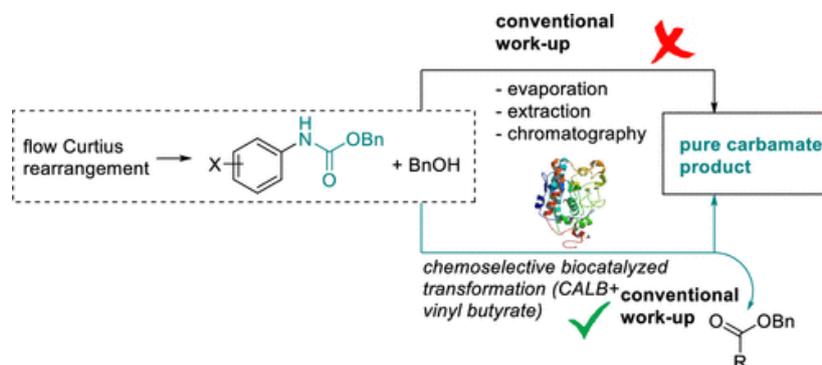


Figure 1. Enzyme mediated purification strategy following a Curtius rearrangement

The resulting telescoped flow process was effectively applied across a series of acid substrates rendering the desired carbamate structures in high yield and purity. The derivatization of these products via complementary flow-based Michael addition reactions furthermore demonstrated the creation of β -amino acid species [5]. This strategy thus highlights the applicability of this work towards the creation of important chemical building blocks for the pharmaceutical and speciality chemical industries.

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Cascade biocatalysed process for the synthesis of bio-based plasticizers

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The present work deals with the optimization of the synthesis of bio-plasticizers and bio-lubricants starting from oil extracted from the seeds of cardoons (*Cynara cardunculus*), a typical plant of the Mediterranean area. [1] The oil was characterized by NMR, observing a high content of unsaturated fatty acids, especially linolenic and oleic acid, which can be substrate of chemo-enzymatic epoxidation. [2,3]

Lipases immobilized on different carriers were tested in the hydrolysis of the oil and then in the epoxidation of the fatty acids. Both adsorbed and covalently immobilized lipases were developed making use of rice husk as renewable enzyme carrier. An integrated cascade biocatalysed process was designed, in order to combine the epoxidation and esterification of the fatty acids.

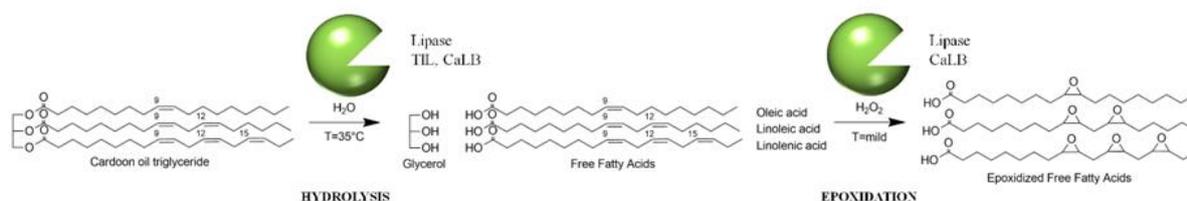


Figure 1. Enzymatic hydrolysis of triglycerides and subsequent epoxidation.

The solvent-free epoxidation of the different unsaturated fatty acids was obtained with quantitative conversion and the possibility to synthesize the corresponding esters was demonstrated.

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Please Biogenic amines production in flow from the sustainable fermentation of aromatic amino acids converting glucose in minimal medium.

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Bacterial tyrosine decarboxylases have been previously identified^[1, 2, 3] however the soluble expression of these enzymes in heterologous hosts is challenging. On the contrary, the soluble expression of tyrosine decarboxylase from *Lactobacillus brevis* (LbTDC) was reported on 2011.^[4] Here, we have successfully expressed, purified and immobilized LbTDC on different supports. As the soluble enzyme, immobilized LbTDC is active not only towards its natural substrate, L-Tyrosine, but also L-Dopa.^[4] These last ones have been obtained by whole cell biotransformation (*E. coli* VH33 Δ tyrR_DOPA),^[5] starting from glucose, ubiquitous and sustainable starting material, within the cost-effective minimal medium in a 2L bioreactor. After a purification step, the fermentation broth, rich in L-Tyrosine and L-dopa, enter the flow system where LbTDC catalyses the complete conversion to Tyramine and Dopamine.

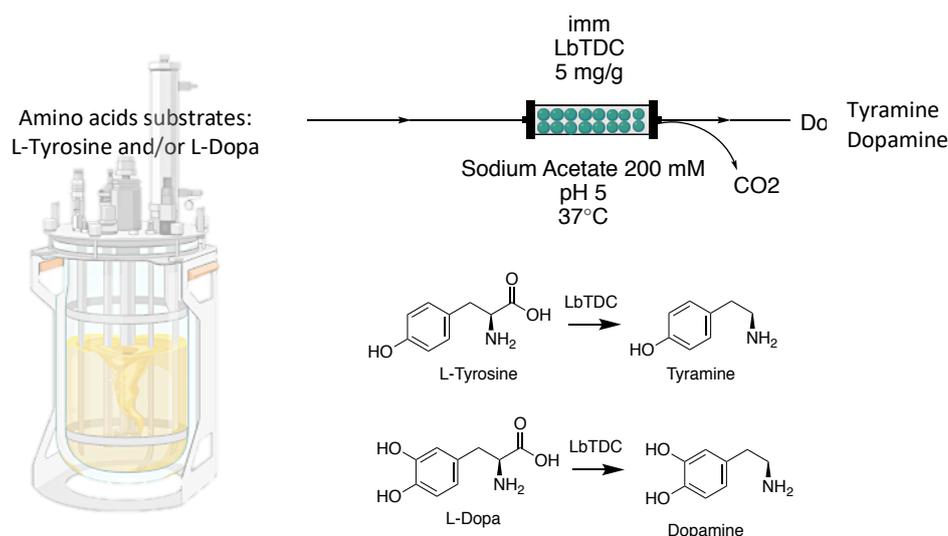


Figure 1. Biocatalytic conversion from L-tyrosine and L-dopa to Tyramine and Dopamine through decarboxylation catalyzed by immobilized LbTDC.

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Chemoenzymatic cascade reactions to unnatural aminoglycoside derivatives

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Aminoglycoside antibiotics (AGAs) are highly potent bactericidal drugs¹. Despite this, they have had some problems clinically owing to toxicity issues and growing antibiotic resistance¹. Therefore, there is considerable interest in developing novel synthetic approaches to AGA derivatives that are safer but also highly effective against resistant organisms.

Aminoglycosides are poly-hydroxylated and poly-aminated species which makes total chemical synthesis challenging, as it is difficult to establish the required regio- or stereochemistry. Consequently, traditional chemical syntheses require the use of extensive protection and deprotection steps which lead to long, atom- and step-inefficient synthetic routes²⁻⁴. Enzymatic synthesis of aminoglycosides is of interest because it can yield more concise, sustainable and enantioselective synthetic routes and can generate novel derivatives through enzymatic and chemoenzymatic cascade reactions.

With this in mind, we aimed to explore the biosynthetic potential of aminoglycoside transaminases and their integration into novel cascade reactions. In particular, we focussed on 6'-transaminases, as previous studies have shown that there is considerable potential for structural modification at this position while maintaining potency^{2,5,6}. As such, seven 6'-transaminases from *Streptomyces*, *Micromonospora* and bacilli aminoglycoside producers were explored.

Previous studies have shown that aminoglycoside 6'-transaminases show considerable substrate promiscuity^{7,8}. Here, it was demonstrated that 6'-transaminases can deaminate both pseudodi- and trisaccharide AGAs (Figure 1) and can tolerate the presence or absence of hydroxyl groups on the 3'- and 4'-positions. Additionally, sugar substituents on the 6-OH were accepted but not on the 5-OH. The aldehyde products can be further upgraded through chemoenzymatic cascade reactions. This approach not only allows facile access to novel aminoglycoside compounds without the use of atom-inefficient protecting and deprotecting steps but may also potentially allow the development of greener, sustainable and more cost-effective synthesis of novel AGAs.

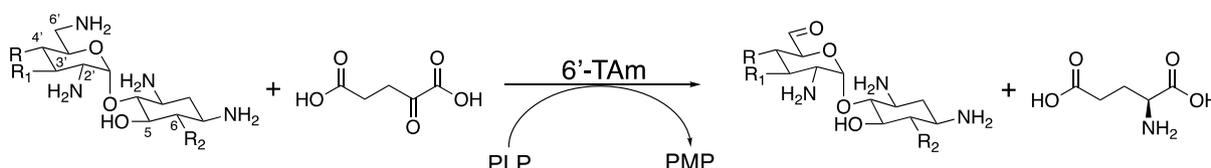


Figure 1. Schematic of the deamination reaction of pseudodi- and trisaccharides explored in this study.

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A novel fungal peroxygenase works as an efficient oxidation catalyst

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The use of enzymes for catalytic C-H oxyfunctionalization reactions is of high interest for synthetic applications due to the potential to cut short synthetic routes and streamline processes towards complex molecules [1]. Unspecific peroxygenases (UPOs) have shown high potential for selective oxyfunctionalization and stand out due to their simplicity compared to their well-known P450 counterparts since they do not rely on nicotinamide cofactors or electron transport chains [2]. Here we report a novel unspecific peroxygenase from *Hypoxylon sp.* (*HpUPO*) which has been structurally characterized possessing a melting temperature of 77 °C and performs hydroxylation, alcohol oxidation and epoxidations on various small molecules by solely using hydrogen peroxide as cosubstrate.

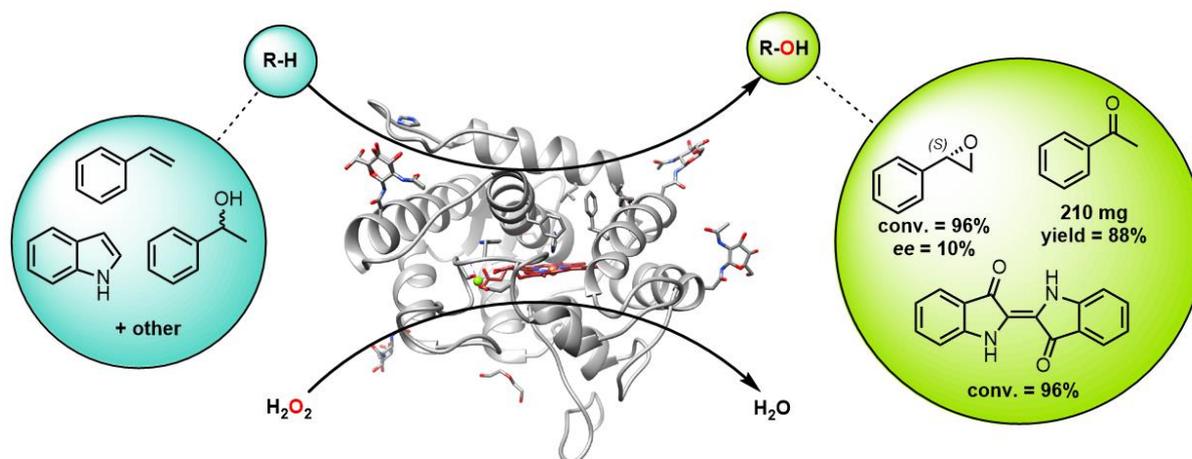


Figure 1. Schematic representation of the biotransformations of selected substrates with *HpUPO*.

To characterize the reaction potential of *HpUPO*, various substrates representing a broad scope of functional groups were investigated. Notably, this biocatalyst oxidized both enantiomers of racemic 1-phenylethanol in a semi-preparative scale reaction (2 mmol) to yield 210 mg of acetophenone (88%, GC-yield = 92%, TON = 13143) indicating a high potential for the oxidation of benzylic *sec*-alcohols. *HpUPO* was also able to efficiently produce the natural dye indigo through the oxidation of indole with conversion of up to 96 % and less than 1 % side oxidation products. This highlights *HpUPO* as an efficient, thermotolerant biocatalyst and as a promising asset for the development of designer peroxygenases targeting specific substrates.

This work was supported by the Federal Ministry of Science, Research and Economy (BMWFW), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, and the Government of Lower Austrian and Business Agency Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.

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Ene-reductase transformation of massoia lactone to δ -decalactone in continuous-flow reactor

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Increasing attention is being paid to the origin of food additives, and those with natural origin are preferred. Compounds obtained by biotransformation, according to European Union and United States regulations, are regarded as natural. [1]

δ -Decalactone, with its creamy, coconut-like, sweet, milky, fruit flavor, is of great interest to the industry. It is used to augment and enhance the aroma and taste of food preparations, beverages, toothpastes, perfumes and detergents. [2]

The key concepts of modern industrial biotechnology are green chemistry and process intensification. In this view, biocatalysis and continuous processing are among the most promising green research areas for sustainable manufacturing of food ingredients, pharmaceutical intermediates and fine chemicals. These two approaches can be combined by immobilization of the biocatalysts and their use in packed bed flow bioreactor.

The main goal of this project was application of selected ene-reductase Old Yellow Enzyme 3 for the reduction of C=C bond of natural (*R*)-(-)-massoia lactone to obtain enantiomerically pure (*R*)-(+)- δ -decalactone (Figure 1). For this purpose, biocatalysts in the form of purified enzyme, whole cells, alginate-encapsulated cells, and lysate were used. The biotransformation performed in batch mode, using Ca²⁺-alginate immobilized cells of *Escherichia coli* BL21(DE3)/pET30a-OYE3, furnished the desired product with complete conversion in 30 minutes.

The process was intensified using a continuous-flow reactor-membrane filtration system (flow 0.1 mL/min, substrate concentration 10 mM, pH 7, 24°C) with cell lysate as biocatalyst combined with cofactor regeneration system, which allowed obtaining > 99% bioconversion of massoia lactone.

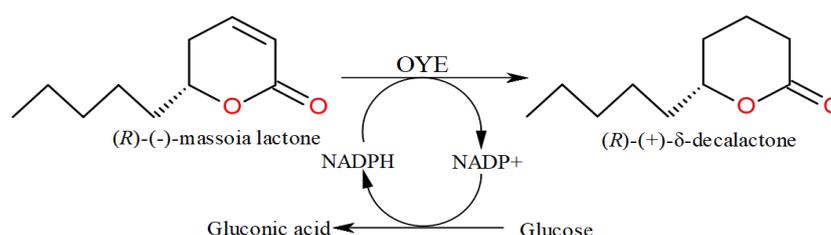


Figure 1. Bioreduction of natural massoia lactone.

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Immobilization of *Geotrichum candidum* alcohol dehydrogenase on novel support materials

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Alcohol dehydrogenase has been a promising green catalyst for asymmetric reduction of prochiral ketones which is beneficial for producing valuable compounds in various industries. Our research group reported the utilization of a novel alcohol dehydrogenase, acetophenone reductase from *Geotrichum candidum* (*GcAPRD*), that could reduce ketones to their corresponding (*S*)-alcohols with excellent enantioselectivity.¹ Although the *GcAPRD* presented its superior properties such as thermostability, organic solvent tolerance, and broad substrate specificity, there is still a limitation in recyclability that needed to be solved. Thereby, enzyme immobilization could be an approach to overcome the issue.

We have developed three methods to immobilize the *GcAPRD* (**Figure 1**). First, the organic-inorganic nanocrystal formation immobilizes the enzyme *via* cobalt ion (Co^{2+}) and *GcAPRD*'s His-tag affinity to yield *GcAPRD* nanocrystal.² Second, *GcAPRD* immobilization *via* physical adsorption on reduced graphene oxide (rGO)³ to obtain rGO-*GcAPRD*. Last, the development of 3D-printed bioreactors which were used as the support for *GcAPRD* immobilization *via* covalent attachment.

To our delight, it was found that all the developed immobilization methods could improve the properties of *GcAPRD*. For example, the recyclability limitation was overcome. The *GcAPRD* nanocrystal and rGO-*GcAPRD* could be recycled to reduce acetophenone up to 7 times with noticeable high yield (>99%) and excellent enantioselectivity (>99% *ee*, *S*). In addition, the temperature profile of the *GcAPRD* nanocrystal and rGO-*GcAPRD* was improved compared with that of free *GcAPRD*, and the broad substrate specificity of the *GcAPRD* was retained after immobilization. They also reduced 'difficult to resolve' aliphatic ketone, 3-hexanone, with excellent enantioselectivity, and produced drug intermediates such as (*S*)-1-(3',4'-dichlorophenyl)ethanol with high yield and excellent enantioselectivity. For the 3D-printed bioreactor, we successfully established the batch bioreactor of *GcAPRD*. The immobilized *GcAPRD* could be recycled to reduce acetophenone with excellent enantioselectivity. The robust properties of the immobilized *GcAPRD* *via* various methods make it a potential candidate for further applications in green and sustainable chemistry.

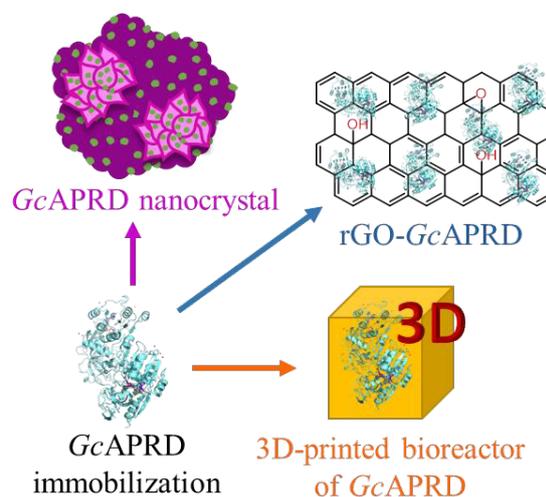


Figure 1. *GcAPRD* immobilization on novel support materials.

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Redesigning the active pocket of ω -transaminase in the Amino alcohol synthesis

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ω -transaminase is based on the coenzyme pyridoxal 5'-phosphate, which can transfer amino groups from amino donors to amino acceptors used as chiral amino and unnatural amino products. It is commonly used to catalyze chiral intermediates and non-natural amino acids in the pharmaceutical market.

Although the organic synthesis method occupies a large proportion in the acquisition of chiral amines, in the process of chemical synthesis of chiral amines, it is often accompanied by excessive intermediate by-products, which cannot be purified later. In recent years, amino alcohol was used as the pharmaceutical intermediates. The production of chiral amino alcohol by ω -transaminase can effectively avoid the high pressure, high temperature, high energy consumption and high pollution of traditional chemical methods, which accords with the green production and manufacturing concept of circular economy. At this stage, nearly 40% of the drugs are amino-containing drugs, such as α -tetrahydronaphthylamine, sitagliptin.

To reduce the size of mutation libraries of random mutagenesis, other mutagenesis methods methods are concentrated to improve the enzyme activity. In this research, two methods of directed evolution were surveyed to redesign the activity pocket of ω -transaminase from *Kordiimonas lacus*. One was based on the sequence alignment and screening of primary structure and the tool I-muant 3.0 was used to predict the protein stability changes. The other is to predict hot spots in structural analysis to obtain the active sites and intermolecular forces (using Hotspot Wizard and Discovery Studio). By limiting the library to variants containing NNK mutation strategy, mutation sites were designed to modify the range of active site of Kord-ATA. In the first round of the Kord-ATA evolution, the site Y56W, A218T, Y149F, T228A were chosen to obtain the high reaction activity towards amino alcohols. In the second round, several sites were combined and enzyme activity is effectively improved by 3-10 fold. By redesigning molecular choreography of active pockets in the Amino alcohol synthesis in silico, those methods could be chosen to make great progress in activities in addition to reducing screening.

Bioamination of prochiral methylketones employing amine transaminases

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Amine transaminases (ATAs) are pyridoxal-5-phosphate (PLP)-dependent enzymes that catalyze the transfer of an amino group from an amino donor to an aldehyde and/or ketone. When the starting ketones are prochiral, they produce optically active amines by asymmetric synthesis. [1-3] In this work was investigated the asymmetric synthesis of primary amines employing ATAs, focussing on studying how the presence of α - β -unsaturated methylketones and benzyloxy ring would affect the ATA-catalysed reaction. The use of different amino donors was also investigated. It was evaluated a set of five wild type ATAs, three of them (*S*)-selectives, from the microorganisms *Vibrio fluvialis* JS17 (Vfl-TA), *Chromobacterium violaceum* (Cvi-TA) and *Ruegeria pomeroyi* (Rpo-TA), and two of them (*R*)-selectives from *Mycobacterium vanbaalenii* (Mva-TA) and *Aspergillus terreus* (Atr-TA). The best results obtained were for the benzyloxy ring and the saturated system, with moderated to high conversions rates (57->99%) and high *ee* (93->99%). The α,β -unsaturated system reduced dramatically the enzymatic reactivity leading to conversions smaller than 10%. Although the conversions in these cases were low, the enantioselectivity was good, with *ee* varying of 90-99%. Docking experiments were also carried out, and the docking results corroborate those obtained experimentally. The benzyloxy system, which had high conversion rates, makes a π -cation type interaction at the active site, which facilitates the nucleophilic attack of lysine. For the α,β -unsaturated compound, there is an increase in the distance for the nucleophilic attack of lysine in relation to the saturated one, which could explain the lower conversions obtained for this compound.

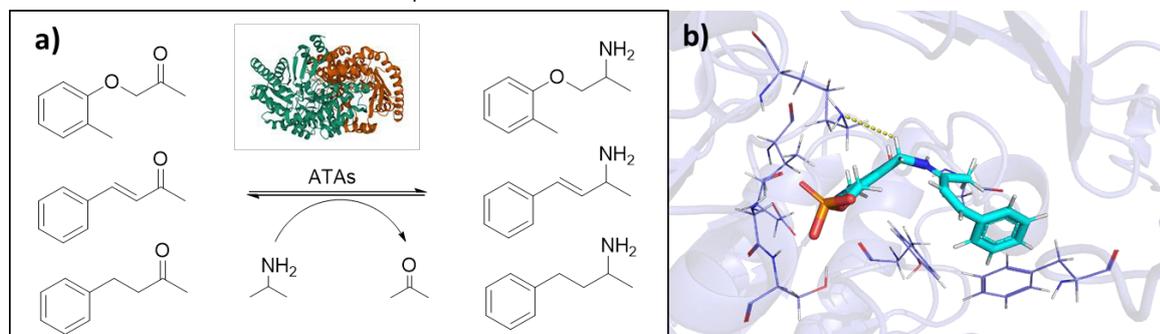


Figure 1. (a) The ATA catalysed reaction employed in this work. (b) Substrate docking experiment with compound 1b

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Pig Liver Esterase-Catalyzed Kinetic Resolution of Cyclic Carbonates

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Due to their low toxicity and environmentally benign behaviour, cyclic carbonates find numerous applications in industry, such as electrolytes in lithium-ion batteries and green solvents in organic synthesis.[1] They are also widely employed as monomers in the production of polycarbonates and synthons for the synthesis of several chemicals.[2,3] Despite their synthesis has been developed to a sophisticated level, few studies are reported for their enantioselective preparation from racemic mixtures. A possible approach for this is the kinetic resolution of epoxides using chiral metal complexes, which convert selectively the epoxide to the corresponding carbonate.[4] As an alternative to this, we propose the direct kinetic resolution of the cyclic carbonates by means of an esterase-catalyzed reaction which hydrolyses selectively the C-O bond in a clean reaction releasing only CO₂ (Figure 1). This will ideally afford a mixture of enantiomerically pure carbonates and diols, which is of high interest since chiral diols play an important role in the synthesis of natural and biologically active substances.[5]

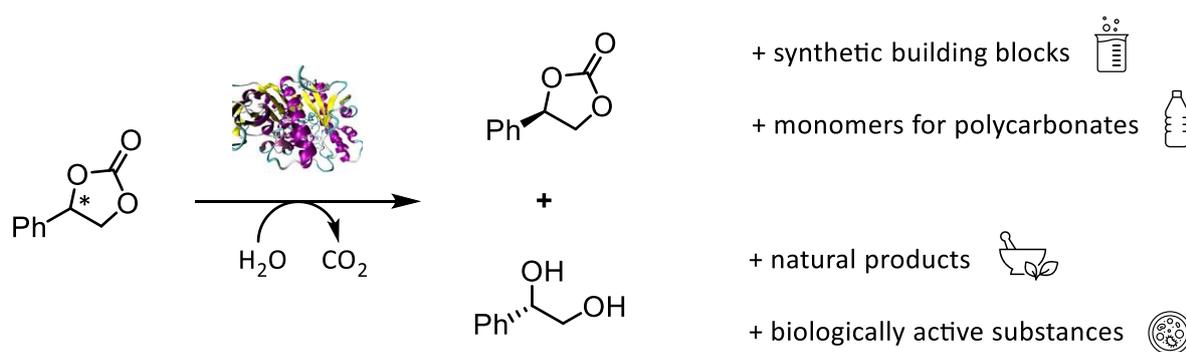


Figure 1. Kinetic resolution of cyclic carbonates via enantioselective enzymatic hydrolysis.

In our first studies we used classical pig liver esterase (PLE) due to its high efficiency and wide applicability for the kinetic resolution of esters.[6] PLE showed to be active for several substrates and gave outstanding results for (*R*)-styrene carbonate that could be isolated with 99% *ee*. Based on this, the synthetic concept was transferred to similar cyclic carbonates, which were also enantioselectively converted. This motivated us to further explore a variety of hydrolases since their high selectivity, stability and broad substrate scope make them promising enzymes for this reaction.

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Building enzyme nanohybrids for remote triggering

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The immobilization of enzymes by entrapment in silica matrix has been widely reported in literature [1]. This synthetic procedure leads to the production of nanostructured particles that are easy to prepare in mild and biocompatible conditions, through the formation of a silica matrix via catalysis of a polyamine molecule in presence of silicic acid. Recently, it has been demonstrated for the first time the possibility to incorporate magnetic nanoparticles into the biohybrids [2]. In this work, we reported the experimental conditions required for the co-entrapment of different MNPs with Horse Radish Peroxidase (HRP), using MNPs with different organic coatings, with the final goal to activate the entrapped enzyme thanks to the capability of the MNPs to generate heat in presence of an external alternating magnetic field (AMF).

The physical-chemical characterization confirms the versatility of this encapsulation methodology for the co-encapsulation of an enzyme with different types of MNPs. Indeed, we obtained nano-hybrids with good co-entrapment efficiencies: HRP immobilization, expressed activity and entrapped iron yields were around 90 %, 50 % and 65 % respectively. As the MNPs used in this work have different AMF-triggered heating efficiencies, these results open the path to find the most adequate one to locally reach the optimal HRP temperature (45°C).

Thus, such versatility could expand the applicability of this type of biohybrids for the development of a new format of Prodrug Enzyme Therapy remotely controlled by magnetic heating.

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Biocatalysis in deep eutectic solvent for menthol derivatization

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Terpenes derivatization based on the biocatalytic (enzymatic) process is a widely used method due to the good efficiency, high enantio-specificity and also cost-effectiveness, especially for the use of immobilized enzyme. However, the polarity of the aqueous phase requested by the enzyme activity is in contrast with the low water solubility of the reagents (e.g. menthol). This is an important limitation aspect especially for the industrial esterification/ transesterification process. Enzymatic processes are not only restricted by low solubilities of the interested substrates and solvent-based inhibition of the enzyme activity, but also by a lack of enzyme stability in the chosen solvent.

To overcome these drawbacks, enzymatic reactions have been performed in non-conventional reaction media (e.g. ionic liquids, supercritical fluids, deep eutectic solvents - DES). DESs are eutectic mixtures of at least two components coupled by hydrogen bond interactions. The resulted mixture is liquid at room temperature, comfortable used as solvent and with lower freezing point compared to the initial constituents. DES involvement in enzymatic biocatalysis has been first described by Abbott and co-workers in 2003 and gained attention as alternative reaction media for biocatalytic process.

We developed a DES-based biocatalytic method for (-)-menthol acylation with methyl fatty acids (FAME) using immobilized lipase enzyme as biocatalyst. The mixture of menthol and FAME in a certain molar ratio was the DES solvent phase of the proposed biocatalytic process. Chemical interaction between DES components started by adding a lipase in DES phase. Optimization and validation of the developed method has been performed. The experimental data and their scientific message will be detailed in a poster presentation.

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Biocatalysis based on cold-active lipase for silymarin derivatization

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Silymarin is a natural mixture of flavonolignans extracted from *Silybum marianum* L. Gaertn (milk thistle) seeds, a species originated from Europe and Asia and extensively cultivated worldwide. *In vivo*, Silymarin is synthesized by radical coupling between flavonoids and coniferyl alcohol [1], determining from a compositional point of view a great diversity of related compounds such as silybin or silybinin (33.4%), silychristin (12.9%), silydianin (3.5%) and isosilybin (8.35%) [2]. These flavonoid-like compounds are responsible for the therapeutic activity of the plant extract [3].

Silybin, as one of the most biologically active compounds within the silymarin mixture, has two diastereoisomers (silybin A and silybin B, 1:1) [4]. Their molecular structure (Figure 1) is particularly composed of a chromone fragment responsible for the weak acidic properties, that enables donor-acceptor interactions with basic compounds. Meanwhile, the presence of polyphenol hydroxyls provides the ability to form complexes with transitional metal ions in the 3,4- or 4,5-positions, as in this way high antioxidant activity is imprinted to the molecule. Moreover, this active ingredient is tolerated by animals and humans even at very high doses [5].

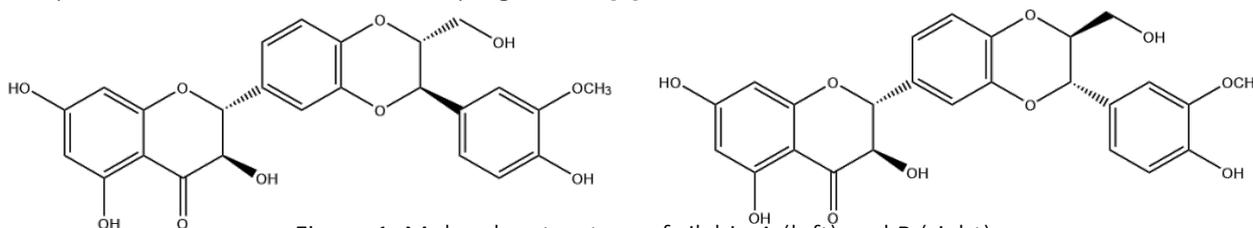


Figure 1. Molecular structure of silybin A (left) and B (right).

Silybin has low liposolubility, thus consisting its drawback on cellular adsorption in the context of bioavailability strategy. So that, silybin fatty acid esters could be a valuable alternative. We propose a biocatalytic cold-active lipase-mediated system for the acylation of silybin A/B with proper fatty acids or esters. The biocatalyst consists of the protein material extracellularly produced by a novel *Psychrobacter sp.* extracted from perennial ice deposits of Scarisoara Ice Cave (Romania). The lipolytic effect of the protein material is considered for the catalysed reaction, as many extracellular putative lipases are attributed to species draft genome. The relative enzyme activity was evaluated for both free and immobilized biocatalyst specimens and the optimization of the biocatalytic system has been achieved. All of these experimental aspects will be detailed during the lecture.

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Formate oxidase from *Aspergillus terreus* (AtFOx) for *in situ* H₂O₂-formation in UPO-driven oxyfunctionalization reactions

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Selective oxyfunctionalization of organic substrates under mild conditions is a desired reaction in chemical synthesis. Unspecific peroxygenases (UPOs), which are driven by hydrogen peroxide (H₂O₂), are promising biocatalysts for these oxyfunctionalizations reactions due to their simplicity and independence from reduced nicotinamide cofactors and complex electron transport chains found in cytochrome P450 monooxygenases.[1,2] However, UPOs are also inactivated by an excess of H₂O₂, so it is essential to keep controlled levels of this compound being high enough for the catalytic activity but low enough to avoid enzyme inactivation.[3,4] For this reason, various approaches for the *in situ* generation of H₂O₂ have been proposed, but many of the systems have drawbacks regarding their cost and the amount of waste that is generated.[4]

Formate oxidase from *Aspergillus oryzae* (AoFOx) has been shown to be suitable for the *in situ* generation of H₂O₂ and stands out in comparison to the other oxidases, in terms of its simplicity, where it only uses formate as substrate and produces H₂O₂ and CO₂ as by-products, as well as its excellent performance.[5] AoFOx also exhibited methanol oxidase activity, which would be very promising because methanol is easily obtained, easy to handle, and can be fully oxidised to CO₂, providing three equivalents of H₂O₂ per one equivalent of methanol.[6]

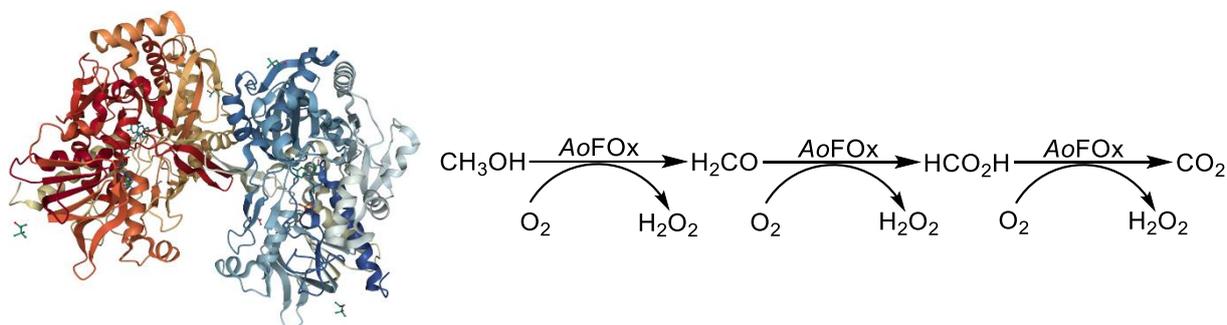


Figure 1. The structure of formate oxidase (PDB 3Q9T), together with the full oxidation of methanol to CO₂, generating three equivalents of H₂O₂. [6]

Here we introduce a new formate oxidase from *Aspergillus terreus* (AtFOx) and report its heterologous expression in *Escherichia coli*, as well as its activity against the substrates sodium formate, methanol and formic acid with the production of H₂O₂ and its kinetic parameters. The results are compared to previously described formate oxidases from literature.

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Tailoring the sol-gel entrapment method of enzymes by design of experiments for solvent-free ester synthesis

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Immobilized lipases are excellent biocatalysts for the enzymatic synthesis of short- and medium-chain fatty esters used as food flavour compounds; however, their catalytic activity greatly depends on the selected immobilization method. While numerous studies are referring to lipase immobilization, there still are no straightforward protocols concerning the optimal method for each enzyme type. Thus, it is necessary to customize the immobilization procedure to the selected enzyme and envisioned applications. [1,2]

The aim of this study was the optimization of the sol-gel entrapment of the *Candida antarctica* lipase B, a generous gift from GenoFocus (South Korea), using organically modified silanes (ORMOSILs) with epoxy functional groups. We hypothesised that the epoxide functional groups could lead to the formation of supplementary covalent bonds between the enzyme and the sol-gel matrix, serving to further stabilize the enzyme (in addition to its entrapment in the sol-gel matrix).

For this purpose, we used design of experiments for the optimization of the key immobilization parameters. First, a Plackett-Burman design was employed for the screening of different ORMOSILs (at a molar ratio of 1:1 with tetramethoxysilane), catalysts, and sol-gel additives. Subsequently, the Box-Behnken design was used to further optimize immobilization parameters such as the molar ratio of the ORMOSIL/TMOS silane precursor system, enzyme loading, and gel ageing time of the obtained xerogels. The immobilized enzyme was used in the synthesis of pentyl hexanoate under solvent-free reaction conditions.

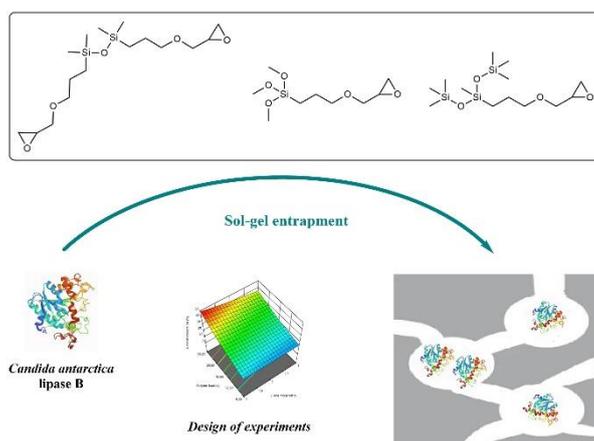


Figure 1. Stabilization of *Candida antarctica* lipase B (CalB) through entrapment in tailored sol-gel matrices.

Acknowledgements

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Cell-free cascade biosynthesis of ω -hydroxyacids from diols

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Cell-free multi-enzyme systems are gaining popularity among the scientific community because they selectively catalyze chemical reactions under mild conditions, increasing the sustainability of the chemical processes. Step-wise biocatalysis in one-pot offers significant advantages over classical step-by-step approaches by i) reducing purification steps, ii) increasing overall production yields and efficiency and iii) pulling the reaction equilibrium towards the final product. Using isolated multi-enzyme systems facilitate the creation of artificial biosynthetic routes without genomic regulation burdens that often limit the titers and productivities, overall when artificial routes are introduced in living chassis.

Manufacturing of ω -hydroxyacids (ω -HA) exhibits a multitude of application in chemical industry since they are used in the production of polyesters, resin, plasticizers and lubricants.¹ It is in polymer industries where ω -HAs show a higher potential as precursors for the next generation of polyethylene-like hydroxyalkanoates that can be used for a variety of commodity plastic applications (i.e. biomedical applications). Long- and medium-chain ω -HA have been synthesized by multienzymatic systems.²⁻⁴ However, the access to short-chain ω -HA (<6 carbon) is yet to be enzymatically achieved from bio-based raw materials.

In this work we present an unprecedented cell-free multi-enzyme system to access short-chain ω -HAs from bio-based diols, such as 1,4- or 1,5 butanediol (Figure 1). Our system incorporates NADH-dependent ADH oxidizing ω -diols to lactones and those lactones can be concurrently hydrolyzed by a lactonase to yield the corresponding hydroxyacids. These diols can be easily produced by microbial fermentation using either renewable sugars from biomass.⁵ Furthermore, using substituted diols (i.e. 3-methyl-1,5-pentanediol), this innovative pathway can also access to substituted ω -HAs (i.e. 5-hydroxy-3-methyl-pentanoic acid). In this system, the redox balance is allowed by incorporating a NADH oxidase (NOX) that replenishes the NAD⁺ pool yielding H₂O₂ as by-product that must be *in situ* eliminated by incorporating a catalase (CAT). Up to now, our highest yield titer achieves 100% of ω -HA starting from 100 mM of diol in 24 h.

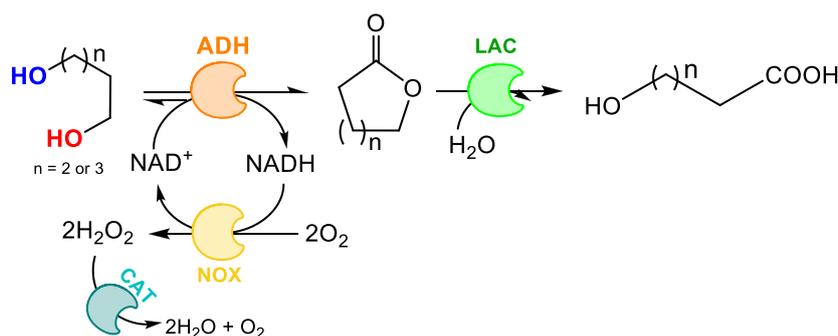


Figure 1. Cell-free cascade biosynthesis of ω -hydroxyacids from diols.

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Stereoselective synthesis of *Aerangis* Lactone mediated by a chemo-enzymatic approach

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Aerangis Lactone (4-methyl-5-decanolide, **I**) is a natural fragrance and it is the main odour component of the African 'moth orchids' (*Aerangis confusa* and *Aerangis kirkii*). We report on the stereoselective synthesis of two individual isomers (**Figure 1**). Possessing optically active forms of *Aerangis* Lactone is of high demand, therefore in our studies biotransformations involving whole cells of bacteria characterized by high alcohol dehydrogenases activity were applied.

After accomplishing the chemical synthesis of the racemic form of *Aerangis* Lactone, we tried to separate the two diastereoisomers exploiting the *vicinal* disubstituent effect.¹ Indeed, the rate of intramolecular reactions, affording cyclic molecules, is often accelerated by introducing a *vicinal* disubstituent group into a linear precursor. In this case, it should be noted that this effect is stereoselective, since the *syn*-diastereoisomer ring-closes more rapidly than the *anti*-isomer, allowing the resolution of the two diastereoisomers with good *de* (>95%).

After the isolation of the diastereoisomers, our aim was to prepare enantiomerically pure isomers of *Aerangis* Lactone. Thus, we chemically reduced the lactones into corresponding diols, the latter were subjected to microbial transformations into chiral *Aerangis* Lactone.² The most promising preliminary results, so far achieved, were obtained using *R. erythropolis* DSM44534, which oxidized the *syn*- and *anti*-diols giving (4*S*,5*S*)-(-)-*cis* and (4*S*,5*R*)-(+)-*trans*-isomers respectively with excellent selectivity (*ee*=98% and 96%).

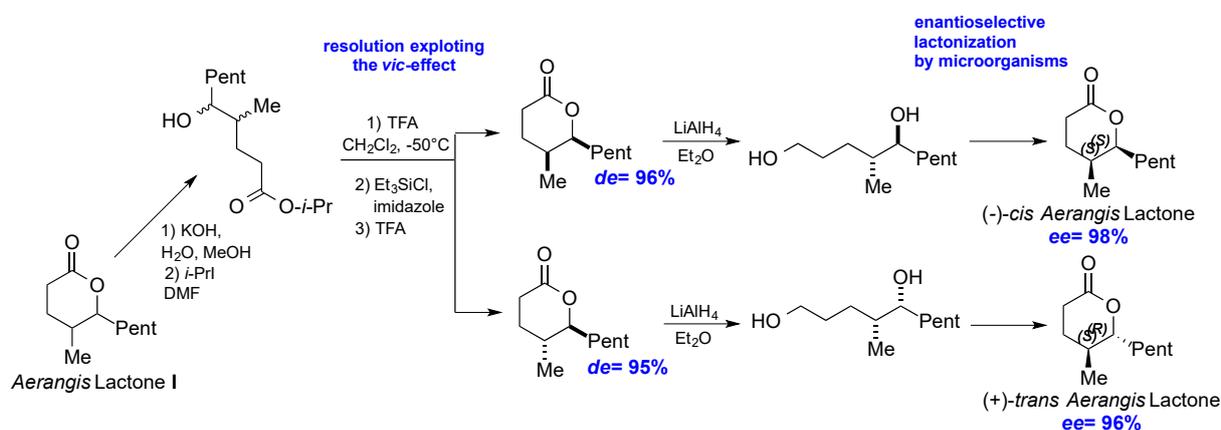


Figure 1. Stereoselective synthesis of optically active isomers of *Aerangis* Lactone.

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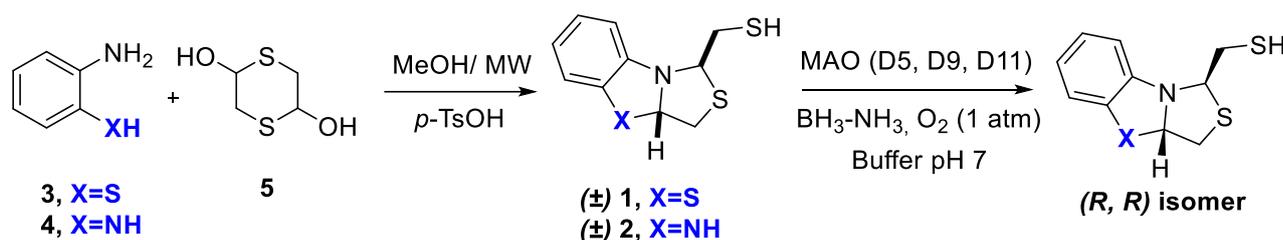
Kinetic resolution of benzothiazolines and benzoimidazolines using Monoamine Oxidase enzyme

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Benzothiazolines **1** and benzoimidazolines **2** are tricyclic compounds designed as penicillin analogs, which demonstrated competitive inhibition of all Metallo-beta-lactamases (MBL) subclasses, with K_i values in the micromolar range. These compounds are obtained in a single step starting from 2-aminothiophenol **3** or 2-aminoanilines **4**, respectively, and dithiane **5**, see Scheme 1.



Scheme 1. Synthesis and enantiomeric resolution of **1** and **2** using MAO-N.

Despite **1** and **2** resulted to be very good inhibitors against all subclasses of MBL, they are obtained as a racemic mixture. Crystallographic studies demonstrated that (R, R) isomer binds preferably with NDM-1 and VIM-2 enzymes, both MBL of clinical importance. For that reason, we aimed to develop a strategy for enantiomeric resolution of **1** and **2**.

Since the mercaptomethyl group present in **1** and **2** is essential for MBL inhibition, it must be preserved during the deracemization process. Compared to non-enzymatic chemical catalysts, biocatalysts are known to present some interesting and advantageous features: high efficiency, mild environmentally-friendly operation conditions, versatility and high selectivity.[1]

Turner's group reported a method for deracemization of primary, secondary and tertiary amines using variants of monoamine oxidase (MAO) from *Aspergillus niger* which display remarkable substrate scope and tolerance for sterically demanding motif.[2,3] We envisioned that this methodology could be useful and applied for enantiomeric resolution of our products. Thus, the racemic mixture was subjected to a deracemization protocol using *E. coli* whole cells expressing three different MAO-N variants and MAO-N purified enzymes. No resolution was found for **1** when using all three variants. On the other hand, all three D5, D9 and D11 variants resulted to be enantioselective for **2**. D11 variant showed the best result, providing the optically enriched product in excellent enantiomeric excess (90%) after 4 hours of reaction. Currently, we are optimizing the parameters to scale up the reaction.

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An integrative biodegradation-bioluminescence toolbox for synthesis of firefly luciferins and expansion of bioluminescence applications

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Halogenated and nitrophenols are widely used as agro- and industrial chemicals. These toxicants are also found as metabolites of pesticides and herbicides in the environment, and they have adverse effects on human health. The flavin-dependent monooxygenase, HadA, catalyzes dehalogenation and denitration of nitro- and halogenated phenols to benzoquinone [1-3]. The HadA reaction can be applied in one-pot reactions to synthesize D-luciferin and luciferin derivatives by coupling with the chemical condensation with D-Cys [4,5]. D-luciferin, a valuable chemical widely used in biomedical applications, can be used as a substrate for the reaction of firefly luciferase to generate bioluminescence. Moreover, the luciferin derivatives from one-pot reactions of HadA can generate bioluminescence with a longer wavelength and the compounds can generate more stable light than the regular luciferin which is useful for bioimaging and biodetection applications. As nitro- and halogenated phenols are key indicators of human overexposure to pesticides commonly used worldwide and indicators of pesticide contamination, the technology provides a sensitive and convenient tool for improved biomedical and environmental detection at ppb sensitivity and specificity in biological samples without the requirement for any pre-treatment. This newly developed methodology provides the dual-pronged advantage of waste biodegradation to produce a valuable chemical as well as a smart detection tool for environmental and biomedical detection.

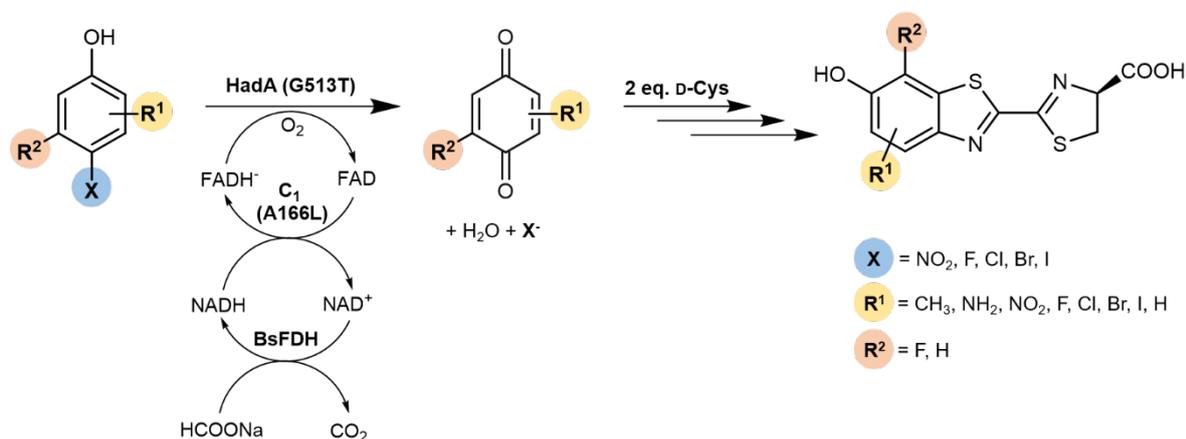


Figure 1. Bioconversion of hazardous phenols to D-LH2 derivatives by the HadA chemo-enzymatic cascade reaction

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Parallel assay for estimating the enzyme promiscuity and hydrogen peroxide in the enzyme-mediated perhydrolysis of *p*-nitrophenyl esters

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Enzyme promiscuity as a result of evolution, enables us to understand the ability of Nature for building complex molecules. Although this strategy is introduced into organic synthesis, no example of a fast and versatile sensor of the enzyme promiscuity is known so far. Therefore, we designed an efficient assay for direct evidence of enzyme promiscuous activity. The protocol presents the comparison of the kinetic studies on the hydrolysis and perhydrolysis for various *p*-nitrophenyl esters. In addition chiral esters were used for the determination of the enzymatic enantioselectivity.[1]

For the first time, the numerical contribution of enzyme promiscuity with respect to the native activity was estimated. Furthermore, the designed system is a parallel method combining probe for hydrogen peroxide detected in a wide range of concentrations (up to 1 ppm). This methodology based on promiscuous multi-sensor provides significant knowledge of peracid formation, profitable in the biological and chemical industry.

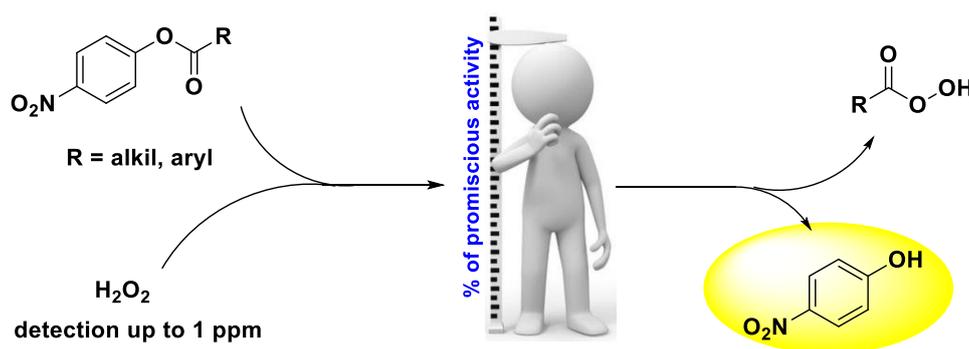


Figure 1. The estimation of enzyme promiscuous activity via perhydrolysis reaction.

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Hydrogen-driven benzyl alcohol production by oxidoreductases from *Aromatoleum aromaticum*

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The betaproteobacterium *Aromatoleum aromaticum* degrades various organic acids, aldehydes and alcohols under anaerobic denitrifying conditions. Some oxidoreductases involved in the respective degradation pathways have application potential for biotechnological reduction of organic compounds. A good example is the production of benzyl alcohol from benzoic acid via benzaldehyde, where the cascade reaction is catalysed by a W-dependent aldehyde oxidoreductase (AOR) and benzyl alcohol dehydrogenase (BDH). Both enzymes were produced by recombinant overexpression and purified to homogeneity by affinity chromatography (as Strep-tag® fusions).

The oxygen-sensitive W-dependent aldehyde oxidoreductase from *A. aromaticum* catalyses the oxidation of aldehydes to carboxylic acids and the respective reverse reaction, reduction of non-activated carboxylic acids to aldehydes. The AOR hosts in its active site a tungsten ion coordinated by two metallopterin cofactors. The tungsten cofactor is connected via an electron-transfer chain of 5 Fe-S clusters to a FAD cofactor that facilitates electron transfer to NAD⁺ as electron acceptor. [1] Unexpectedly, our recent study revealed that the AOR enzyme is also a novel hydrogenase, which uses a molecular hydrogen as electron donor for the reduction of carboxylic acid as well as for NAD⁺ reduction.

Benzyl alcohol dehydrogenase (BDH) from *A. aromaticum* catalyses the NADH or NADPH-dependent reduction of several aldehydes to alcohols and the respective reverse reactions. The enzyme exhibits homotetrameric composition and belongs to the Zn-dependent alcohol dehydrogenase family.

We exploited the unique capabilities of AOR as hydrogenase in a cascade process, employing AOR for reduction of benzoic acid and NADH regeneration and BDH for the further reduction of AOR-produced benzaldehyde with AOR-regenerated NADH. Comparison of the reaction rates in such a cascade system to a system without NADH regeneration (utilizing NADPH as electron donor for BDH) gave even further insight into the complex mechanism of AOR as a bi-functional hydrogenase.

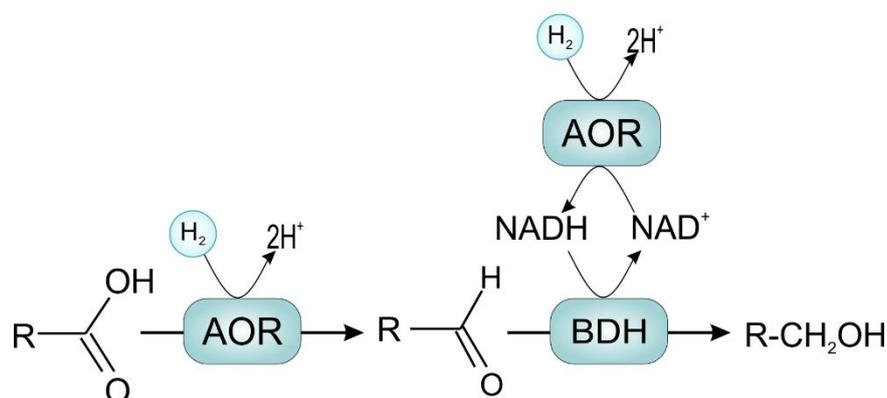


Figure 1. Scheme of reactions occurring in the reactor for bioalcohol production catalysed by AOR and BDH enzymes with hydrogen (2.5 % v/v) as a sole electron donor for the system. R - benzene ring.

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Sustainable synthesis of levetiracetam: enzymatic dynamic kinetic resolution and an ex-cell anodic oxidation

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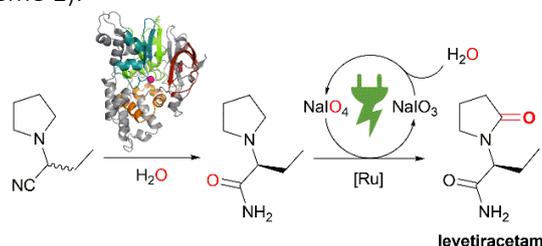
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Levetiracetam is an active pharmaceutical ingredient widely used as a medication for epilepsy. Synthetic routes to this chiral amino amide are low yielding and wasteful. We designed a short and highly efficient route to levetiracetam (Scheme 1).



Scheme 1. Enzymatic dynamic kinetic resolution teamed up with electrochemistry

Herein we describe the enantioselective preparation of the levetiracetam precursor molecule 2-(pyrrolidine-1-yl)butaneamide. The cobalt-dependent thermotolerant nitrile hydratase from *Comamonas testosteroni* (CtNHase) was engineered by directed evolution. To screen for improved variants, we tailored a high throughput screening method that allowed to distinguish clones with improved (*S*)-selectivity or activity on colony level and screened more than 50,000 clones. Key positions were identified and combined, delivering a set of highly *S*-selective CtNHase variants. Racemic nitrile was applied in a fed-batch reaction to a final concentration of 167 mM and converted by to the desired (*S*)-amide in a dynamic kinetic resolution (Fig. 1). The amide was obtained in 73% conversion and 95.2% enantiomeric excess. For the final oxidation to levetiracetam, a ligand-free ruthenium-catalysed method at a low catalyst loading was used. The oxidant was electrochemically generated in 86% yield [1].

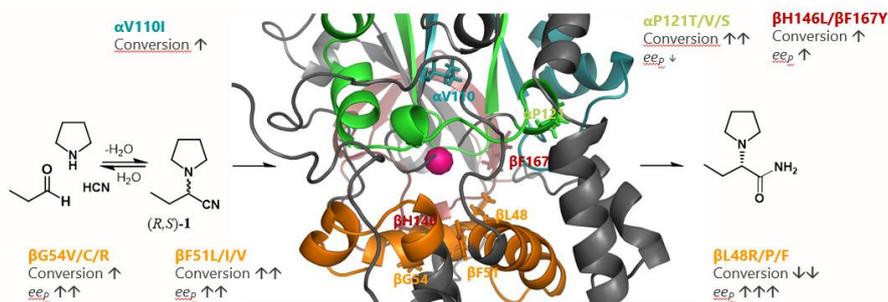


Figure 1. Dynamic kinetic resolution of aminonitrile by engineered nitrile hydratase

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Scaling up asymmetric hydrogenation of substituted alkenes with a potential industrial workhorse: the thermostable Old Yellow Enzyme *TsOYE*

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Scaled-up biocatalytic asymmetric hydrogenation reactions towards industrial use are scarce to find in literature. Implementing ene-reductases from the Old Yellow Enzyme family (OYE) that catalyse such a reaction to produce chiral products[1], would be an interesting application for Industry wishing to move away from traditional chemo-catalysts. The challenge with scaling up biocatalytic reactions is finding the reaction conditions to obtain both high enzyme stability and product turnover with the guiding principles to achieve, within 24 hours, a >95% conversion, >99.5% enantiomeric excess (*ee*), >100 g/L of substrate, while having a substrate to enzyme ratio of >50 and minimizing cofactor <0.5 g/L [2]. Here we demonstrate upscaling of a thermostable OYE that is resistant to co-solvents and pH changes. *Thermus scotoductus* Old Yellow enzyme (*TsOYE*) is a robust, thermostable, flavin-dependent enzyme with optimum activity at 65 °C and performs asymmetric *trans*-hydrogenation of activated alkenes[3]. Compared with previous OYE scale-ups[4][5] an example 50 mL scale afforded a 98% bioreduction of 1 M (*S*)-carvone using *TsOYE* at 40° C and a glucose dehydrogenase recycling system with NADP⁺ (Figure 1), the TON was 112,370, with 90% isolated yield.

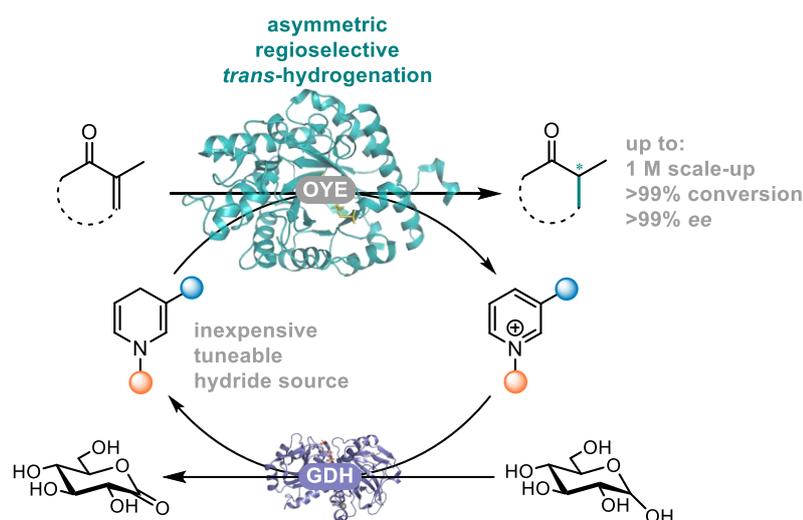


Figure 1. *TsOYE* bioreduction of 1 M (*R*)-carvone to (*2R,5R*)-dihydrocarvone using synthetic co-factor BNAH.

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Chemical-enzyme cascade produce *L*-menthol

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Since menthol propionate contains three chiral centers, chemical synthesis of menthol propionate is a mixture of 8 isomers (Picture 1). Among the eight isomers, *L*-menthol has a unique mint aroma and also has the effects of exciting analgesia, sterilization and itching, making it promising in industrial and pharmaceutical applications.[1]

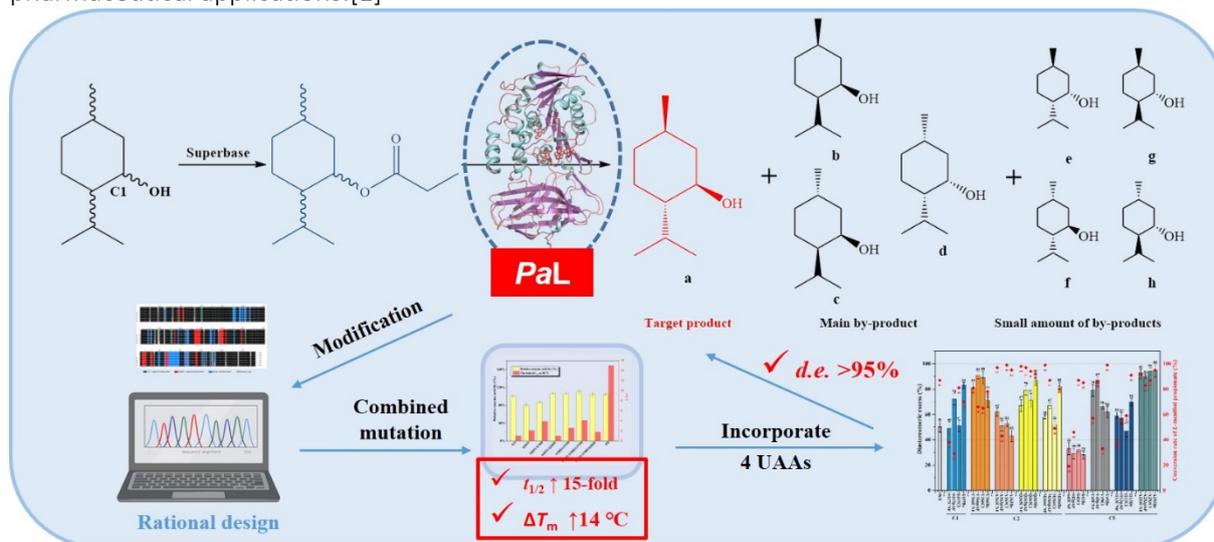


Figure 1. Chemical-enzyme cascade produce *L*-menthol.

We determined lipase from *Pseudomonas alcaligenes* (PaL) crystal structure and identified four key single mutations S205Y, S78H, G379D and Q289E using three computational design strategies. The 4M variant containing four mutations showed high thermostability with a 15-fold improved half-life, T_m increased by 14 °C and a comparable activity with the wild type.

Based on high thermostable variant 4M, the unnatural amino acids were site-specifically incorporated into PaL to explore its diastereopreference mechanism. Menthol propionate has three chiral centers, eight isomers in total. Molecular dynamics (MD) simulations were first applied to analyze the interactions between the active sites of PaL and the target substrate *L*-menthol propionate. Furthermore, the four UAAs (o-bromophenylalanine, o-chlorophenylalanine, p-cyanophenylalanine and p-aminophenylalanine) were substituted for 9 amino acid sites that potentially influenced three chiral centers and several variants with significant improvement in the diastereopreference were obtained. The diastereomer selectivity of beat variant at Ala253 was 100% higher than that of the wild-type. A linear relationship was found between volume, flexibility of the active center and diastereoselectivity.[2]

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Chemoenzymatic synthesis of 3-cyanopropanoic acid from glutamate

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The production of biobased chemicals often requires the removal of (oxygen) functionalities from biomass-derived starting materials^[1] as in case of the oxidative decarboxylation of amino acids in the production of biobased nitriles. The oxidative decarboxylation of L-glutamic acid (Glu) the most abundant amino acid in biomass^[2] generates the corresponding nitrile, 3-cyanopropanoic acid (CPA). CPA is a potential starting material for a range of products such as acrylonitrile, succinonitrile or pharmaceuticals.

The oxidative decarboxylation of amino acids can be mediated by hypobromite (HOBr). In order to minimise undesired oxidative side-reactions, using HOBr in low concentrations is advisable. For this, next to some chemocatalytic^[3] or electrochemical methods^[4] also an enzymatic approach has been developed (Figure 1).^[5]

High selectivity (>99%) and full conversion of L-glutamic acid into 3-cyanopropanoic acid was observed for the enzymatic procedure.^[5] The substrate loadings, however, were as low as 5 mM, which is neither economic feasible nor environmentally acceptable.

The aim of this research was to scale up the conversion of L-glutamic acid into 3-cyanopropanoic acid by increasing the substrate loadings.

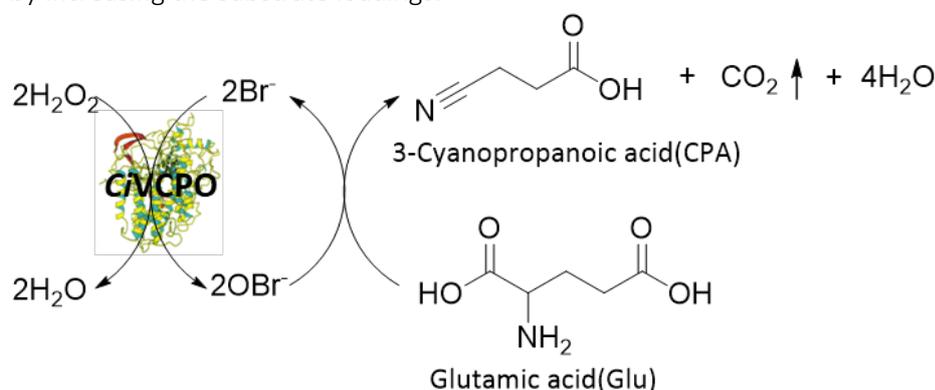


Figure 1. Oxidative decarboxylation of L-glutamic acid yielding 3-cyanopropanoic acid using the vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (ClVCPO) and catalytic amounts of bromide

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Site directed confinement of a laccase in a porous scaffold towards robustness and selectivity

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Laccases variants, graftable either in the vicinity of the T1 Cull site or in the vicinity of the T2/T3 trinuclear cluster, were site-directed immobilized onto macrocellular Si(HIPE) monoliths for continuous flow catalysis. Monoliths were prepared with a classical method based on Schiff based forming reaction between the enzyme and the glutaraldehyde functionalized foam. Both immobilized enzymes exhibit good reusability and stability toward dyes decolorization up to 60 % remaining operational activity after thirteen consecutive decolorization performances and one-year storage. When addressing the catalysts efficiencies we were able to assess the influence of the laccase orientation. We propose that orientation of the enzyme is mediated by the location of the surface lysine covalently bound to the support. Variations observed with different substrates or at different pH support a modulation of the enzyme activity by the artificial environment created close to the Cull T1 site.

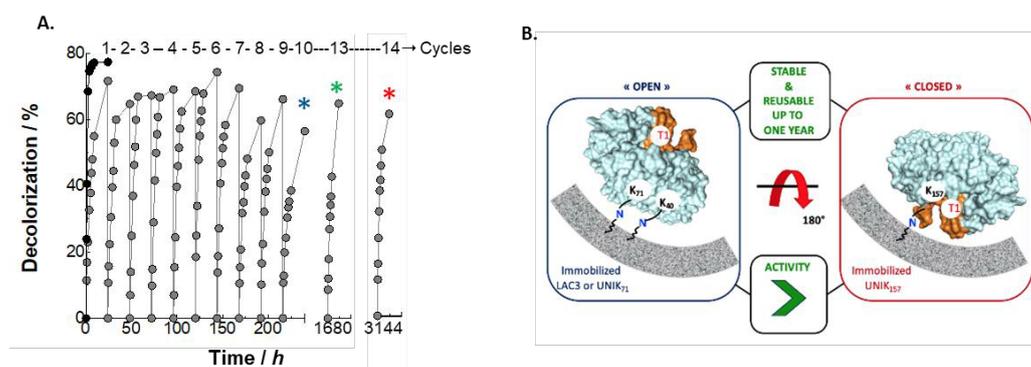


Figure 1. Decolorization of RB5 by LAC3 immobilized onto HCO@Si(HIPE) A. Kinetic traces obtained for (●) LAC3 in homogeneous conditions and (○) LAC3@HCO@Si(HIPE) after several decolorization cycles and up to one year storage. B. Explanation for the effect of enzyme orientation.

Dehydrogenase-catalysed selective oxidation of aminopolyols for the synthesis of iminocyclitols

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Iminosugars are high-value natural products and pharmaceutical targets that possess the ability to inhibit glycosidases. A retrosynthetic analysis quickly identifies sugars as plausible precursors for the iminocyclitol scaffold however, the benefit of using sugars as highly functionalized, readily available starting materials with rich stereochemistry is overshadowed by the limitations in synthetic methods, which are heavily reliant on protecting group chemistry to achieve regioselectivity, restrict conformers and reduce the polarity of the polyhydroxylated species.

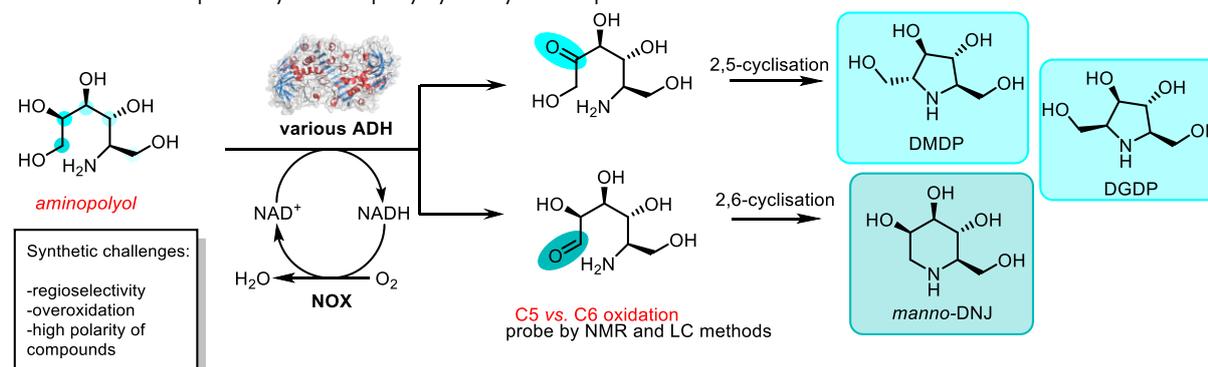


Figure 1. The selective oxidation of aminopolyols by alcohol dehydrogenase (ADH).

The regio- and stereoselectivity of enzyme-catalysed transformations enables protecting-group-free sugar chemistry, and herein, we propose a three-step enzymatic cascade whereby simple sugars undergo transamination, oxidation and reduction *via* transaminase (TA), alcohol dehydrogenase (ADH) and imine reductase (IRED) biocatalysts. It was recently reported that TAs can accept simple aldoses and ketoses. [1,2] Furthermore, a gene cluster responsible for 1-deoxynojirimycin biosynthesis in *Bacillus amylofaciens* expresses both TA and ADH, thus supporting the proposed synthetic pathway. [3,4]

The zinc-dependent ADH from the gene cluster is explored for its ability to accept unprotected aminopolyols. Assisted by a genome mining approach, we selected a panel of homologs with high sequence similarity and achieved heterologous expression and purification in *E. coli*. The spectrophotometric assay to monitor NAD⁺ reduction establishes substrate scope and the best enzyme candidates put forward for engineering. Cofactor recycling with a novel water-forming NADH oxidase (*StNOX*) [5] allows for preparative-scale biotransformations, and a combination of 2D NMR experiments can probe the site of oxidation. [6] A cohort of oxidation enzymes with different regioselectivities for the asymmetric, selective oxidation of aminopolyols will unlock key intermediates for a range of pyrrolidine and piperidine iminosugars and access a library of diverse derivatives.

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Chemoenzymatic Syntheses of Lisofylline Enantiomers *via* Lipase-Catalyzed Kinetic Resolution and Optical Inversion Approach

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Lisofylline (LSF) exhibits strong anti-inflammatory and immunomodulatory properties, thanks to which it is promising drug candidate for the treatment of disorders associated with both respiratory and cardiovascular systems [1].

Moreover, recent studies confirmed that administration of lisofylline improves patient response to chemo- and radio-therapy of the cancers [2] as well as it can be useful in effective prevention and treatment of insulin-dependent Type 1 diabetes and/or after islet cell transplantation [3].

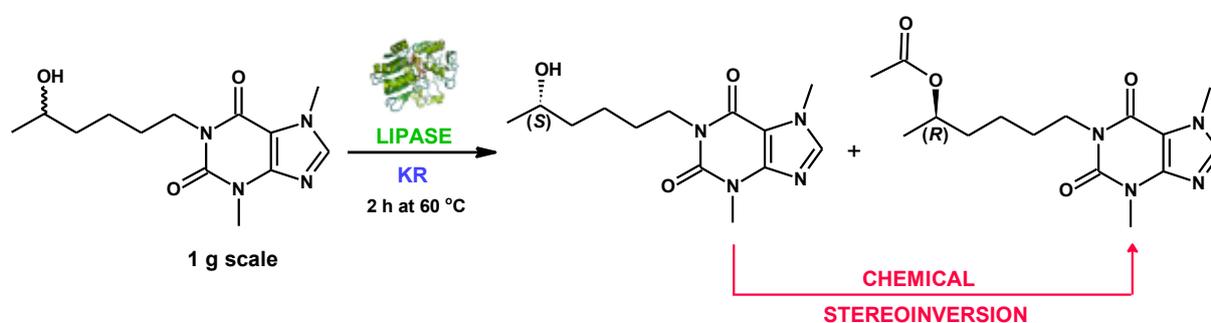


Figure 1. Chemoenzymatic syntheses of (*S*)- and (*R*)-lisofylline utilizing CAL-B as biocatalyst.

In this study, optimization of the reaction conditions for the enzyme-catalyzed enantioselective transesterification of racemic lisofylline under kinetically-controlled conditions was performed. This task allowed to select the most efficient biocatalytic system, which consisted of immobilized lipase type B from *Candida antarctica* (Chirazyme L-2 C-3, CAL-B) suspended in ethyl acetate and vinyl acetate as acetyl donor. The 1 g-scale kinetic resolution (KR) of the racemate carried out for 2 h at 60 °C furnished both KR products in homochiral form (>99 %) with the 50 % conversion. In order to convert (*S*)-lisofylline toward its more biologically-relevant (*R*)-enantiomer, a two-step stereoinversion procedure was applied. In this regard, acetolysis of the respective optically pure (*S*)-mesylate using cesium acetate and catalytic amount of 18-Crown-6 in dry toluene was coupled with K_2CO_3 -mediated methanolysis of the (*R*)-acetate to afford desired counterpart with only slight drop in enantiomeric excess (96% ee). The elaborated lipase-catalyzed KR together with the applied stereoconvergent strategy resulted in development of useful chemoenzymatic protocol toward lisofylline enantiomers obtained with excellent optical purities (>99 % ee) and in satisfactory yields (47-50 %), respectively. In addition, the first crystal structures of lisofylline enantiomers performed by X-ray analyses are reported. Moreover, to enlighten the ability of CAL-B to catalyze the enantioselective transesterification of racemic lisofylline molecular docking simulations were performed.

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Immobilization of multi-enzyme systems through artificial cellulosomes in solid phase

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Synthetic Scaffolds such Cohesin and Dockerin domains enable the spatial organization of enzymes in multi-enzymatic reactions with one of the strongest protein-protein interaction that has been reported.[1] The spatial organization facilitates the direct transfer of a substrate from one enzyme to the next one, avoids accumulating intermediates and maximize the chemical fluxes towards the target products.[2]

Using the Cohesin and Dockerin system, two types of biocatalysts in solid-phase were generated. In the first biocatalyst, Dock-wTA-Dock and Coh-ADH were separately immobilized on two different microbeads, so the intermediates must travel from one bead to the other in order to complete the chemical cascade

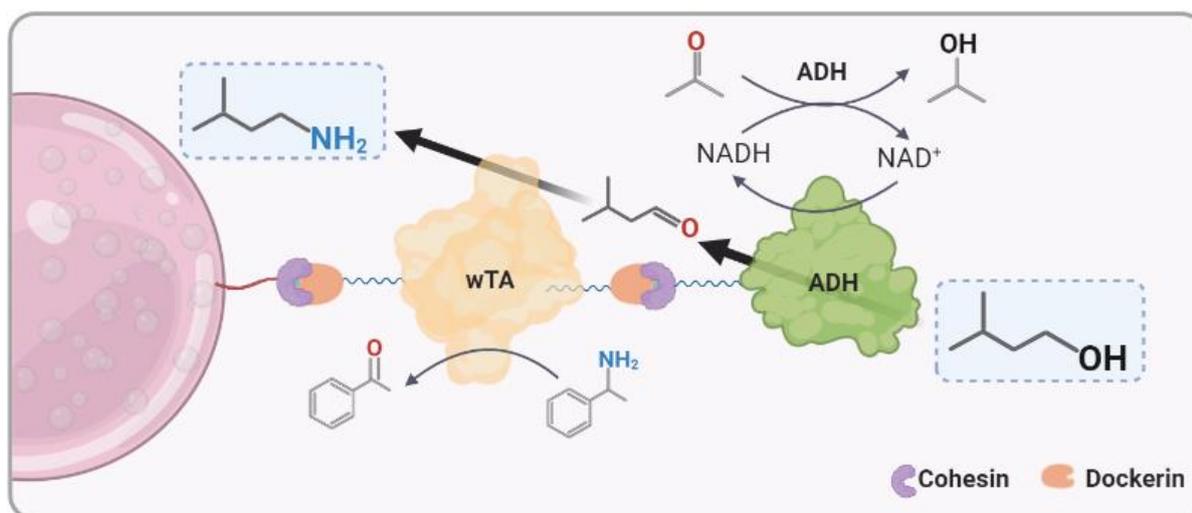


Figure 1. Scheme of enzymatic cascade

The biocatalysts are able to produce acetophenone and isopentylamine from methylbenzylamine and 3-methyl-1-butanol, while the cofactor needed for the first oxidation reaction is in situ recycled by ADH using acetone as co-substrate. We demonstrate the sequential immobilization and the strength of the specific interaction. Our results showed higher performance obtained when both enzymes are co-immobilized versus when enzymes were immobilized apart on two different carriers.

