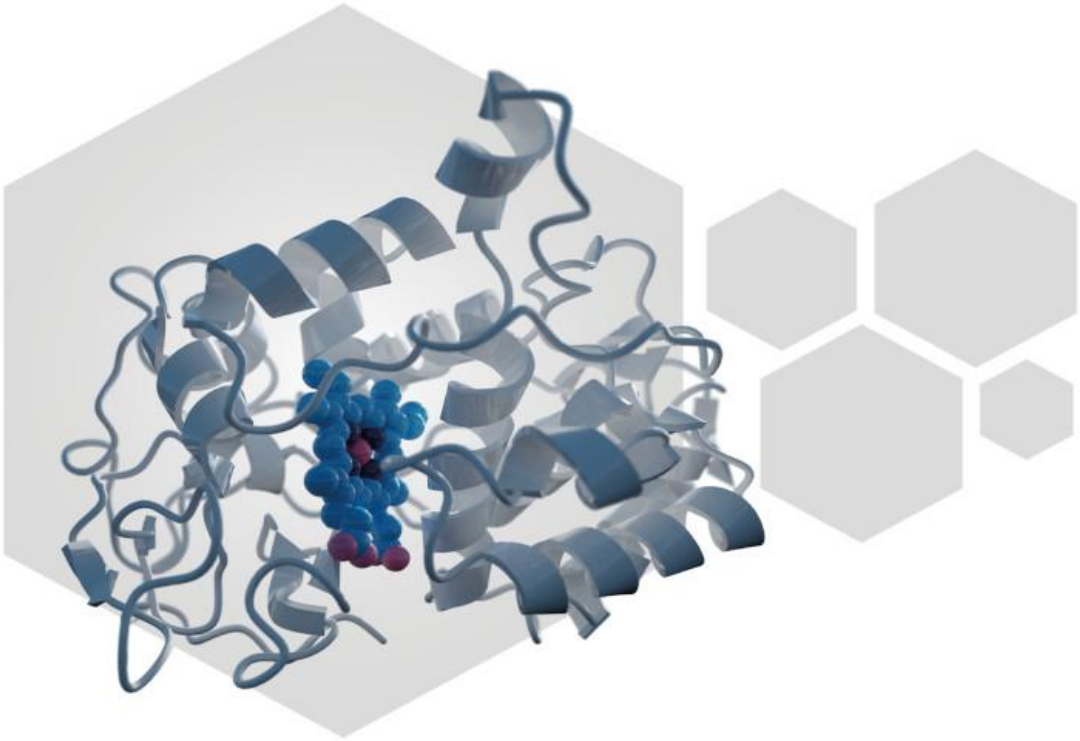

NextGenBiocat

Book of Abstracts

NextGenBiocat 2026

An International Young Investigator Symposium

Munich, 10-11 June 2026

With generous support from our sponsors:

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WELCOME TO NEXTGENBIOCAT 2026

We are delighted to welcome you to the 6th edition of the NextGenBiocat symposium on June 10th and 11th 2026, in Munich, Germany!

Biocatalysis and the investigation of the function of enzymes are exciting and vibrant topics, with new results being published on a daily basis. The NextGenBiocat symposium is organized by young researchers in this area from TU Munich, University of Groningen, University of Milan, University College London, NordicBlue ApS, and University of Bern. Its aim is to bring together young researchers, PhD candidates, postdocs and early-career PIs to feature their research in the field of (but not limited to):

- Applied biocatalysis
- Elucidation of enzymatic mechanisms
- Protein, reaction and metabolic engineering (including computational tools)
- Light-promoted enzymatic processes
- Ultra-high-throughput screening methods for enzyme discovery and optimization
- Design of novel multi-enzyme cascade reactions

Additionally, the afternoon of the second day will be dedicated to a satellite event hosted by the MSCA network BiodeCCodiNNg, which focuses on advancing carbon–carbon (C–C) and nitrogen–nitrogen (N–N) bond-forming enzymes—referred to as CCzymes and NNzymes.

All participants will have the opportunity to showcase their research amongst other young researchers, discuss ideas, and create or reinforce collaborations and career experiences. We are looking forward to exciting interactive sessions with you!

Lastly, we would like to express our gratitude to you for your participation, and to all our generous sponsors and members of the scientific committee for the support. Thank you!

With our best wishes,

The NextGenBiocat 2026 Organizing Committee

Ana I. Benítez-Mateos

Christian M. Heckmann

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HISTORY OF NEXTGENBIOCAT

NextGenBiocat originated as a fusion of two biocatalysis symposia organized by young researchers in 2021. NextGenBiocat2021 was organized by Christoph Winkler, Sandy Schmidt and Caroline Paul in Graz, Austria in February, whereas NGB2021 was organized by Ana Benítez-Mateos in Bern, Switzerland in May. Due to the COVID pandemic, both events took place online. The two organizing committees teamed up to launch the NextGenBiocat symposium as an annual series dedicated to young researchers in biocatalysis.

1st NextGenBiocat	**2021**	*Online*
2nd NextGenBiocat	**2022**	*TU Delft, The Netherlands*
3rd NextGenBiocat	**2023**	*TU Graz, Austria*
4th NextGenBiocat	**2024**	*University of Crete, Greece*
5th NextGenBiocat	**2025**	*POLIMI-UNIMI, Milano, Italy*
6th NextGenBiocat	**2026**	*TUM, Munich, Germany*

Announcement NextGenBiocat 2027

We hope to meet you at the 7th NextGenBiocat in 2027! Save the date!

The date and location of the next edition will be revealed at the end of the conference.

CONFERENCE PROGRAMME

Oral presentations: 15 min + 5 min Q&A **Poster presentations:** A0, portrait

Day 1 — Wednesday, 10 June 2026

8:30–9:00	*REGISTRATION*
9:00–9:10	**WELCOME ADDRESS AND OPENING**
Session 1 · Chair: Michele Crotti	
9:10–9:50	**INVITED LECTURE – IL1** *Lena Barra (University of Konstanz)* Enzyme and Natural Product Discovery in Non-Canonical Pathways
9:50–10:10	**ORAL PRESENTATION – OP1** *Philip Troycke (Technical University of Munich)* Structural and mechanistic studies on bacterial sesterterpene synthases
10:10–10:30	**ORAL PRESENTATION – OP2** *Chunyu Gao (Bielefeld University)* Chiral Alcohols from Alkenes and Water: Directed Evolution of Styrene Hydratases
10:30–11:30	*COFFEE BREAK – POSTER SESSION (odd numbers)*
Session 2 · Chair: Marianna Karava	
11:30–11:50	**ORAL PRESENTATION – OP3** *Anton Natter Perdiguero (University of Zurich)* Genetic incorporation of diverse non-canonical amino acids for histidine substitution
11:50–12:10	**ORAL PRESENTATION – OP4** *Angelique Pothuizen (Delft University of Technology)* Exploring H_2O_2-dependent inactivation of rAaeUPO
12:10–12:30	**ORAL PRESENTATION – OP5** *Andrea Suárez-Herrera (Instituto de Quimica Avançada de Catalunya)* Synthesis of tertiary alcohols via aldol reactions of ketones mediated by 2-oxoacid aldolases
12:30–13:30	*LUNCH BREAK*
Session 3 · Chair: Christian Heckmann	
13:30–14:10	**INVITED LECTURE – IL2** *Laura Leemans (Novartis Pharma AG)* Unlocking a new synthesis: when enzyme engineering and process design co-evolve
14:10–14:30	**ORAL PRESENTATION – OP6** *Cristina Lía Fernández Regueiro (inSEIT AG)* Bioinformatics-Guided Immobilization of a Peptide Ligase for Continuous Flow Chemoenzymatic Synthesis of Semaglutide
14:30–14:50	**ORAL PRESENTATION – OP7** *Federico Zappaterra (University of Ferrara)* Applied Biocatalysis for the Sustainable Synthesis of High-Solubility Prodrugs: From Mechanistic Insight to Scalable Processes
14:50–15:10	**ORAL PRESENTATION – OP8** *Glenn Bojanov (University of Bern)* Going Full Circle: Dynamic Covalent Enzyme Immobilisation via Visually Trackable Boronate Esters
15:10–16:00	*COFFEE BREAK – POSTER SESSION (odd numbers)*
Session 4 · Chair: Fabio Parmeggiani	
16:00–16:20	**ORAL PRESENTATION – OP9** *Ammar Al-Shameri (Technical University of Munich)* Flipping Hydrogenase Reactivity: A New Paradigm for O_2-Free Oxidative Biocatalysis
16:20–16:40	**ORAL PRESENTATION – OP10** *Mikas Sadauskas (Vilnius University)* A Cofactor-Independent Dioxygenase Reaction Catalysed by lifA
16:40–17:00	**ORAL PRESENTATION – OP11** *Manuel Pérez-Soto (Technical University Munich)* Intrinsically Disordered Proteins Enable Robust and Enhanced Enzymatic Biocatalysis under Harsh Conditions
19:00	*CONFERENCE DINNER at Gasthof Neuwirt* *Münchener Str. 10, 85748 Garching bei München*

Day 2 — Thursday, 11 June 2026

Session 5 · Chair: Beatrice Rassati	
9:00–9:40	**INVITED LECTURE – IL3** *Noelia Ferruz (Centre for Genomic Regulation)* Controllable Protein Design with Language Models
9:40–10:00	**ORAL PRESENTATION – OP12** *Manu Suvarna (University of Greifswald)* Active learning maps the activity-selectivity Pareto front in enzymatic PET upcycling
10:00–10:20	**ORAL PRESENTATION – OP13** *Aqza Elza John (University of Girona)* Decoding electrostatic optimization in designed retroaldolases
10:20–11:20	*COFFEE BREAK – POSTER SESSION (even numbers)*
Session 6 · Chair: Silvia Donzella	
11:20–11:40	**ORAL PRESENTATION – OP14** *Sangeet Dhiman (Bar-Ilan University)* From Poses to Pathways: RxnNet Pathway Generation with MCS-Based EnzyDock Docking
11:40–12:00	**ORAL PRESENTATION – OP15** *Martina Bigliardi (University of Milan)* From biomass-derived phenolic acids to valuable aromatic building blocks through a sustainable and tunable multi-enzymatic cascade
12:00–12:20	**ORAL PRESENTATION – OP16** *Julian Schilke (Technical University of Braunschweig)* Increasing phenylpropanoid yield from enzymatic lignin depolymerization
12:20–13:20	*LUNCH BREAK*
13:20–14:00	*POSTER SESSION (even numbers)*
Session 7 · Chair: Ana I. Benítez-Mateos	
14:00–14:20	**EDITORIAL LECTURE** *Laura Woodward-Heni (Wiley-VCH)* Opening the Editor's Black Box: Insider Tips for Successful Submissions
14:20–15:05	**ROUND TABLE: Career Paths**
15:05–15:30	**CLOSING REMARKS AND AWARD CEREMONY**

Day 3 — Friday, 12 June 2026 · BiodeCCodiNNg Satellite Event

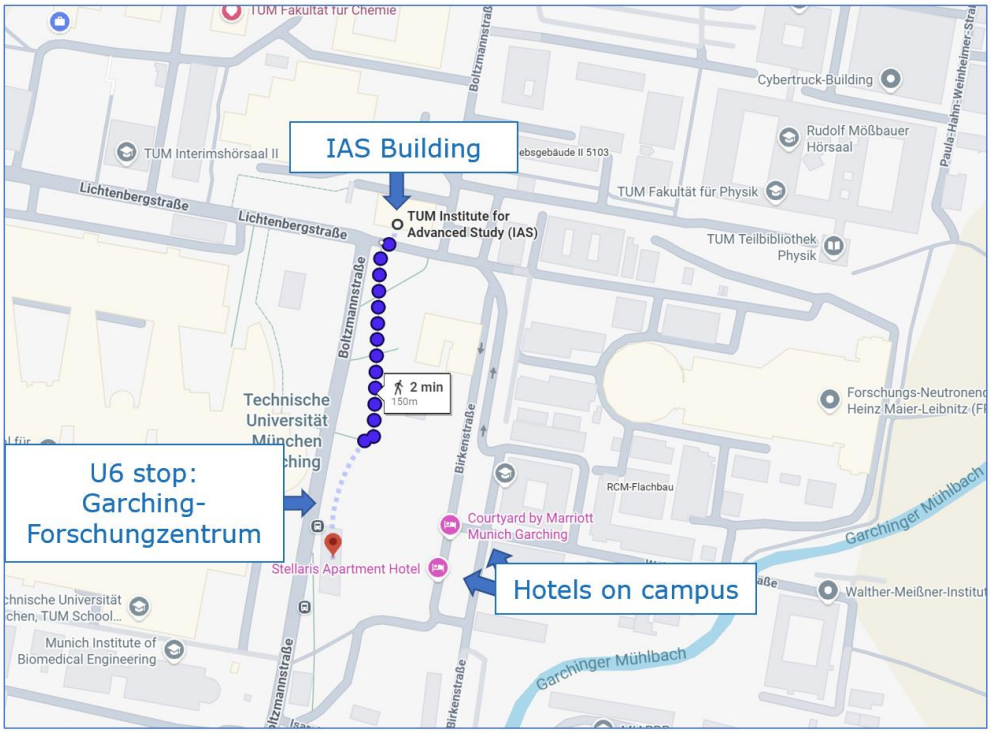
9:00–9:10	**Opening: Sandy Schmidt**
9:10–9:50	**KEYNOTE LECTURE** *Dr. Joerg Schrittwieser* Building molecular complexity: biocatalytic key bond formations for organic synthesis
BiodeCCodiNNg WP1 Session · Chair: Nikita Pal, Lindelo Mguni	
9:50–10:10	**Simon Schröder (DC1)** N-N bond-forming piperazate synthases: Characterization, Engineering and Application
10:10–10:30	**Carina Kipp (DC2)** Discovery and exploration of enzymes for Morita-Baylis-Hillman reactions
10:30–10:50	**Corina Šljubura (DC3)** Construction of artificial metallolyases for C-C bond forming reactions
11:00–11:30	*COFFEE BREAK*
BiodeCCodiNNg WP2 Session · Chair: Flora Maria Bindreiter, Lan Julij Zadravec	
11:30–11:50	**Annika Hein (DC4)** Engineering N-N bond forming enzymes for novel reactions
11:50–12:10	**Nikita Pal (DC5)** Structure and conformational dynamics of N-N bond-forming piperazate synthase
12:10–12:30	**Suresh Rohan (DC6)** Mechanistic and Structural Insights into enzymes with non-natural carboligation activity
12:30–12:50	**Lilla Gal (DC7)** Novel biocatalytic methodology for formylation of aromatics
13:00–14:00	*LUNCH BREAK*
BiodeCCodiNNg WP3 Session · Chair: Suresh Rohan, Simon Schröder	
14:00–14:20	**Lindelo Mguni (DC8)** Novel N-hydroxylating enzymes in biocatalytic cascades
14:20–14:40	**Lan Julij Zadravec (DC9)** Reaction engineering aided enzymatic synthesis of piperazic acid
14:40–15:00	**Flora Maria Bindreiter (DC10)** Stereoselective access of 4-hydroxylated aromatic amino alcohols from renewables via a modular multi-enzyme catalysed reaction
15:00–15:10	**Closing remarks: Sandy Schmidt**

CONFERENCE LOCATION

The NextGenBiocat 2026 conference will take place at the TUM Institute for Advanced Study (IAS) at the Technical University of Munich, Germany.

Lichtenbergstraße 2a, 85748 Garching bei Munich, Germany

Google Maps



Getting There

By Plane — From Munich International Airport (MUC), take the S1 train to Neufahrn, then transfer to bus 690 to Garching-Forschungszentrum.

By U-Bahn — From Munich Hauptbahnhof take U5 to Odeonsplatz, then U6 to Garching-Forschungszentrum (terminal stop). The IAS is a 5-minute walk.

By Car — From the A9 motorway take exit 70 (Garching-Nord) and follow signs to Garching-Forschungszentrum. Parking is available on campus.

CONFERENCE DINNER – VENUE

Gasthof Neuwirt

Münchener Str. 10, 85748 Garching bei München

Wednesday, 10 June 2026 · 19:00

How to get there?

The Gasthof Neuwirt is about 8 minutes by U6 from the IAS Building.

Google Maps

INVITED SPEAKERS

IL-1 – Lena Barra

University of Konstanz, Germany

Lena Barra obtained her PhD under the supervision of Prof. Jeroen S. Dickschat at the University of Bonn in 2018. Thereafter, she worked as a postdoctoral researcher in the group of Prof. Ikuro Abe at the University of Tokyo. In May 2022 she was appointed to a Tenure-Track professorship for Chemical Biology at the University of Konstanz. Her present research focuses on the discovery of novel enzyme chemistries for biocatalytic production of active pharmaceutical ingredients, with a particular emphasis on non-canonical secondary metabolic pathways combining genomics-driven computational methods with functional, structural, and mechanistic investigations.

IL-2 – Laura Leemans Martin

Principal Scientist, Novartis Pharma AG

Laura Leemans Martin obtained her PhD under the supervision of Prof. Anett Schallmey at the Technical University of Braunschweig. She joined Novartis Pharma in 2020 as a postdoctoral researcher focusing on enzyme immobilization and flow biocatalysis. Since 2022, she has been part of the CHAD Biocatalysis team in Development, where she works as a biochemistry expert and enzyme-evolution technical lead, contributing to the engineering of biocatalysts for the efficient synthesis of active pharmaceutical ingredients.

IL-3 – Noelia Ferruz

Centre for Genomic Regulation (CRG), Barcelona, Spain

Noelia Ferruz is a group leader at the Centre for Genomic Regulation (CRG), Barcelona, leading the AI for Protein Design team. Her research combines generative AI, explainable models, and experimental validation to create functional proteins for medicine, sustainability, and biotechnology. She studied Chemistry at the University of Zaragoza, completing her MSc and PhD in Bioinformatics at Pompeu Fabra University, with further training at the University of Cambridge, Pfizer, and the University of Bayreuth. In 2024, she received a €1.5M ERC Starting Grant for the ATHENA project, a transparent AI platform for protein design.

Editorial – Laura Woodward-Heni

Chemistry – A European Journal & ChemBioChem, Wiley-VCH

Laura Woodward-Heni obtained her PhD under Jim H. Naismith at the University of St. Andrews in 2017. She subsequently worked at EMBL Heidelberg as a postdoctoral researcher in the group of Carsten Sachse, before becoming an editor at Angewandte Chemie in late 2018. In 2024, she joined the editorial teams of Chemistry – A European Journal and ChemBioChem as Deputy Editor, where she plays an active role in both the peer-review process and the strategic development of both journals.

LIST OF POSTERS

P	Presenting author ■ Institution
P1	**Stefanie Baldauf** · Technical University of Graz, Austria
P2	**Sadia Batool** · Otto Von Gueriecke University, Germany
P3	**Flora Maria Bindreiter** · Forschungszentrum Jülich GmbH, Germany
P4	**Annkathrin Bohne** · Technische Universität München, Germany
P5	**Matias Brezina Borges** · University of Groningen, Netherlands
P6	**Fabrizio Casilli** · University of Stuttgart, Germany
P7	**Craig Chamney** · University College Dublin, Ireland
P8	**Darly Concha** · Universitat Autónoma de Barcelona, Spain
P9	**Ivan Dimitri Gómez** · Universitat Autònoma de Barcelona, Spain
P10	**Nina Egeler** · Technical University of Munich, Germany
P11	**Sara Foiadelli** · Università degli studi di Milano, Italy
P12	**Parthena (Renia) Fotiadou** · University of Crete, Greece
P13	**Mosè Simone Galluzzo** · University of Graz, Austria
P14	**Lilla Gal** · University of Graz, Austria
P15	**Martí Garçon** · University of Cambridge, United Kingdom
P16	**Xenia Georgiadou** · Graz Technical University, Austria
P17	**Stefano Giuliani** · University of Milan, Italy
P18	**Catharina Gronkowsky** · Heinrich Heine University Düsseldorf, Germany
P19	**Marianne Bore Haarr** · University College Dublin, Ireland
P20	**Christian Heckmann** · NordicBlue ApS, Denmark
P21	**Annika Hein** · Rijksuniversiteit Groningen, Netherlands
P22	**Jiayi Hou** · Forschungszentrum Jülich GmbH, United Kingdom
P23	**Jia-Ying Huang** · Delft University of Technology, Netherlands
P24	**Grzegorz Jamróg** · Technical University of Munich, Germany
P25	**Shiny Joseph Srinivasan** · University of Manchester, United Kingdom
P26	**Carina Kipp** · University of Groningen, Netherlands
P27	**Bence Attila Kucsinka** · TU Delft, Netherlands
P28	**Ilaria Magrini Alunno** · IUSS Pavia, Italy
P29	**Lindelo Mguni** · Ruhr University Bochum, Germany
P30	**Faith Molloy** · University College Dublin, Ireland
P31	**Rahel Mühlhofer** · Technische Universität München, Germany
P32	**Maria Nabatova** · Zymvol Biomodeling, Spain
P33	**Nikita Pal** · University of Graz, Austria
P34	**Fabio Parmeggiani** · Politecnico di Milano, Italy
P35	**Beatrice Rassati** · University of Bern, Switzerland
P36	**Suresh Rohan** · Georg-August-Universität Göttingen, Germany

P37 **Sara Salehi** · Karl-Franzens-Universität Graz, Austria

P38 **Ferran Sancho** · Zymvol Biomodeling, Spain

P39 **Max Scholz** · Ruhr University Bochum, Germany

P40 **Joerg Schrittwieser** · University of Graz, Austria

P41 **Simon Schröder** · University of Groningen, Netherlands

P42 **Anna Schrüfer** · Technical University of Graz, Austria

P43 **Luzia Schütz** · University of Freiburg, Germany

P44 **Beatrice Tagliabue** · TU Delft, Netherlands

P45 **Jan Taubitz** · University of Bern, Switzerland

P46 **Laia Vila-Vidal** · Technical University of Denmark, Denmark

P47 **Nils Weindorf** · Ruhr University Bochum, Germany

P48 **Gloria Zucchi** · University of Milan, Italy

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P58 **William Olsen** · Technical University of Denmark, Denmark

P59 **Kieran Didi** · Oxford University, United Kingdom

P60 **Johan Sebastian Portilla-Pulido** · The University of Queensland, Denmark

P61 **Jiayu Jiang** · DTU, Denmark

P62 **Visnja Kosic** · University of Belgrade, Institute of Chemistry, Technology and Metallurgy, Serbia

P63 **Oren Bachar** · Max Planck Institute for Terrestrial Microbiology, Germany

P64 **Clàudia Ferrer Carbonell** · Delft University of Technology, Netherlands

P65 **Marianna Karava** · Austrian Center of Industrial Biotechnology, Austria

P66 **Ana Robles-Martín** · Nostrum Biodiscovery, Spain

P67 **Lan Julij Zadravec** · University of Zagreb Faculty of Chemical Engineering and Technology, Croatia

P68 **Tim Schlosser** · University of Freiburg, Germany

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Laura Woodward-Heni	Wiley-VCH, Germany
Lan Julij Zadravec	University of Zagreb, Croatia
Federico Zappaterra	University of Ferrara, Italy
Gloria Zucchi	University of Milan, Italy

ABSTRACTS

IL-1

Enzyme and Natural Product Discovery in Non-Canonical Pathways

Lena Barra[1]
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lena.barra@uni-konstanz.de

Natural products have been and continue to be of tremendous importance for human health purposes. They have captivated researchers for decades with their remarkable structural complexity, bioactivity, and the intricate enzymatic reactions underlying their biosynthesis. While much attention has been focused on traditional classes such as non-ribosomal peptides, polyketides, and terpenoids, our research program specifically aims to expand upon these systems by uncovering and investigating distinct specialized metabolic pathways to unlock access to novel chemical entities and the associated enzyme-catalyzed chemical transformations.

The talk will present our ongoing efforts to explore non-canonical enzyme chemistry derived from *S*-adenosylmethionine (SAM) by integrating genomics-driven computational approaches with functional, structural, and mechanistic investigations (**Figure 1**). Particular emphasis will be placed on the discovery and characterization of non-classical terpene biosynthetic pathways that generate methyl-modified terpenes, extending beyond the classical "biogenic isoprene rule". These pathways rely on SAM-dependent carbon methylation reactions that produce methylated prenyl diphosphate intermediates and thereby provide unique entry points for the discovery and engineering of methyl-modified terpenes and subsequent exploration and exploitation of a potential "magic-methyl effect" within terpene chemistry.[1,2]

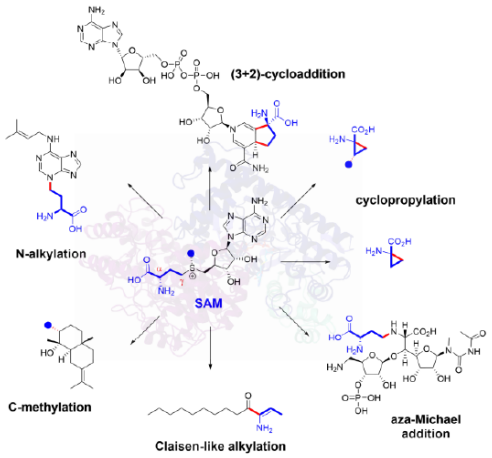

Figure 1. Function of SAM in non-canonical natural product biosynthesis.

[1] L. Zhou, T. Reuter, K. Schumann, M. Mayer, D. M. Hanauska, L. Barra, *Angew. Chem. Int. Ed.* **2025**, *64*, e17837.
[2] T. Reuter, L. Dieminger, S. Steidle, K. Zoller, M. Holocher, L. Zhou, D. M. Hanauska, K. Racz, L. Barra, *Angew. Chem. Int. Ed.* **2024**, *64*, e202418613.

IL-2

Unlocking a new synthesis: when enzyme engineering and process design co-evolve

Laura Leemans Martin[1]
[1] *Novartis Pharma AG*
Basel, Switzerland
Laura.leemans_martin@novartis.com

Iterative alignment between enzyme engineering and process design can be key to developing efficient and sustainable biocatalytic processes.

We describe the development of a ketoreductase (KRED)-enabled dynamic kinetic resolution for the synthesis of a chiral drug intermediate with two stereogenic centers. Route scouting identified an efficient biocatalytic strategy replacing sequential establishment of the stereogenic centers in a consecutive two-step approach by a one-step dynamic kinetic resolution. To overcome limitations due to slow racemization and maximize both conversion and stereopurity, enzyme engineering provided KREDs with increased activity and selectivity, while parallel process development identified enzyme-compatible conditions that accelerated racemization.

Iterative feedback between enzyme evolution and process optimization ultimately enabled identification of a highly active and selective KRED suitable for implementation, unlocking a more sustainable and cost-effective synthesis pathway.

IL-3

Controllable Protein Design with Language Models

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Self-supervised protein language models have opened new possibilities for protein design. In this talk, I will present our work on generative models such as ZymCTRL and REXzyme, which can create protein and enzyme sequences with natural-like structures and functions while exploring distant regions of sequence space. I will discuss applications to several enzyme families, including triose phosphate isomerases, lactate dehydrogenases, α-amylases, formate dehydrogenases, fluorinases and dehalogenases.
I will then focus on a key limitation of these models: their dependence on the distribution of the training data, which restricts their ability to generate proteins with out-of-distribution properties. To address this, I will describe a strategies for steering protein language models toward user-defined phenotypes: reinforcement-learning (RL) approaches, including Direct Preference Optimisation. I will continue by showing these methods provide a route toward designing enzymes and binders with properties that go beyond those observed in natural sequence space, showing the example of the engineering of EGFR binders and formate dehydrogenases. I will finalize by stating current limitations and opportunities for future approaches.

Editorial

Opening the Editor's Black Box: Insider Tips for Successful Submissions

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This talk will give an overview of the publishing process from the perspective of a Deputy Editor of *ChemBioChem* and *Chemistry – A European Journal.*

ChemBioChem and *Chemistry – A European Journal* are Chemistry Europe journals and Chemistry Europe is an association of 16 chemical societies from 15 European countries. *ChemBioChem* and *Chemistry – A European Journal* publish a mixture of Research Articles, Reviews, and Perspectives.

This talk will cover what editors do and how editors make decisions, and concrete tips on how you can optimize manuscripts for submission to a journal.