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Exploring selectivity in enzymatic [4+2]-cycloaddition reactions in the spirotetronate cyclase family

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In recent years, more and more enzymes have been identified which perform [4+2]-cycloaddition reactions in the biosynthetic pathways of complex secondary metabolites.[1] A sub-group of natural pericyclases has been identified in the bacterial polyketide synthase (PKS) gene clusters for the biosynthesis of spirotetronates and spirotetramates.[2] The formation of the spirotetronate/ spirotetramate moiety is achieved enzymatically through an intramolecular Diels-Alder reaction in which both a cyclohexene ring and a larger macrocycle is formed (Figure 1A). Representatives of this family are AbyU[3] and AbmU[4] from the abyssomicin biosynthetic pathways of *Verrucosispora maris* and *Streptomyces koyangensis*, respectively. They share a sequence homology of only 31%, but are conserved in their protein fold, which is mainly composed of a β -barrel (Figure 1B). More than such 10 spirotetronate/spirotetramate cyclases have been already reported in the literature, but the understanding of this cyclases is still limited to their individual natural role. We want to improve the understanding of this enzyme family and investigate features contributing to their high selectivity.

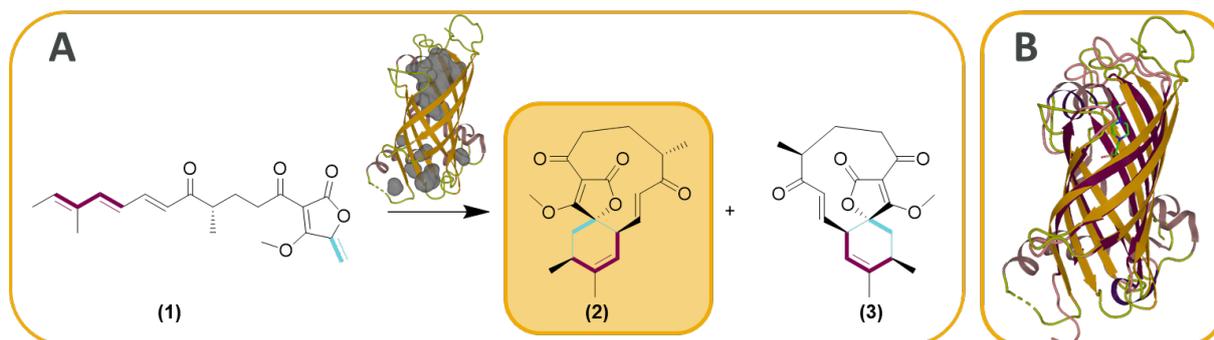


Figure 1. (A) The tetronate substrate **(1)** found in the biosynthetic pathway of (neo-)abyssomicines in *Streptomyces koyangensis* (*abm*) is catalyzed stereo-selectively to the spirotetronate product **(2)** by AbmU. (B) Structural alignment of AbyU (5DYV, purple) with AbmU (6LE0, Orange) and a HEPES molecule (green) bound in the substrate binding pocket.

For this purpose, we performed genome mining and created sequence similarity networks (SSN) by which we were able to predict a diverse set of putative Diels-Alderases of this family. As a consequence, we built a small spirotetronate cyclase library containing representatives from the literature and the SSN analysis data. For their characterization, we first established a streamlined-workflow to express these enzymes recombinantly in *Escherichia coli* with a subsequent two-step purification protocol. The newly obtained enzymes were screened towards derivatives of natural tetronate substrates revealing shared behaviour in activity and selectivity of different members of this group.

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Biocatalytical access to amides

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Amide formation is one of the most important reactions in industrial pharmaceutical synthesis.^[1] Although the synthetic approaches appear to be simple, they suffer in fact from many drawbacks.^[2] So far, many strategies to obtain amides have been published, but several problems have not been solved yet.^[3,4] Challenges are for example the direct transformation of carboxylic acids or esters with amines. Known chemical strategies require the activation of the substrate via SOCl_2 leading to a reactive acyl chloride which reacts with the amine. The reaction suffers from poor atom economy, danger and racemization of the α -center in the acid. For these challenges biocatalysis may provide a solution, especially because biocatalysis is known for using harmless reagents and working under mild conditions.

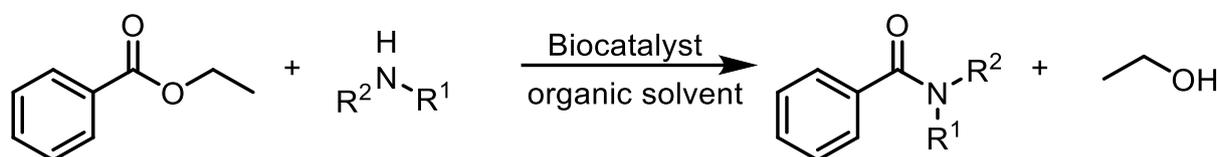


Figure 1. Amide formation starting from a bulky acyl donor.

Our aim is to identify a biocatalytic option for amide formation, especially by the help of hydrolases starting from bulky acyl donors. Screening several hydrolases, one promising candidate has been discovered so far, namely the lipase from *Sphingomonas sp.* HXN-200 also known as SpL.^[5] The lipase was discovered and expressed by Li and co-workers in 2018. SpL shows high activity in aminolysis towards various esters with amines in organic solvents. New substrates have been tested and functional studies on this protein shall be conducted to understand its reaction behaviour.

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Photocatalyzed Wacker-Tsuji oxidation of allylarenes and combination with enzymes in one-pot sequential protocols

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Nowadays, multicyclic systems are considered essential tools for the development of synthetic routes, as they provide elegant solutions for the design of efficient cascade transformations. Remarkably, the integration of biocatalytic reactions in these systems has enabled the access to a large family of chiral products with high efficiency and selectivity.[1] In addition, the combination of photocatalysis along with enzymes is currently gaining a great number of adepts, allowing the access to (optically active) organic molecules under mild reaction conditions.[2]

In the context of metal-catalyzed reactions, Wacker-Tsuji oxidation is a pivotal organic transformation which affords carbonyl compounds from alkenes, using a palladium(II) salt as catalyst in combination with a redox regeneration system, usually composed by a copper(II) salt and molecular oxygen.[3] Searching for the development of more sustainable approaches, this transformation has also been accomplished by applying light irradiation in the presence of photocatalysts.[4,5]

Taking advantage of the versatility of carbonyl groups as precursors of, e.g. enantioenriched amines, our research group has recently demonstrated the compatibility of palladium and amine transaminases for the development of Wacker-Tsuji oxidation-steroselective biotransamination sequences in aqueous medium.[6] Herein, we disclose an improvement of this methodology by performing a metal- and photocatalyzed Wacker-Tsuji oxidation of a series of allylarenes followed by the stereoselective bioreduction or biotransamination of the resulting 1-arylpropan-2-one intermediates. Thus, the combined use of an easily accessible commercial palladium(II) catalyst under blue led irradiation and enzymes will be deeply discussed as displayed in Figure 1, providing us not only highly valuable 1-arylpropan-2-amines but also 1-arylpropan-2-ols in good yields and excellent enantiomeric excess.

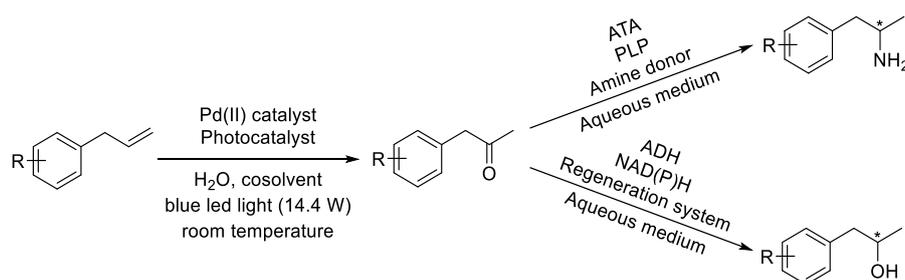


Figure 1. Sequential photo-metal-biocatalytic cascades to obtain optically active 1-arylpropan-2-amines or 1-arylpropan-2-ols.

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Cascade Reactions in Tetrahydroisoquinoline Synthesis

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Alkaloids are historically significant plant-derived natural products as they have been used by humans to treat a variety of conditions for centuries.[1] As a subset of alkaloids, tetrahydroisoquinolines (THIQs) and their derivatives exhibit a range of biological activities, for example as drugs effective against hypertension, suppressing coughs and have even been investigated for their anti-malarial effects.[2, 3] One example of a natural product containing this moiety is (*S*)-norcoclaurine, an important intermediate in the benzyloisoquinoline alkaloid biosynthesis.[4]

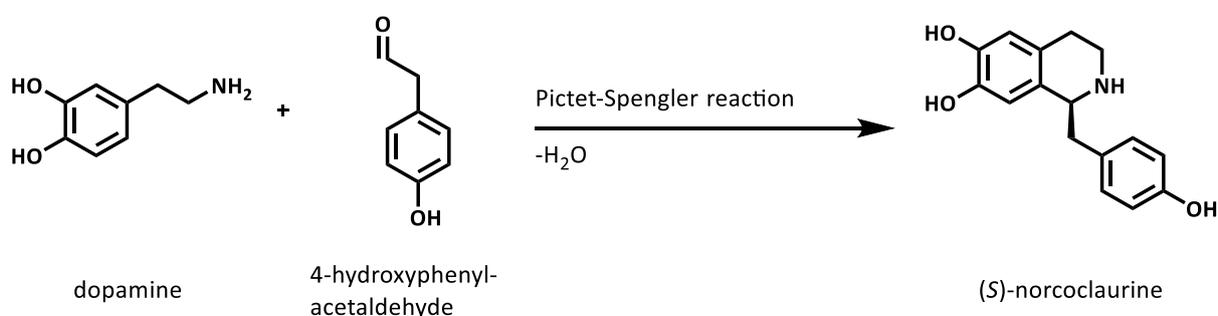


Figure 1. The biosynthesis of the natural product (*S*)-norcoclaurine involves a Pictet-Spengler reaction.

The THIQ scaffold can be generated in a *Pictet-Spengler* reaction in which an aldehyde and a β -arylethylamine react in a cyclisation reaction. This reaction can, for example, be catalysed enzymatically or by potassium phosphate buffer.[5,6]

Considering that aldehydes are often volatile or cannot be stored over a long period of time, their *in situ* generation for cascade reactions is an interesting possibility to bypass these problems. Here, we present a one-pot cascade reaction where in a first step, an aldehyde is chemoenzymatically produced from an alcohol. In the second step, an amine is added and a tetrahydroisoquinoline is formed.

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Biocatalytic Routes Towards a *N*-Methylphenylethylamine Derivative

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Proteins are built from a repertoire of 20 naturally occurring amino acids, rendering the latter as the building blocks of life. Moreover, natural and nonnatural amino acids can serve as valuable starting materials for pharmaceuticals. The enzyme tyrosine phenol lyase (TPL) which catalyzes the conversion towards the naturally occurring amino acid tyrosine can also be used in the reverse direction to induce the carbon-carbon coupling towards tyrosine starting from phenol, pyruvate and ammonia.[1] Furthermore, the use of TPL in combination with amino acid deaminase (AAD) in cascades was reported, of which the latter represents an enzyme which is able to convert amino acids towards prochiral α -keto acids.[1] Herein, the enzymes TPL and AAD were used in combination with selected reductive aminase enzymes in a cascade-fashion towards a *N*-methyl tyrosine derivative starting from the phenol derivative guaiacol and pyruvate (Figure 1).

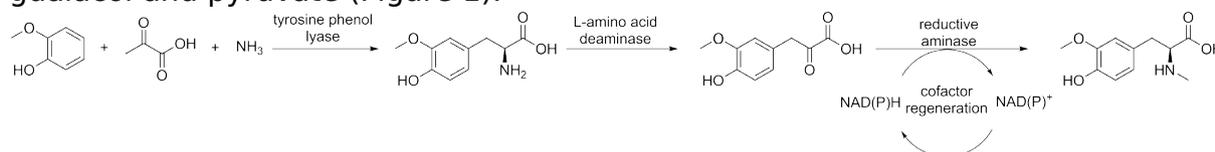


Figure 1. Amide formation starting from a bulky acyl donor.

First, substrate loading studies were performed in order to determine the most productive titers, which was found to be between 70 and 80 mM guaiacol for the first cascade step. Moreover, it was found that the first reaction steps, namely the amino acid formation and the following deamination, do not show compatibility for the use in a one-pot system, since the formation of the amino acid derivative leads to the inhibition of the AAD. For the last reaction of the route, namely the reductive amination of the α -keto acid, several enzymes which were reported in literature were screened in order to find suitable catalysts for the reductive amination using methylamine as the nucleophile instead of ammonia.[2,3] By screening five different enzymes it was found that two Δ 1-pyrroline-2-carboxylate reductases (Pip2CR) (from *Pseudomonas syringae* pv. tomato and *Pseudomonas putida*) as well as ureidoglycolate dehydrogenase (from *Pseudomonas syringae*) were active on the substrate of interest. Enzymatic cascades serve as valuable systems for the production of various structures due to, e.g. rendering purification steps unnecessary. The herein shown approach could serve as a powerful alternative to chemical synthesis for the production of an amino acid building block needed for the organic synthesis of *N*-methylated amino acids.

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Regiodivergent and Stereoselective Hydroxyazidation of Alkenes by Biocatalytic Cascades

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Asymmetric functionalization of alkenes paves the way for the direct synthesis of many chiral compounds. Vicinal hydroxyazidation of alkenes provides a desirable approach to 1,2-azidoalcohols, however existing methods are limited by the control of stereoselectivity and regioselectivity. Herein, we describe a biocatalytic cascade strategy for regiodivergent and stereoselective hydroxyazidation of alkenes, yielding various enantiomerically pure 1,2-azidoalcohols in good to high yields. The biocatalytic cascade process is designed by combining styrene monooxygenase-catalyzed asymmetric epoxidation of alkene and halohydrin dehalogenase-catalyzed regioselective ring-opening of epoxide with azide. Additionally, a one-pot chemo-enzymatic route to chiral β -hydroxytriazoles from alkenes is developed via combining the biocatalytic cascades and Cu-catalyzed azide-alkyne cycloaddition.

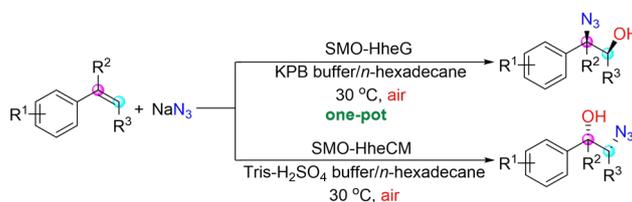


Figure 1. Direct synthesis of chiral 1,2-azidoalcohols via biocatalytic cascade-catalyzed regiodivergent and stereoselective hydroxyazidation of alkenes.

We initially constructed a recombinant *Escherichia coli* (SMO-GDH) strain for co-expression of a styrene monooxygenase and a glucose dehydrogenase.

With the optimum conditions in hand, we next explored the scope of the two biocatalytic cascades for asymmetric hydroxyazidation of alkenes.

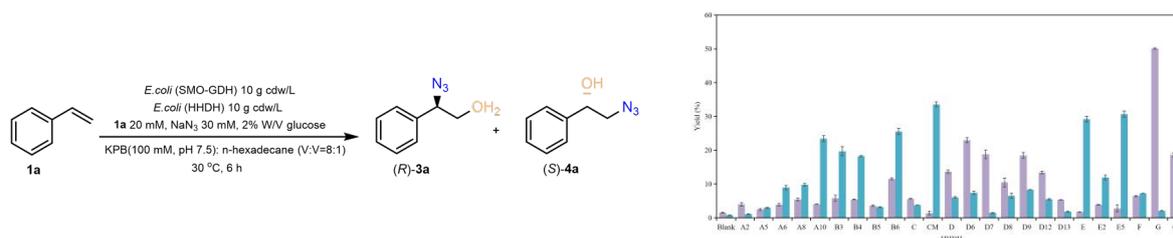


Figure 2. Screening of biocatalytic cascades for stereo- and regioselective hydroxyazidation of 1a.

In summary, we have developed a highly efficient method for regiodivergent and stereoselective hydroxyazidation of alkenes by two novel biocatalytic cascades, providing a direct and green approach to various enantiopure 1,2-azidoalcohols. The reaction is featured by its high regioselectivity, excellent stereoselectivity, good efficiency, broad substrate scope, easy operation and mild conditions. We also demonstrated that direct preparation of chiral β -hydroxytriazoles from alkenes is feasible by a one-pot chemo-enzymatic synthesis. We anticipate that this biocatalytic cascade strategy could impact the development of asymmetric difunctionalization of alkenes.

Development of an enzyme cascade process for biocatalytic production of chiral beta amino acids

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Chiral β -amino acids are valuable building blocks for pharmaceuticals and fine chemicals [1]. Within this project chiral β -amino acids are to be produced applying a modified hydantoinase process using racemic dihydropyrimidines as educts. The process is to be based on two enzymes. A dihydropyrimidinase will be used for hydrolytic cleavage of the dihydropyrimidine ring followed by the reaction of a linear amidase able to decarbamoylate *N*-carbamoyl β -amino acids.

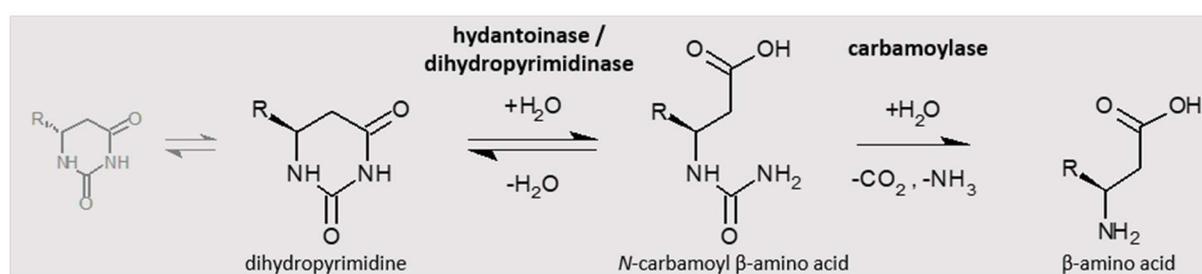


Figure 1: Scheme of the proposed modified hydantoinase process

It was already demonstrated in previous work that hydantoinases/dihydropyrimidinases can hydrolyze racemic 6-substituted dihydropyrimidines to the corresponding *N*-carbamoylated β -amino acids [2]. For several hydantoinases the determination of kinetic parameters for the model substrate phenyldihydrouracil, the evaluation of optimal reaction conditions and immobilization experiments are ongoing.

Within a screening novel decarbamoylating enzymes were discovered, recombinantly expressed and characterized. It was shown that these enzymes hydrolyze several different novel *N*-carbamoyl- β -amino acids to their corresponding β -amino acids.

In upcoming work, the enzyme cascade consisting of hydantoinase and decarbamoylating enzyme is to be assembled, characterized and optimized in order to produce chiral β -amino acids.

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Haloperoxidases: halogenating biocatalysts for chemoenzymatic cascade reactions

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The installation of a halogen group equips active agents with biochemical and biophysical properties that greatly differ from the non-halogenated congener. These altered interactions can be exploited for syntheses of pharmaceuticals or precursors for the synthesis of commonly used building blocks.[1] While chemical halogenation requires the use of energy-inefficient and toxic elemental halogens, halogenating enzymes are capable of halogenating electron-rich substrates using halides.[2]

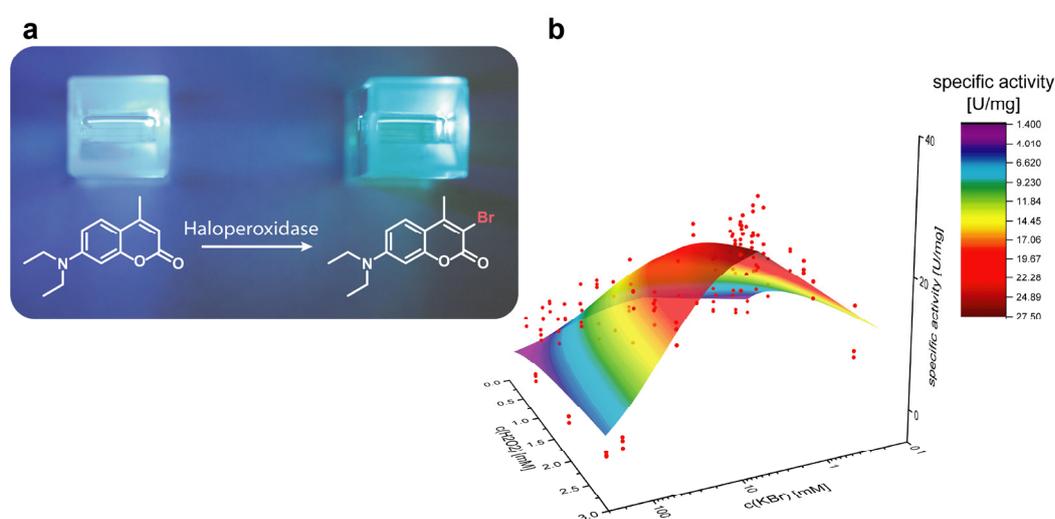


Figure 1. Fluorescence-based haloperoxidase assay. **a:** General assay scheme. **b:** 2D-kinetics of *C. inaequalis* haloperoxidase

Although haloperoxidases do not convey regio- or stereoselectivity beyond that of hypohalides, the use of the cheap oxidant hydrogen peroxide as well as a high tolerance against harsh conditions, renders the subclass of haloperoxidases as a very promising biocatalyst.

This project is about finding and establishing suitable haloperoxidases for cascade reactions. Using a controllable environment, halogenation of substrates may occur with strictly defined reaction parameters, allowing consecutive modifications in one process.

To characterize the enzymes and determine their kinetic parameters, a fluorescence-based assay system was developed to quantify the activity of haloperoxidases in an accurate manner (a). Using a two-dimensional calibration approach, the concentration of starting material and product could be calculated simultaneously. The system was validated by determining the first overall kinetic parameters of the vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (b).^[3] These findings have then been used to evaluate the catalytic profile of the haloperoxidase reaction using a simple GC-MS readout. Using this knowledge, chemoenzymatic cascade reactions are in the production pipeline.

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Biocatalytic cascade synthesis of unnatural amino acids and other valuable building blocks from electron-rich biomass-derived substrates

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Phenylalanine ammonia lyases (PALs) have shown to be effective biocatalysts in the hydroamination of a broad range of substituted cinnamic acids [1]. However, the substrate scope of PALs has been limited to electron-poor aromatic systems. We screened metagenomic libraries and identified a new clade of PALs which have yet to be fully characterised, but have shown novel activities to accept a large scope of demanding electron-rich substrates. Furthermore we engineered the PAL enzyme using our attractive direct infusion of biotransformations to a mass spectrometer (DiBT-MS) technique which semi-quantitatively detected variants with high conversions, achieving sample throughputs equivalent to ~40s per sample [2].

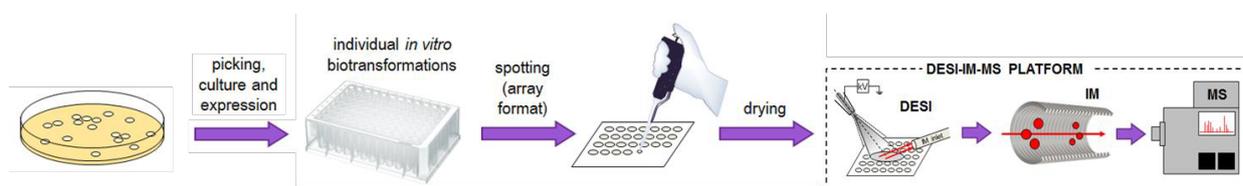


Figure 1. Screening of PALs for the conversion of Electron-donating group cinnamates.

While the hydroamination reaction does not proceed with naturally occurring lignin monomers we have achieved excellent isolated yields 80% and perfect enantioselectivity (ee >99%) with the alkylated 3,4-dimethoxycinnamic acid as a substrate (the presence of -OH substituents effect the complex stereoelectronic factors and the binding affinity in the enzyme active site). We sought to protect natural occurring hydroxycinnamates via methylation of the *para* -OH group with an engineered *O*-methyltransferase (OMT) from *Eriobotrya japonica* which was recombinantly expressed in *E.coli* cells [3] and performed the subsequent PAL reaction to generate the pharmaceutically relevant L-veratrylglycine from 3,4-dimethoxycinnamic acid [4].

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When Gold(I) and Alcohol Dehydrogenase meet: Design of a chemoenzymatic cascade towards optically active allylic alcohols

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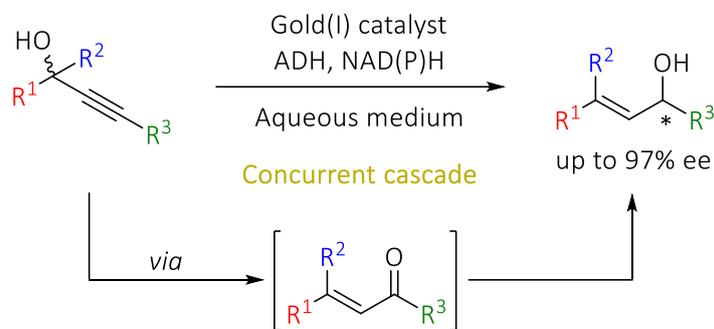
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Gold catalysis has emerged in the last two decades as a versatile tool in organic synthesis, especially to develop the activation of multiple C–C bonds under mild reaction conditions.[1] Remarkably, the use of gold species has been demonstrated along recent years in both organic and aqueous media, allowing their possible combination with enzymatic processes for the design of one-pot sequential[2] or cascade transformations,[3] involving for instance, the action of alcohol dehydrogenases (ADHs), amine oxidases or hydrolases as selective biocatalysts. In this context, the combination of gold(III) catalysts with enzymes has been more developed, while the use of gold(I) complexes is still in its infancy.

Gold(I) species are non-toxic and highly efficient catalysts, although have a great drawback since their application in asymmetric synthesis is a very tricky task due to their linear geometry.[4] For that reason, merging gold(I) and enzymatic catalysis could represent a straightforward methodology to induce chirality and increase molecular complexity.

Herein, a concurrent cascade is described to achieve the isomerization of different racemic propargylic alcohols through the Meyer-Schuster rearrangement, involving a N-heterocyclic carbene gold(I) complex, followed by the ADH-catalyzed stereoselective bioreduction of the allylic ketone intermediates (Scheme 1).[5] Hence, the preparation of a series of chiral β,β -disubstituted allylic alcohols has been achieved in aqueous medium and under mild reaction conditions, representing the first concurrent cascade involving gold(I) catalysts and oxidoreductase enzymes. The approach resulted very general, providing access to both (*S*)- or (*R*)- β,β -disubstituted allylic alcohols depending on the specificity of the selected ADH.



Scheme 1. Meyer-Schuster rearrangement and bioreduction cascade for the asymmetric synthesis of β,β -disubstituted allylic alcohols starting from racemic propargylic alcohols.

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Chemoenzymatic oxysulfonylation-bioreduction sequence for the stereoselective synthesis of β -hydroxy sulfones

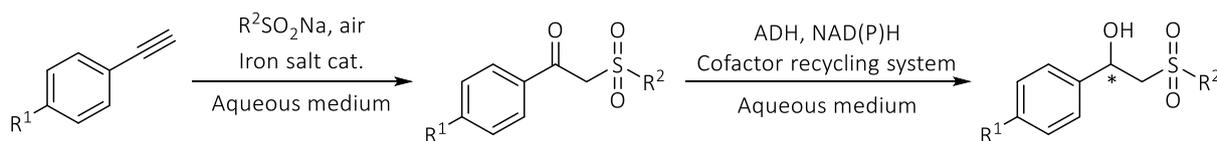
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β -Keto sulfones, also known as 2-oxo-sulfones, are privileged motifs in organic chemistry due to their synthetic versatility to produce a wide family of sulfur compounds.[1] Among the synthetic methods available to produce β -keto sulfones, the metal-catalyzed oxysulfonylation of alkynes has received special attention in recent years due to its possible performance in both aqueous and organic media (Scheme 1, first step).[2] Interestingly, the synthesis of chiral β -hydroxy sulfones has been described in the last years through one-pot processes, by combining conventional chemical transformations, such as nucleophilic substitution of α -bromo ketones with sulfinates, or alternatively oxysulfonylation of alkynes, with metal-catalyzed asymmetric transfer hydrogenations employing chiral ruthenium catalysts.[3]



Scheme 1. Metal-catalyzed oxysulfonylation of aryl acetylenes followed by stereoselective bioreduction to produce optically active β -hydroxy sulfones.

With the prospect of developing a more sustainable procedure, the combination of the exquisite chemoselectivity displayed by metal catalysts with the stereodiscrimination displayed by enzymes is here accomplished towards the asymmetric synthesis of β -hydroxy sulfones (Scheme 1, second step). Thus, we report the design of a chemoenzymatic sequential strategy based on the use of an iron salt to catalyze the oxysulfonylation reaction of a series of alkynes under aerobic conditions, and subsequent stereoselective bioreduction of the transiently formed ketone intermediates using alcohol dehydrogenases (ADHs). The use of enzymes displaying opposite stereoselectivity allowed us to prepare both antipodes of various β -hydroxy sulfones with excellent selectivity (Scheme 1).

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Optimization of a lignin-depolymerizing enzyme cascade

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Lignin is one of the three major components of lignocellulose. Due to the fact that lignin is produced in the pulp and paper industry with yields up to 130 million tonnes per year, it is considered to be a renewable energy source as well as a promising future source of aromatic building blocks. Valorization of lignin for the production of desired mono-aromatics, however, requires a highly selective lignin depolymerisation approach. Lignin is composed of the three monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol which are linked via a variety of different linkage types, with β -O-4-aryl ether bonds accounting for 45-60% of all linkages. Therefore, the selective cleavage of β -O-4-aryl ether bonds represents a major target in lignin biorefinery [1,2].

In this project, a previously described enzyme cascade composed of enantiocomplementary α dehydrogenases and β -etherases, a glutathione lyase as well as a glutathione reductase for internal cofactor recycling (Fig. 1) has been studied for the selective formation of syringyl-hydroxypropanone (SHP) and guaiacyl-hydroxypropanone (GHP) from lignin polymer [2]. Reaction conditions have been optimized with regard to the applied cosolvent for lignin solubilization and the lignin concentration. Furthermore, different enzyme combinations and enzyme concentrations have been tested to optimize GHP and SHP yield per gram of lignin polymer.

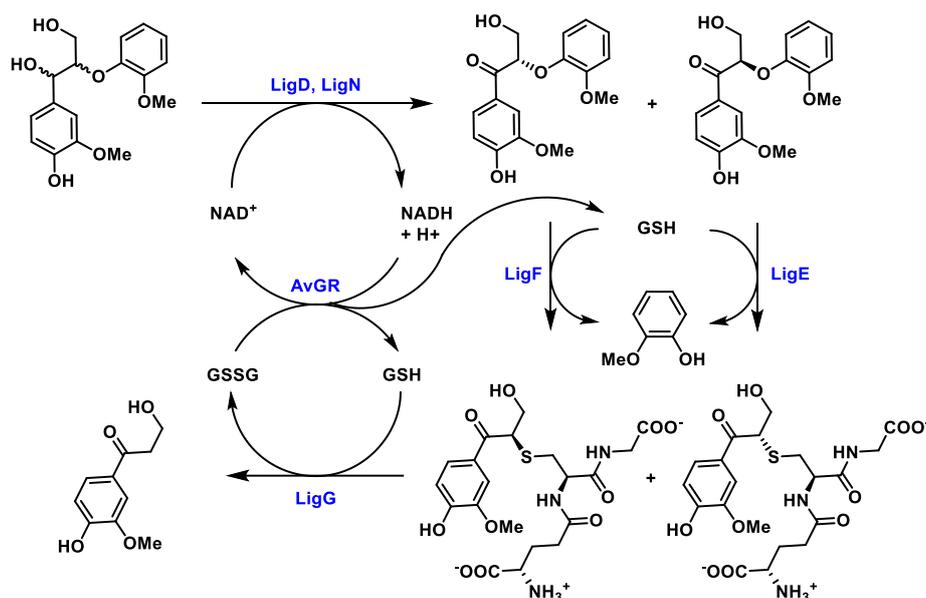


Figure 1. Enzyme cascade with internal cofactor recycling for the cleavage of β -O-4 aryl ether bonds in lignin [LigD, LigN: α dehydrogenases; LigE, LigF: β -etherases; LigG: glutathione lyase; AvGR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione] [1,2].

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Synthesis of chiral *N*-arylamines using a sequential transaminase Buchwald-Hartwig amination cascade

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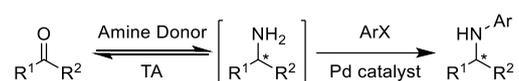
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While enzymes are powerful tools and often offer stereo- and regio-selectivities that are unmatched by chemical means, their integration into synthetic routes is often challenging. One reason for this is the incompatibility of reaction environments—typically aqueous for the enzymatic step and organic for the chemical step—necessitating purification of intermediates and reducing the efficiency of the overall process. Thus, an increasing effort is taking place to develop chemo-enzymatic cascades.^[1] Chiral *N*-arylamines, key building blocks in many APIs, are an attractive target for such chemoenzymatic processes.^[2] Enzymes are often able to produce chiral amines with enantiomeric excesses currently out of reach for even state-of-the-art chemo-catalysts.^[3] On the other hand, the palladium catalysed Buchwald-Hartwig-amination (BHA) is an extremely powerful chemical tool to arylate primary and secondary amines, as well as amides.^[4] Recently, the Turner group reported sequential cascades involving imine reductases and amine dehydrogenases followed by a BHA. However, they were unsuccessful in developing a transaminase-BHA cascade that did not require extraction of the amine product prior to the BHA.^[2]

Herein, a cascade employing a transaminase to afford primary amines from pro-chiral ketones in high *ee*, which are then arylated using the BHA, is described (Scheme 1). A key challenge, in addition to the compatibility issues of the reaction environment, is the presence of excess amine donor which is required in the transaminase catalysed step. Using the right amine donor, palladium catalyst, and reaction system, the desired amine can be selectively arylated in the presence of excess amine donor, avoiding the need to isolate the intermediate and streamlining the synthesis. To highlight the synthetic potential of this cascade, as well as limitations, the substrate scope is explored both with regards to the pro-chiral ketone and the aryl halide.



Scheme 1: Synthesis of chiral *N*-aryl amines: A prochiral ketone is transaminated using a transaminase (TA) biocatalyst and a sacrificial amine donor, which is then arylated in a BHA, ideally without isolation of the intermediate primary amine. X = Cl, Br, or I. R¹, R² = H, alkyl, or aryl.

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From carboxylic acid to nitrile: a chemoenzymatic route in one pot

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The direct conversion of a carboxylic acid to the respective nitrile has been described in biosynthesis pathways towards deazapurine-containing compounds.^[1] The 7-cyano-7-deazaguanine synthases activate the carboxylate at the expense of ATP. Ammonia acts as a nucleophile, and an intermediate amide reacts with a second ATP to the nitrile.^[2] The conceptual elegance of this enzymatic reaction cannot be exploited for synthesis, because the responsible enzymes are strictly specific for their natural substrates.^[3] Biogenic nitriles other than the deazanucleoside preQ₀ are derived from amino acids in an enzymatic cascade involving three enzymes.^[2] In this case, CO₂ is released and the carbon chain shortened.

Herein, we report an artificial pathway from carboxylic acid to nitrile, in which the carbon atom of the acid eventually becomes the nitrile carbon and the carbon chain length is retained. In the first step, a carboxylic acid reductase (EC 1.2.1.30, CAR) reduces the carboxylate to the respective aldehyde^[4], which is trapped in a chemical step with hydroxylamine. The resulting oximes undergo enzymatic dehydration catalyzed by aldoxime dehydratase (EC 4.99.1.5 -7, Oxd). The first step in the envisaged cascade reaction is CAR-mediated acid reduction of carboxylic acid to the respective aldehyde in a whole cell system. In this setup, oxygen is required for cell viability and constant ATP formation, the co-factor that is needed for carboxylate activation. The presence of hydroxylamine in the reaction medium should lead to scavenging the aldehyde as aldoxime, the substrate for the second enzymatic step. Oxds are heme-dependent enzymes that transform primary oximes to the respective nitriles. Oxds were reported to be most active under anaerobic conditions, which maintained the catalytically essential ferrous state of the heme.^[5] OxdBr1 from *Bradyrhizobium sp.* LTSPM299 does not require strictly anaerobic conditions.^[6] This fact was important in light of the oxygen requirement of the CAR-containing living cell biocatalyst and rendered OxdBr1 a promising candidate for the cascade. We report the proof of concept of the successful nitrile formation via this sustainable chemoenzymatic one-pot route.

Acknowledgement

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Chemoselective *N*- and *O*-alkylation by the usage of different SAM-dependent methyltransferases from plants

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Methylation reactions are involved in many biosynthetic pathways. *S*-adenosyl-L-methionine (SAM) serves as cofactor. The transfer of a methyl group onto anthranilate catalyzed by an anthranilate *N*-methyltransferase (ANMT) is a crucial step in the biosynthesis for acridone alkaloids and therefore essential for the growth of many plants. The closest relatives to ANMTs are caffeate *O*-MTs (COMTs) with amino acid identities over 50% and similarities over 70%.

The substrate ranges of those enzymes differ from each other, though. ANMTs accept substrates containing amino groups while COMTs catalyze methylation reactions of substrates containing hydroxyl groups. Hence, the mechanisms of the reactions and the amino acids, involved in substrate binding, are assumed to be different.[1,2]

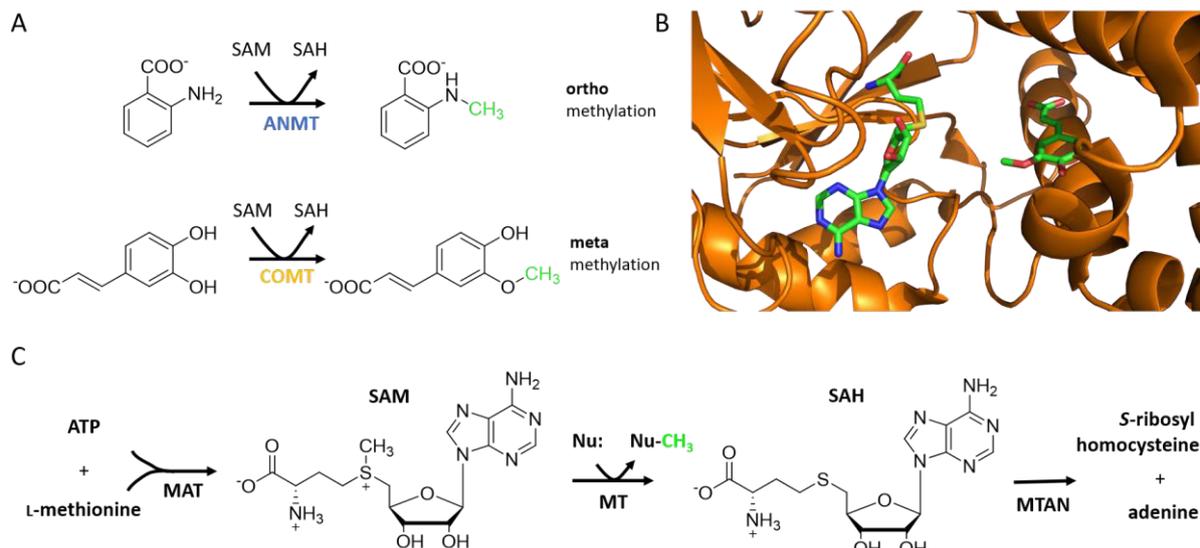


Figure 1. A: MT reactions with natural substrates. ANMT methylates anthranilate. COMT methylates caffeate. B: Representation of the active site of COMT from *Medicago Sativa* forming a complex with the methylated substrate (caffeate) and SAH. C: Three-enzyme cascade for methylation reaction. MAT: methionine adenosyltransferase; MTAN: methylthioadenosine/SAH nucleosidase; ATP: adenosine 5'-triphosphate.

SAM-dependent MTs can also be used as biotechnological tools for chemoselective alkylation reactions. To understand the reaction mechanism, we are investigating the substrate range of different ANMT and COMT enzymes. A linear supply cascade is crucial for *in vitro* assays to avoid degradation of the cofactor and inhibition of MTs by the reaction by-product *S*-adenosyl-L-homocysteine (SAH) (Figure 1C).[3]

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One-pot bi-enzymatic cascade with an Old Yellow Enzyme and a native amine dehydrogenase to synthesize chiral amines

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Chiral amines are highly desired compounds in the pharmaceutical and fine chemical industry for their use as building blocks.[1] Current methods involving the use of precious metals, such as ruthenium, require costly ligand and only provide products with low stereoselectivity. Organocatalysis is a sustainable alternative but requires high loadings of catalyst. Biocatalytic approaches involve the use of lipases, imine reductases, transaminases or amine dehydrogenases, among others. We designed and developed a proof-of-concept bi-enzymatic cascade, consisting of an ene reductase from the Old Yellow Enzyme family (OYE) [2] and a native amine dehydrogenase (AmDH),[3] to afford chiral diastereomerically enriched amines in one pot. By studying the substrate profiles of both reported biocatalysts, unsaturated aldehydes and ketones were assayed with an OYE/AmDH duo. Starting with 10 mM substrate, low (5%) to high (97%) conversion rates were obtained in addition to enantiomeric and diastereomeric excess up to 99%. We expect our established bi-enzymatic cascade to allow access to chiral amines with both high enantiomeric and diastereomeric excess from varying alkene substrates depending on the combination of enzymes.

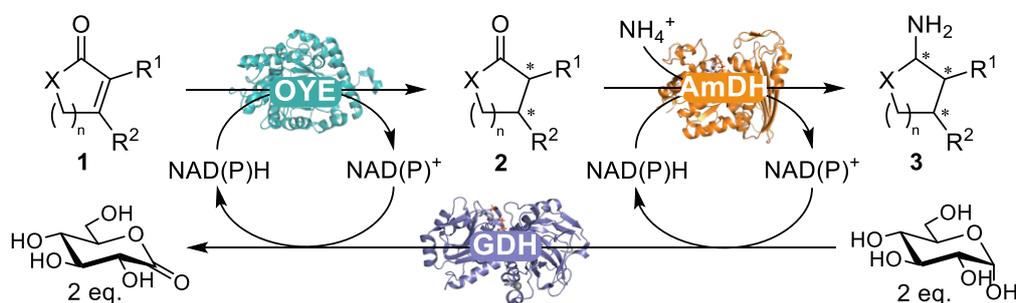


Figure 1. Bi-enzymatic cascade with OYE and AmDH to produce chiral amines from unsaturated ketones and aldehydes, using an efficient GDH cofactor recycling system.

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Are green whole-cell biocatalysts scalable?

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With their excellent selectivity and often very mild reaction conditions, oxidoreductases play an important role in the chemical and pharmaceutical industries. Widely-used enzymatic redox transformations such as C-H oxyfunctionalization or C=C double bond reduction require a stoichiometric supply of electrons, which is usually provided from petrol-based or agricultural co-substrates such as isopropanol or glucose. The use of an equivalent amount of organic cosubstrates for a process is highly problematic for its atom economy, an important metric for sustainability. Whole-cell redox biocatalysis in cyanobacteria uses electrons from photosynthetic water-splitting and saves organic cosubstrates (Scheme 1) [1]. Several examples demonstrated the feasibility with high selectivity and reaction rates exceeding 100 U/g. Yet, a further increase of the productivity and the space-time-yield of the approach is required for application.

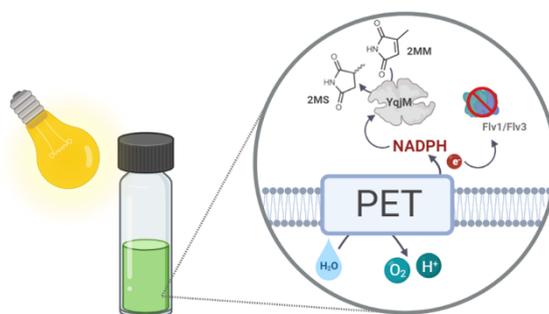


Figure 1. Cyanobacterial biotransformations use photosynthetic water-splitting to provide reduction equivalents for selective redox biocatalysis. PET: Photosynthetic electron transport.

This raises the question to which extent the photosynthetic electron transport chain can be deviated towards an oxidoreductase as heterologous electron sink. We present an integrated strategy that combines cell engineering and reaction engineering to increase both productivity and volumetric yields of cyanobacterial biotransformations. Metabolic engineering of the photosynthetic electron flow achieved a 50% rate increase of a stereoselective C=C double bond reduction [2], underlining the importance of NADPH-supply to achieve high productivity. A second example deals with the investigation of an internally illuminated photobioreactor as scalable system for cyanobacterial biotransformations.

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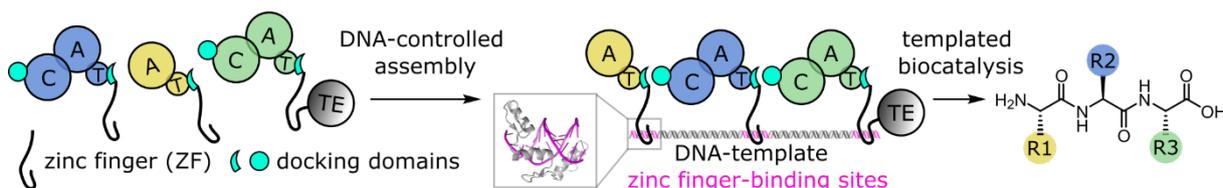
Engineering DNA-templated nonribosomal peptide synthesis

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Nonribosomal peptide synthetases (NRPSs) protect microorganisms against environmental threats by producing siderophores or antibiotics, for instance, and are predisposed for biosynthetic engineering because of their modular molecular structure. We have explored several strategies for the redesign of NRPS specificity. Notable examples are the incorporation of a clickable amino acid through targeted binding pocket mutagenesis [1] or specificity transfer through swapping of small protein fragments [2, 3]. Incorporation of clickable amino acids has further enabled a strategy for high-throughput sorting of mutagenized NRPSs displayed on yeast [4]. Here, we demonstrate the addition of DNA templates to nonribosomal peptide synthetases to facilitate NRPS reprogramming [5]. We have split the NRPS for the cyclic decapeptide gramicidin S into modules. Up to four modules were later reassembled on a DNA template using DNA binding domains with high specificity and affinity, and loosely binding intermodular docking domains. The complex nonribosomal machinery showed astonishing tolerance for structural variations when the DNA spacers between modules were altered in length. In the future, DNA programmable NRPS might allow to write the sequences of natural product-like peptides into short DNA templates to speed up NRPS design.



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Top-Down and Bottom-Up Assembly of compartmentalized Enzyme Cascades – Towards Experiments and Simulation on multiple Scales

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Nature has evolved efficient principles to control a complex network of chemical reactions within living cells. These unique principles include biological compartmentalization and cascading, where for example catalytically active components are organized into spatially defined multienzyme complexes. The understanding of these networks and their transfer to *in vitro* systems provides an extraordinary potential of innovation for the next generation of modular production processes in industrial biocatalysis.¹ On the micro- to centimeter length scale, enzymes can be incorporated into microfluidic devices to harness the precise controllability of reaction parameters for the continuous production of value-added molecules. To this end, we have developed methodologies for the mild and efficient immobilization of recombinant enzymes that are genetically encoded with fusion tags, such as Halo-tag or Snap-tag. Using this approach, stereoselective ketoreductases were integrated as self-immobilizing biocatalysts in microfluidic packed-bed reactors.² In an effort to gain deeper insights into the enzymatic mechanisms and reactor behavior, we are combing experimental data acquisition with mathematical modeling.³ With this approach, we demonstrate an increased biocatalytic process efficiency along with the reduction of material and costs. Furthermore, to fully exploit the concept of enzyme cascades and to understand diffusion and mass transport processes on the nanometer length scale, it is essential to have accurate control over stoichiometry and spacing of multiple interacting enzymes. We demonstrate that supramolecular fabrication of such well-defined enzyme assemblies can be achieved by using concepts of structural DNA nanotechnology.⁴

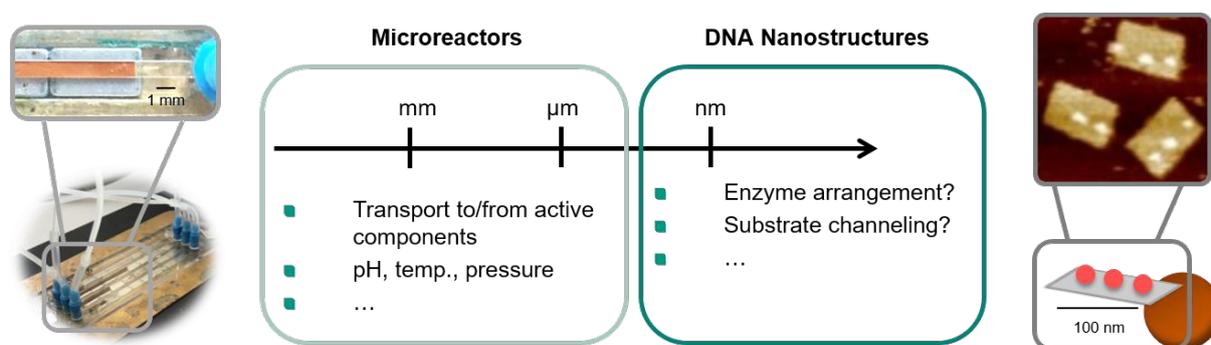


Figure 1. Understanding and reconstruction of highly ordered biochemical processes (compartments and cascades) on multiple scales.

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In situ synthesis of acetaldehyde for C-C bond forming reactions

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4-oxalocrotonate tautomerase (4-OT) is a versatile enzymatic catalyst for various promiscuous carbon-carbon bond forming reactions, which utilize acetaldehyde as a nucleophile. However, the inherent high reactivity of acetaldehyde causes several practical challenges for synthetic applications. Here, we explore three enzymatic synthesis routes for the *in situ* generation of acetaldehyde in one-pot cascade reactions together with 4-OT.[1] Practical acetaldehyde concentrations were obtained using either an environmental pollutant, *trans*-3-chloroacrylic acid, or bio-renewable ethanol, as substrates. The route I uses the enzymes chloroacrylic acid dehalogenase (CaaD) and malonate semialdehyde decarboxylase (MSAD) to generate acetaldehyde starting from *trans*-3-chloroacrylic acid. Route II is generating acetaldehyde via the oxidation of ethanol catalysed by the alcohol dehydrogenase from *Saccharomyces cerevisiae* (ScADH). One-pot Michael-type additions and aldol condensations catalysed by 4-OT were achieved in combination with these two enzymatic routes. The presented cascade reactions are an initial step towards the construction of more complex synthetic pathways for the production of valuable chemical commodities.

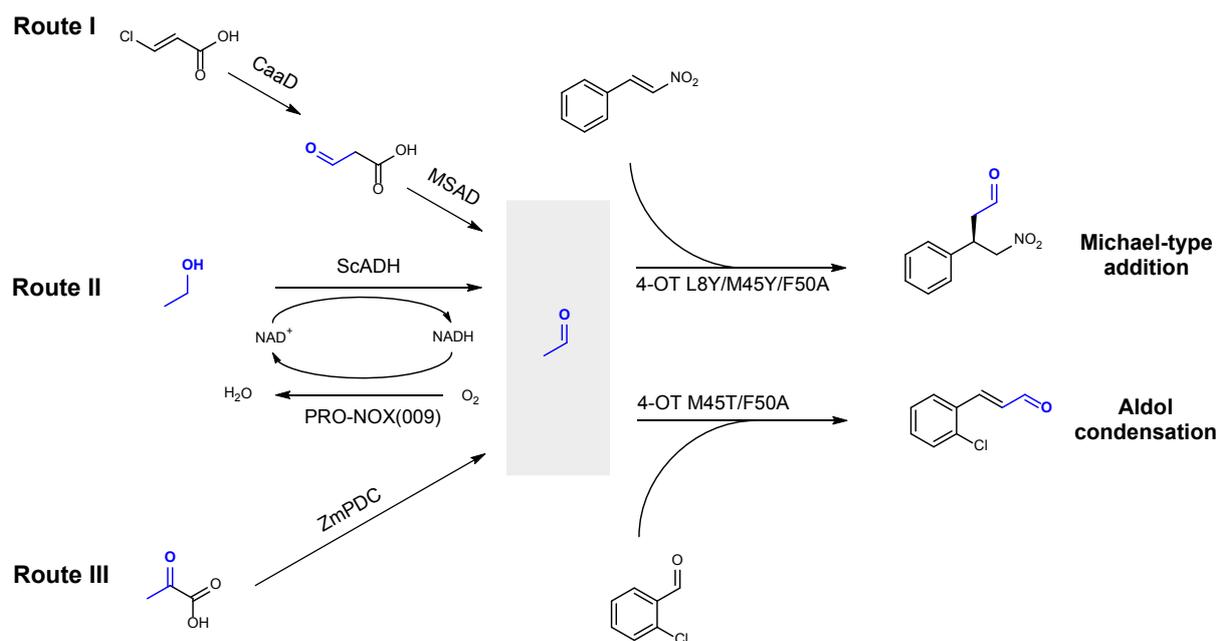


Figure 1. Enzymatic cascade reactions for *in situ* synthesis of acetaldehyde combined with Michael addition and aldol condensation reactions catalysed by the promiscuous enzyme 4-OT.

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Chemoenzymatic synthesis of chiral halohydrins from haloalkynes combining N-heterocyclic carbene gold(I) and ketoreductase catalysis

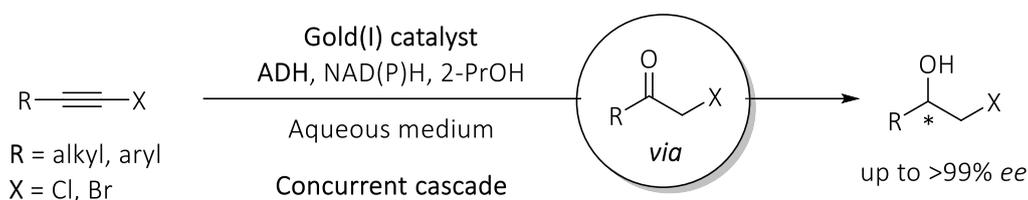
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Merging metal- and enzyme-catalysis is nowadays an extremely powerful synthetic tool due to the exquisite selectivity and complementary reactivities displayed by both catalyst types.[1] Hence, since decades this marriage has enormously broadened the repertoire for organic chemists as can be exemplified in the development of multicatalytic processes using individual metal and enzymatic steps, ideally in one-pot, or alternatively creating hybrid catalysts. Thus, currently the combination of metal complexes and biocatalysts in multistep cascade transformations has become a useful methodology for organic chemists.[2] In this field, palladium chemistry represents a pivotal area because of its enormous potential when a C–C bond formation process is required. Unfortunately, due to incompatibility of Pd complexes and enzymes, (one-pot) sequential approaches are usually mandatory. Among the trends that have recently emerged in organometallic catalysis, the employment of gold complexes can be highlighted, especially in the field of multiple C–C bond activation.[3] Importantly, these catalysts have demonstrated their efficiency in aqueous media under very mild reaction conditions, opening the door for the development of, not only sequential,[4] but also concurrent cascade designs.[5]

Following our interest related to the combination of N-heterocyclic carbene (NHC) gold(I) species and oxidoreductases,[6] herein we describe the stereoselective synthesis of a series of aliphatic and aromatic halohydrins, valuable intermediates due to their high and broad reactivity, starting from readily available haloalkynes (Scheme 1). In this stereodivergent concurrent cascade process, the regioselective hydration of the alkyne is accomplished by the gold(I)-catalyst, providing the α -halo ketone intermediate which is subsequently reduced by an alcohol dehydrogenase (ADH). The use of stereocomplementary ADHs has allowed the access to both halohydrin antipodes with excellent conversions and enantiomeric excess.



Scheme 1. One-pot haloalkyne hydration and stereoselective bioreduction cascade for the synthesis of chiral halohydrins.

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Electrochemical patterning of enzymes for use in flow reactors

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The development of flow reactors is of significance in a number of sectors and in particular in the pharmaceutical industry. The sector faces a significant challenge in its reliance on batch processes, with the synthesis of active pharmaceutical ingredients (API) occurring via individual reactions, methods that are not necessarily well suited to modern, flexible, manufacturing processes that need to be agile and responsive to changing needs.^{1,2} The advantages of continuous flow technology over batch synthesis include the achievement of rapid and efficient mixing, shortened reaction times and improved product yields. Adamo et al. recently described a refrigerator-sized demonstration system that can perform the continuous-flow synthesis and formulation of active pharmaceutical ingredients such as diazepam at a scale of hundreds of thousands of doses per day [1]. In biological systems, enzymes catalyse a wide range of cellular processes where the location of enzymes within the same spatial region brings advantages that include spatial confinement of reactive intermediates and reduction of the rate of (wasteful) diffusion of products outside of this region. A range of multi-enzyme cascade systems have been described that include the enzymatic regeneration of the co-factor NAD⁺/NADH [2], a one-pot 12-step reaction sequence for the synthesis of alkaloids [3] and a cascade system for the peroxygenase catalysed hydroxylation of ethyl benzene [4]. A challenge with cascade reactors lies in the design of reactors where the location of the enzyme can be precisely patterned. Electrochemical approaches can be used to specifically modify the electrode area and the electrode then used in an electrochemical system or simply as a support for the enzymes and/or other catalysts. As an example of the use of electrochemistry for the specific immobilisation of enzymes, we have recently described a cell for the specific and localised release of pharmaceutical materials [5] and have electrochemically patterned a protein (cytochrome c) on to three separate electrodes in sequence in the same solution [6]. Here we extend this work and demonstrate that the three enzymes, alcohol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase can be separately and uniquely immobilised on three electrodes in the same solution (Figure 1). The three enzymes remain catalytically active. We extend this work to the description of a flow system based on *Candida antarctica* lipase B [7] and to an enzyme cascade reaction based on the production of H₂O₂ for redox transformations.

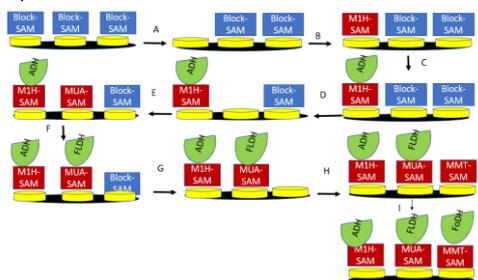


Figure 1. Schematic representation of the patterning of alcohol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase on separate electrode surfaces.

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In situ biosynthesis of aryl acetaldehydes en route to β -hydroxy- α -amino acids

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Multi-enzyme biocatalytic cascades have emerged as practical routes for the synthesis of complex bioactive molecules. The synthetic complexity of biomolecules that can be made from such cascades, however, is often limited by the relative sparsity of water-stable carbon electrophile. Herein, we use styrene oxide isomerase (SOI) to convert aryl epoxides into α -aryl acetaldehydes through a Meinwald-like rearrangement.[1] These unstable aldehyde intermediates are then directly converted into β -hydroxy- α -amino acids by the L-threonine (Thr) transaldolase, ObiH.[2] We capitalized on the facile synthesis of epoxides via the Corey-Chaykovsky reaction to probe the substrate scope of each enzyme in the pathway. Surprisingly, large biaryl and fluorenyl substrates reacted efficiently in the cascade, dramatically expanding the scope of known substrates for both SOI and ObiH. These enzymes were co-expressed in *E. coli* and deployed as whole cell catalysts to synthesize complex α -amino acids with excellent yields and diastereoselectivity. Given the utility of α -aryl acetaldehydes, we used this cascade to clarify the mechanism of SOI. Reactions with isotopically labeled and homochiral styrene oxides ruled out the intermediacy of an enol or benzylic cation. Instead, we introduce an alternative mechanism wherein a 1,2-hydride-shift occurs *in concert* with epoxide ring opening and aldehyde formation. These studies establish a versatile route to α -aryl acetaldehyde formation and add a valuable, promiscuous C–C bond forming cascade to the biocatalytic toolbox.

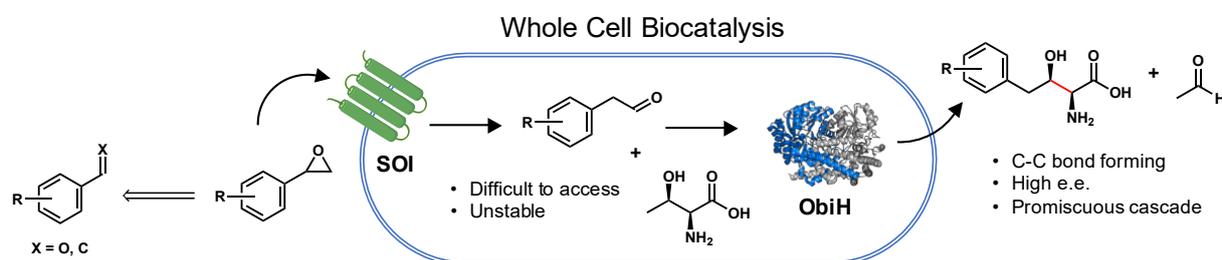


Figure 1. *E. coli* co-expressing both styrene oxide isomerase (SOI) and the L-threonine (Thr) transaldolase, ObiH, serve as whole cell biocatalysts for the production of β -hydroxy- α -amino acids from aryl epoxides.

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An improved SAM regeneration system for recycling of homocysteine to methionine

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Biological methylation reactions catalysed by *S*-adenosylmethionine-dependent methyltransferases (SAM-dependent MTs) are of great interest because of the exquisite chemo-, regio- and stereospecificity of these enzymes. [1] The inhibitory effect of the SAM-derived by-product *S*-adenosylhomocysteine (SAH) and the high cost, instability and stoichiometric requirement of SAM limit the use of MTs for biocatalytic conversions. [2–4] In the cyclic SAM regeneration system, SAH is removed by SAH hydrolase (SAHH) to form adenosine and homocysteine and subsequent phosphorylation of adenosine to ATP is mediated by adenosine kinase (ADK) and polyphosphate kinases 2 (PPK2s) with inorganic polyphosphate (polyP) as phosphate donor. Condensation of ATP and methionine by methionine adenosyltransferase (MAT) generates SAM *in situ*, which is directly used for MT reactions. [5] The free thiol of homocysteine formed by SAHH reaction can have potential inhibitory effect on enzymes of the SAM regeneration system. Hence the system was further extended by methylation of homocysteine to regenerate methionine using either *L*-homocysteine *S*-MT (HSMT) or betaine-*L*-homocysteine MT (BHMT) with *S*-methylmethionine (SMM) or betaine as methyl donor. [6]

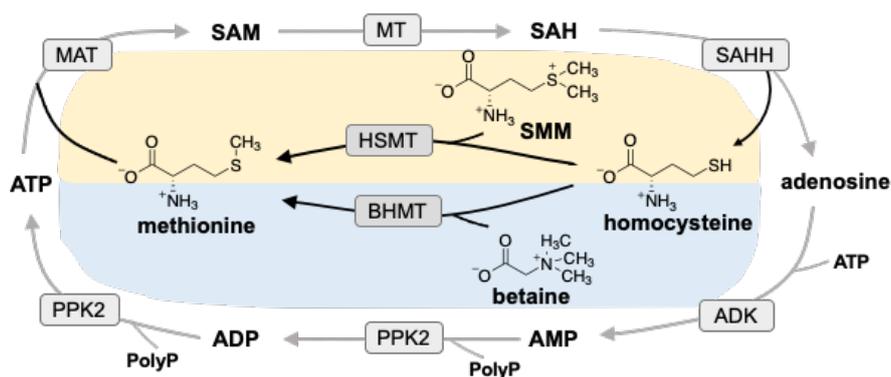


Figure 1. SAM regeneration system with recycling of homocysteine to methionine.

The methyl donor SMM can be used efficiently to recycle homocysteine in the system. In comparison to the SAM regeneration system, the bicyclic system with an additional regeneration of methionine from homocysteine using SMM showed improved performance for *O*- and *N*-MTs with respect to substrate conversion and total turnover.

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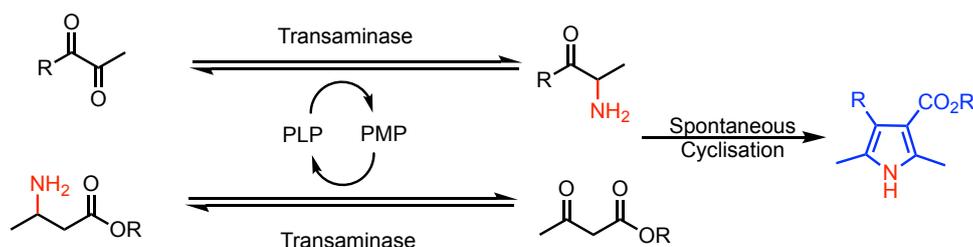
A Transaminase Approach to Amine Borrowing *via* Shuttle Catalysis

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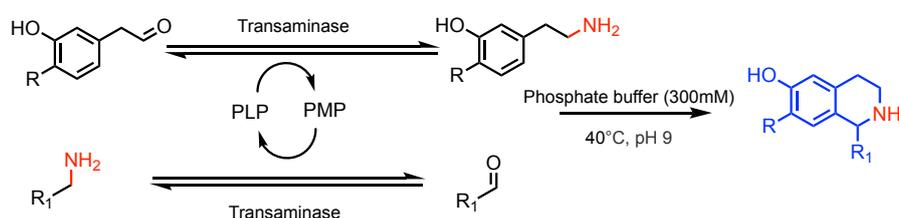
The aim of this work is to develop a new catalytic approach for the synthesis of alkaloid structures with transaminases. We take influence from the idea of 'shuttle catalysis', a relatively new methodology for catalytic functionalization reactions that relies on the reversible transfer of small functional groups from donor to acceptor molecules. This methodology allows for the addition of reactive functional groups without the need for toxic reagents. [1] By applying this thinking to biocatalysis and transaminases in particular, we are developing an 'amine borrowing' concept. This is the shuttling of amine functionality from amine donor to acceptor to form reactive species *in situ* which then undergo an intermolecular reaction. This downstream event reunites the borrowed amine with the original amine donor.

This concept has been demonstrated using the Knorr-Pyrrole synthesis wherein α -amino ketones and β -keto esters are generated *in situ* from the corresponding β -amino esters and diketones *via* transamination. These generated substrates undergo a spontaneous cyclisation, which also pushes the thermodynamics of the transaminase reaction towards the product direction. This reaction occurs with up to 95% conversion to the product and an 80% isolated yield.



Scheme 1. A Knorr pyrrole reaction demonstrating the concept of bio-catalytic amine borrowing. The transaminase substrates are shown in black, and the transaminase products are shown in blue. The 'borrowed' amine is reincorporated in the product.

The Pictet-Spengler reaction is also used to investigate 'amine borrowing'. This named reaction was chosen as it has been recently shown to be catalysed by phosphate buffer, ideal mild conditions for enzymatic reactions. [2] We have shown that the condensation can occur in up to 79% conversion at 40°C and with a reduced cosolvent requirement of 25%.



Scheme 2. A typical Pictet-Spengler reaction used to demonstrate amine borrowing, where $R_1 = H, OH$ and $R =$ range of alkyl/aromatic substituents.

Amine borrowing has the potential to enable highly atom efficient reactions for the synthesis of complex alkaloid structures and indeed expand the range of transformations available via shuttle (bio)catalysis.

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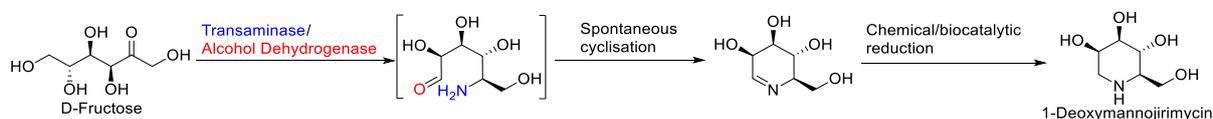
Development of biocatalytic cascades employing designer enzymes for the conversion of simple monosaccharides to iminosugars.

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Iminosugars are naturally-occurring polyhydroxylated secondary and tertiary amines which have been identified as pharmaceutical targets for various different diseases. Iminosugars work by disrupting carbohydrate processing enzymes due to their ability to resemble monosaccharide sugars. There are many difficulties associated with the chemical synthesis of iminosugars including the need for high levels of stereospecificity, expensive metal catalysts and the use of organic solvents.[1] These problems can be circumvented with the development of a biocatalytic cascade which would involve a transaminase (TA), alcohol dehydrogenase (ADH) and a reduction step (Scheme 1). This cascade would be done in green solvents and only require the appropriate co-enzymes.



Scheme 1. A target route for the synthesis of 1-Deoxymannojirimycin from D-fructose.

This project aims to develop an enzymatic route from monosaccharides to biologically active iminosugars, using engineered enzymes. A panel of designer transaminases will be generated using *in silico* semi-rational evolution to increase the enzymes rate of conversion and range of substrates. An *R*-selective TA-derived from *Mycobacterium vanbaalenii* (*Mv*-TA) and a *S*-selective TA acquired from *Halomonas elongata* (HEWT) were the enzymes of choice for the generation of mutant libraries using a combination of directed evolution and rational design. The enzymes *Mv*-TA and HEWT were selected as they have previously reported activity on ketoses and aldoses, respectively.[2,3] Due to the large libraries of variants produced using this engineering strategy, a robust selection assay will be used, which allows the rapid identification of catalysts with desired properties from large libraries of variants. A colorimetric solid-phase screening assay will be used on the transaminase variants in combination with a background depletion method using methylbenzylamine as previously reported by Paradisi *et al.*[4] The most promising mutants from each library will be used to establish the first stepping stone in the biocatalytic cascade for the production of iminosugars.

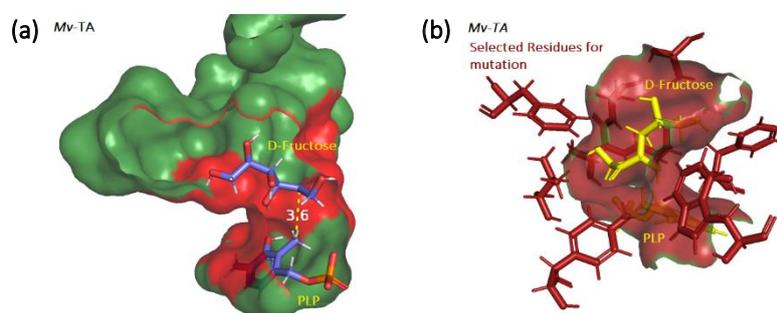


Figure 1. In silico presentation of (a) the active site in *Mv*-TA and (b) the selected residues for mutation in *Mv*-TA.

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Combining Photocatalytic C-C Bond Formation with Enzymatic Ketone Reduction for the Synthesis of Chiral γ -Lactones

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Given its strong potential and rapid growth in various biotechnological applications, cascade reactions represent an attractive branch of synthetic chemistry. Compared to conventional single-sequence transformations, the cascade approach involves several advantages from the perspective of saving resources, reagents, time, and effort, which is usually the consequence of tedious intermediate work-up reactions [1]. Moreover, direct consumption of unstable or toxic intermediates in the subsequent reaction step can result in improved formation of the desired products [2]. In recent years, photo(chemo)enzymatic cascade reactions have been successfully designed to generate industrially important products. Despite the apparent advantages, such as being a green, sustainable, and energy-efficient process, the potential use of photocatalytic biotransformations can be hampered due to incompatibility reasons between photocatalysts and biocatalysts [3]. However, the newly developed and applied strategies could overcome the compatibility challenges of enzyme-coupled photocatalytic reactions driven by light [4].

Herein, we intended to create a photochemoenzymatic tandem reaction for the direct conversion of simple starting materials such as aldehydes to chiral γ -lactones. In the first step of the cascade, tetrabutylammonium decatungstate (TBADT) [5], which is a proven multi-functional and robust photocatalyst, has been used for the photocatalytic C-C bond formation of an aldehyde and an acrylate to yield the corresponding substituted ketone. Subsequently, the intermediate ketone is further converted by a carbonyl reductase to yield the chiral alcohol, which undergoes lactonization to the desired γ -lactone. To implement this strategy, several alcohol dehydrogenases from different organisms were initially screened to determine the best candidate for the enzymatic functionalization step. Herein, we present the feasibility of a novel photo(chemo)enzymatic cascade reaction for the enantioselective synthesis of diverse aliphatic and aromatic γ -lactones. To overcome currently observed limitations, future investigations will focus on the optimization of the reaction conditions for this photochemoenzymatic cascade.

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One-pot synthesis of chiral *N*-arylamines by combining biocatalytic amination with Buchwald-Hartwig *N*-arylation

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The combination of biocatalysis and chemo-catalysis increasingly offers chemists access to more diverse chemical architectures. Here, we describe the combination of a toolbox of chiral-amine-producing biocatalysts with a Buchwald–Hartwig cross-coupling reaction [1], affording a variety of α -chiral aniline derivatives (Figure 1). [2]

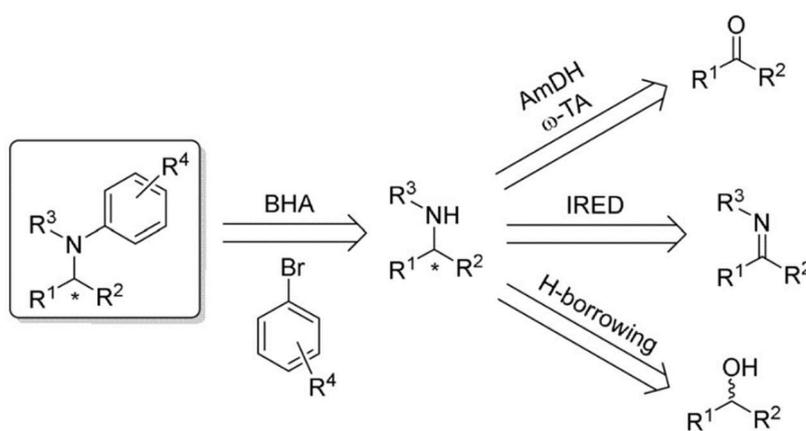


Figure 1. Retrosynthetic strategies for the chemo-enzymatic formation of chiral *N*-arylamines (BHA = Buchwald–Hartwig amination, AmDH = amine dehydrogenase, ω -TA = ω -transaminase, IRED = imine reductase).

The use of a surfactant [3] allowed reactions to be performed sequentially in the same flask, preventing the palladium catalyst from being inhibited by the high concentrations of ammonia, salts, or buffers present in the aqueous media in most cases. This new approach to the synthesis of chiral *N*-arylamines by combining biocatalytic reductive amination, transamination and imine reduction with surfactant enabled Buchwald-Hartwig cross-coupling afforded good to excellent (up to 90%) conversions to the corresponding *N*-arylated amines. Importantly, the asymmetric centre established in the biocatalytic step was shown to be unaffected by the subsequent cross-coupling reaction. The methodology was also extended further by combining it with a previously reported dual-enzyme biocatalytic hydrogen-borrowing cascade [4] in one pot, to allow for the conversion of a racemic alcohol to a chiral aniline. It can be envisaged that this biocompatible, surfactant-enabled cross-coupling approach will broaden the application of chemo-enzymatic processes for the synthesis of high-value compounds.

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Exploring the scope of methyltransferase biocatalysis

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S-adenosylmethionine (SAM)-dependent methyltransferases constitute a large family of enzymes that can catalyze regio-, chemo- and stereospecific methylation of complex natural products. [1] These enzymes could be very useful tools for chemoenzymatic production and diversification natural or artificial compounds. Until recently, preparative applications of methyltransferases in vitro were limited because of the requirement for SAM as a stoichiometric methyl donor. Introduction of a simple SAM-regeneration process based on the ability of halide methyltransferases to transfer methyl-groups from methyl iodide to S-adenosylhomocysteine (SAH) has highlighted a general strategy to harness enzyme-catalyzed alkylation in biocatalysis.[2 - 5] In this presentation we discuss our latest efforts to explore the potential of this approach.

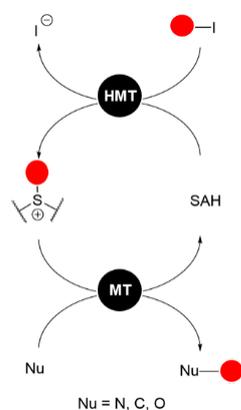


Figure 1. Scheme of HMT-MT cascade transfers alkyl-containing group to C-, N-, O-nucleophile.

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Immobilization Strategies for Compartmentalized Reactions in Flow Reactors

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Keywords: 3D printing, directed evolution, enzymes, flow catalysis, hydrogels

The compartmentalization of chemical reactions is a basic principle in nature, which can be implemented in technical processes by performing reaction cascades with physically separated enzymes. For the development of novel approaches in biocatalysis this principle is a major source for innovations and is therefore mimicked in several ways. The immobilization of biocatalysts in a fluidic setup is one way to achieve compartmentalization and thus precise control over artificial reaction cascades. Many established state-of-the-art technologies to arrange enzymes for sequential reactions require chemical modifications of the target enzymes, which can negatively influence the activities or specificities of the immobilized enzymes. We recently demonstrated the encapsulation of unmodified thermostable enzymes in a 3D printed, agarose-based thermoreversible hydrogel [1,2] and the site-selective immobilization of enzymes on beads.[3]

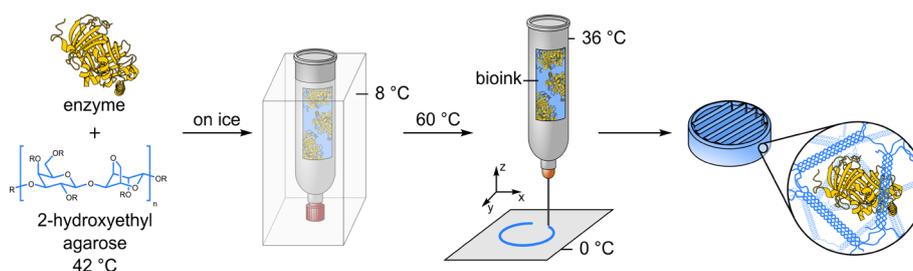


Figure 1. Schematic manufacturing process for encapsulation of unmodified enzymes in 3D printed hydrogel structures.

Both immobilization strategies allow implementation in continuous flow systems and have been successfully utilized for multi-step sequential biotransformations. To test the feasibility of the encapsulation strategy, we used an esterase and an alcohol dehydrogenase from thermophilic organisms as well as a ketoisovalerate decarboxylase from a mesophilic organism as exemplary biocatalysts. The latter was thermostabilized by rational protein engineering and directed protein evolution.[1,4] After the successful prove-of-concept study, we further expanded the scope of this system by integrating encapsulated phenacrylate decarboxylases into a chemoenzymatic workflow.[2] As an alternative, the site-selective immobilization strategy was employed to combine alcohol dehydrogenases and a cofactor regenerating glucose 1-dehydrogenase in continuously operated chip microreactors to produce *meso* diols from ketones.[3] Currently we are expanding the scope of this approach by integrating a novel thermostable benzaldehyde lyase into a custom-made packed-bed reactor for the flow production of α -hydroxy ketones.

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Enantiocomplementary 4-hydroxy-2-oxoacids furnished from L-amino acids in a biocatalytic one-pot cascade

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The replacement of fossil sources by renewable resources will improve the sustainability of chemical processes. Increasing prizes, the predicted shortage thereof and environmental implications are leading to a frantic search for methods to exploit biomass derived resources.[1,2] Biocatalytic in vitro cascades provide the required tool for functionalization of the carbon source material and simultaneously allow the selective conversion to desired products for direct valorization.[3] While most biocatalytic transformations are only functional group interconversions, C-C bond forming catalysts such as aldolases exhibit an attractive class of biocatalysts which enable the construction of more complex carbon frameworks out of the biomass derived starting material.[4]

Hitherto, there are only a few biocatalytic cascades with aldolases in which biomass derived molecules are directly converted to high-value products. 2-Keto acids, on the other hand, are easily derived from renewable resources such as amino acids,[5] while they also act as promising synthons for aldolase mediated carbonylation reactions.[6]

An investigation on the activities of different Type II aldolase variants revealed selectivity of the employed biocatalysts on the corresponding 2-keto acids of canonical as well as non-canonical amino acids. Both enantiomers of the furnished aldol products were yielded within a simultaneous one-pot cascade containing an amino acid deaminase and an (*R*)- or (*S*)-selective aldolase. The overall substrate conversion was optimized by balancing aldol adduct formaldehyde and substrate loading. Subsequently, downstream processing was investigated for the furnished 4-hydroxy-2-keto acids.

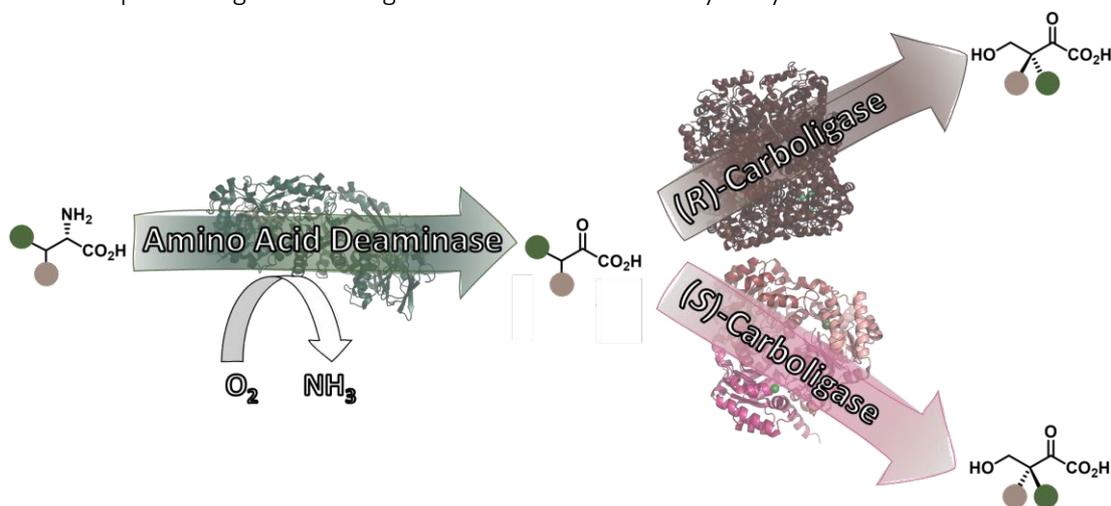


Figure 1. One-pot simultaneous multi-enzyme system for the synthesis of enantiomerically enriched (*R*)- or (*S*)-4-hydroxy-2-oxo acids starting from L-amino acids..

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Bicyclic regeneration systems for SAM and nucleobase analogues for efficient methylation

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S-Adenosylmethionine (SAM)-dependent methyltransferases (MTs) are interesting tools for the specific methylation of various compounds. Nonetheless, SAM has several disadvantages: it is unstable and expensive; therefore, a regeneration system is required for efficient *in vitro* biocatalysis.[1,2]

Methionine adenosyltransferases (MATs) are responsible for the *in situ* synthesis of SAM from methionine and adenosine 5'-triphosphate (ATP). SAM can directly be used by MTs for methylation resulting in the byproduct S-adenosylhomocysteine (SAH) that acts as a feedback inhibitor on MTs. SAH can be removed by SAH hydrolase (SAHH) resulting in adenosine and homocysteine. Adenosine is subsequently re-phosphorylated to ATP by using the combination of adenosine kinase (ADK) and polyphosphate kinases 2 (PPK2s) with inorganic polyphosphate (polyP) as phosphate donor, while homocysteine is regenerated to methionine using homocysteine S-MT (HSMT) with S-methylmethionine as methyl donor. This results in a genuine bicyclic SAM regeneration system, where no by-products accumulate.[3,4]

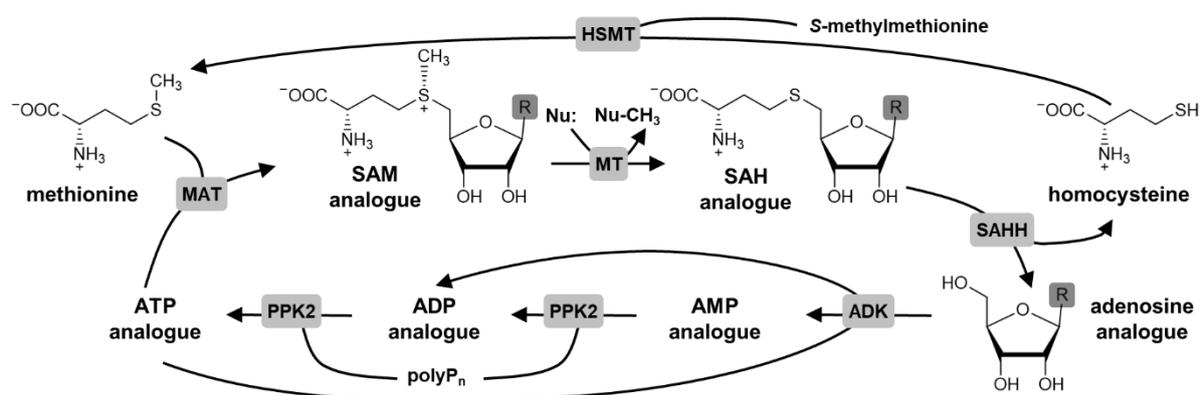


Figure 1. Bicyclic regeneration system for SAM and different nucleobase analogues to methylate various substrates.

The system reached up to >99% conversion for the MT-catalysed reaction with 200 turnovers with *O*- and *N*-MTs. The system also worked starting from different nucleosides and nucleotides containing pyrimidine and purine nucleobases – a promising starting point bioorthogonal systems. Using purified enzymes, as well as crude lysates, was able to achieve high conversion rates, indicating the potential of this system for up-scaling after further optimisation steps.

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Assay Cascade for Highly Specific Measurement of Sialidase Activity

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Sialic acids are acidic nine-carbon sugars and part of cell-surface glycoconjugates. Their negative charge and exposed position support their role in cellular recognition via carbohydrate-protein interactions. [1] Sialidases of viral and bacterial origin play critical roles in pathogen infection of human hosts, rendering specific sialidase inhibitors (e.g. *oseltamivir*) attractive targets for pharmaceutical developments. Sia-(α 2,3)-Gal-(β 1,4)-Glc is the triglycoside moiety of GM3, the most common membrane bound glycosphingolipid. A new coupled enzymatic assay was developed to examine the substrate promiscuity of two bacterial sialidases towards non-natural sialoconjugates. Four *neo*-sialoconjugates, carrying functional modifications at the terminal chain of the Sia unit, were tested relative to the native GM3 triglycoside.[2] The conjugates were also evaluated as potential inhibitors of sialidase activity. Sialidases cleave terminal sialic acids from sialoconjugates, liberating a terminal galactose unit. Subsequent enzymatic degalactosylation liberates glucose, which upon oxidation with glucose oxidase forms hydrogen peroxide as a coupled product. The well-known horseradish peroxidase assay then provides a stoichiometric response against hydrogen peroxide, which is colorimetrically quantified using a microtiter plate reader. (Fig. 1)

Since exo-glycosidases cannot hydrolyze internal galactosides and the employed oxidase acts specifically on the free monosaccharide only, a specific colorimetric signal can be induced only as a consequence of successful sialic acid cleavage by sialidase. If sialidase activity is the overall limiting factor, the principle allows for continuous measurement of its activity.

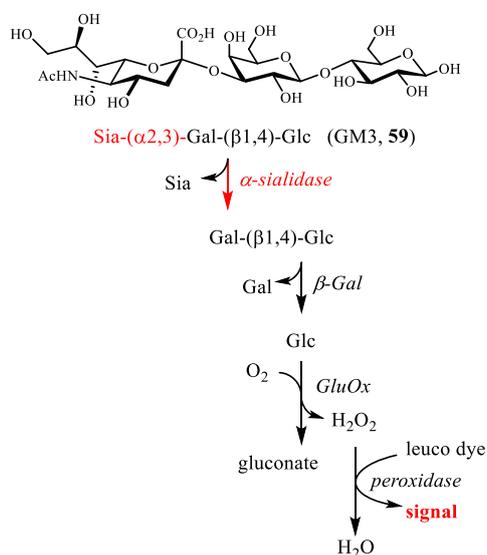


Figure 1. Schematic representation of the cascade process

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Whole cell-enzyme biosynthetic cascade for the synthesis of Serinol

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The need for alternatives to fossil fuels has escalated the production of biodiesel in recent years, which is expected to reach 41.4 billion litres in 2025. Crude glycerol is the main by-product of this industry as 1 kg of glycerol is generated every 10 kg of biodiesel. Its poor quality, significantly low price and contaminant disposal fuels the seek for new biotechnological processes for crude glycerol valorisation. Biocatalytic cascades afford the development of economically sustainable and green processes. Particularly, crude glycerol can be upgraded to serinol through a two-step cascade comprising *Gluconobacter oxydans* and an ω -Transaminase from *Pseudomonas fluorescens* (Pf-ATA). Serinol is a promising and versatile prochiral molecule that can be used as a synthetic block for sphingosine/ceramide synthesis and the production of pharmaceuticals and X-ray contrast agents. Herein we examined the unprecedented co-immobilisation of these biocatalysts to manufacture up to 36 mM serinol, the highest titre ever reported for a non-fermentative biosynthesis. To that end, several strategies with the biocatalysts immobilised separately or together were studied, considering different immobilization matrixes. The use of alginate beads for the co-immobilisation of resting *G. oxydans* cells with Agarose-Co₂+ microbead-immobilised Pf-ATA showed the highest serinol production. The effect of the L-Ala:DHA ratio in the transamination reaction was studied, highlighting the importance of L-Ala excess. More importantly, the industrial by-product crude glycerol was evaluated as a substrate, demonstrating the possibilities of this hybrid heterogeneous biocatalyst for valorising bio-based raw materials.

The design and construction of an artificial, cell-free metabolic pathway to access chiral bio-based fine chemicals.

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Bio-based fine chemicals such as β -hydroxy acids (linear and branched) can be accessed starting from acyl-CoA derivatives through an enzymatic cascade that includes a non-decarboxylative Claisen condensation as a key step, mediated by a thiolase (THL).

Thiolases are a class of enzymes able to catalyse a C-C bond forming step and they have been successfully exploited for the synthesis of α -branched alcohols and acids.[1][2][3]

The condensation product undergoes a dehydrogenase (ADH) mediated reduction followed by a hydrolysis catalysed by a thioesterase (THE) to obtain the desired compound.

Preliminary results have shown the successful *in vitro* synthesis of the linear product 3-hydroxy butyric acid starting from acetyl-CoA.

The introduction of cofactor recycling enzymes into the enzymatic cascade allows the production of the desired product in significant quantities.

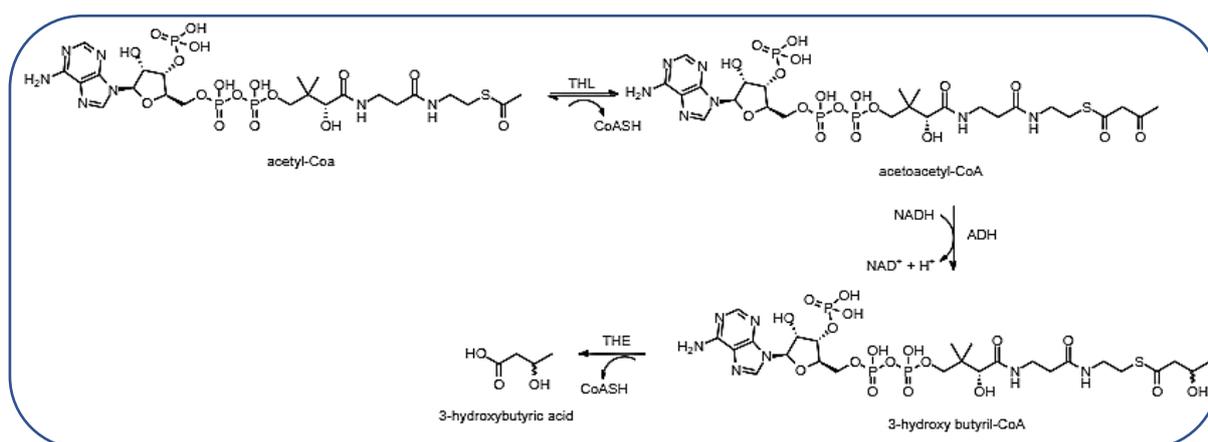


Figure 1. Synthesis of 3-hydroxy butyric acid through an enzymatic cascade.

This innovative approach will lead to the access of a wide range of chiral β -hydroxy acids by the structural modulation of the acyl-CoA derivative involved in the condensation step.

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Smart combination of oxidation bio/chemistry for the synthesis of highly valuable fragrance and flavour aldehydes

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Effective utilization of a wide spectrum of naturally abundant compounds is a vital strategy in achieving sustainability in the chemical industry. This is however restricted by the varying usability of different classes of natural compounds as synthetic building blocks in combination with usually inefficient methods for their chemical transformation. Herein, we demonstrate a proof of concept that enables the transformation of naturally occurring phenyl propene derivatives (e.g., Eugenol, Safrole) to valuable aromatic aldehydes (e.g., Vanillin, Piperonal) by means of two different strategies (see Figure 1).

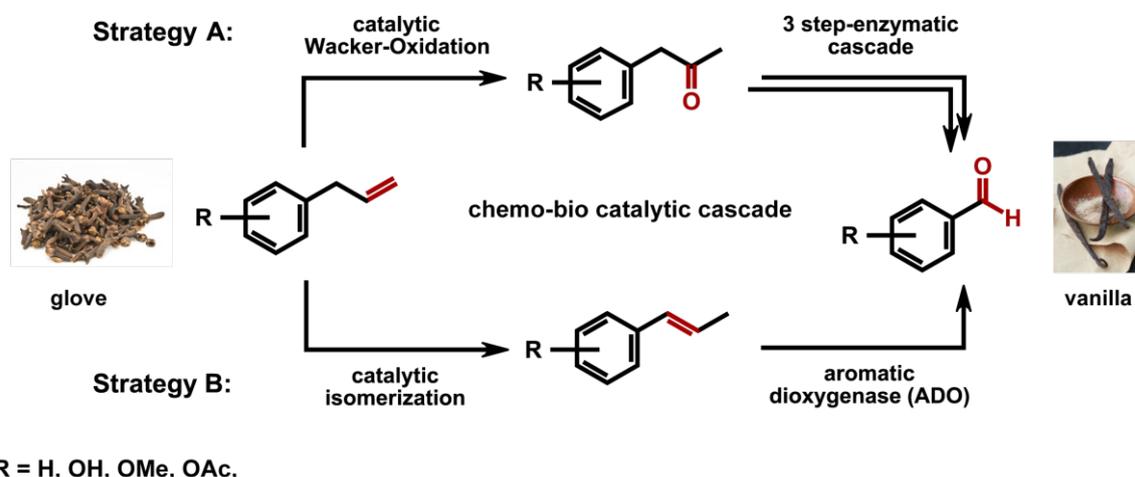


Figure 1: Two different concepts, exploiting oxidation bio/chemistry for the synthesis fragrance and flavour aldehydes.

The common denominator between these two methods is the effective one-pot coupling of a chemical (**Strategy A.** Wacker-Oxidation or **Strategy B.** catalytic isomerisation) and an enzymatic-transformation (**Strategy A.** three-step enzymatic cascade or **Strategy B.** one-step enzymatic “shortcut” transformation). The enzymatic cascade reaction (**Strategy A**) is realized by the combination of a Baeyer-Villiger-Monooxygenase (TmCHMO) together with an Esterase (PFE_I) and an alcohol dehydrogenase (AlkJ). For the “shortcut” enzymatic transformation (**Strategy B**) a coenzyme free aromatic dioxygenase (ADO) is employed [1].

Both strategies were realized in a one-pot fashion and yielded the desired aldehydes in good to very good yields.

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Combining bio- and chemocatalysis in hybrid systems using unconventional reaction media

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Aliphatic vicinal diols can be utilized as potential precursors for bio-based fuels, as substrates for the chemocatalytic production of high-value dioxolanes, and its pure stereoisomers can be processed as essential building blocks. Here, we present a 2-step reaction route from bio-based aldehydes (acetaldehyde, propanal, butanal), obtained by microbial fermentations, for the production of the aliphatic vicinal diols 2,3-butanediol, 3,4-hexanediol, and 4,5-octanediol, respectively.

In the two-step enzymatic cascade, two aldehyde molecules are ligated in the first step using a lyase to form the corresponding 2-hydroxy ketone. The carbonyl group of this intermediate is reduced in the second step using an alcohol dehydrogenase or butanediol dehydrogenase. Enzyme selection is dependent on the desired product stereoisomer. Both, the selectivity of the enzymes concerning product formation and preference of the oxidoreductases towards the 2-hydroxy ketone stereoisomer, has to be taken into consideration.

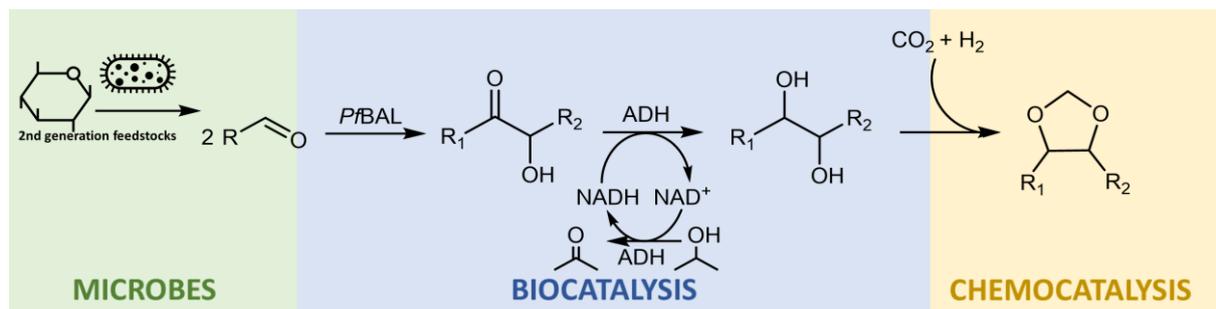


Figure 1. Scheme of biocatalysis in combination with microbial biotransformation and chemocatalysis for the production of dioxolanes via aliphatic, vicinal diols. $R_{1,2}$ = methyl-, ethyl-, propyl-group. PfbAL – benzaldehyde lyase from *Pseudomonas fluorescens*, ADH – Alcohol dehydrogenase

In addition to using diols directly, the biocatalytic transformation can be combined with a chemocatalytic production of dioxolanes. For this synthesis, either plastic monomers (polyoxymethylene monomers) or CO_2 and H_2 can be used to form cyclic acetals.[1] Since the diol is based on sugar-derived aldehydes and the dioxolane additionally add CO_2 into the molecule, the potential fuel derived from this route is considered a bio-hybrid fuel.[2] We demonstrated that the enzyme cascade can be performed in cyclopentylmethyl ether (CPME) as an unconventional reaction medium. CPME is not only an organic solvent, which allows high biocatalytic activities, but is also a good option for the further chemocatalytic transformation. In addition, the usage of an organic solvent for the enzymatic conversion also simplifies downstream processing significantly, since the solvent is distilled off efficiently and the catalyst, formulated as lyophilised whole cells, is removed by filtration. In summary, CPME is a suitable reaction environment in several respects, as it allows enzymatic transformation, chemocatalysis, and straightforward downstream processing.

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Methyltransferases for the diversification of THIQ alkaloids in multienzymes one-pot cascades

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The availability of tetrahydroisoquinolines (THIQs), an important class of biologically active alkaloids, in quantities for study and clinical use is limited as they can frequently only be harvested in small amounts. This has encouraged the development of strategies aimed not only at the enantioselective synthesis of natural occurring THIQs but also non-natural analogues with different substitutions at C-1.[1,2,3] A successful approach which generally offers better enantioselectivities than traditional synthetic methods, includes the development of *in vitro* cascades to mimic their biosynthetic pathways.[4] Methyltransferases (MTs) are crucial in the biosynthesis and diversification of THIQs and their application is limited *in vitro* by the high cost of (*S*)-adenosyl methionine (SAM) and low substrate scope. In this study, we describe the use of methyltransferases in cascades for the enantio- and regioselective diversification of natural and non-natural THIQs on a laboratory preparative scale. We report the integration of these enzymes into up to 7-step cascades for the strategic diversification of bioactive compounds with the incorporation of an *in vitro* cofactor supply system,[5] demonstrating the capacity of such an approach in ambitious biocatalytic applications.

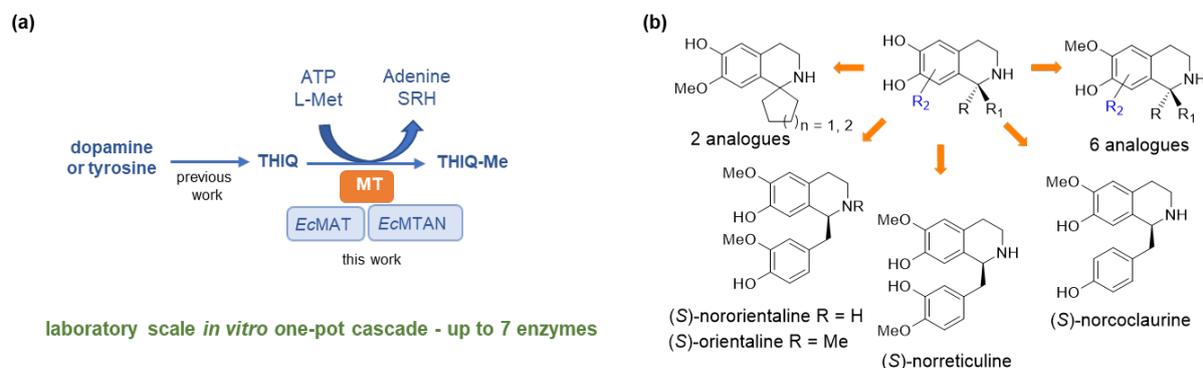


Figure 1. (a) One-pot *in vitro* cascade for the synthesis of methylated THIQ alkaloids including an efficient SAM supplying system; (b) Biologically relevant methylated THIQ alkaloids prepared on a laboratory preparative scale.

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Synthesis of α -Hydroxyketones, Primary, and Secondary Amines via Reactive Aldehyde Intermediates in *E. coli*

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Synthetic enzyme cascades in living cells offer a great possibility for the production of value-added chemicals [1,2]. Hereby, aldehydes represent valuable intermediates and precursors for numerous chemical compounds. However, highly reactive aldehyde species are toxic to the organism which leads to the formation of undesired byproducts (the respective alcohols and carboxylic acids) by endogenous enzyme activities [3]. Previous research has shown that when applying whole-cell biocatalysis, primary alcohols and carboxylic acids can be converted into aldehydes by alcohol dehydrogenases (*AlkJ*, *P. putida*) and carboxylic acid reductase (*CAR_{Ni}*, *N. iowensis*, co-expressed with *PPTase_{Ec}* for posttranslational modification of *CAR_{Ni}*) respectively in *E. coli* resting cells [4, 5]. At the same time, the aldehyde is freely available for further reaction. To expand the spectrum of this concept and to prove that it can be applied to synthesise a wide range of compounds, new enzyme classes were incorporated into the cascade: A pyruvate decarboxylase (*PDC_{Ap_mutant}*, *A. pasteurianus*), ω -transaminases (ω -*TA_{Vf}*, *V. fluvialis*) and imine reductase (*IREDC_{Cf}*, *C. ferrugineus*). For the co-expression of the pathway enzymes, the genes of interest were combined via molecular cloning and co-transformation of two plasmids. After confirmation of co-production of the enzymes, three new biocatalytic pathways were tested. Either the alcohol or the carboxylic acid was employed as starting material for the cascade reaction. In this way, the biocatalytic concept was expanded to yield α -hydroxy ketones, primary, and secondary amines as final cascade products.

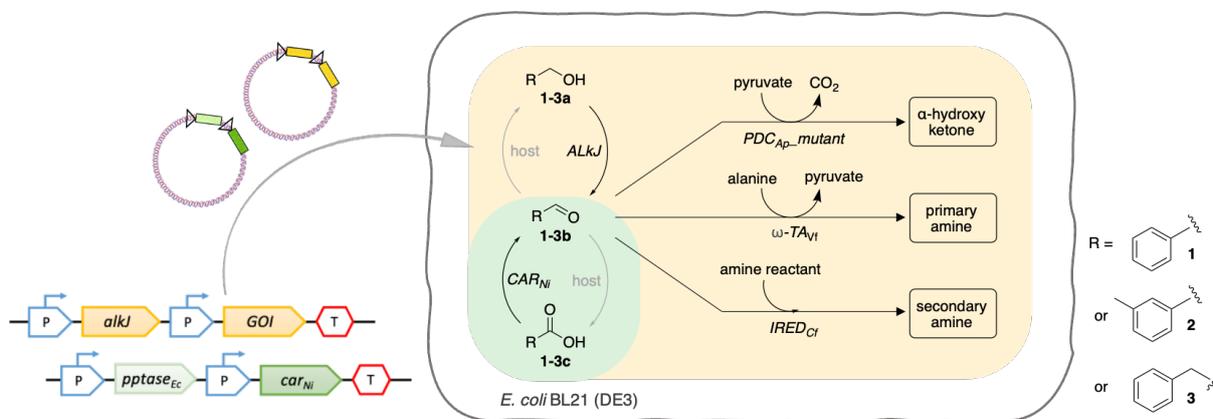


Figure 1. Synthetic pathways to produce α -hydroxyketones, primary, and secondary amines in *E. coli*. Genetic configurations (organisation in pseudo-operons; P..promoter, T...terminator) of the two plasmids are depicted in a simplified manner.

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In vivo and *in vitro* cascades towards a substituted tetrahydroisoquinoline

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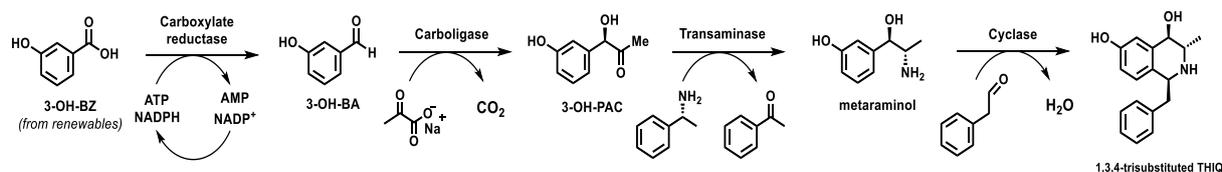
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The tetrahydroisoquinoline (THIQ) moiety is a “privileged scaffold” and it is found in numerous bioactive natural products. THIQ-containing compounds display various bioactivities (e.g. antitumor, antiparasitic, and anticholinergic properties) and are therefore used for pharmaceutical applications.^[1,2] Chemical syntheses of these compounds are possible but they do not give high stereoselectivities. Besides, they often depend on the use of toxic or environmentally harmful chemicals. Thus, novel synthetic approaches towards THIQs are of significant interest.^[2]

In vitro and *in vivo* biocatalysis provide viable methods of producing complex THIQs in high stereoselectivities and under mild conditions. For instance, a three-step enzymatic cascade to access a 1,3,4-trisubstituted THIQ has been already established.^[2] In this approach, the commercially available 3-hydroxybenzaldehyde serves as starting material. Although this aldehyde is quite cheap, it is still mainly obtained from petroleum resources. Thinking in a sustainable and economically competitive bioeconomy, alternative approaches employing renewable materials are well envisioned. Therefore, herein we propose a four-step enzymatic cascade towards the same trisubstituted THIQ starting with 3-hydroxybenzoic acid, which can be obtained by microbial cell factories from renewable resources like glucose and/or xylose.^[3] The complete cascade scheme is shown below (Scheme 1).



Scheme 1. Four-step enzymatic cascade towards (1*S*,3*S*,4*R*)-1-benzyl-3-methyl-1,2,3,4-tetrahydroisoquinoline-4,6-diol.

The novelty of this cascade is the reduction of 3-hydroxybenzoic acid (3-OH-BZ) into 3-hydroxybenzaldehyde (3-OH-BA) by a carboxylate reductase.^[4] In this step, the implementation of an efficient cofactor recycling system of both ATP and NAD(P)H was crucial. For this, an *in vitro* cofactor recycling approach using only purified enzymes^[5] and a whole-cell approach were set-up and compared. Both approaches showed to be promising and high conversions of the acid into 3-hydroxybenzaldehyde were achieved (>80%), but the whole-cell system showed superior performance because it allowed the combination of the first and the second steps of the cascade in an one-pot system with very high conversion (>95%) into the intermediate 3-hydroxyphenylacetylcarbinol (3-OH-PAC).

The third and fourth steps of the cascade had to be performed separately due to cross-reactivity and formation of several by-products. Metaraminol could be formed with good conversions (>70%) applying either purified or whole-cell transaminase from *Chromobacterium violaceum* (Cv2025). The cyclisation step was performed using the norcoclaurine synthase from *Thalictrum flavum* ($\Delta TjNCS-A79I$), leading to the formation of the 1,3,4-trisubstituted THIQ product.

Overall, we were able to demonstrate the applicability of enzymatic cascades for the formation of a complex chiral compound bearing three chiral centers from low-cost renewable starting materials.

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Development of a novel enzyme-coupled cofactor regeneration system for NADPH-dependent oxidoreductases

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Oxidoreductases (EC1) offer fundamental biosynthetic pathways toward value-added chemicals.^[1a] The challenge related to EC1 enzymes is that about 90% of the known ones require nicotinamide cofactors NAD(H) or NADP(H) at stoichiometric amounts for catalysis.^[1b] The high price of NAD(H) or NADP(H) makes their stoichiometric use on industrial scale impracticable.^[1c] As a consequence, different systems for *in-situ* cofactor regeneration have been developed, which includes the coupled-substrate and coupled-enzyme systems.

This study elucidates the potential of a novel coupled-enzyme system by combining a thermostable enzyme pair denoted E1 and NADPH-dependent oxidoreductases. The E1 is able to reduce the NADP⁺ to NADPH in the presence of the cosubstrate dithiothreitol (DTT). We successfully coupled the E1-System with an alcohol dehydrogenase from *Lactobacillus brevis* (*LbADH*) to catalyze the reduction of acetophenone (**Figure 1a**) and a cyclohexanone monooxygenase from *Acinetobacter sp.* NCIMB 9871 (*AcCHMO*) to execute the oxidation of cyclohexanone (**Figure 1b**). Design of Experiment (DoE) software was utilized to recognize significant reaction parameters and accordingly to regulate amounts of (co)substrates or enzymes to achieve the highest yields toward the target products.^[2]

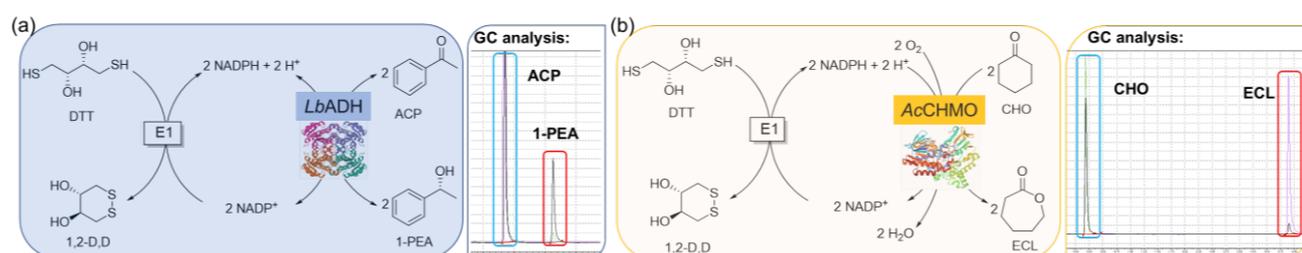


Figure 1. (a) The reduction of acetophenone (ACP) to (*R*)-1-phenylethanol (1-PEA) catalyzed by *LbADH* and (b) the oxidation of cyclohexanone (CHO) to ϵ -caprolactone (ECL) catalyzed by *AcCHMO* promoted by the E1 regeneration system.

Both systems show promising preliminary results, giving rise to accomplished cofactor regeneration, high overall stability, and high product yields of 69% and 94% by *LbADH* and *AcCHMO*, respectively. The systems are currently further optimized with DoE. These findings reveal an alternative NADPH regeneration way to complement the common cofactor regeneration systems.

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An extensive molecular dynamics based methodology to understand the enzyme substrate complexes

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Computational approaches have been extensively used in biocatalysis research especially molecular dynamics (MD) simulations. MD simulations are very potent tools to understand the enzyme dynamics, deciphering the substrate-binding modes, predicting the transition state complexes, and so forth. We hereby, using MD simulations followed by in-depth trajectory analysis presenting a novel computational approach focussing on oxophytodienoate reductase (OPR3) enzyme, a class of ene-reductases. Our methodology is capable of resolving the residue-wise energetic contribution for every observed substrate binding mode in the active site of OPR3. Benchmarking explains the higher acceptance of longer substrates by OPR3 active site and putative mutational sites to catalyze the reductive C-C coupling.

Identification of hidden meta-stable conformational states key for catalytic activity of fungal Monoamine Oxidase

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Aspergillus niger Monoamine Oxidase (MAO-N) is a homodimeric enzyme responsible for the oxidation of amines into the corresponding imine (Figure 1a). Directed evolution experiments provided an extensive library of variants with different substrate scope and catalytic activities, being of great interest for the obtention of chiral amine building blocks at industrial scale, when used in combination of a non-selective chemical reductant.[1,2] In this work, we focus on the study of the conformational dynamics of both wild-type and evolved D5 variant, which includes mutations in the active site but also at distal positions. Molecular dynamics (MD) simulations reveal the existence of different conformational states of MAO-N, that arise from the motion of an important β -hairpin located at ca. 20 Å far from the active site.[3] In this project, the Free Energy Landscape (FEL) of the wild-type and some laboratory-evolved variants were fully reconstructed. We use Markov State Models to characterize the kinetics of the transitions between the different conformational meta-stable states as well as their relative populations (Figure 1b). Our work demonstrated how distal mutations regulate MAO-N activity by stabilizing some catalytically important conformational states, but also by modulating the communication pathway between both MAO-N subunits. Accelerated molecular dynamics were used to elucidate the potential interplay between the β -hairpin conformational dynamics and catalytic activity in MAO-N wild-type and its evolved D5 variant.[4] In both MAO-N wild-type and the laboratory evolved D5 variant, the β -hairpin conformation in one of the monomers affects the productive binding of the substrate in the active site of the other subunit. Our study demonstrates the existence of a delicate communication between both MAO-N subunits that impacts the active site architecture, and thus its catalytic efficiency.

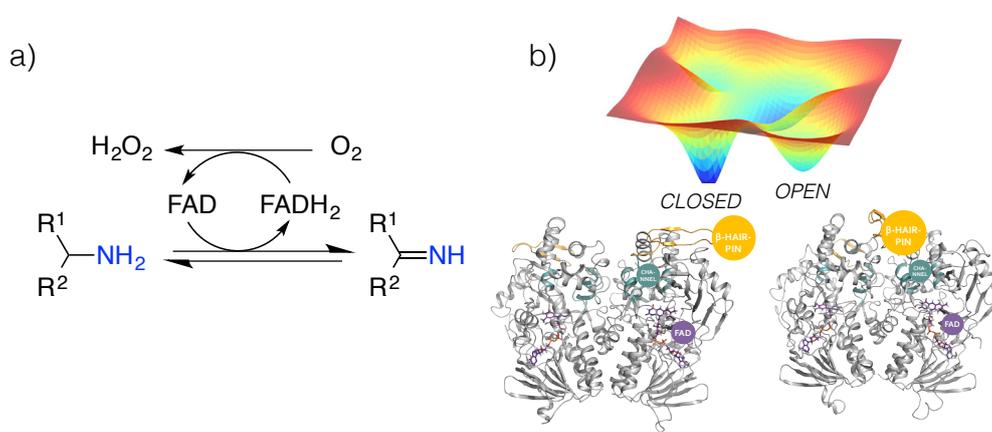


Figure 1. a) MAO-N reaction scheme. b) Free Energy Landscape describing the opening motion of the highlighted β -hairpin

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MODAMDH: identification of diverse Amine Dehydrogenases by screening biodiversity using sequence and structure-based approaches

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The current boom in environmental genomics data provides a huge resource of new sequences of potential biocatalysts. Through the MODAMDH project, we focus on one of the key biocatalysts named amine dehydrogenases (AmDHs) which enable the access to amines that are important entities in the chemical industry [1].

We started from a previously described NAD(P)H-dependent AmDH family from which several members were experimentally characterized [2,3]. This family was first expanded, up to 27k sequences, by mining very large metagenomic databanks in search of the conserved catalytic domain. We then applied structural modelling and active site classification to define subfamilies. We also generated a pool of ~100k candidate families containing more than 20M NAD(P)-binding protein sequences from which we found, using HMM-HMM profile comparison, >30 families sharing distant homology with the reference AmDH family. Furthermore, catalophores (i.e. minimal active site topologies) will be designed from native AmDH structures and used to find active site analogs in the candidate NAD(P)-dependent families. Most interesting enzymes will be further characterized through enzymatic and crystallographic assays, with the purpose of finding new biocatalysts for reductive amination displaying diverse structures and features, particularly in terms of substrate spectrum and complementary stereoselectivities. This work is supported by the Agence Nationale de la Recherche (ANR-19-CE07-0007).

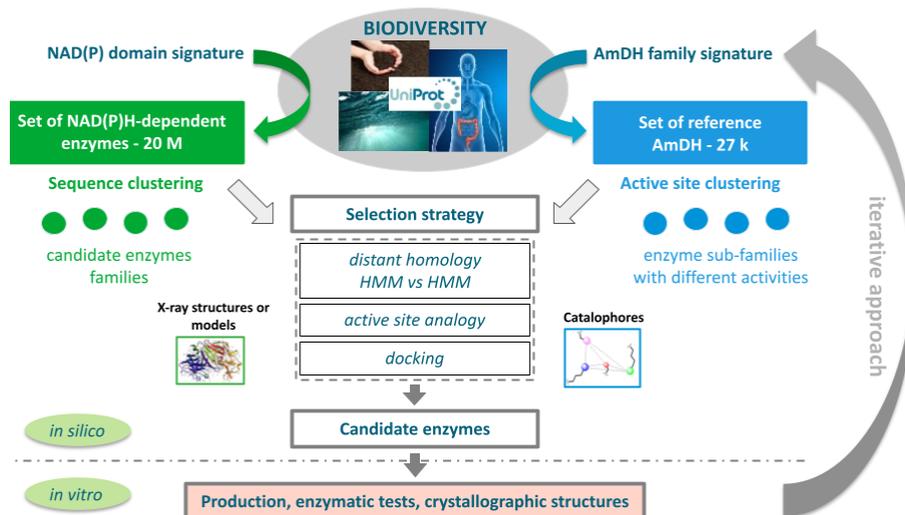


Figure 1. MODAMDH workflow for the discovery of new AmDH enzymes.

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RetroBioCat – computer-aided synthesis planning and a community-driven biocatalysis database

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RetroBioCat is an intuitive and accessible tool for computer-aided design of biocatalytic cascades, freely available at retrobiocat.com (Figure 1). To do this, RetroBioCat uses expertly encoded reaction rules encompassing the enzyme toolbox for biocatalysis, and molecular similarity to identify relevant literature precedent where available. RetroBioCat offers ‘human-led’ computer-aided synthesis planning through ‘Network explorer’. In contrast, ‘Pathway explorer’ offers fully automated cascade design, which we have validated using a test-set of cascades described in the literature [1].

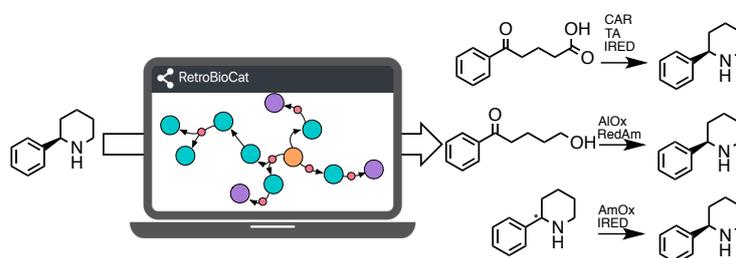


Figure 1. RetroBioCat allows the user to enter any target molecule. Automated biocatalytic retrosynthesis can then be carried out through ‘Network explorer’, or ‘Pathway explorer’.

To identify literature precedent during synthesis planning, we have created a database for synthetic biotransformations, as this data is not well captured in other databases. To further leverage this data, we are developing a set of tools for automated meta-analysis, offering a global view of the chemical and sequence space characterised for an enzyme class (Figure 2). For example, enzymes in the database are automatically blasted against UniRef50 followed by the automated construction sequence similarity networks (SSNs), which are accessible through an interactive visualisation. These tools allow the biocatalysis database to illustrate what an enzyme class is capable of, identify the most promising enzymes for a particular reaction, or to highlight where the gaps in knowledge are. Efficient entry of data into the database is also crucial, for which we are developing a set of semi-automated data curation tools integrated into the RetroBioCat web platform (Figure 2). We demonstrate this platform for selected enzymes. In addition, we make the platform available to the community for the curation and analysis of all biocatalysis data, which is currently scattered throughout the literature.

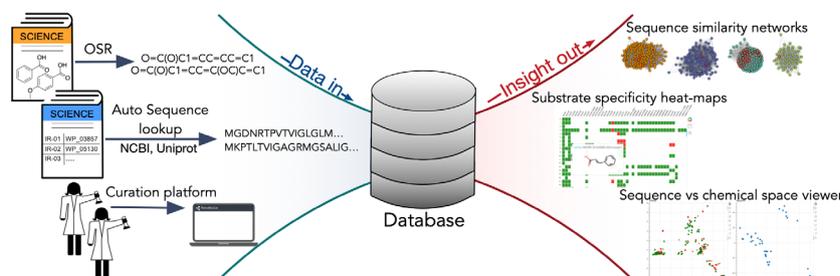


Figure 2. The biocatalysis database offers automated tools to extract insight from biocatalysis data, and automated tools to accelerate data curation from the literature.

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Active site cavity-based identification of new BBE-like monolignol oxidoreductases

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Flavoproteins are a diverse protein class employing an isoalloxazine ring for catalysis in form of the flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD). Among them is the berberine bridge enzyme-like (BBE-like) protein family. Monolignol oxidoreductases from this protein family catalyse the oxidation of monolignols to the corresponding aldehydes. It has been shown that AtBBE-like protein 15 belongs to the group of monolignol oxidoreductases. For these enzymes, a set of residues was identified, which is characteristic for monolignol oxidases and necessary for their catalytic function. [1] This set of residues and the properties of their active site cavities were used to identify new potential monolignol oxidoreductases in the group of BBE-like proteins of different plants. In this work new potential BBE-like monolignol oxidoreductases were identified in *Citrus sinensis* (orange), *Glycine max* (soy), *Solanum tuberosum* (potato), *Populus trichocarpa* (poplar) and *Arabidopsis thaliana*.

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Predicting Enzymatic Reactions with a Molecular Transformer

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The implementation of biocatalytic steps in synthetic processes remains challenging because it is very difficult to predict whether a particular substrate might actually be converted by an enzyme to the desired product. Computer-assisted synthetic planning (CASP) comprises a range of artificial intelligence approaches to predict reaction products from reactant or reagents, or vice-versa, and to plan retrosynthesis[1]. Here we asked the question whether CASP might be exploited to predict the outcome of enzymatic reactions for organic synthesis.

We set out to use multi-task transfer learning combining the 1 million USPTO reaction dataset as a source of general chemistry knowledge with 32.000 enzymatic reactions collected from the scientific literature as a source of specialized knowledge (Figure 1). Such transfer learning strategy was recently shown to enable the Molecular Transformer[2] to predict complex regio- and stereo-selective reactions at the example of carbohydrates[3]. One of the novelties of this work is that we combined SMILES language for the substrates with human language for the enzyme descriptions[4]. Hence, Enzymatic transformer not only learns to interpret the SMILES language but also natural language, as used by human experts to describe enzymes and their mutations. We obtained 62% accuracy when using multi-task transfer learning based on the full description of the enzymes. However, model performance was limited by database size and was lower with enzymes for which only few examples were available.

Furthermore, analysis of successes and failures showed that model performance is also limited by the occurrence of database entry errors. Model performance can probably be increased by using larger and higher quality training dataset. However, our approach should be generally useful to develop models capable of assisting chemists in implementing biotransformations for chemical synthesis.

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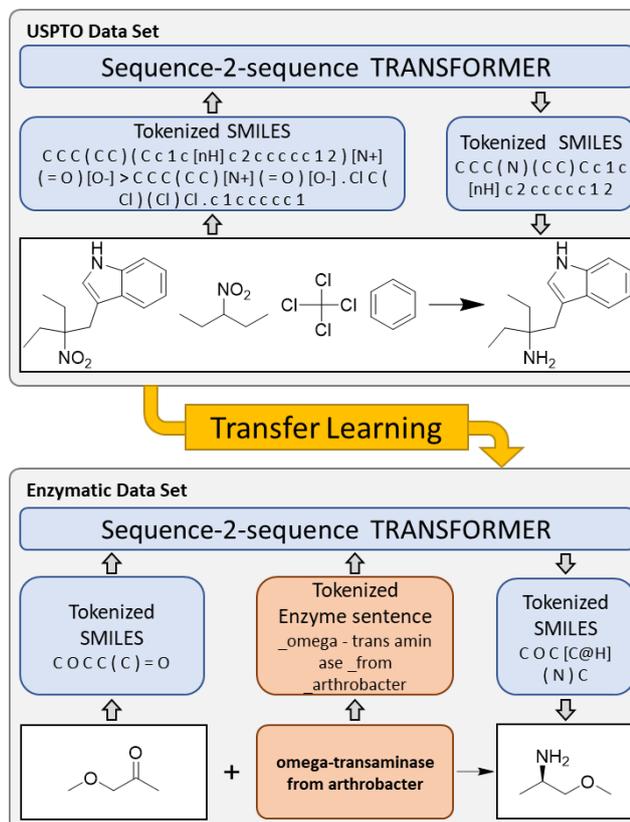


Figure 1. General concept of the Enzymatic Transformer training.

Predicting Enzymatic Reactions with a Molecular Transformer

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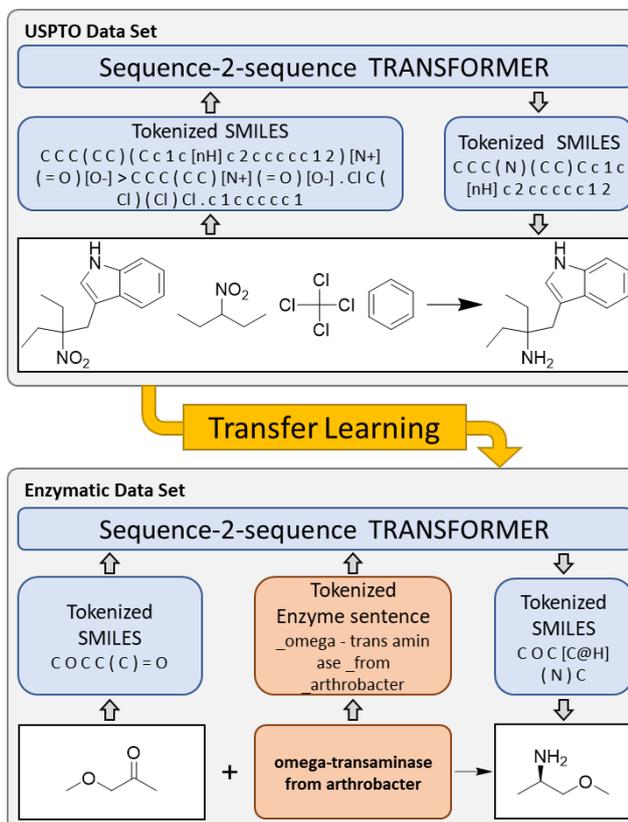


Figure 1. General concept of the Enzymatic Transformer training.

Hacking *Streptomyces*: uncovering two novel imine reductases using a diverse set of bioinformatic tools

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The genus *Streptomyces* is host to a manifold of biosynthetic routes and biocatalytic activities. It is not surprising then that the first reported imine reductases (IREDs) were described within this genus.[1,2] From this original work up to now, several novel enzymes have been reported and characterized, including IREDs as well as reductive aminases (RedAm).[3] Several recent works nicely illustrate how this area has progressed over the last years.[4,5]

In this work we explored a collection of native *Streptomyces* strains isolated from Uruguayan soil for imine reductase activity. A preliminary analysis of five genomes was performed by searching for the VWNR conserved motif. Refinement of the initial search was performed by comparison with previously reported sequences. Finally, 9 potential IREDs were classified according to their predicted stereoselectivity using the criteria described by Fademrecht.[6] Two IREDs, IRED-R and IRED-S were selected for cloning, expression, characterization and bioinformatic analysis.

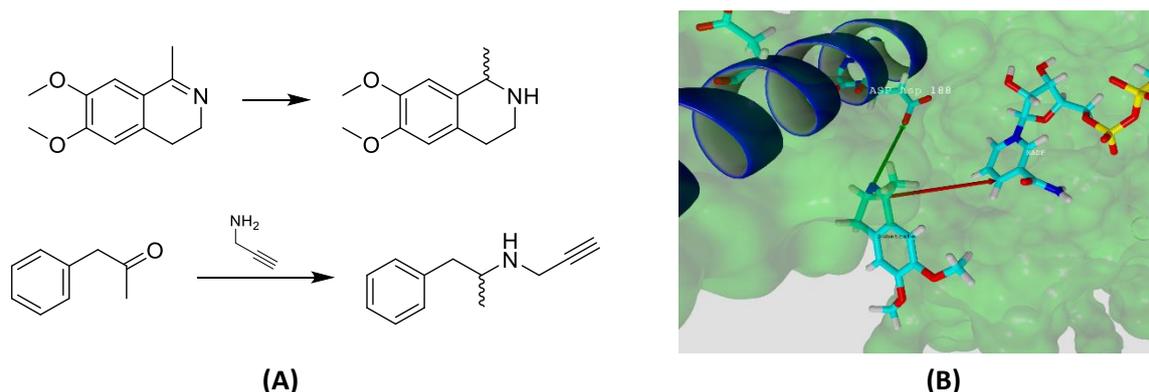


Figure 1. Characterization of IRED-R and IRED-S regarding imine reductase and reductive aminase activity. (A): two example reactions; (B): docking of an imine substrate in the active site of IRED-S.

The characterization was performed with a set of 4 reactions (Figure 1A). IRED-S presented a classical imine reductase behaviour, while IRED-R clearly depicted preference for RedAm activity. This experimental outcome was not foreseen based on the original sequence based analysis; thus, a structural analysis was performed to explain the observed differences in activity (Figure 1B).

A deeper *in-silico* analysis using refined homology models and docking tools proved to be valuable for predicting IRED or RedAm activity with selected substrates and can be used as an additional computational tool on the guided search for novel enzymes.

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BioCatHub, a platform for FAIR data acquisition in biocatalysis

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Reproducing experiments based on published experiments is challenging. The FAIR (Findable, Accessible, Interoperable, Reusable) data principles [1] are important tools to improve the reproducibility of experiments. Nevertheless, it can be challenging for experimenters to implement FAIR compliant research data management in addition to everyday lab work. An increasing number of publishers, institutes and funding agencies demand data acquisition strategies according to the FAIR data principles which enhances the pressure on the experimenter.

To improve this, BioCatHub is being established. The aim is to develop a research data management concept for biocatalysis as an easy-to-use web-interface (available at <https://biocathub.net>). The user interface is designed as wizard like structure and guides the experimenter through the different steps of an experimental procedure, collecting information describing the experiment (metadata) and measurement data. The collected data can be exported to the standardised file format EnzymeML (Enzyme Markup Language) for local storage or uploaded to the Zenodo platform [2], completing the data acquisition according to the FAIR data principles.

The overall purpose of BioCatHub is to develop a FAIR data principles compliant research data management plan for biocatalysis and to provide this in form of an easy-to-use solution for experimenters in biocatalysis. In this way, BioCatHub aims not only to simplify experimental data collection, but also to increase the availability of both positive and negative experimental data.

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Analysis of Cytochrome P450 diversity and the disparity between CYP sequence space and structure space

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Cytochrome P450s (CYPs or P450s) are a ubiquitous superfamily of heme thiolate proteins. CYPs have become attractive options as biocatalysts as they catalyse a large variety of stereo- and regio-selective oxidations under mild conditions. They are, however, limited by their low activities and their need for a redox partner. CYPs have also shown promise as drug targets due to their involvement in the degradation of xenobiotics.[1,2]

Over 40 000 CYP genes from all domains of life have been classified into families and sub-families by Dr Nelson according to his own classification system. Over 300 000 CYP genes remain unclassified, however.[3] Dr Nelson's CYP nomenclature system classifies CYPs according to amino acid sequence identity. Families are determined to have 40% or more sequence identity. Sub-families have 55% or more sequence identity and alleles have 97% or more sequence identity.[4]

The table below illustrates the disparity between CYP sequence space and CYP structure space. It is important to note there are less than 200 PDB entries that represent specific unique CYP sub-families. Many of the non-unique PDB entries are structural studies performed on CYP2 - CYP6 which belong to humans as well as CYP101 and CYP102 which belong to Bacteria.

Table 1. CYP distribution among domains and available crystal structures.[3]

Taxon	Classified CYPs	Unclassified CYPs	CYP families	Protein Data Bank entries
Animals	13 231	1522	556	365
Plants	16 219	168 303	277	
Fungi	7925	77 178	805	
Protozoa	602	0	63	
Bacteria	2979	59 620	591	667
Archaea	64	84	14	14
Viruses	28	0	6	1
Total	41 048	306 707	2252*	1047

*60 families are present in more than one taxon and are included only once

Table 1 illustrates for example that Fungi are the most diverse taxon, with over 800 CYP families. However, the available PDB structures include representatives of only two fungal CYP families namely CYP51 and CYP55. This means that although Fungi have the most CYP families, these promising biocatalysts have the least structural data coverage.

The aim of this study is to analyse in detail the disparity between currently available sequences, activity information and structure data for the cytochrome P450 superfamily.

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The Catalophore™ platform – Enzyme and drug discovery for future applications in biotech and pharma

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Highly efficient and selective enzymes find applications in future-oriented areas of chemistry and biology. The discovery of new and improved enzymes represents a growing field with many potential applications, including chemicals, pharmaceuticals, food, biopolymers, flavors and fragrances, and many more. The Catalophore™ platform combines bioinformatics methods with artificial intelligence to search structural enzyme and drug databases for potential enzyme candidates for industrial processes, reactions, and medical applications in a wide range of application areas.[1] The implemented search algorithm does not focus on traditionally known methods, such as structural or sequential comparisons to the parent enzyme, but on the analysis of the binding sites, the so-called active sites, or cavities of enzymes.[1] These active sites are mapped using the patented 3D point-cloud technology on the Catalophore™ platform covering 19 physico-chemical properties (electrostatics, hydrophobicity, accessibility, potential energies, hydrogen bonding potential, elasticity, etc.).

Herein we present how our Catalophore™ technology, usually applied for the discovery of novel enzymes or proteins particularly in pharmaceutical and biotechnology research areas, such as searching for new active ingredients, the prediction of side effects, or the repositioning of drugs, has spurred recent developments around the Covid-19 pandemic.[2]

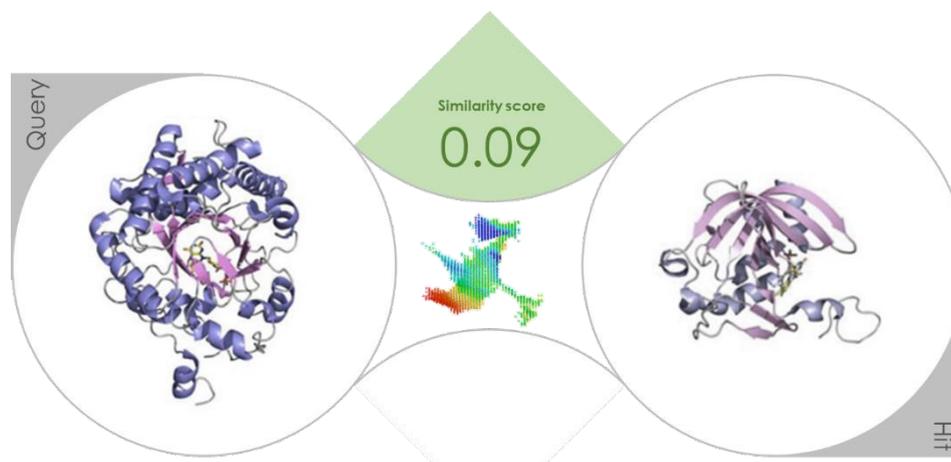


Figure 1. The Catalophore™ platform using the carrier of the catalytic function to search for novel, not yet discovered enzymes that are independent of the overall structure, protein fold and sequence.

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Rational design of natural deep eutectic solvents for biocatalytic preparation of chiral drugs

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Natural deep eutectic solvents (NADES) and biocatalysis's synergistic use fit logically to the efficient and sustainable production of enantiomerically pure compounds. The biotechnological approach ensures catalysing otherwise difficult transformations in high regio-, chemo- and enantioselective, at mild and economic conditions. NADES can serve as the green support for modulating/directing the reaction route to obtain the desired product. Papers dealing with the use of NADES as a medium for enantiopure compounds preparation/recovery implied that the unique properties of these solvents make them a promising candidate for reactions catalysed either by isolated enzymes or whole-cells through improved substrate/product solubility; enhanced enzyme activity, and stability; enhanced reaction yield; possibility to tailor reaction enantioselectivity and regioselectivity; the possibility of solvent NADES recycle and reuse [1,2]

The abundance of possible NADES formulations, makes it impossible to prepare and characterize all of them, pointing out the need to develop predictive NADES structure-activity mathematical models. Approaches used so far for screening appropriate/ideal NADES (out of an enormous pool of structural possibilities) have been governed by time-consuming empirical methods. The systematic investigation in NADES structure-activity relationship, as a backbone for these solvents' rational design, is still lacking. Current literature suggests COSMOtherm, software that can predict thermodynamic properties and phase equilibrium, which would help design the best possible solvent for a specific application. Thus, the trial-and-error method of NADES preparation can be avoided.

Based on those mentioned above, this work aims to apply COSMOtherm in NADES screening for application in *Saccharomyces cerevisiae* - catalysed asymmetric reduction of prochiral ketones for chiral building block synthesis. By using COSMOtherm, the most suitable NADES for biocatalytic reaction is selected among 100 different NADES from our database. Further steps in rational design include optimization of reaction conditions, development of downstream protocol and scale-up.

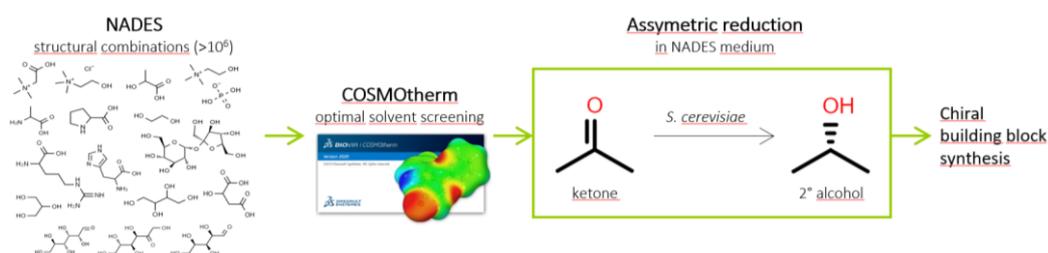


Figure 1. Graphical abstract

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EnzymeML – a data exchange format for biocatalysis and enzymology

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EnzymeML is an XML-based data exchange format that supports the comprehensive documentation of biocatalytic data by describing reaction conditions, time courses of substrate and product concentrations, the kinetic model, and the estimated kinetic constants. EnzymeML is based on the Systems Biology Markup Language (SBML), which was extended by implementing the STRENDA Guidelines. An EnzymeML document serves as a container to transfer data between experimental platforms, modelling tools, and databases. EnzymeML supports the scientific community by introducing a standardised data exchange format to make enzymatic data findable, accessible, interoperable, and reusable according to the FAIR data principles. An Application Programming Interface in Python supports the integration of applications. The feasibility of a seamless data flow using EnzymeML is demonstrated by creating an EnzymeML document from a spreadsheet, by kinetic modelling using COPASI, and by uploading to STRENDA DB and SABIO-RK.

Documentation and software of the EnzymeML project are freely available for non-commercial and commercial users at <https://EnzymeML.org>.

CapiPy: a bioinformatic tool for the rational immobilization of enzymes

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Protein immobilization has become one of the main tools for biocatalysis and yet, its application is now based on a trial and error approach [1, 2]. CapiPy is a python-based software based on Biopython [3] and PySimpleGUI (<https://pysimplegui.readthedocs.io/>) which aims to help give prior information to the user in order to rationalize protein immobilization. It can be installed and run in an Anaconda environment (<https://www.anaconda.com/>) or as a package from Python Package Index (<https://pypi.org/>). It is freely available and is designed to be clear and easy to use for users with no experience with python command line applications, but knowledge on protein immobilization and can be installed in all main operating systems (Windows 10, macOS Catalina and Ubuntu 20.02 LTS). A simple representation of CapiPy's workflow is depicted in Fig. 1.

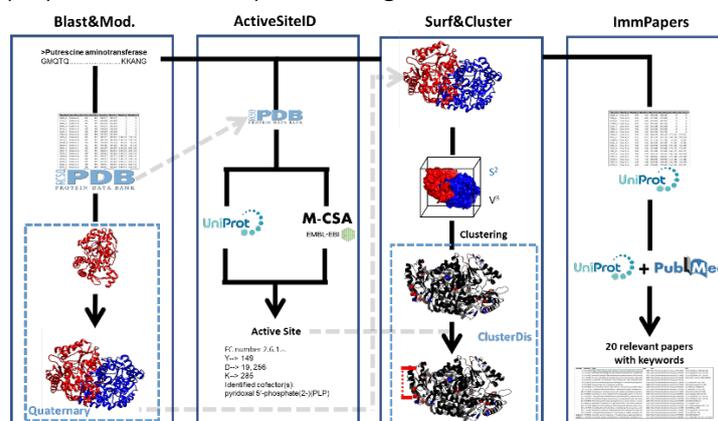


Figure 1. Schematic representation of CapiPy workflow.

The first module allows the user to create a model of the query protein using BLAST [4] and Modeller [5] starting from only the one-letter sequence of the protein. The created model can then be used by the second module to map the active site of the protein, based on homology to registered proteins in the Mechanism and Catalytic Site Atlas (<https://www.ebi.ac.uk/thornton-srv/m-csa/>) [6]. Finally, the most informative module for protein immobilization is the Surface and Cluster analysis. In here, the created model is analysed and the exposed amino acids on its surface are identified based on their half-sphere exposure [7]. The exposed residues are further classified into clusters (defined as groups of 3 or more amino acids of relevance for immobilization strategies (positively charged, negatively charged, hydrophobic, lysines, histidine or cysteine) and the results can be easily visualized using PyMOL [8]. The last available module allows the user to retrieve up to 20 publications with relevant information on the immobilization of related proteins.

CapiPy's performance has been tested with three independent sets of 150 proteins from the UniProt database, with more than 70% success.

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PyPEF – an Integrated Framework for Data-driven Protein Engineering

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Protein engineering is nowadays used routinely in developing biocatalysts for biotechnological, biomedicine, and life sciences applications. Recently, data-driven strategies attracted attention in protein design and engineering because of advances in large experimental databanks of proteins, next-generation sequencing (NGS), high-throughput screening (HTS) methods, and the development of artificial intelligence algorithms [1-3]. However, the reliable prediction of beneficial amino acid substitutions, their combination, and effect on functional properties still pose significant challenges in machine learning assisted protein engineering [4]. In this presentation, we describe a general-purpose framework (PyPEF: Pythonic Protein Engineering Framework^[5]) for data-driven protein engineering by combination of machine learning methods with signal processing and statistical physics techniques. PyPEF assist in the identification and selection of beneficial proteins of a given sequence space by either systematically or randomly exploring the fitness of variants and by sampling random evolution pathways. The predictive accuracy and throughput performance of PyPEF was evaluated based on four public protein and enzyme datasets using common regression models. It turns out that the PyPEF could efficiently predict the fitness of protein sequences for different target properties (predictive models with coefficient of determination values ranging from 0.58 to 0.92). By combining machine learning and protein evolution, PyPEF enabled the screening of proteins with various functions reaching a screening capacity of more than 500,000 protein sequence variants in the timeframe of only a few minutes on a standard PC. PyPEF exhibited significant accuracies on four public datasets (different proteins and properties) and underlined the potential of integrating data-driven technologies for covering different philosophies by either predicting the fitness of the variants to the highest accuracy or capturing the general trend of introduced mutations on the fitness in directed protein evolution campaigns. In essence, PyPEF provide a powerful solution to current sequence exploration and combinatorial problems present in protein engineering through exhaustive *in silico* screening of the protein sequence space.

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Activity and Thermal Stability of Biocatalysts in the Presence of Co-solvents

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After establishing a biotransformation on laboratory scale the addition of organic co-solvent during process intensification might be needed.[1] For instance, a low solubility of the substrate in the aqueous phase might require the solvent as additive. Consequently, the biocatalyst is exposed to organic solvents which might reduce its activity or even lead to a complete inactivation. Why one enzyme can tolerate a co-solvent while another one loses its activity entirely is not understood in detail, yet.

We aim to decipher the solvent resistance of different biocatalysts towards different solvents and shed light onto enzyme and solvent features that define the (in-)stability. To achieve this we combine experimental evaluation of stability with computational methods. In order to have a good data set for the computational analysis we have identified a representative set of ene reductases for which solvent stability was assessed.[2] We have measured the thermal stability in the presence of co-solvents and the influence of the co-solvents on the initial activity.

The addition of the tested organic solvents led to a decrease of the melting temperature in all cases. The degree of thermal destabilization seems to be dependent on the solvent type and concentration while the initial activity under the same conditions behaves different. In some cases the addition of the co-solvent led to an increase in the initial activity while it led to a decrease for other ene reductases or in combination with other co-solvents. Thus, a thermal destabilization does not necessarily go together with a reduction of the initial activity.

To understand which characteristics lead to a boost of initial activity in the presence of co-solvent, we have calculated a number of physicochemical properties from the enzymes's sequence and structure. Up until now we could not identify any measures that correlate with the observed activities. To decipher the complex behavior, we are now running MD simulations under co-solvent conditions and hope to identify details that help to understand which enzymes are positively or negatively influenced. This knowledge will enable to optimize enzymes for increased co-solvent tolerance.

Acknowledgment: This work was funded by BASF SE.

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Radical C-C coupling catalyzed by glycyl radical enzyme, benzylsuccinate synthase from *Thaurea aromatica*

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Glycyl radical enzymes (GREs) catalyze some of the most remarkable and challenging free radical reactions encountered in biology. One of these particularly important reactions is the addition of toluene to the double bond of fumarate, catalyzed by benzylsuccinate synthase (BSS) (Figure 1). This reaction allows anaerobic activation of toluene and its derivatives, providing carbon sources for many anaerobic chemosynthetic bacteria [1-3].

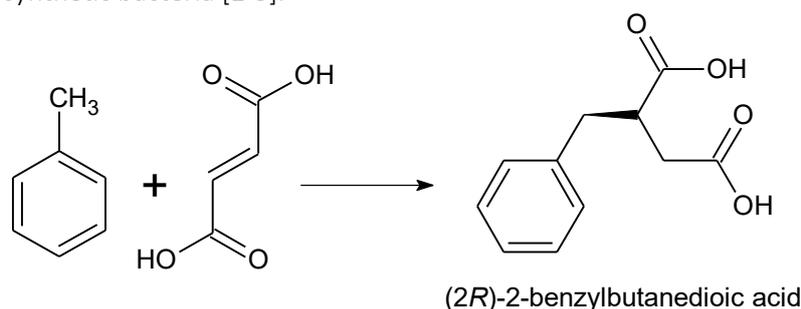


Figure 1. The reaction catalyzed by BSS leading to the formation of (R)-benzylsuccinate ((2R)-2-benzyl-butanedioic acid)

BSS from *Thaurea aromatica* is a heterohexameric enzyme ($\alpha_2\beta_2\gamma_2$) with a characteristic catalytic dyad (Gly828 and Cys492) located in the active site of the α subunit. The Gly is activated to a radical state, which can be transferred to Cys, creating a catalytically relevant enzyme-bound thiyl radical. As the structure of the BSS-substrate complex was determined [2], we started to study the reaction mechanism in more detail by a combination of experimental and modeling techniques. We derived a mechanistic model [3] and studied the dynamic behavior of known BSS substrates as well as derivatives that are not converted by the enzyme using MD. Based on the understanding of structural features that influence substrate binding and comparative analysis of sequences of BSS analogs, we introduced mutation of the crucial residues. The introduction of mutations was possible due to the development of a recombinant production system of activated BSS in *Azoarcus evansi*. We were able to expand the substrate spectrum of the enzyme to m-xylene, which was previously not converted by the wild-type enzyme as well as introduce an ability to synthesize 3-benzyl-4-ketopentanoate from toluene and the fumarate analogue 3-acetyl acrylate [4]. The QM:MM modeling revealed an intricate net of conformational changes involved in the transportation of the radical from Gly828 through Cys492 to toluene. We were able to reexamine the previously described mechanism [3] with full information on the substrate-enzyme interaction enabled now by multiscale QM:MM modeling.

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Structural characterisation of a fungal hydroxylase from the CYP505 family and engineering of its regioselectivity by comparison with CYP102A1

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The CYP102 and the CYP505 family are evolutionarily related. While CYP102s are found in bacteria, the CYP505 family contains the eukaryotic counterpart of these self-sufficient CYP450 monooxygenases.[1] Enzymes in both families generally accept medium-chain alkanes, alcohols, and fatty acids as substrates and hydroxylate them sub-terminally, yielding a product mixture of ω -1 to ω -3 hydroxylated compounds.[2-4]

We recently solved the X-ray crystal structure of the haem domain of a fungal hydroxylase from the CYP505 family. With substrate bound in the substrate channel, the enzyme crystallised in the closed conformation. The structure shows a similar overall fold as seen in CYP102A1 bound to palmitoleic acid and a similar substrate orientation in the active site. We, therefore, explored if we could rationally transfer the knowledge we have on active site mutations in CYP102A1 to evolve the regioselectivity of the CYP505.

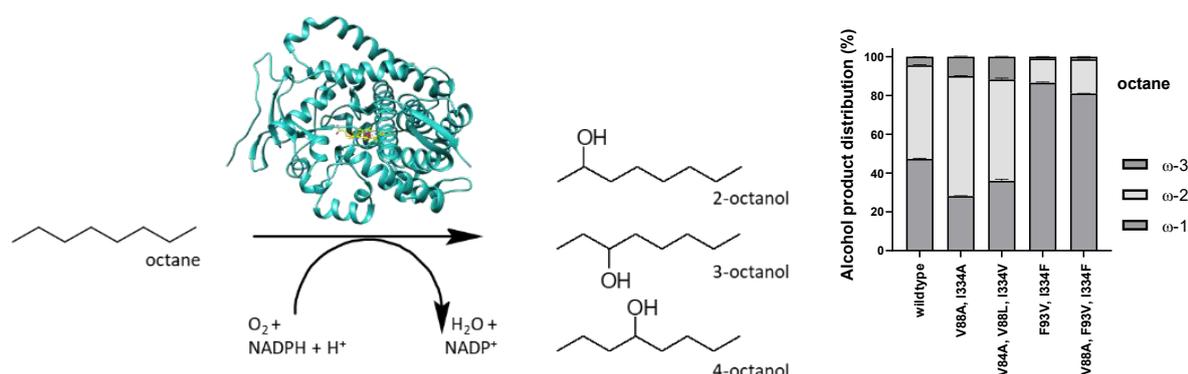


Figure 1. Hydroxylation activity of the CYP505 yields a mixture of ω -1 to ω -3 hydroxylated alcohols from octane. Mutations in the active site increased the regioselectivity.

Using site-directed mutagenesis, we introduced mutations in the active site to change the regioselectivity of the CYP505, based on previous studies done with CYP102A1.[5-7] Cell-free extract biotransformations with octane and decane were used to analyse the product profile of the wildtype enzyme and the mutants. An F93V, I334F double mutant showed an increase in regioselectivity for the ω -1 position to over 80 and 90 % for octane and decane, respectively.

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Functional expression of unspecific peroxygenase from *Daldinia* sp. by directed evolution.

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Unspecific peroxygenases (UPOs, EC 1.11.2.1) are fungal biocatalysts that bring about oxygen-transfer reactions to inactivated hydrocarbons. The fact that UPOs rely solely on hydrogen peroxide as co-substrate and are able to accept a diverse repertoire of organic molecules (more than 350 currently described) has drawn excitement about their application in green processes of organic synthesis [1, 2].

UPOs occur in nature as stable extracellular enzymes involved in detoxification processes and lignin degradation. A great majority of them exhibit a cleavable signal peptide at its N-terminal end that directs their extracellular exportation [2]. Despite there are more than 4300 sequences annotated as putative UPOs, only a handful number of them have been thoroughly characterized. One of the reasons that precludes gaining insights into novel UPOs is their lack of expression in heterologous systems [2]. Therefore, here we present the functional expression of a UPO from an ascomycete fungus *Daldinia* sp. (*DspUPO*) in the heterologous host *Saccharomyces cerevisiae*.

Aiming to obtain variants with increased expression titers in *S. cerevisiae*, *DspUPO* was subjected to a directed evolution campaign in which random mutations were targeted to specific chunks of its sequence by means of MORPHING (Mutagenic Organized Recombination Process by Homologous In vivo Grouping) [3] (Figure 1a). MORPHING permits mutating the enzyme's N-terminal signal peptide without disturbing the active site environment (Figure 1b). In order to select functional expression variants, a dual colorimetric screening assay was used to avoid the selection of mutants with an activity bias towards one of the substrates. A collection of functional expression mutants was disclosed, which constitutes the departure point for prospective engineering efforts.

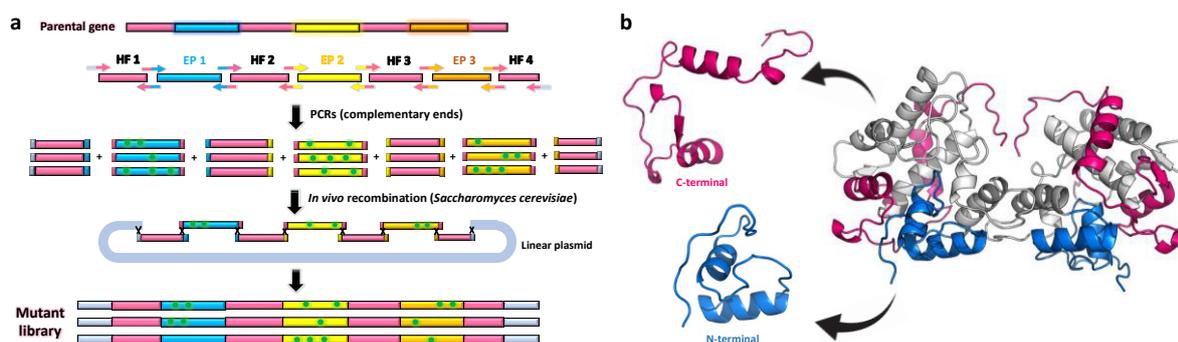


Figure 1. MORPHING exploits *S. cerevisiae* recombination machinery to selectively introduce random mutation at specific regions of a protein sequence. **(a)** MORPHING diagram. **(b)** *DspUPO* model with mutagenic MORPHING fragments coloured in blue (N-terminal) and magenta (C-terminal). The modelled structure appears as a dimer.

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Disulfide engineering of tryptophan 6-halogenases

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Flavin-dependent halogenases allow halogenation of electron-rich aromatic compounds under mild reaction conditions. Unlike classical chemical halogenation strategies, flavin-dependent halogenases are highly regioselective even at electronically disfavored positions. The resulting haloarenes are of interest to the pharmaceutical and agricultural industries and are also suitable for further functionalization, such as cross-coupling reactions. Due to the limited substrate spectrum, the low activity and limited stability of halogenases, further research in this area is necessary.

Previous reports showed that several tryptophan halogenases, like PyrH, RebH and PrnA tend to form dimers in solution and in crystal structure. The tryptophan-6-halogenase Thal, while forming dimers in all published crystal structures, was found to exist as a monomer in solution. ESI-MS analysis of Thal and thermostable Thal variants have shown that the thermostable Thal variants have a higher affinity for homodimer formation than the wild type.[1]

Inspired by these results, the influence of dimerization on thermostability and activity was investigated using disulfide engineering. To generate a covalently bridged Thal dimer, two amino acids at the prospective dimer interface were mutated to cysteines to facilitate the formation of disulfide bridges. Using SDS gel electrophoresis under non-reducing conditions and mass spectrometric analysis, it was demonstrated that the generated Thal variant exists as a disulfide-bridged dimer in solution. The mutant shows substantially increased thermostability over the wildtype while maintaining activity. In combination with a known thermostable variant [1], a further increase in thermostability was achieved while retaining the increased activity of the known variant. The generated variants are a good starting point for further enzyme engineering campaigns to increase activity or expand the substrate spectrum.

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Thermostability engineering of a class II pyruvate aldolase from *Escherichia coli* by *in vivo* folding interference

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The use of enzymes in industrial processes is often limited by the unavailability of biocatalysts with prolonged stability. The intrinsic properties of thermostable enzymes make them suitable for a broad range of applications since they present a longer lifespan -with the consequent reduction of enzyme production cost-, and higher resistance to proteolysis, detergents, chaotropic agents and organic solvents. Thermostable enzymes can be obtained from thermophilic organisms (thermozymes). However, a thermophilic counterpart for each enzymatic activity cannot always be found. In this case, we can opt for protein engineering, which enables the modification of enzymes properties to confer thermostability.