OP 1

Structural and mechanistic studies on bacterial sesterterpene synthases

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Terpenoids, compromising well over 100 000 compounds, make up the single largest group of natural products [1]. Many of which exhibit bioactivity and are of considerable interest as fragrances, flavourings and pharmaceuticals [2],[3]. Central to this chemical diversity are terpene synthases, which utilize achiral oligoprenyldiphosphates to generate complex, oftentimes polycyclic, compounds with multiple stereogenic centres [4]. A small subgroup of this family are sesterterpene synthases utilizing geranylfarnesyldiphosphate (C_{25}) as their primary substrate [5]. To gain deeper insights into substrate-binding modes and enzymatic mechanism, we investigated three bacterial sesterterpene cyclases (**Figure 1**), following two different initial cyclisation pathways. By crystallizing them with unreactive substrate analogues, the enzymes were trapped in a pre-catalytic state with ligands positioned in a productive conformation in line with the proposed cyclisation mechanism. These structures served as foundation for targeted mutagenesis of the active-site to pinpoint catalytically relevant residues and widen the product spectrum. This lead to mutants with significantly altered activity compared to the wildtype as well as the generation of new sesterterpene products. Among these were products with inverted stereochemistry, sesterterpene alcohols, and deprotonation products of intermediates formed throughout the catalysis, providing additional support for the previously proposed mechanism [6],[7].

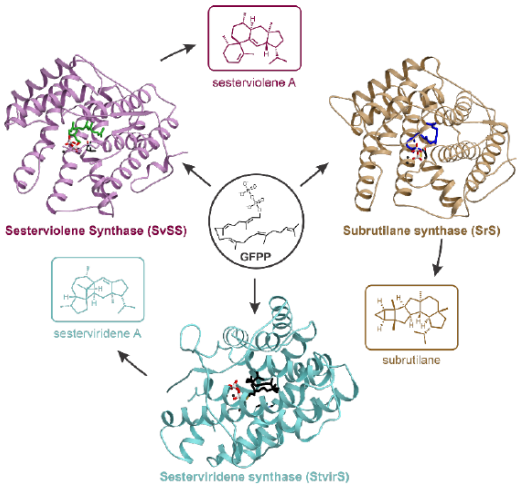

Figure 1 Crystal structures of investigated sesterterpene synthases together with their respective products

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OP 2

Chiral Alcohols from Alkenes and Water: Directed Evolution of Styrene Hydratases

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Chiral alcohols are important intermediates and products in the synthesis of pharmaceuticals and agrochemicals.[1] Yet their direct enantioselective formation by alkene hydration remains a major challenge in catalysis.[2,3] We identified and engineered an oleate hydratase from *Marinitoga hydrogenitolerans* for the highly enantioselective hydration of styrenes to chiral benzyl alcohols. Early engineered variants (styrene hydratase 1.0) showed excellent enantioselectivity but suffered from low activity and required the addition of hexanoic acid to mimic oleate substrate binding.[4]

Through further directed evolution using site-saturation and random mutagenesis, we have now developed hydratase variants (M1-M5) that are hexanoic acid-independent, much more efficient and catalyze alkene hydration under more practical conditions with excellent enantioselectivity (>99% ee).[5] These advances provide access to chiral benzyl alcohols simply from styrenes and water, bringing this long-sought transformation closer to synthetic applications. We will highlight the screening strategy, the evolved catalysts, and their activity and selectivity profiles.

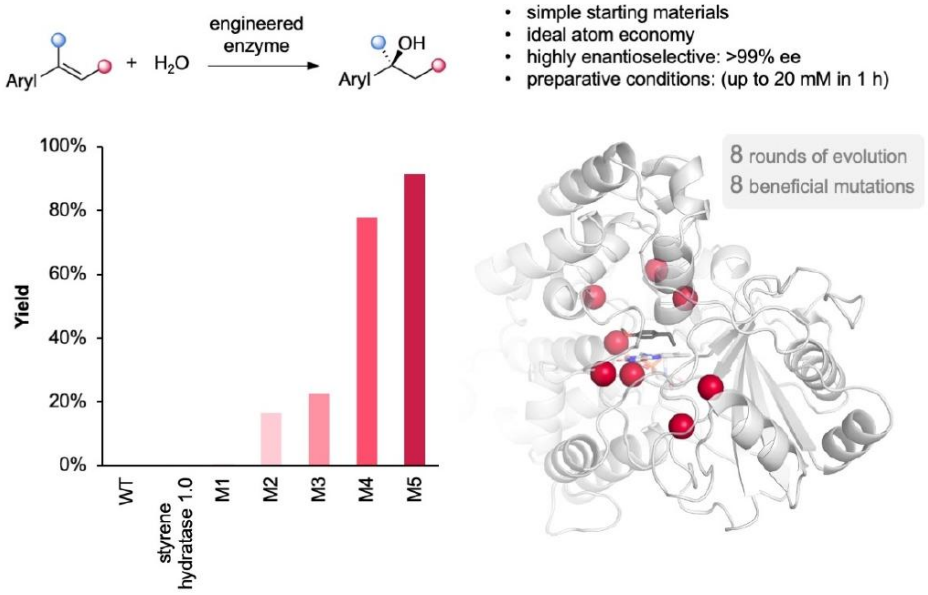

Figure 1. Engineered enzyme for asymmetric hydration of styrenes.

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OP 3

Genetic incorporation of diverse non-canonical amino acids for histidine substitution

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Among proteinogenic amino acids, histidine exhibits the highest catalytic propensity and is frequently found in the active site of enzymes [1]. However, the study of its functional role is fundamentally limited using traditional mutagenesis because its structure and chemistry are distinct within the proteinogenic amino acids, such that substitution with other proteinogenic acids induces complex changes that are often difficult to disentangle. Alternatively, a given histidine residue can be substituted by a histidine-like non-canonical amino acids (ncAA). Genetic code expansion endows researchers with the ability to introduce ncAAs into proteins site-specifically by hijacking ribosomal protein synthesis [2]. Despite the importance of histidine in catalysis, the scope of histidine-like ncAAs that can be incorporated by genetic code expansions remains limited.

We report the genetic encoding of a panel of diverse histidine-like ncAAs in *Escherichia coli* [3]. Using a combination of approaches, we identify nine novel aminoacyl-tRNA synthetase (aaRS)/tRNA pairs for the site-specific genetic incorporation of a panel of twelve histidine-like ncAAs—substantially expanding the tools available to study this privileged catalytic residue (**Figure 1**). The encoded ncAAs feature broadly tuned nitrogen pK_aH, alternative heterocycles, varying sigma-donor properties, and substitution patterns. We further investigate the substrate scope of each aaRS, from which we identify several surprising features and identify mutually orthogonal substrate-aaRS combinations that enable dual histidine-like ncAA incorporation within a single protein.

Together, these tools provide a platform to systematically study and engineer histidine-dependent proteins, and we will present initial applications in enzyme active site interrogation and biocatalyst optimization, enabling new approaches to probe catalytic mechanisms as well as engineer and design protein function with expanded chemical precision.

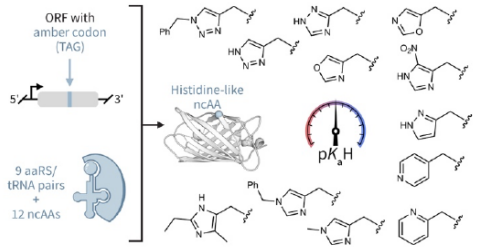

Figure 1. Genetic code expansion with orthogonal aaRS/tRNA pairs is leveraged to genetically encode a broad range of histidine-like non-canonical amino acids (ncAAs) with tuned pK_aH values. The side-chains of the respective ncAAs are shown.

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OP 4

Exploring H_2O_2 – dependent inactivation of *rAae*UPO

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Unspecific Peroxygenases (UPOs) have received significant interest over the past decades due to their ability to selectively catalyze oxidation of a broad range of substrates. UPOs are especially interesting because their reactivity relies on the use of H_2O_2, resulting in a relatively simple reaction mechanism that does not require expensive cofactors to perform the oxidation reactions. However, their dependance on H_2O_2 also comes with some issues, including H_2O_2-dependant oxidative inactivation of the enzyme. While this inactivation can be somewhat mitigated by optimization of reaction conditions, it is still a problem that needs to be addressed to apply these enzymes at larger scale.

This work focuses on the laboratory evolved variant of the UPO from *Agrocybe aegerita* (r*Aae*UPO) also known as PaDa-1. This evolved version of the wildtype *Aae*UPO shows exceptional activities and stabilities in high concentrations of various organic solvents, highlighting its industrial potential.[1] However, even this improved variant still suffers from oxidative inactivation which again highlights the importance of improving our understanding of this problem.[2,3]

We have carefully inactivated the r*Aae*UPO using two different sets of reaction conditions. The first method of inactivation uses the catalase reaction where H_2O_2 is decomposed to O_2 and H_2O as a protective mechanism in absence of an organic substrate.[2] The second method of inactivation uses the oxidative reaction mechanism that occurs when the enzyme is supplied with H_2O_2 and an organic substrate. After purification and subsequent inactivation of r*Aae*UPO, the enzyme was subjected to enzymatic digestion and HPLC-MS analysis. This approach allows us to identify oxidized amino acid residues throughout the UPO and to look at amino acid residues that show increased oxidation over time as the UPO loses more of its activity. So far, the samples that have been inactivated with only H_2O_2 have been fully analyzed. Results show evidence of oxidation of several important amino acid residues including some residues in the substrate access channel and residues that play a role in coordination of the heme cofactor.

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[3] T. Hilberath, R. Van Oosten, J. Victoria, H. Brasselet, M. Alcalde, J. M. Woodley, F. Hollmann, “Toward Kilogram-Scale Peroxygenase-Catalyzed Oxyfunctionalization of Cyclohexane” *Org. Process Res. Dev.* **2023**, *27*, 1384–1389.

OP 5

SYNTHESIS OF TERTIARY ALCOHOLS VIA ALDOL REACTIONS OF KETONES MEDIATED BY 2-OXOACID ALDOLASES

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Tertiary alcohols are important structural units present in biologically active compounds and drugs, such as Atorvastatin and Bedaquiline. Biomolecules containing these units are gaining significant relevance in medicinal chemistry because they expand chemical diversity, improve metabolic stability, and exhibit better solubility and bioavailability.[1]

The synthesis of stereoselective tertiary alcohols is complex due to the steric hindrance of the carbon substituents, which has driven the development of new chemical methodologies. In this context, biocatalysis for carbon-carbon bond formation stands out as an efficient strategy due to its high selectivity, mild reaction conditions, and use of simple starting materials.[2,3]

The main objective of this project is to carry out the stereoselective synthesis of chiral electron-withdrawing group-substituted tertiary hydroxyl-based carboxylic acids via direct aldol reactions using enzymes as biocatalysts. In this project, we have studied the potential capabilities of one Class I aldolase, specifically HBPA, and four Class II aldolases, KPHMT, YfaU, A5VAX1 and B4XH86, with the aim of synthesizing chiral molecules from pyruvate and activated ketones, which are used as building blocks in organic chemistry (**Figure 1**). Furthermore, aldolase mutants were synthesized through rational design to enhance substrate acceptance and increase reaction conversion.

The enzymatic reactions were performed using these aldolases under controlled conditions. Reaction parameters, such as pH, substrate ratio, enzyme and metal loading, were optimized to maximise the reaction conversion. The substrate scope was explored using structurally diverse activated ketones. Product formation was monitored by RP-HPLC analysis, and stereoselectivity was determined using Chiral HPLC analysis.

The results obtained showed high catalytic efficiency and great stereoselectivity on a variety of ketone substrates. Optimized conditions led to improved conversions and yields, highlighting the versatility of the biocatalytic system. Furthermore, the enzymatic approach significantly reduced the need for harsh reagents compared to conventional methodologies. Additionally, the methodology proved scalable without significant loss of catalytic performance.

In conclusion, this work demonstrates the potential of enzymatic aldol reactions as efficient and sustainable tools for stereoselective carbon-carbon bond formation. The study reinforces the applicability of biocatalysis in green synthetic methodologies and supports the application of enzyme-mediated reactions in modern organic synthesis.

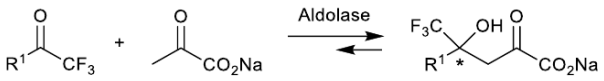

Figure 1. Aldol reaction of pyruvate and activated ketones by aldolases.

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[2] C.J. Moreno, K. Hernández, S.J. Charnok, S. Gittings, M. Bolte, J. Joglar, J. Bujons, P. Clapés, *ACS Catal.*, **2021**, *11*(8), 4660-4669.
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OP 6

Bioinformatics-Guided Immobilization of a Peptide Ligase for Continuous Flow Chemoenzymatic Synthesis of Semaglutide

Cristina Lía Fernández Regueiro[1], Marco Macis[2], Claudia Arnofi[2], Ana Toplak[2], Walter Cabri[1], Francesca Paradisi[3], Antonio Ricci[2]*, David Roura Padrosa[1*]

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Chemoenzymatic peptide synthesis (CEPS) offers a sustainable alternative to solid-phase peptide synthesis (SPPS) for manufacturing therapeutic peptides such as semaglutide, dramatically reducing waste generation [1,2,3]. However, industrial implementation requires enzyme immobilization to enable lower costs through catalyst recovery and higher productivity with continuous processing [4]. To date, no systematic study has demonstrated immobilized peptide ligase application for API manufacturing in continuous flow.

Here, we present the first bioinformatics-guided immobilization of a peptide ligase for continuous flow semaglutide fragment ligation. Computational tools (inSEIT Protein Suite) were used to predict optimal immobilization sites based on surface charge distribution and reactivity analysis. Systematic screening of covalent and non-covalent methods on various supports identified aldehyde chemistry as superior. EP400/SS-Aldehyde (Figure 1a) emerged as the optimal support, achieving >20% recovered activity and maintaining >80% activity after 10 consecutive batch reuse cycles. Translation to a 1.4 mL packed-bed reactor at 10 mg/g enzyme loading reached 85–89% conversion but revealed substrate hydrolysis as a competing side reaction. Optimization of protein loading to 2.5 mg/g suppressed this unwanted reaction, achieving 95% conversion with 30-minute residence time, superior to solution-phase benchmarks (Figure 1b).

This work establishes a robust, generalizable framework for data-driven immobilization, in this case applied to peptide ligases and demonstrates their successful integration into continuous flow bioprocesses for sustainable pharmaceutical manufacturing.

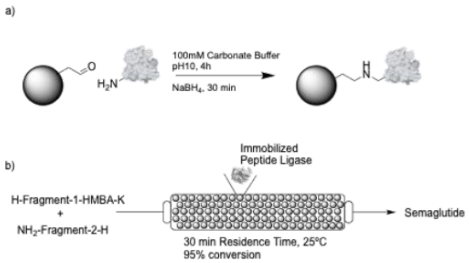

Figure 1. a) Covalent immobilization of peptide ligase on EP400/SS-Aldehyde support via reductive amination b) Continuous-flow ligation of semaglutide fragments using the immobilized peptide ligase in a packed-bed reactor.

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[2] L. Ferrazzano et al., Green Chem., 2022, 24, 975–1020.
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[4] C.L. Fernández Regueiro, D. Roura Padrosa, F. Paradisi, 2025 Comptes Rendus Chim. 2025, 28 (G1), 349–359

OP 7

Applied Biocatalysis for the Sustainable Synthesis of High-Solubility Prodrugs: From Mechanistic Insight to Scalable Processes

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Enzyme technology is emerging as a powerful driver of industrial biotechnology, redefining the synthesis of high-value molecules through unmatched selectivity, process efficiency, and sustainability. In this context, our work outlines a biocatalytic platform designed to transform poorly soluble bioactive compounds into industrially attractive, high-solubility prodrugs, bridging molecular innovation with scalable bioeconomy-oriented manufacturing. We present an integrated strategy, spanning molecular docking, reaction engineering, and process optimization, for the enzymatic esterification of phenolic acids and selected active pharmaceutical ingredients. Our targets include cinnamic acid derivatives such as ferulic and caffeic acids, together with ibuprofen [1] and ursodeoxycholic acid [2] compounds of strong pharmaceutical and cosmeceutical relevance whose application is often constrained by low solubility and limited bioavailability. By exploiting immobilized *Candida antarctica* lipase B (CALB) in non-aqueous media, we achieved highly selective esterification with polyols including erythritol and xylitol, opening access to novel derivatives with improved formulation potential. Molecular docking provided mechanistic insight into the regioselectivity of the reactions, clarifying how hydroxyl substitution patterns and structural unsaturation govern substrate accommodation within the catalytic pocket. These molecular-level findings were translated into robust operating conditions, enabling conversions above 95% under industrially meaningful parameters, including catalyst loading, temperature, molar ratio, and reaction time, while minimizing solvent use and ensuring high reproducibility [3]. Supported by Design of Experiments methodologies, the process demonstrated clear scalability and compatibility with continuous-flow intensification. Overall, this work advances a versatile enzymatic platform for sustainable prodrug manufacturing, contributing to industrial biotechnology, circular bioeconomy integration, and next-generation green chemical production (**Figure 1**).

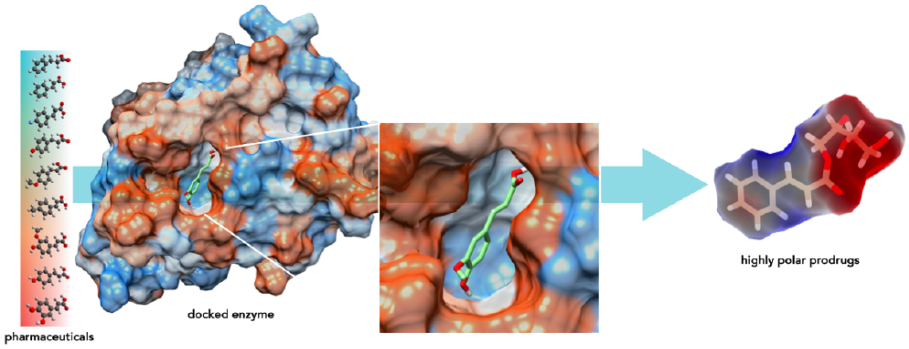

Figure 1. Docking and experimental optimization of enzymatic processes for the green synthesis of enhanced biobased prodrugs.

[1] F. Zappaterra, F. Presini, V. Venturi, L.A. Lerin, P.P. Giovannini, S. Costa, *Appl. Sci.*, **2023**, *13*, 9852.
[2] F. Zappaterra, S. Costa, D. Summa, B. Semeraro, V. Cristofori, C. Trapella, E. Tamburini, *Molecules*, **2021**, *25*, 1–15.
[3] F. Zappaterra, D. Meola, F. Presini, S. Aprile, V. Venturi, C. Nosengo, C. De Luca, M. Catani, L.A. Lerin, P.P. Giovannini, *Curr. Res. Biotechnol.*, **2024**, *8*, 100231.

OP 8

Going Full Circle: Dynamic Covalent Enzyme Immobilisation *via* Visually Trackable Boronate Esters

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Enzyme immobilisation on solid supports enables biocatalyst recycling in industrial biocatalysis, yet the implementation remains materially linear: carriers are discarded when enzyme activity declines. Extending support lifetime is a direct lever to reduce cost and waste. However, existing reversible approaches suffer from enzyme leaching, while irreversible covalent bonds prevent carrier regeneration. Here we report a reversible immobilisation strategy based on dynamic covalent boronate ester formation between alizarin-functionalised enzymes and boronic acid-modified supports (**Figure 1**). Alizarin-methyliminodiacetic acid (alizarin-IDA) serves dual roles as both a pH-responsive binding handle and a visual reporter, enabling real-time colourimetric tracking of enzyme loading (red solution to orange resin), immobilisation completeness, and pH-triggered release. A universal labelling protocol was established and successfully applied to four structurally diverse enzymes representing different fold families and oligomeric states (monomer to octamer, 25 to 188 kDa), all retaining 77–95% of native activity. All alizarin-labelled enzymes achieved greater than 90% immobilisation yield on different supports and could be quantitatively removed by acidic treatment with full regeneration of the supports. The load–use–cleave sequence was repeated five times without loss of binding capacity, enabling more than 50 catalytic cycles per support across multiple enzyme lifecycles with identical performance [1]. This work opens new perspectives for sustainable biocatalytic process design, where both the enzyme and the carrier participate in a truly circular lifecycle [2].

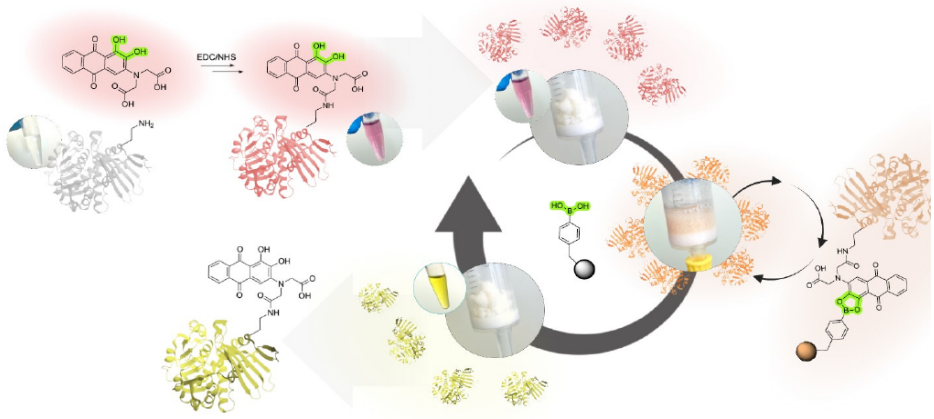

Figure 1. Graphical overview of the reversible enzyme immobilisation strategy via boronate ester formation. Boronic acid-functionalised resins bind alizarin-IDA-labelled enzymes through dynamic covalent chemistry, with a visual colour sequence (red → orange → yellow) tracking each process step.

[1] G. Bojanov, J. Swit, F. Paradisi, *Chem. Sci.*, **2026**, DOI: 10.1039/d5sc08585c.
[2] R.A. Sheldon, S. van Pelt, *Chem. Soc. Rev.*, **2013**, *42*, 6223–6235.

OP 9

Flipping Hydrogenase Reactivity: A New Paradigm for O_2-Free Oxidative Biocatalysis

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Oxygen-dependent biocatalysts are central to oxidative biotransformations, yet their industrial implementation remains constrained by intrinsic O_2-related limitations, including low aqueous solubility, poor mass-transfer rates, and the need for specialized aeration systems. These challenges not only complicate process design but also restrict reactor scalability and operational robustness.[1] Here, we present a conceptually distinct strategy that eliminates the dependency on O_2 by exploiting the thermodynamically unfavored oxidative direction of the soluble hydrogenase (SH). Instead of relying on an O_2-driven NADH oxidase (NOX) system, our approach uses SH to regenerate NAD^+ *via* H_2 evolution, transforming an enzymatic side activity typically considered negligible into a powerful and sustainable cofactor-recycling apparatus. We demonstrate that hydrogenase-driven NAD^+ regeneration can directly replace NOX in multi-enzymatic oxidative cascades, enabling the efficient conversion of sugars into value-added ketoacids. The hydrogenase-based system achieves up to 44,000 mol NAD^+ recycled per mol of enzyme, surpassing NOX in catalytic productivity, scalability, and operational simplicity, generating only "green" H_2 as a benign, potentially reusable by-product.[2]

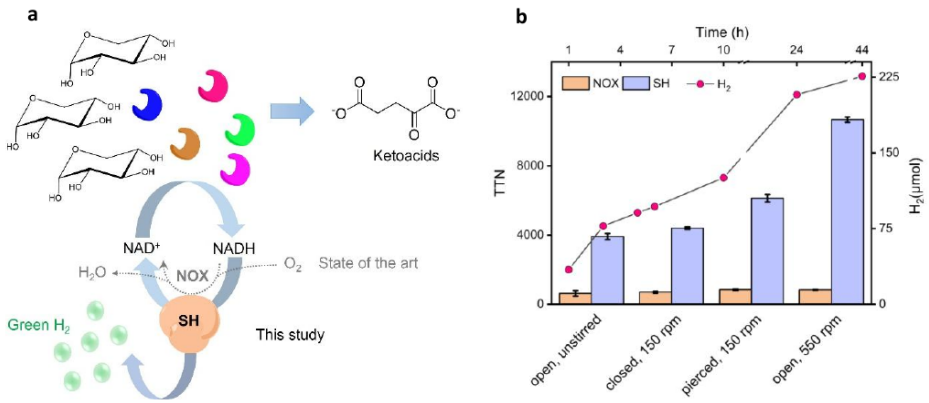

Figure 1. a) Cell-free biotransformation of D-xylose to α-ketoglutarate using hydrogenase-mediated NAD+ regeneration. The hydrogenase system oxidizes NADH with 100% atom efficiency, requiring no external oxidants and releasing only H_2 as a clean by-product. Conventional NAD^+ regeneration systems, including NADH oxidases (NOX), the most established and cleanest to date, still rely on O_2. **b)** Total turnover numbers (TTN) of soluble hydrogenase (SH) versus NOX across different reaction configurations and the cumulative $H_{2(g)}$ production during the D-xylose conversion to α-ketoglutarate at a 10 mL scale.

Overall, this work establishes hydrogenase oxidation as an innovative biocatalytic platform that resolves key limitations of O_2-dependent processes. By turning the "unfavored side" of hydrogenase catalysis into a functional advantage, we open a new route toward flexible, O_2-independent, and environmentally sustainable oxidative biotransformations.

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OP 10

A Cofactor-Independent Dioxygenase Reaction Catalysed by IifA

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Oxygen activation in enzymatic catalysis is most commonly mediated by transition metal cofactors; however, a growing class of cofactor-independent oxygenases challenges this paradigm [1, 2]. Here, we describe the biochemical and mechanistic characterization of IifA, an enzyme that catalyses the oxidative cleavage of 3-hydroxyindolin-2-one to yield anthranilate and carbon dioxide.

Isotopic labelling experiments establish the origin of atoms in the reaction. Using C2-^{13}C-labeled substrate, we confirm that CO_2 is derived from the C2 position. Reactions performed under $^{18}O_2$ demonstrate incorporation of oxygen from molecular oxygen into the products. Mass spectrometric analysis supports product identification and reveals a characteristic dehydration fragment (−18 Da).

Biochemical analysis shows a pH optimum around 6, suggesting involvement of a catalytic residue with a pK_a in this range. Site-directed mutagenesis identifies a conserved histidine as essential for activity. Based on these findings, we propose that this residue acts as a general base, generating an enolate-like intermediate via deprotonation at C3. This activated species is proposed to react directly with O_2, forming a peroxy intermediate that may proceed through a transient C2–C3 endoperoxide, followed by bond cleavage to yield anthranilate and CO_2.

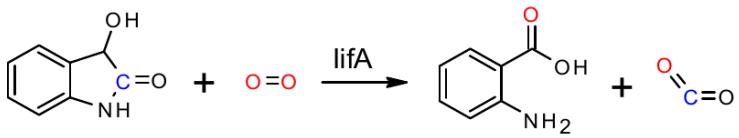

Figure 1. Proposed reaction of cofactor-independent dioxygenase IifA. Conversion of 3-hydroxyindolin-2-one to anthranilate with release of CO_2.

Although intermediates have not been directly observed, the combined isotopic, mutagenesis, and pH-dependence data support a cofactor-independent oxygen activation mechanism. This work expands the scope of metal-independent oxygenases and highlights IifA as a potential biocatalyst for transformations of indole-derived compounds.

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OP 11

Intrinsically Disordered Proteins Enable Robust and Enhanced Enzymatic Biocatalysis under Harsh Conditions

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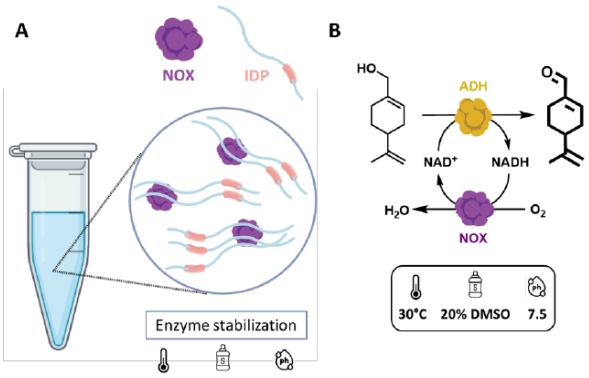
Enzymes are highly efficient biocatalysts, valued for their specificity and compatibility with sustainable processes, which has driven their growing use in biotechnology and biomedicine [1]. Nonetheless, their performance is often challenged by the reaction environments such as fluctuations in temperature, pH or the presence of organic co-solvents [2]. In this work, we present an alternative approach using intrinsically disordered proteins (IDPs) to modulate enzymatic function under such demanding conditions [3]. Our findings show that enzymes combined with IDPs sustain improved the stability across a range of different abiotic stresses: temperature, pH and solvents.

We evaluated the NADH oxidase (NOX), a widely used enzyme for cofactor recycling, stability across pH (4.0-9.0), temperature and solvent exposure. NOX exhibited substantial activity loss even under near-optimal pH, with more pronounced deactivation at extreme pH due to unfolding and aggregation. In contrast, supplementation with IDPs significantly improved stability, preserving up to fivefold higher activity, particularly under acidic conditions. Similarly, at 37 °C, optimal temperature for activity but detrimental for stability, IDPs effectively mitigated activity loss. Consistent stabilization trends were observed in the presence of a variety of organic co-solvents (**Scheme 1**). The applicability of this bioinspired platform is further demonstrated in a bi-enzymatic cascade (NOX and ADH: alcohol dehydrogenase) for the synthesis of a natural product, where the presence of IDPs leads to a threefold increase in product yield compared to the non-supplemented system. Together, these results highlight the potential of IDP-based systems to substantially improve enzyme stability under industrially relevant conditions.