The enzyme YfaU is a class II pyruvate aldolase from *Escherichia coli* K12 that catalyses the aldol addition of pyruvate into different aldehydes producing 4-hydroxy-2-ketoacid derivatives, which are important building blocks for the synthesis of amino acids, hydroxyl carboxylic acids and chiral aldehydes.^[1,2] Due to the interest of YfaU in industrial processes, we have thermostabilized this enzyme creating a library by error-prone PCR and selecting the thermostable variants using the folding interference principle as an activity-independent method in the thermophilic bacterium *Thermus thermophilus*.^[3]

The application of this method allowed us to select two variants showing an increased thermodynamic stability, but they did not present retro-aldol activity. However, the segregation of all mutations from these two variants, led to two single mutants, Q107R and Q141L, with increased thermodynamic, kinetic and operational stability, as well as improved kinetic parameters for the aldol addition of pyruvate to formaldehyde.^[4] These results illustrate that the folding interference *in vivo* is an useful approach for selection of thermostable variants of any protein of interest.

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Acknowledgments

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Surface engineering of transaminases to tailor protein immobilization on microreactors

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Nowadays, the production of chemicals involves larger amount of money, energy, time and complex downstream process for the isolation of the final products that generate the majority of the waste worldwide¹. To overcome this problem, it is necessary to develop new production strategies that combine awareness with sustainability criteria. Remarkably, nature uses smart and efficient synthetic methods through the use of biocatalysts in multi-step pathways avoiding the intermediate isolation and purification step².

Biocatalysts in vitro generally demand for an immobilization step to simplify the downstream processing and to enable the re-use of the enzymes. In addition, the combination of functional materials and protein engineering in flow reactors will trigger in heterogenous biocatalysts³. The use of cell free systems for multistep reactions immobilized in the microreactor will facilitate the manipulation and the complexity of the process. Moreover, this system showed a high productivity for sustainable manufacture being often the preferred option to implement³.

The aim of this project is to design enzyme immobilization strategies based on protein engineering to create biocatalyst evaluation microsystems employing functionalized microreactors that integrate *an in vitro* cell-free enzyme system. This would improve the robustness, spatial orientation, and heterogenous biocatalysts process requirements for the value-added products for diverse applications in industry. To achieve this goal, it would be necessary to develop a toolbox for material design, surface modification techniques, protein engineering methods to fulfil the demand for preparation and implementation of heterogeneous biocatalysts.

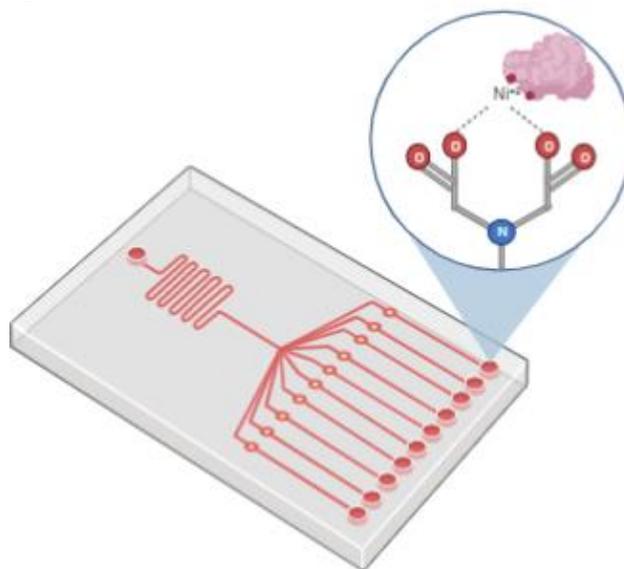


Figure 1. Prototype of the microreactor where surface engineered enzymes will be immobilized.

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Engineering iron-sulfur enzymes to unlock main Methylerythritol Phosphate (MEP) pathway bottlenecks.

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IspG is an iron-sulfur (FeS) cluster enzyme involved in isoprenoid-precursors' biosynthesis, particularly in the methylerythritol phosphate (MEP) pathway (Figure 1). As most of FeS enzymes, its catalytic activity is constrained due to their dependence on external cofactors and complex maturation pathways hindering its functional expression in foreign hosts, and preventing its implementation in industrial biotechnology [1,2].

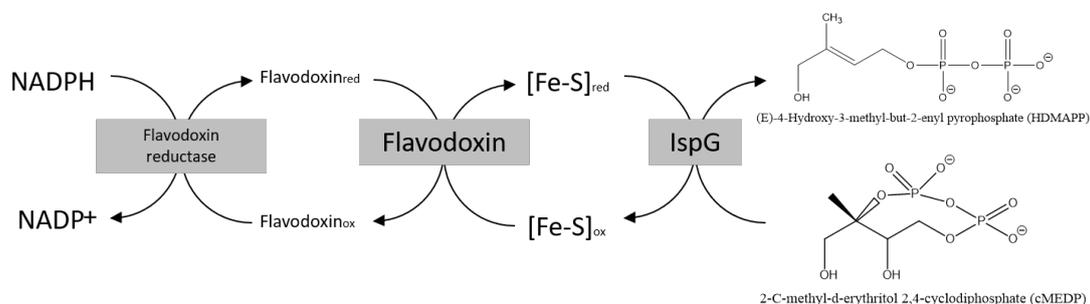


Figure 1. Reaction mechanism of the conversion of 2-C-methyl-d-erythritol 2,4-cyclodiphosphate (cMEDDP) to 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HDMAPP) carried out by IspG enzyme, a rate-limiting step for MEP pathway.

In this study, we focus on IspG from *Escherichia coli* and *Bacillus subtilis*, aiming to shed light on the key components for the catalytic activity, functionality and heterologous expression of IspG. First, directed evolution coupled to a biological selection system were performed upon *E. coli*IspG. Our strategy allowed us to spot several mutants of *E. coli*IspG with a higher catalytic activity. The potentially beneficial substitutions introduced could be more precisely assessed by the use of an impaired *E. coli*IspG variant. On the other hand, chimeras between *E. coli*IspG and *B. subtilis*IspG were designed and their functional expression was estimated. Since the functionality of IspG is essential for bacterial growth; evaluating bacterial growth over time, we were able to detect several chimeric mutants functionally expressed. Overall, our novel results will certainly contribute to elucidate the key components for the catalytic activity and heterologous expression of IspG as well as of other FeS enzymes and, eventually, overcome the main bottlenecks for the biotechnological implementation of MEP pathway in isoprenoids production.

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Engineering of Fatty Acid Hydratases for Modulation of Regioselectivity and for Enzymatic Synthesis of Novel Hydroxy Fatty Acids and Its Derivatives

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Fatty acid hydratases (FAHs) are hydro-lyase enzymes that catalyze regio- and stereospecific water addition to isolated carbon-carbon double bonds of unsaturated fatty acids to generate hydroxy fatty acid (HFAs), oleochemicals with various uses in materials and cosmetics industries [1]. Besides, HFAs and their derivatives possess various biological activities, including anti-inflammatory and anti-diabetic effects. FAHs present a green enzymatic route for production of HFAs from naturally abundant fatty acids. However, most FAHs have a narrow substrate scope and a limited regioselectivity. A unique FAH homolog from *Lactobacillus acidophilus*, namely FA-HY1, has been recently shown to have broad substrate scope and increased regiodiversity, hydrating various double bonds of C16 to C22 fatty acids, leading to a diverse set of HFAs [2].

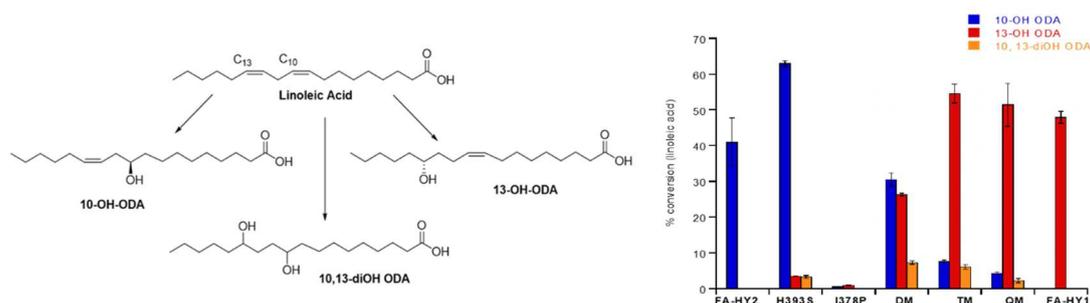


Figure 1. Shift in regioselectivity of single, double (DM), triple (TM) and quadruple (QM) mutants of FA-HY2 with linoleic acid

L. acidophilus possesses one other FAH homolog, FA-HY2, with 76 % similarity to FA-HY1. FA-HY2 produces only 10-hydroxy HFAs from C16-C18 fatty acids. We performed comparative sequence and structure analysis of the two enzymes to shed light on promiscuity and regioselectivity differences. Four amino acid residues within the substrate binding site attracted our attention. When these residues in FA-HY2 were mutated to corresponding ones in FA-HY1, resulting variants exhibited remarkable shift in regioselectivity and substrate promiscuity in a way that is similar to FA-HY1 (Figure 1) [3]. Our further rational and semi-rational engineering studies on the two enzymes led to FAH variants with altered regioselectivities and novel HFA products [4]. We bring explanation for the effect of mutations based on structural, statistical and kinetic analysis. Moreover, mutational scanning of polar active site residues provided valuable information on the mechanism of hydration, for which there is a discrepancy based on two different structures. Furthermore, we demonstrated practical utility of wild-type and engineered FAHs in preparative scale HFA production, in conversion of renewable microalgae fatty acids and in a bi-enzymatic cascade reaction for production of fatty acid esters of HFAs.

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Evolution of Phosphorylases from *N*-Acetylglucosaminide Hydrolases in Family GH3

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Glycoside phosphorylases hold great potential as catalysts for the synthesis of valuable sugars, glycosides, and glycans or for the development of energy-efficient microbial cell factories. However, the number of available phosphorylase specificities is rather limited. We show that it is possible to establish significant phosphorylase activity in GH3 glycoside hydrolases from *Pseudomonas aeruginosa* and *Bacillus subtilis*. Single-site substitutions could introduce the ability to produce glycosyl phosphates, and a combinatorial saturation study demonstrated that this promiscuous side activity can be further optimized through various mutational paths. These findings suggest that future endeavors for the development of phosphorylases could start from hydrolases as engineering templates. In addition, we provide further insights into the elusive determinants of phosphorylase activity in natural GH3 phosphorylases.

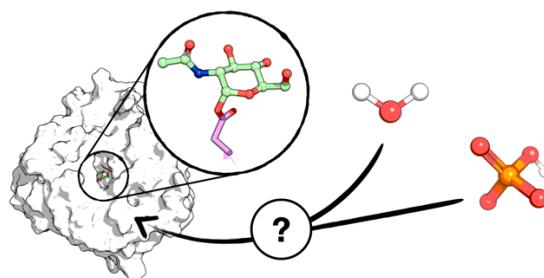


Figure 1. Figure abstract. The covalent glycosyl-enzyme intermediate of *N*-acetylglucosaminidases is intercepted by water, but after engineering, it can also be intercepted by phosphate.

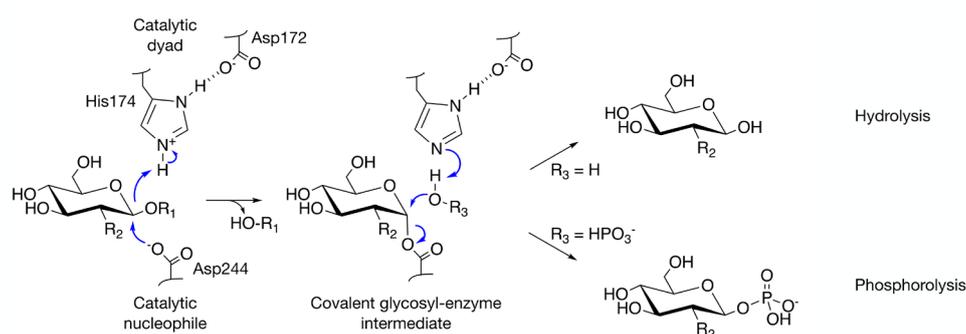


Figure 2. Double displacement mechanism of retaining β -glycoside hydrolases and phosphorylases in the hexosaminidase subgroup of CAZy family GH3 ($R_2 = \text{OH}$ or *N*-acetyl for glucosides and *N*-acetylglucosaminides, respectively).

Engineering a lipoxygenase from *Cyclocybe aegerita* towards long chain polyunsaturated fatty acids

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Lipoxygenases (LOX) are non-heme iron dependent dioxygenases that catalyze the insertion of molecular oxygen at a (1Z,4Z)-pentadiene motif occurring in polyunsaturated fatty acids (PUFAs), in a regio- and stereospecific manner. In fungi of the phyla Basidiomycota and Ascomycota, C18-PUFAs and especially linoleic acid (LA) are predominant, whereas C20-PUFAs like arachidonic acid and eicosapentaenoic acid (ETA) have been found in very low amounts, such as in basidiomycete *Cyclocybe aegerita* (syn. *Agrocybe aegerita*). In general, studies on basidiomycetous LOX are scarce described, with only three LOX to date, all of which have a high preference towards C18-PUFAs of which LA was converted with the highest preference. All primarily produce 13-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPOD) and minor levels of 9-hydroperoxy-10E,12Z-octadecadienoic acid (9-HPOD). With increasing chain length, the activity decreases drastically. To date, no study has addressed the question of which amino acids are the determining factors of the inefficient oxygenation of C20-C22-PUFAs. Here, we are presenting site-directed mutagenesis of three amino acid residues located in the substrate tunnel of the Lox1 from *C. aegerita*, which affect the specific activities towards different PUFAs. The W330L variant showed a 20% increased specific activity towards ETA, while a 2.5-fold increased activity against DTA was accomplished by the V581A variant. Only the combination of promising variants did not lead to a better variant, indicating that the increasing "fitness" cannot be generated by a simple addition and that the landscape is more complex.

Ancestral sequence reconstruction of poly(ethylene terephthalate) hydrolases

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Plastics are an essential product in our everyday life. Their intense use and our neglect in their end-of-life management make them one of the major threats to our ecosystems [1]. There is an urgent need for new strategies to prevent the release of plastic to the ecosystem, while boosting the circular plastic economy by transforming plastic waste into valuable products for the society.

Our work focus on enzymatic biotransformation of poly(ethylene terephthalate) (PET), the most abundant polyester plastic. Our final goal is to obtain an optimal biocatalyst able to perform the complete biodegradation of PET into its monomers (terephthalic acid (TPA) and ethylene glycol (EG)). In this work, we have engineered PET hydrolase [2] to obtain a robust enzyme with improved activity and stability. We exploited ancestral sequence reconstruction (ASR) strategy to infer, based on modern sequences, the protein sequence of an ancient hydrolase by phylogenetic analysis [3]. Our approach can be further exploited to obtain improved PET hydrolase enzymes that meet a cost-efficient PET biotransformation process.

Acknowledgements: This study is funded by the Comunidad de Madrid project 2019-T1/BIO-13207.

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Capture of enzyme aggregates by covalent immobilization on solid supports. Relevant stabilization of enzymes by aggregation.

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Enzymes are soluble and usually unstable biocatalysts. In order to use them in industrial processes, it is important to develop strategies of enzyme stabilization using immobilization techniques [1]. In this paper, a novel procedure for the immobilization and stabilization of enzymes is proposed: multipoint covalent attachment of bi-molecular enzyme aggregates in the presence of moderate concentrations of polyethylene glycol (PEG).

Immobilization of amine oxidase from *Pisum sativum* (AO) in the presence of different amounts of PEG was studied as a model for multimeric enzyme immobilization by promoting aggregation processes. Recently, our group achieved the immobilization of this dimeric enzyme by simultaneous covalent attachment of both amino-termini on a highly activated glyoxyl-agarose support at pH 8.5 [2]. However, while immobilization at this orientation seems to be very promising, immobilization rate, recovered activity, and stability of this interesting industrial enzyme could be improved with this new procedure.

Results shown that in the presence of low concentrations of PEG (30%), AO forms soluble bi-molecular aggregates which were qualitatively analyzed by Dynamic Light Scattering (DLS) and quantitative determined by full chemical loading of a mesoporous support (10 % agarose gels activated with glyoxyl groups). The soluble bi-molecular aggregate was immobilized by multipoint attachment on glyoxyl-agarose at pH 8.5 though the four amino termini of the two dimeric molecules (figure 1) and further incubated at pH 10. The aggregated derivative was 40-fold more stable than a similar derivative of the isolated enzyme and 200 times more stable than native enzymes in experiments of thermal inactivation.

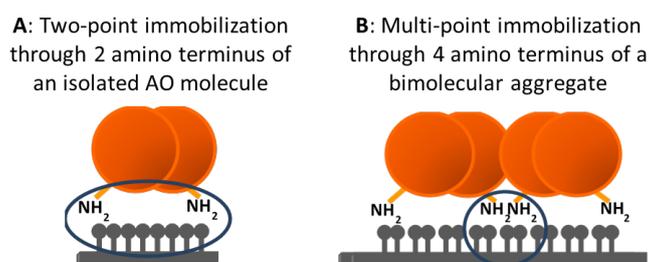


Figure 1. Schematic representation of immobilization of Amine oxidase from *Pisum sativum* at pH 8.5 (by its amino terminus). **A:** immobilization of an isolated enzyme, **B:** immobilization of a bi-molecular aggregate.

Therefore, enzyme aggregation has an important effect on enzyme activity and stability. In this way, the immobilized aggregated structure cannot undergo any movement (translational or rotational) after multipoint attachment and the aggregate is “fixed” on the support surface even after the removal of PEG improving its stability. Furthermore, this orientation allows the region with the highest density of lysine residues to remain unaltered, permitting further stabilization protocols with polymers [3].

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Engineering Imine Reductases for the Preparation of Pharmaceutical Amines

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Chiral amines are key moieties in many drug molecules; In fact, up to 40 % of active pharmaceutical ingredients (APIs) contain a chiral amine in their structure [1], demonstrating their significance in modern healthcare. Traditionally, synthetic routes to chiral amines require the use of harsh conditions including unsustainable metal catalysts with chiral ligands. However, imine reductases (IREDs) offer a biocatalytic alternative to these traditional synthetic routes, and the potential of engineered IREDs for use in industrial processes has already been realised by GSK [2]. A common limitation to the use of IREDs in a broad range of syntheses is their relatively narrow substrate scope and inability to catalyse reactions with larger substrates. Pfizer have identified IREDs with a broader substrate scope and the potential to catalyse reductive aminations with bulky amine groups [3]. One of these IREDs of interest shows novel, significant activity with the bulky amine isoindoline. We have carried out crystallographic studies on this IRED of interest which have guided rational protein engineering to produce a mutant IRED with improved activity towards a range of bulky amines. These experiments have resulted in mutants which will be further engineered by random mutagenesis for ultimate applications in the industrial synthesis of bulky chiral amines.

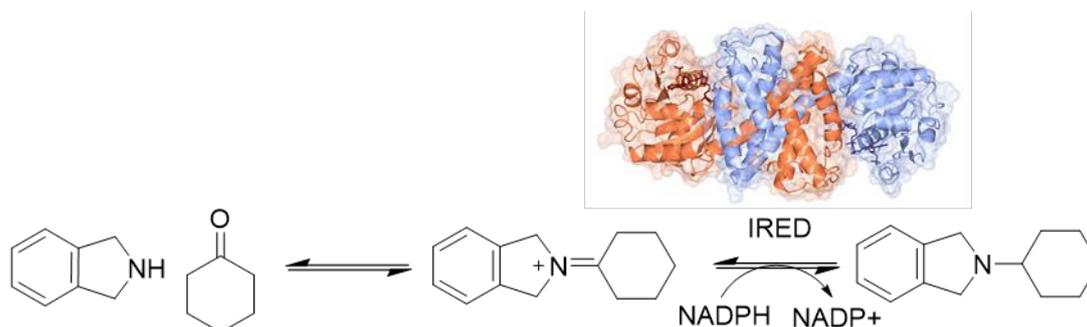


Figure 1. IRED of interest can catalyse the reductive amination of the bulky amine isoindoline with cyclohexanone.

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Functional expression in yeast of two unusual acidic peroxygenases from *Candolleomyces (Psathyrella) aberdarensis* by adopting evolved secretion mutations

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Fungal unspecific peroxygenases (UPOs) are emergent biocatalysts that perform highly selective C-H oxyfunctionalizations of organic compounds, yet their heterologous production at high levels is required for their practical use in synthetic chemistry [1]. Here, we achieved functional expression in yeast of two new unusual acidic peroxygenases from *Candolleomyces (Psathyrella) aberdarensis* (*PabUPO*) and their production at large scale in bioreactor. Our strategy was based on adopting secretion mutations from *Agrocybe aegerita* UPO mutant -PaDa-I variant- designed by directed evolution for functional expression in yeast [2], which belongs to the same phylogenetic family as *PabUPOs* -long UPOs- and that shares 65% sequence identity. After replacing the native signal peptides by the evolved leader sequence from PaDa-I, we constructed and screened site-directed recombination mutant libraries generating two recombinant *PabUPOs* with expression levels of 5.4 and 14 mg/L in *S. cerevisiae*. These variants were subsequently transferred to *P. pastoris* [3] for overproduction in fed-batch bioreactor, boosting expression levels up to 290 mg/L with the highest volumetric activity achieved to date for a recombinant peroxygenase (60,000 U/L, with veratryl alcohol as substrate). With a broad pH activity profile, ranging from 2.0 to 9.0, these highly secreted, active and stable peroxygenases are promising tools for future engineering endeavours, as well as for their direct application in different industrial and environmental settings.

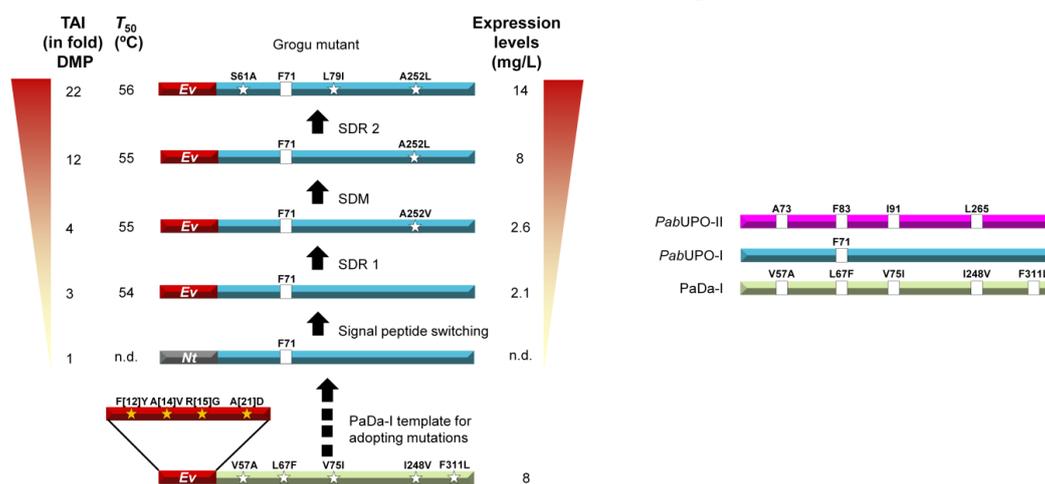


Figure 1. Functional expression of *PabUPO-I* (Left) and positions mutated in PaDa-I and their corresponding amino acids in *PabUPOs*. Stars indicate mutations and the square (F71) represents the exact match between wildtype *PabUPO-I* and PaDa-I. TAI: total activity improvement measured with 2, 6-dimethoxyphenol as a substrate.

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Oxygen-Free *O*-Demethylation By Veratrol-*O*-demethylase

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The *O*-demethylation of inert aryl methyl ethers [1] is a valuable transformation in synthetic chemistry for deprotection by unmasking the reactive phenolic functionality [2]. Several phenolic compounds are present in a variety of pharmaceutical and natural products. Unlike the chemical demethylation that requires harsh reaction conditions, nature provides environmentally friendly alternatives involving enzymes as a biocatalyst. In general, oxidative enzymes such as monooxygenases [3], P450s [4], or fungal peroxygenases [5] are applied for the demethylation by using molecular oxygen or hydrogen peroxide. These enzymes are limited by an oxygen atmosphere in which polymerized and precipitated products [6] are spontaneously formed.

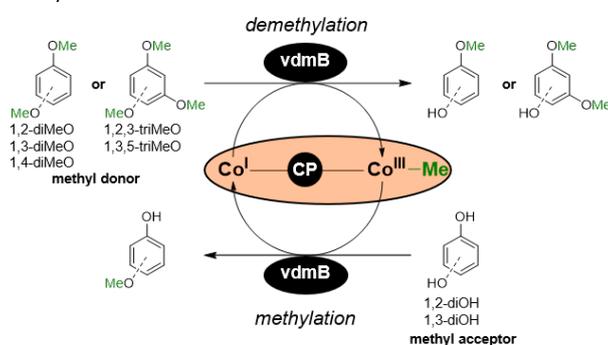


Figure 1. Simplified methyl transfer by veratrol-*O*-demethylase (vdmB) with the corrinoid protein (CP).

Here, we present an oxygen-free enzymatic system utilizing the cobalamin (coenzyme B12) dependent veratrol-*O*-demethylase (vdmB) and a corrinoid protein (CP) as methyl shuttle protein originating from the anaerobic bacteria *Acetobacterium dehalogenans* and *Desulfitobacterium hafniense*, respectively (Scheme 1) [7]. VdmB catalyzes the regioselective mono-demethylation of aryl methyl ethers with two functional methoxy moieties in either 1,2-position, 1,3-position or 1,4-position like 3,4-dimethoxy phenol and 1,3,5-trimethoxybenzene. Moreover, pharmaceutical drugs like papaverine and *rac*-yatein were accepted as well. While demethylation by vdmB abstracts a methyl group from the methyl donor substrate, cobalamin-containing dhaf4611 transfers this methyl group (Me-Cob^{III}) to the methyl acceptor for subsequent methylation (Cob^I). This approach provides an alternative to common chemical and oxygen-requiring enzymatic demethylation methods and expands the synthetic repertoire for modified pharmaceuticals and diversified natural products.

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Regio- and stereoselective hydroxylation of steroids by an engineered P450 monooxygenase

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We engineered the cytochrome P450 monooxygenase CYP107D1 (OleP) from *Streptomyces antibioticus* for the stereo- and regioselective 7 β -hydroxylation of lithocholic acid (LCA) to yield ursodeoxycholic acid (UDCA). OleP was previously shown to hydroxylate testosterone at the 7 β -position but LCA is exclusively hydroxylated at the 6 β -position, forming murideoxycholic acid (MDCA). Structural and 3DM analysis, and molecular docking were used to identify amino acid residues F84, S240, and V291 as specificity-determining residues. Alanine scanning identified S240A as a UDCA-producing variant. A synthetic “small but smart” library based on these positions was screened using a colorimetric assay for UDCA. We identified a nearly perfectly regio- and stereoselective triple mutant (F84Q/S240A/V291G) that produces 10-fold higher levels of UDCA than the S240A variant. This biocatalyst opens up new possibilities for the environmentally friendly synthesis of UDCA from the biological waste product LCA.[1]

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Protein engineering of resveratrol O-methyltransferase from *Vitis vinifera* for the biosynthesis of the nutraceutical pinostilbene

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Stilbenes are phenolic compounds derived from plants that participate in their constitutive and inducible defense mechanisms [1]. Resveratrol is one of the most studied stilbenoids due to its health promoting properties but undergoes from a rapid metabolization and has low bioavailability [2]. It has been shown that a modification of its hydroxyl moiety by O-methylation increases stability and bioavailability [3]. In plants, these reactions are performed by O-methyltransferases (OMTs), yet only a few efficient OMTs that monomethylate resveratrol to yield pinostilbene have been described so far. Here, we report the engineering of resveratrol OMT from *Vitis vinifera* (VvROMT), which has the highest catalytic efficiency [4] in di-methylating resveratrol to yield pterostilbene (**Figure 1**) in a sequential two-step methylation reaction.

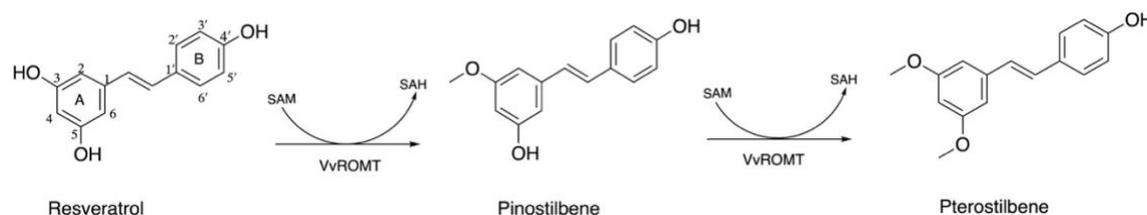


Figure 1. Representation of the VvROMT sequential di-methylation reaction. VvROMT catalyzes methylation at the 3/5-OH position on resveratrol to yield pterostilbene.

In absence of a crystal structure of VvROMT we constructed a three-dimensional protein model with MODELLER, in a closed and catalytically competent conformation in complex with the co-substrate S-adenosylmethionine (SAM) and resveratrol. Then, by applying different *in silico* approaches we identified four critical binding site residues. We performed site directed mutagenesis in these positions generating W20A, F24A, F311A, and F318A variants, which greatly reduced resveratrol's enzymatic conversion. Finally, we rationally designed eight variants through comparison of the binding site residues of our model with other stilbene OMTs. We successfully modified the native substrate selectivity of VvROMT. The variant L117F/F311W showed the highest conversion to pinostilbene, and variant L117F presented an overall increase in enzymatic activity [5]. These variants could be potentially integrated into a synthetic pathway for sustainable production of pinostilbene in an existing metabolically engineered system. Furthermore, our results suggest that VvROMT can be tailor-made to diversify methylated stilbenes and other related phenolic compounds.

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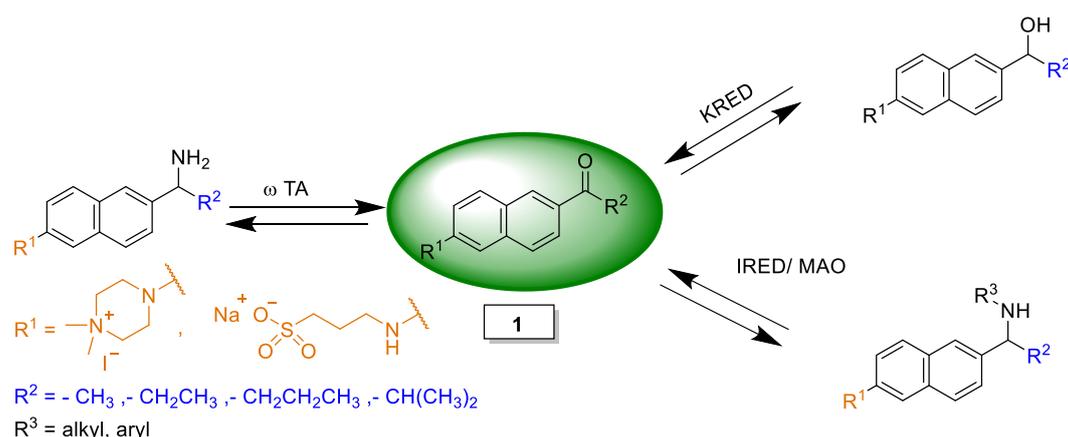
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Versatile fluorogenic assay substrates for ultra-high-throughput screening of pharmaceutically relevant enzyme classes

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The chemical synthesis of pharmaceutically relevant enantiomerically pure building blocks is expensive and laborious. Enzyme-catalyzed asymmetric synthesis is an elegant alternative to implement chirality in complex molecular scaffolds with high stereoselectivity under sustainable conditions. For this purpose, enzyme classes such as ketoreductases (KREDs), imine reductases (IREDs), monoamine oxidases (MAO) and ω -aminotransferases (ATAs) are in the focus of current industrial process research. The discovery and engineering of enzymes having the desired enantioselectivity, substrate promiscuity, and process stability requires suitable assay technology for rapid screening of candidates from genomic or metagenomic libraries. Fluorescence-based assays show very high sensitivity and offer simple opportunities to determine enzyme activity and kinetic parameters. The strong fluorescence of the core ketone structure **1** renders functional derivatives suitable for use as surrogate substrates for fluorogenic high-throughput screening (HTS) of enzymes from KRED, ω -TA, MAO and IRED classes (Scheme 1).[1,2]



Scheme 1. Common assay principle based on type **1** ketones for green fluorescent readout.

In addition, these substrates are promising for use in fluorogenic HTS for analysis in coupled enzyme cascade reactions.[3,4] To be applicable in aqueous solution without co-solvent the hydrophobic fluorophore must be functionalized with an ionic tag, and then seems appropriate for use in (w/o/w) double emulsion droplets with FACS readout for fluorescence-based ultra-HTS.

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Pilot scale enzymatic preparation of chiral amines in neat substrate

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Chiral amines are important compounds and widely used in chemical industries. To obtain these products in high chiral purity, cumbersome resolution or recrystallization steps are required, which generate a large amount of waste, resulting in high cost and environmental impact [1]. Biocatalysts like amine transaminases (ATA) provide a good application in the synthesis of chiral amines compounds, but often possess insufficient activity, stability or stereoselectivity with wild-type ATAs. Based on such a wild-type ATA, Enzymaster has been able to develop a series of engineered ATAs with improved properties using its proprietary enzyme evolution technology, which meet the process requirements for industrial synthesis. As a model product, the preparation of (*R*)-phenylethylamine (*R*-PEA) from acetophenone and isopropylamine on an industrial scale was chosen using whole cells containing the engineered ATA (Figure 1) [1].

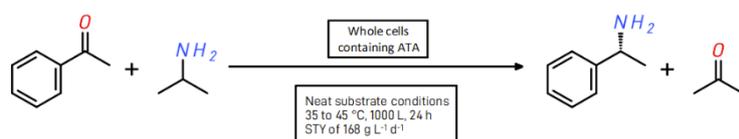


Figure 1: Industrial scale preparation of (*R*)-phenylethylamine from acetophenone and isopropylamine under neat substrate conditions with Enzymaster's engineered ATA.

The biocatalyst lost most of its activity when used in a biphasic aqueous-organic solvent system [2] but showed good conversion in a neat substrate system with only small amounts of water. This implies that, if a significant loss in biocatalyst activity is observed when shifting from an aqueous to a biphasic system, one should not be discouraged. In a further shift to a non-aqueous system, the biocatalyst could show good activity again [3] (Figure 2).

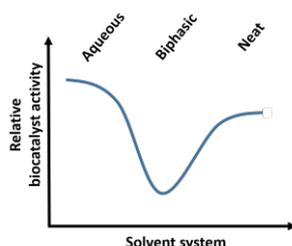


Figure 2: Graphical representation of the activity of the ATA on acetophenone and isopropylamine in different solvent systems taken from the example described above. While the biocatalyst showed good activity in an aqueous buffer with water-miscible cosolvent, only low conversions were observed when the substrate was added in amounts that form a second phase. When the biocatalyst was introduced in a neat substrate system, however, an enhanced activity was retrieved.

To further improve the system, a continuous reactor setup was designed where the biocatalyst and remaining acetophenone could be separated from the product and reused. This not only decreased the cost and waste generation of the process, but also made it possible to achieve very high product concentration of up to 168 g L⁻¹ of *R*-PEA per day in an industrial pilot scale setup due to a shift in the reaction equilibrium [4], which may as well be applied to a large range of biocatalytic transamination reactions.

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Creating hyperstable terpene cyclases using ancestral sequence reconstruction

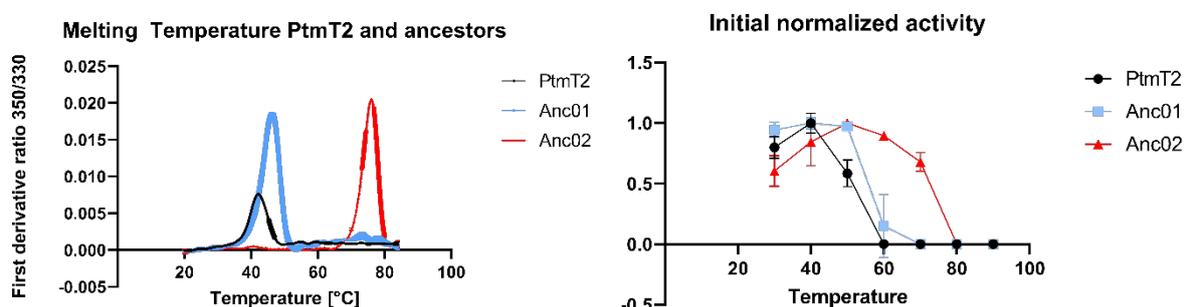
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Ancestral sequence reconstruction (ASR) is a method which uses statistical methods to determine the likelihood of substitutions to arrive at extant proteins[1]. We are interested in the evolution of PtmT2, a terpene cyclase of *Streptomyces Platensis*. Terpenes are one of the most abundant molecules in nature with a large variety of applications [2]. The molecule consists of an extended linear carbon backbone which is cyclized to form for example, precursors of antibiotics. PtmT2 is a type II terpene cyclase which cyclizes the phosphorylated carbon chain but leaves the pyrophosphate intact [3]. Using a homology search a phylogenetic tree was constructed illustrating a potential evolutionary path of PtmT2. Two potential ancestral sequences were reconstructed and tested.

Through ASR we were able to create a hyperstable terpene cyclase with a ΔT_m of 40 °C (figure 1). The oldest ancestor (Anc02) has a sequence identity of 75% which equals over 100 amino acids mutated. Despite this large change in sequence, it has retained the ability to catalyse the same reaction. The optimal temperature of the ancestors does undergo a shift. Anc01 has the highest activity in a range from 30 °C to 50 °C and Anc02 has an optimum of 50 °C whereas the WT protein unfolds at 45 °C and has an optimum of approximately 40 °C.



With the use of ASR we were able to create hyperstable terpene cyclases with an activity at high temperatures. This work demonstrates the capability of ASR as an enzyme engineering technique to create hyperstable functioning proteins.

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Structural studies of Deoxypodophyllotoxin synthase (2-ODD-PH), a C-C bond forming enzyme.

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Deoxypodophyllotoxin synthase (2-ODD-PH) is a member of the 2-oxoglutarate dependent dioxygenase superfamily that performs a ring closing, C-C bond forming reaction. The product of this reaction, deoxypodophyllotoxin, is a polyphenol lignin ultimately derived from tyrosine via the phenylpropanoid pathway. The enzyme was discovered in 2015 during an investigation into the biosynthesis of podophyllotoxin in *Podophyllum hexandrum* (mayapple), but podophyllotoxin can also be found in several other plants.¹ It is used in the clinic as a topical antiviral as well as being a precursor to several topoisomerase II inhibitors. The enzyme is also under investigation for its industrial potential.^{1,2} Carbon-carbon bond formation is of particular interest to biocatalyst development as it is a core aspect of organic chemistry and therefore fine chemical production. Members of the 2-ODD superfamily are already used in the industrial production of antibiotics and modifying 2-ODD-PH will allow the synthesis of deoxypodophyllotoxin derivatives, which may have altered bioactivities.

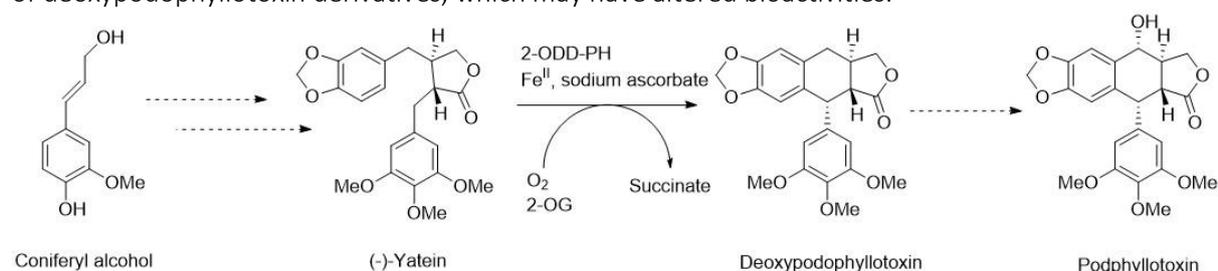


Figure 1: The ring closing reaction catalysed by 2-ODD-PH

Previous work on 2-ODD-PH has demonstrated that the enzyme has some degree of substrate promiscuity.³ In this poster I will present the structure of the enzyme and docking studies with the natural product. The structure was solved using X-ray crystallography to a resolution of 1.64 Å. The crystals were obtained in the space group *P*₂₁ with a single monomer in the asymmetric unit displaying the squashed β-barrel fold common to the 2-ODD superfamily. The open end of this supports the active site with the iron centre coordinated by the “facial triad” of His184, D186 and H239. AutoDock Vina was used to conduct docking studies to determine how the natural substrate (yatein) and product (deoxypodophyllotoxin) may bind.

The structure will permit rational site directed mutagenesis for further mechanistic work and engineering of substrate specificity.

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Enhancing the thermostability of evolved glucose-6-oxidase by directed evolution for glucose monitoring biosensors

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The copper-dependent galactose oxidase (GaOx) has been well researched because of its relevance in oxidations for industrial chemistry. Its natural substrate, galactose, is oxidized at the C6 hydroxyl group to yield the corresponding aldehyde. Over the years, this enzyme has demonstrated substrate promiscuity ranging from various primary and secondary alcohols, oligosaccharides, polysaccharides, and benzylic alcohols indicating a versatile enzyme with promising capabilities[1], [2].

The pioneering research carried out by Arnold group[3] allowed for GaOx to be converted into a glucose-6-oxidase (Glu-6-Ox), with regioselectivity not shown in nature for the first time. This sensitivity to glucose makes GaOx a candidate in the application of a glucose monitoring biosensor. However, a key element in engineering a stable GaOx for biosensors is to enhance its stability. In doing so, laboratory-directed evolution, as well as computational methods, are employed to yield a robust glucose-sensitive GaOx. Consequently, this robustness of the evolved GaOx has the potential to translate into durable immobilization on an electrode. In this communication, we present our platform to enhance the thermostability of the evolved glucose-sensitive GaOx by using computational as well as conventional directed evolution methods to provide a reliable variant for the application of glucose monitoring biosensors.

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Enhancement of cytochrome P450 BM3 activity through genetic fusion with different dehydrogenases

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Flavocytochrome P450 BM3 is a fatty acid hydroxylase that is capable of oxidizing inert C-H bonds with high activity [1]. Numerous studies have been conducted to establish mutated variants of P450 BM3 as biocatalysts for the production of various chemicals such as pharmaceutical metabolites or aroma compounds [2-4]. However, the dependence on the nicotinamide cofactor NADPH and low turnover of non-physiological substrates are among the factors that limit broad application of this cytochrome P450.

To address these limitations, we created bifunctional enzymatic units via genetic fusion of P450 BM3 with either a formate dehydrogenase or an alcohol dehydrogenase. In the fusions between P450 BM3 and formate dehydrogenase, cofactor regeneration was enabled through the turnover of cheap and readily available formate. Intriguingly, an improved catalytic efficiency was determined for both P450 BM3 and formate dehydrogenase in the fusion constructs and substrate conversions achieved with the fusions were up to 7-fold higher than with the individual enzymes. Fusions of P450 BM3 and an alcohol dehydrogenase were created for the synthesis of the flavour (+)-nootkatone from abundantly available sesquiterpenoid (+)-valencene via two consecutive oxidation steps. The P450-ADH fusions catalyzed the cascade reactions more efficiently with a space-time-yield of up to 21 mg L⁻¹ h⁻¹ leading to 420 mg L⁻¹ (+)-nootkatone compared to 10.2 mg L⁻¹ h⁻¹ resulting in 200 mg L⁻¹ product formed by the separate enzymes. Conclusively, genetic enzyme fusion of P450 BM3 and dehydrogenases is a viable strategy to endow P450 BM3 with efficient cofactor regeneration and to simultaneously enhance the catalytic performance.

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Structure elucidation of an (*R*)-selective amine transaminase and protein engineering for bulky substrate acceptance

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Despite having a good understanding of the (*S*)-selective amine transaminases (ATAs) and ways to evolve them, the (*R*)-selective ATAs are not well studied, especially concerning the evolution of their substrate scope. The ATA from *Gamma proteobacterium* was one of the first (*R*)-selective ATAs identified, but its application is still hindered from the lack of structural insight [1]. We were able to elucidate its structure, which exists in a homo-hexameric form, while the active site is formed on the interface of the dimers, as observed in most ATAs. Herein, we present some structural information on this specific ATA and we investigate the formation of the binding pockets with bioinformatic analysis. Starting from the experimentally resolved 3D structure, we prepared (*R*)-1-phenylethylamine's quinonoid intermediate, which is the accepted enantiomer of this benchmark substrate. Subsequently, we used the YAMBER3 force field to get a refined 3D structure [2]. This intermediate helped us to orient in the active site and define the residues that form the small binding pocket (Figure 1). Based on this analysis, we designed four variants, aiming the acceptance of bulkier substrates such as butyrophenone. Herein, the biochemical characterization of the wild type and the variants in kinetic resolution of amine racemates, and in asymmetric synthesis will be presented [3].

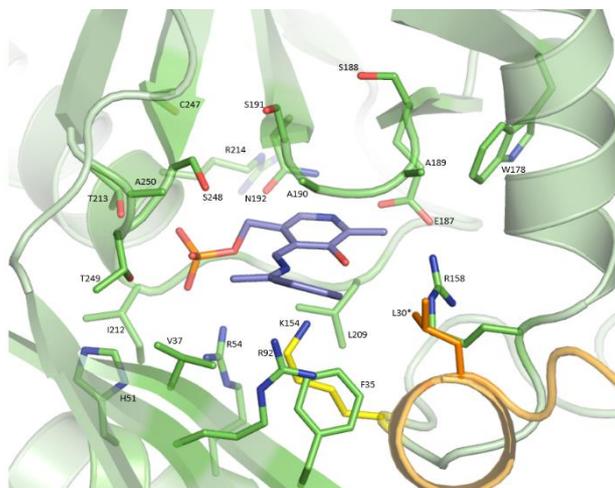


Figure 1. *Gamma proteobacterium* (*R*)-selective ATA's active site, accommodating (*R*)-phenylethylamine's quinonoid intermediate (purple sticks). The side chains of residues within 4 Å from the quinonoid are presented as sticks, including catalytic lysine (K154, yellow). The majority of the active site is formed from one monomer (green), and only a loop from the second monomer (orange) is in close proximity to the active site.

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Acknowledgements

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Polyethylene Terephthalate hydrolase's evolution platform

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Plastics are widely used in modern life mainly due to their well-known thermal and chemical properties. Polyethylene terephthalate (PET) constitutes one of the most commonly used in bottles manufacture. Despite the increasing efforts on its collection and recycling, most post-consumer plastics are still disposed without control into the environment [1].

PETase, an enzyme which is able to perform the enzymatic depolymerization of PET, it has been identified as a serine-hydrolase superfamily's enzyme, with strictly conserved catalytic triad S131-H208-D177, and α/β canonical hydrolase's folding. However, the PETase exhibits a higher flexibility than other serine-hydrolase's enzymes, and some unique features, like a second disulphide bond [2,3]. These differences resulted in the constitution of a new enzyme's family lead by PETase as reference.

Nevertheless, in a practical way, PETase is a labile enzyme, and its low stability is a clear handicap against other alternative enzymes, like cutinases [4], in PET recycling industry where researchers have been focus for years. In this work, we have developed a directed evolution platform to improve PETase enzyme stability and other properties with high relevance in the industrial field.

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Design and development of a chimera employing a glutamate and a formate dehydrogenase as an efficient tool for the quantification of ammonium

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The determination of ammonium in solution may be essential in many applications, as to assess the quality of water samples, for monitoring the ammonia levels in body fluids, for the analysis of bioreactions forming this cation. An enzymatic tool has been developed to quantify low and high concentrations of ammonium by following the production of glutamic acid. The system consists of the hexameric glutamate dehydrogenase from *Clostridium symbiosum*, which consumes ammonia for the amination of α -ketoglutaric acid using NADH, and the dimeric formate dehydrogenase from *Candida boidinii*, that has been coupled with the aim to recycle continuously the cofactor.^[1,2] However, the creation of a unique protein presenting the characteristics of the two separate enzymes would allow a facilitation in the usage of this enzymatic tool moving from two to one single protein with a huge impact on costs and time spent for the manipulation. Moreover, this would improve the efficiency of the system for the higher proximity of the two biocatalysts. Therefore, a fusion protein or chimera has been created which assembles the glutamate- and the formate dehydrogenases, by rationally designing the genetic construction, followed by the cloning of the plasmid DNA, that is then employed for the overexpression of the chimera in *E. coli* cells. After that, the protein is purified and characterized. Drawbacks like the expression of unfolded proteins in very poor amount that leads to inactive enzymes because of conformational changes have been successfully overcome and an efficient chimera has been obtained as a new unique biocatalyst that would highly optimize the great tool offered by the combination of these two enzymes. In addition, the chimera has been immobilized to enhance the efficiency of the bifunctional system, which was additionally proven by performing biotransformations.

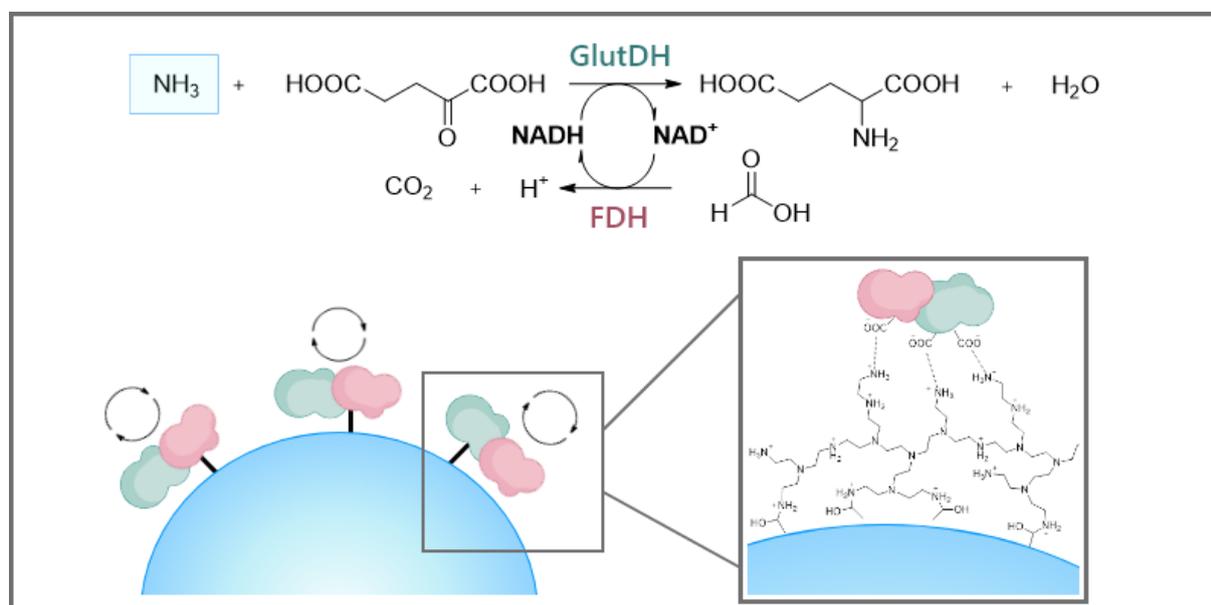


Figure 1. Reaction performed by the new developed chimera and chemistry of immobilization.

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Fungal glycosidases for the preparation of antimicrobial chitooligosaccharides

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Chitooligosaccharides (COS) are $\beta(1\rightarrow4)$ -linked oligomers of *N*-acetylglucosamine, which are increasingly being recognized as bioactive molecules featuring beneficial biological activities such as antifungal, as they act as elicitors of the defence response in plants. Plant diseases are a large issue and they account for losses up to 40% of the annual crop production worldwide. In this study, the route to the effective enzymatic synthesis of chitooligomers with degree of polymerization (DP) higher than 5 by a combined action of novel fungal chitinase and mutant hypertransglycosylating β -*N*-acetylhexosaminidases is investigated.

We discovered a fungal chitinase from *Talaromyces flavus* (*TfChit*), whose sequence was inserted into the yeast expression vector pPICZ α A containing the yeast α -factor sequence for extracellular targeting of the produced enzyme. Robust and high-yielding method of protein expression using methylotrophic yeast *Pichia pastoris* followed by one-step purification was used. The prepared chitinase was biochemically characterized and employed in the hydrolysis of chitin yielding a mixture of chitooligomers with DP of 2 and 3 as the final hydrolytic products.

In a parallel study, a panel of nine transglycosidase mutants of β -*N*-acetylhexosaminidase from *Aspergillus oryzae* (*AoHex*) was designed and their transglycosylation activities were tested. Two of these *AoHex* variants (Tyr445Asn; Val306Trp/Tyr445Phe) were found especially suitable for the synthesis of insoluble chitooligomers with DPs of 6-11 with yields reaching over 50 %. Thus, we have developed an effective cascade of glycosidase-catalyzed reactions for the high-yielding synthesis of rare and highly added-value COS by remodelling the abundant biowaste chitin.

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Directed evolution of the unspecific peroxygenase *MthUPO*

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Unspecific peroxygenases (UPOs) are secreted, fungal haem-thiolate proteins. They catalyse a large variety of reactions types mainly hydroxylations and epoxidations comparable to P450 monooxygenases. There have been more than 4000 putative UPO genes annotated demonstrating a vast available diversity. This diversity together with their broad substrate scope and impressive activities renders UPOs highly interesting biocatalysts.[1] However, although UPOs are able to highly regio- and stereoselectively oxyfunctionalise certain substrates, they have been recently described as “more stereo- than regioselective”. [2] Another challenge is their difficult heterologous expression in fast-growing organism to enable protein engineering. Recent work in our group has aimed to construct a modular yeast UPO expression system allowing the shuffling of a panel of versatile signal peptides for optimal secretion. This approach led to the efficient production of already described as well as novel UPOs in *S. cerevisiae* and *P. pastoris* by a modular cloning system, which was made available via AddGene (Yeast Secrete and Detect).[3, 4] The usage of the host *P. pastoris* is of special interest as it is extensively used as industrial host for large-scale protein productions.[5]

Herein, we utilise the new heterologous expression systems to focus on improving the selectivity and activity of UPOs with the major focus on *MthUPO* from *Myceliophthora thermophila*. [6]

We want to establish and further improve the enzyme engineering process in *P. pastoris* using the model substrate NBD. This will enable us to further enhance our understanding of the role of different amino acid residues within the substrate entrance channel and the active site.

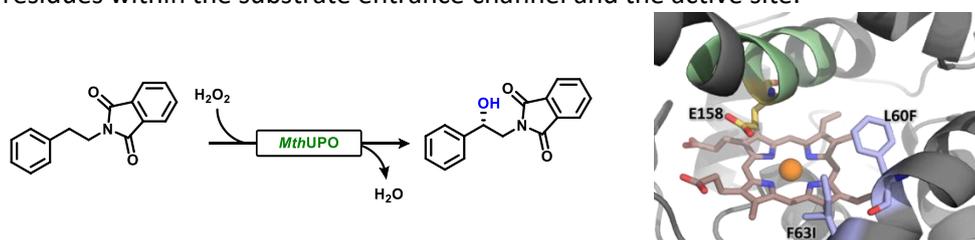


Figure 1. Benzylic hydroxylation of a phenethylamine derivative catalysed by *MthUPO*. Active site of *MthUPO* with mutated residues (blue) and residues selected for the next round of directed evolution (green).

In the future, we plan to transfer our experience in directed evolution in *P. pastoris* to phenethyl amine derivatives. We already showed that *MthUPO* hydroxylates those substrates at the benzylic position with high enantioselectivities >98 %. This enantioselective benzylic hydroxylation leads to pharmaceutically important substances such as beta-blockers and sympathomimetics. We were able to perform this reaction with wild-type *MthUPO* on preparative scale with 57% yield of the hydroxylated product.[3] From this, we want to increase the turnover-number through enzyme engineering and finally produce larger amounts of those valuable products e.g., through fed-batch fermentation.

The poster will describe the heterologous expression in *P. pastoris*, the directed evolution of *MthUPO* and its application in the selective transformation of phenethylamine.

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Protein engineering of phenolic acid decarboxylase from *Bacillus subtilis*

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In the emerging bioeconomy of the twenty-first century, enzymes are of great importance for a wide range of applications, including the sustainable, green production of fine chemicals and biofuels. Many strategies have been developed in an effort to improve enzymes properties and enhance the economic viability of the biocatalytic reactions, including immobilization, chemical modification, and protein engineering¹. Among the desired properties of the enzymes for their industrial application, thermostability is a highly important characteristic due to implementation of biocatalytic reaction at higher temperature can improve not only the yield but also allow higher substrate loading. In this work, we study protein engineering of phenolic acid decarboxylase from *Bacillus subtilis* (BsPAD) to improve enzyme thermostability and enhance its usability.

BsPAD is a cofactor independent decarboxylase that catalyzes the cleavage of carbon dioxide from bio-based hydroxycinnamic acids to yield *p*-hydroxystyrene derivatives, interesting precursors for antioxidants, polymers, and other bio-based products². Interestingly, BsPAD remains highly active and stable in deep eutectic solvents allowing thirty-fold higher substrate loading compared to reaction in buffer conditions³. Different protein engineering approaches were investigated in an effort to identify variants with improved properties compared to the extant PAD. From the protein engineering studies variants with better thermal stability than the extant PAD were identified. In particular, many of them showed melting temperature as high as 10°C compared to the wild type BsPAD, enhancing the industrial application of the enzyme.

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Site-Saturation Mutagenesis for Modulation of β -*N*-Acetylhexosaminidase Substrate Specificity

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β -*N*-Acetylhexosaminidases (GH20; EC 3.2.1.52) are *exo*-glycosidases naturally catalysing cleavage of both *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) from glycostructures [1]. They can also synthesize the glycosidic bond but the synthesis of complex *N*-acetylhexosamine oligosaccharides is complicated by their dual substrate specificity. Substitution of selected amino acid(s) in the active site by site-directed mutagenesis leads to the suppression of the hydrolytic activity in favour of synthesis [2, 3] or to the change the enzyme's substrate specificity [4].

We present here engineering of β -*N*-acetylhexosaminidase from *Talaromyces flavus* (*TfHex*), a promiscuous enzyme with a broad substrate specificity and a high synthetic potential. With the aim to modify its dual substrate specificity (i.e., GalNAcase/ GlcNAcase activity ratio), we selected the Glu332 residue as a mutagenesis hot-spot. Molecular modeling revealed that this residue is located close to the C-4 hydroxyl of the substrate. Site-saturation mutagenesis produced mutant variants in the *Pichia pastoris* expression system, which we screened in a microtiter-plate format for their GalNAcase and GlcNAcase activities. The colonies producing variants with a significantly altered GalNAcase/GlcNAcase ratio were further characterized. The chromosomal DNA isolation, amplification of the mutated *TfHex* gene, ligation, and sequencing allowed identification of the inserted mutation.

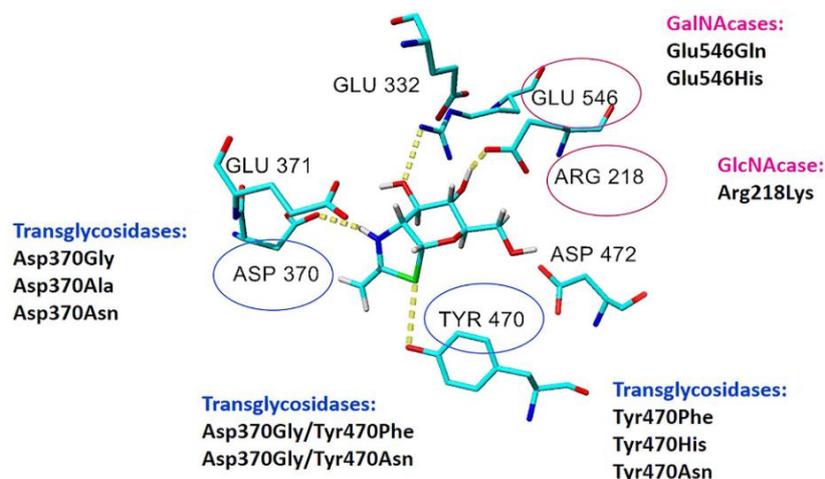


Figure 1. Model of active site of β -*N*-acetylhexosaminidase from *T. flavus* with docked β -*N*-acetylhexosaminidase inhibitor GlcNAc-thiazoline showing possible mutant variants of this enzyme [5].

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Protein engineering on the thrombolytic enzyme ADAMTS13 guided by computational methods

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In this poster we present the first results and future perspectives on the protein engineering campaign of the metalloproteinase ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13)[1] which should result in the generation of a new thrombolytic drug.

The role of ADAMTS13 in haemostasis is highly relevant: among different actors, the von Willebrand factor (vWF) and its physiological partner ADAMTS13, have been proven to mediate the thrombolysis of occlusions that are associated with ischemic stroke and that are resistant to recombinant tissue plasminogen activator (tPA), the standard drug in thrombolytic treatment. In particular, ADAMTS13 counterbalances the pro-thrombotic vWF by means of cleaving it into smaller, less adhesive multimers within nascent platelet-rich thrombi. This prevents vWF to bridge subendothelial collagen at the site of vascular injury with platelets' glycoprotein Ib α . Deficiency in persistent ADAMTS13 activity has been shown to cause disorders like the thrombotic thrombocytopenic purpura[2], which is characterized by increased levels of vWF and an abnormal number of thrombi. Additionally, the use of recombinant ADAMTS13 in patients with severe COVID-19 (performed via enzyme replacement therapy) has balanced normal vWF/ADAMTS13 levels[3]. Altogether, the use of ADAMTS13 as a thrombolytic drug would definitely open new opportunities in therapy.

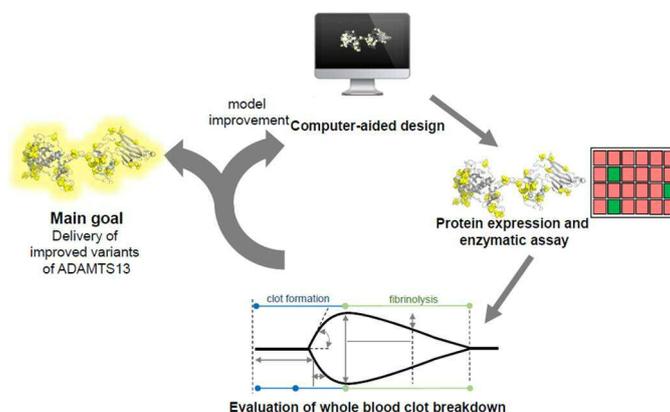


Figure 1. General strategy of this project.

Our project aims to investigate the impact of the glycosylation pattern as well as of the redox state of ADAMTS13 on its enzymatic activity (Figure 1). In this poster we will show our full-length all-atom model of ADAMTS13 built by means of homology modelling and molecular dynamics (MD) simulations. In addition, we will illustrate first steps in the expression and purification of the full-length protein and in the determination of the enzymatic activity of ADAMTS13 using *in vivo* and *ex vivo* assays.

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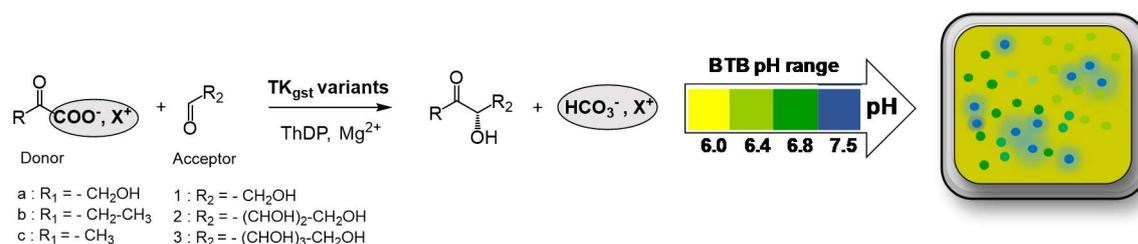
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High-Throughput Solid-Phase Assay for Substrate Profiling and Directed Evolution of Transketolase

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Transketolases (TK) belong to the family of thiamine diphosphate (ThDP) enzymes and catalyze *in vivo* the transfer of a ketol group of a donor to the carbonyl carbon atom of an aldehyde creating a novel asymmetric carbon in the (S) configuration. For biocatalytic application, an α -keto acid is required as a nucleophile rendering the reaction irreversible due to the release of carbon dioxide upon decarboxylation of the ketoacid (scheme 1). TKs from different microbial sources were evolved to broaden substrate scope by directed or site saturation mutagenesis on a few positions.¹⁻⁷ Transketolases became into strategic focus to be applied as efficient biocatalysts for the preparation of optically pure hydroxyketones highly valuable intermediates or products in the pharmaceutical, chemical and agricultural industries.¹⁻⁷ To extend the diversity of TK variants, other techniques could be used such as semi-rational approaches on several positions or on particular sequences by cassette mutagenesis or random approaches, which inevitably causes huge library sizes predominantly containing inactive or less active variants. To speed up a such directed evolution process an efficient assay is crucial to avoid time-consuming and technically demanding analysis of each variant.

We propose in this work an *in vitro* colonies solid-phase screening (SPS) assay based on colorimetric changes detected with the naked eye (Scheme 1). This strategy offers an attractive alternative to liquid phase assay because of the simplicity of operation avoiding enzyme extraction and high-throughput nature not requiring specialized equipment. This SPS assay takes advantage of TK-catalyzed reaction which releases carbon dioxide from the ketoacid donor causing the pH to rise.⁸ To visualize the variation of pH, bromothymol blue (BTB) which changes its colour from yellow to blue was used. The strategy was developed with TK from *Geobacillus stearothermophilus*⁹ (TK_{gst}) that we discovered a few years ago and that we are broadening its substrate specificity by directed evolution, because of its thermostability offering great advantages for further biocatalytic applications.³⁻⁷



Scheme 1. Principle of the SPS assay for TK activity detection in *E. coli* cells.

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Screening of stevia glycoside glycosyltransferase based on sugar receptor motif

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The structure of GT-B family was highly conserved, especially the C-terminal region binding the UDP-sugar nucleotide domain. The N-terminal region is variation to adapt to different receptors. The specific substrate glycotransferase could be obtained more accurately by analysing the relationship between substrate and tertiary structure and primary sequence of the variable N-terminal region than simple sequence alignment or homology analysis. Hence, a system, based on sugar receptor motif, was described to get stevia glycosides glycosyltransferase candidates. Through the analysis of 14 template sequences, the C - terminal and N - terminal motifs were obtained for screening the potential of steviol glycosides. Five potential sequences were obtained by screening, protein expression and glycoylation detection, all of which could catalyze rubusoside, stevioside, reb-A, reb-E, reb-D and reb-E. The catalysis product of LABUGT2 was detected by mass spectrometry, and it was found that it could add two glucose groups successively to rubusoside, stevioside and reb-A.

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Lignin-based amino acids

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Lignin, the second most abundant organic substance in the biosphere, immediately after cellulose, has been produced as technical waste with the commercialization of cellulosic ethanol in recent years. Because of its plenty of heterogeneous aromatic compounds, lignin is considered as the potential feedstock for the production of valuable macromolecules, such as carbon fibers, binders, and emulsifiers. Although the underused lignin attracts recognition as a building block for generation fine chemicals, e.g. vanillin. The lack of efficient conversion of lignin is still a challenging problem. Considering the increase in mortality rates of cancer and the growing demands for corresponding drugs, we reported a valorization strategy for the enzymatic conversion of the lignin into the valued-added optically pure β -tyrosine, which is regarded as a significant precursor for oncology drugs.

By redesigning the binding sites of aromatic aminomutase, these designed enzymes will be developed to catalyze asymmetric addition of ammonia to para-coumaric acid and produce β -tyrosine, which is the motif of the antitumor drug. Based on the original X-ray crystal structure, the results from amino acid sequence analysis, and calculations via PyRosetta for enzyme design, the mutant library in limited number have been generated without the experimental evolution in large-scale. This topic highlights the new strategy of the valorization of lignin with computationally designed enzymes, which have the tendency to produce the building blocks of oncology drugs.

Insights into PET degrading enzymes

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Plastic pollution poses a serious risk to terrestrial and aquatic ecosystems as well as human health. The global production of plastics, excluding synthetic fibres, reached 368 million metric tons p.a. in 2019 and is rapidly increasing.[1] Therefore, efficient disposal strategies are urgently needed for sustainable treatment of plastic waste. Recycling of the existing plastic waste presents the most sustainable and viable solution in context of reducing CO₂ emission as well as of the fuel and energy savings.[2] Research achievements in the last two decades empowered the applicability of selected enzymes and microbes as an alternative eco-friendly plastic recycling technology.[3] Especially for polyethylene terephthalate (PET), which is widely used to produce food packaging, beverage bottles, and synthetic fibres, the biotechnological recycling of plastics that allows the recovery of their monomeric building blocks has been demonstrated both at laboratory and industrially relevant scales.[4,5]

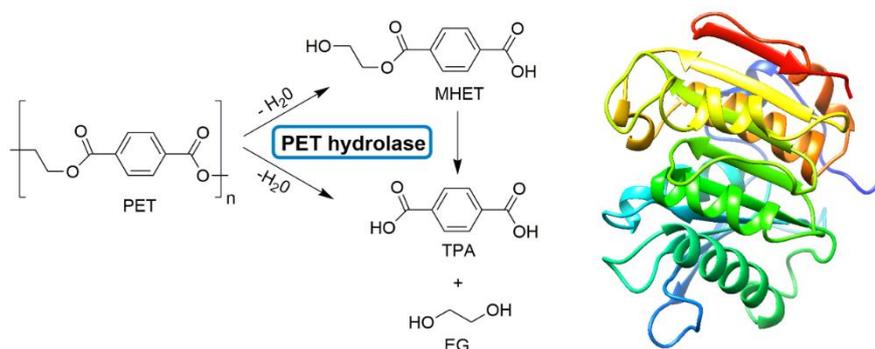


Figure 1. Degradation of PET. The enzymatic hydrolysis of PET yields mono-(2-hydroxyethyl)terephthalic acid (MHET) as dimeric product which is further degraded to PET's monomeric building blocks terephthalic acid (TPA) and ethylene glycol (EG).

Based on recently elucidated crystal structures of a novel thermostable PET hydrolase, mutation hotspots potentially influencing its thermal stability and activity against PET were identified using different computational modelling-based approaches. By means of this semi-rational redesign, enzyme variants with markedly improved stability and catalytic properties allowing rapid depolymerization of post-consumer PET waste at an industrially relevant condition were constructed and characterised. This work provides novel insights in the molecular mechanism of PET degradation and furthermore highlights potential mutation hotspots to which protein engineering can also be applied to optimise other homologous PET hydrolases.

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Enhancing the diversity of fungal peroxygenases (UPOs) for C-H oxyfunctionalisations: A modular high throughput secretion and directed evolution system in *Pichia pastoris*

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Fungal Peroxygenases (UPOs) have gained outstanding interest in recent years within the field of biocatalysis due to their versatile C-H oxyfunctionalisation chemistry, while solely relying on hydrogen peroxide as inexpensive co-substrate^{1,2}. Despite their tremendous potential, their challenging production as secreted, glycosylated, disulphide linked proteins substantially hampers the widespread use of UPOs within the field of biocatalysis². This fact is further reflected by a currently small panel of available UPOs, which can be produced in a heterologous host amendable for directed evolution.

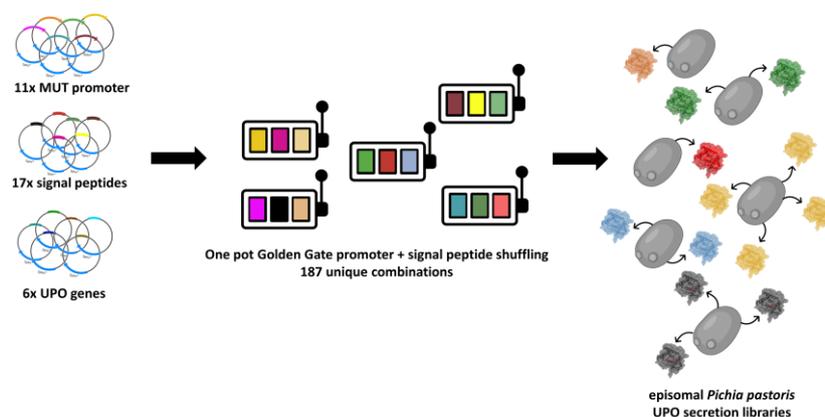


Figure 1. Concept of the modular promoter/signal peptide shuffling system for UPO secretion in *Pichia pastoris*

In our previous work we have built a Golden Gate cloning based site saturation mutagenesis system coined *Golden Mutagenesis*, which allows for the rapid construction of diverse mutagenesis libraries³. Building upon this modular logic we have developed a signal peptide shuffling system for the secretion of novel UPOs in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*⁴. Harnessing this system, we could produce four UPOs for the first time in both yeast organisms and apply these enzymes for stereoselective hydroxylation reactions on a preparative scale. Based upon the outcome of this work one novel UPOs could be further engineered towards selective benzylic hydroxylation combining the modular yeast secretion system and targeted enzyme engineering via *Golden Mutagenesis*⁵. In a recent study we further expanded the episomal *Pichia pastoris* system, being a heterologous host of outstanding industrial interest. By expanding the shuffling concept to strong methanol regulated promoters in combination with signal peptides (Figure 1) we achieved the production of three novel UPOs in yeast as well as the overall highest reported volumetric UPO shake flask titres to date⁶. To foster widespread use of the constructed modular Golden Gate system we have deposited all plasmids with the non-profit plasmid repository Addgene (Kit # 1000000166). Currently we are further improving the episomal secretion rates and evolving enzymatic activity and stability of various UPOs.

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NAD⁺ regeneration with a glucose oxidase-based chemoenzymatic catalyst

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Nature has shown us that conducting cascade reactions in confined architectures (*e.g.*, cell organelles) increases the reaction efficiency significantly. In our lab, we have mimicked Nature's approach to stabilize and arrange multiple (bio)catalysts in well-differentiated compartments within a unique polymeric nanomaterial. For the synthesis, the surface of the enzymes is chemically engineered, and a porous polymeric mantel of ca. 2-3 nm is formed.[1-4] Then, the polymeric shell is selectively decorated with chemical catalysts giving rise to chemoenzymatic nanoreactors.[5] Herein, we have fabricated a chemoenzymatic nanoreactor in which glucose oxidase is wrapped by a polymer with NADH peroxidase-like activity. Using glucose as co-substrate, the nanoreactors can regenerate NAD⁺ in a highly efficient manner, reaching 100% recovery yield of bioactive NAD⁺. The chemoenzymatic nanoreactors are perfectly suitable for one-pot reactions in conjunction with NAD⁺-dependent enzymes (*e.g.*, alcohol dehydrogenase from *Bacillus stearothermophilus* (BsADH)) due to the *in situ* and controlled H₂O₂ production for NADH peroxidation. Importantly, the nanoreactors are able to keep the cofactor pool always in the oxidized form, which is key for reaching a high substrate conversion (see **Figure 1**).

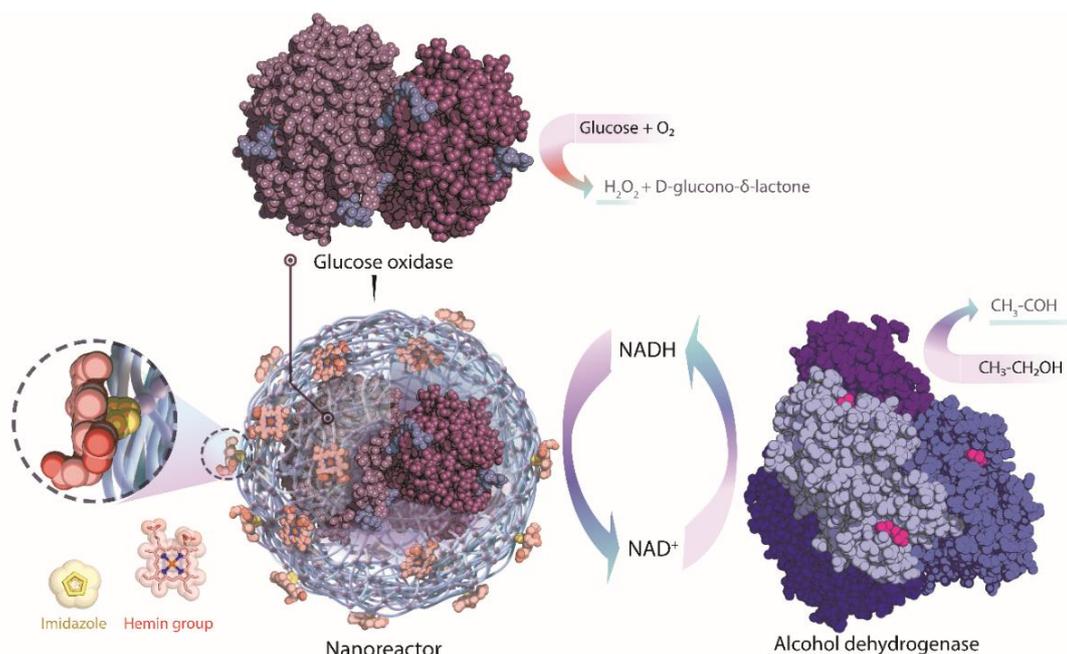


Figure 1. Schematic representation of the synthesis procedure of the glucose oxidase-based chemoenzymatic catalyst and the one-pot reaction together with BsADH for the benzaldehyde production.

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Immobilized biocatalyst engineering (IBE): a novel strategy to obtain improved biocatalyst

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Our research group proposed and validated a novel enzyme improvement platform called **Immobilized biocatalyst engineering (IBE)** [1,2]. IBE is based on the simultaneous integration of Protein engineering and Enzyme immobilization, with a unique combination of improvement through amino acid substitutions and attachment to a support material. To establish this strategy, the following methodologies were developed: direct immobilization of enzymes from microbial supernatant [3], miniaturized and massive enzyme immobilization in microtiter plates (high throughput enzyme immobilization - HTEI) [2], and integration of immobilization in screening [2]. For IBE validation, *Bacillus subtilis* lipase A was for the first time successfully and selectively immobilized directly from cell culture supernatant in mesoporous silica [3]. The enzymatic parameters evaluated between traditional immobilization and HTEI show similar results, however, using the HTEI approach, up to 96 experiments can be performed simultaneously on each microtiter plate and several plates can be evaluated, with a coefficient of variation below 10%.