Scheme 1. (A) Mixture of the enzyme with intrinsically disordered proteins (IDPs). (B) Reaction scheme illustrating a bi-enzymatic cascade (ADH and NOX) for the production of perillaldehyde.

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OP 12

Active learning maps the activity-selectivity Pareto front in enzymatic PET upcycling

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Biocatalytic transformation of polyethylene terephthalate (PET) presents a sustainable strategy for plastic upcycling.[1] While enzyme engineering dominate current efforts, process optimization to desired products/intermediates remain relatively underexplored.[2] Herein, we target bis(2-hydroxyethyl) terephthalate (BHET), a key PET hydrolysis intermediate and platform chemical, whose yield (Y_{BHET}) and selectivity (S_{BHET}) exhibit an intrinsic trade-off, when catalyzed by the LCC_{ICCG} enzyme. To address this challenge, we implement an active learning strategy, that integrates Bayesian optimization algorithm in experimental biocatalysis workflows.[3,4] This cross-disciplinary strategy efficiently explores the vast reaction parameter space,[4] to simultaneously maximize both Y_{BHET} and S_{BHET}.(**Figure 1a**).

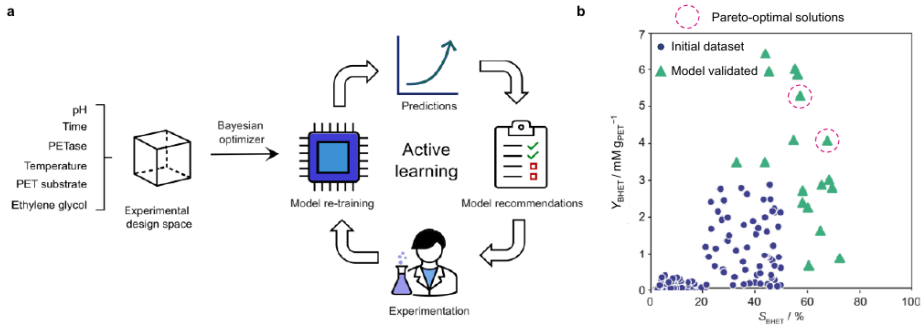

Figure 1. A closed loop active-learning cycle in enzymatic PET hydrolysis

Beginning with a constrained in-house dataset, in five active learning cycles with 25 new experiments, our approach uncovers multiple Pareto-optimal candidates, identifying Y_{BHET} = 5.4 mM g_{PET}^{-1} and S_{BHET} = 59% , along with Y_{BHET} = 4.2 mM g_{PET}^{-1} and S_{BHET} = 70% as the best trade-off solutions (**Figure 1b**). The model attains overall R^2 = 0.86, reduces the experimental search efforts by over 50%, and almost doubles the overall catalytic performance from its starting point. Feature-importance analysis reveals solvent concentration as the key driver affecting activity-selectivity trade-off, complementing insights with Michalis-Menten kinetics revealing substrate inhibition beyond a threshold solvent amount.[5] Lastly, we validate the workflow on PHL7, confirming enzyme-level transferability of our approach.

Overall, the predictive toolkit developed in this study effectively maps the Y_{BHET} and S_{BHET} and guides experimental researchers to perform data-informed experiments to achieve desired biocatalytic performance. In a broader context, our study delivers a scalable multi-objective optimization framework integrating data-driven insights with experimental design to accelerate biocatalytic PET upcycling.

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OP 13

Decoding electrostatic optimization in designed retroaldolases

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Enzymes are powerful biocatalysts, and designing efficient biocatalysts is essential for sustainable chemical synthesis. Their catalytic performance depends on the interplay of several factors, including active-site geometric preorganization, conformational dynamics, and electrostatic preorganization within the protein scaffold. The computationally designed retroaldolase (RA95.0) catalyzes the cleavage of a methodol substrate with initial retro-aldol activity through a multistep reaction mechanism [1]. Extensive directed evolution produced highly active variants, with RA95.5-8F displaying the greatest catalytic efficiency due to the emergence of a new catalytic tetrad precisely placed in the active site [2]. Previous studies have shown that distal mutations introduced along the evolutionary pathway reshape the conformational dynamics of the enzyme [3]. In parallel, QM/MM calculations have highlighted the key role of electrostatic interactions in stabilizing transition states and intermediates [4], and that rate-limiting step shifts upon evolution [5]. However, how laboratory evolution reshapes the electrostatic environment to enhance catalysis remains largely unknown.

Here, we present a computational workflow to analyse and quantify how mutations optimize catalysis by reorganizing enzyme electrostatics along a complete reaction pathway, considering the retroaldolase system as a benchmark (**Figure 1**). The protocol combines molecular dynamics simulations to sample relevant conformations, electric-field analysis to measure electrostatic preorganization in the active site and quantum mechanical calculations to connect scaffold-generated fields with stabilization of transition states and intermediates. Our results show that distal mutations influence flexible loop dynamics, promoting optimal electric field orientation for the rate-limiting C–C bond cleavage [6]. Analysis of the full reaction pathway indicates that the enzyme electric field is tuned not only to stabilize the rate-determining transition state but to guide and favour the entire reaction pathway. We then transfer this protocol to a second computationally designed retroaldolase scaffold containing the same catalytic tetrad. This comparison shows that catalytic residues alone are insufficient to explain activity; surrounding protein environment is crucial for organizing electrostatics and determining catalytic efficiency [7]. Overall, this work provides a general computational strategy to identify electrostatic signatures of efficient catalysis and guide the design of improved biocatalysts.

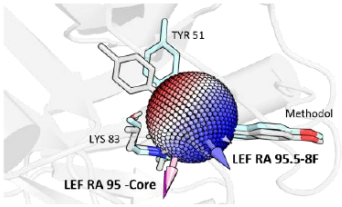

Figure 1. Local electric field in RA95.5-8F oriented to lower the energy barrier of the rate-limiting C-C bond cleavage step.

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OP 14

From Poses to Pathways : RxnNet Pathway Generation with MCS-Based EnzyDock Docking

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Terpene synthases (TPSs) are fascinating enzymes responsible for producing a wide variety of terpenoids in plants, fungi, and prokaryotes. Their catalytic mechanisms are complex, involving reactive carbocation intermediates, multistep cyclizations and rearrangements, and stereochemically rich products. Traditional docking tools fall short in capturing these mechanistic intricacies.

EnzyDock, a mechanistic docking platform [1], overcomes these limitations through multistate, multiscale docking that generates catalytically relevant poses. EnzyDock enables mechanistic docking by allowing substrates, intermediates, transition states, and products to be docked in a stepwise, mechanistically informed manner (i.e., multistate docking). We implemented template-based maximum common substructure (MCS) docking in EnzyDock [2], which uses a crystal structure or docked ligand as a template. In MCS-based docking, EnzyDock employs an automatic atom-matching algorithm that identifies the MCS between the ligands and the reference ligand.

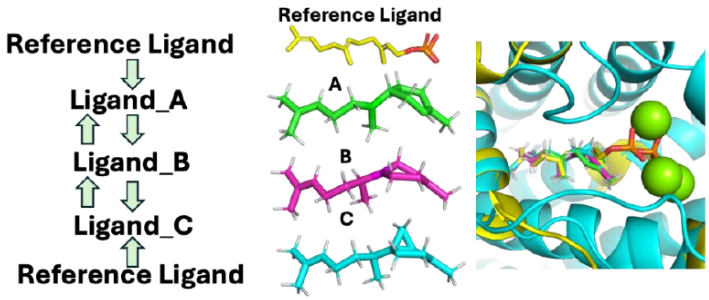

Figure 1. MCS-based EnzyDock docking maps RxnNet-generated intermediates onto catalytically relevant conformations within the TPS active site.

In the current work, RxnNet [3], an AI-assisted reaction generator tool—generated the reaction mechanisms for a series of TPSs and constructed the initial structures. The structures were docked in EnzyDock using crystallized bound ligands as templates for MCS-docking across complete reaction pathways. Detailed analysis of the docking outcomes provided valuable insights into how TPS active sites stabilize carbocation intermediates, shedding light on their catalytic roles in terpene biosynthesis.

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OP 15

From biomass-derived phenolic acids to valuable aromatic building blocks through a sustainable and tuneable multi-enzymatic cascade

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Phenolic acid decarboxylases (FDC/PAD, EC 4.1.1) have attracted growing interest for the valorisation of phenolic acids from renewable resources such as lignocellulosic biomass [1]. These enzymes enable the cofactor-independent decarboxylation of phenolic acids into 4-vinylphenol (4VP) derivatives, which are valuable flavour, fragrance, and industrial intermediates [1]. However, the high reactivity and propensity of 4VPs toward spontaneous polymerisation represent a major bottleneck, requiring rapid and controlled downstream conversion.

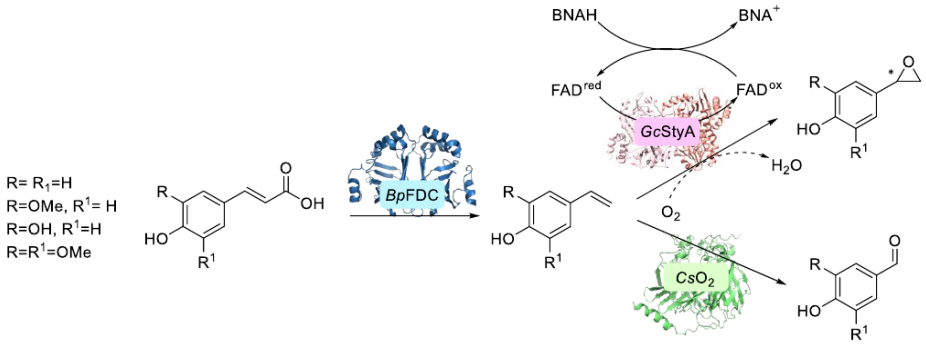

Figure 1. Multi-enzymatic cascade scheme.

Enzymatic cascade reactions (**Figure 1**) represent a powerful and sustainable strategy, enabling the *in situ* generation and direct transformation of unstable intermediates into value-added products. The robust phenolic acid decarboxylase from *Bacillus pumilus* ATCC 14884 (*Bp*FDC) efficiently converts hydroxycinnamic acids to 4VPs (>99–30% conversion in 2 h at 30 °C). This enzyme readily integrated with complementary downstream biocatalysts. For instance, the cofactor-independent dioxygenase *Cs*O2 (EC 1.13.11) from *Caulobacter segnis* ATCC 21756 enables the formation of aromatic aldehydes, providing access to high-value compounds such as favours and fragrances (*e.g.*, vanillin) [1].

Building on this approach, we report for the first time a one-pot, two-step enzymatic cascade for the synthesis of *para*-hydroxystyrene oxide derivatives. Following quantitative decarboxylation by *Bp*FDC, the resulting 4VPs undergo asymmetric epoxidation catalysed by the styrene monooxygenase *Gc*StyA from *Gulosibacter chungangensis*, completed in less than 2 hours. Notably, this system employs an innovative cofactor regeneration strategy based on the non-enzymatic reduction of FAD using 1-benzyl-1,4-dihydronicotinamide (BNAH), thereby avoiding the need for auxiliary enzymes. *Gc*StyA enables the formation of valuable chiral epoxides, which can be further transformed in a one-pot chemical step to yield optically active molecules via regioselective ring-opening reactions [2].

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OP 16

Increasing phenylpropanoid yield from enzymatic lignin depolymerization

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Lignin is the most abundant aromatic heteropolymer on earth and a major component of lignocellulose. As such, it can serve as a sustainable source for aromatic platform chemicals. In nature, multiple organisms have evolved biocatalytic pathways to degrade lignin and its oligomers. A reductive pathway has been found in *Sphingobium* sp. SKY-6, which targets the β-O-4-arylether linkages in lignin. This pathway involves NAD^+-dependent Cα-alcohol dehydrogenases that specifically oxidize the Cα-hydroxyl group adjacent to β-O-4-arylether linkages, which allows glutathione-dependent β-etherases and lyases to cleave this linkage type [1]. Applying this pathway as an *in vitro* cascade (Figure 1), with implemented cofactor recycling [2], for lignin depolymerization under mild conditions specifically yields up to three different phenylpropanoids depending on the lignin source and composition. So far, however, the application of this cascade for lignin depolymerization has only been demonstrated on analytical scale with phenylpropanoid yields of 48-130 mg L^{-1} [3].

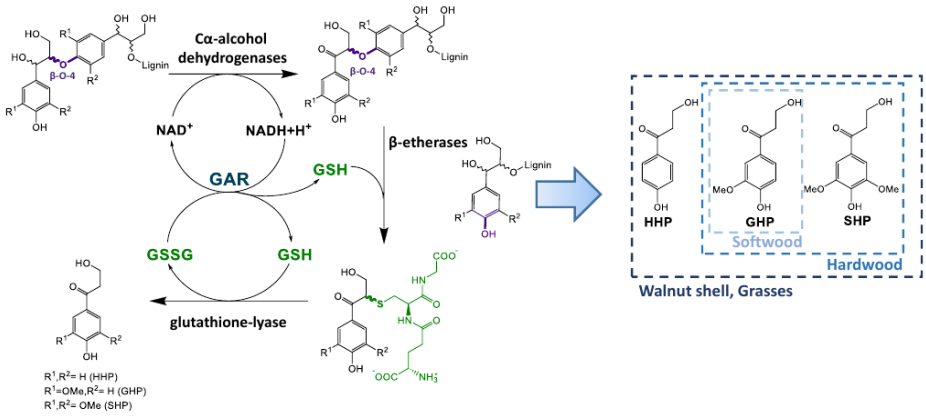

Figure 1. Lignin depolymerization via reductive β-O-4-arylether cleavage using an enzyme cascade, and its major monoaromatic products [GAR: glutathione amide reductase, GSH: reduced glutathione, GSSG: oxidized glutathione].

To markedly increase those yields from enzymatic lignin depolymerization, and to enable their isolation on a preparative scale, a variety of reaction parameters were carefully studied through DoE and ultimately optimized. These experiments revealed that the lignin type and its concentration, the cofactor concentration, the specific enzyme combinations and their concentrations, as well as the type of cosolvent [4] had the greatest impact on the volumetric yield of SHP and GHP. Thus, ethylene glycol proved to be a valuable alternative to DMSO for lignin dissolution. Moreover, the use of cell-free extract instead of purified enzymes further simplified the approach and increased product yield, achieving up to 800 mg L^{-1} of SHP and GHP. Based on the determined optimal conditions, the reaction was scaled up, and an MPLC-based two-step purification method was developed. This enabled the isolation of 500 mg SHP and GHP for further chemical conversion into valuable products [5].
Our results highlight that lignin can be effectively depolymerized using this enzyme cascade on a preparative scale, enabling the selective production of phenylpropanoids as platform chemicals for further valorization.

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P 1

Functional and mechanistic diversity in fungal BBE-like enzymes

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Flavin-dependent oxidases catalyze a wide variety of oxidative transformations, yet the function of many fungal berberine bridge enzyme (BBE)-like enzymes remains uncharacterized. Understanding their structure–function relationships is essential to uncover their roles in metabolism and biocatalysis [1].

Three BBE-like enzymes from the filamentous fungus Neurospora crassa were selected, including representatives from two biosynthetic gene clusters (#13 and #2) and one orphan enzyme. The proteins were heterologously expressed in Pichia pastoris, purified, and characterized using UV–Vis spectroscopy, stopped-flow measurements, X-ray crystallography, and activity assays.

All three enzymes (NcBBE-like 3, 14 and 17) were successfully produced and purified. NcBBE-like 3 exhibits a red-shifted absorbance spectrum, indicating a charge-transfer interaction with its FAD cofactor, and shows activity with a synthetic substrate. NcBBE-like 17, from an uncharacterized gene cluster (#2), oxidizes benzyl alcohol. In contrast, NcBBE-like 14, an orphan enzyme with a solved crystal structure and monocovalently bound FAD, shows no activity with standard BBE substrates.

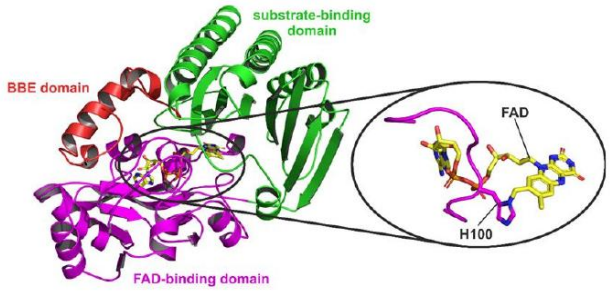

Figure 1: Overall structure of NcBBE-like 14. The inset shows the monocovalent linkage.

These findings demonstrate that structurally related BBE-like enzymes within one organism can display distinct biochemical behaviors, highlighting the variability within this enzyme family and the limitations of predicting function from sequence or structure alone.

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P 2

INVESTIGATING SUSTAINABLE APPROACHES FOR BIODEGRADATION OF PLASTICS AT INDUSTRIAL SCALE

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Fossil-based plastics make up the vast majority of traditional plastics today, and present several major drawbacks, mainly plastic pollution and a high carbon footprint[1]. Transitioning to a bio-based solution may help to mitigate these challenges. Poly(ethylene furanoate) (PEF) is 100% bio-based synthetic polymer that is similar in structure to poly(ethylene terephthalate) (PET)[2]. Enzymatic degradation of PEF by PET-degrading enzymes has shown promising results. Enzymes can hydrolyse the ester bonds in PEF to produce 2,5 furandicarboxylic acid (FDCA) and ethylene glycol (EG). The process is similar to that of PET degradation, where esterases and cutinases hydrolyse the catalytic cleavage of ester bonds (Figure 1)[3]. Enzymatic immobilisation for scaling up the degradation of PEF will allow the recovery of the enzyme in order to reuse it and lower the cost of the reaction. This study aims to optimise the enzymatic degradation of PEF on a large scale.

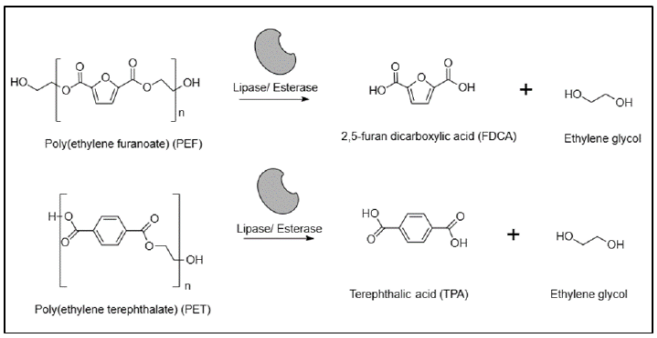

Figure 1. A schematic degradation reaction of PEF and PET

PEF degradation was tested at different temperatures and with different enzymes. LCC^{ICCG} and *Is*PETase N233C/S282C were used during testing, and the reaction was carried out for five consecutive days at different temperatures: 60°C, 65°C, 70°C, and 75°C. HPLC was used to measure the concentration of different products. Moreover, an attempt was made to immobilise the enzyme on Kollicoat beads (a pH sensitive polymer) to reuse it in batch processes.

The results suggest that the *Is*PETase N233C/S282C showed higher degradation activity compared to LCC^{ICCG} at all different temperatures. Among different temperatures, the highest activity was observed at 60 °C. Both enzymes were successfully immobilised on beads, and when tested for PEF degradation, they efficiently showed the degradation activity.

To conclude, scaling up the degradation of PEF for industrial use will be a viable option. Immobilisation of the enzyme will also benefit the process economically and environmentally.

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P 3

A multi-enzyme catalysed process enables the stereoselective access to 4-hydroxylated aromatic amino alcohols

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Stereoselective access to 4-hydroxylated aromatic amino alcohols is of interest in fine chemical synthesis for pharmaceutical intermediates [1]. Yet, enzymatic approaches have been underexplored. Our approach takes the whole value chain into account - from fermentatively obtainable 4-hydroxybenzoic acid [2] to the isolated stereopure amino alcohols. We focus on the stereoselective synthesis of two of the four possible stereoisomers, one of them in (1*R*,2*S*)- and the other one in the chemically less explored (1*R*,2*R*)-configuration. **Figure 1** shows the devised cascade in detail. Building on previously reported proof of concepts of single steps [3,4], we now combined them in a cascade, identified optimal overall process conditions and limiting factors. Therewith we could increase the product concentration and isolate the final products.

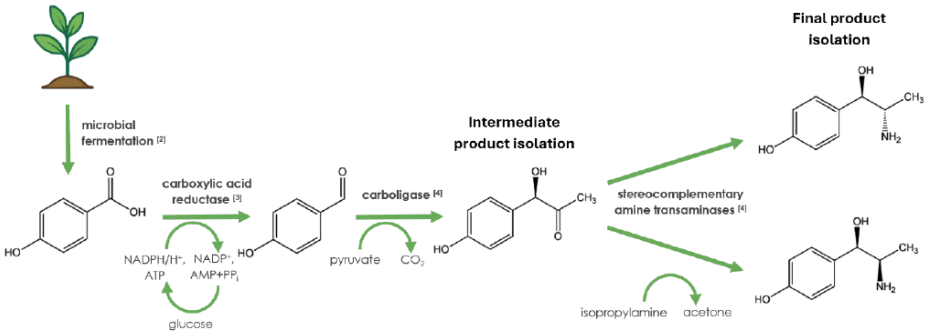

Figure 1. Scheme of the devised two-pot, three-step enzymatic cascade.

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P 4

Mechanistic Investigation of Iterative β-Alanine Chain Assembly in Closthioamide Biosynthesis

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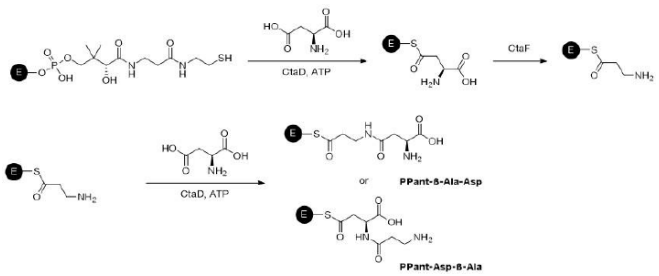

Figure 1. Two alternative mechanisms could account for the second aspartate incorporation step in Chlosthioamide biosynthesis.

Chlosthioamide (CTA) is a polythioamide natural product whose scaffold contains an unusual oligomeric β-alanine backbone. The enzymatic logic governing construction of this poly-β-alanine chain has remained incompletely understood. Here we report a mechanistic investigation into the iterative β-alanine chain assembly catalyzed by CtaD, an ATP-grasp enzyme, and CtaF, a PLP-dependent decarboxylase, acting together with the peptidyl carrier protein CtaE.

Prior in vitro studies established that CtaD and CtaF cooperate to load aspartate onto holo-CtaE and decarboxylate it to β-alanine, generating a tethered $(\beta\text{-Ala})_3$ chain iteratively. A central unresolved question was whether chain growth proceeds in a proximal or distal direction during subsequent elongation cycles, corresponding to the intermediates PPant-β-Ala-Asp and PPant-Asp-β-Ala, respectively [1].

To probe whether a covalent CtaD intermediate is involved, a Michael acceptor-functionalized PPant mimic was synthesized and loaded onto CtaE. Co-incubation with CtaD produced a crosslinked species consistent with a CtaD-CtaE adduct. Mutation of three active-site cysteine to alanine did not abolish crosslink formation, and the slow, non-specific crosslinking pattern suggests the absence of a highly reactive active-site cysteine in CtaD.

SNAC-OH and PPant-OH were employed as oxygen-ester analogs of the native PPant thioester. Incubation with CtaD, CtaF, and aspartate yielded products from two elongation cycles, but not a third. Stepwise ESI-MS analysis confirmed accumulation of PPant-O-β-Ala-Asp as a detectable intermediate, consistent with distal chain extension.

Definitive evidence came from experiments using SNAC-NH_2 as a substrate mimic. Under these conditions, CtaD and CtaF produced a three β-alanine chain, a result explicable only if each new aspartate unit is appended distally to the growing chain rather than at the PPant attachment point. This establishes that iterative chain assembly in CTA biosynthesis proceeds by progressive extension at the free terminus of the nascent β-alanine oligomer.

In summary, these results establish that β-alanine chain elongation in CTA biosynthesis proceeds in a distal fashion, with CtaD and CtaF acting iteratively at the free terminus of the growing oligomer. This work provides a mechanistic framework for polyamide scaffold assembly in polythioamide natural products and informs the chemoenzymatic synthesis of CTA analogs with modified chain lengths.

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P 5

AI-Guided Rational Design For Improvement of Protein Solubility

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Many enzymes of biotechnological interest are membrane-embedded or carry solvent-exposed hydrophobic patches that drive nonspecific association with membranes or hydrophobic cellular structures. These enzymes can often be expressed, extracted, and purified, but only with the aid of detergents, which complicates downstream processing, characterization, crystallization, and industrial application. Here, we present a computational workflow for solubilizing such detergent-dependent enzymes and demonstrate it on a bacterial heme-containing peroxygenase T3G1, the first characterized bacterial homolog of plant caleosins, identified and patented by GECCO Biotech **[1]**. Despite sharing only 36% sequence identity with Arabidopsis Peroxygenase 1, structural prediction using Boltz-2 confirmed a conserved fold and the key catalytic residues shared with plant caleosins (**Figure 1**) . When produced in *E. coli*, T3G1 bound heme and showed peroxygenase activity, but required detergents for handling due to its hydrophobic surface patches.

To convert this enzyme into a soluble biocatalyst, we applied a computational pipeline combining multiple AI tools, with soluble ProteinMPNN as the core engine for redesigning the hydrophobic patch residues (**Figure 1**). The suggested mutations were filtered based on predicted solubility and sequence fit to the original structure. A diverse library of 88 variants, each carrying an average of 12 surface mutations, was expressed and screened for soluble peroxygenase activity.

All 88 variants were active and soluble in the absence of detergents, completely overcoming the wild-type limitation. By reshaping the enzyme surface to eliminate hydrophobic patches while preserving catalytic function, we generated a soluble and active mutants, opening a path toward structural characterization of the caleosin fold and demonstrating a generalizable workflow for any enzyme whose utility is limited by detergent-dependent handling.

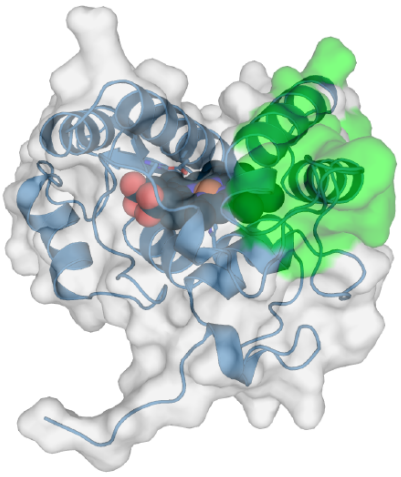

Figure 1. Boltz-2 predicted structure of T3G1 with heme. Residues targeted for redesign on the solvent-exposed hydrophobic patch are highlighted in green. The protein is shown in cartoon and surface representation. Heme is shown in space-filling representation.

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P 6

Engineering the Dynamics of a Designer Enzyme using Combinatorial Mutagenesis

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The structure landscape of enzymes is very diverse and decorated with many local optima of fitness. Classical evolution campaigns often investigate only those paths that can provide a step-wise increase in fitness in every generation. However, to investigate new and possibly better solutions, it is fundamental to move "sideways" in the landscape by adding neutral mutations, which can reshape conformational dynamics without immediate fitness gains. For their discovery, directed evolution campaigns require combinatorial libraries. However, a suitable high-throughput (HT) screening method needs to be available.

We focused our attention on the designer enzyme LmrR_pAF that features the non-canonical amino acid p-aminophenylalanine (pAF) into the protein scaffold LmrR. A classical directed evolution campaign identified the triple mutant LmrR_RMH to have a 75-fold improvement in enzyme efficiency towards a colourful new-to-nature hydrazone formation reaction [1]. We previously identified two remote mutations (>10 Å from pAF) that allosterically modified the dynamics of the enzyme favouring productive conformations [2].

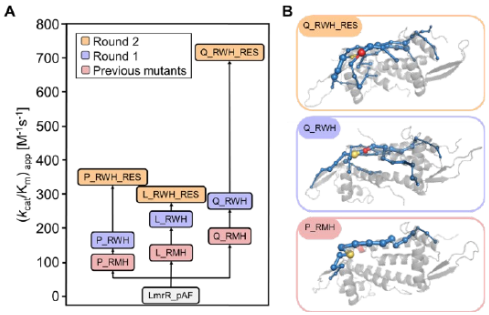

Figure 1. (**A**) Catalytic efficiency changes observed across the evolution trajectory in a pseudo-first order regime. (**B**) Shortest path maps of P_RMH (bottom), Q_RWH (center) and Q_RWH_RES (top) along the full simulation trajectory. Residue 88 and 85 are highlighted in red and yellow respectively. The size of the nodes and thickness of the edges represent the importance of the node or edge in the map.