IBE allows evaluating thousands of variants in a short time through an integrated screening, and selection can be made with more information, resulting in the detection of highly stable and active heterogeneous biocatalysts [2]. Variants of interest were chosen such as P11A11 (Q29R, N98D, N148D), showing higher soluble performance compared with Wt_BSLA, however, it evidenced poor performance as an immobilized biocatalyst. On the other hand, using IBE, variants with non-remarkable performance in their soluble form were, but improved as immobilized biocatalysts were identified. This is where IBE provides an interesting contribution, by detecting prominent variants that otherwise would not have been selected through traditional screening (which is based on the soluble performance of enzymes). For example, variant P6C3 (M137R), shows a remarkable immobilized activity, although its soluble performance was poor. And P5G3 (N89D, R121Q), whose thermal resistance performance immobilized biocatalyst is outstanding.

IBE proposed that enzyme mutant libraries meant to be used as immobilized catalysts should be screened as an integrated process, since variants in their soluble version would be discarded, whereas they could have the desired performance as immobilized biocatalysts. IBE allowed obtaining active and robust heterogeneous biocatalysts, with high catalytic activity against high temperature and non-aqueous solvent conditions. These biocatalysts are unlikely to be obtained through traditional strategies (soluble screening and subsequent immobilization). Using IBE, we aim to come closer to satisfy industry demands and generating ready-to-use biocatalysts. Additionally, IBE shows the benefit of approaching current challenges through a multidisciplinary and cooperative work.

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Phenotypic Selections for Biocatalyst Design and Evolution

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A concerted effort of biocatalysts in microbes and synthetic catalysts that perform new-to-nature transformations promises the production of value-added compounds from readily-available precursors or the degradation of pollutants on-demand. To make such cellular factories to order, we are using biosensors that enable both the discovery of biocompatible small-molecule catalysts and the directed evolution of biotechnologically-relevant enzymes. Specifically, these biosensors are based on the incorporation of chemically or enzymatically synthesized non-canonical amino acids (ncAAs) into proteins that elicit a readily-apparent phenotype (i.e. fluorescence or survival).

To showcase the versatility of this approach, we have developed a 96-well screening platform that enables the identification and optimization of biocompatible, small-molecule catalysts for a diverse range of new-to-nature transformations (Figure 1).[1] Such synthetic catalysts can function in concert with living cells and can be readily applied toward the biocontainment of genetically-modified organisms.[2]

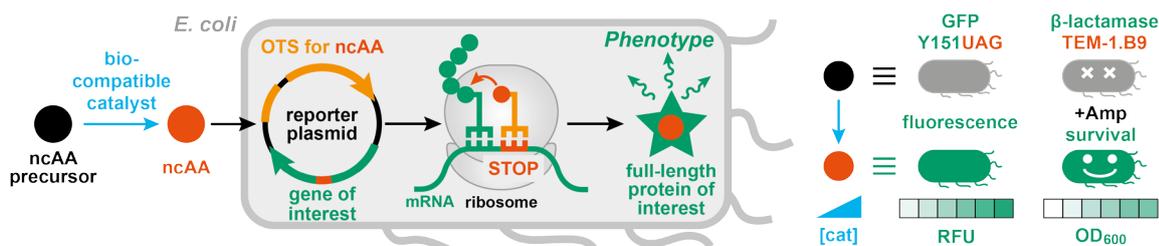


Figure 1. Blueprint for repurposing orthogonal translation systems (OTs) as functional links between the activity of synthetic catalysts and observable phenotypes. Relative fluorescence units (R.F.U.) and cellular density (OD₆₀₀) as quantifiable readouts.

Finally, the identification of enzymes that are fit for industrial applications and/or catalyze desirable new-to-nature reactions is a laborious undertaking, as enzyme variants typically need to be evaluated one-by-one. By linking enzymatic activities to readily-apparent and quantifiable traits, such as fluorescence or survival, we aim to overcome one of the most persistent bottlenecks of directed evolution campaigns (Figure 2). The flexibility of our strategy has the potential to allow us to harness the extremely high-throughput of phenotypic selections for mechanistically-diverse and biotechnologically-relevant biocatalysts.

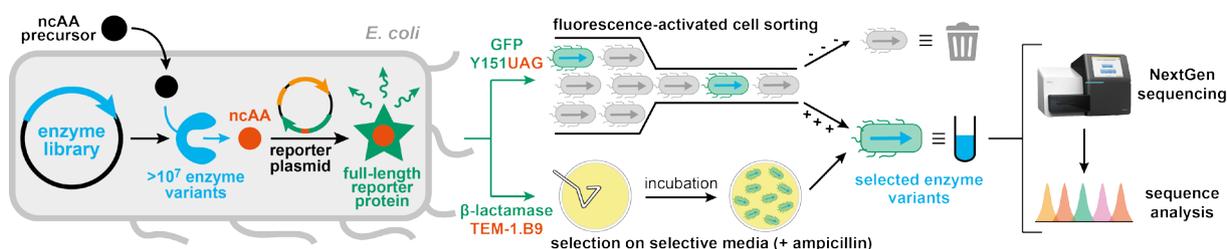


Figure 2. Schematic representation of the envisioned phenotypic selection platform. Improved variants from enzyme libraries can be either identified by FACS (for GFP) or through a life/death selection (for TEM-1.B9).

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Heterologous expression of fusion SUMO/Trx proteins in *Escherichia coli* to explore the natural diversity of unspecific peroxygenases

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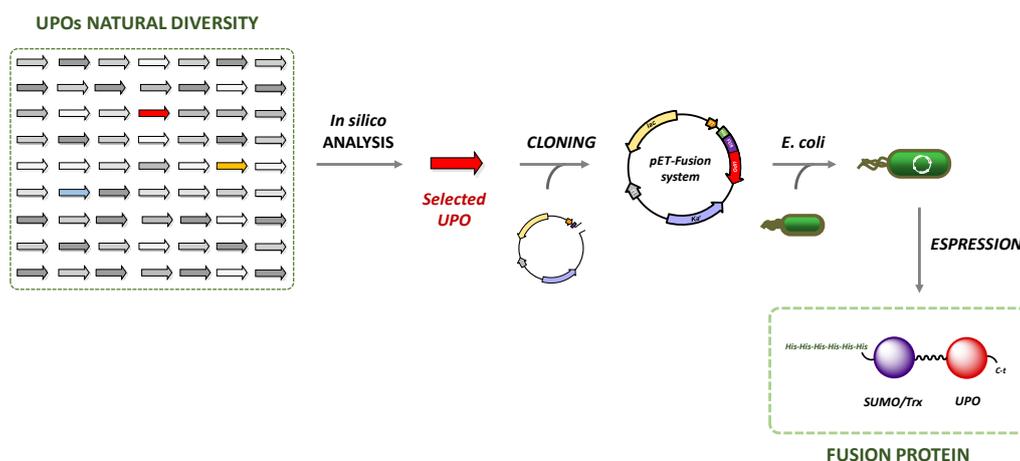
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Unspecific peroxygenases (UPOs, EC 1.11.2.1) are a family of enzymes which has attracted the interests of the organic chemist in recent years because of its versatility in organic synthesis. UPOs are able to catalyse a broad variety of oxyfunctionalization reactions, inserting oxygen into unactivated carbon atoms, and using hydrogen peroxide (H₂O₂) as only oxygen donor (and electron acceptor). In addition, this family of enzymes displays an extensive substrate specificity, both on aliphatic and aromatic compounds: more than 400 compounds have been reported as UPO substrates.[1]

The natural physiological function of UPOs currently remains unidentified, but it is known that they are produced and secreted almost entirely by fungi. Currently, more than 4300 putative UPO sequences from different fungi have been identified, but only a few have been isolated and unequivocally characterized.[1] This enormous difference between theoretically assigned and practically obtained UPOs resides in the fact that the heterologous functional expression of this group of enzymes is arduous: the development of efficient expression systems requires an optimal combination of adequate host microorganism, recombination methods and effective signal peptides. In addition, it is frequently required extensive protein engineering, such *in vitro* directed evolution, to achieve reliable expression levels.[2] The use of more simplified expression systems, like the ones involving *Escherichia coli* as host microorganism, have only been reported for three of these enzymes: *Marasmius rotula* UPO (MroUPO), *Collariella virescens* UPO (rCvirUPO), and *Daldinia caldariorum* UPO (rDcaUPO) albeit with limited expression yields.[3,4]

In the presented work, the use of functional fusion proteins for the efficient heterologous expression of UPOs in *E. coli* host-based systems has been explored, in order to simplify the discovery of new UPO activities from the broad natural diversity. Specifically, the SUMO (small ubiquitin-like modifier) and Trx (thioredoxin) fusion technologies have been assayed with different UPO sequences, to optimize alternative assembling methods and fermentation strategies.



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Heterologous expression of fusion SUMO/Trx proteins in *Escherichia coli* to explore the natural diversity of unspecific peroxygenases

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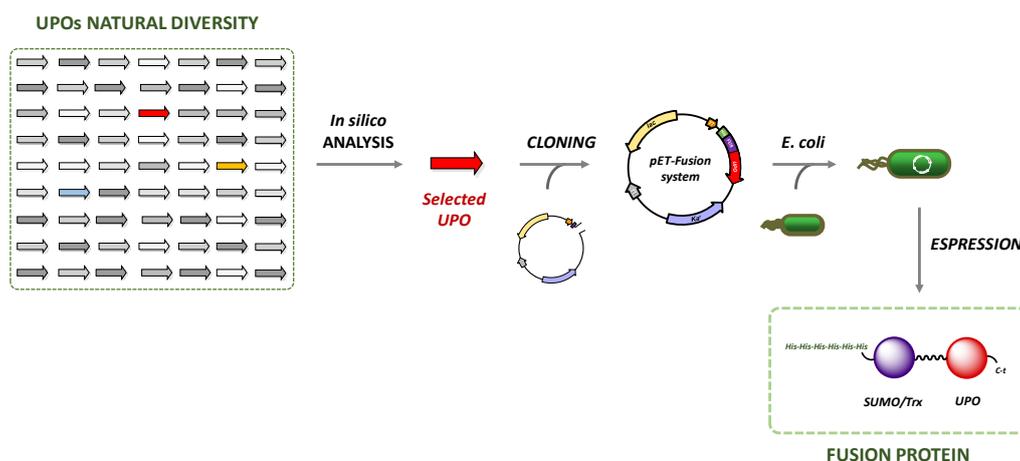
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Linker's influence on the catalytic behavior of fused microbial lipolytic enzymes

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Lipases and carboxylesterases produced by thermotolerant *Geobacillus* bacteria are industrially promising biocatalysts and can be used as models for protein engineering experiments including fundamental analysis of fused lipolytic enzymes [1]. Fused enzymes are artificial biocatalysts developed via protein engineering as a class of novel biomolecules with multifunctional properties [2]. One strategy to construct fused enzymes is end-to-end fusion, allowing the N-terminal to C-terminal linkage of two different domains. However, the close proximity of proteins to each other might sometimes result in unfavored folding, resulting in loss of activity of one or both catalytic domains. A way to avoid this problem is by the addition of linker sequences that allow extended spatial conformation and stability [3]. Nevertheless, the most effective fusion strategy and influence of added linkers on the catalytic behavior of lipolytic biocatalysts has not been explored sufficiently.

In previous studies, a fused lipolytic enzyme GDEst-lip, composed of *Geobacillus* GD-95 lipase and GDEst-95 esterase, was created [4]. The most interesting yet undiscussed question was which strategy (fusion by using flexible or rigid linker, or via unique restriction site only) is the most effective to construct chimeric lipolytic enzymes. For this reason, GDEst-lip variants fused via flexible and rigid linkers as well as in a reverse order fused variant GDLip-Est were created. All new constructed enzymes were expressed, purified and the main physicochemical and kinetic characteristics were analyzed.

After synthesis of fused lipolytic enzymes, yield of GDEst-flexible-lip lipase was very low and high level of proteolysis was detected. Due to GDEst-flexible-lip instability, the physicochemical and kinetic analysis with this variant was not performed. It was showed that GDEst-rigid-lip lipolytic enzyme displayed lower K_M and slightly higher catalytic efficiency than GDEst-lip enzyme. However, the specific activity and V_{max} of GDEst-rigid-lip and GDLip-Est were significantly lower than GDEst-lip biocatalyst. The analysis of catalytic behavior of chimeric enzymes at different temperatures showed that GDEst-lip biocatalyst possessed improved activity at 70 – 75 °C compared to GDEst-rigid-lip and at 60 – 70 °C temperature range compared to GDLip-Est. Newly constructed GDEst-rigid-lip enzyme exhibited similar thermostability as GDEst-lip lipolytic biocatalyst. Significant difference was detected only at 70 °C temperature, where GDEst-lip lipase-esterase possessed approximately 50 % of its activity instead of 20 % activity of GDEst-rigid-lip. The fusion with rigid linker improved the activity of GDEst-lip enzyme toward *p*-NP myristate and *p*-NP palmitate and in reversed order fused GDLip-Est variant possessed an improved ability to hydrolyze *p*-NP dodecanoate and *p*-NP palmitate.

This research highlighted that fusion via simple restriction site or by using a short rigid linker is a preferable fusion strategy for lipolytic enzymes compared with the fusion by using a flexible linker. However, linker's nature and domain composition in fused enzymes should be optimized for each individual case.

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New hypertransglycosylating β -N-acetylhexosaminidase variants for the synthesis of chitooligomers

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The β -N-acetylhexosaminidase (EC 3.2.1.52, GH20) from *Aspergillus oryzae* (AoHex) has previously shown a remarkable synthetic ability. However, in the wild-type enzyme the yields of the transglycosylation reactions are significantly lowered by the concurrent hydrolysis of both the substrate and products. To solve this problem, four putative transglycosidase variants of AoHex were designed, aiming at suppressing the hydrolytic activity and retaining transglycosylation activity of these enzymes. At first, two types of mutants were generated: Y445F and Y445N mutants of the active-site tyrosine residue stabilizing the oxazoline reaction intermediate, and two mutants were designed at the aglycon-binding site (F453W, V306W) introducing tryptophan residues to improve binding of the acceptor sugar in the transglycosylation reaction. The transglycosylation activity of the recombinant enzymes was tested with *p*NP-GlcNAc employed both as a donor and acceptor. All of the prepared enzymes exhibited decreased hydrolytic activity and catalyzed transglycosylation reactions with higher yields than the parent enzyme. Based on the acquired results, five combined double mutants of AoHex were prepared, four of them proved to be highly efficient transglycosidases. This is the first report on the hypertransglycosylating mutants of the aglycon-binding site of a GH20 β -N-acetylhexosaminidase able to synthesize valuable chitooligomers.

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Heterologous Expression and Engineering of the Nitrogenase Cofactor Biosynthesis Scaffold NifEN

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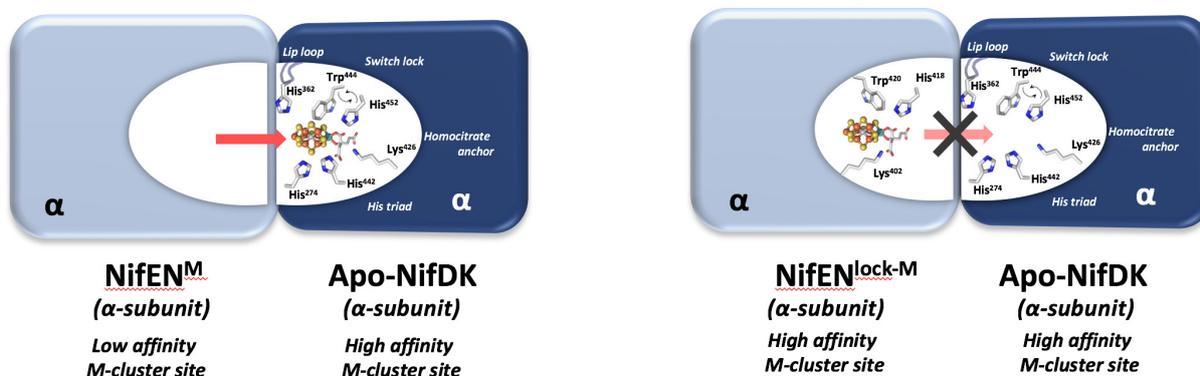
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Nitrogenase is a key player in the biogeochemical cycling of the essential nitrogen element, catalyzing the conversion of atmospheric N₂ to bioavailable NH₃ [1]. NifEN plays a crucial role in the biosynthesis of nitrogenase, catalyzing the final step of cofactor maturation prior to delivering the cofactor to NifDK, the catalytic component of nitrogenase. The fact that NifEN is highly homologous, yet clearly distinct from NifDK presents a unique opportunity to use NifEN as a template to stepwise restore the missing features and generate a functional equivalent of NifDK. Success along this line will combine the functions of both NifEN and NifDK in one engineered protein, thereby simplifying the strategies for the heterologous expression of nitrogenase by omitting the need to express NifDK.

Recently, we have reported the expression and engineering of *Azotobacter vinelandii* NifEN in *Escherichia coli* [2]. Biochemical and spectroscopic analyses demonstrate that the NifEN expressed in *E. coli* has the same subunit and metallocluster compositions as its native counterpart expressed in *A. vinelandii*, and it is fully functional in maturing the cofactor and delivering it to NifDK. More excitingly, these studies reveal that an NifEN variant engineered in *E. coli*, NifEN^{lock}, which has the cofactor “lock” mechanism of NifDK restored, retains the ability of NifEN to mature the cofactor but mimics the ability of NifDK to retain the cofactor at an analogous cofactor-binding site.



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Cross-linked enzyme crystals (CLECs) of halohydrin dehalogenase HheG as immobilized biocatalyst

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HheG from *Ilumatobacter coccineus* is highly attractive for application in biocatalysis due to its ability to accept cyclic as well as other sterically demanding epoxides as substrates in epoxide ring opening reactions [1,2]. Beside this exceptional substrate scope, HheG wild-type exhibits only low thermal stability with an apparent melting temperature of 38 °C, which is a drawback for application. Thermostabilization of HheG by protein engineering has previously been reported [3]. An alternative to protein engineering for reaching an increased enzyme stability is immobilization. Hence, we aimed to stabilize the enzyme by carrier-free immobilization in the form of cross-linked enzyme crystals (CLECs), while retaining high enzymatic activity. Due to an arginine in the catalytic triad of HheG, the commonly used glutaraldehyde was not a suitable cross-linker for CLEC formation. Instead, we aimed to use alternative, cysteine- or lysine-specific cross-linkers. Based on the crystal structure of HheG [1], several amino acid positions facing each other in the crystal interface were selected for mutagenesis to insert additional cysteine or lysine residues on the surface of the HheG homotetramer (Figure 1). Purified mutants were crystallized and cross-linked using either the cysteine-specific cross-linker bis-maleimidoethane (BMOE) or a lysine-specific cross-linker mix of different linker lengths. Resulting CLECs were studied regarding their catalytic activity as well as stability.

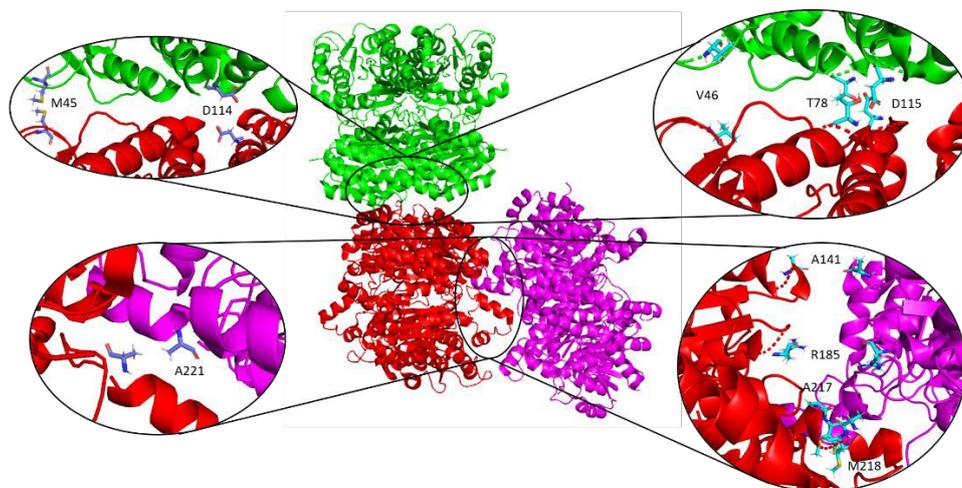


Figure 1. Overview of amino acid positions of HheG selected for mutagenesis into cysteine (left hand) or lysine (right hand) for creating new cross-linking sites.

Several mutants displayed improved crystallizability compared to wild type HheG, without negatively affecting specific activity and apparent melting temperature. Cross-linking with lysine- or cysteine-specific cross-linkers resulted in stable CLECs that retained catalytic activity. Initial tests regarding long-term stability indicated that CLECs of HheG variants could be reused in biocatalytic reactions over several weeks while retaining high residual activity.

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Rational Enhancement of Artificial Metalloenzymes by Computational Design and High-throughput Screening

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Artificial metalloenzymes (ArMs) emerged as attractive alternatives to both chemical catalysts and natural enzymes due to the great potential of protein engineering. To assemble such hybrid catalysts, an organometallic cofactor is anchored within a protein environment, thus extending the enzymatic repertoire with enhanced reaction rates, new-to-nature reactions and reaction cascades combined with improved (enantio)selectivity.¹

Meanwhile, rational and de novo design of enzymes has developed into a valuable tool. Using rational design, a protein can be fine-tuned for specific purposes and novel functions.²

This project aims at the rational design of the homotetrameric protein streptavidin (Sav) which has proven as a versatile host for ArM assembly thanks to its tight binding to biotin which can be utilized to introduce biotin-linked cofactors. The loop between beta sheets 3 and 4 of Sav was selected for rational design to shield the active site from solvent exposure and introduce favourable contacts to the cofactor (Figure 1). Such contacts can ultimately be used in directed evolution campaigns for further improvements.

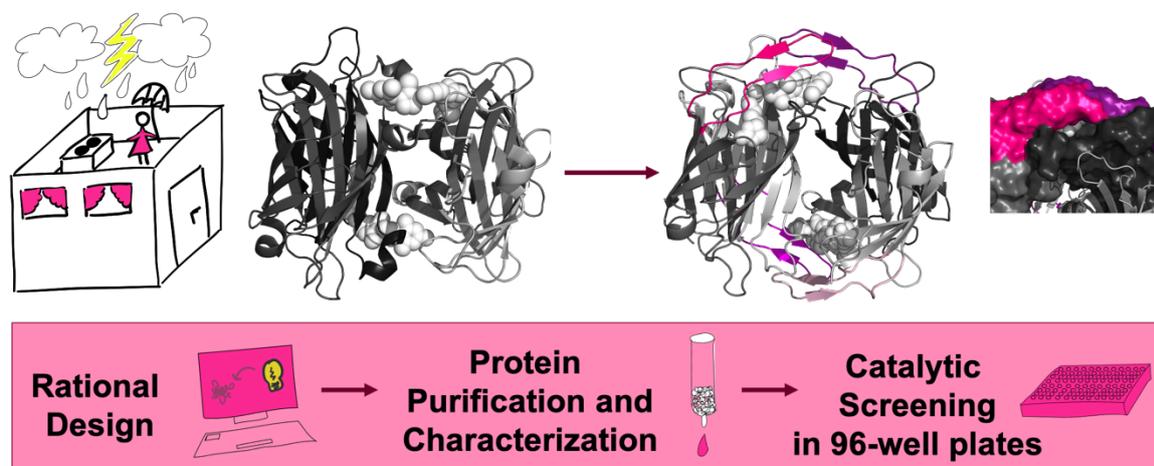


Figure 1. Rational design of Sav to shield the active site. Cooking in a house without a roof is difficult because of exposure to the environment – so is catalysis with an ArM that exposes its cofactor to the surrounding. The 3/4 loop of Sav is used to close the active site of Sav by domain grafting resulting in 135 selected variants. Rational design is followed by protein purification and characterization and subsequent high-throughput screening in 96-well plates.

The variants that arose from the rational design are being screened for three industrially-relevant reactions, namely ring closing metathesis, transfer hydrogenation and deallylation.

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Biocatalytic alkylations using an evolved halide methyltransferase

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Biocatalytic alkylations are important reactions to produce chemo-, regio- and stereoselectively alkylated products. They can be achieved by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs) with SAM analogues as alkyl donors. However, SAM analogues are rarely found in nature and difficult to synthesise.[1] It was recently reported that a halide methyltransferase (HMT) can synthesize SAM directly from *S*-adenosyl-L-homocysteine (SAH) and methyl iodide.[2] We investigated activities of HMTs for the production of SAM analogues using SAH and alkyl iodides.

Based on a sensitive colorimetric assay for the detection of iodide, a by-product of SAM analogue synthesis, we performed directed evolution of the *Arabidopsis thaliana* HMT (*At*HMT). Site-saturation libraries were created at 10 positions in the active site pocket. A single mutant, *At*HMT-V140T, had fivefold higher activity than the wild-type enzyme towards ethyl iodide.[3] The ethyl, propyl, and allyl analogues of SAM were produced on preparative scale using *At*HMT-V140T, achieving 90%, 50%, and 71% conversion of 15 mg SAH, respectively (Figure 1).[3]

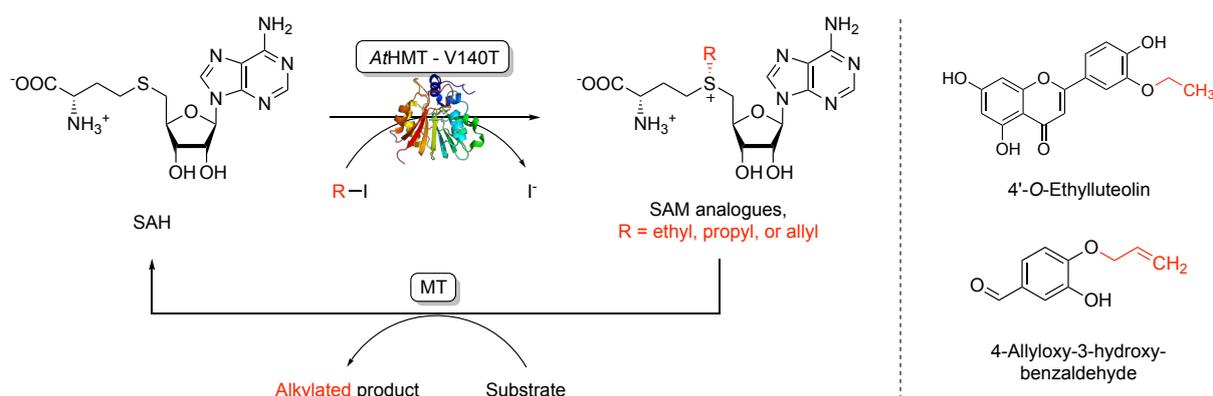


Figure 1. *At*HMT-V140T-catalysed production of SAM analogues from SAH and alkyl iodides, and subsequent MT-catalysed alkylation of substrates. Structures of the alkylated products 3'-*O*-ethyl-luteolin and 4-allyloxy-3-hydroxybenzaldehyde are shown on the right.

Combining the HMT and MTs in cascade reactions also enables direct regeneration of SAH.[2] SAM analogues produced by HMT can be used directly by MT to produce alkylated products, after which the by-product SAH is transformed back to SAM analogues by the HMT (Figure 1). The regioselectively mono-alkylated products 3'-*O*-ethyl-luteolin and 4-allyloxy-3-hydroxybenzaldehyde were produced using *At*HMT-V140T and the corresponding MTs, with 40% and 50% conversion of 20 mg luteolin and 10 mg 3,4-dihydroxybenzaldehyde, respectively (Figure 1).[3]

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Defined synthesis of cellotriose by cellobiose phosphorylase mutant

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Cellodextrins are non-digestible oligosaccharides that have attracted interest from the food industry as potential prebiotics [1,2]. They are typically produced through the partial hydrolysis of cellulose, resulting in a complex mixture of oligosaccharides with a varying degree of polymerisation (DP). In our work [3], we explored the defined bottom-up synthesis of cellotriose as a product since this oligosaccharide is believed to be the most potent prebiotic in the mixture [1]. To that end, the cellobiose phosphorylase (CBP) from *Cellulomonas uda* and the cellodextrin phosphorylase (CDP) from *Clostridium cellulosi* were evaluated as biocatalysts, starting from cellobiose and α -glucose 1-phosphate as acceptor and donor substrate, respectively. The CDP was shown to rapidly elongate the chains towards higher DPs, even after extensive mutagenesis. In contrast, an optimised variant of CBP was found to convert cellobiose to cellotriose with a molar yield of 73%. The share of cellotriose within the final soluble cellodextrin mixture (DP2-5) was 82%, resulting in a cellotriose product with the highest purity reported to date. Remarkably, the reaction could even be initiated from glucose as an acceptor substrate, which could further decrease the production costs. Further research on the affordable and environmentally sustainable enzymatic synthesis of speciality carbohydrates such as cellotriose could result in the production of healthier carbohydrates (i.e., functional foods), now more relevant than ever due to the global rise in obesity and related health problems.

Acknowledgements

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The content of this abstract reflects only the author's view; the EU-Commission is not responsible for any use that may be made of the information it contains.

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Rational design of the type B feruloyl esterase from *Aspergillus terreus*: improving its selectivity towards hydroxylated hydroxycinnamates

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Feruloyl esterases (FAEs) are a subclass of the carboxylic acid esterases, which hydrolyze the ester bond between hydroxycinnamic acid (ferulic, *p*-coumaric, caffeic and sinapic acid) and sugars present in plant cell walls. According to Crepin, feruloyl esterases are classified into four subclasses (types A, B, C and D) based on their preference to hydrolyze hydroxycinnamic methyl esters and their sequence homology [1]. FAEs can be used for the synthesis of some bioactive hydroxycinnamic esters, mostly derived from ferulic acid [2]: the most abundant hydroxycinnamic acid found in vegetables and cereal grains [3]. However, hydroxylated hydroxycinnamic esters have proved to have important biological activities such as antitumoral [4] and antioxidant [5] and thus the generation of selective biocatalysts for the production of such molecules is of industrial importance. According to Crepin *et al*, type B FAEs prefer to hydrolyze methyl esters of hydroxylated hydroxycinnamic acids (*p*-coumaric and caffeic), however, they still can accept ferulic acid as substrate, which could be a problem when a selective reaction towards hydroxylated substrates is desired. In this work, the type B FAE of *Aspergillus terreus* (AtFaeB) was genetically modified to hydrolyze selectively butyl caffeate (BC) in competition with butyl ferulate (BF). A model of AtFaeB based on the crystallographic structure of the type B FAE of *Aspergillus oryzae* (PDB: 3WMT) was built using Swiss Model and the binding cavity of the protein was analyzed. We observed two phenylalanines (Phe217 and Phe337) at each side of the substrate (Fig. 1.A), which we hypothesize could influence the enzyme selectivity towards BC and BF. A steric hindrance effect that could affect the ferulic coupling was sought by mutating the phenylalanines for tryptophans, generating the mutations F217W (Fig 1. B) and F337W (Fig 1. C). When these mutations were evaluated for the competitive hydrolysis of BC and BF, the selectivity BC/BF of the F217W mutant increased 3.8-fold compared to the native enzyme (Fig. 1. D). These results correlate with the molecular docking simulations where it can be seen that the methoxy group of the BF is oriented towards Phe217 in the native enzyme and the F337W mutant (Fig 1. A, C), however in the F217W mutant, the methoxy group is oriented towards Phe337 (Fig 1. B), probably generating a decrease of the BF hydrolytic activity.

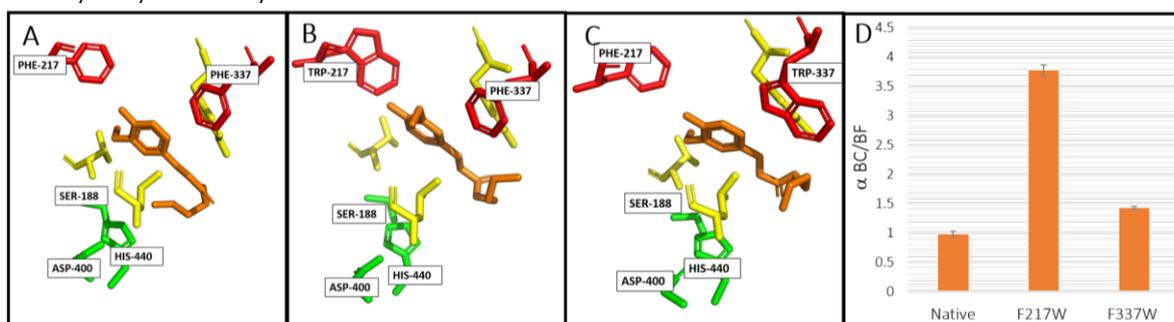


Figure 1. Site directed mutagenesis of the AtFae B. Molecular dockings of butyl ferulate and **A.** the native enzyme. **B.** F217W. **C.** F337W. **D.** Selectivity (α) of butyl caffeate (BC) over butyl ferulate (BF).

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Flourescence-based pH assay for universal measurement of enzyme activity: Application in engineering the scope of transketolase

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It is essential to develop simple and convenient assays that can enable continuous monitoring of enzyme activity with high-throughput parallelization [1]. A number of enzymes important for biocatalyst development or as drug targets are associated with a pH change during their catalytic reaction, owing to the release or uptake of protons. In this context, we have evaluated an enzyme assay (Fig. 1) employing the ratiometric fluorescent pH indicator 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) [2] as a universal system for activity determination of enzymes from multiple classes. With minor modifications in the reaction buffer and ionic strength, the assay was tunable to reliably quantify the activity of various representative enzymes, including kinases, pyruvate decarboxylase, transketolase, acetylcholinesterase, metagenomic decarboxylases and phosphoryl transferases.

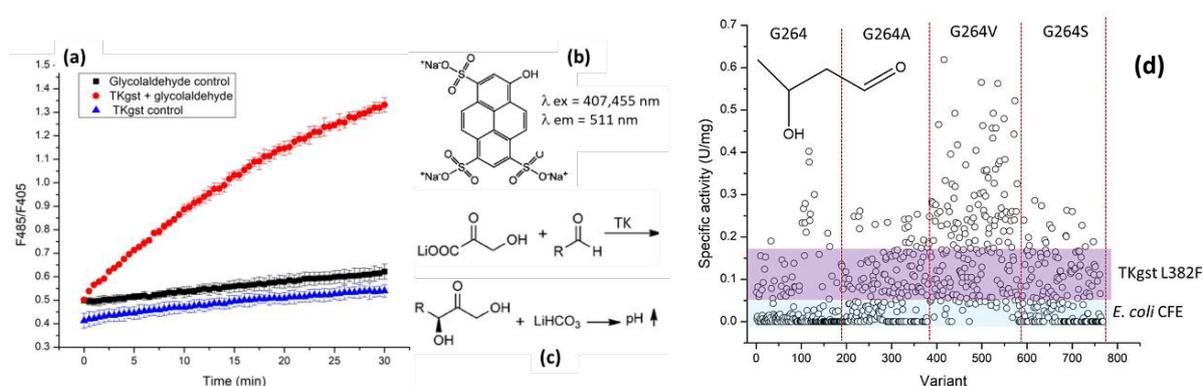


Figure 1. (a) Measurement of TK_{gst} activity with glycolaldehyde and hydroxypyruvate using the pH assay. (b) Structure and fluorescence properties of HPTS. (c) Transketolase reaction scheme. (d) Activity screening of TK_{gst} L382F/G364X/S385X variant library using 3-hydroxybutanal as substrate.

The general nature of the assay also facilitates the determination of specific activities on different substrates, with applications in initial high-throughput screen in enzyme discovery and engineering. To this end, the assay was used to screen directed evolution variants of transketolase. Transketolase catalyzes the addition of a 2-carbon ketol unit to aldehydes, and the thermostable enzyme from *Geobacillus stearothermophilus* (TK_{gst}) [3] has been modified to accept non-phosphorylated non-hydroxylated alkyl and aryl aldehyde acceptors [4]. In this study, the fluorimetric assay was employed in an attempt to engineer substrate promiscuity and remote stereoselectivity on 3-hydroxylated alkyl and aryl aldehydes (Fig. 1). The fluorimetric pH assay showed good sensitivity (LOD = 0.015-0.04 mM) and could possibly be adapted for ultrahigh-throughput microfluidic technology in biocatalytic or inhibitor screening applications.

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Direct electrochemistry of enzymes via cytochrome domains

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Third-generation biosensors are based on highly efficient direct electron transfer (DET) between enzymes to electrodes. Cellobiose dehydrogenase (CDH) is one of the few oxidoreductases capable of DET [1]. It consists of a catalytic dehydrogenase domain (DH) and an electron transferring cytochrome domain (CYT). The two domains are joined by a flexible linker of around 20 amino acids that allows the two domains to come in close contact for the internal electron transfer (IET). [2,3] One important factor to achieve efficient IET within CDH enzymes is a suitable linker that spatially mobiles the CYT to the DH domain. The lengths and amino acid compositions of these linkers in different CDHs vary to a great extent. In this study, linkers of different lengths and properties were designed and inserted into the model enzyme *Neurospora crassa* CDHIIA and compared to the wild type enzyme in regards to their IET by biochemical and electrochemical approaches. In order to study the function of the linker on the electron transfer rate between the flavodehydrogenase domain and the cytochrome domain of CDH, we constructed seven *NcCDHIIA* variants with different linker modifications, which govern the distance between both domains. Biochemical and electrochemical methods were used to study the electron transfer kinetics. We found that shortening the linker length resulted in a more drastically reduced electron transfer rates than lengthening it. Surprisingly, the IET rate in the SS+4AT (*NcCDHIIA*: *p.Ile219Cys,Asn460Cys; c.216_217insATAT*) variant is higher than the wild type CDH. The presented results provide a basis for optimizing direct electron transfer rates and for maximizing their performance in bioelectrocatalytic processes.

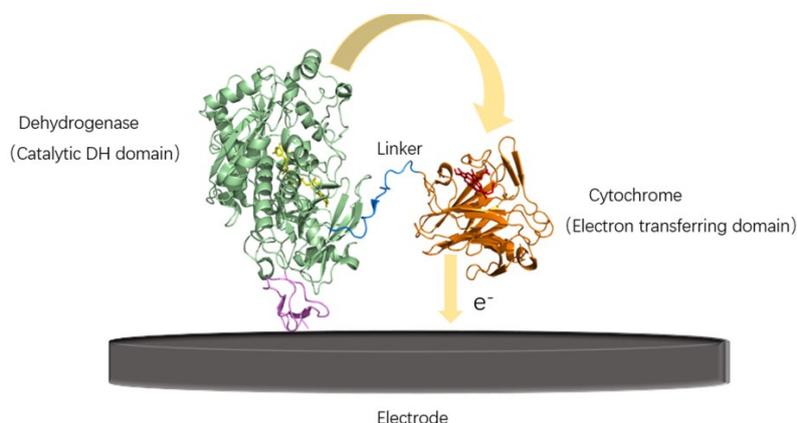


Figure 1. Direct electron transfer between *NcCDHIIA* and electrode

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Synthesis of α -glycerol glycosides by sucrose phosphorylase

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2-O- α -D-glycerol glucoside (2-O- α -D-Glu-Copyright-SN-glycerol) is a glycosidic substance connected by glycosidic bonds between glucosyl and glyceryl groups, and plays an important role in the fields of cosmetics, food, medicine, health care products and so on. The chemical synthesis of 2-O- α -D-glycerol glucoside is usually not used in industry due to its low yield and complex process. The microbial synthesis method utilizes cyanobacteria and other microorganisms to synthesize 2-O- α -D-glycerol glucoside under salt stress, and the highest yield reported so far is only about 0.3g/L, so the method still has no industrial application value. The synthesis of 2-O- α -D-glycerol glucoside catalyzed by sucrose phosphorylase has the advantages of less substrate material and simple product, but it still has the problems of long conversion time, poor stability of enzyme in vitro, easy inactivation, low catalytic activity, etc. In this study, sucrose phosphorylase was used to catalyze the synthesis of 2-O- α -D-glycerol glucoside, and the initial conversion was only about 20%. Therefore, the reaction conditions were optimized and detected by high performance liquid chromatography. The reaction temperature gradient was set as 20 , 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, the optimal reaction temperature was detected as 35°C. Set the reaction pH gradient to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, the optimal reaction pH is 10.0. The substrate conversion rate was tested after gradient reaction time 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 16 h, 24 h, the result indicated that reaction activity of sucrose phosphorylase after 12 h was the highest. The optimal conditions for catalysed reactions were 35 °C at pH 10.0 after 12 h, the reaction conversion rate was increased to 50%. The purpose of effective utilization of the enzyme was achieved, thus improving the yield of 2-O- α -D-glycerol glucoside.

Mechanistic implications of oxygen uncoupling in two Rieske non-heme iron dioxigenases

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Rieske non-heme iron dioxigenases (RDOs) are the key enzymes responsible for initial steps of aerobic biotransformation of numerous persistent aromatic contaminants in the environment. Knowledge of the catalytic mechanism, substrate specificity, and activity of RDOs enables one to assess which environmental contaminant can be fed into common metabolic pathways through dihydroxylation steps leading to *cis*-dihydrodiols and catechol-type products. However, while the strategies of O₂ activation and control of reactive Fe-oxygen species of many mononuclear non-heme iron oxygenases have been studied in detail, the efficiency of substrate oxygenation is largely unknown. Current hypotheses suggest that RDO substrates other than the native ones are hydroxylated only poorly and thus give rise to so-called O₂ uncoupling and concomitant formation of reactive oxygen species [1]. Given that RDO-expressing microorganisms are exposed to complex mixtures of structurally similar aromatic compounds at contaminated sites, O₂ uncoupling could even be the predominant outcome of enzymatic activity.

In this study, we explored the relevance of O₂ uncoupling pathways of RDOs as well as its implications for the kinetics and mechanisms of O₂ activation and substrate oxygenation. We studied two closely related nitroarene dioxigenases, namely nitrobenzene dioxigenase (NBDO) and 2-nitrotoluene dioxigenase (2NTDO) with a wide range of substrates [2,3]. Substrate-specific uncoupling was observed through measurements of O₂ consumption relative to product formation in purified enzyme assays and revealed an extent of O₂ uncoupling of 30% to 100% of the activated O₂. The efficiency of oxygenation by NBDO showed preference for *meta*-substituted nitroarene substrates as opposed to *ortho*-substituted substrates for 2NTDO. Conversely, ¹⁸O kinetic isotope effects (KIEs) used for characterization of reactive Fe-oxygen species were between 1.015 and 1.025 which is indicative of Fe(III)-peroxo species formation and lacked any substrate specificity. ¹³C KIEs of substrate hydroxylation correlated with the extent of O₂ uncoupling of NBDO whereas these numbers were generally close to unity for 2NTDO. Our observations suggest catalytic mechanisms of RDOs in which the timing of O₂ uncoupling and release of seemingly unreacted substrate are both substrate- and enzyme-specific. The high share of unproductive O₂ activation challenges the widely made assumption of enzyme evolution towards an efficient oxygenation of preferred substrates.

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The Heptagonal Box: fingerprints for functional annotation and enzyme engineering of NDP-sugar active SDR enzymes

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Short-chain Dehydrogenase/Reductase enzymes that are active on nucleotide sugars (abbreviated as NS-SDR) are of paramount importance in the biosynthesis of rare sugars and glycosides. Some family members have already been extensively characterized due to their direct implication in metabolic disorders or in the biosynthesis of virulence factors. Here, we present an in-depth analysis of all of the different NS-SDR families (169,076 enzyme sequences). Through structure-based multiple sequence alignment of enzymes retrieved from public databases, we identified clear patterns in conservation and correlation of crucial residues for each reactivity (epimerization, dehydration, reduction, decarboxylation) and known sugar specificity (e.g., UDP-galactose 4-epimerase, dTDP-glucose 4,6-dehydratase, UDP-glucuronate decarboxylase). This analysis resulted in a specificity model in which seven conserved regions surrounding the NDP-sugar substrate serve as fingerprint for each specificity: the heptagonal box. Consequently, this model will be beneficial for functional annotation of the large group of NS-SDR enzymes and form a guide for future enzyme engineering efforts focused on the biosynthesis of rare and specialty carbohydrates. To illustrate its applicability, first examples of enzyme discovery, functional annotation and engineering guided by the heptagonal box will be presented.

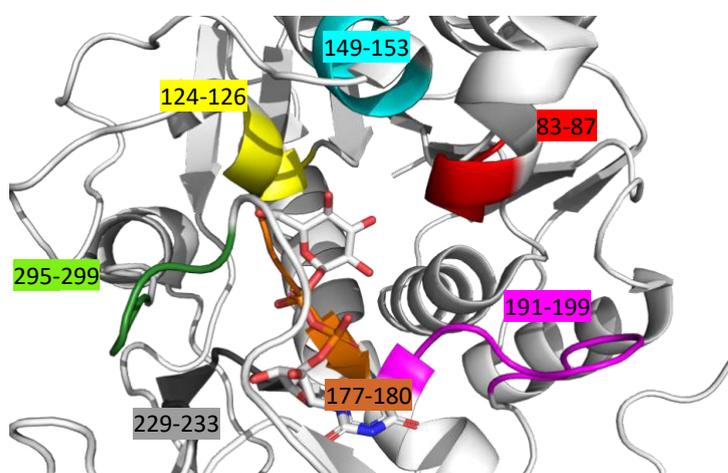


Figure 1. Heptagonal Box in NS-SDRs. Each wall of the box contains a range of conserved residues

The catalytic machinery of the FAD-dependent *At*BBE-like protein 15 for alcohol oxidation

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Monoglignol oxidoreductases are members of the berberine bridge enzyme-like (BBE-like) protein family (pfam 08031), they are flavin dependent enzymes that follow the PCMH- also known as VAO-topology [1]. Recently, we have published the structural and biochemical characterization of two monoglignol oxidoreductases from *Arabidopsis thaliana* [2]. We have now conducted a site directed mutagenesis study for one of the enzymes, *At*BBE-like 15, from the results we propose that the reaction is facilitated by the deprotonation of the allylic alcohol prior to the hydride transfer from the C α -atom of the alcohol to the flavin. Tyr479 and Tyr193 are proposed to act concertedly as catalytic base to facilitate the proton abstraction. Lys436 is indirectly involved in the deprotonation as this residue determines the position of Tyr193 via a cation- π interaction. Additionally, Tyr117, Gln438, Asn411 and Arg292 form a hydrophilic cavity to accommodate the alkoxide intermediate and the transition state. The respective mechanism is shown in Figure 1.

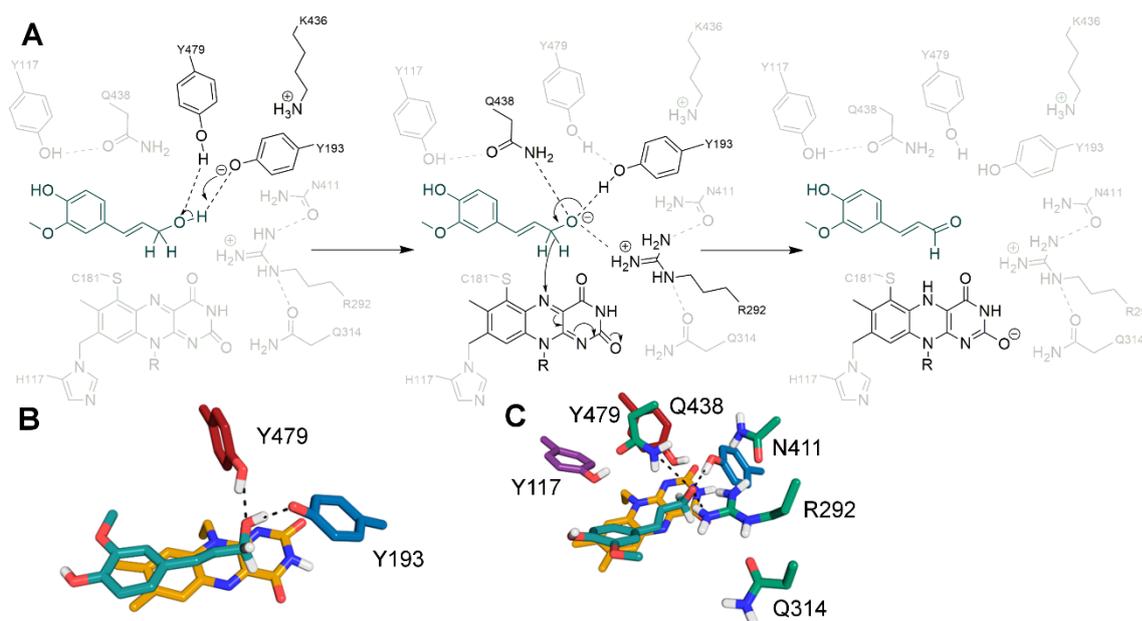


Figure 1: Reaction mechanism of the alcohol oxidation of *At*BBE-like protein 15; A: Schematic reaction mechanism, B: Binding mode alcohol, C: Binding mode alkoxide

The alkoxide binding residues Gln438 and Arg292 were found to be highly conserved, while the proton abstraction can also be realized via a Glu or Asp positioned corresponding to Asn411 in combination Tyr479. We propose that due to the high degree of conservation the presented reaction mechanism can be anticipated as universal for BBE-like proteins oxidizing alcohols.

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Investigation of the dual function of a GH30_7 xylanase through structural studies

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Plant cell-wall polysaccharides are abundant in nature and can be utilized as a source to produce sustainable fine chemicals and biofuels. Xylan, the main hemicellulosic component of plant biomass, is considered a major source of renewable carbon and can be degraded with the assistance of xylanolytic enzymes. The most important among them are endo- β -1,4-xylanases, which are glycoside hydrolases (GHs) that depolymerize the polysaccharide into smaller fragments. Fungal xylanases belonging to family GH30_7, were initially categorized as endo-glucuronoxylanases. However, recent studies have shown GH30_7 family includes members that differ both in terms of substrate specificity, as well as mode of action [1]. Recently, *TtXyn30A*, a GH30_7 xylanase from *Thermothelomyces thermophila*, was shown to possess dual activity, acting on the xylan backbone in both endo- and exo- manner [2]. In an effort to identify the structural characteristics that append these functional properties to the enzyme, we have solved the crystal structure of the enzyme, alone, and in complex with the reaction product. Co-crystallization experiments, using *TtXyn30A* inactive mutants and various substrates are underway. Our aim is to observe any structural rearrangements in the enzyme active site, that could explain its double functionality. This knowledge could contribute in considerably the effort of designing biocatalysts that can be implemented in biotechnological processes that involve biomass exploitation.

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Characterization of fungal cytochrome P450 reductases to potentially expand class II cytochrome P450 catalysis

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Cytochrome P450 reductases (CPR) are diflavin oxidoreductases.[1] This family of enzymes transfers reducing equivalents from NAD(P)H to different protein acceptors such as class II cytochrome P450 monooxygenases (CYP), haem oxygenases as well as cytochrome *b5*. [2] CPRs regulate reaction velocities and product specificities with different efficiencies. Generally, the characterization of CPRs is achieved through reduction assays with non-physiological electron acceptors such as cytochrome *c* and ferricyanide.[3]

Previous work showed that the truncation of the N-terminal anchor of a CPR from *Candida tropicalis* facilitated soluble expression in *Escherichia coli* while retaining its reducing activities.[4] In this study, we characterized two novel Cytochrome P450 reductases from *Cystobasidium minutum* and *Ustilago maydis*. Evolutionary relatedness obtained through amino acid sequence alignment showed conserved key motifs.

Truncated versions of the enzymes were recombinantly produced in *E. coli*, purified, and their kinetic parameters for the reduction of cytochrome *c* were determined (Figure 1). As expected, the kinetic properties determined were comparable to CPRs across all domains of life.[4-8] In addition, we evaluated their capability to support CYP activity.

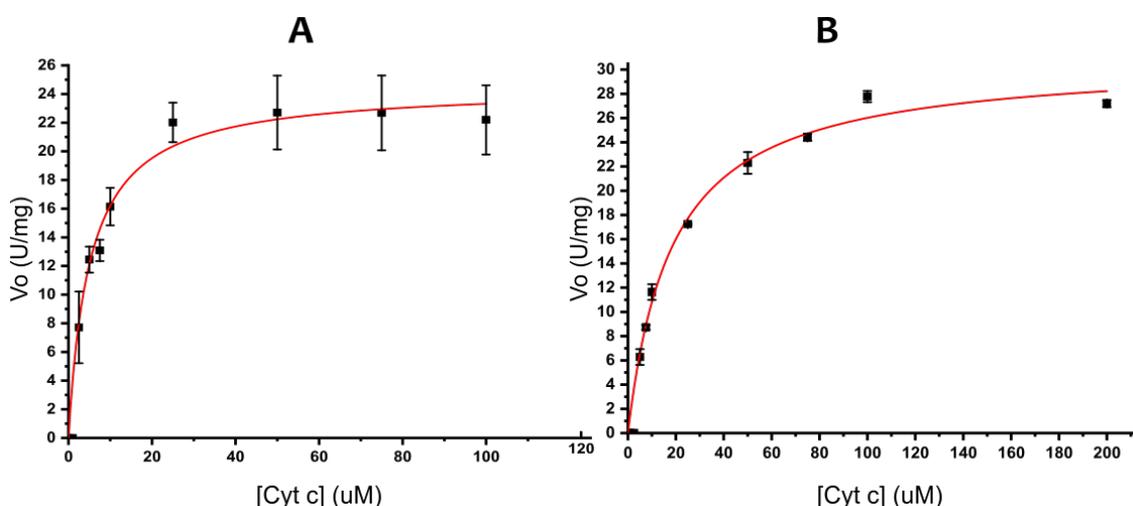


Figure 1: Michaelis-Menten plot to determine the kinetic parameters of cytochrome *c* reduction by truncated *U. maydis* CPR (A) and *C. minutum* CPR (B).

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Class II Pyruvate aldolases: Promiscuity and Selectivity

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Aldolases are grouped based on their strict donor specificity. To expand the use of these versatile enzymes it is essential to identify aldolases that utilize different donors. Recently a number of pyruvate aldolases that accept hydroxypyruvate was identified [1]. The class II hydroxy ketoacid aldolase from *Sphingomonas wittichii* RW1 (SwHKA) accepts hydroxypyruvate and was expressed for detailed studies. This revealed a phosphate dependence of the enzyme and a preference for Mn (II) [2]. A thorough investigation including X-ray crystallography allowed new insights into the mechanistic details. F210 enables the enzyme to accept other donors than pyruvate. SwHKA is thus promiscuous for the donor, it accepts pyruvate, hydroxypyruvate and the halopyruvates as donor molecules. This expands the range of products that can be synthesized with aldolases. Overall SwHKA enables with its promiscuous properties a significant extension of the aldolase toolbox (Figure 1). Surprisingly it was found that the metal ion shifts between two positions in the active site, depending on the presence or absence of the donor molecule. This unusual metal mobility will be discussed in detail.

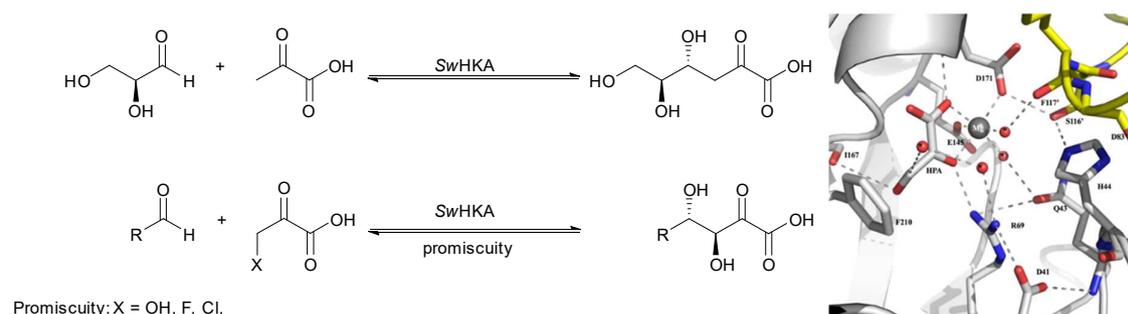


Figure 1. SwHKA is a promiscuous aldolase that accepts a range of donors and acceptors.

Interestingly, the substituted pyruvates yield products with not just one but two new stereocentres. Therefore the diastereoselectivity was studied. This revealed a general rule for the diastereoselectivity of catalysts: Thermodynamics rule the diastereoselectivity in reversible reactions [3].

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Regulation of the catalytic behavior of (*S*)-selective amine transaminases through interactions with graphene oxide

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Amine transaminases (ATAs) are a class of PLP-dependent enzymes that attracted significant industrial interest over the last years [1]. Their excellent enantioselectivity places them among the best routes towards the synthesis of optically pure amines. For their industrial application, ATAs require operational and thermal stability. Enzyme immobilization can be used as a stabilization technique in such cases, while it also allows the reuse of the enzymes. Nanomaterials is a class of materials of significant interest for this application, due to their high surface area which can possibly lead to high enzyme loading, while their surface can be modified. Graphene Oxide (GO) is a 2D hydrophilic derivative of graphene that has been used as an immobilization matrix, due to its high surface area and the many functional groups present on its surface (i.e. epoxides, hydroxyls, carbonyls). Despite its use as support for immobilization for more robust enzymes [2], the application with ATAs can be challenging. ATAs are homodimers, with the active site localized in the interface of the two subunits, and so far there are scarce or no data about the effect of layered nanomaterials on their activity and on the structure of the enzyme.

In this framework, and to understand the interactions manifesting the non-covalent immobilization of ATAs onto GO, we studied the effect of GO on the activity, stability and structure of 6 (*S*)-selective ATAs, namely ATAs from *Ruegeria* sp. TM1040, *Vibrio fluvialis*, *Chromobacterium violaceum* and *Sagittula stellata* E-37. The specific activity of all ATAs in the kinetic resolution of 1-phenylethylamine using pyruvate as amine acceptor was determined, in the absence and in the presence of GO at various concentrations [3]. The effect varies for the different ATAs; while the ATAs from *Ruegeria* sp. were activated by the presence of nanomaterials, the ATA from *C. violaceum* was significantly deactivated. The effect seems to be triggered even from small amounts of nanomaterials, something that seems related to the high surface area of GO. The structural changes were evaluated with circular dichroism at far UV and the melting temperature (T_M) was calculated based on the ellipticity at 222 nm. Again, the presence of the nanomaterial seems to affect the structural stability of the ATAs, however the effect is not universal. These results indicate that the immobilization of ATAs onto GO and related materials can be an interesting approach to facilitate the reuse of the enzyme and improve their performance, however, a thorough characterization of the ATA of interest with the GO.

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The research project was supported by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the "1st Call for H.F.R.I. Research Projects to support Faculty Members & Researchers and the Procurement of High- and the procurement of high-cost research equipment grant" (Project Number:664).

Core Facility for Crystallographic and Biophysical Research to support the development of medicinal products

Jan Kutner*, Maria Górna, Maura Malińska, Monika Wanat, Daria Dawidziak, Katarzyna Polak, Mikołaj Kuska, Weronika Lidwin, Marlena Kisiała, Szymon Sutula, Krzysztof Woźniak*

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As a result of TEAM-TECH Core Facility Project from the Foundation for Polish Science, we have established the Core Facility for Crystallography and Biophysics (CFCB) at the Biological and Chemical Research Centre, University of Warsaw, under the supervision of Professor Krzysztof Woźniak (Head) and Jan Kutner, Ph.D. (Deputy Manager).

The mission of the Core Facility is focused on the analysis of proteins and small molecule compounds leading to crystallization trials for academic and commercial users. The project enables studies of challenging biochemical and pharmaceutical problems, with emphasis on drug development. Research at CFCB is carried out in an interdisciplinary way, including both wet biology ("BIO") and chemical crystallography ("CHEM") techniques as well as theoretical approaches including structure modelling, bioinformatics and computational methods. Biology and chemistry team members work in synergy complementing their knowledge, skills and experience. Apart from services and collaborations, postdoctoral and Ph.D. researchers carry out their research projects dedicated either to small-molecule or protein crystallography.

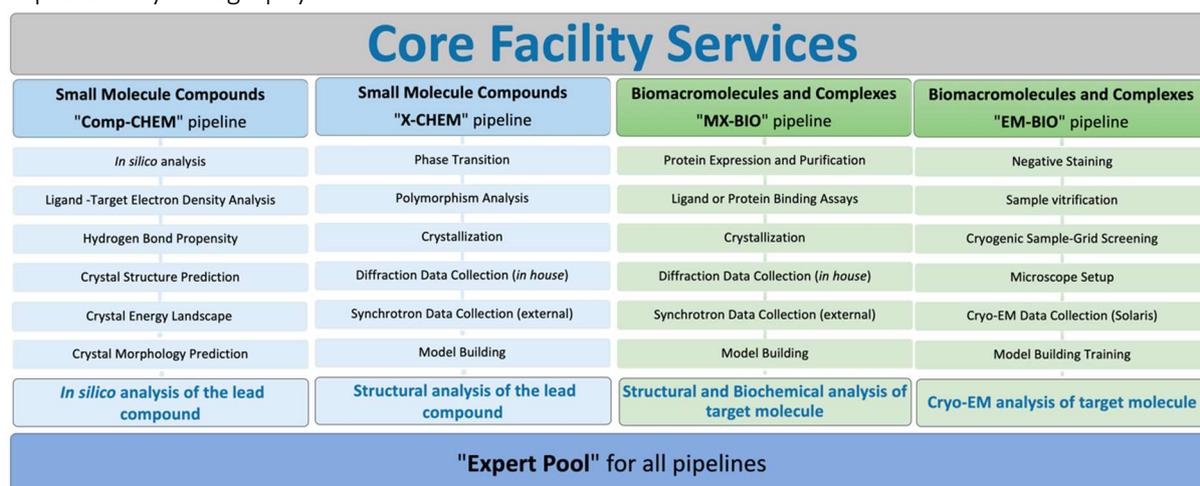


Figure 1. The main pipelines of the CFCB

Work in the Facility includes collaboration with other research groups and biotech/pharmaceutical companies, such as the WPD Pharmaceuticals, Cellis, Leaderna Biostructures, OncoArendi Therapeutics, Pikralida, Bio-Rad and Innvigo.

Moreover, we cooperate with Dr. Sebastian Glatt and Dr. Przemysław Grudnik (Structural Biology Core Facility, Jagiellonian University, Cracow) under the TT CF extension concerning on the commercial aspects (The Integrative Platform for Accelerated Drug Discovery – IPADD).

We are open to different forms of collaborations with individual researchers, research groups or biotech/pharma companies.

Acknowledgments

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Structure-activity relationships for glycosylations by bacterial and plant O-glycosyltransferases

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Glycosyltransferases catalyse the glycosylation of different molecules in many bacteria and plants leading to a structural diversity of compounds, but also to novel physicochemical and biological properties. In clinical research, glycosylation is used to increase the pharmacological activity of some drugs and enable an easier administration to the patient by making them more hydrophilic. [1,2]

Bacterial and plant O-glycosyltransferases catalyse the formation of glycosidic bonds between activated nucleotide sugars, such as UDP-glucose, and a suitable acceptor substrate with a nucleophilic oxygen mostly from a hydroxyl substituent. [2,3] The bacterial oleandomycin glycosyltransferase OleD wild type and its triple mutant PSA and the plant glycosyltransferase UGT71E5 are known to O-glycosylate a broad variety of organic substances such as steroids, peptide antibiotics and cofactors. Their high substrate promiscuity is easily explained by their structure in the form of a GT-B fold. The two domains of this fold are loosely associated and adopt two $\beta/\alpha/\beta$ Rossmann-like folds with the active site located in the resulting cleft. [2,3,4] Yet little is known about their glycosylation of ortho- and para-phenol derivatives with different pK_a values and the dependence of enzyme activity and specificity on the pK_a value. To identify a possible relationship between enzyme k_{cat}/K_M and acceptor substrate pK_a , we used a range of ortho- and para-phenol derivatives with different pK_a values as acceptors and UDP-glucose as donor substrate.

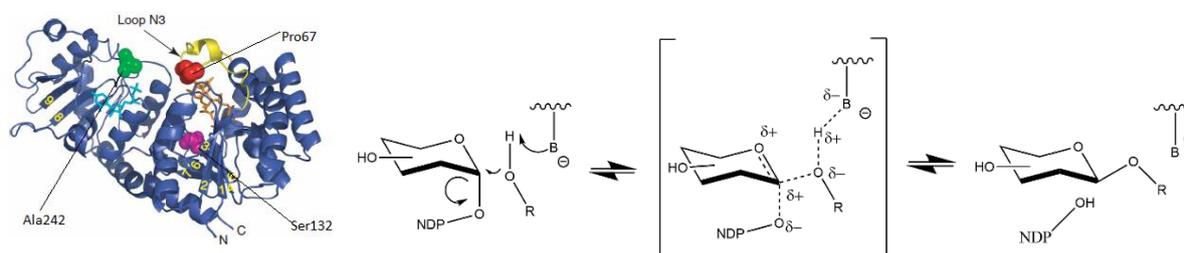


Figure 1. Enzyme structure of OleD mutant with its three mutated amino acids. [1] **Figure 2.** Proposed reaction mechanism of O-glycosyltransferases. [5]

O-glycosyltransferases show a great diversity of mechanisms for transfer due to their loose configuration of the two domains. The common proposed reaction mechanism of O-glycosyltransferases involves a S_N2 -type reaction with a single displacement step and the formation of an oxocarbenium ion-like transition state. A catalytic base residue in the active site of the O-GTs, such as Histidine, increases the nucleophilicity of the acceptor-atom attacking to generate the linkage. Leaving group departure is facilitated metal-ion independent. The overall negative charge developed in the reaction is stabilized by helix dipole and interactions with side chain hydroxyl and imidazole groups. [4]

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Activation and cosubstrate utilisation of a fungal lytic polysaccharide monooxygenase

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Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent oxidoreductases that use an oxidative mechanism to decrystallise organic polymers [1]. These enzymes hold great promise to improve the saccharification of plant biomass in biorefinery settings. LPMOs require a suitable reducing agent and an oxygen-containing cosubstrate for activity [2,3]. Recent kinetic studies show that LPMOs can also utilise H₂O₂ as cosubstrate, resulting in a highly efficient peroxygenase reaction [4]. Using a turbidimetric activity assay, we probed the peroxygenase reactivity of LPMO9C from the model fungus *Neurospora crassa* [5]. Employing various systems for the *in situ* generation of H₂O₂, we demonstrate that the supply of H₂O₂ is the limiting factor for the depolymerisation activity of the enzyme.

Furthermore, stopped-flow and EPR spectroscopy were used to study the activation and cosubstrate utilisation of LPMO9C during catalysis [6]. Our data provide clear evidence that the LPMO required only a single 'priming' electron transfer reaction, which supported up to 20 H₂O₂-driven catalytic cycles. Additionally, we observed that the presence of substrate stabilised the Cu⁺ state of LPMO9C and prevented uncoupling reactions that could otherwise damage the enzyme. In conclusion, these data provide further insights into the reaction mechanism of LPMOs and support the function of H₂O₂ as their relevant cosubstrate.

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Structural analysis and reaction mechanism of kievitone hydratase from *Nectria haematococca*

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Tertiary alcohols are required for the production of pharmaceutical compounds, including the antiviral compound efavirenz or intermediates for the semisynthetic production of taxol.[1] Their synthesis, however, is still a difficult task due to issues such as low yields, poor selectivity or harsh reaction conditions. Therefore, biocatalytic processes are a good alternative, when inefficient, expensive or environmentally unsafe methods need to be avoided. Currently, enzymes from the class of hydrolases are used for the production of tertiary alcohols, such as esterases, proteases, lipases and epoxide hydrolases.[1] Alternative enzymes that have received increasing attention of both academic and industrial research are oleate hydratases and kievitone hydratases.[2,3,4] Kievitone hydratases (KHS) are found in fungal pathogens, where they catalyze the detoxification of the plant phytoalexin kievitone (KV) by hydrating the carbon-carbon double bond, forming the less toxic tertiary alcohol hydroxy-kievitone (HO-KV).

The KHS from *Nectria haematococca* (Nha-KHS) was expressed in *Pichia pastoris* (*Komatagaella phaffii*) and its activity was confirmed by detection of HO-KV in the supernatant with HPLC-MS.[5] In order to obtain functional information on this hydratase, we determined its crystal structure to 1.5 Å resolution. Structural analyses supported by docking calculations with both stereochemical variants of KV pinpointed putative residues relevant for the activity. Mutagenesis experiments underlined the importance of certain amino acid residues and allowed – in combination with the structural results – a proposal of a reaction mechanism. In addition, we compare this mechanism with mechanism proposed for other hydratases, which are able to hydrate C-C double bonds in unpolar substrates.

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Structure and Mechanism of Tyrosine Phenol-lyase

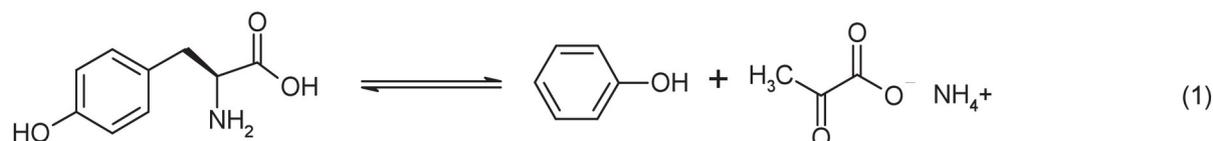
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Tyrosine phenol-lyase (TPL) catalyzes the reversible pyridoxal-5'-phosphate (PLP) dependent elimination of L-tyrosine to give phenol and ammonium pyruvate (Eqn. 1).



TPL has been used to prepare a number of analogues of L-tyrosine with pharmaceutical and biochemical applications [1-3]. We have recently obtained x-ray crystal structures of wild-type and mutant TPL with bound substrates and inhibitors [4, 5]. The resulting structures include all of the proposed *gem*-diamine, aldimine, quinonoid, and aminoacrylate intermediate complexes (Figure 1). The rate of formation of the aminoacrylate intermediate from L-tyrosine shows nonlinear temperature and pressure dependencies, suggesting that the enzyme motion is coupled with the C-C bond cleavage step [5]. These structures will aid in the future design of mutant TPL with broad substrate specificity and enhanced catalytic activity.

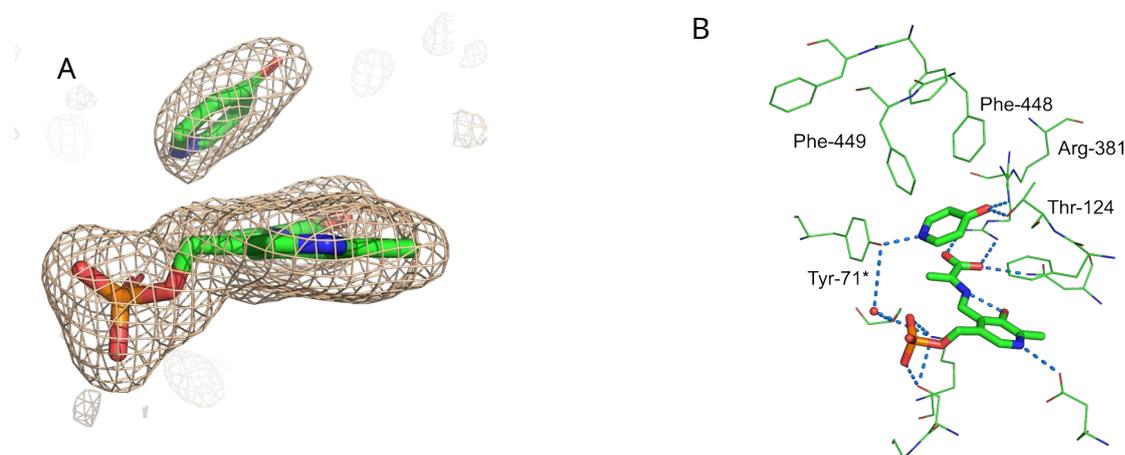


Figure 1. **A.** Sim omit mFo-DFc electron density map at 3σ of the TPL aminoacrylate-4-hydroxypyridine complex. **B.** Hydrogen bonding contacts of the TPL aminoacrylate-4-hydroxypyridine complex.

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Old Yellow Enzymes as oxime reductases: investigating the reaction mechanism with crystallography and bioinformatic.

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Old Yellow Enzymes (OYEs) are a family of ene reductases, reducing C-C double bonds in α,β -unsaturated compounds bearing an electron-withdrawing group [1]. It has been recently showed that oximes can be reduced by OYEs to the relative amines. Finally, the amines spontaneously dimerise and oxidise to a tetrasubstituted pyrazine [2]. Although the biotransformation results successful, several aspects are still unknown, including the reaction mechanism of the enzymatic reduction and the reduced acceptance of substrates with R₁ chain larger than a methyl group.

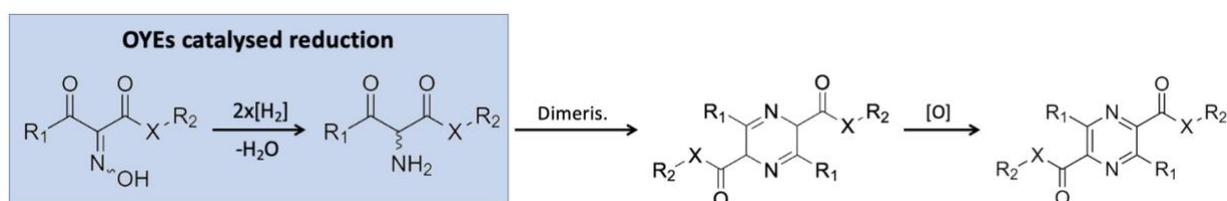
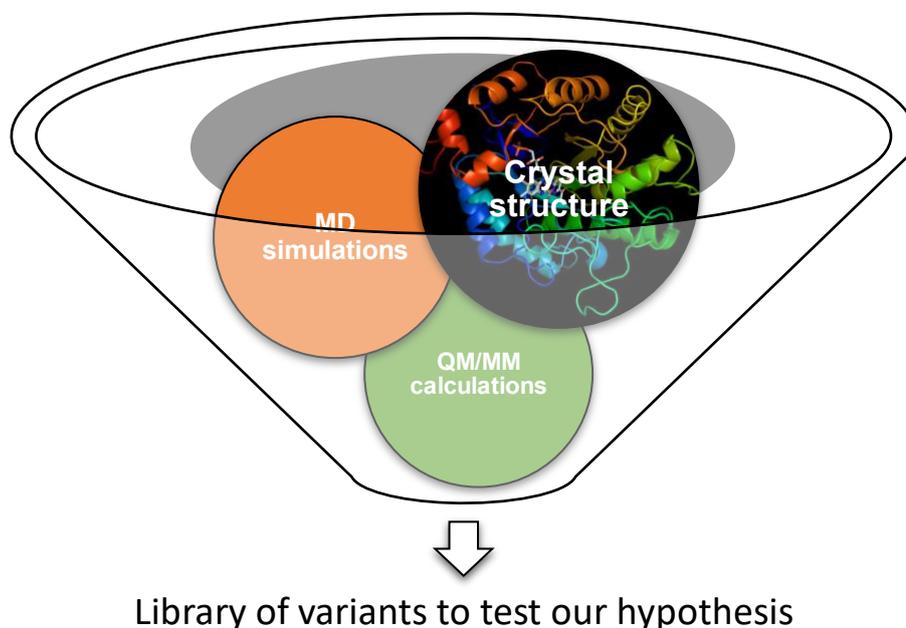


Figure 1. Biotransformation of oximes to tetrasubstituted pyrazines.

In order to investigate the possible reaction mechanism and improve the knowledge about this reaction with non-natural substrates, we worked to obtain the **crystal structure** of two of the investigated OYEs (**OPR3**, **XenA**) in complex with three of these substrates. We finally combined the crystallographic information with **MD simulations** and **QM/MM calculations** to propose a reaction mechanism and possible strategies to expand the substrate acceptance.



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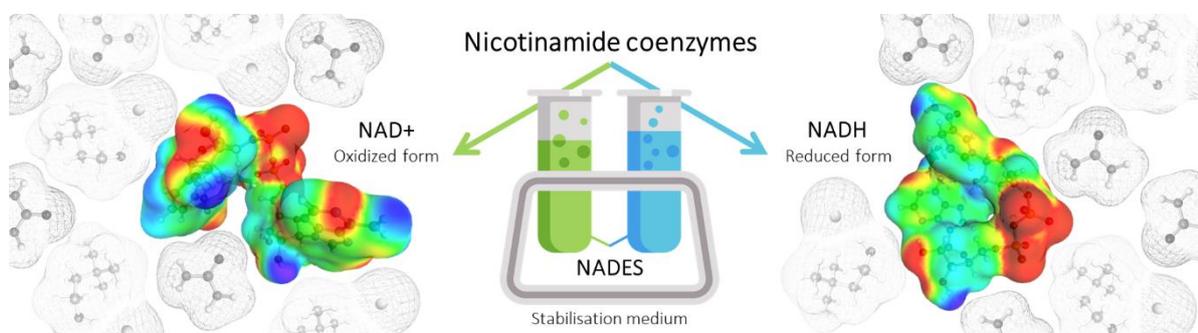
Natural Deep Eutectic Solvents (NADES) as a stabilising medium for nicotinamide coenzymes

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Coenzymes are an indispensable part of oxidoreductase-catalyzed reactions, with nicotinamide coenzymes NAD⁺ and NADH being most frequently present. They have found increasing application in biocatalysis, especially in the reduction of prochiral compounds. When designing such processes, the major drawbacks are the high coenzyme price, its instability in aqueous solution, pH dependence and recycling.[1] Because coenzymes are known for their labile nature and short-term stability, new and fresh stock solutions need to be prepared prior to the reaction. Therefore, finding a solvent that could simultaneously stabilise both nicotinamide coenzymes in oxidoreductive biocatalytic reactions and minimize their degradation during storage could be of great significance.



In the last decade, new natural deep eutectic solvents (NADES) have arisen as a promising tool to shape already green biocatalysis into an even more ecological and sustainable process. NADES are solvents with valuable characteristics such as reduced emissions and toxicity, low volatility, biodegradability, but most importantly easy tunability. Their structural flexibility offers a possibility for rational solvent design to fulfill specific purposes and industrial requests.[2] One of their applications has also been shown for solubilisation and stabilisation of a wide range of biomolecules.[3]

In this work, NADES were investigated as a stabilisation medium for nicotinamide coenzymes, NAD⁺ as an oxidized and NADH as a reduced form. Results showed that coenzyme stabilisation strongly depends on the hydrogen bond donor nature and water content. Cholinium-based NADES containing amide urea as hydrogen bond donor, stabilise both coenzyme forms to a much greater extent than reference buffers. The observed stabilisation effect is significant considering that these two coenzyme forms prefer media with different pH values. Moreover, long term stability of the coenzymes within studied NADES up to 50 days was confirmed. Several approaches were investigated to better understand the coenzyme stabilisation phenomena in NADES by implementing UV/VIS spectroscopy, NMR analysis and molecular dynamics simulations.

This discovery can open a new chapter in coenzyme recycling and reuse during continuous biocatalytic reactions and most importantly, it could solve coenzyme storage shortcomings.