Here, we developed an experimentally straightforward HT agar-plate assay capable of screening >10^4 mutants that does not require specialized equipment, and applied it to the evolution of LmrR_pAF. We simultaneously randomized six residues over two rounds of evolution (three per round), resulting in a hexamutant (Q_RWH_RES) with a 391-fold higher catalytic efficiency. The allosterically active remote mutation N88Q showed a large epistatic effect crucial for this improvement (**Figure 1A**).

Molecular dynamics simulations and graph analysis of the dynamic network revealed a progressively more complex and diffused connectivity acquired during evolution, which allows the ready exploration of catalytically productive conformations (**Figure 1B**).

These findings highlight the importance of epistasis and dynamic allosteric networks in enabling new-to-nature catalysis and corroborate the idea that "dynamic engineering" [3] is crucial to yield highly active designer enzymes.

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P 7

Exploiting Iminium Ion Intermediates Using Biocatalysis

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The repurposing and design of artificial enzymes is emerging as an exciting frontier in biocatalysis.[1] Unlocking new enzymatic reactivity through engineering or the incorporation of non-canonical amino acids into protein scaffolds offers opportunities for developing new-to-nature chemical transformations.[2, 3] This work aims to hijack the imine reductase (IRED) native mechanistic pathway at the iminium ion intermediate and proceed down alternative reaction pathways, unlocking new reactivities, while also exploiting reactive iminium ion intermediates using enzyme-trigged transformations.

The panel of IREDs were unsuccessful in catalysing the *aza*-Prins cyclisation via iminium ion catalysis; however, the substrate scope of the enzymes was expanded to the novel amine, 3-buten-1-amine, and a range of carbonyls with 2-53% yield (**Figure 1**). Using 4-nitrobenzaldehyde as the carbonyl uncovered a nitroreductase – IRED cascade when IREDs were employed as the *E. coli* whole cell. No NTR activity was detected using IRED lysate due to NTR aggregation during lysis.

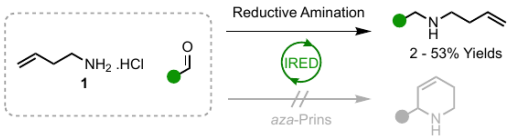

Figure 1. Schematic highlighting attempted biocatalytic *aza*-Prins cyclisation.

An enzyme triggered reaction to generate the iminium ion intermediate *insitu* was envisaged, which could subsequently undergo cyclisation via a Pictet-Spengler reaction. Oxidation of **2** by an alcohol dehydrogenase (ADH) led to the further oxidation of the hemiaminal intermediate **5**, forming the lactam **7** with a 17 % yield and 70 % *e.e.* (**Figure 2**). Oxidation of compound **3** with a transaminase (ATA) enabled the reaction to proceed via a phosphate catalysed Pictet-Spengler cyclisation affording **8**, uncovering a divergent synthesis towards valuable heterocycles.

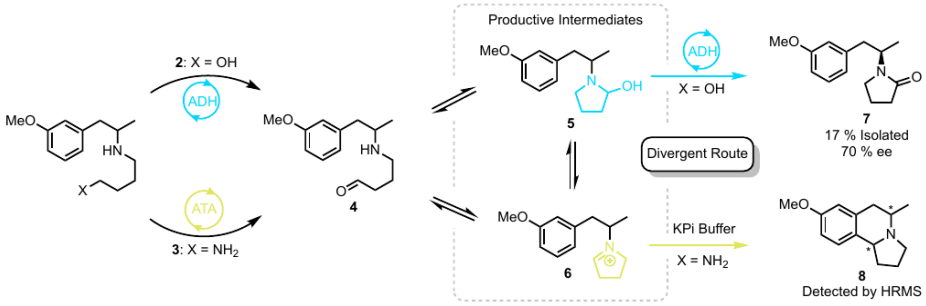

Figure 2. Divergent synthesis of lactam **7** or heterocycle **8**, depending on whether an ADH or ATA to generate the reactive intermediate **4**.

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P 8

Enhanced Biocatalytic FDCA production from Crude HMF via immobilized HMF Oxidase.

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The transition from petrochemical-based plastics to poly(ethylene 2,5-furandicarboxylate) (PEF) necessitates sustainable and scalable routes for the synthesis of its precursor, 2,5-furandicarboxylic acid (FDCA). The enzymatic conversion of 5-hydroxymethylfurfural (HMF) to FDCA using engineered variants of hydroxymethylfurfural oxidase (HMFO) represents an environmentally friendly approach, as the enzyme catalyzes the three consecutive oxidation steps required for FDCA synthesis under mild conditions [1].

This study presents an intensified biocatalytic system for the sustainable production of FDCA, employing the engineered enzyme 8BxHMFO [2] fused with a carbohydrate-binding module [3] (CBM3) and immobilized on microcrystalline cellulose (Perloza MT100) to mitigate oxygen-induced interfacial inactivation [4]. Initial optimization using purified HMF yielded high conversion rates (>95%) and an FDCA concentration of 7.2 g·L-1. To evaluate the industrial relevance of the system, 50 mM crude HMF extract was employed as substrate (**Figure 1**). The biocatalyst maintained its catalytic performance in the presence of impurities of crude HMF, exhibiting a highly competitive FDCA yield (70.6%) and titer (5.7 g·L-1). Furthermore, FDCA recovery was carried out reaching 72% efficiency using an ethanol-based extraction process. These findings highlight the potential of this biocatalyst system to enable cost-effective and environmentally sustainable FDCA production, advancing the development of bio-based plastics.

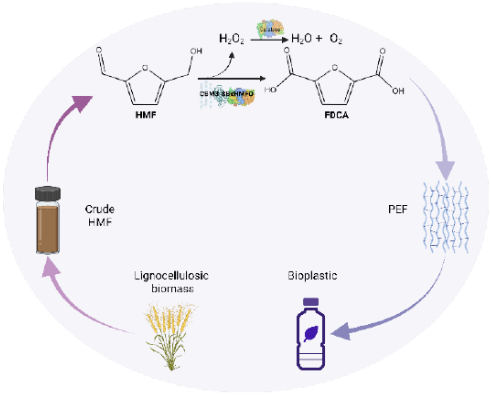

Figure 1. Schematic of the biocatalytic production of FDCA from 50 mM crude HMF extract to evaluate the industrial relevance of the immobilized system.

Acknowledgements:

- FURENPOL- Grant PLEC2021-007690 funded by MICIU/AEI/10.13039/501100011033 and by the European Union.
- NextGenerationEU/PRTR-Generalitat de Catalunya, 2021 SGR 00143.
- ROBUSTOO: Funded by the European Union, Grant agreement No 101135119.
- DEMUBI: PID2022-139725OA-I00 funded by MCIN/AEI/10.13039/501100011033/FEDER, UE.

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P 9

From Nanofibers to Monoliths: A Unified CBM-Driven Approach for Simple and Versatile Cellulose Biocatalysis

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The industrial implementation of enzymes requires immobilization strategies that are robust, cost-effective, and environmentally compatible, avoiding the limitations of conventional chemical functionalization. In this work, cellulose, an abundant, non-toxic, and chemically inert biopolymer, is evaluated as a platform for enzyme immobilization using two complementary architectures: electrospun cellulose nanofiber membranes (CNFMs) and regenerated cellulose monoliths. CNFMs provide a high surface-to-volume ratio that minimizes diffusional constraints, while monoliths offer a rigid, highly permeable scaffold suitable for high-throughput operation with reduced backpressure. Consequently, these supports enable the tailoring of biocatalytic systems to meet specific kinetic and hydrodynamic requirements.

A one-step purification and immobilization strategy was implemented using carbohydrate-binding modules (CBMs) to exploit these structural advantages without hazardous activation chemistries[1]. CBM3 from *Clostridium thermocellum* and CBM9 from *Thermotoga maritima* were fused to the N-terminus of an N-acetylglucosamine oxidase (NagOX) variant with glucose preference[2], allowing enzymatic activity to serve as an indicator of immobilization efficiency.

CBM specificity followed a consistent trend across both supports, with CBM3 emerging as the superior module. At low-loading conditions, CNFM-based biocatalysts achieved a recovered activity of 99.88%, compared to 63.01% for CBM9. Furthermore, when evaluating maximum loading capacities, the CNFMs and the monoliths exhibited maximum capacities of 14 mg·g support^{-1} and 21 mg·g support^{-1}, respectively, while maintaining high recovered activities. These results represent a competitive and sustainable alternative to conventional covalent immobilization, avoiding the activity losses typically associated with chemical functionalization.

The hydrodynamic performance of both platforms was characterized, demonstrating stable operation at high flow rates for CNFM-packed membranes and in-situ synthesized monolithic columns. To validate this strategy, a proof-of-concept multi-enzymatic reaction was conducted in both systems, in which the HPI catalase from *E. coli*, also fused to CBM3, was co-immobilized to mitigate matrix degradation, resulting in sustained glucose conversion and enhanced biocatalyst operational stability under continuous flow conditions.

Overall, these findings demonstrate the robustness of the CBM-mediated strategy on cellulose-based scaffolds, establishing a sustainable platform readily applicable to other enzymatic systems for the development of high-performance flow biocatalysts. To the best of our knowledge, this work provides the first successful evaluation of CBM-mediated immobilization in regenerated cellulose monoliths and one of the first demonstrations in CNFMs.

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The authors acknowledge funding from the Ministerio de Ciencia e Innovación, the Agencia Estatal de Investigación, and the European Regional Development Fund (FEDER) (**PID2022-139725OA-I00: DEMUBI**), the Generalitat de Catalunya (**2021 SGR 00143**), and the European Union's Horizon 2020 research and innovation programme through the OXIPRO project (**grant agreement No. 101000607**). Ivan Dimitri also acknowledges AGAUR (Generalitat de Catalunya) for his predoctoral fellowship (**Joan Oró – 2022FI_B 00955**).

P 10

Increased Terpene Production Through Balanced Expression of MEP Pathway Enzymes

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Terpenes are a diverse group of chemical compounds with applications across various industries, such as pharmaceuticals, food, cosmetics, and fragrances [1]. Microbially, terpenes can be produced via the mevalonate and methylerythritol phosphate (MEP) pathways. While both pathways have been the subject of numerous engineering efforts, they remain attractive targets for metabolic engineering to increase terpene production. Especially the MEP pathway, as it is energetically balanced and, according to stoichiometry, more efficient in transforming glucose into terpenes than the mevalonate pathway [2].
In a previously established *E. coli* production system in which MEP pathway genes *dxs*, *idi,* and *crtE* were already overexpressed, *ispG* and *ispH* were additionally overexpressed to further enhance terpene production [3]. Gene expression of MEP pathway genes was controlled by a set of ribosome-binding sites (RBSs) with increasing expression strength (A >>> F) [4]. Expression of *ispG* and *ispH* was tested in combination with different RBS to determine the optimal expression strength that balances MEP pathway enzyme expression for maximum terpene production (**Figure 1**). MEP pathway plasmids with different RBS were tested together with terpene synthase CotB2, which produces cyclooctat-9-en-7-ol. The terpene product was quantified using GC-FID after extraction from shake-flask cultures.

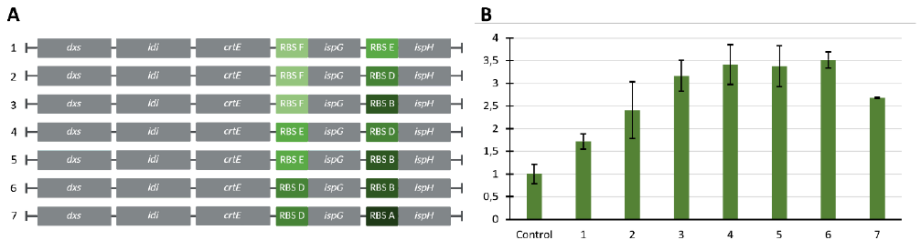

Figure 1. Terpene yield of different RBS and gene combinations. **A:** Combinations of RBS and *ispG* and *ispH* genes on the expressed plasmid. **B:** Cyclooctat-9-en-7-ol yields of the MEP pathway plasmids normalized to final OD and control plasmid without overexpression of *ispG* and *ispH*.

In shake-flask experiments, the overexpression of *ispG* and *ispH* increased terpene production as compared to the control plasmid without *ispG* and *ispH*. By balancing gene expression through RBS strength, an improvement of up to 3.5 times could be achieved, representing an important step towards microbial production of terpenes with industrial significance.

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P 11

One-Pot Cascade Oxyfunctionalization of Aromatic Hydrocarbons by Engineered CYP153A6-*Acetobacter malorum*

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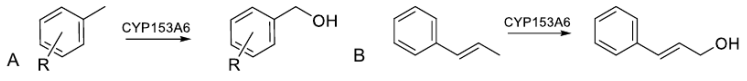
Biocatalytic oxyfunctionalization of aliphatic and aromatic hydrocarbons represents an attractive strategy for the selective transformation of chemically inert molecules into high-value functionalized derivatives. CYP153A6, a cytochrome P450 monooxygenase from *Mycobacterium sp*., is known for catalyzing the highly selective terminal hydroxylation of linear and alicyclic alkanes [1].

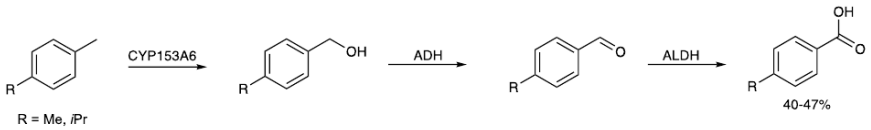
In this study, CYP153A6 was heterologously expressed in *Escherichia coli* and evaluated as a crude cell extract against a panel of aromatic hydrocarbons (**Figure 1**).



R = H, Me, *i*Pr, OMe, OH, Cl, O_2N, CH_2OH

Figure 1: Hydroxylation of A) toluene derivatives and B) Trans ß methylstyrene catalyzed by CYP153A6

The enzyme selectively catalyzed the hydroxylation of the tested substrates at the benzylic position. Notably, in the case of 4-xylene, hydroxylation occurred exclusively at one of the two benzylic methyl groups, highlighting the regioselectivity of the biocatalyst. Among the investigated substrates, 4-methylanisole exhibited the highest reactivity, reaching a specific activity of 1.75 U mg^{-1}, whereas no detectable activity was observed toward 4-cresol, 4-methylbenzyl alcohol, or 4-nitrotoluene.



R = Me, *i*Pr

Figure 2: Sequential oxidation of toluene derivatives using CYP153A6-Acetobacter malorum

To further exploit this catalytic system, *Acetobacter malorum* DSM 112354, an acetic acid bacterium (AAB) previously isolated in our laboratory [2], was engineered to express CYP153A6 using the plasmid pSEVA331Bb carrying the monooxygenase together with its redox partners, ferredoxin and ferredoxin reductase, to support efficient electron transfer. AAB are well known for their strong oxidative metabolism, efficiently converting primary alcohols into carboxylic acids through the activity of native membrane-bound alcohol dehydrogenases (ADHs) and aldehyde dehydrogenases (ALDHs) [3].
By integrating the selective hydroxylation activity of CYP153A6 with the endogenous oxidative machinery of *A. malorum*, the engineered strain enabled a one-pot cascade biotransformation of aromatic hydrocarbons into the corresponding carboxylic acid derivatives at substrate concentrations ranging from 5 to 35 mM (**Figure 2**).

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P 12

Insights into the mechanistic and regioselective properties of Isoeugenol-*O*-methyltransferase from *Clarkia breweri*

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Methylation is an important reaction in the synthesis of pharmaceutical and food-related compounds. Isoeugenol-*O*-methyltransferase from *Clarkia breweri* (IeOMT) and several variants are known to act on various phenolic substrates [1]. In this study, we investigated the mechanistic role of specific amino acid residues in the methylation reaction catalyzed by methyltransferases, as well as their contribution to determining the methylation pattern (regioselectivity). Particular emphasis was placed on active-site residues involved in substrate binding, orientation, and catalytic mechanism (**Figure 1**) [2]. Site-directed mutagenesis was employed to introduce targeted mutations at selected positions within the enzyme. These positions were chosen based on structural and bioinformatic analyses, which suggested their potential involvement in shaping the active site and mediating interactions with the substrate. The resulting enzyme variants were expressed, purified, and evaluated in terms of their catalytic performance and methylation selectivity. Our results revealed that certain amino acids play a critical role in the catalytic mechanism, significantly affecting methylation rates. In parallel, other mutations led to distinct changes in the methylation pattern, indicating that these residues are key determinants of substrate positioning within the active site and, consequently, of the regioselectivity of the reaction. Overall, this work provides important insights into the structure–function relationships of isoeugenol-*O*-methyltransferases with a coumarin-like substrate, and highlights how specific amino acid residues govern both catalytic efficiency and product distribution.

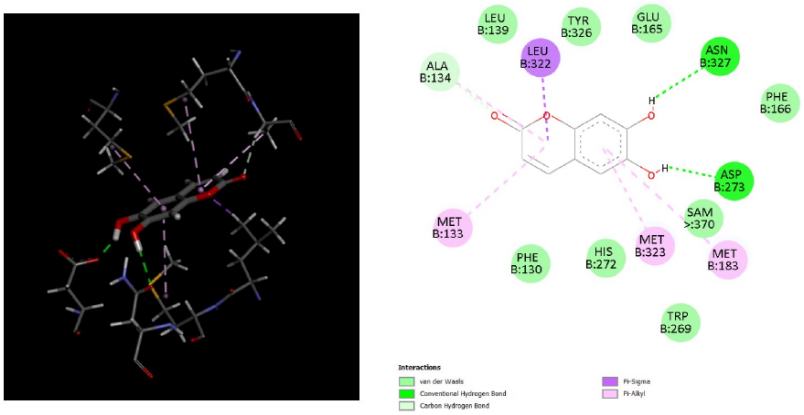

Figure 1. Docking of IeOMT (wt) with esculetin. The C atom of esculetin is in grey and the O atom in red.

Keywords: *O*-methyltransferase, regioselectivity, enzyme engineering, catalytic mechanism.

Acknowledgements: This work was supported by the European Union's HORIZON-WIDERA-2023-ACCESS-04 programme under grant agreement 101159534 (WIDEnzymes).

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P 13

Nitration Of Aromatic Renewables With Oxidative Enzymes

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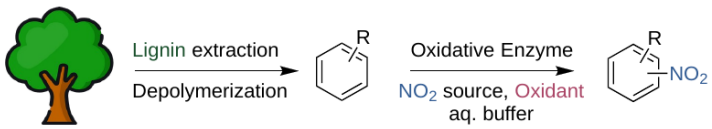
Nitroaromatic moieties are frequently found in industrially relevant compounds, such as pharmaceuticals, dyes and polymers, however their synthesis still relies on harsh methods with poor selectivity. While a few enzymatic methods have been reported, mild and selective approaches to bio-nitration applicable to a broad panel of substrates are still widely underexplored. Biocatalytic nitration reactions can be divided into three main categories: **amine** oxidation, **direct** oxidative nitration, and **nucleophilic** substitution[1].

The goal of this research is to explore the **direct** oxidative nitration of aromatics using various oxidative enzymes. Active enzymes include heme-iron-dependent enzymes such as TxtE[2] or RufO[3], which catalyze the oxidation of aromatic amino acids. In this project, we will focus on enzymes able to regioselectively nitrate aromatics derived from renewables, such as lignin-based monomers. Our progress toward the production of industrially relevant molecules will be presented.



Scheme 1: Schematic depiction of the downstream process from biomass to bio-nitrated aromatic molecules. Tree icon designed by Freepik via Flaticon

Acknowledgements
This research was funded in whole or in part by the Austrian Science Fund (FWF) 10.55776/COE17 within the Cluster of Excellence Circular Bioengineering.

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P 14

Regioselective C-formylation of polyphenolic substrates

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The development of sustainable and efficient methods for C–C bond formation remains a central objective in contemporary synthetic chemistry [1]. In recent years, biocatalysis has emerged as a powerful platform for C–C bond formation, enabling diverse transformations such as aldol-type reactions, acylations, and carboligations [2–6]. Within this context, the introduction of a formyl group (–CHO) represents a particularly valuable transformation for the synthesis of pharmaceuticals and other high-value compounds [7]. Despite the availability of numerous chemically mediated C-formylation strategies [8], a corresponding biocatalytic alternative remains largely unexplored, with formolase representing the only reported example [9].

Here, we report a biocatalytic strategy for the regioselective C-formylation of polyphenols under mild and sustainable conditions (**Figure 1**). Exploiting the promiscuous activity of the cofactor-independent acyltransferase from *Chromobacterium sphagni* (*Cs*ATase) enabled efficient C-formylation.

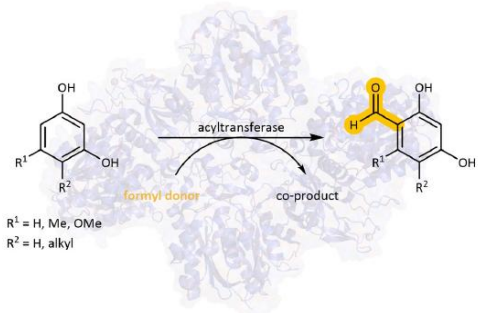

Figure 1. Formylation of resorcinol catalyzed by acyltransferases (PDB:5m3k).

High-resolution crystal structures of *Cs*ATase provided mechanistic insight into substrate binding and regioselectivity. Reaction optimization identified effective formyl donors and conditions, affording high conversions. Resorcinol derivatives underwent selective monoformylation, whereas phloroglucinol was additionally converted to a diformylated product, a key precursor of antibacterial sideroxylonals. Notably, *Cs*ATase-catalyzed reactions translated efficiently to preparative scale, delivering moderate to excellent yields (15–92%) [10].

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P 15

Harnessing non-heme Fe enzymes for artificial reactivity

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Non-heme Fe dependent enzymes are a growing family of enzymes with immense potential in biocatalysis. Their diversity in natural metabolism, varied reactivity and potential for engineering makes them an attractive platform for the development of non-natural reactivity.[1]
Expanding the scope of a biocatalytic transformation beyond molecular scaffolds that are similar to the native substrates is still a significant challenge. Herein, we have developed a strategy for the biocatalytic halogenation of terpenoids by which an anchoring group leads them to masquerade as the native substrate to access valuable halogenated compounds with excellent chemoselectivity and stereoselectivity.[2]

Using substrate engineering and directed evolution, we managed to achieve a significant expansion of the scope of substrates that undergo chlorination by αKG-dependent halogenases to aliphatic scaffolds far from those of the native substrates.

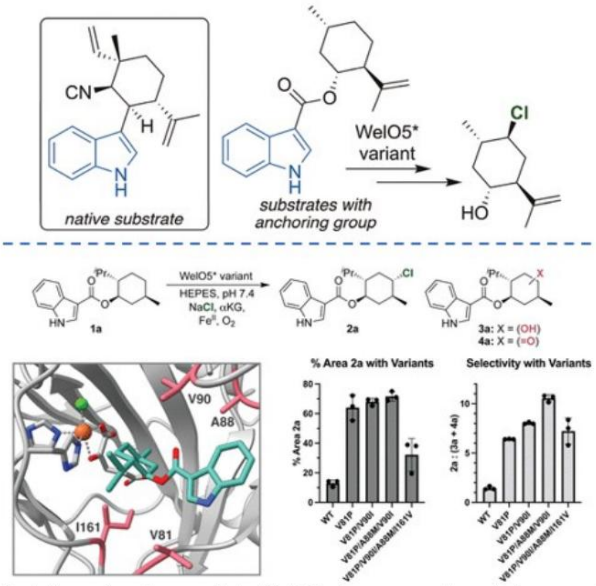

Figure 1. Top: Non-native halogenation of terpenoids by WelO5* using a removable anchoring group. Bottom: Active site of WelO5* with selected residues for mutagenesis and impact on reaction selectivity.

This study will likely guide future efforts to leverage the structure of the native substrates of non-heme iron, αKG-dependent enzymes to enable the functionalization of unnatural structures that require precise substrate positioning and that would be challenging to chemically functionalise in a site- and stereoselective fashion.

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P 16

Mutational landscapes of enzymatic solvent preference

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Deep eutectic solvents (DES) represent a promising solvent alternative for process intensification in biocatalytic transformations involving hydrophobic substrates. DES have been shown to alter enzyme behaviour not only in terms of stability and activity [1–3], with computational studies suggesting that these effects may be linked to reduced enzyme flexibility [4], but also with respect to substrate affinity and enantioselectivity [1,5].
This project aims to investigate the correlation between amino acid composition and DES-induced changes in enzyme behaviour. To this end, enzyme performance in different DES compositions will be compared to performance in buffer. Elucidating the structural enzyme characteristics that render biocatalysts more suitable for use in DES could open new opportunities for enzyme engineering tailored to specific solvent environments. Phenolic acid decarboxylase (PAD), a cofactor-free decarboxylase, is the focus of this study.
Increased enzyme stability as well as a shift in substrate selectivity have been reported for PAD variants in DES [1,4]. To systematically investigate such solvent-induced effects, a spectrophotometric high-throughput screening approach based on the assay published by Terholdsen et al. [6] is being established for screening mutant libraries of *Bacillus subtilis* PAD. In the first year of the project, the main focus has been to define suitable screening conditions, including DES compositions compatible with the enzyme, substrate and assay. DES compositions containing betaine or sarcosine as hydrogen bond acceptors and glycerol as the hydrogen bond donor, along with a 40 wt.% buffer, have shown promising results with respect to enzyme thermal stability and mixing efficiency in 96-well plates.

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P 17

Development of a recombinant *Acetobacter Malorum* strain for optimized fatty acid bioconversion

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This study explores acetic acid bacteria (AAB) as a versatile biocatalytic platform for the synthesis of building blocks for green biopolymers.
AAB are rod-shaped, obligate aerobic bacteria characterized by an exceptional efficiency in the periplasmic oxidation of a broad range of sugars, alcohols and polyols [1]. This oxidative capacity is given by specialized membrane-bound dehydrogenases that ensure chemo- and regioselectivity in the reaction.
In this work, the newly isolated strain A*cetobacter malorum* DSM 112354 [2] was evaluated for the oxidation of ω-hydroxycarboxylic acids into their corresponding $C_7 - C_{12}$ dicarboxylic acids, achieving high conversion rates (ranging from 42 to 97%). Moreover, *A. malorum* DSM 112354 cells were immobilized within calcium alginate beads, facilitating biocatalyst separation from the reaction mixture, product isolation and simplifying downstream processing for reaction scale-up.
To further expand the substrate scope, we designed a novel fusion protein.
This construct integrates a triple-mutant monooxygenase from *Marinobacter aquaeolei*, selected for its high catalytic efficiency and ω-specificity toward medium to long fatty acids [3], with the BM3 redox partner for its coupling efficiencies. The introduction of this enzymatic system into *A. malorum* enables an integrated one-pot oxidation cascade that converts fatty carboxylic acids into the corresponding dicarboxylic acids (**Figure 1**).
The work highlights the potential of engineered AAB for sustainable industrial processes, providing a bio-based alternative to the traditional routes for plastic precursor.

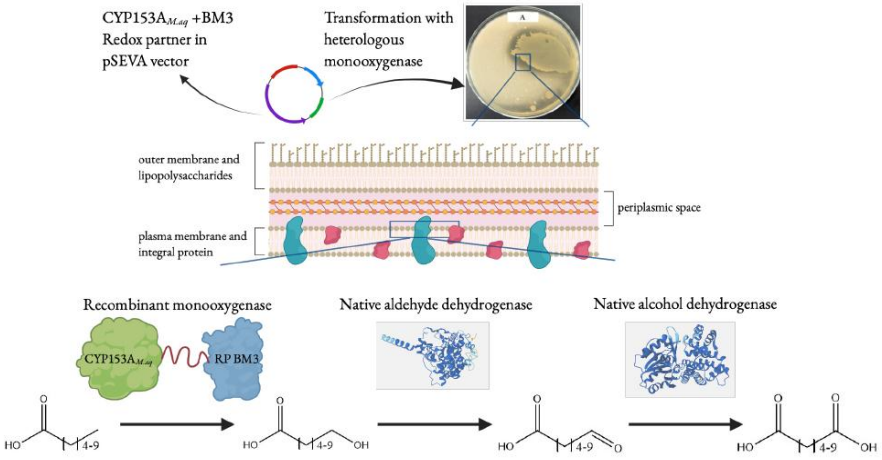

Figure 1. One-pot cascade preparation of dicarboxylic acids using engineered *Acetobacter malorum.*

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P 18

Single-Step Immobilization of Tryptophan Halogenases: A Leap Towards Sustainable Halogenation

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Halogenated compounds are ubiquitous in daily life. Active ingredients like cetirizine or natural compounds like pyrrolnitrin often contain halogen substituents in the final structure (**Figure 1A**) [1,2]. Due to their properties such as high electronegativity or distinctive size, halogen substituents can impact the behaviour of these compounds significantly [3,4]. Within chemical synthesis, halogens also serve as versatile starting points for **further modifications** like cross-couplings, prenylations, or cyclizations (**Figure 1B**) [5,6].

Traditional chemical halogenation using molecular halogens require harsh conditions and the production of chlorine gas *via* the chloralkali process is highly energy-demanding and generates toxic wastes [4]. Alternatives for **more sustainable halogenation reactions** are therefore needed and should be investigated. Halogenating enzymes, especially tryptophan halogenases, are interesting candidates that offer environmentally friendly routes to integrate halogen atoms. These enzymes exhibit a high regioselectivity and they address a broad substrate scope. Furthermore, tryptophan halogenases employ simple halide salts such as chloride, bromide, and iodide as halogenating agents [7]. Despite these advantages, conversion rates and stability remain limited, however, modern enzyme immobilization techniques open up new ways to overcome these challenges [7].

Our work showcases current research on selected flavin-dependent halogenases which enable **regioselective halogenation of tryptophan**. For addressing the challenges of low conversion rates and poor enzyme stabilities, we have designed versatile enzyme constructs that employ chemical immobilization and catalytically active inclusion bodies for **single-step immobilization approaches**. The set of constructs include various immobilization-promoting tags, linkers, and enzyme variants. Therefore, this work underscores a potential approach for utilizing tryptophan halogenases as robust biocatalysts in modern and sustainable bioorganic synthesis and application within chemical industry.

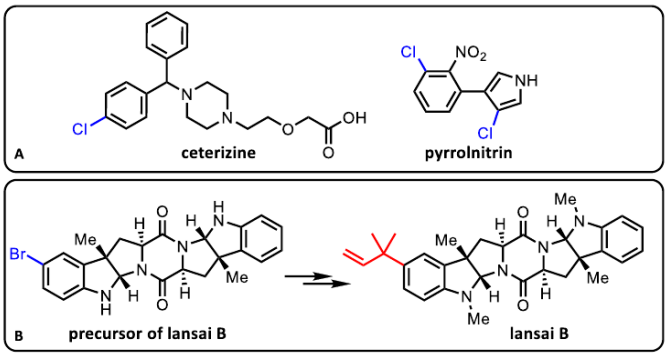

Figure 1. Selected compounds with halogen substituents and further modification of halogen moieties.

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P 19

Pairing Enzyme-Triggered Reactions with Resolution for the Construction of Complex Chiral Compounds

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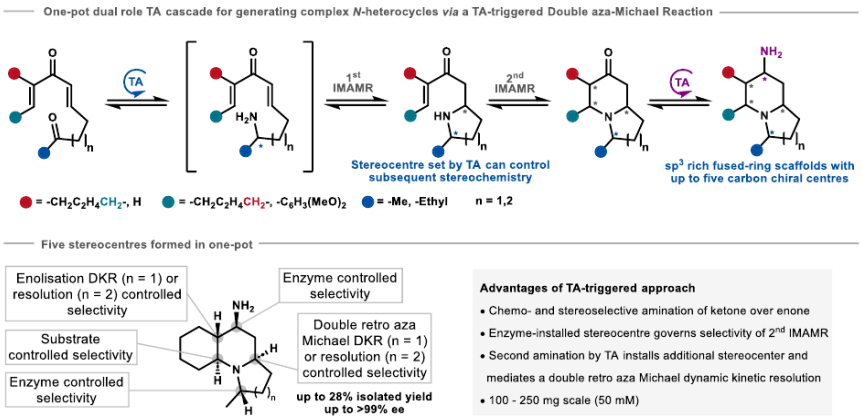
In many cases, biocatalytic transformations involve functional group interconversions, such as redox and amination reactions, but do not lead to significant complexity generation in the molecule. Enzyme-triggered reactions (ETRs), where the enzymatic transformation triggers a subsequent spontaneous reaction inter/intramolecularly, are a powerful approach for building molecular complexity and three-dimensionality from simple, easily accessible starting materials, in a single transformation.[1]
Despite its rapid complexity building, only the biocatalytic step of the ETR is stereoselective, and when targeting compounds with multiple stereocentres, the ETR strategy often produces a mix of stereoisomers arising from the spontaneous step. We herein present strategies for chemical and biocatalytic resolutions of the stereoisomers, including examples of dynamic kinetic resolution using the retro aza-Michael as an epimerisation strategy (**Scheme 1**).[2,3]



Scheme 1. Transaminase-triggered cascades for the synthesis and dynamic kinetic resolution of chiral *N*-heterocycles.

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P 20

Enzymatic Denim Dyeing

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Global production of synthetic indigo amounts to 80,000 tons, produced exclusively from petrochemicals. The synthesis and use of indigo come with major environmental and health implications, due to the need to use toxic and harsh reducing conditions to solubilize indigo for denim-dyeing (Figure 1). At NordicBlue, we are developing an enzymatic synthesis toward indican, a water-soluble natural precursor to indigo, as well as a dyeing process utilizing indican. By deploying indican in existing dyeing-infrastructure, we can side-step the need for the harsh reduction step without requiring major infrastructure upgrades, providing a clear path for adoption of this new technology.

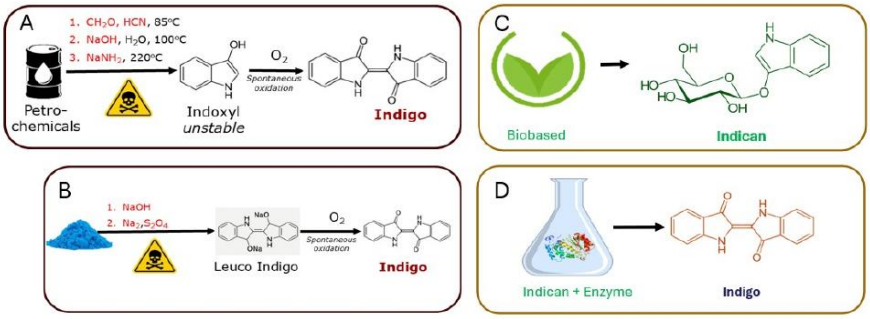

Figure 1. A: Current chemical route to indigo. B: Current reduction of indigo. C: enzymatic indican synthesis. D: enzymatic denim dyeing.

P 21

Exploration Of Novel Hydrazine Synthetases For The Synthesis Of Nitrogen-Nitrogen Bonds

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Nitrogen–nitrogen (N–N) bond formation represents a significant challenge in chemistry due to the strong electronegativity of nitrogen atoms. Nevertheless, nature has evolved multiple enzymatic strategies to overcome these constraints, producing over 300 natural products containing N–N bonds [1]. These compounds display broad biological activities, including antiviral, antibacterial, and anticancer effects. The increasing number of N–N bond–containing molecules approved by the U.S. Food and Drug Administration (FDA) in recent years underscores their pharmaceutical relevance, particularly in the treatment of cancer and cardiovascular diseases [2, 3].
While traditional chemical synthesis of hydrazine and its derivatives allows N–N bond construction, such methods often suffer from instability, toxicity, and safety concerns that limit industrial scalability. In contrast, biocatalytic systems offer a promising and sustainable alternative for N–N bond formation under mild, environmentally friendly conditions. Naturally occurring examples include both linear and cyclic hydrazines ($R_2N–NR_2$) and their derivatives, such as hydrazides. Enzymes like the piperazate synthase KtzT, which catalyzes the formation of L-piperazic acid [4], and hydrazine synthetases such as Apy9 [5], which generate linear hydrazine intermediates, illustrate nature's enzymatic ingenuity in N–N bond biosynthesis. Despite their importance, these enzymes remain insufficiently characterized at both biochemical and structural levels.
This project aims to deepen the understanding of hydrazine synthetases through comprehensive biochemical and structural characterization. Additionally, directed enzyme engineering will be employed to expand their substrate range and reaction scope, thereby advancing the biocatalytic toolkit for sustainable N–N bond formation and paving new roads in synthetic biology and drug discovery.

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P 22

Developing High-throughput Screening for Immobilised Biocatalysts Engineering for Carbon Up-cycling

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Formolase (FLS) is an artificial enzyme that converts formaldehyde (1-carbon) into dihydroxyacetone (3-carbon) in a carbon-ligation reaction, allowing the conversion of electrochemically reduced CO_2 into valuable multi-carbon compounds via the "formolase pathway". However, FLS exhibits low activity and is the bottleneck step in this pathway. The high concentrations of formaldehyde required due to low substrate affinity are also incompatible with in vivo systems. On one hand, FLS can be improved through protein engineering. On the other hand, higher concentrations are achievable *in vitro,* and by using immobilised enzyme flow reactors, the scalability of these *in vitro* pathways can be significantly improved. However, previous studies have shown that immobilisation affects mutants of the same enzyme differently; some are slowed, while others become faster. To avoid the unpredictability of immobilisation, it is desirable to engineer the enzymes directly in their immobilised form. Thus, in this project, we aim to engineer FLS in its immobilised form to ensure direct transferability to the flow reactor using a droplet microfluidic mutant screening platform.

To achieve this, we combined monoclonal rolling circle amplification (RCA) and cell-free protein synthesis (CFPS), polyacrylamide hydrogel beads for immobilisation, NAD-Display for determining mutant activity, and microfluidics for droplet manipulation (**Figure 1**). The hydrogel beads serve as the carrier for: hyperbranched RCA products of mutant plasmids, expressed and immobilised mutant proteins from CFPS, and NAD-Display complexes for generating a fluorescent signal, which include SNAPTag-Tamtavidin-SpyCatcher for immobilising biotin-NADH and the NAD/NADH fluorescent protein sensor SoNar-SpyTag. The beads, after catalysis, can be sorted using fluorescence-activated cell sorting (FACS) to establish the genotype-phenotype linkage of mutants with higher activities.

We identified and optimised the activity assay using glycerol dehydrogenase (GldA) for FLS. We also showed the successful integration of NAD-Display protein complexes with polyacrylamide beads through SNAPTag–benzylguanine linkage, and confirmed it by flow cytometry. Immobilisation of FLS through HaloTag–chloroalkane linkage was demonstrated and Biotin-NADH conversion was shown. Currently, we are working on assay optimisation and demonstration of the whole workflow.

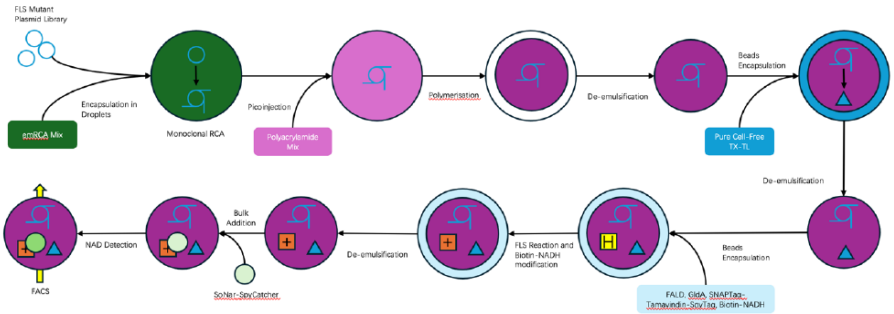

Figure 1. Schematic of the workflow under development.

P 23

Piece by Piece: SCHEMA-guided Combinatorial Assembly of Functional Chimeric Enzymes

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Genetic diversity is the essential element for protein evolution in biotechnology, enabling the production of more robust proteins that can be used in industries like pharma. "Homologous recombination" is a powerful way to create genetic diversity. By chopping homologous proteins (parents) and switching the fragments, the recombinant offsprings (chimeras) might generate special properties in addition to inheriting the beneficial traits of the parents[1].This approach generates a high degree of diversity, whilst maintaining structural and functional integrity, covering a large area of sequence space (Fig 1A). However, identifying structurally compatible cutting points (crossover points) plays a decisive role in assembling functional chimeras, since the suboptimal crossovers might lead to the loss of crucial inter-residue contacts and thus folding and function.

A recent method, combinatorial assembly and design of enzymes (CADENZ), produces large libraries of chimeric enzymes by recombining sequence optimized enzyme fragments with *in silico* screening through Rosetta and ML-based scoring[2]. Inspired by CADENZ, we developed a computationally cheap approach for design of combinatorial chimera libraries of a size suitable for low-throughput characterisation. Our method adapts SCHEMA, an efficient sequence/structure based pipeline, to optimise crossover points across homologs based on primary sequences, which are converted to mutually compatible Golden Gate overhangs[3]. Library sizes can be further reduced through computational filtering through structural prediction and Rosetta-based scoring. Deoxy-2-ribose-5 phosphate aldolase (DERA), a class I aldolase, was employed to verify this pipeline. We tested only 16 chimeras from 5 parents with 3 crossover, with most being stable and active and found two chimeras with higher stability and activity than any of the parents.

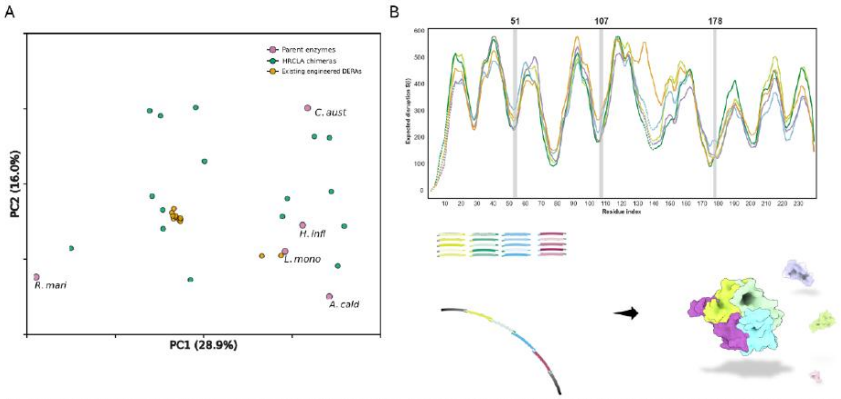

Figure 1. SCHEMA-guided Combinatorial Assembled enzyme. A: diversity of tested parent enzymes and chimeras; B: crossover point identification and fragments ligation *via* Golden Gate Cloning.

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P 24

AI-Guided Engineering of Hydropyrene Synthase

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Terpenes are a diverse class of natural compounds that play essential roles in many biological processes. In addition to their structural and ecological functions, they have attracted strong interest in biomedical research because of their wide range of therapeutic activities, including antimicrobial and wound healing properties [1]. Among these, pseudopterosins, which are diterpene glycosides derived from Caribbean soft coral *Antillogorgia elisabethae*, exhibit anti-inflammatory properties proved in clinical studies [2]. Nevertheless, due to unsustainable and cost-intensive generation of these diterpenes, their applications are limited.

Hydropyrene synthase, a member of the diterpene synthase family, converts geranylgeranyl diphosphate into four distinct products. One of them is isoelisabethatriene A, which is a key intermediate in the biosynthesis of pseudopterosins [3]. To facilitate active site engineering aimed at shifting the product profile toward isoelisabethatriene A, a high-resolution crystal structure is required. However, the enzyme shows low stability and poor solubility, which limits its expression and hinders crystallographic studies (**Figure 1**).

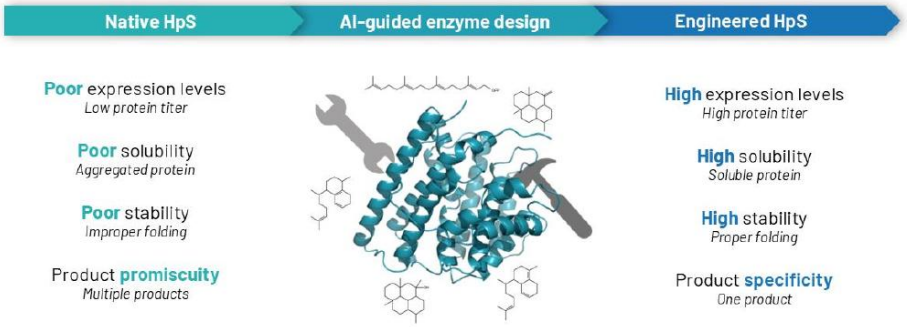

Figure 1. Schematic overview of AI-guided enzyme engineering for improving hydropyrene synthase (HpS). The enzyme's structure was predicted using AlphaFold.

Latest advancements in AI-based enzyme design provide new opportunities to improve enzyme stability, specificity and catalytic performance [4]. In this approach, computational modeling is integrated with experimental validation to engineer and characterize hydropyrene synthase with desired properties, resulting in conformationally stable and product-specific biocatalyst. Recent efforts have involved the use of a structure-based, self-supervised learning model to predict stability changes upon mutation. The predictor successfully identified stabilizing point mutations that maintained catalytic efficiency of the enzyme, as confirmed experimentally.

The iterative nature of this strategy enables the design of a biosynthetic generator of marine drug precursors, providing a sustainable, scalable and economically efficient route for their production.

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P 25

Mild and selective laccase-TEMPO catalysed oxidation of carbohydrates: towards polysaccharide modification

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Polysaccharides (PS) are abundant, renewable biopolymers whose properties can be tailored through selective modifications, enabling exciting applications in medicine and sustainable consumer products. Achieving controlled and selective transformations under mild and energy-efficient conditions is crucial both for preserving polymer integrity and for understanding structure-function relationships. This project focuses on selectively oxidising primary alcohols in PS to aldehydes or carboxylic acids, generating reactive handles that facilitate site-specific modifications, for example installing amines via reductive amination.[1] Traditional TEMPO-mediated oxidations rely on super-stoichiometric amounts of harsh oxidants like NaOCl, which can compromise polymer structure.[2]

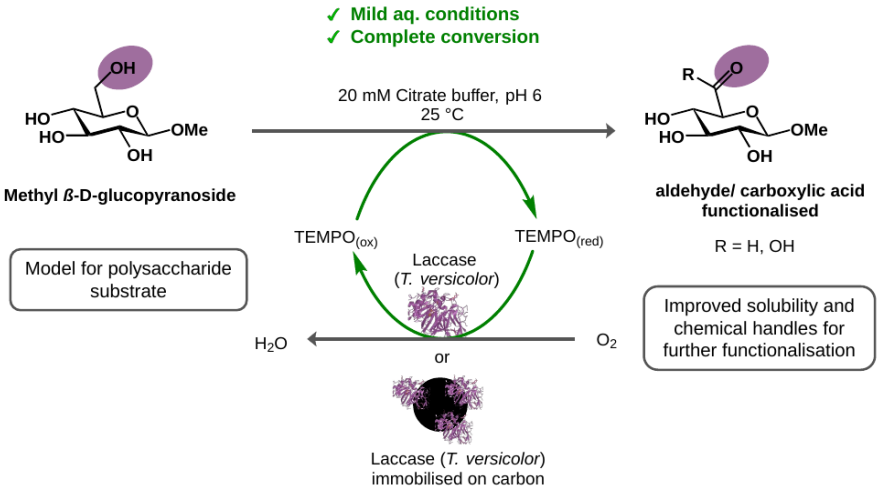

Figure 1. Laccase-TEMPO catalysed selective primary alcohol oxidation of methyl *β*-D-glucopyranoside

Biocatalytic oxidation offers a milder and greener alternative (**Figure 1**). Laccases can regenerate the TEMPO mediator using molecular oxygen under aqueous conditions enabling selective oxidations under environmentally friendly conditions.[3] Here, we applied the laccase/TEMPO/O_2 system to methyl *β*-D-glucopyranoside as a model substrate. Reaction conditions were optimised and products were analysed by NMR, GC-MS, and HRMS. To further improve enzyme stability and recyclability, laccase was immobilised on carbon and applied successfully to complete oxidation of the model compound. These results provide a proof-of-concept for extending this strategy to PS modification.

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P 26

DIRECTED EVOLUTION OF AN ENZYME FOR ASYMMETRIC MORITA-BAYLIS-HILLMAN REACTIONS AND KINETIC RESOLUTIONS

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The Morita-Baylis-Hillman (MBH) reaction represents a significant tool for carbon-carbon bond formation in organic synthesis. Catalyzed by a tertiary amine or phosphine, a carbon electrophile, such as an aldehyde, reacts with a α,β-unsaturated alkene forming a highly functionalized allylic alcohol with a novel stereo center[1–3]. The resulting MBH adducts have been demonstrated to serve as valuable intermediates in the synthesis of a diverse array of compounds with biological significance[4–6]. The atom-economical coupling is typically performed by small catalytic nucleophiles such as DABCO, quinuclidine and cinchona-derived alkaloids with no enzymes known to perform this reaction naturally[1–3,7,8]. Nevertheless, a small number of proteins have proven to perform it in a promiscuous manner, albeit with relatively low turnover numbers and stereoselectivity[9–11]. Recent work by the group of Anthony Green has opened new ways to enzymatically access the MBH reaction. This was achieved by combining enzyme engineering with the incorporation of non-canonical amino acids into a computationally designed enzyme by the Baker group, resulting in high enantioselectivities[12–14].

We here report the redesign of 4-oxalocrotonate tautomerase into a proficient MBH enzyme to promote the asymmetric MBH reaction between α,β-unsaturated aldehydes and various substituted benzaldehydes, as well as kinetic resolutions of MBH adducts, expanding the toolbox of catalysts for this attractive C-C bond-forming reaction.

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P 27

Engineering New Mechanisms in Thiamin Dependent Enzymes

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Thiamine diphosphate (ThDP)-dependent enzymes are highly versatile biocatalysts that catalyse a wide range of reactions.[1] Members of this class, including benzaldehyde lyase (BAL) from Pseudomonas fluorescens, benzoylformate decarboxylase (BFD) from Pseudomonas putida, and pyruvate decarboxylase (PDC) from Saccharomyces cerevisiae, can mediate a diverse range of C–C bond-forming transformations.[2] A prominent example is the benzoin reaction, in which two aldehydes are coupled to yield 2-hydroxy ketones with high enantioselectivity.[3] This process is proposed to proceed *via* a so-called Breslow intermediate, which converts the aldehyde into a nucleophile and enables the addition of the second aldehyde.[4]

In certain cases, ThDP-dependent enzymes such as PfBAL have been shown to form an "extended Breslow" or homo-enolate intermediate. However, reaction still takes place at the carbonyl carbon resulting in the formation of 2-hydroxy ketones.[2] More recently, a radical-based route has been discovered in which the C–C bond formation occurs at the β-carbon of the enolate through a single-electron transfer (SET) mechanism.[5] Reactivity proceeding *via* a two-electron pathway has not yet been reported enzymatically. By combining enzyme mining with directed evolution, we aim to identify and characterise ThDP-dependent enzymes capable of catalysing reactions at this unfavourable site. Enabling this non-natural pathway will unlock new strategies for C–C bond formation and potentially improve the efficiency of such transformations compared to NHC organocatalysts.

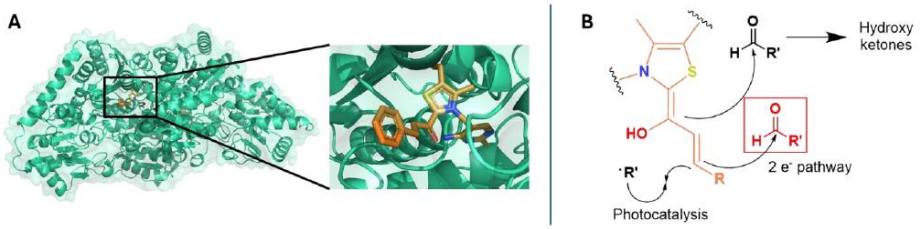

Figure 1. (A) Predicted structure of PfBAL with the "extended Breslow" intermediate located in the active site. (B) Proposed reaction pathways proceeding via the "extended Breslow" intermediate.

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P 28

Flow-based chemoenzymatic synthesis of chiral piperidine derivatives

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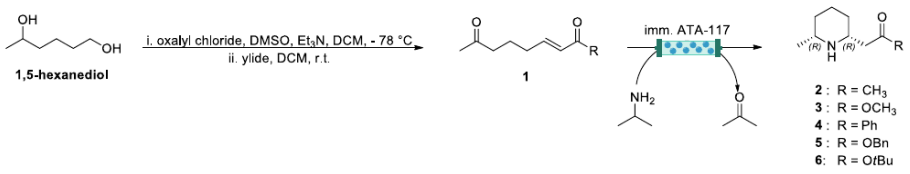
Chiral amines constitute key building blocks in approximately 40–45% of small-molecule pharmaceuticals and are widely used in the production of fine chemicals and agrochemicals.[1] Increasing environmental regulations, together with the growing demand for enantiopure compounds as high-value products, have driven the integration of traditional synthetic methodologies with greener (bio)catalytic approaches.[2]

Nitrogen-containing heterocycles are privileged motifs in many active pharmaceutical ingredients (APIs).[3] In this work we report the asymmetric synthesis of enantiopure piperidines as valuable scaffolds for alkaloid synthesis.[4] A pyridoxal 5'-phosphate (PLP)-dependent transaminase (ATA-117), immobilized for this purpose, enables a stereoselective transamination, followed by a spontaneous intramolecular aza-Michael reaction (IMAMR) (**Scheme 1**), ultimately affording the natural product (−)-pinidinone (**2**).[5]

The synthetic route began with two batch chemical steps to prepare substrate **1**, namely an oxidation followed by a Wittig olefination using commercially available ylides. After enzyme expression and purification, several immobilization strategies were evaluated. Covalent immobilization on Eupergit® C was identified as optimal, enhancing both operational stability and reusability of the (*R*)-selective biocatalyst. Subsequently, the reaction was optimized under continuous flow conditions by varying substrate concentration, isopropylamine equivalents, temperature, residence time, and the type and amount of cosolvent. Finally, the developed protocol was extended to a range of substrates, enabling the synthesis of diverse 2,6-disubstituted chiral piperidines (**3**-**6**).



Scheme 1: Chemo-enzymatic asymmetric flow synthesis of 2,6-disubstituted piperidines.

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P 29

Scaled up reaction in 200 mL SpinChem reactor for N^5-Hydroxy-L-ornithine batch production.

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N-hydroxylating monooxygenases (NMOs) are a group of flavin dependent enzymes that utilize oxygen and an external reductant such as NADPH to oxidize terminal amine groups of L-ornithine, L-lysine and other primary aliphatic diamines to their respective *N*-hydroxy compounds[1]. NMO reaction mechanism consists of a reductive reaction, NADPH binding reducing the FAD and stabilizing FAD intermediates, and the oxidative reaction, where FAD intermediates hydroxylate the substrate. Dissociation of FAD intermediates before substrate binding leads to unproductive H_2O_2 formation. After hydroxylation, the hydroxylated product, $NADP^+$ and H_2O are released and FAD reverts to its oxidized state. To enhance the activity of NMOs, a constant supply of NADPH is necessary to lower the production of H_2O_2 as opposed to a high supply and the removal of accumulating H_2O_2 is essential[2]. Enzyme cascade set ups as shown in **Figure 1** below, enable efficient *N*-hydroxy compound formation, which are used as important intermediates in the synthesis of complex molecules. In this study, we aimed to employ a cascade reaction that consists of immobilized *Thermocrispum agreste* NMO, *Pseudomonas* sp. 101 FDH and *Parageobacillus toebii* catalase for the bioconversion of L-ornithine to N^5-Hydroxy-L-ornithine. The enzymes were immobilized on Immobead His-tag carriers from ChiralVision BV. The cascade reactions set up was scaled up to 200 mL in a SpinChem® rotating bed reactor (RBR) and we achieved 90 % conversion.

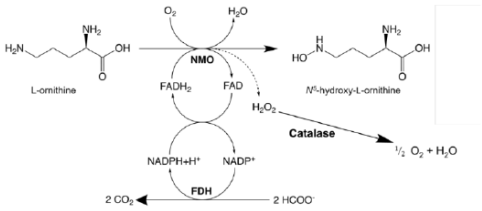

Figure 1: N^5-Hydroxy-L-ornithine production in the cascade reaction using three different enzymes (NMO, FDH and catalase).

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P 30

Towards the Biocatalytic Synthesis of Industrially Valuable Diamines from Commercially Available Starting Materials

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Chiral amines decorate scaffolds which appear in commonly prescribed drugs, increasing the need for stereoselective synthetic routes towards such motifs. There is a wealth of existing research on chemical synthesis of vicinal diamines, popular for their range of applications, spanning from catalytic ligands and auxiliaries for asymmetric synthesis to therapeutic ligands for cancer drugs [1]. However, the less-researched 1,3-diamine motif has been identified as medicinally valuable, with various medicinal candidates recently coming to light interacting with a broad range of receptors. Certain candidates are inhibitors of janus kinase 1 (JAK-1), overactivity of this protein involved in autoimmune, inflammatory and hyperproliferative diseases [2]. A recently approved 1,3-diamine derived drug abrocitinib lessens immune response as it blocks JAK-1 and is used to treat atopic dermatitis [3]. An engineered reductive aminase was used by Pfizer to derive a 1,3-aminoketone intermediate in the synthesis of abrocitinib [3].