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Unraveling mechanistic similarities of sesquiterpene cyclases PenA, Omp6/7, and BcBOT2 by a non-natural FPP ether derivative

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The sesquiterpene cyclases (STCs) pentalenene synthase (PenA) from *Streptomyces exfoliatus* UC5319 and two fungal Δ^6 -protoilludene synthases Omp6 and Omp7 from *Omphalotus olearius* are capable of converting a non-natural FPP ether derivative into several new tetrahydrofuran-bearing terpenoids, one of which is also formed as the main product by the presilphiperfolan-8 β -ol synthase (BcBOT2). [1,2] These results show that the three STCs PenA, Omp6/7 and BcBOT2 follow closely related catalytic pathways and induce similar folding of their diphosphate substrates despite low amino acid sequence similarities. Some of the new terpenoids show pronounced olfactive properties. Finally, first derivatizations with one of the isolated products show how preparative biotransformations using STCs can be advantageously combined with semisynthesis, thus extending the accessibility to structural diversity of non-natural terpenoids. [3]

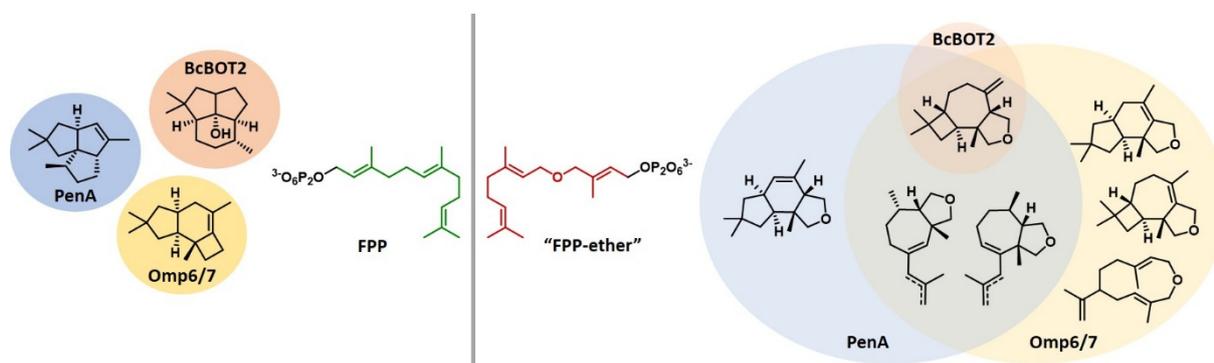


Figure 1. Natural products of PenA, Omp6/7 and BcBOT2 and the resulting non-natural terpenoids from biotransformation of "FPP-ether".

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Study of immobilization in different materials for assembling a heterogenous biocatalyst for non-selective oxidation of diols

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Industrial application of free enzyme faces several difficulties in operational stability and long-term use. To solve those problems, enzyme immobilization let us generate a robust heterogenous biocatalyst which can work on both batch and flow reactions, easy-recovery and maintain enzymatic structure [1]. Despite a wide range of enzymes have successfully been immobilized, enhancing their capabilities, the effects of the immobilization on structures remains to be further investigated. Despite being prove effectively the co-immobilization of several enzymes in one pot to perform a multi-step reaction [2] several aspects involving enzymatic immobilization remains to be elucidate.

Some of the areas that can give us clues for selecting the appropriate chemistry and material for enzymatic immobilization are basic parameters (thermal stability, spatial distribution and conformational changes), kinetic parameters and spatial orientation of the selected enzyme on the surface of the material.

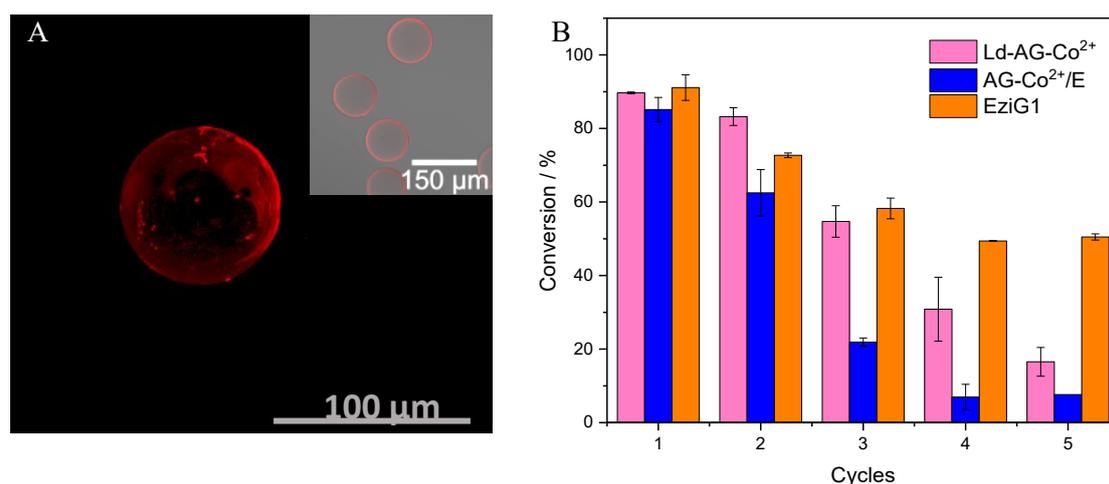


Figure 1. A: Confocal image of spatial distribution of labelled with Rhodamine BsADH. B: Operational stability of the selected biocatalyst in non-selective oxidation of the 1,5-Pentanediol.

Hence, we present the study of the immobilization of NAD⁺-dependent alcohol dehydrogenases from bacillus stearothermophilus. (BsADH) attached reversible and irreversible way. Firstly, three of all carriers tested were selected under thermal stability, distribution, and immobilization parameters, putting them to face an operational stability study in a non-selective oxidation of a selected diol. Secondly, we studied the kinetic parameters of the reaction of both substrates (diol and Nicotinamide cofactor, NAD⁺) for the selected carriers. And finally, we realized proteomic studies of three biocatalysts, and we compared the results with computational data to elucidate spatial orientation of BsADH on the surface of materials.

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Natural variation in the 'control loop' of BVMO_{AFL210} and its influence on regioselectivity and sulfoxidation

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Baeyer-Villiger monooxygenases (BVMOs) are flavoenzymes that use NADPH and molecular oxygen to convert ketones and cyclic ketones to esters and lactones, respectively, as well as catalyse sulfoxidations. The structures of Type I BVMOs have been extensively studied, indicating that these flexible enzymes undergo large domain movements during catalysis [2,3]. Due to this malleability, BVMOs often accept an extensive range of substrates. In the active site, structure transitions of the 'control loop' play a crucial role in the positioning of NADP(H) and substrate during catalysis [4]. The control loop of BVMOs usually contains a conserved tryptophan residue that interacts with NADP(H); however, BVMO_{AFL210} from *Aspergillus flavus* contains a threonine (T513) in this position. Previous studies have indicated that mutating the tryptophan decreases activity and peroxyflavin stability significantly [1,5]. Despite this, BVMO_{AFL210} is highly active and converts a wide range of substrates.

Here we present the crystal structure of BVMO_{AFL210} bound to NADP⁺ in the 'open' and 'closed' conformations, revealing that T513 does not interact with NADP⁺. To further probe this, we used site-directed mutagenesis to create T513W, Y and G mutants and performed whole-cell biotransformations with a panel of substrates. The substrate scope was not significantly altered by any of the mutants; however, the sulfoxidation and regioselectivity of the enzymes were modified in a substrate-specific manner.

As expression levels varied, the mutants were purified, and the turnover frequencies (TOFs) were determined. A general decrease in activity was observed for the mutants in the order of wildtype (T) > G > Y > W, which was more pronounced in the conversion of cyclic and aromatic ketones. In addition, the bulkier substitutions, Y and W, showed a large decrease in peroxyflavin stability. Thus, both the activity and regioselectivity do not only depend on the amino acid at this position but also the substrate evaluated, opening the door to new possibilities of directed-evolution studies for improved activity and/or regioselectivity.

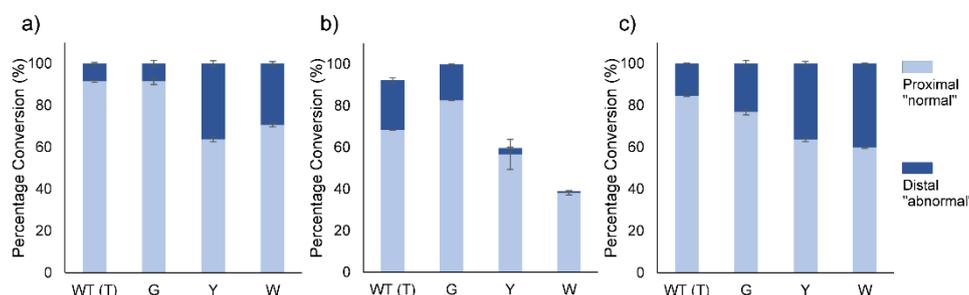


Fig. 1. Regioselective conversion of 2-methylcyclopentanone (a), 2-methylcyclohexanone (b) and *cis*-bicyclo[3.2.0]hept-2-en-6-one (c) by BVMO_{AFL210} wildtype (T) and T513G, Y and W mutants.

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Δ^1 -Dehydrogenation of 3-ketosteroids by 3-ketosteroid dehydrogenase from *Sterolibacterium denitrificans* – structural and mechanistic studies

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3-Ketosteroid Δ^1 -dehydrogenase (AcmB) is a FAD-dependent enzyme (KSTD) that plays an important role in bacterial cholesterol metabolism and shows good sequence similarity to other KSTDs from different bacterial strains. AcmB catalyzes oxidative dehydrogenation between C1 and C2 of androst-4-en-3,17-dione and other steroids such as testosterone and progesterone that are used in the pharmaceutical industry. It naturally occurs in *Sterolibacterium denitrificans* and most probably is located on the cytoplasmic side of the bacterial inner membrane. AcmB isolated from native bacteria, as well as from recombinant *E. coli* forms massive aggregates (>600 kDa).[1,2]

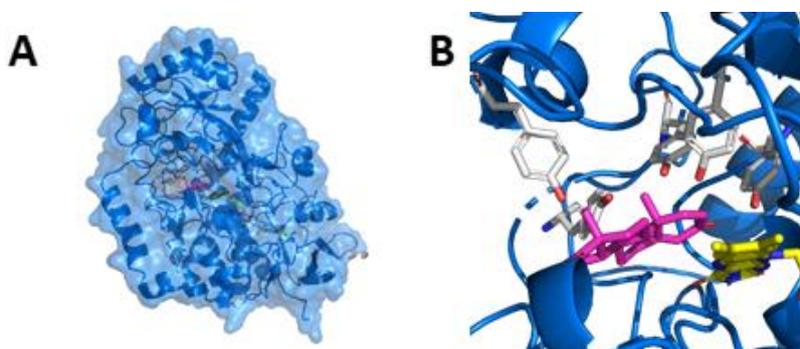


Figure 1. The overall structure of AcmB (A) and the active site (B).

We optimized the AcmB expression in *E. coli* and purification by means of the detergents – Triton X-100 and Tween 20. Due to the initial crystal screening on the commercially available crystallization screens, we were able to obtain crystals of AcmB that diffract X-rays to 2.8 Å and 1.8 Å resolution. Finally, the AcmB structure was solved by molecular replacement with the structure of 3-ketosteroid Δ^1 -dehydrogenase from *Rhodococcus erythropolis* (PDB: 4C3Y).[3] Based on the homology model and the resolved crystal structures we determined the amino acid residues that could play a crucial role in the catalysis. Then site-directed mutagenesis was used to probe the role of them in the active site. As a result, we demonstrated the significant impact of Y115, Y118, Y363, Y467, Y536 and G540 mutations on the enzyme activity. Moreover, to determine the dehydrogenation rate-limiting step we measured the catalytic rate constants of reductive and oxidative half-reactions and studied substrate and solvent kinetic isotope effects. For further investigation of the reaction catalyzed by AcmB we used QM:MM methods. Activation free energy was calculated by generating the corresponding potential of mean force (AM1:AMBER) with interpolated spline corrections at a higher level of theory (B3LYP:AMBER). The calculations suggest that reaction is initiated by proton abstraction by tyrosyl anion (Y363), then transient carboanion is formed. In the last step, the hydride is transferred to the FAD.

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Photosynthetic machinery and glycolytic pathways: two sources of reductants for biotransformations via cyanobacteria

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The chemical processes in pharmaceutical industries can produce up to hundred-fold more waste than the desired product. Detrimental reductants and oxidants are often utilized especially in the case of redox reactions. Among different applications, cyanobacteria can be interestingly utilized in presence of light as source of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to fuel heterologous oxidoreductases which convert the substrates into the corresponding products [1, 2]. Nevertheless, the limit of the exploitation of these photosynthetic prokaryotes for photo-biotransformations is still undetermined. Our results importantly demonstrate how the initial catalytic rates of reactions under light via YqjM ene-reductase are depending on both of photosynthetic NADPH reduction and glycolytic pathways.

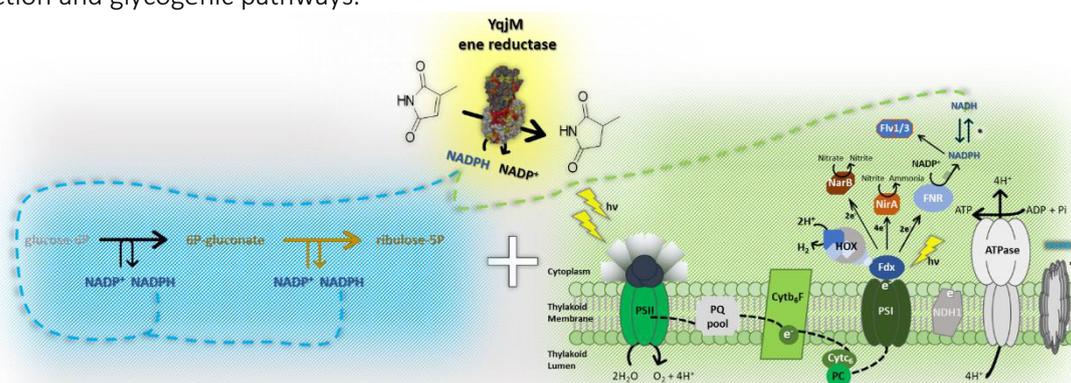


Figure 1. Overview of the main sources of reduced cofactors to fuel heterologous oxidoreductase in cyanobacteria.

The effect of glucose on photosystem I (PSI) and photosystem II (PSII) under production conditions have been observed: both photosynthetic machineries have not been inflicted, acting simultaneously with the light-independent pathways. Reduced cofactors have been available from both light-dependent and -independent routes, a unique feature of photosynthetic microorganisms like cyanobacteria, differently from heterotroph bacteria as *Escherichia coli*. Among the glycolytic routes in *Synechocystis* sp. PCC 6803, the Entner-Doudoroff (ED) pathway has been recently elucidated [3]. Furthermore, we investigated its impact on the ene reductase YqjM-based reactions. Photo-biotransformations have also been performed with strains unable to breakdown stored glycogen – $\Delta glgP1\Delta glgP2$ $P_{cpcB}::yqjm$, and not possessing active pyridine nucleotide transhydrogenase which regulate NADPH-NADH intracellular equilibrium – $\Delta pntA$ $P_{cpcB}::yqjm$ [4]. Glucose acts as external source of reduced cofactors when this is supplemented in parallel to the substrate addition (e.g. 2-methylmaleimide), in all the strains investigated during this study. Importantly, an increment of the enzymatic activity equal to 50 % has been recorded. Moreover, the product formation is instead approximately 2-fold diminished when the cells are cultivated in photomixotrophy before the catalytic reaction.

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Investigation of enzymatic degradation of marine polysaccharides

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Current environmental changes result in extensive grow of algae biomass, which negatively affects the coastal ecosystems. However, the algae-based components are of interest as a raw material for a bio-based economy. Macroalgae derived polysaccharides are already used as gelling agents due to their hydrocolloidal properties. Additionally, sulphated oligosaccharides are desired targets for their high valued bioactivity. The degradation of marine polysaccharides, to access the valuable sugar components, is crucial for the process applicability [1]. High structural complexity of algal polysaccharides requires suitable enzymatic tools to obtain rare oligo- and monosaccharides. Recently, the excellent study described degradation pathway of the polysaccharide ulvan (extracted from *Ulva* sp.) using enzymes from the marine bacterium *Formosa agariphila* KM3901 [2]. Bio-processing of extracted monosaccharides can serve as an enzymatic toolbox to generate and analyse marine poly- and oligosaccharides.

Our project focusses on the elucidation of further ulvan and marine xylan degradation pathways by carbohydrate-active enzymes (CAZymes) derived from marine Bacteroidetes. An additional focus of the investigation is characterization of novel enzyme activities involved in the marine polysaccharide degradation.

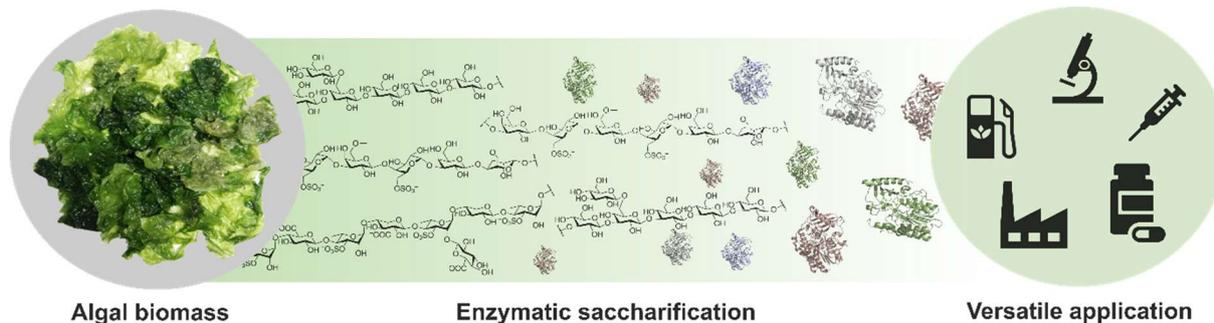


Figure 1. Overview of the conversion and enzymatic degradation of marine polysaccharides [1]

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Microbial Biotransformation of Hesperidin by *Enterococcus durans* ATCC 19432 and Cytotoxic Effects of Its Metabolites on HEK293 Cell Line

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Flavonoids form an important class of plant compounds. Conjugated form with sugar molecules is the most common form of flavonoids. The ability of flavonoids to be produced by tissue engineering techniques depends on successful modification and enantioselectivity [1]. This can be achieved by using biotransformation reactions which are designed to obtain bioactive substances, and using appropriate analytical techniques. In this study, hesperidin was selected for the purpose of developing more active drug candidates because of the completion of basic research phase and exploration phases. Hesperidin is a classical herbal medicine used worldwide for a long time with an excellent safety profile. Hesperidin is a well-known herbal medication used as an antioxidant and anti-inflammatory agent. Available shreds of evidence support the promising use of hesperidin in prophylaxis and treatment of COVID 19 [2]. After biotransformation process by *Enterococcus durans* Collins et al. (ATCC® 19432), the metabolites were separated from the bacterial culture medium by extraction and they were clarified by UV, IR, 1D-NMR, 2D-NMR, ¹³C-NMR and MS techniques after purification by chromatographic methods. At the end of the study, diosmin, hispidulin, penduletin, sinensetin, apigenin, crysin, naringin, naringenin, luteolin, and nepetin were obtained as metabolites.

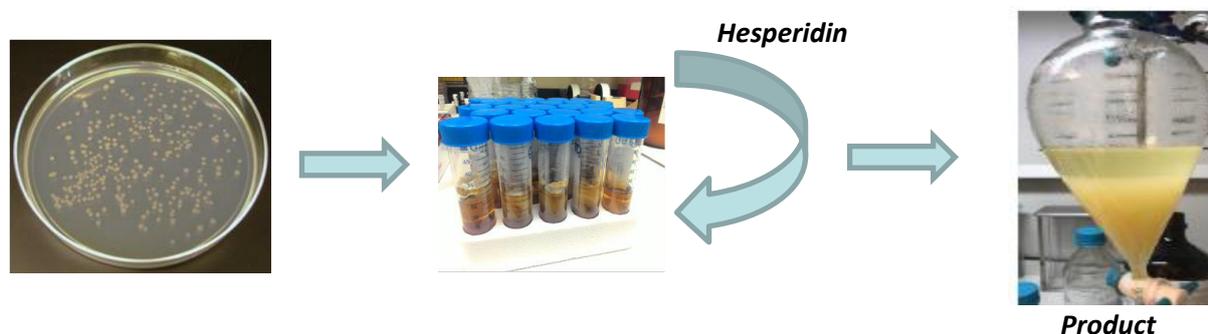


Figure 1. Biotransformation process of Hesperidin

Since the metabolites were biotransformed by *Enterococcus durans* bacteria, they were different compounds than the starting compounds. Finally, anticancer properties were examined by determining the cytotoxic activities of hesperidin and its metabolites by using LDH and MTT assays on HEK293 (human embryonic kidney) ATCC CRL-1573 cell line [3]. Our report highlights a protocol for rapid production of diosmin, hispidulin, penduletin, sinensetin, apigenin, crysin, naringin, naringenin, luteolin, and nepetin from *E. durans* and the applicability of obtained molecules with decreased cytotoxicity activities.

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Development of Controlled Gene Expression System for (*Para*)*Geobacillus* spp. Bacteria

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Parageobacillus and *Geobacillus* spp. bacteria are Gram-positive spore forming thermophiles growing at high temperatures in the optimum range 50-75°C. They are valuable source of various thermostable enzymes and other bioactive compounds, which are significant in biotechnological industry. Moreover, these bacteria can be applied for production of bioethanol, and other chemical compounds at the industrial level[1]. Despite high potential their application in biotechnology is limited due to narrow choice of genetic engineering tools for (*Para*)*Geobacillus* spp. bacteria[2], [3].

The aim of this study was development of new heterologous gene expression system for (*Para*)*Geobacillus* spp. bacteria. For this purpose, bacteriocin (antibacterial peptide) biosynthesis regulation system and its components were used. Our developed system is similar to the “NICE” protein expression systems, which have been already established and adopted for *Lactococcus lactis* host. The NICE system is based on bacteriocin nisin biosynthetic machinery components[4].

We have chosen to adopt geobacillin I biosynthesis machinery[5] to construct inducible and regulated gene expression system for thermophiles. This bacteriocin is encoded in thermophilic bacteria and is similar to nisin. We have constructed the system using two-component signal transduction system genes *geoRK*, responsible for regulation of the expression of the geobacillin I, and bacteriocin promoter *PgeoAI*. Our results showed that the system is working in thermophilic bacterium *Parageobacillus thermoglucosidasius* DSM 2542. Using bacteriocin geobacillin I as an inducer at sub-inhibitory amounts, we were able to induce the biosynthesis of GFP in this bacterium.

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Rhamnosylation of flavonoids by UDP-dependent rhamnosyltransferase expressed in *Escherichia coli*

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Flavonoids are a large group of over 10 000 natural compounds identified in plants. They have a proven antioxidant, anticancer, antibacterial and antiviral activity and are widely used in drugs and food additives designing [1]. Yet, their application can be limited by poor bioavailability and bad water solubility. This issue can be easily enhanced by glycosylation. Sugar groups attached to flavonoids aglycones have also positive effect on their biological activity and stability. Flavonol rhamnosides e.g. have better antiviral and antibacterial activities [2].

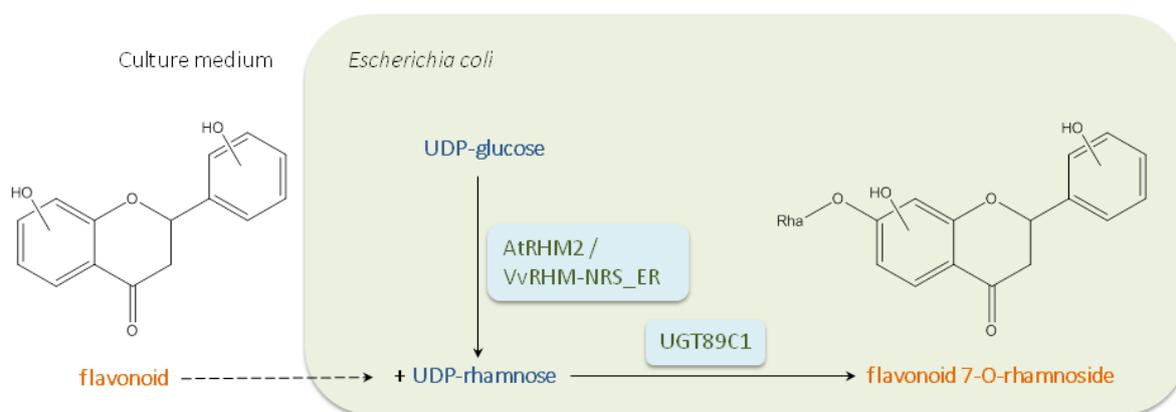


Figure 1. Rhamnosylation of flavonoid in *E. coli*. Rha – rhamnose; UGT89C1 - UDP-dependent rhamnosyltransferase from *A. thaliana* ; AtRHM2 – UDP-rhamnose synthase from *A. thaliana* ; VvRHM-NRS_ER – hybrid UDP-rhamnose synthase.

Rhamnosylation of flavonoids were performed by whole cell biotransformation using modified *Escherichia coli* harbouring UDP-dependent rhamnosyltransferase (UGT89C1) from *Arabidopsis thaliana*, which specifically recognizes UDP-L-rhamnose as sugar donor [3]. Due to absence of UDP-rhamnose in bacterial cells a co-expression of UDP-rhamnose synthase is required [4]. For this purpose, two enzymes were selected and compared for activity: RHM2 from *A. thaliana* [5] and VvRHM-NRS_ER hybrid enzyme constructed by fusion of the N-terminal region of VvRHM from *Vitis vinifera* and bi-functional NRS/ER from *A. thaliana* [6].

Biotransformation resulted in the regioselective production of variety of 7-O-rhamnosides of flavanones, flavonols, and flavones.

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Investigation of *Kluyveromyces marxianus* as a host for the expression of bacterial laccase

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With the growing potential for the use of biocatalysts and the need to substitute chemical catalysts, the effort is focused on the development of new and/or more efficient enzymes in sufficient quantities in order to apply for targeted processes. Laccases are one of the widely investigated biocatalysts, which have a broad substrate range, therefore, their application is explored in a variety of industrial areas - textiles, paper, food, pharmaceuticals, biomass pre-treatment, bioremediation and etc [1]. However, for the wider application of laccases, the main challenges are their commercial availability, which closely relates to cost and stability. Thus, laccases still are greatly investigated for their synthesis in various expression systems [2].

By this study the yeast *Kluyveromyces marxianus* was selected for laccase expression, which is GRAS (generally recognized as safe) microorganism, has such properties as a rapid growth, thermostability, use of various carbon sources, etc [3]. Bacterial laccase gene was isolated from *Bacillus pumilus* strain. Several attempts were made to express the bacterial laccase in various yeast such as *Pichia pastoris*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, however, the obtained protein amount was very low and formed poorly soluble aggregates. Therefore, we have focused the research on the expression of laccase using chaperones, which, when synthesized together with the target laccase, should lead to the successful production of an active target protein without the formation of aggregates. High hydrophilicity MBP from *E. coli* and modified *K. marxianus*-derived proteins EPG (endopolygalacturonase) and TPI (triose phosphate isomerase) were used for the study. The latter two chaperones were never investigated for laccase expression. The obtained results in more detail will be presented during the poster session.

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Enzymatic degradation of mycotoxins in animal nutrition

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The prevalence of mycotoxins in almost all types of grains and agricultural feedstuffs represents a genuine risk to livestock production. Even at concentrations below guidance values, mycotoxins may affect animal health and productivity. Two of the most prevalent mycotoxins frequently infecting crops worldwide are fumonisins (FUM) and zearalenone (ZEN), mainly produced by *Fusarium verticillioides* and *F. graminearum*. Human exposure to FUM is linked to esophageal cancer and neural tube defect, while classic mycotoxicoses in domestic animals are porcine pulmonary edema and equine leucoencephalomalacia. At molecular level, FUM inhibit ceramide synthase, a key enzyme in sphingolipid metabolism. The resulting imbalance of sphingolipids and sphingoid bases is responsible for toxic effects. The resemblance between zearalenone and the primary female hormone, estrogen, leads to interference with the endocrine system especially of swine, causing hyperestrogenism and impaired fertility. Our technological goal is to develop enzyme-based feed additives to biotransform and thereby detoxify FUM, ZEN and other mycotoxins in the gastrointestinal tract of animals.

We identified the fumonisin degrading bacterium *Sphingopyxis* sp. MTA144 and carboxylesterase FumD was found to catalyze the first step in the FUM breakdown pathway. It hydrolyzes two tricarballic acid (TCA) side chains from the FUM molecule. Since fumonisins interfere with the sphingolipid metabolism, their toxic effects can be monitored by using the increased ratio of sphingoid bases sphinganine to sphingosine (Sa/So) as a biomarker. We were able to directly prove efficacy of FumD to degrade FUM *in vivo* by following the restoration of the sphingoid base ratio after supplementation of fumonisin contaminated animal feed with FumD.

Discovery of a zearalenone hydrolase started with isolation of ZEN degrading microorganisms from a soil sample. We isolated *Rhodococcus erythropolis* strain PFA D8-1, exhibiting lactonase activity against ZEN. We purified the previously elusive metabolite hydrolyzed zearalenone (HZEN) and confirmed lack of estrogenicity for HZEN in assays using MCF-7 breast cancer cells. The zearalenone hydrolase encoding gene *zenA* was identified by cloning a genomic library of *R. erythropolis* PFA D8-1 and functional screening in ZEN degradation deficient strain *R. erythropolis* PR4. Subsequently, we identified, expressed and characterized several ZEN lactonase homologs and confirmed their activity *in vivo* by quantifying ZEN and HZEN in urine and feces samples using tandem mass spectrometry.

By combining directed evolution and rational enzyme engineering based on atomic coordinates resolved by X-ray crystallography, we successfully increased thermostability and catalytic activity of FumD and ZenA, in order to withstand feed processing conditions and to efficiently degrade mycotoxins in the challenging gastrointestinal environment of pigs, poultry and ruminants.

FUM carboxylesterase FumD (FUMzyme[®]) is the first purified enzyme EU-authorized as technological feed additive for mycotoxin detoxification and part of Biomin's Mycofix[®] product line. Recently, ZEN hydrolase ZenA (ZENzyme[®]) was launched as another enzymatic solution in Biomin's products for mycotoxin risk management.

FUMzyme[®] and ZENzyme[®] are the proof that hydrolysis of mycotoxins by recombinant enzymes in the gastrointestinal tract of animals is possible and economically viable. Development of additional enzymes for degradation of mycotoxins of major concern is continued.

Discovery of a novel GDP-glucose 4-epimerase in archaea reveals an uncommon promiscuity

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Nucleotide sugar epimerases form a very interesting group of enzymes, as they can invert the configuration of a specific hydroxyl group through a single reaction and without prior activation or protection steps. Within this group, UDP-galactose 4-epimerase (GalE, EC 5.1.3.2) is by far one of the best studied members[1] due to its essential role in the Leloir pathway in which it interconverts UDP-galactose and UDP-glucose[2]. GalE deficiency is responsible for galactosemia, a hereditary disease, highlighting its vital importance[3]. Although GalE was widely studied throughout all domains of life[4–6], ranging from eukaryotes to archaea, its biochemical characterization was often limited to UDP-hexoses, neglecting the possibility that GalE might be promiscuous towards other NDP-sugars and derivatives thereof. In this study, we identified a novel GalE displaying an unprecedented specificity on guanosine diphosphate (GDP) sugars. Indeed, a detailed biochemical investigation performed on GalE from *Pyrococcus horikoshii* (phGalE) revealed that it is a GDP-glucose 4-epimerase. In addition, we confirmed that it accepts a variety of other NDP-sugars including L-sugars moieties, such as GDP-L-Gal/Glc as well as their 6-deoxysugars counterparts GDP-L-fucose and GDP-L-quinovose, respectively.

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An efficient approach for CO₂ capture from industrial sources by a hybrid amino acid ionic liquid: amine: carbonic anhydrase blend

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Carbon Capture and Utilization (CCU) has potential to neutralize CO₂ emissions from the whole lifecycle of traditional energy-intensive industries such as the cement industry, being a promising technology to combat climate change. Traditional capture technologies are based on absorption where use of the primary alkanolamine, monoethanolamine (MEA) is the gold standard. However, MEA suffers from several issues during operation, including high-energy requirements for stripping; high enthalpy of reaction, low absorption capacity, oxidative and thermal degradation and piping corrosion. An alternative to highly reactive MEA is the tertiary methyl diethanolamine (MDEA) that is characterized by high equivalent weight, high stability, and low regeneration energy but shows low reactivity and absorption rates [1]. Carbonic anhydrase (CA; EC 4.2.1.1) is a metallo-enzyme and one of the fastest enzymes found in nature. Due to its high activity, CA-promoted aqueous solvents show significant improvements in CO₂ absorption rate as this enzyme catalyzes the rate-limiting step that is CO₂ hydration. Nevertheless, integration of CA with amine scrubbing has been limited due low enzyme stability in operating conditions (temperature, pH and ionic strength). To address these issues, strategies such as protein engineering have been applied to improve CA properties such as thermal stability [2].

Aim of this work is to demonstrate the potential of a hybrid CO₂ capture process employing a novel thermostable CA [4] and a novel hybrid amino acid amine blend [5]. This approach will enable a reduction in energy costs of the capture process by achieving high absorption rates comparable to MEA and low desorption energy comparable to MDEA. Large-scale CO₂ capture was performed using a packed-bed absorption column and CO₂-rich industrial off gas (GCPV, Spain). The hybrid amino acid ionic liquid: amine: CA blend offered an initial CO₂ absorption rate 5 times higher than MDEA and 82% of that of MEA, underlining its potential as a competitive alternative to conventional amine scrubbing. This work was supported by the EU's Horizon 2020 research and innovation programme BioRECO2VER (grant agreement No 760431).

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Synthesis and investigation of a novel aromatic amine for laccase activity screening on high-throughput agar plates

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Laccases are multi-copper oxidases (EC 1.10.3.2), containing T1, T2 and T3 copper sites. Though the years of investigating various laccases, a lot of information has already been accumulated: catalytic mechanism, redox potentials, enzyme sources, reorganization energies. These enzymes catalyse a unique oxidation of phenols, aromatic amines and thiols by using oxygen as a co-substrate and produce water as a by-product.[1] This process has an enormous potential for food, textile, paper, biofuel, cosmetics, paints, furniture industries and organic synthesis, nanobiotechnology and bioremediation.[2] By employing laccase-mediator systems, and thus expanding their substrate spectrum they could replace hydrogen peroxide and other chemical oxidators at industrial scale. However, tangible application of laccases is hindered by the unresolved drawbacks such as poor stability, commercial unavailability, lack of efficient expression systems, low immobilization yields, etc.

One of the shortcomings for discovering laccases with new and/or novel features is a lack of substrates suitable for high-throughput screening and functional analysis. Currently, the most common compounds used for laccase functional analysis are 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) under the trivial name ABTS and 4-[[2-[(3,5-dimethoxy-4-oxocyclohexa-2,5-dien-1-ylidene)methyl]hydrazinyl]methylidene]-2,6-dimethoxycyclohexa-2,5-dien-1-one known as syringaldazine.[3,4] Spectrophotometric activity assays with these substrates give acceptable results, but these compounds have poor stability, low specificity and are rather expensive. Their use for high-throughput screening on agar plates is very hindered due to their thermal instabilities

By our investigation, we present a new substrate named ferbamine for laccase activity screening on high-throughput agar-plates. This compound was synthesized using relevantly low priced aromatic diamine – N,N-dimethyl-p-phenylenediamine dihydrochloride. The latter substrate was tested on substrate-agar plates with commercially available laccase Novozym 51003 from *Aspergillus oryzae* and with the collection of wild fungi protein extracts provided by Jožef Stefan Institute (Fig. 1) [5]. The results in more detail will be presented during the poster session.

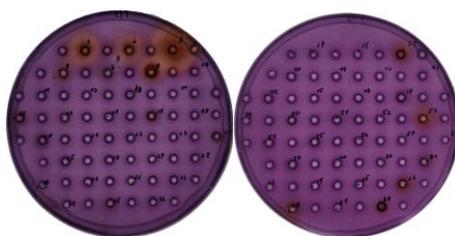


Figure. 1. The results of screening for laccase activity with the collection of wild fungi protein extracts on agar plates with 830 μ M substrate.

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Differential hydrolysis of the flavonoid hesperidin by *Actinoplanes missouriensis* 431^T glycosidases

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Actinoplanes missouriensis 431^T is a soil isolate bacterium, well known for its ability to degrade flavonoids. Using an activity-based screening in combination with a sequence analysis approach, two enzymes active on the flavonoid hesperidin (hesperitin-7-*O*- β -rutinoside) were discovered and recombinantly expressed: the diglycosidase hesperidin 6-*O*- α -L-rhamnosyl- β -D-glucosidase (α R β G, EC 3.2.1.168) and a α -L-rhamnosidase (α R, EC 3.2.1.40) (Figure 1)[1,2]. While α R β G hydrolyzes hesperidin at the heterosidic bond releasing the disaccharide rutinose (6-*O*- α -L-rhamnosyl-D-glucose) and the aglycone hesperetin, α R recognizes the glycosidic bond between the rhamnose and glucose moieties, releasing rhamnose and glucosylated hesperetin (Figure 1). In both cases, the enzymes obeyed typical Michaelis-Menten kinetics with hesperidin as the substrate (α R β G: K_M 1.6 \pm 0.5 mM, V_{max} 0.0013 \pm 0.0002 μ mol min⁻¹; α R: K_M 0.8 \pm 0.2 mM, V_{max} 0.061 \pm 0.003 μ mol min⁻¹). Regarding substrate recognition, the α R β G showed specificity for 7-*O*-rutinosylated flavonoids and only trace of activity with the monoglycoconjugate *p*-nitrophenyl β -D-glucopyranoside. In contrast, the α R exhibited higher promiscuity, with activity against the disaccharide rutinose, 7-*O*-rutinosyl-flavanones, 3-*O*-rutinosyl-flavonols, and rhamnose-containing polymers.

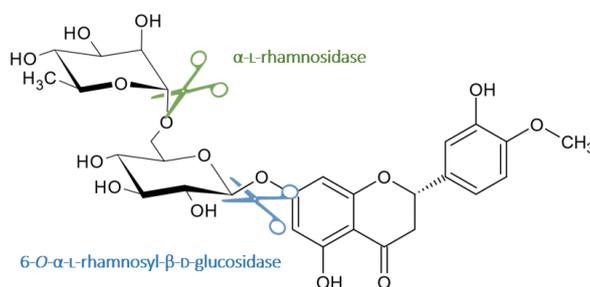


Figure 1. Schematic hydrolysis pattern of hesperidin by the glycosidases α R β G and α R from *A. missouriensis* 431^T

The use of the glycosidases with different specificities represents an interesting strategy for the structural diversification of plant secondary metabolites. The enzymes α R β G and α R from *A. missouriensis* 431^T are thus a contribution to the toolkit of carbohydrate-active enzymes for biocatalytic applications.

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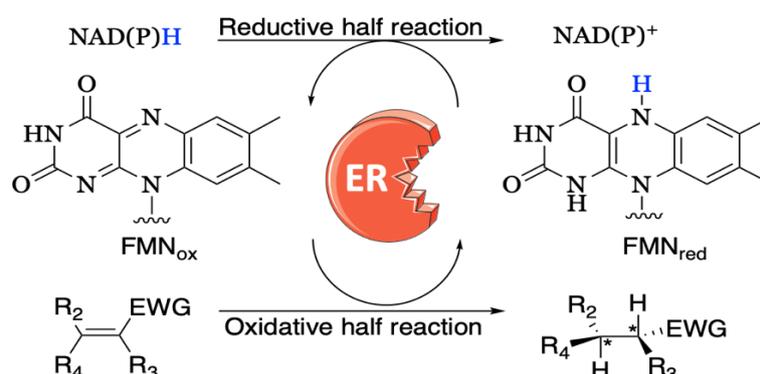
Description of four novel OYE enzymes from the fungi *Aspergillus niger* and *Botryotinia fuckeliana*

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Asymmetric alkene hydrogenation in industrial processes exploits ene-reductases (ERs) from the family of the Old Yellow Enzyme (OYE; E.C. 1.6.99.1). These are nicotinamide-dependent flavoproteins catalyzing the asymmetric hydrogenation of a wide panel of activated alkenes (e.g., α,β -unsaturated ketones, aldehydes, nitroalkenes, carboxylic acids, and derivatives).



In recent years, unexpected reactivities due to the chemical versatility of the flavin cofactor have also been reported for this class of enzymes [1-6]. As a result, the discovery and the characterization of new ERs is being driven not only by the interest for classical C=C-bond reductions but also the curiosity to better understand the potentialities of this class of biocatalysts. Not at least, the research aiming at expanding the toolbox of ERs is also fueled by the necessity of tackling some limitations still encountered in their application (low stability in the harsh industrial conditions, modest turnover numbers, poor substrate tolerance and, in some cases, low enantioselectivity) [7].

These enzymes are ubiquitous in nature and have been found in yeasts, bacteria, plants and parasitic eukaryotes [8]. More than 100 ERs have been already identified and their number is constantly increasing, causing an almost continuous revision of their phylogenetic classification [9,10]. To the protein family of OYEs, we add now four new members of fungal origin, identified in the ascomycete species *Aspergillus niger* and *Botryotinia fuckeliana* (better known as *Botrytis cinerea*). We report here on the strategies adopted to succeed in expression and purification, and on the biochemical and biocatalytic characterization of these enzymes, namely *AnOYE2*, *AnOYE8*, *BfOYE1* and *BfOYE4*.

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A novel stereo-specific and thermostable aldolase from *Acinetobacter baumannii* (*AbHpal*) as a promising green biocatalyst for aldol condensation

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Aldolases catalyze reversible reactions of aldol condensation and cleavage and have great potential in synthesizing chiral compounds.[1,2] Here, we investigated a new pyruvate-specific metal (Class II) aldolase from the *p*-hydroxyphenylacetate degradation pathway in *Acinetobacter baumannii*, 4-hydroxy-2-keto-heptane-1,7-dioate aldolase (*AbHpal*) which has various properties suitable for biocatalysis including stereoselectivity/stereospecificity, broad aldehyde utilization, thermostability and solvent tolerance. Interestingly, *AbHpal* uses Zn^{2+} as a native divalent cofactor as well as other metals including Co^{2+} , Mn^{2+} , Mg^{2+} which is different from other known enzymes in this class. Zn^{2+} also increases thermostability of the enzyme the highest (T_m of 87 °C) compared to other metals. *AbHpal* can also tolerate 20% (v/v) of MeOH, ACN or DMSO with small perturbation in protein stability. For catalysis, all *AbHpal*• M^{2+} complexes prefer to cleave (4*R*)-2-keto-3-deoxy-D-galactonate ((4*R*)-KDGal) over (4*S*)-2-keto-3-deoxy-D-gluconate ((4*S*)-KDGLu) with *AbHpal*• Zn^{2+} displaying the highest *R/S* stereoselectivity ratio, 6-fold higher than other metals. In aldol condensation, *AbHpal*• M^{2+} only specifically forms (4*R*)-KDGal, not (4*S*)-KDGLu. *AbHpal* also prefers aldol formation over cleavage by ~40 folds. The X-ray structures of *AbHpal* in complex with metals and ligands clearly indicate that Zn^{2+} forms an octahedral geometry with Glu151 and Asp177, pyruvate and waters and Arg72 in the Zn^{2+} -bound form governs the stereoselectivity/stereospecificity in *AbHpal*. In contrast to other metals, Ca^{2+} does not take part in catalysis but participates in structural integrity by binding between trimer interface *via* interaction with Asp51. Moreover, *AbHpal* also can utilize aliphatic and aromatic aldehydes for aldol condensation. Altogether with its substrate stereospecificity, utilization, preference towards aldol formation and protein robustness, the characteristic of *AbHpal*• Zn^{2+} is distinctive from their homologues and have properties attractive for industrial biocatalytic applications. [3,4]

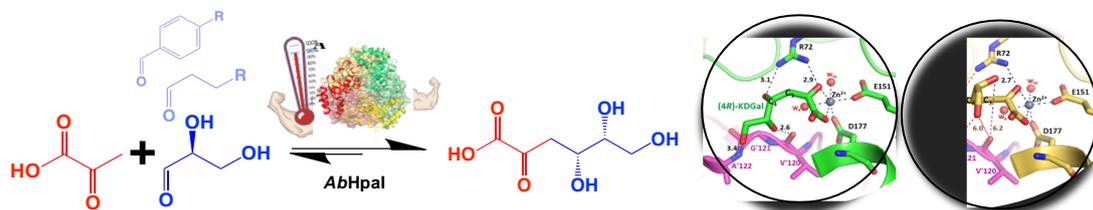


Figure 1. Reversible aldol condensation of pyruvate and (D)- glyceraldehyde catalyzed by *AbHpal* to form (4*R*)-KDGal. The active site of *AbHpal* showing binding interactions between ligands and catalytic residues.

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An Antarctic polyester hydrolase degrades polyethylene terephthalate at moderate temperatures

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Polyethylene terephthalate (PET) is one of the most-commonly used synthetic plastic in the packaging industry¹. Several microorganisms have been described to encode enzymes that catalyze a depolymerization of PET^{2,3}. While these thermophilic PET hydrolases require reaction temperatures between 60°C to 70°C for an efficient hydrolysis of PET, a partial hydrolysis of amorphous PET at lower temperatures by the polyester hydrolase *IsPETase* from the mesophilic bacterium *Ideonella sakaiensis* has also been reported⁴. We show that polyester hydrolases from the Antarctic bacteria *Moraxella* sp. strain TA144 (Mors1) and *Oleispira antarctica* RB-8 (OaCut) were able to hydrolyze the aliphatic polyester polycaprolactone as well as the aromatic polyester PET at a reaction temperature of 25°C. Mors1 caused a weight loss of amorphous PET films by 0.5% within 24 h of reaction and is thus constitutes a PET-degrading psychrophilic enzyme. Comparative modelling of Mors1 showed that the amino acid composition of its active site resembled both thermophilic and mesophilic PET hydrolases. Bioinformatic analysis of Antarctic metagenomic samples demonstrated that members of the Moraxellaceae family carry candidate genes coding for further potential psychrophilic PET hydrolases.

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Biocatalytic Asymmetric Thiol-Michael additions

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4-oxalcrotonate tautomerase (4-OT) is a tremendously versatile biocatalyst able to catalyze a wide range of C-C bond-forming reactions, which find applications in the synthesis of various pharmaceutical precursors and proceed *via* an enzyme-bound enamine intermediate.^[1-4] Recent protein engineering efforts allowed to unlock new asymmetric reactions catalyzed by 4-OT through enzyme-bound iminium ion intermediates, namely the C-C bond-forming Michael-type addition of nitromethane to cinnamaldehyde derivatives^[5,6] and the C-O bond-forming epoxidation of cinnamaldehydes using peroxides.^[7] In this work, we investigated the possibility to extend the reaction scope of 4-OT to the C-S bond-forming Thiol-Michael addition reaction. Two engineered 4-OT variants were found able to effectively catalyze the asymmetric nucleophilic addition of *tert*-butylmercaptane to cinnamaldehyde. Reaction conditions were optimized to avoid the enzyme-catalyzed racemization of the product and the substrate scope was enlarged to a set of aliphatic thiols yielding (R)- β -thioenals with conversions up to 99 % and product e.r. up 95:5.

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Exploration of GH94 sequence space for the discovery of novel phosphorylase specificities

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The substantial increase in DNA sequencing efforts has led to a rapid expansion of available sequences in glycoside hydrolase families over the past few years. The continuously increasing sequence space presents considerable opportunities for the search for novel enzyme specificities.

In this work, the sequence-function space of glycoside hydrolase family 94 (GH94) was explored in detail, using a combined approach of phylogenetic analysis and sequence similarity networks. Selection and experimental screening of unknown clusters led to the exposure of an enzyme from the soil bacterium *Paenibacillus polymyxa* that was found to act as a β -1,4-glucosyl-galactose phosphorylase (GGalP), a specificity that has not been reported to date.

In-depth characterisation indicated that its kinetic parameters, and temperature and pH optima are in line with those of other known phosphorylases. Additionally, the reverse phosphorolysis reaction was exploited to produce a small amount of 4-*O*- β -D-glucosyl-D-galactopyranoside, leading to the isolation of approximately 1 g of crystalline product with a purity exceeding 99 %.

Our current work emphasizes the strength of sequence space exploration in the search for and discovery of novel enzyme specificities. In addition, the discovery of this novel activity broadens the pool of available glycoside phosphorylases, and paves the way for the efficient and highly specific synthesis of 4-*O*- β -D-glucosyl-D-galactopyranoside and 4-*O*- β -D-glucosyl-L-arabinopyranoside, novel disaccharides with potentially beneficial properties.

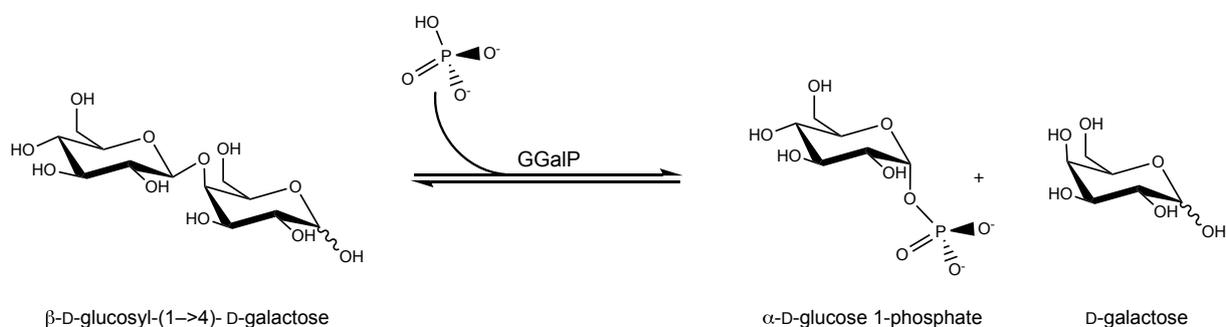


Figure 1. Reversible reaction catalysed by the glucosylgalactose phosphorylase (GGalP) from *Paenibacillus polymyxa*.

Full characterization of a highly active alcohol dehydrogenase from *Arabidopsis thaliana* and two rationally designed variants for biocatalytic reduction

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Saturated and unsaturated alcohols are used extensively as building blocks in synthetic chemistry, i.e. API synthesis. Their accessibility often relies on the use of expensive metal catalysts and highly reactive agents such as hydrogen gas and LiAlH₄ that pose a significant danger on a laboratory or industrial scale and result in large amounts of waste. Enzymatic reductions offer a green alternative and alcohol dehydrogenases in particular have been used in the past to access convenient synthesis routes even for industrial applications.^[1; 2] Plant ADHs are least frequently investigated and characterized as biocatalysts. They may therefore find application in the biotransformation of less commonly considered substrates.

RNA of the sole alcohol dehydrogenase found in *Arabidopsis thaliana* was isolated from leaves and used as a template for the construction of the AtADH/pET28a(+) vector. Expression and purification was optimized and active enzyme was isolated from *E.coli*. AtADH was then fully characterized in terms of optimal pH and temperature as well as tolerance towards different solvents which is crucial for synthetic application. Activity was retained after treatment with up to 70°C and pH values between 5.5 and 10. 5% v/v of different water miscible solvents were tolerated in varying degrees and could be increased up to 20 % before total loss of activity was observed. The substrate scope for the wild-type AtADH as well as a sequence-optimized variant and two mutants AtADH_S49T and AtADH_R48H_S49T_H52Y was defined. Sequence optimization led to a 2-fold increase in activity towards acetaldehyde as substrate. The combined substrate scope shows a wide acceptance of aliphatic, olefinic as well as linear and branched aldehydes, however, no conversion of ketones was detected. Mutations simulating the cofactor binding site of HLADH did not widen the scope to include ketones as suggested in literature but led to increased activity towards large substituents and higher acceptance of NADPH as a non-native cofactor.^[3] Transformation on a preparative scale implementing substrate coupled cofactor regeneration was shown and quantified by GC-MS. α,β -unsaturated aldehydes were also accepted and converted on a preparative scale. Further experiments may also explore these enzymes' ability to resolve racemic mixtures by selective reduction.

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Selective flavanoid ring A *ortho*-hydroxylation by novel FMO from *Pseudomonas putida*

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Flavonoids are important phenolic compounds, plant secondary metabolites that act as antioxidants, free radical scavengers, electron acceptors and metal-ion chelators [1]. Microorganisms can oxidize flavonoids as a mechanism of detoxification or simply utilize flavonoids as a carbon and energy source [2]. In the course of *in silico* screening for possible flavonoid hydroxylases we have identified the FMO active on C7-OH flavonoids. The health-promoting activities of flavonoid compounds are primarily associated with their antioxidant abilities. Flavonoids with a C7-C8 catechol moiety indicated much higher antioxidant activity compared with the C7-OH analogues. [3]

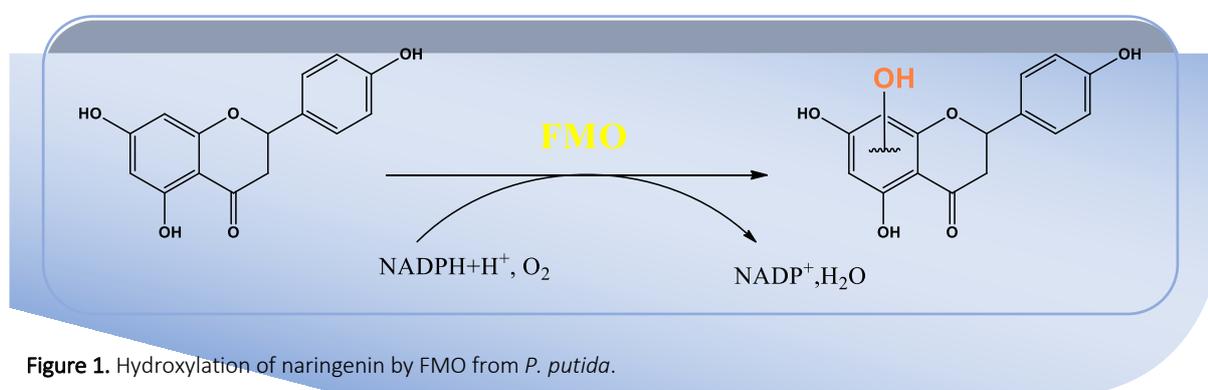


Figure 1. Hydroxylation of naringenin by FMO from *P. putida*.

Flavin-dependent monooxygenases (FMO) (E.C. 1.14.13.8) represent a group of flavoproteins with tightly bound FAD or FMN cofactor. Flavin cofactor has to be in the reduced form for reactivity with molecular oxygen. [4] FMO are involved in a wide range of biological process. [5] These oxidation reactions are often difficult to perform, if not impossible, to be achieved using chemical approaches. Many of these enzymes are highly selective, which makes them attractive as regio- and enantioselective biocatalysis. [4]

Herein, we would like to report broad biochemical characterisation of this novel flavonoid hydroxylase from *P.putida*, including substrate specificity and further application for flavonoid modification, especially that resulting catechol moieties greatly enhances flavonoid antioxidant properties.

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Enrichment of CYP5035 in *Polyporales*: Functionality of an understudied P450 family

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Bioprospecting for innovative basidiomycete cytochrome P450 enzymes (P450s) is highly desirable due to the fungi's enormous enzymatic repertoire and outstanding ability to degrade lignin and detoxify various xenobiotics.[1] The biocatalytic potential of the majority of thus annotated P450 sequences usually remains concealed, although functional profiling identified several P450 families with versatile substrate scopes towards various natural products.[2][3] Functional knowledge about the CYP5035 family, for example, is largely insufficient.[4]

In this study the families of the putative P450 sequences of the four white-rot fungi were assigned and the CYPomes revealed an unusual enrichment of CYP5035, CYP5136 and CYP5150. In order to address the knowledge gap on CYP5035 functionality, a representative subgroup of this P450 family of *Polyporus arcularius* was expressed and screened against a test set of substrates. Thereby, the multifunctional enzyme CYP5035S7 was discovered and its activity towards several plant natural product classes was determined.[5]

Aligning CYP5035S7 to 102,000 putative P450 sequences of 36 fungal species from JGI-provided genomes located hundreds of further CYP5035 family members, which subfamilies were classified if possible. Exemplified by these specific enzyme analyses, this study gives valuable hints for future bioprospecting of such xenobiotic-detoxifying P450s and for the identification of their biocatalytic potential.

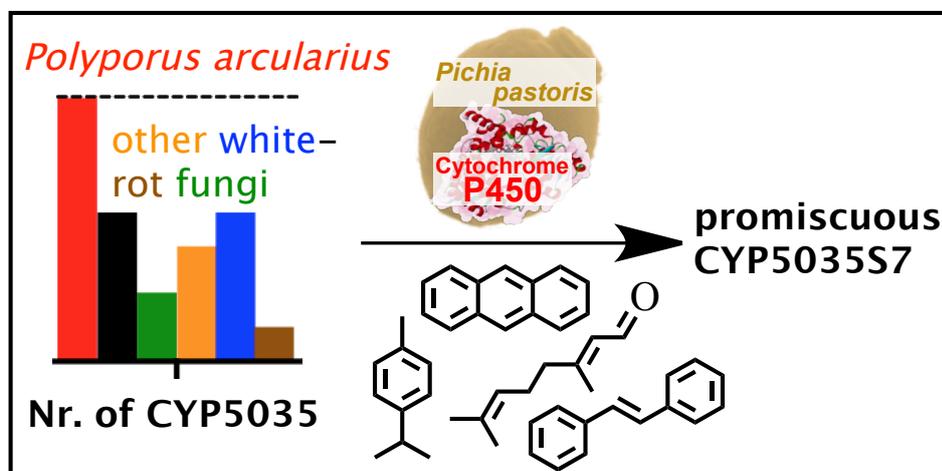


Figure 1. Identification of a P450 enzyme of the CYP5035 family with promiscuous activity towards several natural products classes.

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Biocatalytic hydrolysis of chemically stable lactams using a genomic-based approach

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The lactam motif is abundantly present in nature and usually embedded in larger functionalized molecules such as penicillins, cephalosporins, alkaloids or oxindole derivatives.

Depending on the ring size of the lactams, their chemical stability varies. For instance, five- and six-membered lactams - γ - and δ -lactams, respectively - are much more resistant to (bio)hydrolysis compared to β -lactams. This is due to the reduced ring strain compared to smaller β -lactams, a class of antibiotics that can be easily hydrolyzed by β -lactamases. Due to the increased resonance stabilization of the amide bond [1], and higher partial C-N double bond character in monocyclic γ - and δ -lactams [2,3], no cleaving enzyme active on such structures has been reported yet. In contrast, the chemical ring opening of γ - and δ -lactams requires harsh conditions (e.g. boiling in concentrated strong acid solution), which limits the further application of such methods. In line with growing environmental concerns and awareness for sustainable technologies, milder alternatives are needed.[4]

We could recently identify significant catalytic activity on monocyclic γ - and δ -lactams in bacterial strains grown under metabolic pressure, thereby demonstrating the existence of enzymatic machinery able to cleave such resistant amide structures. Conversions to the amino acid products in up to 86% were obtained when the corresponding lyophilized whole cells were used in the hydrolysis of 10 mM lactams under mild reaction conditions.

In order to identify the enzymes responsible for the ring opening of lactams, the genomes of these bacterial strains were sequenced. Based on genomic data analysis, a few enzymes were selected, cloned, and over-expressed and the recombinant proteins were tested in the hydrolysis of γ - and δ -lactams. Detection of the amino acid products in up to quantitative yield indicated successful identification of active enzymes. The substrate scope is currently being investigated, with a focus on a kinetic resolution approach toward enantiopure amino acids (Figure 1).

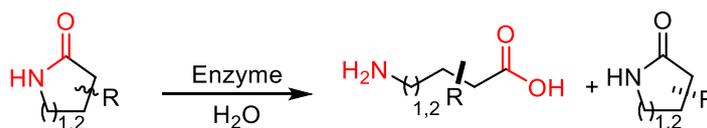


Figure 1. Envisaged kinetic resolution of substituted *rac*- γ - or δ -lactams by enzymatic hydrolysis

Acknowledgements

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Biochemical characterization of yeast-derived potato patatin isoforms

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The rise in global population has led to a growing demand for an adequate supply of protein for food applications. This in turn has motivated the search and development of new protein sources, from insects and plants to recombinant protein expression in microorganisms. Of the plant-based protein alternatives, potato proteins have gained great interest due to their exceptional essential amino acid composition compared to most plant-based proteins, and versatile functional properties. They have excellent gel-forming ability and superb foamability, making them attractive for food applications such as meat and dairy replacements, as well as in beverages, mousses and sauces [1, 2]. Potato tuber dry matter contains only 4% protein, of which approximately 40% are storage glycoproteins called patatins. Unlike other plant storage proteins, patatins possess lipid acyl hydrolase, esterase, acyltransferase, phospholipase A2, glucanase and xylosidase activities. Due to their limited abundance in potato tubers, isolation of pure and functional patatins has challenged researchers over recent years.

To overcome agricultural dependency and purification challenges, and to characterize the biochemical properties of individual patatin isoforms, our group successfully cloned and expressed two potato patatin isoforms in *Pichia pastoris*. The recombinant patatins, rPatB2 and rPat17, were expressed with a C-terminal hexa-Histidine tag, secreted to the media and isolated in a single purification step on a nickel affinity column. Purified rPatB2 and rPat17 were characterized biochemically and compared to commercial patatin isolated from potatoes (Solanic® 200). While both rPatB2 and rPat17 had superior purity and more distinct isoelectric forms compared to commercial patatin, each isoenzyme presented better properties in different attributes. rPatB2 possessed higher specific activity than commercial patatin while specific activity of rPat17 was lower than the commercial protein. rPatB2 was also the only protein to retain activity (40%) after incubation at acidic conditions. However, substrate specificity with pNP-esters containing different chain lengths showed rPat17 had higher relative activity on pNP-Palmitate. rPat17 also had the highest denaturation temperature in both calorimetric measurement and fluorescence differential scanning and was the only protein to remain active (>90%) after 2 hours at 60°C. All-in-all, we have shown that recombinant patatins are comparable to potato isolated patatins in several aspects, and even surpass them in their stability under extreme pH and temperature conditions.

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Continuous colorimetric screening assays to explore both acceptor and donor substrates of amine-Transaminases

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Transaminases (TA) which convert a prochiral ketone into a chiral amine gained more and more interest in the past few years.[1–3] The development of efficient screening methods is still challenging to highlight new TAs in enzyme collections. Therefore, in the course of our research directed to the design of original synthetic processes involving TAs,[4–6] we have developed two continuous colorimetric assays for amine-TA. These new complementary methods are suitable for the high throughput screening of TA collections with both donor and acceptor substrates series. As shown below, the first one allows the assay of a variety of acceptors with L- or D-Ala as generic donor for amine-TAs.[7] Moreover, the use of hypotaurine (HPT) as irreversible amino donor in the auxiliary reaction brings an equilibrium shift while producing sulfur dioxide which is easily detected by spectrophotometry at 412 nm using Ellman's reagent (DTNB). The second one allows the assay of a variety of amine donors with pyruvic acid (Pyr) as generic acceptor which is converted to L- or D-Ala. In the case where L-Ala is produced, it is converted to the D-isomer using an Ala-racemase. Subsequently, the DAAO catalyzed oxidation of D-Ala produces H₂O₂ which is easily detected by spectrophotometry at 498 nm using horse radish peroxidase (HRP), vanillic acid and 4-aminoantipyrine (4-AAP).[8]

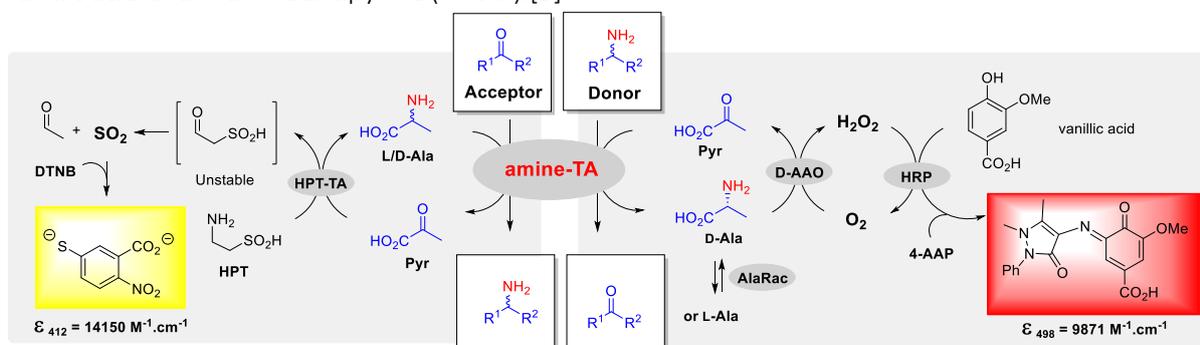


Figure 1. Continuous colorimetric screening assays towards donors and acceptors substrates.

We will present the development and applications of these two methods for the screening of a collection of 549 amine-TA from biodiversity.

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Engineered Myoglobin for Atom Transfer Radical Polymerisations

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Controlled Radical Polymerisations (CRP) have revolutionised the field of radical polymer chemistry. They offer control over molecular weight, chain end functionality, and dispersity to produce well defined polymers.[1] Enzymatic ATRP (bioATRP) has been reported and proofed by our group as an alternative solution, where the catalyst is sustainable protein, reaction is under mild condition and the polymer products could get rid of the metal pollution.

Enzymes that can catalyse ATRP reactions have been termed 'ATRPases'.[2] Several metalloproteins including hemoglobin, horseradish peroxidase and laccase have been successfully employed to the non-natural ATRP reaction in our group. Recently, our study revealed that myoglobin is another promising platform for bioATRP. To repurpose myoglobin as a well-adapted catalyst, we designed a semi-rational strategy for further engineering work: cofactor replacement and protein scaffold modification. The native cofactors of myoglobin, protoporphyrin IX, has been replaced with artificial cofactors, thus we could evaluate the effect of metals and macrocycles. The generated myoglobin variants have been screened in both of ATRP and peroxidase reactions. We tried to setup the correlation between them, so the colorimetric assay of peroxidase activity could be extended as benchmark screening method for further ATRPase mutants library, but the performance in bioATRP does not correlate to the activity in peroxidase reactions, suggesting two different reaction mechanisms. Protein scaffold modification has been achieved by genetic mutagenesis. We have identified several amino acid residues, which are supposed to affect the initiation and propagation phases during polymer synthesis. Further library design and screening work will help to unveil the mechanism behind bioATRP and to optimize artificial biocatalyst for controlled polymer synthesis.

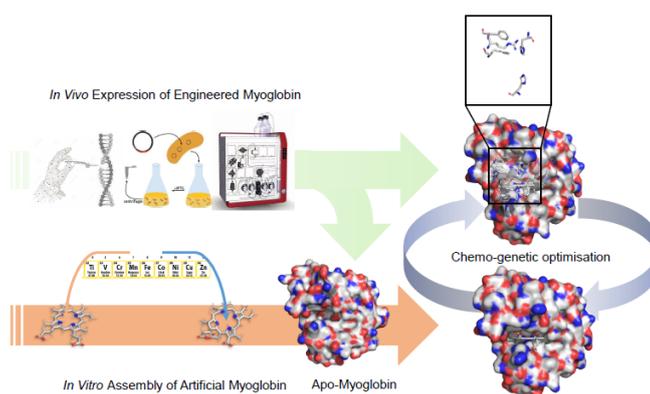


Figure 1. Working flow of engineering myoglobin as ATRPase

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Characterization and Immobilization of *Pycnoporus cinnabarinus* Carboxylic Acid Reductase - A Prospective Reusable Biocatalyst

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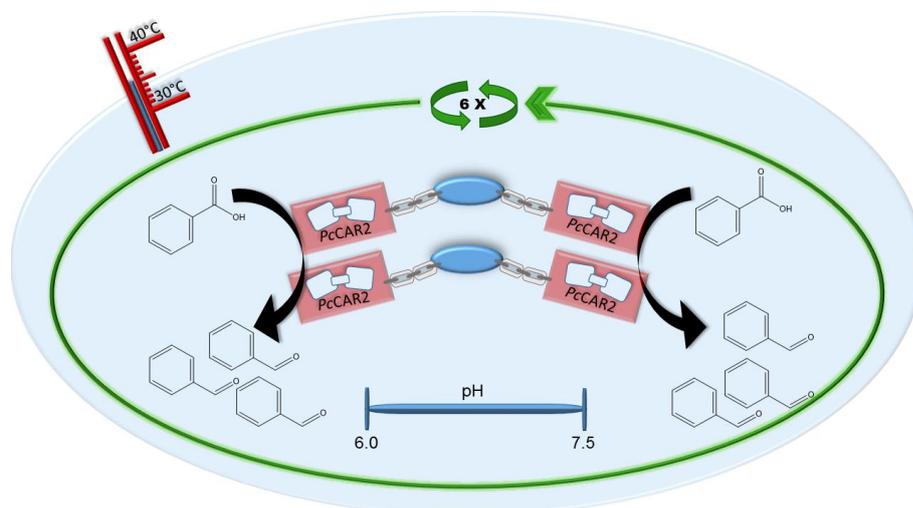
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Carboxylic acid reductases (CARs) are well-known for their eminent selective one-step synthesis of carboxylic acids to aldehydes [1]. To date, however, few CARs have been identified and characterized, especially from fungal sources and further, no immobilization studies using CARs have been conducted to the best of our knowledge. In this study, the CAR from white rot fungus *Pycnoporus cinnabarinus* (PcCAR2) was expressed in *Escherichia coli*. After purification, its biochemical properties were explored *in vitro*, revealing a melting temperature of 53°C, while the temperature optimum was at 35°C. In the tested buffers the enzyme showed a pH optimum of 6.0 and notably, a similar activity up to pH 7.5. PcCAR2 was immobilized to track its performance on benzoic acid and to explore its potential as a reusable biocatalyst. PcCAR2 showed no critical loss of activity after six cycles, with an average conversion to benzaldehyde of 85 percent per cycle (Scheme 1). All in all, our research contributes to the characterization of a thermostable fungal CAR, as well as the demonstration of a more sustainable use of the precious biocatalyst.



Scheme 1. Immobilized PcCAR2 performed 6 cycles with no critical loss of activity, demonstrating its potential as a reusable biocatalyst.

Acknowledgements

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New Advances in DERA substrate specificity

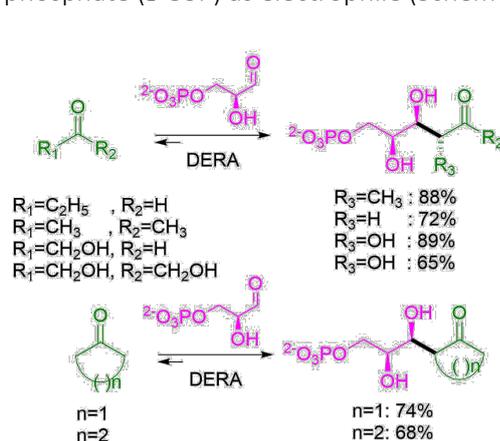
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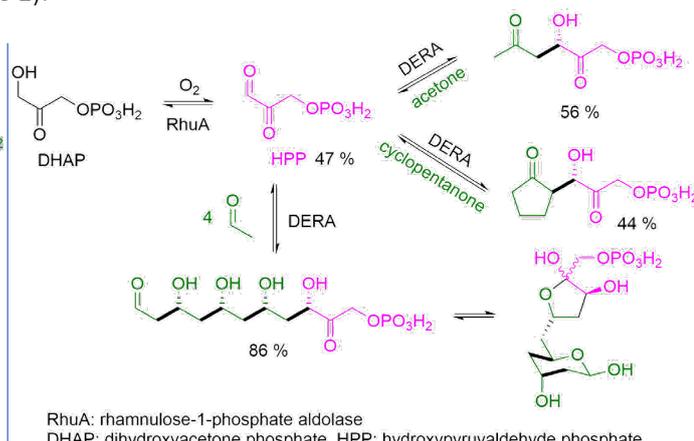
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C-C bond formation is a challenge in organic chemistry. The use of biocatalysts conciliates both a more environmentally friendly chemistry and the access to optically pure compounds. Aldolases are widely described in the literature, to synthesize molecules of interest with a high chemo-, regio- and stereo-selectivity. Among them, 2-deoxy-D-ribose-5-phosphate aldolase (DERA) is of particular interest since both the substrate and the product are aldehydes. Like other aldolases, it is specific for the nucleophile component, acetaldehyde, but more versatile towards the electrophile. Another interesting feature is the ability of DERA to accept the first aldol product as new acceptor in a second aldolization, enabling sequential cross aldol reactions. This property has been extensively developed for industrial preparations of statins and epothilones, anti-cancer and cholesterol-lowering drugs respectively.

In a will to break the dogma of its strict nucleophilic substrate specificity, we have explored the biodiversity by a sequence-driven approach and highlighted new DERAs able to convert ethanal, propanone and even glycolaldehyde, dihydroxyacetone, cyclobutanone and cyclopentanone, leading to rare monosaccharides from the L-series with good to excellent yields, using L-glyceraldehyde-3-phosphate (L-G3P) as electrophile (Scheme 1).^[1]



Scheme 1: Synthesis of rare monosaccharides, from the L-series, involving unusual DERA nucleophiles



RhuA: rhamnulose-1-phosphate aldolase
DHAP: dihydroxyacetone phosphate, HPP: hydroxypyruvaldehyde phosphate

Scheme 2: hydroxypyruvaldehyde phosphate as an acceptor platform for DERA catalysed reactions

In addition, we have explored the electrophile side and found that hydroxypyruvaldehyde phosphate (HPP), the oxidized analogue of G3P, prepared from promiscuous oxidation of DHAP with rhamnulose aldolase, has proven to be an efficient DERA substrate. When coupled in one pot-two step cascade reaction with DERA from *E. coli* or *Arthrobacter chlorophenolicus*, employing respectively acetone or cyclopentanone as the donor, an uncommon access to a 1,4-dicarbonyl unit was revealed through the isolated diuloses.^[2] Surprisingly, changing for the natural nucleophile, in the presence of DERA from *E. coli*, an unexpected quadruple acetaldehyde addition took place, never observed before (Scheme 2).^[3]

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Genome Mining for expanding pyruvate aldolases scope

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Aldolisation reaction is a staple of C-C bond creation in organic synthesis. Among the plethora of existing aldolisation methods, processes involving aldolases offer the benefit of working in eco-friendly conditions, avoiding protection and deprotection steps as well.^[1] In addition, the usefulness of these biocatalysts mainly resides in their capabilities to control the configuration of the newly created chiral centre(s). Nevertheless, the use of aldolases was both hampered by their high specificity towards their natural nucleophile substrate and the use of only aldehydes as electrophiles, leading invariably to secondary alcohol. With the objective to push forward these boundaries, pyruvate aldolases were reported to be able to accept other nucleophiles than pyruvate, like fluoropyruvate, but mainly class I pyruvate aldolases were concerned, *ie* generating an enamine as reactive intermediate with an active site Lys residue. On the contrary, few is known on class II pyruvate aldolases, *ie* harboring a metal cation leading to an enolate nucleophile.

Through a biodiversity research approach, we have shown in recent years that some class II pyruvate aldolases are able to use more polar nucleophiles than pyruvate (hydroxypyruvate)^[2] but also less polar (2-oxobutanoate,^[3] 2-oxopentanoate), demonstrating the great versatility of these biocatalysts. In addition to the flexibility of their active site towards the nucleophile, these aldolases were able to sufficiently activate the ketones and have accepted some of them as electrophiles, thus leading to tertiary alcohols.^[4] We will present herein a compilation of our results, thanks in some cases to the implementation of a pre-screening of potential nucleophiles by an NMR isotopic exchange technique. The powerful enzymes were then tested with several electrophiles, at the analytical scale, in the presence of the selected nucleophile, and finally involved in syntheses at hundred milligram scale. Stereoselectivities and yields of the best aldolases will be discussed.

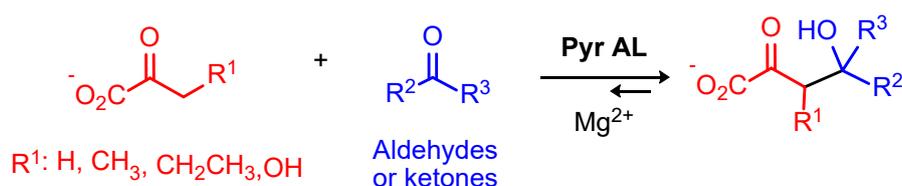


Figure 1. pyruvate aldolase as a must-have biocatalyst for eco-friendly preparation of chiral tertiary alcohols.

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Kinetic Characterization of Cytochrome c Catalysed Organoborane Synthesis

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Organoboron compounds are molecules that are synthesized from an organic component and a boron derivative by C-B bond formation. Due to the generated boron-containing carbon stereocenter, organoboranes perform as strong electrophiles with a remarkable reactivity.[1] Thus, especially organoboronic acids and organoboronic esters embody frequently used chemical precursors and synthesis intermediates. Polymerization catalysts, fuel supplements, antioxidants and pharmaceuticals are only a few examples for possible applications. The conventional chemical strategies to synthesize organoboranes are characterized by environmentally offensive methods, low enantioselectivity and poor total turnover numbers (TTNs). In contrast to the chemical methods, Arnold et al. reported a sustainable and green alternative for organoborane (OB) synthesis. Within this biocatalytic approach, an engineered variant of cytochrome c (Cyt c) catalyzes the biotransformation of a N-heterocyclic carbene borane (NHCB) and ethyl-2-diazopropanoate (DAC) to the corresponding OB product under mild reaction conditions with impressive selectivity and high TTNs in analytical scale (Figure 1).[2] However, before a synthesis in a reactor scale can be targeted, a characterization and optimization of the reaction has to be executed, guiding biocatalytic borylation to an industrial relevant alternative.[3]

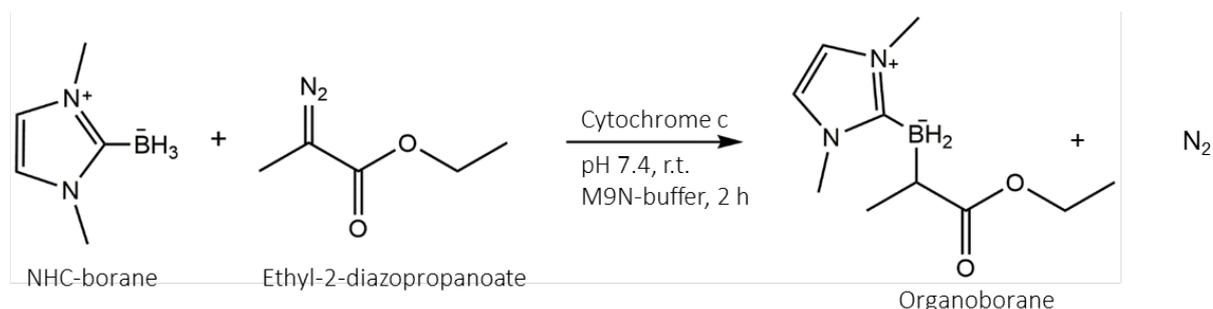


Figure 1. Reaction scheme of biocatalytic organoborane synthesis under moderate reaction conditions in minimal-9-nitrogen buffer using whole cell biotransformation in *E. coli* harbouring genetically engineered Cyt c Rma cyt c BOR^{R1}.