A fully protein-catalysed synthesis of any diamine subclass is not detailed in literature, to our knowledge. This research explores the suitability of biotransformation as an approach to access a panel of chiral five- and six-membered cyclic 1,3-diamine targets from cyclic diketone starting materials (**Figure 1**). A variety of commercially available transaminase enzymes have been screened for primary amine installation on dicarbonyl substrates, as they are involved in several robust syntheses of chiral amines from carbonyls in industry (**Figure 1a**) [4]. Reductive aminases are also of interest due to their versatility and ability to produce primary, secondary and tertiary amines utilising a plethora of amine substrates (**Figure 1b**) [4]. These enzymes were chosen to be screened for activity on the 1,3-dicarbonyl motif as successful implementation of reductive aminases broadens the number of potential products accessible and therefore the scope of the project.

Bearing in mind the possibility of mono-amination, di-amination, failed amination, or formation of mixed product profiles, care was taken to preserve contents of the reaction vial during analysis. This was achieved by employing water-suppression NMR to provide insight to the range of chemical species in aqueous buffer without subjecting potential products to acidic or basic conditions associated with traditional workup procedures. Implementation of rigorous control reactions enabled assessment of reaction success and identification of side-reactions, for example undesirable aldol reactions, which may occur due to relatively high pH biotransformation conditions.

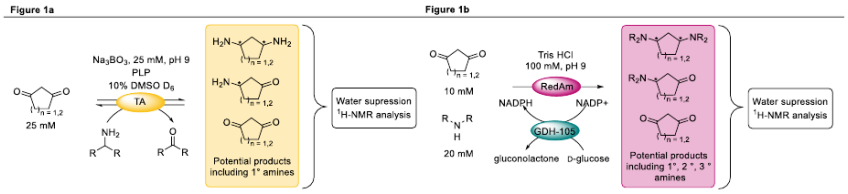

Figure 1. Biotransformation of cyclic diketones with transaminase and reductive aminase enzymes.

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P 31

An Evolvable *In Vivo* Selection System

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In vivo selection systems are powerful tools for the directed evolution of enzymes. In contrast to screening methods, they enable the simultaneous evaluation of millions of variants without relying on time-consuming analytical methods such as HPLC or LC-MS. However, their broader application is limited by the difficulty of coupling enzymatic product formation to host cell survival. Consequently, there is a need for a general and easily adaptable *in vivo* selection system that can be quickly evolved for the use with new enzymatic reactions.

Recently, the plant PP2C hormone sensing system (PYR1/HAB1) has been shown to be highly evolvable for binding a variety of non-native ligands [1,2]. Upon ligand binding, PYR1 undergoes a conformational change that promotes interaction with a phosphatase (HAB1/ABI1). This ligand-induced dimerization can be harnessed to create biosensors by fusing PYR1 and the phosphatase to split reporter systems, such as a yeast two-hybrid system [3] or a split T7 RNA polymerase system[4].

Here, we demonstrate the use of these adaptable biosensors for the directed evolution of enzymes in *E. coli*. As proof of concept, we first evolved sensors for different carboxylic acid products and subsequently utilized one of those sensors for the directed evolution of the BS2 esterase in a dual *in vivo* selection system.

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Computational Design and Engineering of Biocatalysts for Sustainable Polymer Transformations

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Achieving circularity for robust polymer systems requires overcoming substantial thermodynamic and kinetic barriers, both in the sustainable synthesis of engineering thermoplastics such as polyamides, including nylon, and in the depolymerization of complex biopolymers such as lignin [1]. In these contexts, current enzymatic methodologies often fall short of the stability, tolerance, and substrate scope required under industrially relevant conditions. Within the EU-funded COMENZE MSCA Doctoral Network, this research establishes a deep-learning-guided workflow to design novel biocatalysts and enzymatic cascades addressing both "beginning-of-life" processes, including monomer generation and sustainable synthetic routes, and "end-of-life" processes, including polymer recycling and valorization. For polyamide synthesis particularly, we focus on the design of enzymatic cascades that enable the formation of polyamide-relevant monomers and intermediates under mild conditions, thereby developing sustainable entry points for polymer production. In parallel, we extend the same design framework to the depolymerization and valorization of polyamides and lignin. Our approach integrates detailed physics-based modeling with Zymvol's in-house computational pipelines, Zymevolver and BioMatchMaker, combining well known methods such as molecular docking, molecular dynamics, and quantum chemistry to rationalize substrate access, quantify key enzyme-substrate interactions, and prioritize mutational hotspots for improved reactivity toward bulky polymer-derived motifs. This workflow is applied to identify and engineer enzyme variants, particularly transaminases and alcohol dehydrogenases, toward expanded substrate scope and new-to-nature reactivities [2]. This work's primary outcome is a mechanistic rationale linking active-site modifications to changes in enzyme reactivity, selectivity, and substrate acceptance across different polymer-relevant monomers and intermediates. Future work will expand the chemical space explored, including additional amine donors, alcohol substrates, carbonyl precursors, and other building blocks relevant to amide bond formation. By bridging computational prediction with experimental enzyme engineering (**Figure 1**), this research aims to accelerate the development of sustainable biocatalysts for both the production of performance materials and the valorization of polymer waste.

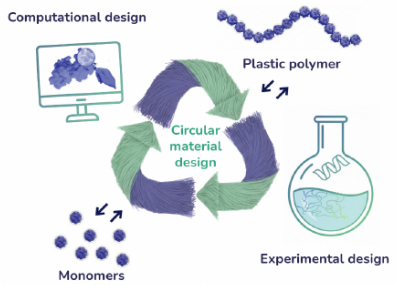

Figure 1. An integrated framework for circular material design and polymer valorization.

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Structure-guided engineering of N-N bond-forming enzymes

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Piperazic acid (Piz) is the sole natural amino acid known to contain an N-N bond [1]. The formation of N-N bonds is a relatively uncommon occurrence in chemistry. Numerous compounds containing N-N bonds are known for their anti-cancer, anti-apoptotic, and antibacterial properties and are actively used as drugs [2]. Our research centers on piperazate synthase (PZS), a Piz-producing enzyme.
The biocatalytic synthesis of Piz ((S)-hexahydropyridazine-3-carboxylic acid) derivatives starting from L-ornithine utilizes an FAD-dependent monooxygenase (NMO) and a heme-dependent PZS. The NMO hydroxylates the δ-amino group of ornithine, while the PZS catalyzes the formation of an N-N bond, resulting in a 6-membered cyclic structure of Piz [2, 3, 4].
We present a detailed structural and computational characterization of SbPZS, a PZS from *Streptomyces* sp. High-resolution crystal structures of the apo- and the holoprotein were determined at ~2.1 Å, enabling detailed characterization of cofactor binding. To further understand the Piz biosynthesis mechanism, molecular dynamics simulations of the protein-substrate complex were performed. This provides insights into the protein-substrate interactions and conformational dynamics. By integrating structural analysis, bioinformatics, and MD simulations, we identified putative engineering hotspots to enhance catalytic efficiency and broaden substrate scope [5]. Rationally engineered SbPZS variants are currently under experimental evaluation using enzyme activity assays and LC-MS to validate these predictions.

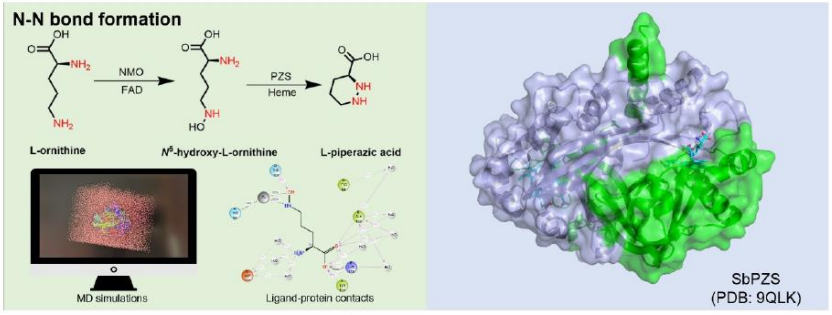

Figure 1. The biosynthesis of L-piperazic acid from N^5-hydroxy-L-ornithine has been investigated through the utilization of structural details of SbPZS, alongside the monitoring of protein-substrate dynamics and ligand-protein interactions.

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Chemoenzymatic platform for the synthesis of L-(hetero)arylalanines

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Non-canonical amino acids are extremely valuable building blocks used for the synthesis of bioactive molecules, as biophysical probes, and for the incorporation into peptides and proteins to introduce new or enhanced functions [1]. However, the stereoselective synthesis of structurally diverse amino acids remains a significant challenge using conventional chemical methods.

Biocatalysis offers an attractive alternative due to its high selectivity and mild reaction conditions. In this context, enzymes such as phenylalanine ammonia lyases (PALs) [2] and tryptophan synthases (TrpS) [3] have emerged as powerful tools for the synthesis of non-canonical amino acids. By exploiting the substrate promiscuity of these enzymes, a variety of L-heteroarylalanines can be accessed from readily available precursors. As case studies, we recently focused on the synthesis of substituted thienylalanines [4] and tryptophans [5] (**Figure 1**).

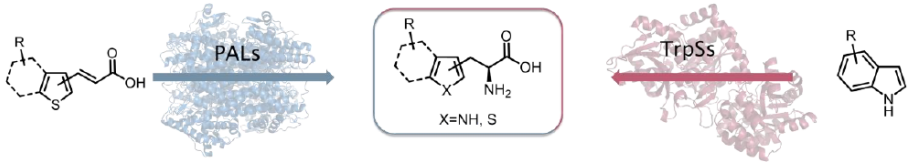

Figure 1. Chemo-enzymatic strategies for the synthesis of substituted L-(hetero)arylalanines.

Furthermore, the combination of enzymatic steps with chemical transformations in chemo-enzymatic cascades enables the conversion of these heteroaryl amino acids into structurally diverse amino acids and peptides, such as linear and branched aliphatic homologues [4]. This strategy expands the accessible chemical space of amino acid derivatives obtained biocatalytically and provides a versatile platform for the sustainable synthesis of valuable non-canonical amino acids.

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P 35

In Silico-Guided Optimization of TEV Protease Surface Reactivity for Covalent Immobilization

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Covalent immobilization is widely used to improve enzyme stability and reusability in biocatalytic processes, especially in flow applications. However, these benefits often come with a reduction of enzymatic activity. Since immobilization outcome depends on multiple interdependent variables, its optimization still relies heavily on resource-intensive experimental screening [1].

In this study, we investigated whether *in silico* approaches can support the rational optimization of enzyme immobilization by guiding the modification of reactive surface composition to minimize activity loss. As a model system, we selected a 224-amino-acid Tobacco Etch Virus protease (TEVp) variant [2,3,4,5], which achieves full substrate cleavage within 1 hour at a TEVp:substrate ratio of 1:10 and remains active over a pH range of 6 to 10.

Molecular dynamics (MD) simulations were used to characterize residue solvent exposure and pKa values of surface residues. Cysteine and lysine residues were identified as potential anchoring points for thiolsulfonate- and glyoxyl-supports, respectively. Among cysteines, Cys130 emerged as the most exposed residue and displayed the lowest predicted pKa, making it a promising candidate for single point attachment. TEVp variants to enhance Cys130 nucleophilicity through thiolate stabilization, as well as lysine-depleted TEVp variants aimed at assessing the contribution of other surface nucleophiles in aldehyde-based immobilization chemistry, were generated *in silico* and evaluated by MD simulations to confirm their structural stability prior to experimental testing.

These work aims at highlighting the potential of computational approaches to guide the design of immobilization-optimized enzyme variants with minimal impact on catalytic activity.

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Structural & Functional Insights into Enzymatic Non-Natural Carboligation

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Chiral γ-aminobutyric acid (GABA) analogues are pharmaceutically important compounds, several of which are marketed as antidepressants and anxiolytics. Prominent examples include phenibut, fluorophenibut, baclofen, and pregabalin. These compounds can be synthesized from the corresponding γ-nitroaldehydes through a two-step process. [1]

This work focuses on structural and mechanistic studies of enzymes engineered to catalyze the production of γ-nitroaldehydes. Specifically, we investigate variants of 4-oxalocrotonate tautomerase (4-OT) engineered to catalyze asymmetric Michael additions of nitroalkanes to cinnamaldehyde **(Figure 1)**. [2,3]

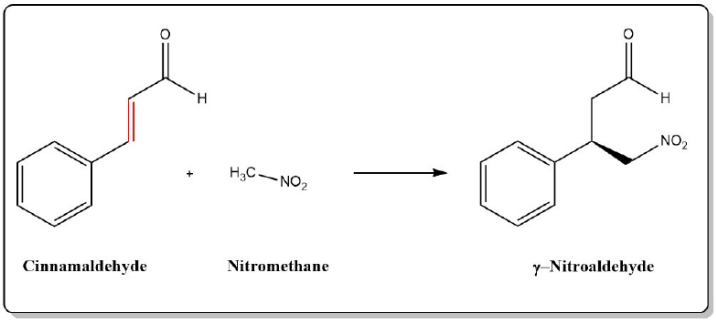

Figure 1. Asymmetric carboligation reaction leading to the formation of a gamma-nitroaldehyde.

The workflow of this research is summarized as follows:

1) High-resolution protein crystallography of enzyme-substrate complexes to capture reaction intermediates within the active site and enable sequential structural mapping of the enzymatic catalysis.
2) Molecular dynamics simulations based on experimentally determined structures to identify the roles of key residues in enzymatic catalysis and characterize interactions between active-site residues and the covalent enzyme-substrate complex.
3) Structure-based enzyme evolution aimed at expanding the substrate scope of the enzyme and enabling the catalysis of mechanistically related carboligation reactions.

Additionally, this work investigates the origin and functional implications of asymmetry in the arrangement of monomers within the trimeric enzyme complex.

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Regio- and Stereoselective C-H Functionalization of Renewables Using α-KG Dependent Enzymes

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This project aims to identify α-ketoglutarate (α-KG) dependent enzymes capable of the regio- and stereoselective functionalization of renewables like fatty acids (FA) and others. FAs, for instance, are considered readily available starting materials derived from natural resources.[1] Chemical methods for regioselective functionalization of FAs such as hydroxylation lead mostly to inseparable mixture of products, in case the α-position is not in the focus.[2, 3] Furthermore, there are only a few reported biocatalytic examples for selective functionalization of FAs. These include the regioselective C5 hydroxylation of decanoic acid with cytochrome P450 monooxygenase,[4] the selective hydroxylation at the ω−2 positions of the unsaturated FA myristoleic acid using a recombinant peroxygenase[2] as well as the regio- and stereoselective α-hydroxylation of FAs with the bacterial peroxygenases from the CYP152 family.[5] Unspecific peroxygenases (UPOs), however, are generally not regarded as regioselective.[6] To the best of our knowledge, there is no specific method available for a regio- and stereoselective hydroxylation at C3, C4 or C6. In the biocatalytic field, most reported α-KG dependent enzymes catalyze the regioselective hydroxylation of amino acids, e.g., lysine,[7, 8] leucine,[9] isoleucine[10] or proline.[11] Recently, the α-hydroxylation of aromatic FAs catalyzed by this enzyme family was described as well.[12] That being said, α-KG dependent enzymes have also demonstrated catalyzing numerous other reaction such as ring expansions,[13] oxidative C-C bond formations,[14] and selective halogenations.[15] Within this study a library of α-KG dependent enzymes will be established based on literature, BLASTP and SSN search. As a first instance, the enzymes in the library will be evaluated for their ability to hydroxylate, aminate or chlorinate fatty acids. Subsequently, the distribution of the regioisomers will be analyzed and the stereochemistry of the newly introduced hydroxy group or other functionality will be determined. Reaction conditions and the enzyme may be improved to achieve a sustainable applicable process and to reach a highest possible TON and highest substrate concentration. In a subsequent step, stereocomplementary enzymes will be searched for.

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Expanding the biocatalytic toolbox: A glutathione *S*-transferase for aliphatic epoxide resolution

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Epoxides are crucial compounds in daily life, serving as intermediates in agrochemicals, appear in different fabrics or adhesives and are used in various pharmaceuticals. In particular, chiral epoxides are essential building blocks for stereochemically defined molecules [1]. The need for efficient and selective ways to access enantiopure epoxides has fueled interest in biocatalytic approaches. In this context, the newly discovered Actino-like glutathione *S*-transferase (GST) class has emerged as a promising source of biocatalysts for the production of enantiopure epoxides. Notably, a recently described GST exhibits promising potential in enzymatic kinetic resolution with aromatic epoxides, however it shows limited performance towards aliphatic ones [2]. This limitation highlights the need for GSTs with improved enantioselectivity towards aliphatic epoxides.

Herein, we investigated a GST from the isoprene degrading *Rhodococcus opacus* PD630 (*Rho*IsoI) of the Actino-like class, as a candidate for the enantioselective conversion and kinetic resolution of aliphatic epoxides.

Combining phylogenetic analysis with multiple-sequence alignment, the sequence-function relationships of *Rho*IsoI with those of recently reported Actino-like GSTs were compared. The enantioselectivity of the GST toward several epoxides was tested *via* chiral GC-FID, followed by investigation in enzymatic kinetic resolution.

Phylogenetic analysis based on amino acid sequences indicated that *Rho*IsoI is distantly related to the recently described GST from glutathione-dependent styrene degradation pathway and can be assigned to the Y-type GSTs. Given that the enzyme showed activity with a preference for the (*R*)-enantiomer of isoprene monoxide (47.58 ± 3.26 U mg^{-1}) and the (*S*)-enantiomer of 1,2-epoxyhexane (9.6 ± 2.15 U mg^{-1}), we proceeded to assess the potential of this GST further. The enzymatic kinetic resolution of these aliphatic epoxides was conducted, resulting in an *s*-factor of 11 for isoprene monoxide and 10 for 1,2-epoxyhexane. Optimization of reaction conditions allowed improved resolution performance for respective substrates, demonstrating the applicability of the enzyme as a biocatalyst.

These findings highlight actinobacterial GSTs as promising candidates for selective epoxide biotransformation and lay the groundwork for methodical engineering to improve selectivity and achieve practical yields.

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Flow-Injection Mass Spectrometry (FIA–MS): A Rapid and Versatile Method for Screening Enzymatic Reactions

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The screening of enzymatic reactions traditionally relies on photometric and fluorometric assays, which offer high throughput but are restricted to substrates with the required optical properties, or on chromatographic methods, which are very general but require analysis times of several minutes per sample. Mass spectrometry (MS) without chromatographic separation has the potential to bridge the gap between these two approaches, as it is broadly applicable and relatively fast (analysis times ≤1 min/sample). Powerful MS-based high-throughput screening methods have been reported in recent years [1–3], but these rely on specialised or even custom-built equipment, which is accessible to only few research groups.

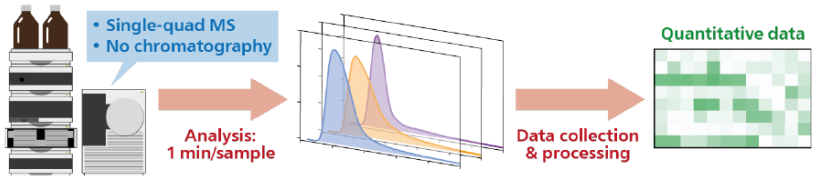

Figure 1. Schematic overview of the flow-injection mass spectrometry workflow.

We have found that flow-injection mass spectrometry (FIA–MS) performed on a common instrumental platform, single-quadrupole HPLC–MS, can be used for the qualitative and quantitative analysis of diverse biotransformations (**Figure 1**) [4]. Samples are injected into the eluent flow of a liquid chromatograph with no column installed and are thus introduced into the electrospray MS with a throughput of one sample per minute. Common organic buffers (*e.g.*, bicine, tricine, MOPS) present in the biotransformations can fulfil the function of an internal standard, allowing a linear quantification of analytes over 1–2 orders of magnitude in concentration. We demonstrate the application of FIA–MS to the screening of reactions catalysed by imine reductases, transaminases, methyltransferases, and a strictosidine synthase, using crude biocatalyst preparations (lysates or whole cells) and straightforward, plate-based liquid handling workflows. In each case, FIA–MS rapidly generated actionable insights into enzyme activity and selectivity that were readily confirmed by chromatographic re-analysis. We are, therefore, convinced that FIA–MS will become a useful additional tool for the screening of enzymatic reactions.

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Characterization, structure, and application of piperazate synthases for biocatalytic N–N bond formation

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Enzymatic nitrogen–nitrogen bond formation has become increasingly characterized in natural product discovery within the current decade [1]. Since the first "NNzyme" was reported in 2017 [3], these biocatalysts have largely remained subject of natural product elucidation, and their potential in applied biocatalysis remains largely untapped. Piperazate synthase (PZS) is a heme-dependent enzyme that catalyzes the intramolecular condensation of N^5-hydroxy-L-ornithine to yield L-piperazic acid, a non-proteinogenic cyclic amino acid found in various secondary metabolites, many with antibiotic activity [4].

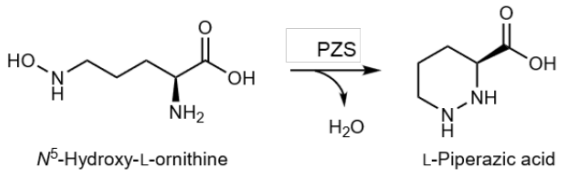

Figure 1. Reaction catalyzed by piperazate synthases (PZS).

Recently, the piperazate residue has gained attention as a shared moiety in a new class of "molecular glue" drugs (e.g. daraxonrasib) targeting cancers driven by the oncogenic and previously "undruggable" KRAS G12X mutations [5]. Because aliphatic, disubstituted hydrazines are challenging to synthesize chemically, the full potential of such building blocks in drug design remains unrealized. PZSs offer a route to *de novo* hydrazine formation as engineerable, reusable biocatalysts with excellent atom economy. Realizing this potential requires biochemical characterization of the enzyme family, insight into structure, function and mechanism, and establishing an engineering workflow.

We selected and produced 16 putative PZS sourced from sequence-similarity networks, extremophilic hosts, and consensus-deviation analysis. We developed quantitative LC-MS and colorimetric activity assays and used them to profile selected candidates across more than 100 conditions each, assessing ideal buffer composition, substrate scope, solvent tolerance, and thermal activity and stability. We identified a PZS from a *Streptomyces* strain with a 10-fold higher specific activity than the next-best enzyme in our panel, solved its crystal structure, and applied it in an immobilized cascade to produce piperazate from L-ornithine. These methods and findings lay the groundwork for structure-guided promiscuity engineering and biotechnological implementation of PZSs in the development of novel N-N bond-containing pharmaceuticals.

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De novo design of thermostable LPMO

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Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes that oxidatively cleave recalcitrant polysaccharides such as cellulose, making them important targets for enzyme engineering [1]. In this project, we designed novel LPMO variants with the aim of improving stability and maintaining catalytic activity. The variants were expressed in *Escherichia coli*, purified where possible, and tested in preliminary activity and stability assays. Initial results suggest that several engineered variants retain activity and show improved thermal stability.

We also expressed and purified cellulose-targeting variants containing a C-terminal cellulose-binding module (CBM). CBMs can enhance interactions with insoluble polysaccharide substrates and may improve substrate targeting [2]. However, the cellulose-based activity assays tested so far have not produced reliable activity readouts, so further assay optimization is needed. As a next step, we plan to test an alternative bacterial CBM.

In parallel, we determined a high-resolution crystal structure of one engineered LPMO variant using X-ray diffraction data collected at the European Synchrotron Radiation Facility (ESRF). This structure will help interpret the engineered features and guide future design improvements.

[1] Vaaje-Kolstad et al., 2010; Forsberg et al., 2019 — LPMO oxidative cellulose degradation.
[2] Armenta et al., 2017; Courtade et al., 2018 — CBMs and LPMO substrate targeting.

P 43

Improving *In Vivo* Methylation in *E. coli*

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Methylation and alkylation are important for the synthesis of small molecules and natural products, as they strongly influence physicochemical and pharmaceutical properties [3,4]. Methyltransferases (MTs) provide a sustainable alternative to conventional chemical methods, offering high regio- and chemoselectivity while reducing environmental impact. However, their use is limited by the need for stoichiometric amounts of the unstable and costly cofactor *S*-adenosyl-L-methionine (SAM). Employing MTs in whole-cell systems such as *Escherichia coli* enables cofactor regeneration *in vivo*, representing a promising strategy to overcome this limitation [3]. Here, we address the limiting factor in SAM synthesis, supply of the precursor methionine (Met), to accelerate methylation activity in *E. coli* [6].
Two metabolic engineering approaches were explored (**Figure 1**). First, the formate assimilation machinery from *Methylorubrum extorquens,* including the formyl-H_4F ligase *Mex*FTFL, was introduced. This allowed the use of formate as a renewable C_1 source, resulting in more than 50% of transferred methyl groups originating from formate (FA). Incorporating C_1-auxotrophy and increasing the FA supply could exceed the FA-derived methyl groups to more than 70%, highlighting the importance of methyl group availability for efficient *in vivo* methylation [1]. Second, introduction of the direct sulfurylation pathway from *Saccharomyces cerevisiae*, including *O*-acetylhomoserine sulfhydrolase (*Sc*OAHS) and homoserine *O*-acetyltransferase (*Sc*MET2), offered an alternative route for Met biosynthesis from methane thiol. This strategy not only improved methylation activity but also enabled the production of methionine analogues through the use of alternative alkyl mercaptans [2,5].
Overall, this work highlights methionine supply as a key determinant of in vivo methylation activity and demonstrates the potential of integrating renewable one-carbon sources and alternative biosynthetic pathways. Future work will focus on expanding the use of sustainable C1 feedstocks such as methanol or CO_2, further optimizing methionine synthesis and methylation activity.

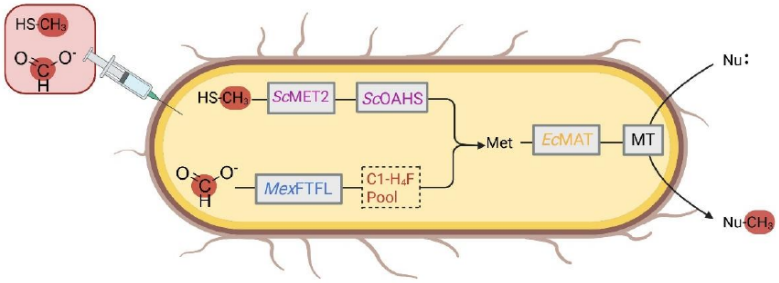

Figure 1. Improving In Vivo Methylation in *E. coli* by Introducing the Direct Sulfurylation Pathway of *S. cerevisiae* and the Formate Assimilation Pathway of *M. extorquens.* Schematic Illustration of the introduced pathways: In the direct sulfurylation pathway homoserine is acetylated by *Sc*MET2 to *O*-acetylhomoserine which, when supplied with methanethiol, is converted to methionine by *Sc*OAHS. In the formate assimiliation pathway formate is converted to formyl-H_4F by *Mex*FTFL and thereby introduced into the C_1-H_4F pool as a C_1-compound, which also leads to the generation of methionine. Synthesis of SAM by *Ec*MAT follows with subsequent MT-dependent methylation of the respective substrates (Nu).

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P 44

Characterisation of gallate decarboxylases across microbial species

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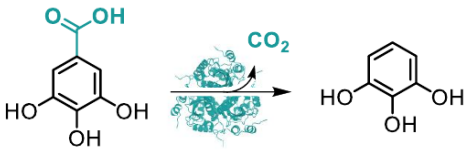
The agrifood system accounts for around one-third of the global greenhouse gas emissions and plant-based proteins have the potential to satisfy the global demand for protein while limiting the environmental impact of the food system. [1] However, consumers' acceptance of plant-based food products remains limited, mainly due to the presence of off-flavours often linked to beany, green and bitter attributes.[2] Gallic acid released from tannic acid degradation and other phenolic acids have been linked to bitter, astringent and sour flavour attributes, and fermentation is a promising strategy for converting them into less flavour-active compounds.[2] Understanding these key enzymatic steps remains one of the main challenges.

Degradation of gallic acid has been characterized in aerobic conditions to undergo ring cleavage by a gallate dioxygenase.[3] However in anaerobic conditions, gallic acid is decarboxylated to pyrogallol, catalysed by the cofactor-independent gallate decarboxylase (GDC, Figure 1). Few enzymes are reported as GDCs,[4] making it challenging to retrieve sequences and structural information. By mining new sequences from UniProt and NCBI databases for the creation of sequence similarity networks, we aim to identify different clusters of organisms with this enzymatic activity that does not seem to be common across microbial species.

Selected enzymes will be characterised *in vitro*, and their substrate scope will be evaluated to discover promising candidates for the decarboxylation of gallic acid and potentially other benzoic acids. These results could set the basis for identifying new microbial species with gallic acid degradation potential and for expanding the enzyme family available for the production of pyrogallol. The application of pyrogallol as antioxidant, therapeutic agent and in functional materials provides further motivation for the investigation of its biosynthesis and biodegradation.



Gallic acid **Pyrogallol**

Figure 1. Decarboxylation of gallic acid to pyrogallol by gallate decarboxylases.

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P 45

Data-Driven Acceleration of C–H Bond Biocatalysis

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Selective C–H bond functionalization remains a central challenge in synthetic chemistry. Although transition metal-catalyzed C–H activations have enabled powerful transformations, they often require harsh reaction conditions and can suffer from limited selectivity. In contrast, enzymatic approaches offer exceptional chemo-, regio-, and stereoselectivity under mild conditions [1]. Among these, Fe(II)/α-ketoglutarate dependent dioxygenases (αKGDs) have emerges as versatile biocatalysts for both late-stage functionalization [2] and the synthesis of valuable building blocks [3]. However, the broader application of these enzymes is often hindered by their narrow substrate scope and the labor-intensive nature of traditional screening and engineering workflows [4].

The discovery of novel biocatalytic activity relies on the ability to detect subtle chemical transformations within complex biological matrices. To address this challenge, we developed EnzyMS, a Python-based pipeline that automates the analysis of high-resolution LC-MS data. In contrast to conventional metabolomics workflows, EnzyMS is specifically designed for biocatalysis applications, enabling reliable discrimination between genuine enzymatic products and background signals.

The utility of this platform was demonstrated through the discovery of a previously unreported selective oxidative demethylation of soraphen A by engineered WelO5* variants. By integrating EnzyMS with a "dock-n-design" computational approach, we achieved a 3-fold improvement in demethylation activity while experimentally testing only three predicted variants. These results highlight how robust analytical pipelines, combined with data-driven enzyme engineering, can substantially accelerate biocatalyst optimization [5].

Building on the high-throughput analytical capabilities of EnzyMS, we next aimed to move from reaction discovery to predictive biocatalysis. To this end, we systematically screened a library of 90 αKGDs against 75 structurally diverse substrates, identifying 37 accepted compounds. The resulting dataset, consisting of 3300 enzyme-substrate pairs, combined with data on αKGD-substrate pairs reported in literature, enabled the development of CH-Predict, a structure-informed deep learning model for predicting αKGD substrate activity.

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A Process-Driven Approach to Designing P450 Biocatalysis

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Cytochromes P450 are promising biocatalysts that have the potential to unlock selective oxidation, a reaction that has long been sought after by the chemical industry. As monooxygenases, P450s can functionalise non-activated carbons in complex molecules with great regio- and stereo-selectivity [1]. This trait makes them attractive catalysts for the manufacturing and modification of active pharmaceutical ingredients, allowing for the production of new drugs with improved pharmacokinetic and physicochemical properties [2].

Despite the potential, examples of implemented industrial processes harbouring P450 are scarce, and most of the research happens at the 0.1-50 mL scale. Although much effort has been put into increasing the volume of reactions that were promising in the lab, most of these examples have resulted in insufficient performance in terms of volumetric rate, titre, and stability in the stage of process development [3,4].

Here, we propose that a process-driven approach to P450-based bioprocess development, in which an overview of the system is used to determine the compositional sub-systems in a reverse-engineering fashion, can enable a more scalable process for the pharmaceutical industry (Figure 1). We have previously identified requirements of the P450 enzyme complex as well as different improvement opportunities for the components of the system. On this occasion, we present a workflow where tools such as flowsheet development or simple technoeconomic analyses are used to identify empirical inputs from the lab, to constrain choices in upstream design, and eventually at the enzyme level. Finally, we present how to apply this framework to the hydroxylation of diclofenac using ancestral P450 enzymes as a case study.

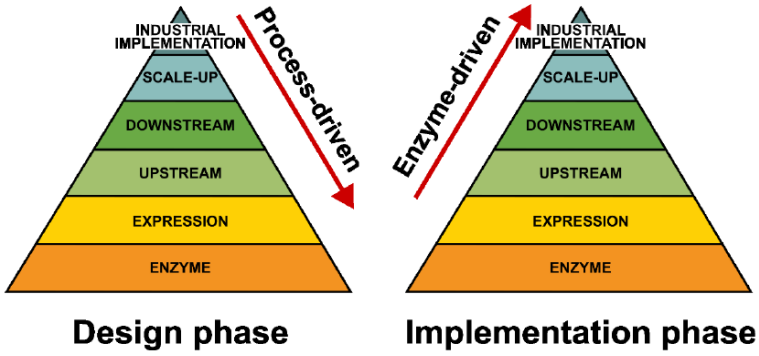

Figure 1. Proposed workflow for process-driven design of biocatalytic reactions.

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Immobilization in Cascade Reactions

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Immobilization is a widely used technique to enhance the stability of biocatalysts under more challenging reaction conditions while simultaneously facilitating their easy recycling. While various immobilization methods exist, such as non-covalent binding to a solid support through adsorption or ionic interactions, or covalent linking of enzymes to a solid support using glutaraldehyde, these approaches often result in reduced activity compared to their free counterparts. Moreover, non-covalent immobilization can lead to leaching of the biocatalyst during reactions, compromising its recyclability. In contrast, metal affinity-based immobilization (IMAC), which utilizes His-tags commonly employed for purification, offers more reproducible immobilization with a potentially lower impact on the biocatalyst's activity, likely due to its directional nature, while also maintaining the biocatalyst's stability during reactions due to the high affinity of the tag for the metal ions. Here, we explored the application of IMAC-based immobilization in cascade reactions. We selected a simple cascade consisting of an oxidase that produces hydrogen peroxide as a byproduct and a catalase responsible for its removal and oxygen regeneration.

We first evaluated the immobilization of the two enzymes individually using a set of 17 different IMAC-based carriers (IB-His, ChiralVision B.V., Den Hoon, The Netherlands) in terms of loading capacity, retained activity, and recyclability. It was discovered that the combination of enzyme and carrier material is crucial, as both enzymes exhibited significant differences in loading capacity, retained activity, and total turnover number (TTN).
For the oxidase, three carriers, IB-His 15, IB-His 17, and IB-His 22, were identified as suitable carriers. These carriers retained activities ranging from 50-70% compared to the free oxidase, with loading capacities ranging from 18.9-31.2 mg/g of carrier. Notably, the TTN could be improved from 648,000 to over 1,000,000 when the oxidase was immobilized on IB-His 15. For the catalase, IB-His 3, IB-His 16, IB-His 17, and IB-His 21 were identified as suitable carriers. However, the retained activities for the catalase were only between 30-50% compared to the free biocatalyst, and the loading capacities ranged from 21.2-45.8 mg/g of carrier. However, quick inactivation of the catalase was observed for IB-His 16, IB-His 17 and IB-His 21, while a TTN of 127,000 could be observed when the catalase was immobilized on IB-His 3. In contrast as it performed well for the oxidase in terms of retained activity and TTN, IB-His 15 showed only about 20% retained activity but had a higher TTN of over 266,000. For both, the oxidase and catalase, immobilization enhanced the tolerance of the biocatalysts to organic solvents.

We then evaluated the combination of the two immobilized biocatalysts in an upscaled reaction. Since each biocatalyst exhibited different performance on respective carriers, we first immobilized each biocatalyst individually on the most effective carriers. Subsequently, we combined the immobilized biocatalysts. However, the diffusion between the different solid phases can be considered a limiting factor. To address this potential limitation, we compared the performance of the two biocatalysts when immobilized together on a selected carrier that showed reasonable performance for both.

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Enzyme-Driven Valorization of Lignocellulosic Phenolics into Functional Aromatics

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Lignocellulosic agricultural residues represent an abundant and underexploited source of aromatic building blocks, among which *p*-hydroxycinnamic acids (*p*-HCAs) stand out as renewable chemical precursors [1]. Their selective conversion into value-added molecules with applications in polymer production, as well as antioxidant, anti-inflammatory, and antimicrobial activities [2], makes them highly attractive for the pharmaceutical, food, and cosmetic [3] sectors, although efficient and sustainable transformation routes remain a key challenge.
Here, we report the development of an enzymatic platform for the transformation of *p*-HCAs into industrially relevant aromatics. A ferulic acid decarboxylase (FDC) from *Bacillus pumilus* was employed to convert *p*-HCAs into 4-vinylphenols, while a cofactor-independent dioxygenase (CsO2) from *Caulobacter segnis* was investigated for downstream oxidative cleavage toward *p*-hydroxybenzaldehydes.

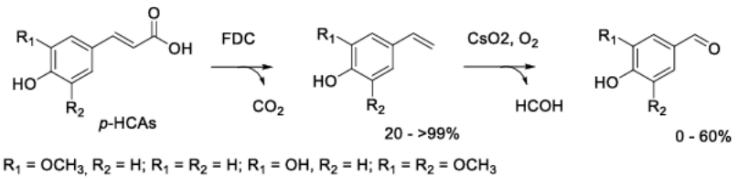

Figure 1. Enzymatic platform for the valorization of *p*-HCAs into valuable aromatics

To enhance catalyst recyclability, FDC was immobilized (40 mg/g) on superparamagnetic iron oxide nanoparticles [4], retaining activity over multiple cycles under aqueous conditions, whereas CsO2 proved less compatible with immobilization.
Based on these findings, the decarboxylation of ferulic acid (25 mM) to 4-vinylguaiacol was selected as a benchmark reaction. A solvent scope study was performed to evaluate the combined effect of solvent type and solvent/buffer ratio on reaction performance with conversions in 24 h quantified by UPLC analysis.
Green solvents such as anisole, phenetole, eucalyptol and 2-MeTHF were compared with DMSO as reference. The results highlighted a strong dependence of enzymatic activity on solvent properties: aromatic ethers (anisole and phenetole) showed comparable behavior; eucalyptol resulted in slightly lower conversion yet maintaining overall good activity, whereas DMSO significantly impaired conversion.
Overall, these findings underline the importance of the reaction medium in biocatalytic processes and demonstrate how the use of immobilized enzymes, combined with solvent scope exploration, can support the development of efficient and sustainable routes for the valorization of lignocellulosic phenolics.

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High-Throughput Profiling of Prenylelongases Enables the Assembly of Modified Prenoids

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Modified (non-natural) prenoids are useful as chemical biology tools, designer fragrances and building blocks of bioactive compounds. However, their laborious synthesis generally limits their widespread application. Although prenylelongases offer potential biocatalytic access to modified long-chain prenoids (**Figure 1**), these enzymes have remained underexplored since i) they are difficult to assay in high throughput and ii) their pyrophosphate substrates are challenging and expensive to synthesize. To address these gaps, we recently developed methods for the continuous high-throughput interrogation of prenylelongases using readily accessible monophosphates of their native and modified substrates [1]. This approach bypasses the need for laborious pyrophosphate synthesis and permits the rapid profiling of libraries of prenylelongases. We demonstrated the utility of this method for the biochemical characterization of a panel of diverse wild-type prenylelongases, their profiling with non-native substrates, and two exploratory engineering campaigns. We also demonstrated that wild-type PEs and their variants can be used to assemble modified prenoids selectively at the (semi-)preparative scale. As such, our work simplifies the engineering of elongases toward expanded substrate scopes and facilitates biocatalytic access to modified prenoids and derivatives thereof.

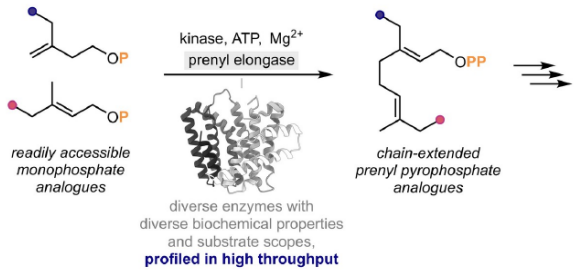

Figure 1. Kinases and elongases assemble small monophosphate building blocks into long-chain pyrophosphate synthons.

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P 50

Metal-Driven Stereocontrol in KPHMT and YfaU Aldolases for the Synthesis of Hydroxypipecolic Acid Precursors

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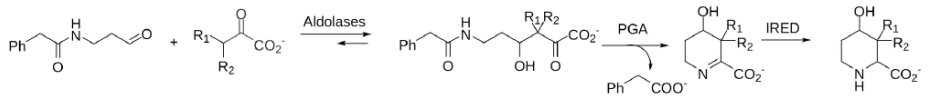
Aldolases are powerful biocatalysts for asymmetric carbon-carbon bond formation under mild aqueous conditions and are therefore valuable tools for sustainable synthesis. Among them, Class II aldolases employ a divalent metal ion as a Lewis acid cofactor to activate donor substrates through enolization, enabling highly stereoselective aldol additions. 3-Methyl-2-oxobutanoate hydroxymethyltransferase (KPHMT) and 2-keto-3-deoxy-L-rhamnonate aldolase (YfaU) from Escherichia coli are metal-dependent Class II aldolases that catalyze aldol addition reactions of 2-oxoacid derivatives with aldehydes [1].

Here, we investigate the influence of metal identity and metal loading on the stereochemical outcome of aldol additions between pyruvate, 2-oxobutyrate, 2-oxoisovalerate, 2-oxopentanoate, 2-cyclobutyl-2-oxoacetate, or 2-cyclopentyl-2-oxoacetate and phenylacetyl-3-aminopropanal, affording precursors of 4-hydroxypipecolic acid derivatives. Apo-KPHMT was generated and reconstituted with substoichiometric amounts of non-native metal ions, and both conversion and stereoselectivity were evaluated. In parallel, computationally designed KPHMT variants (S46A, S46F, S46T, I202F, F229W, F229Y, and I202F/F229W), the YfaU variant W23V, and metagenomically identified YfaU homologues were examined in holo and apo forms supplemented with a selected metal ion.

The effects of enzyme scaffold and metal type (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) on conversion and stereochemical outcome will be discussed. These results provide proof of concept for an artificial metalloenzyme strategy in which replacement of the native metal in KPHMT and YfaU scaffolds generates active, stereoselective catalysts with tunable diastereoselectivity. This approach offers direct access to precursors of hydroxypipecolic acid derivatives (**Figure 1**) and related nitrogen-containing heterocycles, underscoring the synthetic potential of KPHMT- and YfaU-based artificial metalloenzymes for stereocontrolled C-C bond formation under environmentally benign conditions.



R_1=R_2=H; R_1=H R_2=CH_3; R_1=R_2=CH_3; R_1=R_2=$(CH_2)_3$; R_1=R_2=$(CH_2)_4$

Figure 1. KPHMT-catalyzed aldol route to 4-hydroxypipecolic acid derivatives.

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P 51

Application of an Enzymatic Cascade Using Immobilized Enzymes in Droplet Microfluidics

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Cascade biocatalysis enables the production of complex products starting from fairly simple substrates without isolation of reaction intermediates. Thus, higher product yields can be achieved, and waste production is reduced, while time and other resources can be saved. However, the implementation of an enzyme cascade is often challenging due to differing requirements of the individual enzymes regarding reaction conditions. This is especially difficult if different reaction media are required in each step. We therefore aimed to implement an enzyme cascade in flow using droplet microfluidics [1], with each enzymatic step confined in a separate microreactor. As a model cascade, the production of different β-substituted alcohols starting from cyclic alkenes was studied. In the first step, lipase B from *Candida antarctica* (CAL-B) is employed for alkene epoxidation in organic media, while a halohydrin dehalogenase (HHDH) is applied in the second step for enantioselective epoxide ring opening in a two-phase system (Figure 1).

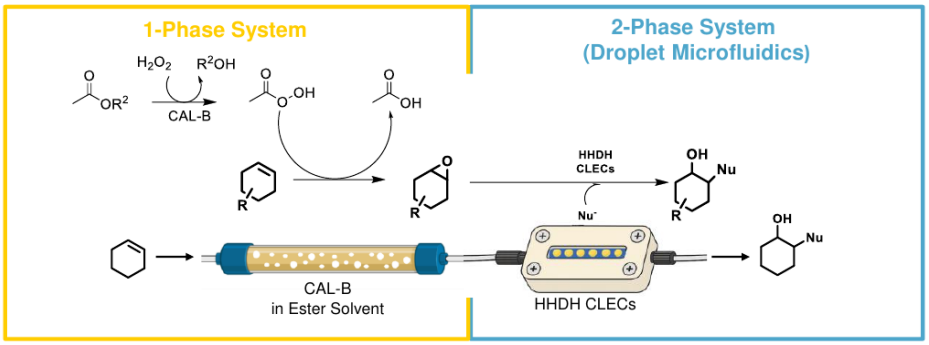

Figure 1. Application of an enzymatic cascade composed of a lipase (CAL-B) and a halohydrin dehalogenase (HHDH) that facilitate the epoxidation of cyclic alkenes followed by subsequent epoxide ring opening. The two reactions steps are compartmentalized by immobilizing the individual enzymes in separate microreactors.

While immobilized CAL-B is commercially available, immobilization of the HHDH in a microreactor had to be studied first. The use of cross-linked enzyme crystals (CLECs) [2,3] turned out to be much more effective compared to surface-based immobilization on the microchannel wall. This CLEC generation enabled both a high enzyme loading in the microreactor and a stable immobilization under flow conditions. The HHDH microreactor exhibited high operational stability, with only a slight decrease in specific activity over 20 days of continuous operation while maintaining a high product enantiomeric excess. Moreover, the HHDH microreactor also enabled the realization of two-phase reactions using microdroplets of different organic solvents, demonstrating the compatibility of immobilized enzymes with droplet microfluidics. The combination of the CAL-B and HHDH-catalyzed reaction steps in flow, to construct a continuously operated cascade, is currently investigated. A respective batch setup of this two-step cascade using immobilized enzymes yielded up to 98% total conversion under optimized conditions, with the concentrations of hydrogen peroxide and organic solvent as the key parameters influencing overall productivity.

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P 52

Electronic and Dynamic Control of Abiological Lactonization vs. Lactamization in Heme-Dependent Enzymes

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Enzymatic catalysis emerges from the interplay between a given reaction mechanism (active-site controlled chemical step), protein dynamics, and environmental effects, which collectively determine catalytic efficiency and selectivity. In (metallo)enzymes, this complexity is amplified by the formation of highly reactive, short-lived intermediates that can access competing pathways, leading to catalytic promiscuity while also creating opportunities for designing new challenging biocatalytic transformations [1]. Experimental characterization of these "fleeting" reactive intermediates remains challenging of the limited spatial and temporal resolution of available structural and spectroscopic techniques. In this context, multiscale computational approaches provide a powerful strategy to characterize these intermediates with atomic-level and real-time resolution [2].

In this work, we present an integrative multiscale computational framework combining Density Functional Theory (DFT) and Molecular Dynamics (MD) simulations to uncover the origins of divergent abiological reaction pathways in iron-heme-dependent enzymes. DFT calculations characterize key transition states and competing reaction pathways, while MD simulations capture the positioning of reactive intermediates and the influence of surrounding active site residues on controlling reactivity. Here, we apply this approach to dioxazolone-derived nitrene transfer reactions catalysed by iron–heme enzymes[3], focusing on the mechanistic divergence between lactamization and lactonization pathways from a common iron-nitrenoid reactive intermediate (**Figure 1**). Our results identify the molecular basis of pathway selectivity arising from fundamental differences in the reaction mechanism, showing that subtle changes in active-site preorganization, conformational dynamics, and intermediate stabilization disfavour lactamization and promote lactone formation. These findings are consistent with experimental observations and provide a detailed mechanistic rationale for the observed reactivity patterns. Overall, this study highlights how multiscale modelling can elucidate the interplay between reaction mechanism and enzyme dynamics in controlling reactive intermediates, offering valuable insights for the rational engineering of next-generation biocatalysts with enhanced selectivity and tailored reactivity.

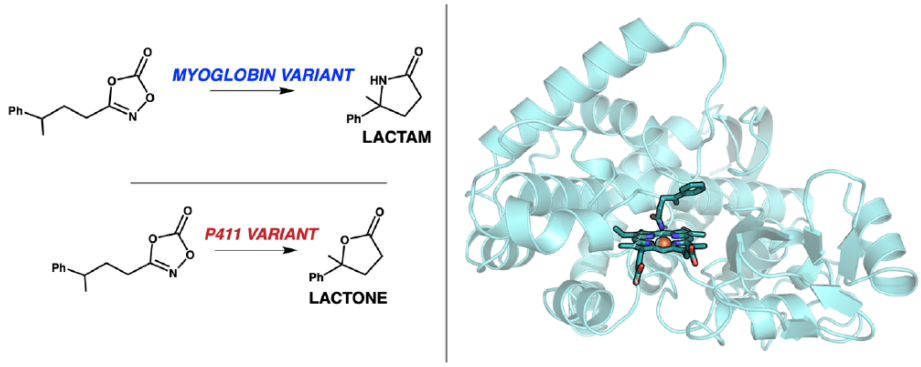

Figure 1. Heme-dependent enzymes control selectivity in dioxazolone transformations.

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P 53

Exploitation of a novel aromatic prenyltransferase for the synthesis of prenylated chalcones as bioactive compounds

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Prenylated polyphenols are a prominent class of natural compounds characterized by the presence of one or more prenyl $(-C_5H_8)_n$ groups within their structure, and are widely distributed across the plant kingdom, particularly in species used in traditional medicine. Prenylation often enhances biological properties of polyphenols with respect to their parent compounds, largely due to the improved lipophilicity [1]. In nature, the biosynthesis of prenylated aromatics is catalyzed by aromatic prenyltransferases (aPTases), enzymes capable of transferring prenyl moieties to aromatic substrates with high regio- and stereo-selectivity under mild aqueous conditions [2]. Among these, prenylated chalcones stand out for their widespread occurrence and their broad spectrum of beneficial properties, such as anticancer, antinflammatory and antifungal activities [3].
In this work, we present AmaPT, a novel prenyltransferase from Aspergillus melleus, successfully heterologously expressed in E. coli and evaluated against a panel of 52 aromatic substrates using DMAPP as prenyl donor. AmaPT shows a notable activity toward chalcones, a feature that can attributed to π–π interactions within the enzyme active site. Notably, the enzyme also exhibits exceptional stability, retaining full activity after more than two months when stored at 4 °C.
To further increase its operational robustness under reaction conditions, different immobilization methodologies were evaluated, assessing both immobilization yield and recovered activity of the immobilized enzyme. The most promising immobilized systems were subsequently implemented in a SpinChem® MiniRBR reactor (Figure 1), enabling the efficient transformation of a panel of chalcones to produce a library of prenylated products to be tested as antimicrobial compounds.

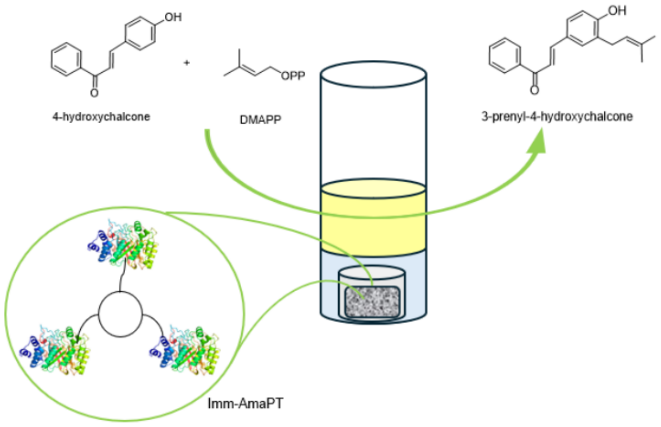

Figure 1. Schematic representation of the SpinChem® MiniRBR reactor.

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P 54

Minimal Structural Switch Enables Ketone Acceptance in ThDP-Dependent Carboligation Enzymes

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Thiamine diphosphate (ThDP)-dependent enzymes are powerful biocatalysts for C–C bond formation via the addition of donor substrates to electrophilic aldehydes. In contrast, their ability to accept less reactive ketones remains rare and poorly understood, despite the synthetic potential for accessing enantioenriched tertiary α-hydroxy ketones.[1]

To uncover the mechanistic basis for ketone acceptance, we analysed selected ketone-accepting ThDP-dependent enzymes (*Pp*YerE, JanthE, ErwE)[2–4] using structural analysis and targeted mutagenesis. We pinpointed conserved active-site residues critical for ketone conversion, as their mutation diminished activity with ketones while preserving reactivity toward aldehydes.
Strikingly, we successfully transferred these insights to *Pf*BAL, one of the most efficient and extensively studied ThDP-dependent enzymes: a single active-site substitution enabled ketone acceptance (**Figure 1**). Therefore, we reveal a minimal structural switch that governs this reactivity and define a direct mechanistic link between active-site architecture and substrate scope.

Altogether, our findings provide the structural basis for ketone reactivity in ThDP-dependent enzymes and establish a framework for predicting and engineering next-generation carboligation biocatalysts with expanded substrate scopes from genomic data.

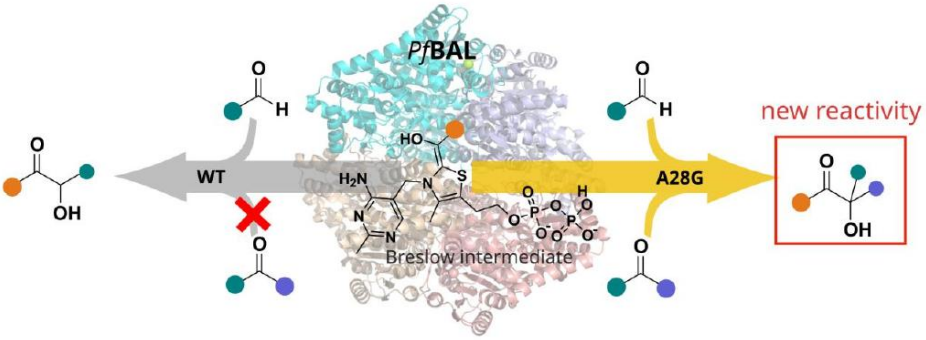

Figure 1. Reactivity of *Pf*BAL wildtype (WT) versus single-point variant.

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P 55

Safer and more sustainable synthesis of natural ingredients for food industry

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The growing consumer demand for natural, sustainable, and high-quality food additives has significantly increased the need for innovative production strategies in the agri-food sector. However, this trend is accompanied by a rise in food fraud, particularly involving the adulteration of natural flavouring agents, posing serious risks to food safety and consumer trust [1]. In this context, the SNIF project, funded by Open Innovation Lombardia, aims to address both challenges by combining advanced analytical methodologies with sustainable biotechnological approaches.
This work proposes the development of innovative biocatalytic routes for the synthesis of bioactive glycosides with potential application as flavour enhancers. These compounds are designed to improve flavour profiles while meeting the increasing demand for naturally derived ingredients. The approach relies on the use of sucrose phosphorylases (EC 2.4.1.7) and glycosyltransferases (EC 2.4.x.x), selected according to the target glycoside, to enable efficient and selective glycosylation reactions [2]. Model compounds include flavour-active molecules such as L-carveol glycoside and ethyl maltol glycoside [3]. In addition, exploiting the substrate promiscuity of some variants of these enzymes, the study explores the synthesis of bioactive glycosides such as arbutin and salidroside, which are known for their antioxidant, antimicrobial, and pharmacologically relevant properties (**Figure 1**). The use of enzymes and whole-cell systems enables highly selective transformations under mild conditions, reducing energy consumption and minimizing environmental impact compared to traditional chemical or thermal processes.
In addition to the above-described activities, SNIF project also focuses on the authentication of natural flavourings via isotopic and metabolomic analyses, enabling the detection of advanced adulteration practices. It further addresses the production of extended shelf-life foods through the extraction of bioactive compounds from renewable biomass, aimed at inhibiting microbial growth and supporting circular economy approaches.

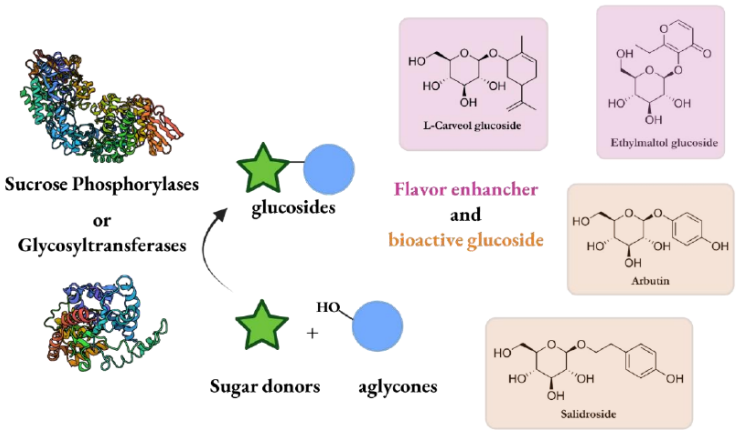

Figure 1. Biocatalytic synthesis of glucosides.