Objective:

This project aims at the characterization and optimization of the biocatalytic synthesis of organoborane compounds in order to provide approaches for an improved conversion, yield and productivity. As Cyt c represents the main part for biocatalytic borylation, the foundation of this study is a detailed kinetic characterization, to investigate the influence of different factors on enzymes performance. For this purpose, basic properties such as temperature and pH were evaluated, as well as the overall kinetics and inactivation effects of DAC. The collected data were used to setup a kinetic model in order to identify optimal reaction conditions, reactor concepts and operation points, which are verified in scaled-up batch and fed-batch modes as well as continuously operated processes. Here, a further investigation of the influence of the side reaction and substrate stability on the reaction process is carried out.

Acknowledgement:

We are grateful to the California Institute of Technology and Prof. Frances Arnold for providing us with the genetically engineered cytochrome c variant *Rma cyt c BOR*^{R1}.

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A New Metagenomic Panel of Epoxide Hydrolase Enzymes

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Biocatalytic epoxide opening is a useful synthetic tool for creating high value intermediates for many pharmaceutical compounds and fine chemicals (**Figure 1**). Epoxide hydrolases (EHs) are enzymes that are ubiquitous in nature, capable of catalysing stereoselective opening epoxides to form the corresponding vicinal diols. Currently, few EHs demonstrate the ability to utilise substrates that differ significantly from their natural substrates styrene and limonene epoxides. Recently, the use of metagenome mining to discover novel enzymes has become of increasing interest in biocatalysis. The advantages of metagenome mining include: discovering enzymes with novel activities; different selectivities; and tolerance to variable reaction conditions such as temperature, pH etc.¹⁻³ Previously, a panel of twenty EH enzymes had been discovered from metagenomic data and two showed activity on a variety of substrates as well as enhanced tolerance to solvents such as methanol, which is important for industrial application.⁴

In this collaboration with Prozomix and AstraZeneca we generated a new EH panel containing over 50 α/β EHs and Limonene EHs (LEHs) derived from various metagenomic samples across the U.K. These enzymes are capable of transforming a broad range of epoxides into their corresponding diols, notably demonstrating high activity on eight different bulky epoxides with varying R groups with almost quantitative conversion when screened by HPLC.

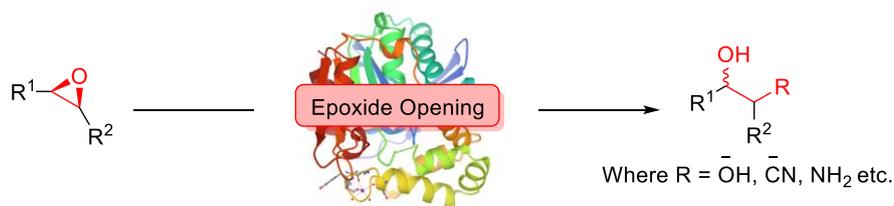


Figure 1. General scheme for biocatalytic epoxide opening reactions.

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Fingerprint of UPOs – Determining Chemo-, Regio- and Stereoselectivity of UPOs with different substrates

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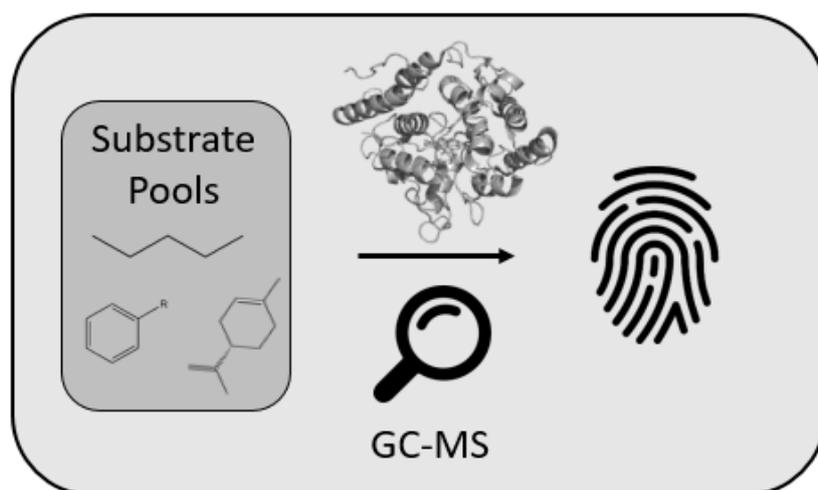
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Unspecific peroxygenases (UPOs) can oxyfunctionalise a broad set of substrates only requiring hydrogen peroxide as a co-substrate. High turnover numbers, stabilities and excellent selectivities render UPOs exciting enzymes for C–H activations. However, their heterologous expression and currently only few regioselective transformations present important challenges.^[1] With a modular secretion system in *P. pastoris* we were able to substantially broaden the available UPO scope and discover 18 yeast secreted new UPOs variants and chimeras.^[2]

To explore the active site of one of the discovered peroxygenases, *MthUPO* from *Myceliophthora thermophila*, we developed a high throughput GC-MS method.^[4] This method enabled the analysis of up to three substrates simultaneously in one reaction mixture. A mutant library was analysed and novel enzymes with enhanced chemo- and regioselectivity were discovered.



Scheme 1: Creating a fingerprint of UPOs.

We wish to apply a similar principle to fingerprint the available set of new or poorly characterised UPOs (Scheme 1). We envision to identify features, activities and selectivities of the different UPOs creating a detailed understanding of structure-substrate relationships.

Protein engineering campaigns can be based on the fingerprint using the most suitable variant as starting point. Additional, insights into the structure-substrate relationships simplifies the identification of critical amino acid residues involved in substrate binding and conversion.

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Novel reductions with an ene/yne-reductase from *Cyclocybe aegerita*: a new tool in biocatalysis

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The applications of ene-reductases (ER) in the chemical landscape are very diverse. Especially ERs of the flavin mononucleotide (FMN) depending Old Yellow Enzymes (OYE) were extensively studied in recent years. Nonetheless, ERs in general are still of great interest in sustainable and green chemistry.^[1] However, currently known ERs can reduce numerous structures with C=C bonds but a variable biocatalyst that is also able to perform “new-to-nature”-like reduction of C≡C bonds has only been shown once for solely one compound.^[2] Current methods in organic chemistry to reduce activated alkynes include the use of transition metal catalysts, organic solvents or complex techniques.^[3, 4] Taken together, the biocatalytic landscape lacks a reductase targeting C≡C bonds that opens up an uncovered field of biocatalysis. A promising portfolio of ERs with novel activities was shown via whole cell biotransformations of α,β -unsaturated substrates in various fungi from the phyla Ascomycota and Basidiomycota.^[5] This implicates a huge enzyme library with great potential in substituting conventional synthesis with environmentally friendly biocatalysts. Hence, we present the first ER from a filamentous fungi of the phylum Basidiomycota as a member of the MDR-superfamily displaying novel biocatalytic activities. This new biocatalyst shows highly efficient and regioselective reductions of $\alpha,\beta,\gamma,\delta$ -unsaturated aldehydes. In addition, it is able to reduce activated alkynes such as oct-2-ynal, 4-phenylbut-3-yne-2-one and 3-phenylprop-2-ynal to their saturated compounds which is the first report on such biocatalytic reductions of ERs.^[6] Taken together, with this ene/yne-reductase the biocatalytic gap of C≡C reductions is filled, that enables a new field of opportunities and challenges for protein engineering approaches, aiming for novel biocatalytic cascades.

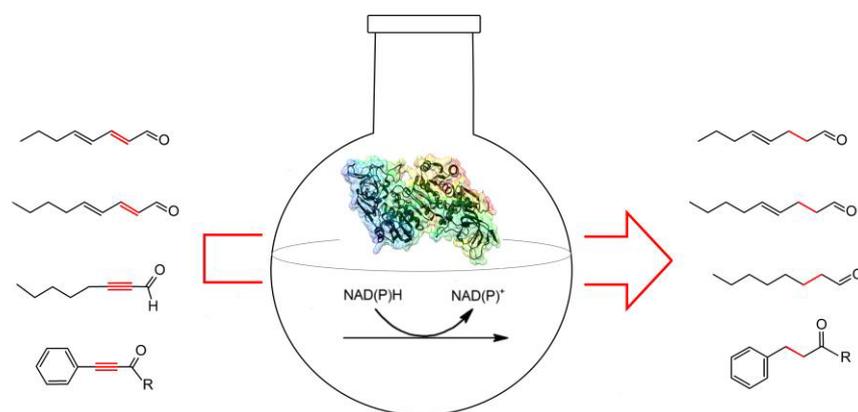


Figure 1. Novel biocatalytic reductions by CaeEnR1 from *C. aegerita* including $\alpha,\beta,\gamma,\delta$ -unsaturated aldehydes and activated aliphatic and aromatic alkynes.^[6]

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Identification and expression of urethane-bond hydrolysing enzyme

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A great amount of industrial and domestic wastes is still neither recycled, nor used in any other profitable way. Currently, intensively promoted Circular Economy model implicates that newly generated waste has to re-enter the production line and save valuable resources. Each waste material requires specific treatment, and a unified process may not be applied. For example, polyurethane (PU) is an excellent material that finds its way into many industrial and domestic products – from insulation to sleeping mattress. Also, nearly half of global unrecycled PU waste finds its way into landfills. PU represent nearly 8 % of 360 mln tons of plastics produced annually. Treating polyurethane wastes by hydrolysis, glycolysis, pyrolysis etc. demand a substantial amount of energy. Chemical hydrolysis still has not been applied on a commercial level because it is economically not favourable. Biocatalysis can be foreseen as one of the alternative treatment methods to for such type of waste. [1,2]

Currently, there are no biocatalytic or green chemical polyurethane utilization technologies or methods. Some polyurethanes are susceptible to enzymatic and microbial degradation.[3] The aim of this research is to investigate enzymes that could potentially hydrolyse urethane bond in PU. Various environmental samples have been screened and PU degrading microorganisms were isolated. Urethane bond hydrolysing enzyme urethanase (GenBank: MK757456.1) was found in *Lysinibacillus* sp. strain TBS 101. This bacterium was isolated from Lithuanian soil samples. Recombinant urethanase was successfully cloned and synthesized. Its ability to hydrolyse a model substrate - ethyl carbamate (urethane) - has been confirmed by Berthelot reaction. Specific enzyme activity reached up to 4.4 U/mg in *E.coli*. Further research is directed towards increasing the amount of urethanase by using heterologous expression systems, such as yeasts. The attained results in more detail will be presented during the poster session.

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Oxidoreductases as a versatile enzymatic system for pharmaceutical asymmetric synthesis

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Our research aims to develop new enzyme systems for the production of chiral synthons as single enantiomers. These synthons are components in the synthesis of biologically active compounds, as well as synthetic intermediates and fine chemicals. Isolated recombinant enzymes are of great interest for chemo-, regio- and enantioselective biotransformation of prochiral molecules. Therefore, there is a need to develop new enzymes with significantly improved catalytic efficiency that can mediate highly efficient and stereoselective chemoenzymatic transformations of various organic compounds.

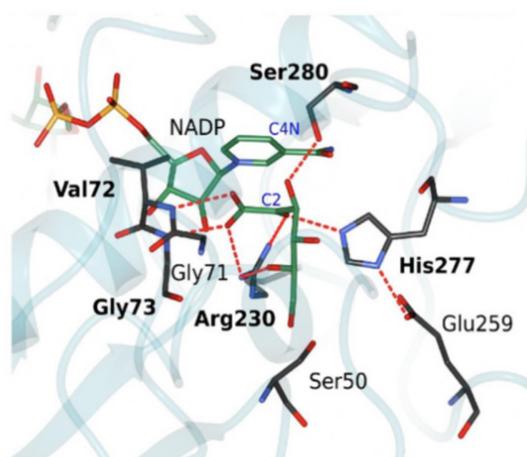


Figure 1. The catalytic site of ternary complexes of *SmGhrB* with 2-keto-D-gluconate and NADP [Kutner et al., 2018]

To this aim, new enzymes from the most numerous group of oxidoreductases, involved in both reductions and oxidations reactions, will be tested. Oxidoreductases are the leading class of enzymes used in the organic synthesis of optically pure chemical compounds. We have focused on new D-2-hydroxyacid dehydrogenases (2HADHs), which catalyze the NAD(P)H cofactor-dependent reduction of the prochiral 2-ketocarboxylic acids to a single enantiomer i.e. (R)-2-hydroxycarboxylic acid. We plan to use a new approach to change the properties of the tested enzymes, including their substrate specificity and kinetics, through enzyme engineering and solving the 3-dimensional structures (3D) of selected oxidoreductases, associated with their substrates or products, we will be able to look at the mechanism and of the catalyzed reaction at almost atomic resolution.

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Identification and Characterization of Microbial Catechol Dehydroxylases

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The human gut microbiota is a major component of the metabolism of endogenous and exogenous molecules and has a significant impact on human health. Efforts to predict and manipulate these metabolic pathways are often hindered by the limited understanding of the biochemical reactivity at the molecular level. Such a challenge is underscored by the uniquely microbial catechol dehydroxylation. Over the last 60 years, a wide range of biologically relevant molecules have been found to undergo dehydroxylation in the presence of different gut organisms. Yet, the identity of specific catechol dehydroxylases was unknown. The Balskus group recently identified dopamine dehydroxylase (Dadh) as the enzyme responsible for the conversion of dopamine to *m*-tyramine in the gut microbiota. [1] Subsequent work by the Balskus group and Turnbaugh group also linked genes encoding Dadh homologs to the metabolism of other catecholic substrates. [2,3] These studies provided a platform to begin a comprehensive investigation into microbial catechol dehydroxylases. Moreover, the discovery of Dadh and its homologs also propels effort to identify other phenolic motifs that are susceptible to microbial dehydroxylation. Herein, we present our effort in identifying and confirming the biochemical activity of other catechol dehydroxylases. In addition, preliminary studies into the mechanism of these novel enzymes will be discussed.

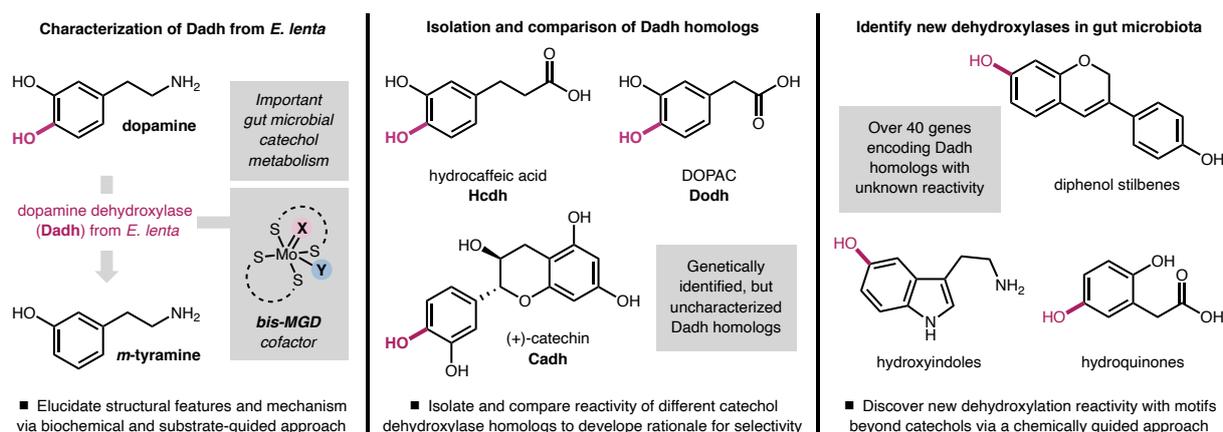


Figure 1. Recent discovery and current effort in the identification and characterization of microbial catechol dehydroxylases

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Development of myoglobin-based catalyst for reactions with organic radicals

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Enzymes can perform clean stereoselective transformations very efficiently under mild physiological conditions, however the scope of these reactions is limited by those essential for life. Adaptation of existing enzymes for the catalysis of reactions not previously observed in nature is one of the ways to use the enzymes for human needs.[1]

Since some porphyrin-based proteins[2] and catalysts[3] can perform Atom Transfer Radical Polymerizations(ATRP), we hypothesized that heme-containing proteins can catalyze organic radical based reactions with small molecules, such as Atom Transfer Radical Cyclisation(ATRC). Out of the range of heme containing proteins we selected myoglobin due to its well characterized structure, stability to mutagenesis and other engineering modes, like cofactor substitution.[4, 5] After introducing mutations in the heme coordination sphere, and therefore adjusting the pocket dimensions and/or polarity, metal redox properties we were able to engineer ATRC activity increasing the TON of the catalytic system from 0 to up to 150. The resulting system displays multiple advantages, including chemoselectivity towards ATRC compared to other observed reaction modes and opens up a possibility for further protein engineering in order to improve TON and ee.

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Towards novel polyethylene terephthalate (PET)-ases: Whole-cell biotransformation of PET-related substrates

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With more than 350 million tons of plastic being produced annually and a 3% annual increase in production, plastic waste has become a global environmental and health problem. Polyethylene terephthalate (PET) is a ubiquitous plastic and is the most commonly used packaging material (indicated by the recycling code '1') and used in the manufacture of 60% of fast fashion clothing. While post-consumer PET beverage bottles are highly commercially valuable, due to the high purity grade PET content, mixed multi-layered PET packaging, pots, tubs and trays as well as textiles are largely unrecyclable and present a considerable waste burden. Post use, only 9% of all plastic ever produced was recycled, the majority ends up in landfills, incinerators or the environment. Research in recent years has identified many enzymes with the potential to degrade plastic, however, only a handful are efficient enough to be used on an industrial scale. Advances in protein engineering of mentioned enzymes have largely improved their activity but a need for discovering new enzymes remains. In this work, we aimed to identify novel plastic degrading biocatalysts by screening novel microorganisms and employing a set of specific PET-related substrates, including PET- dimer and trimer.

A total of 251 microorganisms were isolated from contaminated and uncontaminated sites using four different growth media, in order to promote the diversity of isolated microorganisms. All of the isolated strains were screened for their potential to degrade synthetic polymers. The screening was carried out on Mineral Salt Medium (MSM) agar plates with various plastic polymers and monomers as the sole carbon source. Strains able to produce clearing halos on such plates were selected for further experiments. Seven strains produced clearing halos on bis(2-hydroxyethyl) terephthalate (BHET) a monomer of polyethylene terephthalate (PET).

In order to gain access to potentially novel PET hydrolases, eight PET-related substrates were used for biotransformation reactions in a whole-cell biocatalytic system. Three commercially available substrates (phthalic acid, terephthalic acid and BHET) and five newly synthesized PET-related esters named: M(HET)1, M2(HET)1,5, M(HET)2, M2(HET)2,5, M(HET)3 (Djapovic et al., 2021). Reactions were carried out with resting cells in MSM medium, incubated at 37°C for 72 h and monitored chromatographically with products characterized by NMR.

New biocatalysts showed markedly different patterns of cleaving. Some strains were able to hydrolyze terminal ethylene glycol moieties from a range of substrates and couldn't hydrolyze terminal methyl moieties. However, when M(HET)2 (PET dimer) was used as a substrate, some biocatalysts were able to specifically hydrolyze internal ester bonds.

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Identification and characterization of novel polysaccharide-degrading enzymes from metagenomic libraries of extreme environments

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Marine macroalgae have gained the attention of the scientific community due to their abundant biomass [1] and the numerous applications they can be used for.[2] Seaweeds consist mostly of polysaccharides with high structural complexity and high chemical and thermal stability, making them challenging for processing and refinement.[2a,b] An efficient way to gain access to the high-value products derived from seaweeds are polysaccharide-degrading enzymes.[3] Macroalgal carbohydrates are new products to industry and, therefore, there is a lack of enzymes active on these molecules.[2e,4] Hence, we focused our attention on discovering novel robust, thermophilic enzymes, originating from marine microorganisms living in extreme environments.

Via the collection of Hellenic Centre for Marine Research, we have access to genomic and metagenomic libraries from the Santorini volcanic complex of the Hellenic Volcanic Arc, Aegean Sea.[5] The libraries were constructed from microbial mat samples covering the seafloor of the Santorini-Kolumbo volcanic system and the surfaces of Kolumbo chimneys, from depths ranging from 300 to 500 meters. This volcanic system is characterized by high temperatures (of up to 220 °C in chimneys emitting fluids), high concentrations of heavy metals and low pH values. A methodology was developed on identifying polysaccharide-degrading enzymes of industrial interest. Based on our bioinformatics analysis we identified candidate genes of potentially thermostable proteins in the aforementioned libraries. As expected, the soluble expression of these enzymes from marine origin in *E. coli* turned out to be a challenging goal. Herein, we present the expression optimization, by utilizing chaperons and different *E. coli* strains, as well the subsequent characterization of the selected proteins of interest.

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BioWF – A natural fused biocatalytic system displays unexpected substrate promiscuity

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Biotin is an important vitamin (vitamin H) utilised as a cofactor by a number of carboxylase enzymes in essential metabolic processes such as fatty acid synthesis, amino acid metabolism and gluconeogenesis.^{1,2} Microbial biotin biosynthesis is divided into two stages, firstly, the synthesis of the C7 dicarboxylate pimelate moiety as a free pimeloyl-CoA thioester or bound to an acyl carrier protein (ACP). Secondly, the biotin bicyclic ring structure is formed, which is believed to be evolutionary conserved between different organisms. The biotin biosynthetic pathway has been studied in various organisms, with two distinct pimeloyl-CoA/ACP pathways; the more common BioC-BioH pathway found in *E. coli* and the much rarer BioW-dependent route which was discovered in *B. subtilis*.²

BioW is an adenylating enzyme responsible for the production of the C7 pimeloyl-CoA thioester in an ATP-dependent manner (Fig. 1).^{3,4} This is followed by the reaction of the pimeloyl-CoA intermediate with L-alanine through a decarboxylative, C-C bond forming, Claisen-like condensation. This step produces the key intermediate 8-amino, 7-oxononanoic acid (AON) and is catalysed by the pyridoxal 5'-phosphate (PLP)-dependent, class II aminotransferase AON synthase (BioF).⁵ Our group discovered that the *B. subtilis* BioW displays precise selectivity, only accepting the C7 pimelate substrate that controls the structure of biotin.³ However, using the x-ray crystal structure, *Bs*BioW was rationally engineered by replacing a conserved Tyr that defined the acyl-chain length selectivity of the enzyme. A simple Tyr to Phe mutation expanded the fatty acid substrate scope and enabled the synthesis of various acyl-CoAs.³

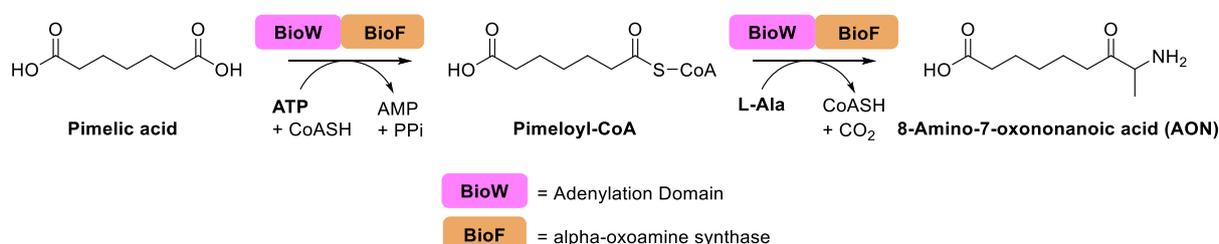


Figure 1. Schematic of the BioWF mechanism of action, highlighting the domains involved in each catalytic step.

In our search for novel biocatalysts, we identified an unusual, BioWF didomain fusion in the bacterium *Corynebacterium amycolatum*. In contrast to the highly selective *Bs*BioW, the *Ca*BioW displayed unexpected promiscuity, accepting a range of mono and di-fatty acid substrates. Sequence and structural analysis revealed a natural Phe residue in place of the highly conserved Tyr in the predicted active site. Based on our *Bs*BioW engineering, this promiscuity is due to the Tyr/Phe swap. However, the *Ca*BioWF catalysed formation of various AON derivatives suggesting this substrate promiscuity extends to the fused BioF domain. Such unexpected broad substrate scope suggests that *Ca*BioWF is a naturally promiscuous biocatalyst that can be utilised to prepare a number of AON analogues.

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Functional characterization and synthetic exploitation of novel transaminases from extremophiles (meta)genomes

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Pyridoxal-5'-phosphate (PLP)-dependent transaminases (TAs, EC 2.6.1.x) catalyze the transfer of an amino group from an amine donor substrate to a ketone or aldehyde acceptor substrate. These enzymes operate under environmentally friendly conditions and usually show an excellent stereoselectivity in the amination of prochiral substrates. Therefore, they have been widely studied during the last decades for the preparation of chiral amines in the pharmaceutical and fine chemical industries.[1]

Recently, novel TAs were identified by *in silico* screening of hot spring metagenomes prepared from samples collected at temperatures ranging from 55 to 95 °C. Specifically, three novel (*S*)-selective TAs, namely Is3-TA, It6-TA, and B3-TA, were discovered in the metagenome of samples collected from hot springs in Iceland and in Italy, cloned from the corresponding metagenomic DNAs and overexpressed in recombinant form in *E. coli*. [2] All the new TAs showed a thermophilic character, as well as a good potential for practical synthetic applications.

Moreover, a new TA, namely Ms-TA2, was recently identified by us from a *Meiothermus* strain capable of using β -phenylalanine for growth and isolated by culture enrichment of samples collected in hot environments. [3] After recombinant production in *E. coli* Rosetta(DE3) cells, Ms-TA2 showed a good thermostability along with high activity towards (*S*)- β -phenylalanine and other (*S*)- β -amino acids, as well as a preference for α -ketoglutarate and aromatic aldehydes as amino acceptors.

Herein we will present our recent results in the preparation and functional characterization of these novel biocatalysts. In particular, the recombinant expression of some extremophilic TAs has been significantly improved by exploiting either a SUMO fusion protein system or chaperonins co-expression, while the substrate scope of the novel TAs was further defined by screening different amino donors and acceptors. Selected reductive amination reactions of synthetic interest have been optimized to maximize the results in terms of conversions and selectivity.

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Single-step reaction and enzyme cascade – Biocatalytic synthesis routes for novel cyclic dinucleotides

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Cyclic-GMP-AMP (cGAMP) is a secondary messenger in metazoans and functions as inducer for the production of type I interferons as strong agonists of the stimulator of interferon genes (STING). cGAMP is of relevance in immunology research and a promising candidate in clinical phase immune oncology studies. The biocatalytic cGAMP synthesis with cGAMP synthase (cGAS) offers some advantages compared to chemical synthesis, such as shorter synthesis time and the avoidance of complex protective group chemistry. Nevertheless, the catalytic capacities and potential of cGAS as biocatalyst are not fully known and by far not exhausted.

Numerous cGAS homologs are predicted in protein databases covering a broad range in the field of vertebrates. However, the catalytic activity towards cGAMP synthesis has been proven for only few of them. Cell-free protein synthesis (CFPS) was used as a rapid pre-screening system to investigate if cGAS homologs originating from higher organisms can be efficiently expressed in an *Escherichia coli*-based expression system.[1] All tested variants were expressible and, interestingly, most of them catalyzed cGAMP-synthesis, even though with a vast difference in their activity. One of these cGAS homologs was characterized in more detail with regard to its substrate acceptance of a broad spectrum of nucleotide derivatives (Figure 1).[2] The products, cyclic dinucleotide (CDN) derivatives, are highly valuable for pharmaceutical applications as a few of them are known to be more hydrolysis-resistant or having a higher affinity towards the STING receptor. Human cGAS turned out to be promiscuous catalyzing the synthesis of a variety of novel CDN derivatives. Most of the investigated substrate derivatives, which had modifications at the nucleobase, ribose, and the α -thio phosphate, were converted into novel CDNs. Selected derivatives were subsequently produced on a preparative scale demonstrating the utility of cGAS for the biocatalytic synthesis of CDN derivatives in a one-step reaction.

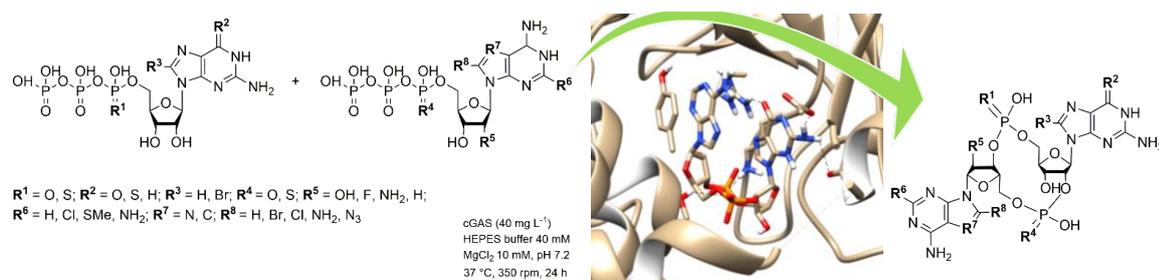


Figure 1. cGAS-catalyzed biotransformation of nucleotide derivatives into cyclic dinucleotide derivatives.

This enzyme was also investigated in combination with three kinases to develop a one-pot enzyme cascade route for cGAMP production, which starts from less expensive adenosine instead of ATP.[3] The four-enzyme cascade was successfully established and optimized. Thus, the achieved synthesis rate of cGAMP was comparable to the one-step syntheses with ATP as initial substrate. The cGAS-catalyzed reaction was hence running close to its optimum. This proves the successful application of enzyme cascades for the synthesis of pharmaceutically relevant CDNs.

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Structural and biochemical studies enlighten the unspecific peroxygenase from *Hypoxylyon* sp. EC38 as an efficient oxidative biocatalyst

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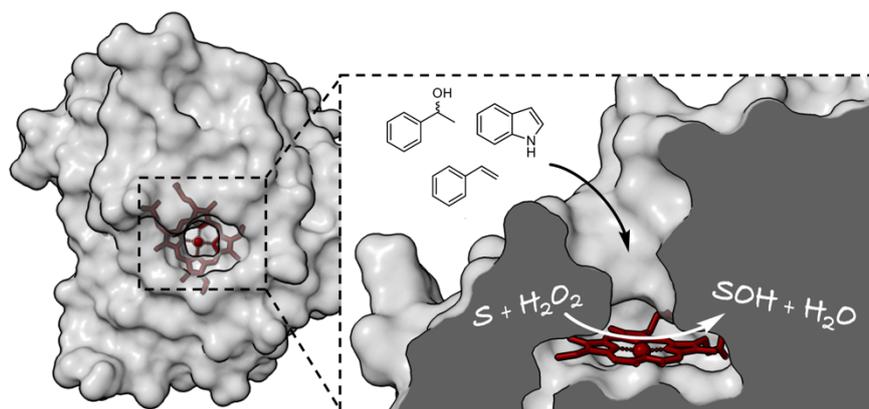


Figure 1. Overall representation of HpUPO structure.

Unspecific peroxygenases (UPO) are glycosylated fungal enzymes that can selectively oxidize C-H bonds. Differently from cytochrome P450s, UPOs employ hydrogen peroxide as oxygen and electron donor and do not need an additional reducing agent [1]. With such an easy-to-handle co-substrate, UPOs are emerging as convenient oxidative biocatalysts. Here, a novel unspecific peroxygenase from *Hypoxylyon* sp. EC38 (*HpUPO*) was identified in an activity-based screen of six putative peroxygenase enzymes that were heterologously expressed in *Pichia pastoris*. The enzyme was found to tolerate selected organic solvents such as acetonitrile, which even boosted the activity up to six-fold. *HpUPO* turned out to be a rather versatile catalyst performing various reactions, such as the oxidation of *prim*- and *sec*-alcohols, epoxidations and hydroxylations. Semi-preparative biotransformations were demonstrated for the non-enantioselective oxidation of racemic 1-phenylethanol to reach completion (TON = 13000), giving the product with 88% isolated yield, and the oxidation of indole to give indigo (TON = 2800) with 98% isolated yield. *HpUPO* features a compact and rigid three-dimensional conformation that wraps around the heme and defines a funnel-shaped tunnel that leads to the heme iron from the protein surface. The tunnel extends along a distance of about 12 Å with a fairly constant diameter in its innermost segment. Its surface comprises both hydrophobic and hydrophilic groups for dealing with small-to-medium size substrates of variable polarities. The structural investigation of several protein-ligand complexes revealed that the active site of *HpUPO* is accessible to molecules of varying bulkiness and polarity with minimal or no conformational changes, explaining the relatively broad substrate scope of the enzyme. With its convenient expression system, robust operational properties, relatively small size, well-defined structural features, and diverse reaction scope, *HpUPO* is an excellent candidate for peroxygenase-based biocatalysis.

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Novel Levansucrase enzymes: catalytic properties, kinetic parameters, and end-product profile

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Novel prebiotics fructooligosaccharides (FOSs) can be obtained in a highly regio- and stereo-controlled manner through enzymatic synthesis. The biocatalytic approach based on Levansucrase (LS)-catalysed transfructosylation reaction is of particular interest for the synthesis of well-defined FOSs (figure 1). The most interesting feature of LSs results from their ability to use the free energy of cleavage of sucrose to synthesize β -(2-6)-levan and FOSs [1]. Despite, the progress that has been made, the use of LS is currently hampered by their poor availability, thermal stability, their unfavourable hydrolytic activity, and the limited modulation of FOS structures.

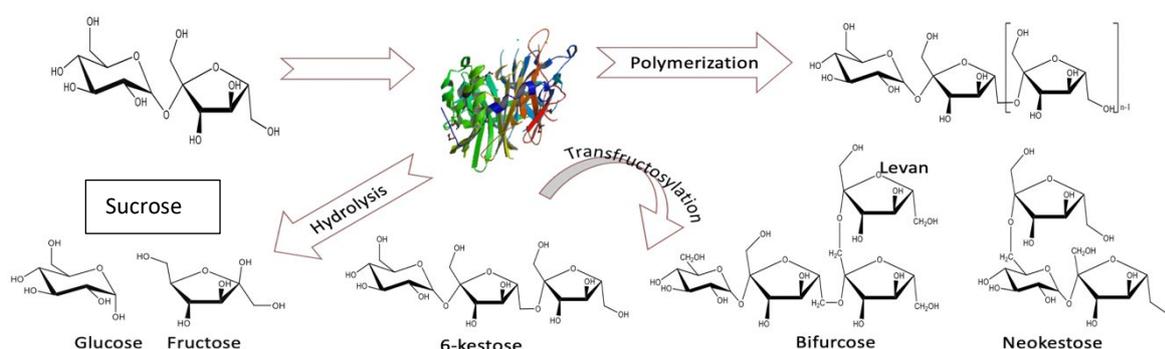


Figure 1. Levansucrase's ability to use energy of cleavage of sucrose to catalyse 3 reactions: Hydrolysis, Transfructosylation and Polymerization.

The present study aims at characterizing the catalytic properties and efficiency of new LSs, expressed in *E. coli*, from *Novosphingobium aromaticivirans*, *Vibrio natriegens*, *Gluconobacter oxydans* and *Burkholderia graminis*. To determine the optimal temperature and the activation energy (E_a) for the transfructosylation and hydrolytic activities of selected LSs, the enzymatic reactions were carried out at a broad range of temperature varying from 15 to 60° C. While the optimal pH was investigated in the pH range of 3 to 9 using different buffer systems. LSs from *N. aromaticivirans* and *V. natriegens* exhibited a maximum ratio of transfructosylation to hydrolysis activity at 45° C; while the ratio for LS from *G. oxydans* and *B. graminis* was the highest at lower temperatures (30 and 35° C, respectively). The E_a of LS from *G. oxydans* (51.84 kJ/mol) was higher than that of LS from *V. natriegens* (28.03 kJ/mol), *N. aromaticivirans* (33.88 kJ/mol) and *B. graminis* (18.24 kJ/mol). This indicates the high-temperature sensitivity of the reaction-catalyzed by LS from *G. oxydans*. As for the optimal pH, LS from *V. natriegens* and *G. oxydans* exhibited a maximum ratio of transfructosylation to hydrolysis activity at the acidic pH of 5, whereas the ratio for LS from *N. aromaticivirans* and *B. graminis* showed a maximum value at neutral pH of 6 and 7 respectively. The catalytic efficiency was determined for each of the enzymes' transfructosylation and hydrolysis activity, showing that LS from *G. oxydans* has the highest transfructosylation catalytic efficiency (1948 s⁻¹ mM⁻¹) to hydrolysis (974 s⁻¹ mM⁻¹) compared other LSs. Our results highlight the end-product profile of each LS using sucrose or raffinose as a donor substrate and reveal the its chemical diversity.

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Screening of fungal unspecific peroxygenases for functional expression in *Pichia pastoris*

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Keywords: unspecific peroxygenases, *Pichia pastoris*, heterologous expression

Discovered in 2004 unspecific peroxygenases (UPOs, EC 1.11.2.1) represent a novel group of secreted fungal enzymes with the ability to introduce oxygen from peroxides to organic substrates [1]. The most attractive reactions catalyzed by UPOs are hydroxylation, epoxidation and dealkylation, which can be used for the synthesis of valuable chemicals. To date a number of UPO genes have been identified, and more than hundreds of substrates accepted by these enzymes have been reported [2]. This substrate spectrum overlaps to some extent with that of cytochrome P450 monooxygenases (P450s). Compared to UPOs, P450s have some intrinsic drawbacks, e.g. their intracellular occurrence that entails low stability, generally low turnover numbers and their dependence on the cofactors NAD(P)H and additional redox partner proteins. In contrast, UPOs are mainly secreted enzymes that use only H₂O₂ as oxygen donor and electron acceptor. Nevertheless, broad exploitations of UPOs is limited by their difficult heterologous expression and secretion in recombinant hosts [2].

Within this project new putative UPO genes have been identified by database analysis and tested for heterologous expression in the methylotrophic yeast *Pichia pastoris*. The genes were inserted with their native signal sequence for secretion under control of the methanol inducible *AOX1*-promoter into *P. pastoris* X-33. *Pichia* transformants were screened for activity towards 2,2'-Azino-di(3-ethylbenzthiazolin-6-sulfonsäure) after expression in shaking flasks. Among others, a UPO from *Aspergillus* sp. has been identified using a BLAST database search with the previously described *Cg/UPO* from *Chaetomium globosum* as template [3]. The new UPO with an amino acid sequence identity to *Cg/UPO* of around 50 % could be successfully expressed in *P. pastoris*. The recombinant UPO was produced in a fed-batch cultivation of recombinant *P. pastoris* and purified for subsequent characterization.

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Development of carboxylic acid reductases towards challenging amide bond formations

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Amide bonds are recognised as the most prevalent motifs found in drugs. Therefore, the development of selective and eco-friendly amidations is paramount in synthetic chemistry. Biocatalytic solutions for amide bond formations provide timely alternatives to conventional approaches. Despite the plethora of strategies in nature, amide bond forming enzymes still suffer from narrow acid and amine scopes as well as low efficiencies, thus limiting their applications in synthesis. Re-tasking of carboxylic acid reductases (CARs) unveiled novel promiscuous, biocatalysts for the activation of carboxylic acid moieties. Engineering and design of modular cascades allows for the selective synthesis of challenging, pharmaceutically relevant amides under aqueous conditions.

In their native reaction, CARs, consisting of three domains, catalyse the reduction of carboxyl groups into aldehydes by initial activation of the carboxylic acid in presence of ATP leading to an acyladenylate (Fig. 1A). The latter species is subsequently reduced to the aldehyde by NADPH.[1] It was shown that CARs are able to accept amine nucleophiles when employing an excess of amine whilst omitting NADPH.[2] The activated acyladenylate formed in the adenylation (A-) domain is intercepted by the amine resulting in the corresponding amide. Isolated, catalytically active A-domains can be generated by truncating the full-length enzymes providing versatile tools for amidation reactions. Applying the truncated CAR from *Mycobacterium marinum* (CAR_{mm}-A) facilitated selective monoacylation of symmetrical diamines. Moreover, by optimising and employing an ATP recycling system up-scaling succeeded making the synthesis of an array of pharmaceutically relevant amides possible (Fig. 1B).[3] Recently, the panel of truncated CARs amenable for amide bond formation has been expanded to address a wider substrate scope. We emphasised on the integration of CAR A-domains into enzyme cascades that allows to exploit the broad carboxylic acid tolerance of newly established CAR variants. By broadening the reaction scope and using alternative nucleophiles, streamlined reaction pathways were developed that enable synthetically viable, challenging amide couplings, e.g., involving bulkier amines and anilines. A modular approach utilising the promiscuity of CAR A-domains for the synthesis of complex amides towards pharmaceutical key compounds is being presented.

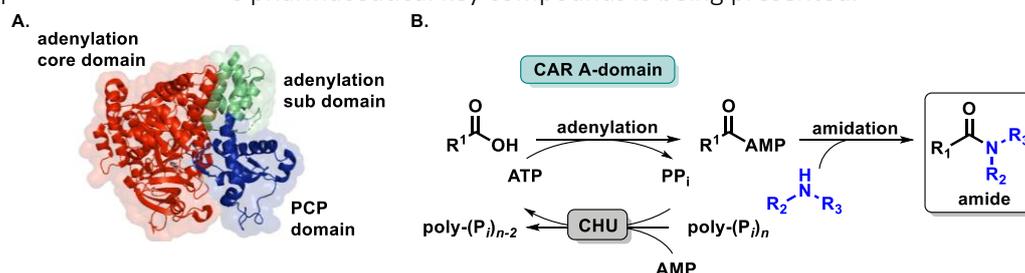


Figure 1. A. Domain organisation of CARs; adenylation and PCP domain are depicted (PDB: 5MSS). Amide bond formation using isolated CAR A-domains along with a polyphosphate kinase for ATP recycling.

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Functional characterization and comparison of novel NADH oxidases for efficient recycling of oxidized nicotinamide cofactor

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Oxidoreductases are widely applied in industry for different transformations. Many industrially interesting oxidoreductases dependent on nicotinamide cofactors to accomplish these transformations[1]. Considering the cost associated with these cofactors, regeneration of these cofactors becomes a necessity. Cofactor regeneration can be accomplished in several ways, but the most common approach for cofactor recycling in oxidation reactions is enzyme coupling with NADH oxidase[2]. The number of available NADH-oxidases is still limited to carry out efficient cofactor recycling of oxidized nicotinamide cofactor. The research focuses on the characterization of novel NADH oxidases and exploring the feasibility of using these enzymes for cofactor regeneration. To benchmark the new enzymes an already known and described enzyme was used as a reference, the water-forming NADH oxidase from *Lactobacillus pentosus* [3].

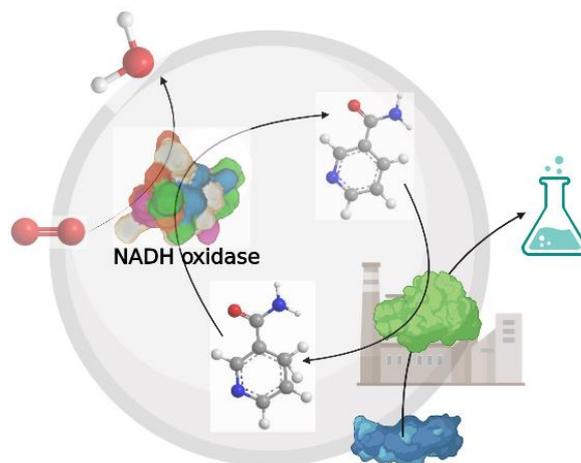


Figure 1. Cofactor regeneration in a chemical synthesis process using NADH oxidase from *Herbaspirillum seropedicae*

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Highly efficient synthesis of chiral amino alcohols using novel (*S*)-selective amine transaminases

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Stereoselective amination of prochiral acyloins and aldols is a current gap in the biocatalysis toolbox aiming at the environment-friendly improvement of industrial chemical processes. The chirality of the amine moiety often determines biological activity in numerous natural and synthetic metabolites of pharmaceutical and agrochemical value. Among several groups of enzymes capable of performing this reaction, transaminases (TAs) are enticing catalysts due to their robustness, outstanding enantioselectivity and independence on consumable co-factors (Figure 1).

Despite prominent achievements in the engineering of (*R*)- and (*S*)-selective TAs for industrial applications,[1, 2] a complementary genomic and metagenomic search likewise provides an abundant supply of excellent enzyme candidates with high efficiency towards natural and artificial substrates.[3]

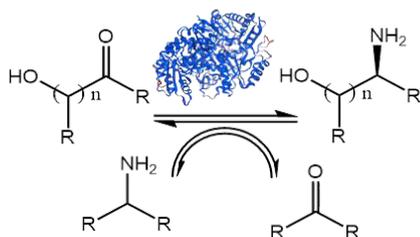


Figure 1. (*S*)-Stereoselective enzymatic transamination of prochiral hydroxycarbonyl substrates.

Screening a commercial TA panel allowed the identification of a set of the novel (*S*)-selective amine transaminases (ATAs) with outstanding activities towards non-aromatic hydroxycarbonyl substrates. The selected biocatalysts are temperature and pH tolerant, accept a broad range of linear and cyclic mono- and polyhydroxylated ketones and aldehydes, and resist high concentrations of isopropylamine, an industrially attractive amine donor. These characteristics facilitate nearly semimolar synthesis of enantiopure amino alcohols during a reasonable (1-2 days) incubation period. Additionally, potent candidates for diastereoselective resolution of vicinal amino alcohols were identified in the TA library and characterized.

Broad substrate promiscuity and process robustness make the discovered ATAs promising candidates for the preparative stereoselective synthesis of chiral amino alcohols, as well as attractive objects for protein engineering towards further improvement in their catalytic capacities.

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Expansion of the substrate and nucleophile scope of HheG homologues

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Halohydrin dehalogenases (HHDHs) are biocatalytically relevant enzymes as they allow for the synthesis of various β -substituted alcohols by nucleophilic epoxide ring opening with formation of new C-C, C-N, C-O and C-S bonds [1]. Recently, HheG from *Ilumatobacter coccineus* was shown to display a remarkable activity in the ring opening of cyclic epoxides such as cyclohexene oxide and limonene oxide, which is unique within the family of HHDHs [2]. Moreover, we could demonstrate that HheG converts also a range of acyclic vicinally di-substituted epoxides [3]. We have now identified and characterized two HheG homologues (HheG2 from *Ilumatobacter nonamiensis* and HheG3 from *Actinobacteria bacterium*) displaying a similar substrate scope as HheG. Moreover, all three enzymes have been studied in the conversion of a larger set of cyclic epoxides using five different nucleophiles.

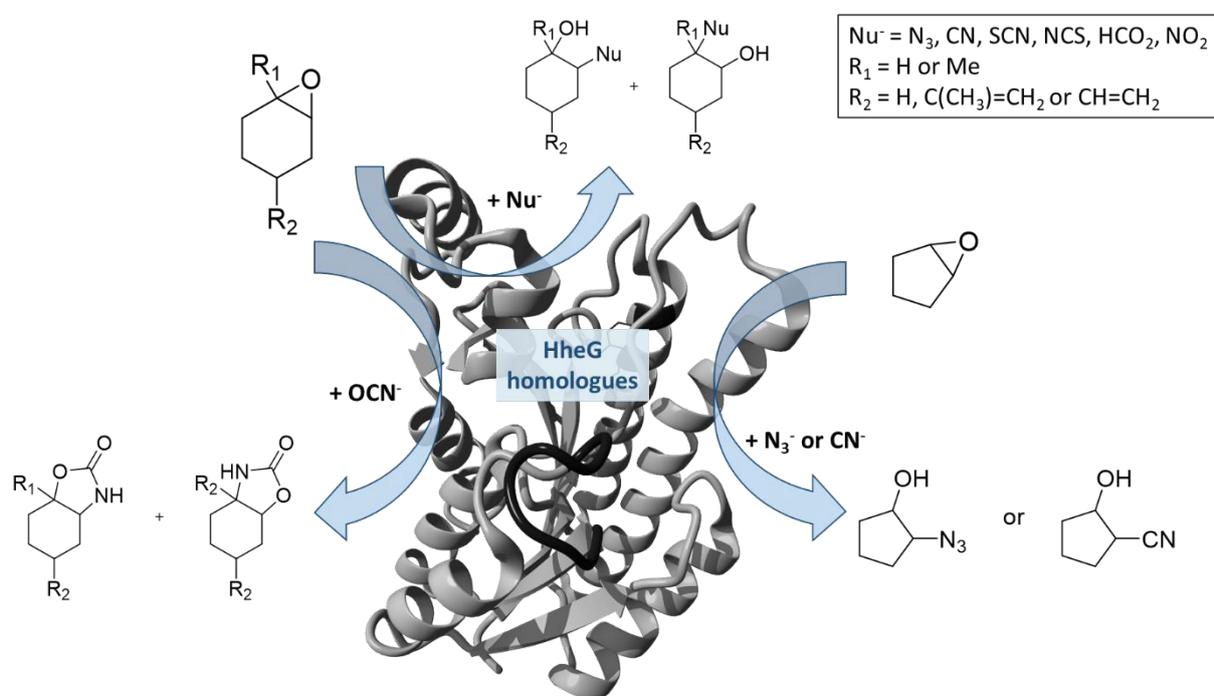


Figure 1. HheG-catalyzed epoxide ring opening using various substrates and different nucleophiles.

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Hydrogenase is a robust flavin recycling catalyst: demonstrated for atom-economical, selective alkene hydrogenations by ene-reductases

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Flavoenzymes catalyse a wide range of useful chemical reactions owing to the diverse redox chemistry enabled by flavin cofactors.[1] Valuable flavoenzymes for chemical synthesis include ene-reductases (alkene reduction), monooxygenases (epoxidation, hydroxylation, Baeyer-Villiger oxidation), and halogenases (halogenation). The application of flavin-dependent enzymes in industrial processes remains under-utilised, perhaps due to the complexity in recycling reduced flavin to supply to these enzymes. Traditionally, these enzymes are supplied with external flavins that are reduced by an NAD(P)H/flavin reductase system, and NAD(P)H is in turn recycled using a waste-intensive glucose/GDH system. Other novel methods for flavin recycling include photochemical, electrochemical or metal-catalysed reductions which can suffer from biocompatibility and selectivity issues or photodecomposition of the flavins themselves.[2,3] We recently made the discovery that the robust [NiFe] Hydrogenase 1 (Hyd1) from *E. coli* reduces flavins, FAD and FMN, using electrons from H₂ oxidation. We employed this as a simple, atom-efficient cofactor recycling system, and accomplished stereoselective alkene hydrogenations by coupling to a suite of Old Yellow Enzyme (OYE) ene-reductases. Conversions of up to 100% with >99% *ee* were observed (Figure 1.)[4] The O₂-tolerant Hyd1 was active across a broad temperature range, stable for >5 days, and Hyd1 turnover numbers of up to 20,200 were recorded. The straightforward reaction setup, excellent atom-economy, and high enzyme stability highlight hydrogenase as a useful flavin-recycling catalyst that has potential for implementation with flavoenzymes in industrial biocatalysis.

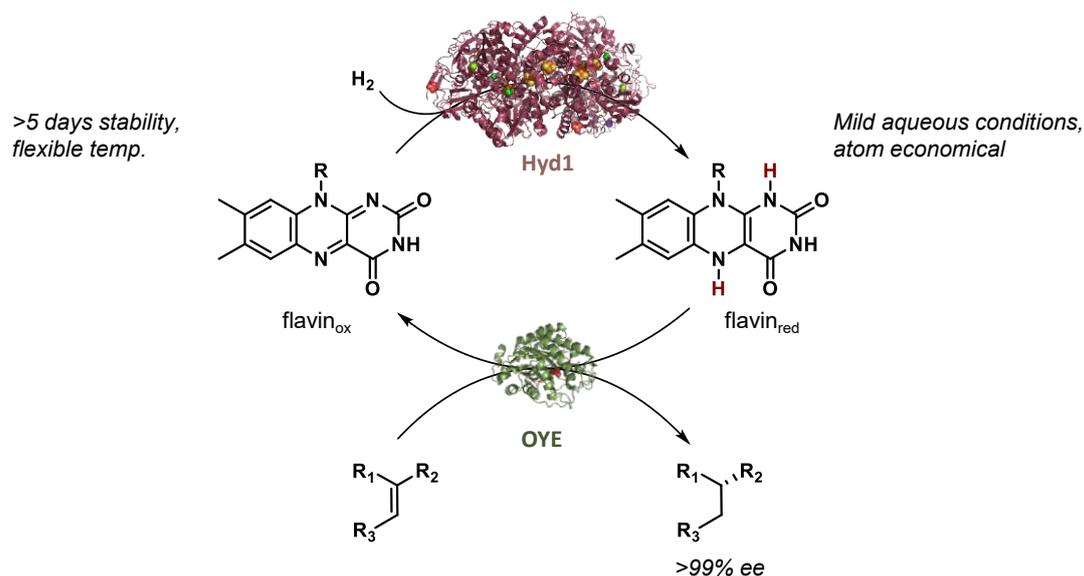


Figure 1. Hyd1 catalysed flavin reduction used for the selective synthesis of alkenes catalysed by OYE ene-reductases

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Repurposing of plant-biomass hydrolyzing enzymes for the degradation of polyethylene terephthalate (PET)

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The progress of modern society has been highly dependent on the advancement of polymer science that, starting in the 1950s, created materials with extraordinary properties. These materials found their place in our daily lives and have shaped modern society as we know it today. However, the overuse of plastics, especially single-use plastics, and their inherent resistance to degradation have created the problem of plastic accumulation in the environment. Single-use plastic bottles, for example, that are widely used as water and beverage containers, are made of polyethylene terephthalate (PET). Even though PET is a fully recyclable polymer, less than 28% of PET bottles were recycled in the United States in 2018, while 57% of the produced bottles were disposed in landfills.

Phytopathogenic microorganisms (mostly bacteria and fungi) play an important role in the planet carbon cycle by utilizing the plant biomass, that was created through photosynthesis. In order to do so, they have developed very sophisticated mechanisms to tackle all plant defenses. Plant cell-wall is composed of non-starch polysaccharides (cellulose and hemicellulose), as well as a non-carbohydrate phenolic-based recalcitrant polymer called lignin. On top of these, most aerial parts of plants are covered with an extra barrier; an oxygenated fatty-acid cross-linked polyester, called cutin. Through millions of years of evolution, these microorganisms have become capable of producing enzyme cocktails that act on these recalcitrant crystalline and/or hydrophobic polymers and break them down to their monomers.

Since plastic materials are relatively new to this planet, the natural biomass degraders have not yet evolved specialized mechanisms for their degradation. Nonetheless, one can detect similar properties and in some cases linkages of natural polymers with some of the manmade ones. Hence, enzymes dedicated to plant polymer degradation can be repurposed for the breakdown of synthetic polymers. One example that is known to the literature for a while now, is the enzymes of the cutinase family, that have been tested for their ability to degrade various natural and synthetic polyesters.

The activity of various (mostly bacterial) cutinases has been tested against amorphous PET with promising results. On the contrary, reports of cutinases with the ability to hydrolyze crystalline PET (similar to bottle-material) are scarce. Additionally, synergistic activity of the enzymes may prove more efficient for polymer degradation.

In the present work, we utilized two cutinases originating from phytopathogenic fungi *Humicola insolens* and *Fusarium oxisporum* and combined their action with carboxylesterases of different families, whose natural substrate is lignocellulose. The synergistic effect of these enzyme cocktails was assessed on PET degradation and their action mechanism was studied on PET-model compounds.

Our goal is to identify such synergistic relationships and then use these enzymes as templates for directed evolution in order to speed up Nature's process by a few thousand years and address plastic biodegradation in a more efficient way today.

Lipase activity of plant latices from Apocynaceae species

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It is well known that lipases are among the most used enzymes in different industries, ranging from biorefinery to pharma and food processing. Commercial enzymes are mainly of microbial origin. However, their cost is usually very high and, therefore, a cheaper alternative to lipases such as Novozym® 435 and Lipozyme® IM is of utmost interest [1]. Within this context, plant lipases represent a very attractive alternative, since extracts with lipolytic activity are relatively easy to obtain [2]. Latex is a fluid containing a mixture of compounds that plants use for defence when attacked by insects. Although its composition depends on plant species, different enzymes can be found in latex. Among them, lipases and esterases were detected in the most emblematic plant latex with enzymatic activity, *Carica papaya* latex. Similarly, lipolytic activity was found in other plant latices [3].

In this contribution, we report the biochemical characterization of lipolytic and esterification activity of the insoluble gums obtained from the latices of two species belonging to the Apocynaceae family, *Araujia serificera* (Fig. 1) and *Calotropis procera* (Fig. 2).



Figure 1



Figure 2

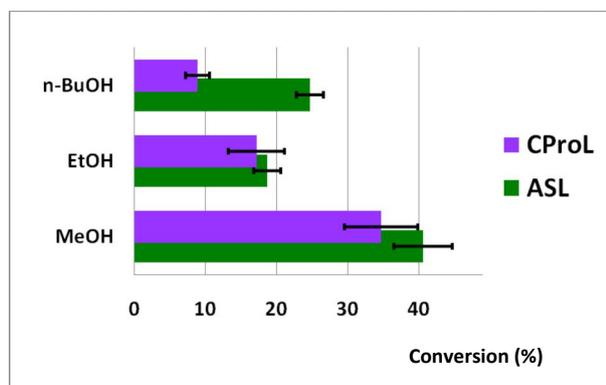


Figure 3

Both *Araujia serificera* lipase and *Calotropis procera* lipase (ASL and CProL, respectively) showed hydrolytic activity with optimum pH in the alkaline range (8 to 12 for ASL and 9 to 11 for CProL). Esterification activity was tested using oleic acid (20 mM) and three alcohols (methanol, ethanol and n-butanol, 40 mM) in three different organic solvents (n-hexane, n-heptane and isooctane). After 1 h of reaction at 40 °C, the best conversions were observed using isooctane as cosolvent. Fig. 3 shows the preference for the alcohols. Both biocatalysts revealed the highest preference for methanol. CProL demonstrated a low affinity for n-butanol, whereas ethanol was the poorest nucleophile for ASL in this set of experiments. The active sites of ASL and CProL were explored by inhibition assays using tetrahydrolipstatin (THL), a lipase inhibitor that binds covalently to the Ser residue of the catalytic triad. The results showed that 1.2 nmoles THL/mg of ASL and 0.6 nmoles THL/mg of CProL inhibited up to 67% of the hydrolytic activity of each biocatalyst using *p*-nitrophenol laurate as substrate. Similar behaviour was observed for other plant latex lipases [4, 5], making ASL and CProL interesting biocatalysts for further studies.

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A novel multifunctional aryl-aldehyde dehydrogenase for lignin valorization

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Lignin is the second most abundant natural source of non-fossil organic carbon in our biosphere. It is an aromatic heteropolymer found in plant cell walls and figures as a promising source of renewable aromatic compounds for the chemical industry. Thousands of tons of lignin are generated as a by-product in paper and cellulosic ethanol industries every year, but its leading destination has been burning for energy production. Lignin conversion to higher value-added products faces considerable technical challenges due to its recalcitrance and structural heterogeneity. Biotechnological solutions to the heterogeneity issue have been prospected in bacteria capable of funneling the complex mixture of depolymerized lignin compounds into target chemicals. However, our knowledge about the diversity of biocatalytic strategies for lignin transformation is still elusive and limited to studies on few species.

In this work, we identified an aryl-aldehyde dehydrogenase from *Xanthomonas*, an under-exploited genus of lignin-catabolizing bacteria, and reveal the multi-specificity of this enzyme which is active on at least four lignin-derived compounds. Its gene (XAC0354) was identified in the genome of *X. axonopodis pv citri* 306 close to the operon encoding enzymes from the protocatechuate catabolism, a key intermediary in the metabolism of aromatic compounds derived from guaiacyl (G) and p-hydroxyphenyl (H) subunits of lignin. The protein encoded by XAC0354, named here *XacALDH1*, has about 50% identity with benzaldehyde dehydrogenases II from *Acinetobacter* species [1, 2], but its substrate specificity and biochemical properties were unknown so far. *XacALDH1* was expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography followed by size-exclusion chromatography. Our results show that *XacALDH1* is an NAD⁺-dependent dehydrogenase, with optimal activity at 30 °C (pH 7 to 10) and the following substrate specificity profile ($k_{cat}^{app} / K_{0,5}^{app}$): vanillin (5,30 x 10⁴ M⁻¹.s⁻¹) ~ 4-hydroxybenzaldehyde (5,02 x 10⁴ M⁻¹.s⁻¹) > coniferaldehyde (3,56 x 10⁴ M⁻¹.s⁻¹) > 3,4-hydroxybenzaldehyde (1,41 x 10⁴ M⁻¹.s⁻¹) ~ benzaldehyde (1,30 x 10⁴ M⁻¹.s⁻¹). No significant activity was observed against syringaldehyde.

These data show that *XacALDH1* can oxidize multiple aldehydes derived from G and H subunits of lignin, allowing their funneling towards the central carbon metabolism from which bioproducts such as itaconic acid and polyhydroxyalkanoates can be made [3]. *XacALDH1* was inhibited above certain substrate concentrations for all tested compounds, except benzaldehyde, but the biological meaning for this inhibition remains to be determined. The molecule 3,4-dihydroxybenzaldehyde had the highest inhibitory effect, followed by coniferaldehyde, 4-hydroxybenzaldehyde, and vanillin. The substrate-specificity profile presented by *XacALDH1* has particularities compared to homologs already characterized, suggesting divergent molecular adaptations that might be useful for biotechnological purposes. Moreover, the observed promiscuity of substrates favors the exploration and optimization of this enzyme through enzymatic engineering or directed evolution, expanding the portfolio of biocatalysts available to design microbial platforms for lignin valorization.

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An Engineered Cofactor-Independent Non-natural Peroxygenase Catalyzing Enantiocomplementary Epoxidation Reactions

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Peroxygenase are heme-dependent enzymes that utilize peroxide-borne oxygen to catalyze a broad range of oxyfunctionalization reactions. In this study, we engineered an unconventional cofactor-independent peroxygenase using a promiscuous tautomerase as template. The engineered peroxygenase accepts both *t*-BuOOH and H₂O₂ to achieve enantiocomplementary epoxidations of various α,β -unsaturated aldehydes (citral and substituted cinnamaldehydes), obtaining both enantiomers of the corresponding α,β -epoxy-aldehydes with high conversions (up to 98%), high enantioselectivity (up to 98% ee), and good product yields (50% - 80%). The tuning of enzyme's activity and selectivity by protein engineering is permitted by the formation of a reactive enzyme-bound iminium ion intermediate. Our results highlight the potential of catalytic promiscuity for the engineering of new cofactor-independent oxidative enzymes. [1]

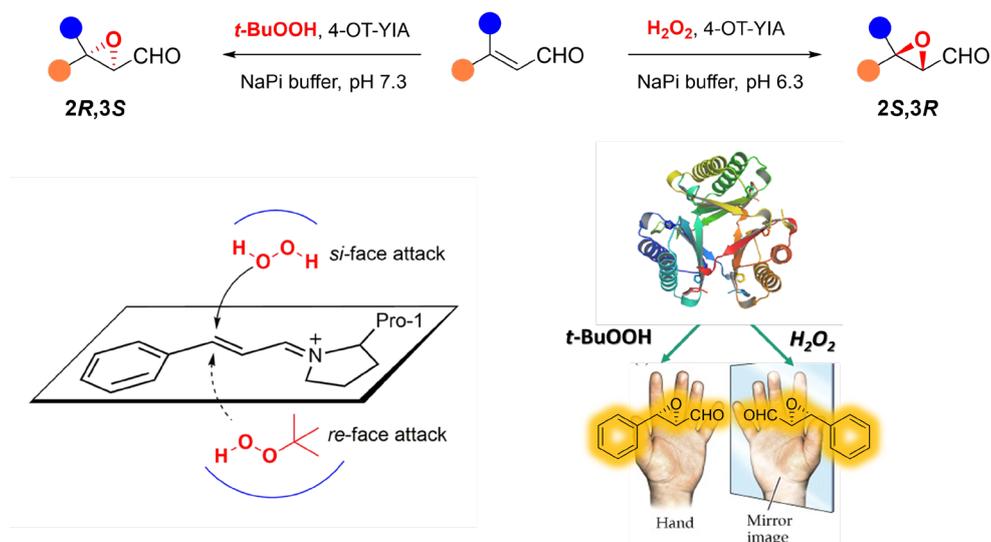


Figure 1. Enantiocomplementary epoxidation reactions catalyzed by an engineered cofactor-independent peroxygenase; the reactions likely proceed via a reactive enzyme-bound iminium ion intermediate.

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Catalytic NADH conversion by a halo-tolerant reductase of a methane monooxygenase

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Methane is an important energy source and has the potential to serve as an attractive C1 building block for a future bioeconomy.^[1] In nature, the soluble methane monooxygenase (sMMO) catalyses the NADH-dependent conversion of methane to methanol. The sMMO consists of three protein moieties: i) an active site-containing hydroxylase (MMOH), ii) a NADH-dependent reductase (MmoC) and iii) a regulatory protein (MmoB).^[2]

Herein, we present biochemical and spectroscopic insights into a novel halo-tolerant reductase MmoC. We recombinantly produced MmoC from the marine methanotrope *Methylomonas methanica* MC09 in *Escherichia coli* and purified it via affinity chromatography, resulting in high yield and purity (manuscript in preparation). The presence of its prosthetic groups, namely a flavin adenine dinucleotide (FAD) and [2Fe2S] cluster, were revealed by reconstitution experiments, iron determination and spectroscopy. After reduction with NADH, electron paramagnetic resonance spectroscopic data showed the semiquinone form of FAD and a quantitatively reduced [2Fe2S]⁺ cluster. Furthermore, we investigated the optima parameters for NADH-oxidising activity with benzyl viologen as an artificial electron acceptor. Interestingly, at 2 M NaCl, MmoC still showed 50% of its specific activity as a true halo-tolerant enzyme, while its optimal NaCl concentration for activity was 0.25 M. At optima reaction conditions, isolated MmoC showed a high turnover rate of 76 s⁻¹ and a K_m value of 11.6 μM for NADH.

This study represents an important step for future recombinant MMOH production. Moreover, the robust performance at various reaction conditions makes MmoC a promising enzyme for prospective NADH regeneration^[3] using graphite particles modified with a hydrogenase and MmoC.

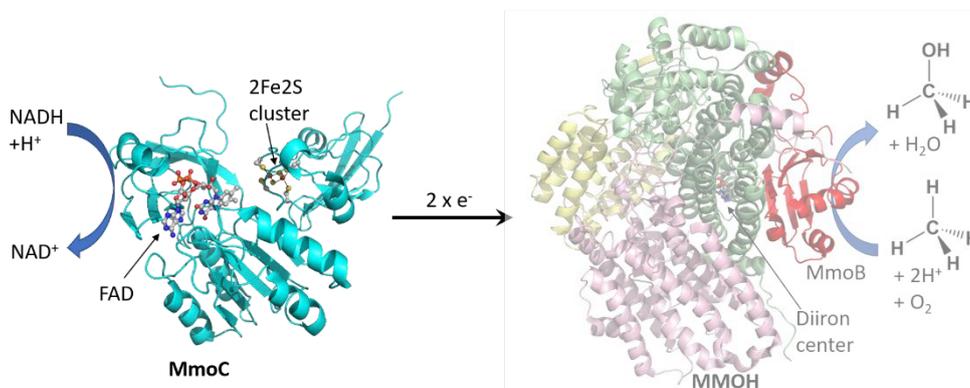


Figure 1: NADH dependent electron transfer by the reductase MmoC to the hydroxylase component of the soluble methane monooxygenase. Crystal structure of the NADH dependent reductase MmoC (PDB: 1TVC, 1JQ4) and the monomeric MMOH-MmoB complex from *Methylococcus capsulatus* (Bath) (transparent, PDB: 4GAM) are shown.

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Coimmobilization of a Thermophilic Alcohol Dehydrogenase with a Formate Dehydrogenase in Yttria-Stabilized Zirconia for Continuous Flow Process

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Scalability, process control or modularity are some of the advantages which are converting flow biocatalysis into a reference in green chemistry. Rigid porous monoliths expand the toolbox available for the design of different reactors for continuous processes. Yttrium-stabilized zirconia (YSZ) membranes has been applied in catalysis but scarcely used as enzyme support. [1,2]

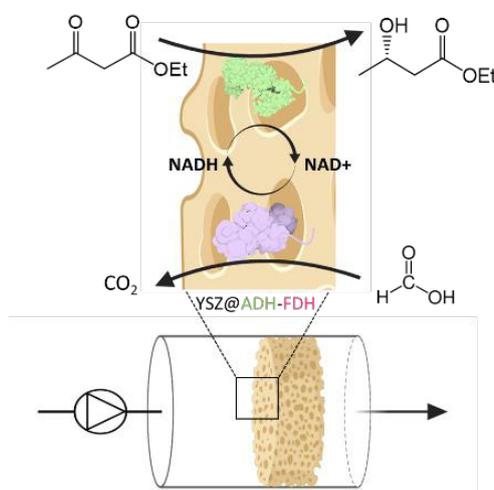


Figure 1. YSZ@ADH-FDH Scheme

An alcohol dehydrogenase from *Thermus thermophilus* HB27 (ADH) fused with a histidine tag was immobilized through affinity coordination on a YSZ membrane support. The maximum enzyme load and the higher catalytic performance at a wider range flow rates guided us to select pure YSZ membranes over alternatives of YSZ doped with Nickel Oxide (II) nanoparticles. An XPS analysis confirmed the presence of protein in the membrane surface and highlight the role of Yttrium in the interaction between YSZ and the enzyme. The activity was tracked during the asymmetric reduction of ethyl-acetoacetate to ethyl (S)-3-hydroxybutyrate, achieving a 100% NADH conversion and recovering almost 70% of soluble enzyme activity. To achieve *in-situ* cofactor regeneration a Formate Dehydrogenase from *Candida boidinii* (FDH) was co-immobilized in YSZ@ADH-FDH; the spatial distribution of both enzymes was studied by fluorescence labelling. The bienzymatic system makes possible the recycling of cofactor molecules improving the total product yield. Finally, the operational stability of the system was tested [3]

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Dual-revalorization of lignin through its use as a versatile and renewable matrix for enzyme immobilization and (flow)bioprocess engineering

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Lignin has emerged as an attractive alternative in the search for more eco-friendly and less costly materials for enzyme immobilization. In this work, we harnessed the terephthalic aldehyde-stabilization of lignin carried out during its extraction [1,2] to develop a series of functionalized lignins with a range of reactive groups (epoxy, amine, aldehyde, metal-chelates). This expanded the immobilization to a pool of enzymes (carboxylase, dehydrogenase, transaminase) via different binding chemistries obtaining 64-100% of immobilization yields. As a proof of concept, a ω -transaminase reversibly immobilized on polyethyleneimine-lignin was integrated in a packed-bead reactor. The stability of the immobilized biocatalyst was tested in continuous flow deamination reactions maintaining the same conversion for 100 cycles. These results outperform previous stability tests carried out with the enzyme covalently immobilized on methacrylic resins,[3] with the advantage that the reversibility of the immobilized enzyme allowed the recycling and reuse of lignin beyond the enzyme inactivation. Additionally, an in-line system based on lignin was added in the downstream process. Firstly, we exploited the hydrophobic properties of lignin to purify the reaction product (acetophenone) by using a catch-and-release column filled with lignin (70% of yield). Secondly, the cofactor (PLP) was purified by using a second scavenger column filled with PEI-lignin (>99% of yield) enabling the cofactor recycling. These results demonstrate a fully closed-loop sustainable flow-biocatalytic system based exclusively on lignin.

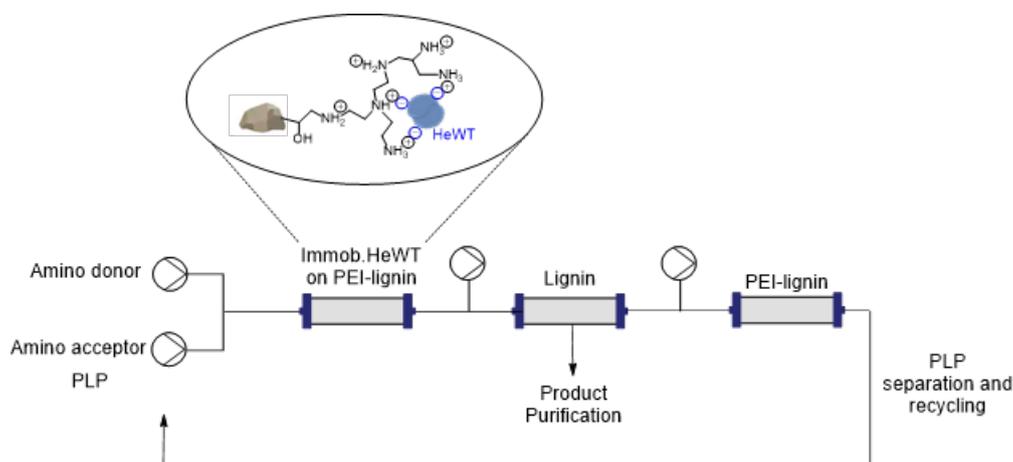


Figure 1. Integration of an immobilized transaminase in a PBR and downstream processes using scavenger columns containing lignin.

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Simulation of a two-step cascade reaction to yield (*R*)-4-chloro-3-hydroxybutyronitrile

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In comparison with traditional organic synthesis methods, cascade multi-step biocatalytic reactions have many advantages. A few of them are sustainability, simple and cheaper reactor set-up, mild reaction conditions and lower environmental impact. [1,2] Advancements in biocatalytic processes are achieved through reaction engineering. Combined kinetic and reactor modelling can significantly contribute to the choice of reactor mode, design and optimal operating conditions. [3,4] Using modelling and simulations helps us study the effects of different variables on the process, which increase our knowledge and understanding of the system. [5]

In this work, a two-step cascade reaction was studied (Figure 1). Both steps are catalysed by halohydrin dehalogenase (HheB2), mutant T120A. The first reaction was a ring closure of a substrate 1,3-dichloro-2-propanol (DCP) to (*R*)-epichlorohydrin (ECH). The second reaction was ring-opening of (*R*)-ECH to (*R*)-4-chloro-3-hydroxybutyronitrile (HBN) with cyanide ion (CN⁻) as a nucleophile. (*R*)-HBN is a precursor to many valuable chemicals, such as L-carnitine, which plays a vital role in human metabolism. [6,7]

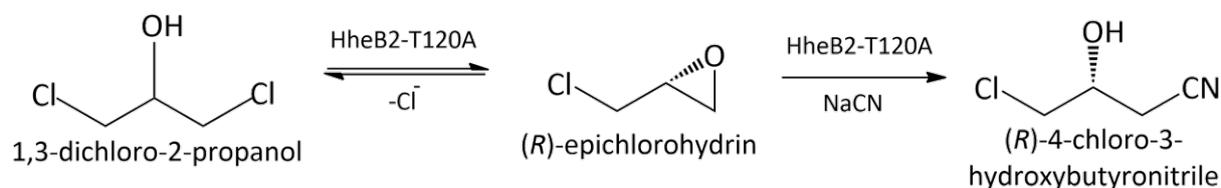


Figure 1. A two-stage cascade reaction of DCP conversion through (*R*)-ECH to (*R*)-HBN.

By using mathematical modelling and simulations, optimal reaction conditions were found for this system. Kinetics research has shown that the best results are achieved when both reaction steps are carried out simultaneously in one pot reaching the maximum product yield, and shifting the equilibrium to the right side. High product concentrations cannot be achieved in a batch reactor because of the substrate inhibition. However, in the fed-batch reactor concentrations of the inhibitors can be controlled which makes it an ideal solution.

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***Chlorella variabilis* fatty acid photodecarboxylase immobilization for its application in the synthesis of drop-in fuels**

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It is widely known that worldwide energy consumption is on the rise, and since most of this energy comes from non-renewable sources, we are facing a very urgent challenge: it is necessary to meet this growing demand of energy, while minimizing environmental impact. In this context, the decarboxylation of fatty acids (FAs) appears as a very attractive solution since they are abundantly found in nature and can be produced in a sustainable way.[1] Also, the alkanes obtained do not differ from petroleum-derived hydrocarbons, as the name 'drop-in' suggests. Unfortunately, chemical decarboxylation of FAs requires relatively harsh reaction conditions, such as a high H₂ partial pressure, temperatures over 350 °C, and the use of heavy metal catalysts,[2] which affects the environmental- and eco-efficiency of the process. On the other hand, using a recently discovered photoactivated fatty acid decarboxylase from *Chlorella variabilis* (CvFAP) [3,4] it is possible to perform this step in a clean and mild way using visible blue light.

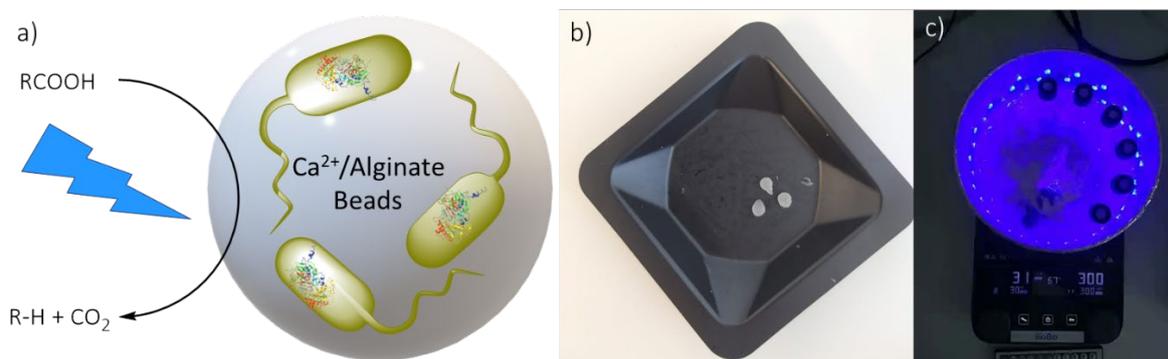


Figure 1. a) Illustration of the photodecarboxylation of a fatty acid using whole cells containing CvFAP entrapped in calcium alginate beads. b) Beads just after filtration. Diameter: ~2mm. c) Photoreactor used for the reaction under illumination with blue light (LED).