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P 56

Engineered Sucrose Synthases with Enhanced Robustness led to More Sustainable Glycosylation of Natural Products

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Sucrose synthase (SuSy) plays a key role in the glycosylation of natural products via uridine-dependent glycosyltransferases (UGTs), enabling efficient recycling of UDP-glucose (UDP-Glc) while minimizing UDP-induced inhibition of UGT activity [1]. However, for this approach to be techno-economically viable, biocatalysts must be robust enough to operate under the high temperatures and co-solvent concentrations required for substrate solubilization. In this work, we applied semi-rational engineering strategies to improve the thermostability and solvent tolerance of SuSy. After screening over 200 variants, we obtained a set of SuSy variants with improved properties compared to the wild type (**Figure 1**). These include increased melting temperatures (Tm) reaching approximately 75°C (ΔTm = 13°C), and significantly enhanced half-lives of 7 h and 31 h at 60 °C and 55 °C, respectively (123- and 12-fold improvements). The novel variants also exhibited enhanced DMSO tolerance, retaining up to 70% activity in 25% v/v DMSO, compared to just 9% of the wild type. Additionally, soluble expression of SuSy was improved 3-fold compared to wild type. We further tested these variants in the glycosylation of indoxyl and methyl anthranilate, where SuSy has been identified as the main reaction bottleneck [2, 3]. In both reactions, the best-performing SuSy variant yielded approximately 50% higher conversions, eliminating SuSy as the reaction-limiting factor. Through preliminary techno-economic and life-cycle assessments, the implementation of robust SuSy variants resulted in up to a 2-fold reduction in costs and environmental impacts compared to wild type. This study highlights the application of semi-rational enzyme engineering strategies to enhance SuSy robustness, thereby improving process performance and reducing costs and environmental impacts. The engineered SuSy variants developed here offer a promising tool for enabling more efficient and sustainable natural product glycosylation via biocatalytic UGT-SuSy cascades.

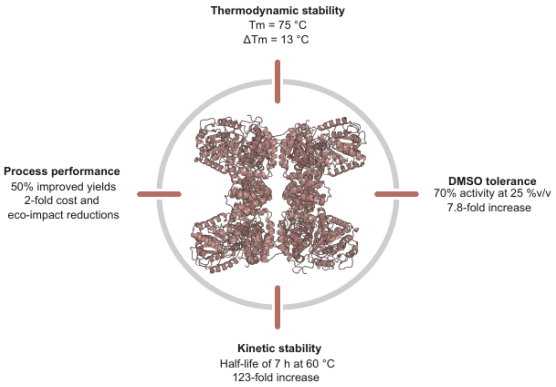

Figure 1. Improvements observed after engineering sucrose synthase, compared to the wild type. Tm refers to the apparent melting temperature. Dimethyl sulfoxide (DMSO) tolerance is measured as relative activity at 25% (v/v) DMSO concentration. Process performance benefits reflect improved conversion yields, reductions in cost and environmental impacts of methyl anthranilate and indoxyl glycosylation cascades.

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P 57

De novo copper enzymes for photoredox catalysis

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Biocatalysis and photocatalysis have immense impact, both in academia and industry. Until recently, these two fields of catalysis have been explored separately. However, researchers have recently started to combine the broad scope of photoreactions with the precise selectivity of protein scaffolds, thereby substantially increasing the potential for application. Recent examples used either naturally occurring photoactive cofactors such as flavin mononucleotide (FMN) [1] or genetically encodable organophotocatalysts such as benzophenone [2].
To extend this, transition metal complexes based on ruthenium or iridium are among the most common photocatalysts, but copper complexes are becoming more appealing photoredox catalysts because of their price, sustainability, versatility and potency [3].
My PhD project aims to develop copper-dependent artificial photoenzymes. We create copper binding sites by incorporating suitable ligands such as 2,2'-bipyridine or 1,10-phenantroline using genetic code expansion or chemical protein modification. Robust protein scaffolds, including *de novo* designed proteins like NTF2 [4] or the highly engineered retroaldolase RA95.5-5 (**Figure 1**) [5] make for diverse tuneability and adaptability.

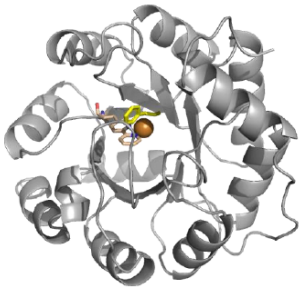

Figure 1. RA95.5_A210BpyA docked with Cu^{2+} and substrate trans-β-methylstyrene

The resulting copper-binding proteins are characterized and tested for catalysis in a selection of photoreactions, such as oxoazidation (**Figure 2**) [6].

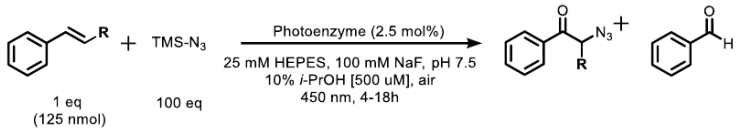

Figure 2. General reaction conditions for photocatalysed oxoazidation

A screening platform (expression, purification and photoreaction) for directed evolution of the resulting photoenzymes is also established in order to improve protein stability, as long as yields and stereoselectivity for the oxoazidation reaction.

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P 58

Enzymatic Detoxification of Deoxynivalenol (DON) for Low-Impact Agriculture

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The contamination of staple food and feed crops with mycotoxins represents one of the most significant and persistent challenges to global food security and public health. It is also an often-overlooked consequence of climate change, which creates environmental conditions that favor fungal growth and infection. Deoxynivalenol (DON) is a mycotoxin produced by Fusarium spp. fungi on crops used for both human and animal consumption. The current global annual crop production of the major crops affected by mycotoxins is approximately 2819M tons, which at current European crop prices equates to $750B [1]. On a global scale, it is estimated that over 20% of all food crops are affected by mycotoxin contamination [2]. In addition to the economic losses, the environmental impact includes increased land use, loss of biodiversity, and increased CO2-equivalent emissions. These losses translate directly into losses in livestock agriculture through increased crop prices and a reduction in livestock production. A sustainable and easy-to-implement method for removing or detoxifying DON and other mycotoxins is sorely missed. We propose that the detoxification of DON in crop products can be addressed through the discovery, engineering, and development of enzymes that are specifically optimized to target and degrade toxins. Several degradation pathways have already been discovered but the majority remain relatively unknown.

In this project, we will develop and optimize a robust enzyme-based system for DON detoxification, with the goal of integrating it into an industrially relevant process. To achieve this, we will utilize the more unknown enzymatic DON degradation pathways as a foundation for a comprehensive search to identify high-activity candidates. This will include conventional homology-based searches alongside state-of-the-art machine learning tools to predict activity and stability. The results of this initial screening study will be presented along with a detailed plan for in vitro testing of promising candidates that will be produced, screened and undergo an engineering campaign to further enhance promising characteristics, using computational design strategies.This work will be done in close collaboration with industry-partner Patent CO, which specializes in feed and feed additive production.

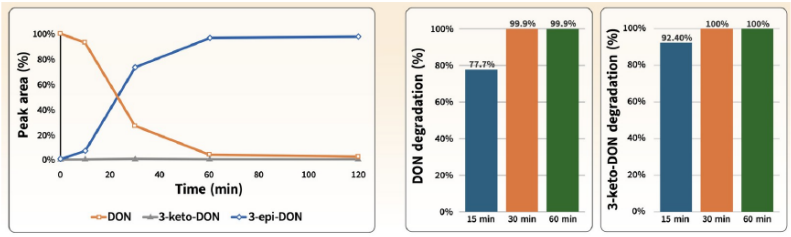

Figure 1. Preliminary activity assays of an enzyme homolog mining campaign, showcasing high activity for enzymes belonging to the DepA/DepB DON epimerization cascade. These results highlights that despite being a relatively well-known cascade, high-performance homologs can easily be identified. This gives us great confidence that other detoxification cascades can similarly be explored with great results.

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P 60

Engineering Oxidative Robustness in Ancestral P450s: Insights from Protein Stability and Molecular Dynamics

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Cytochrome P450s (P450s) are powerful biocatalysts for selective hydroxylation reactions in industrial biotechnology. However, their reliance on auxiliary electron-transfer partners often results in inefficient coupling and the generation of reactive oxygen species (ROS), accelerating enzyme inactivation. While peroxide-driven catalysis circumvents this limitation, oxygen surrogates like cumene hydroperoxide (CuOOH) and hydrogen peroxide (H_2O_2) introduce severe oxidative stress, leading to haem bleaching and the degradation of susceptible amino acid residues. To overcome these constraints, this project investigates the oxidative robustness of resurrected ancestral P450 variants[1,2]. By combining *in vitro* spectral stability assays with molecular dynamics (MD) simulations, we assess the susceptibility of ancestral versus extant P450s to peroxide-induced degradation. MD simulations are utilised to map non-covalent interaction patterns, identifying specific structural vulnerabilities and oxidation-prone regions near the haem moiety. These mechanistic insights drive the targeted engineering of highly stable ancestral scaffolds. Ultimately, securing this oxidative stability is a prerequisite for industrial scale-up, enabling these robust engineered enzymes to be placed into whole-cell biotransformation for efficient metabolite production in stirred bioreactors.

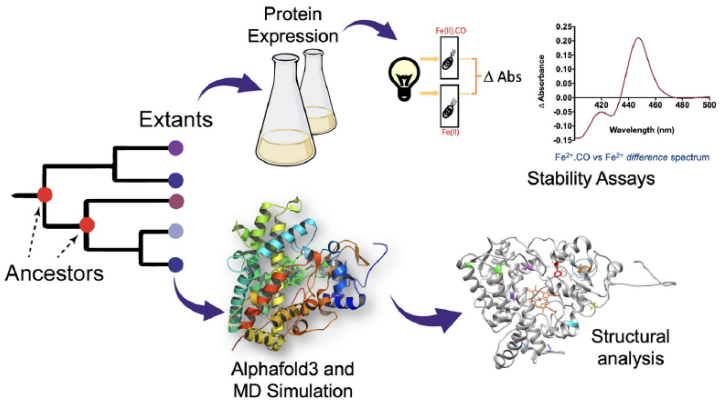

Figure 1. Integrated workflow for the engineering and characterisation of robust ancestral P450s. The approach combines phylogenetic resurrection of ancestral scaffolds followed by protein expression and stability assays in presence of oxygen surrogates (i.e. cumene hydroperoxide, hydrogen peroxide) using the Fe^{2+}.CO vs Fe^{2+} difference spectroscopy. In parallel, AlphaFold3 modelling and molecular dynamics (MD) simulations identify structural features and oxidation-prone regions to guide targeted engineering for industrial scale-up.

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Machine Learning-Guided Identification of Extremophilic Glycosyltransferases

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Enzymatic glycosylation, primarily catalyzed by UDP-dependent glycosyltransferases (UGTs) of glycosyltransferase family 1 (GT1), is a powerful approach for improving the solubility, stability, and therapeutic efficacy of natural products [1]. However, most characterized UGTs are derived from mesophilic sources and exhibit limited stability under industrially relevant conditions, such as high temperatures, extreme pH, and elevated substrate or solvent concentrations [2]. Furthermore, despite the presence of over 73,000 GT1 sequences in public databases, fewer than 0.5% have been experimentally characterized, leaving an enormous underexplored sequence space, especially those from extremophilic organisms.

This study aims to bridge the gap between the massive uncharacterized sequence diversity of GT1s and the industrial demand for robust biocatalysts. The primary objective is to identify, characterize, and develop hyperstable UGTs into industrially relevant biocatalysts, with a focus on thermophilic and halophilic candidates.

In this study, we employed a machine learning (ML)-guided sequence mining strategy to establish a curated, non-redundant dataset of GT1 enzymes from public databases. A VAE model was trained on a multiple sequence alignment of these GT1 enzymes, and the resulting latent space was used to map the full sequence space. Furthermore, a set of informed sequence annotations enabled the identification of candidate UGTs with potential stability-related features. Based on this approach, a set of extremophilic UGTs was selected for experimental validation. More than 50 candidates have been subjected to heterologous expression, with soluble proteins successfully obtained for some enzymes. Further biochemical characterization and substrate-scope screening of these candidate enzymes are currently in progress.

This work demonstrates the feasibility of using ML-guided sequence mining to efficiently identify promising extremophilic UGTs. Ongoing functional validation is expected to provide insights into their catalytic properties and stability, ultimately contributing to the development of robust glycosylation biocatalysts for industrial applications.

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P 62

Engineering Robust Pullulanase Biocatalysts through Computational Design and Sustainable Immobilization

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Pullulanase (EC 3.2.1.41) is an industrially important debranching enzyme widely used in starch saccharification and starch modification processes due to its ability to selectively hydrolyze α-1,6-glycosidic linkages. Beyond its established role in glucose syrup production, pullulanase has attracted increasing interest for the generation of resistant starch and other functional food ingredients, as enzymatic debranching facilitates starch restructuring and promotes retrogradation [1]. However, broader industrial application remains limited by insufficient thermal and operational stability, as well as challenges associated with enzyme reuse under process conditions. Our research focuses on improving pullulanase performance through an integrated strategy combining computational protein engineering and sustainable immobilization approaches.

A designated computational protein engineering pipeline combined with complementary structure-based analyses was employed to identify mutations associated with enhanced thermostability while preserving catalytic activity. Selected variants were experimentally evaluated through thermal shift analysis and residual activity measurements to assess their thermostability profiles. Engineered variants exhibited markedly improved thermostability at 50 °C, showing approximately 5–60-fold increases in half-life compared to the wild-type enzyme, while also demonstrating improved storage stability.

In parallel, immobilization on pillared acid-activated bentonite clay was investigated as a sustainable and cost-effective approach for improving pullulanase stability and operational performance. Comparative studies between free and immobilized enzymes included evaluation of kinetic parameters, pH and temperature optima, and operational stability under process-relevant conditions used in starch-processing applications. The immobilized biocatalyst retained catalytic performance over 10 consecutive reaction cycles, indicating improved operational stability and long-term applicability in starch saccharification and resistant starch production processes. Overall, the integration of computational protein engineering and sustainable immobilization strategies represents an effective approach for developing pullulanase biocatalysts with improved robustness and operational performance.

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P 63

Developing solar-driven new-to-nature cascades for CO_2 fixation using light-harvesting inorganic photocatalysts

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The integration of abiotic nanomaterials with biological systems offers powerful new opportunities for sustainable catalysis and energy conversion. During my PhD, I developed a platform that enables living cells to biosynthesize inorganic nanomaterials, such as Pd [1] and CdS [2] nanoparticles, using engineered variants of a template protein scaffold. These biofabricated nanomaterials endowed cells with novel catalytic and light-harvesting abilities, enabling solar-driven NADPH regeneration and in vivo biomanufacturing of chiral amines. [3] Through this work, I demonstrated both in vitro and whole-cell semi-artificial photosynthetic systems, establishing a foundation for merging biological selectivity with inorganic photoreactivity. [4,5]

Building on this experience at the biotic–abiotic interface, my postdoctoral research in the Tobias Erb Lab at the Max Planck Institute for Terrestrial Microbiology focuses on advancing solar-driven CO_2 valorization, a critical challenge in sustainable chemistry and biotechnology. I have developed solar-driven, new-to-nature enzymatic cascades energized by inorganic light-harvesting modules to fuel cofactor regeneration for highly active CO_2-fixing pathways (Figure 1). By integrating synthetic dyes into photocatalytic systems, we achieved robust, solar-powered multi-enzyme reactions while addressing key limitations such as photostability and high light flux demands. Moving forward, I will design systems in which photoexcited nanomaterials directly fuel ATP-generating enzyme networks, converting solar energy into biochemical energy carriers. Finally, I will construct a (partially) self-sustaining photocatalytic CO_2-fixation platform by coupling nanomaterials to biologically generated quinols, enabling internal recycling of sacrificial electron donors.

By combining my PhD expertise in bio-nanomaterial integration with state-of-the-art synthetic metabolism, this work bridges synthetic biology, materials science, and renewable energy to create next-generation biocatalytic systems for carbon-neutral chemical synthesis.

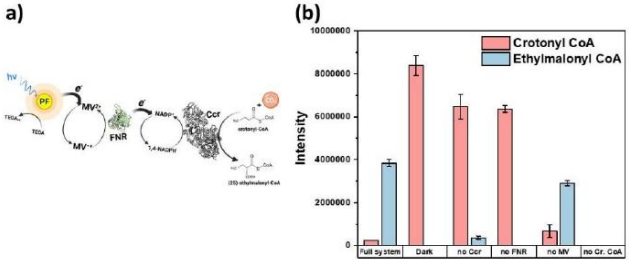

Figure 1. (a) A schematic illustration of the continuous activation of the CO_2-fixing enzyme crotonyl-CoA reductase (Ccr) via photocatalytic NADPH regeneration. This has been achieved by solar-driven activation of ferredoxin NADP+ reductase (FNR). (b) Preliminary qualitative HPLC data showing the cascade performance compared to relevant controls lacking key components.

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P 64

Exploring the biocatalytic reduction of alkynes to alkenes using the Old Yellow Enzyme family

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The selective reduction of alkynes to alkenes is a fundamental transformation in synthetic chemistry, enabling access to valuable building blocks for natural compounds, agrochemicals and pharmaceuticals. Current methods rely on precious-metal catalysts, however biocatalytic alternatives offer more selective approaches operating under milder conditions.

Ene-reductases (EREDs), particularly Old Yellow Enzymes (OYEs), reduce alkyne substrates with electron-withdrawing groups [1,2]. However, achieving high selectivity for the desired alkene product remains challenging, as over-reduction of the corresponding alkane can occur. Accomplishing this alkene selectivity requires identifying EREDs that exhibit a higher catalytic rate constant for the alkyne-to-alkene reduction (k_1) relative to the alkene-to-alkane reduction (k_2) (**Error: Reference source not found**).

In this work, a diverse panel of purified OYEs from different classes was screened with activated alkynes bearing different electron-withdrawing groups (EWG). For 4-phenyl-3-butyn-2-one, OYE2 achieved 89% of the corresponding *E*-alkene with a catalytic efficiency of 16.2 s^{-1} mM^{-1} for the alkyne-to-alkene, compared to only 1.9 s^{-1} mM^{-1} for the alkene-to-alkane reduction. In contrast, for 3-phenyl-2-propynenitrile, OYE3 emerged as the optimal biocatalyst, converting 94% of the alkyne to the *Z*-alkene. Interestingly, these results suggest that the stereochemical alkene outcome may depend on the nature of the EWG.

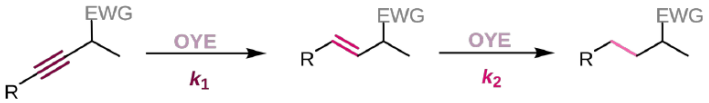

Figure 1. OYE-catalysed reduction of alkynes to alkenes and alkanes.

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P 65

Hydrogen-Driven Whole-Cell Biocatalysis using *Cupriavidus necator* for Sustainable Production of Aldehydes and Alcohols

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The reduction of carboxylic acids to alcohols is a key transformation in industrial chemistry. For instance, fatty alcohols have a yearly production of 3 million tonnes. Conventional processes rely on esterification or transesterification of carboxylic acids followed by metal-catalyzed hydrogenation at elevated temperatures (e.g. 280 °C) and pressures (e.g. 300 bar). In contrast, biocatalysis provides sustainable routes for the synthesis of such compounds using enzymes. Carboxylic acid reductases (CARs) are an important enzyme class that reduces carboxylic acids to aldehydes using NAD(P)H and ATP. [1] Expression of CARs in whole cells offers the advantage of in situ cofactor regeneration. Interestingly, while in vitro CAR mediated biotransformations lead to the formation of aldehydes, in whole-cell biocatalysts, alcohol dehydrogenases (ADH) of the host cell tend to reduce the aldehyde further to the respective alcohol. Herein we report the coupling of CAR catalyzed biotransformations to hydrogen-driven cofactor-recycling in recombinant cells of the hydrogen-oxidizing bacterium *Cupriavidus necator*. [2] The organism's soluble [NiFe]-hydrogenase provides reduced NADH, which subsequently is transformed by the transhydrogenase to NADPH, while the membrane-bound hydrogenase contributes to the proton gradient across the inner cell membrane, which is used for ATP synthesis. Thus, molecular hydrogen, which is a clean and renewable electron source, fuels the supply of both cofactors required for the carboxylic acid reduction, leading to the corresponding aldehyde or alcohol, respectively. Production of the CAR from *Neurospora crassa* together with its maturation factor phosphopantetheinyl transferase in *C. necator*, enabled efficient whole cell reduction of different aromatic carboxylic acids under a $H_2/O_2/CO_2$ atmosphere, achieving conversions up to 89%. Notably, the biocatalytic system supports NAD(P)H- and ATP-dependent biotransformations even under O_2-limited conditions, highlighting its potential for safer bioprocesses. By combining high atom and reaction mass efficiency, this work introduces a novel biocatalytic process for the reduction of carboxylic acids to alcohols using molecular hydrogen as reductant.

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Accelerating Enzyme Engineering Through AI and Physics-Based Molecular Modelling

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Computational protein engineering is transforming the way biocatalysts and functional proteins are discovered, redesigned and translated into industrial applications. At Nostrum Biodiscovery, we combine molecular modelling, artificial intelligence and high-performance computing into modular workflows that support enzyme bioprospecting, *in silico* directed evolution, active-site redesign, mechanistic interpretation and variant prioritization (Figure 1).

Using proprietary and adapted technologies, including PELE-based exploration of protein–substrate interaction landscapes, structure-guided mutagenesis and automated workflow execution, we connect molecular insight with experimentally actionable designs. This platform is illustrated through selected case studies across biotechnology sectors: PluriZyme design for cascade reactions in pharmaceutical intermediate synthesis [1]; engineered protein nanopores for nano-PET deconstruction in polymer recycling and environmental biotechnology [2]; genome rewiring strategies for PET biodegradation and upcycling [3]; and computer-guided laccase evolution for textile, bioremediation and bioelectrochemical applications [4].

Together, these examples demonstrate how scalable computational workflows can expand the functional space of natural proteins, create new catalytic activities and reduce experimental screening burden. While the focus is enzyme engineering, the same infrastructure can be extended to broader protein engineering challenges, including antibody design and vaccine-related protein optimization. This contribution presents an industry perspective on how HPC-backed modelling platforms, workflow automation and scientific consulting can accelerate biocatalyst development from concept to experimental validation.

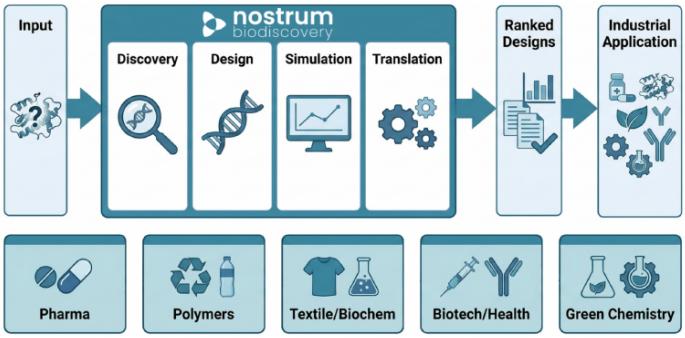

Figure 1. Nostrum Biodiscovery protein engineering platform.

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P 67

Reaction engineering-aided cascade synthesis of L-piperazic acid

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The production of L-piperazic acid using conventional methods of organic synthesis is a complicated, multi-step procedure that requires the use of harsh reaction conditions [1]. The limitations of the conventional synthesis procedure can be overcome with the use of a two-step biocatalytic cascade (Figure 1). In this cascade reaction, L-ornithine serves as the starting material for the production of L-piperazic acid [2]. In the first step of this cascade reaction, a flavin-dependent *N*-hydroxylating monooxygenase (NMO) hydroxylates the δ-amino group of L-ornithine. This enzyme uses NADPH as a source of reducing equivalents, as its FAD cofactor needs to be reduced at the end of the reaction to enable it to be used again [3]. NADPH is regenerated continuously over the course of the reaction, which is achieved *in situ* using an engineered formate dehydrogenase (FDH) that reduces $NADP^+$ to NADPH while simultaneously oxidizing formate to carbon dioxide [4]. The *N*-hydroxylated ornithine is subsequently converted to L-piperazic acid by a heme-dependent piperazate synthase (PZS) [5].

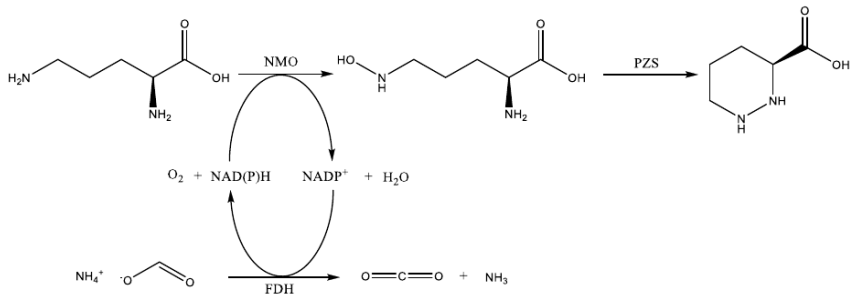

Figure 1. The investigated cascade reaction.

In this work, the reaction kinetics of all three enzymes involved were investigated using the initial reaction rate method. The enzymes' activity was determined at varying concentrations of substrates involved in the cascade reaction and the values of kinetic parameters such as K_M, V_m and K_i were estimated. The parameters that were obtained were used to develop a mathematical model of the cascade reaction. This model was used to carry out process simulations, which enabled the selection of the optimal operating conditions for the cascade reactions. The optimal operating conditions indicated by the model will be used to plan and carry out batch reactions for the synthesis of piperazic acid.

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Investigations on the Potential of different Kinases for Nucleotide Synthesis

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Nucleosides constitute the basic building blocks of nucleic acids and serve as precursors for a wide array of cellular metabolites, signalling molecules and antiviral drugs. In living systems, the main biologically active form is the nucleoside triphosphate (NTP), which powers DNA/RNA synthesis, energy transfer and enzymatic regulation. Nucleosides are first phosphorylated by nucleoside kinases to nucleoside monophosphates (NMPs) before subsequent phosphotransfer reactions, catalysed by polyphosphate kinases (PPKs), extend the phosphate chain to the diphosphate (NDP) and triphosphate levels [1]. PPKs are divided into two families: PPK1, which mainly synthesises inorganic polyphosphate, and PPK2, which generates nucleotides. Within PPK2, subclass II (PPK2-II) enzymes display a kinetic bias toward NDP formation while still being capable of producing NTPs, offering a flexible platform for nucleotide regeneration and synthesis (**Figure 1**) [2,3].

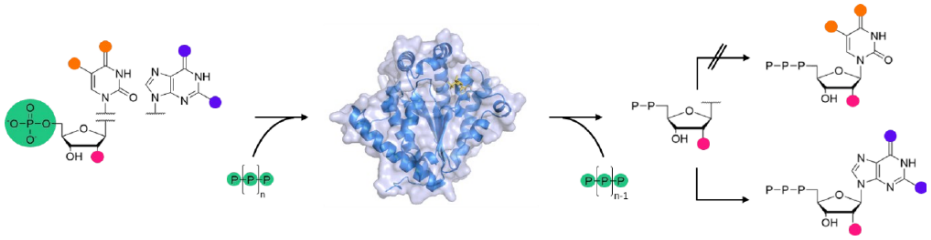

Figure 1. PPK2-II Enzymes catalyse NDP formation from NMPs for both pyrimidines and purines. Purines are further phosphorylated to NTPs.

We screened and biochemically characterised two PPK2-II enzymes [4]. Both readily phosphorylated a broad spectrum of NMPs—including purine, pyrimidine and 2′-deoxy substrates—into their respective NDPs; only purine NMPs were further converted to NTPs. X-ray structures of the enzymes complexed with AMP, ADP and TMP represent the first substrate-bound PPK2-II models and explain the observed selectivity.

These findings highlight the versatility of PPK2-II enzymes as selective biocatalysts for nucleotide production, provide a structural basis for engineering bespoke kinase variants, and help create a new toolbox for the selective synthesis of modified nucleotides for synthetic-biology and pharmaceutical applications in tandem with other enzymes.

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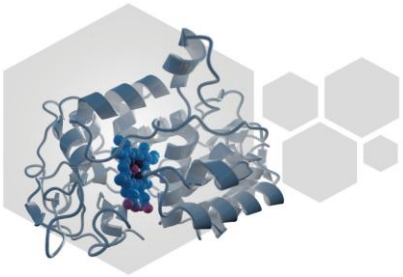

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