With the objective of developing an efficient and robust method for the synthesis of drop-in fuels, in this project we performed the immobilization of *E. coli* cells containing CvFAP in calcium alginate beads, and we applied these beads to the synthesis of the aforementioned fuels. A thorough study of the reaction conditions was carried out to maximize the performance of the system, including different solvents, concentration of cells inside the beads and concentration of beads.

The authors thank for the financial support from the Novo Nordisk Foundation, Light-BioFuels project, grant No NNF19OC0057522.

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Enzyme engineering of acyltransferase LovD and flow simvastatin synthesis

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Pharmaceutical synthesis is progressively replacing the chemical synthesis of cholesterol lowering agents by enzymatic processes. The directed evolution of acyl transferase LovD meant a breakthrough in the synthesis of simvastatin, rising to the most evolved variant LovD9¹.

In this study, we have elucidated the contribution of different groups of mutations scattered in different locations of LovD9 for its kinetic and stability parameters. Through a rational combination of those clusters of mutations, we have generated a minimalist variant (LovD-BuCh) whose catalytic productivity and thermal stability was similar to LovD9 but with 15 mutations less. This work demonstrates the synergic effect between buried and channel mutations to enhance the binding of the acyl donor and the relevance of buried positions to speed up the acyl transfer step.

Furthermore, we identified and quantified for each variant three side reactions which could compete with the synthase activity in an enzymatic industrial process: the inhibition of the first step of the reaction by one of the substrates (monacolin J acid, MJA), the hydrolysis of the acyl-enzyme intermediate and the hydrolysis of simvastatin.

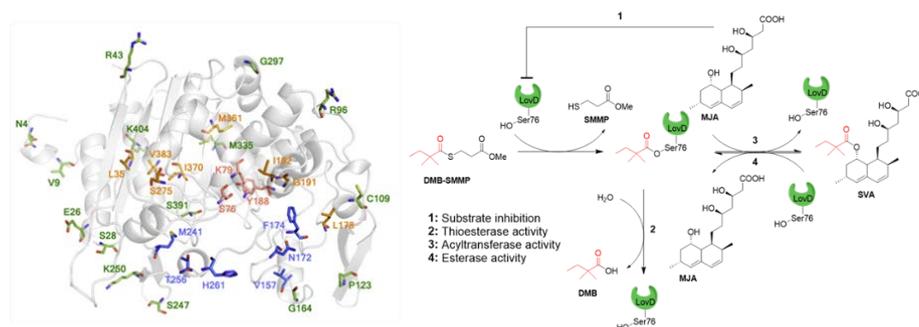


Figure 1. Clusters of mutations in the acyltransferase LovD (left) (in red, catalytic residues, in orange, buried mutations, in blue, channel mutations and in green, surface mutations) and scheme of all the side-reactions catalyzed by the enzyme (right).

In order to design a process optimized towards simvastatin synthesis, we immobilized the LovD-BuCh variant in different supports (Ag-Co²⁺, Ag-Co²⁺/Epoxyde and silica-Fe³⁺). After thermal deactivation assays, enzyme distribution studies through confocal microscopy and 24 h batch reactions, we chose the silica-Fe³⁺ as the best carrier to maintain the optimum activity and stability of the enzyme.

We then performed a flow reaction with this biocatalyzer at different flow velocities, concluding that at a low flow, the hydrolysis of simvastatin avoid high conversion rates, whereas at a high flow, the hydrolysis is appeased. The relation of substrates concentration in the reaction mixture is also fundamental in the final conversion: when MJA is in excess compared to the acyl donor (dimethyl butyryl S-mercaptopropionate, DMB-SMMP), the conversion achieves 30% due to the MJA competitive inhibition, while the excess of DMB-SMMP respect to MJA is traduced in a higher conversion (70 %).

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Exploratory study of new hybrid catalysis systems based on enzymes and gold catalysts supported on layered double hydroxydes

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Hybrid catalysis or chemo-enzymatic catalysis is a challenging topic that aims at realize a scientific breakthrough in the concepts and experimental approaches of chemical synthesis, under the goal of Green Chemistry. Indeed, hybrid catalysis combines enzyme catalysts and chemical catalyst systems to lead to highly efficient one-pot reaction cascades (yield, selectivity, atom economy, minimization of side reactions, reuse of catalysts). This allows access to complex organic molecules in a limited number of steps. This reaction concept is still little explored because of important scientific obstacles concerning i) the choice of catalysts and ii) the compatibility of the protagonists within the same reaction medium. The challenge is therefore to succeed in the association between enzymes and organometallic catalysts. [1,2]

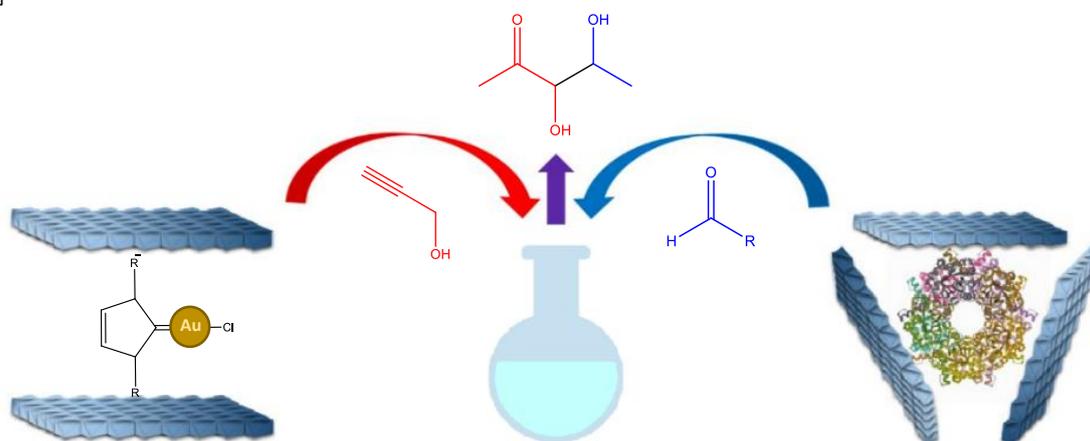


Figure 1. One pot/one step catalysis model

The model catalytic chemo-enzymatic reaction (Figure 1) we are exploring aims at the one pot one step synthesis of chiral monosaccharides. The first step deals with the formation of hydroxyacetone by hydration of propargylic alcohol catalyzed by gold complexes. The second step consists of an aldolisation reaction using an aldolase, namely fructose-6-phosphate aldolase (FSA).[3,4]

We will describe how to find the best compromise between the acidic pH and high temperature required for the chemo-catalyst versus neutral pH for the thermostable aldolase. One option is to confine both in a Layered Double Hydroxide (LDH) structure, as a protection for the enzyme against an acidic medium. The first studies will be presented herein in optimizing LDH composition, morphology and confinement methods.[5]

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Using nanoDSF for Quantification of Salt Effects on Protein Stability

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Specific ion effects on biological macromolecules have attracted the interest of scientists for over 100 years. In 1888 Franz Hofmeister experimented with separated liquid from beaten hen egg white, which contains a broad mixture of proteins. Hofmeister demonstrated that simple addition of different sodium and chloride salts have an impact on solubility of proteins. His results manifested in the well-known Hofmeister series.

Enzyme stability is next to its activity one of the main aspects that need to be optimized for biocatalytic processes. Beyond cosolvent effects on the stability of enzymes [1] we were interested in studying the effects of buffers, salts and other additives on thermodynamic protein stability. In this study it is demonstrated that simple addition of sodium salts can alter the stability of enzymes in solution. Two model enzymes, fructose-6-phosphate aldolase with negative surface charge (FSA_{Ec}) and lysozyme from hen egg white (HEWL) with a positive surface net charge, were utilized to investigate potentially (de)stabilizing effect.

The use of the *Prometheus* instrument [2] allows measuring the shift of intrinsic fluorescence of tryptophan during unfolding, yielding the melting points of 48 samples at once in a temperature range from 0 – 110°C with a temperature ramp of 1 °C/min. The shift of T_m was measured to quantify the (de)stabilizing effect of the sodium salt in a concentration range between 0 – 1 M. The data comparison showed that the surface net charge of the proteins is relevant at low salt concentrations. However, the final ordering of anions at 1 M concentrations was independent of the surface net charge.

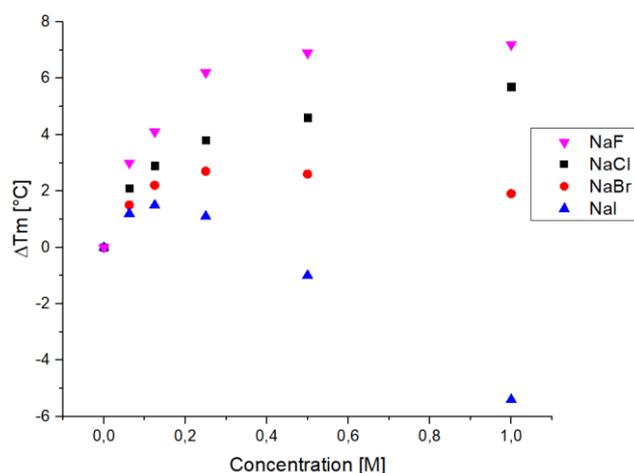


Figure 1. Shift of melting temperature of FSA_{Ec} in the presence of NaF, NaCl, NaBr and NaI.

Acknowledgements. This project has received funding through The German Federal Ministry of Education and Research (BMBF), grant N₀031B0595 and the initiative ERA CoBioTech (European Unions` s Horizon 2020 Research and Innovation Programme, grant N₀722361.

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Nitrilase enzyme engineering and process development for high titre production of a chiral mandelic acid derivative

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Enzyme engineering and process development are two key tools towards industrial biocatalytic solutions. However, the improvements in reaction conditions needed to achieve high titre conversion can often be detrimental to an enzyme, requiring further engineering. Alternatively, lengthy enzyme engineering, performed separately from process development can often lead to an over engineered enzyme, or one that is not suitable for the evolved process. With both areas of investigation typically performed independently and iteratively, the timeframe to develop an economically viable process is often lengthy. Here we detail a parallel approach to the process development and engineering of a nitrilase enzyme (*Burkholderia cenocepacia* J2315 (BCJ2315)) to achieve an economically viable process for the production of a chiral mandelic acid derivative via the development of a one-pot, enantioselective, dynamic kinetic resolution.

Almac's engineering and process development approach has been hugely successful in achieving commercially successful biocatalytic solutions [1-3]. The combination of both approaches resulted in the development of a one-pot procedure to yield the desired enantiomer at 100 g/L scale, with 3 % w/w loading enzyme and a reaction product ee of 97%. Improvements in ee and substrate loading could both be achieved through process development of the wild type enzyme, however, the target ee (97%) relied upon enzyme engineering. Interestingly, the single residue mutants with improved ee in small scale screening reactions were not transferable to the high-titre one-pot procedure due to inactivation, while a double mutation variant delivered the desired outcome at scale with ee > 97 %.

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Engineering of NADPH supply boosts photosynthesis-driven biotransformation

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Conventional cofactor recycling strategies are often associated with a low atom economy and the need for sacrificial cosubstrates. To this end, application of recombinant cyanobacteria as hosts for whole-cell bioreductions remedies these constraints by offering light-driven regeneration of reaction equivalents such as NADPH and reduced ferredoxin via oxygenic photosynthesis. For this purpose, we investigated the heterologous C=C bond reduction of 2-methylmaleimide by the NADPH dependent ene-reductase YqjM as a model reaction in *Synechocystis* sp. PCC 6803. However, the approach is limited by self-shading of cells in high-density cultures which reduces light availability and, thus, photosynthetic activity. To compete with heterotrophic systems, an increase of the cyanobacterial cell productivity is necessary. Herein, we report that the inactivation of flavodiiron proteins (FDPs), which represent a natural electron sink conferring photoprotection, improves electron channelling towards our competing model reaction. In fact, the inactivation of the Flv1/3 hetero-oligomer doubled the productivity of the cells under self-shading conditions, reaching up to 18.3 mmol h⁻¹L⁻¹ and allowed the conversion of 60 mM within 4 h.[1] Our results demonstrate that the optimization of the photosynthetic electron transport by metabolic engineering may be an effective approach leading us closer to a biotechnology that uses biocatalysts produced from carbon dioxide and water for atom-efficient light-driven biotransformations.

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Solvent-free Photobiocatalytic Hydroxylation of Cycloalkanes: Key Factors, Challenges and Opportunities

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A key reaction in the production of many (chiral) chemical building blocks is the hydroxylation of alkanes to activate them for further reaction steps. In the recent years, the use of unspecific peroxygenase (UPO) for hydroxylation reactions has become an up and coming research field. UPO transforms a wide range of substrates and needs only hydrogen peroxide (H_2O_2) as a cosubstrate. While the problem of the enzyme's notorious instability towards H_2O_2 has already been solved with photocatalytic *in situ* H_2O_2 supply [1, 2, 3], one problem remained: Most UPO substrates are rather hydrophobic and therefore hardly soluble in traditional aqueous reaction media. However, high substrate and product titers are important for the industrial applicability of a process. Therefore, we present in this research the photobiocatalytic solvent free hydroxylation of the hydrophobic model substrate cyclohexane (Fig. 1). To sustain the functionality of UPO in organic media, the enzyme was entrapped in calcium alginate beads, which also served as a reservoir for the photocatalyst (Fig.1). This 2-in-1 photobiocatalyst is active for up to seven days under process conditions and synthesizes cyclohexanol.[4]

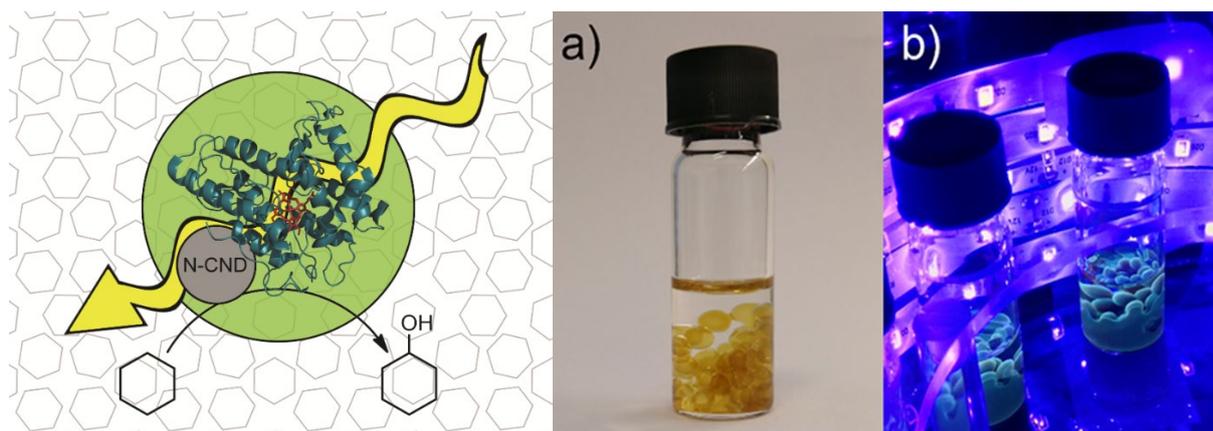


Figure 1. Left: Illustration of the hydroxylation of neat cyclohexane using a 2-in-1 photobiocatalyst. Right: UPO alginate beads (0.8 g, 14 U, 1 nmol) with N-CNDs (4 mg) in cyclohexane shortly after mixing (a) and during the reaction under UV-LED illumination (b).

Beyond the proof of principle, several key factors for the performance of the system were identified: (i) wavelength and (ii) intensity of the applied light source, (iii) ratio of photocatalyst to enzyme and (iv) the size of the beads.

Acknowledgement

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Establishing an internal flow biocatalysis toolbox: CALB-catalyzed kinetic resolution as a case study

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In recent years, the combination of biocatalysis and flow processing has attracted much attention from the scientific community and the pharmaceutical industry.[1-3] Compared to a batch approach, flow biocatalytic reactions can benefit from enhanced mass transfer, a better control of reaction parameters and – when using immobilized enzymes – increased biocatalyst loading and simplified downstream processing. This usually translates into increased space-time yields and total turnover numbers. Furthermore, by tailoring the reactor type to the reaction conditions, substrate or product inhibition may be minimized, affording higher yields.

In our aim to develop more efficient and sustainable processes for the manufacturing of active pharmaceutical intermediates, we set out to establish a new synergistic internal toolbox, combining biocatalysis and flow technology. As a case study we selected the kinetic resolution of styryllyl acetate via a transesterification reaction catalyzed by immobilized *Candida antarctica* lipase B (CALB) (Figure 1), which had been previously investigated by Thomas *et al.* [4]

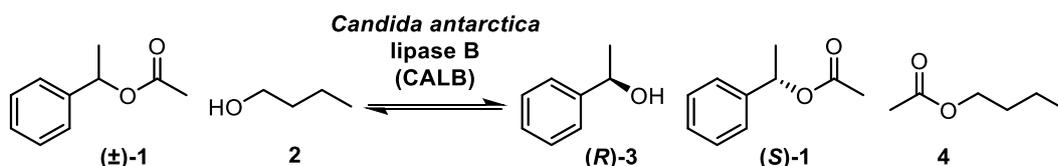


Figure 1. Kinetic resolution of styryllyl acetate (1) via CALB-catalyzed transesterification with butanol (2).

The reaction was first optimized in a conventional batch approach using commercially available immobilized CALB. With the generated data, a kinetic model was constructed, which could reliably predict the effect of reaction variables both in batch and flow. Two different flow setups were investigated: a packed-bed reactor (PBR) and a cascade of continuous stirred-tank reactors (CSTRs). Using a multi-feed approach, substrate inhibition could be partially circumvented and the optimized CSTR cascade was finally operated for 2 days, processing 0.5 Kg of substrate.

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Let it be continuous: Biocatalytic oxyfunctionalization of ethylbenzene in enzyme membrane reactor

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The biocatalytic oxyfunctionalization of substrates with hydrogen peroxide (H₂O₂) as the cosubstrate yielding enantiopure products is a relatively new and promising development.^[1] Yet, the implementation of these enzymatic reactions in an industrial environment is a challenge due to low productivities, solubility issues and difficulties in up-scaling and downstream processing. Continuous operation is a key enabling sustainable manufacturing technology transforming catalytic reactions from analytical to technical scales, and it has recently gained tremendous momentum in biocatalysis.^[2]

In this study, we used the unspecific peroxygenase from *Agroclybe aegerita* (AaeUPO) for the enantiopure synthesis of (*R*)-1-phenylethanol from ethylbenzene and transferred it into a membrane reactor for continuous operation (Figure 1).

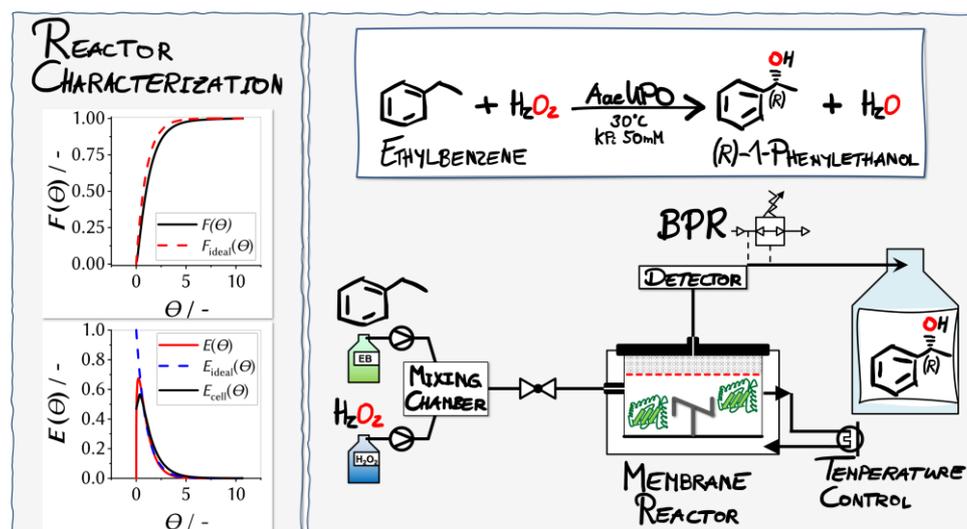


Figure 1. Reactor characterization (left) and oxyfunctionalization of ethylbenzene to (*R*)-1-phenylethanol by unspecific peroxygenase from *Agroclybe aegerita* (AaeUPO) in continuous operation (right). BPR: back pressure regulator.

First, we investigated the residence time (τ_{exp}) distribution of the reactor used in this set-up. We found 1.5 times higher τ_{exp} than theoretical residence times (τ_{theo}). Initial experiments in continuous operation were successfully demonstrated for the first time for UPO catalysis. In addition, we investigated aqueous media, various organic solvents and non-conventional media as mobile phase.

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Utilization of ion exchange resins in downstream-processing concepts of imine reductase- and decarboxylase-catalyzed reactions

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Imine reductases and decarboxylases are valuable biocatalysts being frequently used for the synthesis of a broad spectrum of intermediates for important active pharmaceutical ingredients (APIs). Unfortunately, these synthetically interesting biocatalytic processes suffer frequently from limitations such as poor reaction equilibria, undesired side reactions and downstream-processing issues limiting the overall applicability, productivity, and atom efficiency.

A powerful option to overcome these problems is the integration of suitable separation techniques into the respective biocatalytic processes [1,2]. In this study, we focus on the utilization of ion exchange resins to selectively isolate the product(s), preferably directly from the reaction mixture (Fig. 1, left).

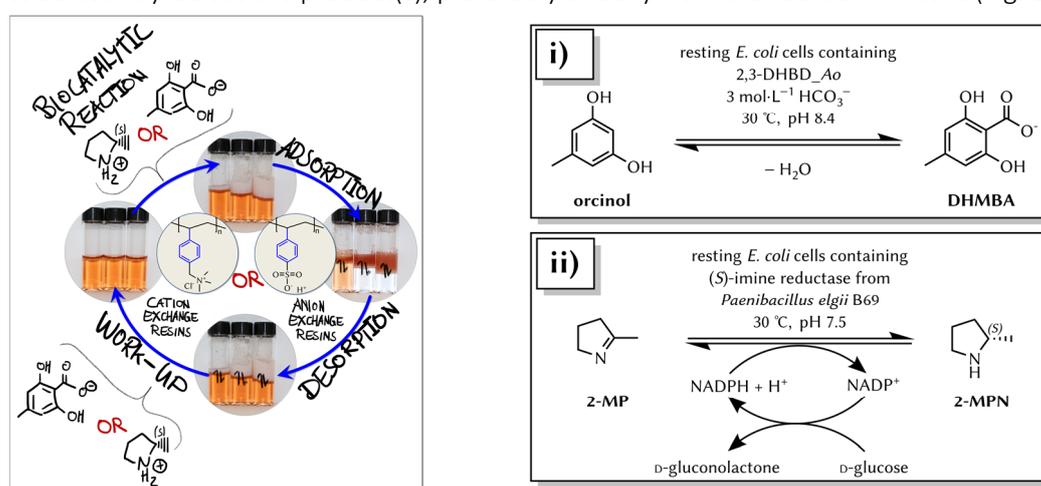


Figure 1. Concept of separation techniques into biocatalytic processes (left) like ortho carboxylation of orcinol (i) or enantioselective reduction of 2-methyl-1-pyrroline (2-MP, ii).

The first example involves the 2,3-dihydroxybenzoic acid decarboxylase from *A. oryzae* (2,3-DHBD_Ao) for the synthesis of 2,6-dihydroxy-4-methylbenzoic acid (DHMBA) being a valuable intermediate for several pharmaceuticals (Fig. 1i) [3]. A utilization of anion exchange resins facilitate an isolation of the product directly from the reaction mixture with a simple desorption yielding the pure product (>99%). The remaining mother liquor, including the biocatalyst, can be recycled. Similarly, we have broadened this concept using the (S)-selective IRED from *P. elgii* B69 for the synthesis of (S)-2-methylpyrrolidine (Fig. 1ii) [4]. The product-amine can be isolated using cation exchange resins and eventually isolated in its pure hydrochloride salt (>99%). In summary, the shown technique allows efficient downstream-processing concepts for biocatalytic processes including a potential re-use of the remaining reaction mixture but may also be used directly as an *in situ*-product removal technique [5].

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Medium engineering of galactose oxidase for its application in biphasic media

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Galactose oxidase poses a promising catalyst for oxidation of a multitude of primary and secondary alcohols to aldehydes and ketones, respectively [1]. In recent years it has come to the spotlight as a valuable catalyst in the realm of furan derivatives, due to its ability to oxidise 5-hydroxymethyl furfural (HMF) to its dialdehyde counterpart diformylfuran (DFF) [2]. However, due to its poor stability and solubility in water, DFF is mostly synthesized in organic solvents [3].

Nevertheless, there is very scarce information on the use of galactose oxidase in organic solvents or biphasic media. The use of a biphasic system can bring about numerous benefits, such as higher substrate loadings, absence of substrate and/or product inhibition, as well as help overcome thermodynamic limitations [4]. Moreover, in this case, it can assist product separation and accommodate the prerequisites of integrating the reaction in a cascade. Therefore, the aim of this study was to assess the possibility of using galactose oxidase in biphasic systems of various water contents, with seven different organic solvents (Figure 1).

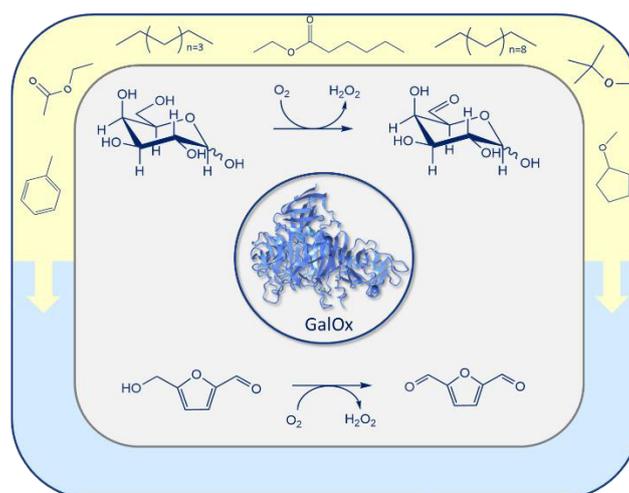


Figure 1. Exploring the potential of application of galactose oxidase (GalOx) in biphasic systems with various organic solvents and water contents.

Herein we present that galactose oxidase retained its activity upon exposure to the majority of screened conditions. The activity remained significant after contact of the enzyme with all seven organic solvents, and moreover, the reduction in water contents did not have a severe adverse effect on the enzyme. The results were confirmed using both D-galactose and HMF as substrates.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860414.

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Use of a suspension-to-suspension reaction concept in a (semi-) continuously operated enzymatic reaction-crystallization system

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Amine transaminases (ATAs) are important and well established biocatalysts for the synthesis of valuable chiral amines from the corresponding prochiral ketones.[1] Unfortunately, a major disadvantage of ATAs is the unfavorable reaction equilibrium for the asymmetric synthesis of chiral amines. Classical chemical concepts to overcome this limitation exist, but suffer from secondary complications.[2]

In this study we present an alternative suspension-to-suspension reaction to overcome the unfavorable reaction equilibrium of a ATA reaction using an *in situ*-product crystallization (ISPC) approach.[3] A crystallization agent (donor salt) with a relatively low solubility was applied to crystallize the product (product salt) from the reaction in a semi continuous process, which possesses an even lower solubility (Figure 1). The overall amine concentration remains constant in solution, while the product stoichiometrically accumulates in the solid phase.

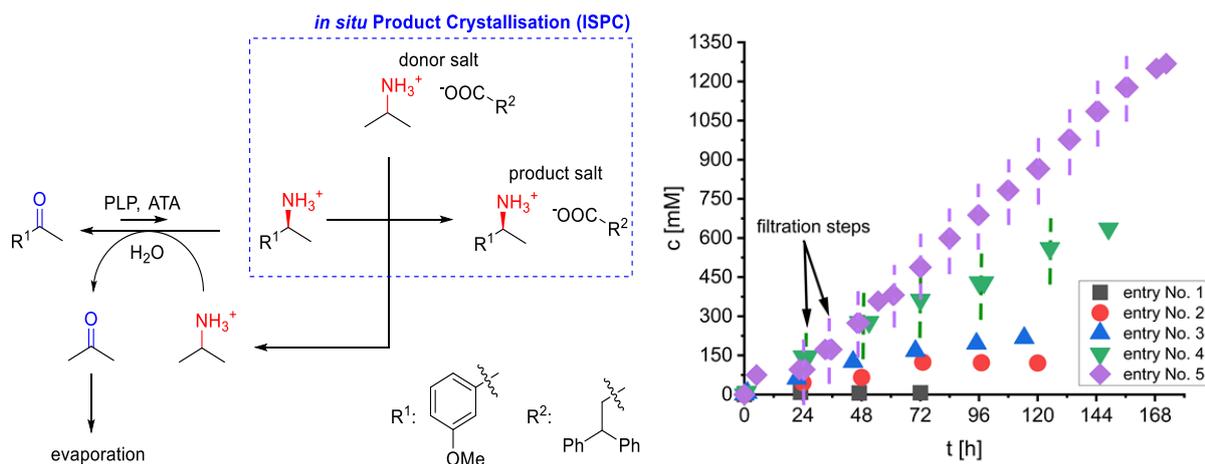


Figure 1. Left: Amine transaminase-catalyzed reaction concept with an *in situ*-product crystallization using a slightly soluble donor salt; Right: semi-continuous reactions at semi-preparative scale.

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Electrochemical recycling of glutathione reductase in ionic liquids

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Biocatalysts have great potential as sustainable catalysts due to their high selectivity and mild operating conditions. However, their use in industrial synthesis is hindered by biocatalysts being incompatible with common industrial conditions. This is predominately due to many biocatalysts having limited stability in the presence of non-aqueous solvents or elevated temperature, limiting the window of usability. Consequently, there is a need to develop new methodologies that improve the solubility and stability of biocatalysts in nonaqueous media [1].

Chemical modification of enzymes has been demonstrated as a method for stabilizing enzymes against temperature, aggregation, and non-aqueous environments [2]. Recent work has shown that it is possible to form solvent-free liquid proteins via chemical modification of the protein surface. The resulting biomaterials have been shown to be soluble in both hydrophilic and hydrophobic ionic liquids, with enzyme structure preserved in the non-aqueous environment. Using the enzyme glucosidase, Brogan *et al.* were able to demonstrate that in ionic liquids, the optimal temperature for enzyme activity shifted to 110 °C accompanied by activity towards water insoluble cellulose. Solvent-induced promiscuity showed that it was possible to reduce the number of enzymes require for cellulose degradation from 3 to 1 [3]. As a result, this nascent technology in conjunction with emerging solvent systems such as ionic liquids could provide a versatile platform for industrial biocatalysis.

Here, recent work has focused on synthesising an enzyme-surfactant nanoconjugate containing the NADPH-dependent flavoprotein glutathione reductase. The aim of this is to demonstrate that it is possible to control the enzyme electrochemically in the absence of nicotinamide cofactors and make use of the broad electrochemical window of ionic liquids. It is hoped that the methods used to modify and control glutathione reductase will be applicable to other flavin containing proteins allowing for a range of biocatalytic transformations to be driven electrochemically. Other flavoproteins, such as ene reductases, may also benefit from improved substrate solubility and a wider substrate scope in ionic liquids.

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Medium and Reaction Engineering of a Chemoenzymatic Route towards Styrene and Stilbene Derivatives

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The idea to use renewable raw materials for the production of bio-based substitute materials or even new types of products with high added-value continues to gain momentum. This also applies to phenolic acids, which are typical constituents of biomass. These compounds can be decarboxylated by either chemical or biocatalytic means yielding styrene derivatives. Latest developments demonstrate a variety of options, including cross coupling, metathesis, acetylation, silylation, and polymerization. [1-6]

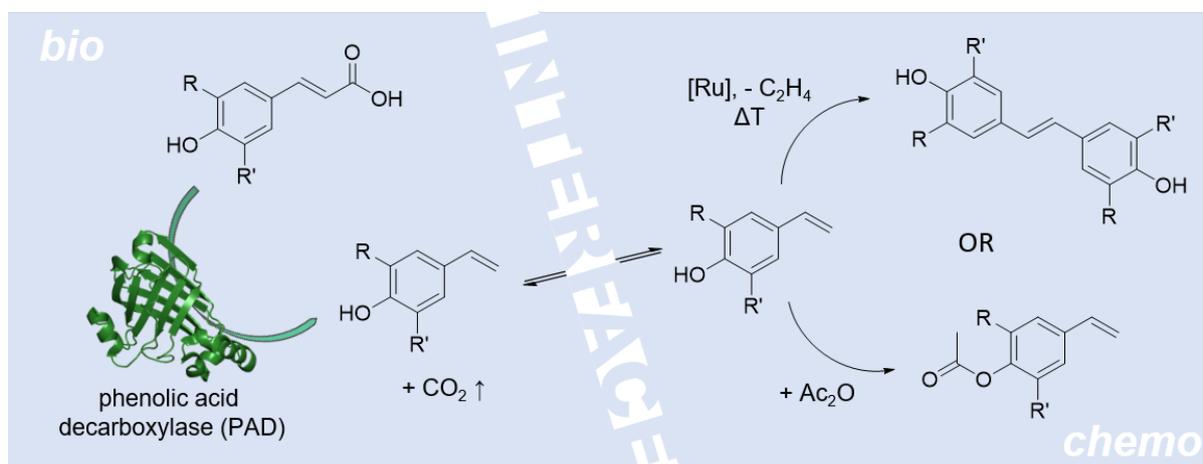


Figure 1. Illustration of the chemoenzymatic cascade to be optimized.

Although all these recent contributions underline the potential of this approach, it faces problems like low substrate solubility in aqueous media, poor stability of hydroxy styrene intermediates, low tolerance of the biocatalyst towards organic solvents and of the chemical steps towards water. Up to now these problems were already addressed in various ways *e.g.*, by compartmentalization of the biocatalyst in gel beads [1], continuous extraction of the intermediate [2] or sequential packed-bed reactors with multi-component solvent mixtures sustaining both steps. [4] However, despite all these measures, product titers have not reached dimensions of technical relevance, which is why we set out to optimize the envisioned reaction cascades (Figure 1) with respect to economic productivity. For this, we combine experimental evaluations of enzymatic activities in both conventional and non-conventional media with computational tools to estimate substrate and product solubilities and distribution coefficients. By these means we obtain insights that facilitate systematic and efficient medium and reaction engineering.

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***In vitro* Characterization and Immobilization of Uridine Diphosphate-Glycosyltransferase from *Polygonum tinctorium* (PtUGT1)**

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Indigo dye is one of the oldest dyes ever used by humans and is still in great demand, especially for the manufacture of denim clothing. [1] With regards to its ecological footprint, two aspects shall be highlighted: First, its synthesis is typically based on non-renewable resources and second, its synthetic precursor indoxyl is not stable under ambient conditions, quickly undergoes autoxidation and thus requires excess amounts of reducing agent to re-solubilize indigo for the actual dyeing step. [2] While a number of routes for the production of indoxyl from renewable resources have been reported in literature [3] they do not address the second problem. In 2018 Hsu *et al.* [2] presented a bioinspired dyeing strategy for which indican was produced from L-tryptophan employing engineered *E. coli* cells. From the stable alternative indican, indoxyl can then be liberated on demand and in a catalytic fashion – e.g. by a β -glucosidase – eliminating the hyperstoichiometric formation of corrosive wastewaters.

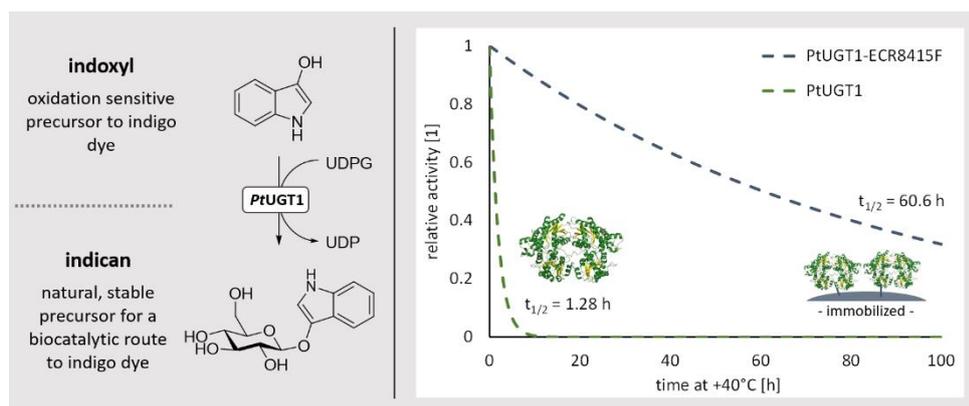


Figure 1. Illustration of the underlying project goal of biocatalytic indican synthesis via glycosylation using PtUGT1 (left) and the achieved stabilization of PtUGT1 upon covalent immobilization on amino-functionalized methacrylate resin ECR8415F (right).

In pursuit of intensifying the key glycosylation step of this approach, we investigated free UDP-glycosyltransferase PtUGT1 using the model substrate 3,4-dichlorophenol. Our results revealed the short-lived nature as well as dilution-induced destabilization of the free enzyme. As part of a subsequently initiated immobilization study we identified a commercial carrier that gives immobilization yields $\geq 98\%$ and a stabilization factor of 47. We also show the multi-cycle reuse of the immobilized enzyme with activity losses between consecutive batches in the lower single-digit percentage range. [4]

Acknowledgement This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 860414 and from The Novo Nordisk Foundation Grant number: NNF10CC1016517.

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***In situ* two-step modular surface display for biocatalysis**

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The immobilization of enzymes on cellular surfaces has been studied for three decades, but the identification of a suitable membrane anchoring domain for a given passenger still relies on painstaking trial and error experiments. To solve this problem, we developed a generalizable, modular approach for the functional anchoring of enzymes on cells. This approach is based on bioconjugation mediated by tag/catcher pairs such as the SpyTag002/SpyCatcher002 split protein. In a first step, the catcher is presented on the cellular surface. In a second step, the addition of fusion proteins composed of the catalysts of interest and the corresponding tag leads to isopeptide bonding. For a proof of concept, a two-enzyme system involving an ene reductase and a formate dehydrogenase was immobilized. Therefore, different fusion proteins of the oxidoreductases and SpyTag002 were constructed. After coupling to soluble SpyCatcher002, the purified fusion proteins preserved catalytic activity. An analysis of the coupling kinetics revealed rate constants ($\sim 6000 \text{ M}^{-1} \text{ s}^{-1}$) in the same order of magnitude as described for reference proteins in literature [1]. Hence, the oxidoreductase-SpyTag002 fusion proteins were next coupled to SpyCatcher002 displayed on the surface of *E. coli* by intimin as described in literature [1]. As SDS-PAGE and photometric evaluation of the surface localized enzymatic activity revealed successful coupling, the surface-anchored catalysts were applied in the bioreduction of (*R*)-carvone. The space time yield ($0.41 \pm 0.012 \text{ mM h}^{-1}$ for $10^9 \text{ cells mL}^{-1}$) equaled about half of the catalytic performance achieved by the corresponding whole cell biocatalysts underlining the potential of the surface anchorage strategy [2]. However, to circumvent the need for external enzyme supply required in this strategy, we combined the surface anchorage with protein E-mediated cell lysis, which leads to the release of the cytoplasm through a single opening in the cell wall [3]. Thus, overexpressed enzymes with SpyTag002 are set free into the medium surrounding the cellular envelopes which allows for an *in situ* enzyme immobilization. We developed a vector architecture allowing the independently tunable expression of SpyTag-fusion proteins in the cytoplasm and intimin-SpyCatcher002 on the cell surface. As a proof of concept we used this architecture in a fed-batch process in a stirred-tank reactor on a litre scale to demonstrate the expression of SpyTag002mClover3, a fluorescence protein, in the cytoplasm and intimin-SpyCatcher002 on the cell surface during the biomass formation phase. Upon induction of protein E-mediated cell lysis, SpyTag002mClover3 was released into the surrounding media and coupled covalently to the cellular envelopes displaying intimin SpyCatcher002 (shown by fluorescence measurements and western blotting). Currently, we are enhancing the quantity of molecules which can be immobilized on the cell surface by presenting additional SpyCatcher domains per intimin. Moreover, we are adapting the process to the oxidoreductases in order to perform preparative bioreductions of (*R*)-carvone. Preliminary experiments showed that the cellular envelopes yielded (2*R*,5*R*)-dihydrocarvone with a substantially higher optical purity compared to the whole cell biocatalysts since an undesired side reaction was prevented due to the elimination of the cytoplasm. This underlines the potential of this concept for biotransformations suffering from host-cell mediated side reactions.

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Enzyme Catalysis at High Pressure

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High Pressure Application

Below 200 MPa several enzymes show a pressure induced change of their structure that affects their performance in terms of activity, stability and selectivity. [1,2,3] The stability of enzymes against thermal deactivation may be increased by high hydrostatic pressure. [4] A detailed kinetic study of enzymes from different enzyme classes will help to understand enzyme class specific behaviour under pressure as a complementary process parameter. For this purpose, a continuously operating high-pressure reactor was designed using immobilized enzymes to investigate enzyme kinetics and the influence of pressure.

Effects of Pressure on Stabilization and Activity

The effects of high pressure on the stability, activity and selectivity will be investigated on the transesterification of vinyl acetate and 1-phenyl-2-propanol to 1-phenyl-2-propanyl-acetate by *Candida rugosa* lipase (EC 3.1.1.3) shown in **Figure 1**. Enzyme kinetics under different temperatures and pressures are determined.

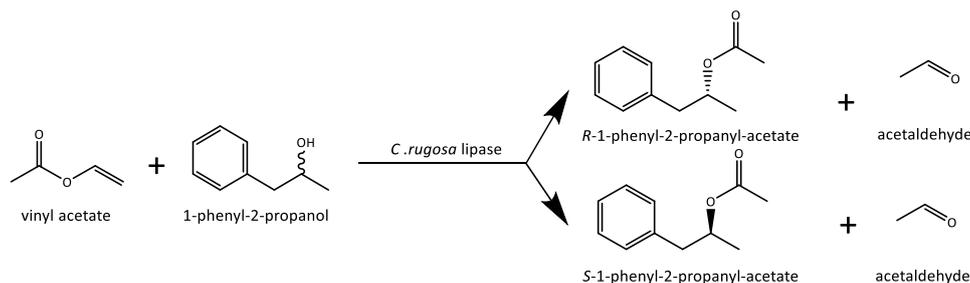


Figure 1. Transesterification catalyzed by *Candida rugosa* lipase (CRL)

Objective

In this work enzyme kinetics will be determined in a novel conceptualized high pressure reactor to understand pressure induced changes in enzyme performance. The gained knowledge can be used to determine optimal reaction conditions and to improve enzymatic reaction system.

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Immobilization of novel α -keto acid-dependent dioxygenases enables catalyst recycling, increases initial rate activity and process stability

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α -keto acid- and Fe^{2+} -dependent dioxygenases (KDOs) catalyze a range of oxidation reactions, making them an interesting target especially for the oxidation of non-active C-H bonds.

Recently novel KDOs were discovered [1,2] that catalyze the stereoselective C-H oxidation of the L-lysine side chain in 3- and 4-position (Fig. 1). The resulting chiral hydroxy-L-lysines find versatile application as precursors for active pharmaceutical ingredients, building blocks for biopolymers, and as chiral auxiliaries.

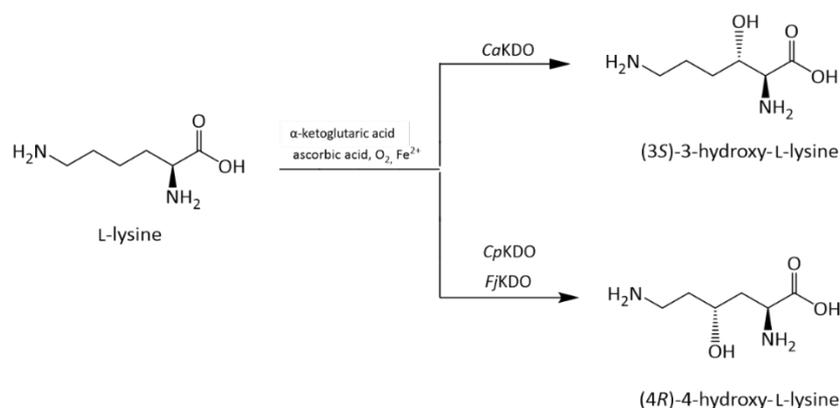


Figure 1: Schematic presentation of the stereoselective C-H oxidation of the L-lysine side chain in 3-position catalyzed by KDO from *Catenulispora acidiphila* (CaKDO) and 4-position catalyzed by KDO from *Chitinophaga pinensis* (CpKDO) or KDO from *Flavobacterium johnsoniae* (FjKDO).

In their isolated form, these weakly active enzymes were exclusively used in analytical scale. Purification turned out to be difficult, since specifically loss of Fe^{2+} from the active site occurs, inactivating the enzymes [3]. Further, some enzymes show precipitation at higher concentration upon chromatographic purification. Application in biotransformations is further limited by the low stability of KDOs in isolated form.

We demonstrate an integrated reaction engineering approach to render KDOs a suitable catalyst for the production of hydroxy-L-lysines at preparative lab scale. In order to increase process stability and enable catalyst recycling CaKDO, CpKDO and FjKDO were genetically fused to the HaloTag[®] and covalently immobilized on HaloLink[™] Resin directly from the crude cell extract [3,4]. Immobilization increased initial rate activity and process stability significantly, enabling full conversion of up to 100 mM L-lysine in a preparative lab scale. In addition, we demonstrated the recyclability of immobilized CpKDO and FjKDO in repetitive batches.

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Biotechnological processes for the high efficiency conversion of CO₂ from industrial waste streams into PHAs (BIOCON-CO₂)

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Increasing levels of carbon dioxide (CO₂) in the atmosphere, land and water are a serious concern and are causing serious effects on climate changes. However, if from the environmental point of view CO₂ is waste that has to be minimized, it could also be seen as a cheap and abundant C1-building block. In this context, utilizing CO₂ as a substrate for the conversion into high value products has been gaining more and more attraction over the last years for the reduction of greenhouse gases emissions.

In this frame, BIOCON-CO₂ project aims to develop and validate flexible and versatile platforms to biologically transform CO₂ from gas streams into added-value chemicals. The versatility and flexibility of the platform is based on 3 main stages: 1) CO₂ solubilisation, 2) bioprocess and 3) downstream. The use of the enzyme carbonic anhydrase (CA) and a trickle bed reactor (TBR) are potential strategies to increase the CO₂ solubility and feed the carbonate to the engineered strain *Cupravidus necator* for its conversion to poly3-hydroxypropionic acid (P-3HP). The capability of the CA to accelerate CO₂ hydration dramatically will be useful for promoting the absorption rates of CO₂ from gas streams. To avoid the high costs of using the commercial human CA isoform II (hCAII), a recombinant hCAII has been produced and purified. Moreover, 30 variants have been identified *via* FRESCO protocol and have been tested and two variants with enhanced activity and stability have been tested. On the other hand, a TBR has been designed with the purpose of developing an absorption process to enhance CO₂ solubility. To improve the bacterial fermentation at fixed TBR systems, 11 different packing materials have been tested for biofilm formation and one has been selected for the final set-up. These tools will be further developed and combined as puzzle pieces to establish a platform for the conversion of CO₂ into P-3HP.

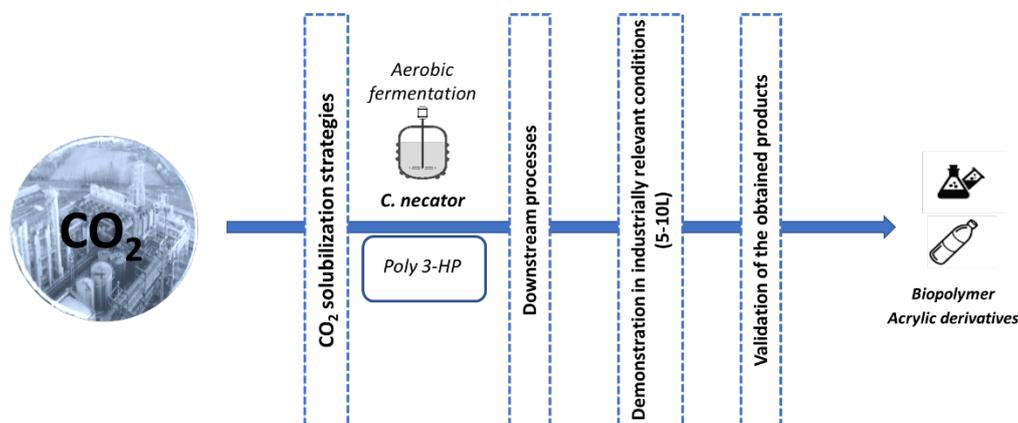


Figure 1. Overall strategy for the development of the aerobic microbial cell factory within the BIOCON-CO₂ project.

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Screening of reaction conditions in the photobiocatalytic decarboxylation of palmitic acid using a custom-built photoreactor

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Photoenzymes represent enzymes which require a continuous flux of light in order to catalyze a chemical reaction. To date, only four classes of natural photoenzymes are known, the most recently discovered being photodecarboxylases [1]. Fatty-acid photodecarboxylase from *Chlorella variabilis* (CvFAP), an FAD-dependent photoenzyme, was first described in 2017 and has shown promise for new sustainable methodologies in the synthesis of biofuels [2,3,4]. Herein, the optimization of reaction conditions in the CvFAP-catalyzed photodecarboxylation of palmitic acid using a custom-built photoreactor is described. The enzyme was heterologously expressed in *E. coli* and applied as cell-free extract. Screening of eight different wavelengths, and variation of light intensity and reaction time were performed. The obtained results, indicate that the constructed photoreactor provides reliable control of wavelength, light intensity, and temperature for reaction screening on 1 mL scale, enabling future examination of CvFAP using a more diverse library of substrates and reaction conditions.

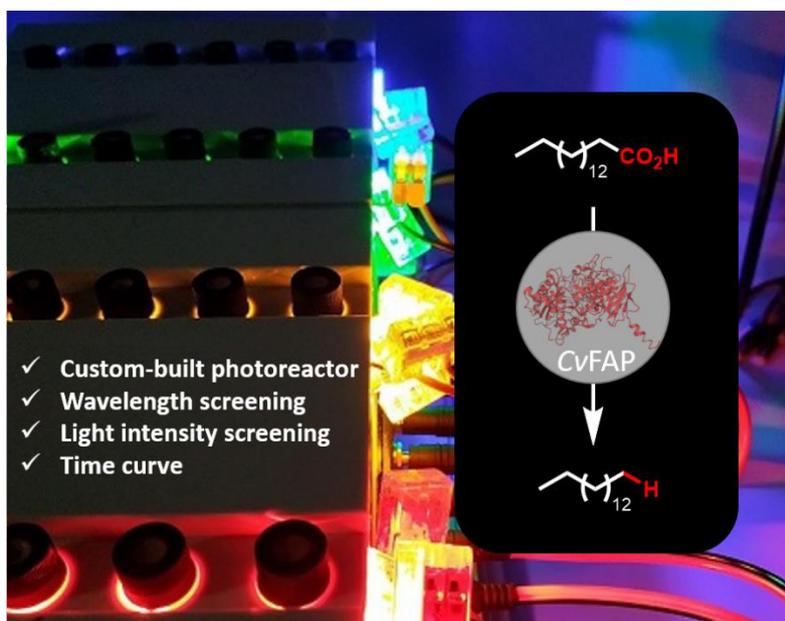


Figure 1. Parallel screening of four different wavelengths in the CvFAP-catalyzed photodecarboxylation of palmitic acid to pentadecane

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 862081.

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Internal illumination to overcome the cell density limitation in the scale-up of whole-cell photobiocatalysis

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The development of environmentally benign processes to produce chemicals and materials is an urgent challenge for the chemical and pharmaceutical industries. In recent years, cyanobacteria have been established as promising hosts for the sustainable production of chemicals.[1] A highly attractive approach is cyanobacterial whole-cell biotransformation that relies on carbon dioxide and water to produce highly selective biocatalysts, and photosynthesis to regenerate cofactors.[2] The major bottleneck for the scale-up of these light-driven biotransformations is light availability that decreases with longer light pathways or cell densities above a few grams per liter.

Here we show that internal illumination in a bubble-column reactor is a promising approach for the scale-up of light-driven biotransformations. As a model reaction, we utilized a strain of *Synechocystis* sp. PCC 6803 expressing the gene of the ene-reductase YqjM for the reduction of 2-methylmaleimide (2-MM) to (*R*)-2-methylsuccinimide ((*R*)-2-MS) with high optical purity (>99% *ee*). Compared to external illumination, internal illumination in a bubble-column reactor equipped with wireless light emitters (WLEs) allowed a more than two-fold increase in product formation rates. Further optimization of the conditions for the biotransformation, resulted in product formation rates up to 3.7 mM h⁻¹ and specific activities of up to 65.5 U g_{DCW}⁻¹, allowing the reduction of 40 mM 2-MM with 650 mg isolated enantiopure product (*R*)-2-MS (73 % yield).[3].

The results demonstrate the power of internal illumination to overcome the intrinsic cell density limitation of cyanobacterial biotransformations, obtaining high reaction rates in a scalable photobioreactor.

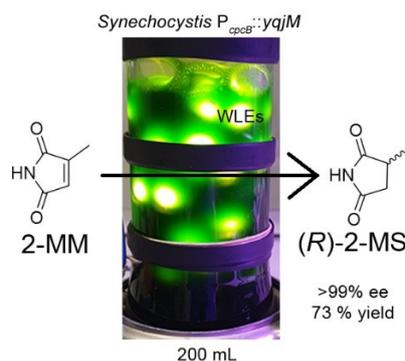


Figure 1. Photo-biotransformations performed in the bubble-column reactor with internal illumination provided by floating wireless light emitters (WLEs) in 200 mL scale. 2-MM: 2-methylmaleimide, (*R*)-2-MS: (*R*)-2-methylsuccinimide.

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Synthesis of piperonal by chemo-enzymatic oxidative cleavage of isosafrole

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Piperonal or heliotropine has a relevant role in the flavor industry [1] and is an essential starting material for the synthesis of fine chemicals such as the antiparkinsonian agent piribedil [2]. In general, it is prepared by chromic acid oxidation or ozonolysis of isosafrole [3], obtained in turn by isomerization of safrole. Since the traditional route is characterized by a considerable environmental impact because of the use of toxic and/or halogenated reagents and problematic solvents, as well as high energy consumption, an enzymatic approach would be desirable. Thus, we developed a chemo-enzymatic three-step procedure: first, lipase-mediated perhydrolysis of EtOAc in the presence of H₂O₂ was employed to generate peracetic acid in situ and promote the epoxidation of isosafrole. Then, the reaction mixture was submitted to methanolic KOH treatment to recover the corresponding mixture of vicinal diols that were successfully oxidized to piperonal using MnO₂, periodically regenerated at the expense of tert-butylhydroperoxide (TBHP). The use of continuous-flow conditions using a continuously-stirred tank reactor for the epoxidation, and a packed bed reactor for the final oxidation improved the productivity and stability of the whole method.

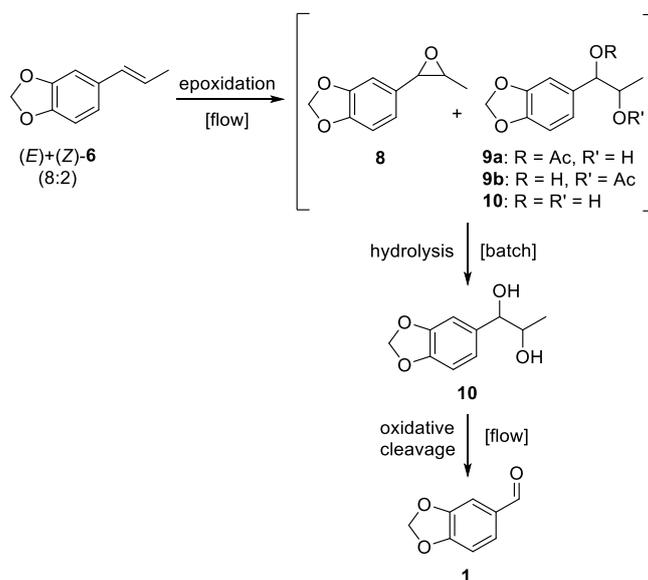


Figure 1. Chemo-enzymatic conversion of isosafrole (6) into piperonal (1) described in this work.

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Biocatalyst- and reaction engineering of biocatalytic oxyfunctionalizations

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The use of enzymes for lactonizations has been shown to be an environmentally friendly and resource efficient approach alternative to conventional chemical methods.^[1] In this context, artificial redox-neutral cascades to synthesize lactones have attracted great attention which provides self-sufficient multi-enzymatic systems in terms of cofactor regeneration.^[2] Recently, Fraaije and coworkers reported on the use of fusion proteins of cyclohexanone monooxygenase (CHMO) and alcohol dehydrogenase (ADH) in a linear cascade fashion for lactonizations.^[3] Their study applied CHMO from *Thermocrispum municipal* (TmCHMO)^[4], a recently discovered highly stable CHMO compared to the well-known and widely applied CHMO from *Acinetobacter* sp. In parallel, the use of a type-II flavin containing monooxygenase (FMO-E) and horse liver ADH (HLADH) was shown for the first time in predominantly organic media using only 5% (v/v) external water added.^[5] For implementation of CHMO-catalyzed lactonizations at high volumetric productivities (g/L/h), here presented study aims to develop heterogeneous enzymatic oxyfunctionalizations in non-conventional media with reaction engineering strategies based on enzyme kinetics^[5]. Within this project, TmCHMO will be coupled with ADH from *Thermoanaerobacter brockii* (TbADH) for *in situ* cofactor regeneration. Both fused and non-fused proteins are to be evaluated for their (co)immobilization and further application in a rotating bed reactor (Figure 1).

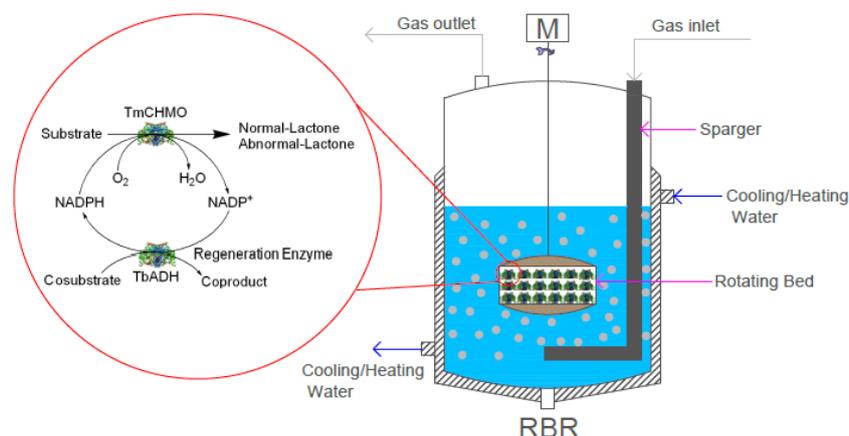


Figure 1. Heterogeneous bi-enzymatic oxyfunctionalizations towards the synthesis of lactones. Left: The generalized reaction scheme. Right: Rotating Bed Reactor (RBR, a stirred-tank reactor with a filter basket for the immobilized enzyme). TmCHMO: *Thermocrispum municipale* cyclohexanone monooxygenase, TbADH: *Thermoanaerobacter brockii* alcohol dehydrogenase.

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Implementation and scale-up of *in situ*-product crystallization concepts in transaminase-catalyzed reactions

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Transaminases (TAs) are highly selective catalysts for the synthesis of valuable chiral amines, used either in the kinetic resolution or asymmetric synthesis.[1] Major challenges in asymmetric synthesis are the unfavorable reaction equilibria of many TA-catalyzed reaction systems and frequently also downstream processing issues that need to be overcome for effective implementation on larger scales.

In this process design study we aim to tackle both issues and present the integration of a selective crystallization step into TA-catalyzed reactions and its development towards pilot plant scale. The induced crystallization selectively removes the desired chiral product amine from the reaction mixture as a sparingly soluble salt, which itself leads to an apparent shift of the reaction equilibrium to the product side. Simultaneously an enriched product stream after a simple filtration step is obtained (Figure 1).[2]

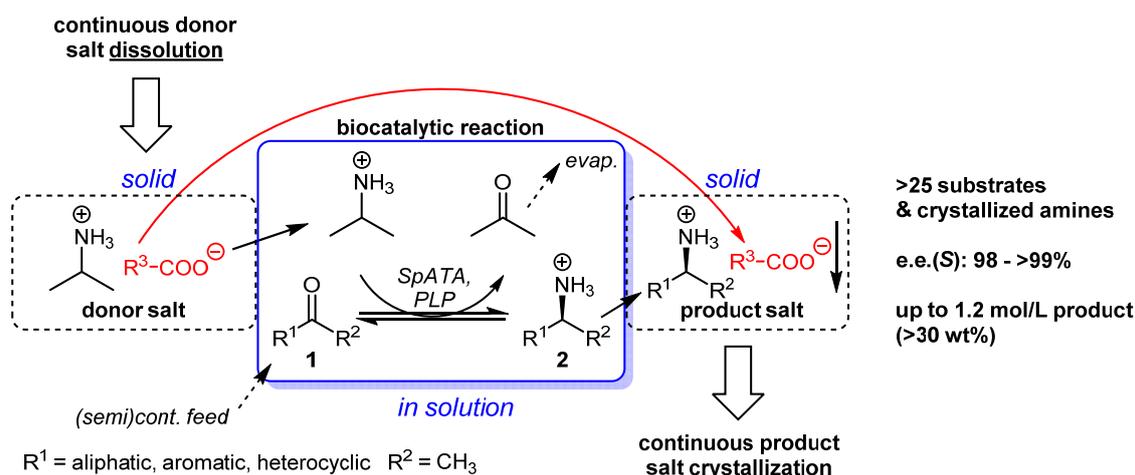


Figure 1. Continuously operated integrated crystallization reaction using the amine transaminase from *Silicibacter pomeroyi* (SpATA)

The process concept was developed from an early study[3] on a relatively small scale for >25 substrates (<0.08 mol/L product, batch reactor) up to a semi-continuous process[4] towards pilot plant scale. At the example (*S*)-1-(3-methoxyphenyl)ethylamine, a potential precursor of rivastigmine, product concentrations of >1.2 mol/L were achieved using essentially only stoichiometric amounts of the donor isopropylamine. In a later study also a fully sequential reaction design was established using two reaction vessels and a separate enzyme membrane reactor, allowing a continuous enzymatic product synthesis.[5]

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Immobilisation Strategies in Use – a Comparison

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Enzymes are versatile and valuable catalysts for synthesis. However, the production of viable biocatalyst can be laborious and costly. Immobilisation tackles this problem by improving reusability, simplifying the product workup and enabling the use in continuous flow synthesis. [1] The question raises: How should one's enzymes be immobilised? Simple covalent methods need pure enzyme, increasing the labour required. Affinity based methods usually lack covalency and render the produced immobilisate susceptible to leeching - the continuous loss of enzyme by elution. [2]

For the general use of 2-deoxy-D-ribose-5-phosphate aldolase (DERA) in flow cascades we investigated the suitability of some readily available immobilisation strategies. The HaloTag®-System is ideal for fast creation of pure and covalent immobilisate in small batches. [3] This system proved reliable for our needs so far. [4] For potential scale up, we looked for alternative methods. To benefit from the ease of simultaneous purification and immobilisation, we also tested the use of metal ion affinity and the use of Strep-tag® - Strep-Tactin® interaction. [5,6] Although more laborious, the immobilisation on epoxy activated carrier was tested also, yielding covalent immobilisation for a small price tag. [7]

The systems were evaluated based on the performance of the immobilisate under standardised conditions with respect to the aldol addition reaction between hexanal and acetaldehyde. To be more specific, we compare the initial activity and the loss of activity for prolonged use for this demanding reaction. With this poster we will present our most recent findings and reveal which immobilisation strategy performs best.

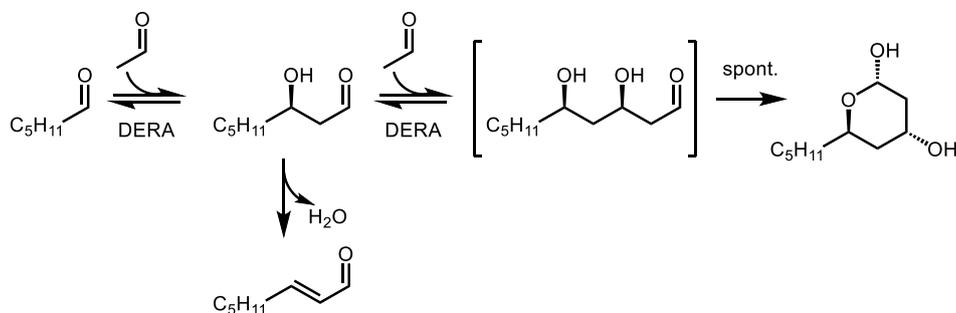


Figure 1: investigated model reaction of the DERA-catalysed transformation between acetaldehyde and hexanal.

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Tailored polymeric emulsion for efficient biocatalysis in organic solvents

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Emulsion biocatalysis has long been considered as an effective tool for synthetic transformation in organic solvents.[1] A typical traditional emulsion is created typically by small surfactants that, however, are hardly removed after operation, and thus have limited usability for catalysis. To improve it, we design three types of block copolymers, which possess sufficient amphiphilicity for emulsion formation while allowing ready separation after reactions. Therefore, they have been successfully applied to encapsulate enzymes for diverse types of biotransformations in organic solvents.

The first polymer, poly(ethylene glycol)-block-poly(ϵ -caprolactone), is synthesized by ring open polymerization.[2] This polymer can stabilize water-in-oil emulsion to encapsulate benzaldehyde lyase (BAL) for the benzoin condensation, resulting in 300-time higher enzyme activity than the biphasic control. The second polymer is enzyme-polymer conjugates that are prepared by grafting *N*-isopropylacrylamide from enzymes like BAL via *in situ* atom-transfer radical polymerization (ATRP) (Fig. 1A). The resultant block polymer allows for the formation of oil-in-water emulsions for not only single-step condensation reactions but also synthetic cascades with high efficiency.[3] Lastly, we have prepared block copolymers that are responsive to multiple stimuli, such as, temperature, CO₂, and pH (Fig. 1B).[4] The multiple stimuli-responsiveness allows for an on-demand switch between emulsion and demulsification upon the environmental changes, which is utilized for the easy exchange of enzymes and products from the emulsion. Therefore, the responsive emulsion can be used for sequential cascade reactions, whereby three enzymes are applied for three-step reactions in their individual optimal conditions, accordingly achieving about 97% theoretic conversion.

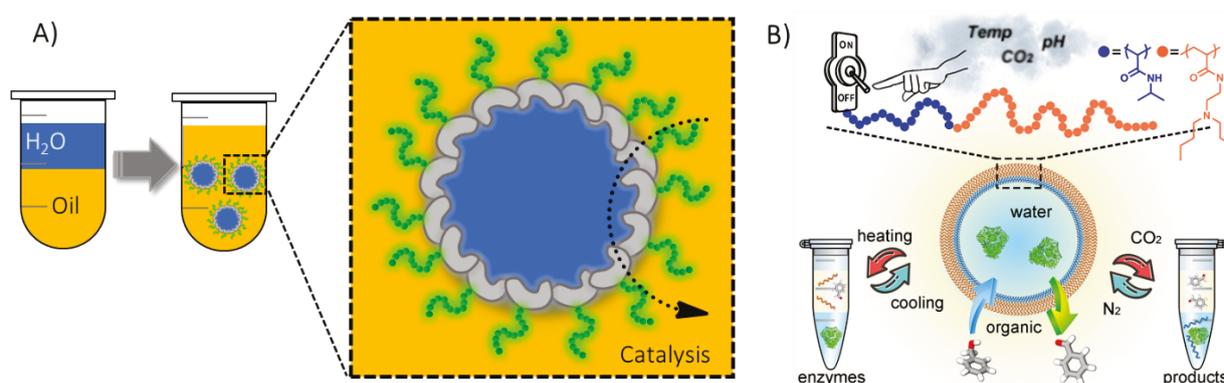


Figure 1. A) The use of enzyme-polymer conjugates for emulsion catalysis, and B) stimuli-responsive emulsions for multienzyme cascades.

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Intensification of photobiocatalytic decarboxylation of fatty acids for the production of biodiesel

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Biodiesel is an important pillar of the ongoing global transition from fossil energy carriers to renewable alternatives. Different from the conventional transesterification of natural fats and oils, decarboxylation is an irreversible reaction producing real biodiesel with higher caloric value. The promising production approach is to simply decarboxylate fatty acids into the corresponding (C1-shortened) alkanes using the fatty acid photodecarboxylase from *Chlorella variabilis* (CvFAP). Yet, light-driven biocatalytic processes are notoriously hampered by poor penetration of light into the turbid reaction media which makes the up-scaling and intensification required for technical implementation difficult. [1-3]

Compared with the conventional external illumination setup with LEDs, performing the reaction using the internal wirelessly powered light-emitting diodes (WLE) resulted in a more than 22-fold acceleration of product formation rate. In this study, WLE are found to represent an efficient and scalable approach for process intensification of the photobiosynthetic production of diesel alkanes from renewable fatty acids. At the productivity achieved in this study, the process could produce 264 mL of pentadecane per liter of reaction volume each day, clearly pointing towards larger scale implementation.

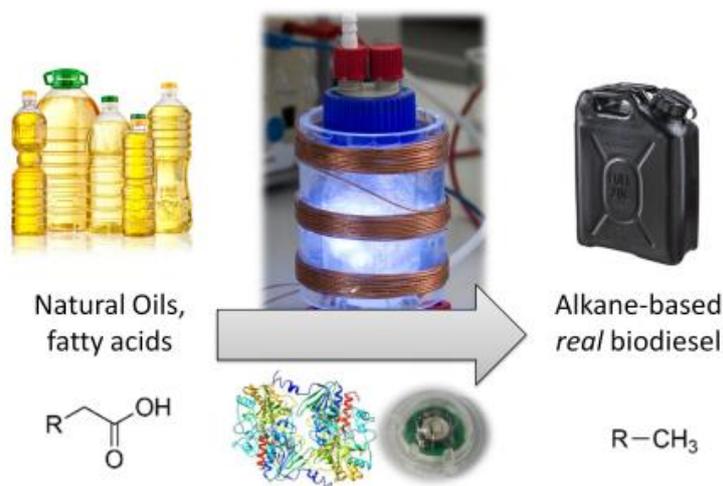


Figure 1. The photoreactor setup employed for the production of biodiesel.

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Understanding alcohol dehydrogenase catalysis in deep eutectic solvents: Joining forces from experiments and molecular dynamics simulations

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Oxidoreductases (EC1) are gaining tremendous interest for their use in non-conventional reaction media.^[1a] In this context, deep eutectic solvents (DESs), being coined as ‘the solvents of the 21st century’ owing to their numerous merits, have attracted great attention.^[1b] However, the systematic and complete analysis of the effects of DESs on oxidoreductases has not been reported yet.

In this study, the effects of DESs on structural and functional changes of a model oxidoreductase have been investigated by assessing the catalytic performance of horse liver alcohol dehydrogenase (HLADH) in chosen DES-water mixtures with the support of molecular dynamics (MD) simulations (**Figure 1**). The MD simulations were performed to quantify the molecular flexibility of HLADH and the hydration layer on the surface of the enzyme in DES-water mixtures with different thermodynamic water activities (a_w) and dynamic viscosities (η).^[2]

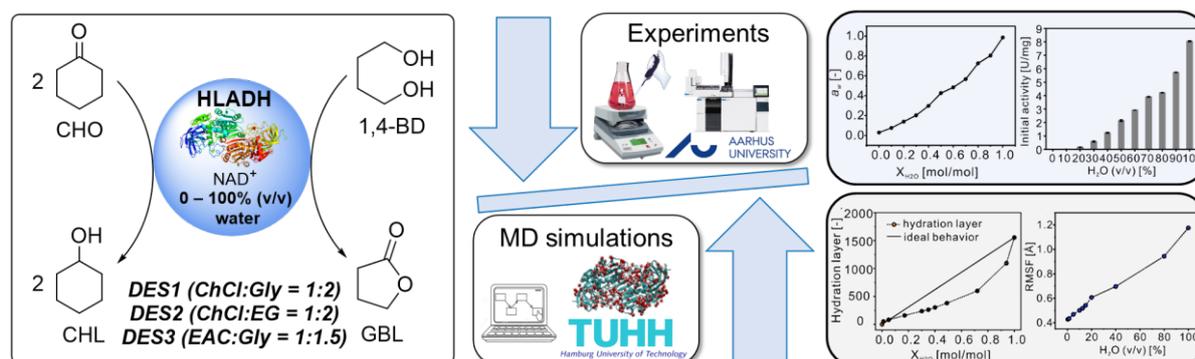


Figure 1. Reduction of cyclohexanone (CHO) catalyzed by HLADH to cyclohexanol (CHL) promoted by 1,4-butanediol (1,4-BD) in DES-water mixtures. HLADH: horse liver alcohol dehydrogenase, ChCl: Choline chloride, Gly: glycerol, EG: ethylene glycol, EAC: ethanol ammonium chloride.

The combination of the experimental and computational analysis disclosed the activity and stability of enzymes were positively related to a_w due to the changes of the solvation layers surrounding enzymes. The individual components of DESs can impose positive (Gly) or negative (ChCl) effects on enzymes. The finding opens a new avenue for the depth-study of the effects of non-conventional media on enzymes, which can guide the selection of the optimal solvents for defined enzymes.

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Can deep eutectic solvents sustain oxygen dependent bioprocesses? – Measurements of oxygen transfer rates (OTRs)

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Oxidoreductases (EC1) are gaining tremendous interest for their use in non-conventional reaction media.^[1a] In this context, deep eutectic solvents (DESs), being coined as ‘the solvents of the 21st century’ owing to their numerous merits, have attracted great attention.^[1b] Given oxidation reactions demanding molecular oxygen (O₂) as a reaction partner, accurate knowledge of O₂ transfer is critical for the control of many bioprocesses.^[1c] In this context, the transfer rate of O₂ from air into DESs has not been reported yet.

In this study, the oxygen transfer rates (OTRs) from air into DESs and DES-water mixtures were evaluated by measuring the volumetric mass transfer coefficient (k_La) using a dynamic method (Figure 1).^[2]

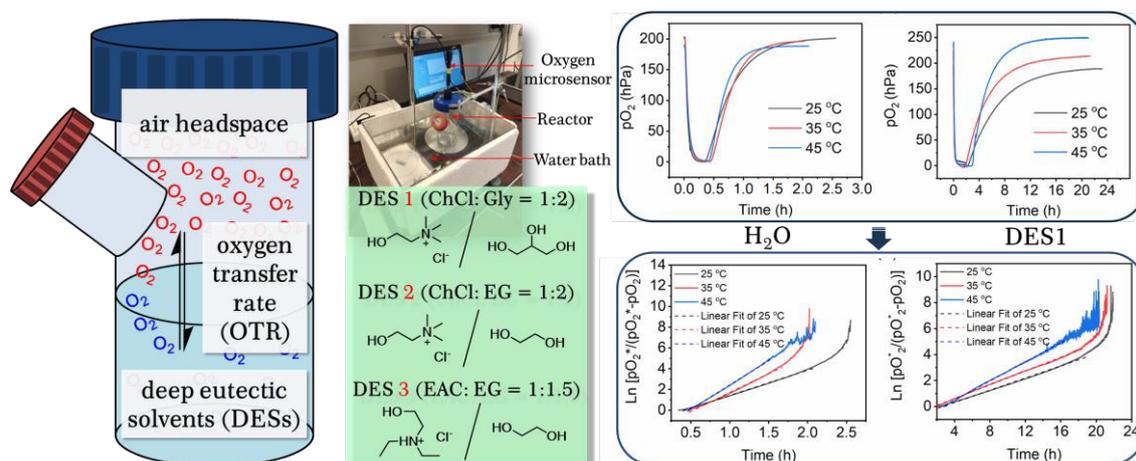


Figure 1. The measurement of oxygen transfer rates (OTRs) from air to diverse DESs at three different temperatures (25 °C, 35 °C, and 45 °C). ChCl: Choline chloride, Gly: glycerol, EG: ethylene glycol, EAC: ethanol ammonium chloride.

This study revealed up to 11-fold lower k_La values for DES2 and 6-fold lower for DES3 when compared to water at 25 °C. The nature of the DESs has a big influence on the oxygen transfer and it is possible to tune OTRs in this way. In addition, the increment of temperature and the addition of water highly assisted the oxygen transfer processes by decreasing the viscosity, leading to higher k_La values. The method can be applied in the further gaseous substances (e.g., CO₂ or H₂) due to their importance in bioprocesses.

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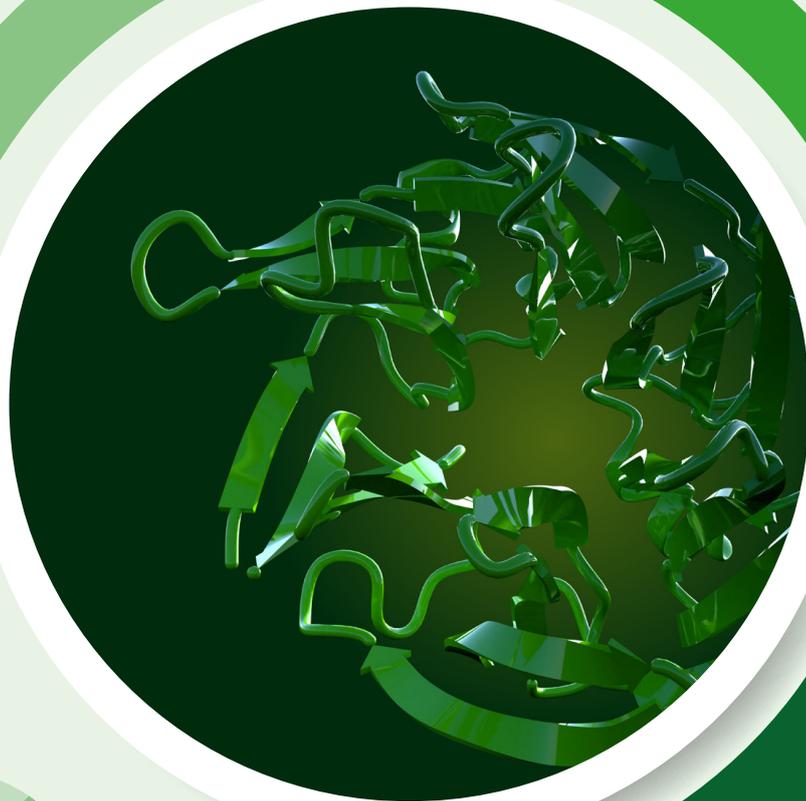
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